Discussion
Oilseed crops play an important role in the national economy and they rank second in importance after food grains. The oil content of oilseeds ranges from about 20 per cent for soybean to over 40-55 per cent for groundnut. Groundnut (*Arachis hypogaea* L), a legume, is a major edible oilseed crop of importance at the global level. Groundnut seeds contain 40-55% oil and 22-30% protein on a dry seed basis. In addition, they are a good source of minerals (phosphorus, calcium, magnesium and potassium) and vitamins (E, K and B group) (Dwivedi *et al*., 1993). Productivity and quality of groundnut is severely affected by various biotic and abiotic factors.

Moisture stress during the crop growth period, account for about 30 to 70 per cent loss in productivity in the country. In India as in many other parts of semi-arid regions of the world, 78 per cent of the area under agriculture is rainfed and is inescapably linked to the vagaries of the monsoon.

Groundnut is a rainfed crop and its productivity is constrained by moisture stress the crop experiences in most of the cultivated areas (Awal and Ikeda 2002, Reddy *et al*., 2003). Inspite of its wider adaptability under intermittent moisture stress conditions, the realized yields are still substantially low. It is demonstrated that groundnut has low water mining activity with relatively low root to shoot ratios. However, stomatal conductance under controlled stress conditions suggests that groundnut has reasonably higher water conservation ability (Craufurad *et al*., 2000). Further, comparative empirical assessment across a few species based on survival and recovery showed that groundnut has moderate level of stress tolerance.

Drought being a very complex abiotic stress, efforts in breeding to develop crop varieties tolerant to moisture stress has only been partially successful. Plants have evolved diverse adaptive strategies to cope with water-limited environments. These mechanisms range from cellular level tolerance leading to adaptation in response to stress and/or specific growth behaviors (inherent traits) that avoid stress effects (Bray, 1997; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005). The tolerance mechanisms that sustain growth under water limited environments need greater attention. Therefore, the
Discussion

key to progress towards breeding for drought tolerant crops requires better characterization of the stress environments and understanding the stress response of crop plants to identify the relevant drought adaptive mechanisms and/or traits.

Drought tolerance can be improved only by bringing together the relevant drought tolerance mechanisms and many being multigenic, the approach has been to identify genotypes with desirable drought traits, genes and QTLs that impart drought tolerance mechanisms and finally pyramid them by molecular approaches. When crops experience intermittent or residual moisture stress, besides the crop phenology; reproductive biology, partitioning, and remobilization the primary traits of importance are those associated with plant water relations. The importance of total transpiration and hence water mining and the water use efficiency is now well elucidated (Vadez et al., 2007). These two physiological process largely drive the crop growth not only under non stress condition but also when crop experiencing moisture stress. From this context, only potion to pyramid these traits is by either exploiting the QTL (markers) regulating these traits or use genotypes with these traits as trait donor parents. There are convincing evidences that field level tolerance and improved productivity can be achieved through efficient water mining and improved use efficiency (Richards et al., 2002; Condon et al., 2003; Sheshshayee et al., 2003). The molecular mechanisms and the genes that are associated with these complex constitutive traits associated with water mining and WUE are poorly elucidated.

In drought prone rainfed environments despite water mining and water conservation mechanisms, plants experience stress when transpiration exceeds the uptake. Besides, desiccation stress is common during seedling establishment. Under these conditions cellular tolerance mechanisms (CLT), which brings about alteration in cell metabolism for adaptation assumes significance. CLT is believed to be very critical to maintain cell metabolism under stress. The CLT broadly involve ionic/osmotic homeostasis, anti-oxidant scavenging (damage/injury control), protein turnover, protein folding/unfolding and regulation of cell cycle. In addition, sustaining protein synthesis and subsequent structural conformation, is critical to maintain the metabolic activities. In the direction, there has been increasing research efforts to identify the genes, which
modulate the process of CLT. Modern biological tools and molecular techniques has been useful in understanding and profiling the stress-induced transcriptome to characterize the genes that regulate the acquired tolerance mechanisms.

To date, there has been a skewed emphasis on characterizing the stress transcriptome and validating the functional significance of stress responsive genes. Unlike the initial focus on validating a few functional genes regulating different metabolic pathways, recent approach has been on characterization of regulatory genes identified through stress transcriptome profiling. Many stress specific transcription factors have been identified and their role in plant drought response has been characterized (Vinocur and Altman, 2005; Bartels and Sunkar, 2005). Transcription factors have proven quite useful in improving stress tolerance in transgenic plants, through co-ordinated expression of a number of stress-related target genes (Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005).

In CLT, protection offered by molecular chaperons (HSps/CSps) is vital (Diamant et al., 2001). Similarly, dehydrins/LEAs are yet another class of proteins that play a role in protein stability (Vinocour et al., 2005). Many stress transcriptional regulators bZIP, NAC, members of AP2/ERF (DREBs, HARDY), Zn-finger, NF-Y and WRKY) known to induce the production of compatible amino acids and over-expression of chaperons like small HSPs, ER luminal binding proteins (BiPs, a member of HSP70) hence relevant in imparting stress tolerance (Chen et al., 2002; Karaba et al., 2007; Nelson et al., 2007). Helicases play a pivotal role in stress management in plants. DNA and RNA helicases are known to be called as molecular motors and are involved in many of the cellular processes for protein turn over and protection. These helicases are involved in translational initiation regulation like eIF4A (Svitkin et al., 2001), 4EBP and S6Kinase has been elucidated with probable improvement in transcription and translation process. Seki et al., (2001) reported that in Arabidopsis, helicase gene was induced by cold treatment, suggesting that helicases might play a role in plant stress signaling. The scientific strategy of this study was to adapt a transgenic approach to introgress traits associated with water relations and mechanisms involved in CLT. Several mechanisms for the involvement of helicases in plant stress tolerance have been proposed, such as their participation in mRNA transport,
secondary structure removal (Gong et al., 2005; Vashisht et al., 2005), regulation of gene expression (Pham et al., 2000; Gong et al., 2002; Sanan-Mishra et al., 2005), and enhancing or stabilizing protein synthesis (Vashisht et al., 2005). Recently, two stress responsive helicases, OsBIRH1 (Li et al., 2008) and MH1 (Luo et al., 2008) are cloned and validated.

With this goal, the research emphasis was to identify trait donor genotypes with superior water mining and WUE and develop transformation protocols, subsequently develop transgenics with the validated upstream genes that might improve cellular level tolerance.

Towards this direction, the results obtained are discussed as,

1. Developing in planta transformation protocol in groundnut using EPSPS gene as a selectable marker.
2. Cloning and characterization of a stress responsive helicase from finger millet.

**Development of in planta transformation protocol in groundnut using EPSPS gene as a selectable marker**

The success in the generation of transformants lies with the regeneration response. This hampered the improvement of some of the crops that are called, 'recalcitrant'. These crops are not amenable to tissue culture. However, the advent of non-tissue culture methods of transformations in Arabidopsis changed the scenario of transgenic technology (Bent, 2006).

Groundnut, being a legume is also less amenable to tissue culture. Still, efforts have been made towards development of regeneration protocols. However, there are limited reports of efficient rooting and establishment of plants in the green house. Probably this was the reason for limited progress in genetic transformation of groundnut. However, few reports published on transgenic groundnut plants were using particle bombardment (Deng, Wei and An, 2001) Agrobacterium-mediated transformation (Bhatnagar- Mathur et al., 2004). The frequency of transformation was less and often
subsequent rooting was a major constraint. In this direction, development of a transformation protocol that avoids tissue culture steps is an alternative. Yet, there are reports of agronomically important traits being introduced into groundnut and \textit{AtDREB1A} gene was stably integrated and evaluated (Bhatnagar-Mathur \textit{et al.}, 2007). Still, serious efforts are needed to explore regeneration and transformation protocols to engineer agronomically desirable genes in groundnut. In this direction, development of a transformation protocol that avoids or minimizes tissue culture is beneficial.

The efficiency of transformation is genotype dependent. Our earlier studies suggests that the groundnut genotypes are not readily amenable for transformation. In this view, development of genotype-independent transformation protocols is difficult-to regenerate species has significant practical relevance. Transformation techniques that evade tissue culture (Graves and Goldman, 1986) therefore become important in crops like groundnut. In the present study, a tissue culture-independent \textit{in planta} transformation protocol, earlier developed, was used to develop transformants (Sankara Rao and Rohini, 1999; Rohini and Sankara Rao, 2000a; Rohini and Sankara Rao, 2000b; Rohini and Sankara Rao, 2001, Keshamma \textit{et al.}, 2008, and Manoj \textit{et al.}, 2009). Such \textit{in planta} transformation techniques have also been standardized in other crops like, \textit{Arabidopsis thaliana} (Feldmann and Marks, 1987), buckwheat (Kojima \textit{et al.}, 2000), mulberry (Ping \textit{et al.}, 2003), kenaf (Kojima \textit{et al.}, 2004), soybean (Chee \textit{et al.}, 1989) rice (Supartana \textit{et al.}, 2005), tomato (Abida Yasmeen \textit{et al.}, 2008) etc. In the present method, \textit{Agrobacterium} is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, \textit{Agrobacterium tumefaciens} transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary transformants (T$_0$) being chimeric in nature. This is the reason for the analysis of the transgenic plants to be carried out in the T$_1$ generation. The chimeric plants producing the stable transformants in the T$_1$ generation depends on the type of cells that were transformed in the T$_0$ plants. If the transgene is integrated into undifferentiated meristematic cells which are destined to develop branches, seeds obtained from these reproductive structures of these branches would produce stable transformants in T$_1$. Therefore, this requires generation of a large number of T$_0$ plants which would eventually give rise to a larger number of T$_1$ plants.
This requires a stringent screening of the T<sub>1</sub> generation plants for the selection of not only putative transformants but also those that are high expressing.

Different methods of screening are being followed for the evaluation of T<sub>1</sub> generation plants. In the in planta transformation strategies, often, the T<sub>1</sub> seeds are germinated in agar media with selectable marker at levels that are lethal to the wild type (Feldmann and Marks, 1987). Such screening approaches are amenable with small seeded crops. Often, longer duration of exposure results into etiolated seedlings since the light intensities under in vitro conditions are often low. Further, such seedlings show high percent mortality during subsequent hardening under green house conditions. Alternate options to screen the autotrophic T<sub>1</sub> plants raised in the green house condition needs to be explored. One of the options is to screen for the expression of the transgene preferably by immunoassays or by assessing the bioefficacy of the transformants depending on the nature of the transgene. From this context, a herbicide selectable marker has specific advantage to screen the T<sub>1</sub> plants generated by in planta transformation protocol.

Glyphosate (N-phosphonomethylglycine) is a non-selective herbicide, capable of inhibiting growth of a broad range of crops and weeds. Glyphosate interferes in the shikimate metabolic pathway by inhibiting synthesis of 5-enolpyruvyl-3-phosphoshikimate (EPSP) by competitively inhibiting EPSPS. This prevents the subsequent synthesis of the aromatic amino acids (Herman and Weaver 1999).

A number of EPSPS genes of plant and bacterial origin have been used as plant selectable markers (Howe et al., 2002). The CP4 gene and glyphosate insensitive plant EPSPS genes have been used as selectable markers in several studies (Zhou et al., 1995; Clement et al., 2000; Howe et al., 2002; Zhao et al., 2006). One of the most successful gene was isolated from Agrobacterium sp. Strain CP4, which encodes an EPSPS enzyme with an extremely high Ki for glyphosate and low Km for the substrate PEP (Padgette et al., 1996). The EPSPS gene in the present study was codon optimized for plant species, custom synthesized and used for the standardization of in planta transformation protocol in groundnut. The EPSPS transgenics developed earlier in the Department of Crop physiology, UAS, Bangalore, was further characterized in this study to explain the stability of transgene across generations and finally came out with an in planta
transformation protocol in groundnut. The stability of tolerance to 1000 ppm glyphosate was assessed successfully upon swabbing the T$_3$ plants developed in green house (Fig. 15). Repeated screening of the transformants allowed identification of stable transformants. Molecular analysis of all the T$_3$ generation plants confirmed the stable integration and expression of EPSPS in groundnut, indicating robustness of the protocol to develop and identify transformants.

Further, the stability and effectiveness of the EPSPS gene in the T$_4$ generation plants was also confirmed in this study. PCR analysis of the T$_4$ generation plants using EPSPS primers amplified a 500 bp fragment confirming the stable inheritance of the transgene in the selected plants. Besides transgene integration, expression of the transgenes EPSPS and nptII was confirmed by RT-PCR. Transcript accumulation of EPSPS gene was more in the transgenic plants compared to wild type (Plate 3). The plants exhibited tolerance to 1000 ppm and some up to 3000 ppm glyphosate when swabbed (Fig. 17). Bio-efficacy of EPSPS transgenics showed varied response to glyphosate though all the plants were PCR positives. Hence the approach provides an option not only to identify the transformants, but also the events with differential expression.

The present investigation, clearly demonstrated the transformability of groundnut and the stability of integration using the in planta transformation protocol and utility of EPSPS as the selectable marker for efficient selection of the putatative transformants using glyphosate.

**Cloning and characterization of a stress responsive helicase from finger millet**

On the onset of drought stress, a range of cellular responses are initiated which can lead to the alteration of the cellular environment thereby imparting plant tolerance. Cellular level tolerance (CLT) can be brought in by osmotic homeostasis, scavenging cytotoxic compounds, protein protection, protein turnover and protein folding and unfolding. Sustaining protein synthesis and structural confirmation of proteins is one of the important mechanisms to improve cellular level tolerance. In these mechanisms, transcription activators and chaperones play an important role. In this direction, transcription activators that maintain the protein turnover has been the major focus. The
genes encoding DEAD-box helicases are reported to play a key role in various abiotic stresses, including temperature, light, oxygen, and salt stress (Liu et al., 2008).

Helicases are the molecular motors that transiently catalyse the unwinding of nucleic acids. They are also involved in unfolding the secondary structures of RNA, transcription, translation, ribosome biogenesis, splicing, RNA transport, etc (Cordin et al., 2006). Nucleic acid metabolism is the sensitive to abiotic stress (Vashisht et al., 2005). However, the molecular targets responsible for this sensitivity need to be demonstrated. Various stress responsive helicases have been reported in plants. Pea DNA helicase 47 (PDH47) is a stress responsive helicase, induced by cold (4°C) and salt (300mM) (Vashisht et al., 2005). A salt-responsive helicase, AvDH1, was isolated from the halophyte, Apocynum venetum. It is induced in response to NaCl stress (400mM) and low temperature (4°C). MH1 when expressed in Arabidopsis showed improved tolerance to drought, salt and oxidative stress (Luo et al., 2008). Similarly, OsBIRH1 has been showed to enhanced oxidative stress and disease tolerance in Arabidopsis (Li et al., 2008). Hence, it is beneficial to identify, isolate and validate the stress responsive helicase from the stress adapted species. In the present study, cloning of an RNA helicase has been attempted from finger millet.

The main aim has been to follow transgenics as an approach to bring about drought tolerance. Helicases are transcription activators and based on the cellular process they regulate, they are called as DNA and RNA helicases. RNA helicases unfold the secondary structures in RNA and are involved in transcription ribosome biogenesis and translation initiation. The present study envisaged the cloning of a RNA helicase (Ec-helicase) from the stress specific library of Eleucine coracana. L. The cloning of full length gene was based on the identification of a 807 bp RNA helicase EST which shared 94% homology with rice helicase. A phylogenetic tree was constructed with the published and validated helicases. Ec-helicase share homology with most of the validated helicases. So the major emphasis was to construct full length gene of a stress responsive DEAD box RNA helicase clone identified from the subtracted cDNA library of finger millet. The initial sequence of the EST was 801 bp and the predicted full length sequence was approximately 2 Kb.
Various strategies are being utilized in different full length cDNA SMART utilizes the Power script Reverse Transcriptase property of terminal transferase activity ie it adds ‘c’ residues when the RT reaches the end of mRNA. Secoisolariciresinol dehydrogenase (Lan, et al., 2010) and HDR gene (Chen et al., 2010) were cloned using SMART™ RACE cDNA Amplification Kit (Clontech, USA). Though the methods have produced successful results they have their own disadvantages.

The method followed in the present investigation was by using Gene Racer™ Kit from Invitrogen which follows the basic principle of RACE PCR. 3’ 5’ RACE is commonly used to isolate the 3’ and 5’ end of the cDNA clone. As a continuation of the 801 bp, towards the 3’ end, 194 bp fragment was successfully cloned. 5’ end of Ec-helicase was also cloned and sequenced. But the sequence did not match the related helicase sequence. However, further cloning of 5’ end is in progress. A phylogenetic tree was constructed for the partial sequence of Ec-helicase with the cloned 3’ end additional sequence with the other published and validated helicases. It revealed the homology of EC-helicase with PDH45 (Fig. 6).

In view of this, Pea DNA Helicase 45 (PDH45), which was validated earlier, was out sourced from ICGEB, New Delhi, and used for the development of transgenics in groundnut for improving cellular level tolerance in the selected genotype K-134, which has superior water relation traits.

**Development and analysis of PDH45 transgenics**

PDH45, a pea DNA helicase, homologous to the eukaryotic initiation factor eIF4A was found to be up regulated in response to abiotic stress (Sanan-Mishra et al., 2005). The envisaged approach was therefore to introduce the stress responsive DNA helicase into the background of a genotype with superior water relation traits (roots, WUE). Phenotyping groundnut genotypes for drought adaptive traits identified K-134 as a genotype as a moderate root type and WUE. Therefore, K-134 was selected as the recipient genotype and transgenics with PDH45 helicase were developed following the in planta transformation protocol. As explained earlier, because of the reason that only a sector of T₀ transformants may contain T-DNA resulting in chimeric shoots (Mc Kently et al., 1995), transformants were analyzed in T₁ generation plants. The strategy for the
selection of promising transformants was based on integration of the transformed gene, desirable phenotype, response to stress and productivity under stress.

**Analysis of the transformants over expressing PDH45**

(i) **Phenotype of PDH45 transgenics**

The genes that confer tolerance to abiotic stress are expected to change the phenotype of the plant thereby helping the plant to combat stress. There are reports showing that over expression of constitutively active form of DREBIA/DREBIB under the constitutive promoter resulted in growth retardation of the plants (Bhatnagar-Mathur et al., 2007). Transgenic rice plants constitutively expressing OsDREBIB showed stunted phenotype (Ito et al., 2006). Several other transgenic plants constitutively expressing transcription factor genes have shown growth retardation (Haake et al., 2002; Dubouzet et al., 2003; Kasuga et al., 2004; Cong et al., 2008). Arabidopsis transgenic plants constitutively expressing OsDREBIA have shown growth retardation but at the time of bolting only (Dubouzet et al., 2003). However, Oh et al. (2005) reported that the transgenic plants exhibited neither growth inhibition nor visible phenotypic alterations despite the constitutive expression of AtDREBIA/CBF3 in rice. From these various reports, it is inferred that expression of regulatory genes and transcription factors alter the plant growth.

From this context, in the present study, we examined the phenotypic variation in groundnut PDH45 transformants. Considerable variation in the phenotype was observed under water deficit stress, which ranged from spreading plants, tall plants, stay green type and production of three seeded pods. Three promising lines, H-5, H-8 and H-19 showed the visual stay green phenotype till the end of the crop period. H-15 and H-19 showed three seeded pods (Fig. 26). It can be presumed that, the introduced PDH45 helicase has resulted in the change in the plant architecture such as increase in LAD and development of erect canopy under stress, resulting in the maintenance of chlorophyll stability. Retardation of the stress induced senescence at later stages of plants growth is a desirable trait at canopy level for improved pod growth. Based on this assumption, selection and advancement of transformants was mainly based on molecular characterization, stress response and productivity under water deficit stress.
(ii) Molecular characterization of PDH45 transformants for the integration of the transgenes (PDH45, hptII and uidA)

Transgenic plants were analyzed for the selection of putative transformants and stability of transgenes in the advanced generations by PCR analysis initially. The plants were analyzed for the amplification of gene of interest, selectable and screenable markers. The gene of interest, PDH45 was amplified using promoter- gene specific primers to amplify a fragment of 827 bp and nested primers were used to amplify a 483 bp fragment. hptII primers were used to amplify a 500 bp fragment of hptII gene and a 750 bp fragment of uidA gene using promoter- gene specific primers. All the selected transformed plants showed amplification with various primers. The reproducibility of the PCR amplification in all the generations not only proved stable integration but also inheritance of the transgenes. These results were in agreement with the Sanjaya et al., (2005) over expressed the AV2 gene in cotton against cotton leaf curl virus and confirmed the T-DNA integration by PCR with nptII and AV2 gene specific primer to prove the transgenic nature of cotton in T1 and T2 generations. PCR analysis confirmed the stable inheritance of the transgenes in the selected plants.

Further, the 827 bp helicase amplified product was cloned, sequenced and aligned with the known gene sequence (Plate 20). The PCR product was also restriction analyzed and digested with HindIII to produce fragment sizes, 308 bp and 172 bp. These experiments unequivocally proved the integration of PDH45.

The number of copies of the T-DNA integration in 8 selected promising lines using HindIII as the single cutter, showed that most of the plants had the T-DNA integrated as a single copy (Plate 21). Similarly, single copy integration was reported in transgenic bell pepper plants expressing uidA gene developed by using in planta transformation protocol (Manoj kumar et al., 2009). This demonstrated that stable transformants can be generated using in planta transformation protocol

RT PCR analysis of the PDH45 gene provided evidence for the transcript accumulation of the integrated transgene. The transgenic lines showed increased transcript accumulation than wild type (Plate 22). This indicated the efficacy of the transgene in the transgenic lines.
These evidences proved that large number of events can be generated using *in planta* transformation protocol. Besides, the approach is able to generate the transformants with stable integration and expression even in the advanced generations.

(iii) **Stay green nature and chlorophyll stability of the transgenics**

(a) **Stay green phenotype**

Functional leaf area and LAD influences the crop growth rate (CGR). Besides Leaf Area Index (LAI), leaf senescence is important determinant of LAD. Stay green phenotype substantially increases the LAD and also maintains higher carbon gain with leaf age. Therefore, a desirable phenotype to combat abiotic stress would be to increase the LAD by a stay green phenotype which results in the increase in the total biomass through its possible effect on CGR and therefore the yield. In addition maintaining chlorophyll stability under stress is also important, as it is related photosynthetic rate. Although there is an argument about whether a higher chlorophyll content (i.e., stay green trait) contributes to yield under drought conditions or not (Blum, 1998), many studies indicated that stay-green is associated with improved yield and transpiration efficiency under water-limited conditions in sorghum, maize and wheat (Benbella and Paulsen, 1998; Borrell *et al.*, 2000; Haussmann *et al.*, 2002; Verma *et al.*, 2004).

A few transgenic approaches to retard senescence also substantiate the fact that stay green nature is a desirable trait. Rivero *et al.*, 2007 demonstrated that it is possible to enhance the tolerance of plants to drought stress by delaying the drought induced leaf senescence during the drought episode by over expressing *Iso-Pentenyl transferase* gene (*IPT*) under senescence inducible promoter, *P*SARK. Similarly, the accelerated leaf senescence in petunia transgenic plants due to drought stress was prevented by over expressing *IPT* under a senescence inducible promoter, *P*SAG12 (Clark *et al.*, 2004).

(b) **Etherel induced senescence and chlorophyll stability**

In the present study, stay green phenotype was observed under water deficit stress in three transgenic lines H-5, H-8 and H-19, over expressing *PDH45*. These lines showed retardation in leaf senescence till the end of the crop growth period (Fig. 41). In addition, *in vitro* leaf disc bio assay was carried out to assess the response of transgenics to
ethylene induced senescence. The transgenic lines showed a lesser extent of etherel induced senescence and maintained higher chlorophyll content compared to wild type (Fig. 30B). The extent of senescence reflected in the degree of bleaching in the leaf discs treated with etherel after 72 hrs (30A). Tolerance of the transgenic lines to etherel induced leaf senescence, corroborated with the stay green nature of the promising lines.

(c) Stress induced chlorophyll stability

Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate (Guo and Li, 1996). Under desiccation stress, chlorophyll is prone to degrade and hence maintaining chlorophyll stability under desiccation stress, improves the photosynthetic capacity, thereby yield. Transgenic petunia over expressing Iso-Pentenyl Transferase (IPT) gene under senescence inducible promoter showed higher chlorophyll content under drought stress. The higher chlorophyll levels was likely due to the increased expression of IPT gene, resulting in the higher cytokinin levels which would prevent the chlorophyll degradation (Clark et al., 2004). Transgenic plants in the present study had higher chlorophyll retention capacity under desiccation stress compared to wild type plants (Fig. 22). The stability index of chlorophyll was also more in the transgenics compared to wild type (Fig. 32). This evidenced by their stay green nature.

(iv) Performance of the PDH45 transgenics to various stresses

Transgenics over expressing PDH45 driven by a constitutive promoter, showed tolerance to 300mM NaCl (Sanan-Mishra et al., 2005). Similarly, MH1 showed improved tolerance to drought, salt and oxidative stress (Luo et al., 2008). Besides, OsBIRH1 is reported to enhance tolerance not only to oxidative stress but also disease tolerance. In view of this, the emphasis in the present investigation, has been to examine the response of transgenics over expressing PDH45 to various stresses.

Stress response

All cellular activities are not equally sensitive to drought stress and the changes in cellular metabolism in the transgenic plants can be assessed by various physiological screens. In the present study, the stress response of the transgenics was analyzed by
estimating the variation in physiological and biochemical parameters under stress. The observed variations could be due to both the variations in water relations and also due to changes in cellular metabolism induced by transgene.

(a) Membrane integrity

A major impact of plant water deficit stress is cellular membrane modification, which results in altered membrane permeability. Estimation of membrane dysfunction under stress by measuring cellular electrolyte leakage from affected leaf tissue into an aqueous medium reflects stress resistance (Zhou et al., 2008). Measurements of damage to the membrane in groundnut PDH45 transgenic plants under desiccation stress showed that maintenance of cell membrane stability was more in the transgenic lines (Fig. 23 and 33), inferring lesser stress induced membrane damage in the transgenic plants.

(b) Survival and recovery

The in vitro physiological and biochemical measurements are another option to assess the stress response of the transgenics under different stresses like salinity, PEG. As the stress received is same for both wild-type and transgenics and any variability in tolerance is because of the cellular level tolerance induced by the expressed gene.

Response to salt stress

The performance of transgenic plants was tested by in vitro leaf disc experiments in which the leaf segments were subjected to salinity stress. The transgenic showed lesser salinity induced loss of chlorophyll compared to wild type in leaf disc bio assay at 300mM Nacl concentration (Fig. 29B). The damage is reflected in the degree of bleaching of the leaf discs, after 72 hr of incubation (Fig. 29A). The results were similar to the tobacco transgenic lines over expressing PDH45 showed better salt stress (300mM) tolerance compared to wild type (Sanan-Mishra et al., 2005).

Response to PEG induced osmotic stress

High-molecular-weight polyethylene glycol (PEG) has been examined in many early studies that attempted to impose a controlled water deficit. PEG is viscous and hence decreases O₂ movement to roots so that the roots become O₂ deficient (Mexal et al., 1975;
It interferes with ion transport (Yeo and Fllowers, 1984) and therefore affects root growth. Stress response using PEG has been examined in several transgenic studies. Over-expression of *Cicer pinnatifi dum* dehydrin DHNI gene in tobacco plants positively affected growth of the seedlings under artificial water stress induced by PEG (Beck et al., 2006).

The transgenic seedlings over expressing *PDH45*, performed better under the PEG induced osmotic stress. Growth of the transgenic seedlings was studied under PEG treatment. Percent reduction of the root length of transgenics over the respective seedlings without treatment was relatively less when compared to wild type (Fig. 46).

Response of the transgenics to salt stress and PEG induced osmotic stress, infers the efficiency of the transgenics to maintain intrinsic cellular level tolerance.

**Root growth recovery**

Under desiccation stress, root growth of *PDH45* transgenic groundnut plants was more than the wild-type. Many environmental and endogenous factors were reported to affect the process of root growth. In the present study, root growth in terms of root volume was studied under water deficit stress. The percent increase in the root volume over wild type was more in the some of the transgenic lines (Fig. 45). Increase in root growth in transgenics could be due to improved cellular metabolic activities under stress. Several regulatory genes have been shown to bring about such response. Over-expression of Arabidopsis *NAC1* increased the root growth and down-regulation reduced lateral root formation. In another study *DREB1A* transgenic plant of groundnut were shown to have enhanced root growth, particularly under water deficit conditions increasing water uptake up to 20 to 30 per cent more than wild type (Vadez et al., 2007). Alteration in the levels of phytohormone ABA or even the mutations in the corresponding signaling pathways would influence the root growth (Brady et al., 2003; Tian et al., 2004).

**Water Use Efficiency (WUE) of the transgenic plants**

WUE and water conservation have significant relevance. Achieving tolerance and improved productivity through efficient water mining and improved water use efficiency
are crucial mechanisms (Sheshshayee et al., 2003). The Carbon Isotope Discrimination ($\Delta^{13}C$) determines the WUE. A study by Sivamani et al., (2000) reported an increased WUE in the transgenic wheat. In the present study, the $PDH45$ transgenics showed relatively low $\Delta^{13}C$ values compared to wild type under water deficit stress. This indicates the improved WUE of the $PDH45$ transgenic lines.

The various physiological response assays showed improved intrinsic cellular level tolerance in transgenics over wild type plants.

(v) Effect of $PDH45$ on growth and yield attributes in transgenic groundnut

The productivity of a plant under stress is influenced by various growth and yield factors. In groundnut, the major factors affecting yield are, development of pods and kernels. Trung et al., (1985) found that under severe water stress yield of groundnut was reduced. A plant would be productive under stress when the leaf area duration is maintained with increased chlorophyll stability. However, it ultimately depends on the effective production of pods from pegs and subsequent production of filled pods. In the present study, improvement in yield in transgenics over wild type was one of the factors for the selection of promising transgenics in all generations. The transgenic lines were measured for various growth and yield attributes like total dry matter (TDM), pod number and pod weight under moderate and severe water deficit stress. All the selected transgenic lines showed improved biomass and yield compared to wild type. A significant positive correlation existed between the TDM and pod weight among the transgenic lines against wild type, indicating that the transgenics showed enhanced growth rates which in turn improved the peg to pod ratio and pod growth. Average yield (pod dry weight) in the promising transgenic lines ranged from 36-43.1 g/plant compared to 29.1g/plant in wild type in all the experiments under water deficit stress (Fig. 47A). The study showed that the transgenic plants showed 15-30% increase in yield under moderate stress conditions and 30-40% increase in yield under severe stress conditions (Fig. 47C). Among the identified promising events, H-5, H-8 and H-19 showed 35-40% increase in yield over the wild type (Fig. 47B). As discussed earlier, the stay green phenotype of the plants due to the introduction of $PDH45$ helicase and the resulting increase in photosynthetic rate could be the possible explanation for the increase in yield. However, the reason for the three
seeded nature of H-19 has to be deciphered. Similarly, H-10, H-11 and H-15 exhibited an erect tall phenotype. These plants showed approximately 40% increase in yield possibly owing to the increased canopy photosynthesis (Fig. 47B).

However, scoring the selected 8 lines for the performance under different physiological screens and yield under stress identified H-19 as a promising event with better performance in both physiological screens and yield. The transgene PDH45 in H-19 unequivocally demonstrated tolerance to stress and improvement in yield (Fig. 48). This was further emphasized in the strong positive correlation of the event demonstrated in the various parameters. The event was also the most water use efficient among the events.

Conclusion

Since drought stress response is multigenic, the upstream genes which might bring in cellular level tolerance under stress have relevance. The present study is an attempt to understand the relevance of transcription activators, i.e., helicases in plant stress tolerance. The major focus was to isolate and characterize the stress induced helicases. It was hypothesized that the stress genes from stress adapted species are functionally more superior in bringing about tolerance to dehydration. Hence an attempt was made to clone Ec-helicase from the stress adapted species, finger millet. Since the cloning of the Ec-helicase was incomplete, a validated PDH45 helicase was used to improve drought tolerance in groundnut. This work presents an evidence for the amenability of the in planta transformation procedure in groundnut using a selectable marker gene, EPSPS. Using the in planta transformation protocol, transgenics in groundnut over expressing PDH45 were developed. Based on the stress responses arrived at using various stress screens, experimental evidences were provided to show that PDH45 gene imparts drought stress tolerance, besides improving yield under stress. The study showed that helicases are the potential candidate genes to impart stress tolerance. The present investigation also provides an approach to improve drought tolerance in trait donar genotype by pyramiding the traits associated with water relations and mechanisms involved in cellular level tolerance.