Studies on the development of abiotic stress tolerance in groundnut (*Arachis hypogaea* L.) by genetic transformation

Thesis Submitted for the degree

of

Doctor of Philosophy

In Biotechnology

By

Pooja Bhatnagar-Mathur

Center for Biotechnology
Institute of Science and Technology
Jawaharlal Nehru Technological University
Hyderabad
2006

Dedicated to my

Ammaa and Maa...

Dedicated to

Farmers of the semi-arid tropics,
The ultimate beneficiaries of the products of biotechnology

CERTIFICATE

Certified that the entire work embodied in this thesis entitled **"Studies on the development of abiotic stress tolerance in groundnut (*Arachis hypogaea* L.) by genetic transformation"** has been carried out by **Ms. Pooja Bhatnagar-Mathur** for the degree of Doctor of Philosophy, under my supervision in the Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. This work is original and has not been submitted so far, in part or in full, for the award of any degree or diploma of any University.

Dr. Kiran K Sharma
Principal Scientist
Genetic Transformation Laboratory
ICRISAT, Patancheru
Andhra Pradesh 502 324, India

DECLARATION

I hereby declare that this thesis entitled **"Studies on the development of abiotic stress tolerance in groundnut (*Arachis hypogaea* L.) by genetic transformation"**, comprises of my own work except where specifically stated to the contrary, and it is not substantially the same as my thesis that has been submitted for any degree to any other university.

Pooja Bhatnagar-Mathur

Acknowledgements

This thesis is the result of four years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I now have the opportunity to express my gratitude for all of them. My heartfelt thanks go to my mentor, **Dr. KK Sharma**, for his warm personality, continual encouragement, support, and perseverance in guiding me through the entire research and dissertation-writing process I am especially indebted for his role in developing the research project and for his patience in handling my shortcomings, which have helped me immensely towards developing my research skills, promoting self-confidence and autonomy. I thank him for giving me the important lessons in conducting an independent and fruitful research and for the strong scientific background in genetic transformation technology.

My heartfelt thanks go to **Dr. Vincent Vadez** for the planned physiological experiments .His overly enthusiasm and integral view on research and mission for providing 'only high-quality work and not less' has made a deep impression on me. I would also like to thank **Dr. S. N. Nigam** and **Dr. R. Serraj** for their valuable suggestions during this study I greatly acknowledge **JIRCAS, Japan** and **Dr. Kazuko Yamaguchi-Shinozaki** for providing the gene constructs used in this study

I appreciate the assistance and support from **Dr. Lakshmi Narasu**, Head Deptt. of Biotechnology, JNTU for the valuable guidance and instructions throughout my research period

I sincerely thank the Learning Systems Unit (LSU), **ICRISAT**, for permitting me to avail the research facilities at ICRISAT. I thank **ICRISAT** for providing me with sophisticated infrastructure, and **ICRISAT Library** staff for their help and co-operation

I thank all my colleagues in research group at the Genetic Transformation Laboratory for the motivating work atmosphere. I owe a great debt of gratitude to my colleagues at ICRISAT for creating stimulating work environment. I am grateful to my colleagues **Sreelatha, Mamta, Srinivas Reddy, S. Chander Rao, Krishna, Sreedhar, Vani, Sowmini, Prashanti** and all others for their active moral support and participation in this study. I would specially like to mention name of my colleague **Jyostana** for offering her help in this research.

I greatly appreciate the day-to-day assistance and technical support from **C. Lakshminarayana, D. Pandary, Mohd. Yousuf, JaganMohan Reddy, Manamma and laxmi** in the lab. without whom the experiments could not have been completed.

No words are enough to express my heartiest thanks to my **Amma**, who has always buoyed up my higher education. I can't thank her enough. The happy memory of my **mom** still

provides a persistent inspiration for my journey in this life. I feel a deep sense of gratitude for my ***father****, who formed part of my vision and taught me the good things that really matter in life. He has been extremely strong and supportive for me over the years.*

No words are enough to express my heartiest thanks to my sisters ***Priyanka*** *and* ***Madhureema*** *for the help rendered in all ways throughout the period of my research. I thank them for their everlasting support in every way and encouragement over the years.*

I owe lots to my husband ***"Vaibhav"*** *for being extremely indulgent during these years and for the enormous support I have received from him without which this work would never have even been contemplated. You have helped me to understand the meaning of an old Chinese proverb: "Love is greater than truth". I should thank my son* ***"Vedant"*** *for remaining nice and healthy.*

I thank my ***in-laws*** *who have always been a support and have been very patiently waiting for me to get this thesis done. I am also grateful to* ***Garima, Siddhartha, Gaurav*** *and* ***Samita*** *for their active moral support during this study. The chain of my gratitude would be definitely incomplete if I would forget to thank the rest of my family members for having confidence in me. Also, I wish to thank all those who have helped me complete this work directly and indirectly.*

I am deeply indebted to ***Dr. William D. Dar, Director General ICRISAT****, for providing me an opportunity to undertake my Ph.D work at ICRISAT, Patancheru and making me understand the real meaning of* ***Science with a human face****.*

My deepest and sincere gratitude towards the ***"Heavenly Power"*** *for inspiring and guiding me!*

CONTENTS

List of Tables

List of Figures

ABBREVIATIONS

°C	degree Celsius
µg/L	microgram per liter
µl	microliter
µM	micromole
2,4-D	dichlorophenoxyacetic acid
2-iP	2-[isopentenyl] adenine
BA	N^6-benzyladenine
bp	base pair
cDNA	complementary deoxyribonucleic acid
cm	centimeter
dNTP	deoxy nucleotide triphosphate
d	day (s)
DEAE	diethyl amino ethyl cellulose
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
FTSW	fraction of transpirable soil water
GA_3	gibberellic acid
GUS	β-glucuronidase
h	Hour
ha	hectare
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kb	kilo basepair
kg	kilogram
Kn	kinetin
L	litre
LB	Luria Broth
LD_{50}	lethal dose at 50% mortality
M	molar
mg/L	milligram per liter
min	minutes

ml	milliliter
mm	millimeter
MS	Murashige and Skoog's medium
NAA	α-naphthaleneacetic acid
NTR	normalized transpiration rate
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	negative logarithm of H^+ ion
RIM	root induction medium
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s	second (s)
SAT	semi-arid tropics
SCMR	SPAD chlorophyll meter reading
SEM	shoot elongation medium
SIM	shoot induction medium
SLA	specific leaf area
SSC	sodium chloride and sodium citrate
TAE	Tris acetate-EDTA
T-DNA	transfer DNA
TDZ	thidiazuron [1-phenyl-3-(1,2,3-thidiazol-5- yl) urea]
TE	Tris-EDTA
Ti	tumour inducing plasmid
Tris	Tris(hydroxymethyl) methylamine
uv	ultraviolet
vol	volume
w/v	weight per volume
wk	week
wt	weight
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YEB	Yeast extract broth

1. INTRODUCTION

INTRODUCTION

Grain legumes, together with cereals, have played a key role for the development of modern agriculture. Since the dawn of civilization, many legume species have been used as human food (e.g., soybean, common bean, pea, peanut, lentil, pigeonpea, chickpea etc.), edible oils (peanut and soybean), animal fodder and forage (alfalfa and clover). Legumes are second in importance for human and animal dietary needs (Vietmeyer, 1986). They provide 33% of mankind's nutritional nitrogen requirements. Worldwide, legumes are grown on about 15% of the arable land (270-300 million hectares). Demand for grain legumes is increasing over the years due to high population growth. Moreover, the per capita availability of cropland has decreased tremendously during the last few years in the developing countries such as India, China, Pakistan, Bangladesh, and Philippines in addition to many countries in the Middle East and Africa, indicating a need to increase the production of legumes and pulses in these regions of the world. During the last century, most agronomic research and production have focused on increasing yields of food and fiber crops (Abelson, 1994).

Globally, efforts have been made to improve the productivity of grain legumes to allow more widespread cultivation of these protein-rich crops. Genetic variation in legume species and their wild relatives has been an important component for successful breeding of improved crop cultivars with added value and durable resistance to insect-pests and diseases. However with the intensification of agriculture due to the ever-increasing demands for food due to increased population pressures, the currently available genetic variation in the genetic pool of cultivated crops and their wild relatives may not be sufficient for further breakthroughs in crop

improvement. Also, there are some complex issues to tackle, such as the abiotic stresses, that conventional approaches have not been able to solve. Efforts involving conventional and biotechnological approaches for the genetic enhancement have immense potential to overcome that complexity and develop resistance/tolerance to various biotic and abiotic constraints affecting the productivity of important crop plants of the semi-arid tropics (SAT) of the world.

The application of biotechnological methods for the improvement of crop plants has been shown to hold great potential. These include (1) Molecular markers and marker-assisted selection where it is possible to identify and map the factors controlling characters as intransigent as yield (Thoday, 1961). Loci which account for a significant genetic variation in traits are mapped using molecular markers based on random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), DNA amplification polymorphism (DAF), sequence characterized amplified regions (SCAR's), sequence-tagged sites (STS), expressed sequence tags (EST's), and amplicon length polymorphisms (ALPs). (2) Genetic transformation approaches have provided opportunities for increasing the productivity of grain legumes for sustaining the food security worldwide. Genetic Engineering approaches have been shown to be comparatively precise and fast, leading to better isolation and cloning of desired traits for combating biotic and abiotic stresses. Genetic improvement for drought tolerance is crucial in many places of the world where agriculture depends on scarce water resources.

Groundnut *(Arachis hypogaea* L.) is one of the important food legume crops of the semi-arid tropics of the world. Groundnut is an annual oil seed belonging to the family of leguminosae and sub-family papillionacea and a native to South America.

However, the geographical classification of groundnut is delineated into six regions: the America, Africa, Asia, New East Asia, Europe and Oceania (Gregory et al., 1980). Groundnut comprises of diploid (2n=20), tetraploid (2n=40) and octaploid (2n=80) and amphidiploid species. Groundnut seed contains over 50% of high quality edible oil and 25% protein (Norden, 1980). The crop is utilized in several ways; the edible oil is an important source for human consumption and the meal is used for livestock feed. It is also used directly for food in industrial countries including USA, Canada and the European Union. The oil may be extracted and used for cooking and the residual cake is used most commonly in animal feeds while the shells may be ground and used as filler in animal feed (Nigam et al., 1991). It is cultivated on 24.8 million ha land area with a total production of 32.8 million t with an average productivity of 1.32 t ha^{-1}. Developing countries account for 96.9% and 93.8% of total production mainly concentrated in Asia and Africa. Of about 100 countries involved in the production of groundnut, India ranks first. On the global scale India is the major producer of groundnut with a total production of 8.9 million tons per year. Other important countries in the order of their production are China, USA, Indonesia, Senegal, Nigeria, Myanmar, Sudan, and Argentina (FAO, 2000).

Since the mid-70's edible groundnuts have increased in both domestic consumption and export trade. In contrast, the production in Africa has declined by 17% during the last two decades. The major reasons for such low production are various biotic and abiotic stresses (Cummins and Jackson, 1982). The disease caused by *Aspergillus flavus* that produces aflatoxins, for which no adapted genotype with sustained resistance is available, adversely affects groundnut commodity and quality. Foliar diseases such as early and late leaf spots caused by *Cercospora arachidicola* and *C. personatum,* respectively, are the most damaging diseases (Subramanyam et

al., 1985). Amongst the insect pest, Spodoptera, legume podborer, aphids and thrips cause the greatest losses to the groundnut crop (Wightman and Ranga Rao, 1994). About 80% of world's production of groundnut is from resource poor smallholder farmers in developing countries who obtain low yields of 500-800 kg per ha. In many cases, the poor yields are because of water scarcity primarily due to unreliable rainfall patterns with frequent droughts, lack of high yielding adapted cultivars, damage by diseases and pests, poor agronomic practices and limited use of inputs.

The major constraint in the production of groundnut is the unpredictable and unseasonal rainfall (Nageshwara Rao and Nigam, 2001). Approximately 19% of the world's agricultural land is subjected to salt stress and 5% to drought stress (FAO 1996). Annual estimated losses in groundnut productivity, equivalent to over US$ 520 million are caused by drought (Sharma and Lavanya, 2002). Drought is a major abiotic stress factor affecting yield and quality of rainfed groundnut worldwide. A major problem is the pre-harvest contamination of groundnut with aflatoxin due to prolonged drought conditions. Yield losses due to drought are highly variable in nature depending on timing, intensity, and duration coupled with other location-specific environmental stress factors such as high irradiance and temperature. Groundnut is grown mostly under rainfed conditions and is usually facing intermittent drought conditions, i.e. episodes of water scarcity of differing length between rainfall events

The conventional methods of combating abiotic stress in groundnut include (A) agronomic methods where the adaptive response to drought can be increased by exposing the crop to short duration drought during the vegetative phase, which may enhance the root development and reduce transpirational losses by limiting the leaf area development thus allowing the plant to utilize the soil moisture from the deeper

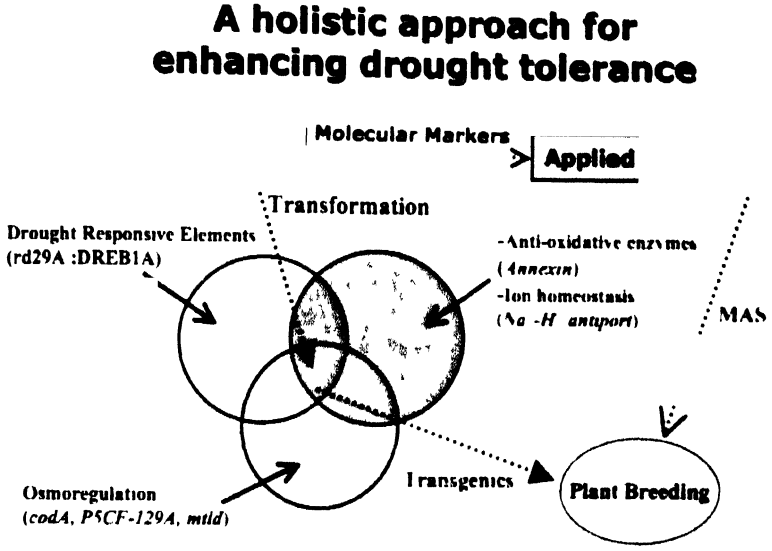
soil profile (B) plant breeding methods which select the genotypes based on their fit to the historical weather and soil data of target location, thereby improving the overall efficiency of the crop. However, the major drawback of such drought management methods is that the available stress tolerant traits in the natural system are not amenable for breeding strategies due to species barrier and also the lacking of proper methods and techniques for screening in the segregating populations thus obtained.

In recent years, the genes that are responsible for low-molecular-weight metabolites have been shown to confer increased tolerance to salinity or drought stresses in transgenic dicot plants (mainly tobacco). Metabolic traits, especially pathways with few enzymes have been characterized genetically and are more amenable to manipulations than structural and developmental traits. Various transgenic technologies have been used to improve stress tolerance in plants (Allen, 1995). The physiological responses to the stress usually arise out of change in cellular gene expression (Shinozaki and Yamaguchi-Shinozaki, 1999). As a result, changes in the integrity of the cellular membrane, imbalance in the homeostatic conditions and finally the decline in growth or death of the plants are usually noticed (Zhu, 2001). Plants can be tailored to tolerate stress if the damage that occurs at physiological and cellular level is known. Certain genes are expressed at elevated levels when a plant encounters stress. These genes can be classified into: i) Single gene product which directly protects the cells from the damage. Genetically engineered plants for single gene products include those encoding for enzymes required for the biosynthesis of osmoprotectants (Tarczynski et al., 1993; Kavikishore et al., 1995; Hayashi et al., 1997), or ii) modifying membrane lipids (Kodama et al., 1994; Ishizaki-Nishizawa et al., 1996), iii) LEA proteins (Xu et al., 1996), and iv) detoxification enzymes (McKersie et al., 1996). Similarly, many genes involved in stress response can be

simultaneously regulated by using a single gene encoding stress inducible transcription factor (Kasuga et al., 1999), which activates or induces a whole cascade of gene products in response to stress thus offering possibility of enhancing tolerance towards multiple stresses including drought, salinity and freezing. The gene products of second group include transcriptional factors (bZIP, MYC, MYB and DRE B, etc.), protein kinases (MAP kinase and CDP kinase, receptor protein kinase, ribosomal-protein kinase and transcription-regulation protein kinase, etc.), proteinases (phosphoesterase and phospholipase C, etc.) which are involved in signal transductions of stresses and the expression controls of stress-tolerant genes. Transgenic plants may give higher yields due to more efficient metabolism or synthesis of specific compounds, or due to a decrease in loss caused by various abiotic stresses. However, there are still a number of unanswered questions such as (i) how plant cells sense the water deficit resulting from drought, high salt or cold? (ii) How these stress signals are transduced to nuclear transcription factors? and iii) how the expression of the downstream functional genes are controlled?

Research approaches utilizing transgenic crops may offer new means to improve agriculture, in particular in dry areas, as genes specifically involved in the response to drought have been identified (Liu et al., 1998). The results of transgenic modifications for biosynthetic and metabolic pathways have so far indicated that higher stress tolerance can be achieved by genetic engineering and that the transfer of a single trait only marginally increases the tolerance, and that multiple mechanisms to engineer water stress tolerance must be utilized (Bohnert et al., 1995). Therefore, a holistic approach integrating physiological and molecular dissection of the tolerance traits is needed to understand the mechanisms underlying drought tolerance, and

eventually to integrate such traits into agronomically desirable germplasm (Subba Rao et al 1995)



A schematic representation of an integrated approach employing biotechnological and traditional plant breeding tools to develop abiotic stress tolerance in crops (source Sharma and Lavanya 2002)

Transgenic approaches may thus offer powerful means to better understand and then minimize loss of yield in this important crop due to the various abiotic stresses Hence, it will likely be necessary to transfer several potentially useful genes into this legume plant in order to obtain a higher degree of tolerance to drought stress Further, it is also possible to control the timing tissue-specificity and expression level of transferred genes for their optimal function Induction of stress tolerance through engineering for over-expression of genes encoding for transcription factors is emerging as an attractive proposition for imparting abiotic stress tolerance in groundnut First, the approach wisely considers that drought stress tolerance may not

be the fact of one single gene The novelty as well as importance of this approach stems from the fact that the *cis*-acting promoter sequences of different stress responsive genes induced in response to the same stress are similar to an extent and thus can be possibly governed at the same time by modulating the transcriptional factors.

Keeping in view these facts, the present study was undertaken to induce abiotic stress tolerance by transforming drought responsive elements and transcription factors like *DREB1A* in groundnut

The main objectives of this study were as follows

- To standardize the regeneration and transformation protocols for selected groundnut cultivar with DREB1A by *Agrobacterium*-mediated gene transfer
- To carry out molecular characterization of the transformants to verify the introduction and expression of the introduced genes
- To carry out studies on the physiological and biochemical factors involved in the development of possible tolerance to water stress under greenhouse conditions and selection of transgenic events exhibiting improved perfomance under water limiting conditions

Table 1A. Effect of various explants and hormones selection on in vitro shoot regeneration in groundnut.

Explant	Genotype	Medium	Growth regulators	Morphogenic response	Reference
Apical meristem	Not known	MS+ B5 vitamins	NAA (10 µM) + BA (0.1 µM)	Single shoots with many roots	Kartha et al., 1981
	Not known		NAA (10 µM) + BA (1 µM)	Shoots without any further development	Kartha et al. 1981
Mesocotyl	Not known	MS	IAA (11 µM) + kinetin (2.3 µM)	Shoots with roots	Bajaj 1982
Epicotyl	Not known	MS	IAA (11 µM) + kinetin (2.3 µM) + casein hydrolysate	Multiple shoots roots	Bajaj 1982
	New Mexico Valencia	MS	BA (10 mg l) + NAA (1 mg l)	Organogenesis	Cheng et al. 1992
De-embryonated cotyledons	ICG 4367 U S 48 TMV 2	MS	2,4-D (2 mg l) + kinetin (2 mg l)	Multiple shoots	Narasimhulu and Reddy 1983
	TG 17	Moist cotton wool	BA (1ppm)	Multiple shoots	Bhatia et al. 1985
Cotyledonary nodes	Not known	MS	NAA (1 mg l) + BA (3 mg l)	Multiple shoots	Banerjee et al. 1988
Mature cotyledons	JL 24 TMV 2 ICGS 11 ICGS 44 Robut 33 1	MS + B5 organics	BA (20 µM) + 2,4-D (10 µM)	Adventitious shoot buds	Sharma and Anjaiah 2000
Immature leaflets	Not known	MS + B5 vitamins	NAA (1 mg l) + BA (1 mg l)	50% shoots	Pittman et al. 1983
	JL-24	MS	NAA (4 mg l) + BA (5 mg l)	....	Chengalrayan et al. 1994
	NC 7	MS	NAA (2 mg l) + BA (4 mg l)	Shoots	Otomo et al. 1996
Embryo axis	Not known	MS	None	Shoots regenerated into plantlets	Atreya et al. 1984

Immature embryos	New Mexico Valencia	-	TDZ (10 mg l)		Kanyand et al 1994
	Tifrun, Florunner, Sunbelt runner, VRG26, GA119-20, Ser56-15	B5	Picloram (0 5-1 mg l)	Shoots with roots	Ozias-Akins et al , 1993
Embryos	MK 374, M 13, TMV 2, Robut-33-1	MS or Whites medium	None	Whole plants	Sastri et al , 1980
	Span cross, Dixie Spanish	MS	IAA (0 5 mg l) + GA_3 (0 05 mg l) + zeatin (0 5 mg l)	Shoots	Ozias-Akins and Branch, 1990
Ovules		MS	kinetin+ GA	Shoots and roots	Martin, 1970
Ovaries	MK 374, M 13, TMV 2, Robut 33-1	MS	BA (0 5 mg l)+ NAA (2 mg l)		Sastri et al , 1980

Table 1B: In vitro shoot organogenesis in groundnut via callus.

Explant	Cultivars	Medium	Growth regulators	Morphogenic response	Reference
Epicotyl	ICG 4367, US 48, TMV 2, TG 19B	MS	none	9-28% shoots	Narasimhulu and Reddy, 1983
De-embryonated cotyledons	MK 374, M 13, TMV 2, Robut-33-1	MS	zeatin (4 mg l) or kinetin (4 mg l)	Multiple shoots	Sastri et al., 1980
Leaflets	Not known	MS	NAA (1mg l) + BA (1mg l)	Organogenic callus	Mroginski et al., 1981
	TMV 2	MS	BA (2 mg l) + NAA (0.5mg l)	Shoot primordia	Venkatachalam et al., 1999
Plumule	Okrun	MS	BA (30 µM) + NAA (5 µM) + Brassin (1 µM)	Multiple Shoots	Ponsamuel et al., 1998

Table 2. Status of genetic transformation in groundnut.

Explant	Mode of gene transfer	Strain/ Plasmid	Gene of interest	Transformation frequency	Reference
Leaf	At	pBI121	*gus*, *npt*II and *35S*	0.2 – 0.3%	Cheng et al., 1997
Embryonic axis	At	EHA 101 pMON9793	*uid*A, *npt*II	9%	McKently et al., 1995
	Pb	pAC2MR pACH2MR	Mercuric ion reductase		Yang et al., 2003
Embryonic axis, cotyledon slices; leaf and petiole explants from 7-day plantlets	At	pTiBo542 pTiT37	*uid*A, *npt*II	transformation dependent on specific bacterial strain-plant cultivar interactions.	Lacorte et al., 1991
Mature cotyledons	At	PBI121 pROK II:IPCVcp	*IPCV* (coat protein)	55%	Sharma and Anjaiah, 2000
	At		*H* protein gene		Khandelwal et al., 2003
Cotyledons	At	LBA4404 pBI121	*uid*A, *npt*II	47%	Venkatachalam et al., 2000

	Pb	PCAMBIA-1301	*uidA*, *hph*	1.6%	Deng Xiang Yang et al., 2001
	Pb	pMOG617 / pxVGH	*uidA*, *hph*	168 independent transgenic lines recovered	Wang et al . 1998
Embryogenic callus	Pb			1%	Ozias-Akins et al., 1993
	Pb	pDO432 pHyg[1] pGIN	*luc*, *hph*, *hph*	54 independent transgenic lines obtained; 53% were fertile	Livingstone and Birch, 1999
Somatic embryos	Pb	pCB13-N and pCB13-N	*hph* gene nucleocapsid protein N gene of TSWV	52 hygromycin resistant cell lines (49 *hph* ; 45 N gene positive and 3 escapes)	Yang et al., 1998a
Leaf, Epicotyl	At	EHA 101	*uidA*	12-36% (leaves) 15-42% (Epicotyl)	Egnin et al., 1998
Leaf discs	At	pBI121	*uidA*, *nptII*	6.7% putative shoots; but were sterile.	Eapen and George 1994
Epicotyl from mature embryo axis	Pb	pKYLX80-N11 pTRA140 /	*uidA*, *hph*		Magbanua et al., 2000

[1]At: *Agrobacterium tumefaciens*; Pb: Particle bombardment; El: Electroporation

2. REVIEW OF LITERATURE

REVIEW OF LITERATURE

Conventional plant breeding mutation breeding and wide hybridization have played a significant role in the improvement of groundnut crop during the last five decades Novel approaches like plant biotechnology including tissue culture and genetic engineering can complement and circumvent the limitations of conventional breeding and other methods for enhancement of quality and productivity of this very important legume of the semi-arid tropics (SAT) of the World Developments in the field of plant genetic engineering and recombinant DNA technology and the accelerated impetus given to the conventional breeding programs by marker assisted breeding methods such as RFLP (Restriction fragment length polymorphism) RAPD (Random amplified polymorphic DNA) AFLP (Amplified fragment length polymorphism) SSR (Single sequence repeats) markers have greatly enhanced the potential in developing the genetically modified groundnut plants for resistance to both biotic and abiotic stresses as well as for the nutritional enhancement

2.1 Genetic transformation

Genetic transformation for the incorporation of novel genes into groundnut gene pool has opened up novel opportunities for crop improvement in this important legume This could be of particular interest given the low level of genetic polymorphism in groundnut which makes a MAS approach difficult The transformation protocols for groundnut are now well established Transformation efficiencies frequently are directly related to the tissue culture response and therefore highly regenerative cultures are often transformation competent

2.1.1 Tissue culture and regeneration of groundnut

The basis of an efficient tissue culture system is that the somatic cells are totipotent and can be stimulated to regenerate into whole plant as first proposed by Schwann and Schleiden (Gautheret, 1983) The tissue culture technology offers means of rapid clonal propagation, possibilities of wide hybridization by embryo, ovule or ovary culture, somatic hybridization, haploid production through anther and pollen culture, cell line selection against various pathogens, and a number of other applications The achievements in developing efficient and reproducible tissue culture and regeneration protocols have been reported in groundnut (Bhatia et al., 1985, Mckently et al., 1990, Mckently, 1991 Cheng et al., 1992 Kartha et al., 1981 Chen et al., 1990, Sharma and Anjaiah, 2000) In vitro regeneration in groundnut occurs through embryogenesis or organogenesis Regeneration by primary organogenesis occur by the development of shoots directly on the surface of cultured explants or by intervening callus phase i e the development of shoots directly from the callus tissue

Direct regeneration system in groundnut has an advantage in the developmental studies, due to the rapidity of morphogenesis and no requirement of frequent subculture, besides, de novo production of shoot primordia, is extremely rapid and synchronous Such a regeneration system favors easy accessibility of *Agrobacterium* to the meristematic cells, which are mainly surface cells during the initial co- cultivation for the genetic transformation (Sharma and Anjaiah, 2000) Organogenesis in groundnut, shoot formation in particular, can be freely induced and complete plants can be obtained from cultures of a wide range of explants The in vitro response in groundnut cultures appears to be strongly influenced by genotype, age of source explant and hormone content of the tissue culture medium Prolific

organogenesis has been obtained from a number of explants such as petiole, epicotyl, hypocotyls, mesocotyl, young leaflets, shoot tips, leaf tissue, seed parts mostly the cotyledons or tissue surrounding the cotyledonary node, mature and immature embryos and whole seeds (Bhatia et al., 1985, Mckently et al., 1990, Mckently, 1991, Cheng et al., 1992, Kartha *et al*., 1981, Chen et al., 1990). Rapid shoot formation and plant regeneration in groundnut via organogenesis has been achieved with varying concentrations of Thidiazuron (TDZ), a substituted phenylurea derivative, added to the growth medium (Li et al., 1994). The use of TDZ has shown to increase the frequency of shoot development (55-60%) with hypocotyls and cotyledon explants (Kanyand et al., 1994). Split halves of mature cotyledons of groundnut responded with a high frequency (95.5 %) of multiple adventitious shoot buds, while producing a greater number of adventitious shoot buds per explant on a modified MS medium (MMS) with MS inorganic salts (Murashige and Skoog, 1962), B5 organics (Gamborg et al., 1968), and 3 % sucrose supplemented with BA (20 µM) and 2,4-D (10 µM) (Sharma and Anjaiah, 2000).

Groundnut cultures are also reported to produce multiple shoots via an intervening callus phase. Earlier reports on groundnut in vitro regeneration have indicated that differentiation of shoots from callus can be induced from a variety of explants (Narasimhulu and Reddy, 1983). Callus derived from an explant may differentiate into multiple shoots on callus induction medium or may require a subsequent subculture on shoot induction medium. Ponsamuel et al., (1998) reported that plumular explants of groundnut cv. Okrun cultured on a preconditioned medium consisting of B5 salts and vitamins along with 2,4-D (10 µM) and kinetin (1 µM) produced direct shoot buds on transfer to a medium enriched with brassin (a synthetic analog of brassinolide which has been shown to promote cell division, cell elongation

and plant growth at very low concentrations) Similarly Venkatachalam (1999) reported shoot bud regeneration from leaflet-derived callus of groundnut on MS medium containing NAA (2.0 mg L) and kinetin (0.5 mg L) upon subsequent transfer to a medium supplemented with a combination of BAP (2.0 mg L) and NAA (0.5 mg L)

The recent developments in the genetic transformation of groundnut have emboldened researchers to pursue the development of transgenic plants capable of producing high quality groundnuts resistant to various diseases insect- pests and abiotic stresses While groundnut tissues are susceptible to infection by wild type strains of *A. tumefaciens* (Lacorte et al 1991) several methods for DNA transfer have been used for the transformation of groundnut Novel genes can be introduced into actively growing groundnut cells using *Agrobacterium* mediated transformation or the direct delivery methods such as electroporation and microprojectile bombardment However *Agrobacterium*-mediated gene transfer is the most broadly applied system in groundnuts which has proven to be very successful

2.1.2 Agrobacterium *mediated transformation methods*

Initial studies on groundnut transformation showed gene transfer into the calluses following the co-cultivation of seedling-derived hypocotyl explants with wild-type *Agrobacterium* (Dong et al 1990 Lacorte et al 1991 Mansur et al., 1993) Later, the *Agrobacterium*-mediated transformation by using leaf explants of groundnut was shown to result in a transformation frequency of 2% (Lapen and George, 1994) Immature embryonic axis was also been employed as an explant for *Agrobacterium*-mediated transformation in groundnut where Mckently et al (1995) obtained a small number of stably transformed shoots Similarly, Xiaoping (1996) obtained a very low frequency of transformation with leaflet explants of 4-day-old

seedlings of groundnut cv. Ehua4 following co-cultivation with *A. tumefaciens* strain AGL1 harbouring the binary plasmid pBI121 for 2 days and selection on 100 mg l kanamycin for selection. Cheng et al (1997) obtained fertile transgenic plants of groundnut with a 0.3 % frequency by using leaf segments. The pre-culture of groundnut cotyledons on medium for 3 days prior to co-cultivation with *Agrobacterium* strain LBA 4404, harboring the plasmid pBI121 containing *uid*A and *npt*II genes for two days, followed by transfer to the embryo induction medium (MS medium supplemented with 0.5 mg l NAA and 5.0 mg l BAP) resulted in the production of transformed somatic embryos at a frequency of 47%. However, the final recovery of transformed plants was not reported (Venkatachalam, 2000, Khandelwal et al., 2003). Rinsing the leaf and epicotyl explants of var. New Mexico in half-strength MS medium prior to co-cultivation has been reported more amenable to *Agrobacterium* transformation than the 'runner' type cultivars. The transient transformation efficiency significantly increased from 12 % to 36 % for leaf explants and 15 % to 42 % for epicotyls (Egnin et al., 1998).

The recovery of transgenic shoots with genes of interest at high frequencies has been a major bottleneck in groundnut. The nucleocapsid gene of tomato spotted wilt virus along with the *uid*A and *npt*II marker genes were attempted with the groundnut variety New Mexico Valencia by using *Agrobacterium*-mediated transformation (Li et al., 1997). To circumvent the problem of low recovery of transformed shoots, a non-tissue culture based transformation method involving direct co-cultivation of cotyledon attached embryo axis with *Agrobacterium* treated with wounded tobacco leaf extract resulted in a stable 3% transformation frequency (Rohini and Rao, 2000). However, a highly efficient transformation system from the cotyledon explants of pre-soaked mature seeds was reported by Sharma and Anjaiah

(2000) This system offers regeneration of adventitious shoot buds with over >90 % frequency resulting in an effective transformation frequency of 55%. Here a number of independently transformed groundnut plants with the coat protein gene of IPC V were produced that resulted in the recovery of morphologically normal and fertile plants with the transplantation success rates of up to 95%. This method was also shown to be independent of the genotype and tested with several Spanish and Virginia type groundnuts. More recently the *Agrobacterium*-mediated transgenic groundnut plants expressing the hemagglutinin (H) protein of *Rinderpest virus* have also been developed as an expression system for the delivery of recombinant subunit vaccine through fodder as a means of mass immunization of domestic ruminants as well as wild life (Khandelwal et al. 2003).

2.1.3 *Direct gene transfer methods*

Direct DNA transfer methods can circumvent the genotype dependency of *Agrobacterium* infection. Direct gene transfer has been accomplished by several methods such as microprojectile bombardment, electroporation of protoplasts and intact tissues, microinjection of protoplasts or meristems and polyethylene glycol mediated transformation of protoplasts.

Particle bombardment developed by Sanford and his co-workers (Sanford et al. 1987, Klein et al. 1988) has been successfully used for direct introduction of genes into a number of plant species including groundnut. Explant choice for bombardment can be made on the basis of criteria such as regeneration potential, favorable metabolic conditions for the expression of a particular genetic construction, or cellular organization that facilitates unambiguous selection of the transformants. Transient expression and stable transformation has been observed in callus lines from immature groundnut leaflet tissue bombarded with micro carrier particles carrying the

plasmid DNA (Clemente et al., 1992). Of 875 leaflets of the cultivar 'PI PN 4' bombarded, 202 kanamycin resistant calluses were recovered but only 1 untransformed shoot was produced. Similar observations were reported by Schnall and Weissinger (1995) where the regenerated plants from slow growing brown callus as well as green clusters formed following the bombardment of leaflets did not result in any stable transformants. However, bombardment of 1-2 year old embryogenic callus derived from immature embryos followed by stepwise selection for resistance to hygromycin B in the semi-solid and liquid media produced transgenic shoots at a frequency of 1 % (Ozias-Akins et al., 1993). Similarly, the shoot meristems of mature embryonic axis produced transgenic plants at a relatively low transformation frequency of 0.9-1 % (Brar and Cohen, 1994). Transgenic groundnut plants expressing the *cry1Ac* gene for resistance to the Corn stalk borer (*Elasmopalpus lignosellus*) have been reported by using the somatic embryos from immature cotyledons of groundnut following bombardment with vectors containing codon modified *Bacillus thuringiensis* cry1Ac gene along with the hph gene for antibiotic resistance with an efficiency of 0.85 to 2.3 transgenic events per bombardment (Singsit et al., 1997). The transformants expressed cry1Ac protein up to 0.18 %, of the total protein as detected by ELISA that was also correlated with various levels of resistance to *E. lignosellus* in insect bioassays. In biolistic studies, the transient gene expression as assayed by GUS histochemistry has been found to be affected by both particle size and amount of DNA used for coating. The latter was found to be positively correlated with gene copy number (Lacorte et al., 1997). Efficient biolistic-mediated transformation of both Spanish and Virginia types of groundnut by using the embryogenic callus derived from mature seeds was obtained by following single step selection for hygromycin B resistance (Livingstone and Birch, 1999). In this study, 3

to 6 independent transformants were obtained after the bombardment of 10 cm^2 embryogenic calluses where the copy number of the introduced genes ranged from 1 to 20 with an average of four copies per recovered transformant. Recent reports, however, show further increase in transformation efficiencies that range from 2.6±3.5 to 19.8±18.5 per bombardment of 5 cm^2 embryogenic callus with fertility rates of 32 % (Wang et al., 1998).

Among the different genes of interest that have been introduced by particle gun bombardment are 2S albumin gene from Brazil nut (Lacorte et al., 1997), improved essential sulphur containing amino acid methionine and synthetic antifungal peptide gene (Yang et al., 1998b). A high frequency transformation and regeneration of somatic embryos via microprojectile bombardment has been achieved with the nucleocapsid protein gene (N-gene) from the lettuce isolate of tomato spotted wilt tospovirus (Yang et al., 1998a). Primary transformants containing a single copy of the transgene expressed the N protein, indicating that a gene silencing existed in the primary transgenic lines with multiple gene integration. More recently, groundnut transgenics exhibiting a high level of resistance to the *peanut stripe virus* (PStV) were obtained following co-bombardment of embryogenic calluses derived from mature seeds of the commercial cultivars Gajah and NC7 with one of the two forms of the PStV coat protein (CP) gene, an untranslatable, full length sequence (CP2) or a translatable gene encoding a CP with an N-terminal truncation (CP4) (Higgins et al., 2004).

Pre-culture and osmotic treatments have been shown to have an important effect on the transformation efficiency. The pre-culture process influences the competence for transformation of the bombarded epidermal and sub-epidermic cells on the adaxial surface of groundnut cotyledons. Cotyledons pre-cultured for 3 days on

half strength MS medium followed by 3 h treatment in an osmotic medium before particle bombardment with a plasmid containing a chimeric *nph* and *uid A* genes resulted in a high transformation frequency (Yang et al 2001). The biolistic based systems for gene delivery into embryogenic calluses and embryo axes is labor intensive and requires the bombardment of a large number of explants to obtain a few transformed cell lines (1 %) which produce transgenic plants at low frequencies that are often chimeric or result from a few transformation events. Moreover the success with recovery of fertile transgenic plants following particle bombardment has generally been low.

Besides biolistics different methods based on biological or direct DNA transfer have also been developed for the production of transgenic groundnut over the last few years. Padua (2000) employed electroporation of DNA directly into the intact embryonic leaflets of groundnut in a modified electroporation buffer supplemented with 75 µM NaCl. A positive effect on the number of shoots and regeneration efficiency was observed by using electric strengths of 500-625 v cm. However such methods have not yet resulted in efficient recovery of transgenics for routine applications.

2.2 Groundnut and abiotic stress

Abiotic stresses like drought, low temperature and salinity are environmental factors that dramatically limit plant growth and productivity where in the case of drought, unpredictable and unseasonal rainfall is a major constraint to the yield in groundnut (Boote and Ketring 1990; Rao and Nigam, 2001). While the area and production of groundnut has been increasing globally, the total productivity has remained almost constant over the past decades (Patel and Golakiya, 1988). Low

rainfall and prolonged dry spells during the crop growth period are the main reasons for low average yields in India. Annual estimated losses in groundnut production equivalent to over US$ 520 million are caused by drought (Subbarao et al., 1995, Rao and Nigam, 2001, Sharma and Lavanya, 2002). A major additional problem is the contamination of groundnut with aflatoxin due to the soil pathogen, *Aspergillus flavus* following drought conditions during maturity and harvesting. Yield losses due to drought are highly variable in nature depending on timing, intensity, and duration besides other location-specific environmental stress factors such as high irradiance and temperature. Although, the conventional plant breeding along with better management practices can improve tolerance to abiotic stresses, a major drawback can be the non-amenability of the available stress tolerance traits due to their complexity or species barrier. Moreover, the lacking of proper methods and techniques for screening for drought tolerant traits in the segregating populations further complicates breeding for abiotic stress tolerance.

2.2.1 *Methods for combating abiotic stress in groundnut*

2.2.1.1 *Conventional approaches*

Briefly, in conventional methods genotypes are exposed to drought conditions, usually intermittent drought spells and progeny selection is made based on the yield achieved under drought. The major drawback of that approach is that yield integrates the many different components that contribute to the overall performance under water deficit conditions. The importance of each component may differ from environment to environment, which introduce a lot of genotype by environment interaction.

Agronomic management options Various aspects like providing supplementary irrigation, timing, method and intensity of irrigation would affect the

yield of the crop (Wright and Nageshwara Rao, 1994). Since in legumes the pod filling stage is very sensitive to drought, tackling the crop at such stage with proper care would increase the yield. The pod yield in groundnut can be increased by 13-19% if the crop is irrigated adequately during the pre-flowering phase (Nageshwara Rao et al., 1985). The adaptive response of groundnut to drought can also be increased by exposing the crop to short duration drought during the vegetative phase, which may enhance the root development and reduce transpirational losses by limiting the leaf area development allowing the plant to utilize the soil moisture from the deeper soil profile (Rao and Nigam, 2001). However, this may not always be easy to implement under rainfed conditions when rain are very unpredictable.

Plant Breeding. In conjunction with the agronomic management, genetic management options in groundnut can prove to be a better long-term strategy to improve the yield and performance of the crop in water limiting and drought prone regions. Selecting the genotypes based on their fit to the historical weather and soil data of target location can also improve the overall efficiency of the crop. Alternatively, genotypes that thrive well under limited moisture conditions show 12 % to 144 % pod yield superiority. Screening groundnut genotypes for better vegetative growth and pod yield under severe end-of-season and mid season drought has been an option (see Rao and Nigam, 2001). Genotypic variations for traits such as deep root system (Ketering, 1993; Wright et al., 1991, Wright and Nageshwara Rao, 1994), lower mean surface leaf area (SLA) that contributes to higher transpiration efficiency have also been observed (Wright and Nageshwara Rao, 1994). Another way is to evaluate wild species for physiological traits associated with drought and aflatoxin resistance and identify suitable DNA markers for drought resistance gene[s] for use in inter-specific breeding to develop drought resistant lines (Rao and Nigam,

2001) However, the molecular tools in groundnut are still in a very preliminary state to implement this sort of approach, at least in the short to mid-term

2.2.1.2 Biotechnological approaches

Judicious application of biotechnological tools hold great potential in alleviating some of the major constraints to crop productivity Transgenic crops offer new means to apply science to improve agriculture in areas of the world where sub-optimal rainfall and other abiotic stresses are important constraints for crop productivity However, a major challenge in using genetic engineering for plant improvement of abiotic stress tolerance is to identify the nature and the number of genes that must be introduced to have a significant impact on plant phenotype Techniques based on molecular biology and biotechnology enable the selection of successful genotypes, better isolation and cloning of favorable traits and the development of transgenic crops of importance to agriculture Genetic engineering for drought tolerance has been suggested to provide a major component of an integrated approach for stabilizing and improving crop production in drought-prone environments (Subba Rao et al , 1995 Udayakumar et al 1998 Saxena, 2001) Besides, genetic engineering has been shown to be comparatively fast for the introduction of novel genes contributing to better drought tolerance (Jaczynski et al 1993, Bohnet and Jensson, 1995, Pilon-Smits et al , 1995, Xu et al , 1996, Bohnert and Shen,1999, Kasuga et al ,1999, Sivamani et al 2000)

2.3.Genetic transformation technology and abiotic stress

Stress induced gene expression can be broadly categorized into three groups genes encoding proteins with known enzymatic or structural functions, regulatory proteins, and proteins with as yet unknown functions Stress-induced proteins with known

functions include water channel proteins, key enzymes for osmolyte (proline, betaine, sugars, and polyamines) biosynthesis, detoxification enzymes, and transport proteins. While most of the regulatory proteins are involved in signal transduction following stress perception, the order in which they act is not known.

2.2.2 Structural genes

2.3.1.1 Osmoprotectants

Accumulation of certain organic solutes (known as osmoprotectants) is a common metabolic adaptation found in diverse taxa. Many plants inhabiting saline and arid regions respond to stress with an increased synthesis of osmoprotectants such as glycine-betaine, thus allowing the maintenance of turgor pressure during water, salt or cold stress (Sakamoto and Murata, 2001). Plants like spinach and other members of chenopodiaceae which are moderately water stress tolerant, accumulate glycine betaine and related compounds in their vacuoles or cytoplasm. They also stabilize macromolecules during cellular dehydration and protect the proteins and membranes against damage by high concentrations of inorganic ions. Some osmoprotectants also protect the metabolic machinery against oxidative damage such as choline O sulfate or beta-alanine betaine (Hanson et al., 1994). Many major crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress tolerant organisms. These osmoprotectants based on their chemical structures can be classified into three types.

- Betaine and its related compounds such as glycine betaine and choline
- Sugars and polyols, such as mannitol, trehalose, fructans, ectonine, and ononitol
- Amino acids such as proline, arginine decarboxylase and glutamine synthetase

However, the metabolic machinery of many crop plants lacks the active pathways to produce these osmoprotectants. One strategy involved in obtaining stress tolerant transgenic plants is to overexpress such osmolytes in plants through engineering. Therefore introducing osmoprotectant synthesis pathways is a potential route to breed stress-tolerant crops. The first step involved in obtaining stress tolerant transgenic plants is to engineer genes that encode enzymes for steps in the synthesis of the osmolytes which reduce the osmotic potential inside the cell due to their accumulation inside the cytoplasm (Bray 1993). Genetic transformation has allowed the introduction of new pathways for the biosynthesis of various compatible solutes into plants. It is believed that osmoregulation would be the best strategy for this kind of abiotic stress especially if osmoregulatory genes could be triggered in response to drought salinity and high temperature. Various strategies are being pursued to genetically engineer increased osmoprotection in plants that possibly operates through oxidative detoxification by preventing damage to the cellular structures. Many osmoprotectants like glycine-betaine (Ishitani et al. 1997, McNeil et al. 2000), proline (Delauney and Verma 1993, Nanjo et al. 1999a), choline oxidase (Sakamoto et al., 2000), glutamine synthetase (Hoshida et al. 2000), arginine decarboxylase (Roy and Wu, 2001), mannitol, onnonitol, fructans, ectoine, and other gene products have been studied which play a role in osmoregulation thereby protecting the membrane and protein complexes (Yang et al., 1996).

Glycine-betaine. Glycine-betaine appears to be a crucial determinant of stress tolerance. Accumulation of this osmolyte is induced under various environmental stress conditions including drought, salinity, cold, and heat. Choline being a precursor of glycine-betaine, plants and bacterial genes for choline oxidation have been used to engineer glycine-betaine synthesis in *Arabidopsis thaliana* (Hayashi et al., 1997,1998,

Alia et al., 1998, 1999, Sakamoto et al., 2000). *Nicotiana tabacum* (Lilius et al. 1996, Holmstrom et al., 2000), *Brassica* species(Huang et al., 2000, Prasad et al., 2000, Gao et al., 2000), *Oryza* sp (Sakamoto et al., 1998, Takabe et al., 1998, Mohanty et al., 2002) and many other plants lacking these enzymes. The gene *codA* encoding for choline oxidase from *Arthrobacter globiformis* resulted in accumulation of high levels of betaine and acquired significant tolerance to salt, cold and heat stress in transgenic *Arabidopsis thaliana* (Hayashi et al., 1997, 1998, Alia et al., 1998, 1999, Sakamoto, 2000). Transgenic tobacco expressing the *bet A* gene encoding choline dehydrogenase from *E. coli* accumulated betaine in transgenic plants that also exhibited increased tolerance to salt stress as well as tolerance to photoinhibition at low temperature (Holmstrom, 2000). An improved protection of the photosynthetic apparatus was thought to be associated with the increased stress tolerance due to the introduced expression of *betA* gene.

Proline. Free proline is regarded as having multiple roles in stress tolerance in plants that include osmotic adjustment (Handa et al., 1986), a stabilizer of subcellular structures (Schobert and Tschesche, 1978), a scavenger of free radicals (Pardha Saradhi et al., 1995), a buffer in cellular redox potential, and a major constituent of cell wall structural proteins that may provide mechanical support for cells (Nanjo et al., 1999b). Transgenic *A. thaliana* plants expressing the proline dehydrogenase (*AtProDH*) gene in antisense orientation accumulated higher levels of proline resulting in an increased tolerance to both salt and freezing stress (Nanjo et al., 1999a). However, the tolerance to salt stress in this study was based on the ability of transgenic plants to avoid lodging only for 6 more minutes over the non-transformed plant, and not based on any dry weight accumulation data under salt stress. Besides, tobacco plants expressing the *P5CSF129A* (a mutated form of *P5CS*, whose feedback

inhibition by proline was removed by site directed mutagenesis) resulted in about two-fold increase in proline accumulation than the plants expressing wild type form (*Vigna aconitifolia, P5CS*). The elevated level of proline significantly enhanced the ability of transgenic plants to grow in medium containing up to 200 mM NaCl (Hong et al., 2000).

Mannitol: Transgenic tobacco plants expressing a foreign gene encoding for mannitol dehydrogenase (*mtlD*, from *E. coli*), leading to mannitol accumulation showed improved salinity tolerance (Tarczynski et al., 1993). Salt stress (150 mM NaCl) reduced the dry weight of wild type plants by 44% but had no effect on the dry weight of transgenic plants. Subsequently, in transformed tobacco was transformed with a construct where *mtlD* enzyme was targeted to the chloroplasts, the presence of mannitol in the chloroplasts resulted in enhanced resistance to oxidative stress due to increased capacity to scavenge hydroxyl radicals (Shen et al., 1997). Ectopic expression of the *mtlD* gene for the biosynthesis of mannitol in wheat was also shown to improve the tolerance to drought and salinity stresses (Abebe et al., 2003).

Fructan: Introduction of fructans in non-fructan producing species has been shown to mediate enhanced tolerance to drought stress in the transgenic plants. *Nicotiana tabacum* plants transformed with *SacB* gene for Levansucrase (an enzyme generating fructan from fructose from *Bacillus subtilis*) fused to the vacuole sorting signal of carboxypeptidase Y from yeast and placed downstream of the 35S promoter of cauliflower mosaic virus was shown to perform significantly better than the untransformed controls under drought conditions by exhibiting 55 % more rapid growth with 33% fresh weight and 59% greater dry weight (Pilon-Smits et al., 1995). Subsequently, transgenic sugar beet *(Beta vulgaris* L.) plants containing *SacB* gene was also shown to accumulate fructan to an extent of 0.5% of the dry weight in both

roots and shoots under drought conditions thus exhibiting improved performance under drought conditions (Pilon-Smits et al., 1999). These improved performance might be related to higher water uptake in the transgenics due to fructan production and lower osmotic potential in the roots when grown with PEG (-0.4 to -0.8 Mpa). However, in the absence of any data on plant transpiration in this study, it is doubtful if such results will have any use under natural soil stress conditions that have higher speed of soil dehydration.

Trehalose: Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress in bacteria, fungi, and invertebrates. Trehalose affects the sugar metabolism as well as osmoprotection against several environmental stresses including high temperature and dessication. Transgenic tobacco expressing the gene for the trehalose-6-phosphate synthase (*TPS1*) subunit of yeast trehalose synthase driven by the *rbcS* gene promoter from *Arabidopsis* accumulated 0.8-3.2 mg g DW trehalose and resulted in improved drought tolerance (Holmstrom et al., 1996). Further, the expression of *TPS1* driven by drought inducible promoter *rd29* also improved drought tolerance of the resultant trehalose accumulating tobacco plants (Zhao et al., 2000). Other genes for the overexpression of trehalose have been used to generate trehalose-accumulating plants. Transgenic tobacco plants expressing *otsA* and *otsB* genes from *E. coli* encoding for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase respectively accumulated low levels of trehalose as compared to the non-structural carbohydrates (Pilon-Smits, 1998). Rice transformation with a trehalose-6-phosphate synthase/phosphatase (*TPSP*) fusion gene that includes coding region of *E. coli otsA* and *otsB* genes resulted in 3 to 10-fold increase in trehalose accumulation without any accompanying negative pleiotropic effects. Increase in trehalose levels using either

the tissue specific or stress-inducible promoters resulted in high photosynthetic efficiency and a decrease in the photo-oxidative damage during stress (Garg et al., 2002). Besides, trehalose-producing transgenic rice (*Oryza sativa*) plants were also generated by the introduction of a gene encoding a bifunctional fusion (*TPSP*) of the trehalose-6-phosphate (T-6-P) synthase and T-6-P phosphatase (TPP) of *E. coli* under the control of the maize ubiquitin promoter (*Ubi1*). Trehalose accumulation in Ubi1:TPSP plants resulted in increased tolerance to drought, salt and cold.

Sorbitol: Sorbitol has also been shown to have potential in improved osmoregulation under abiotic stress conditions. The Japanese persimmon transformed with apple cDNA encoding NADP-dependent sorbitol-6-phosphate-dehydrogenase (S6PDH) resulted in high, medium and low sorbitol producing transgenic events. However, the loss of photosynthetic activity was found to be low in high sorbitol producing lines, indicating an increase in the ability of sorbitol producing plants to tolerate salt stress (Gao et al., 2000).

2.3.1.2 *Polyamines*

The polyamines are organic compounds having two or more primary amino groups - such as putrescine, cadaverine, spermidine, and spermine. Though it is seen that polyamines are synthesized in cells via highly-regulated pathways, their actual function is not entirely clear. In plants, polyamines accumulate under several abiotic stress stimuli, including salt and drought. Polyamines are believed to have an osmoprotectant function in plan cells under water deficit. Typically, the cellular levels of either putrescine or spermidine or both increase in response to the application of different forms of abiotic stress to cell cultures as well as whole plants. Ten-days-old *Zea mays* plants salt-stressed for eight days increased the content of putrescine and spermidine in their roots and leaves, and the increase in leaves was higher than in

roots. A number of stress factors such as potassium deficiency, osmotic stress, low pH, nutrient deficiency or light have been shown to stimulate the accumulation of polyamines, and particularly putrescine in plants. Transgenic cells of carrot (*Daucus carota* L.), overexpressing a mouse ornithine decarboxylase (ODC) cDNA to withstand salt stress and osmotic stress over short periods of 0-48 h. The transgenic cells produced 2-4 fold higher levels of putrescine. Transgenic rice expressing oat adc cDNA and *Tritordeum Samdc* cDNA under control of an ABA-inducible promoter showed increase in biomass under salinity stress condition compared to the control (Roy and Wu, 2001).

2.3.1.3 *Late embryogenesis abundant (LEA) proteins*

LEA proteins represent the category of high molecular weight proteins that are abundant during late embryogenesis (Galau, 1987). These plant proteins accumulate during seed dessication in response to water stress during seed maturation. Amongst the several groups of LEA proteins, those belonging to the group 3 are predicted to play a role in the sequestration of ions that are concentrated during cellular dehydration. These proteins have 11-mer amino acid motifs with the consensus sequence TAQAAKEKAGE repeated as many as 13-times (Dure, 1993). The group 1 LEA proteins are predicted to have enhanced water-binding capacity, while the group 5 LEA proteins are supposed to sequester ions during water loss. Constitutive over expression of the HVA1, a group 3 LEA proteins from barley conferred tolerance to soil water deficiency and salt stress in transgenic rice plants (Xu et al., 1996). Transgenic wheat plants containing the *HVA1* gene showed constitutive expression of the transgene resulting in improvement of growth characteristics under drought conditions (Sivamani et al., 2000). However, the water use efficiency (WUE) reported in this study was extremely low compared to other data reported in wheat cultigens.

Transgenic rice (TNG67) plants expressing a wheat LEA group 2 protein (*PMA80*) or the wheat LEA group 1 protein (*PMA1959*) genes resulted in increased tolerance to dehydration and salt stresses (Cheng et al., 2002). A *HVA1* gene from barley expressed under the control of a constitutive or a stress-inducible promoter in a recalcitrant scented rice variety, Pusa Basmati 1 showed increased stress tolerance in terms of cell integrity and growth after the imposed salt- and water-stress treatments when compared to the untransformed control plants (Rohilla et al., 2002).

2.3.1.4 *Transporter genes*

An important strategy for achieving greater tolerance to abiotic stress is to help the plants to re-establish homeostasis in stress environments by restoring both ionic and osmotic homeostasis. A number of abiotic stress tolerant transgenic plants have been produced by increasing the cellular levels of proteins (such as vacuolar antiporter proteins) that control the transport functions. Transgenic melon (Bordaçs et al., 1997) and tomato (Gisbert et al., 2000) plants expressing the *HAL1* gene showed a certain level of salt tolerance as a result of retaining more K^+ than the control plants under salinity stress.

In *Arabidopsis*, a vacuolar chloride channel, *AtCLCd* gene that is involved in cation detoxification has been cloned (Hechenberger et al., 1996). More recently, the *AtNHX1* gene of *Arabidodpsis*, which is homologous to the *Nha1* gene of yeast, has been cloned and over expressed in *Arabidopsis* to confer salt tolerance by compartmentalizing the Na^+ ions in the vacuoles (Apse et al., 1999). Transgenic *Arabidopsis* and tomato plants that over express *AtNHX1* gene accumulated abundant quantities of the transporter in the tonoplast and exhibited substantially enhanced salt tolerance (Apse et al., 1999; Quintero et al., 2000; Zhang and Blumwald, 2001). SOS1 (Salt Overly Sensitive 1) locus in *A. thaliana*, which is similar to the plasma

membrane Na+ H+ antiport from bacteria and fungi and encodes a putative Na+ H+ antiporter was cloned and over expressed constitutively by using the CAMV 35S promoter The upregulation of *SOS1* gene was found to be consistent with its role in Na+ tolerance possibly by providing a greater proton motive force that is necessary for elevated Na+ H+ antiporter activities (Shi et al 2000).

2.3.1.5 Heat shock genes

The heat shock response the increased transcription of a set of genes in response to heat or other toxic agent exposure is a highly conserved biological response, occurring in all organisms (Waters et al 1996) The response is mediated by heat shock transcription factor (HSF) which is present in a monomeric non-DNA binding form in unstressed cells and is activated by stress to an trimeric form which can bind to promoters of heat shock genes The induction of genes encoding heat shock proteins (Hsps) is one of the most prominent responses at the molecular level of organisms exposed to high temperature (Kimpel and Key 1985 Lindquist 1986 Vierling, 1991)

Genetic engineering for increased thermo tolerance by enhancing heat shock protein synthesis in plants has been achieved in a number of plant species Increased thermo-tolerance by constitutive expression of carrot *hsp17.7* gene was achieved in transgenic carrot cell lines and plants (Malik et al 1999) Transgenic tomato plants harboring an *Arabidopsis thaliana HsfA1b* (*AtHsfA1b*) and *β glucuronidase* (*gusA*) fusion gene under the control of a constitutive *CaMV35S* promoter accumulated higher levels of protein product of heat-shock induced genes than those of the wild-type and showed a significantly higher level of thermal and chilling tolerance (Li et al 2003) More recently, transformed rice plants constitutively expressing the *hsp101*

gene from *A thaliana* showed enhanced tolerance to sudden shifts in extreme temperature regime better than the controls (Kativar-Agarwal, 2003)

2.3.1.6 *Detoxifying genes*

In most of the aerobic organisms, there is a need to effectively eliminate the Reactive oxygen species (ROS) generated as a result of environmental stresses Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms Plants have developed a complex antioxidant system by which they scavenge the ROS thereby protecting the cell from oxidative attack In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge the ROS and these antioxidant systems include various enzymes and non-enzymes, which may also play a significant role in ROS signaling in plants (Vranova et al , 2002) A number of transgenic improvements in the abiotic stress tolerance have been achieved through the detoxification strategy Transgenic plants over expressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases have been developed with improved resistance to salinity and desiccation (Zhu et al , 1999; Roxas et al., 1997) Tobacco transgenics over expressing *SOD* gene in the chloroplast, mitochondria and cytosol have been generated that showed enhanced tolerance to oxidative stress induced by methyl viologen (MV) in the leaf discs Bowler et al., 1991, Van Camp et al , 1996). Over expression of chloroplast *Cu/Zn SOD* isolated from pea exhibited improved photosynthetic performance (~90 %) under chilling stress conditions in transgenic tobacco (Sen Gupta, 1993) whereas, the tomato *Cu/Zn SOD* gene enhanced the tolerance to methyl viologen in potato transgenic plants (Perl et al., 1993). Tobacco transgenic plants over expressing

MnSOD rendered enhanced tolerance to oxidative stress only in the presence of other antioxidant enzymes and substrates (Slooten et al., 1995) thus showing that the genotype and the isozymic composition also has a profound effect on the relative tolerance of the transgenic lines to abiotic stress (Rubio et al., 2002). Transgenic alfalfa (*Medicago sativa*) plants cv. RA3 over expressing *MnSOD* in chloroplasts showed lower membrane injury (McKersie et al., 1996). Lower concentrations of the reactive aldehydes and increased tolerance against oxidative agents and drought stress were observed by overproducing alfalfa aldose reductase gene (*MsALR*) in tobacco transgenics (Oberschall et al., 2000).

2.3.2 *Signal transduction component genes*

Genes involved in stress signal sensing and a stress-signalling cascade in *A thaliana* have been of recent research interest (Wincov and Bastola, 1999; Shinozaki and Yamaguchi-Shinozaki, 1999). Components of the same signal transduction pathway are also shared by various stress factors such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki, 1999). There are multiple pathways of signal-transduction systems operating at the cellular level for gene regulation. Abscisic acid (ABA) is well known as one such component acting in one of the signal transduction pathways. Expression of some of the genes in the stress signal transduction cascade is mediated by ABA while others act independent of ABA. The early response genes have also been known to encode transcription factors that activate downstream delayed response genes (Zhu, 2002). Although specific branches and components exist (Lee et al., 2001), the signalling pathways for salt, drought, and cold stresses all interact with ABA, and even converge at multiple steps (Xiong et al., 1999). Abiotic stress signaling in plants involves receptor- coupled phosphorelay, phosphoionositol-induced Ca2+ changes, mitogen activated protein kinase (MAPK) cascade and

transcriptional activation of stress responsive genes (Xiong and Zhu, 2001). A number of signaling components are associated with the plant response to high temperature, freezing, drought and anaerobic stresses (Grover et al., 2001).

Alteration of these signal transduction components in a way to reduce the sensitivity of cells to stress conditions or such that a low level of constitutive expression of stress genes is induced has been attempted by many workers (For a review, see Grover et al., 1999). Over expression of functionally conserved At-DBF2 (homologue of yeast DBf2 kinase) showed striking multiple stress tolerance in *Arabidopsis* plants (Lee et al. 1999). Salt stress-tolerant transgenic plants were obtained by over-expressing calcineurin (a Ca^{2+} Calmodulin dependent protein phosphatase), a protein phosphatase known to be involved in salt-stress signal transduction in yeast (Pardo et al., 1998). Transgenic tobacco plants produced by altering stress signaling through functional reconstitution of activated yeast calcineurin has not only opened up new routes for study of stress signaling but also for engineering transgenic crops with enhanced stress tolerance (see Grover 1999).

2.3.3 *Regulatory genes*

Many genes respond to multiple stresses like dehydration and low temperature at the transcriptional level are also induced by ABA that protect the cell from dehydration (Mundy and Chua, 1998. Dure et al., 1989. Skriver and Mundy 1990). In order to restore the cellular function and make plant more tolerant to stress, transferring of a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels. To overcome such constraint, enhancing tolerance towards multiple stresses by a gene encoding stress inducible cis-acting or trans-acting transcription factors that regulate a number of genes down stream or upstream of it may prove to be a promising technology (Yamaguchi-

Shinozaki et al., 1994). The transcription factor then activates a cascade of genes that act together in enhancing the tolerance towards the multiple stresses.

2.3.3.1 *Transcription factors*

Transgenic *Arabidopsis* plants over-expressing a cold inducible transcription factor (*CBF1*) constitutively showed a freeze tolerance similar to that of cold-acclimated non-transgenic control plants by inducing expression of cold regulated (*cor*) genes (Jaglo-Ottosen et al., 1998). There was no negative effect on the growth and development of these transgenic plants. *CBF1* cDNA when transformed into the tomato (*Lycopersicon esculentum*) genome under the control of a CaMV35S promoter improved tolerance to chilling, drought and salt stress but exhibited dwarf phenotype and reduction in fruit set and seed number (Hsieh et al., 2002). Another transcriptional regulator, *Alfin1*, when overexpressed in transgenic alfalfa (*Medicago sativa* L.) plants regulated endogenous *MsPRP2* (a NaCl-inducible gene) mRNA levels, resulting in salinity tolerance comparable to the available salt tolerant mutant lines (Winicov and Bastola, 1999). The induction of thermo-tolerant by de-repressing the activity of *ATHSF1*, a heat shock transcription factor leading to the constitutive expression of heat shock proteins at normal temperature has been reported in *Arabidopsis* plants (Lee et al., 1995). Several stress induced *cor* genes such as *rd29A*, *cor15A*, *kin1* and *cor6.6* are triggered in response to cold treatment, ABA and water deficit stress (Thomashow, 1998). Over expression of *Arabidopsis CBF1* (CRT/DRE binding protein) has been shown to activate *cor* homologous genes at non-acclimating temperatures (Jaglo et al., 2001). Enhanced tolerance towards multiple stresses such as cold, drought and salt stress in crops other than the model plants like *Arabidopsis*, tobacco and alfalfa has also been reported (Kasuga et al., 2004; Pellegrineschi et al., 2004; Behnam et al., 2006). Transgenic tomato (*Lycopersicon esculentum*) plants

with tolerance to chilling, drought and salt stress using a stress inducible ABRC1 promoter from barley *HAV22* gene to drive the expression of *Arabidopsis CBF1* (Lee et al., 2003). A cis-acting element, dehydration responsive element (DRE) identified in *Arabidopsis thaliana* is also involved in ABA-independent gene expression under drought, low temperature and high salt stress conditions in many dehydration responsive genes like *rd29A*, which are responsible for dehydration and cold induced expression (Nordin et al., 1991; Yamaguchi-Shinozaki and Shinozaki, 1993; Iwasaki et al., 1997). The cDNAs encoding the DRE binding proteins, DREB1A and DREB2A have been isolated from *A. thaliana* and proteins shown to specifically bind and activate the transcription of genes containing DRE sequences (Liu et al., 1998). DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression.

The transcriptional activation of stress-induced genes has been possible in transgenic plants overexpressing one or more transcription factors that recognize promoter regulatory elements of these genes. The transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes, which has also been shown in *Arabidopsis* (Shinozaki & Yamaguchi, 1997). DREB1A cDNA under control of 35S promoter in transgenic plants give rise to strong constitutive expression of the stress inducible genes and also increased tolerance to freezing, salt and drought stresses (Liu, et al., 1998). Strong tolerance to freezing stress was observed in transgenic *Arabidopsis* plants that overexpress *CBF1* (DREB1B) cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Jaglo-Ottosen et al., 1998). Overexpression of *DREB1A* improved drought- and low-temperature stress tolerance in tobacco, wheat (Kasuga et al., 2004;

Pellegrineschi et al., 2004). The use of stress-inducible *rd29A* promoter minimized the negative effects on the plant growth in these crop species.

2.4 Physiological evaluation of stress effects on plants

A large number of studies evaluate different transgenic constructs in different plants species, and to different stresses, including drought, salinity, or cold. The expression of the genes inserted as well as the level of metabolite increase due to the construct have been reported in great detail. However, a major challenge to transgenic research still lies in properly evaluating the phenotypic responses of the material to any environmental stress and in the understanding the physiological expression of inserted genes at the whole-plant level.

2.4.1 Means of stress impositions and evaluation

Stress conditions used to evaluate the transgenic material in most of the reports so far (Shinwari et al., 1998; Nanjo et al., 1999; Garg et al., 2002), are usually too sharp, which the plants are very unlikely to undergo in a real field condition. Also, the means of evaluation are often dubious. For example, Pellegrineschi et al., (2004) compared the performance of DREB1A transformed wheat seedlings grown in 5x5cm pots against the wild parent by withholding water to 2 weeks. While untransformed plants end up dying within 10-15 days of stress imposition, transgenic plants survived. The evaluation of the transgenic material was carried out on their performance after the stress period by re-watering the plants untill maturity, instead of the biomass accumulated during the stress. Hence most of the growth accounts for the well-watered period, with control plant suffering a dramatic initial setback, something they are unlikely to suffer in real field conditions.

In a separate study, the transgenic plants were exposed to an osmotic stress by using PEG in hydroponic conditions (Pilon-Smits et al., 1996, 1999). While this method was, to an extent useful to test certain responses of the plants under a given osmotic potential, it offers relatively different conditions than in the soil where the water reservoir is by definition finite. In such conditions, assessment of the plant performance based on the differences in water uptake would differ in the field conditions where the water available is limited.

Increased water use efficiency (WUE) in the transgenic wheat has been reported by Sivamani et al. (2000). However, in this study, there was no control over the water loss due to evaporation from the pots, which probably accounts for most of the water loss. Besides, the evaluation of their transgenic plants involved addition of a given quantity of water every other day from 2 to 10 wk disregarding the fact that water requirements increase dramatically during the period, and doing so would probably expose the plants to an initial flooding before a very severe stress. Similarly, other transgenic evaluation protocols investigating drought responses by using fresh weight (Sun et al., 2001) and other indirect estimates of performance like growth rate, stem elongation (Pilon-Smits et al., 1995; Lee et al., 2003), or survival (Pardo et al., 1998) are likely to give inconsistent results. Also, in many experiments, plants are grown in culture chambers with often limitation in the light intensity

2.4.2 Adequate protocols to apply drought

The drought response of plants cannot be investigated without thorough understanding of the different phases that a plant undergoes under drought in natural conditions. Also, a complete understanding of the qualitative and quantitative relationships between soil water and leaf exchange is a must (Sadras and Milroy, 1996). Two major issues that are typically need to be addressed in stress response

evaluation of plants include: (i) the means of stress imposition, details about the stress, and growth conditions (size and water content of pots, including the intensity, quickness of imposition, etc.), and (ii) hard data on the response of tested materials to support conclusions (comparison within the same species). Besides, precise details about the protocols used to evaluate the performance of plants to any given stress, should also be given as to assess the performance of materials. Various steps in evaluating the plants for water stress tolerance have been described in great detail (Ritchie et al., 1981; Sinclair and Ludlow, 1986). In this type of experiments, plant transpiration is evaluated gravimetrically and uses a soil water component to assess the transpiration response (not a number of days after imposition of water stress, as in most water stress studies). Exposure to water deficit is also gradual, to mimic the type of stress that plants would normally face in a field environment. In phase I, the water is abundant and plant can take up all the water required by transpiration and stomata are fully open. During this stage, the water loss is mostly determined by the environmental conditions to which the leaves are exposed. During phase II, the roots are no longer able to supply sufficient water to the shoot and stomata progressively close to adjust the water loss to the water supply so that leaf turgor is maintained. In phase III, roots have exhausted all the available water resulting in full stomatal closure and inhibition of virtually all the physiological processes contributing to growth, including photosynthesis. The fraction of transpirable soil water (FTSW) is used as a covariate for soil moisture available to compare the response of different physiological mechanisms to soil drying, and it has been successfully used across a wide range of species and plant processes (Ritchie, 1980; Sinclair and Ludlow, 1986; Weisz et al., 1994; Ray and Sinclair, 1997,1998.). This has been used to design dry-down experiments to study the response of plants to drought, where FTSW is taken as

a function of the fraction of soil water moisture available to plant for the comparison of stress imposed. This protocol has the advantage of mimicking the situation that a plant would face in the typical field conditions, i.e. a progressive soil drying.

Water use efficiency (WUE) is one of the major traits that have been associated with drought tolerance in groundnut. It is so because WUE is one of the three major component of the yield architecture, as defined by Passioura, 1977, as Y = T x TE x HI, where TE stands for the transpiration efficiency, a term somewhat similar to WUE. It is an essential trait which is due to the ability of groundnut to maintain high photosynthetic activity even under low stomatal conductance without showing impact on carbon assimilation and yield (Wright et al., 1994). The practical difficulties associated with the measurement of water use efficiency have prompted the exploitation of other easily measurable traits such as SCMR, Specific leaf area, leaf nitrogen and C13. Water use efficiency has an established relation with the various easily and non-destructively measurable traits like SLA, SCMR, C13 and O18. Bindumadhava et al. (2003) and Rao et al. (2001) observed that the transpiration efficiency (TE) that is an alternate measure of water use efficiency was positively correlated significantly with the SCMR and inversely related with SLA and leaf nitrogen. Farquhar et al. (1982) also reported that carbon isotope discrimination is closely related with TE and is highly correlated with specific leaf area (Wright et al., 1994). This correlation of water use efficiency with carbon isotope discrimination, specific leaf area and transpiration efficiency enables using all these or either one of these traits for the identification of genotypes with greater transpiration efficiency (Hubick et al., 1986; Wright, et al., 1988; Wright et al., 1994). These surrogates are particularly important to assess TE where it is difficult to assess the transpiration rate such as field conditions.

2.5 Differential antioxidative responses to abiotic stresses

To cope with environmental fluctuations and to prevent invasion by pathogens, plant metabolism must be flexible and dynamic. Active oxygen species, whose formation is accelerated under stress conditions, must be rapidly processed if oxidative damage to the plant cells and tissues is to be averted. The lifetime of active oxygen species within the cellular environment is determined by the antioxidative system, which provides crucial protection against oxidative damage and comprises of numerous enzymes and compounds of low molecular weight. Under optimal conditions, cellular homeostasis is achieved by the coordinated action of many biochemical pathways. However, different pathways may have different molecular and biophysical properties, making them different in their dependence upon external conditions. Thus, during events of suboptimal conditions (stress), different pathways can be affected differently, and their coupling, which makes cellular homeostasis possible, is disrupted. This process is usually accompanied by the formation of reactive oxygen intermediates (ROIs) because of an increased flow of electrons from the disrupted pathways to the reduction of oxygen (Halliwell, 1989; Noctor and Foyer, 1998; Asada, 1999; Dat et al., 2000; Mittler, 2002).

The reactive oxygen species (ROS) react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, inactivation of enzymes, thus affecting cell viability. The antioxidative system of plants comprises several enzymes and low molecular weight quenchers that are principally constitutive and vary in plants at cellular and subcellular levels. Superoxide radicals generated in plant cells are converted to H_2O_2 by the action of SOD. The accumulation of H_2O_2, a strong oxidant, is prevented in the cell either by catalase or by the ascorbate-glutathione cycle where APX reduces it to H_2O. The enzymatic mechanism of

detoxification involves dehydroascorbate reductase, glutathione reductase and other enzymes. Ascorbate and glutathione, other components of the antioxidative defence system, are found to increase under stress conditions. To counter the effects of stress, plants undergo a process of stress acclimation. This process may require changes in the flow of metabolites through different pathways, the suppression of pathways that may be involved in the production of ROI during stress, and the induction of various defense genes such as heat shock proteins (HSPs) and ROI scavenging enzymes (Vierling, 1991; Dat et al., 2000; Mittler, 2002). The complexity of signaling events associated with the sensing of stress and the activation of defense and acclimation pathways is believed to involve ROI, calcium, calcium-regulated proteins, mitogen-activated protein kinase cascades, and cross talk between different transcription factors (Liu et al., 1998; Xiong et al., 1999; Bowler and Fluhr, 2000; Kovtun et al., 2000; Knight and Knight, 2001; Chen et al., 2002). Interestingly, different stress conditions such as drought and cold can result in the activation of similar stress response pathways (Seki et al., 2001; Chen et al., 2002). Thus, a high degree of overlap may exist between gene clusters activated by different stresses. This overlap may explain the well-documented phenomena of "cross tolerance," in which a particular stress can induce resistance to a subsequent stress in plants which may be different from the initial one (Bowler and Fluhr, 2000).

2.6 Chlorophyll a fluorescence as a tool for rapid screening during abiotic stress

Drought stress is known to inhibit photosynthesis through alterations in the proportion of photochemical and energy-dependent quenching as a result of inhibition of the enzymatic sites that consume ATP and NADPH. The fluorescence quenching, predominantly caused by photochemical and energy-dependent mechanisms, is

strongly influenced by the utilization of NADPH and ATP in photosynthesis (Krause and Somersalo, 1989).

Chlorophyll-a fluorescence (CF) emitted by green plants reflects the photosynthetic activities and facilitated insight into the mechanism of fluorescence emission (Krause and Weis, 1991). This indication of the fate of excitation energy in the photosynthetic apparatus has widely been used as an indicator of stress (Willits and Peet, 2001). A lot of information has been obtained from the fluorescence transient (Govindjee et al., 1986; Krause and Weis, 1991, Govindjee, 1995). Transients recorded with high time-resolution fluorimeters, e.g. with the Handy-PEA, have provided accurate information (Strasser and Govindjee, 1992a, 1992b, Strasser et al., 1995), on precise detection of the initial fluorescence F0. The shape of the O-J-I-P transient has been found to be very sensitive to stress caused by changes in different environmental conditions, e.g. light intensity, temperature, drought, atmospheric CO_2 or ozone elevation and chemical influences (Srivastava and Strasser, 1995, 1996, 1997; Tsimilli-Michael et al., 1995, 1996, 1999, 2000, Van Rensburg et al., 1996; Krüger et al., 1997; Ouzounidou et al., 1997, Clark et al., 1998, 2000, Force et al., 2003), as well as by senescence (Prakash et al., 2003). The quantitative analysis of the O-J-I-P transient was introduced (Strasser and Strasser, 1995) and further developed as the 'JIP-test' after the basic steps of the transient, by which several selected phenomenological and biophysical structural and functional parameters quantifying the PSII behavior were calculated. The JIP-test, has proven a very useful tool for the in vivo investigation of the adaptive behavior of the photosynthetic apparatus and, especially, of PSII to a wide variety and combination of stresses, as it translates the shape changes of the OJ- I-P transient to quantitative changes of the

selected parameters (Strasser and Tsimilli-Michael, 2001a,b; Tsimilli-Michael and Strasser, 2001).

2.7 Conclusion

Over the last few years, there have been increasing research efforts in engineering stress-tolerant crops. While conventional plants breeding techniques and methodologies have been somewhat successful in imparting tolerance against abiotic stresses in groundnut, the next step, i.e. the use of marker assisted selection (MAS) for groundnut breeding, is hampered by the lack of genetic diversity in cultivated groundnut. Genetic engineering approaches could lead to simpler and more effective gene based alternatives for combating biotic and abiotic stresses. Several gene transfer approaches have been attempted to improve tolerance to abiotic stresses in different plants species (Holmberg and Bulow, 1998). Stress-induced proteins with known functions include water channel proteins, key enzymes for osmolyte (proline, betaine, sugars, and polyamines) biosynthesis, detoxification enzymes, and transport proteins (Tarczynski et al., 1993; Pilon Smits et al., 1995; Xu et al., 1996; Sivamani et al., 1996; Bohnet and Jensson, 1996; Bohnert and Shen, 1999; Kasuga et al., 1999).

However, in the case of such a complex stress as drought stress, and to restore the cellular function and make plants more tolerant to stress, transferring of a single gene encoding a single specific stress protein may not show the required stress levels. To overcome such constraints, enhancing tolerance towards multiple stress by a gene encoding a stress inducible cis-acting or trans-acting transcription factor that regulates a number of genes and activates a cascade of genes that act together in enhancing the tolerance towards the multiple stresses seems to be a promising technology (Yamaguchi-Shinozaki and Shinozaki, 1999). Drought being a complex trait that involves many genes, the strategy of switching on a transcription factor regulating the

expression of several genes related to abiotic stress seems to be an attractive target category for manipulation and gene regulation in groundnut, which can survive for a critical period under stress conditions. As the DRE related regulatory element is known to exist in a number of crops, the DREB1A cDNA and rd29A promoter may be useful to improve the stress tolerance in groundnut by gene transfer

3. MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Tissue culture

3.1.1 Plant material

Healthy seeds of groundnut (*Arachis hypogaea* L.) were obtained from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), Patancheru, India and stored at 10 °C until use. All the experiments were carried out with the cultivar JL 24.

3.1.2 Explant preparation and shoot regeneration

The seeds of groundnut cultivar JL 24 were surface sterilized with 70% ethanol for 2 min and washed with 0.1% (w/v) aqueous mercuric chloride containing 1 to 2 drops of Tween-20 for 8 min on a rotary shaker. The sterilized seeds were rinsed four to five times in sterile distilled water and soaked in sterile water for 4 h. The seed coat was then removed and the cotyledons were separated using pointed forceps. The embryonic axis was removed surgically and each cotyledon was cut into vertical halves to obtain the cotyledon explants for use in genetic transformation. The regeneration and transformation system used for adventitious shoot development using cotyledon explants was as developed earlier at ICRISAT by Sharma and Anjaiah (2000).

3.1.3 Culture conditions

For all the experiments on the regeneration, elongation and rooting of shoots, a modified MS basal medium (Murashige and Skoog, 1962) containing MS inorganic salts, B5 organic constituents and 3% sucrose was used. pH of the medium was adjusted to 5.8 prior to the addition of 0.8% agar and was autoclaved at 15 psi

pressure for 15 min. The regeneration experiments were carried out in 90 mm X 16 mm sterile disposable plastic petri-plates sealed with Parafilm®, while the elongation and rooting of the in vitro-formed shoots was carried out in 15 mm X 25 cm long glass culture tubes plugged with non-absorbent cotton plugs wrapped in one layer of cheese cloth. Cultures were maintained at 26±1 °C with 23 h photoperiod provided by white cool fluorescent lamps having 60 $\mu Em^{-2}S^{-1}$ light intensity and 1 h of dark period.

3.2. Genetic transformation

3.2.1 Bacterial strain and plasmids

The *Agrobacterium tumefaciens* strain C-58 carrying the genes of interest in a binary plasmid was used for transformation experiments. The recipient *A. tumefaciens* strain C 58 had chromosomal resistance to rifampicin and kanamycin and routinely maintained on the selection medium. For use in plant genetic transformation experiments, the binary plasmid containing *DREB1A* driven by the drought-inducible promoter from *rd29A* gene of *A. thaliana* (rd29A:DREB1A) and CaMV 35S constitutive promoter were mobilized into *Agrobacterium* by electroporation (Fig. 1 A,B). These plasmids were same as reported by Kasuga et al. (1999) and were kindly provided by Dr. K Yamaguchi-Shinozaki, JIRCAS, Japan. The plasmid rd29A:DREB was constructed by Liu et al. (1998).

3.2.2. Isolation of plasmid DNA

Plasmid DNA was isolated according to the procedure reported by Sambrook et al. (1989). Various steps in the procedure were as follows:

Components

- GTE buffer: 50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)
- Lysis buffer: 0.2 N NaOH and 1% SDS (freshly prepared)
- 5 M Potassium acetate, pH 5.2
- RNase (10 mg/ml)
- Chloroform
- Isopropanol
- 70% ethanol
- TE buffer: 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0)

Method

Plasmid construct pBI29ApNot was maintained in *E. coli* strain DH5α and grown on LB (Appendix 5) agar plates containing 100 µg/ml ampicillin. Single isolated colonies were grown in 10 ml of LB medium overnight at 37 °C on a rotary shaker at 220 rpm. 10 ml of the bacterial suspension was pelleted by centrifuging for 10 min at 6000 rpm. The bacterial pellet was suspended in 600 µl of GTE buffer and placed on ice for 5 min. To the resuspended bacterial solution, 1200 µl of freshly prepared lysis buffer was added and the samples were placed on ice. After 5 min 900 µl of 5 M potassium acetate was added to the lysed bacterial suspension and the samples mixed well by inverting the tubes slowly and placed on ice for 5 min. The solution was centrifuged for 10 min at 14,000 rpm and the supernatant was transferred to fresh tubes to which 3 to 5 µl of RNase (10 mg/L) was added followed by incubation at 37 °C for 30 min. Equal volumes of phenol: chloroform was added to remove proteins present in the DNA mixture following brief centrifugation. After removal of the organic phase, equal volume of chloroform was added to the aqueous phase and centrifuged for 30 sec. The aqueous phase was collected in fresh microfuge tubes to which 0.8 volumes of isopropanol was added and mixed by gently inverting to precipitate the nucleic acids. The sample was centrifuged at 10,000 rpm for 10 min.

The pellet containing the plasmid DNA was briefly washed with 70% ethanol air-dried and dissolved in 30 µl of TE.

3.2.3. *Agrobacterium-mediated transformation*

A single colony of *Agro bacterium* strain C 58 harboring binary vectors was grown overnight in a yeast extract broth (YEB) containing 50mg ml kanamycin on an incubator shaker. The OD of the overnight grown culture was ensured to be in a range of 0.6 to 1.0. Aliquots of 12.5 ml of the culture was taken in a 30 ml tube and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the precipitated cells were washed with 10 ml of sterile half MS medium followed by centrifugation at 5000 rpm to collect the cell precipitate. The precipitated cells were then resuspended in 25 ml of sterile half MS and used for co cultivation

Agrobacterium-mediated transformation of groundnut was carried out by using the cotyledon explants as described by Sharma and Anjaiah (2000). The freshly excised cotyledons were briefly immersed with their proximal cut ends into the suspension of *Agrobacterium* containing the plasmid with 35S DREB1A or rd29A:DREB1A for a few seconds and implanted on SIM with cut ends embedded in the medium for 72 h. The co-cultivated cotyledons were transferred to SIM supplemented with filter-sterilized cefotaxime (250 mg/l) at a density of five explants per plate for two wk until multiple shoots appear on at least 70% of the explants. Utmost care was taken to embed the cut ends into the medium. The explants bearing shoot buds were transferred to SIM containing 250 mg/l cefotaxime and 100 mg/ l kanamycin to initiate selection and enrichment of the transformed cells. After 2 wk the proximal parts of the explants containing multiple adventitious shoot buds were excised and transferred to SEM containing 125 mg/l kanamycin for two to three subcultures of 4 wk duration each. The elongated shoots (3-4 cm) were cultured on

RIM without any antibiotics for rooting of the shoots. It takes about 2 wk to produce multiple adventitious roots on shoots cultured on RIM.

3.3. Acclimatization and transplantation of putative transgenic plants

Rooted plants were transferred to pots (6 inch) containing a mixture of sand and soil (1:1) and incubated in a growth chamber at 25 to 28 °C under high humidity (~89%) conditions for about 2 wk for hardening. Initially, the potted plants were covered with polythene bags for about 5 d. The well-established plants were transferred to bigger pots (13 inch) containing autoclaved sand and soil (1:1) and maintained in the containment greenhouse for further growth and maturity. In the greenhouse, irrigation was stopped about 10 d before harvesting.

3.4. Molecular analysis of putative transgenics

The primary transformants (T0) were analyzed for presence and expression of the introduced genes following transfer to greenhouse. Molecular analysis of the putatively transformed plants was carried out to determine whether the material was transgenic as well as to determine the copy number and or the integrity of the DNA insert and transgene expression.

3.4.1. Extraction and purification of genomic DNA from peanut leaves

The genomic DNA was extracted from the putative transformants following the method described by Sharma et al. (2000). This method is based on the Dellaporta DNA extraction method followed by a DEAE-cellulose purification protocol. Briefly, protocol for the extraction of genomic DNA from groundnut was as follows:

Solutions

- Extraction buffer: 100 mM Tris-HCL, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol.

- 20% SDS
- 5M potassium acetate
- Isopropanol
- 10 mg/ml RNAse A
- TE buffer: 10 mM Tris-HCL, pH 8.0, 1 mM EDTA, pH 8.0
- DEAE-cellulose suspension: 7.5% Whatman DE 52, 2 M NaCl, TE buffer
- Wash Buffer: 400 mM NaCl, TE buffer pH 7.5.
- Elution Buffer: 2 M NaCl, TE buffer (10 mM), pH 7.5

Young leaf tissue (0.5 g) is collected from the putatively transformed groundnut plants growing in the containment greenhouse. The samples are freeze dried by immersing in liquid nitrogen and lyophilized for 10-15 min before homogenization to a fine powder with a pestle and mortar. The frozen powder was transferred to a 25 ml polypropylene tube to which 15 ml of the extraction buffer and 1 ml of 20% SDS were added and mixed gently prior to incubation in a water bath at 65 ^{0}C for 15 min. The samples were then brought to room temperature before adding 5 ml of potassium acetate (pH 5.0) followed by incubation on ice for 30 min. The tubes were then centrifuged for 20 min at 13,000 rpm at room temperature. The supernatant was collected and transferred to another tube avoiding disturbance of the interphase. The genomic DNA was further precipitated with 0.6 volumes of isopropanol, mixed gently and incubated for 30 min at -20 ^{0}C before centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and pellet washed in 70% ethanol followed by air-drying.

Prior to the purification of DNA, 700 µl of TE (pH 8.0) and 10 µl of RNAse (10 mg/ml) was added to the dried pellet and incubated at 37 ^{0}C for 2-3 h. The DNA was diluted with two volumes of sterile distilled water before adding 1 ml of DEAE-cellulose suspension with gentle mixing for 3 min so as to maximize the interaction

between the DNA and DEAE-cellulose matrix. The mixture was centrifuged for 30 sec at 3,000 rpm to allow the sedimentation of DEAE-cellulose particles binding the nucleic acids. The supernatant was carefully discarded and the pellet resuspended in 1.2 ml of the wash buffer to eliminate the proteins, polysaccharides, and secondary metabolites not bound to DEAE-cellulose. This step was repeated twice for better results. 0.5 ml of elution buffer was then added to the DEAE-cellulose pellet and mixed gently prior to centrifugation at 3000 rpm for 30-45 sec. The supernatant was collected in a fresh eppendorf tube and this step was repeated with 0.3 ml of the elution buffer. The supernatants were carefully pooled and precipitated by using 0.6 volumes of isopropanol followed by centrifugation at 10,000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet washed with 1 ml of 70% ethanol and centrifuged at 13000 rpm for 2 min. The pellet was air-dried and dissolved in 50-100 µl of TE buffer (pH 8.0) for long-term storage at -20 °C.

3.4.2. *Rapid mini preparation of genomic DNA for quick PCR analysis*

Plant DNAzol is an extra strength DNAZol® reagent (Invitrogen® U.S.A.) that is specifically formulated for the isolation of genomic DNA from plants. The procedure for DNA extraction by using plant DNAzol reagent is based on the use of a novel guanidine-detergent lysing solution, which hydrolyzes RNA and allows the selective precipitation of DNA from a variety of plant tissues. The whole protocol can be carried out at room temperature and time involved in the extraction of the DNA is very less and the method is very efficient and quick.

Components

- DNAzol® reagent
- Chloroform
- DNAzol® + ethanol mix (1: 0.75)

- 70 and 100% ethanol
- TE buffer: 10 mM Tris-HCL+1 mM EDTA, pH 8.0

Young leaf tissue (100 mg) was taken and ground in liquid N_2 to a fine powder. To the ground tissue 300 µl of DNAzol® reagent was added and incubated at 25 °C with gentle shaking. After 5 min, 300 µl of chloroform was added to the above mixture and vortexed for few seconds. The mixture in the eppendorf tube was further incubated for 5 min at 25 °C before being centrifuged for 10 min at 12,000 rpm. The clear supernatant was transferred to the fresh tubes with addition of two volumes of absolute ethanol. The samples were mixed well by inverting the tubes for 6 to 8 times and were stored at room temperature for 5 min and centrifuged for 4 min at 5,000 rpm. The supernatant was discarded and 300 µl DNAzol® + ethanol mix was added to the pellet to precipitate the DNA and followed by vortexing and storage at room temperature for 5 min. The samples were centrifuged for 4 min and the supernatant was discarded. The pellet was washed with 70% ethanol and the samples were centrifuged at 5,000 rpm for 4 min. The ethanol solution was discarded and the pellet was air dried and dissolved in 70 µl of TE.

3.4.3 *PCR analysis of putative transformants*

Initial screening of the putative transformants was done by PCR for the presence of *nptII* and *DREB1A* gene sequences. The 700 bp region of *nptII* gene was amplified by using 22-mer oligonucleotide primers as reported by Hamill et al. (1991) as follows: forward primer: 5'-GAG GCT ATT CGG CTA TGA CTG - 3' and the reverse primer: 5'-ATG GGG AGC GGC GAT ACC GTA – 3'.

The integration of the *DREB1A* cDNA into the genomic DNA was also analyzed by using the 28-mer primers designed to obtain a 500 bp amplicon. These

included, the forward primer: 5'-CGG GTC GTA AGA AGT TTC GTG AGA CTC G-3', and the reverse primer: 5'- TCC GCC GTG TAA ATA GCC TCC ACC AAC G- 3'. To differentiate the introduced *DREB1A* sequence from the possible native *DREB* sequences, a 769 bp rd29 DREB1A junction fragment was amplified by using 24-mer primers including the forward primer: 5'-GGC CAA TAG ACA TGG ACC GAC TAC-3', and the reverse primer: 5'-GTT GAT TCC GGG ATT CGG AGT CTC-3'.

The PCR reaction was performed with 50 µl of a total reaction mixture containing 150 ng of genomic DNA, 5 µl of 10X PCR buffer (- $MgCl_2$), 1.5 µl of 50 µM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of 10 µM Primer I, 1 µl of 10 µM Primer II, and 0.25 µl of 1.25 units of Taq DNA polymerase. The total volume was made up to 50 µl with sterile distilled water. The control devoid of the template DNA was used in each set of reactions with each primer. The amplification reactions were carried out by using the Eppendorf® thermal cycler by using the following conditions denaturation at 94 °C for 60 sec, annealing at 58.5 °C (*npt*II), 50 °C (rd 29A), 55 °C (*DREB1A)* and 64 °C (rd29-DREB1A junction fragment) for 45 sec and extension at 72 °C for 90 sec for 32 cycles with the final extension at 72 °C for 4 min (one cycle).

Fidelity of the amplicons was verified by resolving the amplified fragments on to a 1.2% agarose gel followed by transfer to Hybond +ve nylon membrane (Amersham) and probing with non-radiolabelled *npt*II gene, the *Hind*III fragment containing 965 bp coding sequence of *rd29A* promoter and *Bam*HI fragment with 648 bp *DREB1A* cDNA region respectively by using Alkphos direct system (Amersham).

3.4.4. *Southern blot analysis*

Steps involved in Southern blotting were as described by Sambrook et al (1989): Digestion of the genomic DNA with an appropriate restriction enzyme, gel electrophoresis of the restriction fragments, transfer of the DNA to a nylon or nitrocellulose membrane, hybridization of the blot with a labeled probe, and autoradiography to detect the signal.

Materials

- Denaturation buffer: 1.5 M NaCl and 0.5 M NaOH
- Neutralization buffer: 1.5 M NaCl and 0.5 M Tris, pH 7.5
- 20X SSC (175.5 g/L of NaCl and 88.2 g/L Na-citrate)
- 250 mM HCL
- Blotting tray
- 3M Whatman paper
- Hybond-N$^+$ Nylon membrane (Pharmacia^{R})
- UV crosslinker (Pharmacia^{R})
- Paper towels
- Parafilm^{R}
- 500 gm weight
- Pasture pipette

Digestion of the genomic DNA with restriction enzymes and electrophoresis

Restriction of the genomic DNA was carried out overnight by incubating the samples at 37 °C. The untransformed genomic DNA was taken as control sample. The genomic DNA (25-30 µg) from each of the putative transformants was separately digested with *Bam*HI that has two restriction sites and *Eco*RI with a single site within

the plasmid pBI29NotAP DNA to ascertain the integration pattern based on size separation and number of copies of insert respectively. The reaction mix comprised of the following:

10-25 µg genomic DNA; 5 µl of 10X restriction buffer, 2 units in 2.5 µl of restriction enzyme; 7.5 µl of sterile distilled water to make the final volume of 50 µl. The digested genomic DNA was electrophoretically separated on 0.8% agarose gel casted in 1X TAE and electrophoresis was performed at 40 volts for 3 to 4 h.

Transfer of the DNA to a nylon or nitrocellulose membrane

The agarose gel resolving the restricted DNA fragments was placed in a plastic tray and depurinated in 250 mM HCl with gentle shaking at 10 to 20 rpm for 15 min. The gel was rinsed with distilled water and denaturated in solution consisting of 1.5 M NaCl and 0.5 M NaOH for 15 min with gentle shaking. The above denaturation step was repeated with fresh solution for another 15 min. The denaturation solution was drained off and the gel was gently rinsed twice with distilled water and the gel was placed in a neutralization solution (1.5 M NaCl and 0.5 M Tris, pH 7.5) for 15 min. The neutralization step was repeated again with a fresh solution for another 15 min. To carry out the capillary blotting to transfer the restricted DNA from the gel on to the nylon membrane, the nylon membrane and 3 sheets of a 3M Whatman filter paper were cut to the exact size of the gel to be transferred. The nylon membrane was soaked in 20X SSC. A blotting tray was taken and a glass plate was placed on the blotting tray. A filter paper was placed in such a way that both ends of the filter paper wick were immersed into the 20X SSC placed in blotting tray. A sheet of a 3M Whatman® filter paper of gel size was placed onto the blotting paper. The gel was placed carefully on top of the filter paper starting from one side and with the well side facing down with two additional sheets of filter

papers. On this, pre-soaked nylon membrane was placed on the gel with marked side facing the gel. Air bubbles from the gel were removed by gently rolling a glass pipette over the gel and a sheet of Whatman® paper cut to the size of the gel was placed over the gel. The area all around the gel was covered with Parafilm® to prevent contact of the top paper towels with the wick. A stack of paper towels was placed on top of the Whatman paper placed over the gel and a weight of 500 gm was kept on top of the papers. The blotting was performed overnight for complete capillary action. Later the blot was dried at room temperature for 10 min and the DNA was cross-linked by placing the blot DNA side down on a UV transilluminator for 3 to 6 min.

DNA hybridization with non-radioactive probes

The hybridization step was carried out by using the commercially available Alkphos Direct labelling and Detection System (Pharmacia), a non-radioactive chemiluminiscent system. The probe was labeled with alkaline phosphatase enzyme that reacts with the added substrate. CDP-Star™ (Pharmacia) and emits photons in the form of signals that can be identified on an X-ray film. Temperature in the hybridization oven was set at 55 °C. The Blot was placed in the hybridization bottle to which a pre-heated pre-hybridization solution was added and kept for hybridization. Probe DNA of 10 µl was taken in an eppendorf tube and heated at 100 °C by placing the sample in a boiling water bath for 5 min and immediate transfer of the sample on ice for 5 min to denature the DNA. The sample was briefly spinned down prior to use. To this denatured DNA, 10 µl of reaction buffer, 10 µl of cross linker (2 µl of cross linker added to 8 µl of distilled water) and 2 µl of enzyme labeling reagent was added followed by a brief spin. Subsequently, the sample was placed in a water bath set at 37 °C for 30 min. The blots were probed with non-radioactive labelled (Alkphos Direct labelling and Detection System; Pharmacia) PCR amplicons.

The above labeled solution was added to the pre-hybridization buffer (Appendix 8) present in the hybridization bottles without touching the membrane. After overnight hybridization at 55 ^{0}C, the used probe was discarded and the membrane washed twice with the primary wash buffer (Appendix 9) at 55 ^{0}C for 10 min each. Subsequently, the secondary wash was given for 5 min at room temperature followed by 30 min storage in the secondary wash buffer (Appendix 10) prior to autoradiography.

Autoradiography and X-ray film development

Drops of chemiluminescent detection solution (CDP-Star^{TM}) were added to the blot and excess solution of CDP-Star^{TM} was drained off by touching the tip of the blot on to the tissue paper where excess droplets can be soaked. The blot was placed in a plastic wrap and an X-ray film was placed over it in an exposure film cassette in a dark room and exposed for 45 min.

X-ray film development

The X-ray film was removed from the cassette and placed in a tray containing the X-ray Kodak GBX developer for 60 to 120 sec followed by washing with water for 30 sec. The film was further placed in a tray containing the Kodak GBX fixer for 60 to 120 sec. The film was rinsed with water for 2 min followed by air-drying.

3.4.5. RT-PCR analysis

Reverse transcription followed by the polymerase chain reaction (RT-PCR) leading to the amplification of specific RNA sequences in cDNA form is a sensitive means for detecting RNA molecules; a means for obtaining material for sequence determination and a step in the cloning of a cDNA copy of the RNA. Various strategies that can be adopted for first strand cDNA synthesis include the reverse

transcriptase reaction that can be primed by the down stream PCR, primer annealed to the RNA by random hexamers or by an oligo dT primer at the polyA tail of mRNA (Kawasaki, 1990). RT-PCR analysis of the putative transformants growing in the greenhouse was carried out by using the Thermoscript RT-PCR system (Pharmacia). RT-PCR analysis was carried out on 18 independent transformed plants of T_0 generation (P1 to P18). Total RNA from the putative transformants was isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol (Appendix 11) and the amplified products separated on 1.2% agarose gel. Primer sequences and PCR conditions for the *nptII* transcripts were the same as described in PCR analysis. For the *DREB1A*, the forward and reverse primers used were 5'-CGAGTCTTCGGTTTCCTCAG-3' and 5'-CAAACTCGGCATCTCAAACA-3' respectively (Pellegrineschi et al., 2004) with the PCR profiles of an initial denaturation at 94 °C for 5 min followed by 34 amplification cycles (94 °C for 45 s, 64 °C for 1min, and 72 °C for (1 s) with a final extension cycle at 72 °C for 5 min.

3.5. Inheritance of transgenes in T_0 and T_1 generation

T_0 plants were selfed to get T_1 progeny. A total of 40 seeds, 5 seeds from each primary independently transformed transgenic event (RD2, RD4, RD11, RD12, RD13, RD14, RD15, RD19, RD20, RD21, RD23, RD25, RD28, RD30) were sown for further inheritance studies in T_1 generation (a total of 70 T_1 plants were taken). Inheritance studies were carried out based on PCR analysis for the presence of *nptII* gene in plants transformed with the plasmid pBI29ApNot containing the rd29A:DREB1A gene construct. PCR positive plants were further tested by Southern blot analysis for gene integration and for determination of the copy number. Self-fertilized progeny of T_1 plants were germinated and raised to maturity to get T_2 progeny. Eight seeds of five independent transformed events of T_1 generation (RD2,

RD11, RD12, RD19, RD20) were sown and the replicates were tested for gene segregation, which were carried out based on the PCR positive results and analysis. The transgene integration was further confirmed through PCR analysis for *nptII* and *DREB1A* genes.

3.6. Dry down experiment for screening the performance of transgenic groundnut

The water stress was created in dry-down experiments following the procedure described by Sinclair and Ludlow (1986). For this, 8" pots were filled with dry red soil up to the brim. Initially three seeds per pot were sown and later thinned to one plant per pot. The plants were subjected to natural solar radiation with regulated air and temperature (night/day) in the glasshouse. The experiment was started 21 d after seed germination. The pots were fully saturated and left overnight to drain the excess of water. Subsequently, the pots were covered by placing the entire pot in a plastic bag, bunching the bag opening around plant stem and strictly twist tying it. This was to prevent soil evaporation, so that water loss from the pots was only due to transpiration. A tube was fitted in all the pots, which protruded out of bag so that the pots could be irrigated when required. The initial (target) weight was recorded after the pots were covered with polythene bag so that target weight corresponded to when the soil was at 100% water saturation (field capacity). On the subsequent days, the pot weights were recorded and the transpiration of each plant was calculated by subtracting the present day pot weight from the previous day's pot weight, plus water possibly added (see after) on the previous day. The weights of the pots were recorded daily in the morning. The water lost per day in the control pots was added back. However, in order to expose the drought-stressed (DS) plants to a progressive water

deficit, the drought stressed plants were allowed to loose a maximum of 70 g of water per day. Any transpiration in excess of 70 g per day was added back to the plant

The experiment was terminated when the ratio of the transpiration of the stressed plant to average transpiration (three days) of control plants reaches 0.1. For each genotype, the daily transpiration of each stressed plant in the water-deficit regime was divided by the average mean transpiration of the well-watered control plants.

TR = transpiration of stressed plant average transpiration of control plants

Then a second normalization was done by dividing that ratio of each individual plant by the mean ratio of the first 3-4 days of the experiment. The latter would provide an estimate of the ratio for each plant under well-watered conditions (no stress until 6th or 7th days after submitting plants to water deficit = stage 1), and would then allow correcting for plant-to-plant variations. Total transpirable soil water (TTSW), i.e. the total amount of water that the plant was able to make available for transpiration, was calculated as the difference between the saturated weight and the weight at the end of the experiment (when normalized transpiration of water stressed plants is below 10% of the transpiration of controls). The values of FTSW for each pot was calculated by subtracting the daily pot weight from the initial pot weight (saturated) and dividing the difference by the total transpirable soil water (TTSW).

$$FTSW = \frac{\text{daily pot weight-initial pot weight}}{\text{Initial pot weight-final pot weight}}$$

The FTSW threshold value, the point from where plant's transpiration begins its decline was calculated by a plateau regression procedure with SAS (SAS Institute, 1996) by using NTR as a function of FTSW and plotting a graph with FTSW on X-axis and NTR on Y-axis. The data of NTR-FTSW and the number of days to end

point was subjected to average linkage cluster analysis for preparing dendrogram by using Euclidian distance of NTSYS pc. (Version 2.10 d).

3.6.1 SPAD chlorophyll meter reading

SPAD (Soil Plant Analysis Development) Chlorophyll meter (SPAD-502, Minolta) reading is a unit less value which corresponds to the relative amount of chlorophyll concentration in the leaves by estimating the variation between light disintegration at 430 nm, where chlorophyll a and b have peak wavelength to that at 750nm of near infrared without transmittance. SPAD chlorophyll meter reading was been taken on the secondary leaf from the internodal position on the main branch Readings were evaluated at three different intervals of time at start of stress, after 7 days of stress imposition, and at the harvest. Eight readings were made on each plant and then averaged.

3.6.2 Specific leaf area (SLA)

At the time of harvest, leaflets of the whole plants were collected and processed to measure the leaf area with the LI 3100-leaf area machine. Later, the leaves were oven dried at 80 ^{0}C and dry weights were taken. The ratio of leaf dry weight to the leaf area is defined as a specific leaf area of the respective plant.

3.6.3 Carbon isotope discrimination

The carbon isotope discrimination, $\Delta^{13}C$ of the remaining leaf ground samples was measured by mass spectrometry method. The carbon isotope discrimination values were computed as follows assuming the isotopic composition of atmospheric CO_2 as -8 per million by using the formula, $\Delta^{13}C = (\delta^{13}C \text{ air}-\delta^{13}C \text{ product})/(1+\delta^{13}C \text{ product}/1000)$.

3.6.4 VPD (Vapor pressure deficit)

The daily temperature and relative humidity (RH) in the contained greenhouse chambers was carefully recorded on a daily basis to calculate the vapour pressure deficit (VPD). The VPD values for each line were calculated for the number of days the last plant was harvested in the dry down treatment and were averaged thereafter.

3.7. Chlorophyll–a-fluorescence measurements (O JIP)

The quantification of the CF F0-J-I-P transient (JIP test) is based on the polyphasic fast phase fluorescence rise from two extreme fluorescence intensities, F0 to Fm at ca. 30 ms (Strasser et al., 2000). CF transients of dark-adapted intact leaves of the plants were measured by using a Plant Efficiency Analyser (Hansatech Ltd, King's Lynn, Norfolk, UK). The transients were induced by red light (600 mmolm 2 s_1 excitation intensity) at 650 nm provided by an array of six light-emitting diodes The Chl fluorescence measurements were done on pre-darkened plants For the measurements the plants (60 pots) were covered with a large black cloth after nightfall on the evening before taking the measurements. The measurements were taken between 08:00 and 10:30 next morning and the data were transferred to a computer where all calculations were performed using the "BIOLYZER" computer programme (Maldonado-Rodriquez, 2000) according to the JIP test equations (Strasser and Tsimilli-Michael, 2001).

3.8. Biochemical aspects involved in possible tolerance to abiotic stress

Leaf tissues were harvested at different stages of stress under a typical dry down cycle. These were collected at the stages where the plants reached the NTR 0.8, 0.6, 0.4, and 0.2 along with well-watered control (NTR 1.0). Separate extractions were made for determining the concentrations of proline, MDA (Malondealdehyde)

as well as each antioxidant enzyme. The specific enzyme activities were expressed on the basis of the total soluble protein of the samples.

3.8.1. Estimation of total soluble protein

The fresh leaf tissue collected at different stages of dry-down cycle was homogenized in a pre-chilled mortar at 4 °C in protein extraction buffer pH 7.0 (Tris HCl 30 mM, DTT 1 mM, ascorbic acid 1 mM, Na_2EDTA 1 mM, $MgCl_2$ 5 mM, PMSF 1 mM with PVP 6 mg/ml). The homogenate was centrifuged at 12, 000rpm for 15 min at 4 °C. The total soluble protein was estimated by Bradford's method (Bradford, 1976). The assay mixture contained 100 µl of the protein extract to which 5 ml of the diluted dye binding solution was added. The tubes were stored for 5 min for the development of colour after mixing. The blue color developed was measured by using a spectrophotometer (Beckman DU® 530) at 595 nm. The protein content was determined by calculating the standard curve drawn for the pure commercial bovine serum albumin (BSA).

3.8.2. Estimation of proline

The content of L-proline in the leaf samples was estimated by the method described by Bates et al. (1973). For this, 500 mg of the leaf tissue was homogenized in 10 ml of 3% sulphosalicylic acid (w/v). The homogenate was filtered through Whatman filter paper No. 1 and the filtrate was further used for assay of proline. Two ml of the filtrate was added with equal volume of acid ninhydrin (50 mg of ninhydrin + 1.2 ml glacial acetic acid+ 0.8 ml 6 M o-phosphoric acid) and incubated for 1 h at 100 °C in a boiling water bath. After the colour development, the tubes were placed on ice for 5-10 min for the termination of the reaction. 4 ml of toluene was thereafter added to the reaction mixture and vortexed well for 5-10 sec. The two immiscible

layers formed were carefully separated by using a pipette. The upper layer containing the toluene was used for the estimation of praline. The pink colour was measured by using a spectrophotometer (Beckman DU® 530) at 520 nm absorbance. A standard curve was prepared by using the commercial praline to calculate the proline concentration in the samples.

3.8.3. Estimation of lipid peroxidation

Lipid peroxidation was measured in terms of MDA content (Dhindsa et al., 1981). 1 g of the leaf tissue was homogenized in 5 ml of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 12, 000 rpm for 15 min at 25 °C. A 2 ml aliquot of the supernatant was mixed with equal volume of 20% TCA containing 0.5% thiobarbituric acid. The mixture was heated at 100 °C for 30 min, quickly cooled followed by centrifugation at 10000 g for 10 min. The absorbance of the supernatant was recorded at 532 nm (Beckman DU® 530). The non-specific turbidity was corrected by A_{600} subtracting from A_{532}. The concentration of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

3.8.4. Estimation of Superoxide dismutase *(SOD; E. C. 1.15.1.1)*

SOD (EC 1.15.1.1) activity was assayed by the photochemical method described by Giannopolitis and Ries (1977). 500 mg of the freshly harvested leaf tissue was homogenized in chilled 0.1 M phosphate buffer pH 7.0 containing 0.1mmol/L–1 EDTA. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was used as enzyme source for the estimation of SOD activity by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Dhindsa et al., 1981). Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 2 mM riboflavin, 75 mM NBT, 100 nM

EDTA, and 0–200 ml of the enzyme extract. After the final addition of riboflavin, the tubes were shaken and placed in the reaction assembly. The reaction was started by switching on the light and was run for 10 min. Identical tubes with the reaction mixture were kept in the dark that served as blanks. The light was switched off when the tubes were covered with a black cloth to stop the reaction. Enzyme activity (units/ml) was proportional to (V v-1), where V equals the change in absorbance per min in the absence of SOD, and v equals the change in absorbance per min in the presence of SOD. One unit of SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT (ñ-nitro blue tetrazolium chloride) reduction measured at 560 nm. SOD activity values were expressed in units per mg of protein.

3.8.5. Estimation of Glutathione reductase *GR (EC 1.6.4.2)*

500 mg fresh leaf tissue was used for the extraction in an ice bath in 10 ml of 0.1 m potassium phosphate buffer (pH 7.5) containing 0.1 M EDTA, 200 mg PVP (MW= 25,000), 1% w/v Bovine serum albumin (BSA) and 200 µM β mercaptaethanol. The leaf extract was filtered through Whatman filter paper (No.1) and further used for assay. GR activity was estimated following the oxidation of NADPH at 340 nm as described by Schaedle and Bassham (1977). The assay buffer contained 0.5 ml phosphate buffer (pH 7.5), 0.1 mM NADPH and 0.1 mM GSSG to a final volume of 1 ml. The reaction was initiated by adding 100 µl enzyme to the cuvette and the decrease in the absorbance at 340 nm was recorded. The enzyme activity was expressed as µM NADPH oxidized per min per gm of the tissue.

3.8.5. Estimation of Ascorbate peroxidase APOX *(EC 1.11.1.11)*

Total activity of APOX was measured spectrophotometrically by the method of Chen and Asada (1989). The extraction medium for leaf APOX contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% PVP-10, 1 mM ascorbate, and 0.1% Triton X-100. The leaf extract was filtered through Whatman filter paper No. 1 before being used for the enzyme assay. For assay of the enzyme activity, the rate of hydrogen peroxide-dependent oxidation of ascorbic acid was determined in a reaction mixture that contained 50 mM Hepes-KOH (pH 7.0) containing 1 mM ascorbate, 1% (v/v) Triton X-100 and enzyme extract (Chen and Asada, 1989). The reaction was initiated by the addition of 10 µl of 10% (v v) H_2O_2 and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm (Beckman DU® 530) for 3 min.

3.9 Statistical analysis

For the phenotypic evaluations ANOVA and Tukey's test procedures were executed to analyze the data for the averages and F predicted value for comparison of different transgenic lines with the wild type cultivar JI 24. The mean regression analysis was derived for each trait by regressing the mean values of all lines for particular trait. In the biochemical experiments, the data was scored and analyzed based on mean and SE values (standard error) using student's t- test

4. RESULTS

RESULTS

The present study was carried out by *Agrobacterium*-mediated genetic transformation of groundnut (*Arachis hypogeae* L.) by using the transcription factor *DREB1A* driven by the constitutive *CaMV* 35S promoter as well as a drought responsive promoter *rd29A* for improving abiotic stress tolerance. The results obtained in this study have been mainly organized under tissue culture and transformation studies, molecular characterization of the putative transgenic plants followed by physiological and biochemical evaluation of the transgenic groundnut plants

4.1. Tissue culture and Transformation

Co-cultivation of the cotyledon explants with the *A. tumefaciens* strain C 58 carrying the plasmids of interest (Fig. 1A, B) for 48 to 72 h resulted in the production of large number of putative transgenic shoot buds from over 90% of the explants (Fig. 2B-E). Each responding explant produced multiple adventitious shoot buds that (Fig 2E) differentiated within 2 to 3 wk of culture initiation on a modified MS containing 20 µM BA and 10 µM 2,4-D (shoot induction medium, SIM). Multiple shoot buds differentiated at the proximal cut end (Fig. 2C-E) within 14 days in over 80% of the explants. Transfer to SIM supplemented with cefotaxime (250 µg/ml) and kanamycin (50 µg/ml) at this stage resulted in the development of these adventitious shoot buds (Fig. 2 E). However, no further elongation of the shoot buds was observed thereafter on SIM. Hence, the explant portion bearing shoot buds were cut into two to four pieces (Fig. 2 F) and transferred on to the shoot elongation medium (SEM) containing MS with 2 µM BA and kanamycin (100 µg/ml) for at least three passages of 4 week each when elongated shoots were rescued at the end of each passage (Fig. 2 G).

Frequently, four to eight shoots were recovered from each responding explant. After 3 to 4 passages of subculture, >85% of the shoots transformed with rd29A:DREB1A plasmid construct attained a size of 3 to 4 cm. However the shoots having *35S:DREB1A* transgene did not elongate well in vitro conditions and showed retarded symptoms (Fig. 3 A, B). The shoots exhibiting bleaching were discarded at each stage and only the healthy ones were maintained for rooting. The elongated shoots (4-6 cm in length) were rooted on root induction medium (RIM) containing MS medium with 5 µM NAA but devoid of any selection antibiotic. Roots appeared within 2 wk after culture on RIM in most of the shoots and were allowed to develop further for 4 wk (Fig. 2 H) before their transplantation to the greenhouse following their acclimatization (Fig. 2 I). Over 90% of the rooted shoots transformed with the rd29A:DREB1A construct survived and appeared to be phenotypically normal, while the those containing 35S:DREB1A showed stunted growth and high rates of mortality (Fig. 3 B). Only 10% of the rooted shoots from the latter could be recovered for transplantation.

4.2. Transfer of the plants to greenhouse

The rooted shoots were transplanted to pots containing a mixture of sand and soil (1:1) and maintained under high humidity conditions in a plant growth cabinet before being transferred to the P2 level containment greenhouse until harvest. Fifty independently transformed plants with the rd29A:DREB1A and 18 plants with 35S:DREB1A constructs were successfully transplanted to the greenhouse (Fig. 2 I) and their T1 generation seed collected. Thirty transgenic events of rd29A:DREB1A and all 18 events of 35S:DREB1A were advanced to T2 generation 35S.DREB1A plants exhibited stunted growth even under greenhouse conditions (Fig. 2 C) and

approx 16 % (3 out of 18 primary transformants) of these plants were sterile with no seed set.

4.3. Phenotypic analysis of 35S:DREB1A and rd29A:DREB1A plants

The growth pattern of 35S:DREB1A groundnut tranformants was compared with those with rd29A:DREB1A and wild type JL 24 plants under both in vitro and greenhouse conditions. The 35S:DREB1A plants exhibited acute growth retardation and a very low survival percentage as compared to the rd29A:DREB1A plants (Fig. 3 B, C). T0 generation plants with 35S:DREB1A after being transferred to the greenhouse showed greater mortality rates. The plants of both the types were further advanced to subsequent generations and their seed collected. A significant difference was observed in the number of seeds produced by both the types; 35S:DREB1A plants producing less number of seeds as compared to the rd29A:DREB1A plants. The germination pattern also varied amongst the two types of plants in T1 generation where 40 % of the 35S:DREB1A plants demonstrated severe growth retardation within 7 d of emergence; however, 70% of these resumed growth thereafter. The rd29A:DREB1A plants appeared to be visually normal in their growth pattern.

4.4. Analysis of transgenics

Overall, 69 regenerants recovered from a total of 125 cotyledon explants co-cultivated with rd29A:DREB1A gave a regeneration frequency of 40 %. Although, 69 regenerants survived the in vitro selection, PCR analysis for *nptII* and *DREB1A* genes confirmed gene integration only in 50 of these regenerants giving a transformation frequency of over 72 %. This indicated that there were very few escapes that survived the selection process. Besides, the transgenic T0 plants obtained with 35S:DREB1A-containing construct were relatively lower in number as compared to the

rd29A:DREB1A containing transformants. Results of 35S:DREB1A transgenic T0 plants revealed that total of 30 T0 regenerants were obtained from an initial 129 cotyledon explants resulting in a low regeneration frequency (22 %). Furthermore, PCR analysis showed that only 18 of the 30 primary regenerants contained the *DREB1A* transgene. Overall, the transformation frequency of the 35S driven DREB1A plants was 62 %. Because of high mortality and retarded growth of the 35S:DREB1A plants, all the subsequent experiments were carried out with the putative rd29A:DREB1A transformants only.

4.4.1. Molecular analysis of transgenics

Thirty independently transformed plants of rd29A:DREB1A (RD1 to RD30) in T0 generation were analyzed by PCR, Southern blot analysis, and RT-PCR. PCR analysis of T0 plants was carried out for the amplification of the coding regions of *npt*II and *DREB1A* genes. Based on the PCR analysis, it was concluded that 72-74% of the plants of T0 generation showed the amplification of the expected size of the respective gene fragments (Fig. 4 A, B). These amplicons were further confirmed by Southern blot analysis by transferring the PCR amplified fragments to the nylon membrane and probing with non-radiolabelled *npt*II gene, the *Hind*III fragment containing 965 bp coding sequence of *rd29A* promoter, and 500 bp *Bam*HI fragment of *DREB1A*. This was necessary to ascertain the fidelity of the PCR generated amplicons and identify the positive clones that might have produced very small amplification products that might have been recorded as negatives in ethidium bromide stained gels.

The PCR results were further confirmed in T2 generation plants by amplification of a 769bp *rd29A-DREB* junction fragment, which eliminated the

probability of false positives due to the endogenous *rd29A DREB1A* gene in groundnut (Fig. 7 B).

4.4.1.1. RNA extraction and RT PCR analysis

Total RNA extracted from the transgenic samples showed intact bands of 25S, and 17S RNA, when analyzed on 1.2% formaldehyde agarose gels. The A260 280 ratio of the total RNA was 1.9 to 2.0 in all the tested samples thus indicating very little contamination of the RNA by protein and polysaccharides. Yields in the range of 500-600 µg per 1.0 g of leaf sample were obtained in all the samples extracted. PCR amplification of the extracted RNA by using *npt*II gene primers was negative, thus confirming the absence of genomic DNA contamination in the samples. RT-PCR was carried out on all the independent events of 35S:DREB1A where thirteen plants expressed *npt*II *and DREB1A* genes. RT-PCR studies on the cDNA from the selected transgenic plants containing rd29A:DREB1A showed positive amplification of the 700 bp *npt*II gene fragment in all plants under unstressed as well as stressed conditions. (Fig. 6 A). However, none of these events expressed the *DREB1A* gene under normal well-watered conditions. The plants were thereafter subjected to water stress conditions following a typical dry-down protocol and expression of DREB1A was studied. The RT PCR resulted in the amplification of the 500bp *DREB1A* transcript only after 3-5 days of withholding water (Fig. 6 B).

4.4.1.2. Inheritance studies

Fourteen independently transformed T0 events (RD2, RD4, RD11, RD12, RD13, RD14, RD19, RD20, RD21, RD22, RD25, RD28, RD30 and RD33) containing rd29A:DREB1A were advanced to T1 generation and T2 generations. In order to study the Mendelian inheritance pattern in the T1 progeny, 5 seeds of each

T1 event were sown and PCR analysis was performed on a total of 70 (5*14) T1 plants for the amplification of *npt*II gene (Fig. 5 A1, A2). Of these 70 plants, 46 showed amplification of *npt*II gene fragment that followed the Mendelian ratio of 3:1(Table 3; Fig. 5 A). However, progeny from the event RD21 did not show the Mendelian segregation ratio (χ^2 8.06). Gene integration pattern in the nuclear genome of the putative transformed plant containing rd29A.DREB1A was verified through Southern blot analysis.

The PCR positive plants in the T1 generation were further advanced to T2 generation where 8 seeds per T1 event were sown for further study on the inheritance pattern. Of the 72 T2 plants from 9 independent T0 events, 57 showed the amplification of a 700bp *npt*II gene fragment (Table 4; Fig. 7 A). The putative transformants were also analyzed for the presence of the *rd29A* promoter region by using Southern blot analysis where 7 events showed the integration of the *rd29A* promoter (Fig. 8 A). Further, the Southern blot analysis of T1 individuals indicated that the number of copies of the *DREB1A* transgene in the tested plants varied from one to four (Fig. 8B). Amongst these, 6 events (RD2, RD11, RD12, RD14, RD19, RD20) were further studied for the gene integration pattern by using T2 generation plants where plants from 5 events (RD2, RD11, RD12, RD19 and RD20) showed single copy inserts while those from the event RD14 showed four copies of the transgene (Fig. 8 B).

The results of the molecular analysis of the transformants produced through *Agrobacterium*-mediated gene transfer method indicated that the transgenic plants showed the Mendelian inheritance (3:1) of the introduced genes in the T_1 and T_2 generation while the Southern blot analysis indicated that the copy number of the transgene varied from one to four. These plants also exhibited stable gene expression

based on RT-PCR analysis of the respective genes The transgenic events having one to two copies of the transgene were selected for subsequent gene expression and phenotyping studies

Table 3. Inheritance of *nptII* gene in T_1 generation groundnut plants transformed with rd29A DREB1A gene construct

Event No.	No. T_1 generation plants tested**	Number of PCR positive plants	Number of PCR negative plants	3:1 segregation* χ^2
RD2	5	4	1	0.06
RD4	5	3	2	0.6
RD11	5	4	1	0.06
RD12	5	5	0	1.67
RD13	5	3	2	0.6
RD14	5	2	3	3.26
RD19	5	4	1	0.06
RD20	5	3	2	0.6
RD21	5	1	4	8.06*
RD22	5	3	2	0.6
RD25	5	2	3	3.26
RD28	5	4	1	0.06
RD30	5	5	0	1.67
RD33	5	3	2	0.6

*Significant at 5% probability at 1 degree of freedom, where the tabulated χ^2 value is 3.841

**Plants were T_1 progenies derived from independently transformed T_0 events

Table 4. Inheritance of *npt*II gene in T_2 generation groundnut plants transformed with rd29A DREB1A gene construct

T_1 Plant No.*	No. T_2 plants tested**	No. of *npt* II PCR positive plants	No. of *npt* II PCR negative plants	Genotypic ratios	3:1 segregation** χ^2
RD2-2	8	5	3	2 5 1 5	0 66
RD4-3	8	7	1	3 5 0 5	0 66
RD11-2	8	6	2	3 1	0 00
RD12-4	8	5	3	2 5 1 5	0 66
RD19-1	8	6	2	3 1	0 00
RD20-2	8	8	0	4 0	2 66
RD25-4	8	7	1	3 5 0 5	0 66
RD28-2	8	7	1	3 5 0 5	0 66
RD30-1	8	6	2	3 1	0 00

* The tabulated χ^2 value at 5% probability at 1 degree of freedom is 3 841
**Plants were T2 progenies derived from independently transformed T1 events

4.4.2. Preliminary physiological screening

Plants of 14 transgenic events with rd29A DREB1A in T-2 generation along with the untransformed wild type control JL 24 were evaluated for their physiological performance by exposing them to progressive water limiting conditions in the greenhouse in dry-down conditions (Fig 9 A, B) A plateau regression procedure using NTR as a function of FTSW was calculated by SAS to find the threshold at which the stomatal closure began to occur, i e when transpiration begins its decline upon progressive soil drying This allowed the calculation for the number of days between initial decline of transpiration and end of the dry-down experiment for the plants of each transgenic event Initial assessment of the 14 transgenic events showed that they differed in their transpiration responses to soil drying (Fig 9 C, D) Plants of

the wild type JL 24 started to show wilting symptoms (loss of turgor) by 21 days of stress thereafter showing severe symptoms. While it took 27 days for the WT JL 24 to reach stage III (NTR<0.1), the transgenic events did not show any wilting symptoms even after 21 days. Thereafter, the transgenic events varied in their response to drying conditions where a few transgenic events including RD2, RD4, RD11 and RD19 showing no symptoms, while events RD14, RD22 and RD25 showed reduced level of symptoms when compared to WT JL 24. The transgenic events differed largely in the number of days to reach the end point; RD14 reached the end point in 29 days, about the same as WT JL24, while RD4 reached the end point by 52 days. All events tested were compared based on the FTSW threshold where transpiration declined and found that all the transgenics had a decline starting at lower FTSW than the WT JL 24. A dendrogram based on the similarity in FTSW threshold values and the number of days to reach the end point under water deficit conditions revealed that these transgenic events could be broadly classified into four groups (at a similarity index value of 0.6). This clearly distinguished the water use pattern amongst these events, thus indicating that they differed in their transpiration response to water deficit conditions (Fig. 10).

4.4.3. Evaluation of the transgenic plants by using dry down methodology

Five events from across different clusters of the dendrogram (Fig. 10), having transpiration responses ranging from very similar to very different with respect to WT JL 24 were selected for subsequent experiments to study the various parameters for their physiological evaluation as follows:

4.4.3.1. Plant growth and development under well-watered conditions

Average shoot weight (g plant^{-1}), root weight (g plant^{-1}) and leaf area (cm^2 plant^{-1}) of the tested transgenic events and their wild type parent were measured for the pre-

treatment biomass (initial) and the post-treatment biomass (final) of WW plants (Table 5). There were differences in the initial shoot weight between the transgenic events and their wild type. Significant differences were seen in events RD2 and RD11 that showed a lower initial shoot growth than WT JL 24 and RD20 whose initial shoot weight was comparable. Although the events RD12 and RD19 had a somewhat lower initial shoot growth it was not significantly different from the WT JL 24 and RD20 Regarding the final shoot weight, the transgenic events RD12, RD20 and RD11 did not vary significantly with respect to the final shoot weight of the WT JL 24 However, the transgenic events RD2 and RD19 were observed to have accumulated higher final shoot biomass.

In terms of the initial root weight, except to the event RD 20, there were no differences in of any of the tested transgenic genotypes Moreover, there were also no significant differences between the final root growth of the transgenic events and the WT JL 24. Observations revealed a larger initial leaf area of RD20 than in RD2 and RD11, whereas it was intermediate in WT JL 24 and other transgenic events

By contrast, there was no significant different in the final leaf area between any of the genotypes. Overall, the initial leaf area of the event RD 20 and JL 24 were the highest.

4.4.3.2. Transpiration under well-watered conditions

A considerable range in transpiration was observed amongst the tested events under well-watered conditions (Table 6). The pattern of transpiration was essentially similar in all the genotypes except for the fact that the transpiration of WT JL 24 was among the highest. The cumulated transpiration differed significantly between the transgenics and the wild type.

Table 5. Average shoot weights, root weights and leaf area of the different transgenic lines and their wild type parent under well watered conditions in pot experiment. Data are those of the pre-treatment harvest at 28 DAS (initial), and those of the post-treatment harvest (final)*

Event No.	Shoot Wt. (g/plant)		Root Wt. (g/plant)		Leaf area (cm^2/plant)	
	Initial	Final	Initial	Final	Initial	Final
WT JL 24	5.79^{a}	10.85^{b}	0.56^{ab}	1.65^{a}	427.53^{ab}	1206.22^{a}
RD 19	4.15^{ab}	15.55^{a}	0.42^{b}	1.80^{a}	465.81^{ab}	1449.18^{a}
RD 12	4.38^{ab}	13.90^{ab}	0.48^{b}	1.62^{a}	461.50^{ab}	1522.20^{a}
RD 20	5.77^{a}	14.14^{ab}	0.88^{a}	2.09^{a}	689.03^{a}	1334.47^{a}
RD 2	3.65^{b}	14.83^{a}	0.37^{b}	1.75^{a}	398.38^{b}	1318.28^{a}
RD 11	3.29^{b}	13.93^{ab}	0.44^{b}	1.70^{a}	357.15^{b}	1476.75^{a}
Grand Mean	4.51	13.87	0.529	1.770	467	1385

*Values followed by the same letter are not significantly different at the 5% level by using Tukey's test

Events RD2, RD11 and RD12 had a lower cumulated transpiration than WT JL 24. Overall, the cumulative transpiration of event RD20 and WT JL 24 were similar and significantly higher than in RD11. Since large differences in cumulated transpiration in the experiment were observed despite similar leaf areas, the stomatal conductance of different transgenic events was measured under well-watered conditions. The stomatal conductance of RD2 and RD11 was over two-fold lower than the WT JL 24. Although the stomatal conductance of RD20 and WT JL 24 was similar, that of RD12 and RD19 was significantly lower than that of the untransformed controls. The transpiration efficiency (TE) of the well-watered plants was computed and a significant genotypic variation in TE was found which was significantly higher in

RD2, RD11, RD12, and RD19 than in WT JL 24. These results indicated that all transgenic lines except RD20 had a significantly higher TE. However, non-significant differences could be observed with respect to their TE under well-watered conditions amongst the transgenic events.

Table 6. Stomatal conductance (Gs), average biomass produced (delta DW) and transpiration efficiency (TE) of different transgenic lines of groundnut their wild type parent under well-watered conditions in pot experiments*.

Event number	Delta biomass (g /plant)	Stomatal conductance	Transpiration efficiency
WT JL 24	6.25^{b}	387^{a}	2.05^{c}
RD 19	12.92^{a}	257^{b}	4.31^{ab}
RD 12	10.79^{a}	200^{b}	5.13^{a}
RD 20	9.69^{ab}	289^{ab}	3.19^{bc}
RD 2	13.06^{a}	156^{b}	4.09^{ab}
RD 11	12.01^{a}	176^{b}	4.96^{ab}
Grand Mean	*10.79*	*307*	*3.96*

*Values followed by the same letter are not significantly different at the 5% level by using Tukey's test.

4.4.3.3. Plant growth and development under water deficits

The effect of drought stress on the growth of the plants was observed by examining the final plant dry biomass at the end of the experiment that was found to be about two-third of those in well-watered conditions (Table 7). Although, there were no significant differences in the initial biomass of the pre-harvested plants under water limiting conditions, event RD20 showed a higher shoot dry weight than WT JL 24. However, event RD11 and RD12 had a lower shoot biomass than the rest of the transgenic lines but not significantly different from WT JL 24. On the other hand, the

root dry weight was significantly higher in RD20 and W T JL 24 than the remaining transgenic events The leaf area of RD2 was lowest of all although not significantly different from RD11 In contrast the leaf area of RD20 was on a higher side followed by WT JL 24 and RD19

Table 7. Average shoot weights root weights and leaf area of the different transgenic lines and their wild type parent after undertaking a progressive drought stress in pot experiments*

Event number	Shoot wt. (g /plant)	Root wt. (g/ plant)	Leaf area (cm^2 plant)
WT JL 24	10 27^{bc}	2 38^{a}	1102 69^{ab}
RD 19	11 23^{ab}	1 70	1082 78^{ab}
RD 12	9 52^{c}	1 30	1048 01^{bc}
RD 20	11 92^{a}	2 08^{bc}	1204 71^{a}
RD 2	11 44^{ab}	1 62^{cd}	892^{cd}
RD 11	9 08	1 33^{bc}	952 ^{d}
Grand Mean	10 58	1 739	1047

*Values followed by the same letter are not significantly different at the 5% level using Tukey's test

4.4.3.4. NTR-FTSW relationship

The transgenic events differed in the response of NTR to FTSW and were clearly distinguishable from WT JL 24 (Fig 11 A-F) Overall the transpiration of all the transgenic events started declining at lower FTSW values (drier soil) under drought stress than WT JL 24 A typical "slow wilting' phenotype was observed towards end of the dry-down water stress treatment (Fig 9 E) The NTR of events RD2, RD11 and RD19 dropped at significantly smaller FTSW values than RD20 and WT JL 24 (Table 8) While the WT JL 24 showed a decline in transpiration at high

Table 8. The fraction of transpirable soil water (FTSW) point at which the stomata closure begins to occur as estimated by plateau regression in the glasshous experiments*

Event number	FTSW - Threshold	SE	Confidence limits
WT JL 24	0.5472[a]	±0.0211	0.5053-0.590
RD 19	0.3655[bc]	±0.0128	0.3399-0.3911
RD 12	0.4461[abc]	±0.021	0.4043-0.4880
RD 20	0.4935[ab]	±0.0171	0.4595-0.5276
RD 2	0.2826[c]	±0.015	0.2529-0.3124
RD 11	0.3981[abc]	±0.0209	0.3565-0.4397
Grand Mean	0.416		

*Values followed by the same letter are not significantly different at the 5% level using Tukey's test

FTSW values (0.55, high soil moisture content) the transgenic event RD2 showed a decline in transpiration at a much lower FTSW threshold (0.28 lower soil moisture content) Overall, all the tested transgenic events closed their stomata at lower FTSW values (0.28-0.49) under drought stress in comparison to WT JL 24

4.4.3.5. Water extraction and cumulated transpiration

At the end of the drought stress period, the total amount of water extracted from the soil by transpiration (TTSW) was measured by subtracting the final pot weight from the initial pot weight There was no significant difference in TTSW between any of the genotypes tested throughout the drought stress period (Table 9) Similarly, the cumulative transpiration was computed as the sum of daily transpiration from initiation until the plant depleted all the soil water (FTSW 0.1) Overall, while

the cumulative transpiration of RD20 was the highest, RD2 and RD11 had a lower cumulative transpiration under drought stress than did WT JL 24 (Table 9).

The total biomass produced during the dry down cycle (Δ biomass) showed significant differences among the transgenic events thus indicating apparent differences in the biomass produced per unit of water used (TE). The increase in biomass produced during the dry down period (Δ biomass) in RD2 than the WT JL 24, RD12, and RD11. While analyzing TE of the transgenic events tested, it was observed that the WT JL 24 (4.21) had the lowest TE than the other transgenic events, although, at par with the RD12 (4.25). Moreover, while the events RD2 and RD19 showed significant differences in their TE when compared to the untransformed JL 24, RD20 and RD11 and WT JL 24 did not vary significantly with respect their TE under drought stress.

Table 9. Average transpirable soil water (TTSW), cumulative transpiration (cumulative T), average biomass produced (delta DW) and transpiration efficiency (TE) of different transgenic lines of groundnut throughout the drying cycle.

Event number	TTSW (g/plant)	Cumulative T (g/plant)	Delta biomass (g/plant)	TE
WT JL 24	850.8^{a}	1651^{ab}	6.98^{bc}	4.21^{c}
RD 19	870.4^{a}	1661^{ab}	8.74^{ab}	5.24^{ab}
RD 12	838^{a}	1558^{b}	6.56^{c}	4.25^{c}
RD 20	901.8^{a}	1716^{a}	7.65^{abc}	4.43^{bc}
RD 2	904.4^{a}	1645^{ab}	9.53^{a}	5.79^{a}
RD 11	899.3^{a}	1530^{b}	6.95^{bc}	4.59^{bc}
Grand Mean	877.5	1627	7.74	4.75

*Values followed by the same letter are not significantly different at the 5% level by using Tukey's test.

4.4.4. Exploring surrogates of TE

4.4.4.1 *SLA, SCMR and $\Delta^{13}C$ analysis of pre harvest and well-watered treatments*

Observations revealed that for the pre-harvest treatment the specific leaf area of the transgenic events did not vary significantly among themselves (Table 10) However, significant differences were observed in the specific leaf area of events RD2, RD19 and RD20 when compared to the WT JL 24 Similarly, in the well-watered treatment also, there were no differences in the specific leaf area of the transgenic events and the WT JL 24. The trait SCMR was not significantly different under well-watered conditions (Table 10).

Results for the carbon isotope discrimination $\Delta^{13}C$ trait indicated that in the pre-harvest treatment, the event RD11 showed a significant variation in the $\Delta^{13}C$ values in contrast to the RD19, RD20, and RD 2 and WT JL 24 while it was intermediate for the event RD12. However, under well-watered conditions, RD20 had a higher Carbon isotope discrimination ($\Delta^{13}C$) followed by RD2 and RD11 that was significantly different from the others.

4.4.4.2 *SCMR, SLA and $\Delta^{13}C$ under drought stress treatment*

Specific leaf area analysis of the transgenic events as well as the WT JL 24 revealed that there were no significant differences in the SLA of either of these under drought stress treatment (Table 11). The SCMR readings were taken at three different times throughout the stress treatment. At the beginning of the stress treatment, SCMR for event RD2 was significantly higher than the WT JL 24. Events RD 11 and RD20 were intermediate at this stage, whereas, RD12, RD19 and WT JL 24 had lower values for this trait (Table 11). Again, during the mid-experiment measurements, RD2 showed a significant variation in contrast to the WT JL 24.

Table 10. Average values of Specific Leaf Area (SLA), SCMR and $\Delta^{13}C$ of the different transgenic lines and their wild type parent under well-watered conditions in pot experiment. Data are those of the pre-treatment harvest at 28 DAS (initial), and those of the post-treatment harvest (final)

Line No.*	SLA (g/cm^2)		SCMR		$\Delta^{13}C$	
	Initial	Final	Initial	Final	Initial	Final
	119.7^{b}	187.9^{a}	41.37^{a}	36.30^{a}	21.23^{b}	20.69^{b}
RD 19	200.4^{a}	187.1^{a}	37.62^{ab}	35.48^{a}	20.86^{b}	20.47^{b}
RD 12	189.4^{ab}	202.2^{a}	41.62^{a}	36.73^{a}	21.40^{ab}	20.72^{b}
RD 20	211.4^{a}	202.5^{a}	34.90^{b}	37.11^{a}	20.87^{b}	21.92^{a}
RD 2	230.4^{a}	192.1^{a}	40.35^{ab}	38.08^{a}	21.34^{b}	20.99^{ab}
RD 11	184.4^{ab}	201.0^{a}	37.87^{ab}	37.10^{a}	22.26^{a}	20.87^{ab}
Grand Mean	189.3	195.5	38.95	36.80	21.33	20.95

*Values followed by the same letter are not significantly different at the 5% level by using Tukey's test.

Finally, at the end of the drought stress period, the SCMR differed significantly in the transgenic plants when compared to the untransformed JL 24. Overall, a significant variation was observed amongst the all transformed events including WT JL 24 for the SCMR trait for the first two sets of readings with the maximum value for RD 2 and minimum for WT JL 24. A non-significant asymmetry for SLA measurement amongst all the events was observed. However, no significant differences were observed for the Carbon isotope discrimination $\Delta^{13}C$ in the transgenic events as well as the WT JL 24 during drought stress treatment

Table 11. Average values of Specific Leaf Area (SLA), SCMR and $\Delta^{13}C$ of different transgenic lines of groundnut throughout the drying cycle. Data are those of the post-treatment harvest (final) after the termination of the experiment

Event number	SLA (g/cm^2)	$\Delta^{13}C$	SCMR		
	Post treatment harvest	Post treatment harvest	Beginning of the drying cycle	Middle of the drying cycle	Post treatment harvest
WT JL 24	213.7[a]	21.57[a]	36.15[c]	39.12[b]	39.12[b]
RD 19	193.9[a]	21.49[a]	36.83[c]	41.35[ab]	46.85[a]
RD 12	202.1[a]	20.98[a]	36.47[c]	41.35[ab]	45.68[a]
RD 20	205.5[a]	21.77[a]	37.28[bc]	41.33[ab]	45.12[a]
RD 2	188[a]	21.39[a]	42.03[a]	44.73[a]	49.80[a]
RD 11	193.1[a]	21.38[a]	39.63[ab]	42.32[ab]	47.33[a]
Grand Mean	199.4	21.43	38.07	41.70	45.65

*Values followed by the same letter are not significantly different at the 5% level by using Tukey's test

4.4.4.3 *Relation among the traits SCMR, SLA and C13*

A significant negative correlation was observed between the SCMR and SLA under drought stress conditions ($r=0.9470$, $P<0.01$ Fig. 12 A) and with the well-watered conditions ($r=0.8110$, $P<0.05$, Fig. 12 B). There was, however, no significant relationship between the SLA and SCMR with $\Delta^{13}C$ under drought stress and well-watered conditions (Figs. 13, 14).

4.4.4.4 *Relation of TE with SLA, SCMR and* $\Delta^{13}C$

Results indicated that the reciprocity of the TE with its surrogate traits including SLA, SCMR and $\Delta^{13}C$ was non-significant under well-watered regime

(Figs 15 A, 16 A, 17 A). However, unlike in well-watered conditions, there was a significant variation amongst the tested events under drought stress for TE ranging between 4.211 and 5.796 that had a significant negative correlation with SLA (r=0.8237; Fig. 15 B) and a positive correlation with SCMR(r 0.7359, Fig 16 B) However, the TE did not significantly correlated with $\Delta^{13}C$ (Fig 17 B)

4.4.4.5 *Relationship between TE and FTSW-threshold*

Results obtained in the dry down experiment strongly indicated the existence of a strong correlation between the TE and FTSW threshold values of the transgenic events as well as the WT JL 24 (r=0.9124, P< 0.001, Fig 18) Hence, the events that closed their stomata in drier soils (low FTSW threshold value) utilized the water more efficiently than the others thus resulting in a higher TE

4.4.5. *Effect of water limitation on Photosystem II (PSII)*

Drought-induced decrease in the fluorescence as derived by Fv/Fm is a measure of the cumulative photo oxidative damage to PSII that is considered to be an important parameter for evaluation of the response of plants to oxidative stresses including drought. The present dry-down setup, however, did not show any negative effect on the Fv/Fm of the transgenic groundnut plants after subjecting them to soil drying conditions. The results thus obtained indicate that there is no effect on the Fv/Fm-value as long as the effect of drought is reversible i.e. NTR < 0.1 when all physiological processes in the stressed plants are virtually seized There also did not seem to be any effect of drought stress on the fluorescence transient in both the transgenic events and the WT JL 24 (Fig. 19 A, B), thereby indicating that even under conditions where FTSW declined to a value of 0, the photosynthetic electron transport was operating well. Control and drought-stressed transients had very similar

amplitudes, which indicated that there was little drought-induced decrease in the size of the photosynthetic apparatus. In general, the drought stress did not seriously affect the photosynthetic system and Fv/Fm values of the transgenic groundnut plants as well as the WT JL 24 under the dry down set up used in this study.

4.4.6. Biochemical characterization

The effect of water stress on several key biochemical parameters were also studied in the WT JL 24 and the selected 5 independent transgenic events. These included the major antioxidant enzymes such as *Superoxide dismutase, Glutathione reductas and Ascorbate peroxidase*. The levels of proline which is implicated as having the role of osmoregulation as well as an antioxidant were also determined immediately after the imposition of water stress and at different intervals following this (0, 3, 6, 9 and 12 days) in the leaf tissues. To study the level of lipid peroxidation, MDA (Malondealdehyde) levels were estimated which is an indication of the free radicals.

4.4.6.1 Effect of water stress on Superoxide dismutase *activity (E. C. 1.15.1.1)*

The superoxide dismutase (SOD) activity, which is responsible for the elimination of superoxide radicals in the cells following stress imposition did not statistically vary under well-watered conditions (Table 12). However, the activity of SOD increased significantly in the transgenics after the initiation of drought under a mild water stress (3 DAS) that showed a higher activity in the transgenic events (5.205-6.33 U/mg protein) when compared to the WT JL 24 (4.562 U/mg protein). Thereafter, on progression of the stress, the transgenic events RD2 and RD11 maintained a higher SOD activity as compared to WT JL 24; RD2 showed a significantly higher activity at 6 DAS in contrast to the WT JL 24 (Table 12). At

higher water stress levels (9 and 12 DAS) all the transgenic events showed a significant increase in their SOD activity levels in contrast to the WT JL 24. It was also observed that almost all the transgenic lines except RD20 showed a significantly higher ($P \leq 0.001$) enzyme activity at higher levels of stress (9 and 12 DAS) in contrast to the that at 0 DAS (Fig. 20). In contrast, there were no significant differences in the SOD activity in WT JL 24 throughout the dry-down cycle

'able 12. Mean activities of *Superoxide dismutase* (SOD) in whole leaf extracts of transgenic as well as wild type groundnut plants during progressive dry down cycle. (The SOD activities are expressed as units mg^{-1} protein where N=5; Mean values ± SE).

Event number	Unstressed	3 DAS	6 DAS	9 DAS	12 DAS
RD 2	5.343 ± 0.1979	6.338 ± 0.2147	7.691 ± 0.3573	8.308 ± 0.5957*	11.230 ± 0.3626***
RD 11	5.236 ± 0.1702	5.548 ± 0.172	6.290 ± 0.1955*	7.387 ± 0.3501	9.960 ± 0.3913***
RD 12	5.346 ± 0.0468	5.205 ± 0.1946	7.281 ± 0.3170***	7.702 ± 0.1734*	9.828 ± 0.2581***
RD 19	5.576 ± 0.0509	5.532 ± 0.154	6.226 ± 0.3078	6.192 ± 0.208	9.645 ± 0.3931***
RD 20	5.469 ± 0.2548	5.880 ± 0.090	5.631 ± 0.2478	5.975 ± 0.2929	6.990 ± 0.4316*
WT JL 24	5.121 ± .2179	4.562 ± 0.1091	5.414 ± 0.2071	5.886 ± 0.7642	5.226 ± 0.1045

* Indicates the level of significance from the mean values for the untransformed JL 24. ($P < 0.05$; $P < 0.01$) by using student's t test.

Table 13. Mean activities of *Ascorbate peroxidase* (APOX) in whole leaf extracts of transgenic as well as wild type groundnut plants during progressive dry down cycle. (The APOX activities are expressed as μM mg^{-1} protein where N=4; Mean values ± SE).

Event number	Unstressed	3 DAS	6 DAS	9 DAS	12 DAS
RD 2	0.5136 ± 0.0310	0.4567 ± 0.0323	0.7036 ± 0.0886*	0.7480 ± 0.0711*	0.8895 ± 0.0475*
RD 11	0.4126 ± 0.0434	0.4278 ± 0.0474	0.6060 ± 0.0408*	0.6515 ± 0.1063	0.8409 ± 0.0973*
RD 12	0.4899 ± 0.0617	0.4506 ± 0.2154	0.5867 ± 0.0618	0.5806 ± 0.0661	0.7889 ± 0.2648*
RD 19	0.5465 ± 0.0285	0.3491 ± 0.0180	0.4626 ± 0.0342	0.5239 ± 0.0411	0.9912 ± 0.13532*
RD 20	0.4439 ± 0.0753	0.4667 ± 0.0340	0.4490 ± 0.0527	0.5029 ± 0.0479	0.4878 ± 0.0381*
WT JL 24	0.4336 ± 0.0395	0.3798 ± 0.0134	0.3884 ± 0.0784	0.4907 ± 0.0648	0.5758 ± 0.0507

*Indicates the level of significance from the mean values for the untransformed JL 24 ($P < 0.05$; $P < 0.01$) by using student's t-test

4.4.6.3 Effect of water stress on **Ascorbate peroxidase** *activity (EC 1.11.1.11)*

Water stress showed a distinct effect on the *Ascorbate peroxidase* (APOX) activity in the transgenic events as well as the WT JL 24 during the dry down experiment (Table 13) The differences in the APOX activity were not significant under well-watered conditions at the beginning of the experiment However, after 3 DAS, APOX activity in the leaves of the transgenic plants exposed to progressive soil drying showed a 20% increase as compared to the untransformed control The specific activity of APOX was significantly higher in RD2 (1 8 fold) and RD11 (1 56- fold) than the WT JL 24 plants at 6 DAS Further, at 9 DAS the APOX activity of RD2 was approximately 1.5 fold higher (0 7480 µM mg protein) than the WT JL 24 (0 4907 µM/mg protein). However, a significantly higher APOX activity was observed in RD19 followed by RD2 in contrast to WT JL 24 at 12 DAS Unlike the observed SOD activity, the APOX activity in the transgenic events RD12, RD19 and RD20 did not show a significant increase when compared to that observed at 0 DAS (Fig 22) However, differences in the APOX activity were observed between RD2 and RD11 at 12 DAS. Again, the WT JL 24 did not show any significant differences in APOX activity at different stages of water stress A reduction in APOX activity at 3 DAS observed in RD19 was unexpected

4.4.6.2 Effect of water stress on **Glutathione reductase** *activity (EC 1.6.4.2)*

Glutathione reductase (GR) that catalysis the NADPH-dependent reduction of oxidized glutathione did not show any significant increase in activity under no or mild water stress during 0 and 3 DAS (Table 14) The transgenic events RD11 and RD12 showed a significant increase ($P \leq 0.05$) in GR activity when the plants faced water stress for 6 days (6 DAS) The GR activity increased thereafter where the events

RD11, RD19 and RD20 showed a higher activity at 9 DAS in contrast to the WT JL 24. All of the transgenic events showed a significantly higher GR activity at 12 DAS ($P \leq 0.001$ for RD2 and RD 19) and ($P \leq 0.01$ for RD11, RD12, RD20). The GR activity in the transgenic events was found to be significantly higher at 6, 9 and 12 DAS at least at $P \leq 0.05$ when compared to 0 DAS (Fig. 21). The GR activity in the untransformed JL 24 was not significant throughout the stress period.

4.4.6.4 Effect of water stress on Proline levels

There were no significant differences in the proline content of the transgenic events and the WT JL 24 under well-watered conditions as well as at the beginning of the stress at 3 DAS (Table 15). However, its content increased at 6 DAS thereby indicating significant differences ($P \leq 0.01$) in the proline content of all the transgenic events (869.8-916.3 um g) except in RD20, which did not significantly different from the untransformed control (797.2 µM g). The elevated proline levels in all transgenic events with the exception of RD20 were significantly higher than the WT JL 24 (1.2-1.4-fold at $P \leq 0.01$) at 9 DAS. Similarly, the elevated proline level of the transgenic events at lower NTR value at 12 DAS showed a significantly higher increase (1.25-1.3-fold $P \leq 001$) over WT JL 24. Since 3 DAS, a significant increase was observed in the proline level of all transgenic events as well as the WT JL 24, indicating the accumulation of proline soon after encountering water stress (Fig. 23).

Table 14. Mean activities of *Glutathione reductase*(GR) in whole leaf extracts of transgenic as well as wild type groundnut plants during progressive dry down cycle. (he GR activities are expressed as units mg^{-1} protein where N=4; Mean values ± SE).

Event Number	Unstressed	3 DAS	6 DAS	9 DAS	12 DAS
RD 2	0.4451 ± 0.0776	0.5762 ± 0.1035	0.7729 ± 0.065	0.8532 ± 0.055*	0.8619 ± 0.0385***
RD 11	0.4204 ± 0.0454	0.513 ± 0.0653	0.7682 ± 0.0165	0.8019 ± 0.0678*	0.8875 ± 0.0745**
RD 12	0.4317 ± 0.0678	0.5641 ± 0.0721	0.7772 ± 0.058	0.8273 ± 0.1377	0.8292 ± 0.0635**
RD 19	0.4406 ± 0.04	0.5416 ± 0.0645	0.6983 ± 0.0954	0.7830 ± 0.0418*	0.8324 ± 0.0392***
RD 20	0.4391 ± 0.0591	0.6065 ± 0.0926	0.7180 ± 0.0644	0.7719 ± 0.0397*	0.7841 ± 0.0729**
WT JL 24	0.4148 ± 0.0645	0.6378 ± 0.07633	0.5914 ± 0.0456	0.5417 ± 0.066	0.4586 ± 0.0329

* Indicates the level of significance from the mean values for the untransformed JL 24 ($P < 0.05$; $P < 0.01$) by using student's t test

4.4.6.5 Effect of water stress on free radicals (MDA; Malondealdehyde)

An increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. The level of MDA (one of the major TBA reactive metabolites) increased in the drought stressed plants. In the present study, the MDA levels did not change in the transgenic events as well as the untransformed control under unstressed (well watered) or a mild stress (3 DAS) conditions (Table 16). However, WT JL 24 at 6 DAS showed a significant increase in MDA when compared to the transgenic events RD2, RD12, RD19 and RD 20. The MDA levels in the WT JL 24 at 9 DAS and 12 DAS increased significantly in contrast to the transgenic events ($P \leq 0.001$). Significantly, there were no differences in the MDA content in RD2, RD11 and RD12 throughout the drying cycle. However, significant differences in the MDA level was observed in RD19 and RD20 as well as the WT JL 24 at 9 and 12 DAS in comparison to the unstressed plants (0 DAS) thereby indicating that the increase in MDA content was dependent on the specific response of plants to water stress (Fig. 24).

4.4.6.6 Ratio of Antioxidants

The balance between SOD and APOX (and or CAT) activity in cells is considered to be crucial for determining the steady state level of O_2^- and H_2O_2. Results based on the ratio of specific activities of SOD and APOX indicated that until 9 DAS, these were identical during all the stages of stress in the transgenic events (10.56-12.1) and WT JL 24 (11.2-12.0). However, a decrease in the SOD APOX ratio was observed at 12 DAS in WT JL 24 (8.5) as compared to the average ratio of all the transgenic events (11.2).

Table 15. Proline content in whole leaf extracts of transgenic as well as wild type groundnut plants during progressive dry down cycle. (The proline activities are expressed as μM gm^{-1} tissue FW where N=4; Mean values ± SE).

Event number	Unstressed	3 DAS	6 DAS	9 DAS	12 DAS
RD 2	380.6 ± 23.72	596.3 ± 5 45	875 3 ± 17 61*	1037.2 ± 18.76***	1111 ± 12.34***
RD 11	385.5 ± 6.94	557.9 ± 12 5	891 5 ± 32 49*	1012 4 ± 15.57***	1090 5 ± 14 69***
RD 12	357 ± 9 2	552 9 ± 14 65	916 3 ± 25 03**	952 8 ± 9 19***	1088 6 ± 22 7***
RD 19	354 5 ± 15 2	593 2 ± 17 92	869 8 ± 14 26*	880 9 ± 10 19**	1133 ± 5 95***
RD 20	362 ± 15 37	599 4 ± 12 78	797 8 ± 23 51	807 8 ± 18 01	1091 7 ± 12 5***
WT JL 24	388 6 ± 15 80	565 3 ± 14 19	797 2 ± 17 8	811 5 ± 11 25	877 8 ± 19 91

* Indicates the level of significance from the mean values for the untransformed JL 24 (P < 0 05, P< 0 01) by using student's t test

Table 16. MDA levels in whole leaf extracts of transgenic as well as wild type groundnut plants during progressive dry down cycle.

(The MDA levels are expressed as units mg^{-1} protein where N=4; Mean values ± SE).

Event number	Unstressed	3 DAS	6 DAS	9 DAS	12 DAS
RD 2	24.17 ± 0.879	25.67 ± 2.009	24.81 ± 1.946*	21.97 ± 0.6758***	23.20 ± 0.2321***
RD 11	25.57 ± 0.765	26 ± 1.298	24.49 ± 1.943	24.98 ± 0.6585***	23.47 ± 0.5217***
RD 12	25.03 ± 3.124	25.51 ± 0.507	24.71 ± 1.957	26.75 ± 1.058*	23.90 ± 0.796***
RD 19	26.96 ± 0.647	25.24 ± 0.53	21.91 ± 0.6962***	21.48 ± 0.5616***	23.42 ± 1.113***
RD 20	27.93 ± 0.749	26 ± 0.696	25.35 ± 0.873*	22.13 ± 0.8086***	24.55 ± 6.118
WT JL 24	26.43 ± 1.243	27.82 ± 1.553	30.94 ± 1.247	32.06 ± 0.386	35.29 ± 1.418

* Indicates the level of significance from the mean values for the untransformed JL 24 ($P< 0.05$; $P< 0.01$) by using student's t test

5. DISCUSSION

DISCUSSION

The explosive increase in world population, along with the continuing deterioration of arable land, scarcity of fresh water, and increasing environmental stress pose serious threats to global agricultural production and food security (Garg et al., 2002). Although conventional plant breeding has led to some successes in breeding crop varieties with improved adaptations to abiotic stresses such as drought, high salinity and temperature, further progress in breeding for abiotic stress tolerance may require the use of modern molecular tools. Classical methods such as positional cloning and insertional mutagenesis have been used with success to identify genes having a major effect on the phenotypic variation. However, these methods are limited by genomic size and/or by the lack of transposons in the species being studied. Therefore, there is a continuous need to integrate biotechnological approaches such as transgenics and genomics (transcriptomes, proteomics, metabolomics) with plant physiology and plant breeding so as to build an infrastructure for applying these tools for crop improvement. However, in the absence of molecular tools to introgress drought tolerance traits into germplasm, transgenics offer attractive opportunities for incorporating novel genes that may regulate specific abiotic stress-linked processes in plants (Sharma and Lavanya, 2000).

Several gene transfer approaches have been attempted to improve tolerance to abiotic stresses in different plants species (Holmberg and Bulow, 1998; Vinocur and Altman, 2005; Umezawa et al., 2006). Depending on the developmental stage and the external stimuli, the stress responsive genes are classified as dehydrin-induced, ABA-responsive, or late embryogenesis abundant (LEA). Stress-induced proteins with known functions include water channel proteins, key enzymes for osmolyte (proline,

betaine, sugars, and polyamines) biosynthesis, detoxification enzymes, and transport proteins (Tarczynski et al., 1993; Pilon Smits et al., 1995; Xu *et al.*, 1996; Bohnert and Jensson, 1996; Sivamani et al., 1996; Bohnert and Shen, 1999; Kasuga *et al.*, 1999). However, tolerance to drought is very unlikely to be the effect of a single gene. Hence, a more appropriate strategy would be to switch on regulatory genes (*COR, rd29A*) or a transcription factor regulating the expression of several genes related to abiotic stress (Bartels and Sunkar, 2005; Chinnusamy et al., 2005). Many genes that are induced by various environmental stresses have been identified, cloned and characterized which include several families responsive to desiccation (Yamaguchi-Shinozaki et al., 1992), low temperature (Nordin et al., 1991; Welin et al., 1995), cold (Gilmour et al., 1992; Horvath et al., 1993) and others stresses (Tarczynski et al., 1993; Xu et al., 1996; Sivamani et al., 2000; Bohnert and Shen, 1999). Genetic transformation of plants by using genes encoding for stress inducible cis-acting or trans-acting transcription factors are known to regulate a number of genes. These have been shown to activate a cascade of native genes that are involved in response to stress and may be effectively deployed for abiotic stress tolerance in crop plants (Shinozaki and Yamaguchi-Shinozaki, 1999).

Groundnut *(Arachis hypogaea* L.) is one of the important food legume crops of the semi-arid tropics (SAT). The molecular tools that could help assisting the breeding activities for complex traits such as drought and other abiotic stresses are not yet available in groundnut. Due to the lack of sufficient polymorphisms in groundnut, marker assisted selection is rate limiting. Besides, QTL mapping in groundnut is also limited by the non-availability of molecular markers and difficulties in its genotyping. Therefore, in our efforts to improve the drought tolerance of groundnut, an ABA-independent transcription factor *DREB1A* driven by the stress-responsive promoter

from *rd29A* of *A. thaliana* was used to develop transgenic plants following *Agrobacterium*-mediated genetic transformation. The present work, to the best of our knowledge is the first ever attempt at engineering groundnut for abiotic stress tolerance. The results obtained in this study are being discussed here

Regeneration and transformation studies

Regeneration potential in tissue cultures is affected by the type of explant, culture conditions, and age of the explants (Sharma et al., 1990). *Agrobacterium*-mediated genetic transformation of groundnut by using the de-embryonated cotyledon explants was carried out successfully following the transformation protocol reported earlier from ICRISAT (Sharma and Anjaiah, 2000). The regeneration and transformation system showed high regeneration potential and frequency of transformed shoots. As previously reported by Sharma and Anjaiah (2000), the transformation procedure is simple, rapid and resulted in a large number of transformants as compared to several other reported systems for groundnut (Kartha et al., 1981; Bhatia et al., 1985; Chen et al., 1990; Mckently et al., 1990, Mckently, 1991; Cheng et al., 1992). This system, based on cotyledon explants from mature seeds favours efficient *Agrobacterium*-mediated genetic transformation due to the rapidity of morphogenesis and no requirement of frequent subcultures. The target cells for transformation are those at the proximal cut surfaces of the explants. These types of explants that produce adventitious shoot buds through direct regeneration have been shown to be excellent for obtaining transgenic fertile plants in several other crop species as well (Moloney et al., 1989).

In the present study, the *DREB1A* gene was driven either by the *CaMV* 35S promoter for constitutive expression or by the promoter of *rd29A* gene from *A*

thaliana for drought stress-responsive expression *DREB1A* has been shown to bind to the cis-acting DRE and regulate the expression of many stress-related genes under drought, salt and cold stress conditions in *Arabidopsis* (Liu et al., 1998). DRE is also known to be involved in the ABA independent gene expression under stress conditions in many dehydration responsive genes like *rd29A* that are responsible for dehydration and cold induced expression (Yamaguchi-Shinozaki and Shinozaki, 1993; Iwasaki et al., 1997, Nordin et al., 1991). However, a variation in the transformation efficiency was observed in the transgenic events for the two different constructs used in this study. A large number of putative transgenic groundnut plants (T0 generation) were regenerated that included 18 events from 35S DREB1A (constitutively expressed) and 50 events from rd29A DREB1A (stress-inducible) constructs. These differences might be due the fact that the 35S DREB1A plants expressed *DREB1A* constitutively, thus resulting in the alteration of structure and physiology of the plant. In the present studies, it was observed that constitutive expression of *DREB1A* had adverse implications on the shoot elongation as well as rooting of these plants in vitro resulting in a low recovery of the transformants. Previous reports on constitutive overexpression of the DREB1C/DF genes in plants also showed an undesirable dwarf phenotype (Liu et al., 1998, Gilmour et al., 2000). However, there are no reports on the negative effects of constitutive expression of *DREB1A* on the overall transformation frequency.

In the present study, constitutively expressed *DREB1A* (35S DREB1A) plants exhibited stunted growth even under control conditions in vitro as well as in the greenhouse. Moreover, 16% (3 out of 18 primary transformants) of the 35S DREB1A plants turned out to be sterile with no seed set. Similar observations have been reported earlier in *A. thaliana* where the constitutive expression of *DREB1A* in

Arabidopsis transgenics resulted in improved stress tolerance at the expense of growth and productivity (Kasuga et al., 1999). In contrast, all the 50 independent primary transformants (T0) with rd29A:DREB1A showed a normal phenotype and were completely fertile and produced viable seeds. These plants appeared normal and their phenotype closely resembled that of the regenerated or untransformed control plants. The phenotypic differences observed in the constitutively expressed *DREB1A* plants might be due to the fact that the genes were being expressed all the time, whether or not the plant is under stress. These results are in accordance with those previously reported by in tomato expressing *Arabidopsis CBF1* (Hsieh et al., 2002). Overexpression of *CBF1*, severely reduced growth in tomato, thus suggesting that heterologous *CBF1* also affects developmental processes in transgenic plants. Such plants showed a decrease in fruit size, seed number, and fresh weight as compared with the wild-type plants under normal conditions. These results gave a strong indication that constitutive expression of the *DREB1A* transgene in plants results in stress tolerance at the expense of growth and productivity (Kasuga et al., 2004). However, a recent report on the constitutive expression of DREB1A by using revealed that Ubi1:CBF3 *(DREB1A)* and Ubi1:ABF3 plants displayed normal growth and seed-set (Oh et al., 2005). The use of a stress inducible promoter in the present work appeared to minimize the negative effects on the plant growth where no significant differences in the growth pattern were observed in rd29A:DREB1A plants under non-stress conditions. This observation also support earlier reports indicating that the *rd29A* promoter as stress inducible and allows gene expression during exposure to stress conditions (Shinwari et al., 1998). However, this is in contrast to a recent report on transgenic potato expressing *DREB1A* gene that showed growth retardation under the influence of the *rd29A* promoter (Behnam et al., 2006).

Nevertheless, considering that rd29A DREB1A did not effect the growth of groundnut plantlets in vitro, all further work was restricted to these transgenic plants

Stable integration and expression of foreign genes in transgenic plants are of critical importance for the successful application of genetically engineered crops in agriculture A variety of molecular and biochemical tools including polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) Southern hybridization, Northern hybridization, ELISA, In-gel assays, and Western immunoblotting have been extensively used to confirm the presence copy number and expression of transgenes (Cao et al , 1991) The most common and powerful ways of detecting the presence of transgene are PCR and DNA gel blot hybridization In the present studies the presence of *nptII* and *DREB1A* genes was confirmed in the primary rd29A DREB1A transformants by PCR analysis An additional the fidelity of PCR amplicons was ascertained by transferring the PCR products to the nylon membranes followed by hybridization with the gene fragment Further confirmation of the integration pattern and copy number of the transgenes was carried out through Southern blotting where the putative transformants carrying the *DREB1A* gene showed one to four copies of the insert

Instability in the transgene expression is one of the major problems in the transgenic plants In the present study, a few of the T0 transformants failed to express the transgenes (*DREB1A* and *nptII*), but were found to be positive in PCR for these genes This might be attributed to the gene silencing and interactions between multiple copies of the same transgene or different transgenes, which result in unexpected expression patterns of foreign genes Additionally, several other factors such as transgene rearrangements and silencing may prevent the transgene expression (Kumpatla et al., 1998, Matzke et al , 1994)

The expression of the *npt*II and *DREB1A* genes before and after the water stress treatment was determined in individual plants of the T1 transgenic events by RT-PCR. As expected, *npt*II was constitutively expressed in all plants before and after water stress, while *DREB1A* driven by the *rd29A* promoter was not induced under stress-free conditions. Transcripts of the *DREB1A* gene driven by the *rd29A* promoter were detected after only 5 d of water stress. These observations indicate that the *A thaliana rd29A* gene promoter is an effective water-stress-inducible promoter in groundnut. Earlier work on histochemical analysis for expression of the *rd29A uid*A fusion showed that the *uid*A gene expression was induced by desiccation in transgenic *Arabidopsis* rosettes (Shinwari, 1999). Similarly, in wheat also the *DREB1A* gene expression under *rd29A* promoter showed a level of expression, which was, similar to that observed in *Arabidopsis* (Pellegrineschi et al., 2004)

Stable integration and faithful inheritance of the transgenes is of immense importance in application of transgenic technology (Mohanty et al., 2002) To verify the inheritance of transgenes in the present study, 14 independent T0 events were self-pollinated to obtain segregating T1 progeny for genetic analysis In the T1 progeny, 13 transgenic events showed a segregation pattern of 3:1 for the *npt*II marker gene These studies revealed that the putative transgenic plants generally followed the Mendelian segregation ratios for the transgenes in the T_2 generations as well, thus confirming the success of the transformation procedure.

Evaluation of transgenics

Stress conditions used for the evaluation of transgenic material in most of the studies reported so far (Shinwari et al., 1998; Nanjo et al., 1999, Garg et al., 2002), have been usually too sharp, which the plants are very unlikely to undergo in a real

field condition The overexpression of *DREB1A* has been shown to delay plant death following withdrawal of irrigation in transgenic wheat (Pellegrineschi et al , 2004), while an improved tolerance to salinity in potato (Behnam et al 2006) and low-temperature has been reported in tobacco (Kasuga et al 2004) However, the protocols used for the evaluation of transgenic plants for abiotic stresses often involved the use of young plants grown in small pots disregarding water content in pots that are usually maintained under inappropriate light and growth conditions (Tarczynski et al , 1993, Pilon-Smits et al 1996 Xu et al 1996 Pellegrineschi et al , 2004) However, such procedures to impose stress for the phenotypic evaluation of transgenic plants to study their response to drought and other stresses have been questioned (Sinclair et al , 2004) In the present study a more realistic physiological response to progressive soil drying has been adopted so as to include a proper control of soil moisture depletion This also ensured that the test plants were exposed to stress levels and kinetics of water-deficits similar to those likely to occur under field conditions This dry down procedure involved using the fraction of transpirable soil water (FTSW) as a covariate for soil moisture availability for comparing the response of different physiological mechanisms to soil drying that has also been used successfully across a wide range of crop species (Ritchie 1980 Sinclair and Ludlow 1986, Weisz et al , 1994, Ray and Sinclair 1997 1998)

Initial assessment of 14 transgenic events under the dry-down conditions showed that they differed in their transpiration responses to soil drying The dendrogram based on similarity in FTSW threshold values and the number of days to end point under water deficit conditions revealed that the events could be broadly classified into four groups of 0 6 SI (similarity index) This index clearly distinguished the water use pattern among the tested events with the genotypes

located in the upper part of the dendrogram (including JL24) using the water at a faster rate that those located in the bottom part, thus suggesting that these events differed in their transpiration response to water deficit. These results confirm that the drought responsive element (*DREB1A*) inserted in the transgenic groundnut events were probably associated with stomatal regulation. Contrasting transgenic events can be further used to assess the physiological response of stomata under drought; event RD4 could withstand drought for longer period while the event RD14 was similar to the untransformed control. A selection of contrasting transgenic events in this study selected from different locations in the dendrogram, was further used in detailed experiments to confirm these results and to investigate the link between the differences in stomatal closure and TE. This approach is in contrast to most other studies that chose extremes based on phenotype or gene copy number (Behnam et al., 2006).

There seemed to be a limited effect of the transformation on the overall shoot biomass. The physical appearance did not differ in the transgenic events when compared to their wild type parent. Only slight differences in the early growth were observed in the transgenic events RD2, RD11, and RD19. It was interesting to note that the event RD11 showed somewhat lower growth than the other tested transgenic events under well-watered conditions. This could possibly be due to gene expression modulation by the position of gene integration. Similar effects have also been reported in other transformation systems (Jackson et al., 2004). In fact, in the present study, the overall growth was better in most transgenic events than in untransformed control. In previous studies, it has been found that constitutive expression of *DREB1A* transgene in *Arabidopsis* plants resulted in stress tolerance at the expense of growth and productivity (Kasuga et al., 1999).

The PCR and RT-PCR analysis used for verification and expression of the transgenes in the transgenic plants revealed that the *DREB1A* gene was specifically induced under water stress conditions. Therefore, there is still an unanswered question as why large phenotypic differences in TE and CS were observed under well-watered conditions. According to previous work (Shinozaki and Yamaguchi-Shinozaki, 2000), it has been shown that *DREB1A* transcripts were induced only for a short period after stress imposition while the transcripts of various genes activated by *DREB1A* could be detected for a longer period. Hence, it is possible that in the present study, even the plants under well-watered conditions might have experienced some sort of stress at some point before the initiation of the experiment, e.g., a slight temperature increase on a hot day that could trigger a stress response, where the transcript could not be detected.

Under well-watered conditions, it was observed that none of the transgenic events had smaller roots than in in the untransformed controls. In contrast, the root dry weight of all the transgenic events, except RD20 was lower than that of the untransformed controls following water stress. According to the overall framework for plant performance under water deficit being defined as: Yield = T x TE x HI (where T is the total transpiration, TE is transpiration efficiency and HI is harvest index; (Passioura, 1977), the performance of transgenics in this study would have a high TE component, but their limited root growth under drought might undermine their T component, and could be a cause for concern. However, we found no differences between the transgenics and the wild type in the amount of water extracted from the pot, thus suggesting that despite their roots being smaller under drought, they were efficient at extracting water from the water-limiting soil conditions. In fact, in this type of system, roots are usually not in limited amount to

fully extract the soil moisture. Further investigation would be needed to explore the root growth of the different transgenic events in a system allowing their growth in deep soil layer, such as long and large PVC cylinders.

The present study clearly indicated that there were no significant differences between the selected transgenic events in the leaf area of the pre-treatment plants and or of the post-treatment harvest under the well-watered moisture regime. However, drought stressed plants of events RD2 and RD11 had lower leaf areas than the drought stressed plants of WT JL 24. This suggested that the leaf expansion might have stopped at lower FTSW values in untransformed controls (dryer soil) than in the events RD2 and RD11 (Devi et al., unpublished results). Since reducing leaf expansion or reducing leaf area is an efficient way for drought-adapted species to limit evaporation under water-limited environments (Nageswara Rao et al., 2001), it is likely that this mechanism could also contribute to water economy in the transgenic plants tested in the present study.

Water use efficiency (WUE) is one of the major traits that have been associated with drought tolerance in groundnut (Rao and Nigam, 2001). Transpiration efficiency (TE; biomass produced per unit water transpired), an alternate measure of water use efficiency is an essential trait due to the ability of groundnut crop to maintain high photosynthetic activity even under low stomatal conductance without showing impact on carbon assimilation and yield (Wright et al., 1994). The most striking result of the present work is that most transgenic events had higher TE under well-watered conditions than in WT JL 24, with some events showing up to 70% increase compare to WT JL 24. Also, one event, RD2, had also about 50% higher transpiration efficiency under drought stress conditions. These differences can be considered as very large, compare to the range of variation usually

found for TE between germplasm accessions in other crops. In fact, this study is, as far as we know, one of the first to test the range of phenotypic variations for TE by using isogenic materials. Such type of material is likely to be of great interest to re-explore the physiological mechanisms involved in high TE in groundnut.

It seemed that the differences in TE under well-watered conditions were well related with the stomatal conductance data. There was a significant negative relation between TE and Gs data R^2 = 0.76) under well-watered conditions. This is in agreement with the theory on transpiration efficiency, which states that high TE is achieved under conditions of low stomatal conductance (Farquar, 1982; Condon et al 2002). Indeed, Sinclair et al. (2005) have recently established through model analysis that a maximum daily transpiration rate, imposed by stomatal closure during the high irradiation period of midday would lead to substantial water saving and increased TE in sorghum.

Efforts to breed groundnut genotypes for high TE, i.e., the major trait conferring tolerance to intermittent drought in groundnut (Wright et al., 1994) have obtained limited success. This is in part because the molecular markers that could assist the breeding activities for such a complex trait as TE are not yet available in groundnut. Moreover, several surrogate traits that show a relationship with TE that have been used in the breeding activities ((Hubick et al., 1986; Wright, et al., 1988, 1994; Wright et al., 1994).) do not always show a very tight association with TE. In fact, all the work done in relation to TE in groundnut has used the germplasm material with inherent variation probably hiding the genuine traits related to TE. Among the surrogate traits underlying TE, it has been proven that TE has an established relation with the various easily and non-destructively measurable traits like SLA and SCMR (Wright et al., 1994; Nageswara Rao et al., 2001, Bindumadhava et al., 2003), $\Delta^{13}C$

(Farquhar et al., 1982; Udayakumar et al., 1998) and $\Delta^{18}O$ (Bindumadhava et al., 2003). The question remains as which of these surrogates are really relevant?

One of the objectives of the current study was to examine that relationship amongst the five selected transgenic events and untransformed JL 24, and to re-explore the relations between the surrogate traits and TE. In this study, consistent TE advantage in few of the transgenic events have been observed in comparison to the wild type parent that ranged from 50 to 100% and both under well-watered and water-deficit treatments. Preliminary data indicated that the rd29A:DREB1A transgenic events usually had more conservative use of water that is in agreement with inferences interpreted from other *DREB1A* transgenic materials like wheat (Condon et al., 2002; Pellegrineshi et al., 2004).

TE of the transgenic events selected for this study had a significant negative correlation with SLA(r=0.8237). These results are similar to those reported by Wright et al. (1994) and Nageshwara Rao and Wright (1994) for non-transgenic groundnut that showed a positive correlation (r = 0.90 to 0.93**) between specific leaf area (SLA, ratio of leaf area to leaf dry weight) and $\Delta^{13}C$, and a negative relationship with TE, thereby confirming that SLA can be used as a surrogate measure of TE in groundnut. Although, a close correlation of SLA with TE has been established in controlled experiments, the strength of correlation varied (r=0.71 to 0.94) between SLA and $\Delta^{13}C$ (Wright et al. 1994) when tested over a range of groundnut genotypes and environments (Wright et al. 1996).

Since TE in groundnut is controlled mainly by mesophyll rather than stomatal factors (Roy, 2001; Wright et al. 1994; Rao et al. 1995; Sheshshayee et al 1998; Udayakumar et al., 1998), parameters such as SCMR, which is strongly linked with mesophyll efficiencies, should also be linked with TE. Indeed, a strong relation is

usually found between TE and SCMR. In the present study, TE also had a significant positive correlation with SCMR (r=0.7359). These observations are in accordance with the earlier study by Rao et al., (2001) that show significant correlations between the SPAD Chlorophyll Meter Readings (SCMR), SLA and specific leaf nitrogen (SLN) in groundnut and suggested that SCMR could be used as a rapid, low-cost, non-destructive technique to screen large breeding populations for SLA or SLN.

Despite the several reports on relationships between carbon isotope discrimination ($\Delta^{13}C$) and TE (Farquhar and Richards, 1984; Farquhar et al 1989) and increasing the scope for using $\Delta 13C$ as an indirect selection tool to assess the genetic variability in TE in groundnut (Hubick et al 1986; Wright et al 1988, 1994; Roy et al., 1995; Sheshshayee et al. 1998; Udayakumar et al., 1998), the relationship between TE and $\Delta^{13}C$ could not be established in the present study. This could be due to the fact that the transgenic material used in this study was basically isogenic to untransformed JL 24 and differed only due to the insertion of a *DREB1A* gene. This probably suggests that another mechanism conferring differences in TE operates in these transgenic events. Furhter investigations on this aspect are currently on going.

Drought-induced decrease in the fluorescence parameter Fv/Fm, which is a measure of accumulated photo-oxidative damage to PSII, is considered to be an important parameter for evaluation of the transgenic plants. However, a decrease in Fv/Fm may be a result of different effects of a particular stress. In the first case, this might be due to the PSI fluorescence contribution to Fo (up to 30%). As a result, the chloroplasts with a lot of PSI, the PSI-contribution to Fo will be relatively high and as a consequence the Fv/Fm-value will be lowered. In the second case, the spillover affect due to de-stacking of thylakoids where the antennae of PSII and PSI can make contact would result in an energy flow between the two antennae (energy flows from

PSII to PSI). However, in case of higher plants the membranes are stacked and only PSII can be in stacked membranes, while the PSI is in the destacked parts. This leads to a physical separation between PSII and PSI and limits the energy loss of PSII due to spillover. Thirdly, during photo-inhibition, the light energy knocks out PSII reaction centers, but they do not immediately disappear from the membrane As a consequence, the variable fluorescence is lost but there is still a contribution to Fo This would result in a decline of Fv Fm-value that is proportional to the oxygen evolution activity.The present dry down set up, however, had no effect on the Fv Fm of the groundnut plants. The gradual soil drying treatment did not affect the function of PSII of the groundnut plants. In a previous study, Garg et al (2002) estimated photo-oxidative damage to the PSII reaction center by measuring the Fv Fm in the rice leaves that were dark-adapted for 10 min prior to the measurements at regular intervals (25, 50, 75, 100 h) during 100 h of continuous drought stress Here, the measurements were made only after a 10 min dark adaptation, in contrast to overnight dark-adaptation in our study. The results thus obtained were the development of the Fv/Fm as a function of the time. Therefore, the patterns are different for the different events over the 100 h stress period. In our study, overnight dark-adaptation prior to making measurements in the groundnut plants was more than enough to recover from any photo-inhibition. Therefore, it can be concluded that the drought treatment needs to be lengthened in order to detect an effect on the Fv/Fm of the transgenic events under water stress conditions. The point where this value decreases might, therefore, result in a predictive threshold value for the drought resistance of the transgenic plants.

Many plants accumulate free proline in response to the imposition of environmental stress such as high salinity, low temperature and drought (Hare et al.,

1998). In response to osmotic stress, many organisms accumulate compatible osmolytes to adjust their intracellular osmotic potential and to protect sub-cellular structures against stress damage (Ammengaud et al., 2004). No significant difference in the accumulation of proline levels were detected in the leaves of wild type JI 24 and transgenic plants carrying rd29A:DREBIA when grown under standard stress-free condition. However, proline accumulation was more pronounced and increased significantly in the leaves of the transgenic events than in the WT JI 24 plants exposed to water deficit conditions. The high concentration of proline in transgenic plants under severe water stress suggested that the production of proline is an adaptive response to osmotic stress. It has been shown that there is an increased accumulation of proline in the *sos1* mutant under salt stress, which probably reflects increased cellular damage by salt stress in the mutant (Liu and Zhu, 1997). Similar increase in proline content was also shown in transgenic tomato plants over-expressing *AtNHX1* under high salinity conditions (Fujita et al., 1998; Zhang and Blumwald, 2001). Moreover, the overproduction of proline in tobacco leads to an increased tolerance to osmotic stress (Kavi Kishore et al., 1995). In bacteria, several studies have shown that proline accumulation confers stress tolerance (Leisinger, 1996). It seems that in plants also, the accumulation of proline could be a part of stress tolerance mechanism (Hare and Cress 1998; Nanjo et al., 1999). In addition, the overexpression of the transcription factor *CBF3* lead to elevated levels of proline and soluble sugars that are normally associated with cold acclimation (Gilmour et al., 2000).

The accumulation of proline in plants under stress can offer multiple benefits to the cell (Hong et al., 2000). In the present study, we found that free radicals are formed during water stress, as measured by an increase in the MDA production. MDA, a lipid peroxidation product, has been used widely to assess the levels of free

radicals in living cells (Kunert and Ederer, 1985). These free radicals can react with many cellular constituents, including DNA, proteins and lipids, leading to radical chain processes, peroxidation, membrane leakage and the production of toxic compounds (Davies, 1998). This confirms earlier observations by Ahn et al. (1993) on the production of free radicals under salinity stress where the results indicated a significant negative correlation between the proline levels and MDA production, thus suggesting that the elevated proline also reduced free radical levels in response to osmotic stress, thereby, significantly improving the ability of the transgenic plants to better survive under water stress (Fig. 25). These results in accordance with those reported by Hong et al. (2000) on the role of proline in reducing oxidative stress induced by osmotic stress, in addition to its accepted role as an osmolyte.

Central to signal transduction pathways related to drought and other stresses are reactive oxygen species (ROS), which are molecules formed by the incomplete one-electron reduction of oxygen. Drought stress leads to the disruption of electron transport systems. Therefore, under water deficit conditions, the main sites of ROS production in the plant cell are organelles like chloroplasts, mitochondria, and microbodies that have highly oxidizing metabolic activities. ROS are generally damaging to essential cellular components, and plants have evolved various ROS scavenging mechanisms (Shigeoka et al., 2002). These include the enzymes *Superoxide dismutase* (SOD), Catalase, and Peroxidases, as well as oxidized and reduced glutathione. Comparisons of drought-susceptible and tolerant cultivars of herbaceous species have suggested that increased tolerance is correlated with induction of higher levels or higher responsiveness antioxidative defenses to stress (van Rensburg and Krüger, 1994; Jagtap and Bhargava, 1995; Sairam et al., 1998). Similarly, the tolerant species maintained lower concentrations of O_2^- than drought-

susceptible species, thereby diminishing the risk of oxidative injury (Quartacci and Navaro-Izzo, 1992, Quartacci et al , 1994)

Transgenic plants over-expressing various antioxidant enzymes and showing tolerance to drought and chilling have been reported earlier (Roxas et al 1997 Noctor and Foyer, 1998, Zhu et al , 1999 Oberschall et al 2000) Overexpression of a tobacco *glutathione-S-transferase* (GST) and *glutathione peroxidase* (GPX) in ransgenic tobacco seedlings under a variety of stresses resulted in increased glutathione-dependent peroxidase scavenging and alterations in glutathione and ascorbate metabolism, leading to reduce oxidative damage (Roxas et al , 1997) Wu et al (1999) isolated cDNA-encoding chloroplastic Cu /n- SOD and mitochondrial Mn-SOD from wheat where the Northern blot analysis showed that Mn-SOD genes were stress-inducible. Though Cu Zn-SOD gene did not increase under drought there was increase in expression on reversion to normal condition The results show that both Mn-SOD and Cu/Zn-SOD play definite roles in stress tolerance Studies with transgenic rice over-expressing yeast Mn-SOD showed increased levels of *Ascorbate peroxidase* and chloroplastic SOD in the transformed rice compared to the wild type The transformed rice also showed more salinity tolerance than the wild type Transgenic alfalfa (*Medicago sativa*) expressing *Mn-superoxide dismutase* cDNA tended to have reduced injury from water-deficit stress, and this improvement was also seen in field trials in yield and survival

In the present study, the antioxidant responses of SOD and associated antioxidant enzymes such as APOX and GR to water stress was compared in wild type and transgenic groundnut plants containing rd29A DREB1A The increase in APOX activities in leaves is probably a response to the enhanced production of reactive oxygen species, in particular H_2O_2 under water stress (Elstner et al , 1987,

Smirnoff and Colembe, 1988). This work was deemed of interest because previous work had showed that in water stressed pea leaves there was a correlative increase between the cytosolic CuZnSOD and APOX activities, thereby suggesting the coordinated expression of both enzymes (Mittler and Zilinskas, 1994).

Although the transgenic plants in the present study maintained a ratio of SOD:APOX specific activity that was essentially identical to that in wild type JL 24 in plants under well-watered conditions, this ratio differed in the transgenics and the wild type plants at extreme water stress (12 DAS). Since, the transgenic plants survived drought for a longer period and also accumulated biomass throughout the stress, this could indicate that a high SOD:APOX ratio, combined with increased activity of these enzymes, is necessary for optimum stress resistance. Sen Gupta et al (1993) in their studies on transgenic tobacco plants also speculated that APX gene expression could be upregulated as a direct or indirect response to a constitutive increase in H_2O_2 putatively associated with SOD overexpression in the SOD$^+$ plants. Besides, there is a commonality among oxidative stress tolerance mechanisms that are co-regulated (Neill et al., 2002; Shigeoka et al., 2002). Thus, the elevated levels of APOX and GR in drought-stressed plants could suggest that the drought stress in these groundnut transgenic events may lead to acclimation tolerance.

In conclusion, this study has been efficiently successful in developing transgenic events of groundnut with the *DREB1A* transcription factor that is specifically expressed under the influence of a stress responsive promoter from *rd29A* gene. The stress inducible expression of *DREB1A* in these transgenic plants did not result in growth retardation or visible phenotypic alterations as reported earlier (Kasuga et al., 1999, 2004). The transgenic events, characterized at the molecular level revealed the integration as well as expression of the transgenes. The

transformation frequency was high ≥72-74% with plants exhibiting a low copy number of the insert, establishing the efficacy of the cotyledon based transformation system in groundnut (Sharma and Anjaiah, 2000) Physiological evaluation of these transgenic events showed the promise of transformants of groundnut, with respect to enhanced tolerance to drought stress. The events exhibited a diversity of stress response patterns, especially with respect to the NTR-FTSW relationship All the selected transgenic events differed from the wild type parent in their normalized transpiration rate response to FTSW, showing a decline in transpiration at lower FTSW values (dryer soil). Most of the tested transgenic events performed relatively well under water limiting conditions by maintaining a higher TE, which is an important component of plant performance under limited moisture conditions, two of the selected events showed up to 50% higher TE under water deficit. Further, the linkage of other physiological traits such as SLA, SCMR and $\Delta^{13}C$ to drought tolerance provided more insights into the mechanisms of stress tolerance TE was positively correlated with SCMR and negatively correlated with the SLA, which are in accordance earlier reports suggesting SCMR and SLA as rapid, low-cost, non-destructive techniques to screen large populations for drought in transgenic plants. However, the usual relation between TE and $\Delta^{13}C$ was not found, suggesting that further investigation is needed on what could potentially be a novel mechanism to explain differences in TE. Besides, biochemical comparisons of transgenic plants and its wild type parent suggested that increased drought tolerance was correlated with induction of higher levels of antioxidative defenses. The evaluation of these results under field conditions will provide the usefulness of this approach. The overall results of this study demonstrated that engineering for abiotic stress tolerance by using the

6. SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

6.1. Summary

Environmental stresses in arid and semi-arid tropics (SAT) are the major factors of yield instability in crop production. Plants are constantly being subjected to external stresses that require them to respond in an appropriate manner. Abiotic stresses directly or indirectly affect the physiological status of an organism by altering its metabolism, growth, and development. Despite focused efforts to improve major crops for resistance to abiotic stresses (1) such as drought, excessive salinity, and low temperature by traditional breeding, success has been limited. The multigenic and quantitative nature makes it difficult to breed for abiotic stress tolerance. Therefore, understanding the responses of plants to their external environment is of importance with respect to basic research, but it is also an attractive target for improving stress tolerance. Rapid advances in genomic technologies are leading to an increased understanding of global gene expression in plants. Investigation of the molecular mechanisms involved in the abiotic stress response of plants has made substantial progress in recent years (for reviews, see Knight and Knight 2001; Zhu 2001; Seki et al. 2003). Stress resistance pathways are inter-related, some genes induced by different stresses and the over expression of certain regulatory genes are demonstrated to confer resistance against a number of abiotic stresses (Shinozaki et al., 2000).

The legume family is one of the most important groups of plants worldwide, as an important source of protein in the human diet, of fodder and forage crops for animals, of oil crops, and for available nitrogen in the biosphere. Groundnut is principal legume crop, which has rich source of edible oil (43-55%) and protein (25-28%) content. It is presently cultivated in 108 countries of the world on 24.8 million

hectares with an average productivity of 1.36 tons per hectare. Most of the cultivated area accounts for developing countries enacting about 80% of the world production, even though they are unable to produce substantive amount because of the drought as a major restraint. Pre-harvest aflatoxin contamination is a common occurrence in peanuts that are grown under non-irrigated conditions and exposed to prolonged drought and elevated soil temperatures during seed development.

The mechanism of drought response has been extensively investigated in the model plant *Arabidopsis thaliana*. However, little is known about gene expression of groundnut under drought. Groundnut maintains a high photosynthetic activity even under low stomatal conductance without showing impact on carbon assimilation and yield making the Transpiration efficiency (TE) an essential trait (Wright et al., 1994) for drought tolerance. However, efforts to breed groundnut genotypes for high transpiration efficiency (TE) and stomatal conductance have obtained limited success. This is in part because the physiological data and information about the molecular events underlying the abiotic stress response in groundnut are limited.

Genetic engineering approaches could lead to simpler and more effective gene based alternatives for combating biotic and abiotic stresses in this important legume crop. The present study deals with the production of transgenic groundnut with improved tolerance to drought stress by using the *DREB1A* transcription factor driven by either the *CaMV 35S* promoter for constitutive expression or by the promoter from *A. thaliana rd29A* gene for stress responsive expression.

The first objective of this work was to test if the genetic engineering of groundnut for abiotic stress tolerance can be achieved by stress inducible expression of transcriptional factor *DREB1A* without any detrimental effects on plant growth and

development. The second objective was to investigate and document the molecular characterization of the transgenic events of groundnuts under water limiting conditions I the greenhouse. Finally, the third objective was to carry out a thorough phenotypic and biochemical evaluation of *DREB1A*-containing groundnut transformants for physiological characterization of possible tolerance to water stress under greenhouse conditions, and the identification of transgenic events exhibiting improved tolerance to water limiting conditions. The experiments involved studies on tissue culture and transformation, molecular and biochemical characterization of the transgenic material, and phenotyping studies. The salient features of this study are as follows:

1. The present study involved *Agrobacterium*-mediated genetic transformation of groundnut variety JL 24 by using the cotyledonary explants from pre-soaked mature seeds.

2. The *Agrobacterium tumefaciens* strain C 58 carrying the binary plasmids containing *AtDREB1A* driven by the stress-inducible promoter from *rd29A* gene of *A. thaliana* (rd29A:DREB1A) and *CaMV* 35S constitutive promoter (35S:DREB1A) for transformation experiments. The plasmids contained *nptII* as a selectable marker.

3. Co-cultivation of the cotyledon explants with the *A. tumefaciens* carrying the rd29A:DREB1A and 35S:DREB1A constructs for 48 to 72 h resulted in over 70% transformation frequency. The proximal cut ends of the cotyledon explants resulted in the induction of adventitious shoot buds after 2 wk of culture on shoot induction medium (SIM). The explants bearing shoot buds were transferred to the shoot elongation medium (SEM) containing 250 mg/L

cefotaxime and 100 mg/ L kanamycin to initiate selection and enrichment of the transformed cells for another 2 wk. Thereafter, the proximal parts of the explants containing multiple adventitious shoot buds were excised and transferred to SEM containing 125 mg L kanamycin for two to three subcultures of 3 to 4 wk duration each.

4. The elongated shoots (3-4 cm) cultured on the root induction medium (RIM) without any selection pressure were found to be effective for efficient induction of adventitious roots within 3 wk. The rooted shoots were transferred to pots containing autoclaved sand and red soil resulted in further acclimatization with 95-100% success rate.

5. While over 90% of the rooted shoots transformed with the rd29A.DREB1A construct survived and appeared to be phenotypically normal, the shoots containing 35S:DREB1A showed stunted growth and high rates of mortality (up to 80%).

6. Fifty independently transformed plants with rd29A:DREB1A and 18 plants with 35S:DREB1A were successfully transplanted to the greenhouse and were advanced to T2 generation.

7. Under greenhouse conditions, over 40% of the 35S:DREB1A events showed delayed germination and severe growth retardation within 7 d of seedling emergence where 70% of seedlings resumed sub-optimal growth thereafter. In contrast, the seed from rd29A:DREB1A plants showed normal germination and growth behavior.

8. PCR analysis and Southern hybridization of the amplicons with the target genes carried out for *npt*II and *DREB1A* genes in T0 transformants amplified the

expected sizes of gene fragments from 75% of the analyzed putative tranformants. Transgene integration as confirmed by Southern blotting for *rd29A* promoter region revealed presence of the gene in the T0 generation plants. Southern blot analysis of the 6 tested T1 events with rd29A:DREB1A indicated single insert in 5 events, while only 1 event showed two copies of the transgene.

9. Self-fertilized T0 events advanced to T1 and T2 generation were studied for inheritance patterns of the transgenes. Segregation analysis of the T1 and T2 generation progeny for the *npt*II indicated segregation in a Mendelian ratio (3:1).

10. RT-PCR studies on cDNA from the putative transgenic plants (35S.DREB1A and rd29A:DREB1A) showed positive amplification of the *npt*II gene fragment in all the selected transgenic events under unstressed as well as water-stressed conditions. The transcripts of *rd29A*-driven *DREB1A* were detected only in the plants subjected to 5 d of water deficit.

11. Initial assessment of 14 transgenic events carried out by using the soil drying experiments showed differences in their transpiration responses to soil drying. Wild type (WT) JL 24 started to show wilting symptoms (loss of turgor) much earlier (55 % of FTSW) followed by severe desiccation symptoms and death when compared to the rd29A:DREB1A transgenic events(28-49% FTSW).

12. A dendrogram based on similarity in FTSW threshold values and the number of days to end point under water deficit conditions revealed that these transgenic events could be broadly classified into four groups (at a similarity index value of 0.6). Five events from across different clusters of the dendrogram with

transpiration responses ranging from very similar to very different with respect to WT JL 24 were selected for subsequent experiments on their physiological evaluation.

13. Under well-watered (WW) treatment, the transgenic events RD2 and RD19 accumulated higher final shoot biomass although there were no significant differences in the final leaf area between any of the tested genotypes

14. The cumulated transpiration differed significantly between the transgenic events and the WT JL 24; events RD2, RD11 and RD12 had a lower cumulated transpiration than WT JL 24.

15. The stomatal conductance (Gs) of several transgenic events measured under WW conditions was over two-fold lower than in WT JL 24.

16. There were no significant differences in the initial biomass of the pre-harvested plants under water limiting conditions. The leaf area of RD2 was lowest of all, although not significantly different from RD11. In contrast, the leaf area of RD20 was on a higher side followed by WT JL 24 and RD19.

17. The transgenic events differed in the response of NTR (normalized transpiration rate) to FTSW (fraction of transpirable soil water) and were clearly distinguishable from WT JL 24, with transgenic events declining their transpiration in dryer soil than WT JL 24. The transpiration rate of all the transgenic events started to decline at lower FTSW values (drier soil) under drought stress than in WT JL 24. Overall, all the tested transgenic events closed their stomata at lower FTSW values (0.28-0.49) under drought stress in comparison to WT JL24.

18. A significant genotypic variation in TE was observed under drought stress treatment that was significantly higher in transgenic events RD2, RD11, RD12, and RD19 than in WT JL 24. These results indicated that all transgenic events except RD20 had a significantly higher TE.

19. The total biomass produced during the dry down cycle (Δ biomass) showed significant differences amongst the transgenic events, thus indicating apparent differences in the biomass produced per unit of water used (TE). RD2 showed an increase in the biomass produced during the dry down period (Δ biomass) when compared to the WT JL 24 and transgenic events RD12 and RD11

20. A significant negative correlation was observed between the SCMR and SLA under drought stress conditions ($r=0.9470$, $P<0.01$) when compared to the WW conditions ($r=0.8110$, $P<0.05$). There was, however, no significant relationship between the SLA and SCMR with $\Delta^{13}C$ under drought stress and WW conditions. The relationship of TE with its surrogate traits including SLA, SCMR and $\Delta^{13}C$ was non-significant under well-watered regime in all the tested transgenic events including WT JL 24.

21. Unlike in WW conditions, under the drought stress treatment (DS) there was a significant variation amongst the tested events for TE that ranged between 4.211 and 5.796 that showed significant positive correlation with SLA ($r=0.8237$) and SCMR($r=0.7359$). However, the TE did not significantly correlated with $\Delta^{13}C$, thus suggesting a lack of relationship between TE and $\Delta^{13}C$.

22. A strong correlation was observed between the TE and FTSW threshold values of the transgenic events as well as the WT JL 24 ($r=0.9124$, $P<0.001$) under drought stress treatment. Hence, the events that closed their stomata in drier

soils (low FTSW threshold value) utilized the water more efficiently, thus resulting in a higher TE.

23. Drought stress did not seriously affect the photosynthetic system and Fv/Fm values of the transgenic groundnut plants as well as the WT JL 24 under the dry down set-up used in this study. Drought stress did not affect the fluorescence transient in both the transgenic events and the WT JL 24. Control and drought-stressed transients had very similar amplitudes, which indicated that there was little drought-induced decrease in the size of the photosynthetic apparatus.

24. All the transgenic events showed a significant increase in their SOD activity levels at higher water stress levels (9 and 12 DAS) in contrast to the WT JL 24. The differences in SOD activity in the transgenic and the wild type control plants were found to be statistically significant ($P < 0.001$ by t test) for the transgenic events RD2, RD11, RD12 and RD19 when the plants were exposed to 12 days (DAS) of water stress.

25. *Glutathione reductase* (GR) activity in the transgenic events was found to be significantly higher at 6, 9 and 12 DAS at least at $P<0.05$ when compared to 0 DAS. The GR activity in the untransformed JL 24 was not significant throughout the stress period.

26. The high concentration of proline content in transgenic plants under water stress suggested that the production of proline is an adaptive response to osmotic stress. Proline levels increased significantly after 3 DAS in all transgenic events as well as the WT JL 24, indicating that proline accumulated soon after encountering water stress. However, the increase in MDA content was dependent on the specific response of plants to water stress. There were no

differences in the MDA content in RD2, RD11 and RD12 throughout the drying cycle whereas its levels significantly increased in the WT JL 24 at 9 DAS and 12 DAS ($P \leq 0.001$).

27. The transgenic plants maintained ratios of 10.56-12.1 for SOD APOX specific activity that was essentially identical to WT JL 24 (11.2-12.0) plants under well WW conditions. This ratio differed in the transgenics (11.2) and the wild type plants (8.5) at extreme water stress (12 DAS) levels only.

6.2. Conclusions

The transgenic events of groundnut with the *DREB1A* transcription factor that is either constitutively expressed or specifically expressed under the influence of a *CaMV* 35S promoter or a stress responsive *rd29A* promoter from *Arabidopsis thaliana* were successfully developed in the present study. The stress inducible expression of *DREB1A* in these transgenic plants did not result in any growth retardation or visible phenotypic alterations as was observed with the 35S DREB1A plants. The transgenic events with relatively low copy number (one to two) of the transgene were used for phenotyping. Fourteen transgenic events in T3 generation were phenotypically evaluated in dry down experiments to study various physiological parameters including plant responses to soil drying as measured by the fraction of transpirable soil water (FTSW), stomatal conductance and transpiration efficiency (TE). The selected events exhibited a diversity of stress response patterns, especially with respect to the NTR-FTSW relationship. All the selected transgenic events differed from the wild type parent in their normalized transpiration rate response to FTSW, showing a decline in transpiration at lower FTSW values (dryer soil). Various transgenic events exhibited increased transpiration efficiency (TE),

which is an important component of plant performance under limited moisture conditions. Two of the selected transgenic events showed up to 50% higher transpiration efficiency under drought stress, which is an important component trait of crop water productivity and plant performance under water-limited conditions. The differences in TE observed in the transgenic groundnut events, both under well watered and water stress conditions offered an ideal material to re-explore the relation between the different surrogate traits and TE. There was a significant variation amongst the tested events under drought stress for TE ranging between 4.211 and 5.796 that had a significant positive correlation with SCMR(r 0.7359) and a negative correlation with SLA(r=0.8237). However, the TE did not significantly correlated with $\Delta^{13}C$ thus suggesting a lack of relationship between TE and $\Delta^{13}C$. This relationship between TE and $\Delta^{13}C$ could not be established in this study, which could be due to the fact that the transgenic material used in this study was basically isogenic to JL 24 and differed only due to the insertion of *DRLB1A*

A significant negative correlation between the proline levels and MDA production indicated that the elevated proline reduced the free radical levels in response to osmotic stress, and significantly improved the ability of the transgenic plants for better survival under water stress. Proline accumulation was more pronounced and increased significantly in leaves of the transgenic events than in the wild-type JL 24 plants exposed to water deficit conditions. The transgenic plants maintained a ratio of SOD: APOX specific activity that was essentially identical to that in wild type JL 24 plants under well watered conditions. However, this ratio differed in the transgenics and the wild type plants at extreme water stress (12 DAS). Thus elevated levels of APOX and GR levels in drought stressed plants suggested that drought stress in these groundnut transgenic lines may lead to acclimation tolerance.

Based on the results observed in the present study, it can be concluded that engineering for abiotic stress tolerance by using the *DREB1A* gene of *A. thaliana* has the potential to contribute towards knowledge on the physiological basis of stress tolerance as well as the development of drought stress tolerant genotypes of groundnut in the near future.

BIBLIOGRAPHY

BIBLIOGRAPHY

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.

Abelson, P.H. (1994) Continuing evolution of U.S. *Agron. Sci.* 264: 1383.

Alia, H., Kondo, Y., Sakamoto, A., Nonaka, H., Hayashi, H., Pardha Saradhi, P., Chen, T.H.H. and Norio, M. (1999) Enhanced tolerance to light stress of transgenic *Arabidopsis* plants that express the *codA* gene for a bacterial choline oxidase. *Plant Mol. Biol.* 40: 279-288.

Alia, H., Sakamoto, A. and Murata, N. (1998) Enhancement of tolerance of *Arabidopsis* to high temperature by genetic engineering of the synthesis of glycine betaine. *Plant J.* 16: 155-161.

Alia, Saradhi, P. P. and Mohanty, P. (1993) Proline in relation to free radical production in seedlings of *Brassica juncea* raised under sodium chloride stress *Plant Soil* 155: 497-500.

Allen, R.D, (1995) Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.* 107: 1049-1054.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vascoular Na^+/H^+ antiport in *Arabidopsis* *Science* 285: 1256-1258.

Armengaud, P., Breitling, R. and Amtmann, A (2004) The potassium dependent transcriptome of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. *Plant Physiol.* 136: 2556-2576.

Asada, K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annu. Rev. Plant. Physiol. Plant. Mol Biol.* 50: 601-639.

Bartels, D. and Sunkar, R. (2005) Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.* 21: 1-36.

Bates, L.S., Waldren, R.P., Teare, I.D. (1973) Rapid determination of free proline for water stress studies. *Plant and Soil* 39: 205–207.

Behnam, B., Kikuchi, A., Celebi-Toprak, F., Yamanaka, S., Kasuga, M., Yamaguchi-Shinozaki, K. and Watanabe, K. N. (2006) The *Arabidopsis DREB1A* gene driven by the stress-inducible *rd29A promoter* increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (*Solanum tuberosum*). *Plant Biotech.* 23: 169-177.

Bhatia, C.R., Murty, G.S.S. and Mathews, V. H. (1985) Regeneration from de-embryonated peanut (Arachis hypoagea) plants from elite cultivars using ACCELL technology. *Plant J.* 5: 745-753.

Bhatnagar-Mathur, P., Devi, M. J., Serraj, R., Yamaguchi-Shinozaki, K., Vadez, V. and Sharma, K.K. (2004) Evaluation of transgenic groundnut lines under water limited conditions. *Int. Arachis Newslett.* 24: 33-34.

Bindumadhava, H., Sheshshayee, M.S., Shankar, A.G., Prasad, T.G. and Udayakumar, M. (2003) Use of SPAD chlorophyll meter to assess transpiration efficiency in peanut. In: Breeding of drought-resistant peanuts. *ACIAR Proc* 112: 3-9.

Bohnert, H.J. and Jensen, R.G. (1996) Strategies for engineering water stress tolerance in plants. *Trends Biotechnol.* 14: 89-97.

Bohnert, H.J. and Shen, B. (1999) Transformation and compatible solutes. *Sci. Hort.* 78: 237-260.

Bohnert, H.J., Nelson, D.F. and Jenson, R.G. (1995) Adaptation to environmental stresses. *Plant Cell* 7: 1099-1111.

Boote, K. J. and Ketring, D. L. (1990) Peanut. In *Irrigation of agricultural crops*, B. A. Steward and Nielsen, D. R. (eds.) Agronomy Series No. 30. Madison, Wisconsin: American Society of Agronomy.

Bordás, M., Montesinos, C., Dabauzam, M., Salvador, A., Roig, L.A., Serrano, R. and Moreno, V. (1997) Transfer of the yeast salt tolerance gene *HAL1* to Cucumis melo L. cultivars and in vitro evaluation of salt tolerance. *Transgenic Res.* 5: 1-10.

Bowler, C. and Fluhr, R. (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends Plant Sci.* 5: 241-246.

Bowler, C., Slooten, L., Vandenbranden, S., Rycke, R.D., Botterman, J., Sybesma, C., van Montagu, M. and Inze, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* 10: 1723-1732.

Bradford, M. M. (1976) A refined and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Analy. Biochem.* 72: 248.

Brar, G.S. and Cohen B.A. (1994) Recovery of transgenic peanut plants from elite cultivars utilizing ACCELL technology. *Plant J.* 5: 745-753.

Bray, E. A. (1993) Molecular responses to water deficit. *Plant Physiol* 103:1035-1040.

Cao, J.W., Zhang, W. G., McEroy, D. and Wu, R. (1991) In: G. S. Khush and G. Toenniesseu (eds.) Rice Bio Technology, International Rice Research Institute, Manila, Phillipines, PP. 106.

Chen, G. and Asada, K. (1989) *Ascorbate peroxidase* in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant Cell Physiol.* 30: 987-998.

Chen, J-H., Le, S-Y., Shapiro, B.A., Currey, K.M., and Maizel, J.V. (1990) A computational procedure for assessing the significance of RNA secondary structure. *Comput. Appl. Biosci.* 6: 7-18.

Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G. and Whitham, S.A. (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* 14: 559-574.

Cheng, M., Hsi, D.C. H. and Phillips, G.C. (1992) In vitro regeneration of Valencia-type peanut (*Arachis hypogaea* L.) from cultured petiolules, epicotyl sections and other seedling explants. *Peanut Sci.* 19: 82-87.

Cheng, M., Jarret, R. L., Li, Z., and Demski, J. W. (1997) Expression and inheritance of foreign genes in transgenic peanut plants generated by *Agrobacterium* mediated transformation. *Plant Cell Rep.* **16:** 541 544.

Cheng, W.H., Endo, A., Zhou, L., Penney, J., Chen, H.C., Arroyo, A., Leon, P., Nambara, E., Asami, T. and Seo, M. (2002) A unique short-chain dehydrogenase/ reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* 14: 2723-2743.

Chinnusamy, V., Jagendorf, A. and Zhu, J.K. (2005) Understanding and Improving Salt Tolerance in Plants. *Crop Sci.* 45: 437-448.

Chinnusamy, V., Schumaker, K. and Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. *J Exp Bot* 55 225–236.

Clark, A.J., Landolt, W., Bucher, J. and Strasser, R.J. (1998) The response of *Fagus sylvatica* to elevated CO2 and ozone probed by the JIP-test based on the chlorophyll fluorescence rise OJIP. In: L.J. De Kok and I Stulen (eds), Responses of Plant Metabolism to Air Pollution and Global Change, Backhuys Publishers, Leiden, pp. 283–286.

Clark, A.J., Landolt, W., Bucher, J. and Strasser, R.J. (2000) Beech (*Fagus sylvatica*) response to ozone exposure assessed with a chlorophyll a fluorescence performance index. *Environ. Poll.* 109: 501- 507l.

Clemente, T.E., Robertson, D., Isleib, T.G., Beute, M.K. and Weissinger, A.K. (1992) Evaluation of peanut (*Arachis hypogaca* L.) leaflets from mature zygotic embryos as recipient tissue for biolistic gene transfer. *Transgenic Res* 1. 275-284.

Condon, A.G., Richards, R.A., Rebetzke, G.J. and Farquhar, G.D. (2002) Improving intrinsic water-use efficiency and crop yield. *Crop Sci.* 42: 122-131.

Cummins, D.G. and Jackson, C.R. (1982) World peanut production, utilization and research. Special publication, Number16. Athens, Georgia, USA.

Dat, J.F., Lopez-Delgado, H., Foyer, C.H., Scott, I.M. (2000) Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. *J Plant Physiol.* 156: 659-665.

Davis, T., Yamada, M., Elgort, M.G. and Saier, M. (1998) Nucleotide sequence of the mannitol (*mtl*) operon in *Escherichia coli*. *Mol. Microbiol.* 2, 405-412.

Delauney, A.J., Verma, D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. *Plant J.* 4: 215-223.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 32: 93-101.

Dong, J.D., Bi, Y.P., Xia, L.S., Sun, S.M., Song, Z.H., Guo, B.T., Jiang, X.C and Shao, Q.Q. (1990) Teratoma induction and nopaline synthase gene transfer in peanut. *Acta Genet Sin* 17: 13-16.

Dure, L III, Crouch, M., Harada, J., Ho, T-H.D and Mundy, J. (1989) Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol Biol.* 12: 475–86.

Dure, L. III (1993) A repeating 11-mer amino acid motif and plant desiccation. *Plant J.* 3: 363-369.

Eapen, S and George, L. (1994). *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.). *Plant Cell Rep* 13: 582-586

Eapen, S. (2003) Regeneration and genetic transformation in peanut: current status and future prospects. *In*: P.K. Jaiswal and R. P. Singh (eds). Applied Genetics of Leguminosae Biotechnology. Kluwer Acad. Publ., pp. 165-186.

Egnin, M., Mora, A. and Prakash, C.S. (1998) Factors enhancing *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogaea* L.). *In Vitro Cell. Dev. Bio. -Plant* 34: 310-318.

Elstner, E.F. (1987) Metabolism of activated oxygen species. In: Davies DD (ed.), Biochemistry of plants, Vol. 11.Academic Press, London, pp. 253-315

FAO (Food and Agriculture Organization of the United Nations) (1996) FAO Production year book, FAO, Rome, Italy.

FAO (Food and Agriculture Organization of the United Nations) (2000) FAO Production year book, FAO, Rome, Italy.

Farquhar, G.D., and Richards, R.A. (1984) Isotopic composition of plant carbon correlates with water use efficiency of wheat genotypes. *Aust. J. Plant Physiol.* 2: 539-552.

Farquhar, G.D., Ehleringer, J.R. and Hubick, K.T. (1989) Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol Plant Mol Biol.* 40: 503-537.

Farquhar, G.D., O'Leary, M.H. and Baxter, J.A. (1982) On the relationship between carbon isotope discrimination and intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.* 9: 121-137.

Force, L., Critchley, C. and van Rensen, J.J.S. (2003) New fluorescence parameters for monitoring photosynthesis in plants. 1. The effect of illumination on the fluorescence parameters of the JIP-test. *Photosynth. Res.* 78: 17-33.

Fujita, T., Maggio, A., Garcia-Rios, M., Bressan, R.A. and Csonka, L.N. (1998) Comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for D1-pyrroline-5-carboxylate synthetase from tomato. *Plant Physiol.* 118: 661-674.

Galau, G.A., Bijaisoradat, N. and Hughes, D.W. (1987) Accumulation kinetics of cotton late embryogenesis-abundent (Lea) mRNAs and storage protein mRNAs: coordinate regulation during embryogenesis and role of abscisic acid. *Dev. Biol* 123: 198-212.

Gamborg, O. L., Miller, A. A., and Ojima, K. (1968) Nutrient requirements for suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.

Gao, M., Sakamoto, A., Miura, K., Murata, N., Sugiura, A. and Tao, R. (2000) Transformation of Japanese persimmon (*Diospyros kaki* Thunb.) with a bacterial gene for choline oxidase. *Mol. Breed.* 6: 501-510.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.C., Kochian, L.V. and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci* USA 99: 15898-15903.

Gautheret, (1983) *Bot. Mag.* Tokyo 96: 393-410.

Giannopolitis, C.N. and Ries, S.K. (1977) Superoxide dismutases: II. Purification and quantitative relationship with water-soluble protein in seedlings. *Plant Physiol* 59: 315-318.

Gilmour, S. J., Sebolt, A. M., Salazar, M.P., Everard, J. D. and Thomashow, M. F (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation *Plant Physiol.* 124: 1854-1865.

Gilmour, S.J., Artus, N.N. and Thomashow, M.F. (1992) cDNA sequence analysis and expression of two cold-regulated genes of Arabidopsis thaliana *Plant Mol Biol.* 18: 13-21.

Gisbert, C., Rus, A.M., Bolarin, M.C., Lopez-Coronado, M., Arrillaga, I., Montesinos, C., Caro, M., Serrano, R. and Moreno, V. (2000) The yeast HAL1 gene improves salt tolerance of transgenic tomato. *Plant Physiol* 123: 393-402

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll *a* fluorescence. *Aust J. Plant Physiol.* 22: 131–160.

Govindjee, Amesz, J. and Fork, D.C. (eds) (1986) Light Emission by Plants and Bacteria. Academic Press, New York, pp. 291-328.

Gregory, N.C., Krapovickas, A. and Gregory, M.P. (1980). Structural variation, evolution and classification in *Arachis*. Advances in Legume Science, Royal Botanical Garden, Kew, UK, pp 469-481.

Grover, A., Kapoor, A., Satya Lakshmi, O., Agrawal, S., Sahi, C., Katiyar-Agarwal, S., Agarwal, M. and Dubey, H. (2001) Understanding molecular alphabets of the plant abiotic stress responses. *Curr. Sci.* 80: 206-216.

Grover, A., Sahi, C., Sanan, N. and Grover, A. (1999) Taming abiotic stresses in plants through genetic engineering: current strategies and perspective *Plant Sci* 143: 101-111.

Halliwell, B. (1989) Superoxide, iron, vascular endothelium and reperfusion injury. *Free Radic Res Commun.* 5: 315–318.

Hamill, J. D., Rounsley, S., Spencer, A., Todd, G. and Rhodes, M. J. C. (1991) The use of Polymerase chain reaction in plant transformation studies. *Plant Cell Rep.* 10: 221-224.

Handa, S., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. (1986) Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiol.* 80: 938-945.

Hanson, A.D. and Burnet, M. (1994) Evolution and metabolic engineering of osmoprotectant accumulation in higher plants. In J.H. Cherry (ed), Cell Biology: Biochemical and Cellular Mechanisms of Stress Tolerance in Plants. NATO ASI Series H, Springer, Berlin, pp. 291-302.

Hare, P. D. and Cress, W.A. (1998) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* 21: 79-102.

Hare, P.D., Cress, W.A. and Van Staden, J. (1998) Dissecting the role of osmolyte accumulation during stress. *Plant Cell Environ.* 21: 535-553.

Hayashi, H., Alia, Sakamoto, A., Nonaka, H., Chen, T.H.H. and Murata, N. (1998) Enhanced germination under high-salt conditions of seeds of transgenic *Arabidopsis* with a bacterial gene (codA) for choline oxidase. *J Plant Res* 111: 357-362.

Hayashi, H., Mustardy, L., Deshnium, P., Ida, M. and Murata, N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase accumulation of glycine betaine and enhanced tolerance to salt and cold stress. *Plant J.* 12: 133-142.

Heath, R.L. and Packer, L. (1968) Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophy* 125: 189–198.

Hechenberger M., Schwappach, B., Fischer, W.N., Frommer, W. B., Jentsch, T. J. and Steinmeyer, K. (1996) A family of putative chloride channels from Arabidopsis and functional complementation of a yeast strain with a CLC gene disruption. *J Biol. Chem.* 271: 33632-33638

Higgins, C.M., Hall, R.M., Mitter, N., Cruickshank, A. and Dietzgen, R.G. (2004) Peanut stripe potyvirus resistance in peanut (*Arachis hypogaea* L.) plants carrying viral coat protein gene sequences. *Transgenic Res* 138: 1-9.

Holmberg, N. and Bülow, L. (1998) Improved stress tolerance in plants by heterologous gene transfer: Recent achievements and future prospects. *Trends Plant Sci.* 3: 61.

Holmstrom, K.O., Mantyla, E., Welin, B., Mandal, A., Tapio Palva, E., Tunnela, O.F. and Londesborough, J. (1996) Drought tolerance in tobacco. *Nature* 379: 683-684.

Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, E.T. and Welin, B. (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J. Exp. Bot.* 51: 177-185.

Hong, Z., Lakkineni, K., Zhang, Z. and Verma, D.P.S. (2000) Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase. *Aust. J. Plant Physiol.* 22: 875-884.

Horvath, D.P., McLarney, B.K. and Thomashow, M.F. (1993) Regulation of *Arabidopsis thaliana* L. (Heynh) *COR 78* in response to cold. *Plant Physiol* 103: 1047-1053.

Horvath, D.P., McLarney, B.K., Thomashow, M.F. (1993) Regulation of *Arabidopsis thaliana* L. (Heyn) *cor78* in response to low temperature. *Plant Physiol* 103: 1047–1053.

Hoshida, H., Tanaka, Y., Hibino, T., Hayashi, Y., Tanaka, A., Takabe, T. and Takabe, T. (2000) Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Mol. Biol* 43: 103-111.

Hsieh, T.H., Lee, J.T., Charng, Y.Y. and Chan, M.T. (2002) Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. *Plant Physiol.* 130: 618-626.

Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlindl, J.K., Keller, W.A. and Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol* 122: 747-756.

Hubick, K.T., Farquhar, G.D. and Shorter, R. (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. *Aust. J. Plant Physiol.* 13: 803-816.

Ishitani, M., Xiong, L., Stevenson, B. and Zhu, J.K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell* 9: 1935-1949.

Ishizaki-Nishizawa, O., Fujii, T., Azuma, M., Sekiguchi, K., Murata, N., Ohtani, T. and Toguri, T. (1996) Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase. *Nat Biotechnol.* 14: 1003-1006.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant J.* 27: 325-333.

Iwasaki, T., Kiyosue, T. and Yamaguchi-Shinozaki, K. (1997) The dehydration-inducible rd17 (*cor47*) gene and its promoter region in *Arabidopsis thaliana* *Plant Physiol.* 115: 128.

Jackson, M. W., Stinchcombe, R., Korves, T. M. and Schmitt, J. (2004) Cost and benefits of cold tolerance in transgenic *Arabidopsis thaliana* *Mol. Ecol.* 13: 3609-3615.

Jaglo, K.R., Kleff, K.L., Amundsen, X., Zhang, V., Haake, J.Z., Zhang, T., and Thomashow, M.F. (2001) Components of the *Arabidopsis* C-repeat dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.* 127: 910-917.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M. F. (1998) Arabidopsis CBF1 over expression induces cor genes and enhances freezing tolerance. *Science.* 280: 104-106.

Jagtap, V. and Bhargava, S. (1995) Variation in antioxidant metabolism of drought tolerant and drought susceptible varieties of *Sorghum bicolor* (L.) Moench. Exposed to high light, low water and high temperature stress. *J. Plant Physiol.* 145: 195–197.

Kanyand, M., Dessai, A. P. and Prakash, C. S. (1994) Thidiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea*) plants in vitro. *Plant Cell Rep.* 14: 1-5.

Kartha, K.K., Pahl, K., Leung, N.L. and Mroginski, L.A. (1981) Plant regeneration from meristems of grain legumes, soyabean, cowpea, peanut, chickpea and bean. *Can. J. Bot.* 59: 1671-1679.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress inducible transcription factor. *Nat. Biotechnol.* 17: 287-291.

Kasuga, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2004) A Combination of the *Arabidopsis* DREB1A Gene and stress-Inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant and Cell Physiol.* 45: 346-350.

Katiyar-Agarwal, S., Agarwal, M. and Grover, A. (2003) Heat-tolerant basmati rice engineered by over-expression of hsp101. *Plant Mol. Biol* 51: 677-686.

Kavi Kishor, P.B., Hong, Z., Miao, G.H., Hu, C.A.A and Verma, D.P.S. (1995) Over-expression of-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* 25 1387-1394.

Kavi Kishore, P.B., Hong, Z., Miao, G.U., Hu, C.A.H. and Verma, D.P.S (1995) Overexpression of -pyrroline-5-carboxylate synthase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* 108: 1387-1394.

Kawasaki, S., C. Borchert, M. Deyholos, H. Wang, S. Braazille, K. Kawai, D Galbraith, and Bohnert, H. J. (2001) Gene expression profile during the initial phase of salt stress in rice. *Plant Cell* 13:889-905.

Ketring, D.L. and Reid, J.L. (1993) Growth of peanut roots under field. *Agron. J.* 85: 80-85.

Khandelwal, A., Vally, K. J. M., Geeta, N., Venkatachalam, P., Shaila, M. S. and Lakshmi Sita, G. (2003). Engineering hemagglutinin (H) protein of rinderpest virus into peanut (*Arachis hypogaea* L.) as a possible source of vaccine. *Plant Sci.* 165: 77- 84.

Kimpel, J.A. and J.L. Key. (1985) Heat shock in plants. Trends Biochem. *Sci.* 10: 353-357.

Klein, T.M., Fromm, M.E. and Gradziel, T. (1988) Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. *Biotechnol.* 6: 923-926.

Knight, H. and Knight, M. R. (2001) Abiotic stress signaling pathways: Specificity and cross talk. *Trends Plant Sci.* 6:262-267.

Knight, H. and Knight, M.R. (2001) Abiotic stress signaling pathways: specificity and cross-talk. *Trends Plant Sci.* 6: 262–267.

Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M. and Iba, K. (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast omega-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol.* 105: 601-605.

Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Nat. Acad. Sci.* USA 97: 2940–2945.

Krause, G.H. and Somersalo, S. (1989) Fluorescence as a tool in photosynthesis research: application in studies of photoinhibition, cold acclimation and freezing stress. *Philos. Trans. R. Soc.* Lond. 323: 281-293.

Krause, G.H. and Weis, E. (1991) Chlorophyll fluorescence and photosynthesis: the basics. *Annu Rev Plant Physiol Plant Mol Biol.* 42: 313–49.

Krüger, G.H.J., Tsimilli-Michael, M. and Strasser, R.J. (1997) Light stress provokes plastic and elastic modi.cations in structure and function of Photosystem II in camellia leaves. *Physiol. Plant.* 101: 265–277.

Kumpatla, S.P., Teng, W., Buchholz, W.G., and Hall, T.C. (1997) Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. *Plant Physiol.* 115: 361-373.

Kunert, K. J. and Ederer, M.(1985) Leaf aging and lipid peroxidation: the role of the antioxidants vitamin C and E. *Physiol. Plant.* 65: 85-88.

Lacorte, C., Aragu, F.J.L., Almeida, E.R., Mansur, E. and Rech, E.L. (1997) Transient expression of GUS and 2S albumin gene from Brazilnut in peanut (*Arachis*

hypogaea L.) seed explants using particle bombardment *Plant Cell Rep* 16 619-623.

Lacorte, C., Mansur, E., Timmerman, B. and Cordeiro, A.R. (1991) Gene transfer into peanut (*Arachis hypogaea* L.) by *Agrobacterium tumefaciens* *Plant Cell Rep* 10: 354-357.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. and Zhu, J K (2001) The *Arabidopsis HOS1* gene negatively regulates cold signal transduction and encodes a RING-finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. *Gene Dev.* 15: 912-924.

Lee, J.H., Hubel, A. and Schoffl, 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, J.H., Van Montagu, M. and Verbruggen, N. (1999) A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells *Proc Natl Acad. Sci.* USA 96: 5873-5877.

Lee, S.S., Cho, H.S., Yoon, G.M., Ahn, J.W., Kim, H.H and Pai, H.S (2003) Interaction of NtCDPK1 calcium-dependent protein kinase with NtRpn3 regulatory subunit of the 26S proteasome in *Nicotiana tabacum* *Plant J* 33 825–840.

Leisinger, T. (1996) Biosynthesis of proline. *In* F. C. Neidhardt (ed.) *Escherichia coli* and *Salmonella*: cellular and molecular biology. American Society for Microbiology Press, Washington, D.C. pp 434-441.

Li, H.Y., Chang, C. S., Lu, L. S., Liu, C. A., Chan, M. T. and Chang, Y. Y (2003) Over-expression of *Arabidopsis thaliana* heat shock factor gene (*AtHsfA1b*) enhances chilling tolerance in transgenic tomato *Bot. Bull Acad Sin* 44. 129-140.

Li, Z., Jarret, R. L. and Demski, J. W. (1997) Engineered resistance to tomato spotted wilt virus in transgenic peanut expressing the viral nucleocapsid gene. *Transgenic Res.* 6: 297-305.

Li, Z., Jarret, R.L. Pittman, R.N. and Demski, J.W. (1994) Shoot organogenesis from cultured seed explants of peanut (*Arachis hypogaea* L.) using thidiazuron. *In Vitro Cel. Dev. Biol.-Plant* 30: 187-191.

Lilius, G., Holmberg, N. and Bülow, L. (1996) Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase. *Biotechnol.* 14: 177-180.

Lindquist, S. (1986) The heat-shock response. *Annu. Rev. Biochem* 55: 1151-1191.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10: 1391-1406.

Livingstone, D. M. and Birch, R. G.(1999) Efficient transformation and regeneration of diverse cultivars of peanut (Arachis hypogaea L.) by particle bombardment into embryogenic callus produced from mature seeds. *Mol Breed.* 5:43 51.

Livingstone, D.M. and Birch, R.J. (1995). Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogea* L.) *Aus. J. Plant Physiol.* 22: 585-591.

Maldonado-Rodriquez,(2000)Biolizerprogram, www.unige.ch sciences biology/bioen.

Malik, M.K., Solvin, J.P., Hwang, C.H. and Zimmerman, J.L. (1999) Modified expression of a carrot small heat-shock protein gene, *Hsp 17.7* results in increased or decreased thermotolerance. *Plant J.* 20: 89-99.

Mansur, E., Lacorte, C. and De Freitas, V.G. (1993) Regulation of transformation efficiency of peanut (*Arachis hypogaea* L.) explants by *Agrobacterium tumefaciens*. *Plant Sci.* 99: 89-91.

Matzke, M.A., Moscone, E.A., Park, Y.D., Papp, I., Oberkofler, H., Neuhuber, F., and Matzke, A.J. (1994) Inheritance and expression of a transgene insert in an aneuploid tobacco line. *Mol. Gen. Genet.* 245: 471-485.

Mc Kently, A.H., Moore, G.A., Doostdar, H. and Niedz, R.P. (1995) *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. *Plant Cell Rep.* 14: 699-703.

McKently, A.H. (1995). Effect of genotype on somatic embryogenesis from axes of mature peanut embryos. *Plant Cell Tis Organ Cult.* 42: 251-254.

McKently, A.H. Moore, G. A. and Gardner, F.P. (1990.) In vitro plant regeneration of peanut from seed explants. *Crop Sci.* 30: 192-196.

Mckently, A.H., Moore, C.A., and Gardner, F.P. (1991) Regeneration of peanut and perennial peanut from cultured leaf tissue. *Crop Sci* 31: 833-837.

Mckersie, B.D., Bowley, S.R., Harjanto, E. and Leprince, O. (1996) Water deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* 111: 1177-1181.

McNeil, S.D., Nuccio, M.L. and Hanson, A.D (1999) Betaines and related osmoprotectants: Targets for metabolic engineering of stress resistance *Plant Physiol.* 120: 945-949.

McNeil, S.D., Nuccio, M.L., Rhodes, D., Shachar-Hill, Y. and Hanson, A.D (2000) Radiotracer and computer modeling evidence that phosphobase methylation is the main route of choline synthesis in tobacco. *Plant Physiol* 123 371-380

Mittler, R. (2002) Oxidative stress, antioxidants, and stress tolerance *Trends Plant Sci.* 9: 405-410.

Mittler, R. and Zilinskas, B.A. (1994) Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. *Plant J* 5: 397-405.

Mohanty, A. H., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N. and Tyagi, A. (2002) Transgenics of an elite indica rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. *Theor Appl Genet* 106: 51-57.

Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in Arabidopsis thaliana: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. *Plant Cell Physiol.* 33: 217-224.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vector. *Plant Cell Rep.* 8: 238-242.

Mundy, J. and Chua, N-H. (1998) Abscisic acid and water-stress induce the expression of a novel rice gene. *EMBO J.* 7: 2279-86.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassys with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

Nageswara Rao, R.C. and Wright, G.C. (1994) Stability of the relationship between specific leaf area and carbon isotope discrimination across environments in peanut. *Crop Sci.* 34: 98-103.

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 1 Yield response. *Agro. J.* 77: 782-786.

Nageswara Rao, R.C., Talwar, H.S. and Wright, G.C. (2001) Rapid assessment of specific leaf area and leaf N in peanut (*Arachis hypogaea* L.) using chlorophyll meter. *J. Agro.Crop Sci.* 189: 175-182.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K and Shinozaki, K. (1999a). Antisense supression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett* 461 205-210.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinosaki, K. and Shinozaki, K. (1999b) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J.* 18:185-193.

Narasimhulu, S.B. and Reddy, G.M. (1983) Plantlet regeneration from different callus cultures of *Arachis hypogaea* L. *Plant Sci Lett.* 31: 157-163.

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D. and Hancock, J T. (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants *J Exp Bot* 53. 1237–1247.

Nigam, S.N., Dwivedi S.L., Rao Y.L.C. and Gibbons R.W. (1991) Registration of ICGS-I Peanut cultivar. *Crop Sci.* 31: 1382–1383.

Noctor, G., Foyer, C.H. (1998) A re-evaluation of the ATP:NADPH budget during C_3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity. *J. Exp. Bot.* 49: 1895-1908.

Norden, A.J. (1980) Use of the pedigree breeding method to develop multiline peanut varieties. *Proceedings of Peanut Breeding Symposium. Amer Peanut Res And Educ. Soc. 1980. Research Report No 80*, North Carolina State University, Raleigh, USA.

Nordin, K., Heino, P. and Palva, E.T. (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* 115: 875-879.

Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Dudits, D. and Hovarth, G.V. (2000) A novel aldose aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stress. *Plant J.* 24: 437-446.

Ouzounidou, G., Moustakas, M. and Strasser, R.J. (1997) Sites of action of copper in the photosynthetic apparatus of maize leaves: Kinetic analysis of chlorophyll fluorescence, oxygen evolution, absorption changes and thermal dissipation as monitored photoacoustic signals. *Aust. J. Plant Physiol* 24 81-90.

Ozias-Akins, P., Schnall, J.A., Anderson, W.F., Singsit, C., Clemente, T.E., Adang, M.J. and Weissinger, A.L. (1993) Regeneration of transgenic peanut plants from stably transformed embryogenic callus. *Plant Sci* 93: 185-194.

Padua, V.L.M., Pestana, M.C., Margis-Pinheiro, M. and De Oliveira, D.E. (2000) Electroporation of intact embryonic leaflet of peanut: gene transfer and stimulation of regeneration capacity. *In Vitro Cell. Dev Biol Plant*, 36: 374-8.

Pardha Saradhi, P., Alia, Arora, S. and Prasad, K.V.S.K. (1995) Proline accumulates in plants exposed to UV radiation and protects them against UV induced peroxidation. *Biochem. Biophys. Res. Commun.* 201: 1-5.

Pardo, J.M., Reddy, M.P. and Yang, S. (1998) Stress signaling through Ca2+/ Calmodulin dependent protein phosphatase calcineurin mediates salt adaptation in plants. *Proc. Natl. Acad. Sci.* USA 95: 9681-9683.

Passioura, J.B. (1976) Physiology of grain yield in wheat growing on stored water. *Aust. J. Plant Physiol.* 3: 559–565

Patel, M.S. and Golakiya, B.A. (1988). Role of potassium in counteracting the effect of cyclic drought on groundnut. *J. Potassium Res.* 4: 163-167.

Pellegrineschi ,A., Reynolds, M., Pacheco, M., Brito, R. M., Almeraya, R., Yamaguchi-Shinozaki, K. and Hoisington, D. (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* 47: 493-500.

Perl, A., Perl-Treves, R., Galili, S., Aviv, D., Shalgi, E., Malkin, S and Galun, I. (1993) Enhanced oxidative stress defense in transgenic potato expressing tomato Cu, Zn superoxide dismutases. *Theor. Appl. Genet.* 85: 568-576

Pilon-Smits EAH, Terry N, Sears T & van Dun K (1999) Enhanced drought resistance in fructan-producing sugar beet. *Plant Physiol Biochem* 37: 313-317

Pilon-Smits, E.A.H., Ebskamp, M.J.M., Jeuken, M.J.W., van der Meer, I.M., Visser, R.G.F., Weisbeek, P.J. and Smeekens, J.C.M. (1996) Microbial fructan production in transgenic potato plants and tubers. *Ind Crops Prod* 5: 35-46.

Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, J.W., Weisbeek, and Smeekens, S.C.M. (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol* 107: 125-130.

Pilon-Smits, E.A.H., Terry, N., Sears, T., Kim, H., Zayed, A., Hwang, S., Van Dun, K., Voogd, E., Verwoerd, T.C., Krutwagen, R.W.H.H. and Goddijn, J.M. (1998) Trehalose-producing transgenic tobacco plants show improved growth performance under drought stress. *J. Plant Physiol* 152: 525-532.

Ponsamuel, J., Huhman, D.V., Cassidy, B.G. and Post-Beittenmiller, D. (1998) In vitro regeneration via caulogenesis and brassin-induced shoot conversion of dormant buds from plumular explants of peanut (*Arachis hypogaea* L. cv 'Okrun'). *Plant Cell Rep.* 11: 373-378.

Prakash, J.S.S., Srivastava, A., Strasser, R.J. and Mohanty, P. (2003) Senescence-induced alterations in the Photosystem II functions of *Cucumis sativus* cotyledons: Probing of senescence driven alterations of Photosystem II by chlorophyll *a* fluofescence induction O-J-I-P transients. *Indian J. Biochem. Biophys.* 40: 160–168.

Prasad, K.V.S.K., Sharmila, P., Kumar, P.A. and Partha Saradhi, P. (2000) Transformation of *Brassica Juncea* (L.) Czern with bacterial *codA* gene enhances its tolerance to salt stress. *Mol. Breed.* 6: 489-492.

Quartacci, M. and Navaro-Izzo, F. (1992) Water stress and free radical mediated changes in sunflower seedlings. *J. Plant Physiol.* 139. 621-625

Quartacci, M.F., Sgherri, C.L.M., Pinzino, C. and Navari-Izzo, F. (1994) Superoxide radical production in wheat plants differently sensitive to drought. *Proceed. Royal Society, Edinburgh.* 102: 291–294.

Quintero, F.J., Blatt, M.R. and Pardo, J.M. (2000) Functional conservation between yeast and plant endosomal Na(+)/H(+) antiporters. *FEBS Lett* 471 224-228

Rao, R.C.N. and Nigam, S.N. (2001) Genetic options for drought management in groundnut. *In*: N.P. Saxena (ed.), Management of Agricultural Drought-Agronomic and Genetic Options. Oxford & IBH Publishing Co. Pvt. Ltd New Delhi. pp. 123-142.

Rao, R.C.N., Udayakumar, M., Farquhar, G.D., Talwar, H.S. and Prasad, T.G. (1995) Variations in carbon isotope discrimination and its relationship with specific leaf area and Rubisco content in groundnut genotypes. *Aust. J. Plant Physiol* **22:** 545–551.

Ray, J. D. and Sinclair, T. R. (1997) Stomatal closure of maize hybrids in response to soil drying. *Crop Sci.* 37: 803-807.

Ray, J.D., and Sinclair, T.R. (1998) The effect of pot size on growth and transpiration of maize and soybean during water deficit stress. *J. Exp. Bot.* 49:1381-1386.

Ritchie, J. T. (1980) Climate and soil water. In: Murphy, L. S., Welch, L. F. and Doll, E. C. (Eds), Moving up the yield curve: advances and obstacles. Madison, Wisconsin: ASA Special Publication Number, pp. 1-24.

Ritchie, J.T. (1981) Water dynamics in the soil-plant-atmosphere system. *Plant Soil* 58: 81–96.

Rohilla, J.S., Jain, R.K. and Wu, R. (2002) Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley Hva1 cDNA. *Plant Sci.* 163: 525-532.

Rohini, V.K. and Rao, K. S. (2000) Transformation of Peanut (*Arachis hypogaea* L.): a non-tissue culture based approach for generating transgenic plants. *Plant Sci.* 150: 41-49.

Roxas, V.P., Smith, R.K. Jr, Allen, E.R. and Allen, R.D. (1997) Overexpression of glutathione-S-transferase/ glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat Biotechnol* 15: 988-991

Roy, M. and Wu, R. (2001) Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice *Plant Sci* 160: 869-875

Roy, R.N. (1995) In: Integrated Plant Nutrition System, FAO Fertilizer, and Plant Nutrition Bulletin No.12, pp. 49-66

Rubio, M.C., González, E.M., Minchin, F.R., Webb, K.J., Arrese-Igor, C., Ramos, J and Becana, M. (2002) Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases *Physiol. Plant.* 115: 531–540.

Sadras, V.O., and S.P. Milroy. (1996) Soil-water thresholds for the responses of leaf expansion and gas exchange. *Field Crops Res* 47: 253-266

Sairam, R. K., Deshmukh, P.S. and Saxena D C. (1998) Role of antioxidant systems in wheat genotype tolerance to water stress *Biol Plant* 41: 387-394

Sakamoto, A. and Murata, N. (2001) The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress-resistant transgenic plants *Plant Physiol.* 125: 180-188.

Sakamoto, A., Alia and Murata, N. (1998) Metabolic engineering of rice leading to biosynthesis of glycine betaine and tolerance to salt and cold *Plant Mol Biol* 38: 1011–1019

Sakamoto, A., Valverde, R., Alia, Chen, T.H. and Murata, N. (2000) Tranformation of *Arabidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. *Plant J.* 22: 449-453.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sanford, J. C., Klein, T. M., Wolf, E. D. and Allen, N. (1987) Delivery of substances to the cells and tissues using a particle gun bombardment process *Particle Gun Technol.* 5: 27-37.

SAS Institute (1996) SAS Users Guide: Statistics (SAS Institute, Cary, NC).

Saxena, N.P.(2001) Management of drought in chickpea- a holistic approach In: N.P. Saxena (ed.), Management of Agricultural Drought - Agronomic and Genetic Options. Oxford & IBH Publishing Co. Pvt. Ltd.: New Delhi.

Schaedle, M., and Bassham, J.A. (1977) Chloroplast glutathione reductase *Plant Physiol.* 59: 1011-1012.

Schnall, J.A. and Weissinger, A.K. (1995) Genetic transformation in *Arachis hypogaea* L. Vol 34. *In*: Biotechnology in Agriculture and Forestry Y.P.S Bajaj (ed) Springer-verlag Berlin, Heidelberg, New York, pp. 135-144

Schobert, B. and Tschesche, H. (1978) Unusual solution properties of proline and its interaction with proteins. *Biochem. Biophys. Acta* 541: 270-277.

Seki, M. , Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K , Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001) Monitoring the expression pattern of 1,300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* 13: 61-72.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi- Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA Microarray. *Plant Cell* 13: 61-72.

Sen Gupta, A., Heinen, J.L., Holady, A.S., Burke, J.J. and Allen, R.D (1993) Increased resistance to oxidative stress in transgenic plants that over-express chloroplastic Cu/Zn superoxide dismutase. *Proc. Nat Acad Sci* USA 90: 1629-1633.

Sharma, K. K. and Anjaiah, V. (2000) An efficient method for production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens*-mediated genetic transformation. *Plant Sci* 159: 7-19

Sharma, K. K., Lavanya, M. and Anjaiah, V (2000) Method for the isolation and purification of genomic DNA from peanut that is suitable for analytical applications. *Plant Mol. Biol. Rep.* 18: 393a-393h.

Sharma, K.K. and Lavanya, M. (2002) Recent developments in transgenics for abiotic stress in legumes of the semi-arid tropics. In. M. Ivanaga (ed.) Genetic

Engineering of Crop Plants for Abiotic Stress. JIRCAS Working Report No. 23, JIRCAS: Tsukuba, Japan, pp. 61-73.

Sharma, K.K. and Ortiz, R. (2000) Program for the application of genetic transformation for crop improvement in the semiarid tropics: *In Vitro Cell Dev Biol.-Plant* 36: 83-92.

Sharma, K.K., Bhojwani, S.S. and Thorpe, T.A. (1990). Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.). *Plant Sci.* 66: 247-253.

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.

Sheshshayee, M. S., Bindumadhava, H., Prasad, T. G., Lakshminarayana, M R and Udayakumar, M. (1998) Oxygen isotope enrichment ($\Delta^{18}O$) as a measure of time-averaged transpiration rate. *J.Exp. Bot.* 56: 3033-3039.

Shi, H., Ishitani, M., Kim, C. and Zhu, J.K. (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+ H+ antiporter. *Proc Nat Acad Sci.* USA 97: 6896-6901.

Shigeoka , S. , Ishikawa, T. , Tamoi, M. , Miyagawa, Y. , Takeda, T , Yabuta, T. and Yoshimura, K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *J. Exp. Bot.* 53: 1305-1319.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water stress response. *Plant Physiol* 115: 327-334.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1999) Molecular responses to drought stress. *In* Shinozaki, K. and Yamaguchi-Shinozaki, K. (eds) Molecular Responses to Cold, Drought, Heat, and Salt Stress in Higher Plants. R.G. Landes Company, Texas, pp.11–28.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperatures: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* 3: 217-23.

Shinozaki, K., Yamaguchi-Shinozaki, K., Urao, T. and Koizumi, M. (1992) Nucleotide sequence of a gene from Arabidopsis thaliana encoding a myb homologue. *Plant Mol. Biol.* 19: 493-499.

Shinwari, Z.K., Nakashima, K., Miura, S., Kasuga, M., Seki, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem. Biophys. Res. Commun.* 50, 161-170

Sinclair, T.R. and Ludlow, M.M. (1986) Influence of soil water supply on the plant water balance of four tropical grain legumes *Aus. J. Plant Physiol.* 13 329-341

Sinclair, T.R., Purcell, L.C. and Sneller, C.H. (2004) Crop transformation and challenge to increase yield potential. *Trends Plant Sci.* 9 70-75

Sinclair, T.R., Hammer, G. L. and Oosterom, E. J. V. (2005) Potential yield and water-use efficiency benewfits in sorghum from limited maximum transpiration rate. Func. Plant. Biol. 32: 945-952.

Singsit, C., Adang, m. J., Lynch, R. E., Wang, A., Cardineau, G. and Ozias-Akins, P (1997) Expressions of a *Bacillus thuringiensis cryIAc* gene in transgenic peanut plants and its efficiency against lesser cornstalk borer *Transgenic Res.* 6: 169-176.

Sivamani, E., Bahieldin, A., Wraith, J.M., Al-Niemi, T., Dyer, W.E., Ho, T.H.D. and Qu, R. (2000) Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. *Plant Sci.* 155: 1-9.

Skriver, K. and Mundy, J. (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 5: 503-512.

Slooten, L., Capiau, K., Van Camp, W., Montagu, M.V., Sybesma, C. and Inzé, D. (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. *Plant Physiol.* 107: 737–75.

Smirnoff, N. and Colombé, S.V. (1988) Drought influences the activity of enzymes of the chloroplast hydrogen peroxide scavenging system. *J. Exp. Bot.* 39: 1097-1108.

Srivastava, A. and Strasser, R.J. (1995) How do land plants respond to stress temperature and stress light? *Archs Sci Genève* 48: 135-145.

Srivastava, A. and Strasser, R.J. (1996) Stress and stress management of land plants during a regular day. *J Plant Physiol.* 148: 445-455.

Srivastava, A. and Strasser, R.J. (1997) Constructive and destructive actions of light on the photosynthetic apparatus. *J. Sci Ind Res* 56: 133-148

Steinmeyer, K. (1996) A family of putative chloride channels from *Arabidopsis* and functional complementation of a yeast suain with a CLC gene disruption *J Bio.Chem.* 27: 33632-33638.

Stockinger, E., Gilmour, S.J. and Thomashow, M.F (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription response to low temperature and water deficit *Proc Nat Acad Sci. USA* 94: 1035–1040

Strasser, B.J. and Strasser, R.J. (1995) Measuring fast fluorescence transients to address environmental questions: The JIP-test In P Mathis (ed) Photosynthesis From Light to Biosphere, Vol V, Kluwer Acad Publ., Dordrecht, pp 977 980

Strasser, R.J. and Govindjee (1992a) The F0 and the O-J-I-P fluorescence rise in higher plants and algae. In: Argyroudi- Akoyunoglou J.H (ed), Regulation of Chloroplast Biogenesis, Plenum Press, New York, pp 423 426

Strasser, R.J. and Govindjee (1992b) On the O-J-I-P fluorescence transient in leaves and D1 mutants of *Chlamydomonas Reinhardtii* In: Murata N (ed) Research in Photosynthesis, Vol. 4, Kluwer Acad Publ, Dordrecht, pp 29 32

Strasser, R.J., Srivastava, A. and Govindjee (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. *Photochem Photobiol.* 61: 32-42.

Strasser, R.J., Srivastava, A., Tsimilli-Michael, M. (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P, editors. Probing photosynthesis: mechanisms, regulation and adaptatio.n. Taylor and Francis Publishers, London pp. 445-483

Strasser, R.J., Tsimilli-Michael, M. (2001) Stress in plants from daily rhythm to global changes, detected and quantified by the JIP test. Chim. Nouvelle (SRC) 75: 3321-3126.

Subbarao, G.V., Johansen, C., Slinkard, A.E., Nageshwara Rao, R.C., Saxena, N.P. and Chauhan, Y.S. (1995) Strategies for improving drought resistance in grain legumes. *Crit. Rev. Plant Sci.* 14: 469-523.

Subramanyam, P., Moss, J.P., McDonald, D., Subba Rao, P.V. and Rao, V.R. (1985) Resistance to leaf spot caused by *Cercosporidium personatum* in wild *Arachis* species. *Plant Dis.* 69: 951-954.

Sun, W., Bernard, C., van de Cotte, B., Montagu, M.V. and Verbruggen, N. (2001) At-HSP17.6A, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon overexpression. *Plant J.* 27: 407-415.

Takabe, T., Hayashi, Y., Tanaka, A., Takabe, T. and Kishitani, S. (1998) Evaluation of glycinebetaine accumulation for stress tolerance in transgenic rice plants. In Proceedings of the international workshop on Breeding and Biotechnology for environmental Stress in Rice. October 26-29. Sapporo, Japan. pp. 63-68.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993) Stress protection of transgenic tobacco by production of osmolyte mannitol. *Sci.* 259, 508-510.

Thoday, J. M. (1961) Location of polygenes. *Nature* 191: 368-370.

Thomashow, M. F. (1998) Role of cold responsive genes in plant freezing tolerance. *Plant Physiol.* 118: 1-7.

Tsimilli-Michael, M. and Strasser, R.J. (2001) Fingerprints of climate changes on the photosynthetic apparatus' behaviour, monitored by the JIP-test. In. Walther G-R, Burga CA and Edwards PJ (eds) 'Fingerprints' of Climate Changes-Adapted Behaviour and Shifting Species Ranges; Kluwer Acad Plenum Publ. New York, pp 229–247.

Tsimilli-Michael, M., Eggenberg, P., Biro, B., Köves-Pechy, K., Vörös, I. and Strasser, R.J. (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the chlorophyll *a* polyphasic fluorescence transient O-J-I-P. 42 CF *App. Soil Eco.* 15: 169-182.

Tsimilli-Michael, M., Krüger, G.H.J. and Strasser, R.J. (1995) Suboptimality as driving force for adaptation: A study about the correlation of excitation light intensity and the dynamics of fluorescence emission in plants. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, Vol V. Kluwer Acad Publ., Dordrecht. pp .981-984.

Tsimilli-Michael, M., Krüger, G.H.J. and Strasser, R.J. (1996) About the perpetual state changes in plants approaching harmony with their environment. *Archs. Sci Genève* 49: 173–203.

Tsimilli-Michael, M., Pêcheux, M. and Strasser, R.J. (1999) Light and heat stress adaptation of the symbionts of temperate and coral reef foraminifers probed *in hospite* by the chlorophyll *a* fluorescence kinetics O-J-I-P. *Z. Naturforsch* 54 C: 671-680.

Udayakumar, M., Sheshashayee, M.S., Nataraj, K.N., Bindumadhava, H., Devendra, R., Aftab Hussain, I.S. and Prasad, T.G. (1998) Why has breeding for WUE not been successful? An analysis and alternate approaches to exploit this trait for crop improvement. *Curr. Sci.* 74: 996-1000.

Umezawa, T., Okamoto, M., Kushiro, T., Nambara, E., Oono, Y., Seki, M., Kobayashi, M., Koshiba, T., Kamiya, Y. and Shinozaki, K. (2006) CYP707A3, a major ABA 8'-hydroxylase involved in dehydration and rehydration response in Arabidopsis thaliana. *Plant J.* 46: 171-182.

Van Camp, W., Capiau, K., Van Montagu, M., Inzé, D. and Slooten, L. (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiol.* 112: 1703-1714.

Van Rensburg, L. and Krüger, G. (1994) Evaluation of components of oxidative stress metabolism for use in selection of drought tolerant cultivars of *Nicotiana tabacum* L. *J. Plant Physiol.* 145: 730-737.

Van Rensburg, L., Krüger, G.H.J., Eggenberg, P. and Strasser, R.J. (1996) Can screening criteria for drought resistance in *Nicotiana tabacum L.* be derived from the polyphasic rise of the chlorophyll a fluorescence transient (OJIP)? *S. Afr. J. Bot.* 62: 337-341.

Venkatachalam, P., Geeta, N. and Jayabalan, N. (1998) Induction of somatic embryos and plantlet development in cell suspension cultures of *Arachis hypogaea* L. *Breed. Sci.* 48: 231-236.

Venkatachalam, P., Geeta, N., Khandelwal, A., Shaila, M. S. and Lakshmi Sita, G. (2000) Agrobacterium-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. *Curr. Sci.* 78: 1130-1136.

Venkatachalam, P., Geetha, N., Khandelwal, A., Shaila, M. S. and Lakshmi Sita, G. (1999) Induction of direct somatic embryogenesis and plant regeneration from mature cotyledon explants of *Arachis hypogaea* L. *Curr. Sci.* 77: 269-273.

Vierling, E. (1991) The roles of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 42: 579-620.

Vietmeyer, N.D. (1986) Lesser-known plants of potential use in agriculture and forestry. *Science* 232: 1379-1384.

Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr. Opin. Biotechnol. 16: 123-132.

Vranova, E., Inze, D. and Van Breusegem, F. (2002) Signal transduction during oxidative stress. *J. Exp. Bot.* 53: 1227-1236.

Wang, A., Fan, H., Singsit, C. and Ozias-Akins, P. (1998) Transformation of peanut with a soybean VspB promoter-*uid*A chimeric gene. 1. Optimization of a transformation system and analysis of GUS expression in primary transgenic tissues and plants. *Physiol. Plant.* 102: 38-48.

Waters, E.R., Lee, G.J. and Vierling, E. (1996) Evolution, structure and function of the small heat shock proteins in plants. *J. Exp. Bot.* 47: 325-338.

Weisz, R., Kaminski, J. and Smilowitz, Z. (1994) Water deficit effects on potato leaf growth and transpiration: Utilizing fraction extractable soil water for comparison with other crops. *Amer. Potato J.* 71: 829-840.

Welin, B. V., Olson, A. and Palva, E. T. (1995) Structure and organization of two low-temperature-induced dhn/lea/rab-like genes in *Arabidopsis thaliana* L. Heynh. *Plant Mol. Biol.* 29: 391-395.

Wightman, J. A. and Ranga Rao, G. V. (1994) Groundnut pests. In J. Smartt (ed.), The groundnut crop: a scientific basis for improvement. Chapman and Hall, London, pp. 395–479.

Wightman, J.A. and Ranga Rao, G.V. (1993) Groundnut insects. *In*: Smart, J (Ed.) The groundnut crop. Chapman Hall, London, pp. 394-469.

Willits, D.H. and Peet, M.M. (2001) Measurement of chlorophyll fluorescence as a heat stress indicator in tomato: laboratory and greenhouse comparisons. *J Am Soc. Hort. Sci.* 123: 188–94.

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 Bastola, D.R. (1999) Transgenic overexpression of the transcription factor Alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves salinity tolerance of the plants. *Plant Physiol* 120 473-480

Wright, G. C., Nageswara Rao, R. C. and Basu, M. S. (1996) A physiological approach to the understanding of genotype by environment interactions - a case study on improvement of drought adaptation in peanut. In: M. Cooper and G. L. Hammer (eds.), Plant adaptation and crop improvement CAB Int., Wallingford, UK, pp. 365–381.

Wright, G.C and Nageshwara Rao, R.C. (1994) Groundnut water relations. In. J. Smart (ed.), The Groundnut Crop: A scientific basis for improvement. Chapman & Hall, London. U.K., pp.281- 335.

Wright, G.C., Hubick, K.T. and Farquhar, G.D. (1988) Discrimination in carbon isotopes of leaves correlate with water use efficiency of field-grown peanut cultivars. *Aust. J. Plant Physiol.* 15: 815–825.

Wright, G.C., Hubick, K.T. and Farquhar, G.D. (1991) Physiological analysis of peanut cultivar response to timing and duration of drought stress. *Aust J Agri. Res.* 42: 453–470.

Wright, R.B. (1998) The effects of experimental and natural flooding on the distribution and availability of soil phosphorus in a southeastern floodplain forest. Ph.D. diss. George Mason Univ., Fairfax, VA.

Xiong, L., and Zhu, J.K. (2001) Plant abiotic stress signal transduction: molecular and genetic perspectives. *Physiol. Plant.* 112: 152-166.

Xiong, L., Ishitani, M. and Zhu, J.K. (1999) Interaction of osmotic stress, temperature, and abscisic acid in the regulation of gene expression in *Arabidopsis*. *Plant Physiol.* 119: 205-211.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T. H.D. and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.* 110: 249-257.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993) Characterisation of the expression of a dessication-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Mol. Gen Genet.* 236: 331-340.

Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Kiyosue, T. and Shinozaki, K. (1994) Function and regulation of genes that are induced by dehydration stress in *Arabidopsis thaliana*. *JIRCAS J.* 1: 69-79.

Yang, D.X., Wei, Z. M. and An, H.L. (2001) Transgenic peanut plants obtained by particle bombardment via somatic embryogenesis regeneration system Cell Res 11: 156-160.

Yang, G., Rhodes, D. and Joly, R.J. (1996) Effects of high temperature on membrane stability and chlorophyll fluorescence in glycine betaine-deficient and glycine betaine containir.g maize lines. *Aust. J. Plant Physiol.* 23: 437-443.

Yang, H. Y., Singsit, C., Wang, A., Gonsalves, D. and Ozias-Akins, P. (1998a). Transgenic plants containing nucleocapsid protein gene of tomato spotted wilt *tospovirus* show divergent levels of gene expression. *Plant Cell Rep* 17: 693-699.

Yang, H.Y., Gill, R. and Ozias-Akins, P. (1998b) Transformation of peanut with a synthetic antifungal peptide gene. In: Congress on in vitro Biology, Israel.65 p.

Zeyong, X. (1992) Groundnut production and research in East Asia in the 1980s. In Nigam, S.N. (Ed.), Groundnut- a global perspective: proceedings of an international workshop, 25-29 Nov 1991. ICRISAT Center, India. Patancheru, A.P., India. pp. 157-165.

Zhang, H.X. and Blumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotechnol* 19: 765-768.

Zhao, H.W., Chen, Y.J., Hu, Y.L., Gao, Y. and Lin, Z.P. (2000) Construction of a trehalose-6-phosphate synthase gene driven by drought responsive promoter and expression of drought-resistance in transgenic tobacco. *Acta Bot Sinica* 42. 616-619.

Zhu, J.K. (2001) Plant salt tolerance. *Trends Plant Sci.* 6: 66-71.

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Physiol. Plant Mol. Biol.* 53: 247-273.

Zhu, L., Tang, G.S., Hazen, S.P., Kim, H.S. and Ward, R.W. (1999) RFLP-based genetic diversity and its development in Shaanxi wheat lines. *Acta Bot Boreali Occident. Sin.* 19: 208-213.

FIGURES

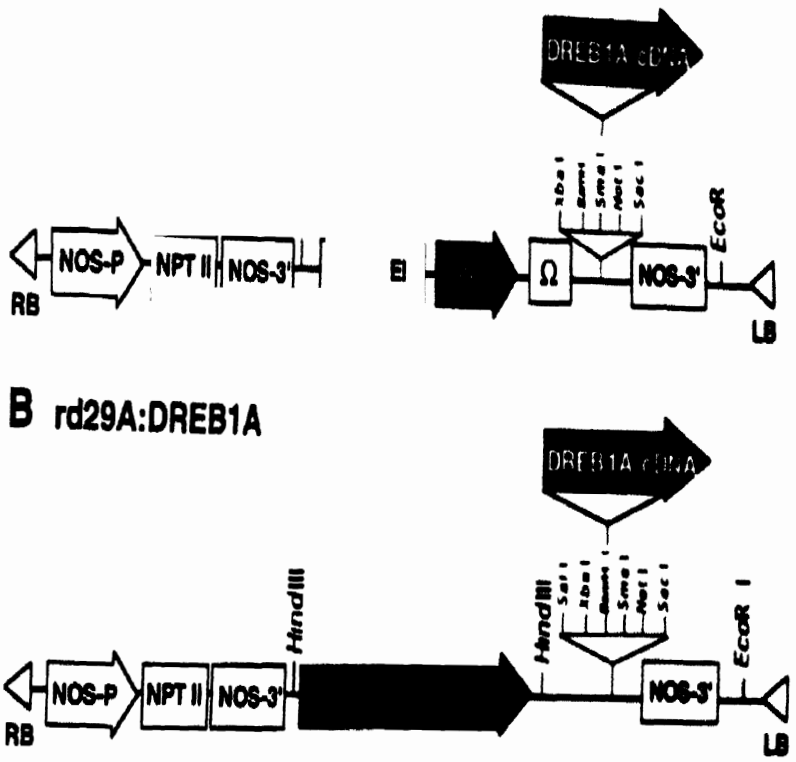


Figure 1A,B. T-DNA regions of the binary plasmids used for *Agrobacterium tumefaciens*-mediated transformation. (A) Construct containing *npt*II and *DREB1A* genes under the control of 35S promoter. (B) rd29A:DREB1A binary plasmid containing *npt*II and *rd29A* driven *DREB1A* gene (LB, left border; RB, right border; *npt*II, neomycin phosphotransferase).

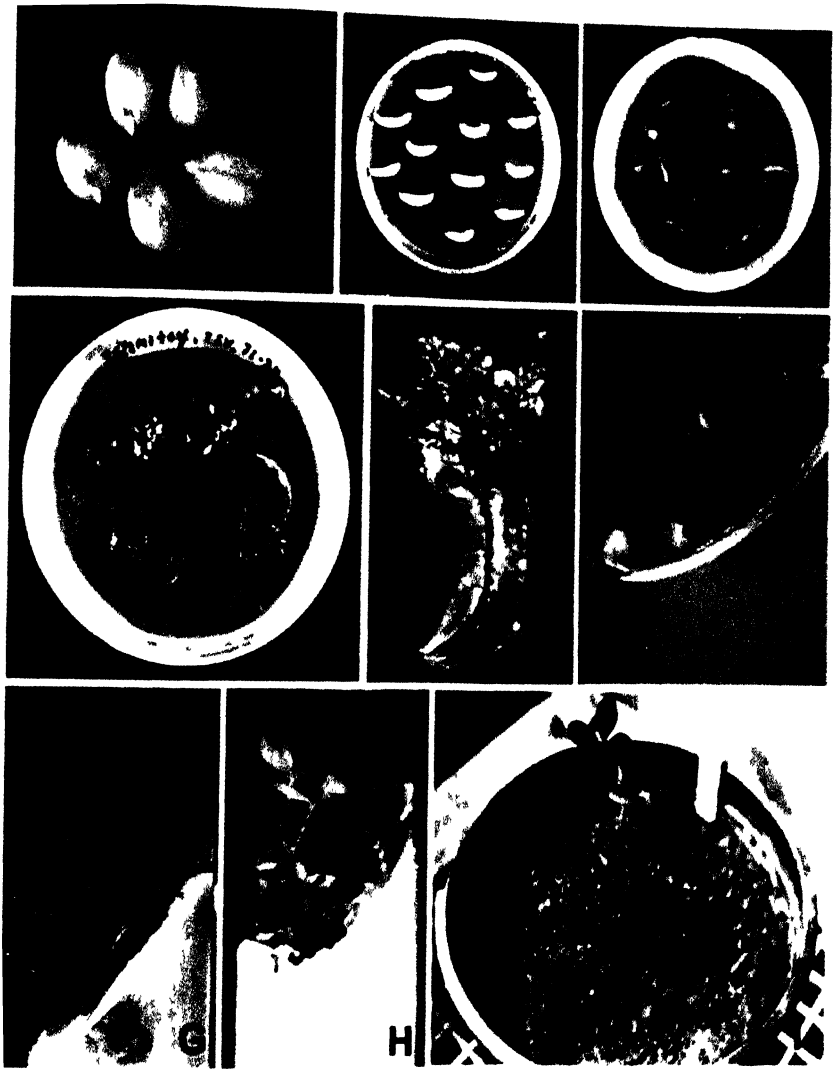


Figure 2A-I. Regeneration of adventitious shoots from cotyledon explants of *A hypogaea* L (A) Healthy seeds of groundnut variety JL 24 (B) Embryo axis is removed surgically from the healthy groundnut seeds and each cotyledon is then cut into vertical halves to obtain cotyledon explants and co cultivated for 48-72 h (C) Greening of cotyledons and initiation of shoot bud formation from the explants after 7-9 d of culture (D) Development of multiple adventitious shoots from the cut ends of cotyledon explants after 3 wk (E) Cotyledon explants bearing multiple shoot buds after 4 wk of culture (F) Development of multiple adventitious shoots after culture on shoot elongation medium (SEM) after 10 d of culture (G) Elongation of shoot cultured on SEM after 2 wk (H) Production of multiple adventitious roots on elongated shoots after 2-3 wk of culture on root induction medium (RIM) (I) A rooted plant transplanted to a pot and maintained under greenhouse conditions after 2 wk of transplantation

Figure 3A-C. Phenotypes of the plants transformed with 35S DREB1A and rd29A DREB1A constructs and growing under controlled *in vitro* and glass house conditions (A) Growth differences in 45 d old plants grown in test tube conditions rd29A DREB1A (right) and 35S DREB1A (left) (B) Severe growth retardation of 3-wk old 35S DREB1A plants (right) as compared to rd29A DREB1A plants (left) of the same age under in vitro conditions (C) Phenotypic differences observed in rd29A DREB1A (right) and 35S DREB1A (left) plants under greenhouse conditions

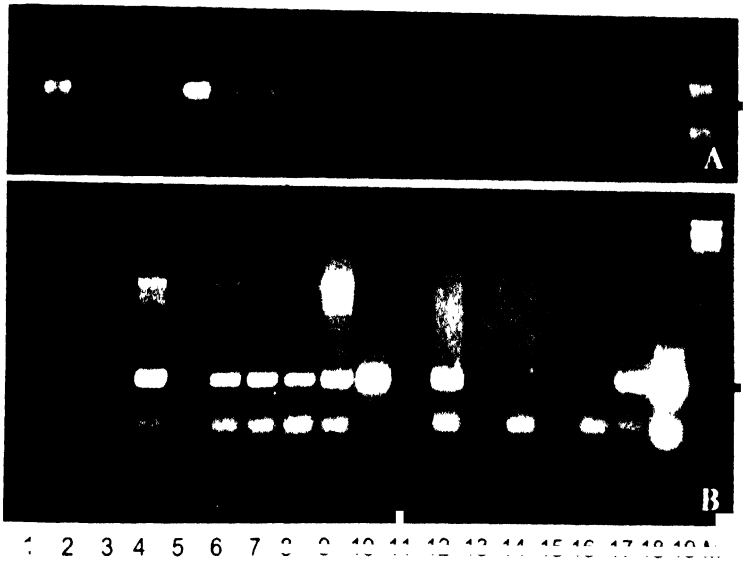


Figure 4A,B. PCR amplification of the *nptII* and *DREB1A* genes in T_0 progenie of independent transgenic events (A) PCR amplification of genomic DNA from 1 T_0 plants showing amplification of 700 bp fragment of *nptII* gene lanes 1-18 carr samples from putative transformants, lane 19 is the untransformed JI 24 and lan 20 is the 100 bp marker ladder. (B) PCR amplification of genomic DNA from 1 T_1 plants showing amplification of the 500 bp DREB1A cDNA, lanes 2-18 carr samples from putative transformants, lane 1 is the untransformed control, lane 1 has the plasmid DNA, and lane 20 is the 100 bp marker ladder

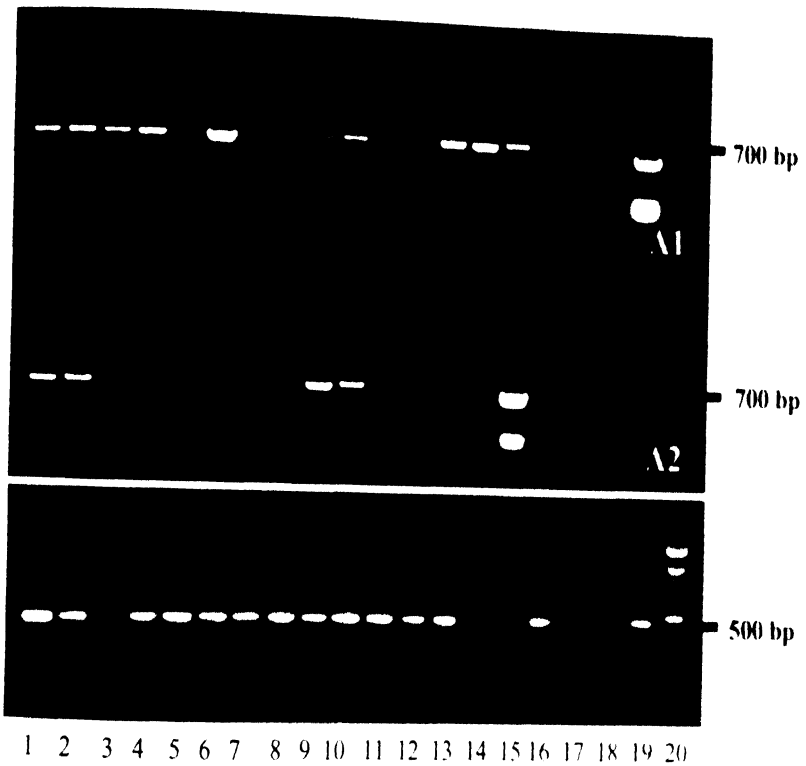


Figure 5A,B. PCR analysis of *nptII* and *DREB1A* genes in T1 progeny of putative transgenic plants. (A) PCR amplification of the *nptII* gene in T_0 representing 8 independent transformed events of T1 generation. (A1) Lanes 1-17 carry samples from putative transformants along with lane 18 with wild type negative control; lane 19 has the plasmid DNA with lane 20 having 100 bp marker DNA (A2) Lanes 1-14 carry the DNA from transgenic plants and lane 15 has plasmid DNA as positive control. (B) PCR amplification of genomic DNA from 16 T_0 plants showing amplification of 499 bp fragment of *DREB1A* gene lanes 1-16 carry samples from putative transformants, lane 17-18 are the untransformed JL 24 controls; lane 19 has the plasmid DNA and lane 20 having100 bp marker.

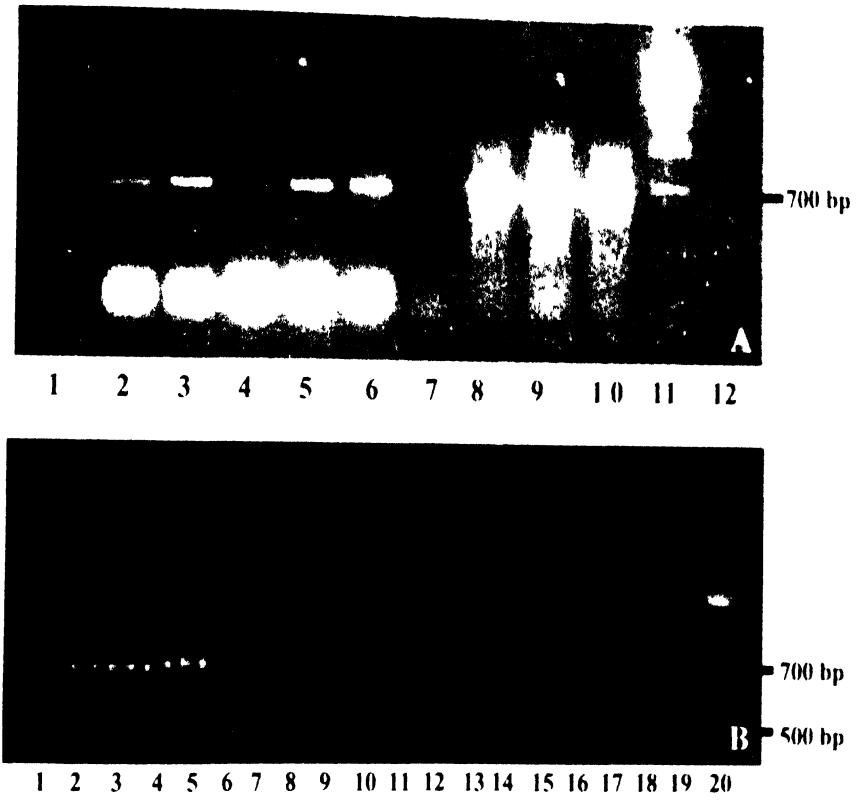


Figure 6A,B. RT PCR analysis for *nptII* and *DREB1* genes under water stressed and unstressed conditions (A) RT-PCR analysis of *nptII* gene expression in untransformed and transgenic plants under non stress conditions lane 2-7 carries the sample RNA from transgenic events whereas lanes 1 and lanes 8-10 carry the RNA samples of untransformed JL 24 and positive control respectively (B) RT PCR of *DREB1A* gene expression in control and transgenic plants carrying rd29A DREB1A before and after water stress treatment Lane 1-8 carries RNA isolated from plants grown under non-stress conditions with non-transgenic control in lane 9 lanes 10-19 has RNA isolated from plants after 5 d of water stress lane 20 has a 100 bp ladder

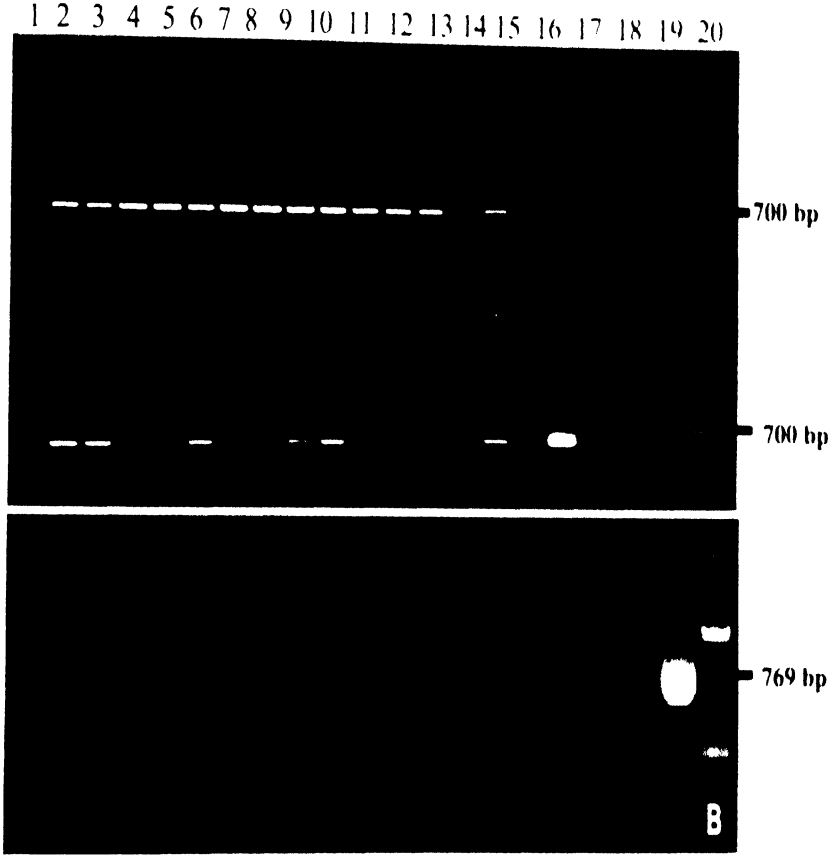


Figure 7A,B. PCR amplification of the *nptII* and *DREB1A* genes in T_1 progenies of independent transgenic events (A) Lanes 1-16 carry samples from putative transformants, lanes 17 and 18 are untransformed controls, lane 19 is the DNA from plasmid rd29A:DREB1A, and lane 20 is the 100 bp marker ladder (B) PCR amplification of genomic DNA from 16 T_1 plants showing amplification of the 769 bp fragment of rd29A:DREB1A junction region. lanes 1-17 carry samples from putative transformants, lane 18 is the untransformed control, lane 19 has the plasmid DNA, and lane 20 is the 100 bp marker ladder

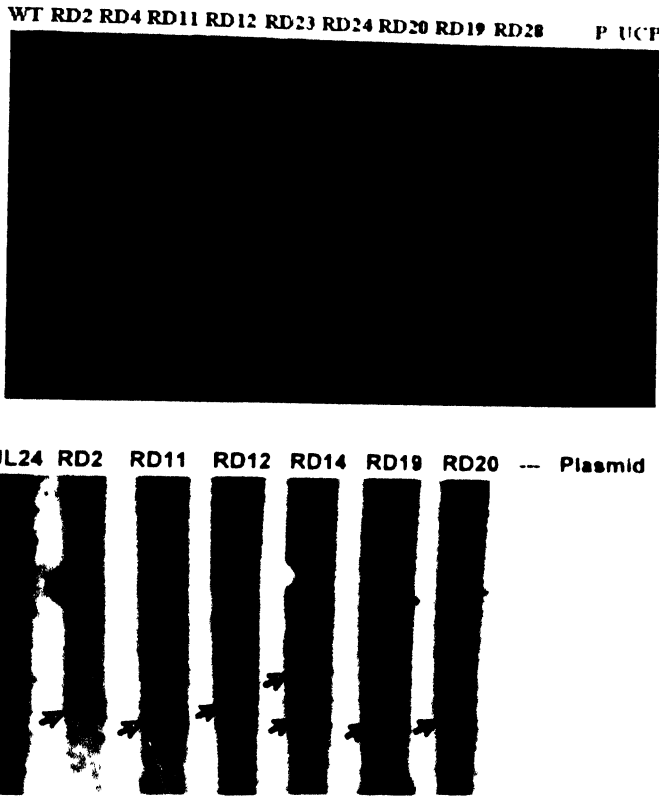


Figure 8A,B. Southern blot analysis of *DREB1A* gene in the genomic DNA of T1 and T2 generation groundnut plants transformed with *A. tumefaciens* carrying the rd29A:DREB1A gene construct.(A) Genomic DNA was restricted with *Hind* III which gives a double cut and was probed with non radiolabeled *rd29A* gene fragment from the plasmid DNA(Lanes 2-9 carry RD2 RD4 RD11 RD12 RD23 RD24 RD20 RD19 and RD28, lane 1 contained the WT JL 24 and Lanes 11 and 12 carried digested as well as uncut plasmid DNA respectively (B) The DNA was restricted using *Eco* RI to provide a single cut within the T-DNA. The blots were probed with Biotin labeled *DREB1A* fragment of 499 bp (Lanes 2-7 carry genomic DNA from events RD2, RD11, RD12, RD14, RD19 and RD20, lane 1 and lane 9 carry DNA from the untransformed controls and a plasmid carrying the rd29A:DREB1A construct, respectively)

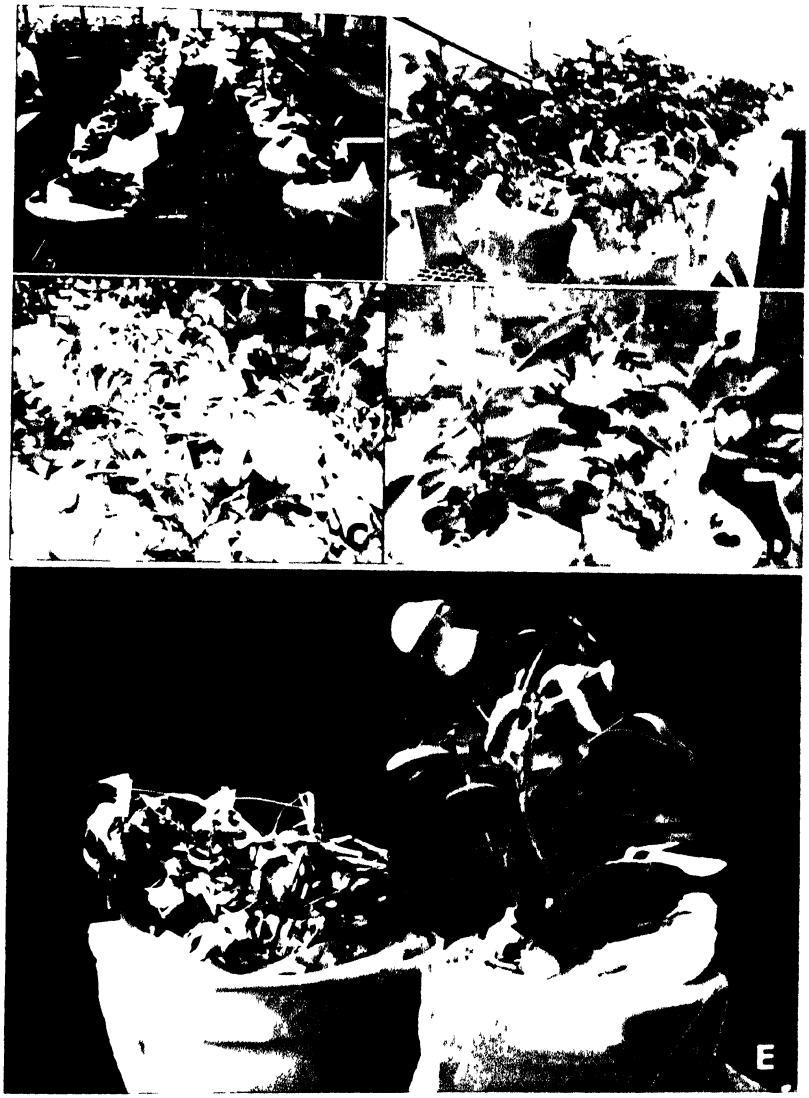


Figure 9A-E. Progressive soil drying in pot experiments conducted in containment greenhouse conditions (A) A typical dry down setup showing the bagging of pots and their arrangement under greenhouse conditions (B-D) A randomized drying down set up in the greenhouse (E) Phenotype of the wild type parent JI 24 (left) and a *DREB1A* containing event RD2 (right) after 12 days of progressive soil drying

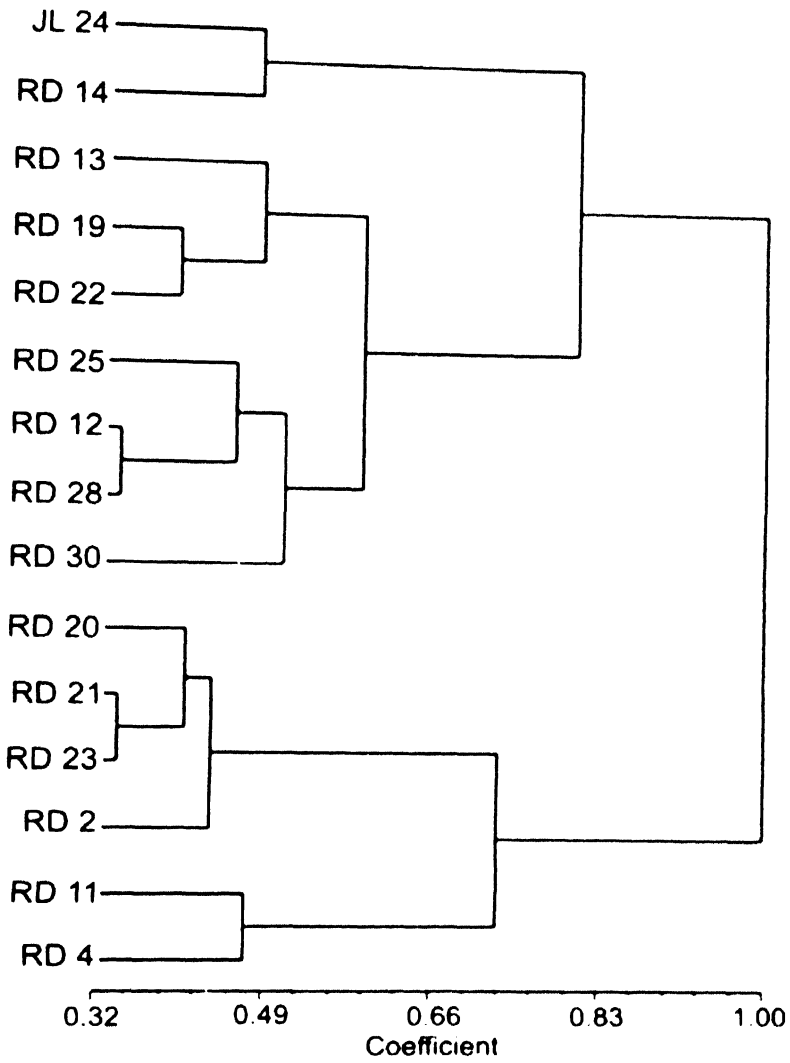


Figure 10. Dendrogram showing relative similarities in 15 independent groundnut events (including wild type JL 24) based on FTSW threshold values and the number of days to end point under water deficit conditions.

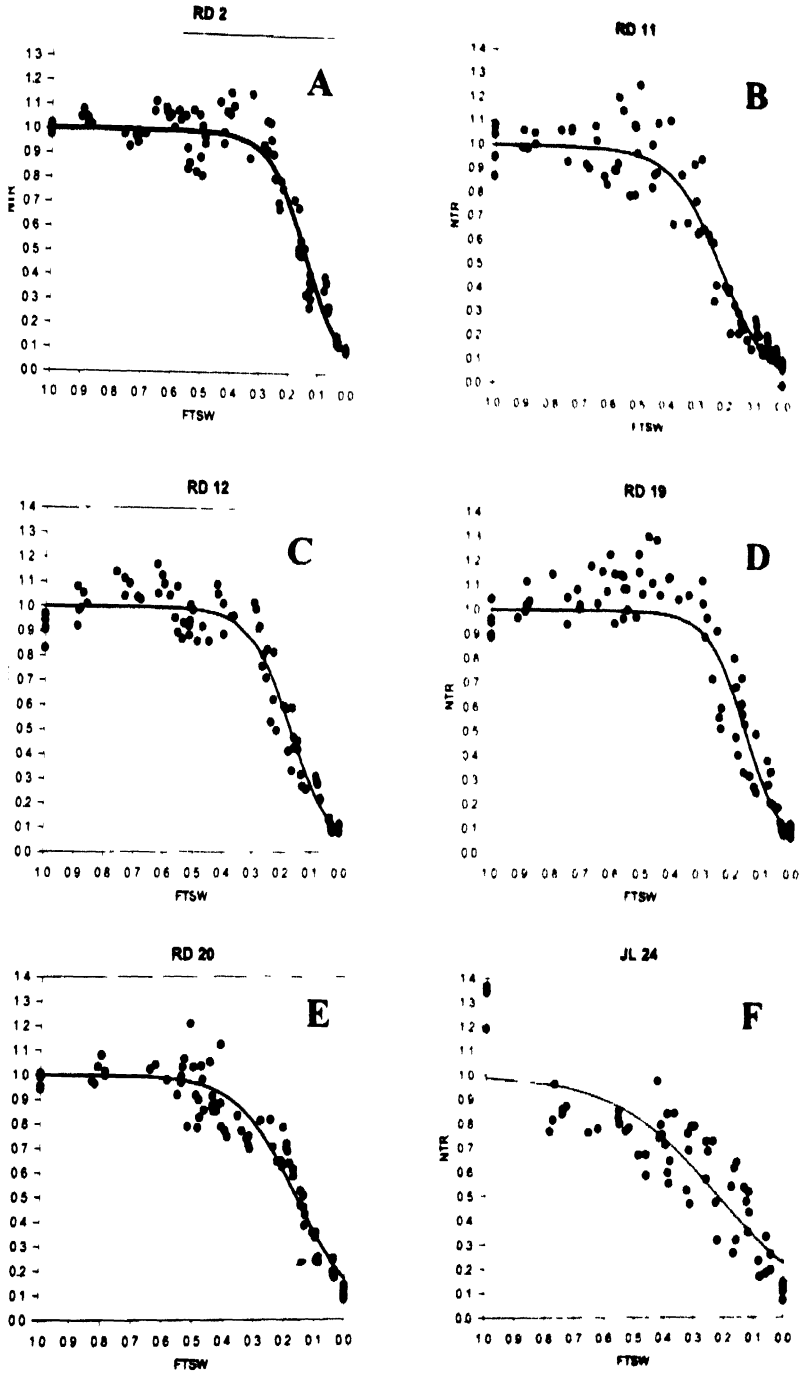
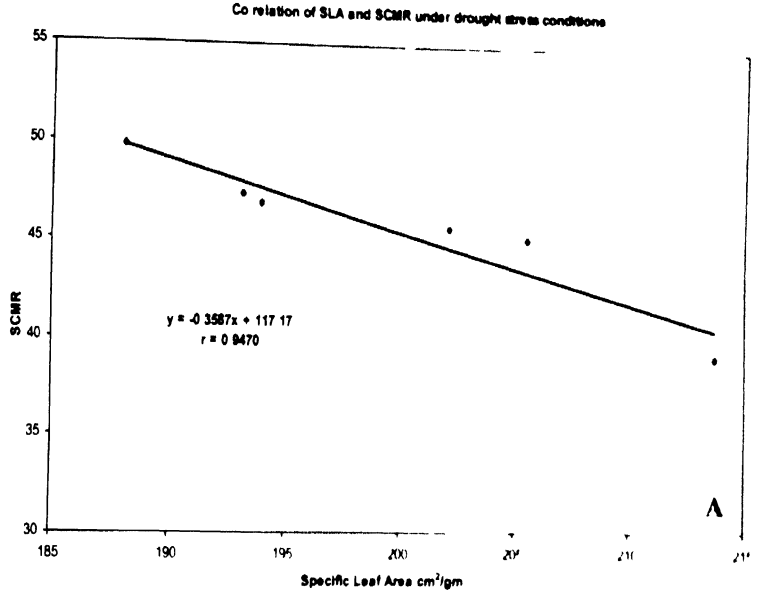


Figure 11A-F. Typical response of NTR to FTSW in transgenic events and untransformed JL 24 under water deficit and respective curve fit.



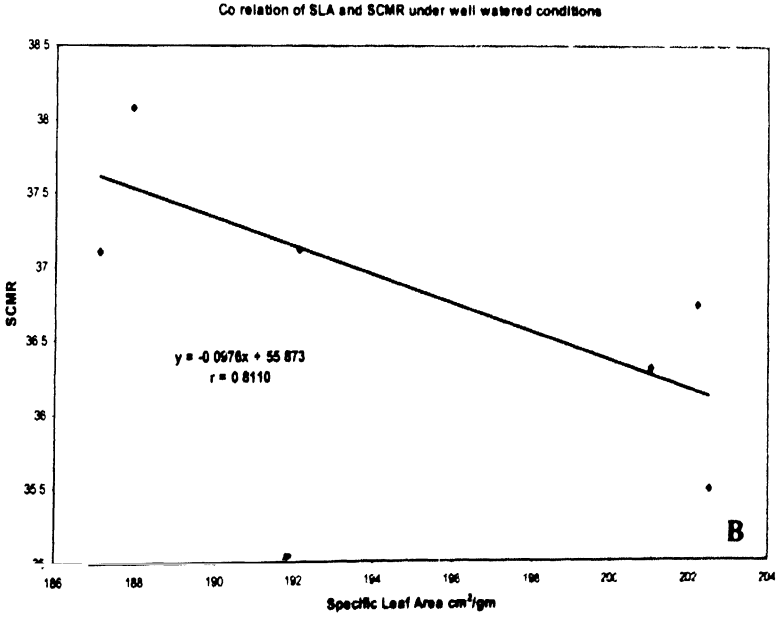


Figure 12A,B. Relationship of SLA and SCMR under well watered and drought stress conditions in the transgenic events and the untransformed JL 24 under drying down cycle.

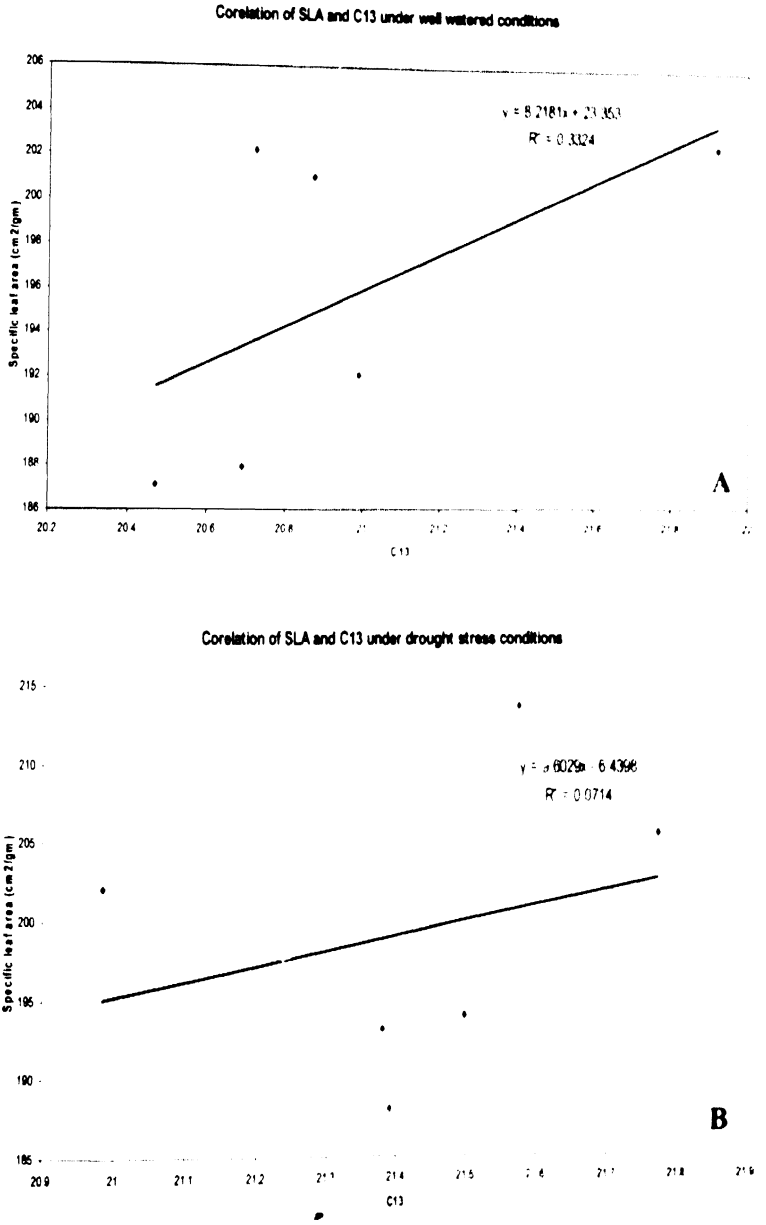


Figure 13A,B. Relationship of SLA and $\Delta^{13}C$ under well watered and drought stress conditions in the transgenic events and the untransformed JL 24 under drying down cycle.

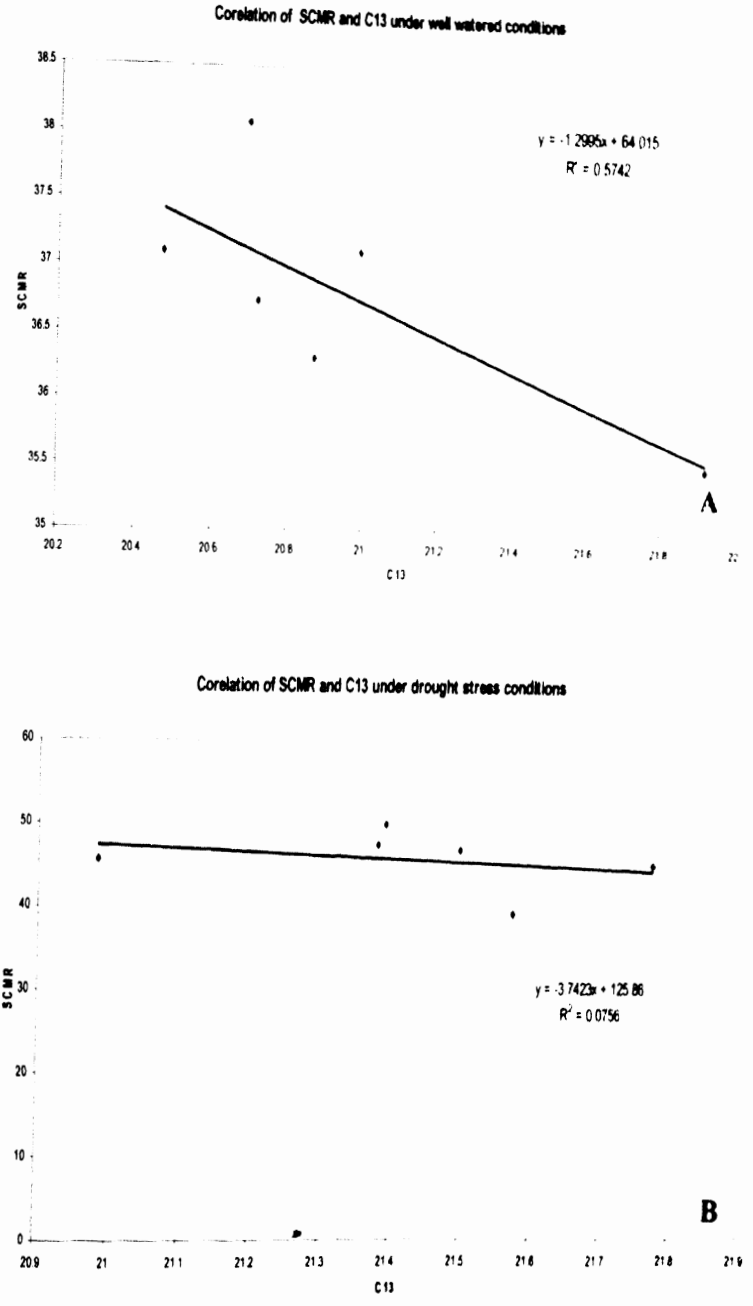
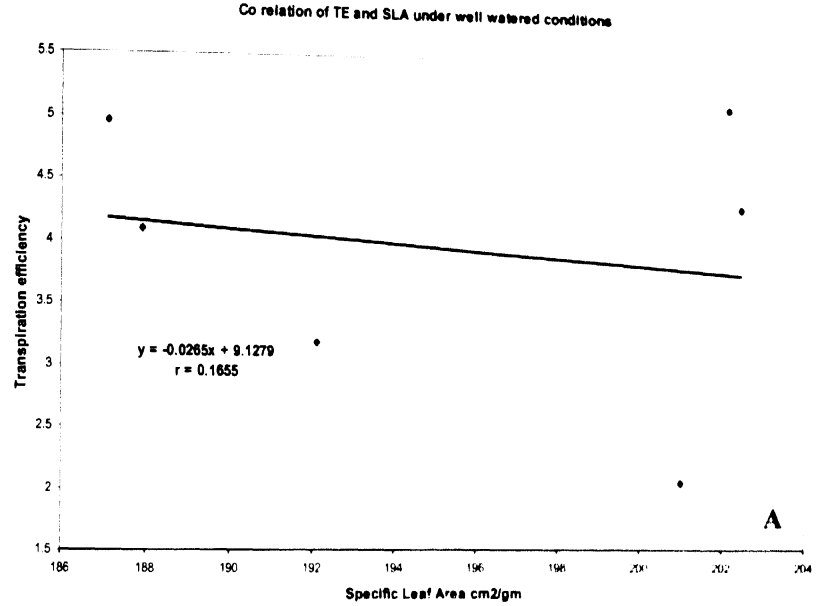


Figure 14A,B. Relationship of SCMR and $\Delta^{13}C$ under well watered and drought stress conditions in the transgenic events and the untransformed JL 24 under drying



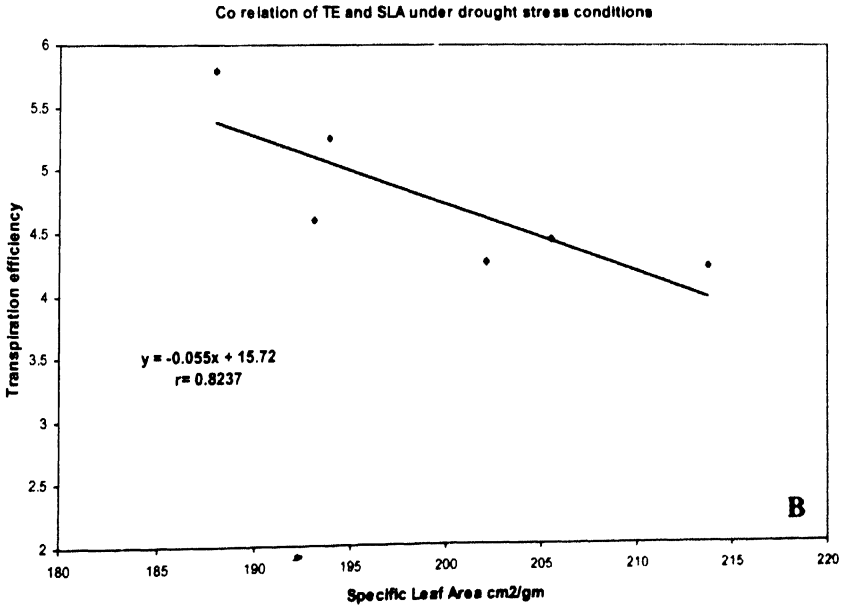
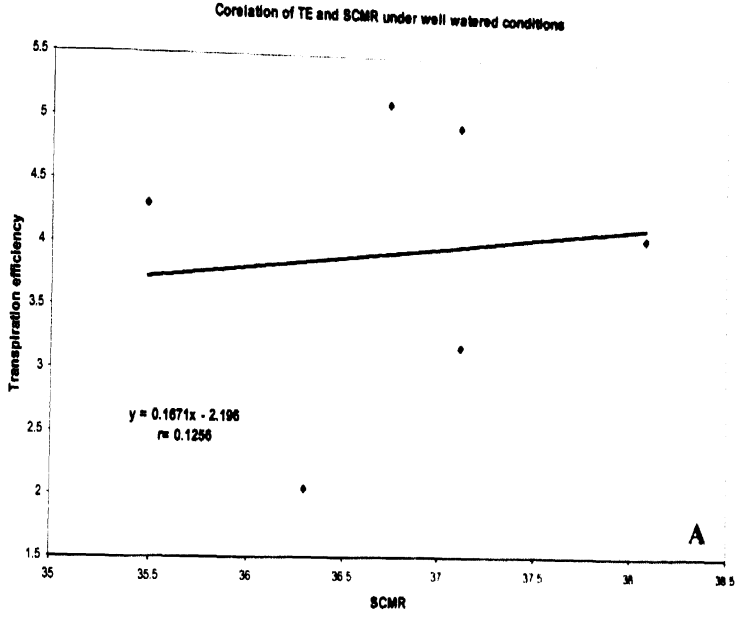


Figure 15A,B. Relationship of TE and SLA under well watered and drought stress conditions in the transgenic events and the untransformed JL 24 under drying down cycle.



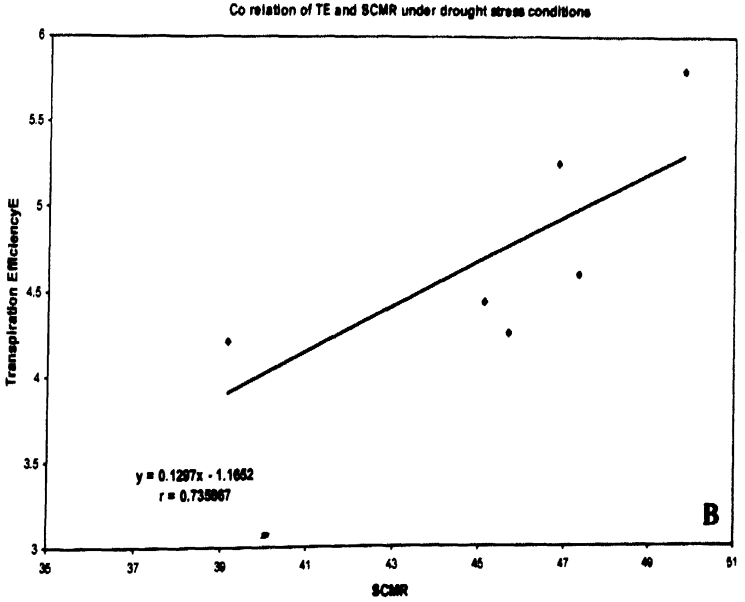
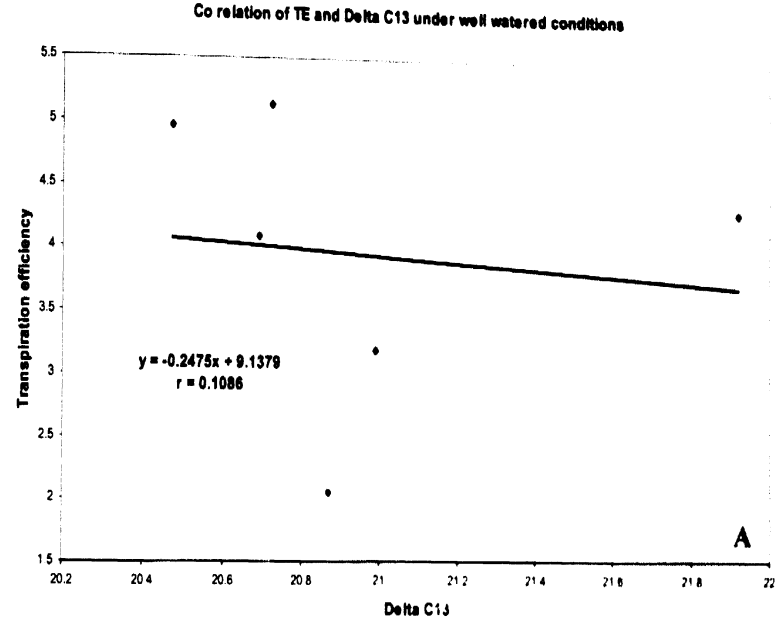


Figure 16A,B. Relationship of TE and SCMR under well watered and drought stress conditions in the transgenic events and the untransformed *JL* 24 under drying down cycle.



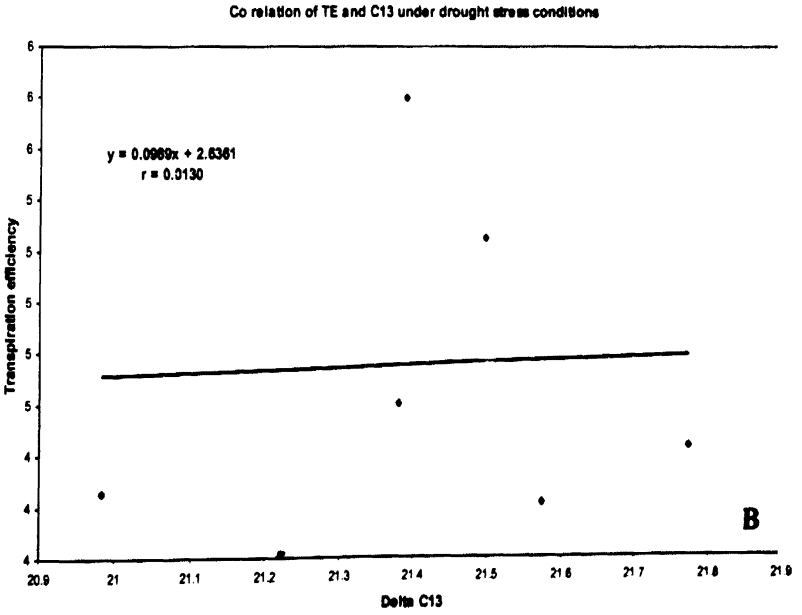


Figure 17A,B. Relationship of TE and $\Delta^{13}C$ under well watered and drought stress conditions in the transgenic events and the untransformed JL 24 under drying cycle.

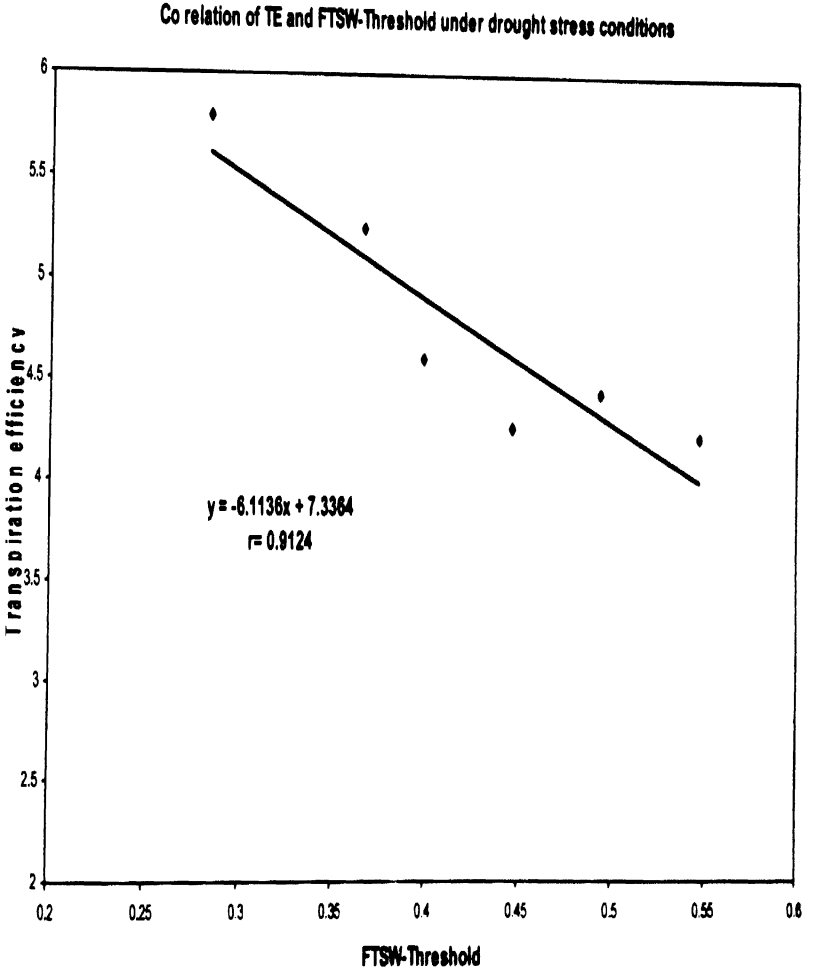
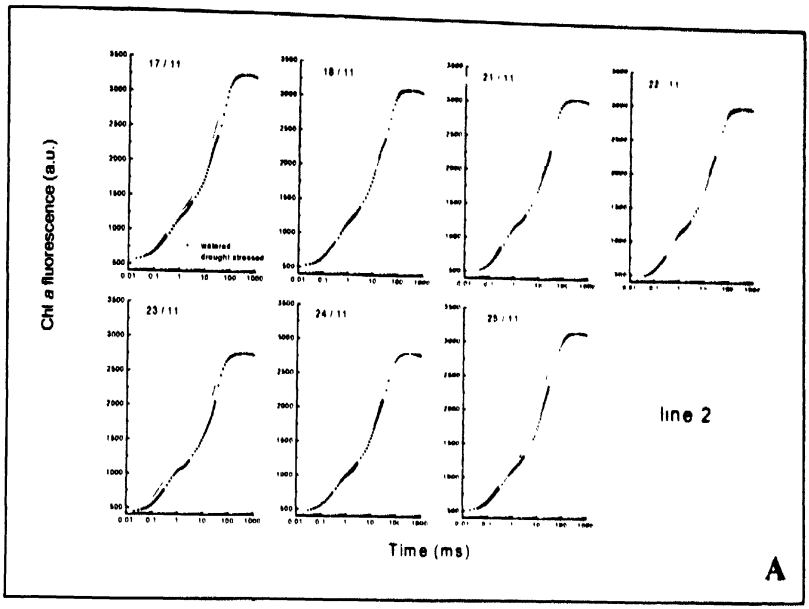


Figure 18. Relationship of TE and FTSW-threshold value under drought stress conditions in the transgenic events and the untransformed JL 24 under the drying cycle.



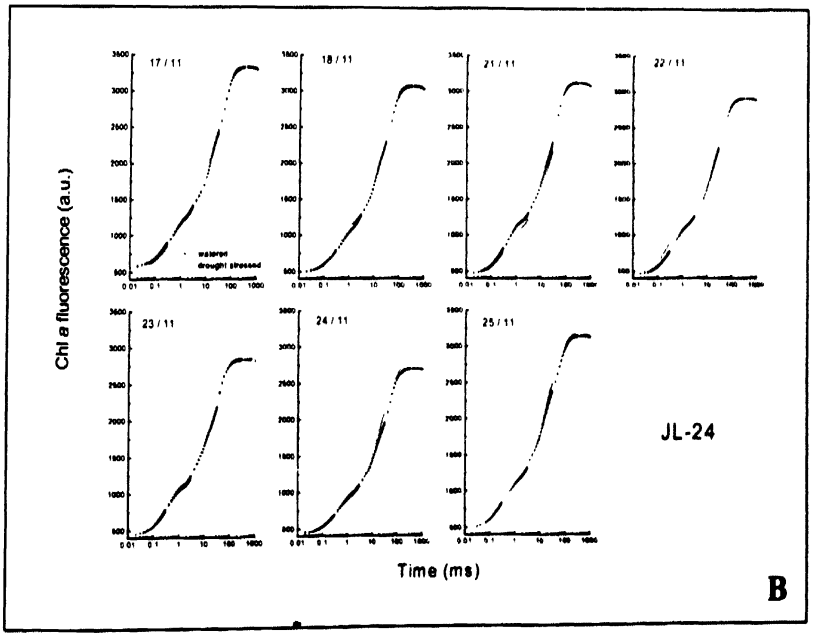


Figure 19A,B. Effect of soil drying on the fluorescence transient of RD 2 and the untransformed JL 24 based on CF F0-J-I-P transient (JIP test).(A) RD2 (B) WT JL 24

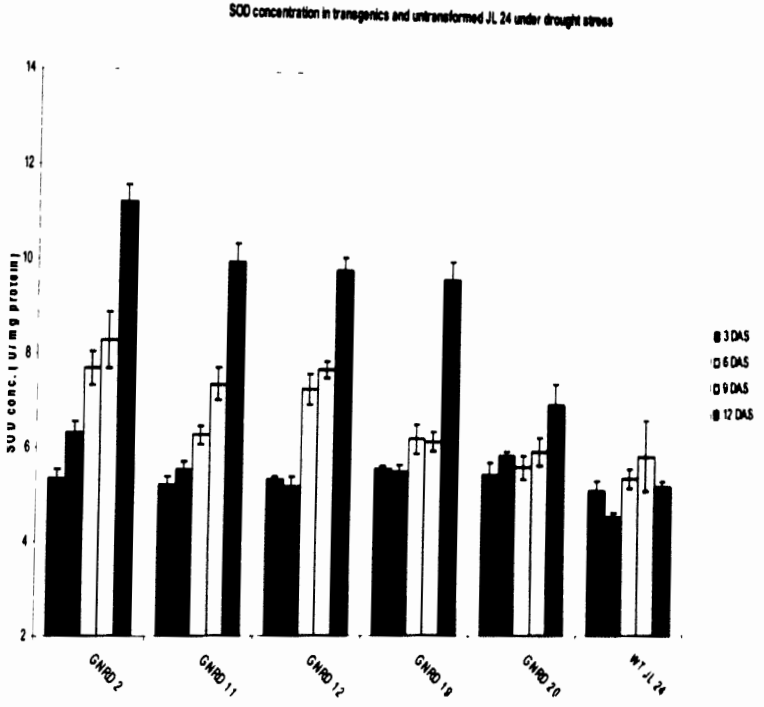


Figure 20. Effect of drought stress conditions on *superoxide dismutase* (SOD) concentration in leaves of transgenic events and untransformed JL 24 under drying down cycle.

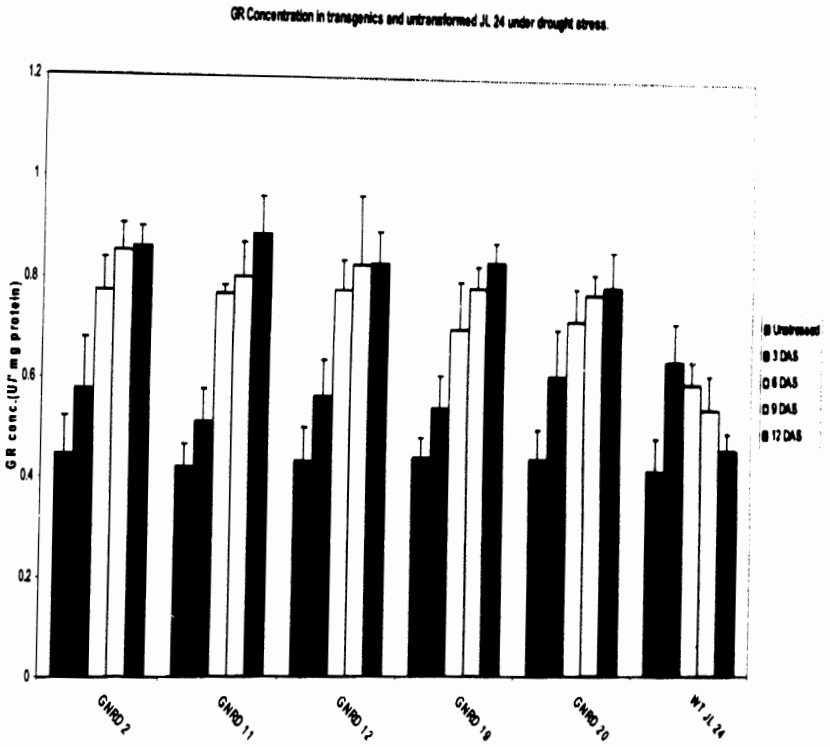


Figure 21. Effect of drought stress conditions on *glutathione reductase* (GR) concentration in leaves of transgenic events and untransformed JL 24 under drying down cycle.

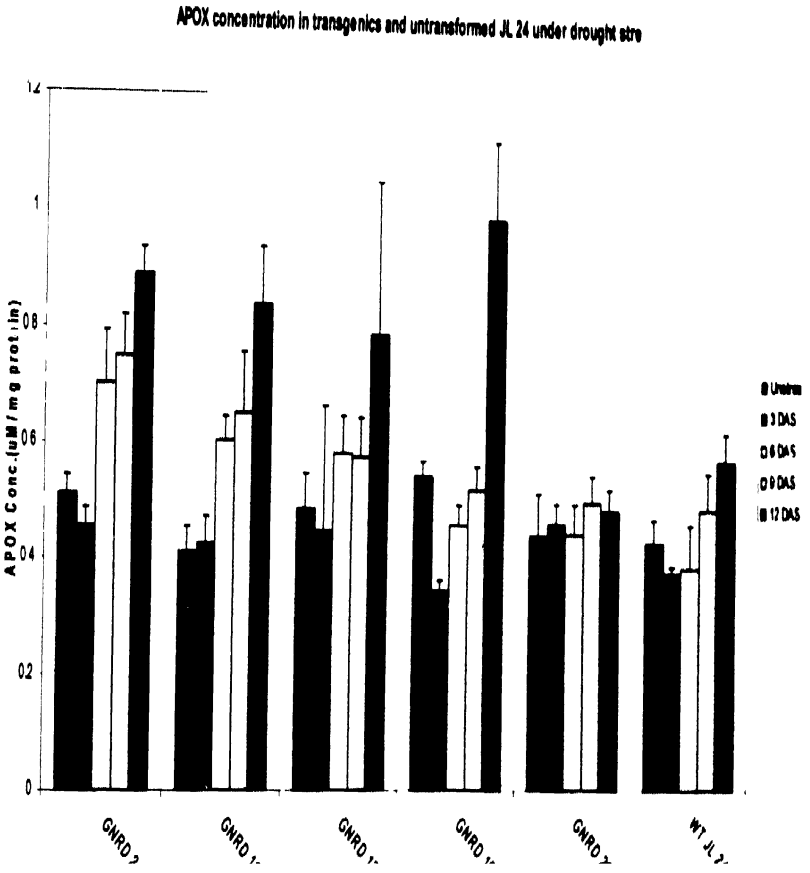


Figure 22. Effect of drought stress conditions on *ascorbate peroxidase* (APOX) concentration in leaves of transgenic events and untransformed JL 24 under drying down cycle.

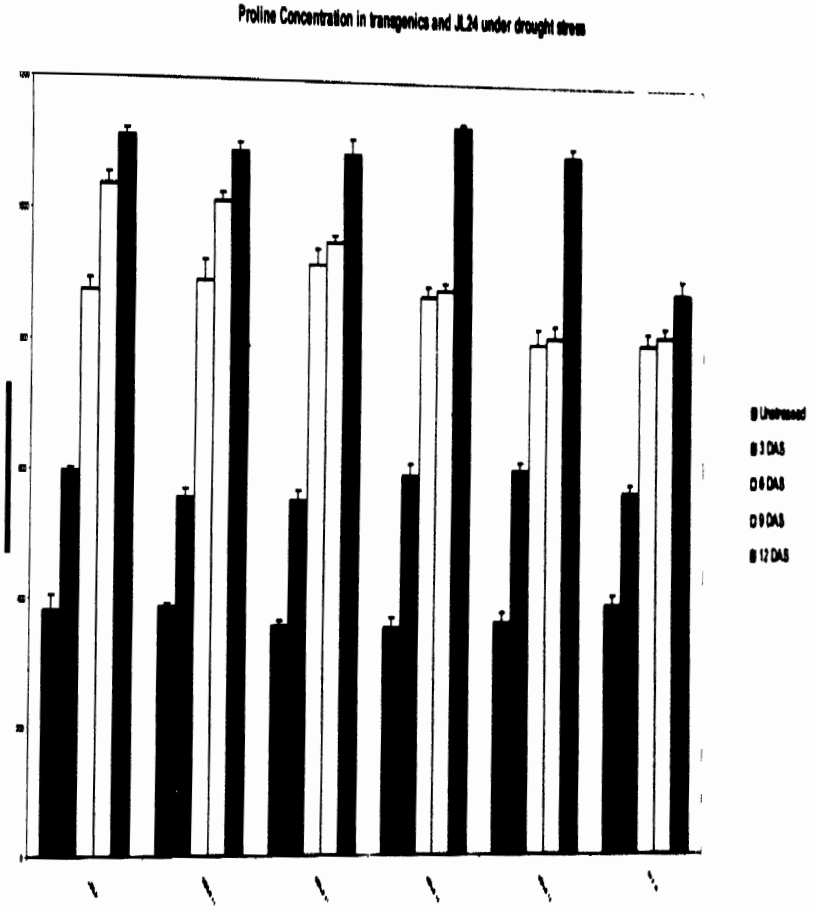


Figure 23. Effect of drought stress conditions on proline concentration in leaves of transgenic events and untransformed JL 24 under drying down cycle.

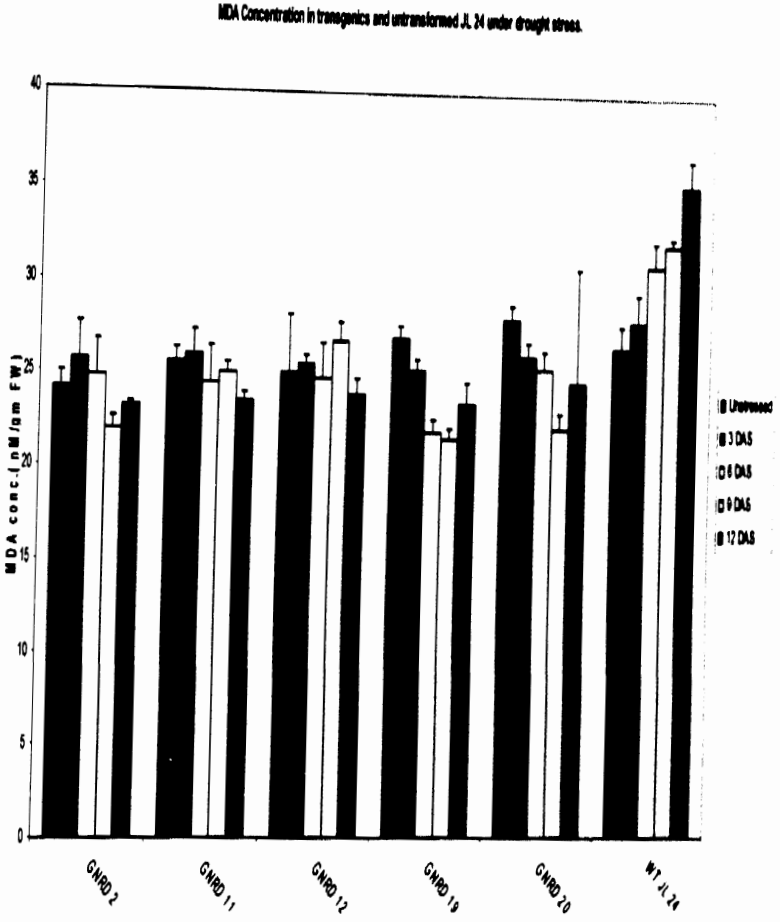


Figure 24. Effect of drought stress conditions on MDA levels in leaves of transgenic events and untransformed JL 24 under drying down cycle.

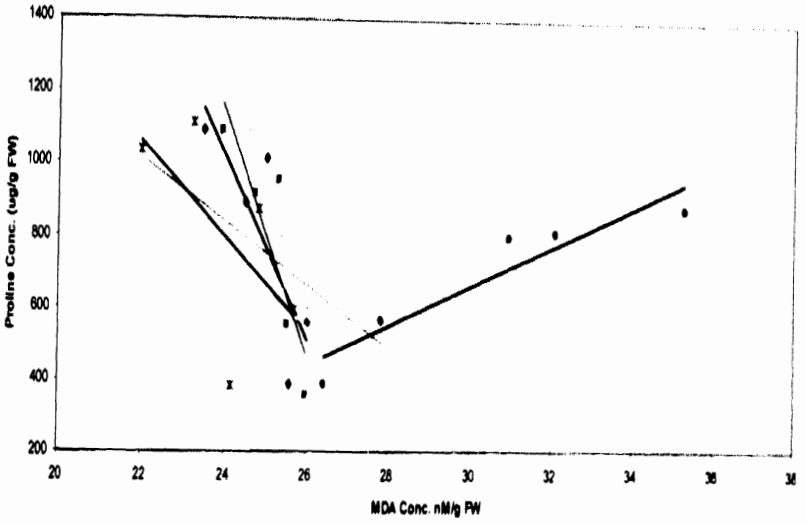


Figure 25. Correlation of proline accumulation and MDA levels under drought stress conditions in the transgenic events and the untransformed JL 24.

APPENDICES

Composition of chemicals and stock solutions for modified MS medium (MMS) (modified from Murashige and Skoog, 1962)

Chemical	Concentration	Stock
MS-Major Salts (50 X)		
*NH_4NO_3	1650	66.0 g 400 ml
*KNO_3	1900	38.0 g 400 ml
*$MgSO_4.7H_2O$	370	17.6 g 400 ml
*KH_2PO_4	170	14.8 g 400 ml
*$CaCl_2$	440	6.8 g 400 ml
***MS-Minor Salts** (100X)		* in 1000 ml
KI	0.83	83 mg
H_3BO_3	6.2	620 mg
$MnSO_4.4H_2O$	22.3	2250 mg
$ZnSO_4.7H_2O$	8.6	860 mg
Na_2MoO_4 $2H_2O$	0.25	25 mg
$CuSO_4.5H_2O$	0.025	2.5 mg
$CoCl_2.6H_2O$	0.025	2.5 mg
***B5 Organics** (100X)		* in 1000 ml
Nicotinic acid	2.0	200 mg
Pyridoxine mono HCl	2.0	200 mg
Thiamine HCl	25.0	2500 mg
***MS- Organics** (100X)		* in 1000 ml
Glycin	2.0	200 mg
Nicotinic acid	0.5	50 mg
Pyridoxine mono HCl	0.1	50 mg
Thiamine HCl	0 5	100 mg
***MS Fe-EDTA (100 X)**		* in 1000 ml
$EDTA.2H_2O$	37.3	3.73 g/l
$FeSO_4.7H_2O$	27.8	2.78 g/l
MS-Inositol (100X)		
*Myo-inositol	100.0	10.0 g/l
Growth regulators		
*BAP	2.25	22.53 mg/ 100 ml
*2,4-D	2.20	22.1 mg / 100 ml
*NAA	1.86	18.6 mg /100 ml
*IAA		

MMS media used at various stages of groundnut regeneration

Amount of stocks* (table) per liter

Item	SIM (36)	SEM (36-2)	RIM
*NH_4NO_3	10 ml	10 ml	10 ml
*KNO_3	20 ml	20 ml	20 ml
*$MgSO_4.7H_20$	10 ml	10 ml	10 ml
*KH_2PO_4	10 ml	10 ml	10 ml
*$CaCl_2$	10 ml	10 ml	10 ml
*MS-Minor	10 ml	10 ml	10 ml
*B5-Organics	10 ml	10 ml	10 ml
*MS- Organics	----	----	----
*MS Fe-EDTA	10 ml	10 ml	10 ml
*MS-Inositol	10 ml	10 ml	10 ml
Sucrose	30 g	30 g	30 g
Agar	8 g	8 g	7.5 g
PH	5.8	5.8	5.8
*BAP	20 ml	2.0 ml	----
*2,4-D	10 ml	----	----
*IAA	----	----	----
*NAA	----	---	5.0 ml

Preparation of chemicals and buffers used in Southern hybridization techniques

1. Composition of DNA extraction buffer

Component	Stock conc	Working conc for 100 ml
Tris	1 M	20 ml
NaCl	5 M	56 ml
EDTA (pH 8.0)	100 mM	40 ml
CTAB	10 %	40 ml
β- mercaptoethanol	0.3 %	300 µl (added just before use)
H_2O	-	40 ml

2. Preparation of Pre-hybridization buffer

- Hybridization buffer (Alkaline phosphatase) - 25 ml
- 0.5 M NaCl - 0.73125 gm
- Blocking reagent - 1 gm
- Add NaCl of 0.73125 gm and blocking reagent of 1 gm to 25 ml of hybridization buffer and keep it for thorough mixing on a magnetic stirrer for 1 hr.

3. Preparation of Primary wash buffer for 500 ml

Chemical	*Chemical to be added for 500ml*
Urea- 2 M	60 gm
SDS-0.1%	500 mg
0.5 M $NaPO_4$ - 50 mM	pH 7 (50 ml)
NaCl- 150 mM	4.32 g
$MgCl_2$ -1M	500 µl
Blocking reagent	1 gm

4. Preparation of 20X Secondary wash buffer for 500 ml

- 1 M Tris base-60.5 gm
- 2 M NaCl-56 gm
- pH set at 10

5. Extraction of RNA through TRIzol method

- Grind the leaf tissue in liquid N_2 (100 mg). Homogenize the tissue in 1 ml of TRIzol reagent.
- Incubate the sample for 5 min at 15 to 30 °C.
- Add 200 µl of chloroform per 1 ml of TRIzol reagent.
- Shake vigorously for 15 sec and incubate at 15 to 30 °C for 2 to 5 min.
- Centrifuge at 14000 rpm for 15 min at 2 to 8 °C till three phases' form.
- Collect the upper aqueous phase (3/4 vol. only) into fresh tubes and precipitate it with 500 µl of isopropanol.
- Incubate the samples at 15 to 30 °C for 10 min and later centrifuge at 14000 rpm for 10 min at 2 to 8 °C.
- Decant the supernatant.
- Wash the pellet with 70% ethanol (1 ml)/ vortex it.
- Spin at 10000 rpm for 5 min at 2 to 8 °C.
- Dry the pellet in vacuum drier for 5 min.
- Dissolve the pellet in RNase free (30 µl) of water.

1. Preparation of chemicals and buffers useful in genetic transformation studies

- 2 N NaOH: Dissolve 8 gm of NaOH in 100 ml of SDW.
- 5 M Potassium acetate (pH 5.2): Dissolve 49.07 gm of Potassium acetate in 100 ml of water. Make up the pH to 5.2 with acetic acid.
- 5 M NaCl: Dissolve 29.22 gm in 100 ml of water
- 0.5 M Sodium phosphate: Dissolve 6.9 gm of Sodium phosphate in 100 ml of water. Adjust the pH to 7 with 5 M HCl
- 1 M Tris: Dissolve 12.1gm of Tris-HCL in 100 ml of water. Adjust the pH to 8 with 1N NaOH
- 100 mM EDTA (pH 8): Dissolve 3.72 gm of EDTA in 100 ml of SDW water. Adjust the pH to 8 with 1N NaOH.
- 10% CTAB: Dissolve 10 gm of CTAB in 100 ml of SDW.
- 10% SDS: Dissolve 10 gm of SDS in 100 ml of SDW.
- 2.5 M $CaCl_2$: Dissolve 36.75gm of $CaCl_2$ in 100 ml of SDW.
- 3 M Sodium acetate (pH 4.8): Dissolve 24.61 of Sodium acetate in 100 ml of SDW. Adjust the pH to 4.8 with acetic acid.

2. Preparation of 50X TAE.

Chemical	Wt in gm	Vol. to be added
Tris-HCL	242	
Na_2 EDTA.$2H_2O$	372.2	
Acetic acid		57.1 ml

Adjust the pH to 8.5; make up the volume to 1L with SDW.

3. Preparation of varied % of ethanol.

Component	Working %	Vol. of ethanol to be added (ml)	Final Volume
Ethanol	10	10	Make up the vol to 100 ml with SDW.
Ethanol	30	30	- do -
Ethanol	50	50	- do -
Ethanol	70	70	- do -

4. Preparation of LB medium (1 L)

Chemical	Wt in gm
Bacto–peptone	10
Yeast extract	5
Sodium chloride (NaCl)	10
Agar	15
pH	7