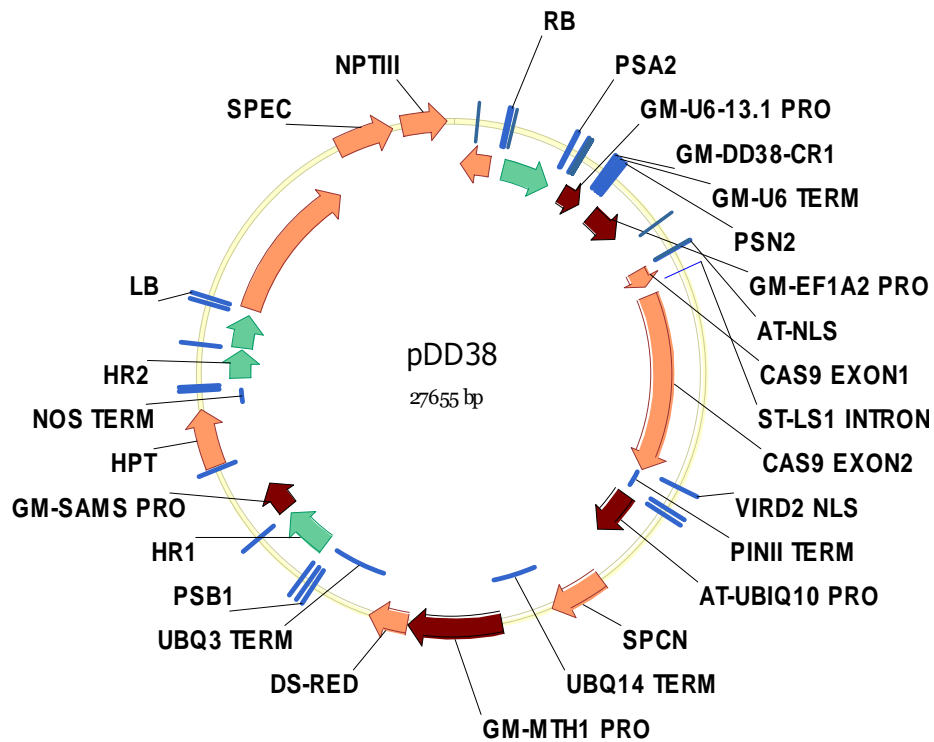
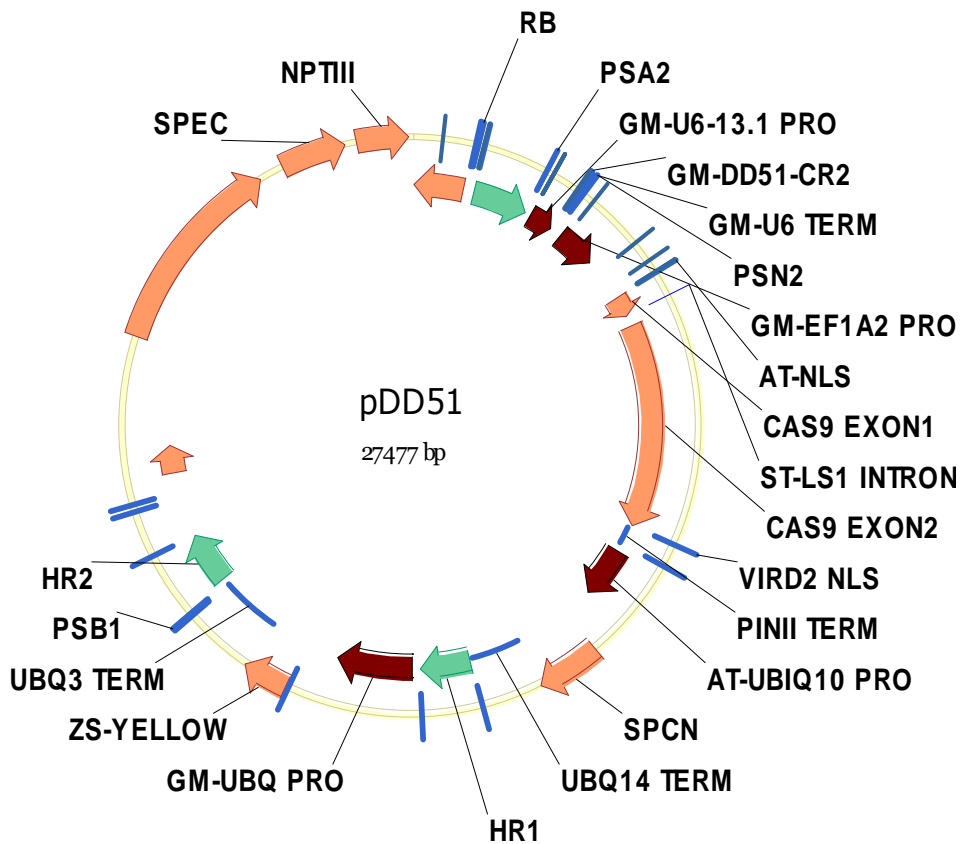


A.



B.



Supplementary Figure S1. Plasmid maps of the constructs pDD38 (A) and pDD51 (B) containing donor template for gene targeting at soybean DD38 and DD51 genomic site, respectively. The full description of the elements is given in Supplementary Table 1.

Supplementary Figure S2: Guide RNA spacer and homology sequences used in pDD38 and pDD51 plasmids. GM-DD38-CR1 and GM-DD51-CR2 are the 20-21 bp targeting spacer sequences of a guide RNA targeting the DD38 and DD51 sites on chromosome 4 as shown in supplementary Figure 1 and described in supplementary Table 1. HR1:DD38 and HR2:DD38 are Homology arm1 and Homology arm2 sequences of DD38, respectively. Similarly, HR1:DD51 and HR2:DD51 are Homology arm1 and Homology arm2 sequences of DD51, respectively. The physical and genetic locations of DD38 and DD51 sites are described in the Materials and Method section.

GM-DD38-CR1

GGTGCTAAGCACGACATCCA

HR1:DD38

CGACCCAGTGATGGCAACTAAGTGGATCAACAGCAACTTTTGTCCGATTTGCGATATAATTTGTCAAAACATGC
CTCTAAAATCACTCTAACATCAAATTTCTGAATAACAATTTTATACACTCCCCTCCTCCAACCTTGTTATGTGCCCTTC
TGACCCTTCTGTTTTGTTTGCTAGTTAACCATGTAGGAAAAAGTATCACTCCACTCAATAGTATTTTCACTTCATCT
GGCATAAATAATTTTGGCCTAATTCAGCATCACGTATCAGGAGTCAATAAGCTTGTCTGTCCGTCTGTATTTATCA
TCTCTTTCGTGCTACTATCACCTCCCCTTTTCTTATGGATTTTTCTGTAAAAAACAAAAAGGAACAGGTAACCCA
CTTGAATTAGGACACCAATGTAGGTGCCACCACCCGTTTCCTTCTGTTGAGTGTGCACGCTTATTGGTTCAACCTCA
GAATTAATTTCTCATAATCCCCTGTTGAGAGAAAATTATAATAGACAAATCAAATTGTGTGGACTTGCCATTTCCC
AAAACATTTTCATTTTGTGCGCGTGTGGGTGGTGGGGTGCCTTTTATTAGAATGAAGACTGTAAAAAGAGGAGT
GTAGGATGATAAGAAGTAGTAAATGTCAGTTGTCAAAAAGATGAAAAGCAACGGAGGAAGGGACCAAACCTTGAC
CTATGGAAAAGTGTGCTATGGTAATTATTGTCACAGGATTTAGGATGATTAATAATAGTTCGGACCACAAATAA
ACAGCTTTAAGAAGTTCAACTTTCCCCACGGATAGCAAATTTGTTGGACCATCTTTGATGAAAGTCCCAATCTC
ATAATTGCCAACCTTTTTTATACGAAACTAGTATTTTATAATTATCAAGAATCTTCAACTCATCAGTGATCGCTCAT
TTCTGATTAAGAAAAAAAAGGGTGAGTAAATGAATCCATTTTGTCTGTTCTCCATGG

HR2:DD38

ATGTCGTGCTTAGCACGTGATGCATCATAGATGCGGTAAATTACTTTCTGTGACTGGGACAATGTGCTCCTCCA
GTGGCCACGTATCGGTAAACCTTGTGCCAGAACACTAATATTTATATAATCAACAATAAAGAATGGTAAGCATTAG
ACAAAGACAAAAACAATTTTTAATAATTTACCTGTACTAATTAGAAGGGTTTGATTTAATTTTATAAACTTAACAT
GTGCAATATGTCCCTTTGTTTAGAGGAAAACAATTACGGTATTTCTTACTAACGTAAACTCTGAATATAATCATA
ATCTGTATTCTGAAAAAGAAAAAGAACTAGAGATAAAAAGCGTAGAAACAACAGTATGTGGTCATAGTCATACAC
GTAAAGGGATGAAAATTGAAAATAGAACCTGCAGGAATAAGTTTGCATTTAATTTGGATATATATCTTGAAGTGT
AAGGTTAACCTCTCAGCTATTAATAGTACTACTACTAACTCAAATAGTACTTCTACTAGCACTCACGGTAACAA
TCTCTTAGTAGGGATGGGAATAGGCCAGATCGGCCTACAGGGGTCTACGACTTGA

GM-DD51-CR2

GTAATGTACGTTGTTGTGTG

HR1:DD51

AAGACCTACAAAACAATGATCCCGACGCTAAAGTAAGTACGAGAGTTAAGAGAATAAATGGGAAAATATGCATA
CATGATTAGGTGTGTGTTCTCAAGAAAGTACGAATGAATATGGTGTGTTGTAGTACATGAATGATGTGTTTT
GAGGGTTCAAGGGAAATTGATATTTATAGAGTGAATGGAACCAGAGGTCTTTGTTGACAAGGGTTGTTATGACT
CTTGCAAATAATTAAGCTTATAAATAATAGCCAATAACTTATTATAGATAGAGTTAGAGATAATATATAGCTAAA
TTTGAACAAGGCATACAAAACAATAATGCTAAATATGAATAAGACAATCAAATTTAGTGTGATGTTCAACTCTTT
GTCGTTGAAGAACTTGTGTCAGTGGTATAGTAAATGGGTGTGAGTGCAGTGTCTACCCATCTCACACCACACAA

CCAACCTCATATCTAAAGATATTGTCGCTGAATACAAAATTGAGTTATGGAATATACAATTCATAATATAGATACGA
AAAATCATTCTTACAAAACATTCAATCAAAAATTATTCAAACATAATTCTAGATTAAGTAATCCGAAGTACAAGTT
AGTATCCTAGATCCGTTAATTTAAAATTATGTTTGCATAATTTGGATTTGGTGTCTATAAGGGCACAATTTGTTC
ATTCTTACAAGTTTGTCAATTCTAAAATATATGCAAATTTGAAGAAAAAAAATTTACGAATGTGTCTCAAACAATAA
CTTAATGGGAGGAGAATGAGGGATGAAGAAGCTCAAAATTACCAACGCCTTCTACCTCAAGAAGCTACTTCACAC
AAAATATGACTGGCGGAAGGATAGGGGACAACCGATAACGAGAAGGAGATACATAAGGTAATGTACGTTGTTGT

HR2:DD51

GTGAGGTACACAATTATGGGGATGAAGAAGTTCAACTTTAGTCGAAAAAATGTTTGAGAGGAACTTATGAAGAAT
GTAATGGCAATTAAGATTTTTAAAATTATGAAGGTGCAGATAATAAATTGTGGGATGTAGGAAGCAAGTGCCTTA
TACTTGAACCTTACTGCCAACATTCGGGACCCAAAAACAAATTCTCCAAAGTCCAAATCATTAGAATTTCTTGTG
AATGATCTTAAAATTCTTAATTGTAACTTTTAACTTTTCATTAGCACTAATACTAAAAGAAGCTAAACAAATTTGTAA
CTCAGCTTAGCATTGCCTGCAATCGCACAAAGCATGGGTGATTGATCCATTTCTTTGATTGACCATATTTTTTTCCCA
GTTTCTGAGGTAAAAACACAAGCATTGCGTATTGATCCATTTCTTTGATTGTCACATTTTTTTCCCTCCAGCTTACT
ATACCAGGTTGTAAGAATTAAGGTTAAGTTTCCAATGGTTTGTGCTGATCCGCACCAAGATGTTCAATTTGTGTTG
AAAAATCCATACAATTACGCCATTCTCTCGACAAGCATGGTCAAAATCATCATCTTGCTTGTCAAAAAATTACCGC
TTCGACGATAGTATCTCCATTTTAAATTGACCAATGTTTTAAGTGTTTTACATTGATTAAGAAGAATCATAAATAA
GTTATTTATTGAAGTAGTTTTACCAAATATTTATACTTTGTATAAATGTATTACCCGATTTTATTGGGTGATTTATG
TACTAGTTGAAAAATTAATAAACACAATACTAAAAAGCTATTATATTGGTGAATTTATTGGATAACTTTAAAAT
AAAATTTAATATCTCATTGAAAACAAAAATATTAGTTAACATTTTTTAAATTAATTAATTTAGCCATTTTAGAAT
AGAGGGAATAAGTATTA

GM-U6 TERM

TTTTTT

Supplementary Table S1: Description of vector components

Component type	Label	Description	Reference/Sequence
Promoter, introns and 5' Sequences	GM-EF1A2PRO/5'UTR/INTRON1	Putative promoter 5'UTR and intron of a soybean elongation factor EF1A homolog PS0333268.	U.S. Patent No. 8697857
	GM-MTH1 PRO	Putative promoter for soybean gene Glyma.07G132000 (Alias GlymaD7g15800) which encodes a metallothionein protein homolog	US20120005791A1
	GM-UBQ PRO/5'UTR/INTRON1	Putative promoter, 5'UTR and intron of a soybean	NCBI Accession D16248
	AT-UBI10 PRO/5'UTR/INTRON1	Arabidopsis Ubiquitin 10 promoter, 5'UTR and intron	[1]
	GM-U6-13.1 PRO	Soybean U6 snRNA gene polymerase III promoter	US20190376074A1 (SEQ ID. 120)
	GM-SAMS PRO	Soybean 5-adenosyl-L-methionine synthetase promoter and	US20030226166A1
	ST-LS1 INTRON2	POTATO LS1 INTRON 2	[8]
Terminator and 3' Sequences	UBQ3 TERM	Ubiquitin-3 (UBQ3) 3'UTR and terminator from <i>Arabidopsis thaliana</i>	NCBI Accession L05363
	GM-U6 TERM	Soybean U6 snRNA gene polymerase III terminator	Supplementary Figure 2
	UBQ14 TERM	Ubiquitin-14 (UBQ14) 3'UTR and terminator from <i>Arabidopsis thaliana</i>	NCBI Accession NM_001203736
	NOS TERM	Nopaline synthase terminator from <i>Agrobacterium tumefaciens</i>	[7]
	PINII TERM	The potato proteinase inhibitor II (pinII) 3' sequence	[2]
Coding sequences	Zsyellow	The Zs-Yellow1 N1 gene encoding a yellow fluorescent protein from <i>Zaanthus sp.</i>	[3]
	Dsred	The red fluorescent protein (DSRED) derived from <i>Discosoma spp.</i> , from Clontech	[3]
	SPCN	Spectinomycin	Genebank protein ID - AAD50455.1.
	CAS9 EXON1	First 375 bps of soy codon optimized CAS9 gene from <i>Streptococcus pyogenes</i>	[4]
	CAS9 EXON2	Last 3732 bps exon 2 of soy codon optimized CAS9 gene from <i>Streptococcus pyogenes</i>	[4]
	AT-NLS	Arabidopsis gene encoding peptide PKKKRKV that is a nuclear localization signal;	At3g04980 (amino acid 3118-324)
	VIRD2 NLS	Canola cotton optimized C-terminal nuclear localization signal from <i>VIRD2 Agrobacterium tumefaciens</i>	[5]
HPT	Hygromycin phosphotransferase gene from <i>E. coli</i>	[6]	
Other sequences	GM-DD38-CR1	20 bp targeting spacer sequence of a guide RNA targeting the DD38 site on chromosome 4	Supplementary Figure 2
	GM-DD51-CR2	21 bp targeting spacer sequence of a guide RNA targeting the DD51 site on chromosome 4	Supplementary Figure 2
	HR1:DD38	Homology arm1 sequence used in DD38 donor	Supplementary Figure 2
	HR2:DD38	Homology arm2 sequence used in DD38 donor	Supplementary Figure 2
	HR1:DD51	Homology arm1 sequence used in DD51 donor	Supplementary Figure 2
	HR2:DD51	Homology arm2 sequence used in DD51 donor	Supplementary Figure 2

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Supplementary Table S2. Primers and probes used for qPCR detection of transgene

Q-PCR Assay*	Amplicon size (bp)	Forward Primer	Reverse Primer	Probe
PSA2	63	CATGAAGCGCTCACGGTACTAT	TCGTACGCTACTGCCACCAA	FAM-ACGGTTAGCTTCACGACT
PSN2	86	CTAAAAACGGCCCTAACTAACG	GCCCCTAGTCGTCGTGTGT	FAM-TTGCCCGCTAGATTAGTCCCCCGTT
PSB1	66	TGATTCCGATGACTTCGTAGGTT	GCTAATCGTAAGTGACGCTTGGA	FAM-TAGCTCAAGCCGCTCG
SPCN	86	CTGCCCGCAATGCTCTTT	ATTACCACTGGACCGTCACAGA	FAM-ACAGTTGCGAACCAAGGTCTCCCG
HPT	65	CAGCGAGAGCCTGACCTATTG	CAGGCAGGTCTTGCAACGT	FAM-ATCTCCCGCCGTGCACAGGG

*Labels match maps in Supplementary Figure 1

Supplementary Table S3. Primers and probes used for gene targeting and mutation analyses

Site	Assay	Forward Primer	Reverse Primer	Probe
DD38	HR1 junction PCR	CTTCAGTTGTTACCATCTGTGTTTTCC	AGTATGATTGGTAAGGAAGATATCCATG	None
	HR2 junction PCR	GATTAGAGTCCCGCAATTATACATTTAATACGCG	TTGAGCCTAATCTTTTAAGTAAATAGGTC	None
	Mutation	TCCATTTTGTCTGTTCTCCATGGATG	CACATTGTCCCAGTCACAGAAAG	FAM-AGATGCCGGTAAATTAT
DD51	HR1 junction PCR	F1:ATGTCGAAGTGAGAGTGATTTTATTGA F2:CATGAAGCGCTCACGGTACTAT	R1:AGACATGCAATGCTCATTATCTCTAG R2:GGAACTATAGCTAAGTCGTACGCTACTG	FAM-ACGGTTAGCTTCACGACT
	HR2 junction PCR	F1:TGATTCCGATGACTTCGTAGGTT F2:GCTAATCGTAAGTGACGCTTGGA	R1:TGGGGTTGAGTTAGGTCTCTCATC R2:TGATTCCGATGACTTCGTAGGTT	FAM-CTAGCTCAAGCCGCTC
	Mutation	GACAACCGATAACGAGAAGGAGAT	GCCATTACATTCTTCATAAGTTCCTCT	FAM-CGTTGTTGTGTGAGGTAC VIC-GACTAAAGTTGAACTTCT)