Smart Plant Breeding for Potato in the Post-genomics Era

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Sana Khalid, Riffat Siddique, and Allah Bakhsh

Abstract

Ceaseless development of plant breeding and genetic endeavors have resulted in accidental plant selection, successive harvest training plus urge of food and food item. The headway made toward this objective explained plant genome composition and prompted deciphering the sequence of full DNA of plant genomes controlling the whole plant life. Each crop improvement program is based on broad usage of wild germplasm and opening the genetic diversity repository. Potato (*Solanum tuberosum* L.) is considered as the most important non-grain vegetation worldwide. It is a significant staple food and has the potential to provide a lot of macro/micronutrients and vitamins when contrasted to other potential food crops, especially in many developing countries. These characters enable engineered potato to gain the scientific attention for nutrition improvement. Very few genes with their known functions have been reported, while the Potato Genome Sequencing Consortium has recognized many genes having unknown functions. Therefore, it is important to assign systematically the functions of expected genes in order to improve the potato cultivars by using different functional genomic techniques. Such loss-of-function and gain-of-function experimental techniques are helpful for producing the mutants with phenotypic variation. So, potato cultivars improve as "future feed" by generating the desired cultivar after revealing the unknown function of mutants. The commercial

S. Khalid
Department of Botany, Lahore College for Women University, Lahore, Pakistan

Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

R. Siddique
Department of Botany, Lahore College for Women University, Lahore, Pakistan

A. Bakhsh (✉)
Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

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deployment of engineered products has become a challenge due to administrative/moral limitations and consumer inclination. In this specific situation, new smart breeding technologies have been discussed to create sans transgene items in a more meticulous, expeditious, and viable way along with their advantages and limitations. Hence, this effect could significantly contribute to the genetic improvement of potato with reference to nutritional/food security.

Keywords

Genomic era · Potato · Conventional plant breeding · Smart plant breeding

13.1 Introduction

Potato (*Solanum tuberosum* L.) is autotetraploid and heterozygous, ranked as the third most important staple food next to wheat and rice, and originates from the Andean Mountains of South America (Zaheer and Akhtar 2016). Potato tubers are storage organ and adaptable, used in vegetative propagation. Currently, ~5000 varieties are cultivated in 125 countries worldwide, showing the global production to be >374 million tons (www.cipotato.org). Interestingly, developing countries are more prone to produce potatoes as compared to economically developed countries. In 2009, China was the leading potato-harvesting country producing 91.81 million tons trailed by India (50.19 million tons), collectively accounting for almost one-third of the potato production worldwide (www.fao.org/faostat). In nutritional point of view, starch in potato supports the growth of beneficial gut microflora after fermentation, acts as prebiotics, decreases insulin and glucose responses, and helps in small fatty acid chain production. In addition to carbohydrates, potatoes are also rich in proteins, fats, vitamins B6/C, potassium, magnesium, and dietary fibers (Zhao et al. 2018). The year 2008 was declared as the "International Year of the Potato" by the UN regarding its contribution to global food security. It is the most favorite edible and used in the paper/wood, textile, and pharmaceutical industries (Clasen et al. 2016; Fritsch et al. 2017). Potato cultivars are prone to many devastating pests and pathogens. Moreover, their germplasms are very limited, and they have weak genetic basis (Xu et al. 2011). So, the need of the hour is to establish novel genetic techniques for the successful breeding of potatoes by understanding the genetic traits. Likewise, nutritionally ironic potatoes can alleviate the global hidden hunger, but many biotic/abiotic factors cause hindrance in such inferences. In recent years, many genomic studies, *viz.*, transcriptome profiling for novel candidate genes, expression of genes involved in biotic-abiotic stress resistance, development of tubers, and starch metabolism, have been conducted (Jeevalatha et al. 2017; Singh et al. 2015; Tiwari et al. 2015; Siddappa et al. 2014; Ferreira et al. 2010). However, smart plant breeding techniques for potato production are required in the post-genomic era. For this purpose, many high-throughput and functional technologies have been implied by producing the functional mutants, either loss or gain of functions, to elucidate the relevant functional gene/s as shown in Table 13.1. Loss-

Table 13.1 Advantages and disadvantages of different functional post-genomic era techniques in potato

Post-genomic era techniques	Advantages	Disadvantages
Techniques involved in loss of functions: VIGS, RNAi, SIGS, TILLING, ZFNs, TALENs	1. Rapid	1. Off-target effects
	2. Heritable in RNAi, VIGS, TILLING	2. May require transformation
	3. Applicable in polyploid crops	3. Silencing is variable
	4. Targeted (RNAi, VIGS, SIGS, ZFNs, TALENs)	4. Require prior knowledge of sequence
	5. No PAM requirement	5. Effectiveness of SIGS is dependent on the dsRNA uptake by the pathogens
Mutagenesis and CRISPR	1. Heritable/ nonheritable	1. Screening is difficult, time consuming in mutagenesis
	2. May lead to complete knockout	2. PAM motif is required in CRISPR
	3. Capable of incorporating mutations at multiple sites	3. May obtain unintended effects if genome sequence
	4. CRISPR is used for precision genome engineering	4. Information is not available in both the cases
	5. May upregulate the expression of genes	
Techniques involved in loss of functions: activation tagging	1. Heritable	1. Require large number of transformants
	2. Dominant phenotypes	2. May produce complex phenotype
	3. Useful for terminated gene function identification	
	4. Gain-of-function phenotypes are easily identified	

of-function [(virus-induced gene silencing (VIGS), RNA interference (RNAi), spray-induced gene silencing (SIGS), TILLING] and gain-of-function techniques (activation tagging) have faced limitations that have led to the introduction of clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) that confers both gain of function and loss of function by enabling targeted insertion, replacement, or disruption of genes in plants. But this technique is poorly understood in clonally propagated polyploids like potato. Here, in this chapter, we provide an understanding of nine different smart functional genomic techniques

along with their pros and cons that lead to the introduction of improved and resistant varieties as shown in Table 13.1.

13.2 VIGS

For potato functional genomics, virus-induced gene silencing (VIGS) is a current and high-throughput technique that encompasses low cost. It can be explained by means of figuring out a lack of partticular phenotype of specific gene inside single generation only. Additionally, it can allow the large-scale selection for a particular evaluation in polyploidy plants. VIGS lets potato in the era of genotypically indistinguishable silenced plants, especially for those cultivars that are challenged to transform (Burch-Smith et al. 2004; Singh et al. 2018). So due to this targeted silencing, a solo potato plant is adequate to trail the phenotype, thus making VIGS an effective tool for deciphering the practical significance of genes (Becker and Lange 2010). In VIGS, a binary vector (modified viral genome devoid of pathogenicity-determined gene) is cloned with cDNA of targeted gene, multiple cloning sites (MCS), and CaMV35S promoter, followed by transfection in host plant via DNA bombardment, *Agrobacterium tumefaciens*-mediated transformation, or virus sap inoculation. After that, PTGS is activated due to degradation of dsRNA (plant Dicer-like enzyme) into homologous siRNA target gene that leads to loss of gene function (Voinnet 2001; Ramegowda et al. 2014).

Potato virus X (PVX) and TRV are the most appropriate VIGS-mediated silencing viral vectors in potatoes. Resistance/candidate (R) genes [RB in *S. bulbocastanum*; R1 and Rx in *S. tuberosum*] have been silenced and assessed functionally with VIGS system by obtaining the susceptible phenotypes (Brigneti et al. 2004; Faivre-Rampant et al. 2004). It is found that suberization-associated anionic peroxidase and lipoxygenase gene provide resistance to potato against *Phytophthora infestans* by using leaf detached assay and TRV vector (Du et al. 2013). In future, heat tolerance and tuber signaling in potatoes will be assessed by using VIGS technology (Tomar et al. 2021).

Nevertheless, VIGS has certain limitations; obtained phenotypes are not applicable to genetic engineering as they are nonheritable. Potatoes are tetraploid in nature, so it is impossible for the gene function to be completely knocked down. Moreover, production of functional protein and expression of phenotypes in silence plants could be achieved with less gene expression, and silencing level fluctuates between the growth conditions and construct-dependent experiments and potato varieties. However, many potato varieties are non-acquiescent for VIGS system for *in vivo* experiments (Burch-Smith et al. 2004; Gilchrist and Haughn 2010; Senthil-Kumar and Mysore 2011).

13.3 RNAi

Downregulation of genes by mRNA degradation is a natural RNA-mediated interference (RNAi) multistep process, also called PTGS, and depends on the entry of DNA construct and production of dsRNA complementary to target genes, which are later chopped into 21–25-nts-containing short fragment (siRNAs) overhangs by Dicer or DCL enzymes through 2 nts at 3′. siRNA contains sense (passenger) strand, which later due to cytoplasmic cellular events is broken, and other antisense (guide) strand that triggers RNA-induced silencing complex (RISC) that will now be part of siRNA-RISC complex, ultimately binding to targeted complementary mRNA. Another vital component of RISC complex, named Argonaute (AGO) endonuclease, hinders translation of target mRNA after chopping it (Majumdar et al. 2017). Another 21-nts-containing ssRNA fragment named microRNA (miRNA) is formed by DCL1 from particular hairpin precursor transcripts causing nonspecific PTGS of many mRNA, while siRNAs target single mRNA in a homology-dependent way, showing that both siRNAs and miRNAs are variable in biogenesis and effects (Lam et al. 2015; Moin et al. 2017). Artificial miRNA (amiRNA) and hpRNA are other RNA silencing means in plants for better understanding of gene functions and genetic engineering for crop improvements. The construct of hpRNA includes the sense and antisense sequences of the target gene mRNA in the form of inverted repeats (IR) lying in terminator and promoter regions of a plant.

A noncomplementary spacer region separates the inverted repeats to stabilize the construct in order to increase the silencing potential. Hairpin RNA structure is developed via complementary antisense and sense sequences of transcribed RNA and gets treated by DCL4 creating 21-nts-long siRNAs, later guiding to RISCs for target gene's inactivation (Guo et al. 2016). Development of amiRNA construct is done by the substitution of endogenous miRNA as well as miRNA* sequences part of miRNA precursor along cautiously developed amiRNA* and amiRNA sequences via PCR overlapping, while stem-loop structural preservation is required for the actual precursor of miRNA.

In amiRNA construct, miRNA is complementary to the targeted mRNA sequence, although the miRNA* strand's sequence is developed to preserve miRNA precursor's duplex (miRNA*:miRNA) structure (Guo et al. 2016). So, naturally found miRNA is used for gene silencing by amiRNAs. For appropriate AGO binding, designing of amiRNA requires cautious assortment of sequence (Guo et al. 2016).

Importance of RNAi is due to its heritable expression observed in T1 generation, dominant character turning potato transformant selection easy, sequence-specific nature which does not require a huge number of individuals for screening, and invention and validation of numerous gene functions accompanied by genetic engineering-based studies, which can never be denied. By using merely one construct, several genes can easily be silenced, so for a polyploidy crop like potato, gene discovery and its usefulness for various resistant cultivar-developing programs are increasing. Genetically mended cultivars have offered appropriate solutions to risk assessment issues and are commercially sanctioned (Arpaia et al. 2020) (Small 2007;

Eamens et al. 2008; McGinnis 2010). Alongside this technique, there has also been a formation of severity-varying phenotypes due to partial characteristics, functional loss, as well as analysis of vital genes whose suppression can cause extremely severe phenotypes or lethality (Small 2007; Eamens et al. 2008; McGinnis 2010).

Along with the advantages of RNAi, we have to also face a few of its disadvantages as in plant transformation, RNAi constructs act as transgenes, which require approval of GMO regulatory compliance policies for commercial use (Arpaia et al. 2020). However, due to variable gene copy numbers in genome, silencing is divergent leading to partial target silencing; sometimes, trials for screening of gene function turn unfavorable due to creation of modified phenotypes because of unwanted target's silencing (Wang et al. 2005; Small 2007; Gilchrist and Haughn 2010). Because of its double-stranded structure, exogenous RNA cannot silence a few genes; sometimes, due to sequence homology with some other genes, it can bind off target genes, thus making it less reliable and increasing regulatory concerns (Gebremichael et al. 2021).

The RNAi approaches in *S. tuberosum* have been extensively used for imparting resistance as well as recognizing genes accountable for defense mechanism against viruses, insects, and other pests which cause yield losses (Table 13.1). By considering its importance, highly resistant transgenic lines against three strains of potato virus Y (PVY) were developed by Missiou et al. in 2004 via hpRNA using 3′ end of viral coat protein-encoding gene. Bhaskar et al. in 2009 exhibited a double-agroinfiltration protocol for RNAi-dependent silencing construct that can be applied for the identification of such genes that contribute to the pathway for late blight resistance-facilitated gene RB. Eschen-Lippold et al. (2012) in another study have analyzed the utilization of processes like callose deposition and vesicle fusion for defense responses as secretions against *Phytophthora infestans* in potato.

With this, they developed transgenic lines exhibiting RNAi-based constructs, which were targeted against the potato's plasma membrane-localized syntaxin-related 1 gene (StSYR1), which led to *P. infestans* growth reduction in potatoes. This increase of late blight resistance was due to the downregulation of gene (syntaxin) expression in potatoes. Later cytological investigations exposed that infections produced by *P. infestans* were correlated with anomalous callose deposition and decreased papilla formation, representing secretory defense response due to syntaxins's contribution to potatoes. Furthermore, other fungi including oomycete plant pathogens synthesize various proteins like effectors into potato cells, modulating innate immunity of host as well as infection occurrence. Avr3a is a virulence gene against late blight-causing *P. infestans*. Silencing of Avr3a effector gene was utilized in popular cultivars of potato for development of resistant varieties by Sanju et al. (2015) via hpRNA construct. They used siRNA against merely Avr3a gene-conversed limited *P. infestans* resistance, so broader resistance creation requires targeting of cumulatively many effector genes.

In another study conducted in 2015 by Jahan et al., they indicated that they designed and introduced GFP marker-based hpRNA construct in potato. Work performed by them indicated that hp-PiGPB1 silences pathogenicity causing β-subunit of G-protein and retarded disease. Likewise, silencing of Avr3a effector

gene via amiRNA turned *P. infestans* into nonvirulent or imparted late blight resistance and caused its death (Thakur et al. 2015). Transgenic potato cultivars exhibiting nearly 100% resistance than severely infected untransformants were developed by Hameed et al. in 2017 using coding sequences of PVX, PVY, and potato virus S (PVS) protein coat via hairpin loop configuration to develop dsRNA expression cassette.

In another study, hairpin loop construct was developed by Tomar et al. (2018) to create resistance against the curling disease of apical leaf in potato, via PTGS of ToLCNDV-potato virus AC1 gene, which is required for replication. StSP6A, a gene in potato for tuberization signal SELF PRUNING 6A, was silenced to understand relatedness among flower bud development as well as tuberization; an observation noted by Plantenga et al. in 2019 indicated that flower bud development was inhibited by StSP6A signal occurrence and vice versa. Recently, ecdysone receptor (EcR)-encoding gene in Colorado potato beetle accountable for molting was significantly silenced using the RNAi construct harboring Agrobacterium transformation; when the larvae of beetle fed on these plants, tremendous reduction in EcR product supported dsRNA significance (Hussain et al. 2019).

Additionally, in transgenic plants, regulation of spatial and temporal gene silencing was observed, based on the expression of synthetic promoter (it encompasses synthetic motif and core promoter as well as ensures transgene expression specificity and strength and is considered ideal for engineering studies of potato) in dsRNAs by Liu and Stewart in 2016.

Li et al. (2013) utilized pCL synthetic promoter to get antisense expression of target gene (acid vacuolar invertase, StvacINV1) obtained from potato. In transgenic potato, sweetness caused by low temperature was inhibited due to the expression and particular activity regulation of target gene.

13.4 SIGS

Spray-induced gene silencing (SIGS) is an environment-friendly, reckless, and RNAi-based strategy to support the gene function in plants. As the name indicates, dsRNA/sRNA* related to pathogenic genes are sprayed on plant surfaces topically in order to confer the effective crop protection, leading to infection inhibition by silencing the target gene. This technique is especially designed for RNAi harboring pests and pathogens. After spraying, dsRNA either digested into sRNA by DCL of pathogen cell directly or spread systemically in plant and dice into sRNA by DCL (Wang and Jin 2017). In potatoes, SIGS is considered as a highly efficient method in controlling insecticidal action of CPB mesh gene (dsMESH) by spraying dsRNA that resulted in a high rate of beetle larval mortality in laboratory experiments (Petek et al. 2020). Recently, late blight of potatoes has been effectively reduced by topical spraying of targeted dsRNA to *Phytophthora infestans* genes causing sporulation and infection (Sundaresha et al. 2021).

SIGS method has many advantages due to its environment-effective nature. Moreover, it is administered and ethically legal, contrary to GMOs, and applicable

to those potato cultivars by identification and validation of responsible disease development genes, which are difficult for gene editing. For instance, dsRNA sprays are target sequence orientated and so are harmless to mutated strains of pathogens (Vetukuri et al. 2021). Nonetheless, dsRNA is less stable in the environment and difficult for uptake. Furthermore, length and duration of dsRNA sprays also affect the topical application (Dubrovina et al. 2019). Sundaresha et al. (2021) reported that irrespective of the difficult and expensive application of nanoparticle-based dsRNA sprays, it is helpful for the less progression of late blight in potatoes by enhancing and boosting the RNAi delivery and action correspondingly. Further research is required for efficient delivery of RNAi-based dsRNA for authentication of gene function to control the diseases in potatoes.

13.5 Mutagenesis

In an organism, various means as chemical, physical, and biological heritable alterations can be done, which are called mutagenesis; mostly, various breeding programs use two (insertional and induced) mutagenesis processes. According to Oladosu et al. in 2016, induced mutagenesis is activated by using various chemical compounds like ethyl nitrosourea, sodium azide, methyl methanesulfonate, and ethyl methane sulfonate, and X-rays, fast neutron, and gamma radiations. Single-nucleotide polymorphisms (SNPs) or one-nucleotide-based alteration is created more conveniently by chemicals. The effect of such radiations is variable; small deletion-based point mutations are created by gamma rays, while large deletions, chromosomal loss, and translocation are caused by fast neutron exposure. The random distribution of such induced mutations is turning gene function identification more easy in genome due to its high saturation in mutant population (Gilchrist and Haughn 2010).

According to Li et al. (2005), different characters like increased microtuber harvest bearing potato mutants were created by γ-irradiation, and improved textural and histological characters (Nayak et al. 2007), novel allele identification, and improved synthesis of starch were obtained using ethyl methanesulfonate (Muth et al. 2008).

In some other way, DNA randomly gets inserted as transposons; T-DNAs and retrotransposons in chromosomes are type of insertional mutagenesis, which can be used for endogenous gene's activation, new gene introduction, or specific gene function disruption and identification, as mutagen. Radhamony et al. (2005) and Tadele (2016) suggest that due to identified insertional element sequence, it is easy to restore disruptive gene via thermal asymmetric interlaced PCR (TAIL-PCR) technique. Various phenotypes as plant stature and morphology of leaf were acquired in *S. chacoense* (diploid wild tuber) Tnt1-carrying lines, during mutation screening caused by Tnt1 retrotransposon insertional mutagenesis created by Duangpan et al. in 2013. They also explained insertional mutation library development, which offers access to allele for gene function studies; gene contribution to

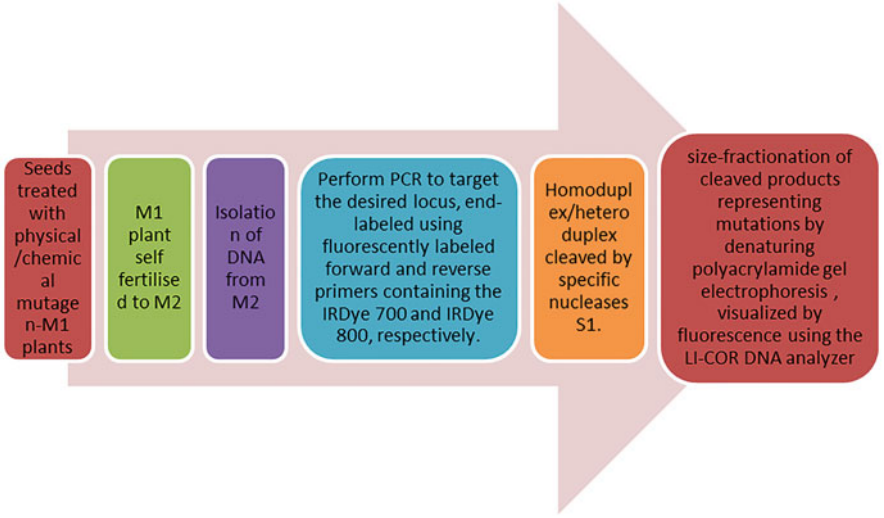
organ; biochemical, cellular, and tissue-based trait control; and tagging of every gene in potato genome (Duangpan et al. 2013; O'Malley et al. 2015).

Conclusively, these mutagenesis approaches are a valuable tool not only in being different from the tedious transformation technique but also in knocking out gene function and for low cost, innateness, easy PCR-based recognition of mutation, exploration of variation to improve traits, as well as correlation of biological function with gene sequence (Oladosu et al. 2016; Penna and Jain 2017; Kolakar et al. 2018). Along usefulness in some cases, as in a polyploid crop, the insertions may end up in one or some of the four homologous chromosomes, keeping other genes intact, which may lead to unexpected phenotypes. Though mutations are randomly dispersed in the whole genome, special care is necessary while handling different mutagens; also gene function analysis requires huge mutagenized population, making this process labor intensive and difficult (Kutscher and Shaham 2014).

13.6 TILLING

Targeting-induced local lesions in genomes (TILLING) is used to screen induced point mutants among chemically/physically mutagenized individuals using reverse genetics (Tadele 2016). It is the combination of traditional mutagenesis with high-throughput mutation discovery.

The detailed procedure is depicted in the following figure (McCallum et al. 2000; Elias et al. 2009; Fondong et al. 2016). Moreover, sequence-based TILLING and EcoTILLING are the extensions of TILLING to recognize INDEL (insertion/deletion) and SNPs. In potatoes, these techniques are employed for mutation identification (gain/loss of function, missense, and nonsense) and characterization of tetraploid germplasm to determine the functions of gene (Elias et al. 2009).



TILLING can be used for identifying those mutations unable to be detected from forward genetics and helps to use headstrong potato cultivars in contrary to transgenic varieties. However, its limitation includes the requirement of locus-specific amplification sequence and complex system to detect mutations because of polymerase slippage (Fondong et al. 2016; Tadele 2016).

13.7 Genome Editing

Genome editing is nifty and engaged for knockdown or overexpression of genes, assisted by protein-guided nucleases, viz., transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) systems. In genome editing, gene function is identified and studied after ensuring mutation in target sites by modifying and deleting the genes, thus inducing DSBs* which are repaired by homologous and nonhomologous recombination.

13.7.1 ZFNs

ZFN is a specific, efficient, and highly targeted technique, introduced with the discovery of IIS- and FokI-engineered endonucleases to separate the DNA-binding and cleavage domains. This is successfully useful in the gene modification of maize, Arabidopsis, and tobacco (Lloyd et al. 2005; Osakabe et al. 2010; Townsend et al. 2009; Shukla et al. 2009). ZFNs identify a unique ββα-configuration of 30-amino-acid Cys2-His2 ZF domain (Pavletich and Pabo 1991; Kim et al. 1996; Pabo et al. 2001) and manipulate the targeted gene by reducing nontarget cleavage as shown in Fig. 13.1.

13.7.2 TALENs

TALENs replace the ZFNs due to its rapid T-DNA integration and *Agrobacterium*-mediated delivery for targeted genes in potato cultivars that encode "acid invertase" and "starch branching enzymes" (Forsyth et al. 2016; Ma et al. 2017). TALENs are time effective and easy to design and generate in large numbers. The basis of TALENs is shown in Fig. 13.2. Recently, sterol side chain reductase 2 (SSR2) gene that controls the level of toxic metabolites in potatoes has been targeted by a very dynamic platinum TALEN expression vector construction system (Yasumoto et al. 2019). In order to reduce the anti-nutritional sterol glycoalkaloid production in potato tubers, four alleles of SSR2 have been knocked out by Sawai et al. (2014). Additionally, acetolactate synthase 1 (ALS1) gene knockout lines of tetraploid potatoes were developed by the transient expression of TALENs (Nicolia et al. 2015). TALENs also successfully enhance the processing traits and cold storage of "Ranger Russet" potato tubers by targeting vacuolar invertase (VInv) and having

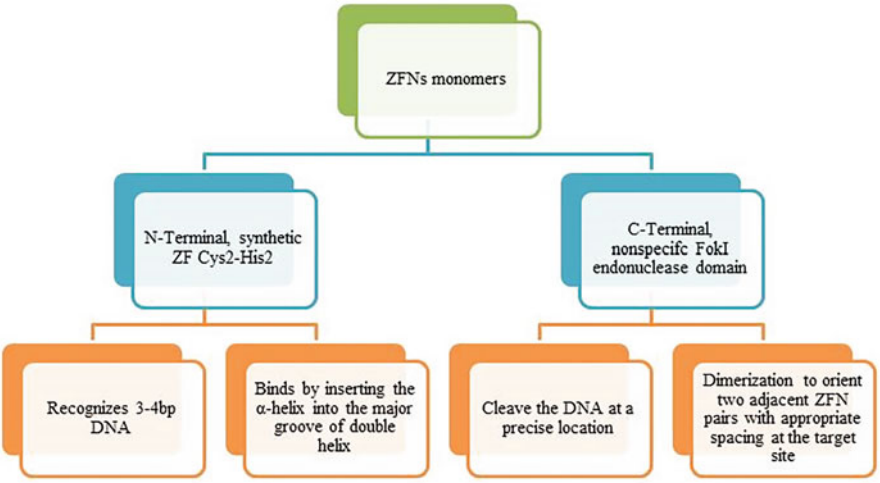


Fig. 13.1 Illustration of ZNFs. *DSBs: double-stranded breaks

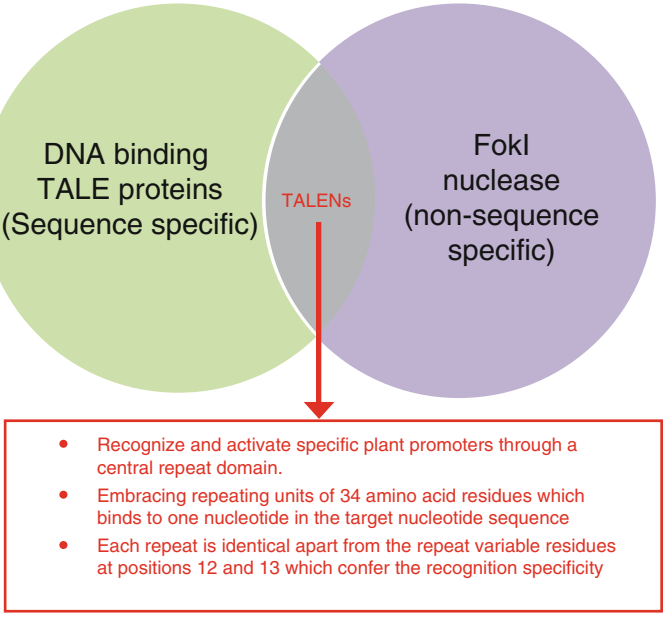


Fig. 13.2 Illustration of the basis of TALENs

reduced and undetectable level of acrylamide and reducing sugars, respectively (Clasen et al. 2016).

13.7.3 CRISPR

A specific, efficient, and easy substitution to TALENs and ZFN for target-specific genome editing induction, CRISPR/Cas, an RNA-dependent DNA cleaving system, represents a novel genome editing tool that has been newly developed. Its the characteristic of bacteria and archea to recognize the complementary sequences of invading phages, viruses and plasmids and cleave them (Wiedenheft et al. 2012; Malzahn et al. 2017). Based on the configuration of Cas genes and target, CRISPR/Cas is of three types, *viz*., type I, type II, and type III DNA editing performing types; except type I, the other two types can also target RNA. Type II complex has one Cas9 protein with two dissimilar RNA-based subunits; it is conspicuous and commonly utilized for gene editing of eukaryotes as here only one huge Cas9 protein is enough for identification and target DNA cleavage, while type I as well as type III contain one RNA subunit with several Cas proteins (Makarova et al. 2011; Unniyampurath et al. 2016).

CRISPR RNA (crRNA), Cas nuclease, as well as transactivating crRNA (tracrRNA) are three components constituting CRISPR/Cas of bacteria. A complex of tracrRNA and crRNA is developed via base pairing that stimulates and guides Cas9 toward targeted genes, which have 20 nucleotide sequences complementary to the crRNA. Cas9 has RuvC and HNH, two nuclease domains, and is an endonuclease introducing DSB into the targeted part of DNA. RuvC domain cleaves similar strand of the double-stranded DNA, while HNH cuts the complementary strand of the crRNA. Cas9 for efficient cleavage relies on protospacer adjacent motif (PAM) sequence downstream of targeted DNA having 5′-NGG-3′ sequence (Soda et al. 2018). In bacteria, CRISPR/Cas system can recognize among self and nonself sequences through interference by PAM recognition. This process led to the development of gene editing tool labeled as CRISPR/Cas system relying on crRNA target specificity and tracrRNA structural characteristics in a chimeric single-RNA guide (sgRNA), thereby reducing the system using Cas9 and sgRNA in place of three components (Doudna and Charpentier 2014). Target DNA sequence can easily be reprogrammed by altering 20 nts in sgRNA. Various mechanisms as homology-directed repair (HDR) as well as nonhomologous end joining (NHEJ) were commenced after DSB generation; mostly for gene knockout, NHEJ repairs DSB, creating gene INDELs and mismatches. HDR causes replacement of gene or knock-in of foreign DNA, when homology-bearing oligo template is near DSB sequence (Liu et al. 2017). By altering Cas proteins, CRISPR/Cas is further mended to make a more effective technique covering many genes. A more effective and simple gene editing alternative of CRISPR/Cas is CRISPRCpf1; here, Cpf1 is an RNA-guided nuclease making DSB specified via only required crRNA and introduces 5 bp cut, to find PAM, present at 5′ end protospacer (Alok et al. 2020; Zetsche et al. 2015). It is found to be remarkable for epigenetic modulation, multiplex gene targeting, base

editing, and transcription (Safari et al. 2019). Another variant is dCas9 (dead or catalytically inactive Cas9), which can strongly and specifically attach to the target via sgRNA guidance. Amendment of the action of target sequence is being done by the fusion of transcription activators and repressors to CRISPR/dCas9 through transcription increase or inhibition (Adli 2018). Another form, dimeric RNA-guided FokI nucleases (RFNs: effective gene editing needs dimerization of RFNs), depends on the fusion of FokI nuclease domain and inactive dCas9 (Khatodia et al. 2016). Similarly, there is another form which is independent of foreign DNA and directly transfers targeting sgRNA- and Cas9-based ribonucleoprotein (RNP) complex by biolistic and transfection method, useful in transgene and marker-free plants (Soda et al. 2018). Recently developed CRISPR-based system after Cas9 fusion with reverse transcriptase (RT; CRISPR/dCas9 H840A) was named prime editing based on new sequence insertion without DNA template and search-and-replace target editing form (Anzalone et al. 2019). It is important for functional genomics; relying on its ability to intrude deletions, 12 types of base substitution and insertion and being independent of DSB construction along need of donor template DNA, many traits of interest conferred via point mutation keeping intact gene loss of function led to accurate editing (Anzalone et al. 2019).

Butler et al. (2015) and Wang et al. (2015) described the usefulness of CRISPR/Cas for targeting StIAA2 gene encoding for Aux/IAA protein as well as ALS1 gene in various potato cultivars. In order to improve starch quality of tetraploid tuber, all four alleles of granule-bound starch synthase (GBSS) were knocked out using CRISPR/Cas9 by Andersson et al. (2017). In 2018, they again created amylopectin starch potatoes after inhibition of amylose formation via CRISPR/Cas9 RNP by knocking out amylose-producing GBSS enzyme. Moreover, GBSS gene was further exploited by Johansen et al. (2019) at protoplast level for editing efficiencies by substituting endogenous U6 promotor in potato with U6 promoter of *Arabidopsis* thus to drive the expression of CRISPR component. In another example, Ye et al. (2018) previously synthesized self-compatible diploid potatoes using CRISPR/Cas9 technique, when self-incompatibility causing Stylar ribonuclease S-RNase gene was knocked out.

After this, González et al. (2020) demonstrated the use of CRISPR/Cas9 for the polyphenol oxidase (PPO) mutation induction, which converts phenolic substrates to quinones responsible for dark-colored potato's precipitation by targeting StPPO2 gene, which results in less PPO activity in potato leading to browning reduction. In a very recent study by Kieu et al. (2021), loss of gene function-mediated CRISPR/Cas9 system was described; three genes StDMR6-1, StCHL1, and StDND1 were found accountable for late blight susceptibility, representing a pavement for novel resistant potato cultivar breeding. Likewise, prime editing via transversion and transition mutations was also utilized in potato's ALS1 gene, which encodes enzyme for branched-chain amino acid biosynthetic pathway (Veillet et al. 2020).

Lots of studies have shown evidences for the usefulness of CRISPR/Cas for potato's genome editing, due to its fast, efficient, and easy handling and being a potent source of multiple gene editing through one transformation, in addition to targeting methylated DNA and creating stable mutation for next generation (Cong

et al. 2013; Gaj et al. 2013; Xiong et al. 2015; Feng et al. 2014). Additionally, chances of off-target effects need genome scanning for mutation detection at sites where sequence matches to the gRNA target sequence that could be hard to get detected (Boettcher and McManus 2015; Unniyampurath et al. 2016; Malzahn et al. 2017). Significantly, gRNAs, due to potatoes' high heterozygosity, are required to be selected from the gene's conservative regions. So, editing creates biallelic, homozygous, heterozygous, and chimeric plants; homozygous state is more required but it is difficult to have in potato because of polyploidy (Fizikova et al. 2021).

13.8 Activation Tagging

For gene function analysis, activation tagging is the prevailing method used for screening of gene function's mutant loss in some plants by providing gain-of-function mutants. Hayashi et al. (1992) indicated the use of first T-DNA based on CaMV 35S gene's multimerized transcriptional enhancers (four tandem copies) inserted in T-DNA, which enhances either side flanking region expression after insertion into plant genome. Kakimoto (1996) identified cytokinin signaling pathway-based genes in the tissue culture of Arabidopsis. Weigel et al. (2000) described that huge, transformed plants conferring resistance against herbicide glyphosate or kanamycin were developed via such novel vectors. Various genes controlling special traits in important crops like tomato, barley, petunia, poplar, and Arabidopsis can be characterized (Weigel et al. 2000; Zubko et al. 2002; Mathews et al. 2003; Aylife et al. 2007; Busov et al. 2011). Regan et al. (2006) described that in Canadian Potato Genome Project, activation-lagged potato lines were developed and screened for tuber health and quality-based traits. In another study on chromosome 12, flanking genes and T-DNA insertion were used to observe cytosine methylation in mutant, revertant, and wild plants. Similarly AT615 (potato-activation tagged mutant) was characterized that differentially expressed 1632 genes in wild and mutant types (Aulakh et al. 2014, 2015). Currently used activation technology has turned out to be very useful for possible T1 generation dominant phenotype screening as well as redundant genes, which require only single allele to be activated to express phenotypically. Use of 35S enhancers rather than constitutive promoters could avoid ectopic expression, and gain-of-function screens can easily be recognized for further exploitation as compared to conventional screening.

For functional analysis of whole genes, a complete set of tagged mutants is difficult to get (Tomoko et al. 2010); expression of many genes simultaneously makes gene saturation difficult due to complex phenotypes, and its dependency on laborious transformation protocol hinders mutant line creation (Ichikawa et al. 2003).

13.9 Conclusion and Future Prospects

Potato genome is quite unknown in spite of its major role in global food security. Consequently, knowing the biological functions of candidate gene of potato genome has now become a big challenge. In order to address the issues related to hidden hunger and introduction of new biotic/abiotic stress-resistant varieties, many novel functional genomic techniques are coming into being to develop new breeding programs. Comparative phenotypic analysis of wild type and mutant is a key to identify the function of genes. Many techniques with their advantages and disadvantages have been discussed in this chapter. Among them, VIGS and RNAi are the cost-effective techniques used to identify the unknown function of genes by creating the mutants of targeted genes. However, sequence-based off-target effects are still puzzling for RNAi in spite of its ability to silence multi-targeted genes. This problem could be curtailed by using *in silico* tools for designing the construct. In SIGS, RNA is directly taken up by pathogens, but more optimized methods and delivery vectors are required for a range of pathogens. Complete gene knockdown is achieved by mutagenesis, but this technique is hard and time consuming due to the screening of large mutagenized population for succeeding the genome saturation. TILLING requires background information for locus-specific sequence amplification. Irrespective of these, ZFNs, TALENs, and CRISPR are genome editing techniques and are efficient, cost effective, and specific for knockout potato-targeted genes. Ever since potato has been characterized with genome heterozygosity and tetraploidy, avoidance of off-targets during gRNA selection has been very difficult. Many gain-of-function mutations could be achieved by activation tagging that could be recognized easily besides redundant gene analysis. Therefore, abovementioned smart plant breeding techniques after the sequencing era have the potential to breed resistant, healthy, and nutrition-rich potato cultivars.

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