



CRISPR/Cas mediated genome editing in potato: Past achievements and future directions

Aytug Tuncel^{a,*}, Yiping Qi^{a,b,**}

^a Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, USA

^b Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD, USA

ARTICLE INFO

Keywords:

CRISPR/Cas

Genome editing

Potato

Protoplast

Agrobacterium mediated transformation

ABSTRACT

Genome engineering has been re-shaping plant biotechnology and agriculture. Crop improvement using the recently developed gene editing techniques is now easier, faster, and more precise than ever. Although considered to be a global food security crop, potato has not benefitted enough from diverse collection of these techniques. Unique genetic features of cultivated potatoes such as tetrasomic inheritance, high genomic heterozygosity, and inbreeding depression hamper conventional breeding of this important crop. Therefore, genome editing provides an excellent arsenal of tools for trait improvement in potato. Moreover, using specific transformation protocols, it is possible to engineer transgene free commercial varieties. In this review we first describe the past achievements in potato genome editing and highlight some of the missing aspects of these efforts. Then, we discuss about technical challenges of genome editing in potato and present approaches to overcome these difficulties. Finally, we talk about genome editing applications that have not been explored in potato and point out some of the missing venues in literature.

1. Introduction

Potato ranks as the 4th most produced crop with 370 million tonnes per year worldwide after maize, wheat, and rice. It is, however, 3rd to rice and wheat in terms of consumption (FAO, 2019). Being rich in carbohydrates, vitamins (C and B6) and minerals, potato plays a key role in human nutrition. Starch in tubers is also utilized in food and non-food related industrial applications including manufacturing of additive, paper and textile products (Kraak, 1992). Unlike other major crops such as maize, which benefit from about 1% genetic yield gain each year (Duvick, 2005), potato yield improvement over the last century has been marginal (Douches et al., 1996). This is because traditional breeding of potato is a slow process that is hindered by many factors. Most of the cultivated potatoes are tetraploids and have highly heterozygous genomes. Moreover, there are about fifty traits that influence the value of a commercial cultivar (Eggers et al., 2021). This genetic complexity, along with relatively long generation cycles and inbreeding depression (Zhang et al., 2019a), make it extremely difficult to obtain the desired allelic combination in the progeny. Likewise, introduction of new traits by outcrossing to wild cultivars can lead to loss of allelic combination. In

addition, potato production suffers from yield losses due to pests and pathogens including late/early blight, bacterial wilting, and several types of potato viruses. Overall, all these factors, together with ever growing world population and deteriorating climate, necessitate the use of new and accelerated breeding techniques to engineer novel traits into potatoes.

Genome editing of potato was first accomplished using transcription activator-like effector nucleases (TALENs) (Sawai et al., 2014) (Table 1). It was, however, surpassed later by the more recently developed clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR associated protein (Cas) system (Jinek et al., 2012). This is mainly because TALENs require specific protein engineering, which can be costly and time consuming. In contrast, CRISPR/Cas systems are more versatile, easy to design and are capable of multiplexing (Malzahn et al., 2017). The most widely used CRISPR/Cas system comprises Cas9 nuclease from *Streptococcus pyogenes* (SpCas9) and a chimeric single guide RNA (sgRNA) consisting of a 20-nt protospacer that determines site specificity and a scaffold sequence essential for Cas9 binding. Cas9 acts as a sequence specific nuclease (SSN) and introduces a double stranded break (DSB) at the target site, usually 3-bp upstream of the

* Corresponding author.

** Corresponding author at: Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, USA.

E-mail addresses: atuncel@umd.edu (A. Tuncel), yiping@umd.edu (Y. Qi).

5'-NGG-3' protospacer adjacent motif (PAM). This DSB can be repaired by two pathways: less common homology directed repair (HDR) or more frequent non-homologous end joining (NHEJ) mechanism which often introduces insertions/deletions (indels) at the target site, creating gene knockout (KO) (Puchta, 2005). In addition to traditional CRISPR/Cas9 more sophisticated CRISPR/Cas methodologies, including base editing, prime editing, and transcriptional activation/repression, have been successfully employed in plants (reviewed in Pan et al., 2021; Molla et al., 2021; Zhang et al., 2019b).

CRISPR/Cas9 mediated genome editing of potato was first reported in 2015 by two different studies. One study (Wang et al., 2015) targeted the auxin/indole-3-acetic acid family member gene (*StIAA2*) and the other (Butler et al., 2015) targeted the acetolactate synthase 1 gene (*ALS1*) using both a conventional T-DNA or a modified geminivirus T-DNA to express genome editing agents. These two proof-of-concept studies paved the road for succeeding endeavours of genome editing in potato including base editing and prime editing (Table 1). Since then, various CRISPR/Cas mediated genome editing approaches have been applied in potato to alter traits including tuber quality, abiotic stress resistance, herbicide tolerance, cooking properties and self-compatibility.

2. Applications of CRISPR/Cas in potato

2.1. Starch quality

Starch in potato tubers constitutes 80% of the dry matter making it a major caloric supply as well as an abundant resource for industrial applications. It is composed of amylose and amylopectin which are linear and branched chains of glucose, respectively. Amylose consists of glucose molecules mainly linked through α -1,4 bonding, whereas amylopectin also includes clustered branching formed by α -1,6 linkage. Amylose/amylopectin ratio of tuber starch differs between cultivars, but roughly equals to 1:4 (Zeeman et al., 2010). This ratio determines chemical and physical properties of starch allowing it to be processed depending on the desired application. For example, amylose free waxy starch is used in paper industry as a bonding agent and in food industry as a bulking, thickening, or coating agent (Sarka and Dvoracek, 2017; Maurer, 2009). In contrast, amylose rich, digestion resistant starch can

be consumed as a healthier alternative to reduce caloric intake, improve insulin resistance, and promote gut health (Keenan et al., 2015; El Kaoutari et al., 2013).

Thus far, efforts to manipulate starch quality of potato tuber by CRISPR/Cas mediated genome editing predominantly focused on knocking out granule bound starch synthase 1 (*GBSS1*), the gene responsible for amylose synthesis (Table 2). The rationale behind creating waxy phenotype tubers is to reduce the number of chemical and physical post-harvest treatments required to separate amylose and amylopectin, thereby making the whole starch processing economically and environmentally more feasible. *GBSS1* has been an attractive target and model gene for many genome editing studies in potato due to technical reasons as well. Although complete KO of all the alleles is required to observe the waxy phenotype, it is still relatively easy to engineer this trait as *GBSS1*, unlike many other starch related genes, is the only isoform that controls this phenotype. Moreover, amylose free starch granules, when stained with iodine, can be easily distinguished by their red-brown color from wildtype (WT) granules which produce a dark blue color due to presence of amylose.

GBSS1 has been targeted using anti-sense RNA (Visser et al., 1991) and RNAi (Otani et al., 2007) approaches, and more recently using CRISPR/Cas9 with the aim of generating transgene free waxy tubers. To achieve this, plasmid DNA coding Cas9 and sgRNAs was transiently transformed into protoplasts, and plants were regenerated (Andersson et al., 2017). 29 of the mutated lines were genotyped in detail and only three of them (10%) were identified to harbour inserts of plasmid origin. Moreover, one of the regenerants coming from a particular transformation event had all copies of the *GBSS1* gene mutated and starch from microtubers of this line displayed the waxy phenotype. In a subsequent study (Andersson et al., 2018), the authors used Cas9 ribonucleoproteins (RNPs) including either synthetically (cr-RNP) or in vitro transcribed RNA (ivt-RNP) to transform protoplasts, thus avoiding foreign DNA integration. Frequencies of total KOs (with all copies of *GBSS1* mutated) obtained by transformation of plasmid DNA, cr-RNP and ivt-RNP were 3%, 2% and 25 or 9%, respectively, with the experimental conditions tested (25% or 40% PEG). All the cr-RNP lines (including those with mono-, bi-, tri or tetra-allelic mutations), as expected, were transgene free, but just one of the lines was a total KO. In contrast, none of the KOs generated by plasmid DNA transformation

Table 1
Technical milestones in potato genome editing.

Pre-2015	2015	2016	2017	2018
Sequence of potato genome revealed by Potato Genome Sequencing Consortium (Potato Genome Sequencing et al., 2011)	TALEN mediated targeted mutagenesis in potato through transient expression in protoplasts and subsequent regeneration of mutant plants (Nicolia et al., 2015)	TALEN mediated targeted T-DNA insertion into potato genome (Forsyth et al., 2016)	CRISPR mediated gene editing in potato through transient expression in protoplasts and subsequent regeneration of transgene free mutant plants (Andersson et al., 2017)	CRISPR mediated gene editing in potato through ribonucleoprotein delivery into protoplasts and subsequent regeneration of transgene free mutant plants (Andersson et al., 2018)
TALEN mediated gene editing in potato (Sawai et al., 2014)	CRISPR mediated gene editing in potato (Wang et al., 2015; Butler et al., 2015)	Gemini virus replicon mediated homologous recombination in potato using TALEN and CRISPR (Butler et al., 2016)		Base editing (C to T) in potato protoplasts using nCas9 and human APOBEC3A (A3A-CBE) fusion (Zong et al., 2018)
2019	2020	2021	2022	Future directions
Cas9 mediated multiplex gene editing in potato through transient expression in protoplasts and subsequent regeneration of mutant plants (Tuncel et al., 2019)	Targeted mutagenesis and base editing (C to T) in potato using Cas9 and/or PmCDA1-CBE from <i>Staphylococcus aureus</i> (SaCas9) (Veillet et al., 2020)	Cas9 mediated homology directed repair using donor plasmid with geminivirus origin (Hegde et al., 2021)	Prime editing in potato (Perroud et al., 2022)	- Targeted insertion - Epigenome editing - Regulation of gene expression (e.g., CRISPR mediated gene activation or interference) - Tissue specific gene editing - Organelle genome editing - Commercial non-transgenic potatoes with: - Improved yield - More nutritional content (e.g., vitamins, antioxidants) - Reduced SGAs - Enhanced tolerance to abiotic stress (e.g., drought, cold)
Base editing (C to T) in potato using PmCDA1-CBE and generation of transgene free mutant plants through <i>Agrobacterium</i> mediated transformation (Veillet et al., 2019b)				
Cas13a mediated RNA targeting in potato (Zhan et al., 2019)				

Table 2

Summary of CRISPR/Cas applications in potato genome editing.

Target gene	Delivery method	Editing agent	Genotype	Phenotype	Cultivar	Reference
Starch quality						
<i>GBSSI</i>	Protoplast (plasmid DNA)	Cas9	Tetra allelic KO in 2% of regenerants	Decrease in amylose content	Kuras	(Andersson et al., 2017)
<i>GBSSI</i>	Protoplast (RNP)	Cas9	Tetra allelic KO in 2–3% of regenerants	Decrease in amylose content	Kuras	(Andersson et al., 2018)
<i>GBSSI</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic mutation in 28% of regenerants	Decrease in amylose content	Sayaka	(Kusano et al., 2018)
<i>GBSSI</i>	Protoplast	A3A-CBE	Heterozygous mutations	N/A	Desiree	(Zong et al., 2018)
<i>GBSSI/SBEII</i>	<i>A. tumefaciens</i>	Cas9	N/A	Low/high amylose	Xushu22, Taizhong6	(Wang et al., 2019)
<i>GBSSI</i>	Protoplast (plasmid)	Cas9	Tetra allelic mutation in 35% of regenerants	Decrease in amylose content	Desiree & Wotan	(Johansen et al., 2019)
<i>GBSSI</i>	<i>A. tumefaciens</i> , protoplast	Cas9, PmCDA1-CBE	Tetra allelic mutations with both methods	Decrease in amylose content	Desiree, Furia	(Veillet et al., 2019a)
<i>GBSSI</i>	<i>A. tumefaciens</i>	Cas9, PmCDA1-CBE	No mutation	N/A	Desiree	(Veillet et al., 2020)
<i>GBSSI</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic	Decrease in amylose content	Yukon Gold, TXYG79	(Toinga-Villafuerte et al., 2022)
<i>SBE1, SBE2</i>	<i>A. tumefaciens</i> , protoplast	Cas9	Tetra allelic mutations in both genes	Diverse range of starch phenotypes	Desiree	(Tuncel et al., 2019)
<i>SBE1, SBE2</i>	Protoplast	Cas9	Tetra allelic mutations in both genes	High amylose starch	Desiree	(Zhao et al., 2021)
<i>SBE3</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic	Low amylose	Sayaka	(Takeuchi et al., 2021)
<i>SS6</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic	N/A	Desiree	(Sevestre et al., 2020)
<i>GWD1</i>	Protoplast	Cas9	N/A	N/A	Wotan, Saturna	(Carlsen et al., 2021)
Carotenoid biosynthesis						
<i>PDS</i>	<i>A. tumefaciens</i>	Cas9	N/A	Bleached (albino) leaves	Desiree	(Banfalvi et al., 2020)
<i>PDS</i>	<i>A. tumefaciens</i>	Cas9	Biallelic (7%)	Bleached whole plantlets	DMF1	(Butler et al., 2020)
Cooking properties (browning)						
<i>PPO2</i>	Protoplast	Cas9	Tetra allelic (24%)	Reduced browning	Desiree	(Gonzalez et al., 2019)
<i>PPO2</i>	<i>A. tumefaciens</i> , protoplast	Cas9	Tetra allelic (46%)	Reduced browning	Desiree	(González et al., 2021)
Glycoalkaloid content						
<i>16DOX</i>	<i>A. rhizogenes</i>	Cas9	Tetra allelic (8%)	No detectable SGAs	Mayqueen	(Nakayasu et al., 2018)
<i>SSR2</i>	<i>A. tumefaciens</i>	Cas9	Multi allelic	66% of WT tuber	Atlantic	(Zheng et al., 2021)
Biotic stress tolerance						
<i>P3, CI, NIB, CP</i>	<i>A. tumefaciens</i>	Cas13a	N/A	Improved resistance to PVY	Desiree	(Zhan et al., 2019)
<i>eIF4E1</i>	Protoplast	Cas9	Tetra allelic	Partial resistance to PVY	Desiree	(Lucioli et al., 2022)
<i>DMR6-1</i>	<i>A. tumefaciens</i>	Cas9, PmCDA1-CBE	Multiallelic	N/A	Desiree	(Veillet et al., 2020)
<i>DMR6-1</i>	Protoplast	Cas9	N/A	N/A	Wotan, Saturna	(Carlsen et al., 2021)
<i>CCOAOMT</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic HDR in 1% regenerants	Improved resistance to late blight	Russet Burbank	(Hegde et al., 2021)
<i>DND1, CHL1, DMR6-1</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic (0.7–18%)	Improved resistance to late blight	Desiree, King Edward	(Kieu et al., 2021)
<i>RNase III</i>	<i>A. tumefaciens</i>	Cas13	N/A	Improved resistance to sweet potato virus disease	Xushu 29	(Yu et al., 2022)
Abiotic stress tolerance						
<i>MYB44</i>	<i>A. tumefaciens</i>	Cas9	Multiallelic	No phenotypic effect	Desiree	(Zhou et al., 2017)
Herbicide tolerance						
<i>ALS1</i>	<i>A. tumefaciens</i> , geminivirus replicon (GVR)	Cas9	Multiallelic	N/A	Desiree, MSX914	(Butler et al., 2015)
<i>ALS1</i>	<i>A. tumefaciens</i> , geminivirus replicon (GVR)	Cas9	Multiallelic	Improved herbicide resistance	Desiree, MSX914	(Butler et al., 2016)
<i>ALS1</i>	<i>A. tumefaciens</i>	PmCDA1-CBE	Multiallelic	Improved herbicide resistance	Desiree	(Veillet et al., 2019b)
<i>ALS1</i>	<i>A. tumefaciens</i>	Prime Editor 2	Monoallelic	Improved herbicide resistance	Desiree	(Perroud et al., 2022)
Self-compatibility						
<i>S-RNase</i>	<i>A. tumefaciens</i>	Cas9	Multiallelic	Self-compatible plants	Phureja S15–65	(Ye et al., 2018)
<i>S-RNase</i>	<i>A. tumefaciens</i>	Cas9	Multiallelic	Self-compatible plants	DRH-195 and DRH-310	(Enciso-Rodriguez et al., 2019)
<i>Sli</i>	<i>A. tumefaciens</i>	Cas9	Multiallelic	Self-incompatible plants	B665 and B663	(Eggers et al., 2021)
Development						
<i>IAA2</i>	<i>A. tumefaciens</i>	Cas9	Multiallelic	N/A	Doubled monoploid	(Wang et al., 2015)
<i>IT1</i>	<i>A. tumefaciens</i>	Cas9	Tetra allelic	Loss of tuberization	Phureja S15–65	(Tang et al., 2022)

were transgene free, an outcome that differed from the previously reported results (Andersson et al., 2017). Interestingly, majority of the ivt-RNP KO lines had also fragments of DNA insertions originating from either the template DNA used to transcribe sgRNA or chromosomal DNA. Only one of the ivt-RNP KOs was transgene free indicating that even small amounts of DNA carried over from sgRNA transcription can be integrated into genome during NHEJ repair. These two pioneering and proof of concept studies described that *GBSS1* can be fully mutagenized by delivery of plasmid DNA or RNPs into protoplasts and that transgene free *waxy* tubers can be obtained by subsequent regeneration of potato plants. However, because the authors primarily focused on the editing outcomes and generating transgene free plants, neither study investigated any possible off-target editing scenario. In addition, both studies lacked in depth biochemical analyses including quantification of amylose, amylopectin, and starch content from tubers of mature plants. Such biochemical measurements, including amylose content and viscosity properties of starch, were performed in a more recent study (Toinga-Villafuerte et al., 2022). The authors generated a stable KO line of *GBSS1* by *Agrobacterium* mediated transformation, which essentially had no detectable amylose in tubers. This study, however, did not report whether total starch amount in the edited tubers was affected due to the decrease in amylose content.

GBSS1 was also targeted as a model gene to assess editing efficacy of various Cas9 mutagenesis systems in potato. For instance, one study (Kusano et al., 2018) fused translational enhancer sequence dMac3 (Aoki et al., 2014) to Cas9 gene to improve editing at the *GBSS1* locus. This significantly improved both the number of mutagenesis events and number of lines carrying mutations in all alleles of *gbss1*, presumably due to the increase in available Cas9 protein. However, direct evidence showing an enrichment in Cas9 mRNA and/or protein levels was missing. Although amylose content in tubers of selected KO lines decreased compared to that of WT, there was still considerable amount amylose present in the tubers. This was most likely due to presence of alleles with small in frame deletions that could express a fully/partially functional protein. Moreover, the authors tested dMac3 in stable lines generated by *Agrobacterium* mediated transformation. It would be interesting to see if dMac3 could also improve editing efficacy during plasmid-based transient expression in protoplasts. In a parallel study (Johansen et al., 2019), the authors tested if replacing the AtU6 promoter, which is conventionally used to express sgRNAs in dicots, with the native StU6 promoter could boost sgRNA expression, thus enhance editing efficacy at the *GBSS1* gene. In fact, editing frequency improved about 9-fold at protoplast level, which in turn translated into 35% of the regenerated plants being tetra-allelic mutants.

First base editing (C to T) in potato was demonstrated at protoplast level by targeting *GBSS1* along with the *ALS* gene (Zong et al., 2018). The authors attached human cytidine deaminase APOBEC3A and uracil glycosylase inhibitor (UGI) to Cas9 nickase (nCas9) to create the A3A-CBE fusion. A3A-CBE and six sgRNAs, targeting the *GBSS1* gene, were transiently expressed from cauliflower mosaic virus (CaMV) 35 S and AtU6 promoters, respectively. Two heterozygous mutants, out of 31 regenerated plants, were identified to have base conversions at one of the target sites (StGBSS-T6). Although the mutant plants did not have any indels or undesired edits at the target site, frequency of C to T conversion was low and no tetra-allelic mutants were recovered. Hence, no further biochemical analysis of starch was reported. In a subsequent study (Veillet et al., 2019a), *GBSS1* was targeted by a fusion of nCas9 and *P. marinus* cytidine deaminase (PmCDA1) to introduce precise point mutations in functionally important motifs that could lead to loss of function of the enzyme. Two mutants, out of 48 stable regenerants, had unusual C₁₇ to G₁₇ conversion in all alleles and had no other unintended mutations at the target site. This nucleotide substitution resulted in L99V mutation in KTGG motif and in turn, mutant microtuber sections stained red-brown with iodine and lacked amylose as determined by size exclusion chromatography (SEC). Interestingly, if the authors included an UGI in their construct, typical C to T base editing would be

observed which would in turn result in C₁₇T₁₆A₁₅ to T₁₇T₁₆A₁₅ silent mutation (L99L). Recently, superior performance of the AtU3 promoter over the AtU6 promoter was reported for base editing in other dicot plants like poplar (Li et al., 2021) and tomato (Randall et al., 2021). It is promising to use AtU3 promoter to drive sgRNA expression for further improved base editing efficiency in potato.

Amylose rich potatoes are also valuable due to their potential health benefits. When cooked, potato starch with unmodified amounts of amylose and amylopectin (1:4 ratio) is easily digested in the upper gut and consequently trigger rapid release of glucose molecules which spike blood sugar level. Repeated cycles of this scenario can eventually lead to development of insulin resistance and worsen conditions of people with obesity and type II diabetes. Starch with high amylose content is more resistant to digestion due to reduced availability of non-reducing ends, sites where α -amylases attack and release glucose. In addition, linear amylose molecules, upon cooking (heating) and cooling, form α -helices that are also resistant to degradation by α -amylases in the upper gut. Therefore, consuming resistant starch with high amylose content (and amylopectin with longer chains of glucose) can help better control of glucose homeostasis and reduce caloric intake as well (Keenan et al., 2015). Moreover, resistant starch that is not digested in the small intestine serves as food and energy source for healthy bacteria in the lower gut. Its fermentation by gut microbiota produces short chain fatty acids (SCFAs) which are considered crucial for gut-brain communication and are also essential for maintenance of gut, and overall metabolic health (Blaak et al., 2020; Silva et al., 2020).

Efforts to generate potato tubers containing digestion resistant starch aimed to reduce the activity of starch branching enzyme 1 and 2 (SBE1, SBE2) that are responsible for introduction of α -1,6 glycosidic bonds in amylopectin (Table 2). Large reductions in total SBE activity in tubers were achieved through antisense RNA (Schwall et al., 2000) and RNAi (Andersson et al., 2006) approaches. Starches from tubers described in these studies had altered properties, were high in amylose content and resistant to digestion (Schwall et al., 2000; Zhao et al., 2018; Karlsson et al., 2007; Blennow et al., 2005). Recently, SBEs were targeted by Cas9 mediated mutagenesis in two different studies to generate transgene free tubers with amylose rich, resistant starch. In the first study (Tuncel et al., 2019), the authors targeted *sbe1* and *sbe2* genes either individually or together through *Agrobacterium* mediated transformation of stem explants and through transient expression of plasmid DNA in protoplasts. Tetra allelic mutants of either isoform was obtained through *Agrobacterium* transformation, but no double KO line was recovered with this approach. Despite starch granules from tubers of these stable lines had altered morphologies, they had only minor changes in the level of branching. Although amylose content was not measured directly, one of the protoplast regenerated lines (identified as double KO) had granules with deep fissures across the hilum, a phenotype indicative of high amylose content which was similar to those generated by antisense RNA (Schwall et al., 2000) or RNAi (Hofvander et al., 2004) approaches. The degree of starch branching in this extreme phenotype was half that of the WT. Interestingly, both *sbe1* and *sbe2* isoforms, presumably, retained partial enzyme activity as one of the alleles of each isoform had six bp long deletions that were in frame with the coding sequences. Moreover, this line was determined as transgene free based on the absence of Cas9 gene, though possible integrations from other parts of the plasmid were not investigated. In a second study (Zhao et al., 2021), RNP delivery of CRISPR/Cas9 into protoplasts was used to generate three groups of mutants with two of the mutants in group three identified to harbour tetra allelic mutations in both isoforms. Starches from tubers of these lines were essentially composed of amylose (>95%) and had no detectable amylopectin. Consequently, these lines had significantly decreased tuber yield, tuber size and dry matter.

2.2. Tuber browning

Browning of potatoes can happen through cold induced sweetening,

accumulation of reducing sugars such as glucose and fructose during cold storage of tubers, and subsequent exposure of these sugars to heat processing (e.g., cooking). The brown tissue changes texture and flavour that is not preferred by consumers. Moreover, the reducing sugars can react with free amino acids at elevated temperatures and form acrylamide through nonenzymatic Maillard reaction. Acrylamide is a cancer-causing agent, therefore poses serious health concerns (Bethke and Bussan, 2013; Sowokinos, 2001). One of the enzymes involved in cold induced sweetening is vacuolar invertase (VINV) which converts sucrose to glucose and fructose (Sowokinos, 2001). The VINV gene was targeted by TALEN mediated mutagenesis to produce potatoes with improved cold storage and processing traits. Transgene free potatoes, with tubers accumulating significantly lower amount of reducing sugars and acrylamide, were obtained through protoplast regeneration (Clasen et al., 2016).

In addition to non-enzymatic browning, potatoes can also undergo enzymatic browning through oxidation which also reduces nutritional quality of tubers. This process occurs when quinones, produced by the action of polyphenol oxidases (PPOs), self-polymerize or react with free amino acids to form brown-colored molecules (Taranto et al., 2017). PPO2, the main isoform that accounts for 55% of total PPO activity in tuber (Chi et al., 2014), was targeted by RNP delivery of Cas9 and sgRNAs into protoplasts to reduce browning (Gonzalez et al., 2019). 10% of the regenerants had tetra allelic mutations, and reductions up to 69% and 73% in PPO activity and browning were achieved, respectively (Table 2). Comparable results were obtained in a follow-up study where the authors compared editing efficacy of different transformation approaches (*Agrobacterium* vs protoplast) using PPO2 as the model gene (González et al., 2021). The question arises whether further reductions in browning can be achieved by multiplexed editing of other isoforms (PPO1, PPO3 and PPO4) that make-up rest of the PPO activity in tuber (Chi et al., 2014), or whether both types of browning (enzymatic and non-enzymatic) can be eliminated by targeting PPO and VINV genes together.

2.3. Steroidal glycoalkaloid content

Like browning, another undesired trait in potatoes is the presence of high concentrations of steroidal glycoalkaloids (SGAs) which are mainly composed of α -solanine and α -chaconine in commercial cultivars. Although these compounds are primarily found in flowers, sprouts, and leaves, they also accumulate in tubers, mainly in peel (Friedman, 2006). However, SGA content can increase several folds in tuber flesh due to wounding, mechanical stress, or high light during post-harvest handling and storage (Friedman and McDonald, 1999). Since they can be toxic to humans at high concentrations, 200 mg/kg of tuber fresh weight is considered as the safe threshold for consumption. Therefore, it is important to create varieties with low tuber SGA content.

Because cholesterol serves as precursor in production of SGAs, sterol side chain reductase 2 (SSR2), the enzyme responsible for synthesis of cholesterol, was targeted using TALEN (Sawai et al., 2014). Only one stable line with tetra allelic mutations in the SSR2 gene, and not those retaining a WT copy, had significantly reduced SGA content (10% that of the WT) in leaves. However, SGA content from tubers of this line was not reported. In a recent study, SSR2 was targeted using CRISPR/Cas9, but tetra allelic mutants could not be obtained (Zheng et al., 2021). Though SGA reductions up to 34% in tuber flesh was reported, some of the lines had more SGA in tubers and leaves compared to WT (Table 2). In another study, 2-oxoglutarate dependent dioxygenase (St16DOX), an enzyme involved in later steps of SGA synthesis, was targeted using CRISPR/Cas9 (Nakayasu et al., 2018). Two edited lines containing tetra allelic mutations had no detectable α -solanine and α -chaconine in hairy roots. Overall, no transgene free potatoes with tubers containing significantly reduced SGAs have been produced so far. In addition, there will be more genes available to target as our understanding of the SGA pathway expands.

2.4. Biotic stress tolerance

Substantial yield losses in potato can occur due to biotic stress. One of the most devastating pathogens of potato is potato virus Y (PVY) which can cause up to 80% yield loss (Quenouille et al., 2013). Because PVY is an RNA virus, CRISPR/Cas systems that target RNA can be used to engineer plants resistant to this pathogen. One such system, composed of Cas13a from *Leptotrichia wadei* (LwaCas13a), was described being as efficient as traditional RNAi to knock-down select genes with more specificity in mammalian cells and rice protoplasts (Abudayyeh et al., 2017). Another Cas13a variant from *Leptotrichia shahii* (LshCas13a) was adopted to potato to target four different RNA regions that are all conserved among three different strains, PVY^O, PVY^N and PVY^{N:O}, and that encode for proteins P3 and CI (viral factors), NIb (viral replicase) and CP (capsid) (Zhan et al., 2019). Each RNA region was targeted separately using four different constructs. Stable transgenic lines with the highest expression of LshCas13a/sgRNA were challenged against the PVY strains and no disease symptoms were observed upon infection of the leaves. Interestingly, lines with intermediate expression of LshCas13a/sgRNA displayed mild symptoms of PVY infection, indicating an inverse correlation between the transgene expression and severity of symptoms (Table 2). Broad spectrum resistance to different potato viruses (e.g., PVA, PVS and PVY) can be engineered using the same methodology with multiplexing. It should, however, be noted that this type of virus resistance trait requires transgene integration. Alternatively, a recent study targeted the translation initiation factor eIF4E1, a host protein that is used by PVY for replication after infecting the cells, and therefore can provide recessive resistance when mutated (Lucioli et al., 2022). Interestingly, the first generation of transgenic potatoes had only two of the eIF4E1 alleles mutated, thus were susceptible to PVY^{NTN}. Re-transfection and re-generation of the protoplasts isolated from the first generation of mutant plant yielded two full KO lines which were partially resistant to PVY^{NTN} infection. In a parallel study (Yu et al., 2022), the authors targeted RNase III viral protein of sweet potato chlorotic stunt virus (SPCSV) which, by co-infection with feathery mottle virus, causes sweet potato virus disease (SPVD) that can lead to significant yield losses (Loebenstein, 2015). Stable lines expressing another Cas13 variant, Cas13d from *R. flavefaciens* (RfxCas13d), and gRNA targeting RNase III did not show viral symptoms when infected.

Potato late blight, undoubtedly the most devastating disease that caused the historic Irish famine and can practically wipe out an entire field, is caused by the pathogen *Phytophthora infestans* and, typically, controlled by several series of pesticide applications. One way to tackle late blight disease is to reduce the activity of susceptibility genes (S-genes) in potato that are triggered upon infection and colonization by *P. infestans*. Two such genes, *StDMR6-1* (downy mildew resistance) and *StCHL1*, were identified as best S-gene candidates for future breeding applications (Kieu et al., 2021). Stable transgenic lines that are tetra allelic mutants of these genes displayed enhanced resistance to *P. infestans* infection but were not fully resistant to the disease. Therefore, the obvious next step would be to generate double KOs of both genes through plasmid or RNP delivery to obtain transgene free, fully resistant potatoes.

Another way to fight late blight disease is to introduce gain of function traits into commercial cultivars. Conventionally, this can be achieved by introgression of resistance genes (R-genes) from wild potato relatives that are resistant to late blight (Zhu et al., 2012; Ghislain et al., 2019). However, this is a time-consuming process and can result in trait loss in the progeny. In a recent study, Cas9 mediated HDR was used to replace an SNP in caffeoyl-CoA O-methyltransferase (*StCCoAOMT*) gene, which eliminated a premature stop codon and restored full-length protein (Hegde et al., 2021). This, in turn, increased expression of downstream R-genes and conferred partial late blight resistance in transgenic plants. The authors used two different plasmids, one to express Cas9/sgRNA and another to provide repair template, in 1:1 ratio during *Agrobacterium* mediated transformation. The HDR repair

template was expressed using a geminivirus based vector to increase its abundance and availability during the double stranded break repair (Baltes et al., 2014). Seven transgenic lines (out of 145) were identified as HDR positive in all four alleles, of which two had proper integration of template without any indels. The ratio of correct HDR events (1.4%) suggests that HR happens at relatively low frequency in potato, albeit at a sufficient level for a tetraploid organism. It is, therefore, intriguing to ask if the HDR outcome can be improved by use of protoplasts.

2.5. Herbicide tolerance

Engineering herbicide resistance into potato through CRISPR/Cas, so far, solely focused on the *ALS1* gene with the aim of assessing various genome editing tools rather than generating herbicide resistant potatoes. Certain mutations in ALS protein confer reduced herbicide susceptibility (Nandula et al., 2020; Okuzaki et al., 2007; Yu et al., 2010; Kochevenko and Willmitzer, 2003) which enables easier screening of successful editing events by using herbicides as a secondary selection pressure. Therefore, the *ALS* gene became a playground for testing more precise editing techniques such as HDR, base editing, and prime editing.

In one of the early studies, two such mutations were introduced into *StALS1* through HDR by expressing SSNs (TALEN and Cas9) and the repair template from plasmids with geminivirus replicon (GVR) (Butler et al., 2016). However, mutants with desired mutations were obtained when the repair template also contained the *nptII* gene allowing only regenerants with the proper template integration to survive on kanamycin selection media. This clearly shows that HDR frequency was very low, and that a secondary selection marker was needed to identify HDR events causing plants to be transgenic. Interestingly, modifications in *StALS1*, thus herbicide susceptibility, improved in offspring events propagated from parental regenerants indicating the somatic nature of the HDR.

ALS was also targeted to test the efficacy of cytidine base editing in potato (Veillet et al., 2019b). Both isoforms of *StALS* (*StALS1* and *StALS2*) were targeted at a conserved region using one sgRNA to create Pro186 and Pro184 mutations, and a base editor with target activation induced cytidine deaminase (Target-AID) (Shimatani et al., 2017). The authors replaced kanamycin selection with herbicide chlorsulfuron after two weeks to enrich transgene free mutant population. Twenty regenerants were recovered with majority of the mutations (75%), not surprisingly, being indels due to lack of a UGI in the construct. One of the indel free mutants had C₋₂₀ of the target region converted in all eight alleles. Therefore, co-editing of an *ALS* gene, together with a primary target, seems to be a promising approach to generate transgene free plants.

In a more recent study, Pro186Ser mutation in *StALS1* and 2 genes was attempted using prime editing (Perroud et al., 2022). This time the authors switched majority of explants to chlorsulfuron selection one week after the *Agrobacterium* transformation. No mutation was detected at the target loci of stable regenerants that were selected by kanamycin. Only one plant coming from the chlorsulfuron selection had the intended mutations in one of the alleles and appeared to be mosaic. These results clearly indicate that the prime editing in potato occurred at a very low frequency and that further optimizations are necessary to improve editing efficacy.

2.6. Self-compatibility

Commercially produced potato cultivars can be self-compatible (SC) but conventional inbreeding is a long and tedious process in these cultivars due to tetrasomic inheritance which makes obtaining the best allele combinations extremely difficult. Out-crossing with wild relatives to introduce desired traits (e.g., disease resistance) into elite cultivars can also result in loss of existing traits and introduction of undesired ones (e.g., high SGA content). In contrast, most wild species of potato are diploid which makes them ideal for inbreeding as it is relatively easier to

stack favourite traits in progeny with diploids. Tuber yield, quality and resistance could be improved if selfing was possible. Majority of wild species, however, are self-incompatible (SI) rendering self-pollination virtually impossible (Watanabe, 2015). SI is controlled by the single, multi-allelic S-locus composed of the S-locus RNase (*S-RNase*) and S-locus inhibitor (*Sli*) genes. The *S-RNase* protein is expressed in style and inhibits growth of self-pollen tubes, hence self-fertilization. *Sli* genes encode S-locus F-box proteins in pollens, which in SC species or cross pollinators, counter act *S-RNase* protein thereby allowing pollen tube growth and fertilization at ovary (Porcher and Lande, 2005; Takayama and Isogai, 2005; Kubo et al., 2010).

Taking these facts into consideration, it could be possible to convert SI species into SC varieties by knocking-out the *S-RNase* gene. Indeed, two different studies targeted conserved regions of *S-RNase* alleles to knock out this gene using CRISPR/Cas9 (Enciso-Rodriguez et al., 2019; Ye et al., 2018). The resulting mutants had multi-allelic mutations in the *S-RNase* gene and were SC. The plants were able to self-fertilize and subsequently generated berries. Moreover, the trait was heritable and the Cas9 gene was segregated out in some of the T1 seeds indicating that non-transgenic progeny can be obtained. In a following study (Eggers et al., 2021), function of the *Sli* gene was investigated by targeting it with Cas9 to convert SC varieties into SI. Furthermore, the authors identified a 533 bp insert in the promoter of the *Sli* gene from SC varieties that enabled its expression in pollens. This region can be introduced into promoters of the *Sli* genes of SI potatoes by targeted insertion to make them SC in future breeding efforts.

3. Technical challenges in delivery methods and genome editing of potato

3.1. *Agrobacterium* mediated delivery

Agrobacterium mediated transformation of stem or leaf explants is one of the most widely used methods for delivering genome editing agents into potato. This technique, like any other method, has pros and cons for generating mutant plants (Fig. 1). In general, *Agrobacterium* mediated transformation is a well-established and robust technique which can provide high transformation efficiency with many cultivars. Since T-DNA is integrated into the host genome, Cas protein and sgRNA (s) are expected to be expressed perpetually which can help improve editing in succeeding clonal propagates. This can be beneficial, especially, if multiplexing is aimed for and complete modification of all alleles (e.g., total KO) is desired, because it is relatively harder to obtain a T0 mutant with all the intended modifications due to tetraploidy of many cultivars. Such a phenomenon was observed in plants with somatic mutations from secondary events displaying reduced herbicide susceptibility (Butler et al., 2016). However, continuous expression of Cas protein and sgRNA(s) might also increase the chances of off-target modifications (Zhang et al., 2015). It is also possible that editing level might not improve within the clonal progeny due to transgene silencing (e.g., methylation or multiple T-DNA copy integration) (Finnegan and McElroy, 1994; Gelvin, 2017).

The main disadvantage of using *Agrobacterium* mediated transformation with potato is stable integration of T-DNA since tubers are vegetatively propagated. Back-crossing with parental line to segregate the transgene is time-consuming and can result in trait loss. One way to avoid stable integration during *Agrobacterium* transformation is to remove the selection pressure associated with the T-DNA from culture media shortly after transformation. This might, however, lead to incomplete editing (non-tetra allelic mutations) and chimerism, though both can also happen in plants regenerated under continuous selection (Banfalvi et al., 2020). Alternatively, diploid varieties that are already engineered to be SC can be used as model plants to perform stable transformation and, later, backcrossed to obtain transgene free plants. Another drawback of stable transformation is that some potato species are recalcitrant to *Agrobacterium* transformation, a problem that can be

<i>Agrobacterium</i>	Protoplast (RNP/plasmid DNA)	VIGE
• Well established and robust • High transformation efficiency • Less prone to somaclonal variation • Editing might improve in clonal progeny • T-DNA integration can be avoided by removal of selection pressure	• Transgene free plants • Less off-target effects • Can observe editing shortly after transformation • Mutant population can be enriched through cell sorting • No plasmid design needed if RNPs are used	• Fast and easy application • Transgene free plants • Less tissue culture work • Less prone to somaclonal variation
• Integration of T-DNA (transgenic plants) • More likely to have off-targets • Transgene silencing can occur • Some species are recalcitrant to transformation	• More prone to somaclonal variation • More tissue culture work • Requires better maintenance of aseptic conditions	• Not well established in potato • Possible virus RNA contamination • Cargo limitations

Fig. 1. Pros and cons of each delivery/transformation method used in potato genome editing.

overcome using alternative *Agrobacterium* strains and/or plant tissue. For example, one study (Butler et al., 2020) used *A. rhizogenes* strain MSU440 to transform hairy roots, tissues that can grow hormone-independent and have high genetic stability.

3.2. Protoplast transformation

In contrast to stable transformation, transient delivery of plasmid DNA or RNPs into protoplasts provides the opportunity to obtain transgene free potatoes. Moreover, editing at off-targets are significantly reduced, particularly with the RNP approach (Svitashev et al., 2016; Liang et al., 2017) (Fig. 1). One of the shortcomings of the protoplast approach is the number of regenerants that needs to be screened since selection pressure cannot be applied during tissue culture. A fluorescent protein can be attached to Cas nuclease and fraction of transformed protoplasts can be enriched by cell sorting before proceeding with regeneration to reduce subsequent screening workload (gDNA extraction and sequence analysis of target regions). In addition, screening can be performed at the callus stage to determine mutations ahead of plantlet regeneration. It should also be noted that physical separation of protoplasts (e.g., embedding into sodium alginate) after transformation is important to keep edited cells from mixing with untransformed cells. Otherwise, heterozygous calli composed of edited and unedited cells can lead to chimeric shoots, a phenomenon explained by multicellular origin of organogenesis (Faize et al., 2010; Poethig, 1989; Marcotrigiano, 1986). Recent evidence indicates that somatic embryos are formed by rearrangement of group of peripheral callus cells rather than originating from a single totipotent cell (Feher, 2019; Su and Zhang, 2014). Therefore, it is possible for a mixed population of edited and untransformed/unedited cells to cluster together when grown in liquid media and develop into a heterozygous callus that can eventually give rise to chimeric shoots.

The major drawback of the protoplast approach is the prevalence of somaclonal variation which occurs due to prolonged exposure of plant material to tissue culture conditions. These variations include single nucleotide deletions (Miyao et al., 2011), epigenetic changes (Stroud et al., 2013; Han et al., 2018), chromosomal deletions and/or rearrangements, and aneuploidy (Lee and Phillips, 1988). Drastic changes in genome structure can be easy to detect at the phenotypic level (e.g., in potato: thicker, smaller, greener and crinkled leaves, stronger stem, and

stunned overall plant growth). Regenerants with minor variations can be phenotypically indistinguishable from parental lines, although they might also possess genetic instability. Indeed, aneuploidy and structural changes in chromosomes were observed in all the fifteen protoplast regenerated potato plants, whereas only 18% of the plants regenerated from *Agrobacterium* mediated transformation had large scale copy number changes (Fossi et al., 2019). Therefore, it is particularly essential to investigate the presence of potentially adverse somaclonal changes in a genome edited plant obtained through protoplast regeneration. Extended periods of tissue culture should be avoided and use of hormones that are known to promote somaclonal variation (e.g., 2, 4-dichlorophenoxyacetic acid) should be minimized to reduce genomic instability (Karp, 1990). Alternatively, one can simultaneously edit a target gene and activate morphogenic genes that can promote faster plant regeneration in the absence of hormones (Pan et al., 2022).

3.3. Virus induced genome editing

Regardless of the method of choice (*Agrobacterium* or protoplast), generating genome engineered plants requires months of labor-intensive tissue culture work. Recent developments in virus induced genome engineering (VIGE), however, are promising to significantly shorten the timeframe and to reduce the tissue culture workload. In a pioneering study (Maher et al., 2020), new meristematic growth was induced at cut shoot apices of *N. benthamiana* by inoculating the wound site with *Agrobacterium* carrying viral replicons, which expressed certain developmental regulators, along with luciferase reporter and sgRNA targeting the *PDS* gene. Because the plants were already (constitutively) expressing Cas9 the newly emerged shoots from the cut site had the *PDS* gene edited. The experiment was replicated in potato using the luciferase gene only but the newly developed shoots, although expressed the reporter gene, had abnormal phenotypes indicating that optimization of regulator(s) expression is necessary. In a follow-up study (Ellison et al., 2020), the sgRNA was mobilized by fusing it to Arabidopsis Flowering Locus T (FT) mRNA which can move from leaves to apical meristem (Li et al., 2018). Inoculation of the same Cas9 transgenic *N. benthamiana* leaves with *Agrobacterium* containing the mobilized sgRNA, which was expressed through tobacco rattle virus (TRV), resulted in *PDS* mutagenesis in the seeds and T1 progeny.

The problem of not being able to deliver Cas9 gene and sgRNAs

together due to cargo limitations of viral vectors was overcome by using an engineered *Sonchus yellow net virus* (SYNV) (Ma et al., 2020). Modified SYNV vector harbouring the *PDS* sgRNA and Cas9 was delivered into *N. benthamiana* leaves through *Agrobacterium* infiltration. Systemic mutagenesis of *PDS* was observed, except in the seeds, and heritable mutations were obtained through tissue culture of infected somatic cells. Intriguingly, in 10% of the mutant regenerants no viral RNA was detected based on RT-PCR results indicating that these lines were virus free, and that the virus was eliminated in progenitor cells after infection and mutagenesis. In another study (Ariga et al., 2020), potato virus X (PVX) was utilized to deliver both gRNAs and Cas9 together into *N. benthamiana* leaves. 60% of the shoots regenerated from agroinfiltrated leaves under non-selective conditions had targeted mutations with 18% of them having Cas9 gene integrated into genome. Interestingly, 12% of the shoots were negative for PVX RNA, as determined by RT-PCR, and for Cas9 gene as determined by PCR, indicating that they were in fact virus and transgene free.

In light of the abovementioned studies, conducted employing *N. benthamiana* as the model organism, VIGE could be also applied to potato provided that potato is a compatible host for the viruses. One strategy, as previously proposed (Dinesh-Kumar and Voytas, 2020), is to express the mobile sgRNAs together with Cas gene from SYNV or PVX vectors. This could enable, upon infection of the leaves, movement and expression of the editing elements in the shoot apical meristem leading to newly grown shoot tips to have the desired mutations. The shoot tips could then be excised, re-grown in rooting medium and clonal propagates (regenerated individually from auxiliary buds) of these edited plants could be screened for absence of virus RNA and transgene. This strategy, however, would require maintenance of aseptic conditions throughout the process which can be a challenge. It is also possible, though, that the editing could occur in stolon meristem resulting in newly formed tubers to be gene edited which would eliminate the need for sterile conditions. This could be achieved by hijacking mobile factors in potato such as self-pruning 6 A (StSP6A) protein, homolog of the *Arabidopsis* FT, and BEL1-like transcription factor (*StBEL5*) mRNA. Both StSP6A protein and *StBEL5* mRNA are expressed in leaves, then move to stolon tips and initiate tuberization (Navarro et al., 2011; Banerjee et al., 2006; Hannapel et al., 2017). StSP6A protein (partially or fully) could be attached to Cas and/or *StBEL5* RNA could be fused to sgRNA to facilitate the transport of these editing elements into stolon meristem. Another approach could be to induce callus directly from the infected leaf using a selection free media as recently described (Ariga et al., 2020). The regenerated shoots could then be screened for the presence of mutations and, absence of transgene and virus RNA. Although this approach still requires tissue culture, regenerants would be less likely to suffer from somaclonal variation compared to protoplast derived plants (Fig. 1).

3.4. Complications due to genomic architecture of potato

Potato genome is highly complex due to its polyploidy and heterozygous allele composition. In a recent study (Sevestre et al., 2020), the authors constructed SNP map of Desiree, a tetraploid model cultivar, by re-sequencing its genome and comparing it to that of the doubled-haploid clone (Potato Genome Sequencing et al., 2011). The comparative analysis revealed high sequence divergence between the genomes of two different cultivars as well as differences within the genome of Desiree including variations of 1 SNP per 68 bp, 1 deletion per 1753 bp and 1 insertion per 1173 bp. This level of heterozygosity complicates sgRNA design as extra caution is needed to make sure that the sequence of a target region is identical between all alleles. Therefore, simple sequence analysis of PCR amplicons covering the target region prior to any genome editing experiment in potato is highly recommended. Presence of SNPs between different alleles of a gene, however, can also be exploited in numerous ways. Allele specific KOs can be created to investigate the function of a particular gene copy or to explore dosage effects in many traits. For instance, potato plants that were tetra

allelic mutants for the *StDND1* gene were resistant to late blight but had a weaker phenotype (Kieu et al., 2021). Bi- or tri-allelic mutants of this S-gene can be generated by allele specific targeting that could result in plants with a similar level of resistance, yet WT like morphology. In fact, RNAi lines of *StDND1* did not have a weaker phenotype, presumably due to incomplete silencing (Sun et al., 2016). Likewise, a gradient enzyme activity can be attained by targeting certain alleles of SBEs thereby fine tuning the starch structure.

Editing efficacy is influenced by many factors including GC content and structure of sgRNA, target locus, and epigenetic status of target region (Bortesi et al., 2016; Weiss et al., 2022). Related research within the context of potato genome editing has been limited to few studies. One such study with protoplasts investigated whether targeting 5' is more effective than targeting 3' region of a gene (Carlsen et al., 2021). The rationale behind the authors' hypothesis was that 5' end of a gene would be more accessible to Cas9 and sgRNAs because this region, in general, has a relatively more open chromatin structure compared to the middle and 3' end which are more tightly packed and, therefore, are less accessible (Li et al., 2007; Schubeler, 2015). Two genes, *DMR6-1* and *GWD1* (glucan, water dikinase 1), were targeted by RNP delivery of Cas9 and several different sgRNAs. Editing efficacy at 5' region was significantly higher for *GWD1* but not for *DMR6-1*. The authors explained the lack of difference in editing efficiency between the 5' and 3' ends by speculating that *DMR6-1* chromatin structure could already be in open state or that it could be transcriptionally activated in response to cell wall removal and re-synthesis during protoplast treatment. Although this study investigated an interesting hypothesis, it was difficult to reach a decisive conclusion, because the sample group was limited to two genes and editing efficiencies conferred by different protospacers were not accounted. Nevertheless, it is crucial that multiple gRNAs are tested for effectiveness at the protoplast level before proceeding with the actual gene editing experiments.

4. Prospects of CRISPR/Cas mediated genome editing of potato

Majority of the CRISPR/Cas mediated genome editing attempts in potato, so far, used SpCas9 to knock out gene(s) of interest with relatively good success rates. Still, SpCas9 system can be further optimized for targeted mutagenesis. This is essential particularly if the genome edited, transgene free potatoes are aimed for commercialization. Currently, the most feasible path to such an end-product appears to be through genome editing by RNPs at the protoplast level followed by plant regeneration. However, this approach, as discussed in the previous section, is vulnerable to somaclonal variation which might reduce plant vigour and yield in the field due to inadvertent genomic instabilities. Therefore, several regenerants with the desired mutations need to be obtained and screened to identify the ones with the least (none, if possible) somaclonal variation. This requires tremendous tissue culture work which can be reduced, in one way, using highly efficient sgRNAs. Chemical modification of sgRNAs can provide better protection against degradation by RNases and enhance their stability, thus improve overall editing efficacy (Allen et al., 2021). Another approach to boost editing efficacy in potato could be through enhancing activity of Cas9 by temperature optimization (LeBlanc et al., 2018).

Although loss of function mutagenesis through conventional SpCas9 targeting has proven to be essential for functional genomics and for altering traits to a certain extent, more efficient, precise, and diverse CRISPR/Cas systems are needed to be tested in potato. One study used *Staphylococcus aureus* Cas9 (SaCas9) which has a 5'-NNGRRT-3' PAM requirement (Veillet et al., 2020). The authors also developed a cytidine base editor (CBE) based on SaCas9 to target *StGBSSI* and *StDMR6-1* genes. However, no plants with mutations in *StGBSSI* gene were obtained and only a handful of mutants for *StDMR6-1* were recovered, indicating the need for more robust Cas9 variants with diverse PAMs that can efficiently edit target genes in potato. Likewise, there are currently only a few proof-of-concept studies that utilized base and

prime editors with limited success. Therefore, base editing and prime editing systems in potato require significant improvement with regards to efficacy and precision (Molla et al., 2021). With the development of highly efficient CBEs such as those based on A3A-Y130F (Li et al., 2021; Randall et al., 2021; Ren et al., 2021a) and adenine base editors (ABEs) such as ABE8e (Yan et al., 2021; Ren et al., 2021b), we anticipate such high-performance base editors will further promote base editing applications in potato.

Unlike Cas9 based systems, Cas12a, a class 2 type V endonuclease, has not been utilized in potato genome editing so far, although the most popular Cas12a from *Lachnospiraceae* bacterium, LbCas12a, has been demonstrated in various crops including rice (Wang et al., 2017; Xu et al., 2017), maize (Lee et al., 2019), tomato (Bernabé-Orts et al., 2019), and citrus (Jia et al., 2019). In contrast to Cas9, Cas12a recognizes canonical TTTV PAM or a relaxed version, TTV (Zhang et al., 2021), and uses a short CRISPR RNA (crRNA) which makes it suitable for multiplexing, and cost efficient for RNP delivery (Zhang et al., 2022). In addition, Cas12a introduces DSBs distant from PAM enabling recurrent cutting/editing that results in larger deletions (Zetsche et al., 2015; Tang et al., 2017). Furthermore, Cas12a offers more specificity and less off targeting compared to Cas9 (Kim et al., 2016; Tang et al., 2018). Although Cas12a shows reduced nuclease activity at lower temperatures (e.g., 22 °C) that are relevant for plant tissue culture and genome editing, improved editing was achieved by growing *Arabidopsis* and maize plants at elevated temperatures (e.g., 28–29 °C) (Malzahn et al., 2019) or by using a temperature tolerant LbCas12a (ttLbCas12a) variant (Schindele and Puchta, 2020). These properties make Cas12a an attractive tool for genome editing in potato, particularly with respect to multiplexing, RNP approach, and if larger deletions are desired. For example, expression of a particular gene can be fine-tuned by targeting its promoter and generating small to large deletions in this region using Cas12a, as previously done using Cas9 (Rodríguez-Leal et al., 2017).

The current and novel CRISPR/Cas tools could be used for improving yield and nutritional content of tubers. One such venue that has not been necessarily explored in potato is abiotic stress tolerance (Table 2). For instance, down regulation of the gene coding for cap-binding protein 80 (CAP80) using artificial microRNAs resulted in transgenic potato plants with improved drought tolerance (Pieczynski et al., 2013). The *CBP80* gene can certainly be targeted using CRISPR/Cas9 to generate transgene free plants. Although *PDS* gene was targeted in potato to assess transformation protocols owing to its easily detectable albino phenotype in knock-out plants (Banfalvi et al., 2020; Butler et al., 2020), carotenoid pathway has not been truly manipulated using novel genome editing approaches to enhance vitamin content of tubers. Most commercial potatoes are not rich in vitamin A and boosting its level in tubers could help fighting vitamin A deficiency. One way to increase beta-carotene level, the potent pro-vitamin A metabolite, could be to target beta-carotene hydroxylase (*BCH*) gene. In fact, silencing of *BCH* by using RNAi lead to tubers with more than 300 µg of beta-carotene per 100 g fresh weight (Van Eck, 2007). Likewise, ascorbate (vitamin C) levels in tubers were increased up to three-fold when polyubiquitin promoter was used to express GDP-L-galactose phosphorylase (*GGP* or *VTC2A*) gene that is responsible for catalyzing the first committed step of ascorbate biosynthesis in plants (Bulley et al., 2012). The same promoter (or a different one) from potato can be replaced with the native promoter of *GGP* gene by CRISPR/Cas mediated HDR or targeted insertion. In addition, tuber yield could be improved by boosting tuber formation. Tuberization is a complex process that involves many genes which either promote or suppress the pathway (reviewed in (Hannapel et al., 2017; Dutt et al., 2017). For instance, down-regulation of sucrose transporter 4 (*StSUT4*) gene, a negative regulator of tuberization, by RNAi resulted in improved tuber yield (Chincinska et al., 2008) making it a promising candidate for targeted mutagenesis. Recently, a transcription factor named identity of tuber 1 (IT1) was identified to be exclusively expressed in the stolon and interact with the StSP6A protein (Tang et al., 2022). CRISPR/Cas9 mediated mutagenesis of the *StIT1* gene caused

branching of the stolons instead of tuberization indicating its key role in tuber initiation. It is intriguing to ask whether up-regulation of the same gene (i.e., through CRISPR/Cas mediated promoter engineering) would stimulate tuberization and increase tuber yield.

Moreover, CRISPR/Cas systems can aid regulation of gene expression (activation and/or interference), epigenome editing, and tissue specific editing. Recently, TALE based organelle genome editing has been reported (Molla et al., 2021; Nakazato et al., 2022; Forner et al., 2022; Kazama et al., 2019). Applications of these tools will further enhance genome engineering capability in potato. For example, it will be interesting to test the use of CRISPR-Combo (nulla) for improved genome editing in potato by augmenting plant regeneration. Furthermore, delivery of editing reagents by viral vectors and nanomaterials (Demirer et al., 2021) will expand the opportunities for obtaining transgene free potatoes that are less likely to harbour somaclonal variations. Development and optimization of these novel genome editing platforms for potato breeding will provide researchers with more flexibility to engineer new traits with higher efficiency and accuracy. After US, UK, Japan, and India's looser policies on the regulatory restrictions of genome edited crops and with more public acceptance, other countries are likely to follow. This will open-up a new era in plant breeding and present opportunities for trait improvement more than ever.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by the NSF Plant Genome Research Program (award no. IOS-1758745 and IOS-2029889), the USDA-NIFA Biotechnology Risk Assessment Program (award no. 2020-33522-32274), the FFAR Crop of the Future Collaborative Program (award no. 21010111), and the USDA-AFRI Agricultural Innovations Through Gene Editing Program (award no. 2021-67013-34554).

References

- O.O. Abudayyeh, et al., RNA targeting with CRISPR-Cas13, *Nature* 550 (2017) 280–284.
- D. Allen, M. Rosenberg, A. Hendel, Using synthetically engineered guide RNAs to enhance CRISPR genome editing systems in mammalian cells, *Front. Genome Ed.* 2 (2021).
- M. Andersson, et al., Targeted gene suppression by RNA interference: an efficient method for production of high-amylose potato lines, *J. Biotechnol.* 123 (2006) 137–148.
- M. Andersson, et al., Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts, *Plant Cell Rep.* 36 (2017) 117–128.
- M. Andersson, et al., Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery, *Physiol. Plant.* 164 (2018) 378–384.
- H. Aoki, et al., Enhanced translation of the downstream ORF attributed to a long 5' untranslated region in the *OsMac1* gene family members, *OsMac2* and *OsMac3*, *Plant, Biotechnol* 31 (2014) 221–228.
- H. Ariga, S. Toki, K. Ishibashi, Potato virus X vector-mediated DNA-free genome editing in plants, *Plant Cell Physiol.* 61 (2020) 1946–1953.
- N.J. Baltes, J. Gil-Humanes, T. Cermak, P.A. Atkins, D.F. Voytas, DNA replicons for plant genome engineering, *Plant Cell* 26 (2014) 151–163.
- A.K. Banerjee, et al., Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway, *Plant Cell* 18 (2006) 3443–3457.
- Z. Banfalvi, E. Csakvari, V. Villanyi, M. Kondrak, Generation of transgene-free *PDS* mutants in potato by *Agrobacterium*-mediated transformation, *BMC Biotechnol.* 20 (2020) 25.
- J.M. Bernabé-Orts, et al., Assessment of Cas12a-mediated gene editing efficiency in plants, *Plant Biotechnol. J.* 17 (2019) 1971–1984.
- P.C. Bethke, A.J. Bussan, Acrylamide in processed potato products, *Am. J. Potato Res.* 90 (2013) 403–424.

- E.E. Blaak, et al., Short chain fatty acids in human gut and metabolic health, *Benef. Microbes* 11 (2020) 411–455.
- A. Blennow, et al., Structure function relationships of transgenic starches with engineered phosphate substitution and starch branching, *Int. J. Biol. Macromol.* 36 (2005) 159–168.
- L. Bortesi, et al., Patterns of CRISPR/Cas9 activity in plants, animals and microbes, *Plant Biotechnol. J.* 14 (2016) 2203–2216.
- S. Bulley, et al., Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase, *Plant Biotechnol. J.* 10 (2012) 390–397.
- N.M. Butler, P.A. Atkins, D.F. Voytas, D.S. Douches, Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system, *PLoS One* 10 (2015), e0144591.
- N.M. Butler, N.J. Baltes, D.F. Voytas, D.S. Douches, Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) using sequence-specific nucleases, *Front. Plant Sci.* 7 (2016) 1045.
- N.M. Butler, S.H. Jansky, J. Jiang, First-generation genome editing in potato using hairy root transformation, *Plant Biotechnol. J.* 18 (2020) 2201–2209.
- F.M. Carlsen, et al., Strategies for efficient gene editing in protoplasts of *Solanum tuberosum* theme: determining gRNA efficiency design by utilizing protoplast, *Front. Genome Ed.* 3 (2021), 795644.
- M. Chi, et al., Reduced polyphenol oxidase gene expression and enzymatic browning in potato (*Solanum tuberosum* L.) with artificial microRNAs, *BMC Plant Biol.* 14 (2014) 62.
- I.A. Chincinska, et al., Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response, *Plant Physiol.* 146 (2008) 515–528.
- B.M. Clasen, et al., Improving cold storage and processing traits in potato through targeted gene knockout, *Plant Biotechnol. J.* 14 (2016) 169–176.
- G.S. Demirer, et al., Nanotechnology to advance CRISPR-Cas genetic engineering of plants, *Nat. Nanotechnol.* 16 (2021) 243–250.
- S.P. Dinesh-Kumar, D.F. Voytas, Editing through infection, *Nat. Plants* 6 (2020) 738–739.
- D.S. Douches, D. Maas, K. Jastrzebski, R.W. Chase, Assessment of potato breeding progress in the USA over the last century, *Crop Sci.* 36 (1996) 1544–1552.
- S. Dutt, et al., Key players associated with tuberization in potato: potential candidates for genetic engineering, *Crit. Rev. Biotechnol.* 37 (2017) 942–957.
- D.N. Duvick, The contribution of breeding to yield advances in maize (*Zea mays* L.), in: *Advances in Agronomy*, Academic Press, 2005, pp. 83–145.
- E.J. Eggers, et al., Neofunctionalisation of the *Sli* gene leads to self-compatibility and facilitates precision breeding in potato, *Nat. Commun.* 12 (2021) 4141.
- A. El Kaoutari, F. Armougom, J.I. Gordon, D. Raoult, B. Henrissat, The abundance and variety of carbohydrate-active enzymes in the human gut microbiota, *Nat. Rev. Microbiol.* 11 (2013) 497–504.
- E.E. Ellison, et al., Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs, *Nat. Plants* 6 (2020) 620–624.
- F. Enciso-Rodriguez, et al., Overcoming self-incompatibility in diploid potato using CRISPR-Cas9, *Front. Plant Sci.* 10 (2019) 376.
- M. Faize, L. Faize, L. Burgos, Using quantitative real-time PCR to detect chimeras in transgenic tobacco and apricot and to monitor their dissociation, *BMC Biotechnol.* 10 (2010) 53.
- FAO, *FAO Statistical Databases, Food Balances*, (2019).
- A. Feher, Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? *Front. Plant Sci.* 10 (2019) 536.
- J. Finnegan, D. McElroy, Transgene inactivation: plants fight back!, *Nat. Biotechnol.* 12 (1994) 883–888.
- J. Forner, et al., Targeted introduction of heritable point mutations into the plant mitochondrial genome, *Nat. Plants* 8 (2022) 245–256.
- A. Forsyth, T. Weeks, C. Richael, H. Duan, Transcription activator-like effector nucleases (TALEN)-mediated targeted DNA insertion in potato plants, *Front. Plant Sci.* 7 (2016) 1572.
- M. Fossi, K. Amundson, S. Kupp, A. Britt, L. Comai, Regeneration of *Solanum tuberosum* plants from protoplasts induces widespread genome instability, *Plant Physiol.* 180 (2019) 78–86.
- M. Friedman, Potato glycoalkaloids and metabolites: roles in the plant and in the diet, *J. Agric. Food Chem.* 54 (2006) 8655–8681.
- M. Friedman, G.M. McDonald, Postharvest changes in glycoalkaloid content of potatoes, *Adv. Exp. Med. Biol.* 459 (1999) 121–143.
- S.B. Gelvin, Integration of *Agrobacterium* T-DNA into the plant genome, *Annu. Rev. Genet.* 51 (2017) 195–217.
- M. Ghislain, et al., Stacking three late blight resistance genes from wild species directly into African highland potato varieties confers complete field resistance to local blight races, *Plant Biotechnol. J.* 17 (2019) 1119–1129.
- M.N. Gonzalez, et al., Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 system, *Front. Plant Sci.* 10 (2019) 1649.
- M.N. González, et al., Comparative potato genome editing: *Agrobacterium tumefaciens*-mediated transformation and protoplasts transfection delivery of CRISPR/Cas9 components directed to *StPP2D* gene, *Plant Cell Tissue Organ Cult.* 145 (2021) 291–305.
- Z. Han, et al., Heritable epigenomic changes to the maize methylome resulting from tissue culture, *Genetics* 209 (2018) 983–995.
- D.J. Hannapel, P. Sharma, T. Lin, A.K. Banerjee, The multiple signals that control tuber formation, *Plant Physiol.* 174 (2017) 845–856.
- N. Hegde, S. Joshi, N. Soni, A.C. Kushalappa, The caffeoyl-CoA O-methyltransferase gene SNP replacement in Russet Burbank potato variety enhances late blight resistance through cell wall reinforcement, *Plant Cell Rep.* 40 (2021) 237–254.
- P. Hofvander, M. Andersson, C.T. Larsson, H. Larsson, Field performance and starch characteristics of high-amylose potatoes obtained by antisense gene targeting of two branching enzymes, *Plant Biotechnol. J.* 2 (2004) 311–320.
- H. Jia, V. Orbovic, N. Wang, CRISPR-LbCas12a-mediated modification of citrus, *Plant Biotechnol. J.* 17 (2019) 1928–1937.
- M. Jinek, et al., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, *Science* 337 (2012) 816–821.
- I.E. Johansen, et al., High efficacy full allelic CRISPR/Cas9 gene editing in tetraploid potato, *Sci. Rep.* 9 (2019) 17715.
- M.E. Karlsson, A.M. Leeman, I.M.E. Björck, A.C. Eliasson, Some physical and nutritional characteristics of genetically modified potatoes varying in amylose/amylopectin ratios, *Food Chem.* 100 (2007) 136–146.
- A. Karp, Somaclonal variation in potato, in: Y.P.S. Bajaj (Ed.), *Somaclonal Variation in Crop Improvement I*, Springer Berlin Heidelberg, 1990, pp. 379–399.
- T. Kazama, et al., Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing, *Nat. Plants* 5 (2019) 722–730.
- M.J. Keenan, et al., Role of resistant starch in improving gut health, adiposity, and insulin resistance, *Adv. Nutr.* 6 (2015) 198–205.
- N.P. Kieu, M. Lenman, E.S. Wang, B.L. Petersen, E. Andreasson, Mutations introduced in susceptibility genes through CRISPR/Cas9 genome editing confer increased late blight resistance in potatoes, *Sci. Rep.* 11 (2021) 4487.
- D. Kim, et al., Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells, *Nat. Biotechnol.* 34 (2016) 863–868.
- A. Kochevenko, L. Willmitzer, Chimeric RNA/DNA oligonucleotide-based site-specific modification of the tobacco acetolactate synthase gene, *Plant Physiol.* 132 (2003) 174–184.
- A. Kraak, Industrial applications of potato starch products, *Ind. Crops Prod.* 1 (1992) 107–112.
- K. Kubo, et al., Collaborative non-self recognition system in S-RNase-based self-incompatibility, *Science* 330 (2010) 796–799.
- H. Kusano, et al., Establishment of a modified CRISPR/Cas9 system with increased mutagenesis frequency using the translational enhancer dMac3 and multiple guide RNAs in potato, *Sci. Rep.* 8 (2018) 13753.
- C. LeBlanc, et al., Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress, *Plant J.* 93 (2018) 377–386.
- K. Lee, et al., Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize, *Plant Biotechnol. J.* 17 (2019) 362–372.
- M. Lee, R.L. Phillips, The chromosomal basis of somaclonal variation, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39 (1988) 413–437.
- B. Li, M. Carey, J.L. Workman, The role of chromatin during transcription, *Cell* 128 (2007) 707–719.
- G. Li, S. Sretenovic, E. Eisenstein, G. Coleman, Y. Qi, Highly efficient C-to-T and A-to-G base editing in a Populus hybrid, *Plant Biotechnol. J.* 19 (2021) 1086–1088.
- T. Li, et al., Domestication of wild tomato is accelerated by genome editing, *Nat. Biotechnol.* 36 (2018) 1160–1163.
- Z. Liang, et al., Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes, *Nat. Commun.* 8 (2017).
- G. Loebenstein, Control of sweet potato virus diseases, *Adv. Virus Res.* 91 (2015) 33–45.
- A. Luciola, et al., CRISPR-Cas9 targeting of the eIF4E1 gene extends the potato virus Y resistance spectrum of the *Solanum tuberosum* L. cv. Desiree, *Front. Microbiol.* 13 (2022), 873930.
- X. Ma, X. Zhang, H. Liu, Z. Li, Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9, *Nat. Plants* 6 (2020) 773–779.
- M.F. Maher, et al., Plant gene editing through de novo induction of meristems, *Nat. Biotechnol.* 38 (2020) 84–89.
- A. Malzahn, L. Lowder, Y. Qi, Plant genome editing with TALEN and CRISPR, *Cell Biosci.* 7 (2017) 21.
- A.A. Malzahn, et al., Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice, maize, and *Arabidopsis*, *BMC Biol.* 17 (2019) 9.
- M. Marcotrigiano, Origin of adventitious shoots regenerated from cultured tobacco leaf tissue, *Am. J. Bot.* 73 (1986) 1541–1547.
- H.W. Maurer, Starch in the Paper Industry, in: J. BeMiller, R. Whistler (Eds.), *Starch*, third ed., Academic Press, San Diego, 2009, pp. 657–713.
- A. Miyao, et al., Molecular spectrum of somaclonal variation in regenerated rice revealed by whole-genome sequencing, *Plant Cell Physiol.* 53 (2011) 256–264.
- K.A. Molla, S. Sretenovic, K.C. Bansal, Y.P. Qi, Precise plant genome editing using base editors and prime editors, *Nat. Plants* 7 (2021) 1166–1187.
- M. Nakayasu, et al., Generation of alpha-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the *St16DOX* gene, *Plant Physiol. Biochem.* 131 (2018) 70–77.
- I. Nakazato, et al., Targeted base editing in the mitochondrial genome of *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. U. S. A.* 119 (2022), e2121177119.
- V.K. Nandula, D.A. Giacomini, J.D. Ray, Resistance to acetolactate synthase inhibitors is due to a W 574 to L amino acid substitution in the *ALS* gene of redroot pigweed and tall waterhemp, *PLoS One* 15 (2020), e0235394.
- C. Navarro, et al., Control of flowering and storage organ formation in potato by *FLOWERING LOCUS T*, *Nature* 478 (2011) 119–122.
- A. Nicolai, et al., Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts, *J. Biotechnol.* 204 (2015) 17–24.
- A. Okuzaki, T. Shimizu, K. Kaku, K. Kawai, K. Toriyama, A novel mutated acetolactate synthase gene conferring specific resistance to pyrimidinyl carboxy herbicides in rice, *Plant Mol. Biol.* 64 (2007) 219–224.
- M. Otani, et al., Inhibition of the gene expression for granule-bound starch synthase I by RNA interference in sweet potato plants, *Plant Cell Rep.* 26 (2007) 1801–1807.
- C. Pan, et al., Boosting plant genome editing with a versatile CRISPR-Combo system, *Nat. Plants* 8 (2022) 513–525.

- C.T. Pan, S. Sretenovic, Y.P. Qi, CRISPR/dCas-mediated transcriptional and epigenetic regulation in plants, *Curr. Opin. Plant Biol.* 60 (2021).
- P.F. Perroud, et al., Prime Editing in the model plant *Physcomitrium patens* and its potential in the tetraploid potato, *Plant Sci.* 316 (2022), 111162.
- M. Pieczynski, et al., Down-regulation of CBP80 gene expression as a strategy to engineer a drought-tolerant potato, *Plant Biotechnol. J.* 11 (2013) 459–469.
- S. Poethig, Genetic mosaics and cell lineage analysis in plants, *Trends Genet.* 5 (1989) 273–277.
- E. Porcher, R. Lande, Loss of gametophytic self-incompatibility with evolution of inbreeding depression, *Evolution* 59 (2005) 46–60.
- C. Potato Genome Sequencing, et al., Genome sequence and analysis of the tuber crop potato, *Nature* 475 (2011) 189–195.
- H. Puchta, The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution, *J. Exp. Bot.* 56 (2005) 1–14.
- J. Quenouille, N. Vassilakos, B. Moury, Potato virus Y: a major crop pathogen that has provided major insights into the evolution of viral pathogenicity, *Mol. Plant Pathol.* 14 (2013) 439–452.
- L.B. Randall, et al., Genome- and transcriptome-wide off-target analyses of an improved cytosine base editor, *Plant Physiol.* 187 (2021) 73–87.
- Q. Ren, et al., Improved plant cytosine base editors with high editing activity, purity, and specificity, *Plant Biotechnol. J.* 19 (2021a) 2052–2068.
- Q. Ren, et al., PAM-less plant genome editing using a CRISPR–SpRY toolbox, *Nat. Plants* 7 (2021b) 25–33.
- D. Rodriguez-Leal, Z.H. Lemmon, J. Man, M.E. Bartlett, Z.B. Lippman, Engineering quantitative trait variation for crop improvement by genome editing, *Cell* 171 (2017) 470–480, e478.
- E. Sarka, V. Dvoracek, Waxy starch as a perspective raw material (a review), *Food Hydrocoll.* 69 (2017) 402–409.
- S. Sawai, et al., Sterol side chain reductase 2 is a key enzyme in the biosynthesis of cholesterol, the common precursor of toxic steroidal glycoalkaloids in potato, *Plant Cell* 26 (2014) 3763–3774.
- P. Schindele, H. Puchta, Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing, *Plant Biotechnol. J.* 18 (2020) 1118–1120.
- D. Schubeler, Function and information content of DNA methylation, *Nature* 517 (2015) 321–326.
- G.P. Schwall, et al., Production of very-high-amylose potato starch by inhibition of SBE A and B, *Nat. Biotechnol.* 18 (2000) 551–554.
- F. Sevestre, M. Facon, F. Wattebled, N. Szydlowski, Facilitating gene editing in potato: a single-nucleotide polymorphism (SNP) map of the *Solanum tuberosum* L. cv. Desiree genome, *Sci. Rep.* 10 (2020) 2045.
- Z. Shimatani, et al., Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion, *Nat. Biotechnol.* 35 (2017) 441–443.
- Y.P. Silva, A. Bernardi, R.L. Frozza, The role of short-chain fatty acids from gut microbiota in gut-brain communication, *Front. Endocrinol.* 11 (2020).
- J.R. Sowokinos, Biochemical and molecular control of cold-induced sweetening in potatoes, *Am. J. Potato Res.* 78 (2001) 221–236.
- H. Stroud, et al., Plants regenerated from tissue culture contain stable epigenome changes in rice, *eLife* 2 (2013), e00354.
- Y.H. Su, X.S. Zhang, Chapter two - the hormonal control of regeneration in plants, in: B. Galliot (Ed.), *Current Topics in Developmental Biology*, Academic Press, 2014, pp. 35–69.
- K. Sun, et al., Silencing of six susceptibility genes results in potato late blight resistance, *Transgenic Res.* 25 (2016) 731–742.
- S. Svitashv, C. Schwartz, B. Lenderts, J.K. Young, A. Mark, Cigan, Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes, *Nat. Commun.* 7 (2016) 13274.
- S. Takayama, A. Isogai, Self-incompatibility in plants, *Annu. Rev. Plant Biol.* 56 (2005) 467–489.
- A. Takeuchi, et al., Creation of a potato mutant lacking the starch branching enzyme gene *StSBE3* that was generated by genome editing using the CRISPR/dMac3-Cas9 system, *Plant Biotechnol.* 38 (2021) 345–353.
- D. Tang, et al., Genome evolution and diversity of wild and cultivated potatoes, *Nature* 606 (2022) 535–541.
- X. Tang, et al., A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants, *Nat. Plants* 3 (2017) 17018.
- X. Tang, et al., A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice, *Genome Biol.* 19 (2018) 84.
- F. Taranto, et al., Polyphenol oxidases in crops: biochemical, physiological and genetic aspects, *Int. J. Mol. Sci.* 18 (2017).
- S. Toinga-Villafuerte, M.I. Vales, J.M. Awika, K.S. Rathore, CRISPR/Cas9-mediated mutagenesis of the granule-bound starch synthase gene in the potato variety yukon gold to obtain amylose-free starch in tubers, *Int. J. Mol. Sci.* 23 (2022) 4640.
- A. Tuncel, et al., Cas9-mediated mutagenesis of potato starch-branching enzymes generates a range of tuber starch phenotypes, *Plant Biotechnol. J.* 17 (2019) 2259–2271.
- J. Van, Eck, et al., Enhancing beta-carotene content in potato by rna-mediated silencing of the beta-carotene hydroxylase gene, *Am. J. Potato Res.* 84 (2007) 331–342.
- F. Veillet, et al., The *Solanum tuberosum* *GBSSI* gene: a target for assessing gene and base editing in tetraploid potato, *Plant Cell Rep.* 38 (2019a) 1065–1080.
- F. Veillet, et al., Transgene-free genome editing in tomato and potato plants using *Agrobacterium*-mediated delivery of a CRISPR/Cas9 cytidine base editor, *Int. J. Mol. Sci.* 20 (2019b).
- F. Veillet, M.P. Kermarrec, L. Chauvin, J.E. Chauvin, F. Nogue, CRISPR-induced indels and base editing using the *Staphylococcus aureus* Cas9 in potato, *PLoS One* 15 (2020), e0235942.
- R.G. Visser, et al., Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs, *Mol. Gen. Genet.* 225 (1991) 289–296.
- H. Wang, et al., CRISPR/Cas9-based mutagenesis of starch biosynthetic genes in sweet potato (*Ipomoea batatas*) for the improvement of starch quality, *Int. J. Mol. Sci.* 20 (2019).
- M. Wang, Y. Mao, Y. Lu, X. Tao, J.K. Zhu, Multiplex gene editing in rice using the CRISPR–Cpf1 system, *Mol. Plant* 10 (2017) 1011–1013.
- S. Wang, et al., Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system, *Plant Cell Rep.* 34 (2015) 1473–1476.
- K. Watanabe, Potato genetics, genomics, and applications, *Breed. Sci.* 65 (2015) 53–68.
- T. Weiss, et al., Epigenetic features drastically impact CRISPR-Cas9 efficacy in plants, *Plant Physiol.*, (2022).
- R. Xu, et al., Generation of targeted mutant rice using a CRISPR–Cpf1 system, *Plant Biotechnol. J.* 15 (2017) 713–717.
- D. Yan, et al., High-efficiency and multiplex adenine base editing in plants using new TadA variants, *Mol. Plant* 14 (2021) 722–731.
- M. Ye, et al., Generation of self-compatible diploid potato by knockout of *S-RNase*, *Nat. Plants* 4 (2018) 651–654.
- Q. Yu, H.P. Han, M.M. Vila-Aiub, S.B. Powles, AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth, *J. Exp. Bot.* 61 (2010) 3925–3934.
- Y. Yu, et al., Targeting of SPCSV-*RNase3* via CRISPR-Cas13 confers resistance against sweet potato virus disease, *Mol. Plant Pathol.* 23 (2022) 104–117.
- S.C. Zeeman, J. Kossman, A.M. Smith, Starch: its metabolism, evolution, and biotechnological modification in plants, *Annu. Rev. Plant Biol.* 61 (2010) 209–234.
- B. Zetsche, et al., Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, *Cell* 163 (2015) 759–771.
- X. Zhan, et al., Generation of virus-resistant potato plants by RNA genome targeting, *Plant Biotechnol. J.* 17 (2019) 1814–1822.
- C. Zhang, et al., The genetic basis of inbreeding depression in potato, *Nat. Genet.* 51 (2019a) 374–378.
- X.H. Zhang, L.Y. Tee, X.G. Wang, Q.S. Huang, S.H. Yang, Off-target effects in CRISPR/Cas9-mediated genome engineering, *Mol. Ther. Nucleic Acids* 4 (2015), e264.
- Y. Zhang, et al., Expanding the scope of plant genome engineering with Cas12a orthologs and highly multiplexable editing systems, *Nat. Commun.* 12 (2021) 1944.
- Y. Zhang, et al., Highly efficient genome editing in plant protoplasts by ribonucleoprotein delivery of CRISPR-Cas12a nucleases, *Front. Genome Ed.* 4 (2022), 780238.
- Y.X. Zhang, A.A. Malzahn, S. Sretenovic, Y.P. Qi, The emerging and uncultivated potential of CRISPR technology in plant science, *Nat. Plants* 5 (2019b) 778–794.
- X. Zhao, et al., Amylose starch with no detectable branching developed through DNA-free CRISPR-Cas9 mediated mutagenesis of two starch branching enzymes in potato, *Sci. Rep.* 11 (2021) 4311.
- X. Zhao, M. Andersson, R. Andersson, Resistant starch and other dietary fiber components in tubers from a high-amylose potato, *Food Chem.* 251 (2018) 58–63.
- Z. Zheng, et al., Editing sterol side chain reductase 2 gene (*StSSR2*) via CRISPR/Cas9 reduces the total steroidal glycoalkaloids in potato, *All Life* 14 (2021) 401–413.
- X. Zhou, et al., StMYB44 negatively regulates phosphate transport by suppressing expression of *PHOSPHATE1* in potato, *J. Exp. Bot.* 68 (2017) 1265–1281.
- S. Zhu, Y. Li, J.H. Vossen, R.G. Visser, E. Jacobsen, Functional stacking of three resistance genes against *Phytophthora infestans* in potato, *Transgenic Res.* 21 (2012) 89–99.
- Y. Zong, et al., Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A, *Nat. Biotechnol.* 36 (2018) 950–953.