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REVIEW

CRISPR/Cas systems: opportunities and challenges for crop breeding

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Abstract

Increasing crop production to meet the demands of a growing population depends largely on crop improvement through new plant-breeding techniques (NPBT) such as genome editing. CRISPR/Cas systems are NPBTs that enable efficient target-specific gene editing in crops, which is supposed to accelerate crop breeding in a way that is different from genetically modified (GM) technology. Herein, we review the applications of CRISPR/Cas systems in crop breeding focusing on crop domestication, heterosis, haploid induction, and synthetic biology, and summarize the screening methods of CRISPR/Cas-induced mutations in crops. We highlight the importance of molecular characterization of CRISPR/Cas-edited crops, and pay special attentions to emerging highly specific genome-editing tools such as base editors and prime editors. We also discuss future improvements of CRISPR/Cas systems for crop improvement.

Keywords Base editing · CRISPR/Cas9 · Crop breeding · Genome editing · Molecular characterization · Prime editing

Introduction

Feeding a growing population in a sustainable way is a great challenge to current crop-breeding efforts (Schaart et al. 2016). Traditional breeding technology based on crossing and selection without any knowledge about genetics, even aided by marker-assisted selection, is a very labor-intensive and time-consuming process, which also shows drawbacks with complex genetic outputs (Schaart et al. 2016). Mutation breeding technology based on chemical and physical genotoxins dramatically increases the mutation rate above natural levels; however, artificially induced changes are unquestionably uncontrolled, requiring complex and expensive screening and selection procedures (Pacher and Puchta 2017). Due to the random modification in the crop genome, outcomes of both natural mutation and mutational breeding are unpredictable. The transgenic breeding technique transfers desired trait-coding genes via an exogenous T-DNA cassette into the elite cultivars; its outcome is relatively predictable. However, time and expenses for research and development of a genetically modified (GM) crop with desirable traits free of unexpected insertions are huge to meet demands from safety regulations and social acceptance (Araki and Ishii 2015; Schaeffer and Nakata 2015).

In contrast, site-directed nucleases (SDNs)-based genome-editing technologies increase significantly the precision of gene modification in crop systems (Jaganathan et al. 2018). SDNs include zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats associated protein 9 (CRISPR/Cas9) (Lusser et al. 2012; Zhu et al. 2017). They precisely cut the genomic DNA at the targeted loci to generate double-strand breaks (DSBs), which triggers specialized repair pathways, either homologous recombination (HR) or non-homologous end-joining (NHEJ), and results in indel mutations (Jinek et al. 2012). Besides, simultaneous introduction of several DSBs by genome editing allows to break genetic linkages, to reshuffle entire chromosome orientations, to create inversions, and to permit reciprocal chromosomal translocations or chromosome fragment exchanges (Pacher and Puchta 2017). Thus, SDN-based genome editing holds a great potential for precise crop improvement.

Each SDN has its particular characteristics. ZFNs are artificially engineered chimeric restriction enzymes composed of site-specific DNA binding zinc finger proteins

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(ZFPs) fused with the non-specific DNA cleavage domain of the *Fok*I restriction enzyme (Guo et al. 2010). Typically, ZFNs consist of three-four lower-affinity ZFPs and two tail-to-tail ZFP-binding sites separated by a 5- to 7-bp spacer sequence, therefore, not all crop sequences can be efficiently targeted (Li et al. 2019b). ZFNs have been reported to induce efficient site-directed mutagenesis in several crop species, including maize (Ainley et al. 2013) and soybean (Curtin et al. 2011). However, due to limitations such as low target specificity, time-consuming, and narrow available target sites (Chen and Gao 2013), ZFNs have given way to other SDNs. TALENs have similar principles to ZFNs but harbor different site-specific DNA binding proteins, named transcription activator-like effectors (TALEs). Each effector domain recognizes a single nucleotide pair in TALEN, therefore, compared with ZFNs, TALENs show higher target specificity (Baltes and Voytas 2015). TALENs have been successfully applied to edit several crop species including tobacco (Zhang et al. 2013), rice (Li et al. 2012), and maize (Liang et al. 2014). Despite its advantages over ZFNs, using of TALENs as genome-editing tools still needs the assembly of complex tandem repeats to bind targeted DNA sequences. In addition, large size and tedious nature make the transfer of the TALEN system to plant cell a challenge (Baltes and Voytas 2015).

CRISPR/Cas9 is a RNA-guided nuclease, and its specificity is single-guide RNA (sgRNA) dependent. Theoretically, CRISPR/Cas9 can bind to any DNA sequence that contains a protospacer adjacent motif (PAM) site when sgRNA is present for identifying the target (Jinek et al. 2012). Unlike protein-guided nucleases such as ZFNs and TALENs, CRISPR/Cas9 introduces a blunt DSB, in the case of SpCas9, the cleavage occurs at a site three nucleotides upstream of the PAM (Jinek et al. 2012). Due to its high target specificity, simplicity, ease of use, CRISPR/Cas9 technique has been widely used as a dominant technique of SDNs for gene editing in crops, humans, and animals (Doudna and Charpentier 2014). In crops, CRISPR/Cas9-induced DSBs are repaired mainly via NHEJ; as a result, indel genetic variations are generated (Zhu et al. 2017). So far, CRISPR/Cas9 system has been widely used for crop improvement in rice, sorghum, wheat, maize, soybean, tomato, potato, apple, and banana (Osakabe and Osakabe 2015; Jaganathan et al. 2018; Tripathi et al. 2020). With advancements in the improvement of CRISPR/Cas systems (Anzalone et al. 2019), such as CRISPR/Cpf1 (Chen et al. 2019; Kim et al. 2017) and nucleotide substitutions tools (Shimatani et al. 2017; Zong et al. 2017), CRISPR systems are becoming to be more efficient for crop improvement (Gao 2021; Hong et al, 2021; Miladinovic et al. 2021). Its applications in crops range from boosting yield, resisting against pests and diseases (Wang et al. 2014), to improving nutritive value (Table 1) (Li et al. 2016, 2018b; Ma et al. 2016; Do et al. 2019; Dong et al. 2020). The objective of this review is to summarize the current developments and applications of CRISPR/Cas systems in crop improvement, discuss the regulatory landscape of genome-edited crops, and to propose future prospects.

Current applications of CRISPR/Cas systems in crop breeding

Providing the world with diverse, abundant, nutritive plants and plant-derived products in a sustainable manner cannot be achieved without better understanding of plant biology under both normal and stressful conditions. CRISPR/Cas-mediated genome editing not only revolutionizes crop biology but also providing means for crop improvement (Chen et al. 2019; Kumar et al. 2020a; El-Mounadi et al. 2020). Current applications of CRISPR/Cas systems in crop improvement regarding yield, quality, biotic and abiotic stress tolerance, and herbicide resistance are summarized in Table 1, albeit the fact that increasing excellent reviews are emerging (Gao 2021; Miladinovic et al. 2021). Outputs of CRISPR/Cas9-edited crops, whether targeting a single gene or multiple genes, include mainly small indels or single nucleotide base substitutions; however, high frequency of large deletions and/or reorganizations are also reported (Zhu et al. 2017; Li et al. 2019b; Biswas et al. 2020a). Nevertheless, all mutations can pass faithfully to subsequent generations without any novel modifications (Zhu et al. 2017; Biswas et al. 2020a). Thus, CRISPR/Cas9 finds its way with high potential to be widely adopted for crop breeding (Fig. 1), because it is blurring the boundaries in the GM regulations (Araki and Ishii 2015).

Crop domestication

Compared with their wild ancestors, currently cultivated crops have reduced genetic diversity and resilience to biotic and abiotic stresses (Doebley et al. 2006). On the other hand, compared with cultivated crops, wild crops and/or orphan crops could harbor desired high yield/nutrition traits and favorable resilience that could readily to adapt to changing climates. Therefore, domestication of wild species/orphan crops could be a fascinating way to secure food supply. Conventional domestication is a time-consuming and laborious process (Fernie and Yan 2019; Yu et al. 2021), and the CRISPR/Cas9 system, with its precise, accurate and multiplex genome modification capacity, could accelerate the process of crop domestication. In tomato, targeting six genes of agronomic importance

Table 1 Applications of CRISPR/Cas systems for major crop improvement (since 2019)

Crop species	Purpose	Target gene	Technology	Mutation type	Mutation position	Factors affect	References
Rice	Improving yield	*OsLOLG5*	CRISPR/Cas9 s	Base deletion	Exon: frame shift mutation	Increased grain numbers and weight (1000 seed weight)	Wang et al. (2020b)
		OsPIN5b	CRISPR/Cas9	Base deletion	Exon 1: frame shift mutation	Increased tiller numbers and longer panicles	Zeng et al. (2019)
		OsGS3, *OsGW2*, *OsGN1a*	CRISPR/Cas9	Base deletion	Exon 2, Exon 1, and Exon 4: frame shift mutation	Increased grain length, width and numbers (1000 seed weight)	Zhou et al. (2019)
	Improving quality	*OsAAP10*	CRISPR/Cas9	Base insertion/deletion	Exon 1; frame shift mutation	Improve eating and cooking quality (ECQ)	Wang et al. (2020a)
		Wx	BE	Base substitution	Exon 3; missense mutation	Low amylose content	Xu et al. (2020)
		OsGBSS1	CRISPR/Cas9	Base insertion/deletion	Promoter and 5´UTR	Low amylose content	Zeng et al. (2020)
		Waxy	BE	Base substitution	Exon 5; missense mutation	Low amylose content	Li et al. (2020d)
		SSU-crtl and *ZmPsy*	CRISPR/Cas9	Base insertion	Genomic safe harbors (GSHs) region	Enriched carotenoid	Dong et al. (2020)
		OsPLDα1	CRISPR/Cas9	Base deletion	Exon; truncated mutation	Reduced phytic acid	Khan et al. (2019a)
	Biotic stress resistance	*SWEET11*,*13*and *14*	CRISPR/Cas9	Base insertion/deletion	Promoter region	Bacterial blight resistance	Xu et al. (2019)
	Abiotic stress resistance	*OsSRL1*, *OsSRL2*	CRISPR/Cas9	Base deletion	Exon; frame shift mutation	Drought tolerance	Liao et al. (2019)
		OsRR22	CRISPR/Cas9	Base insertion	Exon 1; frame shift mutation	Salinity tolerance	Zhang et al. (2019a)
		OsDST	CRISPR/Cas9	Base deletion	Exon; frame shift mutation	Drought and salinity tolerance	Kumar et al. (2020b)
	Herbicide tolerance	*OsALS1*	BE	Base substitution	Exon; missense mutation	Herbicide bispyribac-sodium tolerance	Kuang et al. (2020)
		OsALS1	CRISPR/Cas9	Base substitution	Exon; missense mutation	Herbicide bispyribac-sodium tolerance	Ali et al. (2020)
		OsACCase	BE	Base substitution	Exon; missense mutation	Herbicide haloxyfop tolerance	Li et al. (2020c)

Table 1 (continued)

Crop species	Purpose	Target gene	Technology	Mutation type	Mutation position	Factors affect	References
Rice	Herbicide tolerance	*OsACCase*	BE	Base substitution	Exon; missense mutation	Herbicide haloxyfop-R-methyl tolerance	Liu et al. (2020a)
		OsSF3B1	CRISPR/Cas9	Base deletion and substitution	Exon; frame shift mutation and missense mutation	Herbicide GEX1A tolerance	Butt et al. (2019)
		OsTubA2	BE	Base substitution	Exon; missense mutation	Herbicide trifluralin and pendimethalin tolerance	Liu et al. (2020b)
Wheat	Improving yield	*TaCKX2*	CRISPR/Cas9	Base deletion	Exon 1; frame shift mutation	Increased grain numbers	Zhang et al. (2019b)
		TaGW2	CRISPR/Cas9	Base insertion/deletion	Exon 8; frame shift mutation	Improved seed weight	Zhang et al. (2018b)
	Improving quality	*α–gliadin*	CRISPR/Cas9	Base deletion	Exon; frame shift mutation	Low-gluten content	Sánchez-León et al. (2018)
	Biotic stress resistance	*TaNFXL1*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Resistance to *Fusarium graminearum*	Brauer et al. (2020)
	Abiotic stress resistance	*TaDREB2*, *TaERF3*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Drought tolerance	Kim et al. (2018)
	Herbicide tolerance	*EPSPS*	CRISPR/Cas9	Base insertion/deletion	Exon 2; frame shift mutation	Herbicide glyphosate tolerance	Arndell et al. (2019)
Barley	Improving yield	*HvCKX1*	CRISPR/Cas9	Base insertion/deletion	Exon 1; frame shift mutation	Increased tiller and grain numbers	Holubova et al. (2018)
	Improving quality	*HvGBSSIa*	CRISPR/Cas9	Base insertion/deletion	Exon 6; frame shift mutation	Low amylose content	Zhong et al. (2019b)
		D-hordein	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Increased glutenins content	Yang et al. (2020)
	Biotic stress resistance	*HvMORC1*	CRISPR/Cas9	Base insertion/deletion	Promoter	Resistance to *Fusarium graminearum*	Kumar et al. (2018)
		WDV genome	CRISPR/Cas9	–	–	Resistance to *WDV*	Kis et al. (2019)
Maize	Improving yield	*ZmPHYC1*, *ZmPHYC2*	CRISPR/Cas9	Base insertions/deletions	Exon 1; frame shift mutation	Improved early flowering	Li et al. (2020b)
	Improving quality	*GBSS1*	CRISPR/Cas9	Base deletion	Promoter region and 3´ UTR	Waxy corn	Gao et al. (2020)
Crop species	Purpose	Target gene	Technology	Mutation type	Mutation position	Factors affect	References
Maize	Abiotic stress resistance	*ZmHKT1*	CRISPR/Cas9	Base deletion	Exon 1; frame shift mutation	Salinity tolerance	Zhang et al. (2018a)

Table 1 (continued)

Crop species	Purpose	Target gene	Technology	Mutation type	Mutation position	Factors affect	References
Tomato	Improving yield	*SlENO*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Increased in fruit locule number and fruit size	Yuste-Lisbona et al. (2020)
	Improving quality	*SlSGR1*, *SlBlc*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Increased lycopene content	Li et al. (2018b)
	Biotic stress resistance	*SlJAZ2*	CRISPR/Cas9	Base deletion	Exon; frame shift mutation	Bacterial speck resistance	Ortigosa et al. (2019)
		eIF4E1	CRISPR/Cas9	Base deletion	Exon 1; frame shift mutation	Enhanced resistance to *Pepper mottle virus*	Yoon et al. (2020)
	Abiotic stress resistance	*SlNPR1*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Drought tolerance	Li et al. (2019a)
		SlHKT1;2	CRISPR/Cpf1	Base substitution	Exon 1; missense mutation	Salinity tolerance	Vu et al. (2020)
	Herbicide tolerance	*ALS*	BE	Base substitution	Exon; missense mutation	Herbicide chlorsulfuron tolerance	Veillet et al. (2019a)
Potato	Improving quality	*StGBSSIa*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Low amylose content	Veillet et al. (2019b)
		StSBE1, *StSBE2*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	High amylose content	Tuncel et al. (2019)
	Biotic stress resistance	*PVY*	CRISPR/Cas13a	Knockdown	Conserved coding region	Resistance to *Potato virus Y*	Zhan et al. (2019)
Soybean	Improving quality	*GmFAD2*	CRISPR/Cas9	Base deletion	Exon 2; frame shift mutation	High oleic acid contents	Do et al. (2019)
	Abiotic stress resistance	*Drb2a*, *Drb2b*	CRISPR/Cas9	Base deletion	Exon; frame shift mutation	Drought and salinity tolerance	Curtin et al. (2018)
Oilseed rape	Improving quality	*BnITPK*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Decreased phytic acid content	Sashidhar et al. (2020)
		BnTT8	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Improved seed oil and protein content	Zhai et al. (2020)
		BnSFAR4,5	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Improved seed oil content	Karunarathna et al. (2020)
Crop species	Purpose	Target gene	Technology	Mutation type	Mutation position	Factors affect	References
Oilseed rape	Biotic stress resistance	*BnCRT1a*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Resistance to *Verticillium longisporum*	Pröbsting et al. (2020)
Cassava	Biotic stress resistance	*nCBP-1*, *nCBP-2*	CRISPR/Cas9	Base insertion/deletion	Exon; frame shift mutation	Decreased virus load and symptom	Gomez et al. (2019)
Watermelon	Biotic stress resistance	*Clpsk1*	CRISPR/Cas9	Base insertion/deletion	Exon 1; frame shift mutation	Resistance to *Fusarium oxysporum* f.sp. niveum (fon)	Zhang et al. (2020b)
	Herbicide tolerance	*ALS*	BE	Base substitutions	Exon; missense mutation	Herbicide tribenuron tolerance	Tian et al. (2018)

BE base editor

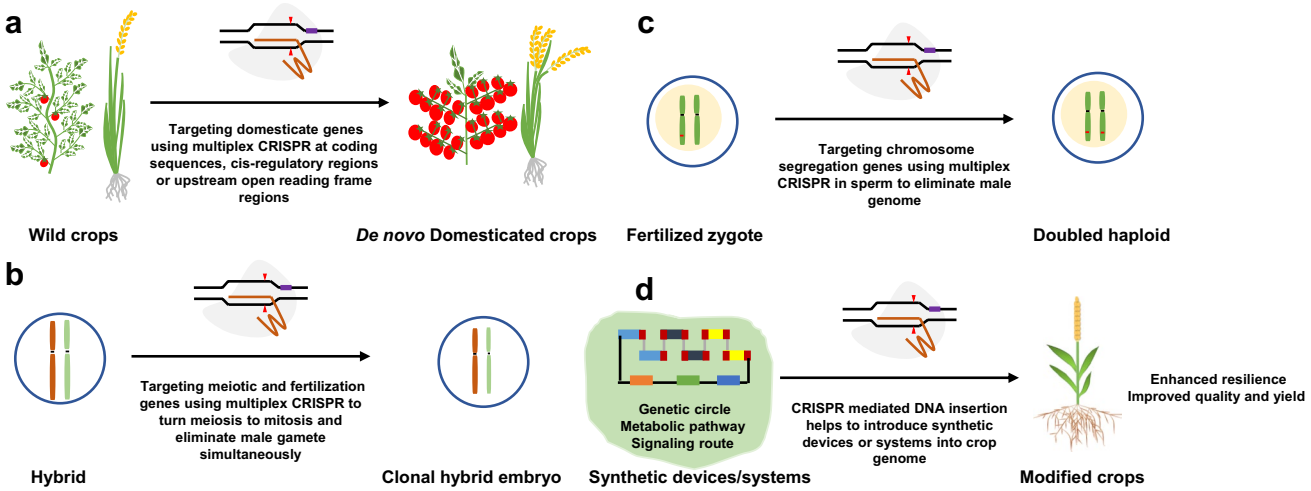

Fig. 1 Principles and methods of CRISPR systems in crops improvement. **a** Crop domestication. **b** Heterosis. **c** Haploid induction. **d** Synthetic biology

present in cultivated tomato by CRISPR/Cas9 system resulted in successful de novo domestication of wild tomato *Solanum pimpinellifolium*, fruit size and fruit numbers in edited wild tomato were significantly increased and lycopene accumulation in edited was remarkably enhanced (Zsögön et al. 2018). Taking advantage of multiple targeting capacity of CRISPR/Cas9 (targeting *SP5G*, *SP*, *SlCLV*3, and *SlGGP1*), several desirable traits were created into four stress-tolerant wild tomatoes; Cas9-free CRISPR/Cas9-edited tomato plants showed domesticated desirable traits (early harvest, determinate shoot architecture, large fruit size, and improved nutritional benefits) while maintained bacterial spot disease and salt tolerance (Li et al. 2018a). Targeting known domestication loci in African landrace Kabre with superior endemic pest resistance and drought and nutrient deficiency tolerance resulted in the reduction of plant stature (targeting *HTD1*) and increase of yields (targeting *GS3*, *GW2* and *GN1A*) in rice (Lacchini et al. 2020). Targeting two domestication-related genes (*qSH1* for shattering and *An-1* for awn length) and two agronomic trait associated genes (*SD1* for height and *GS3* for grain length) through CRISPR/Cas9 demonstrated successful rapid domestication of wild rice polyploidy rice 1 (PPR1), a wild allotetraploid rice *Oryza alta* (CCDD) (Yu et al., 2021). Similarly, simultaneously targeting *Ghd7 and DTH7* using a multiplex CRISPR/Cas9 editing approach in PPR1 significantly altered their heading date (Yu et al. 2021). Those pioneer studies not only proved the concept but also paved the way to utilize the genetic diversity hidden in wild crops for molecular based breeding to achieve rapid de novo domestication of wild crops into staple foods.

In addition, using one-step CRISPR/Cas9 targeting regulator genes of stem length (*SlER*), rapid flowering (*SP5G*) and precocious growth termination (*SP*), vine-like tomato was reconstructed to compact and early yielding plants suitable for urban agriculture (Kwon et al. 2020). This could be a novel application direction of CRISPR/Cas9 for horticultural crop research and development.

Heterosis

Heterosis (hybrid vigor) is the genetic phenomenon in which hybrid offsprings display better performance (in yields/nutrition; stress tolerance, or adaptability) than their parents (Birchler et al. 2010). Heterosis has been widely utilized in modern agriculture, contributing remarkably to food supply worldwide (Schnable and Springer 2013). Heterosis can be maintained only in F1 generation. Farmers have to buy hybrid seeds every year; on the other hand, producing hybrid seeds is time-consuming, laborious, and costly. Thus, fixing desirable hybrid traits is the most challenging neck bottle for using heterosis in crop breeding.

CRISPR/Cas systems show particular promising applications in this area. If the heterozygosity of F1 hybrid can be fixed while haploid seeds can be produced, heterosis can be maintained via self-propagation through such seeds. Genome editing of three meiotic genes (*REC8*, *PAIR1* and *OSD1*) using multiplex CRISPR–Cas9 system produced clonal diploid gametes and tetraploid seeds while editing a fertilization gene (*MATRILINEAL*, *MTL*) produced haploid seeds in hybrid rice. Thus, simultaneously editing of all four genes (*REC8*, *PAIR1*, *OSD1*, and

MTL) in hybrid rice through CRISPR–Cas9 fixed favorite F1 traits (Wang et al. 2019). Overexpressing of *BBM1* (a sperm cell-specific expressed AP2 transcription factor) in egg cells in a triple knockout mutant simultaneously targeting three meiotic genes (*REC8*, *PAIR1* and *OSD1*) has successfully replaced meiosis by mitosis, which resulted in asexual propagation of hybrid rice through seeds (Khanday et al. 2019). These studies demonstrated the feasibility to maintain hybrid rice clonally through seed propagation with the help of CRISPR/Cas systems.

Haploid induction

Haploid induction (HI), the first step of doubled haploid technology, aims to regenerate haploid or spontaneous doubled haploid plants based on intraspecific crossing. Traditional HI, depending on species, is achieved by several approaches, such as androgenesis, gynogenesis or parthenogenesis. It takes time, needs substantial personnel and equipment, and always has unavoidable variability in efficiency (Hooghvorst and Nogués 2020). Targeting genes involved in natural fertilization of female gametic cells using CRISPR/Cas9 impeded fertilization and resulted in haploid embryos generated through egg cells (Hooghvorst and Nogués 20200). CRISPR–Cas9-based HI systems avoided wide ranging adaptation of protocols to different genotypes. Currently, genes involved in chromosome segregation (*MATL*, *CENH3*, and *DMP*) are well-known targets for HI in both monocots and dicots. Targeting *MALT* by CRISPR/Cas9 in wheat obtained 18.9% haploid progeny (Liu et al. 2019a; b). Similarly, targeting *DMP* in maize by CRISPR/Cas9 achieved maternal haploids with the efficacy of 0.1–0.3% (Zhong et al. 2019a, b). To obtain doubled haploid homozygous lines, traditional HI needs six to eight generations, genome-editing mediated HI needs only 1 year (Hooghvorst and Nogués 2020), thus, CRISPR/Cas9 accelerates crop breeding via haploid induction.

Another HI-editing technology (HI-Edit) has been developed to directly edit elite inbred lines of diverse monocot and dicot species by a single cross. In *MALT*-based maternal HI system, the cross between CRISPR/Cas9-edited sperm cells and elite line egg cells leads to successful fertilization off egg cells and edited elite doubled haploid whose chromosomes are exclusively derived from the female parent. In *CENH3*-based paternal HI system, the cross between elite line (pollen donor) with CRISPR/Cas9-edited line results in female genome elimination and doubled haploid (Kelliher et al. 2019). Compared with existed HI systems, HI-Edit avoids the delay and high cost of introgression due to its faster and more effective delivery of edits to advanced breeding materials. HI-Edit provides transgene-free edited inbred lines lacking haploid-inducer parental DNA and the editing machinery as well (Kelliher et al. 2019).

Synthetic biology

Plant synthetic biology integrates engineering principles with biology to design and produce novel biological devices or systems (Wurtzel et al. 2019; Tian et al. 2020). Genome-editing technology could be used for targeted metabolic engineering to produce desirable products through direct knocking out or overexpressing of specific genes, or through the introduction of a combination of existing enzymes. Actually, genome-editing technology could play essential roles in plant synthetic biology to introduce new reactions/pathways that are not present in nature through de novo design, and to renovate endogenous signaling pathways (Tian et al. 2020; Zhang et al. 2020a). Currently, several redesigned/or synthesized novel biological devices or systems have been reported. For example, the rubisco subunits with RAF1 to enhance photosynthesis in maize (Salesse-Smith et al, 2018), a synthetic CETCH cycle constituting a reaction network of 17 enzymes from 9 different organisms of all 3 domains of life to continuously fix CO_2 (Schwander et al. 2016), a synthetic glycolate metabolic pathway to increase C_3 crop yield (South et al. 2019). However, none of them was introduced into plant systems using genome-editing approach. One reason is that CRISP/Cas system has been almost exclusively employed for gene knocking out and deletion but not for gene insertion. A recent study successfully re-oriented a 75.5-Mb-long targeted chromosomal region in maize using CRISPR/Cas9 approach (Schwartz et al. 2020), showing a great potential for the application of CRISPR/Cas9 in synthetic biology for chromosomal engineering to introduce large synthetic device or systems to plants, particularly in the case of overexpression of multiple stacking traits.

Molecular characteristics of genome-edited crops

The outcomes of CRISPR/Cas9 system in crops are affected by various factors, including Cas9 activity, gRNA expression, transformation procedure, callus culture time, and gRNA protospacer sequence (Mikami et al. 2015; Doench et al. 2016; Zhu et al. 2017). While most of the studies focus mainly on transient or early stable transformants (Feng et al. 2014; Wang et al. 2014; Zhang et al. 2014; Zhou et al. 2014; Zhu et al. 2017), very few pay attention to molecular characteristics in the consecutive generations. So far, mutation patterns and inheritability have been investigated mainly in *Arabidopsis* and rice (Feng et al. 2014; Zhang et al. 2014; Čermák et al. 2015). In *Arabidopsis*, a small incidence of homozygous mutation can be identified in the T_1 generation, and rarely off-target mutation is reported (Fauser et al. 2014; Feng et al. 2014; Ma et al. 2015). On the other hand, in rice, homozygous and biallelic mutations appear even in T_0 plants

(Ma et al. 2015), and pass stably to next generations following laws of Mendel inheritance (Zhang et al. 2014).

The isolating transformants and identifying expected mutations are not the only task for molecular characterization of genome-edited crops, particularly for breeding purposes. Generally, exogenous T-DNA elements and Cas9 could be eliminated as early as in T_1 generation (Xu et al. 2015; Zhou et al., 2014). However, if they are not eliminated intentionally at earlier stages, the presence of them in T_2 generation is still high (Biswas et al. 2020a). The presence of exogenous element Cas9 in genome-edited plants could make new mutations in every subsequent generation, making it difficult for mutations transmission analysis and hampering inherit stability. Moreover, it may potentially cause off-target mutations. On the other hand, the presence of other exogenous elements rather than Cas9 in the genome-edited plants could be facing regulatory issues, making it difficult for moving forward to commercialization, because transgene-free is a prerequisite for regulatory approval of commercial utilization of genome-edited plants (He et al. 2018). There are close connections between the presence of exogenous elements and the presence of Cas9 (Biswas et al. 2020a), therefore, screening for the absence of Cas9 in T_1 could help to eliminate the exogenous elements. Nevertheless, the screening for Cas9 cannot replace the screen for other exogenous elements in T_1 or T_2 generation (Biswas et al. 2020a). A potential commercialized product could be heritable, exogenous genetic elements-free, target-modified, and with expected traits that can be influenced by various factors that need to be characterized accordingly (Fig. 2).

Methods for the screening of CRISPR/Cas9-induced mutations in crops

The development of efficient, reliable, and inexpensive methods to effective screening for on-target and off-target genome-edited mutations from a pool of mutants in the early stages helps to speed up further basic and applied studies. To date, many different methods for the screening of genome editing-induced, specifically CRISPR/Cas9-induced indels, have been developed and applied in crops (Table 2).

These methods mentioned in Table 2 are generally PCR based, which are reported to be effective under certain circumstance, and thus, each has its own intrinsic limitations. For example, all of them can reveal mutated genotypes (insertion, deletion or substitution) but cannot reveal the exact nucleotide changes (which nucleotide is inserted, deleted or substituted) without Sanger sequencing. In contrast, many methods based on targeted deep sequencing, such as AGEseq, Cas-Analyzer, CRISPR-GA, CRISPResso and Hi-TOM, have been developed to identify simultaneously mutated genotypes and exact nucleotide changes with high accuracy and sensitivity (see review in Liu et al. 2019a,

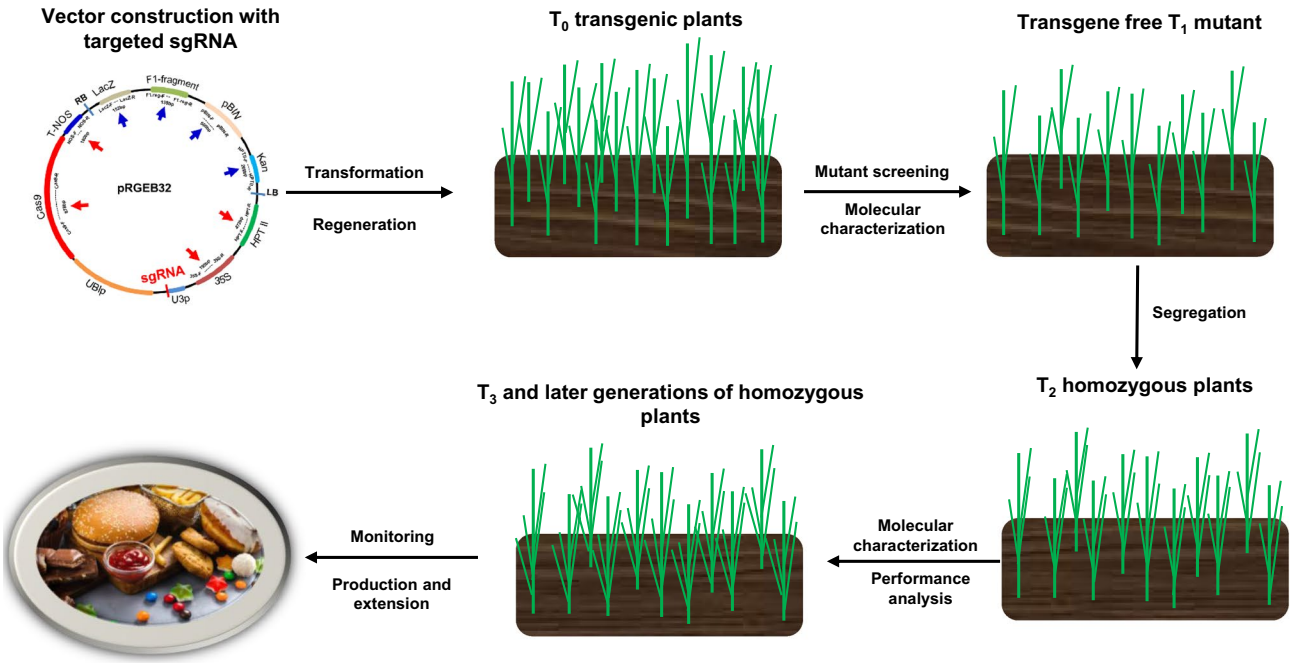

Fig. 2 Molecular characterization of genome-edited crops along the process from the lab to the field

Table 2 Screening methods for CRISPR system-edited mutations in crops

Methods	Principle or procedure	Mutant plant/crops used	Pros	Cons	References
PCR-based	3´ end of the PCR primer is critical to amplifying due to highly complementary. Any mismatches in the 3´ end will affect PCR amplification efficiency. Based on this hypothesis, one pair of primer (mutation hot spot-specific inner primer) and other primer pairs (outside of the mutation hot spot) are designed in this method. Any base mismatches cannot amplify via the inner primer, while the outer primer amplifies both WT and mutant alleles	Rice	Simple, fast, sensitive, and efficiently identify indels down to ± 1 bp	Only useful close to/upstream of the PAM	Biswas et al. (2019)
qPCR	The mutation positions induced by Cas9 nuclease are approximately 3 bp upstream of the PAM. Considering this hypothesis, two differently labeled probes: one positioned outside of the mutation site, and another one located predicted mutation region. Thus, newly arising mutation avert the binding of the second probe, while amplification will not affect the first probe	*Arabidopsis*, rice, sorghum, maize	Simple, sensitive, high-throughput compatible	Expensive, unable to identify large indels	Peng et al. (2018)
EMC	The EMC assay involves denaturing and renaturing the PCR (mixture of WT and mutant locus) products to form heteroduplexes, followed by digesting with an enzyme (T7E1 or Surveyor nuclease) and then gel electrophoresis analysis	Rice, wheat, tobacco	Rapid, sensitive, and cheap	Cannot identify single base replacement, low specificity	Nekrasov et al. (2013); Shan et al. (2014)
MSBSP-PCR	MSBSP-PCR uses Locus-Primer-F and Locus-Primer-R for the first round of PCR. In the second-round PCR, primarily amplified products were used as templates using Target-Primer (mutation site-specific) and Locus-Primer-R. Thus, WT and HE can be amplified in the second-round PCR, while HM can not	*Arabidopsis*, tobacco	Simple, rapid and cheap	Only useful if mutations in close/upstream of the PAM	Guo et al. (2018)

Table 2 (continued)

Methods	Principle or procedure	Mutant plant/crops used	Pros	Cons	References
ACT-PCR	An optimum annealing temperature in conventional PCR restrains mismatched annealing of primer-template, resulting in no amplification	Rice	Inexpensive, rapid, sensitive, and useful to detect HM mutants	Cannot detect HE mutants, cannot identify single base replacement	Hua et al. (2017)
HRMA	HRMA involves the targeted genomic DNA (up to about 200 bp) amplification by qPCR with the fluorescent dye incorporation, followed by amplicon melting curve investigation	*Arabidopsis*	Fast, sensitive and high-throughput compatible	Expensive, unable to identify large indels	Wang et al. (2015)
Amplicon labeling-based	Three primers, including target allele specific-F primer and R primer, additionally 6-FAM (6-carboxyfluorescein)-labeled allele- specific forward (FamF) primer, were used. Fluorophore-labeled amplicon with insertion or deletion will be longer or shorter than WT	Rice	Fast, sensitive, high-throughput compatible	Cannot identify single base replacement	Biswas et al. (2019)
qPCR-HRM	qPCR-HRM includes two parts; one qPCR procedure and second HRM analysis, and could be finished in a single step	Rice	Useful to identify small indels (down to ± 1 bp)	Cannot identify single base replacement, requires special instruments	Li et al. (2020a)
MLPA	The MLPA-based method includes paired half-probes, located adjoining to the predicted mutated region (targeted site). Therefore, any mismatches at the putative mutation site would impair the paired half-probes' ligation and following probe amplification, and thus, mutations can be detected via capillary electrophoresis	Rice	Highly efficient to identify indels down to ± 1 bp, and single base replacement	Cannot cover whole genome analysis	Biswas et al. (2020b)

b). Obviously, as compared with targeted deep sequencing-based methods, PCR-based methods are cheaper and more suitable for screening purpose. Combined with Sanger sequencing, these abovementioned screening methods can also be used for identification purpose. In this case, desirable mutation genotypes (homozygous insertion, deletion or substitution mutants) are first screened from plenty of lines by these PCR-based methods and then confirmed with Sanger sequencing. Among methods mentioned in Table 2, multiplex ligation-dependent probe amplification (MLPA)-based method, when combined with Sanger sequencing, could be the most suitable and accurate approach to screen mutants. It has multiplex capabilities (about 60 different target sites in a single assay), high sensitivity (down to ± 1 bp and single nucleotide replacement) and reliability (suitable for different targets), and multiple functions (on- and off-target detection simultaneously) (Biswas et al. 2020b).

Newly emerging genome-editing tools in crops

During past few years, several new CRISPR systems have been developed to improve the specificity and overcome the bottlenecks of the CRISPR/Cas9 system for more effective genome editing (Fig. 3), which continue to drive major advances in crop sciences and breeding. These emerging technologies could be essential tools for molecular crop-breeding purposes are discussed in below.

Cas12a/Cpf1 nuclease

The class 2 type V-A Cas protein Cpf1, also known as Cas12a with RNA-guided DNA endonuclease activity, has been widely applied in genome editing (Chen et al. 2019; Kim et al. 2017). Cpf1 uses a T-rich PAM sequence to recognize the target site in genomic DNA, which prolongs the editing sites behind those of G-rich PAM preferred by Cas9. The guide RNA of Cpf1 is shorter (about 43 bp) than sgRNA of Cas9 (about 100 bp), and the Cpf1 target site is positioned distal and downstream of the PAM sequence (Chen et al. 2019; Kim et al. 2017). Cpf1 produces staggered-ended DSBs at the distal location of a PAM, which provides further benefits than Cas9 due to knock-in strategies and enhances efficiency for the NHEJ-based gene insertion (Kim et al. 2017; Moon et al. 2018). Genome editing using Cpf1 system in crops has been reported in rice and soybean (Kim et al. 2017; Xu et al. 2017). Whole-genome sequencing analysis results indicated that neither Cas9 nor Cpf1 generates bona fide off-target mutations due to continued expression of Cas9 or Cpf1 in T_1 rice (Tang et al. 2018). Notably, in vitro studies show that Cpf1 has robust non-specific activated nicking activities, which may lead to off-target editing (Murugan et al. 2020). Further investigations are needed to evaluate the specificity of Cpf1 in vivo in other crops and to improve current Cas12a-based applications (Schindele and Puchta 2020).

Cas12b/C2c1 nuclease

CRISPR-associated Cas12b, a class 2 type V-B nuclease, prefers T-rich PAM, creates staggered ends of DNA DSBs, and requires a crRNA and a trans-activating crRNA (combined as a sgRNA). In addition, Cas12b protein is smaller than Cas9 or Cas12a, which has been regarded as the promising CRISPR system for genome editing in crops. In rice, it recognizes VTTV PAMs, more preferring ATTV and GTTG PAMs. The successful establishment of a compelling Cas12b transcriptional activation system in rice indicated that Cas12b is more adaptable for versatile guide RNA engineering (Ming et al. 2020). Cas12b/C2c1 has been successfully used to induce mutations including to create large deletions at multiple loci, and to perform multiplex genome editing in Arabidopsis, which does not show any mutations at potential off-target sites (Wu et al. 2020). Nevertheless, Cas12b requires higher temperature for optimal activity (Teng et al. 2018), which needs to be modified to make it more practically for crop applications.

Cas13/C2c2 nuclease

Cas13, also known as C2c2, is a newly identified CRISPR effector, specifically cutting single-stranded RNA in eukaryotic cells (Wolter and Puchta 2018). Cas13 protein is assigned into class 2 type VI, which acts solely on RNA because of its unique HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domains are exclusively associated with RNase activity. Notably, there is no strict requirement for PAM sequence for some Cas13 orthologues (Wolter and Puchta 2018). To date, three different Cas13 protein classes, such as Cas13a, Cas13b, and Cas13d, have been applied for RNA editing in plants (Schindele et al. 2019), mainly to target RNA for cleavage, for combating RNA viruses (Aman et al. 2018; Wolter and Puchta 2018). Combining Cas13 with other DNA-directed Cas nucleases opens new opportunities for crop breeding by targeting at both DNA and RNA levels.

Cas14/Cas12f nucleases

Cas14a, a highly compact class 2 type V nuclease, is an RNA-guided DNA nuclease that can be utilized for target-specific single-stranded DNA (ssDNA) cleavage (Harrington et al. 2018; Khan et al. 2019b). Cas14a does not require restrictive sequence to target and cleave ssDNA, which is different from other known class 2 systems (Harrington et al.

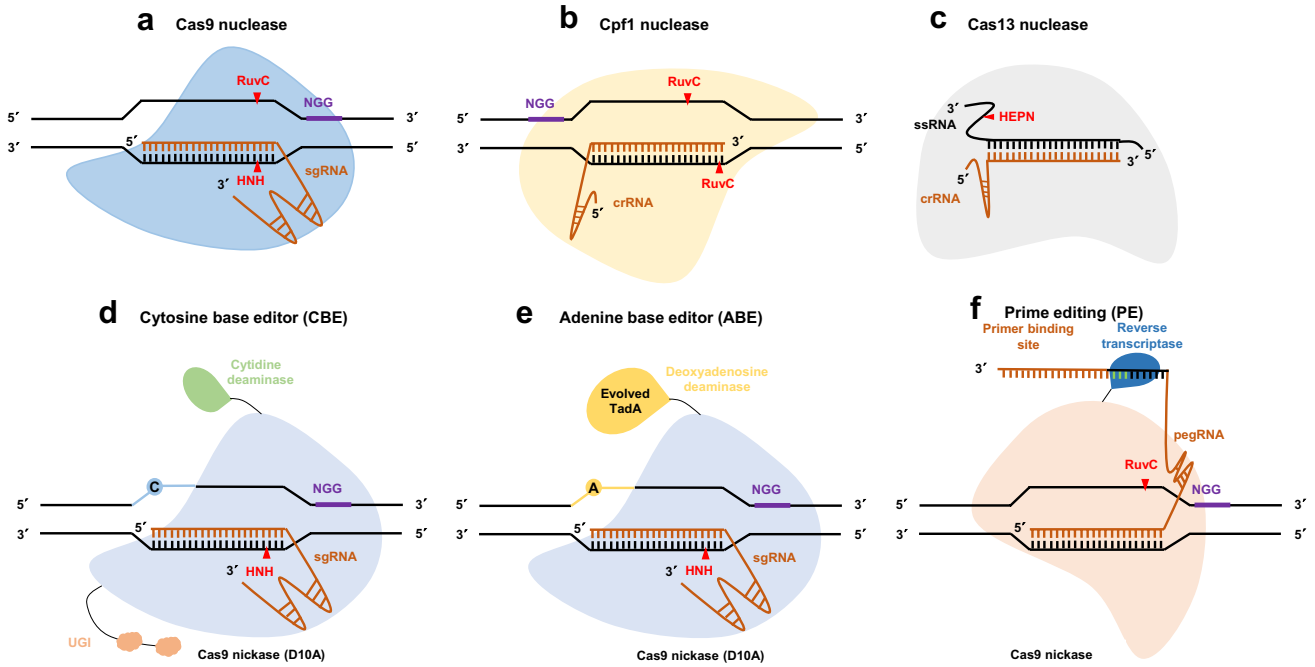

Fig. 3 Genome-editing tools in crop improvement. **a** Cas9 protein forms a crRNA-tracrRNA complex with the sgRNA to bind upstream of the G/C-rich PAM sequence and initiates DSB through its RuvC and HNH domains. **b** Cpf1 and crRNA form a complex to bind the downstream of the A/T-rich PAM sequence and introduces DSB via a RuvC-like nuclease domain. **c** Cas13 targets a single strand RNA (ssRNA) molecule at the outer nuclease surface guided by crRNA in the absence of a PAM recognition site. **d**, **e** Cytosine base editor and adenine base editor, respectively. The complex of nickase Cas9 (nCas9-D10A) fused with cytidine deaminase/deoxyadenosine deaminase converts cytosine (C) or adenine (A) in the target site to uracil (U) or inosine (I), resulting C-T and A-G replacement, respectively. f Prime editor. The nCas9-RT complex formed between Cas9 nickase and engineered reverse transcriptase domains targets to editing sites by engineered prime editing guide RNAs (pegRNAs), and nicks the PAM-containing DNA strand by Cas9 nuclease. *HEPN* higher eukaryotes and prokaryotes nucleotide-binding domains; *HNH* His-Asn-His; *pegRNA* prime editing guide RNA; *sgRNA* single-guide RNA; *UGI* uracil glycosylase inhibitor

2018). Cas14a is the smallest functional CRISPR system to date, which is only one-third size of Cas9 (Harrington et al. 2018). The CRISPR/Cas14a system shows potential application in crops in defense against ssDNA viruses or mobile genetic elements. It has been used to create resistance against ssDNA viruses, including *Geminiviridae* and *Nanoviridae* families, in crops (Khan et al. 2019b).

Altogether, these abovementioned several nucleases (Table 3) enable a wide range of genome-editing applications in crops, with their unique characteristics either at DNA or RNA level. It is worthy to note that except Cas14a (Harrington et al. 2018), LwaCas13a and PspCas13b (Wolter and Puchta 2018), other nucleases require specific PAM sequences for their functions. Two recent studies demonstrate that using SpRY, a modified SpCAS9, does not need all those specific PAM sequences, and realizes PAM-less genome editing in rice (Ren et al. 2021; Xu et al. 2021), which greatly extends the application of CRISPR system in crop genome, and facilitates moving genome-edited crops towards commercialization. Nevertheless, the identification of novel CRISPR–Cas genome-editing systems free of off-target editing activity while maintaining robust on-target editing efficiency and compatibility with crop genomes continues to be a challenge in the future.

Base editors

Different from abovementioned nucleases, base editors (BEs) precisely generate targeted mutations without requirement of DSBs or donor DNA, and independent on homology-directed repair (HDR), providing efficient, simple, well-accepted techniques for specific base replacement at the target site (Chen et al. 2019). BEs are extremely useful when base editing of interested protein-coding genes is needed to generate genetic variants with improved agronomic traits (Li et al. 2020c). Currently, there are two classes of BEs: cytosine base editor (CBE) and adenine base editor (ABE). CBE converts of C-G base pair to T-A base pair while ABE converts of A-T base pair to G-C base pairs (Komor et al.

2016; Gaudelli et al. 2017). CBEs use cytidine deaminases to convert cytosine to uracil (Komor et al. 2016) while ABEs use TadA deoxyadenosine deaminases to convert adenosines to inosines (Gaudelli et al. 2020). Activities of both BEs depend largely on PAM availability, because they both use CRISPR–Cas DNA binding proteins to allow the targeted deamination of single nucleotides at the targeted sites. BEs install transition point mutations but cannot install transversion point mutations, precise insertions or deletions. Both CBE and ABE have been tested in rice, and genome-wide sequencing data indicated that CBE not ABE induces substantial genome-wide off-target mutations, highlighting needs to optimize fidelities of CBEs (Jin et al. 2019). Progresses have been made to increase genome-targeting scope and fidelity of BEs (Anzalone et al. 2020; Yan et al. 2021); hopefully, BEs will play more roles in both random mutagenesis and targeted random mutagenesis (Li et al. 2020c) in crop breeding.

Prime editors

Prime editing, an emerging genome-editing tool, can precisely introduce all possible types of point mutations, and small insertions/deletions without donor DNA or DSBs (Anzalone et al. 2020). Prime editors (PEs) are fused proteins of Cas9 nickase domains with engineered reverse transcriptase domains. PEs target to editing sites by engineered prime editing guide RNAs (pegRNAs), nick the PAM-containing DNA strand by Cas9 nuclease, and prime reverse transcriptions using extensions in the pegRNAs as templates (Anzalone et al. 2020). Plant PEs have been successfully developed and applied to precisely edit several endogenous genes in rice and wheat protoplasts (Lin et al. 2020; Tang et al. 2020), to achieve stable edited lines with desired edits in both exogenous and endogenous genes (Butt et al., 2020); Li et al., 2020e). Nevertheless, this new technology is still at the experimental stages, more studies are needed to apply PEs in crop for different trait improvement.

Safety regulations of genome-edited crops

There is an ongoing argument whether a genome-edited organism obtained by the CRISPR technology is or is not regarded as a genetically modified organism (GMO), and regulated or not regulated as a GMO (Eş et al. 2019). In fact, similar to GMO, genome-edited crops are regulated globally in either technology-based or final product-based manner (Table 4) (Eckerstorfer et al. 2019; Van Vu et al. 2019). Some countries, including European Union (EU), New Zealand, and India, recognize and regulate genome-edited crops as GMOs based on technologies used to generate them (Jouanin et al. 2018; Fritsche et al. 2018; Friedrichs et al. 2019). On the other hand, Argentina, Australia, Brazil, Canada, Chile, Japan, and the USA, recognize and regulate genome-edited crops based on the final products, and if they are free of transgene, they are regarded as non-GMOs (Lema 2019; Eckerstorfer et al. 2019; Eriksson et al. 2019; Smyth 2017; Van Vu et al. 2019; Razzaq et al. 2019). Several countries like Nigeria and Kenya are in their way of developing regulatory policy on genome-editing crops, but many countries have not yet confirmed their positions (Tripathi et al. 2020; Eckerstorfer et al. 2019). In China, according to the current law, GE plants fall in the regulation scope of GMO, specific laws regarding genome-edited products are not yet announced (Gao et al. 2018).

Different countries take different initiative policies regarding regulatory landscapes on genome-edited crops, resulting in an inconsistent global regulatory system, which somehow hinders commercial utilization of genome-edited crops and pragmatic technological improvement. There is a need to establish a more optimistic and more realistic regulatory system regarding genome-edited plants globally, bring the world under the one safety regulation umbrella.

Conclusions and future directions

In addition to applications in basic researches in crops sciences, CRISPR/Cas systems find ways in many aspects of crop breeding. With advances in the development of novel CRISPR/Cas systems that are more specific, accurate, efficient and feasible, CRISPR/Cas systems will play more roles in securing global food supply in a sustainable manner.

However, to be fully applied to crop improvement, further improvements on these versatile tools are needed. These include: (1) the fidelity, where the incidence of off-target effect should be null; (2) the applicability, where the activity is independent on PAM and the system is free of donor DNA; (3) the compatibility, where the delivery into crop cells is independent on species; and (4) the traceability, where any modifications in the genome should be traceable. Molecular characterization of crops generated from any genome-editing tools should be performed before any filed trials. Last but not the least, development of a pragmatic product-based global regulatory policy on genome-edited crops is necessary for speeding up the applications of these tools in crop breeding.

Table 3 Characteristics of different nucleases used in crop breeding

Characteristics	Cas9	Cpf1	Cas13	Cas14	References
Host	*Streptococcus pyogenes*	*Prevotella* and *Francisella 1*	Multiple orthologs; *Leptotrichia shaii*	Uncultivated archaea	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Harrington et al. (2018)
Protein size (aa)	1368	1200–1500	1440	400–700	Jinek et al. (2012); Safari et al. (2019); Garcia-Doval and Jinek (2017); Harrington et al. (2018)
tracRNA/crRNA	Yes/yes	No/yes	No/yes	Yes/yes	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Harrington et al. (2018)
PAM (5′–3′)	5′-NGG-3′	5′-TTTV-3′	Non-G nucleotide at the 3′ protospacer flanking site (PFS)	Not required	Jinek et al. (2012); Safari et al. (2019); Garcia-Doval and Jinek (2017); Harrington et al. (2018)
sgRNA size (bp)	20	20	28	20–25	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Moon et al. (2019)
Substrate	dsDNA	dsDNA	ssRNA	ssDNA	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Moon et al. (2019)
Binding domain	HNH, RuvC	RuvC-like	HEPN	RuvC	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Harrington et al. (2018)
Cleavage site	5′ of PAM	5′ of PAM	gRNA dependent cleavage	gRNA dependent cleavage	Jinek et al. (2012); Moon et al. (2018); Moon et al. (2019)
Cleavage pattern	Staggered 5′-overhang [a]	Staggered 5′-overhang	collateral ssRNA cleavage	Collateral ssDNA cleavage	Moon et al. (2019)
Function	In Cas9 system, the PAM assembles to an NGG consensus which consists of two G:C base pairs	Cpf1 relies on the T-rich 5′-end of the PAM sequence	A single crRNA guide Cas13 protein targets ssRNA and cleave the target via HEP-binding domains	Cas14 utilized for target-specific single-stranded DNA (ssDNA) cleavage without needing restrictive sequences	Jinek et al. (2012); Moon et al. (2018); Wolter and Puchta (2018); Khan et al. (2019b)
Pros/Cons	Mostly used/large protein size	High specificity/unsatisfactory adoption	Flexible/target ssRNA	Small protein size/target ssDNA	Jinek et al. (2012); Moon et al. (2018); Garcia-Doval and Jinek (2017); Harrington et al. (2018)

[a]Subsequent postcleavage trimming activity of RuvC domain produced Staggered 5´-overhang

Table 4 Current regulatory approaches on genome-edited crops in different countries

Country	Agency	Regarded	Basis	References
United States	USDA	Non-GMO	Product based	Razzaq et al. (2019)
Canada	CFIA	Non-GMO	Product and novel trait	Smyth (2017)
Argentina	CONABIA	Non-GMO: if final product is free of transgene	Product based	Lema (2019)
Australia	OGTR	Non-GMO: if final product is free of transgene	Product based	Eckerstorfer et al. (2019)
Japan	MHLW	Non-GMO: if final product is free of transgene	Product based	Van Vu et al. (2019)
Brazil	CTNBio	Non-GMO: if final product is free of transgene	Case-by-case	Eriksson et al. (2019)
Chile	SAG	Non-GMO: if final product is free of transgene	Case-by-case	Eriksson et al. (2019)
European Union	EFSA	GMO	Process based	Jouanin et al. (2018)
New Zealand	HSNO	GMO	Process based	Fritsche et al. (2018)
India	FSSAI	GMO	Process based	Friedrichs et al. (2019)

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Author contribution statement SJ designed the project, supervised the project, and revised the manuscript. BS collected information, did analysis, and wrote the first draft of the manuscript. ZD participated in the discussion.

Declarations

Conflict of interest The authors declare no conflict of interest.

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