Establishing *Zingeria biebersteiniana* (n=2) as a Model System for Plant Biology Research

by

Ryan Patrick Koeth

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved July 2024 by the
Graduate Supervisory Committee:

Changbin Chen, Chair
Tania Hernández
Kathleen Pigg

ARIZONA STATE UNIVERSITY

August 2024

ABSTRACT

Zingeria biebersteiniana (Claus) P.A. Smim is a grass species known for having 2n=2x=4, a trait shared with only four other angiosperms *Haplopappus gracilis* (Nutt.) A. Gray, *Brachycome lineariloba* (DC) Duce, *Brachycome dichromosomatica* C.R. Carter, and *Colpodium versicolor* (Stev.) Woronow. It holds potential as a unique prospect for work as a model species for different cereal studies and as a forgiving gateway to the field of cytogenetics. However, *Z. biebersteiniana* remains an understudied species. In the current study, I have compiled an overview of known collection sites, a description of morphology, and a discussion of past and current cytogenetic work to better document its potential as a model organism. In addition to its unique karyotype, *Z. biebersteiniana* has been demonstrated to undergo callus induction and somatic organogenesis. A silica body analysis was conducted on *Z. biebersteiniana* with regenerated plants derived from tissue culture displaying silica body formation in mature leaf tissue as plants acclimate to soil. This may support the introduction of a silicon nutrient to media, during culture, to promote the health of plants and alleviate transfer shock. In this study, I have developed a protocol demonstrating a pathway for transformation mediated by *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942. In this construct, green fluorescent protein is fused to the F-actin binding domain of mouse talin to label the actin cytoskeleton. This facilitates easier visualization of actin microfilaments under fluorescent light without any need for fixatives or permeabilization agents. Due to the nature of the construct and its reliance on actin microfilament activity, visualization of microfilaments may be limited to a short window of time when observations must be made. After I genotyped *Z. biebersteiniana* using PCR, I analyzed its pollen but failed to

identify the desired phenotype. However, a single novel phenotype was identified but it lacked the typical characteristics associated with the construct. The seeds harvested from the newly transformed line can foster future generations for study but more importantly, their conception has demonstrated a successful pathway for transformation in *Z. biebersteiniana*.

ACKNOWLEDGMENTS

I'd like to acknowledge my advisor Dr. Chen who has helped mentor and motivate me to continue to improve my skills as a researcher. If it wasn't for him, I don't know if I would have the same confidence I do in a lab setting. To Dr. Hernández you have taught me to slow down, appreciate a thought-out effort, and focus on a direct path to the goals ahead. Dr. Pigg has been foundational in my way of thinking when it comes to plants and their evolution and has impacted my confidence when holding professional discussions and their various topics.

TABLE OF CONTENTS

Page

LIST OF FIGURES vi

CHAPTER

1 THE BIOLOGY OF *ZINGERIA BIEBERSTEINIANA* 1

1.1 Introduction 1

1.2 Distribution 2

1.3 Morphology 4

1.4 Cytogenetics 4

1.5 Current and Recent Work 6

2 *IN VITRO* REGENERATION 7

2.1 Introduction 7

2.2 Materials and Methods 8

2.3 Results and Discussion 13

3 SILICON NUTRIENT AND SILICA DEVELOPMENT 19

3.1 Introduction 19

3.2 Materials and Methods 19

3.3 Results and Discussion 22

4 ESTABLISHMENT OF AN *AGROBACTERIUM TUMEFACIENS* TRANSFORMATION IN *ZINGERIA BIEBERSTEINIANA* 25

4.1 Introduction 25

4.2 Materials and Discussion 26

4.3 Results and Discussion 35

CHAPTER Page

REFERENCES 39

LIST OF FIGURES

Figure Page

1. *Zingeria biebersteiniana* and its Distribution 3
2. Callus Induction and Regeneration Progression using Modified Media.....11
3. Abnormal Shoot Development in Media Containing 6-BAP 15
4. Callus Grown in Modified Media 17
5. Result of Dry Ashing Protocol on Leaf Tissue 21
6. Silica Body Development 23
7. Callus Coculture with *Agrobacterium tumefaciens* and Selection Medias 28
8. DNA Extraction and PCR Genotyping 32
9. Anther Size Comparison and Preparation Before the Introduction of PBS 34
10. Transformed Pollen 37

CHAPTER 1

THE BIOLOGY OF *ZINGERIA BIEBERSTEINIANA*

1.1 Introduction

The grass family Poaceae has been integral to our success as a species. From prehistoric evidence found with Ötzi the Iceman to feeding a nation to even the rice found in gas station sushi, the members of Poaceae are a fundamental part of our day-to-day experience. Although some members of Poaceae, particularly grains have had a profound influence on our lives, there are other, lesser-known taxa that offer unexplored potential.

One such member of Poaceae is *Z. biebersteiniana* (Fig. 1A). This relatively benign grass shares a rare trait with only one other grass, *Colpodium veriscolor*. Both contain a chromosome count of 2 (2n=2x=4). (Bennett et al., 1986). These plants share a close relationship with *Catabrosella araratica* a species with a chromosome count of 2n=6x=42. This count may indicate that a two-chromosome grass species is relatively young, having evolved within the last several million years as the result of multiple tandem chromosome fusions. This hypothesis is supported by tracing the internal sites of TTTAGGG telomeric sequences (Kim et al., 2009). An additional desirable trait of Z. *biebersteiniana* chromosomes is the ease of identification through their distinctive morphology, and large size at 4-6 µm in length at miotic metaphase (Bennett et al., 1986).

In this chapter, we will focus on *Z. biebersteiniana* to better understand its previous and current status as a model organism and its potential for further development. Here I provide an overview of its natural distribution based on herbarium records and

records of natural ranges, a discussion of its morphology and previous cytogenetic work, and, lastly, a brief overview of current work being done with the species.

1.2 Distribution

Zingeria biebersteiniana's natural habitat can be roughly identified as endemic to central Eurasia (Fig. 1) falling within Russia, Georgia, Iran, Iraq, Jordan, Lebanon, Kazakhstan, Syria, Ukraine, Armenia, Turkey, Israel, and Palestine (Salih, 2013; Plants of the World (POWO),2024; Euro+Med, 2024). This range on the basis of geopolitical boundaries should be taken with some caution, and as such, data was collected with the help of multiple herbarium specimens and the inclusion of two iNaturalist submissions (iNaturalist, 2019). The known collection sites based on the country are 32 specimens collected in Russia, 4 in Turkey, 4 in Lebanon, 2 in Ukraine, 1 in Armenia, and 1 in Iran. The KEW distribution includes Eastern Europe, Caucus, and Western Asia as a supplementary distribution (POWO, 2024).

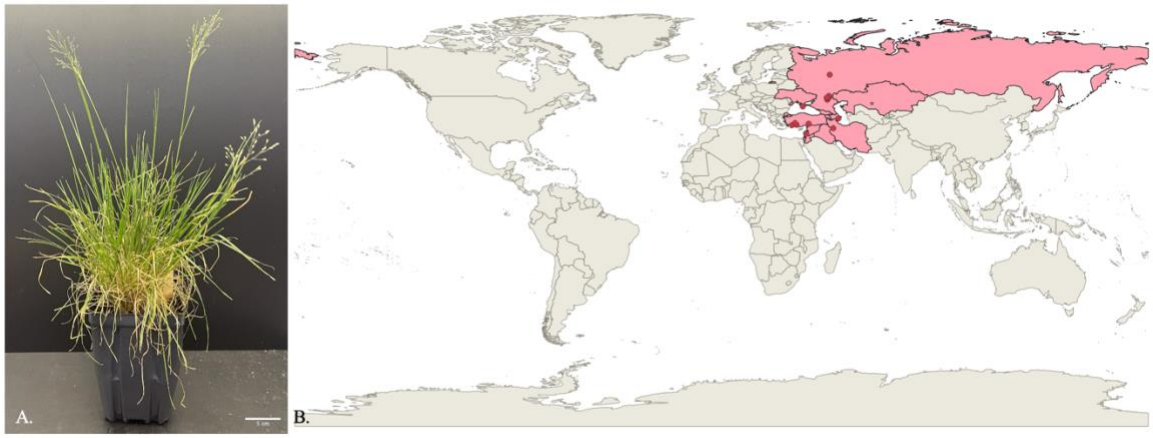

Figure 1: *Zingeria* biebersteiniana and its distribution. A.) *Zingeria biebersteiniana* B.) *Zingeria biebersteiniana global distribution* (Bar = 5cm) . Red nodes represent known collection sites based on herbarium specimens and iNaturalist. Pink countries represent the known geographic ranges.

1.3 Morphology

Zingeria biebersteiniana is an annual, often found in wetlands and rocky areas. Its erect culms are 3-nodded and can extend 8-38cm (Fig. 1A) (Clayton et al., 2006). Leaf blades are flat with an acuminate apex; total leaf length is 0.8-7cm with a width of 0.4-2mm (Clayton et al., 2006). The panicle branches with pedicles 5-13mm in size and glumes lack hyaline margins (Davis, 1985). Spikelets are 1.3 – 2.0 mm in size, dorsally compressed, and comprised of 1 fertile floret lacking a rhachilla extension, The androecium consists of 3 anthers 0.5-0.7 mm long and the fruit is a caryopsis with the seed coat fused to the pericarp, and an embryo comprising 20% of the caryopsis length (Clayton et al., 2006).

1.4 Cytogenetics

Zingeria biebersteiniana has been the subject of cytogenetic work since one of its first karyotype studies conducted by M.D. Bennett and colleagues in 1986. These authors were able to assess *Z. biebersteiniana* chromosomes through root tip squashes and proceeded to describe a nuclear organizing region (NOR) found on chromosome 1 near the centromere (Bennett et al., 1986). With cells that had the maximum number of secondary constrictions, the two chromosome pairs along with the arms of the NOR-bearing chromosome were distinguishable (Bennett et al., 1986). They went on to identify heterochromatin blocks that made up an estimated 28% of the haploid complement. With the use of C-banding, in chromosome 1, pericentromeric heterochromatin was observed to divide unequally, resulting in a heterochromatic segment near the centromere on the long arm that is half as large as the proximal segment

of the short arm. Additionally, chromosome 1 also contained a clear C-band at the short arm telomere, in contrast to chromosome 2 which showed a clear C-band near the short arm telomere (Bennett et al., 1986).

Later S.T. Bennett and colleagues used DAPI or Q-banding with DAPI or Q to aid in the ability to distinguish chromosomes through their fluorescent properties (Bennett et al., 1995). They recommend the use of DAPI or H C-banding which allows for the identification of each chromosome through all miotic stages.

On the hypothesis made by M.D. Bennett et al. (1986), Saunders and Houben (2001) suggested that the two chromosomes comprise at least two or more ancestral chromosomes. However, for this result additional structural events to the genome would have had to occur on the basis of large monocentromeric heterochromatin blocks and the inactivating or combining of original centromeric sites (Saunders & Houben, 2001). They based this hypothesis on their work using FISH analysis to observe the sequences Zbcen1, Zb46, and Zb47A. They identified pericentromeric heterochromatin composed of Zbcen1 tandem repeat family intermingled with copia-like retrotransposon sequences (Saunders & Houben, 2001).

Raskina et al. (2008) later demonstrated that 45s rDNA and tandem telomere sequence TTTAGGG showed small telomeric clusters on the 45s block on chromosome 1. This pattern is aligned with Robertsonian rearrangements, an abnormality where two long arms of a chromosome fuse. The condensed fraction of chromatin found in *Z. biebersteiniana* was 46.4% (Cremonini et al., 2003). Cremonini et al. (2003) noted that highly methylated sites are not always relative to marked C-banding and Q-banding sites

They suggested that this may show evolutionary significance to epigenetic alterations to DNA sequences at specific genomic localities (Cremonini et al., 2003).

1.5 Current and Recent Work

As of 2020 *Z. biebersteiniana* was reassigned taxonomically and is now recognized as *Colpodium biebersteinianum* (Claus) Röser & Tkach. This change was made after a phylogenetic analysis was conducted using plastid DNA, which showed a paraphyletic relationship between *Zingeria* and *Colpodium* (Tkach et al., 2020). Since this is such a recent change, the decision was made to use *Z. biebersteiniana* in this paper as it aligns itself with much of the previous work on the species and hence should be more recognizable, but regardless *Colpodium biebersteinianum* should be acknowledged as the current standing name for the species.

We have obtained a telomere-to-telomere assembly of *Zingeria biebersteiniana*'s genome (Yin et al., unpublished). In this research, evidence has supported genome sizes ranging from 1.3 to 1.4 Gb as opposed to previous estimates of 1.7-1.8 Gb. A complete sequence assembly would supply a vital resource for uncovering the evolutionary history of the species. It would potentially represent the first genome sequenced in the subtribe Coleanthinae.

CHAPTER 2

IN VITRO REGENERATION

2.1 Introduction

The innate ability to regenerate an entire individual from a single cell, known as totipotency, is a characteristic that offers an immense opportunity for research, breeding, and conservation. The proliferation of undifferentiated cells deriving from somatic tissue is an example of tissue culture, the undifferentiated cells which are produced in the process are known as callus or its plural calli. When callus is produced via tissue culture the result is called callus culture. It is through this callus we can manipulate and transform tissue, propagate, and store material that has the potential to produce entirely new individual plants. These regenerated plants can have the advantage of sharing homologous genomic material with the explant source. Plants that are recalcitrant toward standard techniques of genetic transformation can be studied by the process of callus induction, allowing the more susceptible undifferentiated cells to interact with the desired medium. Many grasses require these additional steps and here we will discuss one such individual.

While in theory, all plants can produce viable calli, capable of regeneration, in application there is simply not enough information on hand to prescribe any given species an exact protocol. Often individual species require a specialized regimen to successfully induce callus and others additionally need alterations to regenerate the tissue. There is not one media type that can produce and regenerate callus universally. For *Z. biebersteiniana* callus induction and regeneration were initially demonstrated by T.F. Petrova et al.

(1991). It is their work with *Z. biebersteiniana* that inspired the early phases of my investigation into the species.

Here I will discuss the use and development of three different media types along with methodologies that aim to mitigate common issues found with *In Vitro* work while promoting callus development. With a productive callus culture, a myriad of opportunities become present. Therefore, ensuring healthy induction and regeneration is a necessity.

2.2 Materials and Methods

The starting media used for callus induction was agarose 10 g/ liter, Murashige and Skoog basal medium (MS) 1.33 g/liter, sucrose 30 g/liter, and casein hydrolysate 10 g/liter (Petrova et al. 1991) The phytohormones 2,4-D and 6-BAP were used at concentrations of 10 µM and 2.5 µM respectively (Petrova et al. 1991). Seeds were plated on media lacking phytohormones and were allowed to rest for four days before phytohormones were introduced.

The steps involved with callus induction and maintenance are as follows 1). Prepare media based on the desired quantity of petri dishes, often 20-25ml of media is used per dish. 2) Combine media with 50ml of water, and have on hand forceps, 2 beakers, paper towels, and a 1.5ml microcentrifuge tube for autoclaving. 3) While the autoclave is running sterilize the laminar flow hood by first turning on the airflow and spraying all surfaces with 70% ethanol. 4) Afterward expose all surfaces to UV light, allowing for a full 10 minutes of UV exposure before progressing. 5) Once the autoclave has finished all materials are placed into the flow hood, media should be allowed to cool

for 10-20 minutes, or once it is comfortable to handle pour 20-25 ml of media into each petri dish. 6)After all the media has been poured all surfaces and materials should be exposed to 10 minutes of UV light.

Before sterilization can begin seeds should be dehusked. This is accomplished by placing seeds on paper and rubbing them between your fingers. Be sure to blow off all the husks prior to transferring the seeds to a sterilized 1.5ml microcentrifuge tube. Sterilization of tissue is done by adding 1ml of 1:1 ethanol: hydrogen peroxide to the 1.5ml microcentrifuge tube (Petrova et al. 1991). The tube should be repeatedly inverted to mix the solution with the seeds for three minutes, carefully opening the tube each minute to release any build-up of gas. At exactly three minutes the ethanol: hydrogen peroxide solution should be pipetted out and using sterilized DI water seeds are washed 3-5 times. After rinsing, the seeds should be dried on sterilized paper towels. Using freshly flamed and cooled tweezers, carefully place seeds onto media being sure to flame the tweezers between each petri dish. In the early growth and germination stages, seeds can be tightly placed into 10x10 squares.

Once all petri dishes were plated and sealed with micropore tape, they were placed into a growth chamber with a temperature of 22°C and left in the dark for 4 days. After 4 days seeds are replated on new media containing phytohormones. After 1-2 weeks germinated plants should be present with callus appearing near the base of the shoot often adjacent to the seed coat and sometimes near the tip of the radicle. At this stage plant tissue other than the callus should be removed and the calli should be replated onto new media. This callus can then be grown directly in light ensuring that it is transferred to fresh media every two weeks. Regeneration of callus should occur naturally

and when shooting begins to develop prolifically, plants should be transferred into culture jars, this often occurs within 8-12 weeks. Allow tissue to develop in media until shooting and rooting have been well established, this will be 20-24 weeks after initial plating (Fig. 2).

Once a healthy root structure has been established the plant can be transferred to soil. Soil should be sterilized and when *in vitro* grown plants are transferred all agar should be removed from the roots before planting. Removing agar from roots can be accomplished by using forceps to lightly shake the plants in sterile water. Plants in soil should be placed in a tray and humidome for 1 week, slowly opening the vent holes over the course of the week. If too high of a moisture content is kept fungus may develop and potentially endanger your plants. After the humidome is removed the plant falls into a standard watering regime and the standard fertilization using nitrogen pellets. Once inflorescence begin to emerge carefully monitor spikelets. Once anthers emerge, pollination can be accomplished by performing a clapping motion over the entirety of the inflorescence. Seed harvesting is then done once the plants have undergone reproductive senescence.

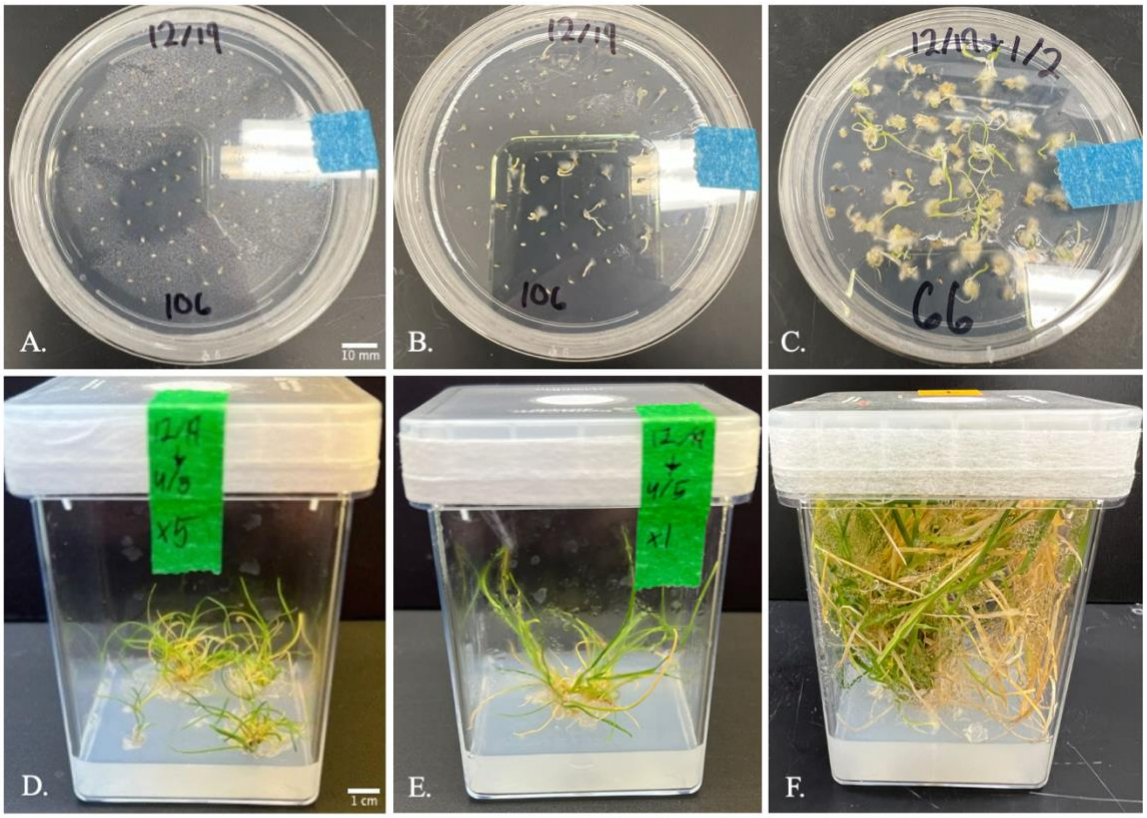

Figure 2: Callus induction and regeneration progression using modified media. A.) 1st day of plating (Bar = 10mm) B.) 1 week of growth C.) 2 weeks of growth D-E.) 4 months of growth (Bar = 1cm) F.) 6 months of growth

A modified media was being developed which consisted of many similarities to the previous media with the addition of plant preservative mixture (PPM), removal of casein hydrolysate, and the removal of 6-BAP. This became the main media type used for the establishment of callus and would often be the product of early images portraying callus culture.

The last media used was based on the A. V. Khromov paper (Khromov & M Yu Cherednichenko, 2022).. The new media consisted of MS 4 g/ liter, sucrose 30 g/ liter, agarose 10 g/ liter, and PPM 2 ml/ liter. Cold stratification at 3°C for three days was implemented on seeds beginning. With the new media came a new phytohormone regimen which was an initial 2,4-D concentration of 4 mg/ liter for 2 weeks, then 2,4-D was transitioned to 0.4 mg/ liter which remained constant until regeneration was underway (Khromov & M Yu Cherednichenko, 2022).

Additional changes were later made to previously listed materials and protocols that became constant for the remainder of the *in vitro* work. These included a third decrease of the 2,4-D concentration in the Khromov media to 0.04 mg/liter. Seeds were directly plated onto 2,4-D for germination. Cultures were grown in complete darkness until callus began shooting or rooting, at which point the culture would be moved in the light to continue growth and progress into complete regeneration. The Khromov media with these additional changes would eventually lead to the first line of plants that were successfully seeded after callus induction.

2.3 Results and Discussion

While conducting tissue culture work there are several key aspects to align yourself with, arguably the most important of which is the ability to work in and maintain an aseptic environment. The initial phases of callus induction require familiarization with the standardized techniques.

What has been listed is the most direct path to callus induction and successful regeneration of *Z. biebersteiniana*, though there was a multitude of caveats that were found using the Petrova media. The first of these may be biased, but the use of casein hydrolysate while not necessarily detrimental to the objective often leads to an increased presence of contamination. This could be a unique experience limited to our supply of the chemical but in the very earlier stages, an alternative media type was being tested. The use of the ethanol: hydrogen peroxide sterilization solution proved to be adequate and became the preferred method for the sterilization of seeds. With *Zingeria biebersteiniana*, the exposure of sterilization solutions would often bleach the seeds; with prolonged exposure, germination success would vary. A thorough rinsing of seeds directly after sterilization should be emphasized to mitigate any loss in germination.

The calli that was produced with the Petrova protocol were demonstrated to be effective in the regeneration of new individuals. However, the callus that was produced was small and often darkened in appearance as opposed to what will be displayed in future media adaptations. The lack of either shooting or rooting did not pose an issue in these tests. Although the roots that were developing in the media were few, careful attention was needed when handling the plants during transfers to new media, ensuring no additional stress was applied to the plants. Regenerated individuals took on a unique phenotype that resembled abnormal shoot development (Fig. 3). This growth formed tight

curls, greatly reducing vertical growth, with only a limited number of shoots expressing normal development. This abnormal growing behavior is believed to be a result of the plant's exposure to 6-BAP. It is also important to recognize that callus was unable to be grown exclusively. Instead, callus would only be present within a short window of time before regeneration would begin. Callus was never observed to develop outside of its initial induction and with these concerns, moving forward in the *in vitro* work for *Z. biebersteiniana* identifying a media type that could produce sizeable callus and maintain a perceived normal growth during regeneration would be of importance.

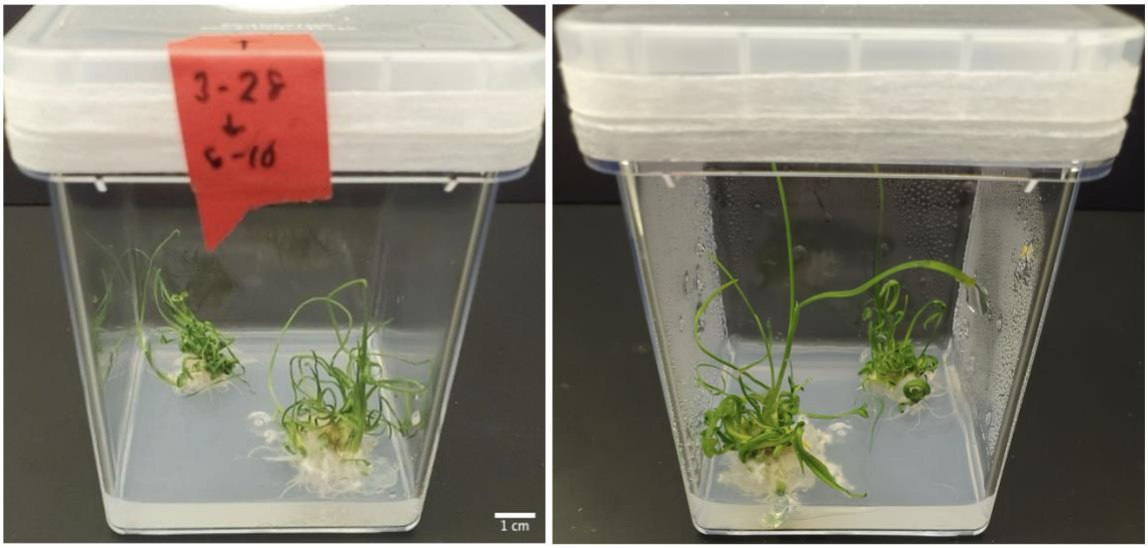

Figure 3: Abnormal shoot development in media containing 6-BAP (Bar = 1cm)

The work on establishing a new media had been in development since the early stages of the study. It was run alongside standard T.F. Petrova media and saw the removal of casein hydrolysate. The introduction of Plant Cell Technology's plant preservative mixture (PPM), which is a proprietary broad-spectrum biocide, was an essential component to maintaining a healthy culture by reducing the chance of contamination. The concern regarding the removal of 6-BAP was that the callus would be unable to successfully regenerate shoots. This however seemed to minimize issues in regeneration. The exclusion of 6-BAP was observed to produce regenerated plants that did not exhibit phenotypes similar to those of the previous media while still maintaining healthy development.

The adjusted 2,4-D concentrations used in the Khromov media led to some of the healthiest calli observed up to this point, with much of the calli exhibiting a hairy root callus type. A hairy root callus is identified through the presence of root hairs encompassing the surface of the callus (Fig. 4 A-D). In addition to the new 2,4-D concentrations, allowing callus to proliferate in the dark allowed increased the time interval before regeneration would begin allowing for an increase in total callus size.

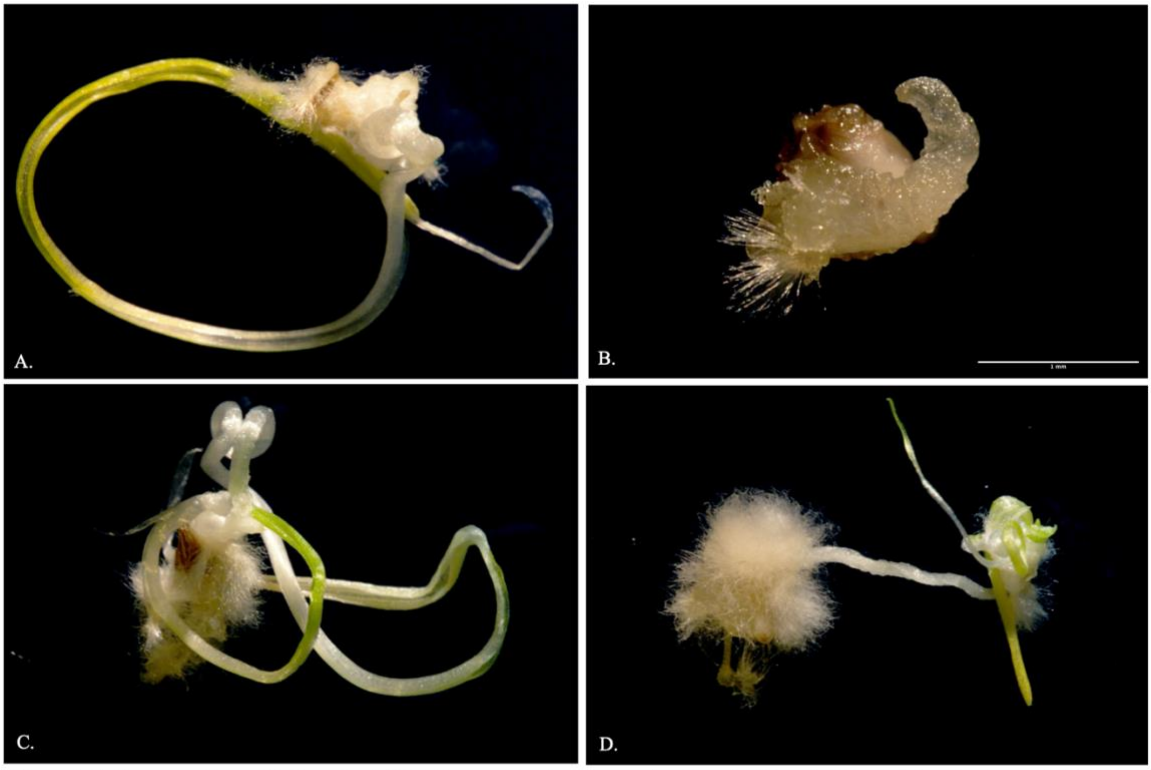

Figure 4: Callus grown in modified media. A & C.) demonstrate a typical growth pattern for *Zingeria biebersteiniana*, having curled shoots with a base segment comprised of hairy root callus B.) represent unhealthy growth, these individuals would often be allowed to grow for the first 2-3 weeks before being discarded (Bar = 1mm) D.) is comprised of two individuals, the leftmost is expressing heavy hairy root callus development while the right most individual is demonstrating a more typical growth pattern.

The ability to repeatedly replicate *Z. biebersteiniana* callus induction and regeneration acted as the catalyst for the development of new pathways that could be explored and new questions that could be answered. The most prominent of which is the potential for genetic transformation.

CHAPTER 3

SILICON NUTRIENT AND SILICA DEVELOPMENT

3.1 Introduction

Silica bodies are silicon structures found in the epidermal tissues of plant leaves. The formation of a silica body requires the presence and uptake of monosilicic acid from the soil which is then accumulated as hydrated silica (Prychid et al., 2003). Silica bodies can serve as a tool for taxonomic applications due to their unique morphology that can vary on the family, genus, or species level paired with their ability to remain preserved in natural conditions (Piperno & Pearsall, 1998; Prychid et al., 2003). In the case of rice, silica body benefits can range from increased resistance to herbivory and bacterial or fungal infections, reduction in cuticular transportation and improved water efficiency, or reductions in the accumulation of toxic concentrations of heavy metals (Agarie et al., 1996). In rice, silica bodies account for 5-20% of the shoots' dry biomass and the absence of available silica has been shown to have stunted or suppressed growth (Agarie et al., 1996).

This study aims to identify patterns of silica body formation post-transferring *Z. biebersteiniana* from *in vitro* to soil. The distribution of silica bodies was assessed using samples collected from *Z. biebersteiniana* that had undergone callus induction in silica-free media, had been regenerated, and then transferred to soil. By identifying silica bodies in mature leaf tissue, we can hypothesize that a pathway exists that facilitates the movement of silicon that is not reliant on new growth.

3.2 Materials and Methods

Dry ashing was used to prepare tissue which resulted in silica bodies that could be visualized under a GFP filter (Fig. 5) (Dabney et al., 2016). Mature leaf cuttings were taken from regenerated plants on the first day after transferring from media to soil and then once a week for eight weeks. Two mature cuttings were taken from each plant and then placed one adaxial and one abaxial side down on a microscope slide (Dabney et al., 2016). A second microscope slide was then placed on top of the leaf cuttings before being moved to a hot plate. Cuttings were heated to 380°C for 45 minutes and then left to cool for an hour. Carefully removing the top microscope slide from the sample (Dabney et al., 2016). Two drops of cedarwood oil were placed on the sample and then covered with a microscope slip sealing the edges with a clear coat of nail polish (Dabney et al., 2016). Slide samples were analyzed using a Nikon Eclipse microscope. Images were taken at 40x magnification with a green fluorescence protein filter.

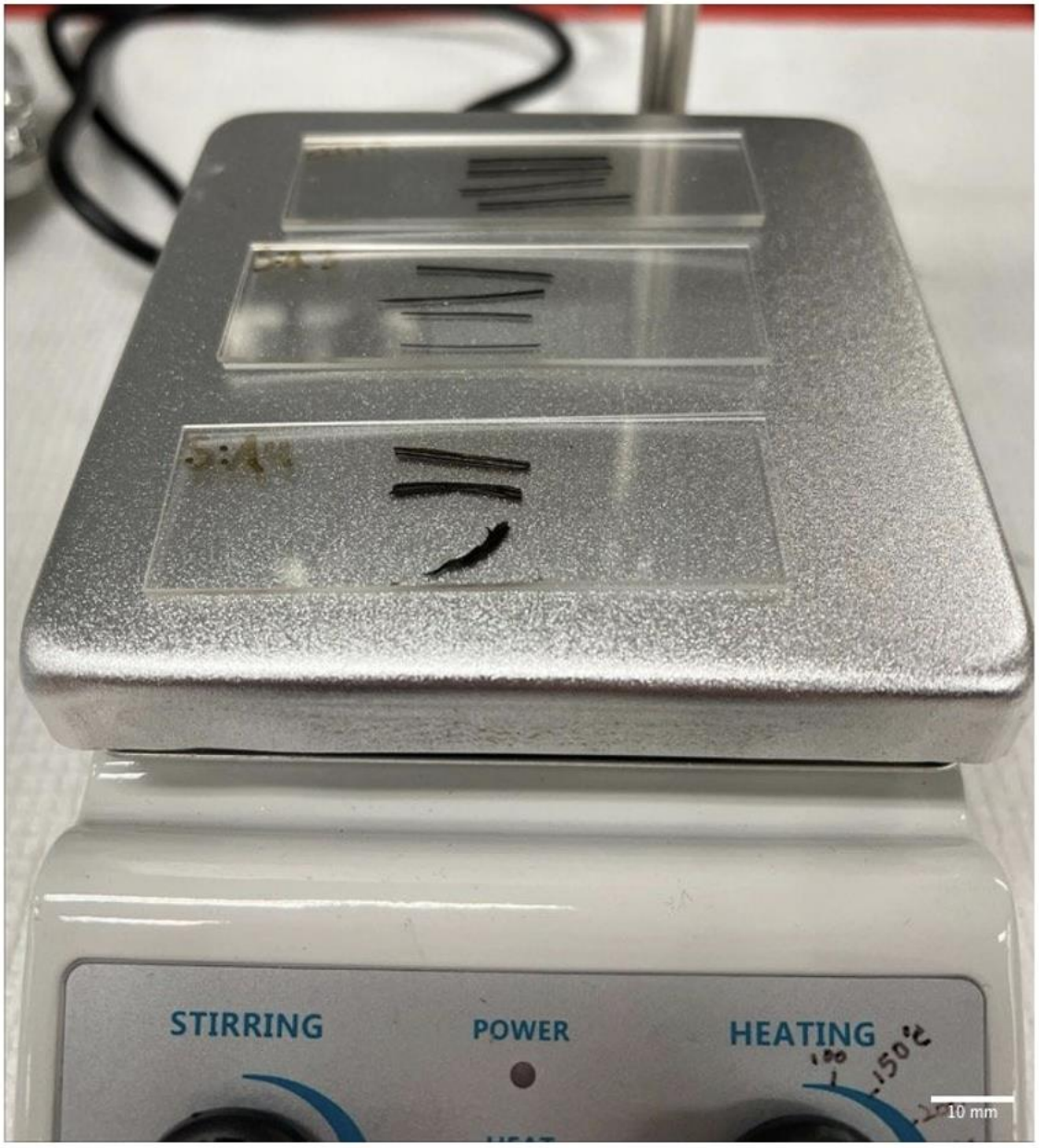

Figure 5: Result of dry ashing protocol on leaf tissue. (Bar = 10mm)

Using NIS-Elements z-stack, images were taken and then compressed in Helicon Focus 8. While in NIS-Elements the conversion for a pixel to metric was recorded at 1 pixel = 0.183μm^2 (Dabney et al., 2016). In Adobe Photoshop the area of the silica bodies was found by first adjusting the contrast of the image to exaggerate the intensity of green given off by the silica bodies against the black backdrop. Highlighting all the silica bodies in the image was accomplished using the magic wand tool. While the silica bodies are highlighted, clicking on the histogram option will show the total pixel count for all the highlighted areas. Silica bodies were then manually counted (Dabney et al., 2016).

3.3 Results and Discussion

Two trends were identified to be occurring within *Z. biebersteiniana*. The first is, that as the plants adjust to soil a preliminary increase in average silica body size occurs, and as time continues the average size decreases. The second is, as time continues the average silica body count increases along with the total area of silica bodies, the latter potentially stabilizing by week 5. In the samples from day 1, week 1, and week 3 silica bodies were absent. Week 1 had a minimal presence of silica bodies with the smallest average of 7.9μm^2. Week 2 had the largest increase in average silica body size to 36.8μm^2, in addition to a moderate increase in silica counts. Week 2 represented the peak in average silica body sizes resulting in a decreasing trend up to week 8 ending with an average size of 10.5μm^2.

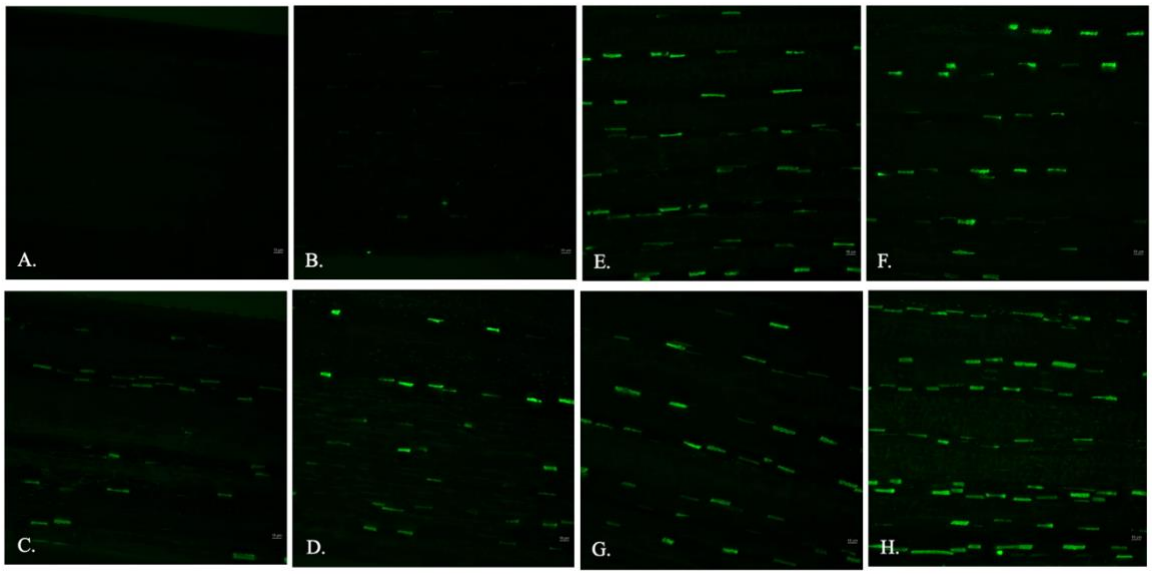

Figure 6: Silica body development. A.) day 0 B.) week 1 C.) week 2 D.) week 3 E.) week 5 F.) week 6 G.) week 7 H.) week 8 (Bar = 10µm)

Average silica body counts were observed to be at a constant increase with the exception of week 5 to week 6. In week 5 the average silica body count was 28 to week 6's average count of 25. This drop in silica bodies could be the result of human error, such as improper selection of leaf material. For week 7 the average silica body count was 48, and then week 8's count of 64 representing the peak in the data. The increase in silica bodies generally supports an increase in the total surface area of silica, up to a point. From week 1 to week 5 the average total area of silica bodies increased from 67.2μm^2 to 675.8μm^2 respectively. For the next two consecutive weeks after week 5, a decrease in total area occurred down to 525.3μm^2 before returning to 646.1μm^2 in week 8. This pattern could correlate to a stabilizing state for *in vitro* plants after acclimating to soil.

The absence of silica bodies in *in vitro* specimens offers a potential for future work investigating how silica body formation deviates with different stressors. The difficulty of transferring *in vitro-grown* plants to soil poses some concern for regenerated plant loss. Work on the influence of silica supplements in media may aid in alleviating these issues in applicable plant species. Implementing additional environmental factors may also be used to gauge the variation of silica body formation in *in vitro* grown plants. Knowing the constant should be an absence of silica bodies before the transfer to soil.

CHAPTER 4

ESTABLISHMENT OF AN *AGROBACTERIUM TUMEFACIENS* TRANSFORMATION IN *ZINGERIA BIEBERSTEINIANA*

4.1 Introduction

With *Z. biebersteiniana* callus induction being successfully replicated and regenerated plants being shown to produce seed, a transition into a new focus for the species could begin. To our knowledge, there has been no previous work on *Z. biebersteiniana* demonstrating a mode for genetic transformation. The goal set forth was to establish a protocol that was capable of generating a transformed line based on the previous protocols and techniques developed.

The study of actin micro-filament interactions within pollen cells can be arduous. Their fragility with common fixatives and staining methods, along with pollen walls which can prevent proper penetration of actin-specific stains, opens a window for an easier delivery of adequate tissue for analysis (Xu et al., 2002). The plasmid used in the study was designed by Shi-Xiong Xu and would facilitate the visualization of actin microfilaments within immature pollen, root epidermis, and root hair cells. The construct used consisted of a rice actin-1 promoter that drives the expression of GFP fused with mouse talin (GFP-mTn) (Xu et al., 2002). The transformation of *Z. biebersteiniana* was mediated using the *Agrobacterium tumefaciens* line C58.

Here I will discuss the techniques established that mediate *Z. biebersteiniana* transformation. Along with a pathway for DNA extraction and PCR genotyping that was successfully applied to *Z. biebersteiniana* and our selected construct. While the desired

phenotype of the transformed line was not identified due to time constraints, the existence of a transformed line has demonstrated a mode for replication.

4.2 Materials and Methods

The constructs used were acquired from Shi-Xiong Xu's lab at the University of Hong Kong and had been inserted into the *Agrobacterium* line C58 before being transferred to my care. The *Agrobacterium* was plated on LB nutrient media which consisted of LB 25 g/ liter, agarose 15 g/ liter, and kanamycin 50 mg/ liter. The *Agrobacterium* was replated each month and left to rest at 28°C for 24 hours before being transferred to 3°C where the plate would rest between co-cultures.

It has been previously demonstrated that *Z. biebersteiniana* was unable to maintain constant growth in a callus state. A successful coculture with *Agrobacterium* could only be achieved within a narrow span of its development before regeneration would begin. The likelihood of producing a transformed line would decrease if the callus was allowed to develop too much. It was found that callus was the most viable 2-4 weeks after the initial plating. At 2-4 weeks callus should be present and nearing its largest size, at which point preparation of *Agrobacterium* suspension would begin. A stock LB broth was prepared using 15 g/liter that was autoclaved and stored at 3°C. From the stock 5 ml of the LB broth with 0.25 mg of kanamycin was added to a falcon tube. Using a pipette tip 2-3 colonies of *Agrobacterium* were collected and added to the falcon tube by dropping the pipette tip directly into the broth. The falcon tube was then placed into a shaker set to 100 RPM and left to incubate at 28°C for 20 hours. After the incubation, 1 ml of the suspension was removed and added to a new falcon tube containing 20 ml of

the stock LB broth and 1 mg kanamycin, the new falcon tube was then placed in the shaker and left to run for 8 hours at 100 RPM and 28°C. After 8 hours the suspension was centrifuged at 3000 RPM for 5 minutes. Pouring off the supernatant, 25 ml sterilized DI water with 50 mg of MS was added to the falcon tube, the pellet was then resuspended. Under the flow hood, callus was placed into the *Agrobacterium* suspension and left for one minute. After one minute callus was dried on sterilized paper towels and replated onto fresh media, when all the callus had been plated the callus was placed in the dark at 22°C for two days.

Following the two-day co-culture callus will need to be replated on selection media. The first step in this process is preparing an antibiotic wash for callus prior to plating. The antibiotic wash consists of sterilized DI water 25 ml, MS 50 mg, hygromycin 1.25 mg, and cefoxitin 2.5mg. Along with the antibiotic wash for the callus, media for continued callus growth was altered with the addition of hygromycin at 50 mg/ liter and cefoxitin at 200 mg/liter (Fig. 7). It should be of note that 200 mg/liter of cefoxitin is double the recommended concentration, this is due to running out of carbenicillin which was initially paired with cefoxitin both of which were used at 100 mg/liter.

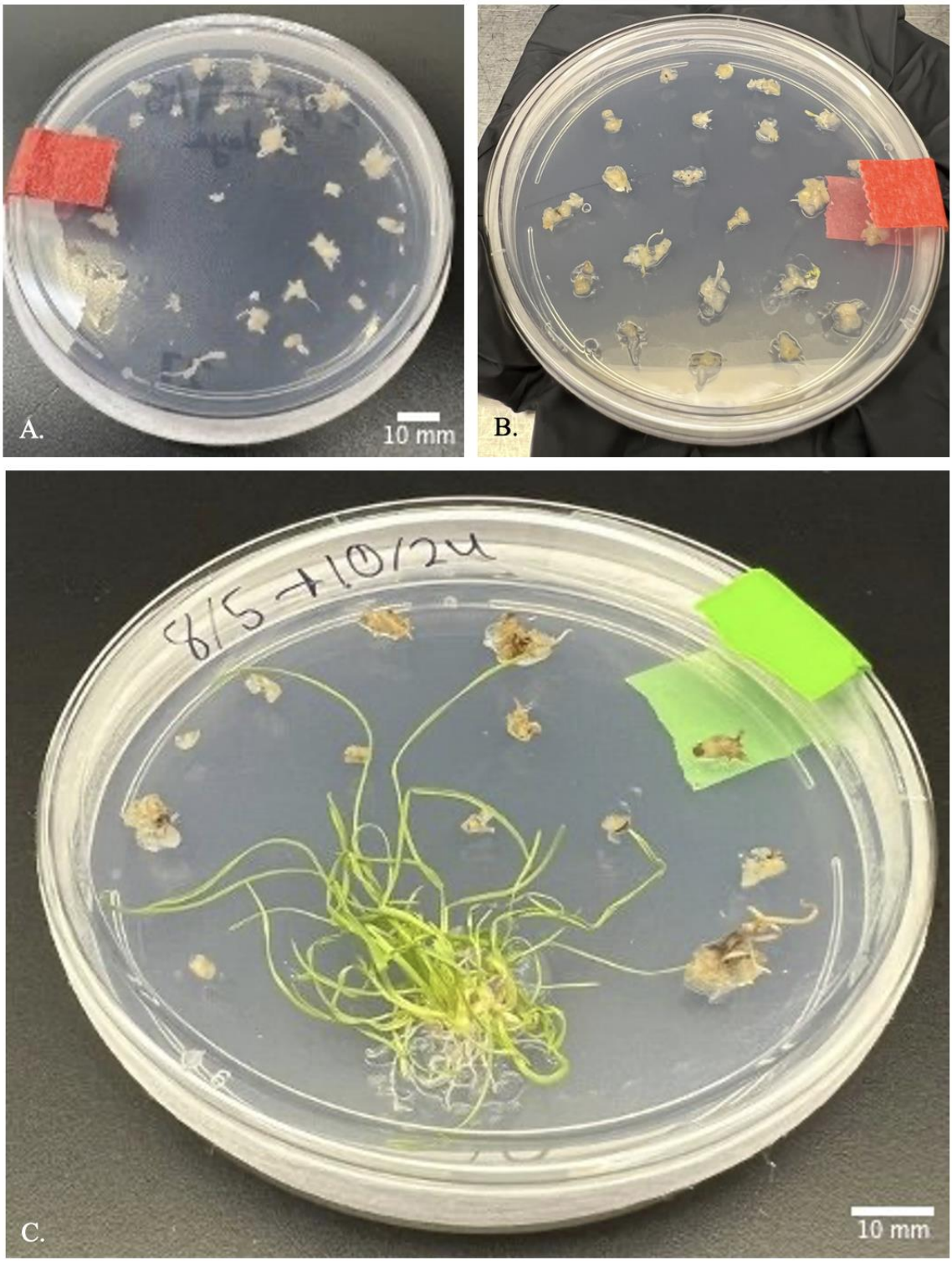

Figure 7: Callus coculture with *Agrobacterium tumefaciens* and selection medias. A.) culture 2 days after introducing *Agrobacterium* (Bar = 10mm) B.) 1st day in selection media C.) 1 month and 1 week in selection media (Bar = 10mm)

The antibiotic dip and selection media were used for the rest of the plant's time in culture. It was found that only running 100 mg/liter of cefoxitin would lead to an increase in *Agrobacterium* contamination after 1 week. The plants are transferred to fresh media every two weeks until they are ready to be transferred to soil. Plants can be propagated off from the original growing mass once shooting and rooting is active. This is done by carefully using sterilized forceps to tear a shoot away from the growing mass, it should be ensured that limited root damage occurs during this process. Once plants display healthy roots and shoots, they can be transferred to soil. The watering tray, pots, soil, and humidome should be sterilized with 70% ethanol and UV lights for 10 minutes. Plants are then dipped a final time in an antibiotic wash and transplanted into soil remaining in the humidome for a single week slowly opening the vent hole over the duration of the week.

After the transplants had acclimated to the soil, genotyping through the use of PCR began. Tissue was collected from mature leaf cuttings and transferred directly to -70°C for storage. Once the tissue was completely frozen 200 mg of tissue was placed into a sterilized mortar and liquid nitrogen was poured in until the tissue was completely submerged (*Home Page*, n.d.). The tissue was then homogenized with the use of a pestle, and 1 ml of CTAB was added to the mortar (*Home Page*, n.d.). Using a sterilized cut pipette tip 500 µl of suspended tissue was transferred to two microcentrifuge tubes. The tubes were then vortexed for 5 seconds and placed in a 60°C bath for 30 minutes (*Home Page*, n.d.). The microcentrifuge tubes were then centrifuged for 5 minutes at 10,500 rpm (*Home Page,* n.d.). The supernatant was transferred to a new microcentrifuge tube where 5 µl of RNase A is added before incubating at 37°C for 20 minutes (*Home Page,* n.d.). After incubation, an equal volume of chloroform/isoamyl (24:1) is added and vortexed

for 5 seconds before being centrifuged at 10,500 rpm for 1 minute (*Home Page*, n.d.). Once centrifuged the aqueous upper layer is transferred to a new tube before the previous step is repeated a single time. The DNA was then precipitated out by adding 0.7 volume of cold isopropanol and placing the microcentrifuge tube directly at -20°C for 15 minutes (*Home Page*, n.d.). After 15 minutes the microcentrifuge tubes are centrifuged at 10,500 rpm for 10 minutes, the supernatant is then removed, and the precipitate is washed with 500 µl of cold 70% ethanol (*Home Page*, n.d.). The ethanol is then removed, and the tubes are left out overnight in the flow hood to dry. The next morning 20 µl of TE buffer is added and the tubes are incubated at 37°C for 5 minutes (*Home Page*, n.d.). After 5 minutes, using a pipette, the DNA is suspended in the TE buffer and stored at -70°C. *Agrobacterium* DNA was isolated by adding 4-5 colonies to 100 µl of molecular grade water in a microcentrifuge tube and incubated at 100°C for 10 minutes. After incubation 900 µl of molecular-grade water was added and vortexed for 10 seconds before being centrifuged for 10 minutes at 12,000 rpm. The supernatant was then pipetted out into a new sterilized microcentrifuge tube and stored at -70°C.

Using extracted DNA from both *Z. biebersteiniana* and *Agrobacterium* PCR began, with *Agrobacterium* acting as a positive control. The oligos used for selection would detect the presence of the hygromycin resistance gene (HPT) with the forward and reverse primers being 5'-CTACAAAGATCGTTATGTITATCGGCA-3' and 5'-AGACCAATGGGGAGCATATAGG-3' respectively (Xu et al., 2002). A PCR master mix was prepared with 2 µl of 10x Buffer, 2 µl of 2mM dNTP, 5µl of 2 µM forward primer, 5µl of 2 µM reverse primer, 1 unit/µl Taq polymerase, 4 µl of molecular grade water, and 1 µl of DNA per sample. Each sample of the master mix was loaded into a

PCR tube strip and ran at 94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 56°C for 40 seconds, 72°C for 2 minutes, and a final cycle of 72°C for 10 minutes (Zhuo et al., 2005). Once the PCR had completed samples were stored at -70°C.

Gel electrophoresis was used to identify sample quality. A gel was prepared using 50ml of a 1x TAE stock with 0.5g agarose. The stock was microwaved for one minute being sure to mix the stock a single time at 30 seconds and again after the full minute. Once the agarose had been completely dissolved in the stock it was left to cool for 5-10 minutes before adding 4 µl of ethidium bromide. The stock was poured into the cassette mold, ensuring no bubbles were formed. After solidifying the cassette was placed into a gel rig and 1x TAE was added until the gel was completely submerged. Each well in the gel was loaded with 3 µl of 6x loading buffer and 6 µl of genomic DNA or 9 µl of PCR product (Fig. 8).

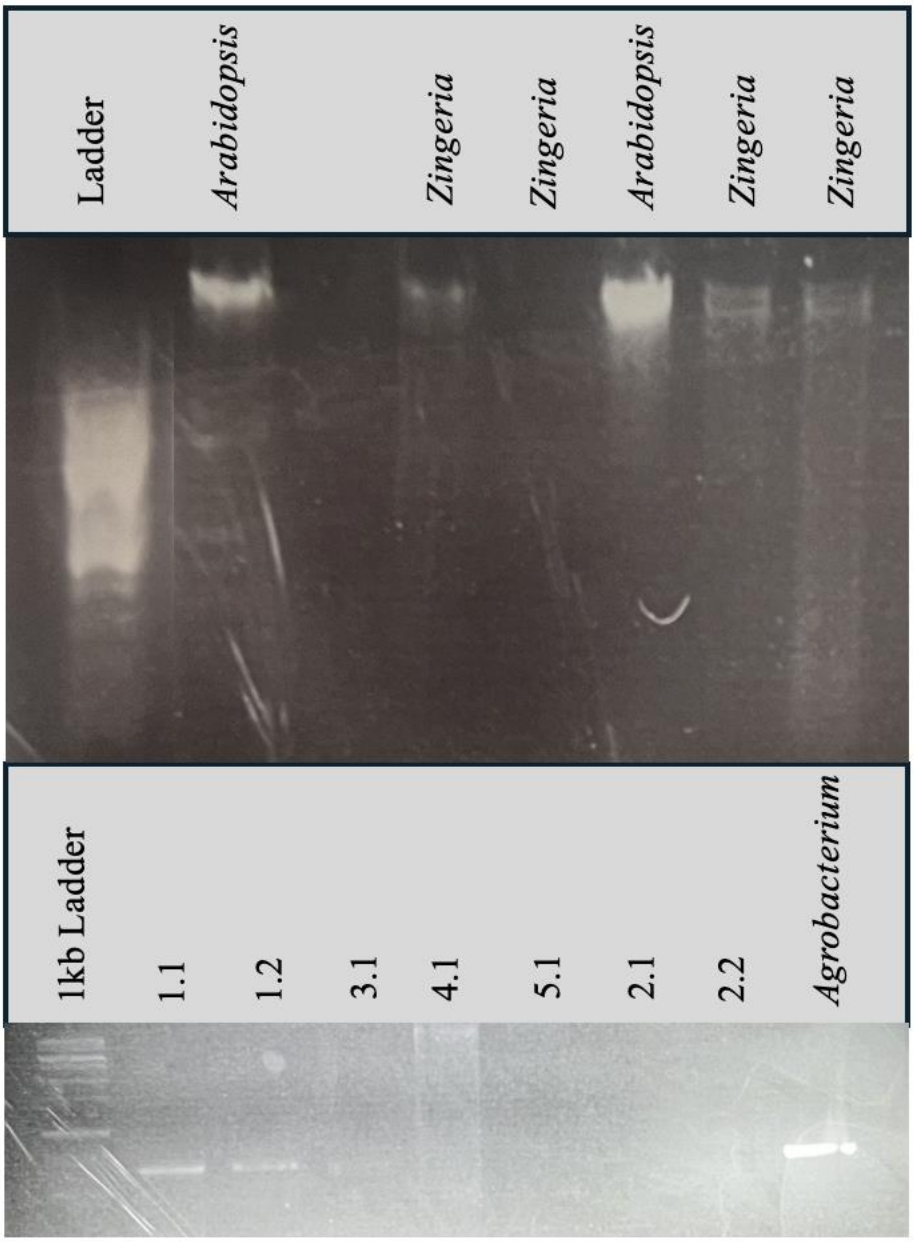

Figure 8: DNA extraction and PCR genotyping. Top: First example of a reliable DNA extraction protocol for *Zingeria biebersteiniana* in this study. Bottom: Positive result from PCR genotyping. Each whole number represents a potential transformed line while the fractional value denotes its accession within that line. *Agrobacterium* was used as a positive control. Line 1 contains two plants both showing a band marker for the HPT gene.

Stamens were collected from *Zingeria biebersteiniana* once spikelets began to open at this stage, there should be spikelets with anthers visible and hanging out of the floral body. Closed spikelets along with open ones were collected and their anthers were removed and placed into 2 drops of PBS on a microscope slide (Xu et al., 2002). The anthers were then sliced and pressed using hypodermic needles to suspend pollen in the PBS. Once all the anthers had been processed anther debris was removed, leaving only the pollen PB suspension on the microscope slide before adding a cover slip (Fig. 9). Pollen was visualized using a Nikon Eclipse microscope with a GFP filter at 20x, 40x, and 80x magnification.

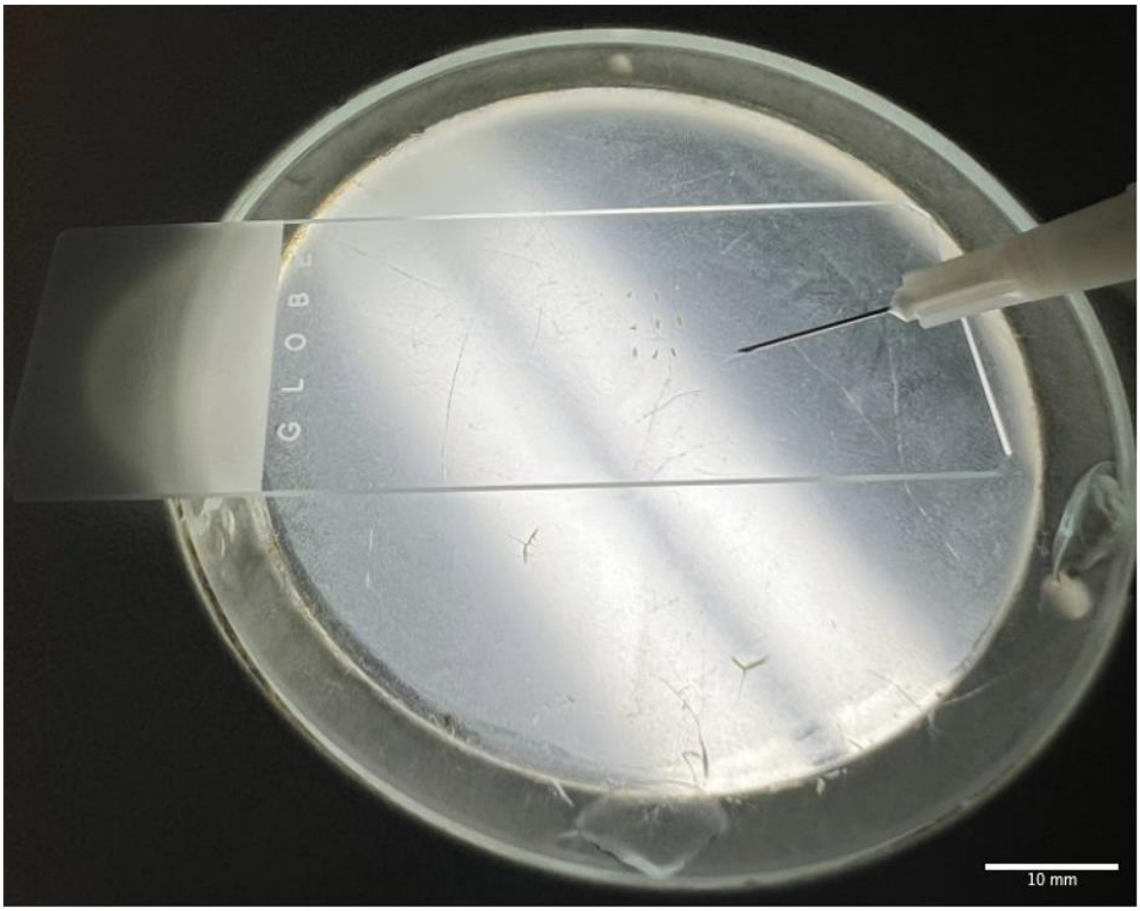

Figure 9: Anther size comparison and preparation before the introduction of PBS. (Bar = 10mm)

4.3 Results and Discussion

In total, 5 potentially transformed lines had been propagated out into 14 total plants. Of the 14 plants, only two came up positive for the HPT gene, both of which were line 1. It should be noted at this point that 12 plants were able to survive in the selection media for over 5 1/2 months, to the point in which healthy rooting and shooting were displayed and plants were deemed safe to transfer to soil. This may pose some level of risk in future efforts to transform the species and hence increasing the total calli and plants in culture is recommended to ensure a successful line is produced. In the run that produced a single transformed line, a total of 194 seeds were initially germinated. Of the two plants that had been transformed seeds were harvested once senescence had been completed and stored at 3°C. The T1 generation is still growing as of writing and will need to develop more to produce enough tissue for genotyping by PCR. Once positive plants have been selected from the T1 generation an analysis of the root epidermis and root hairs will begin, attempting to visualize the GFP expression of microfilaments within the tissues. The risk of tissue collection on roots was not attempted with the T0 line as risking any losses could have been a setback of a minimum of 6 months. Instead screening for GPF expression in the actin microfilaments of pollen was attempted, as it would be a less intrusive analysis.

The pollen analysis did not render any individuals that displayed the phenotype of pollen in the Shi-Xiong Xu study. The phenotypes present in Shi-Xiong Xu's rice study demonstrated clear signals of actin-microfilament which may display a relationship with the vegetative nucleus and generative cell (Zhuo et al., 2005). The early stages of the pollen analysis were conducted without PCR genotyping and as such much of the original

screening of pollen was conducted visualizing wild-type pollen. At the time the two positive plants were confirmed they had already begun senescence and much of the anthers had already dropped off from the spikelets. Though some of the shoots and panicles were still alive, pollen was removed and analyzed from those individuals. The pollen analyzed from the positive plants displayed one unique phenotype not identified in any of the wild-type pollen accessed. Although a second phenotype was identified it requires a deeper investigation to accurately determine whether it is being influenced by the inserted construct. The first phenotype identified was an asymmetrical webbing which appeared to cover the entirety of the pollen. The second phenotype which needs more review, concerns itself with the illuminance given off by the pollen (Fig.10). These individual cells are easily distinguished from other cells, being substantially brighter under fluorescent light and being opaque as opposed to semi-transparent.

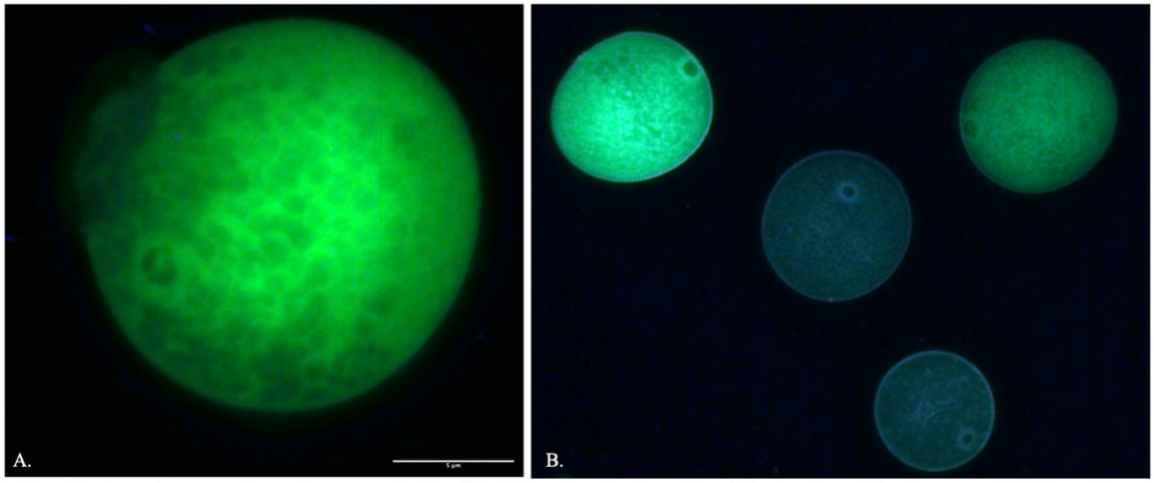

Figure 10: Transformed pollen. A.) Asymmetrical "webbing" encompassing the pollen of the transformed line. (Bar = 5μm) B.)The variance in luminescence of pollen in the transformed line.

Although the phenotypes displayed by the Shi-Xiong Xu's study were not observed in *Z. biebersteiniana*, the examples set in this study create two new precedents. The first of which is, with the T0 generation producing a healthy quantity of seeds studying roots and pollen can be done promptly with the help of PCR genotyping. Observable actin microfilaments may be detected and their interaction with cellular organization may be better studied. The second is, that there is now a working protocol demonstrating the genetic transformation of *Zingeria biebersteiniana* mediated by *Agrobacterium*. To our knowledge, this is the first time that this has been accomplished for the species.

REFERENCES

Salih. (2013, April 26). *IUCN Red List of Threatened Species: Zingeria biebersteiniana*. IUCN Red List of Threatened Species; Name. https://www.iucnredlist.org/species/19618916/19620986

POWO (Plants of the World Online) 2024. Royal Botanic Gardens, Kew. Published on the Internet; http://www.plantsoftheworldonline.org/ Retrieved 21 June 2024.

Euro+Med 2006+ [continuously updated]. : Euro+Med PlantBase - the information resource for Euro-Mediterranean plant diversity. – Published at http://www.europlusmed.org Retrieved 21 June 2024

Tkach, N., Schneider, J., Döring, E., Wölk, A., Hochbach, A., Nissen, J., Winterfeld, G., Meyer, S., Gabriel, J., Hoffmann, M. H., & Röser, M. (2020). Phylogenetic lineages and the role of hybridization as driving force of evolution in grass supertribe Poodae. *TAXON*, *69*(2), 234–277. https://doi.org/10.1002/tax.12204

Clayton, W.D., Harman, K.T., & Williamson, H. (2006). The Board of Trustees, Royal Botanic Gardens, Kew | Feedipedia. (n.d.). www.feedipedia.org. Retrieved June 21, 2024, from https://www.feedipedia.org/node/4565

iNaturalist community. Observations of *Colpodium biebersteinianum* from Lebanon observed on/between May 2008. Exported from https://www.inaturalist.org on July 2024

Davis, P.H. (ed.) (1985). Flora of Turkey and the East Aegean Islands 9: 1-724. Edinburgh University Press, Edinburgh.

Bennett, M. D., Smith, J. B., & Seal, A. G. (1986). The karyotype of the grass *Zingeria biebersteiniana* (2n = 4) by light and electron microscopy. *Canadian Journal of Genetics and Cytology*, *28*(4), 554–562. https://doi.org/10.1139/g86-081

Bennett, S. T., Leitch, I. J., & Bennett, M. D. (1995). Chromosome identification and mapping in the grass *Zingeria biebersteiniana* (2n=4) using fluorochromes. *Chromosome Research*, *3*(2), 101–108. https://doi.org/10.1007/bf00710670

Saunders, V. A., & Houben, A. (2001). The pericentromeric heterochromatin of the grass*Zingeria biebersteiniana*(2*n*= 4) is composed of Zbcen1-type tandem repeats that are intermingled with accumulated dispersedly organized sequences. *Genome*, *44*(6), 955–961. https://doi.org/10.1139/g01-092

Raskina, O., Barber, J. C., Nevo, E., & Belyayev, A. (2008). Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. *Cytogenetic and Genome Research*, *120*(3-4), 351–357. https://doi.org/10.1159/000121084

Cremonini, R., Castiglione, M., Grif, V. G., Kotseruba, V. V., Punina, E. O., Rodionov, A. V., Muravenko, O. V., Popov, K. V., Samatadze, T. E., & Zelenin, A. V. (2003). Chromosome Banding and DNA Methylation Patterns, Chromatin Organisation and Nuclear DNA Content in *Zingeria biebersteiniana. Biologia Plantarum*, *46*(4), 543–550. https://doi.org/10.1023/a:1024863511570

Kim, E. S., Bolsheva, N. L., Samatadze, T. E., Nosov, N. N., Nosova, I. V., Zelenin, A. V., Punina, E. O., Muravenko, O. V., & Rodionov, A. V. (2009). The unique genome of two-chromosome grasses Zingeria and Colpodium, its origin, and evolution. *Russian Journal of Genetics*, *45*(11), 1329–1337. https://doi.org/10.1134/s1022795409110076

Petrova, T. F., Kulinich, A. V., & Abramova, V. M. (1991). Use of *Zingeria biebersteiniana* (2 n= 4) grown in vivo and in vitro as a model species for genetic studies.

Khromov, A. V., & M Yu Cherednichenko. (2022). In vitro culture of *Zingeria biebersteiniana* (Claus) P. Smirn as a method of conservation and expanding its biodiversity. *IOP Conference Series. Earth and Environmental Science*, *981*(4), 042070–042070. https://doi.org/10.1088/1755-1315/981/4/042070

da Costa, F. G. C. M., Pellegrini, M. O. O., & Bove, C. P. (2021). Diversity of silica bodies in *Mourera* Aublet (Podostemaceae) and its phylogenetic, paleobotanical, and taxonomic applications. *Review of Palaeobotany and Palynology*, *295*, 104509. https://doi.org/10.1016/j.revpalbo.2021.104509

Prychid, C. J., Rudall, P. J., & Gregory, M. (2003). Systematics and Biology of Silica Bodies in Monocotyledons. *The Botanical Review*, *69*(4), 377–440. https://doi.org/10.1663/0006-8101(2004)069[0377:sabosb]2.0.co;2

Agarie, S., Agata, W., Uchida, H., Kubota, F., & Kaufman, P. B. (1996). Function of silica bodies in the epidermal system of rice (Oryza sativaL.): testing the window hypothesis. *Journal of Experimental Botany*, *47*(5), 655–660. https://doi.org/10.1093/jxb/47.5.655

Dabney, C., Ostergaard, J., Watkins, E., & Chen, C. (2016). A novel method to characterize silica bodies in grasses. *Plant Methods*, *12*(1). https://doi.org/10.1186/s13007-016-0108-8

Piperno, D. R., & Pearsall, D. M. (1998). The Silica Bodies of Tropical American Grasses: Morphology, Taxonomy, and Implications for Grass Systematics and Fossil Phytolith Identification. *Smithsonian Contributions to Botany*, *85*, 1–40. https://doi.org/10.5479/si.0081024x.85

Xu, S., Wang, L., Qiu, Z., Ye, Y., & Yu, X. (2002, January 1). Actin visualization in living immature pollen of rice using of GFP-mouse Talin fusion protein. *Acta Botanica Sinica*. https://europepmc.org/article/cba/374681

Yin, L. (Unpublished). Genomic Analysis of Zingeria biebersteiniana, a Potential Model System for Grass Genetics and Evolution

Home Page. (n.d.). OPS Diagnostics LLC. Retrieved June 21, 2024, from https://opsdiagnostics.com/notes/protocols/spin_column_plant_protocol.html

Zhuo, Q., Piao, J., Wang, R., & Yang, X. (2005). Refolding and purification of non-fusion HPT protein expressed in Escherichia coli as inclusion bodies. *Protein Expression and Purification*, *41*(1), 53–60. https://doi.org/10.1016/j.pep.2004.12.026