Chapter 7

Biotechnological Interventions for the Improvement of Sugarcane Crop and Sugar Production

Ghulam Mustafa, Faiz Ahmad Joyia, Sultana Anwar, Aqsa Parvaiz and Muhammad Sarwar Khan

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71496

Abstract

Sugarcane, not only fulfills 70% of world sugar needs but is also a prime potential source of bioethanol. It is majorly grown in tropical and subtropical regions. Researchers have improved this grass to great extent and have developed energy cane with ability to accumulate up to 18% sucrose in its Culm. Improvement of this crop is impeded by its complex genome, low fertility, long production cycle and susceptibility to various biotic and abiotic stresses. Biotechnological interventions hold great promise to address these impediments paving way to get improved sugarcane crop. Further, being vegetatively propagated in most of the agroecological regions, it has become more attractive plant to work with. This chapter highlights, how advanced knowledge of omics (genomics, transcriptomics, proteomics and metabolomics) can be employed to improve sugarcane crop. In addition, potential role of *in vitro* techniques and transgenic technology has also been discussed for developing improved sugarcane clones with enhanced sugar recovery.

Keywords: sugarcane, omics, transgenics, crop improvement, biofuel

1. Introduction

Sugarcane is a major contributor to world sugar and more than 70% of global saccharine needs are fulfilled by this sweet grass. It has been cultivated since pre-historic times as a sugar source. Further, it has great potential to be used for the production of bioethanol and has been grown in many countries as an energy crop. Brazil is the top most consumer of sugarcane biofuel followed by USA and fulfill 50% of their energy needs through biofuel. They run more than 5.0 million automobiles on hydrous ethanol at an ethanol content of 95.5% [1]. These facts direct us to strive for the improvement of sugarcane crop so that global energy needs may be fulfilled

sustainably. Various promising varieties have been developed so far but they are posed to certain drastic stresses including biotic as well as abiotic stresses. Similarly, efforts have been made to improve sugar recovery. Since, crop productivity and quality can only be improved by employing innovative technologies. Plant tissue culture and genetic engineering has great potential to resolve problems faced by this crop [2]. Transgenic technology can do a lot to address all the aforementioned yield limiting constraints as any of the alien genes may be introduced into the plant through genetic transformation methodologies. Different methods of transformation i.e. biolistic [3, 4] *Agrobacterium* [5] and electroporation [6] have been employed to engineer valuable agronomic traits like resistance against weedicides [7], viruses [8] and insects [3]. Efforts have been made to engineer metabolic pathways for improved sugar content [9] and for the production of biopolymers and bioplastics. Omics approaches have contributed a lot to understand and explore sugarcane genome to develop improved clones. Milestones in structural and functional genomics are also convincing. Different types of markers have been developed to speed up molecular breeding through early identification of superior genotypes [10]. Thus, biotechnological interventions have great potential to promote sugarcane not only as future energy crop but also a factory house for the production of therapeutics and industrial compounds.

These interventions have been discussed here to focus critical areas of research that can attract researchers for the improvement of this crop. Understanding molecular mechanisms involved

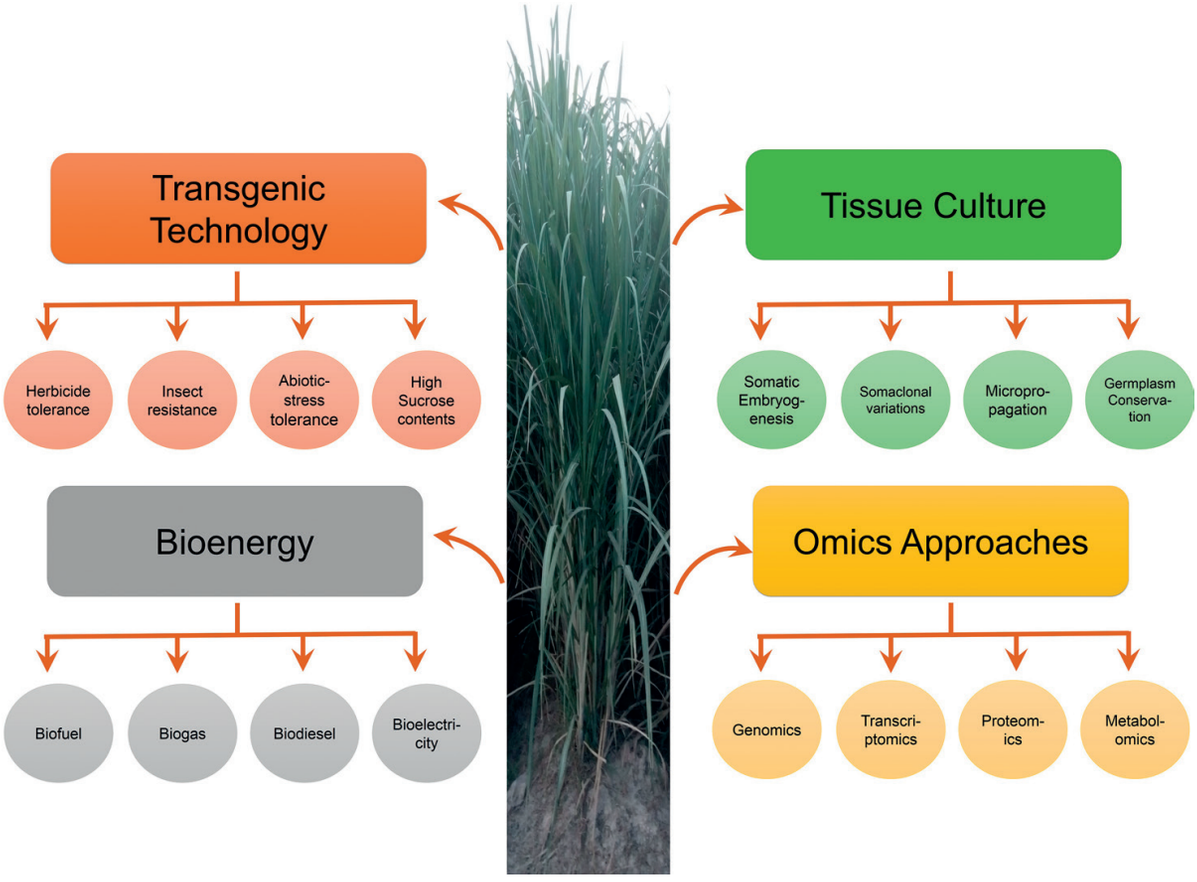

Figure 1. Schematic sketch showing role of biotechnological interventions for the improvement of sugarcane crop and sugar production.

http://dx.doi.org/10.5772/intechopen.71496

in metabolic pathways and sucrose accumulation will prove a real milestone in developing future energy crop. Similarly, importance of *in vitro* techniques have been highlighted that how advancements in tissue culture techniques are important for germplasm conservation, development of somaclonal variants and genetic transformation. Likewise, potential of transgenic technology has been discussed to develop insect resistant, disease resistant and herbicide tolerant plants. Omics, a real potential area of future research, has been overviewed to highlight the role of genomics, transcriptomics, proteomics and metabolomics in sugarcane crop improvement and in developing energy cane (**Figure 1**).

2. Tissue culture based approaches for sugarcane crop improvement

Since the pioneer work on callus induction at Hawaiian Sugar Planters' Association Experiment Station and the method developed by Nickell [11] for root production, sugarcane tissue culture appeared as a valuable tool for diverse research activities. Shortly after this, Heinz and Mee [12] published the first report on sugarcane regeneration. These *in vitro* techniques had huge impact on basic research and also on the research of commercial interest which includes maintenance of elite germplasm, production of agronomically superior somaclones, micropropagation of elite clones, healthy planting material and screening for abiotic/biotic stress tolerance.

2.1. Somatic embryogenesis

Somatic embryogenesis may be divided into two phases: induction and expression. During the induction phase, embryogenic competence is acquired by differentiated somatic cells whereas during expression phase, embryogenic cells differentiate into somatic embryos. Komamine et al. [13] evaluated that embryogenic cells did not require any exogenous stimuli in the form of growth hormones or vitamins at induction state. Rather, competent cells require exogenous stimuli at transitional state in very minute quantities. Somatic embryogenesis may either be direct or indirect. Direct somatic embryogenesis involves development of embryo directly on the surface of explant tissues i.e. stem segments, leaf segments, young inflorescence, zygotic embryo, protoplasts and microspores. Indirect somatic embryogenesis involves an intermediary step of callus induction followed by embryogenesis. Different factors have been found to play key role in the acquisition of embryogenic competence. Exogenous growth regulators promote embryogenic competence by affecting cell polarity, pH gradients and by modifying ionic balance all around the cells. Intracellular pH is very crucial for embryogenic competence acquisition. Another critical aspect is the physical isolation of a cell from others. Embryogenic competence acquisition by somatic cells is regulated by the expression of certain genes which involve either upregulation or down regulation of certain functional genes. In addition, physiological, morphological and metabolic variations are also very important for the acquisition of embryogenic competence. Thus, somatic embryogenesis is a great milestone in sugarcane biotechnology [14]. Originally this method was developed as a substitute of meristem culture and regeneration but now it has become an important component of genetic engineering as well. It has well been exploited for the preservation of mutants and transformed material till

their approval or field establishment. Various high yielding, early maturing, high sucrose content and smut resistant varieties with good ratooning ability have also been developed through somatic embryogenesis.

2.2. Somaclonal variations

Somaclonal variation have been employed to improve cane-quality, sugar recovery, yield, drought tolerance and disease resistance. To increase the incidence of genetic variation and to get positive modifications in the target plant genome, physical (ion beams, gamma rays) and chemical (sodium azide, sodium nitrite and ethylmethane sulfonate (EMS) mutagens have been tested [15]. Introduction of selection pressure at cellular level has been successful to isolate mutants with desired characters. For fungal pathogen resistance inoculation with fungal pathotoxins or culture filtrates has been very effective. Somaclonal variants of sugarcane were developed against eye spot disease by Larkin and Scowcroft. Mutagenesis has been used by various researchers to isolate embryogenic cells and plants tolerant against red rot [16].

Similarly, for abiotic stress tolerance polyethylene glycol, mannitol and sodium chloride has been used for the selection of plants against drought/salinity tolerance [17]. Various studies were conducted to evaluate the level of variability and transmission of variations into the next generation by vegetative propagation. These studies verified the occurrence of considerable variations in *in-vitro* derived plants. However, extensive field experiments showed that tissue culture derived phenotypic variations were often temporary as most of the variants relapsed to the parental phenotype in the first ratoon crop [18]. Few other studies also supported somaclonal variations but to little extent. Chowdhury and Vasil [19] were not able to recognize any considerable variation in the DNA of plants regenerated from cell suspension, protoplasts and callus cultures. Taylor et al. [20] performed random amplified polymorphic DNA (RAPD) analyses of plants regenerated from embryogenic culture. Only a few polymorphisms were observed. Anyhow, there are different opinions about the somaclonal variations and their transmission to next generation. Most of the researchers are of the view that these variations depend upon the genetic makeup and experimental conditions under which plants are screened and selected.

2.3. Micropropagation to produce sanitated plants

Systemic buildup of infections particularly diseases (viral, bacterial and fungal) affect plant vigor and health. Unavailability of an efficient *in vitro* mass multiplication system in sugarcane is a major constraint in the provision of disease free elite germplasm [21]. Sugarcane has long breeding cycle and it requires 10–15 years to complete selection cycle. Fuzz multiplication rate of newly released sugarcane varieties is always slow 1:6–1:8 [22] and diseases accumulate in the seed sets during multiplication. Unavailability of disease free planting material is a major limitation in the improvement of sugarcane crop. Normally, sugarcane reproduces vegetatively but seed propagation is also there under particular climatic conditions [23]. Nodal cuttings are being used for the propagation of commercial sugarcane. In this method of multiplication, meristematic or non-meristematic tissues are used as explant. Sugarcane plants have been regenerated directly from apical and axillary meristems and also from immature leaf tissues [24].

In vitro propagation of sugarcane through meristematic tissues responded better as compared with other types of plant tissues. Therefore, significant efforts have been made to explore meristematic tissues for mass multiplication. In the beginning of twenty-first century, some reports highlighted direct regeneration of sugarcane genotypes through thin layer culture of cells from immature leaf or inflorescence [24]. They reported to lessen the time span required for *in vitro* propagation. Significant efforts have been made to establish protocols for direct or indirect sugarcane regeneration. Almost each part of the sugarcane plant has been exploited for callus induction but only inflorescence and immature leaves [25] responded better to morphogenic callus. Callus based regeneration gained significance with the prediction that *in-vitro* induced mutations can play some key role in sugarcane improvement [26]. *In vitro* induced variability is beneficial for the development of new varieties but it becomes undesirable when true-to-type plants are required. Only fewer examples have been quoted to highlight useful variations in callus-derived plants. Meristem culture was successfully used to eradicate chlorotic streak disease, sugarcane mosaic virus [27], white leaf disease and ratoon stunting disease. Combination of meristem culture and heat treatment have proved very effective to eradicate pathogens of Fiji disease [28], SCMV [27] and downy mildew. It is an effective method to eliminate most of the bacterial and fungal diseases and is commonly used to eradicate diseases of unknown etiology as well. Disease free planting material of sugarcane obtained from apices culture is now routinely used for the international exchange of this crop. Researchers have explored that plants regenerated from thin cell layer culture can be used to produce disease free sugarcane plants from the infected ones with *Leifsonia xyli*, SCMV and FDV. *In vitro* culture techniques are used in Brazil and USA to produce healthy planting material for commercial applications. Cryotherapy has also appeared as a proficient method to eliminate phytoplasma from the crop plants and has also been used for long term storage of germplasm or production of disease free plants [29].

2.4. Germplasm conservation

Another important application of *in vitro* techniques that attracted researchers is germplasm conservation [30]. *In-vitro* storage of sugarcane germplasm had been established at the Centre de Cooperation Internationale en Recherche Agronomique pour le Development (CIRAD) in France, Sugarcane Breeding Institute in India, and BSES Limited (formerly the Bureau of Sugar Experiment Stations) in Australia. More than 200 hybrid clones of *Saccharum* spp. were preserved at 18°C for 12 months and no phenotypic modifications were observed in the recovered plants. However, with the advancements in tissue culture techniques, *in vitro* preservation became more valuable for the preservation of genetic resources especially of sugarcane [31]. The minimal medium used in *in vitro* preservation has been used successfully during short and medium term preservation, especially for meristems and shoot apices. Decline in explants metabolic activity is usually achieved by changing physical environment or composition of the media used [32]. The commonly used approaches are: lowering of incubation temperature and use of osmotically active compounds such as sorbitol, mannitol and sucrose. Moreover, growth inhibitors like abscisic acid (ABA) is also frequently used. Various factors i.e. vitamins, salts, osmotic stress and others have been explored by different researchers. Survival rate varied in all these experiments but nobody exploited genetic or cytological

studies to assess the genetic stability of *in vitro* plants. For diploid species, Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeat (ISSR) have been used successfully to assess genetic stability of *in-vitro* plants [33]. But, for plants like sugarcane which has complex polyploid genome, these tools are inappropriate as interpretation of the results become tricky [34]. Using microscopic techniques for sugarcane is also difficult because of small size and large number of chromosomes and also due to the presence of various cytotypes [35]. In this context flow cytometry has got attraction as it ensures estimation of relative amounts of plants nuclear DNA quickly and precisely [36]. Cytometry is able to discriminate between plants derived different culture techniques and has extensively been used, in many economically important species such as *Gossypium hirsutum*, *Vitis vinifera*, *Passiflora* spp., *Elaeis guineensis*, *Musa acuminata* and *Prunus cerasus* [37]. Flow cytometric analysis of shoots was performed after every 6 months of storage. As a consequence, a discrete behavior of tested varieties was observed during storage and on average approximately 80% cultures were able to recover. From these findings it is concluded sugarcane genotypes can be maintained in minimal growth condition for extensive periods but may lead to genetic variations.

3. Omics approaches to improve sugarcane crop

The word "Omics" has become a broader term and it is impossible to cover it just in one topic. Omics approaches have explored understandings of complex interactions between genes, proteins and metabolites. These integrated approaches heavily rely on analytical methods, bioinformatics, computational analysis and many other disciplines of biology. Using genomics, proteomics, transcriptomics and metabolomics approaches, the consistency and predictability in plant breeding and transgenic technology has been improved. It has helped to produce high quality and stress resilient crops with enhanced nutritional value in less time and lower input usage. Omics has provided insights into the molecular mechanisms involved in insect resistance and tolerance to herbicides, cold, salinity and drought stresses [38]. To interpret the omics (genomics, transcriptomics, proteomics and metabolomics) approaches in sugarcane for higher yield, higher sucrose contents, biotic and abiotic stress tolerance, one should have knowledge about the genome structure, physiology and functional veracity of sugarcane with other related crops.

3.1. Genomics

Sugarcane has a large genome size of 7440 Mb (mega base pairs) having 2n = 100–300. The genome is supposed to be evolved as a result of a complex hybridization event. It is considered that *Saccharum officinarum* is octoploid. The monoploid genome size of *Saccharum officinarum* is 930 Mb and that of *Saccharum spontaneum* is 750 Mb, twice of the size of rice genome (~390 Mb). Geneticists are trying to interpret the associations of complex sugarcane genome with other similar crop plants. The level of genome varies from diploid to decaploid among the *Poaceae* species [39]. The conservation and origin of gene function is suggested by gene order which is maintained by synteny of the genome [40]. The TE (transposable elements) intervening

between coding genes strongly support the extension of genome in grasses. Transposons and retrotransposons are two categories of transposable elements. In plants, the most abundant retroelements are LTR (long terminal repeats) retrotransposons. Transposase proteins are involved in insertion-deletion mechanisms. Active sites of the transcription control the movement of retrotransposons, which reinsert them into the genome after each propagation cycle to increase copy number. Recent studies show that there exists a gene remodeling mechanism which results in the generation of new genes. As a result of gene remodeling, gene expression is altered by new regulatory networks [41]. The study of transposable elements in wheat and barley [42] provided close relationship of transposable elements with genome structure. The transposable elements in sugarcane can be activated and evaluated by functional transcriptomic approaches. The major limitation in sugarcane genetic improvement is its genome size. To sequence the genome of an organism Bacterial Artificial Chromosomes (BAC) are used. BAC (Bacterial Artificial Chromosomes) library was constructed with Hin*d*III partial digestion for sugarcane cultivar 'R570' having more than 100,000 clones with 130 mega base pairs (Mb) insert size [43]. For map-based cloning of sugarcane, BAC resources will be highly esteemed and physical map of sorghum (http://www.genome.clemson.edu/tools/contig_viewer/index.html) will be used as complementary tool.

3.2. Transcriptomics

Transcriptomic approaches have emerged as an effective tool for functional characterization of unknown genes. In combination with proteomics and metabolomics, these approaches are very useful for the development of improved sugarcane clones. It reduces the complexity of data and targets. Only active genes in the cell or tissues are considered at the time of sampling. By employing transcriptomic approaches, one can easily compare similar type of tissues at different developmental stages in different organisms growing in different conditions [44].

3.3. ESTs

Due to large size and complexity of genome, the whole genome sequence of sugarcane was not available. The genome size of its modern cultivar is considered to be more than 10 GB. From 12th July, 2017, NCBI database has 83,138 GSSs (genome survey sequences), 285,216 ESTs (expressed sequence tags) and 13,382 nucleotide sequences including 491 sequences of mRNA under the search of "*Saccharum*". There are three main groups of ESTs including a large group resulting from a modern variety of sugarcane and two small groups from *Saccharum officinarum* and *Saccharum arundinaceum* (**Table 1**). Majority of ESTs belong to six cultivars from different countries including Australia (Q117), USA (CP72-2086), India (CoS 767, Co 1148) and Brazil (SP80-3280, SP70-1143). Most of the ESTs are from mixed tissue samples of Brazilian varieties i.e. P57150-4 x PB5211 or SP83-5077, RB80-5028; SP80-87432, RB855205, CB47-89, RB845298, SP803280 x SP81-5441, SP80-185, SP80-3280 and SP87-396 [45].

Many projects have been executed for sequencing sugarcane ESTs (expressed sequence tags) in Brazil (http://sucest.lad.ic.unicamp.br/en), South Africa and Australia [46]. Until now more than 0.3 million (300,000) ESTs have been generated. A database holding 0.238 million (238,000) ESTs (constructed from diverse organs and tissues) from 37 libraries was erected by

Saccharum species and hybrids	No. of ESTs	Nucleotide sequences
Saccharum officinarum	20,701	7066
Saccharum arundinaceum	341	234
Saccharum hybrid cultivar	284,482	2267
Mixed cultivar of *Saccharum* hybrid	73,778	10
SP80-3280 cultivar of *Saccharum* hybrid	135,534	54
CoS 767 cultivar of *Saccharum* hybrid	25,382	–
Q117 cultivar of *Saccharum* hybrid	9141	54
SP70-1143 cultivar of *Saccharum* hybrid	24,313	8
Co 1148 cultivar of *Saccharum* hybrid	1069	2
CP72-2086 cultivar of *Saccharum* hybrid	7993	4
Co 740 cultivar of *Saccharum* hybrid	310	25
CoC 671 cultivar of *Saccharum* hybrid	315	67
NCo376 cultivar of *Saccharum* hybrid	535	11
H50-7209 cultivar of *Saccharum* hybrid	27	3
F134 cultivar of *Saccharum* hybrid	4	–
Co 62175 cultivar of *Saccharum* hybrid	206	1
Co 86032 cultivar of *Saccharum* hybrid	30	101
Unknown cultivar of *Saccharum* hybrid	3904	339
Total	285,216	13,382

Table 1. ESTs (Expressed sequence tags) and number of nucleotide sequences corresponding to *Saccharum* species and hybrids submitted in db_EST and db_Nucleotide, respectively (NCBI: 12th July, 2017).

SUCEST (the Brazilian ONSA consortium's sugarcane EST project). More than 43 thousand clusters (that may signify distinctive transcripts) were assembled by cluster analysis of the SUCEST. A BLAST search showed that almost 50% of these expressed sequence tag clones had no resemblance with known proteins. In Genbank, almost 40% of the clones represent full length protein sequences. The genes involved in diverse metabolic processes have successfully been recognized by analysis of SUCEST database. These analyses reveal that assemblage of ESTs is highly illustrative and indicate tagging of thousands of sugarcane genes [47]. ESTs represent gene encoding sequences, natural antisense transcripts, transacting siRNA precursors, miRNA and most commonly noncoding RNA. The information provided by EST dataset is an important starting point to know about the genome of an organism. It can also help to determine genes of agronomic importance (tolerance of biotic and abiotic stresses, sugar content and mineral nutrition). EST availability makes possible the analyses of gene expression on a large scale. Numerous studies have been conducted for *in-silico* analysis of transcript enrichment using different cDNA libraries [48].

http://dx.doi.org/10.5772/intechopen.71496

3.4. Proteomics

Proteomics is the large-scale study of proteome (whole protein contents) and diverse properties of proteins. Through proteomics approaches, we can determine the structural and functional details of biological systems under different conditions. Proteomics has been a major field of functional genomics after the completion of many genome sequencing projects. It has also helped to understand the mode of actions, resistance mechanisms and bio-degradation of pesticides. However, in sugarcane, proteome study is a little bit complicated as no standard protein extraction protocol is available [49]. As compared to other monocots, sugarcane proteomics have not gained momentum yet. Finding protein extraction techniques is a stepping stone in shaping up sugarcane proteomics. Earlier, isoenzyme pattern was used as a tool in sugarcane varietal identification and taxonomy. Isoenzyme pattern was analyzed on 1D gradient polyacrylamide gels based on molecular weight differences [50]. *Saccharum* species (*S. sinense*, *S. edule*, *S. robustum*, *S. spontaneum* and *S. officinarum*) were discriminated from other related genus *Eriochrysis*, *Imperata*, *Narenga*, *Eriochrysis*, *Miscanthus* and *Erianthus* by isozyme pattern of acid phosphatases, leucine aminopeptidases and esterases. O'Farrell [51] introduced 2DE which increased interest in sugarcane proteomics. 2DE technique was then used to study sugarcane roots [52], stalks, leaves [53], meristematic cells and suspension cells [54]. Changes in 2DE protein pattern was observed under different stress conditions. Sample preparation is a crucial step and is necessary for reproducible results. Sugarcane tissues are rigid, fibrous in nature, have sucrose, phenolic compounds and other metabolites in its stalk. Protein extraction protocols have been optimized for the extraction of protein from leaves, meristem and cell suspension cultures but extraction of protein from stalk is still a challenge [55]. Since sugarcane stalk is the core site for sucrose metabolism and host-pathogen interaction but no reproducible protocol is available for the isolation of proteins from stalk tissues.

3.5. Metabolomics

Metabolomics is the study of metabolites within the cells, tissues or organism. Proteomics is the study of gene product produced whereas metabolomics explores whether gene products are metabolically active within an organism or not. It also includes role of metabolites in various cellular processes. Hence, metabolites are direct indicators of the performance of a plant under particular biotic or abiotic stresses [56]. Nutritional quality and plant health can be improved by monitoring the changes in metabolite profiling. So, the retrieved informations can effectively be used to develop improved crop varieties as well. Variations in metabolite pattern can also assist to distinguish the mode of action of pesticide which provides critical information for the discovery of new pesticides. Metabolomics may be employed to determine differences and similarities between parents and offsprings on the basis of metabolite composition. Mass spectrometry (MS) and NMR (nuclear magnetic resonance) techniques are used for metabolic profiling, to monitor the metabolic regulations and to analyze the impact of herbicides, pesticides, high temperature, intense light, humidity, soil type, salinity, fertilizers and pests on metabolite composition. One of the fundamental reasons for unavailability of data on sugarcane metabolites is the complexity of sugarcane genome and metabolomes. Most of the research has been focused on differential gene expression. Second constraint is the limited availability of technology due to its sensitivity and labor intensity. Recently,

metabolome (whole metabolites in a specific tissue) has been used as a tool for understanding metabolic regulations. This work was accomplished by some advanced technologies where multiple metabolites were determined in a particular tissue within an hour simultaneously. GCMS (gas chromatography-mass spectrometry) is a vastly used technique that separates the metabolites of different types and identify them on the basis of mass spectral matching and retention time. Identification and extraction methods were optimized for thirty sugarcane metabolites. Hence, metabolome studies are of pivotal importance to understand interaction between the genes and their resultant proteins which can be used to understand mechanisms of sucrose accumulation in sugarcane [57].

4. Transgenic approaches for crop improvement

Transgenic technology is the only technology through which alien genes may be introduced across the species. Sugarcane has well be explored to engineer for certain valuable agronomic traits and for enhanced sucrose contents. Most of the transformation events reported in sugarcane are through biolistic, anyhow *Agrobacterium* and electroporation have also been used. Success of engineered lines depend upon the integration and stable expression of introduced gene/s. Recalcitrancy, low transformation efficiency, transgene inactivation and difficult backcrossing are major bottlenecks in sugarcane transformation. Though limited reports are available for the field plantation of transgenic sugarcane yet a large number of research groups are involved in engineering sugarcane genome [58].

4.1. Developing genetically modified varieties with improved biotic stress tolerance

4.1.1. Herbicide resistance

Herbicide resistance is one of the major traits in transgenic plants. It is so desirable that more than 70% of the transgenic crops growing worldwide are herbicide resistant. Various herbicide resistance genes i.e. *EPSPS*, *bar*, *aroA and BXN* have been have been transformed to crop plants for developing herbicide tolerant crops. Crops resistant to glyphosate and glufosinate have been cultivated since 1990s. Plants having ability to tolerate high dose of glyphosate have been developed through biolistic transformation whereas *Agrobacterium* and other methods of transformation (electroporation) have also been tested [59]. Gallo-Meagher and Irvine [60] reported *bar* gene transformation in sugarcane by biolistic gun. Resultant transformants showed tolerance against basta which authenticated effectiveness of *bar* gene not only for herbicide tolerance trait but also as a selectable marker gene, for the selection of putative transformants. Enriquez-Obregon et al. [61] reported transformation of *uid1* and *bar* genes in sugarcane by *Agrobacterium* mediated transformation. They obtained GUS-positive and BASTA resistant calli. Similarly, BASTA resistant variety (Ja 60-5) of sugarcane has also been developed through *Agrobacterium* mediated transformation. Transformation frequencies reached up to 10–35% by employing different transformation protocols. The *PAT* (phosphinothricin acetyltransferase) and *neo* (neomycin phosphotransferase) genes were reported to be transformed in SP80-180 genotype of sugarcane by biolistic

http://dx.doi.org/10.5772/intechopen.71496

method [62]. The selected transformants were resistant against commercial formulation of ammonium glufosinate. Southern blot analysis was used to confirm the stable integration of *neo* and *PAT* genes. While western blot analysis and RT-qPCR were used to analyze the expression of these genes. Another report was given by Manickavasagam et al. [5]. They developed herbicide tolerant sugarcane plants by *Agrobacterium* mediated transformation. This was first report of *Agrobacterium* mediated transformation in which axillary buds from 6 months old plants were used as explant. *Agrobacterium* with binary vector pGA492 having *β-glucuronidase*, *neomycin phosphotransferase II* and *bar* genes in between the T-DNA regions was used for transformation. This study proved that phosphinothricin (5.0 mg/L) is more effective selective agent as compared with kanamycin and geneticin. Southern blot analysis was used to confirm the transformants. Leibbrandt and Snyman [7] reported the transformation of *pat* gene in NCo 310 genotype of sugarcane which confers resistance to the herbicide Basta. Stable transgene expression was evaluated in glasshouse and field conditions.

4.1.2. Insect resistance

Insect pests are one of the major yield limiting agents which cause serious losses to crop yield. Economically important insect pests of sugarcane can be categorized into borers, sap sucking pests, white grubs and termites. Sugarcane pests show extensive variation in species composition in different tropic and subtropic agro climatic regions. All around the world, sugarcane is facing problems of insect pests and diseases which are seriously affecting sugar production. No exact estimates are available for these cumulative losses caused by the insect pests and diseases. Anyhow, economic losses caused by certain pests has been estimated. Annual loss of $10–$20 million were estimated to sugar industry at Lower Rio Grande Valley of Texas only by *E. loftini*. Similarly, wooly aphid (*Ceratovacuna lanigera*) has been estimated to cause 18.3% yield losses during sixth months [63]. Most of the sugarcane cultivars growing in the field are outcomes of hybridization and selection. Advancements in molecular biology and genetic transformation have helped researchers to develop transgenic sugarcane plants with desired agronomic traits particularly for insect pest resistance. Different types of molecules have been manipulated to produce insect resistant plants such as lectins, proteinase inhibitors, ribosome inactivating proteins, secondary metabolites, delta endotoxins and insecticidal proteins.

Considerable advancements have been made to develop transgenic sugarcane having resistance against lepidopteran borers such as *E. loftini*, *D. saccharalis*, *S. excerptalis* and *C. infuscatellus* by introducing various cry genes. *Bacillus thuringiensis* derived cry genes encoding toxins have been expressed in sugarcane to engineer resistance against insect pests. First transgenic sugarcane was developed by Arencibia [6] against *D. saccharalis*. Five transformation events were selected exhibiting considerable resistance against borer in spite of very low expression (0.59–1.35 ng/mg of soluble leaf protein) of transgene. Truncated *cry1A(b)* gene was expressed in sugarcane under *CaMV 35S* promoter. Lower level of expression was observed in transgenic plants perhaps because of lower activity of the aforementioned promoter in monocots. Low to medium level internode invasions were also observed in the transgenic lines. Transgenic lines were developed with modified GC contents (37.4–47.5%) of *cry1Ac* gene and effect of change in GC contents, was

observed on the expression of transgene [69]. Transgene expression was determined as 1–10 and 0.2–6.0 ng/mg of total soluble proteins in the leaves and stem respectively, which was seven times higher than reported by Arencibia et al. [64]. Plants also showed better resistance to sugarcane stem borer, when tested by *in vivo* and *in vitro* insect bioassay. Expression of the transgene was further increased with increase in GC content of *cry1Ac* gene and was determined as 2.2–50 ng/mg of total soluble proteins when GC content was increased to 54.8%. Hence expression of *cry1Ac* gene in sugarcane increased with increase in its GC content [65].

Proteinase inhibitors (PIs) derived from both the animals and plants had been introduced in sugarcane to confer resistance against borers. A soybean PI was mixed in the artificial diet of insects and it appeared to have detrimental effects on growth of *D. saccharalis*. Falco and Silva-Filho [66] developed transgenic sugarcane expressing Soybean Kunitz Trypsin Inhibitor (*SKTI*) and Soybean Bowman-Birk Inhibitor (*SBBI*) driven by maize ubi-1 promoter. Transgenic lines were evaluated against *D. saccharalis* by feeding excised leaf tissue and by infecting plants with neonate larvae in the green house. Transgenic lines with *SBBI* did not show significant change in the larvae mortality whereas it was slightly higher when fed on the leaves of *SKTI* expressing plants. Leaves of transgenic plants with *SKTI* or *SBBI* inhibited insect growth and metabolism, hence resulted in reduced insect weight. Dead hearts were also observed in almost all of the plants tested in green house. Without knowing the level of expression, it is very difficult to establish relationship between inhibitor content and dead heart to determine their role in borer resistance. Aportinin appeared to be more effective in inhibting gut proteinases of *S. excerptalis* as compared with those of *C. infuscatellus* and *C. sacchariphagus* indicus. In succeeding studies, aprotinin gene under maize ubi-1 promoter was introduced in sugarcane and transgene expression was determined as 0.16–0.50% of the total soluble leaf proteins. *In vivo* screening assays also revealed very low mortality of *S. excerptalis* larvae but weight of insects was reduced by 99.8% hence insect growth was affected to great extent.

Besides proteinase inhibitors and cry toxins, certain other molecules have also been explored for the effective control of insect pests in sugarcane. Plant derived insecticidal proteins (lectins) are more valuable for the control of insects as compared with bacterial insecticidal proteins. Wheat germ lectin, snowdrop lectin (*Galanthus nivalis* agglutinin, *GNA*) and avidin were used as dietary proteins in bioassays against larvae of sugarcane white grubs. Wheat germ lectins and snowdrop appeared insecticidal and growth inhibitor for *Antitrogus parvulus* larvae. Avidin also appeared to inhibit growth of *A. consanguineus* [67]. Such positive results encouraged researchers to exploit potential of these genes for the control of white grubs. Sétamou et al. [68] included extracts of snowdrop lectin expressing transgenic lines (0.89% in leaf sheath) in the artificial diet (0.47% lectin of total extractable proteins) and examined responses of *D. saccharalis* and *E. loftini* larvae. When artificial diet containing 0.50% of transgenic *GNA* was fed to the larvae of *E. loftini*, a considerable decrease in larvae survival, pupation percentage, adult emergence, pupal weight, longevity and fertility was observed. Transgenic sugarcane expressing lectin under phloem specific RSs-1 (rice sucrose synthase-1) or maize ubi-1 promoters were developed via *Agrobacterium* mediated transformation. Reduced survival rate, development, fertility and feeding was observed in the larvae of *C. lanigera* feeding

on transgenic plants. Aphid population density was decreased up to 60–80%, and even up to 95% in some lines [69]. Maximum resistance was observed when phloem specific promoter (RSs-1) was used in transformation against sucking pests like wooly aphids. Field performance of transgenic lines is dependent on the expression level and stability of transgene. The most critical factors in this regard are: promoter strength, codon usage, gene silencing and site of integration. Rice polyubiquitin (*RUBIQ2*) promoter has proved as the strongest promoter for transgene expression in sugarcane [70]. Anyhow, maize ubi-1 promoter has also extensively been used in sugarcane for the optimal expression of transgenes. Though maize ubi-1 is a constitutive promoter but it does not give same level of expression in all plant parts, e.g. leaves, roots and stem. Keeping in view the tissue specificity and feeding habits of insect pests, it is necessary to use tissue specific or wound inducible promoters which will overexpress insecticidal proteins only in the target tissues and will prove more effective for the control of insect pests.

4.2. Developing genetically modified varieties with improved abiotic stress tolerance

Abiotic stresses may alter physiological status of a plant either directly or indirectly by disturbing its metabolism, growth and development. Among abiotic stresses salinity, drought and low temperature are the fundamental factors that significantly influence plant performance. To combat these stresses plant triggers a cascade of physiological and biochemical reactions. Commonly sugars and other osmolytes accumulate in response to abiotic stresses (drought, salinity and low temperature). Water is an essential component for life but its scarcity is increasing day by day throughout the world. Currently almost 65% of fresh water is being used for irrigation, indicating that survival would not be possible without developing drought tolerant varieties in the near future. Sugarcane is a high delta crop and requires heavy irrigation but is now expanding in the regions where water availability is limited, so only drought tolerant varieties can be grown with success [71].

Molecular studies have explored that any of the plant growing in stress conditions strives to withstand those conditions by activating certain stress responsive genes/proteins. They include antioxidant enzymes, late embryogenesis abundant proteins, Arabidopsis *HARDY* genes [72], various transcription factors and certain protease inhibitors. Transcriptomic analyses of sugarcane under drought stress has lead to the identification of stress related genes. These genes should be figured out that how critical they are for stress tolerance. Molecular Systems Biology can be used to characterize regulatory networks using model plant. A novel sugarcane gene *Scdr1* (sugarcane drought responsive 1) was overexpressed in tobacco. Its overexpression resulted in increased tolerance against salinity, drought and oxidative stress as was evident by increased photosynthesis, water content, germination rate, biomass, chlorophyll content and reduced accumulation of ROS. Physiological parameters were also less affected as compared to wild type plants. The relationship between anatomical structure and drought tolerance have also been investigated. In the roots of sugarcane, number of vessels per unit area, more veins, widened vesicles in bulliform cells, thick cuticle and less stomata per unit area have close association with drought tolerance [73]. Drought tolerant varieties have better growth of mechanical tissues around the vascular bundle and their thick-walled cells have higher degree of lignification. Various genes that encode transcription factors *bZIP*, *DREB*, *MYB* [74] and

RD26 have also been evaluated to enhance stress tolerance in plants. Transcription factors of *MYB* superfamily which are in abundant, also play crucial role in growth and development under stress conditions [75]. Expression profiling of sugarcane was performed under stress conditions to identify abiotic-stress-inducible genes. Wild type Q117 sugarcane plants were exposed to salinity, drought and cold stresses. Variations in the expression level of four genes encoding for galactinol synthase (*GolS*), late embryogenesis abundant protein 3 (*LEA3*), early response to dehydration protein 4 (*ERD4*) and pyrroline-5-carboxylase synthetase (*P5CS*) were evaluated by real-time PCR. *P5CS* and *GolS* were strongly induced under salt stress whereas, *LEA3* and *ERD4* were induced under cold and drought stress respectively. Overexpression of *CBF4* (C-repeat binding factor 4) gene from *Arabidopsis thaliana* in Q117 led to a considerable increase in the expression of *P5CS* and *ERD4* as compared with wild-type plants under normal conditions. Anyhow expression of *LEA3* and *GolS* did not appear to be affected in transgenic plants. These results suggested presence of active abiotic stress-inducible genes in sugarcane and that expression of *Arabidopsis CBF4* gene in sugarcane can activate stress tolerance genes under normal conditions [76].

Plants use complex mechanisms to adapt ionic/osmotic stresses and accumulate compatible solutes to cope with these stress conditions. Salt stress has drastic effects on photosynthetic activity which affects crop production, product quality and of course sucrose accumulation in cane stalk. These stresses disturb homeostasis at cellular level and even at plant level. It is very critical for the plant to maintain low level of toxic ions in the cell but under salt stress, Na^+ and Cl^- ions accumulate in the cytoplasm due to their inability to pump them out. The level of Na^+ ion is regulated by specific transporters i.e. plasma membrane Na^+/H^+ antiporter *SOS1*. When *AVP1* (Arabidopsis Vacuolar Pyrophosphatase) gene was overexpressed in sugarcane by Kumar et al. [77], increased tolerance against drought and salinity was observed in the transgenic plants. Profused and longer roots were observed in transgenic plants as compared to control. Concurrently, survival of transgenic plants under drought and salt stress indicated their increased level of tolerance against drought and salinity. Constitutive expression of *AVP1* gene improved plant growth under different abiotic stresses. Many explanations have been anticipated including better vacuolar ion sequestration, enhanced auxin transport, increased heterotrophic growth, and more sucrose transport from source to sink tissues. Mutant plants which lack functional *AVP1* gene and transgenic plants for *AVP1* were used to evaluate its role. It becomes clear that AVP1 is a protein with multiple functions. Systems Biology can be of great help for the complete understanding of these complex biological networks [78].

4.3. Developing genetically modified varieties with improved sugar recovery

Sugarcane has capacity to store more than 25% sucrose of its fresh weight, so a great potential is there to increase sugar recovery. Advancements in biotechnological tools has helped to understand metabolic pathways involved in sucrose accumulation in sugarcane. Enzymes and control points involved in sucrose metabolic pathway, photosynthetic efficiency, degree of phloem loading/unloading, rate of sucrose assimilation and carbon partitioning within the stem and vacuoles are the key targets which needs to be explored for increasing sucrose accumulation. Expression analysis of genes in relation to sucrose accumulation can be of great help to understand role of various genes involved in sucrose metabolism. Until now

http://dx.doi.org/10.5772/intechopen.71496

many studies have been reported on the genes involved in sucrose metabolism directly or indirectly. SPS (sucrose phosphate synthase), SPP (sucrose phosphate phosphatase), SuSy (sucrose synthase), HK (hexokinase), FK (fructokinase) VAI (vacuolar acid invertase), NAI (neutral acid invertase), CWI (cell wall invertase), SAI (soluble acid invertase), PFK (ATP dependent phosphofructokinase), PFP (pyrophosphate dependent phosphofructokinase), UDPase (UDP glucose pyrophosphorylase), ADP-G-PP- (ADP-Glucose pyrophosphorylase) and sucrose transporters (SUT1 and SUT4) are the key enzymes involved in sucrose metabolic pathway [79]. Engineering these enzymes through genetic transformation may lead to increased sucrose accumulation and of course sugar recovery (**Figure 2**).

Fructose 6-phosphate 1-phosphotransferase catalyzes the principal reaction of glycolysis i.e. the reversible conversion of pyrophosphate (PPi) and fructose 6-phosphate (Fru 6-P) into inorganic phosphate (Pi) and fructose 1,6-bisphosphate (Fru 1,6-P2). Pyrophosphate

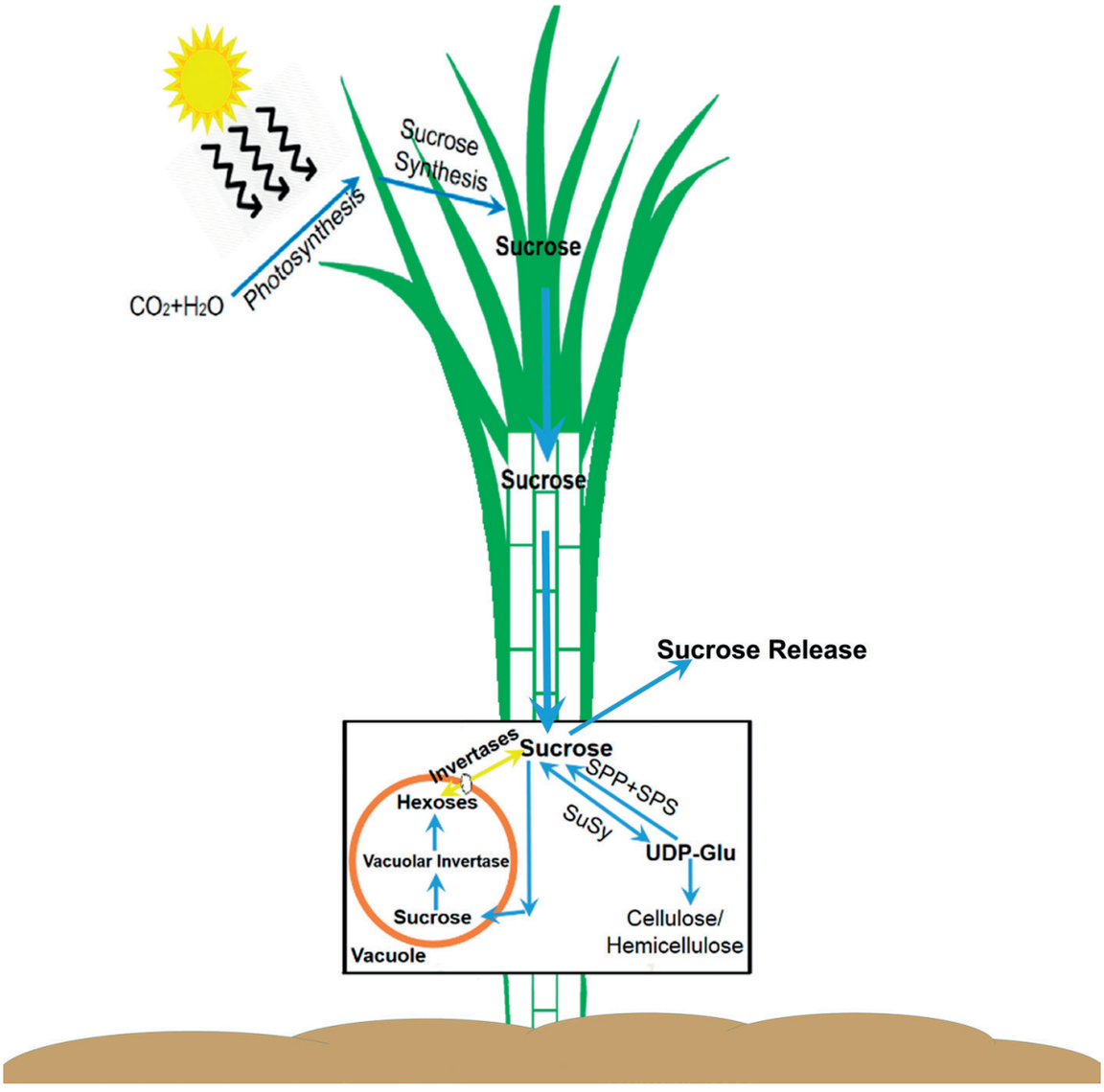

Figure 2. Schematic sketch showing the most critical enzymes involved in sucrose accumulation in sugarcane culmn.

dependent phosphofructokinase (PFP) is also partially responsible for being cycled between the hexose phosphate and triose phosphate pools. This cycling was downregulated by constitutive expression of untranslatable and antisense forms of *PFP-b* gene. Approximately 70% activity was decreased in young internodal tissues and no activity was observed in mature tissues. Hendrik and Botha [80] reported decrease in sugar yield as the result of decrease in the PFP activity. A significant increase in sucrose content (in more than 50% of the lines) was observed in the immature internodes, but even 30% downregulation of Pyrophosphate dependent phosphofructokinase (PFP) activity did not affect the mature internodes as compared to wild type. Mature internodes of most of the transgenic lines showed higher sucrose accumulation but was not significant. Hence Pyrophosphate dependent phosphofructokinase (PFP) activity in internodal tissues of sugarcane has a positive relation with respiration and is inversely related with sucrose content. In transgenic plants, no significant difference was observed in development and growth of plants both under greenhouse and field conditions. So PFP (pyrophosphate dependent phosphofructokinase) influences the sucrose accumulation ability of biosynthetically active and young culm of sugarcane. Equilibrium of glycolytic intermediates (stored sucrose) is restored when ATP dependent phosphofructokinase and the PFP activity is sufficient.

Sugarcane culm is an important experimental system to elucidate biochemical and molecular mechanisms involved in sucrose accumulation or carbon partitioning for the application of gene expression studies in this context [81]. Vacuolar targeted expression of sucrose isomerase gene doubled sucrose accumulation in the culm of greenhouse growing plants. Engineered sugarcane plants not only depicted enhanced sucrose transport but also photosynthesis and sink strengths were improved. These results highlighted importance of sugarcane as an energy crop as more carbon source would result in more biofuel production. Higher level of sucrose and accumulation of isomaltulose (a high value sugar) has also been reported in sugarcane [82]. An experimental study was conducted to explore biosynthesis of isomaltulose (IM) through engineering metabolic pathways. Sucrose (α-D- glucopyranosyl 1,2-D- fructofuranose) is converted into isomaltulose (α-D-glucopyranosyl-1,6-D-fructofuranose) by some bacteria. This sucrose is resistant to several microorganisms as is not metabolized by invertases. Easy digestion (likewise glucose and fructose) by humans is another significant advantage of this sweetener. Instead of salivary invertases, intestinal disaccharidase is involved in the digestion of isomaltulose, so its digestion is relatively slow. Anyhow, it is beneficial because it does not affect insulin and blood glucose levels. Owing to be acariogenic, non-hygroscopic, stable and slowly digestible sweetener, it has mounting market. Biosynthesis of isomaltulose (IM) involves a sucrose isomerase (SI) that does not require cofactor and substrate activation [83]. More isomaltulose (IM) is produced in sugarcane culmn when highly efficient sucrose isomerase (SI) is targeted to vacuole. Further, IM (isomaltulose) could be accumulated without any prominent decrease in sucrose content. Sucrose contents appeared to be doubled in selected transgenic lines but further studies would be required for commercial scale application of this trait i.e. patterns of developmental expression, compartmentation and enzyme stability resulting in high isomaltulose (IM) content. Hence, sucrose isomers can be produced in sugarcane by transgenic technology. Isomaltulose was produced either by expressing sucrose isomerase in the apoplast or in the vacuole. Apoplast-targeted expression did not show any significant

http://dx.doi.org/10.5772/intechopen.71496

increase in isomaltulose (IM) whereas, vacuole-targeted expression of transgene resulted in significant increase in the isomaltulose (IM) accumulation under greenhouse conditions [91]. Hamerli and Birch [84] reported the first field trial of transgenic sugarcane producing trehalulose (TH). Synthetic sweeteners, an alternative to sucrose are produced through fermentation or chemical reactions which are very expensive. Production of sweeteners in sugarcane through targeted expression of transgene in the mature stem can be an economical alternative. For targeted delivery of proteins into the plastids and vacuoles, transit peptides have already been established in transformants. Zhang et al. [85] worked out not only to develop abiotic stress tolerant sugarcane but also on engineering metabolic pathways for improved trehalose (a valuable sugar moiety) content. Directing sucrose accumulation to vacuole in spite of cytosol may prove an effective strategy for enhanced sucrose accumulation because vacuole occupies large space in the cell. Hence, biotechnological interventions can do a lot to improve sugar recovery in this sweet grass.

5. Potential of biotechnology to promote sugarcane as a future energy crop

Plant biomass from grasses including sugarcane, can be used as a renewable source of energy by converting their cellulose, hemicellulose and lignin into bioethanol. Plant derived biofuels reduce dependence on fossil fuels and is of great importance in the countries where oil reserves are limited. Engineering plastid genome of sugarcane may prove a great milestone in this regard [86]. Biofuels produced from plant lignocellulosic biomass (second generation biomass) have advantage over first generation biomass in term of CO2 balance and net energy. Another advantage is that they have no competition for supplies with food industries. As a result, production of bioethanol from 2nd generation biomass is more economical. Sugarcane is one of the most economical source of bioethanol all over the world. Brazil is the leading country in this regard and 50% of the country energy needs are fulfilled by sugarcane ethanol [87]. National fuel alcohol program (ProAlcooL) was launched by Brazil. Major aim of this program was to replace usage of gasoline with bioethanol. In Brazil, 6.19 billion gallons (23.4 billion liters) of ethanol was produced from sugarcane whereas 15% of the total electricity was generated from sugarcane bagasse during 2014 [88]. The genetic foundation of current sugarcane breeding program started with interspecific hybrid varieties originated from early breeding activities in West Indies, India (e.g. Co 206, Co 207) and Indonesia (e.g. POJ 2878, POJ 2364). Sugar yield was increased up to 1–2% per annum by sugarcane programs and most of this increase is attributed to genetics. Conventional breeding in sugarcane has certain limits, as a result desired results could not be achieved. *Saccharum* spp. is genetically complex having 2n = 100–130 with intricate genomic makeup evolved through highly successful interspecific hybridization between *Saccharum spontaneum* and *Saccharum officinarum*, which have well been explored to develop commercial varieties. Ming et al. [89] summarized usage of conventional and molecular approaches for the genetic improvement of sugarcane making it world's most efficient crop in terms of conversion of solar energy into chemical energy.

In sugarcane, about two-third of the photosynthetically fixed carbon is stored in the form of cellulose and hemicellulose. Sugarcane mills produce millions of tons of bagasse annually

in addition to the leaves which are left behind in the field. Sugarcane bagasse is an excellent 2nd generation source for production of ethanol and bioelectricity [90]. High cost of enzymes limit the conversion of hemicellulose and cellulose into cellulosic ethanol. Production cost of enzymes can be decreased by the overexpression of cellulolytic enzymes in GM (genetically modified) plants to meet the demand of sugarcane mills. Adoption of new technologies may help to overcome issues relevant to the stability, storage and overproduction of enzymes in plants. Sainz [91] reported that highly thermostable and hydrolytically efficient enzymes were produced by genetic engineering. Transgenic sugarcane plants overexpressing bacterial endoglucanase (EG) and fungal cellobiohydrolases (CBH I and CBH II) were developed. Targeting EG to chloroplasts and cellobiohydrolases to vacuoles resulted in elevated enzymatic activity in the mature plant leaves. This increased enzyme activity demonstrated that cellulose hydrolytic enzymes can be produced in sugarcane plants [92] and will boost up energy production from cane and its by-products including bagasse. In addition to the traditional agricultural products (food, feed and fiber), plants are emerging as a valuable source of energy, fuel, biomaterials and chemical precursors for the industry. Advancements in research are of pivotal importance to meet the increasing demand of quality raw materials [93]. Genetic engineering techniques are playing important role to achieve this goal and are envisioned to play leading role in the production and processing technology. For instance, input cost can be reduced by producing raw material in plants as plants have proved an effective platform for the production of industrially important compounds. GM microbes are commonly used at industrial scale for rapid conversion of raw materials into desired product. Conversely, a few GM crops have gained commercial status in spite of wide spread eagerness and renowned potential of genetic engineering for crop improvement [94]. Biotechnology occupies a central role in US Department of Energy (DOE) to develop crops with modified cell wall composition. The DOE has received encouraging appreciation because of their research on bioenergy crops and production of valuable processing enzymes by engineering metabolic and biochemical pathways [95]. To successfully attain national goal of bioeconomy, genetic engineering is appearing as a major contributor. A wide range of plants like corn, poplar, switchgrass, canola, sorghum, soybean (*Saccharum* L.) had been used to produce bioenergy but sugarcane is far better choice as is a perennial crop which does not require reseeding after each growing cycle. Hence, sugarcane is the most valuable crop for the production of bioethanol which can further be improved by employing biotechnological innovations.

6. Conclusions

Conventional research has contributed to great extent and has delivered its maximum. So, the only hope to get improved agricultural crops is the implication of advanced research. Adoption of biotechnological interventions have proved their worth and more than 18 million farmers in 26 countries are growing GM crops on an area of 185.1 million hectares (457.4 million acres) which is increasing each year. More than 90% of these crops are either insect resistant or herbicide tolerant resulting a massive decrease in the usage of chemical pesticides by 37%, increased crop yields by 22%, and increased farmer's profits by 68%. Though, biotechnological interventions have produced agronomically improved genotypes yet scientists

are currently working to engineer sugarcane crop as a platform for large scale production of chemicals with industrial as well as therapeutic significance. Hence, Biotechnological interventions hold great promises to develop a better sugarcane crop with improved agronomic traits, sugar contents and biofuel production.

Author details

Ghulam Mustafa*, Faiz Ahmad Joyia, Sultana Anwar, Aqsa Parvaiz and Muhammad Sarwar Khan

*Address all correspondence to: drmustafa8@gmail.com

Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad, Pakistan

References

[1] Wheals AE, Basso LC, Alves DM, Amorim HV. Fuel ethanol after 25 years. Trends in Biotechnology. 1999;**17**:482-487

[2] Sundar AR, Ashwin NMR, Barnabas EL, Malathi P, Viswananthan R. Disease resistance in sugarcane – An overview. Scientia Agraria Paranaensis. 2015;**14**:200-212

[3] Khan MS, Ali S, Iqbal J. Developmental and photosynthetic regulation of *Bacillus thuringiensis* δ-endotoxin reveals that engineered sugarcane conferring resistance to 'dead heart' contains no toxins in cane juice. Molecular Biology Reports. 2011;**38**:2359-2369

[4] Mustafa G. Development of plastid transformation in sugarcane [PhD Diss.]. Pakistan: Quaid-I-Azam Univ.; 2011

[5] Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S. *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Reports. 2004;**23**:134-143

[6] Arencibia AD, Carmona ER, Cornide MT, Castiglione S, O'Relly J, Chinea A, Oramas P, Sala F. Somaclonal variation in insect-resistant transgenic sugarcane (*Saccharum* hybrid) plants produced by cell electroporation. Transgenic Research. 1999;**8**:349-360

[7] Leibbrandt NB, Snyman SJ. Stability of gene expression and agronomic performance of a transgenic herbicide-resistant sugarcane line in South Africa. Crop Science. 2003; **43**:671-677

[8] Gilbert RA, Gallo-Meagher M, Comstock JC, Miler JD, Jain M, Abouzid A. Agronomic evaluation of sugarcane lines transformed for resistance to sugarcane mosaic virus strain E. Crop Science. 2005;**45**:2060-2067

[9] Vickers JE, Grof CPL, Bonnett GD, Jackson PA, Morgan TE. Effects of tissue culture, biolistic transformation, and introduction of PPO and SPS gene constructs a performance of sugarcane clones in the field. Australian Journal of Agricultural Research. 2005;**56**:57-68

[10] Butterfield MK, Barnes JM, Heinze BS, Rutherford RS, Huckett BI. RFLP markers for resistance to eldana and smut from an unstructured sugarcane population. In: Proceeding International Society of Sugarcane Technology Molecular Biology. Workshop, Montpellier, France. Vol. 4; 2003. p. 14

[11] Nickell LG. Tissue and cell culture of sugarcane: Another research tool. Hawaii Plant Records. 1964;**57**:223-229

[12] Heinz DJ, Mee G. Plant differentiation from callus tissue of *Saccharum* species. Crop Science. 1969;**9**:346-348

[13] Komamine A, Kawahara R, Matsumoto M, Sunabori S, Toya T, Fujiwara A, Tsukahara M, Smith J, Ito M, Fukuda H, Nomura K. Mechanisms of somatic embryogenesis in cell cultures: Physiology, biochemistry, and molecular biology. In Vitro Cellular & Developmental Biology-Plant. 1992;**28**:11-14

[14] Lakshmanan P, Geijskes RJ, Wang L, Elliott A, Grof CP, Berding N, Smith GR. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. Plant Cell Reports. 2006;**25**:1007-1015

[15] Koch AC, Ramgareeb S, Snyman SJ, Watt MP, Rutherford RS. An *in vitro* induced mutagenesis protocol for the production of sugarcane tolerant to imidazolinone herbicides. Proceedings of the International Society for Sugar Cane Technologists. 2010;**27**:1-5

[16] Sengar AS, Thind KS, Kumar B, Pallavi M, Gosal SS. *In vitro* selection at cellular level for red rot resistance in sugarcane (*Saccharum sp*.). Plant Growth Regulation. 2009;**58**:201-209

[17] Suprasanna P, Rupali C, Desai NS, Bapat VA. Partial desiccation augments plant regeneration from irradiated embryogenic cultures of sugarcane. Plant Cell, Tissue and Organ Culture. 2008;**92**:101-105

[18] Irvine JE, Benda GTA, Legendre BL, Machado GR. The frequency of marker changes in sugarcane plants regenerated from callus culture. II Evidence for vegetative and genetic transmission, epigenic effects and chimeral disruption. Plant Cell, Tissue and Organ Culture. 1991;**26**:115-125

[19] Chowdhury MKU, Vasil I. Molecular analysis of plants regenerated from embryogenic cultures of hybrid sugarcane cultivars (*Saccharum* spp.). Theoretical and Applied Genetics. 1993;**86**:181-188

[20] Taylor PWJ, Geijskes JR, Ko HL, Fraser TA, Henry RJ, Birch RG. Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. TAG Theoretical and Applied Genetics. 1995;**90**:1169-1173

[21] Kaur R, Kapoor M. Plant regeneration through somatic embryogenesis in sugarcane. Sugar Tech. 2016;**18**:93-99

[22] Jalaja NC, Neelamathi D, Sreenivasan TV. Micropropagation for Quality Seed Production in Sugarcane in Asia and the Pacific. Food and Agriculture Organization of the United Nations. India; 2008

[23] Bakker H. Sugar Cane Cultivation and Management. New York: Kluwer Academic/ Plenum Publishers; 1999

[24] Geijskes RJ, Wang LF, Lakshmanan P, McKeon MG, Berding N, Swain RS, Elliott AR, Grof CPL, Jackson J, Smith GR. Smartsette seedlings: Tissue culture seed plants for the Australian sugar industry. Proceedings of the Australian Society of Sugarcane Technologists, PK Editorial Services. 2003;**25**:6-6

[25] Ali S, Iqbal J, Khan MS. Genotype independent in vitro regeneration system in elite varieties of sugarcane. Pakistan Journal of Botany. 2010;**42**:3783-3790

[26] Mustafa G, Khan MS. Differential role of indolebutyric acid in sugarcane root development. Sugar Tech. 2016;**18**:55-60

[27] Kristini A. The use of tissue culture to eliminate some important diseases in sugarcane [M.Sc. thesis]. Australia: University of Queensland; 2004

[28] Wagih ME, Gordon GH, Ryan CC, Adkins SW. Development of an axillary bud culture technique for Fiji disease virus elimination in sugarcane. Australian Journal of Botany. 1995;**43**:135-143

[29] Wang QC, Valkonen JPT. Elimination of viruses and phytoplasma by cryotherapy of in vitro-grown shoot tips: Analysis of all cases. Advances in Horticultural Science. 2007; **21**:265-269

[30] Bajaj YP. Cryopreservation of plant cell, tissue, and organ culture for the conservation of germplasm and biodiversity. In: Cryopreservation of Plant Germplasm I. Springer Berlin Heidelberg. Germany; 1995. p. 3-28

[31] Martins ERF, Dahmer N, Sforça DA, Silva CBC, Bundock P, Henry RJ, Souza GM, van Sluys MA, Landell MGA, Carneiro MS, Vincentz MAG, Pinto LR, Vencovsky R, Souza AP. SNP genotyping allows an in-depth characterisation of the genome of sugarcane and other complex autopolyploids. Scientific Reports. 2013;**3**:3399

[32] Scherwinski-Pereira JE, Costa FHS. Conservação in vitro de recursos genéticos de plantas: estratégias, princípios e aplicações. In: Barrueto Cid LP, editor. Cultivo in vitro de plantas. Brasília: Embrapa Informação Tecnológica; 2010. p. 177-234

[33] Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK. Ascertaining clonal fidelity of micropropagated plants of *Dendrocalamus hamiltonii* Nees et Arn. ex Munro using molecular markers. In Vitro Cellular and Developmental Biology-Plant. 2013;**49**:572-583

[34] Park JW, Solis-Gracia N, Trevino C, da Silva JA. Exploitation of conserved intron scanning as a tool for molecular marker development in the *Saccharum* complex. Molecular Breeding. 2012;**30**:987-999

[35] Suganya A, Nithiyanantham R, Premachandran MN, Balasundaram N, Govindaraj P, Selvi A, Nair NV. Cytological studies in hybrids between a sugarcane cultivar and three cytotypes of wild *Saccharum spontaneum* L. Journal of Sugarcane Research. 2012;**2**:34-40

[36] Smulders MJM, de Klerk GJ. Epigenetics in plant tissue culture. Plant Growth Regulation. 2011;**63**:137-146

[37] Vujović T, Cerović R, Ružić D. Ploidy level stability of adventitious shoots of sour cherry 'Cacanski Rubin' and Gisela 5 cherry rootstock. Plant Cell, Tissue and Organ Culture. 2012;**111**:323-333

[38] Van Emon JM. The omics revolution in agricultural research. Journal of Agricultural and Food Chemistry. 2015;**64**:36-44

[39] Paterson AH, Bowers JE, Bruggmann R, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Poliakov A, Schmutz J, Spannagl M. The *Sorghum bicolor* genome and the diversification of grasses. Nature. 2009;**457**:551-548

[40] Manetti ME, Rossi M, Cruz GMQ, Saccaro NL, Nakabashi M, Altebarmakian V, Rodier-Goud M, Domingues D, D'Hont A, Van Sluys MA. Mutator system derivatives isolated from sugarcane genome sequence. Tropical Plant Biology. 2012;**5**:233-243

[41] Feschotte C. Transposable elements and the evolution of regulatory networks. Nature Review Genetics. 2008;**9**:397-405

[42] Shirasu K, Schulman AH, Lahaye T, Schulze-Lefert P. A contiguous 66-kb barley DNA sequence provides evidence for reversible genome expansion. Genome Research. 2000; **10**:908-915

[43] Tomkins JP, Yu Y, Miller-Smith H, Frisch DA, Woo SS, Wing RA. A bacterial artificial chromosome library for sugarcane. TAG Theoretical and Applied Genetics. 1999;**99**:419-424

[44] Casu R, Hotta CT, Souza GM, Henry RJ, Kole C. Functional genomics: Transcriptomics of sugarcane-current status and future prospects. In: Genetics, Genomics and Breeding of Sugarcane. Science Publishers. Brazil; 2010. p. 167-191

[45] Souza GM, Berges H, Bocs S, Casu R, D'Hont A, Ferreira JE, Henry R, Ming R, Potier B, Van Sluys MA, Vincentz M. The sugarcane genome challenge: Strategies for sequencing a highly complex genome. Tropical Plant Biology. 2011;**4**:145-156

[46] Casu RO, Dimmock CH, Thomas ME, Bower NE, Knight DE, Grof CH, McIntyre L, Jackson P, Jordan D, Whan V, Drenth J. Genetic and expression profiling in sugarcane. Proceedings of the International Society of Sugarcane Technologists. 2001;**24**:626-627

[47] Grivet L, Arruda P. Sugarcane genomics: Depicting the complex genome of an important tropical crop. Current Opinion in Plant Biology. 2002;**5**:122-127

[48] Simpson AJG, Perez JF. ONSA, the Sao Paulo virtual genomics institute. Nature Biotechnology. 1998;**16**:795-796

[49] Barnabas L, Ramadass A, Amalraj RS, Palaniyandi M, Rasappa V. Sugarcane proteomics: An update on current status, challenges, and future prospects. Proteomics. 2015;**15**:1658-1670

[50] Heinz DJ. Isozyme prints for variety identification. Sugarcane Breed Newsletter. 1969;**24**:8

[51] O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry. 1975;**250**:4007-4021

[52] Pacheco CM, Pestana-Calsa MC, Gozzo FC, Nogueira RJMC, Menossi MTC. Differentially delayed root proteome responses to salt stress in sugar cane varieties. Journal of Proteome Research. 2013;**12**:5681-5695

[53] Zhou G, Yang LT, Li YR, Zou CL, Huang LP, Qiu LH, Huang X, Srivastava MK. Proteomic analysis of osmotic stress-responsive proteins in sugarcane leaves. Plant Molecular Biology Reporter. 2012;**30**:349-359

[54] Ramagopal S, Carr JB. Sugarcane proteins and messenger RNAs regulated by salt in suspension cells. Plant Cell Environment. 1991;**14**:47-56

[55] Que Y, Su Y, Guo J, Wu Q, Xu L. A global view of transcriptome dynamics during *Sporisorium scitamineum* challenge in sugarcane by RNA-seq. PLoS One. 2014;**9**:e0106476

[56] Aliferis K, Chrysayi-Tokousbalides M. Metabolomics in pesticide research and development: Review and future perspectives. Metabolomics. 2011;**7**:35-53

[57] Bosch S, Rohwer JM, Botha FC. The sugarcane metabolome. Proceedings of the South African Sugar Technologists Association. 2003;**7**:129-133

[58] Basnayake SW, Morgan TC, Wu L, Birch RG. Field performance of transgenic sugarcane expressing isomaltulose synthase. Plant Biotechnology Journal. 2012;**10**:217-225

[59] Joyce P, Hermann S, O'Connell A, Dinh Q, Shumbe L, Lakshmanan P. Field performance of transgenic sugarcane produced using *Agrobacterium* and biolistics methods. Plant Biotechnology Journal. 2014;**12**:411-424

[60] Gallo-Meagher M, Irvine JM. Herbicide resistant transgenic sugarcane plants containing the bar gene. Crop Science. 1996;**36**:1367-1374

[61] Enriquez-Obregon GA, Vazquez-Padron RI, Pieto-Samsonov DL, De la Riva GA, Selman-Housein G. Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. Planta. 1998;**206**:20-27

[62] Falco MC, Neto AT, Ulian EC. Transformation and expression of a gene for herbicide resistance in a Brazilian sugarcane. Plant Cell Reports. 2000;**19**:1188-1194

[63] Mukunthan N, Srikanth J, Singaravelu B, Asokan S, Kurup NK, Goud YS. Assessment of woolly aphid impact on growth, yield and quality parameters of sugarcane. Sugar Tech. 2008;**10**:143-149

[64] Arencibia A, Vázquez RI, Prieto D, Téllez P, Carmona ER, Coego A, Hernández L, Gustavo A, Selman-Housein G. Transgenic sugarcane plants resistant to stem borer attack. Molecular Breeding. 1997;**3**:247-255

[65] Weng LX, Deng HH, Xu JL, Li Q, Zhang YQ, Jiang ZD, Li QW, Chen JW, Zhang LH. Transgenic sugarcane plants expressing high levels of modified cry1Ac provide effective control against stem borers in field trials. Transgenic Research. 2011;**20**:759-772

[66] Falco MC, Silva-Filho MC. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: Effects on natural defense against *Diatraea saccharalis*. Plant Physiology and Biochemistry. 2003;**41**:761-766

[67] Allsopp PG, McGhie TK. Snowdrop lectin and wheatgerm lectins as antimetabolites for the control of sugarcane whitegrubs. Entomologia Experimentalis et Applicata. 1996; **80**:409-414

[68] Sétamou M, Bernal JS, Legaspi JC, Mirkov TE, Legaspi BC. Evaluation of lectin-expressing transgenic sugarcane against stalkborers (*Lepidoptera: Pyralidae*): Effects on life history parameters. Journal of Economic Entomology. 2002;**95**:469-477

[69] Zhangsun DT, Luo SL, Chen RK, Tang KX. Improved *Agrobacterium*-mediated genetic transformation of GNA transgenic sugarcane. Biologia. 2007;**62**:386-393

[70] Zhang LH, Weng LX, Jiang ZD. Sugarcane. In: Pua EC, Davey MR, editors. Biotechnology in Agriculture and Forestry Transgenic Crops. Springer. Germany; 2007

[71] Somerville C, Youngs H, Taylor C, Davis SC, Long SP. Feedstocks for lignocellulosic biofuels. Science. 2010;**329**:790-792

[72] Aydin S, Buyuk İ, Aras ES. Expression of SOD gene and evaluating its role in stress tolerance in NaCl and PEG stressed *Lycopersicum esculentum*. Turkish Journal of Botany. 2014;**38**:89-98

[73] Malik KB. Some anatomical characteristics of sugarcane varieties in relation to drough resistance. Agriculture Research. 1986;**11**:43-49

[74] Ahuja I, de Vos RC, Bones AM, Hall RD. Plant molecular stress responses face climate change. Trends in Plant Sciences. 2010;**15**:664-674

[75] Zhang L, Guangyao Z, Jizeng J, Xu L, Kong X. Molecular characterization of 60 isolated wheat MYB genes and analysis of their expression during abiotic stress. Journal of Experimental Biology. 2012;**63**:203-214

[76] McQualter RB, Dookun-Saumtally A. Expression profiling of abiotic-stress inducible genes in sugarcane. In: XXVI Congress, International Society of Sugar Cane Technologists; 2007. pp. 878-888

[77] Kumar T, Khan MR, Abbas Z, Ali GM. Genetic improvement of sugarcane for drought and salinity stress tolerance using Arabidopsis vacuolar pyrophosphatase (AVP1) gene. Molecular Biotechnology. 2014;**56**:199-209

[78] Schilling RK, Tester M, Marschner P, Plett DC, Roy SJ. AVP1: One protein, many roles. Trends in Plant Science. 2017;**22**:154-162

[79] Chandra A, Verma PK, Islam MN, Grisham MP, Jain R, Sharma A, Roopendra K, Singh K, Singh P, Verma I, Solomon S. Expression analysis of genes associated with sucrose accumulation in sugarcane (*Saccharum* spp. hybrids) varieties differing in content and time of peak sucrose storage. Plant Biology. 2015;**17**:608-617

[80] Hendrik JG, Botha FC. Down-regulation of pyrophosphate: Fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. Transgenic Research. 2008;**17**:85-92

[81] Moore PH. Temporal and spatial regulation of sucrose accumulation in the sugarcane stem. Functional Plant Biology. 1995;**22**:661-679

[82] Wu L, Birch RG. Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. Plant Biotechnology Journal. 2007;**5**:109-117

[83] Wu L, Birch RG. Characterization of the highly efficient sucrose isomerase from *Pantoea dispersa* UQ68J and cloning of the sucrose isomerase gene. Applied and Environmental Microbiology. 2005;**71**:1581-1590

[84] Hamerli D, Birch RG. Transgenic expression of trehalulose synthase results in high concentrations of the sucrose isomer trehalulose in mature stems of field-grown sugarcane. Plant Biotechnology Journal. 2011 Jan 1;**9**(1):32-37

[85] Zhang SZ, Yang BP, Feng CL, Chen RK, Luo JP, Cai WW, Liu FH. Expression of the *Grifola frondosa trehalose synthase* gene and improvement of drought-tolerance in sugarcane (*Saccharum officinarum* L.). Journal of Integrative Plant Biology. 2006;**48**:453-459

[86] Mustafa G, Sarwar Khan M. Prospecting the utility of antibiotics as lethal selection agents for chloroplast transformation in sugarcane. International Journal of Agriculture & Biology. 2012;**14**:307-310

[87] Botha FC. Energy yield and cost in a sugarcane biomass system. Proceedings of the Australian Society of Sugar Cane Technologists. 2009;**31**:1-10

[88] Matsuoka S, Ferro J, Arruda P. The Brazilian experience of sugarcane ethanol industry. In Vitro Cellular and Developmental Biology-Plant. 2009;**45**:372-381

[89] Ming R, Moore PH, Wu KK, D'Hont A, Tew TL, Mirkov TE, da Silva J, Schnell RJ, Brumbley SM, Lakshmanan P, Comstock JC, Glaszmann JC, Paterson AH. Sugarcane improvement through breeding and biotechnology. Plant Breeding Reviews. 2006;**27**: 17-118

[90] Arruda P. Perspective of the sugarcane industry in Brazil. Tropical Plant Biology. 2011;**4**:3-8

[91] Sainz MB. Commercial cellulosic ethanol: The role of plant expressed enzymes. In Vitro Cellular and Developmental Biology-Plant. 2009;**45**:314-329

[92] Harrison MD, Geijskes J, Coleman HD, Shand K, Kinkema M, Palupe A, Hassall R, Sainz M, Lloyd R, Miles S, Dale JL. Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. Plant Biotechnology Journal. 2011;**9**:1-13

[93] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T. The path forward for biofuels and biomaterials. Science. 2006;**311**:484-489

[94] Jaffe G. Withering on the Vine: Will Agricultural Biotech's Promises Bear Fruit? Washington, DC: Center for Science in the Public Interest; 2005

[95] Anonymous. US DOE. Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda. DOE/SC-0095. Washington, DC: Office of Science and Office of Energy Efficiency and Renewable Energy, U.S. Department of Energy; 2006