Application of CRISPR/Cas9 in Rapeseed

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Brassica napus L. provides high-quality edible oil and clean energy for humans. For a long time, rapeseed breeders have tried to breed improved varieties through traditional breeding strategies. However, *B. napus* is an allotetraploid species containing many repetitive sequences. It is very inefficient to change traits through traditional genetic methods such as hybridization and random mutagenesis due to gene redundancy. Today, the burgeoning CRISPR/Cas9 technology has been applied in polyploid rapeseed for gene function research and targeted genetic improvement because of its unique advantages of high efficiency and simplicity.

gene-editing CRISPR/Cas9 genetic improvement

1. Advantages of CRISPR/Cas9 Application in Polyploid Rapeseed

Several potential limitations still worry researchers, such as multi-copy gene knockout and off-target effects. *B. napus* is an allotetraploid species, and most genes in *B. napus* are multiple-copy genes with redundant functions [1]. Due to gene redundancy, it is very inefficient to change traits through random mutations. It is often necessary to edit multiple genes to improve one trait. Fortunately, different single-guide RNAs (sgRNAs) can guide the Cas9 protein to specific sites, which offer an opportunity to achieve multiple gene editing by expressing Cas9 along with the multiple sgRNAs [2][3]. CRISPR/Cas9 technology has obvious advantages in polyploid rapeseed because multiple mutations can be induced in one step. In rapeseed, the most common strategy is to introduce mutations at multiple sites or genes concurrently by stacking multiple independent sgRNA-expressing cassettes. At present, there are more and more studies on multi-copy gene knockout in *B. napus* using CRISPR/Cas9, which shows that the prospect of CRISPR/Cas9 in the study of multi-copy gene function is encouraging in *B. napus*. The CRISPR/Cas9 system is an efficient tool and widely used for crop improvement either by a single or multiplex genome editing approach [4]. Analyzing the functions of homologous genes and gene family members with high sequence similarity frequently require multiple gene mutants. Based on many research facts are listed in this entry, different sgRNAs connected in tandem show a huge advantage in multiple gene editing, due to their ability to efficiently generate mutants with multiple gene mutations [5]. The specificity of CRISPR/Cas9 technology for targeted gene editing in plants is also a worrying issue. Generally, to ensure that mutation is as specific as possible, potential off-target sites should be checked. Researchers can adopt some measures to avoid off-target mutations as much as possible. For example, combined with other CRISPR/Cas9 online design tools, BLAST searches against the *Brassica* genome using the full 23 nucleotide sequence (protospacer plus PAM) as a query can allow the selection of guides with no, or a minimal number of, predicted off-targets [6]. Recent studies report that off-target mutations can be tackled by designing precise sgRNA [7]. Therefore, it is essential to design sgRNAs

with minimal off-target activities to avoid unexpected outcomes. Currently, to avoid off-target effects as much as possible, several free online prediction tools, such as CRISPOR [8] and CCTop [9], have been used to help researchers design sgRNA. Also, several machine learning-based methods for identifying sgRNAs with high on-target activity have been developed and applied to sgRNA activity prediction in agronomy [10][11]. The development of these methods helps to identify the activity of designed sgRNA in crops and avoid unexpected results. This is consistent with the conclusion of Matres, et al. [12]: when gRNAs are carefully designed, off-target editing is negligible, and the frequency of occurrence is much lower than naturally occurring diversity in plants. For rapeseed breeders, the off-target mutation is not the main constraint, as any undesirable mutations will be low frequency, predictable, and testable [6]. In summary, CRISPR/Cas9-mediated genome editing technology has been proven to simultaneously alter multiple homologous genes without any off-target editing and generates mutations that are stable and heritable by offspring.

2. Identification of CRISPR Mutants and Detection of Mutation Types in Rapeseed

Amplification of single copies by polymerase chain reaction (PCR) using paralog-specific primers is a common method for identifying multi-copy mutants, followed by sequencing. For complex mutations, the amplicons need to be subcloned into the T-vector, followed by monoclonal sequencing. Mutation analysis work usually requires a large investment in sequencing costs and analysis time for lots of samples by Sanger sequencing. In addition, complex chimeric mutations caused by genome editing are difficult to decode. The Hi-TOM method developed by Liu, et al. [13] is gradually being more widely used because of its simplicity, rapidity, and high throughput. In this method, only two rounds of PCR are needed to complete the construction of a multi-sample mixed sequencing library. After obtaining the sequencing data, the resulting sequencing data are uploaded to the Hi-TOM online tool (http://www.hi-tom.net/hi-tom/) to obtain the detailed mutation sequence of each locus of each sample and the corresponding genotype information. At present, the Hi-TOM method has been applied in the identification of rapeseed mutants [14][15].

3. CRISPR Transformation Receptor Restriction Needs to Be Further Broken

So far, the application of CRISPR/Cas9 in rapeseed has mainly relied on stable transformation by *Agrobacterium tumefaciens* to deliver the CRISPR vector. However, several excellent *B. napus* varieties are usually not easy to transform due to a lack of the traits suitable for culture and regeneration [16]. At present, the reported varieties for *Agrobacterium tumefaciens*-mediated genetic transformation include spring rapeseed varieties and semi-winter varieties, among which the spring varieties include Westar, 862, and Haydn, etc., and the semi-winter varieties include J9707, J9712, and ZS6, etc. Also, there is a problem that more efficient transformation techniques are required for recalcitrant commercial rapeseed varieties [17]. One of the ways to help alleviate the bottleneck is to use morphogenic genes. For maize, this bottleneck has been mitigated by using the morphogenic genes *WUS2* and *BBM* [18]. Over the past three decades, basic research has provided with a detailed understanding of the

genes that control morphogenesis. These insights will continue to provide inspiration for testing morphogenetic genes and, together with new methods to control expression, will lead to continuous improvements that expand the range of different rapeseed varieties suitable for transformation [19]. Moreover, the tissue culture procedures are often technically demanding, time-consuming and laborious. Therefore, developing no-tissue-culture-required delivery methods, such as nanoparticles or virus delivery, will contribute to further extending the application of CRISPR/Cas9 in rapeseed.

4. Application of CRISPR/Cas9 in High-Throughput Gene Editing

The precision, coverage, and flexibility provided by CRISPR/Cas9 are playing an important role in genome editing in rapeseed improvement. In recent years, CRISPR technology has been developed rapidly and has shown unparalleled advantages in high-throughput gene editing. The high efficiency of CRISPR/Cas9 technology is very suitable for high-throughput gene editing in various organisms and cell types [20]. Since the targeting specificity of CRISPR/Cas9 is conferred by a 20bp sgRNA, array-based synthesis of oligonucleotide libraries on a large scale can be easily generated. Such powerful genome-scale CRISPR/Cas9 mutagenesis systems have been successfully used for rice and maize research [21][22]. For example, Lu et al. (2017) used the CRISPR/Cas9 system to perform genome-scale mutagenesis in rice and generated a library of targeted loss-of-function mutants, which provided a useful resource for rice research and breeding. Furthermore, Liu et al. (2020) successfully targeted 743 candidate genes related to traits relevant for agronomy and nutrition by integrating multiplexed CRISPR/Cas9-based high-throughput targeted mutagenesis with genetic mapping and genomic approaches, which provided the guidance and reference for further optimizing experiments on high-throughput CRISPR in plants. In general, their research proves that it is feasible for the powerful genome-scale CRISPR/Cas9 mutagenesis systems to be applied to rapeseed. Utilizing this system to achieve high-throughput targeted gene editing and screen large-scale mutant libraries will be a good way to identify novel genes that can improve target traits in rapeseed varieties.

5. Application Prospect of CRISPR/Cas Precise Genome Editing in Rapeseed Improvement

Current progress in CRISPR technology has offered more and more opportunities for gene function study and genetic improvement that have not been seen before [23][24]. Clustered regularly interspaced short palindromic repeats interference (CRISPRi) and CRISPR-mediated gene activation (CRISPRa), which both derive from the CRISPR/Cas9 technology, can be used in gene functional studies. Both CRISPRi and CRISPRa can repress or activate multiple target genes simultaneously with no detectable off-target activities [25][26]. Furthmore, the CRISPR/Cas system has been applied to live cell chromatin imaging [27][28][29][30] and manipulation of chromatin topology [31][32]. In general, CRISPRi/a technologies lead to transient changes in gene expression [26][33][34]. Epigenetics has been an attractive target for crop improvement as it is a key factor for controlling biological pathways. DNA methylation, histone modification, and non-coding RNAs are all main epigenetic factors that contribute to regulating gene expression. CRISPR provides a genomic targeting system capable of interfacing with

many aspects of the epigenome. CRISPR/Cas-based genome and epigenome editing has been used to improve drought stress tolerance in *Arabidopsis* by fusing dCas9 protein with a histone acetyltransferase (AtHA T1) and using this fused CRISPR/dCas9 system to target the abscisic acid (ABA)-responsive element-binding protein1 (AREB1)/ABRE-binding factor2 (ABF2) [35]. Two powerful emerging technologies, base editing and prime editing, have been applied in plants and have greatly enhanced the effectiveness of gene editing for crop improvement [36] [37]. Desired changes can be installed by both base editors (BEs) and prime editors (PEs) without the donor DNA and a DSB introduction in the genome [38]. BEs and PEs generate edited plants with nucleobase precision as the incidence of DSB is usually low during the editing process [38]. BEs install C•G-to-T•A and A•T-to-G•C transitions [39][40][41] and have been successfully used in plants [42][43]. Several studies reported by Wu, et al. [44] suggest BEs have become an efficient tool for precise genetic modification of important agronomic traits in rapeseed. However, the base editing technologies only can generate the four transition mutations [45] and are constrained by the PAM motif and the editing window [46]. Fortunately, the recent breakthrough of prime editing based on the CRISPER/Cas system can overcome these limitations [47]. Prime editing has enabled search-and-replace editing instead of single base substitutions, conferring precision genome editing by installing small insertions, deletions, point mutations, and combination edits [48]. Prime editing was first applied in human cells [48] and has since been quickly developed for use in plants including rice [49][50][51][52][53], wheat [50], maize [54], and tomato [55] by multiple research groups around the word. Compared to other gene-editing technologies, prime editing can reduce the risk of unwanted off-target activities and poses few restrictions on the edited sequence [56]. With continuous development and progress, the CRISPR technology will likely further revolutionize basic research and precision breeding in rapeseed.

6. Government Regulation and the Future of Gene-Edited Rapeseed

Although the exogenous DNA could be excluded from the gene-edited crops by progeny separation, it is still difficult to commercialize gene-edited crops in many areas, such as the European Union and New Zealand, due to their following a ‘process basis’ that leads to expensive and time-consuming genetically modified (GM) safety tests. Recently, China’s agriculture ministry released preliminary guidelines which will be followed in the safety evaluation of gene-edited plants without foreign genes. Since the release of the new regulations, Chinese researchers have been very excited and eager to submit applications to use their gene-edited crops [57]. The preliminary guidelines state that gene-edited plants without foreign genes can directly apply for a production application safety certificate after an intermediate test when target traits do not increase environmental safety and food safety risks. In China, the process for receiving a biosafety certificate for gene-edited crops under the new guidelines shortens the approval time to one to two years in comparison to the six years needed for a GM crop to get biosafety approval [57]. The new guidelines open the door to the commercialization of gene-edited crops and provide new opportunities for Chinese breeders to develop gene-edited crops with superior traits.

At present, the CRISPR/Cas9 system has not been explored fully yet for trait improvement in rapeseed. One of the main reasons is that most studies have relied on stable transformation by *Agrobacterium tumefaciens*, which leads to randomness of gene insertion and is subject to GMO regulations. Developing transgene-free genome editing

approaches in rapeseed are also required, which could provide a promising means for developing rapeseed varieties with reduced regulatory limitations. In this direction, novel delivery methods that do not introduce exogenous DNA may be able to avoid GM regulation. Recently, researchers have developed an optimized protoplast transient transfection method in rapeseed, which is an efficient solution for delivering CRISPR complexes [58]. This optimized protoplast regeneration protocol will provide important guidance for other rapeseed researchers. In addition, transgenic cotton was successfully produced by transforming pollen with magnetic nanoparticles and then pollinating plants with this magnetofected pollen [59]. Several other nanoparticles, including carbon nanotubes [60][61], DNA origami, and DNA nanostructures [62] have been successfully investigated for unassisted delivery of exogenous DNA. If delivery by nanomaterials without tissue culturing could be successfully applied in CRISPR/Cas9 for rapeseed genome editing, it would be a shortcut to creating non-GM rapeseed while avoiding strict GM regulations. With further development and remaining challenges gradually resolved, the CRISPR/Cas9 technology will produce more excellent rapeseed germplasm resources and create great economic value.

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