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Precision breeding made real with CRISPR: illustration through genetic resistance to pathogens

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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.

31 **Key Words**

32 CRISPR-Cas9, gene targeting, base editing, prime editing, plant/pathogen interactions,

33 precision crop breeding

34

35 **Short Summary**

36 The CRISPR-mediated precision breeding toolbox allows researchers and molecular breeders

37 to fine-tune plant genomes with a high versatility. Application of these genome editing tools

38 to genes involved in plant/pathogen interactions can foster the development of a sustainable

39 agriculture through the production of genetically pathogen-resistant crops.

40

41 **INTRODUCTION**

42 Primary food production across the globe faces the challenge of sustainably feeding a

43 growing population in an accelerating climate change context, while more than 800 million

44 people suffered from undernourishment worldwide in 2017, particularly in Africa and Asia

45 (FAO, 2017). Current agriculture mostly relies on the cultivation of a narrow range of plant

46 species, sometimes in poorly suited locations, far away from their area of domestication

47 (Fernie and Yan, 2019). Labour-intensive and time-consuming conventional crop breeding

48 relying on natural or induced genetic polymorphism has substantially contributed to plant

49 adaptation to new environments and food availability. Recently, the development of genome

50 engineering in plants opened new avenues for precision crop breeding, including the

51 improvement of elite germplasm as well as the molecular domestication of wild species

52 (Zhang et al., 2019).

53 Plant pathogens, including bacteria, fungi and viruses, cause substantial economic losses and

54 threaten food security (Savary et al., 2019). Pathogens rely on diverse strategies to bypass

55 plant immunity. For instance, they produce molecular weapons called effectors that act inside

56 or outside of the plant cell to target diverse host proteins involved in different cellular

57 processes to promote infection through successful colonization of the host.

58 Plants rely on a sophisticated immune system to ward off potential pathogens. Key elements

59 are an arsenal of receptors termed invasion pattern receptors that recognize either microbe- or

60 host-derived signals termed invasion patterns (IPs) that betray the presence of microbial

61 invaders (Cook et al., 2015). IP receptors belong to two main classes: cell surface receptors

62 that are either receptor-like proteins (RLPs) or receptor-like kinases (RLKs) and intracellular

63 receptors that belong to the class of nucleotide-binding leucine-rich repeat domain proteins

64 (NLRs). While NLRs specifically recognize intracellular effectors (Cesari, 2018; Kourelis and

65 van der Hoorn, 2018), RLPs and RLKs perceive microbe-associated molecular patterns

66 (MAMPs) and extracellular effectors, originating from the pathogen, and damage-associated

67 molecular patterns (DAMPs) released by host cells damaged upon pathogen attack (Boutrot

68 and Zipfel, 2017; Kanyuka and Rudd, 2019).

69 The vast majority of disease resistance (*R*) genes cloned from plants code for immune

70 receptors (Kourelis and van der Hoorn, 2018) with NLRs being the dominating class. Another

71 successful strategy to confer plant disease resistance relies on a loss of compatibility through

72 mutations of plant susceptibility (*S*) genes required for pathogen infection and plant

73 susceptibility. As a result, the pathogen will not be able to perform its infectious cycle,

74 resulting in plant disease resistance (van Schie and Takken, 2014).

75 While conventional resistance breeding can be very successful, it may be associated with

76 linkage drag and the resistance conferred by single *R* genes may be rapidly bypassed by fast

77 evolving pathogens. Therefore, the precise engineering of *R* and *S* genes constitutes an

78 exciting track for the development of genetically resistant crops (Langner et al., 2018;

79 Tamborski and Krasileva, 2020; van Wersch et al., 2020), thereby limiting the environmental

80 impact of chemical control. Copying mutations across accessions can also circumvent linkage

81 drags associated with classical breeding, as shown for other characters (Li et al., 2017a).

82 In the last few years, genome editing tools have evolved very quickly with the development of

83 RNA-guided endonuclease systems (Zhang et al., 2019). Until now, genome editing was

84 mostly used to generate loss-of-function alleles through DNA error-prone repair of the target

85 site after double strand cleavage by the classical CRISPR-Cas9 system. For example, this

86 strategy resulted in a powdery mildew resistant tomato by knocking-out the *mildew resistant*

87 *locus O* (*Mlo*) *S*-gene (Nekrasov et al., 2017), while the rice blast resistance was increased

88 due to the loss-of-function of the transcription factor OsERF922 (Wang et al., 2016).

89 However, many traits can be conferred by single or multiple nucleotide substitutions,

90 especially for genes involved in plant/pathogen interactions, where coevolution exerts a dual

91 selective pressure that favours mutations of pathogen effectors to evade recognition, but also

92 mutations of immune receptors to restore perception (Jones et al., 2016). Therefore, genome-

93   editing tools mediating precise and predictable mutations are highly valuable for the
94   production of gain-of-function mutants, which could lead to broader perception of the
95   pathogen and/or host factor evasion from effectors. Of particular interest is the CRISPR-
96   mediated mimicking of natural alleles conferring pathogen resistance (Bastet et al., 2017), as
97   well as directed *in planta* evolution to generate new gene variants that are not present in the
98   natural genetic diversity. In the course of this review, we will refer to the targeted genome
99   alterations, such as nucleotide changes and small deletion, as precision breeding. This process
100 can involve GM techniques but the resulting plant is devoid of transgene (Andersen et al.,
101 2015).

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

110

THE BASIC MACHINERY FOR PLANT GENOME EDITING

112 In the frame of this review, we will focus on genome editing strategies, *i.e.* approaches that
113 will lead to stable modifications in the plant genomic DNA, and result in transgene-free plants
114 through different delivery strategies that are extensively described in recent reviews (Chen et
115 al., 2019; El-Mounadi et al., 2020; Kuluev et al., 2019). We will therefore not cover another
116 important aspect of CRISPR that consists in using nuclease that targets RNA for modification,
117 such as Cas13. More details on this strategy can be found in recent reviews (Burmistrz et al.,
118 2020; Wolter and Puchta, 2018).

119

The CRISPR-Cas9 system

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

124 RNA (sgRNA) that results from the artificial fusion of a crRNA and a trans-activating crRNA

125 (tracrRNA) (Jinek et al., 2012). The sgRNA is composed of a ≈ 80-bp scaffold that mediates

126 binding to the Cas9, and a customizable 20-bp sequence at its 5' end, called the spacer

127 sequence, conferring DNA targeting specificity to the complex (Figure 1A). Binding of the

128 sgRNA to the SpCas9 triggers the transition of the nuclease from an inactive into a DNA-

129 probing state in search for a canonical 5'-NGG-3' protospacer adjacent motif (PAM). Natural

130 and engineered Cas9 variants recognizing alternative PAMs have also been extensively used

131 (Zhang et al., 2019). Recognition of a suitable PAM motif leads to quick interrogation of

132 adjacent DNA, followed by local DNA melting and RNA strand invasion (formation of a R-

133 loop structure) for interrogation of the full spacer sequence (Figure 1A). Perfect base pairing

134 between the so-called seed region (10-12 nucleotides from the PAM) of the spacer sequence

135 and target DNA is required for SpCas9-mediated DNA cleavage, while mismatches in the

136 nonseed region can be tolerated, potentially leading to unwanted off-target activity. While a

137 careful design of spacer sequences is generally considered to be sufficient to avoid off-target

138 activity, some Cas9 variants displaying higher specificity have been developed through

139 protein engineering (Zhang et al., 2019). The gradual base pairing triggers SpCas9

140 conformational changes to an active site, eventually resulting in DNA cleavage by the

141 concerted activity of its HNH and RuvC nuclease domains (Figure 1A). Although SpCas9

142 was thought to only create blunt-ended double strand DNA break (DSB) about 3-bp upstream

143 the PAM (Jiang and Doudna, 2017), recent findings demonstrated that SpCas9 nuclease

144 activity results in both blunt and staggered ends, likely because of the RuvC cutting flexibility

145 (Molla and Yang, 2020). The CRISPR-SpCas9 system is now routinely used in numerous

146 species and can be considered as the golden tool for genome editing in plants (Manghwar et

147 al., 2019).

148

149 **The CRISPR-Cas12 systems**

150 The second leading genome editing tool, the class 2 type V-A CRISPR-Cas12a system also

151 known as CRISPR-Cpf1, displays unique features and constitutes a relevant alternative to the

152 CRISPR-Cas9 system (Zetsche et al., 2015). Cas12a enzymes (1200-1500 amino acids)

153 mostly recognize T-rich 5'-TTTN-3' PAM located upstream of the target sequence. They

154 associate with a short ≈ 43-bp crRNA and only rely on the RuvC-like domain to cleave both

155 DNA strands in a sequential manner, beginning with the non-target strand and resulting in a

156 staggered DNA cleavage with 4-5bp overhangs distal to the PAM (Figure 1B) (Alok et al.,

157 2020; Zaidi et al., 2017; Zetsche et al., 2015). Cas12a orthologs from *Lachnospiraceae*
158 *bacterium* (LbCas12a), *Acidaminococcus* sp. (AsCas12a) and *Francisella novicida*
159 (FnCas12a) have been the most commonly used enzymes in several plant species. They
160 generally display higher specificity and less or no off-targets as compared to Cas9 (Begemann
161 et al., 2017; Endo et al., 2016; Herbert et al., 2020; Kim et al., 2017; Lee et al., 2019; Li et al.,
162 2019a; Tang et al., 2018; Tang et al., 2017; Tang et al., 2019; Xu et al., 2017; Xu et al.,
163 2019a; Yin et al., 2017).

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

177

178 **Evolving CRISPR-Cas systems: going beyond gene knockout**

179 Together, the three CRISPR-Cas systems above-mentioned constitute the base for diversified
180 genome editing tools. So far, most genome editing applications in plants have been focused
181 on the production of knockout mutants for single or multiple genes (Manghwar et al., 2019).
182 This is due to the predominance of error-prone non-homologous end-joining (NHEJ)
183 mechanisms to repair CRISPR-Cas-mediated DSBs in somatic cells of higher plants (Puchta,
184 2005). Contrary to homologous recombination (HR), an endogenous DNA repair mechanism
185 that is responsible for crossovers between homologous chromosomes during meiosis, NHEJ
186 mechanisms mediate DSB repair without the need for a homologous template. While the
187 classical NHEJ (C-NHEJ) pathway appears to be mainly error-free, the alternative NHEJ (Alt-
188 NHEJ) seems to have a key role in error-prone CRISPR-induced DSB repair (Atkins and

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

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

211

212 **PRECISION EDITING: REFINING THE TOOLS?**

213 As many agronomic traits are controlled by single base polymorphisms (Henikoff and Comai,
214 2003), introduction of precise base substitutions and/or predictable insertions or deletions
215 could generate plants with new agronomic properties. For example, the targeted substitution
216 of nucleotide(s) could introduce non-synonymous mutations causing amino acid changes in
217 the encoded protein. Besides, nucleotide substitution can broadly affect the gene by creating
218 or correcting early stop codons or regulating splicing. In the next subsections, we summarize
219 current CRISPR tools for precision editing.

220

221 **CRISPR-mediated gene correction through NHEJ**

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

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

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

251

252 **CRISPR-mediated gene targeting**

253   CRISPR-mediated gene targeting (GT) is a technology relying on HR (Figure 2D) that has

254   been applied for precise nucleotide conversion or precise insertions or deletions in many

255   eukaryotic genomes, including plants. Although it is very promising for genome engineering,

256   HR suffers from low efficiency in plant somatic cells (Puchta, 2005) and the delivery of a

257   sufficient amount of donor template in the vicinity of the target site is still challenging,

258   thereby strongly limiting the use of GT in most higher plant species. An illustration of this

259   challenging task is the high number of CRISPR-mediated GT studies that used phenotypic

260   markers such as herbicide tolerance to facilitate the identification of successful events (Atkins

261   and Voytas, 2020). Nevertheless, a variety of recent improvements allowed to substantially

262   enhance GT in plants (Huang and Puchta, 2019). Of particular interest is the use of

263   engineered geminiviral replicon systems, which use rolling-circle replication to deliver large

264   amount of DNA repair template into the plant cell nucleus. The CRISPR-Cas9 GT-

265   geminiviral replicons strategy was successfully applied for large insertions and/or point

266   mutations in tomato, potato, cassava, wheat and rice (Butler et al., 2016; Cermak et al., 2015;

267   Dahan-Meir et al., 2018; Gil-Humanes et al., 2017; Hummel et al., 2018; Wang et al., 2017).

268   Another interesting approach is the use of Cas12a instead of Cas9 for inducing DSBs.

269   Because Cas12a cuts DNA in the non-seed region distal from the PAM (Figure 1B), allowing

270   multiple rounds of DNA cleavage even after introduction of NHEJ-mediated indel mutations,

271   and produces sticky ends, HR may be favoured (Huang and Puchta, 2019). Consistent with

272   this hypothesis, the CRISPR-Cas12a GT system was successfully applied for targeted

273   insertion or point mutations in rice (Begemann et al., 2017; Li et al., 2019c; Li et al., 2018b).

274   This system was further improved in tomato using a CRISPR/Cas12a GT-geminiviral multi-

275   replicon strategy, allowing the production of transgene-free salt-tolerant plants due to a single

276   amino acid change (N217D) in the *SlHKT1;2* gene (Van Vu et al., 2020). While the

277   geminiviral replicon system allows the delivery of higher amount of donor template in plants,

278   some improvements for GT are still needed in order to spatially and temporally bring the

279   CRISPR components and the repair template at the breaking site, as observed in animals

280   (Aird et al., 2018; Savic et al., 2018). Such a strategy has recently been applied in rice using a

281   fusion between the Cas9 and the *Agrobacterium* VirD2 relaxase (Ali et al., 2020), known to

282   be a key player for ssT-DNA translocation and integration into the plant genome (Gelvin,

283   2017). The CRISPR-Cas9-VirD2 system facilitated GT likely through the delivery of ssDNA

284   repair-template in close vicinity to the Cas9-induced DSB (Figure 2E). This enabled

285   introduction of point mutations in the *OsALS* and *OsCCD7* genes to confer herbicide

286   resistance and to engineer plant architecture respectively, and in-frame insertion of the HA

287 epitope at the C-term of OsHDT (Ali et al., 2020). Together, these recent advances offer new

288 possibilities for precise genome editing, although future progress to increase the efficiency of

289 CRISPR-mediated GT are still needed for a broad and fast adoption in many plant species.

290

291 **CRISPR-mediated base editing**

292 In contrast to GT-mediated gene correction, CRISPR-mediated base editing is a donor

293 template and DSB free approach that induces precise base conversion. Cytosine base editors

294 (CBEs) and adenine base editors (ABEs) are fusion proteins made of a catalytically impaired

295 Cas9 and an enzymatic domain mediating cytosine or adenine deamination, respectively.

296 During the formation of the CRISPR-mediated "R-loop" structure, a small window of the

297 non-targeted ssDNA is exposed and can serve as a substrate for deamination (Figure 3A).

298 CBEs catalyze the deamination of cytosine(s) into uracile(s) in the target region. This triggers

299 the base excision repair (BER) pathway that can result in either an error-free or an error-prone

300 repair leading to a diversification of the edits (C-to-T, C-to-G and C-to-A), albeit at the cost

301 of indels production at a substantial rate (Figure 3B) (Hess et al., 2017). Although varying the

302 edits is interesting for local sequence diversification, predictable targeted base conversions are

303 desirable for precise amino acid changes. Adding an uracil DNA glycosylase inhibitor (UGI)

304 to the CBE architecture that blocks the BER pathway has been developed as a solution to

305 specifically obtain C-to-T conversion with generally low level of by-products (Figure 3C-D)

306 (Komor et al., 2017). Deamination of adenine through ABEs (Figure 4A) does not necessitate

307 the use of alkyl adenine DNA glycosylases inhibitors, because BER of inosine intermediates

308 is inefficient in DNA. ABEs therefore create efficient A-to-G conversion with a very low

309 level of by-products (Figure 4B) (Gaudelli et al., 2017). While first BEs harboured a

310 deadCas9 (dCas9), the incorporation of the edit(s) to the non-deaminated strand was strongly

311 improved by the use of nCas9 with impaired RuvC domain (D10A), that promotes long-patch

312 BER using the edited strand as a model (Komor et al., 2016). Due to the lack of nCas12, the

313 use of Cas12 enzymes for base editing applications remains limited, for the moment.

314 Soon after their development in animals, CBEs and ABEs have been rapidly used in several

315 plant species. The two mostly used cytosine deaminases, PmCDA1 from *Petromyzon marinus*

316 and rAPOBEC1 from rat (both devoid of UGI), have been reported to produce C-to-T

317 transitions, but also C-to-G and C-to-A transversions in *Arabidopsis*, tomato and potato, albeit

318 with a substantial rate of indels, as discussed above (Bastet et al., 2019; Li et al., 2017b; Lu

319 and Zhu, 2017; Shimatani et al., 2017; Veillet et al., 2019a; Veillet et al., 2019b). For an

320 approach requiring a high level of outcome predictability, UGI domain(s) can be added to the

321 CBE architecture, resulting in a higher rate of C-to-T substitutions with lower unwanted

322 mutations (Qin et al., 2019b; Zong et al., 2017). As observed in animals, ABEs produce A-to-

323 G transitions in plants, with a very low rate of indels (Hao et al., 2019; Hua et al., 2020b;

324 Kang et al., 2018; Li et al., 2018a; Li et al., 2019b; Negishi et al., 2019; Yan et al., 2018).

325 These BEs allowed to produce plants with new agronomic traits, including pathogen

326 resistance (Bharat et al., 2020; Mishra et al., 2020). Recently, dual cytosine and adenine BEs

327 were generated to simultaneously mediate C-to-T and A-to-G transitions in the same editing

328 window, increasing the potential outputs for targeted gene modification (Grünewald et al.,

329 2020; Li et al., 2020b). Several different deaminases can also be recruited in the target site

330 through sgRNA-protein interactions, thereby increasing the local amount of catalytic domains

331 for the production of diversified outcomes (Mishra et al., 2020; Zhang et al., 2019).

332 Although base editing constitutes a promising technology, early CBEs and ABEs suffered

333 from some drawbacks. First, their targeting scope is restricted to sequence harbouring a

334 suitable PAM downstream of the targeted sequence, placing the target base in a generally

335 short editing window at the 5'end of the spacer sequence. Much work has been done to use

336 natural Cas9 orthologs with different PAM requirements, such as *Staphylococcus aureus* and

337 *Streptococcus canis* Cas9 (Hua et al., 2018; Qin et al., 2019a; Wang et al., 2020a), or to

338 engineer SpCas9 variants with relaxed PAM recognition, expanding the targeting scope of

339 BEs (Ge et al., 2019; Hua et al., 2019; Niu et al., 2019; Qin et al., 2020; Ren et al., 2019;

340 Veillet et al., 2020; Wang et al., 2018; Zhong et al., 2019). Of particular interest is the recent

341 development in animal cells of new SpCas9 variants that recognize non-G PAMs (Miller et

342 al., 2020) or almost any PAM sequence, as illustrated with BEs harbouring the SpRY variant

343 that are able to target almost any locus, albeit with a preference for sequences upstream of

344 NRN PAMs (R=A or G) (Walton et al., 2020). Due to almost unrestricted PAM recognition, a

345 special attention should be put on limiting sgRNA self-targeting activity when using DNA

346 delivery methods, potentially increasing the off-target risk by introducing mutations into

347 spacer sequences (Qin et al., 2020). Second, the size of the editing window of BEs would

348 benefit from being modular according to the desired editing outcome. The human

349 APOBEC3A cytosine deaminase mediates base conversion inside an extended 17-bp editing

350 window in rice, wheat and potato, thereby increasing the saturated mutagenesis potential of a

351 targeted locus (Zong et al., 2018). In order to increase the affinity of CBEs with their ssDNA

352 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

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

401 While the successful development of highly versatile and precise PEs in mammalian cells
402 holds great hopes, implementation of plant prime editors (PPEs) could also contribute to the
403 improvement of food crops (Zhang et al., 2020c). A few months after its application in
404 animals, prime editing has been adopted by several groups working on cereal crops (Butt et
405 al., 2020; Hua et al., 2020a; Li et al., 2020c; Lin et al., 2020; Tang et al., 2020; Xu et al.,
406 2020a; Xu et al., 2020b). Three different PPEs were assayed for their editing efficiency:
407 PPE2, PPE3 and PPE3b (Figure 5C). While PPE2 only consists on the expression of the
408 nCas9-RT fusion and the pegRNA, PPE3 aims to promote favourable repair by nicking the
409 non-edited strand using an additional sgRNA targeting the edited strand upstream or
410 downstream the editing site. PPE3b also consists of nicking the non-edited strand, but the
411 additional sgRNA targets the new edited sequence so that nicking is restricted only after 3'
412 flap resolution, thereby preventing the formation of DSBs that would lead to higher indels
413 rate (Figure 5C). PPE2, PPE3 and PPE3b systems harbouring an engineered version of
414 Moloney murine leukaemia virus (M-MLV) RT resulted in similar editing efficiencies in rice
415 and wheat protoplasts, as well as in *Agrobacterium*-mediated transformed rice plants. This
416 indicates that nicking the non-edited strand does not necessarily increase prime editing
417 efficiency in plants (Butt et al., 2020; Hua et al., 2020a; Lin et al., 2020; Xu et al., 2020a).

418 PPEs were shown to specifically allow the introduction of all types of single or multiple base

419 substitutions, as well as deletions (up to 40-bp) and insertions (up to 15-bp) (Li et al., 2020c;

420 Lin et al., 2020; Tang et al., 2020; Xu et al., 2020b). As observed in mammalian cells, by-

421 products were mainly pegRNA scaffolds insertions, which likely originate from extensive

422 activity of the RT, and large deletions due to paired nicking of both strands (Lin et al., 2020;

423 Tang et al., 2020). Overall, editing efficiencies in rice and wheat were in the low percentage

424 range, although precise 6-bp deletion and single A-to-T transversion were detected in 21.8%

425 and 31.3% of rice plants regenerated from *Agrobacterium*-mediated transformation

426 respectively (Lin et al., 2020; Xu et al., 2020a).

427 The successful proof-of-concept of CRISPR-mediated prime editing in plants opens up

428 exciting perspectives, although some challenges need to be overcome for a broad use of this

429 new tool. Enhancing prime editing efficiency constitutes an essential track, especially for

430 polyploids and/or vegetatively propagated species. Because a high variability of prime editing

431 activity was observed among targeted sites, the 'copy and replace' mechanisms may be

432 enhanced to promote reliable outcome rates. PPE architecture should be optimized to

433 maximize CRISPR components expression levels (Tang et al., 2020; Xu et al., 2020a; Xu et

434 al., 2020b), and using different RTs that may be more efficient in plant cells is of particular

435 interest, as well as optimizing temperature conditions for reverse transcriptase activity (Lin et

436 al., 2020). The systematic testing of some pegRNA (PBS and RT lengths, esgRNA scaffold)

437 and sgRNA (position of the nicking) designs for new targets is also highly recommended (Li

438 et al., 2020d). While PPEs can accommodate long RT templates and are much less

439 constrained than BEs for PAM availability, the use of Cas9 variants with relaxed PAM

440 recognition may be relevant to localize the edit at putatively favourable position from the

441 ssDNA cutting site. Finally, although prime editing seems to induce lower off-target editing

442 than Cas9 at putative off-target sites in animals (Anzalone et al., 2019), genome wide off-

443 target activity of PPEs needs to be carefully evaluated to assess the capacity of RT to cause

444 Cas9-independant unwanted edits.

445

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

448 Collectively, CRISPR-mediated GT, base editing and prime editing constitute an extended

449 toolbox for precision editing, offering complementary strengths and drawbacks to edit almost

450 any target site. When large DNA sequences need to be precisely inserted or deleted, the

451 classical GT approach is the most suitable tool, as prime editing efficiency decreases with

452 increasing length of the desired insertion or deletion (Lin et al., 2020). However, targeted

453 small insertions and deletions can be efficiently mediated by both the prime editing system

454 and the GT strategy. Besides the utility of such modifications for crop improvement, the

455 possibility to label endogenous proteins with specific tags is of particular interest (e.g. cellular

456 localization, purification, immunoprecipitation). Ali et al. (2020) recently managed to insert

457 the HA-epitope into the C terminus of OsHDT using the CRISPR-Cas9-VirD2 system. It may

458 also be possible to generate such insertion using the prime editing system, provided that the

459 flag length is within the range of possible insertions by PEs.

460 Base editing appears to be generally more efficient than current PPEs for base substitution(s)

461 (Anzalone et al., 2019; Lin et al., 2020). Therefore, early BEs should be used when bystander

462 mutations are acceptable, whereas new BEs harbouring narrowed editing windows should be

463 favoured when bystander edits are not desirable. However, when the desired outcome cannot

464 be generated by BEs (e.g. most transversions or multiple base substitutions), PPEs offer much

465 more versatility. For applications requiring targeted local random mutagenesis, such as

466 directed evolution of proteins, BEs still constitute the most suitable tool. However, PPEs

467 might be modified to randomly insert polymorphism in the target site through low-fidelity

468 reverse transcriptases, thereby providing another source of genetic variability. Because prime

469 editing is only at an early stage of development, we hope that future improvements will

470 considerably enhance the efficiency and widen the targeting scope of PPEs.

471

472 **A CRISPR WAY FOR PATHOGEN RESISTANCE ENGINEERING**

473 Interestingly, CRISPR-Cas can be directly used to target the pathogens'genome, mainly

474 viruses. This could be achieved by either targeting DNA viruses or RNA viruses, but requires

475 the transgenic expression of the CRISPR-Cas machinery and specific gRNA, an approach

476 reminiscent of RNAi strategies. This therefore falls beyond the scope of precision breeding,

477 but the reader can find details on these strategies, as well as their possible caveats, in recent

478 reviews (Pyott et al., 2020; Zhao et al., 2020). Now, it becomes possible to apply precision

479 breeding through CRISPR technology to improve traits conferred by precise and/or punctual

480 sequence variation, with an extraordinary opportunity to develop genetically resistant crops

481 for a sustainable agriculture. CRISPR applications have been predominantly focused on

482   generating loss-of-function alleles, with some successes for the production of pathogen

483   resistant plants (Langner et al., 2018). However, plant-microorganism interactions result from

484   a long coevolution involving a complex molecular dialogue with several key players. As a

485   result, CRISPR-mediated gain-of-function mutations appear to be highly relevant for

486   developing crops with improved resistance to pathogens. In the following, we review current

487   knowledge of CRISPR-mediated precision editing for pathogen resistance and provide

488   interesting tracks that are now within CRISPR reach.

489

490   **Immune receptor engineering**

491   Considerable progress has been made in recent years regarding the molecular mechanisms of

492   action, structural properties and evolution of NLR receptors (Burdett et al., 2019; Kourelis

493   and van der Hoorn, 2018; Tamborski and Krasileva, 2020). This enables novel strategies to

494   improve the capacity of NLRs to induce immune responses, broaden their pathogen

495   recognition spectrum or even create new recognition specificities. However, there are

496   currently very few examples of immune receptors having been improved in this way (Cesari,

497   2018; Grund et al., 2019; Tamborski and Krasileva, 2020). Besides, current NLR engineering

498   strategies essentially rely on either testing modified *NLR* genes in transient expression

499   systems (e.g. by agroinfiltration in *Nicotiana tabacum* or *benthamiana*) or complementing

500   susceptible varieties by stable transformation. Use of a CRISPR-based system for engineering

501   *NLR* genes has not been reported. However, this represents a promising strategy to create new

502   disease resistances directly in elite varieties. The development and quick improvement of a

503   wide range of CRISPR tools pave the way toward these new strategies.

504   One approach for NLR engineering relies on editing of residues required for regulation of

505   these receptors in order to enhance their activation potential and, by this, enlarge their

506   pathogen recognition spectrum. This strategy has been used for the wheat powdery mildew

507   resistance gene *Pm3*, which forms an allelic series mediating the specific recognition of

508   *Blumeria graminis* f. sp. *tritici* (*Bgt*) isolates. By comparing several alleles of *Pm3* that

509   exhibit a broad (*a* and *b* alleles) or narrow (*f* allele) resistance spectrum, Stirnweis et al.

510   identified two polymorphisms in the NB domain that are responsible for enhanced signaling

511   activity and extended resistance spectrum (Stirnweis et al., 2014). CRISPR-mediated prime

512   editing of such regulatory residues in NLRs could create artificial 'trigger happy' variants

513   with broadened resistance spectrum directly in elite cultivars. However, misregulation of

514   NLRs carries the risk of pleiotropic phenotypes and such potential trade-off phenomenon

515   must be taken into consideration in this type of approaches.

516   Alternatively, the recognition spectrum of NLRs can be broadened or modified by changing

517   residues responsible for effector recognition specificity. In allelic NLRs series where distinct

518   alleles exhibit different pathogen recognition specificities (e.g. barley *MLA*, wheat *Pm3*, flax

519   *L* or rice *Pi-2/Piz-t/Pi50*), the LRR domain plays a crucial role in effector recognition

520   specificities (Dodds et al., 2006; Saur et al., 2019). In these cases, an attractive application of

521   CRISPR technology is to provide an elite cultivar with a recognition specificity already

522   existing in other varieties by mutating the specific residues or sequences in LRR domain that

523   determine specificity. This would enable to adapt the pathogen recognition specificities of

524   elite cultivars according to pathogen populations without going through tedious crossing and

525   selection steps. The potential for this type of approach is illustrated by the historical example

526   of the flax NLRs L2, L6 and L10 for which swaps of LRR domains have enabled changes in

527   flax-rust recognition specificities (Ellis et al., 1999).

528   Knowledge-guided engineering of completely new recognition specificities by targeted

529   mutagenesis of specific residues in the LRR domain is for the moment not yet possible. For

530   this, one would require much better insight into the molecular mechanism of NLR activation

531   and specific and precise knowledge on the LRR residues mediating effector recognition and

532   specificity. Investigation of the allelic diversity coupled with structural modelling of LRR

533   domains may help in the identification of polymorphic surface residues that are likely

534   involved in effector binding. Filling this knowledge gap is therefore a priority. Indeed, for the

535   moment, novel recognition specificities by mutations in the LRR domain were only generated

536   by random mutagenesis approaches. For example, in the potato NLR Rx, which confers

537   resistance to potato virus X (PVX), point mutations in the LRR domain were identified that

538   extended the recognition spectrum (Farnham and Baulcombe, 2006). CRISPR-mediated

539   introduction of such mutations identified by random mutagenesis approaches in high

540   throughput screening systems promise to create novel or broadened resistances.

541   Another strategy based on genome editing techniques consists in reactivating pseudogenized

542   *NLR* genes in elite varieties of agronomic interest. This would allow "resuscitation" of

543   resistance without the laborious steps of cloning and complementation and, in many countries,

544   issues related to GMO regulation . This strategy is relevant for *NLRs* where loss of function is

545   due to a limited number of polymorphisms, which can be "repaired" through base editing.

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

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

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

18

579   attention to preserve agronomic traits by avoiding improper regulation of NLRs that can result

580   in autoimmunity, highlighting the need to find a balance between pathogen detection and

581   fitness (van Wersch et al., 2020).

582   In many cases, recognition of effectors by NLRs is indirect and occurs through the detection

583   of effector-mediated modifications of plant proteins, called guardees or decoys (Dangl and

584   Jones, 2001; van der Hoorn and Kamoun, 2008). A promising strategy for resistance

585   engineering consists in modifying such decoys or guardees to trap novel pathogen effectors. A

586   proof for this concept was provided in *Arabidopsis thaliana* using the serine-threonine kinase

587   PBS1, whose cleavage by the bacterial effector AvrPphB is monitored by the NLR RPS5.

588   Transforming *RPS5* plants with a *PBS1* mutant carrying the cleavage sites of other bacterial

589   or viral proteases resulted in recognition of these proteases and novel bacterial or virus

590   resistances (Kim et al., 2016) (Figure 6A). Using genome editing tools such as CRISPR-

591   mediated GT or prime editing, the endogenous locus encoding the 7 residue cleavage site of

592   PBS1 could be readily modified into cleavage sites of other pathogen proteases (Figure 6B),

593   resulting in RPS5-mediated surveillance of these novel effectors (Pottinger and Innes, 2020).

594   PBS1 is highly conserved among flowering plants and NLR-mediated surveillance of its

595   cleavage emerged repeatedly in evolution making it a versatile decoy system in corresponding

596   crops (Carter et al., 2019; Pottinger and Innes, 2020). More generally, similar trap systems for

597   proteases or other effector can probably be engineered with other decoys or guardess in a

598   large spectrum of crops even if they do not possess a PBS1 surveillance system

599   (Giannakopoulou et al., 2016; Kim et al., 2016; Pottinger et al., 2020).

600

601   **Host factor engineering**

602   Because NLR-mediated resistance is often quickly bypassed by pathogens, S-gene

603   engineering constitutes an exciting alternative for diversifying the sources of resistance. S-

604   genes, that can be targeted by pathogen effectors or act independently, facilitate pathogen

605   infection and can either encode proteins involved in host recognition, penetration or

606   metabolism, or act as regulator of plant immunity (Langner et al., 2018). Contrary to R-genes

607   that are generally dominant, loss of susceptibility conferred by engineering S-factors is

608   mainly recessive, meaning that all alleles should be altered to achieve resistance. This is of

609   course a substantial challenge for polyploid plants. To date, most genome editing applications

610   aiming at conferring pathogen resistance consisted in knocking out S-genes (Langner et al.,

611   2018; Zaidi et al., 2018). However, such strategy may be associated with deleterious side-

612   effects as S-genes may encode essential proteins for the host (see below).

613   For example, bacterial and fungal infections lead to a competition for carbon resources at the

614   plant/pathogen interface, in which host sugar transporters play a key role for the outcome of

615   the interaction (Lemoine et al., 2013). In order to increase the sugar supply in the apoplasm,

616   the bacteria *Xanthomonas oryzae* pv. *oryzae* activates the transcription of members of the rice

617   *SWEET* gene family, encoding proteins that mediate passive diffusion of sucrose across the

618   plasma membrane (Figure 7A). This is achieved through the expression of the so-called

619   transcription-activator-like effectors (TALEs) that bind specific regions of the SWEET

620   promoters to activate the transcription, resulting in an enhanced export of sucrose to the

621   apoplast that sustains bacterial growth. Because SWEET proteins are key components of

622   phloem loading for long-distance transport of sucrose (Lemoine et al., 2013), CRISPR-

623   mediated loss-of-function approaches may result in unwanted developmental effects (Chen et

624   al., 2012). In this regard, promoter targeting is an attractive alternative consisting in the

625   introduction of random indel mutations into TALE binding elements. Such a strategy was

626   performed by targeting some *OsSWEET* genes, thereby preventing *OsSWEET* induction by

627   bacterial effectors and conferring bacterial blight broad-spectrum resistance (Li et al., 2020a;

628   Oliva et al., 2019; Xu et al., 2019b). Similarly, CRISPR-Cas9/Cas12a-mediated promoter

629   editing of the *CsLOB1* gene, specifically targeted by bacterial effectors for transcription

630   activation, resulted in the generation of canker-resistant citrus cultivars (Jia et al., 2019; Peng

631   et al., 2017). Because Cas9 nuclease mostly induce small deletions, we postulate that such

632   strategy could be improved using Cas12a and Cas12b nucleases, resulting in a higher rate of

633   larger deletions, as previously discussed. The use of Cas variants with relaxed PAM

634   recognition may also be valuable to precisely target cis-regulatory elements.

635   With the recent expansion of the CRISPR toolbox, it is now possible to edit specific bases

636   leading to predetermined punctual amino-acid change, aiming at developing new or

637   mimicking natural alleles conferring resistance. The *eukaryotic Initiation Factor 4E* (*eIF4E*)

638   genes are key elements of eukaryotic protein synthesis. At the same time, they are also very

639   important susceptibility factors to members of the large *Potyviridae* family, which rely on

640   those factors to perform their infectious cycle in the plant (Bastet et al., 2017) (Figure 7B).

641   Natural resistances found in various plant species often rely on functional resistance *eIF4E*

642   alleles that contain non-synonymous mutations in the coding sequence. Those alleles are

643   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

675 still stand in the way for broad use of CRISPR in crop precision breeding. First, classical

676 delivery methods such as *Agrobacterium*-mediated transformation, protoplast transfection and

677 biolistic mostly target somatic cells and therefore involve subsequent regenerative steps that

678 are time-consuming and highly genotype-dependant (Atkins and Voytas, 2020). Furthermore,

679 delivery and tissue culture methods can cause unwanted changes to the genome, as recently

680 evidenced after protoplast transfection and *Agrobacterium*-mediated transformation in the

681 tetraploid potato (Fossi et al., 2019), and after biolistic transformation in rice and maize (Liu

682 et al., 2019). Secondly, most current delivery methods involve the stable integration of

683 foreign DNA into plant genomes. While these sequences can be segregated out following

684 mendelian inheritance, it would be advantageous to minimize their expression window to

685 avoid off-target effects, especially for base editors. Furthermore, the introduction of DNA

686 intermediates into the plant nucleus may result in genome-wide random insertions, pointing

687 out the necessity to use DNA-free delivery methods. As a result, while we are now able to

688 precisely edit target sites through highly specific CRISPR tools, a special focus should be put

689 on minimizing CRISPR-independent side effects, highlighting the need to develop alternative

690 delivery methods into plant cells to avoid or limit such undesirable effects (Demirer et al.,

691 2019; Maher et al., 2020; Toda et al., 2019), thereby unlocking the full potential of the

692 CRISPR technology.

693 Finally, it is evident that CRISPR technology has great potential for both plant biology

694 research and precision crop breeding. The CRISPR precision toolbox, that is expanding and

695 disseminating at an extraordinary speed, will definitely help us to decipher plant immune

696 responses upon pathogen infection. However, while we are now also able to mimick or evolve

697 immune molecular mechanisms that confer genetic resistance to a broad range of pathogens,

698 with the potential to support food security and safety in a sustainable way through the

699 reduction of chemical use, regulatory frameworks constitute the main obstacle to CRISPR

700 application in food crops, especially in Europe (Zhang et al., 2020c). We expect that a

701 product-based regulatory framework could provide a rational balance between

702 human/environment safety concerns and plant breeding innovation.

703

704 **AUTHOR CONTRIBUTIONS**

705 F.V., T.K., S.C. and J.-L.G. jointly wrote the original manuscript draft. F.V. and M.D.

706 prepared the figures. F.V. and J.-L.G. planned the review outline. All authors contributed to

707 the reviewing and editing of the manuscript.

708

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1171

1172

1173   **FIGURE CAPTION**

1174   **Figure 1**

1175   **CRISPR-Cas systems used for genome editing in plants.**

1176   **(A)** The CRISPR-SpCas9 system made of the endonuclease SpCas9, harbouring RuvC and

1177   HNH catalytic domains, and the sgRNA that guides the complex to an endogenous target

1178   sequence upstream of a G-rich PAM (5'-NGG-3'), leading to blunt and/or staggered DNA

1179   breaks.

1180   **(B)** The CRISPR-Cas12a system involves the endonuclease Cas12a that is guided to the target

1181   locus, downstream of a T-rich PAM (5'-TTTN-3'), by a short crRNA, leading to a staggered

1182   DNA cleavage by a single RuvC domain after conformational changes [(1) and (2)].

1183   **(C)** The CRISPR-Cas12b system relies on a Cas12b endonuclease, harbouring a single RuvC

1184   catalytic domain that mediate staggered DNA cleavage [(1) and (2)], and a sgRNA that target

1185   the complex to a specific site downstream of a T-rich PAM (5'-VTTV-3').

1186   The schemes are not at scale and are for illustrative purposes only.

1187

1188   **Figure 2**

1189   **NHEJ- and HR-mediated DNA mutations after CRISPR cleavage**

1190   **(A)** CRISPR-mediated gene knockout through introduction of indels mutations at the cutting

1191   site after reparation by the error-prone NHEJ repair mechanism.

1192 **(B)** CRISPR-mediated fragment deletion after dual sgRNA-induced DSBs, resulting in

1193 fragment deletion with associated indels after error-prone NHEJ repair.

1194 **(C)** CRISPR-mediated fragment replacement after dual sgRNA-induced DSBs, resulting in

1195 the replacement of a specific locus by a donor DNA sequence, with associated indels due to

1196 error-prone NHEJ repair.

1197 **(D)** CRISPR-mediated gene targeting (GT) for precise and predictable deletions, insertions

1198 and/or DNA substitutions. Homologous recombination (HR) repair pathway occurs through

1199 introduction of available donor templates (mainly dsDNA and ssDNA) harbouring

1200 homologous sequences with both sides of the CRISPR-induced DSB.

1201 **(E)** CRISPR-Cas9-VirD2-mediated GT, allowing to provide the repair ssDNA template in the

1202 vicinity of the cutting site through interaction between the 5' specific sequence (purple) of the

1203 ssDNA donor template and the VirD2 domain. This spatiotemporal delivery of repair

1204 template may increase the rate of precise repair through HR pathway.

1205 The schemes are not at scale and are for illustrative purposes only.

1206

1207 **Figure 3**

1208 **CRISPR-mediated base editing using cytosine base editors (CBEs)**

1209 **(A)** CBEs are composed of a nCas9 (D10A) fused to a cytosine deaminase catalytic domain

1210 (rAPOBEC1, PmCDA1, hAID or hA3A) that mediates cytosine deamination in the so-called

1211 editing window at the 5' end of the non-targeted sequence.

1212 **(B)** After C deamination into U, endogenous uracil DNA glycosylase (eUNG) detect and

1213 remove the U, leading to an abasic site, which is further processed through error-free (U-to-C)

1214 or error-prone repair, producing different base substitutions, albeit at the cost of indels

1215 mutations due to the generation of DSBs through concomitant ssDNA breaks by the nCas9

1216 and endogenous AP lyases (eAP lyase). This system allows the production of C-to-T, C-to-G

1217 and C-to-A conversions.

1218 **(C)** CBE architecture can be upgraded through the fusion of one to several uracil glycosylase

1219 inhibitors (UGIs) to the base editor, with the aim of increasing the rate of C-to-T conversion

1220 while limiting the formation of by-products.

1221 **(D)** After C deamination, UGIs protect the U edits from eUNG, thereby preventing the

1222 formation of abasic sites and mostly producing C-to-T conversion through the nicking of the

1223 non-edited strand and the intervention DNA repair/replication mechanisms, with low level of

1224 by-products such as indels mutations.

1225 The schemes are not at scale and are for illustrative purposes only.

1226

1227 **Figure 4**

1228 **CRISPR-mediated base editing using adenine base editors (ABEs)**

1229 **(A)** ABEs are composed of a nCas9 (D10A) fused to an adenine deaminase catalytic domain

1230 (ecTadA-ecTadA*) that mediates adenine deamination in the so-called editing window at the

1231 5' end of the non-targeted sequence.

1232 **(B)** After A deamination into I (inosine), nicking of the non-edited strand and intervention of

1233 DNA repair/replication mechanisms produce A-to-G conversion, with very low rates of by-

1234 products.

1235 The schemes are not at scale and are for illustrative purposes only.

1236

1237 **Figure 5**

1238 **CRISPR-mediated prime editing**

1239 **(A)** Plant prime editors (PPEs) are composed of a nCas9 (H840A) fused to a reverse

1240 transcriptase (RT), allowing insertions, deletions and all kinds of base substitutions. The

1241 polymorphism of interest is brought through a pegRNA, containing both a sgRNA for target

1242 specificity and a 3' extension that harbours a RNA template bearing the polymorphism,

1243 leading to the targeted writing of new DNA sequences through reverse transcription.

1244 **(B)** Upon cleavage of the non-targeted strand by the HNH domain of the nCas9, the primer

1245 binding site (PBS) sequence of the pegRNA hybridizes with the broken ssDNA upstream of

1246 the cleavage site. This RNA/DNA structure initiates reverse transcriptase activity, copying the

1247 genetic information from the RT template. After resolution of 3' flap ligation, DNA repair

1248 mechanisms permanently install the mutation.

1249 **(C)** Different prime editing strategies can be used to increase the rate of desired outcomes.

1250 The PPE2 strategy only implies the use of the pegRNA, while the PPE3 strategy require the

1251 use of an additional sgRNA to cut the non-edited strand upstream or downstream of the

1252 modified sequence. For the PPE3b strategy the second sgRNA targets the edited sequence, in

1253 order to cut the non-edited strand only after 3' flap resolution, thereby limiting the risk of

1254 indels mutations through the occurrence of DSBs.

1255 The schemes are not at scale and are for illustrative purposes only.

1256

1257 **Figure 6**

1258 **Representative model of the natural and engineered RPS5-PBS1 decoy systems.**

1259 **(A)** RPS5 and PBS1 form an inactive preactivation complex at the plasma membrane. Upon

1260 cleavage of the GDKSHVS motif in the activation loop of PBS1 by the *Pseudomonas*

1261 *syringae* AvrPphB type III protease, RPS5 sense the PBS1 conformational change, leading to

1262 activation of the RPS5-mediated hypersensitive response (HR).

1263 **(B)** Using CRISPR precision editing tools, it is possible to replace the AvrPphB target

1264 cleavage sequence of PBS1 by a motif recognized by another secreted protease, such as the

1265 AvrRpt2 effector that cleaves the VPKFGDW sequence. Gene targeting (GT) or prime editing

1266 (PE) tools can be used to replace the initial target cleavage sequence to confer immunity

1267 toward pathogens (fungi, bacteria and viruses) that secrete proteases with known cleavage

1268 recognition motifs. Alternatively, protein evolution using base editing (BE) can generate

1269 punctual amino acid shifts to generate potential new cleavage sequences. The functionality of

1270 these PBS1 variants can be screened towards pathogens that secrete proteases with unknown

1271 molecular characteristics, potentially conferring new sources of crop resistance.

1272 The schemes are not at scale and are for illustrative purposes only.
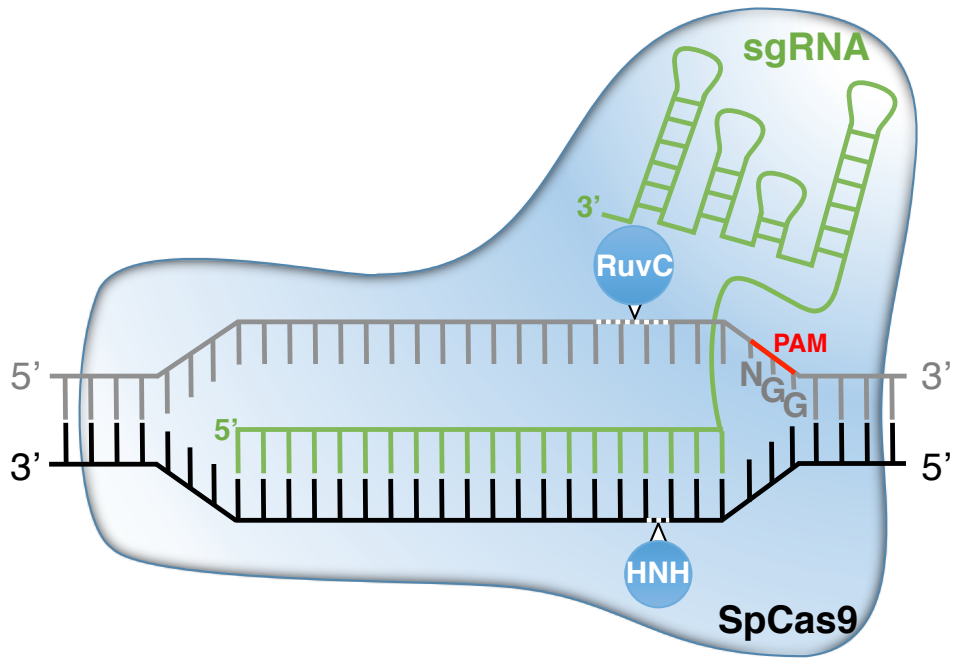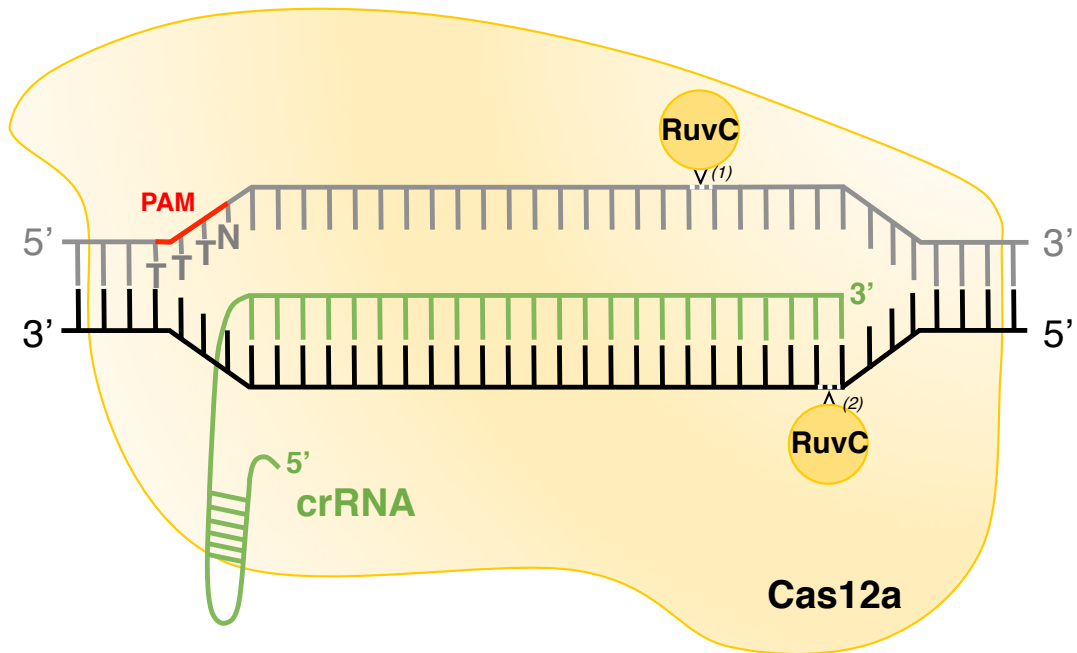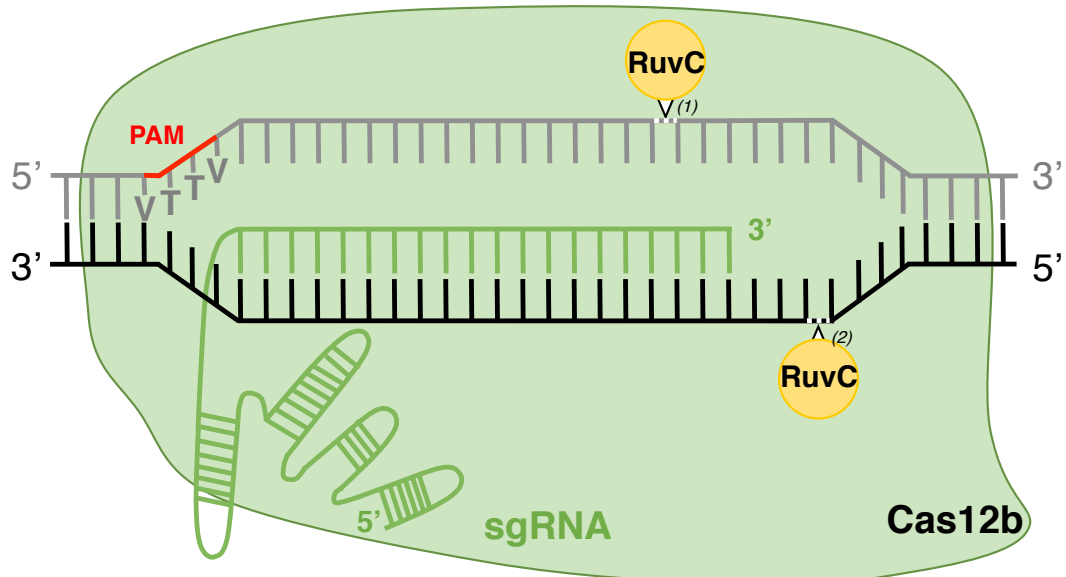
1273

1274 **Figure 7**

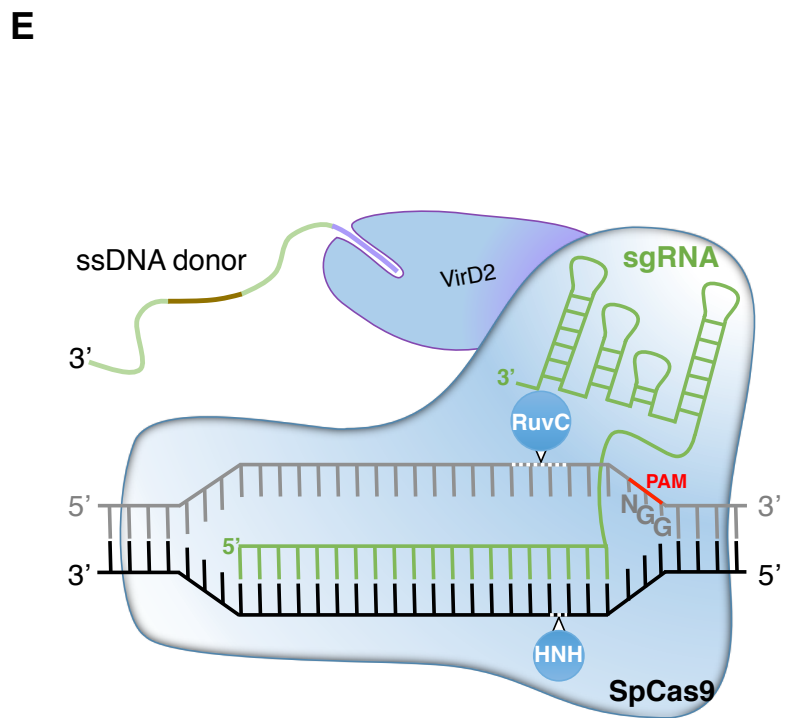1275 **Representative model of editing resistance by loss-of-susceptibility.**

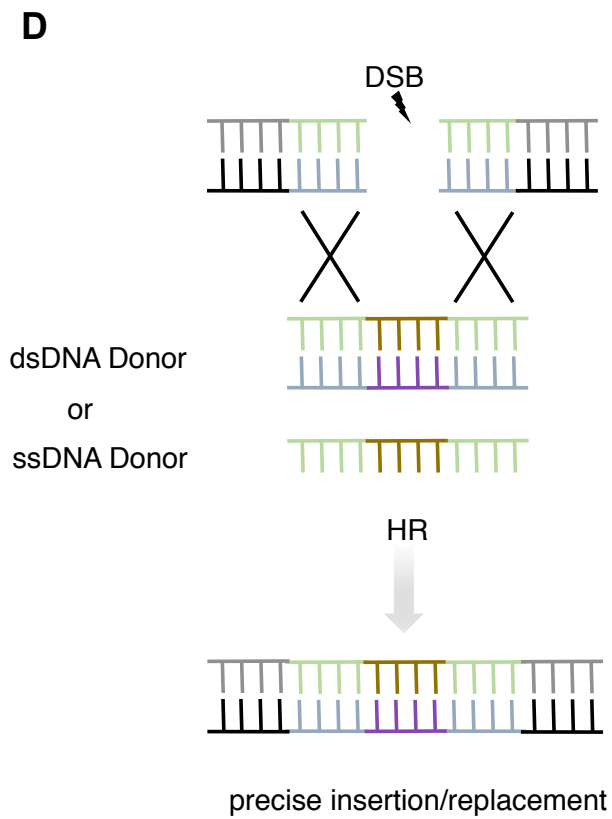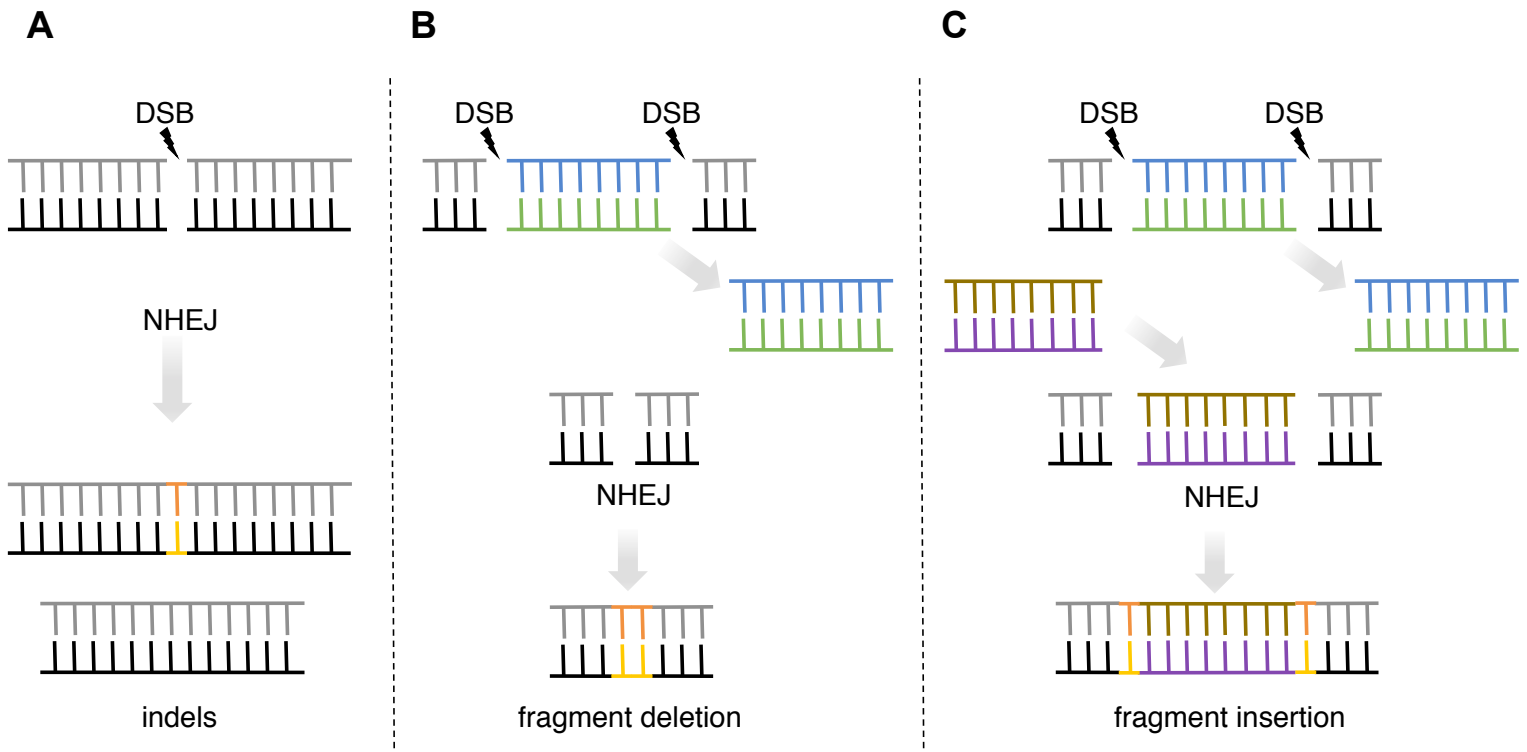1276 **(A)** Resistance to bacteria through the edition of SWEET promoter. During infection leading

1277 to susceptibility (left side), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) bacteria express

1278 Transcription Activator Like effectors (TAL effector) in the plant cell. Those effectors bind

1279 Effector-binding elements (EBE) located in the promoters of the *SWEET* genes that encode
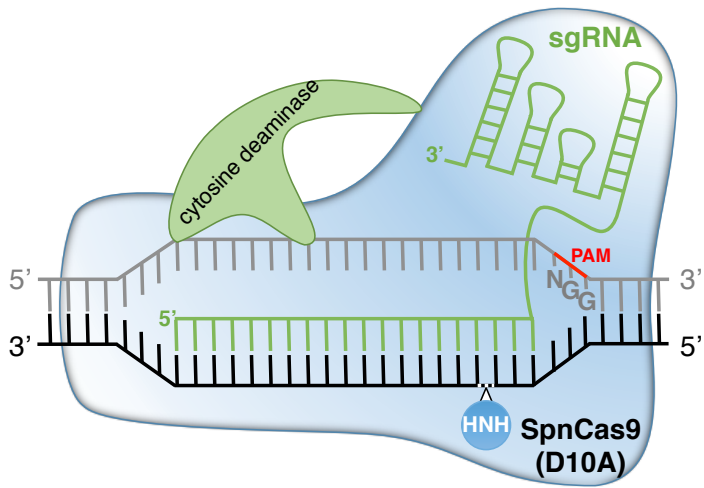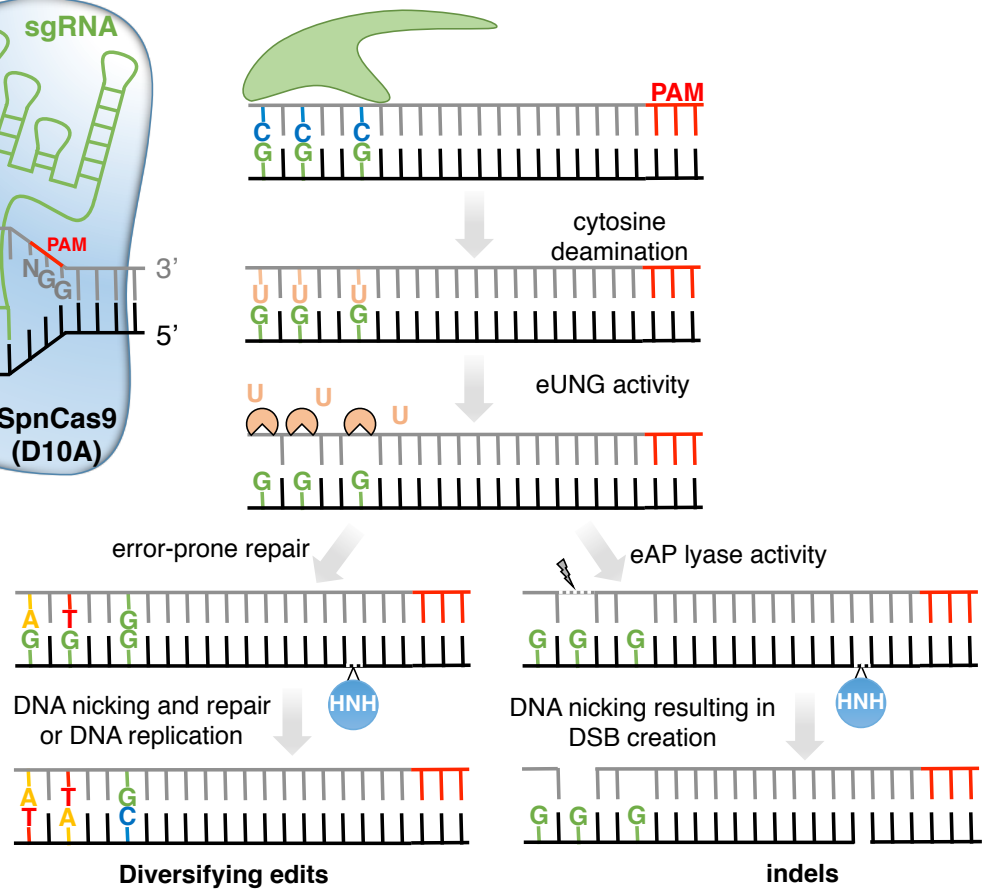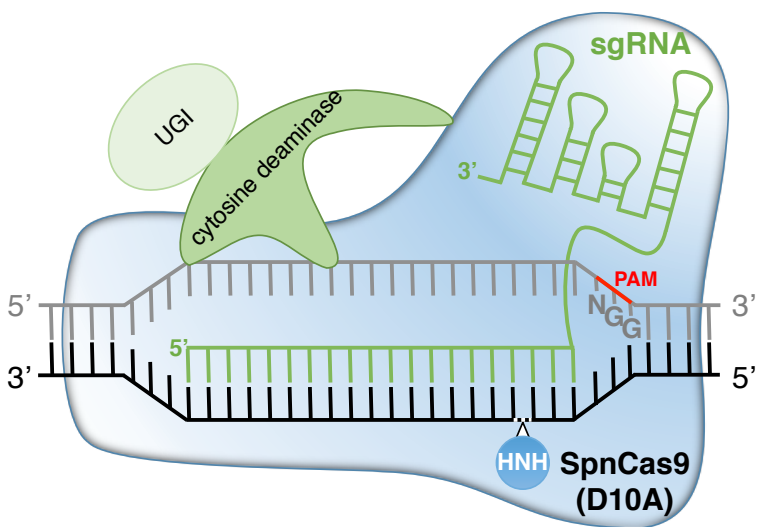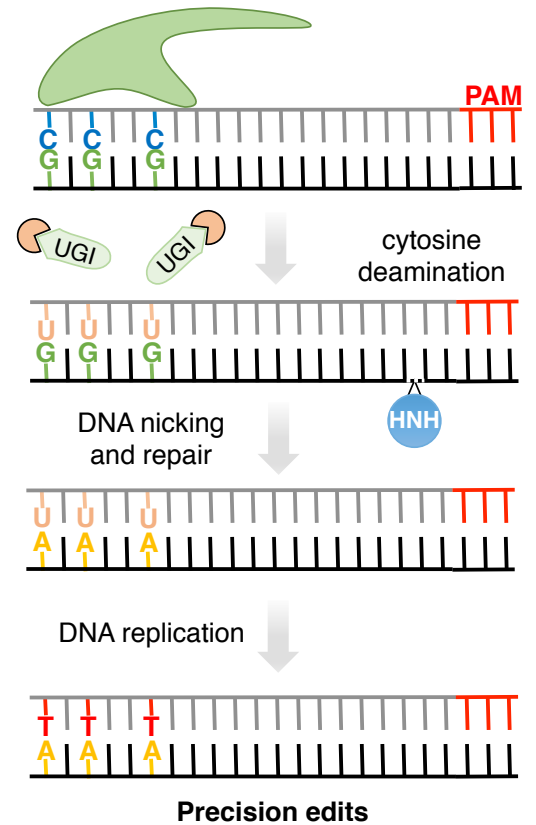
1280   sucrose transporters. The binding triggers the activation of *SWEET* genes, and of the encoded

1281   sucrose transporter, and results in an increase in sucrose content in the apoplasm. The excess

1282   of sucrose benefits to the bacteria and contribute to its multiplication. Genetic resistance can

1283   be engineered (right side) by removing the EBE region (s) from the SWEET promoter region:

1284   the SWEET gene is no longer activated by the TAL effector, sucrose content stay low in the

1285   apoplasm, resulting in resistance.

1286   (B) Resistance to Potyvirus through base editing of the translation initiation factors eIF4E. In

1287   susceptible plants (left side), the translation initiation eIF4E are necessary for the potyviruses,

1288   represented by their ssRNA$^+$ genome linked in 5' to the Viral Protein genome linked or VPg,

1289   to perform their infection cycle. At the same time they are involved in translation initiation of

1290   the host mRNA for protein synthesis. Base editing of the *eIF4E* coding sequence (right side)

1291   can be used to introduce non-synonymous mutations associated with Amino Acid changes

1292   usually found in resistance alleles from the natural diversity of plants. This mutation does not

1293   affect the eIF4E function in translation initiation while suppressing its interaction with

1294   potyvirus, leading to resistance. This allows to develop resistance at no developmental cost.

1295   The translation initiation complex depiction is adapted from Robaglia et Caranta, 2006.

1296   The schemes are not at scale and are for illustrative purposes only.

A

sgRNA

3'

RuvC

PAM

N
G
G

5'   3'

5'

HNH

3'   5'

SpCas9

B

RuvC

(1)

PAM

N

5'   3'

T
T

3'   5'

RuvC

(2)

crRNA

5'

3'

Cas12a

C

RuvC

(1)

PAM

V

5'   3'

V
T
T

3'   5'

RuvC

(2)

3'

sgRNA

5'

Cas12b

A

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

sgRNA

3'

RuvC

PAM

N
G
G

5'   3'

3'   5'

5'

HNH

SpCas9

A

cytosine deaminase

sgRNA

3'

5'

5'

3'

5'

PAM

N G G

HNH

SpnCas9 (D10A)

B

PAM

C C C
G G G

cytosine deamination

U U U
G G G

eUNG activity

U U U U
G G G

error-prone repair

A T G
G G G

eAP lyase activity

G G G

HNH

DNA nicking and repair or DNA replication

A T G
T A C

DNA nicking resulting in DSB creation

G G G

HNH

Diversifying edits

indels

C

UGI

cytosine deaminase

sgRNA

3'

5'

5'

3'

5'

PAM

N G G

HNH

SpnCas9 (D10A)

D

PAM

C C C
G G G

UGI   UGI

cytosine deamination

U U U
G G G

HNH

DNA nicking and repair

U U U
A A A

DNA replication

T T T
A A A

Precision edits

A

sgRNA

3'

adenine deaminase

PAM

5'

N
G
G

3'

5'

3'

5'

HNH

SpnCas9
(D10A)

B

PAM

adenine deamination

HNH

DNA nicking
and repair

DNA replication

Precision edits

A

PBS   RT template

PBS   RT template

PBS   RT template

RT

pegRNA

RuvC

PAM

SpnCas9
(H840A)

B

3' flap   3' flap

5' flap

Flap equilibration

3' flap ligation   5' flap cleavage

DNA repair

Insertion, deletion and any
kind of substitution(s)

C

5' flap   PPE2

5' flap   PPE3

RuvC

5' flap   PPE3b

RuvC

A

THE RPS5–PBS1 DECOY SYSTEM

Apoplast

P. syringae

Cytoplasm

RPS5

PBS1

PBS1

G
D
K
S   V
H
S

AvrPphB

RPS5

PBS1

D   G
K
S   V
S   H
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

G
N
K
L   S
H   I

Protein evolution through
BE for unknwon effectors

RPS5

PBS1

PBS1

HR immunity

HR immunity

HR immunity toward new
pathogens

A

Apoplasm

Cytoplasm

SWEET

Nucleus

SWEET

Plant Susceptibility to *X.o.o*

Promoter targeting
(indels, BE, PE, GT)

= loss of effector
binding elements

SWEET

Plant Resistance to *X.o.o*

●● Sucrose ▪ Effector-binding element ● TAL effector

B

Potyvirus RNA

VPg

Virus cycle =
Susceptibility

Translation initiation

eIF4E

Susceptible allele

GT, BE or PE on *eIF4E1*
coding sequence

= AA change

No virus cycle =
resistance

Translation initiation

eIF4E

Edited resistant allele

● Modified AA