Review
An Updated Overview on Insights into Sugarcane Genome Editing via CRISPR/Cas9 for Sustainable Production

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Abstract: Sugarcane crop constitutes one of the most vital sources of sugar and bioenergy globally; however, higher level of polyploidy makes its genome editing an intricate task. Recently, genome editing has become easier with CRISPR/Cas9 system that uses Cas9 to target sequence-specific regions and introduce double-strand breaks into the target region. This technique has been successfully employed to develop new varieties of sugarcane having desired phenotypic and physiological traits. Several genes can be fused with the CRISPR/Cas9 system leading to successful metabolic engineering and biological improvement for ensuring sustainable enhancement in sugarcane production. This simple RNA-guided genome editing technique has become a revolutionary tool and innovative application in biology that might be effectively employed for inducing specified genomic modifications in plant tissues. This review attempts to synthesize fundamentals of genome editing techniques with an ultimate aim to find out opportunities and challenges of sugarcane genome editing. It also tends to summarize the advances and achievements of gene editing via CRISPR-based genome editing technique for numerous field crops. Lastly, the enormous potential of CRISPR/Cas9 for gene editing in sugarcane, major challenges and future perspectives have been objectively elaborated.

Keywords: biotechnological tools; sugarcane biology; gene editing; molecular markers; sucrose content

1. Introduction

Globally, sugarcane (Saccharum spp.) is one of the most pertinent agricultural cash crops that is widely cultivated in countries having subtropical and tropical climates such as United States, India, Pakistan, China, Brazil, Australia, Cuba, and Philippines [1–3]. A number of industrial high-end products including sugar (75% of sucrose’s global demand is met from sugarcane while remaining comes from sugar-beet crop), biofuel, different types of waxes, and a diverse variety of bio-fibers are obtained from sugarcane worldwide [4]. There are two wild species (S. robustum and S. spontaneum) and ur mainly cultivated species (S. edule, S. barbieri, S. sinense, and S. officinarum) of the Saccharum genus [5]. Saccharum spontaneum has recently emerged as an important genetic resource for utilization in various breeding programs of sugarcane [6,7]. Most of the new sugarcane varieties have been developed from the interspecific hybridization of Saccharum officinarum and Saccharum spontaneum. The resulting varieties tend to be polyploids and aneuploids with chromosome counts ranging from 80 to 120 [8–10].
Historically, sugarcane crop improvement has remained centered toward sucrose content enhancement; however, recently fiber, lignin content, and biomass have become important components of modern breeding strategies [11–14]. It has been established that success of sugarcane breeding programs in order to acquire the desired traits will depend on the genetic diversity in the active germplasm banks [11–14]. For cultivar development, the regular provision of germplasm collections with accessions to diverse genetic backgrounds and quantifying genetic variability in these collections have remained essential tasks for improved management and conservation [15,16]. Recently developed biotechnological tools and approaches have emerged as potent strategies to improving traditional breeding programs, notably for understanding gene structures, genomic locations, and plant transformations. Numerous molecular studies have investigated the sugarcane genome constitution and structure and have revealed the genome size of sugarcane to be over 10 Gbp, with genes that exist in up to 10–12 allelic forms [17–19]. The estimated monoploid genome size ranges from 800–900 Mb, depending on the ploidy level of crop’s variety [20].

Besides intricate genomic background, recently developed cultivars of sugarcane are highly polyploid despite the fact that their mitochondrion (mt) genomes tend to be smaller and simpler along with being easily manageable which hold potential to furnish valuable phylogenetic information [21]. In other words, complex interspecific polyploid genome of sugarcane varieties has resulted in serious hampering of the complete genome exploration (both nuclear genome and organelle genome especially pertaining to mitochondrial genome and chloroplast) [22]. At present, the whole genome of AP85-441, which belongs to *S. spontaneum* (allele-defined genome of tetraploid) has been assembled by Zhang et al. in 2018 [23]. Modern sugarcane cultivar R570 based on bacterial artificial chromosome (BAC) clones have been assembled. The complete chloroplast genome of two sugarcane ancestors *S. officinarum* and *S. spontaneum* were assembled and analyzed [24]. With the development of next-generation sequencing technologies (NGS), a growing number of mt genomes have been assembled. At present, more than 300 complete mt genomes have been submitted to GenBank Organelle Genome Resources. The complete mitochondrial genome of modern commercial sugarcane cultivars has been obtained and analyzed [15,24,25].

Considering the interest in developing sugarcane cultivars for acquiring the desired traits, this review study provides latest insights on biotechnological approaches employed for breeding new sugarcane cultivars. Additionally, the literature has been objectively examined in an attempt to provide an amalgamation of fundamental and recent advances on gene editing technology for knocking out or inserting foreign genes to acquire desired traits in sugarcane. This study places special focus on CRISPR/Cas9 system application, its components delivery into the targeted crop genome, and advancement of CRISPR/Cas9 system for polyploidy crops such as sugarcane along with different factors affecting its efficacy. Finally, current status of genome editing in sugarcane, challenges and future perspectives have been objectively and critically highlighted.

### 2. Fundamentals of Gene Editing Technology

The use of gene-editing technology has provided great opportunities to researchers in the field of molecular biology by facilitating the modification and targeting of specific genes accurately. Through gene editing technologies, plant breeders have been able to develop new cultivars with desired traits. The gene editing processes can successfully knockout or insert any gene for inducing a particular function or trait [26]. Previously, numerous transgenic systems for eukaryotic genome manipulation have been developed including clustered regularly interspaced short palindromic repeats (CRISPR), transcriptional activator-like effector nucleases (TALENs) [27–29], and zinc finger nucleases (ZFNs) [30,31]. Among gene editing techniques, CRISPR/Cas9 system provides a low-cost, simple, and easy-to-use method for gene editing. In CRISPR/Cas9 tools, it is possible to induce site-specific double-strand breaks (DSBs) in related genes to obtain mutations. This has assisted to overcome the limitation of ZFNs and TALENs which have complex technical design processes to achieve specific objectives making them highly time-consuming and onerous in
functioning [32]. However, CRISPR/Cas9 system assist in preparing double strand breaks that can be repaired by the cellular DNA repair mechanisms, namely the non-homologous end junction (NHEJ) or homologous directed repair (HDR) pathways [33]. Figure 1 illustrates few basic and advanced gene editing approaches that are being employed to develop crops varieties having desired agro-botanical traits and physiological characteristics.

Figure 1. The basic genome editing techniques for developing new cultivars having desired traits.

2.1. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) System

Genome-editing via CRISPR is a recent technological advancement to develop new crop varieties having desired phenotypic and physiological traits along with exploring numerous biological phenomenon in crop plants. Besides having different site-directed nucleases for genome editing, the CRISPR/Cas-based genome editing approach offers a variety of advantages such as simplicity, ease of access, low cost, and flexibility [21,29]. There are different types of CRISPR/Cas systems, and each type is distinguished by a different structure of the effector module, including unique characteristic proteins [34]. The CRISPR/Cas9 is the first system for eukaryotic genome editing and is currently the most widely used genome editing system.

There are two components in the CRISPR/Cas9 system including a single guide RNA (sgRNA) and a Cas9 nuclease. The Cas9 is guided to cut the target sequence by sgRNA, which binds to the target sequence. By detecting the 3’-NGG motif (also called original spacer adjacent motif (PAM)) in the target sequence, sgRNA is able to identify the target sequence. The DNA sequences that complement the first 20 bases of sgRNA may be used as targets, but DNA can only be cleaved if PAM exists at the 3’ end of the DNA target. The motif sequence of PAM varies depending on the bacteria from which the CRISPR system was derived and the Cas protein variations used. The PAM sequence of spcas9 is 5’-NGG, and the PAM sequences for different bacteria and Cas9 variants are presented in Table 1. Transcription of sgRNA is typically driven by the U6 promoter, while Cas9 genes are typically driven by CaMV (cauliflower mosaic virus) 35S promoter or the ubiquitin promoter [35]. The Cas9 genes are usually codon-optimized to enhance the expression
of the target plant species [36]. The nuclear localization signal (NLS) is also often fused to the Cas9 gene in order to direct it toward the nucleus with utmost accuracy [35]. The CRISPR/Cas9 system has been successfully used in genome editing of many plant species for producing mutants having requisite traits [37] (Figure 2 and Table 1).

Table 1. Few prominent CRISPR/Cas9 online resources indicating fundamental information and application protocols.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sources</th>
</tr>
</thead>
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<td>Target site design tool</td>
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<td>Guide RNA design tool</td>
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</tr>
<tr>
<td>CRISPR Multi Targeter</td>
<td>Multiplex design tool</td>
<td><a href="http://multicrispr.net/">http://multicrispr.net/</a> (accessed on 2 August 2022)</td>
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<td>Addgene</td>
<td>Reagents and resources</td>
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<td>RGEN Tools</td>
<td>Potential off-target site prediction tool</td>
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<td>CHOPCHOP</td>
<td>Target sites finding tool</td>
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<tr>
<td>CRISPR-PLANT</td>
<td>Genome-wide gRNA prediction tool in plants</td>
<td><a href="http://genome.arizona.edu/crispr">http://genome.arizona.edu/crispr</a> (accessed on 22 July 2022)</td>
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2.2. Delivery of CRISPR Components

The CRISPR components can be delivered into the plant genome such as DNA, mRNA (in vitro transcript), and proteins [38]. The various types of delivery technologies include infection through penetration by Agrobacterium [39,40], gene knockouts or particle bombardment [41], electroporation [42], and virus-based delivery systems [43]. The mRNA can also be used to deliver genome editing reagents as this type of transient delivery is effective in producing the stable transgenic events along with reducing the threat of deviation from targets [44]. Besides above delivery systems, a pre-assembled Cas9 gRNA ribonucleoprotein (RNP) delivery system has also been reported (Figures 3 and 4) involving the direct delivery of RNP complexes which eliminates the possibility of foreign DNA being introduced into the host genome [45].
Figure 2. The basic steps of genome editing in plants using CRISPR/Cas9/sgRNA technology beginning with the selection of target genes.

Figure 3. The CRISPR/Cas applications. (A) Using CRISPR/Cas, mutations can be introduced, which result in indels, gene deletions, and even multiple gene knockouts. (B) Gene insertion and replacement by homologous directed repair or no homologous end connections.
The CRISPR/Cas9 system has been regarded as the simplest, unprecedently efficient, and highly specific that results in fewer off-targeting in comparison to conventional biotechnological approaches [49,52]. Therefore, it has emerged as a promising tool for plant genome modification. This biotechnological system has been anticipated to strategic impact on plant biology’s basic and applied research for crops including sugarcane. This development should also impact crop breeding. Gene editing facilitates the accurate and predictable modification of good varieties or materials directly rather than the arduous backcross procedure used in traditional breeding methods. CRISPR/cas9 is expected to provide a more effective method for pyramid breeding since multiple traits can be modified simultaneously [57]. Gene knockout by NHEJ is the most direct CRISPR/Cas9 technology application. To enhance crop yields and make the host more resistant to pathogens, negative regulators of grain development and disease resistance can be modified. Other gene modification methods such as gene expression regulation and epigenetic regulation may also be employed for crop improvement. Another advantage of CRISPR/Cas9 is introducing target genes into non-GM footprint crops through penetration, viral infection, or
pre-assembled Cas9 protein sgRNA ribonucleoprotein transformation, thus circumventing traditional regulations of genetically modified organisms [58].

With CRISPR/Cas9 technology, it is possible to delete whole chromosomes [59] or specific genes [60] depending on the target traits intended to be acquired in field crops including sugarcane. A 1.6 KB GUS gene was removed by Srivastava et al. [60] via targeting both ends of a gene with Cas9 and two gRNAs. The transgenic plants tend to remove undesirable plant selection genes (such as the kanamycin resistance gene) while transgenic plants may retain few induced genes that has attracted criticism from regulators and consumers. However, the CRISPR technology holds advantage to replace and repair dysfunctional alleles [61] or create a site for gene integration at specific locations that minimized off-targeting [62].

2.4. CRISPR/Cas9 System in Polyploid Crops

Despite the fact that CRISPR/Cas9-mediated genome editing is widely used in plants, its efficiency continues to remain an unexplored subject. Especially in polyploid crops, it is necessary to knock out all copies of genes with the same function at the same time because of functional redundancy between parahomologous genes and homologous genes. Optimization of Cas9 codon, promoter, and target sequence composition (GC content) could impact the mutation efficiency of polyploid crops [63]. The design of sgRNA in polyploid crops is more complex than in diploid species such as Arabidopsis and rice. Some advanced CRISPR/Cas9 tools have been developed for sgRNA design (CRISPR-p and CRISPR-p2.0); however, limitations still exist for genome editing of polyploid plants [64]. To knock out both homologous and paralogous genes simultaneously, it is necessary to design sgRNA that targets all copies of each gene (parahomologous and homologous genes). The sgRNA can be designed manually after sequence analysis to target all genes or specific gene copies.

Interestingly, when there are not many conserved regions in homologous genes, it is necessary to divide these genes into multiple groups and design sgRNAs based on the conserved regions in each group. A new homologous gene from Streptococcus pyogenes spCas9 has been demonstrated to be effective for gene editing in plants, including Streptococcus thermophilus (stCas9) and Staphylococcus aureus (saCas9) [65]. In Streptococcus pyogenes, the RNA-guided endonuclease Cas9 is too large to be used for genome targeting. SPACas9 from Staphylococcus aureus also targets the genome with high efficiency similar to spCas9; however, its size is shorter than spCas9, which makes it easier to use for genome targeting [66]. The CRISPR/cpf1 is a new variant of CRISPR that has shown its effectiveness in editing plant genomes as CRISPR/Cas9 [67]. The CRISPR/CPF1 PAM is tttn that is suitable for targeting complex regions of the genome such as the promoter region. The CRISPR/cpf1 produces DSB with a 5’ sticky end that tends to promote NHEJ gene mutation during the repair process. In addition, several extended PAM sequences have been identified for Cas9 and cpf1 that imparts extension to the recognition sites for the PAM sequences. Moreover, the CRISPR/Cas9 introduces a base editing function by introducing cytidine deaminase into the genome [57,58].

2.5. Factors Affecting the Activity of CRISPR/Cas9

Among many factors that affect the activity of CRISPR/Cas9, the presence of an effective vector constitute the most strategic factor which ensures successful delivery of cas9 protein and sgRNA to the nucleus in CRISPR/Cas9 gene modification. The Cas9 gene and sgRNA gene can be combined on a single plasmid or separate plasmids [68]. The Cas9 has been driven by promoters commonly used in plant transformation, namely ubiquitin and the 35S promoter of cauliflower mosaic virus (CaMV 35S). It is common practice to attach the Cas9 gene to molecular tags in order to detect/purify proteins and nuclear localization signals (NLS) and to facilitate the entry of the Cas9 protein into the nucleus [69]. Xing et al. [70] have demonstrated the effectiveness of pcambia-based sgRNA module vectors in many plant species. The module vectors were constructed by assembling two or more sgRNA expression cassettes using the golden gate or Gibson assembly methods.
Additionally, it has been demonstrated that the vector has a high mutation efficiency (60–95%) in transgenic lines for maize. Moreover, many biallelic mutations can also be efficiently passed on to the next generation [71].

Besides the presence of effective factor, several other factors influence the mutation efficiency of CRISPR/Cas9, including the specificity of the gene target, the location of the PAM sequence, the nature of the sgRNA sequence, the promoter of the Cas9 gene and sgRNA, the tissue of interest, and the conversion technology used. It has been suggested that target genes must be selected carefully because some genes are essential for cell growth and gene knockout can be fatal to plants. Crops usually contain multiple copies of a gene due to rearrangements, polyplody, or replication. Therefore, the nonspecific nature of sgRNA may result in biallelic mutation or chimerism. Among the acetylactate synthase gene family members in maize, there are Als1 and ALS2 on chromosomes 4 and 5, respectively. It was reported that non-gene specific sgRNAs resulted in biallelic mutations in two ALS genes, resulting in the recovery of unstable events. A sgRNA designed for ALS2 based on the polymorphism between Als1 and ALS2 could induce the necessary mutation as inferred by Svitashev et al. [47]. A target site’s GC content can also affect the stability of DNA sgRNA hybridization. The GC content is high, which allows DNA–RNA hybridization to be stable, but a more stable hybridization also increases miss rates. Up to 35% of the GC content in the target region exhibits good Cas9 enzyme activity with little deviation from the target [72]. A PAM sequence is typically NGG, although nag can also be used to reduce nucleases’ ability to bind to genomic DNA [73]. The NRG’s binding efficiency is only one fifth that of NGG’s. Each base in the PAM sequence influences the binding efficiency of核酸lease. The PAM’s first nucleotide is the least conservative, but the G in position two improves binding efficiency by 90%, therefore NRG’s stability is lower than that of NGG’s [74]. The activity of sgRNA also depends on its length. Long sgRNAs of 19 nucleotides are more effective than truncated sgRNAs of 17–18 nucleotides and longer 22–23 nucleotides sgRNAs.

In addition to target sequence and surrounding sequence characteristics, sgRNA may also impact its function [72]. The CaMV 35S promoter has been extensively used to activate the Cas9 gene in dicotyledonous plants. When Cas9 is expressed in maize or other monocotyledonous plants, the expression of the ubiquitin promoter is more often used. sgRNA is generally expressed using plant RNA polymerase III promoters such as U6 and U3. These promoters have defined transcription initiation nucleotides, which are “g” for U6 and “a” for U3 as reported by Belhaj et al. [35]. In terms of RNA polymerase type III preference, the starting nucleotide is not strict, but it may have a significant impact on the mutation efficiency of targeted genes [59].

3. Current Status of Genome Editing in Sugarcane

Previously, sugarcane genome editing has been reported by Jung and Altpeter [75] (2016), who attempted to reduce the lignin content of sugarcane by knocking out the COMT gene. It has been inferred that target mutation in 74% of the transgenic lines might be achieved and it was also observed that 8–99% of the wild-type COMT alleles could be mutated in each line. Transgenic plants with 99% mutation frequency decreased their lignin content by 29–32% in the greenhouse experiment compared to control untransformed plants. Moreover, the mutant phenotypes and mutations did not differ among the clones of vegetative reproduction. Despite the fact that 99% mutation frequency only leads to 29–32% reduction in lignin content in transgenic plants, while biochemical plasticity may be able to save the target activity of knockouts in certain situations. It has been reported by Smits et al. [76] that about 31 of the quantitative targets produced by CRISPR were found to have residual protein expression from low to original levels, with varying degrees of residual expression in 136 different genes produced by CRISPR-induced frame shift in Hap1 cells. Kannan et al. [77] investigated genome-edited strains’ mutation efficiency and field performance. It was inferred that in mutant lines, the proportion of “butyryl guaiacyl” and lignin level were decreased by 19.7%. Compared to the control plants, these
production lines recorded higher saccharification efficiency (up to 43.8%) without affecting the biomass production. According to the sequence of the COMT region of a mutant line CB6, 107 COMT alleles were edited out of a total of 109 copies. The CB6 recorded a decrease in lignin content by over 19% and an increase in saccharification efficiency (54%), while its agronomic characteristics were similar to the control plant. The results have indicated that transgenic tools might be effectively used for the modification of sugarcane genome. However, a complex genome needs to be operated in a highly specific manner to prevent unwanted mutations and cytotoxicity. Additionally, an ideal genome engineering system should be easy to design and must provide accurate targeting and efficient delivery of cells. Despite the fact that talens are easier to generate than ZFNs, they may result in off-target binding [78]. For effective talen-mediated genome editing, two different nuclease genes must heterodimerize around the target, which may reduce the efficiency. In contrast, CRISPR/Cas9 can be designed and implemented more simply than other GE tools. A simple nuclease and a single gRNA are needed that can be transmitted to the host cells. In some cases, it has been demonstrated that the CRISPR/Cas9 system can successfully modify some recalcitrant gene targets against Tarun [30,38]. The CRISPR/Cas9 technology has the potential to serve as an important tool for gene editing of polyplid crops such as sugarcane. Shabbir et al. [1] recently edited the sugarcane genome using the CRISPR/Cas9 system, while this technique has also been used to edit multiple alleles of the magnesium chelatase gene [79]. Oz et al. [80] also reported that CRISPR/Cas9 was employed to co-edit multiple alleles of the acetyllactate synthase (ALS) gene through template-mediated and homologous directed repair (HDR) for developing herbicide resistance. It may be inferred that CRISPR/Cas9 is effective for genome editing of polyplids such as sugarcane, thereby allowing for greater precision in sugarcane genome editing.

4. Prospects and Challenges of Sugarcane Genome Editing

Sugarcane is a highly polyplid crop having chromosomes in the range of 80 to 120 due to aristocracy. The genome’s size is about 10 GB, and the number of copies of homologous genes range between 8 and 12 [17,18]. The haploid genome size has been reported to be 750–930 MB [81]. It may be inferred that sugarcane genetics is complex and therefore difficult to breed regularly using traditional gene editing techniques and approaches. Currently, genetically modified (GM) sugarcane is being produced. As generic engineering (GE) utilizes engineered nucleases to insert, delete, or replace data in the genome, CRISPR/Cas9 makes genome editing much easier than GE. It has been inferred that introducing targeted mutations in sugarcane might help to increase lignocellulosic and ethanol yield [75]. It has become possible to introduce double-strand breaks at multiple sites in the genome using the CRISPR system and therefore, multiple genes can be targeted simultaneously [82]. Recently, Mohan [83] described the main obstacles to sugarcane genome editing which include designing of specific gRNA for specific genes for which genome sequence information is the primary requirement. However, one of the major problems with CRISPR technology is the deviation from the target. In previous studies, the CRISPR/Cas9 technology has been found to have fewer off-target effects in various plant species [35]. In sugarcane, transgene silencing is another major disadvantage of genetic engineering that hinders crop development as transcriptional and post-transcriptional effects of transgene silencing have been reported by Hansom et al. [84], which might be countered through CRISPR/Cas9 technique. It is interesting to note that one of the major components of CRISPR technology is the mutation analysis that constitutes as the greatest challenge in sugarcane genome editing because of the lack of high-throughput screening methods. To cope with this challenge, different tools for CRISPR analysis such as T7 analysis/detection analysis, PCR, restriction analysis, and Sanger sequencing/ngS might be explored further for imparting desired traits in newly developed genetic lines of sugarcane.

Up till now, deploying these methods to analyze sugarcane genome mutations remains a challenging task, and further studies are necessary to identify appropriate analysis tools. However, CRISPR/Cas9 system holds advantage over conventional breeding that is gener-
ally time-consuming and labor-intensive as the reproduction cycle may take 12–15 years from hybridization to release of new variety with improved traits [85]. The conventional breeding process also makes it difficult to manipulate multiple genes or complex metabolic pathways. Transgenic plant technology, particularly CRISPR/Cas9 system, has several advantages for complex polyploid plant species [37]. There are only a few reports on CRISPR/Cas9-mediated sugarcane gene editing. This is partially due to the lack of information about sugarcane genome sequences. Due to Sugarcane’s complex genome and high polyploidy, it has been very complicated to explore accurate genome sequencing and functional genomics [86].

Additionally, one of the biggest advantages of CRISPR/Cas9 technology is it can identify genes associated with specific traits. For instance, by combining phylogenetic analysis, whole genome sequencing, and CRISPR/Cas9 gene knockout technique, Huang et al. [87] identified many rice high-yield genes. Initially, 28 ancestral chromosome fragments found in all high-yielding rice varieties were identified. Subsequently, 57 genes from 123 loci for gene knockout (or knockout) studies were randomly selected as most of them had phenotypic effects on rice production. Functional genomics screening based on CRISPR/Cas9 is also a powerful method of systematically identifying genes associated with specific phenotypes. An experimental CRISPR library containing 25,265 pooled siRNAs targeting 12,786 genes has been constructed by Meng et al. [88]. In addition, sgRNA was cloned to generate a plasmid library for rice transformation, which resulted in more than 14,000 independent, 10-line lines that demonstrated a high edit frequency. Out of the 200 strains tested, 54 changed the morphological phenotype. Over the past few years, the lack of genome sequence data has been a major obstacle in editing sugarcane genomes using GE tools. A genome-wide draft of Brazil’s popular commercial sugarcane hybrid sp80-3280 has been produced using truseq technology from Illumina [89].

The haploid reference genome of sugarcane was published by Garsmeur et al. [20]. During the same year, Zhang et al. [23] sequenced the genome of haploid spontaneous Streptococcus ap85-441 and obtained the genome defined by alleles. Researchers will have better opportunities to accurately locate sugarcane crops using the CRISPR/Cas9 system because detailed genome mapping of sugarcane has become available recently [17,18]. However, having a reference genome may not be enough and additional information of high quality is still required which necessitates conducting further in-depth studies. Even if there is a 1-BP difference between the real genome and the reference genome, it becomes difficult to perform genome editing. Furthermore, the genotype sequence to be edited may differ from the reference genotype. Consequently, the target sequence of a given genotype should be obtained before editing. Another disadvantage of germanium in sugarcane is its time-consuming transformation process. Sugarcane has been successfully transformed numerous times [22,24,25], but its transformation efficiency has remained quite low compared to other crops [90]. Thus, developing an efficient sugarcane transformation system can be of great importance to transport transgenic components and acquire desired trait through effective genome editing [21].

Interestingly, transient analysis techniques such as ag infiltration and protoplast use are not applicable to sugarcane, even though these techniques are typically used for genome editing in a wide array of plant species. Lowe et al. [91] demonstrated that overexpressing baby boom (BBM) and wuschel2 (wus2) gene in maize (maize) can enhance transformation efficiency of monocotyledons. It has also been demonstrated that using this method can increase the transformation efficiency of young embryos of sorghum (two-color sorghum), sugarcane callus (sugarcane), and rice (Oryza sativa SSP indica) as well as produce a high level of transformation in inbred lines of maize that previously were un-transferrable [92–95]. As the use of constitutive promoters often leads to phenotypic abnormalities and sterility in transgenic plants, so the tissue-specific promoter ZM PLTP (phospholipid transferase protein) have been used for stimulating fertilization of transgenic plants [96–99]. Using this method, many embryos can be transformed and germinated directly into plants without undergoing the growth process of a callus [100]. Thus, using
this morphogenetic regulator rather than hormones might lead to significant improvement in the transformation efficiency of monocotyledonous plant species such as sugarcane. Thus, despite the challenges, targeted base gene editing without induction of a foreign DNA donor [9,101–107] or cleavage of double-stranded DNA [108–111] in sugarcane hold bright perspectives to accelerate genome modification (Tables 2 and 3) and breeding for boosting its productivity and sugar recovery on sustainable basis.

Table 2. Some of mutation efficiencies reported in CRISPR/Cas9 systems.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Plant</th>
<th>Gene Targeted</th>
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<th>Indel Frequency</th>
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<td>ALS</td>
<td>Agrobacterium mediated</td>
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<td>[103]</td>
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<td>Tomato</td>
<td>DELLA, ETR1</td>
<td>Agrobacterium mediated</td>
<td>41–92%</td>
<td>16–69%</td>
<td>[105]</td>
</tr>
<tr>
<td>5</td>
<td>Wheat</td>
<td>DEP1, GW2</td>
<td>Particle bombardment</td>
<td>0.4–1.1%</td>
<td>0%</td>
<td>[106]</td>
</tr>
<tr>
<td>6</td>
<td>Rice calli</td>
<td>MPK6, MPK13, SERK2, WRKY45, Tms9-1</td>
<td>Agrobacterium mediated</td>
<td>0–62.26%</td>
<td>0%</td>
<td>[107]</td>
</tr>
<tr>
<td>7</td>
<td>Arabidopsis</td>
<td>FT, PDS3</td>
<td>Agrobacterium mediated</td>
<td>0–85%</td>
<td>NR</td>
<td>[108]</td>
</tr>
<tr>
<td>8</td>
<td>Brassica napus</td>
<td>ALS, PDS</td>
<td>Protoplast transformation</td>
<td>8.8%</td>
<td>&lt;0.1%</td>
<td>[108]</td>
</tr>
<tr>
<td>9</td>
<td>Rice</td>
<td>CDC48</td>
<td>Agrobacterium mediated</td>
<td>44–83%</td>
<td>0%</td>
<td>[109]</td>
</tr>
<tr>
<td>10</td>
<td>Potato</td>
<td>GBSS</td>
<td>Protoplast transformation</td>
<td>6.5%</td>
<td>0%</td>
<td>[110]</td>
</tr>
</tbody>
</table>

Table 3. Genome editing in sugarcane for improvement of different traits.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Targeted Genes</th>
<th>Technique</th>
<th>Improved Traits</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COMT</td>
<td>TALEN</td>
<td>Improvement of cell wall composition for bioethanol production</td>
<td>Jung et al. [108]</td>
</tr>
<tr>
<td>2</td>
<td>ScAPX6, ScNsLTP</td>
<td>CRISPR/Cas9</td>
<td>Methyl jasmonate (MeJA), abscisic acid (ABA) and drought tolerance</td>
<td>Chen et al. [109]</td>
</tr>
<tr>
<td>3</td>
<td>PYL 1</td>
<td>CRISPR/Cas9</td>
<td>Water logging</td>
<td>Miao et al. [111]</td>
</tr>
</tbody>
</table>

5. Conclusions

Plant breeding and genetics have been persistently evolving and genetic modification-based innovations hold the key to meet the challenges of today’s global food and nutritional security. Under changing climate, it has become necessary to continuously introduce new allelic variants to improve crops yield and nutritional quality. However, it has been a challenging task to subject sugarcane to genetic engineering and breeding approaches because of the large genomes and polyploid properties. The application of genome editing technology in plants may provide a wide range of opportunities for breeding sugarcane varieties with desired traits. Among the recently developed technologies, the CRISPR/Cas9 system might be effectively used to generate high frequency mutants in sugarcane having targeted agro-botanical and physiological characteristics for sustainably boosting its productivity under changing climate scenario. It has great potential for facilitating genome-wide functional research in the context of polyploid crops such as sugarcane which have been difficult to breed using conventional breeding techniques. The CRISPR/Cas9 technology has been demonstrated to be efficient in editing multiple alleles of the magnesium chelatase gene in sugarcane. It has also been reported that gene targeting for the acetylactate synthase gene can be achieved through template-mediated and homologous-directed repairs of DNA double strand breaks caused by the CRISPR/Cas9 enzyme, resulting in extensive herbicide resistance. It may be inferred that CRISPR/Cas9 can effectively edit polyploids such as sugarcane, allowing more precise control over its genome editing in order to develop new cultivars having desired agro-botanical traits on sustainable basis.
Author Contributions: Conceptualization, S.H.H., X.L., C.L., M.A.I. and R.A.; methodology, S.H.H., M.D., M.A.I. and X.L.; validation, M.I. and G.H.J.; writing—original draft preparation, S.H.H., M.A.I., R.A., C.L. and X.L.; writing—review and editing, S.H.H., X.L., R.A. and M.A.I. All authors have read and agreed to the published version of the manuscript.

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References
9. Wang, Z.; Ren, H.; Xu, F.; Lu, G.; Cheng, W.; Que, Y.; Xu, L. Genome-Wide Characterization of Lecin Receptor Kinases in Saccharum spontaneum L. and Their Responses to Stagonospora tainanensis Infection. Plants 2021, 10, 322. [CrossRef]
10. Wang, Z.; Xu, F.; Ren, H.; Lu, G.; Que, Y.; Xu, L. Genome-Wide Characterization of NLRs in Saccharum spontaneum L. and Their Responses to Leaf Blight in Saccharum. Agronomy 2021, 11, 153. [CrossRef]


27. Li, C.; Yue, Y.; Chen, H.; Qi, W.; Song, R. The ZmbZIP22 Transcription Factor Regulates 27-kD 


Lo, A.; Qi, L. Genetic and epigenetic control of gene expression by CRISPR–Cas systems. *F1000Research* 2017, 6, 747. [CrossRef]


Khan, I.H.; Javaid, A.; Naqvi, S.F. Molecular characterization of *Penicillium expansum* isolated from grapes and its management by leaf extract of *Chenopodium murale*. *Int. J. Phytopathol.* 2021, 10, 29–35. [CrossRef]

Peng, R.; Lin, G.; Li, J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS J.* 2015, 283, 1218–1231. [CrossRef]


85. Creste, S.; Pinto, L.R.; Xavier, M.A.; Guimaraes, M.; Landell, R. The importance of the germplasm in developing agro-energetic profile sugarcane cultivars. In Sugar Cane Bioethanol RD Product and Sustainability; Editora Edgard Blucher Ltda: São Paulo, Brazil, 2014; pp. 353–358. [CrossRef]
89. Riaño-Pachón, D.M.; Mattiello, L. Draft genome sequencing of the sugarcane hybrid SP80-3280. F1000Research 2017, 6, 861. [CrossRef]


