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REVIEW

Genome editing of potato using CRISPR technologies: current development and future prospective

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Abstract

Potato (*Solanum tuberosum* L.) has tremendous significance due to its nutritional quality. The mounting pressure of increasing population further reinforces its importance as potato is believed to be a vital crop to meet food needs for population growth. Although conventional approaches of breeding, irradiation/mutagens and introgression of quality and yield related traits have improved potato yield, biotic and abiotic stresses continue to impose crop damages. Modern tools such as CRISPR/Cas have assisted plant scientists in accelerating breeding processes by providing new, simple, versatile and robust technologies. These tools make it possible to eliminate traits that are involved in negative regulation of quality and yield parameters. Besides that, genes of interest can also be introduced in close proximity to specific loci that may remain linked throughout the generations. This review focuses on the endeavors, applications and prospects of CRISPR/Cas-based approaches in potato with the potential to increase sustainable crop productivity.

Key message

This manuscript focuses the endeavors, applications and prospects of CRISPR/Cas-based approaches in potato with the potential to increase sustainable crop productivity.

Keywords CRISPR · Gene editing · Potato · NHEJ

Introduction

Potato (*Solanum tuberosum* L.) belongs to the large Solanaceae family of about 98 genera and 2700 species, with around 1000 potato cultivars and near 374.5 million tonnes being generated globally. Potato will be an important crop in the future in securing food resources for the global populace estimated to increase to 9.7 billion by 2050 (Caliskan et al. 2010; Jacobs et al. 2011; FAO 2013). After maize, rice, and wheat, potato ranks fourth among the chief food staples (Hameed et al. 2018). Vulnerable to different types of abiotic stresses such as cold, heat and frost, potato also faces threats arising from global warming leading to water restriction. Potato cultivation can be very challenging where there is erratic rain precipitation and water supply is scarce. Drought stress already has a negative impact on potato crops to some degree with respect to severity and duration (Krauss and Marschner 1984; Hijmans 2003; Easterling et al. 2007; Thiele et al. 2010; FAOSTAT 2012; Simelton et al. 2012). Beddington (2010) identified almost 40% losses of potato arising from insect pests and crop diseases. For such a valuable crop, efforts to develop biotic and abiotic stress tolerant potatoes remain worthwhile. Also, potato tubers need to have better storability and improved quality, which can be addressed via gene editing technologies (Khromov et al. 2018).

For plant breeders, potato possesses both advantages as well as impediments. Being vegetatively propagated, it is exempt from the requirement of being bred from true seeds to generate homogenous plants. At the same time, as a tetraploid, it creates a major hindrance in propagating introduced traits of interest to subsequent progeny (Kennedy

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2008). Additionally, due to the highly heterozygous nature and tetrasomic inheritance of cultivated potato crops, potato research is intricate and time-consuming posing a major challenge when using conventional crossbreeding, rendering application of gene editing necessary (Andersson et al. 2018). Potato is currently unsuitable for chemical mutagenesis such as TILLING (Targeting Induced Local Lesions in Genomes) as seeds are the preferred material for chemical mutagenesis and most strategies of mutagenized population screening use PCR amplification of targets genes followed by sequencing by next generation sequencing (NGS). Cultivated potato is a highly heterozygous tetraploid challenging the current bioinformatic filters used to distinguish true induced mutations from sequencing errors. Although NGS accuracy of both short and long reads can be improved by both sequence coverage and novel enzymatic reactions, induced potato mutants would necessitate multiple successive backcrosses to non-mutagenized plant for removal of unwanted potential mutations in the rest of the genome. Comprehension of the gene function is crucial in improvement of agronomic traits with the use of molecular tools. With cultivated tetraploid potato crops the scrutiny of genes with a functional approach using molecular genetics creates a major impediment. Given that the potato gene transformation is efficient and sequence information of diploid RH and double-haploid DM are readily available, potato is a model crop candidate for gene editing experiments (Wang et al. 2015). There are some impediments in genome editing of potato plants brought about by its complex heterozygosity and tetraploid nature. This creates difficulty in obtaining mutants which are homozygous with all target genes mutated. There are many challenges that need to be addressed in establishing potato mutants using programmable nucleases in just one step (Kusano et al. 2018).

Various synthetic nucleases have been applied in introducing double strand breaks (DSBs) at a precise location in the genome: Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Fig. 1). In potato, TALENs have been used to knockout *vacuolar invertase* gene (*VInv*) in Ranger Russet. All the *VInv* alleles were mutated in five of the potato plants, whereas eighteen other lines had at least one *VInv* allelic mutation (Clasen et al. 2015). However, unlike CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated nuclease 9) technology which involves simple RNA-based nuclease targeting the desired genomic DNA sequence, TALENs and ZFNs involve targeting via protein-DNA interactions with the use of the non-specific cleavage domain of *Fok*I nuclease. ZFNs have been applied to only a few crops such as tobacco, *Arabidopsis* and soybean, but not in potato or horticultural crops. This could be due to lower success and an erratic or lesser mutation rate, elevated off-targeting and design intricacy (Hameed et al. 2018). It was only after the Cas9 protein characterization and the realization that all molecular biology laboratories could easily perform programmable editing of the genomic sites, prompting the rapid adaptation of this technology in the scientific community. In addition, ZFNs and TALENs methods require tedious engineering of the nucleases (Sander and Joung 2014; Zhou et al. 2014).

The CRISPR technology, derived from the bacterial immune system against viruses, has emerged as a breakthrough in gene editing (GE) technology. *Streptococcus pyogenes* SpCas9, the most often used nuclease, induces double strand break (DSB) via its HNH and RuvC domains, 3 to 4 base pairs upstream of PAM (Protospacer Adjacent Motif), together with tracrRNA (*trans*-activating crRNA) and crRNA (CRISPR RNA) seed sequence (~ 20 nucleotide guide sequence) (Zhou et al. 2014) (Fig. 1). The pattern of DSB ends varies depending on the nuclease type: ZFN induces 4–5 nt overhang with staggered cut at the 5′ end, TALENs result in staggered heterogeneous overhangs, SpCas9 induces 1-bp staggered cut (rather than blunt-ended DSB as previously assumed) and 5′ end staggered cut in the case of Cpf1 (Zuo and Liu 2016; Guha et al. 2017). Repair of the DSB is mediated by the non-homologous end joining (NHEJ) pathway usually by insertion or deletion of a small number of nucleotides. This error-prone repair mechanism thereby alters the DNA sequence potentially leading to knock out of the target gene. In the presence of homologous donor template, the DSB is repaired by homologous recombination (HR) introducing a desired sequence without other errors (Ding et al. 2016; Khatodia et al. 2016) (Fig. 1). Nevertheless, NHEJ can introduce a foreign sequence or remove the sequence in a precise manner as well as knocking out genes. Moreover, HR is surpassed by NHEJ in the somatic cells of the plants (Puchta 2017), material that is often used for generating CRISPR lines.

In case of NHEJ repair mechanism, repair proteins do not depend on homology of the DNA sequence, and instead ligate the broken strands (Fig. 1). NHEJ is known to occur mostly at G_1 phase of cell cycle. This mechanism is enhanced via the Shielden complex, which starts from Ku protein complex attaching to the ends of DNA that have been exposed. The broken DNA is later stabilized by DNA-PKcs. End processing of DNA is performed after the recruitment of Artemis nuclease complex. DNA ligase IV then ligates the ends of DNA to repair the ends leading to several base pair long mutations. DNA can also be repaired by another error-prone mechanism known as Alternative End-Joining (A-EJ), or microhomology-mediated end joining (MMEJ), which depends upon 5–25 bp of microhomology to induce Indel mutations, eventually forming 3′ overhangs. Rad 50, Mre11 and Nbs proteins (MRN complex) are involved in removing blockages at the DSB in association with CtIP (C-terminal binding protein interacting protein). HR-repair mechanism

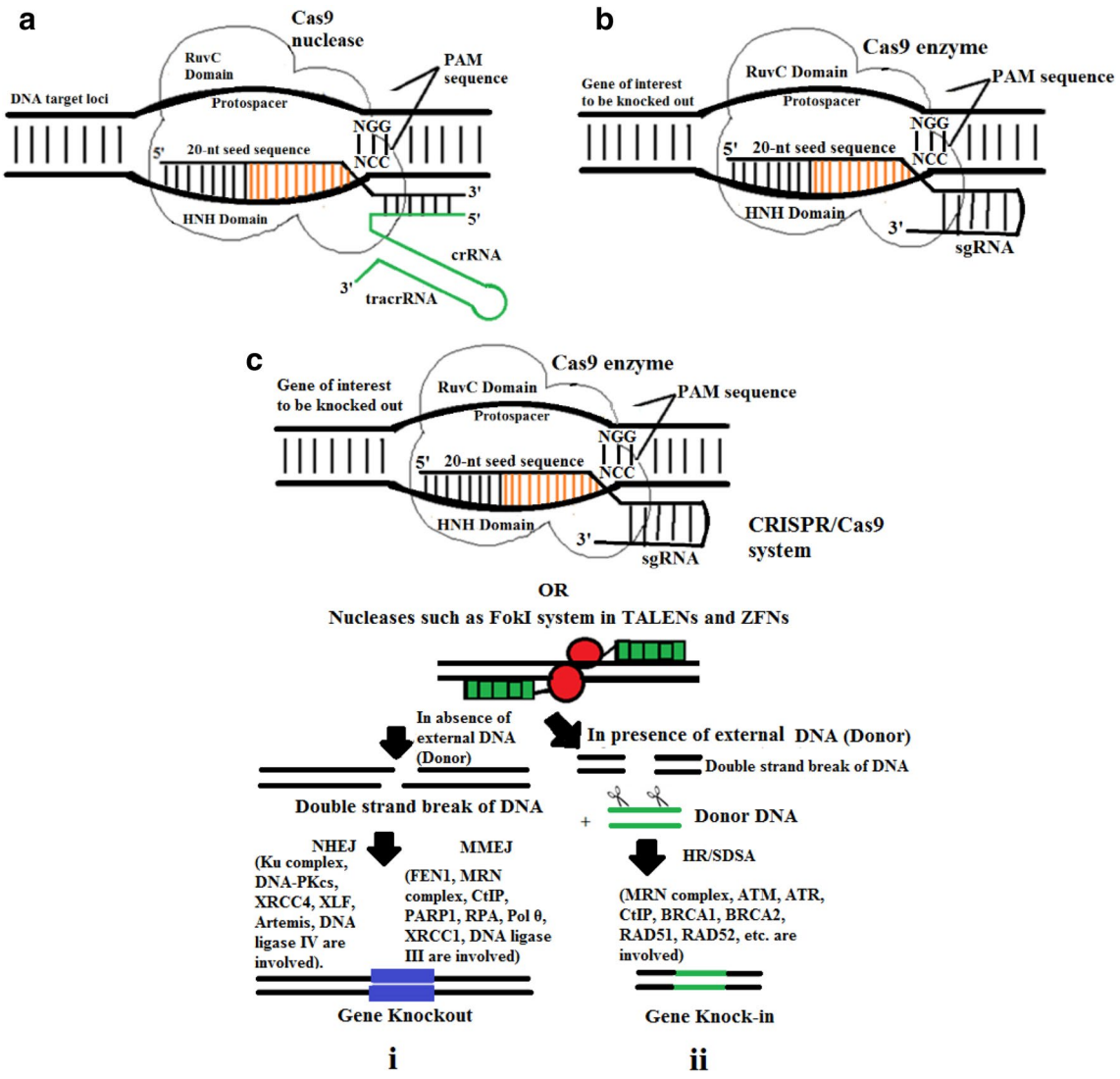

Fig. 1 Synthetic endonucleases and repair mechanism of genome editing: **a** and **b** Adoption of CRISPR/Cas9 system in artificial system to induce target mutation of genes (Reproduced from Bortesi and Fischer 2015); Mechanism of **c** gene knockout via NHEJ or MMEJ (microhomology-mediated end joining) (i) and gene insertion via HR or SDSA (synthesis-dependent strand annealing) (ii) Cubbon et al. (2018) in the cases of TALENs, ZFNs and CRISPR systems. The DSB pattern in ZFNs induces 4–5 nt overhang staggered cut at the 5′ end, staggered heterogeneous overhangs in TALENs, 1-bp staggered cut in case of SpCas9 (rather than blunt-ended DSB as previously assumed) and 5′end staggered cut in case of Cpf1 (Zuo and Liu 2016; Guha et al. 2017)

depends on homologous DNA molecules (Fig. 1), that is sometimes associated with recombinases (RadA, Rad51 or RecA) and regulatory/accessory proteins catalyzed strand invasion. RecBCD and RecA pathways have been identified as two important modes of recombinase-mediated HR. The displacement loop, that is engendered due to the strand invasion, is repaired by other several pathways of DSB repair, break-induced replication (BIR) or synthesis dependent strand annealing (SDSA). Single-stranded template repair (SSTR) is independent of recombinase enzyme and D-loop is not formed. SSTR could be significant in genome-editing based on HR mechanism (reviewed in Cubbon et al. 2018).

Various CRISPR/Cas9-based gene knockout studies in potato

A very limited number of studies have been done in potato with the application of CRISPR/Cas9 technology. Wang et al. (2015) targeted exon 2 of *StIAA2* gene in double haploid DM potato and demonstrated mutation induction in stable transgenic potato. Furthermore, they reported to have obtained homozygous monoallelic and biallelic mutations in the T1 generation.

Butler et al. (2015) utilized the CRISPR/Cas system expressing one of two synthetic single-guide RNAs

(sgRNAs) to target Acetolactate synthase 1 (*ALS*) gene in potato; the mutation inducing ability was tested in callus as well as stable events in diploid MSX914-10 (X914-10) and tetraploid Desiree potato. This was performed using conventional *Agrobacterium*-based transformation with either the classical T-DNA or a geminivirus-based T-DNA viral vector. More than one mutation type was observed in four out of nine primary events and eight of them had targeted mutagenesis retained across clonal generations. Somatic mutations were found to be more apparent in diploid potatoes; and in a single *ALS* locus, at least two mutation types were reported in three out of four primary events. However, in tetraploid potato, four out of five candidates carried a single mutation type. Inherited single targeted mutation was observed through germlines of tetraploid and diploid primary events (87–100% transmission rate). Selfing was done for the tetraploid mutant events and the diploid event was crossed to make the self-compatible diploid M6 line. Cas9-free progenies were found in a proportion ranging from 19 to 37% and transmission of their targeted mutations in the range of 87–100%.

Furthermore, Butler et al. (2016) targeted *Acetolactate synthase 1* (*ALS1*) gene in the tetraploid potato cultivar Desiree and diploid breeding line MSX914-10 (X914-10) via delivery of sequence-specific nucleases by employing geminivirus replicon (GVR). The repair template was designed in a way to introduce a herbicide-inhibiting point mutation within the *ALS1* locus. The benefit of using geminivirus is its ability in gene targeting to serve as a robust repair template and in providing strong heterologous protein expression. This study tested the activity of TALEN and CRISPR reagents targeting GUS and NPTII based reporter assays for facile detection and selection of induced double-strand break events repaired by single-strand annealing (SSA) mechanism. The study reported to have generated a phenotype with diminished herbicide susceptibility only in GVR mediated transformation but not with conventional T-DNA. Additionally, the authors found amelioration in HR gene editing frequency in potato but no improvement of NHEJ mutational frequency with the use of geminivirus. This study demonstrated the potential of GVR transformation in gene editing of vegetatively propagated crops.

Andersson et al. (2017) performed knockout of all four alleles of *granule-bound starch synthase* (*GBSS*) gene in tetraploid potato cv. Kuras without stable integration of plasmid DNA to avoid tedious subsequent crossing to re-establish the genotype of potato with properties of agronomical interest. This was achieved via transient transfection of protoplasts using PEG-mediated transformation method. They induced mutations in all four alleles in 2% of regenerated lines. *StGBSS* gene was targeted at two sites of eighth exon and one in ninth exon. They found mutations in at least one allele in 2–12% of regenerated shoots and multiple alleles (67%) with most mutations being small indels of 1–10 bp. They further confirmed the complete knockout by studying the phenotype of starch under light microscopy in microtubers produced in vitro. Knockout of GBSS enzyme can lead to varied amylose synthesis and elevated amylopectin/amylose ratio which may change the properties of the starch for particular applications.

In another study conducted by Andersson et al. (2018), CRISPR-associated nuclease 9/guide-RNA (Cas9/gRNA) ribonucleoprotein complex (RNP) was delivered into potato protoplasts. This DNA-free gene editing technology was used to target ninth exon of *StGBSS* gene. In this experiment, the authors pre-assembled guide-RNA with Cas9 protein in vitro and obtained up to 9% of indel frequency with no transgene integration in the genome of regenerated plants. Additionally, they obtained 25% mutagenesis frequency when RNP was delivered with IVT-RNP (in vitro transcriptionally produced RNA). Strikingly, at least 80% of regenerated shoots in which indel mutations have been confirmed were found to have insertion of plasmid or chromosomal DNA in the cut site. This result indicates the necessity of removing DNA template at the end of in vitro transcription reactions. Noteworthy of this research was a complete knockout of all four alleles of *StGBSS* gene in 2–3% of regenerated shoots resulting in total absence of enzyme activity.

Nakayasu et al. (2018) assessed the effectiveness of various candidate sgRNAs using CRISPR/Cas9 to knockout *St16DOX* gene in tetraploid potato (cv. Mayqueen). This gene encodes for 16α-hydroxylase which is involved in steroidal glycoalkaloid (SGA) biosynthesis. SGAs are responsible for the bitter taste and are toxic to different organisms. In this study, the authors designed 9 sgRNAs to target *St16DOX* gene and generated two independent lines free of SGA in root hairs.

Khromov et al. (2018) compared in vitro activities of various sgRNAs targeting different regions of *phytoene desaturase* (PDS) from the carotenoid biosynthesis pathway and a *coilin* gene involved in plant resistance. The visual phenotype of PDS knockout makes it convenient for detection and analysis of potato genome editing due to the depigmentation in the absence of PDS. Knockout of *coilin* gene is highly desirable as deterioration of coilin is mainly involved in pathogen resistance and improving tolerance to biotic and abiotic stresses. The authors found that 6 nucleotides proximal to the 3′ PAM sequence directly interacted with Cas9 nuclease; nevertheless, their primary structure and composition did not show any pivotal role in explaining interaction specificity. The authors concluded that the unpaired nucleotides of target DNA with sgRNA can both stimulate or repress the activity of Cas9-sgRNA complex in vitro depending on the position of the mismatch.

After the success in soybean oomycete pathogen *Phytophthora sojae,* attempts have been made to establish the CRISPR/Cas9 system in *Phytophthora infestans*, the oomycete which is a causal agent for potato and tomato late blight diseases. The study targeted *Avr1*, *PiTubA2* and *PiAP5* genes in *P. infestans*. However, no mutagenized transformants were detected in this study with both RNP complex delivery system and the same construct that was being successfully used in *P. sojae*. This failure was due to their incubation temperature at 18 °C, which is lower than the 25 °C used successfully in the other *Phytophthora* species. In addition, the Cas9 isoform used was human codon optimized isolated from *Streptococcus pyogenes*, which is active at 37 °C. This means that the SpCas9 activity declines at lower temperatures. The authors recommend systematic scrutiny of factors that limit the competence of the CRISPR/Cas9 system (Fang and Tyler 2016). Furthermore, an increased rate of induced mutation by CRISPR/Cas9 system was observed when *Arabidopsis* plants were subjected to 37 °C heat stress compared to those grown continuously at the standard 22 °C temperature. Using GFP-based assay, the study observed that upon heat treatment targeted mutagenesis induced fivefold augmentation in somatic tissues and almost 100-fold in germline. They also report a similar event in *Citrus* plants (LeBlanc et al. 2018). Similar work could be done in potato with treatment at 37 °C to induce higher rates of CRISPR/Cas9 mediated mutagenesis.

Design of sgRNAs for potato genome editing

Nakayasu et al. (2018) have used two web tools for the sgRNA in silico analysis, Cas-OT software and CRISPRko (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), to target *St16DOX* gene in tetraploid potato (cv. Mayqueen). However, Khromov et al. (2018) has compared various sgRNAs designed to target genes in potato and found no association of efficiency of sgRNAs predicted using the online tool (https://crispr.cos.uni-heidelberg.de/). The software predicted elevated efficiency for sgRNA4 designed for *coilin* gene; however sgRNA4 failed to show any activity in vitro.

Choosing the best CRISPR/Cas vector for gene knockout in potato

Several CRISPR/Cas vectors are now available from the plasmid repository Addgene or directly from different laboratories. It is important to choose an ideal CRISPR/Cas9 system for potato gene knockout. As potato is a dicot species, appropriate sgRNA promoters derived from dicot plants such as *Arabidopsis* (*AtUp*), potato (*StU6p*) or U3p (Belhaj et al. 2013; Wang et al. 2015) are recommended. Plant codon optimized Cas gene is now available and should be used under the control of a suitable inducible or constitutive plant promoter, such as *CMV*, *EFA*, *LTR*, *UBI*, and *CaMV35S* (Belhaj et al. 2013). It has been shown in *Arabidopsis* that ribozyme based CRISPR technology where both Cas9 and sgRNA are under the control of a single RNA pol II promoter is beneficial. This system avoids the use of RNA pol III promoters such as U6p and U3p thus allowing the use of a wide range of other promoters including tissue specific ones (He et al. 2017).

As cultivated potato has a tetraploid genome, Kusano et al. (2018) investigated developing a more robust genome editing system with the use of a translational enhancer dMac3 of the 5′ UTR of rice *OsMac3* mRNA. This enhancer was fused to the 5′-end of Cas9 to increase its expression level and tested with multiple gRNAs. It was found that the *granule-bound starch synthase I* (*GBSSI*) gene mutant frequency induced by CRISPR/Cas9 system was greatly augmented using dMac3-Cas9 gene. The proportion of transformants with four mutant alleles was found to be about 25% according to Cleaved-Amplified-Polymorphic-Sequence (CAPS) analysis. The mutant plants also showed tubers with lower amylose starch.

CRISPR system delivery into potato cells

Several transformation systems are available for the introduction of CRISPR/Cas elements into the plant cell. These techniques include electroporation, polyethylene glycol (PEG), Agrobacterium floral dip, Agroinfiltration, biolistic inoculation, transit peptides, and viral vectors, some of which have been successfully implemented in potato (Yin et al. 2015; Ma et al. 2016; Sattar et al. 2017) (Table 1).

Butler et al. (2016) harnessed the benefit of geminivirus to test the activity of TALEN and CRISPR reagents resulting in diminished herbicide susceptibility in GVR mediated transformation compared to conventional T-DNA event. Additionally, amelioration in HR frequency without any change in NHEJ mutational frequency was reported. As potato genotypes are highly heterozygous and due to its tetrasomic inheritance, the best strategy would be to transiently introduce CRISPR/Cas elements to generate knockout plants without any exogenous DNA. Elimination of such DNA through classical segregation during fertilization would change the genetic makeup of gene edited potatoes. Andersson et al. (2017) achieved this goal by transiently introducing CRISPR/Cas9 RNP complex using PEG-mediated potato protoplasts transformation.

Table 1 Salient CRISPR/Cas9 based research performed on potato

Gene targeted	Variety used	Mode of delivery	Noteworthy features	Vector features	References
Exon 2 of *StIAA2*	Double haploid DM potato	*Agrobacterium*-mediated transformation	Monoallelic and biallelic homozygous mutants in the T1 generation	Rice codon optimized Cas9 driven by doubled CamV35S promoter. Two NLS each on 5′ and 3′ end of Cas9 gene. *StU6P*::sgRNA with NOS terminator	Wang et al. (2015)
Acetolactate synthase 1 (*StALS*)	Diploid MSX914-10 (X914-10) and tetraploid Desiree potato	Conventional T-DNA transformation mediated by *Agrobacterium* or T-DNA of modified geminivirus	Inherited single targeted mutation was observed through germlines of tetraploid and diploid primary events (87–100% transmission rate)	Arabidopsis codon optimized Cas9 driven by doubled 35S promoter and Rep expression with Nos terminator. Arabidopsis U6 promoter for sgRNA	Butler et al. (2015)
Acetolactate synthase 1 (*StALS*)	Tetraploid Desiree and MSX914-10 (X914-10)	GVR mediated transformation	Ameliorated HR, but no change in rate of NHEJ frequency	Arabidopsis codon optimized Cas9 and Arabidopsis U6 promoter for sgRNA.	Butler et al. (2016)
Exon 8 and 9 of *Granule-bound starch synthase* (*GBSS*)	Kuras	PEG-mediated protoplast transformation	Mutation in all four alleles in regenerated lines (2%)	*U6* promoter of either *Arabidopsis thaliana* or *Solanum tuberosum* origin, 35S CaMV driving Cas9, plant codon-optimized Cas9 gene, NLS on either ends of Cas9 and NOS terminator	Andersson et al. (2017)
Exon 9 of *StGBSS*	Kuras	RNP delivery system	9% of shoot regenerated had indel frequency with no transgenes at all	GeneArt™ system (Thermo Fisher Scientific, Waltham, MA) and Alt-R® CRISPR-Cas9 system (IDT, Coralville, IA) were used	Andersson et al. (2018)
St16DOX	Mayqueen	*Agrobacterium rhizogenes* strain ATCC15834 using electroporation	Complete knockdown of steroidal glycoalkaloids (SGAs) accretion was shown	AtU6-26 *Arabidopsis thaliana* U6 snRNA-26 (U6-26) promoter to drive multiplex gRNAs; 2 × *CaMV35S* promoter with the omega enhancer sequence; Arabidopsis-codon optimized *Streptococcus pyogenes* Cas9	Nakayasu et al. (2018)
Phytoene desaturase and *coilin*	Chicago	In vitro study without delivery in the plant	Found that unpaired nucleotides in DNA with sgRNA not only repressed the activity of Cas9-sgRNA complex, but also stimulated its activity in vitro	Illustrated as 'genetically engineered expression constructs'	Khromov et al. (2018)

Table 1 (continued)

Gene targeted	Variety used	Mode of delivery	Noteworthy features	Vector features	References
Granule-bound starch synthase 1 (GBSSI) gene	Sayaka	*A. tumefaciens* EHA105 mediated transformation	Greater than 28% transformants contained mutation in all four alleles	Different vectors: pZH-OsU7gRNA, pZD-dxCas9, pZD-zeroCas9 with different parameters for each vector were used, each modified with or without: translational enhancer dMac3; MMCas9 coding region driven by double CaMV 35S promoter, pea3A terminator, rice actin1 terminator	Kusano et al. (2018)

Screening of mutant potatoes

Several strategies including Southern blotting, sequencing PCR amplicons, genotypic and phenotypic screening can be used to corroborate mutagenesis (Hua et al. 2017). Wang et al. (2015) performed transcript analysis of Cas9 gene using RT-PCR, and PCR based screening of representative transgenics, followed by cloning and sequencing of the target region. Nakayasu et al. (2018) used simple PCR based analysis of *St16DOX* gene in transgenic potato hairy roots. In this study, dual-gRNA strategy was used to simultaneously target two regions of St16DOX gene generating large deletions that can easily be detected by agarose gel electrophoresis: a single DNA band indicates absence of deletion as in control plants with empty vector, whereas an extra band corresponds to the small PCR fragment reflecting a deletion in the *St16DOX* gene. However, the resolution of this method is not adequate for detecting events where individual target regions are separately mutagenized. PCR based detection followed by cloning was used by Butler et al. (2016), such as Long Template PCR system in detecting circularized GVRs in transgenics and performing quantitative end-point PCR, and other PCR methods using high fidelity DNA polymerase. Butler et al. (2015) performed restriction enzyme digestion assay to screen mutant transgenic potato plants. Andersson et al. (2017) performed high-resolution fragment analysis (HRFA), a PCR based method with a resolution limit of ± 1 bp, to detect potato plants mutated in all four alleles and avoid phenotypic screening of large numbers of lines.

T7 Endonuclease 1 that cleaves only mismatched DNA strands in a DNA fragment can also be used to detect mutations in the target region. In this process, the amplicons of different lines are separately mixed with wild type PCR fragment and the mix denatured and re-annealed resulting in a duplex made of wild type and mutated strands with mismatches at the target site. Following the digestion with T7 Endonuclease 1, the fragments are separated in agarose gel electrophoresis and quantified for efficiency calculation (Wang et al. 2017). Selecting target regions with diagnostic restriction endonuclease sites spanning the nucleotides 3–4 immediately upstream of PAM motif can also help in the screening of regenerated plants since these sites will get mutated by indels upon CRISPR/Cas9 cleavage. Amplicons are digested with the corresponding restriction enzyme and the fragments separated on agarose gel showing one intact fragment for the mutant while the wild type DNA is cleaved into two smaller fragments (Xie and Yang 2013). The other method that can be used to corroborate GE event is the use of Polyacrylamide gel electrophoresis (PAGE) where, using single-stranded conformation polymorphism, DNA of the target loci with mutation in

single stranded form would show varied migration in the gel which arises due to different conformations of DNA (Zhang et al. 2016).

To validate the exact nature of mutagenesis induced by the CRISPR/Cas system one needs to sequence the target region of the gene of interest using Sanger sequencing method which helps in identifying chimeric mutations (Feng et al. 2014). This can be done by direct sequencing of the PCR product and analysis of AB1 files using various deconvoluting softwares. Alternatively, the PCR product can be cloned, and several clones sequenced for each product. Additionally, next-generation sequencing (NGS) or high throughput sequencing can be used to simultaneously screen the generated plants and unravel the mutations both at on- and potential off-targets in a very robust, effective and highly sensitive manner (Feng et al. 2014).

Guo et al. (2018) reported to have developed a simpler, cheaper and faster protocol in screening mutants induced by CRISPR/Cas9 technology via MSBSP-PCR (Mutation Sites Based Specific Primers-PCR), which they have successfully implemented in *Nicotiana tabacum* and *Arabidopsis* to identify biallelic/homozygous mutants.

Avoiding potential off-target mutations in potato

It is desirable to counter any potential off-target mutations that may arise during the GE and affect the normal function of a non-target gene, thereby complicating GE mutational analysis studies as well as inducing undesired changes in the plants. Mismatches in the first eight nucleotides distal to the PAM motif of the 'seed sequence' in the sgRNA to the non-target genes have been shown to be tolerated by Cas9/sgRNA complex. However, such off-targeting can be reduced or even eliminated by good design and test of sgRNA activity (Tang et al. 2018) and use of synthetic proofreading Cas9 variants (Chen et al. 2017). Wang et al. (2015) identified another gene in double monoploid (DM) potato genome containing a sequence identical to *StIAA2* target but without PAM motif. They found no off-targeting effect of their sgRNA:Cas9 construct designed to target *StIAA2* gene.

Various other strategies can be adopted to mitigate against potential off-targets. Cas9 nickase with D10A mutation in RuvC domain introduces single strand cut at the target but can induce double-strand break when used in the presence of two sgRNAs on opposite strands. Similarly, the use of catalytically inactive Cas9 with both mutations D10A and H840A (dCas9) fused to *Fok*I nuclease domain can increase the targeting specificity. Again dCas9-FokI chimeric protein (fCas9) can induce double-strand break for mutagenesis when used with two sgRNAs on opposite strands but in proximity to allow dimerization of the two *Fok*I nuclease domains (Ott de Bruin et al. 2015; Bortesi and Fischer 2015). Such a strategy can be used in potato and other agronomically important plant crops to offset genome-wide off-targeting repercussions. Paired Cas9 nickases has been shown to eliminate off-target mutations in rice, though diminished occurrence of on-target mutation has been reported (Mikami et al. 2016). Cas9/sgRNA ribonucleoproteins (RNPs) have been reported to reduce off-targeting as well as eliminating Cas9 cytotoxic effect when expressed through DNA transfection and the likelihood of integration of plasmid DNA fragments (Kim et al. 2017).

Recently, Tycko et al. (2018) identified that *Staphylococcus aureus* Cas9 (SaCas9) sgRNAs containing 21–22-mer spacer sequences are more active but 20-mer ones are less tolerant to mismatches. The authors also point out that SpCas9 with truncated sgRNAs (17–18 nt pacers) have increased specificity but are less efficient.

Generation of transgene-free potato

To be accepted as non-genetically modified organism (non-GMO) crop by the public and regulatory bodies, the genetic makeup of GE crops must remain undisturbed except for the specifically introduced modification and without any exogenous DNA. In Agrobacterium-based transformation the CRISPR/Cas transgene in the T-DNA is introduced into the cell and integrates in its genome thus changing its genetic makeup. Such expression cassette must be eliminated completely for public acceptance. During generation transition from T_0 to T_2, genetic segregation is utilized in which stably inherited transgene-free plants can be obtained in T2 mutant lines (Ricroch et al. 2017). However, this strategy cannot easily be adopted in tetraploid potato with high allelic polymorphism. RNP delivery into protoplasts is now emerging as an excellent alternative system that avoids DNA intermediates (Arora and Narula 2017). Indeed, purified Cas9 is now commercially available and cheap and gRNA can be easily prepared using in vitro transcription or ordered from specialized companies. Besides, the use of Cas9 mRNA has been shown to be more efficient than the purified Cas9. Although protoplasts can be prepared in any laboratory with tissue culture skills, the regeneration of plants is long and laborious. RNP or Cas9 mRNA/gRNA can also be introduced into any explant material of choice using biolistic transformation but the necessary equipment is costly. GE of potato and other plants will benefit from the expanding field of CRISPR delivery using nanoparticles.

FLP/FRT and Cre/loxP piggyback transposon systems have also been used as molecular tools to get rid of transgenes (Khatodia et al. 2016; Zaidi et al. 2018). Recently, suicide transgenes have been used to exterminate

all the pollen grains and embryos of T_0 plants harboring *Cas9* transgene (He et al. 2018).

The debate over whether GE crops should be classified as GMOs or not has been going on for years and many researchers now accept GE as a clean and precise technology for crop improvement. In many countries around the world, however, GE crops are still being considered as GMO (Ricroch et al. 2017). As the public is very concerned about the GMOs, it is imperative to produce transgene-free GE potatoes in the future for commercialization and persuade the consumers of its safety. Recently, a major blow to the CRISPR gene editing technology came from the European Union (EU) highest court's ruling on gene edited crops. The adjudication read that the GE crops should be treated as stringently as the GMOs, in terms of regulation, assessment of health and environmental impact as well as labeling. Many high-profile scientists have condemned this ruling calling it as being non-scientific. It has been presumed that the repercussion of this ruling would have a lasting negative effect on research on GE crops through lack of funding. It may also leave other countries to follow the lead of EU court's decree. However, the US officials have said that they haven't yet thought of imposing regulations on GE crops like the one used for GMOs (Stokstad 2018; Callaway 2018).

Various other CRISPR technologies that may be used in potato breeding

Modification of gene expression and epigenome with CRISPR/Cas9-based tools

Ectopic regulation of gene expression can be conducted with the use of the CRISPR/Cas9 system. This can give us insights into the function of the genes and to engineer regulatory gene circuits in synthetic biology. dCas9 that retains its DNA binding activity in the presence of gRNA can be fused to different activator/inhibitor domains of transcription factors to regulate gene expression. dCas9 can be used in delivering specific cargos to the target of interest in various locations of the genome such as epigenetic marks and enzymes to edit histone modifications and DNA methylation (Bortesi and Fischer 2015).

CRISPR/Cas12a system

Recently, Cas12a nuclease (previously known as Cpf1; CRISPR as isolated from bacteria *Prevoltella* and *Francisella1*) belonging to class II CRISPR system has been utilized in gene editing of plants such as rice and tobacco resulting in inheritable biallelic mutations. In contrast to Cas9 that recognize PAM motif NGG downstream of the target spacer, Cas12a nuclease supplied with a single crRNA targets T-rich PAM sequence (such as 5′-TTTN-3′) at the 5′-end (Lei et al. 2017; Xu et al. 2017) (Fig. 2). Cas12a acts as a ribonuclease with a processing precursor crRNA isolated from different species. LbCpf1 is a Cas12a isolated from *Lachnospiraceae bacterium* ND2006 among the plethora of Cas12a family. AsCpf1 isolated from *Acidaminococcus* sp. BV3L6 is more effective in the cells of humans, in contrast with the other orthologues of Cpf1 family (Kim et al. 2017).

Two different versions of Cas12a enzymes LbCas12a and FnCas12a have been used to execute homology directed insertion in rice and were reported to have superior HR rates compared to SpCas9 (Begemann et al. 2017). Cas12a nucleases could be a potential option for targeting genes in potato, particularly for those genes with low GC content.

In mammalian cells and zebrafish, improved gene editing efficiency has been reported with the use of engineered Cas12a variants tagged with two different NLS at the C-terminus. Also, the use of a full-length direct repeat of pre-crRNAs containing stem-loop G-C base substitutions has been shown to be beneficial (Liu et al. 2019). In this work, the recombinant proteins FnoCas12a-2xNLS-6xHis and LbCas12a-2xNLS-6xHis were purified and assembled with engineered crRNAs. The delivery of these RNP complexes in elevated amount increased mutagenesis efficiencies with low toxicity. These newly improved Cas12a variants will be easily implemented in potato gene editing.

CRISPR/Cas13 system

CRISPR/Cas13 system has been identified and repurposed into another gene editing technology that can be used in plants. Three Cas13 enzymes namely Cas13a, Cas13b and Cas13c have been reported (Cox et al. 2017). CRISPR/Cas13a (previously known as CRISPR/C2c2) is a Class 2 type VI-A CRISPR effector molecule isolated from *Leptotrichia shahii* and has been demonstrated to be involved in the RNA-guide processing by its RNase activity. Cas13a is guided by a single crRNA molecule, which can be programmed to cleave single stranded RNA targets (Fig. 3). It was furthermore found that LwaCas13a does not display promiscuous RNA cleavage activity in eukaryotic cells including human cells and plant protoplasts (Abudayyeh et al. 2016; Aman et al. 2018). Cas13a has been used to knockdown gene expression as well as RNA detection in mammalian cells and plants (Cox et al. 2017). In the study by Aman et al. (2018), Cas13 was successfully used for interference against Turnip Mosaic Virus (TuMV) expressing green fluorescent protein in *Nicotiana benthamiana* both in stable and transient systems. Several potato RNA viruses like *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Potato leafroll virus* (PLRV) are responsible for low yield of potato (Arif et al. 2011). PVY is one of

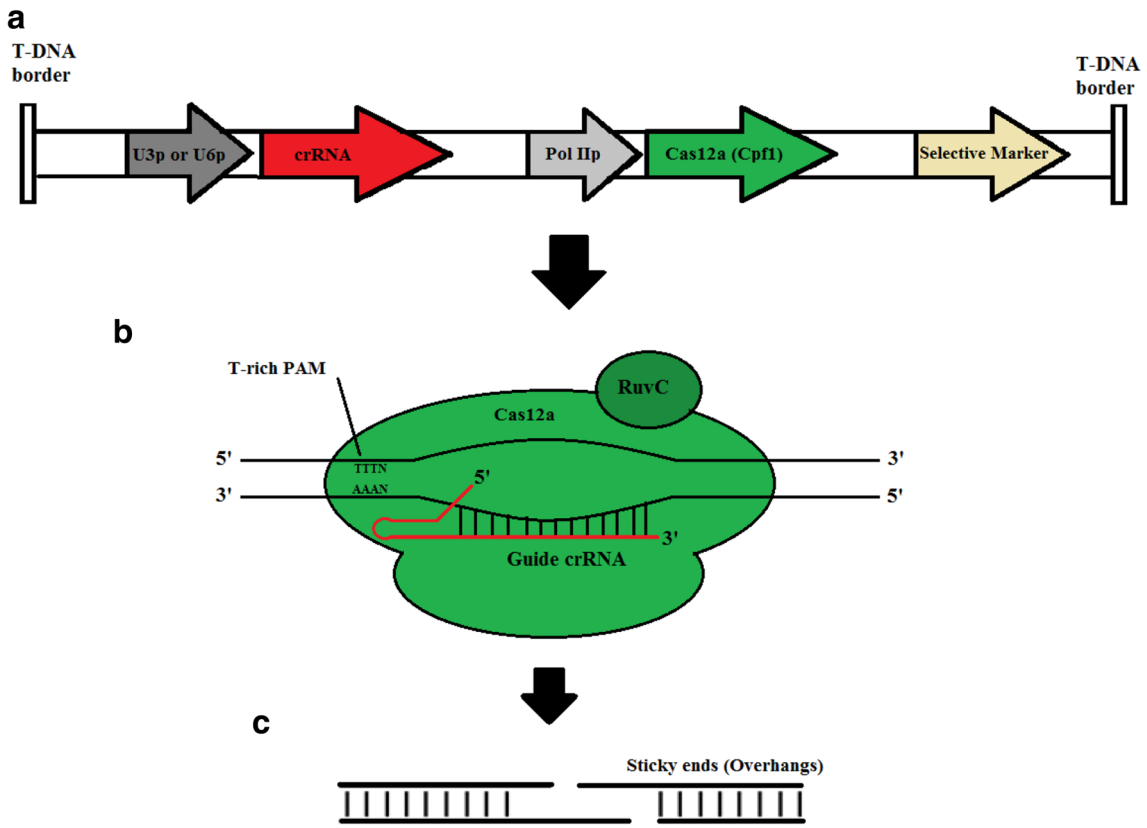

Fig. 2 Illustration of CRISPR/Cas12a system (also known as CRISPR/Cpf1 system). **a** The vector backbone for Agrobacterium-mediated transformation showing various promoters, crRNA, Cas12a nuclease and selective marker within the left border (LB) and right border (RB). **b** The generation of sticky ends via single effector Cas12a nuclease in association with guide crRNA generated by DSB mediated by Nuc and RuvC domains. Reproduced from Xu et al. (2017) and Zaidi et al. (2017)

the most vicious viral pathogens of potato crops (Solomon-Blackburn and Barker 2001). PVM (*Potato virus M*), which has a single-stranded RNA genome (Zavriev et al. 1991) accounts for yield loss of 15–45% in potato (Jeffries 1998). In Kazakhstan, high incidence of PVM (~ 85%) and PVY (100%) were observed (Sozinova et al. 2007), and CRISPR/Cas13a system could be used to target these pathogenic RNA viruses that devastate potato crops.

Furthermore, catalytically inactive Cas13 (dCas13) has been fused to the deaminase domain of human ADAR to direct conversion of adenosine to inosine. This system known as REPAIR (RNA Editing for Programmable A to I Replacement) allows full transcript editing to remove pathogenic mutations making it a breakthrough in basic research, biotechnology industry and therapeutics (Cox et al. 2017).

In addition, type III-B CRISPR-Cas system can be utilized in homology-dependent degradation of RNA which is complementary to the designed crRNA both in vivo and in vitro to mediate the post-transcriptional control of the gene (Bortesi and Fischer 2015).

Programmable base editing without DNA cleavage

Like RNA editing discussed above, target genomic sites can be edited without DNA cleavage. The first generation of such base editors was engineered by fusing dCas9 to cytosine deaminase. This system was followed by Adenine base editor (ABE) that convert A·T to G·C in targeted genomic DNA. It has been shown that introduction of point mutations can be done more efficiently and cleanly with the use of ABEs with less off-target genome modification than the conventional CRISPR/Cas9 based technology. This technology has been shown to be a boon in correction of disease-causing mutations without any double-stranded DNA cleavage (Gaudelli et al. 2017).

Lu and Zhu (2017) performed programmable base editing in rice by synthesis by fusing APOBEC1 from rat to N-terminus of Cas9 (D10A)-NLS separated by a linker XTEN, a peptide of 16 residues. APOBEC1-XTEN-Cas9(D10A)-NLS coding sequence was inserted in a binary vector under the control of ubiquitin promoter. In the presence of guide RNA (gRNA), this new Cas9 derivative edits the gene

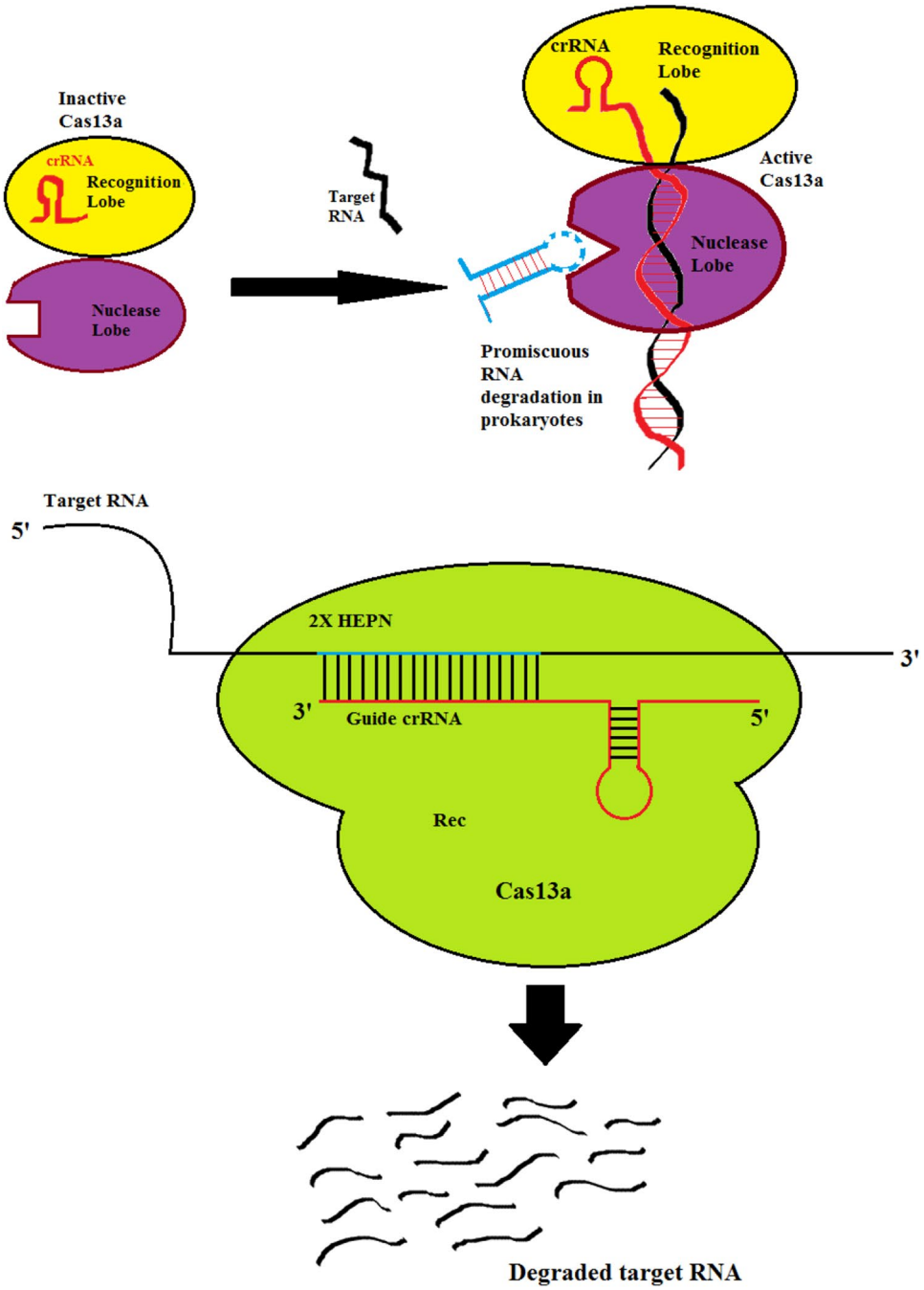

Fig. 3 Illustration of how target-RNA binding confers conformational change in Cas13a in activating promiscuous degradation of non-specific RNA, which is not observed in eukaryotes cells such as human cells or plant protoplast (Abudayyeh et al. 2016; Aman et al. 2018), Cas13a nuclease action in association with guide crRNA on target RNA and degradation of target RNA. Reproduced from Liu et al. (2017)

target by directly converting cytidine (C) to uridine (U). The authors targeted *NRT1.1B* gene that encodes a nitrogen transporter by C/T replacement resulting in Thr327Met amino acid change that can increase nitrogen use efficiency in rice. Their second target was *SLR1* gene which encodes for a DELLA protein, and substitution of amino acid in the vicinity of the TVHYNP motif can reduce plant height. They broadened AID/APOBEC1 system for base-editing of GC-rich regions in rice. This new system provides a robust and simple method in base-replacement for plant research and breeding.

Li et al. (2018) have modified plant adenine base editor (ABE7.10) using tRNA adenosine deaminase fusion to Cas9 nickase. This facilitated the base editors to convert A·T to G·C in up to 59.1% of regenerated wheat and rice plants, and 7.5% in protoplasts of winter wheat Kenong199 and rice Zhonghua11. Using *Agrobacterium*-mediated transformation, they targeted several genomic loci, including *ALS*-T1 gene for which herbicide-tolerant plants with gain-of-function point mutations with no unintended editing at any of the genomic on-target loci of wheat or rice. It could be a boon in potato to be able to base edit genes for gain-of-function

by delivering various base editors described above using geminivirus-based system described for gene editing by Butler et al. (2016). Additionally, this base editing can be done without the introduction of a transgene by using ABE RNPs as suggested by Li et al. (2018).

Conclusion

The potato presents unique challenges and advantages to plant breeders. As a highly heterozygous tetraploid crop, cultivated potato is clonally propagated resulting in identical genotypes. By avoiding allele segregation through sexual reproduction, transfer of desirable traits between cultivars and their stability in subsequent progeny remains a challenge for breeders. Owing to these features, genetic engineering is believed to be an efficient way of introducing desirable traits. CRISPR/Cas technology has opened new avenues in genome editing of crops with the immense potential in resistance against biotic (viral, fungal and bacterial diseases) and abiotic stresses. Furthermore, the advent of different versions of CRISPR/Cas has strengthened efficiency of genome editing of crops through the gain and/or loss of gene(s) functions and potato is no exception. The availability of whole genome sequence of potato enables the scientists of post-genomic era to engineer its genome precisely for crop improvement. CRISPR/Cas approach can also be used to improve potato's nutritional quality especially in reducing its acrylamide contents. The availability of transformation and regeneration protocols of potato can help to achieve genome edited plants without any exogenous DNA in shorter period. Taken all together, CRISPR/Cas based genome editing approach holds promising potential for speeding the breeding programs of potatoes.

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