Utilizing a DNA-Independent CRISPR-Cas9 System to Create Allelic Knockout Variants of *Sorghum bicolor* with Altered Flowering Times

Nancy Lu[1], Elizabeth Li[1], Karl Marback[1], Christopher Fragoso[2], Maria Moreno[3]

[1]Department of Molecular Biochemistry and Biophysics, Yale College, New Haven, CT 06511
[2]Computational Biology and Bioinformatics, Yale University, New Haven, CT 06511
[3]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06511

ABSTRACT

Many plant species have reached yield plateaus in recent years, and one proposed solution is to create allelic variants with longer flowering times. To determine whether this is possible, allelic variants of *Sorghum bicolor* were created using CRISPR-Cas9 technology. Previous studies show that the *Wuschel (WUS)* and *Baby boom (BBM)* genes have been identified as morphogenic regulators that turn plant cells into totipotent cells with improved monocot transformation. The exons of the *WUS* and *BBM* genes (Sb06g031880, Sb03g042810, respectively) were isolated, purified, and stitched with overlap extension PCR technology. The cDNA sequence was then amplified in the Top10 *E. coli* cell line. After purification, the plasmid DNA was sequenced and compared to bioinformatically determined sequences from UniProt. The WUS protein was expressed in the Rosetta2(DE3) cell line and further isolated and purified using immobilized metal affinity chromatography. With quantitative trait loci analysis to synthesize guide RNAs and the recombinant proteins, *Sorghum bicolor* was transformed into an allelic knockout with the CRISPR-Cas9 system. We found that this DNA-independent method of creating a synthetic cDNA sequence in plants to create knockouts is largely effective, though multiple mutations in bacterial plasmid DNA were observed. The main contribution of this experiment to the field is its DNA-independent nature. We conclude that allelic variants can be

created through this methodology, though further research into the effects of mutations on critical regions of the protein and preventing mutations in the first place should be conducted.

INTRODUCTION

One current major agricultural problem is a yield plateau in the existing varieties of domesticated plants, caused partly by an evolutionary bottleneck. One solution is to increase the window of flowering time in order to maximize productivity. It is also known that grain yield is closely controlled by quantitative trait loci (QTL).[1] QTL produce diverse, sometimes opposing, effects based on different gene environments. In this experiment, *Sorghum bicolor*, commonly known as sorghum, is transformed into an allelic variant to knockout gene Sb06g031880.

QTL knockout can be carried out using a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 nuclease, which *Streptococcus pyogenes* uses to prevent unwanted foreign viral RNA.[3] The CRISPR-Cas9 system is a particularly effective technique to replace a small portion of a living cell's genome with a donor DNA sequence.[4] The first step for this targeted gene editing is the creation of a double-stranded break near the modified locus. Two components, the Cas9 nuclease and a guide RNA (gRNA) must be added to perform genomic editing.[6, 7] The gRNA also contains two components: the CRISPR RNA (crRNA) and the transactivating RNA (tracrRNA).[6] Altering the gDNA sequence allows Cas9 to be directed to any specific location. CRISPR replaces other methods of targeting specific genomic sites, such as zinc finger nucleases and transcription activator-like effector nucleases (TALENS).[8] The CRISPR-Cas system is superior because its success depends on simple base-pairing rules rather than complex protein-DNA interactions.

While the CRISPR-Cas system has been extensively tested in bacteria,[1] mice cells,[11] and embryonic stem cells,[12] it has not been extensively tested in plants. After use of the CRISPR-Cas technology, the method of testing whether the gene edit is preserved in later generations will be to make the knockout plant cells totipotent. *Wuschel* genes (*WUS*) and *Baby Boom* (*BBM*) genes have been identified in tomato and *Arabidopsis* crops, respectively, and have also been successfully used for this purpose in *Sorghum bicolor*, the crop of interest in this scenario.[14] *WUS* and *BBM* genes are responsible for healthy, fertile, transgenic crops in approximately 40% of all cases.[14] These genes improve monocot transformation and turn any plant cell into a totipotent stem cell. Second, the exons of the *WUS* and *BBM* genes must be isolated from their respective introns and then stitched together to undergo ligation-independent cloning to create a full-length CDS sequence. Third, the *WUS* and *BBM* genes are cloned into two *E. coli* plasmid vectors, which are also equipped with the SacB cassette, a negative selection marker that selects for CDS insertion. Finally, the bacteria expressing the *WUS* and *BBM* genes will drive plant transformation into a totipotent cell, which can then be regrown and tested to determine whether the gene editing was successful.

The DNA-independent nature of the experiment is its main contribution to the field of CRISPR-Cas technology and has immense implications. An allelic variant of the sorghum is produced rather than a genetically modified organism. This increases the potential of this technology to be used in crop improvement, especially in the face of regulatory authorities, who could limit the use of programmable nucleases if the crops are considered "GMO."[3]

This paper describes the successful DNA-independent synthesis of cDNA sequence which will be used in the transformation process used for sorghum. For the *WUS* gene, primers were first designed for the sequence and ordered for the PCR amplification process. After each

exon was individually amplified, the full-length CDS was then assembled and cloned into an *E.coli* bacterial protein expression vector. For the *BBM* gene, primers were also designed for the sequence. However, the *BBM* gene, unlike the *WUS* gene, contains a gBlock that includes many small internal exons. Thus, only primers for the first two exons and the last exon needed to be designed. Amplification of the gBlock, the first exon, the second exon, and the last exon allowed the full-length CDS to be assembled using ligation-independent cloning. Using a computational interphase technique, guide RNAs (gRNAs) for the CRISPR-Cas system were designed. The gRNAs were then synthesized *in vitro,* such that the RNA-guided endonuclease RNP could be assembled. In future experimentation, using CRISPR-Cas system, the specific gene in sorghum will be knocked out. Using the *WUS* and *BBM* gene, the tissue can be then transformed into a totipotent cell. These cells should then be regenerated into a complete plant that had an expanded flowering time, providing evidence that the gene was successfully knocked out. This implementation has far-reaching implications in gene-editing especially in crops, which may ultimately contribute to an overall increased food supply in the world.

MATERIALS AND METHODS

I: PCR Methods

Amplification of Exon 1 of *S. bicolor* gene

To amplify Exon 1 of the Sb06g031880 *Wuschel* (*WUS*) gene of *Sorghum bicolor,* the Bio-Rad PCR kit (New England Labs) was used as per the manufacturer's instructions for the experimental condition with 1X Phusion Master Mix (Phusion® High-Fidelity DNA Polymerase with GC Buffer, nucleotides, and optimized reaction buffer including $MgCl_2$), 0.52 μM MB17P07 forward primer, 0.52 μM MB17P08 reverse primer, 50 ng (2 ng/μL) genomic DNA, and distilled water. All reactions were properly controlled. The MyCycler thermocycler was used

for the general cycling procedure involving initial denaturation, denaturation, annealing, extension, and final extension. Annealing temperature was 65°C, and extension time was 30 sec, as suggested by the manufacturer's suggestion of 30 sec/kb. The primer sequences and all subsequent primer sequences were synthesized by the W.M. Keck DNA Sequencing Facility at Yale University.

Overlap extension PCR to Stitch *S. bicolor* Exons Together

In order to stitch the three exons together, overlap extension PCR was performed. Exon 2 was gifted by Elizabeth Li and exon 3 was gifted by Karl Marback. The initial concentrations of each exon were 9.4 ng/µL, 24 ng/µL, and 10 ng/µL respectively, but each was diluted to 9.4 ng/µL. Exons 1 and 2 were first annealed with the Bio-Rad PCR Kit using the same conditions in "Amplification of Exon 1" with an annealing temperature of 75°C and an extension time of 45 sec. This fragment is then stitched together with exon 3 at an annealing temperature of 75°C and an extension time of 30 seconds.

Amplification of Complete WUS-CDS Sequence

In order to amplify the complete WUS-CDS of the Sb06g031880 WUS gene, an analogous procedure to "Amplification of Exon 1" was used. The only modification was that MB17P33 and MB17P44 were the forward and reverse primers, respectively.

Amplification of WUS-CDS Insert from Putative Recombinant Bacterial Colonies

In order to screen for putative recombinant clones, 8 bacterial colonies and 1 control condition were analyzed using PCR. The methodology is in accordance with the amplification of the complete *WUS* CDS insert, with the modification that bacterial colonies were used in place of the PCR reaction and no primers were used.

II: Agarose Gel Electrophoresis (AGE) Methods

Diagnostic AGE Gel for Exon 1 of *S. bicolor*

In order to determine whether exon 1 was correctly amplified, AGE was performed on a 0.7% UltraPure agarose gel with SYBR Safe DNA Gel Stain and RapidRun Agarose Buffer (Affymetrix, 100 volt-hours, pH=8.9 ±0.1 at 25°C). The fragment was compared against a 1Kb PLUS DNA Ladder (New England Biolabs). The length of the desired product as well as all subsequent sequencing was determined by the SeqBuilder program (DNASTAR®, Lasergene 14.1). The experimental condition was loaded with 20 μL final volume (1X loading dye, 1X PCR reaction, and water). The control condition was analogous with 1X control PCR reaction. To analyze the AGE, the gel was visualized using the automated image capture system of the Bio-Rad Gel Doc™ XR+ Imager.

Diagnostic AGE Gel for Full WUS CDS

In order to determine whether the correct size fragment for the full WUS CDS was amplified, another gel electrophoresis was run with the same conditions as "Diagnostic AGE Gel for Exon 1 of *S. bicolor*." The experimental well was loaded with 20 μL final volume (1X loading dye, 1X PCR reaction, and water). The full WUS CDS was a gift from Dr. Maria Moreno.

Preparative AGE Gel for Full WUS CDS

To separate the correct *WUS* CDS from other extraneous bands, an AGE procedure of final volume 60 μL (1X loading dye, 1X PCR reaction, and water) was run on a 0.7% UltraPure agarose preparative gel under the buffer conditions specified under "Diagnostic AGE Gel for Exon 1 of *S. bicolor*." The 961 bp full length WUS CDS band was cut out using a UV visualization system.

Diagnostic AGE Gel of PCR Amplification of Recombinant Bacterial Colonies

To determine which bacterial colonies have recombinant DNA, AGE was performed on each of the PCR reactions using the same protocol as above. The presence of the WUS CDS band indicates successful bacterial recombination.

III: Purification and Quantification of PCR Fragments

Purification and Quantification of Exon 1

In order to separate the two bands visible in the AGE of the unpurified Exon 1, the QIAquick Gel Extraction Kit (QIAGEN) was used as per the manufacturer's instructions with 400 mg of gel per spin column. Centrifugation was performed using the Eppendorf Centrifuge 5417C (Fisher Scientific). Absorbance was measured in Au and PCR Product was quantified using the Nanodrop Spectrophotometer (NanoVue^{TM}, version 4.0, GE) as per the manufacturer's instructions.

Purification of the Complete WUS CDS

In order to separate the two bands visible in the AGE of the unpurified complete *WUS* CDS, the QIAGEN QIAquick Gel Extraction Kit was used as per the manufacturer's instructions with 2 spin columns with 300 mg of gel per spin column.

IV: Ligation Independent Cloning (LIC) Methods

Preparation of Vector

In order to digest the LIC vector pNIC28-Bsa4 (Structural Genomics Consortium, Oxford University) and purify it, 1X NEB4 Buffer, 1X BSA (NEB), 0.05 μg/μL plasmid vector, 0.33X BsaI restriction enzyme were mixed. This protocol was from Dr. Opher Gileadi from the Structural Genomics Consortium. After incubation for 2-3 hours at 50 °C, the BsaI enzyme was inactivated by incubating at 65°C for 15 minutes. The digested plasmid vector DNA was then purified using the QIAquick PCR Purification Procedure (QIAGEN).

T4 Polymerase treatment and PCR Insert Preparation

To complete vector treatment with T4 DNA polymerase, 2X BsaI-digested vector, 1X T4 Buffer, 2.5mM dGTP, 1X BSA, 1M DTT, 0.05X T4 DNA polymerase, and water were mixed. BsaI digested enzyme was a gift from Dr. Maria Moreno. The mixture was incubated at 22°C for 30 minutes then at 70°C in the MyCycler thermocycler.

For preparation of the PCR insert, 2X column-purified PCR insert, 1X T4 buffer, 25mM dCTP, 1M DTT, 0.001X BSA (NEB), 0.05X T4 DNA polymerase, and water were mixed. The column-purified PCR insert was a gift from Dr. Maria Moreno. The mixture was incubated at 22°C for 30 min in the thermocycler, then at 70°C for 20 minutes.

Annealing Reaction

To form the annealed DNA complex, 3X BsaI-T4 vector and 2X T4 treated PCR insert were mixed. The mixture was incubated at 22°C for 10 minutes.

Bacterial Transformation

To carry out the bacterial transformation, competent cells (Invitrogen TOP 10) were added the annealed DNA complex as per the Thermos Fisher's instructions. One modification was that the bacteria were stored on ice for 30 minutes rather than 15 minutes. The transformed cells were plated on an LB plate containing kanamycin and 5% sucrose. Efficiency of ligation was assessed by the number of bacterial colonies on the plate.

V: Isolation and Sequencing of Plasmid DNA from Single Identified Recombinant Clones

Isolation of Plasmid DNA

In order to isolate plasmid DNA from a liquid bacterial culture of recombinant isolate, the Plasmid Midi Kit from the Plasmid Purification Handbook (QIAGEN, 2012) was used. One modification to the centrifugation steps 7 and 9 was necessary because the available centrifuge

could not reach the required speed. Instead, the lysate was filtered using Miracloth (EMD, Millipore) and then filtered at a lower speed to remove cell debris.

Sequencing of Plasmid DNA

To determine whether the insert has the correct sequence, 4 sequencing reactions with final volume 18 μL and different primers (3.05 ng/μL plasmid DNA template, 0.44 μM primer, and water) were mixed. The 4 primers were MB17P07, MB17P12, MB3209, and MB3210. The sequencing reaction was analyzed by the Sanger method and the primers were synthesized at the W.M. Keck DNA Sequencing Facility at Yale University.

VI: Design, Purification, and Quantitation of gRNAs

In order to synthesize the three guide RNAs for the CRISPR-Cas system, an R-based computer program (Christopher Fragoso, Yale University) was used to determine the sequence of the gRNA. The sequence was synthesized by the W.M. Keck DNA Sequencing Facility. The sgRNAs were synthesized according to the manufacturer's protocol of the EnGen™ Synthesis kit optimized for production in *S. pyogenes* to create a sequence with the T7 promoter, the target-specific sequence, an overlap complementary to the kit's oligo scaffold. The RNA was then ultra-purified according to the manufacturer's protocol (RNA Clean & Concentrator, "Purification of small and large RNAs into separate fractions"). One modification made to the protocol was final elution of DNA from 25 mL of DNase/RNase free water. The concentration of the RNA was found (Nanovue Spectrophotometer, standard protocol) to be 3837 ng/μL.

VII: Production and Purification of Bacterial Recombinant DNA

The bacterial recombinant DNA was isolated according to the QIAGEN Plasmid Midi Kit, which lyses the bacteria and neutralizes the protein with high salt solution with an optimal

pH. The sample is then ready for purification through binding to the QIAGEN Anion-Exchange resin performed under optimal salt and pH conditions. The plasmid DNA concentration was then determined using the NanoVue spectrophotometer.

VIII: Expression and Purification of Recombinant Proteins in *E. coli*

To express the recombinant proteins in the bacteria, a standard protocol for protein induction with IPTG and cell lysis was used. In order to purify the WUS protein from *E. coli*, cell lysate was run through immobilized metal ion adsorption chromatography according the manufacturer's protocol (His-Spin Trap, GE) using the affinity of the histidine tag to Ni^{2+} ions. One modification is the use of Lysonase instead of the suggested lysis buffer. To determine if the protein was been effectively purified, the flowthrough and the two protein elution fractions were run in an SDS-PAGE to determine if the correct protein size was created.

RESULTS

Construction of the *S. bicolor* Wuschel (*WUS)* Gene

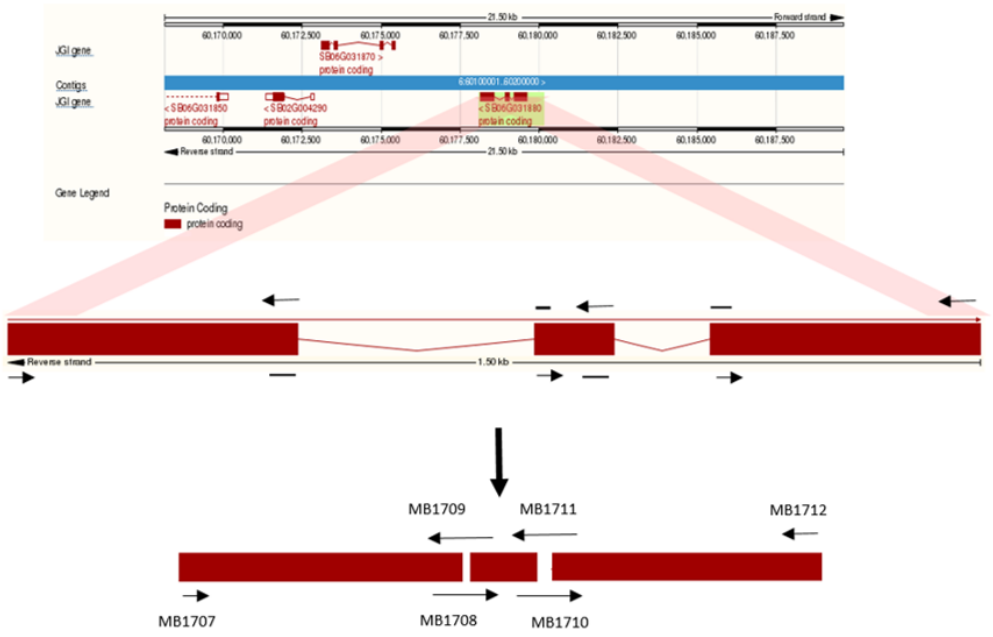

Figure 1A. CDS construction strategy for *Sorghum bicolor* Sb06g031880 WUS gene. Schematic representation of Sb06g031880 gene locus. For the sake of readability, primers are not drawn to scale. Genes and other features are drawn to scale. The dark red boxes represent the three exons of the endogenous locus. The thin red lines represent intron DNA. Labels and arrows indicates positions, names and directions of oligonucleotide primers used in the study.

The sequences of the first exon (134 bp), second exon (712 bp), and g block (1255 bp) fragments containing the Sb03g042810 BBM gene were obtained through Plant Ensembl and UniProt (Fig 1B). Primer sequences were designed from the bioinformatics sequence files (Table 1B) because they were required for the overlap extension PCR reaction (Fig 2).

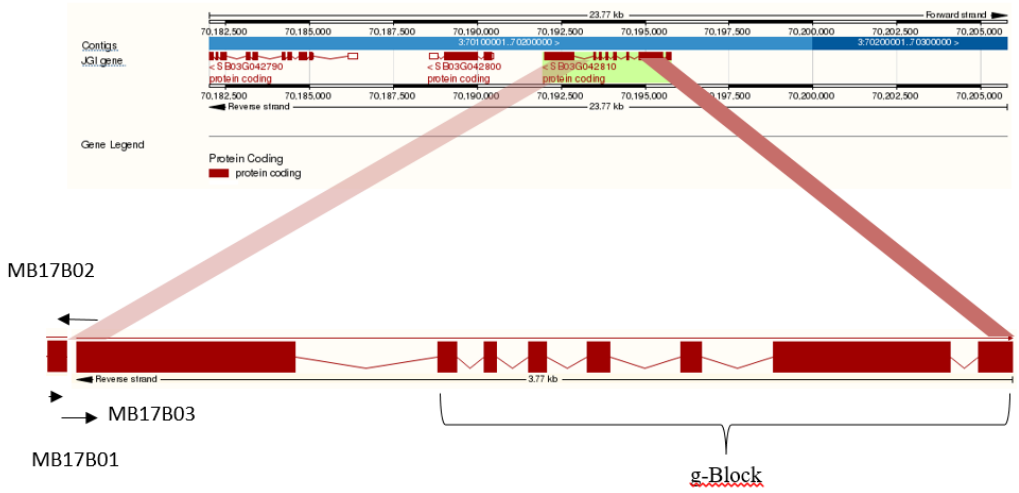

Figure 1B. CDS construction strategy for *Sorghum bicolor* Sb03g042810 BBM gene. Schematic representation of Sb03g042810 gene locus. The same drawing scheme is used in Fig 1A.

Construction and Characterization of the *S. bicolor* Baby Boom (*BBM*) Gene

The three exons (approximately 415 bp, 122 bp, 457 bp) sequences containing the Sb06g031880 WUS gene were obtained through Plant Ensembl and UniProt (Fig 1A). Primers were designed for each exon in order to perform PCR amplification (Table 1A).

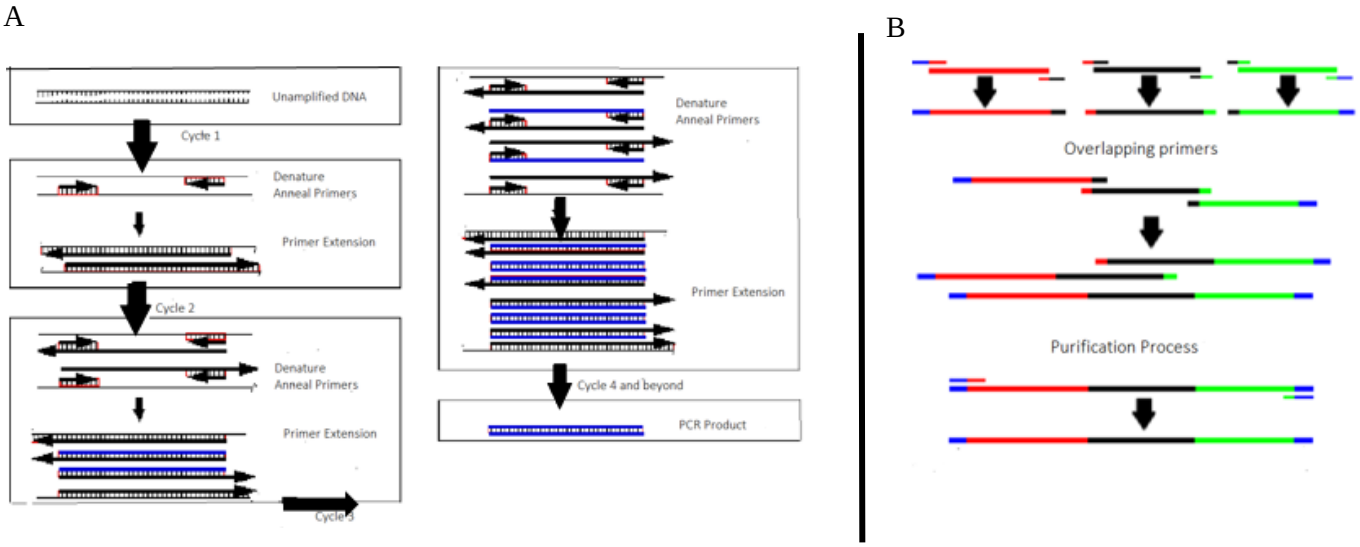

Figure 2: Experimental Strategy of Primer Design for Stitching Together Exons.
A) The 1st extension, 2nd extension, and amplification in the 3rd of the polymerase chain reaction are shown. Arrows refer the direction of synthesis, and each new strand of DNA formed in shown with a different color.
B) Overlapping PCR is used in this experiment to join the three exons together. Exon 1 is red, exon 2 is black, and exon 3 is green. The primers, which are the shorter fragments, have homology between two exons and were designed with that criteria. The overlapping primers helps stitch together the exons for the complete WUS CDS, which can then be purified from other DNA fragments.

The exons were then purified (Quantum Prep PCR Kleen) and analyzed individually with AGE (Fig 3). The NanoVue spectrophotometry revealed that the concentration of exons were 9.4 ng/µL, 24 ng/ µL, and 10 ng/µL, respectively. Overlap extension PCR was performed to obtain the full WUS CDS (994 bp) which was purified (QIAQuick Gel Extraction Kit) and analyzed with another AGE gel (Fig 3B).

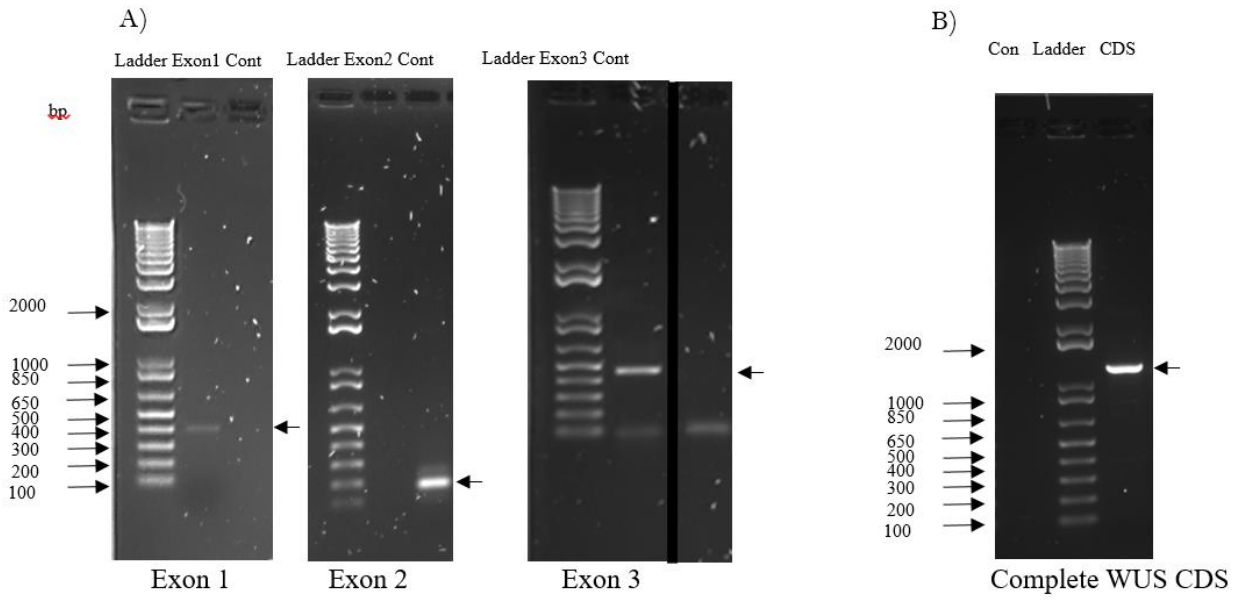

Figure 3: Experimental Evidence for CDS Exon Constructs.
A) Agarose gel electrophoresis (AGE) confirms that PCR amplification and subsequent purification steps were successful for each of the three exons of the sorghum gene. Exon 1 is approximately 415 bp, Exon 2 is approximately 122 bp, and Exon 3 is approximately 457 bp.
B) After the three exons were stitched together and purified, AGE was performed again. The bright white band indicates the complete CDS. The complete WUS CDS is 994 bp. The purified CDS image was a gift from Dr. Maria Moreno.

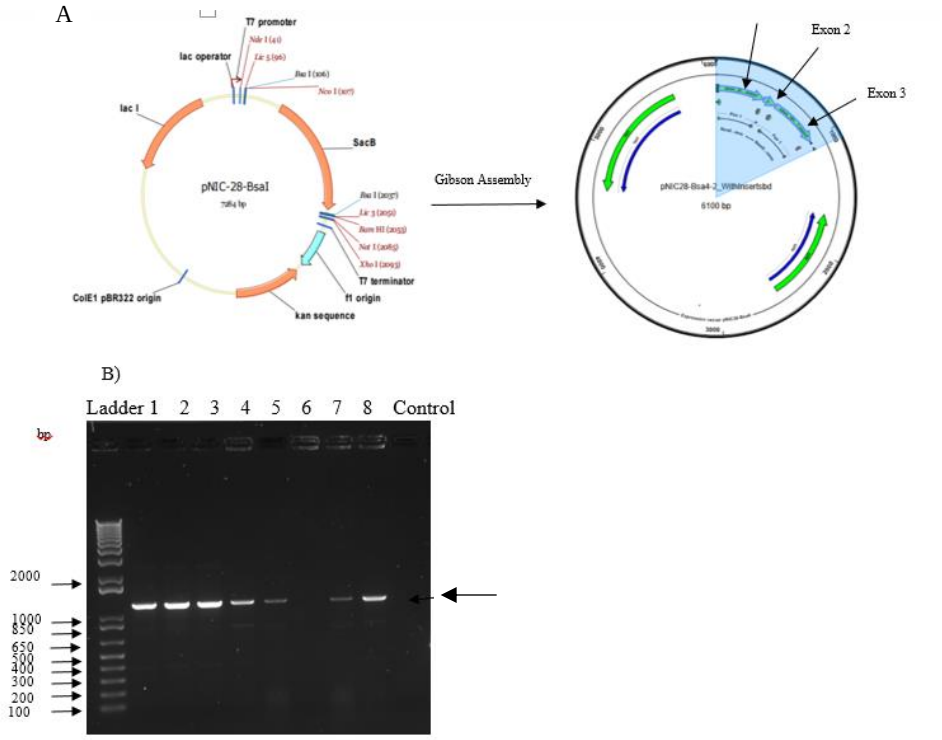

Figure 4: Protein Expression Vector Construction and Screening for Recombinants.
A) On the left, the 7284-bp long pNIC-28-BsaI plasmid is shown. On the right, the region flanked by primers P3209 and P 3210, which contains the Sac-B cassette is removed in order to grow the bacteria in sucrose, and is replaced with the complete WUS CDS via Gibson assembly as shown above.
B) This gel shows the screening for recombinants strategy. Eight colonies of bacteria from two different colony plates were screened using the PCR, and the correct insert, which is 994 bp long, is shown on the image.

The WUS CDS was cloned by ligation independent cloning (LIC) into a pNIC28-Bsa 4 plasmid expression vector (Fig 4). Primers were designed for the LIC process for the WUS CDS (Table 2). Colony PCR revealed that 5 out of 8 bacterial colonies (62.5%) were putative recombinant bacterial colonies that successfully incorporated the CDS (Fig 4B). The plasmid DNA was isolated and sequenced with the Sanger Method. The expression plasmid vector was then moved into the Rosetta2(DE3) bacterial expression cell line which contains a His tag (Fig 4A). The culture was grown to mid-log phase, protein expression was induced using IPTG, and the protein was purified using IMAC affinity chromatography with a divalent Ni^{2+} column,

which binds the His tag effectively. SDS-PAGE was run to separate the proteins that were purified using IMAC chromatography.

A)

Original Amino Acid Sequence

Met L K E L Y Y G C G I R S P S S E Q I Q R I T A M L R Q H G K I E G K N V F Y W F Q N H K A R E R Q K R R L T S L D V N V P A A D A A D A T T S Q L G V L S L S S P S G A A P P S P T L G F Y A G G N G S A V M L D T S S D W G S A A A M A T E T C F L Q D Y M G V M G G A S P W A C S S S S S E D P M A A L A L A P K V T R A P E T L P L F P T G G G G D D R Q P P R P R Q S V P A G E A I R G G S S S S S Y L P F W G A A P T P T G S A T S V A I Q Q Q H Q L M Q M Q E Q Y S F Y S N A Q L L P G T G S Q D A A A T S L E L S L S S W C S P Y P A G P C D S K G D T D P N S S S V D K L A A A L E H H H H H H Stop

New Amino Acid Sequence

Met L K E L Y Y G C G I R S P S S E Q I Q R I T A M L R Q H G K I E G K N V F Y W F Q N H K A R E R Q K R R L T S L D V N V P A A D A A D A T T S Q L G V L S L S S P S G A A P P S P T L G F Y A G G N G S A V M L D T S S D W G S A A A M A T E T C F L Q D Y M G V M G G A S P W A C S S S S S E D P M A A L A L A P K V T R A P E T L P L F P T G G G G D D R Q P P R P R Q S V P A G E A I R G G S S S S S Y L P F W G A A P T P T G Q C H F R C D P A A T P A D A D A R A V Q L L Q Q R P A A A R H R Q P G C S S T S L E L S S A P V L P L T L Q E H V T V S G Y G I R I E L R R Q L A V R T R R H I I Y T E I Q A A Y K P E R K L S W L L H G Stop

B)

Figure 5: Results of the Sequencing of the WUS Construct.
A)The amino acid sequence of the bioinformatically predicted protein and the experimentally created protein are shown. Amino acids in red indicate mutations in the protein. B) Graphical representation of the important domains in the protein. The homeobox domain of the sorghum WUS protein was not mutated. Figure drawn roughly to scale.

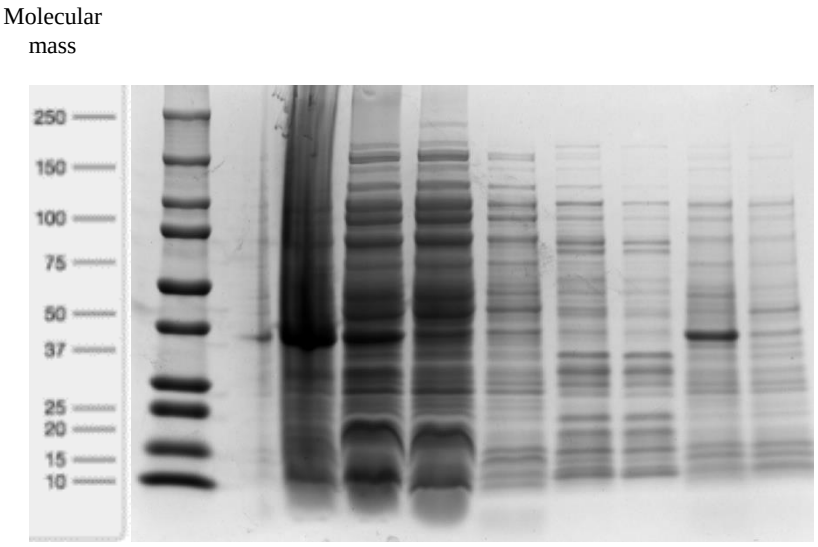

Figure 6: SDS-PAGE Performed for Recombinant Protein Construct.
SDS-PAGE results are shown on each of the cellular fractions. Lane 1: Criterion Gels molecular marker. Lane 2: cellular pellet. Lane 3: supernatant fluid. Lane 4: Flowthough 1. Lane 5: Flowthrough 2. Lane 6: Protein fraction 1. Lane 7: Protein Fraction 2. Lane 8: pYU3205-12 induced cells. Lane 9: pYU3205-12 uninduced cells

Design of gRNA sequences

The R-based program (Fragoso) was used to find the guide RNA sequences (Table 3). The guide RNAs were then synthesized *in vitro* with the *S. pyogenes* using the EnGen sgRNA synthesis kit to create a target-specific oligo sequence. The gRNAs were ultra-purified for use in the CRISPR-Cas9 system.

DISCUSSION

The main contribution of this experiment to the current knowledge of synthetic biology is that the construction of the *WUS* gene demonstrates that construction of a synthetic cDNA in the absence of RNA from specific regions of plant tissue is possible and was successfully performed in this study. The full process was performed on only the *WUS* gene.

According to the results mentioned above, the individual exons were successfully amplified, and had the correct sequence lengths. According to the electrophoresis procedure, the complete *WUS* CDS was successfully created using the overlap extension PCR procedure and has the correct sequence length. After the full *WUS* CDS was transformed in the bacteria, more than 60% of bacterial colonies were putative recombinant, a high success rate.

After these bacterial colonies were isolated for their plasmid DNA, sequencing of the plasmid DNA (Keck Facility, Yale University) revealed approximately a 90% match with the bioinformatically determined sequences from UniProt. The chromatograms themselves yielded good results, with each base pair corresponding to a single peak in the experimental sequences. While there are numerous indel mutations, there was no frameshift mutation, which indicates that the expected protein might be translated and that the polymorphism may not have a significant impact on the structure of the protein. Furthermore, the histidine tag is still present. According to the NCBI conserved domains analysis, the homeobox domains that are critical for expression of the *WUS* and *BBM* genes have been conserved between the *WUS* CDS and the sequenced gene. The BLAST analysis confirms that the sequenced gene corresponds to the *Sorghum bicolor Wuschel* gene.

Finally, SDS-PAGE allowed for a successful purification process of the recombinant WUS protein, which is necessary as a morphogenic regulator. The bacteria that is overexpressing

the protein does appear to be overexpressing the correct protein, though elutions1 and 2 are contaminated with many other bands. One modification to the procedure would be increasing the incubation time for better nickel binding, or a more extensive washing procedure that better removes anything bound loosely in a non-specific manner to the column. One caveat is that many proteins have stretches of histidines that could create a propensity for the Ni^{2+} resin, which means the purification process will likely never be complete. The SDS-PAGE also revealed that the protein is present in both the cellular and supernatant fractions, indicating that the protein is most likely only slightly soluble in water and that it can be dissolved slightly for large-scale analysis.

Immediate further directions include sequencing or mass spectrometry of the amino acids in the protein to confirm that the right protein has been overexpressed. One method is Edman degradation, but this process requires a large quantity of protein. Another method would be to use known proteases to cleave the peptide. Using a ladder of the peptides, the protein breaks apart into smaller pieces, which could be separated by mass spectrometry and analyzed using a database search to confirm the purification. After the creation of the gRNAs and further tests on the protein, the CRISPR-Cas system can then be used to create an allelic variant of the sorghum.

There are several limitations to the current study, which may have led to the differences in the DNA sequencing and the bioinformatically determined results. More specifically, the WUS gene information was predicted from bioinformatics techniques by UniProt rather than experimentally. The experimental cell line might have been different from that used in UniProt's calculations.

The implications of this research are immense because a knockout with altered flowering time would allow for greater food security worldwide. Moreover, there are several further

directions that should be explored. Methods to prevent the point mutations could be explored. Frameshift mutations are particularly detrimental and should be further studied. Application of this procedure could also be applied to *BBM* gene as both *WUS* and *BBM* are morphogenic regulator. Finally, this procedure should be applied to other crops species.

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