

## Review article

## Salinity tolerance in plants: Breeding and genetic engineering

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## Abstract

Salinity stress limits crop yield affecting plant growth and restricting the use of land. As world population is increasing at alarming rate, agricultural land is shrinking due to industrialization and/or habitat use. Hence, there is a need to utilize salt affected land to meet the food requirement. Although some success has been achieved through conventional breeding but its use is limited due to reproductive barrier and scarcity of genetic variations among major crops. The genetic engineering has proven a revolutionary technique to generate salt tolerant plants as one can transfer desired gene from any genetic resource and/or alter the expression of existing gene(s). There are examples of improved salinity tolerance in various crop plants through the use of genetic engineering. However, there is a further need of improvement for successful release of salt tolerant cultivars at field level. In this review, we have given a detailed update on production of salt-tolerant plants through genetic engineering. Future prospects and concerns, along with the importance of novel techniques, as well as plant breeding are also discussed.

**Keywords:** Helicases; Ion transporter; Lea proteins; Osmoprotectants; Salinity stress; Transcription factors.**Abbreviations:** DIGE-differential in gel electrophoresis; GB-glycine betaine; MAPK- mitogen activated protein kinase; QTL-quantitative trait loci; ROS- reactive oxygen species; TILLING- targeted induced local lesions in genome.

## Introduction

Plants are subjected to various abiotic stresses such as low temperature, salt, drought, floods, heat, oxidative stress and heavy metal toxicity during their life cycle. Among all this, salinity is the most typical abiotic stress (Mahajan and Tuteja 2005). Salinity has negative impact on agricultural productivity affecting plant growth and restricting the use of land. It is estimated that 6% of the world's total land and 20% of the world's irrigated areas are affected by salinity (Unesco Water Portal 2007). World population is increasing at an alarming rate and is supposed to reach nine billion by 2050, but our food production is limited (Varshney et al., 2011). As green revolution has already reached its ceiling, there is a major concern over food supply for the ever increasing world population. Rapidly shrinking agricultural land, due to industrialization and/or habitat use is a major threat to sustainable food production. In light of all this, it is almost imperative to raise salt tolerant plants to effectively use salt affected agricultural land for sustainable crop production. Salinity is a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when ion(s) concentration is such that osmotic pressure produced by ion(s) are equivalent to that generated by 40 mM NaCl i.e. 0.2 MPa or more (USDA-ARS 2008). As NaCl is the most soluble and widespread salt, it is not surprising that all plants have evolved mechanisms to regulate its accumulation and to select against it in favor of other nutrients commonly present in low concentrations, such as K<sup>+</sup> and NO<sub>3</sub><sup>-</sup> (Munns and Tester, 2008). Salinity problem is further aggravated by irrigation and is more in hot temperate regions, where there is excessive water loss through transpiration. The initial effect of salt stress is osmotic stress

caused by the presence of ions in rhizosphere which restricts extraction of water by roots and results in reduced plant growth. The secondary effects of salt stress are caused by ionic disequilibrium, which result in inactivation of enzymes, nutrient starvation, ionic toxicity in tissues and oxidative stress. Reactive oxygen species produced due to oxidative stress further damage plants by enhancing lipid peroxidation, DNA damage and inhibition of photosynthesis (Flowers et al., 1977; Greenway and Munns, 1980; Turan and Tripathy, 2012). If the concentration of salt is very high, it leads to plant death (Niu et al., 1995; Yeo 1998; Glenn et al., 1999). There is inter-species and intra-species variability in salinity tolerance in plants (Turan and Tripathy, 2012). Plants adopt different mechanism to resist salinity stress like excluding salts or accumulating ions into different tissue compartments, vacuoles or old leaves (Flowers and Yeo, 1992; Munns, 1993; Yeo, 1998). In most plants, Na<sup>+</sup> and Cl<sup>-</sup> are effectively excluded by roots, while water is taken up from the soil (Munns 2005). In response to osmotic stress, plants produce osmolytes like glycine betaine, trehalose or proline, which protect them from dehydration or protein denaturation. However, oxidative stress-an outcome of ionic stress lead to the production of different enzymatic or non-enzymatic antioxidants, which protect plants from harmful effects of reactive oxygen species (Shao et al., 2007). Plant breeding is being used since long to produce salt tolerant and more productive lines. However, its use is limited due to multigenic nature of salt tolerance and presence of low genetic variation in major crops. In recent times, genetic engineering has played a pivotal role in producing salt tolerant plants. In this review, major emphasis has been given

on different approaches of genetic engineering, used for generating salt tolerance plants. Role of quantitative trait loci (QTLs) and molecular markers in salinity improvement of plants have been briefly described. In the future prospects and concerns section, importance of functional genomics and use of novel techniques along with important cognizant issues, for the successful release of a salt tolerant crop cultivar, at field level are discussed.

### Conventional breeding approach

Plant breeding has been used since long for the production of high yielding and stress tolerant crops. Plant breeders have used genetic variation in crops, at intraspecific, interspecific and intergeneric levels to produce salt tolerant lines. Lots of salt tolerant crop cultivars/lines have been produced by breeding; for example, salt tolerant CSR10, CSR13, CSR27 rice cultivars developed at Central Soil Salinity Research Institute, Karnal. However, breeding has the limitation due to low magnitude of variation in gene pools of most crops. Another problem associated with conventional breeding, is that if the gene is present in a wild relative of the crop, there is difficulty in transferring it to the domesticated cultivar, due to reproductive barrier.

### Role of QTL and molecular markers in engineering salinity tolerance

QTLs are segment of genetic material, in the genome of an organism linked with a particular trait. Salt stress tolerance is a complex trait, so the QTLs related with salt tolerance have significant role in understanding the stress response and generating stress-tolerant plants (Gorantla et al., 2005). There has been progress in methods of identifying genes underlying QTLs, instead of only map based cloning approach (Salvi and Tuberosa, 2005); new approaches like microarray based transcriptional profiling of differential gene expression (Sahi et al., 2006; Walia et al., 2007) or combination of genetic mapping and expression profiling (Marino et al., 2009; Pandit et al., 2010) are being used for identifying genes linked with QTLs. Several QTLs involved in the salt stress responses, have been reported of recent (Cadmac 2005; Ren et al., 2005; Thomson et al., 2007; Ammar et al., 2009; Pandit et al., 2010). The recent developments in molecular marker analysis have made it feasible to analyze both simply inherited; as well as the quantitative traits, and identify individual gene controlling the trait of interest. Molecular markers could be used to tag quantitative trait loci and to evaluate their contributions to the phenotype by selecting for favorable alleles at these loci in a marker-aided selection scheme aiming to accelerate genetic advance. Advanced backcross QTL analysis can be used to evaluate mapped donor introgression in the genetic background of an elite recurrent parent (Tanksley and Nelson, 1996).

### Genetic engineering approach for salinity stress tolerance

Plant breeding strategy for salt tolerance is not much successful due to reproductive barrier and also as it involves the risk of other undesirable traits transfer. So to avoid this problem, genetic engineering strategy is more preferred, as it only deals with the specific gene(s) transferred. Plants try to cope with salinity by inducing various metabolic changes like production of osmolytes, antioxidative enzymes and upregulating various genes involved in stress response like ion transporters, ion channels, transcriptional factors and various signaling pathway components. These plant

responses to salinity have been utilized by scientists to generate transgenic; either by, transferring such stress responsive gene(s) to salt-sensitive crop plant from different genetic background (relatively salt-tolerant plants) or altering the expression of existing genes.

There are a number of gene(s) known which are responsible for salinity tolerance when transferred in plants through genetic engineering (Fig. 1). Details of these genes with their source, target plant and type of gene product are summarized in supplementary Table. S1.

### Gene(s) for osmoprotectants

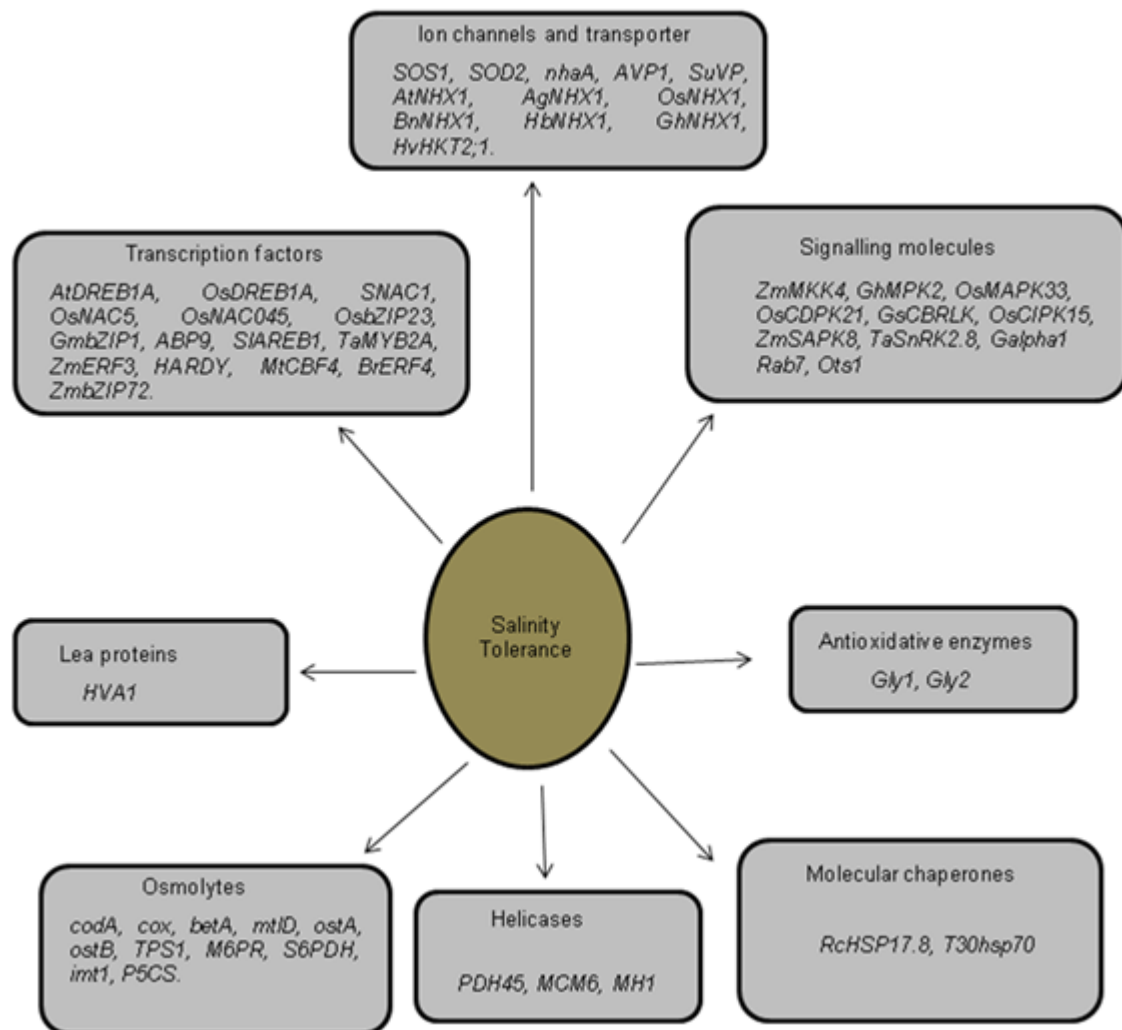
When plants are exposed to stress conditions, metabolic shifts occur and result in changes in the levels of a various cellular metabolites. Such modifications in response to abiotic stress appear to be associated with the enhanced ability to tolerate such stressful conditions. Metabolites that might be expected to contribute to enhanced salt stress tolerance include soluble sugars, amino acids, organic acids, polyamines and lipids (Guy 1990). One important group of such metabolites is so-called 'compatible solutes', which are small organic metabolites that are very soluble in water and are non-toxic at high concentrations.

### Trehalose

Trehalose, a nonreducing disaccharide plays a crucial role in metabolic homeostasis and abiotic stress tolerance in various organisms. In plants, trehalose-6-phosphate synthase (TPS) catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate (G-6-P) to form trehalose-6-phosphate (T-6-P) and uridine diphosphate (UDP). Subsequently, the T-6-P is dephosphorylated into trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958; Goddijn and Smeekens 1998). Li et al., (2011a) have shown that overexpression of *OsTPS1* gene encoding trehalose-6-phosphate synthase in rice improved the tolerance of rice to high salinity and other abiotic stresses. Overexpression of this gene in rice is associated with increased level of trehalose and proline along with upregulation of some of the stress inducible genes including *WS118*, *RAB16C*, *HSP70* and *ELIP*. Transfer of the yeast *TPS1* into tomato resulted in higher chlorophyll, starch content and enhanced tolerance against drought, salt and oxidative stresses (Cortina and Culiáñez-Macià, 2005). Rice plants transformed with *Escherichia coli*'s trehalose biosynthetic gene(s) (*otsA* and *otsB*) as a fusion gene exhibits less photo-oxidative damage and a more favorable mineral balance under salt, drought and low-temperature stress conditions (Garg et al., 2002). Similarly, in tobacco, heterologous expression of *AtTPS1* gene from Arabidopsis increased tolerance to several abiotic stresses such as drought, desiccation and temperature stresses (Almeida et al., 2005). However, the gene transfer for trehalose can also produce aberrations in plant growth such as dwarfism, delayed flowering, abnormal root development and lancet-shaped leaves (Romero et al., 1997; Avonce et al., 2004; Cortina and Culiáñez-Macià, 2005).

### Glycine betaine

Glycine betaine (N, N, N-trimethyl glycine) is a quaternary ammonium compound found in bacteria, haemophilic archaeobacteria, marine invertebrates, plants and mammals (Rhodes and Hanson, 1993; Chen and Murata, 2002; Takabe et al., 2006; Chen and Murata, 2008). GB is synthesized; either by, the oxidation (or dehydrogenation) of choline or by



**Fig 1.** Genes coding for different transcription factors, lea proteins, osmolytes, helicases, molecular chaperones, antioxidative enzymes, signalling molecules, ion channels and transporter which are known to impart salinity tolerance to different plants when overexpressed.

the N-methylation of glycine (Chen and Murata, 2002). It accumulates to osmotically significant levels in many salt-tolerant plants (Rhodes and Hanson, 1993) and halotolerant cyanobacteria (Chen and Murata, 2008). Levels of GB vary considerably among plant species and organs. Plants of many taxonomically distant species normally, contain low levels of GB (these plants are known as natural accumulators of GB), but they accumulate larger amounts of GB when subjected to abiotic stress (Storey et al., 1977). In many other species GB is not detectable under normal or stressful conditions. There are now strong evidences that GB plays an important role in abiotic stress tolerance. The biological functions of GB have been studied extensively in higher plants such as spinach, sugar beet, barley and maize (Rhodes and Hanson, 1993; Chen and Murata, 2008). The availability of GB-accumulating transgenic plants has provided insight into its plant cell protection mechanism. Furthermore, many lines of GB-accumulating transgenic plants exhibit greatly improved tolerance to various types of abiotic stresses and their properties suggest promising strategies for the development of stress-tolerant crop plants. Gene(s) that encoding GB-biosynthetic enzymes have been cloned from different organisms to generate transgenic plants (for recent reviews see: Chen and Murata, 2008; 2011). The transgenic plants accumulate GB at different levels and exhibit enhanced

tolerance to salt and other abiotic stresses. Exogenous application of glycine betaine improves salinity tolerance in many plant species enhancing plant growth and yield (Harinasult et al., 1996; Mäkelä et al., 1999). Transgenic tomato and rice expressing *codA* gene from *Arthrobacter gobiformis* show enhanced salinity tolerance (Goel et al., 2011; Sakamoto et al., 1998). Similarly, transgenic rice plants, for *cox* gene coding for choline oxidase from *Arthrobacter pascens* were found salt tolerant (Su et al., 2006). Genetically engineered tobacco (*Nicotiana tabacum*) plants for *betA* gene from *E.coli* coding for choline dehydrogenase exhibit salt tolerance (Holmström et al., 2000). In the same vein, transgenic plants for enhanced synthesis of glycine betaine have also been produced in Brassica, Arabidopsis and *Solanum tuberosum* showing enhanced salinity tolerance (Hayashi et al., 1997; Hong et al., 2000; Prasad et al., 2000; Sulpice et al., 2003; Ahmad et al., 2008).

#### **Mannitol, Sorbitol and Ononitol**

Bacterial gene *mtID*, which codes for mannitol-1-phosphate dehydrogenase, when expressed in tobacco, causes mannitol accumulation (Tarczynski et al., 1993). Similarly, ectopic expression of *mtID* from *E.coli* in wheat plants results in

enhanced tolerance to salt stress due to protective role of mannitol (Abebe et al., 2003). Arabidopsis plants transformed with celery's mannose-6-phosphate reductase (*M6PR*) gene produced mannitol and were found more tolerant as comparative to wild type under salinity stress (Sickler et al., 2007). Japanese persimmon (*Diospyros kaki* Thunb. cv Jiro) when transformed with apple cDNA for *S6PDH* encoding NADP dependent sorbitol-6-phosphate dehydrogenase, accumulates sorbitol and showed higher salinity tolerance than untransformed plants as reflected by higher ratio of variable to maximum fluorescence (Fv/Fm, Gao et al., 2001). Likewise improved salt and drought tolerance was found in *Nicotiana tabacum*, when transformed with cDNA of *imt1* encoding for myo-inositol-*o*-methyltransferase. This was indicated by accumulation of methylated inositol D-ononitol exceeding 35 mmol/g fresh weights and higher CO<sub>2</sub> fixation capacity in transgenic plants under stress condition (Sheveleva et al., 1997).

### Proline

In plants, proline is synthesized from its precursor glutamic acid and acts as an osmoprotectant under osmotic stress condition (Delauney and Verma, 1993). Two enzymes play important role in the biosynthesis of proline which are pyrroline-5-carboxylate synthase (SPCS) and pyrroline-5-carboxylate reductase (P5CR) (Ashraf and Foolad, 2007). Transgenic rice plants of mung bean *P5CS* gene encoding for pyrroline-5-carboxylate synthase under constitutive or stress inducible promoter showed significant salinity tolerance (Su and Wu, 2004). Likewise transformed *Nicotiana tabacum* plants with cDNA encoding delta-1-pyrroline-5-carboxylate synthetase (*P5CS*) from *Vigna acortifolia* were found more tolerant to salinity and drought stress (Kishore et al., 1995). Similarly, tobacco plants engineered for higher proline production by removing feedback inhibition of rate limiting enzyme in proline biosynthesis showed drought tolerance (Hong et al., 2000).

### Engineering plants for transporters and ion channels

There is a lot of genetic diversity in plants with respect to sensitivity to NaCl. Accordingly, they are classified as halophyte (salt tolerant) and glycophyte (salt sensitive). Halophyte can grow at higher concentration of salt than glycophyte, they do it; either by, excluding Na<sup>+</sup> or accumulating Na<sup>+</sup> in cellular compartments like vacuoles higher K<sup>+</sup>/Na<sup>+</sup> ratio is maintained in the cytoplasm. Excess Na<sup>+</sup> leads to the loss of ionic homeostasis. Potassium acts as a coenzyme for many cytoplasmic enzymes, but when excess Na<sup>+</sup> is present in rhizosphere, it competes for K<sup>+</sup> particularly at low affinity K<sup>+</sup> channels, leading to low K<sup>+</sup>/Na<sup>+</sup> ratio in cytoplasm. Excess Na<sup>+</sup> in cytoplasm is equally harmful to both halophyte and glycophyte. Genetic engineering of genes for antiporter or ion channels have been successful in generation of salt tolerant plants by maintaining higher K<sup>+</sup>/Na<sup>+</sup> ratio. Overexpression of *AtSOS1* encoding a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter which share sequence similarity to Na<sup>+</sup>/H<sup>+</sup> antiporters from bacteria and fungi leads to salt tolerance in Arabidopsis (Shi et al., 2000, 2003). In the same vein, plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter gene *SOD2* from *Schizosaccharomyces pombe* resulted in enhanced salt tolerance in Arabidopsis when overexpressed (Gao et al., 2003). Similarly, *nhaA* of *E.coli* which encodes for Na<sup>+</sup>/H<sup>+</sup> antiporter when expressed in rice improved salt tolerance (Wu et al., 2005). Another strategy of salinity tolerance by plants is to sequester Na<sup>+</sup> ion into vacuole, so as to prevent

cytosol from its toxicity. The transfer of Na<sup>+</sup> into vacuole is driven by a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter which in turn is driven by the electrochemical gradient of protons generated by the vacuolar H<sup>+</sup>-ATPase and H<sup>+</sup>-pyrophosphatase (Blumwald 1987). The overexpression of *AVP1* encoding for vacuolar H<sup>+</sup>-pyrophosphatase in Arabidopsis results in salinity tolerance (Gaxiola et al., 2001). Recently Liu et al. (2011) have isolated and characterized a gene *ScVp* from *Suaeda corniculata*, encoding a vacuolar H<sup>+</sup>-pyrophosphatase (V-H<sup>+</sup>-PPase), whose ectopic expression in Arabidopsis caused salinity tolerance. Genetic engineering of cotton plants with vacuolar H<sup>+</sup>-pyrophosphatase (*AVP1*) from Arabidopsis confers salinity and drought tolerance (Pasapula et al., 2011). Apse et al. (1999) showed that *Arabidopsis thaliana* plants expressing *AtNHX1*, a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter, were salt tolerant. Similarly, overexpression of *AtNHX1* in tomato and Brassica enhances their salt tolerance (Zhang et al., 2001; Zhang and Blumwald, 2001). Likewise when a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene *AgNHX1* from *Atriplex gmelini*, is overexpressed in rice, it enhanced salt tolerance (Ohta et al., 2002). Rice *OsNHX1* encoding for vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter when overexpressed, results in increased salinity tolerance (Fukuda et al., 2004). Transgenic maize and wheat for *AtNHX1* showed higher tolerance to salinity (Xue et al., 2004; Yin et al., 2004). Similarly, the overexpression of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes; *HbNHX1* (barley), *GhNHX1* (cotton) and *BnNHX1* (*Brassica napus*) in tobacco, improved salinity tolerance (Wang et al., 2004; Wu et al., 2004; Lu et al., 2005). Recently, it was found that when *AtNHX5* was expressed in Paper mulberry, it conferred salinity and drought tolerance in transgenic (Li et al., 2011b). Sodium ions enter the cell through several low and high affinity potassium carriers. There are three types of low affinity K<sup>+</sup> transporters: inward rectifying channels (KIRC), outward rectifying channels (KORC) and voltage independent non-selective cation channels (NSCC). Arabidopsis *AtHKT1* carry out circulation of Na<sup>+</sup> in plant by mediating Na<sup>+</sup> loading in leaf phloem and Na<sup>+</sup> unloading from the root phloem sap (Berthomieu et al., 2003). A mutation in *AtHKT* leads to increase of Na<sup>+</sup> concentration in shoot and enhancement of plant Na<sup>+</sup> sensitivity (Maser et al., 2002). Mian et al. (2011) have shown that when barley *HvHKT2;1* was overexpressed, transgenic plants were more tolerant to salt due to increased Na<sup>+</sup> loading into xylem and accumulation of Na<sup>+</sup> into shoot. Therefore, the increased uptake and translocation of Na<sup>+</sup> is also responsible for salinity tolerance.

### Engineering of antioxidative enzymes

Reactive oxygen species (ROS) are produced under normal conditions in plants; but under stress conditions, their level is highly increased. Plants have devised antioxidative defense system to scavenge harmful ROS, and protect plant cells from oxidative injury. This antioxidative defense involves both enzymatic and non-enzymatic metabolites. Various transgenic overexpressing antioxidative enzymes like superoxide dismutase, glutathione reductase, glutathione peroxidase and ascorbate peroxidases; have been generated, which show tolerance to various abiotic stresses (Bowler et al., 1991; Sen-Gupta et al., 1993; Slooten et al., 1995; Van Camp et al., 1996; Roxas et al., 1997; Prashanth et al., 2008). Alfalfa helicase *MHI* when expressed in Arabidopsis enhances salinity and drought tolerance, by improving its antioxidative defense (Luo et al., 2009). Lots of transgenic have been produced by engineering methylglyoxal pathway. Methylglyoxal is a cytotoxic compound which accumulates

to higher concentration in plants during stress conditions. Glyoxalase I and glyoxalase II are the enzymes involved in detoxification of methylglyoxal. Many transgenic plants overexpressing genes; *GlyI* and *GlyII* encoding for enzymes glyoxalase I and glyoxalase II respectively, have been found to show salinity tolerance (Singla-Pareek et al., 2003, 2008; Yadav et al., 2005).

### Engineering for transcription factors

In line with this argument that single gene level management, for stress tolerance is not so affective; efforts were made to raise transgenic plants for stress inducible transcription factors: as a transcription factor regulates many genes. It is also likely that many stress responsive genes, may share a common transcription factor. Various transcription factors belonging to the families of DREB, NAC, MYB, MYC, Cys2His2 zinc finger, bZIP, AP2/ERF and WRKY are known to be involved in salt stress tolerance. They bind to the promoter and/or regulatory elements of genes responsive to stress. Member(s) of different groups may be involved in a single response, and members of the same group may also be responsible for different kind of stress responses. Many transgenic tolerant to salinity stress have been produced through genetic engineering of gene(s) for transcription factors. Transgenic Arabidopsis plants overexpressing *AtDREB1A* were found tolerant to dehydration and freezing (Liu et al., 1998). Similarly, overexpression of rice *OsDREB1A* in Arabidopsis results in freezing dehydration and salt tolerance (Dubouzet et al., 2003). Rice plants overexpressing *OsDREB2A* are comparatively tolerant to salinity and dehydration stress than untransformed plant (Mallikarjuna et al., 2011). The plant specific transcription factor group NAC (NAM, ATAF1/2, and CUC2) is required for its role in plant development and stress response. Transgenic rice plants overexpressing *SNAC1* (stress responsive NAC 1) showed enhanced salinity and drought tolerance (Hu et al., 2006). Similarly, rice plants overexpressing *SNAC2* (a rice NAC transcription factor group), exhibit higher salinity tolerance (Hu et al., 2008). Overexpression of *OsNAC5* in rice and Arabidopsis ectopic expression in enhanced salinity and drought tolerance, while knockdown of this gene in rice by RNAi lead to salt susceptibility (Song et al., 2011). Likewise overexpression of *ONAC045* encoding for NAC transcription factor gene in rice enhanced salinity and drought tolerance (Zheng et al., 2009). In the same vein, *OsbZIP23*, a member of basic leucine zipper (bZIP) transcription factor family from rice, when overexpressed results in drought and salt tolerance (Xiang et al., 2008). Recently a gene, *GmbZIP1*, encoding for a novel bZIP transcription factor from soybean was found to provide multiple abiotic stress tolerance (salt, drought and low temperature) to transgenic plants of Arabidopsis and tobacco, when overexpressed (Gao et al., 2011). The constitutive expression of maize *ABP9*; encoding a bZIP type transcription factor in Arabidopsis, results in enhancement of multiple stress tolerance including high salt, drought, freezing and oxidative stress (Zhang et al., 2011a). Ectopic expression of a maize gene *ZmbZIP72* (a bZIP transcription factor) in Arabidopsis, result in salinity tolerance (Ying et al., 2012). Tomato plants overexpressing *SIAREB1* show drought and salt tolerance (Orlenna et al., 2010). Similarly, *ZFP179* (a salt responsive gene) imparts enhanced salt tolerance after it was overexpressed in rice (Sun et al., 2010). Likewise transfer of wheat *TaMYB2A* in Arabidopsis provides multiple stress tolerance including salt and drought (Mao et al., 2011). Zhang et al. (2009) have shown that Ectopic expression of

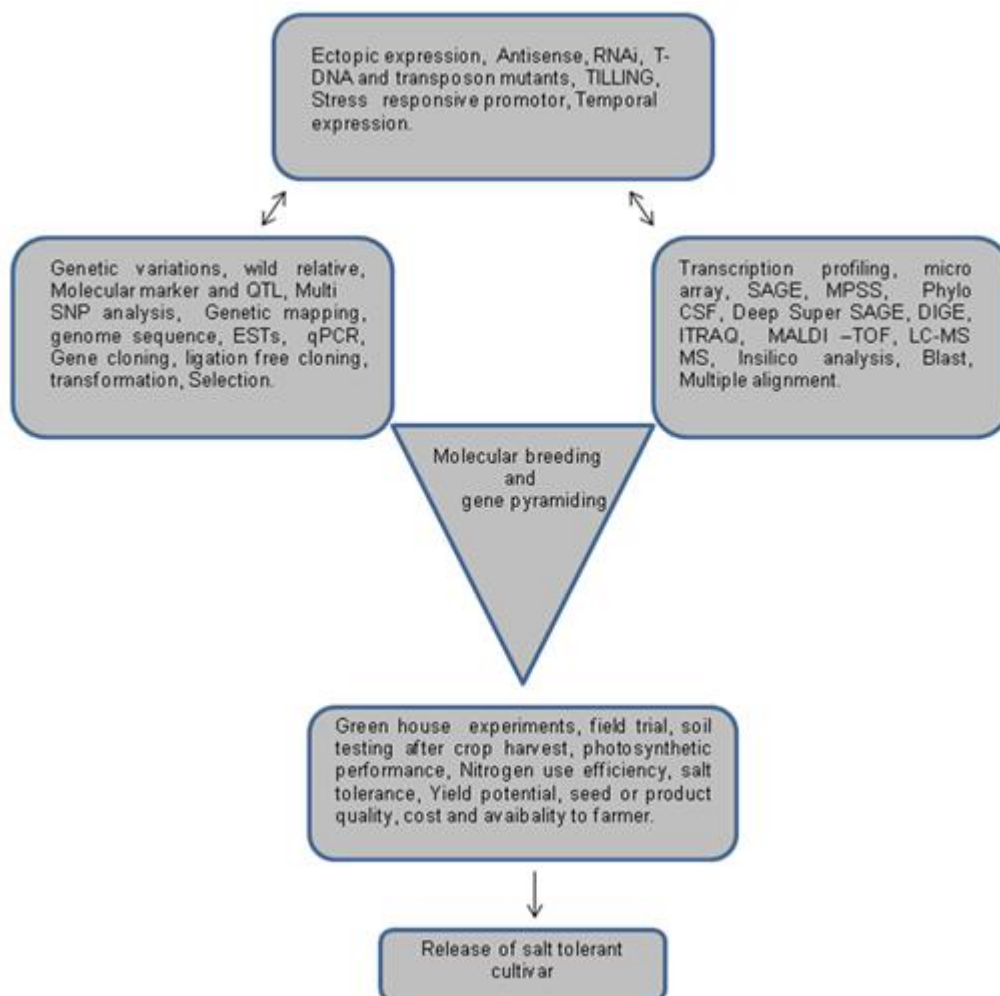
soybean *GmERF3* gene encoding for AP2/ERF transcription factor tobacco enhances tolerance to both biotic and abiotic stresses. Transgenic *Trifolium alexandrinum* L. of a gene *HARDY* from Arabidopsis were found more tolerant to salinity and drought stress (Abogadallah et al., 2011). When MtCBF4 gene from *M.truncatulla* encoding for a transcription factor was overexpressed in Arabidopsis resulted in enhanced salinity and drought tolerance (Li et al., 2011c). Similarly, transgenic Arabidopsis plants overexpressing of *BrERF4* from Brassica showed enhanced salinity and drought tolerance (Seo et al., 2010).

### Transgenic for Helicases

Helicases (RNA or DNA) are proteins involved in unwinding double stranded DNA / RNA. These ATP dependent molecules play a regulatory role in basic genetic processes including replication, transcription, translation and repair or recombination (Lohman and Bjornson, 1996; West 1996; Tuteja and Tuteja, 2004a; b). They have been classified in five superfamilies based on their amino acid sequence, from superfamily 1 (SF1) to superfamily 5 (SF5) (Gorbalenya and Koonin, 1993). Sanan-Mishra et al. (2005) have shown that pea DNA helicase gene (*PDH45*), when overexpressed in tobacco enhances salinity tolerance in transgenic plants without affecting yield. Pea DNA helicase 47 (*PDH47*) transcripts were found induced in both shoot/root in response to salinity and cold. This purified recombinant protein showed ATP dependent DNA/RNA helicase activity and DNA dependent ATPase activity (Vashisht 2005). Liu et al. (2008) have isolated and characterized a salt inducible DEAD box helicase; AvDHI, from halophyte *Apocynum venetum* and suggested its possible role in salt tolerance. Similarly, ectopic expression of a DEAD box helicase (*MHI*) from *Medicago sativa* in Arabidopsis, results in salinity and drought tolerance by enhancing ROS scavenging capacity and osmotic adjustment (Luo 2009). Chung et al. (2009) have isolated salt inducible DEAD box helicase from soybean named GmRH and speculated its role in RNA processing under salinity and chill stress. Dang et al. (2011a) have shown that DNA helicase MCM6 transcript was upregulated in pea during salt and cold stress but not in drought or ABA treatment. Transgenic tobacco plants overexpressing *MCM6* were found salinity tolerant. The investigators also reported stress responsive elements in promoter of *MCM6* (Dang et al., 2011b).

### Engineering of molecular chaperones

Molecular chaperones are a diverse group of proteins involved in various cellular functions comprising folding/unfolding, macromolecular assembly/disassembly, keeping proteins in their native state and preventing their aggregation under various stress conditions, helping in protein synthesis/degradation and targeting to their cellular compartments (Boston et al., 1996). Of late they have been implicated in various physiological processes and plant defense under stress conditions (Chen and Shimomoto, 2011; Gupta and Tuteja, 2011; Hahn et al., 2011; Qi et al., 2011). Reddy et al. (2011) have isolated *pgHsc70* (encoding for cytoplasmic HSP70) from *Pennisetum glaucum* and suggested its probable role in plant salinity tolerance as it imparts salinity tolerance to transformed. Similarly, transformed *E.coli* with salt inducible gene *DcHsp17.7* encoding for a small heat shock protein (SHSP) from *Daucus*



**Fig 2.** Schemataic diagram showing strategies and techniques involved for the production of salt tolerant cultivar at field level.

SNP: single nucleotide polymorphism; ESTs: express sequence tags; qPCR: quantitative real time ployperase chain reaction; SAGE: serial analysis of gene expression; DIGE: differential in gel electrophoresis; iTRAQ: isobaric tag for relative and absolute quantification; MALDI-TOF: matrix assisted laser desorption ionization-time of flight; LC-MS: liquid chromatography coupled with mass spectrometry; ELISA: enzyme linked immunosorbent assay; TILLING: targeted induced local leisions in genome; RNAi: RNA interference.

*carota* L. were found to show enhanced salinity tolerance (Song and Ahn 2011). Jiang et al. (2009) observed that *E.coli*, yeast and Arabidopsis transformed with *RcHsp17.8* (encoding for a SHSP) from *Rosa chinensis* were tolerant to multiple stresses. According to Montero-Barrientos et al. (2010) ectopic expression of *Trichoderma harzianum*'s *T30hsp70* gene in Arabidopsis results in salt, osmotic and oxidative stress tolerance. The heat shock proteins aren't functionally limited to stress conditions, but do play role in normal development and function of plant and various cellular organelles. Constan et al. (2004) have shown that stromal HSP100 protein is required for normal chloroplast development. The mutant of *atHSP93-V* which encodes for the homolog of HSP93, are smaller, paler than wild type and have chloroplasts with less thylakoid membranes.

#### Engineering for lea proteins

Late embryogenesis proteins are a group of hydrophilic proteins produced late during embryo development, and

constitute about 4% of the total cellular proteins. These proteins have been classified into six groups; based on their amino acid sequence, mRNA homology, and expression pattern (Wise 2003). They carry out various functions like acting as hydrating buffers, sequestering ions, helping in renaturation of proteins and acting as chemical chaperones (Dure 1993; Goday et al., 1994). It was found that late embryogenesis abundant (LEA) protein gene *HVA1* from *Hordeum vulgare* L. upon transformation into rice confers salinity and drought tolerance to transgenic plants (Xu et al., 1996). The stress tolerant features (including salt and drought tolerance) of *HVA1* gene transformed plants have further been proven in Basmati rice (Rohila et al., 2002) and *Morus indica* (Lal et al., 2008). In the same vein, ectopic expression of *PM2* from soybean; encoding for a type 3 LEA protein in *E.coli*, results in salinity tolerance and therefore suggesting its probable role in salinity tolerance in plants Liu et al., 2010.

## Engineering plants for signaling molecules

Exposure of any stress to plants causing change in normal plant development is perceived by some kinds of sensor which leads to a signaling cascade resulting in a stress response by plant. These signaling pathways may be specific or non-specific depending upon the type of stress. Signaling pathways for different stresses may crosstalk. These signaling pathways involve many signaling molecules, and may be ABA dependent or independent.  $\text{Ca}^{2+}$  is most common secondary messenger in plants responding to various stimuli (Harper et al., 2004).  $\text{Ca}^{2+}$  is known to be involved in most common pathway for salt stress; Salt Overly Sensitive (SOS) pathway. A change in cytoplasmic  $\text{Ca}^{2+}$  transient is sensed by SOS3; a calcium binding protein (Ishitani et al., 2000). This in the presence of  $\text{Ca}^{2+}$  activates SOS2; a serine-threonine protein kinase (Sanchez-Barrena et al., 2007). SOS3-SOS2 kinase complex regulate the expression, as well as activity of  $\text{Na}^+/\text{H}^+$  exchanger, which is responsible for salinity tolerance (Qiu et al., 2002). Mitogen activated protein kinases (MAPK) are known to be involved in signaling of multiple abiotic stress including salt, drought, temperature, and other physiological processes like cell division (Andreasson and Ellis, 2010; Wu et al., 2010). MAPK cascade involve three tiers of protein kinases: MAPK, MAPK kinase (MAPKK) and MAPKkinase kinase (MAPKKK). MAPK is activated by MAPKK (MAPK kinase) by phosphorylation at two residues and MAPKK in turn is activated by MAPKKK. (Pitzschke et al., 2009). Many transgenic plants have been produced with high salinity tolerance by engineering MAPK cascade. Ectopic expression of *Nicotiana* protein kinase *MAPKKK/NPK1* in maize, leads to the activation of oxidative signal eventually enhancing salt, heat and cold tolerance (Shou et al., 2004). A novel MAPKK from maize, *ZmMKK4*, when overexpressed in Arabidopsis, confers salinity and drought tolerance (Kong et al., 2011). Zhang et al. (2011b) have shown that a MAPK from cotton *GhMPK2* is induced by salt, ABA and drought stress. Its overexpression into tobacco leads to salinity and drought tolerance. In another study it was found that rice plants overexpressing *OsMAPK33* are more sensitive to salt stress than wild type (Lee et al., 2011). There are also calcium dependent protein kinases (CDPKs), which are involved in salt stress response. Asano et al. (2011) have characterized one CDPK gene *OsCDPK21* from rice which when was overexpressed, results in enhanced salinity tolerance in transgenic. Ectopic expression of *GsCBRLK* gene; encoding plant specific calcium-dependent calmodulin binding receptor like kinase from *Glycine soja* in Arabidopsis, enhanced salt and ABA tolerance (Yang et al., 2010). Another signaling molecule, Calcineurin B-like (CBL) proteins, are a group of  $\text{Ca}^{2+}$  sensor in plants. They play an important role in relaying the signal in diverse stress response by interacting with CBL-interacting protein kinases (CIPKs) (Batistic and Kundla, 2009; Weinl and Kundla, 2009). Xiang et al. (2007) have characterized a number of OsCIPKs genes in rice, for their stress inducibility and stress tolerance, out of those *OsCIPK15* was responsible for salinity tolerance in transgenic when was overexpressed. Another family of kinases which play important role in signaling stress response belongs to; sucrose non-fermenting 1-related protein kinase 2 (SnRK2) families (Coello et al., 2011). Recently a gene from maize, *ZmSAPK8* of SnRK2 family, has been cloned. The overexpression of this gene into Arabidopsis confers salinity tolerance, along with upregulation of transcription of other stress marker genes like *RD29A*, *RD29B*, *RAB18*, *P5CS1*, *ABI1* and *DREB2A* (Ying et al., 2011). Similarly, in another study it was found that

ectopic expression of wheat *TaSnRK2.8* of SnRK2 family, in Arabidopsis improves salinity tolerance along with upregulation of transcripts of ABA biosynthesis genes (Zhang et al., 2010). Plant lectin receptor like kinases (LecRLKs) are also known to mediate signaling during stress response (Joshi et al., 2010). G-Protein coupled receptors (GPCRs) are known to perceive extracellular signals, and transduce subsequently to heteromeric G-proteins, which further pass signal to downstream effector (Tuteja 2009; Yadav and Tuteja, 2011). When gene from *Pisum sativum* (*Galpha1*); encoding for G-alpha subunit, was overexpressed in Arabidopsis, it enhanced salinity and heat tolerance (Misra et al., 2007). Ectopic expression of *Rab7* from *pennisetum glaucum*; encoding for Rab-GTPase (a GTP binding protein), in tobacco enhanced salinity tolerance (Agarwal et al., 2008). Conti et al. (2008) have identified two SUMO proteases; OVERLEY TOLERANT TO SALT 1 (OTS1) and OTS2, in Arabidopsis. Double mutant of *ots1* and *ots2* is salt sensitive; whereas, overexpression of *ots1* confers salinity tolerance to the transgenic.

## Future prospects and Concerns

Plant breeding has been mainly used as a tool in the last century to raise abiotic stress tolerant plants and many salt tolerant varieties for different crops were developed. But due to reproductive barrier and narrow genetic variations present in food crops, use of this technique is limited. On the other hand, genetic engineering has successfully utilized the genetic variations present for salt tolerance in different wild relatives of crops and other organisms for the production of salt tolerant plants. There are many genes of unknown function (20-30% in every genome sequenced) which can impart multiple stress tolerance to plants. There is still scope in understanding the functional genomics which will further facilitate the generation of salt tolerant crop plants. The use of "omics" tool and next generation sequencing have promising role in elucidating gene function and response of plant to salt stress. The use of more advanced and less time consuming technologies like Deep Super SAGE, ligation free cloning, Multi-SNP analysis, Glyco-proteomics and Phylo-CSF (a comparative genomics tool to distinguish coding and non-coding region) along with genetic engineering through a systematic approach (Fig. 2) will be time saving and more fruitful in production of salt tolerant crop plants at field level.

Despite progress in technologies and genetic engineering of salt tolerance in plants, success has not been achieved at field level. Majority of the salinity tolerant plants, produced through genetic engineering, are tested for tolerance under laboratory controlled conditions at seedlings stage or reproductive stage in green house. Salinity stress is a multigenic trait which is very complex and often mixed with more than one stress at field level under fluctuating conditions. For developing a successful salt tolerant cultivar at field level, following parameters need be taken into account (of the transgenic plant generated):-

1. How transgenic plant behaves in natural environment under mixed stresses and/or fluctuating environmental conditions?
2. How much it is tolerant at reproductive and/or seed set stage?
3. Effect on yield potential: Transgenic with decreased yield are not desirable.
4. Disease susceptibility.
5. Plant height and root size are of immense importance.
6. Photosynthetic performance and nitrogen use efficiency (NUE) under field conditions.

7. Salt testing of soil after each crop harvest. Whether the transgenic is an ion excluder or not?
8. Finally, the seed cost and availability to farmers.

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