Targeted Precision Nucleotide Substitutions in Sugarcane Following CRISPR/Cas9 and Template Mediated Genome Editing Confer "Gain of Function"

Tufan Mehmet Oz, Ratna Karan, Aldo Merotto, **Fredy Altpeter**
University of Florida - IFAS, Gainesville, FL Contact: altpeter@ufl.edu

Project Goals

We employ the "plants-as-factories" approach, in which biofuels, bioproducts, high-value molecules, and foundation molecules for conversion are synthesized directly in plant stems. This approach circumvents the challenges of developing efficient lignocellulose deconstruction methods, while still retaining residual biomass for deconstruction by traditional or emerging methods. The main thrust of this specific project within the Center for Advanced Bioenergy and Bioproduct Innovation (CABBI) is on genome editing and metabolic engineering of sugarcane. Sugarcane is the world's highest biomass producer with demonstrated potential for accumulation of oil in vegetative biomass after metabolic engineering (Zale et al. 2016).

Abstract

Genome editing tools such as CRISPR/Cas9 and TALEN have been employed in several crop genomes, including sugarcane in the PI's laboratory (Jung and Altpeter 2016; Kannan et al. 2018). They enable precise targeting and introduction of double stranded DNA breaks in vivo. Subsequent cellular repair mechanisms, predominantly non-homologous end joining (NHEJ), act as critical steps to endogenous gene editing or correction. However, there is very limited control over NHEJ, which generates an abundance of random insertions and deletions (indels) near the target site. Frameshift mutations associated with these indels of unspecified size and sequence might result in "loss of function" phenotypes of agronomic importance including improved feedstock quality (Kannan et al. 2018). "Gain of function" mutations, on the other hand, generally require precise nucleotide substitutions in the target locus. This can be accomplished with the aid of a homologous repair template and involves the cellular homology directed repair (HDR) mechanism. We are presenting an efficient HDR mediated genome editing approach conferring herbicide resistance in the highly polyploid sugarcane as an example for "gain of function". This is not only the first report of targeted precision nucleotide substitution in the complex sugarcane genome but also represents a critical enabling technology for multiplexed genome editing to address cane improvement objectives in CABBI.

Construction of vectors and delivery into sugarcane

Sugarcane acetolactate synthase (ALS) gene was selected as a target to modify using CRISPR/Cas9 system. Five plasmids carrying various DNA elements were constructed (**Figure 1**). Expression of *npt*II and codon optimized Cas9 were driven under the control of CaMV35S promoter and HSP70 intron. Termination signals from CaMV35S and AtHSP were located downstream of *npt*II and coCas9, respectively. CRISPR RNA and tracrRNA (sgRNA) were placed under the control of U6 promoter. Donor template with nucleotide modifications to introduce W574L and S653I, and two modified PAM sites (PAM1 and PAM2) to prevent cleavage of the template by sgRNA1 or 2 were designed according to sugarcane ALS sequence (**Figure 1**). Co-delivery of plasmids carrying expression cassettes and donor template was achieved with a biolistic particle delivery system. Treatment conditions were summarized in **Table 1**.

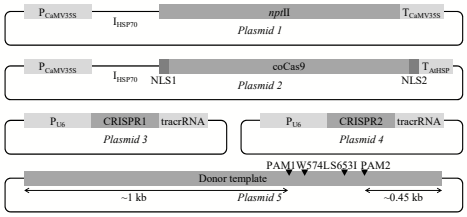


Figure 1. Schematic plasmid maps. Donor template with nucleotide modifications (indicated with arrow heads) were designed according to ALS sequence. Homology arms in donor template were indicated with double headed arrows. Size of DNA elements are not in scale.

Table 1. Treatments in biolistic mediated transformation of sugarcane.

Treatment	Abbreviation (B: Bombardment)	Plasmids 1	2	3	4	5	Molar ratio of components (Plasmids 1 – 4:Plasmid 5)	DNA amount (ng / kb shot)	Number of shots
1	B1	+	+	+	-	+	1:4	1.5	10
2	B2	+	+	-	+	+	1:4	1.5	10
3	B3	+	+	+	+	+	1:4	1.5	10

Sugarcane tissue culture, plant regeneration and phenotyping

Immature leaf whorl explants from commercial sugarcane cultivar CP88-1762 were used to generate embryogenic callus. Biolistic co-delivery of plasmids was followed by recovery period and subsequent selection on media containing 20 mg/L geneticin. Plant were regenerated from media with 20 mg/L geneticin or 0.2 µM bispyribac sodium (BS). Regenerated plants were established in soil and genome edited events (e.g. L2) were sprayed with imazapyr (Arsenal®) at 3 liter per ha (2x the labeled rate) in comparison to the non-edited control (WT) (**Figure 2**).

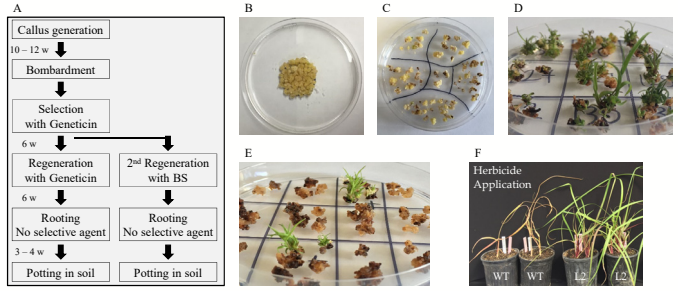


Figure 2. A) General outline of sugarcane tissue culture, plant regeneration and genetic transformation. B) Calli placed at the center of a petri dish for bombardment. C) Calli under selection. D) Regeneration under Geneticin. E) Regeneration under bispyribac sodium. F) Wild type (WT) and genome edited line L2 sprayed with imazapyr (Arsenal®) at 3 liter per ha (twice the labeled rate).

Integration of genome editing tools and selectable marker

Genomic DNA was extracted from leaf tissues of regenerated plants, and integration of transgenes were investigated using conventional PCR with gene-specific primers (**Table 2**).

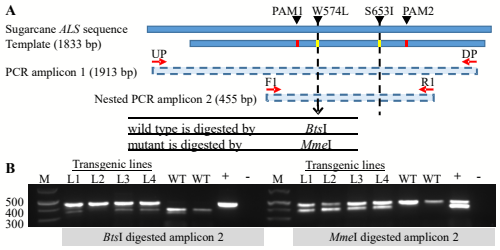
Table 2. Number of transgenic events generated and events with integrated transgenic DNA.

	Number of plants regenerated	*npt*II + (%)†	coCas9 + (%)†	sgRNA1 + (%)†	sgRNA2 + (%)†
Geneticin selection (resistance provided by nptII transgene)					
B1	102	84 (82.4%)	71 (69.6%)	54 (52.9%)	NA††
B2	105	91 (86.7%)	67 (63.8%)	NA††	66 (62.9%)
B3	108	97 (89.8%)	60 (55.6%)	54 (50.0%)	53 (49.1%)
BS selection (resistance provided by edited endogenous ALS gene) with a prior Geneticin selection					
B2	27	23 (85.2%)	16 (59.3%)	NA	15 (55.6%)

† Frequency was calculated as a percentage of events with integrated transgene to all regenerated plants in a treatment.
†† Not applicable.

Screening for mutations with RE digestion patterns

Target nucleotide modification to introduce W574L resulted in modification of the *Bts*I recognition site into *Mme*I. A PCR amplicon was generated with primers UP and DP, the former located outside of template sequence to prevent amplification of randomly inserted templates. Primers F1 and R1 were used to generate shorter amplicons in a nested PCR for subsequent screening by RE digestion (**Figure 3A**).



Wild type (WT) control did not show the restriction pattern that was observed for the template plasmid (+) suggesting that HDR introducing W574L into ALS might be the cause for the diagnostic restriction pattern in lines L1 – L4 (**Figure 3B**).

Figure 3. Restriction enzyme digestion based screening.

Genotyping assay for identification of mutated alleles

Intended mutations were further verified using fluorescent labeled TaqMan® probes which were designed to detect wild type and mutant alleles at both amino acid positions 574 and 653 in two genotyping assays (**Figure 4A**). Allelic discrimination plots constructed with RFU from probes were used to identify transgenic plants with intended mutations. Transgenic lines with targeted mutations were indicated with green triangles whereas lines showing only wild type allele signals were indicated with blue squares. Positive control plasmid (+) was shown with an orange circle. No template control (NTC) did not produce signals (**Figure 4B**). Number of edited events with intended mutations are summarized in **Table 3**.

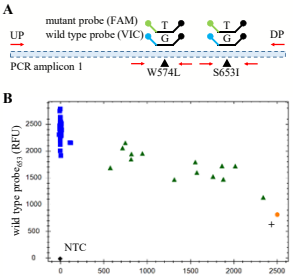


Table 3. Number of transgenic events with mutations W574L and/or S653I.

	Number of events regenerated from 10 shots	Genotyping assay (TaqMan® probe based assay) Number of events analyzed	W574L +	S653I +	W574L + S653I +
Geneticin selection (resistance provided by nptII transgene)					
B1	102	77	37	14	5
B2	105	81	31	23	9
B3	108	85	30	23	10
BS selection (resistance provided by edited endogenous ALS gene)					
B2	27	27	13	9	6

Figure 4. TaqMan® probe based genotyping assay.

Sequencing mutated ALS alleles

Sequence of mutated ALS alleles from edited plants verified that the HDR mediated edits have been achieved in endogenous ALS gene since the sequenced fragments were larger than the donor template (**Figure 5A**). Target HDR mediated edits of W574L and/or S653I and PAM sites were observed in edited line L2. Additionally, indels resulted from NHEJ were observed in edited lines L2 and L3 (**Figure 5B**). Genome edited plants with multi allele conversion of W574L and/or S653I (e.g. L2, **Figure 2F**) were resistant to the herbicide imazapyr (3 liter/ha, twice the labeled rate) in contrast to wild type (WT) sugarcane plants.

Figure 5. Sequencing chromatogram of cloned ALS alleles 1 – 4 from edited line L2 and allele 1 from edited line L3. Numbering according to donor template. CRISPR/Cas9 mediated edits (HDR or NHEJ mediated edits) were shown in red bold face and underlined. Mutated PAM sites (PAM1^{mut} and PAM2^{mut}) were results of template mediated HDR.

Conclusions

- Assays based on RE digestion, genotyping with TaqMan® probes and Sanger sequencing of cloned ALS alleles confirmed precision nucleotide substitutions by homology directed repair (HDR) at multiple target locations in the sugarcane genome.
- Frequency of events with at least one HDR mediated allele replacement ranged from 0.6 to 3.7 events per shot, approaching standard transformation efficiency.
- This efficiency exceeds reports from other crops and will allow multiplexed genome editing for sugarcane improvement.

Funding statement
This material is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Pending Award for CABBI (Center for Advanced Bioenergy and Bioproduct Innovation) Proposal Number 0000227119.

References

1. *Jung, J. H. and F. Altpeter. 2016. TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. Plant. Mol. Biol. 92: 131-142.*
2. *Kannan B., Jung J.H., Moxley G.W., Lee S.-M., Altpeter F. 2017. TALEN mediated targeted mutagenesis of more than 100 COMT copies/alleles in highly polyploid sugarcane improves saccharification efficiency without compromising biomass yield. Plant Biotech. J. (accepted)*
3. *Zale, J., J.H. Jung, J.Y. Kim, B. B. Pathak, R. Karan, H. Liu, X. Chen, H. Wu, J. Candreva, Z. Zhai, J. Shanklin, F. Altpeter. 2016. Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass. Plant Biotech. J. 14: 661-669.*

CABBI CENTER FOR ADVANCED BIOENERGY AND BIOPRODUCTS INNOVATION
UF UNIVERSITY of FLORIDA IFAS
U.S. DEPARTMENT OF ENERGY