Additional file 1

Enhancing powdery mildew resistance in soybean by targeted mutation of *MLO* genes using CRISPR/Cas9 system

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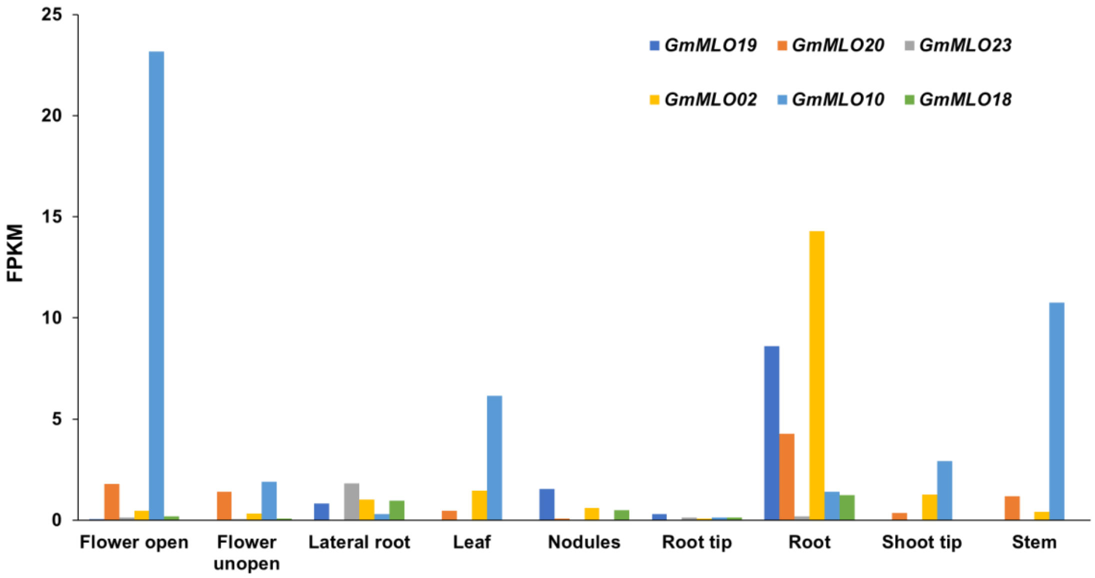
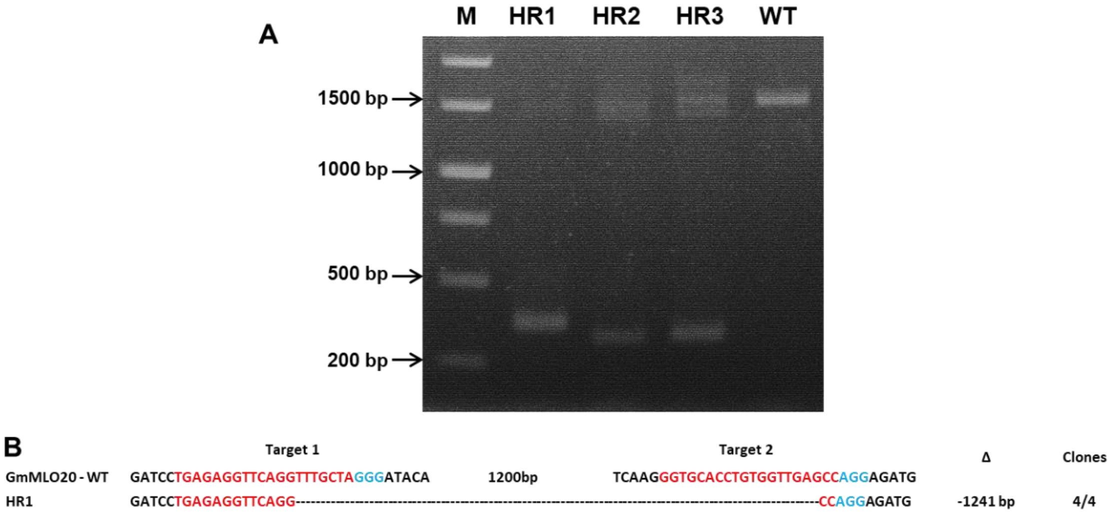


Fig. S1 Transcriptomics analysis of *GmMLO* genes in different tissues of soybean plant. Data were obtained from RNA-Seq Atlas of *Glycine max*



	Target 1		Target 2	Δ	Clones
GmMLO20 - WT	GATCCTGAGAGGTTCAGGTTTGCTAGGGATACA	1200bp	TCAAGGGTGCACCTGTGGTTGAGCCAGGAGATG		
HR1	GATCCTGAGAGGTTCAGG--CCAGGAGATG			-1241 bp	4/4

Fig. S2 Induced mutation analysis of hairy roots. **A** Gel electrophoresis (agarose 1%) of *GmMLO20* edited region in wild-type (WT) and hairy root samples (HR1, HR2, HR3) with large deletions. M: 1 kb DNA marker. Shifted bands in lines HR1, HR2 and HR3 indicated the induced mutations of targeted genes. **B** Sequencing result of the HR1 line for the edited regions of *GmMLO20* compared to wild-type allele. Target sequences and PAMs are indicated in red and blue, respectively. Δ indicates targeted sequence changes: - for deletion. Clones indicate number of colonies with the respective alleles out of total of clones sequenced

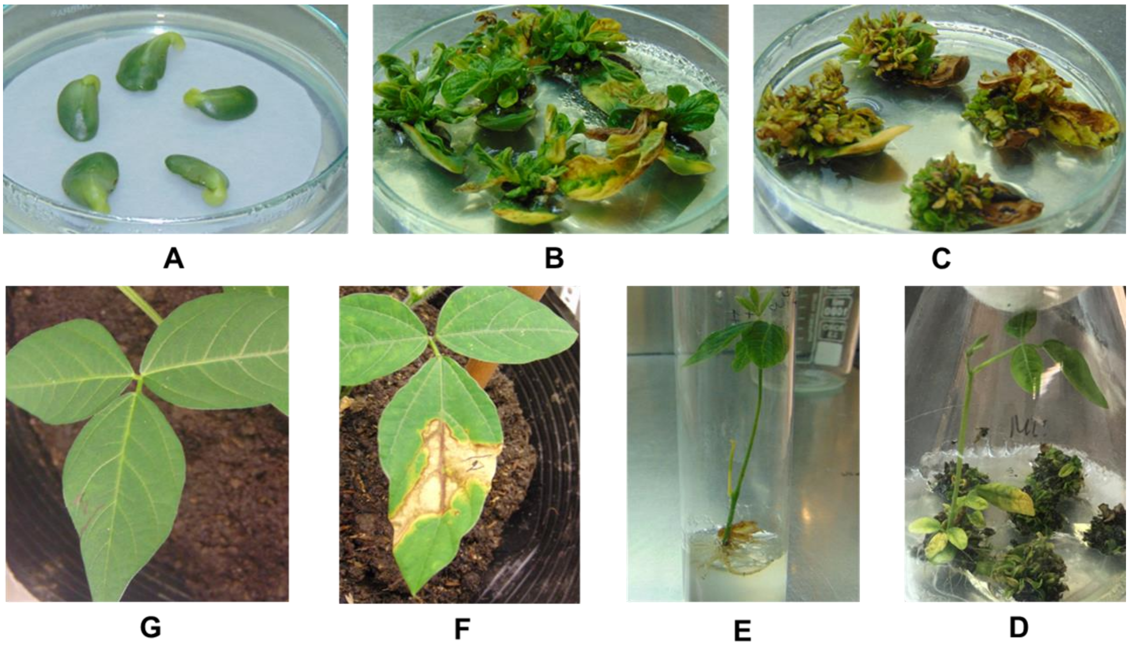


Fig. S3 Soybean transformation procedure. **A** Cotyledons at 5 days on the co-cultivation medium. **B, C** Shoot induction at 14 and 28 days on the selection medium. **D** Shoot elongation. **E** Rooted plants on the rooting medium. **F, G** Leaf painting using 200 mg/L glufosinate

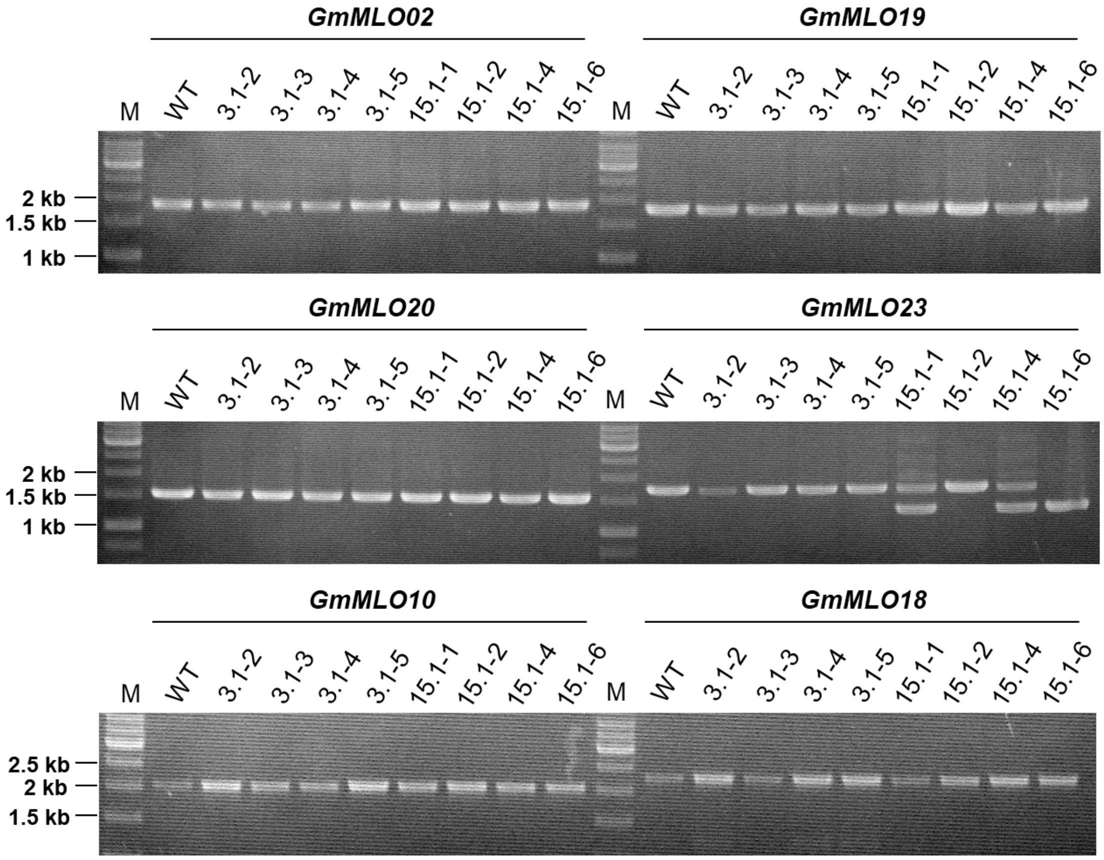
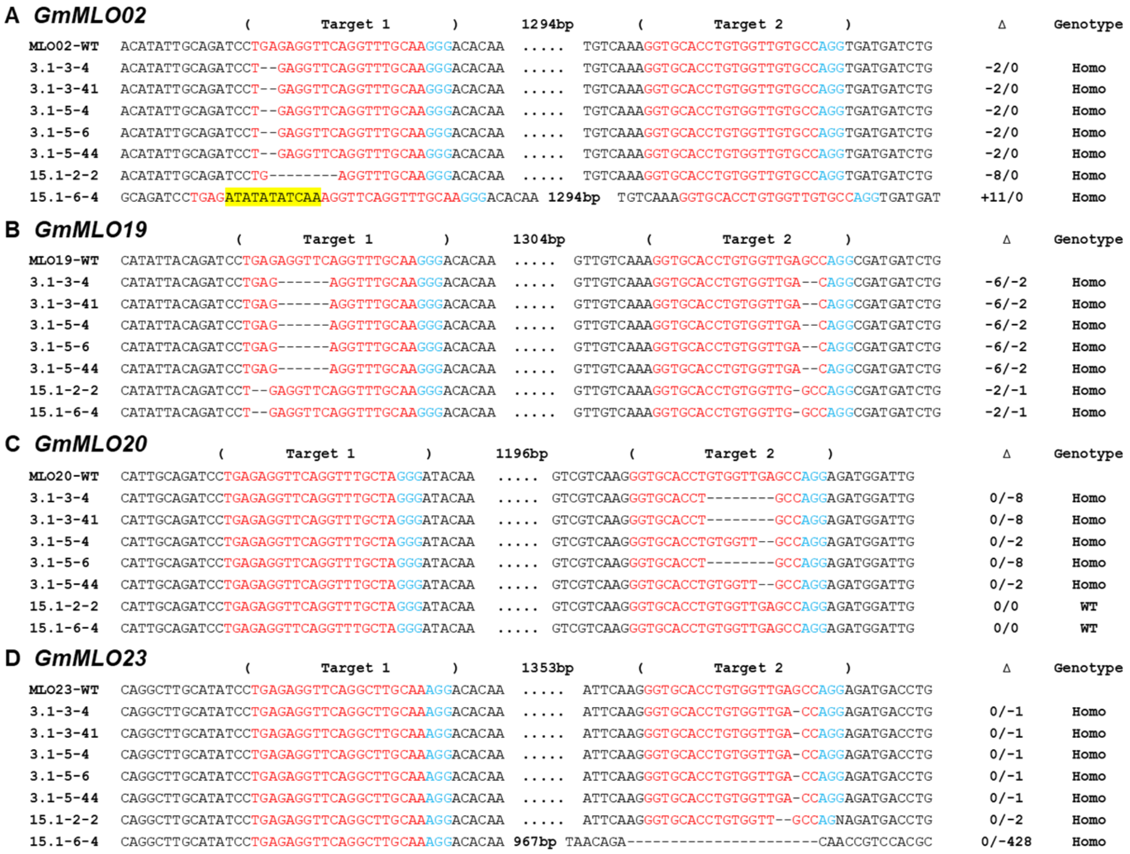


Fig. S4 Gel electrophoresis of PCR-amplicons of *GmMLO* target expanding regions at T1 generation. WT: Non transgenic wild-type plant; 3.1-2 to 3.1-5: T1 plants from 3.1 line; 15.1-1 to 15.1-6: T1 plants from 15.1 line; M: 1 kb DNA ladder. PCR products amplified by specific primers for extended regions of *GmMLO02*, *GmMLO10*, *GmMLO18*, *GmMLO19*, *GmMLO20* and *GmMLO23* genes



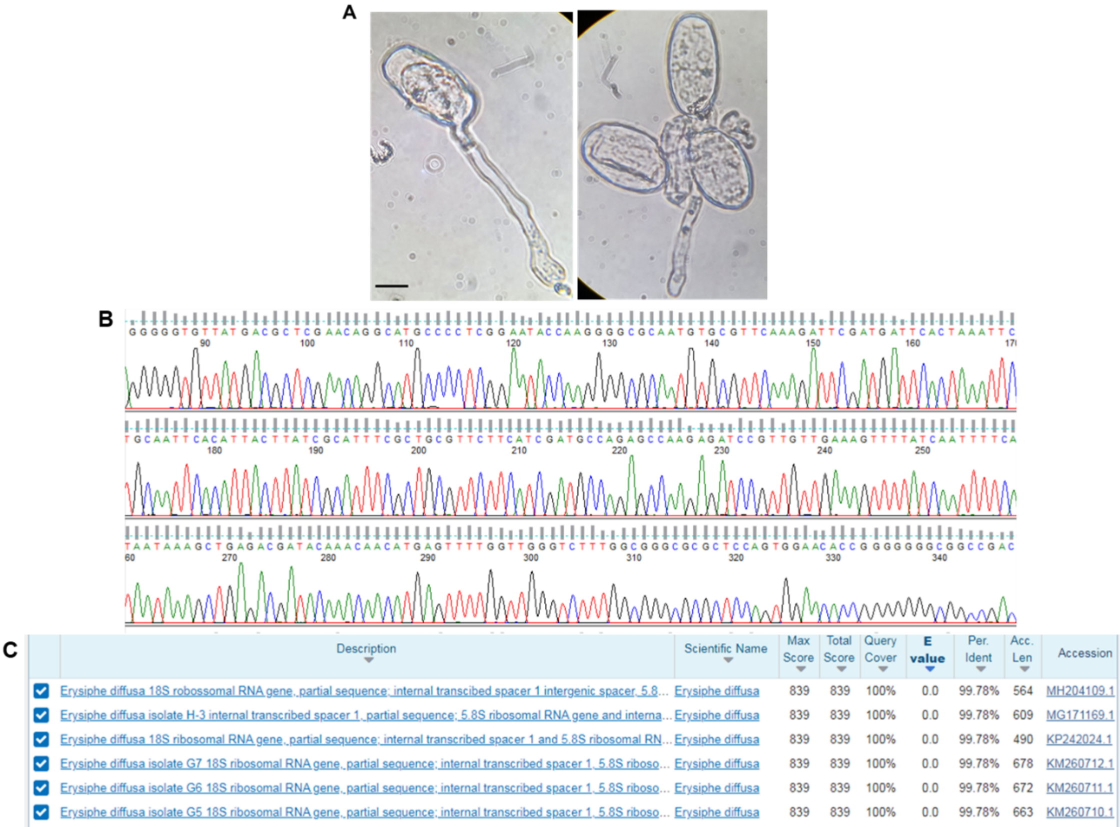
A *GmMLO02*	(Target 1)	1294bp	(Target 2)	Δ	Genotype
MLO02-WT	ACATATTGCAGATCCTGAGAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG		
3.1-3-4	ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-2/0	Homo
3.1-3-41	ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-2/0	Homo
3.1-5-4	ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-2/0	Homo
3.1-5-6	ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-2/0	Homo
3.1-5-44	ACATATTGCAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-2/0	Homo
15.1-2-2	ACATATTGCAGATCCTG--------AGGTTTGCAAGGGACACAA	.....	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGATCTG	-8/0	Homo
15.1-6-4	GCAGATCCTGAGATATATATCAAAGGTTCAGGTTTGCAAGGGACACAA	1294bp	TGTCAAAGGTGCACCTGTGGTTGTGCCAGGTGATGAT	+11/0	Homo

B *GmMLO19*	(Target 1)	1304bp	(Target 2)	Δ	Genotype
MLO19-WT	CATATTACAGATCCTGAGAGGTTCAGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGAGCCAGGCGATGATCTG		
3.1-3-4	CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG	-6/-2	Homo
3.1-3-41	CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG	-6/-2	Homo
3.1-5-4	CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG	-6/-2	Homo
3.1-5-6	CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG	-6/-2	Homo
3.1-5-44	CATATTACAGATCCTGAG------AGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTGA--CAGGCGATGATCTG	-6/-2	Homo
15.1-2-2	CATATTACAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTG-GCCAGGCGATGATCTG	-2/-1	Homo
15.1-6-4	CATATTACAGATCCT--GAGGTTCAGGTTTGCAAGGGACACAA	.....	GTTGTCAAAGGTGCACCTGTGGTTG-GCCAGGCGATGATCTG	-2/-1	Homo

C *GmMLO20*	(Target 1)	1196bp	(Target 2)	Δ	Genotype
MLO20-WT	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGGATTG		
3.1-3-4	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCT--------GCCAGGAGATGGATTG	0/-8	Homo
3.1-3-41	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCT--------GCCAGGAGATGGATTG	0/-8	Homo
3.1-5-4	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCTGTGGTT--GCCAGGAGATGGATTG	0/-2	Homo
3.1-5-6	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCT--------GCCAGGAGATGGATTG	0/-8	Homo
3.1-5-44	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCTGTGGTT--GCCAGGAGATGGATTG	0/-2	Homo
15.1-2-2	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGGATTG	0/0	WT
15.1-6-4	CATTGCAGATCCTGAGAGGTTCAGGTTTGCTAGGGATACAA	.....	GTCGTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGGATTG	0/0	WT

D *GmMLO23*	(Target 1)	1353bp	(Target 2)	Δ	Genotype
MLO23-WT	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGAGCCAGGAGATGACCTG		
3.1-3-4	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG	0/-1	Homo
3.1-3-41	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG	0/-1	Homo
3.1-5-4	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG	0/-1	Homo
3.1-5-6	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG	0/-1	Homo
3.1-5-44	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTTGA-CCAGGAGATGACCTG	0/-1	Homo
15.1-2-2	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	.....	ATTCAAGGGTGCACCTGTGGTT--GCCAGNAGATGACCTG	0/-2	Homo
15.1-6-4	CAGGCTTGCATATCCTGAGAGGTTCAGGCTTGCAAAGGACACAA	967bp	TAACAGA---------------------CAACCGTCCACGC	0/-428	Homo

Fig. S5 Inheritance of induced mutations in *GmMLO02* (**A**), *GmMLO19* (**B**), *GmMLO20* (**C**) and *GmMLO23* (**D**) genes in T2 plants. Target sequences and PAMs are indicated in red and blue color, respectively. Inserted nucleotides are shown in yellow. Δ indicates targeted sequence changes: 0 for no change, - for deletion, + for insertion



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Erysiphe diffusa 18S robossomal RNA gene, partial sequence; internal transcibed spacer 1 intergenic spacer, 5.8...	Erysiphe diffusa	839	839	100%	0.0	99.78%	564	MH204109.1
☑	Erysiphe diffusa isolate H-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and interna...	Erysiphe diffusa	839	839	100%	0.0	99.78%	609	MG171169.1
☑	Erysiphe diffusa 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RN...	Erysiphe diffusa	839	839	100%	0.0	99.78%	490	KP242024.1
☑	Erysiphe diffusa isolate G7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S riboso...	Erysiphe diffusa	839	839	100%	0.0	99.78%	678	KM260712.1
☑	Erysiphe diffusa isolate G6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S riboso...	Erysiphe diffusa	839	839	100%	0.0	99.78%	672	KM260711.1
☑	Erysiphe diffusa isolate G5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S riboso...	Erysiphe diffusa	839	839	100%	0.0	99.78%	663	KM260710.1

Fig. S6 *E. diffusa* isolation and characterization. **A** Conidiophore and conidia of *E. diffusa* isolated from infected leaves. Scale bar = 10 µm. **B** Internal transcribed spacer (ITS) sequences of the collected *E. diffusa*. **C** Nucleotide BLAST result of the *E. diffusa* ITS sequence on GenBank, NCBI

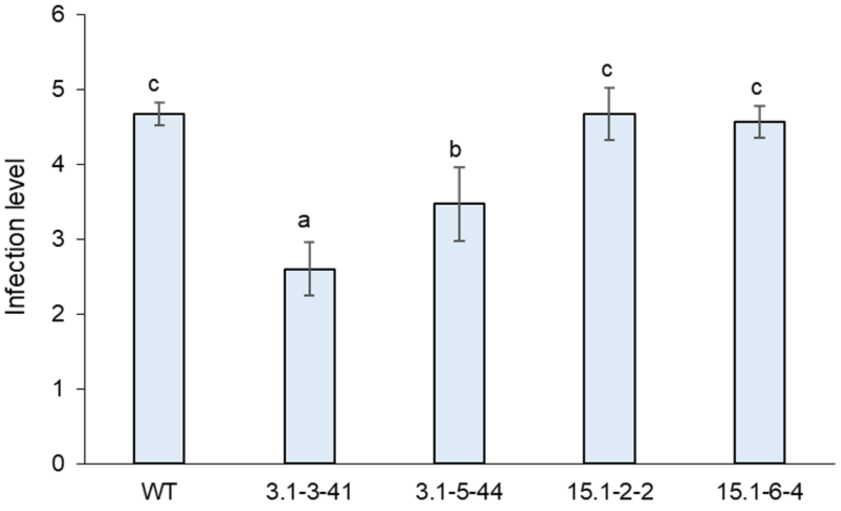


Fig. S7 Powdery mildew resistant assessment of T2 offspring *Gmmlo* mutant lines under the net-house conditions. Infection levels were recorded using a 0 to 5 scale (described by Tran et al., 2015), which according to strong resistance to severe infection. Infection levels were calculated as the average of 20-30 biological replicates and three experiments. Statistical analysis was done using one-way ANOVA followed by a *post hoc* Turkey's test. Significant difference was considered at $P < 0.05$

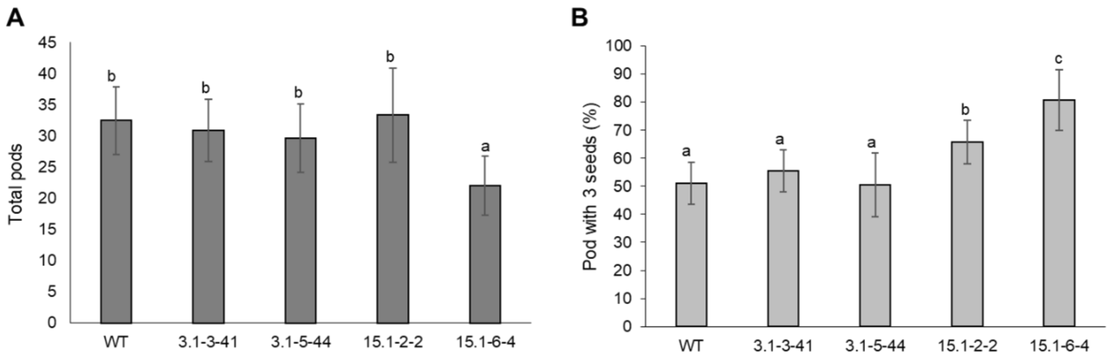


Fig. S8 Soybean seed production under the net-house conditions. **A** The total number of pods per plant. **B** The frequency of pods with 3 seeds. WT: Control line (DT26 cultivar); 3.1-3-41, 3.1-5-44, 15.1-2-2, 15.1-6-4: T2 offspring *Gmmlo* soybean mutant lines. Error bars indicate standard deviations, n = 9-14. Statistical analysis was done using one-way ANOVA followed by a *post hoc* Turkey’s test. Significant difference was considered at $P < 0.05$

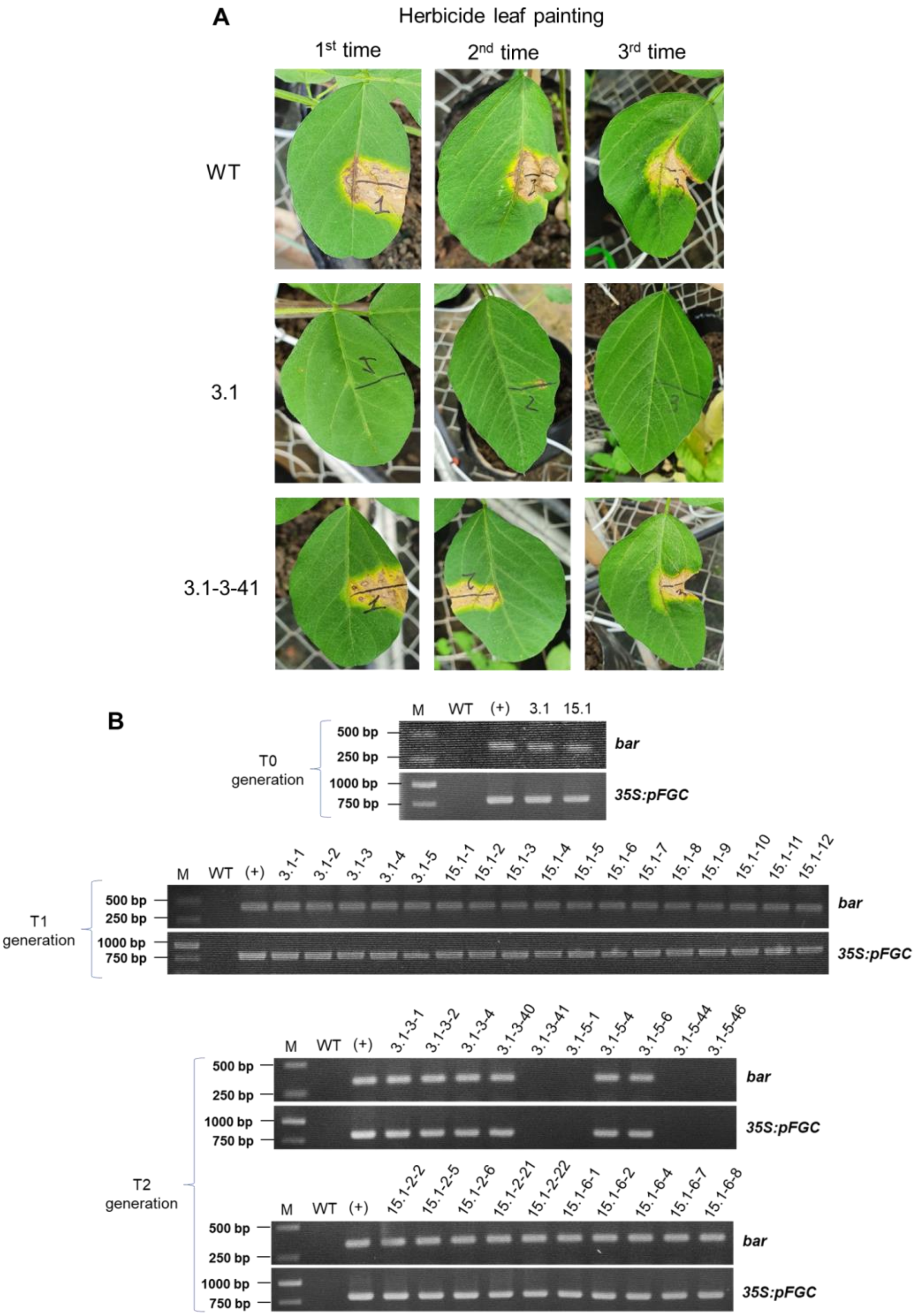


Fig. S9 Transgene inheritance and segregation at different generations. **A** Representative results of herbicide leaf painting with glufosinate solution (200 mg/L) on wild-type (WT), herbicide resistant line (3.1) and herbicide susceptible line (3.1-3-41). **B** Gel electrophoresis of PCR amplicons of transgenes at T0, T1 and T2 generations. M: 1 kb DNA ladder; WT: non transgenic wild-type plant; (+): positive control (CRISPR/Cas9 vector); *bar*: herbicide resistance gene; 35S:pFGC: transgene region spanning pFGC vector and 35SPPDK promoter of pcoCas9.

Table S1 Sequences of oligonucleotides and primer sets used in this study

Oligonucleotides and primers	**5’-3’ sequence**	**Description**
MLO-gRNA1-F	**gttt**TGAGAGGTTCAGGTTTGCAA	Annealed to create gRNA for target 1
MLO-gRNA1-R	**aatc**TTGCAAACCTGAACCTCTCA	
MLO-gRNA2-F	**gttt**GGTGCACCTGTGGTTGAGCC	Annealed to create gRNA for target 2
MLO-gRNA2-R	**aatc**GGCTCAACCACAGGTGCACC	
bar-F	TACCATGAGCCCAGAACGACGCCC	Primer pair specific to *bar* gene.
bar-R	CTTCAGCAGGTGGGTGTAGAGCG	
35S:pFGC-F	TGTGCGTCATCCCTTACGTC	Primer pair specific to a transgene region spanning pFGC and 35SPPDK promoter.
35S:pFGC-R	GAAGGCGGGAAACGACAATC	
MLO-02-F	GCCATTGTTGGTTGGTTCCAT	Primer pair flanking the target region of *GmMLO02* (≈ 1905 bp)
MLO-02-R	TTGTTTCGTGGAAGCAGGAGT	
MLO-19-F	GGGTCTCTGTTTTTGCTTTCGGC	Primer pair flanking the target region of *GmMLO19* (≈ 1820 bp)
MLO-19-R	AGCAGAGAAACGACATGATTCCT	
MLO-20-F	TGTGTCATGCGTCTTGGATGT	Primer pair flanking the target region of *GmMLO20* (≈ 1555 bp)
MLO-20-R	GCATTCTGCAGCACAGAACC	
MLO-23-F	GTCCCATGCTTCTTCATGGTG	Primer pair flanking the target region of *GmMLO23* (≈ 1720 bp)
MLO-23-R	TGCTAATTGAAAGGCATTCTGC	
MLO-10-F	AGCATAGCCTTGTTAATTATGTTGA	Primer pair flanking the target region of *GmMLO10* (≈ 2077 bp)
MLO-10-R	ACTGTAACAAAAACAAAATGATGCG	
MLO-18-F	ACTAACTTGCCAGTGAAAACTACAT	Primer pair flanking the target region of *GmMLO18* (≈ 2317 bp)
MLO-18-R	TGCTAATTGAAAGGCATTCTGC	

Table S2 Genotypes of T0 mutant lines

Events	Gene					
	GmMLO02	*GmMLO10*	*GmMLO18*	*GmMLO19*	*GmMLO20*	*GmMLO23*
3.1	Homozygous	WT	WT	Chimeric	Chimeric	Chimeric
15.1	Biallelic	WT	WT	Homozygous	WT	Chimeric

Notes: Homozygous: genotype that includes two identical mutant alleles; Biallelic: genotype that includes two mutant alleles; Chimeric: genotype that includes one wild-type allele and more than one mutant allele

Table S3 Inheritance and segregation of transgenes at different transgenic soybean generations

Generation	Lines	Herbicide leaf painting	*bar*	35S:pFGC	Generation	Lines	Herbicide leaf painting	*bar*	35S:pFGC
T0	3.1	R	+	+	T2	3.1-3-1	R	+	+
	15.1	R	+	+		3.1-3-2	R	+	+
T1	3.1-1	R	+	+		3.1-3-4	R	+	+
	3.1-2	R	+	+		3.1-3-40	R	+	+
	3.1-3	R	+	+		3.1-3-41	S	-	-
	3.1-4	R	+	+		3.1-5-1	S	-	-
	3.1-5	R	+	+		3.1-5-4	R	+	+
	15.1-1	R	+	+		3.1-5-6	R	+	+
	15.1-2	R	+	+		3.1-5-44	S	-	-
	15.1-3	R	+	+		3.1-5-46	S	-	-
	15.1-4	R	+	+		15.1-2-2	R	+	+
	15.1-5	R	+	+		15.1-2-5	R	+	+
	15.1-6	R	+	+		15.1-2-6	R	+	+
	15.1-7	R	+	+		15.1-2-21	R	+	+
	15.1-8	R	+	+		15.1-2-22	R	+	+
	15.1-9	R	+	+		15.1-6-1	R	+	+
	15.1-10	R	+	+		15.1-6-2	R	+	+
	15.1-11	R	+	+		15.1-6-4	R	+	+
	15.1-12	R	+	+		15.1-6-7	R	+	+
						15.1-6-8	R	+	+

Notes: R: Herbicide resistant; S: Herbicide susceptible; (+): Positive for PCR-amplification with specific primers; (-): Negative for PCR-amplification with specific primers; bar: herbicide resistance gene; 35S:pFGC:transgene region spanning pFGC vector and 35SPPDK promoter of pcoCas9