Review of Literature
2. REVIEW OF LITERATURE

2.1. SUGARCANE AGRICULTURE

Sugarcane is any of six to thirty-seven species (depending on taxonomic system) of tall perennial grasses of the genus *Saccharum* (Family Poaceae, tribe Andropogoneae). Native to warm temperate to tropical regions of Asia, they have stout, jointed, fibrous stalks that are rich in sugar, and measure two to six meters (six to nineteen feet) tall. All sugarcane species are inter-breed and the major commercial cultivars are complex hybrids (Mukherjee, 1957; Daniels and Roach, 1987).

2.2. Cultivation and uses

Sugarcane is grown in over 110 countries with an estimated total production of 1,557 million metric tons in 2008, more than six times the output of sugar beet. Today, about 50 percent of world sugarcane production occurs in Brazil and India (Source: Food and Agriculture Organization of the United Nations. http://faostat.fao.org/ Retrieved 2010-06-17).

Sugarcane products include table sugar, falernum, molasses, rum, cachaca (the national spirit of Brazil), and ethanol. The bagasse that remains after sugarcane crushing may be burned to provide heat and electricity. It may also, because of its high cellulose content, serve as raw material for paper, cardboard and eating utensils that, because they are by-products, may be branded as “environmentally friendly” (Sharpe and Peter, 1998).

Sugarcane is indigenous to tropical south asia and southeast asia (Daniels et al., 1975; Sharpe and Peter, 1998). Different species likely originated in different locations with *Saccharum barberi* originating in India and *Saccharum edule* and *Saccharum officinarum* coming from New Guinea (Daniels et al., 1975). Crystallized sugar was reported 5,000 years ago in India (Sharpe and Peter, 1998).

Around the eighth century A.D., Arabs introduced sugar to the Mediterranean, Mesopotamia, Egypt, North Africa, and Andalusia. By the tenth century, sources state, there was no village in Mesopotamia that did not grow
sugarcane (Daniels et al., 1975). Sugarcane is still extensively grown in the Caribbean. Christopher Columbus first brought it during his second voyage to the Americas, initially to the island of Hispaniola (modern day Haiti and the Dominican Republic). It was among the early crops brought to the Americas by the Andalusians (from their fields in the Canary Islands), and the Portuguese.

"Boiling houses" in the 17th through 19th centuries converted sugarcane juice into raw sugar. These houses were attached to sugar plantations in the western colonies. Made of cut stone, rectangular boxes of brick or stone served as furnaces with an opening at the bottom to stoke the fire and remove ashes. At the top of each furnace were up to seven copper kettles or boilers, each one smaller and hotter than the previous one. The cane juice began in the largest kettle. The juice was then heated and lime added to remove impurities. The juice was skimmed, and then channeled to successively smaller kettles. The last kettle, which was called the 'teache', was where the cane juice became syrup. The next stop was a cooling trough, where the sugar crystals hardened around a sticky core of molasses. This raw sugar was then shoveled from the cooling trough into hogsheads (wooden barrels), and from there into the curing house.

Cuban sugarcane produced sugar that received price supports from and a guaranteed market in the USSR; the dissolution of that country forced the closure of most of Cuba's sugar industry. Sugarcane remains an important part of the economy of Belize, Barbados, Haiti, along with the Dominican Republic, Guadeloupe, Jamaica, and other islands.

Sugarcane production greatly influenced many tropical Pacific islands, including Okinawa and, most particularly, Hawaii and Fiji. In these islands, sugarcane came to dominate the economic and political landscape after the arrival of powerful European and American agricultural businesses, which promoted immigration of workers from various Asian countries to tend and harvest the crop. Sugar was the dominant factor in diversifying the island's ethnic makeup, profoundly affecting their politics and society.
2.3. Distribution of sugarcane production in world during 2008

Brazil is the biggest grower of sugarcane, and produces sugar and ethanol for gasoline-ethanol blends (gasohol) as transportation fuel. In India, sugarcane is sold as jaggery, and also refined into sugar, primarily for consumption in tea and sweets, and for the production of alcoholic beverages.

(Source: Food and Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division)

<table>
<thead>
<tr>
<th>Country</th>
<th>Production (Tonnes)</th>
<th>Remarks</th>
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</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>514,079,729</td>
<td></td>
</tr>
<tr>
<td><strong>India</strong></td>
<td><strong>355,520,000</strong></td>
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</tr>
<tr>
<td>China</td>
<td>106,316,000</td>
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<tr>
<td>Thailand</td>
<td>64,365,682</td>
<td></td>
</tr>
<tr>
<td>Pakistan</td>
<td>54,752,000</td>
<td>P</td>
</tr>
<tr>
<td>Mexico</td>
<td>50,680,000</td>
<td></td>
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<tr>
<td>Colombia</td>
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<td>F</td>
</tr>
<tr>
<td>Australia</td>
<td>36,000,000</td>
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<tr>
<td>United States</td>
<td>27,750,600</td>
<td></td>
</tr>
<tr>
<td>Philippines</td>
<td>25,300,000</td>
<td>F</td>
</tr>
<tr>
<td><strong>World</strong></td>
<td><strong>1,557,664,978</strong></td>
<td>A</td>
</tr>
</tbody>
</table>

P = official figure, F = FAO estimate, * = Unofficial/Semi-official/mirror data, C = Calculated figure, A = Aggregate (may include official, semi-official or estimates).
2.4. Sugarcane agriculture in India

Sugar in India is mainly produced only from sugarcane, as the hot tropical climate of the country is suitable for cane cultivation. Over the past sixty-five years, the yield of sugarcane in tonnes/hectare has more than doubled. (Source: National Portal Content Management Team, http://india.gov.in/govt.php, reviewed on: 14-02-2010).

In India, sugar industry is a second largest industry after the textile and is an agro-based industry plays a vital role in the socio-economic transformation of the country. In India, sugarcane is cultivated in about 51.51 lakh ha and growing area is broadly classified into Sub-Tropical and Tropical regions, the later harvest the better cane yield and sugar recovery. Maharashtra, Uttar Pradesh, Karnataka, Tamil Nadu and Gujarat are the leading sugarcane producing states in the country (Source: National Portal Content Management Team, http://india.gov.in/govt.php, reviewed on: 14-02-2010).

In India, the states of Uttar Pradesh (38.57 %), Maharashtra (17.76 %) and Karnataka (12.20 %) lead the nation in sugarcane production (http://indiabudget.nic.in/es2001-02/chapt2002/tab115.pdf. Retrieved 2008-04-06).

The quality of sugarcane improves with age, reaches a peak, and then gradually starts declining. A rapid deterioration begins from the moment of harvest. To make best use of the sugarcane crop, the cane has to be crushed within 8 to 10 hours of cutting. Every ton of cane cultivation requires approximately 60-70 tonnes of water under controlled conditions. However, the amount of water required varies according to different soils and climatic conditions. Shortfall in rains can be met through irrigation and sprinkling from canals.

2.5. Water-deficit stress

In all societies, progress and development depend upon stable and rich food supplies that then provide the time that allows them to devote their energies to other activities. Early communities were situated near reliable water sources with the consequence that crops became gradually selected/ adapted for growth.
under near optimal conditions of the available water supply (Hodge, 2004; Bressan et al., 2009). On the other hand, soil is also important for agriculture and food supply, and it is the first protecting barrier for man (Gregory, 2006). So both agricultural and ecoenvironmentally sustainable development are closely linked with water and soil in relation to the successful civilization of man (Zhou and Shao, 2008).

Plant growth and productivity is adversely affected by the various abiotic and biotic stress factors that occur naturally. Plants are frequently exposed to many conditions of stress such as low temperature, salt, drought, flooding, heat, oxidation and heavy-metal toxicity. Various anthropogenic activities have accentuated the existing stress factors (Mahajan and Tuteja, 2005).

With the increases in climate change, soil water deficit is one of the biggest challenges not only for crop productivity but also for vegetation diversity (Mittler, 2006; Rodríguez-Loinaz et al., 2008). If a single abiotic stress is to be identified as the most common in limiting the growth of crops worldwide, is low water supply. In the face of a global scarcity of water resources and the increased salinization of soil and water, water-deficit and salinity are widespread in many regions of world, and are expected to cause serious problem of more than 50% of all arable lands by the year 2050 (Vinocur and Altman, 2005).

Being a tropical and subtropical crop, sugarcane produces a large amount of biomass. Its requirements for water and fertilizers are equally very high. Scarcity of freshwater is affecting the productivity and profitability of sugarcane growers and millers in India. Water stress significantly affects sugarcane growth, yield and quality (Wiedenfeld, 1995; Nyati, 1996). During the last 10 years, sugarcane production in India has fluctuated between 233 million tonnes and 355 million tonnes per year. Similarly, the productivity at the farm level is as low as 40 tonnes/ha. With such low yields and fluctuations in production, and predicted increases in the variability of rainfall due to climate change, the industry is in for big trouble.

Soil water stress is just one kind of abiotic stress, which is often subjective and open to various interpretations. The flexibility of the normal
metabolism allows the development of responses to environmental changes, which fluctuate regularly and predictably over daily and seasonal cycles. Thus every deviation of a particular factor from its optimum does not necessarily result in stress. Stress is the altered physiological condition caused by factors that tend to alter equilibrium. Strain is any physical or chemical change produced by a stress (Gaspar et al., 2002). Stress with a constraint or with highly unpredictable fluctuations imposed on regular metabolic patterns causes’ injury, disease or aberrant physiology.

Water stress in general is a mild form of water-deficit, which is a condition where the water status of plant undergoes relatively small changes (Bray, 1997). Water stress may arise as a result of two conditions: either owing to an excess of water or of water deficit. The most common water stress encountered is the water deficit stress, known as the drought stress.

The water deficit stress has a profound impact on ecological and agricultural systems (Rochefort and Woodward, 1992). The reactions of the plant to water stress differ significantly at various organizational levels, depending upon intensity and duration of the stress as well as the plant species and the stage of development (Chaves et al., 2003). Understanding plant responses to drought is of great importance and also a fundamental part of making the crop stress tolerant (Reddy et al., 2004).

2.6. Plant responses to water-deficit stress

Environmental stresses trigger a wide variety of plant responses, ranging from altered gene expression and cellular metabolism to changes in growth rate and plant productivity. Plant reactions exist to circumvent the potentially harmful effects caused by a wide range of both abiotic and biotic stresses, including light, drought, salinity and high temperatures. The biochemical and molecular responses to drought are essential for the perception of plant resistance mechanisms to water-limited conditions in higher plants (Reddy et al., 2004; Zhou and Shao, 2008).
Plants have evolved two major mechanisms to cope with water-deficit stress

1. Stress avoidance;
2. Stress tolerance

2.6.1. Stress avoidance

It may be the formation of seeds before drought conditions prevail; avoidance is also achieved by specialized adaptations in the plant architecture. Morphological adaptations are, for example, the development of specialized leaf surfaces to decrease the rate of transpiration, the reduction of leaf area, sunken stomata or an increase in root length and density to use water more efficiently (Zhou and Shao, 2008).

2.6.2. Water stress tolerance

A complex trait, water stress tolerance appears to be the result of the co-ordination of physiological and biochemical alterations at the cellular and molecular level i.e. the accumulation of various osmolytes and late embryogenesis-abundant (LEA) proteins coupled with an efficient antioxidant system (Zhou and Shao, 2008).

2.7. Approaches to manage water-deficit stress in plants

Water-deficit stress is by far the most important environmental stress in agriculture and many efforts have been made to improve crop productivity under water-deficit conditions.

2.7.1. Natural selection and Breeding approach

Of all the abiotic stresses, water-deficit or drought stress is the most devastating one and the most recalcitrant to breeder’s efforts (Tuberosa and Salvi, 2006). While natural selection has favoured mechanisms for adaptation and survival, breeding activity has directed selection towards increasing the economic yield of cultivated species. More than 80 years of breeding activities have led to some yield increase in drought condition for many crop plants. Comparison of the different cultivars that enabled identification of the main
morpho-physiological traits modified during selection in association with yield improvement as observed in wheat, barley (Cattivelli et al., 1994).

A number of physiological studies have identified some traits associated with plant adaptability to drought-prone environments. Among them, traits such as small plant size, reduced leaf area, early maturity and prolonged stomatal closure lead to a reduced total seasonal evapotranspiration, late heading and flowering and to a reduced yield potential (Van Ginkel et al., 1998; Karamanos and Papatheohari, 1999).

This traditional approach of breeding and selection in crop plants with improved water-deficit or abiotic stress tolerances have so far met limited success (Richards, 2006). This is due to the following factors: (1) the focus has been on yield rather than on specific trait, (2) difficulties in breeding for tolerance traits, which include G × E, (3) interactions and the relatively infrequent use of simple physiological traits as measures of tolerance, have been potentially less subject to G × E interferences, and (4) desired traits can only be introduced from closely related species.

Earlier studies showed that further progress can be achieved via the identification of drought tolerance-related traits and the subsequent manipulation of the corresponding genes using marker assisted selection (MAS) and/or gene transformation in wheat, rice and grass family crops (Cattivelli et al., 2008).

2.7.2. Molecular markers approach

Molecular markers can be used to explore germplasm through segregation and association mapping to identify useful alleles in both cultivated varieties and wild relatives. Most of the studies available up to date on water-deficit tolerance are based on segregation mapping and QTL mapping (Cattivelli et al., 2008). For example, a single gene influencing OA in wheat was mapped on the short arm of chromosome 7A and breeding studies showed increased yield during water-deficit stress condition (Morgan, 2000).

During the last 10 years, the QTL application provided many opportunities to identify QTL regions related to physiological, morphological
and developmental changes observed during water-deficit stress condition. For example, QTL of wheat OA gene (Robin et al., 2003), stay green phenotype (Verma et al., 2004).

Earlier research showed studies on molecular markers in maize (Bolanos and Edmeades, 1996), pearl millet for grain yield (Serraj et al., 2005), rice for deep roots (Courtois et al., 2003), sorghum for isogenic stay green lines (Harris et al., 2007), arabidopsis ERECTA gene for transpiration efficiency (Masle et al., 2005) and barley for drought tolerance (Tondelli et al., 2006).

To date many plant QTLs have been cloned by the positional cloning approach, although alternative strategies based on candidate genes and linkage disequilibrium may represent an interesting shortcut to QTL cloning (Salvi and Tuberosa, 2005; Tondelli et al., 2006).

The contribution of marker assisted selection (MAS) for traits has so far been marginal. Due to the high cost of molecular marker technology and the relative low efficiency in MAS for QTL, the cost effectiveness of QTL-MAS versus standard breeding strategies in commercial programs still needs to be accurately evaluated (Barker et al., 2005).

2.7.3. Genomic approach

Besides being exploited in MAS, major drought related QTLs can be considered for cloning with the aim of manipulating the target trait more directly by genetic engineering (Tuberosa and Salvi, 2006). Genetic engineering strategies for abiotic stress tolerance rely (Reddy et al., 2004) on expression of genes that are involved in signaling and regulatory pathways or genes that encode proteins conferring stress tolerance (Vinocur and Altman, 2005). Current efforts to improve plant stress tolerance by genomics approach have resulted in important achievements, however, the genetically complex mechanisms of water-deficit stress tolerance makes the task extremely difficult. Due to these reasons, biotechnology should be fully integrated with classical physiology and breeding (Wang et al., 2003).
2.7.3.1. Genomics studies on water-deficit stress tolerance

Comprehensive reviews of the drought tolerance engineering using genomics are presented below (Umezawa et al., 2006).

In contrast to plant resistance to biotic stresses, which is dependent on monogenic traits, the genomic complex responses to abiotic stresses are multigenic and thus more difficult to control and engineer (Vinocur and Altman, 2005).

Progress in mass-scale transcriptome, proteome and metabolome profiling has allowed recent 10 years holistic approach in investigations of water-deficit stress tolerance based on the obtained expression pattern of thousands of genes and their products (Tuberosa and salvi, 2006). Plants respond and adapt to these stresses at molecular, cellular, physiological, and biochemical levels. Both forward and reverse genetic approaches have elucidated genes and gene products that are involved in gene expression, signal transduction, and stress tolerance (Tuberosa and salvi, 2006). In the post-genomics era, comprehensive analyses using functional genomics technologies such as transcriptomics, proteomics, and metabolomics have increased our understanding of the complex regulatory networks associated with stress adaptation and tolerance (Pérez-Torres et al., 2009).
2.7.4. Proteomic approaches on water-deficit stress tolerance

In the recent past, extensive research has resulted in impressive achievements in genome and expressed sequence tag (EST) sequencing, yielding a wealth of information for many model organisms, including *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and Medicago, but genome sequence information alone is insufficient to reveal the facts concerning gene function, developmental/regulatory biology and the biochemical kinetics of life. To investigate these facts, more comprehensive approaches that include qualitative and quantitative analyses of gene expression products are necessary at the transcriptome, proteome and metabolome levels.

Although transcriptome analysis using microarray and serial analysis of gene expression technologies are potential tools, mRNA and protein levels (Gygi *et al*., 1999) cannot be correlated due to the inability of total mRNA to translate into protein, whereas proteomics provides a more direct assessment of the biochemical processes of monitoring the actual proteins performing the signaling, enzymatic, regulatory and structural functions encoded by the genome and transcriptome.

Recent improvements in high-resolution two dimensional PAGE (2-DE; Zolla and Timperio, 2005), an increase in the number of sequences of protein and nucleotides, increased capabilities for protein identification utilizing modern mass spectrometry methods, such as matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; Corthals *et al*., 2000) and valuable bioinformatics tools have made the large-scale profiling and identification of plant proteins.

During the last 15 years, research has been carried out on different plant organs (Watson *et al*., 2003) and on sub cellular proteomes such as the chloroplast membrane (Zolla *et al*., 2004), cell wall (Borderies *et al*., 2003) and nuclear envelope (Bae *et al*., 2003), whereas other researchers have focused on individual tissues, including seeds (Gallardo *et al*., 2001), mitochondria (Millar *et al*., 2001), vacuoles (Carter *et al*., 2004), chloroplasts (Kleffmann *et al*., 2004). In addition to analyzing the proteomes at sub cellular and cellular levels, several
studies have been carried out with the same aim, but under different extreme environmental conditions such as salinity (Kav et al., 2004), drought (Salekdeh et al., 2002), cold (Amme et al., 2006), UV light (Casati et al., 2005), visible light (Kim et al., 2005), heavy metals (Ingle et al., 2005), nutrient deficiencies (Kang et al., 2004), fungal (Campo et al., 2004), bacterial (Jorrín et al., 2007) and viral infections (Ventelon-Debout et al., 2003).

These studies all together have given rise to a specific field, ‘environmental proteomics’. Now, there is a good amount of work to answer about the types of proteins under- and/or over expressed during a particular or integrative stress, their impacts on cellular metabolism and the location of the proteins. All these studies reviewed the proteins with important properties, which have been shown to play a crucial role against abiotic environmental stresses directly.

2.7.4.1. Integrated approach of proteomics and genomics

Water deficits are known to alter a variety of biochemical and physiochemical processes, ranging from photosynthesis to protein synthesis and solute accumulation. Among the most common responses are electron leakage through thylakoid membranes, damage to chlorophylls, decrease in antioxidant systems, increase in production of H$_2$O$_2$, O$_2^-$, lipid peroxidation (LP) and decrease in photosynthetic.

A nine-bp conserve sequence, TACCGACAT, called drought-responsive element (DRE), is required for the regulation of the induction of RD29A under drought (Yamaguchi-Shinozaki and Shinozaki, 1994). Protein factor(s) that specifically interact with the 9-bp DRE sequence were detected in nuclear extract prepared from either dehydrated or untreated Arabidopsis plants. Five independent cDNA for DRE/CRT-binding proteins have been cloned using the yeast one-hybrid screening method (Liu and Zhu, 1998). All DRE/CRT-binding proteins (DREBs and CBFs) contain a conserved DNA-binding motif that has also been reported in EREBP and AP2 proteins (EREBP/AP2 motif), which are involved in ethylene-responsive gene expression and floral morphogenesis,
respectively (Liu and Zhu, 1998). In transgenic plants, over expression of the DREB1A cDNA also revealed freezing and dehydration tolerance (Liu and Zhu, 1998). Over-production of 35S CaMV promoter driven DREB1A and CBF1/DREB1B cDNAs, in transgenic plants, showed improved drought stress tolerance.

Drought stress has been found to be overcome by a novel aldose/aldehyde reductase that provides protection to transgenic plants. This enzyme has been found to be active on 4-hydroxynon-2-enal, a known cytotoxic lipid peroxide degradation product in alfalfa. Synthesis of this enzyme in transgenic tobacco plants provided considerable tolerance against various forms of abiotic stress including water-deficit stress (Oberschall et al., 2000).

2.7.5. Metabolomic profiling approaches on water-deficit stress tolerance

The metabolic response of plants to various stresses of biotic or abiotic origin is currently receiving increasing attention (Schauer and Fernie, 2006). Even so, the majority of metabolomic studies are still depended by analysis at transcript level (Fernie, 2004). Alterations in the metabolome of a wide number of species in response to a broad range of stresses have been analyzed recently (Kaplan et al., 2004); many studies will focus on temperature and nutrition stresses (Kaplan et al., 2004; Ishizaki et al., 2005; Daskalchuk et al., 2006).

The stress that has been best characterized using metabolite profiling is the response and acclimation to temperature stress, which has been the subject of several investigations (Kaplan et al., 2004). Various approaches have been used: for example, the kinetic thermo tolerance response and the induction of freezing tolerance were determined by GC-MS in the Colombia ecotype of Arabidopsis (Kaplan et al., 2004), and comparative metabolite profiling using the GC-MS was also carried out in ecotypes Wassilewskija-2 and Cape Verde Islands-1 (Cook et al., 2004). These reports have been complemented using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICRMS; Daskalchuk et al., 2006). Importantly, these studies taken together enabled the identification of more metabolites associated with temperature stresses and showed elements
common between the responses to cold and heat and demonstrated a prominent role for the CBF (C-repeat/dehydration responsive element-binding factor) cold response pathway in the configuration of the low temperature metabolome (Kaplan et al., 2004; Cook et al., 2004; Saito et al., 2006; Daskalchuk et al., 2006).

Stitt et al., (2002) documented the use of metabolite profiling in understanding carbon–nitrogen interactions. Many of these studies were focused on a relatively limited number of metabolites. In combination with recent transcript profiling studies (Wang et al., 2004), this has enabled a characterization of the metabolic response to low nitrogen in tobacco mitochondrial complex I mutant (Dutilleul et al., 2005) and further such characterizations will enable a more complete elucidation of the control points in this metabolic network.

Nikiforova et al., (2004), Bolling and Fiehn, (2005) characterized the plants response to low phosphate and sulfate stress at the metabolite level. Metabolite profiling provides direct functional information on metabolic phenotypes and indirect functional information on a range of phenotypes that are determined by small molecules, for example the discovery of functional threonine aldolases (Broeckling et al., 2005). Given this, metabolite profiling has great potential as a tool for functional genomics in recent years (Achnine et al., 2005; Morikawa et al., 2006).

2.7.5.1. Integrated approach of metabolomics and genomics

Recently, an integrated analysis of the metabolome and transcriptome of the dehydration-stress response was carried using GC-TOF–MS, CE-MS, and DNA microarrays for dehydration-stress response of an Arabidopsis NCED3-knockout mutant and the wild-type plant (Urano et al., 2009). NCED3 plays a role in the dehydration-inducible biosynthesis of ABA (Yamaguchi-shinozaki and Shinozaki, 2006). These metabolite profiling studies revealed that the ABA accumulated during dehydration regulates the accumulation of various amino acids and sugars such as glucose and fructose. In particular, the dehydration
inducible accumulations of BCAAs (branch-chain amino acids), saccharopine, proline, and agmatine are correlated with the dehydration-inducible expression of their key biosynthetic genes (BCAT2, LKR/SDH, P5CS1, and ADC2, respectively), which are regulated by endogenous ABA.

Cramer et al., (2007) also compared the transcript and metabolite profiles during dehydration and salinity stress in grapevine. Those metabolic profiling revealed higher concentrations of glucose, malate, and proline in dehydration-treated plants, compared with salt-stressed plants (Cramer et al., 2007).

These differences in the levels of metabolites were correlated with those of transcript levels of many genes involved in energy metabolism and nitrogen assimilation. Compared with salt-stressed plants, dehydration-treated plants have a greater need to adjust osmotically, detoxify ROS, and ameliorate photo inhibition (Cramer et al., 2007). Rizhsky et al., (2004) also studied the metabolic profiling and showed the sucrose replacement for proline in plants as the major osmo-protectant during the more severe combined dehydration and heat-stress treatment.

2.7.6. Molecular approaches for abiotic stress tolerance mechanism in sugarcane

In spite of the 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 (Raboin et al., 2006) as well as comparisons with closely related diploid genomes such as sorghum (Ming et al., 1998).

Agronomically important traits, like disease resistance (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. D’Hont and Glaszmann, (2001) used the map-based cloning strategy for the cloning of a fungal resistance gene.
Thus, only a limited number of genes related to abiotic and biotic stresses have been reported so far in sugarcane. To identify and study most of an organism’s genes/gene families and their alleles in different genotypes, analysis of expressed sequence tag (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), sugarcane (Vettore et al., 2001; 2003), 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 (Houde et al., 2006). The availability of sequences and analysis tools facilitate broad-based utilization of ESTs for gene structure annotation and comparative genomics (Vettore et al., 2001; 2003).

ESTs represent tags of the expressed portion of a genome and therefore potentially identify genes encoding proteins, miRNA, transacting siRNA precursors, and more generally noncoding RNA. Several sugarcane EST collections have been developed.

The publicly available sugarcane ESTs were assembled into tentative consensus sequences, singletons and mature transcripts, referred to as the Sugarcane Gene Index (SGI: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/giman.pl?gudb=s_officinarum).

Through the SUCEST project in Brazil, Vettore et al., (2003) developed a collection of ~240,000 sugarcane ESTs, generated from 26 tissue and developmental stage-specific cDNA libraries and referred as Sugarcane assembled sequences (SASs). 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), low-temperature-responsive genes (Nogueira et al., 2003), ABA- and MeJA-activated sugarcane bZIP genes (Schlögl et al., 2008).
2.8. Molecular tools for studying water-deficit stress in sugarcane

A variety of methods have been developed to identify the differentially expressed genes associated with a particular phenotype. These methods include differential display, representational difference analysis (Liang and Pardee, 1992), subtractive hybridization (SH) (Barr and Emanuel, 1990), suppression subtractive hybridization (SSH) (Diatchenko et al., 1996), differential screening (Davies and Robinson, 2000) and microarray (Lockhart and Winzeler, 2000) technologies. All these methods have been successfully utilized to isolate differentially expressed genes; however, each method has exhibited inherent drawbacks (Kuhn, 2001). These drawbacks include variability in reproducibility of differential banding patterns, high incidence of false positives, loss of rare transcripts, the inability to simultaneously analyze multiple genes, high costs, and/or reduced availability of a particular resource. To circumvent the limitations of any individual method, some investigators have begun to use two different methods in conjunction (Fuchs et al., 2000; Kim et al., 2001).

2.8.1. Suppression Subtractive Hybridization and Microarray approaches

Diatchenko et al., (1996) developed a new and highly effective PCR based method, termed SSH (Suppression Subtractive Hybridization) to generate subtracted cDNA libraries. This method primarily based on a recently described technique called suppression PCR (Siebert et al., 1995; Chenchik et al., 1996) and combines normalization and subtraction in a single step.

re-watering using cDNA microarray slides containing about 2500 unique cDNAs from immature maize ear.

### 2.9. Studies on stress related genes identified from sugarcane

Sugarcane is a crop of both tropical and subtropical regions produces a large amount of biomass, and hence requires substantial inputs of water and nitrogen to produce maximum yields (Wiedenfeld, 2000). Sugarcane goes through four different growth stages, comprising germination and emergence, tillering and canopy establishment, growth and maturation or ripening. Sufficient water is necessary during crucial stages such as emergence, tillering and canopy establishment (Gascho, 1985).

Smit and Singels, (2006) compared the leaf and shoot phenology, leaf area index and radiation interception under water stress in sugarcane cultivars N22 and Nco376 and its relation to stomatal conductance and leaf water potential. Inman-Bamber and Smith, (2005) reviewed sugarcane water relations and their response to water deficit in terms of water use efficiency. Using the SUCEST database mining, Nogueira et al., (2005) identified 26 non-redundant sugarcane genes encoding NAC domain and demonstrated that SsNAC 23 was strongly induced by extreme chilling stress, water stress and herbivory.

Drought responses vary depending on the duration and intensity of the stimulus and comprehend protective alterations and long term growth changes. Many genes responsive to drought have been catalogued (Ingram and Bartels, 1996; Seki et al., 2001; Oono et al., 2003).

The drought stimulus lead to transient calcium fluxes, the activation of calcium sensors, the accumulation of reactive oxygen species, the activation of the MAPK pathway and the induction of several transcription factors including DREB2A, DREB2B and NAC (Hu et al., 2006). In those studies a MAPK was more expressed in mature internodes and repressed by ABA (SCSBAM1084E01.g) a DREB was induced during culm maturation (SCCCLR1001D10.g) and over forty stress responsive genes were identified (Hu et al., 2006). Lee et al., (2005) showed a dramatic repression of DEHYDRIN
TYPE 1 (SCQGLR1085F11.g) gene regulated by the DREB signaling cascade in mature internodes of high and low Brix plants and induced after 72 h and 120 h drought treatment. Over expression of DREB2A in Arabidopsis thaliana led to the generation of transgenic plants more tolerant to drought (Sakuma et al., 2006).

A detailed organization of sugarcane genes into functional categories (i.e., signal transduction components, regulation of gene expression, development, stress, transposable elements, metabolism, etc. were completed and represent the basis to develop functional genomic approaches. The first report of the use of cDNA arrays to discover 25 down-regulated and 34 up-regulated sugarcane genes modulated by cold stress was analyzed by Nogueira et al., (2003).

Sugarcane homologues to several genes known to be induced by cold stress were found together with genes induced by drought in other species because the cold induces the formation of ice, dehydrating the cell (Menossi et al., 2008). One of the identified gene SsNAC23, is a member of the NAC family of transcriptional factors that are involved in biotic and abiotic stress and development (Olsen et al., 2005). Further, Nogueira et al., (2003) showed the nuclear targeting of SsNAC23 protein. The analysis by using PmmA (Vicentini and Menossi, 2007) revealed a new set of 30 genes as differentially expressed.

To increase the knowledge on the sugarcane responses to drought, Rocha et al., (2007) used the cDNA microarrays to evaluate gene expression in sugarcane subjected to water deprivation. Several plant responses to environmental stress are mediated by phytohormones, with a well-known cross-talk between them (Gazzarrini et al., 2003). Rocha et al., (2007) evaluated the role of ABA spray on sugarcane leaves using cDNA arrays. Phosphatase, small GTPase, Ser/Thr kinases were induced in sugarcane during ABA spray. These findings help to depict an overview of the network of ABA signal transduction in sugarcane. The cross-talk between ABA and MeJA also become evident from the activation of two genes involved in salicylic acid and MeJA biosynthesis in the ABA-treated plants (Rocha et al., 2007). Among the genes differently expressed in (Rocha et al., 2007) studies, transcription factor orthologs of the MYB,
WRKY, NAC, and DREB proteins, which are known to play a role in drought responses of other systems (Abe et al., 1997; Yamaguchi-shinozaki and Shinozaki, 2006) were up regulated. Similarly, the wide array of genes induced by phosphate stress in sugarcane were in line with the complex responses observed in other species, such as tomato, arabidopsis, white lupin, and rice (Menossi et al., 2008).

Transition in gene expression during water deficit is commonly used to unravel the molecular basis of drought stress leading to sensitivity or tolerance (Bray, 1997). In recent years, studies regarding cellular responses and regulation of gene expression during abiotic stresses have been documented, especially in Arabidopsis thaliana (Ingram and Bartels, 1996; Zhu et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002). These includes enzymes required for osmolyte biosynthesis, chaperones, LEA proteins, mRNA binding proteins, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases (Ingram and Bartels, 1996; Seki et al., 2002) as well as cis and trans regulatory factors involved in signal transduction such as protein kinases, transcription factors. The existence of a variety of water inducible genes suggests that the responses of plant to water stress are rather complex (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997; Schaffner, 1998; Ramanjulu and Bartels, 2002; Xiong et al., 2002).

2.10. Transcription factors in water-deficit stress biotechnology

Transcriptome analyses using molecular tools, together with conventional approaches, have revealed that dozens of transcription factors (TFs) are involved in the plant response to drought stress (Vinocur and Altman, 2005; Bartels and Sunkar, 2005). Most of these TFs fall into several large TF families, such as AP2/ERF, bZIP, NAC, MYB,MYC, zinc finger and WRKY (Umezawa et al., 2006). The expression of TFs regulates the expression of downstream target genes that are involved in the stress response and tolerance.

Kasuga et al., (1999) demonstrated the over expression of the DREB1/CBF3 (dehydration-responsive element binding protein/CRT-binding
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factor) TF in *Arabidopsis* for increased tolerance to freezing and high salt exposure. Similarly transgenic plants expressing a drought-responsive AP2-type TF, SHN1-3 or WXP1, induced several wax-related genes and resulted increased drought tolerance (Zhang *et al*., 2005). *AtMYB60*, an *R2R3-MYB* transcriptional repressor in *Arabidopsis*, functions in the regulation of stomatal movements and negatively regulated during drought stress (Cominelli *et al*., 2005).

Recently, Yilmaz *et al*., (2009) integrated and evaluated the SUCEST and PlantGDB (http://planttfdb.cbi.pku.edu.cn/web/index.php?sp=so) EST databases for putative transcription factors and identified 2,406 candidate TFs. These were classified into families and can be found at http://grassius.org. They found twenty-one transcription factors (TFs) regulated during culm development. The great majority was more expressed in the immature internodes, including two members of the Homeobox Knotted1-homeodomain (SCAGLR1021G10.g and SCRLAM1010D08.g), which have been shown to be involved in developmental processes in maize (Vollbrecht *et al*., 1991).

Developmentally regulated genes include a homolog (SCBFAD1046D01.g) to anthocyanin regulatory R-S protein containing a helix-loop-helix (HLH) domain, which controls tissue-specific synthesis of anthocyanin pigments (Ludwig *et al*., 1989). Nine transcription factors were identified as differentially expressed when high brix and low brix genotypes were compared including an *ARF6* (*AUXIN RESPONSE FACTOR6a*) (SCEZLB1010E10.g), a *NAM* (*NO APICAL MERISTEM*) (SCCCLR2003E10.g) and an *EIL* (*ETHYLENE INSENSITIVE3-LIKE*) (SCCCRZ1004H12.g).

The *NAM* transcript was negatively regulated by sucrose and glucose treatment and induced by drought. Further, NAM transcription factors in *Oryza sativa* have been described as important regulators of drought tolerance (Hu *et al*., 2006). This indicates a connection between these signaling pathways (Aoki *et al*., 2007), possible co-regulation associated with sucrose content and cross-talks or signaling overlaps between sugar sensing, sugar mobilization and drought responses.
2.11. MYB transcription factors

Plants are exposed to various abiotic stresses, such as cold, drought and high salinity during the course of their growth and development. To cope with these, plants have evolved complex regulatory mechanisms, which include the expression of stress-related genes. The regulation of these genes during stress depends on the interaction of transcription factors with cis-acting elements and/or with other transcription factors required for gene expression (Grotewold, 2008). A large group of plant transcription factors, such as AP2/EREBP, bZIP, WRKY, MYB and zinc finger proteins have been characterized as a part of the signaling network through which plants respond to changes in environments (Yamaguchi-Shinozaki and Shinozaki, 2006). Among them, MYB family of genes constitute largest group of plant transcription factors (Stracke et al., 2001; Dias et al., 2003; Li et al., 2004; Qu and Zhu, 2006; Yanhui et al., 2006) have been shown to play important role in secondary metabolism and environmental signaling (Martin and Paz-Ares, 1997; Jin and Martin, 1999).

MYB transcription factors have highly conserved DNA-binding domain (MYB domain) consists one to three imperfect repeats 51 or 52 amino acid residues with helix-turn-helix (HTH) structure (Stracke et al., 2001). MYB gene family is implicated in wide range of functions including the phenylpropanoid metabolism, control of cell fate, development as well as abiotic and biotic stress responses (Stracke et al., 2001; Jiang et al., 2004; Yanhui et al., 2006).

2.12. Functional promoters in sugarcane

In practical plant improvement, it is necessary to observe that only a small proportion of transgenic events show stable expression of the introduced gene in precisely the intended pattern to confer the introduced trait in the absence of other undesired changes (Birch, 1997). Sugarcane is an attractive crop for improvement aimed at sustainable biomaterial production, because of high biomass productivity, built-in containment features and an efficient gene transfer system (Birch, 2007).
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Molecular marker studies and sequencing of genomic PCR products indicate high heterozygosity (Jannoo et al., 1999; Cordeiro et al., 2000). Sequences within the sugarcane expressed sequence tag (EST) databases indicate that multiple haplotypes are expressed (Grivet et al., 2001; 2003), but little is known about the differential expression of alleles. This complexity of the sugarcane genome has implications for the isolation of functional sugarcane promoters.

Several sugarcane promoters have been isolated and shown to drive transgene activity in callus or young plants but not in the expected pattern in mature plants (Hansom et al., 1999; Wei et al., 2003; Yang et al., 2003). Hansom et al., (1999) showed several heterologous and synthetic promoters with evidence for both transcriptional and post-transcriptional gene silencing (PTGS) effects. In contrast, the maize Ubi-1 promoter has driven sustained reporter transgene expression in sugarcane (Hansom et al., 1999). Mudge et al., (2009) demonstrated the detailed analysis of a sugarcane gene, SHAQYF subfamily of MYB transcription factor promoter and eight distinct promoter sequences from Q117, tested for ability to drive reporter gene expression in sugarcane.

2.13. Transgenic approach- for enhanced water-deficit stress tolerance

The food security challenge being faced by the world has directed plant scientists towards gene revolution after green revolution due to advances in biotechnology. The gene revolution, in fact, involves modification of qualitative and quantitative traits in an organism by transferring desired genes from one species to another. This strategy is referred to as the transgenic approach.

In contrast to classical breeding, the transgenic approach allows the incorporation of only the specific cloned genes into an organism and restricts the transfer of undesirable genes from donor organism. Through this approach, pyramiding of genes with similar effects can also be achieved. Rapid advance in recombinant-DNA technology, development of precise and efficient gene-transfer protocols have resulted in efficient transformation and generation of transgenic lines in a number of crop species (Gosal et al., 2009).
Transgenic approach is being pursued actively throughout the world to improve traits including tolerance to biotic and abiotic stresses in a number of crops (Ashraf et al., 2008). Ashraf and Foolad, (2007) established the role of compatible organic solutes in drought tolerance. Serraj and Sinclair, (2002) and Ashraf et al., (2008) showed the overproduction of compatible organic osmotica during osmotic stress, engineered the genes encoding the synthesis of such organic solutes in transgenic plants. For example, among the many organic osmolytes known to play a substantial role in stress tolerance, glycine betaine (GB), a quaternary ammonium compound, occurs richly in response to dehydration stress (Ashraf and Foolad, 2007). Transgenic tobacco lines over expressing choline monooxygenase (CMO) have been produced (Zhang et al., 2008) under water limited conditions and hence enhanced drought tolerance. Like glycine betaine (GB), proline is also an important compatible organic osmolyte that plays a key role in stress tolerance. Pyrroline-5-carboxylate synthetase (P5CR) is the key enzyme for proline biosynthesis and many studies showed engineering of this gene in soybean (Ronde et al., 2004), petunia (Yamada et al., 2005), and tobacco (Gubis et al., 2007). Trehalose, a nonreducing sugar, is also a potential organic osmoticum which has a substantial role in the protection of plants against stresses. However, transgenic lines of different crops have been generated using the gene TPS1 for trehalose-6-phosphate synthase in tobacco and rice (Karim et al., 2007). Miranda et al., (2007) developed multiple stress tolerant transgenic line using TPS1–TPS2 fusion gene construct into Arabidopsis thaliana through Agrobacterium using either the 35S or the stress regulated rd29A promoter.

Like other stresses, drought stress leads to increased accumulation of reactive oxygen species (ROS) in plants thus causing an oxidative stress. To counteract these ROS, plants can intrinsically develop different types of antioxidants. Over production of antioxidants in response to drought induced oxidative stress has been found to be associated with the drought stress tolerance of different plant species (Sunkar et al., 2006). Furthermore, genes encoding different types of antioxidants have been engineered in different plants like
potato (Perl et al., 1993), alfalfa (McKersie et al., 1997), and rice (Wang et al., 2005), and tobacco (Eltayeb et al., 2007) for achieving enhanced drought tolerance.

Transcription factors are specific types of proteins that bind DNA and are involved in the regulation of gene transcription, hence gene regulation. Since regulation of genes involved in stress tolerance is important for improving this trait in plants, many efforts are being made these days to identify and characterize transcription factors (regulatory proteins) involved in stress-specific gene regulation. However, several transcription factors have been identified, which are involved in gene regulation in plants under water limited conditions (Bartels and Sunkar, 2005; Vinocur and Altman, 2005). Of a number of transcription factors listed by Gosal et al., (2009), dehydration-responsive element-binding factors (DREB) have attracted the attention of many scientists. Jaglo-Ottosen et al., (1998) and Liu et al., (1998) first reported the up-regulation of many genes in DREB1/CBF transgenic Arabidopsis involved in tolerance to a variety of stresses including drought, salinity, freezing etc. Similarly, transgenic Arabidopsis plants over-expressing DREB1/CBF3 operated by the constitutive promoter CaMV 35S also exhibited improved tolerance to salinity, drought and freezing (Kasuga et al., 1999).

Dubouzet et al., (2003) isolated four rice CBF/DREB1A orthologs, OsDREB1A, OsDREB1B, OsDREB1C and OsDREB1D and exhibited improved tolerance to drought, salinity and freezing. In maize, over-expression of ZmDREB2A under the control of constitutive or stress-inducible promoter resulted in enhanced drought tolerance in plants (Qin et al., 2007). Similarly, peanut plants transformed with rd29A:DREB1A had higher transpiration efficiency than the wild type under drought stress (Bhatnagar-Mathur et al., 2007). Thus, transferring a number of prominent genes effectively involved in stress tolerance to transgenic plants seems to be a logical approach. Although a large number of genes appear to be involved in stress tolerance and most of them have been fully characterized, the function of many of them in the mechanism of stress tolerance is yet to be investigated.