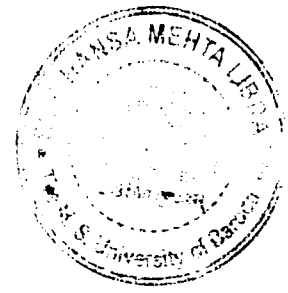

Chapter 1

REVIEW OF LITERATURE

1.0 Introduction

Salinity is one of the major constraints that limit agricultural production world-wide (Boyer, 1982). It is one of the most serious environmental factors limiting the productivity of crop plants (Ashraf, 1999). Nearly 40% of world's surface has salinity problems (Jadhav et al., 2010). At present, out of 1.5 billion hectares of cultivated land in the world, about 77 million hectares is affected by excess salt content (Evelin et al., 2009) of which 45 million hectare is irrigated and 32 million hectare land is non irrigated land (Munns. 2002). The affect of soil salinity on agricultural yield is enormous as it affects the establishment, growth and development of plants leading to huge losses in productivity (Mathur et al., 2007).
Also, Salinization is further spreading to irrigated land due to improper management of irrigation and drainage.

Salinity reduces the ability of plants to absorb water, causing rapid reductions in growth rate (Borsani et al., 2001a). High salt concentration in the soil decreases the osmotic potential of the soil solution and creates water stress for plants. Secondly, it causes severe ion toxicity since Na^+ is not readily sequestered in to vacuole. Eventually, the interactions of salts with mineral nutrition results in nutrient imbalance and deficiencies. Due to high salt concentration, processes such as seed germination, seedling growth and vigor, vegetative growth, flowering and fruit-set get adversely affected leading to plant demise as result of growth arrest and molecular damage (Sairam RK, 2011). Thus salinity affects most aspects of plant physiology.

1.1 Perception of salt stress

The ability of plants to combat environmental stress is determined by the efficiency of the plant to sense the environmental stress and activate its defense machinery. Plants perceive stress as ionic and osmotic stress. Excess Na^+ and Cl^- induced conformational changes in protein structure and

membrane depolarization can lead to the perception of ionic toxicity. Plasma membrane proteins such as Sln1 and Sho1 (Serrano et al., 2001), ion transporters, and/or Na+ sensitive enzymes such as SOS1 (Zhu, 2003) are the sensors of toxic Na+ concentrations in extra and intracellular cites.

1.1.1 Secondary Messengers involved in salt stress signaling

Ca^{+2} acts as signaling molecule in salt stress signaling and prevents Na^{+} entry in to cells (Knight et al.,1997). Thus Ca^{+2} plays important role in maintaining plant growth under saline condition inducing ion channel discrimination against Na^{+} (Schroeder et al., 2001). Cytocsolic Ca^{+2} oscillations during salt stress are regulated through activities of mechanosensitive and ligand-gated Ca^{+2} channels on plasma membrane, endoplasmic reticulum, and vacuole (Zhu, 2003). Excess Na^{+} induced membrane depolarization activate Ca^{+2} channels to generate Ca^{+2} signatures under salt stress. Salt stress also cause, an increased biosynthesis and also accumulation of abscisic acid (ABA) (Koornneef et al, 1998; Taylor et al, 2000) which plays very important role in osmotic stress tolerance (McCourt, 1999; Rock, 2000; Zhu, 2002). ABA is also involved in the control of ion homeostasis. For example, ABA content was higher in the leaves of the salt tolerant rice cultivar versus the susceptible cultivar. This increase in ABA content was accompanied by an improved K^{+} / Na^{+} ratio (Maathuis & Amtamann 1999). Also, the transport and accumulation of K^{+} in higher plant roots were regulated by ABA (Roberts, 1998). Recent reports indicate that ABA regulates K^{+} channel activity in maize and Arabidopsis roots, suggesting that ABA regulation of K^{+} transport in roots is, at least in part, ion channel-mediated (Roberts and Snowman, 2000).

Salt induced water stress leads to reduction in chloroplast stromal volume and generation of reactive oxygen species (ROS) which also plays an important role in inhibiting photosynthesis (Price and Hendry, 1991). ROS can be generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at Photosystem I, in the Mehler reaction (Foyer et al., 2003)

1.1.2 Ion Homeostasis

High salt concentration in the external solution of plant cells produces several deleterious consequences. Salt stress causes an ionic imbalance (Niu et al.,1995; Zhu et al., 1997). When salinity results from an excess of NaCl, which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- ions becomes deleterious to cellular systems (Serrano et al, 1999). In addition, the homeostasis of not only Na^+ and Cl^- , but also K^+ and Ca^{+2} ions is disturbed (Serrano et al, 1999; Hasegawa et al, 2000a, b).As a result, plant survival and growth will depend on adaptations that re-establish ionic homeostasis, there by reducing the duration of cellular exposure to ionic imbalance. Plants achieve ion homeostasis by restricting the uptake of toxic ions and maintaining the uptake of essential ions and by compartmentalization of toxic ions into the vacuole of specific tissue type. In most plants Na^+ is the primary cause of ion toxicity and therefore management of Na^+ concentration is critical for salt tolerance. Na^+ is kept below toxic level in the cytosol by 1) restriction of Na^+ entry at the root cortex cells, 2) excretion of Na^+ from root cells into the soil solutions 3)retrieval of Na^+ from the transpirational xylem stream to re-circulate in to root cells. 4) storage of Na^+ into vacuole of the mature cells 5) Na^+ excretion (Zhu, 2000). Among these mechanisms, Na^+ excretion through glands is significant only in halophytes.

Salt stress is sensed by putative salt stress sensors such as Salt Overly Sensitive 1 (SOS1) or two component Histidine kinase (HK1) proteins (Serrano, 2001). These sensors induce elevation of cytosolic Ca^{+2} concentration. A calcium sensor protein, Salt Overly Sensitive 3 (SOS3) perceives this Ca^{+2} signal and activates SOS2 protein kinase. Activated SOS2 phosphorylates SOS1, a plasma membrane Na^+ /H^+ antiporter, which then transports Na^+ out of the cytosol. The SOS3 dependent SOS2 kinase pathway positively regulates SOS1 transcript level and negatively regulate the Na^+ transporter HKT1. The SOS2 kinase, probably through SOS3 like Ca^{+2} Binding Protein (SCaBPs) dependent pathways, activates the tonoplast Na^+ /H^+ antiporter (NHX1) and vacuolar H^+/Ca^+ antiporter (VCX1), (Chinnusamy et al., 2005).

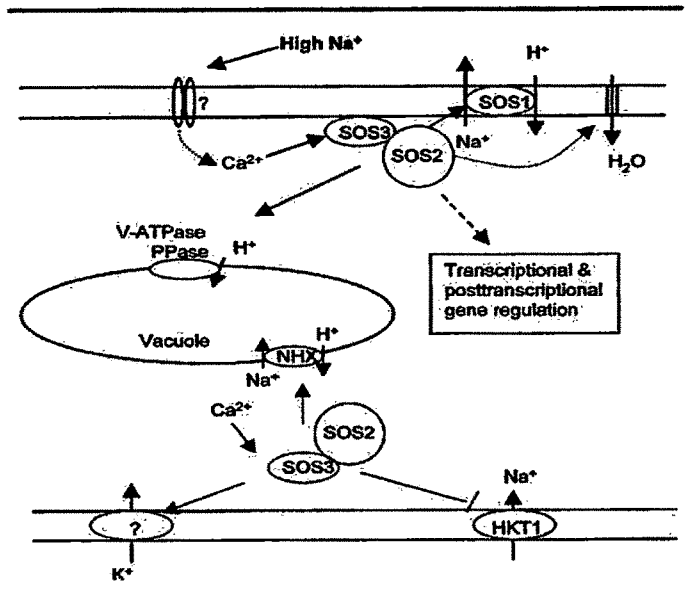

Fig.1.1 Regulation of ion (e.g., Na^+ and K^+) homeostasis by the SOS pathway. High Na^+ stress initiates a calcium signal that activates the SOS3-SOS2 protein kinase complex, which then stimulates the Na^+ /H^+ exchange activity of SOS1 and regulates transcriptionally and posttranscriptionally the expression of some genes. SOS3-SOS2 may also stimulate or suppress the activities of other transporters involved in ion homeostasis under salt stress, such as vacuolar H^+ -ATPases and pyrophosphatases (PPase), vacuolar Na^+ /H^+ exchanger (NHX), and plasma membrane K^+ and Na^+ transporters.

Figure taken from Zhu JK (2002) Salt drought stress signal transduction in plants.

1.1.3 Sodium uptake

Restriction of Na^+ entry in to root cell and then into the transpirational stream is very important to prevent the increase in the toxic level of salt in the shoot. Both glycophytes and halophytes must exclude about 97 % of Na^+ present in the soil at the root surface in order to prevent toxic levels of Na^+ accumulation in shoot (Munns, 2002). Sodium entry into transpirational stream depends on amount of Na^+ uptake by Na^+ and non specific cation transporter and the percentage of water entry in the apoplastic/bypass pathway in the xylem tissue. Na^+ from the soil solution gains initial entry in the root epidermis and the cortex. The casperian strip in the endodermis plays a crucial role in preventing apoplastic Na^+ influx into the root stele (Karahar et al., 2008)

1.1.4 Sodium Efflux

Sodium efflux from root cells is a frontline defense that prevents accumulation of toxic levels of Na^+ in the cytosol and Na^+ transport to the root. Plasma membrane Na^+/H^+ antiporters pump out Na^+ from root cells. In *Arabidopsis*, the plasma membrane Na^+ /H^+ antiporter, SOS1, mediates Na^+ efflux and its activity is regulated by SOS3-SOS2 kinase complex during salt stress (Zhu, 2003). Molecular analysis led to the identification of targets of the SOS3-SOS2 regulatory pathway. One of the target of the SOS pathway is SOS1. The SOS1 has significant protein sequence homology and conserved domains similar to that of plasma membrane Na^+ /H^+ antiporter from bacteria, fungi and animals. Expression of SOS1 is ubiquitous but stronger in epidermal cells surrounding the root tip and in parenchyma cells bordering the xylem. Expression of SOS1-GFP fusion protein and anti-SOS1 antibody confirmed that SOS1 is localized in the plasma membrane of root and leaf cells (Qiu, 2003). The SOS1 mutant plants show hypersensitivity to salt stress (100mM Na^+) and accumulate more Na^+ in roots than do the wild type plants (Shi, 2000).The Na^+ /H^+ exchange activity of SOS1 is regulated by SOS3-SOS2 complex under salt stress. Isolated plasma membrane vesicles from *sos3* and *sos2* mutants showed significantly less Na^+ /H^+ exchange activity than that of wild type plants.

1.1.5 Sodium compartmentation

Soil salinity decreases soil water potential which leads to osmotic stress. To maintain water uptake during osmotic stress, plants have evolved a mechanism known as osmotic adjustment. Osmotic adjustment is active accumulation of solutes such as inorganic ions(Na^+ and K^+) and organic solutes (proline, betaine, polyols and soluble sugars) (Binzel et al., 1987). Vacuolar sequestration of Na^+ is an important and cost effective strategy for osmotic adjustment as it enables at the same time to reduce the cytosolic Na^+ concentration during salinity. Vacuolar Na^+ /H^+ antiporters use the protein

gradient generated by vacouler H^+-adenosine triphosphate (H^+-ATPase) and H^+-inorganic pyrophosphate (H^+-PPase) for Na^+ sequestration in to the vacuole. Hence coordinated regulation of Na^+ /H^+ antiporters, H^+-ATPase and H^+- PPase is crucial for salt tolerance.

1.2 Oxidative Stress Management

High salt concentration also increases the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), super oxide radical (O_2^-), Hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH). Plants possess both enzymic and non enzymatic mechanism for scavenging of ROS. The enzymic mechanisms are designated to minimize the concentration of O_2^- and H_2O_2. The enzymes over produced include super oxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione synthesizing enzymes.

The super oxide radical is regularly synthesized in chloroplast (Elstner et al., 1991) and mitochondria (Rich et al., 1978), although some quantity is also reported to be produced in microbodies (Lindquist et al., 1991). Scavenging of O_2^- by SOD results in production of H_2O_2 , which is removed by ascorbate peroxidase (Asada, 1992) or catalase (Scandalios et al., 1990). However both O_2^- and H_2O_2 are not as toxic as the hydroxyl radical (OH^-), which is formed by combination of O_2^- and H_2O_2 in the presence of trace amount of Fe^{2+} and Fe^{3+} by Haber-Weiss reaction (Haber et al., 1934). The hydroxyl radical can damage chlorophyll, protein, DNA, lipids and other important macromolecules, thus fatally affecting plant metabolism and ultimately growth and yield.

1.3 Synthesis of osmolytes

In plants, a common response to osmotic stress is the accumulation of compatible osmolytes such as proline (Pro), glycine betaine (GB) and sugar alcohols. It has been suggested that compatible osmolytes do not interfere

with normal biochemical reactions and act as osmoprotectans during osmotic stress. Proline is the most common osmoprotectant that accumulates in plants in response to water stress and salinity (Hanson and Hitz, 1982; Yoshiba et al., 1995).

1.4 Halophytes

The halophytes characteristically posses the capacity to grow under high concentrations of NaCl. The biochemical mechanisms leading to salt tolerance in halophytes are regulated in such way that allow a more successful response to salt stress than in other plants (Hasegawa et al., 2000a, b). However, it remains to be tested that whether the mechanisms employed in salt stress tolerance by halophytes can be employed by glycophytes without a loss in productivity or not. The halophytic land plant M. crystallinum has been frequently used as model plant in salt tolerance studies. This plant is now being used in the identification of ESTs differentially expressed after plant salt exposure (Bohnert et al., 2001). A problem in the use of most halophytes is the identification of gene by the use of a genetic approach (i.e. searching for salt hypersensitive mutants). The halophyte plant-*Thellungiella halophila* can survive at seawater-level salinity and its DNA sequence have a similarity of more than 90% of Arabidopsis (Zhu, 2001). Thus Arabidopsis, a closely related species can also be easily transformed allowing insertion tag mutagenesis (Bressan et al., 2001).

1.5 Glycophytes

All major crop plants are glycophytes and the study of these plants as model organisms would uncover processes related to salt tolerance that are specific to these plant species. Of all the glycophytes, Arabidopsis has most often been useful in the determination of processes involved in salt tolerance. Genetic analysis using Arabidopsis as a model is leading to a deeper knowledge of a key signal transduction pathway in salt tolerance, such as the SOS pathway, critical for salt tolerance (Hasegawa et al., 1994; Zhu, 2000).

Another glycophyte employed in genetic analysis using mutagenesis is tomato (Borsani et al., 2001a). Tomato is a widely distributed annual vegetable crop adapted to a large variety of climates. However, in spite of its broad adaptation, production is concentrated to a few warm and rather dry areas (Cuartero and Fernandez-Munoz, 1999). In these areas with an optimal climate for tomato production, salinity is a serious problem (Szabolcs, 1994). For this reason, a large number of physiological studies on salt stress have been performed using tomato as a model plant (Cuartero and Fernandez-Munoz, 1999). Unlike Arabidopsis, direct studies on salinity, adaptation, and molecular changes in this plant can be assessed also for crop yield.

1.6 Identifying determinants for salt tolerance

The first approach employed to ascertain salt tolerance determinants in plants was to identify the metabolic processes critical to tolerate NaCl. Establishment of ion homeostasis is an essential requirement for plants to survive under salt stress conditions. Plant cells respond to salt stress by increasing Na^+ efflux at the plasma membrane and Na^+ accumulation in the vacuole. Therefore, proteins, and ultimately, genes involved in these processes can be considered as **salt tolerance determinants**. Salt tolerance requires not only adaptation to Na^+ toxicity but also the acquisition of K^+ whose uptake by the plant cell is affected by high external Na^+ concentration. The uptake of K^+ is affected by Na^+ due to the chemical similarities between both ions. Potassium is an essential nutrient being the major cationic inorganic nutrient in most terrestrial plants. Therefore, K^+ transport systems involving good selectivity of K^+ over Na^+ can also be considered as an important **salt tolerant determinant** (Rodriguez-Navarro, 2000).

Another metabolic response to salt stress is the synthesis of compatible osmolytes. These organic compounds are thought to mediate osmotic adjustment, protecting sub-cellular structures and oxidative damage by their free radical scavenging capacity (Hong et al., 1992; Smirnoff, 1993; Hare et

al.,1998). Thus, expression of genes regulating the accumulation of these organic compounds can be considered as **salt tolerant determinants**.

1.7 Mechanism of salt tolerance

A common approach used to determine mechanisms of salt tolerance is the identification of cellular processes and genes whose activity or expression is affected by salt stress (Bray, 1993; Botella et al., 1994; Zhu et al., 1997; Hasegawa et al., 2000a, b). Identification of these salt-regulated genes has allowed a better understanding of the complexity of salt tolerance in higher plants (Bray, 1993, Zhu et al., 1997, Serrano et al., 1998, Hasegawa et al., 2000a, b). The generalized assumption was that a gene regulated by salt stress would probably be important in tolerance. Many of these salt-regulated genes have been reported but the most direct procedure to demonstrate their importance in salt tolerance is through functional genetic analysis. This approach is not easily accomplished in crop plants. The genetic identification of mechanisms involved in K^+ nutrition as a process that is critical for salt tolerance in Arabidopsis and tomato suggests that despite the different degree of salt tolerance among these plant species, they share common mechanisms.

These mechanisms include, in addition to K^+ nutrition, synthesis of compatible osmolytes, Na^+ and Cl^- exclusion and compartmentation, increase of toxic radical scavenging capacity and increase in the content of the phytohormone ABA. Besides these common mechanisms, it is clear that glycophytes have the genes necessary to tolerate salt stress but probably the salt tolerance determinants are properly expressed only after adaptation to stress (Hasegawa et al., 1994; Zhu, 2000).

High concentrations of salt impose a hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought.

1.8 DEVELOPMENT OF SALT TOLERANCE IN PLANT

Despite the advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive. In spite of considerable efforts through breeding programmes, progress to enhance salt tolerance has been very slow. Classic genetic studies have demonstrated that the ability of plants to tolerate salt stress is a quantitative trait involving the action of many genes. As a result, it has been difficult to obtain salt tolerance in crop plants by traditional methods (Foolad and Lin, 1997). This situation is complicated by the fact that generally the main character selected in crop plants is productivity, which is also a complex trait. Therefore, the integration of genes required to increase salt tolerance in a specific genotype is difficult without affecting other important multigenic traits like flowering, fruit quality and dry matter production (Flowers et al., 2000).

Plants respond to salt stress at three different levels, i.e., cellular, tissue and whole plant level. Cell-based mechanisms of ion homeostasis and the synthesis of osmoprotectants are essential determinants for salt tolerance. However, as plant cells become specialized during ontogeny, the adaptive mechanisms to tolerate salt stress may be different. Integration and coordination of the responses of cells, tissues, and organs are required for a proper tolerance to salt stress. However, the separate study of each level of response is required to understand the whole picture of salt tolerance.

Moreover, because salt tolerance is regulated throughout the plant development and yet could be a tissue-specific phenomenon, plant tolerance responses at one stage of development may not necessarily be the same at other stages (Johnson et al., 1992; Lauchli and Epstein, 1990). Therefore, the mechanisms of tolerance at specific stages of plant development is required to be studied in order to understand the biochemical events that play important roles in the responses to salt stress (Borsani et al., 2001a).

Therefore, given all the factors determining the pleitropic deleterious effects of salt stress, the adaptation to salinity may involve the modification of a large number of parameters.

1.9 Increasing plant salt tolerance through genetic engineering.

Numerous reports in the literature have shown improvement of salt tolerance via genetic engineering. Genes employed in these studies have been isolated from a number of organisms, ranging from prokaryotic organisms such as E. coli to halophytes or glycophytes (Table 1.1). The improvement of tolerance to salt stress has been analyzed in different plant species, different stages of development and by using different evaluation criteria. These aspects make it extremely difficult to compare the improvement conferred by different genes in salt tolerance. But, the metabolic pathways and mechanisms modified to improve the salt tolerance can be grouped according to the genes employed in transgenic experiments. These genes can be classified into five groups according to their functions:

- Ion homeostasis,
- Oxidative stress,
- Synthesis of osmolytes,
- Protection of cell integrity
-Transcription factors.

1.9.1 Genes involved in Ion homeostasis.

An important approach to generate plant salt tolerance is the introduction of genes that modulate ion transport systems such as *HAL1* and *HAL3*, which are involved in the regulation of K+ and Na+ transport, respectively (Gisbert et al., 2000; Yang et al., 2001). Overexpression of HAL1 from *Sachharomyces cerevisiae* in tomato improved salt tolerance by maintaining a high internal K^+ concentration and decreasing intracellular Na+ during salt stress (Gisbert et al., 2000). The results are similar to those already described when HAL1 when overexpressed in yeast (Serrano et al., 1998) or other plants species

such as Arabidopsis (Yang et al., 2001) and melon (Bordas et al., 1997). This suggests that the mechanisms controlling the positive effects of the HAL1 gene on salt tolerance are conserved among plants and yeast. The yeast HAL3 gene encodes a FMN-binding protein. Also overexpression of AtHal3, the *A. thaliana* HAL3 gene orthologue, in Arabidopsis improves salt tolerance (Albert et al., 2000).

Abundant experimental evidence has shown the involvement of cytosolic Ca^{+2} in salt stress signaling (Lynch et al., 1989). The PP2B phosphatase calcineurin (CaN) is a critical component of a Ca^{+2} dependent signal transduction pathway that mediates Na^+ , Li and Mn tolerance in S. cerevisiae. A truncated activated form of the catalytic subunit and the regulatory subunit of yeast CaN were coexpressed in transgenic tobacco plants to reconstitute an active phosphatase *in vivo* (Pardo et al., 1998). The enhanced capacity to survive Na^+ shock of plants expressing this gene was similar when the evaluation was conducted either on seedling in tissue culture raft vessels or plants in hydroponic cultures (Pardo et al., 1998). The importance of calcium in salt tolerance was also determined by the use of transgenic rice overexpressing the calcium-dependent protein kinase, OsCDPK7. These plants were able to avoid the wilting phenotype observed in control plants exposed to 200 mM NaCl (Saijo et al., 2000). Overexpression of OsCDPK7 enhanced the induction of RAB16 and WSI18 genes that encode group 2 and group 3 LEA related proteins respectively (Saijo et al., 2000). Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles. Overexpression of a vacuolar Na^+ /H^+ antiporter from *A.thaliana* (AtNHX1) in Arabidopsis plants promoted sustained growth and development in soil watered with up to 200 mM NaCl (Apse et al., 1999). Tomato plants overexpressing the same gene were able to grow, flower, and produce fruit in the presence of 200 mM NaCl (Zhang and Blumwald, 2001). Surprisingly, these plants showed high accumulation of Na^+ in leaves but low Na^+content in the fruit. There is also a report on transgenic *Brasssica napus* plants overexpressing the AtNHX1 gene which were able to grow, flower, and produce seeds in the presence of 200

mM of NaCl (Zhang et al., 2001). The seed production and seed oil quality were reportedly unaffected by the soil salt content.

1.9.2 Genes involved in Oxidative Stress Management.

Salinity generates an increase in reactive oxygen species that can induce deleterious effects on cell metabolism (Polle, 1997; Borsani et al., 2001b). Various groups have developed plants that overexpress several oxidative-stress-related genes, with varied results, depending on the tests used to evaluate these transgenic plants (Bajaj et al., 1999). Transgenic plants overexpressing ROS-scavenging enzymes such as superoxide dismutase - SOD (Alscher et al.,2002) ascorbate peroxidase (APX) (Wang et al., 1999) and glutathione S-transferase/glutathione peroxidase (GST/GPX) (Roxas et al., 1997,2000) showed increased tolerance to osmotic, temperature and oxidative stress. The overexpression of the tobacco *NtGST/GPX* gene in transgenic tobacco plants improved salt- and chilling-stress tolerance because of enhanced ROS scavenging and prevention of membrane damage (Roxas et al., 1997,2000).

The potential role of SOD in the protection against salt stress was examined using transgenic rice plants (Tanaka et al., 1999). At high salinity, the transgenic plants had an ascorbate peroxidase activity about 1.5-fold higher than control plants. Total SOD activity was maintained at a high level and ascorbate peroxidase increased upon salt stress. It was found that the PS II activity and the electron transport in the chloroplast were higher in the transgenic plants compared to the wild type plants under salt stress. These results suggest that an increase in the levels of ascorbate peroxidase and chloroplastic SOD are important factors for salt resistance in rice (Tanaka et al., 1999).

1.9.3 Genes involved in Synthesis of osmolytes

The common ubiquitous mechanism in plants to tolerate salinity is the accumulation of certain organic metabolites of low molecular weight that

are known collectively as compatible solutes (Bohnert et al., 1995). Metabolites that serve as compatible solutes differ among plant species and include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen, 1996). The major role of these metabolites is to serve as organic osmolytes with compatible properties at high concentrations; such osmolytes increase the ability of cells to retain water without disturbing normal cellular functions (Yancey et al., 1982). Genetic engineering to increase levels of these compatible solutes is the promising approach in efforts to increase the ability of plants to tolerate environmental stress.

One of the important molecule that can act as osmoprotectant is the polyalcohol mannitol. Tobacco plants transformed with the *mtlD* gene from *E. coli*, which encodes a mannitol 1-phophate dehydrogenase accumulated mannitol and showed increased plant growth under salt stress (Tarczynski et al., 1993). However, later studies revealed that the increase in mannitol content was not enough to explain the tolerance solely based on osmotic adjustment and the scientists assigned to this molecule a possible antioxidant function too (Karakas et al., 1997). Arabidopsis plants transformed with the same gene were able to germinate in the presence of a concentration of salt inhibitory to wild type plants. However, unlike tobacco *mtlD* transformants, Arabidopsis transgenic plants did not tolerate prolonged salt stress (Thomas et al., 1995).

Ectopic expression of a gene encoding myo-inositol O-methyltransferase (IMT1) in tobacco resulted in the accumulation of methylated inositol (D-ononitol), that conferred higher salt tolerance by increasing the photosynthetic activity (Sheveleva et al., 1997). The accumulation of this compound reached up to 600 mM in the cytosol, thus osmotically balancing the high external Na^+ concentration. Arabidopsis plants expressing the enzyme that catalyzes the first committed step in inositol biosynthesis, the D-myo-inositol-3-phosphate (*Ins3P*) synthase, exhibit an increased level of free inositol (Smart and Flores, 1997). Despite a slight increase in salt tolerance, the authors could not detect significant

differences in the phenotype of transgenic plants when a number of characteristics linked to functions of inositol and inositol-derived metabolites were analyzed. These results suggest that the engineering of inositol metabolism to generate salt tolerance may require the manipulation of several genes.

Osmoprotectant	**Gene source**	**Enzyme or gene**	**Plant species engineered**	**Promoter used**	**Product level (%) a**	**References**
GlyBet	*E. coli*	CDH	Tobacco	35S	ND	Lilius et al., (1996)
	E. coli	CDH+BADH	Tobacco	RbcS[c]	1	Holmstrom et al., (2000)
	Arthrobacter	COX	Arabidopsis	35S	5	Hayashi et al., (1997)
	Arthrobacter	COX	Rice,Arabidopsis	35S	5-25	Sakamoto et al., (1998)
	Arthrobacter	COX	Canola ,Tobacco	35S	5-10	Huang et al., (2000)
	SpiNa+ h	CMO	Tobacco	35S	1	Nuccio et al.,(1998)
	SpiNa+ h, beet	CMO+BADH	Tobacco	35S	1	Nuccio et al., (2000a)
Proline	Mothbean	P5CS or deregulated P5CS	Tobacco	35S	100-200	Kishor et al., (1995) & Hong et al., (2000)
Ectoine	Halomonas	ectA+ectB+ectC	Tobacco cells	35S	1	Nakayama et al., (2000)
Mannitol	*E. coli*	MtlD	Tobacco, Arabidopsis	35S	16	Tarczynski et al.,(1992) Thomas et al., (1995)
Sorbitol	Apple	Stpd1	Tobacco	35S	1-260	Tao et al., (1995) Sheveleva et al., (1998)
d-Ononitol	Ice plant	Imtl	Tobacco	35S	10-70	Vernon et al., (1993) Sheveleva et al., (1997)
Trehalose	*E. coli*	TPS or TPS+TPP	Tobacco	35S	1-2	Goddijn et al.,(1997) Pilon-Smits et al., (1998)
	E. coli	TPS or TPS+TPP	Potato tuber	Patatin	1-24	
	Yeast	TPS1	Tobacco	RbcS	5-19	Holmstrom et al., (1996)
	Yeast	TPS1	Tobacco	35S	1	Romero et al., (1997)

a :This column reports the level of osmoprotectant synthesis in osmotically stressed transgenic plants as a percentage of the level found in a representative organism that naturally accumulates the osmoprotectant under similar conditions (Nuccio et al., 1999). ND, not determined

Table 1.1: Examples of Metabolic Engineering of Osmoprotectants in Higher Plants (Rontein et al., 2002)

1.10 Synthesis of Glycine Betaine

Out of various compatible solutes mentioned, GB is by far the most common in the plant kingdom. Plants, which naturally accumulate GB to significant levels, are able to survive severe salt (~300 mM NaCl) and drought stress. (Hayashi et al., 1998).

Betaine is a dipolar but electrically neutral molecule at physiological pH. With respect to protection against osmotic stress, betaine is regarded as being a particularly effective compatible solute (Le Rudulier et al., 1984). The action of betaine *in vivo* is not, however, confined to osmoregulation. Numerous experiments *in vitro* have indicated that betaine acts as an osmoprotectant by stabilizing both the quaternary structure of proteins and the highly ordered structure of membranes against the adverse effects of high salinity and extreme temperatures (Gorham, 1995). In photosynthetic systems, for example, betaine efficiently protects various components of the photosynthetic machinery, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the oxygen evolving photosystem II (PSII) complex, from salt induced inactivaation and dissociation into subunits

Spp	Enzyme/Gene (Source)	Promoter	Subcellular Targeting	Max. [GB]	Type of Stress	Evaluation of tolerance	Reference
Synechococcus	COD/codA (A.globiformis)	conIIc	Cytoplasm	80 mM	0.4 M Na+ 1/8 d	Growth/Photosynthesis/Chlorophyl	Deshniu m et al., (1995)
	CDH, BADH/	Native	Cytoplasm	50 mM	0.4 M Na+ 1/3 d	Growth	Nomura et al., (1995)
Arabidopsis	COD/codA (A.globiformis)	35S	Chloroplast	1.2 mmol / g fresh wt	0.2 M Na+l/5 d	Growth tolerance	Hayashi et al., (1997)
	COD/cox (A. pascens)	2335S	Cytosol	19 mmol / g dry wt	0.1 M Na+ 1/23 d	Growth tolerance	Huang et al., (2000)
O. sativa	COD/codA (A.globiformis) fresh wt	35S	Chloroplast	1.1 mmol / g fresh wt	0.15 M Na+ 1/7 d	Growth tolerance	Sakamoto et al., (1998)
	COD/codA (A.globiformis)	35S	Cytosol	5.3 mmol /g fresh wt	0.1 M Na+ 1/23 h	Photosynthesis tolerance evaluated	Sakamoto et al., (1998)
N. tabacum	COD/cox (A. pascens)	2335S	Cytosol	13 mmol /g dry wt	0.15 M Na+ l	Growth tolerance evaluated	Huang et al., (2000)
	CDH/modified betA (E. coli)	RbcSe	Cytosol	0.035 mmol /g fresh wt	0.15 M Na+ 1/14 d)	Growth tolerance evaluated	Holmstro Èm et al., (2000)
	CMO/not assigned (SpiNa+ ia oleracea)	35S	Chloroplast	0.05 mmol / g fresh wt			Nuccio et al., (1998)
Brassica napus	COD/cox (A. pascens)	35S	Cytosol	13 mmol g21dry wt	0.3 M Na+ 1/10 d)	Growth & Photosynthesis tolerance evaluation under stress	Huang et al., (2000)

Table 1.2. Summary of transgenic cyanobacteria and plants engineered to synthesize GB and evaluation of their stress tolerance (Sakamoto et al., 2001)

The accumulation of betaine in response to salt, drought and cold has been widely recognized in higher plants that are natural accumulators of this compound (Gorham,1995). A positive correlation exists between the accumulation of betaine and the acquisition of tolerance to salt and cold in maize (*Zea mays*) and barley (*Hordeum vulgaris*), respectively (Rhodes et al.,1989; Kishitani et al., 1994) . Genetic evidence also indicates that betaine improves the salt tolerance of these members of Gramineae (Saneoka et al., 1995). Moreover, exogenous application of betaine to leaves or roots has been shown to increase the tolerance to various stresses of several species of plants, including both natural accumulators and non-accumulators (Allard et al., 1998; Hayashi et al., 1998).

In most organisms including plants, betaine is synthesized as a result of the two-step oxidation of choline via betaine aldehyde, a toxic intermediate (Fig. 1.2). In several higher plants from taxonomically unrelated families, the relevant enzymes are choline monooxygenase (CMO), a ferredoxin dependent soluble Rieske-type protein, and betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8), a soluble NAD+-dependent enzyme (Weigel et al., 1986; Brouquisse et al., 1989). These enzymes are found mostly in the chloroplast stroma and their activities, as well as increase levels of betaine, in response to salt stress.

BADH has also been found in several plants that barely accumulate any betaine (Weretilnyk et al., 1990). In mammalian cells and in microorganisms such as *Escherichia coli*, betaine is synthesized by choline dehydrogenase (CDH; EC 1.1.99.1), a membrane-bound oxygen-dependent enzyme, in combination with BADH (Landfald and Strùm, 1986). In contrast to these two pathways that each involve two enzymes, the biosynthesis of betaine is catalysed by a single enzyme, choline oxidase (COD; EC 1.1.3.17), in certain microorganisms, such as the soil bacterium *Arthrobacter globiformis* (Ikuta et al., 1977; Fig. 1.2).

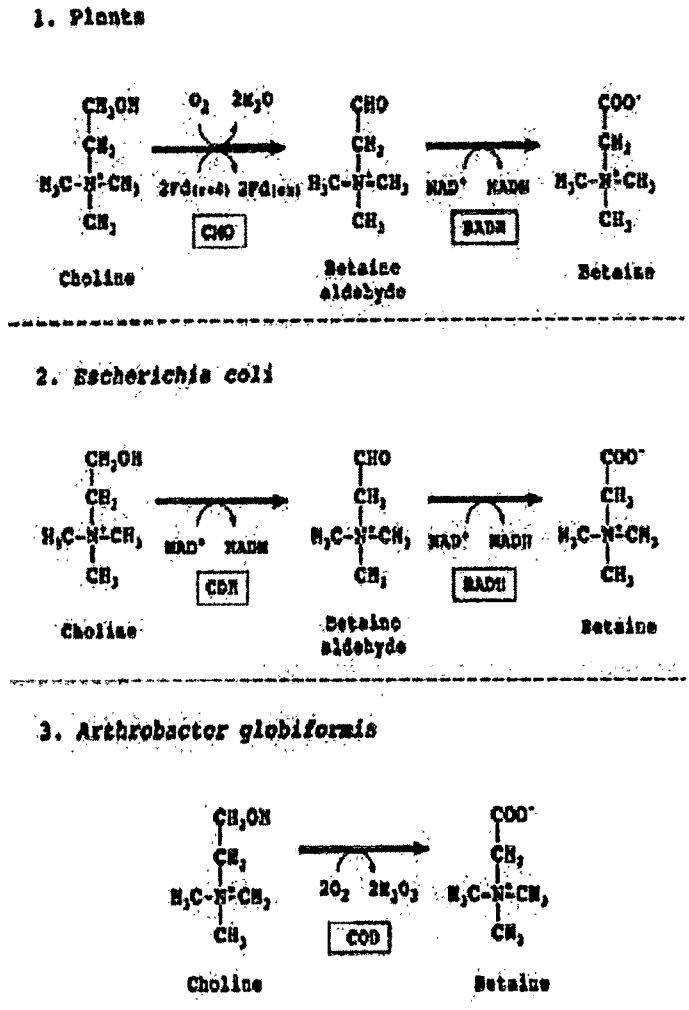

Fig.1.2 Biosynthesis of betaine

The introduction by molecular genetic manipulation of biosynthetic pathways involved in synthesis of betaine has been attempted in non-accumulating species of cyanobacteria (Deshnium et al., 1995; Nomura et al., 1995) and in higher plants (see Table 1.1). Such transgenic plants would hence acquire saline tolerance.

The relevant gene is introduced under the transcriptional control of a strong promoter that ensures high-level expression of the gene in transgenic plants (e.g., the constitutively active promoter of the gene for 35S rRNA from cauliflower mosaic virus or the light-inducible promoter of a gene for the small subunit of Rubisco). For appropriate localization of enzymes of prokaryotic origin, the gene has often been modified so that the encoded polypeptide is transported post-translationally into the chloroplasts of the engineered plants. The transit peptide of the small subunit of Rubisco has been used as a signal for such transport.

1.11 Genetic engineering using the gene for choline oxidase from Arthrobacter

The CODA gene isolated from the soil bacteria *Arthrobacter globiformis*, encodes choline oxidize. The major advantage of using COD, instead of CMO/BADH or CDH/BADH, as a tool for engineering the synthesis of betaine is that the introduction of only a single gene (CODA) for this enzyme is sufficient for the conversion of choline to betaine in transgenic plants. Transformation of A. thaliana with codA enabled the plant to accumulate glycinebetaine and enhanced its tolerance to salt stress (Hayashi et al., 1997). Seeds of the transgenic plants were able to germinate in 300 mM NaCl whereas seeds of wild type did not germinate at all. In addition, transgenic plants had retained wild type Photosystem II activity under salt stress conditions. Transgenic rice plants carrying the codA gene with the encoded protein directed to the chloroplast were more tolerant than the transgenic plants with the protein localized in the cytosol. This result suggested that the protective function of glycine betaine is more efficient when produced in a photosynthetic organelle (Sakamoto et al., 1998). An *Arthrobacter pascens* gene encoding a COX enzyme was used to generate transgenic plants in three different species: *Arabidopsis*, *Brassica napus* and tobacco (Huang et al., 2000). Salt tolerance varied among the species and the

authors could not assign this difference in tolerance to the different glycine betaine accumulation levels. Transgenic tobacco plants expressing the BADH gene accumulated a higher amount of glycine betaine in cytosol and chloroplasts and exhibited increased tolerance to salt stress (Holmstrom et al., 2000). These transgenic plants also showed decreased photoinhibition during salt stress and this caused an increase in fresh weight relative to wild type. Similar results were obtained in transgenic tobacco plants over-expressing a BADH from spinach instead of a BADH prokaryote gene (Sakamoto et al., 1998).

While genetic engineering has allowed engineered plants to produce betaine, there are considerable differences in levels of betaine, on a fresh weight basis, between transgenic plants (0.05±5 mmol g-1 FW) and natural accumulators under stress conditions (4 ±40 mmol g-1 FW) (Rhodes and Hanson, 1993). The amounts of betaine accumulated suggest that the enhancement of stress tolerance can hardly be attributed to any action of betaine that involves osmotic adjustment to the external environment. A major role of betaine might be to protect membranes and macromolecules from the damaging effects of stress. It is also possible that betaine might be compartmentalized within cells such that, at certain sites, the concentration of betaine might be high enough to confer substantial protection against stress even when the overall level of accumulation is low. Such a possibility is supported by the observations that betaine is concentrated exclusively in the cytoplasm, and not in the vacuoles, of leaves of salt-grown halophytes (Matoh et al., 1987) and levels of betaine as low as 5 mmol g-1 FW can protect some natural accumulators from the damaging effects of stress (Arakawa et al., 1990; Ishitani et al., 1993). Other roles of betaine in cells under stress have also been studied. Betaine destabilizes double-helixed DNA and lowers the melting temperature of DNA in vitro. Thus, betaine might play a role in promoting transcription and replication under high-salt conditions (Rajendrakumar et al., 1997). The physiological characterization of transgenic plants (Alia et al., 1999) suggested that betaine might accelerate protein synthesis de novo during recovery from stress. Such possibilities require further detailed examination in future studies Glycine

betaine accumulates in cells of a number of halophytes and bacteria as an adaptive response to high salt.

The possible side effects of the introduction of the gene for COD were examined since the enzyme produces hydrogen peroxide as a by-product of catalysis (Alia et al., 1999). Leaves of transgenic Arabidopsis that expressed COD did have elevated levels of hydrogen peroxide, but the increase were comparable to levels in wild-type plants under stress and non-stress conditions (Alia et al., 1999). Moreover, the activities of enzymes that are responsible for scavenging hydrogen peroxide, namely ascorbate peroxidase and to a lesser extent, catalase, were significantly higher in transgenic plants than in wild-type plants (Alia et al., 1999). These observations suggest that the hydrogen peroxide generated by choline oxidase might have stimulated the expression of scavenging enzymes with the resultant maintenance of intracellular levels of hydrogen peroxide within a certain limited range. Expression of choline oxidase (COD) from Arthrobacter transgenic rice plants were achieved in a fashion similar to the transgenic Arabidopsis plants as described above with COD targeted either to the chloroplasts or to the cytosol. In the former case, betaine accumulated at about 1 mmol g-1 FW in leaves; in the latter plants, it accumulated at about 5 mmol g-1 FW (Sakamoto et al., 1998). Transgenic rice plants with COD targeted either to chloroplasts or to the cytosol exhibited enhanced tolerance to salt or cold induced photoinhibition. Moreover, the photosynthetic machinery was more efficiently protected when COD was targeted to the chloroplasts than to the cytosol of transgenic rice (Sakamoto et al., 1998).This observation indicates that the subcellular site of betaine synthesis might be important in efforts to improve the stress tolerance of plants.

1.12 Aim and scope of the study

In the present thesis, we proposed to undertake studies on the development of saline tolerance in plants with the specific emphasis on synthesis of glycine betaine in the plant of interest through genetic transformation and its subsequent effect on salt tolerance.

The plants of interest for the studies is specifically selected considering regional importance and agro-climatic suitability. The plants under consideration for the studies is Ground nut which is a major cash crop of Gujarat. It is rich source of protein, oil, and fodder and is among the 15 leading food crop of the world. India is the largest producer of groundnut and contributes around 29 % of the total world production. Ground nut has not been specifically transformed for saline tolerance till now.

1.12.1 Work plan for present investigation

1. To study and understand the natural saline tolerances of ground nut.
2. To standardize regeneration protocol using different parts of groundnut plant.
3. To study various biochemical parameters of groundnut under different levels of salinity (NaCl concentrations).
4. To procure gene of interest responsible for synthesis of glycine betaine i.e. COX.
5. Transformation of COX gene into *Agrobacterium tumifaciens.*
6. Co-cultivation of plant parts with *Agrobacterium tumifaciens* containing gene of interest and subsequent regeneration of plants.
7. Selection of successful transformants under appropriate selection pressure and confirmation of transformation.