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Review

Genetic Engineering Technologies for Improving Crop Yield and Quality

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Abstract: Genetic engineering refers to the specific molecular biological modification of DNA sequences. With the rapid development of genetic engineering methods, especially the breakthroughs in guiding endonuclease technology, gene remodeling of crops has become simpler, more precise, and efficient. Genetic engineering techniques can be used to develop crops with superior traits such as high trace elements and high plant nutrients, providing an important tool to meet the needs of nearly 7.6 billion people in the world for crop yield and quality and to achieve sustainable development. This review first introduces transgenic technology and gene editing technology and analyzes the achievements in improving the efficiency of genetic transformation and regeneration in recent years. Then, it focuses on reviewing the applications of related genetic engineering technologies in improving the yield and quality of rice, maize, and wheat. In addition, the problems of genetic engineering technology in crop applications are discussed. The aim is to provide a reference for research on the development of genetic engineering technologies and the improvement of crop yield and quality.

Keywords: genetic engineering; transgenic technology; gene editing technology; crop quality and yield

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1. Introduction

As the global population continues to grow, the demand for food is increasing worldwide. It is undeniable that people in some regions still do not have sufficient food. In addition, the continuous population growth has a certain impact on climate, environment, and available arable land. These factors will eventually affect crop production [1]. Although the basic problem of eating is no longer a concern in some areas, hidden hunger (insufficient intake of essential nutrients and trace elements in the human body) remains a secondary problem [2]. It is estimated that the world's population will reach 10 billion by 2050 [3]. Therefore, the challenge of meeting the crop yield and quality requirements of 10 billion people deserves extensive consideration.

Genetic engineering is the process of using modern biological techniques to modify genomic DNA at the molecular level, thereby changing the genetic characteristics of recipient cells to achieve specific effects [4]. In 1983, genetic engineering was first applied in plants and herbicide-resistant tobacco was produced [5], starting the era of plant genetic engineering. In 1996, the commercial cultivation of the first batch of transgenic crops was realized [6], and genetically engineered crops began to enter the market. Since then, rapidly developing genetic engineering technologies have played an irreplaceable role in improving crop yields and quality.

This review summarizes the principle of several genetic engineering technologies, focusing on the effects of gene editing technology, the differences between gene editing technology and transgenic technology, the efforts of researchers in improving genetic transformation and regeneration in recent years, and the application of genetic engineering technology in improving crop yield and quality. In addition, some remaining problems of genetic engineering technology are also discussed.

2. Genetic Engineering Technology

2.1. Transgenic Technology

Transgenic technology is a method that transmits a piece of DNA in line with the wishes of researchers to the target cell through physical, chemical, biological, or comprehensive methods and integrates it into the target cell genome to achieve its expression [4]. Since the birth of herbicide-resistant transgenic tobacco mediated by *Agrobacterium tumefaciens* in 1983 [5], the research of plant transgenic technology has been deepening constantly. Accordingly, a growing number of technologies have been developed, such as *Agrobacterium tumefaciens* transformation technology, virus-vector-mediated technology, biolistic technology, liposome-mediated transfection, ultrasound-mediated transformation, polyethylene glycol induction, pollen-tube pathway method, ovary injection, etc. [7].

The most commonly used transgenic techniques in plants are *Agrobacterium tumefaciens* transformation and biolistic technology. For the former, the T-DNA region and Vir region of the plasmid are of great significance. With the help of Vir-region-associated proteins and other *Agrobacterium tumefaciens* genes, T-DNA can be randomly inserted into the cell genome of injured plants and expressed [8]. Consequently, using this property, the target sequence can be inserted into the multi-clone site of *Agrobacterium tumefaciens* and integrated into the host cell genome with T-DNA at random. This method is simple and effective, yet obviously limited by the host range. Subsequently, the emergence of biolistic technology to some extent broke the restrictions of the host range [9], in which the third generation utilizes high-pressure inert gas as the driving force to insert gold or tungsten particles wrapped in the target DNA into the recipient cells to achieve transformation [9,10].

The feasibility of this method lies in that the particle size is suitably small (diameter of 0.6 μm) [9]. In addition, the driving force is also sufficiently strong, with impact pressures up to 900 psi [10]. However, it still has apparent defects, for instance, the efficiency of DNA integration into the genome is not high, and multiple particles entering simultaneously may easily lead to a higher number of DNA copies, resulting in more frequent gene inactivation or silencing [11]. In order to develop a technique with wider application and more stable transformation, Ribeiro et al. [10], using cotton hypocotyl as the explant, developed cotton with high resistance to *Anthonomus grandis* with the joint use of *Agrobacterium tumefaciens* transformation and biolistic technology, and this character was found to be stably inheritable. Gurusaravanan et al. [12], using cotton stem tip as the explant, transformed the *uidA* gene in cotton using *Agrobacterium tumefaciens* with the aid of microinjection and ultrasound, and the transformation efficiency was up to 20.25%. In addition, the strategy of creating transgenes based on nanoparticles has also been pursued by researchers owing to its comprehensive advantages such as excellent transformation efficiency, biocompatibility, and less harm to the host [13]. Although it is still in its infancy, its application potential has been proved in a variety of model plants [13].

Transgenic technology developed from traditional breeding technology; they are both essentially the genetic integration of target genes. All kinds of transgenic technologies possess obvious merits and demerits; thus, we can figure out the most suitable transformation method by focusing on the purpose of the research.

Generally speaking, transgenic technology breaks the species restrictions of conventional organisms, and its use can achieve crop improvement more purposefully and efficiently.

2.2. Gene Editing Technology

There are many types of gene editing tools, such as zinc-finger nucleases (ZFNs) [14], transcription-activator-like effector nucleases (TALENs) [15], and clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 protein (Cas9) systems, that are based on biological cutting methods [16] and have broad potential in crop breeding and improvement.

The traditional gene editing process is mainly divided into two steps: First, the target sites of double-stranded DNA are cleaved by a nuclease system with some degree

of engineering modification to produce double-stranded breaks (DSBs). Second, since DSBs are lethal to cells in many cases, to ensure genome integrity, the cells will initiate two endogenous repair pathways (non-homologous end junction (NHEJ) pathway and homologous recombination (HR) pathway) for repair.

Among them, NHEJ has high repair efficiency but poor stability, and it is easy to perform the insertion or deletion of small fragments in the repair site region [17,18]. In addition, in the presence of homologous templates, fractures can be repaired using the HR approach. This repair method is more accurate and at least two orders of magnitude more efficient than the traditional homologous recombination method used for gene shooting [14,17,18]. However, compared with NHEJ repair, HR repair efficiency is still lower [14,17,18]. To make crop improvement more flexible, it is necessary to optimize the efficiency of accurate repair, and researchers have made a series of efforts to improve the efficiency of HR repair. For example, in plants, Matthew et al. [19], in the study based on homologous recombination knock out of the rice *chlorophyll a oxygenase* gene (*CAO1*), used the Cpf1 nuclease to edit *CAO1* and found that the homologous recombination repair efficiency was improved, with an efficiency of 8%. Wang et al. [20] found that, by increasing the number of homologous templates, *endogenous actin 1* gene (*ACT1*) and *glutathione S-transferase* gene (*GST*) were transported by the wheat dwarf virus (WDV) to specific sites in the rice genome cleaved by the CRISPR/Cas9 system, and the homologous recombination repair efficiency reached 19.4%. However, the application of the HR pathway in crops is not as common as that of the NHEJ pathway, mainly because of the cell cycle dependence of the repair pathway, i.e., NHEJ can play a stable role in almost the entire cell cycle, while the HR pathway is only active in the S and G2 phases [18].

2.2.1. ZFNs

ZFNs are the fusion of artificially modified zinc-finger protein (ZFP), having specific binding activity, and Fok I endonuclease, having non-specific cutting activity [21]. Several amino acid residues on the α helix of ZFP play a direct role in the recognition of the target site and can pair with bases adjacent to it. For example, the −1, +3, +6 locus on the α helix of Zif268 (a kind of ZFP) can directly recognize and bind to three adjacent bases on the target sequence [22] (Figure 1). In addition, the binding domain and cutting domain of the Fok I endonuclease can be separated, and the non-specific cutting function of the Fok I endonuclease can be obtained when the binding domain is removed [23]. Finally, under the action of Fok I dimer, the target site will be cut, and DSBs will be generated [22].

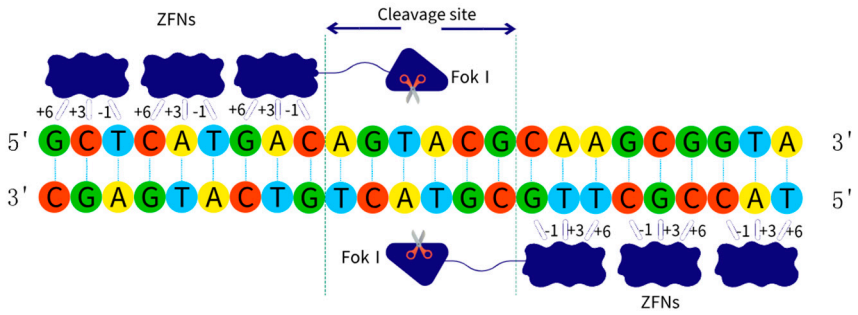

Figure 1. The schematic diagram of ZFNs.

ZFNs, as the first generation of gene editing technology, have been successfully applied in animals at first and have attracted wide attention [14]. Subsequently, ZFNs were applied in corn [24], rice [25], and other crops. However, there are inherent flaws in this system. For example, the inter-ZFP context effect, that is, it is difficult to achieve high efficiency by simply linking specific ZFPs together [14,26]. In addition, there are shortcomings such as unsuitability for multiple editing, high off-target efficiency, and high cytotoxicity, which are gradually being overcome by the later gene editing tools [14,26]. Therefore, its application in crops is not well developed.

2.2.2. TALENs

The structural difference between TALENs and ZFPs is that TALENs use the transcription-activator-like effector (TALE) as their binding domain, and the binding function mainly depends on highly variable amino acids at the 12th and 13th position on the TALE [15,27]. In combination with them, a single base can be specifically identified. The deciphered recognition method can meet the needs of arbitrary base recognition [15,28]. In this system, the non-specific cutting domain is still Fok I endonuclease, and a pair of TALENs will form a Fok I endonuclease dimer after binding to the target site, resulting in DSBs [29] (Figure 2).

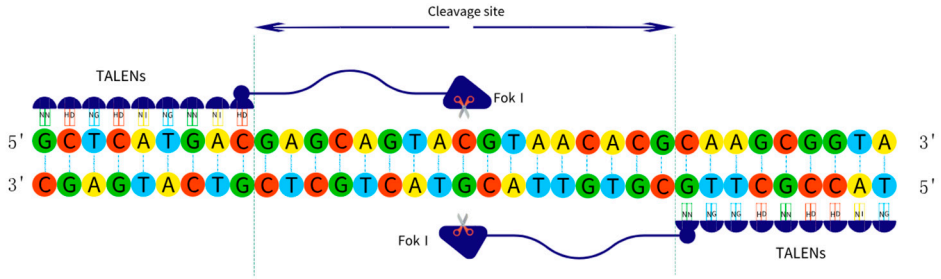

Figure 2. The schematic diagram of TALENs.

TALENs are more targeted, less cytotoxic, have no contextual effect such as the one in ZFPs, and can be assembled in a modular manner [30,31]. Currently, they have been successfully applied in rice [32], corn [33], wheat [34], and other crops. However, TALENs' binding sites are restricted by guanine nucleotides and are not suitable for multiple editing or highly methylated sites [14,35]. But, highly methylated loci are very important in gene regulation research, and multiple editing is of great significance in studying the interaction between genes and improving editing efficiency, so the application of TALENs is limited to some extent.

2.2.3. CRISPR/Cas9 System

The widespread application of gene editing technology in crop improvement at present largely benefits from the emergence of the CRISPR/Cas9 system, which is derived from the immune system of bacteria and archaea [36]. The CRISPR/Cas9 system was synthesized by connecting single-guide RNA (sgRNA) and Cas9 [14]. The sgRNA can bind to the target site after specific modification [14]. The RuvC domain of Cas9 protein can cleave the DNA strand with protospacer-associated motif (PAM) sites, and the DNA strand complementary to sgRNA is cleaved by the HNH domain of Cas9 protein [16]. The cutting site is near the PAM site [16] (Figure 3).

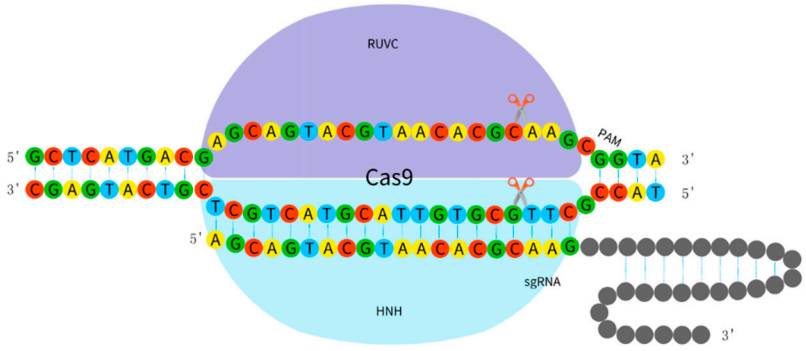

Figure 3. The schematic diagram of CRISPR/Cas9.

In contrast, when using the three gene editing systems for specific editing, ZFNs and TALENs need to modify at the protein level, while CRISPR/Cas9 system only needs to appropriately modify sgRNA at the RNA level for targeted modification, which makes

engineering feasibility stronger. At the same time, the CRISPR/Cas9 system also has the advantages of being suitable for multiple edits and more efficient in generating insertions or deletions [26]. However, its off-target effect and restriction of targeting by PAM sites are its main defects [36,37]. Off-target effects tend to produce unexpected mutations that are often detrimental to the cell. In bacteria and archaea, PAM sites are used to distinguish their own sequences from foreign sequences and are necessary for recognition and cutting [36]. Nevertheless, as a gene editing tool, the dependence on PAM sites limits the system's ability to target genes widely. Therefore, reduction of off-target effects and freedom from confinement to PAM sites can broaden the application range of CRISPR/Cas9. It has been reported that off-target effects can be effectively reduced through Cas9 modification, sgRNA modification, bioinformatics analysis, delivery mode optimization, and other methods [38–43]. The dependence of CRISPR/Cas9 on PAM sites can be reduced through two approaches: directed evolution and structural orientation [44]. On the premise of known enzyme structure, enzyme mutants are usually engineered using a structure-oriented approach.

With the advent of base editors (BEs) and prime editors (PEs), the CRISPR/Cas system has been further expanded. It can be edited accurately without DSBs. Cytosine base editors (CBEs), adenine base editors (ABEs), and glycosylase base editors (GBEs) have been developed successively in animal cells [45–49]. These BEs can realize the substitution of C–T, A–G, C–A, and C–G by selective artificial fusion with deaminase, glycosylase inhibitor, or glycosylase based on Cas9 nickase (nCas9) and sgRNA [45–49]. However, when these tools act directly on crops, they are extremely inefficient and cannot meet the needs of users. Hua et al. [50], based on the research of the David Liu team, developed a single base editor, ABE7-10, for plants by optimizing ABE deaminase. In addition, the introduction of A3A-PBE [51], PhieCBEs [52], pDuBE1 [53], and other base editors can greatly improve the efficiency and scope of application of plant base editing. PEs consist of reverse transcriptase (RT)-nCas9 and pegRNA (composed of sgRNA, reverse transcriptase template, and primer binding site (PBS)) [54]. The RT template was used as donor DNA, and RT-mediated reverse transcription was performed [48,54]. Thus, genes can be accurately knocked in or out while avoiding the inefficient HR repair pathway. In addition, it has the advantages of producing fewer by-products, the editable base substitution of all types, and little restriction by PAM sites [48,54]. These research results were initially realized in animal cells, so it is worth thinking about how to make full use of its powerful function in plants. Lin developed the plant prime editor (PPE) system by optimizing codons, promoters, and editing conditions, which introduced powerful functions into plants for the first time and laid the foundation for subsequent optimization.

Through these efforts, the development of gene editing technology has been deepened, providing an effective tool for accelerating crop domestication, enriching the crop gene pool, and improving crop yield and quality.

3. Effects of Gene Editing Technology

3.1. Gene Knockout

The ultimate goal of gene knockout is to change or delete the proper function of the target gene. Frameshift mutation is one of the commonly used strategies for gene knockout. When DSBs are repaired through the NHEJ pathway, small insertions or deletions are usually generated. If the insertions or deletions appear in the open reading frame (ORF), frameshift mutations are prone to occur, thereby achieving gene knockout. For example, Zhang et al. [55] used CRISPR/Cas9 to enhance the abiotic stress resistance of rice by knocking out the *OsPQT3* gene in rice. Then, the analysis of the three *ospqt3* mutants found that the gene knockout effect was caused by the insertion or deletion of several bases in the ORF resulting in frameshift mutations. In addition, it is also feasible to replace the entire gene or a part of the gene through the HR pathway to achieve the loss of function of the target gene [56]. Although this method is accurate, it is not widely used in plant gene editing knockout due to the low efficiency of the HR pathway. Furthermore, it has been reported that specific editing of parts of non-coding DNA can also achieve gene knockout

because these fragments are closely related to the regulation of gene expression. For example, Komatsu et al. [57] used CBEs to edit the *HIS1* gene, which is negatively related to rice resistance to benzobicyclon, and successfully knocked out the *HIS1* gene by base substitution to deactivate the promoter or introduce the stop codon in advance. It is worth noting that, theoretically, starting from the base substitution of the promoter and the stop codon does not involve the unknown effect of frameshift mutation, which is safer for cells. With the development of BEs and PEs in plants, theoretically, all types of base substitutions can be realized [50,52,53,58]. What is more, BEs and PEs do not need to generate DSBs and are more precise, showing great potential in gene knockout [50,52,53,58]. The application of a multiplexed CRISPR/Cas9 system in plants shows a strong ability of large fragment knockout, which renders the application of gene knockout more flexible [59,60]. In addition, the PEDAR system [61], the PRIME-Del system [62], and the newly developed twinPE system in animal cells provide some reference for how to further knock out super-large fragments in plants and improve the flexibility of gene knockout.

3.2. Gene Knock-In

Gene knock-in can be achieved in two ways, namely the HR repair pathway and the non-HR repair pathway. Conventional gene knock-in is based on the HR repair pathway of the CRISPR/Cas9 system, but its efficiency is generally low [18]. Lu et al. [63], through the optimized development of TR-HDR methods, achieved fragment substitution at five gene loci, with a maximum efficiency of 11.4%, greatly promoting the use of accurate HR repair and promoting the process of directed genetic improvement of crops. The second way is through non-HR repair, with the establishment of the PE system in plants [58], and through optimization to improve the editing efficiency [64] so that the precise insertion of small fragments in plants is more flexible. In addition, the twinPE system combined with Bxb1 integrase has been shown to produce large inserts of 5.6 kb in mammalian cells without obvious off-target effects [65]. It provides a reference for the optimization of the free insertion of large fragments of genes in plants. In conclusion, the improvement of these knock-in schemes will have a positive impact on the study of plant gene function, facilitate gene enrichment, and so on.

3.3. Gene Regulation

Each crop has a rigorous set of molecular mechanisms that selectively produce corresponding effects under different conditions. Nuclease-dead Cas9 (dCas9) is a variant of Cas9 without cleavage ability, which can be used to artificially intervene in crop gene regulation [66]. If dCas9 is fused with a specific transcriptional activator or transcriptional repressor, the target gene can be inhibited or activated by sgRNA targeting the promoter region of a specific gene [67]. Based on this, the sgRNA can be further modified, and some aptamers can be inserted to recruit more regulatory factors, and the regulation will be more efficient in most cases [68]. In addition, epigenetic modifications can be generated by the fusion of epigenetic modifiers with the CRISPR-nCas9/dCas9 system, which in turn affects gene expression [69]. Epigenetic modification is a series of heritable modifications to DNA and proteins under the action of internal and external factors without changing the DNA sequence [70], which affects biological processes, including gene regulation, by influencing structure, molecular affinity, etc. [70]. For example, Papikian and Gallego-Bartolome et al. [71,72], based on the CRISPR-dCas9-Suntag targeting system, recruited the catalytic domain of human TET1 and the catalytic domain of tobacco DRM methyltransferase, respectively, to achieve *Arabidopsis FWA* gene methylation and demethylation of genes to control flowering time in *Arabidopsis* by repressing or activating transcription. Thus, it can be seen that gene editing technology has a variety of gene regulation capabilities.

4. Comparison of Transgenic Technology and Gene Editing Technology

An obvious disadvantage of transgenic technology is that the target gene can only be inserted randomly. The biggest advancement in gene editing technology is the ability to

target the genome. Because of this unique advantage, the two technologies differ in many ways.

Safety: The effect of transgenic technology is inseparable from that of exogenous DNA, but gene editing technology can edit the target site without the presence of exogenous DNA. For example, through the NHEJ pathway, small fragments are randomly inserted or deleted to achieve gene knockout [55]; base editors are used to replace the corresponding bases in the active window to regulate transcription to achieve gene knockout [57]; in addition, editing technology is more controllable and safer than transgenic technology, due to the clearly defined sites of gene editing.

Efficiency: With the deepening of research and application, the requirements for specific site modification of the crop genome are higher. Transgenic technology alone cannot achieve site-specific modification in the true sense because it requires a transgenic library [73]. The cells in the library may be edited at each site. Only through time-consuming and laborious screening, it is possible to find the edited cells at a specific site. Although, combined DNA recombination techniques (such as combined homologous recombination and site-specific recombination) can reduce the burden of screening for target editing [2]. However, its modification efficiency is lower than that of gene editing technology [14]. In addition, gene editing technology can also utilize a more efficient non-HR pathway for precise insertion [58], which further improves the editing efficiency.

Stability: It is easy to insert unintended multi-copy target genes using transgenic technology, which is more common when using biolistic technology for transformation, and the insertion of multi-copy genes can easily cause gene silencing [11]. In addition, the process of using transgenic technology to insert the target gene is random, and the resulting hereditary traits are not stable. Since the transgenic mutation rate will increase, the transgene will be inactivated or silenced in the offspring, whereas, gene editing technology has more stable genetic effects due to its specific targeting.

Applicability: Transgenic technology is used to transfer a target gene fragment into the target genome, and it is generally only suitable for gene knock-in when used alone [10,74]. Gene editing technology uses a series of proteins and nucleic acids to bind and act on specific bases or fragments of the target genome, which can produce fragment knock-out, knock-in, substitution, and other modification types, as well as a wider range of applications [53,58,60].

In conclusion, the advantages of gene editing technology, such as safety, high efficiency, stability, and applicability, make it superior to transgenic technology (Table 1).

Table 1. Comparison between transgenic technology and gene editing technology.

Serial No.	Parameter	Transgenic Technology	Gene Editing Technology	Reference
1	Whether it is targeted	No targeting	Targeting using proteins or nucleic acids	[53,58,60]
2	Is it possible to edit without exogenous DNA	No	Yes	[55,57]
3	The efficiency of target genome modification	Low	High	[2,14,73]
4	Probability of causing gene silencing	High	Low	[11]
5	Genetic stability	Low	High	[11]
6	Types of modifications that can be generated	Single	Diverse	[10,53,58,60,74]

5. Optimization of Genetic Transformation and Regeneration Efficiency

Efficient genetic transformation and regeneration are some of the basic conditions for using genetic engineering technology to cultivate new germplasms and shorten the necessary research time. Over the past 30 years, through people's efforts, the species dependence of genetic transformation has been gradually broken. However, there are still some major defects in crop genetic transformation, such as high genotype dependence,

time-consuming regeneration, and so on. Therefore, the attention of researchers gradually shifted from changing culture conditions to manipulating developmental regulatory factors.

Iwase et al. [75,76] found that overexpression of the AP2/ERF transcription factor *Wound Induced Dedifferentiation1* (*WIND1*) in *Brassica napus (B. napus)* can enhance the ability of cell bud regeneration. At the same time, it has been proved that *WIND1* combined with other developmental regulators can produce a stronger and more specific regeneration effect. Lowe et al. overexpressed maize (*Zea mays*) *Baby boom* (*Bbm*) and maize *Wuschel2* (*Wus2*) genes in multiple maize inbred lines with recalcitrant conversion disorder and achieved high-frequency transformation [77]. Debernardi et al. found that co-transformation of the GRF4–GIF1 protein could significantly improve the efficiency of transformation and regeneration and shorten the duration of the transformation and regeneration process of tetraploid wheat from 91 to 56 days [78]. The practicability of GRF4–GIF1 protein co-transformation has also been verified in rice, citrus, watermelon, and other crops [78,79]. In addition, overexpression of the GRF5 gene has also been found to promote the transformation and regeneration of many monocotyledons and dicotyledons [80]. The expression of these developmental regulatory genes reduced the genotype dependence of genetic transformation to some extent and improved the efficiency of genetic transformation and regeneration. However, these genes can easily cause abnormal callus differentiation, deformity, and fertility problems in regenerated plants, which are relatively cumbersome to treat. Recently, Wang et al. identified the regeneration-related gene *TaWOX* in wheat. Overexpression of this gene can improve the efficiency of regeneration while the callus differentiation process and root development of regenerated plants will not be affected, basically overcoming the genotypic barrier of wheat genetic transformation [81].

Successive regeneration acclimation (SRA) is the second kind of strategy to improve genetic transformation and regeneration. Li et al. used this strategy to obtain Jin668 cotton materials with high genetic transformation efficiency. Further analysis showed that the decrease of gene methylation level during SRA was beneficial for the activation of genes related to somatic embryogenesis, thus improving the crop's regeneration ability [82].

However, post-transformation regeneration is a key speed-limiting step in the research process, and if the tissue culture stage can be skipped, the research efficiency will be greatly improved. In this regard, the crop regeneration dependent on mobile RNA elements is a promising strategy. Flowering Locus T (FT) is transcribed in leaves and then moved to the meristem of the stem tip to induce flowering. With the help of this characteristic, Ellison et al. fused Flowering Locus T (FT) RNA with sgRNA and constructed it into a tobacco rattle virus (TRV) vector. After genetic transformation, *N. benthamiana* [83] with heritable gene editing ability was obtained without tissue culture. In addition, Li et al. [84] fused three RNA mobile elements, i.e., mutated AtFT, a truncated wheat FT RNA sequence, and tRNAMet with sgRNA, and constructed them into a barley stripe mosaic virus (BSMV) vector. After transformation, they successfully constructed gene-edited hexaploid wheat with high efficiency and heredity without tissue culture. In other words, these research results put forward a new scheme for improving the genetic transformation and regeneration efficiency of crops.

6. Application of Genetic Engineering Technology in Improving Crop Yield and Quality

6.1. Transgenic Technology

Wang et al. [85] used transgenic technology to transfer the *TmNAS3* gene into the wheat genome under the mediation of *Agrobacterium* and used a ubiquitin promoter to drive the expression. The gene activating genes related to metal response increased the iron content of wheat grains to 68.75 μg/g, more than double that of the wild type. At the same time, through further studies, they found that the expression of *TmNAS3* also increased the size of wheat grains and, thus, increased yield [85]. Using transgenic technology to insert target genes is full of randomness. However, through homologous recombination technology and specific site recombination technology, etc., the targeted insertion of the target gene can be achieved.

6.2. DNA Recombination Technology

Zhu et al. [2] used DNA recombination technology to develop a multi-gene stacking system. Using eight genes related to anthocyanin synthesis, they designed the biosynthetic pathway of anthocyanins in rice endosperm and developed rice that is rich in anthocyanins in the endosperm. It is worth noting that anthocyanins have strong antioxidant properties and have great application value in the treatment of cardiovascular diseases and some cancers [2]. In addition, parts of RNA have been found to inhibit transcription or translation processes, resulting in RNA-mediated gene silencing techniques [86].

6.3. RNA Interference Technology

In maize, Zhang et al. [87] silenced the *Se1* gene by means of RNA interference, which increased the soluble sugar content by affecting the starch metabolism of the endosperm, making the mutant maize more favorable for intestinal digestion. Wang et al. [88] used MIM168 to inhibit the expression of microRNA168 in rice, increase the expression of the *AGO1* gene, and then increase the number of panicles. Compared with the control rice, the yield was increased by 30–40%.

However, RNA-mediated gene silencing technology can only inhibit gene expression but, generally, cannot completely change the target sequence in the genome. Therefore, the use of gene editing technology further broadens the application scope of genetic engineering.

6.4. Gene Editing Technology

Lu et al. [89] transformed the Per8–Cas9 vector into japonica rice varieties ZH11 and KY131 under the mediation of *Agrobacterium*. Knockout of the *OsAAP6* gene using the CRISPR/Cas9 system then promoted the rice tillering, thereby increasing rice yield. Zhang et al. [90] used the CRISPR/Cas9 system to edit the *Ppo* gene of wheat, which affected the expression of polyphenol oxidase, inhibited dough browning, and improved the quality of wheat. Perez et al. [91] used the CRISPR/Cas9 system to edit the *Wx* gene of rice and reduced the amylose content of rice by downregulating the expression of granule-bound starch synthase I (GBSSI). In the homozygous progeny, the amylose content was as low as 5%, which caused the abnormal cell structure of the aleurone layer and starch granule structure, which affected the quality of rice. The result suggests that editing such genes directly corresponding to the target traits is possibly accompanied by negative effects. Zeng et al. [92], starting from cis-regulatory elements (CREs), successfully developed a kind of soft rice with better quality, which provides another way to regulate gene expression [92]. Moreover, using the CRISPR/Cas9 multiple editing method, one can edit multiple target sites at the same time, which has advantages in shortening the breeding time and making better use of the advantages of quantitative traits. Wang et al. [93] used the CRISPR/Cas9 system to edit the *ZmBADH2a* and *ZmBADH2b* genes of maize and found that only when both genes were mutated could the formation of the aroma of maize popcorn be promoted. They succeeded in creating the world's first aromatic corn. Xu et al. [94] inserted the sequence of the CRISPR/Cas9 multiple gene knockout systems into T-DNA to express it in rice. They then edited the three negatively regulated genes *GW2*, *GW5,* and *TGW6* related to grain weight. The three-gene mutants produced were significantly better than the wild type in grain length, grain width, and grain weight, and the yield could be increased by up to 29.84%. Miao et al. [95] performed a combined knockout of *PYL*-family-related genes and found that the combined knockout of *PYL1-4-6* had the strongest ability to increase yield. Rice yield was increased by 25–31%, while stress adaptability was hardly affected. Furthermore, since Cas proteins are PAM-site dependent, this defect presents a limitation to the achievement of arbitrary target-site editing [36]. Therefore, researchers developed a series of Cas protein variants through the methods of determinate evolution and structure orientation, which have been applied in crop improvement. Huang et al. [96] used the ABEmax–nCas9NG system and the Anc689CEBmax–nCas9NG system, which is less restricted by PAM sites, to edit the intermediate domain of Wx protein, and base

substitutions were performed at five sites in exons 7 and 10, respectively. The *waxy*^{abe2} mutant was screened out, and it was found that the mutant had the advantages of low amylose content (AC) and improved taste and appearance and was very suitable for developing into a new type of soft rice. Liu et al. [97] used ScCas9n^{++}, which has a wider recognition range for PAM sites than nCas9, and fused it with cytosine deaminase PmCDA1 after codon optimization, resulting in a PevoCDA1–ScCas9n^{++} with NNG-PAMs recognition ability and high editing efficiency. By editing exons 11 and 13 of *OsWx*, rice with reduced amylose content was also developed. This Cas9 variant with a lower preference for PAM sites is more important for more refined editing methods, since BEs and PEs are often used for one or more base substitutions.

In conclusion, genetic engineering technology, especially gene editing technology, has powerful capabilities in improving crop yield and quality (Table 2).

Table 2. Application of genetic engineering technology in improving crop yield and quality.

Genetic Engineering Technology	Target	Crop	Crop Benefit	References
Transgenic technology	Knock in *TmNAS3*	Wheat	Increase grain size and Fe content	[85]
Transgenic technology	Overexpress *TaSTT3b-2B*	Wheat	Increase grain weight for higher yield	[98]
Transgenic technology	Knock in *ScAT10*	Maize	Increase the ratio of p-coumaric acid/ferulic acid	[99]
Transgenic technology	Overexpress *ZmCGT1*	Maize	Increase the isoorientin content in maize silk	[100]
DNA recombinant technology	Knock in eight genes related to anthocyanin synthesis	Rice	Increase the rice anthocyanins in the endosperm	[2]
RNA silencing technology	Silence microRNA168	Rice	Increase the number of ears for higher yield	[88]
RNA silencing technology	Silence *Se1*	Maize	Increase soluble sugar content	[87]
RNA silencing technology combined with transgenic technology	Overexpress *CrtB* and silence *TaHYD*	Wheat	Increase the β-carotene content in wheat endosperm	[101]
CRISPR/Cas12a single site editing technology	Edit *TaGW7-B1*	Wheat	Increase grain weight for higher yield	[102]
CRISPR/Cas9 single site editing technology	Knock out *OsAAP3*	Rice	Increase tiller number for higher yield	[89]
CRISPR/Cas9 single site editing technology	Edit *OsSPL4*	Rice	Generate two new high-quality alleles for higher yield	[103]
CRISPR/Cas9 single site editing technology	Edit CREs of *Wx*	Rice	Reduce amylose content appropriately	[92]
CRISPR/Cas9 single site editing technology	Knock out *OsHXK*	Rice	Increase the rate of photosynthesis for higher yield	[104]
CRISPR/Cas9 single site editing technology	Edit *OsBADH2*	Rice	Produce moderate aroma	[105]
CRISPR/Cas9 single site editing technology	Knock out *Ppo*	Wheat	Inhibite dough browning	[90]
CRISPR/Cas9 single site editing technology	Edit *ZmACO2*	Maize	Increase the number of grains per ear for higher yield	[106]
CRISPR/Cas9 multiple sites editing technology	Knock out *GW2*, *GW5*, and *TGW6*	Rice	Increase the volume and the weight of grains for higher yield	[94]
CRISPR/Cas9 multiple sites editing technology	Knock out *PYL*1, *PYL*4, and *PYL*6	Rice	Increase yield	[95]
CRISPR/Cas9 multiple sites editing technology	Knock out *ZmBADH2a* and *ZmBADH2b*	Maize	Produce popcorn aroma	[93]
ABEmax-nCas9NG, Anc689CEBmax-nCas9NG	Substitute base of *Wx*	Rice	Reduce amylose content appropriately	[96]
PevoCDA1-ScCas9n^{++}	Substitute base of *OsWx*	Rice	Reduce amylose content appropriately	[97]

7. Prospects

To make gene editing more flexible and efficient, researchers have made great efforts in searching for Cas protein homologs and engineering targeted modification. The dependence of the Cas protein on the PAM site and the off-target effects that exist during gene editing have been effectively reduced, e.g., via *Streptococcus canis* Cas9 (ScCas9) [107], ScCas9n^{+} [97], ScCas9n^{++} [108], HypaCas9 [109], SpCas9-NG [110], SpG [44], and SpRY [44]. Although the ability to identify the PAM sites of these Cas protein variants has been greatly improved, it is found that there is a preference for PAM sites during practical use [44]. For example, any site can be edited theoretically by using the SpRY system, but its ability to

identify NAN and NGN sites is stronger, and its activity toward NTN and NCN sites is lower. Hence, there are still some limitations in its use [44]. In addition, these variants may also have a self-targeted editing effect, which may easily lead to sgRNA base deletions. Although these sgRNAs still have targeting ability, they can cause higher off-target effects [111]. Therefore, the influence of the self-editing effects should be considered when developing and utilizing these variants and the molecular mechanism of the self-editing effect should be further revealed to improve the editing accuracy while expanding the recognition range of the PAM sites of the Cas protein.

Genetic engineering technology has few applications in directly promoting fruit size and quantity and accumulating trace elements and plant nutrients. One of the reasons may be that the genes controlling good traits in crops are mostly quantitative trait genes, which requires a higher understanding of gene regulatory networks [94]. Therefore, further improvement in the understanding of gene regulatory networks is the basis for better crop improvement. In addition, the method of knocking out target genes through NHEJ has attracted a great deal of attention in improving crop yield and quality. However, gene targeted insertion or substitution can more freely integrate the genes. Moreover, the HR repair pathway is too inefficient, which limits the application of site-specific insertion and substitution. To improve the repairing efficiency, a more promising approach is to use RNA as an HR repair template [112] because RNA repair templates can be generated by transcription in plants, providing more stable templates for repair continuously. Secondly, BE and PE systems should be utilized and optimized. In particular, the reasonable development of a twinPE system suitable for plant editing can more efficiently achieve the deletion, replacement, integration, and inversion of large fragments of DNA at specific sites. Avoiding the HR approach to achieve editing is also expected to improve crops more efficiently and accurately.

In conclusion, genetic engineering technologies make the process of crop improvement more efficient and controllable. With the continuous improvement of related technologies, the process of accurately improving important agronomic traits such as crop yield and quality and, further, directionally creating new crop germplasm has been accelerated.

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References

1. Tian, Z.; Wang, J.; Li, J.; Han, B. Designing future crops: Challenges and strategies for sustainable agriculture. *Plant J. Cell Mol. Biol.* **2021**, *105*, 1165–1178. [CrossRef]
2. Zhu, Q.; Yu, S.; Zeng, D.; Liu, H.; Wang, H.; Yang, Z.; Xie, X.; Shen, R.; Tan, J.; Li, H.; et al. Development of "purple endosperm rice" by engineering anthocyanin biosynthesis in the endosperm with a high-efficiency transgene stacking system. *Mol. Plant* **2017**, *10*, 918–929. [CrossRef] [PubMed]
3. Cucina, M.; Regni, L. New advances on nutrients recovery from agro-industrial and livestockwastes for sustainable farming. *Agronomy* **2021**, *11*, 2308. [CrossRef]
4. Lanigan, T.M.; Kopera, H.C.; Saunders, T.L. Principles of genetic engineering. *Genes* **2020**, *11*, 291. [CrossRef] [PubMed]
5. Herrera-Estrella, L.; Depicker, A.; Montagu, M.V.; Schell, J. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* **1983**, *303*, 209–213. [CrossRef]

6. Mackelprang, R.; Lemaux, P.G. Genetic engineering and editing of plants: An analysis of new and persisting questions. *Annu. Rev. Plant Biol.* **2020**, *71*, 659–687. [CrossRef] [PubMed]
7. Luo, X.; Liu, Z.; Zhang, S.; Shen, S.; Wang, D.; Wang, J. The progress of plant transgenic technology. *Chin. Agric. Sci. Bull.* **2014**, *30*, 234–240.
8. Gelvin, S.B. *Agrobacterium*-mediated plant transformation: The biology behind the "gene-jockeying" tool. *Microbiol. Mol. Biol. Rev. MMBR* **2003**, *67*, 16–37. [CrossRef]
9. Helenius, E.; Boije, M.; Niklander-Teeri, V.; Palva, E.T.; Teeri, T.H. Gene delivery into intact plants using the helios^{TM} gene gun. *Plant Mol. Biol. Rep.* **2000**, *18*, 278–288. [CrossRef]
10. Ribeiro, T.P.; Lourenco-Tessutti, I.T.; de Melo, B.P.; Morgante, C.V.; Filho, A.S.; Lins, C.B.J.; Ferreira, G.F.; Mello, G.N.; Macedo, L.L.P.; Lucena, W.A.; et al. Improved cotton transformation protocol mediated by *Agrobacterium* and biolistic combined-methods. *Planta* **2021**, *254*, 20. [CrossRef]
11. Travella, S.; Ross, S.M.; Harden, J.; Everett, C.; Snape, J.W.; Harwood, W.A. A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. *Plant Cell Rep.* **2005**, *23*, 780–789. [CrossRef]
12. Gurusaravanan, P.; Vinoth, S.; Jayabalan, N. An improved *Agrobacterium*-mediated transformation method for cotton (*Gossypium hirsutum* L. 'KC3') assisted by microinjection and sonication. *In Vitro Cell. Dev. Biol.-Plant* **2020**, *56*, 111–121. [CrossRef]
13. Yan, Y.; Zhu, X.; Yu, Y.; Li, C.; Zhang, Z.; Wang, F. Nanotechnology strategies for plant genetic engineering. *Adv. Mater.* **2021**, *34*, e2106945. [CrossRef]
14. Kim, H.; Kim, J.S. A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* **2014**, *15*, 321–334. [CrossRef]
15. Moscou, M.J.; Bogdanove, A.J. A simple cipher governs DNA recognition by TAL effectors. *Science* **2009**, *326*, 1501. [CrossRef]
16. Nishimasu, H.; Ran, F.A.; Hsu, P.D.; Konermann, S.; Shehata, S.I.; Dohmae, N.; Ishitani, R.; Zhang, F.; Nureki, O. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **2014**, *156*, 935–949. [CrossRef]
17. Lieber, M.R.; Ma, Y.; Pannicke, U.; Schwarz, K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 712–720. [CrossRef]
18. Murray, J.M.; Carr, A.M. Integrating DNA damage repair with the cell cycle. *Curr. Opin. Cell Biol.* **2018**, *52*, 120–125. [CrossRef]
19. Begemann, M.B.; Gray, B.N.; January, E.; Gordon, G.C.; He, Y.; Liu, H.; Wu, X.; Brutnell, T.P.; Mockler, T.C.; Oufattole, M. Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases. *Sci. Rep.* **2017**, *7*, 11606. [CrossRef]
20. Wang, M.; Lu, Y.; Botella, J.R.; Mao, Y.; Hua, K.; Zhu, J. Gene targeting by homology-directed repair in rice using a geminivirus-based CRISPR/Cas9 system. *Mol. Plant* **2017**, *10*, 1007–1010. [CrossRef]
21. Carroll, D. Genome engineering with Zinc-Finger nucleases. *Genetics* **2011**, *188*, 773–782. [CrossRef] [PubMed]
22. Wolfe, S.A.; Nekludova, L.; Pabo, C.O. DNA recognition by cys2his2 Zinc Finger proteins. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 183–212. [CrossRef]
23. Li, L.; Wu, L.P.; Chandrasegaran, S. Functional domainsin *Fok*I restriction endonuclease. *Proc. Natl. Acad. Sci. USA* **1992**, *8*, 4275–4279. [CrossRef]
24. Shukla, V.K.; Doyon, Y.; Miller, J.C.; DeKelver, R.C.; Moehle, E.A.; Worden, S.E.; Mitchell, J.C.; Arnold, N.L.; Gopalan, S.; Meng, X.; et al. Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature* **2009**, *459*, 437–441. [CrossRef] [PubMed]
25. Jung, Y.-J.; Nogoy, F.M.; Lee, S.-K.; Cho, Y.-G.; Kang, K.-K. Application of ZFN for Site Directed Mutagenesis of Rice SSIVa Gene. *Biotechnol. Bioprocess Eng.* **2018**, *23*, 108–115. [CrossRef]
26. Khan, S.H. Genome-editing technologies: Concept, pros, and cons of various genome-editing techniques and bioethical concerns for clinical application. *Mol. Ther. Nucleic Acids* **2019**, *16*, 326–334. [CrossRef] [PubMed]
27. Boch, J.; Bonas, U. *Xanthomonas* AvrBs3 family-type III effectors: Discovery and function. *Annu. Rev. Phytopathol.* **2010**, *48*, 419–436. [CrossRef] [PubMed]
28. Boch, J.; Scholze, H.; Schornack, S.; Landgraf, A.; Hahn, S.; Kay, S.; Lahaye, T.; Nickstadt, A.; Bonas, U. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **2009**, *326*, 1509–1512. [CrossRef]
29. Chandrasekaran, A.P.; Song, M.; Ramakrishna, S. Genome editing: A robust technology for human stem cells. *Cell. Mol. Life Sci. CMLS* **2017**, *74*, 3335–3346. [CrossRef]
30. Mussolino, C.; Morbitzer, R.; Lutge, F.; Dannemann, N.; Lahaye, T.; Cathomen, T. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* **2011**, *39*, 9283–9293. [CrossRef]
31. Briggs, A.W.; Rios, X.; Chari, R.; Yang, L.; Zhang, F.; Mali, P.; Church, G.M. Iterative capped assembly: Rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* **2012**, *40*, e117. [CrossRef]
32. Li, T.; Liu, B.; Chen, C.Y.; Yang, B. TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base Change and Herbicide-Resistant Rice. *J. Genet. Genom. Yi Chuan Xue Bao* **2016**, *43*, 297–305. [CrossRef]
33. Liang, Z.; Zhang, K.; Chen, K.; Gao, C. Targeted Mutagenesis in Zea mays Using TALENs and the CRISPR/Cas System. *J. Genet. Genom.* **2014**, *41*, 63–68. [CrossRef]
34. Luo, M.; Li, H.; Chakraborty, S.; Morbitzer, R.; Rinaldo, A.; Upadhyaya, N.; Bhatt, D.; Louis, S.; Richardson, T.; Lahaye, T.; et al. Efficient TALEN-mediated gene editing in wheat. *Plant Biotechnol. J.* **2019**, *17*, 2026–2028. [CrossRef]
35. Cox, D.B.; Platt, R.J.; Zhang, F. Therapeutic genome editing: Prospects and challenges. *Nat. Med.* **2015**, *21*, 121–131. [CrossRef]
36. Marraffini, L.A.; Sontheimer, E.J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **2010**, *463*, 568–571. [CrossRef]

37. Gohil, N.; Bhattacharjee, G.; Lam, N.L.; Perli, S.D.; Singh, V. CRISPR-Cas systems: Challenges and future prospects. *Prog. Mol. Biol. Transl. Sci.* **2021**, *180*, 141–151. [CrossRef]
38. Sapranauskas, R.; Gasiunas, G.; Fremaux, C.; Barrangou, R.; Horvath, P.; Siksnys, V. The *Streptococcus* thermophilus CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* **2011**, *39*, 9275–9282. [CrossRef]
39. Yusa, K.; Rashid, S.T.; Strick-Marchand, H.; Varela, I.; Liu, P.Q.; Paschon, D.E.; Miranda, E.; Ordonez, A.; Hannan, N.R.; Rouhani, F.J.; et al. Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* **2011**, *478*, 391–394. [CrossRef]
40. Fu, Y.; Sander, J.D.; Reyon, D.; Cascio, V.M.; Joung, J.K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* **2014**, *32*, 279–284. [CrossRef]
41. Guilinger, J.P.; Thompson, D.B.; Liu, D.R. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat. Biotechnol.* **2014**, *32*, 577–582. [CrossRef] [PubMed]
42. Zuo, E.; Sun, Y.; Yuan, T.; He, B.; Zhou, C.; Ying, W.; Liu, J.; Wei, W.; Zeng, R.; Li, Y.; et al. A rationally engineered cytosine base editor retains high on-target activity while reducing both DNA and RNA off-target effects. *Nat. Methods* **2020**, *17*, 600–604. [CrossRef]
43. Neha, D.; Momin, M.; Khan, T.; Gharat, S.; Ningthoujam, R.S.; Omri, A. Metallic nanoparticles as drug delivery system for the treatment of cancer. *Expert Opin. Drug Deliv.* **2021**, *18*, 1261–1290. [CrossRef] [PubMed]
44. Walton, R.T.; Christie, K.A.; Whittaker, M.N.; Kleinstiver, B.P. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science* **2020**, *368*, 6488. [CrossRef] [PubMed]
45. Komor, A.C.; Kim, Y.B.; Packer, M.S.; Zuris, J.A.; Liu, D.R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **2016**, *533*, 420–424. [CrossRef] [PubMed]
46. Gaudelli, N.M.; Komor, A.C.; Rees, H.A.; Packer, M.S.; Badran, A.H.; Bryson, D.I.; Liu, D.R. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* **2017**, *551*, 464–471. [CrossRef] [PubMed]
47. Kurt, I.C.; Zhou, R.; Iyer, S.; Garcia, S.P.; Miller, B.R.; Langner, L.M.; Grunewald, J.; Joung, J.K. CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nat. Biotechnol.* **2021**, *39*, 41–46. [CrossRef] [PubMed]
48. Newby, G.A.; Liu, D.R. In vivo somatic cell base editing and prime editing. *Mol. Ther. J. Am. Soc. Gene Ther.* **2021**, *29*, 3107–3124. [CrossRef]
49. Zhao, D.; Li, J.; Li, S.; Xin, X.; Hu, M.; Price, M.A.; Rosser, S.J.; Bi, C.; Zhang, X. Glycosylase base editors enable C-to-A and C-to-G base changes. *Nat. Biotechnol.* **2021**, *39*, 35–40. [CrossRef]
50. Hua, K.; Tao, X.; Yuan, F.; Wang, D.; Zhu, J.K. Precise A.T to G.C Base Editing in the Rice Genome. *Mol. Plant* **2018**, *11*, 627–630. [CrossRef]
51. Zong, Y.; Song, Q.; Li, C.; Jin, S.; Zhang, D.; Wang, Y.; Qiu, J.L.; Gao, C. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nat. Biotechnol.* **2018**, *36*, 950–953. [CrossRef]
52. Zeng, D.; Liu, T.; Tan, J.; Zhang, Y.; Zheng, Z.; Wang, B.; Zhou, D.; Xie, X.; Guo, M.; Liu, Y.-G.; et al. PhieCBEs: Plant High-Efficiency Cytidine Base Editors with Expanded Target Range. *Mol. Plant* **2020**, *13*, 1666–1669. [CrossRef]
53. Xu, R.; Kong, F.; Qin, R.; Li, J.; Liu, X.; Wei, P. Development of an efficient plant dual cytosine and adenine editor. *J. Integr. Plant Biol.* **2021**, *63*, 1600–1605. [CrossRef]
54. Anzalone, A.V.; Randolph, P.B.; Davis, J.R.; Sousa, A.A.; Koblan, L.W.; Levy, J.M.; Chen, P.J.; Wilson, C.; Newby, G.A.; Raguram, A.; et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **2019**, *576*, 149–157. [CrossRef]
55. Alfatih, A.; Wu, J.; Jan, S.U.; Zhang, Z.S.; Xia, J.Q.; Xiang, C.B. Loss of rice *PARAQUAT TOLERANCE 3* confers enhanced resistance to abiotic stresses and increases grain yield in field. *Plant Cell Environ.* **2020**, *43*, 2743–2754. [CrossRef]
56. Cai, Y.; Wang, N.; Wang, S.; Wang, H. CRISPR/Cas9 mediated homology recombination insertion knockout of human *SH2B3* gene. *Chin. J. Cell Biol.* **2018**, *40*, 252–259. [CrossRef]
57. Komatsu, A.; Ohtake, M.; Shimatani, Z.; Nishida, K. Production of herbicide-sensitive strain to prevent volunteer rice infestation using a CRISPR-Cas9 cytidine deaminase fusion. *Front. Plant Sci.* **2020**, *11*, 925. [CrossRef]
58. Lin, Q.; Zong, Y.; Xue, C.; Wang, S.; Jin, S.; Zhu, Z.; Wang, Y.; Anzalone, A.V.; Raguram, A.; Doman, J.L.; et al. Prime genome editing in rice and wheat. *Nat. Biotechnol.* **2020**, *38*, 582–585. [CrossRef]
59. Shen, L.; Hua, Y.; Fu, Y.; Li, J.; Liu, Q.; Jiao, X.; Xin, G.; Wang, J.; Wang, X.; Yan, C.; et al. Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice. *Sci. China Life Sci.* **2017**, *60*, 506–515. [CrossRef]
60. Duan, K.; Cheng, Y.; Ji, J.; Wang, C.; Wei, Y.; Wang, Y. Large chromosomal segment deletions by CRISPR/LbCpf1-mediated multiplex gene editing in soybean. *J. Integr. Plant Biol.* **2021**, *63*, 1620–1631. [CrossRef]
61. Jiang, T.; Zhang, X.; Weng, Z.; Xue, W. Deletion and replacement of long genomic sequences using prime editing. *Nat. Biotechnol.* **2021**, *40*, 227–234. [CrossRef] [PubMed]
62. Choi, J.; Chen, W.; Suiter, C.C.; Lee, C.; Chardon, F.M.; Yang, W.; Leith, A.; Daza, R.M.; Martin, B.; Shendure, J. Precise genomic deletions using paired prime editing. *Nat. Biotechnol.* **2021**, *40*, 218–226. [CrossRef] [PubMed]
63. Lu, Y.; Tian, Y.; Shen, R.; Yao, Q.; Wang, M.; Chen, M.; Dong, J.; Zhang, T.; Li, F.; Lei, M.; et al. Targeted, efficient sequence insertion and replacement in rice. *Nat. Biotechnol.* **2020**, *38*, 1402–1407. [CrossRef]
64. Lin, Q.; Jin, S.; Zong, Y.; Yu, H.; Zhu, Z.; Liu, G.; Kou, L.; Wang, Y.; Qiu, J.L.; Li, J.; et al. High-efficiency prime editing with optimized, paired pegRNAs in plants. *Nat. Biotechnol.* **2021**, *39*, 923–927. [CrossRef] [PubMed]

65. Anzalone, A.V.; Gao, X.D.; Podracky, C.J.; Nelson, A.T.; Koblan, L.W.; Raguram, A.; Levy, J.M.; Mercer, J.A.M.; Liu, D.R. Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing. *Nat. Biotechnol.* **2021**. [CrossRef] [PubMed]
66. Qi, L.S.; Larson, M.H.; Gilbert, L.A.; Doudna, J.A.; Weissman, J.S.; Arkin, A.P.; Lim, W.A. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **2013**, *152*, 1173–1183. [CrossRef] [PubMed]
67. Lowder, L.G.; Zhang, D.; Baltes, N.J.; Paul, J.W.; Tang, X.; Zheng, X.; Voytas, D.F.; Hsieh, T.F.; Zhang, Y.; Qi, Y. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiol.* **2015**, *169*, 971–985. [CrossRef] [PubMed]
68. Lowder, L.G.; Zhou, J.; Zhang, Y.; Malzahn, A.; Zhong, Z.; Hsieh, T.F.; Voytas, D.F.; Zhang, Y.; Qi, Y. Robust transcriptional activation in plants using multiplexed CRISPR-Act2.0 and mTALE-Act systems. *Mol. Plant* **2018**, *11*, 245–256. [CrossRef]
69. Moradpour, M.; Abdulah, S.N.A. CRISPR/dCas9 platforms in plants: Strategies and applications beyond genome editing. *Plant Biotechnol. J.* **2020**, *18*, 32–44. [CrossRef]
70. Shen, S.; Lu, Z.; Jin, J.; Cai, Y. Genetic modes of epigenetic modification and its research progress. *Chin. Sci. Bull.* **2016**, *61*, 3878–3886. [CrossRef]
71. Gallego-Bartolome, J.; Gardiner, J.; Liu, W.; Papikian, A.; Ghoshal, B.; Kuo, H.Y.; Zhao, J.M.; Segal, D.J.; Jacobsen, S.E. Targeted DNA demethylation of the *Arabidopsis* genome using the human TET1 catalytic domain. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E2125–E2134. [CrossRef]
72. Papikian, A.; Liu, W.; Gallego-Bartolome, J.; Jacobsen, S.E. Site-specific manipulation of *Arabidopsis* loci using CRISPR-Cas9 SunTag systems. *Nat. Commun.* **2019**, *10*, 729. [CrossRef]
73. Ding, Y.; Shao, Y.; Xu, Y.; Chen, F. Screening citrinin mutants from the transformants library of *monascus ruber* M^{-7} by *Agrobacterium*-mediated DNA transfer. *Microbiol. China* **2006**, *4*, 52–57.
74. Wu, K.; Wu, Y.; Zhang, C.; Fu, Y.; Liu, Z.; Zhang, X. Simultaneous silencing of two different *Arabidopsis* genes with a novel virus-induced gene silencing vector. *Plant. Methods* **2021**, *17*, 6. [CrossRef]
75. Iwase, A.; Mita, K.; Nonaka, S.; Ikeuchi, M.; Koizuka, C.; Ohnuma, M.; Ezura, H.; Imamura, J.; Sugimoto, K. WIND1-based acquisition of regeneration competency in Arabidopsis and rapeseed. *J. Plant Res.* **2015**, *128*, 389–397. [CrossRef]
76. Iwase, A.; Mita, K.; Favero, D.S.; Mitsuda, N.; Sasaki, R.; Kobayshi, M.; Takebayashi, Y.; Kojima, M.; Kusano, M.; Oikawa, A.; et al. WIND1 induces dynamic metabolomic reprogramming during regeneration in *Brassica napus*. *Dev. Biol.* **2018**, *442*, 40–52. [CrossRef]
77. Lowe, K.; Wu, E.; Wang, N.; Hoerster, G.; Hastings, C.; Cho, M.J.; Scelonge, C.; Lenderts, B.; Chamberlin, M.; Cushatt, J.; et al. Morphogenic Regulators Baby boom and Wuschel Improve Monocot Transformation. *Plant Cell* **2016**, *28*, 1998–2015. [CrossRef] [PubMed]
78. Debernardi, J.M.; Tricoli, D.M.; Ercoli, M.F.; Hayta, S.; Ronald, P.; Palatnik, J.F.; Dubcovsky, J. A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nat. Biotechnol.* **2020**, *38*, 1274–1279. [CrossRef]
79. Feng, Q.; Xiao, L.; He, Y.; Liu, M.; Wang, J.; Tian, S.; Zhang, X.; Yuan, L. Highly efficient, genotype-independent transformation and gene editing in watermelon (*Citrullus lanatus*) using a chimeric ClGRF4-GIF1 gene. *J. Integr. Plant Biol.* **2021**, *63*, 2038–2042. [CrossRef]
80. Kong, J.; Martin-Ortigosa, S.; Finer, J.; Orchard, N.; Gunadi, A.; Batts, L.A.; Thakare, D.; Rush, B.; Schmitz, O.; Stuiver, M.; et al. Overexpression of the Transcription Factor GROWTH-REGULATING FACTOR5 Improves Transformation of Dicot and Monocot Species. *Front. Plant Sci.* **2020**, *11*, 572319. [CrossRef]
81. Wang, K.; Shi, L.; Liang, X.; Zhao, P.; Wang, W.; Liu, J.; Chang, Y.; Hiei, Y.; Yanagihara, C.; Du, L.; et al. The gene TaWOX5 overcomes genotype dependency in wheat genetic transformation. *Nat. Plants* **2022**, *8*, 110–117. [CrossRef] [PubMed]
82. Li, J.; Wang, M.; Li, Y.; Zhang, Q.; Lindsey, K.; Daniell, H.; Jin, S.; Zhang, X. Multi-omics analyses reveal epigenomics basis for cotton somatic embryogenesis through successive regeneration acclimation process. *Plant Biotechnol. J.* **2019**, *17*, 435–450. [CrossRef] [PubMed]
83. Ellison, E.E.; Nagalakshmi, U.; Gamo, M.E.; Huang, P.J.; Dinesh-Kumar, S.; Voytas, D.F. Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nat. Plants* **2020**, *6*, 620–624. [CrossRef] [PubMed]
84. Li, T.; Hu, J.; Sun, Y.; Li, B.; Zhang, D.; Li, W.; Liu, J.; Li, D.; Gao, C.; Zhang, Y.; et al. Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture. *Mol. Plant* **2021**, *14*, 1787–1798. [CrossRef]
85. Wang, H.; Liao, S.; Li, M.; Wei, J.; Zhu, B.; Gu, L.; Li, L.; Du, X. *TmNAS3* from Triticum monococum directly regulated by *TmbHLH47* increases Fe content of wheat grain. *Gene* **2021**, *811*, 146096. [CrossRef]
86. Fischer, S.E.J. RNA interference and MicroRNA-mediated silencing. *Curr. Protoc. Mol. Biol.* **2015**, *112*, 2611–2615. [CrossRef]
87. Zhang, X.; Mogel, K.J.H.V.; Lor, V.S.; Hirsch, C.N.; Vries, B.D.; Kaeppler, H.F.; Tracy, W.F.; Kaeppler, S.M. Maize *sugary enhancer1* (*se1*) is a gene affecting endosperm starch metabolism. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 20776–20785. [CrossRef]
88. Wang, H.; Li, Y.; Chern, M.; Zhu, Y.; Zhang, L.; Lu, J.; Li, X.; Dang, W.; Ma, X.; Yang, Z.; et al. Suppression of rice miR168 improves yield, flowering time and immunity. *Nat. Plants* **2021**, *7*, 129–136. [CrossRef]
89. Lu, K.; Wu, B.; Wang, J.; Zhu, W.; Nie, H.; Qian, J.; Huang, W.; Fang, Z. Blocking amino acid transporter *OsAAP3* improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnol. J.* **2018**, *16*, 1710–1722. [CrossRef]
90. Zhang, S.; Zhang, R.; Gao, J.; Song, G.; Li, J.; Li, W.; Qi, Y.; Li, Y.; Li, G. CRISPR/Cas9-mediated genome editing for wheat grain quality improvement. *Plant Biotechnol. J.* **2021**, *19*, 1684–1686. [CrossRef]

91. Perez, L.; Soto, E.; Farre, G.; Juanos, J.; Villorbina, G.; Bassie, L.; Medina, V.; Serrato, A.J.; Sahrawy, M.; Rojas, J.A.; et al. CRISPR/Cas9 mutations in the rice *Waxy*/GBSSI gene induce allele-specific and zygosity-dependent feedback effects on endosperm starch biosynthesis. *Plant Cell Rep.* **2019**, *38*, 417–433. [CrossRef]
92. Zeng, D.; Liu, T.; Ma, X.; Wang, B.; Zheng, Z.; Zhang, Y.; Xie, X.; Yang, B.; Zhao, Z.; Zhu, Q.; et al. Quantitative regulation of Waxy expression by CRISPR/Cas9-based promoter and 5′UTR-intron editing improves grain quality in rice. *Plant Biotechnol. J.* **2020**, *18*, 2385–2387. [CrossRef]
93. Wang, Y.; Liu, X.; Zheng, X.; Wang, W.; Yin, X.; Liu, H.; Ma, C.; Niu, X.; Zhu, J.; Wang, F. Creation of aromatic maize by CRISPR/Cas. *J. Integr. Plant Biol.* **2021**, *63*, 1664–1670. [CrossRef]
94. Xu, R.; Yang, Y.; Qin, R.; Li, H.; Qiu, C.; Li, L.; Wei, P.; Yang, J. Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. *J. Genet. Genom. Yi Chuan Xue Bao* **2016**, *43*, 529–532. [CrossRef]
95. Miao, C.; Xiao, L.; Hua, K.; Zou, C.; Zhao, Y.; Bressan, R.A.; Zhu, J. Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 6058–6063. [CrossRef]
96. Huang, X.; Su, F.; Huang, S.; Mei, F.; Niu, X.; Ma, C.; Zhang, H.; Zhu, X.; Zhu, J.; Zhang, J. Novel *Wx* alleles generated by base editing for improvement of rice grain quality. *J. Integr. Plant biology* **2021**, *63*, 1632–1638. [CrossRef]
97. Liu, T.; Zeng, D.; Zheng, Z.; Lin, Z.; Xue, Y.; Li, T.; Xie, X.; Ma, G.; Liu, Y.G.; Zhu, Q. The ScCas9^{++} variant expands the CRISPR toolbox for genome editing in plants. *J. Integr. Plant Biol.* **2021**, *63*, 1611–1619. [CrossRef]
98. Zhu, X.; Rong, W.; Wang, K.; Guo, W.; Zhou, M.; Wu, J.; Ye, X.; Wei, X.; Zhang, Z. Overexpression of *TaSTT3b-2B* improves resistance to sharp eyespot and increases grain weight in wheat. *Plant Biotechnol. J.* **2021**, 1–17. [CrossRef]
99. Fanelli, A.; Rancour, D.M.; Sullivan, M.; Karlen, S.D.; Ralph, J.; Riano-Pachon, D.M.; Vicentini, R.; Silva, T.D.F.; Ferraz, A.; Hatfield, R.D.; et al. Overexpression of a sugarcane BAHD acyltransferase alters hydroxycinnamate content in maize cell wall. *Front. Plant Sci.* **2021**, *12*, 626168. [CrossRef]
100. Sun, X.; Xue, X.; Wang, X.; Zhang, C.; Zheng, D.; Song, W.; Zhao, J.; Wei, J.; Wu, Z.; Zhang, Z. Natural variation of *ZmCGT1* is responsible for isoorientin accumulation in maize silk. *Plant J. Cell Mol. Biol.* **2022**, *109*, 64–76. [CrossRef]
101. Zeng, J.; Wang, X.; Miao, Y.; Wang, C.; Zang, M.; Chen, X.; Li, M.; Li, X.; Wang, Q.; Li, K.; et al. Metabolic engineering of wheat provitamin a by simultaneously overexpressing *CrtB* and silencing carotenoid hydroxylase (*TaHYD*). *J. Agric. Food Chem.* **2015**, *63*, 9083–9092. [CrossRef]
102. Wang, W.; Tian, B.; Pan, Q.; Chen, Y.; He, F.; Bai, G.; Akhunova, A.; Trick, H.N.; Akhunov, E. Expanding the range of editable targets in the wheat genome using the variants of the Cas12a and Cas9 nucleases. *Plant Biotechnol. J.* **2021**, *19*, 2428–2441. [CrossRef]
103. Hu, J.; Huang, L.; Chen, G.; Liu, H.; Zhang, Y.; Zhang, R.; Zhang, S.; Liu, J.; Hu, Q.; Hu, F.; et al. The elite alleles of *OsSPL4* regulate grain size and increase grain yield in rice. *Rice* **2021**, *14*, 90. [CrossRef]
104. Zheng, S.; Ye, C.; Lu, J.; Liufu, J.; Lin, L.; Dong, Z.; Li, J.; Zhuang, C. Improving the rice photosynthetic efficiency and yield by editing *OsHXK1* via CRISPR/Cas9 system. *Int. J. Mol. Sci.* **2021**, *22*, 9554. [CrossRef]
105. Hui, S.; Li, H.; Mawia, A.M.; Zhou, L.; Cai, J.; Ahmad, S.; Lai, C.; Wang, J.; Jiao, G.; Xie, L.; et al. Production of aromatic three-line hybrid rice using novel alleles of *BADH2*. *Plant Biotechnol. J.* **2022**, *20*, 59–74. [CrossRef] [PubMed]
106. Ning, Q.; Jian, Y.; Du, Y.; Li, Y.; Shen, X.; Jia, H.; Zhao, R.; Zhan, J.; Yang, F.; Jackson, D.; et al. An ethylene biosynthesis enzyme controls quantitative variation in maize ear length and kernel yield. *Nat. Commun.* **2021**, *12*, 5832. [CrossRef] [PubMed]
107. Chatterjee, P.; Jakimo, N.; Jacobson, J.M. Minimal PAM specificity of a highly similar SpCas9 ortholog. *Sci. Adv.* **2018**, *4*, eaau0766. [CrossRef] [PubMed]
108. Ma, G.; Kuang, Y.; Lu, Z.; Li, X.; Xu, Z.; Ren, B.; Zhou, X.; Zhou, H. CRISPR/Sc^{++}-mediated genome editing in rice. *J. Integr. Plant Biol.* **2021**, *63*, 1606–1610. [CrossRef]
109. Liu, M.S.; Gong, S.; Yu, H.H.; Jung, K.; Johnson, K.A.; Taylor, D.W. Engineered CRISPR/Cas9 enzymes improve discrimination by slowing DNA cleavage to allow release of off-target DNA. *Nat. Commun.* **2020**, *11*, 3576. [CrossRef]
110. Zhong, Z.; Sretenovic, S.; Ren, Q.; Yang, L.; Bao, Y.; Qi, C.; Yuan, M.; He, Y.; Liu, S.; Liu, X.; et al. Improving plant genome editing with high-fidelity xCas9 and non-canonical PAM-Targeting Cas9-NG. *Mol. Plant* **2019**, *12*, 1027–1036. [CrossRef]
111. Qin, R.; Li, J.; Liu, X.; Xu, R.; Yang, J.; Wei, P. SpCas9-NG self-targets the sgRNA sequence in plant genome editing. *Nat. Plants* **2020**, *6*, 197–201. [CrossRef]
112. Li, S.; Li, J.; He, Y.; Xu, M.; Zhang, J.; Du, W.; Zhao, Y.; Xia, L. Precise gene replacement in rice by RNA transcript-templated homologous recombination. *Nat. Biotechnol.* **2019**, *37*, 445–450. [CrossRef]