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Mutation of *MsSPL8* alleles via CRISPR/Cas9 mediated genome editing leads to superior abiotic stress resiliency and distinct morphological alterations in alfalfa

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Background

• Alfalfa (*Medicago sativa* L.) is one of the world's most important forage species.

• Since the global demand for livestock is predicted to escalate in coming years, there is an urgent need to exploit accelerated molecular breeding technologies to improve productivity and climate resiliency in this species.

• The down-regulation of *SQUAMOSA PROMOTER-BINDING-LIKE8* (*SPL8*) using RNAi has been shown previously to enhance biomass production, as well as drought and salinity tolerance in alfalfa (1).

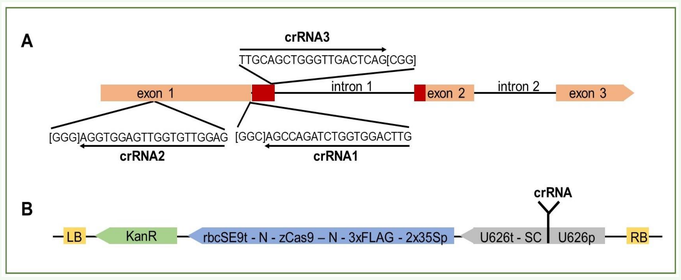
• Despite the potential economic benefit of such germplasm, public concern and regulatory constraints surrounding the use of transgenic crops can be problematic for implementation.

• As such, the aim of this project is to target *SPL8* homologs in the tetraploid, outcrossing *M. sativa* using CRISPR/Cas9 in an attempt to develop novel germplasm with improved yields and abiotic stress tolerance.

Methods and Results

• Three separate guide RNAs (gRNAs; 20 nt) were designed immediately upstream of 5' – NGG -3' PAM sequences within the first exon of two highly similar *MsSPL8* gene copies in alfalfa (MSAD_231217 and MSAD_231229).

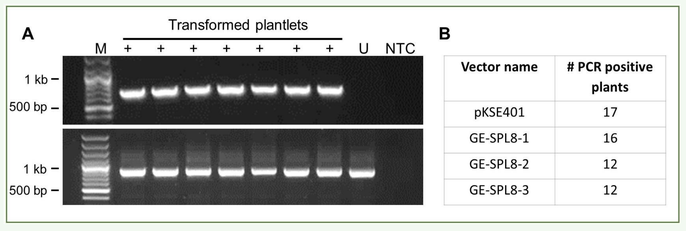
• The pKSE401 vector (2) was used with those three crRNAs seperately to generate binary vectors that target *MsSPL8* alleles.



2x35Sp, 2x CaMV 35S promoter; Bar, phosphinothricin resistance cassette; CaMVt, CaMV 35S terminator; K, kozak sequence; Kan, kanamycin resistance cassette; LB, left border; MtU6p, *M. truncatula* U6 promoter; N, nuclear localization signal; oCas9, *Oryza sativa* codon-optimized Cas9; polyA, polyA signal; RB, right border; rbcSE9t, pea RuBisCO small subunit E9 terminator; SC, sgRNA scaffold; U626p; Arabidopsis U6 gene promoter; U626t; U6 terminator; zCas9, *Zea mays* codon-optimized Cas9.

• The three CRISPR/Cas9 vectors, along with the empty vector control, respectively, were introduced into alfalfa N4.4.2. leaf explants using *Agrobacterium*-mediated transformation (3).

• Kanamycin-resistant alfalfa regenerants were assessed via PCR to confirm introduction of the transgenic cassette in each case

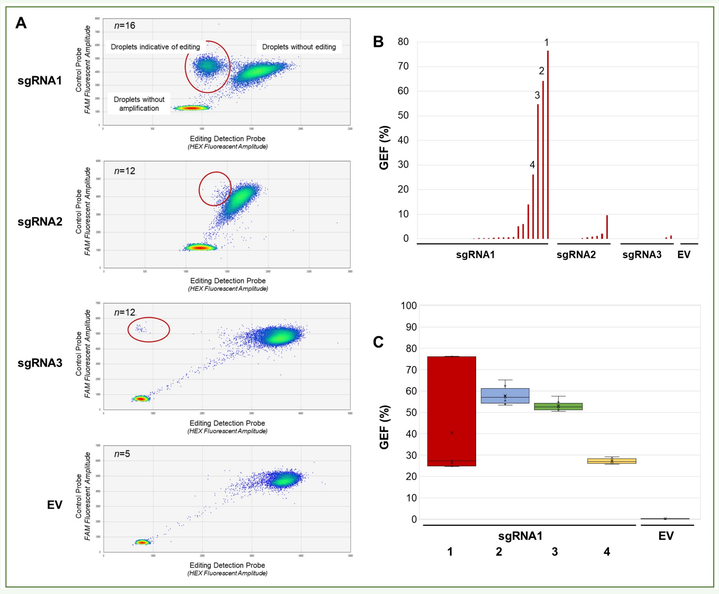


Vector name	# PCR positive plants
pKSE401	17
GE-SPL8-1	16
GE-SPL8-2	12
GE-SPL8-3	12

Alfalfa regenerants were confirmed using PCR. **A** Representative PCR results with top row displaying Cas9-specific fragments and bottom row displaying positive control fragments. **B** Number of transgenic genotypes confirmed in each case. M, molecular weight marker; NTC, no template control; U, untransformed control.

Between 12 and 17 transgenic alfalfa genotypes were obtained bearing each construct, respectively.

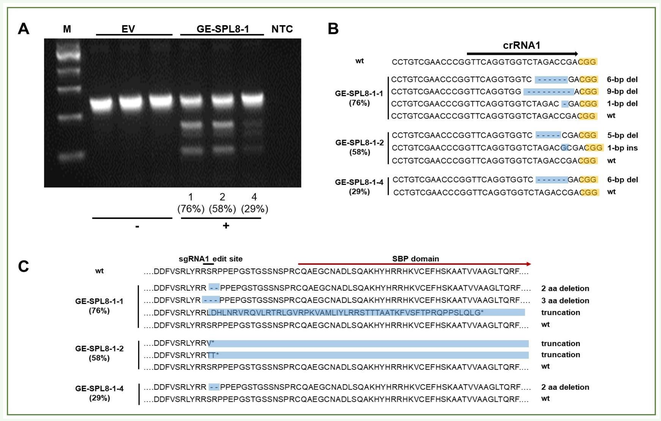
• Gene editing frequency droplet digital PCR (GEF-dPCR) assays were carried out to assess gene editing frequencies (GEFs) in transgenic plants bearing the three GE-SPL8 cassettes, respectively, along with empty vector (EV) controls.



A Ratios of FAM (control probe) and HEX (editing probe) fluorescence following GEF-dPCR of GE-SPL8 transgenic genotypes, as well as a selection of EV controls. Heat maps indicate droplets that were not amplified, as well as those that bound both probes (not edited) and those that only bound the control probe (potentially edited, circled in red). **B** GEF of each transgenic genotype assessed by GEF-dPCR. Numbers denote GE-SPL8-1 genotypes that were selected for screening of separate shoots (shown in C). **C** Evaluation of GEFs in 8 distinct shoots from four edited GE-SPL8-1 genotypes, as well as EV controls. Blocks represent the upper and lower quartiles of the values obtained, x denotes median values, and bars indicate maximum and minimum values.

Targeting with sgRNA1 yielded genotypes with the highest GEF.

• GE-SPL8-1 genotypes with ~75%, 50% and 25% GEFs, respectively, were chosen for further confirmation of editing via T7E1 assays and Sanger sequencing.



A T7E1 assay results from three empty vector (EV) control genotypes and GE-SPL8-1-1, 2 and 4 genotypes. While genotypes with no alterations within the *MsSPL8* crRNA1 target region show a single band, those with genetic alterations within the crRNA1 target region exhibit multiple bands. Percentages indicate GEFs determined via GEF-dPCR. **B** Nucleotide sequences surrounding the *MsSPL8* crRNA1 target region from untransformed (wt), GE-SPL8-1, 2 and 4 genotypes as determined by Sanger sequencing. All results are representative of all genetic variants observed within each genotype following the sequencing of 20 – 24 clones. Percentages indicate GEFs determined via GEF-dPCR. **C** Amino acid sequences surrounding *MsSPL8* crRNA1 target region from untransformed (wt), and SPL8-gRNA1-1, 2 and 4 genotypes.

GEFs of ~75%, 50% and 25% corresponded to mutations in 3, 2 and 1 out of 4 *MsSPL8* alleles, respectively.

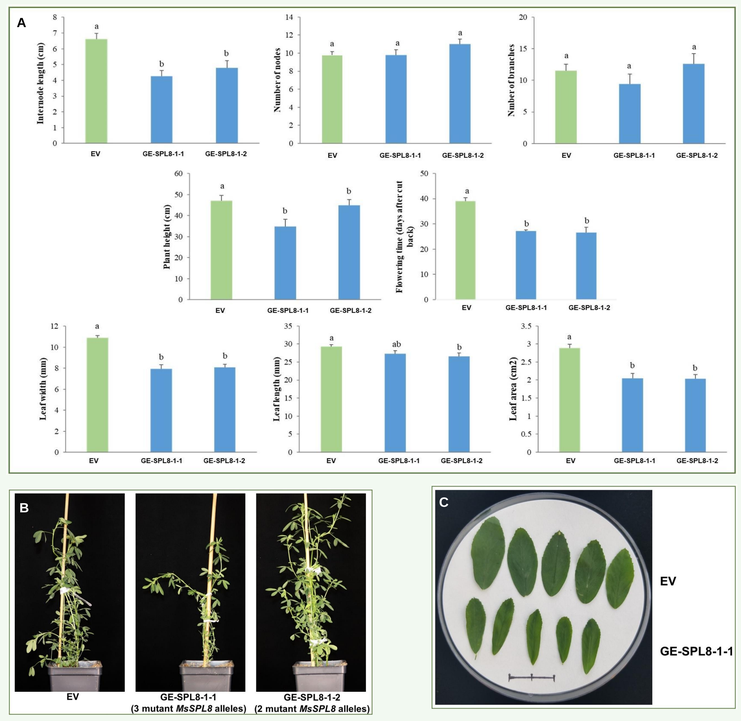
• Possible crRNA1 off-target sites were then selected for downstream Sanger sequencing to assess the specificity of editing events.

crRNA1	GTTCAGGTGGTCTAGACCGA[CGG]
Possible off-target 1	CTTCAGGTGGTTTAGAC--A[TGG]
Possible off-target 2	ATTCAGCT--TCTAGACCGA[TGG]
Possible off-target 3	TTTTAGGTGGT-TAGTCCGA[AGG]
Possible off-target 4	GATCAGGAGTTCTATACCGA[AGG]

8-12 clones were sequenced from each GE-SPL8-1 genotype, as well as empty vector controls and untransformed wild-type plants for each of the four chosen possible off-target sites. No genetic alterations were observed at the putative target site in any clone.

PAM sequences are indicated in square brackets and nucleotides in red denote those that differ from the crRNA1 target site.

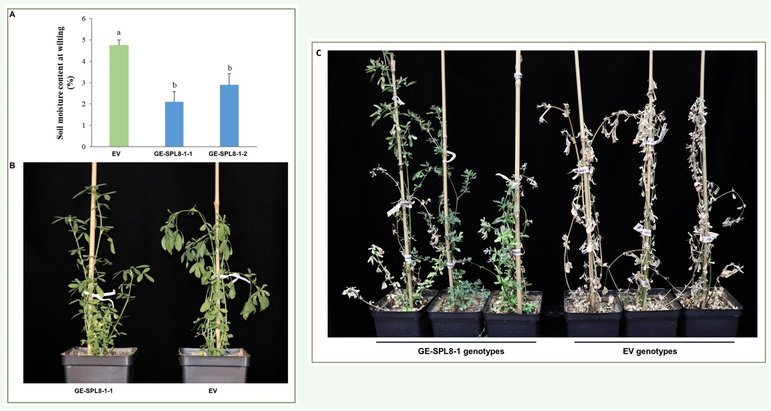
No off-target effects were detected at four possible sites with the highest sequence similarity to the crRNA1 target site.



A Growth and leaf (middle leaflet from the first fully expanded trifoliate) characteristics of empty vector (EV), GE-SPL8-1-1 and GE-SPL8-1-2 genotypes. All measurements included 5 biological replicates of each genotype (generated through stem cuttings derived from a single shoot) with blocks representing means and bars denoting standard errors. Lower case letters indicate statistically significant differences (P≤0.05). **B** Representative images of empty vector (EV), GE-SPL8-1-1 and GE-SPL8-1-2 genotypes under well-watered greenhouse conditions. **C** Representative images of leaves of EV and GE-SPL8-1-1 genotypes.

GE-SPL8-1 genotypes consistently displayed reductions in internode length, height and leaf length/area, as well as accelerated flowering, compared to empty vector controls.

• Empty vector (EV), GE-SPL8-1-1 and GE-SPL8-1-2 genotypes were subjected to drought treatment (withholding of water) and salinity treatment (50 mM NaCl for 2 days, 100 mM NaCl for 2 days, and 150 mM NaCl thereafter) to assess tolerance to each stress.



A Soil moisture levels were recorded at the first signs of wilting in EV, GE-SPL8-1-1 and GE-SPL8-1-2 genotypes. Blocks represent the mean of five biological replicates and bars indicate standard errors. Lower case letters indicate significant differences among groups (P≤0.05) as determined using Tukey's HSD test. Representative images of EV and GE-SPL8-1 genotypes following drought (**B**) and salinity treatment **C**) at a soil moisture level of approximately 2.5% and approximately 10 weeks after salt treatment, respectively.

GE-SPL8-1 genotypes exhibited improved drought and salinity tolerance compared to empty vector controls.

Conclusions and Future Directions

- GEF-dPCR, T7E1 and Sanger sequencing results indicate that successful editing of up to 3 out of 4 *MsSPL8* alleles was achieved in alfalfa.
- Editing efficiencies appeared to be highly dependent on the crRNA.
- No off-target mutations were identified in four regions with the closest homology to the crRNA1 target site.
- Several growth and leaf characteristics were found to be consistently affected in our edited genotypes compared to empty vector controls.
- Drought and salinity tolerance was improved in edited genotypes compared to empty vector controls in preliminary trials.

References:

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2. Xing, Hui-Li, et al. BMC plant biology 14.1 (2014): 327.3.
3. Aung, Banyar, et al. Plant biotechnology journal 13.6 (2015): 779-790.

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