Title: Efficient transgene-free multiplexed germline 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; Liu et al. 2025; Weiss et al. 2025). We recently established a VIGE approach for heritable editing at single loci in *Arabidopsis* by delivering the compact genome editor 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:
TRV is a bipartite RNA virus composed of RNA1 and RNA2. To evaluate TRV-mediated multiplexing capabilities, we co-delivered RNA1 with two RNA2 vectors encoding either *AtPDS3* gRNA12 or *AtCHLI1* gRNA4 to *Arabidopsis* (Figure S1A), two gRNAs with high activity (Weiss et al. 2025). 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.

First, to find the optimal gRNA structure we identified the precise omega RNA (ωRNA) sequence via small RNA sequencing (RNA-seq) in *E. coli*, and found it to be 127-nucleotides (nt) in length (Figures S2A, S2B). In addition, we tested various ωRNA lengths and found that 127-nt gave the highest editing in protoplasts (Figures S2C, S2D). Using the 127-nt ωRNA, we tested multiplexed arrays featuring tRNA, HDV, HDV-HH, or a repeat as gRNA processing elements using an *Arabidopsis* protoplast assay (Figure S2F). Amp-seq analysis showed that while all designs enabled editing, HDV-based designs performed best at simultaneously editing both sites in protoplasts (Figure S2G). Furthermore, polymerase chain reaction (PCR) using primers spanning both sites suggested the occurrence of large deletions between the two target sites (Figure S2H). These experiments identified the HDV and HDV-HH designs as the top

63 performing multiplexing arrays, and that Ymu1 TnpB can generate large deletions
64 between two targeted sites.
65
66 To minimize RNA2 cargo size, the HDV multiplex array was selected for TRV-mediated
67 multiplexed editing *in planta*. Initially, we designed two RNA2 vectors targeting *AtCHLI1*
68 (gRNA4) and *AtPDS3* (gRNA12), incorporating a $tRNA^{Ileu}$ mobility sequence at the 3'
69 end of the cargo to facilitate systemic movement and heritability (Figure S3A). gRNA4
70 targets the gene body of *AtCHLI1* (biallelic edits create yellow tissue sectors) and
71 gRNA12 targets the promoter region upstream of the *AtPDS3* transcription start site (no
72 visible phenotype). After delivering TRV vectors, we did not observe any phenotypic
73 evidence of editing. Suspecting inefficient mobility or processing of the RNA2 cargo, we
74 tested three additional constructs containing a $tRNA^{Ileu}$ downstream of each HDV
75 ribozyme (Figure 1A). After TRV delivery, yellow sectors appeared on leaves for all
76 three vectors, indicating biallelic edits at *AtCHLI1* (Figure 1B). Yellow sectored plants
77 infected with vectors pTW2278 and pTW2279 displayed average editing efficiencies of
78 25.6% for *AtCHLI1* and 30.2% for *AtPDS3* (Figure 1C), and those infected with
79 pTW2498 showed 31.3% for *AtCHLI1* gRNA4 and 5.2% for *AtCHLI1* gRNA6 (Figure
80 1D).
81
82 We recently engineered a highly active Ymu1 variant (Ymu1-WFR) (Zhou et al. 2026).
83 To evaluate Ymu1-WFR efficiency using TRV, we targeted three published *AtCHLI1*
84 target sites: gRNA4, gRNA6, and gRNA9 (Weiss et al. 2025). Infected plants showed a
85 strong yellow phenotype for all three gRNAs (Figure 1E). Amp-seq revealed average
86 editing efficiencies of 18.6%, 14.7%, and 9.8%, respectively, much higher (up to 9.8-
87 fold) than wild type (WT) Ymu1 (Weiss et al. 2025) (Figure 1F).
88
89 To assess the impact of the WFR variant on multiplexed editing efficiency, we replaced
90 the WT Ymu1 sequence in pTW2278, pTW2279, and pTW2498 with Ymu1-WFR
91 (Figure S3B). Following TRV delivery, we observed much more pronounced phenotypic
92 evidence of editing than with WT Ymu1 (Figure 1G compared with Figure 1B). Amp-seq
93 of yellow sectored plants confirmed enhanced editing: pTW2657 and pTW2658

averaged 48.9% for *AtCHLI1* (gRNA4) and 41.3% for *AtPDS3* (gRNA12) (Figure 1H), while pTW2655 averaged 45.3% and 28.1% at the two *AtCHLI1* sites (Figure 1I). Consistent with the protoplast result (Figure S2H), PCR analysis using primers spanning the *AtCHLI1* gRNA4 and gRNA6 sites revealed large deletions (Figures S3C, S3D).

To characterize germline transmission of multiplexed *AtPDS3* gRNA12 and *AtCHLI1* gRNA4 edits, we selected two plants infected with WT Ymu1 (pTW2278_17 and pTW2278_54) and two plants infected with Ymu1-WFR (pTW2658_5 and pTW2658_80). We observed yellow progeny at frequencies of 4.7% and 1.7% using WT Ymu1, and 6.1% and 12.9% with Ymu1-WFR (Figure 1J, S3E, Table S1). Among the yellow seedlings, the majority of them harbored biallelic edits at both loci (Figure 1J, S3F). Additionally, targeting *AtCHLI1* with two gRNAs, gRNA4 and gRNA6 (using pTW2655), resulted in 8.2% and 24.1% yellow seedlings, with 36/158 (22.8%) of the progeny harboring homozygous large deletions between the two targets (Figures 1J, S3G-I). These data demonstrate that TRV effectively delivers Ymu1-WFR and multiple gRNAs for efficient multiplexed germline editing, that biallelic editing at one locus is highly predictive of biallelic editing at the second target site, and that co-targeting the same gene gave progeny with large deletions between the two target sites.

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 many crop species, for example tomato where germline editing has already been demonstrated (Liu et al. 2025). 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 is accessible at NCBI Sequence Read Archive BioProject PRJNA1427739. 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.

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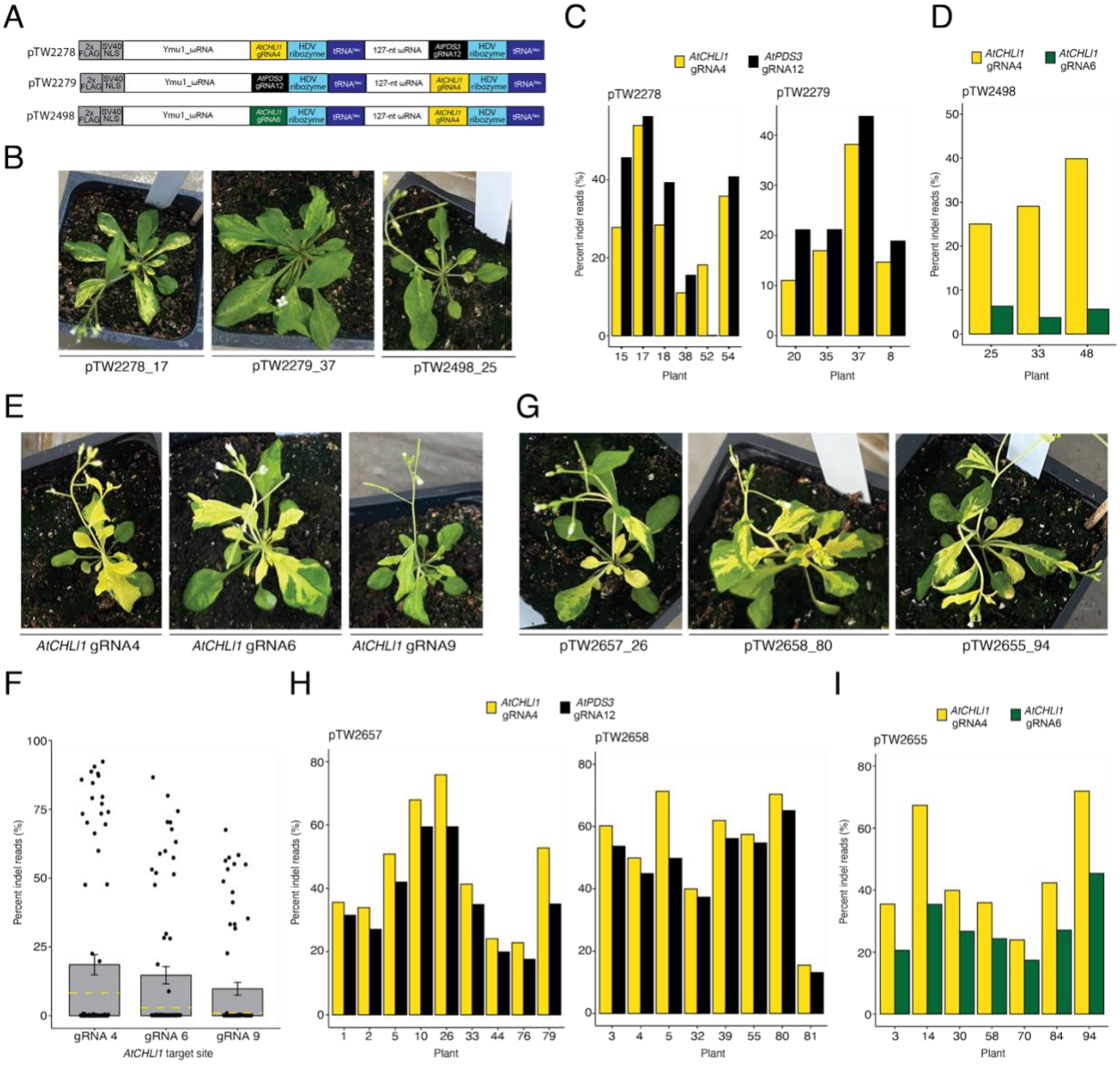

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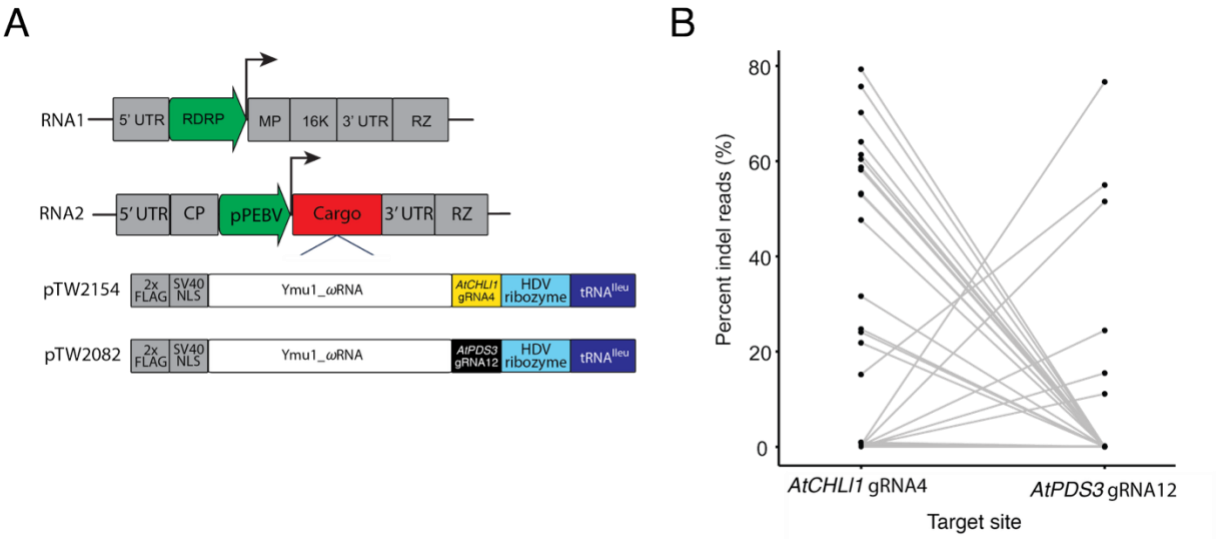
Parent ID	TnpB	# of plants	Yellow plants (%)	-/- *AtCHLI1* , -/- *AtPDS3* (% of yellow plants)	-/- *AtCHLI1* , WT/- *AtPDS3* (% of yellow plants)	-/- *AtCHLI1* , WT/WT *AtPDS3* (% of yellow plants)
pTW2278_17	Ymu1	549	26 (4.7%)	20 (76.9%)	2 (7.7%)	4 (15.4%)
pTW2278_54	Ymu1	541	9 (1.7%)	6 (66.7%)	2 (22.2%)	1 (11.1%)
pTW2658_5	Ymu1-WFR	346	21 (6.1%)	15 (71.4%)	1 (4.8%)	5 (23.8%)
pTW2658_80	Ymu1-WFR	449	58 (12.9%)	26 (44.8%)	20 (34.5%)	12 (20.7%)
pTW2655_14	Ymu1-WFR	503	121 (24.1%)	NA	NA	NA
pTW2655_94	Ymu1-WFR	452	37 (8.2%)	NA	NA	NA

Figure 1: Heritable and transgene-free multiplexed genome editing in *Arabidopsis* via viral delivery of Ymu1. Details provided in Supplementary Materials.

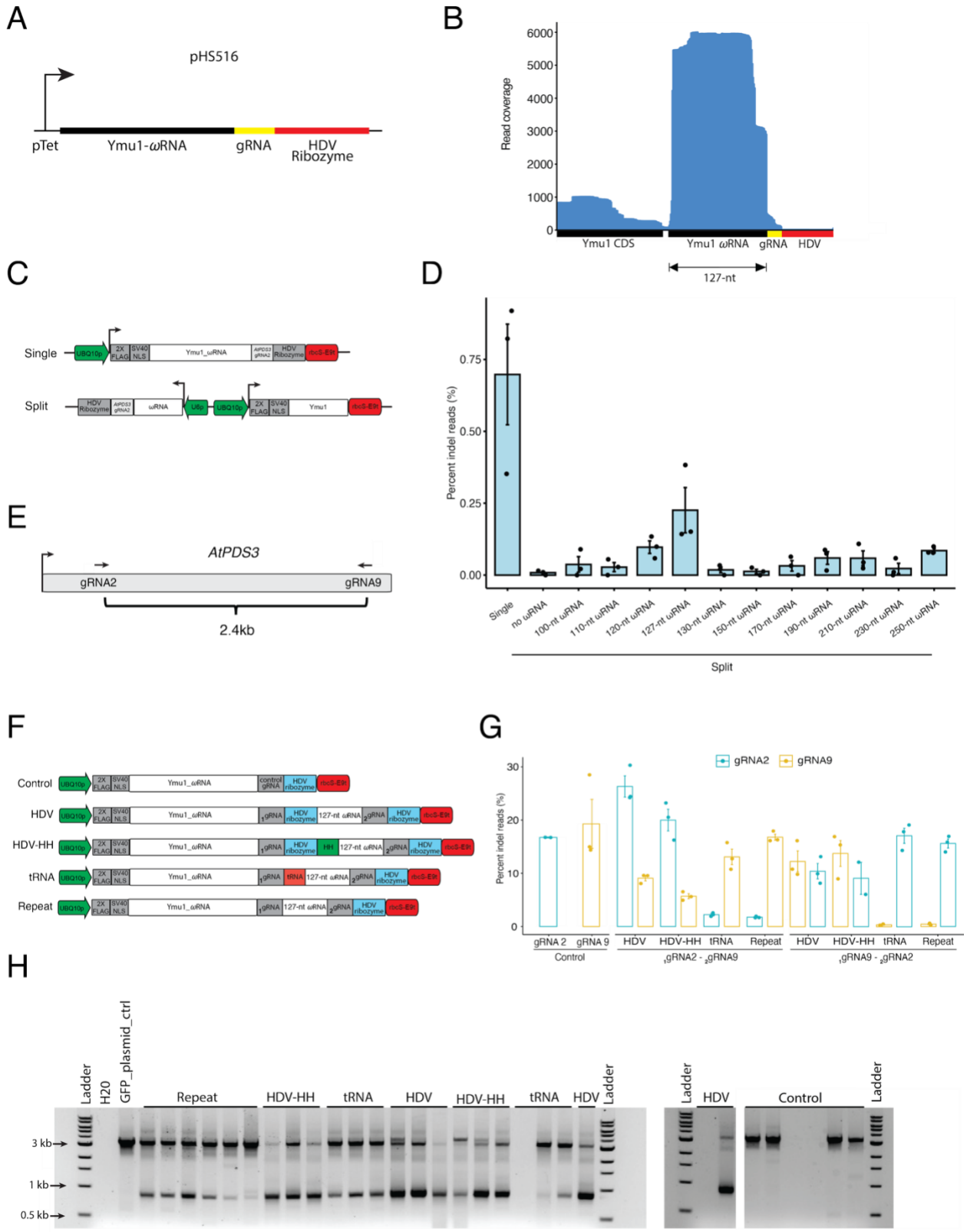
Supplementary Materials

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

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 plasmid ID is listed to the left of each construct. **(B)** Yellow sector phenotype observed from plants infected with the TRV vectors from panel A. The plant ID (construct_plant) is listed below each picture. **(C and D)** Editing efficiency (y-axis) of plants displaying the yellow sector phenotype (x-axis) infected with TRV vectors pTW2278, pTW2279 and pTW2498. **(E)** Yellow sector phenotype observed from plants infected with TRV vectors expressing Ymu1-WFR targeting *AtCHLI1* (gRNA4, gRNA6, and gRNA9). The site being targeted is listed below each picture. **(F)** Editing efficiency (y-axis) of plants infected with TRV vectors expressing Ymu1-WFR targeting *AtCHLI1* (x-axis). Each dot represents an individual plant. The yellow dashed line on each bar indicates the average editing efficiency previously reported (Weiss et al. 2025). **(G)** Yellow sector phenotype observed from plants infected with TRV vectors expressing Ymu1-WFR targeting *AtCHLI1* gRNA4 and *AtPDS3* gRNA12 (pTW2657 and pTW2658) or *AtCHLI1* gRNA4 and *AtCHLI1* gRNA6 (pTW2655). The plant ID (construct_plant) is listed below each picture. **(H and I)** Editing efficiency (y-axis) of plants displaying the yellow sector phenotype (x-axis) infected with TRV vectors pTW2657, pTW2658 and pTW2655. (**J**) Heritability data from plant ID pTW2278_17, pTW2278_54, pTW2658_5, pTW2658_80, pTW2655_14, and pTW2655_94.

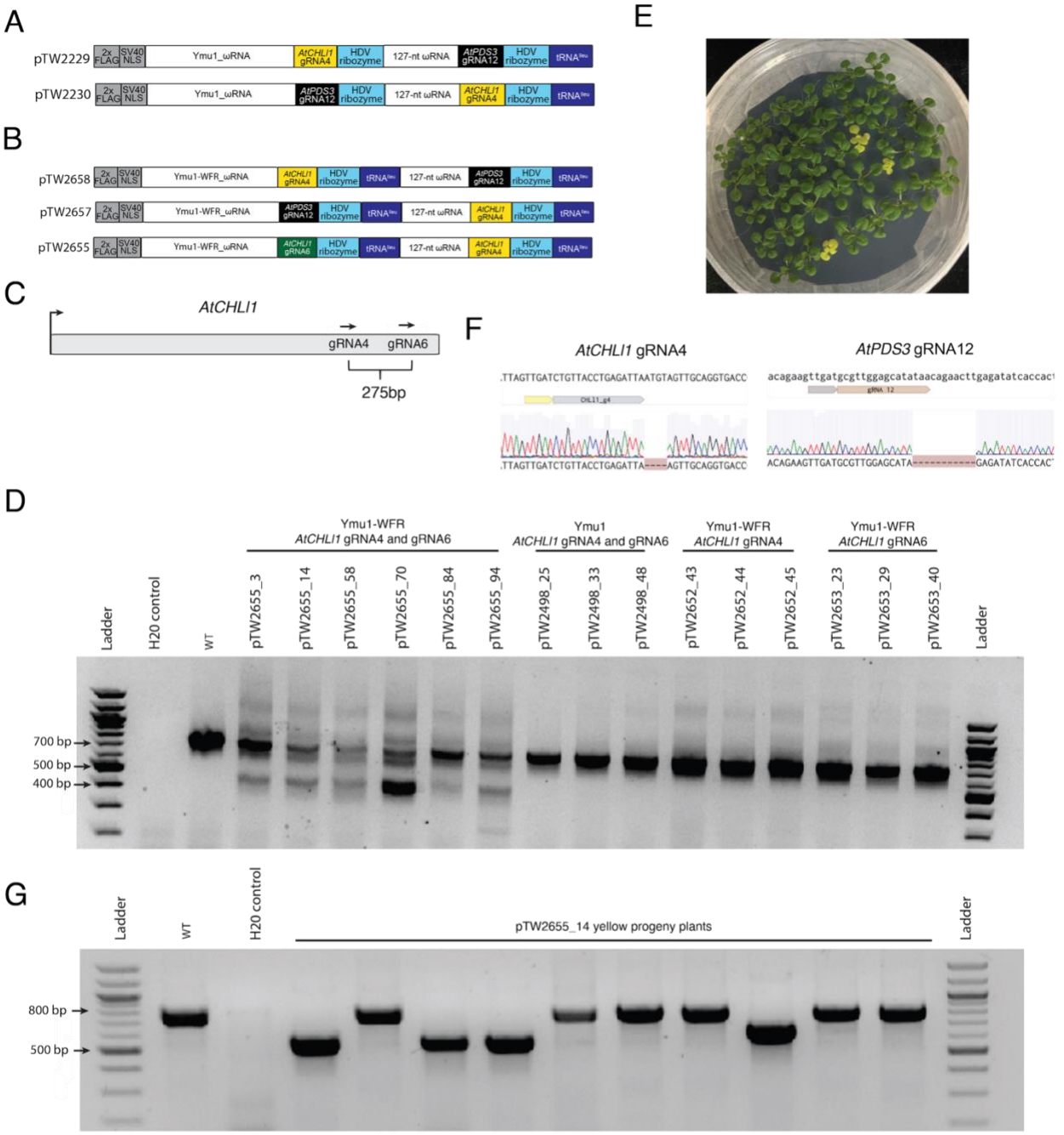


Supplementary Figure 1: Co-delivery of RNA1 and two RNA2 vectors does not enable multiplexed genome editing. (A) Schematic representation of TRV RNA1 and RNA2. The cargo and plasmid ID are listed below RNA2. **(B)** Editing efficiency (y-axis) of plants co-infected with RNA1 and two RNA2 vectors targeting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



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). The plasmid name (pHS516) is listed above the schematic. **(B)** RNA-seq in *E. coli* using the plasmid shown 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)** *AtPDS3* gRNA2 editing efficiency (y-axis) using single expression and split expression constructs with varying lengths of ωRNA (x-axis) as shown in panel C. 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.



Parent ID	# of plants	Yellow plants (%)	Homozygous large deletion (% of yellow plants)
pTW2655_14	503	121 (24.1%)	34 (28.1%)
pTW2655_94	452	37 (8.2%)	2 (5.4%)

Supplementary Figure 3: TRV-delivery of Ymu1 for multiplexed genome editing. (**A**) Schematic representation of the multiplexed cargo being expressed by the PEBV

promoter in TRV RNA2 targeting *AtCHLI1* gRNA4 and *AtPDS3* gRNA12, containing a single 3' $tRNA^{Ileu}$. The plasmid ID is listed to the left of each construct. (**B**) Schematic representation of the Ymu1-WFR multiplexed gRNA 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 plasmid ID is listed to the left of each construct. (**C**) Schematic of *AtCHLI1* gene with the arrows indicating the orientation of gRNA4 and gRNA6, located approximately 275 bp apart. (**D**) Somatic editing analysis of infected plants. 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. (**E**) Representative image of yellow and green progeny seedlings from plant pTW2278_17. All yellow seedlings harbored biallelic edits at the *AtCHLI1* gRNA4 target site. (**F**) Sanger sequencing trace file screenshots from a yellow seedling in panel E, harboring homozygous edited alleles at *AtCHLI1* gRNA4 (4bp deletion) and *AtPDS3* gRNA12 (11bp deletion). (**G**) Representative PCR gel electrophoresis image of progeny from pTW2655_14 using primers spanning *AtCHLI1* gRNA4 and gRNA6 target sites. Image shows the presence of deletion between *AtCHLI1* gRNA4 and gRNA6. (**H**) Heritability data from plants pTW2655_14, and pTW2655_94 infected with TRV targeting *AtCHLI1* gRNA4 and gRNA6 (pTW2655). Large deletions were classified as greater than 275 bp deletion between the two target sites. (**I**) Sanger sequencing trace file screenshot from a yellow plant targeted with *AtCHLI1* gRNA4 and gRNA6 harboring a homozygous large deletion (277 bp).

Parent ID	pTW2278_54 (# of progeny)	pTW2278_17 (# of progeny)
# of green plants sequenced	191	232
WT/- *AtCHLI1*, -/- *AtPDS3*	8	11
WT/WT *AtCHLI1*, -/- *AtPDS3*	9	20
WT/- *AtCHLI1*, WT/- *AtPDS3*	2	5
WT/- *AtCHLI1*, WT/WT *AtPDS3*	2	4
WT/WT *AtCHLI1*, WT/- *AtPDS3*	6	33
WT/WT *AtCHLI1*, WT/WT *AtPDS3*	164	159

387 **Supplementary Table 1**: Genotype characterization of green progeny from plants infected with
388 TRV expressing Ymu1 and gRNAs targeting *AtCHLI1* gRNA4 and *AtPDS3* gRNA12
389 (pTW2278_54 and pTW2278_17).
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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 Ymu1, *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second.
pTW2110	Repeat design multiplex vector expressing Ymu1, *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pTW2137	tRNA design multiplex vector expressing Ymu1, *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2140	tRNA design multiplex vector expressing Ymu1, *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pTW2148	HDV design multiplex vector expressing Ymu1, *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2138	HDV design multiplex vector expressing Ymu1, *AtPDS3* gRNA9 first in the array and *AtPDS3* gRNA2 second
pTW2136	HDV-HH design multiplex vector expressing Ymu1, *AtPDS3* gRNA2 first in the array and *AtPDS3* gRNA9 second
pTW2139	HDV-HH design multiplex vector expressing Ymu1, *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
pTW2684	Cloning vector for PaqCI Golden Gate assembly of Ymu1-WFR guides into TRV2

415 **Supplementary Table 2:** Plasmids and their descriptions used in this study.
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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	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGAGGTTTATCTTGATCGGTTC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCACGGAAATCCTTTGGGTTACTA	NGS amplicon sequencing
AT4G14210	*AtPDS3* gRNA12	gcgttggagcatataa	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaccgacccgagaagagatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtggaatacacacatttgtacaacca	NGS amplicon sequencing
AT4G14210	*AtPDS3* gRNA9	agctttgaaccggttt	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgcgctaaactttataaacccctgatg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtatgattgccagtgagataaagattc	NGS amplicon sequencing
AT4G14210	*AtPDS3*	see above	tgttacacaacttat	ggaagtagccgata	Sanger

	gRNA12		tatgattgggct	acaaaatggag	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 (figure S3D)
AT4G18480	*AtCHLI1* gRNA4 and *AtCHLI1* gRNA6	see above	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaagttaggaacatgattgttgttggt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCACGGAAATCCTTTGGGTTACTA	PCR spanning both target sites and Sanger sequencing (Figures S3G-I)

Supplementary Table 3: Target sites and primers used in this study.

448 **Supplementary Methods:**
449 Plasmids used in this study
450 To generate the Ymu1 plasmids with varying ωRNA lengths, NEB HiFi assembly was performed
451 using two PCR amplicons and pTW2064 (the no ωRNA plasmid backbone). One amplicon
452 contained the U6 promoter and the other amplicon contained the ωRNA. First, pTW2064
453 underwent a restriction enzyme digestion with SpeI (R3133) and Quick CIP (M0525) to linearize
454 the plasmid. Next, both amplicons and the digested and purified pTW2064 plasmid were used in
455 an NEBuilder HiFi DNA Assembly (E2621) reaction.
456
457 Multiplex vectors used in the protoplast experiment expressing wild type Ymu1, *AtPDS3* gRNA2
458 and *AtPDS3* gRNA9 were created using a golden gate reaction. PCR amplicons or gene
459 fragments (Twist Bioscience) containing the first gRNA, processing sequence, 127-nt ωRNA
460 and the second gRNA were inserted into the pMK025 vector (Weiss et al. 2025) using a PaqCI
461 (R0745) golden gate reaction.
462
463 Multiplex TRV2 vectors were created using a golden gate reaction assembly. First, PCR was
464 performed to generate two amplicons; one amplicon contained the first gRNA in the array, an
465 HDV ribozyme and $tRNA^{Ileu}$, and the second amplicon contained the 127-nt ωRNA and second
466 gRNA in the array. Amplicons contained overhangs with PaqCI (R0745) sites to facilitate
467 assembly. Both amplicons and pMK435 (Weiss et al. 2025) were then used in a PaqCI golden
468 gate reaction.
469
470 TRV2 vectors containing the Ymu1-WFR variant were created using NEBuilder HiFi DNA
471 Assembly (E2621). The TRV2 vectors were constructed by digesting the wild type Ymu1 TRV2
472 vectors with HpaI (R0105) and Eco53KI (R0116) restriction enzymes. A gene fragment (Twist
473 Bioscience) containing the Ymu1-WFR sequence was then cloned into the digested plasmid
474 using NEBuilder HiFi DNA Assembly (E2621).
475
476 Following assembly, all plasmids were transformed into NEB 10-beta (C3019) *E.coli* and
477 validated by whole-plasmid sequencing. Plasmid names and descriptions can be found in Table
478 S2. To enable widespread use of single-target and multiplexed TRV2 vectors containing the
479 Ymu1-WFR variant, we developed a TRV2 ccdb guide dropout vector (pTW2684) and a cloning
480 protocol (Supplementary Protocol).
481
482 Plant gene editing experiments
483 *Arabidopsis* mesophyll protoplasts were isolated according to previously described methods
484 (Yoo et al. 2007). For protoplast transfection, 20 µg of plasmid was placed at the bottom of each
485 tube and brought to a total volume of 20 µl with water. Then, 200 µl of protoplasts were added
486 to the plasmid solution, followed by 220 µl of fresh, sterile polyethylene glycol (PEG)-$CaCl_2$
487 solution. The samples were mixed by gentle tapping and incubated at room temperature for 10
488 minutes. Transfection was terminated by adding 880 µl of W5 solution and inverting the tubes
489 two to three times. Protoplasts were subsequently pelleted by centrifugation at 100 RCF for 3
490 minutes, resuspended in 1 ml of WI solution, and plated in 6-well plates precoated with 5% calf
491 serum. The cells were incubated at 26°C for 48 hours. During this incubation period, a 37°C

492 heat-shock treatment was applied for 2 hours, beginning 16 hours post-transfection. At 48 hours
493 post-transfection, the protoplasts were collected for genomic DNA extraction.
494
495 TRV delivery was performed as previously described (Weiss et al. 2025; Nagalakshmi et al.
496 2022). TRV1 and TRV2 vectors were introduced into *Agrobacterium tumefaciens* strain
497 GV3101, and cultures were grown in 200 ml of lysogeny broth (LB) with antibiotics for 18 hours
498 at 28°C. The cultures were centrifuged at 3,500 × g for 20 minutes; the supernatant was
499 discarded, and the pellets were resuspended in 200 ml of sterile water. This suspension was
500 centrifuged again at 2,109 × g for 10 minutes. After discarding the supernatant, the pellet was
501 resuspended in sterile agro-infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 250 μM
502 acetosyringone) to a final optical density OD_{600} of 1.5 and incubated at 23°C for 3 hours with
503 slow shaking. Following incubation, the *Agrobacterium* cultures harboring TRV1 and TRV2 were
504 mixed in a 1:1 ratio (co-delivery experiment used a ratio of two parts TRV1, one part pTW2154,
505 and one part pTW2082). 15 ml of this mixture was delivered to 10-day-old seedlings via
506 agroflood co-culture. After 4 days, the seedlings were transplanted to soil and grown at room
507 temperature for the remainder of their life cycle.
508
509 Approximately 12 weeks after viral delivery, seeds were harvested from TRV-infected plants.
510 Seeds were sown on ½ MS plates supplemented with 3% sucrose and stratified at 4°C in the
511 dark for 5 days. Following stratification, the seeds were moved to a growth room under a 16-h
512 light/8-h dark cycle at 23°C for 10–12 days. A subset of plants was then sampled for genotyping
513 by collecting a single piece of leaf tissue. DNA was extracted using the Invitrogen Platinum
514 Direct PCR Universal Master Mix (A44647500) according to the manufacturer's instructions.
515 Finally, the extracted DNA was analyzed via PCR and Sanger sequencing using the primers
516 listed in Table S3.
517
518 Amplicon sequencing and analysis
519 Genomic DNA was extracted from protoplast samples using the Qiagen DNeasy Plant Mini Kit
520 (Qiagen, 69106). For plants subjected to agroflood, three leaf tissue samples distal to the TRV
521 delivery site were pooled per plant about 3-4 weeks after viral delivery. The ratio of green to
522 yellow tissue samples collected were selected in a manner that most accurately reflected the
523 phenotype of the entire plant. Once collected, all tissue samples were frozen overnight at −80°C
524 and ground. DNA was subsequently extracted using the Invitrogen Platinum Direct PCR
525 Universal Master Mix (A44647500) according to the manufacturer’s instructions. The DNA was
526 then used for next-generation amplicon sequencing.
527
528 Amplicon sequencing was performed using single-end sequencing on the Illumina NovaSeqX
529 platform. Libraries were constructed using a two-step PCR approach. First, target regions were
530 amplified for 25 cycles using primers flanking the TnpB target site (Table S3), followed by
531 purification with 1.0× AMPure XP beads (Beckman Coulter, A63881). Then, samples underwent
532 12 additional cycles of amplification to attach Illumina indexing primers, followed by a 0.7×
533 AMPure XP cleanup. Final libraries were normalized and pooled for sequencing. Amplicon
534 sequencing analysis was performed using the CrispRvariants R package (v.1.14.0) as
535 previously described (Weiss et al. 2025).

536
537 Bacterial RNA expression and sequencing
538 For heterologous RNA expression, *E. coli* transformed with pHS516 (Figure S2A, Table S2) and
539 grown in LB medium to an OD_{600} of 0.6 and induced with 10 mM arabinose at 16°C overnight.
540 Following overnight growth, total RNA from cells was extracted using a hot formamide method,
541 in which pelleted *E. coli* were resuspended in an 18 mM EDTA and 95% formamide solution and
542 lysed by incubating at 65ºC for 5 minutes. After centrifugation to pellet cell debris, the
543 supernatant containing total RNA was purified using the RNA Clean & Concentrator-5 Kit (Zymo
544 Research R1013) following manufacturer protocols. Approximately 200 ng of purified total RNA
545 was subjected to rRNA depletion using the NEBNext rRNA Depletion Kit for Bacteria (New
546 England Biolabs E7850L) following manufacturer protocols. rRNA-depleted samples were again
547 purified using the RNA Clean & Concentrator-5 Kit and subjected to end repair and small-RNA-
548 seq library prep as previously described (Zhou et al. 2026). Sequencing was performed using
549 an Illumina NextSeq 1000/2000 P2 v3 kit. Following sequencing, paired-end reads were
550 trimmed and merged using fastp (v0.23.2) (Chen et al. 2018). Merged reads of 100–150
551 nucleotides were selected with fastq-filter (v0.3.0; https://github.com/LUMC/fastq-filter) and
552 aligned to the Ymu1 locus using BWA (v0.7.17) (Li 2013). Alignments were sorted with
553 SAMtools (v1.17) (Li et al. 2009) and converted to BigWig format using deepTools2 (v3.5.1)
554 (Ramírez et al. 2014), then visualized in the Integrative Genomics Viewer (IGV) (Robinson et al.
555 2011).
556
557
558
559
560
561
562 **Supplementary Protocol:**
563 Protocol for cloning guides into pTW2684
564 pTW2684 is a TRV2 vector containing Ymu1-WFR and the ωRNA driven by the PEBV sub
565 genomic promoter. In place of the 16bp gRNA spacer there is a ~1700 bp insert carrying the
566 toxic ccdb gene and chloramphenicol (CAM) resistance. The insert is flanked by PaqCI Type-II
567 restriction sites, enabling golden gate cloning to replace the toxic ccdb insert with the desired
568 guide sequence.
569
570 **Single target site**
571 1) Order the 16bp guide sequence with 4 bp overhangs as complimentary single
572 stranded oligos from IDT and anneal and phosphorylate them as described below:
573
574 Top Oligo: 5'-TCAAXXXXXXXXXXXXXXXX-3'
575 Bottom Oligo: 3'-YYYYYYYYYYYYYYYYCCGG-5'
576 *Underlined nucleotides are complimentary to the overhangs created by PaqCI digestion
577 of pTW2684
578
579 Phosphorylation and annealing reaction setup:

3 µL 100 µM Top oligo
3 µL 100 µM Bottom oligo
3 µL T4 DNA ligase buffer (with ATP)
2 µL T4 polynucleotide kinase (PNK)
19 µL Water

2) Place samples in a thermal cycler and run the following program: 37°C/30 min + 95°C/5 min + 90°C/1 min + 85°C/1 min + 80°C/1 min + 75°C/1 min + 70°C/1 min + 65°C/1 min + 60°C/1 min + 55°C/1 min + 50°C/1 min + 45°C/1 min + 40°C/1 min + 35°C/1 min + 30°C/1 min + 25°C/hold

3) Next, set up a golden gate reaction to clone the annealed oligos into pTW2684.

Golden gate reaction setup:
50 ng pTW2684
1 µL annealed oligos (diluted 1:25)
0.5 µL PaqCI enzyme
0.5 µL PaqCI Activator
2 µL T4 DNA ligase buffer
1 µL of T4 DNA ligase
Water up to 20 µL

4) Place samples in a thermal cycler and run the following program:
10x(37°C/5min + 16°C/10min) + 37°C/15min + 80°C/5min + 4°C hold

5) Transform 2-5 µL of the reaction into NEB 10-beta (C3019) *E.coli* cells and select for successfully cloned plasmids using kanamycin plates.

Multiplexed vector containing two target sites

Cloning two gRNAs into pTW2684 requires a golden gate reaction with two PCR amplicons. This example describes the cloning strategy to build pTW2658 (*AtCHLI1* gRNA4 and *AtPDS3* gRNA12). To clone different gRNAs you can either first clone each guide separately into pTW2684 using the protocol above, and then use that plasmid as a PCR template, or order synthesized DNA fragments.

1) Perform two PCR reactions and run the samples on a gel to confirm the correct size. (rxn 1 is 192 bp and rxn 2 is 185 bp). Purify the PCR reaction and nanodrop.
Guide RNA
PaqCI overhang
PaqCI binding site
HDV ribozyme
$tRNA^{Ileu}$
ωRNA

1F (5’ – 3’): gtcaccacctgcgatgTCAACTGTTACCTGAGATTAggccgg
1R (5’ – 3’): gactagacacctgcgttcTGCTTCCGGCGGGG
2F (5’ – 3’): gtcaccacctgcgatgAGCACAAACAGGAACCGCAGGAATTG
2R (5’ – 3’): gactagacacctgcgttcGGCCttatatgctccaacgcTTGAAACTC
**Note that the guide sequence in 2R is the reverse complement

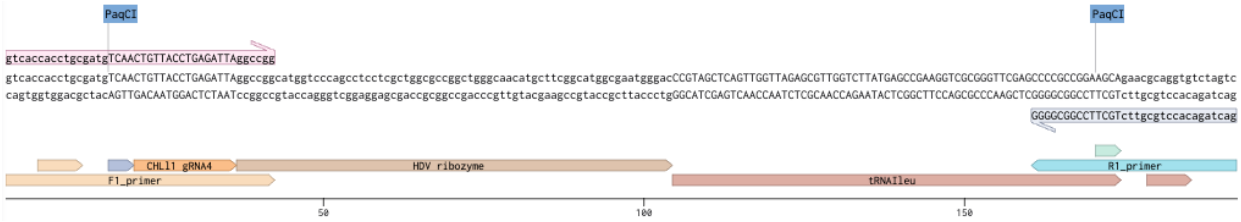
PCR rxn	F primer	R primer	DNA template	Purpose
1	1F	1R	pTW2154	Amplify *AtCHLI1* gRNA4 (the first guide in the array)
2	2F	2R	pTW2082	Amplify *AtPDS3* gRNA12 (the second guide in the array)

PCR reaction
2.5 µl F primer 10 µM
2.5 µl R primer 10 µM
2 ng Template plasmid DNA
25 µl Q5 Hi Fidelity DNA polymerase master mix
Water up to 50 µl

Place samples in a thermal cycler and run using the following settings:

1. 98°C - 30 sec
2. 98°C - 10 sec
3. 55°C - 20 sec
4. 72°C - 20 sec
5. Repeat steps 2-4 35x
6. 72°C - 2 min
7. 4°C - hold

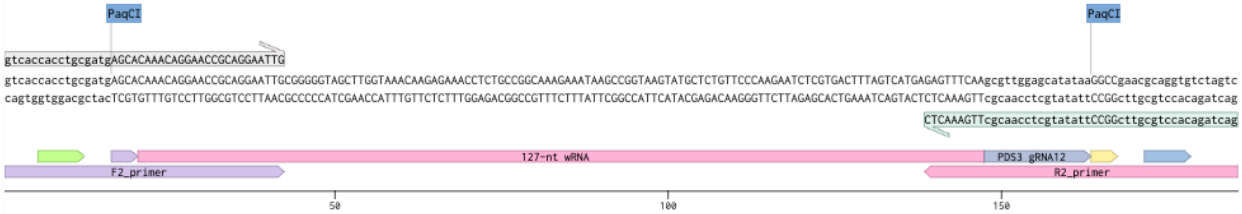
Amplicon 1
Sequence (5’ – 3’):
gtcaccacctgcgatgTCAACTGTTACCTGAGATTAggccggcatggtcccagcctcctcgctggcgccggctgggcaa
catgcttcggcatggcgaatgggacCCGTAGCTCAGTTGGTTAGAGCGTTGGTCTTATGAGCCGAAGG
TCGCGGGTTCGAGCCCCGCCGGAAGCAgaacgcaggtgtctagtc
**Note that the AGCA PaqCI overhang is part of the $tRNA^{Ileu}$ sequence.



Amplicon 2

Sequence (5' – 3'):

gtcaccacctgcgatgAGCACAAACAGGAACCGCAGGAATTGCGGGGGTAGCTTGGTAAACAAGA
GAAACCTCTGCCGGCAAAGAAATAAGCCGGTAAGTATGCTCTGTTCCCAAGAATCTCGTGA
CTTTAGTCATGAGAGTTTCAAgcgttggagcatataaGGCCgaacgcaggtgtctagtc



2) Perform golden gate reaction

100 ng pTW2684
100 ng amplicon 1
100 ng amplicon 2
0.5 µl PaqCI
0.5 µl PaqCI activator
1 µl T4 DNA ligase
2 µl T4 DNA ligase buffer
Water up to 20 µl

Place samples in thermal cycler and run the following program:
10x(37°C/5min + 16°C/10min) + 37°C/15min + 80°C/5min + 4°C hold

3) Transform 2-5 µL of the reaction into NEB 10-beta (C3019) *E.coli* cells and select for successfully cloned plasmids using kanamycin (KAN) plates.

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