Chapter 2

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2. Review of Literature

All plants growing under natural environmental conditions come across various types of stresses during their life cycle (Fig. 1). The response of the plant to these stresses largely depends on plant species and the kind of stress they face. When a threshold limit of a stress is reached, the plant eventually dies. For example, plant diseases and pests decrease the crop yields by less than 10 percent, but abiotic environmental factors on the other hand, can severely affect the crop yield by up to sixty-five percent of the total crop productivity (Serrano, 1999). In crop plants, there are two vital environmental factors that dramatically reduce crop productivity; these are drought and salinity (Zhang et al., 2000; Bray et al., 2002). These factors cause similar reactions in plants i.e. water stress. Salinization is a global phenomenon, as large farmable lands are being affected by salinity. On the other hand, the world population is increasing exponentially and it is expected that in the next 20 years, the world population would increase by 1.5 billion. It is therefore imperative to increase the crop yield under salinity stress conditions by applying various genetic and genomic approaches (Blumwald and Grover, 2006; Ismail et al., 2007).

Figure 1: Stresses affecting the yield of agricultural crops under field conditions.
2.1 Salinity stress in plants: affecting plant productivity and ionic homeostasis

Salt stress is one of the major challenges for plants. It adversely affects the agriculture across the globe, particularly on irrigated farmlands (Rausch, 1996). Large parts of the land have become saline due to poor irrigation practices. The impact of salinity is becoming more significant on everyday basis (Winicov, 1998). Hence, there is an urgent need to improve salt tolerance in plants. There are various factors that contribute to soil salinity and this makes the study of effects of salinity on the plants more complex. Factors such as humidity, temperature, light-intensity, irrigation and soil fertility, all alter the effects of soil salinity (Allen et al., 1994). Plants that grow in natural environments are readily exposed to salt stress. Approximately, one third of the irrigated land worldwide is affected by salinity, but salinity can also occur in non-irrigated land (Allen et al., 1994). However, as the salt concentration increases, yields are severely affected and move towards zero. In the field, the salt level fluctuates seasonally and spatially and variation occurs depending on the circumstances that influence a particular plant. This unevenness makes research much more difficult, which is complicated by the fact that each plant species has its own level of salt tolerance.

Plant response to salt stress is rooted in the transcriptional activation of several defense proteins (Chae et al., 2010). Osmotic stress and ion toxicity are the consequences of salt stress and decrease in chemical activity causes cell to loose its turgor (Serrano et al., 1999). Since plant cell growth depends on turgor that stretches the cell wall, lack of turgor means danger for cell survival. Plants defense mechanism against salinity requires osmotic adjustment and to a certain level, this can be done through synthesis of intracellular solutes (Shoumskaya et al., 2005). Salinity creates ion toxicity and a high sodium concentration is not good for cells. High salt concentrations reduce enzyme activity by impeding the balance of forces that control the protein structure (Liu et al., 2006). Salt toxicity can occur at very low concentration and depending on the plant species;
therefore, homeostasis of sodium is important for the tolerance of plants to salt stress. The stress caused by ion concentrations decreases water gradient, thus making it more intricate for water and nutrients to move through the root membrane (Volkmar et al., 1998). As a result, water uptake slows down and the osmotic effect spreads from the root membrane to the internal membrane – the ion concentration inside the plant changes the osmotic balances (Volkmar et al., 1998). Once high concentrations of salt reach inside the plant tissues and organs, development is severely affected. The accumulated salt causes a slower rate of extension of cells and these compromises the dimension of the leaves (Volkmar et al., 1998). The overall effect of salinity on plants is the final reduction of leaf size, which leads to loss of the leaf and finally the plant. Salinity can also cause reduction in the ATPs and growth regulators required for normal functioning in plants (Allen et al., 1994).

There is broad range of salinity tolerance among higher plants (Robinson et al., 1997), but variation also persists in plants with lower salt tolerance, suggesting that improvements can be made in the tolerance of these plants (Allen et al., 1994). Since salt tolerance limit varies among plants, therefore those with better adaptations may be studied in order to improve other plant species. Halophytes are plants that adapt themselves in saline conditions by altering their physiological and molecular mechanism (Winicov and Bastola, 1997). These plants are ideal for studying the mechanisms they use to hold high salt concentrations. Therefore, scientists can use these plants as model system in research studies aiming to improve the tolerance of non-halophytic plants.

The ability of plants to grow and survive under saline conditions is known as salt tolerance. Plants adapt various processes and mechanisms to respond to salt stress which in turn, is a multigenic response (Nagarajan and Nagarajan, 2010) (Fig. 2). A fundamental two-phase model explains the overall growth response of plants to salinity. An initial water deficit that lasts for few days or weeks followed by a second phase which starts where the ion toxicity initiates leaf death (Rausch et al., 1996). Exclusion of salts from shoot portion of the plant
is a prime form of tolerance in non-halophytic plants. In plant system, most of the sodium going from the root to shoot region is via the xylem stream (Robinson et al., 1997). This means that the rate of accumulation of sodium is directly proportional to the rate of transpiration in plants (Volkmann et al., 1998). Therefore, the stomatal control of transpiration would also control the sodium concentration inside the plant. Inhibition of stomatal opening would regulate the salt level within the shoot region. This stomatal inhibition combined with compartmentalization in vacuoles would thus help to achieve an optimum level of salt inside the cell. These two mechanisms provide feasible pathways to be genetically manipulated for improving salt tolerance of the plant.

The ability of plants to offset stress also depends on the potassium levels available to the plant (Maathius and Amtmann, 1999). Potassium is an important component of plants which works as a balancing charge and is also vital for plants to counter balance the excess salt. On the other hand, sodium is essential only for C4 species where it works as a micronutrient (Maathius and Amtmann, 1999). The optimum availability of sodium is useful for the plants, but high concentrations of the same may cause cellular damage. Since potassium and sodium structures are alike, there is a competition for binding sites that causes potassium deficiency within the cell (Maathius and Amtmann, 1999). The sodium ion that competes for potassium binding sites in the cytoplasm inhibits metabolic processes that largely depend on potassium. Some research studies have shown that plants, which are able to maintain a high level of potassium, also show a possible link with salt tolerance (Volkmann et al., 1998).

Sodium to potassium ratio (Na⁺/K⁺) in cells is regulated by transport systems, which are present on plasma and vacuolar membranes. There are three processes, which are involved in the transportation of these ions. Pumps are transporters fueled by energy and transport across an electrochemical gradient; however, no pumps are found in higher plants (Carden et al., 2003). In the next step, carrier proteins go through a conformational change during transport and in the end, ion channels are proteins that catalyze the dissipation of
trans membrane ionic gradients (Yeo, 1998; Maathius and Amtmann, 1999). The mechanisms mentioned above help in transporting ions across membranes and therefore, could potentially be helpful in altering the salt tolerance in plants by over-expression of genes coding for these proteins.

![Plant cell]

**Figure 2:** Response of plants towards Abiotic Stresses is a multigenic phenomenon that triggers cellular mechanism required to counteract the adverse effect of the same. The lines are different cellular pathways regulated and interacting with each other inside the cell in order to make the plant resistant to stress conditions.

Plants possess several mechanisms to deal with salt stress and to adjust to a saline environment. Various studies have shown that roots play a vital role for short-term adaptation to salt tolerance. It is believed that the morphology of the roots regulate the amount of salt taken into the plant (Maggio *et al.*, 2001). Many studies have also shown that salt stress can also be alleviated by an increased supply of silica and calcium to the growth medium (Rausch *et al.*, 1996; Tuna *et al.*, 2008; Ali *et al.*, 2009). Depending on the concentration, sodium and calcium can replace each other from the plasma membrane and calcium might reduce salt toxicity.
2.2 Bottleneck for raising stress tolerant plants

Growth and development in plants involves gene products of a large number of genes. However, during stress, the biochemical metabolism that functions normally in plants are negatively affected which finally results in low productivity. Salt stress causes both "up regulation" and "down regulation" of numerous genes (Kawasaki et al., 2001). In order to raise transgenic crops, assortment of genes from a given gene pool is required, which is a complex task altogether. Studies related to transgenic plants have advocated that the altered gene expression in plants can lead to improvement in its tolerance limit (Apse et al., 1999). In recent times, efforts have specifically been made to understand the components of both abiotic stress signal recognition and transduction pathways (Zhu, 2001; Chinnusamy et al., 2005) and thus raise transgenic plants which may show improved stress tolerance.

New and efficient tools are now accessible to understand better about the genetics of abiotic stress tolerance (Kasuga et al., 1999) and to identify the complications of stress response that are essential for the production of stress tolerant transgenic plants (Sabharwal et al., 2010). With recent expansion in the knowledge of physiological and molecular basis of tolerance – efficient tools can be now used to manipulate precise components of stress response of crop plants for improving abiotic stress tolerance (Singh et al., 2010).

2.3 Potential sources of "gene of significance" for abiotic stress tolerance

In order to genetically manipulate crop plants to adapt under the abiotic stress conditions, it is imperative to have "genes of interest." These genes of interest can be transferred from one organism to another or a gene of interest from an organism can be altered in vitro and then introduced again into the same organism. In one of the processes, genes have been isolated by exposing the plant to sub lethal stress condition where accumulation of specific
transcripts/proteins in response to water deficit has been noticed (Guerrero et al., 1990; Borkird et al., 1991). Abiotic stress condition leads to several changes in the gene expression, which can be attained by comparing gene expression in non-induced and stress-induced tissues or by comparing genetically different genotypes such as various contrasting cultivars (Sahi et al., 2003; Karan et al., 2009; Kumar et al., 2009; Kumari et al., 2009), natural or induced mutants of the same species (Grover et al., 1992 and references therein), wild tolerant relative of the same species or other species (Das-Chatterjee et al., 2006; Taji et al., 2004; Amtmann et al., 2005; Mahalakshmi et al., 2006), and lower organisms (Thomas et al., 1995; Karakas et al., 1997; Abebe et al., 2003).

2.4 Transgenic plants: an approach for improving tolerance to abiotic stresses

Transgenic approaches have opened up new opportunities to improve tolerance towards abiotic stresses by incorporating genes involved in stress protection from any source into agriculturally important plants (Singh et al., 2010). It had been difficult in the past to develop tolerant cultivars using traditional methods of plant breeding due to non-availability of reliable screening criteria for selecting the desired genotypes in a segregating population. Besides, in the conventional breeding approach, the gene pool remains restricted to closely related plant species, while the genes imparting tolerance to abiotic stresses reside in distantly related wild species and land races, which are in most cases sexually incompatible to the cultivated crop cultivars. Further, many genes which may have undesirable effects are also transmitted to the recipient cultivars along with desired gene(s) while using the conventional breeding techniques. On the contrary, in transgenic approach, only the desired gene(s) is/are transferred to a crop to introduce a particular trait.

The transgenic approach allows scientists to study the mechanisms governing stress tolerance by either over expression or RNAi suppression of the
transgenes into the model plant species and to monitor the phenotypic and biochemical changes before and after a specific abiotic stress treatment. This approach has attained a preliminary success and several varieties of stress tolerant lines have been produced in recent years, few of which have been utilized for further testing under field conditions. Therefore, there is an urgent need to develop more and more transgenic plants with better tolerance to drought, salinity and other environmental stresses so as to increase the crop productivity.

Scientists across the globe have been using constitutive promoters like CaMV35S in order to generate abiotic stress tolerance by deploying these abiotic stress-inducible genes through transgenic approach as listed in Table 1. In addition, several transgenic plants overexpressing genes of signal components, transcription factors, osmolytes synthesis, ROS scavenging enzymes and ion homeostasis have been reported in recent year. There was indeed high expression of the stress-inducible genes coupled to increased tolerance to salt, drought and freezing stresses in the resulting transgenic; however, these effects were associated with phenotypic dwarfing (stunting) of the transgenic progenies. This redundant dwarfing of the transformed plants was thought to be due to the constitutive expression of the transgene. The expression of a protein in amounts more than normal and at stages when it is not needed (i.e. control conditions) is unnecessary and taxing on the energy reserves of the cell and thus it is highly undesirable.

2.5 Understanding the signaling machinery operative in plant species under various abiotic stresses.

When stress-signaling pathways are examined in the laboratory, they are usually considered in isolation from other stresses to simplify interpretation. However, in nature, plants encounter stress combinations concurrently or separated temporally (Sabharwal et al., 2010).
### Table 1: List of Abiotic stress tolerant transgenic developed through genetic modification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source of gene</th>
<th>Transgenic plant</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tpsp (Trehalose synthase)</td>
<td>E. coli</td>
<td>Rice</td>
<td>Drought, salt and cold tolerance</td>
<td>Jang et al., 2002</td>
</tr>
<tr>
<td>Tpsp (Trehalose synthase)</td>
<td>E. coli</td>
<td>Rice</td>
<td>Salinity and drought tolerance</td>
<td>Garg et al., 2002</td>
</tr>
<tr>
<td>codA</td>
<td>Arthrobacter globiformis</td>
<td>Rice</td>
<td>Recovery from a week long salt stress</td>
<td>Mohanty et al., 2002</td>
</tr>
<tr>
<td>CBF3 (Transcription factor)</td>
<td>Arabidopsis</td>
<td>Rice</td>
<td>Drought and salt resistance</td>
<td>Oh et al., 2005</td>
</tr>
<tr>
<td>OsbZIP72 (Transcription factor)</td>
<td>Rice</td>
<td>Rice</td>
<td>Drought tolerance</td>
<td>Lu et al., 2008</td>
</tr>
<tr>
<td>codA (Choline oxidase)</td>
<td>Arthrobacter globiformis</td>
<td>Rice</td>
<td>Water stress tolerance</td>
<td>Kathuria et al., 2009</td>
</tr>
<tr>
<td>ATHK1 (Histidine kinase)</td>
<td>Arabidopsis</td>
<td>Arabidopsis</td>
<td>Drought and salt resistance</td>
<td>Tran et al., 2007; Wolbach et al., 2008</td>
</tr>
<tr>
<td>SOS (Salt Overly Sensitive)</td>
<td>Arabidopsis</td>
<td>Arabidopsis</td>
<td>Salinity tolerance</td>
<td>Yang et al., 2009</td>
</tr>
<tr>
<td>codA (choline oxidase)</td>
<td>Arthrobacter globiformis</td>
<td>B. juncea</td>
<td>Tolerance to stress induced photoinhibition</td>
<td>Prasad et al., 2004</td>
</tr>
<tr>
<td>PgnNHX1 (Vacuolar Na⁺/H⁺ antiporter)</td>
<td>Pennisetum glaucum</td>
<td>B. juncea</td>
<td>Tolerance to salinity stress, exhibited normal growth</td>
<td>Rajagopal et al., 2007</td>
</tr>
<tr>
<td>codA (choline oxidase)</td>
<td>Arthrobacter globiformis</td>
<td>B. napus</td>
<td>Moderate salinity tolerance and enhanced shoot growth</td>
<td>Huang et al., 2000</td>
</tr>
<tr>
<td>AtNHX1 (Vacuolar Na⁺/H⁺ antiporter)</td>
<td>Arabidopsis</td>
<td>B. napus</td>
<td>Salt tolerance, growth, seed yield and seed oil quality</td>
<td>Zhang et al., 2001</td>
</tr>
<tr>
<td>PR10 (Pathogenesis-related)</td>
<td>Pisum sativum</td>
<td>B. napus</td>
<td>Enhances germination and growth in the presence of NaCl</td>
<td>Srivastava et al., 2004</td>
</tr>
<tr>
<td>betaA</td>
<td>E. coli</td>
<td>B. oleracea</td>
<td>Salinity tolerance</td>
<td>Bhattacharya et al., 2004</td>
</tr>
<tr>
<td>Lea</td>
<td>B. napus</td>
<td>B. campestris</td>
<td>Salinity and drought tolerance</td>
<td>Park et al., 2005</td>
</tr>
<tr>
<td>EhCaBP (Calcium binding protein)</td>
<td>Entamoeba histolytica</td>
<td>Tobacco</td>
<td>Enhanced germination and seedling growth under salinity</td>
<td>Pandey et al., 2002</td>
</tr>
<tr>
<td>gly I (Glyoxalase I and gly II (Glyoxalase II))</td>
<td>B. juncea, O. sativa</td>
<td>Tobacco</td>
<td>Salinity, heavy metal tolerance and yield</td>
<td>Singla-Pareek et al., 2003; 2006</td>
</tr>
<tr>
<td>GmERF3 (Transcription factor)</td>
<td>Soybean</td>
<td>Tobacco</td>
<td>Tolerances to salt, drought and diseases</td>
<td>Zhang et al., 2009</td>
</tr>
</tbody>
</table>
Water stress is one of the most essential environmental factors that involve plant growth and development and limit plant production. Plants can take action and acclimatize to water stress by changing their cellular metabolism and raising different defense mechanisms (Zhu, 2002; Boudsocq and Lauriere, 2005). Separate abiotic stress signaling pathways are likely to interact by sharing common elements that are potential nodes for cross talk. It has been observed that all drought-inducible genes are induced by high salinity stress. Most of the drought-inducible genes also correspond to cold stress but some do not and vice versa (Bray, 1997; Shinozaki et al., 2002). Many genes respond to application of ABA whereas some others do not. Different stress conditions may result in the activation of similar stress response pathways. This suggests that high degree of overlap exists between gene clusters activated by different stresses (Chen et al., 2002).

Plant cell generally responds to abiotic stress by first perceiving the stress signal. The signal is then transduced to the nucleus, chloroplast and mitochondria, which subsequently affects the gene expression. Abiotic stresses also result in rise of cytoplasmic calcium ions, cytoskeletal re-organization or protein denaturation, which conveys stress-induced signals to the responding gene through respective signal transduction pathway (Sung et al., 2003). The second ABA dependent pathway induces the production of bZIP proteins under dehydration and salinity stress. These transcription factors bind to the ABRE (ABA responsive elements-PyACGTGGC) in the upstream sequences found in Arabidopsis genes such as rd29B that are induced by osmotic stress and salinity (Uno et al., 2000; Finkel-stein et al., 2002). One of the ABA independent pathways overlaps with that of cold response. In this pathway, CBF1 (CRT binding factor 1) or DREB (dehydration responsive element binding proteins) genes are induced. The DREBs bind to the genes with DRE (dehydration responsive element-TACCGACAT) in their promoter region and induce their expression under stress condition.
Figure 3: Regulation of gene expression in response to drought, salt and cold indicates ABA-dependent and ABA-independent pathways. Four independent signal transduction pathways function in the activation of stress-inducible genes under drought, salt and cold stresses. Cis-acting elements are shown in DNA helixes and transcription factors binds to DNA helixes. Pathways I, II and V are ABA dependent and based on ABRE or MYBR/MYCR cis-acting elements. Pathways III, IV, VI and VII are ABA independent and based on DRE/CRT or still unidentified cis-acting sequences. Both types of pathways are implemented in drought, salt and cold signaling. Some transcription factors, like ABFs and DREB2s, are stress activated to induce gene expression, whereas others like CBFs require biosynthesis (Modified from Boudsocq and Lauriere, 2005).

2.6 Promoters used in plant transformation

The selection of the promoter used in a transgene vector construct is dependent on the objectives of the transformation. These promoters are categorized according to the type or degree of control of gene expression; control in all or virtually all tissues or control depending on the tissue and the developmental stage of the plant (Nakashima and Shinozaki, 2010). Additionally,
promoters may operate in response to external and in some cases controllable stimuli. Thus, they are classified as follows:

• **Constitutive promoters**, which induce the expression of the downstream coding region in all tissues irrespective of environmental or developmental factors.

• **Tissue-specific promoters**, which operate in a particular tissue and at certain developmental stages of the plant.

• **Inducible promoters**, which are only expressed under the presence of inducing factors/compounds. These can be divided into two groups:
  - Chemically-regulated, where chemical compounds, usually not present in plants switch on promoter activity.
  - Physically-regulated, where external factors such as light, heat, mechanical injury and abiotic stress like drought, salinity, high or low temperature and flooding induce the promoter activity.

• **Synthetic promoters**, which comprise consensus DNA sequences of common elements of natural promoter regions.

Various promoters used in plant transformation have been exhaustively reviewed by Potenza et al., (2004). Several patents have been filed for different constitutive and tissue-specific promoters. An exhaustive list of patents filed related to constitutive and tissue specific promoters and also other inducible promoters are listed in a document of CAMBIA, Australia (Roa-Rodriguez, 2003). While different classes of promoters can confer tissue-specific or temporal expression, plant promoters that drive high, abiotic stress related expression have become a valuable tool in plant genetic engineering.
2.7 Salinity tolerance in rice: an overview

Rice is considered as one of the most important staple crops worldwide. Approximately 50% of the world’s population depends on rice. Only in Asia, 30 to 80 percent of the calories are consumed from rice on a daily basis (Narciso and Hossain, 2002). Rice crop has evolved in a semi-aquatic and low radiation environment. However, it remarkably differs in tolerance and susceptibility towards various environmental stresses. It can be grown in water-logged soil and can show submergence level of tolerance. Moreover, it shows moderate tolerance to salinity and soil acidity. But it is highly susceptible to drought and cold conditions (Lafitte et al., 2004). Many rice growing habitats require more tolerance than is actually present in most improved germplasm. In order to meet the requirement for desired abiotic stress tolerance, a rice genotype improved for its tolerance through genetic manipulation could play a significant role in the genome era of plant science. Rice possess a small genome size among most of the cultivated cereals and it has conserved a majority of the gene content and gene order that are present in other species (Gale and Davos 2001). Additionally, it shares a greater synteny with grass family members. The availability of complete genome sequence (Goff et al., 2002), may allow easy identification and localization of genes which are closely related to stress tolerance. The syntenic association between grass genomes has positively encouraged the application of functional genomics approach to rice and to better understand plant processes, disease resistance as well as the tolerance limits of abiotic stresses. Rice shows tolerance to salinity during germination and tillering stage. However, it is quite sensitive during early seedling and reproductive stage (Lutts et al., 1995). The physiological basis of tolerance in the early seedling stage is rather understood, and the main tolerance features include: high seedling vigor, salt exclusion at the root level, ion compartmentalization in new and older tissue, high tissue tolerance, responsive stomata that close just after salt exposure, partially reopen after a period of acclimation and up regulation of antioxidant systems,
predominantly the ascorbate/glutathione pathway of oxidative stress tolerance (Walia et al., 2005). It has been found that tolerant genotypes exclude salt from the flag leaf during reproductive stages (Yeo and Flowers 1986). However, these kinds of traits are autonomous, tolerant genotypes seldom favor these traits and there seems to be a significant variation in the expression of these traits at critical stages of rice development in the cultivated gene pool of rice. Pyramiding of contributing traits at both seedling and reproductive stages is needed for developing resilient salt tolerant cultivars (Moradi et al., 2003). Both conventional approaches as well as genetic manipulation have been used to improve rice germplasm with respect to their salinity tolerance (Singh et al., 2008).

2.8 Salinity tolerance in Brassica: an overview

The family Brassicaceae presents before us a very interesting family comprising of a large number of species across the globe. The model plant Arabidopsis also belongs to the same family. Since genetic mapping has already been performed for this dicot plant, it is an exceptional model for crop plants. The genus Brassica is extremely important for agricultural and horticultural crops than its other counterparts. The most common oil-seed crops grown include rape seeds (B. campestris and B. napus) and mustards (B. juncea and B. carinata). B. napus, B. juncea, and B. carinata are amphidiploids, whereas B. campestris, B. oleracea and B. nigra are diploid. Brassica occupies third rank among oilseed species due to its high economic as well as nutritional value. However, the plant growth, yield and oilseed production plus total biomass are severely affected by salinity stress and therefore, there is an immediate necessity to raise plants which could be appropriate for saline and dry lands in the near future (Ashraf and McNeilly 2004). Most of the Brassica species are considered as moderately salt tolerant, however, amphidiploid species have been found to be relatively tolerant as compared to diploid species (Malik, 1990; He and Cramer 1992; Kumar 1995; Purty et al., 2008). It has been suggested that the higher salt tolerance of
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amphidiploids are due to the presence of the A (B. campestris) or C (B. oleracea) genomes (Ashraf et al., 2001). Specific inter and intraspecific variations have been found for salinity within Brassica that can be exploited through selection and breeding to enhance salt tolerance (Ashraf and McNeilly 2004, Purty et al., 2008).

There are different reports for salinity tolerance at different developmental stages for the Brassica species mentioned above. However, they maintain salt tolerance consistency throughout the plant ontogeny (Ashraf and McNeilly 2004). The accumulation of toxic ions (Na$^+$ and Cl$^-$), in plant tissues that are subjected to saline conditions have been found to be due to the mechanism of partial ion exclusion (exclusion of Na$^+$ and/or Cl$^-$) in most of the species, however, ion inclusion at intraspecific levels has also been found in some cases (Haq et al., 2002). Balance of high tissue K$^+$/Na$^+$ and Ca$^{2+}$/Na$^+$ ratios is crucial as one of the selection criterion for salt tolerance (He and Cramer 1993; Qasim and Ashraf 2006). Moreover, osmotic adjustment has also been found in plants that are subjected to saline conditions especially in salt-tolerant species or cultivars. Organic osmotica like soluble sugars, free proline and free amino acids play a significant role in osmotic adjustment. In order to increase salt tolerance limit in species, breeding crop varieties could be considered as the most promising, energy-efficient and economical approach as compared to traditional approaches such as soil amelioration techniques, which are beyond the scope of marginal farmers (Ashraf and McNeilly, 2004). However, high genetic and additive variation is required for breeding crop varieties in which significant genetic advance occur through repeated selection (Ashraf, 1994). High heritability has been found in forage rape (B. napus) in response to selection for salinity (200 mM NaCl) (Ashraf et al., 1987). While measuring variability in 30 genotypes of the Indian mustard, high heritability and genetic advance were observed for number of siliquae on main shoot, seedling emergence, number of secondary branches per plant and seed yield on saline soil (Kumar, 1993). One of the most important factors required for a successful breeding program is that the
components of the heritable variation be additive (Sharma, 1994). In addition, study on *B. carinata* under saline conditions has also been done. Reports on the above studies have shown that in most of the additive and non-additive variance is very low among the genes controlling this trait. Conventional breeding has been a powerful source in the past for generating varieties of high yield and quality; a few significant salt-tolerant cultivars have also been raised by plant breeders. Central Soil Salinity Research Institute (CSSRI) at Karnal, Haryana has raised several improved *Brassica* species e.g. CS52 and CS54 which have been released to farmers and have shown significant increase in crop yield under saline condition.

However, modern breeding approaches have offered significant success towards the development of salt tolerant crop species. This involves quantitative nature of processes including salt-tolerance trait, which is controlled by polygenes (Ashraf, 2002; Quesada, 2002). However, this approach is time consuming plus labor intensive that allows the transfer of both desirable and undesirable genes. In such conditions, genetic engineering proves to be a potent means of transferring genes efficiently across reproductive barriers.

Only a few reports have been found towards the development of transgenic *Brassica* for salt tolerance. Transgenic *Brassica napus* plants overexpressing AtNHX1, a vacuolar Na⁺/H⁺ antiporter from *Arabidopsis thaliana*, were able to grow, flower, and produce seeds in the presence of 200 mM sodium chloride. Although the transgenic plants grown in high salinity accumulated sodium up to 6% of their dry weight, growth of these plants was only marginally affected by the high salt concentration (Zhang et al., 2001). Transformation of *Brassica oleracea* var. capitata with bacterial betA gene enhances tolerance to salt stress (Bhattacharya et al., 2004). Recently, a complete *Brassica* genome sequencing project has also been started (Yang et al., 2006) which will be very useful for improving its tolerance towards osmotic stresses.
2.9 An overview on SOS pathway

Sensing and responding to ecological perturbations are significant for all living organisms. Plants maintain intercellular ionic homeostasis by keeping the toxic ion concentrations below a threshold limit. Several molecular mechanisms are involved when plant responses to adverse environments such as soil salinity and drought besides others. For cytosolic enzymes to function properly, intracellular K⁺ and Na⁺ homeostasis is crucial, as excess of Na⁺ ions pose toxicity for cellular metabolism (Amtmann and Leigh, 2010). On the other hand, potassium plays important roles in various processes including metabolism, growth and stress adaptation. However, K⁺ and Na⁺ ion homeostasis becomes more important under salt stress conditions. Any disequilibrium of Na⁺ ions due to salt stress leads to disastrous pathologies and severely affects growth, cell survival and division. As a result, soil salinity has become a major environmental stress that limits plant growth and productivity across the globe. One of the crucial aspects for plant cells to become salt tolerant is to keep a low concentration of toxic Na⁺ ions in the cytosol. The JK Zhu laboratory at University of California, U.S.A. recently has pioneered identification of salt tolerance determinants using forward genetics in the plant model Arabidopsis (Hasegawa et al., 2000a; Sanders, 2000; Zhu, 2000). This effort has identified three complementation groups of ion hypersensitive (Salt Overly Sensitive) mutants. Genetic and physiological data indicate that SOS3, SOS2, and SOS1 are components of a signal pathway that regulates ion homeostasis and salt tolerance and their functions are Ca²⁺ dependent. Positional cloning revealed that SOS1 encodes a putative plasma membrane Na⁺/H⁺ antiporter, SOS2 encodes a Suc non-fermenting-like (SNF) kinase and SOS3 encodes a Ca²⁺-binding protein with sequence similarity to the regulatory subunit of calcineurin and neuronal Ca²⁺ sensors (Liu and Zhu, 1998; Liu et al., 2000; Shi et al., 2000). Molecular interaction and complementation analyses indicate that SOS3 is required for activation of SOS2 that regulates SOS1 transcription (Halfter et al.,
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2000; Shi et al., 2000), further confirming that the order of the signal pathway is SOS3→ SOS2→ SOS1 (Hasegawa et al., 2000a; Sanders, 2000; Zhu, 2002).

![Diagram of ion homeostasis in plant cell under salt stress via SOS pathway.](image)

**Figure 4:** Cartoon depicting ion homeostasis in plant cell under salt stress as mediated via SOS pathway. SOS3 is a Ca\(^{2+}\) sensor, SOS2 is a kinase, SOS1 and NHX are Na\(^+\)/H\(^+\) antiporter at plasma membrane and vacuolar membrane respectively. HKT1 is Na\(^+\) transporter (modified from Zhu, 2002).

Plant growth under normal and stress conditions are mediated by chemicals such as calcium ions (Ca\(^{2+}\)) that function as a major secondary-messenger signaling molecule (Boudsocq and Sheen, 2010). However, membrane receptors are the one that first perceives the extracellular stress signal, which then activate complex signaling cascade intracellularly such as the generation of calcium ions. This Ca\(^{2+}\) ion eventually, begins the stress signaling pathways for stress tolerance. Generally, stress signals lead to cytosolic Ca\(^{2+}\) perturbations that are unique and specifically decoded by Ca\(^{2+}\) sensing proteins to pass the signaling cascade. However, the recently discovered calcium sensor, calcineurin B-like proteins (CBLs) and their interacting partners CBL-interacting
protein kinases (CIPKs) have appeared to be vital role player in response to calcium and stress signaling. CIPK starts phosphorylation cascade that again regulate down-stream components for stress tolerance. In Arabidopsis, AtCBL4 is a SOS3 and CIPK24 is a SOS2 member.

2.9.1 SOS1: a plasma membrane Na⁺/H⁺ antiporter

SOS1, a plasma membrane Na⁺/H⁺ antiporter in Arabidopsis, is a salt tolerance determinant critical for the maintenance of ion homeostasis in saline stress conditions. SOS1 mRNA is unstable at normal growth conditions, but its stability is substantially increased under salt stress and other ionic and dehydration stresses (Zhu et al., 2008). SOS1 locus has been isolated through positional cloning. Comparison of the ORF with the genomic sequence has disclosed that SOS1 is comprised of 22 introns and 23 exons. A computational analysis further revealed that SOS1 encodes a polypeptide of 1146 amino acid residues with a molecular mass of 127 kDa. Hydrophobic plot analysis revealed the hydrophobic nature of the N-terminal region and has 12 predicted transmembrane domains. However, the C-terminal region of SOS1 is highly hydrophilic in nature. But this domain was unique and no similarities were found with other antiporters in the GenBank database. In fact, the C-terminal hydrophilic tail makes SOS1 the largest known Na⁺/H⁺ antiporter sequence. Phylogenetic analysis has revealed that SOS1 is closely associated with plasma membrane Na⁺/H⁺ antiporters such as SOD2 (superoxide dismutase), NHA1, NHaA and NhaP. NHA1 functions in the plasma membrane and mediates Na⁺ and K⁺ efflux (Banuelos et al., 1998). Both SOD2 and NHA1 function as plasma membrane Na⁺/H⁺ antiporters to efflux excess Na⁺ out of the cytosol for Schizosaccharomyces pombe and Saccharomyces cerevisae, respectively (Hahnenberger et al., 1996; Dibrov et. al., 1997). Sequence analysis of different SOS1 mutant alleles revealed some residues and regions, which are important for SOS1 function. The sos1-3 and sos1-2 alleles have point mutations in the
membrane spanning region. These mutations are R to C and G to E, correspondingly. Both these mutations affect residues that are conserved in all antiporters and most likely remove the antiporter activity of SOS1 (Shi et al., 2000). Other point mutations such as (sos1-8 and sos1-9) have also been revealed in the hydrophilic tail region. However, these mutations do not affect the transmembrane region and therefore, reveal the importance of both N and C domains for the functioning of SOS1 (Shi et al., 2000). In a functional study, Juliana Martinez-Atienza and coworkers in 2007, have discovered the conservation of SOS pathway in rice. They have recognized functional homolog of the AtSOS1 (Na\(^+\)/H\(^+\) antiporter) protein with that of a rice plasma membrane Na\(^+\)/H\(^+\) exchanger. The rice transporter, OsSOS1 demonstrated the ability for Na\(^+\)/H\(^+\) exchange in plasma membrane vesicles of yeast cells and abridged their net cellular Na\(^+\) content. The Arabidopsis protein kinase complex AtSOS2–AtSOS3, that positively controls the action of AtSOS1, phosphorylated OsSOS1 and stimulated its activity in vivo and in vitro. However, OsSOS1 was found to suppress salt sensitivity of a sos1-1 mutant of Arabidopsis thaliana.

SOS1 has also been isolated from Populus euphratica growing in semi-arid saline areas. PeSOS1 showed 64% sequence identity with AtSOS1 (Ding et al., 2007). In another report, Salt cress (Thellungiella halophila) showed increased expression of SOS1 in the plasma membrane isolated from control- and salt-treated roots and leaves of this plant (Estrella et al., 2005). A recent study on the model plant Arabidopsis thaliana suggested the roles of the SOS1 protein, in addition to its function as a Na\(^+\)/H\(^+\) antiporter, whose disruption affected membrane traffic and vacuolar functions possibly by controlling pH homeostasis in root cells (Oh et al., 2009).

2.9.2 SOS2: Ser/Thr protein kinase

SOS2 gene was first isolated by Jian-Kang Zhu and coworkers in 1997 using positional cloning method. The gene was found to be crucial for sodium
and potassium ion homeostasis and salt tolerance (Liu et al., 1997). Phosphorylation by protein kinases is considered as the most important regulatory mechanism. SOS2-like protein kinase, PKSs or CIPKs are a large family of 25 protein kinases in Arabidopsis thaliana (Guo et al., 2001; Luan et al., 2002; Mahajan et al., 2006). SOS2 and SOS3 are the vital members of a unique protein family in Arabidopsis of 25 kinases containing FISL (also called as NAF) and PPI motifs, the PKS or CIPKs (thereafter CIPKs) (Guo et al., 2001), and of a family of 10 EF-hand-type calcium-binding proteins, the SCaBPs (Guo et al., 2001) or CBLs (thereafter CBLs) (Luan et al., 2002), correspondingly.

2.9.3 Structural domains of SOS2

In case of Arabidopsis, SOS2 encodes serine/threonine protein kinase (446-amino acids) with an N-terminal catalytic domain, which is similar to SNF/AMPK and a novel C-terminal regulatory domain (Liu et al., 2000). In order to make SOS2 functional, both catalytic and regulatory domains are necessary. It has been found that in a yeast 2-hybrid system as well as in vitro, SOS3 physically interacts with SOS2 (Halfter et al., 2000). As salt stress increases inside the cell, the intracellular Ca\(^{2+}\) level increases, which is sensed by calcium binding protein SOS3. After binding with Ca\(^{2+}\), SOS3 conformation changes and interacts with SOS2. This interaction is further supported by sos2sos3 double mutant analysis, which confirms that the two genes function in the same pathway (Halfter et al., 2000). Yeast 2-hybrid and in vitro assays revealed that a 21-amino acid motif in the C-terminal regulatory domain (FISL motif) of SOS2 is crucial and enough for interaction with SOS3. The assay also revealed that N-terminal kinase domain and C-terminal regulatory domain interact with each other and suggested that the regulatory domain might inhibit kinase activity by blocking substrate access to the catalytic site (Guo et al., 2001). In addition, it has also been identified that a protein phosphatase ABI2 (Abscisic acid-Insensitive 2) as a SOS2-interacting protein. Moreover, by deletion analysis, it
was found that a 37-aminio acid domain of SOS2, which is designated as the protein phosphatase interaction motif (PPI) is crucial for interaction with ABI2 (Ohta et. al., 2003). This PPI motif was largely conserved in protein kinases of Arabidopsis SOS2 family. Mutations in the conserved amino acid residues in the PPI motif abolished the interaction of SOS2 with ABI2 suggesting that these two conserved residues in SOS2 are important for interaction with ABI phosphatases.

2.9.4 Biochemical characterization of SOS2

The biochemical characteristics of SOS2 and mechanisms of regulation of its kinase activity are not fully understood. The current evaluation of the biochemical and kinetic properties of the SOS2 has been made possible by the accessibility of recombinant SOS2 mutants that show robust kinase activities. Regulation of protein kinases can be achieved by different mechanisms, including protein phosphorylation by other kinase(s) (Elion, 1998), autophosphorylation (Cooper and MacAuley, 1988; Sato et al., 1996), or control by regulatory domains or subunits. A key feature for regulation in many protein kinases is thought to be the phosphorylation of one or more residues within the activation loop of the catalytic subunit (Vertommen et al., 2000; McCartney and Schmidt, 2001). An unphosphorylated activation loop can block access of substrates to the active site, whereas phosphorylation can cause an outward rotation of the activation loop, making substrate accessible to the active site residues for catalysis (Jeffrey et al., 1995; Sicheri and Kuriyan, 1997; Xu et al., 1999). Recombinant SQS2 protein produced in bacteria exhibits no substrate phosphorylation activity in the absence of SOS3, although it has autophosphorylation activity (Halfter et al., 2000). In the presence of calcium, SQS3 activates the substrate phosphorylation activity of SOS2 (Halfter et al., 2000). The substrate phosphorylation activity of SOS2 could also be activated by a Thr-168 to Asp mutation within the activation loop or by removal of the autoinhibitory FISL motif (Guo et al., 2001; Qiu et al., 2002).
2.9.5 SOS2 interaction with other factors in response to salt stress

The SOS pathway and SOS2 in particular, is also a point of cross talk between salt stress and other stress signals and stress responses (Chinnusamy et al., 2005; Pardo et al., 2006; Zhu, 2003). The SOS2 kinase has been considered as an important regulatory component through its interactions with other signaling proteins. Being a vital part of the SOS signaling pathway, the regulatory region of SOS2 has been shown to interact with SOS3 (Halfter et al., 2000). However, specific interactions have also been found between CIPK and CBL proteins, which are involved in signal transduction controlling abscisic acid (ABA) sensitivity, cold response, sugar response and cellular pH (Cheong et al., 2003; D’Angeolo et al., 2006; Gong et al., 2004; Kim et al., 2003; Pandey et al., 2004). SOS2 also interacts with ABA-insensitive 2 (ABI2) protein phosphatase 2C (PP2C) through a specific protein phosphatase interaction domain (Ohta et al., 2003). It has also been revealed that SnRK3s or CIPK interact with ABI1 or ABI2 but not both. Finding other proteins that interact with SOS2 or other CIPK remains a potential approach towards better understanding of stress signaling. In addition to the conventional role of SOS2 in regulating ion transport (Cheng et al., 2004; Zhu et al., 1998), the interaction of SOS2 with ABI2 suggests a correlation to other aspects of stress signaling (Ohta et al., 2003). Other SnRK3 kinases may also be involved in signaling mechanisms controlling responses to the environment or hormone response, particularly ABA signal transduction (Guo et al., 2002).

Present research shows the interaction of NDPK2 with SOS2 and SnRK3. This kind of interaction along with salt sensitivity phenotype of the sos2-2 ndpk2 double mutant suggests that NDPK2 has a crucial role in salt stress signaling through its interaction with SOS2. NDPK2 plays an important role in the regulation of H$_2$O$_2$ accumulation and sensitivity. In addition, SOS2 has also been found to be associated with catalase 2 (CAT2) and CAT3 and that further
associates SOS2 to $H_2O_2$ metabolism and signaling. Therefore, the interaction of SOS2 with both NDPK2 and CATs reveals a point of cross talk between salt stress response to other signaling factors including $H_2O_2$ (Verslues et al., 2007). Interaction of SOS2 with other factors have been identified using yeast-2 hybrid and proteomic approaches and reported the identification of the V-ATPase as a target of SOS2 interaction and regulation. In response to salt stress, SOS2 is activated and recruited to the plasma membrane by binding to SOS3. The SOS3-SOS2 complex activates the Na$^+$/H$^+$ antiporter SOS1 that reduces Na$^+$ concentration in the cytosol by extrusion to the apoplast. However, on the plasma membrane, H$^+$ is needed to drive Na$^+$ extrusion, which is regulated by H$^+$-ATPase. Moreover, SOS2 also activates tonoplast located NHX-antiporters to compartmentalize Na$^+$ ions to the vacuole and the H$^+$/Ca$^{2+}$ transporter CAX1. SOS2 has been found to regulate these tonoplast transporters independently of SOS3. The H$^+$ gradient needed to drive Na$^+$ transport across the tonoplast is maintained by the tonoplast H$^+$-pyrophosphatase and the V-ATPase. The FISL domain of SOS2 is known to be required for SOS3 binding and could also have a role in the interaction of SOS2 with the V-ATPase either directly or through SCaBPs (Giorgia et al., 2007)

2.9.6 SOS3: calcium binding protein

SOS3/CBL4 is a small myristoylated protein that seems to have no enzymatic activity by itself; Ca$^{2+}$ binding and myristoylation are required for SOS3 function in salt tolerance (Ishitani et al., 2000). SOS3 senses the change in cytosolic Ca$^{2+}$ ion concentration and transduces the signal downstream. As compared to other Ca$^{2+}$ sensors, SOS3 binds calcium with a relatively low affinity (Ishitani et. al., 2000). SOS3 is also called as CBL4 (calcineurin B-like protein) that encodes a novel EF-hand Ca$^{2+}$ sensor (Liu and Zhu, 1998). Each plant CBL harbors four EF-hands with varying degree of conservation, as compared to the canonical EF-hand sequence.
SOS3 shares significant sequence similarity with the regulatory calcineurin B subunit from yeast (Saccharomyces cerevisiae) and neuronal Ca\(^{2+}\) sensors from animals (Klee et al., 1988). In spite of this similarity with calcineurin B at the primary sequence level, it is now clear that Arabidopsis does not have calcineurin and that the SOS3 Ca\(^{2+}\) sensing protein activates a protein kinase and not a protein phosphatase. Only few members of the protein family contain an N-terminal myristoylation motif. SOS3 is myristoylated, which may help target SOS3 and its interacting proteins (e.g., SOS2) to membranes where their target transporters are located (e.g., SOS1).

Soil salinity is a major abiotic stress that decreases plant growth and productivity. Recently, it was reported that plants overexpressing AtNHX1 or SOS1 have significantly increased salt tolerance. From the experimental research, it has been found that transgenic plants overexpressing SOS3 exhibit increased salt tolerance similar to plants overexpressing SOS1. Moreover, salt tolerance of transgenic plants overexpressing AtNHX1-SOS3, SOS2-SOS3, or SOS1-SOS2-SOS3, respectively, appeared similar to the tolerance of transgenic plants overexpressing either SQS1 or SOS3 alone (Yang et al., 2009).

### 2.10 RNAi as a tool for functional genomics

RNAi is one of the emerging tools in the post genomic era. The major driving force in this era is to authenticate the functions of the predicted genes. The earliest and the most significant finding that came into focus was from a serendipitous discovery while attempting to enhance the flower color in Petunia.

In 1990, Napoli et al., attempted to examine Petunia plants for increased anthocyanin production by the overexpression of chalcone synthase (chsA). Surprisingly, instead of getting plants with higher anthocyanin content, variegated flowers with white patches were obtained. This was later on confirmed that the introduction of chsA transgene led to the suppression of endogenous gene expression. This event was named as “co-suppression” and a number of workers...
reported similar occurrences independently. Further studies recommended that this fact did not involve reduced transcription rather degradation of the transcripts through a partial duplex mRNA, that triggered gene silencing. Since this method involves post-transcriptional mRNA degradation, it was termed as post-transcriptional gene silencing in plants (PTGS, Matzke, 1995).

PTGS is supposed to provide a usual function of protecting genome against mobile elements such as viruses. In addition to plants, homology dependent gene silencing was most likely found to occur in fungal systems and these events are termed as "quelling" (Cogoni et al., 1996). Therefore, till now, three phenotypically dissimilar but mechanistically similar forms of RNAi have been found that include PTGS or co-suppression in plants, quelling in fungi and RNAi in animal kingdom. With the accessibility of a large number of eukaryotic genome sequences in the post-genome sequencing era – the present aim is to confirm the functions of all of these predicted genes. In the present time, PTGS is considered as the most preferred technique available for large-scale functional assays of genes (Baulcombe, 2004; Vauchret et al., 2001; Waterhouse et al., 2001).