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ABSTRACT

Since its discovery as a bacterial adaptive immune system and its development for genome editing in eukaryotes, the clustered regularly interspaced short palindromic repeats (CRISPR) technology has revolutionized plant research and precision crop breeding. The CRISPR toolbox holds great promises to produce crops with genetic disease resistance to increase resilience of agriculture and reduce chemical crop protection with strong impact on environment and public health. In this review, we provide an extensive overview on recent breakthroughs in CRISPR technology including the newly developed prime editing system allowing precision gene editing in plants. We present how each CRISPR tool can be selected for optimal use in accordance with its specific strengths and limitations, and illustrate how the CRISPR toolbox can foster the development of genetically pathogen-resistant crops for sustainable agriculture.

Key Words

CRISPR-Cas9, gene targeting, base editing, prime editing, plant/pathogen interactions, precision crop breeding

Short Summary

The CRISPR-mediated precision breeding toolbox allows researchers and molecular breeders to fine-tune plant genomes with a high versatility. Application of these genome editing tools to genes involved in plant/pathogen interactions can foster the development of a sustainable agriculture through the production of genetically pathogen-resistant crops.

INTRODUCTION

Primary food production across the globe faces the challenge of sustainably feeding a growing population in an accelerating climate change context, while more than 800 million people suffered from undernourishment worldwide in 2017, particularly in Africa and Asia (FAO, 2017). Current agriculture mostly relies on the cultivation of a narrow range of plant species, sometimes in poorly suited locations, far away from their area of domestication (Fernie and Yan, 2019). Labour-intensive and time-consuming conventional crop breeding relying on natural or induced genetic polymorphism has substantially contributed to plant adaptation to new environments and food availability. Recently, the development of genome engineering in plants opened new avenues for precision crop breeding, including the improvement of elite germplasm as well as the molecular domestication of wild species (Zhang et al., 2019).

Plant pathogens, including bacteria, fungi and viruses, cause substantial economic losses and threaten food security (Savary et al., 2019). Pathogens rely on diverse strategies to bypass plant immunity. For instance, they produce molecular weapons called effectors that act inside or outside of the plant cell to target diverse host proteins involved in different cellular processes to promote infection through successful colonization of the host.

Plants rely on a sophisticated immune system to ward off potential pathogens. Key elements are an arsenal of receptors termed invasion pattern receptors that recognize either microbe- or host-derived signals termed invasion patterns (IPs) that betray the presence of microbial

invaders (Cook et al., 2015). IP receptors belong to two main classes: cell surface receptors that are either receptor-like proteins (RLPs) or receptor-like kinases (RLKs) and intracellular receptors that belong to the class of nucleotide-binding leucine-rich repeat domain proteins (NLRs). While NLRs specifically recognize intracellular effectors (Cesari, 2018; Kourelis and van der Hoorn, 2018), RLPs and RLKs perceive microbe-associated molecular patterns (MAMPs) and extracellular effectors, originating from the pathogen, and damage-associated molecular patterns (DAMPs) released by host cells damaged upon pathogen attack (Boutrot and Zipfel, 2017; Kanyuka and Rudd, 2019).

The vast majority of disease resistance (*R*) genes cloned from plants code for immune receptors (Kourelis and van der Hoorn, 2018) with NLRs being the dominating class. Another successful strategy to confer plant disease resistance relies on a loss of compatibility through mutations of plant susceptibility (*S*) genes required for pathogen infection and plant susceptibility. As a result, the pathogen will not be able to perform its infectious cycle, resulting in plant disease resistance (van Schie and Takken, 2014).

While conventional resistance breeding can be very successful, it may be associated with linkage drag and the resistance conferred by single *R* genes may be rapidly bypassed by fast evolving pathogens. Therefore, the precise engineering of *R* and *S* genes constitutes an exciting track for the development of genetically resistant crops (Langner et al., 2018; Tamborski and Krasileva, 2020; van Wersch et al., 2020), thereby limiting the environmental impact of chemical control. Copying mutations across accessions can also circumvent linkage drags associated with classical breeding, as shown for other characters (Li et al., 2017a).

In the last few years, genome editing tools have evolved very quickly with the development of RNA-guided endonuclease systems (Zhang et al., 2019). Until now, genome editing was mostly used to generate loss-of-function alleles through DNA error-prone repair of the target site after double strand cleavage by the classical CRISPR-Cas9 system. For example, this strategy resulted in a powdery mildew resistant tomato by knocking-out the *mildew resistant locus O* (*Mlo*) *S*-gene (Nekrasov et al., 2017), while the rice blast resistance was increased due to the loss-of-function of the transcription factor OsERF922 (Wang et al., 2016). However, many traits can be conferred by single or multiple nucleotide substitutions, especially for genes involved in plant/pathogen interactions, where coevolution exerts a dual selective pressure that favours mutations of pathogen effectors to evade recognition, but also mutations of immune receptors to restore perception (Jones et al., 2016). Therefore, genome-

editing tools mediating precise and predictable mutations are highly valuable for the production of gain-of-function mutants, which could lead to broader perception of the pathogen and/or host factor evasion from effectors. Of particular interest is the CRISPR-mediated mimicking of natural alleles conferring pathogen resistance (Bastet et al., 2017), as well as directed *in planta* evolution to generate new gene variants that are not present in the natural genetic diversity. In the course of this review, we will refer to the targeted genome alterations, such as nucleotide changes and small deletion, as precision breeding. This process can involve GM techniques but the resulting plant is devoid of transgene (Andersen et al., 2015).

In this review, we will mostly focus on recent advances in CRISPR technologies used to introduce targeted point mutations in plant genes, including the newly 'search-and-replace' prime editing technology. We will see how the multiple adjustments that have been developed to expand the targeting scope, precision and efficiencies of these CRISPR tools offer complementary strengths and drawbacks that can be mobilized according to specific desired outcomes. The fast adoption and improvement of these precise and versatile genome editing tools in plants open up new avenues for biotechnology and the development of sustainable agriculture, especially through the development of new genetically resistant crops.

THE BASIC MACHINERY FOR PLANT GENOME EDITING

In the frame of this review, we will focus on genome editing strategies, *i.e.* approaches that will lead to stable modifications in the plant genomic DNA, and result in transgene-free plants through different delivery strategies that are extensively described in recent reviews (Chen et al., 2019; El-Mounadi et al., 2020; Kuluev et al., 2019). We will therefore not cover another important aspect of CRISPR that consists in using nuclease that targets RNA for modification, such as Cas13. More details on this strategy can be found in recent reviews (Burmistrz et al., 2020; Wolter and Puchta, 2018).

The CRISPR-Cas9 system

The leading CRISPR-SpCas9 system for genome editing, initially derived from a class 2 type II *Streptococcus pyogenes* adaptive immune system, consists of a two-components complex made of the DNA endonuclease SpCas9 (1368 amino acids) and a customizable single guide

RNA (sgRNA) that results from the artificial fusion of a crRNA and a trans-activating crRNA (tracrRNA) (Jinek et al., 2012). The sgRNA is composed of a ≈ 80-bp scaffold that mediates binding to the Cas9, and a customizable 20-bp sequence at its 5' end, called the spacer sequence, conferring DNA targeting specificity to the complex (Figure 1A). Binding of the sgRNA to the SpCas9 triggers the transition of the nuclease from an inactive into a DNA-probing state in search for a canonical 5'-NGG-3' protospacer adjacent motif (PAM). Natural and engineered Cas9 variants recognizing alternative PAMs have also been extensively used (Zhang et al., 2019). Recognition of a suitable PAM motif leads to quick interrogation of adjacent DNA, followed by local DNA melting and RNA strand invasion (formation of a R-loop structure) for interrogation of the full spacer sequence (Figure 1A). Perfect base pairing between the so-called seed region (10-12 nucleotides from the PAM) of the spacer sequence and target DNA is required for SpCas9-mediated DNA cleavage, while mismatches in the nonseed region can be tolerated, potentially leading to unwanted off-target activity. While a careful design of spacer sequences is generally considered to be sufficient to avoid off-target activity, some Cas9 variants displaying higher specificity have been developed through protein engineering (Zhang et al., 2019). The gradual base pairing triggers SpCas9 conformational changes to an active site, eventually resulting in DNA cleavage by the concerted activity of its HNH and RuvC nuclease domains (Figure 1A). Although SpCas9 was thought to only create blunt-ended double strand DNA break (DSB) about 3-bp upstream the PAM (Jiang and Doudna, 2017), recent findings demonstrated that SpCas9 nuclease activity results in both blunt and staggered ends, likely because of the RuvC cutting flexibility (Molla and Yang, 2020). The CRISPR-SpCas9 system is now routinely used in numerous species and can be considered as the golden tool for genome editing in plants (Manghwar et al., 2019).

The CRISPR-Cas12 systems

The second leading genome editing tool, the class 2 type V-A CRISPR-Cas12a system also known as CRISPR-Cpf1, displays unique features and constitutes a relevant alternative to the CRISPR-Cas9 system (Zetsche et al., 2015). Cas12a enzymes (1200-1500 amino acids) mostly recognize T-rich 5'-TTTN-3' PAM located upstream of the target sequence. They associate with a short ≈ 43-bp crRNA and only rely on the RuvC-like domain to cleave both DNA strands in a sequential manner, beginning with the non-target strand and resulting in a staggered DNA cleavage with 4-5bp overhangs distal to the PAM (Figure 1B) (Alok et al.,

2020; Zaidi et al., 2017; Zetsche et al., 2015). Cas12a orthologs from *Lachnospiraceae bacterium* (LbCas12a), *Acidaminococcus* sp. (AsCas12a) and *Francisella novicida* (FnCas12a) have been the most commonly used enzymes in several plant species. They generally display higher specificity and less or no off-targets as compared to Cas9 (Begemann et al., 2017; Endo et al., 2016; Herbert et al., 2020; Kim et al., 2017; Lee et al., 2019; Li et al., 2019a; Tang et al., 2018; Tang et al., 2017; Tang et al., 2019; Xu et al., 2017; Xu et al., 2019a; Yin et al., 2017).

The recently established class 2 type V-B CRISPR-Cas12b system uses a smaller Cas12b nuclease (≈ 1100 amino acids) than the CRISPR-SpCas9 and CRISPR-Cas12a systems. Like Cas12a, Cas12b prefers T-rich PAMs and induces RuvC-mediated DSBs with staggered ends distal to the PAM (Figure 1C) (Shmakov et al., 2015; Yang et al., 2016). The Cas12b ortholog from *Alicyclobacillus acidiphilus* (AaCas12b), initially characterized as a high specificity nuclease with elevated optimal temperature in mammalian cells (Teng et al., 2018), was reported to be efficient for rice genome engineering, with a 5'-VTTV-3' PAM preference (V=A, C or G) (Ming et al., 2020). In addition, the *Alicyclobacillus acidoterrestris* (AacCas12b) was also successfully used for genome editing in tetraploid cotton plants, displaying an optimal editing efficiency at 45°C and an undetectable off-target activity (Wang et al., 2020b). Although promising, further studies are still required to properly assess the strengths and drawbacks associated with Cas12b compared to Cas9 and Cas12a enzymes for genome editing in plants.

Evolving CRISPR-Cas systems: going beyond gene knockout

Together, the three CRISPR-Cas systems above-mentioned constitute the base for diversified genome editing tools. So far, most genome editing applications in plants have been focused on the production of knockout mutants for single or multiple genes (Manghwar et al., 2019). This is due to the predominance of error-prone non-homologous end-joining (NHEJ) mechanisms to repair CRISPR-Cas-mediated DSBs in somatic cells of higher plants (Puchta, 2005). Contrary to homologous recombination (HR), an endogenous DNA repair mechanism that is responsible for crossovers between homologous chromosomes during meiosis, NHEJ mechanisms mediate DSB repair without the need for a homologous template. While the classical NHEJ (C-NHEJ) pathway appears to be mainly error-free, the alternative NHEJ (Alt-NHEJ) seems to have a key role in error-prone CRISPR-induced DSB repair (Atkins and

Voytas, 2020; Mara et al., 2019). The unfaithful DNA repair eventually creates random small insertion or deletion mutations (indels) at the cleavage site, typically causing frameshift mutations that result in loss-of-function alleles when located in coding sequences. In promoter regions, targeted deletions affecting cis-regulatory elements, can result in altered transcriptional regulation.

An interesting feature of the CRISPR-Cas9 system is that the cutting function can be uncoupled from the target recognition. This opens room for repurposing the system and carry enzymatic domains to a specific locus. Indeed, the inactivation of either the RuvC or HNH catalytic domains by D10A or H840A substitutions produces nickase Cas9 (nCas9) that are only able to cut the targeting and the non-targeting strands, respectively, while introduction of both mutations generates a dead Cas9 (dCas9). Similarly dead Cas12a and dead Cas12b (dCas12a and dCas12b) enzymes are also available, but nickase Cas12 proteins have yet to be reported. However, the fact that DNA cleavage of Cas12 enzymes is sequentially mediated by a single RuvC-like nuclease domain may prevent the development of such nickase Cas12. These impaired Cas proteins keep their DNA-binding properties and thereby allow targeted applications such as epigenome editing or transcriptional regulation through the recruitment of the DNA methylation machinery or transcriptional regulators, respectively (Gallego-Bartolome, 2020; Zhang et al., 2019). Of particular interest is the possibility to bring enzymatic domains that specifically replace nucleotides in genomic sequences and thereby directly edit the sequence of genes. In the next sections, we will mostly focus on such recently developed CRISPR systems that support precise and predictable targeted DNA mutations to confer new traits.

PRECISION EDITING: REFINING THE TOOLS?

As many agronomic traits are controlled by single base polymorphisms (Henikoff and Comai, 2003), introduction of precise base substitutions and/or predictable insertions or deletions could generate plants with new agronomic properties. For example, the targeted substitution of nucleotide(s) could introduce non-synonymous mutations causing amino acid changes in the encoded protein. Besides, nucleotide substitution can broadly affect the gene by creating or correcting early stop codons or regulating splicing. In the next subsections, we summarize current CRISPR tools for precision editing.

CRISPR-mediated gene correction through NHEJ

Although NHEJ-mediated DSB repair upon Cas9 cleavage has been initially considered to result in random mutations, it is becoming increasingly obvious that a fraction of Cas9-induced DSB repair outcomes are predictable. User-friendly web tools with machine learning algorithms have been recently developed to predict repair outcomes in human cells, allowing the selection of suitable guides for the introduction of predictable mutations through NHEJ (Molla and Yang, 2020). The development of such tools in plants would be of great interest, with the possibility to anticipate NHEJ-mediated DSB repair outcomes for predictable mutations in coding or regulatory sequences. While the Cas9 nuclease mainly generates small indels (Figure 2A), Cas12a and Cas12b predominantly produce larger deletions (Bernabe-Orts et al., 2019; Herbert et al., 2020). Whether these different mutation footprints are the result of Cas12 cleavage properties and/or due to the binding time of the nuclease to the broken DNA is still unclear (Chen et al., 2018; Que et al., 2019). Regardless its mechanisms, the cleavage properties of Cas12 enzymes could have specific practical interest compared to Cas9, such as the removal of larger coding or regulatory motifs (Herbert et al., 2020; Li et al., 2020e).

With dual sgRNA approaches, larger DNA fragments can be deleted, allowing to remove complete domains or entire genes (Pauwels et al., 2018) (Figure 2B). The NHEJ-mediated DSB repair approach can also be used for targeted DNA insertion using dsDNA or ssDNA donors without homologous ends. However, this technique introduces small indels at the 5′ and 3′ junctions (Figure 2C) (Wang et al., 2014). This major drawback can be addressed by a strategy where entire exons are replaced by creating DSBs in flanking introns and thereby restricting NHEJ-associated indels to non-coding intron sequences. Such a NHEJ-mediated exon replacement strategy has been successfully applied to the rice *OsEPSPS* gene where the introduction of two amino acid changes created glyphosate resistance (Li et al., 2016).

Although these NHEJ-mediated editing strategies have proved efficient and reliable in many plant species for gene knockouts, the unpredictable outcomes at the cleavage sites limit their applications for precision editing. This drawback is particularly relevant in vegetatively propagated crops, where desirable or undesirable mutations at the target site cannot be segregated through sexual reproduction. Therefore, the predictable and precise introduction of point mutations or indels through NHEJ-independent pathways is of particular interest.

CRISPR-mediated gene targeting

CRISPR-mediated gene targeting (GT) is a technology relying on HR (Figure 2D) that has been applied for precise nucleotide conversion or precise insertions or deletions in many eukaryotic genomes, including plants. Although it is very promising for genome engineering, HR suffers from low efficiency in plant somatic cells (Puchta, 2005) and the delivery of a sufficient amount of donor template in the vicinity of the target site is still challenging, thereby strongly limiting the use of GT in most higher plant species. An illustration of this challenging task is the high number of CRISPR-mediated GT studies that used phenotypic markers such as herbicide tolerance to facilitate the identification of successful events (Atkins and Voytas, 2020). Nevertheless, a variety of recent improvements allowed to substantially enhance GT in plants (Huang and Puchta, 2019). Of particular interest is the use of engineered geminiviral replicon systems, which use rolling-circle replication to deliver large amount of DNA repair template into the plant cell nucleus. The CRISPR-Cas9 GT-geminiviral replicons strategy was successfully applied for large insertions and/or point mutations in tomato, potato, cassava, wheat and rice (Butler et al., 2016; Cermak et al., 2015; Dahan-Meir et al., 2018; Gil-Humanes et al., 2017; Hummel et al., 2018; Wang et al., 2017). Another interesting approach is the use of Cas12a instead of Cas9 for inducing DSBs. Because Cas12a cuts DNA in the non-seed region distal from the PAM (Figure 1B), allowing multiple rounds of DNA cleavage even after introduction of NHEJ-mediated indel mutations, and produces sticky ends, HR may be favoured (Huang and Puchta, 2019). Consistent with this hypothesis, the CRISPR-Cas12a GT system was successfully applied for targeted insertion or point mutations in rice (Begemann et al., 2017; Li et al., 2019c; Li et al., 2018b). This system was further improved in tomato using a CRISPR/Cas12a GT-geminiviral multi-replicon strategy, allowing the production of transgene-free salt-tolerant plants due to a single amino acid change (N217D) in the *SlHKT1;2* gene (Van Vu et al., 2020). While the geminiviral replicon system allows the delivery of higher amount of donor template in plants, some improvements for GT are still needed in order to spatially and temporally bring the CRISPR components and the repair template at the breaking site, as observed in animals (Aird et al., 2018; Savic et al., 2018). Such a strategy has recently been applied in rice using a fusion between the Cas9 and the *Agrobacterium* VirD2 relaxase (Ali et al., 2020), known to be a key player for ssT-DNA translocation and integration into the plant genome (Gelvin, 2017). The CRISPR-Cas9-VirD2 system facilitated GT likely through the delivery of ssDNA repair-template in close vicinity to the Cas9-induced DSB (Figure 2E). This enabled introduction of point mutations in the *OsALS* and *OsCCD7* genes to confer herbicide resistance and to engineer plant architecture respectively, and in-frame insertion of the HA

epitope at the C-term of OsHDT (Ali et al., 2020). Together, these recent advances offer new possibilities for precise genome editing, although future progress to increase the efficiency of CRISPR-mediated GT are still needed for a broad and fast adoption in many plant species.

CRISPR-mediated base editing

In contrast to GT-mediated gene correction, CRISPR-mediated base editing is a donor template and DSB free approach that induces precise base conversion. Cytosine base editors (CBEs) and adenine base editors (ABEs) are fusion proteins made of a catalytically impaired Cas9 and an enzymatic domain mediating cytosine or adenine deamination, respectively. During the formation of the CRISPR-mediated "R-loop" structure, a small window of the non-targeted ssDNA is exposed and can serve as a substrate for deamination (Figure 3A). CBEs catalyze the deamination of cytosine(s) into uracile(s) in the target region. This triggers the base excision repair (BER) pathway that can result in either an error-free or an error-prone repair leading to a diversification of the edits (C-to-T, C-to-G and C-to-A), albeit at the cost of indels production at a substantial rate (Figure 3B) (Hess et al., 2017). Although varying the edits is interesting for local sequence diversification, predictable targeted base conversions are desirable for precise amino acid changes. Adding an uracil DNA glycosylase inhibitor (UGI) to the CBE architecture that blocks the BER pathway has been developed as a solution to specifically obtain C-to-T conversion with generally low level of by-products (Figure 3C-D) (Komor et al., 2017). Deamination of adenine through ABEs (Figure 4A) does not necessitate the use of alkyl adenine DNA glycosylases inhibitors, because BER of inosine intermediates is inefficient in DNA. ABEs therefore create efficient A-to-G conversion with a very low level of by-products (Figure 4B) (Gaudelli et al., 2017). While first BEs harboured a deadCas9 (dCas9), the incorporation of the edit(s) to the non-deaminated strand was strongly improved by the use of nCas9 with impaired RuvC domain (D10A), that promotes long-patch BER using the edited strand as a model (Komor et al., 2016). Due to the lack of nCas12, the use of Cas12 enzymes for base editing applications remains limited, for the moment.

Soon after their development in animals, CBEs and ABEs have been rapidly used in several plant species. The two mostly used cytosine deaminases, PmCDA1 from *Petromyzon marinus* and rAPOBEC1 from rat (both devoid of UGI), have been reported to produce C-to-T transitions, but also C-to-G and C-to-A transversions in *Arabidopsis*, tomato and potato, albeit with a substantial rate of indels, as discussed above (Bastet et al., 2019; Li et al., 2017b; Lu and Zhu, 2017; Shimatani et al., 2017; Veillet et al., 2019a; Veillet et al., 2019b). For an

approach requiring a high level of outcome predictability, UGI domain(s) can be added to the CBE architecture, resulting in a higher rate of C-to-T substitutions with lower unwanted mutations (Qin et al., 2019b; Zong et al., 2017). As observed in animals, ABEs produce A-to-G transitions in plants, with a very low rate of indels (Hao et al., 2019; Hua et al., 2020b; Kang et al., 2018; Li et al., 2018a; Li et al., 2019b; Negishi et al., 2019; Yan et al., 2018). These BEs allowed to produce plants with new agronomic traits, including pathogen resistance (Bharat et al., 2020; Mishra et al., 2020). Recently, dual cytosine and adenine BEs were generated to simultaneously mediate C-to-T and A-to-G transitions in the same editing window, increasing the potential outputs for targeted gene modification (Grünewald et al., 2020; Li et al., 2020b). Several different deaminases can also be recruited in the target site through sgRNA-protein interactions, thereby increasing the local amount of catalytic domains for the production of diversified outcomes (Mishra et al., 2020; Zhang et al., 2019).

Although base editing constitutes a promising technology, early CBEs and ABEs suffered from some drawbacks. First, their targeting scope is restricted to sequence harbouring a suitable PAM downstream of the targeted sequence, placing the target base in a generally short editing window at the 5'end of the spacer sequence. Much work has been done to use natural Cas9 orthologs with different PAM requirements, such as *Staphylococcus aureus* and *Streptococcus canis* Cas9 (Hua et al., 2018; Qin et al., 2019a; Wang et al., 2020a), or to engineer SpCas9 variants with relaxed PAM recognition, expanding the targeting scope of BEs (Ge et al., 2019; Hua et al., 2019; Niu et al., 2019; Qin et al., 2020; Ren et al., 2019; Veillet et al., 2020; Wang et al., 2018; Zhong et al., 2019). Of particular interest is the recent development in animal cells of new SpCas9 variants that recognize non-G PAMs (Miller et al., 2020) or almost any PAM sequence, as illustrated with BEs harbouring the SpRY variant that are able to target almost any locus, albeit with a preference for sequences upstream of NRN PAMs (R=A or G) (Walton et al., 2020). Due to almost unrestricted PAM recognition, a special attention should be put on limiting sgRNA self-targeting activity when using DNA delivery methods, potentially increasing the off-target risk by introducing mutations into spacer sequences (Qin et al., 2020). Second, the size of the editing window of BEs would benefit from being modular according to the desired editing outcome. The human APOBEC3A cytosine deaminase mediates base conversion inside an extended 17-bp editing window in rice, wheat and potato, thereby increasing the saturated mutagenesis potential of a targeted locus (Zong et al., 2018). In order to increase the affinity of CBEs with their ssDNA substrates, Zhang et al. (2020b) fused a ssDNA-binding protein domain between the nCas9

and the deaminases, resulting in highly efficient cytosine base editing in an expanded editing window. On the contrary, CBEs with narrowed editing windows have been developed to avoid bystander mutations, allowing highly precise base substitution (Tan et al., 2019; Tan et al., 2020). Third, the CBE harbouring the rAPOBEC1 deaminase domain fused to an UGI was shown to induce substantial genome-wide sgRNA-Cas9-independent off-target C-to-T mutations in rice, while the ABE did not result in such unwanted effects (Jin et al., 2019). These single-nucleotide variants were especially encountered in genic regions, where single-stranded DNA is generated due to active transcription (Jin et al., 2019). To minimize these CBE-mediated unpredictable genome-wide off-target mutations also observed in animals (Lee et al., 2020; Zuo et al., 2019), engineered CBEs have been developed in animals and still need to be validated in plants (Doman et al., 2020).

Combined with sgRNA libraries, the base editing toolbox holds great promises to drive CRISPR-directed *in planta* evolution of proteins by generating many targeted mutations in a whole gene or specific sequence-encoding domains, allowing the identification of new key amino acid(s) associated with agronomic traits (Capdeville et al., 2020). So far, CRISPR-directed *in planta* evolution has been applied to confer herbicide resistance through amino-acid substitutions in *OsALS1* and *OsACC* genes (Kuang et al., 2020; Li et al., 2020b; Liu et al., 2020), but there is no doubt that this strategy could be used for ecological-friendly purposes, such as the development of pathogen-resistant crops.

The ever-growing base editing toolbox now includes many CBEs and ABEs that could meet various applications for the development of plants with new traits, such as the precise editing of a particular site or *in vivo* directed evolution. However, in addition to the restricted range of outcomes mediated by current base editors, each application needs a proper and careful selection of the most appropriate tool, limiting the wide adoption of base editing and highlighting the need for more versatility.

CRISPR-mediated prime editing

Despite the considerable expansion of the CRISPR toolbox, precise and predictable targeted transversions, insertions, and deletions are still difficult to introduce into eukaryote genomes. Recently, a new ground-breaking technology that directly mediates the writing of new genetic information into a specific locus has been implemented in mammalian cells, unleashing new possibilities for precise genome editing. This ‘search and replace’ technology, called prime

editing, mediates targeted insertions, deletions, and any single or multiple substitutions (transitions and transversions) without requiring a DSB or a DNA donor template (Anzalone et al., 2019). Prime editors (PEs) are composed of a reverse transcriptase (RT) tethered to a nickase Cas9 with impaired HNH domain (H840A) (Figure 5A). The editing protein complex is guided by an engineered prime editing sgRNA named pegRNA and consists of a classical sgRNA fused to a customizable 3' extension that includes a primer binding sequence (PBS) and an RT template bearing the desired polymorphism (Figure 5A). Site-specific ssDNA breakage of the non-targeted strand and annealing of the PBS to the free 3' end of the nicked strand result in priming of the reverse transcription of the RT template. This results in the polymerisation of an edited ssDNA at the free 3'end that is complementary to the RT template and that is called a 3' edited flap (Figure 5B). Subsequent eukaryotic DNA repair mechanisms favour 5' flap excision and 3' edited flap ligation (Keijzers et al., 2015; Liu et al., 2004), thereby producing a heteroduplex between the edited strand and the unmodified strand, which is then resolved to permanently stabilize the desired edit (Figure 5B). Similar to the strategy used for base editing, nicking the non-edited strand substantially increased the efficiency of PEs by favouring the stable incorporation of the edits (Anzalone et al., 2019).

While the successful development of highly versatile and precise PEs in mammalian cells holds great hopes, implementation of plant prime editors (PPEs) could also contribute to the improvement of food crops (Zhang et al., 2020c). A few months after its application in animals, prime editing has been adopted by several groups working on cereal crops (Butt et al., 2020; Hua et al., 2020a; Li et al., 2020c; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a; Xu et al., 2020b). Three different PPEs were assayed for their editing efficiency: PPE2, PPE3 and PPE3b (Figure 5C). While PPE2 only consists on the expression of the nCas9-RT fusion and the pegRNA, PPE3 aims to promote favourable repair by nicking the non-edited strand using an additional sgRNA targeting the edited strand upstream or downstream the editing site. PPE3b also consists of nicking the non-edited strand, but the additional sgRNA targets the new edited sequence so that nicking is restricted only after 3' flap resolution, thereby preventing the formation of DSBs that would lead to higher indels rate (Figure 5C). PPE2, PPE3 and PPE3b systems harbouring an engineered version of Moloney murine leukaemia virus (M-MLV) RT resulted in similar editing efficiencies in rice and wheat protoplasts, as well as in *Agrobacterium*-mediated transformed rice plants. This indicates that nicking the non-edited strand does not necessarily increase prime editing efficiency in plants (Butt et al., 2020; Hua et al., 2020a; Lin et al., 2020; Xu et al., 2020a).

PPEs were shown to specifically allow the introduction of all types of single or multiple base substitutions, as well as deletions (up to 40-bp) and insertions (up to 15-bp) (Li et al., 2020c; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020b). As observed in mammalian cells, by-products were mainly pegRNA scaffolds insertions, which likely originate from extensive activity of the RT, and large deletions due to paired nicking of both strands (Lin et al., 2020; Tang et al., 2020). Overall, editing efficiencies in rice and wheat were in the low percentage range, although precise 6-bp deletion and single A-to-T transversion were detected in 21.8% and 31.3% of rice plants regenerated from *Agrobacterium*-mediated transformation respectively (Lin et al., 2020; Xu et al., 2020a).

The successful proof-of-concept of CRISPR-mediated prime editing in plants opens up exciting perspectives, although some challenges need to be overcome for a broad use of this new tool. Enhancing prime editing efficiency constitutes an essential track, especially for polyploids and/or vegetatively propagated species. Because a high variability of prime editing activity was observed among targeted sites, the 'copy and replace' mechanisms may be enhanced to promote reliable outcome rates. PPE architecture should be optimized to maximize CRISPR components expression levels (Tang et al., 2020; Xu et al., 2020a; Xu et al., 2020b), and using different RTs that may be more efficient in plant cells is of particular interest, as well as optimizing temperature conditions for reverse transcriptase activity (Lin et al., 2020). The systematic testing of some pegRNA (PBS and RT lengths, esgRNA scaffold) and sgRNA (position of the nicking) designs for new targets is also highly recommended (Li et al., 2020d). While PPEs can accommodate long RT templates and are much less constrained than BEs for PAM availability, the use of Cas9 variants with relaxed PAM recognition may be relevant to localize the edit at putatively favourable position from the ssDNA cutting site. Finally, although prime editing seems to induce lower off-target editing than Cas9 at putative off-target sites in animals (Anzalone et al., 2019), genome wide off-target activity of PPEs needs to be carefully evaluated to assess the capacity of RT to cause Cas9-independant unwanted edits.

PRECISION BREEDING, A MATTER OF CHOOSING THE RIGHT TOOL IN THE TOOLBOX

Collectively, CRISPR-mediated GT, base editing and prime editing constitute an extended toolbox for precision editing, offering complementary strengths and drawbacks to edit almost

any target site. When large DNA sequences need to be precisely inserted or deleted, the classical GT approach is the most suitable tool, as prime editing efficiency decreases with increasing length of the desired insertion or deletion (Lin et al., 2020). However, targeted small insertions and deletions can be efficiently mediated by both the prime editing system and the GT strategy. Besides the utility of such modifications for crop improvement, the possibility to label endogenous proteins with specific tags is of particular interest (e.g. cellular localization, purification, immunoprecipitation). Ali et al. (2020) recently managed to insert the HA-epitope into the C terminus of OsHDT using the CRISPR-Cas9-VirD2 system. It may also be possible to generate such insertion using the prime editing system, provided that the flag length is within the range of possible insertions by PEs.

Base editing appears to be generally more efficient than current PPEs for base substitution(s) (Anzalone et al., 2019; Lin et al., 2020). Therefore, early BEs should be used when bystander mutations are acceptable, whereas new BEs harbouring narrowed editing windows should be favoured when bystander edits are not desirable. However, when the desired outcome cannot be generated by BEs (e.g. most transversions or multiple base substitutions), PPEs offer much more versatility. For applications requiring targeted local random mutagenesis, such as directed evolution of proteins, BEs still constitute the most suitable tool. However, PPEs might be modified to randomly insert polymorphism in the target site through low-fidelity reverse transcriptases, thereby providing another source of genetic variability. Because prime editing is only at an early stage of development, we hope that future improvements will considerably enhance the efficiency and widen the targeting scope of PPEs.

A CRISPR WAY FOR PATHOGEN RESISTANCE ENGINEERING

Interestingly, CRISPR-Cas can be directly used to target the pathogens'genome, mainly viruses. This could be achieved by either targeting DNA viruses or RNA viruses, but requires the transgenic expression of the CRISPR-Cas machinery and specific gRNA, an approach reminiscent of RNAi strategies. This therefore falls beyond the scope of precision breeding, but the reader can find details on these strategies, as well as their possible caveats, in recent reviews (Pyott et al., 2020; Zhao et al., 2020). Now, it becomes possible to apply precision breeding through CRISPR technology to improve traits conferred by precise and/or punctual sequence variation, with an extraordinary opportunity to develop genetically resistant crops for a sustainable agriculture. CRISPR applications have been predominantly focused on

generating loss-of-function alleles, with some successes for the production of pathogen resistant plants (Langner et al., 2018). However, plant-microorganism interactions result from a long coevolution involving a complex molecular dialogue with several key players. As a result, CRISPR-mediated gain-of-function mutations appear to be highly relevant for developing crops with improved resistance to pathogens. In the following, we review current knowledge of CRISPR-mediated precision editing for pathogen resistance and provide interesting tracks that are now within CRISPR reach.

Immune receptor engineering

Considerable progress has been made in recent years regarding the molecular mechanisms of action, structural properties and evolution of NLR receptors (Burdett et al., 2019; Kourelis and van der Hoorn, 2018; Tamborski and Krasileva, 2020). This enables novel strategies to improve the capacity of NLRs to induce immune responses, broaden their pathogen recognition spectrum or even create new recognition specificities. However, there are currently very few examples of immune receptors having been improved in this way (Cesari, 2018; Grund et al., 2019; Tamborski and Krasileva, 2020). Besides, current NLR engineering strategies essentially rely on either testing modified *NLR* genes in transient expression systems (e.g. by agroinfiltration in *Nicotiana tabacum* or *benthamiana*) or complementing susceptible varieties by stable transformation. Use of a CRISPR-based system for engineering *NLR* genes has not been reported. However, this represents a promising strategy to create new disease resistances directly in elite varieties. The development and quick improvement of a wide range of CRISPR tools pave the way toward these new strategies.

One approach for NLR engineering relies on editing of residues required for regulation of these receptors in order to enhance their activation potential and, by this, enlarge their pathogen recognition spectrum. This strategy has been used for the wheat powdery mildew resistance gene *Pm3*, which forms an allelic series mediating the specific recognition of *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolates. By comparing several alleles of *Pm3* that exhibit a broad (*a* and *b* alleles) or narrow (*f* allele) resistance spectrum, Stirnweis et al. identified two polymorphisms in the NB domain that are responsible for enhanced signaling activity and extended resistance spectrum (Stirnweis et al., 2014). CRISPR-mediated prime editing of such regulatory residues in NLRs could create artificial ‘trigger happy’ variants with broadened resistance spectrum directly in elite cultivars. However, misregulation of

NLRs carries the risk of pleiotropic phenotypes and such potential trade-off phenomenon must be taken into consideration in this type of approaches.

Alternatively, the recognition spectrum of NLRs can be broadened or modified by changing residues responsible for effector recognition specificity. In allelic NLRs series where distinct alleles exhibit different pathogen recognition specificities (e.g. barley *MLA*, wheat *Pm3*, flax *L* or rice *Pi-2/Piz-t/Pi50*), the LRR domain plays a crucial role in effector recognition specificities (Dodds et al., 2006; Saur et al., 2019). In these cases, an attractive application of CRISPR technology is to provide an elite cultivar with a recognition specificity already existing in other varieties by mutating the specific residues or sequences in LRR domain that determine specificity. This would enable to adapt the pathogen recognition specificities of elite cultivars according to pathogen populations without going through tedious crossing and selection steps. The potential for this type of approach is illustrated by the historical example of the flax NLRs L2, L6 and L10 for which swaps of LRR domains have enabled changes in flax-rust recognition specificities (Ellis et al., 1999).

Knowledge-guided engineering of completely new recognition specificities by targeted mutagenesis of specific residues in the LRR domain is for the moment not yet possible. For this, one would require much better insight into the molecular mechanism of NLR activation and specific and precise knowledge on the LRR residues mediating effector recognition and specificity. Investigation of the allelic diversity coupled with structural modelling of LRR domains may help in the identification of polymorphic surface residues that are likely involved in effector binding. Filling this knowledge gap is therefore a priority. Indeed, for the moment, novel recognition specificities by mutations in the LRR domain were only generated by random mutagenesis approaches. For example, in the potato NLR Rx, which confers resistance to potato virus X (PVX), point mutations in the LRR domain were identified that extended the recognition spectrum (Farnham and Baulcombe, 2006). CRISPR-mediated introduction of such mutations identified by random mutagenesis approaches in high throughput screening systems promise to create novel or broadened resistances.

Another strategy based on genome editing techniques consists in reactivating pseudogenized *NLR* genes in elite varieties of agronomic interest. This would allow "resuscitation" of resistance without the laborious steps of cloning and complementation and, in many countries, issues related to GMO regulation . This strategy is relevant for *NLRs* where loss of function is due to a limited number of polymorphisms, which can be “repaired” through base editing.

Such a strategy has been tested using transcription activator-like effector nucleases (TALEN) editing on the wheat *Lr21* gene, which provides race□specific resistance to leaf rust disease caused by *Puccinia triticina* (Luo et al., 2019). The inactive *lr21Ψ* allele differs to *Lr21* by three nonsynonymous polymorphisms and a single base deletion that disrupts the ORF. By editing the single base deletion, Luo et al. (2019) restored the *lr21Ψ* ORF but this did not reconstitute a functional resistance gene. CRISPR-mediated base editing has been successfully used in rice to reactivate the RLK-coding gene *Pi-d2*, which confers resistance to blast disease (Ren et al., 2018). Rapid progress in the fields of comparative genomics, population genomics and intraspecific detection of NLRs (e.g. by resistance gene enrichment sequencing), which enable the identification of polymorphisms in *NLR* genes associated with disease resistance or susceptibility, will benefit these NLR engineering approaches.

Some NLRs contain unconventional integrated domains (IDs) that interact with pathogen effectors (Bailey et al., 2018; Cesari et al., 2014; Kroj et al., 2016; Le Roux et al., 2015; Sarris et al., 2016; Sarris et al., 2015). Precise engineering of these IDs could result in enhanced and/or broader resistance (Cesari, 2018). Recently, the 3D structures of two IDs in complex with the effectors they recognize have been resolved enabling precise identificztion of the residues for effector binding (Guo et al., 2018; Maqbool et al., 2015). This allowed in the case of Pikp-1 that recognizes the *Magnaporthe oryzae* effector AVR-PikD to perform structure-informed editing of the ID leading to the recognition of the previously not recognized effector allele AVR-PikE (De la Concepcion et al., 2019). This gain of specificity was shown *in vitro* and in transient assays in *N. benthamiana*. Whether the mutations leads to an extended resistance in the homologous rice/*Magnaporthe oryzae* system remains yet to be demonstrated. A CRISPR-mediated base editing strategy in the true host plant would be a real asset in this type of experiments. Although extremely powerful, these approaches remain complicated because of gaps in our knowledge on the mode of action and structure of NLRs, in particular those that operate in pairs. When these gaps are filled, it will be virtually possible to create engineered NLR receptors capable of recognizing a wide variety of biotrophic or hemibiotrophic pathogens.

In the future, CRISPR-mediated directed evolution of NLR domains using base editors or even prime editors, followed by screening for gain-of-resistance mutants, promise to become a powerful strategy for the development of new resistance in crops through completely new effector recognition specificities. However, its development awaits better molecular understanding of NLR function to precisely target the right motifs and will require special

attention to preserve agronomic traits by avoiding improper regulation of NLRs that can result in autoimmunity, highlighting the need to find a balance between pathogen detection and fitness (van Wersch et al., 2020).

In many cases, recognition of effectors by NLRs is indirect and occurs through the detection of effector-mediated modifications of plant proteins, called guardees or decoys (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). A promising strategy for resistance engineering consists in modifying such decoys or guardees to trap novel pathogen effectors. A proof for this concept was provided in *Arabidopsis thaliana* using the serine-threonine kinase PBS1, whose cleavage by the bacterial effector AvrPphB is monitored by the NLR RPS5. Transforming *RPS5* plants with a *PBS1* mutant carrying the cleavage sites of other bacterial or viral proteases resulted in recognition of these proteases and novel bacterial or virus resistances (Kim et al., 2016) (Figure 6A). Using genome editing tools such as CRISPR-mediated GT or prime editing, the endogenous locus encoding the 7 residue cleavage site of PBS1 could be readily modified into cleavage sites of other pathogen proteases (Figure 6B), resulting in RPS5-mediated surveillance of these novel effectors (Pottinger and Innes, 2020). PBS1 is highly conserved among flowering plants and NLR-mediated surveillance of its cleavage emerged repeatedly in evolution making it a versatile decoy system in corresponding crops (Carter et al., 2019; Pottinger and Innes, 2020). More generally, similar trap systems for proteases or other effector can probably be engineered with other decoys or guardess in a large spectrum of crops even if they do not possess a PBS1 surveillance system (Giannakopoulou et al., 2016; Kim et al., 2016; Pottinger et al., 2020).

Host factor engineering

Because NLR-mediated resistance is often quickly bypassed by pathogens, S-gene engineering constitutes an exciting alternative for diversifying the sources of resistance. S-genes, that can be targeted by pathogen effectors or act independently, facilitate pathogen infection and can either encode proteins involved in host recognition, penetration or metabolism, or act as regulator of plant immunity (Langner et al., 2018). Contrary to R-genes that are generally dominant, loss of susceptibility conferred by engineering S-factors is mainly recessive, meaning that all alleles should be altered to achieve resistance. This is of course a substantial challenge for polyploid plants. To date, most genome editing applications aiming at conferring pathogen resistance consisted in knocking out S-genes (Langner et al.,

2018; Zaidi et al., 2018). However, such strategy may be associated with deleterious side-effects as S-genes may encode essential proteins for the host (see below).

For example, bacterial and fungal infections lead to a competition for carbon resources at the plant/pathogen interface, in which host sugar transporters play a key role for the outcome of the interaction (Lemoine et al., 2013). In order to increase the sugar supply in the apoplasm, the bacteria *Xanthomonas oryzae* pv. *oryzae* activates the transcription of members of the rice *SWEET* gene family, encoding proteins that mediate passive diffusion of sucrose across the plasma membrane (Figure 7A). This is achieved through the expression of the so-called transcription-activator-like effectors (TALEs) that bind specific regions of the SWEET promoters to activate the transcription, resulting in an enhanced export of sucrose to the apoplast that sustains bacterial growth. Because SWEET proteins are key components of phloem loading for long-distance transport of sucrose (Lemoine et al., 2013), CRISPR-mediated loss-of-function approaches may result in unwanted developmental effects (Chen et al., 2012). In this regard, promoter targeting is an attractive alternative consisting in the introduction of random indel mutations into TALE binding elements. Such a strategy was performed by targeting some *OsSWEET* genes, thereby preventing *OsSWEET* induction by bacterial effectors and conferring bacterial blight broad-spectrum resistance (Li et al., 2020a; Oliva et al., 2019; Xu et al., 2019b). Similarly, CRISPR-Cas9/Cas12a-mediated promoter editing of the *CsLOB1* gene, specifically targeted by bacterial effectors for transcription activation, resulted in the generation of canker-resistant citrus cultivars (Jia et al., 2019; Peng et al., 2017). Because Cas9 nuclease mostly induce small deletions, we postulate that such strategy could be improved using Cas12a and Cas12b nucleases, resulting in a higher rate of larger deletions, as previously discussed. The use of Cas variants with relaxed PAM recognition may also be valuable to precisely target cis-regulatory elements.

With the recent expansion of the CRISPR toolbox, it is now possible to edit specific bases leading to predetermined punctual amino-acid change, aiming at developing new or mimicking natural alleles conferring resistance. The *eukaryotic Initiation Factor 4E* (*eIF4E*) genes are key elements of eukaryotic protein synthesis. At the same time, they are also very important susceptibility factors to members of the large *Potyviridae* family, which rely on those factors to perform their infectious cycle in the plant (Bastet et al., 2017) (Figure 7B). Natural resistances found in various plant species often rely on functional resistance *eIF4E* alleles that contain non-synonymous mutations in the coding sequence. Those alleles are devoid of associated fitness costs or developmental defects that are associated with loss-of-

function alleles. Moreover it has been shown that the deployment of those functional alleles can reduce the risk of resistance-breaking (Bastet et al., 2017). As a result, conversion of the *Arabidopsis eIF4E1* susceptibility allele into a resistant allele through CBE-mediated single amino acid mutation (N176K) was recently performed at no yield cost (Bastet et al., 2019). It is expected that this approach could be generalized to any crops that are devoid of natural eIF4E resistance allele to potyviruses and related single-strand positive RNA viruses. However, current base editing tools by themselves are quite limited to generate the large range of amino acids changes associated with resistance that could be copied across species. Therefore, it is expected that prime editing could considerably help designing new resistance alleles to mimic more accurately natural resistance alleles that can gather up to 5 independent non-synonymous amino acid changes compared with the susceptible allele. It is expected that this larger number of mutations will help increasing the resistance spectrum as well as the resistance durability associated with this allele (Moury et al., 2014).

Besides translation initiation factors, it is expected that a large number of S genes are available to design new sources of resistances (Hashimoto et al., 2016; van Schie and Takken, 2014). Precise modification of other host factors to prevent their recognition by pathogen effectors, such as auxin response factors (ARFs) that are targeted by *Fijiviruses* proteins, will definitely provide additional resistance mechanisms for crop molecular breeding towards viruses (Zhang et al., 2020a). We expect that several other host factors could be precisely edited in the coming years, providing new molecular mechanisms for the development of elite crops with improved genetic resistance towards a broad spectrum of pathogens.

BOTTLENECKS AND PERSPECTIVES

The CRISPR toolbox for precision breeding in plants greatly expanded in the last few years, allowing the precise and predictable editing of almost any locus in the genome, at least in theory. While improvements of the newly prime editing system are needed, plant scientists have now access to a highly versatile genome editing toolbox for both functional genomics and molecular crop breeding.

However, in addition to the CRISPR system in itself, delivery methods of genome editing reagents into plant cells constitutes the main technical limitation. While transformation of major plant crops such as rice, wheat, tomato or potato is well established, some bottlenecks

still stand in the way for broad use of CRISPR in crop precision breeding. First, classical delivery methods such as *Agrobacterium*-mediated transformation, protoplast transfection and biolistic mostly target somatic cells and therefore involve subsequent regenerative steps that are time-consuming and highly genotype-dependant (Atkins and Voytas, 2020). Furthermore, delivery and tissue culture methods can cause unwanted changes to the genome, as recently evidenced after protoplast transfection and *Agrobacterium*-mediated transformation in the tetraploid potato (Fossi et al., 2019), and after biolistic transformation in rice and maize (Liu et al., 2019). Secondly, most current delivery methods involve the stable integration of foreign DNA into plant genomes. While these sequences can be segregated out following mendelian inheritance, it would be advantageous to minimize their expression window to avoid off-target effects, especially for base editors. Furthermore, the introduction of DNA intermediates into the plant nucleus may result in genome-wide random insertions, pointing out the necessity to use DNA-free delivery methods. As a result, while we are now able to precisely edit target sites through highly specific CRISPR tools, a special focus should be put on minimizing CRISPR-independent side effects, highlighting the need to develop alternative delivery methods into plant cells to avoid or limit such undesirable effects (Demirer et al., 2019; Maher et al., 2020; Toda et al., 2019), thereby unlocking the full potential of the CRISPR technology.

Finally, it is evident that CRISPR technology has great potential for both plant biology research and precision crop breeding. The CRISPR precision toolbox, that is expanding and disseminating at an extraordinary speed, will definitely help us to decipher plant immune responses upon pathogen infection. However, while we are now also able to mimick or evolve immune molecular mechanisms that confer genetic resistance to a broad range of pathogens, with the potential to support food security and safety in a sustainable way through the reduction of chemical use, regulatory frameworks constitute the main obstacle to CRISPR application in food crops, especially in Europe (Zhang et al., 2020c). We expect that a product-based regulatory framework could provide a rational balance between human/environment safety concerns and plant breeding innovation.

AUTHOR CONTRIBUTIONS

F.V., T.K., S.C. and J.-L.G. jointly wrote the original manuscript draft. F.V. and M.D. prepared the figures. F.V. and J.-L.G. planned the review outline. All authors contributed to the reviewing and editing of the manuscript.

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FIGURE CAPTION

Figure 1

CRISPR-Cas systems used for genome editing in plants.

(A) The CRISPR-SpCas9 system made of the endonuclease SpCas9, harbouring RuvC and HNH catalytic domains, and the sgRNA that guides the complex to an endogenous target sequence upstream of a G-rich PAM (5'-NGG-3'), leading to blunt and/or staggered DNA breaks.

(B) The CRISPR-Cas12a system involves the endonuclease Cas12a that is guided to the target locus, downstream of a T-rich PAM (5'-TTTN-3'), by a short crRNA, leading to a staggered DNA cleavage by a single RuvC domain after conformational changes [(1) and (2)].

(C) The CRISPR-Cas12b system relies on a Cas12b endonuclease, harbouring a single RuvC catalytic domain that mediate staggered DNA cleavage [(1) and (2)], and a sgRNA that target the complex to a specific site downstream of a T-rich PAM (5'-VTTV-3').

The schemes are not at scale and are for illustrative purposes only.

Figure 2

NHEJ- and HR-mediated DNA mutations after CRISPR cleavage

(A) CRISPR-mediated gene knockout through introduction of indels mutations at the cutting site after reparation by the error-prone NHEJ repair mechanism.

(B) CRISPR-mediated fragment deletion after dual sgRNA-induced DSBs, resulting in fragment deletion with associated indels after error-prone NHEJ repair.

(C) CRISPR-mediated fragment replacement after dual sgRNA-induced DSBs, resulting in the replacement of a specific locus by a donor DNA sequence, with associated indels due to error-prone NHEJ repair.

(D) CRISPR-mediated gene targeting (GT) for precise and predictable deletions, insertions and/or DNA substitutions. Homologous recombination (HR) repair pathway occurs through introduction of available donor templates (mainly dsDNA and ssDNA) harbouring homologous sequences with both sides of the CRISPR-induced DSB.

(E) CRISPR-Cas9-VirD2-mediated GT, allowing to provide the repair ssDNA template in the vicinity of the cutting site through interaction between the 5' specific sequence (purple) of the ssDNA donor template and the VirD2 domain. This spatiotemporal delivery of repair template may increase the rate of precise repair through HR pathway.

The schemes are not at scale and are for illustrative purposes only.

Figure 3

CRISPR-mediated base editing using cytosine base editors (CBEs)

(A) CBEs are composed of a nCas9 (D10A) fused to a cytosine deaminase catalytic domain (rAPOBEC1, PmCDA1, hAID or hA3A) that mediates cytosine deamination in the so-called editing window at the 5' end of the non-targeted sequence.

(B) After C deamination into U, endogenous uracil DNA glycosylase (eUNG) detect and remove the U, leading to an abasic site, which is further processed through error-free (U-to-C) or error-prone repair, producing different base substitutions, albeit at the cost of indels mutations due to the generation of DSBs through concomitant ssDNA breaks by the nCas9 and endogenous AP lyases (eAP lyase). This system allows the production of C-to-T, C-to-G and C-to-A conversions.

(C) CBE architecture can be upgraded through the fusion of one to several uracil glycosylase inhibitors (UGIs) to the base editor, with the aim of increasing the rate of C-to-T conversion while limiting the formation of by-products.

(D) After C deamination, UGIs protect the U edits from eUNG, thereby preventing the formation of abasic sites and mostly producing C-to-T conversion through the nicking of the non-edited strand and the intervention DNA repair/replication mechanisms, with low level of by-products such as indels mutations.

The schemes are not at scale and are for illustrative purposes only.

Figure 4

CRISPR-mediated base editing using adenine base editors (ABEs)

(A) ABEs are composed of a nCas9 (D10A) fused to an adenine deaminase catalytic domain (ecTadA-ecTadA*) that mediates adenine deamination in the so-called editing window at the 5' end of the non-targeted sequence.

(B) After A deamination into I (inosine), nicking of the non-edited strand and intervention of DNA repair/replication mechanisms produce A-to-G conversion, with very low rates of by-products.

The schemes are not at scale and are for illustrative purposes only.

Figure 5

CRISPR-mediated prime editing

(A) Plant prime editors (PPEs) are composed of a nCas9 (H840A) fused to a reverse transcriptase (RT), allowing insertions, deletions and all kinds of base substitutions. The polymorphism of interest is brought through a pegRNA, containing both a sgRNA for target specificity and a 3' extension that harbours a RNA template bearing the polymorphism, leading to the targeted writing of new DNA sequences through reverse transcription.

(B) Upon cleavage of the non-targeted strand by the HNH domain of the nCas9, the primer binding site (PBS) sequence of the pegRNA hybridizes with the broken ssDNA upstream of the cleavage site. This RNA/DNA structure initiates reverse transcriptase activity, copying the genetic information from the RT template. After resolution of 3' flap ligation, DNA repair mechanisms permanently install the mutation.

(C) Different prime editing strategies can be used to increase the rate of desired outcomes. The PPE2 strategy only implies the use of the pegRNA, while the PPE3 strategy require the use of an additional sgRNA to cut the non-edited strand upstream or downstream of the modified sequence. For the PPE3b strategy the second sgRNA targets the edited sequence, in order to cut the non-edited strand only after 3' flap resolution, thereby limiting the risk of indels mutations through the occurrence of DSBs.

The schemes are not at scale and are for illustrative purposes only.

Figure 6

Representative model of the natural and engineered RPS5-PBS1 decoy systems.

(A) RPS5 and PBS1 form an inactive preactivation complex at the plasma membrane. Upon cleavage of the GDKSHVS motif in the activation loop of PBS1 by the *Pseudomonas syringae* AvrPphB type III protease, RPS5 sense the PBS1 conformational change, leading to activation of the RPS5-mediated hypersensitive response (HR).

(B) Using CRISPR precision editing tools, it is possible to replace the AvrPphB target cleavage sequence of PBS1 by a motif recognized by another secreted protease, such as the AvrRpt2 effector that cleaves the VPKFGDW sequence. Gene targeting (GT) or prime editing (PE) tools can be used to replace the initial target cleavage sequence to confer immunity toward pathogens (fungi, bacteria and viruses) that secrete proteases with known cleavage recognition motifs. Alternatively, protein evolution using base editing (BE) can generate punctual amino acid shifts to generate potential new cleavage sequences. The functionality of these PBS1 variants can be screened towards pathogens that secrete proteases with unknown molecular characteristics, potentially conferring new sources of crop resistance.

The schemes are not at scale and are for illustrative purposes only.

Figure 7

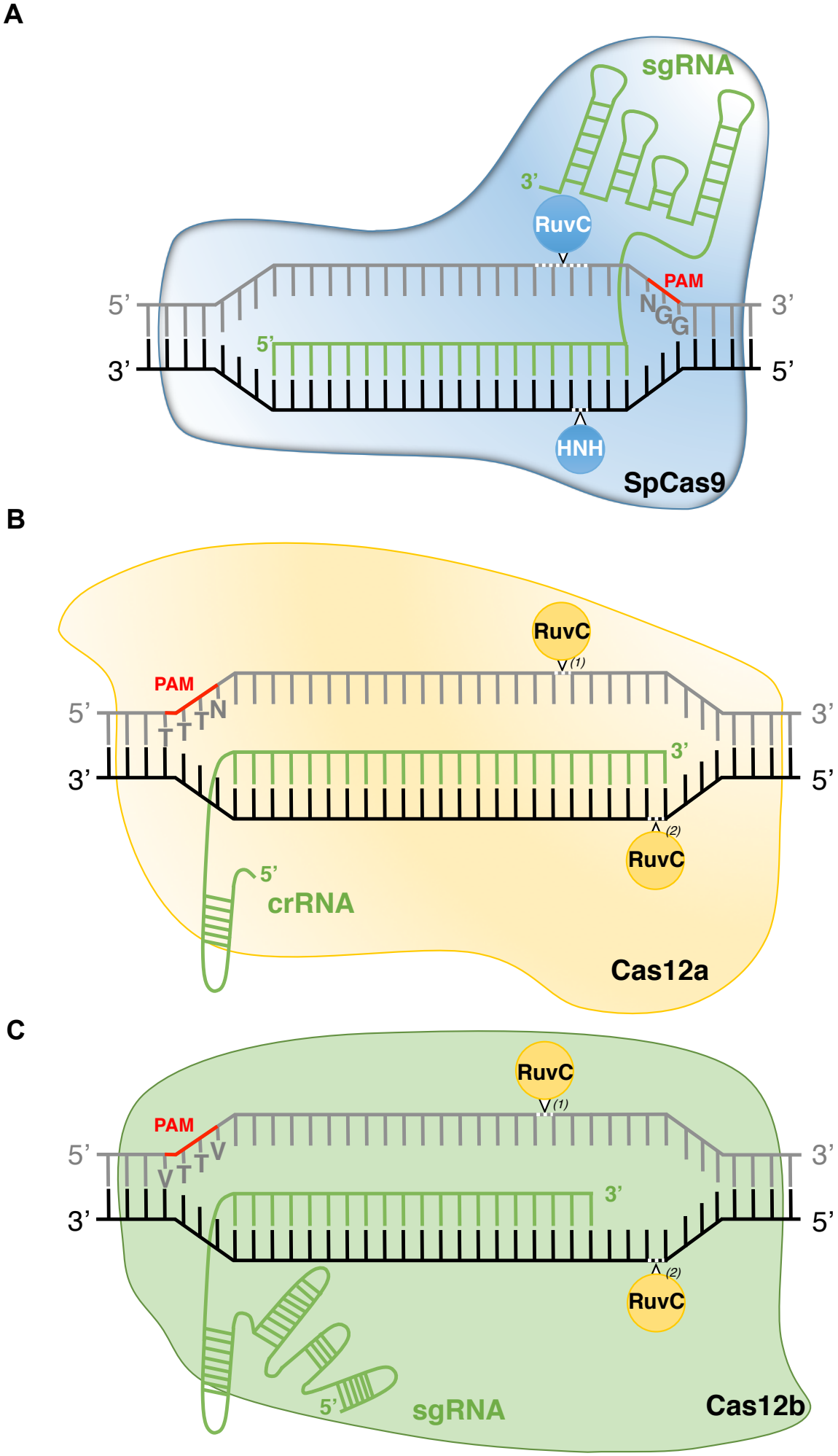
Representative model of editing resistance by loss-of-susceptibility.

(A) Resistance to bacteria through the edition of SWEET promoter. During infection leading to susceptibility (left side), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) bacteria express Transcription Activator Like effectors (TAL effector) in the plant cell. Those effectors bind Effector-binding elements (EBE) located in the promoters of the *SWEET* genes that encode

sucrose transporters. The binding triggers the activation of *SWEET* genes, and of the encoded sucrose transporter, and results in an increase in sucrose content in the apoplasm. The excess of sucrose benefits to the bacteria and contribute to its multiplication. Genetic resistance can be engineered (right side) by removing the EBE region (s) from the SWEET promoter region: the SWEET gene is no longer activated by the TAL effector, sucrose content stay low in the apoplasm, resulting in resistance.

(B) Resistance to Potyvirus through base editing of the translation initiation factors eIF4E. In susceptible plants (left side), the translation initiation eIF4E are necessary for the potyviruses, represented by their ssRNA^{+} genome linked in 5' to the Viral Protein genome linked or VPg, to perform their infection cycle. At the same time they are involved in translation initiation of the host mRNA for protein synthesis. Base editing of the *eIF4E* coding sequence (right side) can be used to introduce non-synonymous mutations associated with Amino Acid changes usually found in resistance alleles from the natural diversity of plants. This mutation does not affect the eIF4E function in translation initiation while suppressing its interaction with potyvirus, leading to resistance. This allows to develop resistance at no developmental cost. The translation initiation complex depiction is adapted from Robaglia et Caranta, 2006.

The schemes are not at scale and are for illustrative purposes only.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RuvC
PAM
N
G
G
5'
3'
5'
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5'
HNH
SpCas9
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(1)
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5'
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crRNA
Cas12a
C
RuvC
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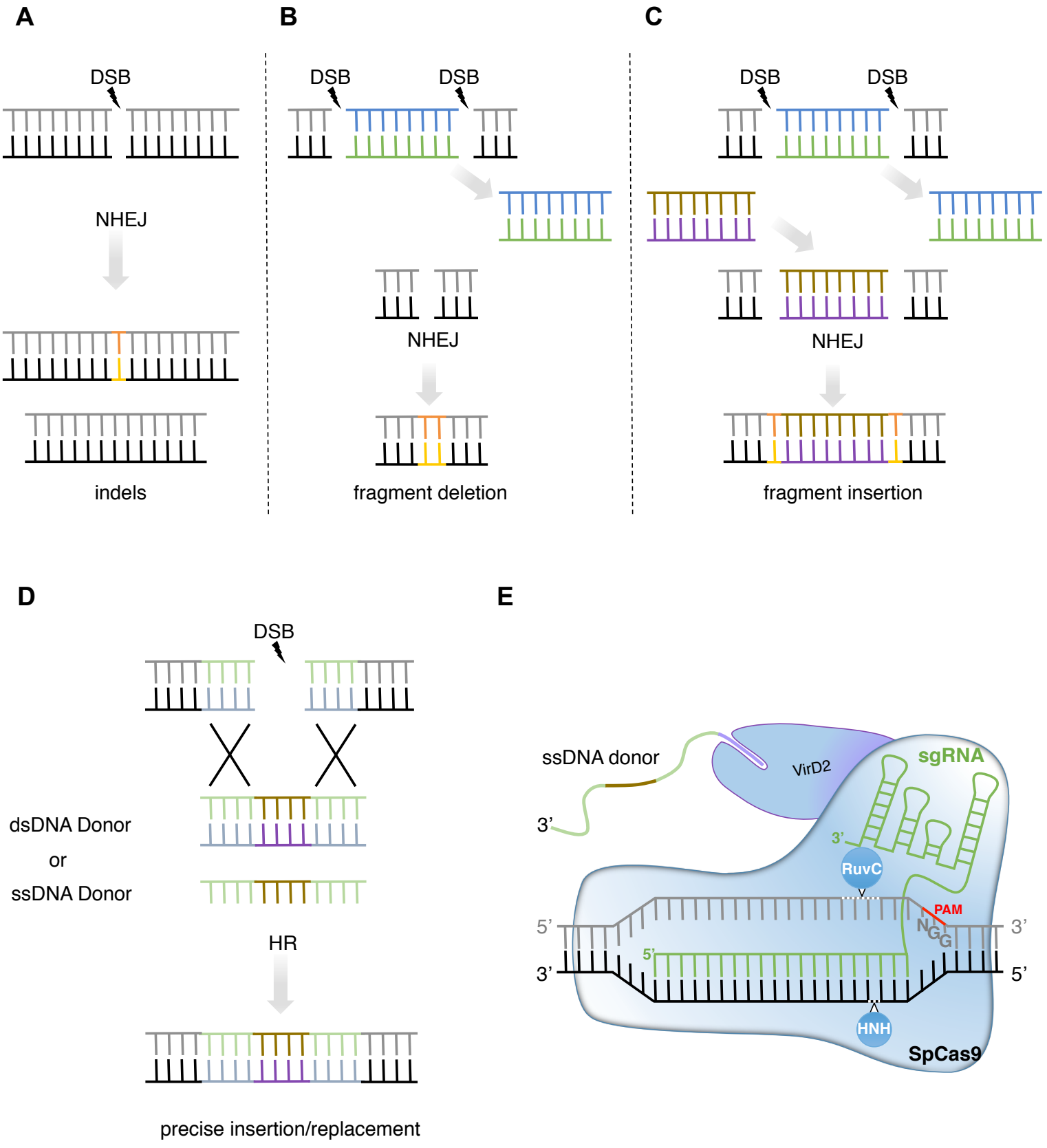
Cas12b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DSB
NHEJ
indels
B
DSB
DSB
NHEJ
fragment deletion
C
DSB
DSB
NHEJ
fragment insertion
D
DSB
dsDNA Donor
or
ssDNA Donor
HR
precise insertion/replacement
E
ssDNA donor
3'
VirD2
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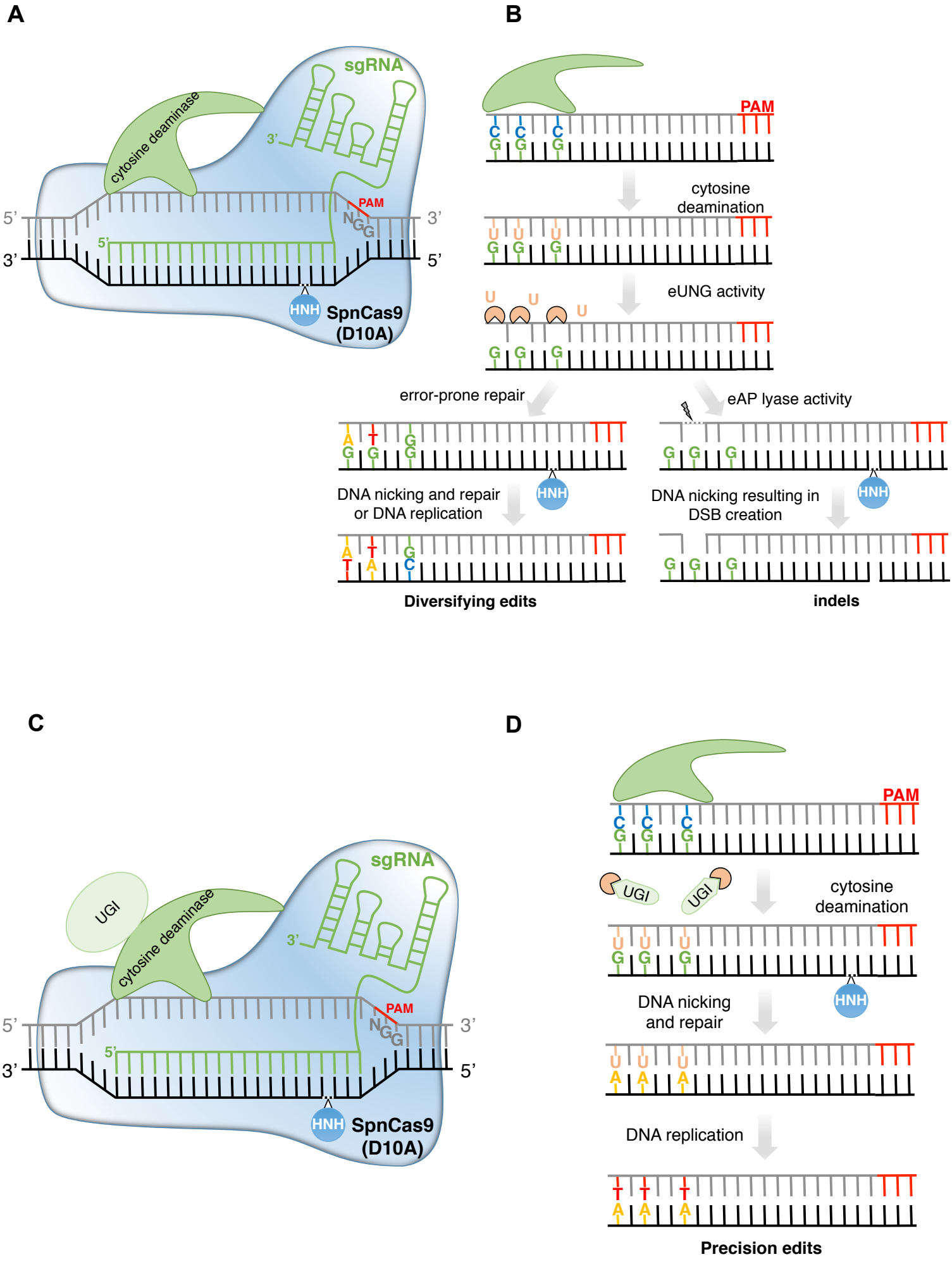
3'
RuvC
PAM
5'
NGG
3'
5'
3'
5'
HNH
SpCas9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sgRNA
cytosine deaminase
3'
PAM
5'
3'
5'
3'
5'
HNH
SpnCas9
(D10A)
B
PAM
cytosine
deamination
eUNG activity
error-prone repair
eAP lyase activity
HNH
DNA nicking and repair
or DNA replication
DNA nicking resulting in
DSB creation
Diversifying edits
indels
C
UGI
sgRNA
cytosine deaminase
3'
PAM
5'
3'
5'
3'
HNH
SpnCas9
(D10A)
D
PAM
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UGI
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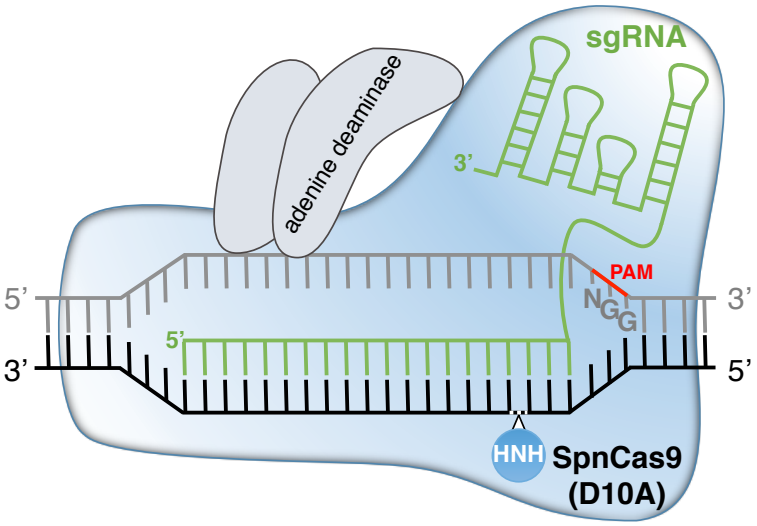
deamination
HNH
DNA nicking
and repair
DNA replication
Precision edits

A



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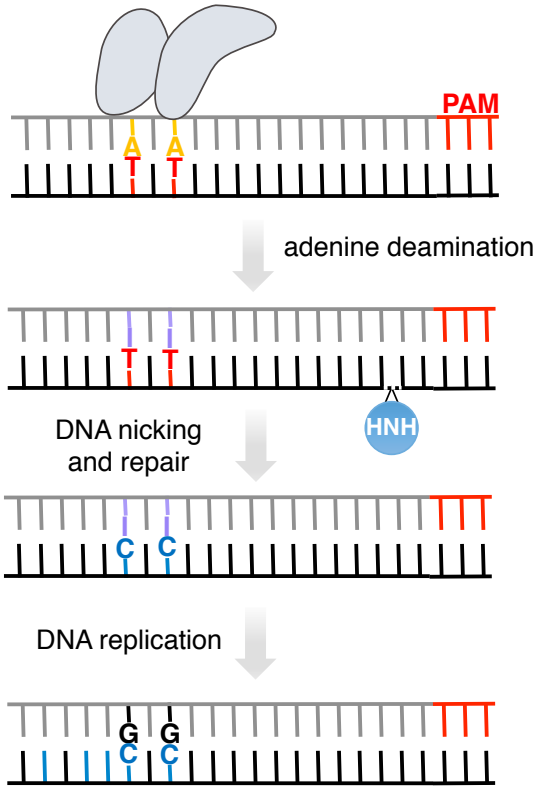
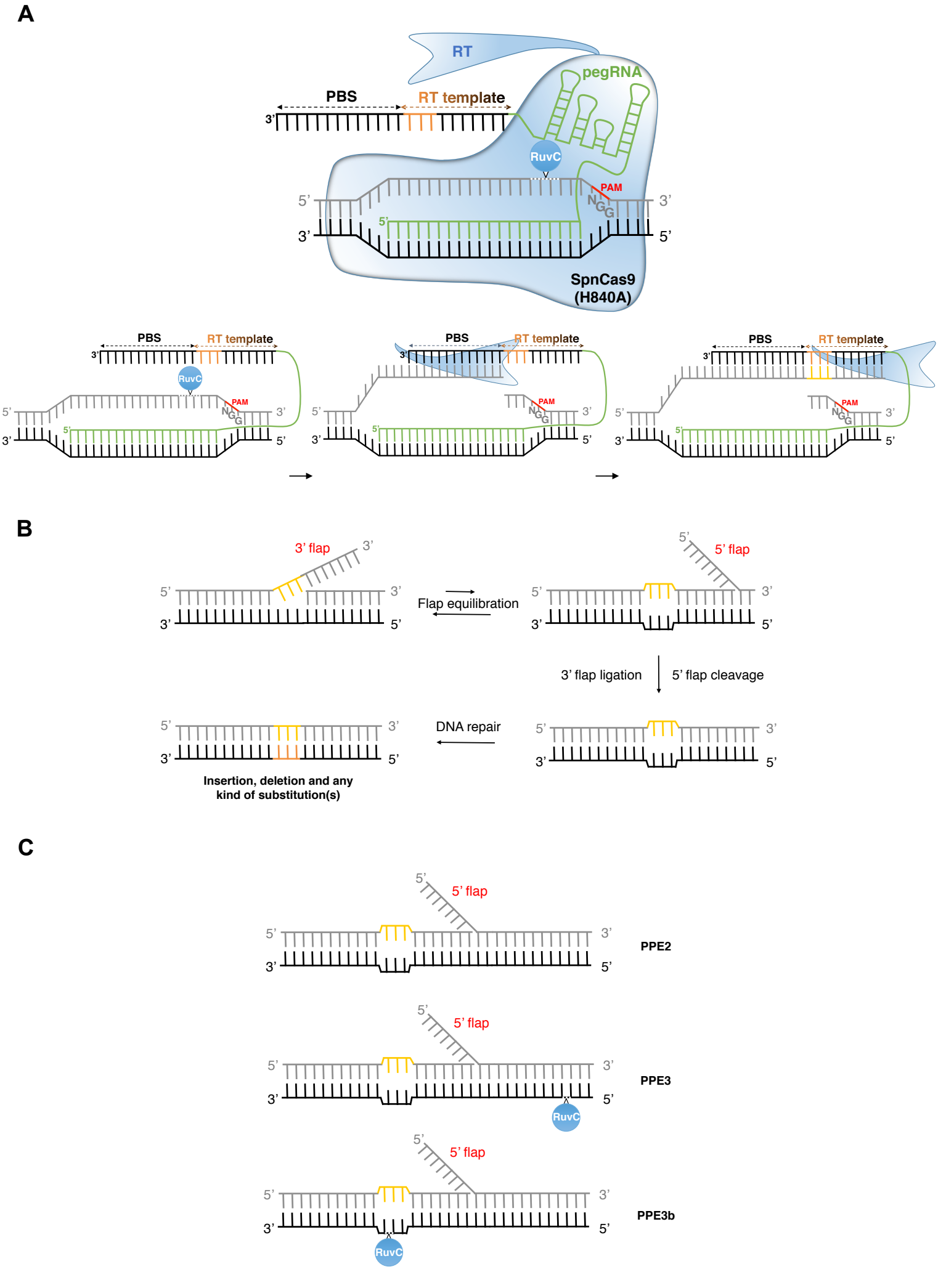


Precision edits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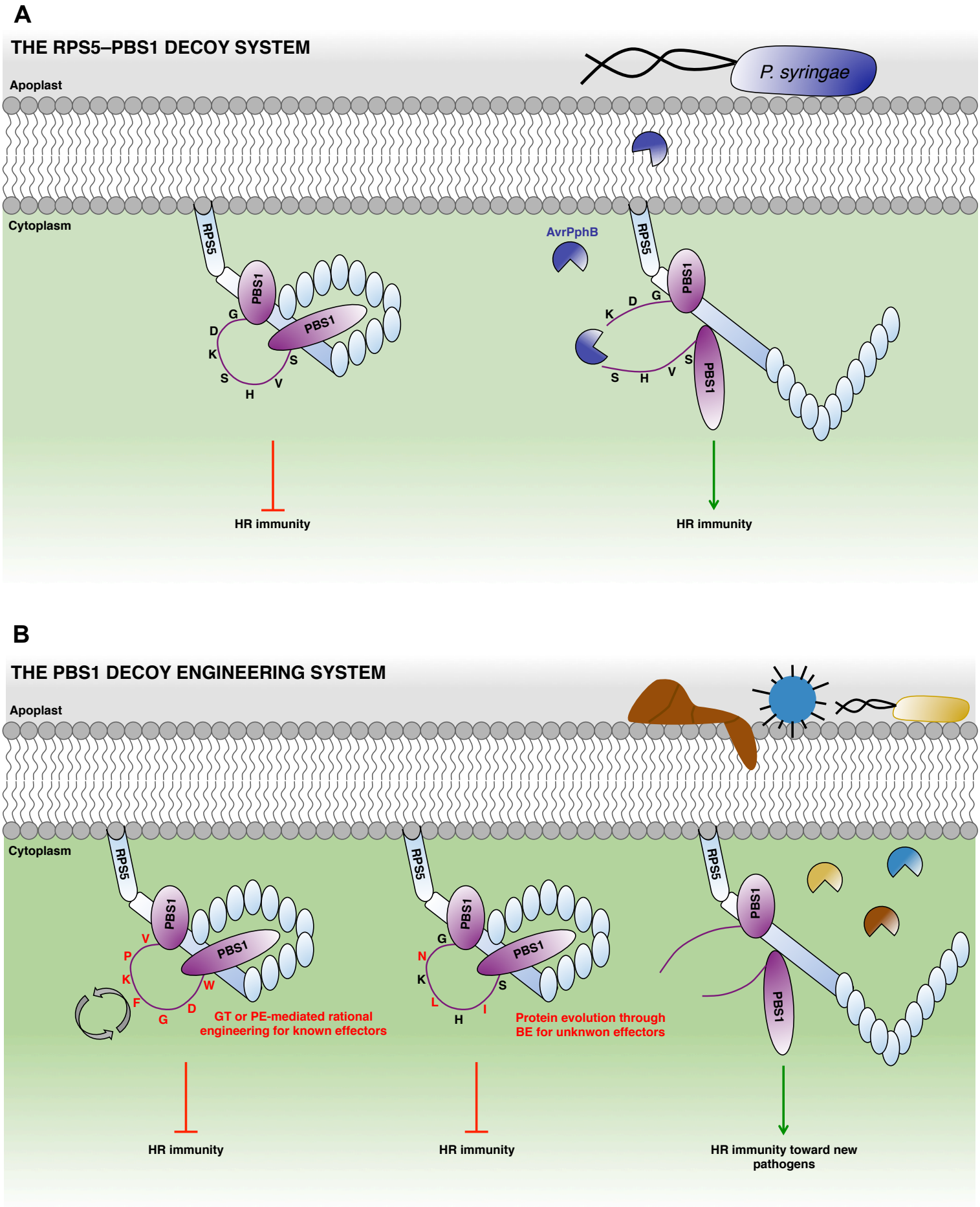
pegRNA
PBS
RT template
3'
RuvC
PAM
NGG
5'
3'
SpnCas9
(H840A)
B
3' flap
5' flap
Flap equilibration
3' flap ligation
5' flap cleavage
DNA repair
Insertion, deletion and any
kind of substitution(s)
C
PPE2
PPE3
PPE3b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THE RPS5–PBS1 DECOY SYSTEM
Apoplast
P. syringae
Cytoplasm
RPS5
PBS1
PBS1
G
D
K
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H
V
S
AvrPphB
RPS5
PBS1
D
G
K
S
S
H
V
PBS1
HR immunity
HR immunity
B
THE PBS1 DECOY ENGINEERING SYSTEM
Apoplast
Cytoplasm
RPS5
PBS1
PBS1
V
P
K
F
G
D
W
GT or PE-mediated rational engineering for known effectors
RPS5
PBS1
PBS1
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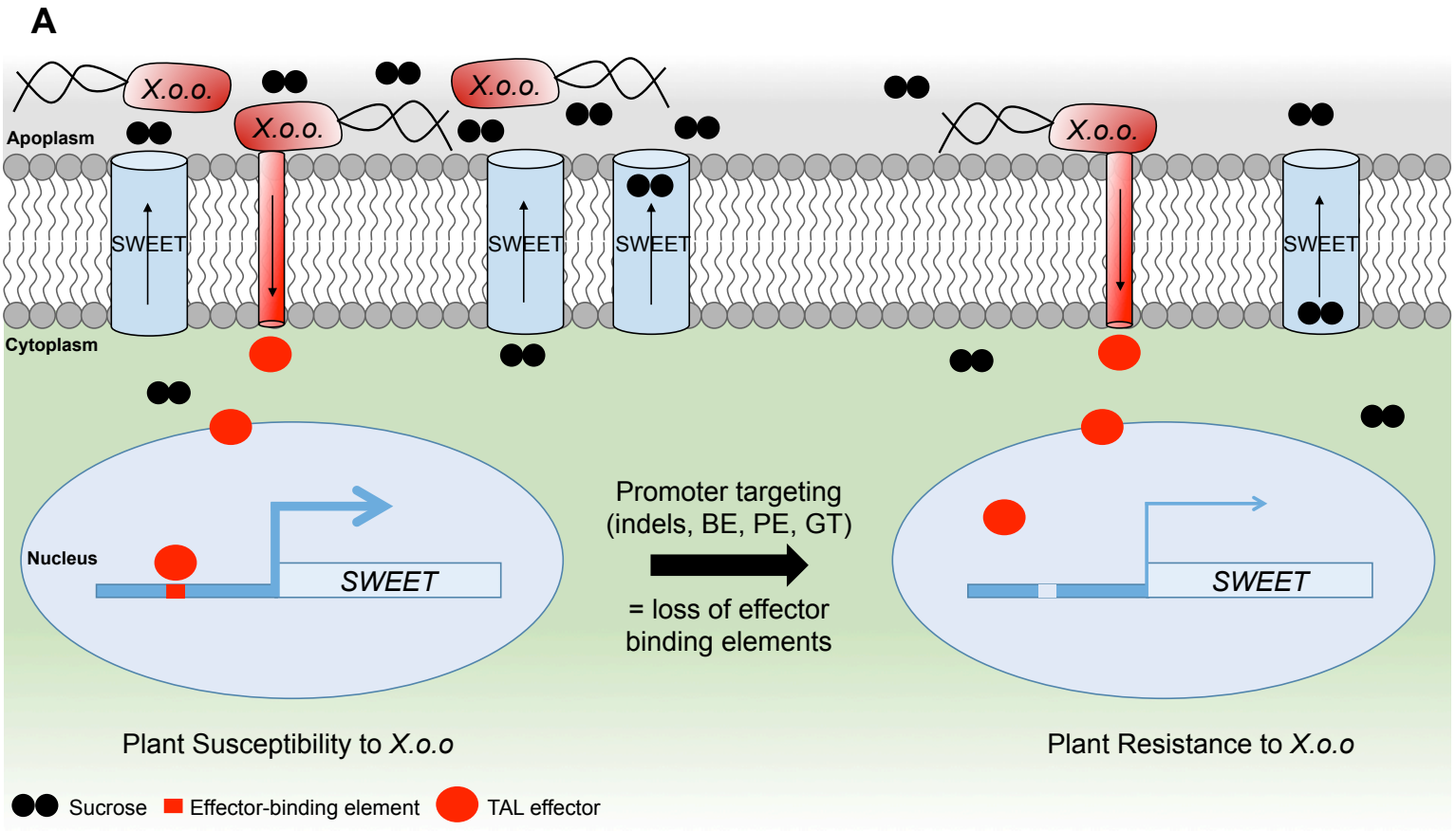
Protein evolution through BE for unknwon effectors
RPS5
PBS1
PBS1
HR immunity
HR immunity
HR immunity toward new pathogens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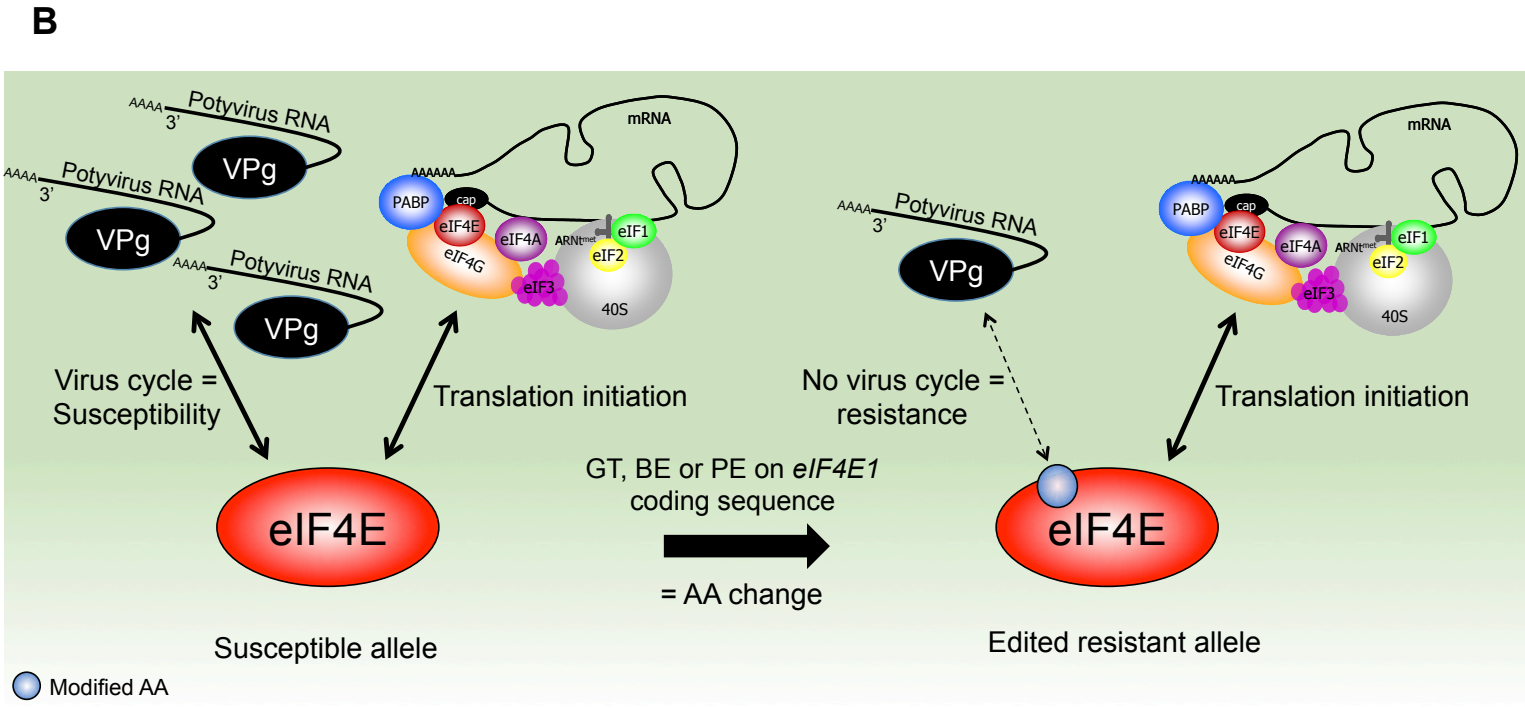
X.o.o.
X.o.o.
X.o.o.
X.o.o.
Apoplasm
Cytoplasm
SWEET
SWEET
SWEET
SWEET
Nucleus
SWEET
SWEET
Promoter targeting
(indels, BE, PE, GT)
= loss of effector
binding elements
Plant Susceptibility to X.o.o
Plant Resistance to X.o.o
Sucrose
Effector-binding element
TAL effector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Potyvirus RNA
VPg
Potyvirus RNA
VPg
mRNA
PABP
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eIF4G
eIF3
eIF1
eIF2
40S
Virus cycle =
Susceptibility
Translation initiation
eIF4E
Susceptible allele
GT, BE or PE on eIF4E1
coding sequence
= AA change
Potyvirus RNA
VPg
mRNA
No virus cycle =
resistance
Translation initiation
eIF4E
Edited resistant allele
Modified AA