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Genome editing for plant research and crop improvement

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Abstract

The advent of CRISPR has had a profound impact on plant biology, and crop improvement. In this review, we summarize the state-of-the-art development of CRISPR technologies and their applications in plants, from the initial introduction of random small indel (insertion or deletion) mutations at target genomic loci to precision editing such as base editing, prime editing and gene targeting. We describe advances in the use of class 2, types II, V and VI systems for gene disruption as well as for precise sequence alterations, gene transcription and epigenome control.

Keywords: CRISPR/Cas, crop improvement, genome editing, plant research

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BRIEF HISTORY OF GENOME EDITING AND THE ADVENT OF CRISPR

Even though the first reports describing CRISPR (clustered regularly interspaced short palindromic repeat) applications appeared only several years ago, two of their main developers, Emmanuelle Charpentier and Jennifer A. Doudna, have just been awarded the 2020 Nobel Prize in Chemistry. Targeted genome editing, in its simplest conception, is a technology that aims to introduce changes in the genome of a cell by guiding DNA nucleases to a very specific locus in a chromosome, producing double strand breaks (DSBs) in the DNA and using the existing cellular DNA repair machinery to introduce mutations. Early approaches included the use of naturally existing meganucleases which specifically target relatively long sequence motifs (>12 bp) but offer no flexibility for design of artificial targets (Paques and Duchateau 2007; Silva et al. 2011). Although limited in the choice of targets, initial experiments using the *I-SceI* meganuclease in the mid 90's reported a 100-fold increase in gene targeting, a remarkable achievement at the time (Rouet et al. 1994; Smih et al. 1995). The discovery of Zinc Fingers, small protein domains that bind to specific 3-bp DNA sequences, and the deciphering of their DNA recognition mechanism provided the needed flexibility and opened the doors to practical genome targeting (Pavletich and Pabo 1991; Boch et al. 2009). Zinc finger domains can be assembled in large modules to recognize longer DNA fragments (multiples of 3 bases) and fused to the non-specific DNA cleavage domain from the FokI nuclease, generating what is known as a Zinc Finger Nuclease (ZFN), to produce DSBs in the genome with extremely high specificity (Kim et al. 1996). Since FokI needs to dimerize in order to create a DSB, two different ZFNs need to be produced targeting the forward and reverse strands. ZFNs were first used to induce mutations in Drosophila and GFP-containing human cell lines and the first reports in *Arabidopsis* and tobacco plants followed soon after (Bibikova et al. 2002; Bibikova et al. 2003; Porteus and Baltimore 2003; Lloyd et al. 2005; Wright et al. 2005). Since then, ZFNs have been used with variable success to engineer many plant species, including rice, maize and soybean (Cai et al. 2009; Shukla et al. 2009; Curtin et al. 2011). Even though ZFNs provided the first practical avenue to achieving genome editing, the technical difficulties in assembling the molecular constructs required for the expression of ZFNs in plant cells limited the adoption of the technology to a few specialist laboratories.

The discovery of the transcription activator-like effector (TALE) proteins in the plant pathogenic bacterium *Xanthomonas* provided the next quantum leap in genome editing (Boch et al. 2009; Moscou and Bogdanove 2009). TALE proteins contain a DNA binding motif with multiple amino acid repeats (33–35 a.a.) almost identical to each other except for two adjacent amino acids in positions 12–13 known as repeat variable di-residues (RVDs). Similar to ZFs, each TALE repeat recognizes and binds DNA but unlike ZFs, a TALE repeat recognizes a single nucleotide with the RVDs providing specificity. The obvious advantage of TALEs over ZFs is the flexibility provided by the single nucleotide recognition that allows a much streamlined design and the possibility to target virtually any DNA sequence. As with ZFNs, TALE-fused nucleases (TALENs) contain an array of DNA binding domains designed to bind a specific chromosomal target, usually 16–19 nucleotides long, fused to the FokI cleavage domain. Also similar to ZFNs, two TALE molecules are needed to create a DSB in the DNA, one binding to the top strand and the other binding to the bottom strand in order to reconstitute a FokI dimer to cleave the double stranded DNA. Assembly of TALEN constructs is much easier than ZFNs and several assembly platforms for plants and animal cells were made publicly available (Cermak et al. 2011; Mussolino et al. 2011) eliciting a wave of excitement among the scientific community and increasing the adoption of the technology. TALEN-based genome editing has been reported in multiple plant species, including Arabidopsis, tobacco, rice, barley, maize, soybean, potato, tomato, wheat and even complex polyploids such as sugarcane (Mahfouz et al. 2011; Li et al. 2012a; Li et al. 2012b; Christian et al. 2013; Shan et al. 2013a; Wendt et al. 2013; Zhang et al. 2013; Haun et al. 2014; Liang et al. 2014; Lor et al. 2014; Wang et al. 2014; Zhang et al. 2014; Cermak et al. 2015; Nicolia et al. 2015; Jung and Altpeter 2016). The flexibility, specificity and technical simplicity of TALEN, compared to ZFNs, made it reign supreme and it was chosen as Natures' method of the year in 2011 (Anonimous 2012; Sprink et al. 2015). Nevertheless, as history goes, TALEN was one of the shortest monarchies ever with the first report of CRISPR-based genome editing appearing in 2013 (Cong et al. 2013; Feng et al. 2013; Jinek et al. 2013; Mali et al. 2013; Mao et al. 2013).

Even though the first CRISPR reports appeared in 2013, the CRISPR history goes 20 years back (Doudna and Charpentier 2014; Lander 2016; Zhang et al. 2020a). The first anecdotal observation of CRISPR repetitive DNA structures in the genome of *Haloferax mediterranei* appeared in 1993 (Mojica et al. 1993), and two years later a more detailed

study of the repeats was published although with a different name from the one used today; it was called Short Regulatory Spacers Motifs (Mojica et al. 1995). By 2000, it was clear that the repeats were present in multiple microbes (Mojica et al. 2000), although their biological functions remained a mystery until 2005 when three different reports proposed an adaptive immunity-related role for the CRISPR repeats (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005) with experimental evidence arriving soon after (Barrangou et al. 2007). In the mist of these developments, it was reported that the immunity to specific phages conferred by CRISPR needed the presence of the Cas5/Csn1 protein (now known as Cas9) in the genome of the bacterium, and the protein contains two putative nuclease domains (Bolotin et al. 2005; Makarova et al. 2006). Most of the subsequent CRISPR developments were centered on the bacteria *Streptococcus pyogenes* and *S. thermophilus,* including the proof that the CRISPR/Cas system can cleave phage and plasmid DNA and that it uses a small molecule of RNA to guide the Cas9 nuclease (Garneau et al. 2010); and culminating with the practical demonstration that a modified CRISPR-based system can be used to efficiently edit the genome of three different types of human cells in 2013 (Jinek et al. 2013; Mali et al. 2013). The extreme technical simplicity provided by CRISPR, in which the recognition of the target is achieved by Watson-Crick base complementarity provided by a short molecule of single stranded RNA resulted in the mass adoption of the system.

BIOLOGY OF DSB-INDUCED CHROMOSOMAL MUTATIONS

Nuclease-based editing tools, such as ZFNs, TALENs and CRISPR, rely on the production and repair of DSBs in chromosomal loci to produce the most commonly sought outcome, the introduction of mutations in the form of indels or base substitutions. Chromosomal double strand breaks are not uncommon events; in fact the cell genome is frequently damaged by internal and external factors such as reactive oxygen species (ROS) and ultraviolet light (UV). Genomic stability is paramount for the survival of the cell and the entire individual, therefore living organisms have developed several cellular machineries aimed at repairing DSBs; the most important being the non-homologous end joining (NHEJ) and the homology-directed repair (HDR) pathways (Wyman and Kanaar 2006; Symington and Gautier 2011; Chapman et al. 2012). These two pathways are fundamentally different in their mechanisms as well as their efficiency and fidelity to faithfully repair the chromosome. NHEJ is mechanistically simpler than HDR, directly

mediating the ligation of two ends at the DSB although it is relatively error-prone resulting in the introduction of small deletions, insertions, or substitutions at the break site after the repair (Lieber 2010). HDR, also called homologous recombination (HR), needs the presence of an intact homologous sequence to serve as a template for repair and it is almost error free (San Filippo et al. 2008). The ultimate choice of repair mechanism by the cell depends on the configuration of the DNA ends as well as the cell cycle phase (San Filippo et al. 2008; Lieber 2010; Symington and Gautier 2011; Chapman et al. 2012). HDR needs the 5' ends of the DSB to undergo partial 5'-3' nucleolytic degradation to generate 3' single stranded overhangs, a process known as 5' resection (Paques and Haber 1999), in order to start the repair process, and is most frequent during the S and G2 phases of the cell cycle after DNA replication since a sister chromatid can serve as template for repair (Ira et al. 2004; Jazayeri et al. 2006). On the other hand, NHEJ is favored to join fragments with intact ends, i.e. with no 5' end resection, and even though it works throughout the entire cell cycle, it is more prevalent in the G1 phase (Ira et al. 2004; Lieber 2010). The two most popular CRISPR-based applications currently are gene disruption and gene targeting that take advantage of the NHEJ and HDR systems respectively (Figure 1).

MEET THE CRISPR FAMILY

CRISPR/Cas is an adaptive immunity system used by many bacteria and archaea. Typical CRISPR/Cas loci contain a CRISPR array and a variable number of CRISPR-associated (Cas) genes. The CRISPR arrays can accumulate up to several hundreds small unique sequence snips (~30−40 bp), called protospacers, acquired from invading DNA molecules separated by exact repeats (~30−40 bp). The function of the protospacers is to guide Cas nucleases to the invaders upon re-infection to produce DSBs on their DNA. CRISPR-mediated immunity is provided in 3 stages: adaptation, CRISPR RNA (crRNA) maturation and interference (Hille et al. 2018). During adaptation, Cas proteins excise a fragment of an invading mobile genetic element (MGE) such as a phage and incorporates it into the CRISPR array. CRISPR arrays are transcribed as a single RNA molecule, the precursor crRNA (pre-crRNA), that is processed during the maturation stage by additional Cas proteins, sometimes with the help of other cellular RNases, to produce individual mature crRNAs which will complex with Cas nucleases. Finally, during the interference stage, the Cas nuclease will bind and cleave invading MGEs with the help of

the associated crRNA to recognize the invading sequence (Hille et al. 2018). Activity of the Cas nuclease is incumbent on the existence of a short sequence motif in the invading DNA, called the protospacer adjacent motif (PAM). The PAM is present in the invading DNA but absent from the CRISPR array, avoiding self-targeting of the chromosomal CRISPR arrays.

Given the extreme diversity of bacterial and archaeal organisms, it is not surprising that CRISPR has evolved to have a large variety of configurations (Koonin and Makarova 2019). Currently known CRISPR systems are divided into two classes with each class containing three different types and multiple subtypes (Makarova et al. 2011; Koonin et al. 2017; Shmakov et al. 2017). The classes are defined by the nature of the 'effector' i.e. the protein used to perform the DSB during the interference stage, with class 1 systems using multi-subunit complexes while class 2 use single protein effectors. The maturation stage is also quite different between classes. Maturation of crRNAs in class 1 systems is complicated, needing the intervention of multiple Cas proteins while class 2 systems adopt a more minimalistic approach. Type II class 2 systems, which use Cas9 as the effector, use the bacterial RNase III plus an additional trans-activating RNA molecule (tracrRNA) present in the CRISPR locus to process the pre-crRNA into mature crRNAs (Deltcheva et al. 2011; Jinek et al. 2012; Jinek et al. 2014). Types V and VI, also belonging to the class 2, are even simpler with pre-crRNA processing performed by the effector proteins Cas12 and Cas13, respectively (East-Seletsky et al. 2016; Fonfara et al. 2016; Liu et al. 2017; Zetsche et al. 2017).

The simplicity of class 2 systems explains their adoption by the wide scientific community for gene editing applications although the effectors belonging to each of the 3 class 2 types have different catalytic activities. In short, the prototypical type II *Streptococcus pyogenes* Cas9 (SpCas9) targets protospacers with an NGG PAM and produces blunt end DSBs, although Cas9 effectors from other bacterial sources recognize different PAMs. Type V effectors, such as Cas12a/Cpf1, recognize T-rich PAMs, produce DSBs with 5 nt 5' overhangs and can process pre-crRNA by themselves without the help of tracrRNAs or RNase III. Type VI Cas13 effectors have the ability to bind and cleave single stranded molecules of RNA at specific sequences determined by the crRNA and can also process pre-crRNA (Koonin and Makarova 2019). More extensive descriptions and applications of each type are provided later in this review. Although most of the focus

has been centered on class 2 systems, class 1 systems are also very diverse and could have potential applications (Koonin and Makarova 2019; Koonin et al. 2020). A recent study has revealed the existence of multiple divergent CRISPR-Cas systems in the archaea *Asgard superphylum*, some of which have been predicted to play non-defense related roles (Makarova et al. 2020).

The CRISPR/Cas system is not restricted to bacteria and archaea, with one well documented case of a phage using its own CRISPR system to overcome the bacterial host resistance (Seed et al. 2013). This single example has been massively expanded by a recent survey of multiple microbiomes reporting hundreds of newly discovered phages with huge genomes (>200 kb). Many of these phages contain CRISPR/Cas systems with a hyper-compact configuration lacking spacer acquisition machinery (Al-Shayeb et al. 2020). It has been hypothesized that some of these phages might hijack the host's CRISPR machinery for their own purposes. Some of the candidate effectors initially identified by Al-Shayeb et al. (Al-Shayeb et al. 2020), named CasΦ, are half the size of Cas9 and have been characterized as functional Cas proteins with the ability to process pre-crRNA and generate crRNA-guided DSBs in dsDNA (Pausch et al. 2020).

THE BASIC TYPE II CRISPR/Cas9 SYSTEM

Typical type II CRISPR loci in bacteria contain the CRISPR repeats as well as an operon encoding for a number of CRISPR-associated (Cas) proteins and an additional gene for a non-coding trans-activating CRISPR RNA (Horvath and Barrangou 2010; Bhaya et al. 2011). The CRISPR array consists of a number of identical repeats separated by unique spacers acquired from the genome of invading MGEs (protospacers) and will eventually protect the bacterium upon subsequent infections. The length of the repeats is conserved within a CRISPR locus but varies between different loci while the length of the spacers is usually similar to the length of the repeats within the same array (Grissa et al. 2007). After transcription of the CRISPR array as a single RNA molecule (pre-crRNA), tracrRNA molecules hybridize to the tandem repeats by base complementarity and recruit RNase III to process the pre-crRNA into mature crRNA molecules. The different crRNA/tracrRNA duplexes remain together after processing and associate with the Cas9 protein. The 20 nucleotides at the 5' end of the crRNA will guide the Cas9 to the complementary protospacer target as long as the target contains a protospacer adjacent motif (PAM), which in the case of the *S. Pyogenes* Cas9 consists of a 3 nt sequence,

NGG. The Cas9 protein contains an HNH nuclease domain and a RuvC-like nuclease domain that cut the DNA strands complementary and non-complementary to the RNA guide sequence, respectively. The cut is very precise and happens between the 3rd and 4th nucleotide upstream from the PAM to create blunt ends (Horvath and Barrangou 2010; Bhaya et al. 2011; Cong et al. 2013).

Engineered CRISPR/Cas9 systems use the same machinery as the bacterial system, although for practical purposes, the tracrRNA and crRNA molecules are replaced by a single hybrid RNA molecule known as single guide RNA (sgRNA or gRNA) (Cong et al. 2013; Mali et al. 2013), reducing the number of components to two: the Cas9 protein that cleaves double stranded DNA and the gRNA that guides Cas9 to the appropriate locus. In order to perform genome editing, it is necessary to deliver both components to the plant nucleus, which is usually achieved by genetic transformation to integrate a cassette containing the Cas9 cDNA and the gRNA under the control of strong promoters into the plant genome. For a given target, genetic constructs are extremely simple to assemble by introducing the 20 bp targeting sequence at the 5' end of the gRNA in the CRISPR cassette. After the recovery of transgenic lines and verification of the existence of mutations in the intended target, the CRISPR transgene can be easily removed by Mendelian segregation, at least in those species with sexual reproduction (Mao et al. 2017).

The typical CRISPR transformation cassettes for plants aim to achieve high expression of the two CRISPR components, Cas9 and gRNA. A codon-optimized version of Cas9 under the control of a strong constitutive promoter such as the cauliflower mosaic virus (CaMV) 35S promoter, for dicots and some monocot species, or the maize ubiquitin promoter for monocots frequently produce heritable mutations (homozygous, hemizygous or biallelic) in the T_0 generation (Shan et al. 2013b; Zhang et al. 2014). A notable exception to this rule is the model plant *Arabidopsis thaliana*, where the CaMV 35S promoter is not always as efficient as in other species with a relatively high abundance of chimeric mutations in the T_1 generation (Mao et al. 2013; Feng et al. 2014; Zhao and Zhu 2016). This is probably due to the *in planta* transformation method used in *Arabidopsis* and can be solved using germline specific promoters such as DD45 and SPL (Wang et al. 2015; Mao et al. 2016). A creative approach to improve heritable editing efficiency via boosting Cas9 and gRNA expression was described by Mao et al. (2018) who discovered

that manipulation of RNA silencing pathways in Arabidopsis could improve gene editing efficiency. As a practical approach, the authors co-expressed the p19 viral suppressor from the tomato bushy stunt virus within the CRISPR cassette, which resulted in an increase in efficiency but also provided a useful visual indicator to identify gene edited lines. Expression of p19 has a distinctive phenotype in *Arabidopsis*, producing curly leaves and increasing to heavily serrated leaves depending on the expression level. Genetic analysis showed that the severity of the phenotype in T_1 lines was directly related to the gene editing efficiency, with lines showing extreme serrated leaf phenotypes having much higher efficiencies than lines with a milder phenotype. The serrated phenotype also provided an easy visible way to identify lines without the transgenes in the T_2 generation by simply looking for individuals without serrated or curly leaves (Mao et al. 2018).

A number of alternatives are available to control the expression of the gRNA. Early reports preferred the use of RNA polymerase III (Pol III) promoters, with Arabidopsis and rice U6 promoters frequently used for dicots and monocots respectively (Jiang et al. 2013b; Li et al. 2014; Zhang et al. 2014; Mikami et al. 2015). The use of endogenous Pol III promoters can increase CRISPR editing efficiency but it is important to keep in mind that there are multiple U6 genes in plants with varied transcriptional efficiencies, therefore when choosing an endogenous promoter it is crucial to test its activity before using it for genome editing (Domitrovich and Kunkel 2003; Wang et al. 2008; Sun et al. 2015; Lu et al. 2017; Ng and Dean 2017; Feng et al. 2018; Long et al. 2018). It is important to emphasize that gRNA efficiency is variable, and there are web-based portals that provide data on the specificity and predicted efficiency of possible gRNAs.

Multiplexing using CRISPR/Cas9 is as simple as expressing multiple gRNAs simultaneously with Cas9 and can be efficiently achieved by building expression cassettes with multiple Pol III promoters (Zhang et al. 2016; Miao et al. 2018; Wang et al. 2018; Zhao et al. 2018) or joining multiple tRNA-gRNA units in tandem under the control of a Pol III promoter and relying on the cellular tRNA processing machinery for gRNA maturation (Xie et al. 2015). Newly developed systems have refined and improved the initial ideas (Cermak et al. 2017). Cermack et al. (2017) used the strong Cestrum Yellow Leaf Curling Virus promoter to drive the expression of relatively long cassettes containing tRNA-gRNA arrays, Csy4-gRNA arrays (Wyvekens et al. 2015), or

ribosomal-gRNA arrays. The toolkit uses Golden Gate cloning of PCR based modules. The authors efficiently targeted up to eight different genes in several plant species and were able to introduce a targeted deletion of 58 kb in *Medicago truncatula* (Cermak et al. 2017).

An obvious limitation of the most popular Cas9, spCas9, is the requirement for an NGG PAM, which limits the number of available targets. This problem has been overcome by engineering spCas9 to produce a number of variants with multiple PAM specificities (Hu et al. 2018; Nishimasu et al. 2018) as well as using nucleases from other sources, such as *S. aureus*, *S. mac*acae, *S. thermophiles*, etc (Gasiunas et al. 2012; Cong et al. 2013; Jiang et al. 2013a; Friedland et al. 2015; Chatterjee et al. 2020).

A large number of examples using CRISPR/Cas9 to introduce agronomically important traits in multiple crops have now been reported and are described in numerous reviews (Zhang et al. 2017a; Hua et al. 2019c; Mao et al. 2019b; Zhang et al. 2019d; Schindele et al. 2020; Tan et al. 2020). Some examples include yield increases in wheat, rice and tomato (Li et al. 2016b; Xu et al. 2016; Li et al. 2017a; Soyk et al. 2017; Zhang et al. 2018c; Miao et al. 2019); resistance to multiple pathogens in wheat, rice, tomato, cucumber, orange and grapefruit (Chandrasekaran et al. 2016; Jia et al. 2016; Wang et al. 2016; Jia et al. 2017; Nekrasov et al. 2017; Peng et al. 2017; Zhang et al. 2017b; Miao et al. 2019; Zhang et al. 2020b); improvement of quality and nutritional attributes in rice, wheat, sorghum, *Carmelina sativa*, tomato and potato (Shan et al. 2015; Waltz 2016; Andersson et al. 2017; Jiang et al. 2017; Sun et al. 2017; Tang et al. 2017a; D'Ambrosio et al. 2018; Li et al. 2018a; Zhang et al. 2018b; Li et al. 2020c); abiotic stresses tolerance (Zhao and Zhu 2016; Liao et al. 2019; Zhang et al. 2019a; Joshi et al. 2020); male sterility in maize, wheat and rice (Zhou et al. 2016; Li et al. 2017c; Okada et al. 2019); fruit parthenocarpy in tomato (Klap et al. 2017; Ueta et al. 2017; Yang et al. 2019); haploid seed formation in rice, maize and wheat (Yao et al. 2018; Zhong et al. 2019; Liu et al. 2020a) and apomixis (Tang et al. 2019; Wang et al. 2019a). CRISPR/Cas9 has been used to delay flowering in soybean, practically expanding the cultivation area for this crop (Cai et al. 2018).

TYPE V Cas12 SYSTEMS

Although Cas12- and Cas9-based CRISPR systems share similar principles and mode of action, they also exhibit important differences that give Cas12 an edge for some applications. Cas12 effectors tend to favor T-rich PAMs instead of the G-rich PAMs of Cas9, providing additional targeting possibilities (Zetsche et al. 2015; Kim et al. 2017c). In addition, Cas12 systems do not need the intervention of a tracrRNA for maturation and interference, using a single RNA molecule that can be engineered to a final length of ~44 nt instead of the ~100 nt in Cas9 gRNAs. Cas12 effectors cleave the target strands at two different positions producing DSBs with staggered ends, instead of the blunt ends produced by Cas9 effectors (Zetsche et al. 2015), a property useful for gene targeting (Begemann et al. 2017; Moreno-Mateos et al. 2017; Li et al. 2018d; Li et al. 2020f). Aside from the DNase activity, Cas12 effectors also have RNase activity used to process the pre-crRNAs into mature crRNAs (Dong et al. 2016; Fonfara et al. 2016). This feature can be exploited in practical terms to simplify multiplexing since it allows to build expression cassettes including multiple gRNAs under the control of a single promoter and use the Cas12 processing activity to produce the individual gRNAs in the cell (Wang et al. 2017b).

The most common Cas12 system is the Cas12a/Cpf1 which was first used for genome editing in 2015 and soon after applied to plants (Zetsche et al. 2015; Endo et al. 2016a). Cas12a/Cpf1 from *Francisella tularensis* novicida (FnCas12a) recognizes a TTN PAM and cleaves the target in two positions, 18 bases on the non-targeted strand and 23 bases on the targeted strand away from the PAM leaving staggered 5 nt overhangs. The PAM for Cas12a from *Acidaminococcus* sp. BV3L6 (AsCas12a) and *Lachnospiraceae bacterium* (LbCas12a) is slightly different, TTTV (V=A, C and G). The first demonstration that Cas12 systems can be applied to plant genome editing was performed in tobacco and rice (Endo et al. 2016a). A codon optimized FnCpf1 under the control of the *Petroselinum crispum* (parsley) ubiquitin promoter was used in combination with 6 crRNAs to target two different tobacco genes, encoding the phytoene desaturase NtPDS and the STENOFOLIA ortholog NtSTF1. Analysis of genomic DNA from T_0 transgenic plants showed ~45% mutation ratios for the two crRNAs targeting the PDS gene, while the crRNA targeting the NtSTF1 gene also produced mutations albeit at a lower efficiency. The FnCpf1 under the control of the maize ubiquitin promoter has been used

to target the rice Drooping leaf (*OsDL*) and Acetolactone synthase (*OsALS*) genes. Hygromycin-resistant calli were recovered and analysis of mutations in the targeted loci revealed very high mutation rates (>60%) for two of the targeted loci while the remaining two showed frequencies ranging from 8% to 25%. T_0 transgenic plants recovered from the two best performing crRNAs had a very high mutation ratio, 85.7% (6/7) for *OsDL* and 90% (9/10) for *OsALS* (Endo et al. 2016a). The nature of the NHEJ-derived mutations generated by Cas12a systems is different from those generated by Cas9, with large indels being more frequently produced by Cas12a (Endo et al. 2016a).

Although Cas12 CRISPR systems are newer, and therefore less explored than the Cas9 system, they have now been used for genome editing applications in multiple plants, including rice (Begemann et al. 2017; Tang et al. 2017b; Xu et al. 2017; Yin et al. 2017; Zhong et al. 2018; Tang et al. 2019), soybean and tobacco (Kim et al. 2017b); *Arabidopsis* (Tang et al. 2017b; Bernabe-Orts et al. 2019), tomato (Bernabe-Orts et al. 2019; Vu et al. 2020); citrus (Jia et al. 2019), maize (Lee et al. 2019c), and cotton (Li et al. 2019a; Wang et al. 2020a).

An important consideration when using Cas12 systems is their temperature dependency (Moreno-Mateos et al. 2017). High temperatures (~37^{o}C) have been shown to improve Cpf1 activity by increasing its ability to access genomic DNA (Moreno-Mateos et al. 2017), which is not a problem for mammalian applications but other organisms such as plants are usually cultured at much lower temperatures (21^{o}C–26^{o}C), at which Cas12 activities are suboptimal. Analysis of AsCas12a, FnCas12a and LbCas12a for their editing efficiencies at different temperatures showed that AsCas12a requires a temperature >28°C for high activity (Malzahn et al. 2019). Applying 'high temperature' growing regimes during the tissue culture stages in rice and maize, the authors reached up to 93% mutation frequency in T_0 transgenic rice lines and up to 100% in maize T_0 lines. In addition, Arabidopsis editing, which is remarkably difficult with Cas12 systems, was substantially improved by growing plants at 29^{o}C for 4 weeks before transferring them to 22^{o}C for recovery (Malzahn et al. 2019). Engineered Cas12a variants have been generated with increased fidelity and editing activity at lower temperatures (Gao et al. 2017; Kleinstiver et al. 2019). New Cas12a variants from *Lachnospiraceae bacterium* (LbCas12a) have been engineered and one of them, harboring a D156R mutation (ttLbCas12a), has shown high efficiency at 22^{o}C (Zetsche et al. 2015), although its

efficiency rises even further at 28^{o}C. It will be interesting to explore whether the combination of the improved ttLbCas12a with the high temperature tissue culture approach used by Malzahn et al. (2019) could have additive effects.

An interesting characteristic of Cas12a is that target binding unleashes unspecific single stranded DNase activity. This trans- cleavage activity has been harnessed to develop highly sensitive and specific diagnostic methods (Chen et al. 2018).

TYPE VI Cas13 SYSTEMS

The immense amount of sequence information available today has allowed the use of data mining to discover putative new CRISPR systems before they can be experimentally tested and this was the case of Cas13a, first identified in 2015 (previously known as C2c2) (Shmakov et al. 2015) and predicted to have some kind of RNase activity due to the presence of two HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domains. Confirmation of such RNase activity came in 2016 when it was confirmed that Cas13a can cleave single stranded RNA (ssRNA) using a gRNA as targeting device in a way similar to types II and V CRISPR systems (Abudayyeh et al. 2016; East-Seletsky et al. 2016). Interestingly, Cas13a is activated upon recognition of the target ssRNA and exhibits non-specific RNase activity after activation, both *in vitro* and in bacterial cells but fortunately such non-specific activity has not been detected in eukaryotic cells (Abudayyeh et al. 2017; Cox et al. 2017). Similar to Cas12a, Cas13a can process pre-crRNA molecules into individual mature gRNAs by itself (Abudayyeh et al. 2016).

Type VI Cas13 systems are relatively new members of the CRISPR family and have not yet been as well characterized as types II and V, but some interesting applications have already been described, not all of them in the gene editing space such as the SHERLOCK diagnostic technology (Gootenberg et al. 2017; Gootenberg et al. 2018). The initial Cas13 characterization work was performed in the type VI *Leptotrichia shahii* Cas13a (LshCas13a) but a screening of 15 orthologs identified a more efficient effector in *L. wadei* (LwaCas13a) (Abudayyeh et al. 2016; Abudayyeh et al. 2017). Interestingly, some but not all Cas13 orthologs require the presence of a protospacer flanking sequence (PFS), similar to a PAM. LwaCas13a-mediated programmable RNA cleavage was achieved with a crRNA encoding a 28 nt spacer and the activity remains with shorter spacer sequences down to 20 nt but needs enhanced Cas13a stability by the addition of a

monomeric superfolder green fluorescent protein (msfGFP) stabilization domain to the Cas13 protein. *In vivo* experiments showed that nuclear localized LwaCas13a achieved higher specific RNA knockdown levels than cytoplasmic LwaCas13a, comparable to those obtained by RNA interference and that the most efficient length for the spacer was 28 nt. *In vivo* specificity was highly dependent on the central seed region of the guide-target duplex and transcriptome wide RNA sequencing for three different genes targeted by either LwaCas13a or RNA interference revealed hundreds of off-targets for the RNAi constructs and virtually none for LwaCas13a as well as no collateral non-specific RNA degradation.

The activity of the system has been shown in rice protoplasts using the rice Actin and U6 promoters to drive the expression of the LwaCas13a cDNA and the gRNA, respectively (Abudayyeh et al. 2017). Transcripts for three different genes, 5-Enolpyruvylshikimate 3-Phosphate Synthase (EPSPS), shikimate *p*-hydroxycinnamoyl transferase (HCT) and elongation factor 1 alpha (OsEF1a) were targeted using three different guides for each gene. Normalized expression values showed that seven out of the nine gRNAs provided >50% knockdown compared to non-targeting guides with the best results reaching 78% knockdown.

An additional CRISPR-associated RNA-guided RNase, Cas13b, was identified in 2017 using bioinformatics analysis searching for putative CRISPR loci lacking either Cas1 or Cas2 within a 10 kb area of the CRISPR array (Smargon et al. 2017). The new class 2 effector from *Bergeyella zoohelcum BzCas13b*, contains HEPN domains and shows collateral RNase activity *in vitro* as Cas13a, but it has a double-sided PFS (at 5' and 3' ends) and additional secondary structure requirements for efficient targeting. Type VIb loci contain one of two small accessory proteins Csx27 (subtype VIb1) or Csx28 (subtype VIb2) that can repress or enhance Cas13b activity respectively (Smargon et al. 2017). A large study of 21 Cas13a and 15 Cas13b orthologs identified a *Prevotella sp. P5-125* Cas13b protein (PspCas13b) with higher *in vitro* knockdown activity than LwaCas13a even in the absence of a msfGFP domain (average of 92.3% and 40.1% for PspCas13b and LwaCas13a, respectively). Similar to *BzCas13b*, PspCas13b has high specificity controlled by the central seed region of the spacer and shows non-specific RNase activity after activation *in vitro* but not in eukaryotic cells.

Type VI-D Cas13 proteins from *Eubacterium siraeum* (Es) and *Ruminococcus* sp. (Rsp) have also been characterized as having many commonalities with subtypes A and B, i.e. they can process pre-crRNAs, possess collateral RNA cleavage upon activation, lack PFS requirements and can cleave ssRNA using a gRNA. The most remarkable characteristic of Cas13d effectors is their small size, on average 190−300 aa smaller than subtypes A & B (Yan et al. 2018b). Another interesting feature of Cas13d systems is the presence of an accessory protein (WYL1) that can stimulate RNA cleavage.

An obvious application of Cas13 effectors is their potential to be used to confer virus resistance. There is a large variety of plant DNA and RNA viruses causing an estimated $30 billion USD in crop losses annually (Sastry and Zitter 2014). Although CRISPR/Cas9 based strategies can be used to control DNA viruses (Ali et al. 2018) most plant viruses have RNA genomes. Aman et al. (2018) used a plant codon optimized Cas13a from *L. shahii*, under the control of the CaMV 35S promoter in transient expression experiments in *N. benthamiana* using agroinfiltration to target a recombinant turnip mosaic virus (TuMV) expressing GFP. Four different crRNA targeting the viral HC-Pro, coat protein and GFP (2x targets) in the viral genome were tested using the tobacco rattle virus (TRV) for expression and the interference activities were tested by GFP visualization seven days after infiltration. The crRNAs targeting HC-Pro and one of the GFP sites reduced GFP levels by 50%, while the remaining two crRNAs produced only modest reduction in GFP fluorescence. These results were validated in transgenic *N. benthamiana* plants overexpressing Cas13a. Transgenic plants were co-infiltrated with TRV expressing each of the four crRNAs and infectious TuMV-GFP clones before quantifying GFP fluorescence levels 7 days after infiltration. Strong reduction in fluorescence intensity was observed with the same two highly efficient crRNAs from the transient assays while the remaining two had only a moderate effect (Aman et al. 2018). In a subsequent study, the same group compared the activities of LwaCas13a, PspCas13b and CasRx variants from *L. wadei*, *Prevotella* sp. P5-125 and *Ruminococcus flavefaciens* XPD3002 respectively for plant virus interference (Mahas et al. 2019). Two versions of each Cas13 variant were prepared containing either an NLS or a nuclear exclusion signal (NES). For this work, the authors used a positive-sense, single-stranded RNA virus engineered to replace the coat protein gene CP with GFP (TRBO-G). As a consequence, the virus is unable to move systemically but can efficiently replicate and express GFP protein in the infected (infiltrated) leaves. For each Cas13 variant, two crRNAs targeting GFP were cloned in

the TRV-based system for transient expression in plants. Each Cas13 variant was co-delivered with the crRNA TRV vectors and the TRBO-G constructs into *N. benthamiana* leaves using *Agro*-infiltration. GFP fluorescence quantification of the leaves showed that the two CasRx versions (NLS and NES) produced the highest interference levels. The fluorescence results were consistent with immunoblot quantification of GFP protein and viral RNA loads measured by RT-qPCR. Targeting of the endogenous replicase gene with three different crRNAs also identified CasRx as providing the highest level of interference with each individual crRNA. The interference ability of the Cas13 variants against a virus capable of systemic spread throughout the plant was tested using the TuMV-GFP virus. The results showed that LwaCas13a-NES, CasRx-NES and CasRx-NLS mediated strong interference against TuMV-GFP in transient assays as well as in transgenic plants constitutively expressing the different Cas13 variants. Finally, simultaneous protection against two viruses was effectively conveyed using CasRx and two crRNAs targeting specific genomic sequences of two different viruses (Mahas et al. 2019). These reports are extremely encouraging as they open the door to multi-viral resistance by co-expression of multiple crRNAs.

BASE EDITING

Most important agronomic traits are determined by single nucleotide polymorphisms (SNPs) or in some cases, a combination of a few SNPs (Doebley et al. 2006; Konishi et al. 2006; Zhao et al. 2011; Morris et al. 2013; Maccaferri et al. 2015; Wu et al. 2017). Introduction of specific SNPs into elite varieties from wild relatives is a long and arduous process but genome editing technologies could greatly accelerate this process. The random nature of the mutations generated by regular CRISPR/Cas systems make this approach impractical, and although it has been used on a number of occasions, gene targeting is still relatively inefficient. An alternative approach is base editing (Figure 2), which allows to perform nucleotide substitutions in the genome using modified Cas effectors with high precision without the need to produce DSBs (Komor et al. 2016). There are two types of base editors (Figure 2), both of which are based on deamination activities: cytidine base editors (CBEs) that convert cytosine (C) to thymine (T) and adenine base editors (ABEs) that convert adenine (A) to guanine (G).

CBEs

CBE-mediated deamination of a cytidine produces uracil (U), which is recognized as a T by DNA polymerase during DNA replication, resulting in a C•G → T•A conversion. Most cytidine deaminases are specific for RNA but some can accept ssDNA as substrate. Fortunately, formation of the Cas9-gRNA-target DNA complex opens the dsDNA strand un-pairing at least 9 nt (Jore et al. 2011), allowing the deaminase to work. The original report described the production of three generations of CBEs using different Cas9 versions and fusion strategies. For the construction of the first system, the authors tested four cytidine deaminases, human AID, human APOBEC3G, rat APOBEC1 and lamprey CDA1, for their efficiencies in deaminating ssDNA, with the rat APOBEC1 (rAPOBEC1) showing the highest enzymatic activity. Different fusions of the rAPOBEC1 to the N and C termini of a 'dead' Cas9 using different linkers were then evaluated for gRNA-dependent C to U conversion with the final result being the BE1 system, in which rAPOBEC1 is linked to the N-terminus of dCas9 using a 16 aa linker (XTEN) (rAPOBEC1:XTEN:dCas9). This system provided efficient catalytic deamination activity in a window of 5 nt *in vitro* (average 44%) but very low efficiency *in vivo* probably due to the efficient removal of uracyl by uracyl DNA glycosilase (UDG) as part of the DNA repair mechanism. Addition of an 83 aa viral protein with strong UDG inhibition activity to the C-terminus of the BE1 effector complex produced the BE2 system (rAPOBEC1:XTEN:dCas9-UGI) with a 3-fold increase in activity *in vivo* (up to 20% conversion efficiency). Finally, the authors tried to take advantage of the eukaryotic mismatch repair machinery, which uses nicks in newly synthesized DNA to repair mismatches, and restored one of the two catalytic nuclease domains in Cas9 by keeping the D10A mutation but reintroducing a His residue in position 840 to produce a nick in the strand containing the G residue pairing with the edited U. The BE3 system (rAPOBEC–XTEN–dCas9(D10A)–UGI) dramatically increased efficiency *in vivo* by an additional 2−6 fold over the BE2 system with a maximum of 37% efficiency in C to T conversion. On the negative side, while the BE1 and BE2 systems exhibited very low frequency of unwanted indel mutations ~0.1%, the indel frequency of BE3 system soared to 1.1%.

Further improvements to the initial BE systems have been reported using relatively small additions or variations to the original theme. In order to increase the accuracy of the

system it was necessary to reduce the deaminase catalytic window, which was achieved by mutating amino acid residues involved in rAPOBEC1 substrate binding. A triple mutation, W90Y+R126E+R132E (named YEE) narrowed the editing window to 1-2 nucleotides when added to the BE3 system (Kim et al. 2017d). A BE4 system used a modified linker to join rAPOBEC1 to Cas9(D10A) and added two UGI groups to minimize unintended edits. Aside from rAPOBEC1, engineered CDA1 and AID deaminases have been used to increase efficiency while addition of the bacteriophage Mu Gam protein to several deaminase-dCas9 complexes, including BE3 and BE4, have reduced the incidence of indel formation (Komor et al. 2017). After discovering that expression levels were a strong limiting factor for efficiency, the BE4 was further improved by adding a different nuclear localization signal (NLS) and optimizing codon usage (BE4max) as well as introducing variations in the deaminase obtained from ancestral deaminases (AncBE4max) (Koblan et al. 2018).

A different approach was taken by Nishida et al (Nishida et al. 2016) who took advantage of the vertebrate adaptive immune system. Antibody generation requires somatic hypermutation to create genetic variation in the variable region of the immunoglobulin locus that produces antibodies. The activation-induced cytidine deaminase (AID) modifies deoxycytidine bases to create such changes. Fusion of the sea lamprey AID ortholog PmCDA1 to the C-terminus of spdCas9 (D10A) nickase produced C to T changes within a window of 3-5 nt and addition of UGI suppressed indel occurrence and further increased efficiency (Nishida et al. 2016). Using a similar approach, human AID mutated to eliminate the nuclear export sequence (AID R190X) and fused to the C-terminus of dCas9 efficiently mutated C nucleotides, but surprisingly only 40% of substitutions resulted in T, with G and A accounting for 30% each (Ma et al. 2016); therefore this method is most useful for producing genetic diversity instead of producing specific changes. Human AID was also combined with dCas9 for protein engineering using yet a different experimental approach (Hess et al. 2016). In this case, the gRNA molecule was modified to contain two MS2 hairpin binding sites (Konermann et al. 2015) and an AID protein was mutated to exclude the nuclear exclusion signal and was fused to the MS2 protein. Recruitment of the MS2-AID fusion to the gRNA MS2 hairpins produced libraries of localized point mutations next to the target sequence (Hess et al. 2016).

Recent work has used phage-assisted continuous evolution methods to develop new deaminases in order to overcome some of the limitations of existing CBE systems (Thuronyi et al. 2019). One of those limitations is the low editing efficiency shown by rAPOBEC1 to edit cytosine in a GC context in the editing window; the evoAPOBEC1-BE4max shows an impressive 26-fold increase in efficiency in cytosine editing in GC contexts. Additional evolved deaminases, such as evoFERNY, are also efficient in all sequence contexts but almost 30% smaller than WT rAPOBEC, while evoCDA1 was especially suited for editing at difficult target sites (Koblan et al. 2018).

ABEs

Theoretically, deamination of adenine should be able to convert adenine to guanine since it produces inosine, which is read as guanine by DNA polymerases during DNA replication. Unfortunately there are no known enzymes with an adenine deaminase activity, therefore Gaudelli et al. (Gaudelli et al. 2017) used an *E. coli* RNA adenosine deaminase (TadA) as a starting point in a series of mutational evolution experiments. Libraries of TadA-Cas9 fusions were created with mutations in the TadA cDNA and introduced into *E. coli* containing a defective chloramphenicol (CamR) resistance gene. Specific A•T → G•C conversion at precise sites in the CamR gene could restore Cam resistance, therefore Cam-resistant colonies were recovered and the sequences of the TadA variants determined. Two specific mutations were abundant among the recovered clones, A106V and D108N. Both mutant sequences were fused to a Cas9(D10A) nickase using the XTEN linker in a similar way to the previously developed BE systems and to an NLS to create the ABE1.2 system (TadA*-XTEN-nCas9(D10A)-NLS) (Gaudelli et al. 2017). Although the efficiency of the ABE1.2 system was only 3.2%, successive rounds of protein engineering and directed evolution vastly improved efficiency on subsequent ABE systems with ABE7.10 showing efficiencies ranging from 34% to 68% when tested on 17 different genomic sites and an average of 53% efficiency (Gaudelli et al. 2017). ABE systems usually showed lower indel mutations than CBE systems (<0.1%) and the frequency of undesired products was also very low, suggesting that enzymatic inosine removal is quite uncommon.

Off target effects and PAM-dependence

Off target effects, i.e. non-intended gRNA-independent base editing events have been amply documented in animal and plant systems (Kim et al. 2017a; Grunewald et al. 2019a; Jin et al. 2019; Kim et al. 2019; Liang et al. 2019; Zuo et al. 2019) (95, 96, 97, 99, 100), including RNA editing events (98), although in the case of plants the ABE system seems to produce less undesired effects (96). Base editors can also edit their own transcripts and those based on nCas9 can still introduce indels due to the unwanted production of DSBs (Komor et al. 2016; Nishida et al. 2016; Lei et al. 2018). As a consequence, efficient methods to detect genome wide off-targets effects have been developed (Kim et al. 2020). New CRISPR-based base editors are nevertheless being developed with reduced RNA off targets and self-editing activities (Grunewald et al. 2019b).

All BE systems developed using spCas9 are dependent on the availability of a G-rich PAM but that problem has been addressed by using spCas9 variants with different PAM recognition sequences (Miao et al. 2018; Nishimasu et al. 2018; Hua et al. 2019a), or Cas9 effectors from different origins, such a *Streptococcus aureus* (saCas9) (Komor et al. 2017). In addition, class 2 type V effectors such as Cas12a/Cpf1 have also been adapted for use in base editing applications further expanding the number of targets for these systems (Zong et al. 2017; Gallego-Bartolome et al. 2018; Hsu et al. 2019). High-fidelity Cas12a variants such as enAsCas12a-HF1 have also been adapted for use in base editing with reduced off-target effects (Kleinstiver et al. 2019). The use of Cas nickase activity in the production of base editors was instrumental in increasing efficiency but it also increased the incidence of unwanted DSB production leading to the introduction of indels. A recent report described a new cytosine base editing system called BEACON (base editing induced by human APOBEC3A and Cas12a without DNA break) that uses nuclease dead Cas12a fused to an engineered human apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (APOBEC3A). Editing efficiency was slightly lower than optimized systems such as BE3 and BE4max but indel introduction as well as RNA off-target editing was minimal with BEACON (Wang et al. 2020b).

RNA editing

The RNA binding ability of class 2 type VI Cas13 systems has been harnessed to develop RNA editing systems. Catalytically inactive Cas13 was fused to an RNA adenosine deaminase (ADAR2) to create the REPAIR (RNA Editing for Programmable A to I Replacement) system (Figure 2) (Cox et al. 2017). Screening of 36 different Cas13a and Cas13b orthologs identified *Prevotella* sp. P5-125 (PspCas13b) as the best starting effector to be fused to the deaminase domain of a hyperactive ADAR mutant ($ADAR2_{DD}$) showing low sequence-dependent constraints and enhanced adenosine to inosine editing rates to create the REPAIRv1 system. REPAIRv1 was able to produce targeted RNA editing of A to I, considered as G by the cellular machinery. Studies in mammalian cells revealed that the system was able to achieve up to 28% editing efficiency when targeted to correct 34 different G-A mutations causing human diseases but genome wide studies revealed the existence of a large number of off-target events. Further protein engineering of $ADAR2_{DD}$ produced the REPAIRv2 system with higher efficiency and, most importantly, vastly improved specificity (>919 fold) (Cox et al. 2017).

A cytidine to uridine RNA editor (RESCUE, RNA Editing for Specific C-to-U Exchange) (Figure 2) has been recently developed (Abudayyeh et al. 2019). The use of enzymes catalyzing the conversion of C to U had been described for DNA base editing (Komor et al. 2016; Nishida et al. 2016) but they have a number of inherent drawbacks such as the relatively elevated rate of off-target effects and the multiple deamination activity over a defined window (Grunewald et al. 2019a; Jin et al. 2019; Zuo et al. 2019). Abbudayyeh et al. (Abudayyeh et al. 2019) used a protein engineering approach to evolve $ADAR2_{DD}$ in order to change its native A to I activity into an C to U activity. For this purpose, $ADAR2_{DD}$ was fused to a catalytically inactive Cas13b from *Riemerella anatipestifer* (dRanCas13b) and residues contacting the RNA substrate in $ADAR2_{DD}$ were subjected to mutagenesis and 16 rounds of evolution resulting in cytidine deamination activity using a 30 nt guide. Interestingly, aside from the newly acquired C to U editing activity, RESCUE also retained the original A to I activity of the non-mutated $ADAR2_{DD}$. This characteristic allows to perform both kinds of editing with a single pre-crRNA construct, taking advantage of the native pre-crRNA activity of Cas13. Initial RESCUE systems were surprisingly efficient, with whole transcriptome profiling showing a ~80% C to U editing efficiency but they also produced 188 C to U off targets

and 1,695 A to I off targets, similar to the off target incidence observed in the version 1 of REPAIR. Further mutagenesis of $ADAR2_{DD}$ produced the RESCUE-S system, which maintained high on target efficiency (~76%) but dramatically reduced off target levels.

Base editing applications in plants

As mentioned above, many useful agricultural traits are conferred by one or a few SNPs (Doebley et al. 2006; Konishi et al. 2006; Zhao et al. 2011; Morris et al. 2013; Maccaferri et al. 2015; Wu et al. 2017), therefore it is not surprising that the first base editing applications in plants were aimed to induce base changes in chromosomal loci known to control important agronomic traits. Japonica rice varieties have lower nitrogen use efficiency (NUE) than indica varieties (Koutroubas and Ntanos 2003) due to a higher nitrate absorption in indica rice (Hu et al. 2015). Genetic analysis discovered a major QTL related to this trait and mapping identified a single SNP associated with the QTL consisting of a C to T change within the CDS of a nitrate transporter that causes a single amino acid substitution of threonine in Nipponbare (japonica) for methionine in IR24 (indica). An CBE base editing approach was performed by Lu and Zhu (Lu and Zhu 2017) in calli of the Zhonghua11 (ZH11) variety to introduce a C to T substitution in the NUE-associated SNP using an APOBEC1-XTEN-Cas9(D10A) fusion protein, achieving a 1.4% efficiency of correct substitutions. The same work reported much higher efficiency (11.5%) when targeting the SLR1 gene near the TVHYNP motif in the protein, which causes reduced plant height (Ikeda et al. 2001; Asano et al. 2009). T_0 lines with the desired base substitution showed the expected reduction in height (Lu and Zhu 2017). This early work highlighted several of the drawbacks of the technology such as the target-dependent variability in efficiency, the incidence of non-intended base replacement within the substitution window (C to G was detected at 1.6% – 3.9% frequencies), and the introduction of indels (1.6% – 7.2%). Similar problems were highlighted by another pioneering plant CBE work targeting 3 loci in the rice OsPDS and OsSBEIIb genes coding for phytoene desaturase and starch branching enzyme, respectively, achieving base replacement efficiencies ranging from 1.0% to 19.2% (Li et al. 2017b). Recently, optimized ABEs were developed by simplifying widely used ABEs, generating ABE−P1S (Adenine Base Editor−Plant version 1 Simplified), which has higher base conversion rates up to 96.3%, and ABE-P2S, which has expanded base editing windows (Hua et al. 2020b). Base editors have also been used in rice for base substitutions to increase yield,

affect nutritional qualities, enhance defense against pathogens, improve herbicide resistance and modify architecture (Li et al. 2017b; Zong et al. 2017; Hua et al. 2018; Ren et al. 2018; Yan et al. 2018a; Hua et al. 2019b; Li et al. 2019b).

An obvious and very popular use of base editing has been the introduction of herbicide tolerance by creating mutations in the amino acid sequences of several proteins targeted by commercial herbicides. Multiplexed base editing of the acetolactate synthase (*ALS*) gene using an activation-induced cytidine deaminase produced multiple mutations leading to herbicide resistance in rice (Shimatani et al. 2017) and CBE-based approaches were also able to successfully produce herbicide resistant Arabidopsis plants (Chen et al. 2017; Dong et al. 2020). The ABE system has also been used to produce herbicide-resistant rice targeting the *ALS* gene (Li et al. 2018b; Wang et al. 2019b). Herbicide tolerance has been incorporated into the allotetraploid oilseed rape targeting the *ALS* gene with a human A3A cytidine deaminase or a rat cytidine deaminase fused to a Cas9 nickase and uracil glycosylase inhibitor (Cheng et al. 2020; Wu et al. 2020). CBE systems have also been used to mutagenize the *ALS* gene to produce herbicide tolerant maize and watermelon (Tian et al. 2018; Li et al. 2020g). Tolerance to sulfonylurea-, imidazolinone- and aryloxyphenoxy propionate-type herbicides has been achieved in wheat by targeting the ALS and acetyl-coenzyme A carboxylase genes with a CBE system (Zhang et al. 2019c). In addition, the efficiencies of co-editing of multiple targets was strongly increased when coupled with a specific editing at the proline residue in the ALS protein which confers moderate tolerance to the nicosulfuron herbicide providing a selectable system (Zhang et al. 2019c). A creative approach was used in tomato and tetraploid potato to produce transgene-free herbicide resistant plants (Veillet et al. 2019). A CBE system was used in a binary vector and Agrobacterium-mediated transformation performed followed by a two week selection period in antibiotic (kanamycin) containing media, after which cultures were transferred to media containing the herbicide chlorsulfuron but no antibiotics. Base edited tomato and potato plants were recovered from tissue culture but, most importantly, 12.9% and 10% of the edited tomato and potato plants respectively did not contain transgenes, an important issue when working with vegetatively propagated species such as potato. Massive use of herbicides in agriculture has accelerated the evolution of herbicide resistant weeds and it is therefore important to develop tolerance to new or infrequently used herbicides in crops. Trifluralin and other dinitroaniline herbicides target tubulin proteins and some mutations in tubulin genes can confer agronomically

significant tolerance (Lyons-Abbott et al. 2010; Chu et al. 2018). Development of dinitroaniline resistance in weeds is quite low compared to other commercial herbicides (Heap 2014). Introduction of a previously known missense mutation in the alpha-tubulin gene of goosegrass (*Eleusine indica*) (Yamamoto et al. 1998) into the rice OsTubA2 gene using an ABE system produced rice lines resistant to otherwise lethal levels of trifluralin and pendimethalin without fitness penalty (Liu et al. 2020b).

Saturation mutagenesis of genes encoding herbicide targets is a promising strategy to find new resistant protein variants. A strategy named base-editing-mediated gene evolution has been used to produce completely new herbicide resistance alleles in rice (Kuang et al. 2020). This method uses Cas9n-based cytosine and adenine base editors as well as a gRNA library tiling the full-length coding region of the targeted gene. When the method was used to target the *ALS* gene, the researchers discovered four amino acid substitutions in two positions of the protein conferring herbicide resistance which were previously unknown by natural or human selection during rice domestication (Kuang et al. 2020). Another strategy to generate *de novo* mutations uses saturated targeted endogenous mutagenesis editors (STEME) with capacity to edit cytosine and adenine at the same target site. The STEME-NG editor is a single fusion protein containing the human cytidine deaminase APOBEC3A, an evolved *E. coli* tRNA adenosine deaminase (ecTadA-ecTadA7.10), the nCas9-NG (D10A) nickase, the uracyl DNA glycosilase inhibitor UGI and two NLS. Li et al. (Li et al. 2020a) used the STEME-NG with a Cas9 variant recognizing an NG PAM, which is less restrictive than the native NGG, and 20 different gRNAs to target a 56 amino acid region of the rice acetyl-coenzyme A carboxylase (OsACC), producing near-saturation mutagenesis. Herbicide resistant mutations were recovered in rice plants subjected to directed evolution of the OsACC gene (Li et al. 2020a). Yet another base editing multiplex approach has been used to target the carboxyltransferase domain of OsACC using an enhanced ABE system and three different base editing libraries to produce transgenic lines in the elite japonica rice variety Feigeng2020 (Liu et al. 2020c). Two new mutations W2125S and C2186R in the OsACC protein conferred tolerance to the commercial herbicide Gallant, belonging to the aryloxyphenoxypropionate herbicide group, in transgenic plants. Unfortunately serious growth retardation and complete sterility were observed in glasshouse grown W2125S and homozygous C2186R plants (Liu et al. 2020c).

PRIME EDITING

Base editors opened a new door into the realm of possibilities in the genome editing sphere but are still limited to four transition mutations; i.e. C•G → T•A for CBEs and A•T → G•C for ABEs. The eight possible transversion mutations, i.e. C→A, C→G, G→C, G→T, A→C, A→T, T→A, and T→G, were not possible to perform accurately until the advent of Prime Editors (PE). The idea behind PEs is to combine a nickase with a reverse transcriptase (RT) and an extended gRNA, the prime editing gRNA (pegRNA), containing the genetic information to correct the target sequence. The 5' end of the pegRNA contains a canonical guide and gRNA scaffold for the nCas9 to bind the correct target and perform a nick in the complementary strand. The 3' of the pegRNA contains an extension with a primer binding site (PBS) that binds to the 3' end of the nicked DNA strand up until the nick site followed by a sequence containing the intended edits which will serve as template for the RT module. Reverse transcription of the template will create an ssDNA extension containing the edited sequence copied from the pegRNA producing an intermediate DNA molecule containing two possible ssDNA flaps; an unedited 5' flap and an edited 3' flap. 5' flaps are more prone to be excised by 5' exonucleases and structure-specific endonucleases, thus favoring the formation of a heteroduplex containing the wild type and edited strands which will be converted into a homoduplex by the DNA repair machinery (Figure 3).

To create the first generation system (PE1), Anzalone et al. (2019) fused the wild type Moloney murine leukemia virus (M-MLV) RT to the C-terminus of the Cas9(H840A) nickase, and used different PBS lengths (8-15 nt). PE1 successfully introduced transversion point mutations at five different genomic loci with efficiencies reaching up to 5.5% and targeted insertions/deletions with 4%-17% frequencies. Efficiency improvements were achieved by engineering the M-MLV RT, introducing mutations previously characterized to enhance RT performance at elevated temperatures, binding to the template–primer complex and enzyme processivity. Introduction of five simultaneous mutations into the WT M-MLV RT produced the best results and the fusion Cas9(H840A)–M-MLV RT(D200N/L603W/T330P/T306K/W313F), named PE2, produced a 1.6 to 5.1 folds of improvement over PE1 in point mutations and consistently better efficiencies in targeted insertions and deletions. Extensive experimental studies failed to develop rigid rules for the design and predicted efficiencies of the pegRNAs,

with no clear relationship between PBS length or G/C content level and editing efficiency, although a PBS of 13 nt was recommended. Different target sites favored different RT template lengths, with no clear relationship between sequence composition and optimal length, although as a rule of thumb an initial 10−16 nt template length was recommended before testing longer and shorter lengths. Interestingly, RT templates starting with C immediately after the gRNA scaffold seemed to produce lower editing efficiencies, making it advisable to avoid extensions starting with C. A third generation PE3 system was produced by introducing an additional nick on the non-edited strand with the intention to direct the DNA repair machinery to use the edited strand as template. Technically, this step is relatively straightforward as it only needs to provide an additional canonical gRNA to guide the existing Cas9(H840A) nickase activity to the additional target site. It is important to position the additional nick away from the original one used for editing to avoid DSBs as they increase the incidence of unwanted indels. Introducing an additional nick in the non-edited strand 40−90 bp 3' of the pegRNA-mediated nick, increased editing efficiency in four out of five target loci to an average of 41%, although the incidence of indels also increased to >20% in some extreme cases. As a compromise, a distance of ~50 bp from the pegRNA-mediated nick was recommended for the secondary nick. In an effort to reduce the incidence of indels, the authors attempted to introduce the secondary nick using a gRNA targeting the edited strand, delaying the secondary nick until the editing had been achieved. With this strategy, termed PE3b, editing efficiencies did not differ from the PE3 system but incidence of indels decreased to 0.74%.

Comparison of PE2 & PE3 with the BE4max CBE system revealed that BE4max provided better efficiencies (2.2 fold) for positions in the center of the editing window while PE3 showed better efficiency at positions outside the center of the window (2.7 fold) and similar results were observed for ABE systems. In terms of accuracy, i.e. production of precise editing events, the prime editing system clearly outperformed BE4max. In genomic contexts containing several cytosines within the window, most of the BE4max contained multiple edits while PE3 was able to produce base substitutions at any site with extreme precision. In summary, when there is only one target within the editing window, or when bystander edits are not problematic, base editors seem to be preferable as they provide higher efficiencies and lower indels incidence. If several cytosines or adenines are present in the editing window and any unwanted conversions

are unacceptable, PE systems should be the preferred option. Of course, for all the edits that are not possible with base editors, prime editors are the only option.

A remarkable characteristic of prime editors is their exceptional low incidence of off-target editing. Off target activity in CRISPR/Cas systems is a not an issue for crop breeding, but is a serious concern for applications in animal gene editing, especially for therapeutic applications. Anzalone et al. (2019) tested the off-target effects of the PE2 and PE3 systems using 16 pegRNAs targeting four genomic loci, each of which having several well-characterized Cas9 off-target sites. Cas9-induced mutations in the top four known Cas9 off-target loci for each sgRNA were observed with average frequencies of 16 ± 16%, 60 ± 26%, 48 ± 28%, and 4.3 ± 5.6%. In comparison, PE2 and PE3 using 16 pegRNAs for the same targets produced off-target editing in only 3 of the 16 off-target sites with only one showing >1% frequencies. This remarkable accuracy might be due to the fact that, in order to produce an editing event, PE systems require three pre-requisites to produce an edit: nCas9 binding requires complementarity between the target DNA and the pegRNA spacer, initiation of the pegRNA-guided RT requires complementarity of the PBS with a different region of the target DNA, and finally flap resolution requires complementarity of the RT product with the target DNA.

The original prime editor, with proper adaptations has been successfully used in rice and wheat protoplasts (Lin et al. 2020). The components of the PE2 and PE3 systems were codon optimized for cereals in order to build the editing vectors and expressed under the control of the maize ubiquitin promoter, named PPE2 and PPE3 for Plan Prime Editor 2&3 respectively. In addition to the M-MLV RT (D200N/L603W/T330P/T306K/W313F) used in PE2 and PE3, two additional RTs were tried, the cauliflower mosaic virus RT (PPE-CaMV) and a retron-derived RT (PPE-retron) from *E. coli* BL21 (Plant et al. 1985; Lim and Maas 1989). Prime editing experiments were performed targeting six genes in rice and six genes in wheat with 21 pegRNAs. The editing efficiency was highly variable ranging from 0.2% to 8.2% for the rice editing targets while wheat showed lower efficiencies with a maximum of 1.4%. Opposite to mammalian observations, PPE3 and PPE3b did not show increased efficiency compared to PPE2, suggesting that the introduction of an additional nick does not have a positive effect in plant protoplasts. PPE editing byproducts, including pegRNA scaffold insertions or replacements were observed at 6 out of the 21 tested targets at frequencies ranging from 0.5% to 4.9%. The

PPE-CaMV and PPE-retron systems successfully produced prime editing events although at lower frequencies than the PPE2 and PPE3 systems. Increasing temperature from 26^{o}C to 37^{o}C increased editing efficiency although the high temperature is not optimal for plant tissue culture, highlighting the need to find RTs with high efficiency at optimal plant culture temperatures. Different experiments tested the effect of pegRNAs, PBS length (6–16 nt), RT template length (7–23 nt), and nicking sgRNA position (−128 to +209). Although all these parameters had strong effects on efficiency, a clear trend could not be observed, indicating the need to optimize all parameters for each new target. A number of transgenic rice lines were produced and prime editing-induced mutations were observed in several of them but unfortunately all plants were chimeras.

The rice ALS gene has been targeted to introduce two nucleotide substitutions, a G → T to convert tryptophan 548 to leucine, providing resistance to the herbicide Bispyribac sodium, useful for the post-emergence control of broad-leaf weeds, and a silent G → A substitution that results in the destruction of the PAM site to prevent successive targeting of the site (Butt et al. 2020). The Cas9(H840A)–M-MLV RT(D200N/L603W/T330P/T306K/W313F) cassette from PE2 was cloned under the control of the rice ubiquitin promoter. A pegRNA containing a 13 nt PBS and a 15 nt RT template was expressed under the control of the OsU3 promoter. Rice calli were transformed using *Agrobacterium* and placed on selection for two weeks before deep sequencing of PCR-amplified target products, which revealed successful editing with efficiencies of 0.26% – 2%. Two additional endogenous genes IDEAL PLANT ARCHITECTURE 1 (OsIPA) and TEOSINTE BRANCHED 1 (OsTB1) were also targeted with pegRNAs containing 13 nt PBSs and 20 nt RT templates. Successful editing was observed for both targets, although partial editing events were observed in the OsTB1 target. The PE3 strategy was also pursued using the polycistronic tRNA system (Xie et al. 2015) to co-express the pegRNA and an additional gRNA targeting a position 55 bp away from the pegRNA nicking site. Transformation of rice calli with the ALS-PE2 and ALS-PE3 systems was followed by antibiotic selection supplemented with Bispyribac sodium to recover herbicide resistant shoots. Successful editing was observed in the recovered plants and, as observed by Lin et al. (2020), similar efficiencies were obtained for the PE2 and PE3 systems.

A plant prime editor, termed PPE3-V01, was constructed with a plant codon optimized nCas9-RT module from PE3, and two additional expression modules for the pegRNA and the additional gRNA under the control of the OsU3 and OsU6 promoters respectively (Tang et al. 2020). Five different sites on four rice genes were targeted using PBS and RT templates of 13nt each and the additional gRNA targeting sites within 100 bp from the editing site. Transfection of rice protoplasts followed by deep sequencing of amplicons revealed the presence of successful edits, albeit at a very low frequency (0.05% − 0.15%). Deletions were also observed probably due to the presence of two simultaneous nicks. The PE3b strategy, using gRNAs matching the edited strand, combined with the use of different RT template guide lengths produced slightly better results with editing frequencies up to 0.4%. Unintended incorporation of extended sequences upstream of the RT template was also observed. A second PE version, named PPE-V02, incorporated additional codon optimization for the nCas9-RT fusion protein, different NLS configuration and a different terminator for the nCas9-RT expression cassette. PPE3-V02 was used to target 3 different loci using different combinations of PBS and RT template lengths in rice protoplasts. The choice of PBS-RT template had an important effect on the editing efficiency although no obvious link between efficiency and PBS-RT template length was observed. The PPE3-V02 system achieved better efficiency than the PPE3-V01 with editing frequencies reaching up to 1.5%. In agreement with the previous two studies, the PPE3 system did not show any improvement in editing efficiency over the PPE2 system. However, in contrast with the observations by Lin et al. (2020), increasing the temperature from 32^{o}C to 37^{o}C did not improve editing efficiency.

An ZmUbi Promoter-NLS-nCas9(H840A)-Linker1(33aa)-M-MLV-RT-Linker2(14aa)-NLSPolyA-E9 cassette was used for prime editing in rice by Li et al. (2020b). The system was used to repair a defective hptII gene containing two artificially introduced stop codons on the premise that successful prime editing of the defective hptII gene after incorporation in the plant genome should provide hygromycin resistance in the transgenic lines. The pegRNA contained a 13 nt PBS and a 28 nt RT template containing two synonymous mutations and two mutations to restore the two stop codons into the original amino acid-encoding codons in the hptII gene. The pegRNA was co-expressed with a gRNA targeting the non-edited strand 50 nt upstream of the pegRNA target using the polycistronic t-RNA strategy. Rice calli was transformed by particle bombardment and calli growing strongly

on hygromycin-containing media regenerated to produce full plants, with three out of the 32 recovered plants (9.38%) containing a correctly edited hptII gene. Using a similar strategy, the authors targeted the 5-enolpyruvylshikimate-3-phosphate synthase (OsEPSPS) gene for prime editing. A pegRNA containing a 13 nt PBS and a RT template containing 3 synonymous mutations as well as additional mutations to replace three amino acids in the sequence of the OsEPSPS protein that confers a high level of resistance to the herbicide glyphosate. The additional nicking gRNA targeted a position 66 nt downstream of the pegRNA target. After transformation, 45 independent T1 transgenic lines were produced with one heterozygous line containing the intended edits. Additional lines containing insertions, deletions and partial edits were also identified and mutations at potential off-target loci were detected.

In contrast with the previous reports, which used mostly protoplasts, Hua et al. (2020a) reported prime editing work results based mostly in regenerated transgenic rice lines. The Cas9(H840A)–M-MLV RT(D200N/L603W/T330P/T306K/W313F) cassette under the control of the maize ubiquitin promoter was used to successfully repair an artificially mutated EGFP gene containing two successive stop codons (TA**G** **T**GA) by prime editing, converting them into TA**C** **G**GA (tyrosine–glycine) in rice (Hua et al. 2020a). The pegRNA was expressed under the control of the OsU6 promoter and contained a 13 nt PBS and a 13 nt RT template targeting the mutated EGFP (Sp-PE2). An additional PE3-type vector was also prepared adding the nicking gRNA as an independent expression cassette driven by the OsU6 promoter. *Agrobacterium*-mediated transformation of rice calli was performed and hygromycin-resistant calli genotyped. Analysis of over 30 lines for each construct showed 17.1% and 15.6% efficiency of successful editing for spPE2 and spPE3 systems respectively, confirming the observations that PE3 does not enhance prime editing efficiency over PE2 in plant systems. All lines showing successful editing also showed restoration of GFP fluorescence. Targeting of five endogenous genes with the SpPE3 system produced successful editing in only one of the targets at a frequency of 9.1%, failing to produce edited transgenic lines for the remaining four genes. The authors studied the efficiency of Cas9 effectors from a different origin by replacing the spCas9(H840A) nickase in the SpPE3 prime editor with SaCas9 (N580A) but the observed prime editing efficiencies were lower than those observed for the spPE3 system. The low editing success observed in this study indicates that the prime editing efficiencies reported for protoplast work do not necessarily translate

when producing full transgenic pants. Consistently, Lin et al. (2020) reported editing in some transgenic lines but all plants containing edits were chimeras, while Butt et al. (2020) and Li et al. (2020b) used positive selection from successful editing events (resistance to herbicide) as an additional selectable marker to enrich the production of plants with successful edits.

Xu et al. (2020) has also reported prime editing results in rice plants using derivatives of the PE3 system. The rice Ubiquitin promoter controlled the expression of the Cas9(H840A)–M-MLV RT(D200N/L603W/T330P/T306K/W313F) cassette, with pegRNA and nicking gRNA under the control of the rice U3 and U6a promoters respectively, generating a prime editor named PE-P1. The OsALS gene was targeted with the prime editor designed to (1) perform a G to T conversion, which is not possible with the existing CBE systems, in order to confer herbicide tolerance by producing a W548L substitution in the protein sequence or (2) a C to T conversion in order to introduce a P171S substitution. Three pegRNAs were designed with different PBS and RT template lengths and the non-edited strand nick was designed to be located 55 nt away from the pegRNA editing site. *Agrobacterium*-mediated transformation was performed and almost 90 transgenic lines were recovered for each pegRNA. Sequence analysis revealed that two of the pegRNAs produced the desired editing (G → T or C → T) at editing efficiencies of 1.1% while the third one failed to produce any edited lines. The same system was used to target the ACCase gene with two pegRNAs intended to achieve single nucleotide conversions (A → G and G → C). No A → G editing was observed in 68 T_0 lines while one editing event was detected for the G → C substitution from a total of 70 T_0 transgenic lines (1.4%). Mutation of the PAM did not increase editing efficiency as reported by Anzalone et al. (2019) with only one out of 83 transgenic lines showing successful editing (1.2%). A second version of the system was achieved by fusing the hygromycin phosphotransferase selectable marker to the 3'end of the nCas9-RT module using the P2A self-cleaving peptide and using an enhanced esgRNA (Li et al. 2018b). All pegRNAs previously used in the PE-P1 system were also used in this modified system to produce transgenic rice lines, and genotyping showed important efficiency increases, ranging from 3 to 22 fold, in virtually all pegRNAs (Xu et al. 2020). Another case of optimized prime-editing tools by optimizing pegRNA expression was recently reported with high efficiency up to 53.2% (33/62) in transgenic maize harboring S621I mutations in *ZmALS1* (Jiang et al. 2020). Although an efficiency of either 26% (in rice) or even

53.2% (in maize) was obtained for one pegRNA, most values were still quite low, reinforcing the observations by Hua et al. (2020a) that optimization of the system is necessary before it can be used to produce edited full plants.

All of the above applications of prime editing have been undertaken in monocots, especially rice plants. For unknown reasons, base editing was found to be much less efficient in dicots compared to monocots like rice and maize (Kang et al. 2018; Mao et al. 2019a). Therefore, it is a question whether prime editors may function in dicotyledonous plants as well as in monocots. Lu et al (2020) answered the question by testing prime editors in tomato protoplasts and plants. The authors found that the use of the ribosomal protein S5A (*RPS5A*) promoter of tomato to drive the expression of nCas9-hMMLV substantially improved the prime editing efficiencies compared to the CaMV 35S promoter, presumably by increasing the nCas9-hMMLV protein level. Prime editing was tested in transgenic tomato plants at three genes, *GAI*, *ALS2* and *PDS1*, with successful editing at *ALS2* and *PDS1* with frequencies of 6.7% and 3.4% respectively. Like most of the studies in rice, the prime edited tomato plants were all chimeras (Lu et al. 2020).

GENE TARGETING

Gene targeting (GT), i.e. the replacement of endogenous genes or DNA regions by homologous recombination, could prove extremely useful for crop breeding and trait improvement but the development of efficient GT systems has eluded plant scientists until recently (Li et al. 2020d). Of the two main DNA repair systems in plants, NHEJ is the predominant one in somatic cells while HDR plays a very minor role (San Filippo et al. 2008). The initial attempts at HDR-mediated GT in plants revealed ridiculously low efficiencies, 10^{-3} – 10^{-7} (Paszkowski et al. 1988; Offringa et al. 1990) and complicated positive-negative strategies only provided modest increases and their application was restricted to a few species (Gallego et al. 1999; Terada et al. 2002). A number of approaches have been developed over the years to increase GT efficiency and although the introduction of DSBs has proven to be promising, the efficiency is still impractically low for most applications (Puchta et al. 1996; Puchta and Fauser 2013; Steinert et al. 2016; Sun et al. 2016a; Miki et al. 2017). Aside from introducing a DSB, it is crucial to have enough amount of donor DNA in order to have efficient HR, and geminiviruses have been extensively used to provide high template quantities. Baltes et al. (2014) provided the first example combining ZFNs, TALENs and CRISPR/Cas9 with the bean

yellow dwarf virus in order to increase GT frequency in tobacco 1-2 orders of magnitude over conventional *Agrobacteriu*m T-DNA methods. HR-mediated insertion of the CaMV 35S promoter in front of the ANT1 gene in tomato was achieved using a similar geminiviral strategy (Cermak et al. 2015) and successful replacement of the ALS gene with a herbicide tolerant variant of the gene was reported in potato (Butler et al. 2016). A monocot geminivirus infecting most cereals, the wheat dwarf virus (WDV), was adapted to provide repair templates in rice, which helped to achieve successful GT (Wang et al. 2017a). The same geminivirus, WDV, was used to attempt GT in wheat cells aiming to introduce a promoterless GFP gene into the third exon of the ubiquitin gene (Gil-Humanes et al. 2017). In this work, WDV vectors containing Cas9 and gRNA expression cassettes, together with the promoterless GFP, flanked by two homology regions to induce HR were bombarded into wheat protoplasts, achieving GT efficiencies up to 3.8%. Bombardment of wheat scutella was also attempted with the number of GFP expressing cells quantified seven days after bombardment. Using this method 65% of bombarded scutella showed GFP fluorescent cells versus 12.1% when using defective geminiviral vectors unable to replicate. Unfortunately, no plants were recovered, raising questions about the practicality of the approach. GT by homologous recombination was reported in soybean using the hygromycin phosphotransferase gene as part of the donor DNA for resistance to hygromycin during the selection process (Li et al. 2015). A few calli suspected to have positive GT events were recovered but only one homology-directed recombination event was transmitted to the T_1 generation. A different strategy was pursued in rice using two sequential transformations, an initial transformation transferring the Cas9 expression cassette was followed by a second one transferring the donor DNA to replace the *ALS* gene with a variant containing two point mutations, W548L and S627I, conferring tolerance to the herbicide bispyribac sodium (BS) (Endo et al. 2016b). Interestingly there were no significant differences in the GT efficiency between Cas9-expressing and not expressing cells. Addition of a gRNA targeting the DNA ligase 4 gene increased GT efficiency with values ranging from 0.15% to 1% (Endo et al. 2016b). Combined delivery of all-in-one vector (containing Cas, sgRNAs and donor DNA) and free donor proved that the sufficiency of the donor DNA is crucial to the frequency of gene replacements or knock-ins via HDR (Sun et al. 2016b; Li et al. 2018c). GT has also been attempted by targeting an intron with Cas9 in an attempt to increase efficiency (Li et al. 2016a). Replacement of the endogenous

5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) with a variant conferring resistance to the herbicide glyphosate was achieved at a 2.0% frequency. It is important to remark that in most of the above examples, GT was achieved with additional help of positive selection provided by the introduction of an antibiotic or an herbicide resistant gene as part of the introduced genetic element involved in the HR event.

A crop improvement approach has been described in maize to increase yield through GT. Maize plants overexpressing ARGOS8, a negative regulator of ethylene responses, under the control of the ubiquitin promoter showed increased yield in field trials under drought conditions (Shi et al. 2015). With the aim to reproduce the drought resilient phenotype of the transgenic plants, Shi et al. (2017) attempted to increase ARGOS8 expression by either introducing the maize GOS2 promoter directly upstream of the *ARGOS8* gene or replacing the native ARGOS8 promoter with the GOS2 promoter. Immature embryos were bombarded with vectors containing Cas9 and either one gRNA (for promoter insertion) or two gRNAs (for promoter swap) and the GOS2 promoter flanked by ~400 bp homologous linkers. From approximately 1,000 bombarded embryos, one single plant was obtained containing the promoter insertion and two plants containing the promoter swap. Field tests showed that the GT plants produced on average five bushels per acre more than WT plants under flowering stress conditions, while no yield differences were observed under well-watered conditions. The GT efficiency in this example, without the use of additional antibiotic or herbicide positive selection was 3 out of 1000 embryos bombarded, stressing the need for further improvements before GT can be used in routine plant breeding.

The staggered ends produced by class 2 type V Cas12 effectors could increase the frequency of HR and some groups have reported the use of Cas12a for GT purposes. Li et al. (2018d) used LbCas12a to induce DSBs in the rice ALS gene and attempted replacement by an ALS variant conferring tolerance to the BS herbicide. Transgenic rice calli resistant to hygromycin were placed in an additional positive selection pressure by growing them in media supplemented with 0.4 μM BS. Out of 165 plants regenerated using this strategy three chimeric individual plants were identified containing one allele with a precise gene replacement. The same group changed some of the parameters in the design of the transgene delivery vectors, achieving an increased GT frequency of 1.8%. Moreover, to overcome the limitation of introducing enough donor DNA into plant cells,

an RNA transcript-template HDR (TT-HDR) approach was developed to produce abundant donor RNA which derved as HDR template and generated desired mutations in the rice *ALS* gene with HDR efficiency of 4.6% and stable heredity (Li et al. 2019c).

An alternative strategy, yielding high GT efficiency in *Arabidopsis* was described by Miki et al.(2018) using germline promoters, rather than the CaMV 35S to drive the expression of Cas9. Four promoters were used to produce transgenic Arabidopsis lines, including two shoot apical meristem promoters (YAO and CDC45) (Stevens et al. 2004; Yan et al. 2015), a pollen specific promoter (Lat52) (Mao et al. 2016) and an egg cell and early embryo-specific promoter (DD45) (Steffen et al. 2007; Wang et al. 2015; Mao et al. 2016). These transgenic lines were then transformed again with a construct containing a gRNA expression cassette and donor DNA using a different selectable marker. For each doubly transformed line, 20-30 seeds were germinated without selection and tested for successful integration events by PCR and seeds from lines testing positive were then individually re-tested. Five experiments targeting different loci with different donor templates showed GT efficiencies between 6.3% and 9.1% (Miki et al. 2018), a remarkable success given that GT events were produced and tested in whole plants without the help of any positive selection. Interestingly, delivery of all elements in a single transformation did not produce any heritable GT events. However, adding the omega translational enhancer from tobacco mosaic virus to the CRISPR/Cas construct to boost the Cas9 protein level made a difference, generating heritable GT events at the ROS1 locus at a frequency of 2.4% (Peng et al. 2020). Wolter et al. (2018) also attempted GT in *Arabidopsis* without the help of positive selection using an egg cell specific promoter but used the *Staphylococcus aureus* Cas9 (SaCas9) instead of the spCas9, achieving 1% GT successful frequency. Using Agrobacterium-mediated immature embryo transformation in maize, Barone et al. (2020) designed a strategy using heat-inducible Cas9 to generate targeted DSBs and simultaneous activation of the selectable marker gene, which produced up to 4.7% non-chimeric heritable GT in one generation.

The GT method mediated by the egg cell- and early embryo-specific DD45 promoter-driven Cas9 (Miki et al. 2018; Peng et al. 2020) is quite efficient in *Arabidopsis* but its usefulness requires floral dipping transformation where Agrobacteria enter the ovule and deliver the donor DNA for HDR in the egg cell or early embryo. However,

most plants cannot be transformed using the floral dipping method. Recently, Lu et al. (2020) reported a method for precise insertion or replacement of sequences of up to 130 bp in rice. The authors firstly developed a method for efficient insertion of double stranded donor DNA in rice through particle bombardment. They found that double stranded DNA flanked by two nucleotides with phosphorothioate-linkage at each end and with 5′-phosphorylation could be efficiently inserted in at the Cas9 DSB site, presumably through the NHEJ pathway. Both short and long sequences, up to 2 kb could be inserted, although the insertion efficiencies were lower for long fragments, likely because long fragments were produced using PCR amplification and consequently only one DNA end was modified with phosphorothioate-linkages. Building on this efficient insertion method, Lu et al. (2020) developed the tandem repeat (TR)-facilitated HDR (TR-HDR) technology for precise sequence insertion or replacement (Figure 4). The approach was based on the observation that production of a DSB in the middle of a tandem repeat such as GUUS or GFFP frequently triggers HDR, producing functional GUS or GFP genes (Mahfouz et al. 2011; Mao et al. 2013). In TR-HDR, a dsDNA donor with phosphorothioate-linkage and 5'-phosphorylation modifications and sequence homology to the target locus is inserted adjacent to the target sequence, using CRISPR/Cas. The donor DNA is designed such that, upon insertion, it generates a target site for the same original gRNA. The second DSB at the regenerated target site would then trigger HDR, causing seamless insertion of part of the donor DNA or precise replacement of the target sequence with the donor sequence. Using this TR-HDR approach, the authors achieved precise replacement of sequences up to 130-bp in length with efficiencies of up to 6.1%. Aside from GT applications, this method could also be useful for in-locus tagging of proteins with FLAG or HA epitopes where seamless insertion is required (Lu et al. 2020).

GENE TRANSCRIPTION CONTROL & EPIGENOME EDITING

The ability to direct Cas effectors to precise chromosomal loci using gRNA molecules can be exploited in a variety of ways to alter gene expression patterns. As an example, dCas9 can be fused to transcriptional activators and repressors to affect the expression of targeted genes (Cheng et al. 2013; Gao et al. 2014). dCas9-based transcriptional activation systems, such as VPR, SAM and SunTag, have been developed for mammalian systems and some of them have been adapted for plant use (Figure 5; Gilbert et al. 2014; Tanenbaum et al. 2014; Chavez et al. 2015; Konermann et al. 2015; Chavez et al. 2016).

The VPR system uses a hybrid VP64-p65-Rta tripartite activator fused to the C-terminus of dCas9 (Chavez et al. 2015), where VP64 is a tetramer of the powerful Herpes simplex viral protein 16 (VP16) (Wysocka and Herr 2003); p65 is the product of the human RELA gene (Nolan et al. 1991) and Rta is a powerful activator from the Epstein-Barr virus (Hardwick et al. 1992). The synergistic activator mediator (SAM) is a three component system comprised of a fusion of dCas9 with VP64, MS2–p65–HSF1 and a modified gRNA (Konermann et al. 2015) and its adaptation to plants is explained below. The SunTag system uses a fusion of dCas9 to the SunTag epitope, a peptide containing GCN4 peptide repeats which can recruit multiple copies of a single chain antibody (scFV) fused to the sfGFP and a VP64 activator (Tanenbaum et al. 2014; Konermann et al. 2015). Although VP64 has proven to be very powerful in mammalian cells, dCas9-VP64 fusions do not seem to provide strong gene activation in plant systems (Piatek et al. 2015; Vazquez-Vilar et al. 2016; Li et al. 2017d; Lowder et al. 2018). Plant based activators such as the EDLL motif found on some ethylene response factor/apetala 2 family members (Tiwari et al. 2012) seem to provide stronger gene activation when fused to dCas9 (Tiwari et al. 2012; Piatek et al. 2015; Li et al. 2017d). Interestingly the combination of VP64 and EDLL fused to dCas9 does not produce significant gene activation (Lowder et al. 2018). A different strategy by the same authors, based on the SAM system, used a modified gRNA architecture introducing aptamers into the gRNA to bind the bacteriophage coat protein MS2 (Konermann et al. 2015), with each of the loops having the capacity to bind two MS2 molecules, and was named CRISPR-Act2.0. Expression of a dCas9-VP64-T2A-MS2-EDLL polypeptide that would ultimately yield two different proteins, a dCas9-VP64 fusion and a MS2-EDLL fusion, produced spectacular results with RNA transcript levels reaching up to 30 and 34 fold increase in two different targeted genes (Lowder et al. 2018). Replacement of the EDLL motif by VP64 produced even better results with up to 50 fold increase in mRNA levels for one of the targeted genes and up to 1,500 fold for the second (Lowder et al. 2018). Li et al (Li et al. 2017d) used 6 copies of the TAL transcription activator domain from *Xanthamonas* TALEs and two copies of VP64 fused to the C-terminus of dCas9, named dCas9-TV system, to produce between 37 and 192 fold increase in mRNA transcript levels in 3 different *Arabidopsis* genes. The Suntag system has been used in *Arabidopsis* to elicit robust and specific activation of several genes in diverse chromatin contexts (Papikian et al. 2019). Given the nature of CRISPR, multiplexing gene activation is as simple as

expressing multiple gRNAs. Co-expression of three sgRNAs targeting WRKY30, RLP23 and CDG1 successfully achieved activation of all targeted genes in *Arabidopsis* using the CRISPR-TV system (Li et al. 2017d). Similarly, transcriptional activation of three rice genes (Os03g01240, Os04g39780, and Os11g35410) was achieved using the CRISPR-Act2.0 system (Lowder et al. 2018). Transcriptional repression can also be achieved simply by directing dCas9 to the gene in a way that interferes with transcription, a technology known as CRISPR interference (CRISPRi) (Larson et al. 2013; Qi et al. 2013). In addition, fusion of transcriptional suppressors to dCas9 and Cas12a/Cpf1 can also interfere with gene expression (Piatek et al. 2015; Tang et al. 2017b).

The above described Cas-based methods to modify gene expression patterns are extremely useful for genetic and gene function studies but have the inherent drawback of needing the presence of a transgene in the genome in order to sustain the modified expression profiles. In order to develop applications for crops and avoid the regulations associated with genetic modification (GM) it would be advisable to use methods in which the expression changes remain after the transgenes are segregated out. Epigenetic modifications could provide such a solution as many of them are stably inherited in plants (Hauser et al. 2011; Paszkowski and Grossniklaus 2011; Holeski et al. 2012; Saze 2012). Even though there has been progress in understanding the complexity of the epigenome, including histone and DNA modifications, the functions and regulation of the epigenome are still poorly understood (Zhang et al. 2018a; Chang et al. 2020). DNA methylation in plant genomes involves the addition of a methyl group to the cytosine C5 carbon to produce 5-methylcytosine (5mC). Methylation is found in the CG, CHG (symmetric) and CHH (asymmetric) sequence contexts (where H is A, C or T), and plays essential functions in growth and development as well as the response to multiple stresses in plants and mammalian systems (He et al. 2011; Zhang et al. 2018a; Chang et al. 2020). The methylation status of promoter and other regulatory regions play a critical role in gene expression, with promoter methylation mostly inhibiting gene expression although in some cases it can also promote expression (Harris et al. 2018; Xiao et al. 2019).

Before the advent of CRISPR-based methylome editing, targeted methylation of the FWA promoter using a zinc finger fused to the RNA-directed DNA methylation factor SUVH9 achieved specific and heritable silencing in *Arabidopsis* (Johnson et al. 2014). The Suntag system has been modified to induce targeted methylation by replacing VP64

with the *Nicotiana tabacum* DRM methyltransferase catalytic domain (NtDRMcd) to introduce CRISPR-based targeted methylation. An *Arabidopsis fwa* mutant lacking methylation in the FWA promoter was used as well as a Suntag peptide containing 5 amino acid residues separating the CGN4 epitope repeats with limited re-establishment of CHH methylation and minimal CG and CHG methylation (Papikian et al. 2019). Further improvement included the extension of the linker to 22 amino acids to avoid steric barriers to the recruited NtDRMcd groups as well as utilizing three different gRNAs targeting the FWA promoter resulting in efficient methylation of the promoter, FWA gene silencing and an early flowering phenotype. Interestingly, non-transgenic T_2 segregant plants from different transgenic lines exhibited different behaviors. While some lines produced progeny maintaining promoter methylation, indicating that the targeted methylation was meiotically heritable, the progeny from other lines did not have enough methylation in the T_2 generation, leading to gene reactivation and late flowering. Nevertheless, maintenance of the transgenic cassette for two generations remedied the problem for most lines establishing strong heritable methylation. In those lines in which methylation was inherited in T_2, the methylation was maintained until the T_4 generation, although further studies will be needed to establish if there is a limit in the heritability of the targeted methylation. In addition to the targeted methylation, genome-wide CHH methylation was also observed in plants containing the transgenes but not in those lacking the transgenic cassette, leading the authors to hypothesize that the NtDRMcd module resulted in non-specific methyltransferase activity. Removal of the NLS from the single chain antibody-NtDRMcd fusion forced the protein to associate with the dCas9-Suntag in order to be transported to the nucleus and greatly reduced non-specific methylation. Similar results were observed when a different promoter, SUPERMAN, was targeted. An important practical message from this work was the importance of producing and characterizing multiple transgenic lines given the inherent variability observed by the authors (Papikian et al. 2019).

Highly specific targeted demethylation of the FWA and CACTA1 promoters has been achieved using the SunTag system by coupling the catalytic domain of the human demethylase TEN-ELEVEN TRANSLOCATION1 (TET1cd) to the scFV-sfGFP peptide (Gallego-Bartolome et al. 2018; Ji et al. 2018). While demethylation of FWA was highly efficient and heritable in the absence of the transgene, the SunTag-TET1cd system was not so efficient in the CACTA1 heterochromatic locus leading to re-methylation and

re-silencing after segregation of the transgene (Gallego-Bartolome et al. 2018). The use of TET1 for demethylation generates stable 5-methylcytosine (5-meC) derivatives which could be independent epigenetic marks on their own and/or have unknown regulatory roles. Plants can follow a different path of active demethylation by directly removing 5-meC using DNA glycosylases/lyases (Parrilla-Doblas et al. 2019). The DNA glycosylase activity of the Arabidopsis REPRESOR OF SILENCING 1 (ROS1) can remove 5-methylcytosine from the DNA backbone before its lyase activity cleaves the DNA backbone at the site of 5-methylcytosine removal (Agius et al. 2006; Zhu et al. 2007). *Arabidopsis* ROS1 homologs DEMETER (DME), DEMETER-LIKE2 and DEMETER-LIKE 3 can also mediate active demethylation (Gehring et al. 2006; Morales-Ruiz et al. 2006; Penterman et al. 2007; Ortega-Galisteo et al. 2008). Interestingly, ROS1 and DME can also work in mammalian systems inducing genome wide methylation changes and targeted demethylation (Mok et al. 2017; Parrilla-Doblas et al. 2017; Morales-Ruiz et al. 2018). Fusion of the ROS1 catalytic domain to dCas9 successfully reactivated a methylation-silenced luciferase reporter gene and induced partial demethylation in a replication-independent manner, although the system has not yet been applied to plants (Devesa-Guerra et al. 2020).

Aside from the multiple practical applications envisioned for crop improvement, targeted methylation and de-methylation can be used in functional studies to understand the regulatory mechanisms of the epigenome, circumventing the complicated interpretation of genome-wide approaches when using mutants or pharmacological drugs. A good example is the study by Li et al. (2020e) who expressed the TET1cd domain directly fused to the C-terminus of dCas9 under the control of the constitutive ubiquitin promoter to induce highly specific targeted de-methylation and demonstrated that histone and DNA methylation marks are critical in determining the ability of RNA-directed DNA methylation target loci to form stable epialleles (Li et al. 2020e). In this study, the demethylation status was heritable in the absence of the transgenes.

Chromatin modification provides another avenue to introduce targeted epigenomic modifications to affect gene expression patterns (Hilton et al. 2015). The *Arabidopsis* histone acetyltransferase 1 (AtHAT1) catalyzes the addition of an acetyl group to some lysines on the N-terminal tail of histone H3 (Schneider et al. 2013), triggering DNA relaxation and exposure of DNA to the transcriptional machinery, which ultimately

stimulates gene expression (Eberharter and Becker 2002). dCas9 has been fused to the catalytic domain of AtHAT1 to create dCas9^{HAT} that was introduced into transgenic *Arabidopsis* lines (Roca Paixao et al. 2019). In an attempt to enhance drought stress tolerance, Roca Paixao et al. (2019) used the system to enhance expression of the ABSCISIC ACID–RESPONSIVE ELEMENT BINDING PROTEIN1 (AREB1) gene. The AREB1 transcription factor binds to the abscisic acid (ABA)–responsive element (ABRE) in the promoter region of ABA-inducible genes to positively regulate the drought stress response in plants (Fujita et al. 2005). Transgenic lines expressing dCas9^{HAT} were retransformed to express two gRNAs, one of them targeting a locus 479 nt upstream of the transcription start site and the second one targeting the 5' UTR (+356 nt). Analysis of transgenic lines revealed a dwarf phenotype and a 2-fold increase in AREB1 expression compared to wild type plants with a concomitant increase in survival rate after exposure to severe drought stress (Roca Paixao et al. 2019). The SAM system has been adapted for histone modification by fusing three different modifier to MS2; (1) the catalytic domain of the human p300 H3K27 histone acetyltransferase, causing gene activation; (2) the catalytic Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain of the human H3K9 histone methyltransferase G9a, triggering gene repression and (3) the SET domain of the Arabidopsis H3K9 KRYPTONITE (KYP) (Lee et al. 2019a). All constructs were targeted to alter the expression of the floral regulator FLOWERING LOCUS T (FT) with only the KYP domain producing statistically significant alterations in gene expression but very minor effects on flowering time (Lee et al. 2019a).

'Classic' CRISPR/Cas9 can also be used to introduce mutations in promoter elements leading to changes in expression and it has been shown that the introduction of indels do not lead to changes in the methylation patterns of the promoter regions flanking the target sequence in either hypermethylated or hypomethylated genes (Lee et al. 2019b).

CONCLUSIONS AND FUTURE PROSPECTS

Some of the genome editing applications, such as gene disruption using Cas9 and Cas12 effectors have reached very high levels of efficiency and specificity and, even tough new improvements will undoubtedly keep arriving, the current state of the technology is adequate in most cases. In the case of GT, impressive advances have been achieved but the current efficiency is still unsatisfactory. Fresh new approaches are needed to increase routine GT frequencies before mass adoption for crop improvement is practical. Base

editing is achieving good efficiency but the nature of the substitution is limited and the capacity to perform individual replacements within the editing window is limited. Prime editing has the potential to address most, if not all the deficiencies of base editing by providing incredible accuracy and decreasing off target effects to almost undetectable levels but it still needs to achieve better efficiencies. It will be important to optimize the different variable parameters in prime editing, such as the lengths of the PBS and the RT templates and minimize the introduction of unwanted indels. Although there are only a few examples available, the use of hybrid Cas effectors to modify the epigenome has immense potential for basic plant biology as well as applied crop improvement.

Achieving improvements on CRISPR-based systems is only as good as the capacity to apply them to elite crop varieties. The current delivery methods are only satisfactory for a small proportion of crops and, with the notable exception of rice, many elite varieties are not always amenable to *Agrobacterium*- or microprojectile-based delivery. Somatic reprograming using morphogenic regulators has proven efficient in stimulating regeneration of difficult crops such as maize and its use could be extended to other crops and recalcitrant varieties (Lowe et al. 2016; Mookkan et al. 2017; Lowe et al. 2018; Gordon-Kamm et al. 2019; Zhang et al. 2019b). *De novo* induction of meristems has also been achieved using morphogenic regulators and it has the potential to develop transgenic plants without the need for tissue culture, although gene edits were only achieved in transgenic lines constitutively expressing Cas9 (Maher et al. 2020). It will be interesting to test if a combination of the above technologies with highly efficient delivery methods, such as nanomaterial-based or high cargo viral vectors, could provide further improvements.

Paradoxically, the main challenge facing genome editing is not scientific, but political. Regulatory barriers to the use and commercialization of genome edited crops can delay or even derail its progress and application to agriculture. Political considerations instead of scientific facts have influenced some jurisdictions to regulate gene edited crops with the same burdening regulations of genetically modified crops. It defies logic why introducing one single mutation in a specific genomic locus with extreme precision using Cas effectors is subject to strict regulations in some countries while introducing thousands of simultaneous mutations in a completely uncontrolled manner by chemical/physical random mutagenesis methods is not regulated. Agriculture is one of the major

environmental polluters and genome editing has the potential to greatly reduce its environmental impact, therefore it is crucial to expedite the adoption of more efficient crops to reduce chemical inputs into the environment and maximize outputs.

The speed at which new genome editing technologies are being developed makes it virtually impossible to predict what the future holds. Integration of the existing technologies with other scientific fields has not been widely explored but can provide important advances. The adoption of nanomaterials for delivery is still in its infancy, while the use of non-invasive technologies, such as near infrared and other wavelength-based emissions for early screening of editing events could facilitate the identification of edited materials and tissues.

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Figure legend

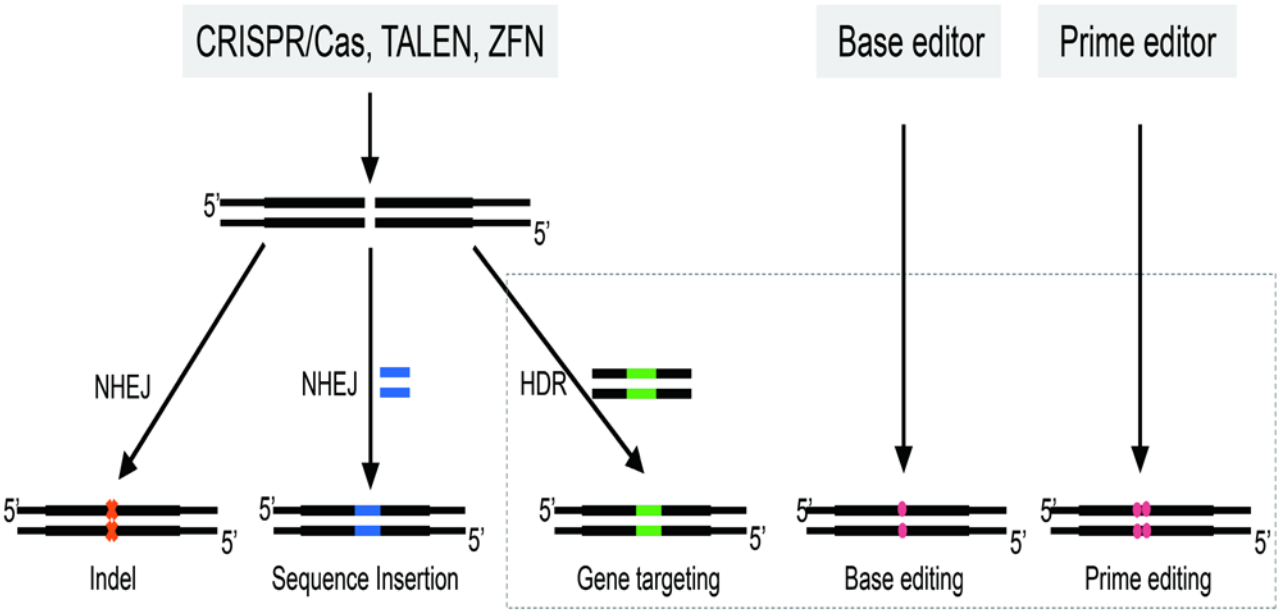

Figure 1. Overview of common genome editing technologies

The most basic applications of genome editing tools such as CRISPR/Cas, TALEN and ZFN is the production of DSBs at target genomic regions. Cellular DSB repair can result in the introduction of random small indels or base substitution mutations through the error prone NHEJ pathway, frequently aimed at producing gene disruption. Sequence insertion (often not precise/seamless) can also be achieved through the NHEJ pathway when a donor DNA fragment is provided, and precise sequence insertion or replacement (i.e., gene targeting) can be achieved through the HDR pathway in the presence of donor DNA with homology to the target sequence. In addition to gene targeting, precision editing (marked with dashed box) can also be achieved by using a base editor to introduce base substitutions or by a prime editor to achieve the substitution of multiple bases.

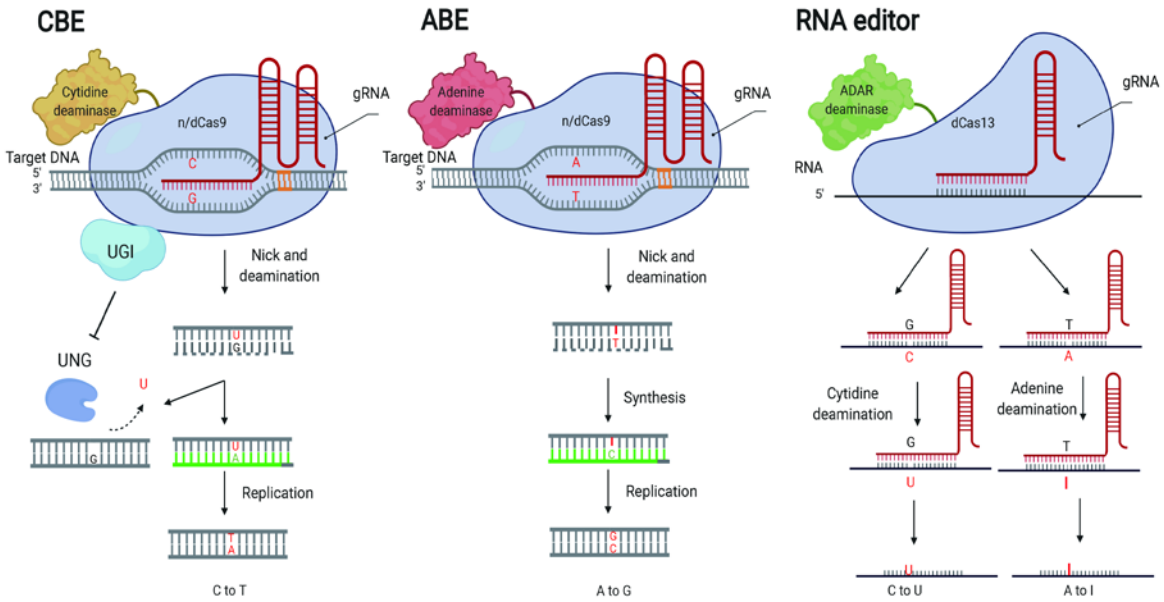

Figure 2. Mechanisms of DNA (CBE and ABE) and RNA (RNA editor) base editors

In the CBE system, nick/dead Cas protein is fused to a cytidine deaminase catalyzing the conversion of cytidine to uracil (U), which is recognized as a T by DNA polymerase during DNA replication, resulting in a C•G to T•A conversion. In the ABE system, an engineered *E. coli* RNA adenosine deaminase (TadA) is fused to a nick/dead Cas protein, converting adenine to inosine (I), that is read as guanine by DNA polymerases during DNA replication, resulting in A•T to G•C conversion. Similarly, in RNA base editors, dCas13 is fused to an RNA adenosine or cytidine deaminase to mediate A-to-I and C-to-U exchange in the RNA molecule, respectively. gRNAs and PAMs are marked with red and yellow lines, respectively. Newly synthesized sequences are indicated in green color.

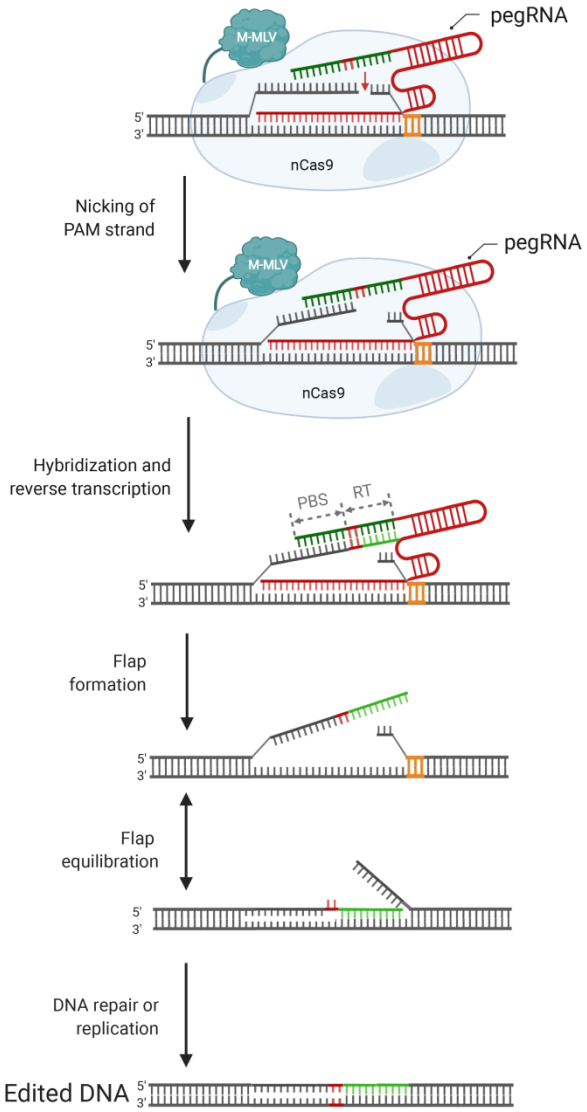

Figure 3. Schematic overview of prime editing

The nCas9/M-MLV/pegRNA complex binds the target DNA and nicks the PAM-containing strand. The 3′ end of the target DNA hybridizes to the PBS, then primes reverse transcription using the RT template of the pegRNA. The newly synthesized 3′ flap is equilibrated with the unedited 5′ flap and is finally processed to produce a stably edited DNA. pegRNA and PAM are marked with red and yellow lines, respectively. PBS and RT sequences in the 3' of pegRNA are indicated in green and contain nucleotides for substitution, deletion or insertion (red).

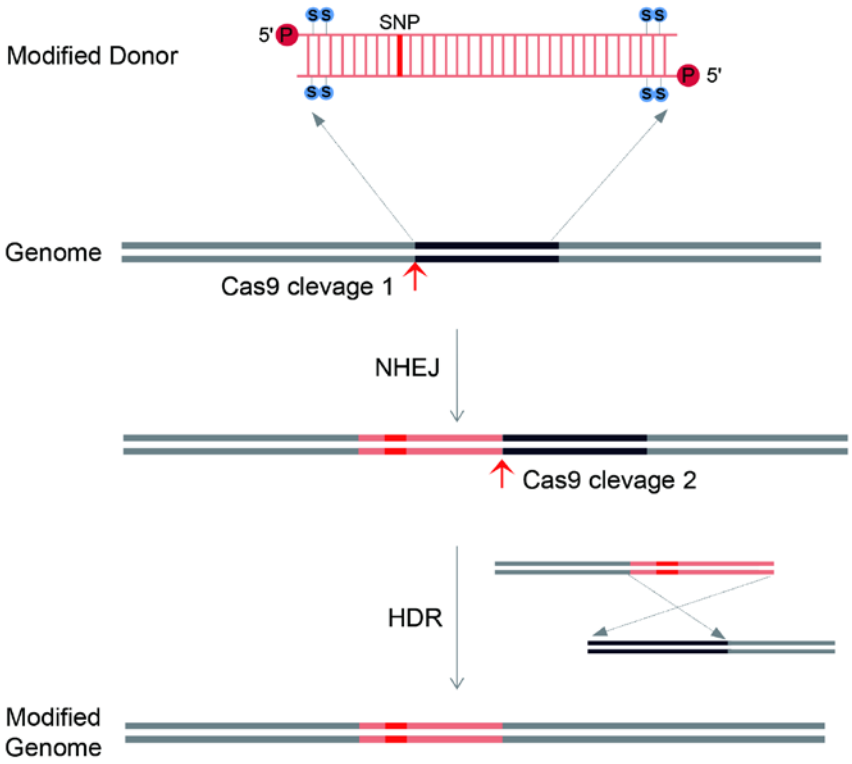

Figure 4. Gene targeting using chemically modified donor DNA and TR-HDR

Double stranded DNA fragment is 5'-phosphorylated (red circles) and contains phosphorothioate linkages (blue circles) at both ends. Edits such as sequence replacement and insertion are introduced through a two-step process involving a first DSB generation (cleavage 1) and donor DNA insertion via NHEJ to form a tandem repeat (TR) between the donor sequence and its homologous target genomic sequence, followed by a second DSB generation (cleavage 2) and homology-directed repair (HDR) to achieve the precise insertion of, or replacement with the provided donor sequence (orange). The desired sequence change is indicated in red.

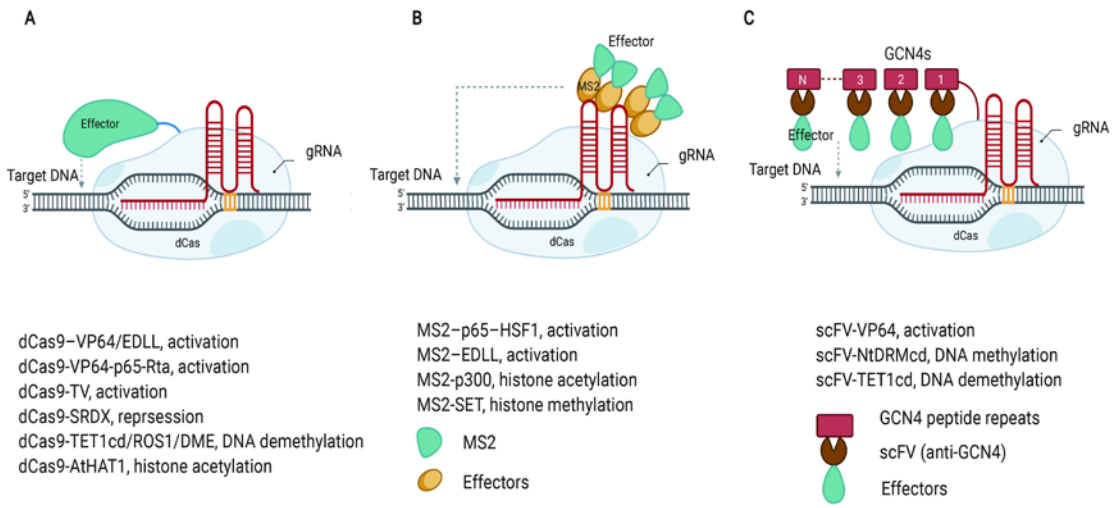

Figure 5. CRISPR-based systems for gene expression/epigenome control

Various effectors can be coupled with dead Cas protein (dCas) via (**A**) direct protein fusion, (**B**) MS2-gRNA interaction and (**C**) the SunTag system, mediating transcriptional activation or repression, DNA methylation or demethylation, histone acetylation or methylation. gRNAs and PAMs are marked with red and yellow lines, respectively. Effectors are indicated in green.