Targeted multi-allelic mutagenesis in polyploid, apomictic bahiagrass (*Paspalum notatum* Flugge) using the CRISPR/Cas9 system

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INTRODUCTION

- **Bahiagrass (*Paspalum notatum* Flugge) is a subtropical grass species widely cultivated in the southeastern US, Central and South America for turf and forage.**
- **The popular bahiagrass cultivar 'Argentine' is apomictic and autotetraploid (2n=4x=40), presenting significant obstacles to genetic improvement using traditional breeding techniques.**
- **Precision gene editing technologies including CRISPR/Cas are potential game-changers for developing improved varieties of polyploid and apomictic or clonally propagated crop species, such as bahiagrass.**

PROJECT OBJECTIVE

- **To establish a rapid-readout system for confirmation of gene-edited events in tetraploid bahiagrass, thereby enabling further optimization of genome editing tools and their delivery in this species.**

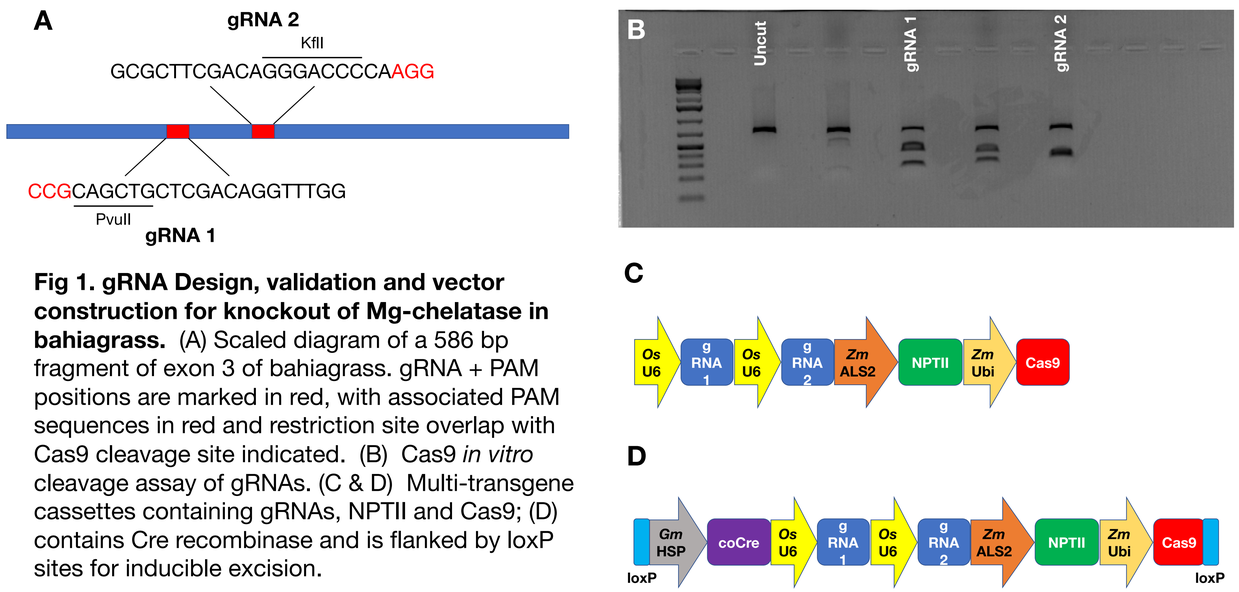

Fig 1. gRNA Design, validation and vector construction for knockout of Mg-chelatase in bahiagrass. (A) Scaled diagram of a 586 bp fragment of exon 3 of bahiagrass. gRNA + PAM positions are marked in red, with associated PAM sequences in red and restriction site overlap with Cas9 cleavage site indicated. (B) Cas9 *in vitro* cleavage assay of gRNAs. (C & D) Multi-transgene cassettes containing gRNAs, NPTII and Cas9; (D) contains Cre recombinase and is flanked by loxP sites for inducible excision.

MATERIALS AND METHODS

- **Translated nucleotide reads from RNA-seq of apomictic tetraploid and doubled diploid sexual bahiagrass genotypes were aligned to the *Sorghum bicolor* Magnesium-protoporhyrin IX chelatase amino acid sequence using tBLASTn, to design PCR primers to amplify and sequence the multiple copies present in apomictic tetraploid bahiagrass cultivar 'Argentine'.**
- **Pairs of gRNAs conserved across all copies of the gene were designed to generate ~100 bp deletions in its third exon (Fig. 1A) and cleavage efficiencies were confirmed in an *in vitro* assay (Fig. 1B)**
- **The selected gRNA pair were cloned into expression vectors containing the NPTII selectable marker and Cas9 (Figs. 1C & 1D), delivered to mature seed-derived callus cultures of 'Argentine' using biolistics, and regenerated in tissue culture (Figs. 2A-2F).**

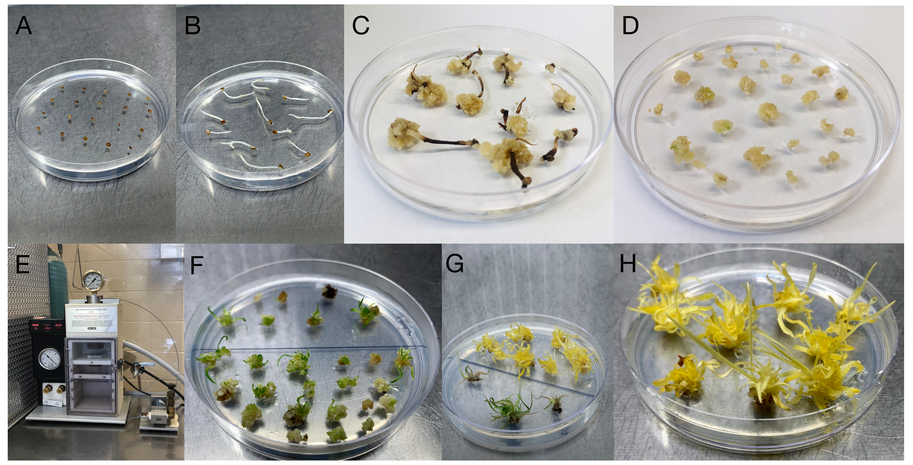

Fig 2. Bahiagrass biolistic transformation workflow. (A) Culture of sterilized mature seeds on CIM media. (B) Germination of seeds on CIM media. (C) Callus formation on shoot meristems and (D) callus division on CIM prior to bombardment. (E) Bio-Rad Model PDS-1000/He Biolistic Particle Delivery System. (F) Regeneration of shoot primordia on SRM with geneticin selection. (G) Emergence of putative edited Mg-chelatase callus line (top half) on SRM with geneticin selection. (H) Shoot elongation of edited line PnMgCTKO.1 on NH medium without selection.

RESULTS

- **A uniform yellow callus line recently emerged in the late regeneration phase of tissue culture (Fig. 2G) and is actively growing (Fig. 2H).**
- **The Mg-chelatase gRNA target sites were amplified with flanking PCR primers using genomic DNA templates from WT Argentine and the regenerated yellow line, and gel electrophoresis results indicated the presence of a 100 bp deletion in the yellow line's Mg-chelatase target region (Fig. 3A).**
- **An initial set of Sanger sequencing reads have confirmed multi-allelic editing in line PnMgCTKO.1 (Fig. 3B) including small indels upstream of both gRNA PAM sites (Pattern E1) and 100 bp deletions between the gRNA cut sites (Pattern E2). No WT reads were recovered from line PnMgCTKO.1.**

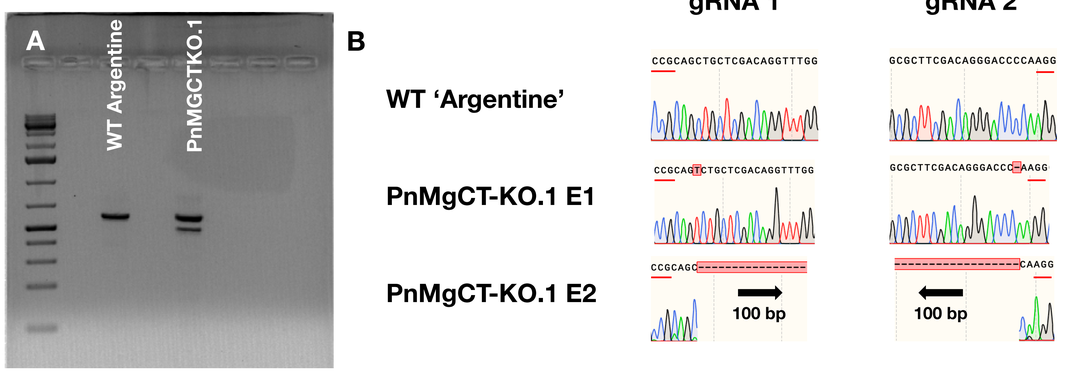

Fig 3. Confirmation of multi-allelic editing in line PnMgCTKO.1. (A) Gel electrophoresis of the Mg-chelatase target amplicons from WT and PnMgCTKO.1. (B) Sanger chromatograms encompassing gRNA 1 & 2 target sites in WT Argentine and PnMgCTKO.1. PAM sequences are underlined in red.

PROSPECTS

- **Our results comprise the world's first report of gene editing in bahiagrass. Further applications of this technology could deliver rapid improvements in monogenic turf and forage quality traits.**
- **Deep sequencing of the Mg-chelatase gRNAs target region using Illumina technology will provide a more comprehensive picture of Mg-chelatase copy number and editing efficiency.**