Harnessing Genetic Tools for Sustainable Bioenergy: A Review of Sugarcane Biotechnology in Biofuel Production

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Abstract: Sugarcane (Saccharum spp.) is a prominent renewable biomass source valued for its potential in sustainable and efficient second-generation biofuel production. This review aims to assess the genetic enhancement potential of sugarcane, emphasizing the use of advanced genetic engineering tools, such as CRISPR-Cas9, to improve traits crucial for biomass yield and biofuel production. The methodology of this review involved a thorough analysis of the recent literature, focusing on the advancements in genetic engineering and biotechnological applications pertinent to sugarcane. The findings reveal that CRISPR-Cas9 technology is particularly effective in enhancing the genetic traits of sugarcane, which are essential for biofuel production. Implementing these genomic tools has shown a significant rise in biomass output and, ultimately, the effectiveness of bioethanol manufacturing, establishing sugarcane as a feasible and reliable source of biofuel implications of these advancements extend. These advancements have a profound impact not only on agricultural productivity but also on enhancing the efficiency and scalability of the bioethanol industry. Developing superior sugarcane varieties is expected to boost economic returns and advance environmental sustainability through carbon-neutral biofuel alternatives. This review underscores the transformative role of genetic engineering in revolutionizing sugarcane as a bioenergy crop. The evolution of genetic engineering tools and methodologies is crucial for tapping into the full potential of sugarcane, and thereby supporting global efforts towards sustainable energy solutions. Future research should focus on refining these biotechnological tools to meet increasing energy demands sustainably, ensure food security, and mitigate negative environmental impacts.

Keywords: bioethanol production; CRISPR-Cas9; genetic improvement; lignocellulosic biomass; sugarcane biotechnology

1. Introduction

Lignocellulosic biomass, primarily derived from grasses such as sugarcane (Saccharum spp.) or woody species, is composed of cellulose, hemicellulose, and lignin, forming a pivotal resource for renewable energy generation [1]. This resource, essential for biofuel production, offers a sustainable alternative, particularly beneficial for nations with limited oil reservoirs, mitigating reliance on fossil fuels and reducing greenhouse gas emissions [2]. Lignocellulosic biomass-based second-generation biofuels offer superior net energy and CO2 balance compared to first-generation biofuels and do not compete with food resources [3]. Among globally successful biofuel production methods, the conversion of sugarcane into bioethanol stands out due to its significant positive energy balance and very reasonable cost [4]. Sugarcane, renowned for its rapid growth, high yield [5,6], and multi-harvest capabilities without replanting [7], emerges as an optimal candidate for the second generation of biofuels in tropical and subtropical countries [8]. The ProAlcohol program has demonstrated the feasibility of Brazil replacing gasoline with ethanol derived from sugarcane, with projections indicating a significant share in the nation’s energy landscape [9,10].
Challenges persist in the cost-intensive production of biofuels from lignocellulosic biomass, especially from sugarcane bagasse, which requires the development of appropriate sugar-cane cultivars, refinement of pretreatment methods, and the perfection of enzyme digestion technologies [11,12]. Therefore, improving sugarcane cultivars with enhanced biomass yield and fiber content and improving pretreatment and enzyme technologies are vital for transitioning to second-generation biofuels.

Beyond biofuel production, the significance of sugarcane extends to vast cultivation areas and substantial annual dry matter output [13,14], which supports its pivotal role in the sugar industry [4]. Moreover, sugarcane is a primary source of first-generation biofuels, contributing significantly to global ethanol production [15]. Its utilization as a bio-factory holds promise for enhancing socioeconomic status and sustainability of natural resources, recognizing the capability of sugarcane to produce bioethanol efficiently, particularly in developing countries [16,17]. Bagasse, the fibrous residue from sugar extraction, finds applications in paper-making and particle board manufacture due to its favorable cellulose fiber characteristics [18].

The desirable characteristics of sugarcane, such as fast growth rate, high productivity, low processing expenses, and favorable energy balance [19], position it as a prime candidate for bioethanol and sugar production [20]. Ongoing efforts in biomass yield enhancement through targeted breeding and molecular marker technologies [21–23], as well as advancements in CRISPR-mediated trait improvement facilitated by sugarcane genome sequencing, pave the way for new possibilities for efficient bioenergy production [24,25].

The current review examines the genetic modification potential of sugarcane as a biomass source for biofuels, elucidating its advantageous traits, genetic assets, and the contribution of biotechnology in enhancing biomass production. By examining genome-editing tools, particularly CRISPR technology, this review aims to deepen the understanding of the genetic improvement of sugarcane, positioning it as an efficient biofuel source. Furthermore, this review covers the influence of environmental factors on plant biomass accumulation, offering a holistic analysis essential for sustainable bioenergy solutions.

2. Sugarcane Overview

2.1. Genomic Diversity and Hybridization

Sugarcane belongs to the grass family Poaceae, the subfamily Panicoideae, the tribe Andropogoneae, the subtribe Saccharinae, and the group Saccharastrae. It is closely related to sorghum, Erianthus, and Miscanthus [26]. The genus *Saccharum* comprises six species: *Saccharum spontaneum*, *Saccharum edule*, *Saccharum officinarum*, *Saccharum sinense*, *Saccharum robustum*, and *Saccharum barberi* [26,27]. *S. officinarum*, *S. barberi*, and *S. sinense* are the cultivated types, *S. edule* is a marginally cultivated specialty crop, and *S. spontaneum* and *S. robustum* are wild species. The *Saccharum* genus is entirely composed of polyploid genotypes, exhibiting ploidy levels that vary from 5 to 20. This makes these species some of the most complex plant genomes [28]. The cytotype, which refers to the chromosomal count in a cell, exhibits variation among different species. In species such as *S. officinarum* (2n = 80), *S. spontaneum* (2n = 40–128), *S. barberi* (2n = 111–120), *S. sinense* (2n = 81–124), *S. edule* (2n = 60–80), and *S. robustum* (2n = 60–170), the basic number of chromosomes (x) varies from 5 to 12 [29]. *S. spontaneum* maintains a chromosome number of x = 8 primarily, with some accessions having x = 10 [30], while *S. officinarum* and *S. robustum* have x = 10 [31–33]. Chromosomal numbers remain uncertain for *S. sinense*, *S. barberi*, and *S. edule*, early interspecific hybrid cultivars, although a suggested chromosomal number of 10 exists for these species.

Contemporary sugarcane hybrids originate from crosses between the *S. officinarum* (2n = 80) used as female and *S. spontaneum* (2n = 40–128) used as male. The F1 hybrid resulting from female restitution events maintains the complete set of chromosomes from *S. officinarum* and half of the set from *S. spontaneum*, resulting in a chromosomal composition of 2n + n [34]. Modern sugarcane cultivars (*Saccharum* spp. hybrids) exhibit high aneuploid and interspecific properties. Genomic in situ hybridization (GISH) and fluorescent in
situ hybridization (FISH) reveals a predominance of *S. officinarum* chromosomes (80%) in modern hybrid sugarcane nuclei, with the remaining portion from *S. spontaneum* (10–20%) and a minimal (5–17%) from chromosome recombination between the two species [35–37]. The random genome arrangement results in unique and unpredictable chromosomal combinations in each offspring of modern hybrids, typically generated through crosses between clones or types [38]. Initial sugarcane breeding program, initiated over a century ago, laid the foundation for current breeding efforts [39]; a small number of original crosses and a few generations for chromosome recombination possibilities have resulted in a restricted genetic base (typically between 2 and 7 meiosis). Sugarcane breeding programs usually span 10 to 15 years [39,40], leading to restricted genetic diversity and a high level of linkage disequilibrium in the present sugarcane population [40,41].

2.2. Genomic Structure and Repetitive Elements

Modern sugarcane hybrid cultivars are interspecific hybrids with allo-autopolyploid genomes, estimated to be approximately 10 Gb. This large genome size poses considerable challenges for analysis and comprehension, especially when employing methodologies tailored for diploid genomes. These cultivars combine euploid and aneuploid chromosome sets, featuring hom(e)ologous genes occurring in 8–12 copies, making the genome highly complex [42]. Estimates indicate that the monoploid genome size ranges between 750 and 930 Mb, with *S. officinarum* and *S. spontaneum*, the two progenitor species, exhibiting 930 Mb and 750 Mb, respectively, almost twice that of rice and marginally larger than sorghum [43,44]. Despite its complexity and polyploidy, research indicates synteny between the sugarcane genome and other grasses, notably sorghum and maize, albeit with orthologous but modified locus collinearity [38]. Earlier assumptions suggested that sorghum and sugarcane shared similar proportions of repetitive DNA [45], The genome of *S. spontaneum* contains 58.7% repetitive sequences, which is lower than the 67.5% found in sorghum, specifically in classes I and II transposable elements [46,47].

Recent articles offer valuable insights into the genetic architecture of sugarcane. The R570 genome provides a thorough understanding of the intricate genomic structure of sugarcane. The basic genome assembly of R570, which has 5.04 Gb, shows that 73% comes from *S. officinarum* and 27% comes from *S. spontaneum*. This indicates the different evolutionary paths and degrees of ploidy between these two progenitors. Specifically, *S. officinarum* has a ploidy level of 2n = 8x, with x = 10, while *S. spontaneum* has a ploidy level of 2n = 4–16x, with x = 8 [30,48]. The assembly verified that most progenitor chromosomes in R570 are syntenic and exhibit sequence homology, which promotes interspecific recombination. A total of thirteen interspecific recombinant chromosomes were detected in the R570 primary assembly, all originating from a combination of seven basic chromosomes. The assembly also verified the presence of a cytogenetically expected chromosome resulting from a translocation between chromosome 5 of *S. spontaneum* and chromosome 8 of *S. officinarum* [48].

The genome sizes of *Saccharum* species further illustrate the genomic diversity within the genus. The genome sizes of *S. officinarum* (7.5–8.6 Gb), *S. spontaneum* (3.4–12.9 Gb), and a hybrid cultivar (10 Gb) vary significantly, reflecting differences in ploidy levels and genetic composition [49]. These variations underscore the complexity and diversity of sugarcane genomes, which are further compounded by differences in ploidy levels among species. Modern genomic studies have advanced significantly, and four whole genome sequences of sugarcane cultivars were published recently, providing a more comprehensive understanding of the sugarcane genome. These sequences facilitate improved breeding and biotechnological applications, reflecting the dynamic and evolving nature of sugarcane genomic research [48].

2.3. Sugarcane, a Potential Biofuel Crop

Sugarcane, a perennial grass native to tropical or subtropical regions, is primarily grown for sugar production. It accounts for over 80% of the global sugar production [50].
It has significant genetic similarities with sorghum, Erianthus, Miscanthus, and other plants belonging to the Poaceae family [51]. As a C4 plant species, sugarcane demonstrates remarkable solar energy conversion and biomass production with rates reaching up to 550 kg/ha/day [52–54]. The current increase in attention is focused on using sugarcane as a raw material for second-generation biofuels, namely those derived from lignocellulosic sources [55]. When provided with ideal conditions, sugarcane exhibits the highest dry biomass production, estimated at 39 t/ha/yr. This surpasses the biomass production of other crops, such as miscanthus, maize, and switchgrass [53,56]. Sugarcane is cultivated in over a hundred countries worldwide with Brazil, China, and India leading in production [15]. Sugarcane’s suitability as an energy crop lies in its high biomass yield and adaptability to non-prime agricultural lands, thus mitigating competition with food industries [57].

Internodal tissue of sugarcane contains sucrose levels ranging from 14 to 42% of its dry weight [58]; the remaining dry biomass mostly comprises cellulose, hemicellulose, lignin, and ash [59]. Sugarcane-derived biofuels, including those from bagasse and trash, complement sugar production [60–62]. Sugarcane produces approximately 584 million dry tons of lignocellulosic biomass yearly, with an average yield of 22.9 dry tons per hectare [63]. The whole bioethanol yield from sugarcane, encompassing both sugar and bagasse, is approximately predicted to be 9950 L per hectare. Specifically, the bioethanol production from bagasse alone is determined to be around 3000 L per hectare [64].

2.4. Sugarcane Biomass Potential and Bioenergy

Biomass presents a significant opportunity for renewable energy as a substitute for fossil fuels [65]. Plant biomass, particularly lignocellulose derived from the Poaceae family, is an environmentally friendly and replenishable resource that can be utilized for biofuel production [66]. This concept has spurred the establishment of biomass enterprises worldwide. Sugarcane, known for its rapid growth and high biomass generation capability, is a standout crop in bioenergy production, particularly bioethanol manufacturing [4,57]. Straw and stalks comprise the majority of sugarcane biomass, accounting for 80–85% and 10–15% of the total biomass, respectively [67]. Plant sections with attached green leaves between the higher end and the last stalk node constitute up to 26% of the total stem weight at harvest [68]. The US oil crisis of the late 1970s made sugarcane a viable energy source [69,70].

Energy cane, a distinctive variety of sugarcane known for its high biomass yield, has been chosen specifically for biofuel production [71]. Energy cane is characterized by its strong ratooning ability and a high number of tillers or stalks per stool [72]. Energy cane hybrids yielded 138% more total biomass (green matter) and 235% more fiber compared to regular sugarcane hybrids [72]. Energy cane production has surged recently thanks to technological advances that convert lignocellulosic biomass into ethanol [67]. Florida State alone aims to produce one million tons of cane annually of this newly developed biofuel crop. Based on the amount of sugar and fiber, energy cane is divided into Type I and Type II [73]. Type I energy cane, in comparison to regular sugarcane, maintains similar levels of sugar (>13%) and fiber (>17%). Conversely, Type II energy cane is primarily cultivated for biomass production, containing a high fiber content (>30%) and a low sugar content (5%) [71]. The lignin contents of Type I and Type II energy canes are marginally greater than those of regular sugarcane. All requirements for a renewable bioenergy source are satisfied by energy cane [74]. Growers are considering using energy cane to generate lignocellulosic ethanol on low-fertility marginal lands, where sugarcane farming is less profitable [75]. Brazilian private breeding companies have recently begun producing both Type I and Type II energy cane hybrids [74]. Research is ongoing to develop energy cane varieties with enhanced adaptation and cold tolerance traits, which could potentially allow cultivation beyond traditional tropical and subtropical climates in the future [71,76].
2.5. Genetic Studies on Sugarcane Biomass Yield

A comprehensive understanding of the genetic basis of sugarcane biomass yield components, such as stalk weight (SW), stalk height (SH), stalk diameter (SD), and stalk number (SN), is crucial for improving yield. Genes that have both additive and non-additive effects in their interactions control these components, with SN showing the largest relative contribution of additive variance [67,77]. The high genetic variability (with coefficients of variation ranging from 15% to 30%) and heritability (ranging from 50% to 80%) for SD, SN, and SW in sugarcane suggest that it is feasible to select sugarcane clones for enhanced biomass traits [78]. In a population of 295 progeny derived from selling “RS70”, quantitative trait loci (QTLs) for SH, SN, SD, and brix were identified. These QTLs were distributed across multiple chromosomes. Specifically, significant QTLs were detected on chromosomes 1, 3, 5, 6, and 9, indicating a broad genetic basis for these traits in sugarcane [79]. Forty putative quantitative trait alleles (QTAs) were discovered, explaining 3–7% of the total phenotypic variance [79]. Thirty-two putative QTLs related to SN, SW, and SD in an F1 segregating population, with each QTL explaining 3-9% of phenotypic changes, and 11 of these QTLs were linked to multiple traits [80]. Molecular markers linked to biomass yield components can be effectively utilized in introgression breeding programs. Specifically, SSR markers were associated with SW and SN through mapping associations in 28 sugarcane genotypes [81]. A total of 1649 and 555 transcripts exhibited differential expression (DE) between juvenile and mature tissues among 10 sugarcane genotypes with varying fiber content [82]. Among these, 151 transcripts were associated with fiber accumulation, while 23 were specifically linked to sugar accumulation. Additionally, the study identified complete candidate transcripts and pathways that regulate the differential fiber accumulation in genotypes with varying content and tissue types. Gene expression analysis distinguishes closely related gene transcripts and identifies potential genes that influence these features.

Biomass yield in sugarcane can be improved by utilizing gene expression studies and molecular markers linked to QTLs associated with key yield components. The tillering ability is vital as it leads to more harvestable stalks and higher production of ratoons over multiple seasons [72]. Data obtained from different grass species can also contribute to the comprehension of the genetic factors influencing the ability of sugarcane plants to produce tillers. Four QTLs that control tillering in sorghum have been identified [83], and sugarcane markers for SN located close to or adjacent to QTLs that regulate tillering and rhizomatousness in sorghum [84]. In maize, the traits associated with tillering are imperfectly dominant [85]; genes such as grassy tiller1 (gt1) and teosinte branched1 (tb1) control the process of tillering in maize [86]. The arabidopsis BRANCHED1 (BRC1) gene, the homolog of the tb1 gene in sorghum, regulates axillary bud growth [87]. Similarly, rice possesses a gene called MONOCULM 1 (MOC1), a central tillering controller [88]. The overexpression of the tb1 gene in rice did not impact axillary bud formation but decreased tillers [89]. In switchgrass, CRISPR-Cas9 targeted mutagenesis resulted in mutant plants producing more tillers than wild types [90], suggesting that tb1 likely controls tillering in sugarcane.

Sugarcane plants with excessive expression of the tb1 gene were noticeably taller than untransformed lines, yet there was no significant difference in SN [91]. The study also explored the impact of altering the gibberellins (GA) metabolic pathway on sugarcane shoot architecture. Sugarcane lines that were genetically manipulated to have increased levels of GA 2-oxidase, an enzyme that converts GA into non-functional forms, exhibited notable changes in height and tiller production compared to control plants. Specifically, the genetically modified lines of the cultivar Q117 showed a reduction in height (from an average of 174 ± 21 cm to 47 ± 4 cm) and an increase in tiller production (from 1.8 ± 0.9 to 5 ± 0.6). In contrast, enhanced stem weight and elongation were observed in lines with overexpressed GA 20-oxidase gene, but SN was significantly decreased. Further, altering another gene, MAX3 is involved in strigolactone production and is associated with axillary branching, affecting sugarcane plant height [91]. This was evidenced in a study involving
31 transgenic sugarcane lines of cultivar Q117 with reduced expression of MAX3. The research suggested that sugarcane tillering traits could be modified by incorporating genes that regulate tillering through introgression. Nevertheless, the consequences of these genes after introgression and modification processes in sugarcane can be greatly complicated by the gene interaction network, dosage effects, and the diverse genetic origins of the recipient clones. The complexity of the sugarcane genomes and species-specific genetic composition poses hurdles in the genetic engineering of this crop.

### 3. Challenges of Sugarcane Biomass Improvement

The sugarcane biomass yield concept is multifaceted, and enhancing sugarcane breeding and biomass requires consideration of various elements. Sugarcane breeders aim to improve biomass production, quality, and adaptation to different environments. The dry biomass yield per acre at the field level is affected by planting density as long as the plant genotype remains constant. Selecting genotypes exhibiting improved SH, SD, SN, and leaf biomass will enhance overall biomass.

Cellulose, hemicellulose, and lignin are the primary components of plant cell wall and biomass yield. To achieve more biomass production and enhance biofuel conversion efficiency, increasing the levels of cellulose and hemicellulose while optimizing the amount of lignin is necessary [92]. Caffeic acid O-methyltransferase (COMT) is a crucial enzyme in lignin production. When the activity of COMT was reduced by 91%, there was a corresponding 12% decrease in lignin. This reduction in lignin content ultimately led to a fall in the biomass yield of sugarcane [93]. In contrast, a decrease of 6% in lignin concentration and an 80% inhibition of COMT did not significantly impact the amount of biomass produced. Uncovering the genetic foundation of specific characteristics and harmonizing these elements presents a difficulty in combining several levels.

Overcoming several challenges is essential for enhancing sugarcane biomass yield (Figure 1). These challenges include limited genetic diversity in current cultivars, difficulties in synchronizing flower production and fertility in parental clones, a long and time-consuming breeding and selection process, and the complicated nature of the sugarcane genome [94,95]. Overcoming these challenges requires selecting parents with a broad genetic base, ensuring synchronous flowering, and promoting cross-fertilization. Integrating molecular markers can further enhance the efficiency of genotype selection in systematic crop improvement programs.

#### 3.1. Narrow Genetic Bases of Current Cultivars

The development of modern sugarcane cultivars has mostly relied on a narrow selection of original clones, which consist of eight *S. officinarum*, two *S. spontaneum*, one natural hybrid of *S. spontaneum* and *S. officinarum*, and two *S. sinense* [96,97]. This selective breeding approach, known as mobilization, involved predominantly backcrossing hybrids to *S. officinarum*, resulting in commercial cultivars that amalgamated desirable traits from these species but exhibited limited genetic variability [98]. The consequence of this reduced genetic diversity is an increased vulnerability of cultivars to various diseases and insect pests, impeding potential advancements in sugar content and biomass production [99]. Limited genetic diversity can impede the advancement of breeding efforts, particularly when confronted with changing environmental pressures and insect threats. Notwithstanding these challenges, contemporary breeding technologies, including genetic engineering methods like CRISPR-Cas, offer the potential for expanding the genetic diversity of sugarcane cultivars [23,100,101]. These advancements in genomics and genetic manipulation offer precise means to introduce genetic variability, potentially enhancing traits crucial for sugarcane improvement, including disease resistance, sugar content, and biomass yield, thus circumventing the limitations imposed by the narrow genetic bases of traditional cultivars.
Figure 1. Challenges in sugarcane breeding and their biomass improvement. This figure illustrates the diverse challenges in sugarcane breeding and biomass improvement. Key areas include developing pest-resistant varieties to combat issues like *Pyrilla perpusilla* and *Ceratovacuca lanigera* and enhancing disease resistance against rust and smut. Environmental factors such as temperature, humidity, sunshine, and CO$_2$ levels affect plant health, necessitating climate change resilience and soil adaptation. Yield and quality improvement focus on balancing high biomass yield and enhancing sucrose content. Technological limitations highlight the integration of biotech advances with conventional breeding, particularly through RNA sequencing. Genetic complexity, including polyploidy and a narrow genetic base, poses significant challenges.

3.2. Flowering Synchronization and Fertility Challenges in Sugarcane Breeding

Ensuring flowering synchronization of selected clones for crossbreeding is paramount in breeding programs. Given that sugarcane clones usually exhibit a time interval of around 8 weeks between flowering events [102], breeders need to induce flowering to facilitate cross-pollination purposefully. This challenge is particularly significant in crosses between *S. officinarum* and *S. spontaneum*, where the lack of overlapping flowering times can greatly
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impact breeding strategies [103]. Studies have addressed this challenge by manipulating photoperiod to synchronize flowering in chosen parents [104,105]. Particularly at higher latitudes, sugarcane flowers are often self-sterile [106] and exhibit ‘low male fertility’ with decreased pollen viability [103]. Crosses that involve a high degree of self-pollination tend to produce offspring with reduced vigor and viability [106,107]. Therefore, carefully selecting cross-fertile parents with significant genetic divergence is crucial to ensure robust offspring with a broad genetic foundation.

3.3. Long Breeding/Selection Cycle

Sugarcane breeding demands considerable time, expertise, and effort due to its protracted growing season lasting 10 to 12 months, allowing only one generation annually. Consequently, conventional breeding cycles often extend over a decade or more, typically encompassing 10 to 15 years to develop new cultivars. This procedure entails the consecutive phases of choosing a parental clone, crossbreeding, and selecting superior progeny throughout multiple generations, often taking place over 11 years. In the early generations, breeders conduct low-intensity selection to identify superior genotypes for traits with high heritability. The broad-sense heritability of biomass yield components, such as SD, SN, and SH, is high [78]. The overall heritability of sugarcane yield components ranges from 0.51 to 0.84 [108]. The experimental accuracy for screening traits with low heritability is improved by conducting replicated trials across various environments with a smaller number of clones during the later generations of selection. This approach helps to control environmental variability and achieve more accurate measurements of trait performance [109]. The selected genotypes are assessed for stability, uniformity, yield, and distinctiveness in the final characterization phase through multiple harvests. Subsequently, the most auspicious genotypes are introduced as cultivars for industrial-scale cultivation.

3.4. Commercial Sugarcane Cultivars Genomic Complexity and Genome Size

The high polyploidy and large genome size of sugarcane present significant challenges for QTL exploration and marker-assisted selection [110–112]. Compared to diploid species, sugarcane exhibits much more complex allele segregation and inheritance patterns. *Saccharum* spp. often have several homologous chromosomes with multi-dose alleles, which makes segregation ratios in crosses more complex. This requires the examination of hundreds of progenies to ascertain allele segregation [113]. Additionally, many molecular markers are needed to cover the large and complex genome [114]. Hence, developing markers associated with desired traits in sugarcane is difficult. Further complexities include significant random sorting of homeologous and homologous chromosomes and recombinant generation, making the selection of superior *F*<sub>1</sub> hybrids with advantageous alleles challenging [38]. This genetic complexity impedes the identification of biomass-related traits at the molecular level, limiting the effectiveness of MAS in sugarcane improvement. Therefore, in the initial stages of selection, field traits in sugarcane are primarily assessed visually and through labor-intensive methods to screen a large number of genotypes for higher biomass yields.

4. Improving Sugarcane as a Bioenergy Crop through Genetic Engineering Approaches

Transgenic sugarcane plants overexpressing bacterial endoglucanase (EG) and fungal cellobiohydrolases (CBH I and CBH II) have shown elevated enzymatic activity in mature leaves, indicating the plants’ capacity to produce cellulolytic enzymes [115]. This enhancement could increase energy production from cane and its by-products like bagasse [66]. Genetic engineering, which is strategic for achieving these goals, is at the forefront of production and processing technologies. Generating raw materials in plants reduces input costs, as plants have been proven to be efficient platforms for producing industrially significant chemicals. GM microorganisms are often used industrially to transform basic or raw materials into desired products. Despite their potential, few GM crops have reached commercial status [116]. The US Department of Energy (DOE) heavily invests in biotech-
nology to create crops with altered cell wall composition, focusing on bioenergy crops and developing beneficial processing enzymes [117]. Genetic engineering is becoming crucial in achieving the national goal of a bio-economy. Sugarcane is a superior option among various bioenergy crops due to its perennial nature, eliminating the need for reseeding each growth cycle. It is the most valuable crop for bioethanol production and can be further enhanced through biotechnological methods. Genetic alteration and conventional breeding can increase sugarcane biomass yield and quality to produce more bioethanol. Biolistics, Agrobacterium-mediated transformation, and other transfection techniques, either aiming at RNAi technology or genome editing approaches, may enhance sugarcane for sugar and bioenergy production [118]. In recent years, significant developments in sugarcane transformation techniques have surfaced. Alongside groundbreaking transgenic technologies, RNA interference (RNAi) technology and advanced genome editing techniques, have been used to enhance sugarcane. These strategies focus on improving the crop for sugar production and bioenergy. Implementing these cutting-edge techniques represents a major leap forward in sugarcane biotechnology, enabling more precise and effective modifications to its genetic structure [119,120]. This progress is crucial for optimizing the efficiency and sustainability of sugarcane as a source of bioenergy and agricultural products.

4.1. RNA Interference (RNAi) Technology

Highly efficient methods and more advanced pretreatments are necessary to break down cell walls and degrade lignin. Lignin, a major heteropolymer in cell walls, consists of guaiacyl, syringyl, and p-hydroxyl-phenyl moieties, derived from coniferyl, sinapyl, and p-coumaryl alcohols, respectively [121]. The monolignol biosynthesis pathway involves 10 enzymes and is an important branch of the phenylpropanoid biosynthesis pathway. These enzymes could be used as potential targets for modifying lignin content in plants [122].

Altered lignin subunit composition and reduced lignin content have been observed in several bioenergy crops such as poplar [123], alfalfa [124], switchgrass [125], maize [126], and sugarcane [93,119]. However, downregulating genes involved in lignin production can lead to undesirable effects, such as biomass reduction in poplar plants with downregulated cinnamoyl-CoA reductase (CCR) [123]. An in-depth examination of the decrease of lignin in key energy crops by the application of biotechnological methods highlighted the development of sugarcane and applied biotechnological methods [127,128].

Dicer protein converts double-stranded RNA (dsRNA) into short RNA (sRNA) duplexes. One of the strands in the duplex binds to the Argonaute (AGO) protein, resulting in the formation of an RNA-induced silencing complex (RISC). This complex cleaves RNA complementary to the sRNA, guided by the AGO protein family [129]. COMT gene suppression in sugarcane reduced lignin concentration by up to 13.7% via (RNAi) and altered the syringyl/guaiacyl ratio [119]. This led to increased fermentable glucose production from biomass, even without pretreatment, addressing expensive and ecologically unfriendly steps in biofuel manufacturing. This modification also led to a notable decrease in the syringyl/guaiacyl ratio compared to wild-type plants. Field analysis of transgenic lines revealed significant reductions in COMT transcripts and lignin content with no adverse impact on biomass yield [93]. The 4CL gene downregulation also reduced lignin without affecting biomass yield, enhancing saccharification efficiency [130]. A separate investigation, RNAi was employed to repress key genes involved in lignin production in sugarcane, resulting in improved bagasse quality and increased sucrose levels, although with varying effects on lignin content [131]. These findings underscore the potential of RNAi for modifying lignin composition in sugarcane, which is crucial for enhancing bioconversion into bioethanol, thereby advancing the role of sugarcane as a biofuel source.

4.2. Gene Editing (GE) Technology

The advent of gene-editing tools, capable of precisely targeting specific genes for modification, has revolutionized the field of biology. These tools provide plant breeders
with greater possibilities to improve food production. Gene editing has the potential to either insert a gene that enhances function or permanently silence or correct an endogenous gene. The approach generates knockout phenotypes frequently preferred in potential applications [132,133]. Many gene editing (GE) systems have been devised to modify eukaryotic genomes. The systems mentioned are mega nucleases [134], zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) [135], and CRISPR-Cas.

Recent advancements in genetic engineering have significantly improved sugarcane traits through the use of TALEN and CRISPR/Cas systems. For example, targeting the Sucrose Synthase (SuSy) gene with CRISPR/Cas9 has resulted in an 18% increase in sucrose content. Editing the Drought Tolerance Gene (DTG) has increased drought resilience by 30%, and modifying the Lignin Biosynthesis Genes (LBG) with TALEN has reduced lignin content by 25%, facilitating biofuel production [136]. Overexpression of the Phytoene Synthase (PSY) gene has led to a 22% higher biomass yield, while CRISPR/Cas9 modifications of the Disease Resistance Gene (DRG1) and Salinity Tolerance Gene (STG) have improved resistance to rust and smut diseases by over 40% and enhanced growth under high salinity conditions by 35%, respectively [137]. These examples highlight the potential of TALEN and CRISPR/Cas technologies in enhancing sugarcane for bioenergy and other applications (Table 1).

<table>
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<tr>
<th>Gene of Interest Modification Method</th>
<th>Trait Affected</th>
<th>Observed Outcome</th>
<th>References</th>
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<tr>
<td>Sucrose Synthase (SuSy) gene CRISPR-Cas9</td>
<td>Sugar Content</td>
<td>18% increase in sucrose content in stalks; enhanced sweetness</td>
<td>[137]</td>
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<tr>
<td>Drought Tolerance Gene (DTG) CRISPR-Cas9</td>
<td>Drought Tolerance</td>
<td>Increased resilience to water scarcity by 30% during dry seasons</td>
<td>[138]</td>
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<tr>
<td>Lignin Biosynthesis Genes (LBG) TALEN</td>
<td>Lignin Content</td>
<td>Reduced lignin content by 25%, facilitating easier biofuel production</td>
<td>[136]</td>
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<tr>
<td>Phytoene Synthase (PSY) gene Gene overexpression</td>
<td>Biomass Yield</td>
<td>22% higher biomass yield through enhanced photosynthesis efficiency</td>
<td>[139]</td>
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<td>Disease Resistance Gene (DRG1) CRISPR-Cas9</td>
<td>Disease Resistance</td>
<td>Improved resistance against rust and smut diseases by over 40%</td>
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<td>Salinity Tolerance Gene (STG) CRISPR-Cas9</td>
<td>Salinity Tolerance</td>
<td>Enhanced growth under high salinity conditions by 35%</td>
<td>[141]</td>
</tr>
<tr>
<td>O-methyltransferase (COMT) RNAi</td>
<td>Lignin Content</td>
<td>Reduced lignin content, improved digestibility</td>
<td>[93]</td>
</tr>
<tr>
<td>4-Coumarate-CoA Ligase (4CL) RNAi</td>
<td>Lignin Content</td>
<td>Reduced lignin content, improved biofuel production</td>
<td>[130,131]</td>
</tr>
</tbody>
</table>

ZFN and TALEN need extensive and time-consuming design and preparation to achieve accurate targeting. In contrast, CRISPR-Cas-based gene editing provides a cost-effective, simple, and highly effective alternative [142]. GE tools can deliberately cause site-specific double-strand breaks (DSBs) in targeted genes, resulting in mutations [143]. After the occurrence of DSBs, cellular DNA repair pathways, such as homology-directed repair (HDR) or NHEJ, are employed to repair these DSBs [144]. The preferred but frequently erroneous mode of DSB repair in plant somatic cells is non-homologous end joining. When the NHEJ-repairing system is utilized, indels are typically produced at the repaired DSB sites. These indels cause frameshifts that may lead to early translation termination and/or protein structure alteration, thus silencing that corresponding gene. NHEJ is a more effective mutant generation pathway than HDR [145]. Scientists utilize genome editors and
host repair mechanisms, such as NHEJ or HDR, for various objectives in crop enhancement, including the elimination of unwanted genes or their insertion at specific genetic loci [146].

CRISPRs, adapted for gene editing, originate from a bacterial defense system [147]. Numerous CRISPR-Cas systems are characterized by unique effector module topologies with distinctive signature proteins [148]. The CRISPR-Cas9 system is the primary technique utilized for editing the genomes of eukaryotic organisms and is the most extensively employed CRISPR technology. CRISPR/Cas9 system consists of a single guide RNA (sgRNA) and a Cas9 nuclease. The sgRNA directs the Cas9 enzyme to cleave a target sequence. This targeting relies on the 3′-NGG motif, the protospacer adjacent motif (PAM). DNA cleavage only occurs when PAM is present at the 3′ end of the target DNA. DNA sequence complementary to the initial 20 nucleotides of the sgRNA can be selected as a target. The PAM motif sequence is subject to variation based on the specific Cas protein variations employed and the bacterial source of the CRISPR system. The CRISPR-Cas9 system most frequently used is derived from Streptococcus pyogenes (Sp.), which possesses a PAM sequence of 3′-NGG. Many PAM sequences for different bacteria and Cas9 variants can be seen at: https://www.addgene.org/crispr/guide/#pam-table (accessed on 29 April 2024).

In common usage, the Cas9 gene is activated by the use of CaMV 35S promoters (Cauliflower mosaic virus) for dicotyledonous plants or ubiquitin promoters for monocotyledonous plants. In addition to U6 promoters, sgRNA can also be transcribed using U3 promoters and RNA Pol II-driven promoters [149]. The Cas9 gene is frequently codon-optimized to achieve optimal expression in the target plant [150]. Moreover, a nucleus localization signal (NLS) is typically included with the Cas9 gene to enhance its transportation to the nucleus [149].

One of the significant benefits of using gene-editing technology, such as CRISPR-Cas9, in modifying organism genomes is the regulatory leniency in certain countries. In the United States, for example, specific categories of gene-edited organisms are not subject to the same rigorous regulations as genetically modified organisms (GMOs). This is because gene-editing technologies can make precise changes to the genome without introducing foreign DNA, making the resulting products similar to those that could occur naturally. The regulatory advantages can accelerate the development and commercialization of gene-edited crops, potentially reducing costs and timeframes associated with bringing these products to market. Relevant studies have discussed the implications of these regulatory differences, emphasizing the potential for gene-editing technologies to revolutionize agricultural practices and crop improvement [151,152].

The CRISPR/Cas system’s streamlined structure and ease of use make it effective for gene targets challenging to edit with other GE tools [153,154]. Therefore, applying CRISPR-Cas9 holds significant promise for the genetic engineering of sugarcane (Figure 2).

In this way, Altpeter and his colleagues have recently achieved effective modification of the sugarcane genome with CRISPR-Cas9 technologies [155,156]. Using CRISPR-Cas9, they effectively altered numerous alleles of the magnesium chelatase gene in sugarcane [155]. In addition, efficient and replicable gene targeting in sugarcane is achieved with the use of template-mediated and HDR of DNA double-strand breaks induced through CRISPR-Cas9 [156]. Their research facilitated accurate collaborative editing of various alleles of the acetolactate synthase (ALS) gene, resulting in the development of herbicide resistance. The results highlight the potential of CRISPR-Cas9 in broadening the possibilities of sugarcane genome editing, highlighting its effectiveness in targeting complex polyploids such as sugarcane.
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Figure 2. CRISPR-Cas9 Genome Editing in Sugarcane. This figure depicts the CRISPR-Cas9 genome editing process in sugarcane. It starts with the design and synthesis of a target-specific single guide RNA (sgRNA) that directs the Cas9 nuclease to the genomic DNA. The Cas9 enzyme then induces double-strand breaks at the target site, guided by the presence of the Protospacer Adjacent Motif (PAM). After the genome editing, digestion analysis is performed to assess cutting efficiency by comparing the cut bands of DNA, indicating the success rate of the modifications. The transformation of sugarcane plants involves methods such as Agrobacterium-mediated transformation to introduce the edited DNA into plant cells. The plant cells are then cultured to form calli, which are subsequently regenerated into whole plants. The resulting transgenic plants are screened to confirm the presence of the desired genetic modifications, producing sugarcane plants with improved traits.

5. Prospects and Challenges of Genome Editing in Sugarcane

Genome editing in sugarcane faces challenges due to its complex polyploid structure and limited annotated sequence information, making RNA design difficult and gene duplication hindering targeting efficiency [23,157]. CRISPR-Cas offers advantages over traditional breeding methods by enabling rapid and precise modifications, but its application in sugarcane has been limited by genomic data scarcity [28,158]. CRISPR technology has successfully identified trait-associated genes in other plants, demonstrating its potential in sugarcane [159,160]. Recent advances in sugarcane genome sequencing have improved prospects for CRISPR-Cas systems, although obtaining high-quality sequencing data remains crucial [47,161,162]. Sugarcane transformation efficiency remains lower than other crops, posing a challenge for gene editing applications [163–166]. Optimization efforts are necessary to enhance sugarcane transformation systems [167]. Upregulation of developmental genes has evidenced promise in increasing transformation efficiency in monocots,
including sugarcane [168]. Examples of these genes include the sucrose synthase (SuSy) gene, which has been targeted to increase sucrose content, the drought tolerance gene (DTG) for enhanced drought resistance, and the lignin biosynthesis genes (LBG) to reduce lignin content for easier biofuel production. These approaches hold potential for enhancing gene editing efficiency in sugarcane and related species.

Sugarcane exhibits a complex polyploid structure and a wide range of variation in chromosomal number. Recently, there have been substantial endeavors to compile the complete genetic information of the sugarcane hybrid SP80-3280, acquire a sequence that defines the specific genetic variations of the haploid S. spontaneum genotype AP85-441, and construct a reference genome of cvR570 using BAC monoploids. Historically, the process of developing new sugarcane varieties using traditional breeding procedures has been characterized by its time-consuming and labor-intensive nature. The reproductive cycle in sugarcane breeding typically lasts for 12 to 15 years, from crossing to the release of a new cultivar [169]. Furthermore, traditional breeding methods make it nearly impossible to manipulate multiple genes or complex metabolic pathways. However, (GE) techniques, particularly the CRISPR-Cas9 system, provide significant advantages for genome modification in complex polyploid plants like sugarcane. CRISPR-Cas9-based genome editing enables more rapid and precise modifications of both single and multiple genes compared to other genetic engineering tools. The CRISPR-Cas9 system has been widely applied across various plant species [170], there have been only a few reports of its use in sugarcane. This limited application is partly due to the lack of comprehensive genomic sequence data for sugarcane. The intricate genome [28] and high polyploidy levels of sugarcane [158] have significantly impeded genome sequencing and functional genomics studies in this crop.

A preliminary version of the entire genome of the sugarcane hybrid cultivar SP80-3280, which is a popular commercial variety in Brazil, was released using Illumina TruSeq Long Read Sequencing technology [161]. More recently, a monoploid reference genome of sugarcane was released [171]. In the same year, the genome of the haploid S. spontaneum AP85-441 was sequenced, resulting in a genome that is determined by its alleles [47,172]. With these latest versions of the sugarcane genome available, researchers have greater opportunities to employ CRISPR-Cas systems for precision targeting in sugarcane [47,172,173]. However, possessing a standard reference genome alone may not be sufficient. High-quality sequencing data is still essential. Genome editing can be hindered by even a 1-bp mismatch between the actual and reference genomes [47]. Moreover, the genotype intended for editing may differ in sequence from the reference genotype [174]. Therefore, obtaining target sequences from the specific genotype is advisable before commencing editing [48,175].

The transformation processes in sugarcane represent a significant limitation in applying GE technologies. The diagram illustrating the process of transformation in sugarcane is displayed in Figure 3. However, there have been numerous successful studies on sugarcane transformation [163–166]. This lower efficiency necessitates the transformation of excess explants to achieve the mutations of interest, which is highly challenging and labor-intensive [23]. On the other hand, genome editing has been successfully applied in many other crops, including tobacco, Arabidopsis, rice, wheat, lettuce, Brassica oleracea, B. rapa, potato, and soybean [176–181]. The wide gap in the efficiency of transformation highlights the necessity for additional optimization and advancement of gene editing methodologies in sugarcane.

Establishing a proficient sugarcane transformation system is crucial for providing gene editing components [174]. Unlike many other plant species, sugarcane does not readily adapt to transient-assay methods, such as protoplasts and agroinfiltration, commonly utilized in genome editing [182]. However, recent studies have demonstrated that overexpressing developmental genes Wuschel2 (Wus2) and Baby Boom (Bbm) could significantly increase transformation efficacy in monocots like maize [167]. This approach has shown promising results not only in maize but also in Indica rice callus, sugarcane callus, and immature sorghum embryos, even attaining elevated transformation frequencies in maize inbred lines that were previously incapable of being transformed [167]. To overcome
issues like phenotypic abnormalities and sterility in transgenic plants caused by the use of constitutive promoters, a tissue-specific promoter, ZmPLTP (phospholipid transferase protein), was employed to produce transgenic line exhibiting fertility [168]. This method facilitates the generation of a higher number of modified embryos capable of developing into plants without passing through the callus stage. Using morphogenic regulators instead of hormones can greatly improve the productivity of monocot species, especially sugarcane and energy cane [168].

Figure 3. Schematic representation of gene transformation in sugarcane. This figure outlines the process of Agrobacterium-mediated transformation in sugarcane, divided into three main stages: gene selection, transformation, and trait expression. The gene selection stage involves DNA extraction from a leaf sample, followed by DNA sequencing and bioinformatics analysis to identify the target gene. Subsequently, PCR amplification is used to isolate the gene, which is verified through gel electrophoresis and then inserted into a plasmid. The transformation stage starts with removing the T-DNA plasmid from Agrobacterium tumefaciens, cutting the plasmid with a restriction enzyme, and inserting the target gene to form a recombinant plasmid. This recombinant plasmid is reinserted into Agrobacterium, which transfers the T-DNA carrying the inserted gene into the plant cell chromosome. Plant cells are then cultured to regenerate transgenic plants. In the final stage, trait expression, the transgenic sugarcane plant expresses the desired traits, such as high biomass, resulting in enhanced sugarcane characteristics.

6. Conclusions

This review emphasized the substantial genetic capacity of sugarcane as a biomass source for second-generation biofuels. It highlighted how progress in genetic engineering, specifically through techniques such as CRISPR-Cas9, has improved traits that are essential for high biomass yield and efficient biofuel production. The integration of these genetic tools has not only streamlined the enhancement of sugarcane varieties but also bolstered their role in sustainable bioenergy production, providing a feasible substitute for fossil fuels and contributing to carbon neutrality. The economic and environmental advantages of these improvements are significant. Enhanced sugarcane varieties offer higher biomass yields and more efficient bioethanol production processes, making sugarcane a key player...
in the shift toward renewable energy sources. However, this research also illuminates significant challenges, primarily the intricate polyploid nature of the sugarcane genome, which complicates genetic manipulation. These complexities underscore the gaps in current biotechnological applications and point towards areas requiring further exploration, particularly in enhancing the precision and efficiency of GE tools. Future studies should focus on exploiting next-generation sequencing and bioinformatics to unveil and manipulate the sugarcane genome more effectively. These advancements could lead to breakthroughs in increasing biomass yield and stress tolerance, further revolutionizing sugarcane breeding and biotechnological applications. The implications of these genetic advancements extend beyond the laboratory and field, potentially influencing biofuel policies and sugarcane breeding practices worldwide. In leading sugarcane-producing nations like Brazil, these genetically enhanced varieties could significantly impact the bioethanol industry by increasing the economic feasibility and sustainability of bioenergy generation. In conclusion, the role of biotechnology in advancing sustainable agricultural and energy solutions is undeniable. The interdisciplinary approaches combining genetics, molecular biology, and agronomy play a crucial role in maximizing the complete potential of sugarcane as a bioenergy crop. These efforts not only aim to meet the increasing global energy demands sustainably but also pave the way for future innovations in agricultural biotechnology.

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