REVIEW OF LITERATURE

2.1 SUGARCANE: An important cash crop

Sugarcane is tall perennial grass of the genus *Saccharum*, with originally soft, watery culm sugarcane acquired through human selection a distinctive feature of partitioning carbon into sucrose in the stem. This striking ability of accumulating levels of sucrose that can reach around 0.7 m in mature internodes (Moore, 1995) is an almost unique feature in cultivated plants. All sugar cane species interbreed, and the major commercial cultivars are complex hybrids.

Sugarcane is cultivated in more than 20 million hectares in tropical and subtropical regions of the world, producing up to 1.3 billion metric tons of crushable stems. It has served as a source of sugar since hundreds of years, represents an important renewable biofuel source, which could turn into a global commodity and important energy source (Pandey et al., 2000). It is generally used to produce sugar, accounting for almost two thirds of the world’s production and has lately gained increased attention because of ethanol which is derived from cane. Sugarcane bagasse (the major waste product generated by sugar mills after extraction of the sucrose from cane juice) is largely used for energy cogeneration at the mill or for the production of animal feed increasing the overall efficiency of the crop system. Recently, there has been increased interest in using bagasse for processes such as paper production, as a dietary fiber in bread, as a wood substitute in the production of wood composite, and in the synthesis of carbon fibers (Sangnark and Noomhorm, 2004). It is expected that enzymatic and hydrolytic processes that allow the bagasse carbon units from cellulose and hemicelluloses to be fermented, will soon be scaled up for ethanol production, turning sugarcane into an efficient crop for energy production as well (Paiva et al., 2004; Han and Wu, 2004).

Botanically, Sugarcane belongs to the Andropogonae tribe of the family Gramineae, order Glumiflorae, class Monocotyledoneae, subdivision Angiospermae, division Embryophyta siphonogama. The subtribe is Sacharae and the genus *Saccharum*, derived from the Sanskrit "sarkara = white sugar", a reminder that the plant reached the Mediterranean region from India. Sugarcane belongs to the genus *Saccharum* L.
composed of hybrids (Price, 1965; Arceneaux, 1967) derived from *Saccharum officinarum* (Noble clones), *S. sinense* (Chinese clones), *S. barberi* (North Indian clones), and *S. spontaneum* (Roach, 1972). The hybrids are highly polyploidy and aneuploid and on average contain 100-120 chromosomes with an estimated somatic cell size of 10,000 Mb (D’Hont and Glaszman, 2001). The number of chromosomes can vary in commercial cultivars, the basic genome size ranges from 760 to 926 Mb, which is twice the size of the rice genome (389 Mb) and similar to *Sorghum’s* (760 Mb) (D’Hont, 2005).

Sugarcane cultivation requires a tropical or temperate climate, with a minimum of 60 centimetres (24 in) of annual moisture. It is one of the most efficient photosynthesizers in the plant kingdom. It is a C-4 plant, able to convert up to 2 percent of incident solar energy into biomass. In prime growing regions, such as India, Pakistan, Peru, Brazil, Bolivia, Colombia, Australia, Ecuador, Cuba, Philippines and Hawaii, sugarcane can produce 20 lb (9 kg) for each square meter exposed to the sun. Although sugarcanes produce seeds, modern commercial sugarcane cultivation relies on vegetative propagation through stem cuttings which has become the most common reproduction method.

Sugarcane has essentially four-growth phase’s viz., germination phase, tillering (formative) phase, grand growth phase, maturity and ripening phase (http://www.sugarcane-crops.com/crop_growth_phases). A brief understanding of these growth phases would help in better management of the crop.

**Crop Growth Phases**
First stage: Germination and establishment phase

- Under field conditions germination starts from 7 to 10 days and usually lasts for about 30-35 days after planting (DAP).
- In sugarcane, germination denotes activation and subsequent sprouting of the vegetative bud.
- The germination of bud is influenced by both external as well as internal factors.
- The external factors are the soil moisture, soil temperature and aeration.
- The internal factors include the bud health, sett moisture, sett reducing sugar content and sett nutrient status.

Second stage: Tillering phase

- Tillering starts from around 40 DAP and may last up to 120 days.
- Tillering is a physiological process of repeated underground branching from compact nodal joints of the primary shoot, providing the crop with appropriate number of stalks required for a good yield.
- Various factors viz., variety, light, temperature, irrigation (soil moisture) and fertilizer practices influence tillering.
- Early formed tillers give rise to thicker and heavier stalks. Late formed tillers either die or remain short or immature.
- Cultivation practices such as spacing, time of fertigation, water availability and weed control influence tillering.
- Encouraging good tillering is important to build adequate population.

Third stage: Grand growth phase

- Grand growth phase starts from 120 DAP and lasts up to 270 days in a 12-month crop. During the early period of this phase tiller stabilization takes place. Out of the total tillers produced only 40-50% survives by 150 days to form millable cane.
- This is one of the most important phase of the crop where in the actual cane formation and elongation and thus yield build up takes place.
- Under favourable conditions stalks grow rapidly almost 4-5 internodes per month.
Fourth stage: Ripening and maturation phase

- Ripening and maturation phase in a twelve-month crop lasts for about three months starting from 270-360 days.
- Sugar synthesis and rapid accumulation of sugar takes place during this phase and vegetative growth is reduced.
- As ripening advances, simple sugars (monosaccharide viz., fructose and glucose) are converted into cane sugar (sucrose, a disaccharide).
- Cane ripening proceeds from bottom to the top and hence bottom portion contains more sugars than the top portions.
- Ample sunshine, clear skies cool nights and warm days (i.e., more diurnal variation in temperature) and dry weather are highly conducive for ripening.

Sugarcane area and productivity differ widely from country to country. Today, sugarcane is grown in over 110 countries. In 2009, an estimated 1,683 million metric tons were produced worldwide which amounts to 22.4% of the total world agricultural production by weight. About 50 percent of production occurs in Brazil and India. Brazil has the highest area (5.343 million ha), while Australia has the highest productivity (85.1 tons per ha. Out of the total white crystal sugar production, approximately 70% comes from sugarcane and 30% from sugar beet. India ranks second in the world, after Brazil, in terms of sugarcane growing area (4.1 million ha) and production (348 million Mt) (FAO, 2009; http://www.fao.org/docrep/011/ai482e/ai482e07.htm). Sugar industry is second largest in our country in the agro-processing sector worth $6.8 billion and over 45 million farmers are involved in sugarcane cultivation and about 7.5% rural population directly or indirectly is dependent on the sugar industry (http://www.iisr.nic.in).

In most countries where sugarcane is cultivated, there are several foods, drinks and popular dishes derived directly from it under different local names such as syrup, gannekarrass, guarab, sayur nganten, cachaça, rum, falernum, jaggery, panela, molasses, rapadura, rock candy etc.
2.2 **ABIOTIC STRESS: Increasing environmental constraints**

An environmental factor that limits crop productivity or destroys biomass is referred to as stress or disturbance (Grime, 1979). Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. Low temperature, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production (Xiong et al., 2002). Among the abiotic factors that have shaped and continue shaping plant evolution, water availability is the most important, while light is the best studied environmental factor in plant research with respect to molecular details. The quality and quantity of light that affects photosynthesis and growth of plants is well studied by many researches (Grover et al., 2001). Water stress in its broadest sense encompasses both drought and salt stress. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Bray et al., 2000; Lee et al., 2009).

Salinity in soil or water is one of the major stresses, especially in arid and semi-arid regions, severely limiting crop production (Shannon, 1998). The remarkable ability of plants to adapt different adverse environments is a fascinating process. The cellular and molecular responses of plants to environmental stress have been studied intensively (Hasegawa et al., 2000). Research into the physiology and metabolism of so-called extremophiles has not only foster better understanding of the evolutionary processes that have created the diversity of life as it exists on earth, but also has economic implications for agricultural biotechnology and the development of novel products. On the other hand, sugarcane production is expected to reduce by 30% in the future due to climate change, as revealed in a recent four-year study conducted by the World Bank (http://www.sri-india.net). The capacity to sequence genomes and the availability of novel molecular tools have now catapulted biological research into eras of genomics and post-genomics, creating an opportunity to apply genomic techniques to extremophile models (Amtmann et al., 2005), which is a dire need of time to feed the increasing global population through significant increase in agricultural production.
2.3 SUGARCANE RESPONSES TO ABIOTIC STRESS: Salinity

Plants are classified as halophytes, which can grow and reproduce under high salinity (>400 mM NaCl), and glycophytes, which cannot survive high salinity. Crops such as bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), corn (*Zea mays*), potato (*Solanum tuberosum*), and sugarcane (*Saccharum officinarum*) are highly susceptible, with a threshold EC of <2 dS m\(^{-1}\), whereas sugar beet (*Beta vulgaris*) and barley (*Hordeum vulgare*) can tolerate an EC up to 7 dS m\(^{-1}\) (Maas, 1990; http://www.ussl.ars.usda.gov/saltoler.htm).

Sugarcane being a glycophyte shows high sensitivity to salinity at various growth stages. As for glycophytes, sodium toxicity represents the major ionic stress associated with high salinity, enforcing ion imbalance or disequilibrium, hyperionic and hyper-osmotic stress, thus disrupting the overall metabolic activities and causing plant demise (Zhu, 2001). Sugarcane varieties differ in their responses to soil salinity and acidity. Germination and early growth stages are more sensitive than later stages of crop growth, besides ratoon crop is more sensitive to salinity than plant crop. Moreover, in India 3.3 million ha and 2.46 million ha of land is affected due to salt and water-logging conditions, respectively (Jain et al., 2007), whilst one million ha of irrigation-induced water-logged saline area is confined to northwest India alone. The economic loss due to environmental degradation through these twin problems is about $37 million, threatening the sustainability of agricultural production in India (Datta and Jong, 2002).

2.3.1 Management of soil salinity

Salinity may occur when there is irregular irrigation, inadequate drainage, wrong fertilizer application, and extremely increases particularly in protected cultivation. Every year more and more land becomes unproductive due to salt accumulation (http://www.indiaagronet.com/indiaagronet/soil_management.htm). Generally plants growing in saline conditions come across with major drawbacks. The deleterious effects of salinity on plant growth are associated with (1) low osmotic potential of soil solution (water stress), (2) nutritional imbalance, (3) specific ion effect (salt stress), or (4) a combination of these factors (Ashraf, 1994). All of these causes adverse pleiotropic
effects on plant growth and development mainly due to changes at the molecular level (Mansour, 2000) ultimately effecting physiological and biochemical levels (Munns, 2002; Yildirim et al., 2008). Morphological symptoms includes stunted growth and necrotic leaves with scorched tips and margins, poor tillering and root growth, reduction in internodal length and girth of cane, impaired cane quality with reduced juice purities, problems in processing for jaggery and sugar in sugarcane due to soil salinity stress have been reported by many researchers (Hussain et al., 2004; Naidoo et al., 2004).

### 2.3.2 Soil salinity estimations
Soil salinity is estimated from the electrical conductivity (EC) of a soil (Rhoades, 1996). Salinity measurements provide information about the ability of a site to support plant growth as well as some information regarding potential leaching and drainage problems. Electrical conductivity is a gross measure of dissolved salts in soil solution, but provides no information as to which salts are present and in what proportion. For non-sensitive plants, EC measurements <4 dS m\(^{-1}\) are satisfactory. Soils with EC >4 dS m\(^{-1}\) are considered saline and plant growth may be inhibited (Pierzynski et al., 2005). While salinity levels >4 dS m\(^{-1}\) can be detrimental to plant growth, additionally high levels of Na\(^+\) (sodicity) in soil solution can cause soil structure problems such as dispersion, and drainage problems. Excessive Na\(^+\) in soil can destroy soil structure, permeability and reduce water infiltration (Brady and Weil, 2001).

### 2.3.3 Stress affecting the physio-biochemical nature of plants
#### 2.3.3.1 Salt stress and biochemical response of plants
Salt stress creates both ionic as well as osmotic stress on plants and these stresses can be distinguished at several levels (Tester and Devenport, 2003). Membrane deterioration commonly reported phenomenon during abiotic stress promoted strong fall in chlorophyll level affected significantly the chlorophyll a, b and ultimately total chlorophyll content in salt stressed plants. Other explication is the photosynthetic reduction through stomatal closure and consequent lower entrance of CO\(_2\) in leaf tissue, as well as degradation of photosynthetic pigments promoted by oxidative stress caused due to excessive saliniazation (Santos and Carlesso, 1998). The analogous results were reported in
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Sorghum, barley, rice and *S. grandiflora* seedlings during high levels of salinization conditions (Sharma and Hall, 1991; Ashraf and Bhatti, 2000; Dhanapackiam and Ilyas, 2010) and water deficit promoted decrease in chlorophyll levels in sorghum cultivars (Younis et al., 2000; Neto et al., 2009).

The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity and drought, despite a significant decrease in net CO$_2$ assimilation rate (Murakoezy et al., 2003). Carbohydrates such as sugars and starch accumulate under salt stress (Parida et al., 2002), playing a leading role in osmoprotection, osmotic adjustment, carbon storage and radical scavenging. The increase in total soluble carbohydrates is due to accumulation of organic solutes as glucose, fructose and sucrose contribute 50% of the total osmotic potential in glycophytes subject to saline condition (Cram, 1976). Furthermore, the increase of sugar concentrations is a result of the starch hydrolysis which needs intense hydrolytic enzyme activities. The recovery of stressed plants and seeds of several higher plants are examples of the accumulation of carbon compounds as strategy to tolerate the water deficit (Hoekstra, 2001; Phillips, 2002). Compatible osmolytes are potent osmoprotectants that play role in counteracting the effects of osmotic stress. The accumulation of nontoxic compatible solutes, such as proline, betaines and sugar alcohols, is a widespread response that protects plants against environmental stress (McNeil et al., 1999). They do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress (Chen and Murata, 2002). Initially it was thought that compatible solutes have their main role in osmotic adjustment playing a primary role of turgor maintenance (Yancey et al., 1982), but there is increasing discussion of other roles like stabilizing proteins and cell structures as well have adaptive value in many metabolic pathways (Hasegawa et al., 2000, Serraj and Sinclair, 2002) further it has been hypothesized that compatible solutes are also involved in scavenging reactive oxygen species by few researchers (Shen et al., 1997a, b; Hong et al., 2000; Akashi et al., 2001; Chen and Murata, 2002).

Proline (Pro) one of the most common compatible osmolytes accumulates in large amounts than other amino acid (Abrham et al., 2003) in a different plant species in
response to stresses such as drought, salinity and extreme temperatures. Pro accumulates in the cytosol and vacuole during stress and was shown to protect plant cells against damages caused by \( ^1O_2 \) or \( HO^- \) (Hong et al., 2000; Matysik et al., 2002). Although its role in plant osmotolerance remains controversial, proline is thought to contribute to osmotic adjustment, detoxification of reactive oxygen species and protection of membrane integrity in plants during stress. Accumulation of proline has been noticed in many plants like citrus, rice during salt stress (Lima-Costa, 2010). Another fascinating positive correlation of proline accumulation as osmoregulator and aroma generation in the aromatic rice grown in the regions of dry and increased salt conditions have been reported (Yoshihashi et al., 2002). Moreover, Suprasanna et al., (1998) reported that the supplementation of L-proline into the culture medium can yield increase in aroma production in callus cultures of aromatic rice. Consequently, it has been demonstrated that proline has wide role during stress as osmoprotector, detoxifier of reactive oxygen species, enzyme protectant.

### 2.3.3.2 Reactive oxygen species (ROS) protection responses

Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Mittler, 2002; Candan and Tarhan, 2003; Gara et al., 2003; Vaidyanathan et al., 2003). ROS are partially reduced forms of atmospheric oxygen, which are produced in vital processes such as photorespiration, photosynthesis and respiration (Mittler, 2002; Uchida et al., 2002). To produce water in these processes, four electrons are required for perfect reduction of oxygen. But ROS typically results from the transference of one, two and three electrons, respectively, to \( O_2 \) to form superoxide\((O2^-)\), peroxide hydrogen \((H_2O_2)\) and hydroxyl radical \((HO^-)\) (Mittler, 2002). These species of oxygen are highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins, nucleic acid, etc., causing lipid peroxidation, protein denaturing and DNA mutation (Breusegem et al., 2001; Karabal et al., 2003; Quiles and Lopez, 2004).

Evidence suggests that cells and organelles membranes are the primary sites of injury during salinity (Candan and Tarhan, 2003). Peroxidation of plasma lemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane
damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplasts (Scandalios, 1993). There are a plethora of antioxidants, all of which forms the network of reactions that is important in vivo which provides comprehensive control of ROS in cells, rather than the action of any single reaction. These networks include the movement of antioxidant compounds by specific transporters between and within cells, as well as the use of membrane-spanning redox couples (Horemans et al., 2000; Foyer and Noctor, 2003). In plant cell, antioxidant enzymes such as superoxide dismutase (SOD, EC: 1.15.1.1) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and play important role in defensive mechanism, peroxidase (POD, EC: 1.11.1.7) is widely distributed in higher plants where it is involved in various processes, including lignifications, auxin metabolism, salt tolerance and heavy metal stress (Passardi et al., 2005). Therefore, POD has often served as a parameter of metabolism activity during growth alterations and environmental stress conditions. Catalase (CAT, EC: 1.11.1.6), have been considered as a defensive team involved in the degradation of hydrogen peroxide into water and oxygen, whose combined purpose is to protect cells from oxidative damage (Willekens et al., 1995; Mittler 2002).

Many studies found a positive correlation between salt stress and the abundance of these antioxidants in plants cells (Badawi et al., 2004; Cavalcanti et al., 2007), increased SOD, POD and CAT activities as reported by various researches during salinity in plants like potato, Cassia, maize, sesame, Jatropha, rice (Agarwal and Pandey, 2004; Rahnama and Ebrahimzadeh, 2005; Azevedo-Neto et al., 2006; Koca et al., 2007; Gao et al., 2008; Roychoudhury, 2008). It has been reported that the changes in activities depend on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Chaparzadeh et al., 2004).

2.3.4 Potential sensors for abiotic stress signals and signal transduction
Plants react to external stimuli by initiating signalling cascade which activate the expression of appropriate responses. These signalling pathways comprise a network of protein-protein reactions and signalling molecules (for example, ROS, Ca2+ etc.). In
contrast to signal perception various components of the signal transduction have been identified, although it is largely unknown how the different molecules interact with each other and where they are positioned in the complex signalling network (Bartels and Sunkar, 2005). Several classes of protein kinases and phosphatases work as signal transducers and are involved in osmotic stress signaling (Munnik et al., 2000).

Major studies on plant salt tolerance have been focused on Arabidopsis. As a consequence, several important pathways involved in salt stress signal transduction have been identified, although some key components in these pathways still remain to be defined (Xiong et al., 2002). These pathways include salt over sensitive (SOS) pathway (SOS3-SOS2-SOS1) which regulates ion homeostasis under salt stress and results in Na\(^+\) efflux and vacuolar compartmentation (Sanchez-Barrena et al., 2005). Protein phosphorylation is one of the major mechanisms for controlling cellular functions in response to external signals. Transient increases in cytosolic Ca\(^{2+}\) are perceived by various Ca\(^{2+}\) binding proteins. In the case of abiotic stress signaling, evidence suggests that the calcium dependent protein kinase (CDPK) pathway plays an important role in osmotic stress (Sanchez-Barrena et al., 2005). CDPKs are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to four EF-hand motifs that can directly bind Ca\(^{2+}\). The Arabidopsis genome encodes at least 34 putative CDPKs (Harmon et al., 2001). A number of studies have shown that CDPKs are induced or activated by abiotic stresses, suggesting that they may be involved in abiotic stress signaling (Pei et al., 1996; Tahtiharju et al., 1997; Hwang et al., 2000). In rice plants, a membrane-associated CDPK was activated by cold treatment (Martin and Busconi, 2001). In addition, over expression of OsCDPK7 resulted in increased cold and osmotic stress tolerance in rice (Saijo et al., 2000). Similary, the mitogen activated protein (MAP) kinase cascades, are common signaling modules which are important for counteracting both abiotic and biotic stresses (Nakagami et al., 2005). At least 20 MAPK, 10 MAPKK and 60 MAPKKK genes have been identified in Arabidopsis on the basis of sequence similarities (Riechmann et al., 2000; Ichimura et al., 2002).
2.3.4.1 Abscisic acid response elements (ABREs)

Salt, drought, and to some extent, cold stress cause an increased biosynthesis and accumulation of ABA, which can be rapidly catabolized following the relief of stress (Liotenberg et al., 1999; Taylor et al., 2000). In addition to ABA, ethylene also plays an important role in the complicated story of abiotic stress and, consequently, cross-talk between these two hormones has been reported (Tanaka et al., 2005). ABA-dependent pathways are known to mediate gene expression in plants during osmotic stress. The distinction is largely based on cis-elements that exist in the promoters of ABA-inducible genes. The ABA-dependent pathways are thought to mediate the gene expression through an ABRE-element and b-ZIP transcription factors (Busk and Pages, 1998), while the other pathways through MYC and MYB elements and transcription factors. Numerous reports have shown that different pathways are interconnected and coordinately regulate the plant response to biotic and abiotic stresses (Ma et al., 2004; Ludwig et al., 2005).

2.3.5 Transcription factors (TFs) and its co-activators modulated by stress

Transcription factors (TFs) bind to DNA through specific cis-regulatory sequences either activate or repress gene transcription and act as control switches in stress signaling. Plants need a large number of transcription factors governing proper and strict transcriptional regulation in response to developmental and environmental cues (Saibo et al., 2009). Regulation of a transcription factor is achieved by reversible phosphorylation or by de novo synthesis of transcription factors. The current analysis of identified transcription factors suggests that different stress signaling pathways may overlap or converge at specific points. A large no of stress related TFs have been identified such as DREBs, ERF, Zinc Finger, WRKY, MYB, HLH, b-ZIP, HD-Zip and NAC (Yanagisawa, 1998; Archana et al., 2009).

Riechmann et al., (2000) reported that over 5% of the Arabidopsis genome is devoted to encoding more than 1,500 transcription factors. A complex network of transcription factors orchestrates the response of plants to changes in environmental conditions (Chen et al., 2002). These include WRKY and other zinc-finger proteins (72 WRKY genes and more than 600 zinc finger proteins in Arabidopsis; Eulgem et al., 2000), MYB
transcription factors (133 genes in *Arabidopsis*; Stracke et al., 2001), and heat shock transcription factors (21 genes in *Arabidopsis*; Nover et al., 2001). However, only a few of these transcription factors appear to respond in a similar manner to all or most of the different environmental stress conditions tested in *Arabidopsis*. To activate or repress transcription, TFs must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus. Therefore, environmental signals that regulate transcription factor activity may affect any one or a combination of these processes. A collection of known and predicted transcription factors of *Oryza sativa* L. sp. *indica* and *Oryza sativa* L. sp. *japonica* contains 2,025 putative transcription factors (TFs) in *indica* and 2,384 in *japonica*, distributed in 63 families currently deposited in ‘The Database of Rice Transcription Factors’ (DRTF) (Gao et al., 2005). Recently, within the soybean genome 5,035 TF models have been estimated and grouped into 61 families (Tran and Mochida, 2010). Moreover the ‘Sugarcane Transcription Factor Database’ has collective 1,177 predicted transcription factors (TFs). The assembled transcripts data of ‘Plant Genomic Database’ (GDB) have extensive annotations for those plant TFs, including similarity searches against major databases (Uniprot, RefSeq, EMBL, TRANSFAC etc) along with InterPro domain information and EST expression information extraction from UniGene (http://planttfdb.cbi.pku.edu.cn:9010/web/index).

Transcriptional co-activators play a crucial role in eukaryotic gene expression by communicating between transcription factors and/or other regulatory components and the basal transcription machinery. They are divided into two classes: transcriptional co-activators that recruit or possess enzymatic activities that modify chromatin structure (e.g. acetylation of histone) and transcriptional co-activators that recruit the general transcriptional machinery to a promoter where a TF is bound (Naar et al., 2001). ‘Multiprotein bridging factor 1’ (MBF1) is a highly conserved transcriptional co-activator involved in the regulation of diverse processes (Brendel et al., 2002; Liu et al., 2003). The flowering plant *Arabidopsis* (*Arabidopsis thaliana*) contains three different genes encoding MBF1, of which steady-state level of transcripts encoding MBF1c (At3g24500) is specifically elevated in *Arabidopsis* in response to pathogen infection, salinity, drought, heat, hydrogen peroxide, and application of the plant hormones abscisic acid or
salicylic acid (Rizhsky et al., 2004; Tsuda and Yamazaki, 2004). The level of transcripts encoding MBF or its orthologs elevated in response to a combination of drought and heat in Arabidopsis, tobacco (Nicotiana tabacum), and the desert legume Retama raetam (Pnueli et al., 2002; Rizhsky et al., 2004). However, the relative contribution of MBF to biotic and abiotic stress tolerance is still unknown.

2.4 GENETIC ENGINEERING OF TOLERANCE TRAITS

Plants can employ numerous physiological and biochemical strategies to cope with adverse conditions by altering the functioning of a number of genes (Lee et al., 2009). Therefore, the identification of these key genes involved in biotic or abiotic stress responses is a fundamental step in understanding the molecular mechanisms of stress responses and developing transgenic plants with enhanced tolerance to stress. In transgenic approach to improve plant stress tolerance has appreciable results over the years, moreover, engineering the synthesis of compatible solutes has been proved a relatively successful approach obtaining stress tolerant plants. Some reports demonstrated the over production of proline in genetically modified tobacco plants showed tolerance to NaCl (Hong et al., 2000).

Nanjo et al., (2003) demonstrated that introduction of antisense proline dehydrogenase cDNA in Arabidopsis, over expresses proline and shows tolerance to freezing temperatures (-7°C) and salinity up to 600mM NaCl. Results in recent published reports indicates that SOD, peroxidase, catalase over expression may be involved in the increase of stress protection observed in some transgenic plants like tobacco, chinese cabbage, potato (Yiu and Tseng, 2005; Tseng et al., 2007; Ahmad et al., 2010). Transgenic tobacco plants with increased mannitol production targeted to the chloroplast showed increased scavenging capacity of HO· enhancing their resistance to oxidative stress (Shen et al., 1997a; b). The recent genomic approaches provided nucleotide sequences of Cu/Zn superoxide dismutase, ascorbate peroxidase and mono-dehydroascorbate reductase, NAC transcription factor from Avicennia marina and antiporter gene from Porteresia coarctata enabled the production of abiotic stress tolerant transgenic plants, more specifically for salt and/or drought stress tolerance in rice. Important role of many stress-response
transcription factors is demonstrated abiotic stresses tolerance in many other plant species through transgenic approach but are limited in sugarcane to moisture stress only as many in house candidate genes in relation to salt stress from sugarcane and its relative species has not been isolated or either studied. (Kasuga et al., 1999; Mishra et al., 2002; Maruyama et al., 2004; Vogel et al., 2005; Molinari, 2007).

2.5 GENOMICS APPROACHES IN RELATION TO SALT STRESS TOLERANCE

The identification of differentially expressed genes and examination of their patterns of expressions are important to gain information about the functions relevant to processes such as cell differentiation, morphological or metabolic changes, and disease development (van den Berg et al., 2004). Many scientists have suggested that selection is more convenient and practicable if the plant species possesses distinctive indicators of salt tolerance at the whole plant, tissue or cellular level (Munns, 2002; Ashraf, 2002). Physiological criteria are able to supply more objective information than agronomic parameters or visual assessment when screening for component traits of complex characters (Yeo, 1994). Munns (1993) suggested that plant physiologists could improve the salt tolerance of plants only by defining genes or characters for geneticists or breeders to exploit. However, knowledge of heritability and the genetic mode of salinity tolerance is lacking because few studies have not yet been conducted in these areas. Indeed, genetic information is lagging behind the physiological information. Despite a great deal of research into salinity tolerance of plants, mainly on water relations, photosynthesis, and accumulation of various inorganic ions and organic metabolites (Munns, 2002), the metabolic sites at which salt stress damages plants is little studied till date in non model plants such as sugarcane.

In recent years, researchers have become interested in the use of genomic tools to identify and isolate genes involved in the tolerance of crop plants to various abiotic stress factors. The first step to understand and evaluate such genetically complex responses is to sequence randomly selected cDNA clones or expressed sequence tags (ESTs) from the plants exposed to the environmental stress (Zhang et al., 2001). Analyses of the identities
and expression levels of these genes that were high throughout could be conducted with the aid of different molecular biology tools, which has provided a better understanding of the role of the genes to plant stress adaptation and will form basis for effective engineering of the non-model plants for improved stress tolerance. Various molecular techniques have been used for studying differential gene expression in many plant species includes representational difference analysis (RDA), suppression subtractive hybridization (SSH), differential display, differential hybridization, subtractive library construction, serial analysis of gene expression (SAGE), cDNA-RAPD and cDNA microarrays (Lisitsyn and Wigler 1993; Velculescu, 1995; Diatchenko, 1996; Schummer, 1997, Kawar et al., 2010).

2.5.1 Ribotyping and differential gene expression

cDNA-AFLP technique was used by few researchers for ribotyping towards identification of transcripts that are strongly accumulated and are induced de novo in response to salinity stress in Spartina alterniflora L. (Baisakh et al., 2006), using the same cDNA-AFLP technique genes induced during Medicago sativa nodule development have been identified as well as a variety of genes involved in nodule development and functions induced during symbiotic root nodule development in alfalfa, by comparing the cDNA-AFLP patterns of infected nodules and uninfected roots have been accomplished by Xie et al., (2006). Though it is robust technique and widely used by researches for ribotyping it has some limitations of its own, is expensive, laborious and needs harmful radioactive materials along with skilful handling.

Moreover McClelland and Welsh (1994) devised a method of RNA fingerprinting called cDNA-random amplified polymorphic DNA (cDNA-RAPD), in which cDNA is used as a template, is an extremely efficient, less labor-intensive and does not require expertise to handle as in other highly technical activities and easily accessible to small laboratories. This method provides a complex phenotype reflecting changes in the abundance of hundreds of RNAs under various stress conditions as that of cDNA-AFLP. Comparison of RNA fingerprints from different treatment groups allowed drawing inferences regarding gene regulation. The ratio of the intensity of RAP-PCR products between
samples correlates with the ratio of abundances of the corresponding RNA. Whereas the intensities of different bands within the same fingerprint vary independently of each other, the intensity of a band between fingerprints appears to be proportional to the concentration of its corresponding template sequence (Welsh and McClelland, 1990). By using cDNA-RAPD technique differentially expressed cDNA fragments have been obtained by differential screening in photoperiod sensitive genic male sterile (PGMS) rice (Jiang, 1999). A novel drought resistance gene was identified by comparing the gene expression profile of *Gossypium hirsutum* by employing the same approach (Selvam et al., 2009).

Application of cDNA-RAPD and -AFLP for isolation of differentially expressed transcripts in chickpea roots, three cDNA libraries by cDNA-RAPD and cDNA-AFLP were successfully constructed and isolated transcripts either differentially expressed or up-regulated in resistant chickpea cultivar challenged by *Fusarium oxysporum* (Nimbalkar et al., 2006). Despite the recent development of high-throughput full genome expression systems like microarray, which rely on comparison of two samples and prior knowledge of gene sequences, cDNA-RAPD/AFLP would remain a useful technique since several transcript pools can be compared in the same experiment. The cDNA-RAPD technique a very useful tool in the global survey of the genes expressed during stress conditions. The technique allowed identifying many transcripts involved in the host-pathogen interactions in chickpea (Nimbalkar et al., 2006), and in another effort permitted isolation of SCGS phytoplasma genes and genes up regulated due to SCGS infection in sugarcane (Kawar et al., 2010). DNA fingerprints obtained using AP-PCR in *Phaseolus vulgaris* has been employed to detect DNA damage caused by environmental chemicals such as para-nitrophenol (PNP) (Enan, 2007).

### 2.5.2 Suppression subtractive hybridization (SSH) and differential gene expression

Subtractive hybridization is an attractive method for enriching differentially expressed genes. This method was first used by Bautz and Reilly, (1966) to purify phage T4 mRNA in the mid-1960. Earlier pure subtractive methodologies are of limited use due to the need for a large quantity of mRNA to drive hybridization to completion as well as the
difficulty in cloning the tiny amount of cDNA remaining after hybridization. The method was greatly improved when Duguid and Dinauer, (1990) adapted generic linkers to cDNA allowing the selective PCR amplification of tester cDNA between hybridization cycles. Diatchenko et al., (1996) further introduced the technique of suppression subtractive hybridization PCR (SSH-PCR) in which differentially expressed genes could be normalized and enriched over 1000-fold in single round of hybridization.

The SSH-PCR select is the first technique to be widely used for the purpose of identifying differentially expressed genes on a global scale (Moody, 2001). The method is designed to selectively amplify differentially expressed transcripts while suppressing the amplification of abundant transcripts, thus eliminating the need to separate single- and double-stranded molecules. In addition, SSH normalizes target transcripts to approximately equal abundance. Advantages of the technique include the ability to isolate genes with no prior knowledge of their sequence or identity and the use of common molecular biology techniques that do not require specialized equipment or analyses (Ji et al., 2002). Several limitations of the original protocols, such as requirements of large quantities of RNA and bias toward abundant genes, have been overcome by incorporation of PCR into the SSH technique. However, SSH remains applicable only to pair-wise treatment comparisons, and the methods is time-consuming and do not allow the level of enrichment of a transcript to be quantified (Moody, 2001), on the other hand the recent commercialization of an SSH-PCR kit by Clontech (Clontech Laboratories, Palo Alto, CA, USA) has lead to its increasing popularity in biological research laboratories and reduced the time period towards its standardizations (Bahn et al., 2001).

SSH is evidenced as an effective method to isolate genes that are specifically and differentially transcribed under various conditions or in response to various biotic and abiotic stresses. Previously SSH has not frequently been used to study plant interactions with pathogen or elicitors up to year 2000; one of the first was by Dellagi et al., (2000) who characterized the gene St-WRKY1 that was up-regulated in potato leaves after inoculation with Erwinia carotovora. SSH has been used in cloning differentially expressed cDNAs in Dunaliella salina under salt stress (Zhang et al., 2002), subtracted
cDNA library, some specific cDNA clones of *Haloxylon ammodendron* seedlings induced by osmotic stress have been successfully identified and isolated (Jiang et al., 2004). Kong et al., (2005) inoculated *Triticum aestivum*, one of the few wheat cultivars with pathogen *Fusarium graminearum*, and demonstrated that some defence related genes, like chitinase, involved in resistance. Degenhardt et al., (2005) reported that many genes were differentially transcribed in apple leaves depending on whether the leaves were resistant or susceptible to *Venturia inaequalis*. Some typical defence genes, such as β-1,3 glucanase, cysteine protease inhibitor and superoxide dismutase, were over transcribed only in the resistant cultivar. Potential important or novel genes involved in the early stage of *Solanum lycopersicum* responses to severe salt stress were identified (Ouyang et al., 2007). Early stage SSH library of wheat under the stress of *Puccinia recondita* have been constructed (Yan et al., 2009). In order to identify genes induced during the salt stress response in *Medicago truncatula* L. seedlings (Kang et al., 2010) and differentially expressed cDNAs in *Ceratoides lanata*, during cold-stressed (Zeng et al., 2010) have led to isolation of valuable antifreeze, heat-resistant, drought-tolerant and alkali-salt-tolerant genes from various crop species and will be an asset for genetic manipulation of these traits.

Especially in sugarcane, the key genes related to regulatory events controlling responses to drought, metal stress, phosphate deficiency, and phytoplasma diseases has been studied in detail recently using the SSH approach. Aluminum stress and phosphate deficiency, which causes important constraints on yield in sugarcane, has been studied in detail to identify some important key genes to cope with tolerance of these conditions (Watt, 2003; Menossi et al., 2008). The genes expressed in response to sugarcane grassy shoot phytoplasma (SCGS) infection and albeit 60% genome of phloem restricted phytoplasma genome has been effectively isolated and characterized using SSH technique (Kawar et al., 2010). Further more recently water deficit stress response related regulatory network revealing, and consequently isolation of alternatively spliced *ScMYBAS1* gene from stress tolerance sugarcane was successfully executed (Prabu et al., 2010). Thus SSH technique has provided new and interesting information about the genes involved in the defense reaction during environmental stress over the last few years.
2.5.3 Expressed sequence tags (ESTs) and gene discovery

The advances in DNA-sequencing technology and greatly reduced costs of DNA sequencing allowed large-scale random sequencing of partial cDNAs (expressed sequence tags or ESTs), making it possible to isolate many genes with faster and more cost-effective methods such as cDNA-RAPD and SSH (Adams et al., 1991; Okubo et al., 1992). A gene is often represented by many ESTs; the more a gene is expressed in a given tissue, the more ESTs for that gene will be found in the cDNA library. Hence, the number of ESTs representing the same gene is a rough estimation of the expression level of the gene in the tissue from which the cDNA library was derived. The ESTs generated are then compared with the databases of identified genes to decide which of the isolated genes would be interesting enough for further study. Extensive EST sequences have been generated from *Arabidopsis thaliana*, maize, rice, soybean, potato, tomato and *Medicago trunculata* and publicly available in databases on the world wide web; [http://www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997), [http://blast.jcvi.org/euk-blast/plantta_blast.cgi](http://blast.jcvi.org/euk-blast/plantta_blast.cgi) (Childs et al., 2007).

To identify and study most of an organism’s genes/gene families and their alleles in different genotypes, analysis of EST library is proving fruitful. Sequencing and analysis of ESTs have been an efficient approach for identifying a large number of genes expressed during different developmental stages or in response to a variety of environmental conditions in plants such as apple (Newcomb et al., 2006), cocoa (Argout et al., 2008), corn (Alexandrov et al., 2009), coffee (Lin et al., 2005), pepper (Kim et al., 2008), soybean (Umezawa et al., 2008), and spruce (Ralph et al., 2008). This approach has also served as a resource for functional genomics of abiotic stress in plants (Rensink et al., 2005, Houde et al., 2006). The availability of sequences and analysis tools facilitate broad-based utilization of ESTs for gene structure annotation and comparative genomics (Ma et al., 2004). Sequencing of ESTs does not require broad molecular information of the target gene(s), and ESTs can be used for the identification of cell type-specific or tissue-specific genes or the regulatory networks of metabolic pathways, characterization of a genome of an organism, discovery of novel genes. There after use this information to
develop different trait specific molecular markers to be used in breeding programs to follow the inheritance of specific trait in the progenies.

Conversely only a limited number of genes related to abiotic and biotic stresses have been reported so far in sugarcane and hence it is the first priority to develop gene data set either in tissue specific or trait specific manner the recent genomic approaches as cDNA-RAPD and SSH are proving very fruitful. Although the SUCEST project in Brazil have a collection of ~240,000 ESTs, generated from 26 tissue and developmental stage-specific cDNA libraries in sugarcane but not publically accessible to sugarcane researchers and most of the ESTs developed in this program are in tissue specific manner only (Vettore et al., 2001, 2003). Though these sugarcane ESTs have aided in the identification and transcription profiling of signal transduction-related genes in sugarcane tissues (Papini-Terzi et al., 2005), molecular chaperon-related sequences in the sugarcane expressome (Borges et al., 2007). And hence to develop transcriptomic data in trait specific manner is a need of time, although few researchers made considerable efforts and reported sugarcane ESTs related to low-temperature stress (Nogueira et al., 2003), and ABA- and MeJA-activated sugarcane bZIP genes (Schlogl et al., 2008), response of sugarcane to red-rot and phytoplasma infection by Indian subtropical variety of sugarcane (Gupta et al., 2010; Kawar et al., 2010), water deficit stress (Prabu et al., 2010). Still substantial efforts needs to be focused to generate genomics data for salt, water logging and other traits to be used for genetic engineering these traits and strengthen the breeding programs through molecular marker approaches especially for sugarcane improvement.

2.5.4 Molecular markers and stress tolerance trait

In recent decades, considerable improvements in salinity tolerance have been made in crop species through conventional selection and breeding techniques (Ashraf, 2002). Biomarkers to identify and characterize the drought and salt tolerant sugarcane lines have been sought for many decades. These markers fall under three broad categories: morphological, cytogenetic, and biochemical. Morphological markers are based on the traditional botanical descriptions of visible characters and were the first markers to be utilized. They are of limited value because in sugarcane they are not inherited in a simple
Mendelian manner (Hogarth, 1987). Cytogenetic markers observed from mitotic and meiotic chromosomes provide additional information, but the variable size of sugarcane chromosomes and their abnormal pairing behavior make difficulty in cytogenetic observations (Sreenivasan, 1987). Biochemical markers became a popular tool in plant genetics, and studies utilizing such markers were also initiated in sugarcane (Ramagopal, 1990).

Protein and secondary metabolites of leaves from variety of plants have been examined and were found to be promising as markers, such as ASR (ABA-water stress-ripening-induced) protein (Riccardi, 1998), dehydrin (Jiang and Huang, 2002; Lopez et al., 2003), superoxide dismutase (Zang, 2007). Molecular markers can be used to identify the genotype of the individual plant and to identify and map the genes affecting complex plant traits such as yield and resistance to biotic or abiotic stresses. The common methods employed for the identification of DNA markers are: random amplified polymorphic DNA (RAPDs); simple sequence repeats (SSRs), sequence tagged marker sites (STMS), inter simple sequence repeats (ISSRs), restriction fragment length polymorphism (RFLP); and amplified fragment length polymorphism (AFLP), single nucleotide polymorphisms (SNPs) and very recent targeted region amplification polymorphism (TRAP). Most of these markers are used for tagging and mapping of monogenic and polygenic trait related genes and are more often targeted on DNA as template and needs considerable breeding efforts to develop mapping populations such as recombinant inbred lines (RILs), near isogenic lines (NILs) and double haploid (DH) progenies and are tedious and time consuming (Semagn et al., 2006).

The advent of high-throughput sequencing technology has generated abundant information on DNA sequences for the genomes of many plant species. This includes the completion of the draft of the whole genome sequences for the model plant Arabidopsis thaliana, in 2000 (The Arabidopsis Genome Initiative, 2000) and for rice, one of the most important food crops, in 2002 (Goff et al., 2002; Yu et al., 2002). In addition, the ESTs of other important crop species have been generated, and powerful bioinformatics tools have annotated thousands of sequences as putative functional genes. During the past few
decades, advances in molecular genetics have led to the identification of multiple genes or genetic markers associated with genes that affect traits of interest in livestock, including genes for single-gene traits and QTL or genomic regions that affect quantitative traits. This has provided opportunities to enhance response to selection, in particular for traits that are difficult to improve by conventional selection as few limitations associated such as low heritability or traits for which measurement of phenotype is difficult, expensive, only possible late in life, or not possible on selection candidates. The task of bridging this DNA sequence information with particular phenotypes relies on molecular markers. Consequently, there is a strong demand for better marker techniques to better utilize the existing sequence information.

2.5.1 Candidate genes as molecular markers

The choice of molecular markers is considered carefully, based on the purpose of the application in the breeding programme, as it is not possible to select a marker system that fits all the requirements for germplasm characterization (Creste et al., 2010). In spite of its immense economic importance, sugarcane genetics has received relatively little attention as compared to other crops, mainly due to its highly heterozygous, polyploid and frequently aneuploid nature, complex genome, poor fertility, and the long breeding/selection cycle. However, the sugarcane genome is beginning to be unraveled by genetic mapping using molecular markers (Hoarau et al., 2001; Raboin et al., 2006) as well as comparisons with closely related diploid genomes such as sorghum (Dufour et al., 1997; Ming et al., 1998). Agronomically important traits, like disease resistance (Daugrois et al., 1996; Rossi et al., 2003) and sucrose yield (Ming et al., 2001), have been studied in sugarcane but complexity of the genome has impeded identification of candidate genes. Map-based cloning strategy is also being utilized for the cloning of a fungal resistance gene (D’Hont and Glaszmann, 2001).

Candidate genes (CG) are sequenced genes of known biological action involved in the development or physiology with the manifestation of the trait. They may be structural genes or genes in regulatory or biochemical pathways, which affect trait expression. One CG hypothesis states, “The significant proportion of the quantitative trait loci (QTL)
affecting trait variation are in fact CGs associated with that trait” (Rothschild and Soller, 1997). The CG approach involves choosing the desired CG, obtaining primer sequences to amplify the gene, uncovering polymorphism, developing a convenient procedure for large scale genotyping, identifying a population for association studies, carrying out an association study of the CG with trait phenotype and verifying the uncovered associations. The CG approach has been utilized successfully to determine the biotic and abiotic characters in rice and other cereals (Faris et al., 1999; Ramalingam et al., 2003; Zheng et al., 2003).

Moreover some limitations are associated with these approach, sometimes we may not get polymorphisms at DNA level in the compared accessions, as it is well known fact that gene may be present in all the accessions but their expression is derived by the promoters, enhancers and other group of elements especially when trait is polygenic or QTL based (Kloosterman et al., 2010). But still seeing over the advantages this system and usefulness to develop an efficient marker system to screen the germplasm or progeny if developed. We intended to exploit this approach to utilize the gene based sequence data generated in this study by cDNA-RAPD and SSH approaches with other data available in sugarcane in conjunction with the gene sequence data available in database for other related plant species towards development of an efficient marker system for sugarcane to screen salt tolerance trait in sugarcane germplasm and early selection in sugarcane breeding programs.