Improving Water Use Efficiency through CRISPR/Cas9-mediated *HT1* Knockout in *Sorghum bicolor*

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ABSTRACT

In this study, we used CRISPR/Cas9-mediated genome editing to perform gene knockout in *Sorghum bicolor* (sorghum). The gene of interest was *HT1*, which encodes a protein kinase that expresses under low-CO_2 conditions in stomatal guard cells, leading to decreased stomatal aperture. This gene has two homologs in sorghum, *HT1.1* and *HT1.2*. By knocking out these genes, we aimed to generate plants with closed stomata to prevent water loss by transpiration and increase water use efficiency (WUE). WUE is an important agricultural trait to consider in the face of groundwater depletion and worsening droughts due to climate change, especially in widely grown cereal crops. Two guide RNAs for each gene were designed, targeting different loci on the exons of each gene. Using Agrobacterium-mediated transformation with immature embryos (IEs) as explants, we regenerated edited sorghum plants through tissue culture. The resulting plants were genotyped by PCR and Sanger sequencing. The resulting editing efficiency was 94.6%. Analysis of the sequencing results also indicates that a high percentage of these edits are frameshift mutations or large deletions that would likely result in functional knockout of the *HT1* genes.

KEYWORDS

Crop editing, *Wuschel2*-assisted transformation, immature embryo transformation, stomatal manipulation, water use efficiency

INTRODUCTION

As climate change and global warming increase temperatures on our planet, the frequency and intensity of droughts are expected to increase as well (Cook et al. 2018). This will have a costly impact on agricultural production worldwide, costing farmers billions each year (Logar and Van Den Bergh 2013). One way to mitigate this issue is to reduce the amount of water necessary to grow their crops, specifically by improving water use efficiency (WUE). WUE is defined as the amount of carbon assimilated per unit of water used by a plant (Hatfield and Dold 2019) and is dependent on two factors: the rate of photosynthesis relative to the rate of transpiration.

Plant stomata present an opportunity to manipulate these factors and potentially improve WUE. Stomata are apertures, found most commonly in plant leaves, that are responsible for mediating gas and water exchange between the plant and its external environment (Bhattacharya 2019). Stomata are made up of guard cells which control the size of the opening in response to external stimuli, such as atmospheric gases or light (Lawson and Blatt 2014). Under elevated atmospheric CO_2, plants naturally close their stomata, which increases their leaf temperature and decreases the amount of water lost by transpiration (Medlyn et al. 2001).

In order to harness the power of stomatal conductance for improving water use efficiency, we can look to the *high leaf temperature 1 (HT1)* gene which is responsible for CO_2 responsive stomatal regulation (Hashimoto et al. 2006, Takahashi et al. 2022). *HT1* negatively regulates CO_2-induced stomatal closing, so reducing its function or knocking out the gene altogether will lead to stomatal cells remaining closed, even at higher CO_2 concentrations (Lopez et al. 2023). Reducing *HT1* gene function is expected to improve WUE, as shown in the model plant, *Arabidopsis thaliana* (Ruggiero et al. 2017).

Given the significance of *HT1* in CO_2-mediated stomatal closing, manipulating its expression provides a possible opportunity for optimizing WUE. While *HT1* editing has been studied in Arabidopsis, there have been no published studies researching the effects of reducing *HT1* function in major crop plants, and none in plants that are drought-tolerant. Thus, sorghum is an attractive option for studying the effects of reduced *HT1* function on WUE, drought tolerance and plant development, as it is considered a model C4 plant with relatively robust drought resistance (Mullet 2014, Harris-Shultz 2019).

In my study, I aimed to understand how *HT1* knockout would affect sorghum. To explore the effects of *HT1* knockout, I examined 1) what kinds of edits were regenerated from our knockout and 2) how our editing process affects plant growth. Because there are two *HT1* homologs in sorghum, I expect that we would obtain plants with knockout of one gene (single knockout) and plants with knockout of both genes (double knockout). I expect that editing could lead to poor growth in plants, both because of the stress of tissue culture regeneration and knockout of the *HT1* gene that controls a crucial plant function.

METHODS

Plant Materials

IEs between 1-2 mm were harvested from sorghum variety RTx430 panicles. Panicles were cut from plants in the greenhouse and brought to the lab. Immature seeds were removed from the panicle and the sepal and other debris was discarded. After selecting 150 seeds, they were sterilized twice in 50 mL Falcon tubes with 75% ethanol for 2 minutes, then rinsed with double distilled water 4 times. After this initial wash, they were spun gently on a tube rotator in a sterilization solution of 20% bleach and 0.1% Tween20 for 20 minutes, then rinsed with double distilled water 5 times.

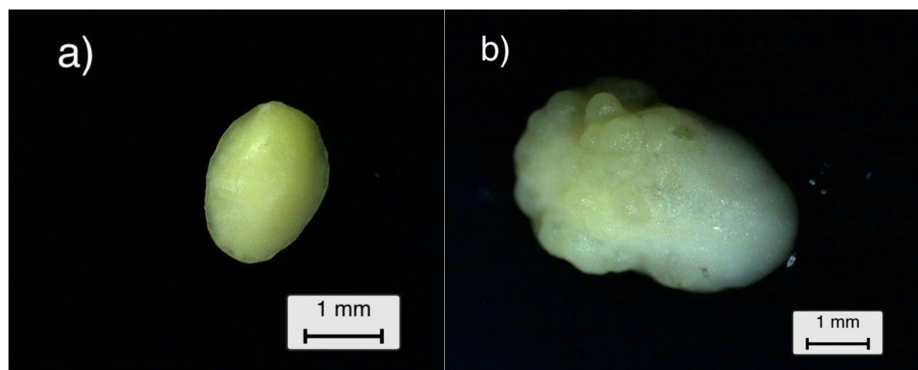

Figure 1: (a) Sorghum IE after extraction from an immature seed, and (b) IE after 1 week on Co-cultivation media.

Once the immature seeds were sterilized, the back of a scalpel was used to apply gentle pressure and extract the IE (Figure 5) from the micropylar end of the seeds. Any damaged embryos or those smaller than 1 mm in size were discarded. IEs were placed on filter paper moistened with

Agrobacterium Infection Media in a petri dish. To avoid drying out, once all IEs were isolated, they were placed in a 1.5 mL Eppendorf tube containing Agrobacterium Infection Media with 200 µM acetosyringone.

Construct Design and Creation

To facilitate *HT1 knockout*, a construct was designed containing a CRISPR expression cassette targeting the *HT1.1* and *HT1.2* genes, the two *HT1* homologs in sorghum (Figure 2). The construct (Figure 3) contains *Wus2*, a morphogenic gene shown to improve CRISPR/Cas9 editing efficiency in sorghum (Che et al. 2022), as well as increasing general transformation efficiency and reducing regeneration time (Aregawi and Shen et al. 2021). It also contains hygromycin phosphotransferase as a selectable marker (Blochlinger and Diggelmann 1984). Finally, it contains *ZsGreen*, a green fluorescent protein for use as a visible marker (Wenck et al. 2003). Terminators and buffer sequences are provided between each cassette to prevent overactive expression due to strong promoters.

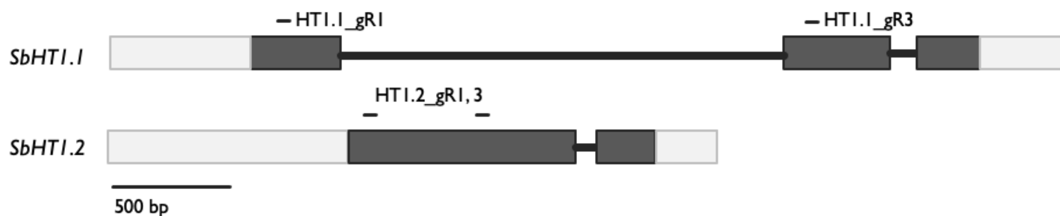

Figure 2: *SbHT1.1* and *SbHT1.2* gene maps. Light grey regions denote the 5' and 3' UTRs, respectively, dark grey regions represent exons, and black lines represent introns. The gRNA sites are noted above their location on the gene.

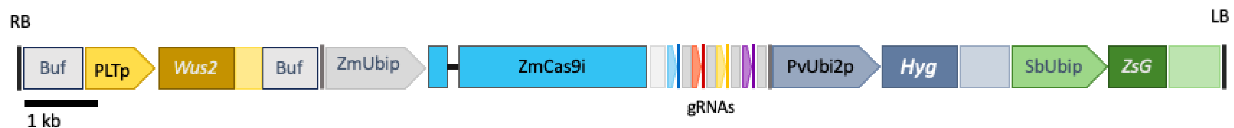

Figure 3: SbHT1 editing construct. The maize phospholipid transfer protein promoter drives *Wus2*. The maize polyubiquitin promoter drives the intronized Cas9 sequence. The switchgrass polyubiquitin promoter drives *Hyg*, the hygromycin resistance gene. The sorghum polyubiquitin promoter drives *ZsG*, the green fluorescent protein.

The CRISPR/Cas9 expression cassette (Figure 4) includes the maize codon-optimized Cas9 gene and gRNAs that direct the Cas9 nuclease to specific sites within the genome. We used an intronized version of Cas9 (ZmCas9i) to improve editing efficiency through increased protein

accumulation (Grützner et al. 2021). It contains four gRNA sequences in total, two gRNAs target the HT1.1 gene and two target the HT1.2 gene (Appendix B, Table B1). The cassette is flanked by attL4 and attL3 sites, which were necessary for a Gateway® recombination reaction that allowed insertion of DNA fragments into the larger construct (Reece-Hoyes and Walhout 2018).

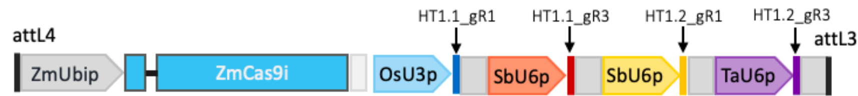

Figure 4: CRISPR/Cas9 expression cassette within the SbHT1 construct. Lengths are not to scale in order to highlight the promoter types. Sorghum U6 promoters drive HT1.1 gRNA3 and Ht1.2 gRNA1, rice U3 promoter drives HT1.1 gRNA1, and wheat U6 promoter drives HT1.2 gRNA3.

Agrobacterium Strain Preparation

Jianqiang Shen designed and created the *SbHT1* plasmid and inserted it into an *Agrobacterium tumefaciens* LBA4404 Thy- strain (Anand et al. 2017). This strain was treaked onto a Yeast Extract Peptone (YEP, Table A1) plate containing 60 mg/L spectinomycin, 50 mg/L thymidine, 25 mg/L gentamicin, and 50 mg/L streptomycin. This plate was incubated at 28°C for 2 days. After 3 days, 5-6 colonies were picked and streaked onto a fresh YEP plate containing the same antibiotics and thymidine. This working plate was incubated at 28°C overnight.

Transformation

On the day of transformation, 3-4 scoops of bacterial cultures from the working plate were suspended in Agrobacterium Infection Media (Table A2) containing 200 µM acetosyringone. The OD_{550} was measured using a spectrophotometer and adjusted to OD_{550} = 700 using excess Agrobacterium Infection Media. After the concentration was properly calibrated, 100 mg/L thymidine and 20 µL/L Silwet L-77 were added. IEs were incubated in this suspension for 5 minutes at room temperature while inverting gently.

Tissue Culture Cultivation

After transformation, immature embryos were placed on Co-Cultivation media (Table A3) with their scutellum side facing upward. Roughly 50 embryos were placed on each plate. The

plates were sealed with parafilm and wrapped in foil to prevent light exposure. They were incubated at 25°C for 24 hours, then 28°C for 6 days. Every 2-3 days, the IEs were monitored for phenolic accumulation and any IEs with high amounts of phenolics were moved to a fresh media plate.

After 1 week on Co-Cultivation media, the IEs were transferred to Resting media (Table A4). The plates were kept at 28°C for 7 days in the dark, and monitoring for phenolics continued. Following one week on Resting media, the IEs were transitioned to Embryo Maturation Media (EMM, Table A5). From this stage onwards, the embryos were plated onto fresh media weekly for 4-6 weeks.

Starting from 4 weeks on EMM, shoots longer than 1.5 cm in length were transferred to Rooting Media (Table A6) in Solo cups. Plantlets on Rooting Media were placed in a 25°C growth room on a 16:8h light:dark cycle. Each embryo tissue was assigned a corresponding number for tracking purposes. Plantlets were maintained in these Solo cups until they reached about 10 cm in height or approached the top of the cup, at which point they were transferred to pots containing SuperSoil potting mix and moved to growth chambers. Three biological replicates were performed, but DNA was only harvested from the first 2 replicates due to the volume of plants regenerated through tissue culture.

Screening by Fluorescence Microscopy

ZsGreen fluorescence screening was conducted on the tissues using a Zeiss AxioZoom V16 low magnification epifluorescence microscope. Starting 1 week after transformation, the tissues were observed under fluorescence at weekly intervals. The total number of tissues with any amount of ZsGreen expression was counted to obtain a percentage of tissues that were putatively transformed. The cutoff point for counting fluorescence was set at four weeks after transformation, as hygromycin selection had become effective by this point, and it was no longer necessary to screen for fluorescence.

DNA Extraction

Genomic DNA was isolated from young sorghum leaves from plants on Rooting Media. 1-3 leaf segments of 2 cm length were harvested from the plants, frozen in liquid nitrogen, and ground in 2 mL tubes with 2 grinding balls each. 700 µL of Urea Buffer (Table A7) and 10 µL of Invitrogen PureLink™ RNase A was added to each tube. The tubes were incubated with the buffer at room temperature for 15 minutes. After incubation, 600 µL of 1:1 phenol:chloroform was added and the tubes were shaken for an additional 15 minutes. They were centrifuged for 15 minutes at 12,000 RPM, then the supernatant was transferred to a new 1.5 mL tube containing 400 µL of isopropanol and 60 µL of sodium acetate. The tubes were kept at -80°C for one hour, then centrifuged for 15 minutes at 12,000 RPM. After centrifugation, the DNA pellet was washed with 500 µL ethanol and dissolved in 50 µL of sterile distilled water.

Genotyping by PCR and Sanger Sequencing

Primers were designed to amplify the regions of the *HT1.1* and *HT1.2* genes containing our gRNAs (Appendix B, Table B2). The resulting PCR product was purified using the GeneJET PCR Purification Kit from ThermoScientific. Gel electrophoresis was performed using 1% agarose gel made with SuperPure Agarose LE from U.S. Biotech Services. UV visualization confirmed the presence of an amplicon and its size. Sanger Sequencing was performed by the U.C. Berkeley DNA Sequencing Facility and ElimBiopharm. Results were analyzed using Synthego™ ICE analysis.

RESULTS

Transformation Efficiency Determination by Fluorescence Microscopy

Fluorescence microscopy, performed while the tissues were on EMM, revealed that the majority of tissues showed *ZsGreen* expression at some points in the first 28 days after transformation. Replication #1 showed the highest average transformation percentage of 82.92%, while Replication #3 showed the lowest average at 58.51% (Table 1, Figure 5). *ZsGreen* transient expression in the tissues peaked sharply at 14 days after transformation (DAT) and steadily

decreased in the following weeks. *ZsGreen* expression was most apparent in somatic embryos, which manifest as round, white tissues that grow from the immature embryo (Figure 6). Dark brown or otherwise unhealthy tissues usually did not exhibit any fluorescence. As the tissue aged, it grew shoot structures that also exhibited strong *ZsGreen* expression. Shoots without any *ZsGreen* expression were discarded during the next media refresh. The third replication was screened by fluorescence microscopy, but ultimately was not used for plant regeneration because of the success of the first two replications.

Table 1. Percentage of tissues expressing *ZsGreen*. Values recorded at 7, 14, 21, and 28 days after transformation across the 3 replications.

	Replication #1	Replication #2	Replication #3	Average
7 DAT	75.35%	62.39%	50.00%	62.58%
14 DAT	89.44%	87.18%	67.52%	81.38%
21 DAT	84.51%	81.20%	66.88%	77.53%
28 DAT	82.39%	78.63%	49.64%	70.22%
Average	82.92%	77.35%	58.51%	72.93%

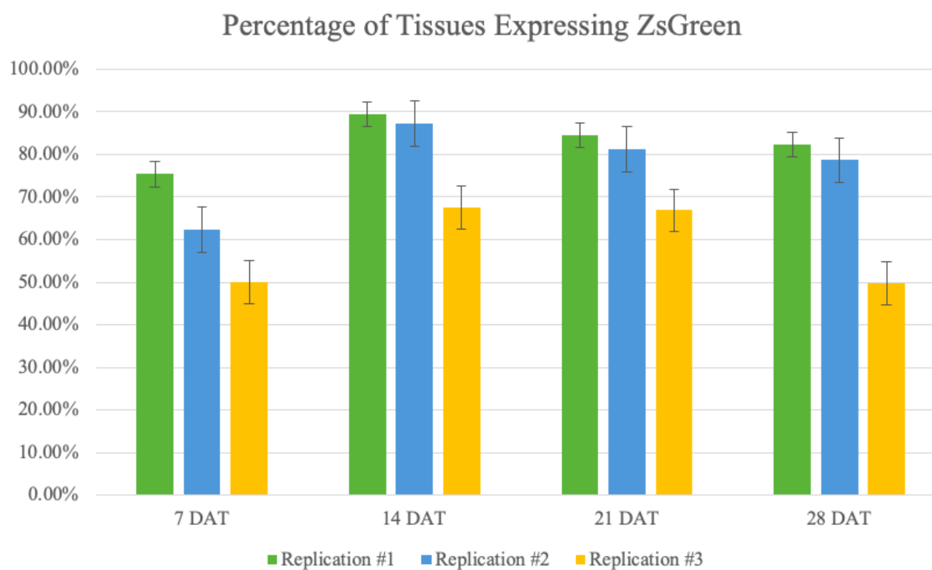

Figure 5. Bar chart showing the percentage of tissues expressing *ZsGreen* at various time points.

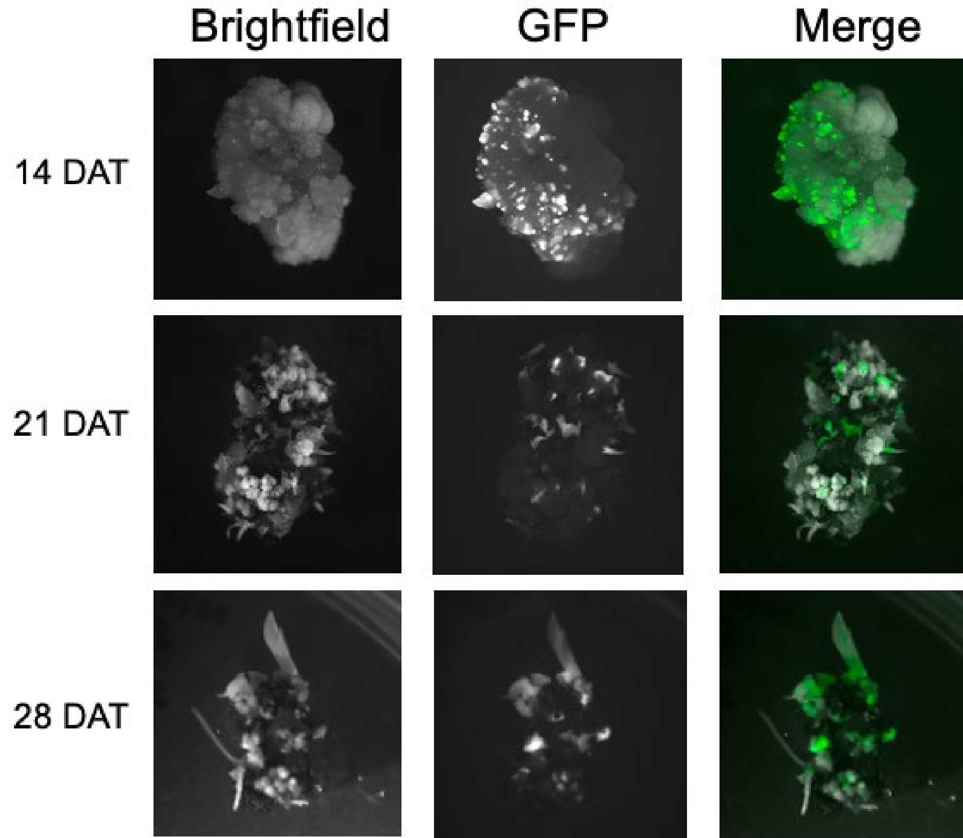

Figure 6: Fluorescence images of putative edited tissues. Images were taken at 14, 21, and 28 DAT under brightfield and fluorescence microscopy. The merge is an overlay of the brightfield and fluorescence images.

PCR and Synthego ICE Analysis

Sequencing results indicate that editing was successful at three out of the four gRNA-directed cut sites (Table 2). Synthego Inference of CRISPR Expression (ICE) analysis indicated that HT1.2 gRNA1, driven by the SbU6 promoter, had the greatest average knockout (KO) score, with a predicted 75.18% of sequences leading to loss of gene function. The OsU3 promoter, driving HT1.1 gRNA1, was unsuccessful as indicated by 0% editing across all sequenced samples. High editing efficiency also indicates relatively successful Hygromycin selection, as only 3 escapes were observed (5% escape rate).

Table 2: KO score by gRNA from Synthego ICE analysis.

Sample #	HT1.1 gR1	HT1.1 gR3	HT1.2 gR1	HT1.2 gR3
HT1E1-1	0	61	73	18
HT1E1-2	0	96	38	93
HT1E1-3a	0	0	0	0
HT1E1-4	0	97	98	76
HT1E1-5a	0	81	73	32
HT1E1-5b	0	65	83	36
HT1E1-6a	0	83	81	38
HT1E1-6b	0	78	91	16
HT1E1-7	0	95	100	100
HT1E1-8a	N/A*	N/A*	40	33
HT1E1-8b.2	0	98	89	70
HT1E1-9a	0	0	0	0
HT1E1-10a	0	90	69	45
HT1E1-11a	0	76	83	88
HT1E1-17a	0	85	51	23
HT1E1-18b	0	94	89	35
HT1E1-19b	0	96	98	72
HT1E1-21a	0	86	85	40
HT1E1-22a	0	30	82	100
HT1E1-23b	0	60	N/A*	50
HT1E1-24a	0	59	68	19
HT1E1-25c	0	69	N/A*	N/A*
HT1E1-26b	0	73	89	21
HT1E1-27	0	97	53	80
HT1E1-28b	0	76	69	32
HT1E1-30	0	68	87	46
HT1E2-1a	0	70	91	20
HT1E2-2a	0	51	64	11
HT1E2-3a	0	93	99	65
HT1E2-4a	0	20	36	0
HT1E2-6b	0	61	92	27
HT1E2-6d	0	84	76	22
HT1E2-7a	0	33	N/A*	86
HT1E2-9-2a	0	96	87	19
HT1E2-10b	0	98	86	100
HT1E2-12-2a	0	75	0	0
HT1E2-14a	0	79	N/A*	18
HT1E2-15a	0	66	95	39
HT1E2-17a	0	92	91	46
HT1E2-18a	0	94	90	78
HT1E2-19a	0	69	70	8
HT1E2-20d	0	94	99	84
HT1E2-23c	0	78	87	61
HT1E2-24a	0	72	89	30
HT1E2-26a	0	97	87	48
HT1E2-26b	0	Undetermined**	100	84
HT1E2-27a	0	0	0	0
HT1E2-29a	0	96	92.5	45
HT1E2-30b	0	91	95	32
HT1E2-31a	0	95	92	35
HT1E2-34a	0	97	90	30
HT1E2-49c	0	97	93	52
HT1E2-50a	0	44	N/A	88
HT1E2-57b	0	88	92	17
HT1E2-60a	0	84	78	42
HT1E2-61a	0	64	61	0
HT1E2-63a	0	97	88	46
Average	0	74.33	75.18	42.79

N/A* indicates low sequencing quality

Undetermined** indicates low R^2 value for samples with low KO-score confidence

Plant Growth

Plants were generally observed for the health of their leaves, including their broadness and size, as well as overall height of the plant. Plants kept in the growth chamber were all genotyped, and those with high knockout scores were moved to the greenhouse Of the first 14 plants moved to the greenhouse at around 3 months old, 4 exhibited short stature and dried, curling leaves (Figure 7).

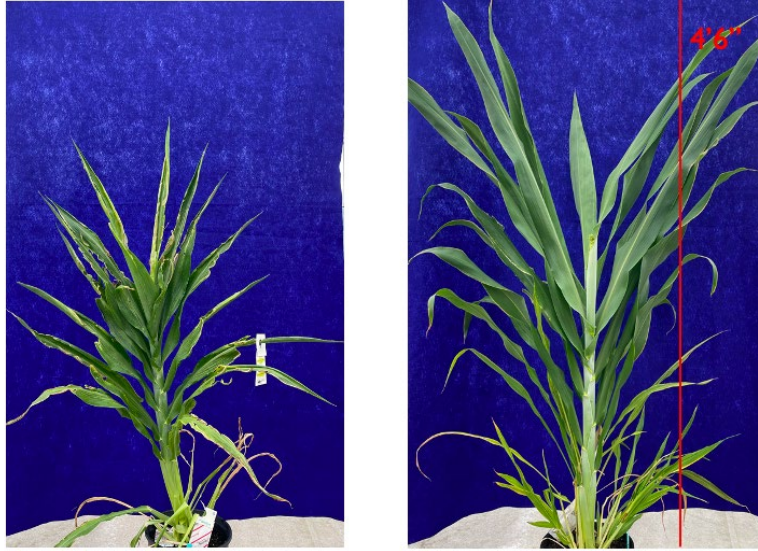

Figure 7: Two *HT1* knockout plants 119 days after being moved to soil.

DISCUSSION

Transformation and Editing Efficiency

Transformation efficiency was calculated as the number of immature embryo tissues expressing ZsGreen fluorescence. The average of all 3 replications was 70.22%, indicating high transformation efficiency. The variation between replications was possibly due to the quality of the original IEs, as unhealthy embryos are less likely to survive and regenerate during transformation and tissue culture processes. Editing efficiency is calculated as the number of samples with edits, as inferred from sequencing results, divided by the total number of samples. Genotyping results indicate a very high editing efficiency of 94.6%, with 54 out of 57 sequenced plants showing knockouts at one or both of the *HT1* genes.

The high transformation and editing efficiency observed was likely due to the use of morphogenic gene *Wus2* in the *SbHT1* editing construct. *Wus2* is a transcription factor and helps promote stem cell proliferation, which generates somatic embryo formation in tissue culture (Zhang et al. 2017). Somatic embryos are key to regenerating healthy plants from the tissue culture process.

Genotypes of Edited Plants

The OsU3 promoter was unsuccessful for expression of *SbHT1.1* gRNA1, as indicated by a 0% knockout score for all samples. This promoter has been shown to be less effective in generating mutations compared to an OsU6 promoter in rice (Mikami et al. 2016), consistent with our results, Other gRNAs driven by SbU6 and TaU6 promoters showed much higher expression levels, as indicated by the knockout score at their respective sites.

Because the *SbHT1.2* gene is relatively short in length as compared to *SbHT1.1* (2,597 bp and 4,107 bp, respectively), we were able to design gRNAs close enough that they could be sequenced with a single read. This is useful in determining if there is a dropout, or a large deletion, between the two gRNA sites. Larger deletions are significant because they are more likely to be passed to the next generation, as they are more difficult to repair during regeneration. A large deletion at the kinase domain would likely lead to total loss-of-function for the gene. Our sequencing results showed 4 samples (HT1E1-2, 4, and 22a, HT1E2-23c) for which there was truncation between the two *SbHT1.2* gRNA sites. Dropout rate is calculated as the number of samples with a large dropout divided by the total number of samples, so the 4 samples from a total of 57 indicate a dropout rate of 7%.

***Wuschel2* Phenotypic Effects**

Adverse effects on plant growth have previously been observed in plants transformed with *Wus2,* especially difficulty in fertility and generating seed (Gordon-Kamm et al. 2019). The negative phenotypes that I observed, including short stature and curling leaves, were likely due to ectopic expression of *Wus2*. Because all of the plants moved to the greenhouse had relatively high knockout scores, I cannot conclude that the poor growth was due to *HT1* knockout as some plants were still healthy with presumably very low or no *HT1* function. Although *Wus2* likely led to poor growth in about 30% of the plants, its disproportionate improvement to editing and transformation efficiency makes it an attractive option for use in sorghum editing. It is just one of the many tools in plant biotechnology that has helped turn sorghum, once considered a very recalcitrant crop (Sharma et al. 2020), to a model crop.

Limitations and Future Directions

A major question left is the effect of *HT1 knockout* on gas exchange and leaf temperature in sorghum. Although edited plants were generated, I have yet to determine whether *HT1 knockout* leads to a closed stomata phenotype and therefore improved WUE, as predicted. However, phenotypic changes are difficult to measure in the T_0 generation due to possible chimerism in the plant and possible epigenetic effects. Chimerism, wherein adjacent cells may have different genotypes (Frank and Chitwood 2016), makes it difficult to draw conclusions from measurements taken because it is impossible to know the genotype of every single cell. As such, phenotypes will be observed in the T_1 generation after tDNA segregation. It is also unclear how *HT1 knockout* affects plant growth, although I hypothesize that a closed stomata phenotype will lead to slower growth due to the possibility of lessened CO_2 intake.

In the T_1 generation, we will be able to screen out the tDNA to avoid possible negative effects of the transgenes (such as *Wus2*). This will allow us to make conclusions about how *HT1* knockout affects water use efficiency in sorghum. By measuring the stomatal conductance (CO_2 and H_2O exchange) under various CO_2 concentration conditions, we can determine the water use efficiency of *HT1* knockout plants. We can also examine the leaves by thermal imaging to determine if *HT1* knockout leads to closed stomata and high leaf temperatures due to lack of evaporative cooling. For these experiments in T_1, control measurements should be taken from other T_1 plants with an unedited (WT) genotype. Although T_1 will no longer have tDNA, there still may be epigenetic effects of tissue culture that a true WT plant would not present, so the true WT plant would not be a direct phenotypic comparison.

As climate change worsens, fresh water will continue to be a critical resource for farmers around the world looking to grow their crops. Genetic editing is just one way to attempt to improve water use efficiency and decrease the reliance on intensive irrigation practices. *HT1*, a critical gene for stomatal manipulation, and sorghum, a drought tolerant and hardy crop, present two great opportunities for learning more about improving water use efficiency. Further studies in either of these areas may lead to commercially viable lines with high water use efficiency.

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APPENDIX A

Media and Buffer Recipes

Table A1: Yeast Extract Peptone (YEP) Media.

	Per 1 L
Reagent	**Amount**
Bacto Peptone	10 g
NaCl	5 g
Yeast extract	10 g
Agar	15 g

Table A2: Agrobacterium Infection Media, pH 5.8.

	Per 1 L		
Reagent	**Amount**	**Stock**	**Final concentration**
MS Salts w/ vitamins	4.43 g		4.43 g/L
Thiamine HCl	0.1 mL	10 mg/mL	1 mg/L
2,4-D	1.5 mL	1 mg/mL	1.5 mg/L
Sucrose	68.5 g		68.5 g/L
Glucose	36 g		36 g/L
Post-autoclave additives			
***Acetosyringone	1 mL	200 mM	200 µM
***Thymidine	1 mL	100 mg/mL	
***Silwet L-77	20 µL		

Post-autoclave additives were added after cooling to < 55°C.
***Acetosyringone was added immediately before transformation to improve infection. Thymidine and Silwet L-77 were also added immediately before, and only after adjusting OD_{550}.

Table A3: Co-cultivation Media, pH 5.8.

	Per 1 L		
Reagent	**Amount**	**Stock**	**Final concentration**
MS Salts w/ vitamins	4.43 g		4.43 g/L
Thiamine HCl	0.1 mL	10 mg/mL	1 mg/L
2,4-D	2 mL	1 mg/mL	2 mg/L
Sucrose	20 g		
Glucose	10 g		
L-Proline	0.7 g		
MES Buffer	0.5 g		
Post-autoclave additives			
Acetosyringone	1 mL	200 mM	200 µM
Ascorbic acid	1 mL	10 mg/mL	10 mg/L
Thymidine	1 mL	100 mg/mL	100 mg/L

Table A4: Resting Media, pH 5.8.

	Per 1 L		
Reagent	**Amount**	**Stock**	**Final concentration**
MS Salts w/ vitamins	4.43 g		4.43 g/L
Thiamine HCl	0.1 mL	10 mg/mL	1 mg/L
2,4-D	2 mL	1 mg/mL	2 mg/L
Sucrose	20 g		
Glucose	10 g		
L-proline	0.7 g		
MES Buffer	0.5 g		
Phytagel	4 g		
Post-autoclave additives			
Ascorbic acid	1 mL	10 mg/mL	10 mg/L
Carbenicillin	0.25 mL	100 mg/mL	25 mg/L

Table A5: Embryo Maturation Media, pH 5.6.

	Per 1 L		
Reagent	**Amount**	**Stock**	**Final concentration**
MS Salts w/ vitamins	4.43 g		4.43 g/L
Zeatin	0.5 mL	1 mg/mL	
Copper sulfate	1.25 mL	1 mg/mL	
L-proline	0.7 g		
Sucrose	60 g		
Phytagel	4 g		
Post-autoclave additives			
IAA	1 mL	1 mg/mL	1 mg/L
ABA	0.1 mL	1 mM	0.1 μM
Thidiazuron	0.1 mL	1 mg/mL	0.1 mg/L
BAP	1 mL	1 mg/mL	1 mg/L
Carbenicillin	0.25 mL	100 mg/mL	25 mg/L
Hygromycin	0.2 mL	100 mg/mL	20 mg/L

Table A6: Rooting Media, pH 5.7.

	Per 1 L	
Reagent	**Amount**	**Final concentration**
MS Salts w/ vitamins	2.15 g	2.15 g/L
Sucrose	20 g	20 g/L
Phytogel	3 g	3 g/L

Table A7: Urea Buffer for DNA Isolation.

	Per 1 L		
Reagent	**Amount**	**Stock**	**Final concentration**
Urea	120g		
NaCl	70 mL	5M	0.35 M
Tris-Cl, pH8	50 mL	1M	50 mM
EDTA	40 mL	0.5M	20 mM
Sarkosyl	50 mL	20%	1% (v/v)

APPENDIX B

gRNA and Primer Sequences

Table B1: Four gRNA sequences used to direct Cas9 cleavage.

gRNA Name	**Sequence**
HT1.1_gR-1	AAGTACCTCGTAGCGCCGCC
HT1.1_gR-3	GTCTCTATGAAGTATACCCT
HT1.2_gR-1	GAAGAGCCCGTGGCCTCCCC
HT1.2_gR-3	GTACATCCGGAGCGTGCCCT

Table B2: Primers used to amplify regions of *SbHT1.1* and *SbHT1.2* by PCR.

Primer Name	**Sequence**
SbHT1.1-GT F	GCCTGCAGTGCTTCAAGCAGAGC
SbHT1.1-GT R	CTCCCACATGACGATTCCAAAGCTGTACAC
SbHT1.2-GT F	TCTTGATACAAGCTGGCGCCTCGC
SbHT1.2-GT R	TCGTTGAGCAGCAGGTTCTGGGACTT