# Physiology and Molecular Biology of Salinity Stress Tolerance in Plants

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# Physiology and molecular biology of salinity stress tolerance in plants

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**The productivity of plants is greatly affected by various environmental stresses. Soil salinity affects plant growth and development by way of osmotic stress, injurious effects of toxic $Na^+$ and $Cl^-$ ions and to some extent $Cl^-$ and $SO_4^{2-}$ of $Mg^{2+}$ and nutrient imbalance caused by excess of $Na^+$ and $Cl^-$ ions. Salinity stress response is multigenic, as a number of processes involved in the tolerance mechanism are affected, such as various compatible solutes/osmolytes, polyamines, reactive oxygen species and antioxidant defence mechanism, ion transport and compartmentalization of injurious ions. Various genes/cDNAs encoding proteins involved in the above-mentioned processes have been identified and isolated. The role of genes/cDNAs encoding proteins involved in regulating other genes/proteins, signal transduction process involving hormones like ABA, JA and polyamines, and strategies to improve salinity stress tolerance have also been discussed.**

EXCESS amount of salt in the soil adversely affects plant growth and development. Nearly 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity[1]. Processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by high salt concentration, ultimately causing diminished economic yield and also quality of produce.

Plants are classified as glycophytes or halophytes according to their capacity to grow on high salt medium. Most plants are glycophytes and cannot tolerate salt-stress. High salt concentrations decrease the osmotic potential of soil solution creating a water stress in plants. Secondly, they cause severe ion toxicity, since $Na^+$ is not readily sequestered into vacuoles as in halophytes. Finally, the interactions of salts with mineral nutrition may result in nutrient imbalances and deficiencies. The consequence of all these can ultimately lead to plant death as a result of growth arrest and molecular damage[2]. To achieve salt-tolerance, the foremost task is either to prevent or alleviate the damage, or to re-establish homeostatic conditions in the new stressful environment. The growth rate must resume, albeit at a reduced rate[1]. However, barring a few exceptions, the conventional breeding techniques have been unsuccessful in transferring the salt-tolerance trait to the target species. A host of genes encoding different structural and regulatory proteins have been used over the past 5–6 years for the development of a range of abiotic stress-tolerant plants. The appreciation is growing that usage of regulatory genes is a more effective approach for developing stress-tolerant plants. Thus, understanding the molecular basis will be helpful in developing selection strategies for improving salinity tolerance. Identification of molecular markers linked to salinity/drought-tolerance traits has provided plant breeders a new tool for selecting cultivars with improved drought-tolerance. The present review deals with proteins/genes, which are induced in crop plants in response to salinity stress. Information on salt-responsive proteins/genes is crucial for improving salt-tolerance through genetic engineering techniques.

## Salt response is a multigenic trait

Salt-stress and dehydration stress show a high degree of similarity with respect to physiological, biochemical, molecular and genetical effects[3]. This is possibly due to the fact that sub-lethal salt-stress condition is ultimately an osmotic effect, which is apparently similar to that brought in by water deficit and to some extent by cold as well as heat stresses[4].

The halophyte *Mesembryanthemum crystallinum* has emerged as a model system for understanding the molecular response to salt-stress. This plant switches from $C_3$ photosynthesis to crassulacean acid metabolism (CAM) in response to salt or drought stress. Organic acids, oxalate and malate are important osmolytes in plants with CAM. A cDNA clone encoding NAPD-malic enzyme has been isolated from common ice plant[5,6]. A large number of genes are concomitantly up- and down-regulated for this switch to be operational[7]. More than a hundred genes are induced and probably transcripts three times that number are repressed in response to salt-stress in *M. crystallinum*[8]. Employing two-dimensional protein gel electrophoresis, it has been noted that application of salt to plants brings about a major change in the protein profile[9]. Therefore, not only is it imperative to ask how many and which genes but also in what hierarchical order do they express. Salinity is a quantitative trait, and arrays of salt-induced genes have been isolated[10].

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## Salinity stress and plant response

### *Compatible solutes*

Under osmotic stress, an important consideration is to accumulate osmotically active compounds called osmolytes in order to lower the osmotic potential. These are referred to as compatible metabolites because they do not apparently interfere with the normal cellular metabolism. Molecules like glycerol and sucrose were discovered by empirical methods to protect biological macromolecules against the damaging effects of salinity. Later, a systematic examination of the molecules, which accumulate in halophytes and halo-tolerant organisms, led to the identification of a variety of molecules also able to provide protection[11,12]. Characteristically, these molecules are not highly charged, but are polar, highly soluble and have a larger hydration shell. Such molecules will be preferentially solubilized in the bulk water of the cell where they could interact directly with the macromolecules.

The biochemical pathways producing them are now better known. Genes rate-limiting these steps have been cloned and transferred into crop plants to raise the level of osmolytes. Osmolytes for which some progress has been made are indicated in Table 1.

*Mannitol*: Tobacco plants have been modified by introduction of *Escherichia coli mtlD* gene, which encodes mannitol-1-phosphate dehydrogenase[13]. It is not normally produced by wild-type tobacco. However, many organisms, including some plants synthesize and accumulate mannitol. Transgenic tobacco plants synthesize mannitol-1-phosphate from fructose-6-phosphate. In the absence of salt-stress, wild and transformed plants have similar height and fresh weight gains, but in the presence of 250 mol $m^{-3}$ salt, the *mtlD* gene-transformed plants have a growth advantage over the wild type in terms of better height grain, less fresh-weight loss and more new leaf and root production. Binzel *et al.*[14] found that tobacco cells adapted to 428 mM NaCl could maintain cytosolic $Na^+$ and $Cl^-$ level at less than 100 mM. Though mannitol only partially decreases the amount of inorganic ion accumulation in the cytosol, its protective effect as a compatible solute may be sufficient to give marginal growth advantage observed in transformed plants. Su *et al.*[15] obtained three rice transgenic lines with bacterial *mtlD* and demonstrated that biosynthesis and accumulation of mannitol in plants are correlated with salt-stress tolerance of plants. These solutes are widely believed to function as a protector or stabilizer of enzymes or membrane structures that are sensitive to dehydrations or ionically induced damage. *Arabidopsis thaliana* plants transformed with bacterial *mtlD* encoding mannitol-1-phosphate dehydrogenase have higher mannitol content and were able to withstand NaCl salinity up to 400 mol $m^{-3}$, whereas the wild type seeds ceased to germinate at 100 mol $m^{-3}$ NaCl[16].

**Table 1.** Important osmolytes that accumulate in plants during drought and salinity

| Carbohydrate | Nitrogenous compound | Organic acid |
|---|---|---|
| Sucrose | Proteins | Oxalate |
| Sorbitol | Betaine | Malate |
| Mannitol | Glutamate | |
| Glycerol | Aspartate | |
| Arabinitol | Glycine | |
| Pinitol | Choline | |
| Other polyols | Putrescine | |

*Pinnitol/ononitol*: The cyclic sugar alcohols, pinnitol and ononitol are stored in a variety of species, which are consistently exposed to saline conditions or accumulate in tolerant species when exposed to saline environments[17]. Facultative halophyte such as *M. crystallinum* accumulates these compounds only when subjected to water and salinity stresses. The proposed synthetic pathway consists of methylation of myo-inositol to the intermediate ononitol followed by epimerization to pinnitol[18]. An inositol methyl transferase (*Imt*) cDNA was isolated from transcripts induced in *Mesembryanthemum* plants by NaCl[19]. Transgenic tobacco for inositol methyl transferase has been obtained[20]. Similar to plants transformed with mannitol-1-phosphate dehydrogenase, growth of wild and *Imt*-transformed plants is not distinguishable in the absence of stress, but the latter have growth advantage over control plants in the presence of salt.

*Sorbitol*: This sugar alcohol of glucose is found in a variety of plant species, usually as a constituent of seeds. Sorbitol accumulation has been reported in seeds of many crop plants[21]. In Rosaceae species, it functions as a translocated carbohydrate and is also reported in vegetative parts in the halo-tolerant *Plantago maritima*[22]. Increasing salinity from 0 to 400 mol $m^{-3}$ resulted in an eightfold increase of sorbitol concentration in shoot tissues and a 100-fold increase in root tissues. Accumulation in *P. maritima* serves an osmo-regulatory function and its accumulation in plant seeds suggests that it may contribute to the desiccation tolerance of the mature embryo. *In vitro* protection by sorbitol of the restriction enzyme *pst*1 from desiccation has been reported earlier[23]. The conversion of glucose to its sugar alcohol is catalysed by aldose reductase. An aldose reductase-like protein accumulates during the period of embryo maturation in barley when desiccation tolerance is obtained[24].

*Proline*: In organisms ranging from bacteria to higher plants, there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity. It may also serve as an organic nitrogen reserve that can be utilized during recovery. In *Lathyrus sativus*, a hardy grain legume which can withstand drought, high proline

accumulation was observed in leaves and roots under water stress[25].

Although proline can be synthesized from either glutamate or ornithine, glutamate is the primary precursor in osmotically stressed cells. The biosynthetic pathway consists of two important enzymes, viz. pyrroline carboxylic acid synthetase and pyrroline carboxylic acid reductase. Transcripts corresponding to both cDNAs accumulate in response to NaCl treatment. Both these regulatory steps are keys to developing strategies for overproducing proline in a selected plant species.

Besides, the intermediates of proline biosynthesis and catabolism, such as glutamine and *d*-1-pyrroline-5-carboxylic acid could increase the expression of several osmotically regulated genes in rice[26]. There is also evidence that degradation of proline in the mitochondria is directly coupled to respiratory electron transport system and ATP production. A pyrroline-5-carboxylate synthetase (P5CS) cDNA from moth-bean was introduced into rice. Expression of this P5CS transgene under the control of a stress-inducible promoter led to stress-induced overproduction of the P5CS enzyme and proline accumulation in transgenic rice plants. Second generation (RI) transgenic plants showed an increase in biomass under salt and water stress conditions[27].

*Glycine-betaine*: Levels of glycine-betaine in *Poaceae* species are correlated with salt-tolerance. Highly tolerant *Spartina* and *Distichlis* accumulated the highest levels, moderately tolerant species accumulate intermediate levels and sensitive species accumulate low levels or no glycine-betaine[28]. Glycine-betaine is synthesized from choline in two steps, the first being catalysed by choline monooxygenase leading to synthesis of betaine-aldehyde, which is further oxidized by betaine-aldehyde dehydrogenase. Salinity stress induces both the enzyme activities[6,29]. Genetic evidence that glycine-betaine improves salinity tolerance has been obtained for barley and maize[28,30]. Isogenic barley lines containing different levels of glycine-betaine have different abilities to adjust osmotically. Transgenic rice plants expressing betaine-aldehyde dehydrogenase converted high levels of exogenously applied betaine-aldehyde to glycine-betaine than did wild-type plants. The elevated level of glycine-betaine in transgenic plants conferred significant tolerance to salt, cold and heat stress.

Huang *et al.*[31] reported metabolic limitation in betaine production in transgenic plants. *Arabidopsis thaliana*, *Brassica napus* and *Nicotiana tobbacum* were transformed with bacterial *Choline oxidase* cDNA. The levels of glycine betaine were 18.6, 12.8 and 13.0 μmol $g^{-1}$ dry weight in *A. thaliana*, *B. napus* and *N. tobbacum* respectively, 10–20 fold lower than the levels found in natural betaine producers. A moderate stress tolerance was noted in some transgenic lines based on relative shoot growth in response to salinity, drought and freezing. However, choline-fed transgenic plants synthesized substantially more glycine-betaine, suggesting that there is need to enhance the endogenous supply of choline to support accumulation of physiologically relevant amount of betaine.

### *Polyamines*

A number of stress factors such as potassium deficiency, osmotic stress, low pH, nutrient deficiency or light have been shown to stimulate the accumulation of polyamines, and particularly putrescine in plants. Putrescine accumulation during environmental stress is correlated with increased argenine decarboxylase (ADC) activity in oats. Recent studies with transgenic carrot cells over-expressing ornithine decarboxylase (ODC) cDNA showed that these cells were significantly more tolerant to both salt-stress as well as water stress[7].

Polyamines have recently gained importance in the escape of seedlings from the adverse effect of salinity. Suppression of polyamine biosynthesis by cyclohexylamine has been reported to result in increased ethylene synthesis as well as seed germination[32]. This suggests a cross-linking of pathways of polyamine and ethylene biosynthesis. Lin and Kao[33] reported an increase in the level of spermidine under salinity, but a low level of putrescine in the shoot and roots of rice seedlings. Accumulation of spermidine and spermine with the activity of ADC in rice seedlings plays a specific role in salt-tolerance.

The diamine putrescine and the polyamines spermidine and spermine are present in probably all plants, whereas the diamine cadaverine occurs within the Leguminosae. Putrescine can be formed from argenine and one of the key enzymes is ADC. Polyamines such as spermine and spermidine are derived from methionine and ornithine. The first step is the decarboxylation of ornithine catalysed by ODC. Under salinity and drought conditions, polyamines as well as their corresponding enzyme activities are substantially enhanced[34]. Whereas nuclear DNA is stabilized by histones in eukaryotic organisms, putrescine and polyamines take over the role of histones in bacteria. DNA in plant mitochondria and chloroplasts is regulated and stabilized by putrescine and polyamines. In addition, many steps of protein biosynthesis are stimulated by polyamines, may be through interaction with nucleic acids. In addition, they can stabilize bio-membranes. Transgenic rice for ADC cDNA showed increase in biomass under salinity stress condition compared to the control[35].

Ten-days-old *Zea mays* plants salt-stressed for eight days increased the content of putrescine and spermidine in their roots and leaves, and the increase in leaves was higher than in roots[36]. Lefevre *et al.*[37] studied the relative importance of ionic and osmotic components of salt-stress on modification of free proline level in rice. They suggested that the ionic component by itself might trigger short-term polyamine accumulation.

*Antioxidants and stress tolerance*

Exposure of plants to unfavourable environmental conditions such as vicissitudes of temperature, high light intensity, water availability, air pollutants or salt-stress can increase the production of reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$). Plants possess both enzymic and non-enzymatic mechanisms for scavenging of ROS. The enzymic mechanisms are designated to minimize the concentration of $O_2^{\cdot-}$ and $H_2O_2$. The enzymes overproduced so far include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-synthesizing enzymes.

Superoxide radical is regularly synthesized in the chloroplast[38] and mitochondria[39], though some quantity is also reported to be produced in microbodies[40]. Scavenging of $O_2^{\cdot-}$ by SOD results in the production of $H_2O_2$, which is removed by APX[41] or catalase[42]. However, both $O_2^{\cdot-}$ and $H_2O_2$ are not as toxic as the ($OH^{\cdot}$), which is formed by the combination of $O_2^{\cdot-}$ and $H_2O_2$ in the presence of trace amounts of $Fe^{2+}$ and $Fe^{3+}$ by the Haber–Weiss reaction[43,44]. Hydroxyl radical can damage chlorophyll, protein, DNA, lipids and other important macromolecules[45–47], thus fatally affecting plant metabolism and ultimately growth and yield. A schematic presentation of production and scavenging of $O_2^{\cdot-}$, $H_2O_2$ and $OH^{\cdot}$, and $OH^{\cdot}$ mediated lipid peroxidation and glutathione peroxidase-mediated stabilization of lipids are presented in Figure 1.

Increase in activities of SOD, APX, CAT and GR under drought, high temperature and salinity, and comparatively higher activity in tolerant wheat genotypes has also been reported by Sairam *et al.*[48–51]. Increase in activity of SOD, APX, GR, DHAR, CAT and POX in response to salinity stress as well as higher antioxidant activity in tolerant species/varieties have also been reported by various workers[52–57]. Sairam and Srivastava[58] reported comparatively higher Cu/Zn-SOD, Fe-SOD, APX and GR activity in chloroplastic fraction and Mn-SOD in mitochondrial fraction in tolerant wheat genotypes in response to salt-stress. Hernandez *et al.* reported NaCl-induced enhanced mRNA expression and activity of Mn-SOD, APX, GR and monodehydro-ascorbate reductase (MDHAR) in tolerant pea cv. Granada, while in salinity-sensitive cv. Chillis, no significant changes in activity and mRNA levels of the above enzymes were observed.

Very little work has been done on the development of transgenic plants over expressing antioxidant enzyme activity under salt-stress. Roxas *et al.*[59] reported over expression of a tobacco glutathione-S-transferase (GST) and glutathione peroxidase (GPX) in transgenic tobacco seedlings under a variety of stresses. Salt-stress treatment inhibited the growth of wild type and caused increased

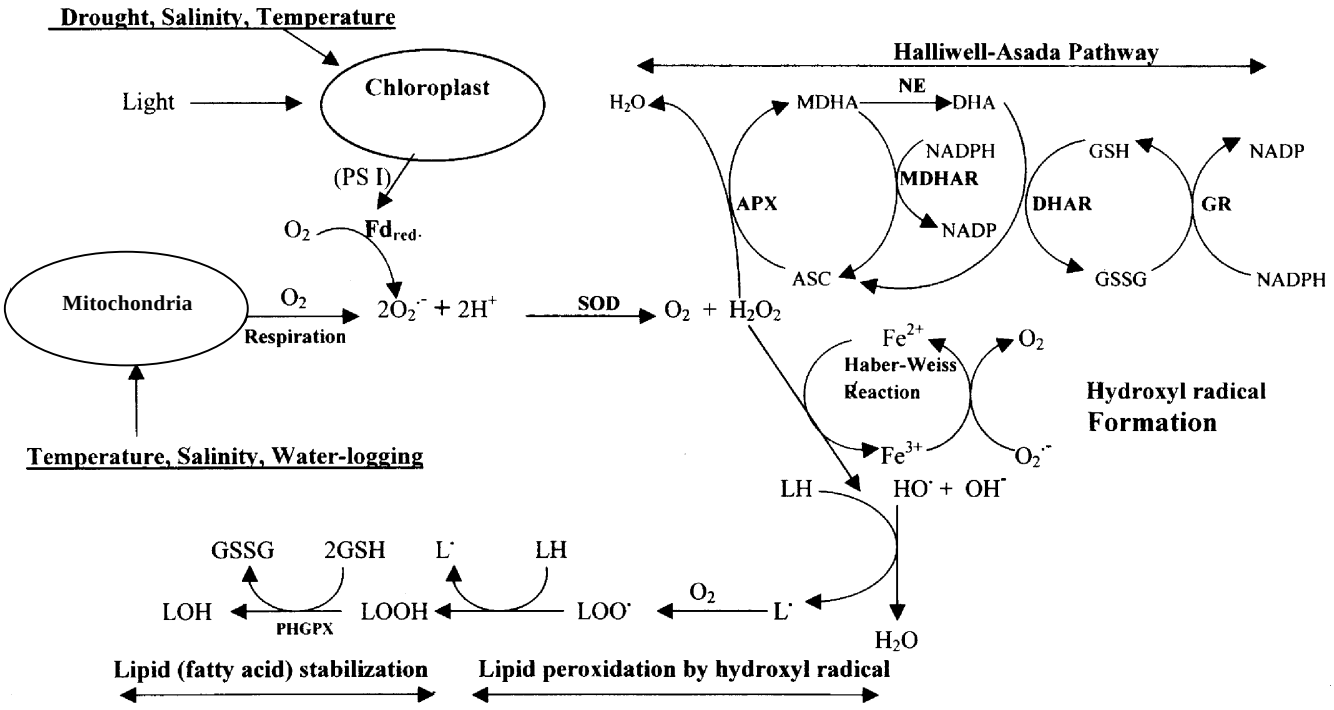


**Figure 1.** Generation and scavenging of superoxide radical and hydrogen peroxide, and hydroxyl radical-induced lipid peroxidation and glutathione peroxidase-mediated lipid (fatty acid) stabilization. APX, Ascorbate peroxidase; ASC, Ascorbate; DHA, Dehydroascorbate; DHAR, Dehydroascorbate reductase; Fd, Ferredoxin; GR, Glutathione reductase; GSH, Red glutathione; GSSG, Oxi-glutathione; $HO^{\cdot}$, Hydroxyl radical; LH, Lipid; $L^{\cdot}$, $LOO^{\cdot}$; LOOH, Unstable lipid radicals and hydroperoxides; LOH, Stable lipid (fatty acid); MDHA, Monodehydro-ascorbate; MDHAR, Mono dehydro-ascorbate reductase; NE, Non-enzymatic reaction; PHGPX, Phospholipid-hydroperoxide glutathione peroxidase; SOD, Superoxide dismutase.

lipid peroxidation, while GST-transformed seedlings did not lead to increased lipid peroxidation. GST/GPX over-expression provides increased glutathione-dependent peroxidase scavenging and alterations in glutathione and ascorbate metabolism, leading to reduced oxidative damage. Studies with transgenic rice over-expressing yeast Mn-SOD showed increased levels of ascorbate peroxidase and chloroplastic SOD in the transformed rice compared to the wild type. The transformed rice also showed more salinity tolerance than the wild type[60].

Transgenic plants over-expressing various antioxidant enzymes and showing tolerance to drought and chilling have been reported by many workers. Poplar (*Polulus tremula* × *Populus alba*) transformed with *A. thaliana* Fe-superoxide dismutase cDNA targetted to chloroplasts resulted in substantial over-expression of foliar SOD and dehydro ascorbate reductase, while no changes were observed in the activities of APX and MDHAR[61]. Reduced glutathione plays an important role as a plant antioxidant, especially in the recharging/reduction of ascorbic acid and *a*-tocopherol. Noctor *et al.*[62] examined the role of enzymes associated with glutathione synthesis in poplar plants transformed with *E. coli g*-glutamyl-cysteine synthetase (*g*-ECS) or glutathione synthetase in the chloroplasts. The transformed plants over expressed the introduced genes (mRNA) and enzyme activity. Enhanced *g*-ECS activity increased *g*-glutamyl-cysteine and GSH levels.

Wu *et al.*[63] isolated cDNA-encoding chloroplastic Cu/Zn-SOD and mitochondrial Mn-SOD from wheat. Northern blot analysis showed Mn-SOD genes were stress-inducible. Though Cu/Zn-SOD gene did not increase under drought, there was increase in expression on reversion to normal condition. The results show that both Mn-SOD and Cu/Zn-SOD play definite roles in stress tolerance, though at different phases. To test the hypothesis that enhanced tolerance to oxidative stress would improve winter survival, two clones of *Medicago sativa* were transformed with Mn-SOD cDNA targetted to the mitochondria or to the chloroplasts. Transformed plants have higher Mn-SOD activity and up to 100% more winter survival and herbage yield[64]. Expression of certain genes in antisense mode has resulted in increased antioxidant activity in transformed plants[65]. *A. thaliana* lines transformed with barley 2-cysteine peroxiredoxin cDNA in antisense mode showed high APX and MDHAR, though the mRNA levels and activity of GPX were lowered.

Sopory and associates have been studying the role of glyoxylase system in stress tolerance. The glyoxylase system is ubiquitous in nature and consists of two enzymes, glyoxylase I and glyoxylase II, which act coordinately to convert 2-oxo-aldehydes into 2-hydroxy acids using reduced glutathione. Their primary function seems to be to remove methyl-glyoxal, primary substrate for glyoxylase I, a cytotoxic compound known to arrest growth and react with DNA and protein. Tobacco plants were transformed with GlyI (Glyoxylase I) cDNA from *Brassica juncea*. Transgenic plants over-expressing glyoxylase I showed significant tolerance to salt-stress, which was correlated with degree of GlyI expression[66]. Post-transcriptional regulation of GR has been studied in maize[67]. It has been suggested that both red-glutathione and oxi-glutathione may function as signal molecules in the hypersensitive response. GR may act as a central determinant of overall cellular redox state, involving redox signalling for the expression of specific genes in optimal and stress condition. The limitations on the regulation of such signalling pathways by the absence of GR may render such tissues, and therefore, maize leaves sensitive to low temperature. Lipoxygenease are non-heme-iron containing enzymes which catalyse the hydroperoxidation of fatty acids. Two- to threefold induction of lipoxygenease expression has been observed in *Lathyrus sativus* under water stress[68].

### *Membrane transport and uptake processes*

While accumulation of compatible solutes contributes to maintenance of cell growth under conditions of increased ion concentration, many organisms have also developed efficient methods to keep the ion concentration in the cytoplasm at low levels. Membranes, their integral and associated components necessary for the uptake and distribution of ions and solutes, are considered determinants in developing stress-resistant crops. Carriers, channels, symporters and antiporters are mainly concerned with the cellular transport phenomenon in plants. Plant cells need to maintain high $K^+$ levels, 100 to 200 mM to maintain normal metabolic reactions. $K^+$ also plays a role in maintaining turgor. $Na^+$ levels on the other hand should be less than 1 mM in cytoplasm, any excess has to be excluded out of the cell or sequestered in the vacuolar compartment. Qadar[69] observed that the concentration of $Na^+$ in the shoot and lamina increased with increasing sodicity levels. Whereas the converse was true for $K^+$, although the total monovalent cations (Na + K) was lower compared with that of control. A low abundance transcript that showed enhanced expression during salt-stress in callus of *M. crystallinum* encoded a protein involved in $K^+$ uptake[70].

Addition of calcium chloride to the culture solution decreased plant Na content and the rate of transport of $Na^+$ from root to shoot and increased the relative growth rate. Addition of $Ca^{2+}$ did not affect the $K^+$ content. Addition of $Ca^{2+}$ could protect the membrane structure under salt-stress[71]. Genes encoding $Ca^{2+}$ ATPase have been isolated from solanaceous species such as tomato. Salinization of tomato enhanced the expression of leaf mRNA three fold and the root mRNA, two fold.

*SOS regulatory pathway for ions*: Regulation of ion ($Na^+$ and $K^+$) homeostasis involving SOS (salt overly sensitive) genes has been recently suggested by the SOS pathway. The input of SOS pathway is due to excessive

intracellular or extracellular $Na^+$, which somehow triggers a cytoplasmic $Ca^{2+}$ signal[72]. The outputs are expression and activity changes of transporters for ions such as $Na^+$, $K^+$ and $H^+$. The input for osmotic stress signalling is likely to be a change in turgor. A myristoylated calcium-binding protein encoded by SOS3 presumably senses the salt-elicited calcium signal and translates it to downstream responses[73]. SOS3 interacts with and activates SOS2, a serine/threonine protein kinase[74,75]. SOS2 and SOS3 regulate the expression level of SOS1, a salt-tolerance effecter gene encoding a plasma membrane $Na^+/H^+$ antiporter[76]. SOS1 by itself can slightly increase the salt-tolerance of a yeast mutant stain lacking all endogenous $Na^+$-ATPase and $Na^+/H^+$ antiporter[77]. Expression of a constitutively activated SOS2 mutant could also increase the salt-tolerance activity of SOS1 in the yeast mutant, implying that SOS2 kinase activity is sufficient for SOS1 activation.

In a complementary study, Qui *et al.*[78] showed that constitutively active SOS2 kinase could enhance a $Na^+/H^+$ exchange activity in purified plasma membrane vesicles from wild type but not in SOS1-1 mutants[79]. In SOS2-2 and SOS3-1 mutants[80], the plasma membrane $Na^+/H^+$ exchange activity is much lower but can be recovered to near wild-type levels by addition of activated SOS2 *in vitro* to the membrane vesicle preparations[78]. The plasma membrane $Na^+/H^+$ antiporter SOS1 has a long tail that is protruded to be on the cytoplasmic side[76]. Membrane transportation with long cytoplasmic tails has been proposed to function as sensor of all solutes they transport. The possibility of SOS1 being both a transporter and a sensor cannot be dismissed. Besides being regulated by SOS2, SOS1 activity may also be regulated by SOS4. SOS4 catalyses the formation of pyridoxal-5-phosphate, a cofactor that may serve as a legend for SOS1, because the latter contains a putative binding sequence for this cofactor[81].

Apse *et al.*[82] have engineered a single endogenous gene (*AtNHX1*) encoding a $Na^+/H^+$ antiporter protein. In *Arabidopsis* a $Na^+/H^+$ antiporter gene has been identified and characterized because of its similarity to bacterial, fungal and mammalian homologues[82,83]. Engineered *Arabidopsis* plants expressed greater levels of *AtNHX1* and showed increased vacuolar uptake of $Na^+$ compared to the wild type. Transgenic plants were significantly more salt tolerant, thriving in soil irrigated with 200 mM NaCl.

### *Salinity-induced changes in gene expression in plants*

There is a wealth of evidence indicating that changes in gene expression occur in plants following an exposure to salt. During the last decade, a number of salt-responsive genes have been isolated and characterized. Within crop species studied, a wide range of tolerance to salinity exists, from very high (*Beta vulgaris*) to extremely low (*Citrus* spp.). Further, within a given crop species, salt-tolerant and salt-sensitive varieties exist. Salt-tolerant species have been frequently employed to isolate genes involved in conferring salt-tolerance, and thereby gain an understanding of the mechanisms that distinguish them from their salt-sensitive counterparts.

A number of approaches have been undertaken to isolate genes whose expression is influenced by salinity in plants. A majority of these experimental approaches involved screening cDNA libraries constructed from mRNA populations isolated from plants or cells that have been exposed to a salt treatment. One of the most common and successful methods used to isolate salt-responsive cDNA clones from these libraries has been differential screening with probes derived from mRNA isolated from salt-stressed and non-stressed plant tissue. In this case mRNA that corresponds to genes that are preferentially expressed in the salt affected plants is isolated and characterized.

More recently, cDNAs that encode proteins that enhance salt-tolerance have been selected using salt-sensitive mutants of yeast that are rescued following transformation with plant cDNA libraries. For example, two cDNA clones of *A. thaliana* were isolated based on their ability to complement yeast cells deficient in the phospho-protein-phosphatase calcineurin[84]. In addition, there are genes which were isolated from plants on the basis of their enhanced expression in response to other environmental stresses, especially water deficit, that are also salt-responsive. This is because both salinity and water deficit confer an osmotic stress upon plants.

Changes in gene expression at the transcriptional and post-transcriptional levels were initially demonstrated by analysis of protein profiles elicited in plants using a salt treatment. These studies revealed both qualitative and quantitative changes in the pattern of polypeptides synthesized following salt treatment[85–89]. Many of these studies were extended to include analysis of mRNA changes effected by salinity as assessed by *in vitro* translation of mRNA populations. These results indicate that salt does elicit changes in relative abundance of mRNA[90,91]. Ramgopal[92] showed accumulation of unique mRNA in the roots of a tolerant barley genotype.

Moons *et al.*[93] were the first to demonstrate the connection among salt-tolerance, the levels of endogenous ABA in salt-treated roots and that of specific polypeptides whose synthesis in roots is induced by exogenous ABA employing salt-tolerant and salt-sensitive varieties of rice. This was the first study to suggest a physiological basis for salt-tolerance involving ABA and the molecular responsiveness of root tissues to ABA.

A pair of polypeptides initially identified on 2D gels of fractionated proteins from salt-treated barley roots was subsequently shown to be similar to germin, a glyco-protein that accumulates in germinating wheat[94]. Germin possesses oxalate oxidase activity[95], responsible for con-

verting oxalate to $H_2O_2$ and $CO_2$. Furthermore, since oxalate is frequently bound to Ca, germin may be involved in the release of not only $H_2O_2$ but also $Ca^{2+}$, both of which are second messenger in plants. In addition, $H_2O_2$ plays a role in the oxidative cross-linking of cell-wall polymers, and therefore, the expression of germin may result in cell-wall modification in salt-stressed plants[96]. Other salt-induced proteins in the roots of rice identified on 2D gels include SalT, a group-3 LEA protein, a group-2 LEA/dehydrin, two pathogenesis-related proteins (PRPs), a peroxidase and a histidine-rich protein[89,93,97,98]. The PRP and peroxidase, like osmotin may play a role in general defence of plants; the group-3 LEA and group-2 LEA/dehydrin may be involved in the protection of cellular components from osmotic stress imposed by salt.

In recent studies, micro-array methods have been employed to catalogue all the changes in gene expression in response to drought, cold and high salt-stress conditions over time. The potential function of approximately 130 genes of *A. thaliana* which have been shown to be up-regulated, point to signalling events, detoxification and other functions involved in cellular response to stress[99]. Seki *et al.*[100] reported the five times increase in transcripts of 53, 277 and 194 genes after cold, drought and salinity treatments respectively. The transcripts of 22 stress-inducible genes responded to all stresses.

*Genes encoding proteins involved in cellular protection*: LEA (late embryogenesis abundance) proteins, which are ABA-inducible group of proteins and originally suggested to be associated with desiccation tolerance during seed maturation, are also induced by salinity and water deficit. LEA proteins have been placed in different groups on the basis of amino acid sequence homology. The *lea* genes encode proteins that are overwhelmingly hydrophilic and soluble upon boiling. Most of these proteins lack cysteine and tryptophan, are not compartmentalized or transported within the cell, and are likely to be located in the cytosol. These proteins protect the cellular structure and components, e.g. membranes and proteins/enzymes from the effects of water loss during a salt-stress.

*Group 1 LEA proteins*: These contain a high proportion of glycine and charged amino acids, and thus are highly hydrophilic. They are related to *Em* gene which encodes wheat EM protein, which is more hydrated than other globular proteins[101,102]. Consequently, the predicted role for group-1 LEA proteins is that of water-binding in order to provide a protective aqueous environment for the cellular components. The expression of members of this group of genes in vegetative tissues is induced by salt[102], water-deficit stress[103,104] and ABA[102].

*Group 2 LEA proteins*: Members of this group are often referred to as dehydrin and or rab (responsive to ABA), the latter term pertaining to the responsiveness of their expression to ABA. There is considerable variability in size and structural properties among members of this family of proteins. However, they are all characterized by a conserved, lysine-rich amino acid domain located in the C-terminus, and at least one more at an upstream position. Suggested roles for these proteins in water-deficit stressed cells include the exclusion of solute from the surface of membranes and cytosolic proteins, thereby preventing denaturation and maintaining the solvation of structural surfaces. The expression of *Leas* in vegetative tissues is responsive to ABA and water deficit-related stresses, including salinity and cold[105,106].

*Group-3 and 5 LEA proteins*: These two groups of LEA proteins are characterized by the presence of tandemly repeated 11-mer amino acid motifs that are repeated many times within the protein [107]. These proteins exist as dimers. The polar face of these dimerized helices would be exposed and capable of binding ions via the formation of salt bridges. As a result, the likely role of these proteins in salt-or water-deficit stressed cells is one of ion sequester action. Genes encoding group-3 LEA proteins are expressed in response to salt, water deficit and ABA in soybean and barley[108,109] and salt and ABA in roots of rice[110]. Naot *et al.*[111] isolated a gene from the salt-tolerant line of Shamuti orange, which encoded a group 5-*lea* homologue in response to salt and water deficit.

*Group-4 LEA proteins*: These proteins contain a conserved N-terminal domain that forms an $\alpha$-helix and a less conserved C-terminus, rich in glycine and amino acids containing hydroxyl groups, forming an unstructured random coil[112]. These have been suggested to bind water molecules and may also act as reverse chaperones, whereby these would stabilize the surface of membranes and possibly proteins by binding water and functioning as solvation film. Genes encoding group 4 LEA are expressed in vegetative tissues in response to salinity, drought, ABA and low temperature[109,113].

*LEA D95*: Galau *et al.*[114] reported this class of proteins in response to water stress in cotton leaves. This protein is unusual, since there are some hydrophobic characters to the protein. LEA D95 is homologous to a cDNA, pcC27-45 from *Craterostigma plantigineum*, where it is expressed in response to salt in callus tissues and in response to desiccation and ABA in both leaves and callus[115].

*Genes encoding proteins involved in ion homeostasis*: Survival in saline environment involves membrane transport leading to exclusion and/or compartmentation of $Na^+$ ions. The transport mechanisms regulate ion transport in order to maintain ion homeostasis and mediate osmotic adjustment via the accumulation and compartmentation of ions within the cell. Sodium, therefore, is actively excluded from the cytosol and sequestered in the vacuole,

thus allowing water to move into the cell. $Na^+/H^+$ antiporters mediate the uptake/exclusion of $Na^+$. The activities of these carriers are coupled to the downhill flux of $H^+$ leading to generation of $H^+$ electrochemical gradient across the plasmalemma or tonoplast, which is catalysed by $H^+$-ATPase in the plasmalemma (P-ATPase) and tonoplast (V-ATPase), and H-pyrophosphatase in the tonoplast. The activities of these ATPases increase in cells exposed to NaCl and the expression of a number of the corresponding genes is also upregulated[116–119].

Salinity also disturbs the levels of $Ca^{2+}$, which increases in the cytosol. Increased cytosolic $Ca^{2+}$ functions as a second messenger, resulting in changes in gene expression and metabolism in salt-affected cells. Elevated $Ca^{2+}$ levels return to their original values through active efflux out of the cell mediated by $Ca^{2+}$ pumps (Ca-ATPase), whose expression increases under salinity. Ca-ATPase is located in the endoplasmic reticulum, plasmalemma and tonoplast, and mediates $Ca^{2+}$ sequestration in the cell[120]. Guerrero and Crossland[121] reported increased expression of genes encoding transmembrane channel proteins, major intrinsic proteins capable of facilitating the movement of water and small molecules across membrane by osmotic stress.

*Genes encoding proteins involved in general defence*: A number of cDNAs isolated as a result of screening libraries expressing under salinity stress were found to be related to previously characterized genes associated with plant defence against pathogens or wounding damage, e.g. PRP from rice[98], endochitinase from tomato[122] and *b*-glucanase from rice[123]. Even osmotin, a polypeptide initially associated with salinity adaptation has been found to be related to a family of PRP. Its expression in salt-affected plants is induced by pathogen attack as well as signalling molecules such as salicylic acid and ethylene, which are produced in plants as a result of pathogen attack or wounding[124].

Protease inhibitor, which is induced upon insect attack, is also induced by salt-stress[125,126]. Several heat-shock protein genes are expressed in response to salt-stress[123,127]. These proteins act as chaperones to prevent denaturation and to help denatured proteins regain their native conformation. Genes for enzymes involved in controlling oxidative stress, such as APX and GPX are also induced by salt-stress. Glyoxylase, which serves to detoxify methylglyoxal, is also induced by salt-stress[128,129].

*Genes encoding proteins involved in osmotic adjustment*: Genes encoding enzymes involved in the biosynthesis of proline, glycine-betaine and the sugar alcohol pinitol/ononitol have been reported from various plants in response to salt-stress. P5CS involved in proline biosynthesis from glutamate has been reported to accumulate in leaves and roots in response to salt-stress in *Pisum sativum*[130], while in *Oryza sativa* and *A. thaliana*, by salt dehydration and ABA[131,132].

Genes and cDNAs encoding choline monooxygenase and betaine aldehyde dehydrogenase, which are involved in conversion of choline to glycine-betaine, have been observed in response to salinity in sugar beet, spinach and barley[133–135]. Ishitani *et al.*[136] reported a six fold increase in the transcript of myo-inositol phosphate synthase, which is the precursor for pinitol under salt stress. Vernon and Bohnert[137] reported increase in activity of myo-inositol-o-methyl transferase, which is involved in the conversion of inositol to pinitol under salt stress. The gene encoding mannitol dehydrogenase, which facilitates its entry into the central carbon metabolism, is downregulated by salt stress[138]. This serves to maintain high concentration of mannitol in salt-stressed cells, to allow it to function as an osmo-protectant.

*Genes encoding proteins involved in metabolism*: *M. crystallinum* is a facultative CAM plant, which switches to CAM metabolism under salt-stress. This switch helps in conserving water, as the stomata remain closed during daytime. There is also induction of genes involved in the synthesis of CAM enzyme proteins. Induced expressions of PEPCase[139], NADP malate dehydrogenase[140] and NADP malic enzyme have been reported in *M. crystallinum* in response to salt-stress. Genes encoding glyceraldehydes 3-phosphate dehydrogenase and phospho-glycero mutase in response to salt-stress have been reported in *M. crystallinum*[123,141].

*Genes encoding proteins involved in regulating gene expression*: Some of the salt-responsive genes are those that encode proteins involved in the regulation of other salt-responsive genes. Salt response regulatory genes are mostly transacting factors[142] and protein kinases[143]. In *A. thaliana*, the expression of receptor-like protein kinase gene is induced in response to salinity and ABA[144]. A receptor protein kinase cDNA was also isolated from rice[111]. These receptors transduce an extracellular signal across the membrane to activate cellular signal transduction pathways. Frandsen *et al.*[145] reported an ABA, salt and desiccation-induced gene encoding a protein containing a conserved $Ca^{2+}$ binding site, suggesting that $Ca^{2+}$-linked signalling occurs in osmotically stressed plants. A gene encoding a phosphatidyl inositol-specific phospholipase-C (PI-PLC) is expressed in response to salinity and desiccation[146]. PI-PLC hydrolyses phosphatidyl inositol 4,5-biphosphate to produce inositol 1,4,5-triphosphate ($IP_3$) and 1,2-diacyl glycerol. $IP_3$ opens $Ca^{2+}$ channels in ER membrane, causing $Ca^{2+}$ efflux to the cytoplasm. This gene is also responsive to ABA, suggesting involvement of $Ca^{2+}$-linked signalling in the mediation of ABA and osmotic stress responses. Mizoguchi *et al.*[143,147] reported a gene in *A. thaliana* encoding components of signal transduction and identified it to be mitogen activated protein kinase (MAPK). Other similar genes are MAPKK kinase and a ribosomal S6 kinase, which all function in the MAPK

cascade. The expression of these genes increases under salt stress.

### *Signal transduction during drought/salinity*

It appears there are multiple pathways of signal-transduction systems operating at the cellular level for gene regulation. Salts first decrease the osmotic potential of soil solution, creating a water stress in plants. The loss of water from the cells, one of the initial events of water deficit, may affect turgor and bring about changes in size and membrane properties.

Although the components of the signal-transduction pathway are difficult to identify, ABA is well known as one such component acting in one of the signal transduction pathways. Experiments conducted so far clearly indicate ABA-dependent and ABA-independent pathways for the induction of stress related genes (Figure 2 *a*).

Evidence indicates that salt- or water-deficit response genes are under complex regulation. Stress-responsive genes can be classified as either 'early response genes' or 'delayed response gene' (Figure 2 *b*). Early response genes are induced quickly (within minutes) and often transiently. This induction does not require new protein synthesis, because all signalling components are already in place. In contrast, delayed response genes, which constitute the vast majority of stress-responsive genes, are activated by stress more slowly and their expression is often sustained. The early response genes typically encode transcription factors that activate downstream delayed response genes[148]. Although specific branches and components exist[149], the signalling pathways for salt, drought, cold and ABA interact, and even converge at multiple steps[150]. This was suggested by a comprehensive mutational analysis in which *Arabidopsis* single-gene mutation was found to affect responses to all or a combination of these signals[151].

Another important component is jasmonic acid (JA). Jasmonate modulates the expression of numerous genes and influences specific aspects of plant growth, development and responses to abiotic and biotic stresses. The JA signal-transduction pathway is mainly unknown. It is presumed that jasmonate interacts with receptors in the cell that activate a signalling pathway resulting in changes in transcription, translation and other responses mediated by JA[152]. It has been the underlying thinking that because of multiplicity of gene action involved, there is not going to be any success against abiotic stresses. But this statement is not entirely true, because though several genes may be involved, action of specified gene(s) may have a decisive effect in controlling genes lower down in the hierarchical level of expression. The appreciation is growing that usage of regulatory genes is a more effective approach for producing more salt-stress-tolerant plants. This is based on the observation that a single regulatory gene leads to altered expression of a number of different downstream structural genes, thus leading to a wide array of altered response. Many *WDS*-inducible/ABA responsive genes contain conserved cis-acting sequences within the 5′ regulatory region to which transcription factors bind directly or indirectly in order to regulate gene expression. The presence of short elements called ABA

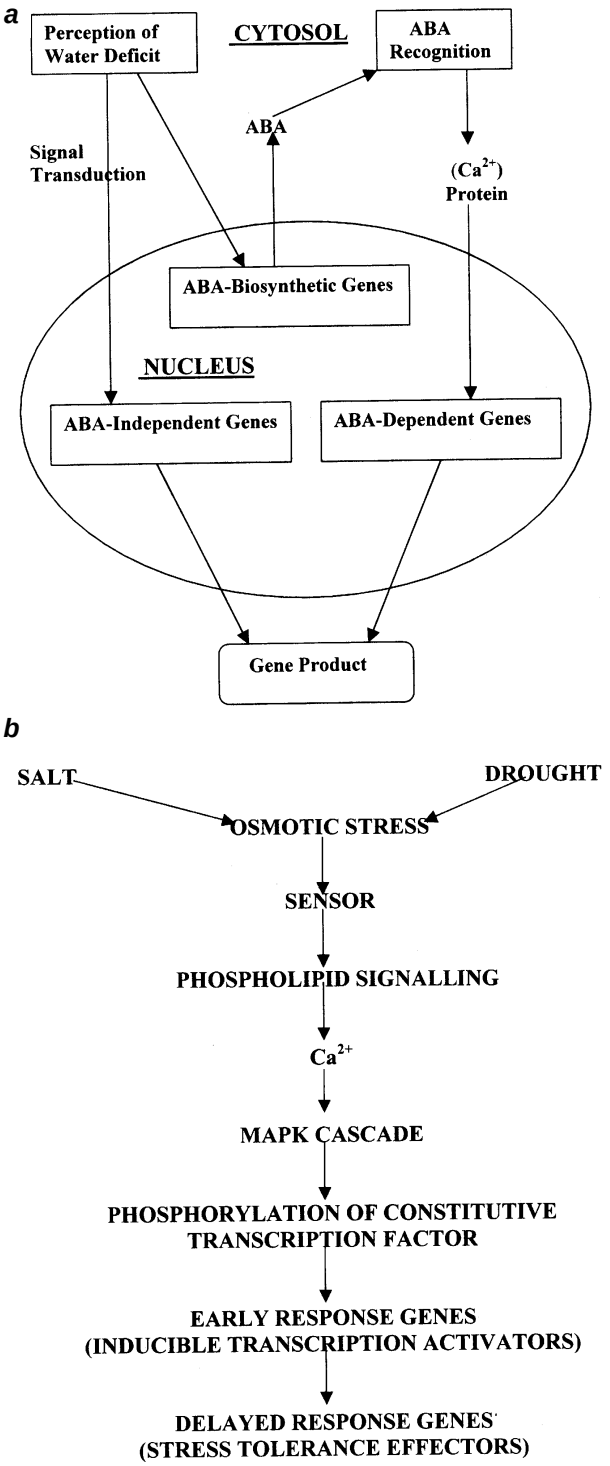


**Figure 2.** ***a***, ABA-dependent and ABA-independent signal transduction. ***b***, Stress-induced regulation of early and delayed response genes.

responsive elements (ABREs) in the 5′ upstream region of the transcription initiation site of the *em* gene of wheat[153] and several other species have been reported.

Another novel 9-bp cis-acting element called DRE (dehydration responsive element) has been isolated from *Arabidopsis*[154]. As the products of abiotic stress and ABA-inducible genes are predicted to play an important role in the mechanism of salt-tolerance, expression of the transcription factor that recognizes ABRE is likely to be regulated when plants are exposed to abiotic stress. Northern blot analysis of total RNA from control and salt treated ten-day-old Pokkali (salt-tolerant) rice plants was performed to find out levels of transcript homologous to a transcription factor that recognizes ABRE. Salinity stress induced accumulation of transcripts[155]. Nakashima *et al.*[156] characterized a gene family for DRE/CRT binding proteins, DREB 2A and DREB 2B in *A. thaliana*. Northern analysis showed that both genes are induced by dehydration and high salt stress. These genes are strongly induced in roots by high salt stress and in roots by dehydration stress. Thus a single regulatory gene leads to altered expression of a number of different downstream structural genes leading to a wide-arrayed altered response.

## Strategies to improve stress tolerance

Recent advances in plant genome mapping and molecular biology techniques offer a new opportunity for understanding the genetics of stress-resistance genes and their contribution to plant performance under stress. These biotechnological advances will provide new tools for breeding in stress environment. Molecular genetic maps have been developed for major crop plants, including rice, wheat, maize, barley, sorghum and potato, which make it possible for scientists to tag desirable traits using known DNA landmarks. Molecular genetic markers allow breeders to track genetic loci controlling stress resistance without having to measure the phenotype, thus reducing the need for extensive field-testing over time and space. Moreover, gene pyramiding or introgression can be done more precisely using molecular tags. Together, molecular genetic markers offer a new strategy known as marker-assisted selection. Another molecular strategy which depends on gene cloning and plant transformation technology, is genetic engineering of selected genes into elite breeding lines. What makes a particular goal attainable or unattainable in genetic engineering experiments is the availability of the following three inputs: (i) the gene of interest, (ii) an effective technique for transferring the desired gene from one species to another and (iii) promoter sequences for regulated expression of that gene. Amongst these, the first is considered a rate-limiting factor. Arrays of stress-induced genes have been isolated[157]. Stress-responsive genes can be analysed following targetted or non-targetted strategy. The targetted approach relies upon the availability of relevant biochemical information (i.e. in terms of defined enzyme, protein, a biochemical reaction or a physiological phenomenon). The non-targetted strategy to obtain a desired gene is indirect. This strategy, for instance, includes differential hybridization and shotgun cloning. The list of genes whose transcription is upregulated in response to stress, is rapidly increasing. Understanding of the mechanisms which regulate gene expression and the ability to transfer genes from other organisms into plants, will expand the ways in which plants can be utilized. To exploit the full potential of these approaches, it is essential that the knowledge is applied to agriculturally and ecologically important plant species.

## Salinity-resistant genotypes/varieties developed by conventional breeding

Development of genotypes/varieties resistant to salt stress has not been successful. However, some genotypes have been developed and released for commercial cultivation in salinity-prone areas. Most of the work on developing salinity-resistant genotypes/varieties has been done at Central Soil Salinity Research Institute (CSSRI), Karnal.

In case of rice, the salinity-resistant varieties have been developed by crossing with Pokkali, a salinity-resistant local land race from Kerala. The improved rice cultivars developed by CSSRI, are CSRI 1, CSR 3, CSR 10, CSR 13 and CSR 27. Other rice varieties developed in India are Damodar, Dasal, Getu (renamed as CRS 3). The former two are moderately tolerant to salinity, while Getu or CRS 3 is comparatively more tolerant to the saline environment. CSA University of Agriculture and Technology, Kanpur has developed another salinity-resistant variety named Usar 1.

In the case of wheat too, the CSSRI, has developed some moderately tolerant to tolerant varieties. The most tolerant wheat variety is Kharchia 65, a selection from local land race of Rajasthan, and most of the improved salinity-resistant varieties have been developed using Kharchia 65 as base material. Some of the salinity-tolerant wheat varieties are CSW 540-2, KRL 1-4, KRL 3-4, and KRL 19. High-yielding wheat variety HD 2009, developed by IARI, New Delhi also shows moderate salinity tolerance.

Development of salinity-tolerant cultivars in other crops has not been successful nor has been attempted with the same zeal. In case of wheat and rice, breeders were also lucky to have local land races (Kharchia and Pokkali) tolerant to salinity. International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad has developed a salinity-tolerant pigeon-pea genotype ICPL 227. In case of mustard, CSSRI has identified CS 52 as the salinity-tolerant cultivar. However, mustard usually is able to tolerate low to medium salinity and only salinity higher than 8–10 dS $m^{-1}$ affects yield significantly.

## Conclusion

Cloned plant genes and transgenic plants have become a standard tool in plant-stress biology. These technologies have mainly been applied to model systems and have greatly enlarged the knowledge of mechanisms of tolerance. The various abiotic stresses cause changes in plant processes at all levels of organization (morphological, physiological, biochemical and molecular). In recent years, attention has focused on alterations in gene expression. The list of genes whose transcription is upregulated in response to stress is rapidly increasing. Functions for some of these polypeptides are close to being identified and their likely role in stress physiology is being determined. The understanding of mechanisms that regulate gene expression and the ability to transfer genes from other organisms into plants will expand the ways in which plants can be utilized. The exploitation of cloned genes to alter the function of gene products in transgenic plants, provides novel opportunities to assess their biological role in a stress response.

The molecular analysis of stress responses has arrived at a stage where research can build upon a large collection of characterized genes. Identification of quantitative trait loci for abiotic stress resistance may well be an effective analytical tool. This approach is promising, considering that saturated DNA marker maps are now available for both genetic model plants and crop plants. The use of novel approaches combining genetic, physiological and molecular techniques should provide excellent results in the near future. Conventional breeding has been of some success only in the case of wheat and rice.


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