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SPECIAL ISSUE ON GENOME EDITING

CRISPR/Cas-mediated genome editing in sorghum — recent progress, challenges and prospects

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Abstract

Sorghum is a versatile crop with great potential as a sustainable food, feed, and bioenergy source. To mitigate the severely negative impact of climate change and population growth on food and energy security, further elevation of the crops stress tolerance is urgently needed. Genome editing technologies such as CRISPR/Cas have great potential to accelerate functional genomics and crop improvement by supporting targeted modification of almost any crop gene sequence. We describe the recent progress in genome editing of sorghum. In addition, we review remaining challenges and prospects of emerging gene editing technologies for rapid precision breeding of this crop.

Keywords Genome editing · CRISPR/Cas9 · Sorghum · Targeted mutagenesis · Crop improvement · Gene transfer · Biotechnology

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the top five cereal crops, used for food, fuel, fodder, and feed and grown on more than 3% of the cultivated land worldwide (Dahlberg *et al.* 2011). Sorghum varieties are categorized as either grain, forage, biomass, or sweet. Grain sorghums are characterized by high grain yields suitable for human and/or animal consumption (Can and Yoshida 1999). Forage sorghums are used for silage, hay production, or animal grazing (Kour and Pradhan 2016). Biomass and sweet varieties are characterized by high lignocellulosic biomass and/or high sugar accumulation, respectively, both of which can be processed into biofuels (Erickson *et al.* 2012; Mathur *et al.* 2017; da Silva *et al.* 2018). As a C4 grass, sorghum displays high photosynthetic efficiency and the ability to maintain rapid, high yielding growth in areas of low fertility (Leff *et al.* 2004; Calvino and Messing 2012). Traits which complement this include a short life cycle and heat and drought tolerance (Kour and Pradhan 2016).

Sorghum is the staple food in hot and dry environments of Asia and sub-Saharan Africa. To sustain the important role of sorghum in food security, its superior heat and drought tolerance needs to be further improved in addition to biotic stress tolerance (Calvino *et al.* 2011; Dhaka and Sharma 2017). Grain varieties also hold great potential as gluten-free alternatives to wheat and barley (Galassi *et al.* 2020).

To mitigate climate change, emphasis is currently being placed on developing renewable sources of energy. Biofuels derived from plant biomass can contribute to a reduction of net greenhouse gas emissions (Mat Aron *et al.* 2020). A shift towards second-generation biofuels, produced from inedible lignocellulosic biomass on marginal land, has also been spurred by emphasizing food security (Schmer *et al.* 2015). Dedicated biofuel feedstocks include sweet and biomass sorghums (Erickson *et al.* 2012; Cifuentes *et al.* 2014). However, scaling up the conversion of lignocellulosic biomass to fermentable sugars is still technically challenging (Singhvi and Gokhale 2019). Therefore, utilizing grasses like sweet sorghum or sugarcane where sugars accumulating in the stem can be easily extracted as juice and directly fermented into ethanol is currently the most promising approach for commercial biofuel production (Mathur *et al.* 2017). Goals for feedstock improvement include increasing biomass and biofuel

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yields on marginal land by improving biomass composition and photosynthetic and water use efficiency.

Traditionally, sorghum improvement has been achieved through conventional breeding practices, using both natural genetic variation and variation obtained through applied mutagenesis (Ulukan 2009; Jordan *et al.* 2011; Hao *et al.* 2021). Multiple breeding programs exist worldwide which have utilized techniques such as S_1/S_2 selection, pedigree, and backcrossing methods to improve sorghum cultivars. The advent of sequencing-based genotyping has accelerated sorghum breeding efforts by enabling genome wide association studies (GWAS), and genomic selection (Hao *et al.* 2021). Sorghum breeding has improved grain and biomass yield, grain and biomass composition, disease and insect resistance, and aluminum, salt and drought tolerance, among other traits, for production of elite varieties (Reddy *et al.* 2010; Kimball *et al.* 2019; Hao *et al.* 2021; Sapkota *et al.* 2020).

Next-generation sequencing technologies in combination with comparative and functional genomics of the extensive genetic sorghum resources have revealed key genetic loci and genes controlling important sorghum traits. For sorghum, a reference genome of the inbred grain variety BTx623 was released in 2009 (Paterson *et al.* 2009). Following this, sorghum has been proposed as a model species for other C_4 grasses, such as sugarcane, due to its small genome size (Calvino and Messing 2012). An improved version of the reference genome was released in 2018 along with a second reference genome obtained from the sweet sorghum variety, Rio (McCormick *et al.* 2018).

Genome editing with programmable nucleases enables functional genomics and creation of genetic diversity for breeding in an unprecedented way. There are four major technologies for targeted genome editing including meganucleases (MegNs; Puchta *et al.* 1993), zinc finger nucleases (ZFNs; Bibikova *et al.* 2003; Porteus and Baltimore 2003), transcription activator-like effector nuclease (TALENs; Boch *et al.* 2009; Moscou and Bogdanove 2009), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9; Jinek *et al.* 2012; Cong *et al.* 2013). All of these editing platforms rely on their ability to make a targeted DNA double-strand break (DSB) *in vivo*, followed by the response of the cellular DNA repair process to rectify the break (Puchta *et al.* 1993; Schmidt *et al.* 2019). These genome editing technologies arose from studies of natural biological processes. For example, CRISPR/Cas is derived from a prokaryotic system of acquired immunity to invading nucleic acids (Garneau *et al.* 2010; Jinek *et al.* 2012). Unlike its predecessors, CRISPR functions through RNA-guided DNA cleavage to create site-specific double-strand breaks (DSBs; Garneau *et al.* 2010; Jinek *et al.* 2012). The break is then restored through one of three cellular repair pathways: template-dependent homology directed repair (HDR), non-homologous end joining (NHEJ), or microhomology-mediated end joining (MMEJ; Liang *et al.* 2005). CRISPR/Cas9 is the most popular genome editing tool due to simplicity of the design, high editing fidelity, and multiplexing ability (Zhu *et al.* 2020).

A range of genome editing tools have been applied successfully for genomics and crop improvement in a wide variety of species (Zhang *et al.* 2018). However progress in sorghum genome editing has severely lagged in comparison to other cereal crops. This review summarizes the research completed in sorghum genome editing to date, which exclusively includes reports for CRISPR/Cas9-mediated editing so far. We also address associated challenges, and future prospects.

Progress in Sorghum Genome Editing

The first successful demonstration of gene editing in sorghum was performed by Jiang *et al.* (2013) using Agrobacterium-mediated transformation of immature embryos (Table 1). An *A. tumefaciens* binary vector (Y158) was prepared with the following independent expression cassettes contained within the T-DNA region: an intentionally frameshifted *red fluorescence protein* (*DsRED2*) under the control of a maize ubiquitin 1 promoter/intron combination and a nopaline synthase 3′ terminator, a synthetic *Cas9* codon-optimized for expression in monocots and under control of the rice Actin1 promoter/intron combination, a guide RNA under control of the rice U6 promoter, and a *GFP-NptII* fusion gene under control of the CaMV 35s promoter with maize hsp70 intron. The latter cassette was designed to function as both a selectable and visual marker. A single sgRNA was designed to target 20-bp upstream of the *DsRED2* coding region, with the aim of repairing the frameshift to enable expression. Therefore, observation of green fluorescence protein indicated stable integration of the T-DNA, whereas expression of the red fluorescence protein indicated successful targeted mutagenesis.

Two weeks post-transformation, *GFP* expression was observed in groups of cells, indicating the presence of stably transformed cells. Five of the 18 cell groups which were *GFP*-positive also expressed *DsRED2*. This approaches the theoretical maximum frequency of one-third for reading frame rescue of *DsRED2* by NHEJ, suggesting that the gene editing in transformed cells was highly efficient. However, although Jiang *et al.* (2013) were successful in demonstrating gene editing using CRISPR/Cas9, the target gene was co-introduced, and was therefore not endogenous to sorghum. The edited cells were not regenerated into plants and sequencing evidence of the edits was not provided.

Following this, the first report of successful endogenous gene editing in sorghum was by Che *et al.* (2018), who employed Agrobacterium-mediated transformation using a ternary vector system to achieve transformation efficiencies between 21 and 25% in the grain inbred var. Tx430 up to 9.4% in the African grain varieties (Macia, Malisor 84-7,

Table 1 Summary of published reports on gene editing in sorghum. Studies are listed in chronological order.

Reference	Genotype	Expl.	Promoter sgRNA/ *Cas*	# of gRNA	SM	Target gene	Delivery method	Edit. effic. (%)*	Mut. type	Phenotype	Transgene & mutation segregation
Jiang *et al.* 2013	NR	IE	*OsU6/ OsAct1*	1	*npt*II	*mDsRED2*	Agro.	NR	NR	*DsRED2* expression (transient in IE cells)	NR
Che *et al.* 2018	Tx430	IE	*ZmU6/ ZmUbi1*	1	*npt*II	*Sb-CENH3*	Agro.	37-40	Indel	NR. Biallelic frameshift mutations potentially lethal	NR
Li *et al.* 2018a	Tx430	IE	*TaU3/ ZmUbi*	1	*npt*II	*k1C* gene family	Agro.	92.4	Indel	Partial opacity in T1 seeds, reduced α-kafirin, improved grain protein digestibility and lysine content	NR
Char *et al.* 2020a	P898012	IE	*OsU6/ ZmUbi1*	2	*bar*	*SbFT SbGA2ox5*	Agro.	33.3 83.3	Indel Indel	Delayed flowering. No phenotype, biallelic mutations potentially lethal	NR
Brant *et al.* 2021	Tx430	IE	*OsU6/ CaMV35S*	2	*npt*II	*SbLG1*	Biolistic	33.3	Indel	Altered leaf inclination angle, ligule and auricle size. Distinct phenotypes for WT, monoallelic and biallelic mutants	Transgene- free, biallelic *lg1* mutant plants in T1

*Calculated as (# of edited events/# of transformed events) * 100; #, number; Expl., explant; Edit. effic., editing efficiency; SM, selectable marker gene; Mut., mutation; IE, immature embryo; NR, not reported; Os, *oryza sativa*; Zm, *zea mays*; Ta, *Triticum aestivum*; CaMV, *cauliflower mosaic virus*; Act1, *actin* 1; ubi 1, *ubiquitin*1, *npt*II, *neomycin phosphotransferase* II; *bar*, bialaphos resistance; Agro., Agrobacterium; Indel, nucleotide insertion and/or deletion; WT, wild type

and Tegemeo). This optimized transformation system was also used for targeted mutagenesis of the sorghum *centromere-specific histone H3* (*Sb-CENH3*) locus. *Sb-CENH3* plays an important role in the regulation of chromosome segregation, and knockouts have potential use for developing haploid inducers. Three sgRNAs, each 20 nucleotides in length, were engineered to target within the *Sb-CENH3* locus and were each delivered independently. *Cas9* and the sgRNAs were under control of the maize ubiquitin 1 and maize U6 promoters, respectively. Editing efficiencies ranged from 37 to 40% over the three sgRNA target sites. Biallelic frameshift mutations of *Sb-CENH3* were not observed and presumed lethal. Events with monoallelic frameshift mutations were regenerated to plants. The impact of the *Sb-CENH3* edit on regulation of chromosome segregation was not reported.

Li *et al.* (2018a) employed CRISPR/Cas9 to edit a whole-gene family simultaneously. The aim of this research was to improve sorghum grain quality, which has notably low lysine content and digestibility. In sorghum seeds, kafirins comprise 70% of the total protein and were previously shown to be a major detriment to grain quality. Out of these, α-kafirins constitute the majority, and are encoded by a single gene family containing 20 highly similar *k1C* gene copies clustered on chromosome five. A sgRNA was designed to target the highly conserved N-terminal ER signal for efficient co-targeting of the entire gene family. CRISPR/Cas9 components were mobilized in a binary vector (pBUN421) and transformed into Tx430 immature embryos via Agrobacterium. *Cas9* and the sgRNA were driven by maize ubiquitin and rice U3 promoters, respectively. Twenty-six T0 events were generated, propagated, and self-pollinated, followed by analysis of the T1 and T2 seeds. T1 seeds of regenerated events harboring the genome editing vector displayed variable partial opacity caused by the reduced thickness of the vitreous endosperm layer, providing the first indication of targeted mutagenesis. A T7E1 assay and Illumina MiSeq analysis were performed to detect and characterize mutations, and SDS-PAGE and Western blot were used to monitor α-kafirin levels. Extensive editing in 25 of 26 events was observed with the majority of events displaying co-editing in two or more *k1C* family genes. One event displayed co-editing of 12 target genes. Kernel flour from T2 seeds exhibited improved protein digestibility and lysine content. This study marked the first report of multiplex target editing in sorghum. As seen in Table 1, a high editing efficiency of 92.4% was reported. In this context, it is important to note that the study targeted 20 genes with one sgRNA, only one of which needed to be edited for the event to be considered an edited event, which contributed to the elevated editing efficiency.

The studies discussed thus far all employed a single sgRNA cassette for gene targeting. However, multiplex gene editing has also been demonstrated using two guide RNAs to simultaneously target two distinct endogenous genes (Char

et al. 2020a). A binary vector containing *Cas9*, directed by the maize ubiquitin 1 promoter, and two guide RNAs, each driven by a rice U6 promoter, was constructed and delivered via Agrobacterium to sorghum immature embryos of var. P898012. The target genes were *SbFT*, hypothesized to underlie flowering time, and *SbGA2ox5*, thought to be involved in plant height. As seen in Table 1, both targets were successfully edited at efficiencies of 33.3% and 83.3% in the T0 transgenic lines, respectively. The relatively high editing efficiency of *SbGA2ox5* is in part a consequence of a single edited event which contained five clones that were each considered as independent lines in the efficiency calculation. Analysis of T1 plants revealed that mutations were inherited by progeny, and novel heterozygous mutations were observed in progeny plants of both edited and non-edited but stably transformed parental lines. Greenhouse trials of *SbFT* mutants revealed a delayed flowering time in comparison to the wild type, confirming the role of *SbFT* in the flowering time. No *SbGA2ox5* biallelic mutants were observed, suggesting lethality of such an event.

Confirming the efficacy of CRISPR/Cas9 constructs can be a time-consuming process, as plants typically need to undergo a period in tissue culture before enough leaf tissue can be excised for analysis. When protocol optimization is still required, as is the case for sorghum, this can be particularly challenging. Therefore, Brant *et al*. (2021) targeted the *Liguleless1* (*LG1*) gene to evaluate if edited events would display a rapidly scorable phenotype. A construct containing *Cas9*, driven by a CaMV 35S promoter with HSP70 intron, and two monocistronic sgRNA cassettes driven by rice U6 promoters were delivered through biolistic gene transfer into immature embryos of var. Tx430. Three transgenic events were generated, of which one displayed a distinguishable upright leaf phenotype in tissue culture. Sanger sequencing confirmed a monoallelic edit in the *LG1* gene with insertion of a single 'A' at one of the sgRNA target sites in the event with the upright leaf phenotype. The edited event was self-pollinated, and biallelic-, monoallelic-, and null-edited progeny were recovered in the T1 generation, including edited lines which exhibited segregation of edits and the transgenes. Both biallelic and monoallelic edited lines exhibited distinct upright leaf phenotypes, with biallelic edited lines demonstrating a more severe upright leaf phenotype completely lacking leaf ligules and auricles. In addition to forming the basis of a rapid phenotyping system for protocol optimization, the *LG1* edited lines have the potential to elevate canopy-level photosynthesis and yield by enabling increased field planting density, as has been demonstrated in maize and rice (Lee *et al*. 2007; Tian *et al*. 2019).

Three additional papers have been published which solely highlight protocols for genome editing in sorghum. Liu *et al*. (2019) described a protocol for biolistic delivery using a particle inflow gun (PIG), stating it was used to produce stable knockouts of the *cinnamyl alcohol dehydrogenase* (*CAD*) gene in var. Tx430. An editing efficiency of 25% was reported; however, no data regarding the lines was published as the paper focused solely on protocol description. Similarly, two protocol papers have been published outlining Agrobacterium-mediated delivery of genome editing tools to sorghum (Sander 2019; Char *et al*. 2020b).

Transient CRISPR/Cas9 Assays

Due to the time and cost involved in generating edited plants, construct functionality and sgRNA efficiency have traditionally been assessed transiently prior to attempting stable transformation, allowing for more rapid execution and optimization of gene editing constructs. The most common transient expression assays for whole vectors include protoplast expression assays and *in planta* expression via agroinfiltration (Abel and Theologis 1994; Lee and Yang 2006). Protoplast transformation allows for the rapid in vivo evaluation of multiple recombinant DNA vectors (Bossche *et al*. 2013). However, this technique requires development of a protocol yielding a high number of protoplast with sufficient viability to enable a high transformation efficiency and subsequent evaluation of gene expression/edits. Agroinfiltration of recombinant DNA constructs is technically less demanding than protoplast transformation. However, agroinfiltration depends on the compatibility between plant and Agrobacterium which is influenced by plant species/genotype, Agrobacterium strain, inclusion of additional virulence genes and suppression of hypersensitivity response (Yang *et al*. 2001; Wroblewski *et al*. 2005).

In sorghum, both protoplast expression and agroinfiltration have recently been successfully used to evaluate genome editing reagents (Meng *et al*. 2020; Sharma *et al*. 2020). Sharma *et al*. (2020) demonstrated agroinfiltration on 3–4-wk-old Tx430 sorghum plants at the three-leaf stage. Similar to the approach taken by Jiang *et al*. (2013), a binary vector was used which contained an intentionally frameshifted *green fluorescence protein* (*GFP*) gene under the control of a CaMV 35S promoter, a plant codon-optimized *SpCas9* under control of the maize ubiquitin 1 promoter, a sgRNA under control of the rice U3 promoter, and a *red fluorescence protein* (*DsRED*) gene under control of nopaline synthase promoter. The frameshifted *GFP* had an engineered 23 nucleotide sgRNA target sequence directly after the start codon, such that successful CRISPR/Cas9 targeting had a theoretical one-third chance to rescue the reading frame through NHEJ. GFP florescence was observed under microscopy 3–4 d after agroinfiltration. Although efficiency was not explicitly reported, it was stated that efficiency was markedly lower than previously observed in tobacco (Sharma *et al*. 2020), and as with the report of Jiang *et al*. (2013), the target gene was not endogenous to the sorghum genome.

Meng *et al.* (2020) developed a protoplast isolation assay from 10–15-d-old green stem tissue of var. BTx623, and achieved editing of the *chlorophyllide a oxygenase* (*CAO*) locus. Protoplasts were transfected in polyethylene glycol, and mutations were validated by T7E1 assay and sequencing. An editing efficiency of 46.7% was reported, with a variety of mutation types observed.

Challenges

Crop improvement by biotechnological approaches including genome editing hinges on the availability of a highly efficient gene transformation system, comprised of tissue culture, DNA delivery, and selection protocols (Altpeter *et al.* 2016, Figure 1). In sorghum, the two most common DNA delivery methods are Agrobacterium-mediated transformation and biolistic delivery. The first transgenic sorghum was generated in 1993 by biolistic delivery, with a transformation efficiency of 0.28% (Casas *et al.* 1993). Seven years later, the first report of Agrobacterium-mediated transformation in sorghum was reported with a transformation efficiency of 2.12% (Zhao *et al.* 2000). Efficiency has progressed since then but is severely limited to a few genotypes and both the reproducibility of reported high transformation efficiencies (Howe *et al.* 2006; Gurel *et al.* 2009; Liu *et al.* 2019) across different laboratories and the event quality still lag behind other major cereal crops (Che *et al.* 2018).

Sorghum regeneration is impeded by limited embryogenic callus induction, excessive production of phenolics, and poor regenerability, all of which are highly dependent on genotype, explant type and age, and medium composition (Elhag and Butler 1992; Jogeswar *et al.* 2007; Indra Arulselvi and Krishnaveni 2009; Raghuwanshi and Birch 2010; Liu *et al.* 2015). The most common choice of explant is immature embryos (IE), which possess high rates of callus induction and regeneration (Liu *et al.* 2015). However, some studies have also utilized shoot apices, leaf whorls, or immature inflorescences (Bhaskaran and Smith 1988; Zhong *et al.* 1998; Jogeswar *et al.* 2007; Silva *et al.* 2020).

As seen in Table 1, the majority of recent studies have utilized the grain sorghum variety Tx430, which is favored for its high regenerability from tissue culture (Miller 1984). Thus, improvements made in Tx430 which are targeted towards biofuel applications need to be transferred to sweet or biomass varieties through conventional breeding methods (Gao 2021). However, progress towards achieving genotype independence has been spurred by the use of morphogenic genes to enhance transformation. Both Lowe *et al.* (2016) and Mookkan *et al.* (2017) demonstrated that ectopic overexpression of the morphogenic genes *Baby boom* (*bbm*) and *Wuschel2* (*Wus2*) yielded high Agrobacterium-mediated transformation efficiencies in previously non-transformable maize varieties (Lowe *et al.* 2016). The use of morphogenic genes, ternary vectors, and auxotrophic agrobacterium strains has significantly improved transformation frequency (Lowe *et al.* 2016; Mookkan *et al.* 2017; Che *et al.* 2018), and has expanded the number of transformable sorghum genotypes (Che *et al.* 2021).

The diverse regulatory landscape for genome-edited crops presents a challenge for the commercialization and global trade of transgenic crops including sorghum. Gene-edited crops are not regulated in the USA, provided any transgenes are segregated out of the final product and the edits are comprised of deletion(s) of any size; or targeted substitutions of a single base pair; or edits correspond to sequences in the plants natural gene pool. A similar regulatory situation is found in Canada, Japan, and several South American countries. In contrast, in Europe and New Zealand, gene-edited crops are legislated as genetically modified organisms (GMOs; Hilbeck *et al.* 2020; Gupta *et al.* 2021). The status of gene-edited crops in some regions such as Africa and Asia remains amorphous (Oloo *et al.* 2020; Zhang *et al.* 2020).

Future Prospects

CRISPR-Cas9 has already revolutionized crop improvement and gene function analysis of major agronomic traits in plants. However, current reports in sorghum are limited to targeted mutagenesis while gene targeting has even greater potential to accelerate crop improvement by introducing precision nucleotide substitutions. Gene targeting with the help of template-mediated HDR has been successful in an increasing number of crops (Huang and Puchta 2019). In addition, novel classes of CRISPR–Cas-derived genome editing agents including base editing and prime editing have been developed and deployed for generation of precision nucleotide substitutions in plants (Zhu *et al.* 2020; Anzalone *et al.* 2019).

Base editing was developed to introduce C:G>T:A and A:T>G:C base transitions at targeted sites using cytidine deaminase (cytosine base editor, CBE) and adenosine deaminase (adenine base editor, ABE), respectively (Zhu *et al.* 2020). In contrast to HDR, CBE and ABE systems are unable to introduce long stretches of nucleotide substitutions. Also, the desired target has to be within a short editing window (Anzalone *et al.* 2019). However, base editors do not require DSBs, donor templates, or HDR. Base editors have already been utilized to enable precise, efficient, single base changes in various species and genes, using CBE or ABE, including rice, tomato, wheat, maize, Arabidopsis, rapeseed, and potato (Shimatani *et al.* 2017; Zong *et al.* 2017; Li *et al.* 2018b; Kang *et al.* 2018; Zong *et al.* 2018). So far, reports on application of CBE or ABE systems in sorghum are lacking.

So far, base editors cannot introduce transversion point mutations (C•G-to-A•T, C•G-to-G•C, T•A-to-A•T and T•A-to-G•C), precise deletions, or precise insertions. In contrast, prime editors can introduce all possible types of point

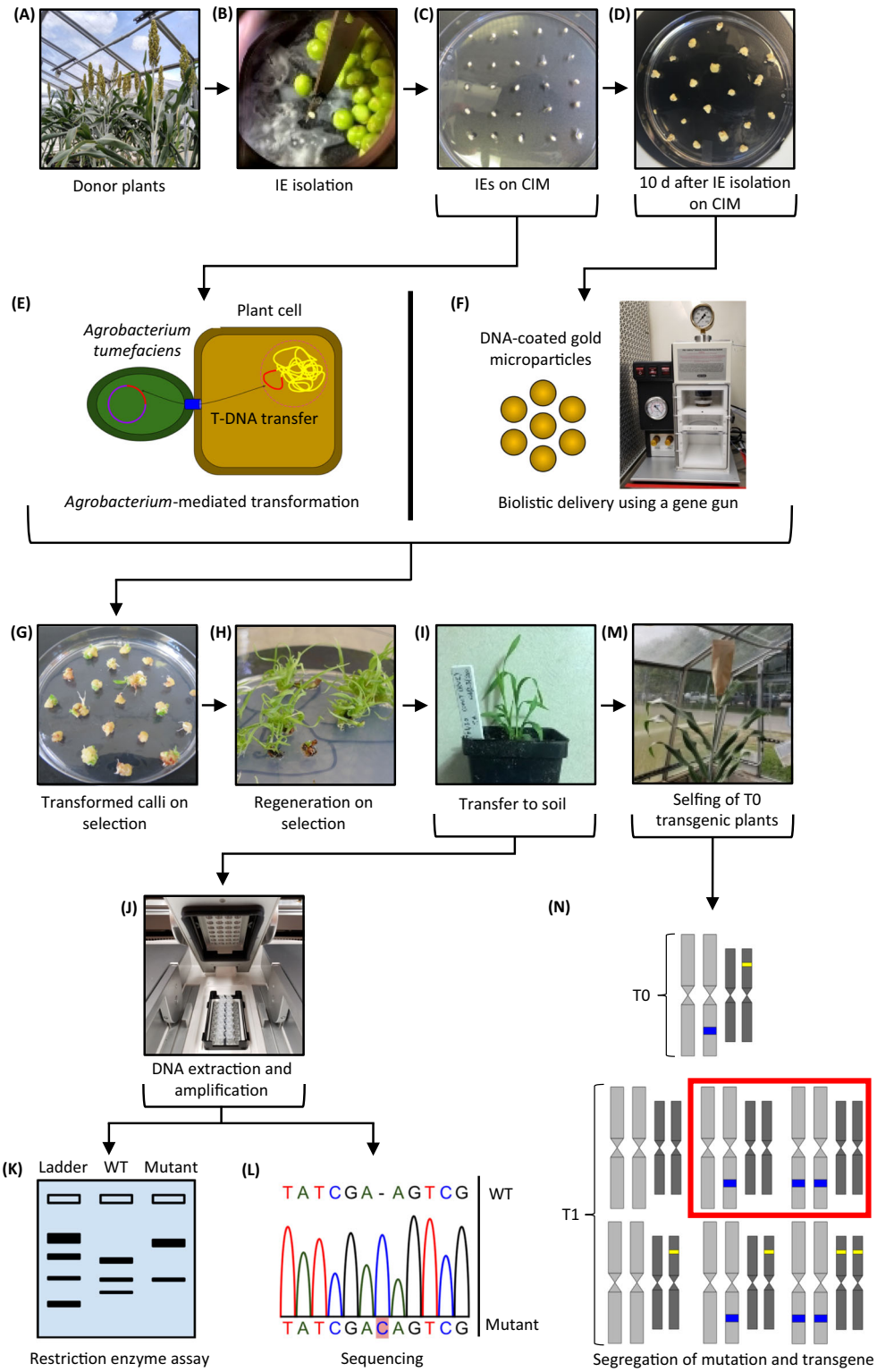

Figure 1. Overview of genome editing in sorghum. (*A*) Sorghum donor plants; (*B*) immature embryo (IE) isolation; (*C*) isolated embryos on callus induction media (CIM) scutellum side down; (*D*) IE on CIM 10 d after isolation; (*E*) inoculation with Agrobacterium containing T-DNA for *Agrobacterium*-mediated transformation; (*F*) biolistic delivery of DNA-coated gold microparticles to IE-derived callus; (*G*) transformed calli on selective media; (*H*) regenerating shoots on selective media; (*I*) regenerated plantlet in soil; (*J*) DNA extraction and PCR amplification for molecular analysis; (*K*) restriction enzyme assay of PCR amplicons of the target gene showing size differences between WT and mutant amplicons; (*L*) DNA sequencing of the PCR amplicons of the target gene showing a single C insertion in the mutant line; (*M*) selfing of mature T0 mutated line using a pollination bag; (*N*) segregation outcomes for the transgene (*yellow band*) and mutation (*blue band*) in T1 progeny. Desired T1 progeny containing the mutation without the transgene are highlighted in *red rectangle*.

mutations in addition to allowing precise insertions of up to 44 bp and precise deletions of up to 80 bp (Anzalone *et al.* 2019). Prime editors possess a Cas nickase domain that is fused to a reverse transcriptase domain. The prime editor protein is directed to the target site by a modified guide RNA (pegRNA), which in addition to specifying the target site also encodes the desired edit in its 3′ spacer. While base editing offers high efficiency and few indel mutations, prime editing allows greater flexibility and precision (Anzalone *et al.* 2020). However, although multiple variations of the prime editing

technology have been explored, it is yet to surpass the moderate efficiency of templated-mediated HDR (Zhu *et al*. 2020). To date, prime editing has only been demonstrated in a few crops (Lin *et al*. 2020; Xu *et al*. 2020a; Xu *et al*. 2020b) and a protocol for sorghum still has to be developed.

In contrast to the pleiotropic effects sometimes seen with gene knockout, gene-expression modulation allows for the creation of elite traits that are tunable and flexible without altering protein-coding sequences. Cas9 variants that are catalytically inactive, known as dead Cas9 (dCas9), do not break DNA but retain sgRNA-mediated sequence-specific DNA binding activity. As dCas9 docks at targeted locations in the genome, it can prevent transcriptional machinery from binding or disrupt RNA polymerase processivity, thereby repressing transcription (Gilbert *et al*. 2013; Shariati *et al*. 2019). Gene expression can also be modified by merging dCas9 or its orthologues with transcription regulators (Lowder *et al*. 2015; Li *et al*. 2017; Lowder *et al*. 2018) or epigenetic modulators (Gallego-Bartolomé *et al*. 2018; Papikian *et al*. 2019). By affecting the dynamics of enhancer–promoter interactions, chromatin structure can also be altered to modulate gene expression.

However, despite being robust, using dCas9 methods requires both dCas9 fusion proteins and sgRNAs to be continuously expressed in the target genome, which poses regulatory concerns. Due to this, targeted editing of *cis*-regulatory elements (CREs) may hold more potential for commercial crop production, as transcriptional regulation can be achieved without constitutive expression of dCas9. An example of this was shown in tomato, where a spectrum of alleles showing varied transcriptional modifications were created using eight sgRNAs targeting CREs in the promoter region of *CLV3* genes. Transgenes were then segregated out in later generations to prevent regulation as GMO (Rodríguez-Leal *et al*. 2017). It has also been demonstrated that use of shorter sgRNAs, between 16bp and 14bp, allows Cas9 to bind and regulate transcription rather than cleave DNA, eliminating the need for dCas9 (Kiani *et al*. 2015). These methods are yet to be explored in sorghum but may prove desirable routes for altering transcriptional regulation.

Although extensive research has been completed regarding the use of traditional CRISPR/Cas9 in targeted mutagenesis of ORFs, its potential for targeting non-coding regions has remained relatively unexplored in plants (Basak and Nithin, 2015; Brant and Budak 2018). For humans, targeting micro RNAs (miRNAs) has been thoroughly studied (Chang *et al*. 2016). However, although many reviews have commented on its potential in plants, very few comprehensive methods have been published. The most notable of these were demonstrated in rice and tomato (Hong *et al*. 2020; Chung *et al*. 2020; Zhou *et al*. 2017). If applied, this could be particularly useful in sorghum, as a number of analyses have already been completed to define the small RNA profiles of various sorghum varieties in relation to desirable traits, such as drought tolerance and sugar accumulation (Calvino *et al*. 2011; Hamza *et al*. 2016).

Multiplexed genome editing typically entails parallel expression of multiple sgRNA or Cas9 sequences, which facilitates modification of multiple genes simultaneously. This includes complex bioengineering applications, and control of regulatory pathways. CRISPR-Cas9 has been applied to plants using many different multiplexed sgRNA systems, such as RNA polymerase III (Pol III)–driven and Pol II–driven systems (Zhu *et al*. 2020). In Pol III–driven systems, multiple Pol III promoters are used (U3 and U6) to drive expression of sgRNAs in a single construct (Xing *et al*. 2014; Ma and Liu 2016). In comparison, in Pol II–driven systems, multiple sgRNAs are simultaneously expressed under a single promoter and processed as a poly-sgRNA-containing transcript. Csy-type ribonuclease 4 (Csy4) processing in plants, which cleaves 20-nucleotide sequences flanking the sgRNAs, was also enhanced following the introduction of a Pol II promoter (Čermák *et al*. 2017). Although multiplexed targeted mutagenesis has been reported in sorghum (Li *et al*. 2018a; Char *et al*. 2020a), future multiplexing approaches will likely also include combinations of targeted mutagenesis and gene targeting.

Novel methods of delivery are also being explored to accelerate testing of sgRNAs for gene function analyses. So far, this has been approached through virus-induced delivery of sgRNAs into lines which had already been engineered to express Cas9 (Ali *et al*. 2015; Mahas *et al*. 2019). Cas9-based synthetic transcription factor components can be co-delivered alongside sgRNAs with viral vectors without exceeding their cargo limitations. This technology has been named ViparíNama (ViN), and has already been demonstrated in tobacco, Arabidopsis, and tomato (Khakhar *et al*. 2021). As ViN can be applied *in planta*, the need for a transformation and tissue culture phase is eliminated, significantly decreasing both the timeline and the limitations currently faced by gene and sgRNA function analyses.

One advantage of CRISPR editing is that it is possible to produce edited varieties which contain no exogenous DNA, thereby circumventing regulations on genetically modified crops (Kumar *et al*. 2019). DNA-free CRISPR/Cas9 protocols have been developed which produce transgene-free edited plants without the need of transgene segregation. As sorghum has a relatively simple genome, transgene segregation from edits can be achieved within a single generation (Brant *et al*. 2021). However, use of DNA-free methods could streamline the process. These protocols involve introducing Cas9 ribonucleoproteins (RNPs) alongside sgRNAs without the addition of a vector backbone or DNA, and have been demonstrated in various crops, including wheat, lettuce, and oilseed rape (Liang *et al*. 2017; Murovec *et al*. 2018; Park *et al*. 2019).

In addition to technical advances in the CRISPR/Cas toolbox, it is important to consider what traits are agronomically

important in sorghum in order to focus future studies. The new generation of editing technology will further accelerate development of genetic variants which can be incorporated into breeding programs for the development of superior germplasm (Hao *et al*. 2021). This will enable improvement of agronomically important traits such as biotic and abiotic stress tolerance, biomass and grain quality and nutritional value, and bioenergy conversion efficiency.

In crops, herbicide resistance is also an economically important characteristic. *Acetolactate synthase* (*ALS*) plays a key role in branched-chain amino acid biosynthesis and is the target of various herbicides, including sulfonylureas and imidazolinones. Several studies have demonstrated that specific amino acid substitutions in the *ALS* gene can lead to herbicide tolerance in plants (Powles and Yu 2010). Recently, using CRISPR/Cas9 in conjunction with HDR, herbicide-tolerant rice, maize, and sugarcane were created by editing ALS alleles (Sun *et al*. 2016; Svitashev *et al.* 2016; Oz *et al*. 2021). Herbicide resistance has also been conferred in rice via CBE base transitions (Kuang *et al*. 2020). Different mutations in *ALS* gene have also been introduced though HDR, CBE, ABE, and NHEJ in maize, soybean, wheat, tomato, potato, among others (Dong *et al*. 2021).

Another herbicide target is *acetyl-CoA carboxylase* (*ACCase*). The enzyme produced by *ACCase* is crucial for lipid synthesis. By inducing an A1992V substitution into the wheat *ACCase*, quizalofop-resistance was induced (Zhang *et al*. 2019). Additionally, editing *EPSPS* (Hummel *et al*. 2018), *PPO* (de Pater *et al*. 2018), *TubA2* (Liu *et al*. 2020a), and *SF3B1* (Butt *et al*. 2019) has been observed to confer resistance to glyphosate, butafenacil, trifluralin, and herboxidiene, respectively. Alongside its agricultural applications, herbicide tolerance is also useful as a selective marker for gene-edited events. The research previously reported in other crops will accelerate the development of herbicide resistance in sorghum. These strategies can be expanded to include CRISPR/Cas-directed evolution. Directed evolution involves first creating a population of mutants, then selecting for desirable phenotypes (Gionfriddo *et al*. 2019). This relies on rapid identification of edits which induce the desirable phenotype without conferring any negative attributes. To date, most directed evolution studies have utilized DNA shuffling or PCR-based methods to introduce random mutations. However, CRISPR/Cas sgRNA libraries can be used to increase through-put and specificity (Zhu *et al*. 2020). For example in rice, C>T and A>G base editors were used with a library of 200 sgRNAs to create new variants of *acetyl-CoA carboxylase 2* (*ACC2*) which conferred herbicide resistance (Kuang *et al*. 2020; Li *et al*. 2020; Liu *et al*. 2020b). However, although CRISPR-Cas-directed evolution holds enormous potential, its application is currently limited to traits like herbicide resistance which are easily selectable, and it is yet to be explored in a wide range of crops.

The studies discussed in this review demonstrate that CRISPR/Cas9 has been successfully applied to create targeted mutations in sorghum. Although routine use of CRISPR/Cas9 in the sorghum breeding toolkit is not yet a reality, it is now evident that this new-generation genome editing technology is poised to make impressive contributions to sorghum improvement.

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Declarations

Disclaimer Any opinions, findings and conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Energy.

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