



# Genome-editing in millets: current knowledge and future perspectives

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## Abstract

Millets are small seeded cereal crops predominantly cultivated and consumed by resource-poor farmers in the semi-arid tropics of Asia and Africa. Millets possess rich nutrients and a climate resilience property when compared to the other cereals such as rice and wheat. Millet improvement using modern genetic and genomic tools is falling behind other cereal crops due to their cultivation being restricted to less developed countries. Genome editing tools have been successfully applied to major cereal crops and, as a result, many key traits have been introduced into rice, wheat and maize. However, genome editing tools have not yet been used for most millets although they possess rich nutrients. The foxtail millet is the only millet utilised up to now for genome editing works. Limited genomic resources and lack of efficient transformation systems may slow down genome editing in millets. As millets possess many important traits of agricultural importance, high resolution studies with genome editing tools will help to understand the specific mechanism and transfer such traits to major cereals in the future. This review covers the current status of genome editing studies in millets and discusses the future prospects of genome editing in millets to understand key traits of nutrient fortification and develop climate resilient crops in the future.

**Keywords** Cereals · CRISPR/Cas9 · Foxtail millet · Functional genomics · Millets · Plant transformation

## Introduction

Millets are small-seeded cereal crops widely cultivated in the semi-arid tropics of Asia and Africa. Millets are key crops for strengthening food and nutrient security in the drought prone areas of these regions. The most frequently cultivated millets are finger millet (*Eleusine coracana* (L.) Gaertn.), foxtail millet (*Setaria italica* (L.) P. Beauvois), pearl millet (*Cenchrus americanus* (L.) Morrone.), kodo millet (*Paspalum scrobiculatum* L.), little millet (*Panicum sumatrense* Roth ex Roem. and Schult.), proso millet (*Panicum miliaceum* L.), and barnyard millet (*Echinochloa crus-galli* (L.) P. Beauvois). Millets have not been utilized frequently for modern genetic and genomic studies since they are cultivated in less developed countries of Asia and Africa. Genome sequences of millets also have only been released in recent years, a few decades after the release of genome sequences of staple cereals like rice [1]. However, millets have started receiving attention in recent years, especially

from researchers in Europe and America, due to their dense nutrients and climate resilient properties [2].

Genome editing has emerged as a central tool for modern plant genetic studies. The recently developed and Nobel Prize winning genome editing tool, clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9), has emerged as the most popular tool for genome editing in a diverse range of organisms [3–5]. The CRISPR/Cas system has been championed to accelerate crop improvement, with an option for precision breeding [6–9]. Many labs around the world have quickly adopted the CRISPR/Cas system and successfully produced mutant plants and scores of reports are available on crop genome editing (reviewed in [10, 11]).

Millets have been generally considered to be orphan crops due to the lower importance they have been awarded for crop improvement programs in the green revolution and modern genetic studies. For example, genetic engineering and genome sequencing works are lagging far behind in millets when compared with other major cereals [1, 2, 12]. Apart from foxtail millet, other millets do not have complete and annotated genome sequences and this hampers modern genetic studies, including the application of genome editing in these crops. Many traits associated with climate resilience

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and nutrient enrichment have not yet been studied in millets with high resolution molecular studies. Studying the molecular mechanism of abiotic stress tolerance and nutrient enrichment with the help of precise genome editing will be helpful to understand the mechanism and transfer such traits to other cereals. The availability of fully annotated genome sequence and efficient transformation system are the key factors to apply the genome editing in millets. In this review, nutrient benefits of millets, the scope of genome editing in millets, the need for genome editing in millets and future prospects for genome editing in millets are discussed.

## Nutritional importance of millets

Nutrient contents of millets are superior to main cereals like rice and wheat [13]. Seeds of millets are the richest source of several nutrients like calcium, magnesium, phosphorus, iron, and proteins [1, 14, 15]. Millets also have high amounts of essential amino acids, dietary fibers, minerals, and vitamins [16]. For example, proso millet is a rich source of protein (12.5%), barnyard millet has highest crude fiber (13.6%) and iron (186 mg/kg dry matter) [17]. Finger millet is a rich source of calcium, magnesium, and potassium [14, 17]. It is noteworthy to mention that the calcium content of finger millet (344 mg) is the highest among cereals [1, 18]. Foxtail millet is also rich in protein (11%) and fat (4%) [19]. Pearl millet has high amounts of zinc, iron, and lysine [20]. Kodo millet is rich in magnesium (1.1 g/kg dry matter) and essential amino acids such as lysine, threonine, valine, and sulfur-containing amino acids [21]. Consumption of millets minimizes the risk of diseases like duodenal ulcers, anaemia, constipation, and atopic dermatitis [22, 23]. It has been predicted that consumption of millets reduced the incidence of cardiovascular complaints, diabetes, and certain cancer diseases [24, 25]. Consequently, millets are considered as nutraceuticals due to their nutrient dense seeds providing potential health benefits. Studying millets with genome editing tools will help to understand key roles of nutrient fortification.

## Genome editing for agricultural improvement

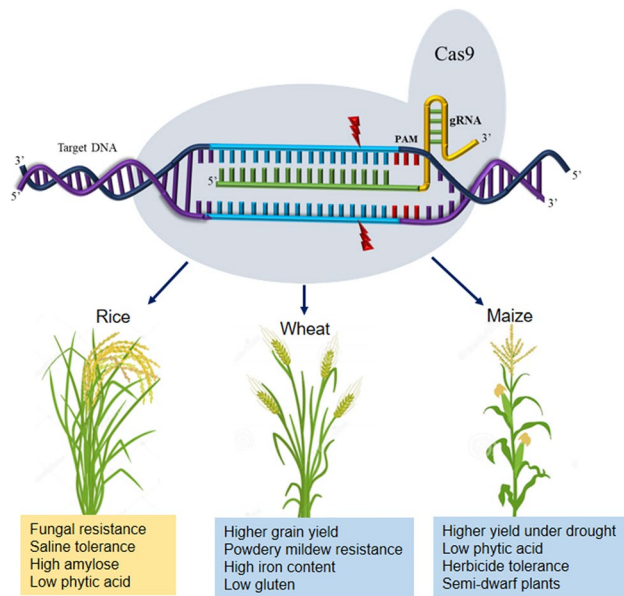
Genome editing is a recent addition to the toolbox for plant breeders to develop new and improved varieties of crops. The commonly used genome editing tools include zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and the most popular, the CRISPR/Cas system [26]. Among these three systems, CRISPR/Cas system has been widely adopted by many labs for efficient genome editing due to its simple, user friendly, and cost effective construct designing [27–29]. Researchers can

choose from the wide choices of constructs available for academic research at Addgene, a non-profit plasmid repository (<https://www.addgene.org/>). Basic plant biotech labs that have expertise with basic cloning and plant transformation experiments can successfully use CRISPR/Cas system-mediated genome editing due to its user friendly construct design option. This has opened a broad scope for the variety of genome editing works in many plant species.

Genome editing tools have been predicted to solve many issues of agriculture. In particular, the CRISPR-Cas9 system has been predicted to play a key role in imparting new traits in crop plants. Many excellent reviews are available on possible application of genome editing for crop improvement [11, 29–32]. Genome editing has been believed to benefit agriculture by imparting key traits including seed quality, drought tolerance, higher yield, disease resistance, improved nutrient uptake, and herbicide tolerance. For example, in rice, through knocking out SWEET genes that are responsible for disease susceptibility, researchers imparted resistance to bacterial blight [33, 34]. Improving yield is the primary objective of plant breeding, and genome editing has been considered to play a key role in this aim. Key genes responsible for yield improvement could help improve the yield. For example, editing genes responsible for tillering is believed to improve the yield of cereals [29, 31, 32, 35–37]. Genome editing could also be harnessed for enhancing the quality of seeds with lower gluten content, enriched carotenoid, and reduced phytic acid levels. Similarly, genome editing could help to overcome other stresses impacting crop production like drought, salinity, and cold, and could offer herbicide tolerance [11]. Imparting these types of novel traits by genome editing for enriching seed nutrient content with yield improvement will ensure both food and nutritional security.

## Genome editing in major cereals

The CRISPR/Cas system has been applied to many cereal crops for crop improvement [7]. Many important traits have been imparted in cereals like rice, maize, and wheat through CRISPR/Cas9-mediated genome editing (Fig. 1). Genome editing tools have been successfully applied to many cereal crops and in particular scores of reports are available on the use of the CRISPR/Cas system (Reviewed in [7, 38, 39, 39]). Many studies were conducted on testing the genome editing tools with non-functional genes in major cereals like rice and wheat [7]. However, a few traits have also been imparted by precision genome editing in rice, barley, wheat, and maize (reviewed in [38]). These include development of fungal resistance in wheat [40–42], blast resistance in rice [43], grain nutrient quality improvement in rice [31, 44, 45] and maize [46], resistance to various biotic and abiotic



**Fig. 1** Application of the CRISPR/Cas9 system for imparting agronomically important traits in rice, wheat, and maize

stresses in wheat [47] and rice [48–51]. Major cereals like rice, maize, and wheat continue to get more attention for genome editing for improvement of various traits.

## Need for millet genome editing

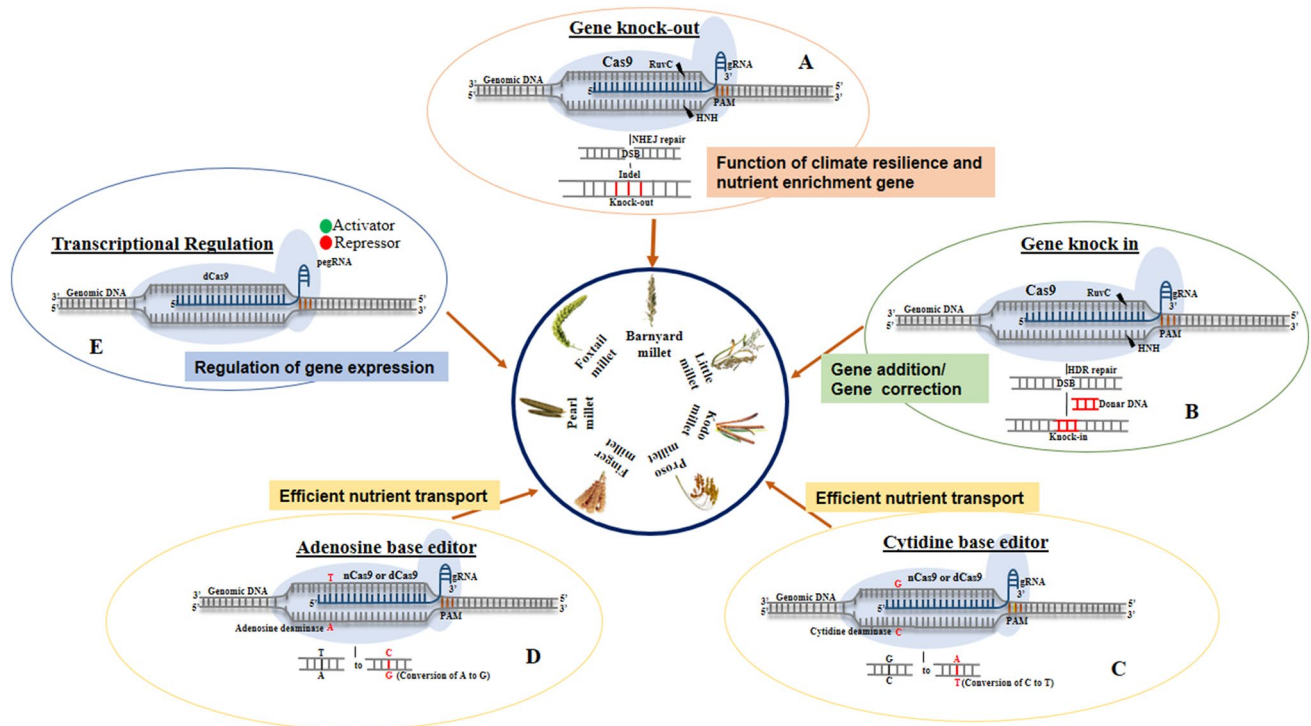
Application of genome editing tools in millets is helpful to improve millet research in the future. Genome editing may help in understanding the mechanism of nutrient fortification and climate resilience and to exchange such useful traits with other cereals. As discussed above, functional genomics studies of millets are lagging far behind when compared to those in main-stream cereals. However, millets possess superior traits to main cereals for agricultural performance and seed nutrient profile. Genes and QTL responsible for such traits need to be dissected through high resolution functional genomic studies with the help of genome editing tools. This will help to identify and transfer such traits to main-stream cereals. Millets show more plasticity in the field under adverse climate conditions [52]. Rice and wheat cannot produce good yield above 37 °C but millets like pearl millet can grow up to 46 °C, a very crucial trait to cope with drought induced by global warming [52]. When compared with main cereals like rice and wheat, millets are short duration crops demanding less maintenance in the field. Similarly, millets require minimum water and rainfall compared to major cereals. These traits have not yet been dissected by functional genomics studies. High resolution functional genomics studies aided by genome editing may help to

unravel the candidate genes responsible for these traits and would help to transfer such traits to main cereals. Exceptionally higher accumulation of mineral nutrients like calcium (finger millet) and iron (barnyard millet) and B-vitamins in millet seeds need to be studied with genome editing tools to identify the transporters and signals responsible for the seed fortification. These traits could be transferred to within and outside the millet cereals to strengthen food security. An illustration on the application of various CRISPR/Cas systems for functional genomics studies and trait improvement in millets is included (Fig. 2).

Although millets possess climate resilient properties, their yield is affected by low nutrient soils since they are mostly cultivated under low input agriculture systems by resource poor farmers. For example, zinc deficiency severely affects the productive stage of pearl millet. It reduces the size of the panicle and delays the development and maturation of the grain [53]. Phosphate and nitrogen deficiencies significantly reduce the growth and yield of foxtail millet [54, 55]. Particularly, phosphate deficiency also significantly influences the size of the panicle and reduces the grain yield of all millets [56]. Precision genome editing like base editing could help to improve nutrient transport and improve the yield of millets under low nutrient soils in semi-arid regions of Asia and Africa.

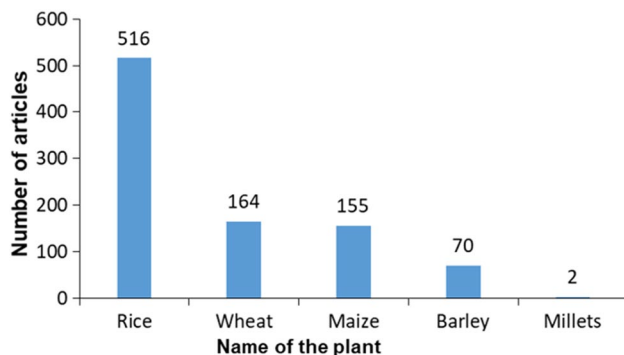
## Current status of genome editing in millets

Among the cereal crops, most reports are available for genome editing in rice. This is followed by wheat, maize, and barley based on a recent Pubmed search. But only 2 reports are available until now on genome editing in millets (Fig. 3). It may be due to lack of funding and the fact that millets are cultivated and consumed by less developed countries in Asia and Africa. It is also one of the reasons for delayed sequencing of millet genomes. Foxtail millet is the only millet which was utilized for genome editing studies. Foxtail millet is considered as a model crop due to its small genome (~450 mb), diploid in nature and has C4 photosynthesis chemistry [57]. Genome sequences of two different foxtail millet genotypes were released in 2012 and the genome is also completely annotated [58, 59]. Many genetic studies have been conducted in foxtail millet as it is a model C4 plant with a small genome. As a result, foxtail millet is the first millet utilized for genome editing studies. The first genome editing study using CRISPR/Cas9 was reported in foxtail millet during 2018 by Lin et al. [60]. They have used the protoplasts of several monocot and dicot plants including foxtail millet to assess CRISPR/Cas9-mediated genome editing in single cells (protoplast). The *phytoene desaturase* gene of foxtail millet was targeted by the plasmid



**Fig. 2** Application of CRISPR/Cas system mediated genome editing in millet improvement. Use of various CRISPR/Cas systems to edit the genomes of various millets to understand functions of genes and improve the millet is illustrated. **A** Gene knock-out studies with the use of the CRISPR-Cas9 system is helpful to study the functions of key genes involved in climate resilience and the nutrient fortification process. **B** Gene knock-in studies with CRISPR-Cas9 aided by homologous recombination (HR) is useful to add novel genes and

replace the defective portion of a gene to improve the performance. Base editing with the help of cytidine (**C**) and adenosine (**D**) base editors may help to change the ligand binding site residues of key nutrient transporters for improving nutrient binding transport in plants growing in low nutrient soils. **E** Expression of genes could be regulated at the transcriptional level using death Cas9 (dCas9) fused with either transcriptional activator or repressor to induce and down regulate respectively the specific genes in millets



**Fig. 3** Number of articles dealing with genome editing in rice, wheat, maize, barley, and millets based on the search at PubMed website on 07-04-2021 at 16.00 to 17.00 h Indian Standard time (IST)

*pCambia1300-35s-Cas9-OsU3-SiPDS*. The targeted mutagenesis was detected by RFLP and sequencing [60]. Both deletion of bases up to 43 with insertion of single base were detected by sequencing results [60]. This is the first report on the successful application of the CRISPR/Cas9 system in foxtail millet.

Recently, CRISPR/Cas9-mediated genome editing has been applied in foxtail millet for the induction of double haploid (DH) lines by targeting the *S. italica Matrilineal (SiMTL)* gene [61]. The exonic region of *SiMTL* was targeted with two different gRNAs and was expressed under the control of the *OsU3* promoter, with Cas9 kept under the control of the *Uq* promoter of maize. Foxtail millet genotype Ci846 was used for this study and the transformation was mediated by *Agrobacterium*. The authors reported a haploid induction frequency of 2.8% in the T2 progeny. It is encouraging to note that foxtail millet, being a model C4 crop, is utilized for genetic studies by genome editing. Apart from foxtail millet, other millets have not yet been used for high resolution genetic studies by genome editing.

## Future prospects of millet genome editing

Considering the geographical area of cultivation, available resources, and the expertise of native researchers, millet genome editing is expected to progress at a slow pace. Due to limited expertise and infrastructure among millet research

labs in developing countries of Asia and Africa, the reach of modern tools like genome editing is delayed. Among the available genome editing tools, the CRISPR/Cas system may play a key role for genome editing in millets due to its user friendly and cost effective construct design and tools like ZFN and TALEN may not be utilized for genome editing in the labs of less developed countries with millet research. Other key aspects influencing millet genome editing are discussed below.

### Whole genome sequence of millet

Availability of a fully annotated genome sequence is the vital prerequisite for undertaking genome editing studies in any organism. A fully annotated whole genome sequence is required for the prediction of target regions and guide RNA (gRNA) in the CRISPR/Cas9-mediated genome editing system. For targeting individual genes, details on the structure of the whole gene, including intronic and exonic regions, are needed. Further, Cas9 from *Streptococcus pyogenes* requires “NGG” at the 3’ end of the target region [3]. So, mining such regions and designing gRNAs targeting the specific sequence demand a fully annotated whole genome sequence. Unfortunately, a fully annotated genome sequence is available only for foxtail millet as of now [2]. Only draft genome sequences were released for finger millet [62], pearl millet [63], and proso millet [64]. Fully annotated genome sequences are not yet available for these millets. Not even a draft genome sequence is reported for other millets (little millet, barnyard millet, and kodo millet) up to now. In genome editing studies, the editing efficiency and type of editing (insertion and/or deletion) are being analyzed by the sequencing of target regions and sometimes by whole genome re-sequencing of the plants especially to detect any off-target effects. Whole genome re-sequencing has been applied in rice [65–67], sorghum [68], soybean [69], and Arabidopsis [70] to find off-target effects of genome-edited plants. Such studies could not be conducted in millets except for foxtail millet since it is the only millet having a complete and annotated genome sequence. So, the availability of

complete and annotated genomes of millets will help in the efficient application of genome editing systems in the future.

### Efficient transformation and regeneration protocols

In addition to having the fully annotated genome, existence of an efficient system for the transformation and regeneration is another important prerequisite for the successful development of mutant plants by genome editing. Generally, monocots are considered as recalcitrant for transformation and regeneration [71] studies. Thanks to the efforts made by labs around the world during the last two decades for the development of an efficient transformation system especially based on *Agrobacterium*, several efficient transformation works were reported for cereals [72, 73]. However, millet crops are lagging behind when compared with mainstream cereals on the transformation studies too, and only a few reports are available on millet transformation (Table 1) [12, 74]. We have recently reviewed the status of millet transformation works [2].

Millets were predominantly transformed by the *Agrobacterium*-mediated system. Finger millet has several transformation reports and transgenic plants were regenerated by both direct [75] and indirect [76, 77] regeneration methods (reviewed in [2]). Following finger millet, foxtail millet has a few reports on transformation studies. The *Agrobacterium*-mediated system was used frequently and it was first reported by Yinghui et al. [78]. Many labs in China used the Jigu11 genotype of foxtail millet for transformation studies (reviewed in [2]). We have also reported a transformation system for the Maxima (Acc. No: Bs 3875) genotype with direct regeneration of transformed explants [79]. Two recent studies of the same period also reported the optimization of conditions for *Agrobacterium*-mediated transformation of foxtail millet [80, 81]. A few reports are also available on genetic transformation of pearl millet and this millet was predominately transformed by the biolistic method (reviewed in [74]). Embryogenic calli were mostly used as the explants for the transformation of pearl millet. Only one old report is available on the transformation of barnyard

**Table 1** Number of transformation studies reported on rice and millets

Name of the millet	Number of transformation studies reported	References
Rice	1067	Reviewed in Fraiture et al. [85]
Finger millet	13	Reviewed in Vetriventhan et al. [2]
Pearl millet	12	Reviewed in Sood et al. [74]
Foxtail millet	10	Reviewed in Vetriventhan et al. [2]
Barnyard millet	1	Reviewed in Vetriventhan et al. [2]
Kodo millet	Nil	Reviewed in Vetriventhan et al. [2]
Proso millet	Nil	Reviewed in Vetriventhan et al. [2]
Little millet	Nil	Reviewed in Vetriventhan et al. [2]

millet by the biolistic method in which the efficiencies of various promoters were tested for the expression of the GUS reporter gene [82]. *Agrobacterium*-mediated transformation of kodo millet was reported recently by Bhatt et al. [83]. They have optimized various parameters for the successful transformation of kodo millet in this study. No report is available on the transformation of little millet and proso millet. This will hamper application of genome editing in these millets. Among the available transformation methods, the *Agrobacterium*-based system is expected to dominate millet transformation and the same could be extended to successful delivery of CRISPR/Cas constructs to millets. Biolistic system-based transformation may not be utilized as many millet research labs in developing countries may not afford this expensive system.

As a leading millet with completely annotated genome with application of genome editing tools, foxtail millet is expected to lead the genome editing studies among millets. To further supplement the genetic studies, a mini foxtail millet variety with a shorter life cycle has been developed recently [84]. This is named as Xiaomi, and has a point mutation in the *Phytochrome C* gene and shows a heading date of 39 days after sowing whereas for wild type plants it takes 82 days. The authors have also assembled the genome and transcriptome of the Xiaomi variety with establishment of an efficient transformation system [84]. This variety of foxtail millet could serve as a model system for functional genomics studies in other millets in future. The Xiaomi variety could be harnessed for several genome editing studies and genes of many other millets could be tested by comparative genomics approaches in this variety to accelerate the gene characterization.

## Conclusion

Millets are nutri-rich cereal crops supplying energy and nutrients to the majority of the people in the semi-arid tropics of Asia and Africa. They also possess good climate resilient properties like tolerance to drought and salinity. Unfortunately, modern genetic research is still far from reaching these crops due to the fact that these are cultivated in less developed countries where the resource for modern molecular research is limited. Application of genome editing has been considered to accelerate crop breeding by helping to impart key traits precisely. Apart from foxtail millet, other millets have not yet been used for genome editing studies. Development of completely annotated genomes and an efficient transformation system for millets may aid high resolution studies using genome editing tools like CRISPR/Cas9 in the near future to help conserve food security in Asia and Africa.

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## Declarations

**Conflict of interest** The author declares that no conflict of interest exists.

**Consent to participate** This article does not involve any clinical studies that need consent of participants.

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