The Nucleus
https://doi.org/10.1007/s13237-024-00485-3

REVIEW ARTICLE

Gene editing tool kit in millets: present status and future directions

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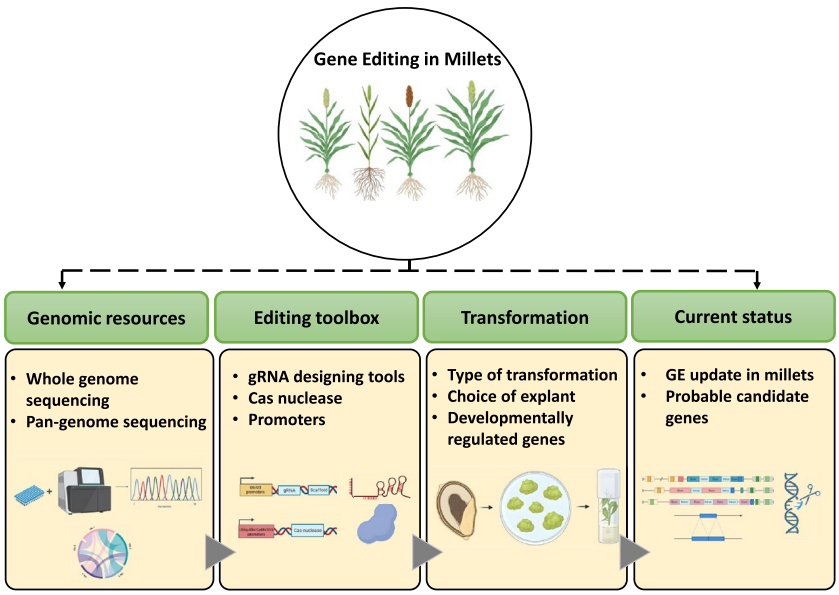
Received: 17 January 2024 / Accepted: 12 March 2024

Abstracts

Millets, the sixth most-grown group of crops in the drylands, support the livelihood of many small-holder farmers in the region. Being one of the most nutritious groups of crops, their production has been increasing since the last decade to meet the demands of the world's ever-increasing population. Since its discovery, CRISPR/Cas-mediated gene editing technology has revolutionized trait improvement in numerous crops by enabling targeted insertions and deletions at specific gene sequences. With advancements like base editing and prime editing, which offer precise modifications at the nucleotide level, this technology holds great promise for enhancing millets by targeting genes responsible for key traits. The updated sequence information in the public domain makes it possible to modify certain genic regions using the CRISPR/Cas-mediated gene editing technology to develop millet crops with improved agronomical properties. The review explores each component of the editing toolbox in millets, including the gRNA designing tools, types of Cas nucleases, and promoters to be considered for enhanced and efficient gene editing in millets. We have discussed fundamental information available to successfully employ CRISPR/Cas-mediated gene editing in millets, such as the availability of genomic information and plant transformation methods. Finally, we have highlighted the limitations of employing this novel technology in millet crops by providing future directions and immediate candidate genes that could be targeted to improve various traits in millet crops.

Graphical abstract



Keywords Gene editing · CRISPR/Cas · Crop improvement · Millets · Plant transformation

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Corresponding Editor: Kutubuddin Ali Molla, Reviewers: Mehanathan Muthamilarasan, Antony Ceasar.

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Published online: 23 April 2024

Introduction

Millets are an important food crop belonging to the family 'Poaceae' grown in the Semi-Arid regions of Asia and Africa. Millets have become an important food and feed crop for people living in dryland regions because of their ability to grow in semi-arid conditions with scarce rainfall, low soil nutrient content, and drought. The commonly cultivated millets include finger millet (*Eleusine coracana* (L.), pearl millet (*Pennisetum glaucum* (L.)), foxtail millet (*Setaria italica* (L.)), kodo millet (*Paspalum scrobiculatum* (L.)), proso millet (*Panicum miliaceum* (L.)), Indian barnyard millet (*Echinochloa frumentacea* (L.)), Japanese barnyard millet (*Echinochloa utilis* (L.) and little millet (*Panicum sumatrense* (L.). Millets are typically small-seeded grains with high nutritional value and are classified into major and minor categories. Major millets include proso millet, finger millet, foxtail millet and sorghum, while minor millets include little millet, barnyard millet, kodo, and teff [122].

Millets ranked as the sixth most-produced cereal crop globally, with a production of 30.86 million tonnes worldwide in 2022; Asia is on the top with an annual production of 15.582 million tonnes, followed by Africa with a yearly production of 14.6 million tonnes. As of the year 2022, millets were cultivated over a 29.85 M ha area across the globe, with India contributing to approximately 28.4% (8.48 M ha) of the total area harvested, followed by Niger and Sudan contributing 22.7% and 8.37%, respectively (http://www.fao.org/faostat). Often called "poor man's crops," millets sustain approximately one-third of the world's population. Their versatility and resilience make them crucial in addressing food security and nutritional needs, particularly in regions facing resource constraints and challenging agricultural conditions [132].

Millets have very high nutritional quality as they are a high source of carbohydrates, proteins, fats, fibers, and minerals. Foxtail, proso, and barnyard millet are rich in proteins, comprising more than 10% of the total grain weight. Additionally, foxtail and little millet have high fat content, with fats accounting for over 4% of the total grain weight. Barnyard millet, little millet, foxtail millet, and fonio are recognized for their high crude fiber content, ranging from 6.7 to 13.6%. Little millet and barnyard millet stand out for their high iron content, containing 9.3 mg and 18.6 mg per 100 g of grain [18, 146]. Millets are gluten-free, making them suitable for consuming people with celiac diseases [5]. It has been established that millets have a lower glycemic index (GI) than wheat and rice, making them an important candidate for exploitation against type II diabetes [143]. Some of the other health benefits of millet include improvement in the health of the muscular system, a reduction in cholesterol, preventing heart disease, and increasing energy [58].

Despite having a huge global impact and the potential to cause a significant economic impact on developing countries, millets are referred to as 'orphan crops' [161]. Various conventional breeding approaches have been applied in millets for trait improvements [172]. However, these methods are labour-intensive, time-consuming, and intricate. Hence, there is a need for more efficient and time-conserving breeding techniques [30]. Recently, much emphasis has been placed on sequence-specific nucleases to utilize them as New Breeding Techniques (NBT) tools for crop improvement [173]. Out of these tools, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system coupled with Cas nuclease has gained much attention for agronomical improvements of plants because of its precision [217]. The CRISPR/Cas-mediated gene editing technology incorporates targeted insertions and deletions at specific regions. The CRISPR/Cas-mediated gene editing technologies comprise the endonuclease protein, whose DNA target selection and cutting activity is controlled by short guide RNA [73]. Advances in this technology have allowed researchers to create Knock-out and knock-in lines using a prime editing tool and knock-down lines using a catalytically dead Cas9 (dCas9), bringing about transcriptional interference [75, 117]. Another advancement of this technology is base editing, which facilitates precise transitions of bases at target sequences [117]. Recent studies proved that CRISPR/Cas-mediated gene editing technology could modify targeted genomic regions in most crops to implant new traits for crop improvement [196].

The CRISPR/Cas-mediated gene editing technology is still in its infancy when millet crop improvement is considered. Using CRISPR/Cas-mediated gene editing technology for precise gene editing in millets, renowned for their climate resilience and high nutritional value, holds promise in identifying and validating various traits and underlying biological mechanisms associated with climate resilience and nutrition. Furthermore, leveraging the knowledge gained from employing CRISPR/Cas-mediated gene editing in other crops can significantly enhance millet crop traits, ultimately improving their resilience to climate variations. Therefore, developing a highly efficient editing toolbox for millets becomes imperative. To our knowledge, no reports explore each component of the CRISPR/Cas-mediated gene editing toolbox or the recent advances in millet gene editing in the context of millet trait improvement. This review explores the CRISPR/Cas toolbox necessary to employ gene editing in millets in the prospects of agronomical improvement. Further, the components of the CRISPR/Cas toolbox have been discussed comprehensively, highlighting recent advances and future directions.

Whole genome sequencing (WGS): pre-requisite for gene editing in millets

Whole genome sequences of plants serve as tools to gather information about coding and non-coding sequences of genes and correlate them to the phenotype for crop improvement. Thus, whole-genome sequences of crops are essential for understanding a plant's genetic makeup. They also help to understand the traits of a particular crop and its possible application in agriculture.

Due to the emergence of next-generation sequencing technologies, the cost of WGS of an organism has been reduced considerably. Foxtail millet is the first millet to be sequenced in the millet family and is considered a model plant because of its smaller genome and C_4 photosynthetic chemistry [134]. The genome assembly covered about 80% of the genome (400 Mb) and contained ~ 35,000 genes [13]. Following this, genomes of other millets were also sequenced, which included millets like Barnyard millet [54], Proso millet [153, 219] and Teff [21]. The genome assembly of finger millet cult. PR202 and ML-365 are still in their scaffold stage [59, 63]. The genome of pearl millet is considerably bigger, consisting of 1.79 GB of the draft genome with 38,579 genes, followed by the finger millet genome (~ 1.2 Gb), which consists of ~ 85,243 genes [63, 169]. In spite of the availability of advanced tools, the genome sequences of millets require reannotation, referencing, and resequencing to fill out the gaps. Additionally, utilizing these advanced tools to sequence genomic information of many minor millets is also required to gain insights into many novel traits. The platinum-grade de novo genome assembly has recently been released for pearl millet, which can help further improve the genomic resources [141]. The recently developed database 'milletdb' (http://milletdb.novogene.com/home) [158] is the most comprehensive database, including multi-omics data on most millet species. Apart from nuclear genomes, complete chloroplast genomes of some of the millets are also available in the public domain, including foxtail millet [182], proso millet [22, 120], little millet [148], and barnyard millet [135, 149, 195].

Sorghum is a widely sequenced crop among all considered crops, with five versions of sequenced genomes available in the public domain. Various genotypes of sorghum have been sequenced, including genotypes like BTx623 [131], BTx642 (https://phytozome-next.jgi.doe.gov/info/SbicolorBTx642_v1_1), Rio [39], RTx430 (https://phytozome-next.jgi.doe.gov/info/SbicolorRTx430_v2_1), SC187 (http://phytozome-next.jgi.doe.gov/info/SbicolorSC187_v1_1) and Wray (https://phytozome.jgi.doe.gov/info/SbicolorWray_v1_1). Older versions of the genome assemblies have been refined to improve order and coverage to get improved genome assemblies like v3.1.1 [113] and v5.1 (https://phytozome.jgi.doe.gov/info/Sbicolor_v5_1).

A strong genomic synteny is noted between the genomes of pearl millet, sorghum, and foxtail millet, with a shared set of 14,398 genes. Specifically, the pairing of pearl millet and sorghum reveals 15,078 common genes, while pearl millet and foxtail millet show 15,887 shared genes. Sorghum and foxtail millet exhibit 16,688 genes in common [147, 169]. The details of genome sequence resources available for millets are summarized in Table 1a.

Pan-genome sequencing offers an advantage over conventional genome sequencing by assembling multiple genotypes, thereby incorporating novel genomic regions into the reference [167]. A pan-genome encompasses the total genes of a biological clade, such as a species, consisting of a set of core genes shared by all individuals and a set of dispensable (or variable) genes partially shared or individual-specific [167]. In the case of millet crops, pan-genome sequencing is still in its nascent stages, with few reports available, primarily focusing on sorghum, pearl millet, foxtail millet, and broomcorn millet (Table 1b). These reports have utilized a map-based approach to generate a pan-genome using de novo sequencing techniques across multiple accessions. The knowledge gained from pan-genomics studies in millets necessitates integration with QTL/GWASs and resequencing studies to identify beneficial genes and alleles for millet crop improvement. The identified alleles could be effectively deployed in a breeding strategy using NBTs such as CRISPR/Cas-mediated gene editing.

CRISPR/Cas-mediated gene editing toolbox in millets

A typical gene editing construct consists of a sgRNA sequence driven by *U6/U3* pol III promoters, a gene coding for a nuclease protein such as Cas9 driven by constitutive promoters such as *ubiquitin*, *CaMV35S* etc., a reporter gene to facilitate the selection of putative transformants such as GFP, RFP etc. and an antibiotic/herbicide resistance gene to select positive transformants in plant transformation. This section summarizes the details of each component for millet crops as a part of the editing tool kit (Fig. 1).

Guide RNA

The success of gene editing depends upon precisely designing sgRNAs and choosing an appropriate target site for editing. The designed sgRNA should be highly specific to the target site, showing minimal off-target specificity. Generally, the sgRNA is designed earlier in the exons to

knock out the targets completely. This could effectively terminate the translation by introducing a premature termination codon (PTC) in the target DNA sequence [58]. However, it is not recommended to design a sgRNA very close to start codon ATG or intron-exon junctions as it does not lead to knockout because of enhanced activity non-sense mediated decay destroying the PTC bearing transcript in a region ≥ 50–55 nt from ATG or intron–exon junctions [137].

The choice of a tool for designing a sgRNA depends upon various factors, one of which is the availability of the considered organism's genome in the selected tool, which allows the design of the guide RNA specific to the considered gene of interest and predicts off-targets. Several online software tools are available to streamline the designing guide RNAs process for various plant gene editing techniques [35, 38, 61, 64, 69, 81, 95, 98, 127, 154]. In designing guide RNAs (sgRNAs) for millets, it is important to note that not all online tools provide genomic resources for these specific plants. Among the online tools, CRISPOR is the most versatile, allowing users to design sgRNAs for various millets, including sorghum, foxtail millet, and green millet (*Setaria viridis*). CRISPR RGEN tools also offer options for designing sgRNAs specific to sorghum and green millet, while CHOPCHOP only allows sgRNA designing for sorghum.

sgRNA designing tools such as RGEN BE-Designer [69] and PnB Designer [154] for base editing and PrimeDesign [64], pegFinder [35] and PlantPegDesigner [95] for prime editing typically do not require specific organism input when designing sgRNAs. Therefore, they can effectively be used for designing sgRNAs in millets without the need for plant-specific genomic resources. This flexibility makes them valuable resources for researchers working on gene editing in millets, where such resources may be limited compared to more extensively studied plant genomes. A significant limitation of these tools is their inability to analyze the off-targets of the designed sgRNAs. Consequently, researchers must manually analyze the off-targets of the chosen sgRNAs using secondary tools and databases containing genome sequence information of millets. Considerable attention must be directed towards integrating newly sequenced genome assemblies of millet crops into sgRNA designing tools. This integration will greatly assist researchers working on millets in effectively designing sgRNAs for various millets and analyzing the off-targets of selected sgRNAs within the same user interface of the tool, facilitating more targeted and accurate gene editing in millet crops.

Cas nucleases

The emergence of CRISPR/Cas9-mediated gene editing marked one of the most pivotal discoveries of this century, stemming from the characterization of the innovative nuclease Cas9. This nuclease, belonging to the class II bacterial adaptive immune system from *S. pyogenes*, revolutionized the field of molecular biology [8, 15]. A typical Cas9 nuclease can recognise a guanine-rich PAM sequence (NGG) and introduce a double-strand break (DSB) at the site of sgRNA binding [15]**.** Since the discovery of CRISPR/Cas-mediated gene editing, the Cas9 endonuclease has been modified for its expression in eukaryotic plant systems and is termed plant-codon optimized Cas9 or pcoCas9. Apart from the optimized codon preferences for plant systems, another essential feature of Cas9 is including a nuclear localization signal (NLS) in its sequence to ensure its localization to the nucleus. Many studies have been conducted in various plants using plant-codon-optimized Cas9 [42, 50, 71, 73, 77, 90, 151]. The pcoCas9 has also been validated and employed to edit genes in millet crops, including genes of sorghum and green millet (Table 2). In addition to Cas9, numerous variants of Cas endonucleases variants have been documented, including Cas12a, which identifies T-rich PAM sequences and generates overhangs at the DSB site [11, 198], and Cas13a, facilitating editing at the single-stranded RNA level [1, 76, 193]. While the utilization of newer forms of Cas nucleases like Cas12a and Cas13 hasn't been documented in millet crops thus far, their potential significance in the millet editing toolbox is noteworthy, particularly in scenarios requiring editing of T-rich regions or RNA interference.

Although CRISPR/Cas9-mediated gene editing technology is highly beneficial, it has certain limitations, as the indels introduced by the technology are random and lack precision. This limitation is overcome by a base editing tool which introduces precise base substitutions in a programmable manner without requiring an external template or causing DNA DSBs [51, 117]. The nuclease used in base editors is a fusion of Cas9 nuclease along with a deaminase enzyme. These editors use Cas9 nickase and a deaminase domain to enable precise A/T to G/C base conversions in the case of Adenine Base Editors (ABE) and C/G to T/A base conversions in the case of Cytosine Base editors (CBE), holding promise for developing new crop traits [171].

Cytosine and adenine base editors have demonstrated their efficacy in a variety of major crop species and model plants, allowing for precise modifications of genes linked to single nucleotide polymorphisms (SNPs) [33, 86, 87, 171, 187, 191, 218]. However, there is only one report on using base editors for crop improvement in foxtail millet, which employs base editing for precise base substitutions in the *acetolactate synthase* (*ALS*) and *acetyl-coenzyme A carboxylase* (*ACC*) genes to enhance herbicide tolerance of the crop [93]. Despite this limited documentation, there is potential for extrapolating the knowledge gained from applying these tools to other plant species. By leveraging the insights acquired through previous reports in other plants, researchers can explore the efficiency of base editing techniques for

Table 1 Genomic resources available for millet crops

A

Crop	Genotype/cultivar	Assembly level	Year of release	References
Foxtail millet	Yugu 1	Chromosome	2012	[13, 158]
Green millet	A10.1	Chromosome	2020	[111]
Pearl millet	PmiG	Chromosome		[158, 169]
	Tift-2017	Platinum-grade de novo genome assemblies	2023	[141]
	PI 521612	Chromosome	2023	[158]
	PI 526529	Chromosome		
	PI 537069	Chromosome		
	PI 583800	Chromosome		
	PmiG	Chromosome		
	Tifleaf 3	Chromosome		
	PI 587025	Chromosome		
	PI 186338	Chromosome		
	PI 343841	Chromosome		
	PI 527388	Chromosome		
	PI 250656	Chromosome		
Finger millet	ML-365	Scaffold	2017	[63]
	PR202	Scaffold	2018	[59, 158]
Proso millet	Landrace (00000390)	Chromosome	2019	[158, 219]
	Longmi4	Scaffold	2019	[153]
Tef	Tsedey (DZ-Cr-37)	Scaffold	2014	[21]
Barnyard millet	STB08	Chromosome	2017	[54, 158]
Sorghum	BTx642	Chromosome	2020	https://phytozome-next.jgi.doe.gov/info/SbicolorBTx642_v1_1
	Rio	Chromosome	2019	[39]
	RTx430	Chromosome	2020	https://phytozome-next.jgi.doe.gov/info/SbicolorRTx430_v2_1
	SC187	Chromosome	2021	http://phytozome-next.jgi.doe.gov/info/SbicolorSC187_v1_1
	Wray	Chromosome	2023	https://phytozome.jgi.doe.gov/info/SbicolorWray_v1_1
	BTx623	Chromosome	2009	[131]

B

Crop	Approach	No. of accessions used for pan-genome assembly	No. of pan-genes	Percentage/ number of core genes	Percentage/ number of private genes	Percentage/ number of dispensable genes	Year of release	Reference
Broomcorn millet	Graph-based pan-genome construction using *de novo* assembly	32	59,332	27,727	5533	24,494	2023	[29]
Pearl millet	Graph-based pan-genome construction using *de novo* assembly.	11	–	46.60–52.08%	0.73–8.73%	39.75–49.94%	2023	[194]

Table 1 (continued)

B

Crop	Approach	No. of accessions used for pan-genome assembly	No. of pan-genes	Percentage/ number of core genes	Percentage/ number of private genes	Percentage/ number of dispensable genes	Year of release	Reference
Foxtail millet	Graph-based pan-genome construction using *de novo* assembly	80	73,528	23.8%	3.9%	29.4%	2023	[62]
Sorghum	Graph-based pan-genome construction using *de novo* assembly	13	44,079	36%	3.3%	37.9%	2021	[165]

A) Status of whole genome sequencing (WGS) resources; B) Pan-genome sequencing studies available for millets

enhancing millet crops, thereby contributing to advancements in millet breeding and crop improvement.

Base editing, limited to C-G to T-A and A-T to G-C substitutions, faces constraints in generating diverse mutations. Prime editing addresses this limitation by allowing eight transversion mutations (C→A, C→G, G→C, G→T, A→C, A→T, T→A, T→G) and four transition mutations, as well as small indels [6]. Prime Editors (PE) are emerging as novel tools exploiting the Homology Directed Repair (HDR) pathway for template sequence insertions in plant genomes for crop improvement. Prime editing tools, comprising Cas9 nickase (nCas9) and reverse transcriptase, form a protein complex with prime editing gRNA (pegRNA) to target specified DNA sequences [6].

Prime editing has been successfully implemented in other plant species for specific insertions [20, 66, 72, 96, 107, 171]. However, the application of this technique concerning millet crops is still in its early stages, with no reported instances exploring its applicability in this context. Nevertheless, the potential exists to extrapolate knowledge from using prime editing tools in other plant species. By drawing insights from these studies, researchers can investigate the efficiency of prime editing techniques for enhancing millet crops, thereby playing a role in millet breeding and crop improvement.

Various Cas nucleases, including Cas9 and advanced versions like Cas12a, base editors, and prime editors, are accessible for enhancing traits in millet crops through gene editing. While Cas9 endonuclease is effective for trait modification by introducing DSBs, base editors and prime editors offer a targeted approach for trait improvement in millet crops.

Pol III promoters to drive the sgRNA expression

CRISPR/Cas9-mediated gene editing primarily depends upon efficient transcription of a cloned single guide RNA [138]. For this purpose, it is imperative to utilize a strong constitutive promoter to drive the expression of sgRNA for efficient editing in millet crops. RNA pol III promoters drive the expression of small-noncoding RNAs such as 5S rRNAs, t-RNAs, and other snRNAs such as U6 and U3 RNAs [41]. The high and stable snRNA transcriptional activity of RNA Pol III promoters makes them a suitable candidate for driving the expression of sgRNA [133]. *U6* and *U3* Pol III promoters have quickly become the choice of researchers to drive the expression of sgRNA because of their distinct definition of transcription initiation site viz, G in *U6* and A in *U3* promoters, as well as their ability to drive the transcription of transcripts of size up to 200 bp [12, 78]. In the past decade, *Arabidopsis thaliana U6* (*AtU6-26*) and *Oryza sativa U6* (*OsU6*) promoters have been extensively used to drive the expression of sgRNAs in dicots and monocots, respectively [49, 71, 213]. Recently, studies have shown that using plant-specific endogenous pol III promoters is more efficient in driving sgRNA expression and ultimately editing the target gene in many plants such as maize [138], wheat [88], soybean [159], grapevine [142], potato [183], camelina [118], chicory [14], apple [25], white spruce [42], lettuce [144] and cotton [102].

In the case of millets, *U6* promoters in sorghum have been characterized for their application in CRISPR/Cas9-mediated gene editing. Replacing *OsU6/U3* promoters with *SbU62.3* or *SbU63.1* significantly increased the editing

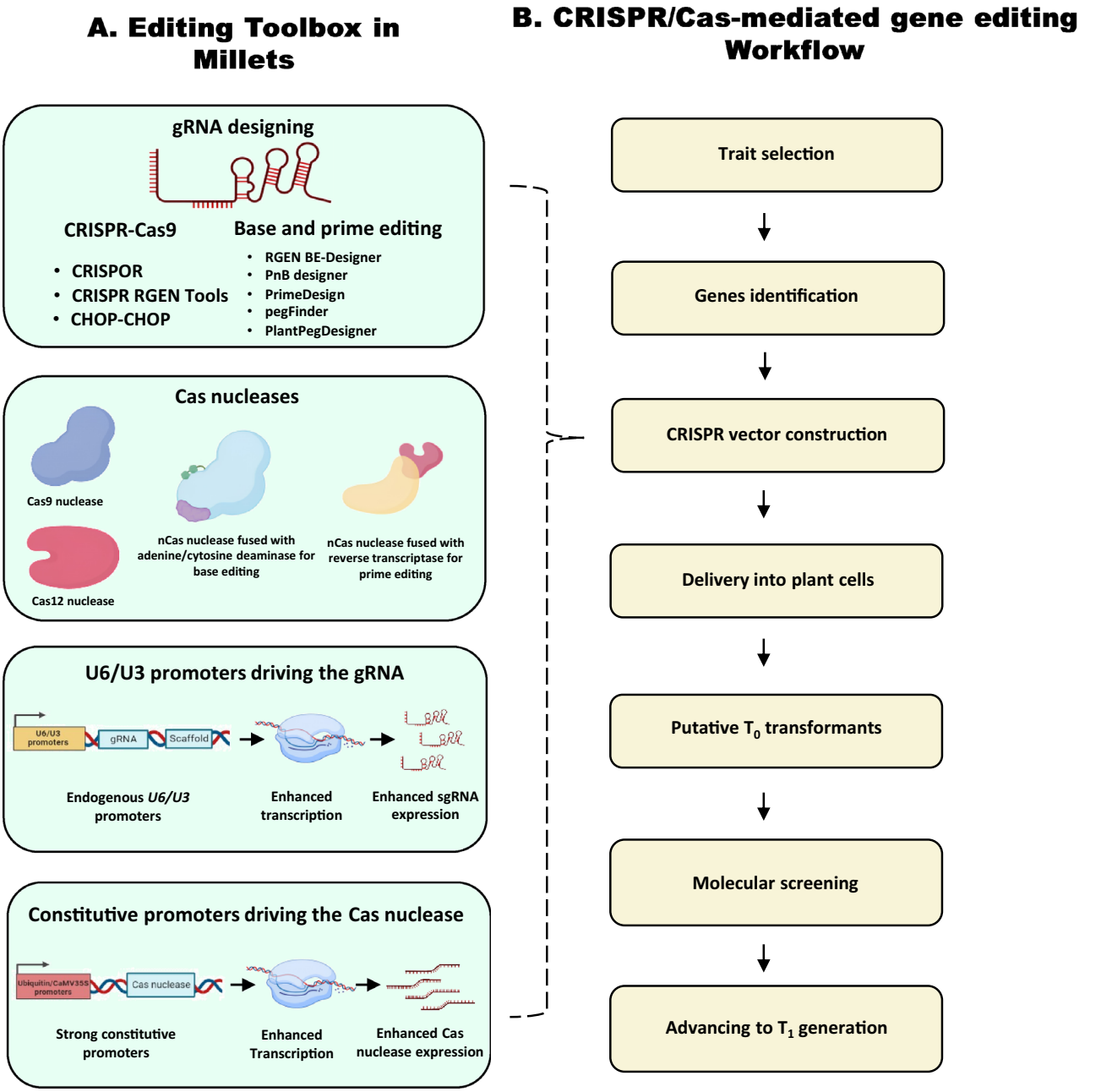

Fig. 1 CRISPR/Cas-mediated gene editing in millets. **A** The editing toolbox in millet comprises bioinformatics tools for designing the guide RNA, different types of Cas nucleases, and promoters. **B** CRISPR/Cas-mediated gene editing workflow in millets

efficiency. The study also demonstrated the higher editing capability of *SbU62.3* as compared to *SbU63.1* [112]. Preliminary reports of single gene editing in foxtail millet have used rice *OsU6/U3* promoters [10, 34, 164, 179, 205, 206]. In contrast, multiplexed gene editing was demonstrated using *the Cestrum yellow leaf curling virus* (*CmYLCV*) promoter in the case of csy4-based editing [189]. In another multi-gene editing approach, three distinct multiplexing approaches were employed utilizing distinct promoters to drive the sgRNA expression viz. multicomponent transcriptional unit utilizing *OsU3*, *TaU3*, and *AtU6* promoters, Csy4 method utilizing *Panicum virgatum PvUbi* promoter to drive expression of multiple sgRNAs separated by Csy4 hairpins and polycistronic tRNA-gRNA method utilizing *OsU3* promoters to drive the expression of multiple sgRNAs [93].

The comprehensive studies of utilized promoters for gene editing in millets suggest that, except for sorghum, the RNA Pol III *U3/U6* promoters have yet to be reported in other

Table 2 Summary of reports for CRISPR/Cas-mediated gene editing studies in sorghum, foxtail millet and green millet. pCoCas9: Plant Codon Optimized Cas9; spCas9: *Streptococcus pyogenes* derived Cas9; zCas9: *Zea mays* codon optimized Cas9; ABE: Adenine Base Editor; CBE: Cytocine Base Editor

Sr. no	Crop	Gene	Trait	Transformation method	Vector	Cas nuclease	Promoter for Cas9	Promoter for gRNA	Editing efficiency	References
1	Sorghum	*DsRED2*	–	*Agrobacterium*-mediated	pCAMBIA1302	pcoCas9	*CaMV35S*	*AtU6–26*	–	[71]
2		*SbCENH3*	Haploid induction	*Agrobacterium*-mediated	pPHP81561	spCas9	–	–	37–40%	[26]
3		*SbKafirin*	Increase in digestibility and protein quality	*Agrobacterium*-mediated	pPZP211	zCas9	–	–	92.4%	[85]
4		*SbCAD* *SbPDS*	–	Particle bombardment	pCUBi1390	spCas9	*ZmUbi*	*OsU3*	–	[97]
5		*SbFT* *SbGA2ox5*	Flowering	*Agrobacterium*-mediated	pTF101.1	spCas9	*ZmUbi–1*	*OsU6*	33.3% 83.3%	[24]
6		*SbLG1*	leaf inclination angle	Particle bombardment	sLGE1925	spCas9	*CaMV35S*	*OsU6*	–	[17]
7		*SbBADH2*	Fragrance	*Agrobacterium*-mediated	pYLCRISPR–Cas9	spCas9	*Ubi*	*OsU6 and OsU3*	–	[200]
8		*Sbk1C5* *SbgKAF1*	Protein digestibility	*Agrobacterium*-mediated	p1C, p2C, p3C	pcoCas9	*Ubi1*	*OsU3*	–	[48]
9		*SbWRKY50*	Inhibition of chlorophyll degradation	*Agrobacterium*-mediated	pYLCRISPR–Cas9–MB	–	–	*OsU6a*	–	[31]
10		*SbCCD7* and *SbCCD8*	Strigolactone biosynthesis	*Agrobacterium*-mediated	pBUN421	spCas9	*Ubi*	*TaU3*	–	[57]
11		*β–kafirin* *GHD7* *γ–kafirin*	Standardizing endogenous U6 promoters	Particle bombardment	pYLCRISPR/Cas9Pubi–B	zCas9	*ZmUbi–1*	*SbU62.3* *SbU63.1*	55.6–100%	[112]
12		*SbLgs1* *SbMTL* *SbKaf*	–	*Agrobacterium*-mediated	pPHP87984 pPHP87098	spCas9	*Ubi*	*U6*	74.2–92.9%	[27]
13	Foxtail millet	*SiPDS*	–	Protoplast transformation	pCAMBIA1301	spCas9	*CaMV35S*	*OsU3*	10.2%	[94]
14		*SiMTL*	Haploid induction	*Agrobacterium*-mediated	–	spCas9	*ZmUbi–1*	*OsU3*	–	[34]
15		*SiNP1*	Male sterility	*Agrobacterium*-mediated	pYLCRISPR/Cas9–MH		*pUbi* *CaMV35S*	*U6/U3*	–	[206]
16		*SiAUX1*	Lateral root development	*Agrobacterium*-mediated	pYLCRISPR/Cas9–MH	spCas9	*pUbi* *CaMV35S*	*OsU6/U3*	–	[164]

Table 2 (continued)

Sr. no	Crop	Gene	Trait	Transformation method	Vector	Cas nuclease	Promoter for Cas9	Promoter for gRNA	Editing efficiency	References
17		*SiFMBP, SiDof4, SiBADH2, SiGBSS1, SiIPK1*	Multiplexing using MCTU system	*Agrobacterium*-mediated	pHUE411	spCas9	–	*OsU3, TaU3, and AtU6*	30.2–42.3%	[93]
			Multiplexing using Csy4 system		pHUE411	–	*PvUbi*			
			Multiplexing using PTG system		pHUE411	spCas9	–	*OsU3*		
			Separate gRNAs for knockout		pHUE411	spCas9	–	*OsU3*		
18		*SiALS SiACC*	Herbicide tolerance	Protoplast Transfection	pHUE411	CBE ABE	*ZmUbi*	*OsU3*	0.01–0.14% 5.16–7.77%	[93]
19		*SiPKS2*	Cytoplasmic male sterility	*Agrobacterium*-mediated	pYLCRISPR/ Cas9-MH	spCas9	*pUbi*	*OsU6a*	–	[205]
20		*SiBADH2*	Fragrance	*Agrobacterium*-mediated	pYLCRISPR/ Cas9–Pubi–H	spCas9	*pUbi*	*OsU6a*	–	[208]
21	Green millet	*SvLes1*	Seed shattering	*Agrobacterium*-mediated	pTRANS_250d	spCas9	*PvUbi1*	*AtU6*	–	[111]
22		*SvDrm1a, SvDrm1b, SvMs26 SvMs45*	Optimization multiplex CRISPR/Cas9	*Agrobacterium*-mediated	pMOD_A1510	pcoCas9 with Csy4	*ZmUbi*	*CmYLCV*	73–100%	[189]
			Optimization multiplex CRISPR/Cas9		pMOD_A1110 (with tRNA system)	pcoCas9	*ZmUbi*	*CmYLCV*		
			Optimization multiplex CRISPR/Cas9		pMG198 pMG199 (tRNA system)	pcoCas9	*ZmUbi*	*CmYLCV*		
			Optimization multiplex CRISPR/Cas9		pMG201 pMG202(tRNA system)	pcoCas9 with Trex2	*ZmUbi*	*CmYLCV*		
23		*SvFON2*	Inflorescence meristem size	*Agrobacterium*-mediated	pTRANS_250d	spCas9	*CaMV35S*	*AtU6*	29%	[216]

millets. This puts forward an immediate need to identify the RNA Pol III genes and their respective promoters in millet crops, especially in crops like foxtail millet, for which the complete genome is already available in the public domain. Following this, the identified promoters could be employed and validated for their application to significantly increase the editing efficiency in millet crops in the future.

Promoters driving the cas nuclease gene expression

Successful integration of the nuclease gene in the plant genome during CRISPR/Cas-mediated gene editing is considered a transgene integration event, and efficient expression of transcript is greatly influenced by the choice of promoter driving the gene [150]. Higher levels of Cas nuclease can increase the editing frequency. Hence, it is imperative to use constitutive promoters to drive the expression of Cas nucleases, which are active in all developmental stages of millet crops. *Cauliflower Mosaic Virus 35S* promoter (*CaMV35S*) has been widely used in transgene expression across many plants, but owing to its lower activity at specific developmental stages, attempts have been made to identify constitutive promoters, especially in monocots [36]**.** Several constitutive promoters have been identified in monocots, which include *Zea mays ubiquitin* promoter *ZmUbi* [36, 37, 40], rice *ubiquitin* promoters *RUBQ1* and *RUBQ2* [180], rice actin promoters, i.e. Rice *Actin1* and *Actin2* [60, 129] rice *OsCc1* promoter [70], the rice *RbcS* promoter [80].

In the case of millets, the trend of using *ZmUbi* constitutive promoter for expression of the nuclease gene is observed to be followed in the limited number of available gene editing studies in foxtail millet and green millet. In the case of foxtail millet, the multiplexing and the CBE/ABE approach have employed the *ZmUbi* promoter to drive the nucleases [93] (Table 2). Similarly, the *ZmUbi* promoter is preferred for Cas9 expression in an attempt to edit foxtail millet [34, 164, 205, 206] (Table 2) and green millet genes [10, 189] (Table 2). Only one study in foxtail millet highlights using *CaMV35S* promoter for gene editing [94] (Table 2), while gene editing studies in sorghum are confined to using either *ZmUbi* or *CaMV35s* promoter for driving the *Cas9* gene [24, 71] (Table 2).

To meet the experimental needs, researchers are increasingly utilizing crop/tissue-specific promoters capable of driving the expression of nuclease genes in particular developmental stages or parts of the plant, such as egg cell-specific promoters or cell-division-specific promoters, etc. [79]. The strategic application of these promoters in millet crops holds immense potential for achieving precise gene editing tailored to specific plant parts or developmental stages. This approach enables targeted modifications and mitigates potential deleterious editing effects at the whole plant level. Similarly, engineered synthetic promoters designed for precise transcriptional regulation of transgenes could be harnessed to drive the *Cas9* gene expression in millets, potentially enhancing the editing efficiency. Alternatively, the efficacy of established constitutive promoters identified within the plant genome could be evaluated by screening their ability to drive reporter gene expression, such as fluorescent protein or GUS, through in vivo assays. Promoters demonstrating the highest expression levels could then be selected to drive Cas9 expression in millets.

Advancements in millet transformation

Plant transformation allows for the development of plants with novel traits unattainable through traditional breeding. Two main gene delivery methods, physical techniques like particle bombardment and *Agrobacterium*-mediated transformation, successfully enhance crop resilience to climate change and aid in sustainable food production. Genetic transformation is crucial for introducing desired traits into crops, facilitated by an optimal in vitro regeneration system. In the context of CRISPR/Cas-mediated gene editing, developing an optimal millet regeneration protocol requires considering factors such as transformation methods, type of explants, strains, and vectors to establish an effective transformation method.

Transformation methods

The investigation into optimal transformation methods for specific millets aims to facilitate the creation of CRISPR/Cas-mediated edited lines. These methods include *Agrobacterium*-mediated and non-*Agrobacterium*-mediated techniques like particle bombardment and electroporation, alongside in vitro regeneration processes. Success relies on establishing favourable conditions for gene transfer and improving transformation efficiency. While studies have detailed gene-edited plants in major cereals, such as rice and wheat, limited reports exist for millets. Notably, CRISPR/Cas9-mediated gene editing has been effectively applied in foxtail millet and sorghum via protoplast transformation and PEG-mediated protoplast transfection [93, 94, 114]. However, similar reports for pearl and finger millet are lacking. Protoplast transfection and particle bombardment hold promise for CRISPR/Cas9, base editing, and prime editing approaches in these millets, suggesting potential avenues for future trait studies.

Biolistic and electroporation gene delivery methods provide genotype-independent transformation for several cereal crops; however, they face limitations in stability, integration, and cost [47]. Researchers increasingly employ precise *Agrobacterium*-mediated transformation to overcome

these challenges, ensuring stable gene integration. Successful studies have reported gene-edited plant development in specific millets like foxtail millet and sorghum through *Agrobacterium*-mediated transformation (Table 2). The success of foxtail millet has positioned it as a potential C_4 model crop, offering insights for other millet transformation methods. Recent research has highlighted the development of gene-edited lines using *Agrobacterium*-mediated transformation in foxtail millet for diverse agricultural traits, including heterosis [206], double haploid lines [34], lateral root production for domestication [164], herbicide resistance [93], and popcorn-like fragrant [208] traits. Similarly, in sorghum, numerous reports demonstrate the transfer of desired genes for traits such as haploid induction [26], flowering time [24], protein digestibility [48, 85], and strigolactone biosynthesis [57] through *Agrobacterium*-mediated transformation. However, there are no reports on gene editing in pearl millet and finger millet, potentially due to the limited protocols for efficient transformation.

Selection of suitable explants

Discovering the optimal pairing of transformation methods and cultural environments for each crop has necessitated assessing various explants, spanning from individual cells like protoplasts to embryonic tissues such as meristems, scutellum, or cotyledons, to seedling-derived tissues like apical/axillary meristems, hypocotyl, or leaves, and ultimately exploring in planta alternatives [181]. Various types of explants suitable for transformation methods were employed in transforming millets. These included highly derived embryonic callus from immature embryos, immature inflorescence, shoot tips, leaf segments, pollen grains, and mature seeds [26, 55, 125, 126]. The preference for immature embryos over somatic embryos was recognized as an effective strategy for transforming recalcitrant crop species, particularly cereals [9]. In developing CRISPR/Cas-mediated edited plants, immature embryos emerged as the most suitable explants, enhancing transformation efficiency through *Agrobacterium*-mediated transformation [26].

Types of Agrobacterium strains

Agrobacterium-mediated transformation effectiveness hinges on selecting the appropriate strain, with commonly used strains for millet transformations including LBA4404, EHA101, and its derivatives like EHA105, AGL0, and AGL1 [136]. Strains with higher virulence, such as AGL1, often exhibit greater transformation efficiency, albeit with potentially decreased event quality [26]. While LBA4404 auxotrophic strains have traditionally been favoured, a thymidine auxotrophic strain (LBA4404 Thy-) has gained preference for sorghum tissue culture to manage bacterial overgrowth [190]. Notably, the successful development of CRISPR/Cas9-mediated gene-edited lines has been achieved in foxtail millet using *Agrobacterium tumefaciens* strains EHA105 [93, 208] and AGL1 [111].

Developmental regulatory (DR) genes

In vitro, regeneration of recalcitrant crops, including millets, has been difficult to manipulate. However, advancements such as using embryogenic tissue cultures, enabling plant regeneration from single somatic cells, have revolutionized plant transformation protocols [170]. This optimization facilitates gene delivery and enhances tissue development, notably through somatic embryogenesis, which has become a cornerstone in genetic manipulation efforts for millets and other crops. Somatic embryogenesis has been widely used to improve genetic manipulation [145]. Recent research has indicated enhancements in the effectiveness of plant regeneration through tissue culture via the overexpression of plant developmental regulators such as *LEAFY COTYLEDON1* [103], *LEAFY COTYLEDON2* [157], *WUSCHEL* (*WUS*) [220], and *BABY BOOM* (*BBM*) [16]. Genes like *BBM* and *WUS* are crucial in somatic embryo formation [104].

Various studies have demonstrated enhanced transformation frequencies in maize and sorghum through differential expression of morphogenic genes and innovative transformation approaches [27, 74]. Similarly, research in dicotyledonous plants has shown the induction of de novo meristems without tissue culture using different combinations of DR genes [110]. However, these methods require further optimization for recalcitrant cereal crops to improve transformation efficiency. Recent advancements, such as utilizing fused protein containing a GRF transcription factor and its GIF cofactor, have significantly boosted regeneration efficacy in monocotyledonous and dicotyledonous plants [45]. This strategy has been successfully applied in wheat, citrus, and grapes, drastically increasing regeneration efficiency [45]. Furthermore, combining morphogenic regulator *ZmBBM* with wheat *GRF4-GIF1* has shown a sevenfold increase in regeneration transformation efficiency in maize [32]. Expanding these approaches to millets using DR genes could optimize stable transformation methods for gene editing and improve regeneration efficiency.

Current status of gene editing in millets

There are considerably fewer reports on GE in millet than other major cereal crops. This could be attributed to the need for more efficient regeneration protocols for most

millet or the limited research funding available for these crops, predominantly cultivated in developing countries across Africa and Asia. The existing reports, outlined in Table 2, can be categorized based on the specific traits under consideration.

Proof of concept studies

The initial proof-of-concept studies, representing the pioneering applications of CRISPR/Cas-mediated gene editing in millets, follow a similar workflow. These studies demonstrate achievable efficiencies in transformation, regeneration, and editing processes. Often, phenotypic controls are selected as target genes, allowing scorable phenotypes to be observed upon editing. One commonly employed phenotypic control in millets is the gene encoding *Phytoene desaturase* (*PDS*), an enzyme crucial for carotenoid biosynthesis. Knocking out this gene typically results in a distinct photobleached or leaf-whitening phenotype attributable to the disruption of the carotenoid biosynthetic pathway [123, 166]. The very first report of gene editing in foxtail millet demonstrates the knock-out of the *PDS* gene in foxtail millet protoplasts with a mutagenesis efficiency of 10.2%; the mutations obtained were confirmed with Sanger sequencing and Restriction Fragment Length Polymorphism (RFLP) [94]. Similarly, using the biolistic bombardment method, the *PDS* gene has also been edited as a proof of concept in sorghum [97].

In early proof-of-concept studies in millets, another approach involved the insertion of an out-of-frame fluorescent protein into plant explants via *Agrobacterium*-mediated T-DNA transfer. This T-DNA also carries a sgRNA targeting the out-of-frame region of the protein. Successful editing of the fluorescent protein gene is expected to render the protein in-frame, resulting in fluorescence in the transformed part. This approach was employed in sorghum to edit an out-of-frame DsRED protein using a specific sgRNA [71]. In a parallel approach, transgenic calli of green millet were generated, incorporating functional Green Fluorescent Protein (GFP) via a transgenic method. Subsequently, these calli were co-transformed with a vector containing sgRNA designed to disrupt the *GFP* gene. Following this, the editing profiles of 10 transgenic events for GFP were screened using Sanger sequencing. Results revealed that 60% of the T_0 events exhibited biallelic editing of *GFP*, with no observed editing in the anticipated off-target loci [10].

Agronomic traits

Double haploids (DH) and cytoplasmic male sterility (CMS)

Double haploid (DH) technology is being actively utilized worldwide to produce hybrid cultivars via haploid induction (HI) of plants [28]. DH technology offers a notable advantage in stabilizing the genetic background of hybrid lines within just two generations [52]. This contrasts the traditional approach, which typically necessitates six to eight generations of selfing to achieve homozygosity. A new HI line has been created in foxtail millet, reporting a 2.1% haploid induction rate by targeting *SiMTL* gene coding for pollen-specific phospholipase via CRISPR/Cas9-mediated mutagenesis [34]. In sorghum, gene editing of sorghum *centromere-specific histone H3* (*SbCENH3*) has been achieved in the prospect of developing haploid inducers with 37–40% editing efficiency with 20–37% knockout (biallelic mutations) efficiency [26]. The '*Matrilineal' (SbMTL)* gene has also been edited in sorghum to check the effect of the *WUSCHEL2* morphogenic gene and ternary vector systems on editing efficiency [27].

Hybrid vigor, a phenomenon widely utilized in agricultural breeding to enhance yields and quality, requires the prevention of self-pollination in the female parent to produce hybrid seeds commercially. Among various strategies to address this issue, inducing male sterility in maternal lines has proven to be the most effective and practical approach [217]. The *SiPKS2* gene has been edited in foxtail millet to generate cytoplasmic male sterile lines [205]. CRISPR/Cas mediated editing of *no pollen 1* (*np1*) gene resulted in proteins with immature stop codons and plants with shrivelled anthers without pollens and complete male sterility [206]. Identifying and targeting genes in related crop species can be replicated in millets to produce DH and CMS lines (Table 3**;** Fig. 2).

Herbicide resistance

Herbicide resistance is another important trait that can be modified using CRISPR/Cas-based gene editing technologies. Herbicides generally function by inhibiting a key enzyme in a metabolically significant pathway, thereby eliminating weeds. The herbicides also deleteriously affect the crop of interest regarding plant growth, yield and quality [46]. Hence, it is imperative to develop millet crops with enhanced herbicide resistance. *Acetolactate synthase* (*ALS*) and *Acetyl co-enzyme A carboxylase* (*ACCase*) genes have been edited in foxtail millet using CBE and ABE. The study employed CRISPR/Cas-mediated gene editing technologies, including multiplexing strategies like MCTU, PTG, and Csy4, to target specific genes related to agronomic traits. Individual gene editing via protoplast transfection yielded deletions, with editing efficiencies reaching 100% in plants' T_0 generation. Protoplast transfection tests demonstrated editing efficiencies ranging from 30.2 to 45.6% across all three multiplexing approaches. A multicomponent transcriptional unit (MCTU) was further applied to stabilize foxtail millet plants. Additionally, base editing techniques were used, resulting in missense mutations and increased

herbicide tolerance in foxtail millet plants, marking the first base editing in millets [93].

While no additional reports of CRISPR/Cas-mediated gene editing studies in millet crops exist, studies targeting specific positions in the *ALS* gene using CBE or HDR have been documented in cereal crops like wheat and rice [20, 92, 160, 177, 203]. Similar investigations have been conducted on target genes such as *ACCase* and *EPSPS* [92, 101, 156]. Moreover, studies targeting genes like *PPO*, *Tub2A*, and *SF3B1* have demonstrated herbicide resistance in rice [19, 100, 130]. Identifying homologs of these genes in millet crops and utilizing gene editing technologies to target them could lead to the development of herbicide-resistant millet crops (Fig. 2) (Table 3).

Domestication related traits

CRISPR/Cas-mediated gene editing technologies are being rapidly utilized in millets to modify domestication-related traits, enabling the creation of elite cultivars in a shorter timeframe than conventional breeding methods. CRISPR/Cas-mediated editing of *Auxin Permease 1 (AUX1),* an auxin influx carrier protein, altered panicle and root development, signifying its relevance to root development in foxtail millet. The study also highlighted the identification of haplotype HAP-2412TT in the promoter region of *SiAUX1,* which is associated with lateral root number and has been selected strongly during domestication [164]. Editing in two endogenous sorghum *SbFT* and *SbGA2ox5* genes underlying the Quantitative Trait Loci (QTLs) for flowering time and plant height in sorghum with the editing efficiency of 33.3% and 83.3%, respectively [24]. Seed shattering is another important domestication trait, specifically in millets with smaller grain sizes, such as green millet, which results in significant yield loss [99]. In an interesting study, a gene responsible for shattering in green millet was identified using GWAS as MYB transcription factor *SvLes1*. CRISPR/Cas-mediated gene editing of *SvLes1-1*, an allele responsible for high seed shattering, resulted in the highest tensile strength with the lowest seed shattering [111]. In a compelling study investigating the domestication trait 'gloom coverage' in sorghum, researchers identified a locus, *GC1*, associated with gloom coverage, which encodes an atypical Gγ subunit of the sorghum G protein. The natural variation (*gc1-a*), characterized by the absence of the C-terminal region of the Gγ subunit, led to reduced gloom coverage [192]. Expanding upon this finding, CRISPR/Cas-mediated gene editing technologies could truncate the natural Gγ subunit at the C-terminal region, potentially decreasing gloom coverage in millet crops. Reducing lignin content to enhance biofuel production represents another significant domestication trait that holds potential for millet crops (Fig. 2). Efforts have been undertaken across various grass species to decrease lignin content and boost biofuel production. Targeted genes in this endeavor include LIM domain transcription factor (*LIM*) in sugarcane [82], *Coniferaldehyde 5-Hydroxylase* (*C5H*) in rice [162], *4-coumarate-coenzyme A-ligase 1* (*4CL*) in switchgrass [128], and *Caffeic acid O-methyltransferase* (*COMT*) in sundangrass and barley [7, 84]. Similar strategies can be applied to millet species, particularly in genotypes with high biomass potential, to develop low lignin mutants, thereby augmenting biofuel production (Table 3).

Nutrition, grain quality and yield-related traits

In spite of being highly nutritious crops, efforts have been made in millet to enhance nutritional qualities, grain qualities, and yield-related traits. An interesting study targeted the α-Kafirin protein gene family in sorghum grains to improve protein quality and digestibility. Using a single guide RNA, editing was achieved in 92.4% of T_1 plants. This resulted in decreased α-Kafirin proteins, improved digestibility, and a visible phenotype of reduced vitreous endosperm layer thickness [85]. In a similar study, *k1C5* and *gKAF1* genes encoding α-Kafirin and γ-Kafirin in sorghum were targeted using three single guide RNAs. Point mutations were introduced at all targeted sites, resulting in seeds with increased in vitro protein digestibility compared to controls despite retaining a thick vitreous endosperm. Further attempts have been made to target the *γ-Kafirin* gene in sorghum in separate studies [27, 112].

Seed aroma is another trait studied in millets using CRISPR/Cas-mediated gene editing technologies. A CRISPR/Cas9-mediated gene editing technology was utilized to target the sorghum *betaine aldehyde dehydrogenase* (*SbBADH2*) gene, resulting in indels that caused amino acid changes or premature stop codons, leading to the truncation of the SbBADH2 protein. Consequently, the edited lines exhibited heightened 2-acetyl-1-pyrroline (2-AP) accumulation in leaves and seeds, contributing to the enhanced fragrance [200]. Similarly, BADH2 has also been targeted in foxtail millet to enhance the content of 2-AP, resulting in heightened fragrance [208].

The revolutionary CRISPR/Cas-mediated gene editing technique could speed up the millet crop improvement process, specifically in grain yield, by building on the knowledge of other crop species with well-established genomic resources (Fig. 2). Several efforts have been undertaken in cereal crops like rice and wheat to enhance yield using the CRISPR/Cas9-mediated gene editing technology. Notable target genes for grain yield in rice include *LOGL5*, a cytokinin activation enzyme [175], *OsAAP3*, an amino acid transporter [105], grain width and weight 2 (*OsGw2*), *Grain*

Table 3 Potential candidate genes for CRISPR/Cas-mediated gene editing in millets for trait improvement including abiotic and biotic stresses, domestication-related traits, agronomic traits, nutrition, grain quality, and yield-related traits

Trait	Gene	Crop	References
Abiotic stresses			
Salinity	*STL1*	Maize	[185]
	SPL10	Rice	[83]
	PQT3	Rice	[4]
	RR22	Rice	[199]
	SOS1	Rice	[106]
	bHLH024	Rice	[3]
	GI	Rice	[184]
Heat	*HSA1*	Rice	[139]
	PYL1, PYL4, PYL6	Rice	[115]
Cold	*MYB30*	Rice	[197]
Heavy metals	*Nramp5*	Rice	[163]
	HAK1	Rice	[121]
	ARM1	Rice	[178]
Biotic stresses			
Fungal pathogens	*EDR1*	Wheat	[207]
	MLO	Wheat	[186]
	ERF922	Rice	[176, 215]
	SEC3A	Rice	[109]
	pi21	Rice	[119, 215]
	bar-d1	Rice	[215]
Bacterial blight	*SWEET*	Rice	[188]
Domestication related traits			
Lateral root development	*AUX1*	Foxtail millet	[164]
Flowering	*FT*	Sorghum	[24]
Plant height	*GA2ox5*	Sorghum	[24]
Seed shattering	*MYB transcription factor Les-1*	Green millet	[111]
Gloom coverage	*gc1-a*	Sorghum	[192]
Reduced lignin content	*LIM*	Sugarcane	[82]
	C5H	Rice	[168]
	4CL	Switch grass	[128]
	COMT	Sundangrass and barley	[7, 84]
Agronomic traits			
Haploid induction (HI)	*MTL*	Foxtail millet and sorghum	[27, 34]
	CENH3	Sorghum and wheat	[26, 108]
	DMP	Maize	[212]
Generating male sterile lines	*PKS2*	Foxtail millet	[205]
	np1	Foxtail millet	[206]
	Ms1	Wheat	[124]
	Ms45	Wheat	[155]
	TMS5	Maize	[89]
	csa	Rice	[91]
Herbicide	*ALS*	Wheat and rice	[20, 92, 160, 177, 203]
	ACCase	Rice	[101]
	EPSPS	Rice	[156]
	PPO	Rice	[130]
	Tub2A	Rice	[100]
	SF3B1	Rice	[130]

Table 3 (continued)

Trait	Gene	Crop	References
Nutrition, grain quality and yield			
Protein digestibility	*Kafirin*	Sorghum	[27, 85, 112]
Seed aroma	*BADH2*	Rice, foxtail millet and sorghum	[68, 200, 208]
Grain yield	*LOGL5*	Rice	[175]
	AAP3	Rice	[105]
	Gw2	Rice and wheat	[209, 214]
	Gn1a, ARE1	Rice and wheat	[201, 214]
	Gs3	Rice	[67, 214]

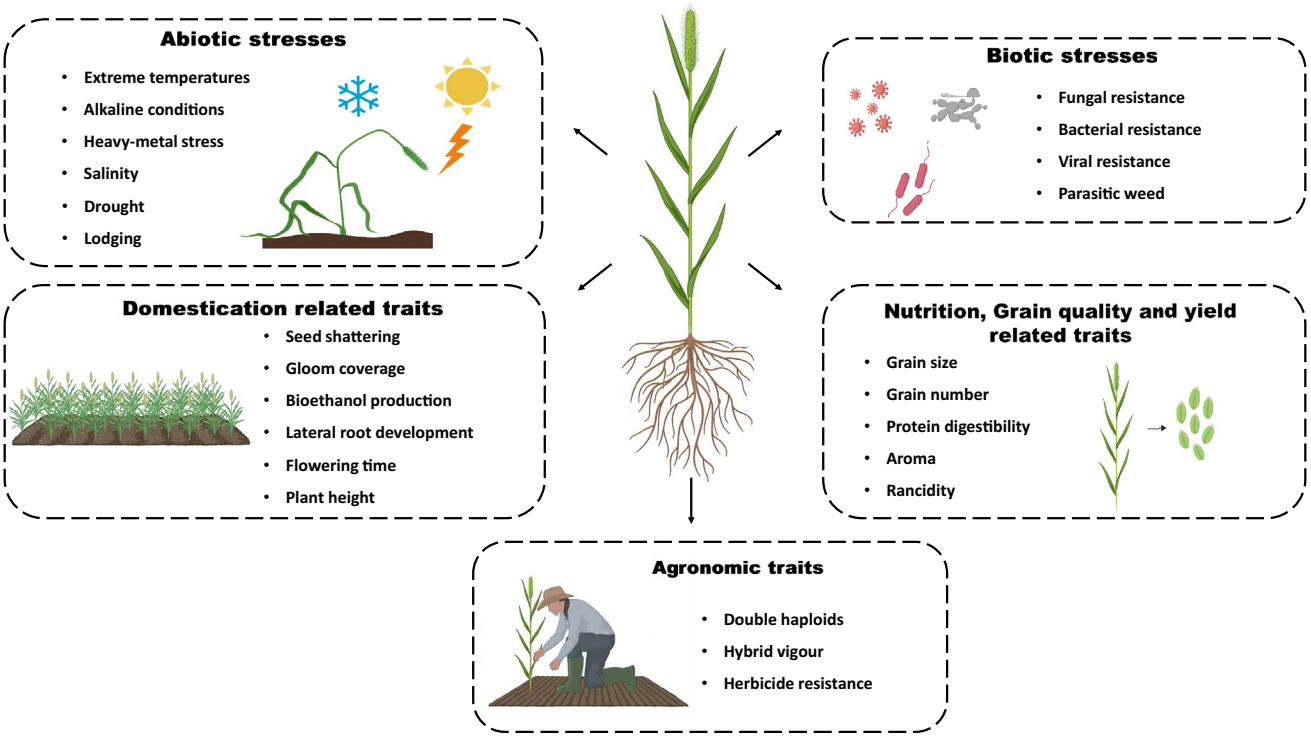

Fig. 2 A list of the potential traits that can be improved in millets through CRISPR/Cas-mediated gene editing

number 1a (*OsGn1a*, a cytokinin oxidase/dehydrogenase), and *Grain size 3* (*GS3*) [67, 214]. In wheat, target genes such as *TaGW2* and *ARE1*, a homolog of *OsGn1a,* have been identified [201, 209, 210]. Of particular interest is the focus on grain size-related genes like *GS3* and *GW2*, which can substantially increase grain size in millet crops, particularly those with smaller grain sizes like barnyard millet and tef (Table 3). Crop-specific grain quality genes can also serve as a potential gene for targeting, such as the *kafirin* gene family, to increase protein digestibility in sorghum, *fatty acid desaturases*, *lipases* and *lipoxygenase* genes in pearl millet to decrease rancidity [2].

Biotic and abiotic stress tolerance

While most millets lack reports highlighting the role of CRISPR/Cas-mediated gene editing for abiotic and biotic stress tolerance, sorghum has a few reports on the subject. In a compelling study, the impact of knocking out *carotenoid cleavage dioxygenase 8* and *7* (*CCD8* and *CCD7*) genes on strigolactone production, plant development, growth, and the parasitic weed 'Striga' was investigated by creating mutants of all gene copies [57]. After editing the *SbCCD8* gene, a significant decrease in orbanchol (a strigolactone precursor) was noted, leading to decreased Striga germination [57].

A notable study identified a significant locus (*AT1*) controlling alkaline tolerance through GWAS in sorghum. Subsequently, CRISPR/Cas9-mediated knockout of this locus in the wheatland background of sorghum led to notably increased alkaline tolerance. The gene *SbAT1* is predicted to encode an unconventional G protein γ subunit, comprising 198 amino acids with a Gγ-like domain at the N-terminus and a cysteine-rich domain at the C-terminus. Extending these findings to foxtail millet, knocking out *SiAT1* resulted in millet plants exhibiting enhanced alkaline tolerance [204].

Genes, the negative regulators of biotic and abiotic stress, have been extensively studied using CRISPR/Cas-mediated gene editing in cereals. The studies include gene editing to create tolerance against lodging [43, 56, 65, 168, 174, 202] and other abiotic stresses such as drought, salinity, heavy metal toxicity, cold and heat [140]. Millets are primarily affected by fungal pathogens, with fewer occurrences of bacterial or viral pathogens [44, 116]. In cereals like wheat, candidate genes such as *Enhanced Disease Resistance 1* (*EDR1*) and *mildew resistance locus* (*MLO*) have been targeted for defense against fungal pathogens [186, 207]. Similarly, in rice, genes like *Ethylene response factor 922* (*OsERF922*), *Subunit of Exocyst Complex 3A* (*OsSEC3A*), *pi2*, and *bar-d1* have been targeted for fungal Blast [109, 119, 176, 215]. Homology-directed repair (HDR) has also been used to confer resistance against Bacterial Blight by inserting specific fragments into the promoter region of *SWEET* genes [188]. Leveraging the existing knowledge of potential candidate genes for abiotic and biotic stress tolerance in other crops, the homologs of these genes could be validated using the CRISPR/Cas-mediated gene editing technology to bolster resistance against both biotic and abiotic stresses in millets (Table 3).

Limitations and future prospects

In a very short period, the CRISPR/Cas-mediated gene editing technology has found its application in various areas of plant sciences, including crop improvement and breeding. The prospect of changing a nucleotide sequence with the aid of the CRISPR/Cas9-mediated gene editing technology or making precise nucleotide changes in the case of base and prime editors has opened new horizons in crop improvement. The application of the CRISPR/Cas-mediated gene editing technology has certain limitations, especially for crops like millet. The tool requires stringent genomic information as a foundation, and the non-availability of proper genomic resources in the case of millet crops makes it particularly difficult to find the application of this technology in these crops. Hence, obtaining complete genome assemblies of millet crops whose genome assembly is in draft versions and updating the genome assemblies of already sequenced millet to unveil more genomic information remain the topmost priorities of millet crop improvement [23].

Despite being regarded as nutritious crops with a significant economic impact on smallholder farmers, research aimed at trait improvement in millet crops is severely hindered by the absence of established gene discovery pipelines. To overcome this limitation, it is imperative to integrate knowledge acquired through advanced genotyping tools, such as pan-genomes encompassing multiple genotypes, with insights obtained from omics approaches, including transcriptomics, metabolomics, proteomics, and epigenomics. Creating an integrated omics database would be a first step in this direction. By leveraging this comprehensive understanding, a highly efficient gene discovery pipeline can be established for millets, facilitating targeted trait enhancement and agricultural advancement.

The lack of efficient transformation methods generating a whole plant that can constitutively express sgRNA and Cas nuclease is one of the major limitations of applying the CRISPR/Cas-mediated gene editing technology in millet crops. Hence, particular emphasis should be placed on developing efficient transformation methods for millet. CRISPR/Cas-mediated gene editing technology is prone to off-target modifications if suitable experimental measures are not followed [53]. These off-target modifications may have a deleterious effect on the plant and can also significantly reduce the editing efficiency of the target region. These off-targets can be avoided by designing a sgRNA with a strong affinity to the target region using updated guide RNA designing tools and a choice of high-fidelity Cas nucleases [211].

The most significant limitation in applying this technique is the constraints on the commercialization of gene-edited products. Owing to the partially transgenic nature of this technique and the threat of potential off-target effects, getting approval to release the gene-edited crop is time-consuming. Nonetheless, the governments of nations like India, the United States of America, Brazil, the United Kingdom, Australia, New Zealand, and many more are taking steps to speed up this process [152].

Acknowledgements PS acknowledges the financial support from the Department of Science and Technology, Government of India, for the support from the core grant research (CRG) grant file no. DST No:CRG/2019/004305. VS acknowledges the DST INSPIRE fellowship from the Department of Science and Technology (DST/INSPIRE Fellowship/IF190079), Government of India, for supporting her doctoral research work. MK acknowledges fellowship support from the Department of Biotechnology, Government of India, in the form of a Junior Research Fellowship (ID: DBT/2022-23/ICRISAT/2065) for his doctoral research work. All the authors have read and approved the manuscript.

Author contributions PSR: Conceptualized the idea and prepared the background information; VS, MK, KY and PSR: Literature survey and

writing. KY and PSR; Editing and evaluation of the draft. All authors read the final version of the manuscript, provided necessary suggestions, and approved it for publication.

Funding Funding was provided by Department of Science and Technology, CRG/2019/004305, Sudhakar Reddy, Department of Science and Technology, DST/INSPIRE Fellowship/IF190079, Vidhi Sapara,Department of Biotechnology, DBT/2022-23/ICRISAT/2065, Mitesh Khisti.

Data availability N/A.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval N/A.

Informed consent N/A

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