Title: Efficient transgene-free multiplexed genome editing via viral delivery of an engineered TnpB.

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Abstract:
Virus-induced genome editing (VIGE) using compact RNA-guided endonucleases is a transformational new approach in plant biotechnology, enabling tissue-culture-independent and transgene-free genome editing (Hu et al. 2025; Weiss et al. 2025; Liu et al. 2025). We recently established a VIGE approach for heritable editing at single loci in *Arabidopsis* by delivering ISYmu1 TnpB (Ymu1) and its guide RNA (gRNA) via Tobacco Rattle Virus (TRV) (Weiss et al. 2025). Here, we greatly improved this approach by devising a multiple gRNA expression system and by utilizing an engineered high-activity Ymu1 variant (Ymu1-WFR) (Zhou et al. 2026) to develop an efficient multiplexed genome editing platform.

Main Text:
To evaluate TRV-mediated multiplexing capabilities, we co-delivered RNA1 with two RNA2 vectors targeting either *AtPDS3* gRNA12 or *AtCHLI1* gRNA4 to *Arabidopsis* (Figure S1A). Amplicon sequencing (amp-seq) revealed editing almost exclusively at one target site or the other (Figure S1B), suggesting viral superinfection exclusion (Perdoncini Carvalho et al. 2022). We therefore sought to develop a system in which both gRNAs could be expressed on a single RNA2 vector.

Through small RNA sequencing (RNA-seq) and protoplast editing assays using various ωRNA lengths, we identified 127 nucleotides as the optimal ωRNA for genome editing (Figures S2A-D). Next, we tested multiplexed arrays featuring tRNA, HDV, HDV-HH, or a repeat as gRNA processing elements. Amp-seq analysis showed that while all designs enabled editing, HDV-based designs performed best at simultaneously editing both sites in protoplasts (Figure S2E-G). Furthermore, polymerase chain reaction (PCR) using primers spanning both sites suggested the occurrence of large deletions between the two targets (Figure S2H).

Next, we tested the HDV multiplexed array design for TRV-mediated editing in planta using vectors targeting *AtCHLI1* (gRNA4) and *AtPDS3* (gRNA12), incorporating a

94 tRNA^{Ile} mobility sequence at the 3' end of the cargo (Figure S3A). gRNA4 targets the
95 gene body of *AtCHLI1* (biallelic edits create yellow tissue sectors) and gRNA12 targets
96 the promoter region upstream of the *AtPDS3* transcription start site (no visible
97 phenotype). After delivering TRV vectors, we did not observe any phenotypic evidence
98 of editing. Suspecting inefficient mobility or processing of the RNA2 cargo, we tested
99 three additional constructs containing a tRNA^{Ile} downstream of each HDV ribozyme
100 (Figure 1A). After TRV delivery, yellow sectors appeared on leaves for all three vectors,
101 indicating biallelic edits at *AtCHLI1* (Figure 1B). Yellow sectored plants infected with
102 pTW2278 and pTW2279 displayed average editing efficiencies of 25.6% for *AtCHLI1*
103 and 30.2% for *AtPDS3* (Figure 1C), and those infected with pTW2498 showed 31.3% at
104 *AtCHL1* gRNA4 and 5.2% at the *AtCHL1* gRNA6 (Figure 1D).
105
106 To characterize the inheritance of edited alleles, we selected two plants: pTW2278_17
107 and pTW2278_54. We observed yellow progeny at frequencies of 4.7% and 1.7%,
108 respectively (Figure 1E, S3B). Among the yellow seedlings, 76.9% (pTW2278_17) and
109 66.7% (pTW2278_54) of them harbored biallelic edits at both loci (Figure 1E, S3C).
110 These data demonstrate that TRV effectively delivers Ymu1 and multiple gRNAs for
111 heritable multiplexed editing, and that biallelic editing at one locus is highly predictive of
112 biallelic editing at the second target site.
113
114 We recently developed an engineered Ymu1 variant (Ymu1-WFR) with improved editing
115 capabilities (Zhou et al. 2026). To evaluate Ymu1-WFR efficiency using TRV, we
116 targeted three published *AtCHLI1* target sites: gRNA4, gRNA6, and gRNA9 (Weiss et
117 al. 2025). Infected plants showed a strong yellow phenotype for all three gRNAs (Figure
118 1F). Amp-seq revealed average editing efficiencies of 18.6%, 14.7%, and 9.8%,
119 respectively, much higher (up to 9.8-fold) than WT Ymu1 (Weiss et al. 2025) (Figure
120 1G).
121
122 To assess the impact of the WFR variant on multiplexed editing efficiency, we replaced
123 the wild type (WT) Ymu1 sequence in pTW2278, pTW2279, and pTW2498 with Ymu1-
124 WFR (Figure S3D). Following TRV delivery, we observed much more pronounced

phenotypic evidence of editing than with WT Ymu1 (Figure 1H compared with Figure 1B). Amp-seq of yellow sectored plants confirmed enhanced editing: pTW2657 and pTW2658 averaged 48.9% for *AtCHLI1* (gRNA4) and 41.3% for *AtPDS3* (gRNA12) (Figure 1I), while pTW2655 averaged 45.3% and 28.1% at the two *AtCHLI1* sites (Figure 1J). Consistent with the protoplast result (Figure S2H), PCR analysis using primers spanning the *AtCHLI1* gRNA4 and gRNA6 sites revealed large deletions (Figures S3E, S3F).

By optimizing the gRNA array design, and incorporating the highly active engineered Ymu1-WFR variant, we developed an efficient multiplexed editing platform that bypasses the need for transgenesis. Given the broad host range of TRV, we anticipate this approach will be adaptable to commercially relevant crop species. Additionally, the ability to generate large deletions should expand this system's utility for regulatory element engineering. Finally, this multiplexed system may enable the study of embryonic lethal genes by utilizing *AtCHLI1* as a visual marker; the yellow somatic sectors should facilitate the identification of tissue harboring biallelic knockout of a gene of interest.

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Author Contributions:
T.W. and S.E.J. designed research. T.W., H.S., J.A.D. and S.E.J. interpreted data. T.W. and S.E.J. wrote the paper. T.W., M.K., H.S., G.W., A.I., J.A., K.V., M.T., Z.L., E.F., N.S., C.A., and K.C. performed experiments.

Data Availability:

Amp-seq data will be accessible at NCBI Sequence Read Archive upon publication. RNA-seq data is available at GEO accession: GSE316183.

Competing interests:

T.W., M.K, H.S., J.A.D. and S.E.J. have filed patents related to this work. S.E.J. is a cofounder and consultant for Inari Agriculture and a consultant for Terrana Biosciences, Invaio Sciences, Sail Biomedicines and Zymo Research. J.A.D. is a cofounder of Azalea Therapeutics, Caribou Biosciences, Editas Medicine, Evercrisp, Scribe Therapeutics and Mammoth Biosciences, a scientific advisory board member at Evercrisp, Caribou Biosciences, Scribe Therapeutics, Mammoth Biosciences, The Column Group and Inari, an advisor for Aditum Bio, the Chief Science Advisor to Sixth Street, a Director at Johnson & Johnson, Altos and Tempus, and has a research project sponsored by Apple Tree Partners.

References:

Hu, Manman, Lingran Zhang, Luis Herrera-Estrella, and Degao Liu. 2025. "Heritable, Tissue Culture-Independent and Transgene-Free Genome Editing in Plants via Viral Delivery of CRISPR/AsCas12f." *Plant Biotechnology Journal*, no. pbi.70315 (September). https://doi.org/10.1111/pbi.70315.

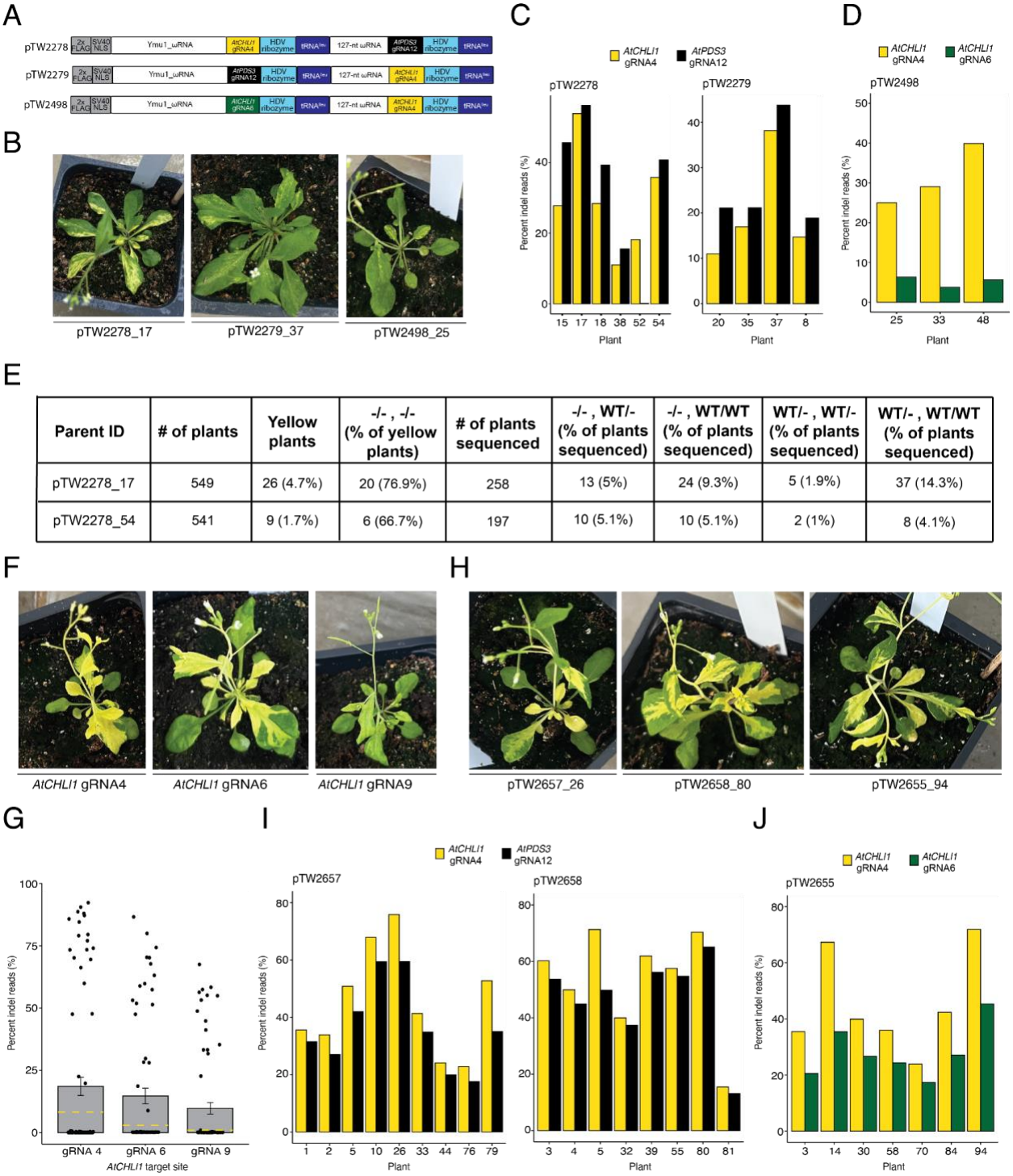
Liu, Ye, Trevor Weiss, Jinhee Lee, et al. 2025. "Virus Induced Transgene- and Tissue-Culture Free Heritable Genome Editing in Tomato." In *bioRxiv*. November 9. https://doi.org/10.1101/2025.11.08.687402.

Perdoncini Carvalho, Camila, Ruifan Ren, Junping Han, and Feng Qu. 2022. "Natural Selection, Intracellular Bottlenecks of Virus Populations, and Viral Superinfection Exclusion." *Annual Review of Virology* 9 (1): 121–137.

Weiss, Trevor, Maris Kamalu, Honglue Shi, et al. 2025. "Viral Delivery of an RNA-Guided Genome Editor for Transgene-Free Germline Editing in Arabidopsis." *Nature Plants* 11 (5): 967–976.

Zhou, Zehan, Iren Saffarian-Deemyad, Honglue Shi, et al. 2026. "Stepwise DNA

Unwinding Gates TnpB Genome-Editing Activity." In *bioRxiv*. BioRxiv, January 9. https://doi.org/10.64898/2026.01.09.698545.



Parent ID	# of plants	Yellow plants	-/- , -/- (% of yellow plants)	# of plants sequenced	-/- , WT/- (% of plants sequenced)	-/- , WT/WT (% of plants sequenced)	WT/- , WT/- (% of plants sequenced)	WT/- , WT/WT (% of plants sequenced)
pTW2278_17	549	26 (4.7%)	20 (76.9%)	258	13 (5%)	24 (9.3%)	5 (1.9%)	37 (14.3%)
pTW2278_54	541	9 (1.7%)	6 (66.7%)	197	10 (5.1%)	10 (5.1%)	2 (1%)	8 (4.1%)

Figure 1: Heritable and transgene-free multiplexed genome editing in *Arabidopsis* via viral delivery of Ymu1. (A) Schematic representation of the cargo being expressed by the PEBV promoter in TRV RNA2. *AtCHLI1* gRNA4 (yellow), *AtPDS3 gRNA12* (black), and *AtCHLI1* gRNA6 (green) were used in the multiplex TRV experiments. The

221 plasmid ID is listed to the left of each construct. **(B)** Yellow sector phenotype observed
222 from plants infected with the vectors from panel A. The plant ID (construct_plant) is
223 listed below each picture. **(C and D)** Editing efficiency (y-axis) of plants displaying the
224 yellow sector phenotype (x-axis) infected with pTW2278, pTW2279 and pTW2498. **(E)**
225 Heritability data from plants infected with pTW2278_17 and pTW2278_54. **(F)** Yellow
226 sector phenotype observed from plants infected with Ymu1-WFR targeting *AtCHLI1*
227 (gRNA4, gRNA6, and gRNA9). The site being targeted is listed below each picture. **(G)**
228 Editing efficiency (y-axis) of plants infected with Ymu1-WFR targeting *AtCHLI1* (x-axis).
229 Each dot represents an individual plant. The yellow dashed line on each bar indicates
230 the average editing efficiency previously reported (Weiss et al. 2025). **(H)** Yellow sector
231 phenotype observed from plants infected with Ymu1-WFR targeting *AtCHLI1* gRNA4
232 and *AtPDS3* gRNA12 (pTW2657 and pTW2658) or *AtCHLI1* gRNA4 and *AtCHLI1*
233 gRNA6 (pTW2655). The plant ID (construct_plant) is listed below each picture. **(I and J)**
234 Editing efficiency (y-axis) of plants displaying the yellow sector phenotype (x-axis)
235 infected with pTW2657, pTW2658 and pTW2655.
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252 **Supplementary Materials:**

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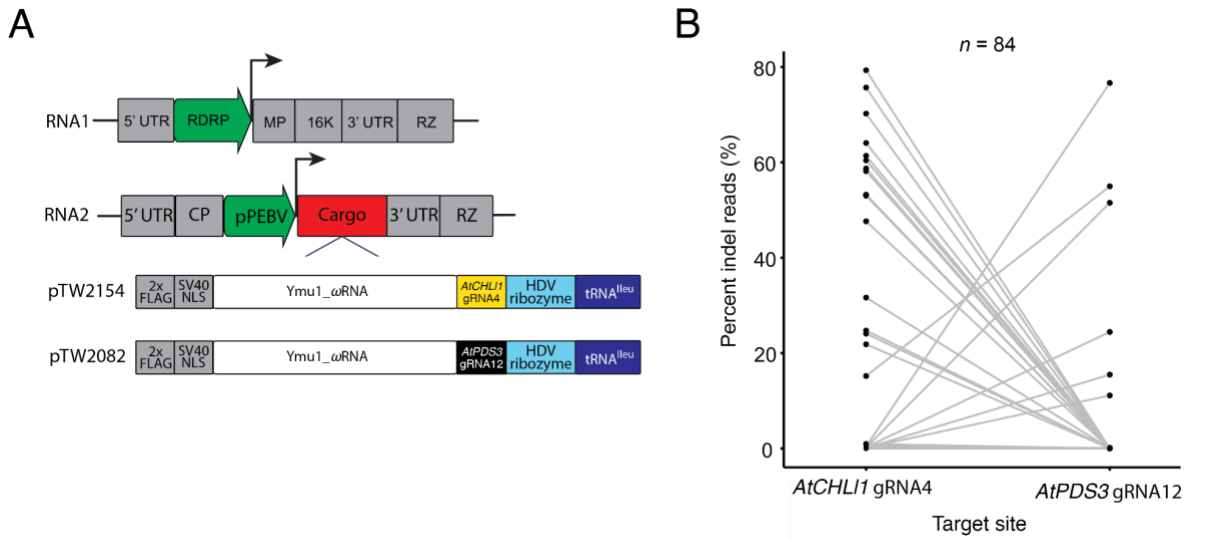

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255 **Supplementary Figure 1: Co-delivery of RNA1 and two RNA2 vectors does not**
256 **enable multiplexed genome editing. (A)** Schematic representation of TRV. The cargo
257 and plasmid ID are listed below RNA2. **(B)** Editing efficiency (y-axis) of plants (*n* = 84)
258 co-infected with RNA1 and two RNA2 vectors targeting *AtCHLI1* gRNA4 and *AtPDS3*
259 gRNA12 (x-axis). Each gray line represents a single plant, with the black dot at each
260 end of the gray line corresponding to the editing efficiency of each target site.

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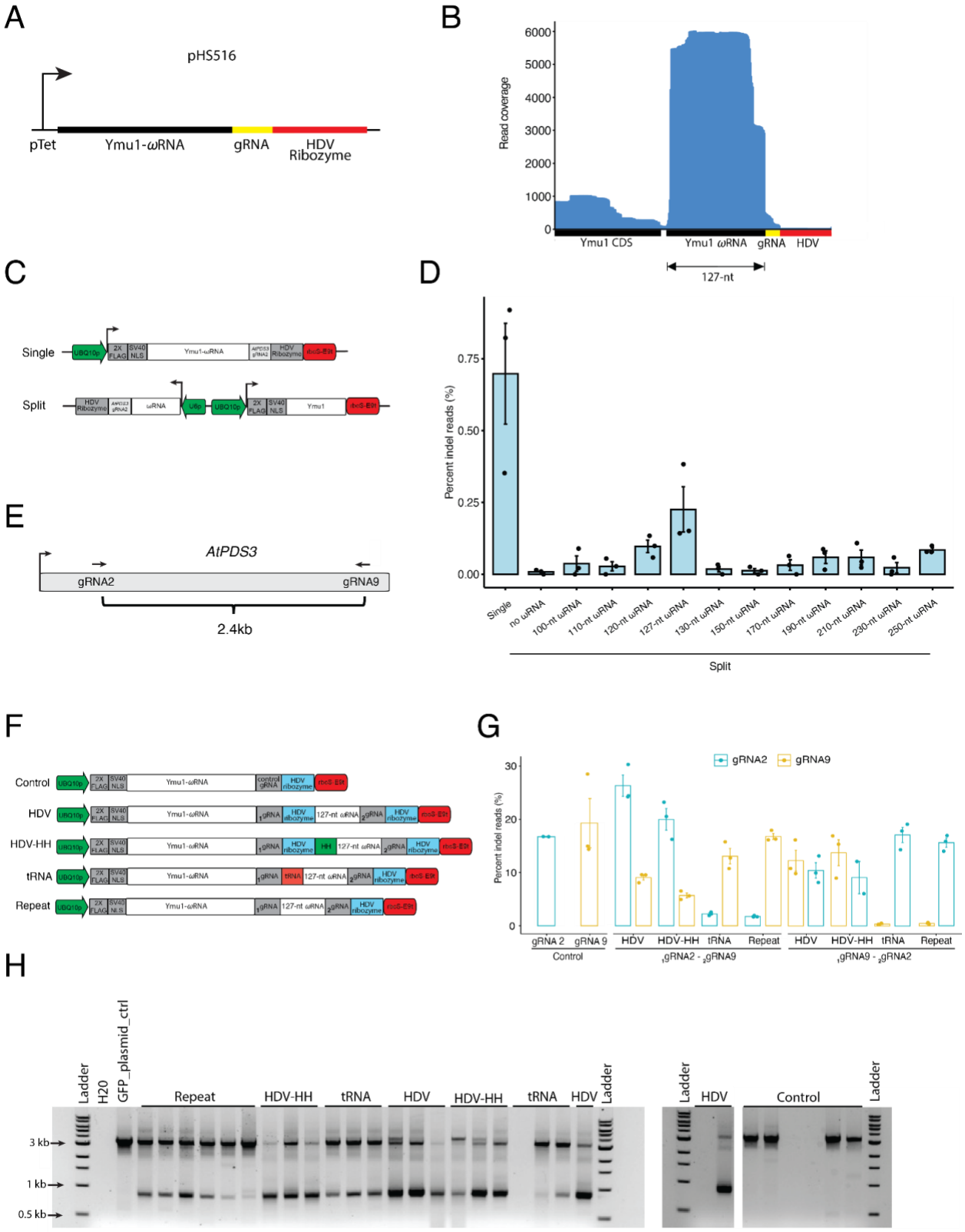
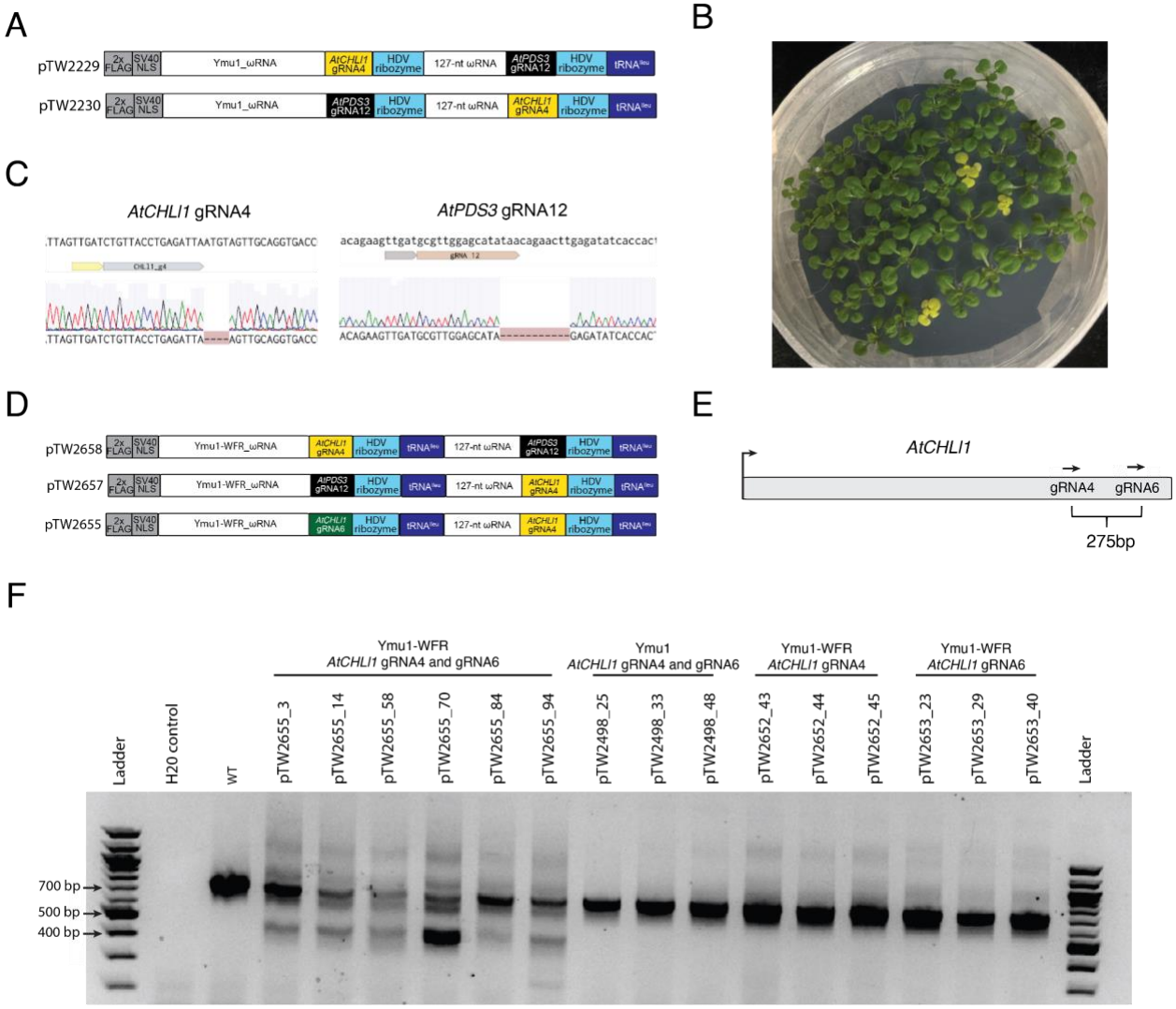


Supplementary Figure 2: Development of Ymu1 multiplexed gRNA arrays for plant gene editing. (A) Schematic of plasmid used in *E. coli* RNA-seq experiment. The

tetracycline promoter (pTet, arrow) was used to express the Ymu1-ωRNA (black), gRNA (yellow), and HDV ribozyme (red). **(B)** RNA-seq in *E. coli* using the plasmid in panel A. Read coverage (y-axis) is displayed for each Ymu1 single transcript feature (x-axis). **(C)** Schematic of plasmids expressing the Ymu1-ωRNA in a single transcript expression format or split expression format targeting *AtPDS3* gRNA2. In the split expression plasmids the Ymu1 coding sequence was expressed by the *AtUBQ10* promoter (UBQ10p) and the ωRNA was expressed using the U6 promoter (U6p). **(D)** Editing efficiency (y-axis) using single expression and split expression constructs with varying lengths of ωRNA (x-axis). Each dot represents an individual transfection with bars indicating the standard error of the mean (SEM). **(E)** Schematic of *AtPDS3* gene with the arrows indicating the orientation of gRNA2 and gRNA9, located roughly 2.4kb apart. **(F)** Schematic of vectors used in the *Arabidopsis* protoplast multiplexed editing experiment. **(G)** Editing efficiency (y-axis) of the multiplex arrays (x-axis) tested in *Arabidopsis* protoplast targeting *AtPDS3* (gRNA2 and gRNA9). The subscript below each gRNA indicates its placement in the multiplex array as depicted in panel F. **(H)** PCR gel electrophoresis image using primers spanning *AtPDS3* gRNA2 and *AtPDS3* gRNA9 target sites. Deletion between the two target sites corresponds to a ~850bp band.



Supplementary Figure 3: TRV-delivery of Ymu1 for multiplexed genome editing. **(A)** Schematic of multiplex vectors targeting *AtCHLI1* gRNA4 and *AtPDS3* gRNA12, containing a single 3' $tRNA^{Ileu}$. **(B)** Representative image of yellow and green progeny seedlings from plant pTW2278_17. All yellow seedlings harbored biallelic edits at *AtCHLI1* gRNA4 target site. **(C)** Sanger sequencing trace file screenshots from a yellow seedling in panel B, harboring homozygous edited alleles at *AtCHLI1* gRNA4 (4bp deletion) and *AtPDS3* gRNA12 (11bp deletion). **(D)** Schematic representation of the Ymu1-WFR multiplexed gRNA cargo being expressed by the PEBV promoter in TRV RNA2. **(E)** Schematic of *AtCHLI1* gene with the arrows indicating the orientation of gRNA4 and gRNA6, located approximately 275bp apart. **(F)** PCR gel electrophoresis image using primers spanning *AtCHLI1* gRNA4 and *AtCHLI1* gRNA6 target sites.

Deletion between the two target sites corresponds to a ~420bp band. The plant ID (construct_plant) is listed above each well. The TnpB (Ymu1 or Ymu1-WFR) and gRNA(s) is listed above the plant ID.

Plasmid	**Plasmid description**
pTW1169	TRV RNA1
pTW2154	TRV2 expressing wild type Ymu1 protein and *AtCHLI1* gRNA4
pTW2082	TRV2 expressing wild type Ymu1 protein and *AtPDS3* gRNA12
pHS516	Wild type Ymu1 single transcript expression plasmid for RNA-seq in E.coli
pTW2064	Wild type Ymu1 with no ωRNA
pTW2071	Wild type Ymu1 with 100 nt ωRNA targeting *AtPDS3* gRNA2
pTW2072	Wild type Ymu1 with 110 nt ωRNA targeting *AtPDS3* gRNA2
pTW2073	Wild type Ymu1 with 120 nt ωRNA targeting *AtPDS3* gRNA2
pTW2074	Wild type Ymu1 with 127 nt ωRNA targeting *AtPDS3* gRNA2
pTW2075	Wild type Ymu1 with 130 nt ωRNA targeting *AtPDS3* gRNA2
pTW2076	Wild type Ymu1 with 150 nt ωRNA targeting *AtPDS3* gRNA2
pTW2077	Wild type Ymu1 with 170 nt ωRNA targeting *AtPDS3* gRNA2
pTW2078	Wild type Ymu1 with 190 nt ωRNA targeting *AtPDS3* gRNA2
pTW2079	Wild type Ymu1 with 210 nt ωRNA targeting *AtPDS3* gRNA2
pTW2080	Wild type Ymu1 with 230 nt ωRNA targeting *AtPDS3* gRNA2
pTW2081	Wild type Ymu1 with 250 nt ωRNA targeting *AtPDS3* gRNA2
pTW2109	Repeat design multiplex vector expressing *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2110	Repeat design multiplex vector expressing *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pTW2137	tRNA design multiplex vector expressing *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2140	tRNA design multiplex vector expressing *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second

pTW2148	HDV design multiplex vector expressing *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2138	HDV design multiplex vector expressing *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pTW2136	HDV-HH design multiplex vector expressing *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2139	HDV-HH design multiplex vector expressing *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pMK061	Wild type Ymu1 and *AtPDS3* gRNA2
pMK067	Wild type Ymu1 and *AtPDS3* gRNA9
pTW2229	TRV2 multiplex vector expressing wild type Ymu1, *AtCHLI1* gRNA4 and *AtPDS3* gRNA12
pTW2230	TRV2 multiplex vector expressing wild type Ymu1, *AtPDS3* gRNA12 and *AtCHLI1* gRNA4
pTW2658	TRV2 multiplex vector expressing Ymu1-WFR, *AtCHLI1* gRNA4 and *AtPDS3* gRNA12
pTW2657	TRV2 multiplex vector expressing Ymu1-WFR, *AtPDS3* gRNA12 and *AtCHLI1* gRNA4
pTW2655	TRV2 multiplex vector expressing Ymu1-WFR, *AtCHLI1* gRNA6 and *AtCHLI1* gRNA4
pTW2278	TRV2 multiplex vector expressing wild type Ymu1, *AtCHLI1* gRNA4 and *AtPDS3* gRNA12
pTW2279	TRV2 multiplex vector expressing wild type Ymu1, *AtPDS3* gRNA12 and *AtCHLI1* gRNA4
pTW2498	TRV2 multiplex vector expressing wild type Ymu1, *AtCHLI1* gRNA6 and *AtCHLI1* gRNA4
pTW2652	TRV2 expressing Ymu1-WFR protein and *AtCHLI1* gRNA4

pTW2653	TRV2 expressing Ymu1-WFR protein and *AtCHLI1* gRNA6
pTW2660	TRV2 expressing Ymu1-WFR protein and *AtCHLI1* gRNA9
pMK435	Cloning vector for PaqCI Golden Gate assembly of Ymu1 guides into TRV2

Supplementary Table 1: Plasmids and their descriptions used in this study.

Gene ID	Target site(s)	Target site sequence	Forward primer sequence	Reverse primer sequence	Purpose
AT4G14210	*AtPDS3* gRNA2	aaggcaaattcgccgc	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaagcagttgtgagttaagttggaga	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttgtcttaagcgcttgagaagtgg	NGS amplicon sequencing
AT4G18480	*AtCHLI1* gRNA4	CTGTTACCTGAGATTA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGGTGTTATGATTATGGGAGATAGAG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCGCAATAACAGGAACTTGCTC	NGS amplicon sequencing
AT4G18480	*AtCHLI1* gRNA6	GAAGTTAATCTCTTGG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCCTTTGAGCCTGGTTTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGGGTGAGAAATCGAAATCCC	NGS amplicon sequencing
AT4G18480	*AtCHLI1* gRNA9	CGGTTTGGTATGCATG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGAGGTTTATCTTGAT	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCACGGAAATCCTTTGGGTT	NGS amplicon sequencing

			CGGTTC	ACTA	
AT4G14210	*AtPDS3* gRNA12	gcgttggagcatataa	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaccgacccgagaagagatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtggaatacacacatttgtacaacca	NGS amplicon sequencing
AT4G14210	*AtPDS3* gRNA9	agctttgaaccggttt	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgcgctaaactttataaaccctgatg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtatgattgccagtgagataaagattc	NGS amplicon sequencing
AT4G14210	*AtPDS3* gRNA12	see above	tgttacacaacttattatgattgggct	ggaagtagccgataacaaaatggag	Sanger sequencing
AT4G18480	*AtCHLI1* gRNA4	see above	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaagttaggaacatgattgttgttggt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCACGGAAATCCTTTGGGTTACTA	Sanger sequencing
AT4G14210	*AtPDS3* gRNA2 and *AtPDS3* gRNA9	see above	ACACTCTTTCCCTACACGACGCTCTTCCGATCTactccattttgttatcggctacttcc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgtgcttccattagtgagtaagaaact	PCR spanning both target sites

AT4G18480	*AtCHLI1* gRNA4 and *AtCHLI1* gRNA6	see above	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATTTGCAGCTATAGTAGGGCA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCACGGAAATCCTTTGGGTTACTA	PCR spanning both target sites

377 **Supplementary Table 2:** Target sites and primers used in this study.

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401 **Methods:**

402 Plasmids used in this study

403 To generate the Ymu1 plasmids with varying ωRNA lengths, NEB HiFi assembly was
404 performed using two PCR amplicons and pTW2064 (the no ωRNA plasmid backbone).
405 One amplicon contained the U6 promoter and the other amplicon contained the ωRNA.
406 First, pTW2064 underwent a restriction enzyme digestion with SpeI (R3133) and Quick
407 CIP (M0525) to linearize the plasmid. Next, both amplicons and the digested and
408 purified pTW2064 plasmid were used in an NEBuilder HiFi DNA Assembly (E2621)
409 reaction.

410

411 Multiplex vectors used in the protoplast experiment expressing wild type Ymu1, *AtPDS3*
412 gRNA2 and *AtPDS3* gRNA9 were created using a golden gate reaction. PCR amplicons
413 or gene fragments (Twist Bioscience) containing the first gRNA, processing sequence,
414 127-nt ωRNA and the second gRNA were inserted into the pMK025 vector (Weiss et al.
415 2025) using a PaqCI (R0745) golden gate reaction.

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417 Multiplex TRV2 vectors were created using a golden gate reaction assembly. First, PCR
418 was performed to generate two amplicons; one amplicon contained the first gRNA in the
419 array, an HDV ribozyme and $tRNA^{Ile}$, and the second amplicon contained the 127-nt
420 ωRNA and second gRNA in the array. Amplicons contained overhangs with PaqCI
421 (R0745) sites to facilitate assembly. Both amplicons and pMK435 (Weiss et al. 2025)
422 were then used in a PaqCI golden gate reaction.

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424 TRV2 vectors containing the Ymu1-WFR variant were created using NEBuilder HiFi
425 DNA Assembly (E2621). The TRV2 vectors were constructed by digesting the wild type
426 Ymu1 TRV2 vectors with HpaI (R0105) and Eco53KI (R0116) restriction enzymes. A
427 gene fragment (Twist Bioscience) containing the Ymu1-WFR sequence was then cloned
428 into the digested plasmid using NEBuilder HiFi DNA Assembly (E2621).

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430 Following assembly, all plasmids were transformed into NEB 10-beta (C3019) *E.coli*
431 and validated by whole-plasmid sequencing. Plasmid names and descriptions can be
432 found in Table S1.
433
434 Plant gene editing experiments
435 *Arabidopsis* mesophyll protoplasts were isolated according to previously described
436 methods (Yoo et al. 2007). For protoplast transfection, 20 µg of plasmid was placed at
437 the bottom of each tube and brought to a total volume of 20 µl with water. Then, 200 µl
438 of protoplasts were added to the plasmid solution, followed by 220 µl of fresh, sterile
439 polyethylene glycol (PEG)-$CaCl_2$ solution. The samples were mixed by gentle tapping
440 and incubated at room temperature for 10 minutes. Transfection was terminated by
441 adding 880 µl of W5 solution and inverting the tubes two to three times. Protoplasts
442 were subsequently pelleted by centrifugation at 100 RCF for 3 minutes, resuspended in
443 1 ml of WI solution, and plated in 6-well plates precoated with 5% calf serum. The cells
444 were incubated at 26°C for 48 hours. During this incubation period, a 37°C heat-shock
445 treatment was applied for 2 hours, beginning 16 hours post-transfection. At 48 hours
446 post-transfection, the protoplasts were collected for genomic DNA extraction.
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448 TRV delivery was performed as previously described (Weiss et al. 2025; Nagalakshmi
449 et al. 2022). TRV1 and TRV2 vectors were introduced into *Agrobacterium tumefaciens*
450 strain GV3101, and cultures were grown in 200 ml of lysogeny broth (LB) with
451 antibiotics for 18 hours at 28°C. The cultures were centrifuged at 3,500 × g for 20
452 minutes; the supernatant was discarded, and the pellets were resuspended in 200 ml of
453 sterile water. This suspension was centrifuged again at 2,109 × g for 10 minutes. After
454 discarding the supernatant, the pellet was resuspended in sterile agro-infiltration buffer
455 (10 mM $MgCl_2$, 10 mM MES, and 250 µM acetosyringone) to a final optical density
456 OD_{600} of 1.5 and incubated at 23°C for 3 hours with slow shaking. Following incubation,
457 the *Agrobacterium* cultures harboring TRV1 and TRV2 were mixed in a 1:1 ratio (co-
458 delivery experiment used a ratio of two parts TRV1, one part pTW2154, and one part
459 pTW2082). 15 ml of this mixture was delivered to 10-day-old seedlings via agroflood co-

460 culture. After 4 days, the seedlings were transplanted to soil and grown at room
461 temperature for the remainder of their life cycle.
462
463 Approximately 12 weeks after viral delivery, seeds were harvested from TRV-infected
464 plants. Seeds were sown on ½ MS plates supplemented with 3% sucrose and stratified
465 at 4°C in the dark for 5 days. Following stratification, the seeds were moved to a growth
466 room under a 16-h light/8-h dark cycle at 23°C for 10–12 days. A subset of plants was
467 then sampled for genotyping by collecting a single piece of leaf tissue. DNA was
468 extracted using the Invitrogen Platinum Direct PCR Universal Master Mix (A44647500)
469 according to the manufacturer's instructions. Finally, the extracted DNA was analyzed
470 via PCR and Sanger sequencing using the primers listed in Table S2.
471
472 Amplicon sequencing and analysis
473 Genomic DNA was extracted from protoplast samples using the Qiagen DNeasy Plant
474 Mini Kit (Qiagen, 69106). For plants subjected to agroflood, three leaf tissue samples
475 distal to the TRV delivery site were pooled per plant about 3-4 weeks after viral delivery.
476 The ratio of green to yellow tissue samples collected were selected in a manner that
477 most accurately reflected the phenotype of the entire plant. Once collected, all tissue
478 samples were frozen overnight at −80°C and ground. DNA was subsequently extracted
479 using the Invitrogen Platinum Direct PCR Universal Master Mix (A44647500) according
480 to the manufacturer’s instructions. The DNA was then used for next-generation
481 amplicon sequencing.
482
483 Amplicon sequencing was performed using single-end sequencing on the Illumina
484 NovaSeqX platform. Libraries were constructed using a two-step PCR approach. First,
485 target regions were amplified for 25 cycles using primers flanking the TnpB target site
486 (Table S2), followed by purification with 1.0× AMPure XP beads (Beckman Coulter,
487 A63881). Then, samples underwent 12 additional cycles of amplification to attach
488 Illumina indexing primers, followed by a 0.7× AMPure XP cleanup. Final libraries were
489 normalized and pooled for sequencing. Amplicon sequencing analysis was performed

using the CrispRvariants R package (v.1.14.0) as previously described (Weiss et al. 2025).

Bacterial RNA expression and sequencing

For heterologous RNA expression, *E. coli* transformed with pHS516 (Figure S2A, Table S1) and grown in LB medium to an OD_{600} of 0.6 and induced with 10 mM arabinose at 16°C overnight. Following overnight growth, total RNA from cells was extracted using a hot formamide method, in which pelleted *E. coli* were resuspended in an 18 mM EDTA and 95% formamide solution and lysed by incubating at 65ºC for 5 minutes. After centrifugation to pellet cell debris, the supernatant containing total RNA was purified using the RNA Clean & Concentrator-5 Kit (Zymo Research R1013) following manufacturer protocols. Approximately 200 ng of purified total RNA was subjected to rRNA depletion using the NEBNext rRNA Depletion Kit for Bacteria (New England Biolabs E7850L) following manufacturer protocols. rRNA-depleted samples were again purified using the RNA Clean & Concentrator-5 Kit and subjected to end repair and small-RNA-seq library prep as previously described (Zhou et al. 2026). Sequencing was performed using an Illumina NextSeq 1000/2000 P2 v3 kit. Following sequencing, paired-end reads were trimmed and merged using fastp (v0.23.2) (Chen et al. 2018). Merged reads of 100–150 nucleotides were selected with fastq-filter (v0.3.0; https://github.com/LUMC/fastq-filter) and aligned to the Ymu1 locus using BWA (v0.7.17) (Li 2013). Alignments were sorted with SAMtools (v1.17) (Li et al. 2009) and converted to BigWig format using deepTools2 (v3.5.1) (Ramírez et al. 2014), then visualized in the Integrative Genomics Viewer (IGV) (Robinson et al. 2011).

Supplementary Materials References:

Chen, Shifu, Yanqing Zhou, Yaru Chen, and Jia Gu. 2018. “Fastp: An Ultra-Fast All-in-One FASTQ Preprocessor.” *Bioinformatics (Oxford, England)* 34 (17): i884–i890.

Li, Heng. 2013. “Aligning Sequence Reads, Clone Sequences and Assembly Contigs with BWA-MEM.” In *arXiv [q-bio.GN]*. March 16. arXiv. http://arxiv.org/abs/1303.3997.

521 Li, Heng, Bob Handsaker, Alec Wysoker, et al. 2009. “The Sequence Alignment/Map
522 Format and SAMtools.” *Bioinformatics (Oxford, England)* 25 (16): 2078–2079.

523 Nagalakshmi, Ugrappa, Nathan Meier, Jau-Yi Liu, Daniel F. Voytas, and Savithramma
524 P. Dinesh-Kumar. 2022. “High-Efficiency Multiplex Biallelic Heritable Editing in
525 Arabidopsis Using an RNA Virus.” *Plant Physiology* 189 (3): 1241–1245.

526 Ramírez, Fidel, Friederike Dündar, Sarah Diehl, Björn A. Grüning, and Thomas Manke.
527 2014. “deepTools: A Flexible Platform for Exploring Deep-Sequencing Data.”
528 *Nucleic Acids Research* 42 (Web Server issue): W187–91.

529 Robinson, James T., Helga Thorvaldsdóttir, Wendy Winckler, et al. 2011. “Integrative
530 Genomics Viewer.” *Nature Biotechnology* 29 (1): 24–26.

531 Weiss, Trevor, Maris Kamalu, Honglue Shi, et al. 2025. “Viral Delivery of an RNA-
532 Guided Genome Editor for Transgene-Free Germline Editing in Arabidopsis.”
533 *Nature Plants* 11 (5): 967–976.

534 Yoo, Sang-Dong, Young-Hee Cho, and Jen Sheen. 2007. “Arabidopsis Mesophyll
535 Protoplasts: A Versatile Cell System for Transient Gene Expression Analysis.”
536 *Nature Protocols* 2 (7): 1565–1572.

537 Zhou, Zehan, Iren Saffarian-Deemyad, Honglue Shi, et al. 2026. “Stepwise DNA
538 Unwinding Gates TnpB Genome-Editing Activity.” In *bioRxiv*. BioRxiv, January 9.
539 https://doi.org/10.64898/2026.01.09.698545.