Chapter

Using of Genome Editing Methods in Plant Breeding

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Abstract

The main task of plant breeding is creating of high-yield, resistant to biotic and abiotic stresses crop varieties with high product quality. The using of traditional breeding methods is limited by the duration of the new crop varieties creation with the required agronomic traits. This depends not only on the duration of growing season and reaching of mature stage of plants (especially the long-period growth plants, e.g. trees), as well as is associated with applying of multiple stages of crossing, selection and testing in breeding process. In addition, conventional methods of chemical and physical mutagenesis do not allow targeting effect to genome. However, the introduction of modern DNA-technology methods, such as genome editing, has opened in a new era in plant breeding. These methods allow to carry out precise and efficient targeted genome modifications, significantly reducing the time required to get plants with desirable features to create new crop varieties in perspective. This review provides the knowledge about application of genome editing methods to increase crop yields and product quality, as well as crop resistance to biotic and abiotic stresses. In addition, future prospects for integrating these technologies into crop breeding strategies are also discussed.

Keywords: genome editing, programmable nucleases, oligonucleotide-directed mutagenesis, base editing, crop yield, food crop quality, crop resistance

1. Introduction

Currently the world population is about 8 billion people. According to the UN data, the number of people experience moderate or severe food shortages has reached 2 billion or 26.4% of the world population [1]. Huge efforts are being made to eradicate hunger and malnutrition in the world actually. Many of them are associated with scientific breakthrough in the life science and agriculture area [2]. However, despite the achievements of plant breeding, the issue of short term creation of new high-yielding and stress resistant varieties of crops is still actual. All of this is aimed to challenge the such problems as crop losses due to climatic changes, reducing of cultivated areas and spread of more aggressive and resistant pathogens. No less important reason is world population growth [2, 3]. At the moment, this is impossible without the use of biotechnological and genetic engineering approaches.

In the 20th century a classical crop breeding approaches were based on either natural mutations or artificially induced mutagenesis [4]. However, the traditional breeding methods have sufficient disadvantage such as long-term period to create

of new varieties with desired agronomic characteristics of any crops. This depends not only on the duration of growing season and reaching of mature stage of plants (especially the long-period growth plants, e.g. trees), as well as is associated with applying of multiple stages of crossing, selection and testing in breeding process. In addition, the following should be mentioned, both natural mutations and conventional methods of chemical and physical mutagenesis do not permit to target the plant genome [4].

At the turn of XXIth century the development and introduction of molecular DNA markers allow to significantly reduce the time required to create new lines and varieties of agricultural crops. In other words, the marker assisted selection approaches were appeared, thus significantly increase the effectiveness of breeding programs to increment in productivity of crops in a wide range of environmental conditions [5, 6]. However, these approaches also do not enable to target the crop genome.

At the same time, advances of next-generation sequencing, multitude of sequenced genomes of major crops and newly identified genes and their functions motivate researchers to pursue targeted breeding of plants. All of this have significantly promoted the development of targeted genome editing (GE) approaches [7–10]. One of the first GE technologies was RNA interference (RNAi) [4, 11, 12]. Despite the successful application of this technology in functional genomics and plant breeding [15–17], this method has a number of disadvantages, such as partial gene function suppression and indefinite insertion place of an RNAi construction into the genome [4].

The solution to these breeding problems was the application of GE methods using sequence-specific nucleases (SSN) to introduce targeted mutations in crops with high efficiency and accuracy [7–10, 12]. Artificially engineered SSNs such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats (CRISPR) associated with the endonuclease Cas (CRISPR/Cas) have been shown to be highly effective in targeting mutagenesis in a wide range of model plants and crops [7–10, 12]. In addition, oligonucleotide directed mutagenesis (ODM) allows to edit the genome at the single nucleotide level. Moreover, base editors (BEs) have recently been developed to replace A-T base pairs to G-C base pairs [12, 13].

Nowadays GE technologies are widely applied both in functional genomics and in the development of new varieties of crops with new valuable properties and resistant to various biotic and abiotic stresses [7–10, 12, 13]. Herewith, despite the fact that modern GE technologies are much more accurate than conventional mutagenesis, the legistation of GE crops remains the main bottleneck [13, 14]. A particular difficulty is associated with the biosafety assessment of such crops, the impossibility of determining the subsequent effect of single base mutations after using ODM and BEs [13, 14].

This review discusses GE mechanisms and their use for crop improvement, as well as the problems associated with these approaches.

2. Genome editing mechanisms

2.1 Programmable nucleases

Currently, there are three main GE methods classified according to the mechanism of action. The most commonly one applied for plant genomics is the targeted generation of double-stranded DNA breaks (DSBs) by SSNs [12, 13]. Whereat this DSBs are recovered by the cell's own endogenous repair mechanisms either through

non-homologous end joining (NHEJ) or homologous recombination (HDR) [7–10, 12–16]. Thereat, the reparation of the target DNA sequence leads to the genesis of single base mutations that changing or shifting of the reading frame and initiating of indels or nucleotide substitutions, as well chromosomal rearrangements [8, 15].

Targeted induction of DSB is possible by programmable nucleases. The prevailing nucleases for GE are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR) associated with the endonuclease Cas (CRISPR/Cas). These three classes of nucleases are different in structure, activity and action mechanism, that leads to differences in target selection, efficiency, specificity and nature of mutation [7–10]. Let's take a closer examine each programmable nucleases types.

2.1.1 Zinc finger nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are the first artificial endonucleases designed for GE [17]. Every ZFN is derived by aggregating of DNA-binding domain containing of a few linked zinc finger (ZF) motifs and the nonspecific endonuclease FokI [12, 17]. Association of ZF motifs promotes to develop ZF proteins (ZFP) consisting of approximately 30 amino acids and having ββα structure stabilized by zinc ions chelation [17]. Combination of ZFP with methylase, FokI and transcription activator/repressor gives rise ZFN [12, 13, 17]. FokI is an endonucleases recognizing of 5-mer non-palindromic sequence 5'-GGATG-3' : 5'-CATCC-3' and cleaving DNA 9/13 nucleotides downstream of the recognition site [12, 17, 18].

By intersecting with DNA, each ZF motif is capable to bind one triplet of nucleotides inserting an α-helix into the major groove of the DNA double helix [12, 18]. It should be also noted that one ZF has not sufficient specificity for binding to the target genome. However, artificial ZFN usualy contains three or four ZFs, which permit to bind 18-24-mer site after FokI dimerization which is necessary for efficient DNA restriction [17]. During FokI dimerization, two ZFNs can bind both forward and reverse DNA strands respectively, and two target sequences - forward and reverse should be separated by a spacer sequence of 5 to 7 bp [7]. In this case, ZFN acts like dimer and generates DSBs with short 5'-cohesive overhangs, which are filled by homologous recombination, that gives rise to indels into the genome [7–10, 12, 17, 18].

It should also be noted that, despite the sufficient binding specificity of ZFNs, they are more likely to make nucleotide mismatch [17]. Heterodimerization of FokI nuclease is used to minimize non-target effects and, accordingly, cellular toxicity of ZFN [19].

According to the first report in 1996, ZFNs have been successfully applied for gene modification in plants [17, 18]. The ZFN technology was used to edit the genome of tobacco and *Arabidopsis* [18]. In tobacco ZFN technology was used to restore the function of the defective reporter gene *GUS:NPTII* [18]. In *Arabidopsis*, the induction of ZFN expression under control of the heat shock protein promoter led to 106 mutations on the DNA, where 83 (78%) were 1-52-mer deletions, 14 (13%) - 1–4-mer insertions and 9 (8%) – deletions accompanied by insertions [18]. Nowadays, there are many studies confirming the possibility of GE in tobacco, *Arabidopsis*, maize, soya, canola and other plants using ZFNs [8, 15, 18]. At the same time, the use of ZFNs permit to introduce mutations in the endochitinase-50 gene (*CHN50*) in tobacco to emergence of resistance to *pat* herbicides [18]. In addition, similar results was got by the target editing of *IPK1* (inositol-1, 3, 4, 5, 6-pentakisphosphate kinase 1) gene, responsible for phytic acid biosynthesis in maize. ZFN-based targeting of *ABI4* (ABA Insensitive-4) gene in *Arabidopsis*, Dicer-like genes (*DCL4a* and *DCL4b*) in soybean and genes of alcohol dehydrogenase and chalcone synthase in *Arabidopsis* have been also reported [18].

However, despite the rather successful use of ZFNs, they have not become widespread as a GE tool due to the presence of a number of disadvantages. Main of them is complexity and high cost intensive technology, constructing of protein domains for each specific locus of the genome [18], likelihood of inaccurate cleavage of the target DNA due to single nucleotide substitutions or incorrect interaction between domains [8, 15, 17–19].

2.1.2 Transcription activator-like effector nucleases (TALENs)

TALENs similar to ZFN are enzymes consisting of specific DNA-binding domains of highly conservative repeats originating from effectors such as transcription activators (TALEs) which associate with FokI [20]. TALEs domains contain 15–30 copies of 33–34 highly conserved amino acid sequences [20]. The exceptions are 12^{th} and 13^{th} amino acid residues, which have high variability (repeat-variable diresidues – RVD) [17, 20]. It permits to establish the recognition code for specific nucleotides using a pair of such amino acids within the repeating peptide chains of a given protein [20]. This code is degenerate, but there is a clearly pronounced preference for some combinations of amino acids [17, 20]. It permit to design recombinant proteins capable of recognizing specific DNA sequences [20]. Activity of TALEN depends on amino acid number between TALE domain and FokI, as well as base number between binding sites [17, 20].

In contrast to ZF each repeat in the TALE domain recognizes one nucleotide [17, 20]. The TALE domains recognize 15-30 nucleotides that is 30-60 nucleotides for each TALEN dimer after FokI dimerization. Moreover, despite the fact that TAL domains have higher binding specificity, they are more likely to allow nucleotide mismatches [12, 13, 17]. As well as ZFN the heterodimerization of FokI is applied to minimize off-target effects by using of TALEN [21].

Analysis of mutations occurred during GE using TALEN shows that deletions are way more than insertions (89% versus 1.6%). The reason is the longer length of the TALEN spacers, which provide more extended protruding ends for the DNA fragments after DSBs [22].

Theoretically, the use of TALEN permit to introduce DSB into any part of the genome. There is one limitation only – the presence of thymidine upstream of the 5' end of the target sequence is needed for the TALEN nuclease recognition sites. However, variation of the spacer length allows to select restriction sites [20].

2.1.3 Clustered regulatory interspaced short palindromic repeats (CRISPR/Cas9)

CRISPR/Cas technology permits to make different changes in the DNA sequence [12–16]. Moreover, this GE technology is much cheaper, faster, more efficient and simple in practical application in comparing to ZFN or TALEN [17, 23]. This technology is based on the use of mechanisms of adaptive "immunity" discovered in bacteria – a specific antiviral defense of bacterial cells based on the complementary binding of viral DNA and their follow destruction [7–10, 12–14].

In this system small guide RNAs (crRNA) are used for sequence-specific interference of foreign nucleic acids. CRISPR/Cas includes a genetic locus so-called CRISPR containing short repeats separated by unique sequences (spacers) [24–29]. The CRISPR complex is predated by the AT-enriched leader sequence and flanked by *cas* genes [24–27].

Depending on the *cas* genes classification CRISPR/Cas systems are divided into two classes. The class 1 of CRISPR-Cas (types I, III, and IV) uses for interference protein complexes with several *cas*, while class 2 systems (types II, V, and VI) – single effector protein [26, 28, 29]. Type I system is characterized by the presence of

Cas3. Type II systems use Cas1, Cas2, Cas9 and Csn2 or Cas4, and type III systems – Cas10, the role of which has not yet been identified [29].

Currently, CRISPR/Cas type II is most often used for genome editing. This type contains the protein Cas9, which is necessary for interference and bacterial immunity [26]. Let's a closer look at this genome editing system.

To edit target genes, the CRISPR/Cas9-based GE requires the occurrence of CRISPR-associated protein 9 (Cas9), CRISPR RNA (crRNA), transactivating crRNA (tracrRNA) and ribonuclease III (RNase III) [12–14]. Thereat the crRNA and tracrRNA coassemble into a single guide RNA (sgRNA) [28].

Cas9 is the endonuclease cleaving a double-stranded DNA (dsDNA) [24–27]. This nuclease were isolated from various bacteria, such as *Brevibacillus laterosporus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus thermophilus* [24–26]. It should be noted that Cas9 from *Streptococcus pyogenes* (SpCas9) is most often used to genome edit [27].

Cas9 contains two domains: His-Asn-His (HNH) and RucV-like domains cleaving dsDNA at 3 bp upstream of the motif adjacent to protospacer (PAM) (5′ NGG or 5′-NAG for SpCas9) [24, 25, 29]. The HNH domain cleaves the complementary crRNA strand, while the RucV-like domain - the opposite strand of dsDNA [47]. Then generated DSBs are repaired by NHEJ or HDR [24–29].

sgRNA is 100-mer synthetic RNA and consists crRNA and tracrRNA. The 5'-end of sgRNA contains a 20 nucleotide guide sequence to identify the target sequence followed by consensus PAM sequence [27]. 3'-end of sgRNA has loop structure which permit to fix the guide sequence to the target site and interact with Cas9 forming ribonucleoprotein complex (RNP) which generates DSB at the target DNA region [27, 29].

Efficient DNA cleavage are provided by ribonucleoprotein complex (RNP) [29]. crRNA plays an important role in the recognition of target DNA due to the sequence that directs RNP to a specific locus by base pairing with target DNA with formation of R-loop structure [29]. R-loop generation activates the HNH and RuvC-like endonuclease domains of Cas9, which cleave dsDNA, creating blunt-ended DSB at 3 bp upstream of the PAM site [25]. Thus, CRISPR/Cas9 performs gene editing in three stages. At the first stage Cas9 is expressed, at the second stage – generation of sgRNA which containing 20 nucleotides complementary to the target region. The third stage requires an NGG PAM recognition site located closer to the 3' end of the target region. The RNP guided by sgRNA generates a blunt-ended DSB at 3 bp upstream of the PAM site [25].

The one limitation of the CRISPR/Cas9 using is the fact that sgRNA design requires the presence of the targeted PAM sequence at the 3' end [24–26]. For SpCas9 this sequence is defined as 5'-NGG-3' [25]. Frequency of PAM sites in the plant genomes revealed by *in silico* is 5-12 sites per 100 bp [30]. This fact underlies a difficulty of identifying target sequence, especially in large genomes with nomerous of repetitive sequences, such as maize, cotton, wheat, etc. [13]. All of mentioned above is one of the factors of CRISPR/Cas9 untargeted effect [30]. To reduce unexpected effects other Cas9 can be used, for example from *S. aureus*, which recognizing less common NNGRRT-PAM [31] or mutant SpCas9 recognizing non-canonical PAM [32].

2.2 Oligonucleotide-directed mutagenesis (ODM)

ODM is site-directed mutagenesis tool using mutagenic DNA fragments 20-200 nucleotides in length [33]. In this approach, the fragment sequence match with a target sequence in the genome, except of a single base pair, which is a putative mutation introduced into the genome [33, 34].

In eukaryotic cells the oligonucleotide for ODM penetrates into the cytoplasm through the cell membrane, then enters the nucleus and complementarily interacts with the target DNA sequence. Herewith, the mismatched nucleotides contribute to initiate a specific change of the sequence that occur in the target gene due to the errors reparation mechanism in the cell. This is two-stage process initially requires annealing of the specific oligonucleotides with target DNA, and subsequently repair of nucleotide mismatch leading to a directed mutation [33]. This system was first demonstrated on mammalian, after that in plants [35]. However, it should be noted that in plants the oligonucleotide does not integrate into the genome because of modifications of the 5' and 3' ends, that prevent DNA ligation, and due to the activity of endonucleases that destroy oligonucleotides [34].

ODM can be accomplished by single-stranded DNA fragments, but their using is limited by a short intracellular half-life [13]. To overcome this disadvantage, the stabilizing modifications for ssODN are necessary. These include chimeraplasts (DNA/RNA duplexes modified by methylation), modification with phosphorothioate, ssODNs with a 5'-tag Cy3 and modified 3'idC reverse base [33]. Additionally, it should be noted the rather low efficiency of ODM, that positive correlate with the length of oligonucleotide fragments. It was shown that by increasing of ssODN length to 200 nucleotides it allows to increase accuracy of editing up to 0.05% in *Arabidopsis thaliana* [33]. The chimeraplasts using did not lead to an increase of mutation frequency higher than the level of spontaneous mutations in *Nicotiana tabacum* or *Brassica napus* [36]. In this regard, to increase the mutagenesis efficiency and the target mutations frequency ODM is often used in combination with non-specific reagents inducing DSB, such as antibiotics or TALENs and CRISPR/Cas9 in *Arabidopsis* and *Linum usitatissimum* [37].

2.3 Base editors (BE)

All programmable nucleases generate DSBs that are repaired by either NHEJ or HDR [7–10]. The common disadvantage of these nucleases is the non-predictable results of DSB repairing due to NHEJ mechanism and the low efficacy of HDR [13]. In this reason it became necessary to develop new methods for the introduction of point mutations excluding of DSBs, which resulted in GE new tools so-called base editors (BE) [38].

BE technologies use nickase Cas9 (nCas9) or functionally inactive Cas9 (dCas9) combined with a cytosine or adenosine deaminase domain, which bring into action transformation bases [39]. For example, cytosine deaminases transform cytosine (C) to uracil (U), which is identified as thymine (T) during following DNA repair and replication, thus providing a C:G to T:A replacement [38, 39]. In the same way, adenine deaminases convert adenine (A) to inosine (I), which polymerases interpreting as guanine (G), creating A:T for G:C replacement [39]. Cytidine deaminase-based BE (CBE) has been used to edit genomes in rice, *Arabidopsis*, wheat, corn, tomato and watermelon [39–41].

In the lack of known adenine deaminases, the substitution of A:T to G:C is a more difficult task than on the contrary [39]. This problem has been solved by bio-engineering of tRNA adenosine deaminases for their adaptation to DNA as a substrate [39–41].

Initially developed BE had an editor window of for several base pairs only, that led to the appearance of unexpected random mutations. But further improvement of these methods allows to create high-precision Cas9-based BEs that control the length and flexibility of the linker and thus able to selectively edit the bases in direct position with high accuracy, efficiency and simplified PAM requirements [39–41].

3. Application of GE approaches

3.1 Application of GE in functional genomics

NHEJ- or HDR-mediated repair of DSBs generated by programmed nucleases leads to the appearance of insertions /deletions, which shift the reading frame [7–10], while the single base changes by ODM [33] or BE [39] promotes targeted replacement of nucleotides. These events lead to changes in the genes activity due to the effects of gene-knockout or gene-knockin, that can be used to reveal their function [17, 29, 34, 42, 43].

Besides gene-knockout and gene-knockin, GE technologies can also be used to regulate gene expression. In this case, genes repression and activation achieved by combining repressors or activators of transcription with the DNA-binding domains of programmed nucleases is most often used [7].

Unlike technologies targeted introducing changes in the DNA nucleotide sequence, gene regulation by GE methods is carried out at the level of transcripts. It allows to reveal the functions of many non-canonical non-coding transcripts without open reading frames [16].

Moreover, the CRISPR/Cas using makes it possible to simultaneously introduce into cells several genetic constructs targeted different regions of the genome [15, 16]. It allows to have an effect on the work of several genes simultaneously and to study intergenic interactions. In this wise, it is possible, for example, to determine the genes involved in the process of crop domestication [29].

In addition, the CRISPR/dCas9 system combined with epigenetic regulatory factors involved in histone acetylation or DNA methylation, can be efficiently used to modulate chromatin activity and gene expression patterns involved in plant development and adaptation to the environment [29].

3.2 Using of GE approaches in plant breeding

Nowadays, GE technologies are effectively used to create new varieties of agricultural crops with improved traits, such as increased yield, product quality and resistance to biotic and abiotic stresses. Such traits improvement is often carried out by introducing target mutations into the corresponding regulatory genes that control the development of undesirable traits leading to the suppression of their activity [7–10].

In this section, we review key advances in crop trait enhancement using GE techniques and discuss their prospects for improving food security.

3.2.1 Crop yields increase

Productivity is one of the most important economically valuable traits of agricultural crops. At the same time, this trait is also one of the most difficult to improve by conventional breeding methods [44]. It's explained by the fact that yield is often a quantitative multigenic trait, the development of which is controlled by multiple quantitative trait loci (QTL) [45]. Additionally, traditional yield-based selection is complicated by QTL introgression between different varieties, which is especially pronounced in the case of closely linked loci [44, 45].

In this regard, GE technologies represent a promising tool for the rapid and directed mutagenesis of target genes [7–10, 13]. Herewith, the most effective way to increase yields using genome editing technologies is to knock out ("turn off") genes negatively affecting the yield [44]. For example, CRISPR/Cas9-based "turn off" of

the functions of yield negative regulators (*Gnla*, *DEP1* and *GS3*) in rice has led to yield improvement, that manifested itself as increased number of grains in panicles and a larger grain size, respectively. It should also be noted that this gene knockout is inherited and observed at least in the T_2 generation inclusive [10, 44].

Additionally, there is evidence that CRISPR/Cas9-based multiplex knockout of the main negative regulators of rice grain weight (*GW2*, *GW5*, and *TGW6*) allowed to significantly increase the weight of grains. Similar results were obtained by CRISPR/Cas9-mediated knockout of the *GASR7* gene (a negative regulator of the wheat grain width and weight). In addition, CRISPR/Cas9-based silencing of *OsGn1a*, *OsDEP1*, *OsGW2*, *OsGW5*, *OsTGW6*, *OsGS3*, *OsIPA1*, *OsPYLs*, *OsCCD7*, *OsLAZY1* and *NtPDR6* genes in wheat allowed to improve the yield-related characteristics [44, 46].

Also, it was shown that the CRISPR/Cas9-xyr5APOBEC1-mediated single base mutations in two rice genes, *NRT1.1B* and *SLR1* improved the efficiency of nitrogen utilization and increased yield [39]. Also, CRISPR/Cas9-mediated knockout of genes contributing to yield improvement allows to amend this economically valuable trait in many other crops [44, 46].

3.2.2 Product quality improving

Products quality is another economically valuable trait, the selection of which by traditional methods is accompanied by significant difficulties. Thereat, selection for this trait is complicated both by the difficulty in obtaining targeted mutations by the methods of chemical and physical mutagenesis, and the presence of negative correlations between the traits of quality and yield [11]. GE technologies allow to cope with the deficiencies of chemical and physical mutagenesis due to the ability to introduce targeted mutations into the genome and improve the nutritional properties of crops [7–10, 44].

Let us consider some examples of the potential application of GE methods for modifying the chemical composition of plants. For example, silencing one of the key genes of phytate biosynthesis *ZmlPK* by TALEN and CRISPR/Cas9 systems allowed to reduce its content in corn (*Zea mays*) [10, 44]. Herewith, the feed value of such corn grain is much higher due to the fact that phytate is considered an anti-nutritional element, reducing availability for digestion of proteins and minerals. Similar results were obtained in barley with TALEN-mediated knockout of the *HvPAphy* gene, which plays an important role in phytate biosynthesis [10, 44].

Also, TALEN-based "turn off" of *VInv* gene encoding vacuolar invertase allows to obtain potatoes (*Solanum tuberosum*) without the potential carcinogen acrylamide, which is formed during frying as a result of the reaction between reducing sugars and free amino acids [10, 44]. Additionally, TALEN system was used to knock out the *OsBADH2* gene in rice that resulted to an increase in 2-acetyl-1-pyrroline [44], which is responsible for the smell of cooked rice. Along with this, TALEN-mediated mutagenesis of the *FAD2-1A/B* gene in soybeans increased the content of oleic acid [44].

CRISPR/Cas9-based targeting of conserved regions in the α-gliadin genes has allowed to create wheat lines with reduced gluten immunoreactivity [44]. At the same time, CRISPR/Cas9-mediated multiplex mutagenesis of *SGR1*, *LCY-E*, *LCY-B1*, *LCY-B2* and *Blc* genes involved in lycopene biosynthesis contributed to the production of tomato lines with an increased content of lycopene [9, 10].

In addition, it was reported that CRISPR/Cas9-mediated knockout of genes responsible for amylose biosynthesis: *GBSS* gene in potatoes and the *Wx1* gene in maize allowed to obtain potato and maize lines with a reduced amylose content [9, 10, 44]. The opposite result was obtained in rice by silencing of the *SBEI* and

SBEIIb genes responsible for the biosynthesis of starch [44]. CRISPR/Cas9 system was also used to decrease the level of linolenic acid and to increase the level of oleic acid in *Camelina sativa* by multiplex knockout of *FAD2* homeologues [46].

3.2.3 Herbicide resistance improving

Herbicides are the class of chemical compounds most widely used in agricultural practice. This is due to the fact that weeds cause significant damage to agriculture, reducing yields from competition with crops for resources [47]. However, despite the success of the herbicide using, their main disadvantage is their non-selective effect. To overcome this disadvantage, herbicide-resistant biotechnological varieties were created using genetic engineering methods. Currently all herbicide-resistant varieties approved for use have been obtained by transgenesis [11]. At the same time, GE methods are an effective tool for creating herbicide-resistant crop lines [7–10].

The main genes targeted by the GE in creating herbicide-resistant lines are the *EPSPS* and *ALS* genes. *ALS* gene encodes acetolactate synthase, participated in the biosynthesis of branched-chain amino acids, and the EPSPS gene encodes 5-enolpyruvylshikimate-3-phosphate synthase involved in the biosynthesis of essential plant aromatic amino acids [47]. Thus, based on the genes functions, it can be assumed that targeting *ALS* gene allows to obtain crop lines resistant to sulfonylurea herbicides, and *EPSPS* gene – to glyphosates [44]. Such lines were obtained by ODM-mediated targeted mutagenesis of the *ALS* gene in tobacco, rice, corn, and wheat [46]. Similar results of obtaining tobacco lines resistant to sulfonylurea were obtained by ZFN [18]. Also, other programmable nucleases (TALEN and CRISPR/Cas9) were used to obtain herbicide-resistant lines of potato, rice, maize, and soybeans [44]. In addition, to obtain herbicide-resistant lines of rice (*Oryza sativa*), wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), and watermelon (*Citrullus lanatus*), single base mutations were introduced into the ALS gene by CBE and ABE [39].

Along with this, CRISPR/Cas9-mediated point replacement of two nucleotides in the *EPSPS* gene allows to obtain glyphosate-resistant flax (*Linum usitatissimum*) and rice lines [44]. The targeting of the protoporphyrinogen oxidase (*PPO*) gene in *Arabidopsis* and exon 2 of inositol-1,3,4,5,6-pentakisphosphate-2-kinase (*IPK1*) gene in maize by ZFN was also used to obtain herbicide-resistant lines [18].

3.2.4 Biotic stress resistance improvement

Biotic stresses are one of the basic factor of crop losses in agriculture [47]. The main biotic stresses affecting crops include phytopathogens (viruses, bacteria, fungi), insect and pests (phytophagous insects, acari or nematodes). The strategy for dealing with biotic stresses is either in increasing of self defense mechanisms in plants or in introducing into the genome of constructs aimed against the pathogens [47, 48].

The producing of crops lines resistant to pathogens and pests using traditional breeding methods is based on increasing the own defense mechanisms in plants, but the introduction of constructs targeted pathogens into the genome is carried out by genetic engineering. The most of biotechnological crop lines resistant to biotic stresses created to date are obtained by transgenesis or RNA interference (RNAi) methods [47].

Nowadays, GE approaches are widely used to create new resistant lines [7–10, 44]. Herewith, it should be mentioned these methods makes possible to use both strategies to deal with biotic stresses [44, 47, 48]. Let us to consider some examples of the use several GE technologies to develop lines and varieties of crops resistant to biotic stresses.

Targeting on plant susceptibility genes. Plant susceptibility genes are essential to successful infection and development pathogens [49]. Thereat, affect these genes brings about development of plant resistance [7, 44, 49]. As targeting of TALEN- and CRISPR/Cas9-based *MLO* homologues providing resistance to powdery mildew allows obtaining resistant lines of barley, wheat, Arabidopsis, tomato and pea [7, 44, 49]. Additionally, the CRISPR/Cas9-mediated directed affecting of *DMR6* gene allows creating tomato lines resistant to *Pseudomonas syringae*, *Phytophthora capsici*, and *Xanthomonas* spp [49].

Targeting of disease susceptibility factors was also used for creation of virus-resistant crops. As CRISPR/Cas9-based silencing of *eIF4e* factors associated with plant infection by positive sense RNA viruses allows creating virus-resistant plants. Herewith, CRISPR/Cas9-mediated disruption of *eIF4Es* gene function in *Arabidopsis* and cucumber promotes to develope potyvirus resistance [49]. Similarly, CRISPR/Cas9-based mutagenesis of *eIF4G* allows obtaining some rice lines resistant to rice tungro spherical virus (*RTSV*) [49].

To creation of crop lines resistant to biotic stress GE approaches are also used to impact on regulatory elements that can affect the process of pathogen proliferation [49]. So as, TALEN- and CRISPR/Cas9-mediated mutagenesis in effector binding site of promoter region of *OsSweet14* gene permits to develop *Xanthomonas oryzae* pv. *oryzae* resistant rice lines [7, 44, 49]. Also it was shown that Cas9/sgRNA-based targeting the effector binding element of *CsLOB1* gene in citrus provided the generation cancer-resistant citrus varieties [50].

Targeting on genes of plant hormonal system. It is known that hormonal signalling plays an important role in immune response of plants [51]. Herewith, salicylate-mediated immune response develops in response to biotrophic and hemibiotrophic pathogens infection, and by jasmonate- and ethylene-mediated – against necrotrophic pathogens. Thus, directed effect on the genes of the hormonal response permit to create pathogen-resistant crop lines [49]. Recently this assumption has been confirmed experimentally. Herewith, CRISPR/Cas9-based targeting of the *SlJAZ2* gene in tomato resulted in genesis of resistance to *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 [48].

Targeting of pathogen genomes. Another advanced strategy for pathogens defence is directed effect on pathogen genome. Currently, this strategy is mainly used against viral plant diseases [7, 44]. Thus, CRISPR/Cas9 system has been successfully applied to increase resistance to DNA-containing viruses, including tomato yellow leaf curl virus (*TYLCV*), beet root curl virus (*BCTV*), Merremia mosaic virus (*MeMV*), beans yellow dwarfism virus (*BeYDV*), cotton leaf curl virus and beet severe curly top virus (*BSCTV*) [44, 48]. It should be noted that one sgRNA designed to targeting a conserved region can mediate interference against a numerous DNA-containing viruses [8, 44]. Additionally, CRISPR/LshCas13a system capable to interfere of viral RNA turnip line is successfully applied to creation of resistant to RNA-containing turnip yellow mosaic virus (*TuMV*) [44].

3.2.5 Abiotic stress resistance improvement

Abiotic stresses are the main factors that negatively affect the yield of most crops [52]. In this regard, a creation of resistant to adverse environmental factors crop varieties is the urgent problem. However, the use of traditional breeding methods to develop such varieties is limited by the fact that the traits of resistance to abiotic stress are multigene controlled and have a complex inheritance type [52, 53]. The disadvantages of traditional breeding can be successfully overcome through the use of GE approaches. Herewith, a literature analysis has shown that the GE application

in various cultures allowed to increase their resistance to abiotic stresses [7, 44, 53, 54]. Let us consider detailed examples of the GE application to obtain varieties of crops resistant to abiotic stresses.

Targeting of structural genes. Structural genes are one of the most convenient targets to increase plant stress resistance. This class of genes can be divided into tolerance genes (T-genes) and sensitivity genes (S-genes). The T-genes encode enzymes of the antioxidant system, while S genes – negative regulators in plant defense mechanisms [54]. Therefore, "turn off" of S-genes allows to obtain drought-resistant crop varieties. This assumption was confirmed by CRISPR/Cas9-based targeting of *ARGOS8* (a negative regulator of ethylene response) in maize, that permit to obtain drought-resistant lines [53]. Similar results were obtained during directed mutagenesis of the *OST2* gene in *Arabidopsis*, *TaDREB2* gene in wheat (*Triticum aestivum*) and *OsDERFl*, *OsEPSPS* and *OsMSHl* genes in rice (*Oryza sativa* L.). In addition, CRISPR/Cas9-mediated targeting of *OsPDS*, *OsMPK2*, and *OsDEPl, OsAOXla*, *OsAOXlb*, *OsAOXlc* and *OsBEL* genes allows to develop rice lines resistant to a wide range of abiotic stresses [10, 44, 53, 54].

Targeting of regulatory genes. Regulatory genes, such as transcription factors, phosphatases and kinases are also involved in the regulation of intracellular signals during abiotic stresses and can be used as target for GE tools to create stress-resistant crop varieties [54]. Presently, CRISPR/Cas9-mediated silencing of *SlMAPK3* regulatory gene in tomato (*Solatium lycopersicum*), *TaERF3* gene in wheat, *OsSAPK2*, *OsPMS3*, *OsRAV2*, and *OsNAC.041* genes in rice permit to create lines of these crops that are resistant to abiotic stresses [44, 53].

Summarize the presented data, it should be noted that GE systems are successfully used to modify a wide range of economically valuable traits in main agricultural crops.

4. Safety assessment and regulatory framework of GE systems

4.1 Off-target effects

One of the main limitation of GE systems is off-target effects, when nucleases, along with target regions, affect other parts of the genome [7–10, 39, 44]. It stems from the fact that the efficiency of DNA cleavage by nucleases depends both on nuclease activity and the availability of the target site and also the affinity of the DNA-binding domain. Moreover, the designed nucleases specificity largely depends on the binding affinity of the nuclease to DNA. In addition, FokI domain dimerization and interaction of Cas9 with PAM may also play an important role [17, 55, 56].

The preliminary comprehensive bioinformatics analysis to choose of specific sites for the introducing of DBS can minimize the off-target effects of the GE system. When choosing the desired sites, it should avoid regions with repeated sequences, as well as regions with high homology to other regions of the genome [17, 55, 56]. In addition, to minimize off-target effects and cellular toxicity of ZFN and TALEN heterodimerization of FokI nuclease is used [19, 21]. Another effective way to reduce the frequency of unintentional mutations is the use of the sgRNA/aptazyme system (ligand-dependent ribozyme) [56].

4.2 Regulatory framework

The legal and regulatory framework in regard to GE plants in different countries has a great impact on their competitiveness. In most countries, the current biosafety

framework is meant to regulate transgenic GMOs. Currently, only Argentina and Brazil have adopted additional legislation for crops obtained using GE techniques [57].

There are two main approaches to defining the regulatory framework for GMOs in global legislation: one is process-based and the other is product-based. In the European Union, a standard is focused on the first approach, while in Canada – on the second. In the United States, a hybrid system is used: the decision on whether a crop belongs to GMOs is based on the first approach, and the risk assessment – on the second. In this case, GE cultures are considered individually [13]. Thus, the same GE culture can be classified in different ways depending on the regulatory framework. For example, in Argentina, crops that are classified as "zero segregants" are not regulated as GMOs [57]. In the EU, GE crops are subject to Directive 2001/18/EC and must undergo a full biosafety assessment procedure, as well as meet the requirements for GMO products [13, 57].

5. Future prospects

GE tools are considered one of the most promising tools for practical agricultural biotechnology because of their high efficiency, relatively low cost, ease to use and multiplexing ability [7–10, 14]. Herewith, directed mutagenesis makes it possible to effectively "turn off" or silencing various genes, that helps to determine the functions of genes [17, 29, 34, 42, 43]. Multiplex targeting allow to reveal the role of individual genes and encoded proteins in intracellular signaling pathways and contributes to the engineering of multigenic agronomic traits in crops [7–10, 44].

Furthermore, CRISPR/Cas9 system may be used for spatial and temporal control of gene expression, as well as tissue-specific regulation of expression [42]. In the furtherance of this goal, influence on the *cis* regulatory elements of the gene promoter region has large potential and allows to change the expression level, expression of patterns, and tissue-specific expression [58, 59]. At the same time, BEs can be used to replace key nucleotides in *cis* elements to modulate the affinity of transcription factors and, accordingly, the level of expression [58]. Additionally, the introduction of mutations using programmed nucleases or BEs into the binding sites of the promoter regions will disrupt their interaction with the virulence proteins of pathogens and, thus, increase the resistance of crops [48, 58, 60].

Epigenomic studies are another promising uses of GE technologies. Herewith, the insertion of transposons into the promoter region due to the NHEJ or HDR mechanisms can affect the epigenetic status and, accordingly, the level of expression of commercially valuable genes [58]. It can be achieved through the using of such dCas9 systems as targeted DNA methylation and demethylation systems [58].

It should also be noted that an advanced uses of GE methods, in particular, CBE and ABE, is the introduction of single base changes into the microRNA (miRNA) binding site of the target gene without alteration of the amino acid sequence of the encoded protein. It will lead to disruption of miRNA/mRNA base pairing and, consequently, to disruption of mRNA cleavage due to the fact that the position and number of mismatches in the miRNA binding site strongly affect the efficiency of miRNA-mediated mRNA cleavage [58]. In turn, such disturbance of miRNA-mediated mRNA cleavage can be used to proper alignment of the target genes expression and, accordingly, can have effect on the development of many agronomical desired trait [58]. In addition, CRISPR/Cas system can be used for NHEJ- or HDR-mediated introduction of translation enhancers into the initiation codon of open reading frames, which provide fine tuning of gene expression at the translation level [58].

Thus, in summary it should be noted that GE systems, especially CRISPR/Cas and BEs, offer great opportunities for crop improvement.

6. Conclusion

In conclusion, it can be noted that GE technology has enormous potential to create new varieties of crops resistant to biotic and abiotic stresses and improved food value and yield. However, for the effective using of these technologies, it is necessary to resolve issues related to the biosafety assessment, including the revision of regulatory frameworks.

Conflict of interest

The authors declare no conflict of interest.

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