The potential applications of site-directed mutagenesis for crop improvement: a review

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Accepted: 16 November 2020 / Published online: 22 December 2020
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Abstract
The search for technologies for crop improvement has been a continuous practice to address the food insecurity to the growing human population with an ever-decreasing arable land and dynamic climate change around the world. Considering the potential technologies for crop improvement could close the rooms of poverty in developing countries in particular and around the globe at large. This review aimed to assess the site-directed mutation creation methods and show the potential tools for future crop improvement programs. Site-directed mutagenesis was found to be an efficient process to create targeted mutation on cereal crops, horticultural crops, oilseed crops, and others. Agronomic traits such as yield, quality, and stress tolerance have been improved using site-directed mutagenesis. Besides, selectable marker elimination was also reported from transgenic crops by targeted mutation. Most of the reports on site-directed mutagenesis is focusing on cereal crops (58.339%) followed by horticultural crops (22.92%). Among the four mutagenic tools that have been reported, the CRISPR/Cas9 technology was found to be frequently used (66.67%) followed by TALENs. This tool is potential, since it is efficient in creating targeted mutagenesis and less likely off-target effect, so it is repeatedly used in different research works. TALENs were used usually to knockout genes with bad traits. Moreover, the mutation created by mutagenic tools was found to be efficient, and the mutated traits proved to be heritable to generations. Hence, site-directed mutagenesis by the CRISPR/Cas9 system is advisable for agricultural development, thereby ensuring food sustainability around the world.

Keywords Cereals · Horticultural crops · Site-directed mutagenesis · Traits

Introduction
Agricultural development has always been on the move towards increasing crop productivity. Sustainable use of natural resources must be wisely managed in combination with the enrichment in the knowledge gained from science and technology (Mohan Jain and Suprasanna 2011). Global food security continues to be the first issue and plant breeders are obliged to sustain food production to meet the demand of the ever-increasing human population of the world (Nestel et al. 2006).

The process of crop improvement has been a fundamental issue for thousands of years ago (Godfray et al. 2010). The ultimate reason to crop improvement is to respond to the huge demand for food for the alarmingly growing human population around the globe (Rashid et al. 2017). Moreover, with the ever-greater demand for a balanced and healthy diet, there must be an on-going effort to develop improved crops using diverse technologies (Caligari and Forster 2001; Rao et al. 2018). Multifaceted and integrated global strategies are required to ensure sustainable food security through crop improvement programs (Godfray et al. 2010; Dobermann and Nelson 2013; Roychowdhury and Tah 2013).

Site-directed mutagenesis is one of the recent tools amongst molecular crop improvement technologies (Sauer...
et al. 2016). The major aim of mutation-assisted breeding is to develop and improve well-adapted plant varieties by modifying one or two major traits (Oladosu et al. 2016). The development of targeted mutation became a source of genetic variation which, in turn, became a resource for plant breeders (Kharkwal and Shu 2009). Therefore, mutation supported plant breeding could play a crucial role in addressing the uncertainties of global climate change and food insecurity challenges (Mohan Jain and Suprasanna 2011). Site-directed mutagenesis aims at a precise change of any coding sequence in vitro or in vivo. Site-directed mutagenesis could be produced using different methods. In vitro targeted mutation could be created by gene vector-based method or PCR-based method (Zheng et al. 2004; Liu and Naismith 2008).

Another method of the site-directed mutation creation method is gene editing using programmable site-directed nucleases (SDN), which are promising for new plant breeding techniques. This method could be achieved by generating a small deletion or insertion at a precisely defined location in the genome (Van de Wiel et al. 2017). These days, programmable nucleases are becoming a method of choice to create a targeted mutation in crops which could, in turn, serve as a platform for molecular breeding (Modrzejewski et al. 2019). Therefore, the purpose of this review is to assess the site-directed mutation creation methods and their potential for crop improvement research.

Site-directed mutagenesis: basics and principles

Site-directed mutagenesis has been used to generate mutation at a single site or multiple sites of the genome (Forloni et al. 2019). So far, three methods of site-directed mutagenesis are known vis. vector-based, PCR-based, and nucleases based site-directed mutagenesis. In the vector-based mutagenesis, either a plasmid or phage vector could be used for the purpose (Saboulard et al. 2006). In this method of mutation, one mutagenic primer and one normal primer could be used (Smith 1982; Zoller and Smith 1984). In PCR-based site-directed mutagenesis, the mutation could occur on double-stranded DNA, and the procedure involves simultaneous annealing of two oligonucleotide primers—one mutagenic and the other normal primer annealed to the denatured double-stranded DNA (Braman et al. 1996). The nucleases based site-directed mutagenesis involves enzymes that cut DNA at a specific sequence.

Site-specific recombinase enzymes catalyze double-stranded DNA exchange between strands with a limited degree of sequence homology. These enzymes attach to the recognition site, which is between 30 and 200 nucleotide length and cleaves the DNA backbone (Coates et al. 2005). The Cre–lox system consists of two components derived from the P1 bacteriophage, the Cre recombinase, and the loxP recognition site. The P1 bacteriophage uses these components as part of the natural viral life cycle and researchers adapted the components for gene manipulation purposes (Araki et al. 2002; Lambert et al. 2007).

Transcription Activator-Like Effector Nuclease (TALEN) is another engineered nuclease, which shows a better specificity and efficiency than ZFN. Similar to ZFNs, TALENs use DNA-binding motifs to direct the same non-specific nuclease to cleave the genome at a specific site, but instead of recognizing DNA triplets, each domain recognizes a single nucleotide (Table 1) (Li et al. 2016a, b. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are the latest exciting development in gene-editing technology. The CRISPR system is an RNA-based bacterial defense mechanism designed to recognize and eliminate foreign DNA from invading bacteriophages and plasmids (Gupta et al. 2020).

Application of site-directed mutagenesis in crop improvement

Feeding the growing human population is an increasingly difficult task. An important part of the solution is the development of improved new crop cultivars with high yield and stress tolerance. As we are facing challenges to increase global agricultural productivity, there is a rapid need to accelerate the development of these traits in crops (Sauer et al. 2016). Among the multitude of approaches that are used in crop improvement, targeted gene mutation technologies using different mutagenic tools are attractive technologies to develop novel traits (Christian et al. 2010; Gaj et al. 2013; Eş et al. 2019).

In recent times, different alternatives are used to bring targeted mutation and producing economic traits in crops or eliminate bad crop traits (Ren et al. 2016; Gupta et al. 2020). The rapid development of the field has allowed the development of highly efficient, precise, and cost-effective means to develop improved crop mutants (Nishizawa-Yokoi et al. 2016). It has been applied and improved the yield, quality, and stress tolerance traits of major food crops including maize, rice, wheat, barley, potato, soybean, carrot, cabbage, and tomato, and became an appeal to molecular breeding.

Maize improvement using site-directed mutagenesis

Maize is a major crop used as food in most of the world. Several research activities have been done to achieve targeted mutations on maize using different site-directed mutagenesis tools (Liang et al. 2014; Cai et al. 2018).

Using targeted mutagenesis on a conserved lysine residue, Lys was replaced by Asn, Glu, or Arg to improve phosphoenolpyruvate enzyme catalytic efficiency and regulatory role on maize using plasmid vectors. As a result, the maximum
| Feature                                      | ZFNs | TALENs | CRISPR-Cas9 | Site-specific recombinase |
|----------------------------------------------|------|--------|-------------|--------------------------|
| **Sources of enzymes**                       | Found in Bacteria and Eukaryotes zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes DNA Cleavage Domain FokI type II restriction enzyme Naturally found in Flavobacterium | Eukaryotes | Bacteria (Streptococcus sp.) | Example CreloxP; It is found from prokaryotes and lower eukaryotes CreloxP is made by integration of bacteriophage lambda and connected with the bacterial chromosome |
| **Length of recognized DNA target**          | 9–18 bp | 30–40 bp | 22 bp + PAM sequence | From a few hundred to tens of thousands of nucleotide pairs |
| **Mechanism of target DNA recognition**      | DNA–protein interaction | DNA–protein interaction | DNA–RNA interaction via Watson–Crick base pairing | Site-specific recombination systems mediate DNA rearrangements by breaking and joining DNA molecules at two specific sites, termed recombination targets |
| **Mechanism of DNA cleavage and repair**     | Double-strand break induced by FokI | Double-strand break induced by FokI | Single- or double-strand break induced by Cas9 | Double-stranded break induced by cre gene enzyme/recombinase |
| **Binding specificity**                      | 3 Nucleotides | 1 Nucleotide | 1:1 Nucleotide pairing | To the spacer of the 13 bp repeats |
| **Mutation rate (%)**                        | 10 | 20 | 20 | From hundreds to thousands of bps |
| **Target site length (bp)**                  | 18–36 | 24–40 | 22 | With an average mutation rate of ~ 11 amino acid substitutions per variant |
| **Double-stranded break pattern**            | Staggered cut (4–5 nt, 5′ overhang) | Staggered cut (Heterogeneous overhangs) | Sp Cas9 creates blunt ends; Cpf1 creates a staggered cut (5′ overhang) | Cleaves double-stranded between 13 bp repeats of loxP |
| **Challenges of the technology**             | High off-target effects | Low | Variable | May not be effective if the Cre gene and the loxP are separately introduced |
| **Dimerization required**                    | Yes | Yes | No | No |
| **Best suited for**                          | Gene knockout, transcriptional regulation | Gene knockout, transcriptional regulation | Gene knockout, transcriptional regulation, base editing | Gene knockout |
velocity ($V_{\text{max}}$) of the enzyme decreased to 22% when Asn was replaced in place of Lys and 2%, and $V_{\text{max}}$ reduction was observed when Lys is replaced by Glu in PEPC enzyme catalytic efficiency thereby enhancing sugar production (Dong et al. 1997). This indicates the potential of site-directed mutagenesis to improve the affinity and catalytic efficiency of PEPC for crop physiology. In a research report when Thr was substituted by Ser using double-stranded plasmid vector site-directed mutagenesis, the regulatory capacity of Pyruvate orthophosphate Dikinase (PPDK) enzyme in maize got improved, while catalytic efficiency remains unchanged (Chastain et al. 1997).

Selectable marker gene elimination from transformed maize was reported using Cre/loxP specific recombination system with the removal of yellow fluorescent protein (yfp), which was used as a selectable marker (Djukanovic et al. 2006). This technology could potentially be used for the efficient removal of other selectable markers from genetically modified crops. In another report, site-directed mutagenesis using engineered endonuclease targeting Lig34-site in the vicinity of the LIGULELESS1 (LG1), induction in the large-scale experiment produced 718 Parental ($T_0$) plants. The 781 $T_0$ transgenic plants were evaluated by PCR, and 23 $T_0$ plants were identified to contain mutations at the LIGULELESS1 locus based on visual screening of the Lig34-site (Gao et al. 2010).

In another work using I-Cel, homing endonuclease enzyme mutation was made at ms26 genomic site of maize that produced small deletion and insertion of EMS26 (fertility gene at chr 5 with 5 exons) 22 bp targeted site. The $T_0$ maize plants carried mutated alleles of MS26 gene which made the maize male sterile (Djukanovic et al. 2013). Targeted site-directed mutation was performed on five gene regions (EMS26 and MS45 fertility genes, ALS1 and ALS2 acetylactate synthase genes, and Linguless1/LIGI gene) of maize using CRISPR/Cas9 system. The mutation occurred at ALS2 that made the crop chlorsulfuron herbicide-tolerant embryo regeneration. Moreover, targeted mutations on EMS26 and MS45 genes produced sterile male maize even at doubled transformation efficiency than done by engineered endonuclease (Svitashev et al. 2015). Stable knockout of the phytoene synthase PSY1 gene from maize using the CRISPR/Cas9 system was reported. The gene knockout increases sugar synthesis and whitens the powder which in turn increases market value (Zhu et al. 2016). By another recent research work, sterile male maize was developed using the CRISPR/Cas9 system on zmms5 gene (chr 2 with exon number 5) mutation and produced thermo-sensitive (32 °C) maize. This is important for out-crossing and to produce improved hybrid seed (Li et al. 2017).

An increment in maize grain yield under drought-stressed condition was reported by changing the promoter of the ARGOSS8 gene (found at chr 5 with exon number 3).

ARGOS8 gene is a negative regulator of ethylene response and modulates ethylene signal transduction and enhances drought tolerance by reducing leaf size and grain yield development. The deletion of 550 bp at genomic DNA fragment between CTS3 and CTSI removes the part of ARGOSS 5' UTR and the upstream promoter sequence. The native maize GOS2 promoter was used to replace the native promoter of gene ARGOSS using CRISPR/Cas9 system, the protein which suppresses ethylene production was performed (Shi et al. 2017). TALENs and CRISPR/Cas9 systems were evaluated for their targeted mutation creation efficiency at a specific site from the maize protoplast genome. Both of the tools achieved a similar mutation rate (13.1%) (Table 2). These tools could be used alternatively for maize genome editing (Liang et al. 2014). In another research using TALENs, a 10% targeted mutation rate was reported (Table 2), and it was proved that the mutation could pass to the next generations (Char et al. 2015). Targeted mutation of Argonate18 (zmAgo18a and zmAgo 18b) and dihydroflavinol-four reductase maize genes (a1 and a4) using CRISPR/Cas9 technology resulted in a 70% mutation rate and was proved that the targeted mutation could pass to the next generations (Char et al. 2017). From different research reports on maize site-directed mutagenesis using vector method and gene-editing mutagenesis, the TALENs and CRISPR/Cas 9 system are found to be promising and repeatedly used tools to improve maize traits (Liang et al. 2014; Char et al. 2017; Shi et al. 2017).

**Rice improvement using site-directed mutagenesis**

High tryptophan rice was established using a vector method of site-directed mutagenesis. The introduction of $SI26F$, $Y367A$, and $L530D$ mutations into OASA2 was performed using a Quick Change II XL site-directed mutagenesis kit (Strata gene). Interestingly, mature seeds of homozygous GT plants accumulated Tryptophan (Trp) levels 230-fold higher than the non-transformants without any apparent morphological developmental change (Table 2) (Saika et al. 2011). This work proved the potential application of site-directed mutagenesis in improving the nutritional benefit of rice for both humans and livestock that was not achieved by conventional mutation methods.

Fragrance increases the marketability of rice (Ashok-kumar et al. 2020). The development of high fragrant rice by knocking of osBADH2 gene (found at chr 5 with 15 exon number) which produces Betain aldehyde dehydrogenase was reported using TALEN technology. TALENs were engineered to target and disrupt the osBADH2 gene and a total of six $T_0$ heterozygous mutant BADH2 rice plants (badh2-1 to badh2-6) were recovered from 20 transgenic hygromycin-resistant plants. Plants badh2-2 and
| Mutagenized crops | Mutagenic tools used | Purpose of mutation                                                                 | Rate of mutation | Sources          |
|-------------------|----------------------|-------------------------------------------------------------------------------------|------------------|------------------|
| Maize             | CRISPR/Cas9 system   | Evaluating the efficiency of the CRISPR/Cas system to create site-specific mutation | 70%              | Char et al. (2017)|
|                   | CRISPR/Cas9 system   | Male sterile maize production by mutating gene zmtnm5                                | Not mentioned    | Li et al. (2017)  |
|                   | CRISPR/Cas9 system   | Mutating on ARGO8 gene reduce ethylene hormone synthesis to increase grain yield by  | 13%              | Shi et al. (2017) |
|                   | TALEN-mediated       | Evaluation of mutation efficiency maize glossy2 (gl2) locus                          | 10%              | Char et al. (2015)|
|                   | Engineered endonuclease from the I-CreI | Mutation rate evaluation at locus liguleless locus (liguleless1)                         | 3%               | Gao et al. (2010) |
|                   | Double-stranded plasmid mutagenesis | To improve photosynthesis enzyme efficiency The enzymatic reaction was regulated and activated when Thr residue is substituted by Ser and Val residue | Not mentioned    | Chastain et al. (1997)|
|                   | I-CreI homing endonucleases | Mutation rate evaluation at EM26-site by agrobacterium delivery made the maize male-sterile for molecular breeding | 8.9%             | Djukanovic et al. (2013)|
|                   | Cre/ loxP            | Marker segregation and removal                                                        | 1–2%             | Djukanovic et al. (2006)|
|                   | TALEN-mediated/ CRISPR/Cas9 | From genes ZmPDS, ZmIPKIA, ZmIPK, and ZmMRP4                                      | 13.1%            | Liang et al. (2014) |
|                   | CRISPR-Cas9 Agrobacterium-mediated transformation | Stable knockout transformants for maize phytoene synthase gene (PSY1) to increase sugar production and the endosperm got white which is color full | The average efficiency of 10.67% | Zhu et al. (2016)  |
|                   | Plasmid vector mutagenesis | Phosphoenolpyruvate carboxylase improved the catalytic nature of the enzyme by replacing lysine by Asn or, Glu | 22% $V_{max}$Asn replace | Dong et al. (1997)|
|                   | CRISPR-Cas9 system particle bombardment gene delivery | Herbicide-resistant maize and male-sterile maize production | 90% Ems26,100% MS45 | Svitashev et al. (2015)|
| Mutagenized crops | Mutagenic tools used | Purpose of mutation                                                                 | Rate of mutation | Sources                          |
|-------------------|----------------------|-------------------------------------------------------------------------------------|------------------|----------------------------------|
| Rice              | CRISPR/Cas9          | Grain weight of rice improvement by knocking the GW2, GW5, and TWG6 genes which negatively regulates the grain size | 27.13–29.84%     | Xu et al. (2016)                 |
|                   | Agrobacterium-mediated |                                                                                   |                  |                                  |
|                   | CRISPR/Cas9          | Enhanced rice blast resistance mutant produced                                    | (42.0%)          | Wang et al. (2016)               |
|                   | Agrobacterium-mediated |                                                                                   |                  |                                  |
|                   | TALEN-mediated       | Mutation efficiency evaluation to the target site                                  | 25% increased    | Zhang et al. (2016)              |
|                   | Agrobacterium-mediated |                                                                                   |                  |                                  |
|                   | CRISPR/Cas9 from     | To evaluate whether the CRISPR/Cas9 from Prevotella and Francisella1 (Cpf1) effective for plant genome editing | Successful       | Endo et al. (2016)               |
|                   | Prevotella and Francisella1 (Cpf1) |                                                                                   |                  |                                  |
|                   | Homologous recombinase | Introduce precise mutations in OASA2—an a-subunit of anthranilate synthase that is a key enzyme of tryptophan (Trp) biosynthesis in rice | 230-Fold higher than in non-transformants | Saika et al. (2011)              |
|                   | TALEN-mediated using particle bombardment gene delivery method | Targeted knockout of the OsBADH2 able to produce a fragrance rice and increases the world market value of rice | 30%              | Shan et al. (2015)               |
|                   | CRISPR/Cas9 Agrobacterium-mediated transformation | Evaluation of site-directed mutagenesis efficiency and heritability | Highly efficient in rice | Zhang et al. (2014)              |
|                   | CRISPR/Cas9 system  | To evaluate mutation efficiency and its inheritable nature                          | High             | Xu et al. (2014)                 |
|                   | Agrobacterium-mediated |                                                                                   |                  |                                  |
|                   | CRISPR/Cas9          | Endogenous 5 enolpyrovete shikimate synthase gene mutation makes glyphosate-resistant rice produced | 2% replacement and 2.2% gene insertion | Li et al. (2016a; b)             |
|                   | Agrobacterium-mediated transformation |                                                                                   |                  |                                  |
|                   | CRISPR/Cas9 system  | The mutation on three-grain-related genes (J809, L237, and CNXJ rice varieties) three genes osGS3, osGW2, and osGlna which regulates negatively the grain size, width and weight and number Yield increased from triple mutates of variety J809 and L237 by 68% and 30% | Not mentioned | Zhou et al. (2019)               |
| Muta-genized crops | Mutagenic tools used | Purpose of mutation | Rate of mutation | Sources |
|-------------------|----------------------|---------------------|------------------|---------|
| Barley            | TALENs mediated Agrobacterium-mediated transformation | Gene knock-out mutations and gfp is mutated through pollen mediated and loss of function was proved | 22% gene knockout with 4–36 nucleotides got deleted | Gurushidze et al. (2014) |
|                   | TALEN Mediated Particle bombardment | Homology directed repair conversion of gfp into yfp, which is associated with a single amino acid exchange in the gene product brought function exchange | Not mentioned | Budhagatapalli et al. (2015) |
|                   | CRISPR/Cas9 Agrobacterium-mediated transformation | Evaluating the effectiveness of RNA-guided Cas9 system to produce homozygous mutants, knockout of Nud gene generates naked grains | Simplex editing hvckx1 locus 88%; multiplex editing HvCKX1 and HvCKX3 it is 21% | Gasparis et al. (2018) |
| Wheat             | CRISPR/Cas9 Agrobacterium-mediated transformation | Evaluating the protocol of gene deletion from TaBCC6, TaNFX1, and TansLTP9.4 genes and deletion from gene TaNFX1 was large and adaptable | Not mentioned | Cui et al. (2019) |
|                   | CRISPR/Cas9 system With Agrobacterium delivery method | To increase yield by increasing grain number by editing four grain regulatory genes TaCKX2-1, TaGLW7, TaGW2, and TaGW8 | 10% without off-target | Zhang et al. (2019a; b) |
|                   | CRISPR/Cas9 system | Editing TaGW7 gene and mutation either of the B and D genome or on both genome increased the grain width and weight of wheat | Dosage increases the mutation rate | Wang et al. (2019) |
badh2-5 with 1-bp and 10-bp deletions, respectively, caused frameshifts at the fourth exon position, and inactivated the gene and favored the biosynthesis of 2-acetyl-1-pyrroline (2AP) (Shan et al. 2015).

Recently, site-directed mutagenesis using the CRISPR/Cas9 system was reported on important rice traits including improved grain weight, glyphosate resistance, and blast resistance (Li et al. 2016a, b; Wang et al. 2016; Xu et al. 2016). Rice grain weight improvement by gene knockout using the CRISPR/Cas9 system was reported. Among the eight-grain weight controlling genes, a mutation on GW2, GW5, and TGW6 genes brought weight gain for rice (Xu et al. 2016). In another report, glyphosate-resistant rice was developed by intron mediated site-specific gene replacement and/or insertion using the CRISPR/Cas9 system. Gene replacement in the rice endogenous gene 5-enolpyruvate shikimate synthase (EPSPS) at a frequency of 2% and gene insertion at a frequency of 2.2% rice harboring the osEPSPS gene with intended substitutions was found to be glyphosate-resistant (Li et al. 2016a; b). Blast-resistant rice was developed by targeted mutation using CRISPR/Cas9 SSN (C-ERF922) targeted mutation on the osERF922 gene (Wang et al. 2016). During the targeted mutagenesis among 50 T₀ rice plants, 21 plants were with targeted mutation (42%) which was blast-resistant.

Moreover, site-directed mutagenesis efficiency and heritability were also assessed by different scholars using TALENs and CRISPR/Cas9 systems (Xu et al. 2014; Zhang et al. 2014, 2016). In a research work using the CRISPR/Cas9 system, 11 rice genes were mutated to which the mutation rate was found to be high and heritable, and to which the mutation result was not important for the agricultural development of rice (Xu et al. 2014). In another report, rice gene mutation using the CRISPR/Cas9 system ranging from 2 to 16% was proved to pass to the next generations (Zhang et al. 2014). Development of sterile male rice enhanced grain yield, and drought-tolerant rice has been achieved by targeted mutation using TALENs. Besides, the mutation rate on targeted genes and the passage of mutant traits to subsequent generations were studied. The target genes were osCSA, osPMS3, osDERF1, osGN1a, osJAD1, osMST7, and osMST8. The mutations on osCSA and osPMS3 genes resulted in photoperiod sensitive male sterility which was used after hybrid seed production and mutation on osGN1A and osDERF1 genes enhanced grain yield (Table 2) and drought resistance, respectively (Zhang et al. 2016).

Barely improvement using site-directed mutagenesis

Research works on barley using site-directed mutagenesis—TALEN and CRISPR/Cas9 systems—reported that site-directed mutagenesis was efficient and was transmitted to the next T1 generations (Gurushidze et al. 2014; Budhagatapalli et al. 2015; Gasparis et al. 2018). The first transformation was made using TALEN and gene knockout through pollen regenerable cells to establish the generation of true breeding of barley. A gfp-specific TALENs via Agrobacterium-mediated gene delivery and 22% homologous primary mutants proved to be knockout of gfp gene, loss of function, and the deletion of nucleotides between 4 and 36pb length (Gurushidze et al. 2014). The work of Budhagatapalli et al. (2015) using TALENs targeted to gfp gene with the single amino acid change, produced yfp using site-specific mutation.

Barely has been modified and the highest mutation rate was reported in simplex editing of the cytokinin oxidase/dehydrogenase HvCKX1 gene with a mutation rate of 88% of the screened T₀ plants (Table 2) using CRISPR/Cas9 system. Multiplex editing of two genes HvCKX1 and HvCKX3 obtained nine plants (21%) of all edited plants. The knockout of the Nud gene produced phenotypically detectable naked barley grains reducing the effort for farm processing. It was proven that the mutation was transmitted to the next generation T1 (Gasparis et al. 2018).

Wheat improvement using site-directed mutagenesis

A couple of independent research works were reported on wheat grain yield-related trait improvement by targeted mutation using CRISPR/Cas9 systems for gene editing (Wang et al. 2019; Zhang et al. 2019a, b). A targeted mutation on four grain negatively regulating genes (TaCKX2-D1, TaGLW7, TaGW2, and TaGW8) and homozygous for 1160 bp deletion in TaCKX2-D1 wheat gene significantly increased grain number spikelet⁻¹ (Zhang et al. 2019a, b). Edition on gene TaGW7 to silent its expression and mutation on either B or D genome or both genomes increased both grain width and grain weight of wheat. The wheat traits that double-copy mutants showed larger yield improvement than single copy mutants (Wang et al. 2019). Using CRISPR/Cas9 system for targeted mutation produced site-specific deletion and the protocol was targeting three genes, TaABCC6, TaNFX1, and TansLTP9.4 in a wheat protoplast assay. The deletion has occurred on two genes amongst the three genes and the edit on gene TaNFX1 with larger deletion found to be successful (Table 2) and adaptable (Cui et al. 2019).

Potato improvement using site-directed mutagenesis

Cold storage of potato tubers is mostly used to reduce sprouting and extending post-harvest shelf life (Alamar et al. 2017). However, cold temperature stimulates the reduction of sugar accumulation in potato tubers (Krause et al. 1998).
In this regard, research work has been reported on potato post-harvest processing improvement using TALENs to knockout VInv gene within the commercial potato variety. From the 600 regenerated plants, 18 plants showed mutation of at least one VInv gene and five of these plants had mutations in all VInv genes. Tubers with full VInv gene knockout (Table 3) plants showed a noticeable level of reducing sugars, and processed chips contained reduced levels of acrylamide and were light-colored. Moreover, seven of the transformed, out of 18 modified, plant lines appeared to contain no TALEN DNA insertion in the potato genome (Clasen et al. 2016). This research output could potentially be used to reduce the post-harvest loss of potato and plays a role in food sustainability programs.

Research works were reported on the evaluation of mutation efficiency and the heritability of the mutation created by CRISPR/Cas9 system and TALEN to the subsequent generations (Wang et al. 2015a, b; Butler et al. 2016; Forsyth et al. 2016). The research work using the CRISPR/Cas9 system for targeted mutation on the stALS1 gene reported mutation ranging from 3 to 60%, and the mutation was proved its heritability to the next generation (Butler et al. 2015). A site-directed mutation on the stIAA2 gene using CRISPR/Cas9 system resulted in a high and efficient mutation, and the change proved was heritable to the next potato generation (Wang et al. 2015a; b).

Using TALENs, site-directed mutagenesis on the stALS gene resulted in a higher mutation rate (Table 3) that was proven to be transferred to the next generations (Forsyth et al. 2016). Starch quality was altered using site-directed mutagenesis on the GBSS gene function using CRISPR/Cas9 technology. In this work, the GBSS gene has been fully knocked out in the protoplast of tetraploid potato, and mutation was produced in all four alleles. At three regions of the gene granule bound starch synthase was targeted and resulted in the mutation of at least one allele in 2–12% regenerated shoots and multiplex mutation was up to 67%. The removal of GBSS enzyme activity leads to starch with altered amylose synthesis concomitant increase in the amylopectin/amyllose ratio (Andersson et al. 2017).

Soybean improvement using site-directed mutagenesis

Soybean oil quality improvement has been reported by targeted mutagenesis of the fatty acid desaturase two gene families (FAD2-1A and FAD2-1B) using TALENs. The desaturase removes hydrogen from fatty acids and makes the poly unsaturation which could be a threat to heart and brain health (Schattenberg and Bergheim 2019). The trans-fatty acids produced through hydrogenation pose a health threat (Park and Koehler 2019). Four of the 19 transgenic soybean line mutations in both FAD2-1A and FAD2-1B were observed in DNA taken from leaf samples. The fatty acid from homozygous mutant seeds of FAD2-1A and FAD2-1B oleic acid which is a monounsaturated fatty acid (18:1 cis-9) was omega fatty acid increased from 20 to 80% and linoleic acid polyunsaturated fatty acid (omega 6 fatty acids) decreased from 50 to 4% (Table 3), and the mutation has proven as heritable (Haun et al. 2014). Another research on soybean oil improvement using CRISPR/Cas 9 system for editing FAD2-2 soybean gene reported a 21% mutation rate with improved oil quality. A considerable oleic acid content (up to 65.58%) and the least production of linolic acid (16.08%) were recorded (Al Amin et al. 2019). These findings showed the potential of site-directed mutagenesis through gene editing for nutritional improvement in food crops.

Recent research work on adaptable soybean to climate change was reported by altering the flowering time of soybean by targeted mutagenesis using the CRISPR/Cas9 system. Cultivar Jack was mutated at a specific site and T1-generation soy bean plants homozygous for null alleles of GmFT2a (chr.16 with four exon number) frameshift mutated by a 1-bp insertion or short deletion resulted in knocking off the gene. The result has produced a trait late flowering period to escape the natural condition to adapt the stress, and the mutation was proved as it is heritable (Cai et al. 2018). Soybean nutritional improvement and viral disease tolerance have been reported using gene-editing targeted mutagenesis. Multiplex gene editing using the CRISPR/Cas9 system on three genes (GmF3H1, GmF3H2, and GmFNSII-1) in soybean which had negative regulation of isoflavone production has been knocked out. The triple gene mutation efficiency was 44.44%. The T3 homologous triple gene mutants increased Isoflavone content in the leaf twice and the crop becomes resistant to soybean mosaic virus due to the increased isoflavone metabolite (Zhang et al. 2020).

The mutation rate and heritability of directed mutagenesis using the CRISPR/Cas9 system with Agrobacterium-mediated transformation of soybean were reported (Li et al. 2015; Kanazashi et al. 2018). Two genomic sites of soybean, DD20 and DD43 mutagenized using the CRISPR/Cas9 system, was reported with a mutation frequency of 59% and 76%, respectively, and the mutation was proven to be heritable to the next T1 generations (Li et al. 2015). By simultaneous site-directed mutagenesis of GmPPD loci using CRISPR/Cas9 system, soybean mutagen in GmPPD confirmed 33% of the T2 seeds that were proven to be heritable (Kanazashi et al. 2018). Among the six research works reviewed on soybean site-directed mutagenesis, five of the works were done using CRISPR/Cas9 technology which was efficient to generate targeted mutation (Fig. 1).
Table 3 Horticultural crops, oilseed crops, and drug crops which have been mutagenized using different technologies

| Mutagenized crops | Mutagenic tools used | Purpose of mutation | Rate of mutation | Sources |
|-------------------|----------------------|---------------------|-----------------|---------|
| **Potato**        | CRISPR/Cas9          | Evaluation mutation of *StALSI* gene and proven the mutation could be heritable | 3–60%           | Butler et al. (2015) |
|                   | Agrobacterium-mediated transformation | | | |
|                   | CRISPR/Cas9 system   | Evaluation mutation of gene Stria2 and proven the change was heritable | High and efficient | Wang et al. (2015a; b) |
|                   | Agrobacterium-mediated transformation | | | |
|                   | TALEN-Mediated       | Vascular invertase (*VInv*) gene knockout avoids browning on tubers | 18 Plants mutated of 5 contained all alleles mutated | Clasen et al. (2016) |
|                   | TALEN-Mediated       | To evaluate gene expression level using target mutation | Not mentioned | Forsyth et al. (2016) |
|                   | CRISPR/Cas9 system   | Altered starch quality with the full knockout of *GBSS* gene improving amylopectin/amylose ratio in potato | Mutations in one allele in 2–12% multiple alleles mutations 67% | Andersson et al. (2017) |
|                   | Agrobacterium-mediated transformation | | | |
| **Soybean**       | CRISPR/Cas9 system multiplex gene-editing technology | Enhancing Isoflavone content by editing the three genes GmF3H1, GmF3H2, and GmFNSH1 in soybean Increased isoflavone content enhanced the leaf resistant to Soybean Mosaic Virus (SMV) | 44.44% triple gene mutation rate | Zhang et al. (2020) |
|                   | CRISPR/Cas9 system Agrobacterium delivery method | Integration/mutation of FAD2-2 gene in soybean to improve oil quality and Considerable oleic acid content up to (65.58%), whereas the least production of linolic acid is (16.08%) were recorded | 21% Mutation rate | Al Amin et al. (2019) |
|                   | CRISPR/Cas9-mediated Agrobacterium-mediated transformation | 1 bp deletions gene GmFT2a (Glyma16g26660) and GmFT5a (Glyma16g04830) produce late-flowering to escape natural conditions | 15.6% GmFT2a and 15.8% GmFT5a | Cai et al. (2018) |
|                   | CRISPR/Cas9-Mediated Agrobacterium-mediated transformation | Evaluation of mutation rate on the two loci GmPPD1 and GmPPD2 and proven it can pass to generations | 33% of T2 | Kanazashi et al. (2018) |
|                   | TALEN- Mediated      | Improved soybean oil quality By mutating both FAD2-1A and FAD2-1B and produce monounsaturated oil | Not mentioned | Haun et al. (2014) |
|                   | CRISPR/Cas9-Mediated Agrobacterium-mediated transformation | Evaluation of mutation on DD20 and DD43 genes from chromosome forgot mutated and proven it can pass to generations | 59% and 76% | Li et al. (2015) |
| **Tomato**        | CRISPR/Cas9          | The mutation on *RIP* gene and prolonging the shelf life of tomato | 0–100%           | Ito et al. (2015) |
|                   | Agrobacterium transformaion | | | |
|                   | CRISPR/Cas9 system   | To evaluate single and multiple site mutation possibility | | Hu et al. (2019) |
| Mutagenized crops | Mutagenic tools used | Purpose of mutation | Rate of mutation | Sources |
|-------------------|---------------------|---------------------|------------------|---------|
| Tobacco           | Cre/Lox Precombinase Agrobacterium transformation | Evaluation excision of coda from the cell expression of codA in plastids made tobacco cells sensitive to 5-fluorocytosine | Highly efficient Marker elimination was observed | Corneille et al. (2001) |
|                   | CRISPR/Cas9 from *Prevotella* and *Francisella* (Cpf1) | To evaluate whether the CRISPR/Cas9 from *Prevotella* and *Francisella* (Cpf1) effective for targeted mutation on *NtPDS* gene and *NtSTF1* gene of tobacco | Successful | Endo et al. (2016) |
| Carrot            | CRISPR/Cas9 system | To knock out anthocyanin pigment synthesizing gene (F3H) and to produce white carrot | Not mentioned | Klimek-Chodacka et al. (2019) |
|                   | CRISPR/Cas9 system | Knock out of the gene *DcPDS* orange carrot generated albino carrot and edition of *DcMYB113*-like gene of purple carrot produce depigmented carrot | 35.3% and 36.4%, respectively | Xu et al. (2019) |
| Cabbage           | TALENs              | Deletion on *FRIGIDA* gene in brassica | Not mentioned | Sun and Zhao (2013) |
|                   | CRISPR/Cas9 system | *BoPDS* gene knock out to produce albino shoot | 1.14% mutation rate | Ma et al. (2019) |
|                   | CRISPR/Cas9 system | *Bnlpat2 and Bnlpat5* genes to improve oil | 17–68% mutation rate | Zhang et al. (2019a; b) |
Tomato improvement using site-directed mutagenesis

Tomato shelf life has been improved by site-directed mutagenesis using the CRISPR/Cas9 system by the agrobacterium gene delivery method. Gene deletion/insertion on the \textit{RIN} gene which encodes an MAD1-box transcription factor regulated fruit ripening. The \textit{RIN} protein defective mutants were found to be effective to make the tomato stay fresh for several months by changing the ripening physiology and ethylene production (Ito et al. 2015). The mutation rate was ranged from 0 to 100%. Targeted mutation employed by CRISPR/Cas9 system as using the Agrobacterium-mediated gene delivery method and single- and multi-site mutagenesis has been reported. In summary, this technology could be employed to produce site-directed mutagenesis on important traits from the same or other crops (Hu et al. 2019).

Tobacco crop site-directed mutagenesis

Incorporation of the selectable marker gene during plant transformation is crucial to know the whereabouts of the gene of interest (Hare and Chua 2002). However, the selectable marker gene especially the old markers remain a public concern (Schaart et al. 2004). Using \textit{CRE–lox} recombinase CoDA selectable marker flanked with two directly oriented lox sites, the highly efficient elimination of the marker gene (Table 3) introduced through pollination was reported (Corneille et al. 2001). In another research report using the CRISPR/Cas9 system as using the Agrobacterium-mediated gene delivery method and single- and multi-site mutagenesis has been reported. In summary, this technology could be employed to produce site-directed mutagenesis on important traits from the same or other crops (Hu et al. 2019).

Cabbage (\textit{Brassica oleracea}) improvement through site-directed mutagenesis

Cabbage is a vegetable and oilseed crop. A research work on Cabbage (\textit{Brassica oleracea L. var}) using customized TALEN-based nuclease constructed using a method “unit assembly” specially targeted the endogenous FRIGIDA gene in \textit{Brassica oleracea L. var} modified the targeted site with deletion. This protocol was proven to bring genetic modification by site-directed mutagenesis (Sun and Zhao 2013). Two other research works were reported on a targeted mutation of the cabbage genome using the CRISPR/Cas9 system (Ma et al. 2019; Zhang et al. 2019). Ma et al. (2019) reported a gene knockout on \textit{BoPDS} gene that resulted in albino cabbage (\textit{Brassica oleracea}) shoot with a mutation rate of 1.14%. The other work by Zhang et al. (2019a; b) reported knockout of \textit{Bnlpat2} and \textit{Bnlpat5} genes with mutation rate ranging from 17 to 68% improved the seed starch content and increased oil bodies of the matured seed of cabbage. These two reports had no off-target edition, which proved the efficiency and potential application of the CRISPR/Cas9 system mutagenesis for crop improvement.

Carrot improvement through site-directed mutagenesis

Two independent research works reported carrot improvement by site-directed mutagenesis using the CRISPR/Cas9 system (Klimek-Chodacka et al. 2019; Xu et al. 2019). In the report by Klimek-Chodacka et al. (2019), a knockout of anthocyanin pigment synthesizing gene (F3H) produced white carrot. The mutation on the F3H gene depigmented the purple color into a white through deletion and removal
of anthocyanin expression. The research by Xu et al. (2019) resulted in knockout CRISPR/Cas9 technology of the gene, DcPDS orange carrot generated albino carrot with a mutation rate of 35.3%. The edition of DcMYB113-like gene of purple carrot produced a depigmented carrot with a mutation rate of 36.4%.

These days, new insights for crop improvement have been added and used as a means to improve crops of interest. New tools and their potency to produce site-specific genetic alteration and the extent of the heritability of the alteration to the proceeding generation have been discussed in several different research findings. In this review paper, 48 original research works on crop targeted mutation have been assessed. In the research works, four mutagenic tools have been investigated with different frequency, in which CRISPR/Cas9 system was found to be repeatedly used to transform different crops—reported as it is an efficient technology to produce the intended specific mutation (Fig. 1) (Chen and Gao 2015; Char et al. 2017). TALENs were found to be the second frequently used tool to transform crops to bring efficiently targeted mutation and it was affirmed by most scholars as it is potent technology for crop transformation (Tables 2, 3).

Furthermore, according to the research reports, these technologies were highly employed in cereal crops (Fig. 2). As monocots are the major staple food crops to humans around the globe (Chen and Gao 2015), improvement efforts targeting cereals in particular and all crops at large could ensure food sustainability.

**Challenges of site-directed mutagenesis**

Site-directed mutagenesis is crucial in crop production as it is important to bring the required change on the target DNA sequence, thereby changing the gene output and the trait of the crop (Oladosu et al. 2016). Site-specific mutagenesis is more powerful than genetic transformation through recombinant DNA technology of crops, since the latter introduce foreign genes to plants at a random place while the former alter the gene at the programmed site. The random integration of the introduction of the foreign gene may silent other important genes or may bring uncommon gene expressions (Belhaj et al. 2013; Agapito-Tenfen et al. 2018). All technologies developed so far might not be equally efficient in bringing the ultimate change in crop improvement using site-directed mutagenesis (Chen and Gao 2015).

Developed technologies for targeted mutation creation showed advances with time. The vector and the PCR methods of site-directed mutagenesis recently are less likely to be used for plant transformation because of their low efficiency and inadequacy in crop agronomic traits improvement (Bryksin and Matsumura 2010). Hence, other new promising and highly applicable technologies have been developed and used for crop transformation through site-directed mutation (Mahfouz et al. 2014; Wang et al. 2015a, b; Eş et al. 2019; Gupta et al. 2020). Recently developed mutagenic tools also have limitations to bring efficient programmed genetic changes to a crop genome (Table 1). Zinc Finger Nucleases (ZFNs) used for site-directed mutagenesis could produce the off-target effect, large size effect to delivery, dimerization, comparatively high cost, and laborious nature.

TALENs is one of highly effective, easy to construct, and less costly tool compared to Zinc Finger Nucleases (ZFNs) to create site-specific deletion or insertion (Shan et al. 2015; Zhang et al. 2016). However, still, it has a dimerization and off-target effect to a lesser extent compared to the CRISPR/Cas9 system (Sun and Zhao 2013; Mahfouz et al. 2014). The CRISPR/Cas9 system possesses several potential advantages over ZFNs and TALENs. The short size of the sgRNA sequence makes it easier to deliver, cheap to construct, not laborious, efficient compared to others. However, these novel nucleases have still little limitations such as it experiences off-target effects (Chen and Gao 2015).
Conclusion

Crop improvement using site-directed mutagenesis employing plasmid vector-based and site-specific nucleases transformation has been summarized. From the reviewed works, CRISPR/Cas9 system was found repeatedly (66.67%) used to improve crop traits by targeted mutagenesis. TALENs were used for knockout of bad trait coding genes. Hence, the CRISPR/Cas9 technology is widely used to improve the crop of their interest and to ensure food sustainability for its efficiency and less off-target effects. A lot of agronomic traits, physiological traits, and stress-tolerant traits of crops have been improved by site-directed mutation.

The site-directed mutagenesis technology is found to be highly applied for cereal crops that were less effective to be transformed using recombinant DNA technology. Cereals were the dominant crops to be transformed by site-directed mutagenesis of which maize and rice are on the front. Finally, the trend of technology usage shows that CRISPR/Cas9 technology has been highly used by researchers around the globe to bring efficient transformation and crop improvement. In addition, for knocking out genes with bad traits, TALENs were found to be ideal. Hence, targeting and doing improvement on the major stable food crops could ensure the world’s food security.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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