SUPPLEMENT MATERIALS FOR CURTIN, et al. "Validating genome-wide association candidates: Selecting, testing, and characterizing genes that control quantitative variation in rhizobial nodulation"

1. Figures
    - S1. The characterization of three Tnt1 mutant lines
    - S2. The characterization of two whole-plant hairpin lines
    - S3. The characterization of the *Pho2-like* CRISPR/Cas9 mutants
    - S4. The characterization of the *Erdj2* CRISPR/Cas9 mutants
    - S5. The characterization of the *Acre-1* CRISPR/Cas9 mutants
    - S6. The characterization of the *Pen3-like* CRISPR/Cas9 mutants
    - S7. The characterization of the *Fmo1-like, Hlz-1 and Mel1-like* CRISPR/Cas9 mutants
    - S8. Nodulation phenotype of *dmi3* and *ipd3-2*
    - S9. The Tnt1 insertion background of the NF12360 Tnt1 mutant line
    - S10. WT and *Pho2-like*, *Pen3-like*, and *Pno1-like* mutant nodules at 14 dpi
    - S11. WT and *Pho2-like*, *Pen3-like*, and *Pno1-like* mutant dissected nodules at 14 dpi
    - S12. The expression analysis of validated genes, derived from the *Medicago truncatula* Gene Expression Atlas ((http://mtgea.noble.org/v3/index.php)

2. Tables
    - S1. List of Tnt1 mutant lines obtained from Noble Foundation Tnt1 mutant database
    - S2. Statistical tests comparing phenotype between mutant and wild-type control plants
    - S3. Primer sequences for PCR assays and vector construction (SEPARATE EXCEL FILE)

3. Supplemental Methods1
    1. CRISPR reagent design and construction

Supplemental Figures

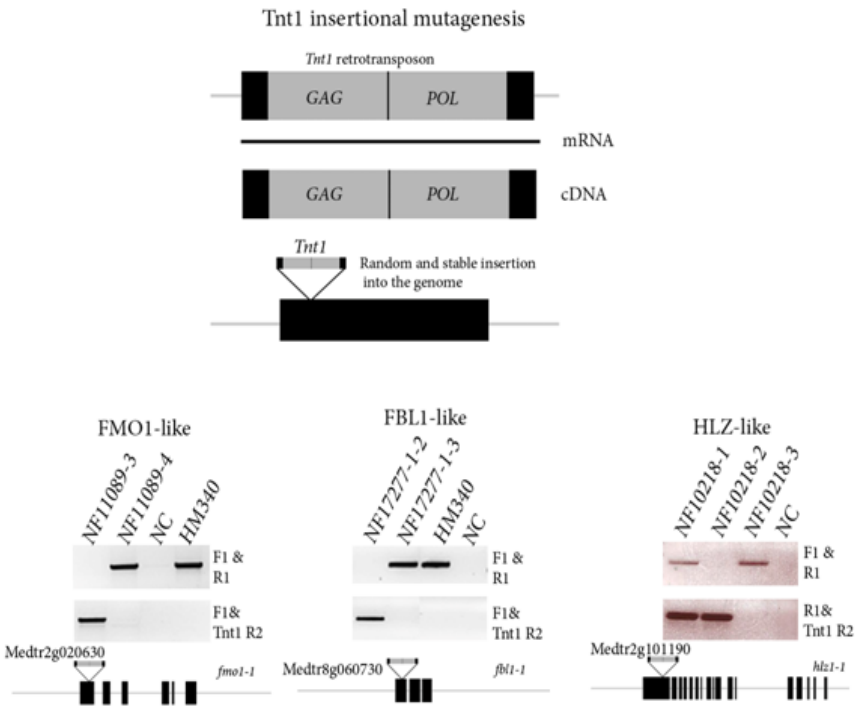


Fig. S1 The characterization of three Tnt1 mutant lines. Primers were designed to span the putative insertion site based on the FST sequences obtained from the Tnt1 mutant database. PCR using candidate gene and Tnt1 specific primers were used to identify homozygous Tnt1 insertion and wild-type plants. Primers sequences can found in the Supplemental Table3.

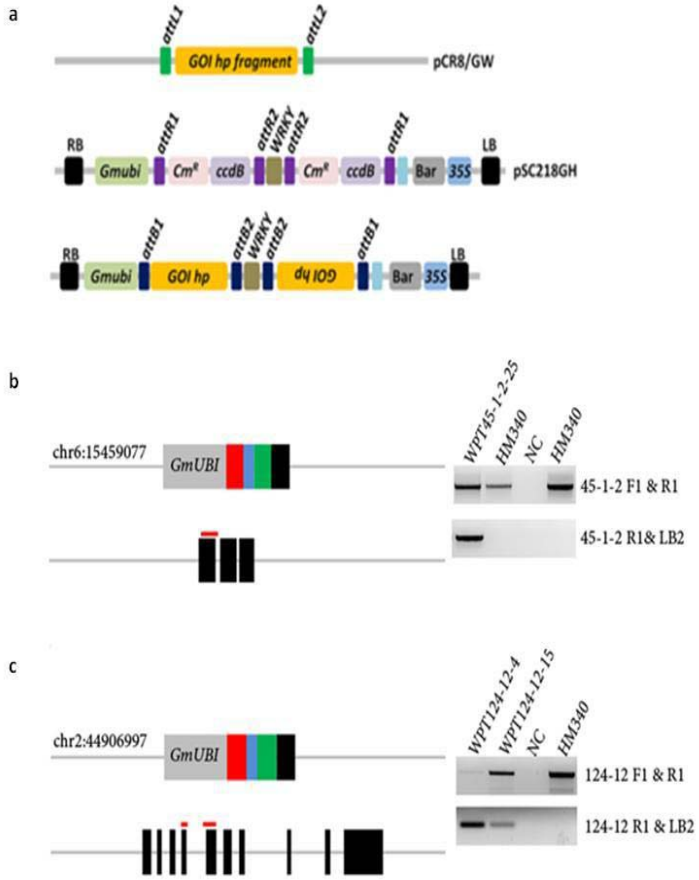


Fig. S2 The characterization of two whole-plant hairpin lines. **a**, Schematic representation of the hairpin vector assembly. **b**, The genomic location on chromosome 6 as identified by TAIL-PCR of a randomly inserted hairpin transgene targeting the *Fbl-like* candidate gene (Medtr8g060730). PCR using DNA template from the progeny of the WPT45-1-2 plant and chr6 transgene-insertion and transgene-specific primers was carried out. Results from this screen failed to conclusively confirm a homozygous plant. A second PCR assay was carried out on all putative mutant plants and the presence of the hairpin transgene was detected in all plants, suggesting the presence of a multi-copy T-DNA insertion that has not been characterized further. All 24 transgene positive mutant plants were used for the phenotype analysis. **c,** The genomic location on chromosome 2 as identified by TAIL-PCR of a randomly inserted hairpin transgene targeting the *Erdj2* candidate gene (Medtr2g044580). A PCR assay using DNA from the progeny of WPT124-12 plant as template and insertion specific primers identified a

homozygous plant (WPT124-12-4) that was selfed, and its progeny seed used for the phenotype analysis.

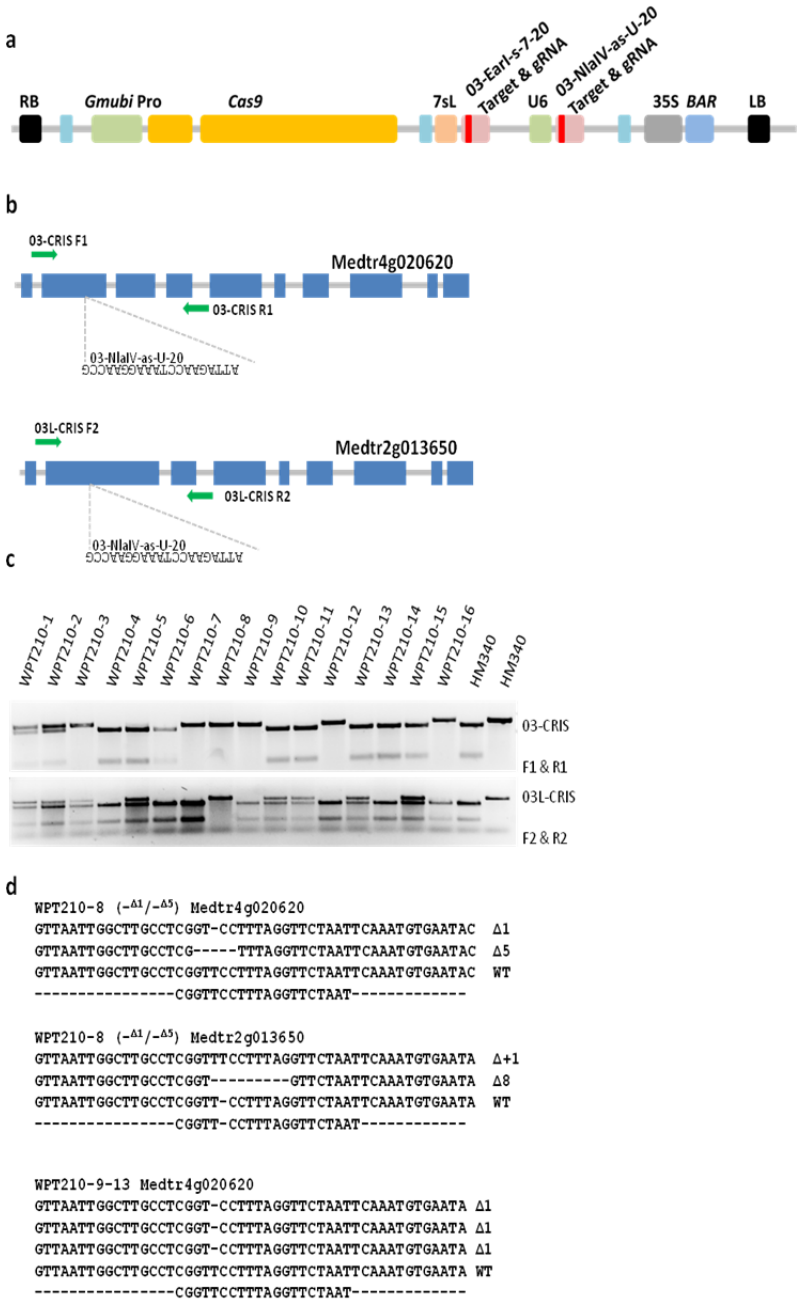


Fig. S3 The characterization of the *Pho2-like* CRISPR/Cas9 mutants. **a**, A schematic representation of the CRISPR/Cas9 reagent used to target the *Pho2-like* gene (Medtr4g020620). Although, not a candidate gene in this study, a homolog of *Pho2-like* located at Medtr2g013650 was also a target of the same CRISPR/Cas9 reagent. **b**, The genomic loci of both *Pho2-like* genes in Medicago and the U6 gRNA target sites at the second exon of both genes. The 7SL targets (not depicted) are typically validated only when U6 targets fail to generate mutations. **c,** A gel

depicting the PCR-digest assay showing both homozygous and heterozygous single and double mutants at the T_0 generation for the *Pho2-like* and homolog genes. The assay was used to determine the mutation frequency of the U6 target in 16 T_0 Medicago plants. Primer pairs specific to either *Pho2-like* gene (Medtr4g020620) or *Pho2-like* homolog (Medtr2g013650) was used to generate a gene-specific amplicon that are digested with the restriction enzyme NlaIV post-PCR. NlaIV-resistant amplicons indicate homozygous mutant plants, two bands indicate wild-type and three bands indicate heterozygous plants. The homozygous T_0 plant *Pho2-like* (WPT210-9) was used for the phenotype analysis in this study. Screening of the WP210-9 T_1 plants identified a non-transgenic plant where the transgene had been removed by genetic segregation (WPT210-9-13). The sequencing of this plant also confirmed heritable transmission of the same 1-bp deletion observed in the T_0 plant and no evidence of wild-type alleles. **d**, Amplicons were cloned and sequenced to confirm T_0 mutations in several of the six T_0 homozygous line. In addition, we also identified a quad-allelic T_0 double mutant of *Pho2-like* (Medtr4g020620) and *Pho2-like* homolog (Medtr2g013650) (WPT210-8), highlighting the striking efficiency and efficacy of the CRISPR reagent.

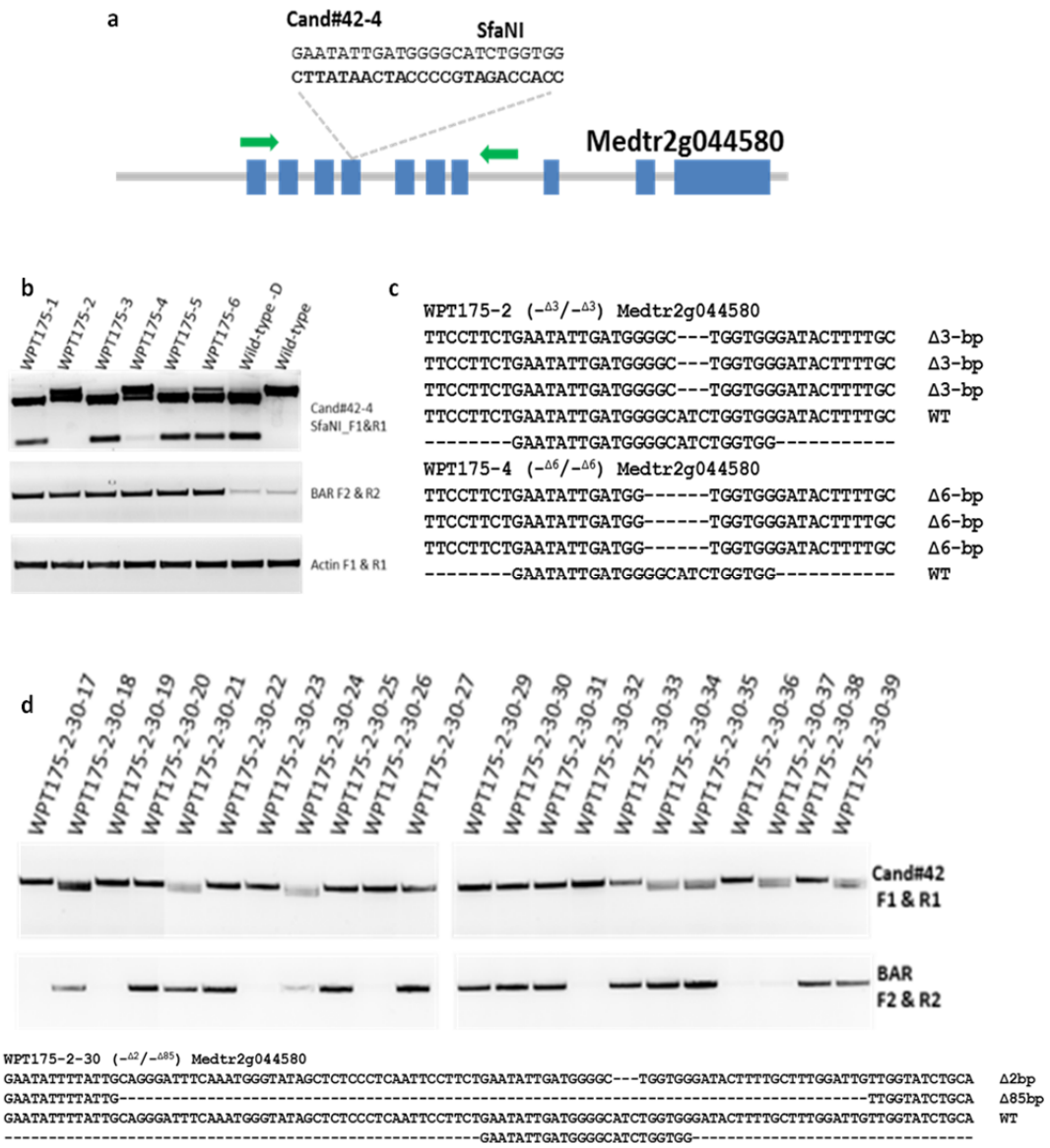
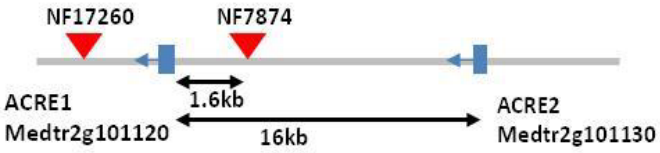


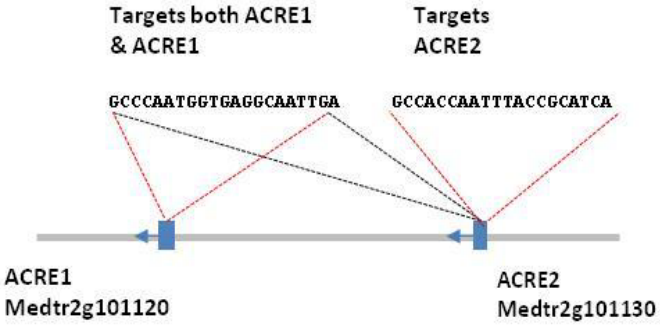
Fig. S4 The characterization of the *Erdj2* CRISPR/Cas9 mutants The genomic loci of the *Erdj2* gene in Medicago and the U6 gRNA target sites at the fourth exon. **b,** A PCR-digest assay spanning the *Erdj2* target site of 6 T_0 plants from the transformation event WPT175. The CRISPR/Cas9 reagent was highly active, generating 2 putative T_0 homozygous mutant plants and one heterozygous plants. Amplicons were digested post-PCR with the restriction enzyme SfaNI. SfaNI-resistant amplicons indicate homozygous mutant plants, two bands indicate wild-type

and three bands indicate heterozygous plants. **c**, Putative homozygous plants WPT175-2 and WPT175-4 were sequenced and a 3-bp and a 6-bp deletions at the target site respectively were confirmed. **d**, We checked the heritable transmission of the 3-bp deletion in the WPT175-2 progeny (WPT175-2-30) and a second mutant allele was observed that was previously overlooked. Sequencing confirmed that this second mutation was a frame-shift 85-bp deletion that can be screened by PCR without digestion, since the amplicons are a combination of the 485-bp amplicon (3-bp mutant allele) and a 403-bp amplicon (85-bp mutant allele). The two alleles can be conveniently visualized by gel electrophoresis separation. Using this PCR strategy we screened forty plants to try to identify a plant harboring both 85-bp frame-shift deletions, however we could not identify this plant, suggesting that the candidate *Erdj2* gene is gametophytic lethal. In addition, we observed removal of the transgene in several plants segregating for the two mutant alleles (3-bp and 85-bp) and confirmed a plant which had 3-bp and 85-bp deletion without a transgene (WPT175-2-30-37).

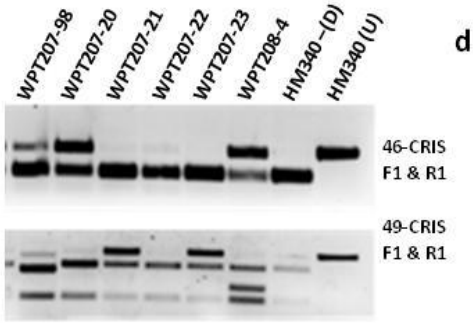


CRISPR

b



c



d

```
WPT208-4 (+/-Δ1) Medtr2g101120
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
GGGCGTAGCCCAATGGTGAGGCAAT-GAGGGTG Δ1
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
-------GCCCAATGGTGAGGCAATTGA-----

WPT207-20 (+/-Δ9) Medtr2g101120
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
-------GCCCAATGGTGAGGCAATTGA-----
```

e

```
WPT208-4-2
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
-------GCCCAATGGTGAGGCAATTGA-----

WPT208-4-16
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGG---------GTG Δ9
GGGCGTAGCCCAATGGTGAGGCAATTGAGGGTG WT
-------GCCCAATGGTGAGGCAATTGA-----
```

120

121

Fig. S5 The characterization of the *Acre-1* CRISPR/Cas9 mutants. a, The Noble Tnt1 mutant database was queried to identify Tnt1 insertion mutants at the *Acre-1* locus (Medtr2g10120) however none were found. Therefore, we attempted to identify insertions by using the gene specific PCR-based reverse screening service provided by the Noble foundation. Two insertion lines were identified from this screen, NF17260 and NF7874, however closer analysis revealed that neither one of these insertions had disrupted the reading frame or promoter region of *Acre-1*. In addition, a second paralog copy of the gene (*Acre-2*) was identified 16kb upstream of *Acre-1*, and there was interest in generating a mutant for this gene in case gene redundancy confounded future phenotype analyses. **b**, A CRISPR/Cas9 reagent that targeted the reading frame of both *Acre-1* and *Acre-2* was constructed with the goal of obtaining a series of single and double mutants, and possibly a large deletion (~16kbp) whereby both loci were removed. Two guide RNAs were engineered, one a U6 gRNA targeting both 5' coding regions of *Acre-1* and *Acre-2* and the second 7SL gRNA targeting the middle region of both genes. **c**, During T_0 screening of plants for targeted mutations in the both genes we noticed a SNP at the U6 target of *Acre-1* and that no mutation activity could be detected at this locus. We therefore screened the 7SL target locus and identified mutations in both genes at high frequency. **d**, We identified a T_0 heterozygous mutant plant and sequenced confirmed a frame-shift 1-bp deletion in the coding region of *Acre-1* (WPT208-4). **e**, The T_0 WPT208-4 was selected for the phenotype analysis and approximately 90 heterozygous plants were PCR-digest screened to confirm heritable transmission of the mutation at the expected segregation frequency. From this assay we recovered approximately 24 mutant plants and observed the expected segregation frequencies. Intriguingly, sequence confirmation of the mutation in T_1 plants revealed a consistent 9-bp in-frame mutation in several plants (WPT208-4-2 & WPT208-4-16), differing from the initial 1-bp deletion observed in the T_0. This observation is not uncommon, and more thorough screening of T_0 plants may be required.

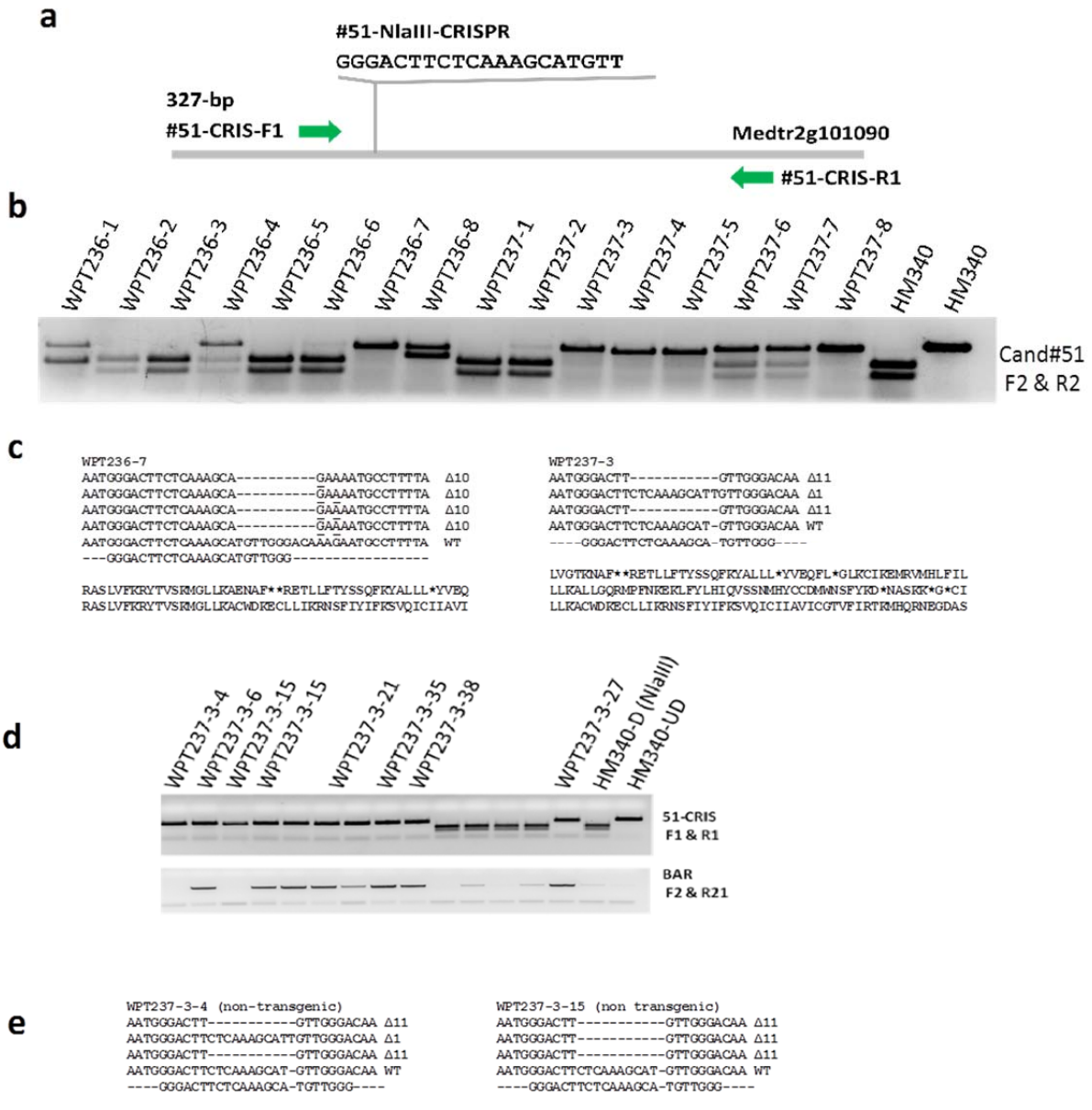


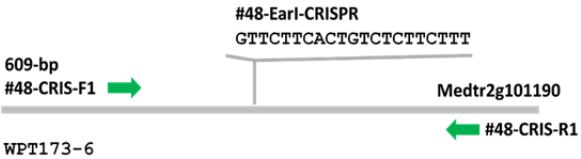
Fig. S6 The characterization of the *Pen3-like* CRISPR/Cas9 mutants in whole plant Medicago. **a,** The genomic loci of the *Pen3-like* gene in Medicago and the U6 gRNA target sites at the third exon. **b,** A PCR-digest assay spanning the *Pen3-like* target site of 16 T_0 plants from two independent transformations, WPT236 and WPT237 was carried out. The CRISPR/Cas9 reagent was highly active, generating five T_0 homozygous mutant plants as well as several heterozygous plants. Amplicons were digested post-PCR with the restriction enzyme NlaIII. NlaIII-resistant

158 amplicons indicate homozygous mutant plants, two bands indicate wild-type and three bands
159 indicate heterozygous plants. **c**, We planned to use the WPT236-7 plants for the phenotype
160 analysis since this plant had identical 10-bp mutant alleles. However, we had difficulty
161 germinating T_1 seed from this plant for reasons not known and therefore used T_1 seed from a
162 second homozygous T_0 plant (WPT237-3). This plant had a segregating bi-allelic mutation of 1-
163 bp (insertion) and 11-bp (deletion), both frame-shift mutations resulting in-frame stop codons.
164 **d**, Ten T_1 mutant plants from WPT237-3 were screened by a PCR-digest assay to confirm
165 heritable transmission of the homozygous mutation. **e**, Amplicons from these mutant plants
166 were sequenced to confirm mutations and same T_0 mutant alleles were observed in the T_1
167 plants. Two homozygous plants, one with a 1-bp and 11-bp biallelic mutation (WPT237-4) and
168 the other with a mono-allelic 11-bp mutation (WPT237-3-15) were identified as non-transgenic
169 resulting from the removal of their transgene by genetic segregation.
170

a

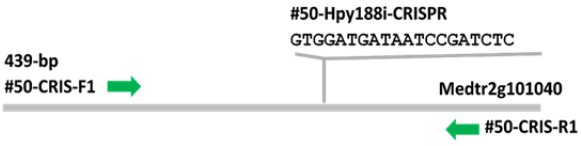
```
WPT205-1 T0
TGTTCGAGACAAAGAAGGT-----------------------------------GTGGGAGCTTTATGTTCA  Δ56
TGTTCGAGACAAAGAAGGT-----------------------------------GTGGGAGCTTTATGTTCA  Δ56
TGT---------------------------------------------------------------CA  Δ88
TGT---------------------------------------------------------------CA  Δ88
TGTTCGAGACAAAGAAGGTTTTGATT//TTGGATGCTTACCTCCTGACCATGGAAA//GTGGGAGCTTTTTGTTCA  WT
-----------------------------GGATGCTTACCTCCTGACCA-------------------------
RAYAVYFDLFKYVMFNYKVVEIKFVRDKEGFDFGCLPPDHGNPLPGRMVWELFVQTNESYVIQRYHFEFIVVCTGK
RAYAVYFDLFKYVMFNYKVVEIKFVRDKEGVGALCSD**IRRHSAVPLRVYSGLHWKIWRYTIDAKISIQQGP*GV
RAYAVYFDLFKYVMFNYKVVEIKFVRLMNQFSGTTSSL*WFALENMEIYH*CQNFHTTRALRCSRVRLCIPLSIVN

WPT205-1-7 T1
TGT---------------------------------------------------CAGACTAATGAATCAGACGTCA Δ88-bp
TGT---------------------------------------------------CAGACTAATGAATCAGACGTCA Δ88-bp
TGT---------------------------------------------------CAGACTAATGAATCAGACGTCA Δ88-bp
TGTTCGAGACAAAGAAGGT-----------------GTGTGGGAGCTTTATGTTCAGACTAATGAATCAGACGTCA Δ56-bp
TGT---------------------------------------------------CAGACTAATGAATCAGACGTCA Δ88-bp
TGTTCGAGACAAAGAAGGTTTTGATT//TTGGATGCTTACCTCCTGACCATGGAAA//ACTAATGAATCATACGTCA
-----------------------------GGATGCTTACCTCCTGACCA-------------------------
```

b




```
WPT173-6
TCAACGTGTTTT-------------------------------------------------------------TCCAATGAAA Δ59
TCAACGTGTTTT-------------------------------------------------------------TCCAATGAAA Δ59
TCAACGTGTTTTCTTGTGTTCTTCGTCTCTTCTTTAGGAAACACGTTTATTTCCGTGCTTAATCTTTTTTTTCCAATGAAA WT
-----------------GTTCTTCGTCTCTTCTTTAGG-----------------------------------------
            EPDRCYHDEVFHKALISKISTCFLVFFTVSSLGNTFISVLKLLFLLPMNFFILKKHL
            EPDRCYHDEVFHKALISKISTCFTSNEFFHLKKALIIGIWIDRTRKFCGW*K*F*KK
```

c




```
WPT209-13 T0
AAAAGGAACCAGGGGGGGAAAATGGGGATAAAAACCCAATCCCCGGGGGGGGTCCTCGCG
AAAAGGAATTTGGG-------------------------------------TCCTCGCG  Δ38
AAAAGGAAGCAGTGGTGGAAGATGTGGATGATAATCCGATCTCCGGTGGCGGTCCTCGCG  WT
----------------------GTGGATGATAATCCGATCTCCGG--------------

      VVNVMETPLKEIKKEAVVEDVDDNPISGGGPRDVYGEDKATE  WT
      VVNVMETPLKEIKKEAVSSRCLWRG*GH*GSLCDSLECFCC*  Δ38

WPT209-13-39 T1
AAAAAGGAAGCAGTGGTGGAAGATGTGGATGATAATC-----TCCGGTGGCGGTCCTCGCG Δ5
AAAAAGGAAGCAGTGGTGGAAGATGTGGATGATAATC-----TCCGGTGGCGGTCCTCGCG Δ5
AAAAAGGAAGCAGTGGTGGAAGATGTGGATGATAATC-----TCCGGTGGCGGTCCTCGCG Δ5
AAAAAGGAAGCAGTGGTGGAAGATGTGGATGATAATCCGATCTCCGGTGGCGGTCCTCGCG WT
----------------------GTGGATGATAATCCGATCTCCGG--------------
```

171

Fig. S7 The characterization of the *Fmo1-like, Hlz-1 and Mel1-like* CRISPR/Cas9 mutants in whole plant *Medicago*. **a**, A *Fmo1-like* homozygous T_0 mutant was generated by a CRISPR/Cas9 reagent designed to target the locus. The T_0 plant consisted of frame-shift bi-allelic mutations of 56bp and 88bp that disrupted the reading frame of the gene. To confirm heritable transmission of the mutations a PCR-digestion assay was performed on all mutant plants used in the phenotype analysis. Results from this assay indicated that mutant plants were homozygous. Several T_1 mutant plants were sequenced to confirm the same mutations observed in the T_0 plant (WPT205-1-7). We also detected activity from the second guide RNA from the CRISPR reagent used to target *Fmo1-like* at Medtr2g020630. The 7SL target inadvertently had target homology to a second *Fmo1-like* gene at Medtr2g043850. Putative double mutant plants have a striking dwarf phenotype and were excluded from the phenotype analysis. **b**, The T_0 heterozygous mutant plant WPT173-6 harboring a 59-bp deletion in the coding region of the *Hlz1-like* gene (Medtr2g101190) was identified. T_1 progeny were screened by a PCR-digest assay and heritable transmission of the mutation was confirmed by the recovery 18 homozygous plants that were used for the phenotype analysis. **c**, The screening of a *Mel1-like* heterozygous T_0 mutant plant generated by a CRISPR/Cas9 reagent revealed a plant with wild-type and a mutant allele with a 38-bp deletion (WPT209-13). We expected this mutation to be heritably transmitted at the expected segregation ratio. However when we screened approximately 90 plants using a PCR digestion assay to detect homozygous plants, only seven mutant plants appeared to be homozygous. Moreover, sequence confirmation of these plants indicated a different (5-bp) mutation observed at the target (WPT209-13-39). Due to the odd heritable transmission of these mutations, a future more robust phenotype analysis should be carried out using completely characterized progeny.

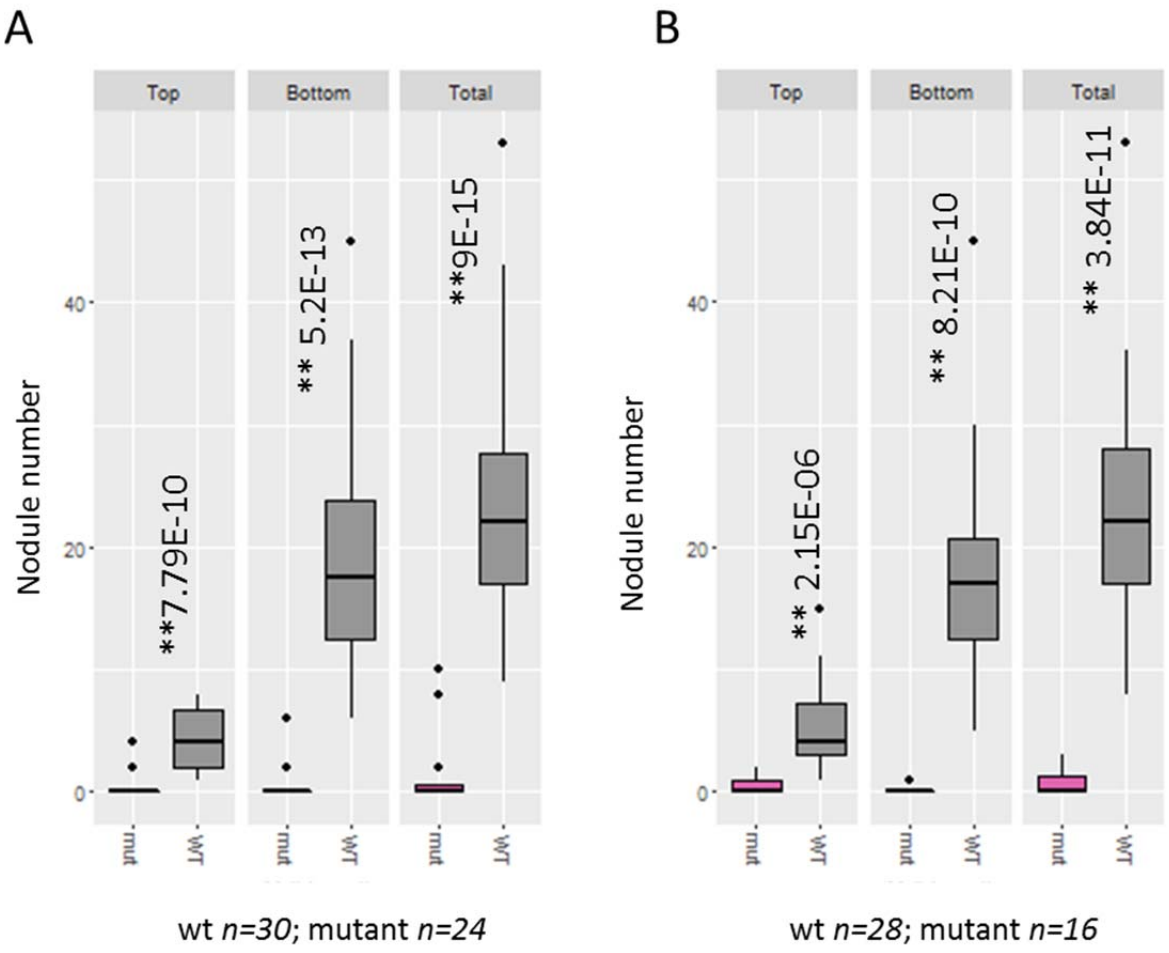
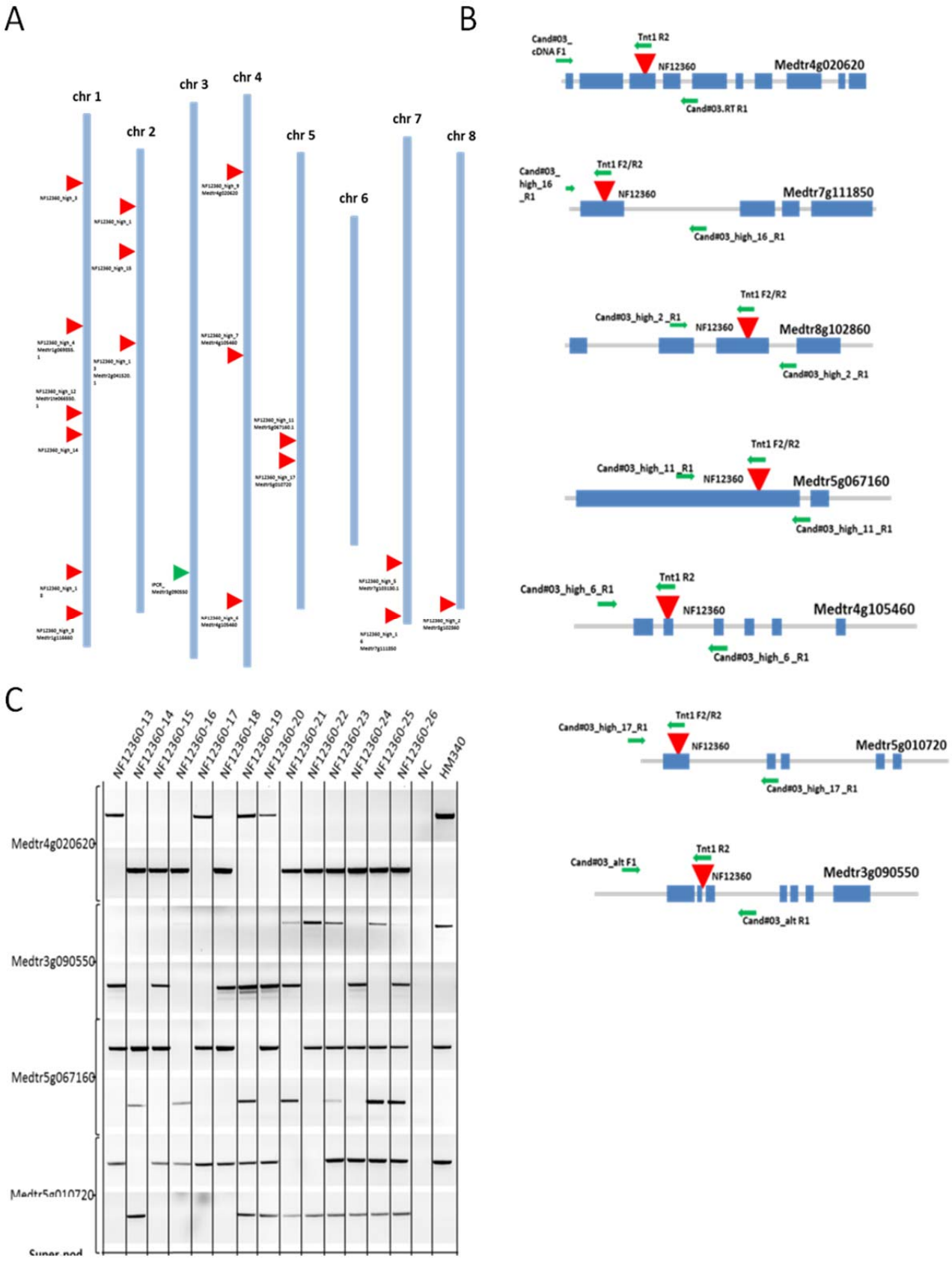


Fig. S8 Nodulation phenotype analysis of two previously characterized nodulation mutants, TRV25 *dmi3* and NF5939 *ipd3-2*. (A)

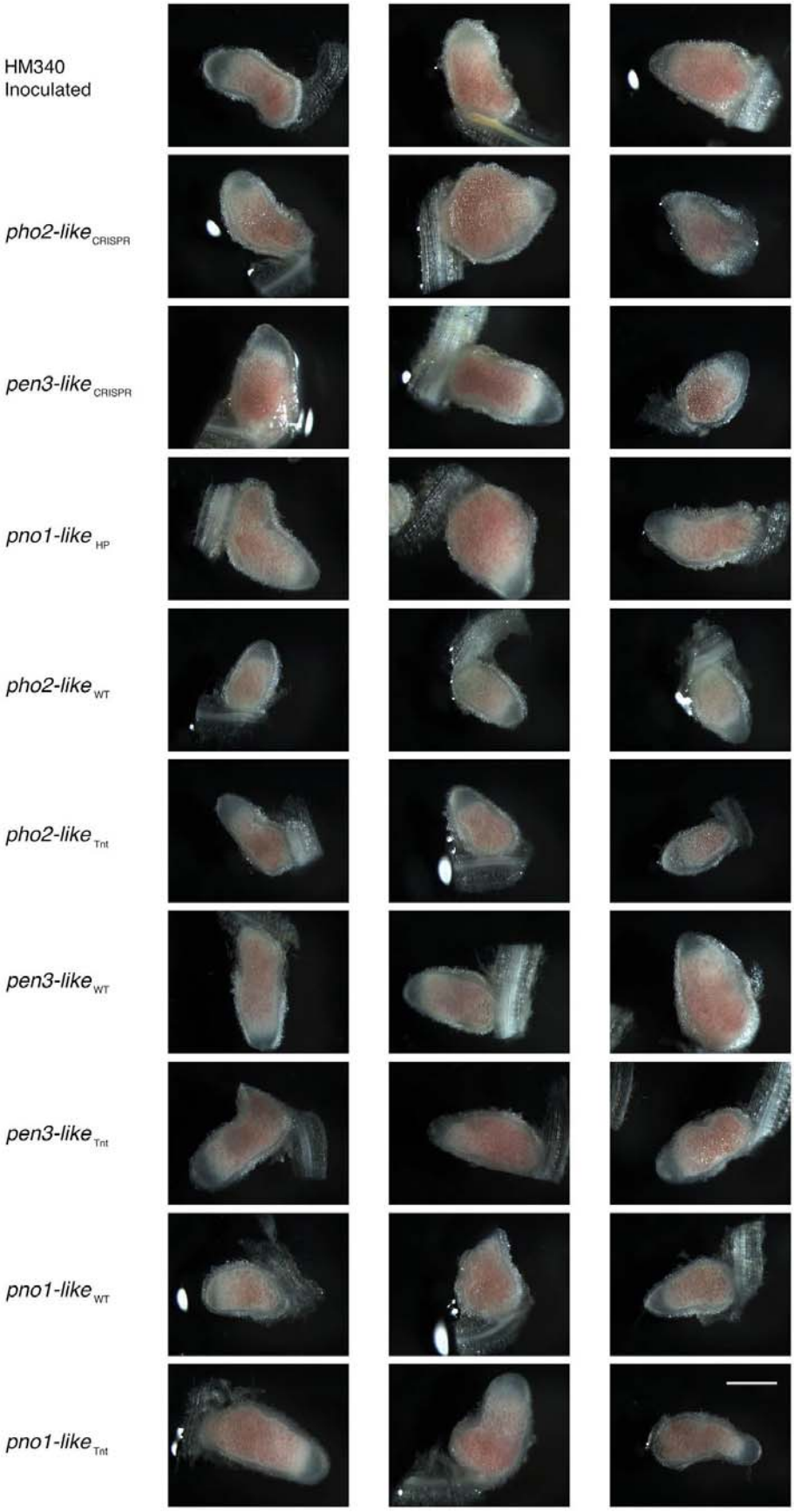

A
chr 1
chr 2
chr 3
chr 4
chr 5
chr 6
chr 7
chr 8
B
Cand#03_ cDNA F1
Tnt1 R2
NF12360
Medtr4g020620
Cand#03.RT R1
Cand#03_ high_16 _R1
Tnt1 F2/R2
Medtr7g111850
Cand#03_high_16 _R1
Cand#03_high_2 _R1
Medtr8g102860
Cand#03_high_11 _R1
Medtr5g067160
Cand#03_high_6_R1
Medtr4g105460
Cand#03_high_6 _R1
C
NF12360-13
NF12360-14
NF12360-15
NF12360-16
NF12360-17
NF12360-18
NF12360-19
NF12360-20
NF12360-21
NF12360-22
NF12360-23
NF12360-24
NF12360-25
NF12360-26
NC
HM340
Medtr4g020620
Medtr3g090550
Medtr5g067160
Cand#03_high_17_R1
Medtr5g010720
Cand#03_alt F1
Medtr3g090550
Cand#03_alt R1

207

Fig. S9 The Tnt1 Insertion background of the NF12360 Tnt1 mutant line. a, The eight Medicago chromosomes indicating the approximate Tnt1 insertion sites obtained from Noble Foundation Tnt1 database. Using this approach we identified 17 Tnt1 insertions in line NF12360. In addition, we used inverse-PCR (IPCR) to identify an extra insertion (green). **b**, Several of these insertion events are located in coding regions of random genes. **c**, Segregation profile of four genes disrupted by a Tnt1, the gel also indicates that many of the insertions are homozygous.


HM340
Inoculated
pho2-like CRISPR
pen3-like CRISPR
pno1-like HP
pho2-like WT
pho2-like Tnt
pen3-like WT
pen3-like Tnt
pno1-like WT
pno1-like Tnt

S10. WT and *Pho2-like*, *Pen3-like*, and *Pno1-like* mutant nodules at 14 dpi. Three plants for each line were harvested at 14 dpi. For each plant the second or third nodule from the top is shown close up. The scale bar indicates 1 mm.


HM340
Inoculated
pho2-like CRISPR
pen3-like CRISPR
pno1-like HP
pho2-like WT
pho2-like Tnt
pen3-like WT
pen3-like Tnt
pno1-like WT
pno1-like Tnt

S11. WT and *Pho2-like*, *Pen3-like*, and *Pno1-like* mutant dissected nodules at 14 dpi. Three plants for each line were harvested at 14 dpi. For each plant the second or third nodule from the top was cut in half longitudinally and examined under a stereo microscope. The scale bar indicates 1 mm.

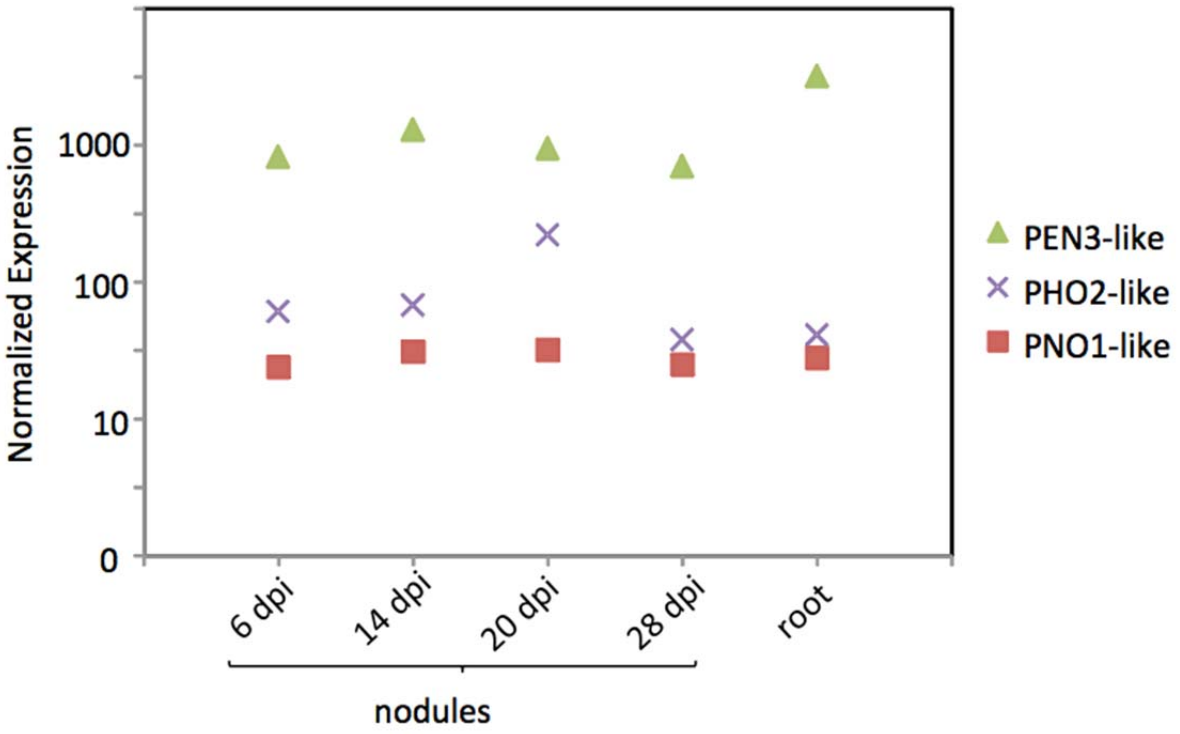


Fig. S12. The Expression levels of three validated candidate genes in roots and nodules. PEN3-like shows high expression in nodules and roots, with expression in nodules, peaking modestly at 14 dpi. PHO2-like is expressed at relatively low levels in roots and nodules, with peak expression in nodules at 20 dpi. PEN1-like is expressed at very low levels in both nodules and root tissue with little change over time. Data are from the *Medicago truncatula* Gene Expression Atlas (http://mtgea.noble.org/v3/index.php). Note vertical axis is on a log scale.

Table S1. List of the Tnt1 mutant lines obtained from the Noble Foundation Tnt1 mutant database. http://medicago-mutant.noble.org/mutant/. Tnt1 insertion could not be identified in several lines. In addition, candidate mutants that could not be identified in the mutant database were identified by a PCR-based reverse screen. Identification in a PCR-based reverse screen often produced false positives and in most cases insertions did not disrupt the gene of interest or could not be found.

Candidate	Gene Name	Line No.		Status
2	Medtr2g020620	NF14883	ordered from database	Insert not found
2	Medtr2g020620	NF2136	ordered from database	Insert not found
3	Medtr4g020620	NF12360	ordered from database	Homozgous and wild-type plant identified
24	Medtr8g060730	NF17407	ordered from database	Insert not found
24	Medtr8g060730	NF17277	ordered from database	Homozgous and wild-type plant identified
24	Medtr8g060730	NF19232	ordered from database	Insert not found
30	Medtr8g018370	NF12320	ordered from database	Insert not found
30	Medtr8g018370	NF20885	ordered from database	Heterozygous plant recovered and bulked
33	Medtr5g088410	NF11825	ordered from database	Homozgous and wild-type plant identified
33	Medtr5g088410	NF13140	ordered from database	Insert not found
34	Medtr8g073550	NF16011	ordered from database	Insert not found
36	Medtr2g020630	NF11089	ordered from database	Homozgous and wild-type plant identified
38	Medtr3g014710	NF15060	ordered from database	Insert not found
38	Medtr3g014710	NF20362	ordered from database	Insert not found
39	Medtr2g059590	NF12197	ordered from database	Insert not found
39	Medtr2g059590	NF20479	ordered from database	Homozgous and wild-type plant identified
39	Medtr2g059590	NF4387	ordered from database	Insert not found
41	Medtr2g044570	NF15195	ordered from database	Heterozygous plant recovered and bulked
41	Medtr2g044570	NF11192	ordered from database	Insert not found
42	Medtr2g044580	None	n/a	None found
44	Medtr2g087630	None	n/a	None found
45	Medtr3g079860	NF11648	ordered from database	Homozgous and wild-type plant identified
46	Medtr2g101120	NF17260	PCR-based reverse screened	Insert not found
46	Medtr2g101120	NF7874	PCR-based reverse screened	Insert not found
47	Medtr2g101180	NF12123	PCR-based reverse screened	Insert not found
47	Medtr2g101180	NF11016	PCR-based reverse screened	Insert not found
48	Medtr2g101190	NF12952	PCR-based reverse screened	Insert not found
48	Medtr2g101190	NF10218	PCR-based reverse screened	Homozgous and wild-type plant identified
49	Medtr2g101130	None	PCR-based reverse screened	None found
50	Medtr2g101040	NF4794	PCR-based reverse screened	Insert not found
50	Medtr2g101040	NF12520	PCR-based reverse screened	Insert not found
51	Medtr2g101090	NF2826	PCR-based reverse screened	Homozygous and wild-type plant identified

Table S2. Results from statistical tests comparing phenotypes (nodule number, N content) of mutant and wild-type control plants. Results for validated genes are in bold.

Candidate	Mutant type	Number of mutant / wt plants	P value nodules	Number of mutant mean (sd)	Number of wt mean (sd)		% N[a]	Paired wild type % N	$t_{df=4}$	P value of N content
PHO2-like										
Medtr4g020620	***pho2-like$_{Tnt}$***	**29 / 36**	**<0.001**	**13 (4.9)**	**23 (9.9)**		3.38	3.40	NA[b]	NA
	pho2-like $_{CRISPR}$[c]	**23 / 24**	**0.002**	**42 (13)**	**53 (10.1)**		4.18	4.15	0.23	0.83
	pho2-like $_{CRISPR}$[c]	**24 / 24**	**0.003**	**25.4 (6.6)**	**33.6 (11.0)**					
FBL1-like										
Medtr8g060730	*fbl1-like$_{hp}$*	22 / 22	0.11	33.6 (10)	39.1(12.6)					
PNO1-like										
Medtr5g088410	***pno1-like$_{Tnt}$***	**22 / 17**	**0.02**	**24 (11.8)**	**34 (14.1)**		3.58	3.91	2.12	0.1
	pno1-like$_{hp}$	**24 / 24**	**0.003**	**27 (8.2)**	**37.3(11.1)**[d]		4.44	4.15	0.85	0.19
FMO1-like										
Medtr2g020630	*fmo1-like$_{Tnt}$*	24 / 24	0.01	35.7 (7.6)	29.7 (8)					
	fmo1-like$_{CRISPR}$	21 / 25	0.93	45 (18.2)	45.4(13.9)					
RFP1-like										
Medtr2g044570	*rfp1-like$_{Tnt}$*	23 / 23	0.94	25.8 (7.4)	26 (7.7)					
ERDJ2										
Medtr2g044580	*erdj2$_{hp}$*	24 / 24	0.15	33 (12.7)	39 (13.6)					
	erdj2$_{CRISPR_6}$	7 / 24	0.23	32.8 (9.3)	37.3(11.1)[d]					
	erdj2$_{CRISPR_3/85}$	23 / 23	0.016	30.3 (7.6)	37.3(11.1)[d]					
	erdj2$_{CRISPR_3/85}$	62 / 11	0.47	36 (11.8)	34 (12.8)					
ACRE1										
Medtr2g101120	*acre1$_{CRISPR}$*	24 / 23	0.60	62 (18.3)	59 (19.1)					
HLE1-like										
Medtr2g101190	*hlz1-like$_{Tnt}$*	25 / 11	0.001	54 (20)	35 (13.2)					
	hlz1-like$_{CRISPR}$	18 / 29	0.35	48 (17.6)	53 (5.7					
MEL1										
Medtr2g101040	*mel1$_{CRISPR}$*[e]	7 / 15	0.21	39 (14.5)	48 (13.6)					
	mel1$_{CRISPR}$[e]	8 / 6	0.60	23 (8.5)	21 (8.7)					
PEN3-like										
Medtr2g101090	***pen3-like$_{Tnt}$***	**24 / 24**	**<0.001**	**24 (7.6)**	**35 (11.2)**		3.20	3.57	0.85	0.44
	pen3-like$_{CRISPR}$	**10 / 21**	**0.03**	**17.1 (8.0)**	**24.1 (8.2)**		4.09	4.15	0.85	0.45
Dmi3		24 / 30	<<0.001	1.08 (2.6)	23.2 (9.8)					
Ipd3		16 / 25	<<0.001	0.6 (1.0)	22.7 (9.9)					

[a] %N was calculated on three replicates per genotype.

[b] No t-test was conducted for *pho2-like$_{tnt}$* due to insufficent replication.

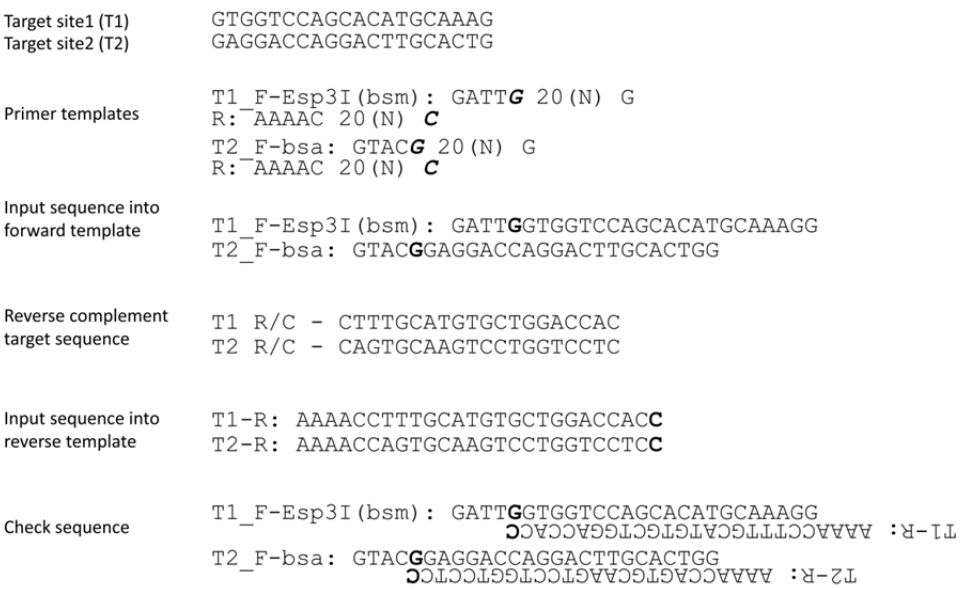
[c] The two CRISPR entries represent independent assays using the same mutant.

[d] The *PNO1-ike* wild-type control plants were used for the statistical tests involving the *ERDj2* CRISPR lines. These controls were grown in a rack next to, but not randomized with, the *ERDj2* plants.

[e] The *MEL1* CRISPR assays were conducted at separate times but the mutants were derived from the same parent plant.

Supplemental Methods Part A: CRISPR reagent design and construction

1. Query the sgRNA designer v.1 for selection of target guide RNAs (www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design-v1)
2. Design the primers for the annealing assay and cloning into the entry vector



3. Order forward and reverse primers.
4. Anneal primers with PNK

Target1 & Target2 30µL PNK primer annealing reaction

3µL 100µM primer#1

3µL 100µM primer#2

3µL 10x T4 ligation buffer

2µL T4 PNK

19µl dH2O

Incubate reaction at 37°C for 90 mins.

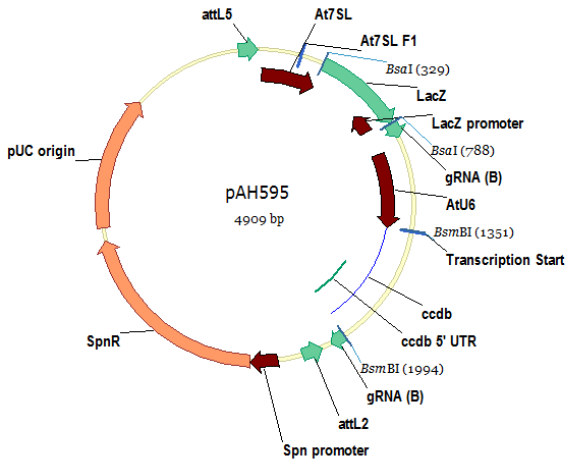
Add 4µL 0.5M NaCl and boil mixture for 5 mins

Cool slowly to RT

Dilute 1:500 with dH2O

Use 1µL of solution in ligate reaction

5. Clone target1 using a golden gate ligation of the target1 annealed primer product into the BsmBI(Esp3I) sites of the entry vector pAH595



<u>Target1 20µL GG reaction</u>

2µL 10x T4 ligation buffer

1µL pAH595

1µL annealed oligo

1µL Esp3I (BsmBI should be avoided since it requires a 50^{o}C digestion temperature)

1µL T4 ligase

14µl dH2O

<u>Golden Gate Run Cycle</u>

37^{o}C for 5 minutes + 16^{o}C for 10 minutes

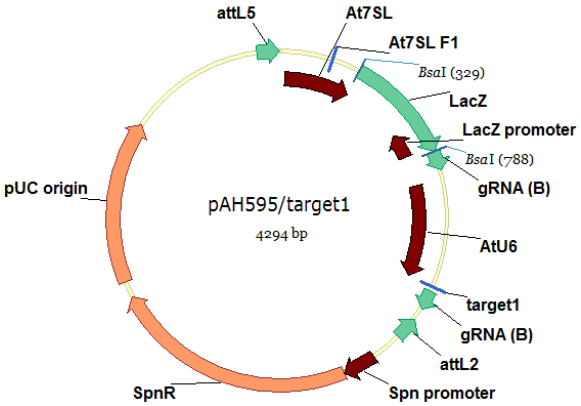
10 cycles (the amount of cycles here can be reduced)

37^{o}C for 15 minutes

80°C for 5 minutes

Transform into DH5α competent cells and plate on Spec100.

Sequence confirm insertion with U6_F1 primer AGAACAATAGTATTTCTTATATAGG



6. Repeat step #5 to introduce target2 into the BsaI sites of pAH595/target1 and sequence confirm vector

<u>Target 2 20µL GG reaction</u>

2µL 10x T4 ligation buffer

1µL pAH595/target1

1µL annealed oligo

1µL BsaI

1µL T4 ligase

14µl dH2O

<u>Golden Gate Run Cycle</u>

37°C for 5 minutes + 16°C for 10 minutes

10 cycles (the amount of cycles here can be reduced)

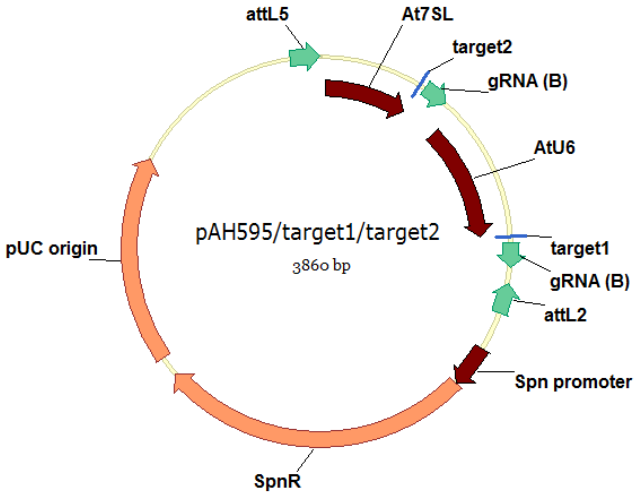
37°C for 15 minutes

80°C for 5 minutes

Transform into DH5α competent cells and plate on Spec100 and X-gal for blue white selection

Select white colonies for mini-prep and sequence confirm.

Sequence confirm insertion with At7SL F1 primer CTATAATGGGACTCAAAATAAGG



7. Carry out a mutli-site LR clonase reaction (Life Technologies) to Introduce the completed guide RNA entry vector and Cas9 entry vector into a destination Gateway™ binary vector such as the pSC218GG used in this report.

8. <u>LR clonase reaction</u>

1µL pNJB184-4 Cas9-Intron entry vector

1µL p595/target1/target2 target guide RNA entry vecto

1µL Destination binary vector (pSC218GG)

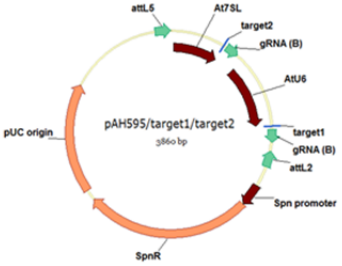
1µL LR clonase

1µL TE buffer

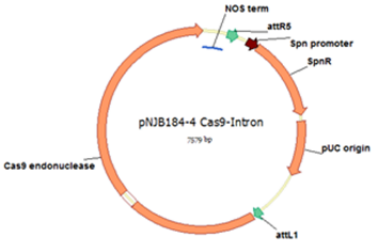
Incubate O/N at room temperature and transform into DH5α competent cells, Plate ~120µL of Kan100 LB plates.

The following day select colonies and mini-prep using a 15mL broth (pSC218GG is low-copy number plasmid).

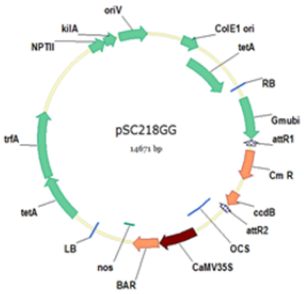
pAH595/target1/target2 entry



pNJB184-4 Cas9 entry



Gmubi destination



A representation of the multi-site Gateway ™ LR clonase reaction used to incorporate Cas9 and the guide RNA entry vector into the final destination vector

Gmubi:Cas9:target1 & 2 guide RNA CRISPR reagent