SI Data

Figure S1. Cloning procedures and vector maps. A, procedure for cloning one sgRNA according to Fauser *et al.*, 2014. Two oligonucleotides are synthesized that have 4-bp overhangs for Type-IIS cloning and contain a guide sequence of 17 to 20 nucleotides. The annealed oligos are cloned in the pEN-C1.1 shuttle vector that contain the Arabidopsis U6-26 promoter driving the sgRNA. The G of the 5' ATTG overhang is the first transcribed base by the RNA polymerase III. After sequence verification, the vectors are recombined with the pDE-Cas9Km destination vector that contains the Cas9 (codon optimized for *Arabidopsis thalaiana*) under control of the *Petroselinum crispum* ubiquitin4-2 promoter (pPcUBI) and the *nptII* selection marker for plants B, dual sgRNA cloning. The pMR217 or pMR218 shuttle vectors used are identical, except for the MultiSite Gateway recombination sites (in blue). AmpR, KmR and SpR are ampicillin, kanamycin and spectinomycin resistance markers for *Escherichia coli*.

Figure S2. Comparison of TIDE spectra between leaves of the same T1 plant. Three true leaves (A, B and C) of a T1 plant were sampled. Genomic DNA was extracted, PCR amplified and sequenced. The indel spectrum is visualized with an estimated overall efficiency and the frequency of each indel using TIDE. Bars indicate the number of sequences with a given indel size. Pale green bars (indel size of zero) represents WT or base substitution alleles. A, example of a highly efficient edited T1 plant with low chimerism. B, example of a highly efficient edited T1 plant with high chimerism. C, example of a T1 plant with low editing efficiency.

Figure S3. *VQ33* dual sgRNA approach. A, genomic structure of *VQ33* and location of the sgRNAs. Dark green boxes designate exons; light green boxes, UTRs; solid lines, introns. B, PCR analysis of T1 lines. Leaf genomic DNA of 16 chimeric T1 plants was PCR amplified. The expected size of the WT *VQ33* amplicon is indicated as well as the expected size of the deletion of 459 bp between Cas9 cut sites. One T1 line having one T-DNA locus that was continued is highlighted with a green box. C, Cas9 PCR for the continued line in T2 generation. Putative Cas9 null-segregants are indicated with green boxes. D, Cas9 null-segregants were genotypes for *VQ33*. The selected plant 11-8 (*vq33-1*) is indicated with a triangle. E, Sequence alignment of the simultaneously targeted loci for Col-0 and *vq33-1*. PAMs are highlighted, the Cas9 cut sites indicated with triangles. A 458 bp deletion was detected and is indicated with dashed lines. The reading frame is marked.

Figure S4. *grxS17-4* is explained by loop out SD-MMEJ. A, *GRXS17* wild-type sequence surrouding the Cas9 cut site, which is indicated with a triangle. Primer repeat (P) regions are indicated in red with P2 break-proximal. Microhomology repeats (MH) are indicated in green. B, End resection by 5'-3' nuclease activity and unwinding by helicase activity. C, loop formation by P1-P2 basepairing. D, templated elongation by polymerase activity. E, unwinding. F, annealing of mh2 with mh1 templated complimentary overhang. G, observed repair product in *grxs*17-4. Inserted nucleotides in blue, resulting direct repeat underlined.

Figure S5. ***CRISPR workflow*****.** Schematic overview of the selection of Cas9 null-segregants with bi-allelic mutations in the T2 generation

Table S1. Oligonucleotides used in this study.

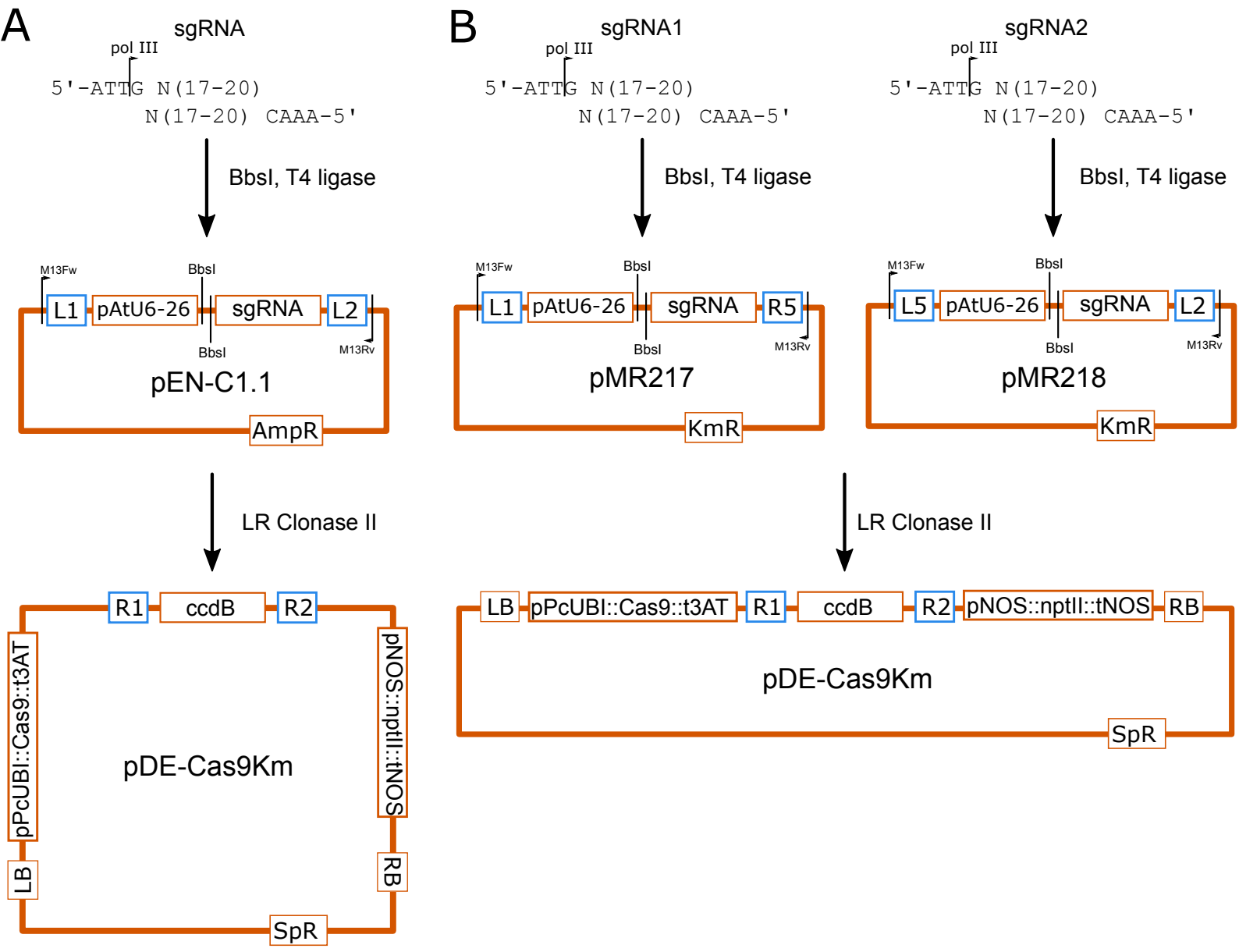

A
sgRNA
pol III
5'-ATTG N(17-20)
N(17-20) CAAA-5'
BbsI, T4 ligase
M13Fw
BbsI
L1
pAtU6-26
sgRNA
L2
BbsI
M13Rv
pEN-C1.1
AmpR
LR Clonase II
R1
ccdB
R2
pPcUBI::Cas9::t3AT
pNOS::nptII::tNOS
LB
RB
pDE-Cas9Km
SpR
B
sgRNA1
pol III
5'-ATTG N(17-20)
N(17-20) CAAA-5'
BbsI, T4 ligase
M13Fw
BbsI
L1
pAtU6-26
sgRNA
R5
BbsI
M13Rv
pMR217
KmR
sgRNA2
pol III
5'-ATTG N(17-20)
N(17-20) CAAA-5'
BbsI, T4 ligase
M13Fw
BbsI
L5
pAtU6-26
sgRNA
L2
BbsI
M13Rv
pMR218
KmR
LR Clonase II
LB
pPcUBI::Cas9::t3AT
R1
ccdB
R2
pNOS::nptII::tNOS
RB
pDE-Cas9Km
SpR

Figure S1

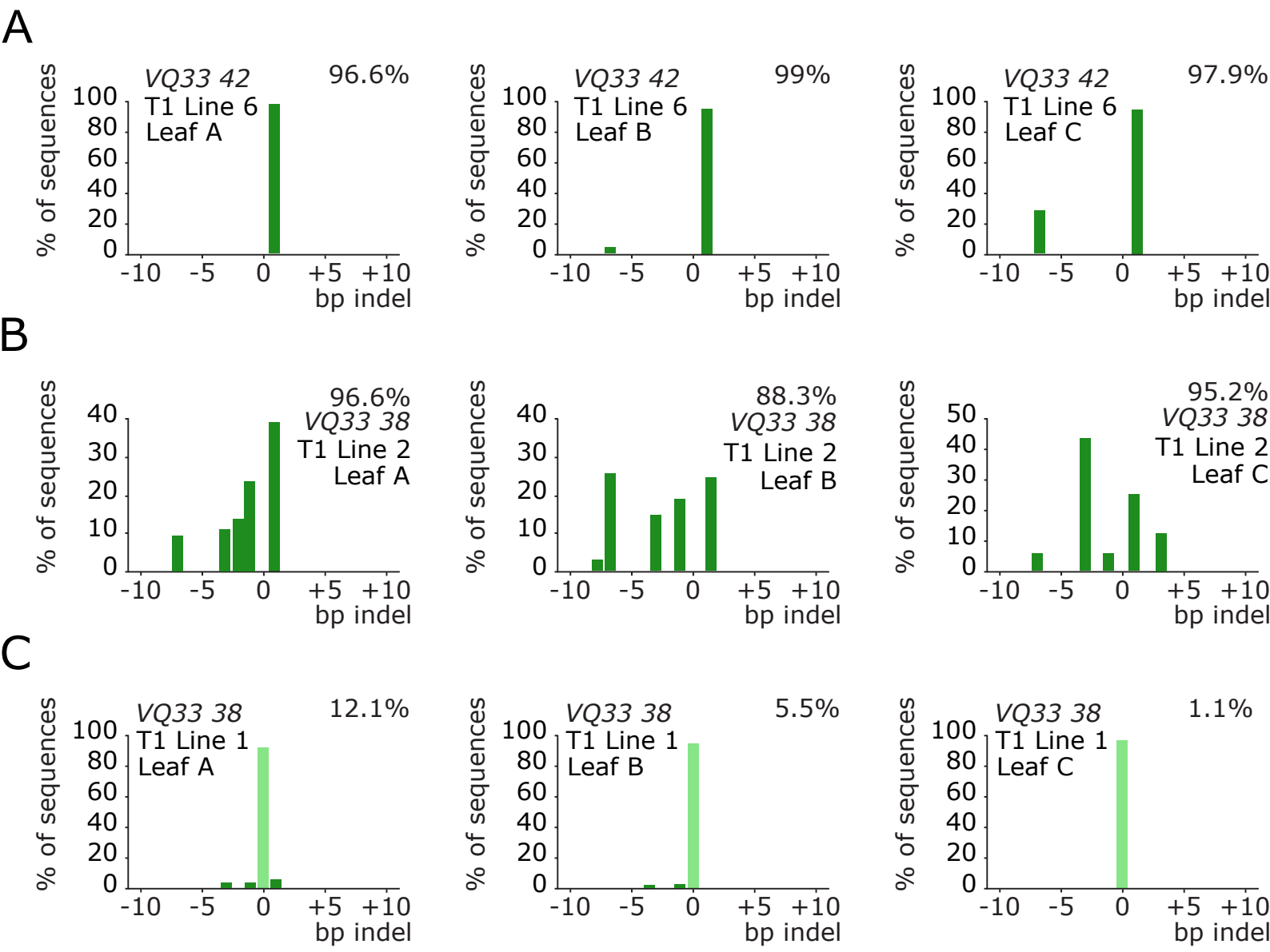

A
VQ33 42
T1 Line 6
Leaf A
96.6%
VQ33 42
T1 Line 6
Leaf B
99%
VQ33 42
T1 Line 6
Leaf C
97.9%
B
96.6%
VQ33 38
T1 Line 2
Leaf A
88.3%
VQ33 38
T1 Line 2
Leaf B
95.2%
VQ33 38
T1 Line 2
Leaf C
C
VQ33 38
T1 Line 1
Leaf A
12.1%
VQ33 38
T1 Line 1
Leaf B
5.5%
VQ33 38
T1 Line 1
Leaf C
1.1%
% of sequences
bp indel
-10
-5
0
+5
+10

Figure S2

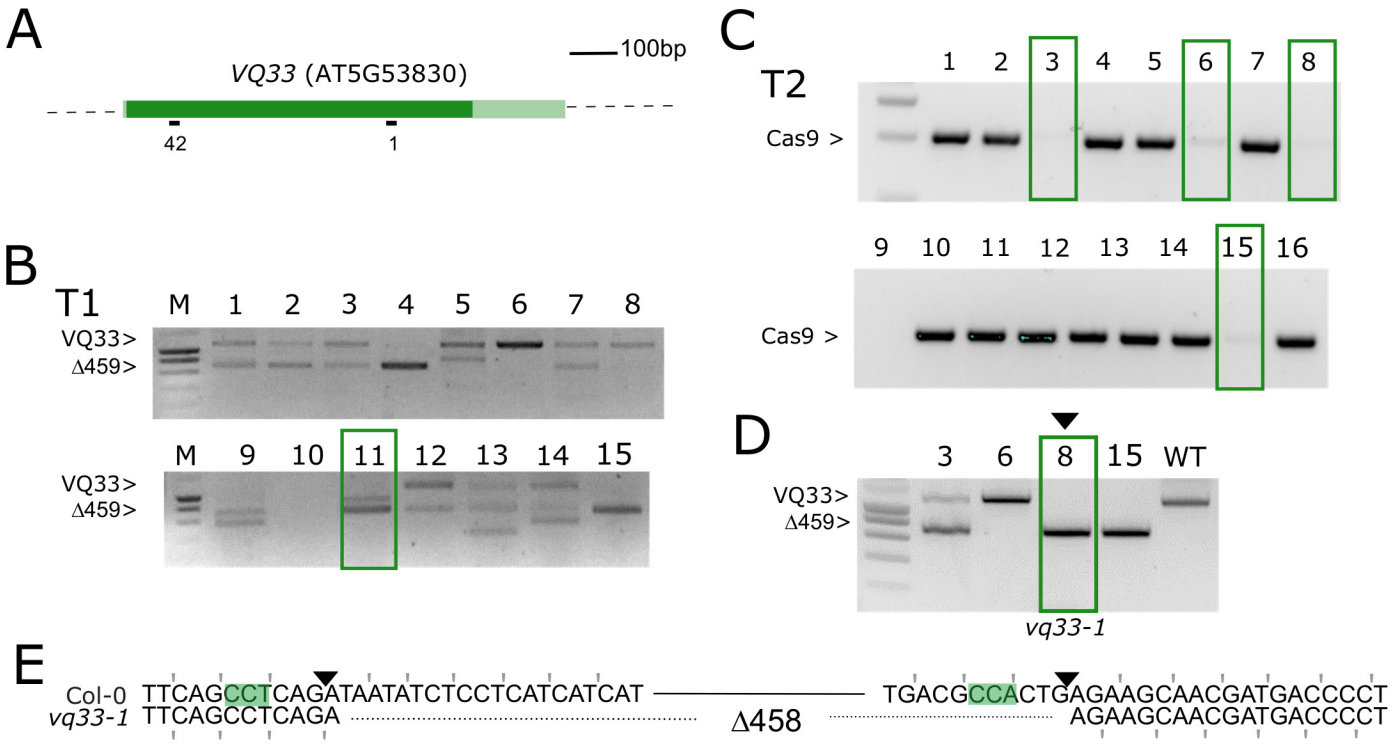

A
100bp
VQ33 (AT5G53830)
42
1
B
T1
M 1 2 3 4 5 6 7 8
VQ33>
Δ459>
M 9 10 11 12 13 14 15
VQ33>
Δ459>
C
T2
1 2 3 4 5 6 7 8
Cas9 >
9 10 11 12 13 14 15 16
Cas9 >
D
3 6 8 15 WT
VQ33>
Δ459>
vq33-1
E
Col-0 TTCAGCCTCAGATAATATCTCCTCATCATCAT
vq33-1 TTCAGCCTCAGA
Δ458
TGACGCCACTGAGAAGCAACGATGACCCCT
AGAAGCAACGATGACCCCT

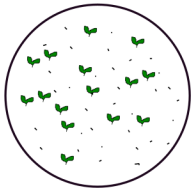
Figure S3


A
ATGAGCGGTACGGTGAAGGATATCGTTTCAAAGGCGGAGCTTGATAACTTGCGCCAGAGCGGCGCACCAGTCGTGCTTC
P1
mh1
P2
mh2
B
ATGAGCGGTACGGTGAAGGATATCGTTTCAAAGGCGG
CTTC
TACTCGCCATGCCACTTCCTATAGCAAAGTTTCCGCC
GTCAGCACGAAG
C
AGCGGTACGGTGAAGGA
ATATCGTTTCAAAG
ATG GCGG
CTTC
TACTCGCCATGCCACTTCCTATAGCAAAGTTTCCGCC
GTCAGCACGAAG
D
AGCGGTACGGTGAAGGA
ATATCGTTTCAAAG
ATG GCGGTAC
CTTC
TACTCGCCATGCCACTTCCTATAGCAAAGTTTCCGCC
GTCAGCACGAAG
E
ATGAGCGGTACGGTGAAGGATATCGTTTCAAAGGCGGTAC
CTTC
TACTCGCCATGCCACTTCCTAT
GTCAGCACGAAG
F
ATGAGCGGTACGGTGAAGGATATCGTTTCAAAGGCGGTACAGTCGTGCTTC

Figure S4

T0

> Floral dip



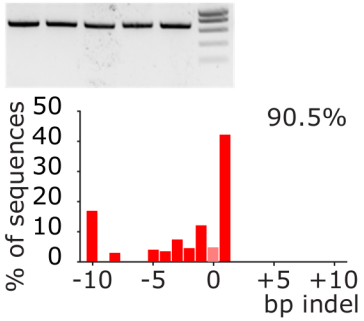
T1

> Select seeds on Km
> Transfer **15 seedlings** to soil

> PCR amplify GOI (~700 bp)

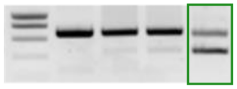
a) 1 sgRNA

- Sequence + TIDE analysis
- Chimeric at this stage
- Evaluate overall efficiency



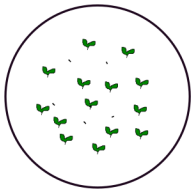
b) dual-sgRNA

- Evaluate efficiency on gel



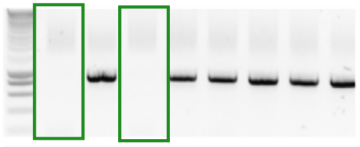
T2

> Harvest seeds of **~5** most promising lines
> Evaluate T2 seedlings on Km
> Select line(s) with 1 T-DNA locus



> Sow **~15 seeds** on soil of the best line
> PCR amplify Cas9

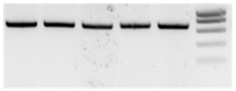
Cas9 >

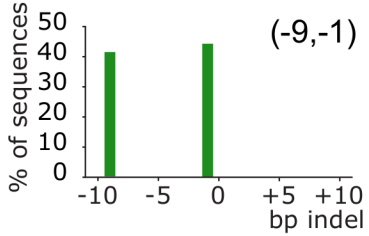


T2 (continued)

> PCR amplify GOI in Cas9 null-segregants

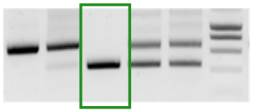
a) 1 sgRNA





- Sequence + TIDE analysis
- Non-chimeric
- Evaluate genotype

b) dual-sgRNA

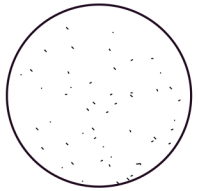


- Non-chimeric
- Evaluate genotype on gel

T3

> Harvest seeds of **ideally a homo-allelic** Cas9 null segregant

> Seeds on Km
> Verify absence of T-DNA (all sensitive)



> Sow **~8 new seeds** on soil
> PCR amplify GOI
> Sequence to determine final genotype

```
Col-0  AGAAAGAGCACAACCATGACCTTGGAGGTCATATAGA
3-11   AGAAAGAGCACAACCATGACCT-GGAGGTCATATAGA
```

Figure S5

Table S1. Oligonucleotides used in this study.

sgRNA cloning		
LAPAU2501	**ATTG**ATGAGGAGATATTATCTG	VQ33-42
LAPAU2502	**AAAC**CAGATAATATCTCCTCAT	VQ33-42
LAPAU2503	**ATTG**CCTTAACGTATTGATCATT	VQ33-38
LAPAU2504	**AAAC**AATGATCAATACGTTAAGG	VQ33-38
LAPAU2505	**ATTG**GGTCATCGTTGCTTCTCAG	VQ33-1
LAPAU2506	**AAAC**CTGAGAAGCAACGATGACC	VQ33-1
LAPAU2507	**ATTG**CGGAGAGTCTGGAGATCTT	VQ19-34
LAPAU2508	**AAAC**AAGATCTCCAGACTCTCCG	VQ19-34
LAPAU2509	**ATTG**GGACTGTTAAGTGCAAGCT	VQ19-6
LAPAU2510	**AAAC**AGCTTGCACTTAACAGTCC	VQ19-6
LAPAU2648	**ATTG**ATAAGGCATCGGTGTTAAG	GLB3-3
LAPAU2649	**AAAC**CTTAACACCGATGCCTTAT	GLB3-3
LAPAU2650	**ATTG**ACCTTCGAGCCGAGCTCGG	GRXS17-4
LAPAU2651	**AAAC**CCGAGCTCGGCTCGAAGGT	GRXS17-4
LAPAU2819	**ATTG**ACTTCCCAAAATGACTCCAG	WRKY20-201
LAPAU2820	**AAAC**CTGGAGTCATTTTGGGAAGT	WRKY20-201
LAPAU2821	**ATTG**TATGGCTGCACAAGAAGAA	WRKY20-39
LAPAU2822	**AAAC**TTCTTCTTGTGCAGCCATA	WRKY20-39
LAPAU2830	**ATTG**GAGATTTGGTTCTCTGTTG	JAM2-109
LAPAU2831	**AAAC**CAACAGAGAACCAAATCTC	JAM2-109
LAPAU2834	**ATTG**TATTGCAGAGAGCCTAAAGA	JAM2-140
LAPAU2835	**AAAC**TCTTTAGGCTCTCTGCAATA	JAM2-140
LAPAU3059	**ATTG**CTTGATAACTTGCGCCAGAG	GRXS17-133
LAPAU3060	**AAAC**CTCTGGCGCAAGTTATCAAG	GRXS17-133
LAPAU3061	**ATTG**ATTATGGAGCTAAGTGAGAG	GRXS17-67
LAPAU3062	**AAAC**CTCTCACTTAGCTCCATAAT	GRXS17-67
TIDE		
LAPAU2759	GTCGTCGCACGACAAAACTA	VQ33-42
LAPAU2760	TCATCGTTGCTTCTCAGTGG	VQ33-42
LAPAU2761	CCTCATCATCATGACCAGCA	VQ33-38
LAPAU2762	GCTAAACAAAATCCGGAGATAGA	VQ33-38
LAPAU2763	CAACAACAACATGTTCGCTAAG	VQ33-1
LAPAU2764	ATTGAGTGGCACACGTTGAA	VQ33-1
LAPAU2765	TTTTCCAAACTATTAGAGGAAACCA	VQ19-34 and VQ19-6
LAPAU2766	TTTTCCAAACTATTAGAGGAAACCA	VQ19-34 and VQ19-6
LAPAU2789	CAGCTGTACTGCAACGGAGA	GRXS17-4
LAPAU2790	GGAAAAGTTGGCCAGTTTGA	GRXS17-4
LAPAU2791	ATATGCCAAAAAGGCCCAAC	GLB3-3
LAPAU2792	TTGAAACCATTCTTCTTCGTCA	GLB3-3
LAPAU3063	TGGAACGAGGACGATAAAGC	JAM2-109
LAPAU3064	AGCTCTAACCGGAGGCAAAC	JAM2-109
LAPAU3065	AAGCGATTGTTGCGTCATTA	JAM2-140
LAPAU3066	GCAAATCCTTGCCGAATATC	JAM2-140
Cas9		
LAPAU3076	TCCCTCATCAGATCCACCTC	Cas9 genotyping
LAPAU3077	CTGAAACCTGAGCCTTCTGG	Cas9 genotyping
qPCR		
LAPAU1470	AGATATCTGAGGCTTACTCTGTTGC	*GRXS17* Exon 2
LAPAU1471	TACCATCCTTGAAGAAGACGAAA	*GRXS17* Exon 2
LAPAU1472	CGCCAAGGCCTTAAAGTGTA	*GRXS17* Exon 3
LAPAU1473	CATAAGCTCGCCTTTCACG	*GRXS17* Exon 3

Overhangs for type IIS cloning are indicated in bold.