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REVIEW

Use of TALEs and TALEN Technology for Genetic Improvement of Plants

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Abstract Genome editing with engineered nucleases has become a powerful tool of targeted genome modifications providing unprecedented control over animal and plant genetic material for precise, robust and highly specific genome engineering. Precise genome editing has been a long standing goal in the field of biology which has been achieved with the help of engineered nucleases like zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated system. These engineered nucleases consist of a binding and a nuclease domain which are generally used in the form of a pair. The binding domain binds specifically to a DNA sequence whilst the nuclease domain creates double-strand breaks (DSBs) which are further used for non-homologous end joining or homologous recombination repair. Creation of DSBs is the principle of this technology which can be further used for gene addition, deletion and modification in the targeted DNA. Besides nuclease activity, TALE (transcription activator-like effector) proteins have also been used along with other effector domains for different purposes like gene activation, gene repression, epigenetic modifications, etc. The use of TALEs and TALENs for precise genome modifications of plants is now a common practice. So far, tens of crop plants have been modified using engineered nucleases like rice, wheat, tomato, potato, tobacco, maize, barley, cotton, etc. The TALE and TALEN technology is being used for development of biotic and abiotic stress-resistant plants as well as yield and quality improvement. In this article, we will briefly review and discuss TALEs and TALENs, their discovery, binding specificity, designing, functional domains, delivery and use for genome editing specifically in plants.

Keywords Targeted genome engineering · Engineered nucleases · TALE and TALEN technology

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Introduction

Precise genetic modifications have been one of the fundamental goals of scientists working in the fields of biotechnology, gene therapy, drug development, molecular breeding and synthetic biology. Engineered nucleases are powerful reagents for creating targeted modifications to genomes in vivo (Bogdanove and Voytas 2011). Precise genome modifications with engineered nucleases can provide a platform to address basic biological questions and to improve biotechnology (Voytas 2013). To date, different kinds of engineered nucleases have been used for precise genome modifications: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas) systems, etc. Scientists have been using engineered nucleases for the last two decades to harness the repair machinery of the cell by creating double-strand breaks (DSBs) in DNA sequences at specific sites (Kim et al. 1996; Durai et al. 2005). Binding of a reprogrammable protein to the desired sequence of DNA and simultaneous cleavage is a prerequisite to generate DSBs (Nakatsukasa et al. 2005).

TALEN technology offers the opening up of a new, swifter and excellent route for crop improvement. TALENs have rapidly emerged as an alternative to ZFNs, by introducing

targeted DSBs for genome editing. TALENs are similar to ZFNs, comprising a nonspecific *FokI* nuclease domain fused to a customizable DNA-binding domain. This DNA-binding domain is composed of highly conserved repeats derived from transcription activator-like effector proteins (TALEs) secreted by *Xanthomonas* spp. to alter gene transcription in host plant cells (Boch and Bonas 2010).

TALENs enable efficient and specific genetic modifications via the induction of a DSB in a specific genomic target sequence, followed by non-homologous end joining (NHEJ) (Moehle et al. 2007) or homology-directed repair (HDR) (Rémy et al. 2010). NHEJ is a rapid and efficient DSB repair mechanism which involves a simple ligation of the two DNA ends that result from a DSB (Lieber 2010). The implications of this technology for crop improvement include the ability to remove unwanted marker genes after transgene integration, which may have important regulatory benefits. Other applications of the technology include the deletion of large regions of highly repetitive DNA and the generation of marker-free plants. For engineering crops to cope with biotic constraints, prior knowledge of gene function and regulation is vital (Liu et al. 2013).

Ever since the emergence of engineered nucleases, the field of genetic engineering has been revolutionized. TALENs are becoming powerful tools in gene targeting, because they produce site-specific results and they have more than 10 % gene-modification efficiencies. TALENs can represent a reliable and robust method for genome targeting addition, deletion and modifications. This paper will give a brief review of TALEN technology, and also look at the basic knowledge about TALEs, their history and specificity.

Tale of TALEs

Initially, a protein, AvrBs3, was discovered in pepper (Bonas et al. 1989), which was later used as a scaffold for DNA binding and gene activation in tomato (Morbitzer et al. 2011b) and other organisms. Hopkins et al. (1992) was the first to derive a family of avirulence genes from *Xanthomonas oryzae* pv. Oryzae. Later, this distinct class of plant pathogenic proteins was named TALEs. These plant pathogenic proteins were also observed in pepper (Römer et al. 2007) and tomato (Conrads-Strauch et al. 1993) which have a mysterious but tremendous function of modulating host gene expression. TALE proteins facilitate bacterial survival and colonization by regulation of host gene expression (Boch et al. 2009; Moscou and Bogdanove 2009). These are type III secretion system-dependent proteins which act like transcription activators, localize in the nucleus and bind double-stranded DNA in the promoter regions of host genes to modulate host gene expression making plants susceptible to disease (Yang et al. 2000; Büttner and Bonas 2002; Römer et al. 2009; White et al. 2009; Gan et al. 2011; Li et al. 2012). The proteins have been purified and deciphered for their DNA binding specificity (Boch et al. 2009; Boch and Bonas 2010; Bogdanove et al. 2010; Yang et al. 2014; Morbitzer et al. 2011b). TALE proteins are type III secretion system-based proteins which are injected into infected plant cells by pathogens of the genus *Xanthomonas* (Bogdanove et al. 2010; Scholze and Boch 2011). Inside the plant cell, they enter the nucleus, bind to effector-specific promoter sequences, and activate the expression of individual plant genes, which can either benefit the bacterium or trigger host defense systems (Kay et al. 2007; Römer et al. 2007). TALE proteins are typically composed of three domains: an N-terminal translocation domain, a central repeat domain that specifically binds to a particular DNA sequence, and a C-terminal transcriptional activation domain. These factors are critical for the recognition of and binding to the target DNA in the host genome and for the activation of the expression of genes necessary for pathogen establishment. The central repeat domain that mediates DNA binding to the target DNA comprises a tandem array of 15.5–19.5 single repeats, each one consisting of 34 highly conserved amino acids. The last module generally contains only 20 amino acids (Scholze and Boch 2010) and is therefore referred to as a 'half-repeat' (Boch and Bonas 2010). TALEs and/or TALEs-like effectors have also been discovered in the pathogenic bacteria *Ralstonia solanacearum* and *Burkholderia rhizoxinica* which have the same modularity and functioning as the previous TALE proteins (Salanoubat et al. 2002; Heuer et al. 2007; Li et al. 2013; de Lange et al. 2014).

DNA Binding Specificity of TALE Proteins

The bewildering specific DNA binding property of TALE proteins was unraveled by Boch's team (Boch et al. 2009; Boch and Bonas 2010). They demonstrated that the DNA recognition by TAL effectors is mediated by tandem repeats, each 33–35 residues in length which specify nucleotides via unique amino acids. The DNA binding specificity of each TALE repeat unit is essentially driven by a polymorphism at positions 12 and 13 within the module (Deng et al. 2012; Mak et al. 2012). The amino acids at positions 12 and 13 are called repeat variable di-residues (RVDs). RVDs dictate the specificity of the corresponding repeat to a single nucleotide, hence establishing a simple 1:1 code for protein-to-DNA interaction (Boch and Bonas 2010; Moscou and Bogdanove 2009) (Fig. 1). More than 20 unique RVDs sequences have been observed in TAL effectors, but just 7, HD, NG, NI, NN, NS, "N*" (in this RVD, the asterisk denotes a missing residue at 13) and

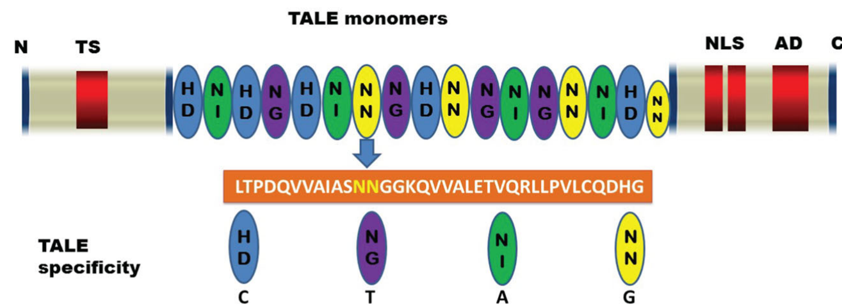

Fig. 1 TALE structure and DNA recognition code. The TALE protein is composed of TALE monomers, N-terminal, C-terminal, *NLS* (nuclear localization signal), *AD* (activation domain), *TS* (T3S signal). TALE monomers are tandem repeats; each repeat having 34–35 amino acids. Each repeat binds to one DNA base on the bases of its RVDs (amino acids at positions 12 and 13) specificity. Each TALE monomer is denoted by the RVDs. There are four RVDs (NN, NG, NI and HD) which bind the G, T, A and C DNA bases, respectively

HG, account for nearly 90 % of all repeats (Boch and Bonas 2010) and, respectively, specify C, T, A, G/A, A/C/T/G, C/T and T (Boch et al. 2009; Moscou and Bogdanove 2009). NH is a rare RVD found in TAL effectors, it uniquely specifies the G (guanine) base of the DNA (Streubel et al. 2012; Cong et al. 2012). It has been observed that the 12th residue of RVDs is N, most commonly, or H. The degeneracy of the code is due to the 13th residual RVD. The NN RVD has 50 % affinity for G/A, so if there is a difference of this base in consensus sequences, NN can be used instead of NK or NH which specifically bind a guanine (G). On the bases of this flexibility, we are targeting the consensus sequence of five viral strains with a single TALEN pair (Khan et al., unpublished data).

These relationships enable the prediction of targets for existing TAL effectors, and the engineering of artificial TAL effectors that bind DNA sequences of choice. Consequently, TAL effectors have received much attention as DNA targeting tools (Bogdanove and Voytas 2011). The modular nature of the DNA binding domain is unique throughout all kingdoms of life.The ‘Golden Gate’ cloning strategy can be used to assemble RVDs with different specificities (Engler et al. 2008; Cermak et al. 2011) in order to target a particular user-defined DNA sequence. Context-dependent effects (between repeating units) as observed with zinc fingers (Cathomen and Joung 2008) have not so far been reported, and a systematic study addressing this aspect of TAL-effectors is still lacking. The designing of customized TALEs to target a given sequence in a genome is facilitated by this particular feature. Owing to the interaction between the N-terminal domain and thymine (Mak et al. 2012), all target sequences are preceded by thymine (5′ T) at the zero position (Miller et al. 2011; Mussolino et al. 2011; Zhang et al. 2011). This could be a possible constraint in designing a TALE for a particular DNA sequence, but the TALE domains with different N-terminal specificities have also been isolated and characterized (Lamb et al. 2013). So, virtually, TALE proteins can be tailored on the basis of their modular nature with different effectors/functional domains to target any DNA sequence for desirable modifications (Fig. 2).

Design and Assembly of TALEs and TALENs

Because designing of TALEs is much easier than zinc fingers and meganuleases, they have been designed and assembled more frequently (Zhang et al. 2013a, b; Ding et al. 2013). Different softwares have been developed for designing and assembly prediction of TALEs and TALENs, but we discuss here the most prominent and useful. These programs are either freely available online or downloadable for off-net use. TALEs and TALEN monomers responsible for binding to specific DNA base can be predicted manually for a short sequence but for large-scale screening and other relevant analysis, the use of in silico tools has become common.

TAL Effector Nucleotide Targeter 2.0

A number of web tools are provided by Cornell University, U S A, for TAL repeat array designing. These programs are freely available online (Doyle et al. 2012) and facilitate in finding TAL repeat arrays to a particular DNA target. The DNA sequence to be analyzed must be provided in FASTA format. Moreover, user-defined ranges for a number of TAL repeats can be adjusted. For guanine-specific RVD selection, NN or NH can be used. Furthermore, the RVDs are designed according to the Golden Gate method. Programs including old and new versions are: TALEN Targeter (design arrays for TALENs to target particular DNA sequence), and TAL Effector Targeter (design a single TAL effector to target specific DNA sequence). A newer, updated version of single TALE Targeter, Target Finder, identifies the best-scoring sites in a DNA sequence for a specified RVD sequence. This new tool combines the

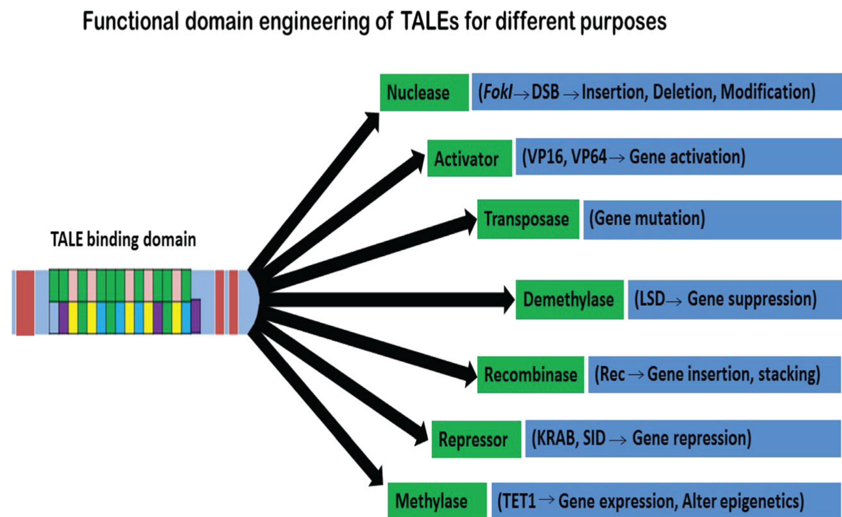

Fig. 2 Fusion of different effector domains with TALEs. The TALE protein can be engineered and fused with different effector/functional domains for targeted genome modifications. Different functional domains are shown which can be fused with the TALE binding domain for DSBs creation, gene activation, gene repression, gene insertion, gene mutation, gene stacking and epigenetic modifications

functions of the older TAL Effector Site Finder and Off-target Site Finder into one tool. Paired Target Finder, identifies the best-scoring sites in a DNA sequence for a pair of RVD sequences. These tools can be accessed at [https://tale-nt.cac.cornell.edu/].

E-TALEN

E-TALEN is also a web-based program used to design TALEN to target various DNA sequences (Heigwer et al. 2013). It can be used to target from single to multiple TALEN target sites (up to 50 target genes). This software can also be used to evaluate existing TALEN designs. E-TALEN has been provided as an easy-to-use web server which does not require any prior knowledge about the sequence or gene to be analyzed for TALEN design. E-TALEN can be accessed at [http://www.e-talen.org/E-TALEN/].

Galaxy TAL Effectors

Galaxy is an online server of Martin Luther University, Halle-Wittenberg, Germany. This server provides different tools for TALEs and TALENs along with other informative and useful programs such as the free public server [http://galaxy2.informatik.uni-halle.de:8976/]. Different programs for TALEs and TALENs prediction and sequence analysis provided by the Galaxy server are TALgetter, TALgetterlong and TALENoffer. TALgetter is used to predict TAL binding sites in a given genome sequence with respect to TAL RVDs (Grau et al. 2013). TALgetterlong is a variant of TALgetter which is designed to analyze large sequences. TALENoffer is used to predict off-targets of TALENs (Grau et al. 2013).

Mojo Hand

Mojo Hand is another web-based program used for the prediction of TALEs and TALENs which further aid in the design and construction of TALEs and TALENs. The tool was proposed by Neff et al. (2013) and predicts TALEs and TALEN design according to the Golden Gate assembly method. Moreover, it also provides restriction site recognition in the spacer region between binding sites of TAL monomers which is further used to analyze DSB results. The software works in a very comprehensive and simple manner. It also facilitates auto-downloading of DNA sequences from the National Center for Biotechnology Information (NCBI). Mojo Hand can be accessed at [www.talendesign.org].

idTALE

idTALE was developed by King Abdullah University of Science and Technology, Saudi Arabia, in 2013 to facilitate TAL designing and construction and to check out off-targets in model organisms (Li et al. 2013). TALE-TF (Transcription Factor) and TALENs binding sites can be predicted easily by using idTALE. It is also useful for genome-wide TAL binding sites screening because there is no restriction on sequence length as in the case of TALE-NT (5000 bp). It provides results for heterodimeric as well as homodimeric TALENs. It also benefits the users with a screening facility of the same TALEN binding sites in other organisms. idTALE can be accessed at [http://idtale.kaust.edu.sa].

SAPTA

Scoring Algorithm for Predicting TALEN Activity (SAPTA) is an online TALENs design tool (Lin et al. 2014) provided by Bao's laboratory (The Laboratory for Biomedical Engineering

and Nanomedicine, Rice University, Texas, U S A). SAPTA also uses NK RVD for more specificity and targeting efficiency. Lin et al. (2014) evaluated existing TALEN design guidelines and quantitatively tested 205 TALENs. It was claimed that the existing guidelines are inadequate and that the TALEN designed through SAPTA increased intracellular monomer activity by 3-fold and 39 % gene modification frequency. SAPTA is first and foremost a tool based on quantitative and experimental validation of TALEN target sites with high cleavage activity. It provides a list of TALENs for a target site with scoring and ranking. SAPTA is accessible at [http://baolab.bme.gatech.edu/Research/BioinformaticTools/ TAL_targeter.html].

CHOPCHOP

CHOPCHOP is provided by Montague et al. (2014). It is the latest web tool for the prediction of binding sites for TALEN and CRISPR/Cas9. Input can be provided as a gene name or sequence, and a TALEN or CRISPR/Cas9 target site can be determined from a growing list of organisms. CHOPCHOP also displays restriction sites at the target locus and allows restricting the search to restriction sites of enzymes from a particular company. The spacer length can be adjusted to be different from the default (14–20 bp). Off-target searching with number of mismatches is also facilitated by scoring. According to assembly protocol, NH RVD in place of NN RVD for guanine can be chosen, as NH is more specific than NN (Cong et al. 2012; Streubel et al. 2012). CHOPCHOP also integrates primer design with a TALEN target site. CHOPCHOP can be accessed at [https://chopchop.rc.fas.harvard.edu/].

ZiFiT Targeter

ZiFiT (Zinc Finger Targeter) was initially developed for the prediction of ZFN target sites by the Zinc Finger Consortium (Sander et al. 2010). Later, it has been updated for TALE, TALENs and CRISPR target site prediction and design. Now, ZiFiT is providing web-based free services for ZFN, TALE, TALENs and CRISPR/Cas9 design in a very efficient and user-friendly manner. ZiFiT provides target design according to Addgene TALEN-encoding plasmid assembly. Moreover, in ZiFiT Targeter version 4.2, tools for designing ZF, TALEs and CRISPR proteins, nucleases and nickases according to context-dependent assembly (CoDA), oligomerized pool engineering (OPEN), restriction enzyme and ligation (REAL), REAL-Fast and ligation-based automatable solid-phase high-throughput (FLASH) assemblies have been provided. ZiFiT targeter can be accessed at [http://zifit.partners.org].

EENs (Engineered EndoNucleases)

Artificial endonucleases are designed to target desirable DNA sequences with TALENs, ZFNs and CRISPR/Cas9 systems. The most frequently used EENs are TALENs and ZFNs. EENdb (Engineered EndoNucleases database) is a database and knowledge base of reported TALENs, ZFNs and CRISPR/Cas9 systems targeting organisms. EENs can be accessed at [http://eendb.zfgenetics.org/].

TALEN*designer*

TALEN*designer* is a web-based tool which is used to find TALEN binding sites. TALEN*designer* genome browser provides convenient access to TALEN target sites in the coding region of the mouse, rat, and the human genome. For example, in the mouse genome, 7.27 million identified target sites are available. Different sequences can be scanned for binding and cleavage sites, while sequences can also be predicted by giving RVDs from the database which can be used as tailor-made. TALEN*designer* can be accessed at [http://www.talen-design.de/index_talen.html].

Assembly of TAL Effectors and TALENs

Golden Gate Cloning

In this method of TALEs and TALENs assembly/construction, type IIS restriction enzymes are used to create several sticky ends. It is a vector-based method in which multiple overhangs are created by restriction enzymes that are used for specific ligation. It is also a restriction-/ligation-based method in which restriction and ligation are performed in a single step and single pot. It requires 1 week for constructing TALEs and TALENs, and is a quick, specific and highly efficient method of TALEN assembly. It needs sequencing to confirm the correct TALE. This method has been developed by different scientists (Morbitzer et al. 2011a, b; Li et al. 2011; Geissler et al. 2011; Weber et al. 2011; Cermak et al. 2011). The Golden Gate assembly protocol provided primarily by Cermak et al. (2011) is very comprehensive and cost-effective, being a three-step process in which a maximum 10 RVDs can be assembled in one reaction. Sakuma et al. (2013) provided a more robust protocol using Golden Gate cloning vectors of Bogdanove and Voytas (2011) namely Golden Gate TALEN and TAL Effector Kit (Addgene) along with their vector which excluded the use of destination vectors of pFUS A, A30A and A30B. The success rate increased up to 100 % compared to 10 % in the case of using six-module assembly instead of ten module-assemblies (Sakuma et al. 2013). A limitation of the protocol of Weber et al. (2011) is that it is limited to assemble up to 17 repeats of TALEs. So, it is not

flexible for building TALEs for multiple lengths of DNA bases.

Golden Gate PCR-Based Assembly

This method of TALE and TALEN construction is a bit different from the simple Golden Gate method because it is PCR-based, while the simple method is vector-based. It is quicker than the conventional Golden Gate method. It needs some ready-made plasmids and primers (Zhang et al. 2011; Sanjana et al. 2012). One basic limitation of this method is that a fixed length (18.5) of TALEs can be produced, so it is less flexible for the construction of multiple lengths of TALE. Moreover, the use of PCR is another prerequisite for this assembly method Tables

REAL/REAL-Fast

The restriction enzyme and ligation (REAL) method was developed by Joung's laboratory, Massachusetts General Hospital, U S A (http://www.jounglab.org/) and provided as "TAL Effector Engineering Reagents". Although this method of TALE assembly is very precise, it is costly and time-consuming because it was based on conventional restriction and ligation. ZiFiT free online software (http://zifit.partners.org/ZiFiT/Disclaimer.aspx) is used to design the TALE repeat array for the REAL/REAL-Fast TALE and TALEN construction methods (Sander et al. 2011).

FLASH Assembly

The fast ligation-based automatable solid-phase high-throughput (FLASH) method of TAL assembly was developed by Reyon et al. (2012). It is a very robust method of TAL assembly but it needs higher preliminary costs. It can be promising for large-scale construction of TALEs. The use of large amounts of preassembled units has decreased its usage and applicability. Furthermore, ZiFiT software is used to design TALE repeat arrays for FLASH assembly.

Iterative Capped Assembly

This method has been developed by Church's laboratory, Harvard Medical School, U S A. It is based on iterative ligations on solid-phase magnetic beads. Full-length TALENs are achieved by blocking incompletely extended chains with capping oligonucleotides. The method is PCR-based and followed by Golden Gate cloning. Primarily, the method was used to develop TALENs. This method can be used to produce multiple length TALEs from 11 to 19 repeats. Incomplete ligation faults can be overcome and, further, it avoids self-ligation of the monomers. It provides rapid and scalable assembly of TALEs from individual monomers (Briggs et al. 2012).

Integrated Chip

This method is a solid-phase synthesis which uses magnetic bead-based TALE assembly. It is a very efficient method by which 100 TALEs of 16–20 repeats can be prepared just in 3 days (Wang et al. 2012). It provides efficient purification of the ligation products. It can be the most efficient and promising TALEs, TALENs and TALE-TF assembly method if, along with time saving, it could be made cost-effective. This protocol also facilitates the construction of TALEs fused with the transcription activation domain VP64 which is used for gene activation.

Unit Assembly

The unit assembly method (Huang et al. 2011) is based on using four basic single unit modules. By using this method, TALE/TALENs can be assembled in 1 week. DNA fragments encoding all four units are synthesized, amplified and cloned in vectors contain unique *SpeI* and *NheI* sites. The method is similar to BioBrick (Synthetic Biology). Using this protocol, a TALE vector of 9–16 repeats can be prepared in three rounds of restriction/ligation in 1 week. Although the method is simple and comprehensive, it is also labor-intensive and tedious compared with Golden Gate and some other methods as it is based on unit assembly preparation and handling. If the vectors once established, could be reused for further repeats assembly, the preparation of further assemblies would be more robust.

USER-Based Ligation Mediated Assembly of TAL Effector (ULtiMATE)

This method is based on the uracil-specific excision reagent (USER) which was used to create sticky ends for specific ligation (Yang et al. 2013). It is a robust method in which TALEs and TALENs can be produced in hours. This is PCR-based method in which pre-assembled trimmers are used to amplify TALEs of 17.5-mer, but for the generation of multiple TALE lengths more labor is required. There is also the need for at least 40 uracil primers. By using this method, a researcher can generally target 18 base pairs of a desired sequence. However, it is not very flexible with respect to multiple TALE lengths and DNA targets. One advantage is that it uses one backbone for a half-/last repeat while in other protocols there are four different backbones for four half-repeats (Geissler et al. 2011; Cermak et al. 2011; Reyon et al. 2012; Sanjana et al. 2012). Moreover, the generation of trimmers for all 64 combinations of 3-bp DNA has made this protocol more time-saving. A JAVA program is also provided to reduce the design efforts for PCR using the ULtiMATE method.

LIC Assembly

This protocol of ligation-independent cloning (LIC) was developed by Schmid-Burgk et al. (2013). In this method, one repeat unit is used to prepare 2-mer fragments, then a further 6-mer fragments are produced in a level 1 backbone which are finally used to prepare the 18-mer assembly. TALENs are assembled in a mammalian expression vector. This protocol is robust and time-saving as TAL constructs of 15.5–18.5 can be produced in 2–3 working days. Schmid-Burgk et al. (2013) have generated a 5-mer fragments library of TALE repeats by which more than 600 TALEN genes can be produced in 1 day. Moreover, the method does not require any PCR, agarose gel preparation or sequencing or ligation reaction which has made it suitable for mass production of TALEN constructs.

The TALEN technology has transformed genome engineering into more robust processes mostly due to simple and cost-effective assembly of TALEs and TALENs constructs. Cermak et al. (2015) have developed a rapid, inexpensive, and user-friendly method to design and assemble TALEN constructs based on the Golden Gate cloning strategy. Using this method, ready-to-use TALENs targeting 13- to 32-bp-long DNA sequences can be constructed within 5 days. In overall comparison, Golden Gate kits can be the most useful and effective and using the least resources for average laboratories (Table 1).

TALENs: Molecular Scissors for Targeted Mutagenesis in Plants

Different mutagenesis techniques have been used in plants to get desirable results. Variation in plant genetic makeup has been used in the past for various purposes like dwarfism, disease resistance, etc. Since Muller (1927) discovered that X-rays can induce mutations in *Drosophila*, a number of physical (radiation) (Stadler 1928), chemical (EMS) (Kim et al. 2006) and biological (transposable) (Greco et al. 2001) mutagens have been used for plant mutagenesis. Use of mutagenic agents has revolutionized the field of forward and reverse genetics. According to FAO/IEAE (2014), from 1930 to 2014 more than 3200 mutagenic plant varietals have been released. All these mutagens create mutations at the cost of a number of off-target effects. So, to obtain a true mutant, a lot of resources and labor are required. TALEN technology is more robust, precise, accurate and highly specific for site-directed mutations. TALEN technology is currently revolutionizing synthetic biology because of higher sequence fidelity and less cytotoxicity compared to other DNA binding proteins (e.g., zinc fingers). TALENs are protein-based DNA-targeting systems which have emerged as an alternative to ZFNs for genome editing, and it has been reported previously that TALENs are more mutagenic than ZFNs (Chen et al. 2013). TALENs are similar to ZFNs and comprise a nonspecific *FokI* nuclease domain fused to a customizable DNA-binding domain to create DSBs (Christian et al. 2010; Mahfouz et al. 2011; Miller et al. 2011) (Fig. 3).

DSBs created by an engineered nuclease can be sealed by either the NHEJ or HDR pathways. NHEJ is an error-prone repair pathway which simply re-joins the broken DNA ends leading to small insertions or deletions at the DSB, while in the case of the HR-based repair pathway, the DSB is faithfully reconstituted using a homologous sequence as a template (Bibikova et al. 2002; Salomon and Puchta 1998; Rouet et al. 1994; Puchta et al. 1993) (Fig. 4). By the introduction of a donor DNA as double-stranded DNA or a single-stranded DNA oligonucleotide (ssODN), a gene or DNA fragment can be inserted based on the HDR mechanism (Soldner et al. 2011; Zhang et al. 2013a, b). NHEJ is generally preferred in the case of eukaryotic cells over HDR, but the frequency of the later can be substantially increased when transferring large amounts of a homologous template sequence to the cell (Lombardo et al. 2007). TALENs have been proved to create DSBs in plant cells at specific DNA sites (Mahfouz et al. 2011; Shan et al. 2013). Moreover, NHEJ can be used for site-specific gene integration. Maresca et al. (2013) demonstrated Obligate Ligation-Gated Recombination (ObLiGaRe) to insert genes in a site-specific manner. In this method, the

Table 1 Commercial available kits/plasmids and services for TALEs and TALEN construction

No.	Company	Web link
1	Addgene	www.addgene.org/talen/
2	Newlife	http://www.nlbiochemex.com/genome-editing.html
3	Cellectic	www.lifetechnologies.com/TAL
4	Life Technologies	https://www.thermofisher.com/pk/en/home/life-science/genome-editing/geneart-tals.html
5	System Biosciences	https://www.systembio.com/ez-tal
6	Labomics	http://labomics.com/index.php/products/rgen-talen-zfn/tale-talens.html
7	Genecopoeia	http://www.genecopoeia.com/product/talen-tal-effector/
8	Transposagen Biopharmaceuticals	http://www.transposagenbio.com/xtn

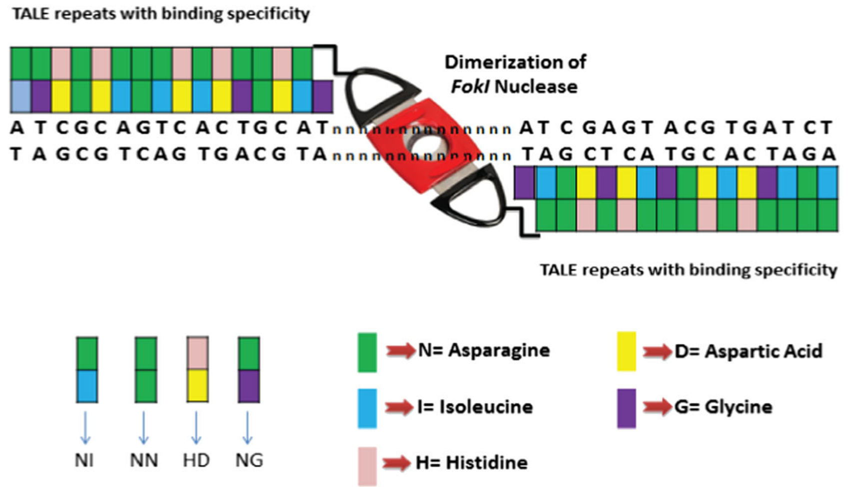

Fig. 3 Artificially engineered TALEN pair with RVDs and their binding specificity. Artificially engineered TALEN pair bind targeted DNA and create DSBs on the dimerization of *FokI* nuclease. Upon dimerization, *FokI* nuclease acts like a cutter and cuts the DNA in the spacer region. Artificial TALENs are designed and assembled on the basis of the binding specificity of RVDs. The four RVDs (NI, NN, HD, NG) with their binding specificity are shown. The di-amino acids on which RVDs consist are also given

heterodimeric property of custom-designed nucleases (CDNs; ZFNs and TALENs) was used for precise gene targeting without cloning homology arms into the donor plasmid. To ligate the exogenous DNA fragment, homology arms binding site of CDNs present in genome was also introduced in the vector, but in an altered orientation to lock the ligation product for further digestion inside homology arms genome. The transformation efficiencies with ObLiGaRe are five times greater than using HR. ObLiGaRe in broadly applicable and provide an additional tool of genetic engineering. Yamamoto et al. (2015) also used ObLiGaRe for precise integration of large DNA into the specified genomic target site. This method is straightforward and highly efficient and does not need any additional gene manipulations. Therefore, ObLiGaRe is applicable to a broad range of organisms. Along with site-specific insertion of genes, ObLiGaRe is very useful for reporter gene insertion, gene tagging, monitoring gene expression and purification of fusion proteins.

TALENs have generated much interest and excitement because they can be designed very easily and rapidly using a simple 'protein–DNA code' that relates modular DNA-binding TALE repeat domains to individual bases in a target-binding site. The DNA binding domain of TALENs is composed of highly conserved repeats derived from TALEs, which are secreted by *Xanthomonas* spp. bacteria to alter gene transcription in host plant cells. On the basis of naturally occurring TALEs, the binding sites for TALEs are expected to occur after every 35 bases of DNA which are more frequent than zinc fingers (Cermak et al. 2011). But recently, it has been demonstrated that some limitations with respect to binding sites for TALEs and TALENs have been compensated by using truncated N-terminal TALEs (Lamb et al. 2013), as the wild N-terminal of TALEs requires a thymine (T) base upstream of a target site (Boch et al. 2009; Moscou and Bogdanove 2009). Doyle et al. (2013) altered the specificity of TAL effectors for the '0' base of the targeted DNA by

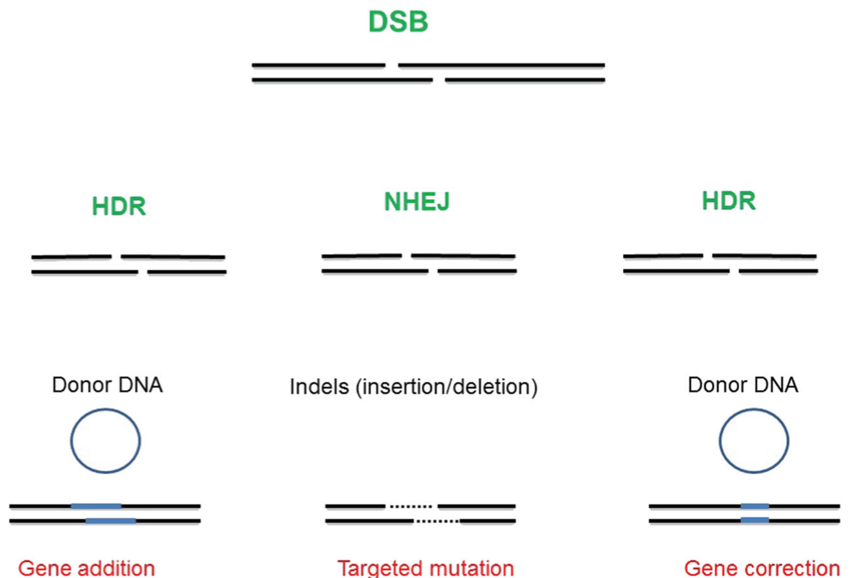

Fig. 4 Targeted genome modifications after DSBs. DSB is utilizes for targeted genome modifications. A general DSB repair mechanism is shown which has been elaborated. The double-strand can be repaired in an error-prone NHEJ manner which results in indels or HDR upon the provision of donor DNA. HDR is used for site-specific gene addition or correction

substituting for tryptophan in the cryptic repeat. With the pace of progress in binding affinity and specificity investigations and research of TAL effectors, it has theoretically been claimed that every sequence of any string of DNA can be targeted using TALEs. TALENs fused with *FokI* nuclease have been used mostly in yeast, animal cells and human pluripotent cells (Richard et al. 2014; Takada et al. 2013; Hockemeyer et al. 2011), likewise ZFNs, but there are also successful reports in plants (Mahfouz et al. 2011; Li et al. 2012).

Targeted mutations at specific sites have been achieved by using TALENs which can be used for gene disruption and silencing. Silencing of unwanted genes with high precision and accuracy has been demonstrated in different plant species (Zhang et al. 2013a, b; Haun et al. 2014). Furthermore, deletion of genes can also be achieved by creating two breaks on both up- and downstreams sites of the desired gene. The Aceto Lactate Synthase (ALS) gene of tobacco has been targeted up to 30 % targeted mutation frequencies using TALEN (Zhang et al. 2013a, b). A comprehensive experimental study to check the binding specificity of four common RVDs of TALEs to potential target sites has revealed that TALENs are highly target-specific, suggesting their applicability as potential engineered nucleases (Juillerat et al. 2014).

TALEN technology has been widely utilized for targeted gene mutagenesis, especially for gene inactivation, in many organisms, including agriculturally important plants such as rice, wheat, tomato and barley. The primary advantage of TALENs over other sequence-specific nucleases, namely zinc finger nucleases and meganucleases, lies in their ease of assembly, reliability of function, and their broad targeting range. TALENs have been used for targeted mutations in barley (Wendt et al. 2013) and the mutations in the targeted promoter region of the *PAPhy-A* gene of barley has been reported by creating DSBs. Application of this technology to generate heritable genome modifications in different crop plants like maize, rice, etc., has also been demonstrated. TALENs were employed to generate stable, heritable mutations at the maize glossy2 (gl2) locus (Char et al. 2015). Transgenic lines containing mono- or di-allelic mutations were obtained from the maize genotype Hi-II at a frequency of about 10 %. The same studies have been carried out in rice by using the TALEN system efficiently (Zhang et al. 2015). Furthermore, TALEN-induced mutations were stably transmitted to the T1 and T2 populations in a normal Mendelian fashion. The vast majority of TALEN-induced mutations (~81 %) affected multiple bases and ~70 % of them were deletions. TALEN has been used recently for a single base substitution in the rice *OsEPSPS* gene (Wang et al. 2014). Moreover, co-transformation of TALEN with chimeric RNA/DNA oligonucleotides (COs) have been demonstrated as a good strategy for single base substitutions. The induced mutations in transgenic generation were also capable of stably passing to the next generation. TALENs along with newly emerged CRISPR/Cas9 have been proved potentially useful for targeted genome engineering of plants. Moreover, the stable inheritance in a Mendelian fashion has made TALENs a profound and efficient approach for targeted mutagenesis in plants. TALEN technology also offers the development of homozygous plants in one generation. Thus, the TALEN technology can be used for efficient, stable and precise mutagenesis in plants. Moreover, for targeted mutagenesis, TALEN can be delivered in the form of proteins (Luo et al. 2015) which further facilitates the development of non-transgenic plants.

Successful TALEN-Based Genome Editing Reports in Agriculture

The use of engineered nucleases has proved a paradigm shift in the field of targeted genome engineering. To date, TALENs have been used to generate targeted modifications in a variety of organisms, such as *Arabidopsis thaliana* (Cermak et al. 2011), tobacco (Mahfouz et al. 2011), rice (Li et al. 2012), yeast (*Saccharomyces cerevisiae*) (Li et al. 2011), fungi (Arazoe et al. 2015), zebrafish (Huang et al. 2011; Sander et al. 2011), rats (Tesson et al. 2011), sheep (Zhao et al. 2016), *C. elegans* (Cheng et al. 2013) and humans (Miller et al. 2011). In addition to custom TALENs, designer TALEs (dTALEs) have also been developed (Mahfouz et al. 2012; Morbitzer et al. 2011b) that activate or repress gene expression *in planta*.

TALEs and TALENs for Next Generation Disease Resistance Development

TALEs and TALENs have been demonstrated as sophisticated tools for gene correction, gene therapy and establishment of disease models and disease resistance in animals and human cell lines (Ding et al. 2013; Choi et al. 2013; Ramalingam et al. 2014; Dreyer et al. 2015). There is a need to develop strategies for next generation disease resistance development based on understanding of host–pathogen interactions in plants. TALEs and TALEN technology have great potential for disease resistance development in plants against bacterial, fungal and viral diseases. The most successful report of using TALEN technology for disease resistance in plants is in rice, in which the rice bacterial blight susceptibility gene *Os11N3* (also called *OsSWEET14*) was targeted for TALEN-based disruption (Li et al. 2012). Mutation of the binding site of foreign pathogenic proteins is a tremendous strategy which is based on basic knowledge of host–pathogen interaction and cross-talk. This rice variety has been made patent in 2015 which has opened up new vistas for the approval of genetically engineered plants with the TALEN technique. This approach of targeting indigenous genes for targeted mutations in which

developmental function remains undisrupted can be used in other plants for the development of resistance against biotic constraints (Table 2).

In the past, Sera (2005) and Takenaka et al. (2007) used artificial zinc finger proteins (ZFPs) against begomoviruses; beet severe curly top virus (BSCTV) and tomato yellow leaf curl virus (TYLCV) respectively. Moreover, Mori et al. (2013) also used artificial zinc finger proteins to inhibit the biding of TYLCV rep proteins to its replication origin. The strategy of targeting *rep* gene or rep protein binding sites to occupy or disrupt the binding sites can be very fascinating using the TALEs and TALEN approach with high specificity. Mino et al. (2014) used the zinc finger nuclease to inhibit DNA replication of human papillomavirus (HPV) by disrupting HPV *ori* plasmid. Moreover, TALENs have also been used efficiently for human immunodeficiency virus (HIV-1) resistance (Fadel et al. 2014; Ye et al. 2014) and hepatitis B virus (HBV) replication inhibition (Bloom et al. 2013; Chen et al. 2014). Recently, it has been demonstrated by Cheng et al. (2015) that artificial TALE proteins can be a platform for broad spectrum resistance against begomoviruses.

Unequivocally, viral diseases are the most dangerous diseases in plants because there is no strategy to directly inhibit the virus rather the vector. The recent studies of using TALEs for begomovirus resistance has become a milestone in the way of achieving disease resistance through viral DNA targeting with specific DNA binding proteins and engineered nucleases (Cheng et al. 2015). Targeting viral DNA or host factors associated with the pathogenesis of viral disease for disruption can be possible strategies for virus suppression and disease resistance. There is a great possibility and progress in the idea of also using TALENs and TALE repressors for antiviral gene therapy to suppress potent viruses causing global mortality and morbidity like HIV (Bloom et al. 2015). So far, different regions of viral genomes have been targeted to inhibit replication and to suppress viruses. But there is a dire need to develop a strategy to target the most conserved nonanucleotide region of viruses which can be the most promising sites for broad spectrum resistance. We are searching for conserved and consensus sequences of a complex of viruses which are causal agents of cotton leaf curl disease in Pakistan. In addition, we are trying to sort out the most promising

Table 2 Examples of genetic modifications using TALEN in plants

Organism	Gene(s)	Editing	Reference
Arabidopsis	*ADH1*	Gene knockout	Cermak et al. 2011
Tobacco	EBE of Hax3	Gene knockout	Mahfouz et al. 2011
Nicotiana benthamiana	Endogenous genes	Gene targeting	Mahfouz et al. 2011
Rice	EBE of AvrXa7 and PthXo3	Gene knockout	Li et al. 2012
Rice	*OsSD1*	Gene knockout	Shan et al. 2013
Rice	*OsBADH2*	Gene knockout	Shan et al. 2013
Brachypodium	*BdABA1*	Gene knockout	Shan et al. 2013
Brachypodium	*BdSPL*	Gene knockout	Shan et al. 2013
Brachypodium	*BdSBP*	Gene knockout	Shan et al. 2013
Brachypodium	*BdCOII*	Gene knockout	Shan et al. 2013
Tobacco	*SurA*, *SurB*	Gene knockout, insertion, replacement	Zhang et al. 2013a, b
Barley	*PAPhy-A*	Gene knockout	Wendt et al. 2013
Brassica oleracea	*FRIGIDA*	Gene knockout	Sun et al. 2013
Soybean	*FAD2-1A*, *FAD2-1B*	Targeted mutagenesis	Haun et al. 2014
Barley	*PAPHY-A*	Gene knockout	Gurushidze et al. 2014
Wheat	*MLO*	Targeted mutagenesis	Wang et al. 2014
Rice	*OsMST8*, *OsMST7*	Targeted mutagenesis	Zhang et al. 2015
Rice	*OsEPSPS*	Targeted mutagenesis	Wang et al. 2015
Maize	*Glossy2* locus	Targeted mutagenesis	Char et al. 2015
Potato	*Vlnv*	Gene knockout	Clasen et al. 2015
Nicotiana benthamiana	*FucT*, *XylT*	Gene knockout	Li et al. 2015
Arabidopsis	*CLV3*	Gene deletion	Forner et al. 2015
Rice	*OsALS*	Gene editing	Li et al. 2016

possible strategies based on potential target sites and options of using artificial proteins and/or engineered nucleases (TALENs and CRIPSPR/Cas9) for suppressing this disease.

TALEs for Gene Activation and Repression

TALEs act like transcription activators in plant cells and play an important role in the virulence of the pathogen (Castiblanco et al. 2012; Marois et al. 2002). As reported a couple of decades ago, these proteins bind specifically to a DNA sequence and make the host susceptible to disease resistance, and the number and/or order of TALEs RVDs have a role in the virulence and avirulence activities (Bodnar et al. 2013). The acid activation domain (AAD), downstream of the nuclear localization signal (NLS), is involved in the recruitment of the host transcriptional machinery (Saijo and Schulze-Lefert 2008). In host–pathogen interactions, there can be secretion of activation domains which activate susceptibility genes, or repression domains which repress vital genes important for resistance in plants. Along with binding domains, different activation and repression domains have also been discovered since the discovery of TALEs. The effector domains, activators and/or repressors fused with TALEs can be used for the activation or repression of a gene (Uhde-Stone et al. 2014; Blount et al. 2012). Activation domains like V16 and V64 (Boch 2011; Miller et al. 2011) and repression domains like KRAB (Kruppel-associated box), GAL4, and SRDX have been discovered and used for tunable expression of genes (Cong et al. 2012; Zhang et al. 2013a, b; Mahfouz et al. 2012; Garg et al. 2012). It has been demonstrated that TAL effectors are versatile tools and can serve as repressors, trans-activators and trans-silencers (Werner and Gossen 2014). Having knowledge about the host–pathogen cross-talk and interaction can be proved beneficial in using TAL technology for gene activation and repression. Taking this view for future plant genome engineering, gene activation and repression would definitely be promising from the GMOs regulatory viewpoint. TALEs along with effector domains can be tailored as remote controls for gene expression in plants. This is a very charming approach of controlling transcription at the most fundamental level by targeting promoters of a gene. Moreover, TALE proteins have been recently fused with riboswitches, called RiboTALE, for tunable control of expression levels of different proteins on recognition of exogenous signals (Rai et al. 2014). As riboswitches are RNA-based regulatory elements, and are involved in post-transcriptional regulation, their use along with TALE proteins would be quite beneficial for strong regulation and tunable expression.

Use of TAL Technology for Crop Quality Enhancement

TALEs and TALEN technology have been used for plant improvement in growth, production, yield and quality. For quality enhancement, different plants of economic importance have been engineered with TALEs and TALENs. In the past, transgenic fruits and vegetables have been developed using the latest technologies of that time: RNAi, antisense, ribozyme, etc. Today's engineered nuclease technology is more precise and specific and gives heritable and higher expression. Moreover, old techniques often result in incomplete gene silencing. Clasen et al. (2015) used TALENs to knockout the VInv gene within the commercial potato variety, Ranger Russet. The gene was silenced by TALEN knock-out system which resulted in low levels of reducing sugars, and hence was prevented from sprouting, while cold storage shelf life was increased. Furthermore, Clasen et al. (2015) also claimed that some of their transgenic do not have TALEN DNA insertions in the genome. TALEN technology can be used efficiently for gene suppression to increase shelf life and sustain the quality of the fruits and vegetables. Besides the development of non-transgenic edible fruits and vegetables would minimize the concerns associated with the use and approval of GMOs.

The mutation of two fatty acid desaturase genes (*FAD2-1A* and *FAD2-1B*) in soybean using TALEN technology for quality enhancement have been reported by Haun et al. (2014). The production of polyunsaturated fats was significantly decreased. In addition to a decrease in polyunsaturated fats, the shelf life and oxidative stability were also improved. It was found that certain soybean lines lacked TALEN transgene which have proved that TALEN can offer a platform for desirable and valuable traits modification in a single generation. It has been demonstrated that plants with heritable and homozygous traits can be produced in one generation which would not have foreign gene integration using TALEN technology (Wang et al. 2014; Zhang et al. 2015). Scientists are using TALEN technology for targeted mutations to obtain commercially valuable cereals, fruits and vegetables with desirable agronomic traits. Recently, a rice variety has been developed with altered fragrance. As fragrant rice is liked all over the world, so indels (insertions/deletions) in the betaine aldehyde dehydrogenase (BADH2) encoding gene (*OsBADH2*) have been produced using TALENs which resulted in the production of 2-acetyl-1-pyrroline (2AP), which is a major fragrance compound (Shan et al. 2015). These recent reports have provided a platform for mutations in the unwanted and undesirable genes of color, flavor or fragrance in fruits and vegetables. Moreover, any gene of desirable flavor or fragrance can

be inserted precisely using TALEN technology. Mendelian inheritance of mutated genes and the use of simple genetic crosses to get rid of TALEN genes have made this technology a favorite for engineering edible cereals, fruits and vegetables.

TALEs and Epigenome Editing

The regulation of expression of the epigenome, even though mysterious, can be tremendously modulated for desirable modifications. Changes in gene expression without any change in DNA sequence have remained an enigmatic conundrum for years, but now the factors which are responsible, epigenetically, for activation or suppression of gene expression have been discovered. Thus, it has become possible with the help of engineered proteins to change at will gene expression epigenetically. So far, ZFPs, TALEs and CRISPR/Cas9 have been used for this purpose (Stolzenburg 2014; Gao et al. 2014; Cho et al. 2015; Hilton et al. 2015). Presently, TALEs and nuclease-deficient Cas9 (dCas9) have been predominantly used for epigenome editing. These proteins, along with different effector domains like 10–11 translocation methylcytosine deoxygenase 1 (TET1) (Maeder et al. 2013), lysine specific demethylase 1A (LSD1) (Joung et al. 2013), methyltransferase and calcium and integrin binding proteins have become potential epigenome editors. Maeder et al. (2013) used zinc finger fused with TET1 (ZF-TET1) for demethylation. TET1 is used for the demethylation of cytosine at CpG sites while LSD1 is used for the demethylation of H3K4me1/2 and deacetylation of H3K27.

DNA methylation normally occurs on repeat regions, promoters, enhancers and the gene body. It also has a role in gene splicing. Mendenhall et al. (2013) used TALE-LSD1 to modify the methylation pattern of different upstream sites. TALE binding specificity for epigenome editing is checked out by chromatin immunoprecipitation (ChIP) followed by Sanger sequencing (Konermann et al. 2013; Maeder et al. 2013; Mendenhall et al. 2013). Gao et al. (2014) has given a comparison of TALEs and CRISPR/dCas9 for epigenome modifications in mammalian cells. It is reported that TALEs, being natural transcription factors, are more promising for gene activation as compared to dCas9. Moreover, they also checked the feasibility of both TALE and dCas9 for gene activation and repression and found promising results which further strengthen the possibility of using designed transcription factors for epigenome erasure and remodeling. TALE kits are available commercially from Addgene to develop constructs having TALE fused with different epigenome modifiers like histone demethylase LSD1 for epigenome editing. Epigenome editing has opened up new avenues of developing non-transgenic modified plants and animals. Precision manipulation and modulation of epigenetic marks without alteration of DNA sequences using modular and sophisticated systems like TALEs would be equally beneficial in plants. Although potential application of TALEs for targeting DNA or histone for epigenome editing has been demonstrated more research is needed for the development and validation of epigenetically modified crops/organisms (EMOs).

TALEs and TALENs for Gene Stacking

Site-specific recombinases are powerful tools for genome engineering. Transcription activators like effectors have been used along with recombinases and transposases as fusion proteins. Homologous recombination is desirable in genetic transformation events. Chimeric TALE recombinases (TALERs) have been constructed by fusing a hyperactivated catalytic domain from the DNA invertase Gin with a TALE central repeat domain. TALERs with optimized architecture recombine DNA efficiently in bacterial and mammalian cells, providing an alternative approach for targeted genome editing (Mercer et al. 2012). Hyperactivated variants of the resolvase/invertase family of serine recombinases function without accessory factors, and thus can be re-targeted to sequences of interest by replacing native DNA-binding domains (DBDs) with engineered ZFPs. However, imperfect modularity with particular domains, lack of high-affinity binding to all DNA triplets, and difficulty in construction has hindered the widespread adoption of ZFPs in unspecialized laboratories. The discovery of a novel type of DBD TALE proteins provides an alternative to ZFPs. Mercer et al. (2012) described TALERs, engineered fusions between a hyperactivated catalytic domain from the DNA invertase Gin and an optimized TALE architecture. They used a library of incrementally truncated TALE variants to identify TALER fusions that modify DNA with efficiency and specificity comparable to zinc-finger recombinases in bacterial cells. Moreover, it has been reported that TALERs recombine DNA in mammalian cells. The TALER architecture provides a platform for the insertion of customized TALE domains, thus significantly expanding the targeting capacity of engineered recombinases and their potential applications in biotechnology and medicine. Moreover, meganucleases have been used for gene stacking in cotton through HDR-mediated targeted genome modification (D'Halluin et al. 2013). Targeted gene stacking of economically important molecular traits can be achieved more precisely using TALENs for targeted HDR-mediated modifications.

Delivery of TALEs and TALENs into Plants

Sequence-specific nucleases enable facile editing of higher eukaryotic genomic DNA; however, targeted modification of plant genomes remains challenging due to ineffective methods for delivering reagents for genome engineering to plant cells.

The method of delivery of engineered nucleases is very crucial to obtaining desirable expression and results. Delivery of TALEs and TALENs in the form of nucleic acid, mRNA and proteins has been successfully carried out in the case of animals (Tong et al. 2012; Tesson et al. 2011; Wefers et al. 2013; Chen et al. 2013; Liu et al. 2014; Jia et al. 2014). TALEN activity largely depends on the choice of delivery material, expression vector and transformation method. Plasmids and viral vectors have traditionally been used for expression of required proteins inside the cell. Baltes et al. (2014) have used geminivirus-based replicons for transient expression of sequence-specific nucleases (ZFN, TALENs and CRISPR/Cas) and the delivery of DNA repair templates. In tobacco (*Nicotiana tabacum*), replicons based on the bean yellow dwarf virus enhanced gene targeting frequencies by one to two orders of magnitude over conventional *Agrobacterium tumefaciens* T-DNA. In addition to the nuclease-mediated DNA DSBs, gene targeting was promoted by replication of the repair template and pleiotropic activity of the geminivirus replication initiator proteins. They demonstrated the feasibility of using geminivirus replicons to generate plants with a desired DNA sequence modification. By adopting a general plant transformation method, plantlets with a desired DNA change were regenerated in less than 6 weeks. These results, in addition to the large host range of geminiviruses, advocate the use of replicons for plant genome engineering.

There are different advantages of using mRNA of TALEN instead of T-DNA. Firstly, in certain cases, particularly pharmaceuticals, mRNA is better from the regulatory viewpoint whereas virus vectors are considered as gene-modified organisms. Secondly, the use of mRNA removes the risk of undesirable integration and mutagenesis and unwanted long-term expression because it is based on transient expression. TALEN mRNA has been injected in animal cells and desirable results have been achieved (Davies et al. 2013; Li et al. 2015; Mock et al. 2015). In mouse, this approach has been proved valuable to get the desired mutations rapidly and efficiently. Gallie (1993) introduced mRNA into the plant protoplast efficiently using PEG-based transformation. Thus, the TALEN mRNA delivery can be more attractive for transient expression in plants to reduce undesirable results and to prompt the regulatory process. Different companies like TriLink BioTechnologies are providing synthetic TALEN mRNAs for genome editing.

The direct injection of TALEN proteins into living organisms is also fascinating for biomedical applications. Jia et al. (2014) used bacterial type III secretion system for direct injection of TALEN proteins into human cell lines which were efficiently functional in the production of targeted mutations. The direct delivery of TALEN proteins may further decrease the risks associated with TALEN mRNA like post-transcriptional and translational constraints in the expression of plasmid TALENs. Luo et al. (2015) reported direct delivery of proteins in *N. Benthamiana* protoplasts using PEG, and claimed it as a non-transgenic genome editing approach. Direct delivery of TALEN proteins into plants has been shown to be the favorite approach for the regulatory approval of edible crop plants.

Comparison of TALE(N)s With Other Engineered Nucleases

TALEs and TALENs are engineered nucleases like meganuclease, ZFNs and CRISPR/Cas9. All technologies have almost the same mode of action and give the same results, but they are different from one another in terms of nature, components, target specificity, target requirements, target limitations, modularity and construction assembly methods. Along with some limitations, TALEs and TALENs also have some advantages over other DNA targeting systems. Firstly, TALEs are natural transcription factors, so their function and activity is well understood and exploited accordingly. Secondly, there is no limitation of target lengths in the case of TALEs and TALENs such as faced in the case of ZFNs and CRISPR/Cas9. Moreover, a very small chance of off-targeting has made TALENs a safer and sound technology for gene targeting. In the case of CRISPR/Cas9, the sgRNA can tolerate up to five mismatches and hence gives a higher frequency of off-targets (Fu et al. 2013). It has also been reported that the perfect match of the ten bases of sgRNA can be sufficient for Cas9 binding to DNA (Kuscu et al. 2014). The use of dCas9 fused with *FokI* endonuclease (RNA-guided *FokI* nuclease or RFNs) or paired nickases to reduce off-targeting has some other complexities like a requirement of a PAM region, spacer length and additional target design constraints (Ran et al. 2013; Mali et al. 2013; Tsai et al. 2014; Shen et al. 2014). TALENs are used in pairs which is compulsory for the dimerization and catalytic activity of *FokI* nuclease. Boissel et al. (2013) developed a rare-cleaving nuclease architecture, designated as megaTALs (TALEs fused with meganuclease), for therapeutic genome engineering to overcome the limitation of using TALEs in pairs. Moreover, the use of TALENs for ObLiGaRe has made it more attractive than CRISPR/CAS9 for NHEJ-based site-specific gene insertion, as CRISPR/Cas9 is not compatible with ObLiGaRe because it produces blunt ends on DSBs.

Here, we have provided a brief review on TALEs and TALENs technology and their applications in the genetic improvement of crop plants. The potential of TALEs as transcription activators and repressors for targeted modifications of epigenome marks has made TALEs technology a method of choice. Although the latest CRISPR/Cas9 is also very comprehensive and provides precise targeted modifications, off-targeting and limitations of target sites still need to be

Table 3 Comparison of three popular engineered proteins/nucleases for DNA targeting

Features	ZF(N)s	TALE(N)s	CRISPR/Cas9
Origin	*Xenopus laevis*	*Xanthomonas* (similar proteins also reported in *Ralstonia solanacearum* and *Burkholderia rhizoxinica*)	*Streptococcus pyogenes* (present in 40 % bacteria and 90 % *Archaea*)
Nature	DNA binding motifs in eukaryotes	Plant pathogenic protein	Prokaryotic defense protein
Function	DNA binding as transcription factors	DNA binding and gene modulation of host plant (act like transcription factors)	Endonuclease that cuts DNA of infecting viruses and plasmids
Target binding	Protein-DNA (one to triplet)	Protein–DNA (one to one)	RNA–DNA (one to one)
Components	DNA binding domain	DNA binding domain Effector domain (activator/repressor)	Endonuclease gRNA
Year of emergence as gene targeting tools	2000	2010	2012
Target length	~9–36 nt	~12–50 nt	~20–23 nt
Target limitations	It binds to a triplet of DNA bases	Needs T base at 5′	Needs PAM region (5′NGG)
Modularity	Low	High	High
Off-targeting	Low	Very few	High
Size	Small	Relatively large (small in case of TALEs)	Large
Mode of action	DNA binding and DSB (NHEJ/HDR)	DNA binding, Expression modulation/ DSB (NHEJ/HDR)	DNA binding and DSB (NHEJ/HDR)
Assembly	Difficult	Technical but easy	Easy
Uses	Gene disruption, gene deletion, gene correction, Gene addition, Tag ligation, ObLiGaRe	Gene activation, gene repression, Gene disruption, gene deletion, Gene correction, gene addition, Tag ligation, ObLiGaRe	Gene disruption, gene deletion, Gene correction, gene addition,
Epigenome editing	Less reported	More reported (natural TFs)	Less reported
Delivery	DNA, mRNA	DNA, mRNA, Protein	DNA
Targeting efficiency and success rate	Low and variable	High	High
Delivery via viral vector	Easy	Easy	Challenging
Delivery as RNA molecule	Easy	Easy	Challenging
Delivery as protein	Easy	Easy	Challenging

addressed. Moreover, for epigenome editing, dCas9 is less efficient compared to TALEs (Gao et al. 2014) which further strengthens the idea that TALE(N)s are still the potential method of choice for targeted genetic and epigenetic modifications. Recently, Cermak et al. (2015) used TALENs and CRISPR/Cas9 for precise modifications of the tomato genome and found that the both TALENs and CRISPR/Cas9 have similar efficiencies of gene targeting. The main features of the most prominent contemporary engineered nucleases are given in Table 3.

Future Perspectives

Even though there are limitations, TALEN technology, owing to its convenience, flexibility and simplicity, still promises to facilitate and enhance genetic manipulations in different organisms and cell types. The main advantage is that they can be produced rationally to bind a DNA sequence of choice, whereas in case of zinc fingers there is the need to select from a library of fingers with the desired binding properties. Although CRISPR/Cas9 is emerging as a best tool for gene targeting, because it is based on simple RNA–DNA interaction according to Watson–Crick rules but the off-target activity is high as compared to TALENs. Recently, TALENs and CRISPR/dCas9 have been evaluated for off-target activity and it has been shown that TALENs have no off-target event as compared to CRISPR/dCas9 (Wang et al. 2015). That is why TAL technology is generally cost-effective and time-saving and guarantees binding sites for every predefined sequence. Functional domain engineering of TALEs as epigenome editors and TALERs has opened up new corridors in the field of genetic engineering. Gene stacking and clean genes can be achieved precisely using TALEs and TALEN technology. Although many interesting and challenging questions remain unanswered, the accessibility and power of TALENs have made this technology an exciting and important subject for future research and development. Targeted genome engineering with engineered nucleases has revolutionized the course of gene engineering, but there is still the need to address some issues to get more precise and desirable results; e.g., choice of vector, explant, transformation protocol, regeneration and tissue culture techniques need to be optimized. In addition, recently, a new site-specific DNA-guided endonuclease *Natronobacterium gregoryi* Argonaute (NgAgo) has been reported (Gao et al. 2016). In contrast to Cas9, NgAgo does not require PAM and moreover it has no off-targeting. Furthermore, it requires a 24-nt target sequence to create DSB. Another astonishing feature of the NgAgo–gDNA system is that along with DSB it also removes some DNA bp from the site, making the cleavage site hard to recover. It is a new system which is being used for mammalian genome editing and further research is needed to explore its potential. One limitation with TALEN is the use of a pair of TALEs which is necessary for dimerization of the *FokI* nuclease; scientists are working on this but there is still less understanding and usage. Recently, it has been reviewed by different authorities like the USDA (United States Department of Agriculture) that some precise modifications, especially NHEJ-induced, produced by engineered nucleases in different organisms do not fall under the GMOs category so the plants carrying these mutations will not be regulated (Waltz 2012). This is an encouraging notion for biotechnologists working in this field. Luo et al. (2015) has demonstrated direct protein delivery to plants for targeted mutagenesis, and have further claimed that the plants would be non-transgenic. It has been suggested by different scientists for the better adaptation of modified crops that the precise modifications in genomes of living organisms should fall under a new category, other than GMOs. Moreover, the concerns associated with the integration of foreign DNA fragments and the regulation of GMOs can be addressed. Scientists are hopeful that the future of targeted genome engineering is bright and positive and will give solutions to the prevailing problems with which we are faced.

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