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

Xian Zhang[1,¶], Lihua Kang[1,¶], Qi Zhang[1,¶], Qiqi Meng[1], Yafei Pan[1], Zhiming Yu[1], Nongnong Shi[1], Stephen Jackson[2], Xinlian Zhang[3], Huizhong Wang[1], Mahmut Tör[4], Yiguo Hong[1,2,4*]

[1]Research Centre for Plant RNA Signaling and Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China.

[2]Warwick-Hangzhou RNA Signaling Joint Laboratory, School of Life Sciences, University of Warwick, Warwick CV4 7AL, UK.

[3]Department of Family Medicine and Public Health, Division of Biostatistics & Bioinformatics, University of California San Diego, La Jolla, CA 92093, USA.

[4]Worcester-Hangzhou Joint Molecular Plant Health Laboratory, Department of Biology, School of Science and the Environment, University of Worcester, WR2 6AJ, UK.

[¶]These authors contributed equally to this work.

[*]Correspondence should be addressed to Y.H. (yiguo.hong@hznu.edu.cn, yiguo.hong@warwick.ac.uk or y.hong@worc.ac.uk).

Running title RNAi suppressor enhances VmGE

Keywords CRISPR/Cas9, FoMV, VmGE, RNAi suppressor

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

Introduction

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

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

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

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

Materials and methods

Construction of FoMV-based expression vectors

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

the *Hpa*I/*Asc*I sites of the binary vector pCambia2300-FoMV (Liu et al., 2016) to generate FoMV/Cas9 (**Fig. 1a**). To produce FoMV/sgRNAnon and FoMV/sgRNApds (**Fig. 1a**), the corresponding DNA fragments were amplified using the high-fidelity KOD-Plus-Neo DNA polymerase, pT-U6p-scaffold-U6t (Yin et al., 2015) or pCVA-gRNA::NbPDS plasmid DNA (Yin et al., 2015) as template together with primers AUT-Hpa-F3 and U6T-ASC-R2 (**Supplementary Table 1**), digested with *Hpa*I/*Asc*I and then cloned into the *Hpa*I/*Asc*I sites of the binary vector pCambia2300-FoMV (Liu et al., 2016). To generate FoMV/P19:sgRNApds (**Fig. 1a**), the p19 gene was amplified using the high-fidelity KOD-Plus-Neo DNA polymerase, pEAQ-HT plasmid carrying the p19 coding sequence (Sainsbury et al., 2008) as template and a set of primers P19-ORF-F and P19-ORF-R (**Supplementary Table 1**), and cloned into the *Hpa*I site of FoMV/sgRNApds. Similarly, the eGFP gene was amplified using primers eGFP-ORF-F and eGFP-ORF-R (**Supplementary Table 1**), plasmid pEGFP (Clonetech) as template, and cloned into the *Hpa*I site of pCambia2300-FoMV to produce FoMV/eGFP (**Supplementary Fig. 1a**). Insertion of *FLAG:NLS:Cas9:NLS*, sgRNAnon, sgRNApds or P19:sgRNApds in these FoMV vectors was verified by PCR using a pair of primers Fomv seq_6830_5K-F and Fomv Seq_7260_SUBP-R (**Supplementary Table 1**), and further confirmed by Sanger sequencing. All FoMV constructs and the binary vector pEAQ-HT (Sainsbury et al., 2008) were transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation (Chen et al., 2018) respectively, confirmed by sequencing plasmid minipreped from *Agrobacterium* culture prior to their use in subsequent agroinfiltration experiments and plant transformation.

Plant transformation

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

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

Virus infection (VmGE), plant growth and maintenance

To prepare FoMV and recombinant FoMV inoculum, *A. tumefaciens* LBA4404 harboring different FoMV constructs (**FIG. 1a** and **Supplementary Fig, 1a**) was cultured to reach a density of 1.0 OD_{600} at 28 ˚C overnight in LB medium containing 0.5 mg/ml streptomycin and 0.5 mg/ml kanamycin, then collected by centrifugation at 3,000 rpm for 10 min, and resuspended in sterile water to give a final density of 0.5 OD_{600}. For leaf-agroinfiltration, *Agrobacterium* was infiltrated into young leaves of WT or transgenic *Nb* plants at six-leaf stage through needleless 0.5-ml syringe. Alternatively, germinating seeds were agroinfiltrated in order to shorten virus infection time. Briefly, dozens of *Nb* seeds were spread onto 3MM Whatman filter paper pre-soaked in sterile water and placed within a petri dish (10 cm in diameter). The petri dish was kept in a growth chamber at 25 ˚C with constant light. After 2 days under such conditions, seeds began to break their coat and germinate. At this stage, seeds were collected and mixed with 5 ml 0.5 OD_{600} *Agrobacterium* in a 50mL-Falcon™ centrifuge tube. Agroinfiltration of seeds was achieved using a vacuum pump under the pressure of 0.085 MPa for 10 min. Agroinfiltrated seeds were then transferred to composts and kept under dark for 24 h. Plants were then grown under 16 h light/8 h dark conditions at 25 ˚C in insect-free growth rooms, regularly examined for development of local and systemic infection, and photographically recorded using a D7000Sony NEX-5R camera. It should be noted that (1) for leaf-agroinfiltration, 6 plants were infiltrated with each FoMV construct or each combination of FoMV constructs in a separate experiment, and such leaf-agroinfitration experiment was repeated 2-4 times; and (2) for seed-agroinfiltration, 50-100 seeds were used for each combination of FoMV constructs in two separate experiments.

Confocal microscopy

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

RNA extraction and RT-PCR

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

Western blot

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

Genomic DNA extraction and molecular characterization of ViGE

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

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

Results and Discussion

To test VmGE, we exploited the FoMV vector originally designed for VIGS (Liu et al., 2016), but recently adapted for VIF (Yuan et al., 2019) and transient gene expression (**Supplementary Fig. 1**). We cloned the coding sequences for 3xFLAG and nuclear localization signal (NLS)-tagged Cas9, *PDS*-targeting sgRNApds (Yin et al., 2015) or a sgRNA lacking any targeting sequence (sgRNAnon), into FoMV and generated FoMV/Cas9, FoMV/sgRNApds and FoMV/sgRNAnon, respectively (**Fig. 1a**). Through leaf co-agroinfiltration, *Nb* plants infected with FoMV/Cas9+FoMV/sgRNAnon or FoMV/Cas9+FoMV/sgRNApds developed no symptoms (**Supplementary Fig. 2a-d**), consistent with latent FoMV infection where viral RNA was detectable by RT-PCR (**Supplementary Fig. 3a-c**). We then extracted genomic DNA from systemic leaf tissue and amplified the sgRNApds target *PDS* gene. Complete *Mly*I-digestion of the resultant PCR products and subsequent sequencing analyses suggest no occurrence of VmGE (**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 sgRNApds in plants.

195 To enhance FoMV infectivity for increasing the level of Cas9 and sgRNApds, 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/sgRNApds 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-*Mly*I screening of 24 infected *p19*-transgenics in 4 separate
203 experiments showed that the sgRNApds target was only partially cleaved by *Mly*I,
204 suggesting occurrence of *in planta* systemic VmGE (**Supplementary Fig. 4f**). Further
205 cloning and sequencing *Mly*I-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:sgRNApds to express both p19 and sgRNApds (**Fig. 1a**). Upon
210 agroinfitration of germinating *Nb* seeds with FoMV/Cas9 and FoMV/P19:sgRNApds,
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, sgRNApds and p19 were expressed from FoMV/Cas9 along with
219 FoMV/p19:sgRNApds. 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:sgRNApds 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 sgRNApds. 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

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

Acknowledgments

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

Completing interests

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

References

Ali Z, Abul-faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, Aouida M, Piatek A, Baltes NJ, Voytas DF, Dinesh-Kumar S, Mahfouz MM. (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol Plant 8: 1288-1291.

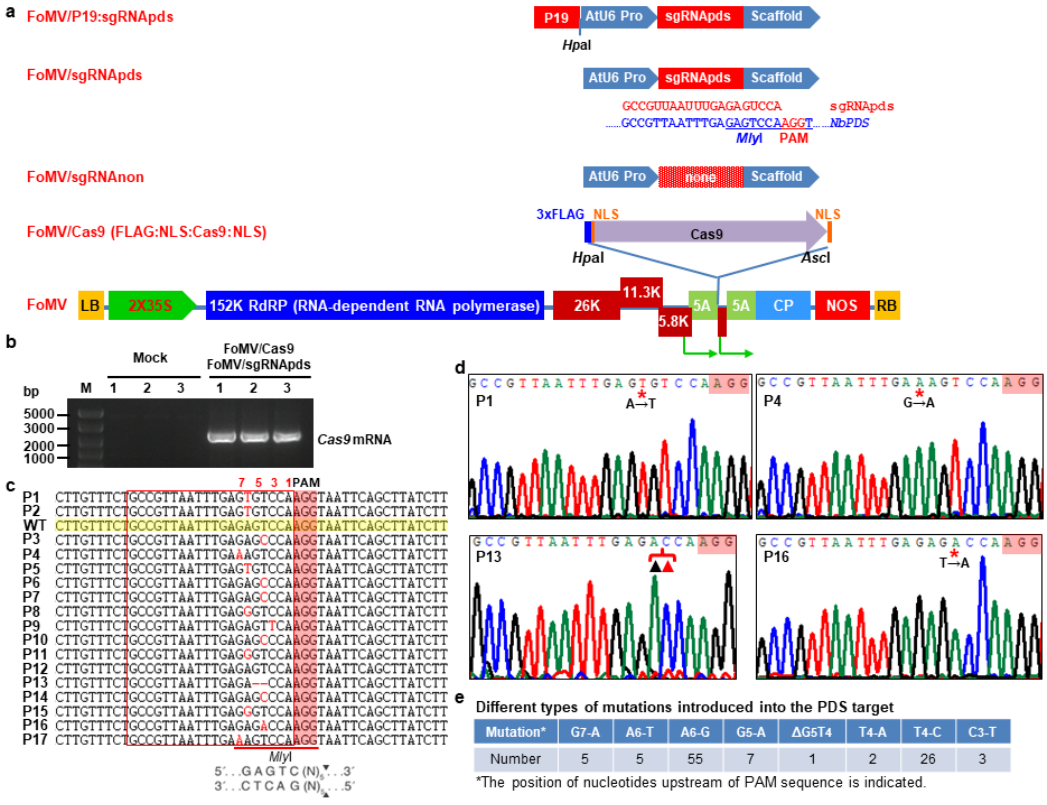
Chen W, Kong J, Qin C, Yu S, Tan J, Chen YR, Wu C, Wang H, Shi Y, Li C, Li B, Zhang P, Wang Y, Lai T, Yu Z, Zhang X, Shi N, Wang H, Osman T, Liu Y, Manning K, Jackson S, Rolin D, Zhong S, Seymour GB, Gallusci P, Hong Y. (2015) Requirement of CHROMOMETHYLASE3 for somatic inheritance of the

281 spontaneous tomato epimutation Colourless non-ripening. Sci Rep 5: 9192.
282 Chen W, Zhang X, Fan Y, Li B, Ryabov E, Shi N, Zhao M, Yu Z, Qin C, Zheng Q,
283 Zhang P, Wang H, Jackson S, Cheng Q, Liu Y, Gallusci P, Hong Y. (2018) A genetic
284 network for systemic RNA silencing in plants. Plant Physiol 176: 2700-2719.
285 Cody WB, Scholthof HB, Mirkov TE. (2017) Multiplexed gene editing and protein
286 overexpression using a Tobacco mosaic virus viral vector. Plant Physiol 175: 23–
287 35.
288 Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W,
289 Marraffini LA, Zhang F. (2013) Multiplex genome engineering using CRISPR/Cas
290 systems Science 339: 819-823
291 Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, Wang Z, Zhang Z, Zheng R, Yang L,
292 Zeng L, Liu X, Zhu JK. (2014) Multigeneration analysis reveals the inheritance,
293 specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis.
294 Proc Natl Acad Sci USA 111: 4632-4637.
295 Hong Y, Saunders K, Hartley MR, Stanley J. (1996) Resistance to geminivirus infection
296 by virus-induced expression of dianthin in transgenic plants. Virology 220: 119–
297 127.
298 Hong Y, Saunders K, Stanley J. (1997) Transactivation of dianthin transgene expression
299 by African cassava mosaic virus AC2. Virology 228: 383-387.
300 Hong Y, Stanley J, van Wezel R. (2003) Novel system for the simultaneous analysis of
301 geminivirus DNA replication and plant interactions in *Nicotiana benthamiana*. J
302 Virol 77: 13315–13322.
303 Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. (2012) A
304 programmable dual-RNA-guided DNA endonuclease in adaptive bacterial
305 immunity. Science 337: 816-821.
306 Kaya H, Ishibashi K, Toki S. (2017) A Split Staphylococcus aureus Cas9 as a Compact
307 Genome-Editing Tool in Plants. Plant Cell Physiol 58: 643–649.
308 Li C, Gu M, Shi N, Zhang H, Yang X, Osman T, Liu Y, Wang H, Vatish M, Jackson S,
309 Hong Y. (2011) Mobile FT mRNA contributes to the systemic florigen signalling in
310 floral induction. Sci Rep 1: 73.

311 Li C, Zhang K, Zeng X, Jackson S, Zhou Y, Hong Y. (2009) A cis element within
312 flowering locus T mRNA determines its mobility and facilitates trafficking of
313 heterologous viral RNA. J Virol 83: 3540-3548.
314 Lin Z, Hong Y, Yin M, Li C, Zhang K, Grierson D. (2008) A tomato HD-Zip homeobox
315 protein, LeHB-1, plays an important role in floral organogenesis and ripening.
316 Plant J 55: 301-310.
317 Liu N, Xie K, Jia Q, Zhao J, Chen T, Li H, Wei X, Diao X, Hong Y, Liu Y. (2016)
318 Foxtail mosaic virus-induced gene silencing in monocot plants. Plant Physiol 171:
319 1801-1807
320 Mao Y, Yang X, Zhou Y, Zhang Z, Botella JR, Zhu JK. (2018) Manipulating plant
321 RNA-silencing pathways to improve the gene editing efficiency of CRISPR/Cas9
322 systems. BMC Genome Biol 19: 149.
323 Qin C, Chen W, Shen J, Cheng L, Akande F, Zhang K, Yuan C, Li C, Zhang P, Shi N,
324 Cheng Q, Liu Y, Jackson S, Hong Y. (2017) A virus-induced assay for functional
325 dissection and analysis of monocot and dicot flowering time genes. Plant Physiol
326 174: 875–885.
327 Sainsbury F, Thuenemann EC, Lomonossoff GP. (2009) pEAQ: versatile expression
328 vectors for easy and quick transient expression of heterologous proteins in plants.
329 Plant Biotechnol J 7: 682–693.
330 Van Wezel R, Dong X, Blake P, Stanley J, Hong Y. (2002) Differential roles of
331 geminivirus Rep and AC4 (C4) in the induction of necrosis in *Nicotiana*
332 *benthamiana*. Mol Plant Pathol 3: 461-471.
333 Van Wezel R, Hong Y. (2004) Virus survival of RNA silencing without deploying
334 protein-mediated suppression in *Nicotiana benthamiana*. FEBS Lett 562: 65-70.
335 Van Wezel R, Liu H, Tien P, Stanley J, Hong Y. (2001) Gene C2 of the monopartite
336 geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity
337 determinant that is localized in the nucleus. Mol Plant Microbe Interact 14:
338 1125-1128.
339 Yu Z, Chen Q, Chen W, Zhang X, Mei F, Zhang P, Zhao M, Wang X, Shi N, Jackson S,
340 Hong Y. (2018) Multigene editing via CRISPR/Cas9 guided by a single-sgRNA

341 seed in Arabidopsis. J Integr Plant Biol 60: 376-381.
342 Zhou T, Zhang H, Lai T, Qin C, Shi N, Wang H, Jin M, Zhong S, Fan Z, Liu Y, Wu Z,
343 Jackson S, Giovannoni JJ, Rolin D, Gallusci P, Hong Y. (2012) Virus-induced gene
344 complementation reveals a transcription factor network in modulation of tomato
345 fruit ripening. Sci Rep 2: 836.
346 Yin K, Han T, Liu G, Chen T, Wang Y, Yu AY, Liu Y. (2015) A geminivirus-based guide
347 RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci Rep 5:
348 14926.
349 Yuan C, Li H, , Qin C, Zhang X, Chen Q, Zhang P, Xu X, He M, Zhang X-l, Tor M,
350 Xue D, Wang H, Jackson S, He Y, Liu Y, Shi N, Hong Y. (2019) Foxtail mosaic
351 virus-induced flowering assays in monocot crops. Plant Biotechnol J under review.

Figures



Mutation*	G7-A	A6-T	A6-G	G5-A	ΔG5T4	T4-A	T4-C	C3-T
Number	5	5	55	7	1	2	26	3

*The position of nucleotides upstream of PAM sequence is indicated.

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

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

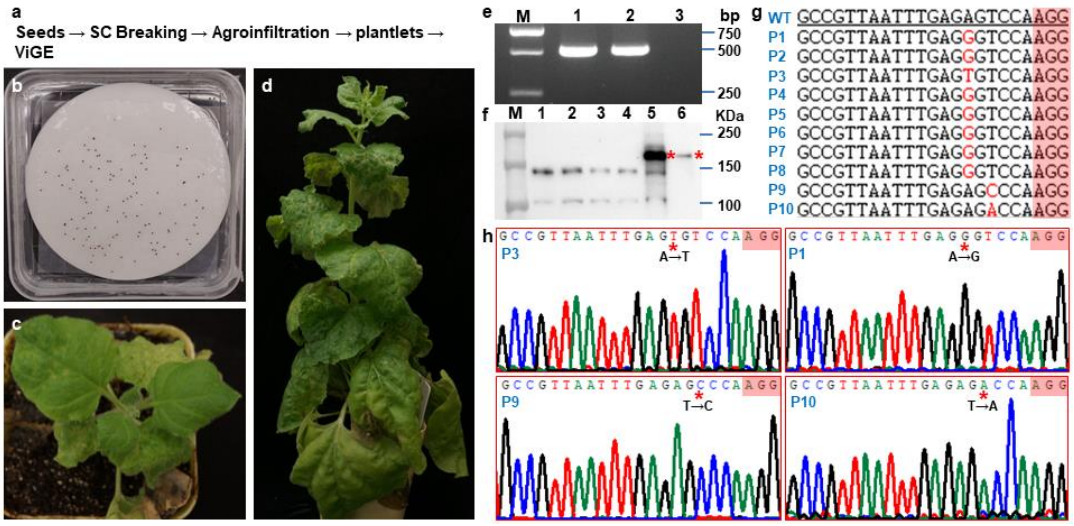


Figure 2 Effective VmGE through co-delivery of Cas9, sgRNApds and p19 from FoMV. **(a-d)** VmGE induction. Experimental strategy is outlined (**a**). Broken seed-coat (SC) seeds were infiltrated with mixed agrobacteria carrying FoMV/Cas9 and FoMV/P19:sgRNApds (**Fig. 1a**) under vacuum-pressure (**b**).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