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REVIEW

Genome Editing of *Sorghum bicolor* (L.) Moench: Current Status and Future Applications

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ABSTRACT: Sorghum is a heat- and drought-tolerant cereal crop used for feed and food purposes in more than 100 countries. Sorghum becomes highly important in conditions of climate warming. The use of genome editing technologies is of paramount importance for this crop, which has a number of constraints in key agronomic traits that are difficult for improvement using conventional breeding. Application of genome editing for sorghum is limited due to problems of genetic transformation; however, recent advances in this field have increased the efficiency of the genome editing procedure in sorghum. This review summarizes recent achievements in the field of sorghum genetic transformation and results of site-directed mutagenesis in this crop, such as obtaining mutants with increased kafirin digestibility, apomixis, resistance to *Striga hermontica*, modification of plant architecture and "stay-green" trait, fragrant leaves and seeds, and others. The review also discusses the biosafety of edited plants and outlines prospects for further work on the genetic improvement of sorghum using genome editing technologies, and its application in practical breeding.

KEYWORDS: Sorghum; genome editing; CRISPR/Cas; sorghum genetic transformation

1 Introduction

The past 10 years have seen significant progress in the development and use of genome editing to address a wide range of plant genetics and breeding challenges [1–3]. The development of CRISPR/Cas9 technology has significantly simplified the task of introducing changes to targeted sites in the genome, compared to genome editing methods based on other types of endonucleases: meganucleases (MNs), zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). These methods had not found wide application due to the difficulty of modifying the activity of these nucleases for the desired sites of the genome.

As is known, the CRISPR/Cas9 technology, based on the use of clustered regularly interspaced short palindromic repeats (CRISPR) and the Cas9 endonuclease from *Streptococcus pyogenes*, has been widely used due to its high efficiency and ease of use. Cas9 is an endonuclease operated by means of RNA-guides, cutting of alien DNA, complementary RNA-guide. The Cas9 endonuclease produces double-strand breaks (DSB) in target DNA located 3 nucleotides upstream of the sequence 5′-NGG-3′, which is called protospacer adjacent motif (PAM) (where N—any nucleotide; G, guanine). These DNA breaks result in nucleotide substitutions, small deletions, or insertions arise at the target site, which can lead to frameshifts and null mutations [1–4].

Cas9 endonuclease is directed to the target DNA region by guide RNA (gRNA), which consists of a constant region (trans-activating crRNA (tracrRNA) binding Cas9) and a variable region (crRNA) (CRISPR RNA), called the spacer, which directs the Cas9 nuclease to a homologous target protospacer location in the genome. Its 5′-end has a 20-nt sequence that is complementary to the target DNA sequence, accompanied by a PAM sequence. Thus, the binding specificity of Cas9 with the target DNA is determined by both PAM and gRNA–DNA base pairing of the target region [1,3,5].

Furthermore, multiplex vectors consisting of the *cas9* gene and several guide RNA sites were developed [6,7]. There are different types of such vectors: in some, the expression of each individual guide RNA is controlled by its own promoter; in others, multiple single gRNAs are fused with a tRNA recognition sequence, which is expressed as a single transcript under a single promoter. In plant cells, these multiple gRNAs are separated into individual gRNAs by endogenous ribonucleases [8]. Such multiplex vectors allow editing of nucleotide sequences of several genes simultaneously, which is of critical importance for modification of complex traits controlled by several genetic systems, such as plant architecture, grain/biomass yield, apomixis and others.

Site-directed mutagenesis using CRISPR/Cas9 technology is currently used to generate mutants in a wide range of major agricultural crops such as wheat, corn, rice, soybeans, potatoes, and many others, including sorghum, an important global agricultural crop whose importance will increase with global warming. A steady trend towards increasing air temperatures, as well as an increase in the duration and frequency of droughts reduces the yield of major grain crops (wheat, corn, barley) and complicates their agricultural production [9–11].

Sorghum (*Sorghum bicolor* (L.) Moench) is a high-yielding, heat- and drought-tolerant grain crop, a reliable source of feed and food grain. It is of particular importance for the regions that regularly suffer from drought, where the sustainable production of traditional grain crops such as wheat, maize and barley is difficult. In global agriculture, sorghum is one of the five most widely cultivated grain crops on Earth and serves as a source of nutrition for more than 500 million people in 100 countries, mainly in Africa and Asia [12]. Besides, sorghum has been actively used for many years as a source of feed for livestock and poultry farming, as well as for the production of ethanol, biofuels, and biogas. Moreover, sorghum grain has attracted attention as a source of healthy nutrition due to its high content of antioxidants (anthocyanins) and other bioactive substances [13,14], and, in this regard, its benefits in combating obesity, cardiovascular diseases, and some types of cancer. In addition, sorghum grain does not contain gluten and can serve as a source of protein for people susceptible to various forms of celiac disease (gluten intolerance), who must adhere to a gluten-free diet [12–14]. The number of such people varies quite widely in different countries: on average, 1% of the population [15], although in some risk group, its frequency reaches 6.6%–16.3% [16]. In 2024, the cultivated area occupied by sorghum crops in all countries of the world amounted to 40.9 million hectares, while global production was 61.3 million tons [17].

In agreement with botanical classification, sorghum belongs to the family *Poaceae*, tribe *Andropogoneae*, subtribe *Sorghinae*, genus *Sorghum* [18,19]. Sorghum is one of the most actively evolving genera, which includes a large number of species that are crossed with each other and produce both fertile hybrids and hybrids with cytoplasmic male sterility. All cultivated sorghum varieties belong to one species—*Sorghum bicolor* (L.) Moench (2n = 20), which includes five basic races: *bicolor*, *guinea*, *caudatum*, *kafir*, and *durra*, and 10 intermediate races, which obviously arose as a result of hybridization. These races are differentiated by the phenotype of their mature panicles and spikelets [18,20]. Based on the areas of practical use, grain sorghum, sugar sorghum, forage sorghum and broom sorghum are distinguished. Grain sorghum varieties and hybrids are distinguished by a shortened stem, a large panicle and, often, an elongated peduncle. Forage sorghum plants have a longer stem length and higher tillering, and therefore are capable of forming large

biomass, which is used as feed for livestock, as well as for the production of biofuel and biogas. Sugar sorghum is distinguished by the ability to accumulate a large amount of soluble sugars in the stem. Many varieties and hybrids of sugar sorghum are also capable of forming a large biomass and, in this regard, are used to produce syrup, alcohol, biofuel and high-sugar feed for livestock [18,19].

However, sorghum has few constraints that hinder its wider distribution and determine the directions of its genetic improvement. One of the most important constraints is a relatively low nutritional value of the grain, due to the low content of essential amino acids in the storage proteins (kafirins) and the resistance of kafirins to protease digestion, which in turn negatively affects starch digestibility [21–25]. The resistance of kafirins to proteolytic digestion is caused by (1) the ability of kafirins to form oligo- and polymeric complexes resistant to proteases [21–23,26,27]; (2) the peculiarities of the structural organization of the protein bodies of the endosperm, in which γ-kafirin, rich in cysteine and forming polymeric complexes with α- and β-kafirins, occupies a peripheral position, complicating the digestion of the main storage protein—α-kafirin, which is located in the inner layers of the protein bodies [21–23,28]; (3) the presence of protease inhibitors in the grain [29,30], as well as polyphenols, anthocyanins, tannins, which interact with kafirins and inhibit their digestion by proteases [21,26,31,32].

Sorghum is characterized by relatively slow initial growth, and in the early stages of ontogenesis, its development is inhibited by weeds [33,34]. Therefore, a very pressing issue in sorghum breeding is developing varieties and hybrids tolerant to herbicides used for weed control.

In the course of development, sorghum plants are faced with attacks from many different pests and diseases. Important factors that reduce grain yield and quality are damage by pathogenic fungi causing head smut (*Sporisorium reilianum* (Kuhn) Clinton), grain mold (*Fusarium spp.*, *Curvularia lunata*, *Alternaria alternata*, and *Phoma sorghina*), and others [35]; insect pests such as sorghum shoot fly (*Atherigona soccata*), stem borer (*Chilo partellus*, *Busseola fusca*, *Coniesta ignefusalis*), and few others [36]. In Africa, significant damage to sorghum crops is caused by the parasitic plant *Striga hermonthica. Striga hermontica* belongs to the Orobanchaceae family. The seeds of Striga germinate under the influence of sorghum root exudates and form haustoria, through which they penetrate the root tissues. As a result of infestation, yield losses can reach up to 65%, which makes it highly important to breed sorghum varieties resistant to *Striga* [37–39].

Being a drought- and heat-tolerant crop sorghum, nevertheless, reduced its yield in conditions of drought stress. Drought leads to poor yields and quality associated with reduced photosynthesis, buildup of reactive oxygen species (ROS), damaged cell membranes, and poor assimilation rates [40–42]. The impact depends on the intensity and duration of the stress, and the stage of plant development exposed to drought. For example, drought stress imposed at the vegetative or reproductive stages reduced grain yield by more than 36% and 55%, respectively [43]. Other estimates indicate that drought stress at the booting and flowering stages led to a sorghum yield loss of 87%, but only long periods of stress at the vegetative stage can result in such a substantial yield loss [44]. Taking into account the forecasts related to global warming [45], the development of drought-tolerant varieties and hybrids is one of the most important areas of sorghum breeding.

With the development of molecular genetics and biotechnology, genetic improvement of sorghum, like many other cultivated crops, has received a powerful impetus. For sorghum, as for other cultivated cereal crops, approaches based on cell selection, genomic selection, and genetic engineering have been developed [46,47]. Molecular markers associated with a number of traits of biological and economic value have been identified [48]. A decisive step forward was the sequencing of the sorghum genome, completed in 2009 [49]. Genome sequencing made it possible to identify the structure of individual genes controlling the expression of agronomically valuable traits and opened up the possibility of targeted modification of

the genotypes of cultivated lines and varieties using genome editing technology to solve key problems of sorghum breeding. Genome editing technology, which emerged from the combination of methods and knowledge from molecular genetics and biotechnology, allows for significant intensification of sorghum breeding in many important areas, which is of great importance in the context of global warming and the increasing global population. However, it should be noted that the use of genome editing technology to change the expression of specific agronomically important traits in sorghum faces a number of limitations, the main ones being the problems of genetic transformation and its strong genotype dependence.

Experimental works performed on sorghum using genome editing has been previously summarized in a number of reviews [50–52]. Detailed protocol for genome editing in sorghum using CRISPR/Cas9 technology have also been published [53,54]. This review summarizes previous and new experimental data rapidly accumulating as a result of using this groundbreaking technology.

2 The Main Steps of Genome Editing Technology in Sorghum

2.1 Designing of CRISPR/Cas System

The use of the CRISPR/Cas system in sorghum, as in other plant species, begins with analyzing the nucleotide sequence of the target gene and creating a genetic construct for its editing. Several web-based programs that help to design gRNA sequences are developed, such as Cas-Designer (http://www.rgenome.net/cas-designer/), CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) CRISPOR (http://crispor.tefor.net/), CHOPCHOP (https://chopchop.cbu.uib.no/) and others [55]. These programs help to predict the number of off-targets, editing activity prediction, and genome editing outcome prediction. In general, gRNA sequence should not induce off-targets for 1 bp and 2 bp mismatches, and contains 45~70% GC.

After selecting the guide RNAs, it is advisable to test their nucleolytic activity *in vitro* using Cas9 protein, the respective gRNA and target DNA [56], or on cultured protoplasts. The methods for testing the efficiency of guide RNA in sorghum protoplasts were reported [57,58].

The next step is to create a genetic construct that includes the *cas9* genes driven by an efficient promoter, and the guide RNA, the sequence of which is also preceded by a promoter. In most successful studies on sorghum genome editing, maize Ubi1 was used as a *cas9* promoter. It was also shown that the use of the *cas9* gene with maize-codon optimized sequence in combination with maize Ubi1-promoter significantly increased editing efficiency in comparison with SpCas9 driven by sorghum Ubi-promoter [59]. Rice U3 or wheat U6 promoters were used as a guide RNA promoter. It has also been reported that the use of the endogenous sorghum U6 promoter (in particular, SbU62.3 promoter) significantly improves gene editing efficiency in sorghum up to 90% of experimental plants [60].

In a number of studies, multiplex vectors consisting of the *cas9* gene and several guide RNAs were used that allow editing of nucleotide sequences of several genes simultaneously [61,62]. This approach appears to be very promising for targeted modification of complex traits controlled by different genetic systems.

2.2 Transformation Tools for CRISPR/Cas System

The introduction of the CRISPR/Cas genetic construct into the sorghum genome is the key and most challenging step in genome editing in sorghum due to problems with genetic transformation. In most studies on sorghum, *Agrobacterium*-mediated transformation was used for these purposes, less often the constructs were introduced by biolistic transformation or using pollen-mediated approach, in which plasmid with CRISPR/Cas9 genetic construct was introduced into the pollen by mild sonication, and then sonicated pollen grains were used for pollination of emasculated flowers [63].

Both *Agrobacterium*-mediated and biolistic transformation approaches are based on robust tissue culture protocols, which allow efficient plant regeneration from transformed tissues. Such protocols were developed in the late 1970s–early 1980s, long before the development of genetic transformation methods using various explants, such as immature embryos [64,65], fragments of young panicles [66], seedling leaves [67,68], and mature embryos [69]. Numerous subsequent studies on plant regeneration in sorghum tissue culture from these types of explants confirmed the general principles that must be taken into account when using tissue culture in studies on genetic engineering and genome editing in sorghum. Namely: (1) only ontogenetically young, meristematically active types of explants exhibit the ability to undergo morphogenesis *in vitro*; (2) isolated sorghum cultures are characterized by significant callus polymorphism, and selective subculturing of embryogenic tissues is necessary to maintain the morphogenic activity of isolated sorghum cultures; (3) embryogenic sorghum calli exhibit a need for the presence of cytokinins in the medium, and, taking these features into account, it is possible to obtain morphogenic cultures that retain their regenerative capacity for years when subcultured on 2,4-D-cytokinin-containing media [70,71]; (4) the capacity for embryoidogenesis and plant regeneration in sorghum is strictly determined by genotypic factors; (5) cultured sorghum tissues produce a significant amount of toxic pigments that cause partial necrosis of explants, but do not interfere with the manifestation of embryogenic potential.

To overcome the toxic effect of pigments, the production of which is significantly enhanced during co-cultivation with agrobacterial cells, frequent subcultures of cultured embryos/calli and/or addition of polyvinyl-polypyrrolidone into the nutrient medium were recommended [72]. The use of media containing lipoic acid was also proposed [73].

To reduce the production of toxic pigments and enhance the embryogenic potential of cultured immature embryos, we developed the M11 nutrient medium, containing increased concentrations of phosphate, proline, and asparagine [74]. This medium reduces the release of phenolic pigments characteristic of cultivated sorghum tissues and increases embryogenic potential. Moreover, the combination of asparagine and proline in some sorghum varieties and lines causes the formation of friable embryogenic callus, which has high embryoidogenic activity and does not produce pigments at all [75].

Further important modification of the nutrient media for genetic transformation and genome editing in sorghum was an increase in the content of copper ions, both in the medium for obtaining embryogenic callus and for regeneration, which significantly improved the development of the root system of regenerants [76].

These modifications substantially improve genetic transformation of sorghum either through biolistic DNA delivery [77,78]; or *Agrobacterium*-mediated genetic transformation [79–82], and were used in many CRISPR/Cas9 genome editing experiments [60,61,83,84].

Another important modification of the nutrient medium that promoted the survival of transformed calli after co-cultivation with agrobacterial cells was the introduction of 6-BAP into the selection medium and the replacement of sucrose with maltose [85].

However, even with the use of modified nutrient media, obtaining transgenic plants in *Agrobacterium*-mediated transformation experiments in sorghum with conventional agrobacterial strains is a serious problem; their frequency rarely exceeds 9–10%. Statistically significant effect on obtaining genetically transformed embryogenic calli in *Agrobacterium*-mediated genetic transformation caused heat-shock treatment of the immature embryos before inoculation with agrobacterial cell suspension [86].

A significant contribution to facilitating *Agrobacterium*-mediated transformation in sorghum has been made by the use of the "hypervirulent" *A. tumefaciens* strains NTL4 [79], AGL1 [85], both in the same C58 strain chromosome background, containing disarmed Ti plasmid, pTiBo542, harbouring additional *vir* genes, and agrobacterial strains containing specially designed ("superbinary" vectors) with additional *vir*-genes

that enhance the transfer of T-DNA from agrobacterial cells to sorghum cells [72,85]. Using super-binary vector pSB1 the frequency of transgenic events in *Agrobacterium*-mediated genetic transformation in some experiments increased up to 33% [85].

Subsequently, a ternary vector system containing a disarmed Ti plasmid, the pVIR as an accessory helper plasmid with additional *vir*-genes, and a T-DNA binary vector was developed [87]. pVIR plasmids (pPHP70298, or pPHP71539, or pPHP7976) contain sets of *vir* genes (*virA*, *virG*, *virE*, *virJ*, *virB*, *virC*, and *virD*) from the hypervirulent pTiBo542 plasmid and a stable high copy number origin of replication pVS1 ORI. pVS1 ORI ensures plasmid stability, high copy number and compatibility with ORI of other plasmids in the same bacterial cell, which contributes to increased transformation frequency [88]. Additional *vir* genes improve T-DNA delivery. Namely, additional copies of *virG* increase sensitivity to acetosyringone, which leads to an "explosive" synthesis of all other Vir proteins [89]. This results in increased production of VirD1/D2 proteins, which recognize the boundaries of T-DNA on the binary plasmid and excise its single-stranded copy. In addition, "superbinary" vectors ensure increased synthesis of VirE2 and VirE3 proteins, which protect T-DNA from degradation by plant nucleases, abundant in cereal cells, and "drag" it into the plant cell nucleus [90–92].

This vector system, pVIR, allowed to obtain transformation frequency up to 29%, with single copy, backbone-free 'quality events' ranging from 45% to 66% of the total events produced, and helped to transform several recalcitrant sorghum cultivars [93].

An important factor that contributed to the increased efficiency of *Agrobacterium*-mediated genetic transformation in these experiments was an auxotrophic mutant LBA4404 Thy-incapable of growth on a media without thymidine, the use of which made it possible to significantly simplify the transformation procedure. Furthermore, pVIR-based vector system has also been successfully used in genome editing experiments [93].

Considerable progress results from the creation of binary vectors carrying the genes encoding morphogenetic regulators BABY BOOM and WUSCHEL, which promote the direct development of embryoids from scutellum cells of immature embryos, and thereby increase the number of regenerants and the frequency of transgenic plants (morphogene-assisted transformation, MAT) [81,94,95]. The use of MAT made it possible to overcome strict genotype limitations for somatic embryogenesis and allowed to obtain genetically transformed plants in recalcitrant genotypes. Moreover, the use of the *Wus2* gene has been shown to enhance the mutagenic effect of genome editing constructs and significantly increase the frequency of genome-edited plants, up to 90% of the number of regenerants carrying gRNA, as demonstrated in three different sorghum varieties and different genetic constructs for editing three different genes [95].

To prevent the negative impact of morphogenetic regulatory genes on the development of edited plants, either excision of these genes using a CRE recombinase driven by a heat-inducible promoter, or "altruistic transformation" was used, in which transformation was carried out jointly by two agrobacterial strains—one with a gene-of-interest (GOI) construct and the other with a construct containing a morphogene [59,94]. Using this strategy, termed altruistic, most transformants had the GOI but lacked morphogenes.

Recently, J. B. Fontanet-Manzaneque and colleagues, by optimization of *Agrobacterium*-mediated infection parameters (optical density, co-cultivation time, pH, and temperature), using the pVS1-VIR2 ternary vector system combined with the morphogenic genes *BABY BOOM* and *WUSCHEL2*, and an excision-based transformation system, reported a remarkable enhancement in total transformation efficiency, reaching up to 164.8% (several transgenic plantlets per transformed embryo) [96]. However, it should be noted that such a high transformation frequency was achieved on the model sorghum line RTx473, and it is not yet known how other varieties and lines will react to these experimental conditions.

Another ternary vector system has been developed recently by Chinese researches [97]. This system is based on using the helper vector encoding a chimeric protein GRF4-GIF1. GRF4 is a growth regulator factor 4, and GIF1 is its co-factor, GRF-interacting protein 1. GRF genes encode transcription factors, and GIFs are proteins that interact with chromatin remodeling complexes. Therefore, the GRF-GIF chimeric protein is a transcriptional activator that can bind to chromatin and induce an open chromatin state, facilitating the expression of genes required for organogenesis and embryogenesis [98]. Experiments on wheat have shown that the GRF4-GIF1 chimeric protein promotes shoot regeneration from transformed calli obtained from immature embryos by replacing or enhancing the effect of cytokinins [99,100]. This effect is based on the ability of the GRF4-GIF1 chimeric protein to stimulate cell proliferation and activate meristem development programs [101,102]. The plasmid containing GRF4-GIF1 increased transformation efficiency in sorghum up to 23–24% that is two-times higher than the control pVS1-VIR2 ternary system. Compared with BBM-WUS, overexpressing GRF4-GIF1 caused no noticeable growth defects in sorghum. The combination of GRF4-GIF1 with helper plasmid pVS1-VIR2 achieved the highest transformation efficiency, reaching 38.28%, which is 7.71-fold of the control experiment performed without using ternary vectors. Introduction of CRISPR/Cas9 system into the GRF4-GIF1 ternary vector allowed achieving highly efficient sorghum genome editing tool: up to 29% mutant frequency in T_0 generation for *phytoene desaturase* (*PDS*) gene, encoding a critical enzyme in carotenoid biosynthesis, mutations in which cause albinism, and 54% for *brown midrib 6* (*BMR-6*) genes.

Thus, there are three approaches for producing transgenic plants carrying genetic constructs of the CRISPR/Cas system that induce mutations: *Agrobacterium*-mediated genetic transformation, biolistic transformation, and pollen-mediated genetic transformation. These systems differ in efficiency, genotype effect, cost, and off-target risk (Table 1).

As an alternative approach to obtaining mutants that do not contain transgene constructs, the use of DNA-free gene editing techniques, including transient expression of plasmid-based CRISPR-DNA, preassembled CRISPR/Cas ribonucleoprotein (RNP) and virus-induced gene editing, has been proposed [103–106]. This approach alleviates public concerns about the possible presence of foreign genetic constructs in genome-edited plants and circumvents the difficulties associated with plant regeneration in tissue culture, which is particularly important for sorghum. Using engineered foxtail mosaic virus (FoMV) the sgRNAs targeting *Phytoene desaturase* (*PDS*), *Magnesium-chelatase subunit I* (*MgCh*), and *4-hydroxy-3-methylbut-2-enyl diphosphate reductase* (*Lw1*) genes were introduced in transgenic sorghum lines expressed *cas9* nuclease gene that results in the induction of chlorophyll-deficient mutant plants with frequencies 35–60%. However, these mutations were not transmitted to the next generation [107].

The introduction of preassembled CRISPR/Cas RNP complexes into plant cells is possible using cultured protoplasts [103,104]. However, this approach is currently unacceptable for sorghum due to the difficulties of plant regeneration from cultured protoplasts. To date, only one paper has been published on the regeneration of sorghum plants from protoplasts [108].

A report on the production of transgene-free genome-edited sorghum plants within a single generation using particle bombardment of immature embryos was recently published, in which co-transformation with two plasmids was used, one of which carried a maize (*Zea mays*)-optimized Cas9 vector, the other—a guide RNA (gRNA) cassette with gRNAs aimed at editing the *PDS* gene [109]. In this work, plants with an edited *PDS* gene sequence that did not carry transgene constructs were detected already in the T_0 generation. Notably, 22.2–38.1% of albino plants were detected in the variant without selection, while in the variant with selection the proportion of such plants varied from 0 to 5.9% [109].

Table 1: Comparison of different methods of genetic transformation of sorghum.

Criterion	*Agrobacterium*-Mediated Transformation	Biolistic Transformation	Pollen-Mediated Transformation
Efficiency	High (up to 36–160%, depending on the strains used)	Average (up to 20–46% in model lines; success depends on the ability of the genotype to form embryogenic callus)	Low and unstable
Genotype effects	High	High due to the genotypic determination of the ability to form embryogenic callus	Low
Cost	Medium (tissue culture reagents are used)	High (expensive equipment, gold/tungsten particles and tissue culture reagents are used	Low (no need for tissue culture)
Off-target risc	Average (determined by the structure of the gRNA and somaclonal variation)	High (gRNA structure + DNA fragmentation + somaclonal variation)	Low (determined by the structure of the gRNA)
Stability of inheritance	High	Medium because of possible gene silencing caused by multiple copies integration	High (if an insertion occurred)

2.3 Approaches for Mutant Isolation

The next steps in using the CRISPR/Cas system to obtain genome-edited sorghum plants are the selection of transformed calli and regenerated plants carrying the introduced genetic construct for editing, and the selection of genome-edited plants among them. To solve this problem, most of the studies included nutrient media containing the aminoglycoside antibiotics such as G418 [56,59,60,93,95,97,109–114], herbicide bialofos or ammonium glufosinate [61,84,115,116], or—less commonly—hygromycin [63]. Accordingly, the editing vectors carried corresponding marker genes (*nptII*, *bar*, *hpt*) conferring resistance to these agents. The use of the *ptxd* gene, a genetic marker that provided the ability to metabolize phosphite in the culture medium, was also reported [58]. The presence of a genetic construct for genomic editing in selected resistant plants is checked using PCR for specific fragments of gRNA and *cas9*. In a number of studies, PCR was also used to check the absence of backbone sequences of the vectors carrying constructs for genomic editing that could weaken the expression of the *cas9* gene.

To identify mutants among the T_0 plants carrying the CRISPR/Cas construct, restriction analysis is used. The target amplicon is digested with restriction enzymes, and the resulting restriction fragments from the original line and the putative mutant are analyzed using gel electrophoresis. As a result of the mutation, a deletion of the restriction site may occur, which will be visible on electrophoresis. This approach was used by Hao et al. [115]. However, a more convincing way to identify an induced mutation is to use sequencing the nucleotide sequences of target genes (Sanger sequencing, NGS, WGS) [53,54].

In addition, expression analysis of target genes is performed in T_0 and T_1 plants to identify the desired phenotype. In the case of induction of a mono-allelic (heterozygous) mutation, the appearance of plants with a mutant phenotype should be expected only in the T_1 generation obtained as a result of self-pollination of T_0 plants. In the case of a bi-allelic (homozygous) mutation, mutants can be identified already in T_0 [53,54].

Another critical step is the final screening of mutant progeny to identify genome-edited plants that lack the CRISPR/Cas construct but exhibit the desired mutant phenotype. For this purpose, either selection in self-pollinated progeny of mutants is used, or crossing with the original cultivar and subsequent selection

in the F_2 generation, in which mutants are analyzed using PCR with primers for the *Cas9* gene and the backbone vector sequences [53,54,105].

However, despite significant progress in methods for producing mutants based on the CRISPR/Cas technology, the number of successful experimental studies on genome editing in sorghum to date is small, in comparison to other crops. The results of published works on site-directed mutagenesis in sorghum using CRISPR/Cas technology are presented in Table 2.

3 Examples of Using CRISPR/Cas Technology for Editing the Sorghum Genome

In the early studies on the use of the CRISPR/Cas9 system for editing the sorghum genome, the researchers' efforts were aimed at developing a technology for introducing genetic constructs to induce mutations and establishing the fact of site-directed mutagenesis using sequencing of target genes. For this purpose, they used constructs aimed at inducing mutations in the genes with an easily registered phenotypic effect: albinos, fluorescent protein reporter genes.

In the first work on the use of the CRISPR/Cas9 system for editing the sorghum genome, a mutant non-functional *DsRED* gene was successfully edited, and, as a result of site-directed mutagenesis, acquired the ability to function [117].

To identify factors affecting the efficiency of genome editing and to develop an optimal protocol, a number of studies used genetic constructs targeting the *pds* gene, which controls the synthesis of phytoene desaturase [59,83]. Mutations in the *pds* gene result in plant albinism. Thus, K. Aregawi and colleagues using two vectors, each containing two guide RNAs to different exons of the *pds* gene, showed that the vector containing the *Streptococcus pyogenes Cas9* (*SpCas9*) gene, which was controlled by the sorghum ubiquitin1 promoter (*Sb-Ubi1pro*), gave a lower yield of plants with an edited genome compared to the vector containing maize codon-optimized *Cas9* under the control of the maize ubiquitin1 promoter (*Zm-Ubi1pro*) (15.5% vs. 22.7%), the frequency of plants with a knockout of the *pds* gene also differed significantly (0 vs. 16.7%) [59].

In subsequent studies, the researchers focused on improving the traits valuable for sorghum breeding, such as plant architecture, grain and biomass quality, tolerance to biotic and abiotic stresses, reproduction systems.

3.1 Plant Architecture

Leaf inclination angle is an important plant trait. Growing plants with altered leaf inclination angle can be used to improve plant productivity at high sowing density. This trait is controlled by the *LIGULELESS-1* (*LG1*) gene, which is responsible for the formation of the ligule and auricle. Using CRISPR/Cas technology, E. J. Brant and colleagues [56] obtained mono- and biallelic mutants in the *LIGULELESS-1* locus. A genetic construct contained two guide RNAs aimed at editing the first exon of the *lg1* gene, each under the control of OsU6 promoter, the *cas9* gene was under the control of the CaMV 35S promoter, and the *nptII* marker, also under the control of the CaMV 35S promoter. This construct was introduced into the genome of the line Tx430 via particle bombardment of immature embryos. Three transgenic plants carrying the *cas9* gene were obtained, one of which had a single nucleotide insertion in the selected target of the *lg1* gene. In the T_1 generation, homo- and heterozygotes for the induced mutation were identified, which differed for leaf inclination angles (2.3°–5.4° and 9.1°–10.7°, respectively). Homozygous (biallelic) mutants completely lacked both leaf ligules and auricles. Remarkably, homozygous mutants lacking *cas9* gene were found in T_1 generation as a result of the genetic segregation of the induced mutation and the genetic construct that induced this mutation [56].

3.2 Grain Quality

Among numerous genes affecting sorghum grain quality, the main focus has been on editing genes controlling kafirin synthesis. Improving the seed protein digestibility of kafirins is one of the key tasks of sorghum breeding. It is believed that the resistance of kafirins to proteolytic digestion is due foremost to their primary structure, since kafirins (especially, γ-kafirin) have a high content of cysteine, which forms intra- and intermolecular disulfide bonds leading to the formation of oligo- and polymers of kafirins resistant to protease digestion [21–23]. A major role is also played by such factors as the structural organization of protein bodies in sorghum endosperm cells, in which γ-kafirin, which is most resistant to proteolytic digestion, form the external "shell" of protein bodies and hinder the access of proteases to the more easily digestible α-kafirins located inside the protein bodies; the interaction of kafirins with non-protein components, in particular, with tannins, which reduce the activity of proteases, and with polysaccharides that are part of the starch granules of endosperm cells; the interaction of kafirins with non-kafirin proteins (glutelins), which also form intramolecular disulfide bonds in the vitreous endosperm of sorghum [118].

The use of genome editing technologies seems to be a fairly promising approach to solving this problem, since the sequences of individual kafirin genes have already been sequenced and deposited in GenBank.

The use of the CRISPR/Cas9 system to induce mutations in the nucleotide sequence of genes encoding the synthesis of 22 kDa α-kafirin was reported [111]. The target was the nucleotide sequence encoding the synthesis of the signal polypeptide responsible for the deposition of α-kafirins in the protein bodies of endosperm cells. Since there are 20 genes encoding 22 kDa α-kafirin in the sorghum genome, forming a gene cluster on chromosome 5, a consensus sequence having homology to all genes of this gene family (*k1C*) was chosen as a target for guide RNA aimed to induce mutations in all 20 genes of this family simultaneously. As a result, 26 transgenic T_0 plants were obtained, 25 of which carried mutations in one or multiple *k1C* family genes. Mutations in the α-kafirin signal sequence were deletions of one to 33 nucleotides and, less commonly, insertions of one to 16 nucleotides. Reduced α-kafirin levels and altered protein body structure were observed in the kernels of T_1 and T_2 plants; some T_2 plants had higher endosperm protein digestibility, increased lysine content, and a completely or partially floury endosperm. After crossing selected mutants to original cv. Tx430, a line, 19Q4-13, was selected [119]. This line did not contain transgenic constructs; the kernels of this line had a similar level of vitreousness to wild-type Tx430 that is important because floury endosperm is known to be a negative trait because it reduces mechanical strength of the kernels and promotes its damage by pathogens [23]. RNAseq-analysis showed that 184 genes were differentially expressed between wild-type and edited line, among which 109 genes were up-regulated and 32 genes down-regulated in the edited line, including 11 alpha-kafirin genes. Among up-regulated genes there were genes involved in lysine biosynthesis and carbohydrate metabolism [119]. Further investigation of two lines obtained in these experiments revealed big deletions (approx. 400 kb) in the *k1C* family in both studied lines encompassing seven active genes. However, no significant decrease was observed in *k1C* expression because non-deleted *k1C* genes had elevated expression and compensated effect caused by deletion. These data illustrate the complexity of editing highly repetitive genome regions such as gene families, whose members can compensate for the effects of CRISPR-induced multigene deletions [120].

In our study, we obtained sorghum mutants with edited sequences of the *k1C5*, which encodes the 22 kDa α-kafirin; these mutants had improved digestibility of endosperm proteins and a vitreous endosperm type [84]. Mutations were induced using a CRISPR/Cas9 vector carrying a gRNA targeted to the signal polypeptide nucleotide sequence within the *k1C5* gene, and a maize-codon-optimized *cas9* nuclease gene driven by the maize Ubi1 promoter. Substitutions of individual nucleotides were induced both within the

selected targets and in close proximity to PAM in the 3′-direction. In T_1, plants were identified that did not contain *cas9*, but with improved protein digestibility (up to 85–92%, while in the original cv. Avans it varied within 63.4 ± 2.3%, $p < 0.05$). Fig. 1 shows the SDS-PAGE spectra of endosperm proteins of sorghum plants with a mutation in the *k1C5* gene before and after pepsin digestion that clearly demonstrate differences in protein digestibility in original cv. Avans and the mutants. Improved grain protein digestibility was inherited in the progeny of some mutants, although the digestibility level in plants from the T_3 generation decreased to 68–74% in the 2C-2.1.1 mutant and 72–84% in the 2C-1.2.5a mutant, significantly exceeding, however, this indicator in the original cv. Avans. Almost all families contained plants in which some portion of kernels had floury endosperm or endosperm with a "blurred" or thin vitreous layer (Fig. 2), i.e., with endosperm types, characteristic of mutants with impaired kafirin synthesis [121].

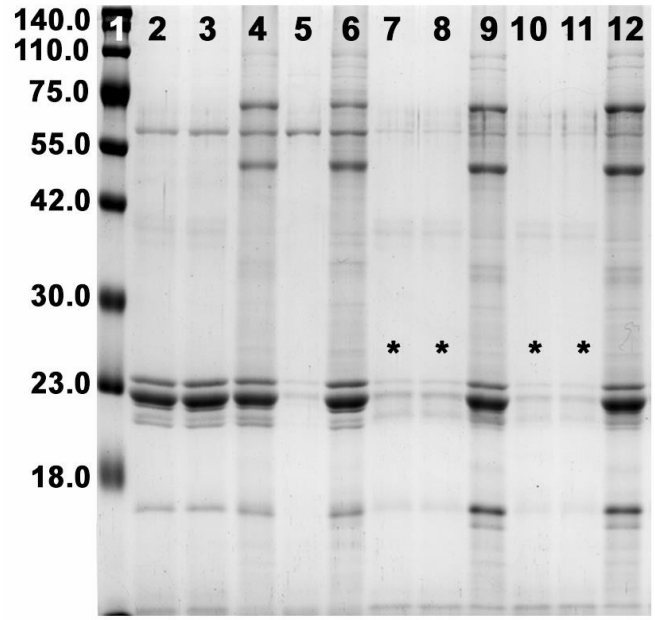

Figure 1: Electrophoretic spectra of flour proteins from kernels of T_1 sorghum mutants obtained by *Agrobacterium*-mediated genetic transformation of immature embryos of cv. Avans with genetic construct for site-directed mutagenesis of the *k1C5* gene encoding 22 kDa α-kafirin. A flour of experimental samples and of original cv. Avans was treated with pepsin solution in a potassium phosphate buffer (pH 2.0). Control samples were incubated in potassium phosphate buffer without pepsin. 1–protein molecular mass markers; 2–4–original cv. Avans; 5, 6–RNAi mutant Avans-1/18 used as a standard of high protein digestibility; 7–9 and 10–12–different plants from the progeny of mutant 2C-1.2.5. Lines 4, 6, 9, 12–without pepsin digestion; lines 2, 3, 5, 7, 8, 10, 11–after pepsin digestion. The spectra of the mutants with significantly higher level of digestibility, in comparison with the original cv. Avans, are marked with asterisks. Reprinted from [84]. © 2023, Crop Science Society of China and Institute of Crop Science, CAAS.

Previous studies have also reported the generation of edited sorghum plants with mutations in the nucleotide sequences of β- and γ-kafirin genes [60]. In this study, the high efficiency of endogenous U6 promoters (in particular, SbU62.3 promoter) was shown to improve gene editing efficiency in sorghum up to 90% of experimental plants. Plants with deletions in the γ-kafirin gene, including one with the completely deleted sequence of this gene, were obtained [60], but nothing was reported on the effect of these deletions on protein digestibility. Mutants with mutations in the β-kafirin gene were characterized by altered endosperm protein composition and protein body morphology. However, this mutation did not improve protein digestibility possibly due to a compensatory increase in the γ-kafirin content [112].

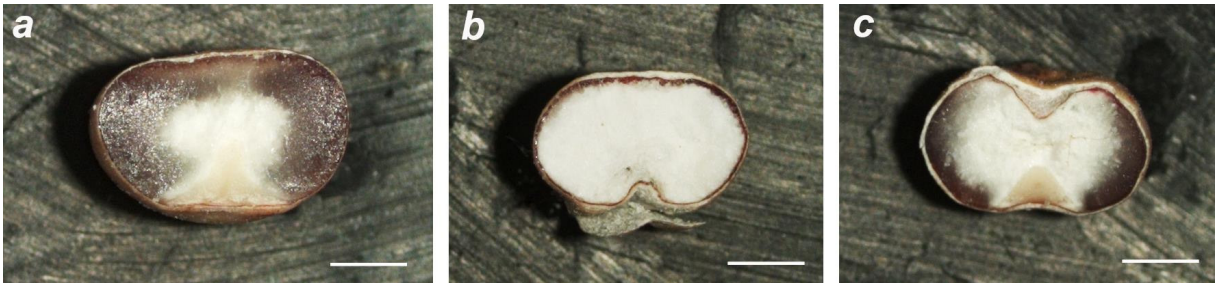

Figure 2: Cross-sections of the kernels from T_3 generation of sorghum mutant 2C-1.2.5a obtained by *Agrobacterium*-mediated genetic transformation of immature embryos of cv. Avans with genetic construct for site-directed mutagenesis of the *k1C5*. (**a**)–normal vitreous endosperm, (**b**) floury endosperm, (**c**) endosperm with a blurred vitreous layer. Scale bar 1 mm. Donor cultivar has kernels with vitreous endosperm, while the mutant has kernels with both vitreous endosperm and kernels with floury or with a blurred vitreous endosperm. Reprinted from [121]. © 2025, Elkonin LA, Gerashchenkov GA, Borisenko NV, Sarsenova SK, Panin VM.

Mutants with mutations in the γ-kafirin gene were also obtained in another study [63]. A guide RNA was designed to introduce mutations in the CDS region that encodes the endoplasmic reticulum signal peptide of γ-kafirin. The pK2GsgRNA/Cas9 vector was transformed into sorghum using the pollen-mediated transformation method. Sequencing of the transformants showed that three out of 24 transgenic plants contain genetic mutations in the targeted region. Compared to the wild type, γ-kafirin levels in mutant plants decreased by 12.7%–19.2%, while raw flour protein digestibility increased by 26.9%–74.3%.

In this regard, suppression of γ-kafirin synthesis through genome editing should lead to the production of mutants with higher nutritional value, since this does not suppress the synthesis of the main storage protein, α-kafirin, but improves its digestibility.

3.3 Forage Quality

Interesting work was carried out by Chinese researchers to obtain fragrant sorghum by editing the *BADH2* gene, which controls the synthesis of the enzyme BETAINE ALDEHYDE DEHYDROGENASE (BADH EC 1.2.1.8), which catalyzes the oxidation of betaine aldehyde to glycine betaine using NAD+ as a coenzyme [116]. The authors constructed a vector carrying two guide RNAs aimed at inducing mutations in two exons of the *BADH2* gene of the grain sorghum cv. Wheatland. As a result, 14 individual edited plants with different mutation types around the targeted sites were obtained (editing efficiency: 38%). 12 edited plants had mutations at both target sites. These mutant plants can be divided into six types according to edited forms, including base deletion, base insertion and substitution, and these mutations lead to amino acid changes in *SbBADH2* or premature translation termination. Notably, in some mutants, the PAM sequences as well as nucleotides 3′ of PAM were deleted. Homozygous lines with premature termination of translation, lacking the *cas9* gene, were selected, with significantly increased content of 2-acetyl-1-pyrroline, which accumulates as a result of the suppression of BADH expression. The resulting lines were characterized by an extraordinary aromatic smell in both leaves and seeds. Animal feeding experiments showed that fragrant sorghum leaves were more attractable than the non-fragrant control line. Later on, mutations in *SbBADH2* using a CRISPR/Cas9 vector were also induced in the sweet sorghum, variety Gaoliangzhe [110].

3.4 "Stay-Green" Trait

"Stay-green" is an important trait for both forage and grain sorghum cultivars and hybrids because it positively influences many plant traits. For example, "stay-green" varieties and hybrids are characterized by higher drought and heat tolerance. "Stay-green" plants manage water more efficiently and resist environmental stress-induced senescence, allowing them to remain functional under harsh conditions. By extending the grain-filling period, "stay-green" hybrids/lines produce higher yields, particularly in post-anthesis stress scenarios where other plants lose green leaf area prematurely. In addition, it improves forage quality: "stay-green" plants retain higher nutrient levels in the leaves and stems, increasing the nutritional value and feeding quality. Sorghum lines and hybrids with delayed leaf senescence are characterized by high yield potential in water scarce environment and produce significantly higher biomass yield. This trait is often combined with lodging resistance: "stay-green" plants often have improved stalk strength because the plant does not need to remobilize as much carbon from the stem to the grain, resulting in stronger plants at harvest [122,123].

The onset of leaf degreening and senescence is governed by a complex regulatory network including environmental cues and internal factors such as transcription factors and phytohormones, in which ethylene is one key inducer. One of the triggers controlling leaf senescence and death is the degradation of chlorophyll and chloroplasts. SbWRKY50, a transcription factor, functions as a negative regulator in leaf senescence through the prevention of chlorophyll degradation in sorghum. This fact was established through the study of a mutant obtained using a CRISPR/Cas genetic construct carrying gRNA targeting the first exon of *SbWRKY50*. Suppression of *SbWRKY50* gene expression in a mutant carrying one mutant allele harbouring 2-bp incertion (*SbWRKY50-KO*) at the target sequence led to early termination of *SbWRKY50* translation, and caused leaf senescence [124].

Table 2: CRISPR/Cas9-induced site-directed mutagenesis in sorghum.

Target Gene/Locus	Encoding Trait/Protein	Mutant Phenotype	Editing Efficiency, %	Delivery	Reference
DsRED2	Red fluorescent protein	Red fluorescence in the mutant with destroyed fluorescence	27.8% of the stably transformed groups of cells contained *DsRED2* sectors	*Agrobacterium*-mediated genetic transformation	[117]
SbCENH3	Centromere-specific histone H3	No data	37–40% of T_0 plants with edits for different gRNAs	*Agrobacterium*-mediated genetic transformation	[93]
k1C, gene family, consensus sequence for 20 genes	22 kDa α-kafirin	Reduced α-kafirin level, increased protein digestibility and lysine content, floury endosperm	96.2% of T_0 plants with mutations in one or multiple *k1C* family genes	*Agrobacterium*-mediated genetic transformation	[111,119,120]
SbFT (*Sobic.010G045100*)	Flowering time	Delayed flowering	33.3% of T_0 plants (one bi-allelic mutant)	*Agrobacterium*-mediated genetic transformation	[61]
SbGA2ox5 (*Sobic.009G230800*)	Plant height	No effect	50% of T_0 plants (one monoallelic mutant)		
pds	Phytoene desaturase involved in carotenoid biosynthesis pathway and chloroplast development	Albino plants	30% of T_0 plants	particle bombardment	[83]

Table 2: *Cont.*

Target Gene/Locus	Encoding Trait/Protein	Mutant Phenotype	Editing Efficiency, %	Delivery	Reference
lg1	Ligule development	Reduction in leaf inclination angle; absence of auricle (in homozygous mutants)	33% of T_0 plants (one monoallelic mutant)	particle bombardment	[56]
Mtl (*Matrilinial, Sobic.001G348600*)	Haploid induction	No data	74.2% of T_0 plants	*Agrobacterium*-mediated genetic transformation, MAT[1]	[95]
γ-Kafirin (*Sobic.002G211700*)	γ-Kafirin	No data	63.6–66.7%, for different gRNAs, of T_0 plants		
Lgs1 (*Low germination stimulant 1, Sobic.005G213600*)	Strigolactone synthesis	No data	83.3–92.9%, for different gRNAs, 52.4% gene-dropout		
Bmr6 (*Brown midrib 6, Sobic.004G071000*)	Brown midrib	No data	93.2–93.7%, for different gRNAs, 43.9% gene-dropout		
pds	Phytoene desaturase involved in carotenoid biosynthesis pathway and chloroplast development	Albino plants	15.5–22.7% for different genetic constructs, 16.7% with knockouts	*Agrobacterium*-mediated genetic transformation, MAT	[59]
Low germination stimulant 1 (*LGS1*)	Strigolactone synthesis	Root exudate from lines with deletion induced lower *Striga hermonthica* germination	60% of T_0 plants with deletion of *LGS1*	*Agrobacterium*-mediated genetic transformation, MAT	[113]
β-Kaf (*Sobic.009G001600*)	β-kafirin	No data	80% of T_0 plants were mutants with bi-allelic indels	particle bombardment	[60,112]
gKaf1 (*Sobic.002G211700*)	γ-kafirin	No data	90% of T_0 plants were biallelic mutants with large deletions or small indels		
SbGhd7 (*Sobic.006G004400*)	Grain number, plant height and heading date	No data	56% of T_0 plants were mutants (3–bi-allelic, 2–mono-allelic)		
SbBADH2	Betaine aldehyde dehydrogenase, which is responsible for 2-acetyl-1-pyrroline (2-AP) production	Aromatic smell in both seeds and leaves	39% of T_0 plants	*Agrobacterium*-mediated genetic transformation	[116]
k1C5 (*Sobic.005G193100*);	22 kDa α-kafirin	Improvement of protein digestibility	66.7% of T_0 plants had mutations in *k1C5*	*Agrobacterium*-mediated genetic transformation	[84]
gKaf1 (*Sobic.002G211700*)	γ-kafirin	Improvement of protein digestibility	12.5% of T_0 plants		
k2G (*gKaf1*?)	γ-kafirin	Improvement of protein digestibility, floury endosperm	12.5% of T_0 plants	Pollen-mediated transformation	[63]
SbWRKY50 transcription factor	Leaf senescence	accelerated leaf senescence	No data	*Agrobacterium*-mediated genetic transformation	[124]

Table 2: *Cont.*

Target Gene/Locus	Encoding Trait/Protein	Mutant Phenotype	Editing Efficiency, %	Delivery	Reference
SbCCD8a, *SbCCD8b*	Carotenoid cleavage dioxygenase involved in strigolactone biosynthesis	Reduction of orobanchol production and *Striga* germination; increased tillering; reduced grain yield	28.6% of T_0 plants	*Agrobacterium*-mediated genetic transformation	[115]
SbBADH2	Betaine aldehyde dehydrogenase	Increased 2-AP content in seeds and leaves	40% of T_0 plants	*Agrobacterium*-mediated genetic transformation	[110]
pds	Phytoene desaturase, involved in carotenoid biosynthesis	Albinism	29% of T_0 plants	*Agrobacterium*-mediated genetic transformation, *GRF4-GIF1* ternary vector	[97]
bmr6 (*Sobic.004G071000*)	Brown leaf midrib	Leaves with brown midrib	54% of T_0 plants		
ccd7, *ccd8*, *max1*, *duf*	Carotenoid cleavage dioxygenases; MORE AXILLARY GROWTH 1; DUF protein	Decreased content of strigolactones in root exudates, delayed or reduced emergence rates of *Striga*	8.1–17.5% of T_0 plants had mutations in different genes	*Agrobacterium*-mediated genetic transformation	[114]
pds	Phytoene desaturase involved in carotenoid biosynthesis pathway and chloroplast development	Albino plants	22.2–38.1% of T_0 plants had mutations in variants without selection	particle bombardment, co-transformation by *Cas9* plasmid and plasmid with gRNA cassette aimed at editing the *pds* gene	[109]

[1]MAT–morphogene assisted transformation.

3.5 Resistance to Biotic Stressors

Improving resistance to biotic stressors—pathogens, insect pests and the parasitic plant *Striga*—are important objectives of sorghum breeding. However, to date, there are no reports in the literature on the use of genome editing to solve these problems, with the exception of works describing the production of sorghum plants with resistance to *Striga hermontica*.

The parasitic plant *Striga hermontica* causes significant damage to sorghum crops in Africa. The seeds of Striga germinate under the influence of sorghum root exudates and form haustoria, through which they penetrate the root tissues. It was found that strigolactones, which are part of the exudate, are an inducer of Striga seed germination. The *LOW GERMINATION STIMULANT 1* (*LGS1*) locus located on chromosome 5 (*Sobic.005G213600*) was identified [113]. This locus encodes strigolactones, hormones that regulate plant development and mediate rhizosphere interactions. Synthesized from β-carotene through a multistep enzymatic pathway, strigolactones modulate key physiological processes, including shoot branching, leaf development, flowering, and root growth. In addition, strigolactones are exuded into the soil, where they facilitate symbiotic relationships with arbuscular mycorrhizal fungi to enable nutrient exchange and are also exploited by parasitic weeds to locate host plants. The synthesis of strigolactones in sorghum is controlled by a number of genes: *D27*, *CCD7*, and *CCD8* encoding, respectively, iron-binding isomerase D27, carotenoid cleavage dioxygenases 7 and 8, which cleave and convert 9-cis-β-carotene into carlactone, the central precursor of all structurally diverse strigolactones, and *MAX1* (*MORE AXILLARY GROWTH*),

controlling subsequent stage of strigolactone synthesis (an oxidase converting carlactone into carlactonic acid) [125,126].

In order to delete the *LGS1* gene in the sorghum cv. Macia using the CRISPR-Cas9 system, two single guide RNAs, were designated and plasmid containing these gRNAs, *cas9* and morphogenes genes *Baby boom* and *Wuschel2* were introduced into immature embryos via *Agrobacterium*-mediated genetic transformation. From 250 infected embryos, 65 T_0 plants were regenerated and 39 T_0 plants were positive for *LGS1* gene deletion (60%). In each of the three independent lines, selected for further analysis, the S_1 plants segregated at ratio of 3:1 for deletion (*LGS1 lgs1* and *lgs1 lgs1*) and no deletion (*LGS1 LGS1*). Root exudate from these *LGS1* deletion lines induced substantially lower *S. hermonthica* germination compared to the control lines, however this effect varied in different environmental conditions [113].

In another study [115], CRISPR/Cas9-mediated mutations in the genes encoding carotenoid cleavage dioxygenase (*SbCCD8a* and *SbCCD8b*), which is involved in biosynthesis of strigolactone were induced. Two out of 7 T_0 plants contained mutations: one plant had mononucleotide insertion in the target motif of the *CCD8a*, and another one had mononucleotide deletion in the target motif of the *CCD8b* that introduced a premature stop codon. Knockout of the *CCD8b* gene significantly reduced orobanchol production and *S. hermonthica* germination. However, in the mutants, changes in plant morphology were observed: altered root architecture, increased branch number, and reduced grain yield.

Recently, S. Kaniganti and colleagues [114] reported the production of sorghum lines with edited sequences of several genes controlling the synthesis of strigolactones (*CCD7*, *CCD8*, *MAX1*), and an uncharacterized gene (*DUF*) localized in the fine-mapped 400 kb *lgs1* region. The induced mutations led to down-regulation of the targeted genes. In different mutants, the *CCD7* expression levels was decreased significantly by 2.6 folds, a decrease in *CCD8* expression by around 2.2–4.8 folds, and *MAX1* expression by 2.3–5.9 folds when compared to wild-type plants. Such decreased expression level of key strigolactone synthesis genes results in lowering amounts of 5-deoxystrigol, sorgomol, and strigol in root exudates by 2.4- and 34-fold less than in the wildtype plants. *Striga* infection experiments conducted in a greenhouse on T_2 generation plants demonstrated delayed or reduced emergence rates of *Striga* in edited lines with lower strigolactones production. However, the edited sorghum plants in the T_1 generation displayed altered agronomically valuable traits, such as reduced plant height and increased tillering, compared to the wild-type plants. Nevertheless, this work demonstrates the potential of using genome editing to produce sorghum lines resistant to *Striga* infestation.

3.6 Plant Reproduction

Genetic systems involved in plant reproduction hold a key position in the genetic improvement of sorghum. Reproduction of sorghum plants is controlled by numerous genes that regulate initiation of flowering, inflorescence structure and size, spikelet formation and its structure, development of flowers, and their structural elements, including micro- and macrosporangia (anthers and ovules), microsporogenesis and microgametogenesis, macrosporogenesis, embryo sac development, double fertilization, endospermogenesis, seed development and its structure. Currently, work on editing the genes that control the reproductive system of sorghum plants is in its infancy. In this regard, it is worth noting the work of Char et al. [61] on editing the *SbFT* gene, which controls flowering time. The same work presents results on editing the *SbGA2ox5* gene, which was believed to control plant height. Among the T_0 plants carrying the constructs for editing these genes, a biallelic mutant with deletions of one and four nucleotides in the *SbFT* gene and a monoallelic mutant with a large deletion (43 bp) in the *SbGA2ox5* gene were identified. The frequency of plants carrying the CRISPR/Cas constructs was low (14.3% among plants carrying the construct for

mutation of the *SbFT* gene, and 13.3% for those carrying the construct for mutation of the *SbGA2ox5* gene). In the progeny of the biallelic mutant, the induced deletions were inherited according to a 1:2:1 segregation, whereas the large deletion in the *SbGA2ox5* gene was present only in the heterozygous state in one T_1 plant. Notably, in the T_1 generation, new mutants were detected in the progeny of T_0 plants carrying the CRISPR/Cas genetic constructs at a frequency of 68% and 24%, respectively [61].

The use of haploids underlies many methods and technologies of modern plant genetics and breeding. Haploid induction is essential for breeders to develop doubled haploids and stabilize the genetic architecture of inbred lines. One effective method for producing haploids is the use of haplo-inducer lines carrying mutations in the *CENH3* (Centromeric Histone H3 variant) gene. *CENH3* plays a crucial role in centromere formation and kinetochore assembly, and mutations in this gene may cause centromere dysfunction or inactivation and induce selective chromosome elimination during hybridization, thereby facilitating haploid formation [127–129]. Site-directed mutagenesis of the *CENH3* nucleotide sequence using CRISPR/Cas genetic constructs can facilitate the production of haplo-inducer lines in sorghum. Che and colleagues conducted experiments to obtain mutants in the *CENH3* gene of sorghum (*Sb-CENH3*) [93]. The frequency of mutants among the plants obtained in these experiments was 37–40% for different gRNAs. However, the authors did not report anything about the acquisition of the ability for haplo-induction in the obtained mutants.

The use of genome editing can facilitate researches on obtaining lines and hybrids capable of apomictic reproduction. Fixation of the heterosis effect in the progeny of F_1 hybrids has been the cherished dream of many generations of geneticists and plant breeders. With the development of genome editing and synthetic biology technologies, it has become possible to construct apomixis *de novo* by combining different genetic systems in one genotype: (1) causing disruption of meiosis and the formation of unreduced egg cells, (2) controlling the ability of the egg cell to undergo parthenogenetic development, (3) determining the autonomous development of the endosperm (or endosperm development can occur through pseudogamy). In this way, by using genetic constructs CRISPR/Cas that induce mutations in three genes involved in the genetic control of meiosis—*Pair1* (chromosome pairing disorder during crossing over), *Rec8* (sister chromatid cohesion in the first division of meiosis, preventing their separation), and *Osd1* (omission of second division), together forming a *MiMe* system (*mitosis instead meiosis*), and the genes encoding morphogenetic regulators *BBM* or *Wus*, which were used as parthenogenesis inducers, apomixis has been successfully engineered in rice [130–132].

Recently, by using similar approach induction of synthetic apomixis was reported in two sorghum hybrids [62]. In this study, meiosis was disrupted by CRISPR/Cas9-induced knockout of the genes *Spo11* (encodes an endonuclease that ensures chromatid breaks during conjugation), *Rec8*, and *OsdL1* (or *OsdL3*). Parthenogenesis was induced in the resultant diploid egg cell by the expression of the apospory-specific genomic region *BABYBOOM-LIKE2* gene *ASGR-BBML2* from the natural apomict, *Cenchrus squamulatus* (syn. *Pennisetum squamulatum*). Apomictic plants were obtained using two-step approach. Initially, transgenic plants of the line Tx430 with genetic construct with *ASGR-BBML2* were obtained. The frequency of parthenogenesis was 30–40% with different genetic constructs differed by promoters used. Then, transgenic plants of Tx430 with *ASGR-BBML2* genetic construct were crossed as female with a wild-type Tx623 sorghum line, and immature embryos were used to introduce CRISPR/Cas9 genetic construct with *MiMe* gRNA. The frequency of editing of each of *MiMe* loci ranged from 45% to 47%. As a result, four independent transgenic events were obtained, which produced diploid parthenogenic progeny with a frequency ranged from 68.0% to 87.5%. Analysis of SNP polymorphism in the progeny of these plants showed that 96% of plants from T_1 progeny were heterozygous on all chromosomes. Similar level of heterozygosity was observed in T_2-T_4

generation obtained by self-reproduction. These data indicate that induced synthetic apomictic seed set is maintained over at least four generations. However, seed set in these apomictically reproduced hybrids was reduced in comparison with F_1 hybrids, thus indicating the need for further research.

4 Conclusions and Prospects

CRISPR/Cas is undoubtedly one of the most effective technologies of modification of plant genes. However, the results of its use in sorghum to date are far from this statement. The main reason for the lag in the use of this technology in sorghum is the complexity of introducing CRISPR/Cas genetic constructs into the genome of this crop. Current achievements in the field of *Agrobacterium*-mediated transformation, based, among other things, on the use of vectors with genes of morphogenetic regulators, allow us to expect rapid progress in this area. However, the widespread use of such vectors in the creation of new varieties and hybrids may encounter restrictions related to patent law.

Legislative restrictions on the use of genome-edited plants in various countries around the world also hinder the advancement of this technology. Due to the complex regulatory constraints on the use of edited plants on account of the possible presence of transgenic sequences, an important promising approach is the development of a transgene-free genome editing systems. Such systems involve editing of target genes as a result of transient expression of a CRISPR/Cas construct, without its stable integration into the plant genome, or by DNA-independent editor delivery, for example, by using particle bombardment of immature embryo with preassembled CRISPR/Cas9-gRNA ribonucleoproteins [103–105]. This approach simplifies the regulatory processes in many jurisdictions around the globe.

A serious problem with using CRISPR/Cas technology is the potential for off-target mutations. CRISPR/Cas technology is considered one of the most effective tools for inducing mutations at strictly defined loci of the plant genome. However, due to the homology of the nucleotide sequences of selected targets to other loci in the genome, off-target mutations can sometimes arise [133–135]. The off-target activity of the genetic constructs used to edit target genes can theoretically pose a risk of mutations elsewhere in the genome, which raises concerns among biosafety experts [136]. It is quite obvious that when using multiplex vectors, the likelihood of off-target mutations increases significantly. However, among the relatively small number of experimental studies on sorghum genome editing, we were unable to find reports on the induction of off-target mutations. Nevertheless, the likelihood of such effects of CRISPR/Cas genetic constructs exists, but it can be reduced by carefully choosing and designing gRNAs for the target genes. Reducing the likelihood of off-target effects is a prerequisite for research aimed at obtaining practical result. In this regard, a promising approach is to increase the specificity of genetic constructs used for genome editing, for example, new types of nucleases with more complicated PAM sequences (Cas12b, Cas12c, eSpCas9, SpCas9-HF). Choosing unique target sequences in the genome can be a strategic approach to reducing off-target effects [135,137]. Another cause of off-target mutations may be somaclonal variation arising during the production of edited plants in tissue culture [138]. It should also be noted that the vast majority of off-target mutations can be detected in subsequent generations during 2–3 cycles of self-pollination of the genome-edited plants and rejected.

The potential for non-targeted genetic changes, as well as the presence of transgenic constructs in edited plants, necessitates the development of biosafety guidelines that should regulate their use. With regard to transgenic plants, existing regulations in different countries of the world are divided into two groups: as either process or product-oriented [139]. Regarding genome-edited plants, the discourse is centered on determining which system is best suited for the regulation of products developed using gene editing techniques. Currently, different countries around the world have different approaches to

regulating the use of genome-edited plants. For example, in North America—the United States and Canada, in South America—Argentina, Brazil, Chile, Paraguay, Colombia, and Ecuador, in Asia—Japan, India, and the Philippines, in Africa—Nigeria, Kenya, and Ghana, as well as in Australia, the genome-edited plants are regulated as conventional new varieties [140].

Another important issue in the practical use of mutant lines obtained in genome editing experiments is the stability of inheritance and expression of induced mutations. Genetic redundancy can lead to the replacement of knocked-out genes by homologous genes present in the genome. This issue is particularly relevant for genes that form gene families, in particular the kafirin genes [120]. On the other hand, field growing conditions, exposure to high temperatures, and drought can modify the expression of induced mutations. In our experiments, a relative decrease in the high digestibility of kafirins was observed in the progeny of mutants grown in the field compared to mutants grown in a greenhouse [121]. However, these problems are common to genetic studies of plant mutants obtained by various methods.

The main areas of further use of CRISPR/Cas technology will be related to overcoming the limitations that restrain sorghum production. Namely: increasing the yield and quality of grain and green mass, increasing resistance to abiotic stresses (drought, soil salinization and acidification), pests and diseases. Since these polygenic traits are governed by complex gene networks, multiplex vectors carrying several guide RNAs are required to target multiple genes simultaneously.

These studies should be based on similar works on other cereal species. For example, in rice, editing the nucleotide sequence of the *OsSPL14* gene, *IDEAL PLANT ARCHITECTURE 1*, led to an increase in shoot number and panicle size [141]; mutation of the *GROWTH REGULATING FACTOR 4* (*OsGRF4*) gene in rice led to an increase in grain size [142].

An important aspect of improving the quality and nutritional value of sorghum grain is the change in the starch composition, namely, a decrease in the amylose content and an increase in the amylopectin content. The digestibility of kafirins as well as the digestibility of starch itself is higher in *waxy* mutants characterized by suppressed amylose synthesis, in which the starch consists mainly of amylopectin [118,143]. This fact is of great importance for sorghum cultivars and hybrids used for both food and feed purposes. The key enzyme controlling amylose synthesis in plants is granule-bound starch synthase (GBSS I, EC 2.4.1.11), which functions in amyloplasts. The occurrence of mutations in the nucleotide sequence of the *GBSS* gene (*Sb10g002140.1*) leads to blocking of the synthesis of the GBSS I enzyme or disruption of its functioning, which completely suppresses or significantly reduces the synthesis of amylose [144,145]. Sorghum varieties carrying the *waxy* mutation, which reduces amylose content, have a higher nutritional value due to improved digestibility of proteins and starch, and therefore are of higher value as sources of feed and food grain [118,146]. The use of genome editing technology seems to be a promising approach to solve this problem, since the *waxy* gene sequence (*GBSS*) has been sequenced and deposited in GenBank. *GBSS* gene knockout mutants have been previously generated using CRISPR/Cas technology in rice [147–149] and maize [150]. These studies can serve as a guide for generating *waxy* mutants in sorghum.

A serious problem in sorghum cultivation is the overgrowth of crops by weeds. In this regard, the use of CRISPR/Cas technology seems very promising in terms of site-directed mutagenesis of the gene encoding one of the essential enzyme involved in amino acid biosynthesis—ACETOLACTATE SYNTHASE (ALS), which is strongly inhibited by several herbicides, such as sulfonylureas, imidazolinones, triazolopyrimidines, and others with a similar chemical structure. Examples of successful application of CRISPR/Cas-mediated site-directed mutagenesis using cytidine base editor for obtaining plants tolerant to these herbicides have been reported for wheat [151], rice [152], and sugarcane [153].

Great opportunities are opening up when using genome editing technology to modify sorghum reproductive systems. Targeted mutagenesis of the centromeric histone H3 gene (*CENH3*) via genome editing triggers chromosome elimination, enabling the creation of haploid inducer lines. Recently, the possibility of creating haplo-inducer lines based on mutations in the *CENH3* gene induced by the CRISPR/Cas system was demonstrated in wheat [154] and maize [155].

Editing nucleotide sequences of CMS fertility restorer genes (*Rf*) can facilitate the generation of sterility maintainer lines and, thus, the development of new CMS-lines used for the production of heterotic hybrids. This approach may be relevant for the A1 type sterile cytoplasm (milo), widely used in the F_1 sorghum hybrid breeding, with the aim of obtaining stable male sterility maintainers among valuable agronomically-important lines.

Moreover, using a mitochondrial-targeted transcription activator-like effector nuclease (mitoTALENs), as recently shown in rice [156] and broccoli [157], opens up the possibility of editing mitochondrial genes involved in the control of CMS. For sorghum, the development of such an approach may have enormous prospects, since targeted induction of mutations in the mitochondriome can contribute to the creation of new CMS types. In addition, editing of mitochondrial genes can contribute to the production of fertile revertants, which are necessary for studying the molecular mechanisms of CMS and for practical breeding.

The use of more efficient CRISPR-associated protein systems, such as Cas9 nickase (nCas9) and Cpf1 (Cas12a), opens up wide possibilities. nCas9, unlike Cas9, can only generate single-strand breaks, a process that can reduce off-target effects of CRISPR/Cas system. Therefore, nCas9 increases editing specificity and is used for base editing [158]. Until now, it has not been used in genome editing experiments in sorghum.

Cpf1 endonuclease from *Francisella novicida* is small in size compared to Cas9 and requires shorter crRNA. Cpf1 is guided by gRNA (20 bp) that binds on T-rich protospacer adjacent motifs 5′-TTTN-3′ or 5′-TTN-3′ and cuts the DNA 18–23 nucleotides away from the PAM by introducing 5 base pair (bp) staggered cuts. By staggering DNA cuts (sticky ends), the Cpf1 system promotes more efficient and precise insertion of new DNA fragments (knock-in) through the mechanism of homologous recombination compared to the blunt ends left by Cas9. In addition, Cpf1 can be used for editing of T-rich genomic sequences contrary to Cas9, which is used for G-rich sequences. The size of the gene encoding for Cpf1 is smaller than that of Cas9, thus reducing the overall size of the plant transformation vector, which makes for easy packaging and transfer into plant cells. The dual activity of Cpf1, cleaving target DNA as well as cleaving its own crRNA, makes it suitable and the easiest way for multiplexing than Cas9. The CRISPR/Cpf1-mediated high genome editing efficiency was shown in wide variety of di- and monocotyledonous plants (tobacco, soybean, maize, rice), although until now it has not been used in genome editing of sorghum [8,158].

Base editors are considered as more precise tools than Cas9. These are chimeric protein comprised of an RNA-guided endonuclease (nCas9 or Cpf1), and an enzyme able to deaminate a cytidine or an adenine base. The fused deaminating enzyme may be cytidine deaminase (C-to-T) or adenine deaminase (A-to-G) [8,158]. Base editors do not produce double DNA breaks and, therefore, have a lower frequency of off-target mutations than ordinary Cas9. Both cytidine (CBE) and adenine base editors (ABE) are currently widely used for targeted mutagenesis in numerous plant species, including rice, wheat, maize, cotton, soybean, and others. For example, CBE was used to generate mutants with new *waxy* alleles in rice [159]. With the use of CBE, wheat, rice, and sugarcane mutants tolerant to herbicides with a point mutation in the *ALS* gene were obtained [151–153].

Prime Editors (PEs) have introduced the possibility of small programmable genomic changes. The PE2 consists of a Cas9 nickase fused to an engineered reverse transcriptase. To introduce a modification in the genome, PEs use a prime editing extended guide RNA (pegRNA), consisting of a 20 nt guide sequence, a

primer binding site (PBS) and a reverse transcriptase template (RTT). The guide directs the Cas enzyme to a target site, the PBS hybridizes to the opposite strand to prime the reverse transcriptase, and the RTT integrates the desired genomic alteration [160]. Prime editing has been used in rice, wheat, maize, and some other plants, but not in sorghum.

An analysis of the results of genome editing in sorghum compared to other cultivated cereal crops—rice, maize, and wheat—indicates a significant lag in sorghum research. Research on the genome editing of rice, maize, and wheat has experienced exponential growth over the past decade, with thousands of publications now available across major scientific databases. The main areas of these researches are editing genes that control yield, grain quality, resistance to biotic and abiotic stresses, and plant reproduction systems. The results of this work have already been presented in the form of varieties ready for field trials or those that have already undergone such trials and are awaiting approval from regulatory authorities. Thus, high-yielding and stress-tolerant rice, created through genome editing, has already undergone field trials in India and has been approved for cultivation [161]. Low-gluten wheat is undergoing field trials in the UK [162]. Waxy corn, created through genome editing of the *Waxy* genes and characterized by increased yield, is ready for the market [163]. These studies serve as guidelines for similar research in sorghum. To date, the practical applicability of mutants created in sorghum has not been proven. Moreover, some of them, such as mutants resistant to *Striga* damage, have reduced yield or morphological abnormalities. Some mutants with improved kafirin digestibility have a floury endosperm, reducing their application in practical breeding. These data indicate the need for further research, the relevance of which, in connection with ongoing climate change, is absolutely obvious.

Thus, the above examples of the use of genome editing technology demonstrate the virtually unlimited possibilities of this approach for creating sorghum cultivars and hybrids with new traits that will make this crop more in demand and adapted to environmental changes and the market.

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