Chapter 5
Sugarcane as a Novel Biofactory: Potentialities and Challenges

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Abstract Sugarcane is the most productive crop plant to date, and its potential of becoming a crucial biofactory for generating high-value bioproducts is emerging only recently. Though it possesses one of the most complex genomes in the plant kingdom, important advances have been made in terms of transgenic approaches to generate new varieties, both by particle bombardment and *Agrobacterium*-mediated transformation. Nevertheless, crucial aspects in breeding programs and molecular technologies have to be developed or improved, before this crop consolidates as the highest productive biofactory. Social and biosafety issues also need to be addressed. Here, we highlight the most recent advances in the biotechnology of sugarcane to produce alternative products such as pharmaceutical proteins, biopolymers, and high-value carbohydrates, and strengthen opportunities and challenges of sugarcane as a biofactory of novel compounds. We conclude that the progress in molecular approaches to develop sugarcane into a sustainable biofactory demonstrates that this crop has tremendous potential and may play an important role in the growing bioeconomy through biopharming. Like no other contemporary crop, sugarcane is facing new paradigms and is expected to contribute at least partially to the development of new generation highly profitable biofactories.

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5.1 Introduction

Modern sugarcane, the main source of sucrose worldwide, belongs to the grass family (Poaceae) and was created about a century ago from the combination of *Saccharum* polyploid species. According to Daniels and Roach (1987), the genus *Saccharum* comprises six different species: *S. barberi*, *S. edule*, *S. officinarum*, *S. robustum*, *S. sinense*, and *S. spontaneum*. Of these, *S. officinarum* (the domesticated sugar-producing species) and *S. spontaneum* (a vigorous wild species with many aneuploidy forms) are thought to be the ancestors of cultivated sugarcane. *S. officinarum* originally derived from *S. robustum*, while *S. barberi* and *S. sinense* are thought to have been derived by crossing *S. officinarum* and *S. spontaneum* (Asano et al. 2004; Sandhu et al. 2012). However, Irvine (1999) suggested only two true species: *S. officinarum* and *S. spontaneum*, and therefore, current sugarcane commercial cultivars are thought to be hybrids with 80–90 % of the genome from *S. officinarum* and 10–20 % of the genome from *S. spontaneum* (Grivet et al. 1996; Hoarau et al. 2002).

The chromosome number of these species ranges from 36 to 200 (Asano et al. 2004; OGTR 2011). The polyploid and aneuploid nature of the genus *Saccharum* has made phylogenetic analyses and, as a result, breeding programs a tough task. Furthermore, the taxonomy and phylogeny of sugarcane is complicated as plants from five genera are thought to share common characteristics and form a closely related interbreeding group known as the "*Saccharum* complex". This complex comprises the genera *Saccharum*, *Erianthus* section *Ripidium*, *Miscanthus* section *Diandra*, *Narenga* and *Sclerostachya* (D'Hont et al. 1998; OGTR 2011), albeit controversial discussions still remain in the scientific community concerning the genetic relationships among genera in this complex and new hypotheses are being formulated. As a consequence, the assumption that *S. officinarum* is a result of a complex introgression between *S. spontaneum*, *Erianthus arundinaceus*, and *Miscanthus sinensis* (reviewed by Daniels and Roach 1987) is being analyzed in the light of new biochemical and molecular approaches. Accordingly, current extant species of the genera *Saccharum*, *Erianthus*, and *Miscanthus* are clearly distinct in their isozyme profiles, nuclear and cytoplasmic restriction fragment

length polymorphisms (AFLPs), and simple sequence repeats (SSRs) and sequence data (reviewed by D'Hont et al. 2008). As a result of these analyses, it has been assumed that the genus *Saccharum* is a well-defined lineage that has diverged over a long period of evolution from the lineages leading to the *Erianthus* and *Miscanthus* genera (Grivet et al. 2006; D'Hont et al. 2008) and that cultivated sugarcanes probably emerged from wild *Saccharum* species, while secondary introgressions with other genera are not likely pathways (D'Hont et al. 2008).

The *Saccharum* species are not only polyploid, but also autopolyploid (hosting more than two sets of homologous chromosomes derived from a single species) and allopolyploid (possessing two or more unlike sets of chromosomes from different species) (Sreenivasan et al. 1987; Besse et al. 1997), which represent a tremendous challenge for breeders that normally base their statistical genetic approaches on models developed for diploid organisms.

A summary of the genetic characteristics of the *Saccharum* species and the "*Saccharum* complex" is shown in Table 5.1.

As a relatively recently domesticated species, sugarcane exhibits little of the available genetic diversity having been incorporated or actively analyzed for introgression into domesticated varieties (Dillon et al. 2007; OGTR 2011; Sreenivasan et al. 1987), and breeding programs in the early 1900s focused on hybridization of *S. officinarum* clones, but quickly progressed to interspecific crosses incorporating *S. spontaneum*. This resulted in improved agronomic traits, such as tilling, stand and trashiness abilities, ratooning and disease resistance, but required a backcrossing program to *S. officinarum*, called "nobilization," to elevate the sucrose content (Dillon et al. 2007; Edmé et al. 2005). Since then, the majority of breeding programs have focused on intercrossing between the hybrids, though in recent decades the larger increases in genetic gains have been made by incorporating more diverse germplasm into the cultivated backgrounds (Edmé et al. 2005; Dillon et al. 2007) not only to increase sucrose production, but also to diversify into other alternative products to regain profitability.

As a C4 carbohydrate metabolism plant having a perennial life cycle, sugarcane is one of the most productive cultivated plants. Apart from producing sugar, this crop has gained increased attention because of its importance as a biofuel source among other value-added products developed from sugarcane biopharming using molecular approaches. Nevertheless, sugarcane has one of the most complex genomes among cultivated plants, which has long hampered the development of crucial areas such as genetics to support breeding for crop improvement programs. With the advent of molecular techniques, the sugarcane genome has become less mysterious, although its complexity has still been confirmed in many aspects (D'Hont et al. 2008).

In this chapter, we review the current status of sugarcane as a potential biofactory focusing particularly on the production of pharmaceutical proteins, biopolymers, and alternative carbohydrates; topics related to the use of sugarcane for bioenergy generation have been thoroughly addressed elsewhere (Arruda 2012; de Siqueira et al. 2013; Kuan et al. 2013; Vermerris 2011) and therefore will not be discussed herein.

Table 5.1 Genetic characteristics of species from the *Saccharum* genus and *Saccharum* complex

Species or genus	Classification	Sugar content	Chromosome number (2n)	Monoploid genome size (Mbp)	References
Saccharum barberi	Ancient hybrid	Low	111–120	3,156–4,121	Asano et al. (2004), Bonnett and Henry (2011)
S. edule	Wild species	Nil	60–80		Asano et al. (2004), Bonnett and Henry (2011)
S. officinarun	Noble cane	High	70–140	985	Asano et al. (2004), Bonnett and Henry (2011), Zhang et al. (2012)
S. robustum	Wild species	Nil	60–200	1,195	Asano et al. (2004), Bonnett and Henry (2011)
S. sinense	Ancient hybrid	Low	80–124	4,183	Asano et al. (2004), Bonnett and Henry (2011)
S. spontaneum	Wild species	Nil	40–128	843	Asano et al. (2004), Bonnett and Henry (2011), Zhang et al. (2012)
Erianthus spp.	Wild sugarcane relative		20–60	980–1,205	Chae (2012)
Miscanthus spp.	Close sugarcane relative		38–76	2,150–2,650	Rayburn et al. (2009), Swaminathan et al. (2012)
Narenga spp.	Wild sugarcane relative		30–34		Sobral et al. (1994), Grivet et al. (2006)
Sclerostachya spp.	Wild sugarcane relative		30–96		Janaki-Ammal (1940), Butterfield et al. (2001), D'Hont et al. (2008)

5.2 Sugarcane Is More Than Sucrose

According to Hoarau et al. (2007), sugarcane converts the sun's energy into carbohydrates more efficiently than any other crop plant and has the unusual ability to store sucrose in stem cell vacuoles. This, along with its high biomass production and ease of cultivation, makes it one of the most interesting and productive agricultural crops. In fact, Waclawovsky et al. (2010) have established that the current world yield average for sugarcane is 80 t ha^{-1}, but the estimated theoretical yield potential is over 380 t ha^{-1}, while Moore (2009) calculated it over 472 t ha^{-1}, which supports the hypothesis of yield gains to be expected in the future.

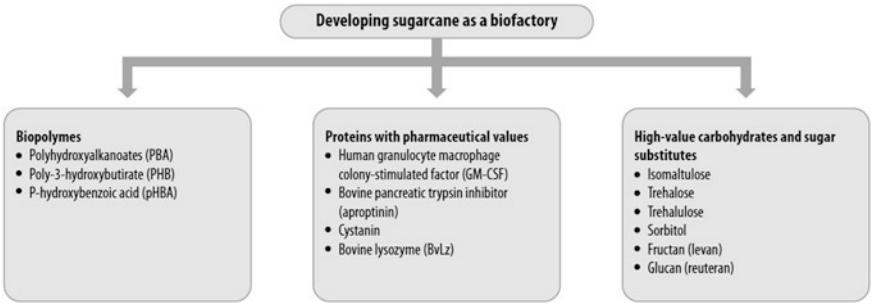

Fig. 5.1 Current main focus areas for developing sugarcane as a biofactory

Flourishing in humid and warm climates, sugarcane is mainly cultivated in tropical and subtropical regions on 25.4 million ha in more than 90 countries; its harvested biomass makes it the world's largest crop with nearly 1,800 million metric tons produced in 2011 as reported by FAO (http://faostat.fao.org). Mainly used to produce sugar, it accounts for approximately 75 % of the total world sugar production, while beet sugar is responsible for 25 %. By-products obtained from sugarcane include a wide range of derivatives (e.g., molasses, alcohol, fuel, livestock feed, paper, particle board) that can be used in the energy, food, chemical, pharmaceutical, and other industries (Hoarau et al. 2007; Tew and Cobill 2008).

Apart from sugar and bioethanol production, in the last few years, sugarcane has also turned into a target crop for biosynthesis of novel products such as proteins with pharmaceutical properties (Holland-Moritz 2003; Wang et al. 2005), biopolymers (Brumbley et al. 2004, 2007; McQualter et al. 2005a; Petrasovits et al. 2012), and high-value carbohydrates and sugar substitutes (Basnayake et al. 2012; Bauer et al. 2012; Chong et al. 2007, 2010; O'Neill 2011; Paterson et al. 2013) (Fig. 5.1).

Biopolymers are considered novel petrochemical substitutes that are environmentally friendly. Proteins with pharmaceutical value may contribute to the alleviation of important human diseases. Novel carbohydrates and sugar substitutes are crucial for developing nutraceutical products that can benefit consumers and other industrial processes.

The plant is well suited for such objectives due to some of its characteristics such as vegetative propagation, absence of flowering in most commercial varieties, production of a large biomass, large amount of carbon partitioned into sucrose (up to 42 % of the stalk dry weight), and the mobile pool of hexose sugars through most of its life cycle (D'Hont et al. 2008).

5.3 Sugarcane Biofactory for High-Value Biopolymers

Nowadays the production of plastics, polymers, surfactants, and other similar synthetic products is dominated by the petrochemical industry, although biotechnology, through metabolic engineering, may account for as much as 15 % of

the US$250 billion polymer market by the year 2015 (Nielsen 2005; Endres et al. 2007). As a matter of fact, traditional chemical industries are already shifting from chemical to biological processes and new opportunities are continuously emerging in the pharmaceutical, food, and biomedical areas. As a consequence, using sugarcane to manufacture plastics has several potential advantages over the traditional methods of production, including higher yields, greater purity, lower energy use, and less waste production (Nielsen 2005).

Importantly, by using bioplastics it is likely to reduce toxic emissions in the environment and also diminish loads of industrial wastes on landfills (Fritz et al. 2001; Seinbüchel 2003). Besides, when plants are used as biofactories, the major limitations of organic synthesis, namely long product lead time and expensive plant design to handle toxic compounds at high pressure and temperature, are overcome (Nielsen 2005). As a consequence, various research groups worldwide have made important progress in the development of sugarcane as a novel biofactory in the last decade.

For instance, Brumbley et al. (2004) engineered the genetic pathway for poly-3-hydroxybutyrate (PHB) in sugarcane. In general, polyhydroxyalkanoates (PHA) which include PHB, have thermoplastic properties and are biodegradable. In a subsequent transgenic approach, McQualter et al. (2005a) reported that transgenic sugarcane plants harboring a chloroplast-targeted version of *Escherichia coli* chorismate pyruvate-lyase (CPL) (Siebert et al. 1996) and a 4-hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonas fluorescens* (HCHL) (Gasson et al. 1998) (both enzymes providing a one-enzyme pathway from a naturally occurring plant intermediate), were able to synthetize p-hydroxybenzoic acid (pHBA, an aromatic hydroxiacid which constitutes monomers of liquid crystal polymers used in the electrical and optical industries),which was quantitatively converted to glucose conjugates by endogenous uridine diphosphate (UDP)-glucosyl transferases and apparently stored in the vacuole. The largest amounts detected in leaf and stem tissue were 7.3 and 1.5 % dry weight, respectively, while there were no evident phenotypic defects. However, as a result of diverting carbon away from the phenylpropanoid pathway, there was a severe reduction in leaf chlorogenic acid, subtle changes in lignin composition, and an apparent compensatory upregulation of phenylalanine ammonia-lyase (McQualter et al. 2005b).

Brumbley et al. (2007) transformed sugarcane with three genes from the bacterium *Ralstonia eutropha* that encode the genetic pathway for the biosynthesis of PHB. In the best transformed line, PHB accumulated at 2.5 % of leaf dry weight. Furthermore, transgenic plants were evaluated as a production platform for pHBA using two different bacterial genes, one from *Escherichia coli* and the other from *Pseudomonas fluorescens*. Each of these genes modifies a different existing biochemical pathway in sugarcane. In the best line, a glycosylated form of pHBA accumulated in the leaf and stem tissue at 7.3 and 1.5 % dry weight, respectively.

Purnell et al. (2007) demonstrated that several transgenic sugarcane lines accumulating the bacterial PHB exhibited a vertical PHB concentration gradient, while the polymer concentration showed the lowest level in the youngest leaves and increased with leaf age. In addition, there was a horizontal gradient along the

length of a leaf, with the PHB concentration increasing from the youngest part of the leaf (the base) to the oldest (the tip). The rank order of the lines did not change over time. Moreover, there was a uniform spatiotemporal pattern of relative PHB accumulation among the lines, despite the fact that they showed marked differences in absolute PHB concentration. Molecular analysis showed that the expression of the transgenes encoding the PHB biosynthesis enzymes was apparently coordinated, and that there were good correlations between PHB concentration and the abundance of the PHB biosynthesis enzymes. The maximum PHB concentration recorded (1.77 % of leaf dry weight) did not result in agronomic abnormalities. Although moderate PHB concentrations were achieved in leaves, the maximum total-plant PHB yield was only 0.79 % (11.9 g PHB in 1.51 kg dry weight).

As plant peroxisomes contain the substrate molecules and essential reducing power for PHB biosynthesis, Tilbrook et al. (2011) generated transgenic sugarcane with the three-enzyme *Ralstonia eutropha* PHA biosynthetic pathway targeted at these cell compartments. PHB accumulated in sugarcane leaves at levels up to 1.6 % dry weight, in both peroxisomes and vacuoles. A small percentage of total polymer was also identified as the copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate). As a result of peroxisomal PHA biosynthesis, no obvious detrimental effect was observed on plants. This study highlights how using peroxisomal metabolism for PHA biosynthesis could significantly contribute to reaching commercial production levels of PHAs in crop plants.

Petrasovits et al. (2012) used different plant and viral promoters, in combination with multigene or single-gene constructs to increase PHB levels in sugarcane. Promoters tested included the maize and rice polyubiquitin promoters, the maize chlorophyll A/B-binding protein promoter, and a Cavendish banana streak badnavirus promoter. At the seedling stage, the highest levels of polymer were produced in sugarcane plants when the Cavendish banana streak badnavirus promoter was used. However, in all cases, this promoter underwent silencing as the plants matured. The rice Ubi promoter enabled the production of PHB at levels similar to the maize Ubi promoter. The maize chlorophyll A/B-binding protein promoter enabled the production of PHB to levels as high as 4.8 % of leaf dry weight, which is approximately 2.5 times higher than previously reported levels in sugarcane. However, the highest PHB-producing lines showed phenotypic differences to the wild-type parent, including reduced biomass and slight chlorosis.

5.4 Sugarcane Biofactory for Protein Products

Regarding pharmaceutical applications, one of the first approaches reported was done by Holland-Moritz (2003), who transformed sugarcane to produce pharmaceutical-grade human structural proteins for human therapeutics. Later, Wang et al. (2005) successfully produced the human granulocyte macrophage colony-stimulated factor (GM-CSF, used in clinical applications for the treatment of

neutropenia and aplastic anemia) in transgenic sugarcane plants. Accumulation of GM-CSF protein ranged from undetectable to 0.02 of total soluble protein. Human bone marrow cells (TF-1), which require GM-CSF for cell division, proliferated when growth media was supplemented with transgenic sugarcane extracts. Comparison to purified commercially produced GM-CSF indicated that sugarcane-produced protein had essentially identical activity levels. In a 14-month field trial, accumulation levels remained stable.

Arvinth et al. (2010) transformed sugarcane cultivars Co 86032 and CoJ 64 with the *cry1Ab* gene driven by maize ubiquitin promoter through particle bombardment and *Agrobacterium*-mediated transformation systems. Gene pyramiding was also attempted by retransforming sugarcane plants carrying the bovine pancreatic trypsin inhibitor (aprotinin, which reduces bleeding during complex surgeries) gene, with *cry1Ab*. Aprotinin-expressing sugarcane pyramided with *cry1Ab* showed reduction in damage by the shoot borer *Chiloinfuscatellus*.

Henrique-Silva and Soares-Costa (2012) generated transgenic sugarcane expressing a His-tagged cystatin (a human protein used as biomarker for the identification and prevention of various diseases) under the control of the maize ubiquitin promoter. A transformed sugarcane plant presented high levels of protein expression and was selected for the purification of this protein through affinity chromatography in nickel columns. Therefore, it was demonstrated that sugarcane can be a viable expression system for recombinant protein production and that the His-tag purification strategy used to isolate the purified protein was effective.

Recently, Barros et al. (2013) generated transgenic sugarcane expressing recombinant bovine lysozyme (BvLz, used to control gram-negative pathogenic bacteria) in order to evaluate the feasibility of extraction and fractionation of recombinant proteins expressed in sugarcane stalks. Partial removal of native proteins was achieved using a 100 kDa membrane, but 20–30 % of the extracted BvLz was lost. Concentration of clarified extracts using a 3 kDa membrane resulted in twofold purification and 65 % recovery of BvLz. Loading of concentrated sugarcane extract on hydrophobic interaction chromatography (HIC) resulted in 50 % BvLz purity and 69 % recovery of BvLz.

5.5 Sugarcane Biofactory for High-Value Carbohydrates and Alternative Sugars

Other research groups have focused on developing sugarcane as a platform for the production of higher value isomers of sucrose such as isomaltulose and trehalose.

Isomaltulose is a natural isomer of sucrose. It is widely approved as a food with properties including slower digestion, a lower glycaemic index, and low cariogenicity, which can benefit consumers. Furthermore, isomaltulose displays reducing properties that make it attractive as industrial precursor for the manufacturing of biosurfactants and biopolymers (Lichtenthaler and Peters 2004;

Ravaud et al. 2007). In turn, trehalose is implicated in anhydrobiosis as a result of its high water retention capabilities, and is used in the food and cosmetic industries. The saccharide may form a gel phase as cells dehydrate, which prevents disruption of internal organelles and may function as an antioxidant as well (Reina-Bueno et al. 2012). Availability of these saccharides is currently limited by the cost of fermentative conversion from sucrose (Mudge et al. 2013).

Wu and Birch (2007) engineered an efficient sucrose invertase isolated from the bacterium *Pantoea dispersa*, with a monocot promoter and a vacuolar targeting sequence (Gnanasambandam and Birch 2004) and transformed sugarcane explants with this construct to produce isomaltulose. Isomaltulose accumulated in sugarcane stem storage tissues of transformed plants without any decrease in the stored sucrose concentration, resulting in nearly doubled total sugar concentrations in harvested juice. Transgenic plants also showed higher photosynthetic activity, sucrose transport, and sink strength, which indicates a possible feedback signal for sucrose biosynthesis, translocation, and storage (Wu and Birch 2007).

In order to develop an efficient *in planta* sugarcane-based production system by coupling the synthesis of alternative products to the metabolic intermediates of sucrose metabolism, Chong et al. (2007) evaluated the biosynthesis of sorbitol (a polyalcohol used as sugar substitute) in sugarcane using the *Malus domestica* sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*). The average amounts of sorbitol detected in the most productive line were 120 mg g^{-1} dry weight (equivalent to 61 % of the soluble sugars) in the leaf lamina and 10 mg g^{-1} dry weight in the stalk pith. The levels of enzymes involved in sucrose synthesis and cleavage were elevated in the leaves of plants accumulating sorbitol, but this did not affect sucrose accumulation in the culm. Sorbitol-producing sugarcane generated 30–40 % less aerial biomass and was 10–30 % shorter than control lines. Leaves developed necrosis in a pattern characteristic of early senescence, and the severity was related to the relative quantity of sorbitol accumulated. When the *Zymomonas mobilis* glucokinase (*zmglk*) gene was coexpressed with *mds6pdh* to increase the production of glucose-6-phosphate, the plants were again smaller, indicating that glucose-6-phosphate deficiency was not responsible for the reduced growth. In conclusion, sorbitol hyperaccumulation affected sugarcane growth and metabolism, but the outcome was not deleterious for the plant.

Interested in the unusual development of the leaves in some transgenic sorbitol-producing sugarcane plants, Chong et al. (2010) compared the polar metabolite profiles in the leaves of those plants against a group of control sugarcane plants. Principal component analysis of the metabolites indicated that sorbitol, gentiobiose (a disaccharide), and gentiobiitol (a sugar alcohol) were strongly associated with sorbitol-producing canes. Gentiobiose and gentiobiitol were positively correlated with sorbitol accumulation.

Trehalulose is also a structural isomer of sucrose that has a sweet taste with similar physical and organoleptic properties to sucrose. Additionally, trehalulose is acariogenic and can be applied in diabetic and sports foods and drinks as its absorption reduces the rate at which monosaccharides and insulin are released into the bloodstream (Ravaud et al. 2007). Hamerli and Birch (2011) reported the

transformation of sugarcane plants with a vacuolar-targeted trehalulose synthase gene modified from the gene in *Pseudomonas mesoacididophila* MX-45 and obtained transgenic lines reaching about 600 mM of trehalulose in mature stem juice. These plants retained vigor and trehalulose production over multiple generations under glasshouse and field conditions.

Sucrose is the translocated photosynthate and the largest soluble carbon store in sugarcane. The capacity to carry stored sucrose into pathways that provide substrates to produce alternative products would be highly advantageous in an efficient biofactory. Hence, a high-yielding sugarcane biofactory system would ideally contain culm tissues that function as a secondary source tissue rather than a sink in terms of sucrose balance (O'Neill 2011). To that end, O'Neill et al. (2012a) demonstrated that sucrose is mobilized from set storage parenchyma via phloem to the growing shoot tissue. Overall, metabolism in storage parenchyma shifts from futile cycling to a more quiescent state during sucrose mobilization. Subsequently, trehalose metabolism in sugarcane was engineered in an attempt to create a significant carbon drain of stored sucrose to impart value-added properties and enhance abiotic stress tolerance (O'Neill et al. 2012b). To that end, two transgenes were introduced into the sugarcane genome: trehalose-6-phosphate synthase-phosphatase (TPSP) to increase trehalose biosynthesis, and an RNAi transgene specific for trehalase to abrogate trehalose catabolism. In RNAi-expressing lines, trehalase expression was abrogated in many plants although no decrease in trehalase activity was observed. In TPSP lines trehalase activity was significantly higher. No events of co-integration of TPSP and RNAi transgenes were observed, suggesting that trehalase activity is essential to mitigate embryonic lethal effects of trehalose metabolism (O'Neill et al. 2012b).

Moreover, transgenic sugarcane plants expressing a vacuole-targeted isomaltulose synthase in seven recipient genotypes (elite cultivars) were evaluated over 3 years under commercial field conditions (Basnayake et al. 2012). Isomaltulose concentration typically increased with internode maturity and comprised up to 217 mm (33 % of total sugars) in whole-cane juice. There was generally a comparable decrease in sucrose concentration, with no overall decrease in total sugars. After several cycles of field propagation, selections were obtained with cane yields similar to the recipient genotypes. Sucrose isomerase activity was low in these transgenic lines, and the results indicate strong potential to develop sugarcane for commercial-scale production of isomaltulose if higher activity can be engineered in appropriate developmental patterns.

Bauer et al. (2012) reported the effect of high molecular weight bacterial fructan (levan) and glucan (reuteran) on growth and carbohydrate partitioning in transgenic sugarcane plants. These polysaccharides are products of bacterial glycosyltransferases, enzymes that catalyze the polymerization of glucose or fructose residues from sucrose. Heterologous expression resulted in reduced total carbohydrate assimilation rather than a simple diversion of biopolymers by competition for substrate.

Lately, transgenic sugarcane plants with developmentally controlled expression of a silencing-resistant gene encoding a vacuole-targeted isomaltulose synthase

were tested under field conditions. High yields of isomaltulose were obtained, up to 81 % of total sugars in whole-cane juice from plants aged 13 months (Mudge et al. 2013). Using promoters from sugarcane to drive expression preferentially in the sugarcane stem, isomaltulose levels were consistent between stalks and stools within a transgenic line and across consecutive vegetative field generations of tested high-isomer lines. Importantly, these data represent the highest yields ever achieved of value-added materials through plant metabolic engineering. The sugarcane stem promoters are promising for strategies to achieve even higher isomaltulose levels and for other applications in sugarcane molecular improvement. Silencing-resistant transgenes are critical for delivering the potential of these promoters in practical sugarcane improvement. At the isomaltulose levels now achieved in field-grown sugarcane, direct production of this disaccharide in plants is feasible at a cost approaching that of sucrose, which should make the benefits of isomaltulose affordable on a much wider scale.

5.6 Potentialities and Challenges of Sugarcane as a Biofactory

Recently, sugarcane has become an important crop for food and energy production, and is emerging as a pivotal biofactory for high-value products. Its ability to accumulate high levels of sucrose in its stems and its distinctive high yield make it a unique crop, showing it to be the highest tonnage crop among cultivated plants. Though breeding programs have focused on improving sugar content, an evolving industry of biofuel and bio-based compounds such as biopolymers, pharmaceutical proteins, and novel carbohydrates may require vast amounts of biomass and, therefore, higher yields as well (Dal-Bianco et al. 2012).

Compared to other major crops, efforts to improve sugarcane are limited, as a consequence of its narrow gene pool, complex genome for molecular breeding, and the long breeding/selection cycle. These constraints, nonetheless, make sugarcane a good candidate for the application of molecular technologies. In recent years, considerable progress has been made in understanding the sugarcane genome, creating transgenic plants with improved agronomic, industrial, or other important traits, developing novel molecular markers, and understanding the molecular aspects of sucrose biosynthesis, transport, and accumulation in greater detail (Lakshmanan et al. 2005; Ming et al. 2006; Paterson et al. 2013). Accordingly, biotechnological routes for sugarcane improvement including technological data available and the use of marker-assisted breeding, genome sequencing, genetic engineering, and gene discovery for traits of interest are being addressed to reach higher productivity goals and develop sustainable molecular pharming.

Although a plethora of advantages of crop plants as biofactories are well documented (Ahmad et al. 2010; Becerra-Moreno et al. 2012; Jacobo-Velázquez et al. 2011; Jenkins et al. 2011; Rigano et al. 2013) as they are renewable resources of lower environmental impacts with balanced carbon emission, these systems also

face technical constraints and have to compete functionally and economically with traditional petrochemical production methods (Nielsen 2005; Goldemberg et al. 2008). Consequently, low-cost raw materials, efficient biocatalysis, and product innovations are all key determinants of success. Accordingly, sugarcane juice is a readily fermentable low-cost feedstock, and the bagasse, representing outstanding sources of low-cost green process energy, and fermentable and aromatic compounds (Chandel et al. 2013; Cheng and Zhu 2013; Nielsen 2005). Moreover, sugarcane has several other traits that give it tremendous potential to become a critical crop for transition from petrochemical-based to bio-based economies (Paterson et al. 2013). Then, using sugarcane as a biofactory of novel environmentally friendly products may also offer possible diversification for cane-growers, as well as reducing the reliance by rural sectors on sugar prices (Nielsen 2005). Currently, important research groups are involved in this form of biopharming projects around the world.

Nevertheless, many impasses must be overcome before sugarcane biofactories can become a commercial fact. To contend with commercial protein production systems that use well-established molecular protocols in plants such as maize and tobacco, approaches will need to bring about much higher levels of protein expression in the transformed sugarcane plants, especially in the stalk. These challenges will demand the identification, isolation, and amplification of new promoter regions (both constitutive and inducible), development of novel vectors, and success with both transcriptional and post-transcriptional gene modification and silencing. Moreover, the protocols for protein extraction and purification at an industrial level from vegetative tissues represent a daunting task that has to be addressed with several innovative strategies. Practical knowledge and skills in this field are in their infancy, and especially for global industries such as sugarcane (Paterson et al. 2013).

Constraints related to the long time required for conventional breeding of sugarcane (i.e., it takes 12–15 years to carry out, test and launch a new variety to the market) and its highly complex genome (polyploidy and aneuploidy) may be overcome by using molecular approaches. However, sugarcane exhibits recalcitrance to genetic transformation and several parameters usually need optimization at the variety level to reach higher transformation efficiencies (Scortecci et al. 2012). Indeed, the first protocol developed for genetic transformation of sugarcane was particle bombardment (biolistic) of cell suspension, embryogenic callus or meristem (Bower and Birch 1992; Snyman et al. 2006), but the efficiency of this method depends on callus formation and plant regeneration, which varies with genotype and culture conditions (Kaeppler et al. 2000; Scortecci et al. 2012). Later, *Agrobacterium tumefaciens*-mediated transformation arose (Arencibia et al. 1998; Brumbley et al. 2008) and was more efficient than biolistics for its higher stability on transgene expression, which derives from the smaller number of transgene copies integrated into the genome (Dai et al. 2001; Scortecci et al. 2012). Nevertheless, *Agrobacterium*-mediated transformation has shown low efficiency and is highly genotype- dependent, so that some in vitro culture parameters

resulted as key factors to improve this transformation method, as well as genotype screening, explant type and quality, selective agents, and *Agrobacterium* strains (Arencibia et al. 1998; Arencibia and Carmona, 2006; Manickavasagam et al. 2004). Importantly, Jackson et al. (2013), van der Vyver et al. (2013) and Mayavan et al. (2013) have recently reported successful results using both transformation methods for sugarcane.

Although no commercial transgenic sugarcane variety is available in the market so far, genes associated with sucrose content (Papini-Terzi et al. 2009), resistance to pests and pathogens, including constructs against insects, bacteria, and viruses (Arencibia et al. 1997, 1999; Falco and Silva-Filho 2003; Ingelbrecht et al. 1999; Weng et al. 2006, 2011; Zhu et al. 2011; Ismail 2013), herbicide-resistance genes as selective markers (Manickavasagam et al. 2004) and drought tolerance (Molinari et al. 2007) have been successfully cloned into some varieties. Besides, none of those reports refers to plastid transformation, even though this technology is considered a valuable tool for improving the containment of the transgene, and enhancing the biosafety of genetically modified (GM) plants (CBU 2007; Gottschamel et al. 2013; Ruf et al. 2007; Scortecci et al. 2012).

The inheritance of the chloroplasts in most plants is maternal, as these organelles are not carried by pollen. The manipulation of the chloroplast genome for crop improvement is therefore a highly promising technology for biosafety reasons. There are several examples of agronomical and biotechnological applications of plastid transformation with enhanced biosafety and higher transgene product yields in C4 plants and green microalgae (Wang et al. 2009; Chen and Melis 2013; Hanson et al. 2013) and new advances are being developed (Gottschamel et al. 2013). Although chloroplast genetic transformation is still very incipient in monocots like rice (Lee et al. 2006) and wheat (Cui et al. 2011; He 2012), and it has not been reported for sugarcane (Scortecci et al. 2012), the research avenue is widely open since the chloroplast genome of sugarcane has been completely sequenced (Calsa-Júnior et al. 2004), which enables recombination-based transformation with huge potential for basic and applied research in molecular pharming.

Importantly, a repertoire of gene promoters that work efficiently and precisely regarding level, timing, and location of expression is a critical element of transgenic cultivar development (Scortecci et al. 2012).

Public opinion currently appears to be biased against foods derived from GM organisms, and the cane industry faces a general community rejection of sugar produced by GM plants (Grice et al. 2003). In other industries, GM cultivars that are environmentally friendly and not designed for human consumption (e.g., *Bt*-cotton) have been accepted reasonably well. One of the main causes of public concern about genetic engineering has been the lack of information about the process and the types of products, particularly nonfood products that can be developed. As a consequence, in many countries GM sugarcane is facing release restrictions (Grice et al. 2003; Cheavegatti-Gianotto et al. 2011; Scortecci et al. 2012), which has to be taken into consideration when designing sugarcane

programs aimed at developing biofactories using GM strategies. Due to the potential for new alternative uses of sugarcane other than food, such as supplying high-value niche markets with a variety of novel products, the need for further analyses into product diversification as a way of increasing industry returns has also been emphasized (Grice et al. 2003).

Thus, despite the convoluted genetic system present in sugarcane, which largely limits the use of traditional genetic markers in breeding programs, it is becoming clear that molecular genetics and genomics will play important roles in sugarcane breeding programs, as transformation techniques become more efficient and more molecular tools (characterization of genes of interest, transformation vectors, specific promoters) become available.

Of economical relevance, Hansen et al. (2011) describe a series of recent patents on methods and techniques involving genes coding for proteins and breeding techniques with agronomic applicability on economically important crops, including sugarcane.

The sequencing of the complete sugarcane genome led by an international research group from Australia, Brazil, China, France, South Africa, and the USA is underway, and will greatly contribute to deciphering vital genetic information controlling crucial desirable traits related to genomic organization, promoters, gene regulators, and gene networks controlling metabolic pathways (Hotta et al. 2010; Scortecci et al. 2012; Dal-Bianco et al. 2012).

Moreover, sugarcane plantations are often criticized as they occupy large field areas of fertile arable land that otherwise could be used for food production, for impacting the environment with deforestation and land degradation, monocultures, as well as pollution (run-off of fertilizers, pesticides and molasses; pre-harvest burning and air pollution) (Scortecci et al. 2012; Uriarte et al. 2009). As environmental and social responsibility issues are being addressed in agriculture more often, it is also criticized that sugarcane production systems rely heavily on low-paid seasonal jobs and labor abuses worldwide (child labor, slavery regimen, hazardous conditions, underpayment) (Martinelli and Filoso 2008; Miranda 2010; Scortecci et al. 2012). Therefore, a need for developing a sustainable sugarcane industry with social responsibility is demanded by society worldwide.

Till date, substantial efforts have been directed toward sugarcane as a biofabric for high-value products. While these achievements are commendable, a greater understanding of the sugarcane genome, cell, and whole plant biology will accelerate the implementation of commercially significant biotechnology outcomes (Lakshmanan et al. 2005; Ming et al. 2006). The rapid progress in molecular biology and emerging biotechnology innovations will play significant roles in future sugarcane crop improvement programs and will offer many new opportunities to develop it as a new generation industrial crop and a sustainable biofactory.

5.7 Conclusions and Future Perspectives

Sugarcane has become an ideal biofactory, as it converts sunlight and water into biomolecules such as sugar, fibers, and waxes in a very efficient manner, making it the most productive field crop among cultivated plants. However, theoretical and technical constraints are yet to be overcome. Accordingly, using sugarcane as a biofactory is an exciting but challenging area of research and innovation that could have a huge influence on the evolution of alternative sugarcane industries worldwide.

Its complex polyploidy and high level of heterozygocity make proper exploitation of sugarcane variability a tough task. Consequently, significant advances in traditional breeding of sugarcane are limited by its narrow gene pool, complex genome, and the long breeding cycle. However, these disadvantages make sugarcane a good candidate for the application of transgenic approaches. Indeed, examples of sugarcane transgenic lines exhibiting improved agronomic and industrial traits have been cited in this chapter.

Thus, the establishment of molecular approaches reviewed herein to develop sugarcane into a biofactory demonstrates that this crop has tremendous potential and may play an important role in the growing bioeconomy through biopharming. Like no other contemporary crop, sugarcane is facing new paradigms and is expected to contribute at least partially to the development of new generation highly profitable biofactories. Social and biosafety issues are expected to be considered in any program aimed at developing a sustainable novel sugarcane biofactory in the future.

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