Establishing an *Agrobacterium*-mediated maize transformation protocol

by

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B.S., Kansas State University, 2017
B.S., Kansas State University, 2020

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Horticulture and Natural Resources
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Approved by:

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Abstract

Maize (*Zea mays*) is one of the top five agricultural crops grown in the world. It is important to keep developing new and improved varieties to counter insecticide-resistant pests, increase yield, and develop varieties that can be grown in areas significantly impacted by climate change. Traditional breeding methods, which tend to be slow, require a large area, and large amounts of fertilizer and pesticide inputs. Novel methods using a vector *in vitro* to transform maize are also possible. Currently, possible ways to transform and increase transformability are done primarily with the use of commercially available genes, Wuschel (WUS2), Baby boom (BBM) and Growth Regulating Factor- GRF-Interacting Factor (GRF-GIF), which can be cost prohibitive. The aim of this project is to establish a transformation system that can be used efficiently and be made freely available as an alternative to currently available systems. To do this, several experiments were conducted. First, which type of maize explant to use was established. To do this, the most common type of explant, immature embryos, was compared to other potential explants, mature seed embryos, and leaf tissue. This was done with three different varieties, A188, B104 and KSU62. The best explant was determined to be the immature maize embryos. To determine the best way of selecting transformed embryos from untransformed, a selection pressure test comparing common herbicides and selection markers was done. This resulted in G418 Sulfide being the best selection pressure over the more common Bialaphos. Once the explant was determined, transformation using a classical protocol was attempted, and it was determined that several medias needed to be reevaluated, as no transgenic lines or events had occurred. Later, a modified Iowa State University's 'QuickCorn' protocol was used as a modern control protocol, and several transgenic lines have been generated from our lab using

this protocol. However, this protocol is not solely our labs, so media was formulated, tested, and compared to the QuickCorn method. The resulting best medias of the preliminary testing have been compiled as a recommended media for a protocol alternative moving forward. However, a modified QuickCorn protocol is a reliable, widely tested way to acquire transformed explants. To test the protocols, transformation needed to occur. Using *Agrobacterium* strains LBA4404 PYLCRISPR/Cas9_WAT1_Pubi-B, LBA4404 PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B,. The first agrobacterium strains tested was plain LBA4404 with Bialaphos resistant single gene and double gene knock out constructs. Later, to update the materials, a ternary vector strain of LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1_Pubi-B, and LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B was used. Also, a strain just encoding the visual marker gene RUBY, LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_PCL101_RUBY, was used to test inoculation media efficacy. From the background experiments it was determined that immature embryos remain the ideal transformation explant. Also, out of the lines tested, A188 and KSU62 had the best regeneration from immature, but B104 is more robust. Finally, it was determined that a reliable method for maize transformation is a modified QuickCorn method, but preliminary data suggests a protocol specifically tailored for our lab that can produce results of equal efficiency and quickness, using G418 as a selection pressure, and adding cysteine, DTT, and acetosyringone to media during the inoculation stage. However, this protocol requires more rigorous testing on different maize varieties using different expressed or excised genes to determine if it is truly a replacement for the known good protocol. Using the modified QuickCorn protocol, transformed maize had been generated, but only for the single gene deletion of WAT1, and only inoculation data for the new protocol has been tested, not generation of mature transgenic plants.

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Acknowledgements

I would like to thank everyone who supported me throughout my time as a graduate research assistant at the Kansas State University Horticulture department.

I would first like to extend thanks to Dr. Sunghun Park for providing this opportunity to me. This has been a great chance to learn and grow my scientific career in a way I have not had up until now. I sincerely appreciate his acceptance of myself into his lab, and the trust he has extended to me over the two years I have been here.

I would also like to thank the members of my committee for their patience and willingness to support me in my research. Dr. Harold Trick has provided valuable insight and offered words of encouragement. Dr. Kimberley Williams has always had something kind to say as well as offering an outside opinion of my research, as it is not her area of expertise, and I am incredibly grateful for that. Without their support, I would not have been able to accomplish what I have.

A sincere thanks to the other members of the lab, Dr. Aaron Avalos, and Gergely 'Gary' Motolai. They have been a delight to work with and have always been there to help me understand concepts and protocols that I didn't have experience with. They have also been very good friends to me and have provided much needed moral support. With their guidance, critiques, and jokes, they have been great mentors and colleagues. I wish them all the best on their future experiences.

I would next like to thank all the professors and other students of the horticulture department for always being friendly and willing to answer questions I might have had and elucidated many areas of research I have not been familiar with before. It is with their help and guidance that I have been as successful as I have been during this period.

Finally, I would like to thank my family and friends. My parents have been a constant source of support throughout my adventures, and that did not stop this time. My sister has been a friend when I needed it and has been much help and support for more things that I could have possibly expected from her, whether it was complaining, helping troubleshoot ANOVA attempts, or just sharing a meal, having a family member close brightened my experience. Ashley Davis has been a caring companion from the time I started college and has been supporting and caring for all my degrees and work attempts. She and the rest of my family have helped me get through the most challenging years of my life so far, and I hope they will continue to be there for many more years to come.

Once again, Thank you all so much.

Dedication

I would like to dedicate this Thesis to my loving and (usually) supporting family, without them I would not have had the strength and confidence to start, let alone finish this degree. I would also like to dedicate this Thesis to all the plants I probably harmed, intentionally or unintentionally, along the way.

Chapter 1 - Protocol Background

Introduction

Maize (*Zea Mays*) is a staple cereal crop that is widely grown. It is highly versatile and adaptable, thriving in diverse climates and soil conditions, which makes it a reliable source of food in various regions around the world. Its nutritional value is another key factor as to why it is so widely grown; maize is rich in carbohydrates, providing a significant source of energy, and it contains essential vitamins, minerals, and dietary fiber (Nuss & Tanumihardjo, 2010). This versatility and ease of cultivation have made maize a cornerstone of global agriculture, ensuring food security for millions of people and supporting both human and animal nutrition. Since maize is a staple crop in many countries, growers constantly face challenges in the climate, water, and inputs required for a good yield. To ensure food stability, new varieties are constantly being generated. To do this in a rapid fashion, genetic modification through tissue culture methodology is used. There are several ways of generating a genetically transformed plant: particle bombardment, *Agrobacterium* mediated, and direct insertion of the gene via nanotubes (Burlaka et al, 2015). Regardless of what method is chosen, an efficient plant tissue is needed, and that plant tissue must be able to regenerate into a full plant capable of reproducing.

Plants have great resiliency and can often recover and regrow from wounds. One big reason why this occurs is due to plant cells having high plasticity for cell differentiation. This often results in callus formation, which are clumps of undifferentiated cells, much like tumor growth in humans. Callus can be separated into two main types, type I and type II. Type I callus is characterized by being compact, relatively hard, a white to yellow color, slow growing and loses regeneration ability quickly. Type II callus is friable, globular, is fast growing, and can

maintain regeneration ability for longer periods of time. This means that type II callus is ideal for *in vitro* tissue culture experiments (Hodges et at, 2012).

Many plant tissues are capable of regenerating into a new plant. The most common method of callus regeneration in maize is from immature embryos. The first described regeneration utilizing maize embryos was by Green and Philips in 1975. Other methods include callus from anthers, leaf segments, seedling segments, shoot tips and shoot apical meristems (Wang et al, 2023), and mature seed embryos (Huang et al, 2004). Callus initiation and plant regeneration can be dependent on plant genotype, environmental conditions during growth and the R2 or blister stage of kernel development, and the size and stage of the embryos. When transforming maize, the most common method of transformation uses immature embryo isolation. Immature embryos are ideal for transformation due to the ease of callus induction and regeneration (Didone et al, 2018). The totipotency of immature embryos is also important as all different cells derived from the embryo will be transformed throughout the plant (Frame et al, 2011).

While useful for transformation, the use of immature embryos has downsides. Immature embryos require exact planning, as there is a small timeframe- about two to three days- during which they can be harvested and are still ideal for transformation (Masters et al, 2020; Frame et al, 2002). This means the entire experiment is dependent on that short period and, if missed, transformation efficiency declines and the experiment might have to be restarted. However, it's not always possible to obtain the embryos during the ideal timeframe, resulting in the need to use mature embryos or leaf tissue derived callus. Being able to use mature embryos for transformation would provide many boons to the biotechnology industry. It would allow for use of seed that has been in storage and would be less time consuming as there would be no need to

grow fresh, new maize embryos for each transformation. The downside of using mature embryos is that the totipotency has decreased. Unless callus is formed, made competent, and then used for the transformation, efficiency is not ideal (Al-Abed et al, 2006). Efficiency can often depend on which variety of maize is used. This can be because of the tendency of each line to produce different amounts of hormones, cytokinin and auxins needed to produce callus, which can necessitate specific media. Different lines can also be predisposed to production of different types of callus that do not regenerate, much like how different plant tissues can produce callus, but that callus might not grow to a full plant. To remedy this issue, morphogenic regulators can be used, as well as different hormone levels in media.

Three maize lines will be compared in this experiment. For this paper, we will be using immature embryos, mature embryos, and shoot apical meristem callus to compare three different maize varieties, A188, B104, and KSU62. The three maize lines each have pros and cons. B104 is an inbred line derived from the public inbred maize line B73, which is widely used reference line that has a fully sequenced genome, and both B104 and B73 come from the Iowa Stiff Stalk Synthetic lines10 (Hallauer et al, 1997). B104 has a low transformation rate, so it is often used to show if target genes during transformation improve efficiency. B104 is shown to develop less callus than KSU62, so it is less ideal for mature seed-based callus derivation. B104 has proven to be a vigorous line that maintains a high yield when grown under greenhouse conditions. A188 is a good transformation line, as it has recently had its full genome sequenced, and was one of the first maize lines transformed. A188 often produces more type I callus when placed on callus induction media (Ishida et al, 1996). KSU62 is a double haploid inbred line originating from an in-house cross of B73 x A188. This line has been made homozygous and has better plasticity

than B104 for transformation. This line has not been registered, as of time of publication. Develops type I callus more often than type II callus, which limits the ability to regenerate.

Immature embryo derived callus requires the growth of maize to maturity, has a very specific time frame for collection, and requires effort to isolate the embryos, which isn't practical on a commercial scale. However, the use of mature seed would allow for samples to be taken from storage and used with only a short preparation time or could be taken from large growing operations as there is little to no need to monitor embryo growth. Like mature seed callus, leaf tissue callus requires minimal monitoring and can use bulk stored seed. While there are many papers that discuss and test the transformation ability of each of these callus methods, or compare 2 of the 3 methods, there is rarely a direct comparison between the 3 methods.

Before attempting to transform plants, but after choosing the explant, it is necessary to select a way to screen the transformed explants from the untransformed explants. Therefore, it is necessary to choose a selection agent, often a herbicide or antibiotic, that, when corresponding genes are inserted into the genome of the transformed plant, provides resistance to the selection agent. There are many different selection agents commonly used, especially when using bacteria, but relatively few are commonly used with working with maize.

The selection agents commonly used in monocot and maize transformation are 3mg/l bialaphos (L-phosphinothricyl-L-alanyl-L-alanine), 25mg/l Hygromycin B, 4mg/l PTT (phosphinothricin tripeptide) and 150mg/l G418 sulfide (Geneticin) (Bashir et al., 2004; Dennehey et al., 1994; Lee et al., 2023; Spencer et al., 1990). Less commonly used in monocots, but prevalent in dicot transformation is kanamycin (100mg/l) (Klein et al., 1989). These five selection agents are commonly used in plant transformations but work in different ways.

Bialaphos and PPT are both phosphinothricin-based selection agents that have resistance conveyed by the *bar* gene (Dennehey et al., 1994). Bialaphos inhibits glutamine synthetase when metabolized by releasing glufosinate. PPT works in a similar way, inhibiting glutamine synthetase by acting as a competitive inhibitor. Hygromycin B is an antibiotic that inhibits chloroplast and mitochondrial protein synthesis, and has resistance conveyed with the *HPT* gene (Walters et al., 1992). Kanamycin and G418 both make use of the *NptII* gene for resistance (Klein et al., 1989; Lee et al., 2023). Kanamycin works similarly to hygromycin by inhibiting protein synthesis in chloroplasts and mitochondria. However, despite using the same gene for resistance, G418 inhibits polypeptide synthesis by preventing elongation. It is important to to select the correct selection pressure for your experiment, as choosing wrong can delay death, or not select at all. Through this experiment we aim to compare these commonly used selective agents through analyzing callus death over time on a constant selection pressure.

However, choosing selection is only one important part about making media for a transformation experiment. The growth media provides essential nutrients, vitamins, and hormones required for the proliferation and regeneration of plant tissues during the transformation process. Historically common base medias used for transformation experiments in maize are Chu N6 basal salt based media and MS (Murashige and Skoog) media, along with modified MS medias (MMS) that are used in more recent popular medias (CHU et al., 1975; Masters et al., 2020; Phillips & Garda, 2019; Zhao et al., 2002). To make an efficient media, it must include a sugar, basal salts and vitamins, and water. To encourage growth and development different hormones and plant growth regulators can be added (Aesaert et al., 2022). Specifically for transformation experiments, where transformed explants should have marker genes for visible distinction or chemical selection, an addition of a selection agent should be added.

Marker genes are genes that are inserted into constructs that indicate if the transformation was successful. There are two common types of marker genes: genes that convey some kind of resistance and genes that allow for visible discernment. Common resistance genes are antibiotic resistance and herbicide resistance. Visible markers are often added along with the resistance markers. The most common marker genes are GFP, mCHERRY, dsRed, and GUS. Some visual markers require a sacrifice of material to see transformation (GUS) or require expensive equipment to view (GFP, mCHERRY, dsRed). A new marker system has been introduced allowing for visible selection without using a fluorescent microscope, or the loss of material, called *RUBY*. *RUBY* was first described in 2020 in rice, tobacco, and Arabidopsis (He et al., 2020). *RUBY* is a combination of genes encoding a pathway that transforms Tyrosine into Betalain, a naturally occurring pigment found in beets, bougainvillea, and many cacti. It is a red or yellow pigment, and in this pathway results in red pigmentation in transformed plants through the use of the QuickCorn protocol (Lee et al., 2023).

The QuickCorn agrobacterium mediated maize transformation protocol was first described in 2020 (Masters et al., 2020). The protocol uses the *BBM:WUS* Cre/*lox* recombination system as well as the Pltp promoter to consistently obtain transgenic plants. The morphogenic genes were excised using heat induction to avoid stunted and malformed plants. This protocol is the current fastest and is now a widely used protocol for maize transformation. A full transgenic plant using traditional methods takes about 2 months minimum, while this protocol shortens the time to 5-7 weeks. This protocol skips the callus induction phase, which could potentially make maize transformation less genotype dependent. For the experiments described, a modified QuickCorn protocol is used and compared to several other methods. One of the modifications is the use of L-Cysteine, an antioxidant, during the co-cultivation stage.

Studies suggest that the use of antioxidants, like DTT and L-Cysteine in cocultivation media during maize transformation can increase efficiency (Vega et al., 2008). This can occur by increasing reactive oxygen species, which can up-regulate genes associated with oxidation reduction processes (Y. Liu et al., 2017). Antioxidant levels have also been shown to reduce tissue browning and necrosis during transformation steps, thus increasing the efficiency (Dan, 2008). There are several already used antioxidants and even more chemicals that are naturally produced by plants already, which haven't been used in media, Lycopene and beta-carotene. Here we test different antioxidants and concentrations of antioxidants to demonstrate any differences

Materials and methods:

Callus and Regeneration Maize Line Comparison

Maize inbred lines B104, A188, and KSU62 were grown in greenhouse conditions, during spring, fall and winter under less-than-ideal light conditions. These plants were started in a no-holes flat filled with potting media, grown until about 15 days post emergence. The plants were then transplanted into 4-inch pots also in potting media. After an additional 20-30 days, the plants were transplanted into either 1-gallon or 2-gallon pots to reach maturity. Upon silk emergence, silk was trimmed to approximately 3 mm below husk tip to ensure fresh silk for pollination. The trimmed silk was covered by semi-transparent treated paper shoot bags and let regrow for 1-2 days. The silk was then pollinated during peak pollen hours. Immature embryos were obtained from greenhouse plants in fall and winter 9-14 days post pollination. Mature seeds were obtained from seed stock stored at 4.5°C for at least 3 weeks.

Media used for this experiment was either modified MS media or modified Chu N6 medias (Murashige, 1962; Chu, 1978). All media is autoclaved to sterilize at 250°C for 20 minutes. Stock preparation information can be found in **Appendix B** in additional figures. To germinate mature seeds for leaf-based callus a modified simple MS media was made containing 2.22 g/L MS basal salts with vitamins, 20g Sucrose, and 5g of Sigma brand Agar was added. The pH was adjusted to 5.6 prior to adding agar and autoclaving the media. Post autoclave, an optional antibiotic can be added, then the media is poured into magenta boxes, at about 50mL/box. For callus initiation from immature embryos and mature seeds, a modified Chu N6 media was used. Callus induction media (CIM) was made containing 3.99 g/L Chu N6 salt with vitamins, 30g/L sucrose, 0.90 g/L Proline, 0.10 g/L Myo-Inositol, 0.50 g/L MES, 0.25 g/L Casein, 7.5 ml/L 2,4-D (2 mg/10 mL), and 12 ml/L Thiamine HCL (40 mg/L). The pH was adjusted to 5.8, and 8 g/L tissue culture agar was added. The media was then autoclaved. After removed from the autoclave, it was allowed to cool to 60°C and 1ml filter sterilized B5 vitamins (1000x) 10 µl filter sterilized silver nitrate (850 mg/10 ml), and 250 mg Clavamox (clavacillin) was added. The mixture was then poured into 100x15 petri dishes. Leaf callus induction media used the callus induction media (CIM) formula as well. The final media mixture used is the regeneration media. Regeneration media is a modified MS media containing 4.43 g/L MS salt with vitamins, 60 g/L Sucrose, 0.5 g/L Proline, 0.2 g/L Casein, 20 ml/L Zeatin (25 mg/100 ml stock), and after raiding pH to 5.6, 3g of Gelrite was added and the mixture was autoclaved. After autoclaving, the media was cooled to 60°C and 1 ml sterile MS vitamin (1000X), 100 µl Filter-sterilized Glycine (20 mg/ml), and 250mg Clavamox were added. The media was then poured into 100x15 petri dishes. Once the media was poured, it was allowed to cool and harden. Upon fully cooling, the plates were bagged and placed into a 4.5°C refrigerator until used. Upon

removal from refrigeration, the media plates have any condensation poured off under aseptic conditions and are allowed to dry for up to 30 minutes, also under aseptic conditions, before use.

Fresh ears were harvested (9-14 days after pollination) and all husk leaves and silks removed, the cobs were then washed by agitation in soapy water. The cobs were then surface sterilized for 20 minutes in 50% commercial bleach (5.25% sodium hypochlorite) with 2-3 drops of Tween-20. After surface sterilization, the ears were washed three times with sterilized water. Finally, the cobs were washed with 70% ethanol for 5 minutes, rinsed with sterile water. After sterilization, the immature embryos were dissected in laminar flow cabinets (embryo sizes, 1.5–2.0 mm) using a flame sterilized spatula. To extract the embryos, the top 1/3rd of the kernel was removed, the spatula was inserted and moved in a counterclockwise direction, which removes the embryo. The fresh embryos were then placed onto CIMS media orientated so the scutellum was facing up and placed into 27°C incubation chamber under dark conditions. Mature seeds for both mature seed callus and leaf meristem callus were taken from storage and were sterilized through a soap wash, 20 minutes in 50% commercial bleach (5.25% sodium hypochlorite) with 2-3 drops of Tween-20 and rinsed 2-3 times. The sterile seeds were then separated into two 50 seed batches, one set of 50 was cut in half (cutting the embryo also in half) under aseptic conditions with the cut side placed on MS media plates, then stored at 27°C incubation chamber in dark conditions. The other 50 seeds were placed in magenta boxes, 5 seeds per box, filled with MS media, and left to germinate.

After placing mature seed and immature embryo explants on CIMS media, callus usually appeared within a week. Once callus was 2-5mm, it was excised from the original explant material, and placed on fresh CIMS media and let to grow again until 6mm- 1cm. If necessary,

the callus was moved to fresh media every two weeks. Once the callus reached ideal size, or ideal type I or type II callus has grown, the callus was moved to regeneration media.

Leaf meristematic tissue was harvested approximately 12-14 days post germination. This was done by cutting and discarding the leaves to get to the meristem using sterilized scissors. The meristem is removed and placed onto a sterile petri dish. Cross-sectional, thin slices were taken off the meristematic tissue using a sharp, sterile scalpel. These cross sections were then placed on the callus induction media (CIM), the plates were sealed with parafilm and placed in a dark 26°C incubation chamber. Once callus had grown, they were placed onto CIMS media and continued to let grow, moving to fresh media as needed, until 1- 2cm where they were then placed onto regeneration media.

Once the callus from all different methods and maize varieties have reached about 1-3cm, they were moved to regeneration media. The new culture was placed in light conditions, under 4500K bright white LED lights emitting 5500 Lumens . They were left under light conditions until differentiation occurs and leaves have grown. These cultures are moved every two weeks to fresh media as required. When shoots have sufficiently grown, about 1-3 cm, the regenerating callus is then moved to magenta boxes with regeneration media. If there were instances of multiple shoots developing from one callus cluster, the shoots were allowed to grow to approximately 7.62cm (3 in), where they were assessed for possibility of separation. If deemed impractical to separate at this time they were allowed to continue to grow until time to remove from the magenta box. Once grown to approximately 12.7 cm (5 in) they are moved to a 4in pots with well-draining seed starting media and placed in high humidity conditions to harden off. In this experiment, the freshly potted plants were placed in a zip lock 1 gallon bag for 2 days with a little bit of water inside the bag. The bag was slowly opened to lessen humidity without wilting

the plant. The plant and bag were placed in a growth chamber with a humidifier. The humidity in the chamber was then gradually lessened over the course of a week. The plant was then removed from the humidity and observed for 15 minutes, if signs of wilting were observed, the plant was placed back into the chamber. If no wilting was observed, the plant was deemed fully hardened. After fully hardened off the plants are let grow to about 30.5 cm (1 ft) before being moved to greenhouse conditions to reach maturity.

To determine significant differences between varieties or maize, and the different methods used, counts of total embryos, seeds, and meristematic tissue clusters were taken. Next, counts of callus derived from each line, and each method were collected, and notes about callus type were taken. The percentage of callus grown was generated, and the same was done for the percentage of regenerated plants.

Selection Pressure Experiment

Lab standard Callus Induction Media (CIM) was prepared, autoclaved, and 200ml was aliquoted into 5 sterile 200ml Erlenmeyer flasks, and the remainder was left in the autoclave bottle. Each aliquot had a commonly used selection agent added, in commonly used concentrations of 3 mg/l bialaphos, 25mg/l hygromycin, 4 mg/l PPT, 150 mg/l G418, and 100 mg/l kanamycin. The flasks and remaining media were then mixed, remaining in their individual containers, by swirling and were subsequently poured into 100x15mm petri-dishes. This results in 6 total selection tests: no selection, kanamycin, bialaphos, PTT, hygromycin, and G418 sulfide.

Previously prepared KSU17, an unreleased homozygous line similar to KSU62, embryos and typeII (friable) callus was placed onto the plates, with each selection agent test having 3 plates with 10 embryos per plate. Each plate contained 5 younger, smaller callus, and 5 older,

larger callus. Callus remained on the same plate for 2 weeks before being transferred to new media of the same concentration for 3 additional weeks. After 3 additional weeks, the callus was transferred to lab standard regeneration media CRM.

Data was collected by taking pictures of each plate, labeled 1, 2, and 3, once a week and observing how many callus remained actively growing. Plates were observed at a minimum of twice a week. Images of the callus were taken once a week and used to identify callus growth and amount of living callus was recorded. At week 5, data was compiled, and a two-way ANOVA was run using Dunnett's multiple comparisons test. This was done comparing all treatments against the Control treatment for each week and run again comparing G418 to all medias for each week.

Co-Cultivation Media Experiment

To compare different antioxidant's impact on transformation, different antioxidants were added to media during the co-cultivation stage. Preliminary data comparing use of acetosyringone, DTT, and L-Cysteine in co-cultivation media. This experiment used a combination of morphogenic regulators BABYBOOM and GRF-GIF (BBGG) expressing dsRed. Embryos were checked for dsRed expression 7-10 days later and counted. The average percentage of transformation events was calculated and graphed. Since this is a preliminary experiment to test theories, there was not enough data to perform statistical analysis. For this first media comparison, the media compared was CIM, CIM with acetosyringone, CIM with acetosyringone, DTT and L-Cysteine, and CIM with DTT and L-Cysteine.

After the first test demonstrated the combination of L-Cys and DTT had the highest transformation event percentage, there was a need to explore DTT and L-CYS separated, and with the modified 562V with L-Cys that has been used in our lab previously. The medias

compared were QuickCorn's 562v +L-Cysteine, PhiB, and modified CIM medias with L-Cys and DTT and acetosyringone separately, with the control being unmodified CIM media. Media components for the base medias can be found in **Appendix A**. The components added to the CIM media for this was 300mg/l L-Cysteine, and 150mg/l DTT. The *agrobacterium* used to inoculate the immature embryos was LBA4404 encoding pCBL-101_RUBY with helper plasmid PKL2299. Expression of RUBY could be seen 2-3 days after transformation and was deemed to not be transitive after 5 days, which was when data was collected. This was done in triplicate. Data collected for each repetition was recorded and the mean was measured. The means were grouped by treatment and a one-way ANOVA with Tukey's multiple comparisons test was ran. Grouped transformed embryos were totaled and divided by the sum total embryos transformed on each plate, and these averages were graphed.

Results

Callus and Regeneration Maize Line Comparison

Immature Embryo Callus

Callus yield from immature embryo harvest was high. All three lines had over 90% of embryo generating callus (**Figure 2A**). The quality of callus varied, as KSU62 generated type 1 callus more than type 2 callus (**Figure 2B**), as well as more roots. B104 and A188 lines generated type 2 callus. A188 generated type 1 callus before type 2 callus (**Figure 2d**). B104 generated type 2 callus more than type 1 callus (**Figure 2C**). There is not a large difference between total callus generation of the three lines when generating from extracted immature embryos. However, callus quality and type varied with immature embryo age and line. Callus development of type 2 callus develops within after type 1 callus and after trimming of any sprouting roots or shoots.

Mature Seed Callus

Comparison of callus derived from mature seed showed some variability in callus generation, but no large difference, as with immature embryos indicated in **Figure 2**. However, there was less callus generation from mature embryos, with the highest percentage being A188 with 87.50%, KSU62 at 77.78%, and lowest B104 at 71.88% **(Figure 3 A)**. KSU62 showed high type 1 callus development, with some type 2 callus (**Figure 3B)**. Line B104 developed mostly type 2 callus, with the callus growing on excised shoot growths **(Figure 3C)**. A188 callus developed ideal type 2 callus, with small type 1 callus growth **(Figure 3D)**.

Leaf Tissue Callus

Leaf meristematic tissue based callus induction and regeneration remains difficult to produce without the use of morphogenic regulator. Across all lines there was callus development (**Figure 4A)**. Some callus developed but did not develop into embryogenic callus. All lines produced callus on about half of the explants. The largest amount and best quality of callus was produced by B104 at producing 58% callus (**Figure 4B**), while the least was KSU62, producing only 47% and had the worst quality of callus (**Figure 4C**). The middle at 55% was A188, which produce about the same quality of callus as B104 (**Figure 4D**). None of the callus developed into type II callus, and no regeneration was seen.

Maize Line Comparison

When inducing callus that can be regenerated, immature embryo isolation remains the best option. With every line tested showing above 90% callus induction and mature seeds callus's highest percentage being 87%, there is a noticeable difference between the two options (**Figure 5A)**. The starkest difference can be seen when comparing the regeneration of the

different explants as the only explant to regenerate was immature embryos(**Figure 5B**). Callus derived from immature embryos developed type II callus that turned green and regenerated (**Figure 5C**). Mature seed callus formed after any shoots and roots that appeared were trimmed. While some callus appeared to be a form of type II as well as type I, none regenerated (**Figure 5D**). The worst callus produced was leaf derived callus, which had the worst high percentage, at 58%, and the worst quality (**Figure 5E**).

Selection Pressure Experiment

The data collected from all samples over the course of 6 weeks (week 0 to week 5) was compiled. Callus remaining alive on the three rounds of each treatment per week was averaged and plotted along with the standard deviation to form a chart showing callus death over time (**Figure 6 A)** . Callus growth and death over time can be seen through imagery (**Figure 6 B**).

To analyze the results, a two-way ANOVA using Dunnett's multiple comparisons test was done. We saw no statistical differences during week 0 and week 1. However, at week 2 G418 had a statistically significant difference between all other treatments, with p-values of 0.0068 for kanamycin and hygromycin, 0.0015 for bialaphos, 0.0003 for PTT, and <0.0001 for control. For week 3 kanamycin and hygromycin are statistically significant from the control, and G418 remains significant for all treatments. Week 4 all treatments are statistically significant compared to the control, but G418 is no longer significantly different from kanamycin and hygromycin. Week 5 we see G418, kanamycin, and hygromycin remaining statistically significant to control, but bialaphos and PTT are not significant to control as the control saw some callus death.

The data collected follows expectations of G418 being the most efficient method, followed by hygromycin, then kanamycin, then bialaphos, PTT, and no selection. The only

exception to expectations was kanamycin, which was anticipated to have little to no selective pressure. However, kanamycin was shown to kill most of the callus within 4 weeks and having few escapes.

Co-Cultivation Media

Preliminary data testing the efficiency of the addition of acetosyringone and of a combination of L-Cysteine and DTT to a media indicated that acetosyringone is essential to transformation, and adding to co-cultivation media outside of the initial inoculation stage greatly increases the chances of successful transformation events, and the combination of DTT and L-Cysteine increased the percentage of transformation events by 11% (**Figure 7 A**). Expression of dsRed from this initial experiment registered in the scutellar epidermis, opposite of the coleorhizal end (**Figure 7 B**). This preliminary data led to the next experiment testing CIM with acetosyringone, CIM with acetosyringone and DTT, and CIM with acetosyringone and L-Cysteine compared to Phi B, a co-cultivation media previously used in transformation, 562V + L-cysteine, a modified QuickCorn co-cultivation media, and the control, CIM media with no additives. The preliminary data comparing these media was collected after 5 days, when RUBY expression was determined to not be transient, and data was collected. Each embryo was scanned and analyzed for quality and quantity of RUBY expression. Collected data was analyzed by a one-way ANOVA using Tukey's multiple comparisons test. The data collected shows that the additions of L-cysteine, and DTT had the same efficiency as 562v + L-Cysteine, while being statistically significant from control, Phi B, and CIM with acetosyringone (**Figure 7 C**). While acetosyringone has expression, it is not substantial, only 4 instances, and barely showed up during the scanning process (**Figure 7 D**). Media with L-cysteine, DTT, and 562V with L-cysteine had expression in all embryos tested (**Figure 7 E, F, G**). Media with DTT had

expression on the scutellar epidermis and parenchyma, but the quality of expression was not of the same quality as media with L-Cysteine or with 562V with L-Cys (**Figure 7 F**). L-Cysteine media had dark red expression on the scutellar parenchyma surrounding the coleoptile, but no expression on the coleoptile (**Figure 7 E**). 562V with L-Cys had similar coloration to L-Cysteine media, but the transformation events were not spread as evenly across the scutellar epidermis and parenchyma, with most transformation events happening at the tip of the scutellar epidermis (**Figure 7 G**). ANOVA data showed that 562V with L-Cys, and all other media with additives show a significant raise in transformation events compared to control and phi B with p-values of <0.001. The media with acetosyringone is another baseline for comparison, as all other medias that has transformation events also had acetosyringone. However, the media with additional additives and 562V with L-Cys showed a significantly higher transformation event percentage, with comparative p-values of <0.001.

Discussion

Callus initiation and regeneration are both important aspects of plant tissue culture and transformation for both dicots and monocots. This is why determining the best explant material is essential for future experiment success. These results suggest that immature embryogenic callus yields higher regeneration rates than callus derived from mature seed and leaf tissues. The B104 line shows higher immature embryo callus production, but not at a statistically significant rate compared to KSU62 and A188. In mature seed callus initiation, B104 had the lowest callus development, and A188 had the highest. Mature seed callus development is less consistent than immature embryos. While callus is generated, there is a much higher chance of improper sterilization, or use of non-viable seed which could possibly skew the generation of callus. However, even when generating callus, the callus generated was often type II, but potentially not

totipotent enough to fully regenerate. While initiation is important, callus type plays a more significant role in the ability to fully regenerate. While KSU62 shows callus development, the main callus type generated was type I, which is more difficult to regenerate. However, this could be due to embryo size being too big at time of isolation or media not being suited for the maize line. Another factor that should be taken into consideration is that the coleoptile often develops rapidly after mature seed is cut, exposing the embryo. There is a chance that cutting this off after it has partially developed could result in hormones being out of the ideal balance for callus formation, so more adjustment to media would need to occur. However, there is evidence that use of mature seeds is viable for a commercial scale by grinding off the endosperm and adding *agrobacterium* to the isolated embryo to transform the coleoptile resulting in transformed plants (Ye et al., 2022).

Leaf tissue callus yield was low. This could be because certain genes are necessary to generate good quality and abundant callus from leaf tissue. In previous studies, morphogenic regulators have been necessary to include for leaf tissue to fully regenerate (Lowe et al, 2016; Wang et al, 2023). Current research done using leaf tissue uses a combination of *WUSCHEL* and *BABYBOOM* genes, which are commonly used commercial morphogenic regulator genes, to generate consistent callus from leaf tissue (Mookkan et al, 2017; Zuo et al, 2002; Boutilier et al, 2002) In order to improve leaf tissue based regeneration, it would be necessary to find morphogenic regulators that can be used publicly, such as *GRF-GIF* fusion proteins, or by searching other species to identify potential new regulators (Debernardi et al, 2020; Gordon-Kamm et al, 2019). With transformation, callus initiation, and regeneration improving genes identified, the use of mature seed or leaf tissue explants would become more relevant.

There are many different lines of maize that are used for transformation. While the three lines tested in this study are widely used, they are only three out of many, so for future transformation experiments it might be necessary to identify new lines with better callus initiation and regeneration. To do this you could use several different methods of genotype analysis: rnaseq, a genome-wide association study (GWAS), quantitative trait loci (QTL), or other methods of genomic selection. Similar methods could be used to identify morphogenic regulator genes found in other species, such as *Arabidopsis*, rice, sorghum, etc. Using bioinformatic techniques, you could then see if any identified genes are present in maize lines. Finding better genes and lines could drastically improve callus and regeneration rates for not only leaf-based callus, but also immature embryo and mature seed callus. There could even be potential for other explant options not currently available in monocot species.

The data found in this study supports that A188 derives better callus overall and KSU62 had higher regeneration rates. This indicates that A188 line and the immature embryo isolation method could possibly have higher transformability. The significance of type II callus development indicates that mature seed could be a reliable source of explant material available year-round, despite not having a higher efficiency than immature embryos. Immature embryos will remain a staple explant for maize callus and transformation experiments, especially if adequate facilities are available to produce embryos all year. Leaf tissue explants might be viable explants if the lines used have specific genes for callus and regeneration (Wang et al, 2023). However, further testing will need to be done to confirm. The results indicate that immature embryos are the best explant for transformation as they are the only explant tested that reliably generated totipotent callus capable of regeneration at a high percentage across all lines.

Historically, bialaphos has been a common selection pressure to use during maize transformation (Lee et al., 2023; Spencer et al., 1990). However, the data collected in this experiment shows that the use of G418 can drastically decrease transformation time to only one round of selection, 7-14 days with no escapes. This result is similar to results shown in a previously published paper (Lee et al., 2023). Hygromycin is also known to rapidly select explants and has potential to be an alternative to G418. An unexpected result appeared in this experiment with kanamycin selection working effectively, as *NptII*, was previously thought to not have an impact on monocots (Wilmink & Dons, 1993). This is why G418 was an important creation for maize transformation, as it also operates through *NptII*. The *BAR* gene selection agents were last in effectiveness but might have had improved efficacy if a gradually increasing selection pressure was applied, as is often used in transformation. Despite this being the case, it is apparent that bialaphos and PTT are the slowest, and least effective selection agents used in the experiment. The rapid and statistically significant death of callus on G418 plates indicates that it is the best selection agent for use in transformation, followed closely by hygromycin B. The kanamycin results shown in this experiment are not what was expected due to the afore mentioned resistance inherent to *Zea mays*. This result could only be for this specific line of maize, which warrants further testing of the line, as well as testing the selection treatments across other lines of maize. However, with the rapid selection with G418 described using B104 in recent publications (Kang et al., 2023; Lee et al., 2023) there is a high possibility for selection across most maize lines. This is why I recommend the use of G418, also known as Geneticin, for future use in maize transformation experiments.

Antioxidants play an important role in many aspects of plant health. The maintenance of reactive oxygen species (ROS) homeostasis can help with seed germination, cell division, and

interaction with certain hormones (Xue et al., 2021). ROS during stress can reduce the auxin-induced signaling in plants, which can reduce growth, and may have an impact on callus development (Mansoor et al., 2022). Many plants produce different antioxidants to maintain this homeostasis, such as lycopene and beta-carotene. However, it is not widely known what happens to ROS homeostasis when *agrobacterium* is introduced. Often during the transformation process, embryos and callus, start browning and turn necrotic which can limit bacterial spread between cells. It has been reported that *Agrobacterium* can cause symptoms of apoptosis caused by ROS signaling proteins, which an addition of antioxidants can prevent by regulating ROS. (Dan, 2008; Hansen, 2000). Two chemicals have been shown to increase transformation levels with their addition to co-cultivation media, L-Cysteine (L-Cys) and dithiothreitol (DTT). L-Cysteine is a non-essential amino acid that is a precursor to the antioxidant glutathione, and DTT is an antioxidant already. Several papers have shown that the addition of L-Cys or L-Cys and DTT have increased transformation events (Du et al., 2010; Frame et al., 2006; Vega et al., 2008). The significant results shown in the co-cultivation experiment gives confidence that the use of combined L-Cys and DTT greatly increases transformation event percentage for the transformation protocol, although it is need of further testing to confirm these findings.

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Figures

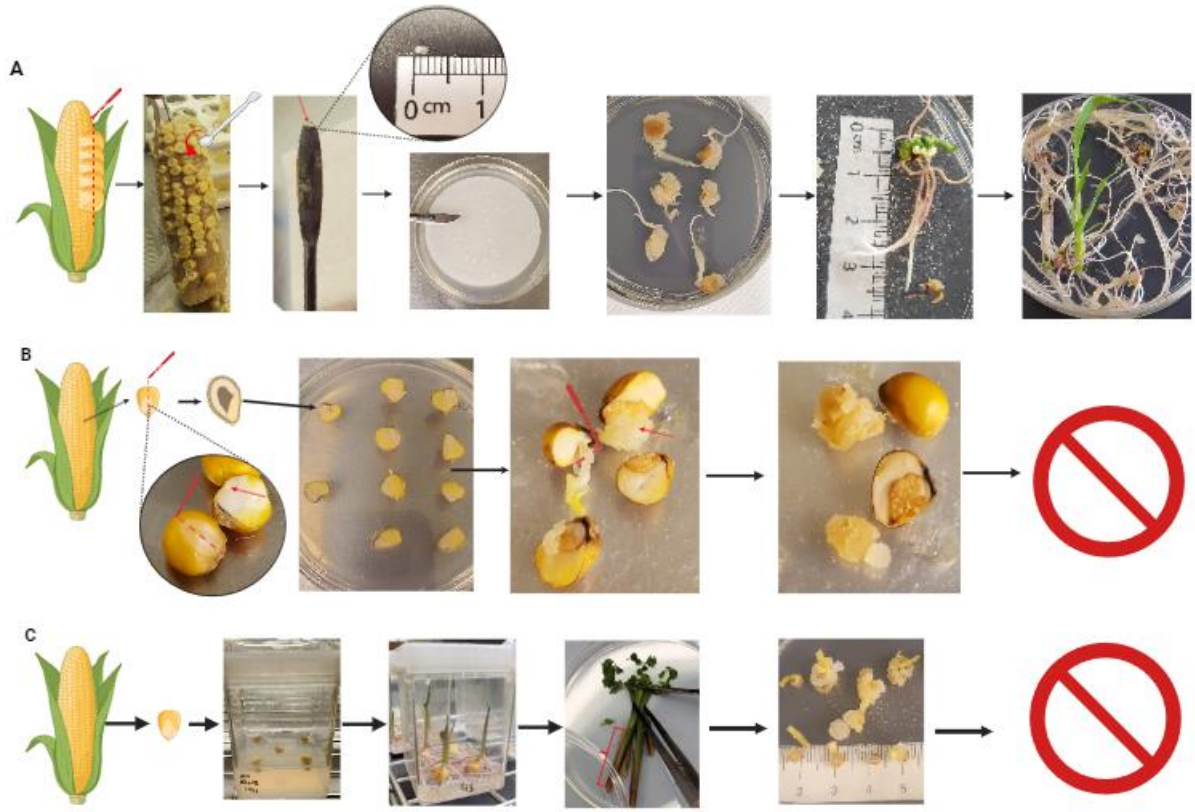

Figure 1 Callus Growth Flow Chart

A step by step flow chart showing the different steps for callus formation to regeneration of all three methods. The process from cob to regenerated plant of the immature embryo method (Figure 1 A). Mature seed to callus formation, as no regeneration occurred (Figure 1 B). Leaf meristematic callus formation occurred, but also no regeneration (Figure 1 C).

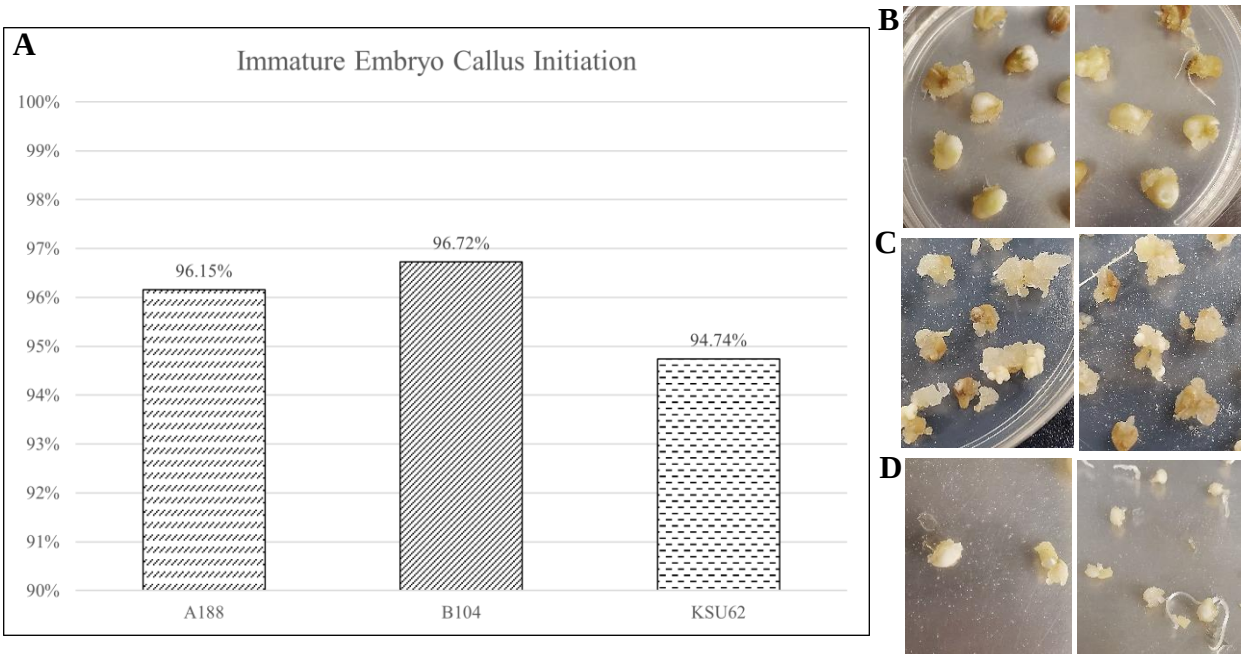

Figure 2: Immature callus comparison

The callus generation of the three different lines of maize A) were all above 90%, B) with KSU62 generating more type I callus and roots, C) B104 generating good type II callus, D) A188 generating type I and type II callus

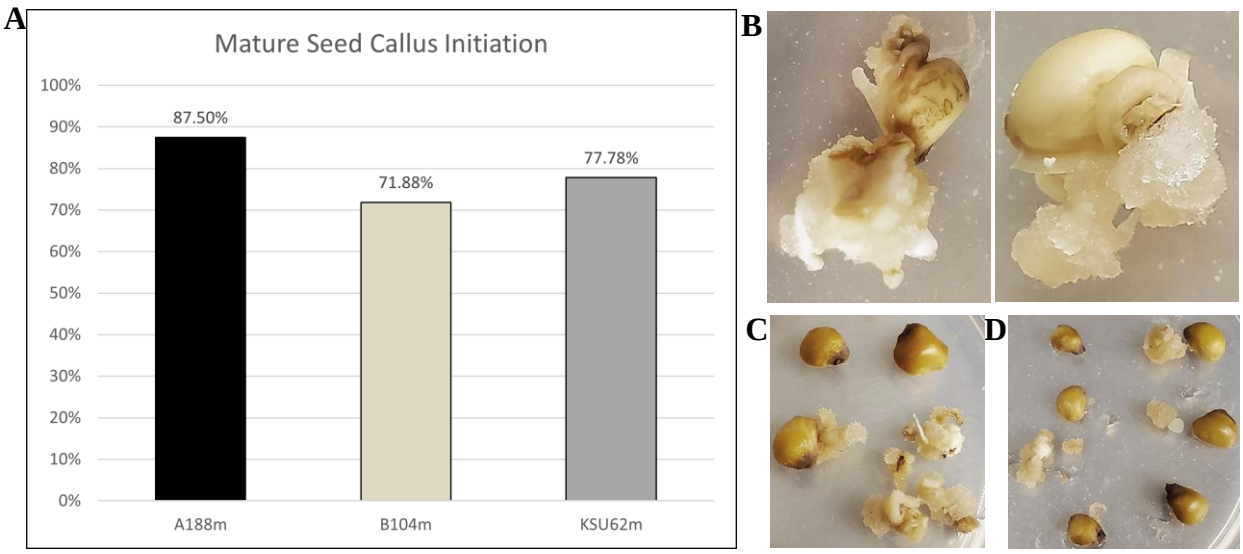

Figure 3: Mature Seed Callus Comparison

Mature seed callus development for each line (**A**) shows similar percentages, with B104 showing the least amount at 71.88% and the highest being A188 at 87.50%. KSU62 showed high root growth but developed type 1 callus after trim (**B**), B104 had high shoot development, but the callus quality suffered (**C**), and A188 had decent callus growth even after excised from the mature seed (**D**).

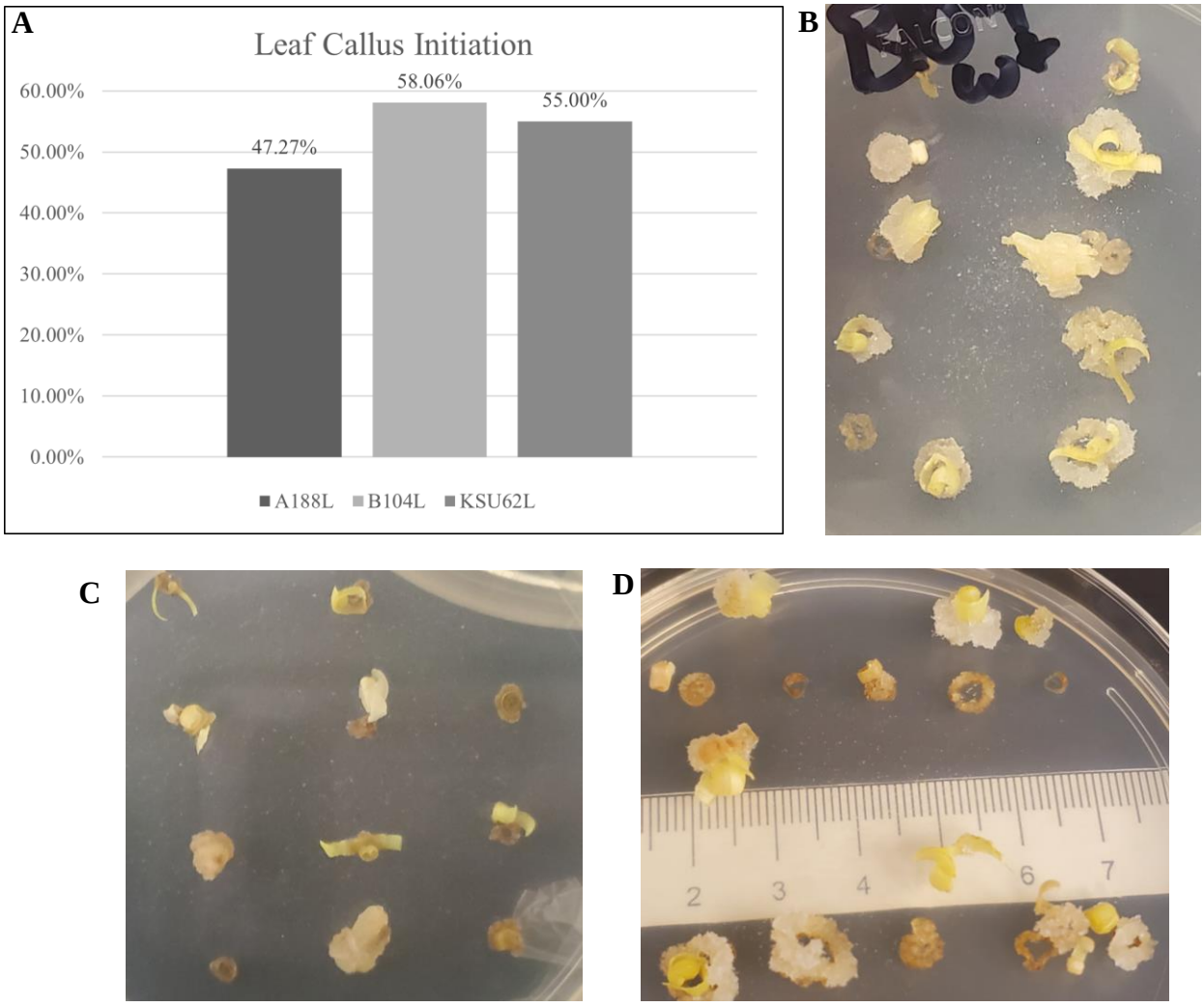

Figure 4: Leaf Callus comparison

Callus development when generated from leaf meristematic cuttings occurred (**A**) but did not develop into regenerable callus. All maize lines developed similar callus, with B104 having the highest generation (**B**), KSU62 having the next highest but also the lowest quality (**C**), and A188 having the least (**D**).

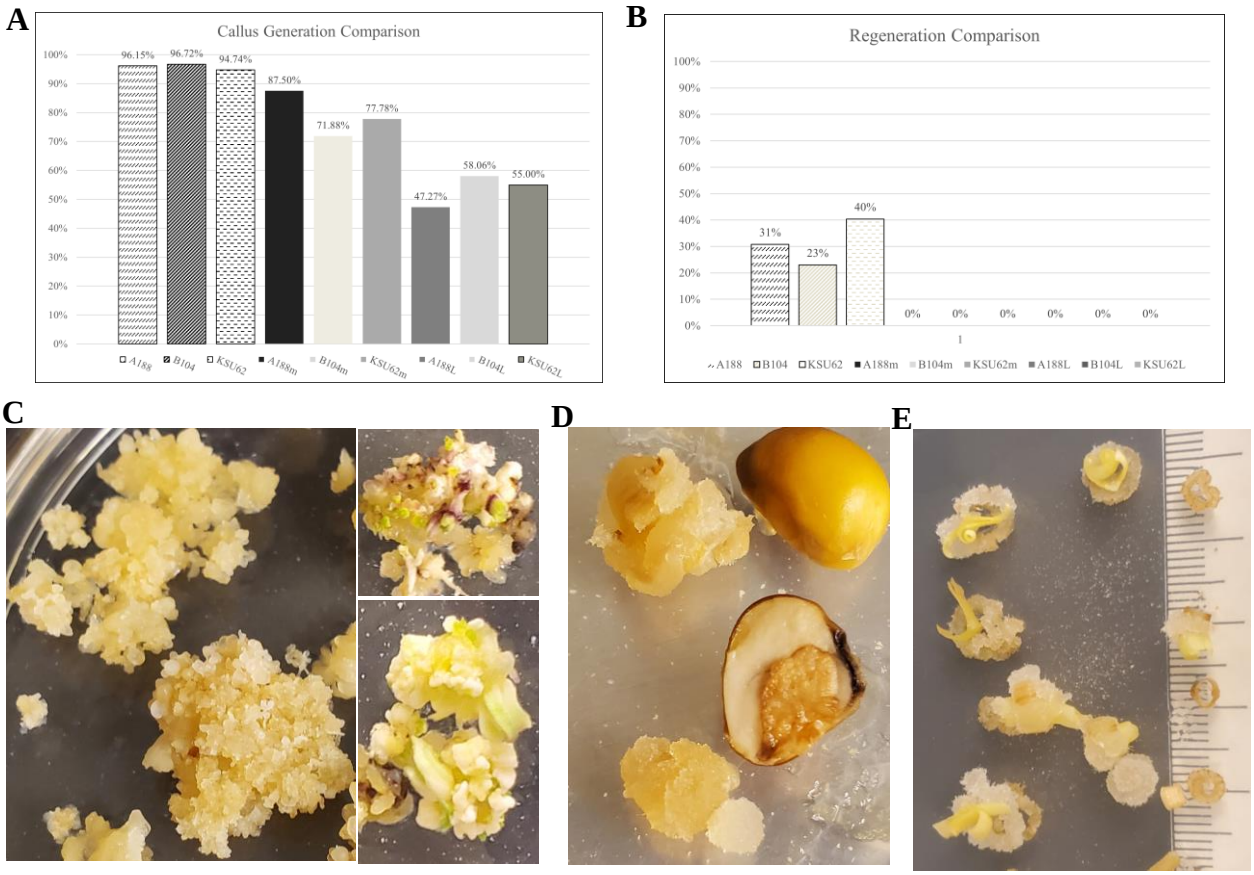

Figure 5: Callus Comparison of all Three methods

A comparison of all callus generation methods (**A**) shows that all different methods can generate callus. Immature embryos have the best callus generation, and are the only explant capable of regeneration (**B,C**). Mature seed callus generates callus only after the immediate shoot development occurs and, while developing good callus, none is embryogenic and regenerates (**D**). Leaf meristematic tissue develops the least amount of callus and is of poor quality (**E**).

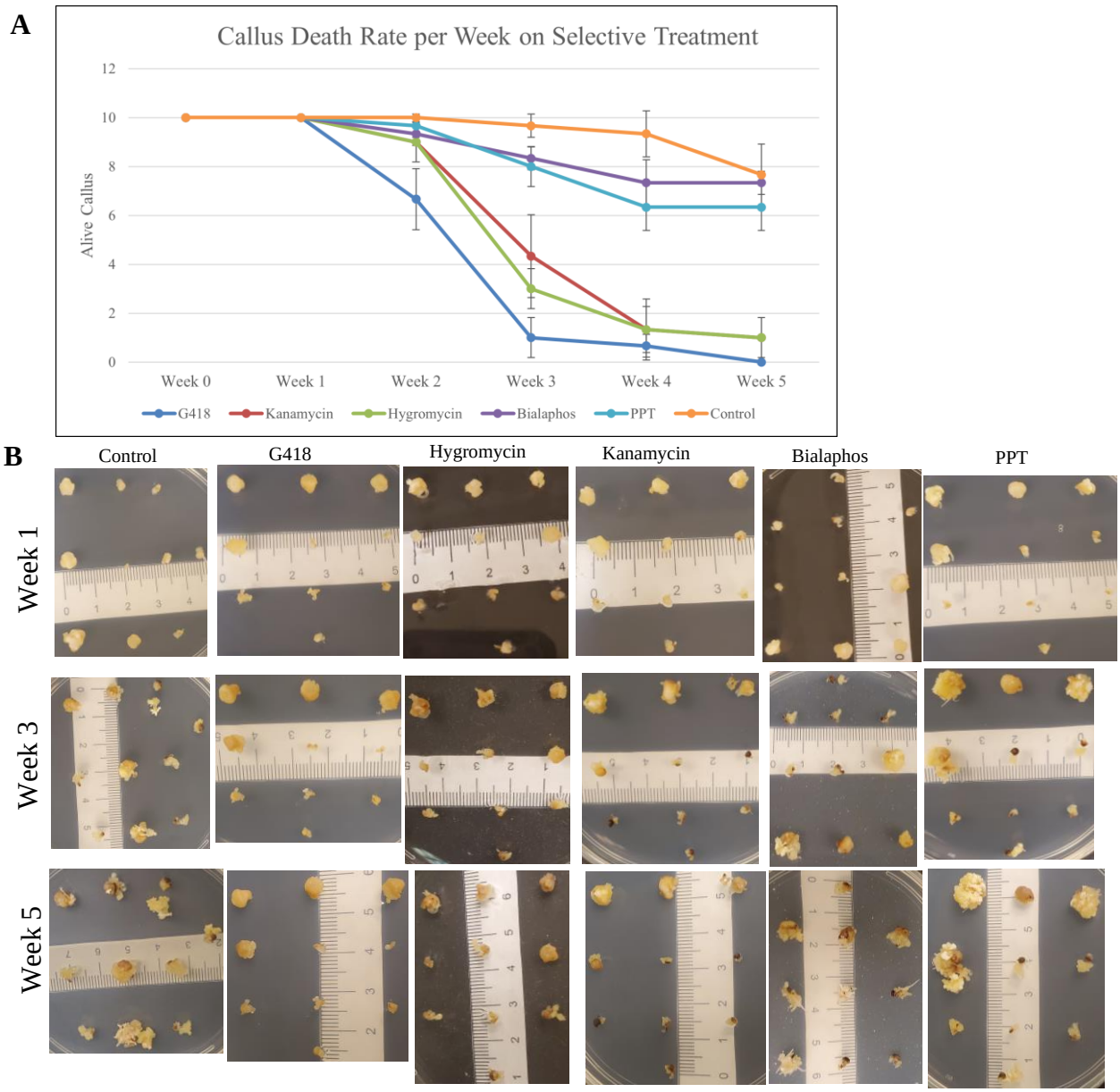

Figure 6: Selection Pressure Comparison

Selection pressure over time occurs in all treatments, but the most effective selection agent is G418. While the fastest and most effective is G418, hygromycin and kanamycin are almost as efficient, while the *bar* selection agents PPT and bialaphos are the least effective the graph plots the average living callus amount per treatment over the trial time, with error bars indicating the standard deviation between the three test plates of each treatment(**A**). Photos of each treatment at 1 week, 3 weeks, and 5 weeks showing the growth and death of callus over time (**B**).

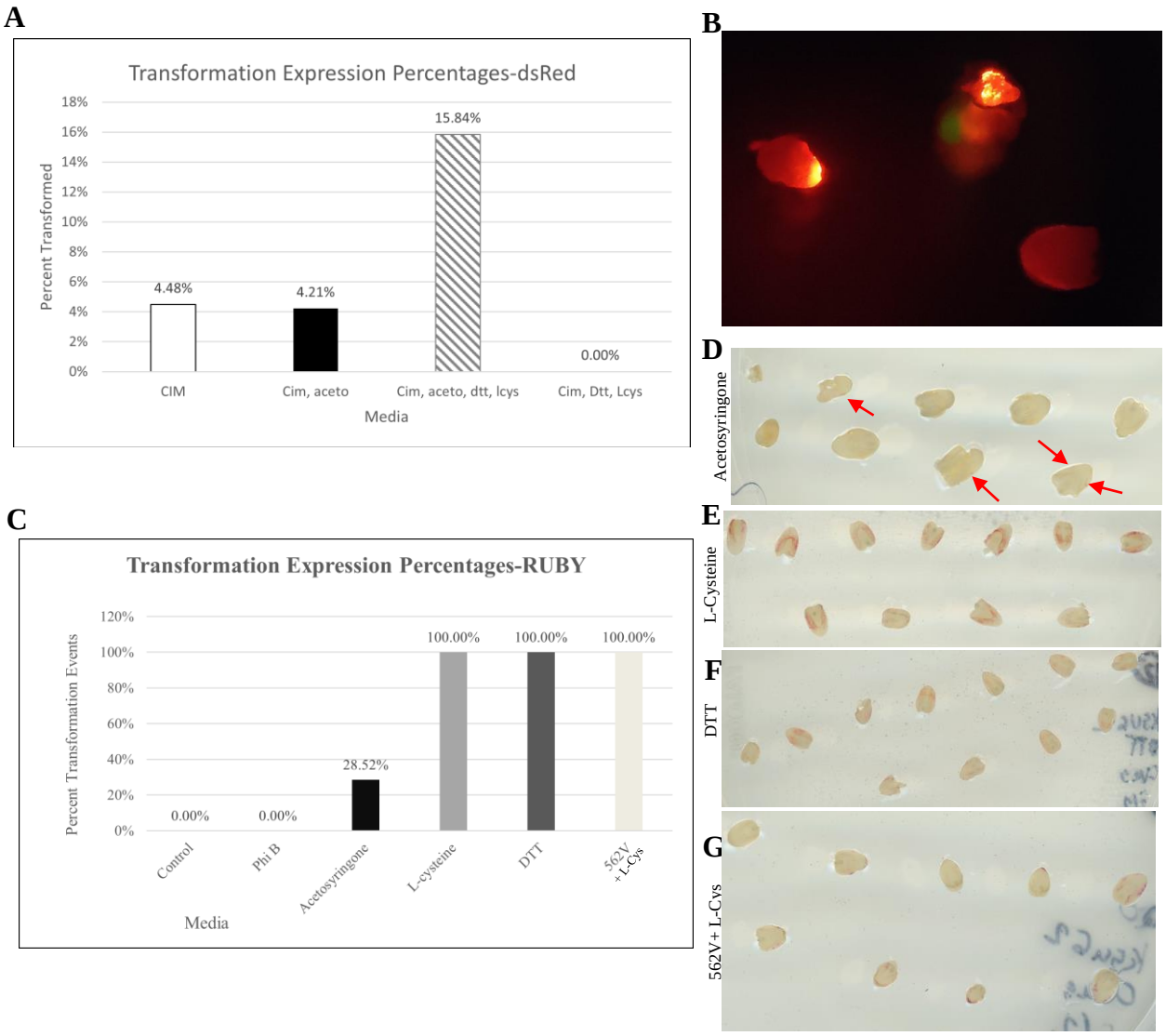

Figure 7: Co-Cultivation Media Comparison

Preliminary data indicates that an addition of the combination of acetosyringone, DTT, and L-cysteine to CIM media yields a higher percentage of transformation events than a CIM media, CIM media with acetosyringone, and CIM with DTT and L-cys no acetosyringone with 11% higher than the next highest yielding (**A**). To show what was counted as a transformation event the photo (**B**) has two dsRed expression transformation events. A comparison of a modified known good co-cultivation media, QuickCorn's 562V + L-Cys, to a previously used media (PhiB), to a modified CIM media containing L-Cysteine and DTT, through the use of visual marker RUBY indicates that the modified CIM media proposed by our lab is as efficient as generating transformation events as 562V+ L-Cys (**A**,**C**). Transformation events were recorded by observing coloration and size of RUBY expressing embryos on each treatment(**D,E,F,G**) (Motolai & Stewart 2024 unpublished data).

Chapter 2 - Transformations

Introduction

Agrobacterium-mediated transformation is a versatile and widely utilized tool for genetic engineering across a diverse array of plant species. Utilizing the natural genetic transfer capabilities of *Agrobacterium tumefaciens*, this technique enables the introduction of desired traits into plants ranging from crops like maize, soybean, and rice to horticultural species like tomatoes and potatoes, as well as ornamental plants and even trees. The process involves the delivery of foreign DNA into plant cells via Agrobacterium, leading to stable integration into the host genome and subsequent expression of the introduced traits (Chilton et al., 1983; de Framond et al., 1983; Fraley et al., 1983). With its efficiency, reliability, and relative simplicity, agrobacterium-mediated transformation has revolutionized plant biotechnology. The use of agrobacterium-mediated transformation has led to advancements in crop improvement, disease resistance, stress tolerance, and the production of pharmaceutical compounds (Cheung et al., 2011; Spencer et al., 1990; Tamang et al., 2021). The relative ease of agrobacterium transformation has become a common technique for introduction and modification of plant genomes. The process begins with the preparation of a binary vector containing the desired gene of interest flanked by sequences recognized by Agrobacterium. The Agrobacterium culture harboring this vector is then co-cultivated with maize embryogenic callus tissue, allowing the bacterium to transfer the genetic payload into the plant cells (Gelvin, 2003). Through the integration of the foreign DNA into the maize genome, transgenic plants expressing the desired trait can be regenerated from the transformed cells. Furthermore, agrobacterium-mediated transformation offers a relatively straightforward and cost-effective approach compared to other methods such as particle bombardment or protoplast transformation (J. Liu et al., 2019; Maas &

Werr, 1989). As researchers continue to refine and optimize this transformation method, its widespread adoption promises to contribute significantly to the sustainable enhancement of maize varieties, addressing the challenges faced by agriculture in the 21st century.

Agrobacterium-mediated transformation has been significantly enhanced through the utilization of ternary vectors (Anand et al., 2018; Kang et al., 2022; Lowe et al., 2018). Ternary vectors exploit the natural virulence machinery of Agrobacterium tumefaciens to efficiently deliver DNA into plant cells. The vir genes encode proteins that facilitate the transfer of the T-DNA from the bacterial cell into the host plant genome. Upon recognition of specific DNA sequences within the T-DNA region, the Vir complexes nick the T-DNA, allowing it to be processed and transferred to the plant cell. The T-DNA is then integrated into the plant genome, leading to stable transformation and expression of the introduced gene. To enhance the virulence factor of agrobacterium vectors, ternary vectors were designed. Ternary vectors offer several advantages, including higher transformation efficiency through the use of accessory plasmids, reduced risk of vector backbone integration, and greater flexibility in gene stacking due to their modular design. Since additional VIR genes have been shown to increase transformation efficiency, using a ternary vector pkl2299 to increase *vir* genes should also benefit a transformation protocol (Ishida et al., 1996; Kang et al., 2022).

Marker genes are important tools in plant transformation experiments. These genes are usually integrated into transformation vectors alongside the gene of interest and serve as indicators of successful transformation events. Common marker genes include antibiotic or herbicide resistance genes, such as the kanamycin and G418 sulfide resistance gene (NPTII) or the phosphinothricin acetyltransferase and bialaphos (bar) gene, which provide transformed cells with resistance to specific antibiotics or herbicides. These genes enable growth in media

specifically prepared to inhibit growth unless these genes are present. Additionally, reporter genes can be proteins with easily detectable characteristics, such as β-glucuronidase (GUS), green fluorescent protein (GFP), dsRED, or most recently, RUBY, a betalain pathway (He et al., 2020; Wang et al., 2023). These reporter genes allow for visual or enzymatic detection of transformed tissues or cells, allowing efficient screening and selection of transgenic plants without the need for additional chemical agents or the destruction of transformed material. Marker genes are essential components of plant transformation protocols. For this reason, several antibiotic resistance genes and herbicide resistance genes were used throughout all of steps of this transformation, with the end goal of conveying bialaphos resistance alongside the CRISPR/Cas9 knock out of genes WAT1 and Gl3 to maize and tobacco.

WALLS ARE THIN 1 (WAT1) is a gene found in Arabidopsis. It is essential in auxin transport facilitation, and auxin homeostasis (Ranocha et al., 2013). Since auxins are crucial to plant growth and development, specifically cell growth and elongation, but also regulation of reproductive and vascular development (Benjamins & Scheres, 2008). Mutations of this auxin down regulating gene can result in auxin being over expressed to the extent of killing the plant, as herbicides such as 2,4-D demonstrate. Since WAT1 hasn't been categorized in maize, it is not known what will happen with a deletion of this gene. However, this gene was chosen to be tested due to it being identified as candidate gene to improve callus generation when knocked out. It was chosen as a candidate gene by RNA comparison between type1 callus and type 2 callus, type 2 callus being ideal, to see which genes were active. Since WAT1 was active in type 1 callus but not type 2 callus, it was selected to be a candidate for testing a CRISPR/Cas knock out transformation system (Ishida et al., 1996; Kang et al., 2022; Tamang et al, 2023). Along with WAT1, a categorized gene, Gl3, was used. Gl3 is a gene that impacts epicuticular waxes and

trichome formation in maize. A knockout of this gene should show significant differences between the transformed plant and wild type. However, since it is unknown what deletion of these genes would have on plants, they were first tested in tobacco.

Tobacco species are model plants for agrobacterium-mediated transformation. Tobacco possesses a high regeneration capacity, allowing for efficient regeneration of whole plants from transformed leaf cuttings. Tobacco plants also exhibit a relatively short life cycle, enabling rapid generation turnover, expediting the analysis of transformed traits. Tobacco's susceptibility to *Agrobacterium tumefaciens* without the need of a wounding chemical, such as acetosyringone, simplifies the transformation process. It has well-characterized genome, when paired with the availability of diverse molecular tools and resources, increases tobacco's utility as a model organism for investigating plant molecular biology and genetic engineering. Specifically, tobacco serves as a model organism for testing novel trait insertions, or gene deletions destined for monocot species in a rapid manner, as monocots have a long turnabout time, and have some unique characteristics that pose challenges for agrobacterium-mediated transformations.

Transformation has become one of the fastest ways to generate new traits in maize. Many new traits have been introduced, including herbicide resistance genes, genes that mitigate environmental stresses such as heat and drought stress, which is increasingly important with climate change. Other genes that have been introduced but are used to increase efficiency of transformation itself are morphogenic regulator genes. Morphogenesis describes the process that causes tissues or organs to develop into specific tissues during stages of embryo development. Morphogenic regulators are genes or proteins that regulate certain pathways that control or help induce different aspects of plant growth. Two of the oldest to be used in maize transformation, yet still relevant morphogenic regulators used in maize transformation are *WUSCHEL* and

BABYBOOM. *WUSCHEL,* referred to as *WUS* or *WUS2*, is a gene that enhances embryogenic competence and organogenesis (Zuo et al., 2002). This most often occurs in the shoots or apical meristems. Plants that express *WUS* can exhibit abnormal growth and be sterile. Another commonly used morphogenic regulator is *BABYBOOM,* or *BBM* (Boutilier et al., 2002). *BABYBOOM* works in a way similar to transcription factors. Ectopic expression of *BBM* increases somatic embryo formation. *BBM* promotes cell proliferation and morphogenesis during embryogenesis. Like *WUS,* expression can cause abnormal growth and sterility. One way to prevent sterility is to use a Pltp promoter which results in expression only in leaf tissues and embryos. Both *WUS* and *BBM* are copyrights of Corteva, so they are unable to be freely used. To dramatically increase the efficiency of transformation, there is a combination of *BBM+WUS* genes recently used to test protocols where regeneration of plants is unnecessary. This combination results in unviable plants when expressed in the full plant. To prevent this, a Cre/*lox* marker gene system allows for the combination of *WUS/BBM* can be excised using a heat bath (Zhang et al., 2003). This allows for fully viable plants to benefit from the morphogenic regulators and fully regenerate into viable plants. The final, most recent, morphogenic regulator discussed in these papers is the fusion protein GROWTH-REGULATING FACTOR 4 (GRF4) and cofactor GRF-INTERACTING FACTOR 1 (GIF1) published by UC Davis (Debernardi et al., 2020). GRF-GIF increases the regeneration speed and efficiency and doesn't cause issues like similar morphogenic regulator WUSCHEL. A remarkable new combination of *BBM:GG* demonstrated in an (as of right now) unpublished paper shows that this combination increases totipotency and regeneration. This combination also results in no stunted or sterile plants. Since *WUS,* and *BBM* are the property of corporations and can cause problems during growth, the candidate genes identified, including WAT1 are hopeful

alternative morphogenic regulators. This is a secondary goal, along with establishing an individual maize transformation protocol.

Materials/methods

Several DNA plasmid constructs were designed or purchased for the transformation experiments (Figure 1). CRISPR/Cas9 based plasmids were obtained by sending a DNA construct to GenScript where they were constructed. Other plasmids (pCBL101-RUBY, PKL2299) were purchased through AddGene. Once obtained the plasmids were inserted into *E.coli* strains Top10 and DH5a. These strains were used to generate plasmids for transferring into agrobacterium.

Primers used for PCR amplification of part of the CRISPR/Cas9 system with the forward primer being 5'TCCATCGGCCTCGAC-3' and the reverse primer being 5'TTCTCGAGACGCCTGG-3' these will be referred to as PartCasFW and PartCasRV. The *Taq* DNA polymerase used was DreamTaq (Thermo Scientific™). This was used to confirm transformed *E. Coli*, agrobacterium, and maize.

The transformation protocol was tested using visible marker LBA4404 harboring PKL2299 and plasmid PCL101_RUBY. Maize transformation using a CRISPR system was done using single gene deletion construct inserted into binary vector LBA4404 harboring plasmid PYLCRISPR/Cas9_WAT1_Pubi-B and a double gene deletion construct inserted into LBA4404 harboring plasmid PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B. Ternary vector constructs: LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1_Pubi-B and double gene deletion construct inserted into LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B. Construct information can be found in Figure 8.

The maize lines used in this experiment were A188, B104, KSU62, and KSU17. All were grown in greenhouse conditions throughout all seasons. Maize was started in a high humidity mist greenhouse or growth chamber until developing 2-3 true leaves and being repotted into 1-gallon pots to reach maturity. Maize plants were grown on drip irrigation lines and watered every 3 days with fertilizing water applied every 2 waters. Plants were sprayed with pesticides if necessary. Plants had silk trimmed a day after appearing and were pollinated the next day. Silk was covered by an opaque wax bag to prevent cross pollination. To further prevent cross pollination, all different lines were grown in different greenhouse units. Upon pollination, the silk was covered by pollination bags until 9 days after pollination, upon which kernels were checked for embryo development.

Media

Tobacco Media

The media used in tobacco transformations for plating transformed explants contained 30g/l sucrose, 4.43g/l MS basal salts and vitamins, 10ml/l myo-inositol (10mg/l), 10ml/l BA (10mg/100ml), 1ml NAA(10mg/100ml), a pH of 5.7 to 5.75, and 8g/l of tissue culture agar. After autoclaving, any selection pressures are added, and the media is poured into sterile plastic petri dishes. The antibiotics used in RUBY encoded LBA4404 tobacco was 1ml/l gentamycin (20mg/ml), 1ml/l kanamycin (100mg/ml), and 1ml/l spectinomycin (120mg/ml).

Maize media

Several different media compositions were used during the transformation of immature maize embryos. Exact media compositions can be found in Appendix A. However, the medias used were a known good media that has generated transgenic lines in our lab, the QuickCorn method from Iowa State (Lowe et al., 2016; Masters et al., 2020), a method previously used by

collaborating labs (Zhao et al., 2002), and media that was developed in our lab specifically for our lab, which is based off of both of these medias.

Bacterial Transformations

Plasmids obtained were transformed into competent *E. coli* cells via the freeze/thaw method and screened by PCR to confirm the presence of the constructs. Upon confirmation that the plasmids had been incorporated into the *E. Coli*, a single colony was taken and grown in liquid LB media containing kanamycin (100mg/ml) and streptomycin (100mg/ml) antibiotics for 24 hours. This *E. Coli* culture then had the plasmids extracted using the QIAprep® Spin Miniprep Kit (QIAGEN®). Plasmids were transformed into *Agrobacterium tumefaciens* LBA4404 and LBA4404 harboring PKL2299 both the freeze thaw method and electrotransformation. The transformed agrobacterium was then plated and confirmed using PCR. Fresh plates of agrobacterium were plated for each maize transformation.

Nicotiana Transformations

Living materials

The plant material used during the confirmation transformation of tobacco were tobacco species Nicotiana tabacum and Nicotiana benthamiana. To confirm the efficacy of the transformed agrobacterium strains LBA4404 PYLCRISPR/Cas9_WAT1_Pubi-B, LBA4404 PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B, LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1_Pubi-B, and LBA4404 harboring PKL2299 and plasmid PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B, the N. tabacum variety KY14 was used. To test LBA4404 harboring PKL2299 and plasmid PCL101_RUBY, N. benthamiana was used. These LBA4404 agrobacterium strains were grown on standard YEP media along with the selection pressures 1ml/l of 100mg/ml kanamycin, 1ml/l of 100mg/ml streptomycin, and for the PKL2299

strains1ml/l of 20mg/ml gentamycin was added with 1ml/l of 120mg/ml spectinomycin. After growing those plates, an individual colony was selected and grown in plain YEP for 1 hour. After 1 hour, 200µL of the liquid is placed on a YEP plate containing the same antibiotics, streaked, and grown overnight. From this plate, a whole loop or scoop of the bacteria was grown in 2-5 ml of plain YEP or inoculation media for 1-2 hours or until an OD500 of .5-.75 if using a larger (4-5ml) amount of liquid. An optional step to increase infection is adding acetosyringone to this liquid prior to inoculation. This liquid will be used to transform tobacco.

Transformation of tobacco

To prepare tobacco species for transformation, the tobacco variety must have leaves of approximately 2.4 cm or larger for the best transformation results. When taking explant leaf cuttings for transformation, a sterile scalpel was used, and cut along the midrib on both sides of the leaf were made. Then, the external edge of the leaf was cut off, and the remaining leaf was split into 2-3 sections of equal size creating wounds on all sides of the cutting. All cut samples were placed into a sterile, empty petri dish and the grown agrobacterium was poured on top of the samples. Sterile forceps were used to make sure the leaf cuttings were completely submerged. Cuttings were left in the agrobacterium for 10-20 minutes. Inoculated leaf cuttings were placed onto sterile filter paper and the top and bottom of the leaf cuttings were dabbed free of the remaining agrobacterium liquid. The cuttings were placed stomata (bottom) side down onto the cocultivation plain tobacco media and remained there for 48 hours. The cuttings were then placed onto tobacco media containing Clavamox and selection media, 3mg bialaphos for CRISPR transformations, and Gentamycin for RUBY transformations. The cuttings can remain on this media until callus and plantlets begin to form or can be moved to fresh media every 2-3 weeks. Once plantlets began to form, and formed 3-4 true leaves, they were moved to rooting

media, still containing Clavamox and selection pressure. Using this method it agrobacterium and constructs can be confirmed to work in under a month with the formation of callus. Further confirmation can be made by conducting DNA extraction of the leaves and running a PCR to confirm the presence of your construct. This was not done in this experiment.

Maize Transformations

Agrobacterium Preparation

Transformed agrobacterium was streaked onto plate with selection about 1.5 weeks prior to transformation. After 2-3 days a colony was taken and grown in liquid with selection for about 2 days. A loop of this liquid was again streaked on selection plate overnight without a need for individual colonies. A loop/scoop was taken of agrobacterium growth off the plate and allowed to grow for 1 hour in inoculation media for the transformation (OD550= 0.5-0.75). An optional step to be done at this state is to add acetosyringone to this step as well as to the embryos.

Cob Sterilization

Fresh ears were harvested between 9-14 days after pollination, when embryos are the ideal size of between 1.5 mm and 2.5 mm, and all husk leaves and silks removed, the cobs were then washed by agitation in soapy water. The cobs were then surface sterilized for 20 minutes in 50% commercial bleach (5.25% sodium hypochlorite) with 2-3 drops of Tween-20. After surface sterilization, the ears were washed three times with sterilized water. After sterilization, the immature embryos were dissected in laminar flow cabinets using a flame sterilized spatula.

Embryo Isolation

To extract the embryos, the top 1/3rd to 2/3^{rds} of the kernel was removed carefully as to not damage the embryo by removing too much. Holding the cob with the top (pointy) side upwards, the spatula was gently inserted on the right side of the kernel and moved in a

counterclockwise direction, which removes the embryo. The embryo will be deep in the kernel so care was taken to fully remove the whole embryo.

Transformation

The isolated embryos were placed into inoculation media (TIM) filled 1.5ml centrifuge tubes 2.5µl of 100 mM acetosyringone was added to the tubes as well. The embryos were transformed within one hour of isolation from cob. Prior to infection with agrobacterium, the embryos were placed in an optional heat bath of 46°C for 3 minutes and were then spun down on a centrifuge at max RPM for 15-30 seconds. Inoculation media was removed and 2.5µl of 100 mM acetosyringone was added. Agrobacterium (OD550= 0.5-0.75) was then added to the embryos, mixed gently but thoroughly, and let infect for 10-15 minutes. The tubes were kept in dark conditions and mixed by gently rotating tubes upside down 2-3 times every 5 minutes. After the infection time of 10-15 minutes, the embryos and liquid were poured onto co-cultivation media (CCM), and a scoop was used to extract any remaining embryos from the tube. The embryos were let rest on the media for 30 seconds to 1 minute before excess liquid was removed from the plates with a pipette. Once the excess liquid was removed, a scoop or a scalpel was used to ensure all embryos had the scutellum on the side not on the media.

The embryos were left on co-cultivation media (CCM, or 562v+L-Cys, or 13329A) for 48-72 hours in dark conditions at 20- 26°C. After which the embryos were moved to resting media (CIM-no selection) for 7-10 days. During the resting stage, any developing coleoptiles and roots that appeared were trimmed. Once done resting, the embryos were moved to selection media (CIM) for 14 days per selection round. A minimum of 2 rounds of selection were done, the first using 2 mg/l bialaphos, the second using 3mg/l bialaphos, and subsequent rounds using 5 mg/l bialaphos. Callus were kept in dark conditions for all selection rounds. If friable, rapidly

growing callus was observed after the second round of selection callus was moved to regeneration media (TRM) containing selection pressure. Callus placed on regeneration were placed in light conditions, under 4500K bright white LED lights emitting 5500 Lumens. They were left under light conditions until differentiation occurs and shoots had grown. These cultures were moved every two weeks to fresh media as required. When callus had developed shoots, they were moved to a rooting media (RM) with selection pressure. The rooting was started on 20x100 petri-dishes but were moved to magenta boxes when they outgrew the plates. Once the plantlets have grown roots and 2-3 true leaves, or have reached the top of the magenta box, they were transplanted into sterile potting media. To transplant the plantlets, they were removed from the box, and gently had all media washed off of the roots to prevent molding and planted in a 4 inch pot of media. The transplanted plantlets were placed in a plastic bag with water in the bottom to maintain a high humidity environment. They were left sealed in the plastic bag for 3 days before starting to harder the plants to lower humidity environments. This was easily done with a humidifier and a growth chamber, or by slowly increasing the opening on the top of the bag. Once the plants were fully hardened, they were removed from the bag and let grow for 2-3 additional days. If the plant had reached about 8-10 inches, they were repotted into a 1-gallon pot and moved to the greenhouse to grow to maturity.

Results

Tobacco transformations:

After transforming the tobacco with LBA4404 harboring PKL2299 and plasmid PCBL101_RUBY, the transformed cells started to produce betalain in 3-5 days (Figure 9 B). Transformed and untransformed callus began to grow (**Figure 9 C**), so the explants were placed on the selection media, where the callus continued to grow about one month. At this point,

untransformed callus grew shoots on top of places that did not touch the media. These shoots were excised and move to selection media **(Figure 9 D**). Ruby Producing callus grew, but only 3 developed what appeared to be shoot-like structures, However, out of these shooting callus, only one produced several transformed plantlets (**Figure 9 D, E**). These plantlets were moved to rooting media to mature. This experiment demonstrated the low regeneration rate that often accompanies agrobacterium mediated transformations. It also demonstrates why tobacco species are a reliable model plant for testing construct design.

Maize transformation:

Over 8000 embryos were transformed in the process of finding a working protocol. For the transformation attempts, only 2 have developed into callus (**Table 1, 2 A, B, E**). During isolation, it is possible to damage the embryo as it is deep within the kernel (Figure 10 A) and is very small. To ensure good contact with the media embryos much be placed scutellum side up (**Figure 10 B**). Callus started growing during the resting stage and continued growing with some developing type I, type II, or both **(Figure 10 C, D**). They were allowed to continue to develop on multiple selection rounds with increasing pressure each round (2mg/l →3mg/l →5mg/l) until either a lot of type II callus had grown, or callus started to green (**Figure 10 D**), upon which the callus was moved to regeneration media where few will green and develop shoots (**Figure 10 E**). A total of 8 callus have been moved to rooting media from regeneration after developing some form of shoots from 24 that were moved from regeneration media (**Table 2 A,B, Figure 10 E, F**). These callus came from two transformations (**Table 2**), and out of those moved to rooting, only 2 have developed leaves along with roots (**Figure 10 E**) This results in a very low possible transformation efficiency of 3.91% (**Table 2 C**) and 1.34% (**Table 2 D**). Potential transgenic plants will be confirmed using PCR and Southern Blotting.

Discussion:

Transformation of tobacco was very efficient. LBA4404 harboring PKL2299 and plasmid PCBL101_RUBY demonstrated the effectiveness of its use as a visual marker for testing transformation. Within 3-5 days, RUBY was visibly expressed in leaf cuttings to the naked eye, which is faster than other reporter genes such as dsRed and GFP and more convenient, as there is no need for equipment to view. However, one problem with the plasmid construct of PCBL101-RUBY is that is uses the constitutive CaMV35s promoter, which causes the gene to always be expressed. This has led to slower growth in transformed plants compared to the control plants. One other potential reason for a slower growth is the fragility of *N. benthamiana* as similar experiments conducted in our lab using *N. tobaccum* had faster growth of transgenic plants. This could be because *N. benthamiana* has a higher metabolite production but a lower yield (Drapal et al., 2021). With the ease of expression of RUBY in tobacco, but at the cost of potential toxicity through over production and accumulation of betalain in leaves causing stunted or retarded growth (Wang et al., 2023). Similar results have been shown in maize, where expression of RUBY can vary in individual plants, sometimes resulting in stunted growth (Lee et al., 2023). With this in mind, continued use of RUBY as a marker for transformation event experiments is recommended, and with a switch to a promoter tied to a stress

The results of transformed embryos developing into full plants is indicative of a low transformation rate at this time, but the use of the ternary plasmid PKL2299 improved the efficiency of generating shoots from greening callus. Future use of a different selection pressure such as G418 would decrease screening time and have less escapes. This would increase time efficiency if not transformation efficiency

Since the WAT1-like gene used in the transformation is undescribed in maize, it is possible that a deletion of this gene causes mutated and deformed growth in plantlets. However, since WAT1 in maize is 72% conserved from Arabidopsis, there is a chance that mutations in the gene will have similar disruptions in function (Reaume & Sokolowski, 2011).

From 2016 when the QuickCorn methodology was first described (Lowe et al., 2016) to the current day, many different papers use the QuickCorn method to transform maize. In the experiment conducted, there is a demonstrated ability that the protocol works for a maize transformation system. The recommended protocol in this thesis is a viable option to the known good QuickCorn protocol (Masters et al., 2020). A modified QuickCorn protocol, with modified co-cultivation media, and using multiple selection rounds of our CIM media, yielded transgenic plants. This suggests that using the QuickCorn protocol is a good baseline to compare the new protocol against. Since the media using L-cysteine and DTT demonstrated an equal, or better number of transformation events, it is a viable option for the new protocol. While further testing is needed for every line of maize used in future experiments, there is the indication that the media and protocol is relatively future-proof and can be easily modified for different selection pressures and hormone concentrations.

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Figures

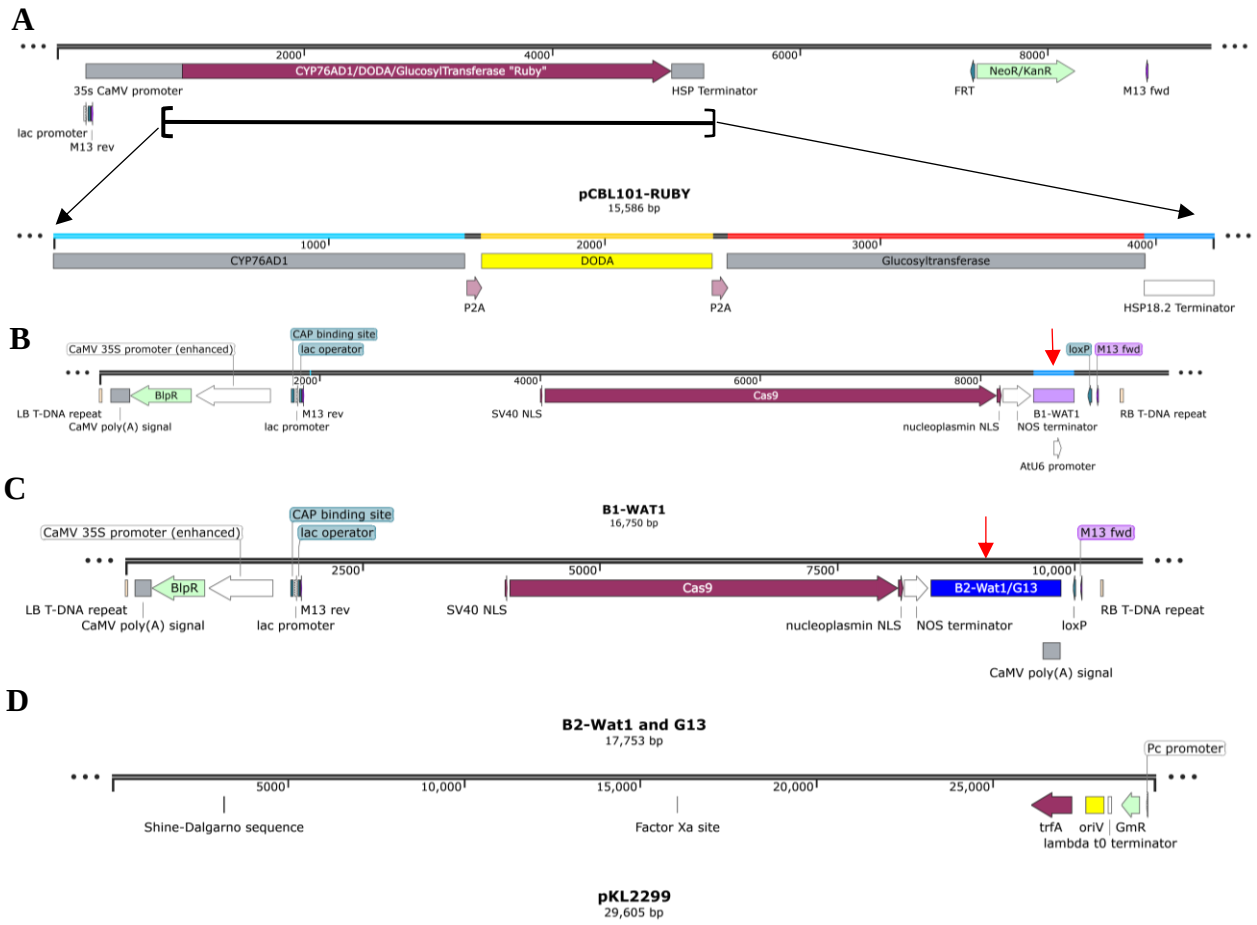

Figure 8: Constructs

Constructs designed for the transformation experiments include: plasmid pCBL101_RUBY (**A**) which encodes a betalain biosynthetic pathway (He et al., 2020), single gene deletion construct PYLCRISPR/Cas9_WAT1_Pubi-B (**B**) to knock out *WAT1* and a double gene deletion construct PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B (**C**) to knock out *WAT1* and *Gl3*. Ternary vector construct helper plasmid PKL2299 (**D**) which only has *vir* genes.

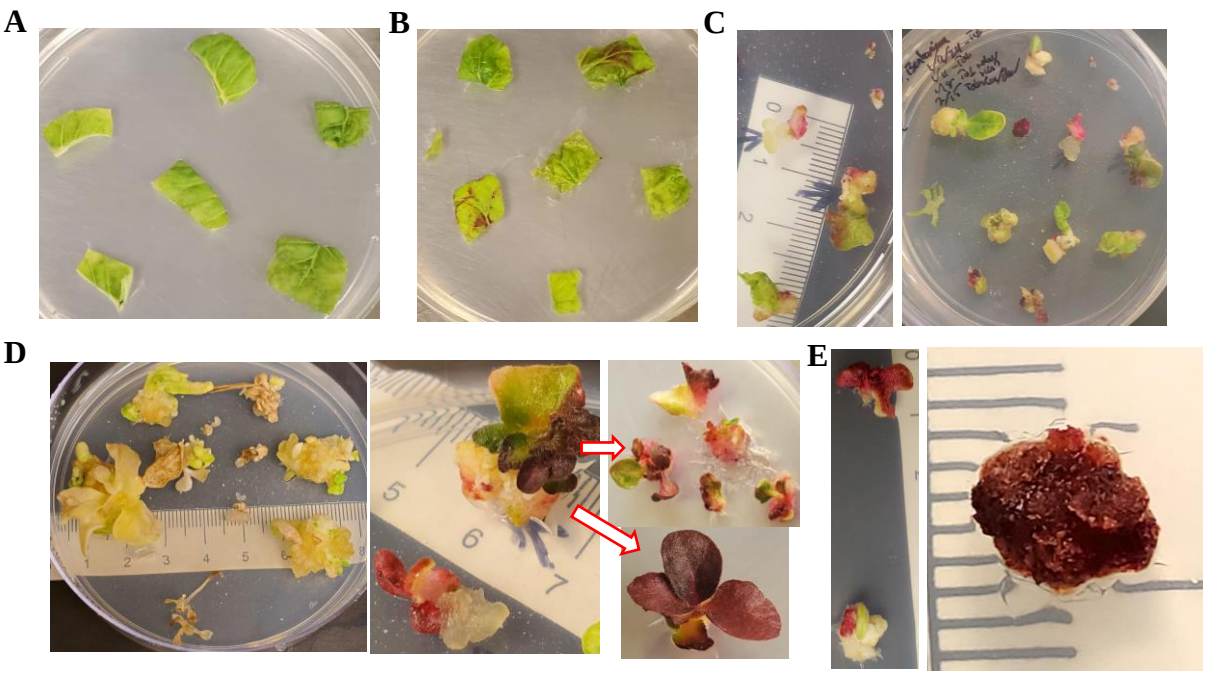

Figure 9 Tobacco Transformation

Tobacco transformation starts with leaf cuttings inoculated with the chosen agrobacterium harboring whichever construct, (**A**) for this figure, RUBY explants were used. Within 3 days, betalain had been expressed, even without callus formation (**B**). Callus formed demonstrated both transgenic and non-transgenic properties depending on media contact, and some started developing shoots(**C**). When excised from leaf tissue and placed on selection media, the non-transgenic plantlets started dying, while new growth could be seen on transgenic plantlets(**D**), but not all transgenic callus developed plantlets (**E**).

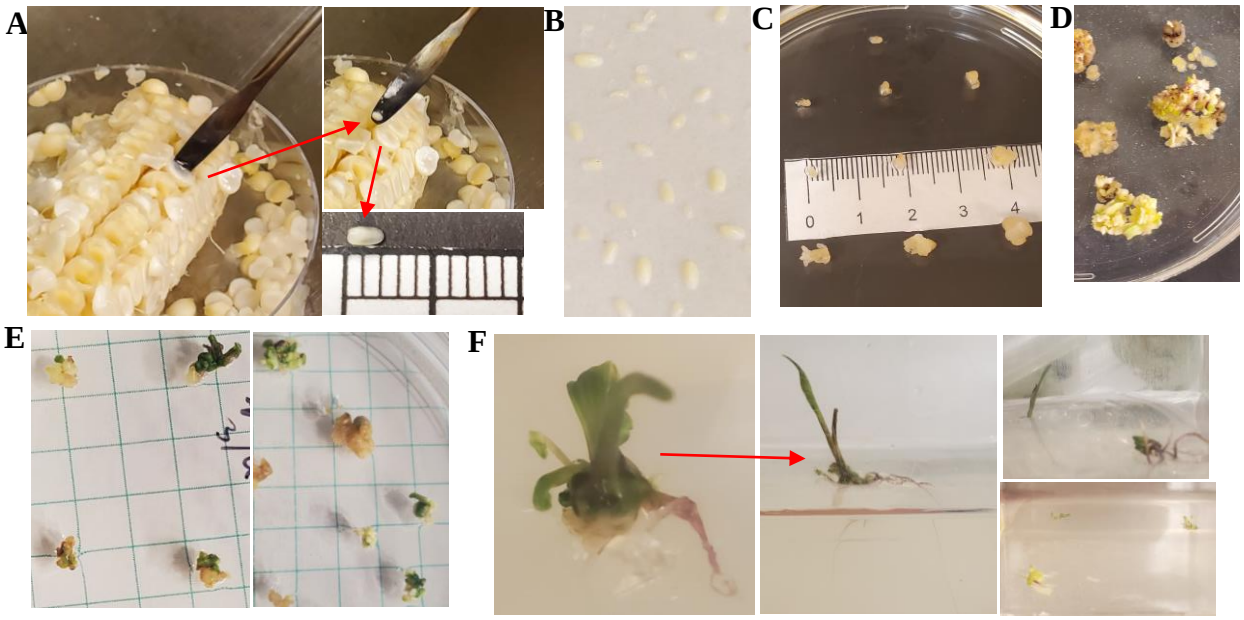

Figure 10 Maize Transformation

Maize Transformation was done by isolating the embryos 9-12 DAP, or when 1.5-2mm (**A**), then inoculating them and placing them scutellum side up on co-cultivation plates (**B**). After co-cultivation, they were moved to resting media for 10 days then onto selection media. During these two steps, callus starts to form (**C**). Once callus has developed into type II callus or starts to turn green, it is moved to regeneration media (**D**). On regeneration media, they are allowed to grow under light conditions until shoots develop (**E**) upon which the transgenic plantlets are moved to a rooting media (**F**).

Tables

Transformation stage and embryo count															
Variety	A188	A188	B104 wt	KSU 62	B104 wt	KSU 62	B104 wt	KSU 62	B104 wt	B104 wt	A188	B104 wt	KSU 62	B104 Wt	
date	14-Feb	21-Feb	3-Mar			8-May			6-May		8.9.23	8.9.23	8.9.23	8.9.23	
Media	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	PHI_	
Stage	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	dead	
Control	9	35	?	32	78	63	92	30	18	25	72	48		13	
Neg Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
B1-1	199	113	514	103	150	92	142	100	167	173	185	78	96	53	
PKL2299 B1															
B2-1	172	96	225	106	209	71	78	106	159	128	49	56	70	48	
PKL2299 B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
OE-GL3	-	-	-	-	-	-	-	-	197	207	-	-	-	62	
AGL-GL3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BBGG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
OE-WUS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
RUBY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Table 1 Table of all Maize Transformations Part 1

The first half of all immature embryo agrobacterium mediated maize transformations. No transgenic plants developed in this half of the experiment.

Transformation Stage and Embryo Count pt2													Total
Variety	KSU 62	A188	A188	A188	A188	KSU 62	KSU 62	KSU 17	KSU 17	KSU 17	KSU 17	KSU 17	-
date	30-Nov	8-Dec	16-Dec	17-Dec	18-Dec	21-Dec	23-Dec	23-Dec	11-Jan	17-Jan	18-Jan	3-Feb	-
Media	-	13329 A	C->Lcys + Dtt + aceto	562v Lcys	C->Lcys + Dtt + aceto	562v	562v + Lcys	562v	562v	562v +aceto paper	562v +aceto paper	562v +aceto paper	-
Stage	dead	regen	regen	regen	regen	regen	regen	regen	N/A	N/A	N/A	Regen	-
Control	40	48	33	22	25	67	35	34	96	86	91	49	1141
Neg Control	23	42	23	15	16	35	33	-	-	-	-	-	187
B1-1	61	154	77	88	-	-	-	-	-	-	-	-	2545
PKL2299 B1	**A**				91	185	**B** 224	62	-	-	-	38	600
B2-1	63	128	48	95					-	-	-		1907
PKL2299 B2	-	-	-	-	80	158	198	50	-	-	-	27	513
OE-GL3	54	-	-	-	-	-	-	-	-	-	-	-	520
AGL-GL3	62	130	112	-	-	-	-	-	-	-	-	-	304
BBGG	-	167	114	-	-	-	136	44	-	-	-	-	461
OE-WUS2	-	-	-	-	-	-	50		-	-	-	-	50
RUBY	-	-	-	-	-	-	-	-	170	165	107	77	519
	C	3.91%				**D**	1.34%					**E**	8747

Table 2: Table of all maize transformations part 2

This table is the second half of the experiment, where different medias were used during the co-cultivation stage. As indicated by the red outlined boxed in the highlighted sections (**A, B**), potential transgenic callus was formed from these steps. Transformation efficiency as a percentage is low, at almost 4% (5 callus) and 1.34% (3 callus) respectively (**C, D**). the total number of embryos transformed (**E**) is over 8000, so a total transformation efficiency is 0.09%.

Appendix A - Media compositions

Appendix Table A.1Transformation media compositions

-	Transformation Media Composition											
-	Inoculation media			Co-Cultivation				Resting/Selection				
-	Quick Corn	Zhao et al/Tej	Novel	QuickCorn	Zhao et al/Tej	Novel	QuickCorn	Zhao et al/Tej	Novel			
-	700A	PhiA	TIM	562V	PhiB	CCM	605T	PhiC/PhiD	CIM			
MS Basal Medium	4.4g/l	-	-	-	-	-	4.3g/l	-	-			
Chu N6 salt with vitamins	-	3.99g/l	3.99g/l	4g/l	3.99g/l	3.99g/l	-	3.99g/l	3.99g/l			
Sucrose	68.5g/l	65.8g/l	68.5g/l	30g/l	30g/l	30g/l	20g/l	30g/l	30g/l			
Glucose	36g/l	36g/l	36g/l	-	-	-	0.6g/l	-	-			
Thiamine HCL	-	12.5ml (40mg/l)	12.5ml (40mg/l)	-	12.5ml (40mg/l)	12.5ml (40mg/l)	0.2mg/l	12.5ml (40mg/l)	12.5ml (40mg/l)			
2,4-D	1.5mg/l	7.5ml (2mg/10ml)	7.5ml (2mg/10ml)	-	10ml (2mg/10ml)	7.5ml (2mg/10ml)	400µl (2mg/ml)	7.5ml (2mg/10ml)	7.5ml (2mg/10ml)			
Acetosyringone	-	-	-	100µM final conc	1ml/l (100mM)	1ml /l (100mM)	-	-	-			
Silver nitrate (AgNO3)	-	-	-	1mg/l	10µl/l (850mg/10ml)	1ml/ l (3.4 mg/ml)	-	10µl/l (850mg/10ml)	1ml/l (3.4 mg/ml)			
Proline	-	2.303g/l	-	-	2.303g/l	0.9g/l	2g/l	2.303g/l	0.9g/l			
Casein	-	0.2g/l	-	-	0.2g/l	0.25g/l	0.3g/l	0.2g/l	0.25g/l			
MES	-	-	-	-	0.5g/l	0.50g/l	-	0.5g/l	0.50g/l			
Myo-Inositol	-	-	-	-	-	0.10g/l	-	-	0.10g/l			
MS vitamins	-	-	-	-	-	-	-	-	-			
B5 vitamins	-	-	-	-	-	1ml/l (1000x)	-	-	1ml/l (1000x)			
Zeatin	-	-	-	-	-	-	-	-	-			
BAP	-	-	-	-	-	-	-	-	-			
Thidiazuron (1mg/ml stock)	-	-	-	-	-	-	-	-	-			
Glycine (filter sterilized)	-	-	-	-	-	-	-	-	-			
Clavamox/antibiotic	-	-	-	-	-	-	250mg/l	250mg/l	250mg/l			
Selection	-	-	-	-	-	-	Yes, after resting	Yes, after resting	Yes, after resting			
Tissue Culture Agar	-	-	-	8mg/l	-	8mg/l	-	8mg/l	8mg/l			
phytagel	-	-	-	-	-	-	2.5g/l	-	-			
gelrite	-	-	-	-	3g/l	-	-	-	-			
Cysteine	-	-	-	-	0.4g/l	300mg/l	-	-	-			
DTT	-	-	-	-	-	150mg/l	-	-	-			
N6 Macro Salts (0.6x)	-	-	-	-	-	-	60ml/l	-	-			
B5 Micro Salts (0.6x)	-	-	-	-	-	-	0.6ml/l	-	-			
Eriksson's Vitamins (0.4x)	-	-	-	-	-	-	0.4ml/l	-	-			
S&H Vitamins (0.6x)	-	-	-	-	-	-	0.6g/l	-	-			
Ferrous Sodium stock (0.6x)	-	-	-	-	-	-	6ml/l	-	-			
KNO3	-	-	-	-	-	-	1.68g/l	-	-			
Dicamba (1mg/ml stock)	-	-	-	-	-	-	1mg/l	-	-			
pH	5.2	5.8	5.2	5.8	5.8	5.8	5.8	5.8	5.8			

-	Transformation Media Composition					
-	Regeneration			Rooting		
-	Quick Corn	Zhao et al/Tej	Novel	Quick Corn	Zhao et al/Tej	Novel
-	13329A	Phi E	TRM	13158	PhiE	RootM
MS Basal Medium	4.4g/l	4.43g/l	4.43g/l	4.4g/l	4.43g/l	4.4g/l
Chu N6 salt with vitamins	-	-	-	-	-	-
Sucrose	60g/l	60g/l	60g/l	40g/l	60g/l	40g/l
Glucose	-	-	-	-	-	-
Thiamine HCL	-	-	-	-	-	-
2,4-D	-	-	-	-	-	-
Acetosyringone	-	-	-	-	-	-
Silver nitrate (AgNO3)	-	-	-	-	-	-
Proline	-	0.5g/l	0.5g/l	-	0.5g/l	-
Casein	-	0.2g/l	0.2g/l	-	0.2g/l	-
MES	-	-	-	-	-	-
Myo-Inositol	-	-	-	-	-	-
MS vitamins	-	1ml (1000X)	1ml (1000X)	-	1ml (1000X)	-
B5 vitamins	-	-	-	-	-	-
Zeatin	0.5mg/l	20ml (25mg/100ml)	0.5mg/l	-	20ml (25mg/100ml)	-
BAP	1mg/l	-	1mg/l	-	-	-
Thidiazuron (1mg/ml stock)	1ml/l	-	-	-	-	-
Glycine (filter sterilized)		100µl/l (20mg/ml)	100µl/l (20mg/ml)	-	100µl/l (20mg/ml)	-
Clavamox/antibiotic	250mg/l	250mg/l	250mg/l	250mg/l	250mg/l	250mg/l
Selection	Yes	Yes	Yes	Yes	Yes	Yes
Tissue Culture Agar	-	-	-	-	-	-
phytagel	2.5g/l	-	-	2.5g/l	-	2.5g/l
gelrite	-	3g/l	3g/l		3g/l	-
Cysteine	-	-	-	-	-	-
DTT	-	-	-	-	-	-
N6 Macro Salts (0.6x)	-	-	-	-	-	-
B5 Micro Salts (0.6x)	-	-	-	-	-	-
Eriksson's Vitamins (0.4x)	-	-	-	-	-	-
S&H Vitamins (0.6x)	-	-	-	-	-	-
Ferrous Sodium stock (0.6x)	-	-	-	-	-	-
KNO3	-	-	-	-	-	-
Dicamba (1mg/ml stock)	-	-	-	-	-	-
pH	5.8	5.6	5.8	5.8	5.6	5.6

Appendix B - Transformation protocol

Park lab Maize Transformation Protocol- Agrobacterium-mediated

Nathan Stewart, 2024

Transformed maize in 2.5-4 months depending on selection agent

- **Maize**
    - Grow maize- 9-14 DAP harvest immature embryos
- **Agrobacterium**
    - Streak transformed agrobacterium onto plate with selection about 1.5 weeks prior to transformation.
        - After 2-3 days take a colony and grow in liquid with selection for about 2 days
        - Streak again on selection plate overnight (doesn't need to be individual colonies)
        - Take loop/scoop of agrobacterium growth off of the plate and grow for 1 hour in inoculation media for the transformation (OD550= 0.5-0.75).
            - **(Optional)** add acetosyringone to this step as well as to the embryos.
- **Transformation**

Infection

- The infection process involves sterilization of maize ear (9-14 DAP, usually 12 DAP) by:
    - Washing ears with a 5% soap to water mixture for 10 minutes
    - Submerge in 50% bleach with few (3-5) drops of 1% of Tween 20 for 20-30 minutes.
    - Follow by washing ears using sterilized Distilled or double distilled water for at least three times for 5 minutes each.
    - After final rinse, keep in DH20 until embryo isolation
- Isolate embryos in an aseptic environment by:
    - Hold cob by the top (pointy) end
    - Using a scalpel to cut off the top $1/3^{rd}$ -$2/3^{rd}$ of the kernels. There is a risk of damaging embryos if cut more than this, or if embryos are larger than than ideal 1.5 – 2.5 mm in size
    - Use scoop to remove embryo by gently inserting on the right side of the kernel and rotating counter clockwise while gently 'popping' the kernel meat out. The embryo should be on the scoop, or on the popped out flesh.

- Place immature embryos in 2 ml tubes filled with TIM media + 2.5µl of 200 mM acetosyringone
    - Prior to infection, spin embryos at max RPM for 15-30 seconds
    - (OPTIONAL) Prior to infection with Agrobacterium, the embryos are heat treated at 46°C for three minutes followed by short spin down
- Immature embryos should be infected with Agrobacterium within 1 hour of isolation to increase efficiency.
- Add Agrobacterium (OD550= 0.5-0.75) to the embryos for 10-15 minutes. Keep dark and mix embryos by rotating tubes upside down 2-3 times every 5 minutes.
- Pour embryos and liquid onto co-cultivation media, using a scoop to remove any remaining embryos from the tube. Let sit for 30 seconds to 2 minutes. Then remove any excess liquid from the plates with a pipette.
- Use a scoop, scalpel, or forceps to make sure the embryos are **scutellum side up** (flat side against media, curved side up).

Co-Cultivation

- Co-cultivate for 48-72 hours in dark conditions at 20- 26°C.

Resting

- Rest for 7 to 10 days.
- Trim any developing shoots/roots that may appear during this time.

Selection

- Do at least 2-3 rounds of selection depending on the selection agent. Observation should be made very often and transgenic calli tend to divide rapidly, have a whitish coloration, and are friable (break/come apart easily).
- Increase selection pressure every 2-3 weeks (2 weeks is optimal)

Regeneration

- Actively dividing calli are transferred to regeneration media. (Optional: keep a section of actively dividing tissue in CIM media as a backup.)

Rooting

- When Calli have greened and started to shoot, move to a rooting medium containing selection pressure
- The start of rooting should be done on 20x100 petri-dishes

- Once roots have formed, move to magenta boxes with the same rooting medium and selection pressure

Growing

- Upon growing 2-3 true leaves, or reaching the top of the magenta box, remove the plantlet from the media, wash roots of media gently but thoroughly.
- Plant the plantlet to potting media (sterilized) and place in a high humidity environment (minimum of 70% humidity) it is recommended to place in a sealable, clear plastic bag and fully seal for a minimum of 3 days. Once the plantlet has recovered from transplanting, slowly open the bag to harden the plant to normal humidity levels. If in a humidity controlled growth chamber, you can slowly decrease the levels until levels reach greenhouse humidity levels.

Media component preparation

Preparing Acetosyringone

Acetosyringone should be prepared and added to media when it is used. Acetosyringone needs to be freshly prepared. Else, store it at -20 °C.

Prepare 5 ml of Acetosyringone (3',5'-demethoxy-4'-hydroxyacetophenone) in DMSO (Dimethyl sulfoxide). A 5-10 seconds vortex should dissolve. Filter sterilize and store in sterile 1.5 ml microcentrifuge tubes. 5 ml is aliquoted to almost 4 tubes with each containing more than 1 ml of acetosyringone. To make 100 mM of Acetosyringone, Dissolve 0.0981 grams of acetosyringone in 5 ml DMSO. For a litre of CCM media, add 1 ml of 100 mM Acetosyringone to make final concentration of 100uM. Storing acetosyringone after filter sterilize is **always -20°C**.

Preparing Silver nitrate stock

Dissolve 34 mg in 10 ml of sterile Double distilled water. This will give a 3.4mg/ml stock.

Dissolve by vortexing and Filter sterilize into 1.5 ml microcentrifuge tubes with each tube containing more than 1 ml**. Protect from light**. For 1 litre media, Add 1 ml of silver nitrate to media after cooling of the media (which means media is already autoclaved).

Selection agent

I usually make a stock of bialaphos (10 mg/ml) and filter sterilize. Store at -20°C. From stock add, 200 µl to make final concentration 2mg/l, increase for each selection round.

Adjust selection agent for future constructs. Possible better agents for quicker turnaround are G418 sulfide or Hygromycin.

Media recipes

Preparation of Transformation Inoculation media (TIM, Liquid media)

	For 1L	For 250 ml
1.	Add 800 ml in 1 litre autoclavable bottle. Add following:	
2.	N6 salt with vitamin ---------- 3.99 grams	0.997 grams
3.	Sucrose ---------- 68.5 grams	17.125 g
4.	Glucose ---------- 36 grams	9 g
5.	Thiamine HCl (40 mg/l stock) ---------- 12.5 ml	3.125 ml
6.	2,4-D (2 mg/10 ml stock) ---------- 7.5 ml	1.875 ml

Dissolve by using magnetic stirrer and make a final volume of 1000 ml by adding water

7. Maintain pH to 5.2 (Use 1 M KOH)

Autoclave and keep it at 4 °C for future use.

Preparation of Co-Cultivation media (1 Liter) (CCM media: solid media)

1. Add 800 ml in 1 Liter autoclavable bottle
2. N6 salt with vitamins …..... 3.99 g/L
3. Sucrose …..... 30 g/L
4. Proline …..... 0.90 g/L
5. Myo-inositol..... 0.10 g/L
6. MES …..... 0.50 g/L
7. Casein …..... 0.25 g/L
8. 2, 4-D (2mg/10ml stock) ..... 7.5 ml/L
9. Thiamine HCL (40mg/L stock) ..... 12.5 ml/L

Once everything dissolved bring to final volume (1000ml)

10. Adjust the pH to 5.8 with KOH
11. TC agar 8 g/L

Autoclave media and let the media to cool to 60°C

12. After cooling add sterile (1000X) B5 vitamins …...1 ml
13. Silver nitrate (3.4 mg/ml) ..... 10 µl
14. Acetosyringone (100mM) ……. 1 ml
15. DTT……..300mg
16. L-Cysteine……..150mg

Pour media into 100X15 petridish

Preparation of callus induction media (1 Liter) (CIM media: solid media)

1. Add 800 ml in 1 Liter autoclavable bottle
2. N6 salt with vitamins …..... 3.99 g/L
3. Sucrose …..... 30 g/L
4. Proline …..... 0.90 g/L
5. Myo-inositol..... 0.10 g/L
6. MES …..... 0.50 g/L
7. Casein …..... 0.25 g/L
8. 2, 4-D (2mg/10ml stock) ..... 7.5 ml/L
9. Thiamine HCL (40mg/L stock) ..... 12.5 ml/L

Once everything dissolved bring to final volume (1000ml)

10. Adjust the pH to 5.8 with KOH
11. TC agar 8 g/LAutoclave media and let the media to cool to 60°C
12. After cooling add sterile (1000X) B5 vitamins …...1 ml
13. Silver nitrate (3.4 mg/ml) ..... 1 ml
14. Clavamox (to kill excess Agro): 250 mg (2 tablets; 1 tablet is usually 125 mg; check label)
15. **Selection marker**: used Bialaphos- 2 to 5mg/l- increase each round of selection

Pour media into 100X15 petridish

Preparation of Transformation Regeneration Media 1 (1 litre) (TRM media: solid media)

1. Add 800 ml in 1 litre autoclavable bottle.

Add following:

2. MS salt with vitamin ---------- 4.43 grams
3. Sucrose ---------- 60 grams
4. Proline ---------- 0.5 grams
5. Casein ---------- 0.2 grams

Dissolve by using magnetic stirrer and make a final volume of 1000 ml by adding water
Maintain pH to 5.6 (Use 1 M KOH)
Add 3 grams of Gelrite
Autoclave and cool down to 60°C. After cooling, Add following:

6. Zeatin ----------0.5mg/l
7. 1 ml sterile MS vitamin (1000X)
8. Filter-sterilized Glycine (20 mg/ml): 100 µl
9. BAP, 1 mg/l (phytotech labs)
10. Clavamox : 250 mg (2 tablets; 1 tablet is usually 125 mg; check label)
11. **Selection marker**: use bialaphos 3 mg/ litre

Pour media into 100X20 petridish.

Preparation of Rooting media (1 litre) (RootM: solid media)
1. Add 800 ml in 1 litre autoclavable bottle.
Add following:
2. MS salt with vitamin ---------- 4.43 grams
3. Sucrose ---------- 40 grams
Dissolve by using magnetic stirrer and make a final volume of 1000 ml by adding water
Maintain pH to 5.6 (Use 1 M NaOH or HCL)
Add 2.5 grams of phytagel
Autoclave and cool down to 60°C. After cooling, Add following:
4. Clavamox : 250 mg (2 tablets; 1 tablet is usually 125 mg; check label)
5. **Selectable marker**: used bialaphos 3 mg/ litre Pour media into 100X20 petridish.

Appendix C - ANOVA data

Appendix Table C.1 Selection ANOVA data

Dunnett's multiple comparisons test	**Below threshold?**	**Summary**	**Adjusted P Value**	**-**	**Dunnett's multiple comparisons test**	**Below threshold?**	**Summary**	**Adjusted P Value**
-	-	-	-	-	-	-	-	-
Week 0				-	**Week 0**			
G418 vs. Kanamycin	No	ns	>0.9999	-	Control vs. G418	No	ns	>0.9999
G418 vs. Hygromycin	No	ns	>0.9999	-	Control vs. Kanamycin	No	ns	>0.9999
G418 vs. Bialaphos	No	ns	>0.9999	-	Control vs. Hygromycin	No	ns	>0.9999
G418 vs. PTT	No	ns	>0.9999	-	Control vs. Bialaphos	No	ns	>0.9999
G418 vs. Control	No	ns	>0.9999	-	Control vs. PTT	No	ns	>0.9999
-	-	-	-	-	-	-	-	-
Week 1				-	**Week 1**			
G418 vs. Kanamycin	No	ns	>0.9999	-	Control vs. G418	No	ns	>0.9999
G418 vs. Hygromycin	No	ns	>0.9999	-	Control vs. Kanamycin	No	ns	>0.9999

G418 vs. Bialaphos	No	ns	>0.9999	-	Control vs. Hygromycin	No	ns	>0.9999
G418 vs. PTT	No	ns	>0.9999	-	Control vs. Bialaphos	No	ns	>0.9999
G418 vs. Control	No	ns	>0.9999	-	Control vs. PTT	No	ns	>0.9999
-	-	-	-	-	-	-	-	-
Week 2				-	**Week 2**			
G418 vs. Kanamycin	Yes	**	0.0068	-	Control vs. G418	Yes	****	<0.0001
G418 vs. Hygromycin	Yes	**	0.0068	-	Control vs. Kanamycin	No	ns	0.4837
G418 vs. Bialaphos	Yes	**	0.0015	-	Control vs. Hygromycin	No	ns	0.4837
G418 vs. PTT	Yes	***	0.0003	-	Control vs. Bialaphos	No	ns	0.8106
G418 vs. Control	Yes	****	<0.0001	-	Control vs. PTT	No	ns	0.9867
-	-	-	-	-	-	-	-	-
Week 3				-	**Week 3**			
G418 vs. Kanamycin	Yes	****	<0.0001	-	Control vs. G418	Yes	****	<0.0001
G418 vs. Hygromycin	Yes	*	0.0258	-	Control vs. Kanamycin	Yes	****	<0.0001

G418 vs. Bialaphos	Yes	****	<0.0001	-	Control vs. Hygromycin	Yes	****	<0.0001
G418 vs. PTT	Yes	****	<0.0001	-	Control vs. Bialaphos	No	ns	0.2225
G418 vs. Control	Yes	****	<0.0001	-	Control vs. PTT	No	ns	0.0831
-	-	-	-	-	-	-	-	-
Week 4				-	**Week 4**			
G418 vs. Kanamycin	No	ns	0.8106	-	Control vs. G418	Yes	****	<0.0001
G418 vs. Hygromycin	No	ns	0.8106	-	Control vs. Kanamycin	Yes	****	<0.0001
G418 vs. Bialaphos	Yes	****	<0.0001	-	Control vs. Hygromycin	Yes	****	<0.0001
G418 vs. PTT	Yes	****	<0.0001	-	Control vs. Bialaphos	Yes	*	0.0258
G418 vs. Control	Yes	****	<0.0001	-	Control vs. PTT	Yes	***	0.0003
-	-	-	-	-	-	-	-	-
Week 5				-	**Week 5**			
G418 vs. Kanamycin	No	ns	0.4837	-	Control vs. G418	Yes	****	<0.0001
G418 vs. Hygromycin	No	ns	0.4837	-	Control vs. Kanamycin	Yes	****	<0.0001

G418 vs. Bialaphos	Yes	****	<0.0001	-	Control vs. Hygromycin	Yes	****	<0.0001
G418 vs. PTT	Yes	****	<0.0001	-	Control vs. Bialaphos	No	ns	0.9867
G418 vs. Control	Yes	****	<0.0001	-	Control vs. PTT	No	ns	0.2225

Appendix D - DNA Data

Appendix Data I: pCBL101-RUBY

AddGene # 199723, (Lee et al., 2023)

GTTTACCCGCCAATATATCCTGTCAAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGATCCAAGCTCAAGCTCCAA
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Appendix Data II: Plasmid PYLCRISPR/Cas9_WAT1_Pubi-B

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Appendix Data III: Plasmid PYLCRISPR/Cas9_WAT1and Gl3_Pubi-B

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Appendix Data IV: Helper/Ternary plasmid PKL2299

AddGene #186332, GenBank ID DQ058764.1 (Kang et al., 2022)

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