Summary
VI. SUMMARY

Plant productivity is severely affected by abiotic stresses such as drought, high salinity, high and low temperature. Among the various abiotic stresses, drought is the major factor that limits crop productivity worldwide and in certain areas the yield loss due to drought alone was estimated to be as much as 30% (Zou et al., 2007). Therefore, improving the productivity of crop plants in rain-fed areas is a global agenda. In this direction, identifying the relevant adaptive traits and pyramiding by molecular genetic approaches has been the focus.

The major objective of the present investigation was to develop groundnut transgenics tolerant to drought. The scientific strategy of the study was to develop transgenics expressing transcriptional activator genes to improve cellular tolerance machinery in a genotype with superior water relations. Field level tolerance to drought can be achieved only when two important adaptive traits, cellular level tolerance mechanism and superior water relation traits are pyramided on to a superior genetic background. The approach is initially to identify (i) validated candidate gene to improve cellular level tolerance, (ii) groundnut genotype with superior water relations and (iii) finally standardize transformation protocols to develop transgenics to pyramid these traits.

**Robust transformation protocol for the development of transgenics in groundnut**

For successful development of transgenics an efficient transformation protocol was developed. The crop of choice, groundnut is a legume and hence a difficult to regenerate crop. As an alternative, tissue culture independent transformation protocols are advantageous. In this direction, *in planta* transformation was standardized in groundnut using *EPSPS* as the selectable marker gene. The procedure involved co-cultivation of differentiated embryo of the germinating seeds with *A. tumefaciens* carrying the transgene. Since the primary selection of putative transformants is done in T1 in *in planta* transformation technique, an efficient screening protocol with a suitable selectable marker was adapted in this study. Over expression of the *EPSPS* gene makes the plant tolerant to glyphosate. Hence, glyphosate was used to screen transgenics harboring *EPSPS* gene by
leaf swabbing bioassay method. Based on the integration and efficacy of the transgenes, transformants were identified in T₁ and T₂ generations.

In the present study, the stability of EPSPS transgenics to glyphosate tolerance was assessed in T₃ and T₄ generation. The transgenic plants in both generations were PCR positives and showed relative tolerance 1000 ppm glyphosate. Some of them were tolerant up to 3000 ppm and retained higher chlorophyll content after treatment. The selected transgenic plants showed higher EPSPS transcript accumulation compared to wild type, confirming the stability of the transgene and its expression, even in the advanced generations. This infers the transformability of groundnut by in planta transformation protocol and utility of EPSPS as a marker gene, to screen the transformants.

**DEAD box helicases, possible candidate genes to improve cellular level tolerance**

The aim of the study was to isolate stress responsive helicases and characterize their relevance in drought stress tolerance. A stress responsive DEAD box RNA helicase (Ec-helicase) was identified from an existing finger millet cDNA library. BLAST and multiple sequence alignment showed that the Ec-helicase was only partial. Full length sequence of any gene is needed for its characterization. So, attempts were made to clone full length 3' and 5' ends of the selected stress responsive gene. Initially, 3' RACE was carried out using Gene Racer™ cloning kit and an additional 194 bp fragment of Ec-helicase was cloned from finger millet. 5' end needs to be cloned to construct the full length Ec-helicase. Phylogenetic analysis revealed homology of Ec-helicase with Pea DNA Helicase (PDH45). Hence, PDH45 was out sourced from ICGEB, New Delhi and used for the development of transgenics in groundnut for improving cellular level tolerance in the selected groundnut genotype K-134, with superior water relation traits.

**Groundnut PDH45 transgenics showed significant phenotypic variability**

Transgenic groundnut plants expressing PDH45 gene were developed following Agrobacterium mediated in planta transformation. The T₁ transformants were characterized initially for phenotype variation and selected transgenics were advanced based on molecular characterization and stress response besides productivity under stress.
Expression of regulatory proteins brings about considerable change in phenotype (Hsieh et al., 2002; Haake et al., 2002; Dubouzet et al., 2003; Kasuga et al., 2004; Ito et al., 2006; Bhatnagar-Mathur et al., 2007; Cong et al., 2008). Even PDH45 helicase groundnut transgenics showed significant variability, ranging from spreading plants, tall plants, stay green type and production of three seeded pods. H-15 and H-19 showed three seeded pods.

One of the striking observations was, some of the transgenic lines showed stay green phenotype. Three promising lines, H-5, H-8 and H-19 showed the visual stay green phenotype till the end of the crop period. One of the associated characters to stay green nature is chlorophyll stability. Etherel and stress induced chlorophyll degradation was significantly less in transgenic lines, especially those which showed stay green character. This evidenced the stay green nature of these transgenic lines.

Besides, stay green nature, Water Use Efficiency (WUE), is an important measure of drought tolerance. WUE is a desirable physiological adaptive trait, when difference in this parameter is brought about by the variation in mesophyll efficiency, i.e., chloroplast carbon assimilation efficiency. Stay green nature and chlorophyll stability, observed in some of the transgenic lines might have improved the mesophyll efficiency. The WUE as measured by Δ^{13}C, a surrogate for this trait, was significantly higher in some of the transgenic lines viz., H-5, H-8, H-15 and H-19.

**Molecular characterization confirmed the stable integration and expression**

The integration, stability and expression of the transgene were assessed in all the generations by molecular analysis. Stability and integration of the PDH45 gene was confirmed by PCR using different set of primers amplifying the 827 bp 35s promoter-PDH45 fragment in selected plants of all the generations. Nested PCR resulting in 483 bp fragment further confirmed PDH45 gene integration. Besides integration of PDH45 gene, stability and integration of selectable marker genes was also confirmed by PCR using gene specific hptII and uidA primers amplifying 500 bp and 750 bp respectively. Further, the authenticity of the PCR product was confirmed by restriction digestion analysis and
Summary

sequencing of the PCR product. Sequence analysis of the cloned PCR product showed the
100% homology with the existing PDH45 sequence.

The integration and copy number of transgene was assessed by the genomic
southern analysis. Most of the selected plants integrated the T-DNA once. Semi-
quantitative RT-PCR analysis of the selected transgenic plants showed enhanced
accumulation of PDH45 transgene (PDH45) as against wild type plants.

Transgenic lines showed improved stress tolerance and productivity under stress

The stress response of the transgenic lines was assessed under water deficit stress.
Membrane damage to groundnut transgenic lines under water deficit stress was assessed.
The transgenic lines showed lesser membrane leakage compared to wild type, indicating a
lesser stress induced membrane damage. The relative tolerance of transgenics to salt stress
was analyzed by performing leaf disc bioassay at 300mM NaCl and the chlorophyll
content of the leaf discs was quantified after treatment. The chlorophyll content was more
in the transgenic lines compared to wild type, inferring the tolerance of transgenics to salt
stress.

Transgenics were assessed for the root growth response under water deficit stress.
The root volume of the transgenic plants grown in different soil moisture regimes, viz.,
moderate and severe stress was measured. The transgenic lines showed improved root
growth under desiccation stress compared to wild type. The transgenic seedlings also
showed improved growth under PEG induced osmotic stress compared to wild type.
Stress response of the transgenic lines shows the ability of transgenic lines to maintain
intrinsic cellular level tolerance.

Based on the stability of integration, expression and stress response, 8 promising
events from 6 T1 back grounds were selected at the end of T5 generation. The promising
PDH45 transgenic lines H-5, H-8 and H-19 showed desirable phenotype like, spreading
type, stay green nature and three seeded pods. H-10, H-11and H-15 were tall plants
showing improved productivity. The selected transgenic lines showed improved yield
compared to wild type under both moderate and severe stress. Percent increase in the
average yield of the transgenic lines over wild type ranged from 10.4-37.5. Transgenics showed 26.75% increase in yield over wild type under moderate stress and severe stress it was 17.2%.

The transgenic line H-19 was identified as a promising line, when scored for the performance in various physiological screens and yield under water deficit stress. Besides the better performance and yield, it showed stay green phenotype with three seeded pods. It also showed improved water use efficiency.

**Outcome**

The study therefore demonstrated the feasibility of *in planta* transformation in groundnut to generate stable transformants. This technique has potential in transgenic technology in groundnut to express gene of interest in desired genotype. Our study provided evidences of the stress specific *helicases* like *PDH45* as a potential candidate gene to improve cellular level tolerance.

The significant outcome of this study is to provide proof of concept that drought tolerance at the field level, can be achieved by pyramiding the cellular level tolerance traits and most relevant water relation traits like water mining and water use efficiency. In this direction, transgenics is a potential option for gene pyramiding.