

**CHARACTERIZATION OF GROUNDNUT
TRANSGENIC PLANTS CO-EXPRESSING THREE
REGULATORY GENES TO IMPROVE DROUGHT
RESISTANCE**

SWETHA, T.N.

PALB 1046

**DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU**

2017

**CHARACTERIZATION OF GROUNDNUT
TRANSGENIC PLANTS CO-EXPRESSING THREE
REGULATORY GENES TO IMPROVE DROUGHT
RESISTANCE**

SWETHA, T.N.

PALB 1046

Thesis submitted to the

UNIVERSITY OF AGRICULTURAL SCIENCES, BENGALURU

in partial fulfilment of the requirements

for the award of the degree

DOCTOR OF PHILOSOPHY

in

CROP PHYSIOLOGY

BENGALURU

SEPTEMBER, 2017



**AFFECTIONATELY
DEDICATED TO
MY PARENTS
MY DEAR HUSBAND
JAIPAL
MY SON VIYAAN**

**DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU 560 065**

C E R T I F I C A T E

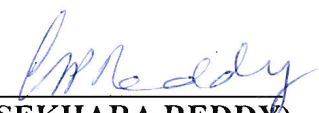
This is to certify that the thesis entitled “CHARACTERIZATION OF GROUNDNUT TRANSGENIC PLANTS CO-EXPRESSING THREE REGULATORY GENES TO IMPROVE DROUGHT RESISTANCE” submitted in partial fulfillment of the requirement for the degree of **Doctor of Philosophy in Agriculture**, in **Crop Physiology** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work carried out by **Ms. SWETHA T.N, ID No. PALB 1046**, under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bengaluru

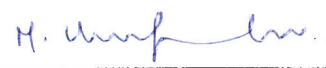
September, 2017


(P. CHANDRASEKHARA REDDY)
Major Advisor

APPROVED BY:

Chairman: 
(P. CHANDRASEKHARA REDDY)

Members:

1. 
(UDAYAKUMAR M.)

2. 
(N. NATARAJA KARABA)

3. 
(P.H. RAMANJINI GOWDA)


(G. RAMA RAO) 4/9/17
External member

4. _____
(H.C. LOHITHASHWA)

ACKNOWLEDGEMENT

With regardful memories

This thesis would not have been possible without the inspiration and support of a number of wonderful individuals – my thanks and appreciation to all of them for being part of this journey and making this thesis possible.

Firstly, I would like to express my sincere gratitude to my chairman Dr. P. Chandrashekara Reddy, Professor, Department of Crop Physiology, UAS, Bengaluru, for the continuous support and advice throughout this PhD programme.

I owe my deepest gratitude to Dr. M. Udayakumar, Project co-ordinator, Department of Crop Physiology, UAS, GKVK, Bengaluru, member of advisory committee for his excellent guidance, constant support, close counsel, valuable suggestions, extreme patience and the encouragement given to me will always remain in my heart with great respect. His enthusiasm, concern, help and moral support have always aroused my spirits to do more, to achieve higher. His care has been a source of strength during the most demanding times. I admit that it has been a great fortune for me to be associated with him during my PhD programme.

I would like to express my sincere gratitude and thanks to the other members of my advisory committee Dr. N. Nataraja Karaba, Professor, Department of Crop Physiology, UAS, Bengaluru, Dr. P. H. Ramanjini Gowda, Professor, Department of Biotechnology, UAS, GKVK, Bengaluru and Dr. H. C. Lohithashwa, Professor, Department of Genetics and Plant Breeding, College of Agriculture, V.C. Farm, Mandya, for their insightful comments and encouragement throughout my research.

I record my respectful indebtedness and gratitude to Dr. Ramu for his understanding, encouragement, valuable suggestions and personal attention, which

have provided good and smooth basis for my Ph.D. tenure. I would like to express my special thanks to Dr. Mahesh Salimat and Dr. Amar for their help to do and understand the metabolomics and proteomics work.

I would like to acknowledge Dr. Mahesh Kulkarni, Dr. Thulasiram, Dr. Gopinath, Genotypic private limited, NCL lab members for helping us to do the omics work.

I would like to thank my beloved senior friends Dr. Mahesh Patil, Dr. Babitha, Dr. Jalendra, Dr. Rajashekara Reddy, Dr. Pruthvi, for their care, constant encouragement and support during my research program.

Above all, I humbly place before the throne of the Almighty, my most sincere gratitude. There grace and tender mercy has renewed me every day, all the way on the journey throughout my life. On a personal note, my deep and sincere gratitude to my father Narasimhamurthy, My mom Sudha, my loving brother Rakesh, My in-laws Annapurnama, Doddaramaiah and Venugopal for their continuous unparalleled love, help, support, blessings, whose prayers were my strength to go ahead with each of the endeavour I had undertaken, whose love and affection have no equivalent and as an everlasting source of inspiration to me. It is wise to remain indebted to them rather than to go in search of words.

My heartfelt thanks to my family members Anatha padmanab, Lalitha, Lakshmi kanth, Kanthamma, Krishna murthy, Rajeshwari and my cousins Kashyap, Shilpa, Champa, Chethan, Kiran, Nachi, and our cuteee pies Chiru, Mansi, Darshan who constantly cheered and encouraged me and lead the helping hand when it was most needed.

I avail this moment to specially thank my husband Jaipal who was always there when I really needed. Thank you doesn't seem sufficient but it is said with appreciation

and love for support, encouragement, care, understanding and for everything. My special special thanks to my sweetu Viyaan you are the god blessings to me.

I gratefully acknowledge to my lab members Parvathi, Sujitha, Dhanya, Sapna, Ashwini, Sajeevan, Rakshitha, Shiva, Madhura, Ugraiha, Nagarjun, Sashi, Supriya, for their wishes, encouragement, unquantifiable love and affection. It has been a worthy privilege for me to have such wonderful lab mates, thanks for being there for me whenever I was in need. Thanks buddies.

I also have been highly fortunate in having many affectionate friends, Seniors and Juniors Manasa, Sangeetha, Asha, Vinutha, Sheelakka, Vanitha, punitha, Nandini, Basvarajeshwari, Pavithra, Sheela, Prathibha, Preethi, Nisarga, Appana, Sashi, Rashmi, Namratha, Shruthi, Pallavi, Gouspak who always stood with me and shared my anxiety, tensions, happy and sad moments without whom life would have been miserable.

On this occasion I whole heartedly thank helpers at GKVK field and lab assistants for the help they rendered for the smooth conduct of the field experiment. I am profoundly thankful to other seniors, junior friends and non-teaching staff of our department for their kind cooperation and help during my research.

I would like to acknowledge the financial support from Department of Biotechnology, HUB programme and PURSE Programme, to support my Ph.D. programme.

Any omission in this brief acknowledgement does not mean lack of gratitude.

*Bengaluru
September, 2017*

SWETHA, T.N.

CHARACTERIZATION OF GROUNDNUT TRANSGENIC PLANTS CO-EXPRESSING THREE REGULATORY GENES TO IMPROVE DROUGHT RESISTANCE

SWETHA T.N.

THESIS ABSTRACT

Adaptation of groundnut crop under rain fed conditions can be achieved by improving suitable plant traits such as efficient water mining and cellular level tolerance (CLT) mechanisms. Pyramiding these drought adaptive traits by simultaneous expression of genes regulating root growth and sustaining cell metabolism by maintaining protein turnover has phenomenal relevance in improving stress. From this context, Groundnut transgenic plants expressing Alfin1, a root growth-associated transcription factor gene, heat-shock factor (PgHSF4) and PDH45 involved in protein turnover and protection have been developed. Emphasis in the present study is to identify superior promising events that possess higher root growth, tolerance and productivity. The transgenic plants showed more root growth at different developmental stages under both stress and non-stress conditions. Further, the transgenic plants exposed to diverse stresses like salinity, DTT and moisture stress, showed significantly superior performance compared to wild type. The productivity of transgenic lines was more compared to wild type under both stress and non-stress conditions. Based on stress tolerance with improved root growth and productivity eight promising lines were identified. Further, to decipher the molecular mechanism associated with improved root growth and tolerance the transcriptome, proteome and metabolome analysis was done in wild type and transgenic plant. From the transcriptome data it is evident that many root related genes and several heat shock genes were up regulated in transgenics under stress condition. In proteomics data, several root related and CLT associated proteins were up regulated. The metabolic profiling is integrated with proteomic and transcriptome data which provided basic leads in identifying key pathways contributing for improved adaptation, tolerance and productivity under stress condition. The data clearly demonstrates that over-expression of two or three regulatory genes improve drought adaptation by improving water relation and CLT mechanisms.

September, 2017

Department of Crop Physiology
UAS, GKVK, Bengaluru

(P. Chandrashekar Reddy)
Chairman

ಶೇಂಗಾ ಕುಲಾಂತರಿ ಸಸ್ಯಗಳ ಬರ ನಿರೋಧಕತೆಯ ಸುಧಾರಣೆಯಲ್ಲಿ ಮೂರು ನಿಯಂತ್ರಿತ ಧಾತುಗಳ ಗುಣಲಕ್ಷಣ

ಶ್ವೇತ ಟಿ. ಎನ್

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ಮಳೆ ಆಶ್ರಿತ ಶೇಂಗಾ ಬೆಳೆವಣಿಗೆಯಲ್ಲಿ ಸಸ್ಯಗಳು ಅನೇಕ ಸೂಕ್ಷ್ಮ ಲಕ್ಷಣಗಳಾದ ಸುಧಾರಿತ ನೀರಿನ ನಿರ್ವಹಣೆ ಮತ್ತು ಕೋಶ ಮಟ್ಟದ ಸಹಿಷ್ಣು ಕಾರ್ಯವಿಧಾನಗಳನ್ನು ಪರಿಣಾಮಕಾರಿಯಾಗಿ ಅಳವಡಿಸಿಕೊಂಡಿರುತ್ತದೆ. ಮೇಲಿನ ಗುಣಲಕ್ಷಣಗಳನ್ನು ಅಳವಡಿಸಿಕೊಂಡು ಪ್ರೋಟೀನಿನ ವಹಿವಾಟನ್ನು ನಿರ್ವಹಿಸುವುದರ ಮೂಲಕ ಬೇರಿನ ಬೆಳೆವಣಿಗೆ ಮತ್ತು ಜೀವಕೋಶದ ಚಯಾಪಚಯ ಕ್ರಿಯೆಯನ್ನು ನಿಯಂತ್ರಿಸುವ ಧಾತುಗಳ ಏಕಕಾಲಿಕ ಅಭಿವ್ಯಕ್ತಿಯಿಂದ ಒತ್ತಡವನ್ನು ಸುಧಾರಿಸುವಲ್ಲಿ ಅದ್ಭುತ ಪ್ರಸ್ತುತತೆಯನ್ನು ಹೊಂದಿದೆ. ಈ ಹಿನ್ನೆಲೆಯಲ್ಲಿ, ಅಲ್ ಫೈ ಎನ್ 1, ಬೇರಿನ-ಬೆಳೆವಣಿಗೆಗೆ-ಸಂಬಂಧಿಸಿದ ಟ್ರಾನ್ಸ್ಕ್ರಿಪ್ಟ್ ಫ್ಯಾಕ್ಟರ್ ನ ಧಾತು, ಹೀಟ್-ಶಾಕ್ ಫ್ಯಾಕ್ಟರ್ (ಪಿಜಿಎಚ್‌ಎಸ್‌ಎಫ್ 4) ಮತ್ತು ಪಿಡಿಹೆಚ್ 45, ಪ್ರೋಟೀನ್ ವಹಿವಾಟು ಮತ್ತು ರಕ್ಷಣೆಯಲ್ಲಿ ತೊಡಗಿರುವ ಶೇಂಗಾ ಕುಲಾಂತರಿ ಸಸ್ಯಗಳನ್ನು ಅಭಿವೃದ್ಧಿಪಡಿಸಲಾಗಿದೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದ ಮಹತ್ವವು ಹೆಚ್ಚಿನ ಬೇರು ಬೆಳೆವಣಿಗೆ, ಬರ ಸಹಿಷ್ಣುತೆ ಮತ್ತು ಉತ್ಪಾದಕತೆಯನ್ನು ಹೊಂದಿರುವ ಉನ್ನತ ಭರವಸೆಯ ಸಸ್ಯಗಳನ್ನು ಗುರುತಿಸುವುದು. ಕುಲಾಂತರಿ ಸಸ್ಯಗಳ ವಿವಿಧ ಬೆಳೆವಣಿಗೆಯ ಹಂತಗಳಲ್ಲಿ ಒತ್ತಡ ಮತ್ತು ಒತ್ತಡವಿರದ ಪರಿಸ್ಥಿತಿಗಳೆರಡರಲ್ಲೂ ಹೆಚ್ಚಿನ ಬೇರಿನ ಬೆಳೆವಣಿಗೆಯನ್ನು ತೋರಿಸಿದೆ. ಇದಲ್ಲದೆ, ಲವಣಾಂಶ, ಡಿಟಿಟಿ ಮತ್ತು ತೇವಾಂಶದ ಒತ್ತಡದಂತಹ ವಿಭಿನ್ನ ಒತ್ತಡಗಳಿಗೆ ಒಡ್ಡಿದಾಗ ಕುಲಾಂತರಿ ಸಸ್ಯಗಳನ್ನು ಕಾಡು ಪ್ರಕಾರದೊಂದಿಗೆ ಹೋಲಿಸಿದರೆ ಗಮನಾರ್ಹವಾಗಿ ಉತ್ತಮ ಕಾರ್ಯನಿರ್ವಹಣೆಯನ್ನು ತೋರಿಸಿದೆ. ಕುಲಾಂತರಿ ಶೇಂಗಾ ಸಂತತಿಗಳ ಉತ್ಪಾದಕತೆಯು ಒತ್ತಡ ಮತ್ತು ಒತ್ತಡ ರಹಿತ ವಾತಾವರಣಗಳೆರಡರಲ್ಲಿಯೂ ಕಾಡು ರೀತಿ ಗಿಂತ ಹೆಚ್ಚಾಗಿರುವುದು ಕಂಡುಬಂದಿದೆ. ಒತ್ತಡ ಸಹಿಷ್ಣುತೆ, ಸುಧಾರಿತ ಬೇರಿನ ಬೆಳೆವಣಿಗೆ ಮತ್ತು ಉತ್ಪಾದಕತೆಯ ಆಧಾರದ ಮೇಲೆ ಎಂಟು ಭರವಸೆಯ ಸಂತತಿಗಳನ್ನು ಗುರುತಿಸಲಾಗಿದೆ. ಇದಲ್ಲದೆ, ಸುಧಾರಿತ ಬೇರಿನ ಬೆಳೆವಣಿಗೆ ಮತ್ತು ಸಹಿಷ್ಣುತೆಗೆ ಹೊಂದಿರುವ ಆಣ್ವಿಕ ಕಾರ್ಯವಿಧಾನವನ್ನು ತಿಳಿಯಲು ಟ್ರಾನ್ಸ್ಕ್ರಿಪ್ಟಮ್, ಪ್ರೋಟೀನಮ್ ಮತ್ತು ಮೆಟಾಬೊಲೊಮ್ ವಿಶ್ಲೇಷಣೆಯನ್ನು ಕಾಡು ಮಾದರಿ ಮತ್ತು ಕುಲಾಂತರಿ ಸಸ್ಯದಲ್ಲಿ ಮಾಡಲಾಗಿದೆ. ಟ್ರಾನ್ಸ್ಕ್ರಿಪ್ಟ್ ಮಾಹಿತಿಯಿಂದ ಒತ್ತಡದ ಸ್ಥಿತಿಯಲ್ಲಿ ಕುಲಾಂತರಿ ಸಂತತಿಯಲ್ಲಿ ಹಲವು ಬೇರಿಗೆ ಸಂಬಂಧಿತ ವಂಶವಾಹಿಗಳು ಮತ್ತು ಹಲವಾರು ಹೀಟ್ ಶಾಕ್ ವಂಶವಾಹಿಗಳು ಪ್ರಚೋದನೆಗೊಂಡಿರುವುದು ಸ್ಪಷ್ಟವಾಗಿದೆ. ಪ್ರೋಟೀನೋಮಿಕ್ಸ್ ದತ್ತಾಂಶದಲ್ಲಿ, ಹಲವು ಬೇರು ಸಂಬಂಧಿತ ಮತ್ತು ಕೋಶ ಮಟ್ಟದ ಸೈರಣೆಗೆ (CLT) ಸಂಬಂಧಿತ ಪ್ರೋಟೀನ್‌ಗಳು ಪ್ರಚೋದನೆಗೊಂಡಿವೆ. ಮೆಟಾಬಾಲಿಕ್ ಪ್ರೊಫೈಲಿಂಗ್, ಪ್ರೋಟೀನೋಮಿಕ್ ಮತ್ತು ಟ್ರಾನ್ಸ್ಕ್ರಿಪ್ಟಮ್ ಮಾಹಿತಿಯೊಂದಿಗೆ ಸಂಯೋಜಿಸಲ್ಪಟ್ಟಿರುತ್ತದೆ, ಒತ್ತಡ ಸ್ಥಿತಿಯಲ್ಲಿ ಸಸ್ಯಗಳ ಸುಧಾರಿತ ಅಳವಡಿಕೆ, ಒತ್ತಡ ಸಹಿಷ್ಣುತೆ ಮತ್ತು ಉತ್ಪಾದಕತೆಗೆ ಸಂಬಂಧಿಸಿದ ಮೂಲಭೂತ ದಾರಿಗಳನ್ನು ಗುರುತಿಸುವಲ್ಲಿ ಪಾತ್ರವಹಿಸುತ್ತದೆ. ಎರಡು ಅಥವಾ ಮೂರು ನಿಯಂತ್ರಕ ವಂಶವಾಹಿಗಳ ಹೆಚ್ಚಿನ ಅಭಿವ್ಯಕ್ತಿಯಿಂದ ನೀರಿನ ಅಳವಡಿಕೆ ಮತ್ತು ಕೋಶ ಮಟ್ಟದ ಸೈರಣೆಯ ಕಾರ್ಯವಿಧಾನಗಳನ್ನು ಸುಧಾರಿಸುವ ಮೂಲಕ ಬರ ಪರಿಸ್ಥಿತಿಗೆ ಹೊಂದಿಕೊಳ್ಳುವುದನ್ನು ಸುಧಾರಿಸುತ್ತದೆ ಎಂದು ಈ ಅಧ್ಯಯನದಿಂದ ಸ್ಪಷ್ಟವಾಗಿದೆ.

ಸೆಪ್ಟೆಂಬರ್, 2017

ಬೆಳೆ ಶರೀರ ಕ್ರಿಯಾಶಾಸ್ತ್ರ ವಿಭಾಗ

ಕೃ. ವಿ. ವಿ, ಜಿಕೆವಿಕೆ, ಬೆಂಗಳೂರು- ೫೬೦೦೬೫

(ಪಿ. ಚಂದ್ರಶೇಖರ ರೆಡ್ಡಿ)

ಮುಖ್ಯ ಸಲಹೆಗಾರರು

CONTENTS

CHAPTER	TITLE	PAGE No.
I	INTRODUCTION	1-6
II	REVIEW OF LITERATURE	7-50
III	MATERIALS AND METHODS	51-72
IV	EXPERIMENTAL RESULTS	73-127
V	DISCUSSION	128-149
VI	SUMMARY	150-159
VII	REFERENCES	160-198
	APPENDICES	

LIST OF TABLES

Table No.	Title	Page No.
1.	Genes / transcription factor associated with root growth and development	13
2.	List of biochemically active DNA helicases from plant cells	29
3.	Five major classes of plant Hsps/molecular chaperones and their subfamilies	33
4.	Validated HSF genes associated with stress tolerance	37
5.	Proteomic studies in different subcellular compartments in plants	42
6.	Root length of wild type and transgenic seedlings under salinity stress	79
7.	Productivity analysis of transgenic and wild type plants under contained managed drought environment (MDE)	88-89
8.	Net assimilation rate (NAR) and crop growth rate (CGR) of wild type and transgenic lines under both control and stress conditions	90
9.	Mean yield and biomass of selected transgenic lines grown in contained field conditions	91-92
10.	Mean pod yield of managed drought environment experiment and contained field experiment	92
11.	Mean yield and biomass of selected transgenic lines grown in contained field conditions at T6 generation	96
12.	Statistics of Denovo transcriptome Assembly of WT and Transgenic groundnut plants	100
13.	List of up-regulated and down-regulated genes in transgenic plants when compared to wild type plants	101-102
14.	Few Genes that are up or down regulated in transgenics under stress and control conditions	102
15.	List of up-regulated and down-regulated genes in transgenic plants when compared to wild type plants using <i>Medicago truncatula</i> as the reference genome	102-103

Table No.	Title	Page No.
16.	List of up regulated genes in transgenics when compared to wild type which are involved in cellular level tolerance	110-111
17.	List of transporters, transcription factors, receptors and hormones up regulated in transgenics when compared to wild type	112-113
18.	List of up-regulated and down-regulated proteins in transgenic plants when compared to wild type plants using <i>Medicago truncatula</i> as the reference genome	115
19.	List of up regulated proteins in transgenics compared to wild type that are involved in cellular level tolerance mechanisms under stress conditions	119
20.	List of standards used for metabolite extraction	121-122
21.	List of constitutively expressed metabolites in transgenics under control condition which are mapped against KEGG pathway	123
22.	List of constitutively expressed metabolites in transgenics under stress condition which are mapped against KEGG pathway	124
23.	Classification of metabolites based on their function in transgenics compared to wild type under control condition	125
24.	Classification of metabolites based on their function in transgenics compared to wild type under stress condition	125
25.	List of up regulated and down regulated transcripts, proteins and metabolites from transcriptomics, proteomics and metabolomics data	127

LIST OF FIGURES

Figure No.	Title	Between Pages
1	Schematic representation of plant traits/mechanisms involved in drought adaptation	10
2	Mechanism of stress tolerance by a helicase	28
3	Suggested cross-talk between the heat shock protein (Hsp)/chaperone network and other stress-response mechanisms	35
4	General structural and regulatory features of HSFs	37
5	Schematic representation of destination vector pKM12GW after LR reaction having different genes	51-52
6	Flow chart depicting generation wise analysis of groundnut transgenics expressing multigene construct	76-77
7	Stress response of groundnut transgenic plants on exposure to mid-season moisture stress	77-78
8	Response of multigene expressing transgenic seedlings to salinity stress	78-79
9	Response of wild type and transgenic lines for salinity stress in hydroponics system	80-81
10	Percent reduction in root length and biomass of wild type and transgenic lines for salinity stress in hydroponics system	80-81
11	Correlation analysis between physiological parameters	80-81
12	Correlation analysis between percent reduction in root length over control in salinity stress at seedling stage experiment and Hydroponics experiment	80-81
13	Growth parameters of wild type and transgenic lines in pots under normal condition	81-82
14	Yield parameters of wild type and transgenic plants under normal condition	81-82
15	Correlation analysis between yield parameters	81-82

Figure No.	Title	Between Pages
16	Correlation analysis between experiments	81-82
17	Z-distribution analysis between experiments	82-83
18	Response of multigene expressing transgenic seedlings to NaCl stress	84-85
19	Percent reduction in root length of wild type and transgenic lines for salinity induction response	84-85
20	Moisture stress recovery response of transgenic and wild type	85-86
21	Response of transgenic lines under moisture stress conditions	85-86
22	Percent reduction in root length and biomass of wild type and transgenic lines for moisture stress	85-86
23	Estimation of biochemical parameters for wild type and transgenic seedlings	86-87
24	Percent reduction in biochemical parameters of wild type and transgenic lines	86-87
25	Total protein content in wild type and transgenic plant under control and stress condition	86-87
26	Correlation analysis between root length and biomass	86-87
27	Correlation analysis between MDA, root length and biomass	86-87
28	Z-distribution analysis between MDA, root length and biomass	86-87
29	Yield parameters of wild type and transgenic plants under Stress and control condition	87-88
30	Percent reduction in pod yield over control in wild type and transgenic lines	88-89
31	Correlation analysis between pod yield and biomass	88-89
32	Growth parameters of wild type and transgenic plants at 50 days after sowing	88-89

Figure No.	Title	Between Pages
33	Leaf are parameters of wild type and transgenic plants at 50 days after sowing	89-90
34	Correlation analysis between various physiological parameters under control condition at 50 DAS	89-90
35	Gas exchange parameters of wild type and transgenic plants under contained field condition	89-90
36	Growth parameters of wild type and transgenic plants under contained field condition at 80 DAS	89-90
37	Leaf are parameters of wild type and transgenic plants at 80 days after sowing	89-90
38	Gas exchange parameters of wild type and transgenic plants under contained field condition at 80 DAS	91-92
39	Correlation analysis between physiological parameters at 80 DAS	91-92
40	Yield parameters of wild type and transgenic plants at harvest	91-92
41	Percent reduction in Pod yield over control in wild type and transgenic lines	91-92
42	Yield parameters of wild type and transgenic plants in T6 generation	95-96
43	Salinity stress response of wild type and transgenic seedlings at whole plant level	97-98
44	Response of wild type and transgenic seedlings to salinity stress at whole plant level	97-98
45	Response of wild type and transgenic to DTT treatment	97-98
46	Comparative assessment of root length in transgenic expressing individual genes and multigenes	98-99
47	Comparative assessment of salinity stress in transgenic expressing single and multi-gene	98-99
48	Differentially expressed transcripts in wild type and transgenic plants under control and moisture stress conditions	101-102

Figure No.	Title	Between Pages
49	Classification of upregulated genes in transgenics using Gene Ontology under control condition	102-103
50	Biological functions of upregulated transcripts representing different classes	103-104
51	Molecular functions of the up regulated genes in transgenic plants under control conditions	104-105
52	Cell function overview in transgenic control v/s wild type control using <i>Medicago truncatula</i> as a reference genome	105-106
53	Classification of upregulated genes in transgenics using Gene Ontology under stress	107-108
54	Biological functions of up regulated transcripts representing different classes in stress conditions	107-108
55	Molecular functions of the up regulated genes in transgenic plants under control conditions	107-108
56	Cell function overview in transgenic stress v/s wild type stress using <i>Medicago truncatula</i> as a reference genome	108-109
57	Venn diagram showing common up regulated and down regulated proteins in transgenic control and stress condition.	115
58	Overview of the proteins that are associated with cell function in transgenic plants compared to wild type under non-stress condition using proteome data	116-117
59	Overview of the proteins that are associated with cell function in transgenic under stress compared to wild type	118-119
60	Solvent concentration standardization for metabolite extraction	121-122
61	Constitutively expressed metabolites in transgenics compared to wild type under control condition	121-122
62	Constitutively expressed metabolites in transgenics compared to wild type under stress condition	121-122

Figure No.	Title	Between Pages
63	MS/MS spectra of spiked standard of Jasmonone compared with sample fragmentation pattern in groundnut transgenic under control condition	121-122
64	MS/MS spectra of spiked standard of 12-OPDA compared with sample fragmentation pattern in groundnut transgenic under stress condition	121-122
65	MS/MS spectra of spiked standard of Tyramine compared with sample fragmentation pattern in groundnut transgenic under stress condition	121-122
66	Mean percent reduction in root length over control in wild type and transgenic lines	134-135
67	Mean percent reduction in pod yield over control	140-141
68	Integration of Omics data sets that respective transcript, proteome, metabolome associated with different drought adaptive mechanisms	145-146

LIST OF PLATES

Plate No.	Title	Between Pages
1	Molecular confirmation of T ₄ transgenic plants by PCR of few representative plants by using different primer combinations	83-84
2	Molecular confirmation of T ₅ transgenic plants by using different primer combinations	88-89
3	Molecular confirmation of promising T ₅ transgenic plants by PCR	93-94
4	Southern blot and expression analysis	93-94

Introduction

I. INTRODUCTION

Plants are continuously exposed to unfavourable environmental abiotic stress factors that affect crop yields. Abiotic stresses include decreased water availability, extreme temperatures (heating or freezing), decreased availability of soil nutrients and/or excess of toxic ions, excess of light and increased hardness of drying soil that inhibit root growth. Among all these factors water availability is the major limitation to plant productivity. Hence drought is the most serious problem for global agriculture, approximately affecting 40% of the world's land area. Even worse, global climate change is predicted to lead to extreme temperatures and prolonged drought in some parts of the world, which will have a dramatic impact on crop growth and productivity (Trenberth *et al.*, 2014). Apart from drought, salinity has emerged as another major factor limiting productivity of crops. It is estimated that approximately 7% of the world's land area is affected by salinity (Rozema and Flowers 2008). Globally drought- and salt-affected soils cover areas of 60 and 10.5 million km², respectively. In India 94 mha of area comes under drought scenario with up to 55% of yield losses due to high water stress (Gupta *et al.*, 2011). The loss of farmable land due to either salinization or drought has posed a major challenge for maintaining world food supplies for the growing population (Xuekun *et al.*, 2014).

Groundnut (*Arachis hypogaea* L.) is an annual legume, third most important oilseed crop of the world. It is an important human food crop because of its high protein and oil content. Groundnut contains on an average 47-48 per cent of edible oil and 25-26 per cent of protein and is a rich source of calcium, iron and vitamin B complex like thiamine, riboflavin, niacin and vitamin A. Therefore, it plays a pivotal role in the oilseed economy of India. The world annual groundnut production is around 35.9 million tons from the 25.2 million hectares of production area (Shinde and Laware 2014). It is largely cultivated as a rain-fed crop, and hence the crop is exposed to intermittent moisture stress.

Water deficit-induced desiccation results in loss of turgor affecting leaf expansion and hence decreases net carbon gain and pod yields. Decline in water status considerably

affects photosystem mediated electron transport mechanisms besides reduced synthesis of photosynthetic pigments. This results in declined light harvesting and photochemical quenching of absorbed light energy that affects photosynthetic rates (Rucker *et al.*, 1995). Under decreased water potential, plants experience oxidative stress triggered by the generation of cytotoxic reactive oxygen species (ROS) leading to lipid peroxidation and production of diverse toxic secondary radicals and reactive carbonyl compounds. ROS further result in oxidative damage to proteins and DNA (Apel and Hirt, 2004). Moisture stress also affects cell cycle genes, protein structure, stability and protein turnover.

Groundnut plants have evolved diverse adaptive strategies to cope with water deficit conditions (Nageswara *et al.*, 1985; Nautiyal *et al.*, 1995) by altering the phenology, maintaining positive turgor and finally maintain cell metabolic activities under decreased cell water potential. Cellular responses to stress include adjustments of the membrane system, modifications of the cell wall architecture, and changes in cell cycle and cell division (Klutz, 2005). In addition, plants alter metabolism in various ways, including production of compatible solutes (proline, raffinose, and glycine betaine) that are able to stabilize proteins and cellular structures and maintain cell turgor by osmotic adjustment, and redox metabolism to remove excess levels of ROS and re-establish the cellular redox balance (Bartels and Sunkar, 2005; Valliyodan and Nguyen, 2006). Improved water relations has been one of the adaptive strategies to sustain growth or improved recovery growth upon stress alleviation. Positive turgor under water limited conditions is achieved by stomatal regulation, increased impermeability of cuticles, improving water use efficiency and more importantly improving water acquisition by roots. Therefore two important mechanisms emerging as relevant are a) improving water relations and b) improving cellular level tolerance.

It is important to maintain plant water relations to sustain the growth under moisture stress. The plant mechanisms associated with traits such as better water mining by superior root architecture, water use efficiency (WUE) and water conservation traits have relevance in avoiding drought stress (Bartels and Sunkar, 2005).

Root traits have been claimed to be critical for increasing yield under soil-related stresses (Serraj *et al.*, 2004). It has been hypothesized that coarse roots have a direct role in drought tolerance because larger diameter roots are related to penetration ability (Clark *et al.*, 2008) and branching (Ingram *et al.*, 1994), and they have greater xylem vessel radii and lower axial resistance to water flux (Yambao *et al.*, 1992). Functional role of several genes in root growth and development has been demonstrated by mutant and overexpression studies (Hodge *et al.*, 2009; Ivashuta *et al.*, 2005; Winicov, 2000). The regulatory genes such as aberrant lateral root formation-4 (ALF4), SOLITARY-ROOT (SLR) and NAC1 (NAM, ATAF1, 2, CUC2) have been shown to play a role in regulating root growth and development (Celenza *et al.*, 1995; DiDonato *et al.*, 2004; Fukaki *et al.*, 2002; Xie *et al.*, 2002). Alfin1 is a PHD finger protein with Cys4 and HisCys3 finger structures that have DNA-binding motif suggesting that they have a role in transcriptional regulation (Bastola *et al.*, 1998). The overexpression of Alfin1 in alfalfa showed significant improvement in root growth (Winicov, 2000). Studies in tobacco also demonstrated improved root growth in Alfin1 overexpressing tobacco plants (Nethra, 2010).

By improving root growth water use efficiency can be improved because it is an important trait to sustain growth under water limited conditions. Water-use efficiency describes a plant's photosynthetic production rate relative to the rate at which it transpires water to the atmosphere. Under water deficit conditions plants maximize WUE by reducing transpiration rate. WUE has greater impact on growth when the differences in WUE are independent of transpiration rate (Cabuslay *et al.*, 2002) and hence a relevant trait to improve plant water relations.

Another important strategy to conserve leaf moisture status is by stress induced stomatal closure and by increasing cuticular waxes. Attempts have been made to exploit the variability in stomatal factors with stomatal index/frequency besides stomatal conductance. However, leaf moisture conservation by increased stomatal resistance often affects carbon gain and hence in recent years the emphasis is on exploiting the variability in waxes. Waxes reflect radiation and hence can significantly reduce the heat load on leaves which in turn leads to the maintenance of lower VPD and hence the plants would

have significantly reduced transpiration at a given stomatal conductance (Prathiba 2012; Mamrutha *et al.*, 2010).

Apart from maintaining superior water relations, another important strategy is enhancing cellular level tolerance mechanisms (CLT) under low tissue water potentials. Often these are referred as acquired tolerance mechanisms achieved by altered gene expression. The relevance of some of the CLT mechanisms like scavenging reactive oxygen species (ROS), osmotic homeostasis, maintaining the stability of the membrane and macromolecules, protein synthesis and turnover have been well documented (Vemanna *et al.*, 2015; Valliyodan and Nguyen, 2006; Krasenski and Jonak, 2012). In this context, one of the important CLT processes which are very important under stress is protein turnover and stability (Foss *et al.*, 2011). Protein turnover is a balance between protein synthesis and degradation which involves different level starting from DNA synthesis, transcription, pre-mRNA processing, translation, protein folding, and protein maturation to finally functional protein. Each level is crucial and decides the protein turnover. Among them, mRNA processing, translation and protein stability are important and several studies have shown relevance of these process under stress (Lu *et al.*, 2009; Sonenberg and Hinnebusch, 2009; Wang *et al.*, 2004).

Although transcription and protein synthesis function in a coordinated manner, it has been shown that under stress, such an association significantly diminishes (Foss *et al.*, 2011). Stress induced secondary mRNA structures act as obstacles for mRNA processing and translation leading to production of a truncated non-functional protein (Amar *et al.*, 2011). The extent and amplitude of attaining secondary structure in mRNA is high under stress conditions. Yiliang *et al.*, (2014) have studied Arabidopsis genome wide profiling of RNA secondary structure under different stress conditions and has shown that there is increase in complex secondary structure formation which inhibits the translation. A few of the genes that regulate these processes have been shown to improve stress tolerance in plants. In this regard, RNA helicases play an important role in translation process by unwinding the energetically stable mRNA secondary structure. Overexpression of a salt stress-responsive Pea DNA helicase (PDH45), which is a homologue of eukaryotic

translation initiation factor eIF-4A, conferred salinity tolerance in tobacco plants (Sanan-Mishra *et al.*, 2005).

Further, stability of synthesized proteins is also crucial which gets affected during process of folding to form native and active structure under stress which give rise to protein misfolding and aggregation formation and finally degraded (Sadana and Dinh, 2001). Folded proteins are generally less prone to aggregation and degradation but partially unfolded or intrinsically disordered regions of proteins can confer functional advantages, as they allow efficient interaction with binding partners and provide a mechanism for the regulation of cellular processes. Several heat-shock protein (HSP) chaperones have been reported to increase protein stability under stress (Liu *et al.*, 2009) and improve adaptation of cells with reduced turgor. Stress-induced transcription factors such as heat-shock factors (HSFs) play a critical role in activating HSPs (Mittal *et al.*, 2011; Swindell *et al.*, 2007). Several reports clearly demonstrated improved tolerance of transgenic plants to diverse abiotic stress by overexpressing several HSFs (Zhu *et al.*, 2006). Overexpression of AtHSFA1 gene enhanced thermo tolerance in Arabidopsis, soya bean and tomato (Zhu *et al.*, 2006). Expression analysis of *Pennisetum glaucum* heat-shock factor (*PgHSF4*) in *P. glaucum* showed maximum increase in expression of *PgHSF4* in response to heat stress within 30 min of exposure (Reddy *et al.*, 2009).

Genetic enhancement of these traits can be achieved by either adopting conventional breeding approaches or by adopting molecular breeding and transgenic approach. Several QTLs and markers have been identified for trait introgression and would perform better under water limited conditions. However, progress made in identifying several QTLs for acquired traits is not encouraging. But fairly good progress has been made in identifying the genes regulating acquired tolerance mechanisms. Several stress specific cDNA libraries, ESTs, micro array data provided leads in this aspect. Significant progress has also been made in identifying stress responsive genes for plant adaptation to stress.

Several mechanisms involved in stress tolerance can be improved by bringing together specific traits. For field level tolerance we require to improve water mining and

cellular level tolerance. With this background groundnut (*Arachis hypogea*) transgenic plants co-expressing PDH45, HSF4 and Alfin1 was developed earlier in the department of crop physiology, UASB. The major emphasis in this study was comprehensive characterization of transgenic lines to assess root growth, stress tolerance and productivity under stress and non-stress conditions. Ultimate aim was to identify most promising events with improved productivity and drought tolerance. The other objective was to generate transcriptome and proteomics data to provide insight into the changes brought about by these transgenes in improving stress tolerance and productivity.

With this background, specific objectives of the present study are;

1. Evaluation of T4 transgenic lines to identify phenotypically superior event co-expressing HSF4, PDH 45 and Alfin1.
2. Comparative analysis of multigene transgenics with single gene transgenics.
3. To examine molecular basis of differential response of transgenic plants co-expressing HSF4, PDH 45 and Alfin1.

Review of Literature

II. REVIEW OF LITERATURE

Groundnut (*Arachis hypogaea* L.) (Leguminosae) is one of the most important legumes in India, China, the United States, Australia, South America, Central America and Africa. One-third of global peanut production is used for food, and the remaining is used for oil production. Peanut is third most important source of vegetable protein, besides serving as a dietary source of vitamin E and phytosterols (FAO, 2010). Globally, peanut is grown on an average of ~21 million hectares (ha) that produces ~33 million metric tons. Two-thirds of the world's peanut is produced by India and China. In developing countries, peanut is largely cultivated as a rain-fed crop, and hence, the crop is exposed to intermittent moisture stress. Water deficit-induced desiccation results in loss of turgor affecting leaf expansion and hence decreases net carbon gain and pod yields. Groundnut plants have evolved diverse adaptive strategies to cope with water deficit conditions (Vemanna *et al.*, 2015). Intermittent moisture stress is a typical stress the crop faces in rain-fed agriculture scenario. Water is the most overriding constraint in agriculture scenario.

Only one-third of the water that annually runs to the sea is accessible to humans. Of this, more than half is already being appropriated and used. This proportion might not seem so much, but demand will double in thirty years. And much of what is available is degraded by eroded silt, sewage, industrial pollution, chemicals, excess nutrients, and plagues of algae. Per capita availability of good, potable water is diminishing in all developed and developing countries. - **Marq de Villiers, *Water*, 2000**

Kramer and Boyer (1995) have summarized four functions of water in plants. Water is arguably the single most important constituent of a plant, comprising more than 90% of the fresh weight of most herbaceous plants. Water is also a solvent with unique biophysical properties – such as high heat of vaporization, high dielectric constant and high surface tension. These unique properties allow water to remain liquid over a broad temperature range and solvate a wide variety of ions, minerals and molecules. In addition to being the solvent, water acts as a reactant in a number of critical biochemical reactions

including serving as the primary electron donor in photosynthesis. Finally, from a physiological perspective, water is the key component in maintaining cell turgor.

Water availability is one of the major limitations to plant productivity (Boyer, 1982), and is one of the major factors regulating the distribution of plant species. Agricultural regions affected by drought can experience yield loss up to 50% or more. Developing crops that are more tolerant to water deficits, while maintaining productivity, will become a critical requirement for enhancing agriculture in the twenty-first century. Understanding how plant cells tolerate water loss is a vital prerequisite for developing strategies that can impact agricultural crop productivity and survival under these conditions of decreasing water availability.

So from this context it is important to improve adaptation of groundnut under aerobic condition by improving water relations and cellular level tolerance mechanisms.

2.1 Plant responses to abiotic stress

Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels. Plant responses to abiotic stresses are dynamic and complex they are both elastic (reversible) and plastic (irreversible). The plant responses to stress are dependent on the tissue or organ affected by the stress. Water deficit inhibits plant growth by reducing water availability into the expanding cells, and alters enzymatically the rheological properties of the cell wall; for example, by the activity of ROS (reactive oxygen species) on cell wall enzymes. In addition, water deficit alters the cell wall non-enzymatically; for example, by the interaction of pectate and calcium. Furthermore, water conductance to the expanding cells is affected by aquaporin activity and xylem embolism, the initial growth inhibition by water deficit occurs prior to any inhibition of photosynthesis or respiration. Water deficit induced desiccation results in loss of turgor affecting the cell expansion and cell size. Leaf area expansion depends on leaf turgor, temperature, and assimilating supply for growth. Water deficits reduce the number of leaves per plant, individual leaf size and leaf longevity. Drought-induced reduction in leaf area is ascribed to suppression of leaf expansion through reduction in photosynthesis due

to impaired photosynthetic machinery (Rucker *et al.*, 1995). Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate. Drought stress results in decreased chlorophyll content due to pigment photo-oxidation and chlorophyll degradation due to loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (Kaiser *et al.*, 1981). Under drought, ROS levels such as O_2^- , H_2O_2 and $\cdot OH$ radicals increase dramatically which attack the membrane lipids and increases lipid peroxidation leading to decreased membrane integrity. ROS further result in oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). One of the earliest metabolic responses to abiotic stresses and the inhibition of growth is the inhibition of protein synthesis and an increase in protein folding and processing. Energy metabolism is affected as the stress becomes more severe (e.g. sugars, lipids and photosynthesis). Thus, there are gradual and complex changes in metabolism in response to stress (Cramer *et al.*, 2011). Protective mechanisms must be in place to limit damage to the protoplasm. Repair mechanisms must be able to repair stress-induced damage. However, no single group of plants employs each adaptation. And most certainly they do not use the same genes to mediate each adaptation. The plant molecular responses to abiotic stresses involve interactions and crosstalk with many molecular pathways. Systems biology and omics approaches have been used to elucidate some of the key regulatory pathways in plant responses to abiotic stress. Usually one kind of stress is accompanied or followed by another stress. For example, heat stress is accompanied by drought stress due to physical loss of water, and cold stress is followed by drought stress due to physiological unavailability of water. Many stress adaptive mechanisms are validated for their relevance under drought conditions. These mechanisms can be broadly classified under two categories. Plant traits and mechanisms, which are related to water relations of plants i.e. Plants maintaining positive turgor when exposed to water deficit conditions. The important traits are associated with water mining such as roots and WUE, which reflects the carbon gain for unit transpiration. The root is the most crucial organ for meeting transpiration demand at a reasonably high leaf water status, on the condition that water is available in the rooting zone. High root length density increases the number of contact points between root and

soil. White and Kirkegaard (2010) showed that root contact as driven by extensive root branching and long root hairs is a prime determinant of moisture extraction from dry soil. Phenomenal literature supports several root characters has relevance for stress adaptation and many reports have shown that genotypes with higher roots maintained the leaf temperature cooler for longer period under stress. Plants have evolved various adaptive mechanisms to maintain positive turgor which mainly involves improving water relations and cellular level tolerance.

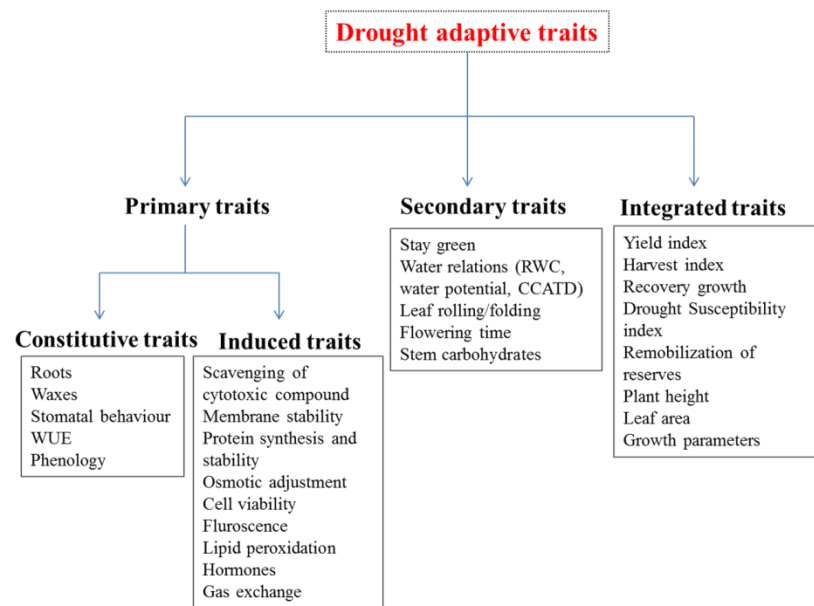


Fig. 1: Schematic representation of plant traits/mechanisms involved in drought adaptation

2.1.1 Water relations

Plant water relations are dominated by cell water relations because most of the water occurs in cells, chiefly in the vacuoles. Mature plant cells are characterized by their relatively rigid walls and large central vacuoles, both of which play important roles in cell water relations.

Water mining: The development of higher plants is dependent on the continuous mining of water and essential mineral nutrients from the soil by a growing root system. Plant root systems are built up by the growth of individual roots and their elongation is the result of cell division and cell expansion (Michal *et al.*, 2004). The plant mechanisms associated

with traits such as better water mining by superior root architecture, water use efficiency (WUE) and water conservation traits have relevance in avoiding drought stress. Many studies have shown that genotypes with superior root system maintained the leaf turgor and cooler leaf temperature for longer periods under continuous soil water-depleted conditions.

The relative water content of a leaf is a measurement of its hydration status (actual water content) relative to its maximal water holding capacity at full turgidity. RWC provides a measurement of the 'water deficit' of the leaf, and may indicate a degree of stress expressed under drought and heat stress. A decrease in the relative water content (RWC) in response to drought stress has been noted in wide variety of plants (Nayyar and Gupta, 2006) that when leaves are subjected to drought, leaves exhibit large reductions in RWC and water potential. Exposure of plants to drought stress substantially decreased the leaf water potential, relative water content and transpiration rate with a concomitant increase in leaf temperature (Siddique *et al.*, 2001). Although components of plant water relations are affected by reduced availability of water, stomatal opening and closing is more strongly affected.

Guard cells are specialized cells in the epidermis of leaves, stems and other organs that are used to control gas exchange. Stomata are induced to close, and leaf growth is reduced in order to limit the surface area from which water can be lost, and these changes occur very sensitively in response to changes in rhizospheric and aerial microclimatic conditions. An increase in xylem and bulk leaf abscisic acid (ABA) concentration is often associated with the drying of the soil around the root, or of the air around the shoot. The synthesis of ABA is stimulated by the dehydration of root and leaf cells and ABA can be translocated around the plant in response to environmental perturbation. Roots sensing a loss of soil moisture often transport more ABA to the shoot via the xylem vessels before the water status of the shoot becomes reduced (Wilkinson and Davies, 2008). Upon drought conditions, plants produce and accumulate more ABA that induces stomatal closure, thus conserving water. ABA serves as a primary chemical signal that induces stomatal closure through second messengers such as reactive oxygen species, nitric oxide, Ca^{2+} , followed by activation and inactivation of protein

kinases/phosphatases that further target the ion channels. Stomatal aperture also plays a role in leaf cooling through latent heat cooling.

Genes associated with roots for abiotic stress tolerance

The growth and development of crops depend on their roots to take up water and nutrient material from soil. The distribution of roots, particularly those that can penetrate deeper in the soil, plays a crucial role in determining the ability of plants to capture key resources such as water and mobile nutrients like nitrate. Root architecture therefore has a profound effect on the growth and yield of crop plants.

Overexpression of AUX1, TIR1, or NAC1 can promote lateral root development (Gray *et al.*, 1999; Xie *et al.*, 2000; Marchant *et al.*, 2002). Overexpression of SINAT5 produces fewer lateral roots, whereas overexpression of a dominant-negative C49S (Cys-49 to Ser) mutant of SINAT5 develops more lateral roots. The lateral root phenotypes correlate with the expression of NAC1 (Xie *et al.*, 2002). As a central regulator, auxin regulates cell division, expansion, and differentiation. It also functions in many other aspects of plant growth and development, such as promoting hypocotyl and stem elongation, mediating root and shoot gravitropism, maintaining apical dominance, and promoting vascular pattern formation (Thimann, 1977; Sachs, 1991; Estelle and Klee, 1994; Hobbie, 1998).

Therefore to see the relevance of root traits under water stress condition, attempts has been made to overexpress a few root trait related genes such as Alfin1, CAP2, CIPK, PIN1, CDPK, OsRAA1, OsORC3 in model plant Tobacco (*Nicotiana tabacum*) which has been studied earlier in other crop species.

The OsRAA1 (*Oryza sativa* Root Architecture Associated 1) encodes a 12.0-kD protein that has 58% homology to the AtFPF1 (Flowering Promoting Factor 1) in Arabidopsis. Constitutive expression of OsRAA1 under the control of maize (*Zea mays*) ubiquitin promoter resulted in phenotypes of reduced growth of primary root, increased number of adventitious roots and helix primary root, and delayed gravitropic response of roots in seedlings of rice (*Oryza sativa*). With overexpression of OsRAA1, initiation and

Table 1: Genes / transcription factor associated with root growth and development

Gene	Source	Study	Phenotype	References
CaCIPK	Chickpea	Over expression	Higher stress tolerance in rice and <i>Arabidopsis</i>	Tripathi <i>et al.</i> , (2009)
CaCAP2	Chickpea	Over-expression	Increased number of lateral roots in tobacco and salinity stress tolerance and <i>Arabidopsis</i>	Shukla <i>et al.</i> , (2006)
CaCaZF	Chickpea	Over-expression	CaZF promoting tolerance against dehydration, salinity and high temperature	Jain <i>et al.</i> , (2013)
Armadillo	<i>A thaliana</i>	Mutation/Over-expression study	Promote the lateral root development	Coates <i>et al.</i> , (2006)
Alfin 1	<i>Alfa alfa</i>	Over-expression study	Increase root growth under normal and saline condition	Winicov (2000)
AP37 (AP2)	Rice	Constitutive over expression	Enhanced drought resistance and grain yield under severe drought conditions	Oh <i>et al.</i> , (2009)
OsPIN1	<i>Oryza sativa</i>	Over-expression /Suppression study (RNAi)	Caused change in tiller no and R/S ratio. Adventitious root emergence & development was significantly inhibited	Xu <i>et al.</i> , (2005)
Crl1(Crown root less 1)	<i>Oryza sativa</i>	Mutation study	Decreased lateral root no, auxin insensitivity in lateral root formation and impaired root gravitropism	Inukai <i>et al.</i> , (2005)
OsRAA1 (Root architecture associated 1)	<i>Oryza sativa</i>	Constitutive expression under maize Ubiquitin Promoter/Over-expression studies	Reduced growth of primary root increased no of Adventitious roots, helix primary root & delayed gravitropic response. Initiation and growth of adventitious roots were more sensitive to the treatment of Auxin.	Lei, <i>et al.</i> , (2004)
RHD3 (root hair defective 3)	<i>A. thaliana</i>	Mutation study	Defective in root hair formation and RHD3 control epidermal cell file rotation, root skewing and waving on hard agar surfaces	Christen <i>et al.</i> , (2005)
AtFPF1(Flowering promoting factor1)	<i>A. thaliana</i>	Constitutive expression under Ubiquitin promoter.	AtFPF1 enhanced adventitious root formation but inhibited root growth in rice during the seedling stage.	Kania <i>et al.</i> , (1997)
NAC1 transcription factor	<i>A. thaliana</i>	Micro RNA mediated study	Reduces lateral root emergence and NAC1 transduces auxin signals for lateral root emergence. It acts downstream of TIR1 to transmit auxin signal to promote lateral root emergence	Guo <i>et al.</i> , (2005)

growth of adventitious root were more sensitive to treatment of auxin. OsRAA1 constitutive expression also caused longer leaves and sterile florets at the last stage of plant development (Lei *et al.*, 2004).

Oryza sativa Origin Recognition Complex subunit 3 (OsORC3) was strongly expressed in regions of active cell proliferation, including the primary root tip, stem base, lateral root primordium, emerged lateral root primordium, lateral root tip, young shoot, anther and ovary. OsORC3 knockdown plants lacked lateral roots and had a dwarf phenotype. OsORC3 plays a crucial role in the emergence of lateral root primordia (Chen *et al.*, 2013).

SHORT-ROOT (SHR) is a well-characterized regulator of radial patterning and indeterminacy of the *Arabidopsis* (*Arabidopsis thaliana*) primary root. The root growth behaviour of the *shr* mutant results from its primary root apical meristem failing to initiate cell division following germination. The inability of *shr* to reactivate mitotic activity in the root apical meristem is associated with the progressive reduction in the abundance of auxin efflux carriers, PIN-FORMED1 (PIN1), PIN2, PIN3, PIN4, and PIN7. However, SHR function is not restricted to the primary root but is also required for the initiation and patterning of lateral root primordia. In addition, SHR is necessary to maintain the indeterminate growth of lateral and anchor roots (Mikael *et al.*, 2011).

Phylogenetic analysis of 32 members of the PIN family, taken from across different species, showed that in terms of evolutionary relationship, OsPIN1 is closer to the PIN1 family than to the PIN2 family. OsPIN1 was expressed in the vascular tissues and root primordia in a manner similar to AtPIN1. Adventitious root emergence and development were significantly inhibited in the OsPIN1 RNA interference (RNAi) transgenic plants, which was similar to the phenotype of NPA (N-1naphthylphthalamic acid, an auxin transport inhibitor) treated wild type plants (Min *et al.*, 2005).

Alfin1

Alfin1 cDNA encodes a novel member of the zinc-finger family of proteins, and its modulation in NaCl tolerance makes it an interesting target for manipulation in plants.

It contains sequence information for adjacent Cys-4 and His/ Cys-3 zinc-finger domains that appear to bind adjacent G-rich triplet motifs in DNA (Bastola *et al.*, 1998). It also contains an acidic region characteristic of DNA-binding proteins that interact with other proteins (Kakidani and Ptashne, 1988) and therefore is likely to function as a transcription factor in plants. Alfin1 is expressed predominantly in roots, appears to be unique or a low-copy gene in the alfalfa genome, and shows conservation among such diverse plants as alfalfa, rice, and Arabidopsis (Winicov and Bastola, 1997). These characteristics, in addition to in vitro binding to promoter fragments of the root-specific MsPRP2 gene that is also NaCl inducible (Winicov and Deutch, 1994; Deutch and Winicov, 1995), suggested that it may have a significant function in plant-root gene expression and contribute to gene regulation in NaCl tolerance. Alfin1 overexpression in transgenic alfalfa (*Medicago sativa* L.) enhances expression of the salt-inducible MsPRP2 gene in roots, suggesting that *Alfin1* functions to regulate gene expression in roots (Winicov, 2000).

The PHD fingers in ING (inhibitor of growth) homologues (AtING) and Alfin1-like (AL) proteins, which are highly similar to those in human ING2 and bromodomain PHD finger transcription factor (BPTF), based on predicted tertiary structures. AtING and AL proteins are nuclear proteins that are expressed in various tissues of the Arabidopsis plant. ING and AL proteins are nuclear proteins that are involved in chromatin regulation by binding to H3K4me3/2, the active histone markers, in plants (Woo *et al.*, 2009).

The Alfin-like (AL) proteins in two *Arabidopsis* species (*A. lyrata* and *A. thaliana*) and a salt-tolerant close relative *Thellungiella halophila*. These AL-like proteins could be divided into four groups and the two known DUF3594 and PHD-finger domains had co-evolved within each group of genes, irrespective of species, due to gene duplication events in the common ancestor of all three species while gene loss was observed only in *T. halophila*. Functional analysis based on transgenic overexpression lines and T-DNA insertion mutants indicated that salt-stress-induced AtAL7 could play a negative role in salt tolerance of *A. thaliana*, suggesting that adaptive evolution occurred in the members of AL gene family (Yu *et al.*, 2013).

The Alfin-Like 6 (AL6) controls the transcription of a suite of genes critical for root hair elongation under low Pi conditions, suggesting a novel physiological function for an Alfin gene in Arabidopsis (Nulu *et al.*, 2013).

Water conservation: Water loss from leaves can be through the stomatal pore or the waxy cuticle. Plants exhibit diverse adaptive mechanisms to prevent water loss from leaf surfaces, such as leaf movement, leaf rolling, sunken stomata, efficient regulation of stomatal movement, reduction in leaf size, changes in leaf orientation and canopy architecture, and heavy cuticle depositions. It has often been postulated that heavy wax deposits of plant surfaces limit transpiration and improve water retention. Moreover, many plants possess the ability to respond to water-limited environments by increasing the deposition of epicuticular waxes, which likely increases the cuticle's ability to function as a hydrophobic barrier (Mamrutha *et al.*, 2010). Progress has been made in understanding the major steps of wax biosynthesis for plant growth and development. Most of the biosynthetic steps were revealed by identifying wax-related genes of the eceriferum (cer) and glossy (gl) mutants in Arabidopsis and maize, respectively (Kunst and Samuels, 2009). However, a few genes linking cuticular wax accumulation with drought stress have been reported. Overexpression of WXP1 and CER1 resulted in enhanced wax production and drought adaptability with decreased water loss and cuticle permeability (Zhang *et al.*, 2005; Bour *et al.*, 2011).

2.1.2 Cellular level tolerance

Crops grown under rainfed conditions, besides water mining, intrinsic cellular level tolerance (CLT) mechanisms are also important to sustain the cell metabolic activities. Cellular responses to stress include adjustments of the membrane system, modifications of the cell wall architecture, and changes in cell cycle and cell division. In addition, plants accumulate various compatible solutes such as proline, glycine betaine, and a variety of sugar and sugar alcohols to lower the osmotic potential and thereby maintain the cell turgor by improving water uptake from drying soil. In order to cope up with excess levels of ROS production plants have evolved a complex enzymatic and non-enzymatic antioxidant system. Cellular level tolerance can be achieved by maintaining

cell cycle regulation, effective scavenging of ROS, osmotic adjustment, osmoprotection, membrane stability and by protein turnover and stability (Bartels and Sunkar, 2005; Valliyodan and Nguyen, 2006).

2.1.2a Accumulation of osmolytes

Osmolytes are low-molecular-weight organic solutes, a broad group that encompasses a variety of compounds such as proline, sugar alcohols and quaternary ammonium compounds. The advantages of the accumulation of osmolytes are that they keep the main physiological functions of the cell active, the induction of their biosynthesis is controlled by environmental cues, and they can be synthesized at all developmental stages. In addition to their role in osmoregulation, osmolytes have crucial functions in protecting subcellular structures and in scavenging reactive oxygen species (Slama *et al.*, 2015).

Proline

Under stress conditions many plant species accumulate proline as an adaptive response to adverse conditions. Although a clear-cut relationship between proline accumulation and stress adaptation has been questioned by some researchers, it is generally believed that the increase in proline content following stress injury is beneficial for the plant cell. Proline accumulation is believed to play adaptive roles in plant stress tolerance. Proline has been proposed to act as a compatible osmolyte and to be a way to store carbon and nitrogen (Hare and Cress, 1997). Proline accumulation during osmotic stress is mainly due to increased synthesis and reduced degradation. Proline can act as a signalling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which is essential for plant recovery from stress (Szabados and Savoure, 2010). Accumulation of proline influences protein salvation and preserves the quaternary structure of complex proteins, maintains membrane integrity under dehydration stress and reduces oxidation of lipid membranes or photo inhibition (Demiral and Turkan, 2004). Furthermore, it also contributes to stabilize sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007). Stress conditions stimulate

proline synthesis while its catabolism is enhanced during recovery from stress. Overexpression of P5CS in tobacco and petunia led to increased proline accumulation and enhanced salt and drought tolerance (Yamada *et al.*, 2005), whereas Arabidopsis P5CS1 knock-out plants were impaired in stress-induced proline synthesis and were hypersensitive to salinity (Szekely *et al.*, 2008).

Sugars and sugar alcohols

Soluble sugars may also function as a typical osmoprotectants, stabilizing cellular membranes and maintaining turgor. Sugars may protect cells during desiccation by forming glasses (Black and Pritchard, 2002). Sugars are also thought to interact with polar head groups of phospholipids in membranes so that membrane fusion is prevented. A complex essential role of soluble sugars in plant metabolism is well known as products of hydrolytic processes, substrates in biosynthesis processes, energy production but also in a sugar sensing and signalling systems. It has been claimed that even sugar flux may be a signal for metabolic regulation (Gibson *et al.*, 2008).

A number of “sugar alcohols” (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of compatible-solute overproduction and overexpression to protect the membrane and protein complexes during stress (Cortina and Culiaz, 2005). Transgenic rice, over and under expressing pyruvate decarboxylase1 (pdc1) gene has also been developed, which showed a positive correlation of higher PDC activities with survival after submergence (Quimlo *et al.*, 2000). Expression of bacterial mannitol-1-phosphate dehydrogenase (mtlD) gene for accumulation of mannitol has been one of the successful transgenic approaches for enhanced tolerance to salt stress in eggplant (Prabhavathi *et al.*, 2002) and wheat (Abebe *et al.*, 2003). Mannitol has been known for long time as free radical scavenger *in-vitro* (Smirnoff and Cumbes, 1989). Transgenic tobacco over expressing mtlD gene showed increased tolerance to oxidative stress, which indicated a potential role for mannitol in radical oxygen scavenging (Shen *et al.*, 1997).

Quaternary ammonium compounds

The role of quaternary ammonium and tertiary sulfonium compounds as compatible solutes has been extensively reviewed (Wyn Jones and Storey, 1981; Anthoni *et al.*, 1991; Rhodes and Hanson, 1993; Gorham, 1995). These zwitter ionic compounds include glycine betaine, choline-O-sulfate, B-alanine betaine, proline betaine and hydroxyl proline betaine, and dimethylsulfoniopropionate (DMSP).

Glycine betaine has been extensively studied as a compatible solute, both by genetically engineering and by exogenous application (Chen and Murata, 2008). When maize plants were transformed with the *betA* gene from *E. coli* that encodes choline dehydrogenase, they accumulated glycinebetaine in tissues and were more tolerant to drought stress than wild-type plants at different developmental stages (Perroud and Rudulier, 1985). Transgenic rice with chloroplast-targeted choline oxidase gave better tolerance under water deficit condition (Kathuria *et al.*, 2009).

2.1.2b Antioxidant mechanisms

The common feature of any biotic or abiotic stress is production of reactive oxygen species (ROS), which are toxic to cells at high concentrations as they cause oxidative damage to membrane lipids, proteins and nucleic acids (Arora *et al.*, 2002). They may act as signalling molecules to trigger and orchestrate plant stress responses (Aebi and Sutter, 1971). The regulation of this antioxidant reaction is very crucial for plant to defend against any stress (Asada, 1992). This defence system is composed of a range of non-enzymatic and enzymatic primary antioxidants. Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and non-enzymatic low molecular metabolites, such as ASH, GSH, α -tocopherol, carotenoids and flavonoids (Mittler *et al.*, 2004). Superoxide dismutase is the key enzyme catalyzing dismutation of oxy free radicles into hydrogen peroxide and oxygen. Increased SOD activity has been positively correlated with tolerances to different stresses such as low/high temperature, drought, salinity, high light intensity, and ozone (Wang *et al.*,

2004; Badawi *et al.*, 2004b). Pea Cu/Zn-SOD protects tobacco plants from oxidative stress (Gupta *et al.*, 1993), tomato Cu/Zn-SOD enhanced protection to potato plants from methyl viologen toxicity (Perl *et al.*, 1993). Over-expression of yeast SOD2 gene in Arabidopsis plants increases salt tolerance (Gomez *et al.*, 1999). Similarly, over-expression of rice cytosolic Cu/Zn- SOD gene enhanced tolerance to salt and water deficit stress in tobacco (Badawi *et al.*, 2004a and 2004b).

APX reduces H_2O_2 to H_2O , the scavenging enzyme of H_2O_2 in chloroplasts (Asada, 1992). APX isoforms are classified according to their subcellular location as: the thylakoid membrane (tAPX) and stroma (sAPX) in chloroplasts, the cytosol (cAPX), the mitochondria (mitAPX) and the peroxisomes (mAPX). Increased activity of APX isoenzymes in response to environmental stresses, such as salinity and drought has been reported in several plant species, indicating the function of APX enzymes in eliminating H_2O_2 from plant cells (Kavitha *et al.*, 2008).

The plant catalases has role in plant metabolism, defense, and signal perception. GPx catalyze the reduction of H_2O_2 as well as inorganic hydroperoxides to water or the corresponding alcohols and they are one the key antioxidant enzyme in animals, whereas their role in plants is still unclear (Herbette *et al.*, 2007; Yoshimura *et al.*, 2004) reported, overexpressing a Chlamydomonas GPx in tobacco and found that transgenic plants displayed increased tolerance to oxidative stress induced by methylviologen application, chilling and salt stress. The overexpression of a GPx (NSGPx) gene increased the resistance of *C. reinhardtii* to photooxidative stress conditions (Ledford *et al.*, 2007). The GPx overexpressing plants are more resistant towards abiotic stresses (Herbette *et al.*, 2011).

2.1.2c Protein turnover and stability

Protein turnover is the balance between protein synthesis and protein degradation. Many cellular proteins are constantly degraded and resynthesized. To facilitate this recycling, a complex system for the controlled turnover of proteins has evolved. Damaged or unneeded proteins are marked for destruction by the covalent attachment of chains of a small protein, *ubiquitin*. Polyubiquitinated proteins are subsequently degraded

by a large, ATP-dependent complex called the *proteasome*. However, stable mRNA is crucial for protein production. It is very well understood that transcription, translation and protein turnover are concomitantly related. There are many regulatory checks and hindrances as well at DNA level, transcription, pre-mRNA processing, translation and protein maturation and stability to get functional protein.

Transcription: The regulation of transcription is a central step in the control of gene expression in plants and it therefore plays an essential role in development and in the way plants cope with environmental influences. A major level at which gene expression is regulated is the initiation of transcription, and this is reflected in the percentage of the genome dedicated to transcription factors in plants and other eukaryotes. An analysis of 1.9 Mb of Arabidopsis genomic sequence from chromosome 4 revealed that about 15% of the genes with predicted or known functions were involved in transcription, a percentage similar to what has been found in other eukaryotes (Bevan *et al.*, 1998). Genomic DNA is complexed with proteins to form chromatin. One of chromatin's major roles is to facilitate the packaging of DNA in the nucleus, but the structure of chromatin also leads to a general suppression of gene activity. For gene activation and transcription to occur, the chromatin in the vicinity of the gene must be remodeled to allow access for transcription factors and the recruitment of the RNA polymerase II (Pol II) transcription-initiation complex (Karam, 1998). Plant scientists have developed many crop species which are tolerant to stress through transgenic approach by over expressing regulatory genes. More than 42 per cent of the total transgenics are targeted to nucleus, indicates the importance of manipulating the transcription under stress (Mohammad-Zaman and Setsuko, 2013).

Transcription factors: It plays important and diverse roles in gene expression, including chromatin remodeling and recruitment/stabilization of the Pol II transcription-initiation complex. Transcription factors, which come in many shapes and sizes, can be divided into a number of functional classes, with some proteins belonging to more than one class. A major class of transcription factors is activators and repressors. These proteins bind to specific DNA sequences found only in certain promoters and are instrumental in giving rise to gene specific regulation. A second class of transcription factors are coactivators or

corepressors. These proteins mediate the transcriptional effects of specific activators/repressors, in some cases by remodeling chromatin. Whereas this group of transcription factors are typically not able to bind to DNA on their own, they can still be promoter-specific as a result of protein-protein interactions with specific activators and repressors. A third class comprises the general transcription factors, which are important components of the Pol II transcription-initiation complex. A fourth class is architectural transcription factors that are also involved in remodeling DNA, e.g. by inducing bends that facilitate the binding of other proteins to the promoter (Karam, 1998). In *Arabidopsis thaliana* genome about 1500 TFs are described which are considered to be involved in stress responsive gene expression (Riechmann *et al.*, 2000). Most of the stress responsive TFs belong to the families bZIP AP2/ERF, NAC, MYB, MYC, HSF, Zinc-finger, HD-ZIP and WRKY (Nakashima and Yamaguchi-Shinozaki, 2005).

m-RNA processing: Pre-mRNA splicing is one of the fundamental processes in constitutive and regulated gene expression in eukaryotes. During splicing, introns present in primary gene transcripts are removed and exons are ligated to produce translationally competent mRNAs. The basic mechanism of intron excision is similar in all eukaryotes. The reaction is mediated by the spliceosome, a large ribonucleoprotein (RNP) complex, which is assembled anew at each intron from small nuclear RNP particles (U-snRNPs) and numerous protein factors. Spliceosome assembly is a highly ordered and dynamic reaction, involving hydrolysis of several ATP molecules and many structural rearrangements. Different classes of proteins, including RNA-binding proteins, RNA dependent ATPases, RNA helicases and protein kinases, are responsible for the precision and regulation of this process. During splicing, exon and intron sequences have to be effectively distinguished and appropriate 5' and 3' splice sites (5'ss and 3'ss) have to be selected and juxtaposed prior to the catalytic step. The recognition of intron and exon borders requires a multitude of cis acting elements, each with relatively low information content, that are recognized by the trans-acting factors (Zdravko *et al.*, 2000). Although many molecular processes related to splicing have been well characterized, we still face a major challenge in understanding how precision and efficiency in splicing are regulated and how we could harness these regulations to enhance cellular functions. *Arabidopsis*

supersensitive to abscisic acid and drought 1 gene (SAD1), which encodes Sm-like protein 5 (LSm5), promotes an inaccurate selection of splice sites that leads to a genome-wide increase in alternative splicing. Over expression of SAD1 showed increased tolerance to stress (Peng *et al.*, 2014).

Translation: Translational control provides cells with a mechanism to rapidly control gene expression in a reversible manner in response to environmental and developmental cues. It involves the dynamic, coordinated activity of numerous factors that direct the synthesis of proteins with precision in space and time. Translational control is primarily regulated at the level of initiation, and as such, mechanisms that regulate translation most often target the initiation machinery. Translation in plants is fundamentally similar to that of other eukaryotes. However, there are significant differences in translation factor isoforms and their associated proteins, and the types of regulation that can act upon these factors. Regulation of translation in plants can involve protein phosphorylation, variable associations of initiation factor isoforms, RNA sequence element interactions, and small RNAs (Muench *et al.*, 2012).

Protein folding and stability: Abiotic stresses like heat, cold, freezing, drought salinity, flooding or oxidizing agents usually cause protein dysfunction. Protein folding stability is undoubtedly one of the most challenging problems in organisms undergoing stress conditions. Efficient protein repair systems and general protein stability facilitate survival upon sudden changes in the environment. As sessile organisms plants need to adopt quickly to overcome various environmental stresses during their lifespan. Recently, most emphasis is being directed towards an understanding of how plants recognize external conditions and initiate protective reactions such as mechanisms through which protein function is protected and maintained. Proteins are biological macromolecules involved in virtually every biological process in a living system. The roles played by proteins are varied and complex. Proteins are used for storage and transport of small molecules or ions and control the passage of molecules through the cell membranes essential for metabolic function. Molecular chaperones, proteins that prevent inappropriate association or aggregation of exposed hydrophobic surfaces of unfolded or partially folded proteins and direct them into productive folding, transport, or degradation pathways, function to

minimize protein aggregation and can promote dissociation of aggregates that have formed. Protein-folding catalysts, conventional enzymes that accelerate the rate-limiting steps in protein folding, allowing folding intermediates to avoid aggregation and non-productive interactions with other proteins, also assist cellular proteins to avoid aggregation by accelerating the rate of correct folding (Jan, 1999). Several proteins have been identified which act as chaperones and assist in folding process under stress like Late Embryogenesis Abundant (LEA) Proteins, Heat Shock Proteins (HSPs), Binding Proteins (BiPs) etc. HSPs and LEA proteins help to protect against stress by controlling the proper folding and conformation of both structural (i.e. cell membrane) and functional (i.e. enzymes) proteins.

2.2 Late Embryogenesis Abundant (LEA) proteins

Late Embryogenesis Abundant (LEA) proteins accumulate at the onset of seed desiccation and in response to water deficit in vegetative plant tissues. The typical LEA proteins are highly hydrophilic and intrinsically unstructured. LEA proteins found in both plant and animals with low molecular weight of about (10-30 kDa) proteins, which are involved in protecting higher plants from damage caused by drought and cold shock. Most LEA proteins identified to date belong to hydrophilins, a widely distributed group of proteins characterized by a high content of charged amino acid residues, as well as glycine or other small amino acids such as alanine, serine, or threonine. Moreover, most hydrophilins lack tryptophanes and cysteines. These physicochemical properties led to propose that hydrophilins are unstructured proteins in aqueous solutions, which has been shown for some LEA proteins. Analyses of LEA protein amino acid sequences recognize seven different groups, each one showing distinctive motifs (LEA1 – LEA7). In particular, for groups 2, 3, and 4 different protein variants have been found showing a particular organization of their corresponding motifs. A variety of studies have shown a potential role for LEA proteins in stress tolerance. Their function in protein protection upon water deficit was demonstrated by in vitro experiments, where different hydrophilins, including LEA proteins from groups 2, 3, and 4, were able to prevent the inactivation of enzymes such as LDH (lactate dehydrogenase) or MDH (malate

dehydrogenase) upon different dehydration levels. Similarly, protective properties were also detected during freeze-thaw in vitro assays (Marina and Alejandra, 2013).

2.3 RNA Binding Proteins (RBPs)

RNA-binding proteins (RBPs) are central regulatory factors controlling posttranscriptional RNA metabolism during plant growth, development, and stress responses. The regulation of RNA metabolism is carried out by either direct or indirect binding of RNA-binding proteins (RBPs) to target RNAs. Determination of protein structures and functional characterization of RBPs in diverse organisms have revealed that RBPs harbor several conserved motifs and domains, including RNA-recognition motif (RRM), zinc finger motif, K homology (KH) domain, glycine-rich region, arginine-rich region, RD-repeats, and SR-repeats. Many nuclear-encoded chloroplast- or mitochondria-targeted RBPs play pivotal roles in RNA metabolism in chloroplasts and mitochondria. The importance of RBPs in intron splicing and plant growth and development has been demonstrated. Among the seven minor spliceosomal small nuclear ribonucleoproteins that are involved in splicing of U12-type introns, U11/ U12-31K and U11/U12-65K proteins play essential roles in the splicing of many U12 introns, which is crucial for normal growth and development of dicot and monocot plants. In particular, the stress-responsive expression and functional roles of RBPs, including GRPs, RZs, CSDPs, RHs, CRMs, SDPs, and PPRs, have been extensively investigated in Arabidopsis, rice, wheat (*Triticum aestivum*), barely (*Hordeum vulgare*), and cabbage (*Brassica rapa*) under diverse environmental conditions. Arabidopsis AtGRP2 and AtGRP7 and rice OsGRP1 and OsGRP4 promote seed germination and seedling growth under low temperatures and confer freezing tolerance to Arabidopsis plants (Kwanuk Lee and Hunseung Kang, 2016).

Among all the processes mRNA processing, protein synthesis and protection are very important and regulation of these processes under stress showed improved protein turnover and stability. The key proteins involved in these processes are RNA helicases and chaperones.

2.4 Helicases

The helicases are ubiquitous enzymes that catalyze the unwinding of energetically stable duplex DNA (DNA helicases) or duplex RNA secondary structures (RNA helicases). Helicases are protein motors that use the energy of NTP hydrolysis to dissociate the hydrogen bonding between the nucleic acid duplexes and also to disrupt other non-covalent interactions between complementary base pairs. RNA helicases represent a large family of proteins that are involved in the modulation of RNA structure and thereby influence RNA synthesis, splicing, replication, translation initiation, editing, rRNA processing, ribosome assembly, nuclear mRNA export, and mRNA stabilization and degradation (Chen *et al.*, 2002, Jankowsky *et al.*, 2001, Linder and Stutz, 2001). With their important role in RNA metabolism, RNA helicases are found in all kingdoms of life, and many viruses express one or more of these proteins to execute their biological functions, for example, to replicate in the host (Jin and Peterson, 1995; Wen *et al.*, 2009).

2.4.1 Classification

A computer-assisted amino acid sequence analysis of helicases from many different organisms has revealed seven short conserved motifs (I, Ia, II, III, IV, V, and VI), called 'helicase motifs'. The helicases have been classified into three super families (SF) namely SF1, SF2, and SF3 based on the extent of similarity and the organization of these conserved motifs. High sequence conservation has been maintained in this large group of helicases, suggesting that the motifs containing helicase genes evolved from a common ancestor. These seven motifs of SF1 and SF2 are usually clustered in a region of 200±700 amino acids and can be called a core-region. These conserved motifs are separated by stretches of low sequence, but high length, conservation. It has been suggested that the divergent regions are responsible for individual protein function, whereas the highly conserved domains are involved in ATP-binding and hydrolysis or binding and unwinding of nucleic acids. Because of the sequence of motif II (DEAD or DEAH or DEXH), the helicase family is also called the DEAD-box protein family. From the post-sequencing genome era of Arabidopsis it is now known that the DEAD-box helicase family contains more than 55 members in Arabidopsis (Tuteja, 2003).

Most helicases are members of DEAD-box protein superfamily that play essential roles in basic cellular processes regulating plant growth and development, such as DNA replication, repair, recombination, transcription, ribosome biogenesis, and translation initiation. It seems, therefore, that DEAD-box helicase might also be playing an important role in stabilizing growth in plants under stress conditions by regulating some stress-induced pathways.

Function: The two possibility of helicases action: 1) at the level of transcription or translation to enhance or stabilize protein synthesis 2) in association with DNA multi-subunit protein complexes to alter gene expression. Removal of the secondary structure in 5' UTR of eukaryotic mRNA for efficient translation and in turn to help in the synthesis of molecules providing stress tolerance. This hypothesis is supported by the up regulation of transcript of eIF4A in response to abiotic stress (Tuteja *et al.*, 2005). RNA helicase not only remove RNA secondary structure, but also simultaneously protect mRNA from degradation under condition where transcription and translation is uncoupled as a result of inefficient translation initiation (Sanan-Mishra *et al.*, 2005). Regarding the second mode of action of helicases, helicase was found to interact with enzymes such as topoisomerases (Tuteja and Phan, 1998). Among eukaryotes, translation initiation process is conserved and regulation of translation occurs at initiation step. The RNA helicase activity of DEAD-box proteins could facilitate translation by altering the nascent RNA structure, a process that can stimulate re-initiation and/or elongation (Vashisht and Tuteja, 2006).

2.4.2 DEAD-box helicases

DEAD-box proteins are mainly known as RNA helicases or putative helicases, but some members are also known as DNA helicases (Tuteja and Tuteja, 2004b; Cordin *et al.*, 2006). RNA helicases unfold the secondary structures in RNA and are involved in ribosome biogenesis, RNA splicing, transport, turnover, transcription, translation initiation, RNA interference, RNA editing, and mRNA stabilization and degradation (Cordin *et al.*, 2006), whereas DNA helicases unwind duplex DNA and are involved in

replication, repair, recombination, transcription, pre-rRNA processing, and translation initiation (Tuteja and Tuteja, 2004b).

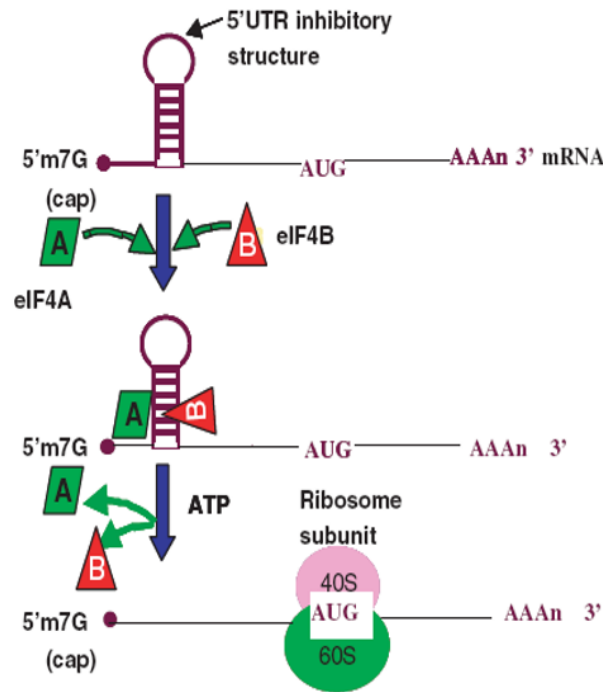


Fig. 2: Mechanism of stress tolerance by a helicase (Vashisht and Tuteja, 2006)

In sorghum, a salt-responsive transcript HVD1 (*Hordeum vulgare* DEAD-box protein), encoding a putative ATP dependent DEAD-box RNA helicase, is induced under salt and cold stress (Nakamura *et al.*, 2004). Two chilling and freezing stress-inducible DEAD-box helicase genes have been identified using Arabidopsis mutants that are impaired in the cold-regulated expression of CBF and their downstream target genes (Gong *et al.*, 2005).

Using cDNA microarray analysis of 1300 Arabidopsis genes, a cold stress-inducible DEAD-box helicase gene was identified. Pea DNA helicase 45 (PDH45) was found to be induced in pea seedlings in response to high salt (NaCl), dehydration, wounding, and low temperature (Pham *et al.*, 2000). When this gene was transformed to tobacco, it provided high salinity tolerance without affecting yield (Sanan-Mishra *et al.*, 2005). Another gene from *Pisum sativum* (PDH47, pea DNA helicase 47) was reported to be induced in response to cold and salinity stress in shoots and roots, and heat and abscisic acid (ABA) treatment in roots (Vashisht *et al.*, 2005).

Table 2: List of biochemically active DNA helicases from plant cells (Tuteja 2003; Vashisht *et al.*, 2005) CDH, chloroplast DNA helicase; ds, double-stranded; DSBs, double-strand breaks; eIF-4A, eukaryotic translation initiation factor 4A; Nd, not determined; PDH, pea DNA helicase.

Name	Molecular mass (kDa)	Nucleotide cofactors	Polarity	Remarks
Lily's U-protein	130 (native)	ATP	Nd	Partially purified. Unwinds DNA from ends, gaps and nicks. Its abundance increases during meiosis.
Soybean chloroplast DNA helicase	Nd	ATP>>G TP>dATP>NTPs	Nd	Partially purified. Shows unwinding activity at higher concentration of Mg ²⁺ (10 mM).
Pea chloroplast DNA helicase I (CDH I)	68; 138 (native)	ATP>dA TP>>NTPs	3'-5'	First plant helicase reported in purified form. It is a homodimer.
Pea chloroplast DNA helicase II (CDH II)	78	ATP=dATP	3'-5'	Purified to homogeneity. Stimulated by fork-like structures.
Pea nuclear DNA helicase I (PDH45)	45	ATP>dA TP>>NTPs	3'-5'	First plant nuclear DNA helicase and its homologous to eIF-4A. It stimulates topoisomerase I activity.
Pea nuclear DNA helicase II (PDH65)	-	ATP>dATP	3'-5'	A plant homologue of human DNA helicase I. Localized in nucleolus .Phosphorylated and upregulated by CK2 and cdc2 protein kinases.
Arabidopsis Ku DNA helicase (AtKu70/A tKu80)	70.3; 76.7	ATP (other NTPs/dNTPs ± Nd)	Nd	It functions as heterodimer that binds to dsDNA. Gene expression is induced by DSBs, may be involved in DSB repair.
Pea nuclear DNA helicase III (PDH120)	54; 66; 120 (native)	ATP>NTPs	3'-5'	Purified to homogeneity. Present at extremely low abundance and contain the high specific activity.
Pea nuclear DNA Helicase47 (PDH47)	47	ATP	3'-5' & 5'-3'	Isolated & characterized from Pea. Transcript induced under both salinity & cold.

Cytosol and nuclear localized Pea p68 member of DEAD-box protein family, is found to be up regulated in response to high salinity in pea. Overexpression of p68 driven by constitutive CaMV35S promoter in tobacco transgenic plants confers enhanced tolerances to salinity stress by improving growth, photosynthesis and antioxidant machinery. Under stress treatment, pea p68 overexpressing tobacco accumulated higher K⁺ and lower Na⁺ level than the wild-type plants. And also ROS accumulation was remarkably regulated by the overexpression of pea p68 under salinity stress conditions (Tuteja *et al.*, 2014).

PDH45 (Pea DNA Helicase 45): PDH45 is a member of the DEAD-box protein family. Although, its deduced amino-acid sequence has the eight common conserved helicase motifs, it is a unique member of this family, containing DESD and SRT instead of DEAD/H and SAT in motifs V and VI respectively (Tuteja, 2003; Pham *et al.*, 2000). The alignment of amino-acid sequences between PDH45 and other DNA and RNA helicases reveals regions of sequence identity only in the eight domains characteristic of helicases (Pham *et al.*, 2000). The amino acids in these eight motifs are variously responsible for ATP binding and hydrolysis, nucleic acid binding, nucleic acid unwinding and RNA binding (Tuteja and Tuteja, 2004b). PDH45 is localized in the nucleus and cytosol and unwinds DNA in the 3'–5' direction using ATP. It also has ATP-dependent RNA unwinding activity (Pham *et al.*, 2000). The amino-acid sequence of PDH45 shows 86% similarity with the tobacco translation initiation factor eIF-4A, and 58% with eIF-4A from *Arabidopsis thaliana*, maize, rice and wheat (Pham *et al.*, 2000). The eukaryotic translation initiation factor (eIF-4A) is also a prototypic member of the DEAD box family. It is an essential component of the cap binding complex that facilitates ribosome assembly on mRNA by unwinding the secondary structures in the 50 untranslated region (UTR) of many proteins (Pause *et al.*, 1994). Antibodies against PDH45 inhibit in vitro protein synthesis. In addition PDH45 has been found to physically interact with and stimulate pea topoisomerase I activity (Pham *et al.*, 2000).

The homolog of PDH45 in *Medicago sativa*, a glycophyte, MH-1 (Medicago helicase I) was shown to be enhanced after mannitol, NaCl or H₂O₂ treatments. The cDNA when over-expressed caused *Arabidopsis* to be both salt and drought-tolerant. The

elevated stress tolerance in MH-1-expressing plants was correlated with an increase of superoxide dismutase (SOD) and ascorbate peroxidase (Apx) activities, indicating that MH1 functions in abiotic stress by elevating the capacities for reactive oxygen species (ROS) scavenging and osmotic adjustment (Luo *et al.*, 2009). To improve the salinity tolerance of rice, a DEAD-box helicase gene isolated from pea with a CaMV35S promoter was transformed into the Bangladeshi rice variety Binnatoa through *Agrobacterium*-mediated transformation. At the reproductive stage, transgenic rice plants expressing PDH45 showed better fertility and produced higher grain yield by 16% compared to WT plants under continuous stress of 6 dS/m from 30 days till maturity (Mahzabin *et al.*, 2012). Over-expression of PDH45 in peanut showed stay-green phenotype and increased chlorophyll stability under stress and reduced chlorophyll retardation under etherel-induced simulated stress conditions. Stress-induced root growth was also substantially higher in the case of transformants. This was reflected in increased WUE (low $\delta^{13}C$) and improved growth rates and productivity. The transgenics showed 17.2 and 26.75 % increase in yield under non-stress and stress conditions over wild type ascertaining the feasibility of trait pyramiding strategy for the development of drought tolerant peanut (Manjulatha *et al.*, 2014).

PDH45, a pea DNA helicase homologous to eIF4A, up-regulated under abiotic stress including salinity and drought, was found to be a potential candidate gene to improve drought stress.

2.5 Heat shock proteins (HSPs)

Heat shock proteins (HSPs), function as molecular chaperones and impart stress tolerance (Schlesinger, 1990). They can prevent the accumulation of protein precursors, accelerate the transport of proteins, and absorb complexes of unfolded proteins to maintain their transport abilities (Timperio *et al.*, 2008). HSPs can also sustain the normal folded state of proteins, degrade misfolded proteins, stabilize polypeptide strands, and prevent protein inactivity. In addition, HSPs participate in regulating the activation and function of target proteins, although they themselves are not components of the target proteins (Pratt and Toft, 2003; Rutherford, 2003).

Ritossa (1962) made the seminal discovery of temperature-induced puffs in polytene chromosomes of *Drosophilla melanogaster* larvae salivary glands. A decade later, it was shown that the puffing pattern corresponded to a robust activation of genes encoding the heat shock proteins (HSPs), which function as molecular chaperones. The heat shock response is a highly conserved mechanism in all living organisms from yeast to humans that is induced by extreme proteotoxic insults such as heat, oxidative stress, heavy metals, toxins and bacterial infections. The HSPs conservation among different eukaryotes suggests that the heat shock response is essential for survival in a stressful environment (Akerfelt *et al.*, 2010).

2.5.1 Classification

Heat shock proteins are grouped in plants into five classes according to their approximate molecular weight: (1) HSP100, (2) HSP90, (3) HSP70, (4) HSP60 and (5) small heat-shock proteins (sHSPs). Higher plants have at least 20 sHSPs and there might be 40 kinds of these sHSPs in one plant species. Molecular Hsps/chaperones are located in both the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and ER. Different classes of molecular chaperones appear to bind to specific non-native substrates and states. Chaperone proteins do not covalently bind to their targets and do not form part of the final product. Aside from these major families, there are other proteins with chaperone functions, such as protein disulfide isomerase and calnexin/calreticulin, which assist in protein folding in the endoplasmic reticulum (ER). Overexpression of Hsp70 genes induces thermal tolerance and increase resistance to environmental stresses (Wang *et al.*, 2004; Hyun *et al.*, 2015) showed importance of small HSPs in light independent germination and improved tolerance to temperature stress of tobacco seeds. These results clearly demonstrate the relevance of HSPs under different stress.

Table 3: Five major classes of plant Hsps/molecular chaperones and their subfamilies (Wang *et al.*, 2004)

Classes	Representative members	Intracellular localization	Major functions
HSP70 Subfamily: DnaK Hsp 110/SSE	Hsp/Hsc70 Hsp70 Bip Hsp91	Cytosol Chloroplast, mitochondria Endoplasmic reticulum Cytosol	Preventing, aggrgeation, assisting, refolding, protein import and translocation, signal transduction and transcriptional activation
Chaperonin/Hsp60 Subfamily: Group I Group II	Cpn60 CCT	Chloroplast, mitochondria Cytosol	Folding and assisting refolding
Hsp90	Hsp90 AtHsp90-1 AtHsp90-5 AtHsp90-6 AtHsp90-7	Cytosol Chloroplast Mitochondria Endoplasmic reticulum	Facilitating maturation of signaling molecules, genetic buffering
Hsp 100/Clp Subfamily Class I Class II	Hsp 100 ClpB, ClpA/C ClpD ClpM, ClpN ClpX, ClpY	Cytosol, mitochondria Chloroplast Chloroplast	Disaggregation, unfolding
sHsp Subfamily I II III IV V VI	Hsp17.6 Hsp17.9 Hsp21 Hsp26.2 Hsp22 Hsp23 Hsp22.3	Cytosol Cytosol Chloroplast Endoplasmic reticulum Mitochondria Membrane	Preventing aggregation, stabilizing non-native proteins

2.5.2 Hsp/chaperone network

The protective effects of Hsps/chaperones can be attributed to the network of the chaperone machinery, in which many chaperones act in concert. During stress, many enzymes and structural proteins undergo deleterious structural and functional changes. Therefore, maintaining proteins in their functional conformations, preventing aggregation of non-native proteins, refolding of denatured proteins to regain their functional conformation and removal of non-functional but potentially harmful polypeptides (arising from misfolding, denaturation or aggregation) are particularly important for cell survival under stress. Thus, the different classes of Hsps/chaperones cooperate in cellular protection and play complementary and sometimes overlapping roles in the protection of proteins from stress. Hsp18.1 from pea (*Pisum sativum*) can stably bind heat-denatured protein and maintain it in a folding-competent state for the further refolding by Hsp70/Hsp100 complexes (Wang *et al.*, 2004).

2.5.3 Cross-talk between Hsps/chaperones and other stress response mechanisms

The ubiquitous Hsp/chaperone system plays pivotal roles in cells, both under normal growth conditions and when stressed. An increasing number of studies suggest that the Hsps/ chaperones interact with other stress-response mechanisms. Osmolytes, or compatible solutes, are a group of low-molecular-weight organic compounds that accumulate in organisms in response to osmotic stress. A study performed in yeast showed that trehalose suppresses the aggregation of denatured proteins, maintaining them in a partially folded state from which they can be reactivated by molecular chaperones. Chaperones of the Hsp90 and Hsp70 families and their co-chaperones were also found to interact with a growing number of signaling molecules, including nuclear hormone receptors, tyrosine- and serine/threonine kinases, and cell-cycle and cell-death regulators, demonstrating that they play a key role in cellular signal transduction networks. The redox status of thiol-containing molecules is important to cellular functions such as the synthesis and folding of proteins, and the regulation of the structure and activity of enzymes, receptors and transcription factors (Wang *et al.*, 2004).

Heat-shock transcription-factor-dependent expression of antioxidants ascorbate peroxidases in *Arabidopsis* suggested that HSFs might be involved not only in Hsp synthesis but also in oxidative stress regulation of antioxidant gene expression. Furthermore, it was shown in *Arabidopsis* that several Hsp genes were upregulated under high light stress, implicating Hsps in the antioxidative response in addition to their chaperone function.

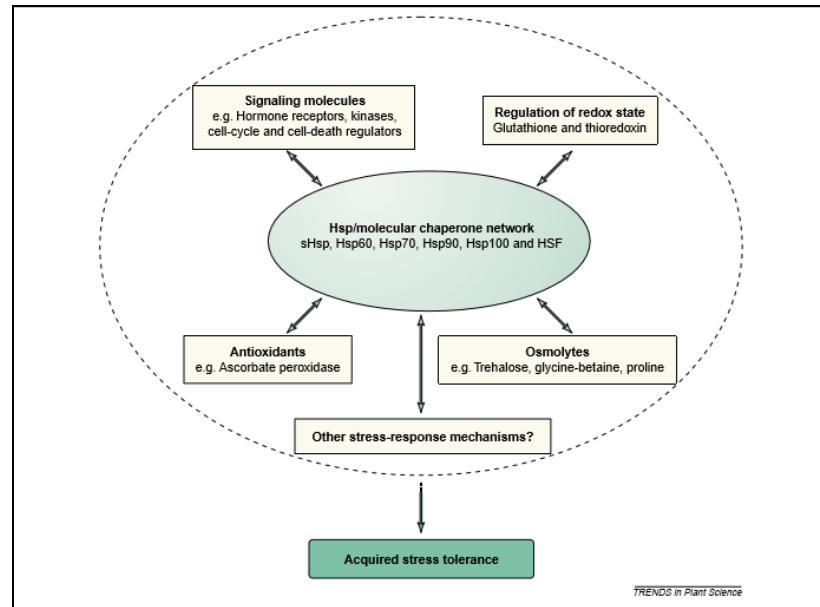


Fig. 3: Suggested cross-talk between the heat shock protein (Hsp)/chaperone network and other stress-response mechanisms (Wang *et al.*, 2004).

Since the expression of these HSP genes is controlled by a heat shock transcription factor (HSF), it is important to study the relevance of HSFs under stress.

2.5.4 Heat Shock factors (HSFs)

Heat shock transcription factors are major regulators mediating heat shock responses. Upon heat shock or under some stress conditions, these regulators would bind to the heat shock elements (HSEs) of the heat shock protein (HSP) gene promoters, regulating their expressions (Xiukun *et al.*, 2013). Under normal growth conditions, HSF is maintained in an inert monomer state through association with molecular chaperones such as Hsp70. In response to heat shock, HSF is converted from a transcriptional inactive monomer to active trimer using oligomerization domains,

which function as sequence specific trimeric DNA binding proteins. Enhanced thermo-tolerance in *Arabidopsis* could be achieved by constitutive expression of AtHSF41 in transgenic plants leading to constitutive expression of certain HSPs (Lee and Chen, 1993). Similar observation was obtained by the constitutive overexpression of HSF3 in *Arabidopsis* (Prandl *et al.*, 1998). However, number of HSFs across different species, depicts the functionality of HSFs Yeast-1, mammals-4, *Drosophilla*-1, *Arabidopsis*-21, soybean-52 loci (Akerfelt *et al.*, 2010) and Rice-25 (Wang *et al.*, 2009).

Plant HSF genes were assigned to three different classes (A, B and C) according to their unique structural characteristics (Nover *et al.*, 2001). Class A HSF proteins comprise the largest group of Hsfs with 15 proteins in *Arabidopsis*. They contain an activation domain at the C-terminus and are thought to be involved in transcriptional activation. Due to considerable redundancy of *Arabidopsis* HSF genes, only few HSF mutants display identifiable phenotypic changes. Class B and C HSFs lack a defined aromatic/hydrophobic/acidic (AHA)-type activation domain. The absence of an activation domain, as well as their inability to rescue the yeast Hsf1 mutation, had led to the assumption that class B HSFs function as repressors (Boscheinen *et al.*, 1997; Czarnecka-Verner *et al.*, 2000; 2004). However, HSFB1 was recently demonstrated to function as a novel co-regulator of the tomato HSFA1 or HSFA2 enhancing the transcriptional activity (Bharti *et al.*, 2000, 2004).

Structure of HSF: Among the various cloned HSF genes, there is an overall sequence identity of 40% with structural conservation in the winged helix–turn–helix DNA binding domain, an adjacent 80 residue hydrophobic repeat (HR-A/B) essential for trimer formation, and the carboxy-terminal transactivation domain. Other features unique to certain HSFs are the presence of an amino-terminal transactivation domain in the *Saccharomyces cerevisiae* HSF and spliced variants of mouse and human HSF2 and HSF4, which have variable transactivation properties. Apart from *S. cerevisiae*, the HSF form present in unstressed cells is a latent monomer that lacks both DNA binding and transcriptional activity (Richard, 2017).

Table 4: Validated HSF genes associated with stress tolerance

Gene name	Transgenics	Tolerant phenotype	References
AtHSFA2	<i>Arabidopsis</i>	Salt, osmotic stress, anoxia and submergence	Ogawa <i>et al.</i> , (2007) Banti <i>et al.</i> , (2010)
TaHSFA4a	Rice	Cadmium tolerance	Shim <i>et al.</i> , (2009)
HaHSFA9	Tobacco	Dehydration	Prieto-Dapena <i>et al.</i> , (2008)
OsHSFA2e	<i>Arabidopsis</i>	Heat and salt stress	Yokotani <i>et al.</i> , (2008)
OsHSF7	<i>Arabidopsis</i>	Thermo-tolerance	Liu <i>et al.</i> , (2009)
HSFB1 and HSFB2	KO <i>Arabidopsis</i>	Pathogen resistance	Kumar <i>et al.</i> , (2009)
OsHSFC1b	KO Rice	Salt tolerance	Schmidt <i>et al.</i> , (2012)
AtHSFA4A	<i>Arabidopsis</i>	Salt and Oxidative stress	Imma <i>et al.</i> , (2014)

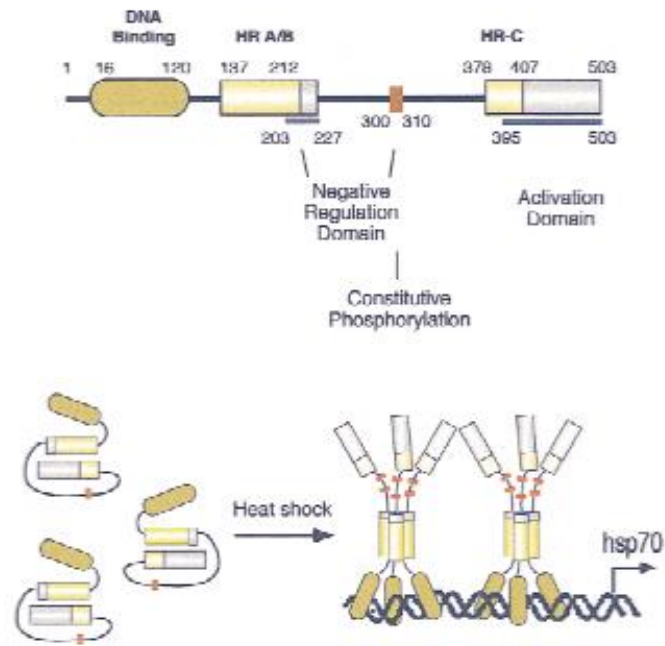


Fig. 4: General structural and regulatory features of HSFs (Richard, 2017)

2.5.5 HSF4 (Heat Shock Transcription Factor 4)

HSF4 has two isoforms: HSF4a and HSF4b. The splicing of HSF4 mRNA is complex, as two alternative 5' splice sites have been found in exons 8 and 9 with a frame shift in the transcript. The original cDNA clone encodes HSF4a, which has been reported to be transcriptionally inactive and to function as a repressor of heat shock gene expression. HSF4b isoform has the potential for transactivating heat shock genes and substituting for the yeast HSF. HSF4 has one unique structural feature: both isoforms lack the HR-C necessary for suppression of HSF trimer formation, leading to constitutive DNA binding activity of HSF4 in vitro (Lila *et al.*, 2001).

The function of the HSFA4 group is not well known. HSFA4A of wheat (*Triticum aestivum*) and rice (*Oryza sativa*) were reported to increase cadmium tolerance (Shim *et al.*, 2009). HSFA4 from tomato (*Solanum lycopersicum*) was shown to activate heat stress-induced genes and interact with HSFA5-generating inactive hetero oligomers. HSFA4 and HSFA5 belong to a separate group within the class A HSFs, sharing similarities in the DNA binding and oligomerization domain and C-terminal conserved sequence motifs (Baniwal *et al.*, 2004; Kotak *et al.*, 2004; Baniwal *et al.*, 2007). In the rice spotted leaf7 mutant, one base substitution in the DNA-binding domain of HSFA4D resulted in the appearance of necrotic lesions on leaves, suggesting stress hypersensitivity (Yamanouchi *et al.*, 2002). Arabidopsis HSFA4A was implicated in regulation of responses to high light and oxidative stress by regulating the transcription of ASCORBATEPEROXIDASE1 (APX1) and ZAT12 genes (Davletova *et al.*, 2005a).

Overexpression of HSFA4a alters the transcription of a large set of genes regulated by oxidative stress. HSFA4a shows homomeric interaction, which is reduced by alanine replacement of three conserved cysteine residues. HSFA4a interacts with mitogen activated protein kinases MPK3 and MPK6 in yeast and plant cells. MPK3 and MPK6 phosphorylate HSFA4a in vitro on three distinct sites, serine-309 being the major phosphorylation site. Activation of the MPK3 and MPK6 mitogen-activated protein kinase pathway led to the transcriptional activation of the HEAT SHOCK PROTEIN 17.6A. In agreement that mutation of serine-309 to alanine strongly diminished

phosphorylation of HSFA4a; it also strongly reduced the transcriptional activation of HEAT SHOCK PROTEIN 17.6A (Salamo *et al.*, 2014).

2.6 System biology approach to abiotic stress

Understanding the function of genes is a major challenge of the post-genomic era. While many of the functions of individual parts are unknown, their function can sometimes be inferred through association with other known parts, providing a better understanding of the biological system as a whole. High throughput omics technologies are facilitating the identification of new genes and gene function.

In the post-genomic era, comprehensive analyses using three systematic approaches or omics have increased our understanding of the complex molecular regulatory networks associated with stress adaptation and tolerance. The first one is ‘transcriptomics’ for the analysis of coding and noncoding RNAs, and their expression profiles. The second one is ‘metabolomics’ that is a powerful tool to analyze a large number of metabolites. The third one is ‘proteomics’ in which protein and protein modification profiles offer an unprecedented understanding of regulatory networks. Protein complexes involved in signaling have been analyzed by a proteomics approach. Integration of the different omics analyses facilitates abiotic stress signaling studies allowing for more robust identifications of molecular targets for future biotechnological applications in crops (Cramer *et al.*, 2011).

2.6.1 Transcriptomics

Transcriptome analysis technologies have advanced to the point where high-throughput DNA sequencers and high-density microarrays such as tiling arrays are readily available. These technologies provide new opportunities to analyze noncoding RNAs and can clarify aspects of epigenetic regulation of gene expression. Similar approaches have revealed the global transcriptomes of plants exposed to abiotic stresses such as dehydration, cold, heat, high-salinity, osmotic stress, and ABA. These analyses indicate that these stresses increase or decrease transcript abundance from not only previously identified stress-responsive genes, but also from thousands of unannotated

non-protein-coding regions (Cramer *et al.*, 2011). Approximately 80% of previously unannotated up regulated transcripts arise from antisense strands of sense transcripts. There was a significant linear correlation between the expression ratios (stress-treated/untreated) of the sense transcripts and the ratios of the antisense transcripts. Interestingly, the data suggested that such stress-responsive antisense transcripts are derived from antisense strands of the stress-responsive genes, RD29A and CYP707A1 (Matsui *et al.*, 2008).

Comparative analysis of differential gene expression in transgenic soybean seed tissue addressing multiple factors that could potentially induce changes in endogenous gene expression (e.g. transgene expression, protein accumulation, etc.). Genes involving responses to wounding, translation, ribosomal constituents, endopeptidase inhibitors, cellular biosynthesis and gene expression were all up regulated while genes involving the nuclear envelope were down regulated. The transcriptomic profiles of transgenic plants can be significantly different than those of wild type controls, and has provided a comprehensive first investigation into gene expression differences resulting from high levels of transgene expression and recombinant protein generation targeted to soybean seed tissues (Kevin *et al.*, 2015).

In rice an in-depth temporal transcriptome analysis was done at early and late stages of Pi deprivation as well as Pi recovery in roots and shoots, using next-generation sequencing. Analyses of 126 paired-end RNA sequencing libraries, spanning nine time points, provided a comprehensive overview of the dynamic responses of rice to Pi stress. Differentially expressed genes were grouped into eight sets based on their responses to Pi starvation and recovery. A reference annotation-based transcript assembly was also generated, identifying 438 unannotated loci that were differentially expressed under Pi starvation. Several genes also showed induction of unannotated splice isoforms under Pi starvation. Among these, PHOSPHATE2 (PHO2), a key regulator of Pi homeostasis, displayed a Pi starvation-induced isoform, which was associated with increased translation activity (David *et al.*, 2013).

2.6.2 Proteomics

Proteomics can be defined as the systematic analysis of proteome, the protein complement of genome. This technology allows the global analysis of gene products in various tissues and physiological states of cells. With the completion of genome sequencing projects and the development of analytical methods for protein characterization, proteomics has become a major field of functional genomics. The initial objective of proteomics was the large-scale identification of all protein species in a cell or tissue. The applications are currently diversified to analyze various functional aspects of proteins such as posttranslational modifications (Park, 2004). The unique feature of proteomics is its feasibility to analyze the changes occurring at the protein level that cannot be predicted from genomic sequence. Proteins undergo post-translational modifications, proteolysis, recycling, multi complex formation and subcellular translocation that are key events to regulate protein functions in cellular processes. Proteomics can eventually reveal all proteins in a cell or tissue at any given time, including those with post-translational changes.

The pattern of protein synthesis during hypoxic acclimation and anoxia in maize roots was analyzed by incorporation of ³⁵S-Met combined with 2-DE and MS (Chang *et al.*, 2000). It showed that protein synthesis during acclimation, but not during subsequent anoxia, is crucial for acclimation. This work shows some of the potential and difficulties of proteomics to study up-and down-regulation of protein expression.

The proteins of the 30S and 50S ribosomal subunits in spinach chloroplasts were identified by a combination of 2-DE, chromatography, MS, and Edman sequencing (Yamaguchi and Subramanian, 2000; Yamaguchi *et al.*, 2000). It was concluded that the spinach plastid ribosome comprises 59 proteins, of which 53 are *Escherichia coli* orthologues and six are non-ribosomal plastid-specific proteins (PSRP-1 to PSRP-6). A number of these proteins were shown to be post-translationally modified.

Table 5: Proteomic studies in different subcellular compartments in plants (Park, 2004)

Subcellular compartment	Summary of studies
Chloroplast	61 luminal and peripheral thylakoid proteins in pea identified by MALDI-TOF MS, ESI MS/MS and Edman sequencing; more than 100 envelope membrane proteins in Arabidopsis identified by LC-MS/MS; 350-kDa ClpP protease complex with 10 different isoforms in Arabidopsis identified by blue-native gel electrophoresis, MALDI-TOF MS and ESI MS/MS; light-harvesting proteins of photosystems I & II in 14 and 9 different plant species, respectively, identified by reverse phase HPLC-ESI-MS
Mitochondria	52 mitochondrial proteins in Arabidopsis identified by immunoblotting, Edman sequencing, MALDI-TOF MS and ESI MS/MS; 91 mitochondrial proteins in Arabidopsis identified by MALDI-TOF MS; protein complexes, ATP synthase complex, cytochrome c reductase complex and TOM complex, in Arabidopsis resolved by 3DE; subunits of TOM complex in Arabidopsis identified by immunoblotting and direct sequencing
Nucleus	184 nuclear proteins in Arabidopsis identified by MALDI-TOF MS
Cell wall	111 cell wall proteins in Arabidopsis identified by MALDI-TOF MS; extracellular proteins responding to fungal elicitors in Arabidopsis suspension cultures identified by immunoblotting and MALDI-TOF MS
Plasma membrane	82 proteins in plasma membrane and soluble fractions in Arabidopsis identified by direct sequencing; 38 plasma membrane proteins associated with cold acclimation in Arabidopsis identified by MALDI-TOF MS
Post-translational modification	thylakoid proteins reversibly phosphorylated in Arabidopsis identified by MALDI-TOF MS and ESI MS/MS; 30 GAPs identified by LC-MS/MS and 248 GAPs predicted by a bioinformatic analysis in Arabidopsis

The soluble and peripheral proteins in the thylakoids of pea were systematically analyzed by using 2-DE, MS, N-terminal Edman sequencing, and bioinformatics (Peltier *et al.*, 2000). After correcting to eliminate possible isoforms and posttranslational modifications, it was estimated that the thylakoid contains at least 200 to 230 different luminal and peripheral proteins. Sixty-one proteins were identified, of which about 40% had no annotated function. It is surprising that about 50% of the identified luminal proteins have a typical twin Arginine motif in the luminal transit peptide, suggesting that they translocate via the so-called TAT pathway, rather than via the Sec translocon (Keegstra and Cline, 1999).

About 40% of the predicted proteins in the Arabidopsis genome have no assigned function. Although reverse genetics will help to determine such functions, redundancy, lethality, and strong phenotypes can often prevent obtaining any insight in gene function. Most proteins have a transient or stable interaction with other proteins and the determination of these interacting proteins often can help to obtain more insight in gene function. Epitope tagging of transgenes, followed by affinity purification, will be very useful to identify (low abundant) protein complexes. For more high-throughput analysis, multidimensional purification schemes can possibly be used for rapid identification of protein-protein interactions (Klaas, 2001).

In the last decade, methodological improvements have allowed comparative proteomic investigations of plants under stress, which have allowed to analyse biochemical pathways and the complex response of plants to environmental stimuli (Qureshi *et al.*, 2007).

2.6.3 Metabolomics

Metabolomics developed from metabolic profiling and is the most recent of the ‘-omics’ approaches to emerge. The word ‘metabolome’ was first suggested in 1998 by Stephen Oliver to designate the set of all low molecular mass compounds synthesised by an organism. Metabolomics is generally defined as a holistic qualitative and quantitative analysis of all metabolites present within a biological system under specific conditions. It differs from the classical or traditional targeted phytochemical analysis in various

fundamental aspects, such as being a data-driven approach with predictive power that aims to assess all measurable metabolites without any pre-conception or pre-selection. In order to attain this goal, advanced analytical tools that provide high degrees of sensitivity, selectivity and reproducibility are required. Metabolomics has become a valuable tool for advancing our understanding of primary and secondary metabolism in plants and is revolutionising the field of plant biology. It is viewed as a complementary technique to other functional genomics approaches such as transcriptomics and proteomics. Furthermore, metabolomics is a cornerstone in the integration of the ‘-omics’ technologies that contribute to a systems biology overview. As such, it assists in providing a holistic understanding of the organisation principle of cellular functions at different levels, and in providing ways of monitoring all biological processes operating as an integrated system. Moreover, metabolomics as a post-genomics tool is often regarded as offering distinct advantages when compared to other ‘-omics’ technologies (Tugizimana *et al.*, 2013).

Although metabolomics is downstream of the other functional genomics (transcriptomics and proteomics), the practical size of the metabolome of a species, unlike transcriptome or proteome, cannot be speculated directly by known genomic information via central dogma. Therefore, metabolomics is used to obtain a large amount of valuable information for the discovery of genes and pathways through accurate and high throughput corollary peak annotation via snapshotting the plant metabolome (Jun *et al.*, 2016).

Maize plants are subjected to water stress and salinity stress separately or concurrently, levels of six metabolites (citrate, fumarate, phenylalanine, valine, leucine, isoleucine) in leaves only change significantly under combined stresses, indicating a crosstalk effect in multiple stresses, but the potential of using those six metabolites as stress markers has not been concluded (Sun *et al.*, 2015).

In rice floral organ development provided mechanistic understandings of the responses of rice floral organs to combined stresses, in which integrative analyses on metabolomics and transcriptomic features of floral organs revealed that sugar starvation

is the determinant of the failure of reproductive success under heat and drought stress in rice (Li *et al.*, 2015). Heat-sensitive (Moroberekan) anther has lower levels of sucrose and myo-inositol but higher level of galactinol and raffinose, while heat-tolerant (N22) anther has lower abundances of glucose-6-P and fructose-6-P (Li *et al.*, 2015). Consistent with metabolomic changes in anther, Moroberekan rice has significantly up-regulated expression of the intercellular sugar transport regulation gene Carbon Starved Anthers (CSA) (Zhang *et al.*, 2010), while N22 rice shows the enhanced expression of MST8, a sugar transporter gene, and INV4, a cellwall invertase gene (Li *et al.*, 2015; Zhang *et al.*, 2010).

The combined cold and dehydration stresses in rice cause the up-regulation of carbohydrate metabolism associated genes, which is consistent with the build-up of glucose, fructose, and sucrose in the aerial parts of the plant (Maruyama *et al.*, 2014). Several sugars such as sucrose, raffinose, maltose, and glucose frequently accumulate in plant cells suffering combinatory stresses, perhaps protecting plants via osmotic adjustment from oxidative damage that usually follows most stress conditions (Rizhsky *et al.*, 2004; Wulff-Zottele *et al.*, 2010). A combined stress normally result in a more extreme condition than that of each individual stress alone, and therefore has profound effects on central metabolisms such as sugars and their phosphates and sulfur-containing compounds (Caldana *et al.*, 2011; Rizhsky *et al.*, 2004; Wulff-Zottele *et al.*, 2010). Interestingly, the combined elevated CO₂ and salinity stress exerts a milder effect on the metabolic physiology of the plants than that of salinity stress alone (Kanani *et al.*, 2010), indicating that there is a complicated crosstalk between different stresses.

Plants possess defense mechanisms against various stresses so they can adapt to unfavorable surroundings. Since the synthesis of specific metabolites is one of the most well-known defense reactions, the investigation of defense reactions with a metabolomics approach can help the holistic understanding of the strategies of plants against stresses (Yozo and Kazuki, 2012).

2.6.4 Integration of omics analysis

Integrated omics analyses have markedly increased our understanding of plant responses to various stresses. These analyses are important for comprehensive analyses of abiotic stress responses, especially the final steps of stress signal transduction pathways.

Integrated analyses of the transcriptome and the metabolome successfully demonstrate connections between genes and metabolites, elucidating a wide range of signal output from ABA under dehydration (Urano *et al.*, 2009) and the DREB1/CBF transcription factors in response to low temperature (Cook *et al.*, 2004; Maruyama *et al.*, 2009). Metabolite profiling reveals that ABA accumulates during dehydration, regulating the accumulation of various amino acids and sugars such as glucose and fructose. Comparative metabolite analysis between *Arabidopsis* responding to heat shock and cold shock revealed that the majority of metabolites in response to heat shock overlapped with those produced in response to cold shock (Maruyama *et al.*, 2009; Salekdeh *et al.*, 2009). The results indicate that a metabolic network of compatible solutes includes proline, monosaccharides (glucose and fructose), galactinol, and raffinose, which have an important role in tolerance to temperature stress. Transcriptomics, metabolomics and enzyme activities were integrated in a comprehensive study of K deficiency (Armengaud *et al.*, 2009). Carbon and nitrogen metabolism were significantly affected by K deficiency. This integrated approach pinpointed that pyruvate kinase activity (not transcription) was inhibited directly by K deficiency and was primarily responsible for the metabolic disorders observed.

Systems biology approaches have given us a more holistic view of the molecular responses. Transcriptomics studies are well advanced, but proteomics analyses are lagging behind, especially the study of post-translational modifications. Plant responses to abiotic stress are dynamic and complex. The integration of multiple omics studies has revealed new areas of interactions and regulation. Time series experiments have revealed the kinetics of stress responses, identifying multiple response phases involving core sets of genes and condition-dependent changes. One consistent trend in response to abiotic stress is the early down regulation of energy metabolism and protein synthesis. This may

indicate a conservation of energy by the plant and may reflect a shift from plant growth to protective mechanisms (Cramer *et al.*, 2011).

2.7 Multi-gene manipulation

Signal transduction under stress involve interaction of many important traits, as well as complex metabolic pathways, depend on interactions among a number of genes, and so genetic engineering must now advance from manipulation of single-gene traits to manipulation of polygenic traits, multiple traits, and multiple gene products (Lin *et al.*, 2003).

In the early years of plant biotechnology, most transgenic plants developed have single gene along with selection marker gene controlled by either constitutive or inducible promoter. Although fairly good success has been made by this approach to improve certain plant characters, alteration of complex interactive metabolic pathways and multiple traits was still a limitation.

The use of transgenic technologies for the genetic manipulation of plant species has had a profound impact on basic plant research and biotechnology. Overexpression of heterogenous genes, for example, is widely used for the introduction of novel traits into transgenic crop plants. Overexpression can also be used in combination with down-regulation and controlled expression (*e.g.* induced, developmentally regulated, or tissue specific) studies as a tool for basic plant research and for functional analysis of native genes in various model plants. Two crucial components are required for the transient and/or stable expression of foreign genes in plant cells. The first is a plasmid vector capable of carrying the foreign gene-encoding sequence and the regulatory elements, i.e. promoter and terminator sequences, needed for its expression in plant cells (thus collectively referred to as an expression cassette). The second is the biological or physical methods by which the vector can be delivered into the target plant cells. With the exception of virus mediated gene expression, where the distinction between the plasmid vector and the vehicle itself is rather blurry, biolistics, polyethylene glycol, and *Agrobacterium* mediated gene transfer all require plasmid vectors, some of which have been specifically designed for optimal use by a particular method (Mery and Tzvi, 2007).

A number of approaches have been used to introduce multiple genes into plant genomes and then to coordinate transgene expression. These approaches include sexual crossing between plants carrying separate transgenes (Bizily *et al.*, 2000), sequential retransformation (Lapierre *et al.*, 1999), and co-transformation with multiple plasmids (Ye *et al.*, 2000) or with single plasmids on which several transgenes are linked (Goderis *et al.*, 2002).

Recently, co-transformation approach was used to introduce multiple genes into plants. In co- transformation approach, multiple transgenes harboured within different T-DNAs in a single *Agrobacterium* strain or harboured separately within different strains are introduced simultaneously into plants via direct (*e.g.*, biolistics) or indirect (*Agrobacterium*- mediated) transformation methods (Chen *et al.*, 1998; Ye *et al.*, 2000). A major advantage of co-transformation is that the co-introduced transgenes tend to co-integrate at the same chromosomal position in a high proportion of transgenics. This ensures that genes are unlikely to segregate apart in subsequent generations. Co-transformation via particle bombardment has also been used to simultaneously introduce three insecticidal genes (the Bt genes cry1Ac and cry2A, and the snowdrop lectin gene gna) into indica rice (Maqbool *et al.*, 2001). However, as the number of transgenes increases, any linked co- transformation strategy with conventional vectors becomes much less efficient owing to vector instability, the lack of unique restriction sites during iterative cloning and the fact that larger input DNA sequences are more likely to fragment.

In the multigene linking strategy, multiple transgenes are introduced simultaneously into plants by linking multiple transgene expression cassettes into a single T-DNA or transformation vector. Gateway based vector systems are an effective tool that can be made use of, which enables multiple transgenes to be directly linked or fused (Multisite Gateway Cloning, Invitrogen, USA). The gateway technology is a flexible and universal cloning approach based on λ phage site specific recombination (Hartley *et al.*, 2000). This exploits the bacteriophage λ recombination system; thereby bypassing the need for traditional ligase mediated cloning.

In multiround gateway, once the DNA fragments of interest are cloned into the two entry vectors, they can be introduced into a destination vector in a defined order and orientation through multiple rounds of gateway recombination reactions (Chen *et al.*, 2006). For multisite gateway, three fragment recombination, three PCR products (gene of interest) flanked by specific attB or attBr sites and three multisite gateway donor vectors are used in separate BP recombination reactions to generate three entry clones. These three entry clones and destination vector are used together in multisite gateway LR recombination reaction to create expression clone containing three DNA elements (genes). Hence, by this technique multiple abiotic stress responsive transgenes can be delivered sequentially and indefinitely into a gateway compatible destination vector through alternate use of two special entry vectors.

Recently, a modified gateway multisite cloning vector has been developed using hybrid cloning strategy. This approach involves both traditional cloning and gateway recombination cloning strategy. It involves initial restriction based cloning to modified gateway entry (pGATE) vectors containing attL sites flanking multiple cloning sites and subsequent cloning into plant expression vector (pKM12GW) with specific recombination sites by LR reaction. Using same the two *Bt* crystal protein genes have been stacked and over expressed in *Arabidopsis* (Vemmana *et al.*, 2013) and also the co expression of *AtbHLH* and *AtWRKY28* enhanced expression of many downstream target genes and transgenics showed improved stress tolerance (Babitha *et al.*, 2013). Therefore, the stacking of upstream regulatory genes is considered as an elite strategy in bringing multiple stress tolerance in crop plants.

Utilizing multigene cassette technology, transgenic groundnut harboring *PgHSF4*:*PDH45*:*OsAlfin1* under different promoters RD29A (inducible), 2XCaMV35S (constitutive), CaMV35S (constitutive) and terminators nos, poly A, poly A respectively have been developed. These transgenics showed wide range of abiotic stress tolerance compared to wild type plants (Vemanna *et al.*, 2015).

Existing information indicates the complex drought adaptive mechanisms/traits. Some of them are put under the category of primary, secondary and inter related traits. Considerable efforts made to manipulate specific traits by using upstream genes. The major goal of this study is to manipulate cellular level tolerance by co-expressing two regulatory genes and in root growth one transcription factor which are well validated and reported in the literature.

Material and methods

III. MATERIAL AND METHODS

The main emphasis of this study is to improve the adaptation of groundnut to drought stress by altering cellular tolerance mechanisms and root traits. The cellular tolerance mechanisms and higher root growth traits significantly contribute to improve the intrinsic tolerance of plant cell and increase the water uptake and mining abilities to sustain growth and development under drought stress. Therefore, genes involved in protein turnover, protein stability and protein folding (PDH45, PgHSF4) and root growth-associated gene (Alfin1) was simultaneously expressed in groundnut variety GPBD4. Modified multigene stacking approach was used to develop multigene construct (Fig. 5).

Location and plant species used in the study

All experiments were conducted in green house/ containment facility of the Department of Crop Physiology, University of Agricultural Sciences, GKVK campus, Bengaluru, Karnataka. The site is situated at 12⁰ 58' North latitude, 77⁰ 35' East longitudes and an altitude of 930m above Mean Sea Level (MSL). The plant species used for the study was groundnut (*Arachis hypogaea*).

In order to achieve this objective the following experiments were conducted

1. Evaluation of T₄ transgenic lines to identify phenotypically superior event co-expressing HSF4, PDH 45 and Alfin1.
2. Comparative analysis of multigene transgenics with single gene transgenics
3. To examine molecular basis of differential response of transgenic plants co-expressing HSF4, PDH 45 and Alfin1.

In this chapter, initially the general protocols and techniques are described. Later, the experimental details used in the study are described.

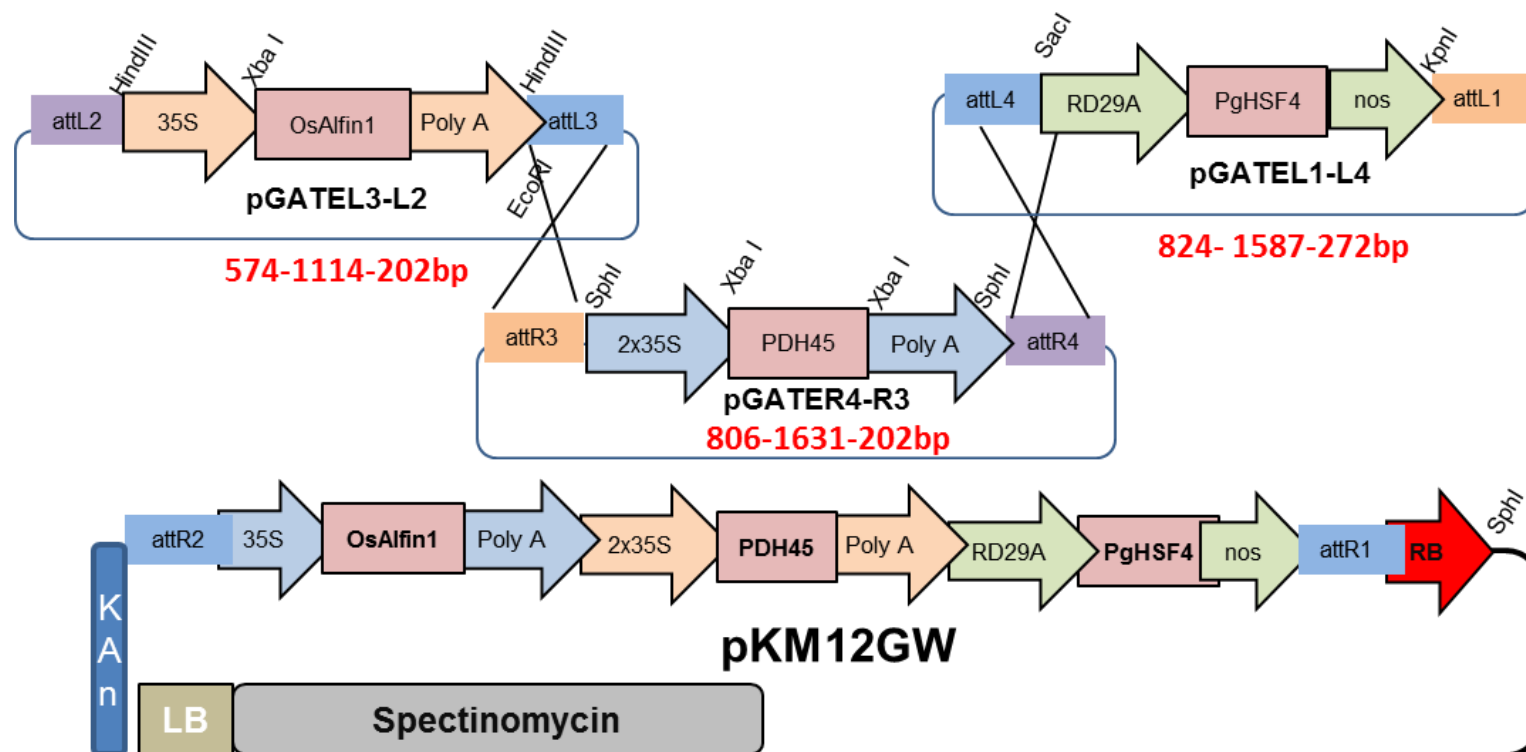


Fig. 5: Schematic representation of destination vector pKM12GW after LR reaction having different genes. Entry vectors pGATEL3L2:OsAlfin1, pGATEL1L4:PgHSF4 and pGATER4R3:PDH45 subjected to LR reaction to develop destination vector

3.1 General protocols and techniques

3.1.1 Molecular biology protocols

3.1.1.1 Genomic DNA extraction by CTAB method

Genomic DNA was extracted from peanut leaf tissue as described by Doyle and Doyle (1989), with some modifications. The plant material (500 mg) was ground in liquid nitrogen and homogenized in 7.5 ml of pre-warmed (65 °C) DNA extraction buffer [2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; and 0.2 % β mercaptoethanol (added *in-situ* before DNA extraction)] and further incubated at 65 °C in water bath for 30 min. An equal volume (7.5 ml) of chloroform: isoamylalcohol (24:1) was added after cooling it to room temperature and centrifuged at 20 °C for 20 min at 6000 rpm. To the supernatant (10 ml) 2/3 volume (6 ml) of chilled isopropanol was added and incubated at 4 °C for 1-2 h. The mixture was later centrifuged at 8000 rpm for 15 min at 20 °C and the pellet resuspended in 5 ml of 70 per cent ethanol and centrifuged again (10 min, 8000 rpm, 20 °C). The supernatant was carefully discarded; the pellet air dried and then resuspended in 500 μ l high salt TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at room temperature. To this mixture 10 μ l RNase A (1mg/ml) was added and incubated at 37 °C for 1 h. An equal volume (500 μ l) of cold phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged for 5 min at 10000 rpm. The supernatant was mixed with an equal volume of chloroform: isoamylalcohol (24:1) and again centrifuged for 5 min at 10000 rpm. To the resulting supernatant, twice the volume of absolute ethanol was added and incubated at -20 °C for 1-2 h. The mixture was centrifuged (10 min, 14000 rpm, 4°C) and the pellet washed twice in 70 per cent ethanol, air dried and resuspended in 100 μ l TE buffer (10 mM) and stored at -20 °C.

3.1.1.2 Polymerase chain reaction

The DNA fragments were amplified from various plasmid DNA/genomic DNA/cDNA in a 20 μ l reaction volume containing 100 ng of template DNA, 2 μ l PCR buffer (10X), 2 μ l dNTPs (2 mM), 1 μ l forward primer (5 pmol/ μ l), 1 μ l reverse primer (5 pmol/ μ l), 1 U Taq DNA polymerase and volume made up to 20 μ l with sterile water. PCR was performed in Master cycler Gradient (Eppendorf AG, Germany). The

optimal number of PCR cycles and the annealing temperature (TA) was determined empirically for each PCR. A standard PCR program followed is as follows;

Step 1: 95 °C for 5 min (initial denaturation)

Step 2: 95 °C for 1 min (denaturation)

Step 3: AT for 30 sec (primer annealing)

Step 4: 72 °C for 1 min/kb fragment (DNA synthesis)

Step 5: Go to step 2; repeat 25-35 cycles

Step 6: 72 °C for 10 min (final extension)

Step 7: Hold at 4 °C; end

The PCR samples were analysed by agarose electrophoresis. The presence of the expected PCR products was determined with the help of DNA ladders (GeneRuler DNA ladders, MBI-Fermentas) (Sambrook *et al.*, 1989).

3.1.1.3 Isolation of total RNA

Total RNA was extracted according to the protocol described by Datta *et al.*, (1989) with some modifications. All care was taken to use RNase free material during RNA isolation. All glassware's and plasticwares were treated with 0.1 per cent (v/v) diethyl pyro carbonate (DEPC) all solutions were prepared using DEPC treated water and were autoclaved. About 1 g of frozen tissue was taken in mortar and pestle and ground to a fine powder in liquid nitrogen. The tissue was homogenized with 10 ml of RNA extraction buffer (0.1 M Tris-HCl, pH 9.0; 0.25 M sucrose; 0.2 M NaCl and 0.01 M MgCl₂), 1 ml of 0.5 M EDTA, 1 ml of 20 per cent SDS followed by 10 ml of water saturated phenol: chloroform (1:1) and β-mercaptoethanol was added to the final concentration of 0.2 per cent. The homogenized mixture was taken in oakridge tubes and incubated at 4 °C for 20 min while shaking. The tubes were centrifuged at 16000 rpm for 30 min at 4 °C. The supernatant was transferred into a fresh tube and equal volume of chloroform: isoamyl alcohol (49:1) added, vortexed and centrifuged at 15000 rpm for 15 min at 4 °C. The aqueous phase was transferred to another tube and 8 M LiCl was added

to the final concentration of 3 M and incubated at -70 °C overnight. The thawed tubes were centrifuged for 30 min at 15000 rpm at 4 °C. The pellet was then washed with 2 M LiCl followed by one wash with 70 per cent (v/v) ethyl alcohol. The resulting pellet was air dried at room temperature and dissolved in 50 µl RNase free water. The concentration and purity of the total RNA was ascertained spectro-photometrically (Nano drop, Thermo-Scientific, USA) at 260/280nm. The quality was also checked on formaldehyde denaturing agarose (0.8 %) gel.

3.1.1.4 Synthesis of complementary DNA (cDNA) from RNA

First strand cDNA was synthesized by oligo (dT) primers using Molony Murine Leukaemia Virus reverse transcriptase enzyme (MMLV-RT; MBI Fermentas, Hanover, MD, USA). About 2 µg of RNA was reverse transcribed in a 20 µl reaction mixture containing 100 U of MMLV, 2 µl of 20 pico moles oligo dT primer, 1X reaction buffer with 10 mM dNTPs mix. The reverse transcription was performed at 42 °C for one and a half hour (Archana *et al.*, 2009).

3.1.1.5 Semi-quantitative RT-PCR analysis

The cDNA was used as template to perform semi quantitative RT-PCR analysis using gene specific primers. Elongation factor (Elf) was used as loading control. PCR analysis was carried out using standardized PCR conditions as follows: Initial denaturation of 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 30 sec, annealing 52-58⁰ for 30 sec, and polymerization for 45 sec (72 °C) followed by a final extension for 5 min (Babitha *et al.*, 2013).

3.1.1.6 Agarose gel separation of DNA

Separation of DNA fragments was performed using horizontal gel electrophoresis. Depending on the requirement 0.8 to 1.2 per cent agarose solutions were prepared in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA and pH-8.0) by heating in a microwave oven. After cooling to 50 °C, ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 µg/ml to the solution and poured into a mold with comb in the apparatus. The comb was carefully removed when the gel was completely set, and the gel

was mounted into the electrophoresis tank containing 1X TAE buffer. DNA samples were mixed with 0.1 volume loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 15 % glycerol) and loaded into the slots of the submerged gel. The DNA fragments were electrophoretically separated (65-70 mA, 45-60 min) using a 1 kb ladder or 100 bp DNA ladder or I-DNA/Hind III digest as the reference marker. The detection of the DNA fragments was carried out under UV-trans-illuminator (302 nm) and photographed using a gel documentation system (Herolab, GmbH Laborgerate, Germany) (Sambrook *et al.*, 1989).

3.1.1.7 Southern blot analysis

Thirty micrograms of purified genomic DNA of transgenic and wild-type plant was digested overnight with EcoRI that cuts once within T-DNA region for the analysis of copy number by Southern hybridization. The digested DNA samples were electrophoresed on a 0.8% (v/v) agarose gel in TAE buffer and blotted on to a positively charged nylon membrane (Amersham GE healthcare Pvt. Ltd., Bangalore, India). The 1 kb length of Alfin1 fragment was restriction digested for labelling by Dig-labelled kit (Amersham, GE healthcare Pvt. Ltd.). Hybridization and washing was carried out according to user manual. The signal was developed using chemiluminescence substrate (CDP-star) reagent and exposed to X-ray and autoradiogram according to Sambrook *et al.*, 1989.

3.1.2 Protocols for stress imposition

3.1.2.1 Salinity induction response

Two days old seedlings of uniform size and adequate numbers (8-10) were placed in a petriplate lined with filter paper. Three such replicated plates form one set and three sets are prepared viz., set-1 (induced), set-2 (non-induced) and set-3 (untreated control). Set-1 seeds were subjected to inducing NaCl concentrations viz., 100mM for 8hrs. After induction the set-1 seedlings were again exposed to 300mM for 8hrs after which they were transferred to water for the next 3 days for recovery. Set-2 seedlings were directly transferred to 300mM NaCl for 8hrs after which they are transferred to water for the next 3 days for recovery. Set-3 was maintained as untreated control (in water) throughout the

experiment. At the end of recovery period, the seedlings from set-1, set-2 and set-3 were observed and root length was recorded.

3.1.2.2 Salinity stress imposition in Hydroponics system

The groundnut transgenic seedlings were raised in hydroponic facility and nutrients were supplied through Hoagland's solution. The 10-day-old seedlings were exposed to NaCl stress by initially treating with 100 mM for 5 days, later 250mM NaCl was given as lethal stress for 3 days and then subjected to recovery for 10 days. The root length of transgenic and wild type plants was measured after recovery and total dry matter (TDM) was recorded for wild type and transgenic seedlings. The plants were photographed to show the root growth.

3.1.2.3 Drought stress imposition by gravimetric method

To impose moisture stress at 20-day-old seedling stage, the plants were raised in pots with 2.5 kg of soil. To arrive at relative comparison in each pot, one plant each of wild type and transgenic event was maintained. Adapting the gravimetric approach, the pots were weighed twice a day to measure the water loss in evapotranspiration and required amount of water was replenished to maintain the desired soil moisture status (FC). Altogether, three sets of plants were raised with five replications, one set of plants were maintained at 100% FC, the second and third set of plants maintained at 100% FC till 20 days, and subsequently, gradual moisture stress was imposed to arrive at desired soil moisture status. For the second set, the gradual stress was imposed till the soil FC reduced to 50% FC on 35th day, and in the third set, to 30% FC. At the end of the stress period, the plants were re-watered to 100% FC, and after a week, recovery growth was recorded.

3.1.2.3 Stress imposition on plants grown in contained field condition

Managed drought environment: The transgenic plants along with wild type were grown in managed drought environment facility (containment facility), rain free conditions in the MDE facilities are created by erecting the rain out shelter. The groundnut transgenic lines along with wild type were sown in this containment MDE facility having semi-

automated irrigation with sprinkler facility adjusted to movable rain-out shelter. Two plots were maintained and were irrigated every alternate day with 10 mm of water through sprinkler system. When the plants were 50-day-old, one of the stress plots received irrigation once in every week (10 mm water). Such irrigation schedule was continued up to harvesting and the total amount of water provided was 180 mm for stress plot and the control plot was given a total amount of 500 mm water.

Contained field condition in summer season: The groundnut transgenic lines were grown in containment field facility (net house facility). The spacing provided was 30×10 cm with 10 plants in each row. When the plants were 50 days old in one of the plot the stress was imposed for a period of 20 days and another plot kept as a control. After 50 days and 80 days after sowing (DAS) gas exchange parameters were recorded and after recording the parameters the plants were pulled out to measure various growth parameters like plant height, number of leaves, total leaf area and total dry matter (TDM). After 120 DAS plants were harvested and analyzed for yield parameters like pod yield and TDM. Measurement of growth and yield parameters were described in 3.1.3.1a and 3.1.3.1b respectively.

3.1.2.5 Salinity stress imposition at whole plant level

Peanut transgenic and wild type plants were raised in pots with three replications each for 15 days and later subjected to salinity stress by gravimetric approach. The pots were weighed regularly to measure the water loss in evapo-transpiration, the amount of water loss by evapo-transpiration was replenished by 50mM NaCl for 3 days and then increased to 100mM NaCl for 3 days. After stress period plants were analyzed for relative water content (RWC), per cent leakage, root length and TDM.

3.1.2.6 DTT stress imposition

Germinated seeds of transgenic and wild type plants were placed on half MS agar medium and supplemented with DTT (5mM) for 2 days and then transferred to water for recovery. The plates were observed for seedling growth after recovery. Photographs were taken after recovery.

3.1.3 Estimation of physiological and biochemical parameters

3.1.3.1 Physiological parameters

3.1.3.1a Measurement of growth parameters

The transgenic and wild type plants were analysed for the phenotypic characters. Different growth parameters were analysed by recording the plant height (cm), total leaf area (cm²), root length (cm), root volume (ml), stem weight (g), leaf weight (g), number of leaves, leaf area index (LAI), net assimilation rate (NAR) and crop growth rate (CGR). Three individual lines from transgenics were taken for measurements.

Plant height was measured from the base of plant above soil to the shoot apex. Leaf area was computed by using leaf area meter. Root length (cm) was measured from the base to the tip of the root. Root volume (ml) was measured by dipping root in measuring cylinder containing known amount of water. The amount of water displaced by the root when it was immersed into known volume of water was recorded. The plants harvested and leaves were separated from the plant and then stem and leaf weight was recorded. Total number of leaves was counted at different stages like 50 and 80 days after sowing.

LAI was calculated using the formula (Watson, 1947)

$$\text{LAI} = \text{leaf area at 50 DAS} / \text{spacing}$$

NAR was arrived for the duration between 50 DAS to 80DAS and calculated by using the method of Gregory (1926) and expressed as g.m⁻².day⁻¹.

$$\text{NAR (g.m}^{-2}\text{.day}^{-1}\text{)} = \frac{D_2 - D_1}{T_2 - T_1} \times \frac{\ln LA_2 - \ln LA_1}{LA_2 - LA_1}$$

where, D₁ and D₂ are total dry weight of plant at times T₁ and T₂, respectively. LA₁ and LA₂ are leaf area at times T₁ and T₂, respectively. ln represents values of natural log to the base 'e'.

Crop growth rate is the amount of biomass accumulated per unit land area per unit time. CGR was calculated for the duration between 50 DAS to 80DAS by using the modified formula suggested by Watson (1952).

$$\text{CGR (g.m}^{-2}\text{.day}^{-1}) = \text{NAR} \times \text{mean LAI}$$

3.1.3.1b Productivity analysis of transgenics

The transgenic and wild type plants were harvested at 120 DAS and productivity was assessed by recording yield and biomass. Biomass was recorded by drying plant samples at 60 °C in drying oven. Filled and unfilled pods was separated and dried and then total filled pod weight was recorded. Three individual lines from transgenics were taken for measurements.

3.1.3.1c Gas exchange parameters

Gas exchange parameters such as photosynthetic rate ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$) and stomatal conductance ($\text{mmol.m}^{-2}.\text{s}^{-1}$) were measured in transgenic and wild-type plants at 50 and 80 days after sowing by using a portable photosynthetic system (LICOR 6400, USA). The measurements were made at an ambient CO_2 concentration of $360 \mu\text{molmol}^{-1}$ and PPFD of $1400 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ using LICOR light source and chamber temperature of 28°C (Nataraja and Jacob, 1999)

3.1.3.1d Relative water content (RWC)

The leaves collected from plants exposed to salinity stress were quantified according to Barrs and Weatherly (1962). Initially, fresh weight (FW) of leaf discs was recorded and the discs were then floated in 5 ml of water for 6 h, and allowed to gain turgidity. Turgid weights (TW) were recorded and dried in oven at 80°C to constant weight to record dry weight (DW). RWC was quantified and expressed in percent using the following formula.

$$\text{RWC} = \left[\frac{\text{FW}-\text{DW}}{\text{TW}-\text{DW}} \right] \times 100$$

3.1.3.1e Membrane damage assessment

The extent of stress induced damage to cellular machinery was assessed by electrical conductivity (EC) of solution containing leaf discs (Sullivan's test) (Campos *et al.*, 2003). The leaf discs collected from the control and stress treatment were washed in distilled water, blotted on Whatman filter paper and air dried. The leaf discs were incubated in 25 ml of water with continuous shaking for 2 h. Initial EC was taken using EC-TDS analyzer (ELICO-CM183). Then the leaf discs were boiled for 30 min and final EC was taken. The cell leakage was computed using the formula,

$$\text{Cell leakage (\%)} = \{(\text{Initial EC})/(\text{Final EC})\} * 100$$

3.1.3.2 Biochemical parameters

3.1.3.2a Methly glyoxal estimation

100 mg each of wild type and transgenic lines of both control and moisture stress samples of peanut were ground in distilled water and centrifuged at 11000 rpm for 10 minutes at 4⁰C and later, supernatant was collected. If supernatants are coloured (which happens when leaf and other plant extracts are used), charcoal at the rate of 10mg/ml was added to decolorize the supernatant. Once decolorized, the supernatants were neutralized by keeping in saturated solution of potassium carbonate at room temperature for 15 minutes and centrifuged again at 11000rpm for 10 minutes. The neutralized supernatant so collected was used for Methyl Glyoxal (MG) estimation. In order to quantify the MG content, 250 µl of 7.2mM of 1,2-diamino benzene (1,2-phenylene diamine), 100µl of 5 M perchloric acid and 650 µl of the neutralized supernatant were added in the same order. After mixing the contents thoroughly, the absorbance was read at 336 nm using spectrophotometer. Against MG standards, the OD values were substituted to quantify MG (Yadav *et al.*, 2005).

3.1.3.2b Measurement of malondialdehyde (MDA) content

Malondialdehyde is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. About 0.5-1.0 g of tissue was homogenized in 5 mL of 5% (w/v) trichloroacetic acid and the

homogenate was centrifuged at 12,000 g for 15 min at room temperature. The supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroacetic acid], and the mixture was boiled for 25 min at 100°C, followed by centrifugation for 5 min at 7,500 g to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the A600. MDA content in leaf tissue was calculated using standard graph (Loreto and Velikova, 2001).

3.1.3.2c Total protein quantification

Leaf material from plants were frozen in liquid nitrogen and ground in 100 mM Tris HCl buffer (pH 7.8) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 5 mM benzamidine and the solution was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was used for quantification of protein by following Bradford method (Bradford, 1976). BSA standard curve was prepared (0.1-1.0 mg/ml) and determined the protein concentration of the plant extract was determined from the regression curve.

3.2 Identification of superior lines by statistical analysis

Since the aim of the study was to identify superior transgenic lines with stress tolerance and high yielding type, correlation and z-distribution analysis were employed.

3.2.1 Correlation analysis

The yield parameters recorded for all the lines were analyzed using correlation analysis. The mean values were calculated for these parameters which consisted of three replications. The values of any two parameters were plotted as X-Y scatter to arrive at the relation between these two parameters.

3.2.2 Z-distribution analysis

The mean value and standard deviation for stress response, yield and biomass was calculated for all the lines and Z-distribution was performed between two parameters using Z-value arrived by the following formula,

$$Z\text{-value} = \frac{(\text{Grand mean value} - \text{specific mean value})}{\text{Standard deviation}}$$

This data provides an option to identify the plants which has highest value for a parameter amongst the whole lot of plants from both the experiments. The values for any two parameters were plotted as X-Y scatter and resulting graph shows four quadrants. The plants which have highest values for both parameters will have highest negative Z-value and falls in the 3rd quadrant (-, -). The plants which shows least values have highest positive Z-value will fall in 1st quadrant (+, +). The plants which shows more negative in one parameter and positive in another parameter will fall in the 2nd quadrant (+, -) and 4th quadrant (-, +). Since, we need lines which are stress responsive and high productivity type, lines coming in 3rd quadrant were selected as superior lines for further analysis in next generation.

3.3 Statistical analysis

Student t-test: The data obtained in different experimental results were analysed using student t-test as per the procedure given by Fisher (1960). The level of significance used in 't' test was $P = 0.05$

3.4 System biology: “Omics” protocols

3.4.1 Transcriptomics

For a comprehensive understanding of the groundnut transgenic plants, transcriptome analysis was performed using groundnut leaf samples by using RNA sequencing technology i.e., Illumina NextSeq500 platform by Genotypic Technology Private Limited, Bangalore. Groundnut wild type and transgenic plants were subjected to moisture and non-moisture stress through gravimetrically and then samples were collected from both wildtype and transgenic plants for transcriptome analysis. Four samples namely wild type control (WC1), wild type stress (WSII), transgenic control (TC1) and transgenic stress (TS1) were analyzed.

The Illumina NextSeq paired end raw reads were quality checked using FastQC 0.8.0. Quality reads were obtained by trimming the raw reads at a minimum PHRED score of Q20. *De novo* assembly of processed reads data was performed using Trinity for default k-mers i.e 25. Transcripts were annotated using NCBI BLAST 2.2.29 with the proteins of Viridiplantae taken from Uniprot database. Differential expression of gene(s) was estimated based on the number of reads mapped against wildtype and transgenic plants using DeSeq tool.

3.4.2 Proteomics

Protein Extraction

Protein extraction was performed according to a modified version of the trichloroacetic acid (TCA) acetone precipitation method (Isaacson *et al.*, 2006). One gram of lyophilized leaf tissues were ground in liquid nitrogen and suspended in the ice cold 10% TCA-Acetone mixture contained 2% of β -mercaptoethanol and incubated for overnight at -20°C . The tubes were centrifuged at 10000 rpm for 20 min at 4°C . The supernatant was discarded and the pellet was kept it for drying. To the pellet 30 mg of PVPP and 5 ml of extraction buffer (0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl, pH 7.5 and 50 mM EDTA+ 2% β -mercaptoethanol and 1% SDS) was added and vortexed for 15 min. The mixture was centrifuged at 10000 rpm for 15 min at 4°C and this step was repeated twice until other residues goes from the supernatant. To the clear supernatant 5 ml of phenol was added and vigorously vortexed for 15 min and centrifuged at 10000 rpm for 20 min at 25°C . The phenolic phase was collected and transferred into fresh tube and added 5ml of extraction buffer and vortexed the tubes for 15 min and centrifuged at 10000 rpm for 20 min at 25°C . The phenolic phase was collected and added 0.1 M ammonium acetate prepared in methanol of 2% β -mercaptoethanol and made the volume up to 12mL. The tubes were briefly vortexed and incubated at -20°C for overnight. The tubes were centrifuged at 10000 rpm for 20 min at 4°C . The supernatant was discarded. The pellet was washed with methanol containing 2% β -mercaptoethanol and this step was repeated. The final pellet was resuspended in methanol containing 2% of β -mercaptoethanol and vortexed vigorously for 15 min. The dispersed pellet was transferred into Low bind centrifuge tubes and centrifuged at 12000 rpm for 20 min at

4°C. Again the pellet was washed with acetone containing 2% β -mercaptoethanol by centrifuging at 12000 rpm for 20 min at 4°C. The above step was repeated. The final pellet was used for solubilization and subsequently for trypsinization. The protein concentrations were determined by the methods of Bradford (1976) using bovine serum albumin as the standard, and then the protein was stored at -80°C.

Trypsinization

Trypsinization of the protein was done according to Kazi *et al.*, (2017) 100 μ g complex protein mixture was taken and washed twice with 500 μ L of 0.1% Rapigest (1 vial diluted in 1000 μ L 50mM AmBic) using a MW 5000 (or MW 3000) cut off spin column. The proteins were denatured by heating to 80°C for 15 minutes. Proteins were reduced and alkylated by 5 μ L of 100mM DTT (made up in 50mM AmBic) and the solution was heated at 60°C for 15 minutes. And 5 μ L of 200mM (made up in 50mM AmBic) Iodoacetamide was added and incubated at 30°C. 1 μ g/ μ l trypsin (made up in 50mM AmBic) was added and the solution was left overnight at 37°C. The trypsin digestion was stopped by adding HCl incubated at 37°C for 20 minutes. The supernatant was collected, passed through 3000 or 5000 Da cut off columns and then the filtrate was transferred to a protein LOBind tube. Digested peptides were cleaned up by means of C18 chromatography using Zip tips (Millipore, Billerica, MA) and concentrated using vacuum concentrator. Peptides were reconstituted in 3% ACN with 0.1% formic Acid.

Liquid chromatography-mass spectrometry analysis (LC-MS/MS analysis)

SWATH MS analysis

All samples were analyzed on Triple-TOF 5600 (AB Sciex; Concord, Canada) mass spectrometer coupled with Micro LC 200 (Eksigent; Dublin, CA) in high-sensitivity mode. In order to generate the SWATH spectral library, equal amounts of peptide samples from each stage were analyzed via Information dependent acquisition (IDA) on LC-MS/MS. Accumulation time for MS was 0.25ms and that for MS/MS was 0.01ms. Fragmentation was done using rolling collision energy. SWATH MS datasets were acquired for 2 biological replicates and 4 technical replicates using beta galactosidase digest (AB Sciex) as an internal standard. Briefly, peptides were injected onto an

Eksigent C18-RP HPLC column (100×0.3mm, 3µm, 120 Å) and then separated using a 95-min gradient of 3% to 35% acetonitrile in water with 0.1% (v/v) formic acid at a flow rate of 8 µL/min. MS1 spectra were collected over the precursor mass range of 400–1250 m/z whereas SWATH MS/MS spectra were collected from 100 to 2000 m/z.

To get spectral library from IDA run, data was analyzed by Protein Pilot software using UniProt containing number of reviewed protein entries. Generated spectral library was used as database for SWATH analysis with 20ppm mass error. SWATH runs were uploaded in swath processing window of PeakView software with 50 ppm error, 5min mass window and 99% confidence. SWATH file was exported to MarkerView software to get quantitative analysis of proteins, peptides and ions in the different stages. The reproducibility of individual acquisitions was evaluated by performing PCA/PLSDA analysis using the web server MetaboAnalyst. Gene ontology analysis of the differentially expressed proteins was performed using UniprotKB

3.4.3 Metabolomics

Sampling and metabolite extraction

Peanut control and moisture stress samples were sampled at the same time (midday). Leaf tissue were harvested and immediately frozen in liquid nitrogen. Frozen samples were stored in -80°C.

Standardization of metabolite extraction protocol was done for the samples obtained from control plants. Organic solvents, HPLC grade methanol and water were used for metabolite extraction. Different concentrations of methanol (100, 80 and 50 %) were prepared, and extraction was done according to Pushpa *et al.*, (2014) with some modifications. Metabolites extracted with different concentration of methanol were fed to HRMS (High resolution mass spectrometry), raw files were analyzed to check the number of peaks to standardize the concentration of extraction solvent.

Extraction of metabolites in wild type and transgenics

Metabolites were extracted from samples 1-2 days ahead of their analysis. The samples stored at -80°C were crushed in liquid nitrogen using a mortar and pestle which was cleaned with methanol and precooled with liquid nitrogen. 100 mg of the powdered sample were placed in a 2 mL microcentrifuge tube that was washed with methanol and precooled with liquid nitrogen; 400 μL of 50% cold methanol was added. To this an internal standard, 1,4-bis(2-ethanesulfonic acid), Piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES) (210pg/ μL), was added for abundance correction and the mixture was stirred using a vortex stirrer. Each sample was sonicated for 15 min at 40kHz in a water bath at room temperature. Sample extracts were centrifuged for 10 min at 13,000 rpm at room temperature. The supernatant was filtered through a 0.22-mm poly (vinylidene difluoride) (PVDF) membrane filter and centrifuged at 13,000 rpm for 10 min. The filtrate was placed in labeled sampling glass vials and stored at -20°C .

Metabolite analysis

The metabolites were analysed using HR/MS, with electrospray ionization, a quadrupole linear ion trap capable of MSn and an Orbitrap electrostatic fourier transform mass spectrometer capable of high mass accuracy and resolution (LC-ESI-LTQOrbitrap). The orbitrap was externally calibrated every day. A 5- μL sample extract was injected. For chromatographic separation of the compounds, a capillary hypersile GOLD column (dimension of 200 X 2.1mm, particle size of 1.9 μ and pore size of 175- $\text{\AA}$) using methanol-water gradient solvent programme of 35 min (0.0 min, 40 % methanol/water; 5.0 min, 50 % methanol/water; 10.0 min, 60 % methanol/water; 25.0 min, 65 % methanol/water; 30.0 min, 90 % methanol/water; 32.0 min, 90 % methanol/water; 34.0 min, 40 % methanol/water; 35.0 min, 40 % methanol/water) with a flow rate of 0.3 mL/min. 0.1 % LC-MS grade formic acid was added to water. MS and MS/MS runs were carried out using the tune method as follows: sheath gas (nitrogen) flow rate 45 units, auxiliary gas (nitrogen) flow rate 10 units, sweep gas (nitrogen) flow rate 2 units, spray voltage 3.60 kV, spray current 3.70 A° , capillary temperature 320°C , s-lens RF level 50, heater temperature 350°C .

HRMS raw file processing and statistical analysis

Thermo Scientific, Xcalibur RAW files were converted to mzXML file using ms converter. Raw data was processed with an user interactive LC/MS data processing tool, MZmine2 (Pluskal *et al.*, 2010). Prior to peak detection, baseline for all the peaks were corrected in relation to the total ion chromatograms (TIC). Chromatograms ($\square$ 0.2 min) were built from the peaks with $\square$ 10000 intensity. Peaks were deconvoluted from the chromatograms with centwave algorithm, aligned across the samples and inconsistent peaks (absent in more than 2 replicates) were removed. Data matrix was exported to MS-Excel for further statistical analysis. The data on relative intensity of peaks (mz=mass/charge ratio with one proton subtracted due to negative ionization) were subjected to student's Ttest, using statistical analysis software (SAS 9.2) to identify peaks which are significantly different among treatment combinations TC v/s WC and TS v/s WS. The peaks significant at P, 0.05 were retained and classified into up-regulated. Then these up-regulated were subjected to metabolite identification base on two main criteria 1) accurate mass match (accurate mass error (AME=((observed mass-expected)/ expected mass) / 10^{-6}) of 5ppm with metabolites reported in different databases: Plantcyc, METLIN, KNAp- SAcK, Plant Metabolic Network (PMN), LIPIDMAPS, KEGG and Massbank) Standards spiking and matching sample fragmentation with standard compound 2) in silico fragmentation verification using Massspec scissors in ChemsSketch (ACD labs, Toronto).

3.5 Experimental detail

3.5.1 Characterization of T₄ transgenics

In T₄ generation 30 lines were selected based on canopy temperature and TDM. These 30 lines were extensively characterized for stress tolerance and productivity by conducting different set of experiments as described below

3.5.1.1 Salinity induction response

Transgenics and wild type seedlings subjected to salinity stress as described in 3.1.2.1 and later root length was recorded and photographed. The percent reduction in root length over control was calculated in wild type and transgenics.

3.5.1.2 Salinity stress in Hydroponics system

The 10 days old seedlings of wild type and transgenics were subjected to salinity stress as described in 3.1.2.2 and later root length and total dry matter (TDM) was recorded. Percent reduction in root length over control was calculated. Correlation analysis was done between root length of control and stress, TDM of control and stress condition, root length and TDM in control and stress condition was analyzed as described in 3.2.1.

The percent reduction in root length of both experiments was calculated and correlation analysis between both experiments was studied to see the variation.

3.5.1.3 Growth and productivity analysis of transgenics

After 120 day after sowing (DAS) transgenic and wild type plants were harvested, growth and yield parameters like plant height, root length, root volume, TDM and pod yield was recorded as described in 3.1.3.1a and 3.1.3.1b respectively. Correlation analysis between root dry weight and shoot dry weight; TDM and pod yield was done as described in 3.2.1.

Correlation analysis was studied between root length of hydroponics experiment and root length of pot grown plants, TDM of hydroponics experiment and TDM of pot grown plants and TDM of hydroponics experiment and pod yield of pot grown plants.

3.5.1.4 Identification of superior lines

Z- distribution analysis was done as described in 3.2.2 between TDM of hydroponics experiment and TDM of pot grown plants and TDM of hydroponics experiment and pod yield of pot grown plants to identify the superior lines.

3.5.1.5 Molecular analysis

Transgenic plants were analyzed for gene integration in transgenic lines by PCR as described in 3.1.1.2.

3.5.2 Characterization of T₅ transgenics

In T₅ generation 17 lines were selected based on stress tolerance and productivity. These 17 lines were again extensively characterized for stress tolerance and productivity by conducting different set of experiments as described below.

3.5.2.1 Salinity induction response

Transgenics and wild type seedlings subjected to salinity stress as described in 3.1.2.1 and later root length was recorded and also photographed. Percent reduction in root length over control was calculated in wild type and transgenics.

3.5.2.2 Moisture stress response of wild type and transgenics

Wild type and transgenic seedlings were subjected to moisture stress by gravimetric approach as described in 3.1.2.3 and then root length and biomass was recorded and also photographed. The percent reduction in root length and biomass was calculated in wild type and transgenics.

Bio chemical parameters like methyl glyoxal, MDA and total protein was estimated in both control and moisture stress samples of wild type and transgenics as described in 3.1.3.2a, 3.1.3.2b and 3.1.3.2c respectively. Correlation analysis was done between TDM and root length under control and stress condition and also correlation was done between root length and biomass under control and stress condition. Correlation analysis was also done between the percent increase in MDA and percent decrease in root length and biomass.

Z- distribution analysis was done as described in 3.2.2 between percent increase in MDA and percent decrease in root length and biomass to identify the superior lines.

3.5.2.3 Yield analysis under managed drought environment

Transgenic and wild type plants were analyzed for productivity by growing them under both control and moisture stress condition as described in 3.1.2.4. After harvest leaf weight, stem weight and pod weight was recorded. Percent increase in pod yield over control was calculated. Correlation analysis was performed between pod yield and biomass to identify the superior lines (3.2.2).

3.5.2.4 Molecular analysis

Transgenic plants were analyzed for gene integration in transgenic lines by PCR as described in 3.1.1.2.

3.5.2.5 Yield analysis under contained field condition in summer season

Transgenic and wild type plants were grown under contained field condition and stress was imposed as described in 3.1.2.4 and various parameters like growth parameters (3.1.3.1a), physiological parameters and yield parameters (3.1.3.1b) were recorded at 40 DAS, 80 DAS and at harvest. Correlation analysis was done between various physiological parameters at different growth intervals.

3.5.2.6 Molecular analysis

Transgenic plants were analyzed for gene integration in few selected lines by PCR as described in 3.1.1.2. In few selected transgenic southern blot was done as described in 3.1.1.7 to identify the copy number and integration of multigene cassette in groundnut. Downstream target gene expression was done as described in 3.1.1.5 20 days old plants were subjected to moisture stress and soil moisture status was maintained for 4 days. Tissue was collected and total RNA was isolated and cDNA was prepared for groundnut samples.

3.5.3 Characterization of T₆ transgenics

3.5.3.1 Yield analysis under controlled condition

Wild type and transgenic plants were grown under rainfed condition and analyzed for yield parameters like pod weight and TDM (3.1.3.1b).

3.5.3.2 Salinity stress response at whole plant level

Transgenic and wild type plants were sown in small pots for 15 days and then salinity stress was imposed to whole plant as described in 3.1.2.5 and then photographed. Various parameters like root length (3.1.3.1a), TDM (3.1.3.1b), relative water content (RWC) (3.1.3.1d) and cell leakage (3.1.3.1e) was recorded.

3.5.3.3 DTT induced stress

Wild type and transgenic germinated seeds were subjected to DTT stress as described in 3.1.2.6 and after recovery photographed.

3.5.3.4 Comparative assessment of transgenic expressing single and multi-gene

3.5.3.4a Root length variation

Groundnut transgenics expressing single gene (Shankar, 2010; Manjulatha *et al.*, 2014) and multi-gene along with their wild type were seen for root length variation in different sets of experiments.

1. At seedling level root length variation was photographed
2. Seeds were sown in small pots for 15 days and then stress was imposed for 3 days and then allowed for recovery for a week and then root length was photographed both under control and stress condition.
3. All the lines were sown in pots and then root length at harvest was photographed.

3.5.3.4b Salinity induction response

Groundnut transgenics expressing single and multi-gene along with their wild type seedlings subjected to salinity stress as described in 3.1.2.1 and later root length was recorded and also photographed.

3.6 System biology: Omics

Groundnut wild type and transgenic plants were subjected to moisture and non-moisture stress through gravimetrically and then samples were collected from both wild type and transgenic plants for transcriptome analysis (3.4.1), proteomics (3.4.2) and metabolomics (3.4.3).

Experimental Results

IV. RESULTS

The challenges of abiotic stress on plant growth and development are evident among the emerging ecological impacts of climate change, and the constraints to crop production exacerbated with the increasing human population competing for environmental resources. The field of plant abiotic stress encompasses all studies on abiotic factors or stressors from the environment that can impose stress. These stressors include extreme levels of light (high and low), temperature [high and low (chilling, freezing)], water (drought, flooding, and submergence), chemical factors (heavy metals and pH), salinity due to excessive Na⁺, deficient or in excess of essential nutrients, mechanical factors, and other less frequently occurring stressors. Since combinations of these stresses such as heat and drought frequently occur under field conditions, and can cause unique effects that cannot be predicted from individual stressors. Since plants are sessile, they have developed complex adaptive mechanisms to cope with unfavorable situation both at physiological and molecular level. Stress signal perception and transduction is a complex process involving various regulators and functional genes. Among the upstream regulators, transcription factors play a prominent role in activating various downstream genes, which are involved in the improvement of cellular tolerance under harmful conditions. The cellular tolerance mechanisms and higher root growth traits significantly contribute to improve the intrinsic tolerance of plant cell and increase the water uptake and mining abilities to sustain growth and development under drought stress condition. Therefore the emphasis of this study is to improve the adaptation of groundnut to drought stress by improving cellular tolerance mechanisms and root traits. Genetic enhancement of these traits can be achieved by either adopting conventional breeding approaches or by adopting molecular breeding and the other option is to initially identify the genes regulating these traits and by co-expressing these genes using molecular approach.

In this study the transgenic technology has been employed to improve the adaptation of groundnut to drought stress by co-expressing the genes PgHSF4, PDH45 and Alfin1 which have been already validated.

4.1 Multigene expressing binary vector pKM12GW with PgHSF4, PDH45 and OsALFIN1 with Kanamycin selection marker was developed using modified multisite gateway technology

a. Heat shock transcription factor 4 (HSF 4): HSFs regulate heat shock response via activating the expression of heat shock protein (HSP) genes by binding to the heat shock elements (HSEs). Under normal growth conditions, HSF is maintained in an inert monomer state through association with molecular chaperones such as Hsp70. In response to heat shock, HSF is converted from a transcriptional inactive monomer to active trimer using oligomerization domains, which function as sequence specific trimeric DNA binding proteins. HSFs have an N-terminal DNA binding domain (DBD) and followed by an oligomerization domain (OD). OD is composed of two hydrophobic repeats (HR-A/B) which allows homo- and hetero-multimerization (Baniwal *et al.*, 2004). A total of 16 and 17 HSF genes were identified from *Arachis duranensis* and *A. ipaensis*, respectively.

HSF4 has two isoforms, namely HSF4a and HSF4b. The splicing of HSF4 mRNA is complex, as two alternative 59 splice sites have been found in exons 8 and 9 with a frame shift in the transcript. HSF4 has one unique structural feature: both isoforms lack the HR-C necessary for suppression of HSF trimer formation, leading to constitutive DNA binding activity of HSF4 in vitro (Lila *et al.*, 2001).

The *PgHSF4* transgenic groundnut plants were developed earlier in the Department of Crop Physiology. Molecular and physiological analysis of these transgenics showed some level of stress tolerance in groundnut (Annexure I).

b. Pea DNA Helicase 45 (PDH 45): RNA helicases play an important role in translation process by unwinding the energetically stable mRNA secondary structure. PDH45 is a member of the DEAD-box protein family, it is a unique member of this family, containing DESD and SRT instead of DEAD/H and SAT in motifs V and VI respectively. The amino-acid sequence of PDH45 shows 86% similarity with the tobacco translation initiation factor eIF-4A, and 58% with eIF-4A from *Arabidopsis thaliana*, maize, rice and wheat (Pham *et al.*, 2000).

Earlier in our department to improve drought tolerance in peanut a stress responsive helicase, PDH45 was overexpressed in trait donor peanut genotype (K-134) with superior water relation traits. The study showed that transgenic lines showed stay-green phenotype and increased chlorophyll stability under stress condition. Stress-induced root growth was also substantially higher in the case of transformants. This was reflected in increased WUE (low $\Delta^{13}\text{C}$) and improved growth rates and productivity. The transgenics showed more yield under non-stress and stress conditions over wild type ascertaining the feasibility of trait pyramiding strategy for the development of drought tolerant peanut (Manjulatha *et al.*, 2014) (Annexure II).

c. Alfin1: Alfin1 is a PHD finger protein with Cys4 and HisCys3 finger structures that have DNA-binding motif suggesting that they have a role in transcriptional regulation. It also contains an acidic region characteristic of DNA-binding proteins that interact with other proteins (Kakidani and Ptashne, 1988) and therefore is likely to function as a transcription factor in plants. Alfin1 is expressed predominantly in roots, appears to be unique or a low-copy gene in the alfalfa genome, and shows conservation among such diverse plants as alfalfa, rice, and Arabidopsis (Winicov and Bastola, 1997).

In our earlier study there was increase in specific leaf area, specific leaf weight, plant biomass, root length and root volume in Alfin1 over expressing tobacco plants indicating better growth than wild type plants and also there was improved root growth in both control and salinity stress conditions (Nethra, 2010) (Annexure III).

4.2 Development of multigene construct over-expressing PgHSF4, PDH45 and OsALFIN1

Earlier in our department the multigene expressing binary vector pKM12GW harbouring three genes driven by individual promoter and terminator, RD29A:PgHSF4:nos (RD29A is a stress-inducible promoter), 2X35SCaMV:PDH45:polyA and 35SCaMV: OsAlfin1:polyA, with kanamycin selection marker was developed using modified Multisite Gateway technology. The individual gene cassettes along with their respective promoter and terminators was subcloned into different pGATE vectors having specific attL recombination sites and subsequently

developed expression clone by LR clonase reaction with pKM12GW. The multigene vector was mobilized to EHA105 *Agrobacterium* strain through electroporation. The transformants were developed by *in planta* transformation method in groundnut (Vemmana *et al.*, 2015).

Strategy to clone or develop multigene cassette: The *in planta* transformation strategy is a tissue culture-independent transformation strategy that targets the *Agrobacterium* to embryonal axes of seeds.

The emphasis of this study is to improve the adaptation of groundnut to drought stress by altering cellular tolerance mechanisms and root traits. The cellular tolerance mechanisms and higher root growth traits significantly contribute to improve the intrinsic tolerance of plant cell and increase the water uptake and mining abilities to sustain growth and development under drought stress. Therefore, genes involved in protein turnover, protein stability and protein folding (PDH45, PgHSF4) and root growth-associated gene (Alfin1) was simultaneously expressed in groundnut. The main aim of the present study is to identify the superior lines under drought stress conditions and also to assess their superiority over wild type in terms of biomass and yield. One of the best lines in terms of stress tolerance and productivity was identified for a deeper understanding of physiologically complex processes and cellular function through system biology approach. Systems biology takes the molecular parts (transcripts, proteins and metabolites) of a transgenic and wild type groundnut sample and attempt to fit them into functional networks or models designed to describe and predict the dynamic activities of the transgenic plant. The overall results generated to meet the envisaged objective are presented in this chapter.

4.3 Development of transgenics

The embryonal axes of groundnut seeds were used to transform the multigene construct using *Agrobacterium* mediated *in planta* transformation method. The flow chart in figure 6 gives the overall steps in developing and advancement of the transgenics over-expressing PgHSF4, PDH45 and OsALFIN1 genes along with marker gene npt II in successive generations. Since the T₀ plants are chimeric, the putative transformants

Multigene expressing binary vector *pKM12GW* with *RD29A:PgHSF4:nos*, *2x35SCaMV:PDH45:polyA* and *35SCaMV:OsALFIN1:polyA* with Kanamycin selection marker was developed using modified multisite gateway technology



102 T₁ plants were identified based on phenotype, kanamycin response and molecular characterization and advanced to T₂ generation



In T₃ generation 99 lines were grown in soil under contained green house facility then based on yield responses , TDM and canopy temperature 30 transgenic lines were selected for further characterization



In T₄ generation- Thirty lines selected were advanced for further study

- a) **Salinity stress response**
- b) **Hydroponics experiment to assess the root growth**
- c) **Yield analysis (pot experiment)**



T₅ generation- Seventeen lines selected

- a) **Salinity stress response**
- b) **Moisture stress response by gravimetry**
- c) **Yield analysis under MDE**



Few promising lines were selected for further study

- a) **Ten best lines selected for yield analysis under contained field condition in summer season**
- b) **Molecular confirmation by PCR and Southern**
- c) **Downstream gene expression under moisture stress**



T₆ generation- Eight lines selected

- a) **Yield analysis**
- b) **Stress experiments- Two lines were selected based on productivity and tolerance for further study : NaCl and DTT**
- c) **Transcriptomics**
- d) **Proteomics**
- e) **Metabolomics**

Fig. 6: Flow chart depicting generation wise analysis of groundnut transgenics expressing multigene construct

were identified in the T₁ generation initially by screening the seedlings on kanamycin. Many of these kanamycin-positive plants showed the amplification of marker gene neomycin phosphotransferase (nptII) indicating the integration of T-DNA into the transformed plants. Based on the phenotype, kanamycin response, temperature tolerance and molecular characterization, 102 plants were further advanced to T₂ generation. In T₂ generation, 20 seedlings from each line were rigorously screened for kanamycin sensitivity and 102 T₂ groundnut transgenic and wild type plants were grown in contained facility and various yield parameters such as pod number, pod dry weight and total dry matter was recorded after harvesting (Annexure IV). The lines that showed more than 90% of survived seedlings on kanamycin and having higher pod yield and biomass were considered for further advancement to T₃ generation. Based on this analysis and phenotypic uniformity, 99 lines were advanced to T₃ generation. In T₃ generation 99 lines were grown in soil under contained glasshouse facility till 50th day, and then, gradual moisture stress was imposed for 20 days. After 10 days of moisture stress imposition, canopy temperature was measured using infrared (IR) thermometer. Many transgenic lines showed lower canopy temperature with more negative crop canopy air temperature difference (CCATD) values compared to wild type plants. Further transgenic and wild type plants were analyzed for yield characters like pod dry weight and biomass. Many of the transgenics showed more pod yield and biomass compared to wild type plants. The biomass was consistently higher in transgenic lines and showed a positive correlation with pod yield. Similarly, a positive correlation was seen between pod weight and pod number, indicating that observed increase in pod yield was due to higher pod number per plant (Fig. 7).

Based on yield response, TDM and canopy temperature 30 transgenic lines were selected for further characterization.

The results obtained in different experiments are briefly described

4.4 Characterization of T₄ groundnut transgenic lines

The identified 30 transgenic lines based on yield response, TDM and canopy temperature were selected for further characterization. The major emphasis has been to

Summary of T3 transgenics

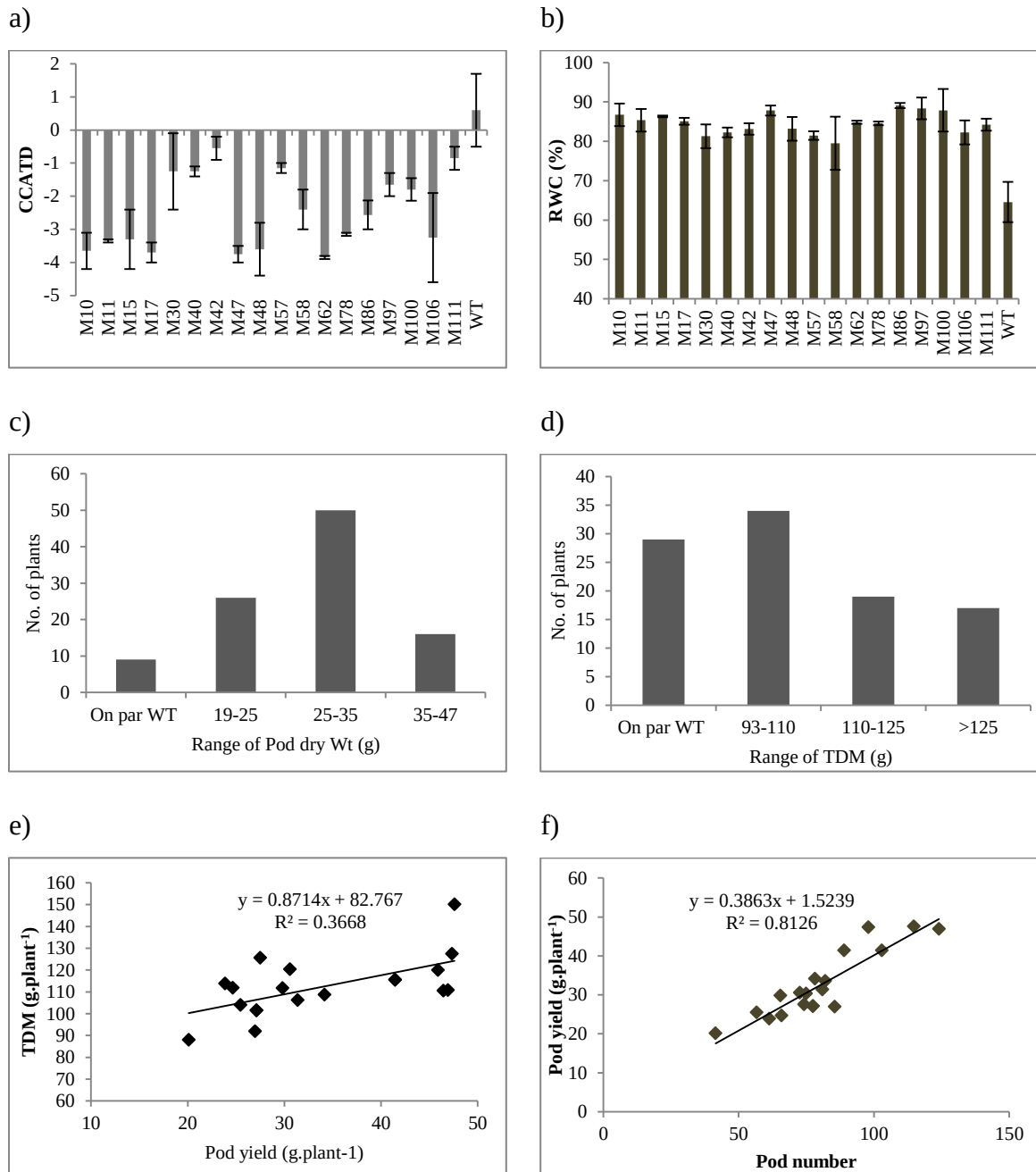


Fig. 7: Stress response of groundnut transgenic plants on exposure to mid-season moisture stress. a) Variation in Crop canopy air temperature difference (CCATD). b) Variation in relative water content (RWC). (c) and (d) Frequency distribution of lines based on pod dry weight (yield) and total dry matter (TDM) per plant, respectively. Correlation analysis between (e) pod yield and biomass. (f) Pod yield and pod number.

Based on yield response, TDM and canopy temperature 30 transgenic lines were selected for further characterization

study the salinity stress response at two different stages (seedling stage and whole plant level) and to study the variation in growth and productivity under non-stress conditions. In the first experiment salinity stress was imposed at seedling level in germinated seedlings. In the second experiment salinity stress was imposed under hydroponics system and in the third experiment the pot grown transgenics were analyzed for growth and productivity under non-stress condition.

4.4.1 Characterization of selected groundnut transgenic lines for salinity stress (T₄ generation)

The salinity stress response was analyzed in thirty lines selected based on CCATD, biomass and productivity. The main aim of the study was to subject plants initially to induction stress treatment followed by exposing to severe salinity stress conditions. Salt at high concentrations can induce osmotic stress, which in turn leads to membrane damage, loss of protein stability, cell death etc., leading to overall decrease in growth and development. Whereas low doses of salt can induce or trigger protective mechanisms that will protect the cells from a lethal dose of NaCl. So two-day-old pre-germinated seeds from transgenic and wild type plants were subjected to 100 mM NaCl for 8 hours and then transferred to 300 mM NaCl for 8 hours and then subjected to recovery for 3 days. Transgenic seedlings showed significantly improved tolerance to salinity compared to wild type plants (Fig.8a). Most of the transgenic lines showed an increase in root length compared to wild type under stress condition (Fig. 8b). Wild type plant root length ranged from 3-3.8 cm under controlled condition and 0.62-0.8 cm under stress condition whereas transgenic lines showed 3-7.52 cm under controlled condition and 0.75-2.93 cm under stress condition (Table 6). Per cent reduction in root length over control was less in most of the transgenic lines compared to wild type (Fig. 8c). The mean per cent reduction in root length over control in some of the transgenics like 2, 17, 29, 57, 58, and 97 was 48.41 % whereas in wild type the per cent reduction was 78.77%.

T4 generation

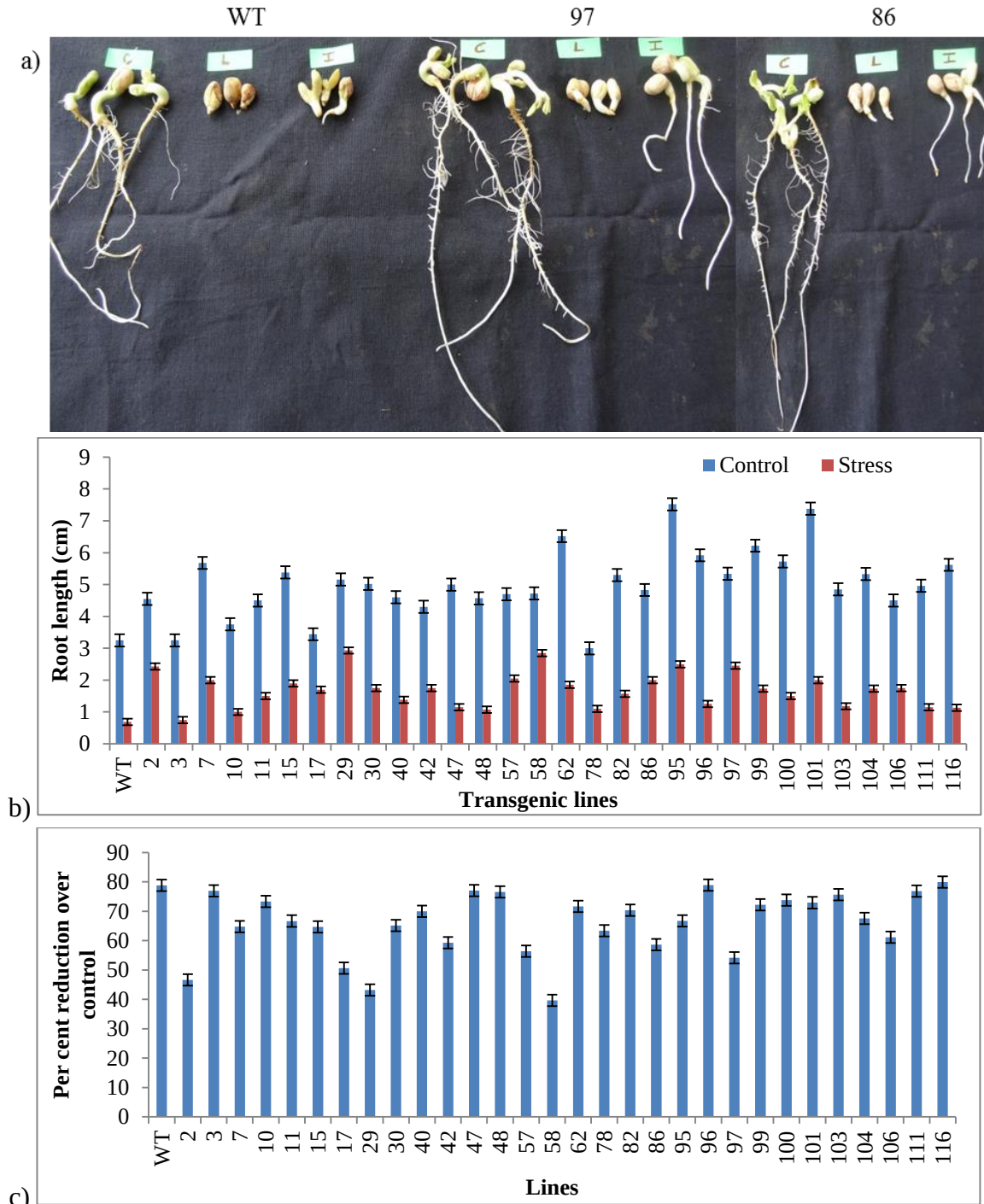


Fig. 8: Response of multigene expressing transgenic seedlings to salinity stress. Two-day-old pre-germinated seeds from transgenic and wild type plants were subjected to 100 mM NaCl for 8 hours and then transferred to 300 mM NaCl for 8 hours and then subjected to recovery for 3 days. a) Representative pictures showing phenotype of seedlings to salinity induction response. b) Graph showing the root length of wildtype and transgenic seedlings. c) Per cent reduction in root length over control. WT-Wild type

T4 generation

Table 6: Root length of wild type and transgenic seedlings under salinity stress.

Wild type and transgenic seedlings were subjected to salinity induction response and root length was recorded after recovery.

N	Control		Stress	
	Mean (cm)	Range (cm)	Mean (cm)	Range (cm)
WT (10)	3.25	3-3.8	0.69	0.62-0.8
Transgenics (30)	5.05	3-7.52	1.70	0.75-2.93

4.4.2 Characterization of selected groundnut transgenic lines for salinity stress under hydroponics system (T₄ generation)

In order to study the salinity stress response of transgenics, the transgenic and wild type plants were raised in cups supplemented with Hoagland's solution. The 10 day old seedlings were exposed to 100 mM NaCl stress along with Hoagland's solution for 5 days later 250mM NaCl was given as lethal stress for 3 days and then subjected to recovery for 10 days. The transgenic lines had better root growth compared to wild type plants (Fig. 9a&b). Few lines such as 17, 48, 86, 97, 99, 100 showed more root length compared to wild type plant (Fig. 9c). Total dry matter (TDM) was also more in case of transgenics compared to wild type plants (Fig. 9d). Percent reduction in root length and biomass over control was less in most of the transgenic lines (Fig. 10a & b). Some of the lines such as 2, 3, 10, 29, 30, 42, 47, 48, 58, 62, 82, 86, 96, 97, 103, 104 and 106 showed less per cent reduction in root length and biomass over control. There was a strong positive correlation between root length under control and stress condition (Fig. 11a). Biomass also showed positive correlation under control and stress condition (Fig. 11b). The transgenic lines which showed higher root length also showed higher biomass. A positive correlation was observed between root length and TDM both under control and stress conditions (Fig. 11c & d).

Correlation analysis between salinity response at seedling stage and salinity response in hydroponics system

The major objective of this study was to observe the salinity stress response at both seedling stage and whole plant level (Hydroponics). The per cent reduction in root length over control was calculated from both the experiment and seen the correlation between the two experiments. The per cent reduction in root length over control was less in transgenics compared to wild type in both the experiments. Positive correlation was seen in per cent reduction in root length over control between both the experiments (Fig. 12). Genotypic response remained same under both the conditions.

T4 generation

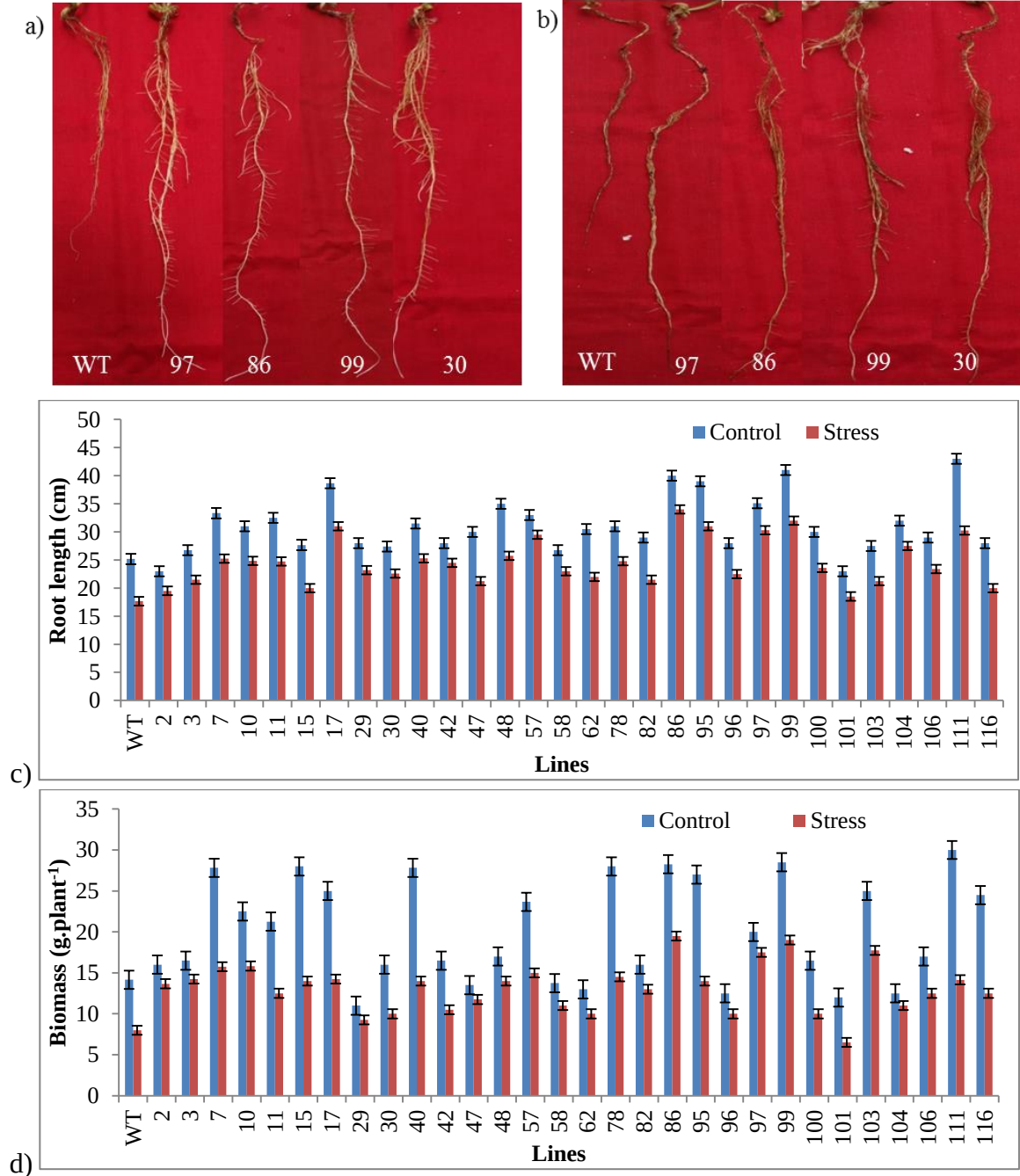
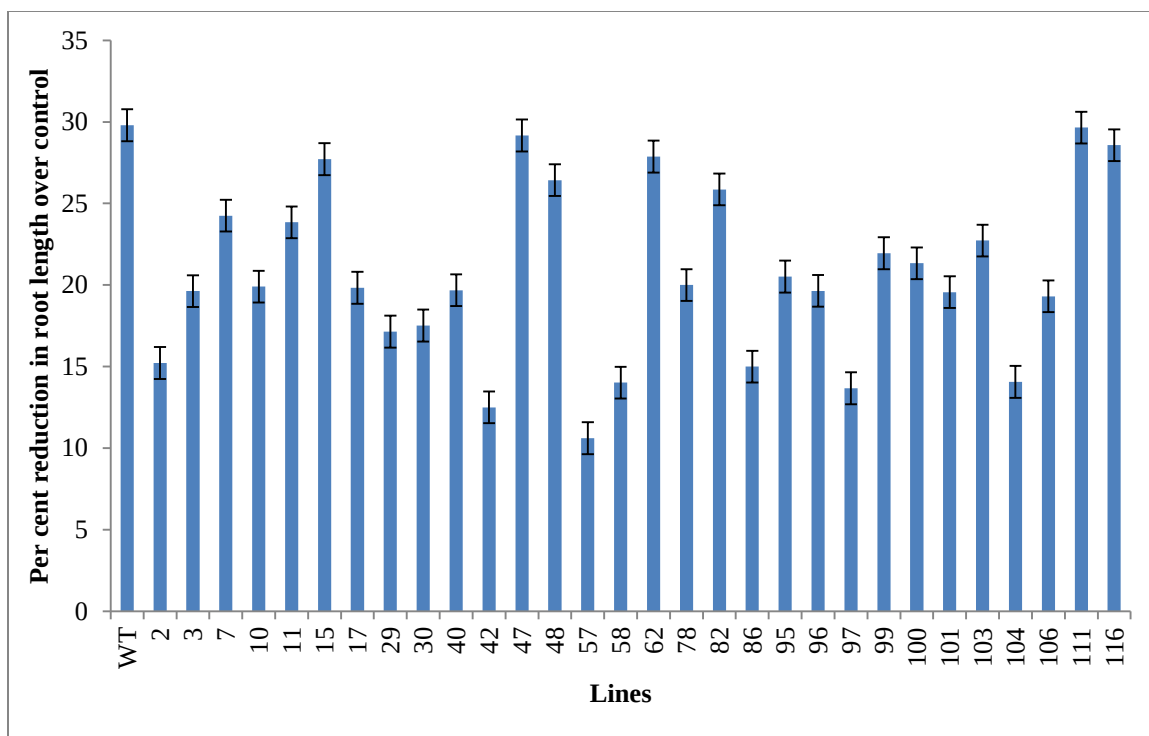
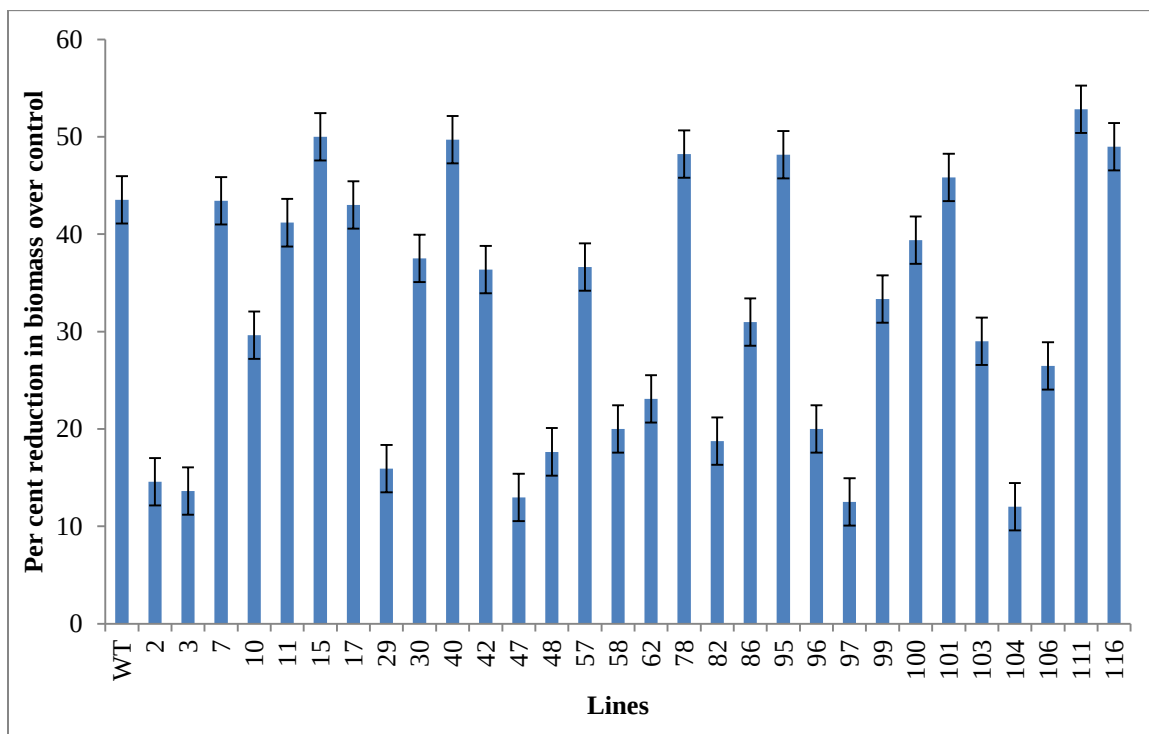


Fig. 9: Response of wild type and transgenic lines for salinity stress in hydroponics system. a) & b) Representative photograph showing root length of wild type and transgenic under control and stress condition respectively c) Root length of wild type and transgenic d) Total dry matter (TDM) of wild type and transgenic lines. 10 day old seedlings were subjected to 100 mM of NaCl initially for 5 days later 250 mM NaCl was given as lethal stress for 3 days and then subjected to recovery for 10 days.



a) Percent reduction in root length over control



b) Percent reduction in biomass over control

Fig. 10: Per cent reduction in root length and biomass of wild type and transgenic lines for salinity stress in hydroponics system

T4 generation

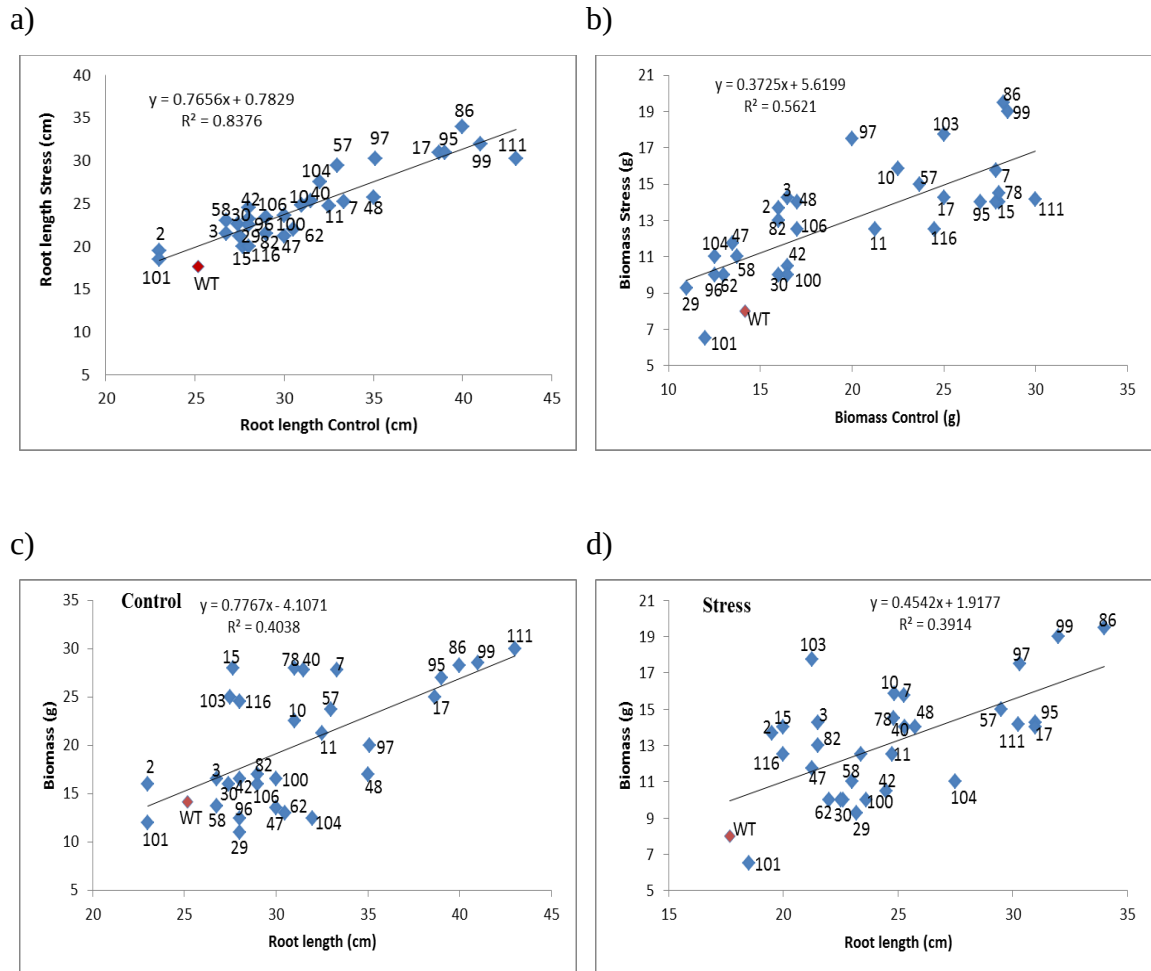


Fig. 11: Correlation analysis between physiological parameters a) root length under control and stress condition b) TDM under control and stress condition c) Root length and TDM under control condition b) Root length and TDM under salinity stress condition. 10 day old seedlings were subjected to 100 mM of NaCl initially for 5 days and then 250 mM of NaCl was given as lethal stress for 3 days and later subjected to recovery for 10 days.

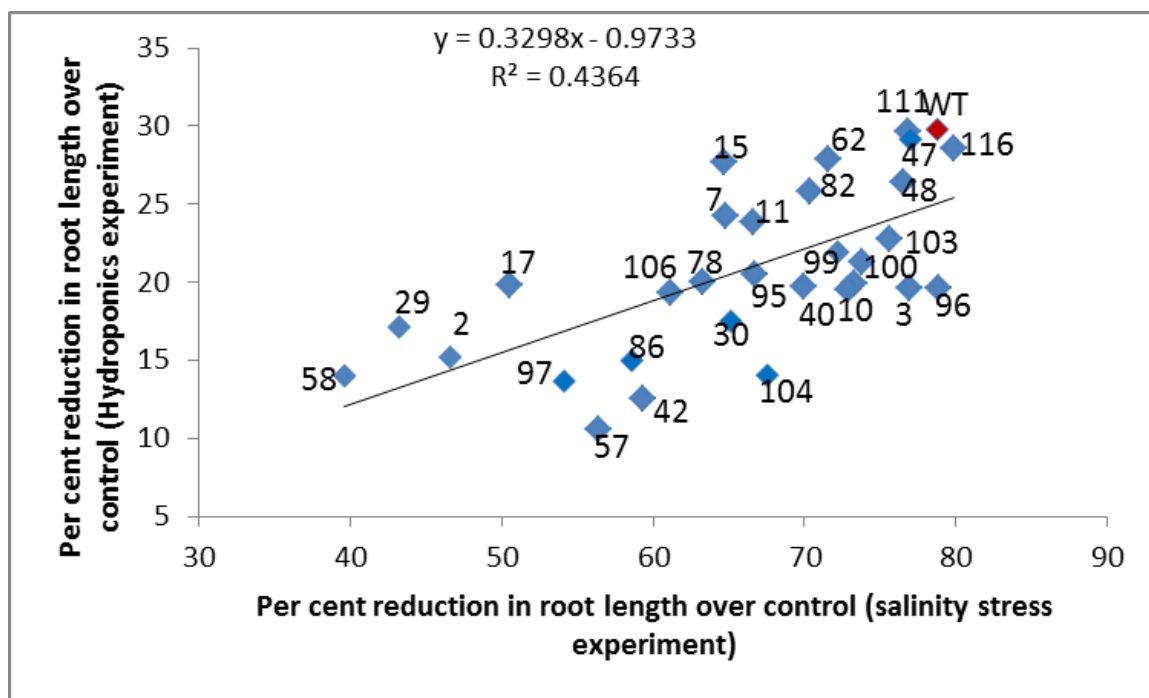


Fig. 12: Correlation analysis between per cent reduction in root length over control in salinity stress at seedling stage and whole plant level (Hydroponics)

4.4.3 Growth and productivity analysis in selected groundnut transgenic lines under net house condition (T₄ generation)

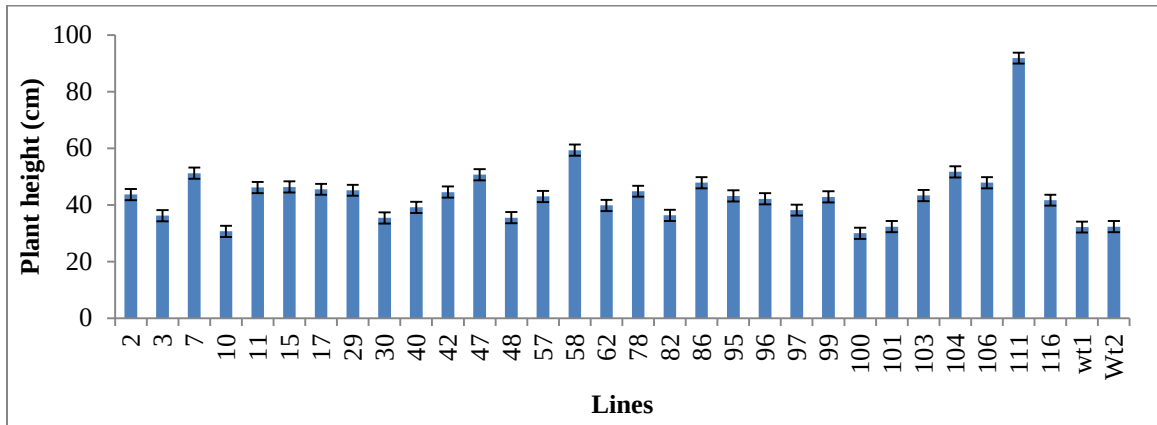
Apart from stress tolerance another important criteria was to select the promising lines with improved growth and productivity. The growth and productivity of selected transgenic lines was studied under non-stress condition. Since in earlier experiment there was more total dry matter (TDM) in transgenic lines compared to wild type the productivity in transgenic lines was examined.

The transgenic and wild type plants were grown in pots and maintained under control condition till harvest. After 120 days wild type and transgenic plants were harvested and some of the growth parameters like plant height, root length and root volume were recorded. Most of the transgenic lines showed improved growth and development as compared to wild type (Fig. 13a, b & c). Yield parameters like pod yield and TDM was also more in case of transgenics compared to wild type plants (Fig. 14a & b). The transgenic lines such as 7, 11, 29, 30, 40, 42, 57, 58, 78, 86, 97, 99, 103, 104 and 111 showed more biomass compared to wild type plant and few transgenics such as 3, 7, 10, 11, 30, 40, 57, 78, 86, 97, 99, 100, 103 and 104 showed more pod yield than wild type plant. There was a positive correlation between root dry weight and stem dry weight (Fig. 15a). Since yield and biomass are the prime factors to be considered for improved crop productivity, there was a strong positive correlation between biomass and yield was observed with $R^2=0.71$ (Fig. 15b).

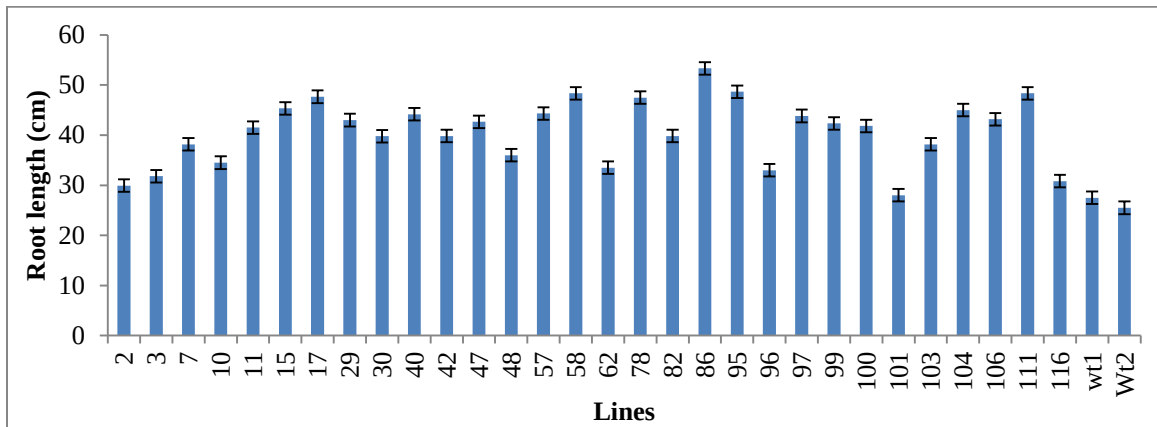
Correlation analysis between yield parameters and salinity stress experiments

To select high productive and tolerant types the productivity of transgenic lines with TDM of hydroponics system, TDM of pot grown experiment with TDM of hydroponics experiment and root length of pot grown experiment with root length of hydroponics experiment were compared. There was positive correlation between root length of pot grown experiment and root growth of hydroponics experiment with $R^2=0.411$ (Fig. 16a). There was no correlation between biomass of pot grown plants and hydroponics experiment (Fig. 16b). The pod yield of pot grown experiment and biomass of hydroponics were compared. It also showed positive correlation but it was not

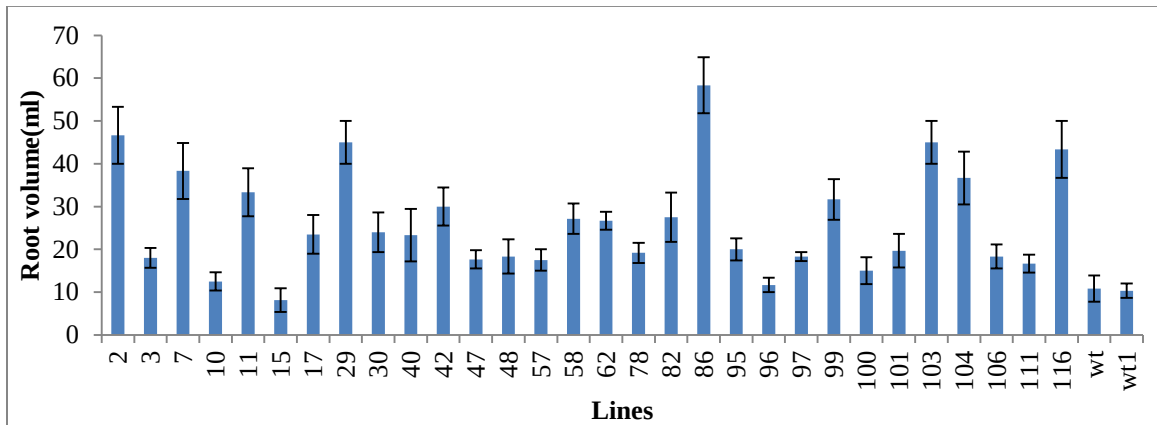
T4 generation



a) Plant height



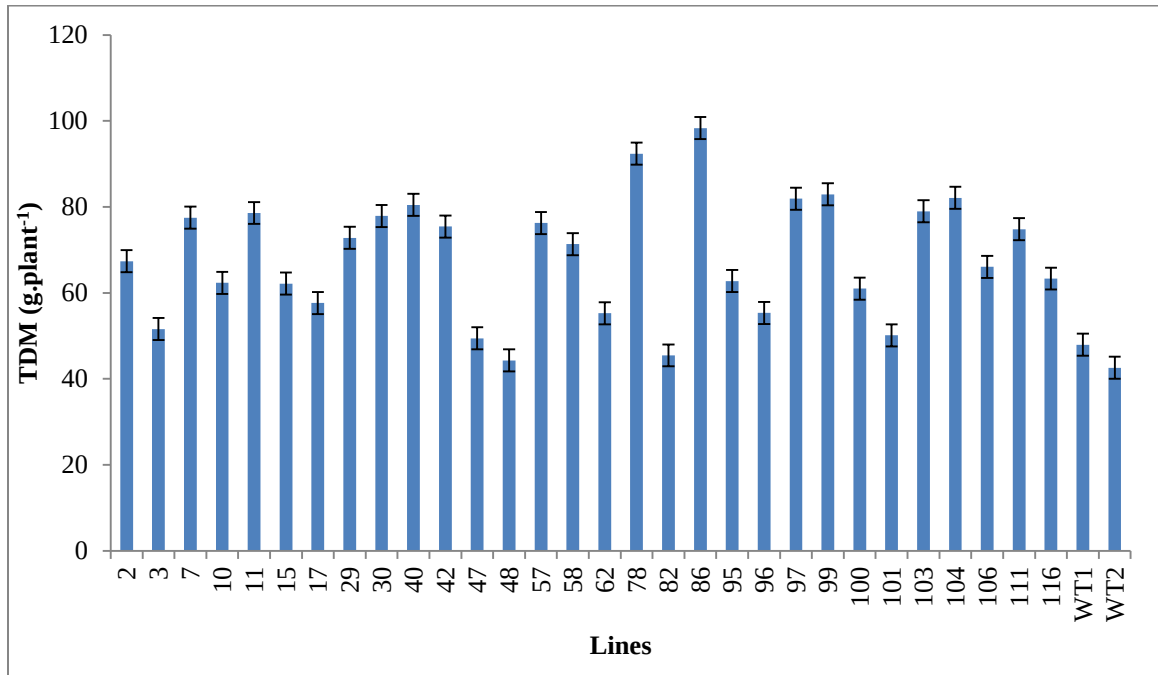
b) Root length



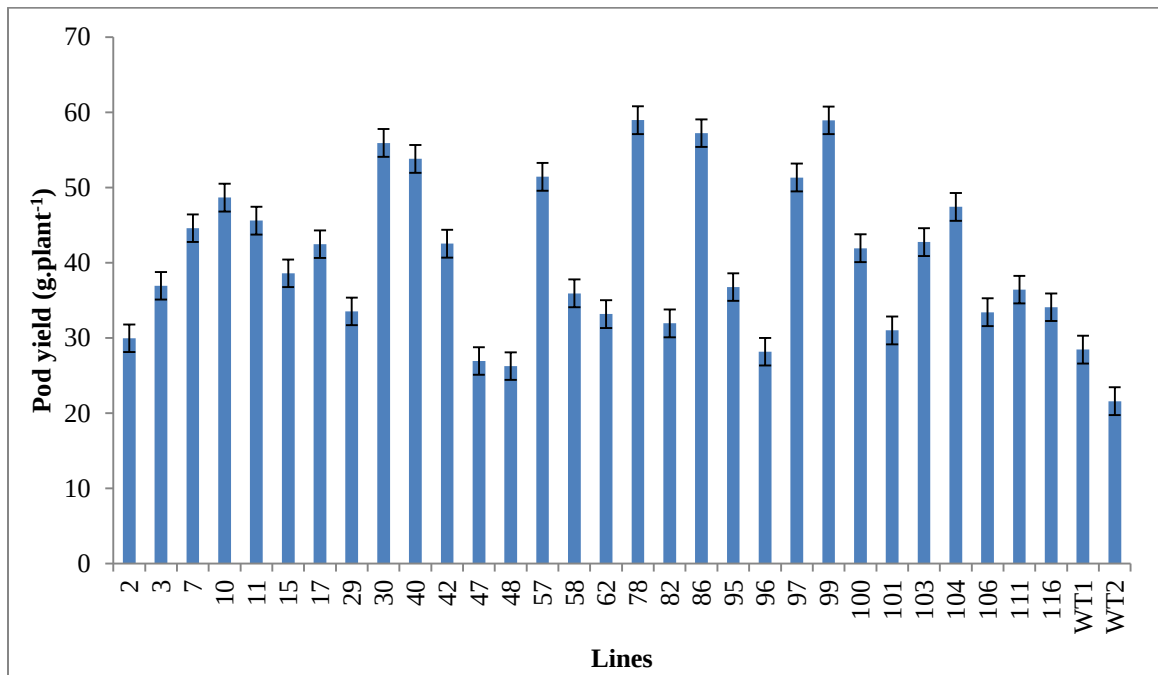
c) Root volume

Fig. 13: Growth parameters of wild type and transgenic lines in pots under normal condition. Wild type and transgenic plants were sown in pots and maintained under controlled condition till harvest and then observations were recorded (n=5).

T4 generation



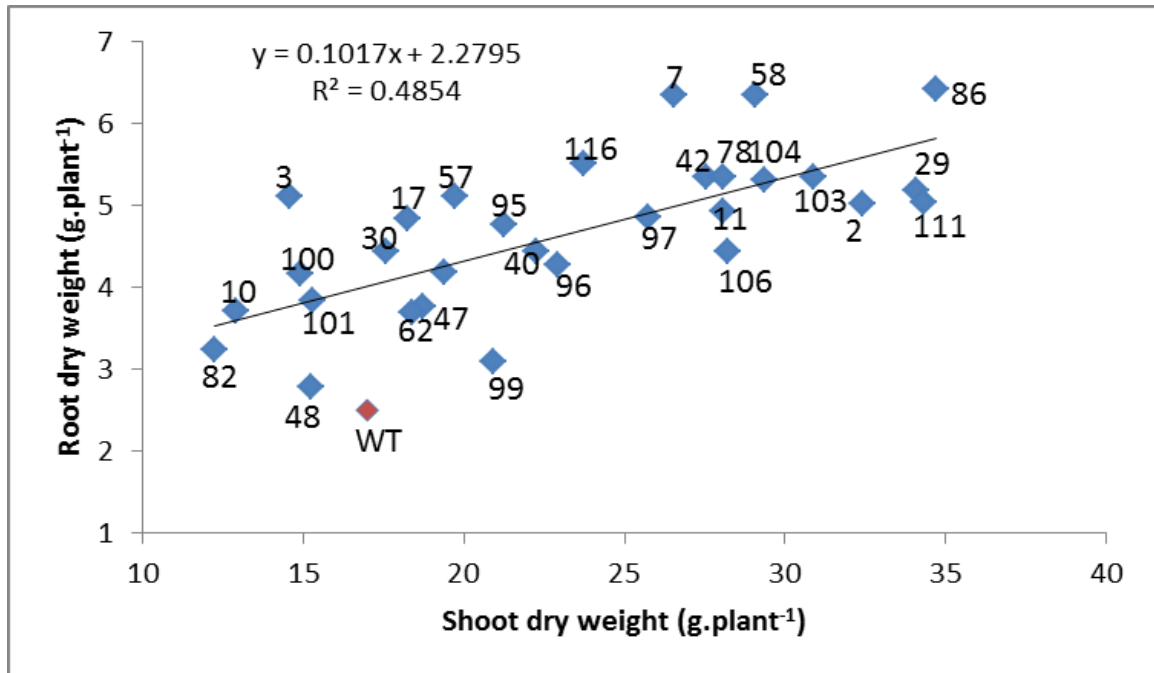
a) Total dry matter



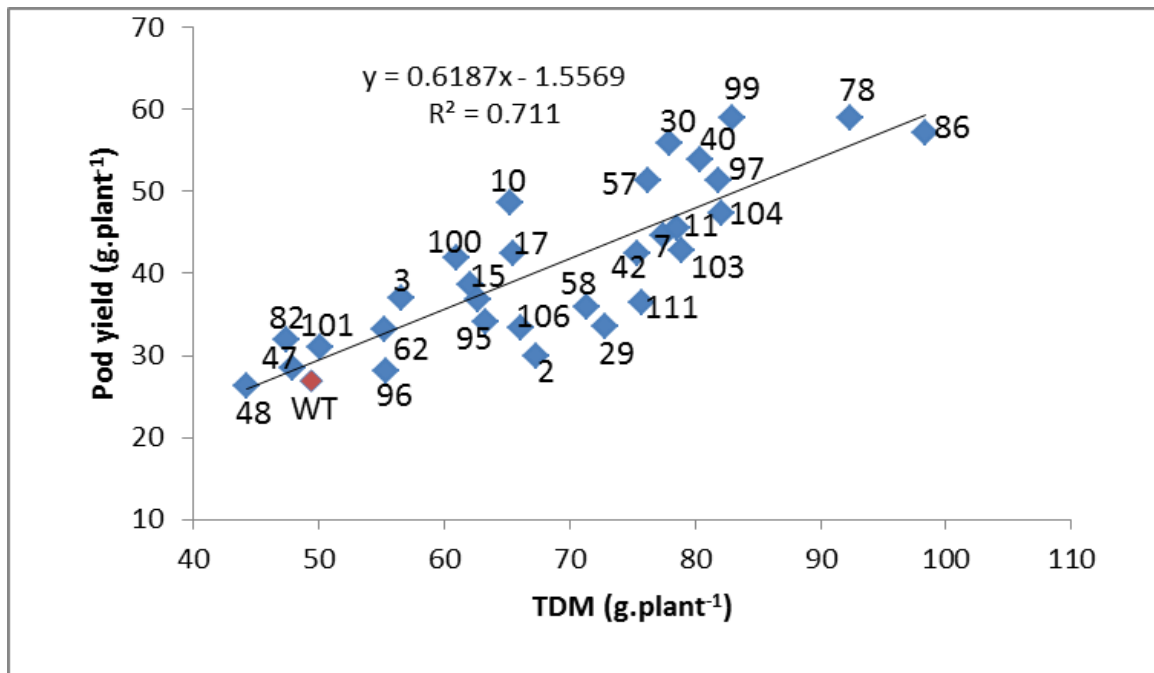
b) Pod yield

Fig. 14: Yield parameters of wild type and transgenic plants under normal condition. Wild type and transgenic plants were sown in pots and maintained under controlled condition (n=5).

T4 generation

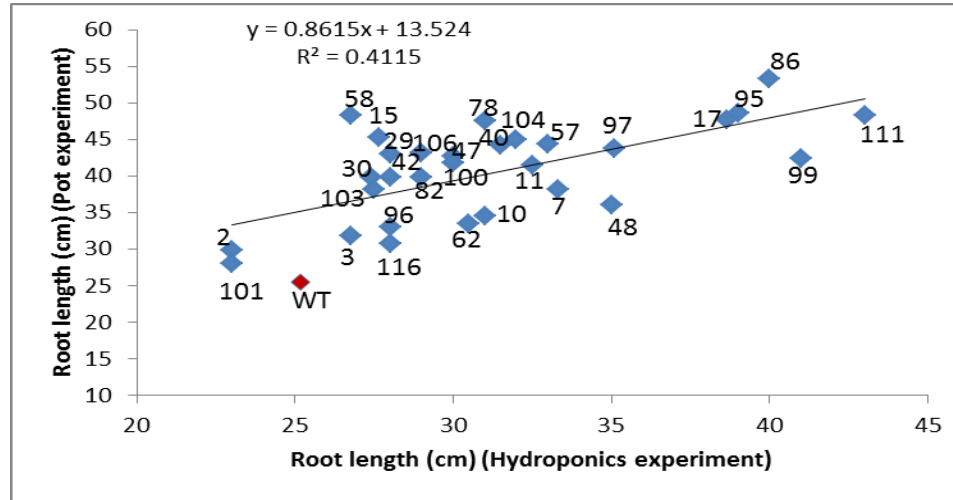


a) Stem dry weight and Root dry weight

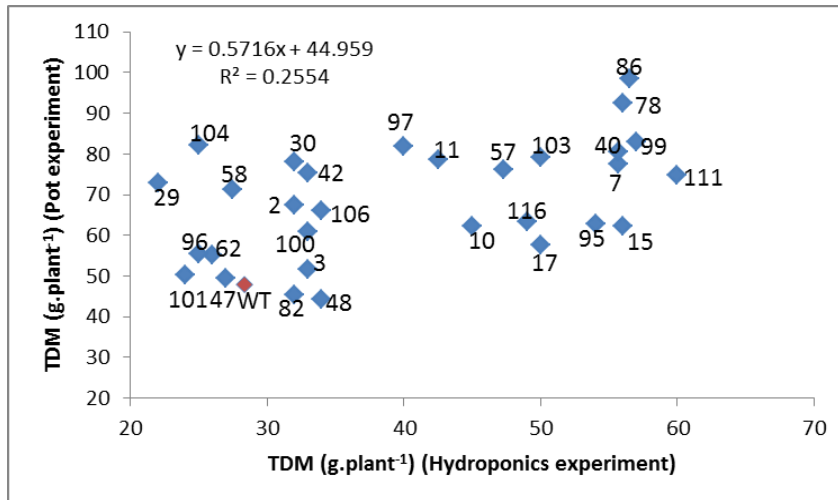


b) Total dry matter and pod yield

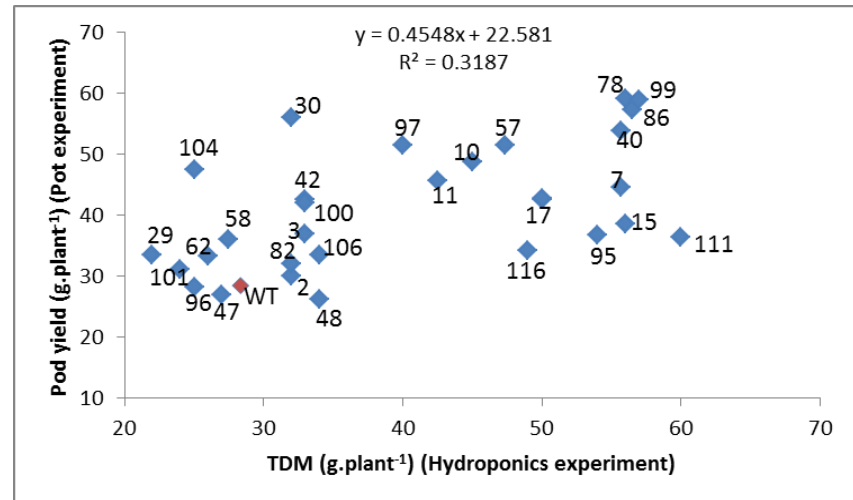
Fig. 15: Correlation analysis between yield parameters. Wild type and 30 transgenic lines were sown in pot and analyzed for correlation between TDM and yield.



a) Root length of pot experiment and Hydroponics



b) TDM of Pot experiment and Hydroponics experiment



c) TDM of Hydroponics experiment and pod yield of pot experiment

Fig. 16: Correlation analysis between experiments

significant (Fig. 16c). So Z-distribution analysis was performed to select high yield type with high tolerance.

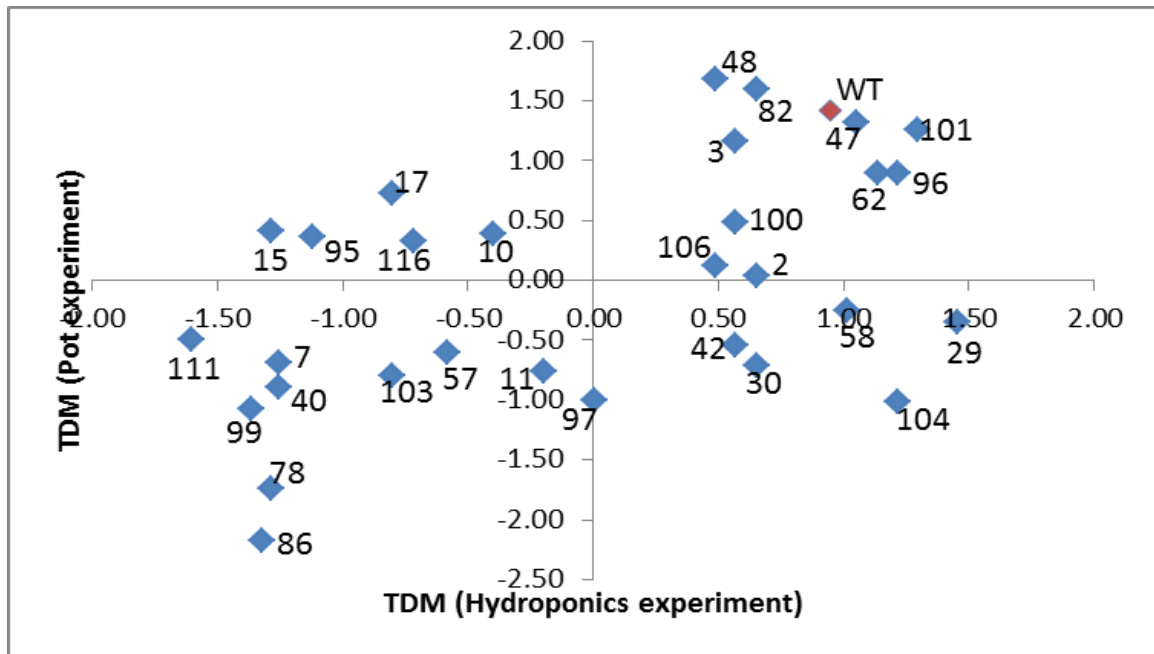
4.4.4 Identification of promising lines from T₄ generation based on Z-distribution analysis

A novel statistical tool i.e. Z-distribution was performed for Z-distribution analysis, the mean value and standard deviation (for root length/biomass/pod yield) of all the lines was calculated. Z-distribution was arrived at using the formula: (mean value – specific line value)/Standard deviation of all the lines. This data provides an option to identify the plants which has highest value for a parameter from both the experiments. The values for any two parameters were plotted as X-Y scatter and resulting graph shows four quadrants. The plants which have highest values for both parameters will have highest negative Z- value and falls in the 3rd quadrant (-, -). The plants which shows least values highest positive Z-value will fall in 1st quadrant (+, +). The plants which shows more negative in one parameter and positive in another parameter will fall in the 2nd quadrant (+, -) and 4th quadrant (-, +).

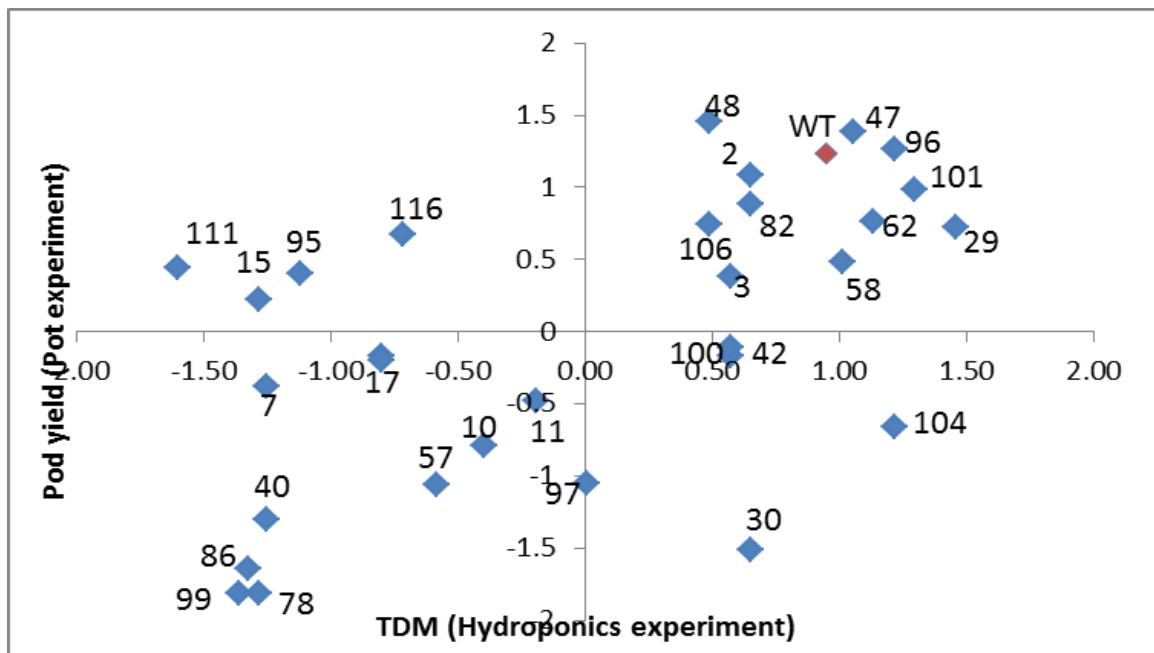
The Z-distribution analysis was performed between TDM of pot grown plants and hydroponics system experiment, the lines falling in the third quadrant will have higher TDM in both pot grown and hydroponically grown plants whereas the lines which fall in first quadrant will have lower TDM in both the experiment and the lines which showed high TDM in any one of the experiment will fall in second and fourth quadrant (Fig. 17a). In pod yield of pot grown experiment and TDM of hydroponics experiment, the lines which are falling in third quadrant will have higher pod yield and higher TDM but lines which fall in first quadrant will have lower TDM and pod yield and in second quadrant the lines which are showing higher yield but lower TDM will fall in second quadrant (Fig. 17b). Therefore the lines falling in second and third quadrant were selected as superior lines which are both stress responsive and high yield type.

Based on Z-distribution analysis 17 lines namely 30, 86, 100, 106, 17, 40, 97, 78, 99, 29, 3, 10, 15, 42, 57, 104 and 111 were selected for further characterization.

T4 generation



a) TDM of hydroponics experiment and pot experiment



b) TDM of hydroponics experiment and pod yield of pot experiment

Fig. 17: Z-distribution analysis between experiments

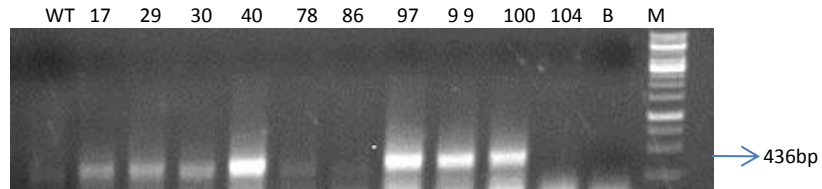
4.4.5 Molecular analysis of the selected groundnut transgenic lines (T₄ generation)

In order to study the stability of all the transgenes in T₄ generation, genomic DNA was isolated from 30 transgenic and wild type plants and PCR amplification was carried out using gene specific primers. The representative transgenic lines molecular confirmation is given in plate 1. The presence of marker gene NPTII was confirmed through gene specific primers which showed an amplification of 436 bp (Plate 1a) indicating the integration of T-DNA in the transgenic lines. The presence of PDH45, OsAlfin1 and PgHSF4 was confirmed through gene specific primers which showed an amplification of 200 bp (Plate 1b), 200 bp (Plate 1c) and 950 bp (Plate 1d) respectively indicating the integration of the transgene in the transgenic lines.

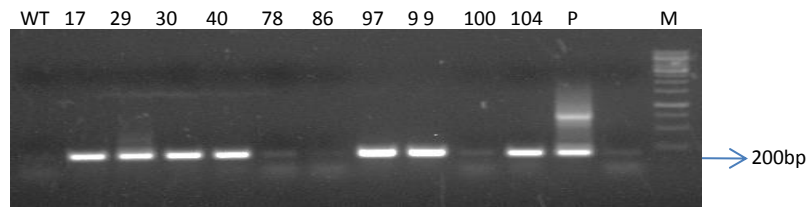
4.5 Characterization of T₅ groundnut transgenic lines

Based on Z-distribution analysis 17 lines which performed better under stress with improved productivity were selected for further characterization. Major emphasis of the study was to reconfirm the salinity tolerance in transgenic lines. Besides moisture stress response was also studied by exposing the plants to defined moisture regimes in a gravimetric experiment. Finally the productivity of these lines was also assessed in two different experiments. In these identified 17 lines using sub set of seeds four different experiments were conducted to assess the stress response and productivity. In the first experiment 17 transgenic lines along with wild type plants subjected to salinity stress response at seedling level. In the second experiment selected 17 transgenic lines were assessed for moisture stress recovery response by gravimetric approach. In third experiment these 17 transgenic lines were studied for growth and productivity in managed drought environment under stress and non-stress condition. In the fourth experiment we selected 10 best promising lines based on productivity of managed drought environment experiment and studied moisture stress response of transgenic lines and also studied yield attributes under stress and non-stress conditions.

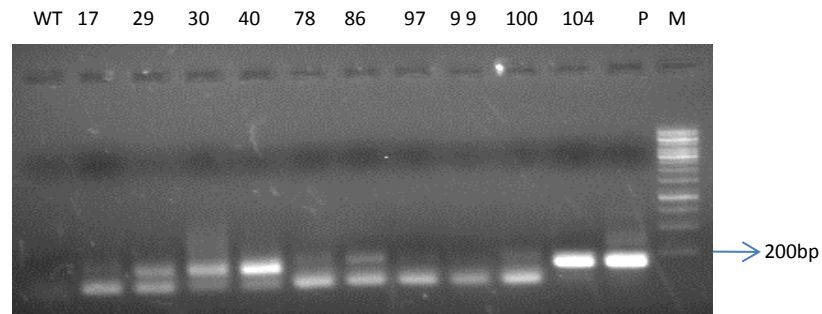
T₄ generation



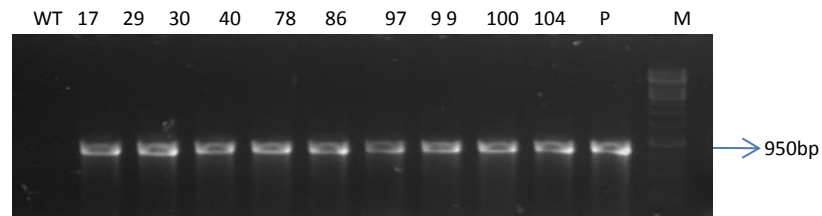
a) *NPT II*



b) *PDH45*



c) *Alfin1*



d) *HSF4*

Plate 1: Molecular confirmation of T₄ transgenic plants by PCR in few representative plants by using different primer combinations for *NPT II*, *PDH45*, *Alfin1*, *HSF4* genes. Lane 2-11 transgenic lines M- 1kb DNA Ladder, P- Positive control (plasmid), WT- wild type, B-Blank

4.5.1 Characterization of selected groundnut transgenic lines for salinity stress (T₅ generation)

The salinity stress response was reanalyzed in the selected 17 lines. To study the salinity induction response, two-day-old pre-germinated seeds from transgenic and wild type plants were subjected to 100mM NaCl for 8 hours and then transferred to 300mM NaCl for 8 hours and then subjected to recovery for 3 days. Transgenic seedlings showed significantly improved tolerance to salinity compared to wild type plants (Fig.18a). All the transgenic lines showed higher root length compared to wild type under control and stress condition (Fig. 18b). Per cent reduction in root length over control under induced condition was less in transgenics compared to wild type. But when seedlings were transferred directly to lethal stress level there was no difference between transgenic and wild type plants (Fig. 19a & b). Transgenic lines such as 86, 17, 97, 78, 29, 3, 42 and 104 showed less per cent reduction in root length over control under induced condition.

4.5.2 Moisture stress recovery response of groundnut transgenic plants (T₅ generation)

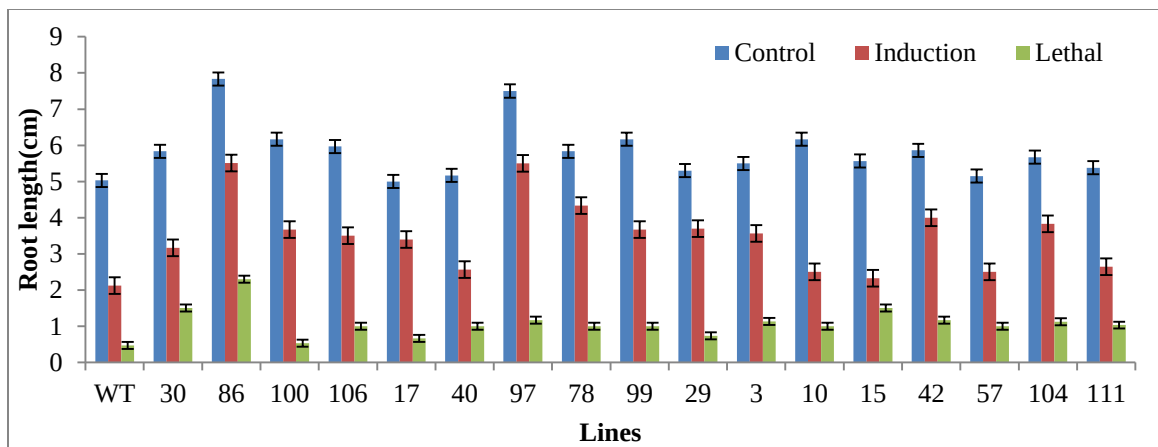
Gravimetry technique to impose moisture stress

This technique was followed to quantify the amount of water lost by evapotranspiration (ET) and replenish the same to maintain the specific soil moisture status. To quantify the amount of water lost by ET, the pots along with plants were weighed daily, using weighing devices. Therefore in this approach irrespective of variations in water utilized for ET, the plants were maintained at specific soil moisture status. In each pot, one plant each from transgenic event and wild type plants was interplanted to assess the relative performance under similar soil moisture status. The main aim of this approach was both wild type and transgenic plants will experience similar moisture stress and it will have similar plant water relations (water content, relative water content (RWC), water potential). In order to impose moisture stress, the amount of water that needs to be added to maintain the required field capacity (FC) was calculated. Plants were exposed to two stress levels, 50 and 30% FC. Control plants were maintained at 100% FC. All the three sets of plants were initially maintained at 100% FC till 10 to 12 days and then for two sets of plants moisture stress was imposed

T5 generation

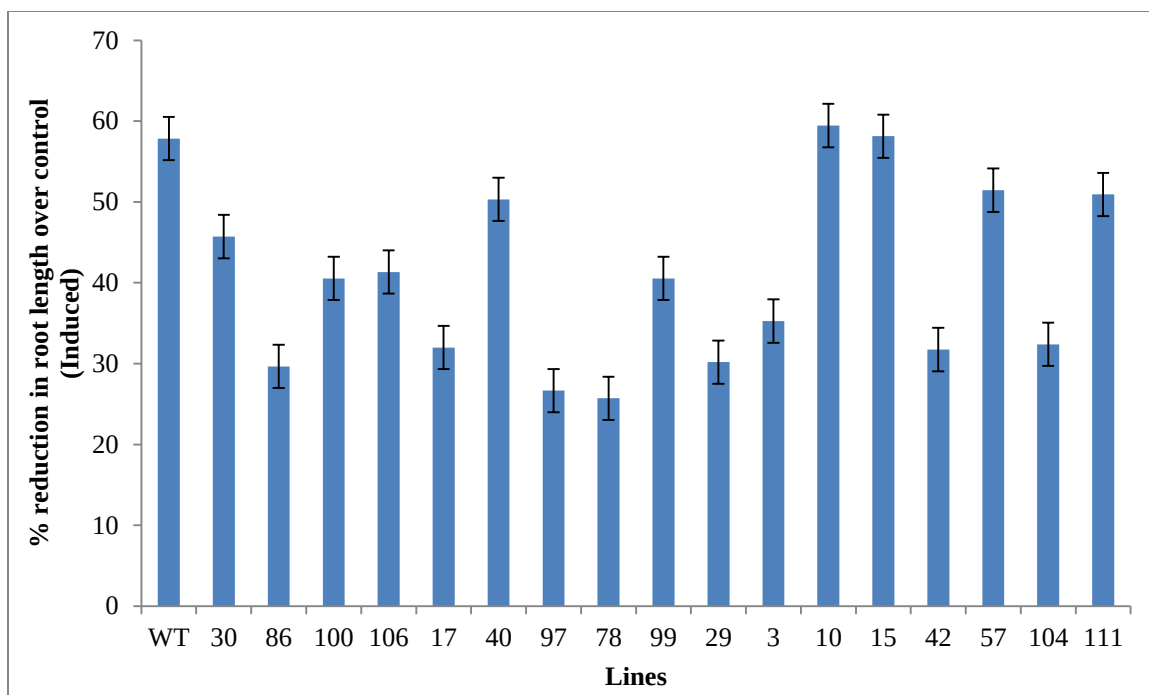


a) Representative pictures showing phenotype of seedlings to salinity induction response

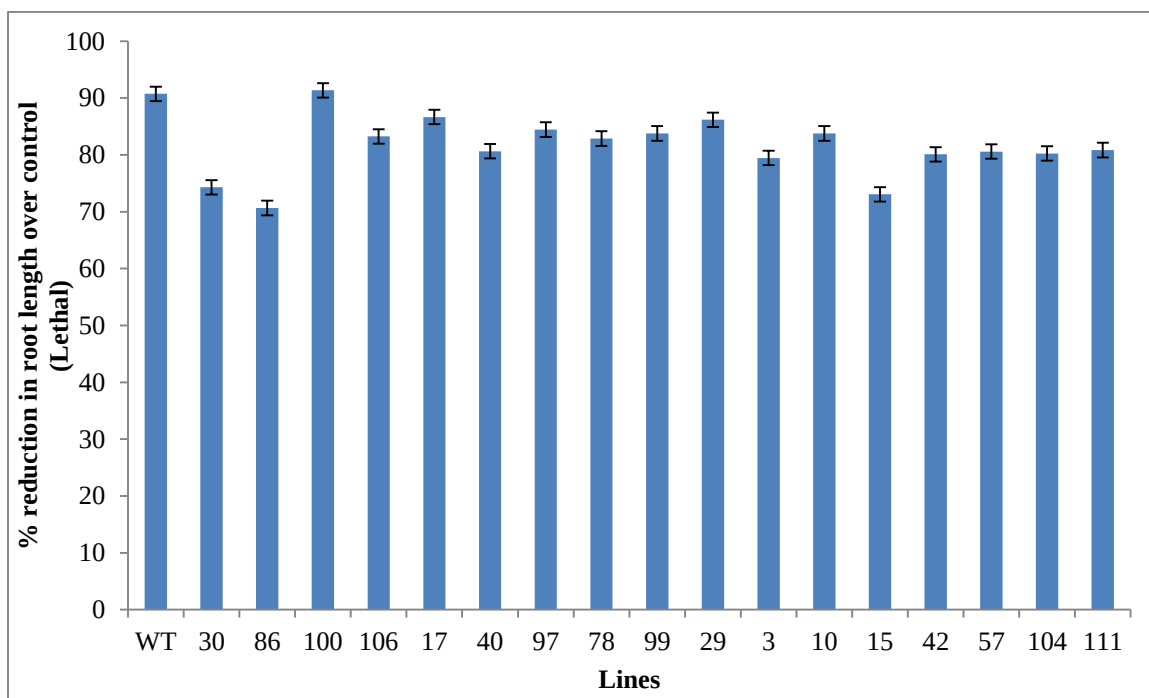


b) The root length of wild type and transgenic seedlings

Fig. 18: Response of multigene expressing transgenic seedlings to NaCl stress. Two-day-old pre-germinated seeds from transgenic and wild type plants were subjected to 100 mM NaCl for 8 hours and then transferred to 300mM NaCl for 8 hours and then subjected to recovery for 3 days WT-Wild type



a) Per cent reduction in root length over control under induced condition



b) Per cent reduction in root length over control under lethal condition

Fig. 19: Per cent reduction in root length of wild type and transgenic lines for salinity induction response

gradually in a pre-calculated manner to simulate natural rate of decline in soil moisture as in field conditions. The decrease in soil moisture status was restricted to 5-7.5% FC to 10% FC per day by irrigating the pots as and when they reach this level. The soil FC is decreased gradually from 100% FC to 50 and 30% FC over a period of 12 days. After achieving specific soil moisture stress, the pots were maintained at required FC's for 5 days and studied for stress response by analyzing different parameters and subsequently brought back to 100% FC, which allowed the plants to recover from stress for a week and then recovery response was photographed and root length and biomass was recorded.

Recovery growth:

The moisture stress response of 20-day-old transgenic plants was assessed by gradually imposing moisture stress gravimetrically. At the end of stress period, transgenic plants showed less severe wilting symptoms compared to wild type plants (Fig. 20a, b & c). The recovery growth of transgenic lines was significantly superior over the wild type plants (Fig. 20d & e). Therefore, variation in survival and recovery of plants at early plant growth is predominantly due to variation in cellular level tolerance mechanisms.

Root growth and biomass: The transgenic plants showed significantly higher root growth and total biomass than wild type plants under both control and stress conditions (Fig. 21a, b & c). Amongst the transgenic lines tested 40, 86, 97 and 100 showed significantly higher root growth and biomass than other transgenic lines. The percent reduction in root length and biomass was less in case of transgenics compared to wild type plants (Fig. 22a & b).

Variation in root growth of transgenic lines was not the major factor for recovery of the plant because similar soil moisture status was maintained in both wild type and transgenic lines. The observed variation in survival and recovery was not due to water mining but due to cellular level tolerance. Some of the transgenic lines such as 17, 40, 42, 78, 86, 97 and 100 showed superior recovery growth and also showed more root length and biomass compared to wild type.

T5 generation

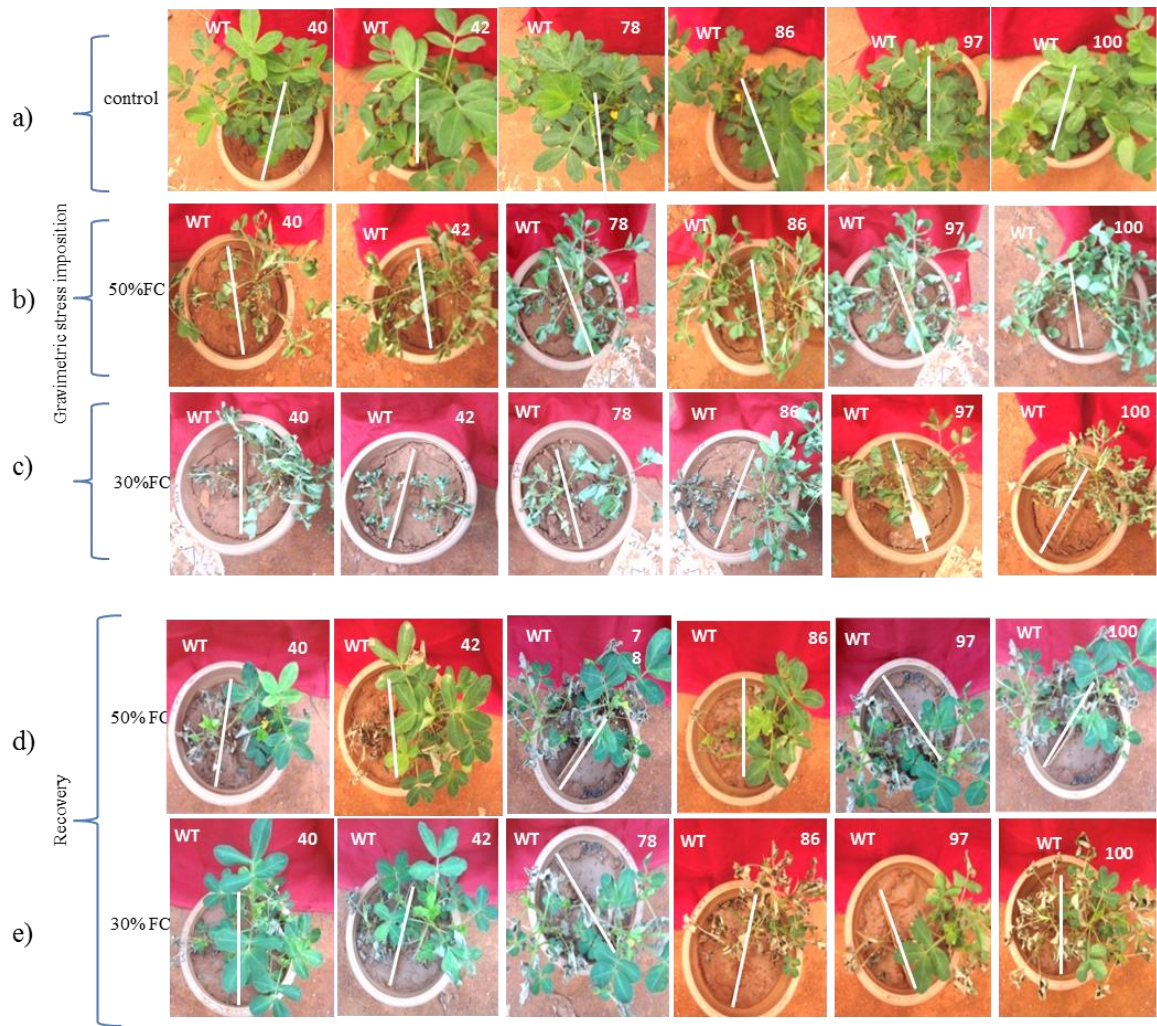


Fig. 20: Moisture stress recovery response of transgenic and wild type a) Under control condition b) Under 50% FC at the end of stress c) Under 30% FC at the end of stress d) Under 50% FC after recovery e) Under 30% FC after recovery. 20-day-old seedlings were raised in pots to arrive at relative comparison in each pot, one plant each of wild type and transgenic event was maintained. By adopting gravimetric approach, the pots were weighed twice a day to measure the water loss in evapotranspiration and required amount of water was replenished to maintain the desired soil moisture status (FC).

T5 generation

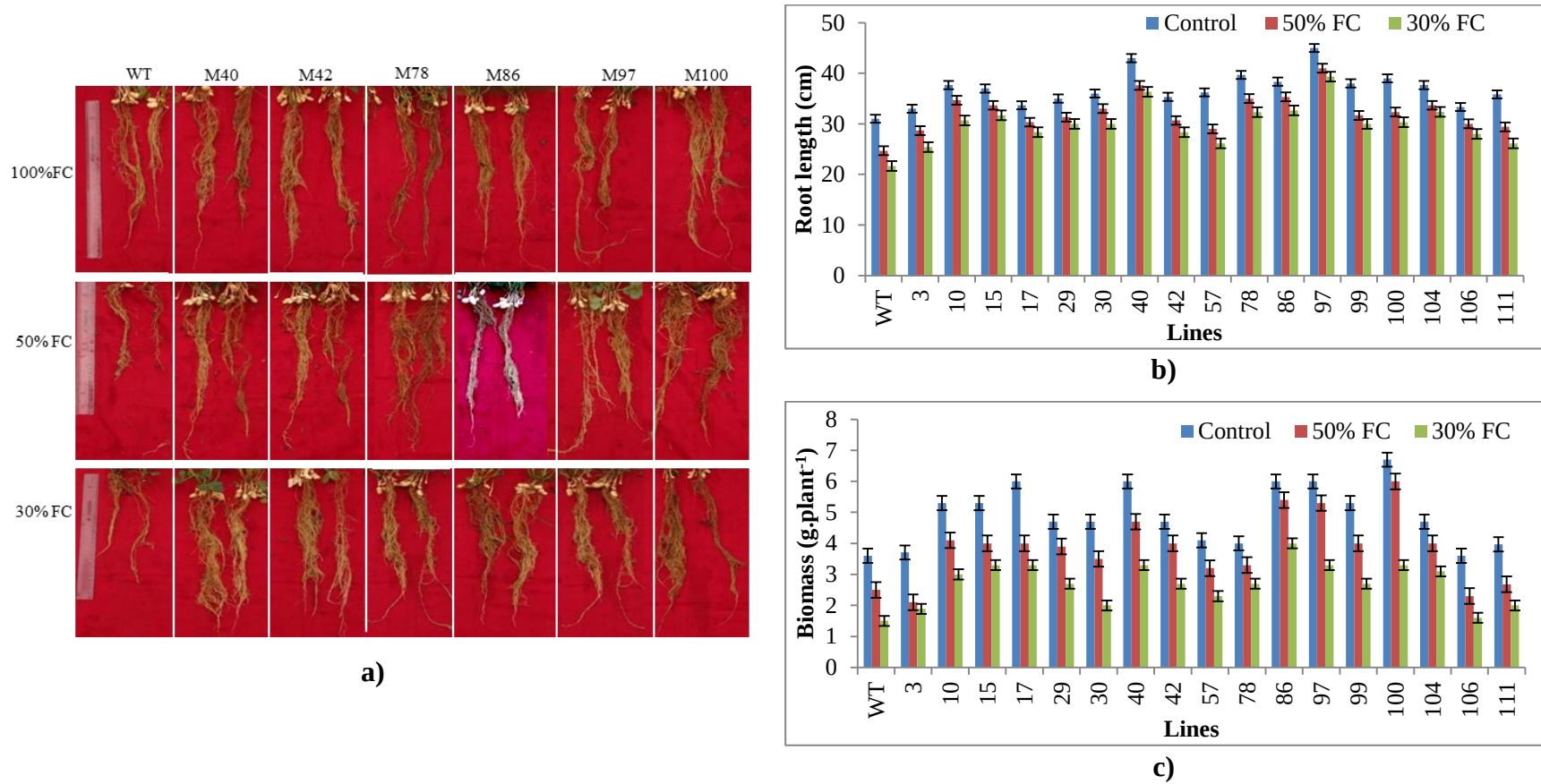
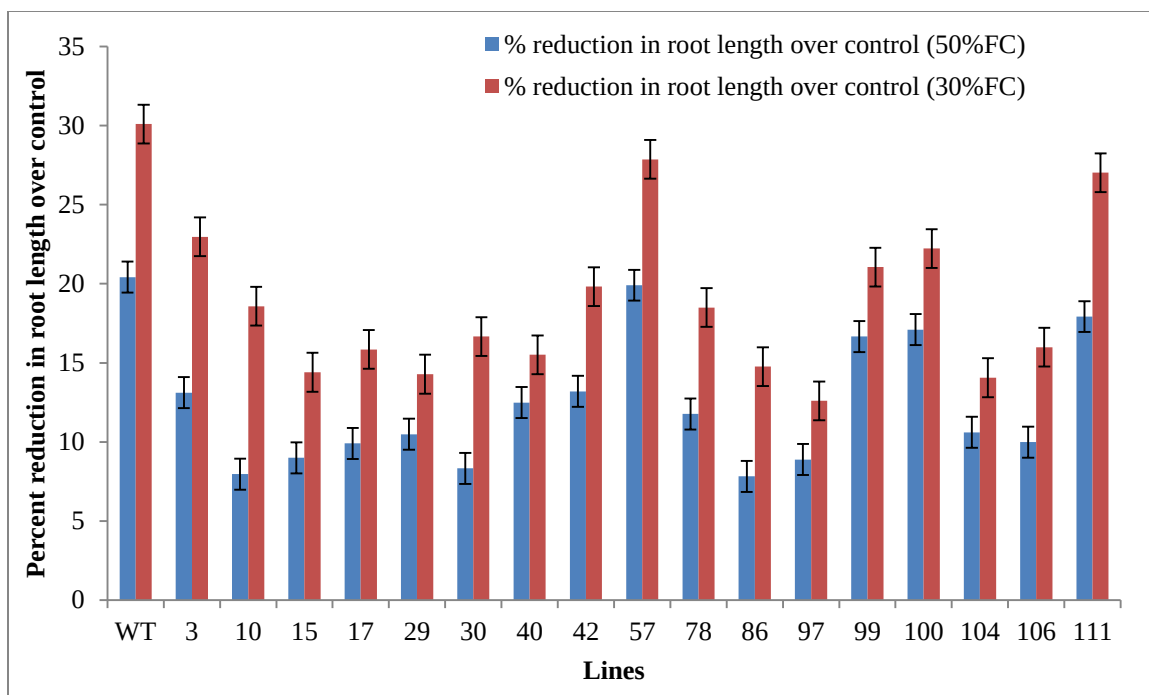
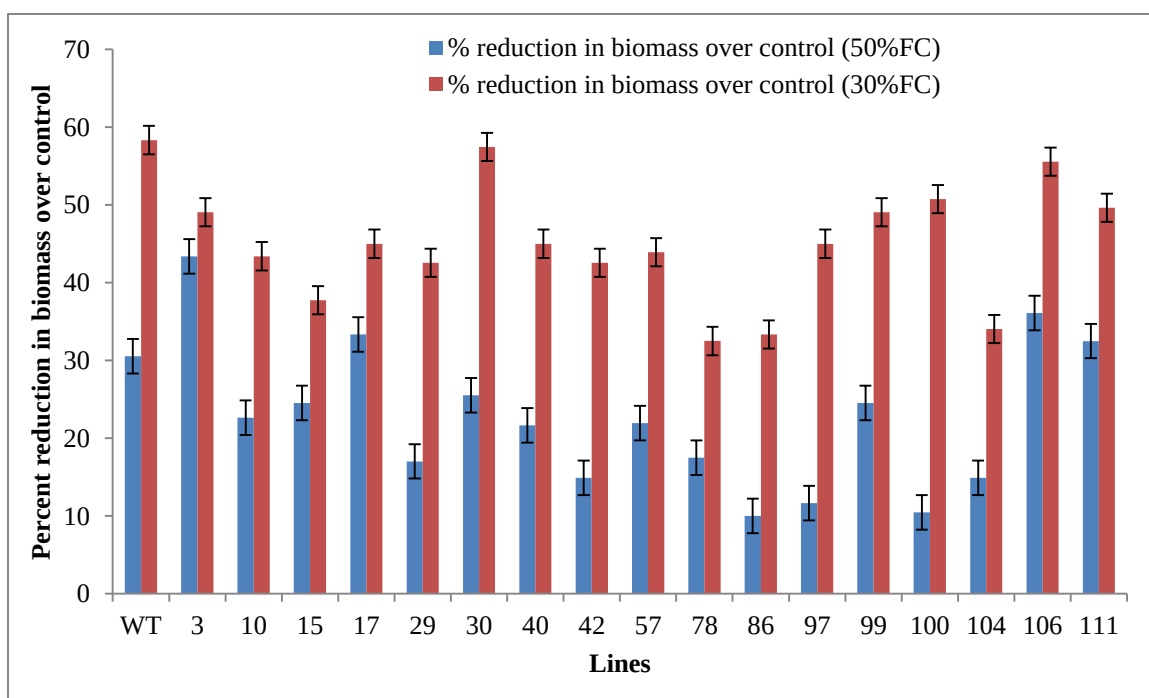


Fig. 21: Response of transgenic lines under moisture stress conditions. Plants were subjected to gradual moisture stress and maintained at different field capacity (FC) of 50% and 30% and allowed for recovery growth. a) Representative photographs of root growth after recovery (35 day old) b) Root length c) Total dry matter of transgenic under different FC (n=3).



a) Per cent reduction in root length over control



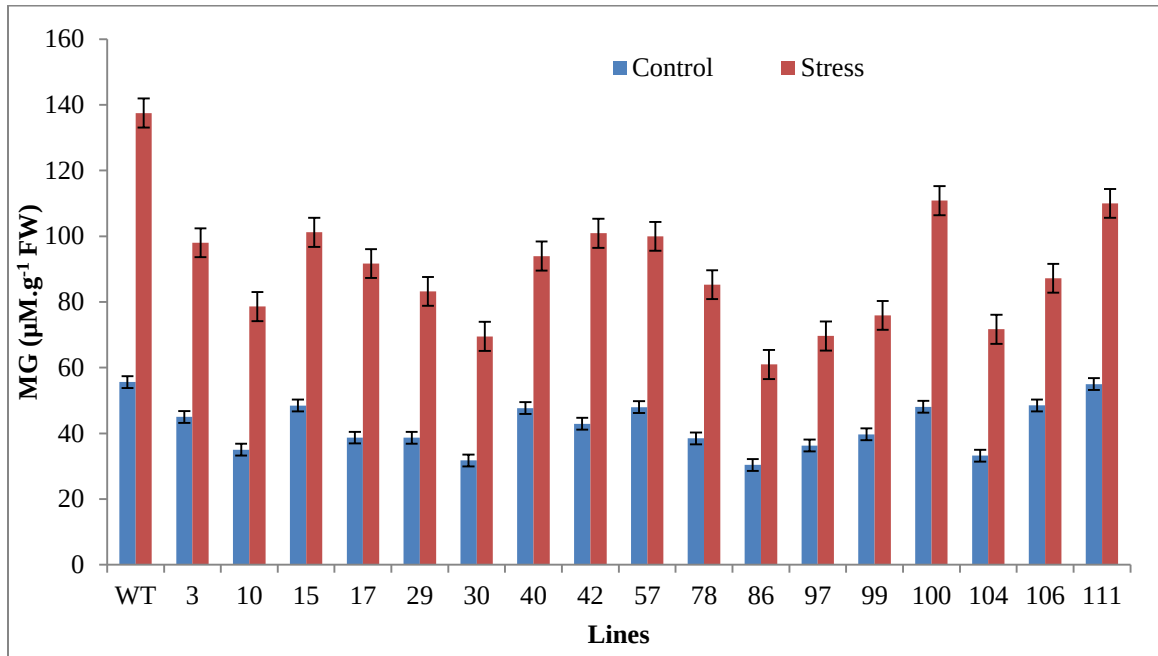
b) Per cent reduction in biomass over control

Fig. 22: Per cent reduction in root length and biomass of wild type and transgenic lines for moisture stress

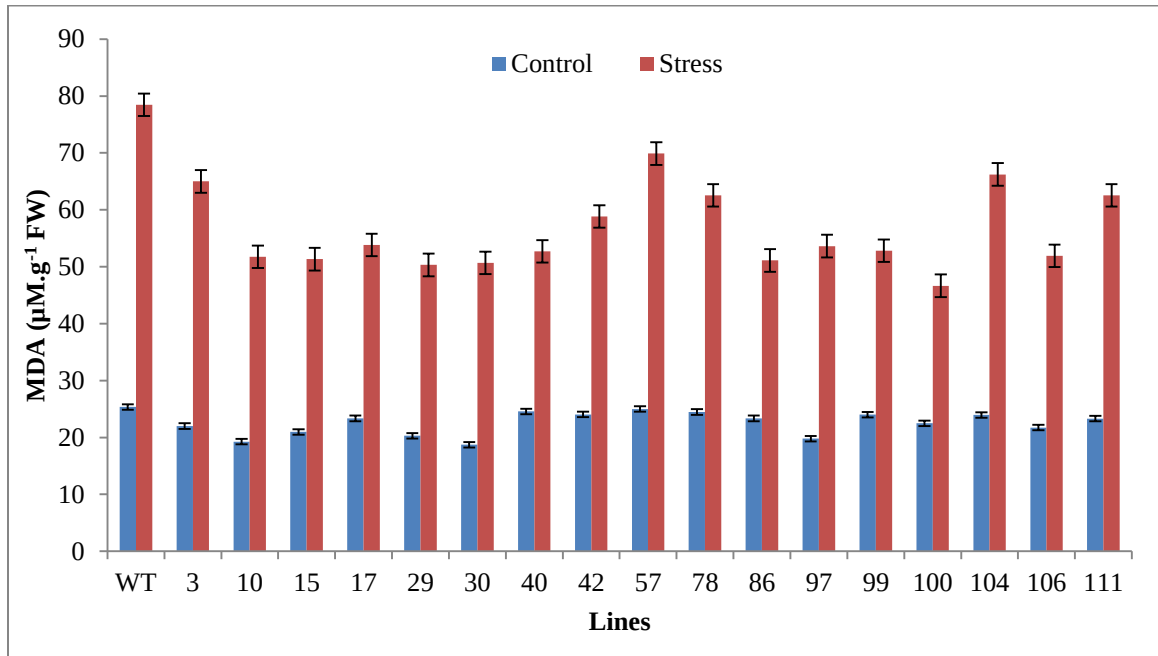
Biochemical parameters: The biochemical parameters like Malondialdehyde (MDA) which is an end product in lipid peroxidation of polyunsaturated fatty acids in phospholipids, an increase in free radicals causes overproduction of MDA which in turn leads to oxidative stress and responsible for cell membrane damage. Methyl glyoxal (MG) is a highly reactive cytotoxic alpha-oxoaldehyde compound, and it accumulates when plants are exposed to abiotic stresses. The stress induced MG functions as a toxic molecule, inhibiting on the function of several macromolecules. The MG and MDA levels are accumulated more in case of wild type than transgenics (Fig. 23a & b). Per cent increase of MG and MDA content over control was relatively high in wild type compared to transgenics but the differences were marginal (Fig. 24a & b). Total soluble protein content indirectly reflects the total protein turnover/synthesis. In few transgenic lines the total protein content was estimated in control and stressed plants and pooled data is presented in the Fig. 25. The results indicated that transgenes maintained relatively higher protein synthesis and have higher protein soluble protein levels.

Correlation analysis: Strong positive correlation was seen between root length under control and stress condition (Fig. 26a) and the same trend was followed in case of biomass under control and stress condition (Fig. 26b). There was positive trend with regards to total dry matter and root length but it was not significant both under control and stress condition (Fig. 26c & d). Similarly positive trend was seen between per cent reduction in root length over control and percent increase in MDA over control but the correlation was not significant (Fig. 27a). Similar trend was also seen in case of per cent reduction in biomass and percent increase in MDA (Fig. 27b). The objective is to identify tolerant types which show less accumulation of cytotoxic compounds and high biomass under stress. Z-distribution analysis was performed between percent decrease in root length over control and per cent increase in MDA over control. The lines which fall in first quadrant will have less per cent reduction in root length and less per cent increase in MDA over control but the lines which fall in third quadrant showed more decrease in root length and more increase in MDA over control (Fig. 28a) similarly for per cent decrease in biomass and percent increase in MDA over control was analyzed. The lines which fall in first quadrant will have less decrease in biomass and less increase in MDA over

T5 generation

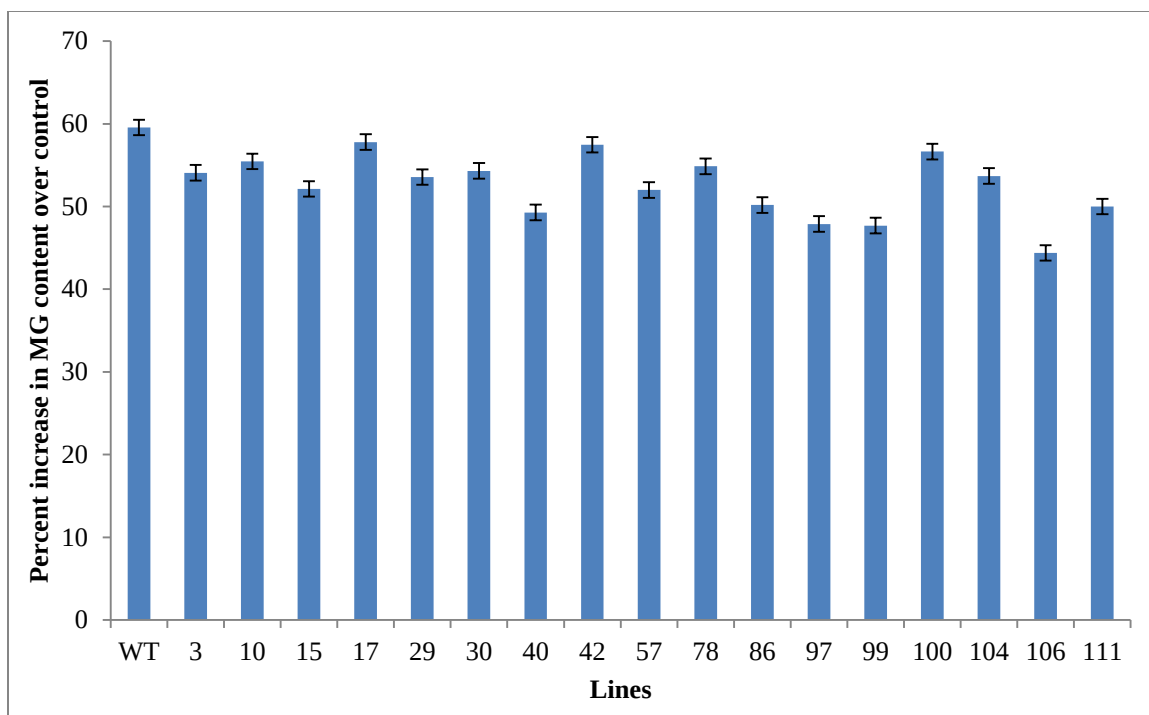


a)

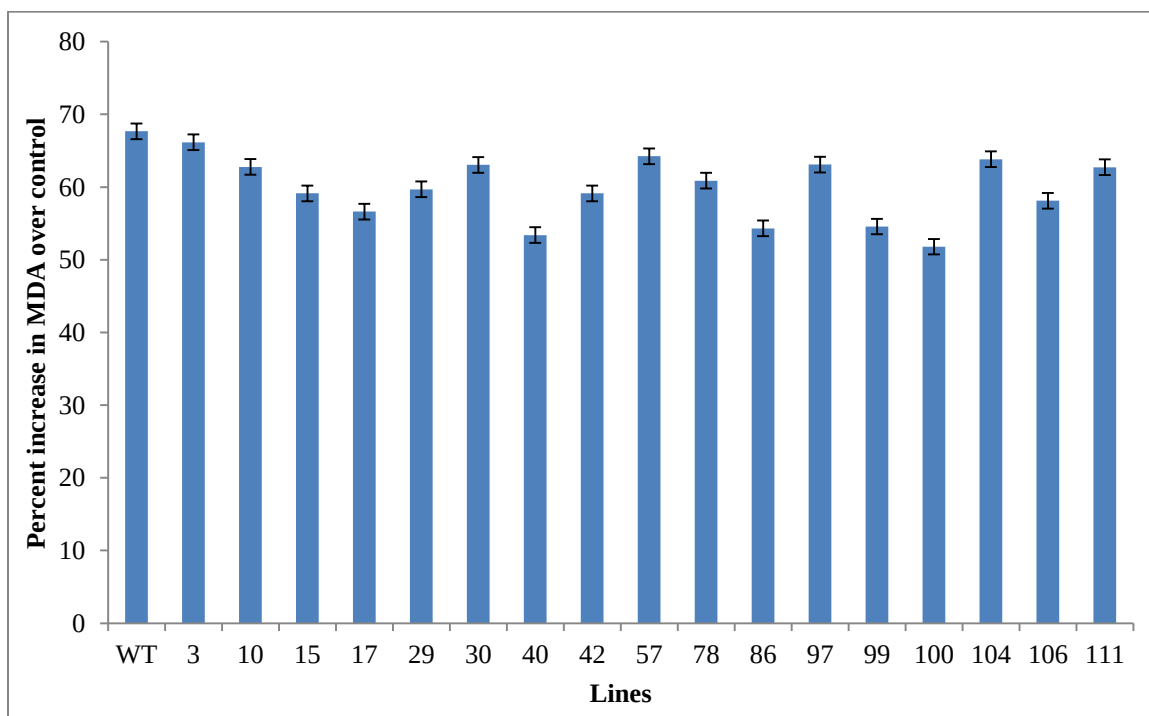


b)

Fig. 23: Estimation of biochemical parameters for wild type and transgenic seedlings. a) Methyl glyoxal b) Malondialdehyde content. Wild type and transgenic samples from the plants subjected to moisture stress by gravimetry were collected for biochemical studies

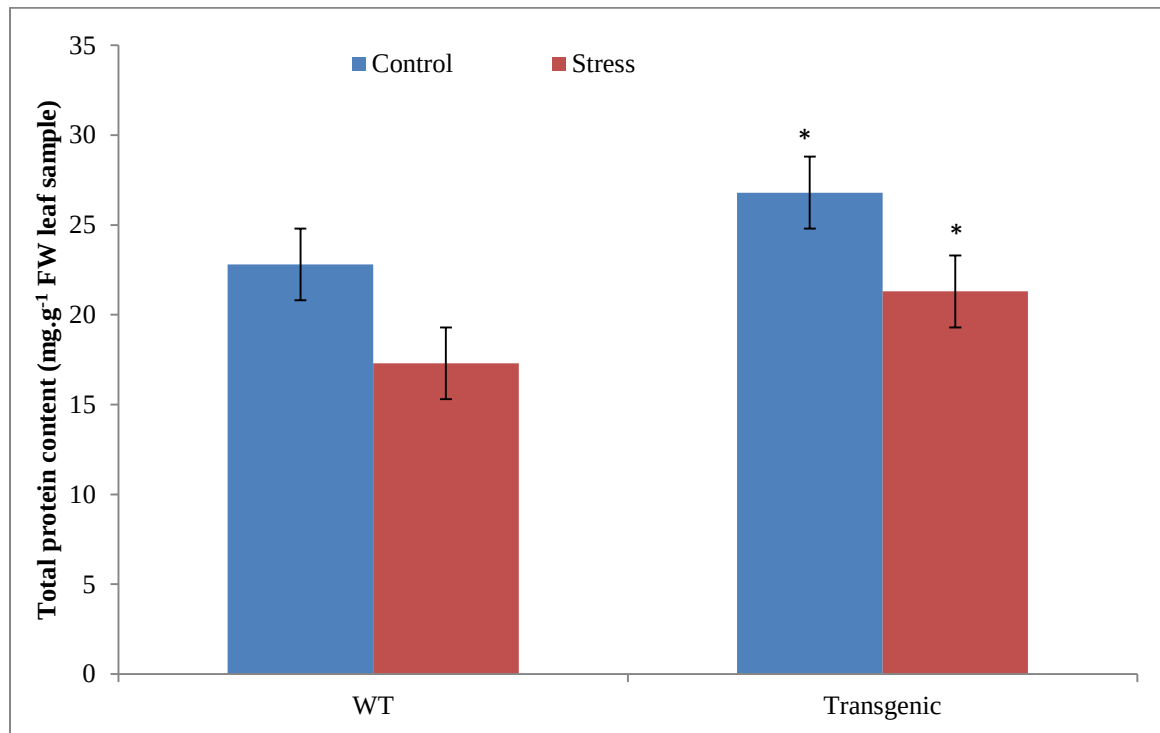


a) Per cent increase in MG over control



b) Per cent increase in MDA over control

Fig. 24: Per cent reduction in biochemical parameters of wild type and transgenic lines



* t-test with $P < 0.05$

Fig. 25: Total protein content in wild type and transgenic plant under control and stress condition. Protein from wild type and transgenic plant under control and stress was extracted and buffer soluble protein was quantified by Bradford method.

T5 generation

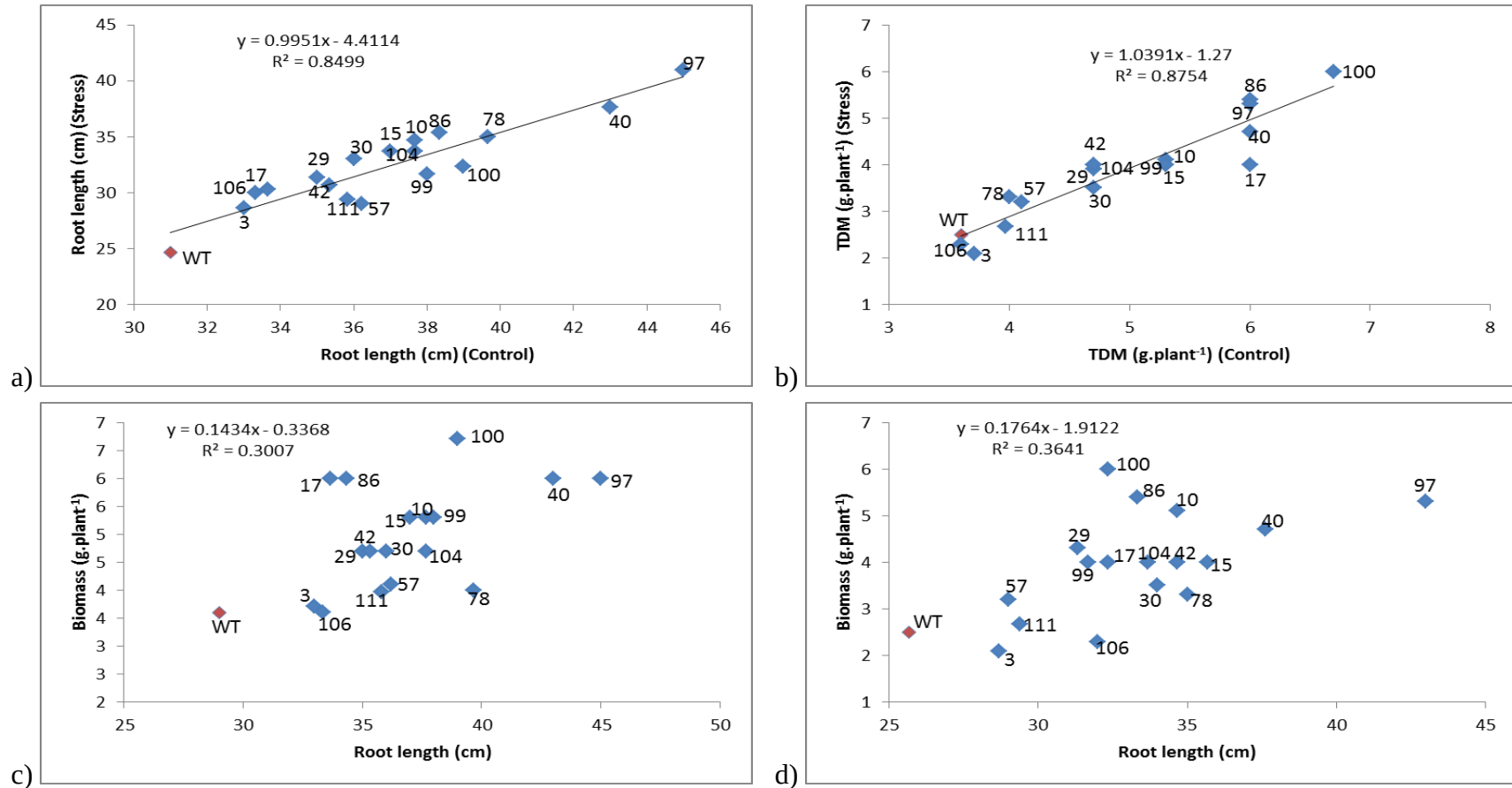
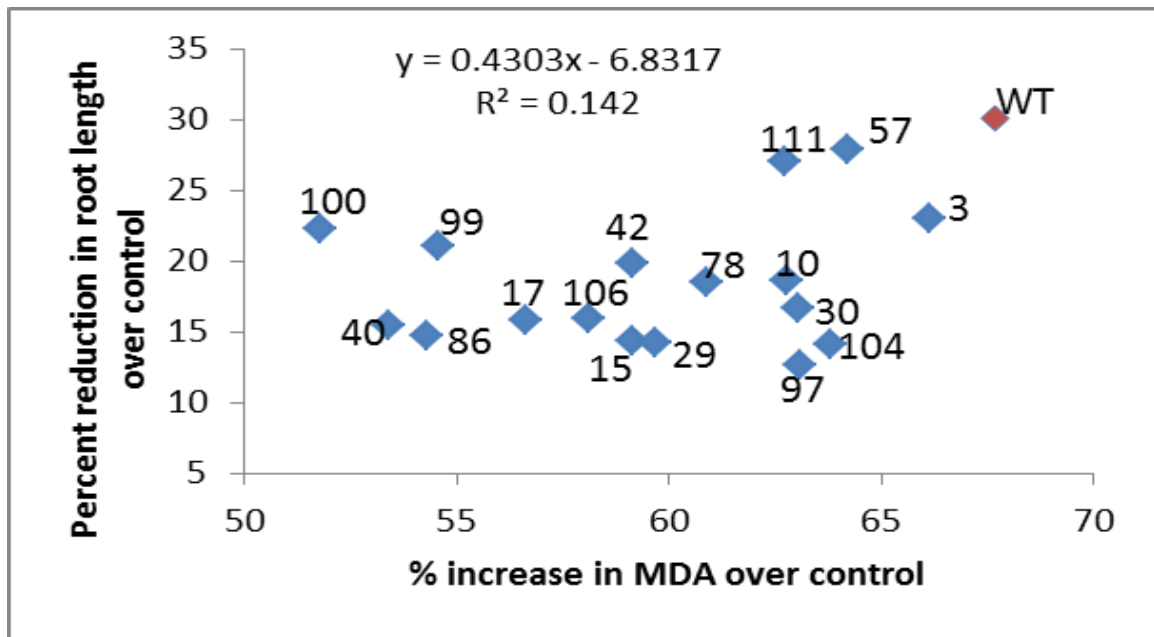
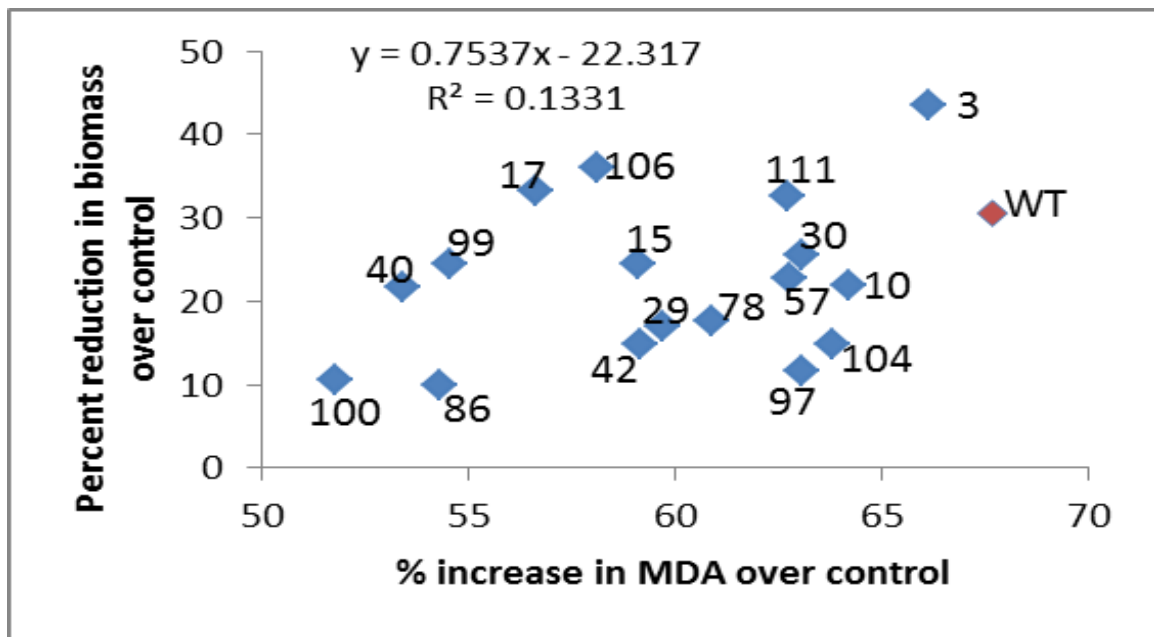


Fig. 26: Correlation analysis between root length and biomass a) Root length under control and stress condition b) TDM under control and stress condition c) Total dry matter and root length of wild type and transgenic plants under control condition d) Total dry matter and root length of wild type and transgenic plants under stress condition. Wild type and transgenic seedlings were subjected to moisture stress by gravimetry approach.

T5 generation

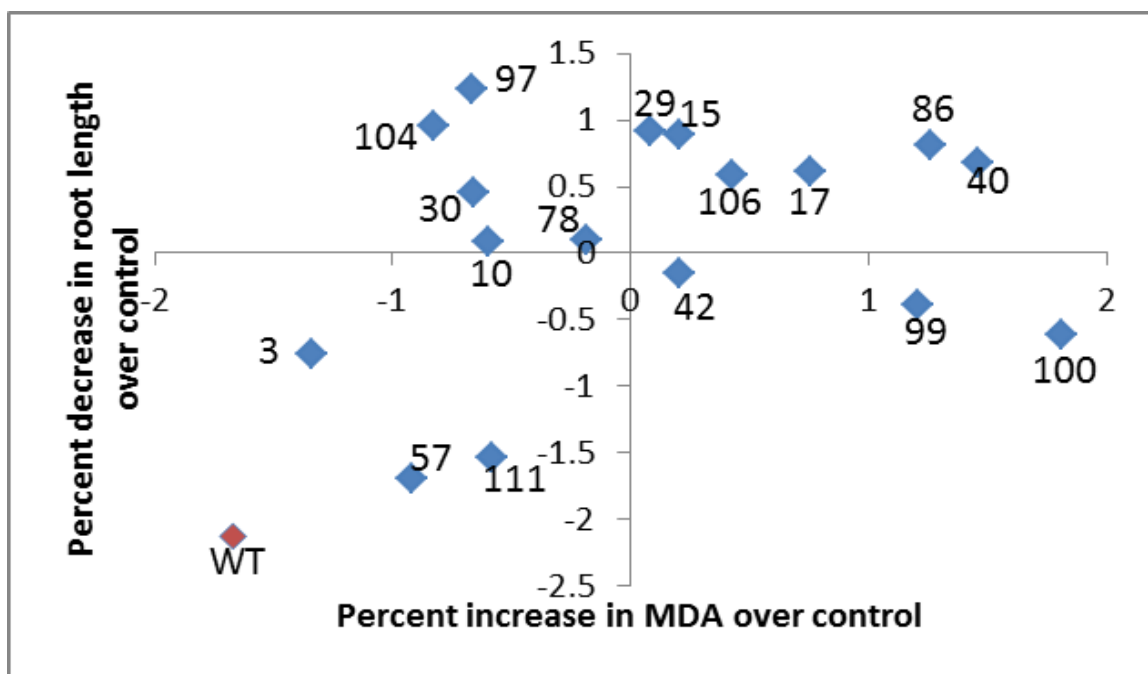


a)

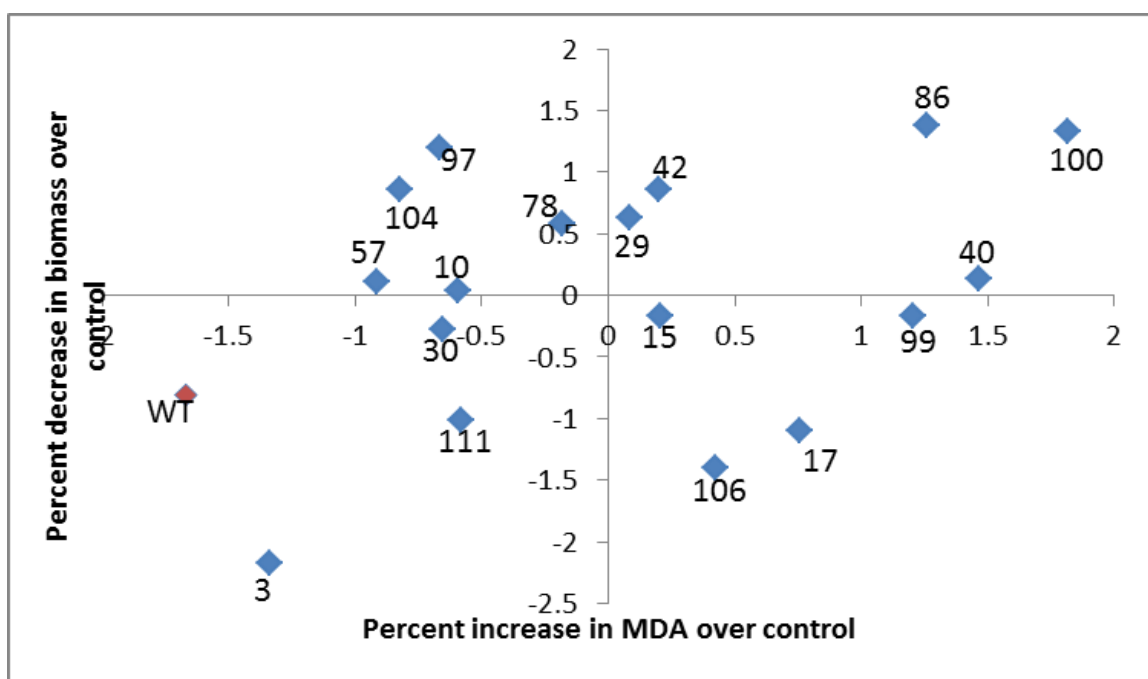


b)

Fig. 27: Correlation analysis between MDA, root length and biomass a) Per cent increase in MDA over control and per cent reduction in root length over control b) per cent increase in MDA over control and per cent reduction in biomass over control Wild type and transgenic seedlings were subjected to moisture stress by gravimetry approach.



a)



b)

Fig. 28: Z-distribution analysis between MDA, root length and biomass a) Per cent reduction in root length over control and per cent increase in MDA over control b) Per cent decrease in biomass over control and per cent increase in MDA over control. Wild type and transgenic seedlings were subjected to moisture stress by gravimetry approach.

control (Fig. 28b). So lines which fall in first quadrant such as 17, 29, 40, 42, 86, 100, 106 are selected as tolerant types.

4.5.3 Yield analysis of groundnut transgenic lines under managed drought environment (T₅ generation)

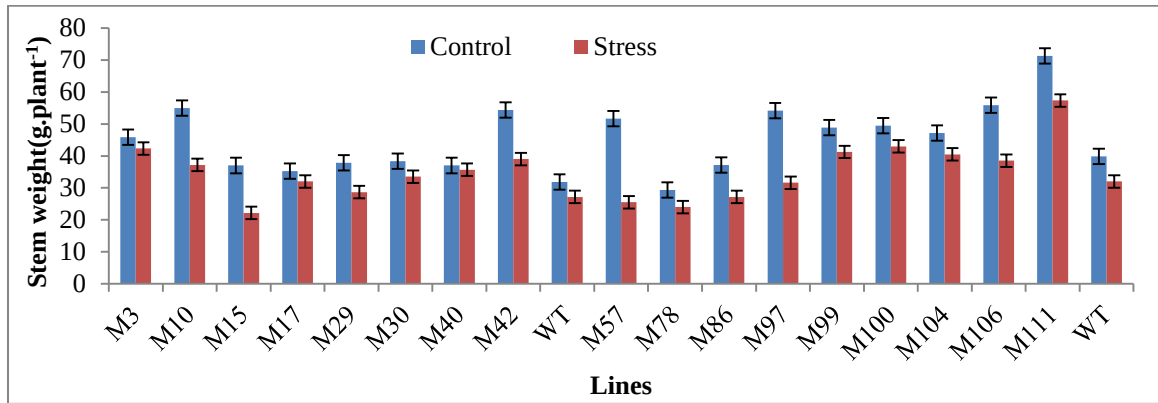
Major objective of this study was to identify productive transgenic lines in soil grown plants under contained condition. The transgenic plants along with wild type were grown in managed drought environment facility, the most widely accepted and relevant method for creating a uniform moisture regime in the field is by establishing a managed drought environment (MDE) facility. Rain free conditions in the MDE facilities are created by erecting the rain out shelter. The MDE is a facility where plants are grown in contained field conditions by providing deserved input of water by sprinklers simulating the natural precipitation. The moisture regimes are maintained based on the pan evaporation (PE) and crop evapotranspiration (ET) rates for a given area and season.

The groundnut transgenic lines along with wild type were sown in this containment MDE facility having semi-automated irrigation with sprinkler facility adjusted to movable rain-out shelter. Two plots were maintained and were irrigated every alternate day with 10 mm of water through sprinkler system. When the plants were 50-day-old, one of the stress plots received irrigation once in every week (10 mm water). Such irrigation schedule was continued up to harvesting and the total amount of water provided was 180 mm for stress plot. The control plot was given a total amount of 500 mm water. The plants were harvested and different yield parameters were computed such as stem weight, leaf weight and pod weight.

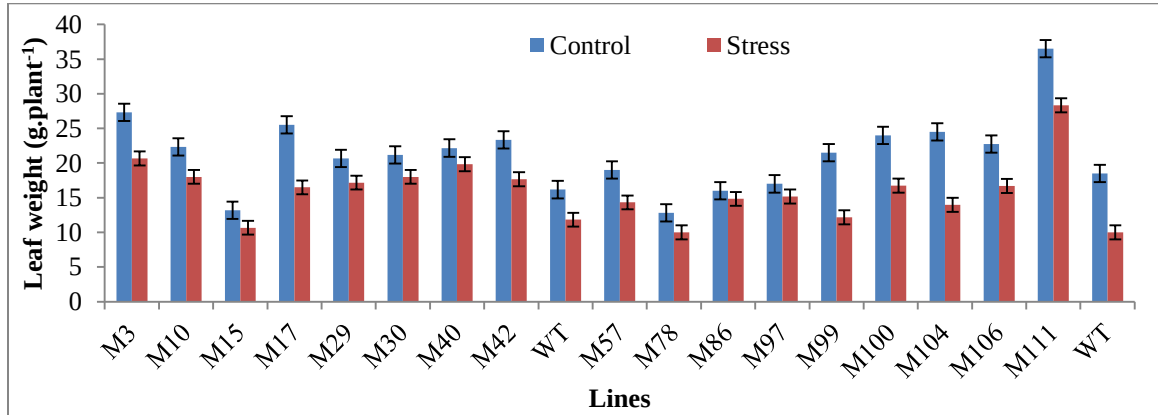
Stem and leaf weight: Most of the transgenic lines like 17, 29, 30, 40, 86, 97, 99, 100 and 104 showed more leaf and stem weight compared to wild type under both control and stress condition (Fig. 29a & b). The mean and range of stem weight and leaf weight are presented in Table 7. The transgenic lines showed high biomass when compared to wild type plant.

T5 generation

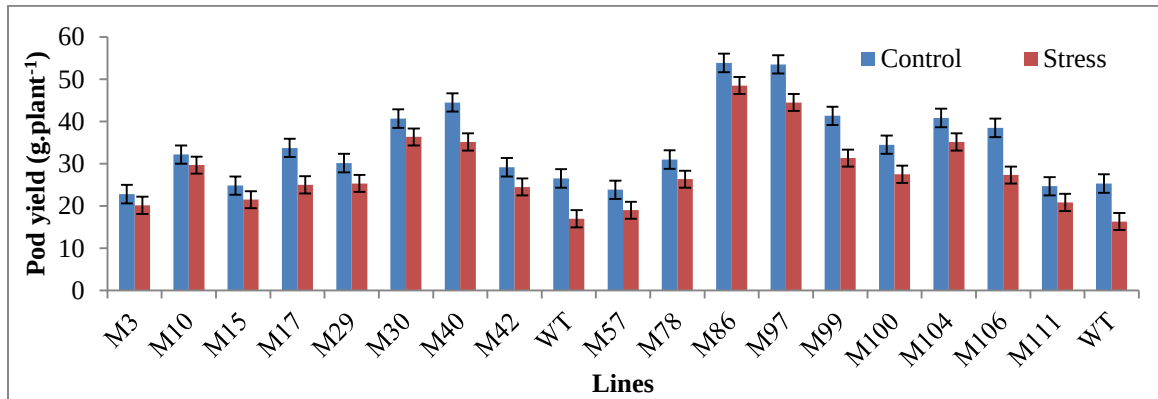
Yield analysis under Managed Drought Environment (MDE)



a) Stem weight



b) Leaf weight



c) Pod yield

Fig. 29: Yield parameters of wild type and transgenic plants under Stress and control condition. Wild type and transgenic plants were sown under MDE and stress was imposed after 50 days after sowing for 20 days and then analyzed for yield parameters at harvest (n=3).

Pod yield: The transgenic lines showed more pod yield compared to wild type plant (Fig. 29c). The transgenic lines showed less per cent reduction in pod yield around 16% over control whereas transgenic lines showed 35% reduction in pod yield over control (Fig. 30). Some of the transgenics such as 3, 10, 15, 29, 30, 42, 78, 86, 97 and 104 showed less per cent reductions in pod yield when compared to wild type.

Correlation analysis: Correlation analysis was performed between Yield and TDM both under control and stress condition to select the promising lines. The pod yield under control and stress condition showed strong significant relationship with $R^2 = 0.91$ (Fig. 31a) and biomass also showed significant relationship both under control and stress condition with $R^2 = 0.80$ (Fig. 31b). Based on pod yield under both stress and non-stress condition the ten transgenic lines such as 17, 29, 30, 40, 78, 86, 97, 99, 100 and 104 were selected for further evaluation.

4.5.4 Molecular analysis of the selected groundnut transgenic lines (T_5 generation)

The integration of all the three genes in 17 transgenic lines along with marker gene was confirmed by PCR analysis using genomic DNA as template. The transgenic lines showed integration of all the three genes by using gene specific primers. The marker gene showed an amplification of 436bp and three regulatory genes i.e. PDH45, Alfin1 and HSF4 showed an amplification of 200bp, 200bp and 500bp respectively (Plate 2).

4.5.5 Growth and productivity analysis under contained field condition during summer season (T_5 generation)

The 10 best transgenic lines were selected and grown under contained field condition till harvest. Two plots are maintained and irrigated every alternate day; one plot was subjected to moisture stress after 50 DAS for 20 days and then maintained till harvest. Growth parameters like plant height, total number of leaves, total leaf area, TDM and gas exchange parameters were recorded at different growth intervals.

50 DAS: After 50 DAS growth parameters like plant height, number of leaves, leaf area and TDM were significantly high in case of transgenic lines compared to wild type plants (Fig. 32). The leaf expansion of the transgenic plants was more compared to wild type

Table 7: Productivity analysis of transgenic and wild type plants under contained managed drought environment (MDE)

		Stem Weight (g.plant ⁻¹)		Leaf weight (g.plant ⁻¹)		Pod yield (g.plant ⁻¹)	
		Mean	Range	Mean	Range	Mean	Range
Transgenics	Control	46.21	29.33-71.33	21.75	12.83-36.5	35.3	22.83-53.83
	Stress	35.26	22.17-57.33	16.52	10.00-28.33	29.3	19.00-48.50
Wild type	Control	35.83	31.83-39.83	17.34	16.17-18.50	25.92	26.50-25.33
	Stress	29.58	27.17-32.00	10.92	10.00-11.83	16.67	16.33-17.00

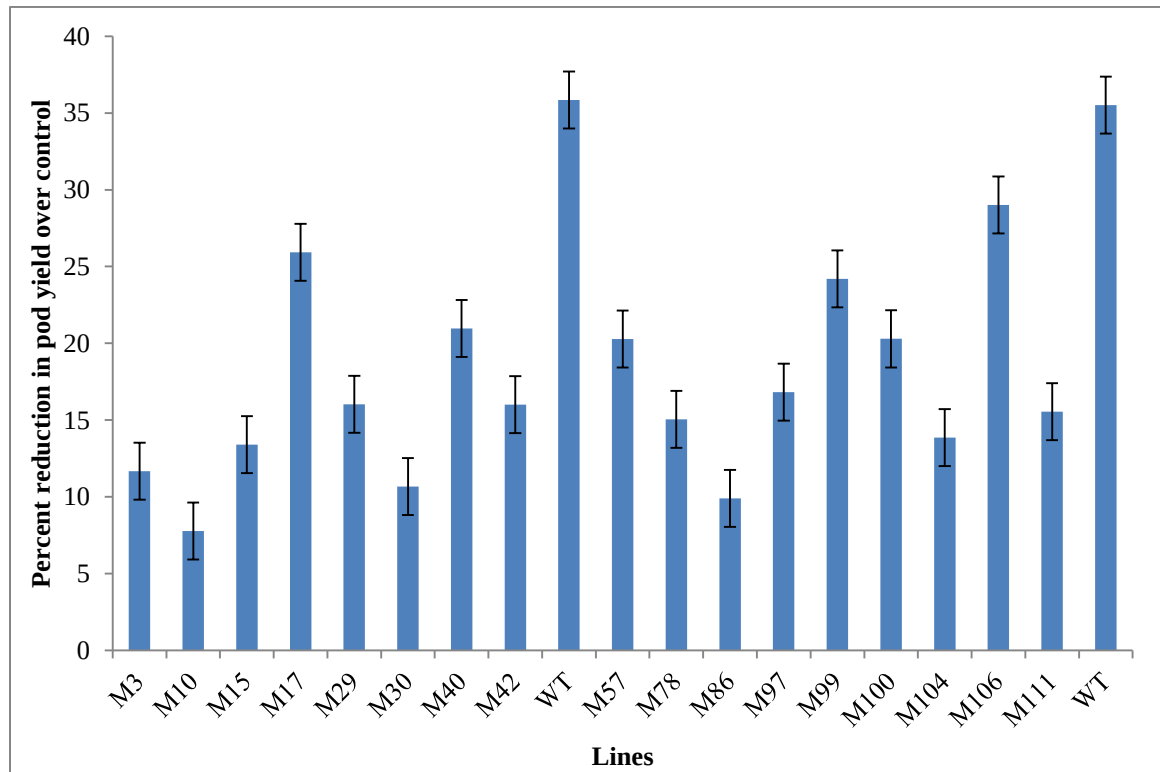
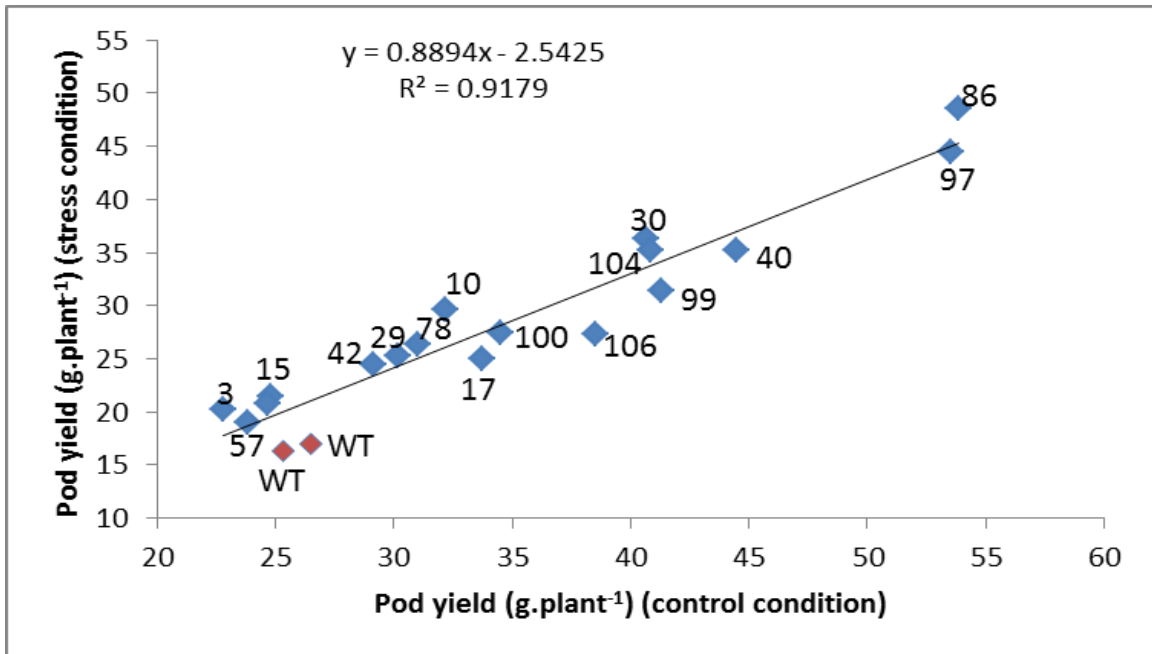
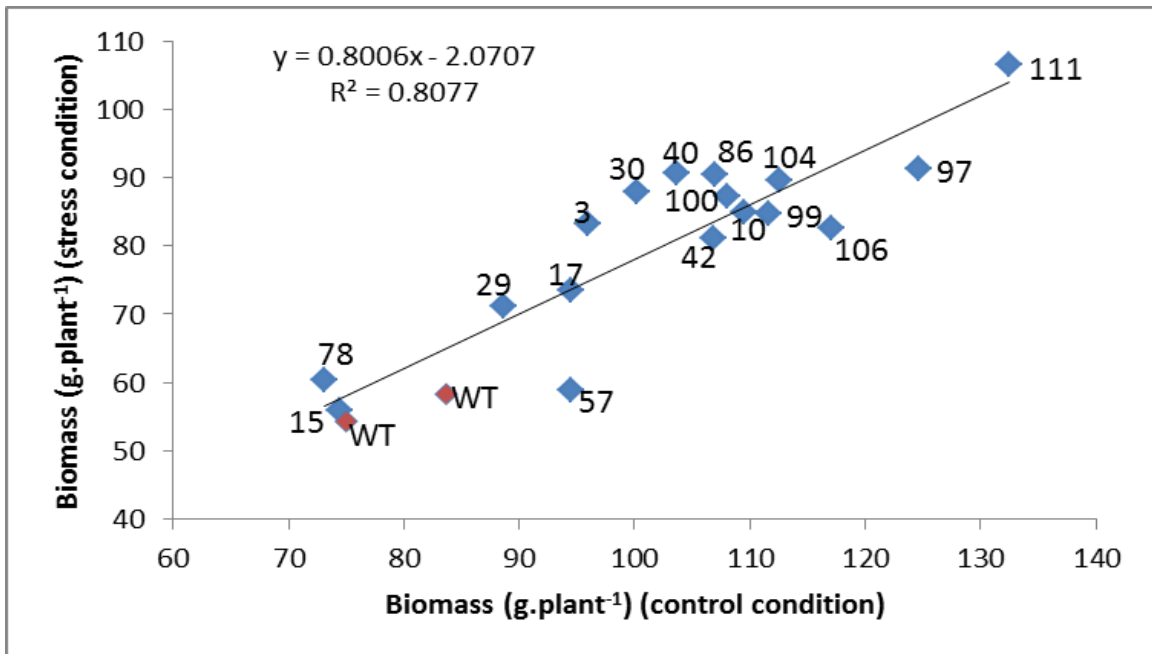


Fig. 30: Per cent reduction in pod yield over control in wild type and transgenic lines. Wild type and transgenic plants were sown under MDE and stress was imposed after 50 days after sowing for 20 days and then analyzed for yield parameters at harvest. (n=3).

T5 generation



a) Yield



b) Biomass under control and stress condition

Fig. 31: Correlation analysis between pod yield and biomass. Wild type and transgenic plants were sown under MDE and stress was imposed 50 days after sowing for 20 days and then analyzed for yield parameters at harvest. N=3 in each line 3 replications were maintained.

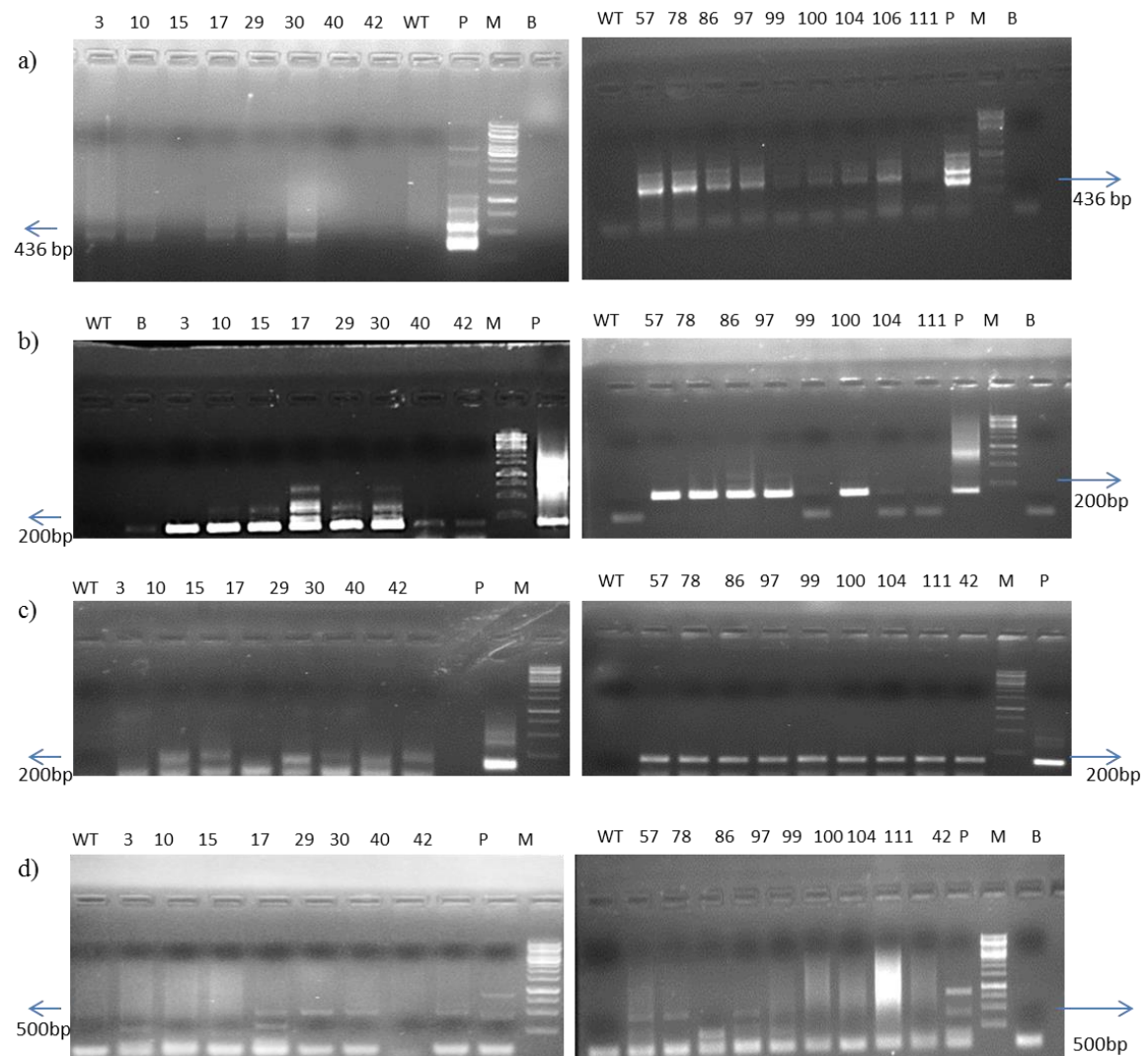
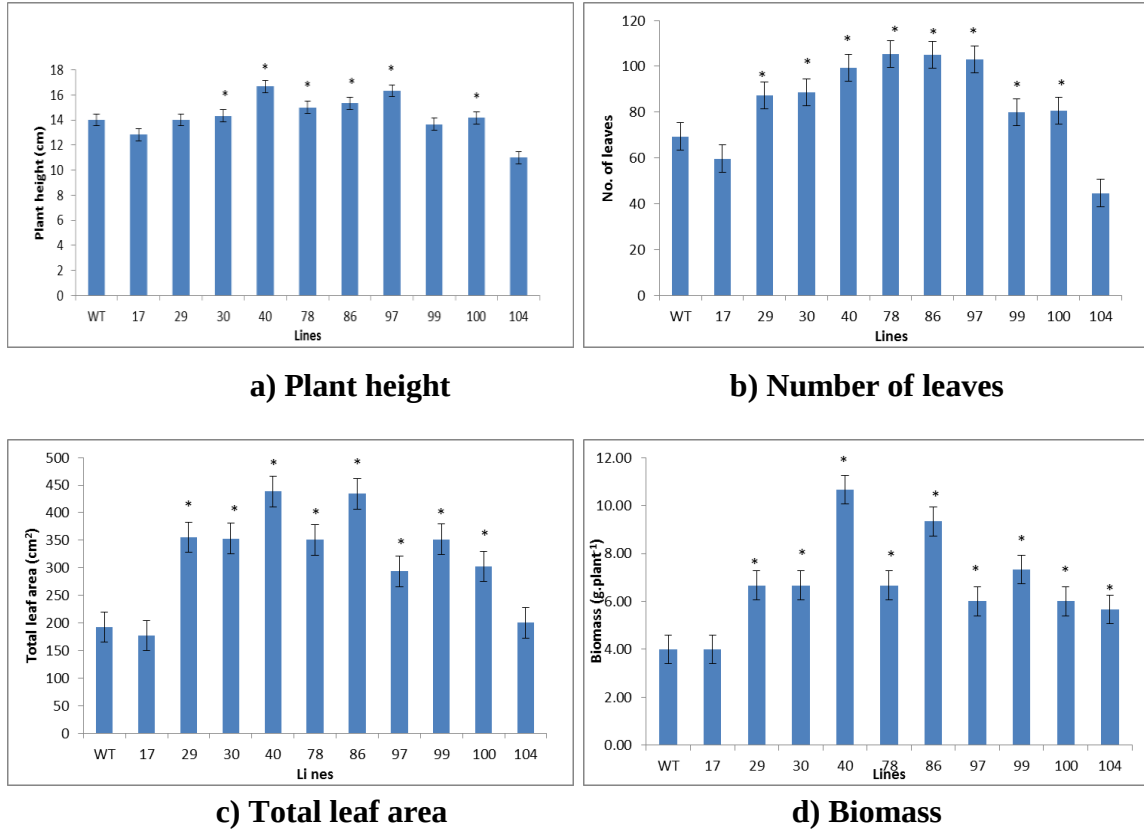


Plate 2: Molecular confirmation of T_5 transgenic plants by using different primer combinations for a) NPT II b) PDH45 c) Alfin1 d) HSF4 genes. M- 1kb DNA Ladder, P- Positive control (plasmid), WT- wild type, B-Blank

T5 generation

Yield analysis under contained field condition in summer season



* t-test with $P < 0.05$

Fig. 32: Growth parameters of wild type and transgenic plants at 50 days after sowing. Wild type and transgenic plants were sown under controlled field condition and observation was recorded at 50 DAS (n=3).

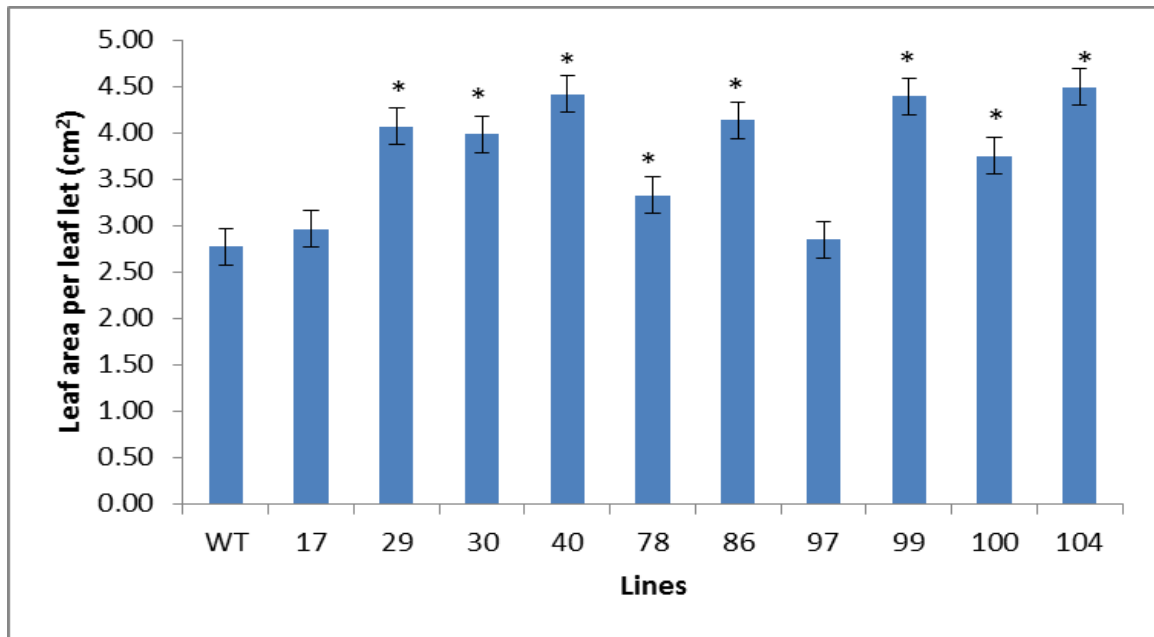
(Fig. 33a) and leaf area index (LAI) of transgenics was significantly high in most of the transgenics compared to wild type plant (Fig. 33b). There was a positive correlation between total leaf area and plant height $R^2 = 0.46$ (Fig. 34a) and there was a strong positive correlation between total leaf area and TDM $R^2 = 0.88$ (Fig. 34b).

Photosynthetic rate at 50DAS: Photosynthetic gas exchange parameters were analyzed using IRGA (LiCOR, USA). Photosynthetic rate in some of the transgenics such as 29, 30, 40, 97, 99 and 106 showed significantly high compared to wild type (Fig. 35a). Stomatal conductance was also high in these transgenic lines compared to wild type plant (Fig 35b).

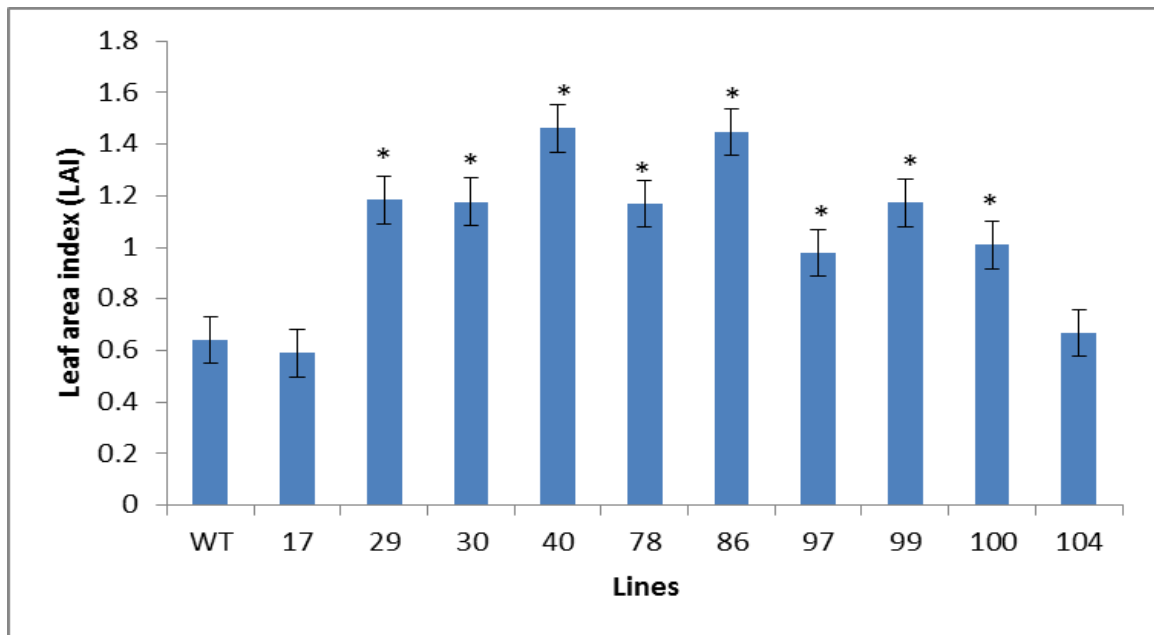
Growth rates under stress and non-stress conditions at 80 DAS: After 80 DAS same growth parameters were recorded under both stress and non-stress condition the transgenic lines showed more plant height (Fig. 36a), Total number of leaves (Fig 36b), Total leaf area (Fig. 36c) and TDM (Fig. 36d) compared to wild type plants. The leaf area per leaf let and LAI was significantly more in most of the transgenics compared to wild type plant (Fig. 37a & b). Net assimilation rate (NAR) in transgenics was almost on par with the wild type. Transgenics showed around $27.38 \text{ g.m}^{-2}.\text{day}^{-1}$ and $18.13 \text{ g.m}^{-2}.\text{day}^{-1}$ mean NAR under non-stress and stress conditions respectively. Whereas wild type showed around $24.39 \text{ g.m}^{-2}.\text{day}^{-1}$ under non-stress condition and $21.11 \text{ g.m}^{-2}.\text{day}^{-1}$ under stress condition there was no much difference between wild type and transgenics under both conditions. Whereas crop growth rate (CGR) was more in case of transgenics compared to wild type. The mean CGR of transgenic lines was $81.15 \text{ g.m}^{-2}.\text{day}^{-1}$ whereas in wild type it showed $44.50 \text{ g.m}^{-2}.\text{day}^{-1}$ under non-stress condition and in stress condition in case of transgenics mean CGR was $40.70 \text{ g.m}^{-2}.\text{day}^{-1}$ when compared to $36.86 \text{ g.m}^{-2}.\text{day}^{-1}$ in wild type plant (Table 8). Dry matter and leaf area ratio (DM/LA) was not high in case of transgenic lines when compared to wild type plant under both conditions (Table 8).

No much variation was observed in NAR and DM/LA ratio between wild type and transgenics under both non-stress and stress condition but variation exists in CGR

T5 generation



a) Leaf area per leaf let

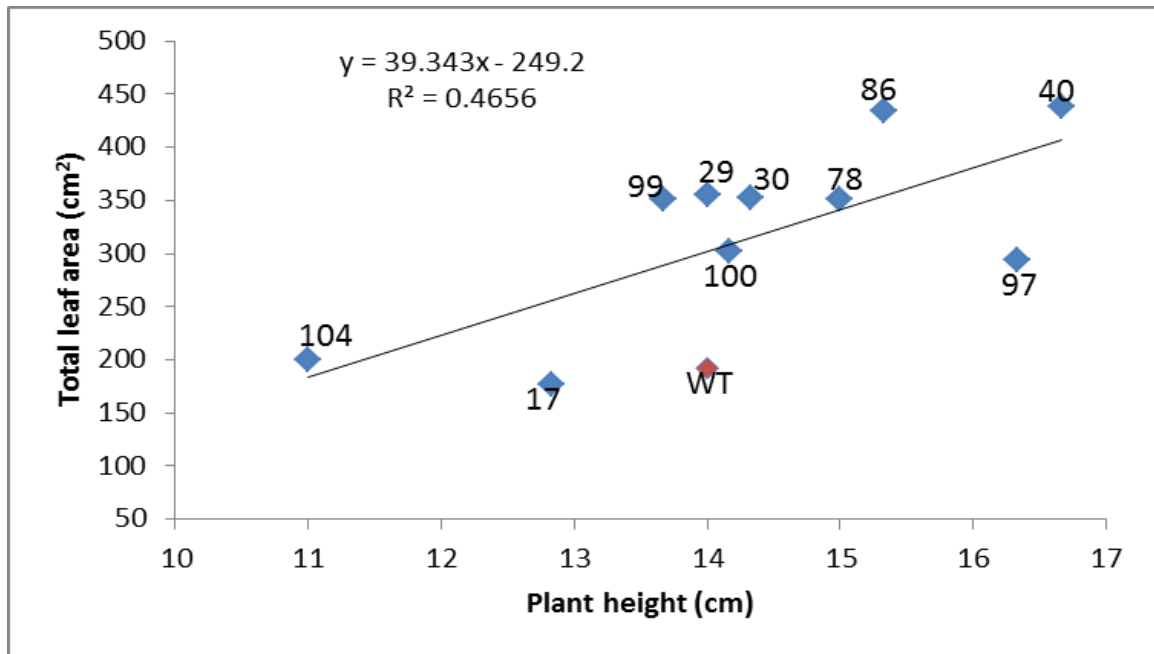


b) Leaf area index (LAI)

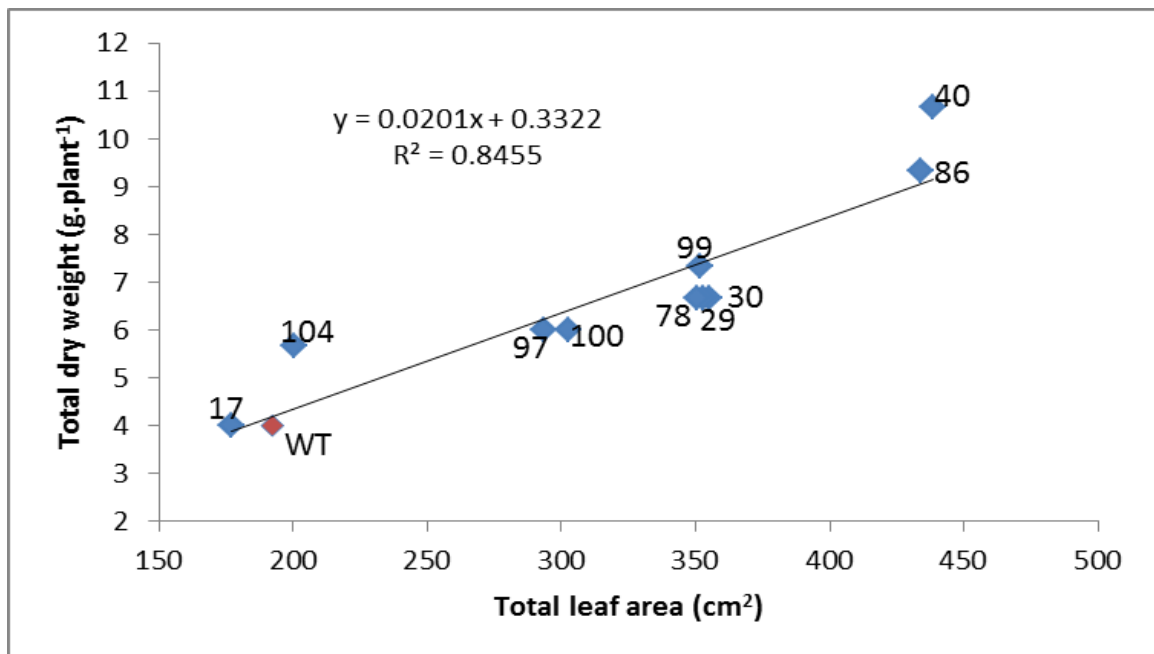
* t-test with $P < 0.05$

Fig. 33: Leaf are parameters of wild type and transgenic plants at 50 days after sowing. Wild type and transgenic plants were sown under controlled field condition and observation was recorded at 50 DAS (n=3).

T5 generation



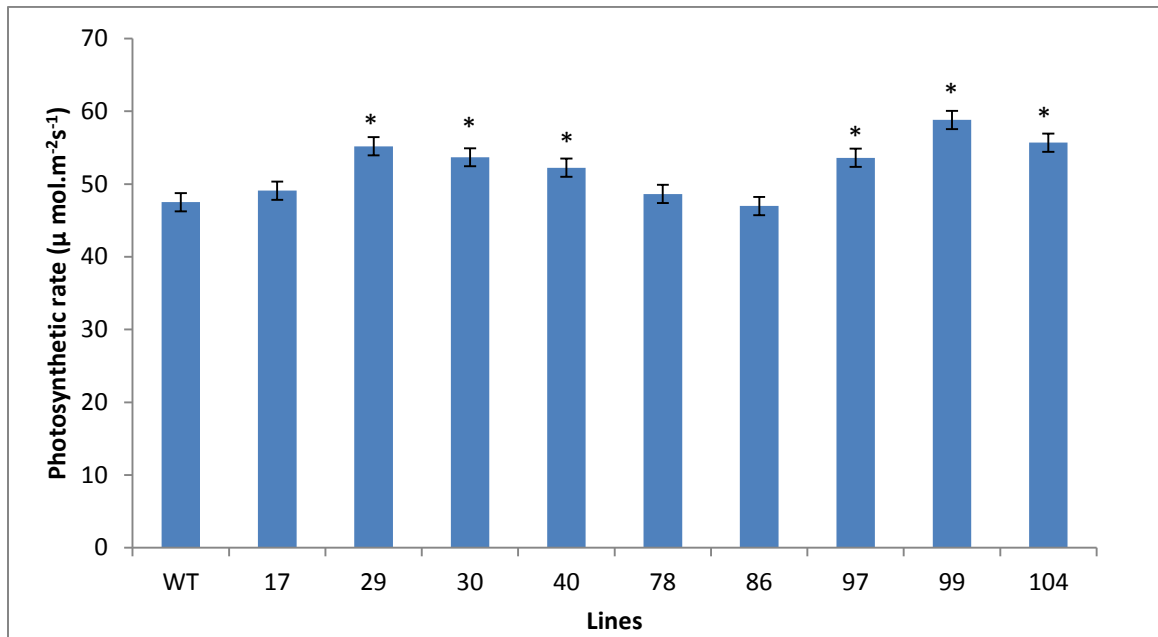
a) Plant height and total leaf area



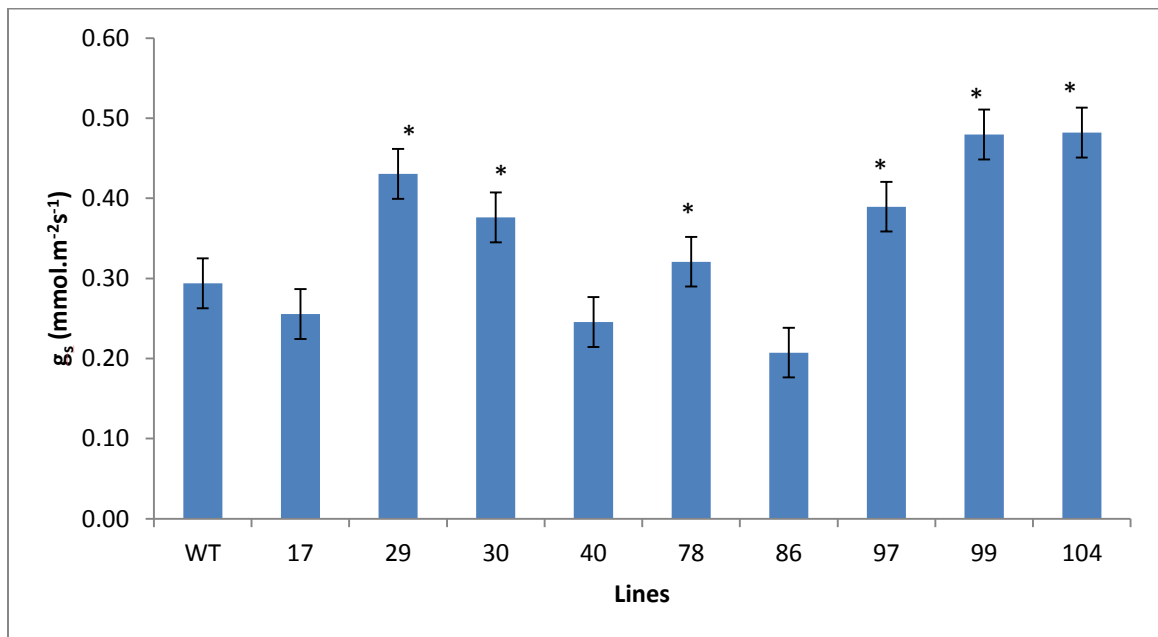
b) Total leaf area and total dry weight (TDM)

Fig. 34: Correlation analysis between various physiological parameters under control condition at 50 DAS. Wild type and transgenic plants were sown under controlled field condition.

T5 generation



a) Photosynthetic rate

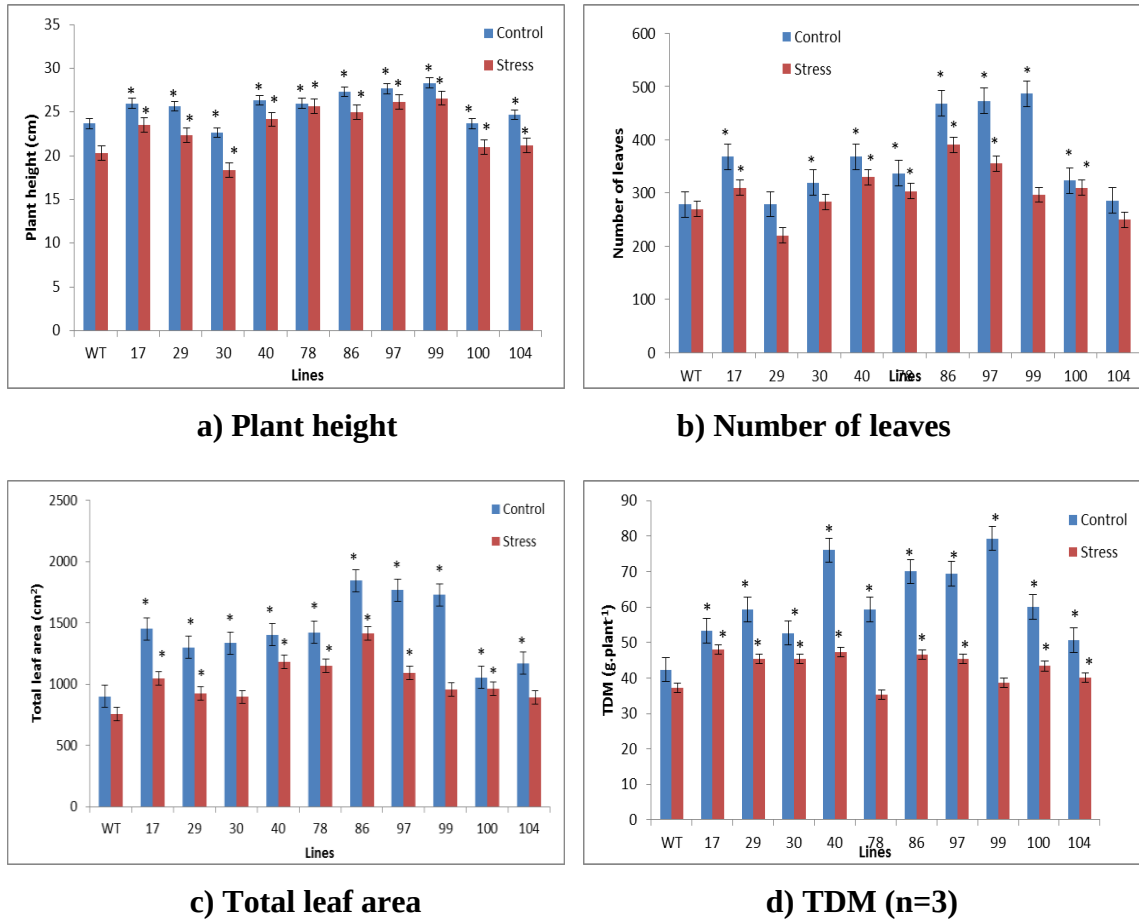


b) Stomatal conductance of wild type and transgenic plants after 50 DAS (n=5)

* t-test with $P < 0.05$

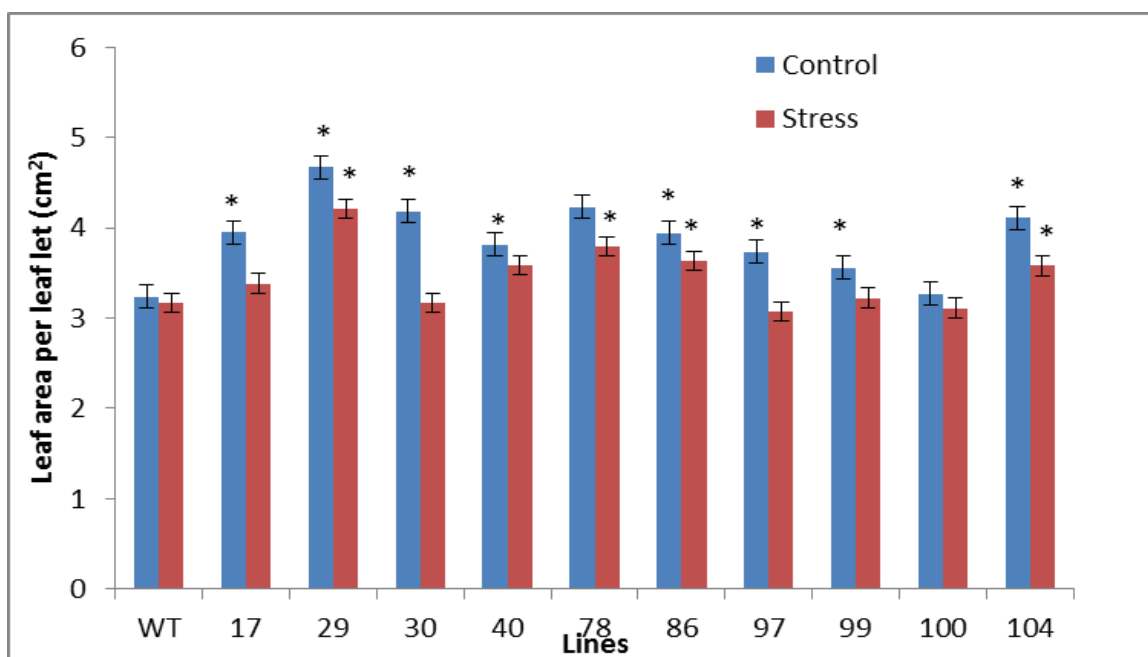
Fig. 35: Gas exchange parameters of wild type and transgenic plants under contained field condition. Wild type and transgenic plants were sown under controlled field condition and photosynthetic efficiency measured using IRGA (LiCOR USA)

T5 generation

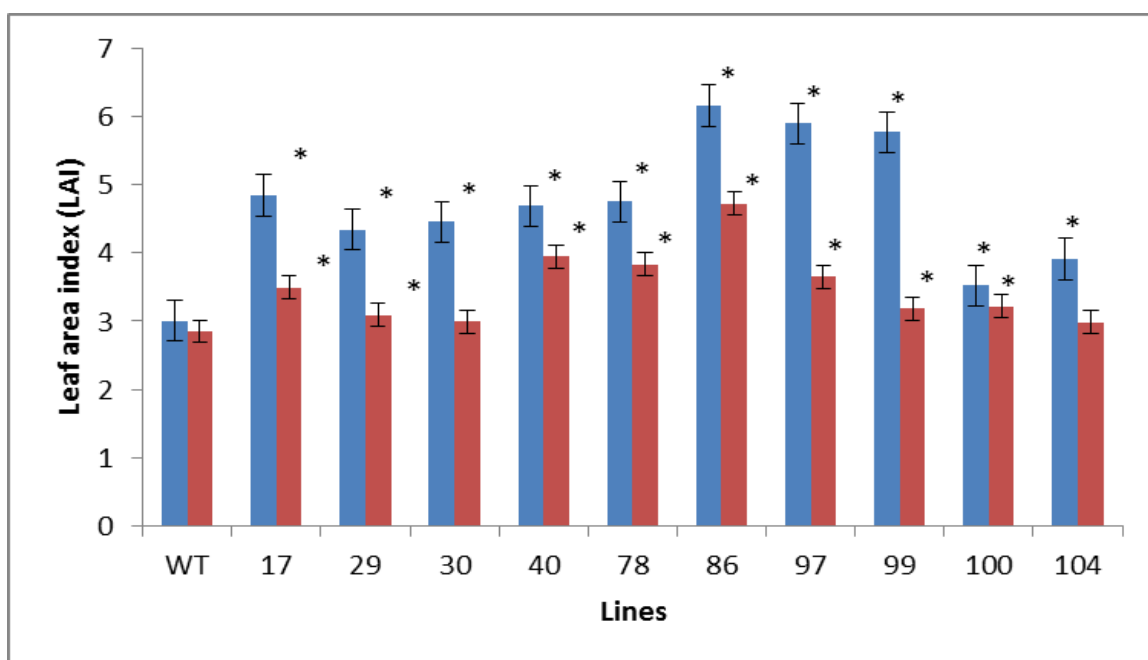


* t-test with $P < 0.05$

Fig. 36: Growth parameters of wild type and transgenic plants under contained field condition at 80 DAS. Wild type and transgenic plants were sown under controlled field condition and stress was imposed after 50 DAS for 20 days observation was recorded at 80 DAS.



a) Leaf area per leaf let



b) Leaf area index (LAI)

Fig. 37: Leaf parameters of wild type and transgenic plants at 80 days after sowing. Wild type and transgenic plants were sown under controlled field condition and stress was imposed after 50 DAS for 20 days observation was recorded at 80 DAS.

Table 8: Net assimilation rate (NAR) and crop growth rate (CGR) of wild type and transgenic lines under both control and stress conditions

Lines	Control	Stress	Control	Stress	Control	Stress
	NAR (g.m ⁻² .day ⁻¹)	NAR (g.m ⁻² .day ⁻¹)	CGR (g.m ⁻² .day ⁻¹)	CGR (g.m ⁻² .day ⁻¹)	DM/LA (g.cm ⁻²)	DM/LA (g.cm ⁻²)
17	26.83	23.93	72.88	48.89	3.67	4.58
29	27.39	20.11	75.66	42.96	4.56	4.89
30	24.53	20.62	69.02	42.96	3.94	5.05
40	26.86	15.07	82.54	40.74	5.41	4.00
78	23.39	12.73	69.23	31.85	4.16	3.07
86	21.85	13.45	83.05	41.48	3.79	3.29
97	30.43	18.90	104.56	43.70	3.92	4.14
99	36.71	15.97	127.39	34.81	4.58	4.04
100	28.41	19.64	64.39	41.48	5.68	4.49
104	27.39	20.90	62.73	38.14	4.32	4.47
Mean	27.38	18.13	81.15	40.70	4.40	4.20
WT	24.39	21.11	44.50	36.86	4.69	4.35
SD	4.12	3.64	20.27	4.80	0.68	0.64
CV %	15.04	20.09	24.98	11.78	15.34	15.19

between wild type and transgenics under both conditions it may be due to the increase in leaf area, LAI, is due to the more number of leaves and leaf size per leaf let is high.

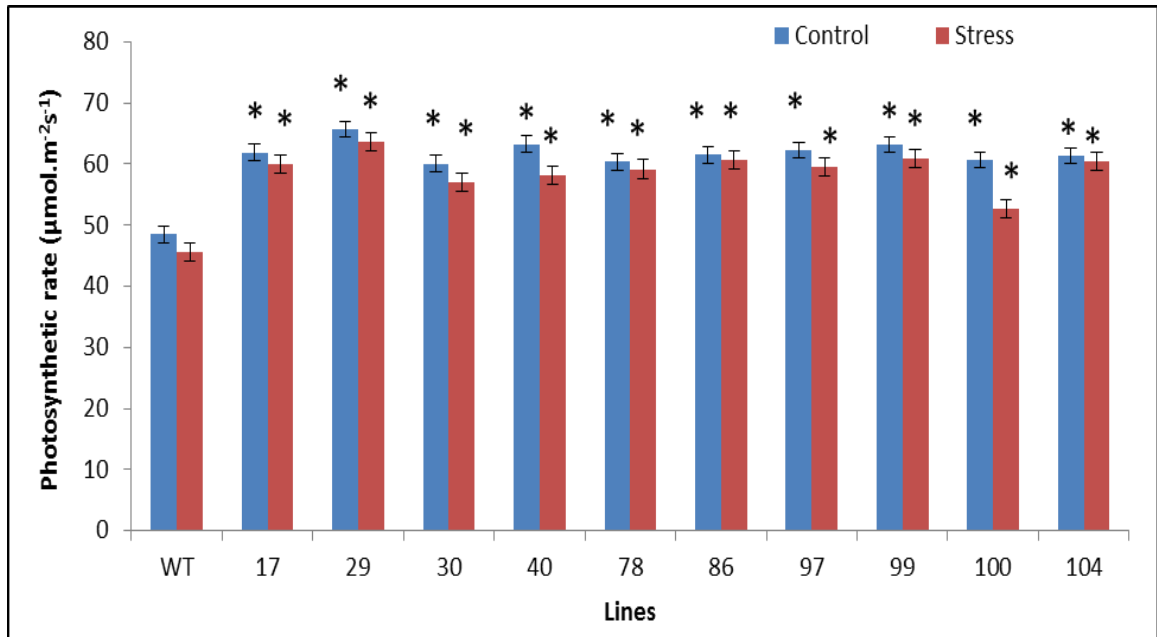
Photosynthetic rate at 80DAS under stress and non-stress conditions: The photosynthetic efficiency and stomatal conductance was significantly high in all the transgenic lines compared to wild type plant under both stress and non-stress conditions (Fig. 38a & b).

Correlation: There was a strong positive correlation between plant height under control and stress condition (Fig. 39a) and also there was a positive correlation between total leaf area under control and stress condition (Fig. 39b). LAI and total leaf area also showed positive correlation but it was not significant (Fig. 39c).

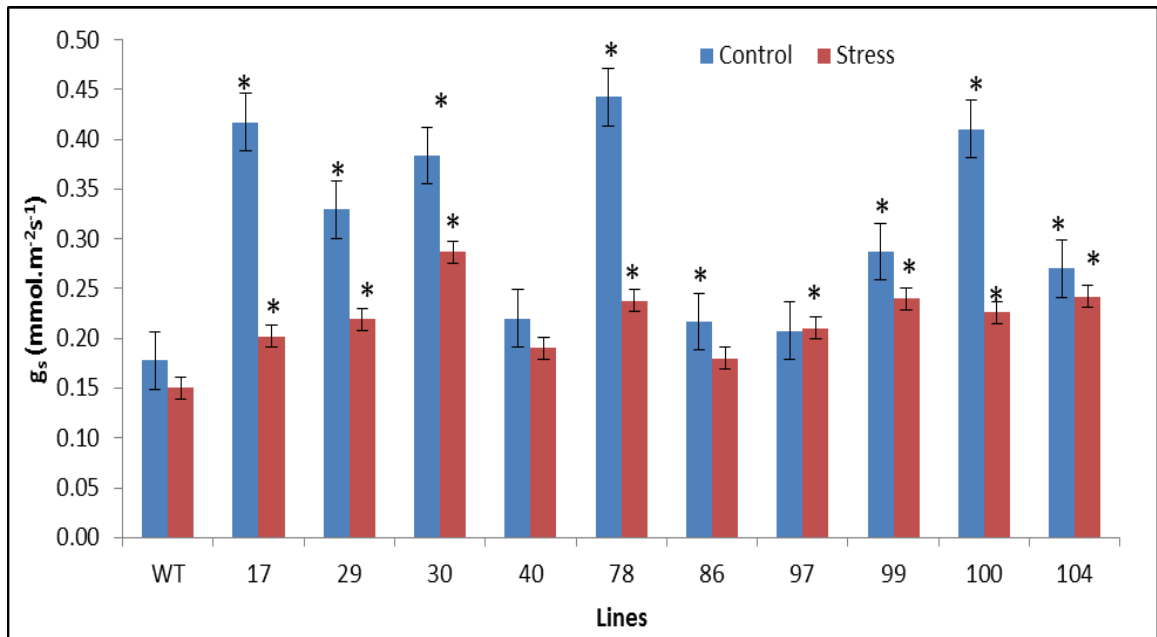
Yield attributes: At harvest yield parameters like pod weight and TDM was recorded in both control and stress condition. The transgenic lines showed more pod weight and TDM when compared to wild type plants under both conditions (Fig. 40a, b & c) (Table 9). The pod yield was significantly more in 29, 40, 86, 97, 100 and 104 under both control and stress conditions. The per cent reduction in pod yield over control was less in most of the transgenics compared to wild type plant (Fig. 41). The mean percent reduction of some of the transgenics such as 29, 30, 40, 86, 97, 100 and 104 was 21.59% whereas in wild type the per cent reduction over control was 32.66%. The productivity of transgenics was more compared to wild type it may be due to increase in crop growth rate but net photosynthetic rate remain same in both wild type and transgenic condition.

The aim of the experiment was to identify few lines with good productivity, hence mean yield of managed drought environment and contained field experiment was calculated. The transgenics which showed high mean pod yield were selected for further study (Table 10). The eight lines selected for further evaluation were 17, 30, 40, 86, 97, 99, 100 and 104.

T5 generation



a) Photosynthetic rate



b) Stomatal conductance

* t-test with P<0.05

Fig. 38: Gas exchange parameters of wild type and transgenic plants under contained field condition at 80 DAS. Wild type and transgenic plants were sown under controlled field condition and stress was imposed after 50 DAS for 20 days and then photosynthetic rate was recorded using IRGA (LiCOR USA) at 80 DAS (n=5).

T5 generation

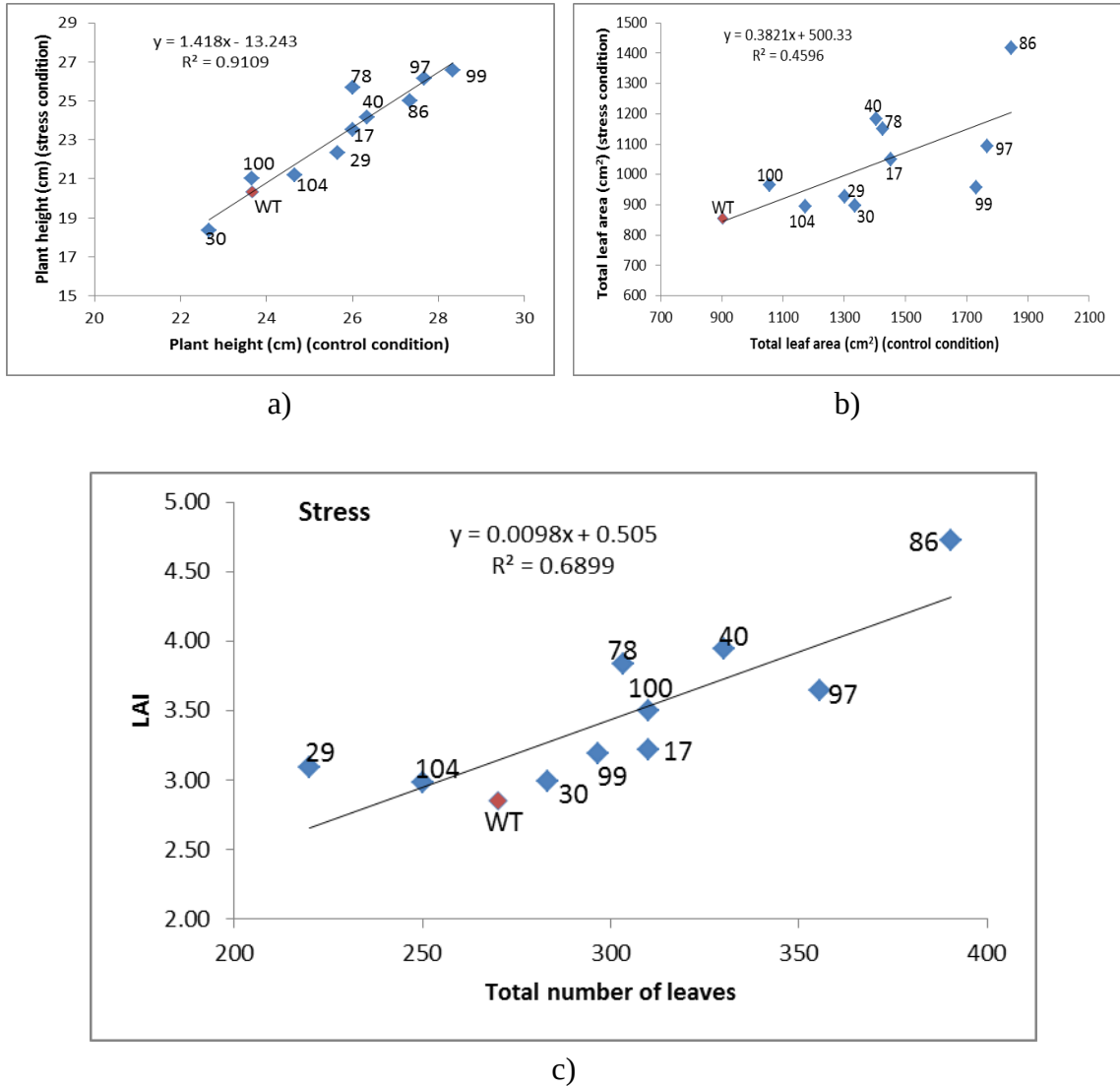
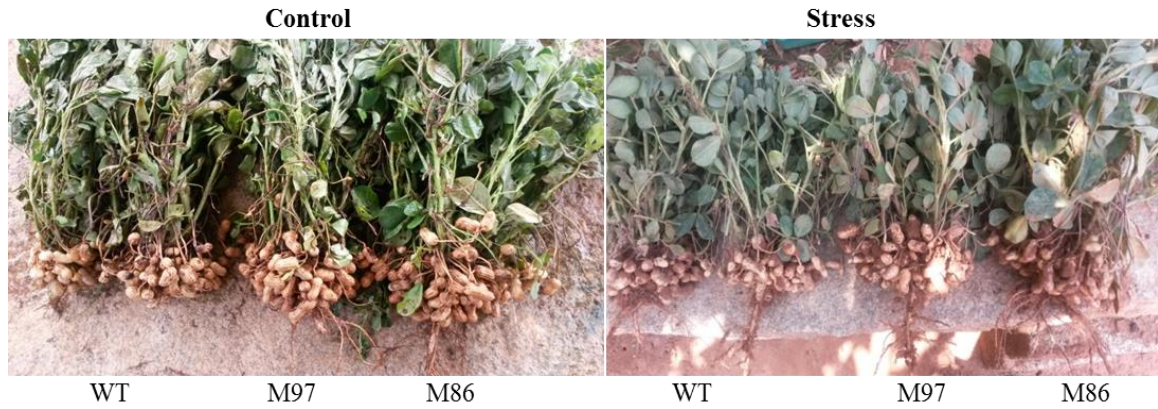
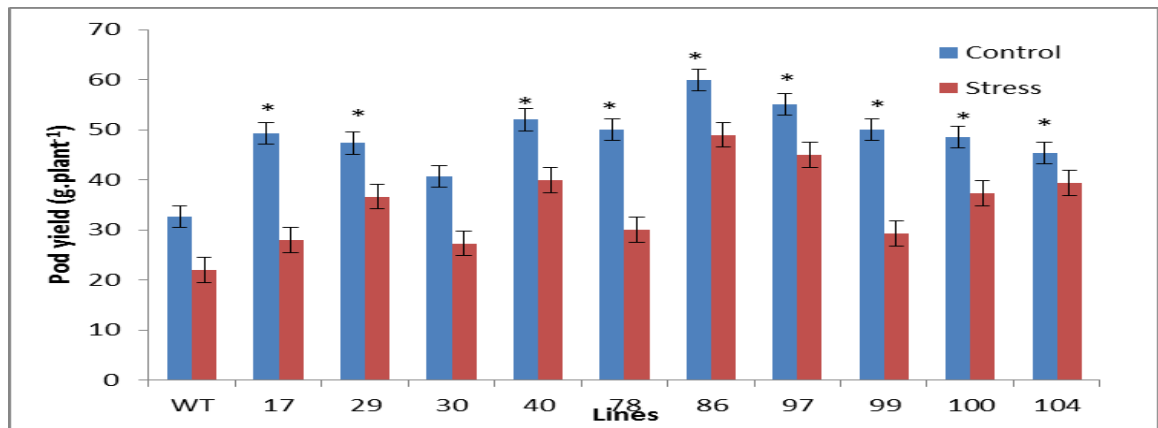


Fig. 39: Correlation analysis between physiological parameters at 80 DAS a) Plant height under control and stress condition b) Total leaf area under control and stress conditions c) Total number of leaves and leaf area index (LAI) under stress conditions. Wild type and transgenic plants were sown under controlled field condition and stress was imposed after 50 DAS for 20 days (n=3). Observations recorded at 80 DAS.

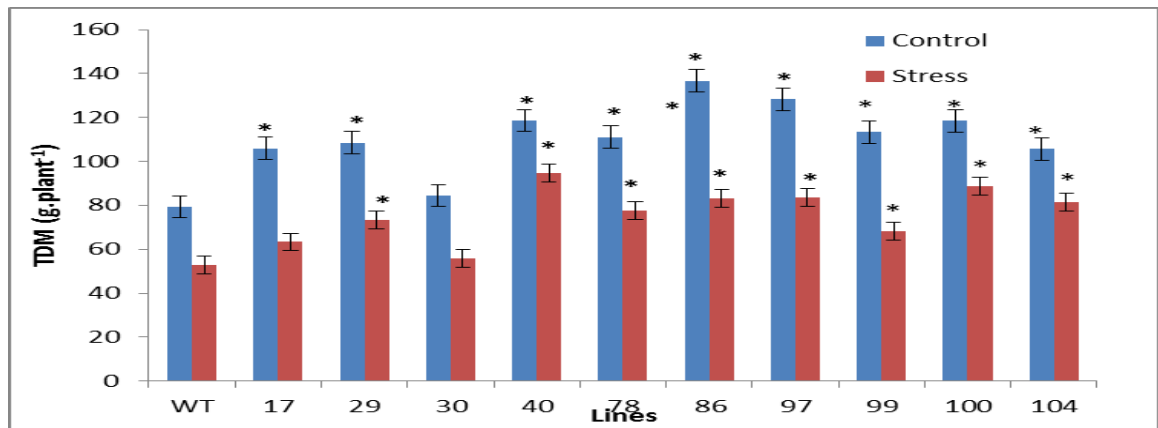
T5 generation



a)



b)



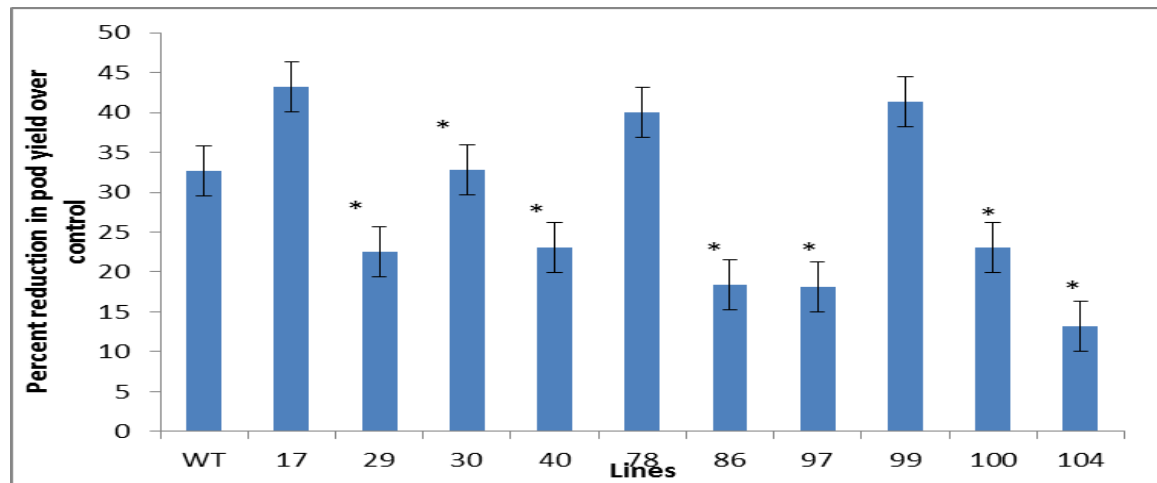
c)

* t-test with P<0.05

Fig. 40: Yield parameters of wild type and transgenic plants at harvest a) Photograph showing yield at harvest under control and stress condition b) Pod weight (g. plant⁻¹) c) Total dry matter (TDM) (g.plant⁻¹). Wild type and transgenic plants were sown under controlled field condition and observation was recorded at harvest (n=3).

Table 9: Mean yield and biomass of selected transgenic lines grown in contained field conditions

	Control	Stress	Control	Stress
	Pod yield (g.plant ⁻¹)	Pod yield (g.plant ⁻¹)	TDM (g.plant ⁻¹)	TDM (g.plant ⁻¹)
17	49.33	28.00	106.00	63.33
29	47.33	36.67	108.44	73.33
30	40.67	27.33	84.45	56.00
40	52.00	40.00	118.67	94.89
78	50.00	30.00	111.11	77.56
86	60.00	49.00	136.67	83.00
97	55.00	45.00	128.33	83.67
99	50.00	29.33	113.33	68.44
100	48.50	37.33	118.50	88.67
104	45.33	39.33	105.77	81.33
Mean	49.82	36.20	113.13	77.02
WT	32.67	22.00	79.34	52.67
SD	5.24	6.87	13.91	10.87
CV %	10.52	20.56	12.50	15.42



* t-test with P<0.05

Fig. 41: Per cent reduction in Pod yield over control in wild type and transgenic lines. Wild type and transgenic plants were sown under controlled field condition and observation was recorded at harvest. N=3 in each line 3 replications were maintained.

Table 10: Mean pod yield of managed drought environment experiment and contained field experiment

	Pod Yield (g.plant⁻¹) (Summer season)	Pod yield (g.plant⁻¹) (MDE)	Mean Pod yield (g.plant⁻¹)
WT	32.67	24.67	28.67
17	49.33	33.75	41.54
29	47.33	30.17	38.75
30	40.67	40.67	40.67
40	52.00	44.50	48.25
78	50.00	31.00	40.50
86	60.00	53.83	56.92
97	55.00	53.50	54.25
99	50.00	41.33	45.67
100	48.50	34.50	41.50
104	45.33	40.83	43.08

4.5.6 Molecular analysis of selected groundnut transgenic lines in T₅ generation

Genomic DNA PCR analysis of selected 10 transgenic lines

In order to confirm the integration of multigene cassette in the transgenic lines, genomic DNA was isolated from selected 10 transgenic lines and wild type plants. The npt II marker gene showed amplification of 436bp indicating the integration of T-DNA in the transgenic lines. PDH45 (200bp), Alfin1 (200bp) and HSF4 (500bp) was confirmed by using gene specific primers (Plate 3).

Southern blot analysis in groundnut transgenic lines (T₅ generation)

The stable integration of transgenes was assessed by genomic Southern blot analysis in three transgenic lines that showed superior survival at the end of stress and high recovery growth on stress alleviation. Thirty micrograms of purified genomic DNA of transgenic and wild type plant was digested overnight with *EcoRI* that cut once within T-DNA region for the analysis of copy number by Southern hybridization. The 1 kb length of *Alfin1* fragment was restrict digested for labelling by Dig-labelled kit (Amersham, GE healthcare Pvt. Ltd.). Hybridization and washing was carried out according to user manual. The signal was developed using chemiluminescence substrate (CDP-star) reagent and exposed to X-ray and autoradiogram according to Sambrook *et al.*, (1989). Southern blot analysis was done in three transgenic plants i.e. 86, 97 and 100 demonstrated that the transgenes were stably integrated into the genome of transgenic groundnut lines (Plate 4a) with single copy of the transgene in these selected three transgenic lines.

Gene expression analysis in groundnut transgenic plants

The transgenic and wild type plant raised in small pots for 20 days and then moisture stress was imposed for 4 days and then samples were collected and total RNA was isolated from stressed plants, cDNA was synthesised and used as template for RT-PCR analysis. *Elongation factor (Elf)* was used as an internal control for normalization of RT-PCR. The transcript levels of a few stress-responsive genes were analyzed by RT-PCR in two transgenic lines.

T5 generation

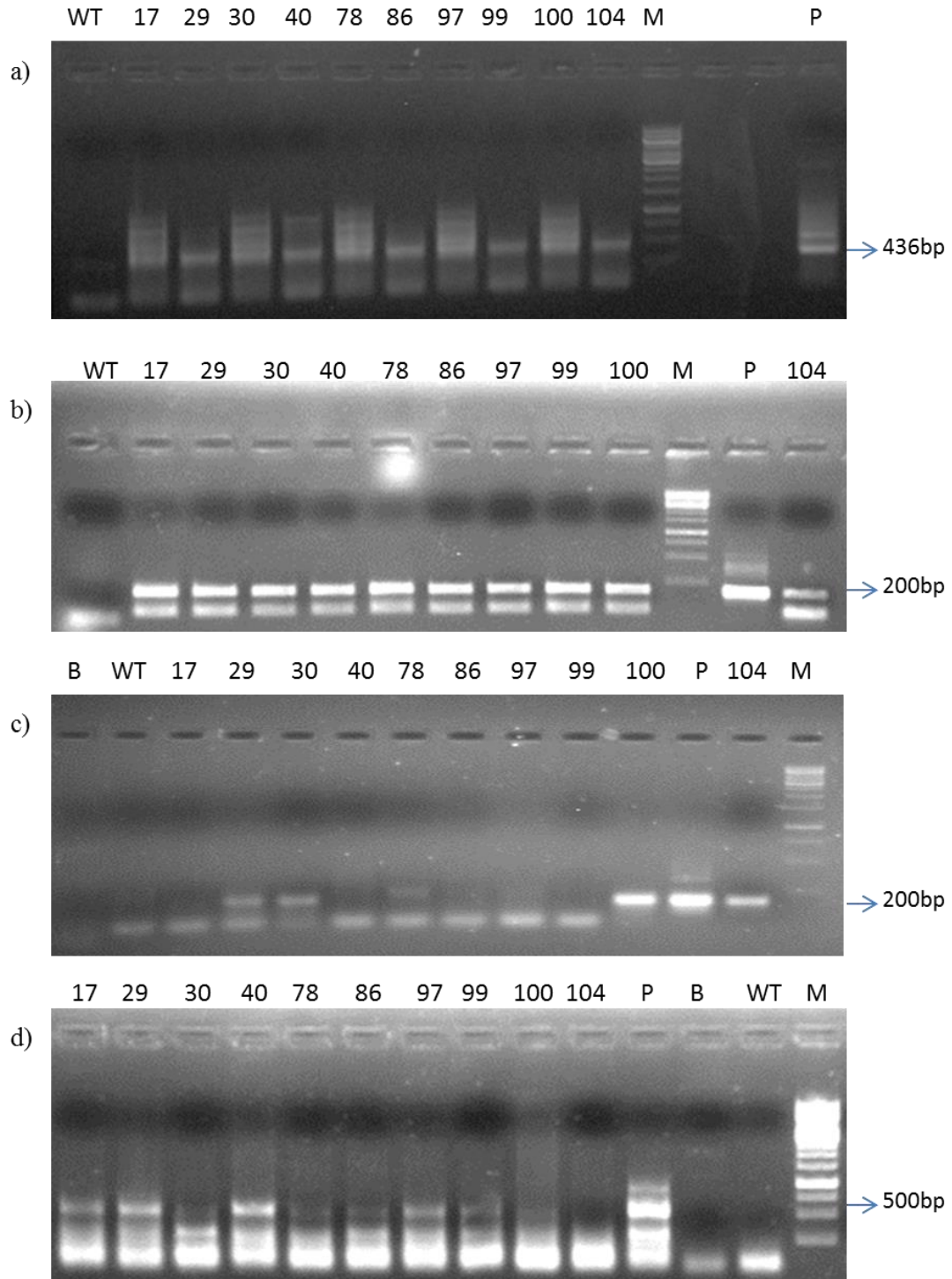


Plate 3: Molecular confirmation of promising T₅ transgenic plants by PCR for a) NPT II b) PDH45 c) Alfin1 d) HSF4 genes. M- 1kb DNA Ladder, P- Positive control (plasmid), WT- wild type, B-Blank

T5 generation

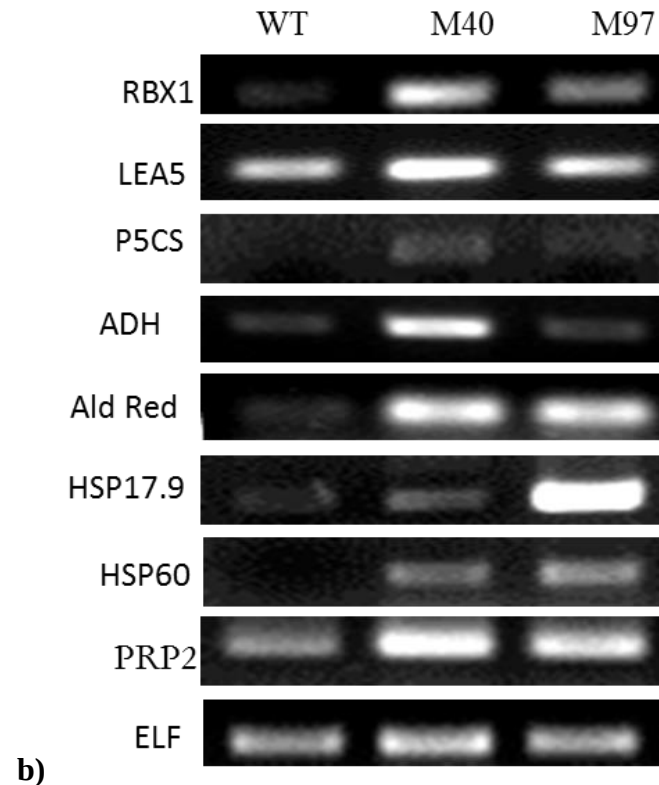
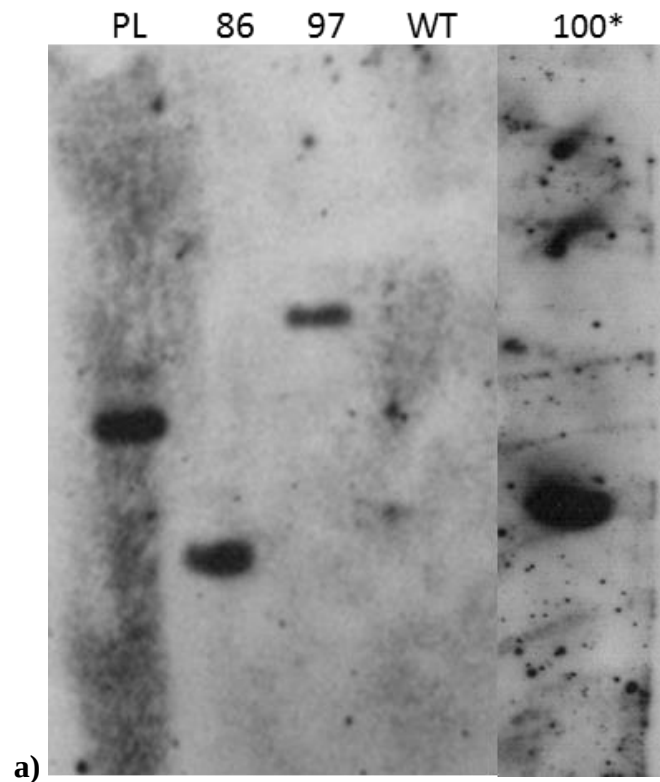


Plate 4: Southern blot and expression analysis a) copy number and integration of multigene cassette in groundnut transgenics - 30µg of genomic DNA from 3 independent plants from each line was digested with EcoRI and Dig labelled(GE-Amersham) Alfin1 probe was used to hybridize the DNA (*- blot was done in a separate experiment) b) Downstream target gene expression under moisture stress -20-day-old plants were subjected to moisture stress and soil moisture status was maintained for 4 days. The tissue was collected and total RNA was isolated and cDNA was prepared.

Specific genes like heat shock proteins like HSP60 and HSP17.9, Aldehyde dehydrogenase, Really interesting gene box-1, Aldose reductase, Pyrroline-5-carboxylate synthase, Late embryogenesis abundant-5 and Proline-rich protein 2 which were shown to get expressed under stress were chosen for expression study in transgenic lines.

Heat shock proteins (HSP 60) play an important role in protein–protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. Small heat shock proteins (HSP 17.9) present in different cell organelles indicate that potentially this gene might act as chaperones in protecting the various cellular compartments under stress condition. Aldehyde dehydrogenase (ADH1) is responsible for oxidation of biogenic aldehyde intermediates as well as for cell detoxification of aldehydes generated during lipid peroxidation. Really interesting gene box-1 (RBX1) a highly stress responsive protein involved in protein degradation under stress by ubiquitin proteasome pathway. Aldose reductase is a cytosolic NADPH-dependent oxidoreductase that catalyzes the reduction of a variety of aldehydes and carbonyl compounds. Pyrroline-5-carboxylate synthase (P5CS) is a bifunctional enzyme it involved in the biosynthesis of proline which is involved in maintaining cell turgour and osmotic homeostasis. Late embryogenesis abundant-5 (LEA5) proteins are associated with tolerance to abiotic stresses, such as drought, salinity and high or cold temperature. Proline-rich protein 2 (PRP2) is a root growth associated protein and it is a downstream target of Alfin1. In transgenic and wild type plants the expression levels of these genes are studied under stress condition.

Most of the genes (heat-shock proteins HSP17.9 and HSP60, aldehyde dehydrogenase (ADH1), Really interesting gene box-1 (RBX1) and Aldose reductase) showed enhanced expression in transgenic plants than wild type under stress except for the transcript levels of ADH1 which was less in the transgenic line M97. The transcript induction of pyrroline-5-carboxylate synthetase (P5CS) was less than other genes, but the transgenic plants still showed higher levels than wild type. However, late embryogenesis abundant-5 (LEA5) gene showed similar expression levels in wild type and transgenic plants even after exposure to stress. Further, the root growth-associated proline-rich

protein2 (PRP2) showed significant up-regulation of transcripts in the transgenic plants (Plate 4b).

4.6 Characterization of T₆ groundnut transgenic lines

Based on stress response and growth and productivity 8 lines were selected for further characterization. The selected 8 lines were studied for productivity in larger plot size with more number of plants under non-stress condition. The two best transgenic lines were selected based on abiotic stress tolerance and less percent reduction in productivity for further evaluation. For selected two lines two experiments were conducted to study the stress response. In the first experiment salinity stress was imposed to transgenic and wild type plants and some of the parameters like RWC, membrane damage, root length and biomass was recorded. In the second experiment transgenic and wild type plant were studied for ER stress response.

4.6.1 Productivity analysis of selected transgenic lines (T₆ generation)

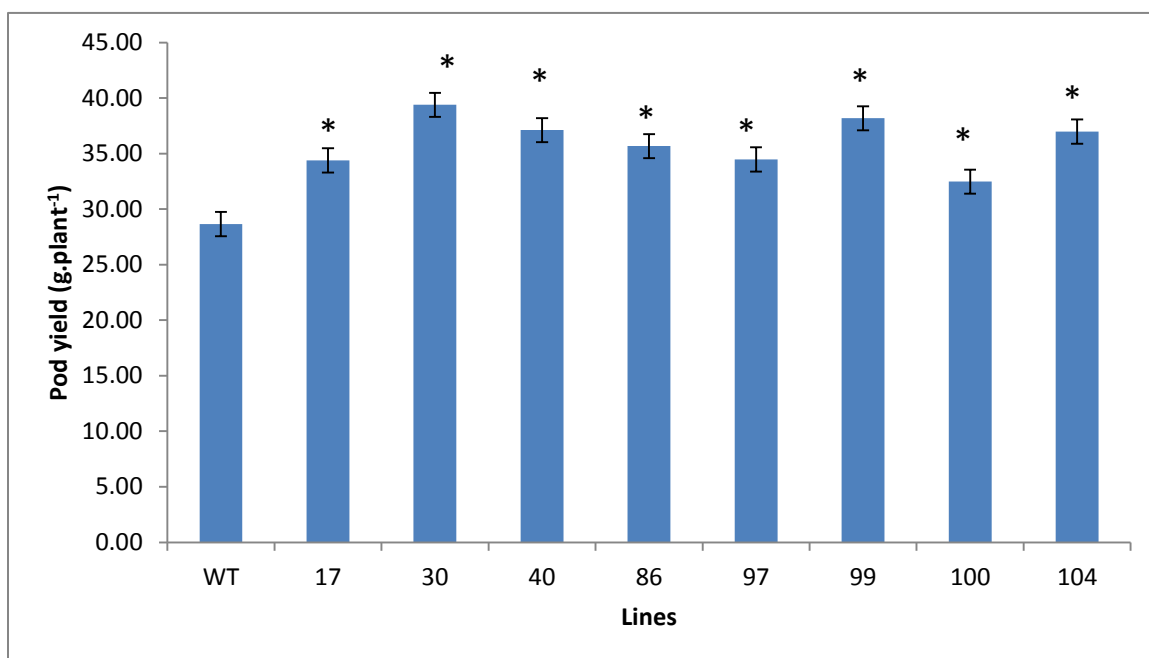
The aim of the study is to reconfirm the productivity levels of the selected transgenic lines in larger plot size with more number of plants under non-stress condition. The transgenic and wild type plants were grown under contained field condition till harvest. At harvest some of the yield parameters like pod yield and TDM was recorded. All the 8 selected transgenic lines showed more pod yield and biomass compared to wild type plants (Fig.42a & b). There was 15-20% more yield in case of transgenic lines compared to wild type plant (Table 11).

The 2 best transgenic lines which showed relatively more tolerance to salinity, moisture stress and also which showed less percent reduction in pod yield was selected for further study.

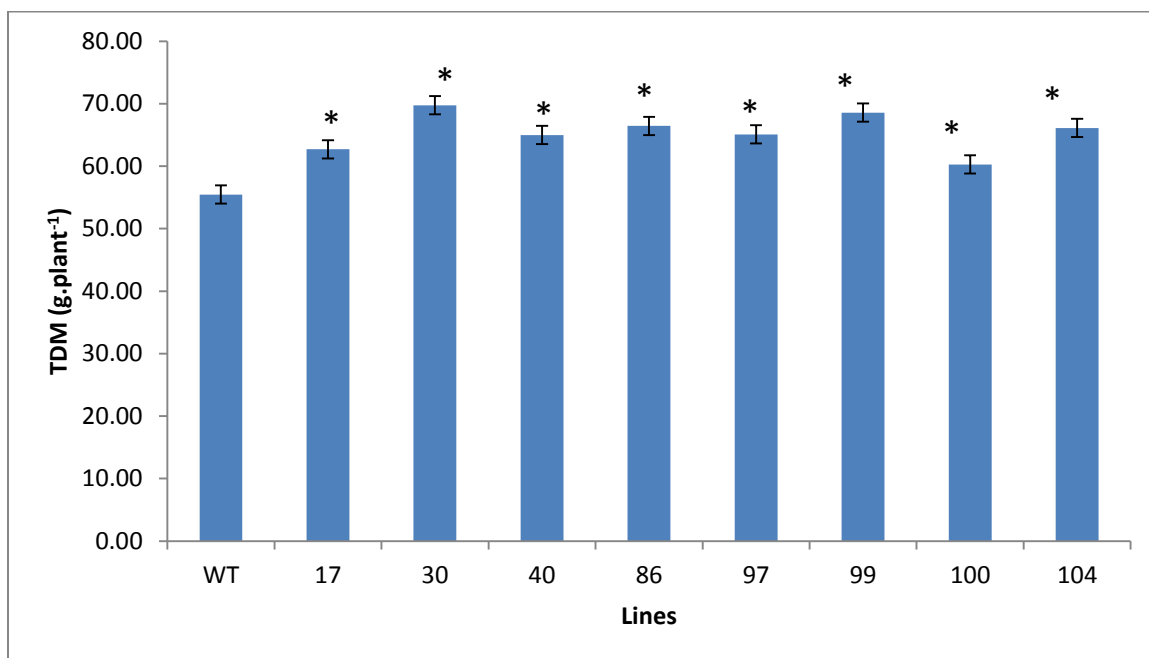
4.6.2 Salinity stress response of selected two transgenic events (T₆ generation)

In order to study the salinity stress response of transgenics, the transgenic and wild type plants were raised in pots for 15 days and later subjected to salinity stress. The pots are weighed regularly to measure the water loss in evapo-transpiration; the amount

T6 generation



a)



b)

* t-test with P<0.05

Fig. 42: Yield parameters of wild type and transgenic plants in T₆ generation. Wild type and transgenic plants were sown under controlled condition and analyzed for yield parameters at harvest a) Pod yield b) TDM (n=3).

Table 11: Mean yield and biomass of selected transgenic lines grown in contained field conditions at T6 generation

Lines	Pod yield (g.plant ⁻¹)	TDM (g.plant ⁻¹)
17	34.39	62.70
30	39.39	69.76
40	37.11	64.98
86	35.67	66.45
97	34.47	65.08
99	38.18	68.58
100	32.47	60.27
104	36.97	66.12
Mean	36.08	65.49
WT	28.66	55.45
SD	2.26	3.04
CV (%)	6.27	4.64

of water loss by evapo-transpiration was replenished by 50 mM NaCl for 3 days and then increased to 100 mM NaCl for 3 days. After stress period plants were photographed for phenotype and root length (Fig. 43a & b). The transgenic lines showed slightly more RWC compared to wild type plants under both control and stress condition (Fig. 44a). Wild type plants showed more membrane damage compared to transgenic lines under stress condition (Fig. 44b). Root length and TDM was more in case of transgenics compared to wild type under both control and stress condition (Fig. 44c & d).

4.6.3 ER stress response of transgenics (T₆ generation)

To study the effect of DTT induced ER stress response, uniformly germinated seeds of transgenic and wild type plants were placed on half MS media supplemented with 5 mM DTT for 2 days and then transferred to water for recovery. After a week transgenic plants were recovered whereas wild type did not recover (Fig. 45).

4.7 Comparative assessment of groundnut transgenic expressing single and multi-gene

Stress response is a polygenic trait and several mechanisms are involved. For instance even the protein turnover depends on stability and synthesis. Earlier in our department over the year's single gene groundnut overexpressing HSF4 and PDH45 were developed. Our objective was to see whether multigene carrying transgenic line will perform better under abiotic stress than single gene carrying transgenic lines and also we wanted to compare the root growth of multigene transgenic line with single gene carrying transgenic lines. So we did couple of experiments to compare multi-gene and single gene with their respective wild types.

4.7.1 Root length variation in single and multi-gene groundnut transgenic lines

To study the relevance of Alfin1 overexpression in root growth, relative improvement of root growth of Alfin1, PDH45 and HSF4 overexpressing multi-gene transgenic plants with HSF4 and PDH45 single gene overexpressing transgenic groundnut plants were compared. These single gene transgenic lines were developed earlier in the department.

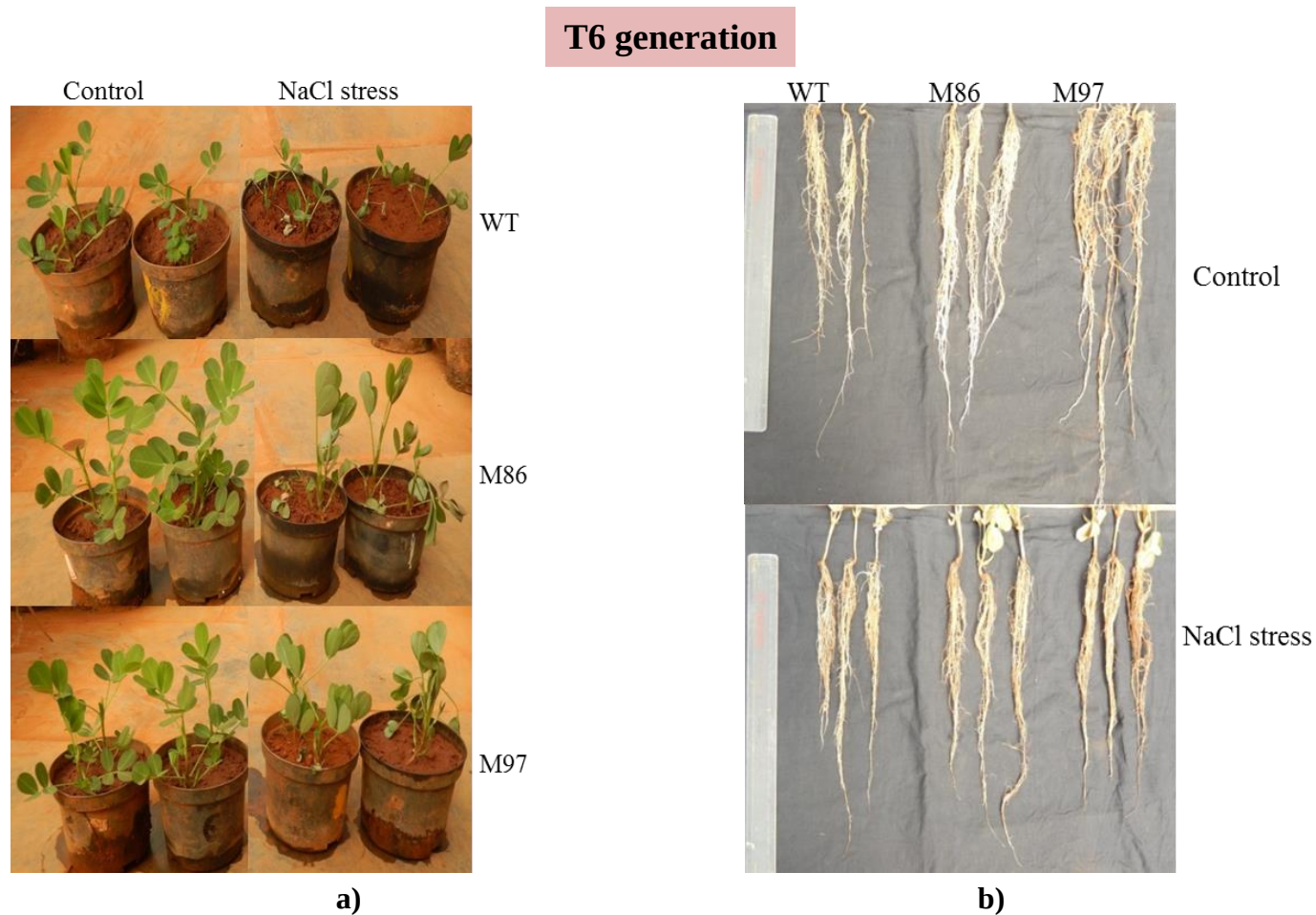
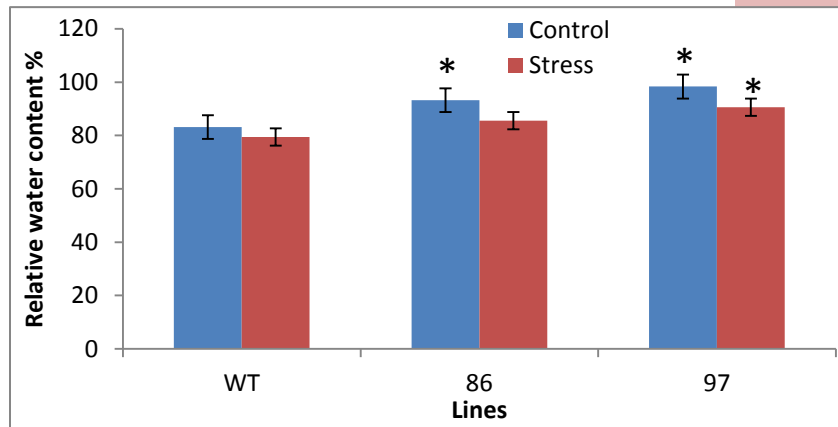
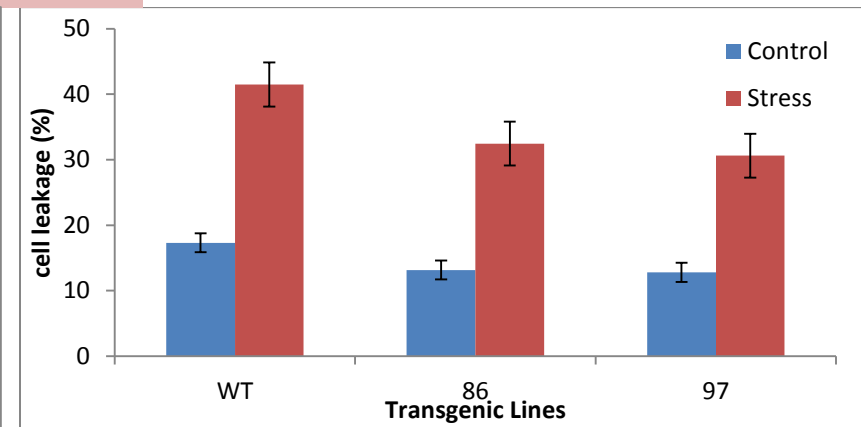


Fig. 43: Salinity stress response of wild type and transgenic seedlings at whole plant level. a) Photograph showing phenotypic response of salinity stress b) Root length of wild type and transgenic seedlings under control and stress condition. Wild type and transgenic seeds were sown in pots and stress was imposed after 15 DAS. Pots are weighed regularly the amount of water loss by evapotranspiration was replenished by 50 mM NaCl for 3 days and then increased to 100 mM of NaCl

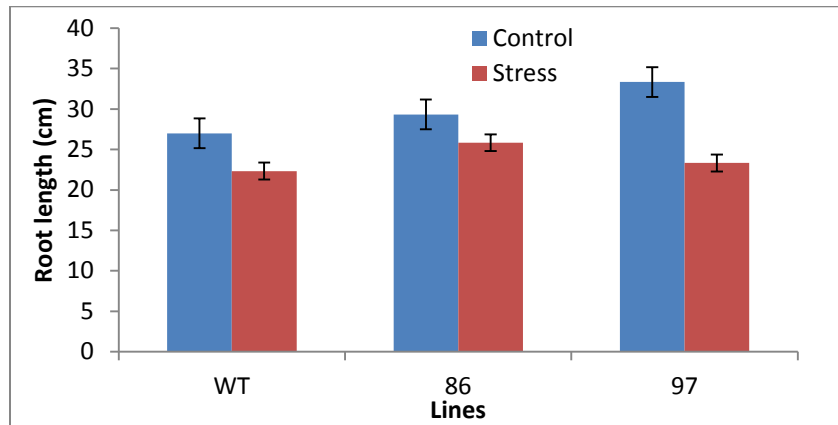
T6 generation



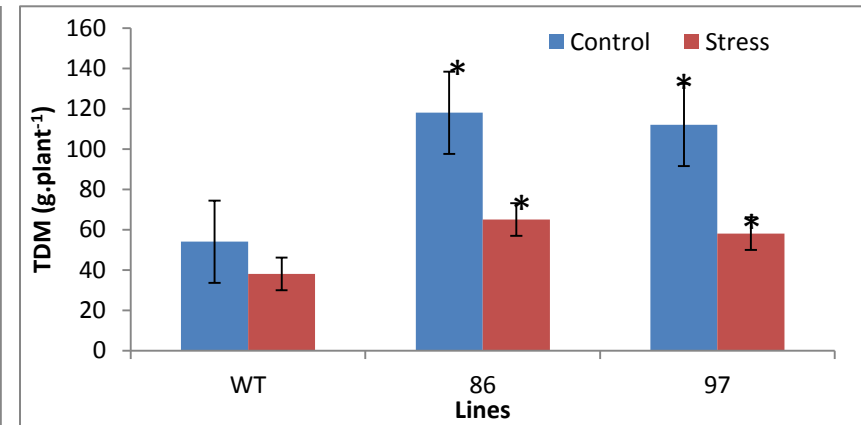
a) Relative water content



b) Percent leakage



c) Root length



d) TDM (g.plant⁻¹)

* t-test with P<0.05

Fig. 44: Response of wild type and transgenic seedlings to salinity stress at whole plant level. Wild type and transgenic seeds were sown in pots and stress was imposed after 15 DAS. Pots are weighed regularly the amount of water loss by evapotranspiration was replenished by 50 mM NaCl for 3 days and then increased to 100 mM of NaCl



Fig. 45: Response of wild type and transgenic to DTT treatment. Wild type and transgenic seedlings was subjected to DTT (5mM) for 2days and then transferred to water for recovery. Seedling growth under a) Water control b) DTT (5mM) treatment.

Root length variation at seedling stage: Multi-gene and single gene carrying transgenic lines with their respective wild type were kept for germination and after 3 days of germination root length was observed. At seedling stage the multi-gene expressing plant showed more root length when compared to single gene expressing plant (Fig. 46a).

Root length variation at harvest: Root length variations in multi-gene and single gene carrying transgenics were observed at harvest and photographed (Fig. 46b). The multi-gene carrying transgenic line showed more root length when compared to wild type and single gene carrying transgenic line.

Root length variation under stress and non-stress conditions: Seeds of both multi-gene and single gene carrying transgenic lines along with their respective wild types were sown in small pots and two sets were maintained. For one set of the pots after 15 days stress was imposed for 3 days and then allowed for recovery for a week and then root length was photographed for both control and stress condition (Fig. 46c).

The multi-gene expressing transgenic plants showed improved root growth in all stages compared to wild type and single gene transgenic plants which are individually overexpressing PDH45 and HSF4.

4.7.2 Salinity stress response in single and multi-gene groundnut transgenic lines

To study the salinity induction response, two-day-old pre-germinated seeds from single and multi-gene transgenics along with their wild type plants were subjected to 100mM concentration for 8 hours and then transferred to 300mM concentrations for 8 hours and then subjected to recovery for 3 days. The transgenic carrying multi-gene showed more tolerance to salinity when compared to single gene transgenics (Fig. 47a). The root length was also more in case of multi-gene transgenics compared to single gene under stress condition (Fig. 47b).

In view of this co-expression of specific genes with diverse functions has significance. To validate this aspect we compared the stress response of multi-gene transgenics with transgenics expressing single genes. Besides another objective is to

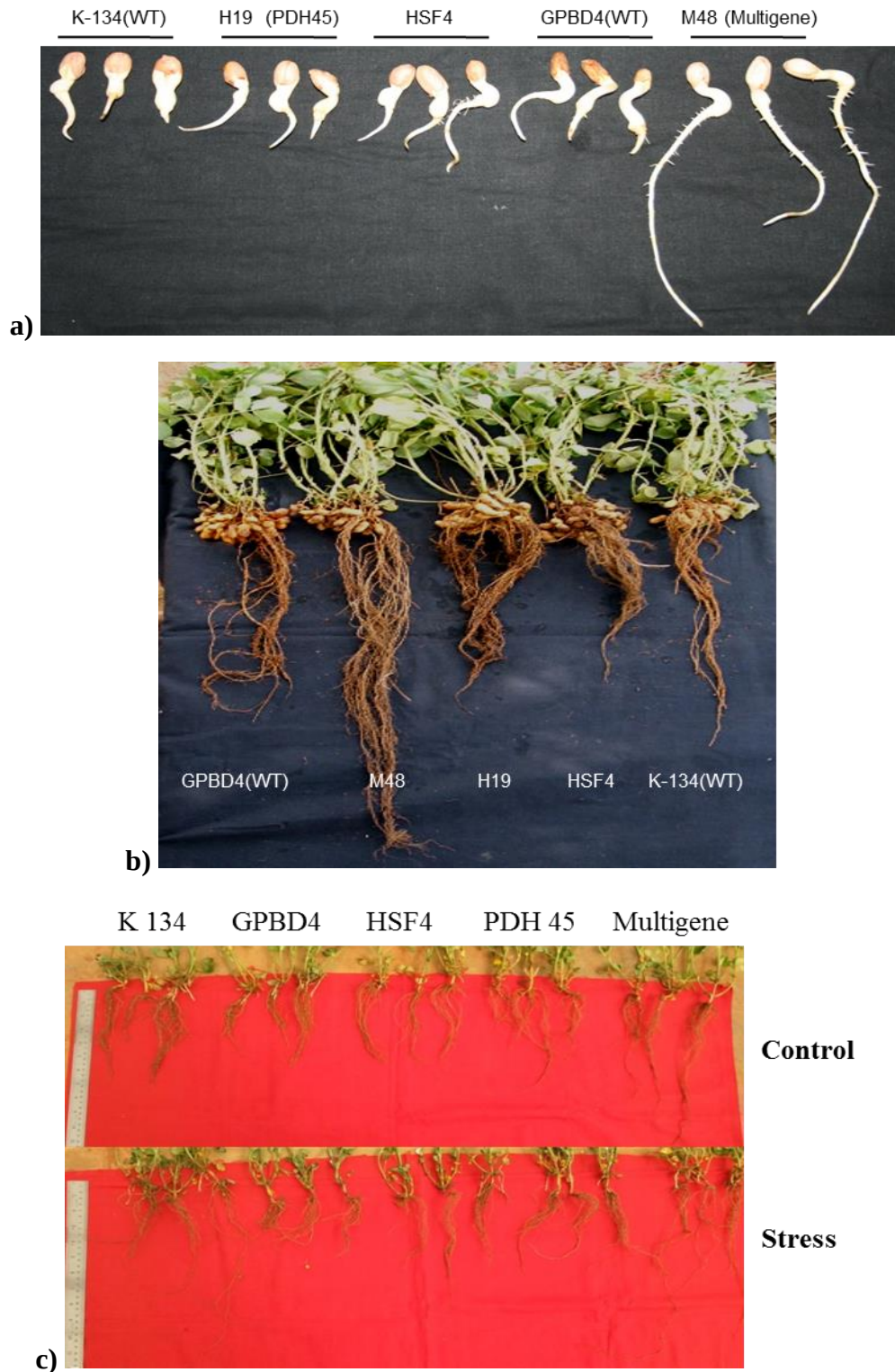


Fig. 46: Comparative assessment of root length in transgenic expressing individual genes and multigenes. Root length variation in groundnut transgenic a) at seedling level b) at the time of harvest c) after 25 days after sowing under control and stress condition. Stress imposed after 15 DAS for 3 days

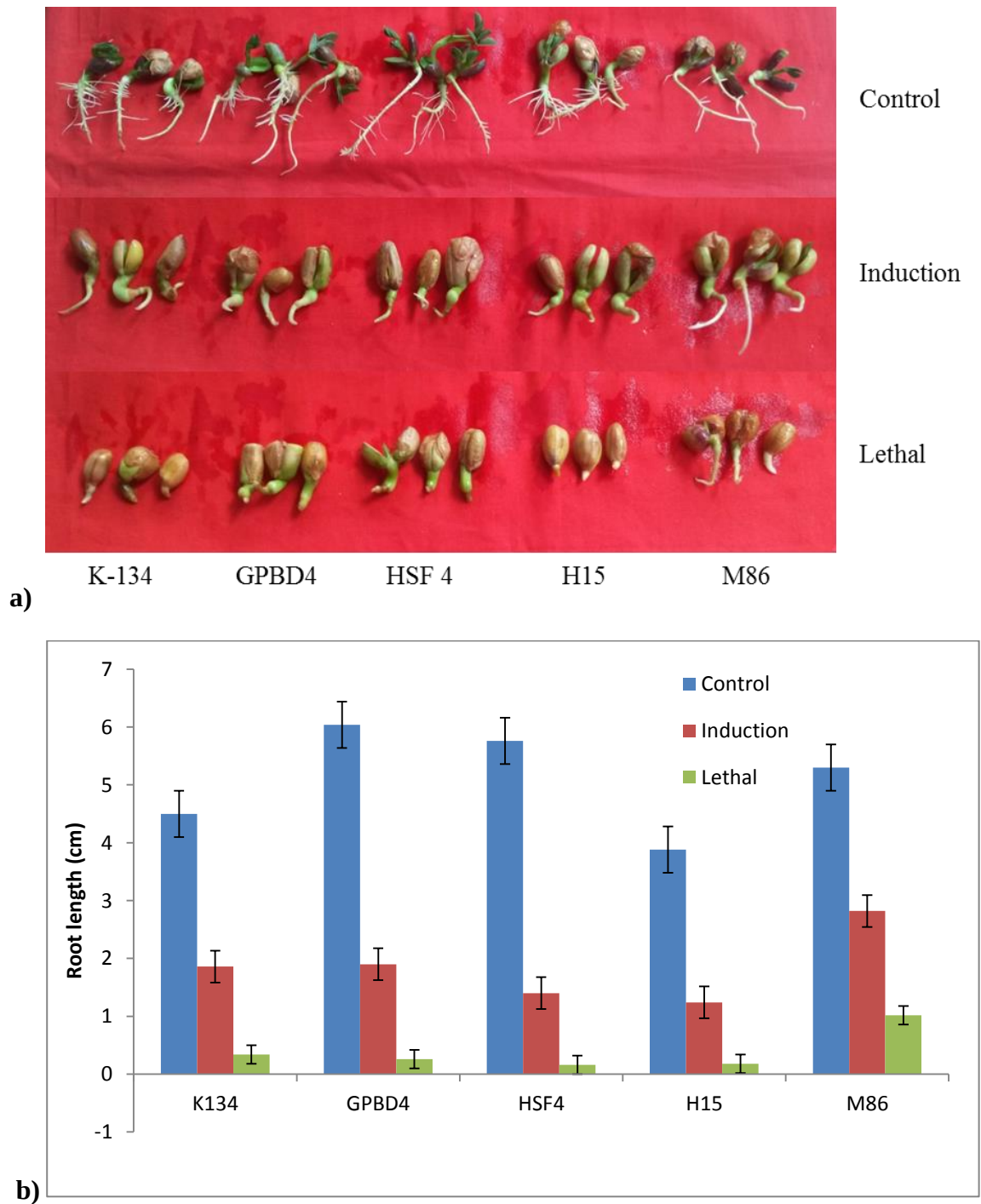


Fig. 47: Comparative assessment of salinity stress in transgenic expressing single and multi-gene. a) Salinity induction response of wild type and transgenic seedlings b) Root length of wild type and transgenic seedlings Two-day-old pre-germinated seeds from multi-gene and single gene transgenics along with their wild type plants were subjected to 100 mM NaCl for 8 hours and then transferred to 300 mM NaCl for 8 hours and then subjected to recovery.

assess the relevance of Alfin1 in improving root growth in comparison with other transgenics expressing genes regulating protein turnover.

4.8 System biology-Omics in groundnut transgenic and wild type plant

One of the best lines in terms of stress tolerance and productivity was identified for a deeper understanding of physiologically complex processes and cellular function through system biology approach. Systems biology takes the molecular parts (transcripts, proteins and metabolites) of a transgenic and wild type groundnut sample and attempt to fit them into functional networks or models designed to describe and predict the dynamic activities of the transgenic plant.

4.8.1 Transcriptome analysis of transgenic groundnut under stress and non-stress condition:

To understand the mechanisms involved in drought tolerance in groundnut transgenic plants, transcriptome analysis was performed using Illumina NextSeq 500 in leaf tissue. Drought stress was imposed by gravimetric approach as mentioned in the Chapter 3, (M& M) and leaf samples were collected for isolating total RNA. The following four samples were analyzed, viz., wild type control (WC), wild type stress (WS), transgenic control (TC) and transgenic stress (TS). The library construction, sequencing and subsequently the differential gene expression analysis were carried out at Genotypic Pvt., Ltd, Bangalore using Illumina NGS platform.

The Illumina Next Seq paired end raw reads were assessed for quality using Fast QC 0.8d Quality reads were obtained by trimming the raw reads at a minimum PHRED score of Q20. *De novo* assembly of processed reads data was performed using Trinity for default k-mers i.e 25. Summary of *Denovo* transcriptome assembly is presented in Table 12.

4.8.1.1 Differential expression of gene(s) (DEG):

Differential expressed gene(s) (**DEG**) were identified based on the number of reads mapped against wild type and transgenic plants using DeSeq tool. Using DESeq

Table 12: Statistics of *de novo* transcriptome assembly of wild type (WT) and Transgenic groundnut plants.

Transcriptome Denovo Assembly Statistics				
Sample Name	TC	TS	WC	WS
Tool used	Trinity			
Hash length	25 mer			
Transcripts Generated	49724	59151	58805	51206
Maximum Transcript Length	7573	10130	11966	9173
Minimum Transcript Length	301	301	301	301
Average Transcript Length	1065.3	1093.4	1118.9	1006.4
Median Transcript Length	1263	907	2261	1110
Total Transcripts Length	52968898	64673032	65795246	51532331
Transcripts >=500 bp	35859	43432	43272	36393
Transcripts > 1 Kb	20751	25631	26022	20032
Transcripts > 10 Kb	0	1	2	0
N50 value	1446	1479	1524	1330

TC: Transgenic control, TS: Transgenic stress, WC: Wild type control and WS: Wild type stress

analysis >2-fold change in transcript abundance were considered as differentially expressed (DE) genes.

Since groundnut genome information was not annotated completely the genes were mapped against different genomes like Arabidopsis, Medicago, Soyabean, French bean etc., using phytozyme database to identify differentially expressed genes. Based on this analysis around 1471 and 2627 were up-regulated and down regulated, respectively, in transgenics compared to wild type under control conditions. In stress conditions 2275 genes were upregulated and 3080 genes were down regulated in transgenics compared to wild types plants (Table 13). Several genes that are differentially expressed have been clustered using multiple expression viewer tool (MeV tool). The heat map clearly showed that many genes were differentially expressed in transgenic under stress as well as control conditions (Fig. 48). Under stress conditions in transgenics few transposon encoding genes were down-regulated (Table 14). In contrast a few transcription factors, protease genes, were up-regulated in stress signifying the tolerance mechanisms in transgenics under drought conditions.

4.8.1.2 Differentially expressed genes in comparison with *Medicago truncatula* as reference genome

Although the DEG were identified in the initial analysis based on Phytozyme database that provided the name of the genes. However, the gene IDs were different for many genes that were assigned based on the respective genomes. To classify the genes the other bioinformatics tools use the single specific genome IDs. From this context the RNA seq data was blast searched against another legume crop *Medicago truncatula* because it is closely related species to *Arachis hypogea*. Based on the blast analysis 57 % genome coverage was matched with *Medicago truncatula* showing differentially expressed genes. There are 625 and 531 genes were upregulated and down regulated respectively in transgenics in control conditions compared to wild type. Whereas under stress conditions 1452 and 1384 genes were up regulated and down regulated respectively in transgenic plants (Table 15).

Table 13: List of up-regulated and down-regulated genes in transgenic plants when compared to wild type plants. Total RNA was extracted from control and moisture stress plants of wild type and transgenic plants were subjected to transcriptome analysis using IlluminaNextSeq500. TS-Transgenic stress, WS-Wild type stress, TC-Transgenic control and WC-Wild type control

	TC v/s WC	TS v/s WS
Up-regulation	1471	2275
Down-regulation	2627	3080

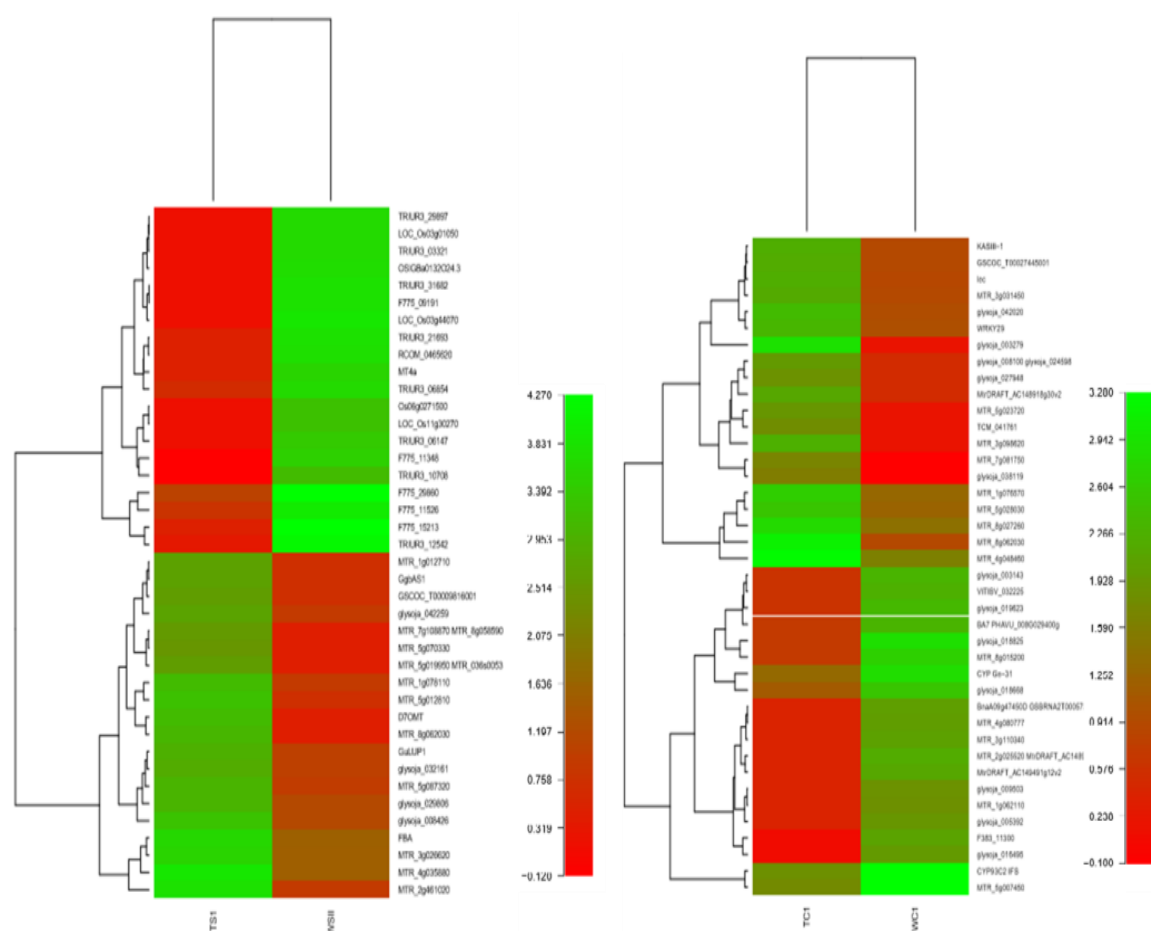


Fig. 48: Differentially expressed transcripts in wild type and transgenic plants under control and moisture stress conditions. To illustrate differences between the transgenic and wild type, a heat map was constructed for DEGs using multiple expression viewer tool in control and stress conditions. Green color indicates up regulated genes whereas red color indicates down regulated genes.

Table 14: Few Genes that are up or downregulated in transgenics under stress and control conditions

Genes that are up or downregulated in transgenics under control conditions	
Gene id	Description
Up regulated	
KASIII-1	3-ketoacyl-acyl-carrier protein (ACP) synthase III-I
MTR_3g098620	Mei2-like protein OS=Medicago truncatula
MTR_3g031450	UDP-glucosyltransferase family protein
MTR_8g027260	Subtilisin-like serine endopeptidase
MTR_8g062030	Replication factor-A carboxy-terminal domain protein
Down regulated	
VITISV_032225	Hypothetical protein/Putative uncharacterized protein
MTR_4g080777	Protein coding, Disease resistance protein (TIR-NBS-LRR class), putative
MTR_1g062110	Leucoanthocyanidin dioxygenase-like protein
MTR_5g007450	Cytochrome P450 family 71 protein
BA7 PHAVU_008G029400g	Anthocyanin acyltransferase
Genes that are up or downregulated in transgenics under stress conditions	
Up regulated	
MTR_1g012710	Protease inhibitor/seed storage/LTP family protein, Protein coding
MTR_5g070330	Vacuolar cation/proton exchanger 3
MTR_5g087320	Receptor-like protein
MTR_3g026620	Glycoside hydrolase family 1 protein
MTR_2g461020	Zinc finger, C3HC4 type (RING finger) protein
Down regulated	
LOC_Os03g01050	Retrotransposon protein, putative, unclassified
LOC_Os03g44070	Transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os11g30270	Transposon protein, putative, CACTA, En/Spm sub-class
F775_29860	Glutamate dehydrogenase 2
F775_15213	ATP-dependent DNA helicase Q5

Table 15: List of up-regulated and down-regulated genes in transgenic plants when compared to wild type plants using *Medicago truncatula* as the reference genome. Transgenic control v/s Wild type control (TCv/sWC) and Transgenic stress v/s Wild type stress (TSv/sWS).

	TC v/s WC	TS v/s WS
Up regulated	625	1452
Down regulated	531	1384

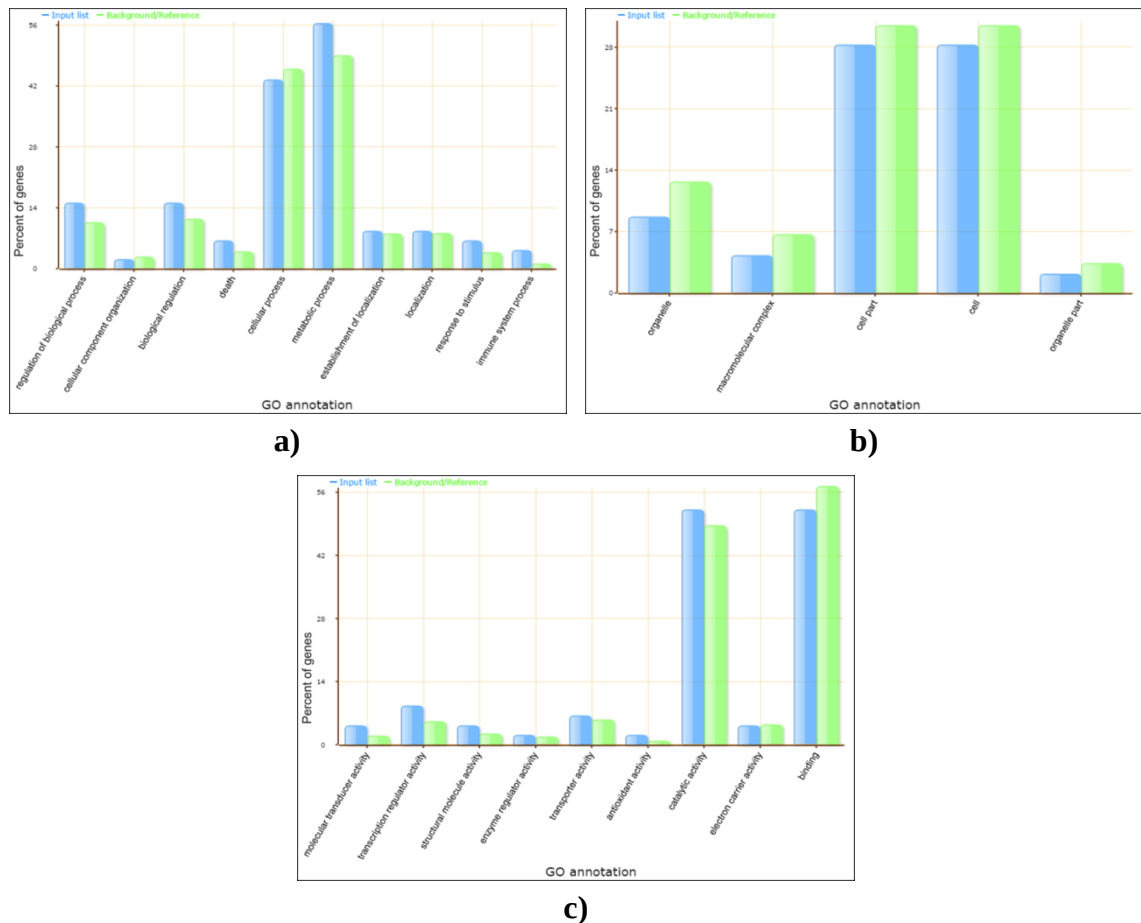


Fig. 49: Classification of upregulated genes in transgenics using Gene Ontology under control condition. Three main categories were summarized: a) biological process b) cellular components c) molecular functions

4.8.1.3 Functional annotation and classification of differentially expressed genes

Differentially expressed genes in transgenic plants compared to wild type under non-stress

Gene Ontology (GO) was predicted using *Medicago truncatula* as the reference genome and the up regulated genes were classified based on biological processes, cellular components and molecular functions using AgriGO analysis with Single Enrichment Analysis (SEA) tool (Fig. 49). Within each of the three main categories of GO classification, the dominant subcategories 42-56% of genes were representing cellular process, metabolic process, catalytic activity and binding activity.

Based on the AgriGO analysis, the genetic networks that involved in regulation of many processes have been identified using biological function of the upregulated genes in transgenics under control conditions. The analysis showed that cofactor metabolic process genes (GO:0051196 -66 genes) were positively regulating the coenzyme metabolic process genes (GO: 0006732- 43 genes). Further these group of genes were positively regulating group transfer associated genes (GO:0006752-21 genes) and coenzyme metabolic process (GO:0009108- 21 genes). In another node a few genes that are involved in cellular and small molecule metabolomics process positively regulated cellular aromatic compound metabolic process (GO: 0006725-50 genes) and monocarboxylic acid metabolic process (GO:0032787- 79 genes). Both these pathway genes further positively regulated the folic acid and derivative metabolic process (GO:0006760-12 genes and GO:0009390-12 genes) (Fig.50). Based on the biological functions of all these genes folic acid metabolism associate genes were highly represented in the transgenic plants under control conditions.

Folic acid, also called vitamin B9, enables plants to regulate their DNA functions. This acid basically helps a plant to produce more nucleic acid (DNA& RNA) and involved in protein synthesis mechanisms. Folic acid additionally enables plants to produce RNA, a nucleic acid that carries information from DNA to plant cell structures known as ribosomes and helps a plant synthesize protein. Regulating a plant's metabolic functions is another important contribution of folic acid. Folic acid enables plants to

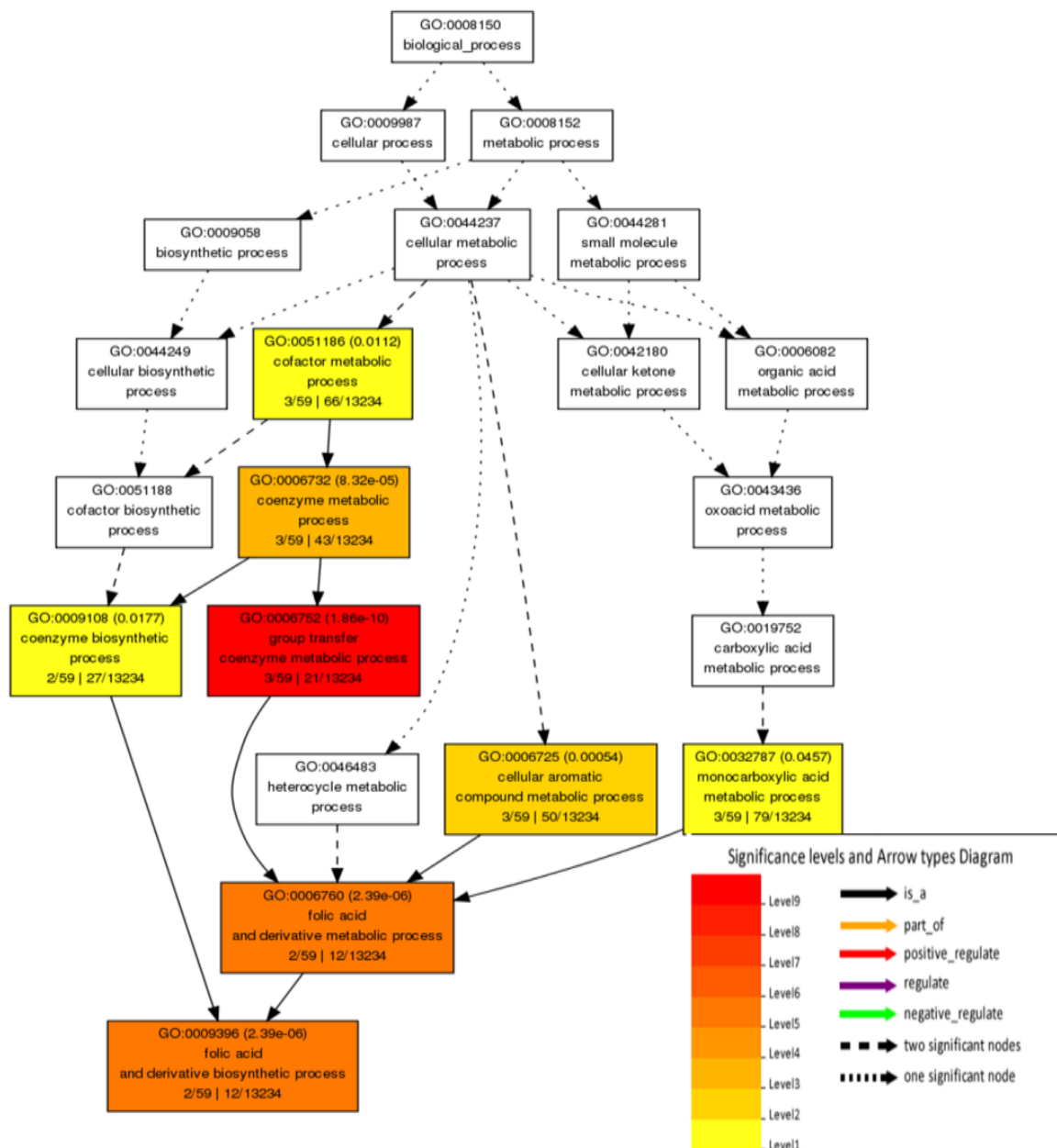


Fig. 50: Biological functions of upregulated transcripts representing different classes. The gene IDs of transcripts were mapped in AgriGO using *Medicago truncatula* as the reference genome. Color code and arrows depicts the level and meaning of representative groups based on the biological functions.

metabolize carbohydrates, proteins and lipids or fatty substances. Folic acid facilitates improved plant growth.

Further to assess the molecular function of the upregulated genes in transgenics, AgriGo analysis showed that many catalytic genes possessing transferase activity which are involved in transferring one carbon groups (GO:0018741-143 genes), that positively regulated a total of 141 Methyl transferases enzyme activity (GO:0008168-141 genes). These different methyl transferases utilize different substrates in methyl transfer reactions. They include protein methyltransferases, DNA/RNA methyltransferases, natural product methyltransferases, and non-SAM dependent methyltransferases. Methylation at DNA level bring in epigenetic modifications, affects transcription, gene stability, and parental imprinting. It directly impacts chromatin structure and can modulate gene transcription, or even completely silence or activate genes, without mutation to the gene itself. Though the mechanisms of this genetic control are complex, hypo- and hypermethylation of DNA is implicated in many diseases. Methylation of proteins has a regulatory role in protein-protein interactions, protein-DNA interactions, and protein activation. Few genes having chaperone binding (GO:0051087-3 genes) suggesting that heat shock proteins are upregulated and protecting the proteins under stress signifying the overexpression of HSF4 transcription factor. About 115 genes showed protein dimerization activity (GO:0046983) positively regulating dimerization many proteins. In another node of genes regulate ATPase regulator function (GO:0060590-3 genes) which are positively regulating adenylyl-nucleotide exchange factor (NEF) activity (GO: 0000774-3 genes). This suggests that in transgenics plants nucleotide exchange factors actively assist in the exchange of depleted nucleoside diphosphates for fresh nucleoside triphosphates. NEFs are specific for the nucleotides they exchange (ADP or GDP, but not both) and are often specific to a single protein or class of proteins with which they interact. These changes in turn affect the structural, enzymatic, or signalling properties of the protein. Further in another node of genes that are up regulated represents ferrous iron binding genes (GO:0008198-3 genes) (Fig. 51) indicating Iron homeostasis may be better in transgenics or heme biosynthesis

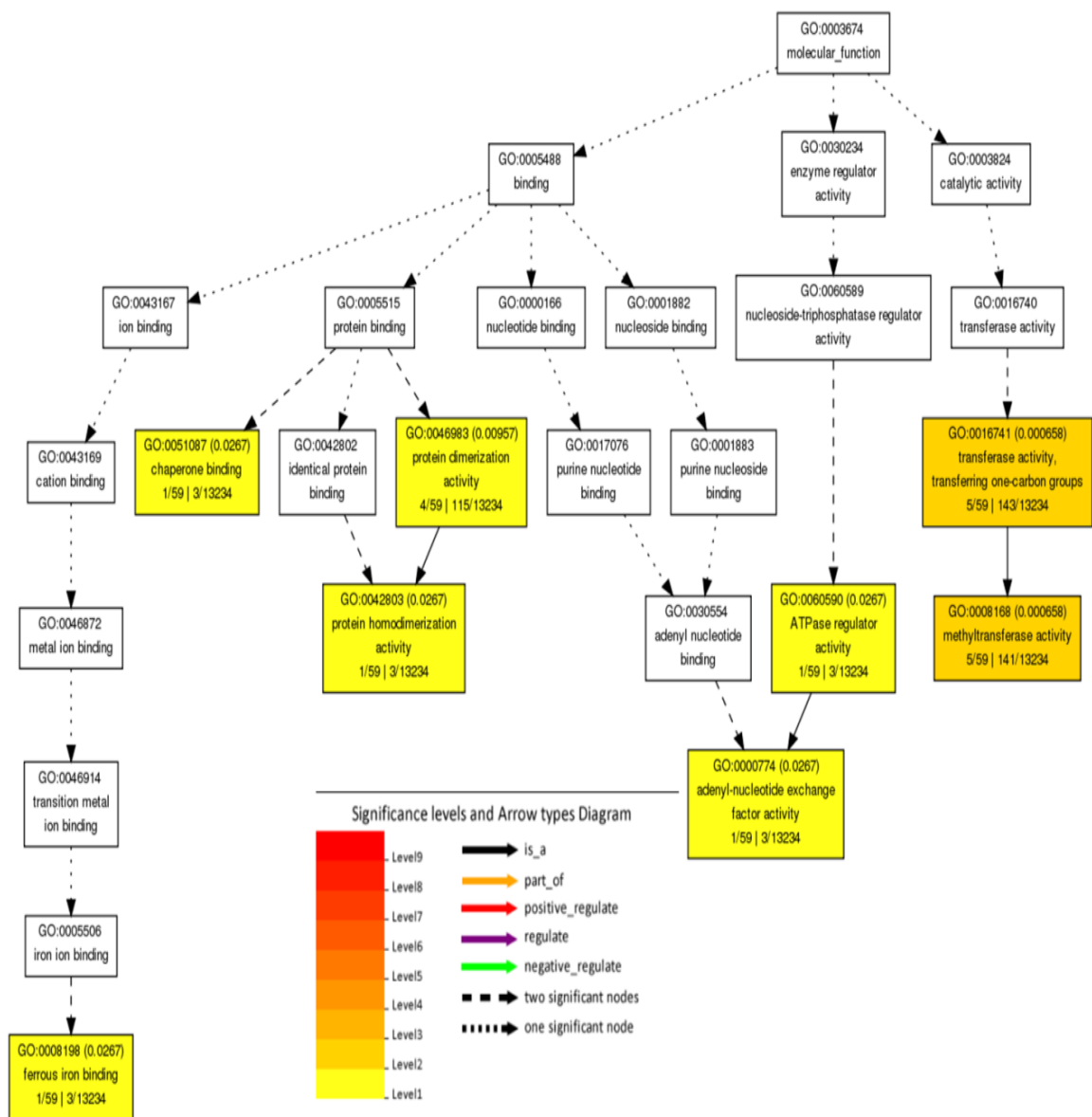


Fig. 51: Molecular functions of the up regulated genes in transgenic plants under control conditions. The gene IDs of transcripts were mapped in AgriGO using *Medicago truncatula* as the reference genome. Color code and arrows depicts the level and meaning of representative groups based on the molecular functions

for chlorophyll synthesis may be contributing for the dark green leaf phenotype of transgenic plants.

Overall, the Agrigo analysis of up regulated genes in transgenic plants shows that Folic acid and its derivative pathway associated genes, methylation associated genes, chaperones that are involved in cell protection, protein stability, Ionic balance genes are highly represented and hence the transgenic may have better protein turnover, protection and sustained metabolism under stress compared to wild type plants.

MapMan Analysis: MapMan is a user-driven tool that displays large datasets (e.g. gene expression data from RNA seq, microarray) onto diagrams of metabolic pathways or other processes. To get more clear understanding of the up regulated genes in transgenic groundnut transcriptome data, MapMann was used to develop metabolic pathways or other processes. The Gene IDs and the fold change values of the transcripts were given as input and mapped against *Medicago truncatula* as a reference pathway. The response was first visualized using the metabolic overview display and screen shots of the displays are presented.

Some of the proteins like drought responsive element binding protein (DREB), bZIP protein, Myb DNA binding domain, F box protein, ribosomal protein, Ethylene responsive element binding protein etc., are up regulated. Whereas some of the proteins like ubiquitin, substilin protease, thioredoxin, cytochrome P450, Isoflavone synthetase etc., are up regulated in transgenic plants (Fig. 52).

Target genes of the transgenes expressed in transgenic plants

The multigene expressing groundnut transgenic plants harboring HSF4, Alfin1, and PDH45, the up regulated genes were grouped based on their direct or indirect regulation.

Probable targets of HSF4: The genes that up regulated in transgenic plants were assessed to identify the Heat-shock proteins (HSPs), because these HSPs may have HSE cis elements in their promoters. Based on the transcriptomic data, Dna J, heat shock

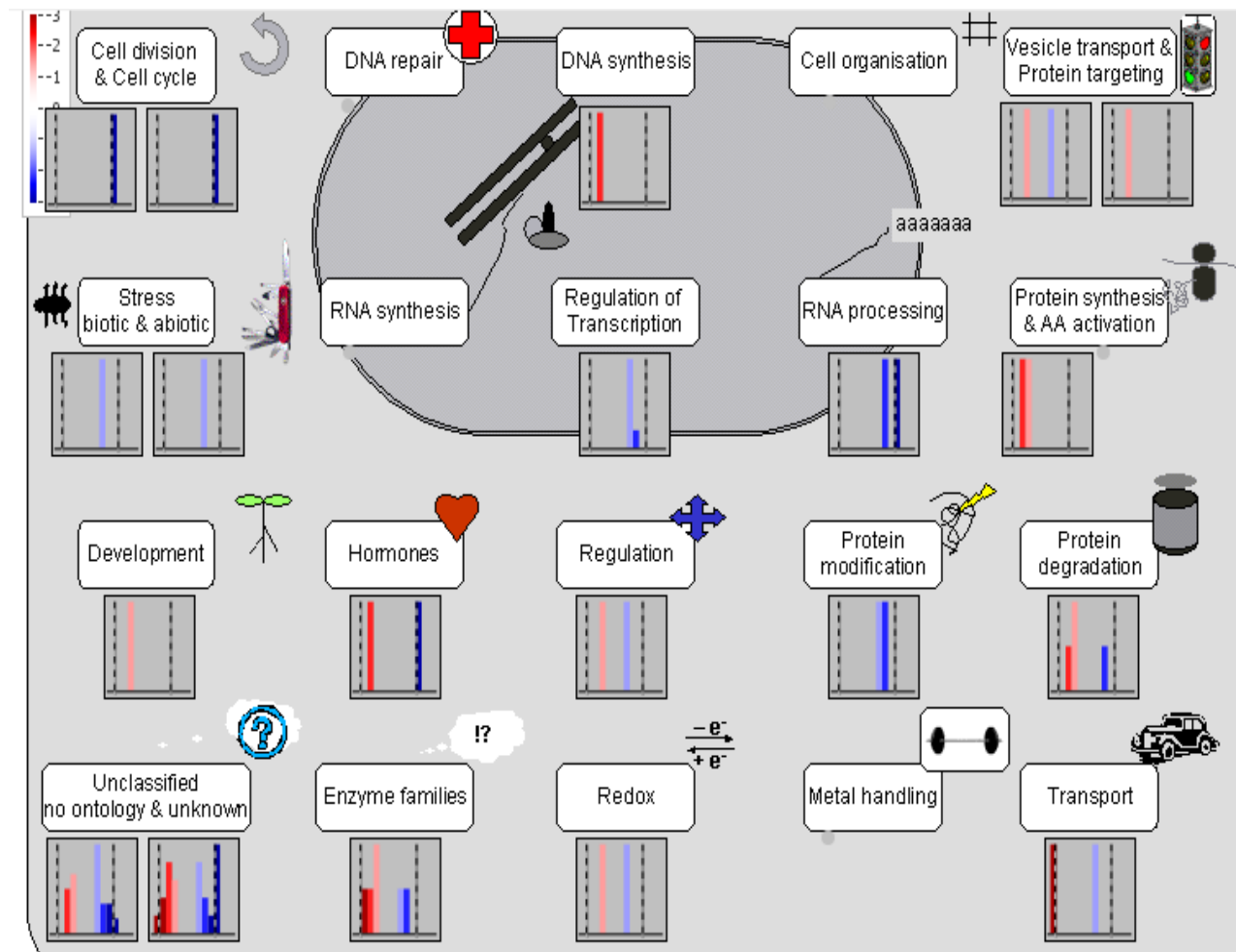


Fig. 52: Cell function overview in transgenic control v/s wild type control using *Medicago truncatula* as a reference genome. Cell division, cell cycle, RNA processing and Regulation of transcription functions were up regulated significantly in transgenics under control condition.

family protein and BAG family chaperone like proteins are found to be up regulated in the transgenic plants under control condition. These small heat shock proteins have cellular protein protection function under diverse stresses.

Root genes: Since the transgenic plants showed improved root growth due to previously characterized Alfin1 TF, the transcriptome data was assessed to identify the probable targets of root associated genes. Around 70 proteins which were known to associated in root growth were assessed based on the literature survey and identified 10 genes in transgenics groundnut transcriptomic data under control condition. Some of the root related genes are up regulated such as Expansin B1, Auxin binding protein, Early nodulin 93, SAUR- like auxin-responsive family protein, Ankyrin repeat protein, Armadillo repeat family protein, Auxin efflux carrier, Root hair defective 3 homolog and Nodule-specific glycine rich peptide. Most of the proteins like Ankyrin repeat, Auxin binding protein, SAUR-like auxin-responsive protein and Armadillo repeat proteins are involved in lateral root initiation and development. Expansin B1 involved in root hair elongation and Early nodulin 93, Nodule-specific glycine rich peptide involved in root nodule formation.

Regulatory proteins: Some of the transcription factors like GRAS family transcription factors, NAC, MYB, TGA5-like protein, transcription initiation factor TFIID and transcription factor KAN2 were upregulated in transgenics compared to wild type under control condition. Some of the ribosomal protein, polymerases, transcription initiation factors, helicase etc., were up-regulated in transgenic plants under control condition.

Transporters: The transporters like sugar porter, amino acid transporter, Inositol transporter 4, ammonium transporter, aluminium-activated citrate transporter, ABC transporter, MFS transporter, high affinity sulfate transporter, White-brown-complex ABC transporter, Nucleobase-ascorbate transporte-like protein, UAA transporter family protein, Calcium transporting ATPase9 and Drug resistance transporter were upregulated in transgenic plants under control condition.

Differentially expressed genes in transgenic plants compared to wild type under stress

To understand the genetic networks that involved in upregulation transcripts in transgenic plants under stress AgriGo analysis suite was used to identify the processes that have different biological function. Transgenic transcripts under stress condition were categorized into 22 functional groups, including biological processes, cellular components and molecular functions (Fig. 53). The cellular process, metabolic process, binding and catalytic activity associated genes were highly represented in the up regulated genes.

Further the genetic network analysis of genes based on biological process shows that the aromatic amino acid biosynthetic process, and prephenate pathway associated genes (GO:009095-8 genes) (Fig. 54) were up regulated in transgenics. This signifies that the metabolic processes are better managed in transgenic plants compared to wild type plants. It is known that to improve the adaptation of crops these pathways have been targeted. In case of development of abiotic resistance crops many groups target these biosynthetic processes signifying the importance of this pathway in sustaining growth and development under stress conditions. From this context the transgenics exposed to moisture stress have greater advantage than wild type plants in sustaining the growth and productivity under stress.

Further the genes encoding deaminase activity (GO:0019239-8 genes), AMP deaminase activity (GO:0003876-2 genes), prephenate dehydrogenase activity (GO:0008977-3 genes) and prephenate dehydrogenase (NADP+) activity (GO:0004665-3 genes) (Fig. 55) were highly represented in transgenics under stress condition. The data suggests that the nitrogen metabolism is better managed in transgenics that may be contributing for higher protein synthesis, sustained plant metabolic processes under stress, accumulation of higher biomass and productivity. The up regulation of these transcripts may bring in more catalytic activity of many metabolic enzyme activity and hence co-ordinating for sustained growth and productivity in transgenics compared to wild type plants.

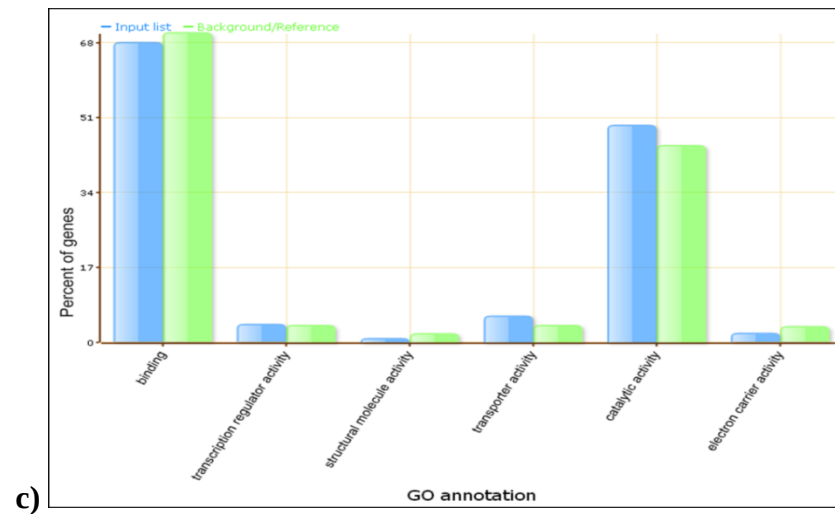
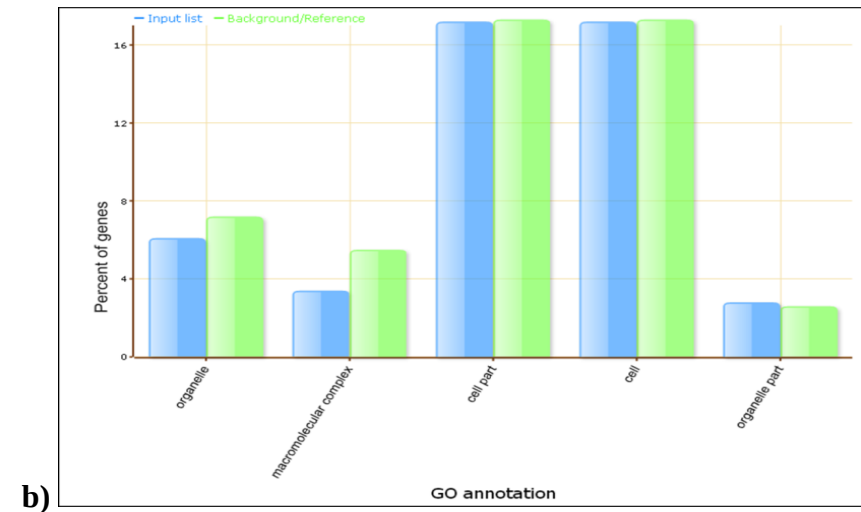
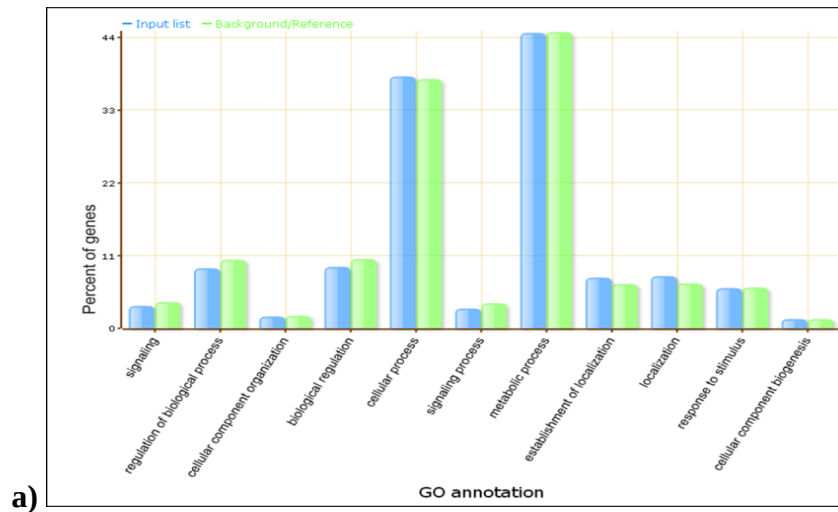


Fig. 53: Classification of upregulated genes in transgenics using Gene Ontology under stress. The results are summarized as three main categories: a) biological process b) cellular components c) molecular functions.

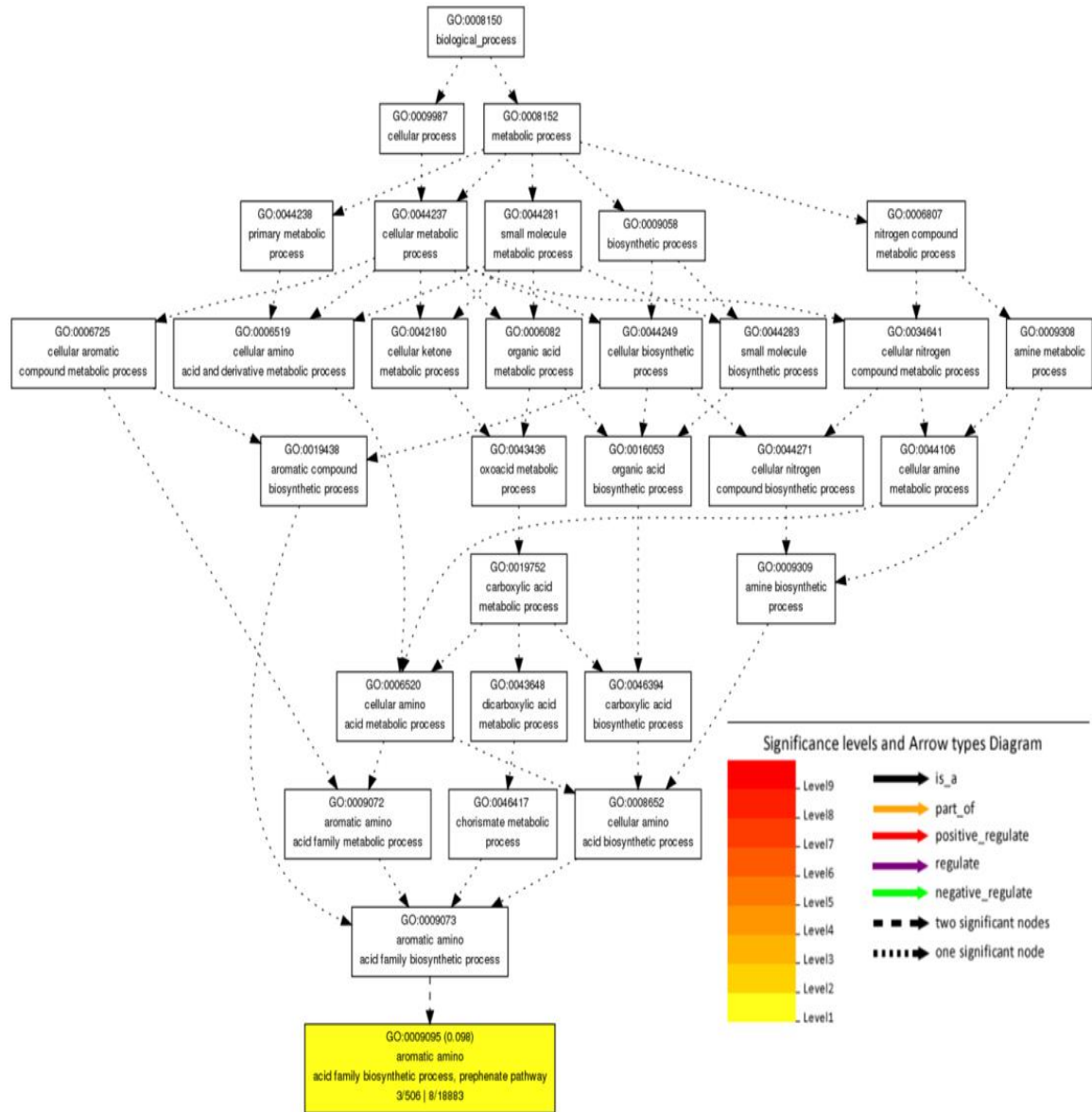


Fig. 54: Biological functions of up regulated transcripts representing different classes in stress conditions. The gene IDs of transcripts were mapped in AgriGO using *Medicago truncatula* as the reference genome. Color code and arrows depicts the level and meaning of representative groups based on the biological functions

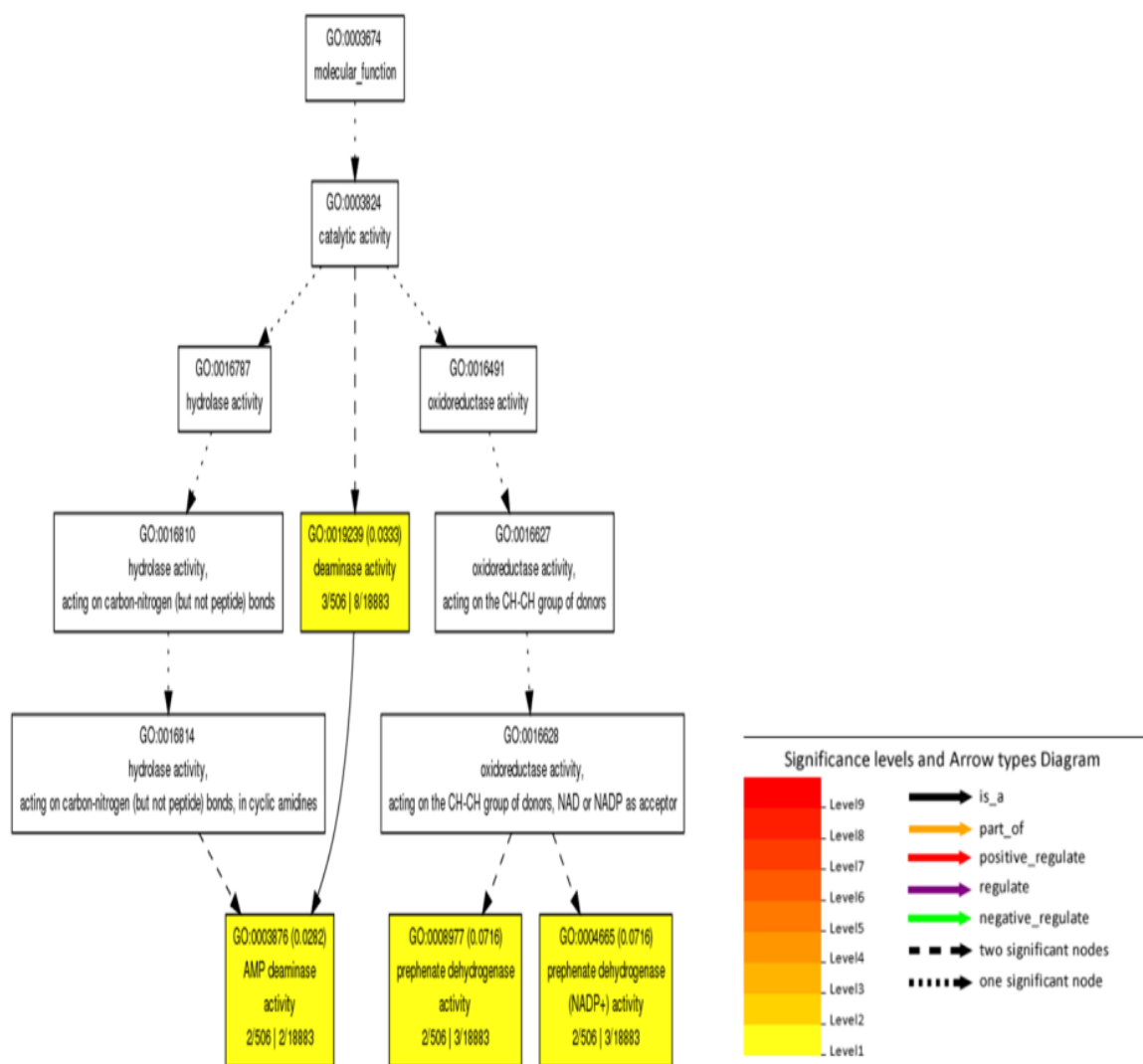


Fig. 55: Molecular functions of the up regulated genes in transgenic plants under control conditions. The gene IDs of transcripts were mapped in AgriGO using *Medicago truncatula* as the reference genome. Color code and arrows depicts the level and meaning of representative groups based on the molecular functions

MapMan Analysis: The differentially expressed genes in transgenics and wild type plants were analysed to know which pathway is highly represented and that may be contributing for the stress tolerance in transgenics. The transcripts data and fold change value was used as input data for mapman analysis and mapped against *Medicago truncatula* as a reference genome.

In cell function overview proteins like Basic helix loop helix domain (bHLH), Zinc finger, Homeobox, NAC1, bZip transcription factor, F-box protein etc. are up regulated in transgenics under stress condition compared to wild type plant (Fig. 56). Some of the proteins like Myb DNA binding domain, aspartokinase, Ribosomal protein, peroxidase 3, cationic peroxidase etc., were down regulated in transgenics.

Target genes that is improving in transgenics

Probable targets of HSF4: There are 5 classes of HSPs in transgenics under stress condition were up regulated. The genes such as 17.5 kDa class I heat shock protein, Heat shock transcription factor A3, cytosolic class I small heat shock protein, calmodulin binding heat shock protein, HSP 70, Heat shock 22 kDa protein, HSP 20 and Dna J class molecular chaperone were up regulated in transgenics compared wild type under stress condition. This data clearly signifying that these proteins under stress conditions protect the proteins and maintain the stability.

Root genes: Around 70 proteins which were associated with roots in *Medicago truncatula* were assessed from literature survey. In transgenics under stress condition 20 genes were up regulated. Ankyrin domain protein, Expansin A1, Early nodulin 93, PHD finger plant-like protein, Armadillo/beta-catenin-like repeat protein, Auxin transporter, Auxin response factor, Nodule specific glycine rich peptide, Ankyrin repeat protein, SAUR- like auxin responsive family protein, Auxin efflux carrier, Hydroxyproline rich glycoprotein family protein, Aconitate hydratase, Armadillo, Root hair defective 3 homolog and Xyloglucan glycosyltransferase were up regulated in transgenics under stress condition when compared to wild type. Further we analyzed these root genes function, proteins like Ankyrin repeats, Armadillo repeats, Auxin response factor, Auxin efflux carrier were involved in lateral root initiation and development. Expansin A1 and

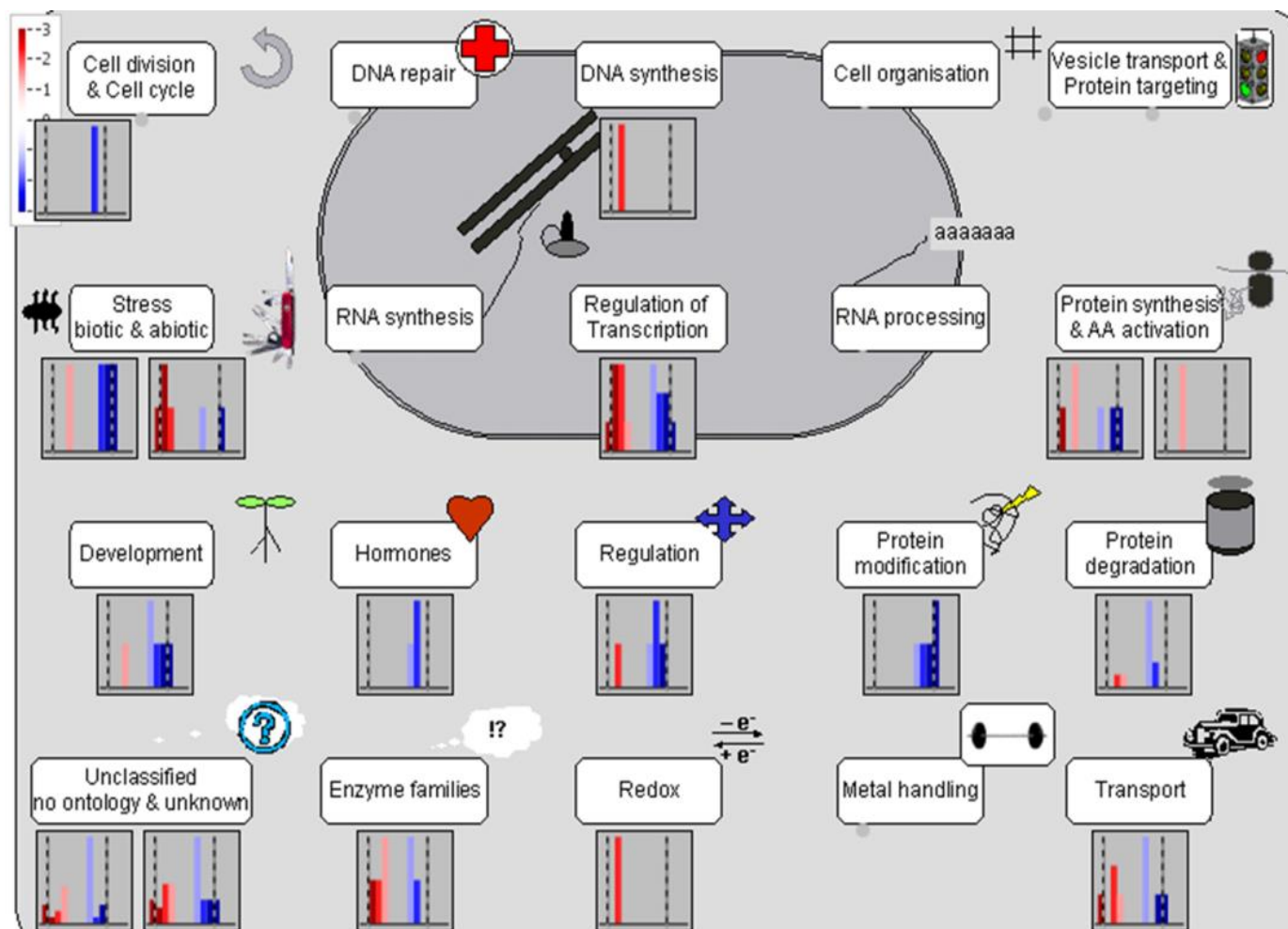


Fig. 56: Cell function overview in transgenic stress v/s wild type stress using *Medicago truncatula* as a reference genome. Cell division, Regulation of transcription, Hormones, Protein modification and degradation were highly assessed in transgenic than wild type under stress condition. The blue color depicts up regulation whereas red depicts down regulation.

Xyloglucan glycosyltransferase involved in root hair elongation. Hydroxy-proline rich glycoprotein involved in root cell elongation. Early nodule 93 and Nodule specific glycine rich peptide involved in root nodule development. The genes involved in water mining are Ankyrin domain protein, Expansin A1, Early nodulin 93, PHD finger plant-like protein, Armadillo/beta-catenin-like repeat protein, Auxin transporter, Auxin response factor, Nodule specific glycine rich peptide, Ankyrin repeat protein, SAUR-like auxin responsive family protein, Auxin efflux carrier, Hydroxyproline rich glycoprotein family protein, Aconitate hydratase, Armadillo, Root hair defective 3 homolog and Xyloglucan glycosyltransferase are upregulated.

Cellular level tolerance: Cellular level tolerance can be achieved by maintaining cell cycle regulation, effective scavenging of ROS, osmotic adjustment, osmoprotection, membrane stability and by protein turnover and stability. In transgenics many of the proteins involved in cellular level tolerance were up regulated when compared to wild type plant (Table 16). Many of the translation related proteins like ribosomal protein, DNA polymerases, RNA binding domain, helicases, DEAD box ATP dependent helicases, pre mRNA splicing factors, transcription initiation factor etc. were up regulated in transgenics under stress condition when compared to wild type. The other genes that are involved in acquired tolerance mechanisms based on differentially expressed genes were identified because the overexpressed genes improved root growth and cellular level tolerance under stress conditions.

Apart from water mining and cellular level tolerance in transgenics various hormones, transcription factors, transporters and receptors also up regulated when compared to wild type (Table 17).

4.8.2 Proteomics data of groundnut transgenic under stress and non-stress condition

The emphasis in the study is to improve protein turnover, stability to maintain normal cellular metabolic processes under stress. The groundnut transgenic plants were developed using the genes associated with protein synthesis mechanisms and protein stability process. The transgenics showed improved drought tolerance and productivity

Table 16: List of up regulated genes in transgenics when compared to wild type which are involved in cellular level tolerance

Scavenging of cytotoxic compounds	Membrane stability	Protein synthesis and stability
Lactoylglutathione lyase/glyoxalase I family protein	TMS membrane protein/tumor differentially protein (Uncharacterized protein)	RNA-directed DNA polymerase (Reverse transcriptase); Ribonuclease H
4-coumarate:CoA ligase-like protein	LMBR1 integral membrane-like protein	17.5 kDa class I heat shock protein
Cytosolic aldehyde dehydrogenase	Plasma membrane H ⁺ -ATPase	17.5 kDa class I heat shock protein
2OG-Fe(II) oxygenase family oxidoreductase	Transmembrane protein, putative	40S ribosomal protein S2-4
Thiol-disulfide oxidoreductase DCC		Eukaryotic translation initiation factor 3 subunit I (eIF3i)
Glutathione S-transferase, amino-terminal domain protein (Uncharacterized protein)		Mediator of RNA polymerase II transcription subunit 36a
Glutathione S-transferase, amino-terminal domain protein		40S ribosomal S3-like protein (Uncharacterized protein)
Lactoylglutathione lyase/glyoxalase I family protein		Hsp20/alpha crystallin family protein
Aldo/keto reductase family oxidoreductase		PHAX RNA-binding domain protein
Cinnamyl alcohol dehydrogenase-like protein		RNA-directed DNA polymerase (Reverse transcriptase); Polynucleotidyl transferase, Ribonuclease H fold
Thioredoxin reductase (EC 1.8.1.9)		Pre-mRNA-splicing factor of RES complex protein
Thioredoxin		40S ribosomal protein S2-4
Lactoylglutathione lyase/glyoxalase I family protein		DEAD-box ATP-dependent RNA helicase-like protein
Glutathione S-transferase		Heat shock transcription factor A3
Cytosolic aldehyde dehydrogenase		Pre-mRNA-splicing factor
Aldo/keto reductase		DNA polymerase (EC 2.7.7.7)
Polyvinylalcohol dehydrogenase-like protein		RNA-directed DNA polymerase (Reverse transcriptase); Zinc finger, CCHC-type; Peptidase aspartic, active site; Retrotransposon gag protein
Respiratory burst oxidase-like protein		DnaJ-class molecular chaperone

Scavenging of cytotoxic compounds	Membrane stability	Protein synthesis and stability
Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein		Tryptophan RNA-binding attenuator-like protein
		Pre-mRNA-splicing factor ATP-dependent RNA helicase
		Transcription initiation factor
		Cytosolic class I small heat shock protein (Uncharacterized protein)
		RNA recognition motif
		Helicase
		Cytosolic class I small heat shock protein
		Calmodulin-binding heat-shock protein
		Heat shock protein 70 (HSP70)-interacting protein, putative
		Small heat shock protein
		Ribosomal RNA small subunit methyltransferase E
		Double-stranded RNA-binding motif protein
		RNA-directed DNA polymerase (Reverse transcriptase); Polynucleotidyl transferase, Ribonuclease H fold
		DExH-box splicing factor-binding site protein
		Heat shock 22 kDa protein, putative
		DEAD-box ATP-dependent RNA helicase-like protein
		Heat shock transcription factor A3 (Uncharacterized protein)
		DNA polymerase III, epsilon subunit-like protein
		50S ribosomal protein L21, related protein
		Transcription initiation factor TFIID subunit 10
		Chaperone DnaJ domain protein
		RNA recognition motif

Table 17: List of transporters, transcription factors, receptors and hormones up regulated in transgenics when compared to wild type

Transporters	Transcription factors	Receptors	Hormones
Cation-transporting ATPase plant	GATA transcription factor	Glutamate receptor	Protein phosphatase 2C-like protein
Polyol/monosaccharide transporter 1	Myb-like transcription factor family protein	Golgi SNAP receptor complex member 1	Ethylene response factor
MATE efflux family protein	WRKY family transcription factor	SPLa/Ryanodine receptor (SPRY) domain protein	Indole-3-acetic acid-amido synthetase
Cation chloride cotransporter	GRAS family transcription factor	B-cell receptor-associated-like protein (Uncharacterized protein)	Abscisic acid receptor PYL9-like protein (Uncharacterized protein)
Drug resistance transporter-like ABC domain protein	Transcription factor jumonji (JmjC) domain protein	LRR receptor-like kinase family protein	Protein phosphatase 2C-like protein
Sulfate/bicarbonate/oxalate exchanger and transporter sat-1	Heat shock transcription factor A3	Receptor-like kinase	Auxin transporter BIG
Nodulin MtN21/EamA-like transporter family protein	AP2/B3 transcription factor family protein	Glutamate receptor	Indole-3-acetic acid-amido synthetase
Nodulin-like/MFS transporter	BZIP transcription factor	Receptor-like kinase	Auxin response factor
Transmembrane amino acid transporter family protein	E2F transcription factor		Ethylene response factor
Heavy metal transport/detoxification superfamily protein (Uncharacterized protein)	NAC transcription factor-like protein		Ethylene receptor ETR2
Ctr family copper transporter	Myb-like transcription factor family protein		SAUR-like auxin-responsive family protein
Inositol transporter 4	BZIP transcription factor		Ethylene response factor (Ethylene-responsive element-binding protein)
White-brown-complex ABC transporter family protein	Transcription factor Pcc1		Auxin efflux carrier family protein
Inositol transporter, putative	Myb-like transcription factor family protein		Zeaxanthin epoxidase, chloroplastic (EC 1.14.13.90)
Transmembrane amino acid transporter family protein	BZIP transcription factor		Protein phosphatase 2C ABI2-like protein

Transporters	Transcription factors	Receptors	Hormones
ABC transporter B family protein	GRAS family transcription factor		GRAM domain protein/ABA-responsive-like protein
Putative sucrose-H ⁺ symporter (Fragment)	WRKY1b transcription factor		
Anion transporter 4, putative	NAC transcription factor-like protein		
Silicon efflux transporter	Transcription factor UNE12-like protein		
Peptide/nitrate transporter	Myb-like transcription factor family protein		
ABC transporter A family protein	MADS-box transcription factor		
Auxin transporter BIG	BZIP transcription factor bZIP124		
Potassium transporter	Transcriptional factor B3 family protein		
Endoplasmic reticulum-type calcium-transporting ATPase	AP2 domain transcription factor		
SUT1-2 (Sucrose transporter)	Myb-like transcription factor family protein		
Vacuolar iron transporter-like protein	BZIP transcription factor		
Major intrinsic protein (MIP) family transporter	GATA type zinc finger transcription factor family protein		
Transmembrane amino acid transporter family protein	Transcription factor Pcc1		
Ctr family copper transporter	Apoptosis antagonizing transcription factor		
Transmembrane amino acid transporter family protein	Myb transcription factor		
MFS transporter	GATA transcription factor		

under stress conditions. Further to understand the proteomic changes in the transgenic plants compared to wild type plants, proteomic analysis was carried out. The plants were subjected to gradual reduction in moisture stress and then samples of wild type and transgenics were collected in control and stress conditions. The total proteins were extracted using trichloroacetic acid (TCA): acetone precipitation method (detailed protocol is given in 3.4.2). Total protein content in transgenics under control and stress condition is significantly higher than wild type plants.

The samples were analyzed on Triple-TOF 5600 (AB Sciex; Concord, Canada) mass spectrometer coupled with Micro LC 200 (Eksigent; Dublin, CA) in high-sensitivity mode LC-MS/MS.

4.8.2.1 Differentially expressed proteins:

The differentially expressed proteins in groundnut transgenic and wild type plant were identified using *Medicago truncatula* as reference genome. The proteins having ≥ 1.5 fold change are considered as up or down regulated proteins. In transgenics under control conditions there are 323 proteins were up regulated and 42 proteins were down regulated compared to wild type plants. Similarly 330 proteins were up regulated and 73 proteins were down regulated in transgenics plants compared to wildtype under moisture stress conditions (Table 18).

Further to know how many genes may have up or down regulated with in the transgenic or wild type under control and stress conditions, the Venn 2.0 tool was used to identify common and unique proteins. In transgenics 145 common proteins were found to up regulated between control and stress plants. There are 125 proteins uniquely expressed in transgenics under normal conditions and 131 proteins are uniquely upregulated in stress conditions. Only 3 downregulated in both stress and control samples, 32 unique proteins under control and 54 proteins under stress were downregulated in transgenics (Fig. 57).

Table 18: List of up-regulated and down-regulated proteins in transgenic plants when compared to wild type plants using *Medicago truncatula* as the reference genome

	Up regulated	Down regulated
TC v/s WC	323	42
TS v/s WS	330	73

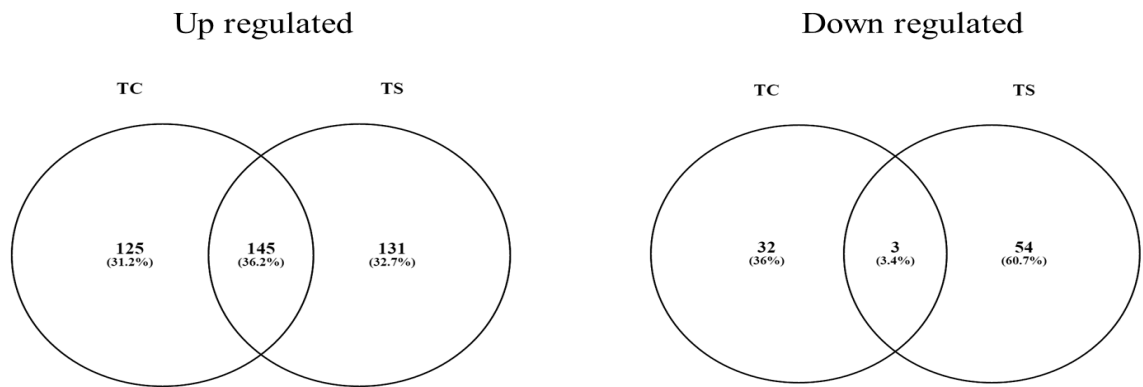


Fig. 57: Venn diagram showing common up regulated and down regulated proteins in transgenic control and stress condition.

4.8.2.2 Transgenic plants showed higher number of proteins expressed compared to wild type in control conditions.

Mapman analysis: The proteome data from the groundnut transgenics were analyzed by using *Medicago truncatula* as the reference genome. The up regulated proteins were assessed in different pathways that are showing specific responses. The overview of cell function associated proteins shows that cell division and cell cycle, DNA synthesis, vesicle transport and protein targeting; regulation of transcription, RNA processing etc. proteins are identified from proteomic studies. The Bromo-adjacent homology (BAH) domain containing protein is highly up regulated in transgenics. The BAH is commonly found in chromatin-associated proteins. It is found in proteins such as eukaryotic DNA (cytosine-5) methyltransferases, the origin recognition complex 1 (Orc1) proteins, as well as several proteins involved in transcriptional regulation. The BAH domain appears to act as a protein-protein interaction module specialized in gene silencing, as suggested for example by its interaction within yeast Orc1p with the silent information regulator Sir1p. The BAH module might therefore play an important role by linking DNA methylation, replication and transcriptional regulation. The ribosomal proteins are also up regulated in transgenics suggesting their improved protein synthesis. In transgenics G- protein, Endochitinase precursor, Glutathione S-transferase proteins were up regulated in control conditions suggesting that the signalling mechanisms and cell protection mechanisms are improved in transgenic plants (Fig. 58). Many proteins which are involved in scavenging of cytotoxic compounds were also upregulated in transgenics. Proteins like Glutathione S-transferase, Peroxidase, Cytosolic aldehyde dehydrogenase, Superoxide dismutase [Cu-Zn], Glutaredoxin C4, L-ascorbate peroxidase, Lactoylglutathione lyase and Catalase were upregulated in transgenics under normal condition.

Since, the co-expressing transgenic lines containing HSF4 that upregulate HSPs. From this context the proteome data was specifically analyzed for some of the HSPs. Heat shock cognate 70 kDa, Heat shock protein 81-2, cytosolic class II small heat shock protein, cpn60 chaperonin family protein, 17.6 kDa small class I heat shock protein, Dna J class molecular chaperone and GroES chaperonin were up regulated in transgenics under control condition when compared to wild type plant. The molecular

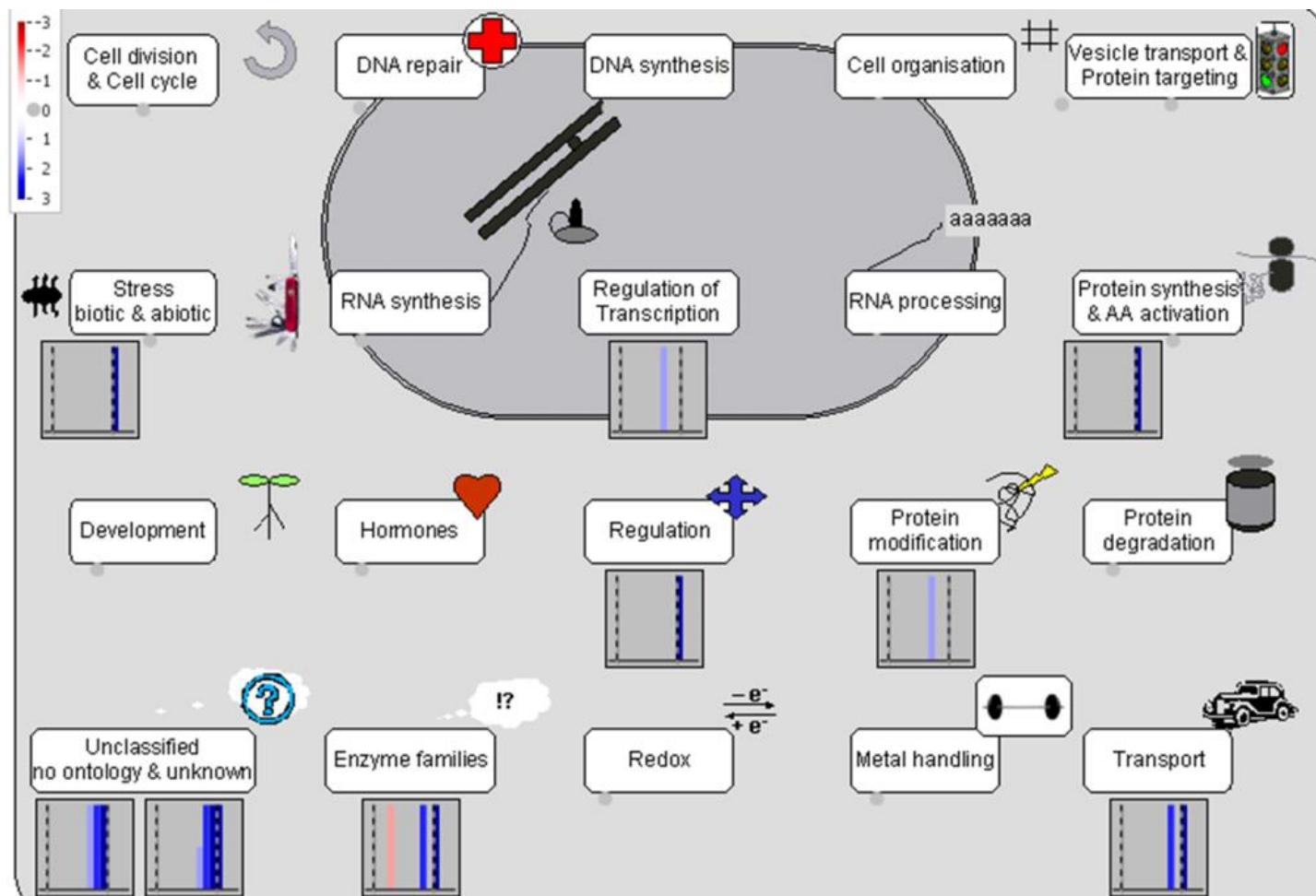


Fig. 58: Overview of the proteins that are associated with cell function in transgenic plants compared to wild type under non-stress condition using proteome data. Regulation of transcription, protein synthesis and amino acid activation, protein modification and transport functions were up regulated significantly in transgenics under control condition.

chaperones/heat-shock proteins (HSPs) are responsible for protein stabilization, proper folding, assembly, and translocation of many proteins under normal and stress conditions showing that transgenics have better mechanisms to protect cellular enzymes under adverse conditions.

The another protein we expressed is PDH45 an helicase have important role in translation mechanism. So we looked into some of the translation related proteins in transgenics under control condition. Some of the proteins like ribosomal proteins, translation initiation factor, DEAD box ATP dependent helicase, DNA/RNA binding protein, GTP binding elongation factor etc. were up regulated in transgenics under normal conditions compared to wild type plants

Photosynthesis related proteins were up regulated in transgenic plants: In proteome data of groundnut there was upregulation of photosynthesis related proteins like Light-harvesting complex I, chlorophyll-A/B-binding protein, Phosphoenolpyruvate carboxylase, Mg-protoporphyrin IX chelatase, Magnesium-protoporphyrin IX monomethyl ester cyclase, NADPH-protochlorophyllide oxidoreductase, Photosystem II protein D1, Photosystem II D2 protein, Photosystem II stability/assembly factor HCF136, Photosystem I reaction center subunit X psaK and RuBisCO large subunit-binding protein under normal condition. During photosynthesis the photosynthetic reaction center is a complex of several proteins, pigments and other co-factors that together execute the primary energy conversion reactions. These processes important to maintain improved growth and productivity.

4.8.2.3 Differentially expressed proteins in transgenic plants under stress conditions compared to wild type stress

Transgenic lines carrying three upstream regulatory genes like Alfin1 (root gene), PDH45 (Removes secondary structures - improves translation process) and HSF4 (protein stability). These genes regulate the water relation and cellular level tolerance, so we looked into water mining and cellular level tolerance proteins in proteome data. Scavenging of cytotoxic compounds, Membrane stability, maintenance of cell turgor, protein turnover, stability and folding are the major factors that sustain cellular tolerance

under stress. Transgenics consists of many of the cellular level tolerance proteins when compared to wild type plant (Table 19).

The proteomics data from the transgenic and wild types groundnut plants under moisture stress conditions were analysed to identify the pathways that are associated using **mapman analysis tool** using *Medicago truncatula* as the reference genome. In cell function overview some of the proteins like WRKY32, Zinc finger type protein, cyclin dependent kinases, Bromo-adjacent homology domain containing protein like, Endochitinase precursor, F-box protein, 26S proteasome regulatory sub particle, peroxidase etc proteins were up regulated in transgenics in stress conditions (Fig. 59).

In transgenic plants, translation related proteins were highly up regulated. Proteins like 33 kDa ribonucleo protein, DNA/RNA binding protein, ribosomal protein, Eukaryotic initiation factor, elongation factor, signal recognition particle, DEAD box ATP dependent helicase etc. were up regulated more than 2 fold in stress conditions. These improved expression of proteins suggested that the protein synthesis in transgenic plants is improved under stress conditions and hence contributing for higher improved biomass.

Some of the HSPs such as HSP20, HSP 81-2, Heat shock 70 kDa protein, cpn 60 chaperonin family protein, Gro-EL-like chaperone and Dna J class molecular chaperone were up regulated in transgenic under stress condition compared to wild type plants. Protein dysfunction is an inevitable consequence of a wide range of adverse environmental conditions and molecular chaperones like heat-shock proteins (HSPs) are responsible for protein stabilization, proper folding, assembly, and translocation. To sum up, in transgenics these chaperones may be playing important role in combating drought stress by maintaining protein stability or re-establishing normal protein conformation to improve cellular homeostasis under stress.

Root genes: The transgenic plants showed improved root growth due to Alfin1 TF, so we looked into some of the root proteins in proteome data. Around 70 proteins which are associated with roots from *Medicago truncatula* was known, whereas as in transgenic

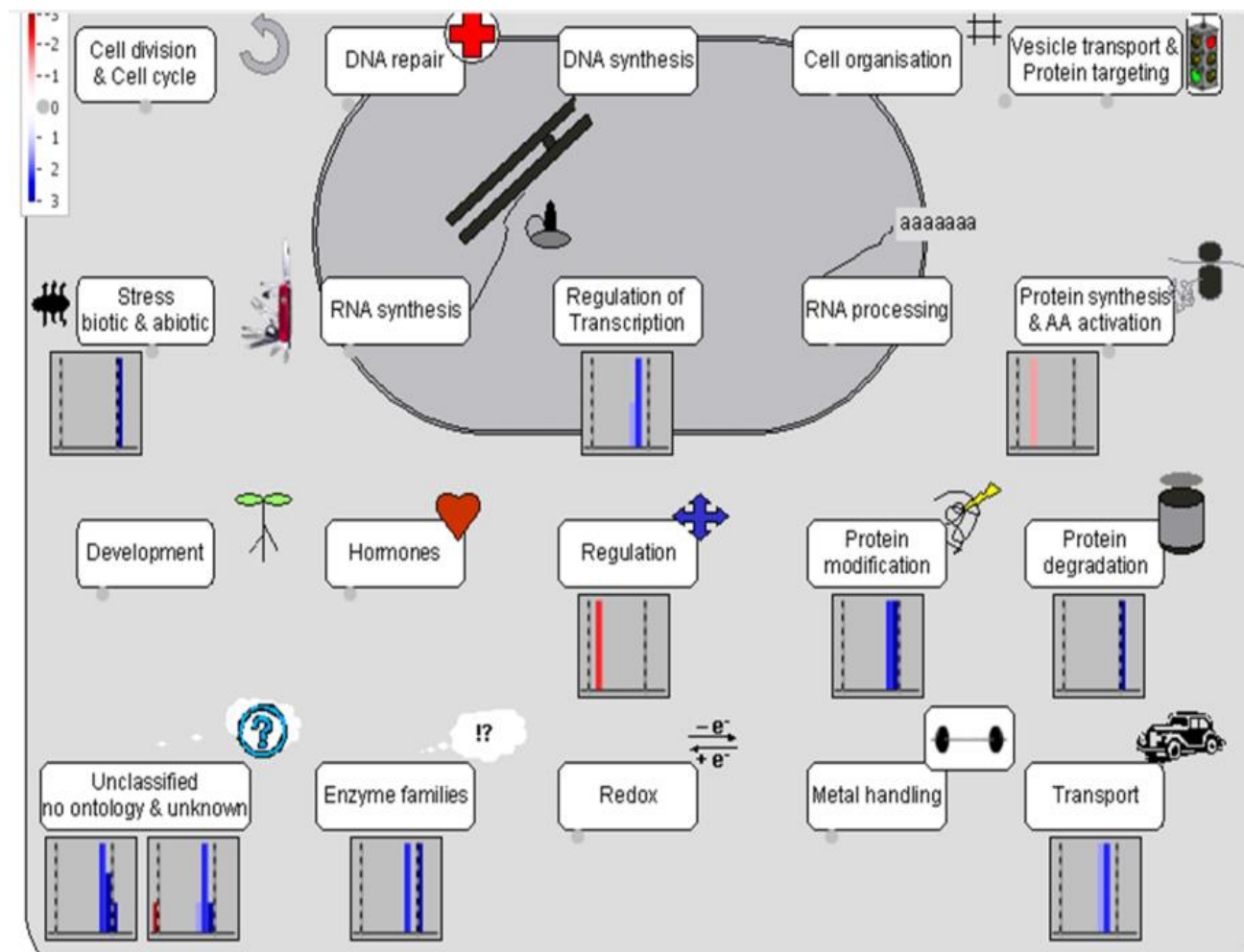


Fig. 59: Overview of the proteins that are associated with cell function in transgenic under stress compared to wild type. Regulation of transcription, protein modification, protein degradation and enzyme families were significantly up regulated in transgenics than wild type under stress condition.

Table 19: List of up regulated proteins in transgenics compared to wild type that are involved in cellular level tolerance mechanisms under stress conditions

Scavenging of toxic compounds	Membrane stability	Protein synthesis and stability
Superoxide dismutase [Cu-Zn]	Plasma membrane ATPase	Heat shock protein HSP20, putative (Fragment)
Aldo/keto reductase family oxidoreductase	Transmembrane protein, putative	Heat shock protein 81-2
Lactoylglutathione lyase	Plasma membrane ATPase	Heat shock 70 kDa protein
Acyl-CoA dehydrogenase		Heat shock 70 kDa protein
Glutathione S-transferase		TCP-1/cpn60 chaperonin family protein
Aldo/keto reductase family oxidoreductase		Heat shock cognate 70 kDa protein
Glutathione S-transferase tau		Heat shock protein 81-2
Aldo/keto reductase family oxidoreductase		Heat shock protein 81-2
Thioredoxin-dependent peroxidase		GroEL-like chaperone, ATPase
L-ascorbate peroxidase		Heat shock cognate 70 kDa protein
Aldo/keto reductase family oxidoreductase		DnaJ-class molecular chaperone
Peroxidase		33 kDa ribonucleoprotein
		Alba DNA/RNA-binding protein
		50S ribosomal protein L5P
		40S ribosomal protein S4
		DEAD-box ATP-dependent RNA helicase-like protein
		Eukaryotic translation initiation factor 3 subunit F
		40S ribosomal protein S15-4
		40S ribosomal protein S23-1
		Small nuclear ribonucleoprotein OS=Medicago truncatula GN=MTR_2g104440 PE=2 SV=1
		Ribosomal protein S13P/S18e
		Small nuclear ribonucleoprotein
		60S ribosomal protein L23a-2
		Signal recognition particle 54 kDa protein
		60S ribosomal protein L24-2
		Elongation factor 1-alpha
		40S ribosomal protein S3
		Poly(RC)-binding-like protein
		Eukaryotic translation initiation factor 4E
		Eukaryotic translation initiation factor 3 subunit I
		40S ribosomal protein S24
		50S ribosomal protein L9
		50S ribosomal protein L2, chloroplastic
		Eukaryotic translation initiation factor 3 subunit A
		30S ribosomal protein S19, chloroplastic
		Elongation factor 1-alpha
		DEAD-box ATP-dependent RNA helicase

under stress condition only 3 up regulated proteins were found. The proteins are Hydroxy proline rich glyco protein family protein which is involved in root cell elongation, Isocitrate dehydrogenase present in root nodules and protect against nitro-oxidative stress and syntaxin protein which is involved in root tip growth was found to be up regulated in transgenic plants.

The photosynthesis and downstream metabolic process associated proteins were up regulated in stress in transgenic plants Proteins involved in photosynthetic pathway like Light harvesting complex I, chlorophyll-a/b binding protein, Photosystem II 10 kDa protein, plastid developmental protein, phosphoenolpyruvate carboxylase, photosystem II cp-43 reaction center, Mg-protoporphyrin IX chelatase and photosystem I reaction center were up regulated in transgenics compared to wild type. This suggests that transgenics maintain the normal growth and development under stressed environment, mainly by upregulating some of the metabolic pathways like photosynthesis, glycolysis and tricarboxylic acid (TCA) cycle, etc. Citric acid cycle proteins like Pyruvate dehydrogenase E1 beta subunit, Pyruvate dehydrogenase E1 component subunit alpha, Citrate synthase, Isocitrate dehydrogenase [NAD] subunit, mitochondrial, Malate dehydrogenase, Malic enzyme, Glyoxysomal malate dehydrogenase and Succinyl-CoA ligase subunit beta were upregulated more than 2 fold in transgenic plants. This clearly demonstrated that glycolysis and TCA cycle proteins are up-regulated that might help the stressed plants to produce more reducing power to compensate high-energy demand and hence improve productivity. Further proteins involved in acquired tolerance mechanisms are also up regulated in transgenic plants.

4.8.3 Metabolomics of groundnut transgenic line under stress and non-stress conditions

Metabolomics is a promising analytical technology that has been used to unravel the metabolic fluctuations of crucial plant molecules and the metabolic flux. Groundnut wild type and transgenic plants were subjected to moisture stress through gravimetric approach and then samples were collected from both wild type and transgenic plants under stress and control conditions for Metabolomics analysis. The following four

samples were analyzed i.e. wild type control (WC), Wild type stress (WS), Transgenic control (TC) and Transgenic stress (TS) with three biological replicates.

4.8.3.1 Standardization of metabolite extraction

Metabolite extraction is a fundamental and critical step in metabolite profiling. Methanol, an organic solvent widely used for metabolite extraction. Different concentrations of aqueous methanol were tested to extract maximum number of metabolites and analyzed using LC/MS. Highest numbers of metabolites were observed in 70% methanol in both positive and negative ionization mode (Fig. 60). For both wild type and transgenic groundnut sample 70% methanol was used as solvent for extraction of metabolites. List of standards used for metabolite extraction is given in Table 20.

4.8.3.2 Metabolic profiling

Differential accumulation of metabolites was compared with transgenic control versus wild type control and transgenic stress versus wild type stress. The metabolites having more than 1.5 fold change are considered for up regulation in transgenic plant. In transgenic, 21 constitutive metabolites were accumulated under control condition (Fig. 61) whereas 27 metabolites were constitutively expressed under stress condition (Fig. 62) when compared to wild type and 15 metabolites were exclusively induced in transgenics under stress condition. These metabolites belong to both primary and secondary metabolites. Amino acids, carboxylic acids, organic acids, sugars and fatty acids were the major group of primary metabolites, while secondary metabolites include terpenoids, flavonoids, alkaloids and phenyl propanoids.

4.8.3.3 MS/MS fragmentation pattern:

The identified metabolites were confirmed by spiking commercially available synthetic standard compounds and matching the MS/MS fragmentation patterns using LC-LTQ-Orbitrap. MS/MS spectra of spiked standard of Jasmone (Fig. 63) compared with sample fragmentation pattern in groundnut transgenic under control condition. 12-OPDA (Fig. 64) and tyramine (Fig. 65) spiked standard were compared with sample fragmentation pattern in groundnut transgenic under stress condition.

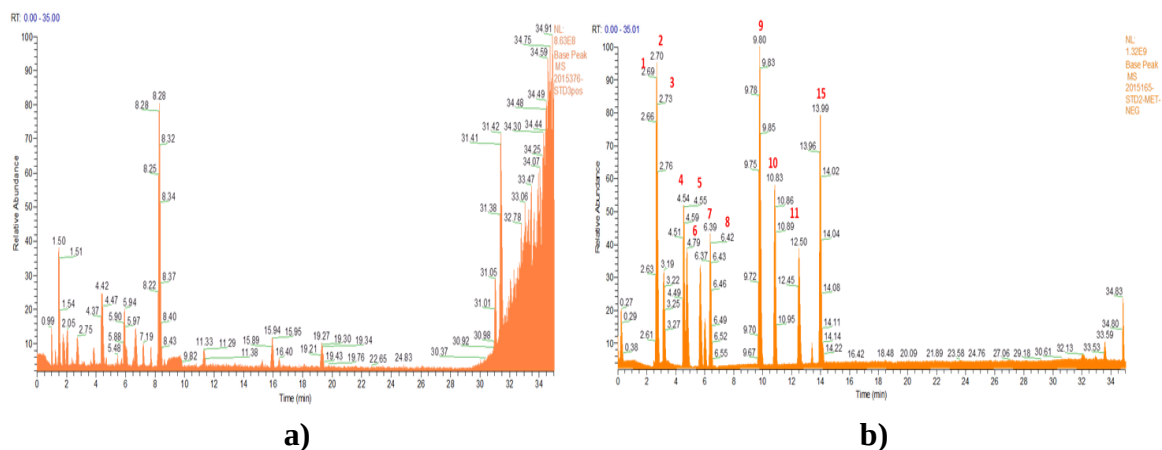


Fig. 60: Solvent concentration standardization for metabolite extraction in both a) Positive ionization mode b) Negative ionization mode

Table 20. List of standards used for metabolite extraction

Sl. No.	Standards	Sl. No.	Standards
1	Methyl jasmonate	23	L-leucine
2	(+)-Jasmonic acid	24	L-Glutamate
3	p-Coumaric acid	25	L-tryptophan
4	Shikimic acid	26	L-tyrosine
5	cis-Jasmone	27	Threonine
6	sinapic acid	28	Serine
7	Coniferyl alcohol	29	L-Aspartate
8	Gibberelic acid	30	L-Arginine
9	ABA	31	L-proline
10	3-indoleacetic acid	32	Asparagine
11	Glutathione	33	L-Glutamine
12	zeatin	34	Glucose
13	indol-3-butyric acid	35	Sucrose
14	melatonin	36	D-Sorbitol
15	kinetin	37	glu-6-po4
16	caffeic acid	38	Fructose 1,6 po4
17	D-(-) Quinic acid	39	L-alpha-phosphotidyl choline
18	Quercetin	40	Glutothion Reduced
19	raffinose	41	Coumarin
20	mannitol	42	Succinic acid
21	SA	43	Fumaric acid
22	L-phenylalanine	44	D-Limonene

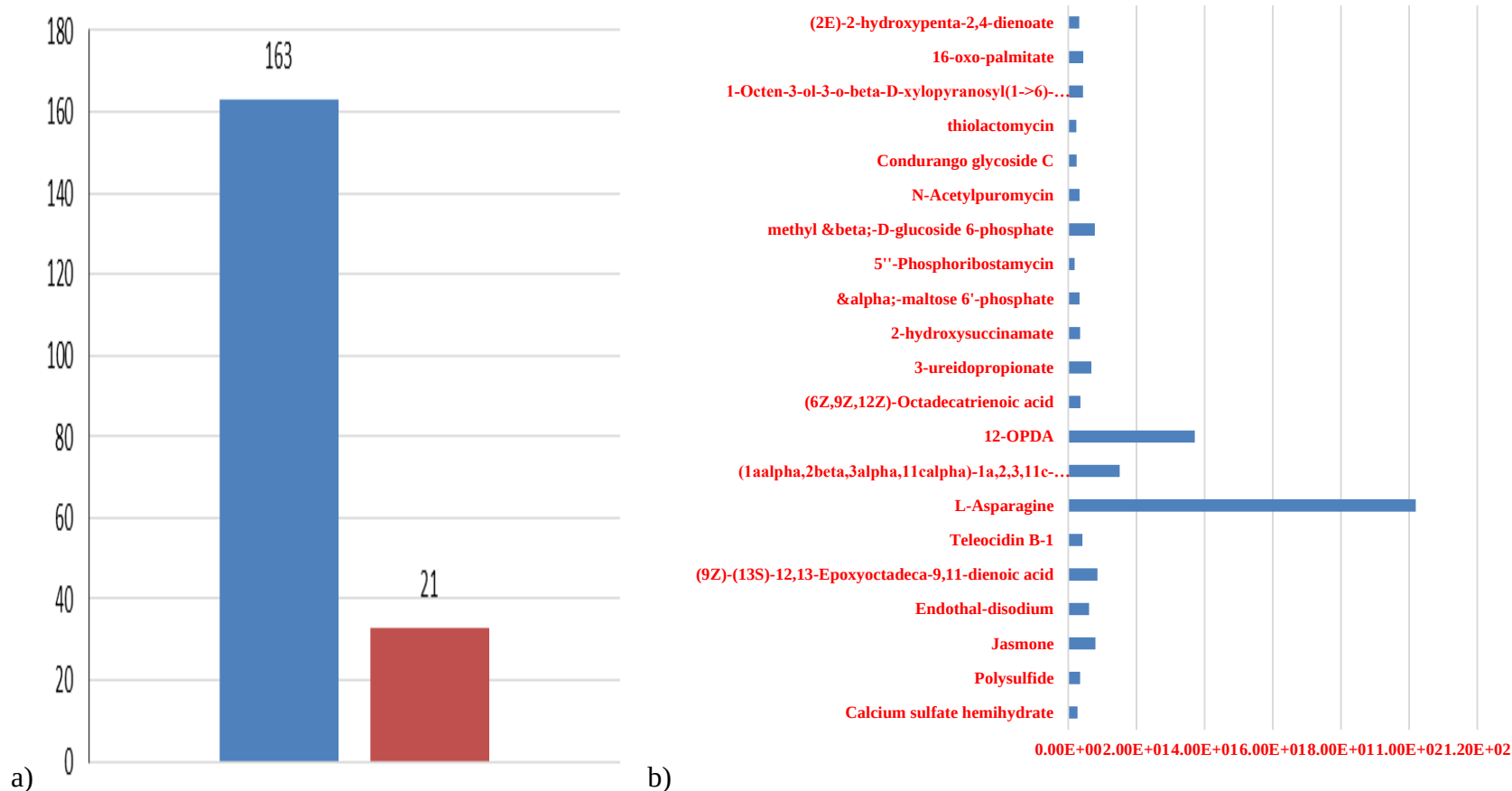
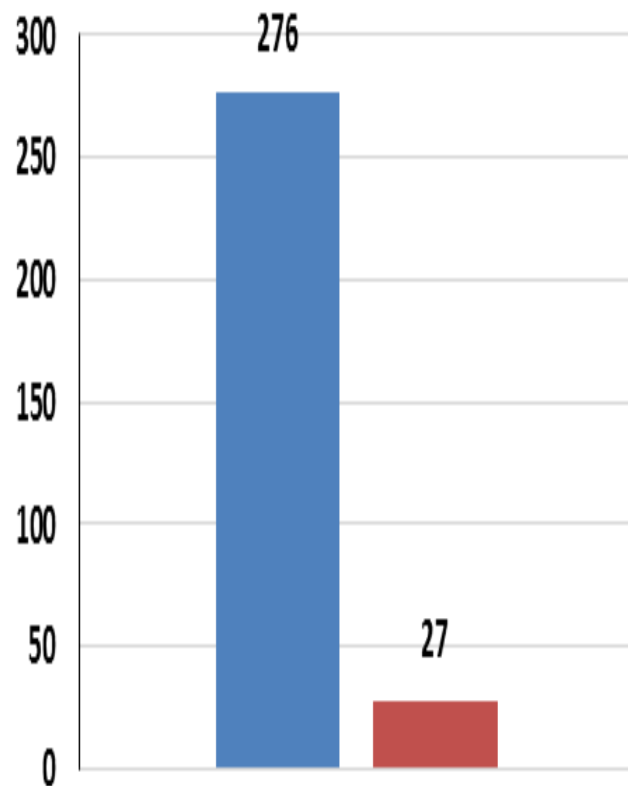
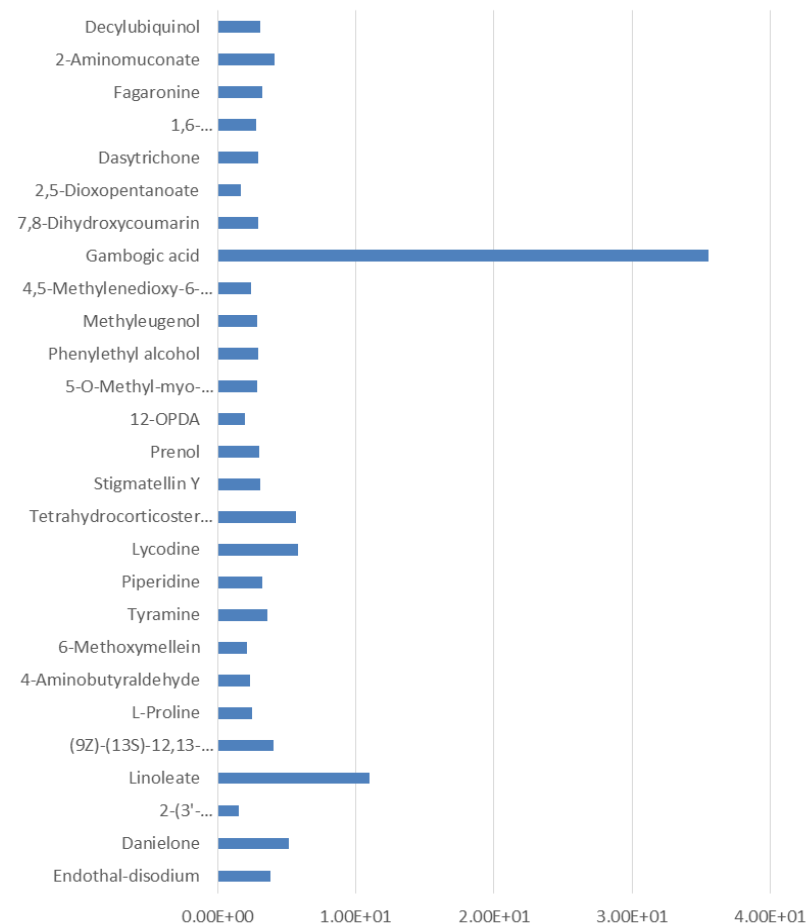


Fig. 61: Constitutively expressed metabolites in transgenics compared to wild type under control condition. a) Graphical representation of constitutively expressed metabolites in transgenics over wild type plant under non-stress condition. In transgenic plants 163 peak area points are identified but only 21 peak area points are annotated from the database b) Annotated metabolites in transgenics over wild type which are having more than 1.5 fold change.



a)



b)

Fig. 62: Constitutively expressed metabolites in transgenics compared to wild type under stress condition. a) Graphical representation of constitutively expressed metabolites in transgenics over wild type plant under stress condition. In transgenic plants 276 peak area points are identified but only 27 peak area points are annotated from the database b) Annotated metabolites in transgenics over wild type which are having more than 1.5 fold change.

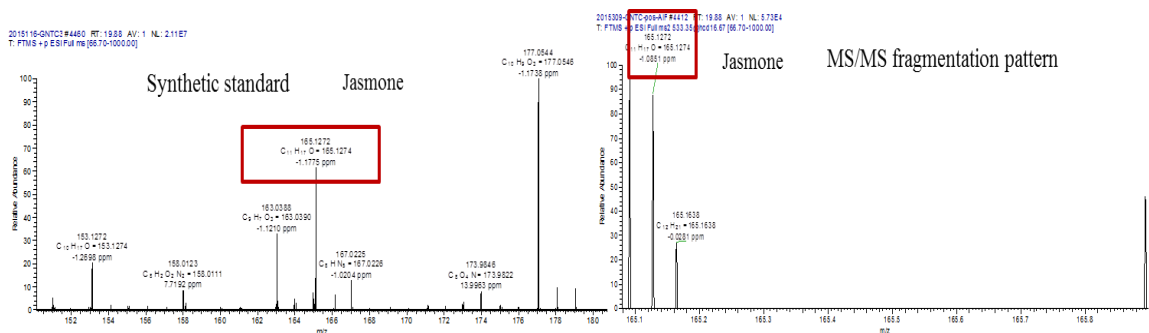


Fig. 63: MS/MS spectra of spiked standard of Jasmine compared with sample fragmentation pattern in groundnut transgenic under control condition

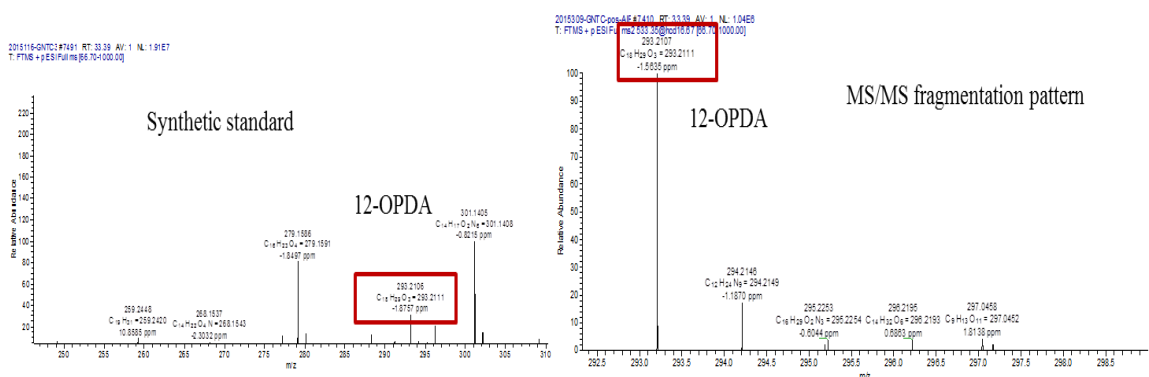


Fig. 64: MS/MS spectra of spiked standard of 12-OPDA compared with sample fragmentation pattern in groundnut transgenic under stress condition

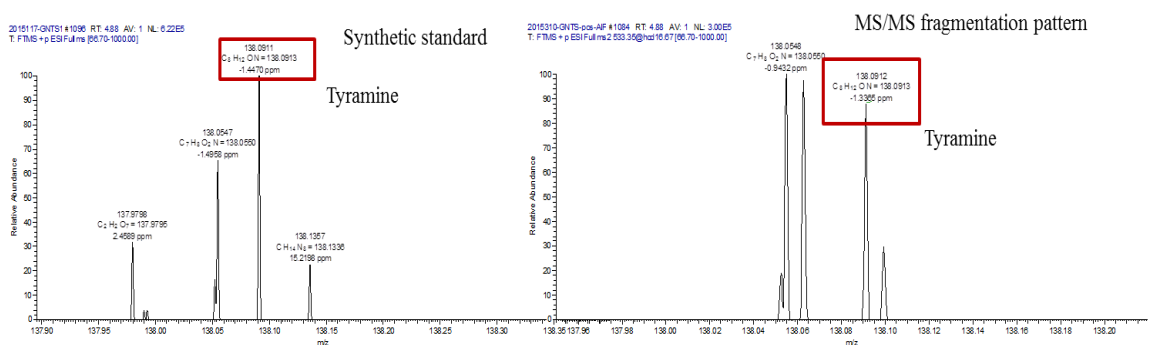


Fig. 65: MS/MS spectra of spiked standard of Tyramine compared with sample fragmentation pattern in groundnut transgenic under stress condition

4.8.3.4 Functional annotation:

Further the metabolites up regulated in transgenic were compared to wild type are mapped in KEGG database and obtained the pathway information of each metabolite. In transgenic plant under control condition out of 21 metabolites only 9 are mapped against KEGG database (Table 21). Whereas in transgenic under stress condition out of 27 metabolites 22 were mapped in KEGG database (Table 22). Further these metabolites in transgenics under control condition were grouped based on their function like amino acids, antioxidants, and maintenance of cell turgour, Plant defense, antibiotics and wax synthesis (Table 23). Whereas in transgenics under stress condition the mapped metabolites are classified into aminoacids, antioxidants, maintenance of cell turgour, osmolytes, secondary metabolites, alkaloids and phytoalexin (Table 24).

4.8.3.5 Induced metabolites in transgenics under stress condition:

In transgenics 15 metabolites were exclusively induced under stress condition they are 5-bromo-3-phenyl Salicylic Acid, Acrovestone, adenylo-succinate, affinisine, aspidin, carnosol, Flavonol 3-O-[alpha-L-rhamnosyl-(1->6)-beta-D-glucoside], Inulicin, Loganin penta acetate, magnesium-protoporphyrin IX 13-monomethyl ester, NADPH, Oxalosuccinic acid, S-Methyl-1-thio-D-glycerate, Spirasine I and Trigonelline. Affinisine, spirasine I and trigonelline are belong to the alkaloids group; and these alkaloids may act as reservoirs for protein synthesis and act as protective substances against the animal or insect attacks. Acrovestone as an antioxidant property, aspidin is a natural anti-fibrotic compound, carnosol is a phenolic diterpene compound and magnesium protoporphyrin involved in chlorophyll biosynthesis.

Plants have evolved various mechanisms for abiotic stress; they include short term avoidance mechanism or long term evolutionary adaptations. Some major cellular level tolerance mechanisms include antioxidant defense, osmoprotectants and maintaining protein synthesis and stability. Some of the metabolites related to cellular level tolerance were up regulated in transgenics compared to wild type. Antioxidants like L-Proline, 4-Aminobutyraldehyde, linoleate, 5-O-Methyl-myo-inositol, 7,8-Dihydroxy Coumarin, 3-bromo-5-phenyl salicylic acid and carnosol were up regulated in

Table 21: List of constitutively expressed metabolites in transgenics under control condition which are mapped against KEGG pathway

Metabolites	Pathway	KEGG ID
Polysulfide	Sulfur metabolism	map00920
L-Asparagine	Protein digestion and absorption	map04974
(1aalpha,2beta,3alpha,11calpha)-1a,2,3,11c-Tetrahydro-6,11-dimethylbenzo[6,7]phenanthro[3,4-b]oxirene-2,3-diol	Metabolism of Xenobiotics by cytochrome P450	map00980
12-OPDA	alpha-Linolenic acid metabolism	map00592
(6Z,9Z,12Z)-Octadecatrienoic acid	Linoleic acid metabolism	map00591
3-ureidopropionate	Pyrimidine metabolism	map00240
N-Acetylpuromycin	Puromycin biosynthesis	map00231
16-oxo-palmitate	Cutin, suberin and wax biosynthesis	map00073

Table 22: List of constitutively expressed metabolites in transgenics under stress condition which are mapped against KEGG pathway

Metabolites	Pathway	KEGG ID
Danielone	Phytoalexin	
2-(3'-Methylthio)propylmalic acid	Glucosinolate biosynthesis	map00966
Linoleate	Linoleic acid metabolism	map00591
(9Z)-(13S)-12,13-Epoxyoctadeca-9,11-dienoic acid	Epoxy fatty acids	
L-Proline	Arginine and proline metabolism	map00330
4-Aminobutyraldehyde	Arginine and proline metabolism	map00330
6-Methoxymellein	dihydroisocoumarin, a phenolic compound	
Tyramine	Tyrosine metabolism	map00350
Piperidine	Tropane, piperidine and pyridine alkaloid biosynthesis	map00960
Lycodine	Alkaloid	
Tetrahydrocorticosterone	Steroid hormone biosynthesis	map00140
Prenol	long-chain isoprenoid alcohols	
12-OPDA	alpha-Linolenic acid metabolism	map00592
Phenylethyl alcohol	Phenylalanine metabolism	map00360
Methyleugenol	Phenylpropanoid biosynthesis	map00940
4,5-Methylenedioxy-6-hydroxyaurone	Flavonoids	
Gambogic acid	Gambogic acid has inhibited the growth of a broad range of tumour cells	
7,8-Dihydroxycoumarin	Coumarins	
2,5-Dioxopentanoate	Arginine and proline metabolism	map00330
Dasytrichone	Flavonoids	
1,6-Naphthalenedisulfonic acid	Naphthalene degradation	map00626
2-Aminomuconate	Tryptophan metabolism	map00380

Table 23: Classification of metabolites based on their function in transgenics compared to wild type under control condition

Amino acids	Plant defense	Antioxidant	Cell Turgour	Antibiotics	Wax synthesis
L-Asparagine	Jasmone	Hercynine	12-OPDA	5"-phosphoribostamycin	16-oxo-palmitate
	Thiolactomycin	2-hydroxysuccinamate		N-Acetylpuromycin	
	Terbinafine	Condurango glycoside C			

Table 24: Classification of metabolites based on their function in transgenics compared to wild type under stress condition

Amino acids	Antioxidant	Osmolytes and cell turgour	Secondary metabolites	Phytoalexin	Carbon metabolism	Alkaloids
L-Proline	L-Proline	L-Proline	2-(3'-Methylthio) propylmalic acid	Danielone	5,10-Methenyltetrahydrofolate	Piperidine
	Linoleate	12-OPDA	Linoleate	6-Methoxymellin		Lycodine
	4-Aminobutyraldehyde		Oblongolide			Tetrahydrocorticosterone
	Deacetoxycephalosporin C		12-OPDA			Prekinamycin
	5-O-Methyl-myo-inositol		Methyleugenol			Bacconarborine A
	7,8-Dihydroxy coumarin		4,5-Methylenedioxy-6-hydroxyaurone			
	2,5-Dioxypentanoate		Dasytrichone			
			Fagaronine			

transgenics. Osmoprotectants like 12-OPDA and L-proline are involved in maintenance of cell turgour and osmotic homeostasis were up regulated in transgenics and 2 of the amino acids L-Proline and L-Asparagine were up regulated in transgenics.

Some of the important citric acid cycle intermediates were examined. Such as, Oxalosuccinic acid and Oxalomalic acid the abundance of these metabolites were high in case of transgenics compared to wild type. Magnesium-protoporphyrin IX 13-monomethyl ester metabolite exclusively induced in transgenic which involved in chlorophyll biosynthesis there by imparting a stay green phenotype in transgenics.

4.8.4 Integration of transcriptomics, proteomics and metabolomics in groundnut transgenic line

In this study transcriptome, proteome and metabolome information was developed in transgenics and an attempt was made to identify differentially expressed transcripts, proteins and metabolites. A comprehensive information was possible to generate from transcriptome data, however with regards to the proteomics, limitations in protein annotations resulted only 10 -20% % of proteins. Similarly the information that could be retrieved from metabolome data was also inadequate because lack of metabolomics information in plant species. However, attempt was made to correlate transcriptional and proteomic changes that are associated with specific pathways/processes. From the omics studies in transgenics and wild type plants under stress and non-stress conditions, it is inferred that, in transcriptomics more number of genes were differentially expressed compared to proteome data. Whereas in metabolomics only few number of metabolites were identified (Table 25). Generally it was assumed that up regulation of a gene also improve higher levels of protein and subsequently contribute for higher metabolome content, but this is not always true.

The omics data assessed to see whether transcriptome, proteome and metabolome correlate with any of the metabolic pathways. Five important pathways were identified such as proline biosynthesis, chlorophyll biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis pathway, alanine, aspartate and glutamate metabolism and citric acid cycle.

Table 25: List of up regulated and down regulated transcripts, proteins and metabolites from transcriptomics, proteomics and metabolomics data

	Transcriptomics		Proteomics		Metabolomics	
	Up regulated	Down regulated	Up regulated	Down regulated	Constitutive	Induced
TC v/s WC	625	531	323	42	34	57
TS v/s WS	1452	1384	330	73	54	141

Discussion

V. DISCUSSION

Groundnut is largely cultivated as a rain-fed crop and hence the crop is exposed to intermittent moisture stress. Intermittent moisture stress is a typical stress the crop faces in rain-fed agriculture scenario. Therefore there is a need to avoid dehydration stress by maintaining superior water relations to sustain the growth under moisture stress conditions. The plant have evolved several mechanisms to maintain water relations such as better water mining by superior root architecture, water use efficiency (WUE) and water conservation traits (Bartels and Sunkar, 2005). Apart from maintaining plant water relation the survival and recovery is also important when stress is severe and during early stress period because root system will not support the water mining from deeper soil layer.

In this context, plants have evolved several adaptive mechanisms and among them improving water relations and cellular level tolerance (CLT) are considered to be important to improve adaptation under moisture stress conditions. Among several CLT mechanisms protein synthesis, protection and turnover are crucial which normally get affected under stress condition (Patel *et al.*, 2002). So our major focus is to improve both water mining and CLT in transgenics by over-expressing PgHSF4, PDH45 and Alfin1.

It is important to maintain plant water relations to sustain the growth under moisture stress. The plant mechanisms associated with traits such as better water mining by superior root architecture, water use efficiency (WUE) and water conservation traits have relevance in avoiding drought stress (Bartels and Sunkar, 2005). Many studies have shown that genotypes with superior root system maintained the leaf turgor and cooler leaf temperature for longer periods under continuous soil water-depleted conditions (Mohammadi *et al.*, 2012; Tuberosa, 2012). Several regulatory genes to improve root system architecture and their role in developing root associated traits have been well studied and transgenics have been developed. CAP2 (Shukla *et al.*, 2006), OsRAA (Ge *et al.*, 2004), Alfin1 a zinc finger transcription factor (Winicov and Bastola, 1997), several ABA mutants, several auxin biosynthesis or responsive genes have been reported to have regulatory role in root development. The regulatory networks involved in root

growth and patterning (architecture) were elucidated in *Arabidopsis* by global gene expression studies (Jung and McCouch, 2013). The regulatory genes such as aberrant lateral root formation-4 (ALF4), SOLITARY-ROOT (SLR) and NAC1 (NAM, ATAF1, 2, CUC2) have been shown to play a role in regulating root growth and development (DiDonato *et al.*, 2004; Fukaki *et al.*, 2002; Xie *et al.*, 2002). Our studies also demonstrated improved root growth and salinity tolerance in Alfin1 overexpressing tobacco plants (Nethra, 2010).

When tissue water status decreases under prolonged soil water deficit condition, cellular tolerance mechanisms help in maintaining the cell metabolic activities leading to the adaptation of plants to water deficit condition (Bandurska, 1998). Cellular tolerance mechanisms during water deficit conditions are many and mainly include osmotic adjustment (Laszlo and Arnould, 2009), cell cycle control, protein chaperoning (Kovacs *et al.*, 2008) and repair, removal of damaged proteins, production of scavenging enzymes and chromatin stabilization (Blum, 2005; Klutz, 2005). Among several CLT mechanisms protein synthesis, protection and turnover are crucial which normally get affected under stress condition (Patel *et al.*, 2002). When proteins are synthesized, several proteins misfolded and finally degraded resulting in reduced protein turnover (Grover *et al.*, 2013).

A few of the genes that regulate these processes have been shown to improve stress tolerance in plants. In this regard, RNA helicases play an important role in translation process by unwinding the energetically stable mRNA secondary structure. Several reports have shown that stress responsive RNA helicases improve stress adaptation (Patel *et al.*, 2002; Owttrim, 2006). Overexpression of a salt stress-responsive Pea DNA helicase (PDH45), which is a homologue of eukaryotic translation initiation factor eIF-4A, conferred salinity tolerance in tobacco plants (Sanan-Mishra *et al.*, 2005). Several heat-shock protein (HSP) chaperones have been reported to increase protein stability under stress (Liu *et al.*, 2013) and improve adaptation of cells with reduced turgor (Liu *et al.*, 2013). Stress-induced transcription factors such as heat-shock factors (HSFs) play a critical role in activating HSPs (Mittal *et al.*, 2011; Swindell *et al.*, 2007). Several reports clearly demonstrated improved tolerance of transgenic plants to diverse

abiotic stress by overexpressing several HSFs (Zhu *et al.*, 2006). Overexpression of AtHSFA1 gene enhanced thermo-tolerance in Arabidopsis, soya bean and tomato (Zhu *et al.*, 2006). Expression analysis of *Pennisetum glaucum* heat-shock factor (*PgHSF4*) in *P. glaucum* showed maximum increase in transcript level in response to heat stress within 30 min of exposure (Reddy *et al.*, 2009).

As stress tolerance of crops can be improved by bringing together diverse adaptive traits/mechanisms. The aim of this study is to improve the adaptation of groundnut to drought stress by altering cellular tolerance mechanisms and root traits. The cellular tolerance mechanisms and higher root growth traits significantly contribute to improve the intrinsic tolerance of plant cell and increase the water uptake and mining abilities to sustain growth and development under drought stress. Therefore the goal is to improve both water relations and intrinsic cellular tolerance mechanisms by co-expressing PDH45, HSF4 and Alfin1.

The simultaneous expression of relevant genes in a single locus necessitates the development of multigene cassette. Earlier in our department we developed a modified vector system to engineer and stack multiple genes into plants to express two to three genes simultaneously (Vemanna *et al.*, 2013). Using this strategy, the simultaneous expression of *AtWRKY28* and *AtbHLH17* transcription factors substantially improved stress tolerance in Arabidopsis under different abiotic stress conditions (Babitha *et al.*, 2013).

Earlier in our department the multigene expressing binary vector *pKM12GW* harbouring three genes driven by individual promoter and terminator, *RD29A:PgHSF4:nos*, *2X35SCaMV:PDH45:polyA* and *35SCaMV: OsAlfin1:polyA*, with kanamycin selection marker was developed using modified Multisite Gateway technology (Fig 5). The embryonal axes of groundnut seeds were used to transform the multigene construct using *Agrobacterium* mediated *in planta* transformation method. The *in planta* mediated transformation procedure can be applied to all genotypes and cultivars of crops which are susceptible to *Agrobacterium tumefaciens* infection, although the efficiency might vary between them.

The putative groundnut transformants were identified in T₁ generation initially by screening the seedlings on kanamycin. Many of these kanamycin-positive plants showed the amplification of marker gene neomycin phosphotransferase (nptII) indicating the integration of T-DNA into the transformed plants. Based on the phenotype, kanamycin response and molecular characterization, 102 plants were further advanced to T₂ generation. In T₂ generation, 20 seedlings from each line were rigorously screened for kanamycin sensitivity (Annexure IV). The lines that showed more than 90% of survived seedlings on kanamycin were considered for further advancement. The distinctly kanamycin-tolerant positive plants were established in pots and advanced to T₃ generation after confirming nptII amplification. Based on this analysis and phenotypic uniformity, 99 lines were advanced to T₃ generation. In T₃ generation 99 groundnut transgenic lines subjected to gradual drought stress showed significant variability in CCATD and also many of the transgenics showed more pod yield and biomass compared to wild type plants. The biomass was consistently higher in transgenic lines and showed a positive correlation with pod yield. Based on yield response, TDM and canopy temperature 30 transgenic lines were selected for further characterization.

The main objective of present study is to extensive characterization of selected 30 transgenic lines. The goal is to identify promising transgenic events with improved tolerance and productivity. As discussed earlier the major emphasis is to improve plant water relations by enhancing root characteristics and improve the drought tolerance by improving genetic enhancement of specific mechanisms associated with cellular level tolerance. One such widely accepted mechanism is sustaining overall protein turnover by enhancing protein stability and improving processes related to translation. The selection and choice of genes in transgenics have shown to improve root growth (Alfin1), protein stability (HSF4) and translation (PDH45).

While characterizing the transgenics in T₄, T₅ and T₆ the major emphasis has been to extensively characterize,

1. Improvement in root growth
2. Tolerance under different abiotic stresses
3. Productivity under both stress and non-stress conditions

Root characteristics in transgenics

Several studies have shown that under field conditions, especially with limited soil moisture status, root characters determine the crop canopy temperatures and facilitate the maintenance of higher transpiration rate (Pinheiro *et al.*, 2005; Balota *et al.*, 2012). Root system architecture had directly affected biomass accumulation and historical yield trends (Ooka *et al.*, 2003). Under drought conditions, drought-resistant plants tend to develop a deeper root system, resulting in roots that can reach into still-moist deeper soil layers (Rich and Watt, 2013). Thus, deeper rooting is thought to be a key factor in enhanced drought avoidance by crops (Fukai and Cooper 1995, Kirkegaard and Lilley, 2007, Manschadi *et al.*, 2006). Therefore, improving root system architectures to increase rooting in deep soil layers would be an effective strategy to avoid the negative impacts of drought on yield (De Dorlodot *et al.*, 2007).

Keeping in view the importance of root characteristics in our study are quantified in transgenic plants grown under control conditions and also in plants which are subjected to different abiotic stresses like moisture stress and salinity stress. Significant improvement in root growth was seen in groundnut transgenic lines at all stages of plant growth.

Root growth at control condition: The root growth of both wild type and transgenics were studied in different experiments at different stages in both T₄ and T₅ generations. The transgenic plants showed relatively more root length in all the experiments under control condition (Fig. 8, 9 and 18).

Root growth under stress: The root length of transgenic lines was more even under stress condition when compared to wild type plant. In T₄ generation salinity stress experiment at both seedling stage and salinity stress in hydroponics system was studied and in T₅ generation salinity stress experiment at seedling stage and moisture stress experiment by gravimetry was studied. In both stresses at all stages the root length of transgenic lines was higher when compared to wild type.

One of our earlier study in groundnut showed that when transgenic lines subjected to gradual drought stress there was significant variability in CCATD. Lower crop canopy temperature and larger negative CCATD values in transgenic lines reflect high transpiration rate and hence higher water mining abilities of these plants. In transgenic lines with cooler canopy temperatures, leaf RWC values were high and proline levels were low indicating the maintenance of high leaf water status (Vemmana *et al.*, 2015). The negative CCATD and improved water relations in the transgenic lines could be mainly due to improved root characters. Under field conditions, especially with limited soil moisture status, root characters determine the crop canopy temperature (Balota *et al.*, 2012; Pinheiro *et al.*, 2005).

The improved root growth in multi-gene groundnut transgenic plants could be due to the expression of *Alfin1*. The *Alfin1* gene has been shown to improve root growth in alfalfa and tobacco transgenic plants (Nethra, 2010; Winicov, 2000). The *Alfin1* silencing plants showed less root growth than *Alfin1* overexpression plants (Winicov and Bastola, 1999). *Alfin1* cis-binding elements were found in promoter region of nitrate transporter 2.1 (NRT2.1) and nitrate-responsive promoters (NRP) (Udvardi *et al.*, 2007) that are involved in plant growth and nitrogen metabolism (Orsel *et al.*, 2004). *Alfin6* transcripts were induced under phosphate deficiency conditions and shown to play a role in root hair elongation. The multigene expressing groundnut transgenic plants showed the enhanced expression of Proline-rich protein (PRP2) in roots under stress (Plate 4b), which has been shown to be a downstream target of *Alfin1* transcription factor (Winicov, 2000). PRPs are cell wall proteins with potential as an anchor to the membrane, and they function as extensions that enable support and extension of cell wall structure. Increased utilization of cell wall proteins would be consistent with increased root growth (Deutch and Winicov, 1995). In *Arabidopsis*, *AtPRP1* and *AtPRP3* were highly expressed in roots and contributing to cell wall structure in root hair bearing epidermal cells (Bernhardt and Tierney, 2000).

In transcriptome data we assessed the probable targets of root associated genes. Some of the root related genes are up regulated in transgenics such as Expansin B1, Auxin binding protein, Early nodulin 93, SAUR- like auxin-responsive family protein,

Ankyrin repeat protein, Armadillo repeat family protein, Auxin efflux carrier, Root hair defective 3 homolog and Nodule-specific glycine rich peptide. Most of the proteins like Ankyrin repeat, Auxin binding protein, SAUR-like auxin-responsive protein and Armadillo repeat proteins are involved in lateral root initiation and development. Expansin B1 involved in root hair elongation and Early nodulin 93, Nodule-specific glycine rich peptide involved in root nodule formation.

In proteomics study also few of the proteins related to roots were up regulated in transgenics such as Hydroxy proline rich glyco protein family protein which is involved in root cell elongation, Isocitrate dehydrogenase present in root nodules and protect against nitro-oxidative stress and syntaxin protein which is involved in root tip growth.

Abiotic stress tolerance of transgenics

Salinity stress response of transgenics: In T₄ and T₅ generation salinity stress experiment at seedling stage was studied. The transgenics showed more tolerance compared to wild type plant. The root length of transgenic lines was more in salinity stress condition when compared to wild type (Fig. 8a, b & 18a, b). The percent reduction in root length over control is less in transgenics when compared to wild type (Fig. 8c & 19a). The consolidated and pooled data of percent reduction in root length over control is presented in Fig. 66.

The transgenic lines and wild type plants were subjected to salinity stress in hydroponics system. The transgenic lines showed more root length and biomass when compared to wild type plant (Fig. 9). The percent reduction in root length and biomass over control is less in transgenics when compared to wild type plant (Fig. 10).

Salinity stress response at whole plant level in two of the promising transgenic lines showed high RWC, root length and biomass when compared to wild type. The membrane damage due to salinity stress was more in case of wild type plant when compared to transgenic plant (Fig. 44).

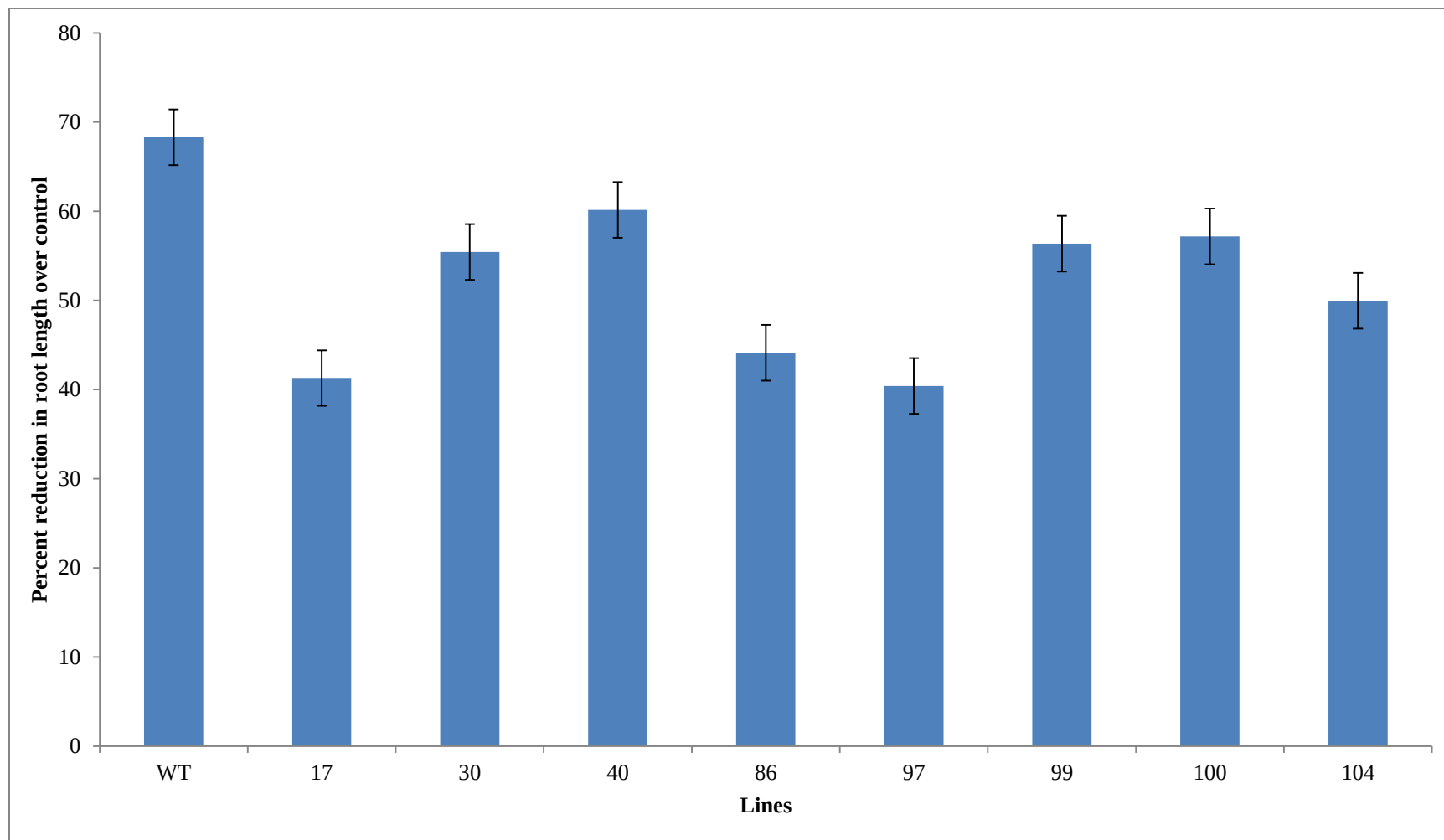


Fig. 66: Mean percent reduction in root length over control in wild type and transgenic lines. Wild type and transgenic lines were subjected to salinity stress in T₄ and T₅ generations and mean percent reduction in root length was calculated for promising lines

The two of the promising transgenic lines along with wild type plant studied for DTT induced ER stress, the transgenic lines showed tolerance to DTT when compared to wild type plant (Fig. 45).

Moisture stress response of transgenics: The moisture stress response of transgenic lines and wild type plant was assessed by gradually imposing moisture stress through gravimetrically. In each pot, one plant each from transgenic event and wild type plants was inter-planted to assess the relative performance under similar soil moisture status. The transgenic plants showed better recovery when compared to wild type plant (Fig. 20d & e). The transgenic plants showed an average of 32 cm root length whereas in wild type the root length was 24.67 cm under stress condition and biomass was showed an average of 3.9 g in case of transgenic plants and in wild type it was around 2.5 g (Fig. 21). The percent reduction in root length and biomass over control is less in transgenics when compared to wild type plant (Fig.22) Further, extent of cytotoxic compound MDA, MG and accumulation of total buffer soluble protein content under moisture stress was assessed the transgenic plants showed lesser accumulation of MDA and MG with more protein content. The transgenic plants showed on an average of $55.98 \mu\text{M.g}^{-1}$ FW whereas wild type showed around $78 \mu\text{M.g}^{-1}$ FW under stress condition. Similarly, the MG content in transgenic plants was $87. \mu\text{M.g}^{-1}$ FW and in wild type the MG content was $137.53 \mu\text{M.g}^{-1}$ FW under stress condition (Fig. 23). The percent increase in MDA and MG over control was more in case of wild type when compared to transgenic lines (Fig. 24). The transgenic plants showed improved tolerance to drought stress with minimal MDA and MG accumulation and increased protein content.

The improved cellular tolerance could be due to the expression of regulatory genes, *HSF4* and *PDH45*. The scientific strategy was to improve the protein turnover and its stability under stress by simultaneous expression of these diverse genes which are involved in the translation and protein stability. There are several studies which supports our results that constitutive expression results in enhanced expression of transgene and also target genes under stress such as overexpression of *HsfA1* in *Arabidopsis* showed enhanced expression of *HsfA1* and also downstream target gene i.e. HSPs under heat, low pH and oxidative stress (Qian *et al.*, 2014). Overexpression of *AtHSFA1* in tomato

provided tolerance to heat stress, and expression of HSF4 in tobacco provided tolerance to salinity, oxidative and high temperature stress (Al-Whaibi, 2010; Nishizawa-Yokoi *et al.*, 2011). HSF4 transcription factor regulates diverse HSPs which act as molecular chaperones and play a crucial role in protein trafficking, folding, refolding and repair mechanisms under high temperature tolerance as well as other stresses (Wang *et al.*, 2004). Under stress conditions, mRNA processing and translation is affected predominantly due to secondary structure, thus leads to decreasing protein synthesis. From this context, RNA helicases are relevant in improving protein turnover, and hence, groundnut transgenic plants expressing *PDH45* showed improved tolerance under stress conditions. The RNA helicases function as molecular motors that rearrange RNA secondary structures which function in RNA maturation or RNA proofreading (Owtrim, 2006). Apart from these there are also evidences showing overexpression of RNA helicase and HSFs resulted in tolerance to diverse stresses. Overexpression of *PDH45* showed tolerance to salinity stress (Sanan-Mishra *et al.*, 2005), *GmRH* to low temperature and salinity stress (Eunsook *et al.*, 2009), *HVD1* to salinity and drought stress (Nakamura *et al.*, 2004), *OsHSF7* to heat and drought stress (Liu *et al.*, 2009). Overexpression of *PDH45* in tobacco and rice conferred tolerance to salinity stress by maintaining RNA stability, processing, transcription and translation initiation (Amin *et al.*, 2012; Sanan-Mishra *et al.*, 2005). In our earlier studies, groundnut transgenic plants overexpressing *PDH45* showed higher water mining and WUE resulting in improved drought tolerance (Manjulatha *et al.*, 2014).

Transgenics showed improved expression of stress responsive genes: The observed improved tolerance in transgenics over wild type could be due to enhanced protein turnover and stability which indirectly might have improved the expression of relevant genes involved in stress adaptation. So one of the objectives was to observe the expression of known stress responsive genes, the altered expression of these genes could be due to direct and indirect effect of the transgenes. The expression pattern of stress responsive genes was studied in transgenics and wild type under moisture stress. The expression of stress responsive genes has increased in the transgenics. The groundnut transgenic plants showed increased transcript levels of *HSP17.9* and *HSP60* under

drought stress conditions (Plate 4b), suggesting improved macromolecule protection in transgenic plants than wild-type plants. Further, enhanced levels of a few stress-responsive genes such as *RBX1*, *aldose reductase (ALR)*, *LEA5*, *P5Cs* and *alcohol dehydrogenase-1 (ADH1)* were observed in transgenic plants under stress conditions (Plate 4b). One of the study showed that the overexpression of *AhRBX1* (having a role in removing unfolded proteins) in tobacco improved tolerance to moisture stress, NaCl and oxidative stress (Lakshmi *et al.*, 2010). Overexpression of *aldose reductase* from *Medicago sativa* in tobacco improved tolerance to different abiotic stresses including temperature and heavy metals (Hegedus *et al.*, 2004). Similarly, *ADH1* from *Lotus japonicas* in yeast provided tolerance to abiotic stresses (Zeng *et al.*, 2011).

Due to over-expression of HSF4 in transgenic lines some of the HSPs were up regulated in transgenic plants. In transcriptome and proteome data some of the genes/proteins which involved in maintaining protein stability such as 17.5 kDa class I heat shock protein, Heat shock transcription factor A3, cytosolic class I small heat shock protein, calmodulin binding heat shock protein, HSP 70, Heat shock 22 kDa protein, HSP 20 and Dna J class molecular chaperone were up regulated in transgenics compared wild type under stress condition. This data clearly signifying that these proteins under stress conditions protect the proteins and maintain the stability.

The transgenic lines showed enhanced tolerance to abiotic stress with improved growth when compared to wild type plants. Three possible reasons could be speculated for the increased plant growth and higher tolerance in transgenic plants is

- 1) Due to enhanced protein synthesis and protection leading to better protein turnover.
- 2) Lesser accumulation of a cytotoxic compound MDA and MG which indirectly reflect the lesser oxidative damage to cell.
- 3) Due to enhanced expression of few stress responsive genes involved in stress.

These results suggest that, groundnut transgenics co-expressing pgHSF4, PDH45 and Alfin1 showed improved tolerance to abiotic stresses.

Growth and productivity:

The promising transgenic lines should have higher tolerance to diverse abiotic stresses besides being productive. Therefore, the objective was to identify lines which are tolerant and also productive. In view of this in this study the relationship between tolerance levels under salinity conditions and productivity of these lines in pot grown plants. The relationship between biomass, pod yield of pot grown experiment and biomass of salinity experiment was positive but not significant (Fig. 16b & c).

Since, the objective is to identify transgenic lines with higher tolerance and productivity; we did a Z-distribution analysis between pot grown experiment and salinity stress (hydroponics) experiment. The lines in third quadrant showed more biomass in both pot grown and salinity stress experiment (Fig. 17a) and also showed more pod yield in pot grown experiment and stress tolerance for salinity stress (Fig. 17b). Based on this analysis we selected 17 superior lines and advanced to T₅ generation.

The selected lines were extensively characterized for productivity under both stress and non-stress condition under contained field conditions in MDE. MDE is a facility where plants are grown in controlled field conditions at a given amount of precipitation. When the plants were 50-day-old, one of the plot was subjected to moisture stress by withholding irrigation were watered once in every week. The objective of this experiment is to identify transgenic lines with higher productivity under control and stress condition. The mean yield of transgenics was of 35.3 g.plant⁻¹ and 29.3 g.plant⁻¹ under control and stress condition respectively whereas in wild type mean pod yield was 25.92 g.plant⁻¹ under control condition whereas under stress condition 16.67 g.plant⁻¹ (Table 7). The percent reduction in pod yield over control was less in case of transgenics (16.91%) compared to wild type (35.68%). There was a significant correlation between pod yield and biomass under control and stress condition (Fig. 30).

Based on these results 10 promising lines with improved productivity both under stress and non-stress conditions were selected and characterized further.

Response of selected promising lines to moisture stress conditions:

The promising ten lines which were showing more biomass and pod yield in both stress and non-stress conditions in MDE experiment were selected for further study and sown in contained field condition till harvest in summer season. Two plots are maintained and irrigated every alternate day; one plot was subjected to moisture stress after 50 DAS for 20 days and then maintained till harvest. The mean pod yield of transgenics was 49.82 g.plant⁻¹ and 36.2 g.plant⁻¹ under control and stress condition respectively. Whereas in wild type 32.67 g.plant⁻¹ and 22 g.plant⁻¹ under control and stress conditions (Table 9). The biomass was also more in case of transgenics when compared to wild type plant. The mean biomass of transgenic plants was 113.13 g.plant⁻¹ under control condition whereas under stress condition it was 77.02 g.plant⁻¹ and in case of wild type the biomass was 79.34 g.plant⁻¹ under control condition and 52.67 g.plant⁻¹ under stress condition (Table 9). The percent reduction in pod yield over control was less in case of transgenics compared to wild type plant.

Substantially the transgenic lines have higher leaf area, number of leaves and LAI there by resulting in improved biomass compared to wild type (Fig. 36). There was a marginal variation in NAR and DM/LA ratio between wild type and transgenics under both non-stress and stress condition but variation exists in CGR between wild type and transgenics under both conditions (Table 8) it may be due to the increase in leaf area, LAI, more number of leaves and leaf expansion. There by resulting in improved productivity in transgenic lines compared to wild type plant.

Several studies showed that CGR strongly contributes to total biomass accumulation of crop plants. Some of the studies had shown the importance of CGR in determining the biomass accumulation (Subramanian, 1980; Chinnavenkata Reddy, 1976; Shankar, 1988). With increase in leaf area, the extent to light interception increases and hence, a positive significant relationship exists between leaf area and total biomass (Vasanth kumar, 1975; Chandrasekhar, 1978; Gurumurthy, 1982; Shankar, 1988; Ankegowda, 1996).

Mean productivity: The mean pod yield of both experiments was calculated and presented in Table 10. The mean percent reduction in pod yield over control in selected 8 promising transgenic lines of both the experiment was less in case of transgenics when compared to wild type (Fig. 67). The transgenic lines showed mean percent reduction of 22.23 % whereas wild type showed mean percent reduction of 34.09 % in pod yield over control.

The increased productivity of these promising lines could be due to improved water relations and also due to cellular level tolerance.

Improved water mining and water relation in transgenic lines showed higher productivity under both stress and non-stress conditions. Several studies shown that vigorous growth of roots with an increase in length and thickness is correlated with drought tolerance and grain yield (Ekanayake *et al.*, 1989). Root-specific overexpression of *OsNAC10* showed improved root growth, enhancing drought tolerance of transgenic plants, which increases grain yield significantly under field drought conditions (Jin *et al.*, 2010).

Adaptation to moisture stress under field conditions can be achieved by improving CLT mechanisms besides improving traits associated with water relations. Among several cellular tolerance mechanisms, the protein turnover and its stability are crucial for sustaining the cellular metabolism under stress. Since, the protein synthesis and stability gets affected under stress, the major focus in this study was to characterize the relevance and significance of RNA helicase, *PDH45* and heat shock transcription factor (*HSF4*) which play a crucial role in removing mRNA secondary structure thereby hastens the translation process and also protect the proteins from degradation.

Overall, this study provides evidence that the simultaneous expression of *OsAlfin1*, *PDH45* and *PgHSF4* genes regulating diverse adaptive mechanisms improves drought tolerance and productivity in groundnut. Maintenance of positive turgor by improved root growth substantially contributed for increased leaf size and positive carbon gain. Transgenic plants also showed improved cellular level tolerance as evidenced by

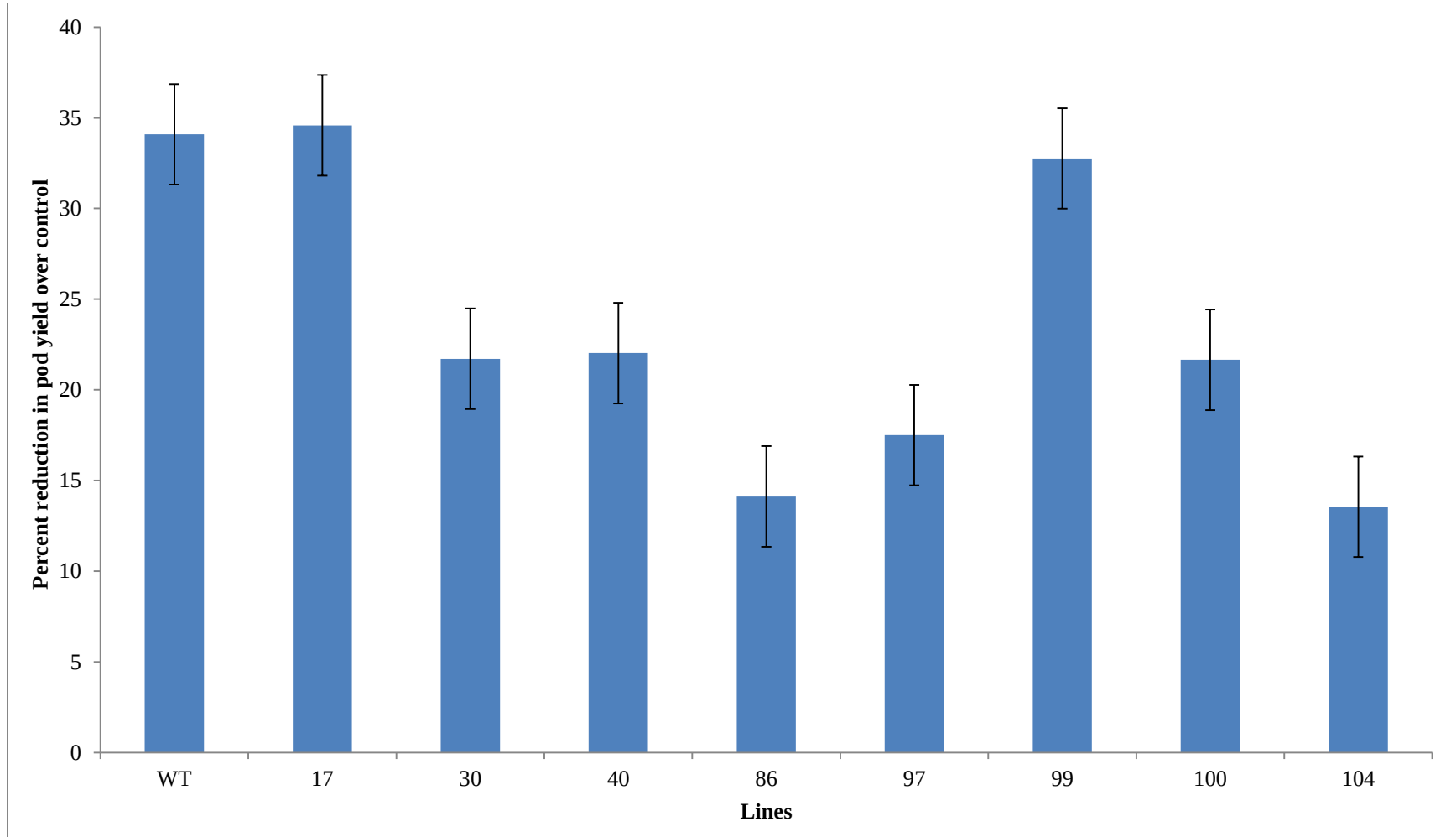


Fig. 67: Mean percent reduction in pod yield over control. The mean pod yield of MDE experiment and summer season experiment was calculated for few promising lines and percent reduction over control was calculated in both transgenics and wild type plant.

tolerance to drought stress through up-regulation of many stress-responsive genes. Thus, provided evidence that improved adaptation to moisture stress can be achieved by pyramiding traits associated with water mining and cellular-level tolerance mechanisms. Finally, our results confirm that transgenic technology is a potential approach to pyramid specific traits by the simultaneous expression of multiple genes.

Molecular analysis: The integration of all the three genes along with marker gene npt II was analyzed in selected transgenic lines in both T4 and T5 generation. The transgenic lines showed amplification of npt II (436bp), PDH45 (200bp), Alfin1 (200bp) and HSF4 (500bp) (Plate 1, 2 and 3). The stable integration of transgenes was assessed by genomic Southern blot analysis in three transgenic lines that showed superior survival at the end of stress. In three transgenic plants i.e. 86, 97 and 100 demonstrated that the transgenes were stably integrated into the genome of transgenic groundnut lines with single copy of the transgene in selected three transgenic lines (Plate 4a). For remaining transgenic lines the southern analysis is in progress. The expression analysis of few stress responsive genes was studied in two transgenic lines along with wild type under stress conditions. Most of the genes (heat-shock proteins HSP17.9 and HSP60, aldehyde dehydrogenase (ADH1), Really interesting gene box-1 (RBX1) and Aldose reductase) showed enhanced expression in transgenic plants than wild type under stress except for the transcript levels of ADH1 which was less in the transgenic line M97. The transcript induction of pyrroline-5-carboxylate synthetase (P5CS) was less than other genes, but the transgenic plants still showed higher levels than wild type. However, late embryogenesis abundant-5 (LEA5) gene showed similar expression levels in wild type and transgenic plants even after exposure to stress. Further, the root growth-associated proline-rich protein2 (PRP2) showed significant up-regulation of transcripts in the transgenic plants (Plate 4b).

Omics approach: To study the mechanisms involved in improved yield and drought tolerance in transgenic lines

The emphasis in the study is to improve protein turnover, stability to maintain normal cellular metabolic processes under stress. The groundnut transgenic plants were developed by co-expressing the genes associated with root growth, protein synthesis

mechanisms and protein stability process. The transgenics showed improved drought tolerance and productivity under stress conditions. To understand the mechanisms involved in improved yield and drought tolerance in groundnut transgenic plants, transcriptome, proteomics and metabolomics was studied. Groundnut wild type and transgenic plants were subjected to moisture stress through gravimetric approach and then samples were collected from both wild type and transgenic plants under stress and control conditions.

Transcriptomics: To understand the mechanisms involved in drought tolerance in groundnut transgenic plants, transcriptome analysis was carried out by RNA-seq analysis. Several differentially expressed genes (DEG) were identified in transgenics compared to wild type under control and stress conditions. In transgenics several metabolic processes were up regulated such as folic acid biosynthesis pathway, transferases activity, chaperone activity, aromatic amino acid synthesis and deaminase activity.

Folic acid known to enables plants to regulate their DNA functions. This acid basically helps a plant to produce more nucleic acid (DNA& RNA) and involved in protein synthesis mechanisms. Folic acid additionally enables plants to produce RNA, a nucleic acid that carries information from DNA to plant cell structures known as ribosomes and helps a plant synthesize protein. This could be another reason that transgenics may have higher protein synthesis due to overexpression of protein turnover associated genes.

In transgenics the methyl transferases showed upregulated that utilize different substrates in methyl transfer reactions. They include protein methyltransferases, DNA/RNA methyltransferases, natural product methyltransferases, and non-SAM dependent methyltransferases. The methylation at DNA level bring in epigenetic modifications, affects transcription, gene stability. It directly impacts chromatin structure and can modulate gene transcription, or even completely silence or activate genes, without mutation to the gene itself (Viswanathan and Jian-kang, 2009). These data clearly shows that transgenics may adapt better for enviornmental changes for improved adaptations.

The aromatic amino acid biosynthetic process, and prephenate pathway associated genes were also up regulated in transgenics. This signifies that the metabolic processes are better managed in transgenic plants compared to wild type plants. It is known that, to improve the adaptation of crops these pathways have been targeted. In case of development of abiotic resistance crops many groups target these biosynthetic processes signifying the importance of this pathway in sustaining growth and development under stress conditions (Iqbal *et al.*, 2015; Chen *et al.*, 2014).

Few of the genes up regulated in transgenics having chaperone binding activity suggesting that heat shock proteins are up regulated and protecting the proteins under stress signifying the overexpression of HSF4 transcription factor (Baniwal *et al.*, 2007).

Some of the target genes are up regulated in transgenics with more than 2 fold change when compared to wild type plant. Some of the HSPs such as 17.5 kDa class I heat shock protein, Heat shock transcription factor A3, cytosolic class I small heat shock protein, calmodulin binding heat shock protein, HSP 70, Heat shock 22 kDa protein, HSP 20 and Dna J class molecular chaperone were up regulated in transgenics. Several studies showed that over-expression of HSPs and HSF resulting in improved stress tolerance in many crops. Enhanced thermo-tolerance in *Arabidopsis* could be achieved by constitutive expression of *AtHSF41* in transgenic plants leading to constitutive expression of certain HSPs (Lee *et al.*, 1995). Similar observation was obtained by the constitutive overexpression of HSF A3 in *Arabidopsis* (Prandl *et al.*, 1998). However, HsfB1 was demonstrated to function as a novel co-regulator of the tomato HsfA1 or HsfA2 enhancing the transcriptional activity (Bharti *et al.*, 2000, 2004).

Similarly several root associated genes were up regulated in case of transgenics compared to wild type. Ankyrin repeats, Armadillo repeats, Auxin response factor, Auxin efflux carrier were involved in lateral root initiation and development. Expansin A1 and Xyloglucan glycosyltransferase involved in root hair elongation. Hydroxy-proline rich glycoprotein involved in root cell elongation. Early nodule 93 and Nodule specific glycine rich peptide involved in root nodule development. Hydroxy-proline rich glycoprotein appear to be not solubilized in cell walls as a response to the stress

(Showalter, 1993; Fowler *et al.*, 1999). They are expressed during wounding and pathogen attack in some tissues and implicated in different stages of plant growth and development, nodule formation, fertilization, cytokinesis, apoptosis, senescence, and cell wall lignification (Ye *et al.*, 1991; Showalter, 1993; Nothnagel, 1997; Wu *et al.*, 2001; Hall and Cannon, 2002; Lamport *et al.*, 2006).

Cellular level tolerance can be achieved by maintaining cell cycle regulation, effective scavenging of ROS, osmotic adjustment, osmoprotection, membrane stability and by protein turnover and stability. In transgenics many of the proteins involved in cellular level tolerance were up regulated when compared to wild type plant. Many of the translation related proteins like ribosomal protein, DNA polymerases, RNA binding domain, helicases, DEAD box ATP dependent helicases, pre mRNA splicing factors, transcription initiation factor etc. were up regulated in transgenics under stress condition when compared to wild type. In sorghum, a salt-responsive transcript HVD1 (*Hordeum vulgare* DEAD-box protein), encoding a putative ATP dependent DEAD-box RNA helicase, is induced under salt and cold stress (Nakamura *et al.*, 2004). Two chilling and freezing stress-inducible DEAD-box helicase genes have been identified using Arabidopsis mutants that are impaired in the cold-regulated expression of CBF and their downstream target genes (Gong *et al.*, 2005).

The overall transcriptome data shows that overexpression of these three important key regulatory proteins brings in up regulation of many root associated genes, improved metabolic associated genes that are contributing for higher protein synthesis and cellular level tolerance mechanisms.

Proteomics: Similar responses were found from proteomics data. Some of the HSPs such as HSP20, HSP 81-2, Heat shock 70 kDa protein, cpn 60 chaperonin family protein, Gro-EL-like chaperone and Dna J class molecular chaperone were up regulated in transgenic under stress condition compared to wild type plants. Protein dysfunction is an inevitable consequence of a wide range of adverse environmental conditions and molecular chaperones like heat-shock proteins (HSPs) are responsible for protein stabilization, proper folding, assembly, and translocation (Wang *et al.*, 2004). Overexpression of

AtHSFA1 in tomato provided tolerance to heat stress, and expression of *HSF4* in tobacco provided tolerance to salinity, oxidative and high temperature stress (Al-Whaibi, 2010; Nishizawa-Yokoi *et al.*, 2011). Root related proteins such as Hydroxy proline rich glyco protein family protein which is involved in root cell elongation, Isocitrate dehydrogenase present in root nodules and protect against nitro-oxidative stress and syntaxin protein which is involved in root tip growth was found to be up regulated in transgenic plants.

Integration of omics

Similarly few metabolite data also supporting that many metabolites were upregulated in transgenic plants. The transcriptome data was correlated with proteome and metabolome data. The major emphasis of the study is to improve water relation and cellular level tolerance (Fig. 68). Several genes that are involved in water relation were identified in transgenics and it could be due to over-expression of *Alfin1* in the transgenic lines. Recent molecular evidences showed that *Alfin1* involved in epigenetic mediated regulation of gene expression and also have a role in improvement of root growth. Several root related genes and proteins are up regulated in transcriptome and proteomics data under stress condition in transgenic plant.

To improve cellular level tolerance mechanisms scavenging the cytotoxic compounds, maintaining membrane stability and protein synthesis and stability play an important role. From transcriptome studies 10 genes were identified that are involved in scavenging cytotoxic compounds. In proteomics also some of the proteins that are involved in scavenging of cytotoxic compound were up regulated with more than 1.5 fold change. Some of the genes/proteins like lactoylglutathione lyase, aldo-keto reductase, glutathione S-transferase and thioredoxin were common between transcriptomics and proteomics. In metabolomic study also identified few metabolites like proline, coumarate, linoleate and 4-aminobutyaldehyde were up regulated in transgenic plants. Linoleate showed 10.99 fold changes in transgenic line compared to wild type.

Another important mechanism in CLT is maintaining the membrane stability, from this context, plasmamembrane ATPase and transmembrane proteins commonly found in proteomics and transcriptomics data. L-Proline and 12-OPDA metabolites that

are involved in maintaining the cell turgour as well as act as osmolytes also up regulated in transgenic plant compared to wild type plants under stress condition. Protein synthesis and stability play an important role maintaining CLT mechanisms, many of the transcripts and proteins that are involved in protein synthesis and stability were up regulated in both transcriptomics and proteomics data.

The omics data assessed to see whether transcriptome, proteome and metabolome correlate with any of the metabolic pathways. Five important pathways are identified such as proline biosynthesis, chlorophyll biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis pathway, alanine, aspartate and glutamate metabolism and citric acid cycle. In proline biosynthesis pathway 2 genes at transcript levels and 2 proteins at proteome level were up regulated in transgenics that also resulted in higher levels of proline metabolite. Recent discoveries point out that proline plays an important role in plant growth and differentiation across life cycle. It is a key determinant of many cell wall proteins that plays important roles in plant development. The role of extensins, arabinogalactan proteins and hydroxyproline- and proline-rich proteins as important components of cell wall proteins that play pivotal roles in cell wall signal transduction cascades, plant development and stress tolerance

One of the protein hydroxylprolinerich-glyco protein (HRGPs) is induced at both transcript and proteome level. These proteins are ubiquitous components of the plant extracellular matrix and comprise up to ~10% (w/w) of the cell wall in dicots. They create covalently cross linked networks, generating negatively charged cushions at the membrane-wall interface, and functions such as barriers against pathogen ingress, adhesives for pollen capture, primers for wall polysaccharide biosynthesis, and regulators of Ca^{2+} signaling. Hydroxylation of the Pro residues and glycosylation occur post-translationally in the Golgi. The amino acid motifs are presumed to be important for secondary and tertiary structure, to which Ser-(Hyp)₄ contributes molecular rigidity and kinks (Ferris *et al.*, 2001), and the multiple Tyr residues are thought to allow both intramolecular and intermolecular isodityrosine cross-linking (Kieliszewski and Lamport, 1994). Four major classes of structural cell wall proteins have been described: HRGPs, Pro-rich proteins, Gly-rich proteins, and arabinogalactans (AGPs) (Showalter,

1993; Cassab, 1998). HRGPs appear to be not solubilized in cell walls as a response to the stress (Showalter, 1993; Fowler *et al.*, 1999). They are expressed during wounding and pathogen attack in some tissues and implicated in different stages of plant growth and development, nodule formation, fertilization, cytokinesis, apoptosis, senescence, and cell wall lignification (Ye *et al.*, 1991; Showalter, 1993, 2001; Nothnagel, 1997; Wu *et al.*, 2001; Hall and Cannon, 2002; Lamport *et al.*, 2006).

In chlorophyll biosynthesis pathway few genes like Protoporphyrinogen oxidase and cytochrome c induced at transcript level with fold change of 2.00 and 2.15 respectively and Magnesium-protoporphyrin IX monomethyl ester cyclase, Mg-protoporphyrin IX chelatase and Light-harvesting complex I, chlorophyll A/B-binding protein were up regulated at protein level at more than 1.5 fold change. Improving in transcripts and proteome levels of this pathway improved Magnesium-protoporphyrin IX 13-monomethyl ester and Divinylprotochlorophyllide at metabolome level. These data is highly correlating with the higher chlorophyll levels in transgenic plants even under stress and hence they maintain stay green phenotype. Stay green phenotype is one of the physiological trait that contributes for higher photosynthesis and productivity.

In ubiquinone and other terpenoid quinone biosynthesis pathway 2 genes, 2 proteins and 2 metabolites were up regulated in transgenics compared to wild type plant. In transcriptomics 4-coumarate Co A ligase (4CL) like protein and ubiquinone biosynthesis protein increased by 2.50 and 2.08 fold change respectively. Whereas in proteomics proteins like geranylgeranyl reductase and NADH ubiquinone oxidoreductase were up regulated in transgenics. The data indicates that primary carbon metabolic pathway is up regulated in transgenics. The flow of carbon from primary metabolism for the biosynthesis of an array of phenylpropanoid secondary products involves a minimum of three enzymatic steps, which are catalyzed by phenylalanine ammonia-lyase (PAL), cinnamic 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL: EC 6.2.1.12). These enzymes collectively form the general phenylpropanoid pathway to maintain a continuous metabolic flux for the biosynthesis of phenylpropanoids. 4CL is the last pivotal enzyme that catalyzes the production of 4-cinnamic acids or their derivatives into corresponding CoA esters to generate precursors that are needed for the formation of a

Integration of Omics in transgenic plant for stress tolerance

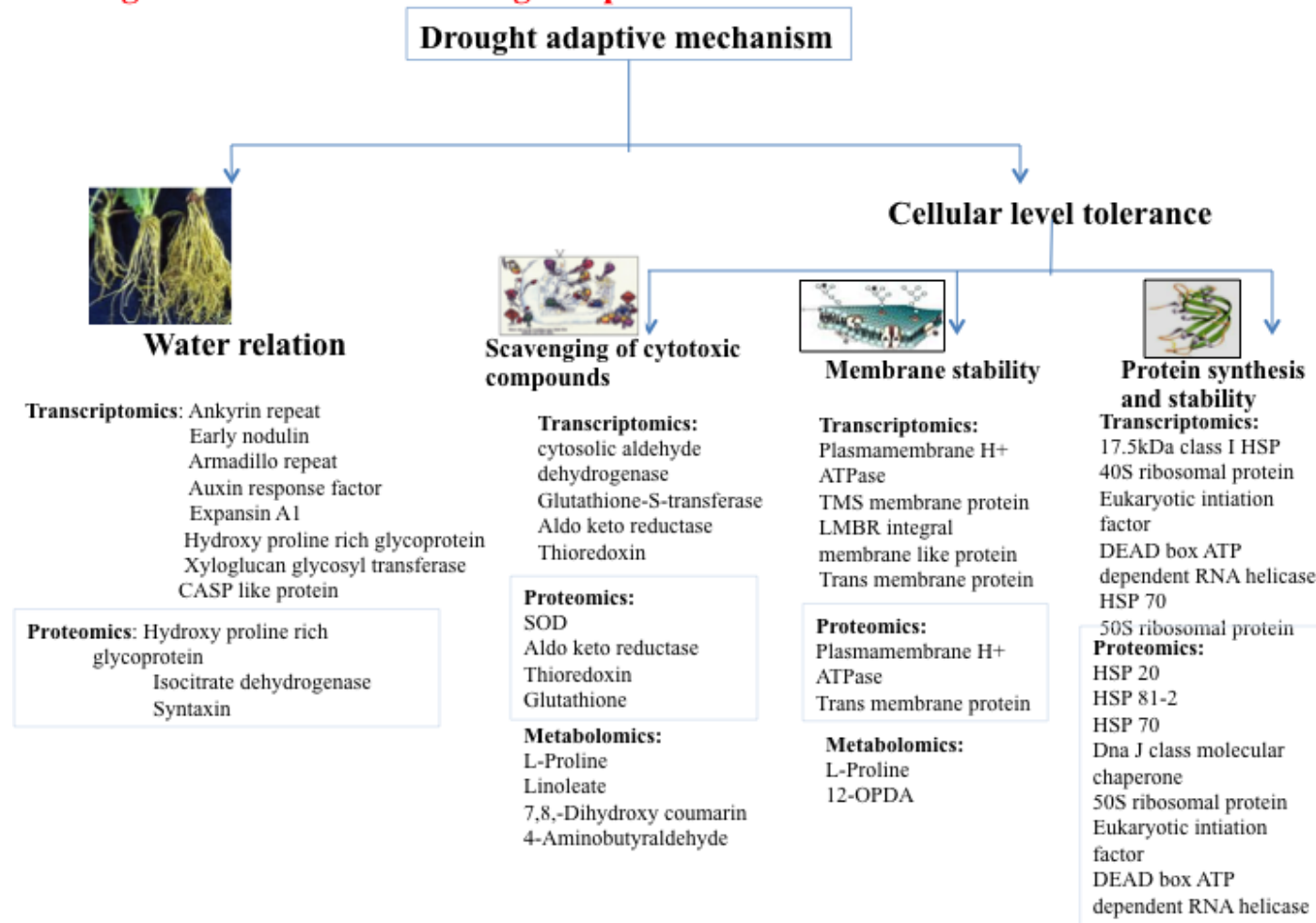


Fig. 68: Integration of Omics data sets that respective transcript, proteome, metabolome associated with different drought adaptive mechanisms

number of natural products, such as flavonoids, stilbenes, and lignin, which serve diverse functions, such as phytoalexins that protect against fungal infections, ultraviolet (UV) protectants, flower and fruit pigments, and structural components of cell walls. 4CL is the key enzyme in the phenylalanine metabolic pathway, and it is regulated developmentally and activated by external stimuli, such as pathogen infections, elicitor treatments, wounding, and UV light irradiation. Additionally, it shows different catalytic activity for various cinnamate derivative substrates, such as cinnamic acid, 4-coumarate acid, caffeate acid, sinapate acid and ferulate acid, and it has a defined substrate preference. The preference is beneficial for metabolism, as it can provide downstream processes with suitable proportions of cinnamoyl CoA mixtures, and it regulates the metabolites flowing to lignin or non-lignin syntheses. Because flavonoids strongly absorb UV irradiation, the induced synthesis of flavonoids can afford plants protection against UV light. Since these pathways are upregulated in transgenic plants the ground plants shows improved ROS scavenging ability and maintain higher CLT.

Alanine, aspartate and glutamate metabolism pathway also up regulated in all the three omics. In transcriptomics glutamate receptors are upregulated with 2 fold change similarly in proteomics aspartate-tRNA ligase with 2.20 fold change increased in transgenics and glutamate dehydrogenase showed 9.64 fold change in transgenics compared to wild type plant. In turn L-Asparagine metabolite increased significantly with 101.92 fold change in transgenics compared to wild type plant.

In citric acid cycle there were 5 genes in transcriptomics such as acetyltransferase component of pyruvate dehydrogenase complex, aconitate hydratase, citrate synthase, malic enzyme and malate synthase up regulated in transgenics with more than fold change. Whereas in proteomics 9 proteins were up regulated in transgenics they are Pyruvate dehydrogenase E1 beta subunit, Pyruvate dehydrogenase E1 component subunit alpha, Citrate synthase, Isocitrate dehydrogenase [NAD] subunit, Isocitrate dehydrogenase [NADP], Malate dehydrogenase, malic enzyme, Glyoxysomal malate dehydrogenase and Succinyl-CoA ligase subunit beta are up regulated with more than 1.5 fold change. Disodium malate, oxalosuccinic acid and oxalomalic acid metabolites were induced mainly in transgenics under stress condition .

The groundnut transgenic over-expressing HSF4 transcription factor were known to upregulate HSPs and improve the protein stability under stress condition, in this context several HSPs transcripts were identified in transgenics under stress condition. Similarly few were also identified in proteome data. Overall data suggested that few of the transcripts correlating with proteome data and subsequently with specific metabolome accumulation from metabolomics data. Suggesting that over-expression of PgHSF4, PDH45 and Alfin1 in groundnut transgenic lines showed that the genes, proteins and metabolites that are involved in maintaining water relations are up regulated. Similarly several CLT associated genes, proteins and metabolities also up regulated in transgenic plants. The data clearly demonstrates that over-expression of two or three regulatory genes improve drought adaptation by improving water relations and CLT mechanisms.

Outcome of the study in a nut shell:

- Over-expression of Alfin1, PDH45 and HSF4 in groundnut transgenic lines improved the expression of several stress responsive genes.
- The transgenic lines showed enhanced root growth at all stages of the crop growth and also under moisture stress condition. The improved root growth in transgenic plants could be due to the expression of Alfin1.
- Over-expression of PDH45 and HSF4 resulted in improved protein turnover and stability under stress conditions.
- Improved water relation by improved root growth substantially contributed for increased leaf size and positive carbon gain.
- Screens - an approach provided an option to identify productive and tolerant types.
- Overall, study provided the proof of concept that improved adaptation to moisture stress and productivity can be achieved by pyramiding traits associated with water mining and cellular level tolerance.

Summary

VI. SUMMARY

The main aim of agriculture scenario is to improve productivity under diverse abiotic stress conditions especially drought. Water availability is one of the major limitations to plant productivity in drought prone areas and is one of the major factor to realize the potential yields. Such a scenario is more common in rain fed crops. Groundnut is one of the major oil seed crop of India, is largely cultivated under rain fed condition. Since the crop is exposed to intermittent moisture stress, productivity is severely affected. To improve the productivity under drought genetic enhancement of relevant adaptive traits is important. From this context it is important to improve adaptation of groundnut under rain fed drought prone condition by improving water relations and cellular level tolerance mechanisms.

Water relation traits that include water mining, WUE, and water conservation are inherent constitutive primary traits. The importance of total transpiration and thereafter water mining and WUE is well elucidated (Vadez *et al.*, 2007a). These two physiological processes largely drive the crop growth not only under non-stress conditions but also when the crop is experiencing moisture stress. There are convincing evidences that field level tolerance and improved productivity can be achieved through efficient water mining and improved WUE (Sheshshayee *et al.*, 2003).

In drought-prone rainfed environments, despite water mining and water conservation mechanisms, plants experience stress when transpiration exceeds the uptake. Under these conditions cellular tolerance mechanisms (CLT), which brings about alteration in cell metabolism for adaptation assumes significance. The CLT broadly involves ionic/osmotic homeostasis, anti-oxidant scavenging (damage/injury control), protein turnover, protein folding/unfolding, and regulation of cell cycle. In addition, sustaining protein synthesis and subsequent structural conformation is critical to maintain the metabolic activities.

Stress tolerance of crops can be improved by bringing together diverse adaptive traits/mechanisms. Therefore, emphasis has been laid on improving water mining, protein

turnover and stability through transgenic approach by overexpressing genes involved in root growth, protein synthesis and protection. The three important criteria to develop transgenic plants are a) choice of trait and genes associated b) robust transformation c) comprehensive characterization of transgenes to assess not only the stress response but also phenology and productivity under defined stress conditions. Subsequently to provide genetic and molecular evidences for the observed variation. The major thrust in the present study is to address the third aspect i.e. comprehensive evaluation of transgenes for stress response and productivity.

Earlier in our department in a comprehensive programme to improve drought tolerance, groundnut transgenics were developed by co-expressing, *OsAlfin1* that regulates root growth, *PDH45* and *PgHSF4* associated with protein turnover and protection. Since *in planta* transformation method was adopted. The putative groundnut transformants were identified in T₁ generation initially by screening the seedlings on kanamycin. Based on the phenotype, kanamycin response and molecular characterization, 102 plants were further advanced to T₂ generation. In T₂ generation, the lines that showed more than 90% of survived seedlings on kanamycin were considered for T₃ generation. In T₃ generation 99 groundnut transgenic lines subjected to gradual drought stress showed significant variability in CCATD and also many of the transgenics showed more pod yield and biomass compared to wild type plants. Based on yield response, TDM and canopy temperature 30 transgenic lines were selected for further characterization.

With this background, the emphasis was to characterize the transgenic events developed earlier to identify most promising few events with improved productivity and tolerance. In successive generations transgenic events were assessed for stress response mainly salinity and moisture stress using different physiological screens. Besides stress tolerance another criteria considered for selection of promising ones was improvement in root characteristics. Finally the major emphasis was to assess the productivity of transgenic events both under control and stress conditions. Since the emphasis is to identify improved tolerance with high productive types we adopted Z-distribution statistical analysis to achieve this objective. Yet another important criteria was selection and advancement of transgenics based on stable integration by PCR, RT-PCR and

Southern analysis. Finally the other major objective was to generate transcriptome and proteomics data to provide insight about the changes brought about by these transgenes to improve tolerance and productivity.

Improved root growth in transgenic lines

One of the co-expressed genes in these transgenics is Alfin1, so in the transgenic lines significant improvement in root growth was observed in all the experiments.

1. Improved root growth was seen in transgenics at all the developmental stages i.e. at seedling stage, at 25 days after sowing and at harvest. Significant improvement in root length and lateral roots was observed in transgenics compared to wild type.
2. Even under stress conditions also the transgenic lines showed improved root growth. Transgenics exposed to salinity both at seedling and whole plant level showed significantly higher root growth. Similarly the transgenics showed improved root growth under moisture stress conditions and there was a less percent reduction in root length over control in transgenics compared to wild type.
3. Increased root growth at all stages lead to increased biomass in transgenic plants under both control and stress conditions and there was a significant positive correlation was observed between biomass and root length.
4. The observed increase in root characteristics was assessed based on differential expression of genes related to root growth and development.
 - a) In semi quantitative RT-PCR analysis transgenic plants showed the enhanced expression of Proline-rich protein (PRP2) in roots under stress, which has been shown to be a downstream target of Alfin1 transcription factor. PRPs are cell wall proteins with potential as an anchor to the membrane, and they function as extensions that enable support and extension of cell wall structure.
 - b) In transcriptome data some of the root associated genes were up regulated in transgenics such as Ankyrin repeat, Auxin binding protein, SAUR-like auxin-responsive protein and Armadillo repeat proteins which are involved in lateral root initiation and development. Expansin B1 involved in root hair elongation and

Early nodulin 93, Nodule-specific glycine rich peptide involved in root nodule formation.

- c) In proteomics study also few of the proteins related to roots were up regulated in transgenics such as Hydroxy proline rich glyco protein family protein which is involved in root cell elongation, Isocitrate dehydrogenase present in root nodules and protect against nitro-oxidative stress and syntaxin protein which is involved in root tip growth.

Groundnut transgenic lines showed improved stress tolerance

The scientific strategy is to improve protein turnover and stability by simultaneous expression of HSF4 and PDH45 which are involved in translation and protein stability. Our study clearly demonstrates that in all the experiments the transgenic showed improved tolerance to abiotic stresses like moisture and salinity stress.

1. Salinity response was studied at both seedling stage and whole plant level. In both the experiments transgenics showed improved tolerance. Not only the percent reduction in several growth attributes was less in transgenics but also the absolute growth was high.
2. Even under moisture stress condition also the survival and recovery growth response was significantly superior in most of the transgenic lines. There was a less percent reduction in root length and biomass over control in transgenics compared to wild type. The transgenic plants showed less accumulation of cytotoxic compounds such as MDA and MG. The total protein content was more in case of transgenic lines than wild type plant.
3. The transgenic lines also showed tolerance to DTT induced ER stress compared to wild type plant.
4. The differentially expressed genes in transgenics related to cellular level tolerance mechanisms were assessed by both semi quantitative RT-PCR analysis and also by RNA-seq. The RT-PCR analysis of selected stress responsive genes showed increased expression of small molecular HSPs, RBX1, aldose reductase (ALR), LEA5, P5Cs and alcohol dehydrogenase-1 (ADH1). The RNA-seq information also showed the

enhanced expression of many genes associated with cellular level tolerance mechanism such as HSPs, Dna J class molecular chaperones, lactoylglutathione lyase, aldo-keto reductase, glutathione S-transferase and thioredoxin in transgenics.

Transgenic plants showed improved biomass and pod yield in stress and non-stress conditions

Improved root growth maintained higher RWC and cooler canopy and also tolerance to abiotic stresses, lead in maintaining higher growth rate and thus resulted in higher productivity under both stress and non-stress conditions.

Biomass:

1. One of the criteria for selection of promising lines was to identify transgenic lines with improved biomass and productivity. In all the experiments where plants were grown to maturity the transgenic lines especially lines which are advanced to T₅ and T₆ showed significantly higher growth rates and biomass.
2. The increased biomass was mainly a due to higher crop growth rate which was predominantly due to higher leaf area and LAI and also due to marginal increase in photosynthetic rates (NAR).

Pod yield:

1. The promising transgenic lines should have higher tolerance to diverse abiotic stresses besides being productive. Therefore, the objective was to identify lines which are tolerant and also productive. Z-distribution analysis was carried out to identify superior lines having higher stress tolerance, yield and biomass. The selected promising lines in T₅ and T₆ generation showed higher pod yields besides being high crop growth rate types with improved tolerance to stress.
2. The selected transgenic lines were evaluated for productivity under both stress and non-stress conditions in T₅ generation. In MDE condition the mean yield of promising transgenics was of 35.3 g.plant⁻¹ and 29.3 g.plant⁻¹ under control and stress condition respectively whereas in wild type mean yield was 25.92 g.plant⁻¹ and 16.67 g.plant⁻¹ under control and stress conditions respectively.

3. In the selected promising ten lines the mean productivity was 49.82 g.plant⁻¹ and 36.2 g.plant⁻¹ under control and stress condition respectively. The transgenics showed 15-20% increase in pod yield compared to wild type.
4. Based on improved root growth, stress tolerance and productivity in both stress and non-stress condition eight lines i.e. 17, 30, 40, 86, 97, 99, 100 and 104 were selected as promising lines.

Molecular analysis

1. In successive generations one of the criteria for selection of promising lines is stable integration of all the three genes. The integration of Alfin1, PDH45 and HSF4 in all the generations was confirmed by using gene specific primers. The lines which showed integration of all the three genes were carried out to further evaluation.
2. Genomic southern blot was carried out in three transgenic plants i.e. 86, 97 and 100 demonstrated that the transgenes were stably integrated into the genome of transgenic groundnut lines with single copy of the transgene in selected three transgenic lines. Multiplex PCR and flanking sequence information is in progress.

Omics approach: To study the mechanisms involved in improved yield and drought tolerance in transgenic lines

To understand the mechanisms involved in improved yield and drought tolerance in groundnut transgenic plants, transcriptomics, proteomics and metabolomics was studied.

With regards to proteomic analysis the gene ontology was done using *Medicago truncatula* as a reference genome, so only 50% of the genes could be annotated. With regards to metabolomics though differentially expressed metabolites accumulated in transgenics, only 12% of metabolites were annotated due to lack of specific standards for identified peak areas. With this background the following inferences were made.

Transcriptomics:

1. Several differentially expressed genes (DEGs) were identified in transgenics compared to wild type under control and stress conditions. In groundnut transgenic line 625 and 531 genes were up regulated and down regulated respectively in control conditions. Whereas under stress conditions 1452 and 1384 genes were up regulated and down regulated respectively in transgenic plants.
2. In transgenic plants many metabolic processes were up regulated such as folic acid biosynthesis pathway, transferases activity, chaperone activity, aromatic amino acid synthesis and deaminase activity. Folic acid known to involve in synthesis of DNA and subsequently in protein synthesis. Similarly up regulation of deaminase pathway genes and aromatic acid biosynthesis genes indicate that higher nitrogen metabolism for improved protein synthesis and turnover contributing for stress adaptation and improved productivity.
3. Some of the transcription factors like HSF A3 is upregulated and further HSPs such as small HSP17.5, cytosolic class I small HSP, calmodulin binding HSP, HSP 70, HSP 22, HSP 20 and Dna J class HSPs were up regulated in transgenics.
4. Several root associated genes such as Ankyrin repeats proteins, Armadillo repeat proteins, Auxin response factor, Auxin efflux carrier, Expansin A1, Xyloglucan glycosyltransferase and Hydroxy-proline rich glycoprotein genes were up regulated in transgenics.
5. Genes associated with cellular level tolerance mechanism such as lactoylglutathione lyase, aldo-keto reductase, glutathione S-transferase and thioredoxin, Plasmamembrane ATPase, Chaperones, DEAD-box ATP dependent helicase etc. were up regulated more than 2 fold change in transgenics.

Proteomics:

1. The differentially expressed proteins in groundnut transgenic and wild type plants were identified using *Medicago truncatula* as reference genome. In transgenics under control conditions there are 323 proteins were up regulated and 42 proteins were down regulated compared to wild type plants. Similarly 330 proteins were up

regulated and 73 proteins were down regulated in transgenic plants compared to wild type under moisture stress conditions.

2. Some of the HSPs such as HSP20, HSP 81-2, HSP 70, Chaperonin 60, Gro-EL-like chaperone and Dna J class chaperones were up regulated in transgenics under stress conditions.
3. Root related proteins such as Hydroxy proline rich glyco protein involved in root cell elongation and Isocitrate dehydrogenase present in root nodules and protect against nitro-oxidative stress. Syntaxin protein which is involved in root tip growth was found to be up regulated in transgenic plants.
4. The proteins associated with scavenging cytotoxic compounds and cellular tolerant mechanisms such as Glutathione S-transferase, Peroxidase, Cytosolic aldehyde dehydrogenase, Superoxide dismutase [Cu-Zn], Glutaredoxin C4, L-ascorbate peroxidase, Lactoylglutathione lyase and Catalase were upregulated in transgenics.
5. The photosynthesis associated proteins and other metabolic processes associated proteins were highly upregulated in transgenics signifying better adaptation under stress conditions.

Metabolomics:

1. In transgenic plant 21 constitutive metabolites were accumulated under control condition whereas 27 metabolites were constitutively expressed under stress condition compared to wild type and 15 metabolites were exclusively induced in transgenics under stress condition.
2. Antioxidants like L-Proline, 4-Aminobutyraldehyde, linoleate, 5-O-Methyl-myoinositol, 7,8-Dihydroxy Coumarin, 3-bromo-5-phenyl salicylic acid and carnosol were up regulated in transgenics.
3. Osmoprotectants like 12-OPDA and L-proline are involved in maintenance of cell turgour and osmotic homeostasis were also up regulated in transgenics.

Integration of omics

1. The transcriptome and proteome data showed several genes that are involved in water relations were up regulated in transgenics and it could be attributed by over-expression of *Alfin1* in transgenic lines.
2. The groundnut transgenic over-expressing HSF4 transcription factor that known to upregulate HSPs and improve the protein stability under stress condition, in this context several HSPs transcripts were identified in transgenics under stress condition similarly few were also identified in proteome data.
3. Transcriptome studies revealed that 10 genes are involved in scavenging cytotoxic compounds. In proteomics also some of the proteins that are involved in scavenging of cytotoxic compound were up regulated with more than 1.5 fold change. In metabolomics study also few anti-oxidant metabolites like proline, coumarate, linoleate and 4-aminobutyraldehyde were up regulated in transgenic plants.
4. Genes/proteins involved in maintaining the membrane stability are plasmamembrane ATPase and transmembrane proteins commonly found in proteomics and transcriptomics data. L-Proline and 12-OPDA metabolites that are involved in maintaining the cell turgour as well as act as osmolytes also up regulated in transgenic plant compared to wild type plants under stress condition.
5. Protein synthesis and stability play an important role in maintaining CLT mechanisms, many of the transcripts and proteins that are involved in protein synthesis and stability were up regulated in both transcriptomics and proteomics data.
6. Five important pathways are identified from transcriptome, proteome and metabolome such as proline biosynthesis, chlorophyll biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis pathway, alanine, aspartate and glutamate metabolism and citric acid cycle.

Overall data suggesting that few of the transcripts correlating with proteome data and subsequently with specific metabolome accumulation from metabolomics data. The results suggest that over-expression of *PgHSF4*, *PDH45* and *Alfin1* in groundnut transgenic lines showed that the genes, proteins and metabolites that are involved in

maintaining water relations are up regulated. Similarly several CLT associated genes, proteins and metabolites also up regulated in transgenic plants. The data clearly demonstrates that over-expression of two or three regulatory genes improve drought adaptation by improving water relation and CLT mechanisms.

Outcome of the study:

1. Drought tolerance is polygenic trait. To improve the productivity under drought genetic enhancement of few relevant adoptive traits is the only option. We provided proof of concept in the study that genetic enhancement of root traits, protein turnover and stability by transgenic approach is a potential option.
2. Simultaneous expression of Alfin1 which is involved in root growth, PDH45 and HSF4 associated with protein turnover and stability substantially improved root characteristics and intrinsic tolerance at cellular level under diverse abiotic stresses.
3. The selected transgenic lines are also productive with improved pod yield under control and stress conditions.
4. In depth characterization of RNA seq and proteomics studies clearly demonstrated differentially expressed genes regulating cellular level tolerance and water mining are highly up regulated in transgenic lines.
5. Overall the results clearly indicate that genetic enhancement of specific traits showed substantially improved stress tolerance with higher productivity.

References

VII. REFERENCES

- ABEBE, T., GUENZI, A. C., MARTIN, B. AND CUSHMAN, J. C., 2003, Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. *Plant Physiol.*, **131**: 1748-1755.
- AEBI, H. AND SUTER, H., 1971. Acatalasemia. *Adv. Hum. Genet.*, **2**:143-199.
- AKERFELT, M., MORIMOTO, R. I. AND SISTONEN, L., 2010, Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Rev. Mol. Cell Biol.*, **11**: 545–555.
- AL-WHAIBI, H. M., 2010, Plant heat-shock proteins: a mini review. *J. King Saudi Univ. Sci.*, **23**, 139–150.
- AMAR, K., KAZUO, F., XIAOHONG, Z., PAYAL, R., CHEN, S., XIAOPING, C., ZHIREN, L., SHE, C. AND JANE Y. W., 2011, RNA helicase p68 (DDX5) regulates tau exon 10 splicing by modulating a stem-loop structure at the 5' splice site. *Mol. Cell Biol.*, **31(9)**:1812.
- AMIN, M., ELIAS, S. M., HOSSAIN, A., FERDOUSI, A., RAHMAN, M. S., TUTEJA, N., ZEBA, I. S., 2012, Overexpression of a DEAD box helicase, PDH45, confers both seedling and reproductive stage salinity tolerance to rice (*Oryza sativa* L.). *Mol. Breeding*, **30**: 345–54 doi: 10.1007/s11032-011-9625-3.
- ANKEGOWDA, S. J., 1996, Canopy temperature, crop canopy air temperature difference as a screening technique for dehydration post ponement under water stress in finger millet (*Eleusine coracana* Gaertn.). Ph.D Thesis, Univ. Agric. Sci., Bangalore.
- ANTHONI, U., CHRISTOPHERSEN, C., HOUGAARD, L. AND NIELSEN, P. H., 1991, Quaternary ammonium compounds in the biosphere - An example of a versatile adaptive strategy. *Comp. Biochem. Biophys.*, **99B**: 1-18.

- APEL, K. AND HIRT, H., 2004, Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, **55**: 373-399.
- ARCHANA, K., RAMA, N., MAMRUTHA, H. M. AND NATARAJA, K. N., 2009, Down-regulation of an abiotic stress related Nicotiana benthamiana WRKY TF induces physiological abnormalities. *Indian J. Biotechnol.*, **8**: 53-60.
- ARMENGAUD, P., SULPICE, R., MILLER, A. J., STITT, M., AMTMANN, A. AND GIBON, Y., 2009, Multilevel analysis of primary metabolism provides new insights into the role of potassium nutrition for glycolysis and nitrogen assimilation in *Arabidopsis* roots. *Plant Physiol.*, **150**(2): 772-785.
- ARORA, A., SAIRAM, R. K. AND SRIVASTAVA, G. C., 2002, Oxidative stress and antioxidative system in plants. *Curr. Sci.*, **82**: 1221-1235.
- ASADA, K., 1992, Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. *Plant Physiol.*, **85**: 235-241.
- ASHRAF, M. AND FOOLAD, M. R., 2007, Roles of glycinebetaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.*, **59**: 206-216.
- BABITHA, K. C., RAMU, S. V., PRUTHVI, V., MAHESH, P., NATARAJA, K. N. AND UDAYAKUMAR, M., 2013, Co-expression of AtbHLH17 and AtWRKY28 confers resistance to abiotic stress in *Arabidopsis*. *Transgenic Res.*, **22**, 327-341.
- BADAWI, G. H., KAWANO, N., YAMAUCHI, Y., SHIMADA, E., SASAKI, R., KUBO, A. AND TANAKA, K., 2004a, Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stresses and water deficit. *Plant Physiol.*, **121**: 231-238.

- BADAWI, G. H., YAMAUCHI, Y., SHIMADA, E., SASAKI, R., KAWANO, N., TANAKA, K. AND TANAKA, K., 2004b, Enhanced tolerance to salt stress and water deficit by overexpressing superoxide Dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. *Plant Sci.*, **166**: 919-928.
- BALOTA, M., ISLEIB, T.G. AND TALLURY, S., 2012, Variability for drought related traits of virginia-type peanut cultivars and advanced breeding lines. *Crop Sci.*, **52**: 2702–2713.
- BANDURSKA, H., 1998, Implication of ABA and proline on cell membrane injury of water deficit stressed barley seedlings. *Acta Physiol. Plant*, **20**: 375–381.
- BANIWAL, S. K, BHARTI, K., CHAN, K. Y., FAUTH, M., GANGULI, A., KOTAK, S., MISHRA, S. K., NOVER, L., PORT, M. AND SCHARF, K. D., 2004, Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *J. Biosci.*, **29**: 471–487.
- BANIWAL, S. K, CHAN, K. Y, SCHARF, K. D. AND NOVER, L., 2007, Role of heat stress transcription factor HsfA5 as specific repressor of HsfA4. *J. Biol. Chem.*, **282**: 3605–3613.
- BANTI, V., MAFESSONI, F., LORETI, E., ALPI, A. AND PERATA, P., 2010, The heat-inducible transcription factor HSFA2 enhances anoxia tolerance in Arabidopsis. *Plant Physiol.*, **152**: 1471-1483.
- BARRS, H. O. AND WEATHERLY, P. E. 1962. A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Austral. J. Biol. Sci.* **15**: 413-428.
- BARTELS, D. AND SUNKAR, R., 2005, Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.*, **24**: 23-58.

- BASTOLA, D. R., PETHE, V. V. AND WINICOV, I., 1998, Alfin1, a novel zinc-finger protein in alfalfa roots that binds to promoter elements in the salt-inducible MsPRP2 gene. *Plant Mol Biol.*, **38**: 1123–1135.
- BERNHARDT, C. AND TIERNEY, M. L., 2000, Expression of AtPRP3, a proline-rich structural cell wall protein from Arabidopsis is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiol.*, **122**: 705–714.
- BEVAN, M., BANCROFT, I., BENT, E., LOVE, K., GOODMAN, H., DEAN, C., BERGKAMP, R., DIRKSE, W., VAN STAVEREN, M., AND STIEKEMA, W., 1998, Analysis of 1.9Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature*, **391**: 485–488.
- BHARTI, K. E., SCHMIDT, E., LYCK, R., BUBLAK, D. AND SCHARF, K. D., 2000, Isolation and characterization of HsfA3, a new heat stress transcription factor of *Lycopersicum peruvianum*. *Plant J.*, **22**: 355-365.
- BHARTI, K., VONKOSKULL-DORING, P., BHARTI, S., KUMAR, P., TINTSCHL-KORBITZER, A., TREUTER, E. AND NOVER, L., 2004, Tomato heat stress transcription factor HSFB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. *Plant Cell*, **16**: 1521–1535.
- BIZILY, S. P., RUGH, C. L. AND MEAGHER, R. B., 2000, Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nat. Biotechnol.*, **18**: 213–217.
- BLACK, M. AND PRITCHARD, H. W., 2002, Desiccation and survival in plants: Drying without dying. Wallingford, UK: CABI. 10.1079/9780851995342.0000.
- BLUM, A., 2005, Drought resistance, water-use efficiency, and yield potential— are they compatible, dissonant, or mutually exclusive? *Aust. J. Agric. Res.*, **56**: 1159–1168.

- BOSCHEINEN, O., LYCK, R., QUEITSCH, C., TREUTER, E., ZIMARINO, V. AND SCHARF, K. D., 1997, Heat stress transcription factors from tomato can functionally replace Hsf1 in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **255**: 322–331.
- BOUR, B., BERNARD, A., FREDERIC, D., PASCAL, S., LEGER, A., ROBY, D., PERVENT, M., VILE, D. AND JOBES, J., 2011, Overexpression of Arabidopsis ECERIFERRUM1 promotes wax very long chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. *Plant Physiol.*, **156**: 21–45.
- BOYER, J. S., 1982, Plant productivity and the environment. *Science*, **218**: 443–448.
- BRADFORD, M. M., 1976, A dye binding assay for protein. *Anal. Biochem.*, **72**: 248–254.
- CABUSLAY, G. S., ITO, O. AND ALEJAR, A. A., 2002, Physiological evaluation of responses of rice (*Oryza sativa* L.) to water deficit. *Plant Sci.*, **163**: 815–827.
- CALDANA, C., DEGENKOLBE, T., CUADROS-INOSTROZA, A., KLIE, S., SULPICE, R., LEISSE, A., STEINHAUSER, D., FERNIE, A.R., WILLMITZER, L. AND HANNAH, M. A., 2011, High-density kinetic analysis of the metabolomic and transcriptomic response of *Arabidopsis* to eight environmental conditions. *Plant J.*, **67**: 869–884.
- CAMPOS, P. S., QUARTIN, V., RAMALHO, J. C. AND NUNES, M. A., 2003, Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *J. Plant Physiol.*, **160**(3): 283–92.
- CASSAB, G. I., 1998, Plant cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol.*, **49**: 281–309.
- CELENZA, J. L. JR, GRISAFI, P. L. AND FINK, G. R., 1995, A pathway for lateral root formation in *Arabidopsis thaliana*. *Genes Dev.*, **9**: 2131–2142.

- CHANDRASEKHAR, K., 1978, Photosynthetic efficiency and translocation and their relationship with dry matter production and yield in five genotypes of ragi (*Eleusine coracana* Gaertn.). M.Sc (Agri.) Thesis, Univ. Agric. Sci., Bangalore.
- CHANG, W. W., HUANG, L., SHEN, M., WEBSTER, C., BURLINGAME, A. L. AND ROBERTS, J. K., 2000, Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment and identification of proteins by mass spectrometry. *Plant Physiol.*, **122**: 295–318.
- CHEN, H. C., LIN, W. C., TSAY, Y. G., LEE, S. C. AND CHANG, C. J., 2002, An RNA helicase, DDX1, interacting with poly (A) RNA and heterogeneous nuclear ribonucleic protein K. *J. Biol. Chem.*, **277**: 40403- 40409.
- CHEN, L., MARMEY, P., TAYLOR, N. J., BRIZARD, J. P., ESPINOZA, C., D'CRUZ, P., HUET, H., ZHANG, S., DE KOCHKO, A., BEACHY, R. N. AND FAUQUET, C. M., 1998, Expression and inheritance of multiple transgenes in rice plants. *Nat. Biotechnol.*, **16**:1060–1064.
- CHEN, Q. J., ZHOU, H.M., CHEN, J. AND WANG, X. C., 2006, A Gateway-based platform for multigene plant transformation. *Plant Mol. Biol.*, **62**: 927–936.
- CHEN, T. AND MURATA, N., 2008, Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.*, **13**: 499–505.
- CHEN, X., JING SHI, XI HAO, HUILI LIU, JIANGHUA SHI, YUNRONG WU, ZHONGCHANG WU, MINGXIU CHEN, PING WU AND CHUANZAO MAO, 2013, OsORC3 is required for lateral root development in rice. *Plant J.*, **74**: 339–350.

- CHEN, Y. A., WEN-CHANG CHI, NGOC NAM TRINH, LI-YAO HUANG, YING-CHIH CHEN, KAI-TENG CHENG, TSAI-LIEN HUANG, CHUNG-YI LIN AND HAO-JEN HUANG, 2014, Transcriptome Profiling and Physiological Studies Reveal a Major Role for Aromatic Amino Acids in Mercury Stress Tolerance in Rice Seedlings. *PLoS One*, **9(5)**: 1-11.
- CHINNA VENKATA REDDY, V., 1976, Effect of soil moisture stress at different growth stages on growth and yield of finger millet, M.Sc (Agri.), Thesis, Univ. Agric. Sci., Bangalore.
- CHRISTEN, Y.L. YUEN, JOHN C. SEDBROOK, ROBYN M. PERRIN, KATHLEEN L. CARROLL AND PATRICK H. MASSON, 2005, Loss-of-Function Mutations of ROOT HAIR DEFECTIVE3 Suppress Root Waving, Skewing, and Epidermal Cell File Rotation in Arabidopsis. *Plant physiol.*, **138**: 701-714.
- CLARK, L. J., PRICE, A. H., STEELE, K. A. AND WHALLEY, W. R., 2008. Evidence from near-isogenic lines that root penetration increases with root diameter and bending stiffness in rice. *Funct. Plant Biol.* **35**: 1163–1171.
- COATES, J. C., LAPLAZE, L. AND HASELOFF, J., 2006, Armadillo-related proteins promote lateral root development in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **103**: 1621-1626.
- COOK, D., FOWLER, S., FIEHN, O. AND THOMASHOW, M. F., 2004, A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **101(42)**: 15243-15248.
- CORDIN, O., BANROQUES, J., TANNER, N. K. AND LINDER P., 2006, The DEAD-box protein family of RNA helicases. *Gene*, **367**: 17–37.
- CORTINA, C. AND CULIA' N EZ-MACIA,F., 2005, Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. *Plant Sci.*, **169**:75–82.

- CRAMER, G. R., KAORU URANO, SERGE DELROT, MARIO PEZZOTTI AND KAZUO SHINOZAKI, 2011, Effects of abiotic stress on plants: a systems biology perspective. *Plant Biol.*, **11**:163-177.
- CZARNECKA-VERNER, E., PAN, S., SALEM, T. AND GURLEY, W. B., 2004, Plant class B HSFs inhibit transcription and exhibit affinity for TFIIB and TBP. *Plant Mol. Biol.*, **56**: 57–75.
- CZARNECKA-VERNER, E., YUAN, C. X., SCHARF, K. D., ENGLISH, G. AND GURLEY W. B., 2000, Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential. *Plant Mol. Biol.*, **43**: 459–471.
- DATTA, S. K., PATEL, H., AND BERRY, D., 1989, Extraction and purification of RNA from crop plants. *J. Exp. Bot.*, **165**:1252.
- DAVID SECCO, MEHDIJA BNOUNE, HAYDEN WALKER, HUIXIA SHOU, PING WU, YVE SPOIRIER AND JAMES WHELANA, 2013, Spatio-Temporal Transcript Profiling of Rice Roots and Shoots in Response to Phosphate Starvation and Recovery. *Plant Cell*, **25**: 4285–4304.
- DAVLETOVA, S., RIZHSKY, L., LIANG, H., SHENGQIANG, Z., OLIVER, D. J., COUTU, J., SHULAEV, V., SCHLAUCH, K. AND MITTLER, R., 2005a, Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. *Plant Cell*, **17**: 268–281.
- DE DORLODOT, S., FORSTER, B., PAGES, L., PRICE, A., TUBEROSA, R. AND DRAYE, X., 2007, Root system architecture: opportunities and constraints for genetic improvement of crops. *Trends Plant Sci.*, **10**: 474-481.
- DEMIRAL, T. AND TURKAN, I., 2004, Does exogenous glycinebetaine affect antioxidative system of rice seedlings under NaCl treatment? *J. Plant Physiol.*, **161**: 1089-1110.

- DEUTCH, C. E. AND WINICOV, I., 1995, Post-transcriptional regulation of a salt-inducible alfalfa gene encoding a putative chimeric prolinerich cell wall protein. *Plant Mol Biol.*, **27**: 411–418.
- DIDONATO, R. J., ARBUCKLE, E., BUKER, S., SHEETS, J., TOBAR, J., TOTONG, R., GRISAFI, P., FINK, G.R. AND CELENZA, J. L., 2004, Arabidopsis ALF4 encodes a nuclearlocalized protein required for lateral root formation. *Plant J.*, **37**: 340–353.
- DOYLE, J. J. AND DOYLE, J. L., 1989, Isolation of plant DNA from plant fresh tissue. *BRL Focus*, **12**: 13-14.
- EKANAYAKE, I., DE DATTA, S. AND STEPONKUS, P., 1989, Spikelet sterility and flowering response of rice to water stress at anthesis. *Ann. Bot.*, **63**: 257–264.
- ESTELLE, M. AND KLEE, H. J., 1994, Auxin and cytokinin in *Arabidopsis*. In EM Meyerowitz, CR Somerville, eds, *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 555–578.
- EUNSOOK, C., CHANG-WOO, C., BO-HYUN, Y., HONG-KYU, C., HYUN-AH, S., SEON-WOO, L. AND JAI-HEON, L., 2009, Molecular cloning and characterization of the soybean DEAD-box RNA helicase gene induced by low temperature and high salinity stress. *Gene*, **443**: 91–99.
- FAO, 2010, Food and Agriculture Organization of the United Nations, Rome (<http://www.fao.org/forestry/fra/fra2010/en/>).
- FERRIS, P. J., WOESSNER, J. P., WAFFENSCHMIDT, S., KILZ, S., DRESS, J. AND GOODENOUGH, U. W., 2001, Glycosylated polyproline II rods with links as a structural motif in plant hydroxyproline-rich glycoproteins. *Biochemistry-US.*, **40**: 2978-2987.
- FISCHER R. A., 1960, The Design of Experiments, 7th Edn Edinburgh: Oliver and Boyd.

- FOSS, J. E., RADULOVIC, D., SHAFFER, S. A., GOODLETT, D. R., KRUGLYAK, L. AND BEDALOV, A., 2011, Genetic variation shapes protein networks mainly through nontranscriptional mechanisms. *PLoS Biol.*, **9**: e1001144.
- FOWLER, T. J., BERNHARDT, C. AND TIERNEY M. L., 1999, Characterization and expression of four proline-rich cell wall protein genes in Arabidopsis encoding two distinct subsets of multiple domain proteins. *Plant Physiol.*, **121**: 1081:1091.
- FUKAI, S. AND COOPER, 1995, Development of drought-resistant cultivars using physiomorphological traits in rice. *Field crop Res.*, **40**: 67-86.
- FUKAKI, H., TAMEDA, S., MASUDA, H. AND TASAKA, M., 2002, Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY- ROOT/ IAA14 gene of Arabidopsis. *Plant J.*, **29**: 153–168.
- GE, L., CHEN, H., JIANG, J. F., ZHAO, Y., XU, M. L., XU, Y. Y., TAN, K. H., XU, Z. H. AND CHONG, K., 2004, Overexpression of OsRAA1 causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity. *Plant Physiol.*, **135**(3): 1502–1513.
- GIBSON, B. R., CHRIS, A. B., WENDY, G. B., NEIL, S. G., STEPHEN, J. L., ROBERT, S. T. AND KATHERINE, A. S., 2008, Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. *Yeast*, **25**: 549–562.
- GODERIS, I. J., DE BOLLE, M. F., FRANCOIS, I. E., WOUTERS, P. F., BROEKAERT, W. F. AND CAMMUE, B. P., 2002, A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. *Plant Mol. Biol.*, **50**: 17–27.
- GOMEZ, J. M., HERNANDEZ, J. A., JIMENEZ, A., DELRIO, L. A. AND SEVILLA, F., 1999, Differential response of antioxidant enzymes of chloroplasts and mitochondria to long term NaCl stress of pea plants. *Free Radical Res.*, **31**: 11-18.

- GONG, Z., DONG, C. H., LEE, H., ZHU, J., XIONG, L., GONG, D., STEVENSON, B. AND ZHU, J. K., 2005, A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis. *Plant Cell*, **17**: 256–267.
- GORHAM, J., 1995, Betaines in higher plants - biosynthesis and role in stress metabolism. In "Amino acids and Their Derivatives in Higher Plants." (RM Wallsgrove ed) Society for Experimental Biology Seminar Series, Vol 56, Cambridge University Press, Cambridge, pp. 173-203.
- GRAY, W. M., DEL POZO, J. C., WALKER, L., HOBBIE, L., RISSEEUW, E., BANKS, T., CROSBY, W. L., YANG, M., MA, H. AND ESTELLE, M., 1999, Identification of an SCF ubiquitin ligase complex required for auxin response in Arabidopsis thaliana. *Genes Dev.*, **13**: 1678–1691.
- GREGORY, F. G., 1926, The effect of climatic conditions on the growth of barley. *Ann. Bot.*, **40**: 1-26.
- GROVER, A., MITTAL, D., NEGI, M. AND LAVANIA, D., 2013, Generating high temperature tolerant transgenic plants: achievements and challenges. *Plant Sci.*, **205–206**: 38-47.
- GUO, A. B., QI XIE, A.C., JI-FENG FEI, A. AND NAM-HAI CHUA, 2005, Micro RNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. *Plant cell*, **17**: 1376-1386.
- GUPTA, A. S., WEBB, R. P., HOLADAY, A. S. AND ALLEN, R. D., 1993, Overexpression of superoxide Dismutase protects plants from Oxidative stress. *Plant Physiol.*, **103**: 1067-1073.
- GUPTA, K. A., PALLAVEE TYAGI AND VINAY K. SEHGAL, 2011, Drought disaster challenges and mitigation in India: strategic appraisal. *Curr. Sci.*, **100**: 1795-1806.

- GURUMURTHY, B. R., 1982, Desirable growth characteristics in relation to productivity in finger millet (*Eleusine coracana* Gaertn.) – A physiological analysis, M.Sc (Agri.) Thesis, Univ. Agric. Sci., Bangalore.
- HALL, Q. AND CANNON, M., 2002, The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in *Arabidopsis*. *Plant Cell*, **14**: 1161-1172.
- HARE, P. D. AND CRESS, W. A., 1997, Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.*, **21**: 79–102.
- HARTLEY, J. L., TEMPLE, G. F. AND BRASCH, M. A., 2000, DNA cloning using in vitro site-specific recombination. *Genome Res.*, **10**:1788–1795.
- HEGEDEUS, A., ERDEI, S., JANDA, T., TOTH, E., HORVATH, G. AND DUDITS, D., 2004, Transgenic tobacco plants overproducing alfalfa aldose/aldehyde reductase show higher tolerance to low temperature and cadmium stress. *Plant Sci.*, **166**: 1329–1333.
- HERBETTE, S., ROECKELDREVET, P. AND DREVET, J. R., 2007, Selenoindependent glutathione peroxidases: more than simple antioxidant scavengers. *FEBS J.*, **274**: 2163–2180.
- HERBETTE, S., DENIS TOURVIEILLE, D.E., LABROUHEB, JOEL, R., DREVET AND ROECKELDREVET, P., 2011, Transgenic tomatoes showing higher glutathione peroxidase antioxidant activity are more resistant to an abiotic stress but more susceptible to biotic stresses. *Plant Sci.*, **180**: 548–553.
- HOBBIE, L. J., 1998, Auxin: molecular genetic approaches in *Arabidopsis*. *Plant Physiol. Biochem.*, **36**: 91–102.
- HODGE, A., BERTA, G., DOUSSAN, C., MERCHAN, F. AND CRESPI, M., 2009, Plant root growth, architecture and function. *Plant Soil*, **321**: 153–187.

- HYUN, J. K., SOO, M.P., KEUN, P. K., MI, C. S., MI, O. L., SEONG, K. L., XIA, X. AND CHOO, B. H., 2015, Small heat shock proteins can release light dependence of tobacco seed during germination. *Plant Physiol.*, DOI:10.1104/pp.114.25284.
- IMMA, P. S., PAPDI, C., RIGO, G., ZSIGMOND, L., VIELELA, B., LUMBRERAS, V., NAGY, I., HORYATH, B., DOMOKI, M. AND SZABADOS, L., 2014, The heat shock factor A4A confers salt tolerance is regulated by oxidative stress and the mitogen-activated protein kinases MPK3 and MPK6. *Plant Physiol.*, **165**: 319-334.
- INGRAM, K. T., BUENO, F. D., NAMUCO, O. S., YAMBAO, E. B. AND BEYROUTY, C. A., 1994, Rice root traits for drought resistance and their genetic variation. In: Kirk, G.J.D. (Ed.), *Rice Roots: Nutrient and Water Use*. International Rice Research Institute, Manila, Philippines.
- INUKAI, O. A, TOMOAKI SAKAMOTO, B., MIYAKO UEGUCHI-TANAKA, A., YOHKO SHIBATA, C., KENJI GOMI, A., IICHIRO UMEMURA, A., YASUKO HASEGAWA, A., MOTOYUKI ASHIKARI, A., HIDEMI KITANO, A. AND MAKOTO MATSUOKAA, 2005, Crown rootless1, which is essential for crown root formation in rice, is a target of an auxin response factor in auxin signaling. *Plant cell*, **17**: 1386-1397.
- ISAACSON, T., DAMASCENO, C. M., SARAVANAN, R. S., HE, Y., CATALÁ, C., SALADIÉ, M., ROSE, J. K., 2006, Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nat Protoc.*, **1(2)**: 769-74.
- IQBAL R.M. KHAN, MEHAR FATMA, TASIR S. PER, NASER A. ANJUM AND NAFEEES A. KHAN, 2015, Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Front. Plant Sci.*, **6**: 462.

- IVASHUTA, S., LIU, J., LIU, J., LOHAR, D.P., HARIDAS, S., BUCCIARELLI, B., VANDENBOSCH, K. A., VANCE, C. P., HARRISON, M. J. AND GANTT, J. S., 2005, RNA interference identifies a calcium-dependent protein kinase involved in *Medicago truncatula* root development. *Plant Cell*, **17**: 2911–2921.
- JAIN, M., MISRA, G., PATEL, R. K., PRIYA, P., JHANWAR, S. AND KHAN, A.W., 2013, A draft genome sequence of the pulse crop chickpea (*Cicer arietinum* L.). *Plant J.*, **74**: 715-729.
- JAN A. MIERNYK, 1999, Protein Folding in the Plant Cell. *Plant Physiol.*, **121**: 695-703.
- JANKOWSKY, J. L., SLUNT, H. H., RATOVITSKI, T., JENKINS, N. A., COPELAND, N. G. AND BORCHELT, D. R., 2001, Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol. Eng.*, **17**: 157-165.
- JIN SEO JEONG, YOUN SHIC KIM, KWANG HUN BAEK, HARIN JUNG, SUN-HWA HA, YANG DO CHOI, MINKYUN KIM, CHRISTOPHE REUZEAU, AND JU-KON KIM, 2010, Root-Specific Expression of OsNAC10 Improves Drought Tolerance and Grain Yield in Rice under Field Drought Conditions. *Plant Physiol.*, **153**: 185–197.
- JIN, L. AND PETERSON, D. L., 1995, Expression, isolation, and characterization of the hepatitis C virus ATPase/RNA helicase. *Arch. Biochem. Biophys.*, **323**: 47-53.
- JUN HONG, LITAO YANG, DABING ZHANG AND JIANXIN SHI, 2016, Plant Metabolomics: An Indispensable System Biology Tool for Plant Science. *Int. J. Mol. Sci.*, **17**: 767-783.
- JUNG, J. K. H. AND MCCOUCH, S., 2013, Getting to the roots of it: genetic and hormonal control of root architecture. *Front. Plant Sci.*, **4**: 186.

- KAISER, W.M., GEORG KAISER, SILVIA SCHÖNER AND SPIDOLA NEIMANIS, 1981, Photosynthesis under osmotic stress, Differential recovery of photosynthetic activities of stroma enzymes, intact chloroplasts, protoplasts, and leaf slices after exposure to high solute concentrations. *Planta*, **153 (5)**: 430-435.
- KAKIDANI, H. AND PTASHNE, M., 1988, Gal4 activates gene expression in mammalian cells. *Cell*, 52: 161–167.
- KANANI, H., DUTTA, B. AND KLAPA, M. I., 2010, Individual vs. combinatorial effect of elevated CO₂ conditions and salinity stress on *Arabidopsis thaliana* liquid cultures: Comparing the early molecular response using time-series transcriptomic and metabolomic analyses. *BMC Syst. Biol.*, **4**: 177.
- KANIA, T., DORIS RUSSENBERGER, STEFAN PENG, KLAUS APEL, AND SIEGBERT MELZER, 1997, FPF1 Promotes Flowering in Arabidopsis. *Plant cell*, **9**: 1327-1338.
- KARAM, B. SINGH, 1998, Transcriptional Regulation in Plants: The Importance of Combinatorial Control. *Plant Physiol.*, **118**: 1111–1120.
- KATHURIA, H., GIRI, J., KARABA N. N., MURATA, N., UDAYAKUMAR, M. AND TYAGI, A. K., 2009, Glycinebetaine-induced water-stress tolerance in codA-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. *Plant Biotechnol. J.*, **7(6)**:512–526.
- KAVITHA, K., VENKATARAMAN, G. AND PARIDA, A., 2008, An oxidative and salinity stress induced peroxisomal ascorbate peroxidase from *Avicennia marina*: molecular and functional characterization. *Plant Physiol. Biochem.*, **46**:794–804.
- KAZI, S. T., SOYOUNG SHIN, HO-LEUNG FUNG, 2017, Cellular interactions between L-arginine and asymmetric dimethylarginine: Transport and metabolism. *PLoS ONE*, **12(5)**: 1-15.

- KEEGSTRA, K. AND CLINE, K., 1999, Protein import and routing systems of chloroplasts. *Plant Cell*, **11**: 557–570.
- KEVIN C. LAMBIRTH, ADAM M., WHALEY, IVORY C. BLAKLEY, JESSICA A. SCHLUETER, KENNETH L. BOST, ANN E. LORAIN AND KENNETH J. PILLER, 2015, A Comparison of transgenic and wild type soybean seeds: analysis of transcriptome profiles using RNA-Seq. *BMC Biotechnol.*, **15**: 89-97.
- KIELISZEWSKI, M. J. AND LAMPORT, D. T. A., 1994, Extensin: Repetitive motifs, functional sites, post-translational codes and phylogeny. *Plant J.*, **5**: 157-172.
- KIRKEGAARD, J. A. AND LILLEY, J. M., 2007, Root penetration rate-a benchmark to identify soil and plant limitations to rooting depth in wheat. *Aust. J. Exp. Agr.*, **47**: 590-602.
- KLAAS J. VAN WIJK, 2001, Challenges and Prospects of Plant Proteomics. *Plant Physiol.*, **126**: 501-508.
- KLUTZ, D., 2005, Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.*, **67**: 225–257.
- KOTAK, S., M. PORT, A., GANGULI, F., BICKER, P. AND VON KOSKULL-DORING, 2004, Characterization of C-terminal domains of Arabidopsis heat stress transcription factors (HSFS) and identification of a new signature combination of plant class A HSFS with AHA and NES motifs essential for activator function and intracellular localization. *Plant J.*, **39**: 98–112.
- KOVACS, D., KALMAR, E., TOROK, Z. AND TOMPA, P., 2008, Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins. *Plant Physiol.*, **147**: 381–390.
- KRAMER, P. J. AND BOYER, J. S., 1995, Water Relations of Plants and Soils. *Academic Press, San Diego*.

- KRASENSK, Y. J. AND JONAK, C., 2012, Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J. Exp. Bot.*, **63**: 1593–1608.
- KUMAR, M. W., BUSCH, H., BIRKE, B., KEMMERLING, T., URNBERGER, F. AND SCHOFFL, 2009, Heat shock factors HSFB1 and HSFB2B are involved in the regulation of Pdf1.2 expression and pathogen resistance in *Arabidopsis*. *Mol. Plant*, **2**: 152–165.
- KUNST, L. AND SAMUELS, A. L., 2009, Plant cuticles shine: Advances in wax biosynthesis and export. *Curr. Opin. Plant Biol.*, **12**: 721-727.
- KWANUK LEE AND HUNSEUNG KANG, 2016, Emerging Roles of RNA-Binding Proteins in Plant Growth, Development, and Stress Responses. *Mol. Cell.*, **39(3)**: 179–185.
- LAKSHMI, G., RAMA, N., GOVIND, G., NATARAJA, K. N., BALI, G., SARANGI, S. K. AND UDAYAKUMAR, M., 2010, Overexpression of peanut RING (Really Interesting New Gene) Box Protein (*Rbx1*) improves abiotic stress resistance in tobacco. *J. Plant Biol.*, **37**: 301-308.
- LAMPORT, D. T. A., KIELISZEWSKI, M. J. AND SHOWALTER, A. M., 2006, Salt stress upregulates periplasmic arabinogalactan proteins: using salt stress to analyse AGP function. *New Phytol.*, **169**: 479-492.
- LAPIERRE, C., POLLET, B., PETIT-CONIL, M., TOVAL, G., ROMERO, J., PILATE, G., LEPLÉ, J. C., BOERJAN, W., FERRET, V. V., DE NADAI, V. AND JOUANIN, L., 1999, Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiol.*, **119**: 153–163.

- LASZLO, S. AND ARNOULD, S., 2009, Proline: a multifunctional amino acid. *Trends Plant Sci.*, **15**: 89-97.
- LEDFORD, H. K., CHIN, B. I. AND NIYOGI, K. K. 2007, Acclimation to singlet oxygen stress in *Chlamydomonas reinhardtii*, *Eukaryot. Cell.*, **6**: 919–930.
- LEE, J. H., HEUBEL, A. AND SCHEOFFL, F., 1995, Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic Arabidopsis. *Plant J.*, **8**: 603–612.
- LEE, S. P. AND CHEN, T. H., 1993, Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in brome grass (*Bromus inermis* Leyss) suspension culture. *Plant Physiol.*, **101**: 1089-1096.
- LEI, G. E., HUI CHEN., JIA-FU JIANG., YUAN ZHAO., MING-LI XU., YUNYUAN XU., KE-HUI, TAN., ZHI-HONG, XU. AND CHONG, K., 2004, Overexpression of OsRAA1 Causes Pleiotropic Phenotypes in Transgenic Rice Plants, including Altered Leaf, Flower, and Root Development and Root Response to Gravity. *Plant Physiol.*, **135**: 1502–1513.
- LI, X., LAWAS, L. M., MALO, R., GLAUBITZ, U., ERBAN, A., MAULEON, R., HEUER, S., ZUTHER, E., KOPKA, J. AND HINCHA, D. K., 2015, Metabolic and transcriptomic signatures of rice floral organs reveal sugar starvation as a factor in reproductive failure under heat and drought stress. *Plant Cell Environ.*, **38**: 2171–2192.
- LILA PIRKKALA, PA IVI NYKA NEN AND LEA SISTONEN, 2001, Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.*, **15**: 1118-1131.

- LIN, L., YAO GUANG LIU, XINPING XU, AND BAOJIAN LI, 2003, Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. *P. Natl. Acad. Sci.*, **100**: 5962–5967.
- LINDER, P. AND STUTZ, F., 2001, mRNA export: Travelling with DEAD box proteins. *Curr. Biol.*, **11**: R961-R963.
- LIU, A. L., ZOU, J., LIU, C. F., ZHOU, X. Y., ZHANG, X. W., LUO, G. Y. AND CHEN, X. B., 2013, Over-expression of OsHsfA7 enhanced salt and drought tolerance in transgenic rice. *BMB Rep.*, **46**: 31-36.
- LIU, J. G., QIN, Q. L., ZHANG, Z., PENG, R. H., XIONG, A. S., CHEN, JM. AND YAO, Q. H., 2009, OsHSF7 gene in rice, *Oryza sativa* L., encodes a transcription factor that functions as a high temperature receptive and responsive factor. *BMB Rep.*, **42(1)**: 16-21.
- LORETO, F. AND VELIKOVA, V., 2001, Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products and reduces lipid peroxidation of cellular membranes. *Plant Physiol.*, **127**: 1781-1787.
- LU, G., GAO, C., ZHENG, X. AND HAN, B., 2009, Identification of OsbZIP72 as a positive regulator of ABA response and drought tolerance in rice. *Planta*, **229(3)**: 605-15.
- LUO, Y., LIU, Y. B., DONG, Y. X., GAO, X. Q. AND ZHANG, X. S., 2009, Expression of a putative alfalfa helicase increases tolerance to abiotic stress in arabidopsis by enhancing the capacities for ROS scavenging and osmotic adjustment. *J. Plant Physiol.*, **166(4)**: 385–394.

- MAHZABIN AMIN, SABRINA M. ELIAS, ALAMGEER HOSSAIN, ALIYA FERDOUSI, MD. SAZZADUR RAHMAN, NARENDRA TUTEJA AND ZEBA I. SERAJ, 2012, Over-expression of a DEAD-box helicase, PDH45, confers both seedling and reproductive stage salinity tolerance to rice (*Oryza sativa* L.). *Mol. Breeding*, **30**: 345–354.
- MAMRUTHA, H. M., MOGILI, T., JHANSI LAKSHMI, K., RAMA, N., KOSMA, D., UDAYAKUMAR, M., JENKS, M. A. AND NATARAJA, K. N., 2010, Leaf cuticular wax amount and crystal morphology regulate post-harvest water loss in mulberry (*Morus species*). *Plant Physiol. Biochem.*, **48(8)**:690-6.
- MANJULATHA, M., ROHINI SREEVATHSA, A. MANOJ KUMAR, CHINTA SUDHAKAR, T. G. PRASAD, NARENDRA TUTEJA AND M. UDAYAKUMAR, 2014, Overexpression of a Pea DNA Helicase (PDH45) in Peanut (*Arachis hypogaea* L.) Confers Improvement of Cellular Level Tolerance and Productivity Under Drought Stress. *Mol. Biotechnol.*, **56**: 111–125.
- MANSCHADI, A. M., CHRISTOPHER, J., DEVOIL, P. AND HAMMER, G. L., 2006, The role of root architectural traits in adaptation of wheat to water-limited environments. *Funct. Plant Biol.*, **33**: 823-837.
- MAQBOOL, S. B., RIAZUDDIN, S., LOC, N. T., GATEHOUSE, A. M. R., GATEHOUSE, J. A. AND CHRISTOU, P., 2001, Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Mol. Breeding*, **7**: 85–93.
- MARCHANT, A., BHALERAO, R., CASIMIRO, I., EKLOF, J., CASERO, P. J., BENNETT, M. AND SANDBERG, G., 2002, AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. *Plant Cell*, **14**: 589–597.

- MARINA BATTAGLIA AND ALEJANDRA, A. COVARRUBIAS, 2013, Late Embryogenesis Abundant (LEA) proteins in legumes. *Front. plant sci.*, **4**:190-201.
- MARUYAMA, K., TAKEDA, M., KIDOKORO, S., YAMADA, K., SAKUMA, Y., URANO, K., FUJITA, M., YOSHIWARA, K., MATSUKURA, S., MORISHITA, Y., SASAKI, R., SUZUKI, H., SAITO, K., SHIBATA, D., SHINOZAKI, K. AND YAMAGUCHI-SHINOZAKI, K., 2009, Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiol.*, **150**(4): 1972-1980.
- MARUYAMA, K., URANO, K., YOSHIWARA, K., MORISHITA, Y., SAKURAI, N., SUZUKI, H., KOJIMA, M., SAKAKIBARA, H., SHIBATA, D. AND SAITO, K., 2014, Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. *Plant Physiol.*, **164**: 1759–1771.
- MATSUI, A., ISHIDA, J., MOROSAWA, T., MOCHIZUKI, Y., KAMINUMA, E., ENDO, T. A., OKAMOTO, M., NAMBARA, E., NAKAJIMA, M., KAWASHIMA, M., SATOU, M., KIM, J. M., KOBAYASHI, N., TOYODA, T., SHINOZAKI, K. AND SEKI, M., 2008, *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.*, **49**(8):1135-1149.
- MERY DAFNY-YELIN AND TZVI TZFIRA, 2007, Delivery of Multiple Transgenes to Plant Cells. *Plant Physiol.*, **145**: 1118–1128.
- MICHAL BASSANI, PETER, M. NEUMANN AND SHIMON GEPSTEIN, 2004, Differential expression profiles of growth-related genes in the elongation zone of maize primary roots. *Plant Mol. Biol.*, **56**: 367–380.

- MIKAE LUCAS, RANJAN SWARUP, IVAN A. PAPONOV, KAMAL SWARUP, ILDA CASIMIRO, DAVID LAKE, BENJAMIN PERET, SUSAN ZAPPALA, STEFAN MAIRHOFER, MORAG WHITWORTH, JIEHUA WANG, KARIN LJUNG, ALAN MARCHANT, GORAN SANDBERG, MICHAEL J. HOLDSWORTH, KLAUS PALME, TONY PRIDMORE, SACHA MOONEY, AND MALCOLM J. BENNETT., 2011, SHORT-ROOT Regulates Primary, Lateral, and Adventitious Root Development in Arabidopsis. *Plant Physiol.*, **155**: 384-398.
- MIN XU, LING ZHU, HUIXIA SHOU AND PING, W. U., 2005, A PIN1 Family Gene, OsPIN1, involved in Auxin-dependent Adventitious Root Emergence and Tillering in Rice. *Plant Cell Physiol.*, **46**:1674-168.
- MITTAL, D, D., ENOKI, Y., LAVANIA., SINGH, A., SAKURAI, H. AND GROVER, A., 2011, Binding affinities and interactions among different heat shock element types and heat shock factors in rice (*Oryza sativa* L.). *FEBS J.*, **278**: 3076–3085.
- MITTLER, R. S., VANDERAUWERA, M., GOLLERY, F. AND BREUSEGEM, V., 2004, Reactive oxygen gene network of plants. *Trends Plant Sci.*, **9**: 490 - 498.
- MOHAMMADI, M., KARIMIZADEH, R., SABAGHNIA, N. AND SHEFAZADEH, M. K., 2012, Effective application of canopy temperature for wheat genotypes screening under different water availability in warm environments. *Bulgarian J. Agric. Sci.*, **18**: 934–941.
- MOHAMMAD-ZAMAN AND SETSUKO, 2013, Subcellular protein overexpression to develop abiotic stress tolerant plants. *Front. Plant Sci.*, **4(2)**.
- MUENCH, D. G., ZHANG, C. AND DAHODWALA, M., 2012, Control of cytoplasmic translation in plants. *Wiley Interdiscip. Rev. RNA.*, **3(2)**:178-94.

- NAGESWARA RAO, R. C., SINGH, S., SIVAKUMAR, M. V. K., SRIVASTAVA, K. L. AND WILLIAMS, J. H., 1985, Effect of water deficit at different growth phases of peanut. L. yield responses. *Agron. J.*, **772**: 782–786.
- NAKAMURA, T., MURAMOTO, Y. AND TAKABE, T., 2004, Structural and transcriptional characterization of a salt-responsive gene encoding putative ATPdependent RNA helicase in barley. *Plant Sci.*, **167**: 63–70.
- NAKASHIMA, K. AND YAMAGUCHI-SHINOZAKI, K., 2005, Molecular studies on Stress-Responsive Gene Expression in Arabidopsis and Improvement of Stress Tolerance in Crop. *Plants Regulon. Biotechnol. JARQ.*, **39**:221-229.
- NATARAJA, K.N. AND JACOB, J., 1999, Clonal differences in photosynthesis in *Hevea Brasiliensis* Müll. Arg., *Photosynthetica.*, **36**: 89-98
- NAUTIYAL, P. C., RAVINDRA, V. AND JOSHI, Y. C., 1995, Gas exchange and leaf water relations in two peanut cultivars of different drought tolerance. *Biol. Plant.*, **37**: 371–374.
- NAYYAR, H. AND GUPTA, D., 2006, Differential sensitivity of C3 and C4 plants to water deficit stress: association with oxidative stress and antioxidants. *Environ. Exp. Bot.* **58**: 106-113.
- NETHRA, P., 2010, Functional validation of root growth associated genes by over expression in model plants and development of transgenics with a suitable candidate gene. Ph.D. Thesis. Bangalore, India: University of Agricultural sciences, GKVK.

NISHIZAWA-YOKOI, A., NOSAKA, R., HAYASHI, H., TAINAKA, H., MARUTA, T., TAMOI, M., IKEDA, M., OHME-TAKAGI, M., YOSHIMURA, K., YABUTA, Y. AND SHIGEOKA, S., 2011, *HsfA1d* and *HsfA1e* involved in the transcriptional regulation of *HsfA2* Function as key regulators for the Hsf signaling network in response to environmental stress. *Plant Cell Physiol.*, **52**, 933–945.

NOTHNAGEL, E. A., 1997, Proteoglycans and related components in plant cells. *Inter.Rev.Cytol.*, **174**: 195-291.

NOVER, L., BHARTI., K, DORING, P., MISHRA, S. K., GANGULI, A. AND SCHARF, K. D., 2001, Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need. *Cell Stress Chaperon.*, **6**: 177–189.

NULU NAGA PRAFULLA CHANDRIKA, KALAIPANDIAN SUNDARAVELPANDIAN, SU-MAY YU AND WOLFGANG SCHMIDT, 2013, ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis. *New Phytol.*, **198**: 709–720.

OGAWA, D.K., YAMAGUCHI, T. AND NISHIUCHI, 2007, High-level overexpression of the Arabidopsis HSFA2 gene confers not only increased thermotolerance but also salt/osmotic stress tolerance and enhanced callus growth, *J. Exp. Bot.*, **58**: 3373–3383.

OH, S. J., YOUN SHIC KIM, CHANG-WOO KWON, HYE KYONG PARK, JIN SEO JEONG AND JU-KON KIM, 2009, Overexpression of the transcription factor AP37 in Rice improves grain yield under drought conditions. *Plant physiol.*, **150**: 1368-1379.

- OOKA, H., SATOH, K., DOI, K., NAGATA, T., OTOMO, Y., MURAKAMI, K., MATSUBARA, K., OSATO, N., KAWAI, J., CARNINCI, P., HAYASHIZAKI, Y., SUZUKI, K., KOJIMA, K., TAKAHARA, Y., YAMAMOTO, K. AND KIKUCHI, S., 2003, Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. *DNA Res.*, **10**: 239–247.
- ORSEL, M., EULENBURG, K. AND DANIEL-VEDELE, A.K.F., 2004, Disruption of the nitrate transporter genes AtNRT2.1 and AtNRT2.2 restrict growth at low external nitrate concentration. *Planta*, **219**: 714–721.
- OWTTRIM, G. W., 2006, RNA helicases and abiotic stress. *Nucleic. Acids Res.*, **34**: 3220–3230.
- PARK, O. K., 2004, Proteomic Studies in Plants. *J. Biochem. Mol. Biol.*, **37**: 133-138.
- PATEL, J., MCLEOD, L.E., VRIES, R.G.J., FLYNN, ANDREA, WANG, X. AND PROUD, C.G., 2002, Cellular stresses profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors. *Eur. J. Biochem.* **269**: 3076–3085.
- PAUSE, A., METHOT, N., SVITKIN, Y., MERRICK, W. C. AND SONENBERG, N., 1994, Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF4F in cap-dependent and cap-independent initiation of translation. *EMBO J.*, **13(5)**: 1205–1215.
- PELTIER, J. B., FRISO, G., KALUME, D. E., ROEPSTORFF, P., NILSSON, F., ADAMSKA, I. AND VAN WIJK, K. J., 2000, Proteomics of the chloroplast: systematic identification and targeting analysis of luminal and peripheral thylakoid proteins. *Plant Cell*, **12**: 319–342.
- PENG, C., SHOUDONG Z., FENG, D., SHAHJAHAN, A. AND LIMING, X., 2014, Dynamic regulation of genome-wide pre-mRNA splicing and stress tolerance by the Sm-like protein LSm5 in *Arabidopsis*. *Genome Biol.*, **15**:R1.

- PERL, A., PERL-TREVES, R., GALILI, S., AVIV, D., SHALGI, E., MALKIN, S. AND GALUN, E., 1993, Enhanced oxidative –stress defense in transgenic in potato expressing tomato Cu-Zn Superoxide Dismutases. *Theor. Appl. Genet.*, **85**: 568 - 576.
- PERROUD, B. AND RUDULIER, D. L., 1985, Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J. Bacteriol.*, **161**(1): 393–401.
- PHAM, X. H., REDDY, M. K., EHTESHAM, N. Z., MATTA, B. AND TUTEJA, N., 2000, A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase 1 activity. *Plant J.*, **24**(2): 219–229.
- PINHEIRO, C., KEHR, J. AND RICARDO, C.P., 2005, Effect of water stress on lupin stem protein analysed by two-dimensional gel electrophoresis. *Planta*, **221**: 716–728.
- PLUSKAL, T., CASTILLO, S., VILLAR-BRIONES, A. AND ORESIC, M., 2010, MZmine 2: modular framework for processing, visualizing and analyzing mass spectrometry-based molecular profile data. *BMC bioinformatics*, **11**: 395.
- PRABHAVATHI, V., YADAV, J. S., KUMAR, P. A. AND RAJAM, M. V., 2002, Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphodehydrogenase gene. *Mol. Breeding*, **9**: 137-147.
- PRANDL, R., HINDERHOFER, K., EGGERS-SCHUMACHER, G. AND SCHOFFL, F., 1998, HSF3, a new heat shock factor from *Arabidopsis thaliana*, derepresses the heat shock response and confers thermotolerance when overexpressed in transgenic plants. *Mol. Gen. Genet.*, **258**:269–278.

- PRATIBHA, M. D., 2012, Genetic variability and relevance of epicuticular waxes: physiological characterization and differential expression of a few wax biosynthetic genes leading to allele mining. M. Sc thesis, University of Agricultural Sciences, Bangalore, India.
- PRATT, W.B. AND TOFT, D.O., 2003, Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.*, **228**: 111–133.
- PRIETO-DAPENA, P., CASTANO, R., ALMOGUERA, C. AND JORDANO, J., 2008, The ectopic overexpression of a seed specific transcription factor, HaHSFA9, confers tolerance to severe dehydration in vegetative organs. *The Plant J.*, **54**: 1001-1014.
- PUSHPA, D., YOGENDRA, K. N., GUNNAIAH, R., KUSHALAPPA, A. C. AND MURPHY, A., 2014, Identification of late blight resistance-related metabolites and genes in potato through nontargeted metabolomics. *Plant Mol. Boil. Rep.*, **32**: 584-595.
- QIAN, J., CHEN, J., LIU, Y. F., YANG, L. L., LI, W. P. AND ZHANG, L. M., 2014, Overexpression of Arabidopsis HsfA1a enhances diverse stress tolerance by promoting stress-induced Hsp expression. *Genet. Mol. Res.*, **13(1)**: 1233-1243.
- QUIMLO, C. A., TORRIZO, L. B., SETTER, T. L., ELLIS, M., GROVER, A., ABRIGO, E. M., OLIVA, N. P., ELLA, E. S., CARPENA, A. L., ITO, O., PEACOCK, W. J., DENNIS, E. AND DATTA, S. K., 2000, Enhancement of submergence tolerance in transgenic rice plants overproducing pyruvate decarboxylase. *J. Plant Physiol.*, **156**:516–521.
- QURESHI, M. I., QADIR, S. AND ZOLLA, L., 2007, Proteomics-based dissection of stress-responsive pathways in plants. *J. Plant Physiol.*, **164**: 1239–1260.

- REDDY, R. A., KUMAR, B., REDDY, P. S., MISHRA, R. N., MAHANTY, S., KAUL, T., NAIR, S., SOPORY, S. K. AND REDDY, M. K., 2009, Molecular cloning and characterization of genes encoding *Pennisetum glaucum* ascorbate peroxidase and heat shock factor: interlinking oxidative and heat-stress responses. *J. Plant Physiol.*, **166**: 1646–1659.
- RHODES, D. AND HANSON, A. D., 1993, Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Plant Mol. Biol.*, **44**: 357-384.
- RICH, S. M. AND WATT, M., 2013, Soil conditions and cereal root system architecture: review and considerations for linking Darwin and Weaver. *J. Exp. Bot.*, **64**(5):1193-1208.
- RICHARD I. MORIMOTO, 2017, Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, **12**: 3788–3796.
- RIECHMANN, J. L., HEARD, J., MARTIN, G., REUBER, L., JIANG, C., KEDDIE, J., ADAM, L., PINEDA, O., RATCLIFFE, O. J., SAMAHA, R. R., CREELMAN, R., PILGRIMM, BROUN, P., ZHANG, J. Z., GHANDEHARI, D., SHERMAN, B. K, AND YU, G., 2000, Arabidopsis transcription factor: genome wide comparative analysis among eukaryotes. *Science*, **290**: 2105–2110.
- RITOSSA, F., 1962, A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, **18**: 571–3.
- RIZHSKY, L., LIANG, H., SHUMAN, J., SHULAEV, V., DAVLETOVA, S. AND MITTLER, R., 2004, When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. *Plant Physiol.*, **134**: 1683–1696.
- ROZEMA, J. AND T. FLOWERS, 2008, Crops for a salinized world. *Science*, **322**: 1478–1480.

- RUCKER, K. S., KVIEN, C. K., HOLBROOK, C. C. AND HOOK, J. E., 1995, Identification of peanut genotypes with improved drought avoidance traits. *Peanut Sci.*, **24**: 14-18.
- RUTHERFORD, S. L., 2003, Between genotype and phenotype: protein chaperones and evolvability. *Nat. Rev. Genet.*, **4**: 263–274.
- SACHS, T., 1991, Pattern Formation in Plant Tissues. Cambridge University Press, Cambridge, UK.
- SADANA, A. AND DINH, T. V., 2001, Biomedical implications of protein folding and misfolding. *Biotechnol.*, **33**:7-16.
- SALAMO, I. P., PAPDI, C., RIGO, G., ZSIGMOND, L., VILELA, B., LUMBRERAS, V., NAGY, I., HORVATH, B., DOMOKI, M., DARULA, Z., MEDZIHRADSZKY, K., BOGRE, L., KONCZ, C. AND SZABADOS, L., 2014, The heat shock factor A4A confers salt tolerance and is regulated by oxidative stress and mitogen activated protein kinases MPK3 and MPK6. *Plant physiol.*, **165**: 319-334.
- SALEKDEH, G. H., REYNOLDS, M., BENNETT, J. AND BOYER, J., 2009, Conceptual framework for drought phenotyping during molecular breeding. *Trends Plant Sci.*, **14(9)**: 488-496.
- SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T., 1989, Molecular cloning: A laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor).
- SANAN-MISHRA, N., PHAM, X. H., SOPORY, S. K. AND N. TUTEJA., 2005, Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proc. Natl. Acad. Sci.*, **102**: 509 –518.
- SCHLESINGER, M. J., 1990, Heat shock proteins. *J. Biol. Chem.*, **265**:12111–12114.

- SCHMIDT, R., SCHIPPERS, J. H. M., WELKER, A., MIEULET, D., GUIDERDONI, E. AND MUELLER-ROEBER, B., 2012, Transcription factor *OsHsfC1b* regulates salt tolerance and development in *Oryza sativa* ssp. japonica. *AoB Plants*: pls011; doi:10.1093/aobpla/pls011.
- SERRAJ, R., KRISHNAMURTHY, L., KASHIWAGI, J. W., KUMAR, J., CHANDRA, S. AND CROUCH, J. H., 2004, Variation in root traits of chickpea (*Cicer arietinum* L.) grown under terminal drought. *Field Crops Res.*, **88**: 115–127.
- SHANKAR, A. G., 1988, Identification of conceptual types with low plant conductances and high photosynthetic rate and assessing their productivity under intermittent moisture stress condition. Ph. D. Thesis, Univ. Agric. Sci., Bangalore.
- SHANKAR., 2010, Development of multiple gene construct and overexpression of multigene cassette for abiotic stress tolerance in plants. MSc Thesis, Department of Crop Physiology, university of agricultural sciences, GKVK, Bangalore.
- SHEN, B., JENSEN, R. G. AND BOHNERT, H. J., 1997, Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. *Plant Physiol.*, **113**: 1177- 1183.
- SHEESHAYEE, M. S., BINDUMADHAVA, H., SHANKAR, A. G., PRASAD, T. G. AND UDAYAKUMAR, M., 2003, Breeding strategies to exploit water use efficiency for crop improvement. *J. Plant Bio.*, **30 (2)**: 253–268.
- SHIM, D., HWANG, J. U., LEE, J., LEE, S., CHOI, Y., AN, G., MARTINOIA, E. AND LEE, Y., 2009, Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. *Plant Cell*, **21**: 4031–4043.
- SHINDE B. M. AND LAWARE S. L., 2014, Screening of groundnut (*Arachis hypogaea* L.) varieties for drought tolerance through physiological indices. *J. Enviro. Res. Devel.*, **9(2)**: 375-380.

- SHOWALTER, A. M., 1993, Structure and function of plant cell wall proteins. *Plant Cell*, **5(1)**: 9-23.
- SHOWALTER, A. M., 2001, Arabinogalactan-proteins: structure, expression, and function. *Cell. Mol. Life Sci.*, **58**: 1399–1417. doi: 10.1007/PL00000784
- SHUKLA, R. K., RAHA, S., TRIPATHI, V., CHATTOPADHYAY, D., 2006, Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant Physiol.*, **142**: 113–123.
- SHUKLA, R.K., RAHA, S., TRIPATHI, V. AND CHATTOPADHYAY, D., 2006, Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant physiol.*, **142**: 113-123.
- SIDDIQUE, M. R. B., HAMID, A. AND ISLAM, M. S., 2001, Drought stress effects on water relations of heat. *Bot. Bull. Acad. Sin.* **41**: 35-39.
- SLAMA, I., ABDELLY, C., BOUCHEREAU, A., FLOWERS, T. AND SAVOURE, A., 2015. Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. *Ann. Bot.*, **115**: 433–447.
- SMIRNOFF, N. AND CUMBES, Q.J., 1989, Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, **28**: 1057- 1060.
- SONENBERG, N. AND HINNEBUSCH, A. G., 2009, Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. *Cell*, **136(4)**: 731745.
- SUBRAMANIAM, P., 1980, Growth and yield analysis in relation to population and planting patterns in ginger millet (*E.coracana* Gaertn.). M.Sc (Agri.) Thesis, Univ. Agric. Sci., Bangalore.

- SUN, C., GAO, X., FU, J., ZHOU, J. AND WU, X., 2015, Metabolic response of maize (*Zea mays* L.) plants to combined drought and salt stress. *Plant Soil*, **388**: 99–117.
- SWINDELL, W. R., HUEBNER, M. AND WEBER, A. P., 2007, Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genom.*, **8**: 125.
- SZABADOS, L. AND SAVOURE, A., 2010, Proline: a multifunctional amino acid. *Trends in Plant Sci.*, **15**: 89–97.
- SZEKELY, G., ABRAHAM, E. AND CSEPLO, A., 2008, Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. *Plant J.*, **53**: 11–28.
- THIMANN, K. V., 1977, Hormone Action in the Life of the Whole Plant. University of Massachusetts Press, Amherst, MA.
- TIMPERIO, A. M., EGIDI, M. G. AND ZOLLA, L., 2008, Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). *J. Proteomics.*, **71**: 391–411.
- TRENBERTH, K. E., A. DAI, G. VAN DER SCHRIER, P. D. JONES, J. BARICHIVICH, K. R. BRIFFA AND J. SHEFFIELD, 2014, Global warming and changes in drought. *Nat. Climate Change.*, **4**: 17–22.
- TRIPATHI, V., PARASURAMAN, B., LAXMI, A. AND CHATTOPADHYAY, D., 2009, CIPK6, a CBL interacting protein kinase is required for development and salt tolerance in plants. *Plant J.*, **58**: 778-790.
- TUBEROSA, R., 2012, Phenotyping for drought tolerance of crops in the genomics era. *Front. Physiol.*, **3**: 347.
- TUGIZIMANA, F., PIATER, L. AND DUBERY, I., 2013, Plant metabolomics: A new frontier in phytochemical analysis. *S Afr J Sci.*, **109**: 1-11.

- TUTEJA, N. AND PHAN, T. N., 1998, A chloroplast DNA helicase II from pea that prefers fork-like replication structures. *Plant Physiol.*, **118**: 1029–1038.
- TUTEJA, N. AND TUTEJA, R., 2004b, Unraveling DNA helicases. Motif, structure, mechanism and function. *Eur. J. Biochem.*, **271**: 1849–1863.
- TUTEJA, N., 2003, Plant DNA helicases: the long unwinding road. *J. Exp. Bot.*, **54**: 2201-2214.
- TUTEJA, N., BANU, S. A., KAMRUL HUDA, K., SINGH GRILL, S., JAIN, P., PHAM, X. H. AND TUTEJA, R., 2014, Pea p68, a DEAD-Box Helicase, Provides Salinity Stress Tolerance in Transgenic Tobacco by Reducing Oxidative Stress and Improving Photosynthesis Machinery. *Plos one*, DOI: 10.1371.
- TUTEJA, N., VASHISHT, A. A. AND TUTEJA, R., 2005. Structural and mechanistic aspect of DEAD-box helicases. *Natl. Acad. Sci. Lett.*, **28**: 313– 323.
- UDVARDI, M. K., KAKAR, K., WANDREY, M., MONTANARI, O., MURRAY, J., ANDRIANKAJA, A., ZHANG, J. Y., BENEDITO, V., HOFER, J. M. I., CHUENG, F. AND CHRISTOPHER, D., 2007, Town update on legume transcription factors. Legume transcription factors: global regulators of plant development and response to the environment. *Plant Physiol.*, **144**: 538–549.
- URANO, K., MARUYAMA, K., OGATA, Y., MORISHITA, Y., TAKEDA, M., SAKURAI, N., SUZUKI, H., SAITO, K., SHIBATA, D., KOBAYASHI, M., YAMAGUCHI-SHINOZAKI, K. AND SHINOZAKI, K., 2009, Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *Plant J.*, **7**: 1065-1078.

- VADEZ, V., KRISHNAMURTHY, L., KASHIWAGI, J. W., KHOLOVA, J., DEVI, J. M., SHARMA, K. K., BHATNAGAR-MATHUR, P., HOISINGTON, D. A., HASH, C. T., BIDINGER, F. R. AND KEATINGE, J. D. H., 2007a, Exploiting the functionality of root systems for dry, saline and nutrient deficient environments in a changing climate. *J. SAT Agric Res.*, **4**.
- VALLIYODAN, B. AND NGUYEN, H. T., 2006, Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curr. Opin. Plant Biol.*, **9**: 189-195.
- VASANTH KUMAR, G. K., 1975, Scheduling irrigation to finger millet varieties using can evaporimeter, M.Sc (Agri.) Thesis, Univ. Agric. Sci., Bangalore.
- VASHISHT AND TUTEJA, N., 2006, Stress responsive DEAD-box helicases: A new pathway to engineer plant stress tolerance. *J. Photo chem. Photo boil.*, **84**: 150–160.
- VASHISHT, A. A., PRADHAN, A., TUTEJA, R. AND TUTEJA, N., 2005. Cold- and salinity stress-induced bipolar pea DNA helicase 47 is involved in protein synthesis and stimulated by phosphorylation with protein kinase C. *Plant J.*, **44**: 76–87.
- VEMANNA S. RAMU, THAVAREKERE N. SWETHA, SHEKARAPPA H. SHEELA, CHANDRASHEKAR K. BABITHA, SREEVATHSA ROHINI, MALIREDDY K. REDDY, NARENDRA TUTEJA, CHANDRASHEKAR P. REDDY, TRICHI GANESH PRASAD AND MAKARLA UDAYAKUMAR, 2015, Simultaneous expression of regulatory genes associated with specific drought-adaptive traits improves drought adaptation in peanut. *Plant Biotechnol. J.*, 1–13.
- VEMANNA, R., BABITHA, K., CHANDRASHEKAR, H. M., RAO, H., SHAILESH, K., SATHYANARAYANAGUPTA, K. S., SARANGI, KARABA, N. N. AND UDAYAKUMAR, M. A., 2013. Modified MultiSite Gateway Cloning Strategy for Consolidation of Genes in Plants. *Mol. Biotechnol.*, **53(2)**:129-138.

- VISWANATHAN CHINNUSAMY AND JIAN-KANG ZHU, 2009, Epigenetic regulation of stress responses in plants. *Curr. Opin. Plant Biol.*, **12**: 133-139.
- WANG, C., ZHANG, Q. AND SHOU, H., 2009, Identification and expression analysis of OsHsfs in rice. *J. Zhejiang Univ. Sci. B.*, **10(4)**: 291-300.
- WANG, W., BASIA VINOCUR, ODED SHOSEYOV AND ARIE ALTMAN, 2004, Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.*, **9**: 1360-1385.
- WANG, Y., YING, Y., CHEN, J. AND WANG, X. C., 2004, Transgenic Arabidopsis overexpressing Mn-SOD enhanced salt-tolerance. *Plant Sci.*, **167**: 671-677.
- WATSON, D. J., 1947, Comparative physiological studies on the growth of field crops I. variation in net assimilation rate and leaf area between species and varieties within and between years. *Ann. Bot. N.S.*, **11**: 41-76.
- WATSON, D. J., 1952, Physiological basis of variation in yield. *Adv. Agron.*, **4**: 101-145.
- WEN, G., XUE, J., SHEN, Y., ZHANG, C. AND PAN, Z., 2009, Characterization of classical swine fever virus (CSFV) non-structural protein 3 (NS3) helicase activity and its modulation by CSFV RNA- dependent RNA polymerase. *Virus Res.*, **141**: 63-70.
- WHITE, R. G. AND KIRKEGAARD, J. A., 2010, The distribution and abundance of wheat roots in a dense, structured subsoil – implications for water uptake. *Plant Cell Environ.*, **33**: 133-148.
- WILKINSON, S. AND DAVIES, W. J., 2008, Manipulation of the apoplastic pH of intact plants mimics stomatal and growth responses to water availability and microclimatic variation. *J. Exp. Bot.*, **59**: 619–631.

- WINICOV, I. AND BASTOLA, D. R., 1997, Salt tolerance in crop plants: new approaches through tissue culture and gene regulation. *Acta Physiol. Plant*, **19**: 435–449.
- WINICOV, I. AND DEUTCH, C. E., 1994, Characterization of a cDNA clone from salt-tolerant alfalfa cells that identifies salt inducible root specific transcripts. *J Plant Physiol.*, **144**: 222–228.
- WINICOV, I., 2000, Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. *Planta*, **210**: 416-422.
- WOO YONG LEE, DAEYOUNG LEE, WON-IL CHUNG AND CHANG SEOB KWON, 2009, Arabidopsis ING and Alfin1-like protein families localize to the nucleus and bind to H3K4me3/2 via plant homeodomain fingers. *Plant J.*, **58**: 511–524.
- WU, H., DE GRAAF, B., MARIANI, C. AND CHEUNG, A. Y., 2001, Hydroxyproline-rich glycoproteins in plant reproductive tissues: structure, functions and regulation. *Cell Mol. Life Sci.*, **58**: 1418-1429.
- WULFF-ZOTTELE, C., GATZKE, N., KOPKA, J., ORELLANA, A., HOEFGEN, R., FISAHN, J. AND HESSE, H., 2010, Photosynthesis and metabolism interact during acclimation of *Arabidopsis thaliana* to high irradiance and sulphur depletion. *Plant Cell Environ.*, **33**: 1974–1988.
- WYN JONES, R. G. AND STOREY, R., 1981, Betaines. In "The Physiology and Biochemistry of Drought Resistance in Plants" (LG Paleg, D Aspinall eds), Academic Press, Sydney, pp 171-204.
- XIE, Q., FRUGIS, G., COLGAN, D. AND CHUA, N. H., 2000, Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev.*, **14**: 3024–3036.

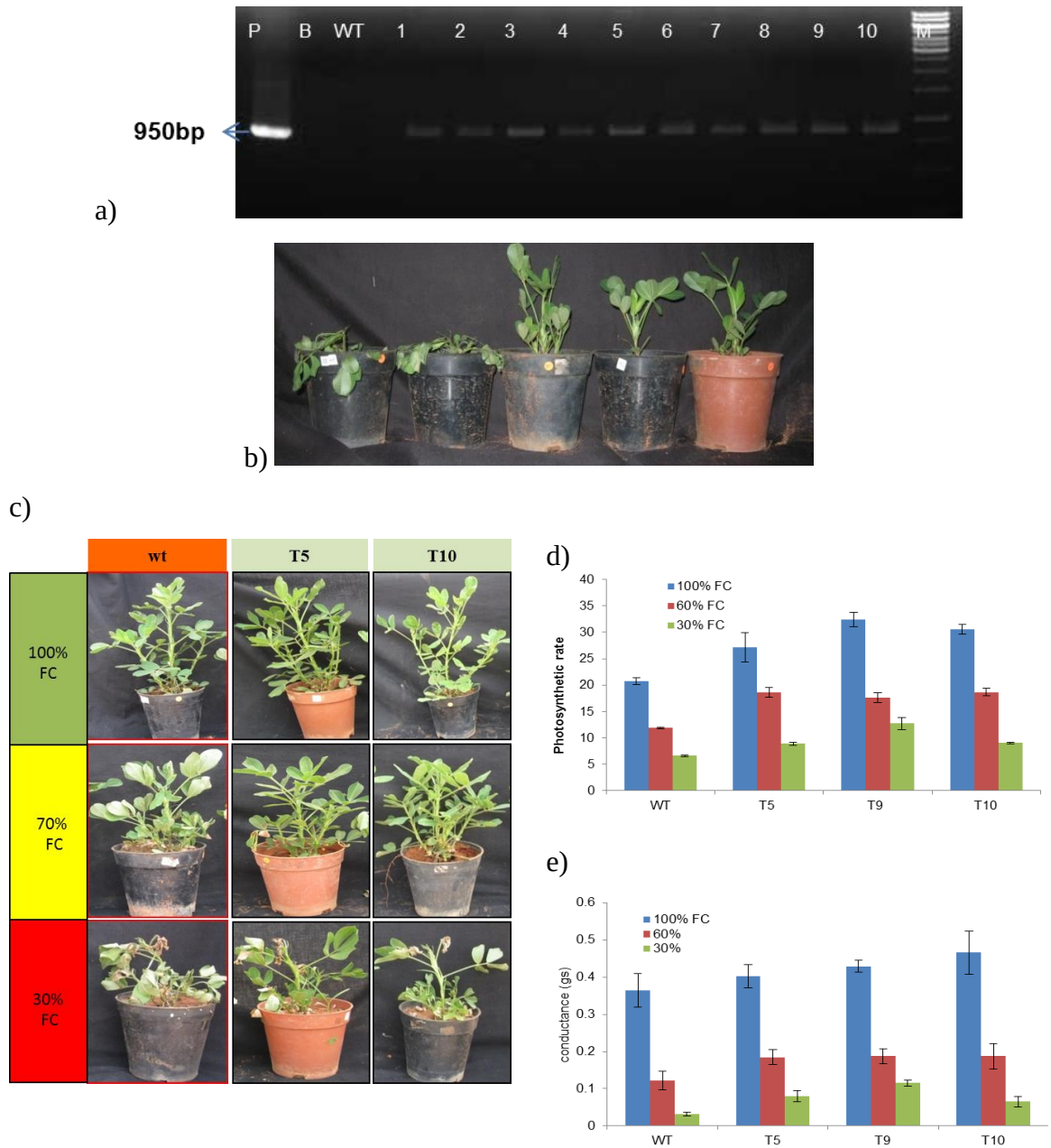
- XIE, Q., GUO, H. S., DALLMAN, G., FANG, S. Y., WEISSMAN, A. M. AND CHUA, N. H., 2002, SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. *Nature*, **419**: 167–170.
- XIUKUN CUI, LEI WANG, JING ZHANG, RONG DU, SHENGJIE LIAO, DUANZHUO LI, CHANG LI, TIE KE, DAVID WAN-CHENG LI, HUA HUANG, ZHAN YIN, ZHAOHUI TANG AND MUGEN LIU, 2013, HSF4 regulates DLAD expression and promotes lens de-nucleation. *BBA-Mol. Cell Res.*, **1832**: 1167-1172.
- XU, M., ZHU, L., SHOU, H. AND WU, P., 2005, A PIN1 family gene, *OsPIN1*, involved in auxin-dependent adventitious root emergence and tillering in rice. *Plant Cell Physiol.*, **46**: 1674–1681.
- XUEKUN ZHANG, GUANGYUAN LU, WEIHUA LONG, XILING ZOU, FENG LI AND TAKESHI NISHIO, 2014, Recent progress in drought and salt tolerance studies in Brassica crops. *Breeding Sci.*, **64**: 60–73.
- YADAV, S. K., SINGLA-PAREEK, S. L., RAY, M., REDDY, M. K. AND SOPORY, S. K., 2005, Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochem. Biophys. Res. Commun.*, **337**: 61–67.
- YAMADA, M., MORISHITA, H., URANO, K., SHIOZAKI, N., YAMAGUCHI-SHINOZAKI, K., SHINOZAKI, K., YOSHIBA, Y., 2005, Effects of free proline accumulation in petunias under drought stress. *J. Exp. Bot.*, **56**: 1975–1981.
- YAMAGUCHI, K. AND SUBRAMANIAN, A. R., 2000, The plastid ribosomal proteins. Identification of all the proteins in the 50 s subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.*, **275**: 28466–28482.
- YAMAGUCHI, K., VON KNOBLAUCH, K. AND SUBRAMANIAN, A. R., 2000, The plastid ribosomal proteins. Identification of all the proteins in the 30 s subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.*, **275**: 28455–28465.

- YAMANOUCHI, U., YANO, M., LIN, H., ASHIKARI, M. AND YAMADA, K., 2002, A rice spotted leaf gene, *Spl7*, encodes a heat stress transcription factor protein. *Proc. Natl. Acad. Sci.*, **99**: 7530–7535.
- YAMBAO, E. B., INGRAM, K. T. AND REAL, J. G., 1992, Root xylem influence on the water relations and drought resistance of rice. *J. Exp. Bot.*, **43**: 925–932.
- YE, X., BABILI, A. S., KLTI, A., ZHANG, J., LUCCA, P., BEYER, P. AND POTRYKUS, I., 2000, *Science*, **287**: 303–305.
- YE, Z. H., SONG, Y. R., MARCUS, A. AND VARNER, J. E., 1991, Comparative localization of three classes of cell wall proteins. *Plant J.*, **1**: 175-183.
- YILIANG, D., TANG, Y., KWOK, C. K., ZHANG, Y., PHILIP, C. B. AND ASSMANN, S. M., 2014, In vivo genome wide profiling of RNA secondary structures reveals novel regulatory features. *Nature*, **505**: 696-700.
- YOKOTANI, N., ICHIKAWA, T., KONDOU, Y., MATSUI, M., HIROCHIKA, H., IWABUCHI, M., ODA, K., 2008, Expression of rice heat stress transcription factor OsHsfA2e enhances tolerance to environmental stresses in transgenic *Arabidopsis*. *Planta*, **227**: 957–967.
- YOSHIMURA, A. K., MIYAO, K., GABER, A., TAKEDA. T., KANABOSHI, H., MIYASAKA, H. AND SHIGEOKA, S., 2004, Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. *Plant J.*, **37**: 21–33.
- YOZO OKAZAKI AND KAZUKI SAITO, 2012, Recent advances of metabolomics in plant biotechnology. *Plant Biotechnol. Rep.*, **6**: 1–15.
- YU SONG, JIE GAO, FENGXI YANG, CHAI-SHAN KUA, JINGXIN LIU AND CHARLES H. CANNON, 2013, Molecular Evolutionary Analysis of the Alfin-Like Protein Family in *Arabidopsis lyrata*, *Arabidopsis thaliana* and *Thellungiella halophila*. *PLoS One*, **8(7)**: 1-10.

- ZDRAVKO J. LORKOVIC, DOMINIKA, A., WIECZOREK KIRK, MARK, H. L., LAMBERMON AND WITOLD FILIPOWICZ, 2000, Pre-mRNA splicing in higher plants. *Trends plant sci.*, **5**: 1360-1385.
- ZENG, T., LIU, S., LUO, R., GONG, P., ZHAO, D. AND FANG, X., 2011, Cloning and expression of an alcohol dehydrogenase from *Lotus japonicus* and characterization of *LjADH1*. *Legume Genomics Genet.*, **2**: 6-13.
- ZHANG, H., LIANG, W., YANG, X., LUO, X., JIANG, N., MA, H. AND ZHANG, D., 2010, Carbon starved anther encodes a MYB domain protein that regulates sugar partitioning required for rice pollen development. *Plant Cell*, **22**: 672–689.
- ZHANG, J. Y., COREY, D. B., ELISON, B. B., MARRY, K. S., LLOYD, W. S. AND ZENG, Y. W., 2005, Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *The Plant J.*, **42**: 689-707.
- ZHU, B., YE, C., LEU, H., CHEN, X., CHAI, G., CHEN, J. AND WANG, C., 2006, Identification and characterization of a novel heat shock transcription factor gene, GmHsfA1, in soybeans (*Glycine max*). *J. Plant. Res.*, **119**: 247–256.

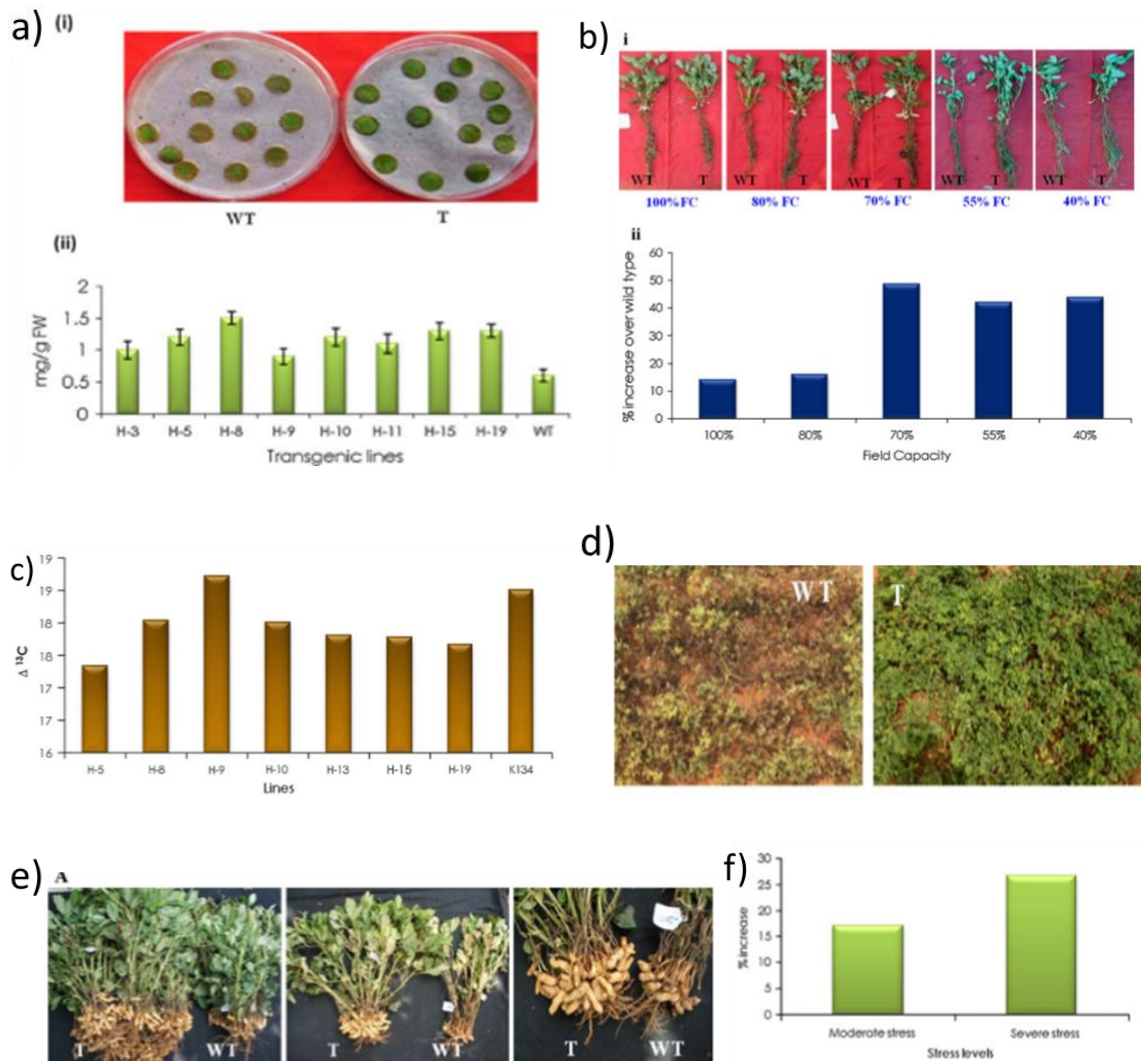
Annexures

ANNEXURE I



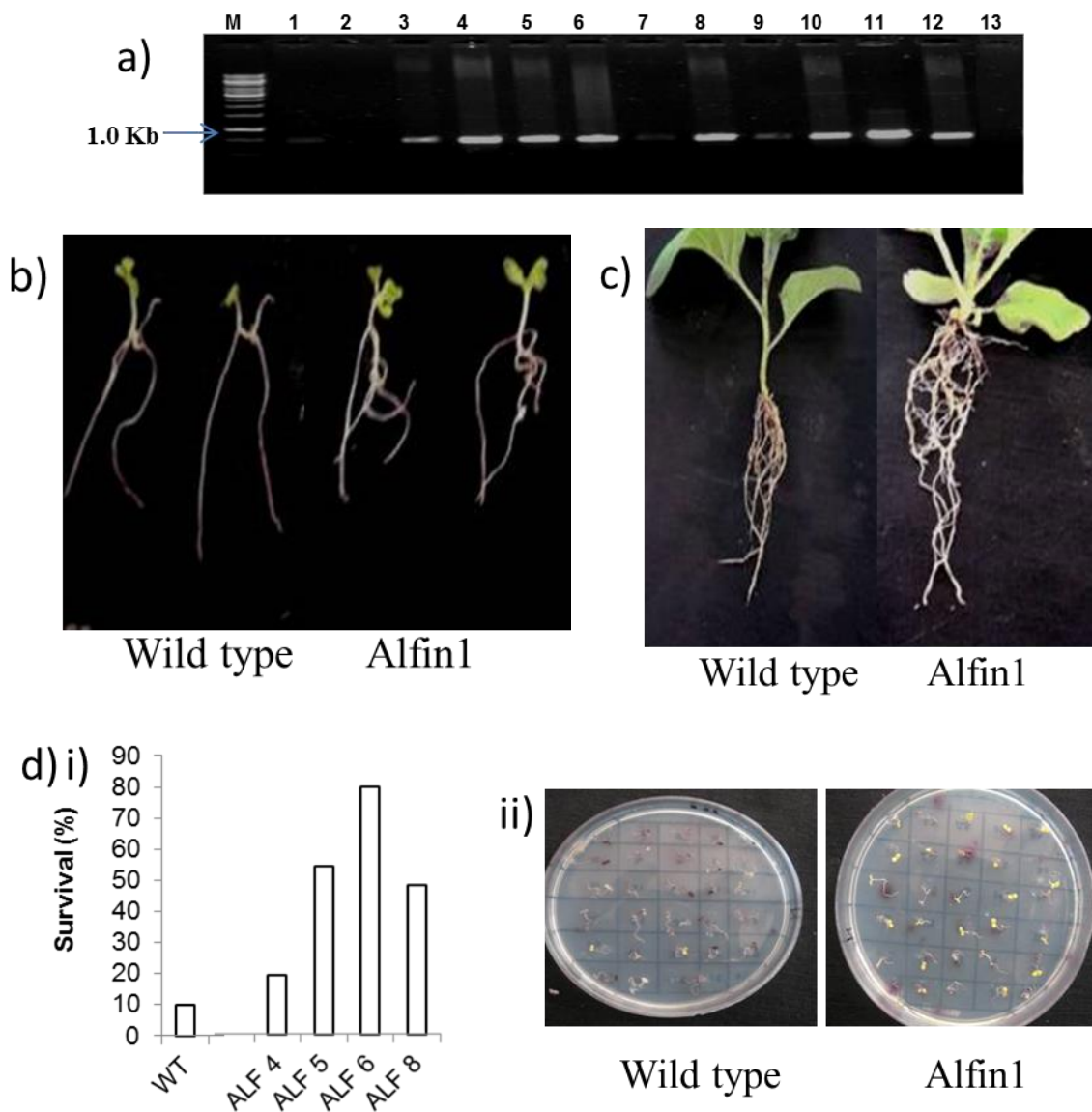
Groundnut transgenic overexpressing *PgHsf4* confers stress tolerance. a) PCR confirmation of transgenic plants by using gene specific primers b) Transgenic plant showing tolerance to high temperature c) Moisture stress tolerance of transgenic plants d) Photosynthetic rate e) stomatal conductance in wild type and transgenic plants under control and stress conditions.

ANNEXURE II



Groundnut transgenic overexpressing *PDH45* confers stress tolerance a) i) Response of the T3 generation transgenic lines to ethylene-induced senescence; variation in the total chlorophyll content of wild type and transgenic lines after treatment with 2,000 ppm etherel. ii) Chlorophyll content in the leaf disks after etherel exposure b) Response of the transgenic event, to desiccation stress i) variation in the growth compared to wild type under different soil water regimes ii) variation in root volume at different water stress levels over wild type c) Estimation of $\Delta^{13}C$, a surrogate for WUE in the transgenic lines and wild type d) Stay-green phenotype observed till the end of crop growth period in transgenic lines e) Improved productivity in the transgenics in comparison with the wild type f) Overall percent increase in yield in the transgenic lines over wild type under moderate and severe stress

ANNEXURE III



Tobacco plant overexpressing Alfin1 showed improved root growth and salinity tolerance. a) PCR analysis of tobacco (T0) putative OsAlfin1 transformants using gene specific primers b) Root phenotype of one week old seedlings of wild type and T1 transgenic tobacco plants expressing *Alfin1* c) Root phenotype of one month old seedlings of T1 transgenic tobacco expressing *Alfin1* d) i) Percent survival of transgenic tobacco seedlings subjected to NaCl (300mM) stress ii) Photograph showing survivability of transgenic seedlings subjected to 300mM NaCl stress. T1 transgenic seedlings (one week old) expressing *Alfin1* under constitutive promoter were subjected to NaCl (300mM) stress for one week and survival (%) was calculated.

ANNEXURE IV

Summary of T2 transgenics



Wild type seedlings treated with 450PPM of kanamycin



Wild type



Transformants treated with 450ppm of Kanamycin



Transgenics

Response of T2 transformants and wild type seedlings to kanamycin. The pre-germinated seeds were treated with kanamycin 450 ppm for 5 hour and subsequently sown in plastic trays containing sterilized quartz sand. The plant growth was assessed over a period of 10 days. Photograph was taken after 10 days of treatment period.

Yield analysis of transgenic and wild type plants. 102 T2 groundnut transgenic and wild type plants were grown in contained facility and various yield parameters such as pod number, pod dry weight and total dry matter was recorded after harvesting.

Plants	n	Pod no		Pod dry wt	
		Mean	Range	Mean	Range
Transgenics	102	63.24±9.5	30.6-104	26.1±7.3	15.5-42
Wild type	10	42.4±14.9	28-47.08	16.55 ±3.22	14-17.5

Based on kanamycin response, phenotype and yield analysis 99 transgenic lines were selected for T3 generation.