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► **To cite this version:**

Gaël Le Trionnaire, Sylvie Tanguy, Sylvie Hudaverdian, Florence Gléonnec, Gautier Richard, et al.. An integrated protocol for targeted mutagenesis with CRISPR-Cas9 system in the pea aphid. *Insect Biochemistry and Molecular Biology*, 2019, 110, pp.34-44. <10.1016/j.ibmb.2019.04.016>. <hal-02154486>

HAL Id: hal-02154486

<https://hal.science/hal-02154486v1>

Submitted on 26 May 2020

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An integrated protocol for targeted mutagenesis with CRISPR-Cas9 system in the pea aphid

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Highlights

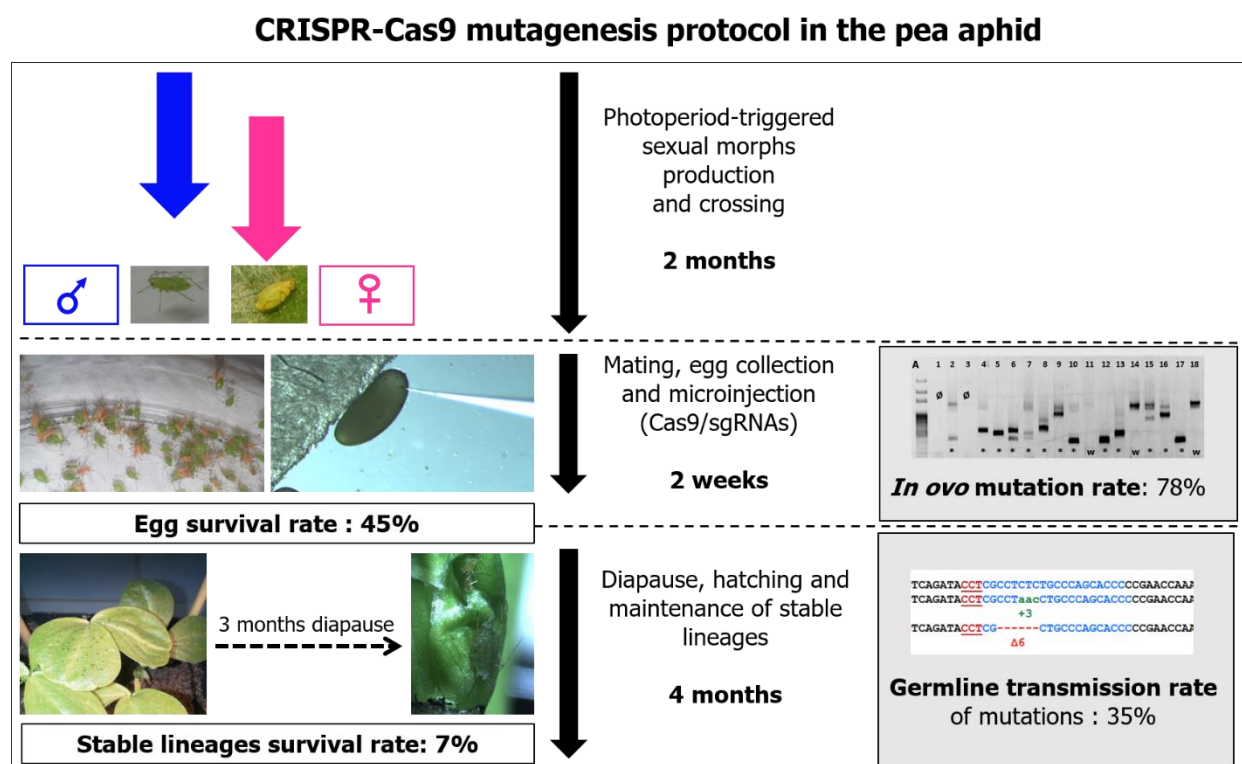
. Aphids are major crop pests that alternate between clonality and sexual reproduction.

. Targeted mutagenesis was set up in the pea aphid to edit *stylin-01* gene by injecting eggs with CRISPR-Cas9 components.

. Key steps include sexual morphs induction and mating, egg injection, diapause, hatching and edited lineages maintenance.

. Germline transmission rates could be estimated at 35% with the generation of six stable lineages edited for *stylin-01* gene.

Graphical abstract



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41 Abstract

42
43 CRISPR-Cas9 technology is a very efficient functional analysis tool and has been developed in several
44 insects to edit their genome through injection of eggs with guide RNAs targeting coding sequences of
45 genes of interest. However, its implementation in aphids is more challenging. Aphids are major pests of
46 crops worldwide that alternate during their life cycle between clonality and sexual reproduction. The
47 production of eggs after mating of sexual individuals is a single yearly event and is necessarily triggered
48 by a photoperiod decrease. Fertilized eggs then experience an obligate 3-month diapause period before
49 hatching as new clonal colonies. Taking into consideration these particularities, we developed in the pea
50 aphid *Acyrtosiphon pisum* a step-by-step protocol of targeted mutagenesis based on the microinjection
51 within fertilized eggs of CRISPR-Cas9 components designed for the editing of a cuticular protein gene
52 (*stylin-01*). This protocol includes the following steps: **i**) the photoperiod-triggered induction of sexual
53 morphs (2 months), **ii**) the mating and egg collection step (2 weeks), **iii**) egg microinjection and
54 melanization, **iv**) the 3-months obligate diapause, **v**) the hatching of new lineages from injected eggs (2
55 weeks) and **vi**) the maintenance of stable lineages (2 weeks). Overall, this 7-month long procedure was
56 applied to three different crosses in order to estimate the impact of the choice of the genetic combination
57 on egg production dynamics by females as well as hatching rates after diapause. Mutation rates within
58 eggs before diapause were estimated at 70 to 80%. The hatching rate of injected eggs following diapause
59 ranged from 1 to 11% depending on the cross and finally a total of 17 stable lineages were obtained and
60 maintained clonally. Out of these, 6 lineages were mutated at the defined sgRNAs target sites within
61 *stylin-01* coding sequence, either at the two alleles (2 lineages) or at one allele (4 lineages). The final
62 germline transmission rate of the mutations was thus around 35%. Our protocol of an efficient targeted
63 mutagenesis opens the avenue for functional studies through genome editing in aphids.

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84 1. Introduction

85

86 The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system has emerged in
87 the last few years as a very powerful genome editing tool allowing the generation of precise and targeted
88 mutational events within the DNA sequence of various metazoans. This technology is derived from a
89 bacterial “adaptive” immune system dedicated to the recognition and the cleavage of invasive viral or
90 plasmid DNA. The unique properties of this mechanism have been used to engineer the “CRISPR-Cas9”
91 system for mediating targeted mutagenesis. This system relies on the use of a single-guide RNA
92 (sgRNA) and a Cas9 nuclease (Doudna and Charpentier, 2014). In short, the sgRNA contains a sequence
93 of 20 nucleotides (nt) corresponding to the genomic region to be targeted, and a universal sequence of
94 120 nucleotides. The folding capacity of the latter allows the formation of the sgRNA-Cas9
95 ribonucleoprotein complex. Once delivered to the cells of the organism of interest, the Cas9-sgRNA
96 complex first scans the genome until the targeted sequence is recognized by the sgRNA specific 20-nt
97 motif through RNA-DNA complementarity. This then promotes the nuclease activity of the Cas9 protein
98 at that site, which also depends on the presence on the genomic DNA of a 3-nt Protospacer Adjacent
99 Motif (PAM) at the end of the 20-nt recognition site. After this recognition event, the endonucleolytic
100 activity of the Cas9 generates a double-strand break (DSB) of the DNA that is then repaired by
101 endogenous cellular systems such as the Non Homologous End Joining (NHEJ) or the Homology-
102 Directed Repair (HDR) system. NHEJ is considered to be error-prone since the reparation sometimes
103 generates mistakes such as insertions or deletions (InDels) of one to several nucleotides, thus causing
104 genomic DNA mutations. These InDels can potentially generate frame shift mutations resulting in
105 premature stop codons or altered proteins. CRISPR-Cas9 technology is commonly used in a wide range
106 of organisms including animal and plant models. In insects, such a system has proved to be efficient in
107 models such as *Drosophila melanogaster* (Basset et al., 2013), *Aedes aegypti* (Kistler et al., 2015),
108 *Bombyx mori* (Wang et al., 2013) or *Tribolium castaneum* (Gilles et al., 2015). Recently, it was also
109 successfully implemented in a wide range of non-model insects ranging from Diptera, Lepidoptera,
110 Orthoptera, Coleoptera, Hymenoptera and other arthropods (for detailed reviews, see Sun et al., 2017,
111 Cui et al., 2017 and Taning et al., 2017). The main delivery method for this technology in insects (and
112 in most animal models) now relies on the microinjection of a ribonucleoprotein (RNP) complex -
113 containing the sgRNAs and purified Cas9 protein - within single cells or early embryos.

114

115 Implementation in non-model insect organisms remains challenging, therefore we aimed to optimize the
116 CRISPR-Cas9 technology in aphids, which are hemipteran phloem-sap feeding insects known as major
117 pests of most crops worldwide. Their life cycle is quite singular since they develop by clonal
118 reproduction (viviparous parthenogenesis) during spring and summer. When summer ends, aphids can
119 perceive photoperiod shortening and integrate this signal towards their developing embryos that in turn
120 switch to a sexual reproductive mode, resulting in the production of males and sexual oviparous females.
121 Sexual individuals then mate and females lay fertilized eggs on plants during the autumnal period. Then
122 these eggs must experience an obligate diapause of three months over winter, before they hatch in the
123 next spring as new parthenogenetic colonies (Le Trionnaire *et al.*, 2013). Aphids display other life
124 history traits (host plant adaptation mechanisms, endosymbiosis, phenotypic plasticity) that allow them
125 to efficiently cope with biotic and abiotic stresses or increase damages on crops (especially as vectors
126 of plant viruses). Deciphering the molecular mechanisms controlling these traits and especially

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127 identifying master regulator genes could potentially provide new molecular targets for specific aphid
128 control strategies. Understanding and validating the function of these genes is thus a necessary pre-
129 requisite. So far the peculiarities of the aphid model have made the development of functional analysis
130 tools particularly complex. RNA interference (RNAi) is used to transiently silence the expression of
131 aphid transcripts (Jaubert et al., 2007). However, silencing lasts only for a few days in most cases and
132 its efficiency depends on the targeted tissue and rarely exceeds 50% (Mulot et al., 2016). EMS
133 mutagenesis is another alternative to generate stable mutants (Tagu et al., 2013). The availability of an
134 efficient genome editing tool in aphids would thus be a major breakthrough in the aim of understanding
135 gene functions in such an important crop pest.

136
137 Taking into consideration all the constraints inherent to the model, a step-by-step protocol for targeted
138 mutagenesis with the CRISPR-Cas9 system was optimized in the pea aphid (*Acyrtosiphon pisum*), one
139 of the model species used commonly in the aphid research community, and for which genomic resources
140 are available (IAGC, 2010 and Legeai et al., 2010). In this study, the candidate gene for developing and
141 validating the efficiency of the method was *stylin-01*, a gene coding for a cuticular protein from the CPR
142 family, which is a prime candidate receptor of noncirculative plant viruses. This protein has recently
143 been identified in the acrostyle, an organ in aphid maxillary stylets containing plant virus receptors
144 across its surface (Webster et al, 2018). In the same study, the authors showed that the viral helper
145 protein P2 of *Cauliflower mosaic virus* (CaMV) and an antibody targeting the surface-exposed peptide
146 of Stylin-01 compete *in vitro* for binding to the acrostyle. Moreover, reducing Stylin-01 expression in
147 the aphid vector *Myzus persicae* impacted its ability to transmit the CaMV. However, transcript levels
148 were only partially reduced, despite numerous attempts made by multiple methods. In addition, a direct
149 P2-Stylin-01 interaction was not observed, due to intrinsic properties of the protein, preventing Stylin-
150 01 from being definitely identified as the receptor of CaMV. A *stylin-01* mutant would undoubtedly help
151 define the precise role of Stylin-01 in noncirculative virus transmission. Here, we will describe in detail
152 the following steps of the protocol: **i**) male and sexual female synchronized photoperiod-triggered
153 induction, **ii**) mating and fertilized eggs synchronization, **iii**) sgRNAs and Cas9 protein microinjection,
154 **iv**) diapause experienced by melanized eggs, **v**) hatching and maintenance of new colonies, **vi**) the
155 mutation detection protocol, and **vii**) the effective germline transmission of mutations within the
156 offspring of hatching colonies. Each critical step in the protocol will then be discussed in the light of
157 our experience. This study represents the first successful genome editing protocol ever developed in
158 aphids to create stable mutant lineages.

159

160 **2. Material and methods**

161

162 *2.1. Single guide RNAs preparation*

163

164 *2.1.1. In-silico single guide RNAs design*

165

166 The CRISPOR algorithm (<http://crispor.tefor.net/>, Concordet et al., 2018) was used to find targets for
167 single guide RNA synthesis within the *stylin-01* coding sequence. Prior to this computational step, the
168 genomic *stylin-01* DNA sequence from the *A. pisum* clones used in this study were checked to identify
169 putative polymorphic sites. CRISPOR software specifically searches for target sequences of a N¹⁶⁻²⁰-

170 NGG format, N¹⁶⁻²⁰ corresponding to a succession of 16 to 20 nucleotides (N=A, T, G or C) and NGG
171 to the PAM motif necessary for *Streptococcus pyogenes* Cas9 activity. This algorithm can also search
172 within *A. pisum* genome for putative off-target sites (within exonic, intronic or intergenic regions)
173 sharing respectively 0, 1, 2, 3 or 4 mismatches with the initial target sequence. A specificity score is
174 then calculated for all target sequences and a measurement of their uniqueness within the genome is
175 provided. Target sequences (up to 4, if possible) with the maximum specificity score (100 being the
176 highest) and the lowest number of off-target sites were then selected. In our case, the selected sequences
177 also need to begin with GG, since these two nucleotides are necessary for *in vitro* transcription using T7
178 polymerase (see below). Considering this additional constraint, target sites selection within *stylin-01*
179 coding sequence were restricted to GG-N⁽¹⁴⁻¹⁸⁾-NGG motifs.

180

181 2.1.2. *In vitro* single guide RNA synthesis

182

183 For single guide RNA synthesis (**Figure S1**), the DR274 plasmid (Plasmid 42250, Addgene) was used
184 as a template. To linearize the plasmid, a digestion with *BsaI* restriction enzyme (New England Biolabs)
185 was performed at 37°C for 24 hours in a total volume of 100 µL, using 80 U of enzyme and 8 µg of
186 plasmid. The digestion quality was checked by electrophoresis on a 1.5% agarose gel containing SYBR
187 safe (Life Technologies) and quantified with a Quantus Fluorometer (Promega). The linearized plasmid
188 was then used for single guide RNA template preparation with specific primers. The Forward primer of
189 65 nt consists in a T7 promoter sequence at the 5'-end (20 nt) followed with the target site sequence
190 defined by CRISPOR software (16-20 nt) and the beginning of the universal sequence of the guide RNA
191 at the 3'-end (25 nt). The Reverse primer corresponds to a 23 nt sequence covering the end of the
192 universal guide RNA sequence (see primers **Table S1**). PCR was subsequently performed using *Taq*
193 polymerase (Promega) with the following conditions: one initial denaturation step of 95°C for 5 min, 35
194 cycles of denaturation step at 94°C for 30 s, one annealing step at 58°C for 30 s and one elongation step
195 at 72°C for 20 s, and one final elongation step of 72°C for 5 min. The primers used for the four RNA
196 guides of *stylin-01* gene are listed in **Table S1**. PCR products were then purified using the GenElute
197 PCR Clean Up kit (Sigma Aldrich) according to the manufacturer's instructions. Purified PCR products
198 were quality checked on an agarose gel, quantified with Quantus Fluorometer (Promega) and
199 concentrated by vacuum drying, before being used as a template for *in vitro* transcription of single guide
200 RNA. 4 µg of purified PCR product were *in vitro* transcribed with the MAXIscript T7 transcription kit
201 (Ambion) following manufacturer's instructions with an additional DNase digestion step. Single guide
202 RNAs were purified once with acid phenol-chloroform (1:1) and once with chloroform (1:1), before
203 isopropanol precipitation (1:1) at -20°C. The RNA pellet was then dried using a vacuum dessicator (or
204 dryer) and resuspended in 15 µL of RNase-free water. RNA quality was checked by electrophoresis under
205 RNase-free conditions and the concentration was estimated with a Quantus fluorometer (Promega).

206

207 2.1.3. *In vitro* single guide RNA cleavage efficiency validation

208

209 The genomic DNA sequence of *stylin-01* was initially amplified by PCR using specific primers (**Table**
210 **S1**) and *Taq* polymerase (Promega) starting with 2 µL of genomic DNA in a 50 µL reaction mix with
211 the following steps: 95°C for 5 min followed by 45 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for
212 20 s and a final step at 72°C for 5 min. The amplification product was checked by electrophoresis on an

213 agarose gel and used as template for an *in vitro* cleavage assay. Briefly, 100 ng of PCR target fragments
214 were mixed with 300 ng of Cas9 protein (CP01, PNA Bio Inc) and 250 ng of each sgRNA individually
215 in NEB buffer 3 containing BSA. The mix was incubated at 37°C for 1 hour. The Cas9 protein was then
216 inactivated at 65°C for 10 min. A negative control without sgRNA was also performed. The cleavage
217 reaction efficiency was analyzed by electrophoresis on an agarose gel.

218

219 2.1.4. Injection mix preparation

220

221 A total volume of 40 µL of injection mix was prepared with 1600 ng of each sgRNA corresponding to
222 a final concentration of 40 ng/µL for each sgRNA, 13.3 µg of recombinant Cas9 protein (CP01, PNA
223 Bio Inc) at a final concentration of 333 ng/µL in RNase free water, with aliquots of 4 µL stored at -80°C
224 until microinjection of fertilized eggs.

225

226 2.2. Egg production and microinjection

227

228 2.2.1. Sexual morphs production

229

230 The ultimate purpose of this part of the protocol is to generate a sufficient number of fertilized eggs for
231 the microinjection steps (see below). To this end, males and females have to be perfectly synchronized
232 in order to optimize their mating once they reach adulthood. The five clones used in this study were
233 initially sampled in eastern France on *Medicago sativa* for L9Ms10, L9Ms13 and L9Ms14, *Trifolium*
234 *pratense* for L7Tp23 (Nouhaud *et al.*, 2014) and *Genista tinctoria* for Gt2.01 (Peccoud *et al.*, 2015).
235 These clones are maintained in a controlled environment as clonal colonies on *Vicia fabae* plants. A
236 Long Day (LD) photoperiodic regime (16 hours of light and 8 hours of darkness at 18°C) allows
237 multiplication by viviparous parthenogenesis. In order to induce the production of sexual morphs, a
238 Short Day (SD) photoperiodic regime (12 hours of light and 12 hours of darkness at 18°C) was applied
239 to the different clones used in this study. Two distinct induction protocols were used: i) a so-called
240 “partial induction protocol” (aphids are moved to SD conditions for a limited number of days and then
241 moved back to LD) that allows production of males, and ii) a so-called “complete induction protocol”
242 (aphids are moved to constant SD conditions) that allows production of sexual oviparous females. The
243 partial induction starts exactly one week before the complete induction, allowing males and sexual
244 females to reach adulthood simultaneously and ensures that they are both mature for mating. Females
245 start to lay fertilized eggs a few days after mating (**Figure 1**). The following cross combinations were
246 produced: L9Ms10 (Female) x L7Tp23 (Male), L9Ms14 (F) x L9Ms10 (M) and Gt2.01 (F) x L9Ms13
247 (M).

248

249 2.2.1.1. Partial induction for male production

250

251 This protocol was applied to L7Tp23, L9Ms10 and L9Ms13 clones (**Figure 1**). Initially reared under
252 LD conditions, 20 synchronized third stage larvae (L3) aphids (Generation 1 or G1) of each clone were
253 moved to SD conditions on *V. fabae* plants (2 aphids/plant) for exactly 9 days. In these conditions,
254 adulthood was reached within 5 days and mature adults started to produce offspring 8 days later. For
255 each adult individual, 2 to 3 L1 (Generation 2 or G2) were kept in order to obtain 40 L1 larvae of the

256 same age. These sexuparae - which are the male-producers - were isolated on plants (1 aphid/plant).
257 After 9 days under SD conditions these G2 individuals were moved back to LD conditions in which they
258 reached adulthood 10 days later. Production of offspring started after 3 days. Each adult was kept on the
259 plant until it produced around 20 individuals (Generation 3 or G3, batch 1) before being moved to
260 another plant to produce around 20 individuals (G3, batch 2). Because of the specificities of this partial
261 induction protocol, batch 1 contained only parthenogenetic females, while batch 2 was mainly composed
262 of males. These newborn G3 males needed another 10 days to reach adulthood. In total, 800 males
263 (40*20) were produced and were ready for the mating step (see below).

264

265 2.2.1.2. Complete induction for sexual females production

266 This protocol was applied to the second clone of the cross and started exactly 7 days after the partial
267 induction of males (**Figure 1**). Initially reared under LD conditions, 20 synchronized L3 aphids
268 (Generation 1 or G1) from the L9Ms10, L9Ms14 or Gt2.01 clone were moved to SD conditions on *V.*
269 *fabae* plants (2 aphids/plant). Since this induction is complete, aphids are never moved back to LD
270 conditions. For each individual, around 4 to 5 L1 were kept (Generation 2 or G2) to obtain 70 L1 larvae
271 of the same age. These sexuparae individuals are the female-producers. They were isolated (1
272 aphid/plant) until they reach adulthood after 10 days and started the production of offspring 3 days later.
273 Each adult was kept on the plant until it produced around 20 individuals (Generation 3 or G3, Batch 1)
274 and adults were discarded afterwards. Because of the specificities of this complete induction protocol
275 all individuals were sexual females. These new born individuals needed another 10 days to reach
276 adulthood. In total, 1400 females (70*20) were produced and ready for the mating step (see below).

277

278 2.2.2. Cross preparation, mating and egg collection

279

280 Thirty adult females and 15 adult males were placed on individual plants for mating, and an average of
281 6 plants were used each day. The mixing of sexual individuals started on the Wednesday before the
282 week of injection (**Figure 2a**). Usually four batches were set up: one on the Wednesday, one on the
283 Thursday, one on the Friday and one on the Saturday. Once put on plants, the first eggs laid by sexual
284 females were observable three days later and the peak of eggs production occurred after 5 days. As a
285 consequence, the 4 successive batches of crosses corresponded to four rounds of injection the next week
286 (from Monday to Thursday). On each day during the week of injections, aphids were placed on a new
287 plant at precisely 9.00 am to allow sexual females to lay eggs for exactly 4 hours. Sexual females usually
288 laid their eggs on the plant, on the plastic bag or on the plastic pot (**Figure 2b, 2c and 2d**). Eggs were
289 collected with a fine paintbrush under a magnifying glass and placed on a microscope slide with wet
290 filter paper (**Figure 2e**). With this protocol, eggs were collected within a 4 hours window so that they
291 were 2 hours old on average. After egg collection, aphids were placed back on the plants before being
292 manipulated exactly the same way on the following day.

293

294 2.2.3. Microinjection

295

296 Twenty eggs were aligned and orientated on a wet filter paper deposited on a microscope slide (**Figure**
297 **2f**). The filter paper was cut diagonally and the “straight” or ventral side of the egg (opposite from the
298 “curved” or dorsal side) was orientated to be in contact with the paper. The anterior pole of the egg was

299 also distinguishable from the posterior pole under visible light using a dissecting microscope, as the
300 anterior pole appears clearer and the posterior pole contains the bacteriocyte dark-green structure. Once
301 aligned, eggs were injected using the FemtoJet Microinjector device (Eppendorf), and Eppendorf™
302 Femtotips™ Microinjection Capillary Tips (inside diameter of 0.5µm and outside diameter of 0.7µm).
303 Capillaries were filled with the injection mix containing the Cas9 protein and the guide RNAs (see
304 above) using dedicated Eppendorf Microloaders™ (Eppendorf). Eggs were injected at the anterior pole
305 by a single pressure on the foot pedal for 0.3 s. An injection pressure of 500 hPa was applied and a
306 compensation pressure of 50 hPa was set up to avoid liquid or cytoplasm reflux. A micromanipulator
307 was used to quickly shift the capillary from one egg to another. After injection, eggs were kept on the
308 microscope slide used for injection, then directly transferred to a Petri dish containing wet filter paper -
309 to prevent eggs from desiccation - and placed in a climatic chamber at 15°C (**Figure 2g**).

310

311 2.2.4. Egg melanization, diapause and hatching

312

313 Melanization occurs at the beginning of embryos development where they slowly acquire a black serosal
314 cuticle necessary for cold resistance. Thus complete melanization indicates a non-disruptive injection.
315 Eggs that did not reach this stage were considered damaged by the injection procedure and discarded
316 (**Figure 3a**). Melanization of injected eggs was complete within 3 to 4 days at 15°C. Melanized eggs
317 were then collected from the microscope slide (**Figure 3b**). At this time, eggs were either directly
318 transferred to plants, or treated before transferring them to prevent them from developing fungal or
319 bacterial contamination during the obligate diapause period. This treatment consisted of an initial 5 min
320 wash step in 5% bleach solution followed by three 5 min washes in ultrapure water. Eggs were then
321 dried out on filter paper and carefully deposited with a fine paintbrush on the upper surface of *V. fabae*
322 leaves (**Figure 3c** and **3d**). Plants were then kept in a climatic chamber at 4°C (LD conditions) for
323 exactly 85 days, a period corresponding to the duration of the obligate diapause they experience before
324 hatching. After this period, plants were moved back at 18°C (LD conditions) to allow diapause to be
325 broken. During two weeks, plants were carefully observed every day under a magnifying glass and
326 hatching aphids were collected. These individuals are the so-called “foundress” and correspond to
327 viviparous parthenogenetic females (**Figure 3e**). Each “foundress” was then individualized on a new
328 single plant until they produced offspring by parthenogenesis. These new clonal lineages were then
329 maintained by isolating two individuals (L2 or L3) every generation to a new plant.

330

331 2.3. Mutations detection

332

333 2.3.1. Mutations detection in injected eggs

334

335 Once injections were completed, sub-samples of 20 to 40 melanized eggs were collected and frozen at
336 -80°C prior to DNA extraction, which was carried out using the ‘salting out’ protocol previously
337 described by Sunnucks et al. (1996). DNA from a single egg was extracted and resuspended in 20 µL
338 of ultra-pure sterilized water. The *stylin-01* genomic region (containing the target sites of the four
339 sgRNAs) was PCR amplified starting with genomic DNA (see above). The sizes of the amplicons were
340 compared to the size of the wild-type genomic fragment by electrophoresis on a 1.5% agarose gel to
341 evaluate the presence of insertions or deletions.

342 2.3.2. Mutations detection in hatched colonies

343
344 Once hatched, “foundress” individuals were isolated on a new plant until they produced offspring. As
345 clones, newborns are all genetically identical. For each newly established lineage, a pool of 5 individuals
346 at the 4th instar stage (L4) was frozen in liquid nitrogen. Genomic DNA was extracted using PureLink™
347 Genomic Plant DNA Purification kit (K183001, ThermoFisher Scientific). A PCR fragment of 1388 bp
348 flanking the targeted region was amplified using Phusion High-Fidelity DNA Polymerase (F530S,
349 ThermoFisher Scientific) with primers BC57F 5’-AGACGCATCCACAATCACAA-3’ and BC57R 5’-
350 CCGATGATCATTTCGAGGAG-3’. PCR cycling conditions included a denaturation step at 98°C for
351 30 s, 35 cycles of 98°C for 10 s, 64°C for 30s, 72°C for 45s followed by a final extension at 72°C for 5
352 min. To analyze the genomic DNA sequence from the 2 alleles of the *stylin-01* gene, blunt-ended PCR
353 products were cloned in pGGz plasmid (derived from pDonR-Zeo) and transformed in *E. coli* DH5
354 strain. Four to 20 colonies were selected by colony PCR (Sandhu et al, 1989) using GoTaq G2
355 polymerase (M7841, Promega, Corporation, Madison, WI, USA) with primers 13F 5’-
356 AACAGGGGCTGGTGAAATG-3’ and 14R 5’-GGCCATATCGGTGGTCATC-3’. PCR
357 amplifications included a denaturation step at 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30s,
358 72°C for 100 s followed by a final extension at 72°C for 5 min. Selected colonies were cultivated
359 overnight, their plasmid DNA extracted using the Wizard Plus SV Minipreps DNA purification System
360 (A1460, Promega, Corporation, Madison, WI, USA) and sequenced using the same primers
361 (GENEWIZ, South Plainfield, NJ, USA). Sequence alignment steps were performed using the Geneious
362 software (<http://www.geneious.com>).

363 364 3. Results

365 366 3.1. Evaluation of egg production dynamics and hatching rate in three genetic backgrounds

367
368 Before applying CRISPR-Cas9 mutagenesis to the pea aphid, it was necessary to select lineages that are
369 able to respond to the photoperiod shortening signal to produce sexual individuals. Three combinations
370 of crosses were tested in this study: L9Ms10 (Female) x L7Tp23 (Male), L9Ms14 (F) x L9Ms10 (M)
371 and L9Ms13 (F) x Gt2.01 (M). Two key parameters were considered for these crosses: **i**) the number of
372 fertilized eggs laid by sexual females after mating and **ii**) egg hatching success after the 85 days of
373 obligate diapause. Females usually start to lay eggs 3 days after mating (d.a.m) for a total period of 6
374 days with an optimum for eggs production at 6 d.a.m. Although the number of eggs laid by single
375 females was not precisely recorded (**Table 1**), the females from crosses L9Ms10xL7Tp23 and
376 L9Ms13xGt2.01 produced higher numbers of fertilized eggs compared to the females from the
377 L9Ms14xL9Ms10 cross (at least 10 per female for the two first crosses, and less than 5 per female for
378 the third one). Thirty six and 35% of the untreated eggs hatched and produced “foundress” lineages for
379 L9Ms10xL7Tp23 and L9Ms14xL9Ms10 crosses, respectively, whereas 77% of eggs hatched in the case
380 of Gt2.01xL9Ms13 cross (**Table 1**). Hatching rates were reduced for bleach-treated eggs, ranging from
381 9% (L9Ms10xL7Tp23), 28% (L9Ms14xL9Ms10) to 33% (Gt2.01xL9Ms13), suggesting that this
382 treatment affects embryos viability and should be removed from the protocol (**Table 1**). Taken together,
383 our results show that the Gt2.01xL9Ms13 cross is the most efficient for producing sufficient numbers
384 of eggs for the microinjection step and post-diapause lineage selection.

385

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386 3.2. Assessment of single guide RNAs *in vitro* cleavage efficiency

387
388 CRISPOR algorithm found four target sites for single guide RNAs synthesis fulfilling the GG-N¹⁴⁻¹⁸-
389 NGG motif constraint within the *stylin-01* coding sequence. The four selected sequences do not share
390 perfect identity with other homologous regions within the pea aphid genome: the single guide RNAs
391 synthesized to target these regions (namely sg1, sg2, sg3 and sg4) were therefore unlikely to generate
392 off-target cleavages. Briefly, *stylin-01* genomic DNA is composed of three exons. Sg1 target site is
393 located within the second exon and the three others are distributed within the last exon (**Figure 4**). These
394 four sgRNAs were tested for their ability to cleave the *stylin-01* genomic DNA sequence *in vitro* (**Figure**
395 **5**). Electrophoresis profiles showed two bands corresponding to the expected cleavage fragments when
396 sg1 and sg3 were present in the reaction, suggesting a high *in vitro* binding efficiency for these guides.
397 Expected cleavage fragments were also observed for sg2 and sg4. However, a band corresponding to
398 uncleaved PCR fragment remained, suggesting an effective but reduced efficiency of these guides *in*
399 *vitro*. Altogether, this experiment ensured that all the designed sgRNAs were functional *in vitro*.

400

401 3.3. Effect of the microinjection on egg survival

402
403 In this study, three independent series of microinjections were performed on eggs originating from three
404 different crosses. For each experiment, eggs were injected with two different mixtures: the first one
405 comprising the 4 guide RNAs (sg1, sg2, sg3 and sg4) and the Cas9 protein; and the second one
406 comprising only one guide RNA, sg4, and the Cas9 protein. Overall, 1698 eggs were injected in 5 days
407 for the first cross (L9Ms10xL7Tp23), 412 eggs in 4 days for the second cross (L9Ms14xL9Ms10) and
408 527 eggs in 2 days for the third cross (Gt2.01xL9Ms13) (**Table 2**). The impact of microinjection on egg
409 survival was estimated with the proportion of melanized eggs reported to the total number of injected
410 eggs, considering melanization as a proxy measurement of survival. Egg survival rate was around 20%,
411 31% and 44.4% for the first, second, and third cross, respectively. These differences in egg survival
412 might either reflect different embryo fitness for the three crosses, or an experimenter effect due to
413 manual microinjection. The survival rate of microinjected eggs could thus exceed 45% under optimal
414 conditions.

415

416 3.4. Estimation of the *in ovo* mutation rate

417
418 To rapidly estimate CRISPR-Cas9 efficiency after microinjection, up to 40 melanized eggs injected with
419 the 4 sgRNAs/Cas9 mixture were collected within the pool of eggs kept for diapause. For
420 L9Ms10xL7Tp23 cross, the PCR amplification was efficient for 36 eggs out of the 40 selected (**Figure**
421 **6**). A single band corresponding to the wild type amplicon was observed for 8 eggs. Although this result
422 may reflect the rate of “non-mutated” eggs, we cannot totally exclude the presence of small
423 insertion/deletion events at the four target sites than cannot be detected with the PCR protocol used. For
424 the 28 remaining eggs, the electrophoresis profiles revealed 1, 2 or even 3 bands with a size lower than
425 the one expected for the wild type PCR fragment, either found alone or together with the wild type
426 amplicon. These short PCR fragments probably arose from the combined action of at least 2 guide RNAs
427 triggering large deletions in *stylin-01* genomic DNA sequence. In addition, small amplicons of various
428 sizes were observed in the eggs, indicating that large re-arrangements occurred between different

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429 combinations of guide RNAs. These 28 eggs were thus assigned as “mutated”, bringing the mutation
430 rate in the first experiment to at least 77.8%. Eighteen eggs were examined as part of the third experiment
431 (Gt2.01xL9Ms13 cross), out of which 12 gave a PCR result. Four samples were assigned as “non-
432 mutated” and 8 samples as “mutated”, indicating a mutation rate of at least 66.7%. These *in ovo* analyzes
433 confirmed the efficiency of the CRISPR-Cas9 system in mutating aphid eggs at early stages post
434 microinjection. They also showed that the injection of several guide RNAs could generate various
435 combinations of large deletions within the genomic sequence of *sylin-01* without knowing - at this stage
436 - if the observed mutations arose from somatic or germline cells.

437

438 3.5. Hatching and stable lineages establishment rate from injected eggs

439

440 After the obligate diapause period, embryos have completed their development and hatch as the so-
441 called “foundress” individuals that initiate new clonal colonies. For the three cross combinations tested
442 in this study hatching rates of injected eggs were very low (**Table 2**) and varied from 1.1% for
443 L9Ms10xL7Tp23 cross (3 hatched eggs/274 eggs injected with sg 4 or with the four sgRNAs), to 4.9%
444 for L9Ms14xL9Ms10 cross (5/101), and 11.7% for Gt2.01xL9Ms13 cross (23/196). These hatching
445 rates were much lower than the ones observed for non-injected eggs in the three genetic backgrounds
446 (36, 35 and 77% respectively, see **Table 1**). This suggests a potential damage occurring during the
447 injection procedure or a toxic effect of the sgRNAs/Cas9 mix impacting embryos survival and hatching.
448 These results also mirror the differences in hatching rates for eggs from the three genetic backgrounds
449 (Table 1), the Gt2.01xL9Ms13 cross showing the highest rate compared with the two other crosses.
450 Altogether, 31 “foundress” individuals out of the 571 injected and melanized eggs were obtained in the
451 three experiments. These 31 “foundress” were installed individually on plants to allow their complete
452 developmental to adulthood. Seventeen “foundress” aphids gave offspring, producing by
453 parthenogenesis genetically identical individuals, leading to the establishment of stable lineages
454 subsequently maintained as clonal colonies. Finally, 55% of the hatched “foundresses” ended up as
455 stable linages (**Table 2**). A potential effect of mosaicism in the “foundress” might in part explain why
456 14 individuals could not produce offspring. The final rate of stable lineages setting is therefore closed
457 to 3% (17/571) in the three combined genetic backgrounds.

458

459 3.6. Germline transmission rate estimation in stable lineages

460

461 All the individuals within the progeny of “foundress” aphids are genetically identical since they arise
462 from diploid oocytes produced clonally. They can potentially originate from one or several germline
463 nuclei within the syncytial early embryo. If only one germline nucleus has been targeted by the CRISPR-
464 Cas9 system within the early-fertilized egg then all the progeny will share the same mutations (one or
465 two different mutated alleles). If there are several germline nuclei at the time of injection, it is possible
466 to detect within the progeny individuals displaying different mutation patterns (more than two mutated
467 alleles). To detect mutated lineages, DNA was extracted from pooled individuals for the 17 stable
468 lineages to amplify the *stylin-01* genomic DNA sequence. PCR fragments were then cloned in a plasmid
469 and sequenced to discriminate between the two parental alleles. A total of 4 lineages (L2, L4, L7 and
470 L15) originating from eggs injected with the four guide RNAs (sg1, sg2, sg3 and sg4) and 2 lineages
471 (L14 and L16) originating from eggs injected with a single guide RNA (sg4) were mutated in the *stylin-*

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472 *01* gene (**Table 2**). In more details (**Figure 7**), L2 lineage displayed multiple re-arrangements in the two
473 mutated alleles and at the four sites targeted by sgRNAs: allele 1 showed a 10 nt deletion at target site
474 1, a 3 nt deletion at target site 2, a 3 nt insertion at target site 3 and a 13 nt insertion / 6 nt deletion at
475 target site 4, while allele 2 showed a large 380 nt deletion at target site 1, a 10 nt insertion / 2 nt deletion
476 at target site 2, a 2 nt insertion at target site 3, and a 7 nt deletion at target site 4. In L4, L7 and L15
477 lineages, insertions or deletions of a few nucleotides were observed at the different target sites for 1 of
478 the 2 alleles. Unexpectedly for these three lineages, only one parental allele was detected by cloning,
479 none of the 20 sequences analyzed for each lineage allowed the detection of the second parental allele.
480 For lineages originating from eggs injected with a single guide RNA sg4, L14 lineage showed re-
481 arrangements on both alleles at the target site: a 3 nt insertion / 2 nt deletion on allele 1 and a 6 nt deletion
482 on allele 2. L16 lineage displayed a 7 nt deletion at target site 4 on allele 1, the other parental allele
483 could not be detected in the set of sequenced clones. Since lineages were maintained clonally, the same
484 protocol was applied several generations later to sequence the 6 lineages and perfectly identical
485 mutational patterns as those previously described were observed (data not shown). Since 6 out the 17
486 established lineages were stably mutated, the germline transmission rate of the mutations was close to
487 35% (**Table 2**). These results thus demonstrate that the CRISPR-Cas9 protocol described here is fully
488 functional in the pea aphid and efficient to generate stable mutant lineages on the two targeted alleles of
489 *styllin-01* gene.

490

491 **4. Discussion**

492

493 *4.1. Difficulties encountered in the aphid model to develop CRISPR-Cas9 mutagenesis*

494

495 Alternation of reproductive mode as well as the obligate diapause fertilized eggs must experience are
496 two specificities of the aphid life cycle that have caused difficulties in developing CRISPR-Cas9
497 mutagenesis in aphids. Direct injection within viviparous parthenogenetic females could be an
498 alternative to egg injection. However, in a recent study, Jamison *et al.* (2018) tried to deliver sgRNA-
499 Cas9 RNP complexes designed to target the *tor* gene - a cuticle pigmentation gene - in viviparous aphid
500 ovaries by electroporation. The analysis did not indicate any mutation (neither in the soma nor in the
501 germline) of the targeted gene in the offspring of the treated individuals. It is indeed very unlikely that
502 both components could efficiently reach germline cells that are embedded between several layers of
503 tissues within the ovaries. Thus, fertilized eggs seem to be the most rational starting point and therefore
504 the production and the collection of a significant number of eggs just after being laid by sexual females
505 is the first critical point of the protocol. This implies the three main parameters we have first optimized
506 in this protocol: **i**) the induction of sexual morphs, **ii**) the synchrony of males and females production
507 and **iii**) a large number (1000s) of manipulated individuals to produce a sufficient number of eggs.
508 Because diapause cannot be avoided, the *in ovo* PCR test is a proxy to estimate the success of the
509 mutagenesis before eggs experience the 3 months of slow development. The second critical point of the
510 protocol corresponds to the *a priori* choice of the cross that conditions genetic compatibility and by
511 extension hatching rates and fitness of stable lineages. The main challenge of the aphid model in the
512 view of developing targeted mutagenesis thus relies on the complexity of the biology of this insect. We
513 discuss in the following paragraphs all the parameters that have been optimized to allow the production
514 of stable mutant lineages in the pea aphid.

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515

516 *4.2. Lineages choice for sexual morphs induction, mating and hatching success*

517

518 In order to produce sexual morphs, the two different lineages chosen for the cross must be able to fully
519 respond to photoperiod shortening and alternate their reproductive mode. The implementation of two
520 distinct induction protocols for the two lineages - one favoring a late production of males and the second
521 an early production of oviparous females - ensures that sexual individuals become adult at the same
522 period and are synchronously mature for mating. Self crosses must be avoided since most of the time
523 they are unfit and usually result in very low hatching rates after diapause. In the current study, the three
524 different cross combinations tested resulted in variable egg production rates thus revealing different
525 genetic compatibility levels between selected lineages. Nevertheless, the production of numerous
526 synchronized sexual females (1400) and males (800) is a way to overcome this issue, and to produce the
527 hundreds of eggs needed for further microinjection. Once melanized, eggs must experience a 3-month
528 period of diapause at low temperature. The process of diapause in the pea aphid – and in many other
529 insects - is a slowing but not a cessation of development (Shingleton et al., 2003). The early stages of
530 embryogenesis progress at a temperature-independent rate whereas later stages of embryogenesis
531 progress at a temperature-dependent rate. However embryos maintained at very high temperatures in
532 the very early stages show severe developmental abnormalities. Diapause is thus an incompressible
533 event that cannot be artificially overcome. Hatching is promoted by placing aphids at 18°C during a 2-
534 weeks period. The embryos originating from the three cross combinations tested in this study displayed
535 various hatching rates from 35 to nearly 80%. The hatching rate, in our hands, corresponds to the most
536 robust indicator of the genetic compatibility between two lineages selected for a cross. It is thus
537 important, when starting to set up a targeted mutagenesis protocol, to optimize the choice of the two
538 lineages that will allow reaching the necessary scale of the experiment.

539

540 *4.3. Egg injection procedure: timing and effect on melanization and post-diapause hatching*

541

542 Once the mating of adult individuals is completed, sexual females start to deposit the first fertilized eggs
543 on plants three days later. Females then continue to lay eggs for approximately one week with a peak in
544 production three days after the first laid ones. Every day of the week of microinjection it is mandatory
545 to repeat the same routine: individuals are placed every morning on a new plant and eggs are collected
546 exactly four hours later. The age of the eggs can thus be estimated between 0 and 4 hours after egg
547 laying (hAEL). Lin et al. (2014) performed a detailed cytological observation of the 10 first cycles of
548 nuclei division of pea aphid early embryos originating from fertilized eggs. The authors estimated that
549 these 10 cycles are completed within 16 hours and that the 5th cycle of division is reached after
550 approximately 6 hours. At that time, the embryo contains 16 nuclei in a syncytial structure. The authors
551 also performed in situ hybridization of ApVas1 mRNA - a germline marker - within embryos.
552 Interestingly, they could not observe the germ plasm after the 3rd nuclei division, which means that none
553 of the eight observable nuclei are predetermined to be a germline nucleus. After the 4th division, the
554 germ plasm is detectable which means that some nuclei will give rise to primordium germ cells. Whether
555 the germline was of a single or multiple origin was not assessed in this study. It is known in drosophila
556 that germ cells originate from three founding nuclei in the syncytial blastoderm embryo and continue
557 amplification before moving into polar buds that give rise to the primordium germ cells (Campos-Ortega

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558 and Hartenstein, 1997 and Lindsley et al., 2016). Therefore in the case of the pea aphid, injecting eggs
559 4 hours maximum after laying guarantees that they contain between 8 and 16 nuclei in a syncytial
560 structure, but we do not know how many nuclei display a germline origin. CRISPR-Cas9 system could
561 thus potentially generate more than two different mutated alleles within the offspring of the “foundress”
562 individual. Nevertheless, we could only observed at most two mutated alleles within the offspring of the
563 six mutated “foundress” suggesting that in these specific cases only one germline nucleus had been
564 targeted. In a protocol aiming at performing germline transformation of various species of mosquitoes
565 (including *A. gambiae* and *A. aegypti*) embryos are collected right after oviposition for 15 min then
566 placed for 30 min under mosquito rearing specific conditions to allow their maturation/melanization.
567 At the time of injection, embryos are thus 30 to 45 min old (Lobo et al., 2006). In *D. melanogaster*
568 injections are also performed around 30 min after embryos collection which corresponds to the syncytial
569 blastoderm stage (Kiehart et al., 2007). In both cases injections take place at the very early stages of
570 embryogenesis to reduce mosaicism levels. As stated above, the early steps of aphid embryogenesis are
571 slower so that a 0-4 hours post laying window is comparable to a 30-45 min post-oviposition window
572 in drosophila or mosquitoes. Another feature of aphid eggs is that bacteriocyte is observable at the early
573 steps of embryogenesis at the posterior pole of the egg and is situated close to the future germline nuclei.
574 To avoid any bacteriocyte damage the capillary was inserted at the opposite pole to allow a diffusion of
575 the CRISPR-Cas9 components within the entire syncytium. Nevertheless, injecting those reagents at the
576 “bacteriocyte pole” could be a way to preferentially reach germline nuclei. It has to be noted that given
577 the large scale of these experiment it was not possible to test different sgRNAs concentrations or
578 compare the impact of the injection of a Cas9 mRNA versus the Cas9 protein. These parameters had
579 already been tested in the mosquito injection protocol (Kistler et al., 2013) so that we decided to directly
580 inject the recombinant Cas9 protein with several sgRNAs at optimized concentrations. After the
581 microinjection step, melanization is a first estimation of egg survival. Only eggs that had completed this
582 step were conserved and placed on plants at low temperature. In our study, melanization rate ranged
583 from 20 to 45% depending on the experiment. In mosquitoes, hatching occurs between 3 and 5 days
584 after microinjection (Lobo et al., 2006) whereas it happens between 2 and 3 days in drosophila (Kiehart
585 et al., 2007). In the case of aphids, due to the obligate diapause, hatching cannot be observed before 85
586 days post-injection. For further comparisons hatching rates were estimated starting from melanized
587 rather than injected eggs. As mentioned above, hatching rate is highly dependent on the genetic
588 compatibility between the two lineages that are crossed. Nevertheless, the comparison of hatching rate
589 of non-injected versus injected eggs mirrors the deleterious effect – including cytoplasm leakage,
590 membrane deterioration and probably zygotic maternal mRNAs gradient perturbation - that the injection
591 procedure can have on embryos fitness. Embryos survival rate after injection is estimated at 15 to 20%
592 in *D. melanogaster* (Bassett et al., 2013) and approaches 10% in *A. aegypti* (Kistler et al., 2015). When
593 considering the best cross combination (Gt2.01xL9Ms13), it appears that a survival rate of 11.7% (23
594 hatched lineages out of 195 melanized eggs) is quite similar to what has been observed in mosquitoes
595 or drosophila. The individuals that hatch from eggs (also called foundress) then produce genetically
596 identical offspring by clonal reproduction. Considering the peculiarity of clonal reproduction in aphids,
597 if the germline nucleus was reached by the CRISPR-Cas9 system at the time of microinjection, the
598 mutation would be fixed and all offspring will be identically mutated (see below). On the contrary, if
599 only somatic cells were affected, then the offspring will not be mutated and somatic mutations would
600 potentially affect the fitness of foundress individuals. This is illustrated by the fact that not all the

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601 hatched lineages gave birth to viable offspring. Indeed for the last experiment 14 out of the 23 hatched
602 lineages gave offspring: these are referred as to stable lineages. The final stable lineages rate is thus of
603 7.1% (14 out of 195 melanized eggs).

604

605 4.4. *In vitro and in ovo guide efficiency validation: two necessary check points before diapause*

606

607 As mentioned above, the whole experiment requires 7 months for completion: 8 weeks for the induction
608 of sexual morphs, 2 weeks for microinjection/melanization, 12 weeks of diapause, 2 weeks for hatching
609 and another 4 weeks for the set up and maintenance of clonal lineages. Considering the complexity of
610 the protocol, it is therefore crucial to verify *a priori* the cleavage efficiency of the sgRNA/Cas9 mixture
611 injected within the egg. Indeed sgRNAs design and prediction mainly rely on the likeliness of the RNA
612 sequence to anneal its target DNA sequence. In the recent years, many different bioinformatics tools
613 were developed for such prediction purposes. In this study, 4 sgRNAs predicted to target the *stylin-01*
614 coding sequence were designed and all of them appeared to efficiently cleave DNA *in vitro* when
615 combined with the recombinant Cas9 protein. It thus appears that CRISPOR software (Concordet et al.,
616 2018) offers a good chance to generate functional sgRNA molecules. Injecting several sgRNAs is a
617 means to generate large deletions within the genomic DNA sequence of a given gene, and the
618 observation of amplicons smaller than the wild-type is thus an easy way to confirm sgRNA efficiencies.
619 As a side effect, the simultaneous injection of several guide RNAs might also increase the risk of off-
620 target effects. In the future, methods such as the Digenome-seq (Kim et al., 2015), could be used to
621 estimate the rate of off-target cleavages caused by specific sgRNAs, but were not investigated in this
622 study. The approximate size of amplicons also appears to be sufficient to predict which combinations of
623 sgRNAs are responsible for the large deletion events observed. In that regard the various patterns
624 observed confirmed that the 4 sgRNAs were efficient to trigger DNA cleavage in early embryos and
625 validated the functionality of the CRISPR-Cas9 system in the pea aphid. Finally, *in ovo* mutation rates
626 could be estimated at 70 to 78% in pea aphid eggs, a rate close to what can be observed in drosophila
627 (50 to 86%, Bassett et al., 2013) but higher than the rates in mosquitoes estimated at 24.6% (Kistler et
628 al., 2013). Sacrificing several eggs in the early steps of the experiment to check the editing efficiency is
629 thus an absolute pre-requisite to include before going through the long and complex steps of diapause
630 and hatching.

631

632 4.5. *Germline transmission rate within established mutant lineages*

633

634 Combining the three successive microinjection experiments performed in this study, 17 stable lineages
635 were eventually generated and maintained clonally. Depending on the efficiency of the system, it was
636 possible to observe either one or the two alleles of *stylin-01* gene mutated and in the latter case to observe
637 different mutations for the two alleles. Amplification, cloning and sequencing of the *stylin-01* genomic
638 DNA region within the offspring of the 17 stable lineages revealed that 6 lineages (4 arising from eggs
639 injected with the 4sgRNAs and two from eggs injected with only one sgRNA) contained an
640 insertion/deletion of a few nucleotides within the target sequence of at least one sgRNAs. In all cases,
641 open reading frames were modified with insertion/deletion events ranging from -10 nt to +13 nt. Large
642 deletions (-380 nt or -75 nt) outside predicted target sites have also been observed in two cases. For two
643 lineages L2 and L14, mutations in both *stylin-01* alleles were detected. In L2 lineage, multiple re-

644 arrangements with insertions and deletions were observed at the four target sites for the two alleles. In
645 the lineage originating from eggs injected with one sgRNA, L14, mutations were also observed on both
646 alleles at sg4 target site. These two lineages correspond therefore to double heterozygous *stylin-01*
647 mutants. For the other four mutated lineages, only one of the two parental alleles could be detected and
648 its sequence analyzed. Surprisingly, the other parental allele could not be detected in these 4 lineages. It
649 is possible that sequencing only 20 clones per PCR product would not guarantee the identification of
650 both parental alleles. A possible cloning bias could also result in the fact that only one of the two alleles
651 was preferentially introduced within the plasmid. In addition, the exact same mutations could be detected
652 in later parthenogenetic generations suggesting the stable character of the mutations produced here (data
653 not shown). In the aphid model, clonality thus allows germline mutations to be directly transmitted and
654 fixed within their offspring. Finally, the germline transmission rate in this study reached 35%. In model
655 organisms, germline transmission rate estimation requires a subsequent crossing generation. Those rates
656 can reach 34.5% in *Drosophila* (Basset *et al.*, 2013) and 18.9% in mosquito (Kitel *et al.*, 2013). Despite
657 the low number of stable colonies generated in this study, the germline transmission rate of the mutations
658 in the pea aphid is very close to what is observed in those models, which fully validates the current
659 CRISPR-Cas9 mutagenesis protocol. In the case of heterozygous mutant lineages, it is possible to obtain
660 homozygous mutant lineages by performing a self-cross. Mutated lineages must be submitted to a
661 decreasing photoperiod treatment to allow the production of sexual morphs and their subsequent mating.
662 After hatching, 25% of the lineages should thus be homozygous for the mutations, 50% heterozygous
663 mutant and the remaining 25% would be of a wild-type genotype.

664

665 In conclusion, the current study provides a detailed step-by-step and reproducible framework of
666 CRISPR-Cas9 mutagenesis to create and maintain stably edited aphid lineages. *Stylin-01* edited stable
667 lineages have been successfully generated and colonies are now well established. Analyzing their
668 phenotypes will require further investigations. To our knowledge this work corresponds to the first
669 example of an efficient targeted mutagenesis in the aphid model. The availability of such a protocol thus
670 represents a major advance for the aphid scientific community to decipher and validate gene function
671 for various life history traits associated with aphids' peculiar biology and strongly responsible for the
672 serious damages they cause within agro-ecosystems.

673

674 **Acknowledgements**

675 This work was funded by an INRA-SPE grant (2016-2017) lead by Gaël Le Trionnaire and Véronique
676 Brault (INRA Colmar, UMR SVQV) and the ANR SexAphid (2018-2020) project (ANR-17-CE20-
677 0010) lead by Denis Tagu. The authors would like to thank Jean-Christophe Simon, Gaëtan Denis and
678 Julie Jaquiéry (INRA Rennes, UMR IGEPP) for their advices on aphid crosses and rearing. Eric Marois
679 and Stéphanie Blandin (CNRS Strasbourg, IBMC Unit) are greatly acknowledged for their advices on
680 mosquito eggs microinjection protocol.

681

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Tables and Figures

Table 1 – Comparison of the egg production level and hatching rate of 3 different crosses

Female	Male	Egg production	Egg treatment	Hatching rate
L9Ms10	L7Tp23	High (at least 10 eggs/female)	Control	36%
L9Ms10	L7Tp23		H2O + Bleach	9%
L9Ms14	L9Ms10	Low (less than 5 eggs/female)	Control	35%
L9Ms14	L9Ms10		H2O + Bleach	28%
Gt2.01	L9Ms13	High (at least 10 eggs/female)	Control	77%
Gt2.01	L9Ms13		H2O + Bleach	33%

Table 2 – CRISPR-Cas9 mutagenesis of *stylin 1* gene in three different genetic backgrounds

Cross	sgRNAs combination	<i>Foundress generation</i>			<i>Foundress offspring</i>	
		Injected eggs	Melanized eggs (%)	Hatched eggs (%)	Stable lineages (%)	Mutated lineages (%)
L9Ms10 (F) x L7Tp23 (M)	sg1-sg2-sg3-sg4 sg4	1059	199/1059 (18.8)	3/159 (1.9)	1/3 (33.3)	0/1 (0)
		639	140/639 (21.9)	0/115 (0)	0 (0)	0 (0)
L9Ms14 (F) x L9Ms10 (M)	sg1-sg2-sg3-sg4 sg4	257	89/257 (34.6)	4/74 (5.4)	1/4 (25)	1/1 (100)
		155	39/155 (25.2)	1/27 (3.7)	1/1 (100)	1/1 (100)
Gt2.01 (F) x L9Ms13 (M)	sg1-sg2-sg3-sg4 sg4	286	119/286 (41.6)	17/101 (16.8)	9/17 (52.9)	3/9 (33.3%)
		241	115/241 (47.7)	6/95 (6.3)	5/6 (83.3)	1/5 (25)

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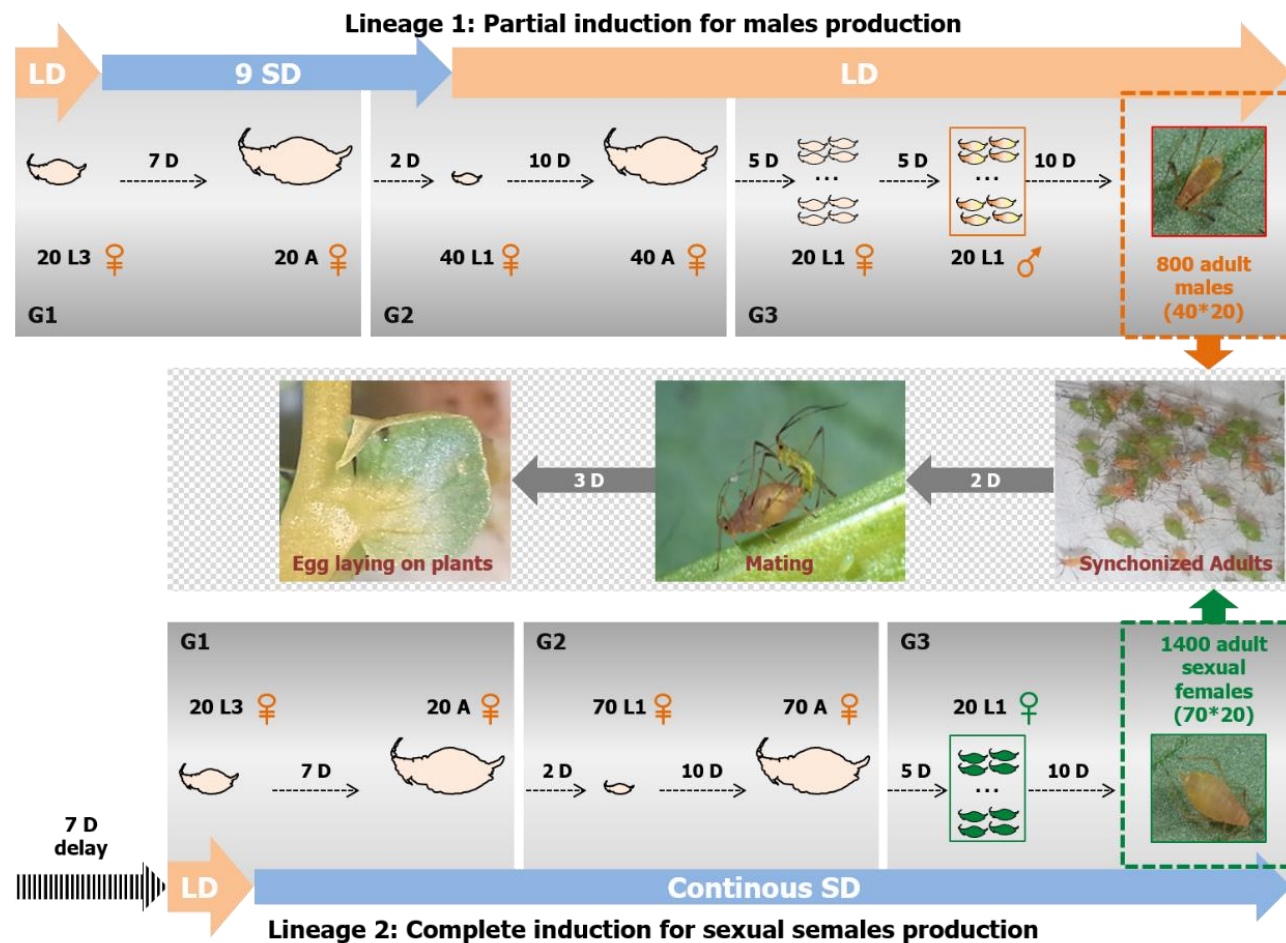


Figure 1 – Sexual morph induction protocol. Lineage 1 undergoes a partial induction protocol where 20 L3 parthenogenetic aphids, initially maintained under long days photoperiod conditions (LD: 16 h of light, 8 h of night), are moved to short days (SD: 12 h of light, 12 h of night) for exactly 9 days before being moved back to LD conditions. Once the adults reproduce, the first 2 L1 offspring per adult are kept until they reach adulthood. Each of these 40 so-called “sexuparae” then produces a first batch of approximately 20 parthenogenetic females which are discarded, followed by a second batch of around 20 males that are kept for a total of 800 males. Seven days after lineage 1 induction has started, lineage 2 undergoes a complete induction protocol where 20 L3 parthenogenetic aphids under LD are moved to constant SD. These are kept until they are adults, and the first 3-4 L1 produced by each adult are then kept until they reach adulthood. Each of these 70 adult sexuparae produces a first batch of 20 sexual females that are kept for a total of 1400 females. These two delayed protocols allow a synchronized production of males and sexual females that all become adults at the same time, after which they are placed together (15 males for 30 females per plant) for mating. Around 5 days later, the first eggs are laid by females and the microinjection procedure can start. Abbreviations: LX: larval stage X, A: adult, GX: Generation X, D: Day.

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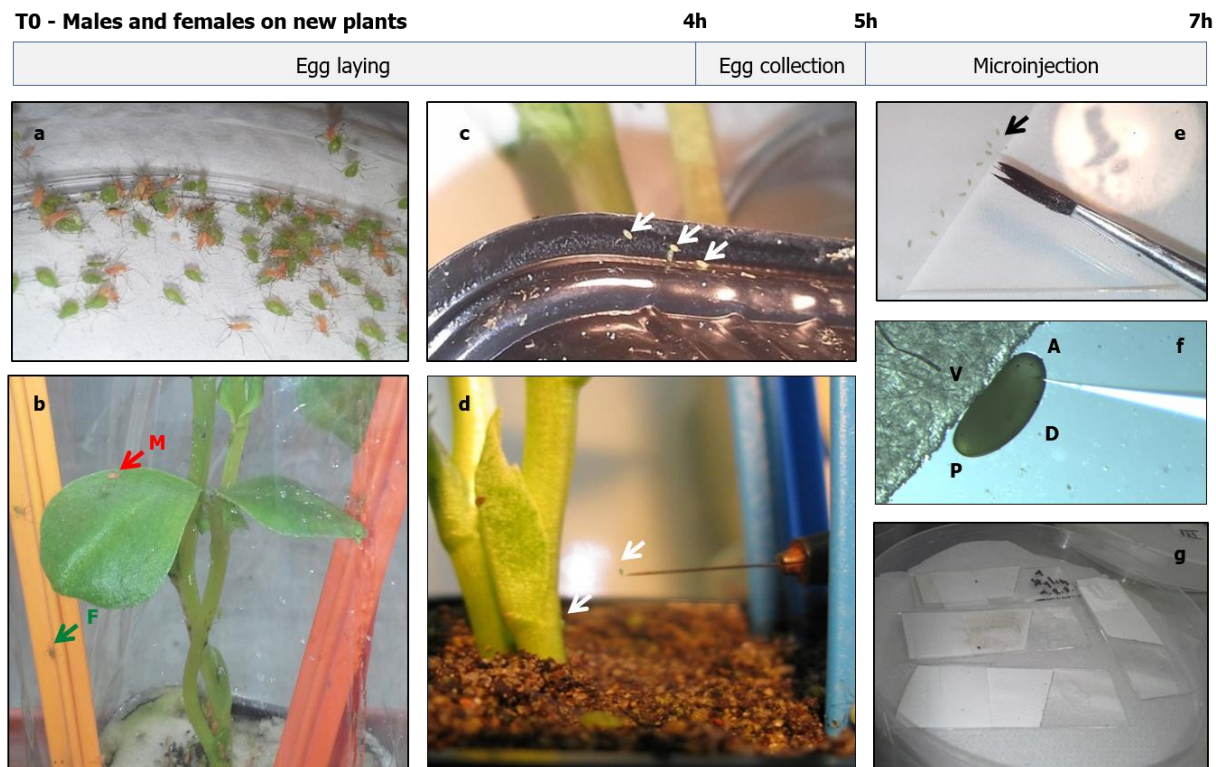


Figure 2 – Egg collection and injection procedure. On each day, during the week of injections, males and sexual females - initially put together for mating a few days before - are placed on a new plant at exactly 9.00 am (a and b). On each plant, 30 sexual females and 15 males are placed together during 4 hours so that females can lay eggs on the plant, the plastic bag, the pot or the tutor (c and d). Precisely four hours later, eggs are collected with a fine paintbrush and aligned (e) on wet filter paper deposited a microscope slide (20 per slide). The ventral face (V) of the egg is placed in contact with the paper and the injection is performed on the dorsal face (D) at the anterior pole (A), which is opposite of the posterior pole (P) defined as the pole where the bacteriocyte is observable (f). Once injection is completed slides are placed into petri dishes containing wet filter paper in climatic chambers at 15°C (g).

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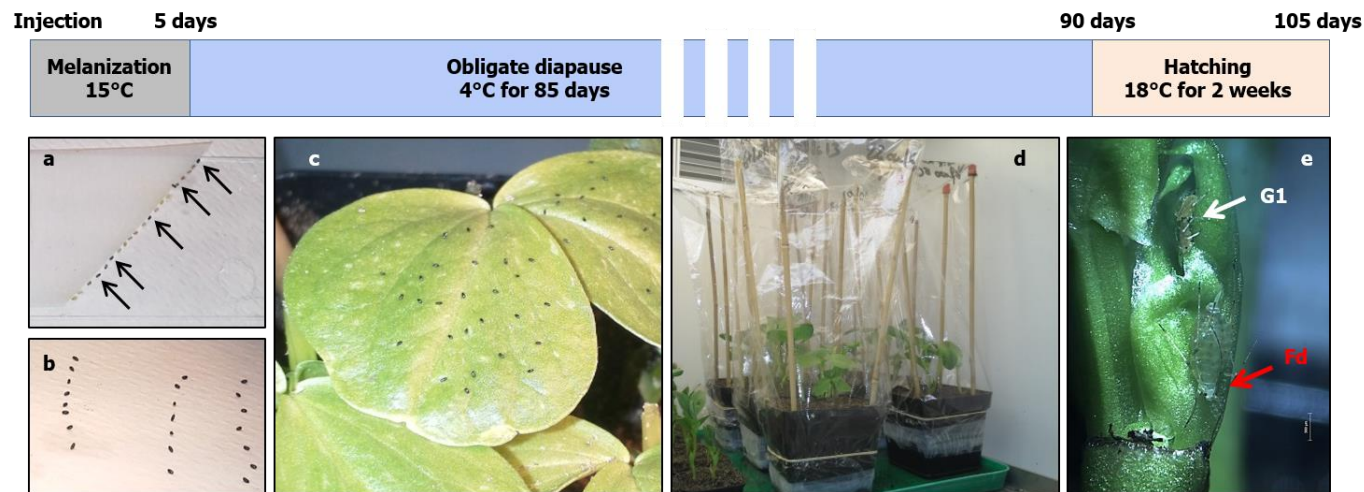


Figure 3 – Diapause and hatching steps. Eggs that were not damaged by the injection usually melanized within 5 days (a). Melanized eggs are then collected directly from the microscope slide and placed on the upper surface of the leaves of a new plant (b and c). Plants are then covered with a plastic bag and placed in a climatic room for 85 days at 4°C for the obligate diapause period (d). Once diapause is completed plants are moved back to 18°C to allow egg hatching. The individual that hatches is called the “Foundress” (Fd) and each of those is placed individually on a new plant until it becomes an adult and start producing an offspring (G1) by parthenogenesis (e).

```
>stylin-01 (ACYPI009006)
ATGCAGgtaagagtctctcgaagtttcgagaatggattgttattcgtttataccggcgaggtaaccggtctcacagtttcacgcttttgacagGTCACCTTTGCCGTATCGTCGTTGCT
GTTAGCTGTCGTCGCCGTCAGCGCGTACCCCGCATCACTGAACCCGGAAATCCAGAGCCGCATCTTGGTCCAAGATTCAGCACCCAAACGCCGATGGATCATTCAAGAACAAGtatgcaca
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CCCAGAAGGACCCGTAGCTGTGTTCCAGGGAGCTTCTGCGTACGTCGCCCCAGACGGCCAGACCAATCCAATCGGATACATCGCCGACGAGAACGGTTACCAGCCGTACGGCGCTCATT
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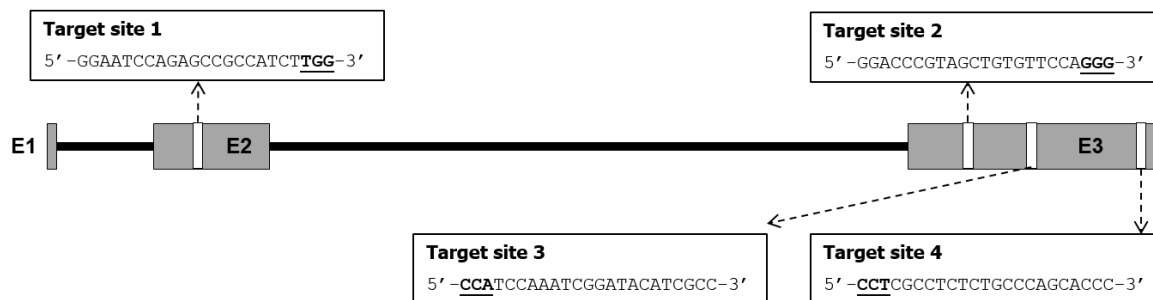


Figure 4 – *Stylin-01* gene model and guide RNAs target sites. *Stylin-01* gene comprises 3 exons (E1, E2 and E3) and 2 introns for a genomic DNA length of 1.35 kb. CRISPOR software was used to identify putative target sites (in grey) comprising NGG motifs (underlined) at the 3' end to allow potential DNA cleavage by sgRNA-Cas9 complex. One target site was predicted in exon 2, and 3 target sites in exon 3.

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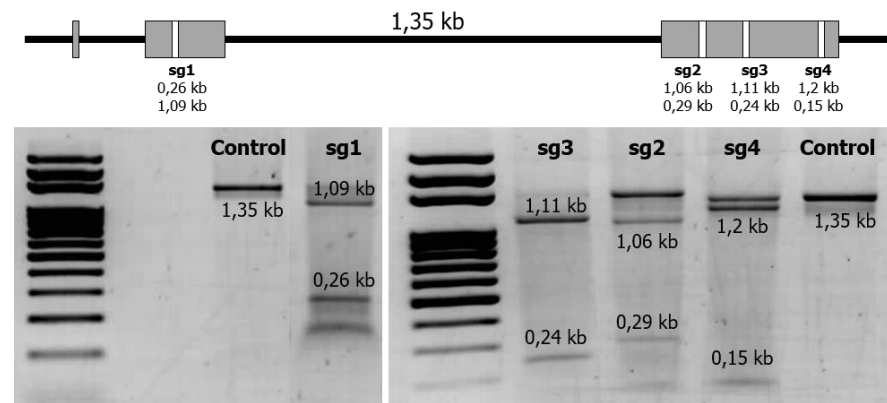


Figure 5 – sgRNAs *in vitro* cleavage assay. In order to test the ability of the 4 synthesized sgRNAs to efficiently cleave their predicted target sequence *in vitro* a PCR product from *stylin-01* genomic DNA (1.35 kb) was mixed with each sgRNA individually and Cas9 protein in its buffer. If the sgRNA actually targets its predicted sequence and slices the DNA fragment, two cleavage fragments will be observed on the agarose gel. The size of expected cleaved fragments are indicated on top of the gel for each sgRNA. The size of the bands that can be observed at the end of the reaction either for the control or for the 4 tested sgRNA are indicated on each lane of the gel.

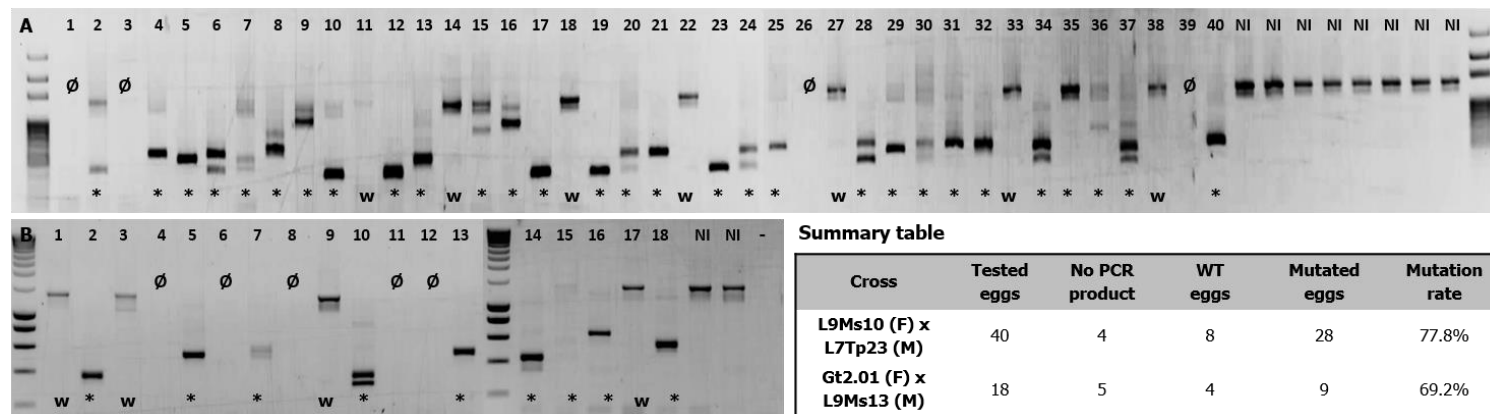


Figure 6 – *In ovo* mutation rates estimation. For two different crosses (A: L9Ms10 x L7Tp23 and B: Gt2.01 x L9Ms13) eggs were injected with a sg1-sg2-sg3-sg4 and Cas9 protein mix. Respectively 40 and 18 melanized eggs from these two experiments were then collected for DNA extraction and for subsequent *stylin-01* genomic DNA region amplification. PCR products were loaded on a gel in order to identify deletion events between the different sgRNAs target sites, revealed by the presence of amplicons having a different size than the wild-type expected size. Eggs are classified into three different classes: wild-type eggs (w), mutated eggs showing deletion events (*) and eggs for which PCR did not work (Ø). In the summary table the ratio [mutated eggs / PCR positive eggs] gives an estimation of the *in ovo* mutation rate.

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A. Stable lineages originated from eggs injected with sg1, sg2, sg3 and sg4 guide RNAs

sg1 target site

WT	CGCATCACTGAACCCGGAATCCAGAGCCGCCATCTGGTCCAAGATT	
L2-Allele1	CGCATCACTGAACCCGGAATCCAGAGCCGCCA-----AGATT	Δ10
L2-Allele2	CGCATCACTGAACCCGGAATCCAGAGCCGCCAT-----	Δ380
L4-Allele1	CGCATCACTGAACCCGGAATCCAGAGC-----CTGGTCCAAGATT	Δ6
L7-Allele1	CGCATCACTGAACCCGGAATCCAGAGCCGCCATCTGGTCCAAGATT	∅
L15-Allele1	CGCATCACTGAACCCGGAATCCAGAGCCG--TCTGGTCCAAGATT	Δ3

sg3 target site

WT	GCCCCAGACGGCCAGACCA <u>TCCAAATCGGATACATCGCCGACGA</u>	
L2-Allele1	GCCCCAGACGGCCAGACCA <u>TCCAA</u> gacAATCGGATACATCGCCGACGA	+3
L2-Allele2	GCCCCAGACGGCCAGACCA <u>TCCAAA</u> aaTCGGATACATCGCCGACGA	+2
L4-Allele1	GCCCCAGACGGCCAGACCC-----ATCGGATACATCGCCGACGA	Δ6
L7-Allele1	GCCCCAGACGGCCAGACCA <u>TCCAAATCGGATACATCGCCGACGA</u>	∅
L15-Allele1	GCCCCAGACGGCCAGACCA <u>TCCAAATCGGATACATCGCCGACGA</u>	∅

sg2 target site

WT	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGTGTTCAGGG</u> AGCTTCTGC	
L2-Allele1	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGT</u> ---CA <u>GG</u> AGCTTCTGC	Δ3
L2-Allele2	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGT</u> agcccgtagcTCCAGGAGCTTCTGC	+10, Δ2
L4-Allele1	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGTGT</u> tgggagTCCAGGAGCTTCTGC	+6
L7-Allele1	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGTGT</u> agCCA <u>GG</u> AGCTTCTGC	+2, Δ1
L15-Allele1	GAAGGCTGGCCAGAA <u>GGACCCGTAGCTGTG</u> --CA <u>GG</u> AGCTTCTGC	Δ2

sg4 target site

WT	AAGAGTCGCTCAGATA <u>CCTCGCCTCTCTGCCAGCACCCCGAACC</u> AA	
L2-Allele1	AAGAGTCGCTCAGATA <u>CCTCGC</u> Cgaaggttaggtat-----AACCAA	+13, Δ6
L2-Allele2	AAGAGTCGCTCAGATA <u>CCTC</u> -----TGCCAGCACCCCGAACCAA	Δ7
L4-Allele1	AAGAGTCGCTCAGATA <u>C</u> -----CTCTGCCAGCACCCCGAACCAA	Δ7
L7-Allele1	AAGAGTCGCTCAGATA----- <u>CCTCTCTGCCAGCACCCCGAACC</u> AA	Δ5
15-Allele1	AAGAGTCGCTCAGATA <u>CCTCGC</u> ---CTGCCAGCACCCCGAACCAA	Δ4

B. Stable lineages originated from eggs injected with sg4 guide RNA

sg4 target site

WT	AAGAGTCGCTCAGATA <u>CCTCGCCTCTCTGCCAGCACCCCGAACC</u> AA	
L14-Allele1	AAGAGTCGCTCAGATA <u>CCTCGCCTCT</u> --aacGCCAGCACCCCGAACCAA	+3, Δ2
L14-Allele2	AAGAGTCGCTCAGATA <u>CCTCG</u> -----CTGCCAGCACCCCGAACCAA	Δ6
L16-Allele1	AAGAGTCGCTCAGATA <u>C</u> -----CTCTGCCAGCACCCCGAACCAA	Δ7

Figure 7 – Sequences of the mutations detected in lineages edited with CRISPR-Cas9. Both *stylin-01* alleles from all the lineages originated from eggs injected with a combination of sg1, sg2, sg3 and sg4 (A) or only sg4 (B) were sequenced. For the first sgRNAs combination (A), four lineages (L2, L4, L7 and L15) were stably mutated whereas for the second sgRNAs combination (B) two lineages were stably mutated. For each sgRNA the target site change relative to the wild type sequence for each allele is indicated: insertion (+) or deletion (Δ). The 3 nucleotides corresponding to the PAM motif are underlined.

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Supplementary Tables and Figures

Table S1 – List of the primers used in this study

Oligonucleotide	Sequence
Stylin1-pF-sgRNA1	GAAATTAATACGACTCACTATAGGAATCCAGAGCCGCCATCTGTTTTAGAGCTAGAAATAGCAAG
Stylin1-pF-sgRNA2	GAAATTAATACGACTCACTATAGGACCCGTAGCTGTGTTCCAGTTTTAGAGCTAGAAATAGCAAG
Stylin1-pF-sgRNA3	GAAATTAATACGACTCACTATAGGCGATGTATCCGATTTGGAGTTTTAGAGCTAGAAATAGCAAG
Stylin1-pF-sgRNA4	GAAATTAATACGACTCACTATAGGGTGCTGGGCAGAGAGGCGGTTTTAGAGCTAGAAATAGCAAG
pR-sgRNA-univDR274	AAAAGCACCGACTCGGTGCCACT
Sty11-BC29F	ATATGAATTC-TTACTGGTATTTTGGTTC
Sty11-BC24R	ATATGAATTC-TTACTGGTATTTTGGTTC
Sty11-BC57F	AGACGCATCCACAATCACAA
Sty11-BC57R	CGGATGATCATTTTCGAGGAG
M13-Fwd	GTA AACGACGGCCAGT
M13-Rev	AACAGCTATGACCATG

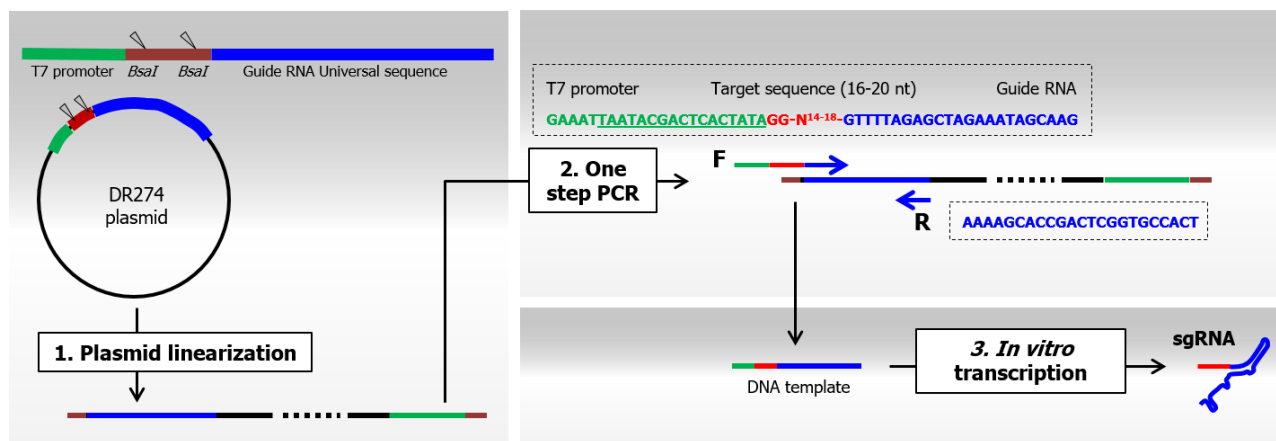


Figure S1 – *In vitro* single guide RNA synthesis. DR274 plasmid contains the guide RNA universal sequence necessary for Cas9 complexing. **1:** this plasmid is first linearized with *BsaI* restriction enzyme and then used as a template for PCR amplification with a reverse primer corresponding to the end of the universal guide RNA sequence and one long forward primer. **2:** this specific primer comprises the T7 promoter sequence followed by the target sequence of the candidate gene and the beginning of the universal guide RNA sequence. PCR amplification with low annealing temperature then allows the production of a DNA template. **3:** This template is used for T7 *in vitro* transcription of the single guide RNA.

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