CHAPTER 4: DISCUSSION

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop, which is a rich source of nutrients and cultivated in more than 100 countries across the globe (Dwivedi and Crouch 2003; Kumari et al. 2012; Pandey et al. 2012). Maximum portion of world total annual production was contributed by Asia (Dwivedi and Crouch 2003). India stands second in terms of world total annual production (USDA, 2014) of which Gujarat state is the largest producer (ASG, 2012). Salinity and drought are the two major abiotic constraints causing significant loss of groundnut productivity. When groundnut faces drought stress, it alone causes loss of approximately 70 % of the crop productivity (Pandey et al. 2012). The Gujarat state constitutes the longest coastline of about 1600 km in India, which are salinity affected (Yadav 2013). Several strategies have been employed using traditional breeding programs to improve the tolerance ability of groundnut to abiotic stresses. But these strategies failed, due to tetraploidy nature and conserved genome of cultivated groundnut (*A. hypogaea* L.) (Pandey et al. 2012). At present, it was proposed that the use of genetic engineering and molecular breeding, and their integration with conventional breeding are the approaches, which can assist in achieving the goal of groundnut quality improvement (Varshney et al. 2011).

Genetic engineering imparts two major steps, tissue culture and genetic transformation. Genetic transformation requires a potential gene source and a gene to be used for transformation. A halophyte, *Salicornia brachiata* grow luxuriantly in salt marshes (Jha et al. 2012), is a potential source of abiotic stress responsive genes, as salinity stress responsive EST database have been developed from it (Jha et al. 2009) and numerous abiotic stress responsive genes have been characterized. The *SbASR-1* has shown upregulation in salinity stress conditions (Jha et al. 2009) and conferred salt tolerance in transgenic tobacco lines (Jha et al. 2012). More than 20 years have been

passed after discovery of *ASR-1* gene from tomato and was characterized from several glycophytic plants (González and Iusem 2014), but still the complete functional analysis of a halophytic *ASR-1* gene cloned from *S. brachiata* is lacking. Therefore, in the present study *SbASR-1* gene was characterized at the genomic level and genetically transformed into local cultivar of groundnut GG 20. The salinity and drought stress tolerance ability of transgenic groundnut was studied in T1 generation.

4.1 Characterization of *SbASR-1*

The putative promoter region of the *SbASR-1* gene cloned from the genomic DNA and gene sequence were discriminated from the promoter region by aligning with the cDNA sequence present in the NCBI database. The 5'-UTR sequence of 106 bp is expected to contain the TSS at +1 position. This assumption was supported by the presence of conserved TATA-box at -32 bp upstream from the expected TSS (**Figure 3.1, 3.2**). *In-silico* analysis of the 843 bp promoter sequence revealed the presence of numerous putative *cis*-acting motifs. Presence of ABRE, MYB and MYC binding elements and light inducible GT1 and GATA elements suggest its inducibility in the abiotic stress conditions. The expression of this gene is regulated by ABA-mediated signalling cascade which may involve ABRE and MYB binding elements (**Table 3.1**). Analysis of *VvMSA* promoter region also showed the presence of several ABA inducible and DRE motifs (Saumonneau et al. 2012). Apart from the stress inducible motifs, *cis*-acting motifs involve in the sugar repression, phytohormones like cytokinin, auxin, GA and SA regulated pathway and pathogen stress inducible bell like homeodomain transcription factors and WRKY binding consensus sequences were identified (**Table 3.1**). The functionality of these motifs was confirmed by the previous reports demonstrating the expression of *ASR* gene transcripts, induced by ABA, salt, drought, PEG-6000, cold and injury and H_2O_2, during fruit ripening, Al

toxicity and also by biotic factors (Amitai-Zeigerson et al. 1995; Jha et al. 2009; Kim et al. 2009; Liu et al. 2010; Hsu et al. 2011; Ricardi et al. 2012; Arenhart et al. 2013b; Hu et al. 2013; Arenhart et al. 2013a). The ASR-1 also plays crucial role in regulation of leaf sugar level and its mobilisation in other organs and plant growth by regulating the GA biosynthesis (Cakir et al. 2003; Dominguez et al. 2013). This report also supports the presence of A-box and GA inducible motifs in *SbASR-1* promoter region. Presence of SA regulated pathway, pathogen stress inducible bell like homeodomain transcription factors and WRKY binding consensus sequences were also in coherence with the report suggesting upregulation of plantain *ASR-1* under biotic stresses (Liu et al. 2010).

Structural elucidation of *SbASR-1* gene revealed its length of 2549 bp. There was only one intron sequence was present in the gene separating the two exons (**Figure 3.1, 3.2**). The intron region is 1611 bp long which is the largest intron sequence in *ASR* gene family yet reported. The genomic structure of *ASR* gene family was reported from the banana, maize and rice genome. All the genes of this family contain either single intron or are intronless (Philippe et al. 2010; Henry et al. 2011; Virlouvet et al. 2011), which are in coherence with our results. Intron lengths in the banana, maize and rice *ASR-1* gene are of 89, 130 and 131 bp, respectively (Philippe et al. 2010; Henry et al. 2011; Virlouvet et al. 2011). In case of *SbASR-1*, intron is several times longer as compared to these reports, this might be due to evolutionary adaptation of gene in *S. brachiata* to cope-up with harsh environmental conditions and further study is required to decipher the reason behind it.

The genomic organization study of the *SbASR-1* gene was performed using Southern Hybridization technique of genomic DNA from *S. brachiata*. Use of partial cDNA of *SbASR-1* gene as a probe strongly suggests the presence of at least four copy

of the *ASR* gene in *S. brachiata* (**Figure 3.3**). This is first kind of study on *ASR* gene from a halophyte. The copy number of *ASR* gene varied in different glycophytic plants. The numbers of paralogues present in the different monocots and dicots plants were reviewed by González and Iusem (2014). They have summarized as, maize genome contains the highest number of paralogues of this gene, 9 (Virlouvet et al. 2011), 4 members in banana (Henry et al. 2011), 4 in tomato (Frankel et al. 2006) and 6 members in rice (Philippe et al. 2010). Exceptionally, grape *ASR* also known as *VvMSA*, is present as a single copy in grape genome (Cakir et al. 2003). Remaining *ASR* genes from other reported plants are either present in single copy or yet not studied with this perspective.

Bioinformatics analysis of *Sb*ASR-1 protein amino acid sequences revealed unique characteristics. There was an ABA/WDS conserved motifs found in the amino acid sequence of *Sb*ASR-1 (**Figure 3.4**) which is characteristic domain of ASR-1 protein. As described by Battaglia et al. (2008), *Sb*ASR-1was categorized in hydrophillins group because of high (25 %) Gly content and hydrophilicity index higher than 1. The *Sb*ASR-1 belongs to Group 7 LEA protein as it contained four of the total five conserved protein motifs as proposed by Battaglia et al. (2008). The four motifs present are 1, 2, 3 and 5 and arranged in order of 3-1-2-5 towards the C-terminal region (**Figure 3.4**). There were few amino acid residues in *Sb*ASR-1 differed from the reported residues in the conserved motifs. Halophytes have evolved unique amino acid composition in their proteins as compared to their homologous glycophytic proteins. These conserved motifs of Group 7 LEA proteins are deduced from the glycophytic ASR-1 protein and this might be plausible reason for the variation of few amino acids in conserved motifs present in *Sb*ASR-1. The motif 4 is His rich motif discovered to be located at N-terminus of some of the ASR-1 proteins but not

necessarily to be present in all ASR-1 proteins. Of these five motifs, presence of motif 1, 2 and 3 are essential to categorize that protein in LEA Group 7 protein (Battaglia et al. 2008).

The multiple alignment of *Sb*ASR-1 protein amino acid sequence with other glycophytic ASR-1 amino acid sequences leads to identification of four of the five functional motifs as described by Virlouvet et al. (2011) (**Figure 3.5**). These motifs are involved in the DNA-binding or other several functions. Of these motifs few motifs are overlapping with the LEA protein Group 7 characteristic motifs or are part of these motifs. At N-terminus the motif A present in glycophytic ASR-1 was absent in SbASR-1 where His residues are replaced with Gly residues (**Figure 3.5**). Most of the ASR proteins have K- or R-rich bipartite NLS on the C-terminal end (Wang et al. 2005). Similarly, C-terminal end of the *Sb*ASR-1 protein also contains conserved bipartite K-rich NLS (**Figure 3.5**).The LLA23 protein has K-rich NLS, and it was found to be distributed in the nucleus and cytosol of the vegetative cells of mature pollen grain (Wang et al. 2005). The *Vv*MSA (Grape ASR protein) was localised in the nucleus and interacted with the *VvHT1* promoter (Cakir et al. 2003).

In phylogenetic tree of *Sb*ASR-1, constructed using amino acid sequences of glycophytic ASR-1, *Sb*ASR-1 showed evolutionary proximity with the Solanaceae family members (**Figure 3.6**). This was also supported by the finding of similar number of copy number of gene in the tomato genome (Frankel et al. 2006; González and Iusem 2014). Comparative analysis of post-translation sites on amino acid sequences revealed an interesting finding, highest number of N-myristoylation site and maximum number of disorder promoting amino acids (**Table 3.2**). Approximately, 30 % of all eukaryotic proteins are either completely or partially disordered (Fink 2005) and these proteins are grouped into a category known as Intrinsically Disordered

Proteins (IDPs). The LEA proteins are found in disordered form and strongly behave like IDPs when present in solution. They attain a fixed conformation while binding with Zinc ion or upon desiccation stress (Goldgur et al. 2007). The IDPs protect other cellular proteins under stress conditions and help in transcriptional regulation of other genes. It can undergo extensive post-translational modifications, such as phosphorylation, acetylation, ubiquitination, sumoylation, etc., allowing easier protein-protein interaction for modulation of biological functions because of their structural flexibility (Uversky and Dunker 2010). These disordered conformations of proteins are attributed by the presence of disorder promoting amino acids in maximum proportion in those particular proteins. Also, FoldIndex program predicted that, the *Sb*ASR-1 protein constituted of 66.3 % disorder promoting amino acids. Similarly, tomato ASR-1 protein contained higher percentage of disorder-promoting amino acid residues (Goldgur et al. 2007). Uversky et al. (2000) reported that the natively unfolded protein or IDPs had generally low M_rs with less than 150 amino acid residues and showed either acidic or basic isoelectric pH. The *Sb*ASR-1 protein exhibited an acidic isoelectric pH (5.3) compared to almost neutral isoelectric pH of tomato ASR protein confirming that *Sb*ASR-1 is also an IDP. The post translational modifications are the essential steps for proteins to become functionally active. One of these modifications, myristoylation occurs by covalent attachment of myristate group at the N-terminal glycine residue of eukaryotes and viruses (Farazi et al. 2001). Myristoylation of protein helps in protein-protein interaction and in regulating signalling pathways during apoptosis and salt stress adaptation (de Jonge et al. 2000). Ishitani et al. (2000) reported that mutation in myristoylation sites makes plants hypersensitive to salt stress. Therefore, it is possible that the presence of glycine-rich region in the *Sb*ASR-1 may help in salt adaptation mediated by N-myristolation.

4.2 Groundnut tissue culture

The regeneration of groundnut via caullogenesis (Joshi et al. 2008) or direct oraganogenesis are achieved by several groups using explants like de-embryonated cotyledon (Radhakrishnan et al. 2000), de-embryonated cotyledon half (Asif et al. 2011), cotyledonary nodes (Iqbal et al. 2012), embryo axes (Swathi-Anuradha et al. 2006), hypocotyl (Swathi-Anuradha et al. 2006), and epicotyles (Shan et al. 2009). In most of the recent reports, the explants used in regeneration or transformation experiments were performed using de-embryonated cotyledon half as explants. Large size of the explants, therefore easy to handle and comparatively more number of shoot buds were regenerated per explants. These are the few advantages, why de-embryonated cotyledon explants are the preferable one for regeneration and transformation. The regeneration potential and morphogenesis of *in-vitro* cultured tissue largely depends upon genotype of mother plant from which the explants were obtained and other culture conditions like nutrients, phytohormone, light, pH, water potential, temperature, gaseous atmosphere etc. (Tiwari and Tulli 2008b). In comparative analysis using three protocols, Radhakrishnan et al. (2000; SM1), Sharma and Anjaiah (2000; SM2) and Tiwari and Tulli (2008b; SM3) for regeneration efficiency of groundnut (*A. hypogeae* L.) cv. GG 20, SM1 gave the best results. The SM1 media showed regeneration in 52.69 % explants (Table…). The SM2 and SM3 gave regeneration in 34.35 and 37.29 % explants, respectively. Original reports showed 57, 95.5 and 91.6 % regeneration efficiencies on SM1, SM2 and SM3 media, respectively (Radhakrishnan et al. 2000; Sharma and Anjaiah 2000; Tiwari and Tulli 2008b). The groundnut cultivar used in SM1 media was J11 while in SM2 and SM3 were JL24 (Radhakrishnan et al. 2000; Sharma and Anjaiah 2000; Tiwari and Tulli 2008b). The SM2 and SM3 media showed almost similar (>90 %) regeneration

efficiencies. These findings suggest that lower regeneration efficiency in local cultivar GG 20 is due to different genotype of this cultivar from those used in earlier reports. While comparing the number of shoot buds per explants SM2 media resulted in highest number of shoot buds per explant and that is 17.67 (**Table 3.3**). The regeneration of shoot buds from de-embryonated cotyledon explants occurred from the meristmatic cell layer on cotyledon from where embryo axes are attached. In whole de-embryonated cotyledon this meristematic layer gone inside the notch and not exposed to the media properly. This might be the plausible reason behind higher shoots per explants obtained in vertical halves of cotyledon explants. The elongation of regenerated shoot buds at EM3 media (13.32 µM BAP) gave the best results. This finding is also supported by the other earlier reports using lower concentration of BAP for elongation (Sharma and Anjaiah 2000; Asif et al. 2011; Tiwari and Tulli 2008b, 2012). Use of rooting media containing 5.37 µM NAA showed rooting in 100 % shoots and similar results were shown by earlier reports (Sharma and Anjaiah 2000; Tiwari and Tulli 2008b).

4.3 Optimization of hygromycin lethal dose (LD_{50})

Selection of putative transgenic plants is important step to reduce or remove the untransformed or chimeras to avoid unnecessary labour to grow them. Kanamycin-based selection is not efficient enough to eliminate the non-transformed cells completely (Sharma and Anjaiah 2000; Dodo et al. 2008; Tiwari and Tulli 2012) and may result in escapes or chimeric plants. The hygromycin is comparatively strong and stringent selective antibiotic resulted in the efficient recovery of numerous putative transformed shoots in a comparatively shorter period (Tiwari and Tulli 2012). This enable shorter period of maintaining plants in culture conditions and thus reduces the probability of inducing somaclonal variation (Olhoft et al. 2003). In this study, the

binary vector contains *hpt* gene coding for hygromycin phosphotransferase enzyme and antibiotic hygromycin was used as selective agent. Before start of the transformation experiments, the hygromycin lethal dose (LD_{50}) was determined. This was essential for obtaining positively transformed transgenic plants and to avoid chimeras. At hygromycin concentration 20 mg/l average of 58 % shoots survived after 3-week of culture and at 30 mg/l only 33 % of shoots were survived (**Figure 3.9a-b**). Therefore, 20 mg/l concentration of hygromycin was considered as lethal dose (LD_{50}; concentration at which 50 % explants died). However, different other reports varied in the concentration of hygromycin for selection of transgenic plants. Iqbal et al. (2012) has used 25 mg/l hygromycin concentration in the elongation media for selection purpose. Wherever, Tiwari and Tulli (2012) found 50 mg/l hygromycin, as a suitable concentration for the selection of putative transformants. This variation in the hygromycin concentration may due to variation in cultivar type or age of the regenerated shoot used in experiments.

4.4 Optimization of transformation parameters

Most of the legumes including groundnut are recalcitrant towards regeneration and transformation. However, recent literature survey yielded a good number of reports on the transformation and regeneration of different cultivars of groundnut across the globe. But the *Agrobacterium*-mediated transformation efficiency varies from genotypes to genotype. The local cultivar GG 20 was developed in the year 1992. Despite of its, long life span, this cultivar is famous among the farmers and used widely in Gujarat state for cultivation. This cultivar has property of large seed size and improved resistance to fungal pathogens (**Table 1.3**). Yet, there is no report on the transformation of GG 20 cultivar. To achieve this goal, transformation protocols reported by Sharma and Anjaiah (2000) and Tiwari and Tulli (2008a, 2012) were

screened. The protocol described by Tiwari and Tulli (2012) shown better result in transient transformation efficiency using GUS histochemical assay of the explants after co-cultivation. While explants transformed according to Sharma and Anjaiah (2000) were co-cultivated for 5 days in dark and cultured on the SM1 media supplemented with 400 mg/l cefotaxime showed regeneration in 62.75 ± 7 % explants but the transformation efficiency was only 50 %. Later on different parameters were optimized to establish an efficient protocol for the transformation in this cultivar. There was no significant difference in transient expression found in transformation with bacterial culture of OD_{600} 1.0 to 1.8. However, the average transient expression efficiency was found highest at 1.8 of culture OD_{600} (**Figure 3.12**). Bacterial cultures in the late log phase (OD_{600} 1.0 to 1.6) of their growth were earlier reported to be used in the transformation (Qin et al. 2011; Tiwari and Tulli 2012). Since the culture at OD_{600} 1.8 has given the best transformation efficiencies, were kept constant and other parameters like acetosyringone and L-cysteine concentration in co-cultivation media were optimized. Acetosyringone is a phenolic compound and well documented as to play crucial role in activating the *Agrobacterium* cell for transfer of T-DNA. Without or at lower concentration on it in co-cultivation media showed no transformation events. But if used at the 200 µM concentration in the co-cultivation media, highest transformation efficiencies (80 %) was achieved (**Figure 3.11b**). Transformation efficiencies above this concentration did not change and found to be similar. Therefore, the optimized concentration of acetosyringone was 200 µM.

Upon infection of the cut surface of de-embryonated cotylrdon explants may leads to production of free radicals causing oxidative stress in explants. This limits the growth or survival of the infected plant cells in co-cultivation medium. Antioxidants used in the co-cultivation media protect cells from oxidative stress by scavenging free

radicals (Dutt et al. 2011; Tiwari and Tulli 2012). Tiwari and Tulli (2012) have suggested that L-cycteine at concentration of 100 mg/l has worked more efficiently in recovery of transformed cells as comparaed to other antioxidants used in the study. Therefore different concentrations of L-cysteine in co-cultivation media with 200 µM of acetosyringone were used to determine the appropriate concentration of it required for GG 20 cultivar. In this study, 50 mg/l L-cysteine concentration was found to be most efficient giving transient GUS expression in 75 % of explants. Further increase in L-cysteine concentration did not change the transformation efficiency significantly (**Figure 3.11c**). The co-cultivation media with this optimized L-cysteine concentration (50 mg/l) gave transient expression approximately 4-fold higher transformation efficiency than that of media without L-cysteine. Use of L-cysteine at time of transformation, successfully reported to minimize oxidative damage of competent host cells during co-cultivation and increased transformation efficiency in crop plants (Olhoft et al. 2003; Kumar et al. 2011; Tiwari and Tulli 2012). It was suggested that L-cysteine could enhance survival of cell from the hypersensitive responses leading to increased survival of embryogenic competent cells after transformation and increased stable transformation efficiency. Use of L-cysteine in co-cultivation media inhibits the action of peroxidases and polyphenol oxidases, which are responsible for induction of hypersensitive response following Agrobacterium infection in explants (Olhoft et al. 2003; Tiwari et al. 2008a). Similarly, transformation efficiency of sorghum was increased by 2.9-fold in the presence of L-cysteine during co-cultivation of immature embryo explants (Kumar et al. 2011).

In an observation as mentioned above, explants transformed according to protocol Sharma and Anjaiah (2000) and cultured on SM3 media showed better regeneration efficiency. This finding suggests the role of BAP and 2,4-D concentration

and their ratio in co-cultivation media regulate the transformation efficiency and regeneration after transformation. Different co-cultivation media were designed with varied concentration of BAP alone or with different concentration 2,4-D. The media in which only BAP was used, showed higher transformation efficiency of 85 % but low regeneration efficiency 17.2 %. But at same concentration of BAP, if 0.25 mg/l 2,4-D was added to it, transformation efficiency reduced to 50 % and regeneration efficiency increased by 3 times (54.76 %) (**Figure 3.12**). In coherence with this finding, Petri et al. (2009) has also found that regeneration efficiency increased by 10-fold in transformed explants, if 2,4-D was used in the regeneration media. However, present finding contrasting the earlier findings that, 2,4-D enhances transformation efficiency (Mannan et al. 2009). This create a hypothesis, that 2,4-D may perturbed endogenous phytohormone which hinders the transformation. Untransformed explants were now cultured on media containing only BAP or in combination with 2,4-D for the same period and in same condition as co-cultivation. Then phytohormones were extracted from them and subjected to HPLC analysis for determination endogenous IAA concentration. It was found that, use of 2,4-D in co-cultivation media induced the endogenous IAA synthesis (**Table 3.4**) which was proved to be potential inhibitor of *vir* gene expression (Liu and Nester 2006). This may be the reason of lower transformation efficiency on co-cultivation media containing 2,4-D.

At last, the washing of explants before infection and co-cultivation played crucial role in transformation. During explant preparation, cut surface of de-embryonated cotyledon explants released certain whitish exudates which may contains phenolic compounds and get oxidised upon exposure to air. After oxidation the explants turns brown and died (Egnin et al. 1998). To avoid this, explants were washed with ½ MSi (half strength MS-salts with 100 mg/l myo-inositol, 2 % glucose and 100

μM acetosyringone) for 3-4 times gently in sterilized conditions (Egnin et al. 1998; Tiwari and Tulli 2012). There was PVP also used in washing media, but it reduced the transformation efficiency significantly. PVP is strong chelating agent and may adsorb the entire phenolic compounds of which some are essential for *vir* gene activation (Egnin et al. 1998). The sucrose in the ½ MSi solutions was replaced by D-glucose. Reducing monosaccharides were reported to assist transformation by two ways, first by enhancing VirA-VirG sensitivity to phenol and second by elevating the saturating concentration of phenol (Lacroix et al. 2011). D-glucose acts as co-inducer and binds with the ChvE protein present in periplasm of *Agrobacterium*. This activated ChvE protein interacts with periplasmic domain of VirA and enhances its ability to activate *vir*-operon for transfer of T-DNA (Lacroix et al. 2011). Tiwari and Tulli (2012) have found 2 % (w/v) D-glucose in ½ MSi solution was optimum to for transformation and the same concentration was adopted. Lower pH (5.6-5.7) has activated the *virG* gene expression by involving two-component regulatory system of chromosomal origin composed of ChvG and ChvI (Charles and Nester 1993). In the present study pH 5.6 to 5.8 were tried and there were no differences in the transformation efficiencies were observed. During transformation of groundnut using de-embryonated cotyledon explants, the pH of ½ MSi solution used was 5.6 (Tiwari and Tulli 2012) and this pH was used as optimum pH of ½ MSi solution.

The cut surfaces of explants facilitate exposure of competent cells with both *Agrobacterium* and shoot induction media. This assumption was supported by Sharma and Anjaiah (2000) where they have reported that vertical halves of de-embryonated cotyledon explants showed better regeneration compared to the full cotyledons. Uranbey et al. (2005) has described the temperature dependency of the *vir* genes expression and T-DNA delivery in plant tissue. The optimum temperature for it varied

in different crops. In case of groundnut, Tiwari and Tulli (2012) have used 21 °C as optimum temperature. In the present study the co-cultivation temperature used was 21 °C for 5 days in dark. After co-cultivation, it is necessary to remove the *Agrobacterium* from the explants. Failure in it resulted in the death of the explants due to hypersensitivity and necrosis. Tiwari and Tulli (2012) have optimized different concentrations of cefotaxime alone or in combination with augmentin. The best activity against *Agrobacterium* without affecting the regeneration efficiency was cefotaxime 200 mg/l along with augmentin 200 mg/l. However, 400 mg/l cefotaxime has also shown efficient inhibition of bacterial growth without affecting the regeneration efficiencies in this study.

4.5 Genetic transformation of groundnut

After optimization of several parameters a new efficient protocol has been developed for transformation and regeneration. De-embryonated cotyledon explants transformed using this protocol showed 85 % transformation efficiency in the transient expression assay. Explants were washed and cultured on the SM3 media supplemented with 400 mg/l cefotaxime. After incubation of 4-weeks 14.65 ± 1.06 % explants were regenerated. This media gave regeneration in 37.29 ± 2.21 % non-transformed explants. Two reasons assumed behind this observation of lower regeneration in transformed explants. First, several times washing of the explants may cause loss of competent cells and second, the transformation of explants may induce hypersensitivity and death of the competent cells. Regenerated shoot buds in bunches were excised from the explants and subjected to selection for 4-round on SLM3 media. Total of 3.815 ± 0.6 % hygromycin resistant explant (bunches of shoots with a node) were obtained while remaining explants died even 3rd round of selection. Differentiated shoots were cut and cultured on elongation media (EM3) supplemented

with hygromycin and the basal node of the hygromycin resistant explants was again cultured on the SLM3 media for multiple shoot bud induction. Total of 37.92 ± 1.52 % transgenic shoots were obtained. The shoots when grown upto 2-3 cm of length were transferred to the RM3 media. Only 14.58 ± 2.95 % of shoots were able to develop roots after 3 round sub-cultures each of 3-week period. There are several reports suggesting poor *in-vitro* rooting, low fertility, low recovery and low survival following acclimatization of transformed plants are the major constraints on genetic transformation of groundnut (Swathi-Anuradha et al. 2006; Dodo et al. 2008; Tiwari and Tulli 2012). Grafting of *in-vitro* grown shoots onto healthy rootstock has emerged as an excellent alternative to enhance the survival of regenerated and transgenic plantlets in the soil. This method was successfully attempted in several crops including cultivated groundnut (Tiwari and Tulli 2012), wild groundnut species (Still et al. 1987), cotton (Jin et al. 2006), chickpea (Chakraborti et al. 2006), citrus (Ballester et al. 2008) and safflower (Belide et al. 2011). Grafting shown 100% survival of the transgenic plantlets in soil and after acclimatization transferred to the Green-House conditions. The most important point behind the success of grafting was, stem to be used as scions should be well developed and the root stocks should not be older than 2-weeks.

4.6 Confirmation of putative transgenic line

The PCR amplification using *SbASR-1*, *hpt* and *gusA* gene specific primers gave amplification of 368, 990 and 1200 bp, respectively which was the correct length of the genes according to primers designed (**Figure 3.15**). Southern blotting and hybridisation with *SbASR-1* probe showed single band in all the transgenic line but there was no band detected in the Wt (**Figure 3.16**). Among five transgenic lines, line A1 and A2 gave signal at same position and similar to it A4 and A5 also gave band at

the same position. The plausible reason behind may be that these lines (A1-A2 or A4-A5) either were same or T-DNA was integrated at same loci in these lines. The RT-PCR of *Ah-actin* and *SbASR-1* genes using RNA isolated from Wt and transgenic lines showed amplification of *SbASR-1* in all transgenic line but not in the Wt plants (**Figure 3.17**). Whereas the actin gene amplification was obtained from Wt as well as transgenic lines and this confirmed the stable integration and expression of transgene in the transgenic lines. Histochemical GUS assay showed strong blue colour development in the leaves from transgenic plants but the leaves from Wt remained colourless (**Figure 3.18**). This blue colour development in transgenic lines is due to accumulation of insoluble indolyl dye by activity of β-glucosidase enzyme on its substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt (X-gluc) (Jefferson et al. 1987). This enzyme in transgenic lines was coded by *gusA* gene transformed with T-DNA and suggests the expression of functionally active proteins from the transgenes.

4.7 Analysis of T1 transgenic lines

Three transgenic lines A1, A3 and A4 were seem to be different transgenic lines based on southern hybridisation in T0 transgenic plants (**Figure 3.16**). Also the histochemical GUS assay of leaves from these lines showed higher level of expression (**Figure 3.19**). Therefore, lines A1, A3 and A4 were selected for the tolerance assay against salt and drought stress. One week old seedlings of Wt and transgenic lines grown in Soilrite were subjected to stress treatment of 250 mM NaCl solution in water on each alternate day and drought stress by withholding the irrigation for 15-days. Since Soilrite has shown superior to the soil for its better nutrient absorption efficiency by plants and therefore chosen as media to grow plants for stress tolerance assay.

Before start of the stress treatment, leaves from the same position of Wt and transgenic lines were harvested for pigment estimation.

4.7.1 Estimation of photosynthetic pigments

Chlorophyll content is studied as one of the markers of cellular stress, and it decreases in plants under stress. Chlorophyll a (Chl a), b (Chl b), total chlorophyll (Chl) and carotenoid content was significantly reduced in Wt as compared to transgenic plants under NaCl or drought stress (**Figure 3.21a-e**). During stress conditions, ROS scavenging mechanisms in chloroplast failed to cope-up the higher rate of ROS generation which inhibits the PSII repair system and synthesis of D1 proteins in chloroplasts. Outcome of this condition resulted in the loss or degradation of chlorophyll (Bartels and Sunkar 2005). Transgenic tobacco plants overexpressing *TaASR1* exhibited higher chlorophyll content in oxidative stress condition induced by methyl viologen (Hu et al. 2013). Similarly, transgenic tobacco lines overexpressing *SbASR-1* gene showed significantly higher chlorophyll content compared to Wt plants growing under NaCl stress (Jha et al. 2012). Recently, ASR-1 proteins from different plants were reported to act as antioxidant, involve in ROS scavenging directly or by enhancing the activity and expression level of several antioxidative enzymes (Kim et al. 2012; Hu et al. 2013; Li et al. 2013). Based on these facts, it was assumed that overexpressed *SbASR-1* may protect the chlorophylls from oxidative damage and thus transgenic lines maintain higher chlorophyll contents. In the present study, the carotenoids contents were also found significantly higher in transgenic lines as compared to Wt plants grown under salinity and drought stress conditions (**Figure 3.21e**). Carotenoids are reported to involve in protection of photosynthetic machinery by stabilizing thylakoid phospholipids and quenching the excited triplet state of chlorophyll and singlet oxygen (Ramel et al. 2012; Parida and Jha 2013). This may

function as a scavenger of reactive oxygen species (ROS), like singlet oxygen, produced under salinity and drought conditions (Parida and Jha 2012). There is substantial evidence for increased carotenoid content under stress in different plant species also (Parida and Jha 2012). This may also contributed in protection of chlorophyll from oxidative damages.

4.7.2 Electrolyte leakage and MDA contents

Electrolyte leakage and MDA are the common stress markers which measure the degree of injury caused by stress in plants. Stress induced ROS are responsible for these leakage and malonaldehyde formation. Malonaldehade (MDA) is the product of lipid peroxidation caused by ROS (Moore and Roberts. 1998) whereas electrolyte leakage is efflux of K^+ through ROS activated cation channels and its counterions Cl^-, $HPO4^{2-}$, NO^{3-}, $citrate^{3-}$ and $malate^{2-}$ (Demidchik et al. 2014). The present study exhibited higher electrolyte leakage and MDA content in Wt plants as compared to *SbASR-1* overexpressing transgenic groundnut lines (**Figure 3.22a, 3.23**) under both salinity and drought stress condition. However, under control conditions, transgenic lines and Wt plants exhibited similar level of MDA and electrolyte leakage. Transgenic Arabidopsis lines, overexpressing banana ASR-1 gene showed lower MDA and electrolyte leakage as compared to that of Wt plants (Dai et al. 2011).

4.7.3 Estimation of proline

Proline is the cellular stress marker accumulated in the osmotic stress condition to maintain the osmotic balance across the membrane. In the present study, the transgenic lines showed lower accumulation of free proline as compared to Wt plants under salinity and drought stress conditions (**Figure 3.22b**). In coherence to this finding, similar results were observed in transgenic tobacco lines overexpressing tomato ASR-1 and *Sb*ASR-1 grown under saline conditions (Kalifa et al. 2004; Jha et

al. 2012). In these reports, it was found that transgenic plant leaves accumulated lower Na^+ as compared to that of Wt plants and assumed that lower proline accumulation was due to reduced stress condition faced by the cells. Similarly, lower accumulation of proline in the drought treated transgenic lines may be assumed due to potential ROS scavenging ability of ASR-1 protein as proposed by Kim et al. (2012).

4.7.4 Relative water content

Battaglia et al. (2008) has proposed a special Group 7 of LEA protein exclusively for ASR-1 proteins based on conserved motifs. Out of five conserved motifs described for this group, the *Sb*ASR-1 contains 4 motifs, 1, 2, 3 and 5 arranged in order of 3-1-2-5 (**Figure 3.4**). However, only three motifs, 1, 2 and 3 are essential for membership of this group. Therefore *Sb*ASR-1 is a Group 7 LEA protein. LEA proteins are highly hydrophilic in nature and have strong water retention ability (Battaglia et al. 2008). The RWC analysis of Wt and transgenic groundnut lines exhibited higher relative water content in the transgenic lines as compared to Wt plants under salinity and drought stress (**Figure 3.22c**). Similar, results were displayed by *Sb*ASR-1 overexpressing transgenic tobacco line in salt treated condition (Jha et al. 2012). Transgenic tobacco plants overexpressing tomato ASR-1 showed lower rate of water loss as compared to that of Wt while subjected to NaCl stress (Kalifa et al. 2004). This ability of ASR-1 or *Sb*ASR-1 is assumed to because it is a LEA protein.

4.7.5 Total soluble and reducing sugar, starch content

Total soluble sugar, reducing sugar and starch content all were found to be significantly lower in the transgenic groundnut lines overexpressing *SbASR-1* gene as compared to that of Wt plants grown under salinity and drought stress (**Figure 3.24a-c**). When *ASR-1* gene was silenced in tobacco, showed higher accumulation of starch, sucrose and glucose in leaves as compared to Wt tobacco leaves (Dominguez et al

2013). Similar to this transgenic potato overexpressing potato *ASR-1* showed lower accumulation of glucose in the tuber (Frankel et al. 2007). The grape ASR-1 is found to bind with promoter of hexose transporter (*Vv*HT1) and regulate their expression (Cakir et al. 2003). When role of ASR-1 in sugar metabolism was studied extensively by Dominguez et al. (2013), it revealed that tobacco ASR-1 increased the transcript expression of hexose transporter, sucrose transporter and vacuolar glucose transporter proteins. These transporters are involved in retrieval of glucose and sucrose from leaves to the phloem for mobilisation of these nutrients to other organs. This study strongly supports the present study in which also similar results were obtained. Finally based on the above reports it was imperative to postulate that *Sb*ASR-1 behaves like a transcription factor, regulate the expression of several sugar transporter proteins and enhance the tolerance ability in transgenic plants through better mobilisation of nutrients to root or other non-photosynthetic organs.

4.7.6 *In-vivo* localisation of H_2O_2 and O_2^- radicals

In-vivo localisation of peroxide and superoxide free radicals was carried out by DAB and NBT staining. Staining of leaves from Wt and transgenic plants grown under control condition showed negligible amount of insoluble brown and blue coloured precipitate. Whereas the leaves from the Wt plants grown under salt and drought treated plants showed higher level of brown and blue coloured precipitate in DAB and NBT staining (**Figure 3.25a-b**). This observation depicted the higher level of peroxide and superoxide free radicals formation in Wt plants under stress conditions. In coherence to our finding, transgenic tobacco lines overexpressing *TaASR-1* also showed lower accumulation of H_2O_2 and O^{2-} free radicals in seedlings grown under mannitol (Hu et al. 2013). They have shown that, *Ta*ASR-1 protein enhanced transcript expression and activity of the antioxidative enzymes like catalase and SOD.

The *Gm*ASR protein was found to chelate the Fe^{3+} ions and thus may control the hydroxyl radical generation which causes damage to the nucleic acids (Li et al. 2013). A litchi *ASR-1* (*LcASR-1*) overexpressing transgenic Arabidopsis lines also exhibited higher transcript expression of catalase, SOD, APX and glutathione reductase enzyme and thus participated in better ROS scavenging in transgenic plants (Liu et al. 2013). In an *in-vitro* experiment, rice ASR-1 protein was found to reduce H_2O_2 in water molecule and the activity was comparable to catalase (Kim et al. 2012). These reports strongly supports the finding of present study and enables an assumption that *Sb*ASR-1 may also involve in ROS scavenging activity directly as antioxidants or indirectly by enhancing the expression of antioxidative enzymes.

4.7.7 Transcript expression analysis of genes coding for antioxidative enzymes

Further to confirm this ROS scavenging or activity as transcription factor of *Sb*ASR-1 protein, transcript expression of *APX*, *CAT* and *SOD* genes were performed in Wt plants and transgenic lines subjected to control or salinity and drought stress. The expression of *APX* was induced in both Wt as well as transgenic lines. But the induction in Wt was higher as compared to transgenic lines (**Figure 3.26a**). The *CAT* transcript expression was also upregulated in Wt plants under stress conditions, whereas in transgenic lines it was downregulated (**Figure 3.26b**). The major role played by APX and CAT is to detoxify the H_2O_2 into water molecules. Kim et al. (2012) has reported that rice ASR-1 showed ability to reduce H_2O_2 in water as comparable to catalase in their *in-vitro* study. Therefore, it was assumed that *Sb*ASR-1 may scavenge most of the H_2O_2 in transgenic plants and lower expression of these genes occurred. The upregulation of *SOD* transcript was higher in transgenic lines as compared to Wt plants. Similar results were found in transgenic tobacco lines overexpressing *TaASR-1* (Hu et al. 2013). The ASR-1 protein behaves as transcription

factor, binding promoter region of several genes directly or by interacting with another transcription factor (Cakir et al. 2003; Saumonneau et al. 2008; Ricardi et al. 2014). This property leads to assumption that *Sb*ASR-1 also acts as transcription factor and regulates the expression of *SOD* gene to detoxify ROS generated under stress conditions.