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1 **An RNAi suppressor activates *in planta* virus-mediated gene editing**

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17 **Running title** RNAi suppressor enhances VmGE

18 **Keywords** CRISPR/Cas9, FoMV, VmGE, RNAi suppressor

19 **Abstract** RNA-guided CRISPR/Cas9 technology has been developed for gene/genome  
20 editing (GE) in organisms across kingdoms. However, *in planta* delivery of the two core  
21 GE components, Cas9 and small guide RNA (sgRNA), often involves time-consuming  
22 and labor-intensive production of transgenic plants. Here we show that *Foxtail mosaic*  
23 *virus*, a monocot- and dicot-infecting potyvirus, can simultaneously express Cas9,  
24 sgRNA and an RNAi suppressor to efficiently induce GE in *Nicotiana benthamiana*  
25 through a transgenic plant-free manner.

## 26 **Introduction**

27 The CRISPR/Cas9 system, an adaptive immune defense against extrachromosomal  
28 DNA and viruses in prokaryotes, creates double-stranded breaks (DSBs) in targeted  
29 regions through a coordinated activity of sgRNA and Cas9 nuclease (Jinek et al. 2012).  
30 DSBs can be repaired by homologous recombination or non-homologous end-joining,  
31 which leads to nucleotide substitutions and/or indels (Jinek et al. 2012). This mechanism  
32 has been exploited for specific and multiplex gene and genome editing (GE) in  
33 eukaryotes (Jinek et al. 2012; Cong et al. 2013; Feng et al. 2014; Yu et al., 2018).

34 In plants, one of the critical challenges to induce GE is the delivery of Cas9 and  
35 sgRNA into cells. Indeed, gene editing is often created in transgenic plants that are  
36 usually transformed with T-DNA based binary vectors expressing Cas9 and specific  
37 sgRNAs either by *Agrobacterium*-mediated transformation or biolistic bombardment  
38 (Mao et al., 2018; Yu et al., 2018; <https://www.nature.com/subjects/transgenic-plants>).  
39 The Cas9 and sgRNA expression cassettes (i.e. transgenes) are integrated into the  
40 genome in transgenic plants. Subsequent segregations of these transgenes need to be  
41 separated from the edited target gene. Apart from its extreme time-consumption and  
42 labor-intensity, this process can be much more complicated and difficult for  
43 out-breeding species in which a specific elite genotype (phenotype) requires numerous  
44 back-crosses to re-constitute. In addition, plant transformation cannot be readily and  
45 easily achieved in all plant species, which is in particular challenging in some  
46 transformation-recalcitrant species. Furthermore, even if an inheritable gene-edited  
47 plant is obtained after the tedious transformation process and lengthy genetic selection,  
48 the resulting line could be still regarded to be transgenic, which may not be acceptable

49 in many countries. Thus development of a non-transgenic strategy is needed to  
50 maximize the potential of GE technology in plant functional genomics and crop  
51 improvement (Feng et al. 2014; Mao et al., 2018; Yu et al., 2018).

52 Transgenic plant-free transient toolboxes such as virus induced gene silencing  
53 (VIGS; Lin et al., 2008; Chen et al., 2015), gene complementation (VIGC; Zhou et al.,  
54 2012) and flowering (VIF; Li et al., 2011; Qin et al., 2017) have been widely used in  
55 plants. To date, a *bona fide* non-transgenic virus-mediated GE (VmGE) platform has not  
56 been established to induce inheritable gene modifications although several RNA/DNA  
57 viruses were utilized to express sgRNA for GE in *Cas9*-transgenic plants (Ali et al.,  
58 2015; Yin et al., 2015; Cody et al., 2017). For instance, *Tobacco rattle virus*, a  
59 bi-genome component single-stranded (ss) RNA virus, was used to deliver sgRNA  
60 specifically targeting the *phytoene desaturase* (*PDS*) gene in *Cas9*-overexpressing  
61 transgenic *Nicotiana benthamiana* (*Nb*) lines resulted in successful *PDS* gene editing  
62 (Ali et al., 2015). Targeted gene editing was also achieved by using a modified ssDNA  
63 geminivirus *Cabbage Leaf Curl virus* to express *PDS*- or *isopentenyl/dimethylallyl*  
64 *diphosphate synthase* gene-specific sgRNAs in stable transgenic *Nb* expressing *Cas9*  
65 (Yin et al., 2015). Moreover, *Tobacco mosaic virus*, a single-genome component ssRNA  
66 virus has been engineered to create multiplexed gene editing (Cody et al., 2017).  
67 However, these RNA/DNA virus-based GEs all occurred in *Cas9*-transgenic plants (Ali  
68 et al., 2015; Yin et al., 2015; Cody et al., 2017), thus they are not transgenic plant-free  
69 GE technology. Here we report non-transgenic VmGE using *Foxtail mosaic virus*  
70 (FoMV), a positive ssRNA potexvirus, to express *Cas9*, sgRNA and the RNAi  
71 suppressor p19 in order to edit *PDS* gene in transgenic-free *N. benthamiana*.

## 72 **Materials and methods**

### 73 Construction of FoMV-based expression vectors

74 The coding sequences for 3xFLAG and nuclear localization signal (NLS)-tagged *Cas9*  
75 (designated *FLAG:NLS:Cas9:NLS*) was amplified using a high-fidelity KOD-Plus-Neo  
76 DNA polymerase (Toyobo), a plasmid carrying *FLAG:NLS:Cas9:NLS* (Yu et al. 2018)  
77 as template and a set of primers *Cas9-3X-NLS-Hpa-MLU-F* and  
78 *Cas9-3X-NLS-XhoI-ASC-R* (Supplementary Table 1). The resultant PCR product of  
79 approximately 4.2 Kb in length was then treated with *HpaI* and *AscI*, and cloned into

80 the *HpaI/AscI* sites of the binary vector pCambia2300-FoMV (Liu et al., 2016) to  
81 generate FoMV/Cas9 (Fig. 1a). To produce FoMV/sgRNA<sub>anon</sub> and FoMV/sgRNA<sub>pds</sub>  
82 (Fig. 1a), the corresponding DNA fragments were amplified using the high-fidelity  
83 KOD-Plus-Neo DNA polymerase, pT-U6p-scaffold-U6t (Yin et al., 2015) or  
84 pCVA-gRNA::NbPDS plasmid DNA (Yin et al., 2015) as template together with  
85 primers AUT-Hpa-F3 and U6T-ASC-R2 (Supplementary Table 1), digested with  
86 *HpaI/AscI* and then cloned into the *HpaI/AscI* sites of the binary vector  
87 pCambia2300-FoMV (Liu et al., 2016). To generate FoMV/P19:sgRNA<sub>pds</sub> (Fig. 1a),  
88 the p19 gene was amplified using the high-fidelity KOD-Plus-Neo DNA polymerase,  
89 pEAQ-HT plasmid carrying the p19 coding sequence (Sainsbury et al., 2008) as  
90 template and a set of primers P19-ORF-F and P19-ORF-R (Supplementary Table 1),  
91 and cloned into the *HpaI* site of FoMV/sgRNA<sub>pds</sub>. Similarly, the eGFP gene was  
92 amplified using primers eGFP-ORF-F and eGFP-ORF-R (Supplementary Table 1),  
93 plasmid pEGFP (Clontech) as template, and cloned into the *HpaI* site of  
94 pCambia2300-FoMV to produce FoMV/eGFP (Supplementary Fig. 1a). Insertion of  
95 *FLAG:NLS:Cas9:NLS*, sgRNA<sub>anon</sub>, sgRNA<sub>pds</sub> or P19:sgRNA<sub>pds</sub> in these FoMV  
96 vectors was verified by PCR using a pair of primers Fomv seq\_6830\_5K-F and Fomv  
97 Seq\_7260\_SUBP-R (Supplementary Table 1), and further confirmed by Sanger  
98 sequencing. All FoMV constructs and the binary vector pEAQ-HT (Sainsbury et al.,  
99 2008) were transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation  
100 (Chen et al., 2018) respectively, confirmed by sequencing plasmid miniprep from  
101 *Agrobacterium* culture prior to their use in subsequent agroinfiltration experiments and  
102 plant transformation.

### 103 Plant transformation

104 A number of primary p19-transgenic lines were generated by leaf disc transformation  
105 of *Nicotiana benthamiana* (*Nb*) with *A. tumefaciens* LBA4404 harboring pEAQ-HT as  
106 described (Hong et al., 1996). Transformation was verified by PCR amplification of  
107 integrated *p19* transgene using specific primers P19-ORF-F and P19-ORF-R  
108 (Supplementary Table 1). Following self-fertilization, T1 and T2 progenies were

109 tested for antibiotic sensitivity by germinating seeds on 0.5 mg/ml kanamycin. Five  
110 independent single-copy homozygous *Nb* lines transformed with the *p19* transgene  
111 were obtained as evidenced by the Mendelian 3:1 segregation ratio between  
112 kanamycin-resistant and sensitive plantlets. Like WT *Nb*, all *p19*-transgenic plants  
113 properly grew and developed.

114 Virus infection (VmGE), plant growth and maintenance

115 To prepare FoMV and recombinant FoMV inoculum, *A. tumefaciens* LBA4404  
116 harboring different FoMV constructs (**FIG. 1a** and **Supplementary Fig, 1a**) was  
117 cultured to reach a density of 1.0 OD<sub>600</sub> at 28 °C overnight in LB medium containing  
118 0.5 mg/ml streptomycin and 0.5 mg/ml kanamycin, then collected by centrifugation at  
119 3,000 rpm for 10 min, and resuspended in sterile water to give a final density of 0.5  
120 OD<sub>600</sub>. For leaf-agroinfiltration, *Agrobacterium* was infiltrated into young leaves of WT  
121 or transgenic *Nb* plants at six-leaf stage through needleless 0.5-ml syringe.

122 Alternatively, germinating seeds were agroinfiltrated in order to shorten virus infection  
123 time. Briefly, dozens of *Nb* seeds were spread onto 3MM Whatman filter paper  
124 pre-soaked in sterile water and placed within a petri dish (10 cm in diameter). The petri  
125 dish was kept in a growth chamber at 25 °C with constant light. After 2 days under such  
126 conditions, seeds began to break their coat and germinate. At this stage, seeds were  
127 collected and mixed with 5 ml 0.5 OD<sub>600</sub> *Agrobacterium* in a 50mL-Falcon™  
128 centrifuge tube. Agroinfiltration of seeds was achieved using a vacuum pump under the  
129 pressure of 0.085 MPa for 10 min. Agroinfiltrated seeds were then transferred to  
130 composts and kept under dark for 24 h. Plants were then grown under 16 h light/8 h  
131 dark conditions at 25 °C in insect-free growth rooms, regularly examined for  
132 development of local and systemic infection, and photographically recorded using a  
133 D7000Sony NEX-5R camera. It should be noted that (1) for leaf-agroinfiltration, 6  
134 plants were infiltrated with each FoMV construct or each combination of FoMV  
135 constructs in a separate experiment, and such leaf-agroinfiltration experiment was  
136 repeated 2-4 times; and (2) for seed-agroinfiltration, 50-100 seeds were used for each  
137 combination of FoMV constructs in two separate experiments.

138 Confocal microscopy

139 To examine eGFP expression from FoMV/eGFP, *Nb* leaves were collected at 4 days  
140 post leaf-agroinfiltration and examined by a Nikon A1 confocal microscope under  
141 488-nm excitation to excite GFP and monitor emission (510 nm) of green fluorescence  
142 (Hong et al., 2003). *Nb* epidermis was also photographed under bright field following  
143 the manufacturer's instructions. Confocal images were processed using the Nikon A1  
144 Nis-Elements software.

145 RNA extraction and RT-PCR

146 RNA was extracted from systemic young *Nb* leaf tissues using RNAPrep Pure Plant Kit  
147 (TIANGEN). First-strand cDNA was synthesized from DNase I-treated RNAs (2 µg)  
148 by M-MLV Reverse Transcriptase using the FastQuant RT Kit (TIANGEN) according  
149 to the manufacturer's instructions. PCR was performed to detect FoMV genomic RNA,  
150 virally expressed Cas9 or p19 mRNAs using cDNA as template together with primers  
151 specific to each of the targets (**Supplementary Table 1**) and analyzed via  
152 1.2%-Agarose gel electrophoresis.

153 Western blot

154 Total proteins were extracted from systemic young *Nb* leaf tissues as described (Hong  
155 et al., 1996). Protein aliquots (20 µg) were separated on 10% SDS-PAGE gel after  
156 electrophoresis under 100 V for 2 hr and transferred to a nitrocellulose membrane  
157 (Bio-Rad). Western blot analyses were performed with 1:2000 mouse anti-FLAG  
158 (Sigma-Aldrich) antibody, detected by 1:5000 goat anti-mouse IgG horseradish  
159 peroxidase-conjugated secondary antibody (Abcam) and the SuperSignal West Femto  
160 Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescent signals  
161 were detected with a ChemiDoc XRS+ imaging System (Bio-Rad) following the  
162 manufacturer's instructions.

163 Genomic DNA extraction and molecular characterization of ViGE

164 DNA was isolated from systemic young *Nb* leaf tissues using DNeasy Plant Mini Kit

165 (Qiagen) following the manufacturer's instructions. Genomic PCR amplification of the  
166 sgRNA target PDS gene (407 bp) was performed using the high-fidelity  
167 KOD-Plus-Neo DNA polymerase, 10-100 ng DNA as template and primers PDS\_MLY  
168 ID-F3 and PDS\_Mly\_ID-R (**Supplementary Table 1**). Subsequently, two approaches  
169 were used to characterize ViGE. In Approach I, genomic PCR products (approx. 400 ng)  
170 were treated with *MlyI* (NEB) at 37 °C for 6 hr and analyzed by 1.5% agarose gel  
171 electrophoresis. Any undigested PCR fragments were purified from gel and cloned into  
172 the pEASY-Blunt3 Cloning Vector (TransGen Biotech) for Sanger sequencing. In  
173 Approach II, genomic PCR products were directly cloned into the pEASY-Blunt3  
174 Cloning Vector. After high-fidelity colony PCR/*MlyI* digestion screening, plasmid  
175 DNA was miniprep'd for sequencing. It should be noted that the high-fidelity  
176 KOD-Plus-Neo DNA polymerase and detections of deletions as well as various  
177 nucleotide substitutions including A→T and T→A ensure that these mutations we  
178 identified were not the result of PCR errors, but were generated from *in planta* VmGE.

## 179 **Results and Discussion**

180 To test VmGE, we exploited the FoMV vector originally designed for VIGS ([Liu et al.,](#)  
181 [2016](#)), but recently adapted for VIF ([Yuan et al., 2019](#)) and transient gene expression  
182 (**Supplementary Fig. 1**). We cloned the coding sequences for 3xFLAG and nuclear  
183 localization signal (NLS)-tagged Cas9, *PDS*-targeting sgRNAs ([Yin et al., 2015](#)) or a  
184 sgRNA lacking any targeting sequence (sgRNA<sub>anon</sub>), into FoMV and generated  
185 FoMV/Cas9, FoMV/s<sub>gRNA</sub>Pds and FoMV/s<sub>gRNA</sub>Anon, respectively (**Fig. 1a**). Through  
186 leaf co-agroinfiltration, *Nb* plants infected with FoMV/Cas9+FoMV/s<sub>gRNA</sub>Anon or  
187 FoMV/Cas9+FoMV/s<sub>gRNA</sub>Pds developed no symptoms (**Supplementary Fig. 2a-d**),  
188 consistent with latent FoMV infection where viral RNA was detectable by RT-PCR  
189 (**Supplementary Fig. 3a-c**). We then extracted genomic DNA from systemic leaf tissue  
190 and amplified the s<sub>gRNA</sub>Pds target *PDS* gene. Complete *MlyI*-digestion of the resultant  
191 PCR products and subsequent sequencing analyses suggest no occurrence of VmGE  
192 (**Supplementary Fig. 2e-h**). We suspected that this initial failure of VmGE might be

193 due to low efficacy of FoMV infection and insufficient viral expression of Cas9 and  
194 sgRNAs in plants.

195 To enhance FoMV infectivity for increasing the level of Cas9 and sgRNAs, we  
196 co-expressed *Tomato bushy stunt virus* p19, a mutated RNAi suppressor with strong  
197 RNAi suppression activity but deprived of pathogenesis function (Sainsbury et al., 2009)  
198 in plants. We generated single copy homozygous *Nb* lines transformed with the  
199 *p19*-expressing cassette (Supplementary Fig. 4a). Transgenic plants infected with  
200 FoMV/sgRNAs and FoMV/Cas9 developed mosaic, chlorosis and leaf curling  
201 (Supplementary Fig. 4b-e), and viral expression of *Cas9* mRNA was readily detectable  
202 (Fig. 1b). Genomic PCR-*MlyI* screening of 24 infected *p19*-transgenics in 4 separate  
203 experiments showed that the sgRNAs target was only partially cleaved by *MlyI*,  
204 suggesting occurrence of *in planta* systemic VmGE (Supplementary Fig. 4f). Further  
205 cloning and sequencing *MlyI*-resistant PCR products identified 104 mutations including  
206 nucleotide deletions and substitutions in the targeted region (Fig. 1c-e). These  
207 discoveries are supported by a finding that GE is increased in *Arabidopsis* defective in  
208 RNAi pathway (Mao et al. 2018). We then generated a new vector  
209 FoMV/P19:sgRNAs to express both p19 and sgRNAs (Fig. 1a). Upon  
210 agroinfiltration of germinating *Nb* seeds with FoMV/Cas9 and FoMV/P19:sgRNAs,  
211 plants grew and developed evident systemic viral symptoms (Fig. 2a-d). Viral delivery  
212 of *p19* markedly enhanced levels of Cas9 protein in young leaf tissues (Fig. 2e,f) and  
213 led to efficient systemic VmGE in the *PDS* target (Fig. 2g,h and Supplementary Fig.  
214 5).

215 Taken together, our data reveal that a concurrent delivery of Cas9, sgRNA and  
216 RNAi suppressor p19 from the RNA virus FoMV can lead to VmGE in plants. The gene  
217 edits reported here were found in primary generation of plants, i.e. in *Nb* plants where  
218 Cas9, sgRNAs and p19 were expressed from FoMV/Cas9 along with  
219 FoMV/p19:sgRNAs. Unfortunately, we do not have any data to show if the edit  
220 would be heritable or not at the moment. However, we would like to mention that there  
221 are three technical challenges for development of a transgenic plant-free VmGE  
222 platform. (1) It is notoriously difficult to use plant virus vectors to express large proteins

223 although many plant viruses have been engineered to express small non-viral proteins  
224 such as GFP in plants (Kaya et al., 2017). (2) A successful gene editing event requires  
225 simultaneous expression of both Cas9 and specific sgRNA in same cells from virus  
226 vectors which are not integrated into the plant genome. (3) Transgenic plant-free VmGE  
227 needs to occur in reproductive cells, leading to the edit heritable to next generation. We  
228 now resolved the first two technical challenges and provided proof-of-concept of  
229 VmGE, evidenced by the efficient expression of Cas9, a protein of more than 160 KDa  
230 and sgRNA together with a silencing suppressor p19 protein, and occurrence of GEs in  
231 wild-type *N. benthamiana* (i.e. non-transgenic plant).

232 FoMV, like *Potato virus X* (PVX), is a monopartite ssRNA virus in genus  
233 *Potexvirus* (Liu et al., 2016). During viral genomic RNA replication of FoMV/Cas9 or  
234 FoMV/p19:sgRNAs in systemic young leaf tissues, these recombinant viruses  
235 produce subgenomic (sg) RNA via the regulation of the duplicated coat protein sgRNA  
236 promoter, a similar strategy found in PVX infection (Hong et al., 1997; van Wezel et al.,  
237 2001; 2002; van Wezel & Hong, 2004; Li et al., 2009). The subgenomic RNA serves as  
238 mRNA for translation of the Cas9 and p19 proteins as well as used as sgRNAs. All  
239 these processes occur in virus-infected cells but do not involve integration of FoMV  
240 genomic or subgenomic RNA into host genome at any stage of the viral life cycle (Liu  
241 et al., 2016). Expression of a large protein such as Cas9 of more than 160kD in size  
242 from FoMV is also rare for any plant virus-based gene expression system (Kaya et al.,  
243 2017). This rapid and effective approach involves neither plant transformation nor  
244 transgenic expression of Cas9 or sgRNA, i.e. it is transgenic plant-free. Thus,  
245 FoMV-based VmGE would produce non GMO plants.

246 FoMV can infect plants of 56 Poaceae species and at least 35 dicot species (Liu et  
247 al., 2016). Considering the broad range of its host species (Liu et al., 2016), the  
248 FoMV-based VmGE should be applicable to various dicots and monocots including  
249 important cereal crops. Indeed, this technique is of particular use to edit genes for  
250 creating new traits and/or for functional genomics in crops in which genetic  
251 manipulation is difficult or no transformation system is yet available. On the other hand,  
252 target genes can be edited to modulate growth, flowering, yield, nutrition as well as  
253 biotic and abiotic stress response for instance. Thus, this technique will also provide a  
254 quick and efficient method for mutating genes so that function of the edited gene can be

255 delineated from phenotypic changes without introducing Cas9 and sgRNA expression  
256 cassette or any genes into the genome of the edited plants using genetic engineering  
257 processes. Dependent on the edited target genes, they can function and act in the control  
258 of plant growth and development, flowering, yield, nutrition as well as innate response  
259 to biotic and abiotic stresses.

## 260 **Acknowledgments**

261 We thank Yule Liu for providing the FoMV vector, George P. Lomonosoff for  
262 providing the pEAQ-HT vector and Feng Hu, Qianqian Zheng and Pengcheng Zhang  
263 for technical supports. This work was in part funded by the Ministry of Agriculture of  
264 China (National Transgenic Program 2016ZX08009001-004), the Ministry of Science &  
265 Technology of China (National Key R&D Program 2017YFE0110900), the National  
266 Natural Science Foundation of China (31872636, 31201490, 31200913, 31370180) and  
267 Hangzhou Normal University (Sino-EU Plant RNA Signaling S&T Platform Initiative  
268 9995C5021841101).

## 269 **Completing interests**

270 A international patent based on results in this manuscript has been filed and a leading  
271 international biotech company has taken the license of this technology for research and  
272 commercial applications.

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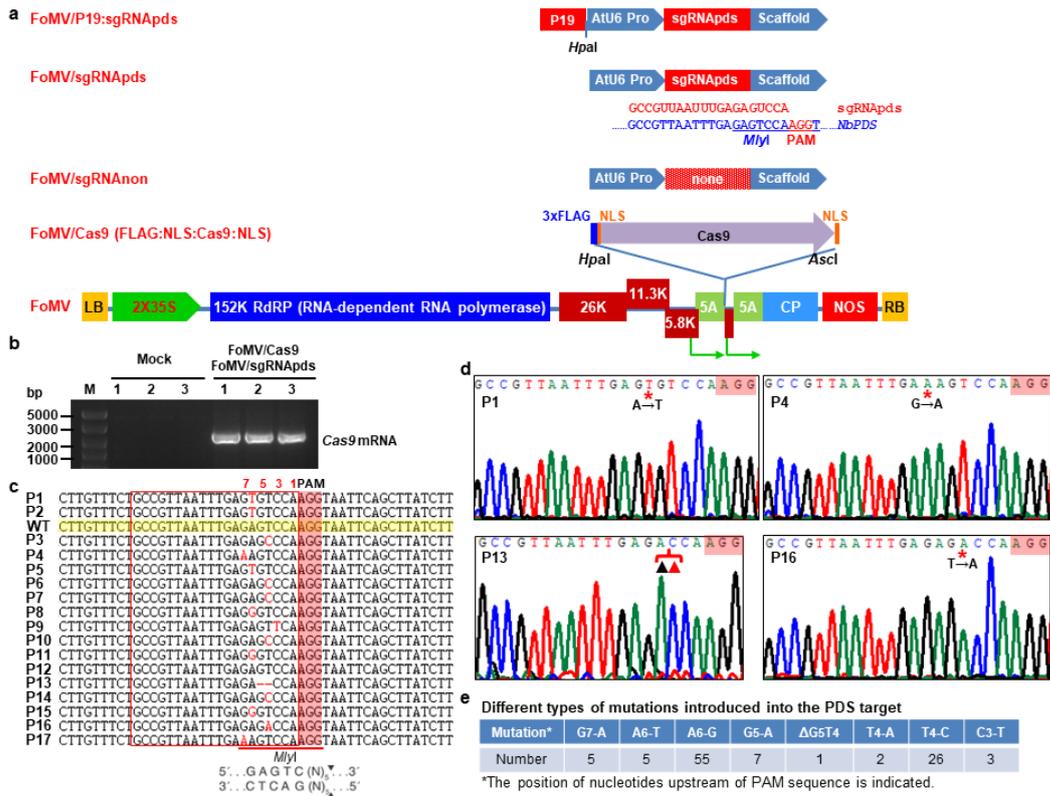
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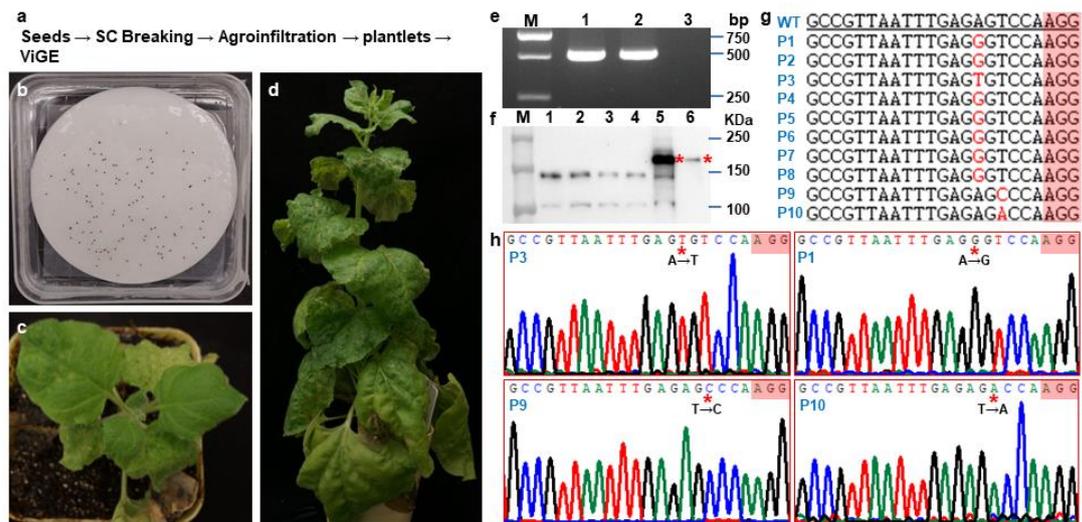
352 **Figures**



353

354 **Figure 1** Viral delivery of Cas9 and sgRNApds induced PDS gene editing in *p19*-transgenic  
 355 plants. (a) FoMV-based Cas9, sgRNApds and p19 expression vectors. FoMV/Cas9,  
 356 FoMV/sgRNApds and FoMV/sgRNAnon are for expression of Cas9 (FLAG:NLS:Cas9:NLS)  
 357 tagged with 3xFLAG at the N-terminal and a NLS at both the N- and C-termini, sgRNA  
 358 targeting PDS gene, and the empty sgRNA scaffold. FoMV/P19:sgRNApds is expected to  
 359 co-express p19 RNAi suppressor and sgRNApds. The sgRNApds sequence, PDS target region  
 360 and PAM as well as the FoMV genome together with regulatory elements (left and right  
 361 borders LB and RB, cloning sites, double 35S promoter 2x35S and NOS terminator) are  
 362 indicated. Blue arrows represent the native and duplicated coat protein (CP) subgenomic RNA  
 363 promoters. (b) Detection of Cas9 mRNA in young leaf tissues of 3 *Nb* plants infected with  
 364 FoMV/Cas9+FoMV/P19:sgRNApds, but not in 3 mock-inoculated controls. Positions and sizes  
 365 of the DNA ladder (M) as well as the position of the detected Cas9 mRNA are indicated. (c,d)  
 366 Detection of FoMV-mediated systemic VmGE in young leaf tissues. Comparison of wild-type  
 367 (WT) and 17 edited PDS sequences (P1 to P17) reveals various point mutations and a 2-nt  
 368 deletion in the sgRNApds targeted region (boxed, c). Representative of Chromas diagrams

369 show the sgRNAs targeted sequences (P1, P4, P13 and P16) with VmGE-mediated  
 370 substitutions (asterisks) and deletions (2 triangles). (d). (e) Summary of various types and  
 371 occurrences of VmGE events in sgRNAs targeted PDS gene. PAMs are highlighted (c,d) and  
 372 positions of the nucleotides upstream of PAM are numbered (c,e).



373  
 374 **Figure 2** Effective VmGE through co-delivery of Cas9, sgRNAs and p19 from FoMV. (a-d)  
 375 VmGE induction. Experimental strategy is outlined (a). Broken seed-coat (SC) seeds were  
 376 infiltrated with mixed agrobacteria carrying FoMV/Cas9 and FoMV/P19:sgRNAs (Fig. 1a)  
 377 under vacuum-pressure (b). Systemic viral symptoms developed in *Nb* plants growing from  
 378 agroinfiltrated seeds (c,d). Seeds at 2 days after spread onto water-soaked filter paper (b) and  
 379 plants at 28 (c) and 60 (d) days post seed-agroinfiltration were photographed. (e) Analysis of  
 380 p19 mRNA. Expression of p19 was detected in virus-infected *Nbs* (lane 1 and 2), but not in  
 381 mock plant (lane 3). Positions and sizes of the DNA ladder (lane M) are indicated. (f) Delivery  
 382 of p19 from FoMV enhances viral co-expression of Cas9 protein in plants. Western blot  
 383 detection of the 160-KDa FLAG-tagged Cas9 (asterisk) in *Nb* young leaf tissues infected with  
 384 FoMV/Cas9+FoMV/P19:sgRNAs (lane 5), and in *p19*-transgenic young leaf tissues infected  
 385 with FoMV/Cas9+FoMV/sgRNAs (lane 6), but not in *Nb* young leaf tissues infected with  
 386 FoMV/Cas9+FoMV/sgRNAs (lanes 1 and 2) or mock controls (lanes 3 and 4). Positions and  
 387 sizes of the protein marker (lane M) are indicated. (g,h) FoMV-mediated VmGE in systemic

388 young leaf tissues. Comparison of wild-type (WT) and 10 edited PDS sequences (P1 to P10) is  
389 shown (**g**). Representative of Chromas diagrams (P1, P3, P9 and P10) show nucleotide  
390 substitutions (asterisks) in the sgRNAs target sequences (**h**). PAMs are highlighted red (**g,h**).