



Progresses of CRISPR/Cas9 genome editing in forage crops

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ABSTRACT

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) mediated-genome editing has evolved into a powerful tool that is widely used in plant species to induce editing in the genome for analyzing gene function and crop improvement. CRISPR/Cas9 is an RNA-guided genome editing tool consisting of a Cas9 nuclease and a single-guide RNA (sgRNA). The CRISPR/Cas9 system enables more accurate and efficient genome editing in crops. In this review, we summarized the advances of the CRISPR/Cas9 technology in plant genome editing and its applications in forage crops. We described briefly about the development of CRISPR/Cas9 technology in plant genome editing. We assessed the progress of CRISPR/Cas9-mediated targeted-mutagenesis in various forage crops, including alfalfa, *Medicago truncatula*, *Hordeum vulgare*, *Sorghum bicolor*, *Setaria italica* and *Panicum virgatum*. The potentials and challenges of CRISPR/Cas9 in forage breeding were discussed.

1. Introduction

Forage grasslands are used to feed livestock and globally, it has been estimated that grasslands represent 26% of the land area and 70% of agricultural area (Conant, 2010). Forages are described as the edible plant portions that can be picked for food or that supply fodder for grazing animals (Allen et al., 2011). Forage crops are plants that are planted especially for being grazed by livestock or preserved as hay or silage (Horrocks and Valentine, 1999). Forage crops aid in achieving production targets for traits like growth or weight increase and to overcoming seasonal imbalances between feed supply and demand. Forage crops can be a valuable tool for farmers, if the proper crop is chosen and correctly maintained during establishment and grazing to guarantee that the maximum output is reached (Stevens, 2009). Additionally, they can be crucial in sustaining ground cover, preventing erosion, building up nitrogen in the soil, and improving land conditions (McGourty and Reganold, 2005). Therefore, forage crops are the foundation for sustainable agriculture (Allen et al., 2011). However, forage crops have received less attention compared to food crops, as evidenced by the lack of research and development initiatives for forage improvement (Katoch, 2022). A better forage supply is needed to

increase sustainability, as planted areas cannot grow, and larger forage patches with one or a few species should not be cultivated (Simeão et al., 2021). Currently, there is a pressing need to increase food production and accelerate the development of sustainable agriculture (El-Mounadi et al., 2020).

Crop improvements are crucial to fulfill the rising global food demand and increase food nutrition (Mishra and Zhao, 2018). Therefore, breeders have paid steadily attention to both enhancing agricultural production and improving food quality. Plant breeders have been improving crops for years through traditional crop breeding, physical and chemical methods (e.g., gamma radiation, ethyl methanesulfonate), or genetic engineering breeding (e.g., T-DNA, transposon insertion) to enhance various agricultural traits resulting from gene mutations, deletions, insertions, or rearrangements (Osakabe et al., 2010; Sikora et al., 2011; Lusser et al., 2012; Wenefrida et al., 2013; Chaudhary et al., 2019; Ramesh et al., 2020).

Plant breeding is one of the earliest sustainable agricultural techniques used to improve the production, quality, and resistance to biotic and abiotic stress (Sharma et al., 2017). Conventional breeding has made it possible for breeders to create better varieties of many crops, increasing food security, crop production, resistance to biotic and

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abiotic stress, and nutrient content (Raza et al., 2019). However, breeders are still facing increasing challenges that are expected to be overcome in the era of climate change and greater consumer demands (Raza et al., 2019). Although conventional breeding of tropical fodder has successfully produced high-yielding, well-adapted cultivars over the past few decades, the genetic gains have been modest given the rising global demand for food (Ashraf, 2010; Tester and Langridge, 2010; Simeão et al., 2021). Conventional breeding techniques have limitations, such as laborious, time-consuming, and causing biosafety concerns (Sharma et al., 2017; Mishra and Zhao, 2018). Therefore, more-effective and time-saving breeding techniques are required.

Advanced techniques for plant genetic engineering have been developed to facilitate plant breeding faster, more predictable, and adaptable to various species (El-Mounadi et al., 2020). The most recent method of genetic engineering is genome editing with programmable endonucleases (Puchta et al., 1993; Puchta, 2005; Symington and Gautier, 2011). In the last decade, the use of genome editing technologies with site-specific nucleases (SSNs) has demonstrated precise gene editing in both animal and plant systems (Jinek et al., 2012; Gaj et al., 2013). These SSNs create double-stranded breaks (DSB) in the target DNA. Nonhomologous end joining (NHEJ) or homology-directed recombination (HDR) pathways repair the DSBs (Puchta et al., 1996; Puchta, 2005; Symington and Gautier, 2011; Jinek et al., 2012).

Plant genome editing currently uses three types of programmable endonucleases including zinc finger nucleases (ZFNs) (Wright et al., 2005; Carroll, 2011; Shah et al., 2018), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010; Mahfouz et al., 2011; Li et al., 2012; Malzahn et al., 2017; Shah et al., 2018), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Cong et al., 2013; Malzahn et al., 2017; Shah et al., 2018; Zhang et al., 2018; Bao et al., 2019). The CRISPR/Cas9 system uses a programmable Cas9 nuclease and a synthetic short guide RNA (sgRNA), in which the sgRNA determines the specificity of DNA targets. CRISPR/Cas9 is more convenient and economical compared to Zinc fingers and TALENs, that can target multiple genes at once/simultaneously (Cong et al., 2013; Mali et al., 2013).

CRISPR/Cas9 has swiftly emerged as the preferred technology for genome editing and has been applied in various crop species for trait improvement (Schaeffer and Nakata, 2015; Svitashv et al., 2016; Wolabu et al., 2020). In this review, we focus on recent advancements in CRISPR/Cas9 genome-editing technology for the forage crops.

2. Overview of CRISPR/Cas9 in plants

A new era of genome engineering is now possible due to genome editing technologies, allowing effective, precise, and rapid engineering of plant genomes (Wada et al., 2020). CRISPR/Cas9 is the most widely used approach that has been modified into a versatile genome editing tool with a wide variety of applications in medicine, agriculture, and fundamental research on genetic modifications (El-Mounadi et al., 2020). CRISPR/Cas9 technology is rapidly evolved to accommodate many applications, such as multiplex gene mutation, transcriptional control and gene replacement, and is becoming a potent tool for targeting gene modifications in a variety of organisms, including plants (Ma and Liu, 2016; Ma et al., 2016; Soyars et al., 2018; Mao et al., 2019; Wolabu et al., 2020). CRISPR/Cas9 is continuously improving, and its applications have been expanded significantly, which is thus considered a revolutionary technology in plant biology (Liu et al., 2017). CRISPR/Cas9 has been used in both the basic and applied plant sciences to increase yield, regulate metabolic processes, and increase stress resistance in numerous plants (Zheng et al., 2021).

CRISPR arrays were first identified in the *Escherichia coli* genome in 1987, but their function was not understood until 2005 (Ishino et al., 1987). However, the function of CRISPR was unknown at the time of its discovery. A key discovery that shed light on the function of CRISPR

came in 2005 when the spacers within CRISPR were found to be originated from invading viruses and plasmids (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Additionally, they found that spacers have a common end sequence, called PAM. This discovery provides evidence that CRISPR/Cas may be involved in prokaryotic adaptive immunity (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). The discovery that viruses cannot infect archaeal cells carrying sequences matching their own genomes led researchers to hypothesize that CRISPR/Cas systems could serve as a defensive immune system protecting owners from pathogens (Mojica et al., 2005).

The first evidence that CRISPR/Cas is an adaptive immune system came from an experiment in 2007 in which researchers found that adding or deleting particular spacers could modify the phage-resistant phenotype of bacteria. It was confirmed that the CRISPR arrays provide protection against invading viruses when combined with the Cas genes. In every infection, new phage DNA is incorporated into the CRISPR array, building the potential to fight the upcoming infection (Barrangou et al., 2007).

CRISPR/Cas9 is composed of two components: a guide RNA (gRNA) that matches the desired target gene and the Cas9 (CRISPR-associated protein 9), an endonuclease that creates a double-stranded DNA break (Jinek et al., 2012; Redman et al., 2016). The CRISPR/Cas9 system uses single-guide RNAs to edit genomes, making it a straightforward, reliable, and potent tool for targeted gene mutagenesis, knockouts, knock-ins, and transcriptional regulation (Hussain et al., 2018). It is possible to deliver the Cas9 gene and gRNA into the plant cells under the control of the appropriate promoters within any vector. The Cas9 protein, also known as RNA guided site-specific nucleases (RGNs), and the transcribed gRNA, is assembled and delivered into the regenerating plant cells. CRISPR/Cas9 requires the presence of 5'NGG3' at the target site, also known as protospacer adjacent motif (PAM). A Cas9 enzyme uses gRNA to cleave the two strands of a target gene or DNA. A double-stranded break can be repaired either by homologous direct repair (HDR) or nonhomologous end joining (NHEJ). NHEJ mediates direct religation of the broken DNA molecules in the absence of a homologous template, frequently resulting in insertion/deletion mutations (INDELS) or substitutions at the DSB site. In contrast, HDR introduces new alleles, even correcting mutations or introducing novel sequences of interest in the presence of a donor DNA. Genome modifications can vary depending on the repair pathway and the availability of the repair template (Fig. 1) (Budman and Chu, 2005; Gong et al., 2005; Zha et al., 2009; Symington and Gautier, 2011; Bortesi and Fischer, 2015; Puchta, 2017).

In August 2013, five reports presented the first applications of CRISPR/Cas9 in plant genome editing (Feng et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013). CRISPR/Cas9 technology brought new opportunities to the field of genetic manipulations in plants and has gained great attention in recent years (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). The CRISPR/Cas9 technology has been applied to genome editing in many plants, including the model organisms *Arabidopsis thaliana* (Feng et al., 2013; Jiang et al., 2013; Li et al., 2013) and *Nicotiana benthamiana* (Jiang et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Upadhyay et al., 2013), and a broad range of crop species, such as *Oryza sativa* (Feng et al., 2013; Jiang et al., 2013; Shan et al., 2013; Xie and Yang, 2013; Liu et al., 2016), *Zea mays* (Liang et al., 2014; Shi et al., 2017), *Triticum aestivum* (Shan et al., 2013; Upadhyay et al., 2013; Wang et al., 2014; Liang et al., 2017, 2018; Howells et al., 2018; Zhang et al., 2018), sorghum (Jiang et al., 2013), *Brassica napus* (Braatz et al., 2017; Zhai et al., 2019), *Hordeum vulgare* (Lawrenson et al., 2015), *Gossypium hirsutum* (Wang et al., 2018), *Solanum lycopersicum* (Brooks et al., 2014; Ron et al., 2014; Soyk et al., 2017a,b), *Solanum tuberosum* (Butler et al., 2015), and *Glycine max* (Cai et al., 2018; Cheng et al., 2019).

CRISPR/Cas9 is showing promise in the field of plant editing as it becomes clear that each component of this system can be optimized to enhance editing results (Soyars et al., 2018). CRISPR/Cas9-based

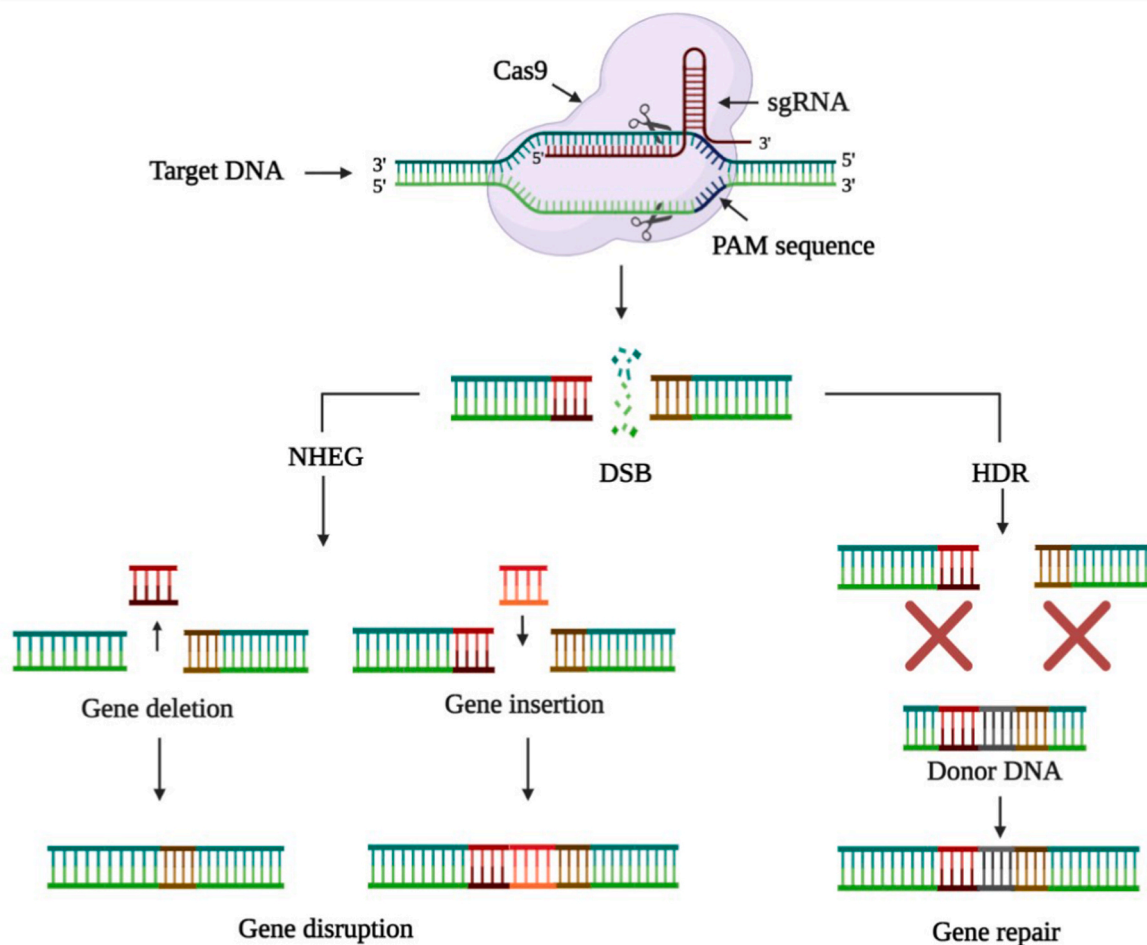


Fig. 1. Illustration of the CRISPR/Cas9 genome editing system in plants. Cas9 is directed to its DNA target based on base pairing between sgRNA and DNA. The Cas9 recognition and cleavage takes place in the presence of the PAM motif downstream of the sgRNA-binding region. The endogenous DSB repair is triggered by Cas9/sgRNA cutting both strands of the target DNA. The first mechanism involves non-homology end joining while the second involves homology direct repair. CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; PAM, protospacer adjacent motif; sgRNA, single guide RNA; DSB, double-stranded break; NHEJ, nonhomologous end joining; HDR, homology direct repair.

technologies have the potential to transform crop trait improvement in a variety of plants to improve yield, quality, nutritional value, and tolerance to biotic and abiotic stresses, among other applications (Schaeffer and Nakata, 2015; El-Mounadi et al., 2020). CRISPR/Cas9 enables the rapid production of transgene-free genome-edited plants also known as “null segregants” (Wada et al., 2020).

The steps involved in CRISPR/Cas9-based genome editing in plants are as follows: A target gene where the mutation is to be introduced; identification of the PAM sequence in a target gene and designing the sgRNA complementary to the expected target sequence; cloning sgRNA and Cas9 under suitable promoters in plant binary expression vector; delivery of CRISPR/Cas9 components to plants employing a suitable transformation method and identifying the mutations in regenerated transgenic plants using PCR, restriction digestion and gel electrophoresis assays, and sequencing (Fig. 2). CRISPR/Cas9 vectors are available in Addgene public plasmid repository (<http://www.addgene.org/crispr-plant/>).

3. CRISPR/Cas9 in forage crops

Agriculture is one of the most critical sectors on the planet as it sustains food for living organisms, and both abiotic and biotic stresses threaten agriculture around the globe (Zaidi et al., 2016; Hillary and Ceasar, 2019). Over the years, crop production has faced numerous

challenges because of climatic change, biotic (bacteria, fungi, insects, and viruses) and abiotic (salinity, drought, flooding, heavy metal toxicity, high temperature) stresses (Bao et al., 2019; Chen et al., 2019), have adversely affected crop plantation. Therefore, plant breeders have always focused on improving yields, quality, stress tolerance and resistance in response to worldwide population explosion, food shortages, and environmental degradation (Zhang, 2007). Forage crop improvement focuses on some of the most critical aspects such as increased yield, resistance to pests and diseases, tolerance to various abiotic stresses, improved shelf life, processing quality, aesthetic value, enhanced nutritional value, etc.

Nowadays, CRISPR/Cas9 genome editing has become a mature cutting-edge biotechnological tool for crop improvement and has been used to improve monocots and dicots crops for a variety of traits, including plant yield, quality, development and morphology, pathogen resistance, abiotic tolerance, secondary metabolism, and fiber development (Ma and Liu, 2016; Zhang et al., 2021). CRISPR/Cas9-mediated gene editing has been established in some forage crops including alfalfa, *M. truncatula*, *H. vulgare*, *S. bicolor*, *S. italica* and *P. virgatum* for which transformation protocols are available.

Genome editing in forage crops by CRISPR/Cas9 is an emerging field, and very few reports have appeared so far are summarized in Table 1. Here, we review the status and prospects for using CRISPR/Cas9 to enhance a variety of forage crop traits.

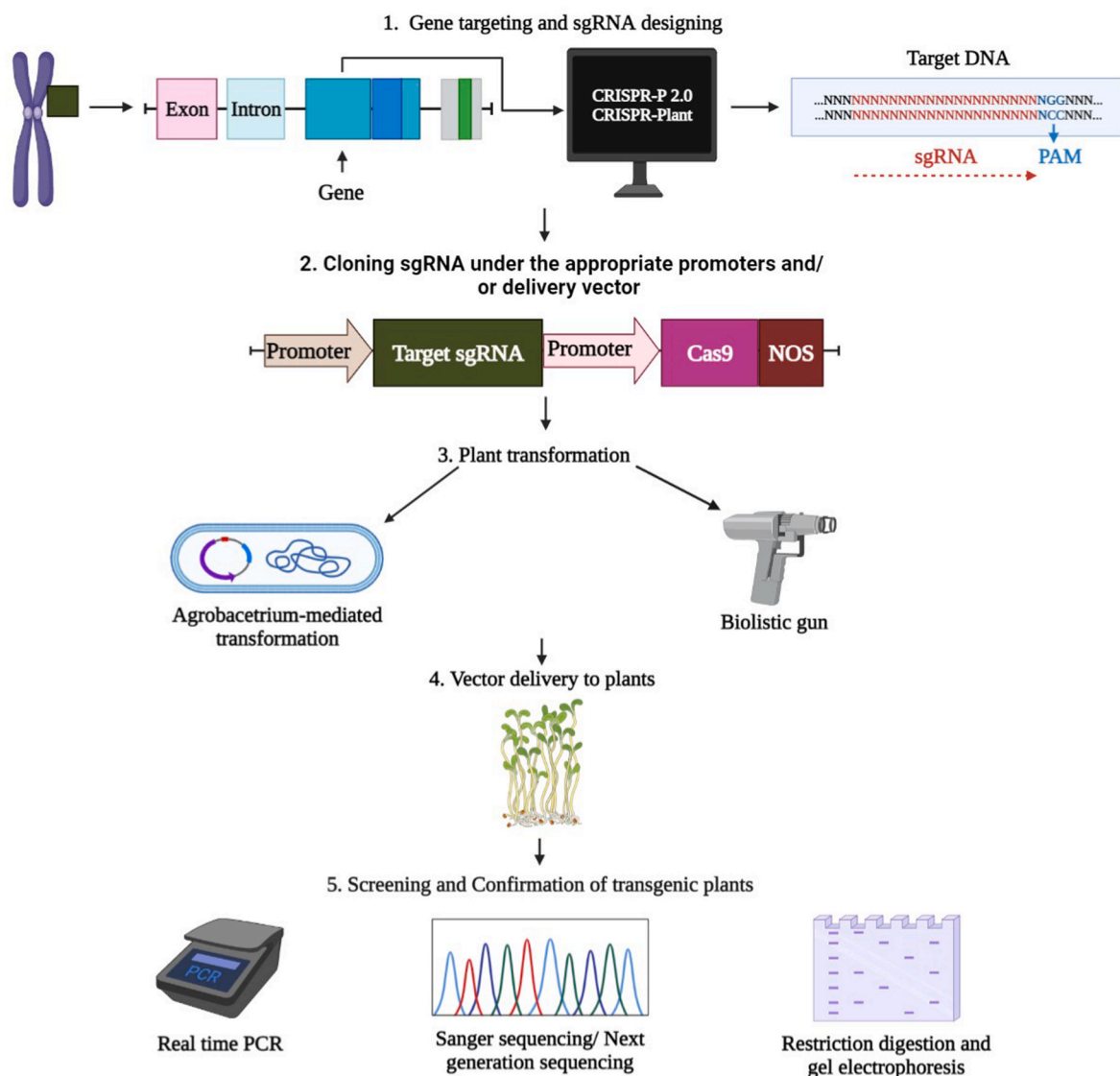


Fig. 2. A schematic layout representing the execution of CRISPR/Cas9 based genome editing in plants. The first step involves selecting a gene and designing a sgRNA, the second step involves cloning the sgRNA into a suitable binary vector, the third step involves the transformation process, the fourth step involves the induction of binary vectors into the host plant, and the fifth step involves screening and confirmation of transgenic plants. PAM, protospacer adjacent motif; sgRNA, single guide RNA.

3.1. Alfalfa

Alfalfa (*Medicago sativa* L.), which is known as “Queen of the Grass”, is a perennial tetraploid legume species of the family Fabaceae (Ibrahim et al., 2016; Badr et al., 2020). Alfalfa is a widely grown legume forage that is grown more than 32 million hectares of lands worldwide (Tesfaye et al., 2006; Kumar, 2011; Wolabu et al., 2020). A CRISPR/Cas9 technique was first used in alfalfa in a high-throughput manner to target the SPL gene (*MsSPL9*), followed by restriction enzyme digestion and PCR amplification and sequencing. The gene-editing frequency detected by ddPCR showed the highest rate of 2.2%, which was relatively low compared to other plant species (Gao et al., 2018).

3.1.1. Development of multiplex genome editing

A single gRNA-CRISPR/Cas9 system in alfalfa generated a total of 1531 independent transgenic lines by targeting stay-green (*MsSGR*), phytoene desaturase (*MsPDS*) and other genes. However, in such many transformants, no mutant phenotype was observed, and the overall genotypic mutagenesis efficiency was only 2.5% with only one or two copies of the target gene mutated. Furthermore, the outcrossing nature

of alfalfa made it impossible to obtain complete knockout in the next generation by selfing (Wolabu et al., 2020). Moreover, subsequent studies have led to substantially higher editing frequencies and consequent phenotypic alterations in alfalfa, with up to 100% allelic mutation frequency observed in the first generation when targeting the phytoene desaturase (*MsPDS*) and *MsPALM1* genes (Chen et al., 2020). Hence, the use of a multiplex gRNA CRISPR/Cas9 system was required to achieve high editing frequencies in alfalfa in certain instances (Wolabu et al., 2020), yet this has not always been the case (Chen et al., 2020).

A multiplex gRNA-CRISPR/Cas9 systems based on tandem arrays of tRNA-gRNA sequences have been proven to be effective in Arabidopsis, rice, and wheat genome editing (Xie et al., 2015; Wang et al., 2016; Zhang et al., 2018). Therefore, a multiplex gRNA-CRISPR/Cas9 vectors by a polycistronic tRNA-gRNA approach was constructed targeting the *M. sativa* stay-green (*MsSGR*) gene to develop an optimized genome editing system in alfalfa (Wolabu et al., 2020). A knockout efficiency phenotype was analyzed using the *MsSGR* gene, as plants with the sgr mutation show a visible greenish appearance during dark or shade treatment due to senescence induction (Ren et al., 2007; Barry et al., 2008; Zhou et al., 2011; Wu et al., 2016).

Table 1
Summary of CRISPR/Cas9-mediated genome editing in forage crops.

Crop species	Gene/Mutant	Transformation method	Function	Reference
<i>Medicago sativa</i>	<i>MsSGR</i>	Agrobacterium-mediated transformation	chlorophyll retention; stay green phenotype; maintains consistently deep green appearance	Wolabu et al. (2020)
<i>Medicago sativa</i>	<i>MsSPL8</i>	Agrobacterium-mediated transformation	small leaf size; reduced internode length; early flowering; enhanced drought resilience	Singer et al. (2021)
<i>Medicago sativa</i>	<i>MsGA30x1</i>	Agrobacterium-mediated transformation	semi dwarf and prostrate phenotype; more lateral branches; high leaf/stem ratio and crude protein; plays an influential role in GA-regulated plant height	Zheng et al. (2022)
<i>Medicago sativa</i>	<i>PHO2</i>	Agrobacterium-mediated transformation	phosphate hyperaccumulation	Miller et al. (2022)
<i>Medicago truncatula</i>	<i>NPD</i>	Agrobacterium-mediated transformation	nodule formation	Trujillo et al. (2019)
<i>Medicago truncatula</i>	<i>MtCEP1/2/12</i>	Agrobacterium-mediated transformation	control lateral root; nodule number	Zhu et al. (2021)
<i>Medicago truncatula</i>	<i>BS1</i>	Agrobacterium-mediated transformation	arrests axillary bud outgrowth; regulate organ size; increased leaf and flower size	Yin et al. (2020)
<i>Medicago truncatula</i>	<i>mtsup-2</i>	Agrobacterium-mediated transformation	twin flowers; flowers with more petals and fewer stamens; deformed carpels with less ovules	Rodas et al. (2021)
<i>Medicago truncatula</i>	<i>CYP93E2</i>	Agrobacterium-mediated transformation	modulates the triterpene saponin biosynthesis	Confalonieri et al. (2021)
<i>Hordeum vulgare</i>	<i>HvCKX1/HvCKX3</i>	Agrobacterium-mediated transformation	affects the regulation of cytokinin metabolism and root morphology	Gasparis et al. (2019)
<i>Hordeum vulgare</i>	<i>HvMORC1; HvMORC6a</i>	Agrobacterium-mediated transformation	plant immunity; transcriptional gene silencing; plant growth; resistance to biotrophic and necrotrophic plant pathogenic fungi; enhance resistance to fusarium root rot and bipolaris spot blotch	Galli et al. (2022a), b
<i>Hordeum vulgare</i>	<i>MTOPVIB</i>	Agrobacterium-mediated transformation	critical for meiotic DSB; accompanied SC and CO formation	Steckenborn et al. (2022)
<i>Hordeum Vulgare</i>	<i>HvARE1</i>	Agrobacterium-mediated transformation	improve nitrogen use efficiency	Karunaratne et al. (2022)
<i>Sorghum bicolor</i>	<i>k1C</i>	Agrobacterium-mediated transformation	improve protein digestibility; high lysine content	Li et al. (2018)
<i>Sorghum bicolor</i>	<i>lg1</i>	Particle bombardment-mediated transformation	alter leaf inclination angle	Brant et al. (2021)
<i>Sorghum bicolor</i>	<i>SbBADH2</i>	Agrobacterium-mediated transformation	aromatic smell in seeds and leaves; produce fragrant sorghum	Zhang et al. (2022)
<i>Setaria italica</i>	<i>SiNP1</i>	Agrobacterium-mediated transformation	heterosis; pollen development; male fertility	Zhang et al. (2021b)
<i>Setaria italica</i>	<i>SiBOR1</i>	Agrobacterium-mediated transformation	regulates panicle development; cell wall integrity; cellular homeostasis	Wang et al. (2022)
<i>Panicum virgatum</i>	<i>Pv4CL1</i>	Agrobacterium-mediated transformation	lignin reduction; improved sugar release	Park et al. (2017)
<i>Panicum virgatum</i>	<i>Pvtb1</i>	Agrobacterium-mediated transformation	enhanced tiller production and biomass yield	Liu et al. (2020)

A multiplex tRNA-gRNA with four alfalfa spacers was inserted into three optimized vectors of pRGE31 to assemble a multiplex gRNA-CRISPR/Cas9 vectors and transgenic plants were obtained through Agrobacterium-mediated transformation (Wolabu et al., 2020). In the multiplex gRNA system, replacing the CaMV35S promoter with the Arabidopsis ubiquitin promoter (AtUBQ10) led to a significant improvement in genome editing efficiency and showed 75% genotypic mutagenesis efficiency, which is 30-fold more efficient than the single gRNA CRISPR/Cas9 system. Furthermore, phenotypic changes were easily observed in the mutants, and the phenotypic mutation efficiency reached 68% (Wolabu et al., 2020).

The multiplex gRNA-CRISPR/Cas9 vector drastically improved the mutagenesis efficiency of the *MsSGR* gene by 23%–49% at the genotypic level and allowed the generation of homozygous mutants with a complete knockout of the four allelic copies in the T0 generation in alfalfa (Wolabu et al., 2020). This multiplex gRNA-CRISPR/Cas9 genome editing system for alfalfa using a polycistronic tRNA-CRISPR-gRNA approach is simple and reliable compared to earlier biotechnology techniques and offers a reliable and effective method of testing gene function by overcoming major barriers in applying genome editing to the improvement of alfalfa, as well as other dicots with complex genomes, particularly legumes (Wolabu et al., 2020).

3.1.2. Improving alfalfa visual appearance

Most of the alfalfa is used to make hay. The color of alfalfa leaves is a crucial characteristic for the assessment of hay value at the market. One of the physical features of premium hay is bright green rather than

looking yellowish. The visual appearance (greenish color) is an important trait of alfalfa hay (Zhou et al., 2011). CRISPR/Cas9 complete knockout of the four allelic copies of *MsSGR* gene generated mutants with an obvious stay-green phenotype (more obvious greenish color) that had excellent chlorophyll retention with a consistently deep green appearance throughout the dark incubation period (Table 1) (Wolabu et al., 2020).

3.1.3. Improving drought resilience

Abiotic stress such as drought severely affects agriculture and causes a major setback to agricultural productivity (Ahluwalia et al., 2021). The effects of drought on crop yield and quality are influenced by numerous factors, including water source reliability, crop type vulnerability to water stress, and socio-economic factors (Rey et al., 2017). Plant-specific transcription factors, known as Squamosa promoter-binding protein-like (SPL) proteins, have a highly conserved zinc-containing DNA binding region known as the SBP-domain that functions in a partially overlapping manner in the regulation of an exceptionally diverse set of processes such as vegetative growth, response to abiotic stress and yield (Yamasaki et al., 2004, 2009; Birkenbihl et al., 2005; Xing et al., 2013; Xu et al., 2016; Gao et al., 2018; Hu et al., 2021; Sun et al., 2022). Alfalfa *MsSPL8* gene is down-regulated by RNA interference (RNAi), which enhances alfalfa biomass, branching, regrowth, and drought tolerance (Singer et al., 2021).

A CRISPR/Cas9 system was used to further characterize *MsSPL8* function in alfalfa. Three gRNAs targeting different regions of *MsSPL8* alleles were inserted into the pKSE401 background vector to yield SPL8-

gRNA1, SPL8-gRNA2, and SPL8-gRNA3 binary vectors. They were then introduced into *A. tumefaciens* LBA4404 using electroporation and transferred separately into the alfalfa genotype through Agrobacterium-mediated transformation of leaf explants (Singer et al., 2021).

CRISPR/Cas9 successfully edited three of four *MsSPL8* alleles in the first generation with a single gRNA and up to 100% of alleles in the second generation. The SPL8-gRNA1 genotypes showed a distinct morphological alteration, including reduced internode length, small leaf size, and early flowering, despite the presence of a wild-type copy of *MsSPL8* in first-generation plants (Table 1) (Singer et al., 2021). Moreover, SPL8-gRNA1 genotypes exhibited a superior ability to survive under water-deficit conditions compared to empty vector (EV) controls, showing that gene editing has enhanced drought resilience (Singer et al., 2021). Therefore, the ability of alfalfa plants with SPL8-gRNA1 genotypes to withstand abiotic stress is an invaluable trait for future climate change (Singer et al., 2021), which provides the foundation for further research exploring drought tolerance mechanisms in forage crops.

3.1.4. Regulating gibberellin signaling and synthesis

Plant height is a key factor of the architecture of crop plants, affecting crop yield and quality (Liu et al., 2018; Eshed and Lippman, 2019). Gibberellin (GA) is an important hormone that regulates the growth and development of higher plants (Hedden, 2001; Yamaguchi, 2008). Disruption of the GA signaling pathway or blockage of the GA synthesis pathway causes semidwarfism (Zheng et al., 2022). Gibberellin (GA) 3-oxidase, a class of 2-oxoglutarate-dependent dioxygenases, catalyzes the transformation of precursor GAs into their bioactive forms, thereby directly influencing the concentrations of bioactive GAs in plants. *GA3ox1* plays a role in vegetative growth and development, as well as reproductive organ development (Mitchum et al., 2006).

MsGA3ox1 was cloned from alfalfa, and two knockout targets were designed. The *msga3ox1* mutants were generated using CRISPR/Cas9 genome editing through Agrobacterium-mediated transformation in alfalfa. Plant height and internode length were significantly reduced in *msga3ox1* mutants. However, these mutants had significantly more lateral branches, a higher leaf/stem ratio, and a higher crude protein content in aerial parts of the plant, resulting in semi-dwarf and prostrate alfalfa plants. Therefore, *MsGA3ox1* plays an influential role in GA-regulated plant height in alfalfa (Table 1) (Zheng et al., 2022). Alfalfa with a prostrate growth can be utilized as mulch in orchards without affecting regular farming and can also provide a high-quality protein source for poultry (Zheng et al., 2022).

3.1.5. Regulating phosphate homeostasis

Phosphorus (P) is a major element and performs vital functions for sustenance, growth, and development of plants (Ahmad et al., 2009). Phosphorus is absorbed by plants in the form of available Pi from the soil. Phosphate ion (Pi) is often present in low concentrations, even though the concentration of phosphorus in the soil can be high (Raghothama, 1999). PHO2 is an important component of the Pi homeostasis pathway and negatively regulates several Pi transporters including PHT1 protein family members, PHO1, and PHF1 at the protein level (Liu et al., 2012; Huang et al., 2013; Park et al., 2014).

CRISPR/Cas9 was used to target PHO2-like genes, *PHO2-B* and *PHO2-C* haplo-alleles in alfalfa. The guide RNA targets were cloned into a one-step binary vector (pDIRECT.22C) and delivered to alfalfa leaf explants by Agrobacterium-mediated transformation. CRISPR/Cas9 generated a suite of haplotype mutant plants of the *PHO2-B* and *PHO2-C* genes in alfalfa. A mutation in PHOSPHATE2 (PHO2) disrupts Pi homeostasis, and the loss of function of *PHO2-B* and *PHO2-C* genes results in a Pi hyperaccumulation trait that increases the concentration of Pi in leaf tissues to levels 2.7- to 5.6-fold higher than wild-type plants (Table 1) (Miller et al., 2022). The resultant alfalfa mutants not only hyperaccumulated Pi but also acted as a promising tool for bioremediation of phosphate-contaminated soils (Miller et al., 2022).

These encouraging developments illustrate that the CRISPR/Cas9-

mediated editing in a multiplex alfalfa genome will ultimately lead to significant advances in crop improvement.

3.2. *Medicago truncatula*

Medicago truncatula emerged as a model legume in 1990, comprising the third largest family of land plants (Burks et al., 2018). *M. truncatula* is a close relative of alfalfa and has several characteristics that make it a good model system, including a diploid and small genome that is easy to modify, a short life cycle, and a high level of natural diversity (Meng et al., 2017). Studies have been conducted extensively on *M. truncatula*, from fundamental molecular, physiological, and developmental mechanisms to translate and apply trait improvement to related economically significant legume crops (Michno et al., 2015; Meng et al., 2017; Čermák et al., 2017; Curtin et al., 2017; Gao et al., 2018; Curtin, 2018).

3.2.1. Modifying nodule morphologies

Nodules are specialized root organs formed during biological nitrogen (N₂) fixation by soil rhizobia (Jones et al., 2007). In legumes, rhizobial symbionts and plant hosts interact to mediate symbiotic nitrogen fixation (Trujillo et al., 2019), and lineage-specific expansions (LSEs) of several small signaling families are known to be associated with nodulation, including nodule-specific cysteine-rich peptides (Mergaert et al., 2003), calmodulin-like proteins (Liu et al., 2006), and glycine-rich proteins (Vandepoel and Van de Peer, 2005; Silverstein et al., 2006).

A CRISPR/Cas9 multiplex genome editing approach was used to generate knockout lines targeting one to five PLAT domain genes (NPD) to probe their potential functions in nodulation in *M. truncatula* (Trujillo et al., 2019). *M. truncatula* was transformed by inoculating leaf explants with *A. tumefaciens* (strain EHA105) using the pSC218 GG vector. A clear difference in external nodule morphology was observed in mutants with three, four, and five *NPD* knockouts. Consequently, mutant lines with various combinations of inactive *NPD* genes displayed progressively smaller nodules, an earlier onset of nodule senescence, or ineffective nodules (Table 1). Therefore, nodule-specific PLAT domain proteins (NPDs) are essential for successful nodule formation (Trujillo et al., 2019).

3.2.2. Regulating organ size

Organ size is a major agronomic trait that determines grain yields and biomass production in crops (Yin et al., 2020). Plant organ growth is controlled primarily by two coordinated developmental processes: cell division and cell expansion (Horiguchi et al., 2006). A fundamental regulator of seed size and weight, BIG SEEDS1 (BS1) was identified in *M. truncatula* (Ge et al., 2016). *BS1* encodes a plant-specific transcription regulator and plays a key role in the control of the size of plant organs, including seeds, seed pods, and leaves, through a regulatory module that targets primary cell proliferation (Ge et al., 2016).

A significant increase in the size of *M. truncatula* organs occurs when *BS1* function is lost, including enlarged seeds, fruits, and leaves (Ge et al., 2016), pointing out that *BS1* has significant potential for legume crop improvement. *SLB1* genetically interacts with *BS1* to control organ size and lateral branching in *M. truncatula* (Yin et al., 2020). The U6 promoter and single guide RNA (sgRNA) scaffold were amplified using primer sets MtU6-F1/MtU6-R1 and BS1-sgRNA-F/R1, respectively for CRISPR/Cas9 vector construction. The U6 promoter and single guide RNA (sgRNA) scaffold were amplified using primer sets MtU6-F1/MtU6-R1 and BS1-sgRNA-F/R1 for CRISPR/Cas9 vector construction and were transformed into plants using *A. tumefaciens* strain AGL1 (Yin et al., 2020). Plants with disrupted expression of *BS1* in the R108 background, generated by CRISPR/Cas9 (*BS1-CR*), displayed arrested axillary bud outgrowth, but exhibited significantly increased leaf and flower size (Table 1) (Yin et al., 2020).

3.2.3. Regulating lateral roots and nodule formation

The root architecture system, which contributes to the perception, absorption, allocation, and transport of nutrients and provides structural support, is essential for the survival of the entire plant (Motte et al., 2019). The CEP peptide (C-terminally Encoded Peptide) is considered to play a central role in balancing root architecture and nodulation under nitrogen-limited conditions (Imin et al., 2013; Patel et al., 2018; Laffont et al., 2020; Zhu et al., 2020). CEPs contain 15 highly conserved amino acids in their mature form, and are involved in a variety of physiological processes, including root growth and symbiotic nitrogen fixation and nitrate uptake (Ohyama et al., 2008; Imin et al., 2013; Tabata et al., 2014). The complex gene family known as CEP includes multiple members and is involved in several physiological processes in plants. It has been demonstrated that *MtCEP1* and *MtCEP7* control lateral root formation or nodulation, but these studies were based only on gain of function or artificial miRNA (amiRNA)/RNAi approaches, never knockout mutants (Zhu et al., 2021).

CRISPR/Cas9-mediated genome editing produced single mutants of *MtCEP1*, 2, 4, 6, and 12 and double mutants of *MtCEP1/2C* and *MtCEP5/8C* in *M. truncatula*, but these mutants did not exhibit any visible changes in their phenotypes. However, the triple mutant *Mtcep1/2/12C* and the quintuple mutant *Mtcep1/2/5/8/12C* generated using binary vector pHSE401 through Agrobacterium-mediated transformation showed more lateral roots and fewer nodules. Therefore, *MtCEP1*, 2, and 12 play crucial roles in regulating lateral root development and symbiotic root nodulation and are redundantly important in the control of lateral root number and nodulation in *M. truncatula* (Table 1) (Zhu et al., 2021). CRISPR/Cas9-mediated mutations in these genes contribute to improving our understanding of CEP biology and regulation of root system architecture (Zhu et al., 2021).

3.2.4. Improving floral development

The floral development of legumes shows early carpel initiation and the establishment of common primordia that are ephemeral meristems from which petals and stamens differentiate. Flowers generate sepals, petals, stamens, and carpels, which are grouped into four floral whorls (Krizek and Fletcher, 2005; Prunet et al., 2009). The floral meristem (FM), which is made up of a collection of stem cells and is maintained only temporarily until it terminates, gives rise to a definite number of whorls and floral organs (Bowman et al., 1989; Schultz et al., 1991; Bossinger and Smyth, 1996). SUPERMAN (SUP) is a gene that participates in the control of the number of carpels and stamens in *A. thaliana* (Sakai et al., 1995; Breuil-Broyer et al., 2016).

A binary vector containing hCas9 controlled by the AtUBQ promoter, hygromycin resistance, and a transcriptional unit harboring three copies of sgRNA downstream the AtU6-26 promoter was constructed and then transformed into *M. truncatula* with Agrobacterium-mediated transformation (Rodas et al., 2021).

CRISPR/Cas-9 generated an additional *mtsup* mutant allele in *M. truncatula*. The molecular analyses of several independent T0 transgenic lines revealed that the MtSUP-sgRNA line 1 (called the *mtsup-2* allele) carried a four-nucleotide deletion as heterozygous at the Cas9 editing site. The *mtsup-2* allele produced twin flowers (76%), while the remaining were single flowers without spikes in all flowering nodes. *Mtsup* alleles produced more flowers compared to the wild type. The *mtsup-2* allele showed flowers with more petals (from six to eight), fewer stamens (from six to nine), and distorted carpels with fewer ovules (Table 1) (Rodas et al., 2021).

MtSUP plays a crucial role in compound inflorescence and flower development and regulates the number of floral organs in the second whorl of the flower, in addition to the inner two whorls in *M. truncatula*. Moreover, *MtSUP* regulates the activity of the secondary inflorescence meristem, which in turn controls the number of flowers produced (Rodas et al., 2021).

3.2.5. Modulating triterpene saponin biosynthesis

Many plant species produce a wide group of triterpene or steroid glycosides known as saponins, exhibiting a broad range of biological and pharmacological activities (Haralampidis et al., 2002; Sparg et al., 2004; Tava and Avato, 2006; Augustin et al., 2011; Moses et al., 2014). Soyasapogenol saponins have a limited level of biological and pharmacological activity because they lack hemolytic properties (Argentieri et al., 2008; Tava et al., 2011; Rafińska et al., 2017; Carelli et al., 2020). Hemolytic saponins from Medicago species are useful specialized metabolites that can be used in the agricultural industry and for pharmaceutical purposes (Bora and Sharma, 2011; Rafińska et al., 2017).

M. truncatula was transformed using the *A. tumefaciens* strain EHA105 harboring the CRISPR/Cas9 binary vector (pDGB3 omega1 nptII-gRNA1-gRNA2-Cas9) targeting the *CYP93E2* gene. After *A. tumefaciens*-mediated transformation, 52 potential *CYP93E2* mutant plant lines were obtained. Of these, fifty-one sequenced plant lines showed 84% editing efficiency (Confalonieri et al., 2021).

CRISPR/Cas9-mediated knock-out mutants of the *CYP93E2* gene did not produce soyasapogenols in the leaves, stems, and roots, and diverted the metabolic flux toward the synthesis of valuable hemolytic saponins in *M. truncatula*. *CYP93E2* mutations had no negative effects on the morphological characteristics of plants under greenhouse conditions. Furthermore, *cyp93e2* mutants showed differential expression of saponin pathway genes compared to control (Confalonieri et al., 2021).

CRISPR/Cas9 generated *CYP93E2* barrel medic mutants with high efficiency and highlighted the potential of converting β -amyrin into beneficial hemolytic saponins by blocking the metabolic flux toward non-hemolytic saponins. Thus, CRISPR/Cas9-based targeted mutagenesis of *CYP93E2* modulates the biosynthesis of triterpene saponins in *M. truncatula* (Table 1). These mutant plants with high hemolytic saponin content are compatible with the potential of industrial saponin extraction for a variety of applications (Confalonieri et al., 2021).

As a result of these findings in *M. truncatula*, CRISPR/Cas9 genome editing will lead to significant improvements in forage crops.

3.3. *Hordeum vulgare*

Barley (*Hordeum vulgare* L.) is the fourth most widely cultivated cereal crop species worldwide after maize, wheat, and rice (Faostat, 2017; Gasparis et al., 2019). Barley is a model crop species with several advantages, such as its whole sequenced genome, genuine diploidy, and well-proven genetic transformation techniques based on particle bombardment and *A. tumefaciens* (Mayer et al., 2012).

3.3.1. Regulating cytokinin homeostasis

Cytokinin is a plant hormone that regulates a broad range of developmental processes and significantly influences grain yields. Genetic manipulation of cytokinin content can be used to enhance barley yields to desired levels (Gasparis et al., 2019). The homeostasis of cytokinin is regulated by members of several multigene families. The cytokinin oxidase/dehydrogenase enzyme, which catalyzes the irreversible degradation of cytokinin, is encoded by the *CKX* genes (Gasparis et al., 2019). Overexpression of *CKX* genes and the resulting decrease in cytokinin levels enabled the root system to grow more rapidly. *CKX* genes regulate the morphology and growth of roots (Werner et al., 2003; Mrázová et al., 2013). Several studies have demonstrated that the RNAi-based silencing of *CKX* genes leads to increased grain yields in some crop species (Gasparis et al., 2019).

Gasparis et al. (2019) prepared two binary vectors, pBract211-Cas9-ckx1sgRNA and pBract211-Cas9-ckx3sgRNA, and used them independently for the Agrobacterium-mediated transformation of immature embryos of the barley cultivar golden promise. The RNA-guided Cas9 nuclease produced knockout mutations in the *HvCKX1* and *HvCKX3* genes in barley, resulting in *ckx1* and *ckx3* mutants. The *CKX* enzyme activity decreased significantly in the spikes of *ckx1* lines but remained relatively unchanged in the *ckx3* lines. Despite these

differences, neither mutant line showed any changes in grain yields. In turn, the root morphology of the *ckx1* and *ckx3* mutants reflected their differences in CKX activity. A decrease in CKX activity in *ckx1* lines corresponded to increased root length, surface area, and root hairs. In contrast, an increase in CKX activity in *ckx3* mutants showed the opposite results (Gasparis et al., 2019). Therefore, an RNA-guided Cas9 nuclease knockout of the *HvCKX1* or *HvCKX3* gene affects cytokinin metabolism and root morphology in barley (Table 1) (Gasparis et al., 2019).

3.3.2. Improving plant pathogen defense

Plant pathogens such as *Fusarium* fungi are widespread worldwide and cause devastating diseases like *Fusarium* head blight, *Fusarium* crown rot, and *Fusarium* root rot (Balmas et al., 2015; Hollaway et al., 2013). In addition, they contaminate grains with mycotoxins, which reduces quality and availability (Gaffar et al., 2019). In both mammals and plants, Microorchidia (MORC) proteins function as essential regulators of genome stabilization, chromatin remodeling, and gene expression (Galli et al., 2022a,b).

MORC proteins play a role in plant defense, but it is highly dependent on the plant species. MORC proteins enhance resistance to pathogens in Arabidopsis and potato, while they negatively affect plant immunity in barley, tobacco, and tomato (Kang et al., 2010, 2012; Langen et al., 2014; Manosalva et al., 2015; Kumar et al., 2018). MORC proteins play a role in microbial pathogen immunity in plants (Koch et al., 2017). Furthermore, the *HvMORC1*, *HvMORC2*, and *HvMORC6a* proteins play a significant role in maintaining genomic stability by suppressing transposable elements (TEs) (Langen et al., 2014; Kumar et al., 2018).

Galli et al. (2022a) employed the CRISPR-Cas9 system from *Streptococcus pyogenes* (CRISPR/SpCas9) to generate *hvmorc6a* KO (knockout) mutants and *hvmorc1/hvmorc6a* dKO (double knockout) mutants through Agrobacterium-mediated transformation to further elucidate the role of *HvMORC6a* and its potential interactors in regulating plant immunity. *HvMORC6a* plays a critical role in resistance to biotrophic (*Blumeria graminis*) and necrotrophic (*Fusarium graminearum*) plant pathogenic fungi, whereas the dKO *hvmorc1/6a* displays the most resistant phenotype. The dKO mutants showed the highest levels of basal pathogenesis-related (PR) gene expression and TEs depression. *HvMORC1* and *HvMORC6a* interact with components of the epigenetic machinery for gene silencing, produce nucleocytoplasmic homo-/heteromers, and repress the activity of transposable elements (TE). CRISPR/SpCas9-mediated double knockout of Microorchidia, *MORC1* and *MORC6a* play a crucial role in plant immunity, transcriptional gene silencing, and plant growth in *H. vulgare* (Table 1) (Galli et al., 2022a).

Similarly, the CRISPR/SpCas9-mediated knockdown of epigenetically active MORC proteins also enhanced barley resistance to *Fusarium* root rot and *Bipolaris* spot blotch caused by *Fusarium graminearum* and *Bipolaris sorokiniana* (Table 1) (Galli et al., 2022b). The use of CRISPR/SpCas9-mediated single and double knock-out mutants resulted in the de-repression of transposable elements (TE) and pathogenesis-related genes and an increase in resistance to biotrophic and necrotrophic plant pathogenic fungi (Galli et al., 2022b).

3.3.3. Forming meiotic double-strand break

The homologous recombination (HR) process in meiosis ensures genetic diversity in sexually reproducing organisms (Hunter, 2015; Mercier et al., 2015). Genetic variations in offspring are ensured by homologous recombination during meiosis. A meiotic double-strand break (DSB) is repaired as a crossover (CO) or non-crossover (NCO) during meiotic recombination. *MTOPVIB* plays a critical role in the formation of meiotic DSB, which is essential for CO-recombination, as well as in meiotic bipolar spindle formation in rice and maize (Steckenborn et al., 2022).

Transgenic barley plants were produced by dissecting immature embryos from surface-sterilized caryopses and inoculating them with *A. tumefaciens* strain AGL1 harboring plasmid pGH615. CRISPR/Cas9

endonuclease generates the *mtopvib* plants, which are completely sterile due to the absence of meiotic DSB, synaptonemal complex (SC), and CO formation, which causes univalents to develop and their unbalanced segregation into aneuploid gametes in *H. vulgare*. Therefore, *MTOPVIB* is essential for the meiotic DSB and subsequent SC and CO formation while being dispensable for meiotic bipolar spindle formation in *H. vulgare* (Table 1) (Steckenborn et al., 2022).

3.3.4. Improving nitrogen use efficiency

Nitrogen significantly influenced the amount and quality of the grains. Improving nitrogen use efficiency (NUE) is fundamental for sustainable agriculture as excessive use of nitrogen fertilizer leads to environmental pollution and high production costs (Karunaratne et al., 2022). *HvARE1* is expressed mostly in leaves and shoots, with low expression in roots under low nitrogen conditions in *H. vulgare*. The Agrobacterium-mediated genetic transformation of immature embryos (cv. Golden Promise) with a single guide RNA targeting the *HvARE1* gene produced 22 T0 plants, and genotyping revealed four T1 lines harboring missense and/or frameshift mutations (Karunaratne et al., 2022).

Mutant *are1* lines exhibited an increase in plant height, tiller number, grain protein content, and yield as well as a 1.5–2.8-fold increase in total chlorophyll content in the flag leaf at the grain filling stage and delayed senescence by 10–14 days. Furthermore, *are1* mutants exhibited high nitrogen content in their shoots under low nitrogen conditions in barley. CRISPR/Cas9 gene editing and natural variation analysis showed that *HvARE1* enhances the nitrogen use efficiency in barley (Table 1) (Karunaratne et al., 2022). These outcomes demonstrated that commercial barley cultivars can be improved using CRISPR/Cas9-mediated gene editing.

3.4. Sorghum bicolor

Sorghum (*Sorghum bicolor* L. Moench) is one of the world's most cultivated cereal crops and the fifth most important cereal worldwide, with a multi-use for feed, food, forage, and fuel (Brant et al., 2021; Aregawi et al., 2022).

3.4.1. Improving nutritional quality

In sorghum seeds, kafirins account for 70% of the total protein and have a significant influence on sorghum protein quality and digestibility (Hamaker et al., 1995; Li et al., 2018). Kafirins also provided the kernel with poor protein quality because they lacked the essential amino acid lysine (Li et al., 2018). Kafirins are divided into α -, β -, γ -, and δ -subunits based on their molecular weight, solubility, and structure (Esen, 1987; Shull et al., 1991). α -kafirin alone comprises about 80% of the total kafirins among all kafirin subunits (Hamaker et al., 1995).

CRISPR/Cas9 gene editing approach was used to create variants with reduced kafirin levels and improved protein quality and digestibility. *S. bicolor* was transformed with the binary vector (pPZP211_zCas9_Kaf.sgRNA) mobilized into *A. tumefaciens* by triparental mating. The sequencing of kafirin PCR products revealed extensive edits in 25 of 26 events in one or multiple members of the *K1C* family. According to the molecular and biochemical analysis of the T1/T2 generation, CRISPR/Cas9 successfully generated kafirin variants with high Lys content and improved protein digestibility in sorghum (Table 1) (Li et al., 2018).

3.4.2. Improving yields

The development of laminar junctions between leaf blades and sheaths is facilitated by *liguleless1* (*LG1*). Each junction is composed of an auricle, the appendage at the base of a blade surrounding the sheath, and a ligule, the membranous outgrowth of the sheath. All these together make up the leaf inclination angle. The *lg1* mutation enhances the upright stature of leaves by preventing the formation of the auricle and ligule (Lee et al., 2007). Previous research in rice and maize revealed that *LG1* is responsible for the formation of the ligule and

auricle which govern the leaf inclination angle (Brant et al., 2021).

Particle bombardment of immature embryos along with a nptII selectable marker was used to deliver the genome editing reagents to sorghum that leads to regeneration of transgenic plants. CRISPR/Cas9-mediated knockout of *Ig1* displayed a visually distinct phenotype for monoallelic and biallelic edits in sorghum. The edited sorghum plants had similar vigor to the null event and the leaf inclination angle indicated that monoallelic *Ig1* knockout events had a moderate leaf inclination angle. Therefore, the CRISPR/Cas9-mediated targeted mutagenesis of *Ig1* alters leaf inclination angle, resulting in a rapidly scorable phenotype in sorghum (Table 1), and can act as a prospective target for sorghum crop improvement. In high-density plantings, altering the leaf angle can increase the yield (Brant et al., 2021).

3.4.3. Improving flavor

Aromatic compounds produced by plants are one of the major attraction factors between animals and plants (Xie et al., 2019). The genetic mutation of *BADH2* in some other plants leads to the production of fragrance. Cucumber fragrance is caused by a single amino acid change in *CsBADH* (Yundaeng et al., 2015). Similarly, a SNP in the conserved region of *GmBADH2* and a 2-bp deletion caused soybean fragrance (Juwattanasomran et al., 2011).

The two sgRNAs with target sequences were transcribed under the control of the OsU3 and OsU6a promoters and cloned into the CRISPR/Cas9 vector pYLCRISPR/Cas9_{P_{ubi}}-B. In immature embryos, Agrobacterium-mediated gene transformation was used to incorporate the vector into the sorghum inbred line wheatland (WT). CRISPR/Cas9-mediated knockout of *SbBADH2* generates *S. bicolor* lines with an extraordinary aromatic smell in both seeds and leaves (Table 1), and animal feeding experiments demonstrated that the fragrant sorghum leaves were attractive (Zhang et al., 2022). As a result, CRISPR/Cas9-mediated quality improvements can be applied to breeding and the development of enhanced varieties of sorghum.

3.5. *Setaria italica*

Foxtail millet (*Setaria italica*) is a significant food crop in Asia and Africa and the second-most widely grown species worldwide after pearl millet. Foxtail millet is a nutritious crop because it contains elevated levels of protein, vitamins, minerals, fiber, and various macro- and microelements compared to other common grains (Lu He et al., 2015; Abdullah et al., 2021). *Setaria italica* is very appealing as a model plant due to the possession of several distinct characteristics, such as short stature and life cycle, good seed production, self-compatibility, high photosynthetic efficiency, resistance to pests and diseases, true diploid nature ($2n = 18$), small genome size, and C4 attributes which can serve as a model for other C4 crops (Zhang et al., 2012; Saxena et al., 2018; Abdullah et al., 2021).

3.5.1. Improving grain yields

Male sterility is a prevalent biological phenomenon in plants, and it has been exploited to generate male-sterile lines, which are essential genetic resources for the development of commercial hybrid crop seeds. The use of heterosis in cereals is an effective strategy to ensure food security by increasing grain yields (Zhang et al., 2021).

A male sterility gene *SiNP1* was cloned using bulk segregation analysis and its function was confirmed through CRISPR/Cas9 genome editing in *S. italica*. A pYLCRISPR/Cas9-MH vector system was used to knock out *SiNP1*. The pCRISPR-SiNP1 plasmid was constructed and introduced into embryogenic calli of the wild type Ci846 through transformation mediated with *A. tumefaciens* strain EHA105 (Zhang et al., 2021).

The *SiNP1* protein was predominantly expressed in the panicle and localized to the endoplasmic reticulum (ER). The *sinp1* mutant showed differential expression of several genes, including proteins putatively involved in carbohydrate metabolism, fatty acid biosynthesis, and lipid

transport and metabolism. These genes are closely related to pollen wall development and play a partially conserved role in pollen development and diverse functions. The identification of *SiNP1* in foxtail millet provides further insight into the mechanism of pollen reproduction in plants and a candidate gene for heterosis utilization (Table 1) (Zhang et al., 2021).

3.5.2. Maintaining boron nutrition

Boron is an essential micronutrient for the growth and development of vascular plants, and adequate B nutrition is crucial for crop production (Camacho-Cristóbal et al., 2008). A pYLCRISPR-Cas9-MH vector was constructed using a target site in the second exon of *SiBOR1* and then transformed into *A. tumefaciens* strain EHA105 for Ci846 (an efficient transgenic genotype) transformation, using mature seeds as the explants to knock out *SiBOR1* gene in *S. italica* (Wang et al., 2022).

CRISPR/Cas9 system generated the knockout transgenic lines confirmed the function of *SiBOR1* in regulating panicle development in *S. italica*. The B content of panicle primary branches decreased because of the induced mutation in *SiBOR1*, and B deficiency-related phenotypes included thicker cell walls and enhanced cell porosity. A transcriptome analysis revealed that *sibor1* was enriched in genes involved in cell wall biogenesis, jasmonic acid synthesis, and programmed cell death response pathways. *SiBOR1* regulates panicle primary branch development to maintain grain yield in *S. italica* (Table 1) (Wang et al., 2022).

These findings demonstrate that the emergence and development of Crispr/Cas9 genome editing technology will provide new possibilities and encourage hope for relevant foxtail millet research.

3.6. *Panicum virgatum*

Panicum virgatum (switchgrass), a perennial C4 grass native to North America, has demonstrated high biomass yields and is well-adapted to marginal land not suitable for food crops (Mitchell et al., 2008; Narasimhamoorthy et al., 2008). Despite its outcrossing nature, switchgrass has a complex, allotetraploid genome ($2n = 4x = 36$), which limits its ability to generate homozygous knock-out plants (Park et al., 2017). The U.S. Department of Energy recognized switchgrass as the model species for herbaceous bioenergy crops in 1991 after more than a decade of research (Wright and Turhollow, 2010; Liu et al., 2018).

3.6.1. Maintaining plant strength

Lignin is a major cell wall component that contributes to cellulosic feedstock's resistance to decomposition and is a barrier to efficient biofuel production by limiting enzyme access to cell wall polymers during the fermentation process (Park et al., 2017). CRISPR/Cas9 genome editing system was developed in switchgrass to target a key enzyme involved in the early steps of monolignol biosynthesis, 4-Coumarate: coenzyme A ligase (4CL). The pRGEB32 carrying a 4CL spacer was transferred into the *A. tumefaciens* strain AGL1. Through Agrobacterium-mediated transformation, transgenic switchgrass plants were produced (Park et al., 2017).

In *Pv4CL1* knockout plants, the thickness of the cell wall was reduced, the total lignin content was reduced by 8–30%, glucose release increased by 7–11%, and xylose release increased by 23–32%. The CRISPR/Cas9 system specifically targeted the selected *Pv4CL1* gene and generated switchgrass knock-out mutant plants with decreased lignin content and reduced recalcitrance. These mutants showed a reduction in lignin and improved sugar release (Table 1) (Park et al., 2017).

3.6.2. Regulates plant biomass yields

High biomass yield is a high priority for switchgrass breeding and tiller number is positively correlated with biomass yields (Das et al., 2004; Boe, 2007; Boe and Beck, 2008; Okada et al., 2010; Casler, 2010; Lu et al., 2013). A transcription factor gene known as *teosinte branched 1* (*tb1*) regulates tillering and branching by integrating environmental and developmental factors, making it an essential part of the

tillering/branching process (Doebley et al., 1997; Whipple et al., 2011; Seale et al., 2017). Switchgrass *tb1* mutant plants were generated using CRISPR/Cas9 but the allelic composition of *Pvtb1* genes in these T0 mutant plants was not fully characterized. Additionally, these primary mutants were chimeric, which prevented an accurate assessment of *tb1* function (Liu et al., 2018).

Therefore, the functional characteristics of the teosinte branched 1 (*tb1*) gene using CRISPR/Cas9 in *P. virgatum* was evaluated by Liu et al. (2020). To obtain primary mutant plants, CRISPR/Cas9 construct comprising two gRNAs targeting the conserved regions of the *Pvtb1a* and *Pvtb1b* genes was introduced into the switchgrass genome through the Agrobacterium-mediated transformation of caryopsis-derived embryogenic callus. They successfully generated nonchimeric mutants from chimeric primary mutants induced by CRISPR/Cas9 using micro-propagation, eliminating the need of obtaining progeny mutants for gene function analysis (Liu et al., 2020).

Furthermore, transgene-free progeny mutants were generated that preserved the phenotypic effects of *Pvtb1* mutations like those seen in T0 mutants. In *P. virgatum*, double biallelic mutants for *Pvtb1a* and *Pvtb1b* genes enhanced tiller production and increased biomass yield, indicating that *Pvtb1* genes negatively regulate tillering in switchgrass (Table 1). CRISPR/Cas9-directed mutagenesis of *Pvtb1* knockdown mutant 52-1 and a wild-type (WT) plant WT-1 suggested that *Pvtb1* genes regulate tillering through multiple pathways. Furthermore, Cas9/gRNA-induced mutations in the progeny of T1 mutants showed that the mutations could be transmitted from generation to generation in *P. virgatum* (Liu et al., 2020).

These findings revealed that multiplex CRISPR/Cas9 platform can edit multiple genes simultaneously, which is beneficial for polyploid plants like switchgrass.

4. Conclusion and future perspectives

Genome editing using CRISPR/Cas9 is a groundbreaking technology with many advantages, including ease of manipulation, high efficiency, and wide applications that can improve the yields, nutrition value, disease resistance, and other properties of forage crops. CRISPR/Cas9 technology evidence of essential functions in genome editing opens many new experimental avenues for gene function analysis and offers significant potential for various plant research projects. CRISPR/Cas9 can now be used to target multiple genes, enabling the improvement of multiple traits in forage crops such as yield, nutritional value, and abiotic stress, and can be used to generate knockout lines for selected genes. This technology has the potential to be applied to both model plants and forage crops for functional genetics. Furthermore, CRISPR/Cas9 can enhance forage crop yields by selecting desirable traits. Despite the possibility of using CRISPR/Cas9 in plant genome editing, there are challenges to overcome, such as reducing off-target rates, finding mechanisms to minimize them, and optimizing their use. Another challenge lies in the use of delivery methods and knock-in/replacement-based precise editing in target plants. In forage crops, research on CRISPR/Cas9 technology has been scanty, and there are ethical concerns that need to be addressed. In addition, CRISPR/Cas9 is still inefficient at inducing mutations in forage crops. Several genes associated with higher yields as well as the nutritional quality of grains have been identified in major cereals. Therefore, their homologs can be detected in forage crops. CRISPR/Cas9 technology can be used to exploit these genes to improve forage. CRISPR/Cas9 mediated gene editing functions must be further studied in forage crops to improve its application in the future, so that its basic and applied capabilities can be further developed.

Author contributions

S.I.U.H. wrote the manuscript. Q.S.Q. revised the manuscript. D.Z., N.F., X.J., F.Q. and J.S.H. commented on the manuscript. All authors read the manuscript and approved its contents.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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