Creating and Characterizing Inducible BBM-WUS Transgenic Sorghum Lines to Improve Editing Efficiency

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ABSTRACT

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop in the world. Genome-editing techniques, such as CRISPR-Cas 9, can be applied to sorghum to improve its carbon fixation efficiency and drought tolerance ability. To increase editing efficiency and reduce the growth time of transgenic tissue arising from transformed immature embryos, morphogenic genes, specifically Baby boom (*Bbm*) and Wuschel2 (*Wus2*), can be incorporated into the transformation construct. Here, we report the creation and characterization of the inducible BBM-WUS transgenic sorghum lines. The inducible system was achieved by the sulfonylurea-responsive repressor that only in the presence of ethametsulfuron will the morphogenic genes be expressed. We observed that 50 ppb ethametsulfuron is optimal to induce the morphogenic genes while maintaining tissue growth. We identified the transgenic embryonic calli by detecting the yellow fluorescence protein but did not find the *Bbm* and *ALS2* through PCR in the calli we sampled. We speculated that the transformation efficiency might be low due to the large size of the construct. We also suggested using antioxidant chemicals, such as ascorbic acid (ABA), polyvinylpyrrolidone (PVP), and lipoic acid (LA), to reduce the necrotic browning in the tissue culture. In the future, more optimization in the transformation procedure is necessary to be able to regenerate T_0 plants, after which we would be able to introduce new constructs into the transgenic plants to test whether the introduction of this inducible system can speed up and simplify the transformation process by being able to introduce editing constructs directly into plants that already have CRISPR-Cas9 machinery.

KEYWORDS

Sorghum transformation, morphogenic genes, *Baby boom*, *Wuschel2*, ethametsulfuron

INTRODUCTION

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop in the world. It is an important food source in semi-arid areas of Asia and Africa. Globally, it is also an important source of animal feed and forage, an emerging renewable biofuel, and a model plant for C4 grasses (Paterson et al. 2009). Harboring traits, such as tolerance to drought, waterlogging, and salinity, makes sorghum a highly productive crop in environmental conditions that restrict the cultivation of and production of other cereals (Hadebe et al. 2017). Genome-editing techniques, such as CRISPR-Cas9, can be applied to sorghum to maximize yield and increase its carbon fixation efficiency and drought tolerance (Silva et al. 2022). Though these technologies have been widely used in plant biology, genome editing is lagging in efficiency in sorghum relative to other cereal crop plants (Aregawi et al. 2021). Sorghum transformation was technically challenging, comparatively costly, time-consuming, and limited to a few genotypes (Silva et al. 2022).

One of the promising tools helping to improve transformation is the use of genes involved in controlling plant growth and development(Altpeter et al. 2016). These genes involved in the developmental process, such as embryogenesis and meristem maintenance, are referred to as morphogenic genes(Gordon-Kamm et al. 2019). A major breakthrough in the use of morphogenic genes in the transformation was made by Lowe et al. in 2016 when the Corteva group incorporated into their transformation constructs the morphogenic genes Baby Boom (*Bbm*) and Wuschel2 (*Wus2*) to promote transformation in monocots.

Bbm, a transcription factor in the AP2/ERF family, was first shown to be upregulated during embryo development in *Brasscia napus* (Boutilier et al. 2002). WUS, a homeodomain protein involved in specifying stem cell fate in shoot and floral meristems, plays a key role during embryogenesis by promoting the vegetative-to-embryogenic transition and maintaining the identity of the embryonic stem cells (Zuo, 2002). Thus, by co-expressing Bbm and Wus2, direct somatic embryogenesis could be realized. It can increase transformation efficiency and reduce the growth time of somatic embryos. Lowe et al. in 2016 successfully demonstrated that the maize *Bbm* and *Wus2* genes can stimulate transformation in sorghum (*Sorghum bicolor*) immature embryos, sugarcane (*Saccharum officinarum*) callus, and indica rice (*Oryza sativa* ssp

indica) callus. Compared to classical sorghum transformation which requires extended callus culturing, morphogene-assisted transformation (MAT) reduces transformation times by nearly half (Aregawi et al. 2021).

However, the constitutive and strong expression of these morphogenic genes often leads to undesired pleiotropic effects, including developmental abnormalities and reduced fertility. Thus, the control of timing and the level of expression for these genes are critical for optimized transformation. Commonly, researchers use an inducible excision system (Figure 1) to remove the morphogenic gene after plant regeneration (Gordon-Kamm et al. 2019). For example, Lowe et al. in 2016 used a desiccation-responsive promoter rab17 to provide highly efficient control of CRE excision of the morphogenic genes. Sometimes, however, the frequency of complete excision is low, and the escape of morphogenic genes may lead to infertile plants. A construct containing morphogenic genes, the excision system, and the gene of interest may also be difficult to create and too large for transformation. These factors reduce successful transformation efficiency.

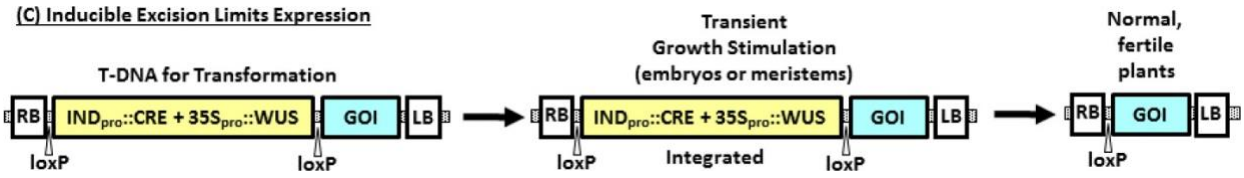

Figure 1. Construct Map for Inducible Excision System (Gordon-Kamm et al. 2019).

Hence, to speed up the process of getting edits in sorghum, it is desirable to create sorghum lines with built-in morphogenic genes, into which editing constructs can be introduced. Chemically induced expression of the morphogenic genes is necessary for this design. Upon the induction by the chemical compound, transient stimulation of the morphogenic genes is provided (Figure 2). The earliest practice of inducible expression was made by Heidmann et al. (2011) to improve the transformation efficiencies and regenerative capacity of pepper (*Capsicum annuum*) varieties. Recent publications about inducible expression include the estradiol-inducible system to control *WOX* gene expression in tobacco (*Nicotiana tabacum*) (Shires et al. 2017) and the DEX-inducible system to control LEC2 gene expression in cacao (*Theobroma cacao*) (Kyo et al.

2018). Pertinent to this thesis work, Corteva, Inc. previously reported sulfonylurea-responsive chemical switches wherein the gene expression is regulated by a sulfonylurea compound (patent no. US 2011 0287936A1). The chemical switch has two components, including a polynucleotide encoding a sulfonylurea-responsive repressor (SuR), and a repressible promoter, operably linked to a polynucleotide of interest. SuR was redesigned from a tetracycline repressor that can specifically recognize sulfonylurea compounds, while retaining its ability to specifically bind tetracycline operator sequences. This patent reported the successful induction of morphogenic genes in maize leaves, corn callus, and rice callus on culture media containing 100 ppb EMS (ethametsulfuron). However, no such inducible system has been practiced in sorghum.

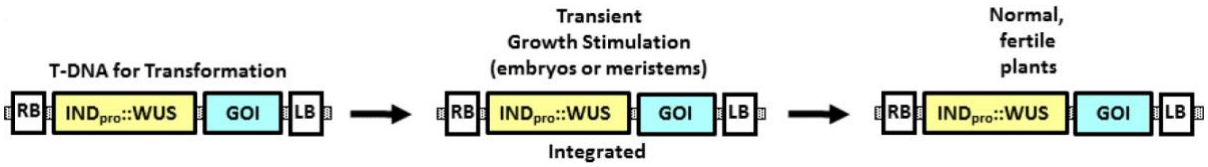

Figure 2. Construct Map for Inducible Expression System (Gordon-Kamm et al. 2019).

In this study, I developed a transgenic sorghum line with an EMS-induced BBM-WUS2 construct created by Corteva, Inc. Experimentation on the tissue culture demonstrated the optimal level of EMS for the induction of SuR. If transgenic plants containing this construct are regenerated in the future, we will introduce new editing constructs into the transgenic BBM-WUS2 plants to test whether this inducible system can speed up and simplify gene editing.

METHODS

Agrobacterium strain and vector. *Agrobacterium tumefaciens* strain LBA4404 (Thy -) was used in this study (US patent no. US8334429B2). Vector pPHP63015 (Figure 3), created by Corteva, Inc, contains five gene cassettes as follows: 1) NOS promoter driving Wuschel 2 (*Wus2*) from maize (*Zea mays*) controlled by the tetracycline operon (TET OP1) repressor; 2) Maize ubiquitin

promoter (Ubi1Zm), with three repeats of TET OP1, driving maize ovule development protein 2 (*Bbm* or *ODP2*); 3) *Z. mays* ubiquitin1 (*Ubi1Zm*) promoter driving the 28.3 kDa ethametsulfuron repressor (ESR); 4) Acetolactate synthase (ALS) promotor from sorghum (*S. bicolor*) RTx430 driving maize acetolactate synthase gene (*Zm-ALS*) to confer resistance to the acetolactate synthase-inhibiting herbicide, imazapyr; and 5) Lipid transfer protein2 (Ltp2) promoter driving yellow fluorescence protein (YFP).

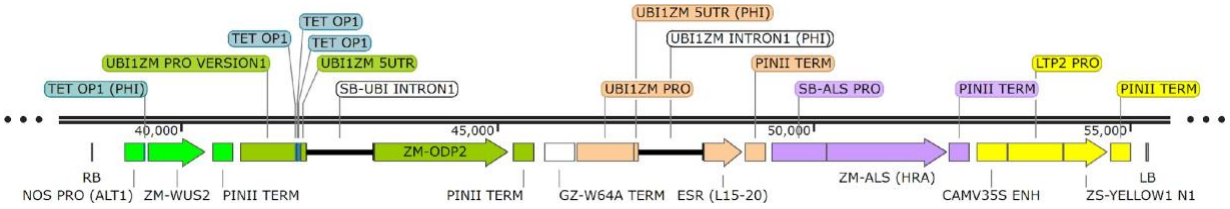

Figure 3. Construct pPHP63015.

Plant material. Sorghum genotype RTx430 was used in this study. All sorghum immature embryo donor plants and transgenic plants were grown in a greenhouse located in the Oxford Tract at the University of California, Berkeley. Greenhouse temperature averaged 29℃ during the day and 20℃ at night with an approximately 16-hour d and 8-hour night photoperiod with supplemental lighting provided, as needed. The components of the medium used in this study are listed in Appendix A.

Agrobacterium transformation. The basic transformation protocol is described in detail in Wu et al. (2014). Briefly, freshly harvested sorghum immature seeds were sterilized with 50% bleach and 0.1% Tween-20 for 30 minutes. Then, rinsed with sterile water five times and the immature embryos were extracted from the seeds and cultured on different media in five sequential steps. (1) Agrobacterium infection: embryos were incubated in the Agrobacterium suspension (OD = 0.7 at 550 nm) with PHI-I medium for 5 minutes. (2) Co-cultivation: embryos were cultured on PHI-T medium following infection for 7 days at 25℃ in the dark. (3) Resting and induction: infected embryos were cultured on DBC3 medium with 100 mg/L carbenicillin and 50 ng/L EMS for 7 days at 28℃ in the dark. (4) Selection: embryos were cultured on DBC3 medium (plus 100 mg/L carbenicillin, 50 ng/L imazapyr, 50 ng/L EMS) at 28℃ in the dark. Every 2 to 3weeks,

embryos were subcultured to new media with a total culturing duration of approximately 2.5 to 3 months. After 8 weeks on DBC3 with EMS, tissues were transferred and recovered on the DBC3 medium without EMS. (5) Regeneration: calli were cultured on PHI-X medium for 2-3 weeks in the dark and then transferred to light (42 $\mu E\ m^{-2}\ s^{-1}$) to stimulate shoot development. If there are young plantlets regenerated, they will be subsequently cultured on PHI-Z medium for 2-3 weeks under lights (16 hours/day) to stimulate root growth.

Fluorescence visualization. Zeiss Lumar v12 epifluorescence stereomicroscope and QImaging Retiga SRV camera were used to observe immature embryos on the resting medium. YFP fluorescence was detected by the YFP filter set. Access to the instruments was provided by the CNR Biological Imaging Facility at the University of California, Berkeley.

DNA extraction. Callus samples (around 3mm x 3mm large) were collected from DBC3 medium and PHI-XM medium, frozen in liquid nitrogen, and ground in an MM300 bead beater (Retsch GmbH, Haan Germany) for 1.5 mins at 25 cps twice. 700 uL of urea buffer (2 M urea, 0.35 mM NaCl, 20 mM Tris-HCl pH 8, 20 mM EDTA, 1% sarkosyl) was added and the mixture was vortexed for 30 secs. 10 uL of RNaseA (20 mg/mL, Invitrogen) was added and the mixture was incubated for 10 mins at room temperature. 700 uL of phenol:chloroform: isoamyl alcohol (25:24:1) was added. The mixture was vortexed for 15 mins and centrifuged at 13800 rpm for 15 mins. The organic phase was discarded and 55 uL of 3 M sodium acetate (pH 5.2) and 367 uL of isopropanol were added to the aqueous phase. To precipitate DNA, the mixture was stored in the 1.5 ml Eppendorf tubes at -20 °C overnight. On the following day, the mixture was centrifuged at 13800 rpm for 5 mins. The supernatant was removed, and the pellets were rinsed in 500 uL 70% EtOH. The excess ethanol was removed, and pellets were air-dried for 5–10 mins in the laminar flow hood. Finally, the pellets were resuspended in sterile distilled water and stored at -20 °C.

PCR analysis. Polymerase Chain Reaction (PCR) was performed on DNA from callus tissue to confirm the integration of Bbm and ALS genes. The information about primers and reaction conditions are listed in Appendix B.

RESULTS

Ethametsulfuron-methyl as an inducing agent for the expression of morphogenic genes

Ethametsulfuron-methyl repressor (ESR) was used as a chemical switch to control the expression of morphogenes, *Baby Boom* (*BBM*) and *Wuschel2* (*Wus*) in pPHP63015. In the absence of EMS, ESR binds to the tetracycline operon (TET OP1) behind the promoters of morphogenes to inhibit the expression of the morphogenes. In the presence of EMS, on the other hand, EMS binds to ESR to prevent it from binding to the TET operon. It thus enables the expression of the morphogenes.

We observed that EMS had a noticeable negative effect on the sorghum transgenic tissues. In the first round of transformation, we cultured transformed immature embryos over DBC3 medium without EMS. We observed a regenerative rate of embryonic calli at 71% (Table 1). The regeneration rate of embryonic calli was calculated by dividing the number of embryonic calli by the immature embryos used at the start of the transformation. In the second round of the transformation when we used 100 ppb EMS to induce the morphogenic genes on DBC3 medium; however, we only observed a regeneration rate of 2% embryonic calli that survived after 6 weeks of induction (Table 1).

Table 1. **Percentage of immature embryos generating callus.**

Round of transformation	Number of immature embryos	Number of embryonic calli	Rate of embryonic calli[a]
1	104	74	71.1%
2	93	2	2.1%
3	103	62	60.2%
4	94	50	53.2%

[a] The regeneration rate of embryonic calli is calculated by dividing the number of embryonic calli by the immature embryos used at the start of the transformation.

To find out the optimal amount of EMS, we cultured the sorghum putative transgenic tissues on DBC3 medium with three different levels of EMS: 0, 50 ppb, or 100 ppb. For each group, the transgenic embryos were grown on the co-cultivation medium for one week and moved to the DBC3 medium with different levels of EMS. After staying on DBC3 medium with EMS for 7 weeks, we observed significant differences in the appearance of the transgenic tissues (Figure 4). As the concentration of EMS increased (Figure 4a,b,c), the sizes of the tissues were smaller, and more tissues showed necrotic morphology. It indicated that EMS had a harmful effect on the tissues.

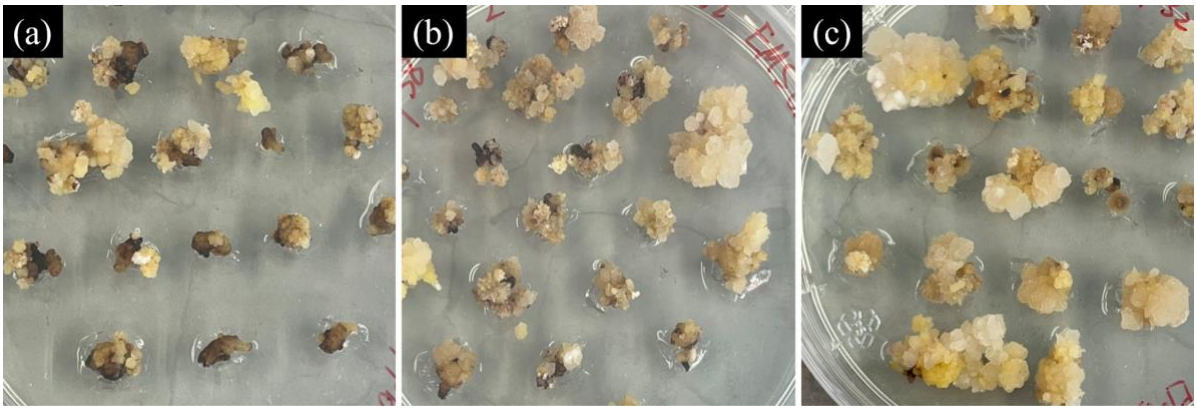

Figure 4. Tissue response to different levels of EMS. Transgenic tissues were exposed to three different levels of EMS on DBC3 medium for 7 weeks: 100 ppb (a), 50 ppb (b), and 0 ppb (c).

Comparing the results among the three groups, 50 ppb appeared to be optimal at inducing the morphogenic genes while maintaining the health of the calli. Based on this, in the fourth round of transformation, we used 50 ppb of EMS to induce the morphogenic genes and observed a regeneration rate of embryonic calli at 53.2% (Table 1). Surviving calli stayed healthy on the DBC3 medium.

Yellow Fluorescence Protein as a Reporter Gene

Yellow fluorescent protein (YFP) was used as the reporter gene in plasmid pPHP63015 (Figure 3**)**. Expression of YFP was observed using the AxioZoom V16 epifluorescence microscope under brightfield and fluorescence conditions in the calli three to eight weeks after transformation (Figure 5). We observed a few embryonic calli expressed YFP (Table 2). The intensity of the fluorescence signal was also very weak. We observed more fluorescence signals at the early stage of tissue regeneration. It could possibly be because the YFP was driven by the lipid transfer protein (Ltp2) promoter, which would only be active at the early stage of the cell division (Kalla et al. 1994, Morino et al. 2004).

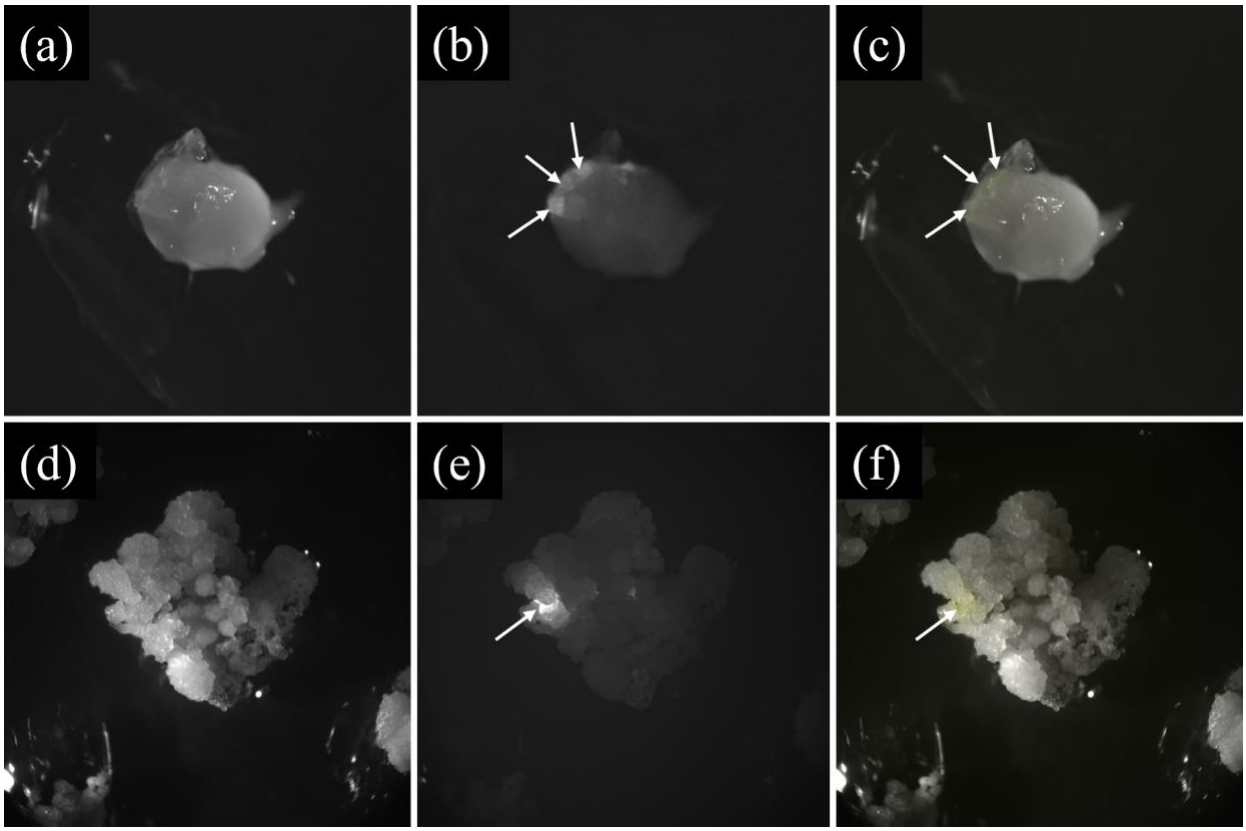

Figure 5. Fluorescent images of transformed callus. Brightfield (a, b), fluorescence (b, e) and merged (c , f) images were taken while tissues were on embryo mature medium on either the 3rd week (a, b, c) or the 8th week (d, e, f) from the transformation. Arrows indicate the YFP expression in somatic embryos.

Table 2. Number of YFP detecting in the embryonic calli.

Round of transformation	Number of immature embryos	Number of embryonic calli
1	104	7
4	94	5

Significant browning during the shoot regeneration phase

To generate transgenic plants from somatic embryo callus, the embryogenic calli were moved from DBC3 medium to PHI-XM to induce shoot formation. However, we observed a significant browning and necrotic morphology of the plant tissues. In the first and third rounds of transformation (Table 1), we observed that only 6.7% and 4.8% of the calli generated small shoots (Table 3), though none of the shoots ultimately survived (Figure 6).

Table 3. Percentage of immature embryos generating shoots.

Round of transformation	Number of embryonic calli	Number of regenerative shoots	Regenerative shoot rate[b]
1	74	5	6.7%
2	2	0	0%
3	62	3	4.8%
4	50	N.D.[a]	N.D.

a. The data was not available at the time of writing.

b. The shoot regeneration rate is calculated by dividing the number of regenerative shoots by the number of embryonic calli.

Figure 6. Significant browning was observed on the shoot regeneration medium (PHI-XM).

Genotyping calli by PCR

Systematic herbicide imazapyr was used to select transgenic tissues that are successfully transformed with the acetolactate synthase gene (*ALS2*), the reporter gene in the plasmid pPHP63015. Failure to transform with this gene would result in the death of the embryonic calli. To identify whether the necrotic morphology observed resulted from the transformation process, we genotyped 3 calli that were at the callus-induction stage (on DBC3 medium) and 3 calli that were at the shoot-regeneration stage (on PHI-XM medium) by doing polymerase chain reaction (PCR) to identify the presence of *ALS2* and *Bbm*. For *ALS2*, the forward and reverse primers chosen were CTTTGGCTCATGGAACGA and ATCTTCTTTATCGCTGCGC, respectively, and they would result in the generation of an expected 576 bp product. For Bbm, the forward and reverse primers chosen were GGTCGTCAAGTCTATTTAGGTGGCT and AAGTAAAGATCCTTGTTCCCTGCAACT, respectively, and they would result in the generation of an expected 299 bp product. The Agrobacterium clone containing the pPHP63015 plasmid was used as the positive control.

Neither gene was detected by PCR **(**Figures 7, 8**)**, which indicates the transformation did not occur in the calli we sampled. Since we observed the yellow fluorescence signal in a few of the calli (Table 2), we hypothesized that the transformation was successful in some of the immature embryos but the transformation efficiency using this plasmid may be very low.

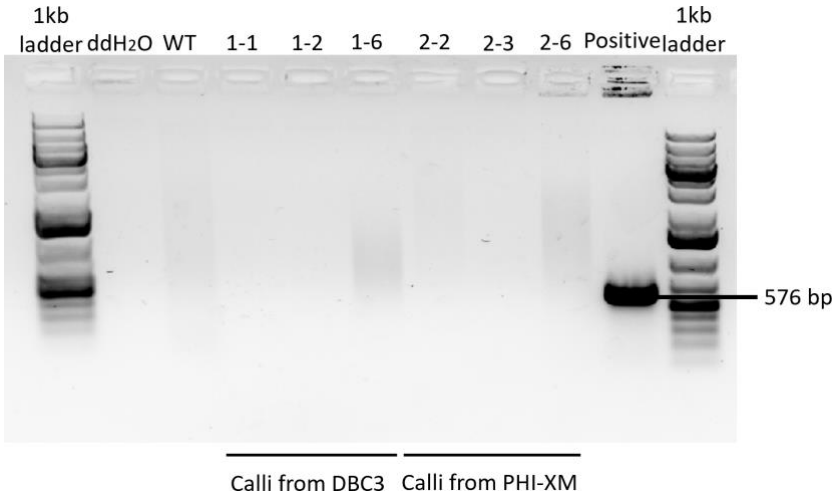

Figure 7. **PCR results for *ALS2*. DNA was extracted from 3 calli on DBC3 medium and 3 calli on PHI-XM medium. The expected product is 576 bp.**

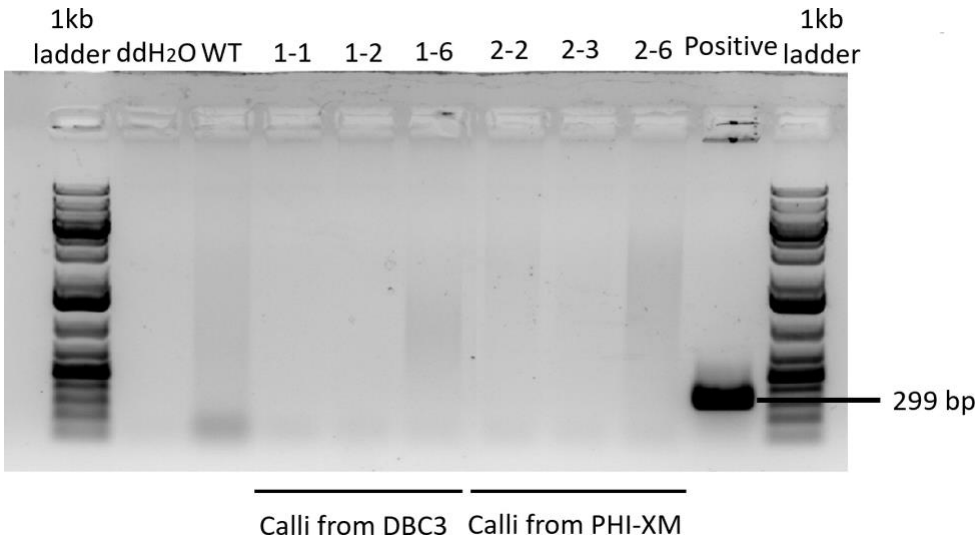

Figure 8. **PCR results for *Bbm* that were extracted from calli on DBC3 medium and PHI-XM medium. The expected product is 299 bp.**

DISCUSSION

In this study, we demonstrate an attempt at using an inducible system to create a transgenic sorghum line that contains editing machinery into which editing constructs can be introduced. By incorporating the sulfonylurea repressor (SuR) and TET binding site, our plasmid, pPHP63015, allows the expression of the morphogenes only upon exposure to the sulfonylurea compound. It is suggested that this method would eliminate the risk of growth abnormalities resulting from the failure of removing morphogenes and would thus speed up the transformation process.

In my study, I found that 50 ppb EMS is optimal for the growth of sorghum callus, which is different from the report of using 100 ppb to induce morphogenes in the Corteva Inc. patent (Fang et al, 2011). Applying 100 ppb to sorghum tissues, as reported in the patent, we observed most of the tissues became small and black. It is probably because sulfonylurea is a commercialized herbicide for gramineous crops (Boldt and Jacobsen 1998). Ethametsulfuron methyl, specifically, is a commercial herbicide developed by DuPont Inc. in 1989 for postemergence broadleaf weed control in Spring oilseed rape (*Brasscia napus*) (Stetter 1994). Thus, to balance the negative effect on tissue culture and the inductive role of morghogenic genes, we think 50 ppb EMS would be optimal for sorghum tissue culture. With regard to the different observations stated in the previous Corteva Inc. reports, we think this may result from

different exposure times to EMS. Corteva Inc. exposed the tissues for a short time (mostly 7 days), but we exposed the tissues for 6-8 weeks to enable complete induction of the morphogenes. The reason to cultivate the tissues with EMS for longer times is that we applied the classical sorghum transformation strategy, which normally takes a longer time to generate T_0 plants. In future experiments, different exposure times might be tested to see if this might lessen the tissue browning.

During the shoot regeneration phase, we observed significant browning on the transgenic calli. We thought this issue may come from the transformation or tissue culture. According to the PCR results, we did not detect *ALS2* and *Bbm* in the calli we sampled. Since we detected YFP in some of the embryonic calli, which indicated the successful transformation, we thought that the transformation efficiency for this construct may be very low. It might be due to the large size of the plasmid (59635 bp). In the future, a more comprehensive sampling of the embryonic calli is necessary to have a more accurate estimation of the transformation efficiency. We also need to optimize the DNA extraction process to reduce polysaccharide contamination and improve the DNA quality.

In regard to the tissue culture, it was thought that browning is related to polyphenol oxidase (PPO) in tissues. When encountering external damage or stress, the cell membrane structure is destroyed and the regional distribution of phenols is disturbed. Enzymes related to the metabolism of the reactive oxygen species, including superoxide dismutase, catalase, and peroxidase, become dysfunctional, and too many free radicals accumulate (Dan et al. 2009, Xiao et al. 2018). It has been suggested to use antioxidant chemicals, including ascorbic acid (ABA) (Ko et al. 2009), polyvinyl pyrrolidone (PVP) (Liu et al. 2015), lipoic acid(LA) (Dan et al. 2009, Xiao et al. 2018), and activated charcoals (Kassahun Bantte and Feyissa 2015), to minimize tissue browning and promote the shoot formation.

Due to the time constraint, we were unable to regenerate the T_0 generation. In the future, we can optimize the transformation process to increase the number of transgenic calli. We will also optimize the shoot regeneration medium by adding different antioxidants to test if they can reduce the lethal browning and enhance regeneration. After successfully generating the T_0 plants, we could introduce new constructs to test if the inducible system can improve the transformation efficiency and simplify the editing process.

SUMMARY AND CONCLUSIONS

It is a pressing issue to improve the transformation and editing efficiency in sorghum in order to do gene function studies. This study outlines an approach to improve the transformation and editing efficiency by creating inducible BBM-WUS transgenic lines into which editing constructs can be introduced. By using a sulfonylurea-responsive repressor, the morphogenic genes will only express in the presence of ethametsulfuron. We found that 50 ppb is an optimal amount of ethametsulfuron to induce the morphogenic genes while maintaining the growth of the tissue. We found transgenic embryonic calli through the detection of the yellow fluorescence protein but did not find the *Bbm* and *ALS2* through PCR. We speculated that the transformation efficiency might be low due to the large size of the construct. We also suggested using antioxidant chemicals, such as ascorbic acid (ABA), polyvinylpyrrolidone (PVP), and lipoic acid (LA), to reduce the necrotic browning in the tissue culture. In the future, more optimization in the transformation procedure is necessary to be able to regenerate T_0 plants, after which we would be able to introduce new constructs into the transgenic plants to test whether the introduction of this inducible system can speed up and simplify the transformation process by being able to introduce editing constructs directly into plants that already have CRISPR-Cas9 machinery.

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APPENDIX A: Medium

Table A. Medium Information

Medium name	**Medium components**
PHI-I (infection medium - liquid)	MS salts plus vitamins, 4.43 g/L, thiamine-HCl 1mg/l, 2,4-D 1.5 mg/l, sucrose 68.5 g/l, glucose 36 g/l, acetosyringone1 39.24 mg/l, pH 5.2.
PHI-T (Co-cultivation, callus induction - solid)	MS salts plus vitamins, 4.43 g/L, thiamine-HCl 1mg/L, 2,4-D 2 mg/l, sucrose 20 g/l, glucose 10 g/l, L-proline 0.7 g/l, MES buffer 0.5 g/l, acetosyringone 39.24 mg/l, ascorbic acid 10 mg/l, thymidine 100mg/l, phytagel 4 g/l, pH 5.8.
DBC3 (Resting, callus maintenance - solid)	MS salts plus vitamins 4.43 g/L, thiamine-HCl 1 mg/L, myo-inositol 0.13 g/L, copper sulfate 1.3 mg/ml, casein hydrolysate 2 g/L, L-proline 0.7 g/L, maltose 30 g/L, 2,4-D 1 mg/L, carbenicillin 25 mg/l, phytagel 4 g/l, pH 5.8.
PHI-X (Embryo-maturation - solid)	MS salts plus vitamins, 4.43 g/L, zeatin 0.05 mg/l, copper sulfate 1.25mg/l, L-proline 0.7 g/l, sucrose 60 g/l, IAA 1 mg/l, abscisic acid 0.026mg/l, thidiazuron 0.1 mg/l, carbenicillin 250mg/l, phytagel 4 g/l, pH 5.6.
PHI-Z (Rooting - solid)	MS salts 4.43 g/l, sucrose 40 g/l, phytoblend 4 g/l, pH 5.6

APPENDIX B: PCR primers and reaction conditions

Table B.1 Primers

Gene Symbol	Protein Name	Primer (F/R)	Amplicon length (bp)	Tm (℃)
ALS2	Acetolactate synthase	CTTTGGCTCATGGAACGA ATCTTCTTTATCGCTGCGC	576	58.2
Bbm	Baby boom	GGTCGTCAAGTCTATTTAGGTGGCT AAGTAAAGATCCTTGTTCCCTGCAACT	299	63

Table B.2 PCR Reaction Conditions

Gene	Initial Denature	Denature	Annealing	Extension	Extension	Hold	Polymerase
ALS2	95℃/ 3min	95℃/ 30sec	54℃/30sec	72℃/60sec	72℃/5min	4℃	EconoTaq PLUS GREEN 2x Master Mix
Bbm			53℃/30sec	72℃/30sec			