Chapter 7

The CRISPR/Cas9 System for Crop Improvement: Progress and Prospects

Kah-Yung Bernard Leong, Yee-Han Chan, Wan Muhamad Asrul Nizam Wan Abdullah, Swee-Hua Erin Lim and Kok-Song Lai

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75024

Abstract

The global demand for high-quality crops is continuously growing with time. Crop improvement techniques have a long history and they had been applied since the beginning of domestication of the first agricultural plants. Since then, various new techniques have and are being developed to further increase the commercial value and yield of crops. The latest crop improvement technique known as genome editing is a technique that enables precise modification of the plant genome via knocking out undesirable genes or enabling genes to gain new function. The variants generated from the genome editing are indistinguishable from naturally occurring variation. It is also less time-consuming and more readily accepted in the market commercially. The usage of genome editing has proven to be advantages and plays a promising role in future crop improvement efforts. Therefore, in this chapter, we aim to highlight the progress and application of genome editing techniques, in particular, the CRISPR/Cas9 system as a powerful genome editing tool for crop improvement. In addition, the challenges and future prospects of this technology for crop improvement will also be discussed.

Keywords: CRISPR/Cas9, crop improvement, genome editing, TALENs, ZFN

1. Introduction

As the agricultural commercial market continues to grow, development of new techniques for crop improvement is always in high demand; conversely, traditional breeding practices for crop improvement are phasing out as some techniques are far too time-consuming and

laborious and usually result in little yield. Examples of such traditional breeding are the selective crossbreeding between plants with the desired trait or classical breeding with induced mutation via radiation or chemicals. Currently, a new methodology that involves genetic engineering was developed and had paved the way to improve the quality of plants with high specificity for the attribute of interest; the application of site-specific nucleases (SSNs) [1].

SSNs are programmable nucleases that have the ability to produce DNA single-stranded or double-stranded breaks (DSBs) that activate the endogenous DNA repair pathways of the cells to repair the DNA damage and this usually leads to targeted mutagenesis [1, 2]. This technology empowers plant scientists to precisely regulate any genes in any plant while directly evaluating the function of that specific gene in the plant [3]. As a result, plants that express characteristics such as higher yield, disease resistance or shorter maturation periods can be generated through this methodology [4–6].

SSN-based genome editing system can be classified into three categories that are the zinc finger nuclease (ZFN), transcription-like effector nucleases (TALENs) and, the recently developed, clustered regularly interspaced short palindromic repeats that are associated with the RNA-guided Cas9 double-stranded DNA-binding protein (CRISPR/Cas9) [1]. The main differences between the categories lie in their mechanism of the double-stranded break induction and their efficiency in targeting their desired sequences [7]. These SSN-based genome editing systems are very powerful and they have undoubtedly revolutionized the agriculture industry.

1.1. Site-specific nucleases (SSNs)

SSNs have two major components, which are the engineered nonspecific endonucleases and the sequence-specific DNA-binding domains. The nonspecific nucleases have the ability to produce DSBs in DNA but they are very random as they lack specificity. On the other hand, DNA-binding domains are proteins that can specifically bind to DNA sequences that are complementary to them [8]. Hence, when DNA-binding domains synergize with the endonucleases, the paired components are able to introduce breaks at any specific target site [9, 10].

As shown in **Figure 1**, after the DNA break is induced, it will trigger the native DNA repair mechanisms of the cell to fix the break either by the error-prone nonhomologous end joining (NHEJ) mechanism or by the homology-directed repair (HDR) mechanism [11]. The NHEJ mechanism can occur during any phases of the cell cycle and due to its high erroneous repair rate, it occasionally forms frameshift mutation. Hence, this phenomenon can be exploited to form what is known as "knocking out" of a specific gene where the functionality of the protein encoded by that gene is lost due to random insertion, deletion or inversion. However, if provided an engineered DNA repair template that is homologous to the upstream and downstream of the target sequence, the HDR pathway can then be activated instead. The HDR mechanism functions to repair the break differently from which it will insert a new strand of DNA as a form of a desired mutation that resembles the DNA repair template [7]. This is also known as gene "knocking in" where the genome now has a new gain-of-function to encode a specific protein.

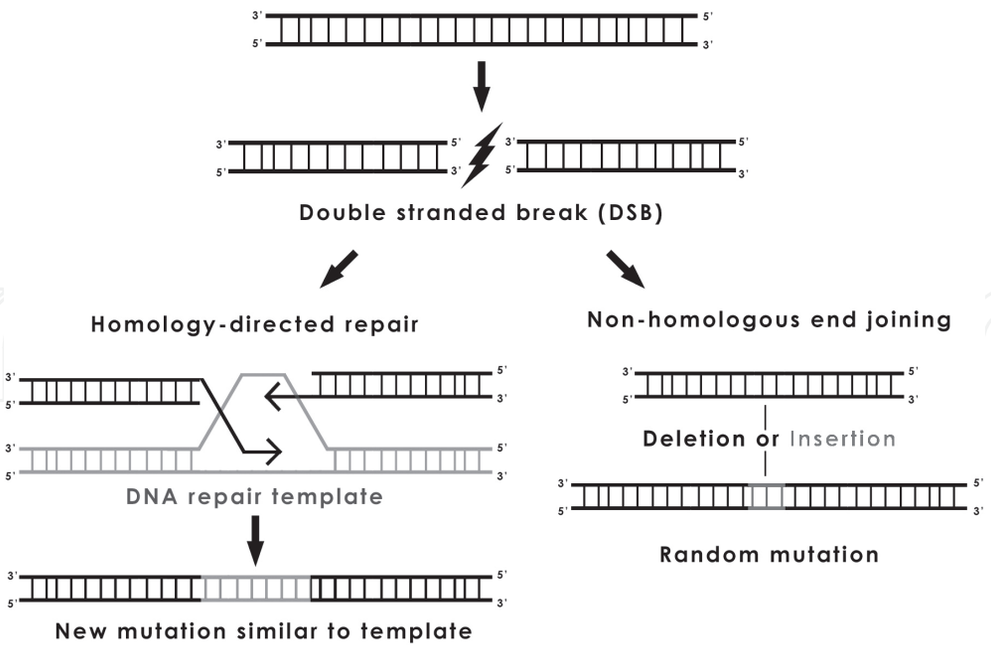

Figure 1. Different DNA repair mechanisms for DSBs. Left: if a DNA repair template is provided, the repair will proceed via the homology-directed repair mechanism where a desired mutation that is based on the template is introduced to the DNA. Right: the double-stranded break can be repaired via error-prone nonhomologous end joining mechanism where random mutation will be introduced to the DNA in the form of deletion or insertion. Adapted from Ott de Bruin et al. [12].

1.1.1. Zinc finger nuclease (ZFN)

One of the earliest SSNs developed for genome editing is known as the zinc finger nuclease (ZFN). ZFN is constructed through the coupling of two major domains, which are the eukaryotic-based DNA-binding domain known as the zinc finger protein (ZFP) and the endonuclease domain of the Fok1 restriction enzyme (**Figure 2**). Consequently, this coupling combines the quality of the DNA-binding specificity of the ZFP and cleaving activity of the Fok1 endonuclease into a single system, thus making ZFN a useful tool for genome editing [10].

Zinc finger proteins (ZFPs) have the ability to specifically bind to a discrete 3-base pairs (bp) sequence of DNA known as codons [12]. In fact, recent studies have shown that each ZFNs can use 3 zinc fingers to bind a 9-bp target sequence, and when made into a ZFN dimer, it can recognize up to 18-bp of a DNA [9, 13]. Furthermore, there are up to 64 possible types of ZFPs that can be produced as there are 64 codon combinations known today [10]. As a result, by determining which type of ZFPs are to be linked into the ZFN, researchers are able to design ZFNs in a way that they would only target desirable sequences. Additionally, studies have also shown that more fingers (up to six per ZFN) can be added to recognize longer and rarer target sequences [10].

Paired with the zinc fingers is the nonspecific Fok1 endonuclease domain, which is an enzyme that can be found naturally in *Flavobacterium okeanokoites* and it has the ability to induce DNA double-stranded breaks [14, 15]. By linking both domains together, they will now be known as the zinc finger nuclease, and the ZFP domain will lead the Fok1 endonuclease domain to the desired DNA sequence to cleave the target site, which is adjacent to it; this equips ZFN with the ability to precisely cut any targeted DNA sequences. In early studies, it has been

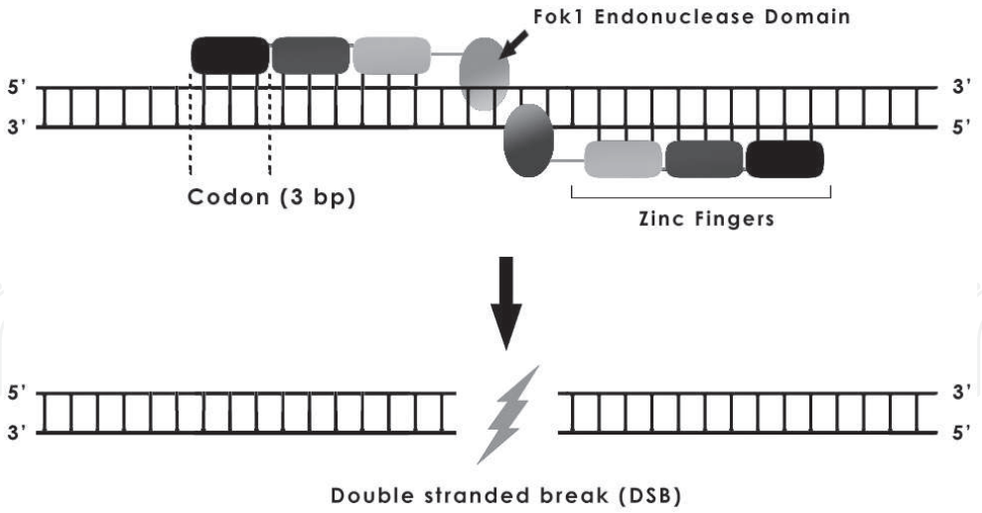

Figure 2. ZFN dimer binding to DNA at each side of the sequence. Different shaded boxes represent the different zinc fingers and each of them binds to a specific codon. Short dotted lines indicate 3-bp long codons. Shaded ellipses indicate the Fok1 endonuclease domain with the ability to induce DNA double-stranded breaks and they are coupled by the zinc fingers to guide them to their cleavage site. The cleavage site will be between the dimer and it is about 4-bp long. Adapted from Carroll [9].

known that the Fok1 endonuclease domain must dimerize to produce the breaks on DNA [13, 16]. However, the interaction between the dimer ZFN can be weak if it is not optimally designed. Thus, the optimal design for ZFN to achieve DNA cleavage is to direct two sets of fingers to neighboring sequences and join each to a Fok1 DNA-cleaving domain. Once the dimers bind to their respective sequences, adjacent cleavage factors will facilitate the dimerization and cleavage of the DNA strand [9]. Nevertheless, the construction of ZFN has proven to be difficult as there are many complicated interference in the interaction between the ZFPs despite many attempts made in the past to simplify them [17–23].

1.1.2. Transcription activator-like effector nucleases (TALENs)

It may seem that ZFN is the most practical method; nevertheless, the challenge lies in the construction as mentioned in addition to less popularity as the success rate for the DNA repair pathway via HDR is still considerably low [7]. Therefore, a more recent genome editing tool known as transcription activator-like effector nucleases (TALENs) had been developed with better modularity [24, 25]. TALENs are quite similar to ZFN in terms of the idea of directing the same nonspecific endonuclease to a specific site with the help of DNA-binding motifs (**Figure 3**).

Unlike ZFN, the DNA-binding domains for TALENs are known as the transcription activator-like effector (TALE) proteins rather than ZFPs and they are found in pathogenic plant bacteria (specifically of genus *Xanthomonas*) instead of in a eukaryotic cell. TALE protein is comprised of a repetitive sequence of a series of 34 amino acid residues, where each TALE protein has the ability to selectively bind to one nucleotide in the DNA target site [1]. Specifically, the pair residue at the 12th and 13th position is the one that determines the nucleotide specificity of the TALE proteins and they are known as the repeat variable domain (RVD).

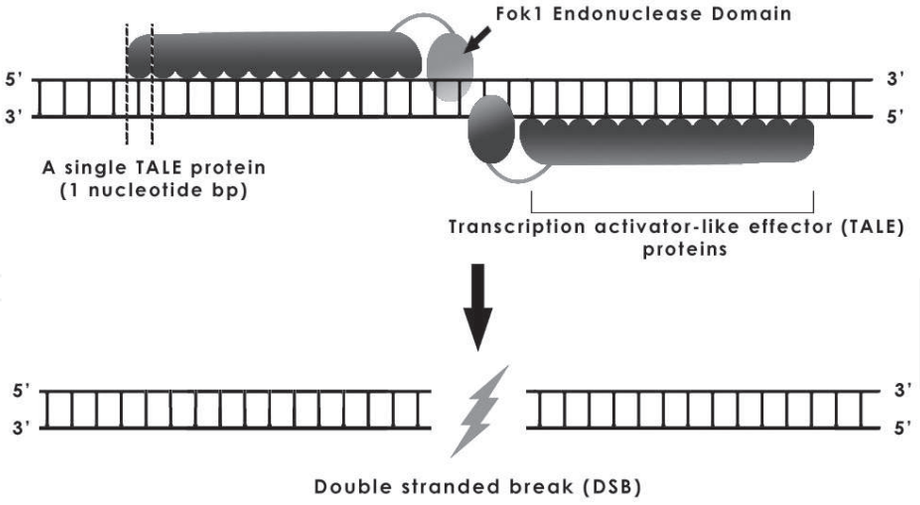

Figure 3. Transcription activator-like effector nuclease (TALEN) dimer bound to DNA. Like the ZFN, it has a Fok1 endonuclease domain that can cleave DNA. Indicated by small dotted lines, each TALE protein can specifically detect a single nucleotide base pair and it can be linked with more TALE proteins to detect longer sequences. By coupling the Fok1 endonuclease domain with the TALE proteins, it is now known as TALEN and it can specifically detect and cleave DNA. Adapted from Gupta [73].

TALE proteins have a different method to detect DNA sequences. They recognize a single nucleotide instead of DNA triplets. In fact, TALE proteins can also be assembled in a way to recognize virtually any DNA sequence that is desired by their users [26]. This attribute enables TALENs to have a higher range of target sites to suit user specifications, making them significantly more flexible and generally more straightforward. Like ZFN, the DNA-cleaving domain for TALENs is the nonspecific Fok1 endonuclease domain, which is highly dependent on the DNA-binding domains to achieve higher specificity of DSBs [1]. In a nut shell, both ZFNs and TALENs function as genome editing tools but they are considerably complex, a challenge to be constructed and less efficient compared to newly developed genome editing tool like CRISP/Cas9 system.

1.1.3. CRISPR/Cas9

The most recently developed SSN in genome editing technology is known as the CRISPR/Cas9 system. The CRISPR/Cas9 system was developed based on the bacterial type-II CRISPR/Cas adaptive immune system that is deployed by the hosts to recognize and eliminate any invading phage or plasmid DNA [27–29]. The three major components of the immune system are the protospacer-containing CRISPR RNA (crRNA), transactivating crRNA (tracrRNA) and the Cas9 endonuclease [27, 30].

During the invasion of foreign DNA, the bacterial type-II CRISPR/Cas system will integrate a short fragment of the foreign DNA, called "spacers," into the CRISPR genomic loci [31]. These spacers act as a form of an acquired immunity memory for the host. Then, the spacers will be transcribed and processed in the form of crRNA. Thus, the CRISPR genome loci act as the library where it can store information to enable the bacteria CRISPR/Cas9 system to retarget any known foreign DNA. In case of another attack by the invader, the crRNAs will then bind to the tracrRNAs that have the ability to trigger the direction of the Cas9 endonuclease

to the target site of the foreign DNA. After that, it will induce DSBs on the foreign DNA that carries the same protospacer sequence, which is accompanied by the protospacer adjacent motif (PAM) to disable the virus [7, 30]. As a matter of fact, recent studies have shown that the specificity of Cas9 protein is highly dependent to the PAM sequence as it licenses the landing of the crRNA-tracrRNA-Cas9 complex [32–34].

The CRISPR/Cas9 system has been redesigned to work more efficiently by fusing the Cas9 endonuclease with an artificial crRNA-tracrRNA chimera known as guide RNA (gRNA) [35–37]. The gRNA maintains the function of both crRNA and tracrRNA where just by itself is able to recognize the PAM-containing target sequence to direct the Cas9 protein for DNA cleaving activity. In fact, studies have shown that the gRNA works more efficiently compared to the combination of crRNA and tracrRNA [38, 39].

Compared to ZFNs and TALENs, the CRISPR/Cas9 system is potentially more efficient and effective with three main advantages [40], which are:

1. *Target design simplicity*: the DNA sequence targeting system of the CRISPR/Cas9 system is based on a formation of RNAs rather than protein or DNA recognition. RNA is much more accessible as it is simple and readily produced to be used to target any sequence in a genome.
2. *Efficiency*: the CRISPR/Cas9 system is a straightforward tool for genome editing. Modification of the target genome can be carried out by directly introducing RNAs that encode the Cas9 protein and gRNA to the host. This method allows researchers to skip all the long and laborious processes of the classical homologous recombination techniques.
3. Multiplexed mutation: several targeted mutations can be carried out in multiple genes at different locations at once by introducing multiple gRNAs at once. For example, Dr. Yang H. and his team have successfully simultaneously introduced mutation in five different genes in mouse embryonic stem cells [41–43].

2. Mechanism of CRISPR/Cas9 system

The CRISPR/Cas9 system is a type II adaptive immune system in bacteria and archaea, protecting them against invading nucleic acid such as virus by cleaving the foreign DNA through specific sequence recognition [29]. The immunity is acquired through the integration of short fragment of the foreign DNA as spacer between two adjacent repeats at the proximal end of a CRISPR locus [29]. The bacterial CRISPR/Cas9 system involves three stepwise processes, namely acquisition, biogenesis and interference.

2.1. Bacterial adaptive immunity: acquisition, biogenesis and interference

The defense mechanism of CRISPR/Cas9 system can be divided into three stages, which are spacer acquisition or adaptation, crRNA biogenesis and interference (**Figure 4**).

Conceptually, the spacer acquisition process can be further divided into two steps, which are protospacer selection and integration of the spacer into the CRISPR array to synthesize new repeat [44]. The protospacer selection step of the spacer acquisition stage is guided by a conserved DNA sequence element, namely protospacer adjacent motif (PAM), that is located downstream of the DNA target, which has the sequence of 5′-NGG-3 [44]. Next, the selected protospacer will be integrated into the leader-repeat boundary of CRISPR array as new spacer and is duplicated in order to synthesize new repeats. Each repeat is a 29 nucleotide sequence, and repeats are interspaced by five intervening 32-nucleotide nonrepetitive sequences [30].

Then, the CRISPR will be transcribed into a long precursor crRNA (pre-crRNA) with the help of Cas proteins (Cas1, Cas2, Cas9 and Cas4/casn2) or simply known as biogenesis. During this process, the tracrRNA will be transcribed from the bacterial genome as it is needed for processing of the pre-crRNA into a mature guide crRNA. Next, the antirepeat sequence of tracrRNA will enable the complementary base pairing with each pre-crRNA repeats, resulting in the formation of a crRNA-tracrRNA duplex [45].

During the target interference stage, the crRNA-tracrRNA duplex recognizes the PAM sequence located downstream of the protospacer in the foreign DNA and triggers the 'non–self-activation,' which prevents self-targeting of CRISPR array. After that, the duplex guides the Cas9 endonuclease to bind and cleave the DNA target, resulting in the formation of DSBs. Additionally, the DNA target specificity duplex is provided by what is called the 'seed sequence,' which is located at approximately 12 bases upstream of the PAM sequence that matches the RNA with the DNA target [29] (**Figure 4**).

2.2. Engineered CRISPR/Cas9 in plant genome editing

The CRISPR/Cas9 system for plant genome editing comprises four steps. First, a gene-specific gRNA is designed and constructed by fusing crRNA and tracrRNA. Many online tools have been developed for computer-based design of gRNA [46]. However, the in silico design of gRNA has not been fully adapted for plants, and large-scale data collection and systematic study of gRNA efficiencies in plant cells are needed to increase the accuracy of computational gRNA selection [46]. Both gRNA and Cas9 expression cassettes are constructed separately. The expression of gRNA is driven by U3 or U6 small nuclear RNA gene promoters with defined initiation and termination site, facilitating the transcription of gRNA by RNA polymerase III. The first nucleotide in the guide sequence is a 'G' if U6 promoter is used or an 'A' if U3 promoter is used [47]. Guide sequence should match the target, except for the first nucleotide (5′ G or A) that does not have to match [47]. In Cas9 expression, nuclear localization of Cas9 requires fusion of a single or dual nuclear localization signal (NLS) to the Cas9 coding sequence, which is 4107-bp in length. Both expression cassettes are then assembled into a vector.

The next step involves the transformation of protoplasts with CRISPR in which the activity of gRNA is best validated before being used in genome editing [46]. This step is followed by the selection of active CRISPR using polymerase chain reaction (PCR) or restriction enzyme digestion. Then, the CRISPR/Cas9 system in the form of vector is delivered into the target

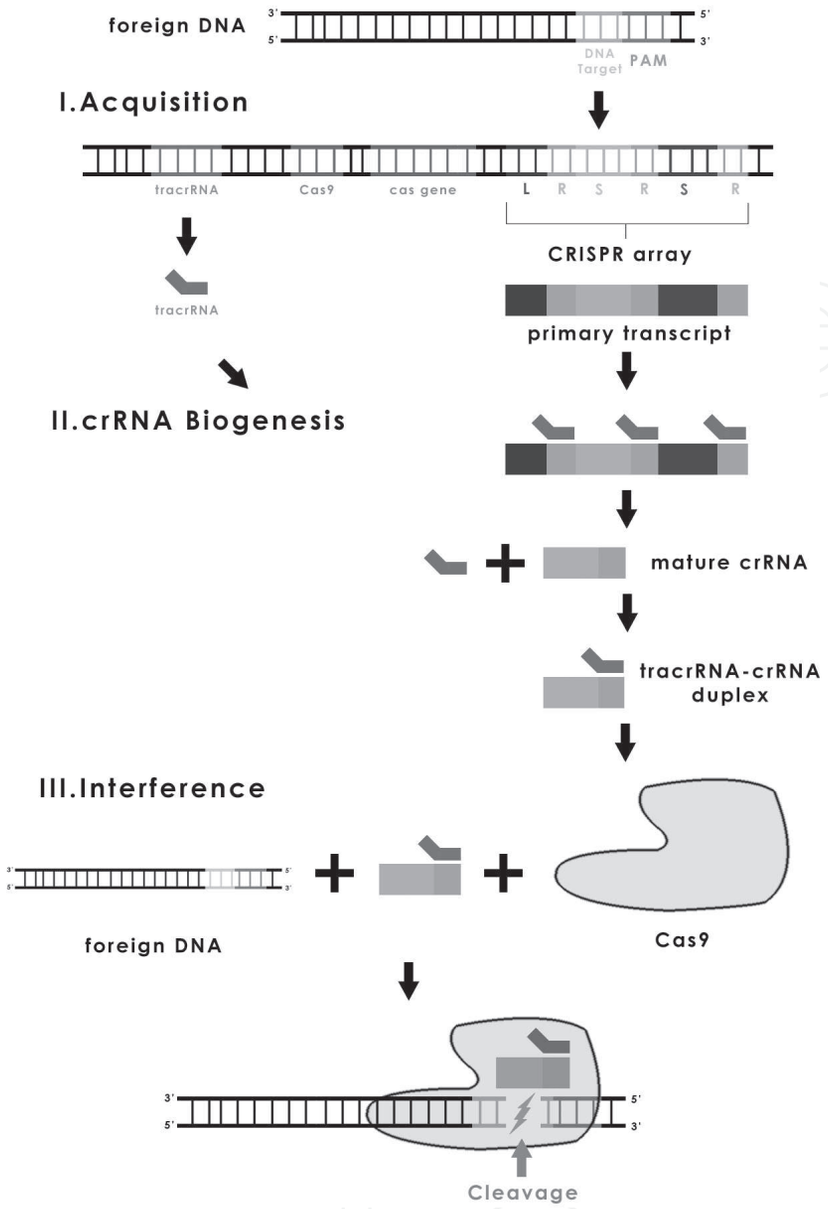

Figure 4. Type II adaptive immunity system by CRISPR/Cas9 in bacteria. The type II adaptive immunity system by CRISPR/Cas9 involves three stages, which are acquisition, crRNA biogenesis and interference to cleavage the DNA target, resulting in the formation of DSBs.

plant cell. The CRISPR/Cas9 system can be delivered via *Agrobacterium*-mediated transformation or particle bombardment [46]. Up to this stage, the expression cassettes are stably integrated into plant genome. Finally, the transformed plants with targeted mutations are screened by polymerase chain reaction (PCR) genotyping and confirmed by sequencing [46].

2.3. RNA-guided DNA cleavage by Cas9

The Cas9 protein consists of six domains, which are REC I, REC II, Bridge Helix, PAM interacting, HNH and RuvC [47]. It remains inactive in the absence of gRNA. The gRNA binds to Cas9 protein and induces a conformational change to form a riboprotein complex. This results

in the activation of the Cas9 protein from a non–DNA-binding conformation into an active DNA-binding conformation. Once the Cas9 protein is activated, it recognizes DNA target by binding with DNA sequence that matches its PAM. Once the Cas9 protein finds a potential target sequence with complementary PAM, it melts the bases immediately upstream of the PAM and pairs them with the complementary sequence on the gRNA. RuvC and HNH nuclease domains will then cleave the DNA target after the third nucleotide base upstream of the PAM to generate blunt-ended DSBs.

2.4. Orthologues of CRISPR/Cas9

To date, the most common Cas9 protein used in plants is from the bacteria *Streptococcus pyrogens* (SpCAs9), which recognizes the NGG-type PAM [46]. Even though this PAM sequence is widely distributed across plant genomes, it does not cover the entire genome of the plant [46]. Many naturally occurring Cas9 orthologues impose distinct crRNA-tracrRNA duplex and PAM requirements [30]. Therefore, exploring orthologous Cas9 proteins with cognate gRNA and PAM sequences would greatly expand possible target sequences in a given genome and add new Cas9 orthologues with unique properties into the CRISPR/Cas arsenal [30]. As the alternative orthologous Cas9 requires different PAM sequences, the total number of possible target sites within a plant genome can be increased. Hence, the CRISPR/Cas9 system is modified by cointroducing multiple Cas9 orthologue-based platforms with different effectors such as nuclease, transcription activator or repressor into the same cell, where they are guided by a specific group of gRNAs to carry out multiplex and complex manipulation of gene activities [30]

3. Application and recent advances

Today, the application of the CRISPR/Cas9 system-based genome editing for crop improvement has already begun its movement despite still being in its infancy. As a matter of fact, there are already numerous publications that reported that this technology has been successfully implemented in a broad range of plant species (as shown in **Table 1**).

The applications of the CRISPR/Cas9 system in plants can be classified into three types, which are the gene disruption, gene insertion and gene regulation.

3.1. Gene disruption

Gene disruption or simply known as gene knockout is a genetic technique that turns one of the genes in an organism to become inoperative. This technique is very powerful as it can inactivate any potential harmful or nonbeneficial gene that downgrades the quality of a plant. Gene disruption is the most applied technique as it can knockout genes by simply introducing small deletion or insertion via NHEJ repair mechanism in CRISPR/Cas9 system [5, 7].

A good example that employed full use of the gene knockout mechanism is the *Waxy* (*WX1*) gene of a maize plant. The maize *WX1* gene encodes a starch-synthesizing protein that is

Species name	Target gene(s)	Gene function	Description	Mode of action	Ref.
Arabidopsis thaliana	*BR11, JAZ1, GAI*	Growth regulators	Transgenic plants displayed retarded growth after being subjected to targeted mutagenesis	Gene disruption	[51]
Brassica oleracea	*BolC.GA4.a*	Gibberellin biosynthesis	Transgenic plants displayed dwarf phenotype after being subjected to targeted mutagenesis	Gene disruption	[52]
Citrus sinensis	*CsPDS*	Carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene regulation	[53]
Cucumis sativus	*eIF4E*	Translation initiation factor	Transgenic plants developed resistance toward a broad range of virus	Gene disruption	[54]
Glycine max	*Bar, GmFE11, GmFE12*, etc.	Root hair growth factors	Transgenic plants displayed higher root hair growth induction after being subjected to targeted mutagenesis	Gene regulation	[55]
Hordeum vulgare	*HvPM19*	Grain dormancy regulator	Transgenic plants displayed signs of dormancy after being subjected to targeted mutagenesis	Gene disruption	[52]
Marchantia polymorpha	*ARF1*	Auxin response factor	Transgenic plants showed no response toward auxins after being subjected to targeted mutagenesis	Gene disruption	[56]
Medicago truncatula	*GUS*	Fluorescence	Transgenic plants displayed no signs of staining after being subjected to targeted mutagenesis	Gene disruption	[57]
Nicotiana benthamiana	*NbPDS*	Carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene insertion	[58]
Nicotiana tabacum	*NtPDS*	Carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene disruption	[59]

Species name	Target gene(s)	Gene function	Description	Mode of action	Ref.
Oryza sativa	*OsPDS, OsMPK2, OsBADH2, etc.*	Carotenoid biosynthesis, growth regulator	Transgenic plants displayed albinism and dwarfism after being subjected to targeted mutagenesis	Gene disruption	[60]
Petunia hybrid	*PDS*	Carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene disruption	[61]
Populus tomentosa	*PtoPDS*	Carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene disruption	[62]
Solanum lycopersicum	*SlAGO7*	Involved in RNA biogenesis regulation	Transgenic plants displayed needle-like or lacking lamina leaves after being subjected to targeted mutagenesis	Gene disruption	[63]
Solanum tuberosum	*StALS1*	Acetolactate biosynthesis	Transgenic plants showed increased resistance on herbicides after being subjected to targeted mutagenesis	Gene insertion	[64]
Sorghum bicolor	*DsRED2*	Fluorescence	Transgenic plants showed signs of red fluorescence after being subjected to targeted mutagenesis	Gene insertion	[65]
Triticum aestivum	*TaINOX, TaPDS*	Inositol metabolism and carotenoid biosynthesis	Transgenic plants displayed albinism expression after being subjected to targeted mutagenesis	Gene disruption	[66]
Vitis vinifera	*IdnDH*	Tartaric acid biosynthesis	Transgenic plants showed no signs of tartaric acid in their fruits after being subjected to targeted mutagenesis	Gene disruption	[67]
Zea mays	*ZmIPK*	Phytic acid biosynthetic pathway catalyst	Transgenic plants showed reduction of phytic acid level after being subjected to targeted mutagenesis	Gene disruption	[68]

Table 1. List of CRISPR/Cas9 system-based genome-edited plants.

involved in the kernel maintenance [48]. Today, there is a known mutant maize that has a deletion in the coding sequence of the WX1 allele [49, 50] that causes it to have an altered grain starch composition [51]. Waxy corns are highly sought after in the commercial market because it provides a variety of benefit such as improved uniformity, stability and texture despite its lower yield compared to elite corns [52]. Up until recently, there have been attempts to introduce the mutant WX1 allele by crossbreeding a nonelite Waxy corn with an elite plant with excellent agronomic qualities. However, this method was unsuccessful as some of the nonelite alleles near the mutant *WX1* gene may be carried along during the introgression process in addition to increased time requirements [52].

Recently, an agricultural company known as DuPoint took this matter with an alternative solution through gene disruption by using the CRISPR/Cas9 system [53]. The gene disruption via CRISPR/Cas9 system is cheap, fast and, most importantly, precise as *WX1* deletions can now be generated directly in the genome of the elite plant to overcome the imperfections that are associated with trait introgression. The gene disruption via CRISPR/Cas9 system works by deleting the entire *WX1* gene with the usage of two Cas9-gRNAs. Each of the Cas9-gRNAs will target two sites, which are the upstream of the transcriptional start site and the downstream of the stop codon. Then, the region is excised and the remaining DNA damage is repaired through the NHEJ, which will bring about the *WX1* null allele with the Waxy phenotype [52].

Another study that utilized the ability of gene disruption of CRISPR/Cas9 system was carried out in wheat, an important staple food in many parts of the world [54]. The team reported that the *inositol oxygenase* (*INOX*) and *phytoene desaturase* (*PDS*) gene of the wheat plant was successfully deactivated at the same time, making it a multiplex mutagenesis. The application of the CRISPR/Cas9 system to the gene causes the gene to have random insertion into its sequence, resulting in gene disruption. Consequently, the phenotype of the wheat changes to express albinism or etiolated leaves.

3.2. Gene insertion

Gene insertion or addition is another famous technique where more than one nucleotide base pairs are added into a DNA sequence. The newly inserted sequence can be designed in such a way where it can specifically encode proteins that bring crucial benefits. However, designing the inserted-to-be DNA sequence is not an easy task as imperfection could pose a risk to the health of the host cell or organism, or it can be simply nulled as it fails to function as predicted [55]. In the terms of gene addition for crop improvement, the desired goal is usually the addition of beneficial traits such as pest resistance, high yield or quality.

In fact, adding multiple genes that confer different trait improvement in a single plant is a common practice to produce elite cultivar. For instance, the TC1507 maize that contains both the *Bacillus thuringiensis* (*Bt*) gene that confers insect resistance and *acetyltransferase* gene that is herbicide tolerance [56]. To date, the most common and efficient method used is to collocate all the desired genes into a single molecular stack, whereby all of the genes will now behave as a single locus or better known as gene stacking [52]. The colocation is no easy task as it has two major limitations. First, each of the genes may potentially affect each other if they are placed adjacently too close [57]. Second, once the transgenes are collocated, they can no longer be moved as they are placed too close to each other to the point where it acts as a single locus.

There is another unconventional substitute to molecular stacks where it can only be generated through the CRISPR/Cas9 system. That substitute is known as complex trait loci (CTL) or quantitative trait loci (QTL) and where transgenes can also be genetically collocated [58, 59]. An example of CTLs is constructed through the CRISPR/Cas9 system by specifically inserting the transgenes into the desired region in the genome through HDR. To start, the transgenes in the CTL can be separated by a larger distance (50 kb to more than 1 Mb) compared to the molecular stacks (few hundred or thousand bp) while retaining their genetic linkage [60]. The changes of distance solves both the limitations of the molecular stacks as adjacent transgene will no longer affect each of their function and they can now be individually moved and swapped.

Similarly, with the help of the CRISPR/Cas9 system, the DsRED2 gene, which encodes a protein that expresses red fluorescence, was also successfully inserted into the genome of an immature sorghum embryo [61]. As a result, the plant now displays red fluorescence.

3.3. Gene regulation

Gene regulation is a technique whereby the gene encoding for its transcription factors is altered to induce changes in its gene expression level [62]. Consequently, plant traits such as the fruit color, size and shape can be controlled and adjusted according to the consumer demands.

The CRISPR/Cas9 system can also be used to regulate the expression of genes for plants [63]. It was carried out by the usage of a catalytically inactive Cas9 known as dead Cas9 (dCas9). The deactivation occurs when rare bacteriophages with anti-CRISPR protein AcrIIA4 binds to the Cas9 of a gRNA that causes its cleaving activity to be disabled [64]. Consequently, the dCas9 is unable to cleave DNAs but it can still bind to specific DNA sequences with gRNA. To be used in gene regulation, the dCas9 must be fused with either a transcriptional activator or a repressor.

For transcriptional activation, dCas9 will be fused with a transcription activator domain such as VP64. For example, there is a study that reported that the paired dCas9-VP64 couple successfully activates the *anthocyanin pigment 1* (*AtPAP1*) gene from *Arabidopsis thaliana,* which encodes the protein involved in the production of anthocyanin pigment 1 [65]. Meanwhile, for transcriptional repression, dCas9 will be fused with a transcription repressor domain such as SRDX instead. Consistently, a study had reported the usage of dCas9-SRDX pair to successfully repress the *A. thaliana cleavage stimulating factor 64* (*AtCSTF64*) gene of a plant of the same species. This technique is still new compared to the previously mentioned gene disruption and gene addition techniques.

3.4. CRISPR/Cas9 system-based genome-edited plants

As the aforementioned plants are successfully genetically modified in the lab, there are actually some of them that are almost readily available in the commercial market. These plants may be new to the market but it is undeniable that they will eventually be able to monopolize the market as they have much more improved traits compared to their relative wild-type plants. As shown in **Table 2**, most of the plants such as the wheat and Ranger Russet potato are important food staples in many parts of the world and this proves that the CRISPR/Cas9 system-based genome editing for crop improvement is definitely on its way to revolutionize the agriculture industry.

Crop	Trait(s) improved	Status	Name of organization	Ref.
White button mushroom	Browning resistant	Submitting for review to Food and Drug Administration (FDA)	Yinong Yang; Penn State College of Agricultural Science	[74]
Waxy corn	Disease resistant Drought tolerant	To be marketed within 5–10 years, pending field trials and applicable regulatory review	DuPont Pioneer	[75]
Wheat	Produce gluten-free wheat by eliminating gliadins in wheat	Working with *gliadin* genes that are still present	Institute for Sustainable Agriculture	[76]
Soybean	Produce healthier oil with reduced unsaturated fat content by increasing the percentage of oleic acid	Inactivation of two genes in soybean	Institute for Basic Research (IBS)	[77]
Ranger Russet Potato	Longer freshness because it does not accumulate sweet sugars at typical cold storage temperature Does not produce acrylamide (carcinogen) when fried	To be grown and sold in 2019	Dan Voytas; Cellectis Plant Sciences	[78]

Table 2. List of CRISPR/Cas9 system-based genome-edited plants that are making their way to the commercial market.

4. Social acceptance and regulation

Genome editing with engineered nucleases (GEEN) has evolved as a highly specific and efficient tool for crop improvement with the potential to rapidly generate useful novel phenotypes. This leads to the emergence of new plant breeding technologies such as to allow the investigation of gene functions and inducing variations for crop improvement. Among these, CRISPR/Cas9 system is now one of the trending applications in plant breeding. Besides the CRISPR/Cas9 system, there are also other plant-breeding technologies that involve cis-genesis and intra-genesis such as transgenic development, whereby unspecific mutagenesis is induced by radiation or chemicals that are much faster and efficient than the conventional breeding method [66]. A question arises as to how genetically edited plants with desired traits will be received by the public and regulated within legislation on genetically modified organism (GMO). According to a recent survey comparing scientist and citizen views on a range of science, engineering and technology issues [67], the most pronounced difference obtained from the study was found on the question addressing the safety of consuming genetically engineered crops; whereby 37% of the public at large responded that GM foods are generally safe to eat, whereas 88% of scientists interviewed recognized GM foods as generally safe [66].

There are two sides to this discussion. Those who take the view that new plant breeding technique (NPBT) such as CRISPR/Cas9 system should be exempted from GMO legislation argued that the products are similar to the products generated from conventional breeding methods. The opponents contend that the process used to generate the plants is in fact genetically modified. As stated in the European Law, the definition of GMO means an organism with the

exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.' Europe Commission (EC) has stressed that the decision to include or exclude a technique from the scope of Directives 2001/18/EC and 2009/41/EC depends only on the interpretation of the definition of genetically modified organisms and genetically modified microorganisms and of the conditions for exemption provided for in the two directives [68]. There are regulators such as the German Consumer Protection Association, or also known as Verbraucherzentrale Bundesverband (VZBV) and Swedish scientists that call for the exclusion of such 'gene editing' from GMO regulation as long as such crops do not contain any 'foreign DNA' [69]. The evaluation is sophisticated because the definition of GMO under European Union (EU) law refers both to the characteristics of organisms and to the techniques used. To date, a clarifying legal opinion of the EC is still pending. Until the legal opinion is released, the legal status of living organisms as well as products deriving from NPBT approaches is unclear [70].

In the United State, the Coordinated Framework for Regulation of Biotechnology (CFRB) determined that it is the final product of genetic engineering that potentially poses a risk to human health and the environment, not the process by which the product is made [71]. The engineered products could be channeled to and handled by regulatory net involving Environmental Protection Agency (EPA), Food and Drug Administration (FDA) and US Department of Agriculture (USDA) depending on what category it falls into [71]. In April 2016, a CRISPR edited, nonbrowning mushroom emerged as the first CRISPR-derived product to be approved by USDA [72].

5. Future prospects

With all the studies done so far, it is undeniable that the CRISPR/Cas9 system is on its way to change the pace and course in the agriculture industry. Perfect plants that have high yield, quality and resistance toward any disease and pests will no longer be impossible with the dawn of this technology. Moreover, CRISPR/Cas9-based gene editing for plants will also be developed to the point where it can be used to replace any defective gene with a normal allele at its natural location. Consequently, all plants will now no longer need to be in danger from any traditional diseases as long as this technique is present and approved for human consumption.

There are still many uncertainties on the usage of plant genome editing. Therefore, in-depth studies are required to ensure this technology will have zero risks while gaining maximum benefits. Besides that, the idea of genome editing might also raise ethical questions from the public; these need to be adequately addressed by researchers and scientists that are well adept in genome engineering. Educational talk or workshop on genome editing should be given to nonscientists to ensure they understand the basics and benefits of this technology. More laws and regulations will also be required for the implementation to ensure CRISPR/Cas9 system is used responsibly without slowing down its development and research. Only when the CRISPR/Cas9 system is well understood and regulated, it will be possible for the application of this technology to be maximized to its fullest potential to achieve previously envisioned ideas in plant science.

Acknowledgements

The authors thank Putra Grant (GP-IPM/2015/9450800) from Universiti Putra Malaysia and Fundamental Research Grant Scheme (FRGS/1/2015/ST03/UPM/02/5) from Ministry of Higher Education, Malaysia, for the support.

Author details

Kah-Yung Bernard Leong[1], Yee-Han Chan[1], Wan Muhamad Asrul Nizam Wan Abdullah[2], Swee-Hua Erin Lim[3,4] and Kok-Song Lai[2]*

*Address all correspondence to: laikoksong@upm.edu.my

1 Faculty of Science, University Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, Perak, Malaysia

2 Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor, Malaysia

3 Perdana University-Royal College of Surgeons in Ireland, Perdana University, MAEPS Building, Selangor, Malaysia

4 Health Sciences Division, Abu Dhabi Women's College, Higher Colleges of Technology, Abu Dhabi, United Arab Emirates

References

[1] Abdallah NA, Prakash CS, McHughen AG. Genome editing for crop improvement: Challenges and opportunities. GM Crops Food. 2015;**6**(4):183-205. DOI: 10.1080/21645698.2015.1129937

[2] Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in drosophila using zinc-finger nucleases. Genetics. 2002;**161**(3):1169-1175

[3] Kim H, Kim J-S. A guide to genome engineering with programmable nucleases. Nature Reviews. Genetics. 2014;**15**(5):321-334. DOI: 10.1038/nrg3686

[4] Puchta H, Fauser F. Synthetic nucleases for genome engineering in plants: Prospects for a bright future. Plant Journal. 2014;**78**:727-741. DOI: 10.1111/tpj.12338

[5] Puchta H. The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution. Journal of Experimental Botany. 2005;**56**(409):1-14. DOI: 10.1093/jxb/eri025

[6] Voytas DF. Plant genome engineering with sequence-specific nucleases. Annual Review of Plant Biology. 2013;**64**(1):327-350. DOI: 10.1146/annurev-arplant-042811-105552

[7] Gaj T. ZFN, TALEN and CRISPR/Cas based methods for genome engineering. Trends in Biotechnology. 2013;**31**(7):397-405. DOI: 10.1016/j.tibtech.2013.04.004

[8] Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th ed. New York: Garland Science; 2002. Site Specific Recombination. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26845/

[9] Carroll D. Genome engineering with zinc-finger nucleases. Genetics. 2011;**188**(4): 773-782. DOI: 10.1534/genetics.111.131433

[10] Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nature Reviews. Genetics. 2010 Sep;**11**(9):636-646. DOI: 10.1038/nrg2842

[11] Wyman C, Kanaar R. DNA double-strand break repair: All's well that ends well. Annual Review of Genetics. 2006;**40**(1):363-383. DOI: 10.1146/annurev.genet.40.110405.090451

[12] Pavletich N, Pabo C. Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 A. Science (80-). 1991;**252**(5007):809-817

[13] Bitinaite J, Wah DA, Aggarwal AK, Schildkraut I. FokI dimerization is required for DNA cleavage. Biochemistry. 1998;**95**:10570-10575

[14] Li L, Wu LP, Chandrasegaran S. Functional domains in Fok I restriction endonuclease. Proceedings of the National Academy of Sciences of the United States of America. 1992;**89**(10):4275-4279

[15] Sugisaki H, Kanazawa S. New restriction endonucleases from Flavobacterium okeanokoites (FokI) and Micrococcus luteus (MluI). Gene. 1981 Dec;**16**(1-3):73-78

[16] Smith J. Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Research. 2000;**28**(17):3361-3369

[17] Carroll D, Morton JJ, Beumer KJ, Segal DJ. Design, construction and in vitro testing of zinc finger nucleases. Nature Protocols. 2006;**1**(3):1329-1341. DOI: 10.1038/nprot.2006.231

[18] Wright DA, Thibodeau-Beganny S, Sander JD, Winfrey RJ, Hirsh AS, Eichtinger M, et al. Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. Nature Protocols. 2006;**1**(4):1637-1652. DOI: 10.1038/nprot.2006.259

[19] Gonzalez B, Schwimmer LJ, Fuller RP, Ye Y, Asawapornmongkol L, Barbas CF. Modular system for the construction of zinc-finger libraries and proteins. Nature Protocols. 2010;**5**(4):791-810. DOI: 10.1038/nprot.2010.34

[20] Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nature Methods. 2011;**8**(1):67-69. DOI: 10.1038/nmeth.1542

[21] Maeder ML, Thibodeau-Beganny S, Osiak A, Wright DA, Anthony RM, Eichtinger M, et al. Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. Molecular Cell. 2008;**31**(2):294-301. DOI: 10.1016/j.molcel.2008.06.016

[22] Kim S, Lee MJ, Kim H, Kang M, Kim J-S. Preassembled zinc-finger arrays for rapid construction of ZFNs. Nature Methods. 2011;**8**(1):7-7. DOI: 10.1038/nmeth0111-7a

[23] Gupta A, Christensen RG, Rayla AL, Lakshmanan A, Stormo GD, Wolfe SA. An optimized two-finger archive for ZFN-mediated gene targeting. Nature Methods. 2012;**9**(6):588-590. DOI: 10.1038/nmeth.1994

[24] Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science (80-). 2009;**326**(5959):1509-1512. DOI: 10.1126/science.1178811

[25] Moscou MJ, Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. Science (80-). 2009;**326**(5959):1501-1501. DOI: 10.1126/science.1178817

[26] Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. Nature Biotechnology. 2012;**30**(5):460-465. DOI: 10.1038/nbt.2170

[27] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (80-). 2012;**337**(6096):816-821. DOI: 10.1126/science.1225829

[28] Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science (80-). 2014;**346**(6213):1258096-1258096. DOI: 10.1126/science.1258096

[29] Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnology Advances. 2015;**33**(1):41-52. DOI: 10.1016/j.biotechadv.2014.12.006

[30] Zhang D, Li Z, Li JF. Targeted gene manipulation in plants using the CRISPR/Cas technology. Journal of Genetics and Genomics. 2016;**43**(5):251-262. DOI: 10.1016/j.jgg.2016.03.001

[31] Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. Nature. 2012;**482**(7385):331-338. DOI: 10.1038/nature10886

[32] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier EA. Programmable Dual-RNA—Guided DNA endonuclease in Adaptice bacterial immunity. Science. 2012;**337**(August):816-822. DOI: 10.1126/science.1225829

[33] Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell. 2014;**156**(5):935-949. DOI: 10.1016/j.cell.2014.02.001

[34] Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature. 2014;**507**(7490):62-67. DOI: 10.1038/nature13011

[35] Miller JC, Holmes MC, Wang J, Guschin DY, Lee Y-L, Rupniewski I, et al. An improved zinc-finger nuclease architecture for highly specific genome editing. Nature Biotechnology. 2007;**25**(7):778-785. DOI: 10.1038/nbt1319

[36] Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;**339**(6121):823-826. DOI: 10.1126/science.1232033

[37] Cho SW, Kim S, Kim JM, Kim J-S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nature Biotechnology. 2013;**31**(3):230-232. DOI: 10.1038/nbt.2507

[38] Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X, et al. Targeted mutagenesis in rice using CRISPR-Cas system. Cell Research. 2013 Oct 3;**23**(10):1233-1236. DOI: 10.1038/cr.2013.123

[39] Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Research. 2014 Sep 29;**42**(17):10903-10914. DOI: 10.1093/nar/gku806

[40] Yeadon J. Pros and cons of ZNFs, TALENs, and CRISPR/Cas [Internet]. 2014. Available from: https://www.jax.org/news-and-insights/jax-blog/2014/march/pros-and-cons-of-znfs-talens-and-crispr-cas [Accessed: 2017-11-01]

[41] Yang H, Wang H, Jaenisch R. Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. Nature Protocols. 2014 Jul 24;**9**(8):1956-1968. DOI: 10.1038/nprot.2014.134

[42] Yan W, Chen D, Kaufmann K. Efficient multiplex mutagenesis by RNA-guided Cas9 and its use in the characterization of regulatory elements in the AGAMOUS gene. Plant Methods. 2016;**12**:23. DOI: 10.1186/s13007-016-0125-7

[43] Ma X, Liu Y-G. CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants. In: Current Protocols in Molecular Biology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016. pp. 31.6.1-31.6.21. DOI: 10.1002/cpmb.10

[44] Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie. 2015;**117**:119-128. DOI: 10.1016/j.biochi.2015.03.025

[45] Ma X, Zhu Q, Chen Y, Liu YG. CRISPR/Cas9 platforms for genome editing in plants: Developments and applications. Molecular Plant. 2016;**9**(7):961-974. DOI: 10.1016/j.molp.2016.04.009

[46] Yin K, Gao C, Qiu J-L. Progress and prospects in plant genome editing. Nature Plants. 2017;**3**(8):17107. DOI: 10.1038/nplants.2017.107

[47] Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. Editing plant genomes with CRISPR/Cas9. Current Opinion in Biotechnology. 2015;**32**:76-84. DOI: 10.1016/j.copbio.2014.11.007

[48] Nelson OE, Rines HW. The enzymatic deficiency in the waxy mutant of maize. Biochemical and Biophysical Research Communications. 1962 Oct 31;**9**(4):297-300

[49] Collins G. A New Type of Indian Corn From China. 1st ed. Bureau of Plant Industry, editor. Washington: Government Printing Office; 1909. 1-30 p

[50] Wessler S, Tarpley A, Purugganan M, Spell M, Okagaki R. Filler DNA is associated with spontaneous deletions in maize. Proceedings of the National Academy of Sciences of the United States of America. 1990 Nov 1;**87**(22):8731-8735

[51] Sprague GF, Brimhall B, Hixon RM. Some effects of the waxy gene in corn on properties of the endosperm Starch1. Agronomy Journal. 1943;**35**(9):817

[52] Chilcoat D, Liu ZB, Sander J. Use of CRISPR/Cas9 for Crop Improvement in Maize and Soybean. Progress in Molecular Biology and Translational Science. Vol. 149. 1st ed. Elsevier Inc.; 2017. 27-46 p. DOI: 10.1016/bs.pmbts.2017.04.005

[53] Steenhuyse L Van. DuPont Pioneer's Next Generation of Waxy Corn Shows the Green Side of CRISPR-Cas9 [Internet]. 2017. Available from: https://biovox.eu/insights/detail/dupont-pioneer-rsquo-s-next-generation-of-waxy-corn-shows-the-green-side-of-crispr-cas9 [Accessed: 2017-11-14]

[54] Upadhyay SK, Kumar J, Alok A, Tuli R. RNA-guided genome editing for target gene mutations in wheat. G3 (Bethesda). 2013 Dec 9;**3**(12):2233-2238. DOI: 10.1534/g3.113.008847

[55] Papapetrou EP, Schambach A. Gene insertion into genomic safe harbors for human gene therapy. Molecular Therapy. 2016 Apr;**24**(4):678-684. DOI: 10.1038/mt.2016.38

[56] Baktavachalam GB, Delaney B, Fisher TL, Ladics GS, Layton RJ, Locke ME, et al. Transgenic maize event TC1507: Global status of food, feed, and environmental safety. GM Crops Food. 2015 Apr 3;**6**(2):80-102. DOI: 10.1080/21645698.2015.1054093

[57] Eszterhas SK, Bouhassira EE, Martin DIK, Fiering S. Transcriptional interference by independently regulated genes occurs in any relative arrangement of the genes and is influenced by chromosomal integration position. Molecular and Cellular Biology. 2002 Jan 15;**22**(2):469-479. DOI: 10.1128/MCB.22.2.469-479.2002

[58] Holland JB. Genetic architecture of complex traits in plants. Curr Opin Plant Biol. 2007 Apr 1;**10**(2):156-161

[59] Collard BCY, Jahufer MZZ, Brouwer JB, ECK P. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. 2005:169-196. DOI: 10.4236/ajps.2014.59125

[60] Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho M-J, et al. Morphogenic regulators baby boom and Wuschel improve monocot transformation. The Plant Cell. 2016 Sep 6;**28**(9):1998-2015. DOI: https://doi.org/10.1105/tpc.16.00124

[61] Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Research. 2013 Nov 1;**41**(20):e188-e188. DOI: 10.1093/nar/gkt780

[62] Liu X, Wu S, Xu J, Sui C, Wei J. Application of CRISPR/Cas9 in plant biology. Acta Pharmaceutica Sinica B. 2017;**7**(3):292-302. DOI: 10.1016/j.apsb.2017.01.002

[63] Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, Al-Shareef S, et al. RNA-guided transcriptional regulation in planta via synthetic dCas9-based transcription factors. Plant Biotechnology Journal. 2015;**13**(4):578-589. DOI: 10.1111/pbi.12284

[64] Shin J, Jiang F, Liu J-J, Bray NL, Rauch BJ, Baik SH, et al. Disabling Cas9 by an anti-CRISPR DNA mimic. Science Advances. 2017 Jul;**3**(7):e1701620. DOI: 10.1126/sciadv.1701620

[65] Lowder LG, Zhang D, Baltes NJ, Paul JW, Tang X, Zheng X, et al. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. Plant Physiology. 2015 Oct 1;**169**(2):971-985. DOI: 10.1104/pp.15.00636

[66] Wolt JD, Wang K, Yang B. The Regulatory Status of Genome-edited Crops. 2016;**4**(October 2015):510-518. DOI: 10.1111/pbi.12444

[67] Funk C, Lee R. Public and Scientists' Views on Science and Society | Pew Research Center [Internet]. 2015. Available from: http://www.pewinternet.org/2015/01/29/public-and-scientists-views-on-science-and-society/ [Accessed: 2017-12-17]

[68] Laaninen T. New Plant-Breeding Techniques Applicability of GM Rules [Internet]. 2016. Available from: http://www.europarl.europa.eu/RegData/etudes/BRIE/2016/582018/EPRS_BRI(2016)582018_EN.pdf [Accessed: 2017-12-17]

[69] Ammann K. How Should Europe Regulate CRISPR, New Breeding Technologies (NBTs [Internet]). 2016. Available from: https://geneticliteracyproject.org/2016/01/15/europe-regulate-crispr-new-breeding-technologies-nbts/ [Accessed: 2017-12-15]

[70] Sprink T, Eriksson D, Schiemann J, Hartung F. Regulatory hurdles for genome editing: Process- vs. product-based approaches in different regulatory contexts. Plant Cell Reports. 2016;**35**(7):1493-1506. DOI: 10.1007/s00299-016-1990-2

[71] Kuzma J. Policy: Reboot the debate on genetic engineering. Nature. 2016;**531**(7593):165-167. DOI: 10.1038/531165a

[72] Waltz E. Gene-edited CRISPR mushroom escapes US regulation. Nature. 2016;**532**(7599): 293-293. DOI: 10.1038/nature.2016.19754

[73] Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. Journal of Clinical Investigation. 2014 Oct 1;**124**(10):4154-4161

[74] Gill C. Penn State Developer of Gene-Edited Mushroom Wins "Best of What"s New' award | Penn State University [Internet]. Penn State News. 2016 [cited 2018 Mar 3]. Available from: http://news.psu.edu/story/432734/2016/10/19/academics/penn-state-developer-gene-edited-mushroom-wins-best-whats-new?utm_source=newswire&utm_medium=email&utm_term=433007_HTML&utm_content=10-19-2016-21-27&utm_campaign=Penn+State+Today

[75] Sander J, Jeschke M. CRISPR-Cas Advanced Plant Breeding [Internet]. DuPont Pioneer. 2016 [cited 2018 Mar 3]. Available from: https://www.pioneer.com/home/site/us/agronomy/library/crispr-cas/

[76] Dormehl L. CRISPR's Latest Achievement? Taking the Gluten Out of Wheat [Internet]. Digital Trends. 2017 [cited 2018 Mar 3]. Available from: https://www.digitaltrends.com/cool-tech/crispr-gene-editing-low-gluten/

[77] Kim H, Kim S-T, Ryu J, Kang B-C, Kim J-S, Kim S-G. CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun. 2017 Feb 16;**8**:14406

[78] Regalado A. A Potato Made with Gene Editing - MIT Technology Review [Internet]. MIT Technology Review. 20115 [cited 2018 Mar 3]. Available from: https://www.technologyreview.com/s/536756/a-potato-made-with-gene-editing/

IntechOpen