Chapter 11
Gene and Genome Editing with CRISPR/Cas Systems for Fruit and Vegetable Improvement

Semih Arbatli, Julia Weiss, and Marcos Egea-Cortines

Abstract Ever since the advent of agriculture, breeding new varieties has relied upon crosses between individuals from a single species, and since the early twentieth century with relatives or via mutagenesis. Two major problems have been found time and again. First, combining genomes to improve a character often times causes decreases in other traits as a result of genetic linkage. The second is that natural variation does not always comprise all the possibilities a genome may have in terms of allelic combinations suitable for further improving a set of characters. In the last twenty years a number of technologies have been developed allowing the perturbation of a single gene. Development of genome editing technologies includes zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Palindromic Repeats (CRISPR). Here we review current methodologies regarding to the use of gRNA targeted gene and genome editing strategies by various CRISPR/Cas9 systems in agriculture. The molecular mechanism of DNA modification by CRISPR/Cas relies on guide RNA molecules comprising 20–25 DNA bases homologous to the target locus. This has opened the possibility of tackling single loci or multiple paralogs in a gene family. Importantly, complex genomes with polyploid structures such as wheat or camelina have been successfully engineered with single guides. This opens a new window of opportunities to engineer gene families, pathways and complex genomes that was unfeasible before the advent of CRISPR/Cas.

Keyword Genome editing · CRISPR/Cas · Multilocus edition

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11.1 From Chromosome Transfer to Single Gene Transfer

Plant breeding refers to the cross-fertilization of two parents in order to obtain desired traits. Throughout the history, humans have modified nearly all plant species of economic interest in order to enhance quality and increase the yield obtained.

At the end of the nineteenth century, the scientific plant breeding studies emerged in order to achieve new and improved genetic variations of the commonly used crop species in all aspects of cultivation (Hallauer 2007). Later on, in the twentieth century, the advancement of plant breeding started to ascend with the formation of agricultural research centers and development of private companies. New and faster methods have been expanded to match raised requirements to agricultural needs. The crosses with wild relatives had a crucial role in the development of the current diversity of cultivars. The introgression of genes for breeding purposes via interspecific hybridization from non-cultivated plants species to a related crop species is an additional method where natural variation was exploited (Goodman et al. 1987).

To illustrate, an early example of interspecific hybridization to a cultivated crop species is wheat. Gene transfer of wheat with the aforementioned method has been achieved in 1930 with the transfer of resistance genes from *Triticum tauschii* into *T. aestivum* (reviewed in [Hoisington et al. 1999]). In 1936, a resistance gene was transferred from *Solanum pimpinellifolium* to the cultivated tomato (*S. esculentum*) (reviewed in [Goodman et al. 1987]). Crop improvement continued with interspecific gene transfer, and in those cases where it was feasible via untargeted mutagenesis (Menda et al. 2004; Sikora et al. 2011). The random mutagenesis using chemicals or ionic radiations has given rise to "Targeting Induced Local Lesions in Genomes" (TILLING) (Comai and Henikoff 2006). There are successful examples of TILLING on various plant species such as *Solanum tuberosum*, *Cucumis melo* and *Cucumis sativus* (Elias et al. 2009; González et al. 2011; Boualem et al. 2014). While TILLING has many advantages, such as obtaining non-transgenic material, an extensive genetic work has to be performed to isolate the single mutant and introduce it in elite germplasm.

In the 1980s, the age of plant biotechnology started. *Agrobacterium tumefaciens* which is a gram-negative, plant pathogenic soil bacterium, made it possible to develop horizontal gene transfer technologies, thus opening a new era for breeding based on different conceptual principles (Chilton et al. 1977; Herrera-Estrella et al. 1983). Thereafter, different methods of gene transfer have been developed such as microinjection process and particle bombardment. These methods are relying on the injection of desired DNA into the target plant or bombarding the plant with tiny particles that contain the gene of interest, thus achieving single gene transfer in plants that are not natural hosts of *Agrobacterium*. But the rationale behind these technologies was to engineer plant genomes one gene at a time, in sharp contrast to the classic breeding programs where one or several genes encompassing chromosome fragments, are

crossed into a line and then it is backcrossed for at least eight generations to clean the original genome from unwanted fragments.

Genetically modified (GM) crops are agricultural crops that express a certain gene or genes inserted in the host genome, that do not possess it naturally. They have been arguably the biggest success in terms of technology development and they amount for over 50% of the total crop area in the World (FAO). They created a large debate based on the presence of resistance genes used during the transformation process as they were thought as a threat to human health if they would be transferred to the human gut microbiome. In a recent study conducted on the cultivated sweet potato clone "Huachano," the presence of an additional transfer DNA (T-DNA) has been detected in 291 tested accessions of cultivated sweet potato (Kyndt et al. 2015). This research also displays the presence of a specific T-DNA among all cultivated sweet potato species, excluding the wild relatives. Hence, the study points out the possibility of an *Agrobacterium* infection on sweet potato in history. It has been suggested that this T-DNA transfer provided a benefit in an agricultural manner, thus made it preferable through its domestication process (Kyndt et al. 2015). A recent work examined 275 dicot species and found presence of T-DNA in 23 species such as *Arachis*, *Nissolia*, *Camellia* or *Dianthus* (Matveeva and Otten 2019). This new data shows that the horizontal gene transfer by *Agrobacterium* is rather common in nature. However, transgenic systems relying on random insertions in the genome such as those based on *Agrobacterium* or biolistic, do show high variability in terms of transgene stability, level of expression and copy number.

11.2 Gene Targeting

Gene targeting relies on Homologous Recombination (HR) whereby a gene sequence is replaced by a nearly identical sequence albeit mutated (Reiss 2003). The HR-based DNA repair is a DNA protection pathway against structural damages involving double-strand DNA breaks. Naturally there are different ways of foreign DNA integration to the original genome. These are improper recombination, Non-Homologous End Joining (NHEJ) and Single Strand Annealing. However, methods relying on HR are limited from certain aspects such as difficult screening strategies, time-consuming experimental setup and potential mutagenic effects due to the low target efficiency and improper binding (Gaj et al. 2013). While homologous recombination worked very well in several genetic models such as yeast or mice, it was not amenable in others where the genetic tools were highly developed such as Drosophila or plants. The only exception is *Physcomitrella patens,* a haploid moss, where homologous recombination was rapidly achieved (Schaefer and Zrÿd 1997).

11.3 First-Generation Genome Editing Technologies

One standard tool in molecular biology labs is DNA digestion with restriction enzymes. They recognize short sequences, mostly palindromic between four and twelve bases long. This made them unsuitable to target one locus as a standard enzyme may digest a single genome into hundreds to hundreds of thousands of fragments. Thus, early efforts were done to obtain engineered nucleases ideally recognizing a single target DNA stretch in a genome. Considering DNA as a random molecule comprising four bases, which is a very gross scientific misconception, one can argue that a DNA fragment of roughly 15–20 bases should suffice to identify a single fragment in the genome just randomly, i.e. the probability of finding precisely 15 bases is $(1/4)^{15}$. This means that obtaining nucleases with specificities of 18–25 bases should give enough target specificity to tackle a single locus for genome engineering.

The use of engineered nucleases (ENs) was first applied in 1981 by Wallace et al. (Bruce Wallace et al. 1981). Since then, four different types of defined nuclease classes have been developed: Zinc Finger Nucleases (ZFNs), Meganucleases, Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based technologies (Metje-Sprink et al. 2019). To date, ZFNs, TALENs and recently CRISPR/Cas9 systems have been commonly used in the field of plant genetics.

The first-generation genome editing technologies rely on a sequence-specific DNA-binding domain and a non-specific nuclease domain. The system relies on the use of site-specific nucleases (SSNs). SSNs are probably the most well-known method in the field of gene editing (Sivanandhan et al. 2016). SSNs cleave the target DNA fragment, thus creating Double-Strand Break (DSB) (Sanagala et al. 2017). These DSBs can be repaired through NHEJ and Homology directed recombination (HDR) pathways, consequently creating insertions or deletions (INDELs) on the targeted region. Gene targeting strategies with DSBs administer an exogenous template for the naturally occurring repair mechanism (Carroll 2011).

NHEJ pathway has been commonly preferred over HDR due to low efficiency rates of conducted studies using HDR (Schindele et al. 2018). ZFN and TALEN-based systems have been applied as a first-generation genome editing systems followed by the CRISPR/Cas-based gene modification approaches due to several aspects explained later on throughout the chapter (Fig. 11.1).

11.4 Zinc Finger Nuclease Genome Editing

ZFNs are a class of nucleases that consist of separate DNA-cleavage and DNA-binding domains (Carroll 2011). Zinc finger (ZF) domains that are attached to the FokI domain recognize the target sequence. *FokI* is a natural type-IIS restriction enzyme, with a non-specific target sequence (Kim and Chandrasegaran 1994). The identification of alterable properties on the *FokI* domain introduced the possibilities of DNA

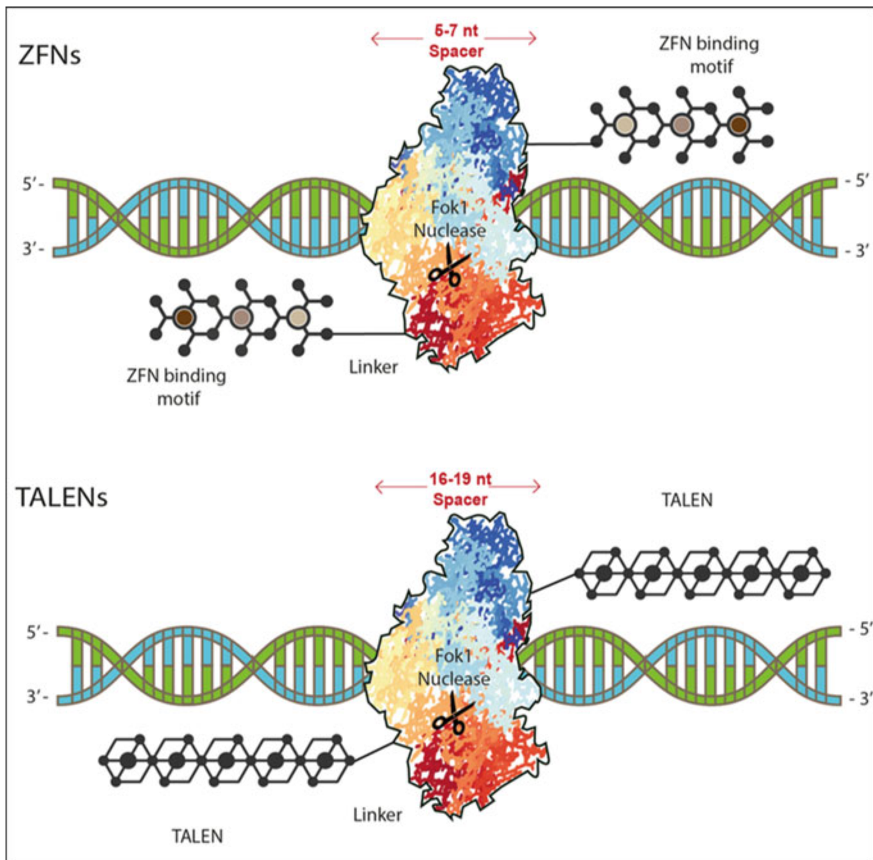

Fig. 11.1 Schematic representation of ZFN and TALEN systems on target DNA fragment resulting with DSBs and creation of INDELS (Fok1 nuclease structure obtained from Protein Database (PDB), [Wah et al. 1997])

cleavage in a site-specific manner, without the necessity of previously engineered sites for the target to bind and function (Sanagala et al. 2017). The advancement of ZFN-mediated gene targeting enables the modification on plant genome specifically by homology directed repair (HDR) of target DSB (Malzahn et al. 2017). ZFNs are able to recognize 18–24 bp of DNA sequences with a 5–7 bp spacer on both ends (Pabo et al. 2001). Binding of ZFN to the target site pursues with the production of DSBs with the Fok1 cleavage domain.

First gene editing reports using ZFN system in plants were published in 2005 by Lloyd and his colleagues (Lloyd et al. 2005). The study conducted with Arabidopsis as a model organism and the ZFN-induced mutations has been characterized showing that the NHEJ-based methods are more likely to be effective in comparison to the HR-based mutagenesis.

11.5 TALEN-Based Genome Editing

The modification of the transcriptional activator-like effector (TALE) domains to achieve successful gene editing strategy resulted in the development of TALENs, that are based on a TALE domain fused with *FokI* nuclease site. TALENs have the capability to recognize 18–20 bp long stretches on the target region. TAL effectors belong to *Xanthonomas sp.* The mechanism simply uses type III secretion system (T3SSs) for the translocation process into the cells (White et al. 2009). T3SSs are bacterial structures providing gram-negative pathogens with the ability to inject effector proteins into host cell cytoplasm. Some of the TAL effectors have been known for their transcriptional activation on target region (Sugio et al. 2007; Li et al. 2011). A repetitive region containing various number of amino acids (~34 bp nuclear localization motif) along with the transcriptional activation domain are the building blocks of the TAL structure (Gürlebeck et al. 2006). These repetitive regions act as the target recognition agents (Moscou and Bogdanove 2009). As they are universally recognized by the cellular machinery, they can be used for gene alteration as a gene modification tool in plants (Boch et al. 2009).

There are different TALE variations with different binding specificities aiming to increase the target specificity (Sprink et al. 2015). For instance, Hax3, which can recognize a target sequence of 12 bp long, has been used for the construction of a genetically engineered nuclease for targeted mutagenesis on *Nicotiana benthamiana* plants (Mahfouz et al. 2011). Since then, TALEN-mediated genome engineering studies have been applied to various plant species (reviewed in [Sanagala et al. 2017]).

Custom engineering of ZFN proteins is a time-consuming process with a low success rate. This hampers the widespread use of the ZFN system and led to the development of new efficient and precise gene editing techniques. (Joung et al. 2010). The problem of low success rate is directly related to the affinity of the particular ZFN. To increase the specificity, each ZFN construct has been designed with at least 3 fingers. However, the contribution of each finger does not occur at the same level, compared to each other. Moreover, at a certain point, the amount of added fingers might also reduce the target binding affinity due to various factors such as complex chromatin structure (Carroll 2011). Currently, ZFNs are the least preferred gene modification tool due to limited number of target-cleaving sites and high number of off-target cleaves leading to the low target specificity and restricted experimental systems.

TALENs are another system that is also based on the TAL gene responsible from the manipulation of host gene expression (Li et al. 2011). TALENs are functional in order to manipulate eukaryotic genomes in the manners of target identification and cleavage. However, there are limited number of modified plant species based on TALENs (Arabidopsis, barley, tobacco, rice, *Brachypodium*, tomato, maize, *Nicotiana bentamiana*, soybean, sugarcane, potato and wheat) (reviewed in [Sanagala et al. 2017; Malzahn et al. 2017]). Another issue regarding to the insufficiency of TALENs as a gene modification tool is its time-consuming procedures as long as

complex and labor-intensive experimental setup. These crucial problems are main inadequate aspects of TALEN-based gene alteration systems.

11.6 The CRISPR/Cas Technologies

CRISPR was first described in *E.coli* by Ishino et al. in 1987 (Ishino et al. 1987). Naturally, CRISPR system is an adaptive defense response of archaea and bacteria to prevent viral invasions (Bhaya et al. 2011). The discovery of CRISPR/Cas9 systems was an important breakthrough in the development of precise gene editing studies. CRISPR-based systems are having a different origin than other gene alteration systems and they are common in various species. The first success achieved by CRISPR/Cas system was in mammalian cells (Jinek et al. 2012). The first plant gene editing study using CRISPR/Cas systems has been published by Feng et al. in 2013 (Feng et al. 2013). This achievement has been followed by its immediate implementation on various plant species such as Arabidopsis, rice, wheat and tobacco (Feng et al. 2013; Upadhyay et al. 2013; Jiang et al. 2013). Later on, different Cas variants isolated from different species such as *Streptococcus thermophilus* have been developed for further gene alteration processes (Steinert et al. 2015). Studies continued with successfully edited CRISPR/Cas plants including but not limited to barley, cotton, dandelion, flax, liverwort, soybean, sorghum, tomato or petunia, to name a few (Malzahn et al. 2017).

The CRISPR/Cas systems ease the whole gene alteration processes and proved itself as a successful gene modification tool in the field of plant genetics, in comparison to the first-generation genome editing techniques based on TALEN or Zinc Finger Nucleases. Cas9-induced mutagenesis has been used to target cis-regulatory-elements (CREs) of quantitative traits (Rodríguez-Leal et al. 2017).

CRISPR system involves two different classes of RNA-guided nuclease effectors. Class 1 effectors are related to the utilization of multi-protein complexes, while Class 2 effectors act as unique agents, single component effector proteins (Zetsche et al. 2015). The Class 1 effectors are consisting of type I, type III and type IV systems while Class 2 effectors are containing type II, type V and type VI (Makarova et al. 2015). Especially the effector modules are distinctive among the various types of CRISPR/Cas systems (Charpentier et al. 2015). The Class 1 effectors are able to form an effector complex including CRISPR RNA (crRNA) and certain Cas proteins, while Class 2 effectors use a large Cas module associated with crRNAs to obtain target specificity. crRNA is the transcribed RNA in the presence of a secondary viral attack after the incorporation of a spacer sequence after the primary intrusion. Each transcribed crRNA is carrying both nucleotide repeats and spacer. Another product of the aforementioned formation is the trans-activating crRNA (tracrRNA) which is another molecule that binds to Cas structure in order to lead it to the target site.

11.7 Types of CRISPR/Cas Systems

11.7.1 CRISPR/Cas9

CRISPR/Cas9 is the best characterized type among all CRISPR/Cas precise gene modification tools. The ortholog of Cas9, derived from *S. pyrogenes* (spCas9), is commonly used in the field of plant genetics. Moreover, another ortholog derived from *Staphylococcus aureus* (saCas9) has been proven to have similar success in order to modify plant genomes, suggesting a second alternative for the common spCas9-based plant gene modification studies (Steinert et al. 2015).

The CRISPR/Cas9 system basically works with a designed, short synthetic guide RNA (gRNA) fragment (~20 bp) responsible for target identification and binding and a nuclease with a capacity to cleave 3–4 bases after the so-called protospacer adjacent motif (PAM) (Jinek et al. 2012). These PAM regions are consisting of the sequence 5' NGG. The Cas nucleases are generally a composition of HNH domain and a RuvC-like domain (Jaganathan et al. 2018). The first system based on the spCas9 was developed by Jinek et al. Later on, various orthologs of Cas9 have been identified and are available for further plant gene editing studies (Fig. 11.2).

11.7.2 Crispr/CAS12a (Cpf1)

CRISPR/CAS12a (formerly known as Cpf1) is a Class 2 type V gene modification system that diverges from the Cas9-based systems in several ways. First of all, target specificity of the CRISPR/Cas12a system requires a minimum of 22 nucleotides (Lei et al. 2017). It is a longer region compared to the traditional CRISPR/Cas9-based gene editing systems and increases the target binding efficiency while reducing the off-target effects (Chen et al. 2018).

Moreover, the Cas12a-based system requires Thymine-rich PAM sequences (5'-TTTV-3') while Cas9 requires Guanidine-rich PAM structures (5'-NGG-3'), therefore increasing the amount of possible approaches by offering a higher number of potential target regions. It is further characterized by the creation of staggered DSB ends distal from the PAM region instead of the blunt-end DSBs obtained by the traditional Cas9 (SpCas9) system (reviewed in [Schindele et al. 2018]). The cleavage of the target strand occurs after the 23rd nucleotide while non-target strand cleavages occur after the 18th nucleotide, producing a region consisting of a 5-nucleotide overhang on the 5' end. SpCas9 requires a gRNA with approximately 100 nucleotides, while CRISPR-Cas12a system requires a crRNA of 43 nucleotides in length (Zetsche et al. 2015).

A variation of Cas12a nuclease isolated from *Leptotrichia shahii* is presenting dual nuclease activity, therefore capable of targeting single-stranded RNA (Zaidi et al. 2017). The first Cas12a-based experiments in plant genetics have been conducted on tobacco and rice (Endo et al. 2016; Xu et al. 2017). Since then, three different

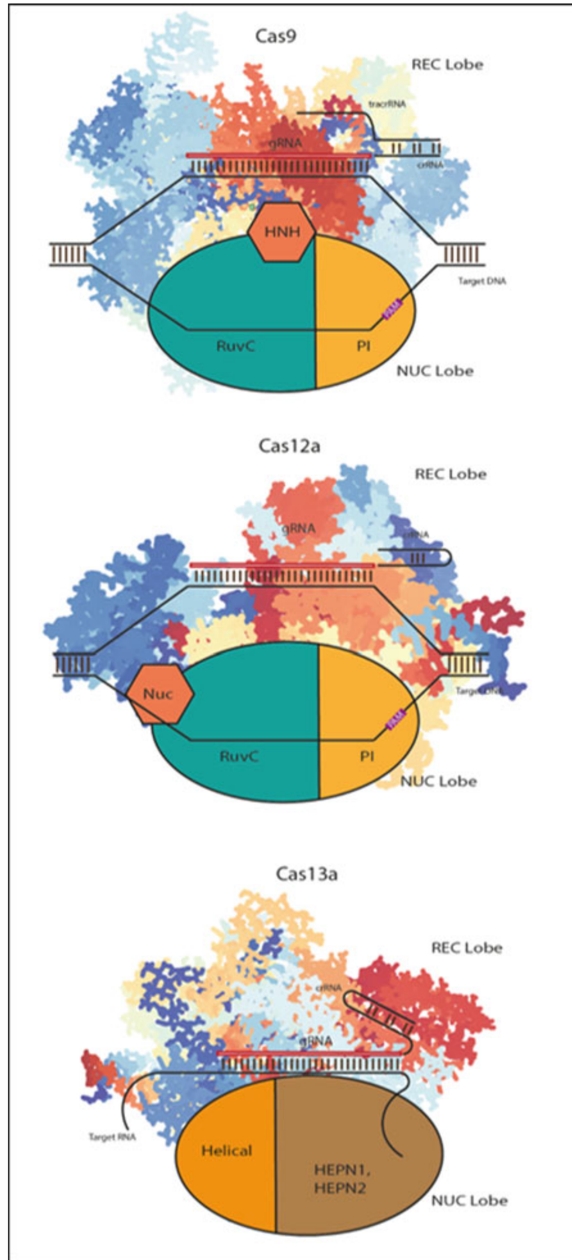

Fig. 11.2 Basic representation of Cas9, Cas12a and Cas13a system (Representative background models for Cas9, Cas12a, Cas13 belong to spCas9, FnCas12a and LbuCas13a, respectively) (Nishimasu et al. 2014; Swarts and Jinek 2019; Liu et al. 2017)

variants of Cas12a have been introduced into various model plant species. These are including but not limited to *Acidaminococcus sp.* Cas12a (AsCas12a), *Francisella novicida* Cas12a (FnCas12a) and *Lachnospiracea bacterium* ND2006 (LbCas12a) (reviewed in [Jia et al., n.d.]).

11.7.3 Crispr/Cas 13(C2c2)

Both Cas9 and the Cas12 have been commonly used in order to create ssDNA breaks in plant DNA. However, CRISPR/Cas-based alterations of single-stranded RNA (ssRNA) fragments have been achieved only with the development of CRISPR/Cas13 (previously called C2c2) systems, entitled as a "new swiss army knife" for plant biologists (reviewed in [Wolter and Puchta 2018]). In 2015, Shmakov et al. identified 3 novel Class 2 effectors named C2c1, C2c2 and C2c3 (Shmakov et al. 2015). Both C2c1 and C2c3 represent characteristics similar to Cas12. However, C2c2 diverges from other Class 2 effectors due to its distinctive features. Therefore, the system was later on classified as a new type of effectors. CRISPR/Cas13 is a Class 2, type VI ribonuclease gene modification system which confers immunity against phage invasions (Schindele et al. 2018).

The Cas13 system comprises a diverse structure with nuclease and recognition domains different from other Class 2 effector systems. Similar to Cas12, Cas13 systems also do not require tracrRNA to process pre-crRNA (reviewed in [Liu et al. 2017]). In Cas13, target RNA cleavage activity and the crRNA maturation process are distinct from each other (reviewed in [Wolter and Puchta 2018]).

Another structural difference compared to Cas9 and Cas12 consists in the position of higher eukaryotes and prokaryotes nucleotide binding domain (HEPN) located on the outer surface. These domains are enabling the protein to cleave target RNA which is positioned on the outside of the binding region. However, this ability also causes the possible non-specific cleavage of any other RNAs that are present in that area (East-Seletsky et al. 2016).

11.7.4 Using Crispr to Modify Single Genes

Dicot plants have been the main objects of gene editing by CRISPR/Cas systems, as they are comprising most of the agricultural plants. Initial experiments were performed by Feng et al. in 2013 (Feng et al. 2013). These studies targeted three different genes of the Arabidopsis genome, *Brassinosteroid insensitive1 (BRRI1), Jasmonate-zim-domain protein 1 (JAZ1)* and *Gibberellic acid insensitive (GAI),* and a significant efficiency (26–84%) of mutagenesis was observed. Since then, many applications of the CRISPR/Cas systems targeting the Arabidopsis genome were reported (Miki et al. 2018).

Tomato (*Solanum lycopersicum L.*) is an important crop model for fruit quality improvement, and the genome structure is well characterized (Pan et al. 2016). Successful CRISPR/Cas9-based interference of the tomato gene *SlAGO7* (*ARGONAUTE*) demonstrated the possibility of precise gene editing in tomato (Brooks et al. 2014). Another study targeting the *SHR* (*SHORT-ROOT*) gene, which regulates *SCARECROW* (*SCR*) transcription factor gene expression, using the CRISPR/Cas system was performed by Ron et al. and results indicate a correlation between the tomato *SHR* gene and both root length and the *SCR* gene expression (Ron et al. 2014). Yet another experiment in tomato, conducted by Ueta et al., showed that the interruption of *SlAA9, a* gene related to parthenocarpy, resulted in the development of seedless fruits and alterations in the leaf morphology (Ueta et al. 2017).

Soybean (*Glycine sp.*) is an important crop containing a high protein content along with physiologically active substances in the seeds. The first CRISPR/Cas-mediated gene modification experiment on soybean was conducted by Cai et al., targeting two genomic sites on chromosome 4 and leading to small deletions and insertions in this region (Cai et al. 2015). Another approach was the interference of *Rj4* gene, which plays a role in nodulation inhibition in many strains of *Bradyrhizobium elkanii*, using the CRISPR/Cas9 system (Tang et al. 2016, p.4).

Cotton (*Gossypium sp.)* is another major crop in many areas. Its uses include but are not limited to fiber and biofuel production (reviewed in [Jaganathan et al. 2018]). The sequencing of the *Gossypium hirsutum* genome was published in 2015 (Li et al. 2015). First targeted gene editing experiments in cotton using CRISPR/Cas9 system have been accomplished by Janga et al. using transgenic cotton bearing an integration of the Green fluorescent protein (GFP) (Janga et al. 2017). The generated GFP regions were targeted by CRISPR/Cas9 and examined in order to identify the pathways related to the DSB utilization. Another study aimed to interfere with two genes of the cotton genome—*Cloroplastos alterados 1* (*GhCLA1*) and *Vacuolar H-pyrophosphatase* (*GhVP*)—and results demonstrated a high mutational efficiency (47.6–81.8%) (Chen et al. 2017).

Grape (*Vitis sp.*) is another plant of high economic importance. Five different CRISPR/Cas9 target sites within the *Vitis vinifera* genome (protospacer adjacent motif or PAM) were identified (TGG, AGG, GGG, CGG, NGG) and found to be uniformly distributed among the grape genome (Wang et al. 2016). The CRISPR/Cas9 system has been used to mediate interference of the *I-idonate dehydrogenase* (*IdnDH*) gene on "Chardonnay" suspension cells following regeneration of grape plantlets, showing the absence of off-target mutations (Ren et al. 2016). Nakajima and his colleagues targeted the *Phytoene desaturase* (*VvPDS*) gene which is related to the albino leaf formation with CRISPR/Cas-mediated mutagenesis (Nakajima et al. 2017). The study demonstrates that old leaves have a high mutation rate compared to the newly formed leaves, suggesting an increased incidence of DSBs or impaired repair mechanisms in the old leaf samples (reviewed in [Jaganathan et al. 2018]). Another study targeting the *MLO*-7, a gene related to an increased resistance to powdery mildew disease on grapevine protoplasts, resulted in the generation of resistant mutants (Malnoy et al. 2016).

Sweet orange (*Citrus sinensis*) is another commonly produced and consumed fruit providing 60% of the total citrus production worldwide (Xu et al. 2013). In a study conducted by Jia et al., sweet orange *Phytoene desaturase* gene (*CsPDS*) related to citrus canker disease resistance, has been targeted with a novel Xcc (*Xanthomonas citri subsp. citri*)-facilitated agroinfiltration. The study resulted in a successful mutagenesis of *CsPDS* genes (3.2–3.9% mutation rate) along with the absence of off-target effects (Jia and Wang 2014).

There are several monocot plant species where CRISPR/Cas gene modification systems have been successfully introduced (reviewed in [Jaganathan et al. 2018]). In a study conducted on barley genome, an interference on the *Endo-N-acetyl-b-D-glucosaminidase* gene (*ENGase*) has been mediated while using both *Agrobacterium*-mediated transformation and particle bombardment technique (Kapusi et al. 2017). The study states that among all observed T0 and T1 mutant barley lines, 78% showed mutational efficiency.

Rice (*Oryza sativa*) is a major crop and plant model, with significant progress of CRISPR/Cas-based studies. In a study conducted in 2013 by Shan et al., *Phytoene desaturase* gene (*OsPDS*) of rice protoplasts, targeted with CRISPR/Cas9, resulted in an efficient, targeted mutagenesis (15%) underlining the adaptability of CRISPR/Cas technique for the rice genome (Shan et al. 2013). Another study published in the same year presents a successful demonstration of type II CRISPR/Cas application in targeting the promoter region of the bacterial blight susceptibility genes (*OsSWEET14* and *OsSWEET11*) (Jiang et al. 2013). Since then there are various CRISPR/Cas-based studies conducted on the rice genome (reviewed in [Malzahn et al. 2017]).

11.8 Using Crispr to Modify Protein Families and Complex Genomes

While the identification of single mutants and their production is a methodology with a long tradition, one characteristic of plant genomes is the large amount of gene families, gene redundancies and polyploid genomes. Under these scenarios, the use of CRISPR/Cas has shown to be a major breakthrough as several genes can be engineered with a single construct, provided there is enough sequence similarity.

Abscisic acid (ABA) is an important regulator of environmental stress responses including but not limited to drought, salinity and heat stresses. ABA directly regulates the control of stomatal closure and organ growth. The pyrabactine resistance 1 (PIR1) and PYR-like (PIL) proteins form a family of fourteen members in the Arabidopsis genome acting as ABA receptors. In a seminal experiment using CRISPR/Cas (Zhao et al. 2018), a set of new mutations were stacked onto a previously sextuple mutant, thus obtaining a duodecuple mutant of PYR/PIL genes in Arabidopsis (Gonzalez-Guzman et al. 2012). This shows that gene families can be engineered to obtain

high-level mutant combinations that would require a very long time to be generated in form of single mutants, even in short-lived plants such as Arabidopsis.

Potato (*Solanum tuberosum*) is an important agricultural plant, ranking as third most important crop in the world with a long history of biotechnological approaches (Barrell et al. 2013). Potato is an essential crop due to its high amount of starch. Importantly, the commercial potato is highly heterozygous and autotetraploid. In a study, CRISPR/Cas system has been used to mediate interference on a gene encoding granule-bound starch synthase *(GBSS)* (Andersson et al. 2017). In another study, targeting of *ACETOLACTATE SYNTHASE1* (StALS1) resulted in the generation of multi-allelic mutagenesis (Butler et al. 2016).

Commercial wheat (*Triticum sp.*) comprises three genomes, but due to its extreme importance it has been targeted by several CRISPR/Cas-based studies, NHEJ-based mutation induced on *MLO* (mildew resistance locus) related to natural powdery mildew resistance (Shan et al. 2013; Wang et al. 2014). Another study shows the successful targeting of both Phytoene desaturase (PDS) which is key enzyme of carotenoid biosynthesis and inositol oxygenase (INOX) gene responsible from the oxidization of myo-inositol into glucuronic acid of *Triticussm aestivum.* The targeting resulted in the production of efficient incidences of indels (insertion & deletion), consequently suggesting the possibility of allohexaploid gene editing with CRISPR/Cas systems.

Camelina is an allohexaploid plant and an emerging crop for high-quality oil. The objective of gene modification studies in *Camelina* is to increase the oleic acid while decreasing the linoleic and linolenic acid content (Weeks 2017). A group of *Fatty Acid Desaturase 2 (FAD2)* genes play a role in both linoleic and linolenic acid biosynthesis. *FAD2* genes, located on three independent pairs of Camelina chromosomes, were targeted for mediated interference (Jiang et al. 2017). The analysis of T4 generation seeds shows an increase of 50% in the oleic acid levels while reducing the polyunsaturated fatty acid levels by 15%. Another study conducted by Morineau et al. (2017) created a large number of *FAD2* gene knockouts, obtaining successful knockout plants of all existing *FAD2* genes (reviewed in [Weeks 2017]).

Recently another approach was performed on the banana genome by Kaur et al. The banana gene phytoene desaturase *(RAS-PDS)*, involved in the pathway of carotenoid biosynthesis, has been targeted for interference mediation and the resulting 13 mutant lines have been examined for chlorophyll and carotenoid content (Kaur et al. 2018).

Oilseed rape (*Brassica sp.*) is a tetraploid plant which is important for its oil content. Two *ALCATRAZ* (*ALC*) homeologs of *Brassica napus* have been targeted with CRISPR/Cas9 to increase shatter resistance, an important issue causing up to 25% seed loss during preharvest (Braatz et al. 2017). It has been observed that the plants with disrupted *ALC* function are lacking the production of specialized silique tissues, leading to reduce disease resistance. The rapeseed plants with knocked out *ALC* function show a lower seed loss in the process of threshing. The targeted mutagenesis of *BnALC* homologs resulted in the mutation of four alleles in a single T1 plant using a single target sequence, indicating the possibility of simultaneous modification of different homoeologous gene copies in polyploid species.

11.9 Conclusions

Fruit and vegetable improvement, both concerning product quality and yield, is a major aspect of plant breeding. Advancements in the field of gene editing strategies allow scientists to rapidly obtain fast and efficient results compared to the traditional gene modification systems. The basic working principle and versatility enable CRISPR/Cas systems to be the most powerful gene modification tool since the beginning of plant breeding. As an example, the development of gRNA libraries increases the speed of the CRISPR/Cas application to discover certain functional genes or the regulatory elements. The development of online tools, providing pooled CRISPR libraries, speed up the process of advancements in this field. For instance, tomato CRISPR libraries have been generated based on the *Agrobacterium*-based T-DNA delivery technique for the generation of mutants for gene families (Jacobs et al. 2017). Consecutively, the web tools enabled scientists to target single genes on a certain plant genome while allowing the identification of the same gene on different species.

The identification of relationships among the traits of interest with selectable markers is crucial in order to improve the accessibility and rapidity of gene modification studies.

The importance of CRISPR/Cas system lies in the fact that it can be easily designed for different purposes such as visual identification of defined regions by the combination of CRISPR technique with fluorescent proteins as well as purification and isolation of proteins and nucleic acids associated with DNA or RNA (Tanenbaum et al. 2014; Fujita and Fujii 2013). Future studies should be carried out in order to enhance the efficiency of gene targeting applications in the field of plant breeding.

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