DEVELOPMENT OF TISSUE CULTURE AND TRANSFORMATION STRATEGIES FOR THE GENUS MIMULUS

By

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(Under the direction of Wayne Parrott and Andrea Sweigart)

ABSTRACT

Monkeyflowers (genus *Mimulus*) serve as model species used in studies on adaptation, speciation, and evolution. The implementation of functional genomics is necessary to increase the utility of *Mimulus* as a model system. In this context, the development of reliable tissue culture and *Agrobacterium*-mediated transformation strategies are essential. This study is to determine suitable genotypes for tissue culture and ultimately provide a protocol for *Agrobacterium*-mediated transformation in the yellow flower clade of *Mimulus*. 15 inbred lines from the yellow-flowered clade of *Mimulus* were tested for their performance in tissue culture using two regeneration systems. From this evaluation, suitable genotypes were chosen for a subsequent experiment testing parameters of transformation including various *Agrobacterium* strains and multiple days of cocultivation.

INDEX WORDS: *Mimulus*, Tissue culture, *Agrobacterium*, transformation

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DEDICATION

I would like to dedicate my success to the people who have been so generous to me with their time, effort, and patience. You have made all the difference in my life and I am sincerely grateful. I will always remember your kindness and I will try my best to pay it forward. Thank you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Mimulus

The genus *Mimulus* has become the subject of many studies of adaptation, speciation, and evolution due to its incredible diversity within and among species (**Figure 1.1**). *Mimulus* as a model system provides a unique opportunity to identify the genetic and developmental basis of important plant phenotypes that are not present in other well studied plant systems (Yuan 2019). *Mimulus* has been studied for over 70 years (Hiesey 1971). The genus, specifically the yellow monkeyflower clade, which includes *M. guttatus* and *M. nasutus,* is becoming increasingly popular with many research groups (Wu, Lowry et al. 2008). However, a reliable transformation protocol is still unavailable for this particular clade.

Plant transformation has been a valuable tool in other *Mimulus* species and other plant systems including *Arabidopsis* (Zhang, Henriques et al. 2006, Yuan, Sagawa et al. 2013, Susič, Bohanec et al. 2014, Ding, Patterson et al. 2020). Plant transformation allows for the elucidation of genetic control of plant phenotypes. Given the considerable diversity in the *Mimulus* genus, as compared to *Arabidopsis*, development of a transformation protocol for the yellow flower clade would be of utmost use to the community that studies *Mimulus*.

For transformation to be successful, the associated protocols often must be optimized for individual species or even genotypes (Li, Cong et al. 2017, Ahmed, Ding et al. 2018). Many other parameters can affect transformation efficiency, including but not limited to strain of *Agrobacterium* used and duration of co-cultivation (Li, Cong et al. 2017, Utami, Hariyanto et al. 2018, Taak, Tiwari et al. 2020). Transformation success can also be affected by choice of explant (Chakraborty, Chakraborty et al. 2020). This has been demonstrated already in *Mimulus aurantiacus* (Susič, Bohanec et al. 2014). For these reasons, testing these parameters can be beneficial for the development of an optimized tissue culture protocol in specific *Mimulus* genotypes.

Mimulus as a model system

The genus *Mimulus* includes over 120 species (Grant 1924) many of which have been used in a model system for genetic and evolutionary studies (Wu, Lowry et al. 2008). Many of these studies focus on adaptation (MacNair 1983, Leclerc-Potvin and Ritland 1994, Lowry, Hall et al. 2009, Hendrick, Finseth et al. 2016, Selby and Willis 2018, Mantel and Sweigart 2019), speciation (Sweigart, Karoly et al. 1999, Sweigart, Mason et al. 2007, Streisfeld, Young et al. 2013, Sweigart and Flagel 2015, Zuellig and Sweigart 2018), species range limits (Ramsey, Bradshaw et al. 2003, Angert and Schemske 2005, Sweigart, Martin et al. 2008, Garner, Kenney et al. 2016, Kerwin and Sweigart 2017) and plant pollinator interactions (Schemske and Bradshaw 1999, Ding, Mou et al. 2017). Because of the diverse array of floral phenotypic traits within and among species in the *Mimulus* genus, an investigation is underway to understand the developmental genetics of certain phenotypic traits (Yuan, Sagawa et al. 2014, Ding and Yuan 2016, Ding, Patterson et al. 2020). Traditionally, *Arabidopsis* has been used as an important model species for identifying and characterizing genes and gene products (Jeffrey Chen, Wang et al. 2004). However, *Mimulus* has arisen as a major model organism for researchers in more recent times due to its unique amount of phenotypic variation.

Members of the genus are found worldwide and are known to vary in habitat preference, pollinator specificity, mating system, ploidy level, and floral morphology (Wu, Lowry et al. 2008). Understanding the genetic basis of these features is one goal of research on *Mimulus*. Members belong to species complexes, meaning this group is largely monophyletic, with some controversial exceptions (Beardsley and Olmstead 2002). *Mimulus* also has other important attributes that make it a good candidate as a model organism. For example, most species are easily propagated and have short generation times with high fecundity. *Mimulus* also have small genomes, and an amenability to both field and laboratory experiments (Wu, Lowry et al. 2008).

Despite interest in *Mimulus*, most species lack protocols for generating transgenic plants, a key resource for functional genomics. There has been some success with *Agrobacterium*-mediated stable transformation, and transient expression having been used reliably in the *M. lewisii* complex to understand genes controlling floral features that play a part in speciation between members (Yuan, Sagawa et al. 2013, Ding and Yuan 2016). There is also a published transformation protocol for *M. aurantiacus* (Susič, Bohanec et al. 2014). However, the majority of *Mimulus* research groups study members of the yellow flower clade, which lacks an efficient and reliable transformation protocol.

Many genomic tools exist for identification of genomic variants in *M. guttatus*, including a sequenced genome (Wu, Lowry et al. 2008). However, an adequate *Agrobacterium*-mediated transformation protocol is still unavailable, which has widely affected research on this group. Stable transformation in *M. lewisii* was achieved by using a combination of floral spray and vacuum infiltration methods (Yuan, Sagawa et al. 2013). To date, this method has been successfully utilized only once in *M. guttatus* but was unable to be repeated (Sweigart et al. unpublished). Difficulty with replication could be because of the shortcomings of the method, which include the need to infiltrate a large number of flowers then spend extra time hand pollinating. The current vacuum infiltration method is also highly inefficient with an overall low rate of successful stable transformants created (Yuan, Sagawa et al. 2013).

Species within the *M. guttatus* complex belong to the yellow monkey flower clade and are known to show unique differences in mating system (Fishman, Kelly et al. 2002, Sweigart, Mason et al. 2007) and local adaptation to distinct environmental conditions (Lowry, Hall et al. 2009, Lowry and Willis 2012, Wright, Lloyd et al. 2013, Selby and Willis 2018, Mantel and Sweigart 2019) Within the complex, *M. nasutus* is a sister species to *M. guttatus*. They often exist sympatrically but are partially reproductively isolated due to the phenological barrier of flowering time (Kiang and Hamrick 1978, Fishman, Sweigart et al. 2014, Kenney and Sweigart 2016). Although *M. guttatus* and *M. nasutus* are mostly distinct, some level of hybridization is currently happening. The existence of a partial barrier to gene flow has led to an understanding of genetic introgression that selects against *M. nasutus* and in favor of *M. guttatus* (Sweigart and Willis 2003, Martin and Willis 2007, Brandvain, Kenney et al. 2014).

Since a major objective of research on speciation is to understand genes that act as barriers to gene flow (Coyne and Orr 2004), a thorough knowledge of the genes that are involved in flowering time would be just one example of an opportunity to understand the evolutionary significance of a trait. Understanding the genetic and molecular mechanisms that control traits of interest would open the door to more research in this area of study (Wu, Lowry et al. 2008). Further development of the genomic tool-kit available in *Mimulus* is necessary to elucidate genes controlling adaptive divergence and will serve as a key next step in *Mimulus* research. Additionally, this functional tool will enable the creation of mutant libraries, which will serve as a resource for the *Mimulus* community.

Genetic transformation

Transformation usually takes place by the use of *Agrobacterium tumefaciens*, a soil-derived plant pathogen that is used in biotechnology as a way to transfer foreign DNA to plant cells (**Figure 1.1**). Many economically important plants have been improved by the use of *Agrobacterium*-mediated transformation (Ke, Khan et al. 2001).

Historically, *Agrobacterium* was studied to understand the culprit behind plant diseases such as crown gall (Smith and Townsend 1907). *A. tumefacien*s is responsible for galls due to specific genetic elements on the tumor-inducing (*Ti*) plasmid (Smith, Agriculture et al. 1911). This portion of the plasmid is referred to as the virulence region since it contains different genes (*vir* A, B, C, D, and E), which vary in function.

Virulence proteins, encoded by *vir* genes, respond to plant stimuli in different ways, depending on which *vir* genes are present (Winans 1992). Basically, *Vir* proteins take on different regulatory roles, such as the formation of pili that aid in the ability to attach to the host cells (Fullner, Lara et al. 1996). However, not all genotypes are susceptible to transformation infection. Plant species vary in their ability to be infected by particular strains of *Agrobacterium*. The variation in infection capacity of the host could be due to the bacteria’s capacity to attach to the host cell, the ability of the T-DNA to transfer into the cytoplasm of the plant cell, and the T-DNA ability to move or integrate into the nucleus (Gelvin 2000, Wroblewski, Tomczak et al. 2005).

Plant stimuli that induce a response from *A. tumefaciens* involve secondary plant metabolites such as phenolic compounds. Phenolic compounds are produced by the plant in response to stress or pathogen invasion (Clé, Hill et al. 2008). The natural phenolic compound acetosyringone can be used externally to induce expression of *vir* genes by acting as a chemical that is produced during wound defense response (Stachel, Messens et al. 1985).

Another key region on the plasmid is referred to as Transfer-DNA (T-DNA). T-DNA border repeat sequences flank this region and are necessary for *A. tumefacien*s' ability to induce tumor growth (Yadav, Vanderleyden et al. 1982). T-DNA and virulence genes are the two main components of *Agrobacterium* that allow the genetic transformation process to occur. Once the bacteria are attached to the host cell, the cell recognizes the signal, which turns on a gene to produce an endonuclease. The endonuclease aids in the excision of a T-strand of T-DNA. This T-strand is trafficked through the host cell and is ultimately transported to the nucleus and integrated into the host chromosome through the use of a type IV secretion system (T4SS) (Zechner, Lang et al. 2012). T-DNA containing the gene of interest is transferred to the host, but *vir* genes are not.

A binary vector system can be used, whereby a disarmed *Ti* plasmid contains the necessary *vir* genes and an *Agrobacterium* replicon plasmid contains the T-DNA (Bevan 1984). The desired gene(s) to be transformed into the host cell are cloned into the T-DNA region of the plasmid. The virulence genes aid in T-DNA processing and subsequent transfer of the gene(s) of interest to the host cell (Lee and Gelvin 2008).

A proper selectable or visual marker is an essential component of stable transformation. One selectable marker that is frequently used is the hygromycin phosphotransferase gene (*hph*), which allows for positive selection of transgenic plants by using hygromycin B (Gheysen, Angenon et al. 1998). A handful of other selectable markers is available, including a spectinomycin resistance gene. Selection using spectinomycin is not widely used but has been successful in flax (Bretagne-Sagnard and Chupeau 1996). A callus assay reveals the correct concentration, as sensitive cells will lose their pigmentation (Angenon, Dillen et al. 1994).

The vector carries the DNA to a host cell and the desired gene(s) can be expressed either stably or transiently. When transformation is performed transiently, the T-DNA is delivered to the nucleus of the host cells where it is integrated so that only these cells are able to create relevant metabolites and the rest of the plant remains unchanged. This method is high-throughput and used for its ease and versatility when testing transgenes (Wu, Liu et al. 2014). Transient expression does not alter the germline of transformed plants, but instead, expression peaks at 3-4 days post transformation, thus no effort is made to recover transformed plants (Janssen and Gardner 1990).

Transient expression can be done without the use of tissue culture. This method is less labor intensive and can use plants that have been grown in non-sterile conditions. In a binary system, a construct is cloned into a binary vector then transformed into a specific strain of *Agrobacterium*. One method uses a bacterial suspension culture at a specific concentration that is then used to infiltrate the underside (stomata) of young and healthy leaves. This transformation method can be done by either using a syringe or by the use of vacuum infiltration (Leuzinger, Dent et al. 2013). Plants are left to incubate and transgene expression can be quantified three days post infiltration (Lee and Yang 2006). This method has been used reliably in *M. lewisii* with the 35s Cauliflower Mosaic Virus promoter to understand expression and localization of fluorescent proteins (Ding and Yuan 2016).

To obtain stably transformed plants, two criteria must be met. Not only must *Agrobacterium* be able to move DNA into the cell as just described, success is dependent upon both the ability of cells to regenerate and efficiency of a selection agent once DNA has been delivered to the cell. The transformed cells must be totipotent, making them able to regenerate adult plants. Lack of cell totipotency from non-responsive genotypes is a major limiting factor in transformation efficiency. Because not all genotypes are amenable to these criteria, some are not able to be stably transformed. Dependence on genotype is one of the major bottlenecks to stable transformation (Harwood 2011). Genotype specificity in tissue culture could mean that not all *Mimulus* genotypes have the ability to be transformed using this system.

Beyond genotype, there are many factors that can affect transformation efficiency, such as explant type, medium composition, time inoculated with *Agrobacterium*, and *Agrobacterium* concentration (Li, Cong et al. 2017). The particular strain of *Agrobacterium* used for transformation can have a high influence on efficiency. Exploring transformation using specific plant genotypes with various strains of *Agrobacterium* can have significant advantages when trying to increase the transformation efficiency (Allahbakhsh, Anayol et al. 2013). The antibiotic can also have an adverse effect on efficiency by affecting the health of the explant. The antibiotic should be at a high enough concentration to be able to differentiate between transformed and non-transformed cells, while having little or no impact on the transformed cells (Sundar and Sakthivel 2008). Other conditions during co-cultivation can be modified to increase transformation efficiency. Lighting, concentration of acetosyringone, and type and concentration of hormones are all parameters that can be altered in the protocol (Pitzschke 2013).

Auxotrophic strains of *Agrobacterium* can be used when rapid bacterial overgrowth on explants is affecting the transformation efficiency. A mutation is rendered in a gene producing an amino acid or other components essential for growth. This mutation makes it so the bacterium is unable to produce that particular compound. This means the mutated strain cannot survive in the absence of supplementation of that compound. Because the bacterium cannot survive, use of auxotrophic strains can be more desirable for biological containment. Another advantage to using auxotrophic strains is that antibiotic selection is not necessarily required, which can increase transformation efficiency (Ranch, Liebergesell et al. 2012). If auxotrophic strain(s) of *Agrobacterium* can produce similar transformation efficiency to wild-type strains, their use could be beneficial to the optimization of *Mimulus* transformation.

Prior to transformation, *Agrobacterium* inoculation medium must be prepared at a specific bacterial density. Once the experimental plants are grown in sterile conditions and are healthy and at the proper developmental stage, an explant is used as the donor material to be inoculated with *Agrobacterium* inoculation medium. The explant can be many types of tissue, including cotyledonary nodes, leaf nodes, epicotyls, hypocotyls, and root and leaf tips, as long as it is totipotent so that it can regenerate. This means that the explant should be vigorous and young enough to contain differentiating tissue, as an inadequate explant can affect transformation and/or regeneration abilities (Li, Cong et al. 2017).

Once explants have been inoculated with *Agrobacterium*, cocultivation takes place, allowing T-DNA to be transferred to plant cells. If callus formation is desired, the inoculated explant is placed onto callus growth medium containing the proper type and concentration of hormones and a selection agent. Direct regeneration can also be performed. For this method, an explant containing meristematic tissue is placed onto a shoot regeneration medium containing a cytokinin (Skoog and Miller 1957). The selection agent can be in the form of an antibiotic or herbicide. This same medium also contains an antibacterial component, which suppresses the growth of *Agrobacterium*. Cells subjected to selection that have taken up and express the T-DNA will produce healthy calli or shoot primordia. In an organogenic system, calli will then be transferred to a shooting medium containing cytokinin and shoot primordia will be transferred to a lower concentration of cytokinin and allowed to proliferate. Once healthy shoots are formed, the tissue is transferred to a rooting medium containing an auxin and eventually to soil.

The two approaches to *in vitro* plant regeneration are organogenesis, as described, and embryogenesis. Most plants work in one or the other pathway. In organogenesis, explants develop apical meristems before other tissues and organs can be established. With the proper conditions, embryonic cells are formed and can be used to undergo another regeneration pathway, embryogenesis (Steward, Mapes et al. 1958). In embryogenesis, somatic embryos with both a shoot and root meristem are formed on a high concentration of auxin before entire plants can be developed and recovered (Ikeuchi, Ogawa et al. 2016). If the pipeline has a callus phase, regeneration efficiency is calculated by percentage of calli that reach the shooting phase and thus form healthy plants. In direct regeneration, shoots that are large enough to move to rooting medium are used to quantify regeneration efficiency (Prehn, Serrano et al. 2003).

Regeneration of transformed plants from meristematic cells can be advantageous to callus culture due to ease and time saved from omitting the callus induction step. However, one caveat to meristematic tissue culture is the formation of chimeric transformants. While this mosaic phenotype is interesting, the resulting gametes may be non-transformed, so the progeny will not carry the transgene (Chen 2011). If *Mimulus* tends to have a high outcome of chimeric transformants, meristem culture may not be the best strategy to use. However, given the ability to circumvent the callus induction phase, use of meristematic tissue may be beneficial for the *Mimulus* tissue culture pipeline.

There are other strategies to *in vitro* stable transformation such as particle bombardment/biolistics and floral dip (Clough and Bent 1998, Bent 2000, Ozyigit and Yucebilgili Kurtoglu 2020). Particle bombardment is the main alternative to *in planta* stable transformation. Small particles coated in DNA are projected at high speed, penetrating cell walls. Biolistics allows for direct transfer of DNA to multiple cell types to produce stably transformed cells without the use of *Agrobacterium*.

The floral dip and vacuum infiltration method is used to inoculate flowers that then produce transformed seed. This approach is desirable because it is very straight-forward and there is no requirement of sterile technique. Floral dip and vacuum infiltration has been successful in *Arabidopsis* (Bechtold 1993, Clough and Bent 1998, Bent 2000) and *M. lewisii* (Yuan, Sagawa et al. 2013). It can be high-throughput, but labor intensive due to the need to infiltrate large numbers of flowers and time spent pollinating in order to get transformed seeds.

NEGAN and *Mimulus* transformation

An *Agrobacterium*-mediated stable transformation protocol has been developed for *M. lewisii* and has been used reliably (Yuan, Sagawa et al. 2013). Most studies that utilize *Mimulus* transformants rely on the floral spray (Chung, Chen et al. 2000) and vacuum infiltration (Bechtold 1993) method, described in Yuan et al 2013. However, this method has produced a low transformation efficiency in *Mimulus* (Yuan, Sagawa et al. 2013).

Because of *Mimulus*' utility in understanding how pollinator preference is affected by floral pigmentation and patterning (Bradshaw and Schemske 2003, Streisfeld, Young et al. 2013), an understanding of genes involved in pigmentation is relevant. Many studies have been conducted to assess the genetic control of flavonoid expression, particularly anthocyanins (Winkel-Shirley 2001). Genes involved in anthocyanin biosynthesis are regulated by a complex known as MYB-bHLH-WD40 (MBW) (**Figure 1.2**) (Zhang, Gonzalez et al. 2003, Carey, Strahle et al. 2004, Matsui, Umemura et al. 2008).

There are many genes involved in the highly conserved transcriptional regulatory complex. MYB and bHLH gene families interact with many regulatory proteins including those that are anthocyanin-activating (Feller, Machemer et al. 2011). Yuan, Sagawa et al. (2014) used *M. lewisii* to identify one bHLH, one WD40, and two MYB transcription factor genes, *PELAN* and *NEGAN*. *PELAN* controls petal lobe anthocyanin whereas *NEGAN* controls nectar guide anthocyanin. These genes are members of gene families that belong to the MBW complex that regulates anthocyanin expression. In this same study, *NEGAN* was verified as a transcription factor which regulates anthocyanin production in the MYB complex. Verification of the function of *NEGAN* was done through *Agrobacterium*-mediated stable transformation on *M lewisii*. Then, it was found that *NEGAN* is also expressed in the yellow flower clade of *Mimulus*, as it is conserved between these two clades (Yuan, Sagawa et al. 2014). Because *NEGAN* expression can be used as a visual marker for successful transformation, it is a dependable gene to use for *Mimulus* transformation.

CHAPTER 2

DEVELOPMENT OF AN EFFICIENT TISSUE-CULTURE PROTOCOL FOR MIMULUS[1]

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[1] Bell, L., Ueno, S., Parrott, W., Sweigart, A. Development of an Efficient Tissue Culture Protocol for *Mimulus*. To be submitted to Plant, Cell Tissue & Organ Culture.

Abstract

Mimulus is an important model organism used for the study of evolution, adaptation, and speciation. Currently, there is a shortage of genomic tools to use in this influential genus. Specifically, the lack of genomic tools for the yellow-flower clade has become a hurdle for the *Mimulus* scientific community. The optimization of a *Mimulus* tissue culture protocol for use in *Agrobacterium*-mediated transformation is a necessary next step for the yellow flower clade of this model organism.

Towards that end, both petiole and nodal explants were evaluated as part of an effort to develop a transformation system for the yellow-flowered clade. These two explant sources were used to explore two regeneration pathways using two basal media components from 15 inbred lines. Suitable genotypes to be used in subsequent transformation experiments were identified in both portions of the experiment.

Introduction

The genus *Mimulus* accommodates an immense array of phenotypically unique and closely related species. Because of this diversity, *Mimulus* has long been used as a model organism for studies on ecological and evolutionary functional genomics (Wu, Lowry et al. 2008). More recently, *Mimulus* has been of importance for the study of developmental genetics of phenotypic traits among its members (Yuan 2019).

The development of genomic tools is necessary for the *Mimulus* community to be able to elucidate the genetic control of phenotypes of interest. An *Agrobacterium*-mediated leaf infiltration transformation protocol has been utilized for *M. lewisii* (Ding and Yuan 2016). The floral spray (Chung, Chen et al. 2000) and vacuum infiltration method (Bechtold 1993) has been employed for recovery of stable transformants of *M. lewisii* as well (Yuan, Sagawa et al. 2013). However, neither of these protocols are suitable for the heavily studied yellow flower clade of *Mimulus*.

Mutant libraries via transformation have also been created for this species and the closely related sister species *M cardinalis* (Yuan, Sagawa et al. 2013, Yuan, Sagawa et al. 2014, Yuan 2019). While there is a possibility to recover stable transformants using the previously mentioned approaches, there is only one protocol published for *in vitro Agrobacterium*-mediated transformation for use in *M. aurantiacus* (Susič, Bohanec et al. 2014) and one publication entailing in vitro work on *M. guttatus* (Ding, Patterson et al. 2020).

Objective

The aim of this experiment was to select for two types of *Mimulus* explants, petioles and nodes, the combinations of genotype and basal medium that performs best in tissue culture. Two basal media and fifteen genotypes were evaluated. For the evaluation, number of live explants, quality of callus, and number of shoots were considered. This experiment exists in two parts for the evaluation of both nodal and petiole explants.

Materials and Methods

Fifteen genotypes from the yellow-flower clade of *Mimulus* were evaluated in this study. These genotypes consisted in seven annual *M. guttatus,* three perennial *M. guttatus*, and four lines of *M. nasutus* (**Table 2.1**).

Table 2.1: Ecotype or species name, respective line name, collector, and seed custodian.

Ecotype/species	**Line**	**Collector**	**Seed custodian**
Annual *M. guttatus*	CAC110	A. Sweigart	Sweigart lab
Annual *M. guttatus*	CAC134	A. Sweigart	Sweigart lab
Annual *M. guttatus*	CAC262	A. Sweigart	Sweigart lab
Annual *M. guttatus*	CAC415	A. Sweigart	Sweigart lab
Annual *M. guttatus*	IM62	J. Willis	Willis lab
Annual *M. guttatus*	IM274	J. Willis	Willis lab
Annual *M. guttatus*	IM767	J. Willis	Willis lab
Annual *M. guttatus*	MED102	B. Blackman	Willis lab
Mostly annual *M. guttatus*	CAC6	A. Sweigart	Sweigart lab
Perennial *M. guttatus*	DUN	D. Lowry/K. Wright	Lowry lab
Perennial *M. guttatus*	SWB		
M. nasutus	CAC9	A. Sweigart	Sweigart lab
M. nasutus	CAC22	A. Sweigart	Sweigart lab
M. nasutus	CAC27	A. Sweigart	Sweigart lab
M. nasutus	SF	K. Ferris/J. Selby	Willis lab

To obtain sterile plants for the experiment, the seeds were sterilized using a protocol developed by Rachel Kerwin (personal communication). The sterilization was performed by placing ~25 seeds in a 1.7-ml microcentrifuge tube with 1.2% sodium hypochlorite and 0.05% detergent solution. This was followed by 10 minutes of inversion and four washes with sterile water. Then, the seeds were suspended in sterile water and vernalized (4 °C) for seven days. The sterile seeds were germinated in 23 °C with a 16/8 hour light cycle using Halco brand T8 fluorescent bulbs. Seeds were kept on 100x20 mm Petri plates containing medium consisting of ½-strength Murashige and Skoog (MS) plus 10 g L^{-1}

sucrose and 3.0 g L^{-1} Gelzan™ at pH 6.0. The plants were allowed to grow in these conditions for a duration of six weeks before use in the experiment.

Petiole explants (**Figure 2.1**) were excised from six-week-old plants before being placed in two different basal medium components with low or high ion concentration: Gamborg's (B5) and MS, respectively to evaluate callus growth. All media employed B5 vitamins. Media was in 100x15 mm Petri plates and also contained 4.44 µM BAP plus 5.37 µM NAA, 20 g L^{-1} sucrose, and 3 g L^{-1} Gelzan™ at a pH of 6.0.

The plates containing the explants were placed in 24 °C with a 16/8 hour light cycle using Philips brand fluorescent 40W T-12 bulbs at PAR 66-95 µE m^{-2} s^{-1}. Explants were kept on this medium for a total duration of eight weeks with a subculturing step at four weeks. Data was collected at both time points.

After a total of eight weeks, viable callus was transferred to 100x15 mm Petri plates containing shoot induction medium and kept at 23°C with a 16/8 hour light cycle using Halco brand T8 fluorescent bulbs. Shoot induction medium consisted of half strength MS salts, 10 g L^{-1} sucrose, and 3.0 g L^{-1} Gelzan™ at a pH of 6.0. Data was once again collected after four weeks on this medium.

The experiment was conducted in completely randomized design and consisted of two replications of each treatment (combination of basal medium and genotype). The experimental unit was composed of nine petiole explants oriented adaxial side up placed in a 100x15 mm Petri dish. In total, 30 treatments (15 genotypes on 2 basal media) were evaluated with two replications (**Figure 2.2**).

Similar to petioles, nodal plant material was obtained using the previously mentioned methods (**Figure 2.1**).

Figure 2.1: CAC415 showing the portion of the plant where the petiole explant (left) and the nodal explant (right) were excised.

Nodal explants were excised from six-week-old plants before being placed in two different basal medium components with low or high ion concentration: Gamborg's (B5) and MS, respectively, to evaluate shoot induction. All shoot induction media employed B5 vitamins. Shoot induction media was in 100x15 mm Petri plates and also contained 4.44 μM BAP, 10 g L^{-1} sucrose, and 3 g L^{-1} Gelzan^{TM} at a pH of 6.0.

Explants were kept on shoot induction media for eight weeks with a subculture step at four weeks. Data was collected at both time points.

Explants were then moved onto 100x20 mm Petri plates containing ½ strength MS or ½ strength B5 supplemented with 0.444 μM BAP, for an additional four weeks of shoot proliferation. Plates were kept in 23 °C with a 16/8h light cycle using Halco brand T8 fluorescent bulbs.

Like petioles, the nodal portion of the experiment was conducted in completely randomized design and consisted of two replications of each treatment (combination of basal medium and genotype). The experimental unit was composed of nine nodal explants oriented adaxial side up placed in a 100x15 mm Petri dish. In total, 30 treatments (15 genotypes on 2 basal media) were evaluated with two replications (**Figure 2.2**).

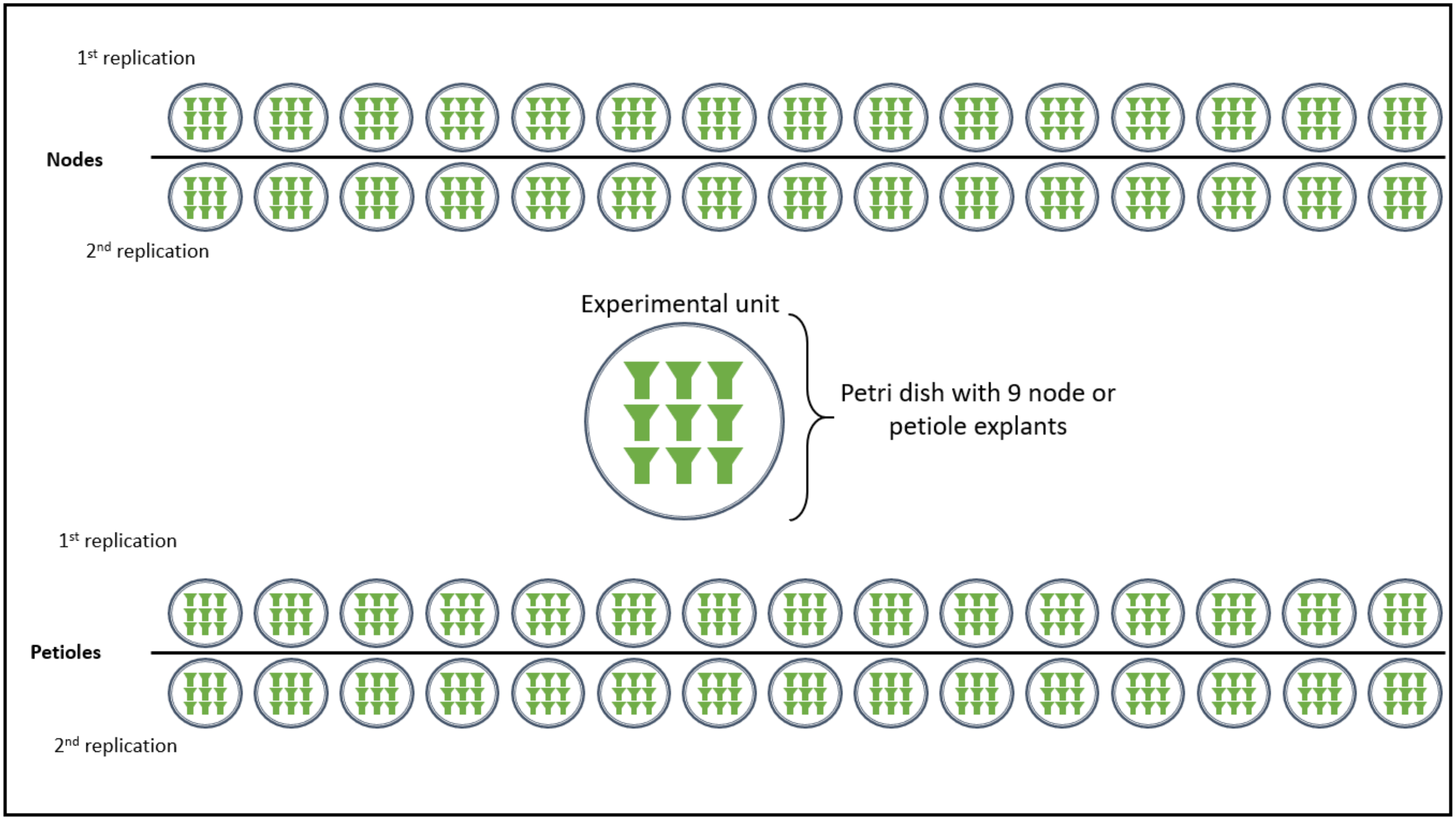

Figure 2.2: Experimental design to evaluate for two types of *Mimulus* explants, petioles and nodes, the combinations of genotype and basal medium that performs best in tissue culture.

The following traits were evaluated in petioles: number of live explants in the end of the fourth week and number of live explants and quality of calli at the end of the eighth week. The quality of the calli was defined by quality scale, ranging between 1 and 5 (**Figure 2.3**). A rating of 1 was given for tissue that was dead. Explants received a rating of 2 if any part of the tissue was living or if the response of the cells was minimal but transparent and ‘watery’. A rating of 3 was given if the tissue was alive and responsive. The growth was not clear/watery and instead green and dense. A rating of 4 was given when growth was adequate, green, and dense. Finally, a rating of 5 was reserved for the best callus.

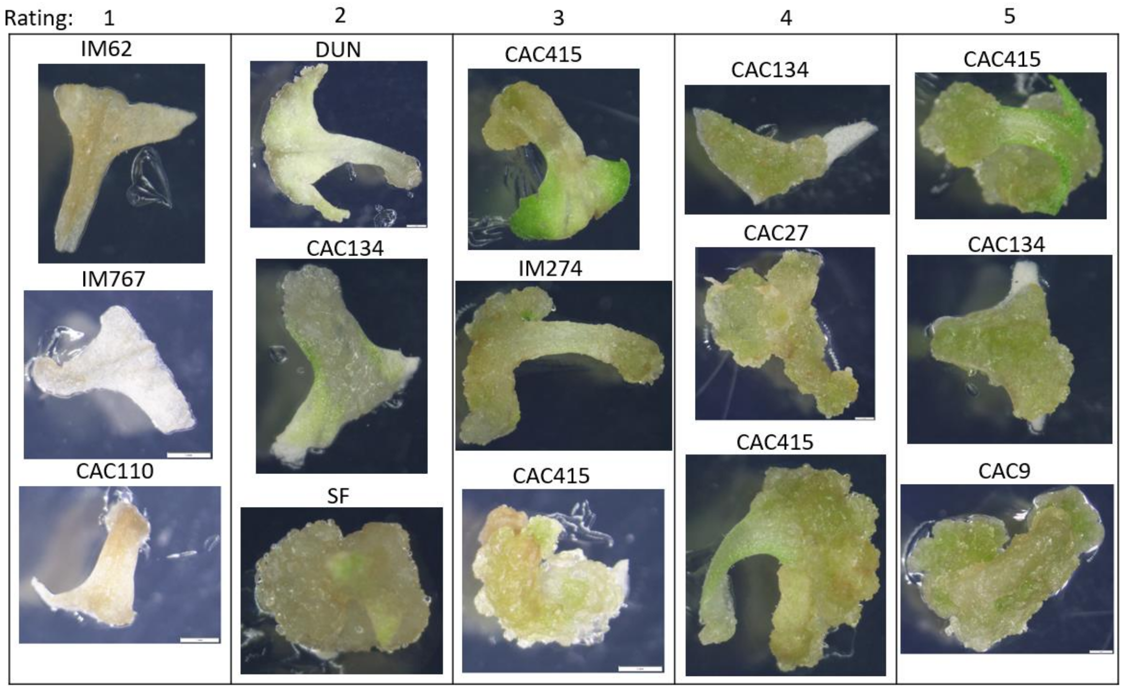

Figure 2.3: Representative callus for each rating. Callus was rated based on quality at four- and eight-week time points.

For nodes as explants, the traits evaluated were number of live explants at the end of the fourth week; number of live explants, quality and number of shoots at the end of the eighth week. The quality of calli was defined by a quality scale, ranging between 1 to 5, set up based on the pictures taken from the experiment (**Figure 2.4**). A rating of 1 was given for tissue that was alive but did not produce shoots. A rating of 2 was given for explants that produced 4 or under shoots or if the explant produced more than 4 shoots but was yellowed. A rating of 3 was used when the explant contained over 4 shoots or if the explant met the rating qualifications for 4 but was yellowed. A rating of 4 was given to explants that contained an uncountable number of shoots or if the explant met qualifications for a rating of 5 but was yellowed. A rating of 5 was reserved for explants containing too many shoot apices to count and the growth of the explant was so large, it was out of frame.

The other rating scale noted the determination of number of shoots by estimation. The following criteria were used: no shoots (1), up to half full of shoots (2), half full or more of shoots (3), full of shoots (4). This means that the evaluation focused on the “fullness” of the explant. If the original explant was less than about 50% visible and thus more than approximately 50% covered in shoots, it received a rating of 2, and so on and so forth.

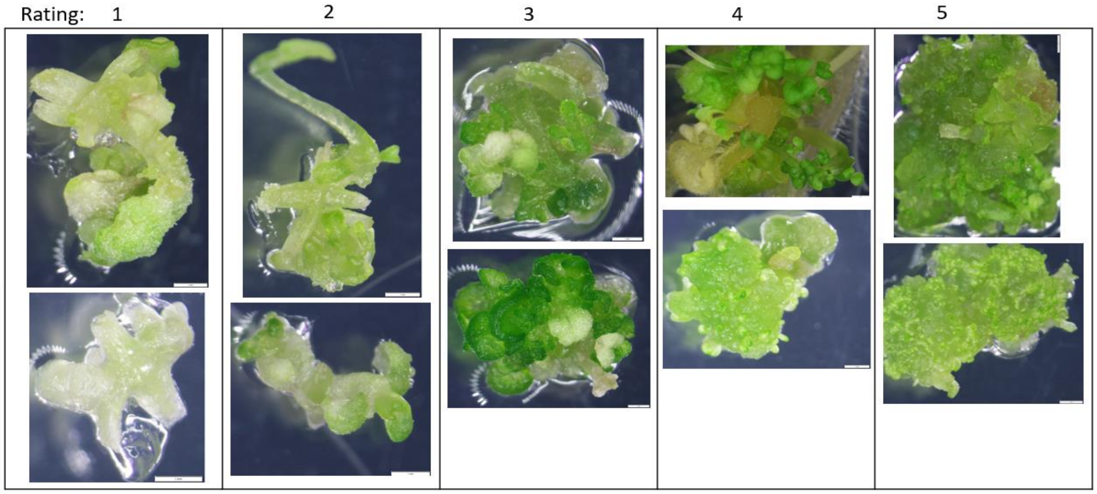

Figure 2.4: Rating scale images used for the node quality scale. Nodes were rated at the end of eight weeks.

To evaluate whether the basal medium, genotype, or its interaction plays a role in the differential number of live petiole explants, an analysis of variance (ANOVA) was performed for the number of live explants at the end of the eighth week. The analysis was conducted with only the eighth week data set due to similarities between the fourth and eighth week's data and also due to the fact that there was no significant interaction between the factor week and the other factors (genotype and basal medium). In addition, four genotypes were excluded from the data set: IM62, SWB, CAC6, and CAC262 because no explants of these genotypes survived on any medium.

Following the ANOVA, Sidak's multiple comparison test (Sidak, 1967) was performed to select the best treatments (combinations between media and genotypes). The ANOVA for quality rating of callus were performed with the best combinations of genotype and medium. For determination of a ranking with the best combinations, Duncan's multiple range test (Duncan 1955) was conducted.

Because the residuals for the number of live nodes did not follow a normal distribution, even after the genotypes that did not survive in any medium were discarded, a Box-Cox transformation was performed. This was followed by a non-parametric analysis. A Kruskal–Wallis test (Kruskal 1952) was employed to verify a significant effect of the medium and genotype combination on the trait. This approach is not capable of verifying the medium and genotype interaction, just the combination of these two factors. Considering this, these following combinations from the data set were excluded: MS_IM62, MS_IM767, B5_SF, B5_IM62, B5_IM767, B5_CAC9, B5_CAC22, B5_CAC27, B5_CAC6, B5_CAC262. None of the explants in these mentioned treatments survived.

To evaluate if the factors medium or genotype play a role in the quality rating of callus and number of shoots, an ANOVA was performed for both traits. Additionally, the Pearson correlation coefficient was used to calculate between quality rating of callus and number of shoots to verify if the treatments could be selected based on only one of these traits. Duncan's multiple range test (Duncan 1955) was applied to determine a ranking with the best combinations. All the statistical analyses were performed using RStudio (RStudioTeam 2020).

Results: Petiole explant experiment

The analysis of variance was conducted with only the eighth week data set due to similarities between the fourth and eighth weeks and because there was no significant interaction between the factor of week and the other factors (genotype and basal medium). The results showed a significant effect of genotype and medium and for their interaction

(**Table 2.2**). On average, the genotypes on MS medium (6.77 live explants, average per plate) performed better than those on B5 (2.09 live explants). Although all genotypes except DUN performed better in MS medium, the significant interaction between medium and genotype could be due to the differences among the genotype's relative performance in both media. Some genotypes suffered a drastic negative impact in B5. Others performed just slightly worse in B5 in comparison to MS (**Figure 2.5**).

Table 2.2: Mean squares and respective significance levels of the analysis of variance for number of live explants at the end of 8th week.

Source of variation	**Df**	**Mean Sq**	**F value**
Medium	1	241.108	93.8851**
Genotype	10	12.740	4.9469**
Medium : Genotype	10	13.413	5.2230**
Residuals	22	2.568	

** Significant at 1% probability by *F* test.

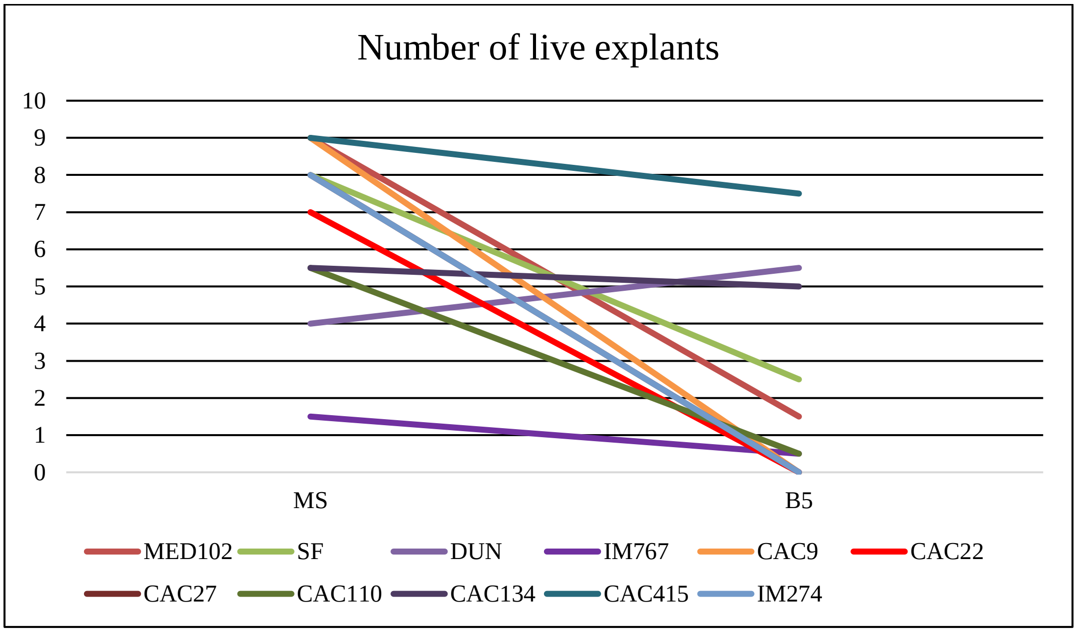

Figure 2.5: Mean of number of live explants of 11 genotypes in MS and B5 basal medium.

From the 22 treatments evaluated, the Sidak's multiple comparison test (**Figure 2.6**) was able to just separate the six best treatments from the worst three treatments. Considering the lack of statistical power to differentiate the treatments, a cutoff among the treatments was established. All treatments that resulted in less than six live explants on average were discarded. The cutoff number can be justified due to the need of selecting suitable genotypes that can produce quality calli in a reasonable number. Using this approach, from the initial 22 treatments evaluated, eight genotype/medium combinations were selected: MS_MED102, MS_SF, MS_IM274, MS_CAC9, MS_CAC22, MS_CAC27, MS_CAC415, and B5_CAC415.

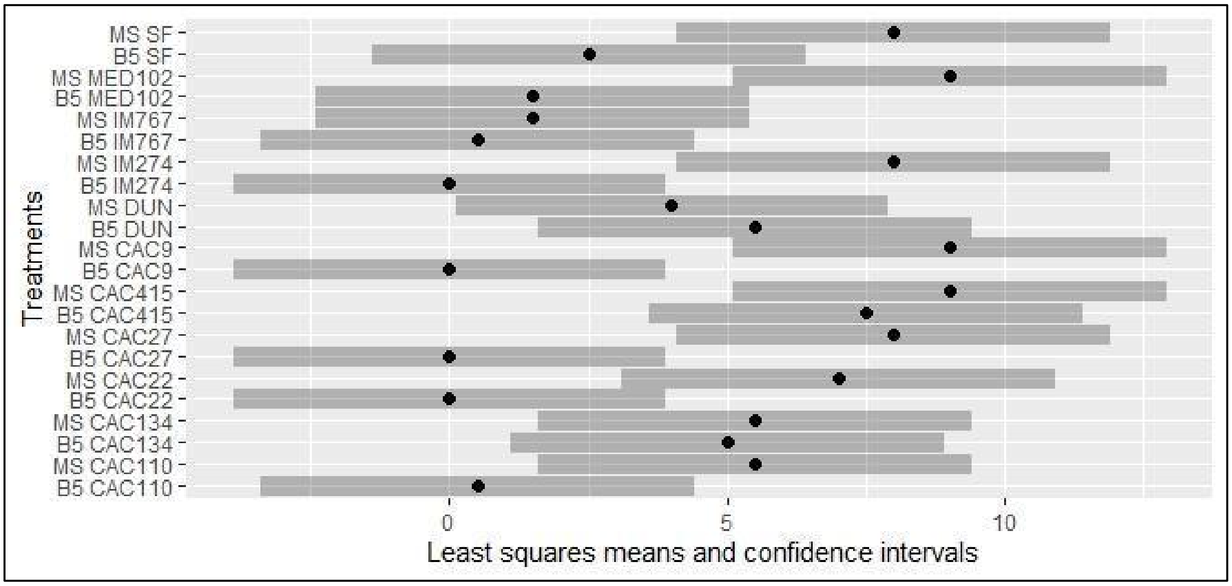

Figure 2.6: Mean of number of live explants for each combination of medium and genotype. Bullets indicate the mean and error bars are +/- 1 standard error. Means overlapping the error bars do not differ significantly at the 95% confidence level based on the Sidak mean comparison method.

The analysis of variance for quality rating of callus revealed a significant effect for medium and genotype combinations (**Table 2.3**). According to the Duncan's multiple range test, the treatments MS_SF, MS_CAC27 and MS_CAC415 performed better than the treatments B5_CAC415, MS_MED102 and MS_IM274. The treatment MS_CA22 performed better than MS_MED102 and MS_IM274 while the treatment MS_CAC9 was not significantly different from any except MS_IM274. The other comparisons did not show significant differences for quality rating (**Figure 2.7** and **Table 2.4**).

Based on this information, the basal medium MS and the genotypes SF, CAC27, CAC415, CAC22 and CAC9 are the most suitable to be incorporated in the *Mimulus* transformation pipeline when petioles were used as explants (**Figure 2.8**).

Table 2.3: Mean Squares and respective significance levels of the analysis of variance for quality rating of callus at the end of 8th week.

Source of Variation	**Df**	**Mean Square**	**F value**
Medium and genotype combination	7	0.9094	6.554**
Residuals	8	0.1387	

** Significant at 1% probability by *F* test.

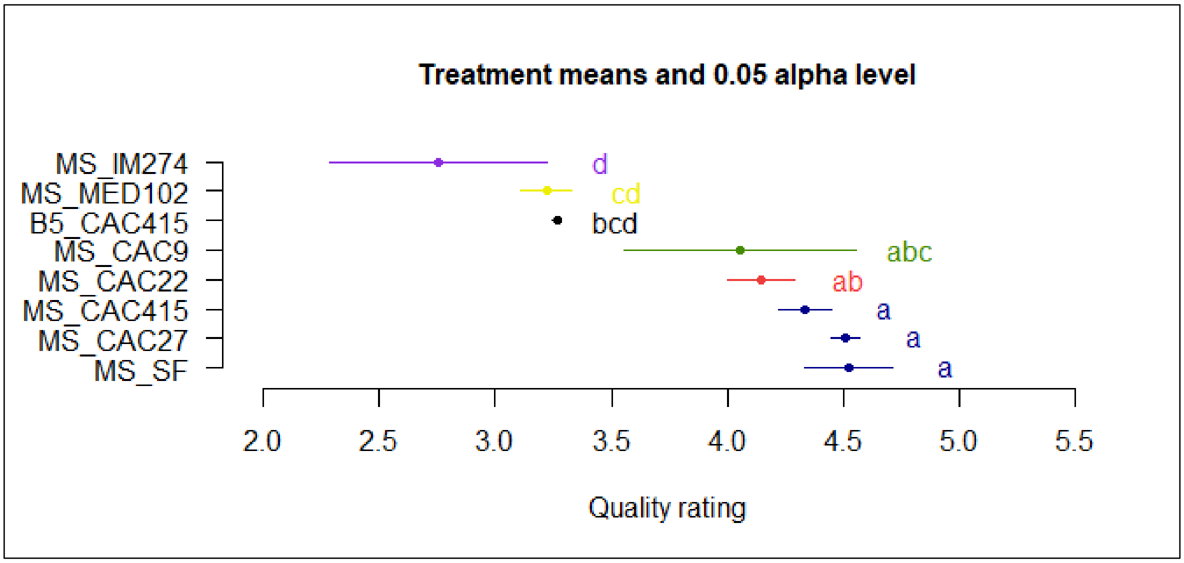

Figure 2.7: Mean of quality rating of callus for each combination of medium and genotype. Bullets indicate the mean and error bars are +/- 1 standard error. Means sharing a letter are not significantly different.

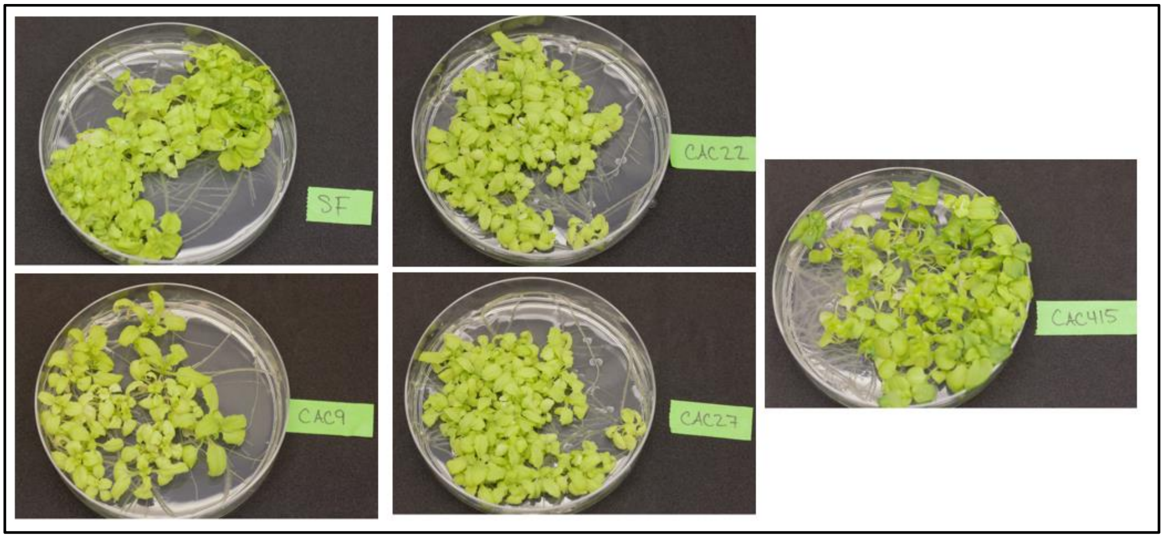

Figure 2.8: Genotypes selected as fit for use as petiole explant sources. Included are four *M. nasutus* and one *M. guttatus*.

Table 2.4: Treatment, average number of live calli, average quality rating, and respective grouping code based on Duncan's multiple range test.

Treatment	**Avg number of live callus**	**Avg quality rating**	**Duncan's Test**
MS_SF	8	4.5238	a
MS_CAC27	8	4.5079	a
MS_CAC415	9	4.3333	a
MS_CAC22	7	4.1429	ab
MS_CAC9	9	4.0556	abc
B5_CAC415	7.5	3.2679	bcd
MS_MED102	9	3.2222	cd
MS_IM274	8	2.7540	d

Results: Nodal explants experiment

On average, the number of live explants per plate was 7.5, ranging from 3.5 to 9. The Kruskal-Wallis test showed no significant effect of medium and genotype combination for the number of live explants (P-value = 0.1821), suggesting no statistical differences in performance among the treatments. Considering the lack of statistical power to differentiate the treatments, the same approach that was applied to petioles was followed, establishing a cutoff among them. All treatments that resulted in less than six live explants on average were discarded. Based on this cutoff, four treatments were excluded: MS_DUN, MS_CAC22, B5_IM274 and B5_SWB.

The ANOVA revealed a significant effect for both quality rating of the explant and number of shoots (**Table 2.5**). These results indicate that at least one of the treatments had a differential performance regarding the quality and number of shoots. Also, the highly significant and positive Pearson correlation coefficient between both traits (R = 0.96, p = $2.2e^{-16}$) suggests that there was no need to use both traits to evaluate the explant performance, one would be enough (**Figure 2.9**). In this case, number of shoots would be more recommended, as it is a more straightforward trait for being evaluated than quality rating, which requires a customized scale set up for every explant in the experiment.

Table 2.5: Sources of variation, degrees of freedom (Df), mean squares (MS), F values and respective significance levels of the analysis of variance for quality rating of callus and number of shoots at the end of the 8th week.

		Quality rating of callus		Number of shoots	
Source of Variation	Df	MS	F value	MS	F value
Medium/ genotype combination	15	2.1512	15.421**	1.4452	9.3279**
Residuals	16	0.1395		0.1549	

** Significant at 1% probability by *F* test.

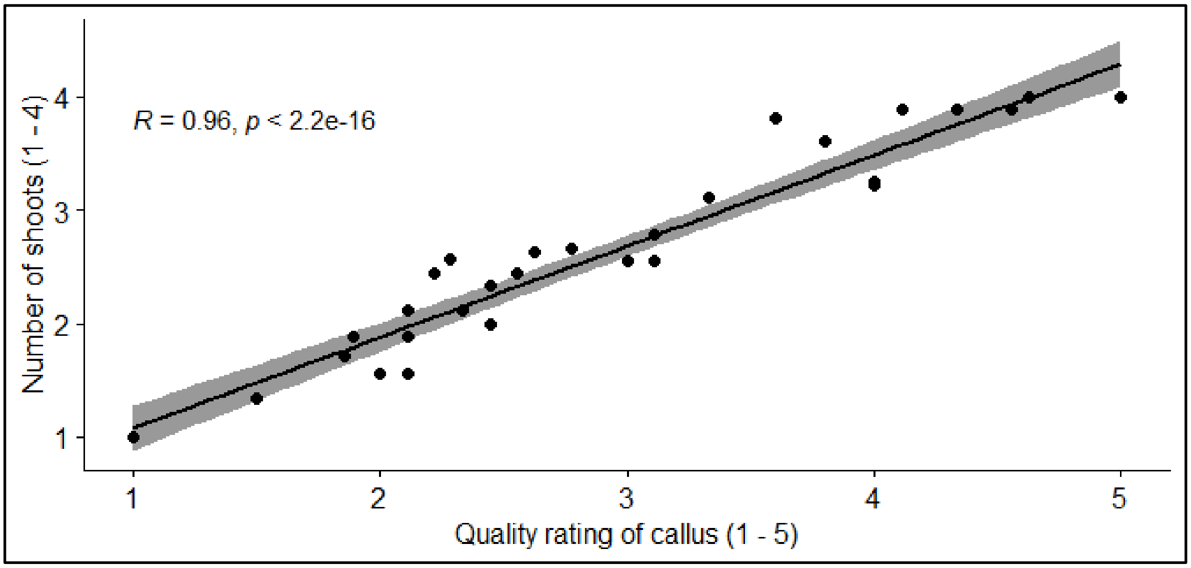

Figure 2.9: Pearson correlation coefficient (R) between quality rating and number of shoots, P significance of the correlation (p).

Considering the number of live explants and the Duncan's Multiple Range test for quality rating and number of shoots (**Figures 2.10** and **2.11**, **Table 2.6**), MS basal medium and the genotypes MED102, CAC9, CAC27, and SF are the most suitable to be used as source of nodes in the *Mimulus* transformation pipeline (**Figure 2.12**).

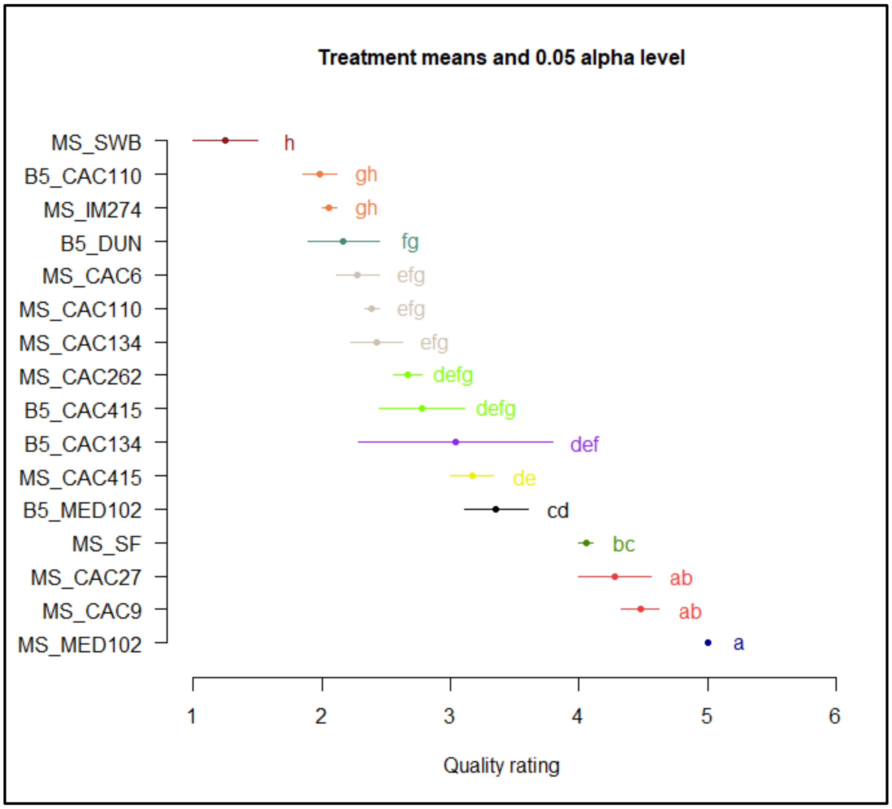

Figure 2.10: Mean of quality rating of explants for each combination of medium and genotype. Bullets indicate the mean and error bars are +/- 1 standard error. Means sharing a letter are not significantly different.

Table 2.6: Treatments, number of live explants, and Duncan's Multiple Range test for quality rating and number of shoots (α=0.05).

Treatment	Avg number of live explants	Avg quality rating		Avg number of shoots	
MS_MED102	6	5.00	a	4.00	a
MS_SF	9	4.06	bc	3.56	ab
MS_IM274	9	2.06	gh	1.56	ef
MS_SWB	7.5	1.25	h	1.17	f
MS_CAC9	8.5	4.48	ab	3.94	a
MS_CAC27	8.5	4.28	ab	3.57	ab
MS_CAC6	9	2.28	efg	2.11	de
MS_CAC110	9	2.39	efg	2.06	def
MS_CAC134	8	2.42	efg	2.53	cd
MS_CAC262	9	2.67	defg	2.56	cd
MS_CAC415	9	3.17	de	2.83	bcd
B5_MED102	7	3.36	cd	3.18	abc
B5_DUN	9	2.17	fg	1.94	def
B5_CAC110	8	1.98	h	1.91	def
B5_CAC134	6	3.04	def	3.09	abc
B5_CAC415	9	2.78	defg	2.39	cde

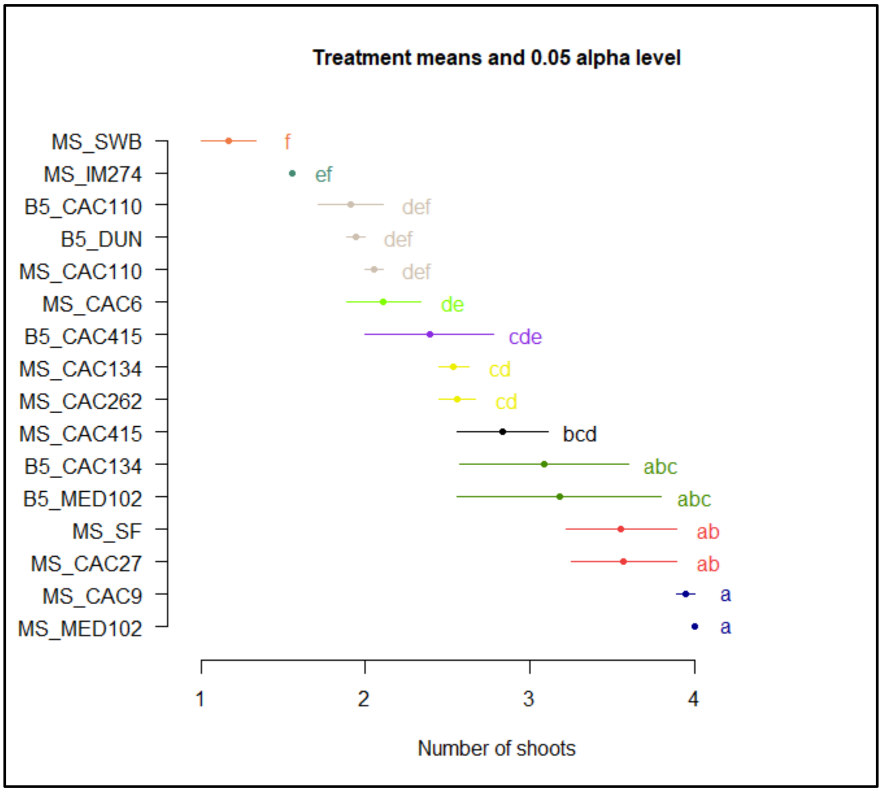

Figure 2.11: Mean of number of shoots for each combination of medium and genotype. Bullets indicate the mean and error bars are +/- 1 standard error. Means sharing a letter are not significantly different.

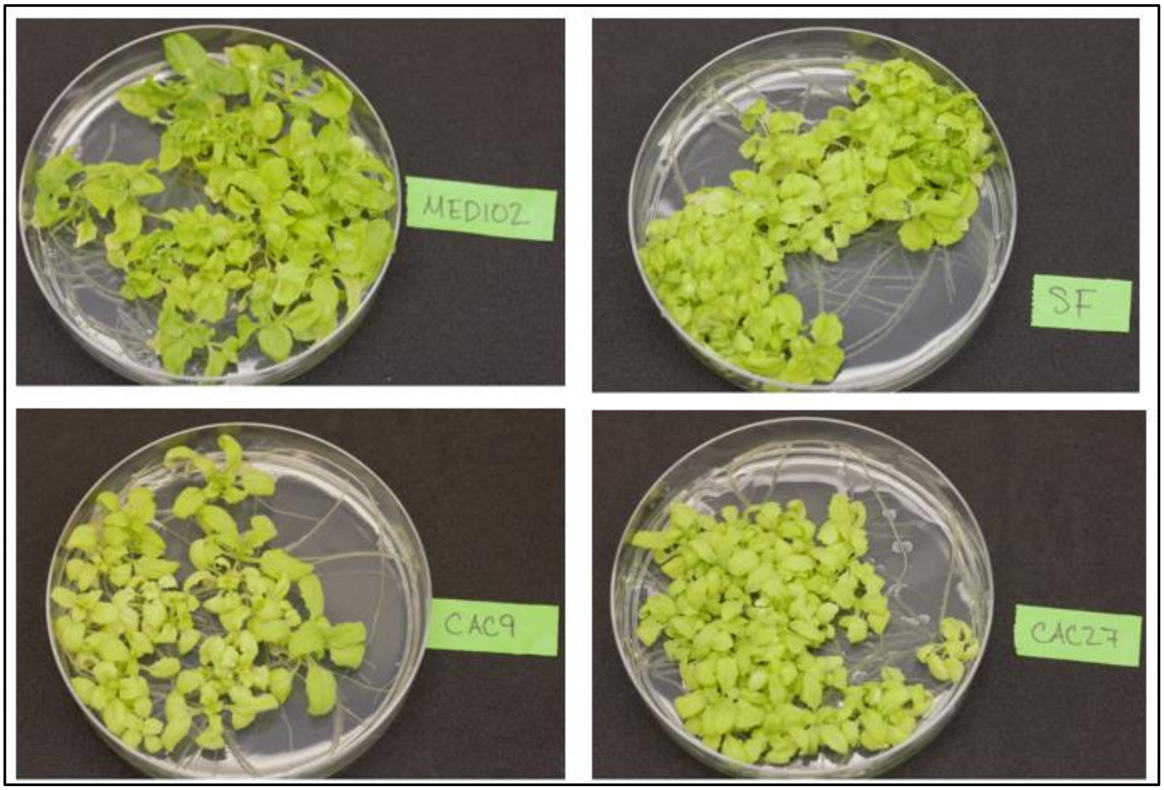

Figure 2.12: Selected genotypes for node explant sources. Included are one *M. guttatus* and three *M. nasutus*.

Discussion

Based on the data, it is obvious that MS is the proper choice for the basal medium component when working with *Mimulus*, regardless of explant source. Petioles from one genotype, DUN, performed slightly better with B5 basal medium. The difference in performance between the two basal media components explains the significance of the interaction between genotype and medium that was found in the ANOVA for quality rating of callus using petiole explants. CAC415 using B5 basal medium was one of the eight highest ranking genotypes using nodal explants. However, CAC415 using MS basal medium performed significantly better. The exploration of different explant sources and thus more than one regeneration pathway is worthwhile considering the ability to circumvent the callus phase in direct regeneration but also the risk of development of chimeric transformants in subsequent experiments. Based on the results of this experiment, genotypes MED102, SF, CAC9, and CAC27 were chosen for transformation.

CHAPTER 3

OPTIMIZATION OF AN AGROBACTERIUM-MEDIATED TRANSFORMATION PROTOCOL FOR MIMULUS[2]

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[2] Bell, L., Ueno, S., Parrott, W., Sweigart, A. Optimization of an *Agrobacterium*-mediated transformation protocol for *Mimulus*. To be submitted to Plant, Cell Tissue & Organ Culture.

Abstract

Interest in the yellow-flowered clade of *Mimulus* has grown in recent times. Specifically, enthusiasm for an understanding of the genetic control of phenotypic traits has been on the rise. Because of this, there is value in a contribution to the genomic tools available to the *Mimulus* community. This experiment tests different parameters of *Agrobacterium*-mediated transformation using two regeneration pathways and includes previously selected genotypes from the yellow-flowered clade of *Mimulus*.

Introduction

Much work has been done in the realm of evolutionary and ecological functional genomics for members of the genus *Mimulus* (Wu, Lowry et al. 2008). *Mimulus* holds value in its phenotypic variation among its members that is unparalleled in the model organism *Arabidopsis*. As enthusiasm increases in the elucidation of phenotypic traits of interest, more genomic tools are needed (Yuan 2019).

More work in the realm of genetic control of unique phenotypes has been done with the species *M. lewisii* and *M. cardinalis* than on other members of the *Mimulus* genus. These species are sister species in the *M. lewisii* complex of *Mimulus*, as opposed to the yellow flower clade (Bradshaw, Wilbert et al. 1995, Bradshaw, Otto et al. 1998, Yuan, Sagawa et al. 2013, LaFountain, Frank et al. 2015) However, members of the yellow flower clade are used for many other molecular studies (Oneal, Willis et al. 2016, Pantoja, Simón-Porcar et al. 2017, Zuellig and Sweigart 2018, Kerwin and Sweigart 2019, Twyford, Wong et al. 2020). For this reason, it would be of utmost use to the *Mimulus* community for plant transformation strategies to exist for the yellow-flower clade.

Plant transformation has long been used as a means to introduce desirable traits, such as resistance to disease (LaFountain, Frank et al. 2015, Dong and Ronald 2019), insects (Sharma, Sharma et al. 2004), and abiotic stresses (Bansal, Singh et al. 2012). Many methods exist including particle bombardment (Sanford 1990) and *Agrobacterium*-mediated transformation (Gelvin 2003). The latter technique has been employed reliably to generate transgenic *Arabidopsis* (Wang, Yaghmaiean et al. 2020). Transformation can take place *in planta* with no tissue culture step involved (Harrison, Mott et al. 2006). Bechtold et al 1998 was responsible for advancing *Arabidopsis* transformation by the use of vacuum infiltration. In this method, the meristematic tissue of an immature inflorescence is the target for transformation, making it so the transgene can be selected for in the progeny. Stable transformants are selected for by applying the proper antibiotic or herbicide for the selectable marker in the germination medium (Bechtold 1993). *In planta* transformation can also be done without the need for vacuum infiltration, where the *Agrobacterium* suspension plus surfactant alone is satisfactory (Clough and Bent 1998). The advantages of this method lie in its ease. The floral spray method requires no tissue culture techniques or regeneration step, which also saves time. *In-planta Agrobacterium*-mediated transformation remains a valuable tool for the creation of transgenic plants (Nakashima, Satoh et al. 1998, Shinwari, Nakashima et al. 1998).

For these reasons, *in planta* transformation has been the main method utilized for *Agrobacterium*-mediated transformation of *Mimulus* species (Yuan, Sagawa et al. 2013).

M. lewisii and *M. cardinalis* are sister species that are often used to study reproductive isolation (Ramsey, Bradshaw et al. 2003). They occur sympatrically but are not known to hybridize in the wild due to their floral morphology's impact on pollinator choice (Bradshaw and Schemske 2003). Fine-scale mapping and *in planta* transformation has been used to characterize the genetic control of the pigmentation of these two species. *ROSE INTENSITY1* (*ROI1*) was found to be the causal gene for a floral anthocyanin QTL in both species. Repression is dominant for this trait, meaning *M. cardinalis* has the recessive allele so that it produces a higher concentration of anthocyanin than *M. lewisii*. To verify its control, an RNAi plasmid was used in wild-type *M. lewisii* (line LF10). This was also demonstrated by the insertion of *ROI* in a homozygous recessive near-isogenic LF10 line (Earley, Haag et al. 2006). *Agrobacterium* strain GV3101 was used with a floral spray and vacuum infiltration protocol, followed by selection with glufosinate (Bechtold 1993, Chung, Chen et al. 2000). While this system was able to produce stable transformants, efficiency was found to be ~0.02% (Yuan, Sagawa et al. 2013).

Genetic regulation of floral anthocyanins in *Mimulus* has been further established using stable transformants of *M. lewisii*. Transcriptome sequencing and analysis was employed to identify three possible genes controlling anthocyanin expression. These three genes were verified through *in planta* transformation. Mutants were identified that have loss of function genes for each member of the MBW anthocyanin regulatory complex. These plants were then sequenced to find that they did have a mutation from each of the three MBW families that were suspected. The genes were subsequently named: *Petal Lobe Anthocyanin* (*PELAN*), *MIWD40a*, and *MIANbHLH1*. Since *PELAN* was found to only affect pigments in the petals but not spots in the nectar guide, a paralog was identified that was expressed in the corolla tube. This paralog was named *Nectar Guide Anthocyanin* (*NEGAN*). It was postulated that *NEGAN* was involved in a self-regulatory feedback loop..

Stably transformed lines expressing *NEGAN* were obtained using the *in planta* method described previously, and *NEGAN* was verified to be the transcriptional activator in an autocatalytic feedback loop regulating spot formation in the nectar guide of *M. lewisii.*

M. guttatus, a member of the yellow flower clade, does not contain anthocyanin in its petal lobes but does express anthocyanin in the corolla. The pigmentation in the corolla is controlled by *NEGAN*, as this gene is conserved between these two clades of *Mimulus* (Yuan, Sagawa et al. 2014). It was later found that *Red Tongue* (*RTO*) is the transcriptional inhibitor in this feedback loop responsible for anthocyanin spot patterning in the nectar guide of both *M. lewisii* and *M. guttatus*. This was verified by the use of an overexpression construct containing *RTO* on *M. lewisii* (LF10) to create a mutant with no anthocyanin spotting in the corolla. To do this, the floral spray method combined with vacuum infiltration was employed (Ding, Patterson et al. 2020). This technique continues to be utilized in other *Mimulus* transformation experiments with an outcome of low efficiency (Zheng, Om et al. 2021).

Ding, Patterson et al (2020) also verified the self-regulatory feedback loop several ways in *M. guttatus,* line MAC. *RTO* and *NEGAN* RNAi and CRISPR constructs were used in *Agrobacterium*-mediated transformation of callus, not *in planta* transformation. The *Agrobacterium* strain EHA105 was used for the transformation. In this method, petiole explants were excised from sterile grown plants and briefly dipped in *Agrobacterium* suspension containing half strength MS salts plus 200 μM acetosyringone. Cocultivation took place in the dark, for three days, on growth medium containing MS salts plus 1 mg L^{-}

^{1} CPPU and 100 μM acetosyringone. Following this, petioles were washed with 100 mg L^{-1} timentin and 50 mg L^{-1} cefotaxime for bacterial elimination. Explants were then cultured on callus induction medium containing MS salts plus 1 mg L^{-1} CPPU and 6 mg L^{-1} phosphinothricin for a total of 25-28 days before transferring to shoot induction medium containing MS salts plus 0.1 mg L^{-1} meta-topolin. To allow rooting, shoots were transferred to MS medium containing 0.1 mg L^{-1} 1-napthalene acetic acid plus phosphinothricin before moving on to soil (Ding, Patterson et al. 2020).

Susič, Bohanec et al. (2014) also achieved *in vitro Agrobacterium*-mediated stable transformation in *M. aurantiacus*. For this study, CAMBIA binary plasmids containing the CaMV 35s promoter were made with a variety of reporter genes and the *hph* marker gene in *Agrobacterium* strain LBA4404. Leaf, petiole, and hypocotyl explants were suspended in *Agrobacterium* plus 200 μM acetosyringone for 15 minutes. Following this, explants were co-cultivated in the dark for four days on MS medium plus 200 μM acetosyringone and 1 mg L^{-1} BAP, 0.5 mg L^{-1} 1-naphthaleneacetic acid. Then explants were washed with 200 mg L^{-1} timentin before being transferred to plates containing MS medium plus 150 mg L^{-1} of timentin and a variable amount of hygromycin, depending on the explant. Explants were kept on this medium for a minimum of eight weeks with intermittent subculturing before moving to MS medium plus 0.1 mg L^{-1} BAP, 0.1 mg L^{-1} IAA and 150 mg L^{-1} timentin. As mentioned, leaf, petiole and hypocotyl explants were used as a means to understand transformation efficiency of all three explant types using this method. Petiole explants had a 23.26% efficiency while leaf explant had a 1.48% efficiency and hypocotyls 1.37% (Susič, Bohanec et al. 2014). This highlights the value in exploration of different explant types.

The previously outlined studies utilize different strains of *Agrobacterium* with different genotypes of *Mimulus*. Choice of *Agrobacterium* strain can affect the success of the transformation. The ability of *Agrobacterium* to infect the plant cell is known to be both genotype and strain-specific in other plant species (Hobbs, Jackson et al. 1989, Delzer, Somers et al. 1990, Mishra, Sreenath et al. 2008, Petti, Wendt et al. 2009, Fu, Li et al. 2015).

Auxotrophic *Agrobacterium* contain a mutation on a gene involved in regulating production of a certain amino acid that is essential for growth, rendering it unable to grow on media that have not been supplemented for the missing component (Collens, Lee et al. 2004). Given that these strains cannot survive without supplementation, they are easier to contain than prototrophic strains (Aliu, Azanu et al. 2020). Prototrophic *Agrobacterium* grows at a faster rate than plant tissue, making it so bacterial overgrowth can be an issue. Considering this, auxotrophic strains can be used when there is a need to limit bacterial overgrowth. Bacterial overgrowth can limit transformation efficiency and thus the use of a slower growing bacteria can be beneficial (Collens, Lee et al. 2004). For these reasons, it is worthwhile to test for the most advantageous strain of *Agrobacterium* with our selected genotypes.

Finally, time of cocultivation is a parameter that affects transformation efficiency (Utami, Hariyanto et al. 2018, Taak, Tiwari et al. 2020). As a result, evaluation of the cocultivation period could be profitable for optimization of Agrobacterium-mediated transformation of *Mimulus*.

Objective

The aim of this experiment is to optimize the *Agrobacterium*-mediated protocol for use in a diverse set of ecotypes and species of *Mimulus*, with emphasis on the yellow-flowered clade.

Materials and Methods

A total of four genotypes from the yellow-flower clade of *Mimulus* were evaluated in this study. These genotypes were selected from the previous genotype screen experiment. These genotypes comprise two sets of ecotypes, and species of *Mimulus*: annual *M. guttatus* (CAC415 and MED102) and *M. nasutus* (SF and CAC27).

To obtain sterile plants for this experiment, the seeds were sterilized using a protocol developed by Rachel Kerwin (personal communication). The sterilization consisted of placing the seeds in a 1.7 ml microcentrifuge tube with 1.2% sodium hypochlorite and 0.05% detergent solution, 10 minutes inversion and four washes with sterile water. Then, the seeds were suspended in sterile water and vernalized (4 ºC) for seven days. The sterile seeds were germinated in 100x20 mm Petri plates at 23 ºC with a 16/8 hour light cycle. The plates contained germination medium consisting of ½ strength Murashige and Skoog (MS) medium, supplemented with 10 g L^{-1} sucrose and 3 g L^{-1} Gelzan^{TM}, at a pH of 6.0. Plants were allowed to grow for six weeks before use.

To select the most suitable *Agrobacterium* strain for the previous selected *Mimulus* genotypes, three *Agrobacterium* strains were evaluated: wild type LBA4404, one mutated strain LBA4404ΔAdeC#2 and one auxotrophic strain, LBA4404ΔMet#5. These last two strains were developed by T. Chappell. The strain numbers were assigned based on the selection of separate colonies at the end of the process (personal communication, March 25, 2021).

The construct containing the *NEGAN* gene in the *Agrobacterium* strains of LBA4404 will be used in this experiment. To build this construct, a PCR amplification (**Table 3.1**) was performed using the primer pair CD_NEGAN_F and CD_NEGAN_R (**Table 3.2**), followed by a gel purification using a Zymo DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) to isolate the DNA. The recovered DNA was then quantified (90 ng/ul) and assembled into an empty CD entry vector, pGGC000, using the Gibson Assembly method. The transformation into Dh5α competent *E. coli* cells was performed using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA, USA). The Plasmid Miniprep Kit (GenCatch Plasmid DNA Mini-Prep Kit, Epoch Life Sciences, Missouri City, TX, USA) was used on the bacterial cultures and the resulting plasmids were then then sequenced by Genewiz.

Once the incorporation of *NEGAN* into the empty CD vector was verified, the Golden Gate Assembly method was used to incorporate *NEGAN* into a pGGP-AG destination vector (Decaestecker, Buono et al. 2019) modified in-house to have a plant-optimized spectinomycin resistance gene (Gift from James Thomson, USDA, Albany, CA) driven by the StUbi3 promoter (Garbarino and Belknap 1994). The terminator was taken from an existing binary vector commonly used in the Parrott lab (p201G2ubi3rfp). The *NEGAN* promoter, Cassava vein mosaic virus (CsVMV), was obtained from the same binary vector. It was cloned into an AB entry vector using the Green Gate Cloning System (Lampropoulos, Sutikovic et al. 2013) and incorporated into the plasmid. Similarly, the

Pisum sativum rbcS terminator (Rubisco) (An, Watson et al. 1985) was cloned into an EF entry vector and incorporated into the plasmid. All the incorporations were performed using the Golden Gate Assembly method (**Tables 3.3, 3.4** and **Figure 3.1**). After the assembly, a chemically competent cells transformation protocol from the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA, USA) was followed using Dh5α competent *E. coli* cells. Finally, the plasmid was electroporated into *Agrobacterium* strains LBA4404 wild type, mutated strain LBA4404ΔAdeC#2 and auxotrophic strain LBA4404ΔMet#5.

Table 3.1: PCR conditions for *NEGAN* amplification.

Temperature (°C)	Time	Number of cycles
98	30 s	1
98	10 s	
55	15 s	25
72	15 s	
62	5 min	1
16	∞	1

Table 3.2: Sequences used to amplify the *NEGAN* gene, from Sigma.

Oligo name	Sequence
CD NEGAN F	5’-AGAAGTGAAGCTTGGTCTCAGGCTCC**ATGGAAAACACACCTGTAGG -** 3’
CD NEGAN R	5’- AGGGCGAGAATTCGGTCTCACTGA**ATTTGGGCCCCAGTAGG -**3’

Table 3.3: Golden Gate Assembly components and their respective fragments.

Component	**Element**
A B entry vector	CsVMV promoter
B C entry vector	Empty (No N-tag or linker)
C D entry vector	*NEGAN*
D E entry vector	Empty (No C-tag or linker)
E F entry vector	Rubisco small subunit terminator
F G entry vector	Empty
Destination vector	pGGP-AG_spec
10X custmart buffer	
10mM ATP	
T4 DNA ligase	
Bsal- HF v2	Restriction enzyme

Table 3.4: Conditions used for Golden Gate Assembly method.

Temperature (°C)	**Time**	**Number of cycles**
37	2 min	20
98	2 min	
98	5 min	1
98	5 min	1
16	∞	1

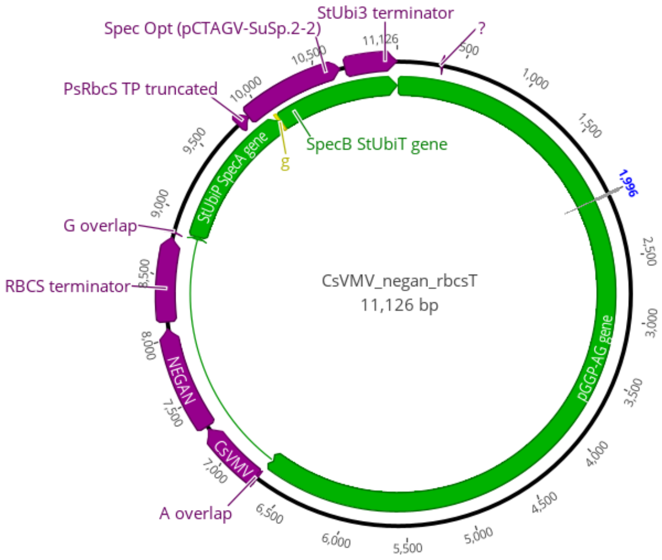

Figure 3.1. CsVMV:*NEGAN*:rbcsT construct.

To optimize the *Agrobacterium*-mediated protocol for use in a diverse set of ecotypes and species of *Mimulus*, four previously selected genotypes (CAC415, MED102, CAC27 and SF) were evaluated for their amenability to transformation utilizing nodal explants. The petiole explant portion of the experiment utilized the genotypes CAC415, MED102, and CAC27. Three *Agrobacterium* strains (wild type LBA4404, mutated LBA4404ΔAdeC#2 and auxotrophic LBA4404ΔMet#5) and three periods of co-cultivation (three, four, and five days) were tested intending to select the best combination of strain and period of co-cultivation for each genotype.

The nodal portion of the experiment (**Figure 3.2**) was a 3x3x3 factorial conducted in completely random design, in two replications. The genotypes comprised 3 levels of the factorial, period of cocultivation comprised 3 levels, and *Agrobacterium* strains comprised 3 levels. For each experimental unit, nine explants were placed in a 100x15 mm Petri dish, totalizing 27 plates with the treatments plus three plates with the negative controls for each replication. In total, 54 observations occurred.

Considering the petiole explant portion of the experiment (**Figure 3.3**), four genotypes were utilized, as described earlier. This experiment was a 4x3x3 factorial conducted in completely randomized design, in two replications. The genotypes comprised 4 levels of the factorial, period of cocultivation comprised 3 levels, and *Agrobacterium* strains comprised 3 levels. For each experimental unit, nine explants were placed in a 100x15 mm Petri dish, totalizing 36 plates with the treatments plus four plates with the negative controls for each replication. In total, 72 observations occurred.

Agrobacterium strains were grown overnight in dark conditions, at 28°C on 100x15 mm Petri plates containing solid Ym medium plus antibiotics. The next day, colonies from each auxotrophic bacterial strain were PCR screened for the respective mutation. Selected colonies from all strains were resuspended in 2 mL liquid Ym medium containing antibiotic selection and incubated at 28°C while rotating 180 rpm for 24 hours. The following day, cultures were transferred to 50 mL Ym medium containing antibiotics and incubated at 28°C while rotating 180 rpm for an additional 24 hours. All bacterial media used with auxotrophic strains contained 0.77 g L^{-1} Ura DO supplement (Takara Bio, Mountain View, CA, USA). Next, bacterial cultures were subjected to centrifugation for 15 minutes at 4500 rpm in order to obtain a pellet. The supernatant was then removed, followed by resuspension in plant inoculation medium containing MS salts and 20g L^{-1} sucrose at pH 5.5, supplemented with 100 μM acetosyringone (AS) until an OD_{600} of ~0.1-0.2 was reached. The bacterial suspensions were then transferred to clean flasks and incubated at 28°C while rotating 180 rpm for 1 hour before being used for the transformation.

Explants were excised from 6-week-old sterile grown plants and placed in sterile Petri plates containing the *Agrobacterium* suspension. These explants were incubated for ten minutes then blotted briefly onto sterile paper to dry. Following the drying, nodal explants were transferred to 100x15 mm Petri plates containing *Mimulus* co-cultivation medium for nodes, which contained MS salts plus 20 g L^{-1} sucrose, 4.44 µM BAP, 200 µM AS and 3 g L^{-1} Gelzan^{TM}. Petiole explants were transferred to 100x15 mm Petri plates containing *Mimulus* co-cultivation medium for petioles, which contained MS salts plus 20 g L^{-1} sucrose, 5.37 µM NAA, 4.44 µM BAP, 200 µM AS and 3 g L^{-1} Gelzan^{TM}.

Explants were kept on this medium for three, four, or five days, depending on the treatment. The infected explants were incubated in 23 °C in the light using a 16/8 hour light cycle using Halco brand fluorescent T8 bulbs.

After the respective period of co-cultivation, the explants were moved to resting medium, which is similar to the *Mimulus* co-cultivation medium except for the addition of 10 mg L^{-1} of meropenem. Explants were kept on this medium for three days for *Agrobacterium* elimination. After this stage, pictures of the explants were taken using the Olympus DP27 Camera with the Olympus SZX12 Stereo Microscope.

To obtain stable transformants of *Mimulus*, explants from all treatments were transferred to respective *Mimulus* co-cultivation medium supplemented with 10 mg L^{-1} meropenem and 20 mg L^{-1} spectinomycin for an additional eight weeks. The plates containing petiole explants were kept at 24 °C with a 16/8 hour light cycle using Philips brand fluorescent 40W T-12 bulbs at PAR 66-95 µE m^{-2} s^{-1}. The plates containing nodal explants were kept in 23 °C in the light using a 16/8 hour light cycle using Halco brand fluorescent T8 bulbs. Both explant types were sub cultured at the four week interval.

Following eight weeks on this medium, transformed petiole tissue will be selected and moved onto 100x15 mm Petri plates containing MS medium with 10 mg L^{-1} sucrose, 4.44 µM BAP, 3 g L^{-1} Gelzan^{TM}, at a pH of 5.5 for a minimum of four weeks. From here, viable shoots will be transferred 100x15 mm Petri plates containing MS medium with 10 mg L^{-1} sucrose, 0.444 µM BAP, 3 g L^{-1} Gelzan^{TM}, at a pH of 5.5 for an additional four weeks. Transformed nodal tissue will be selected and moved onto 100x20 xx Petri plates containing MS medium with 10 mg L^{-1} sucrose, 0.444 µM BAP, 3 g L^{-1} Gelzan^{TM}, at a pH of 5.5 for a minimum of four weeks until adequate shoots develop.

Explants that produce mature shoots can then be transferred to Fabri-Kal^{TM} brand clear plastic cups with dome lids containing MS medium supplemented with 10 mg L^{-1} sucrose and 3g L^{-1} Gelzan^{TM} at a pH of 6.0. Once rooting has taken place, plants will be transferred to sterile soil using GA7 & (Magenta Corp.) boxes.

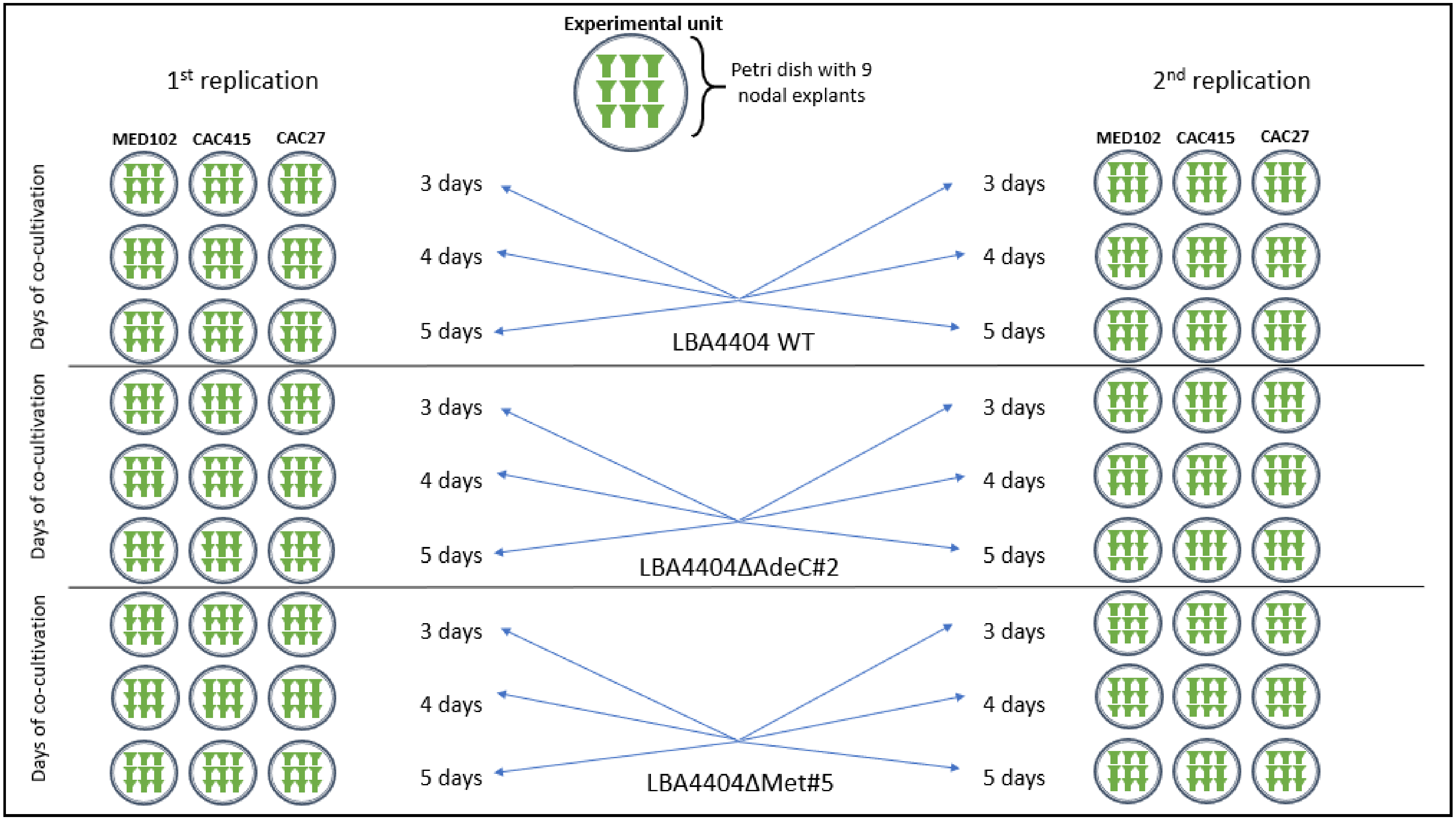

Figure 3.2: Experimental design to evaluate the transformation efficiency of three *Agrobacterium* strains in three different periods of co-cultivation on three *Mimulus* genotypes using nodal explants.

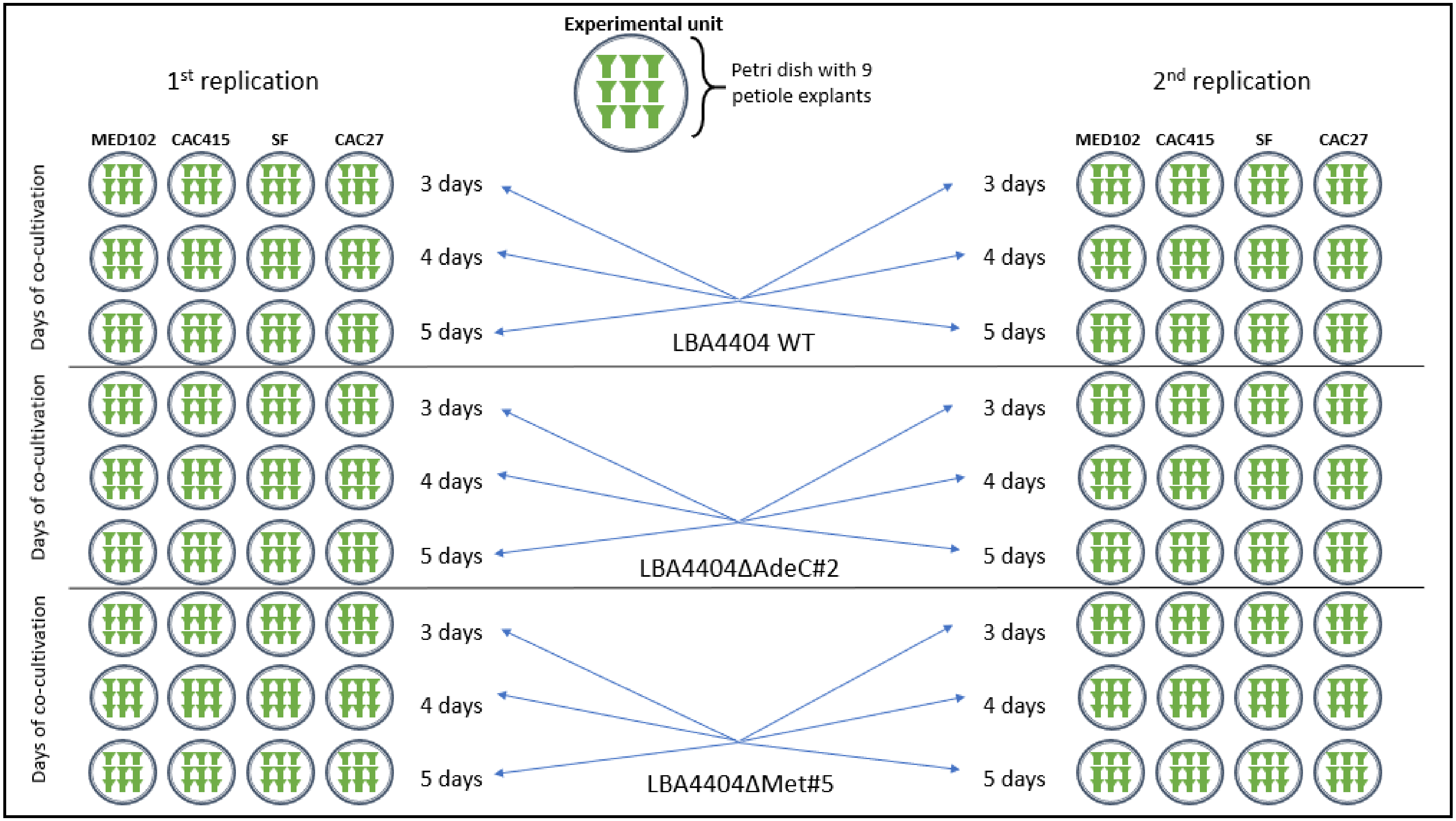

Figure 3.3: Experimental design to evaluate the transformation efficiency of three *Agrobacterium* strains in three different periods of co-cultivation on three *Mimulus* genotypes using petiole explants.

The images taken from the explants were used to calculate the percentage of *NEGAN* expression on the surface in each treatment (**Figure 3.4** and **Figure 3.5**). The explant surface area was measured using the software ImageJ (Abràmoff, Magalhães, et al., 2004).

Two replications of a completely randomized design with a 3x3x3 factorial arrangement was used for evaluation of the percentage of *NEGAN* expression on explants of three *Mimulus* genotypes inoculated with three *Agrobacterium* strains and three periods of co-cultivation, totalizing 27 treatments.

Two replications of a complete randomized design with a 4x3x3 factorial arrangement was used for evaluation of the percentage of *NEGAN* expression on explants of four *Mimulus* genotypes inoculated with three *Agrobacterium* strains and three different periods of co-cultivation, totalizing 36 treatments for each type of explant.

The analysis of variance (ANOVA) and the Tukey test at 5% significance were performed using R Studio (RStudioTeam 2020).

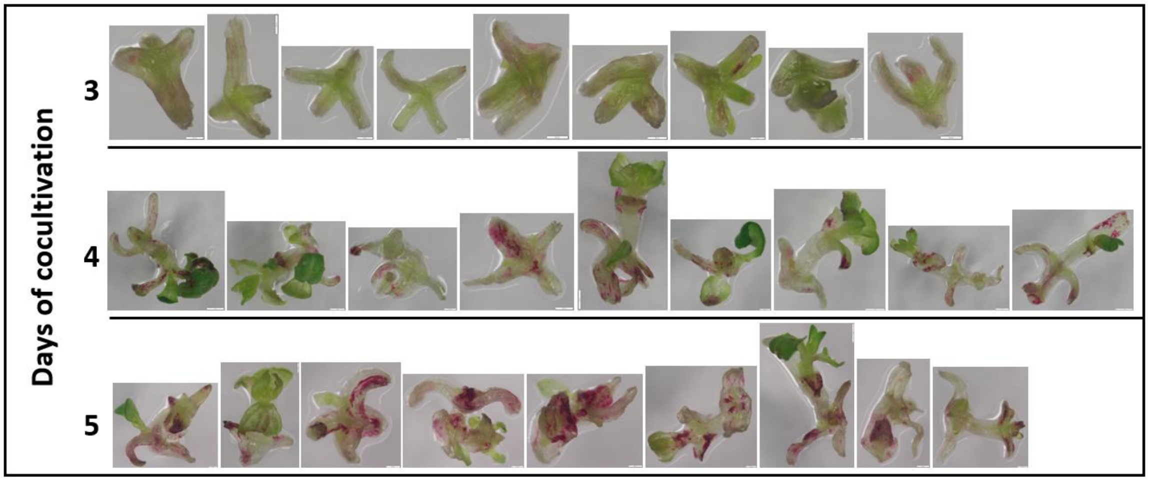

Figure 3.4. One replication of nodes from genotype CAC27 transformed with LBA4404ΔAdeC#2 using 3, 4, or 5 days of cocultivation. Purple color indicates *NEGAN* expression.

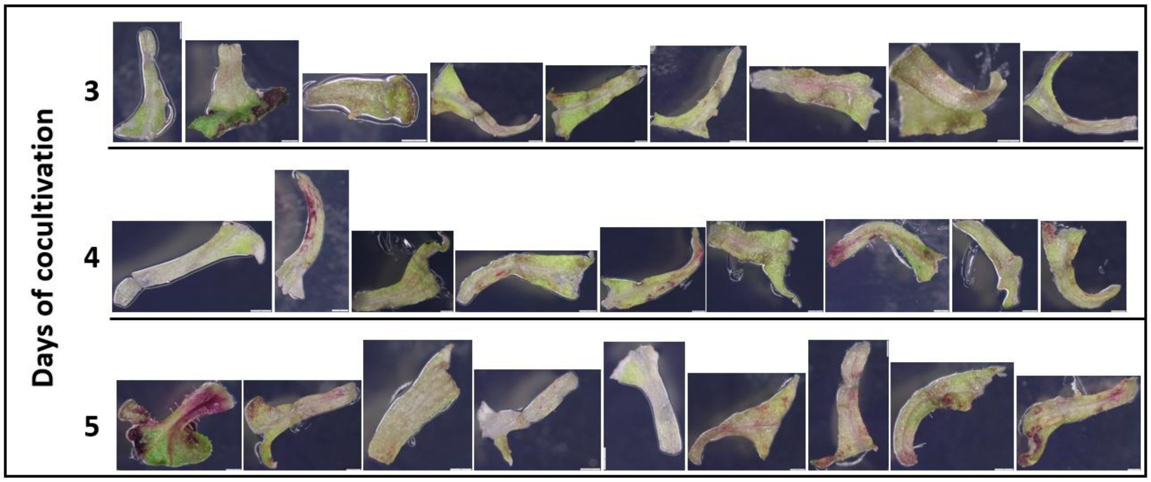

Figure 3.5. One replication of petioles from genotype CAC27 transformed with LBA4404WT using 3, 4, or 5 days of cocultivation. Purple color indicates *NEGAN* expression.

Results

Nodal explant experiment

The analysis of variance was conducted with the response variable as the ratio average for percentage of *NEGAN* in each treatment (**Table 3.5**). Given the statistical power in terms of degrees of freedom, the interactions among the factors were also able to be assessed. The data did not fit the normality assumption of the model. Therefore, it was subjected to a Boxcox transformation for the analysis. The only factor that was found to have a significant effect was *Agrobacterium* strain. It was found that the auxotrophic strain LBA4404ΔMet#5 performed significantly poorer than the other two (**Table 3.6**).

Table 3.5: Mean squares and respective significance levels of the analysis of variance for the ratio average of transformed area of nodal explants

Source of variation	**Df**	**Mean square**	**Pr(>F)**	**F value**
Strain	2	0.3333	0.0008**	9.3256
Co-cultivation (CC)	2	0.0917	0.0954	2.5654
Genotype	2	0.1076	0.0660	3.0103
Strain:CC	4	0.0155	0.7822	0.4349
Strain:Genotype	4	0.0489	0.2711	1.3683
CC:Genotype	4	0.0229	0.6378	0.6411
Strain:CC:Genotype	8	0.0091	0.9752	0.2547
Residuals	27	0.0357		

**Significant at 1% probability by *F* test.

Table 3.6: Strain, ratio average (%), and respective grouping based on Tukey test for nodal explants. Different letters in the same column indicate significant statistical differences ($p<0.05$).

Strain	**Ratio average (%)**	**Groups**
LBA4404 WT	2.313	A
LBA4404ΔAdeC#2	1.993	A
LBA4404ΔMet#5	0.544	B

Results: Petiole explant experiment

The analysis of variance was conducted with the response variable as the ratio average for percentage of *NEGAN* in each treatment (**Table 3.7**). This analysis also included an assessment of the interactions among the factors. Like the nodal explant portion of the experiment, the data were unable to fit the normality assumption of the model. Because of this, a Boxcox transformation was performed. Once the assumptions were met, the model found that all factors had a significant effect, but their interactions did not. Similar to the nodal portion of the experiment, it was found that LBA4404ΔMet#5 performed significantly poorer than the other two strains (**Table 3.8**). In terms of days of cocultivation, the highest transformation occurred with three days of cocultivation. However, this duration did not produce results that were significantly different from the four day period of cocultivation. Three days of cocultivation was significantly different from five days of cocultivation, which was not significantly different from four (**Table 3.9**). The Tukey test in terms of genotype showed that CAC27 and CAC415 were not significant different from each other but were both significantly different from MED102 and SF (**Table 3.10**).

Table 3.7: Source of variation, degrees of freedom, mean squares and respective significance levels of the analysis of variance for the ratio average of transformed area of petiole explants

Source of variation	**Df**	**Mean square**	**Pr(>F)**	**F Value**
Strain	2	0.3335	1.464e-13**	74.9602
Co-cultivation (CC)	2	0.0300	0.00324**	6.7513
Genotype	3	0.0791	3.013e-07**	17.7806
Strain:CC	4	0.0093	0.10341	2.0826
Strain:Genotype	6	0.0113	0.03804	2.5293
CC:Strain	6	0.0049	0.38675	1.0904
Strain:CC:Genotype	12	0.0077	0.09986	1.7344
Residuals	36	0.0045		

**Significant at 1% probability by *F* test

Table 3.8: Strain, ratio average (%), and respective grouping based on Tukey test for petiole explants. Different letters in the same column indicate significant statistical differences ($p<0.05$).

Strain	Ratio average	Groups
LBA4404 WT	1.300	A
LBA4404ΔAdeC#2	1.674	A
LBA4404ΔMet#5	0.1217	B

Table 3.9: Days of cocultivation, ratio average (%), and respective grouping based on Tukey test for petiole explants. Different letters in the same column indicate significant statistical differences ($p<0.05$)

Days of co-cultivation	Ratio average	Groups
3	0.6693	B
4	0.8586	AB
5	1.5681	A

Table 3.10: Genotype, ratio average (%), and respective grouping based on Tukey test for petiole explants. Different letters in the same column indicate significant statistical differences ($p<0.05$)

Genotype	Ratio average	Groups
CAC27	1.260	A
CAC415	1.668	A
MED102	0.757	B
SF	0.4423	B

Discussion

Considering the low transformation efficiency with both petiole and nodal explants using the auxotrophic *Agrobacterium* strain LBA4404ΔMet#5, this strain should be excluded from further experimentation with members of yellow-flowered clade of *Mimulus*. It looks as if the use of either LBA4404 WT or the mutated LBA4404ΔAde#2 strain does not make a difference in terms of transformation efficiency. Because there is no statistical difference between the two, the wild-type strain would be the better choice given that there is no need to perform a screen to verify the mutation prior to the experiment. If the true auxotrophic strain were not significantly different from either of the other two strains, then this would have been preferrable. Use of the auxotrophic strain would require extra steps to verify the mutation. However, the auxotrophic strain holds the advantage of not requiring an antibiotic to control *Agrobacterium* growth, which can ultimately effect transformation success.

While days of cocultivation did not have a significant effect on the transformation efficiency of nodal explants, the effect was significant on the use of petiole explants. It appears that five days of cocultivation is a preferable duration to let the explants be subjected to this stage in the pipeline. However, the use of five days of cocultivation was not significantly different than the use of four days, which was not significantly different from the use of three days.

Finally, the genotype did not have a significant effect with the use of nodal explants, but this factor was significant with petiole explants. CAC27 and CAC415 both showed a higher ratio of *NEGAN* expression compared to MED102 and SF. This was fortunate considering the best expression was in one *M. nasutus* and one *M. guttatus*.

It is interesting to note the relatively low ratio of *NEGAN* expression when using the *NEGAN* construct, regardless of duration of cocultivation, explant type, or genotype. It will be worthwhile to run molecular tests on plants that are recovered through the pipeline to see if there is a correlation between transformation efficiency calculated by evaluating the purple area on the explants.

CHAPTER 4

PRELIMINARY EXPERIMENTS

To familiarize with the *Agrobacterium* mediated transformation technique, two preliminary experiments were carried out. First, a histochemical GUS assay was implemented to evaluate the transformation performance of four *Agrobacterium* strains in two different explant types, petioles and nodes, from the *Mimulus* line, DUN. Second, the MYB complex member *NEGAN* for anthocyanin expression was used to evaluate the transformation performance of four *Agrobacterium* strains in petioles from *Mimulus* line CAC22.

First experiment - Materials and Methods

This experiment was performed using two wild-type (LBA4404 WT and EHA105 WT) and two auxotrophic strains of *Agrobacterium* (LBA4404ΔAdeC#2, and EHA105ΔMet#5). The strains contained plasmid Cambia1305.2 by electroporation. The plasmid has the hygromycin resistance gene (*hph*) for plant selection and kanamycin resistance gene (neomycin phosphotransferase II) for bacterial selection. The vector also contains a GUSPlus^{TM} reporter gene with a catalase intron for preventing expression in prokaryotes.

The strains were used to infect both petiole and node explants, taken from 6-week-old sterile grown plants. These plants were grown described in Chapter 2. *Mimulus* genotype DUN was used for this experiment. The experimental layout was in completely random design and was replicated three times, with each treatment replicate consisting of nine explants. In addition, a negative and a positive control were used to ensure the reliability of the experiment.

Bacterial cultures were grown overnight in 2 mL liquid Ym medium containing antibiotic selection and incubated at 28°C for 24 hours while rotating 180 rpm. The following day, cultures were transferred to 50 mL liquid Ym medium containing antibiotics and incubated at 28°C while rotating 180 rpm for an additional 24 hours. Once bacterial suspensions were ready for use, they were subjected to centrifugation for 15 minutes at 4500 rpm in order to obtain a pellet. The supernatant was then removed, followed by resuspension in plant inoculation medium containing MS salts and 20g L^{-1} sucrose at pH 5.5, supplemented with 100 µM acetosyringone (AS) at an OD_{600} between 0.1-0.2.

The explants were excised and placed in sterile Petri plates containing the *Agrobacterium* suspension. Incubation in the suspension took place for ten minutes then explants were removed and blotted briefly onto sterile paper to dry.

Following the drying, explants were placed in 100x15 mm Petri plates containing *Mimulus* co-cultivation medium. The co-cultivation medium for petioles contained MS salts plus 20 g L^{-1} sucrose, 5.37 µM NAA, 4.44 µM BAP, 200 µM AS and 3 g L^{-1} Gelzan^{TM}. The co-cultivation medium for nodes was as described, except for the omission of 5.37 µM NAA. The duration of co-cultivation was 3 days. Following this, explants were transferred to resting medium in 100x15 mm Petri plates for three days. The resting medium contained same ingredients as the co-cultivation medium plus meropenem at 10 mg L^{-1} for LBA4404 strains and 50 mg L^{-1} for EHA105 strains. Culture conditions were also as described previously. After this period, tissues were stained using a histochemical GUS assay.

Images of the stained tissue were taken using Olympus DP27 microscope and Olympus CellSens software. The blue area and total area of the treatments were obtained using the software ImageJ (Abramoff 2004). The ANOVA was performed using RStudio (RStudioTeam 2020). The ratio area of transformation was calculated as follows:

$$Ratio\ area\ of\ transformation\ =\ blue\ area\ /\ total\ area$$

First experiment - Results

No blue spots were detected in the negative control. and a positive result was obtained in the positive control indicating no contamination and no failure in the GUS assay (**Figures 4.1** and **4.2**). The analysis of variance showed a significant effect from explant type and strain used, but not for the interaction of these two factors (**Table 4.1**). The petioles showed a higher ratio of transformation compared to the nodes. LBA4404WT and LBA4404ΔAdeC#2 showed highest ratio of transformed area, followed by EHA105WT and EHA105ΔMet#5 (**Table 4.2**).

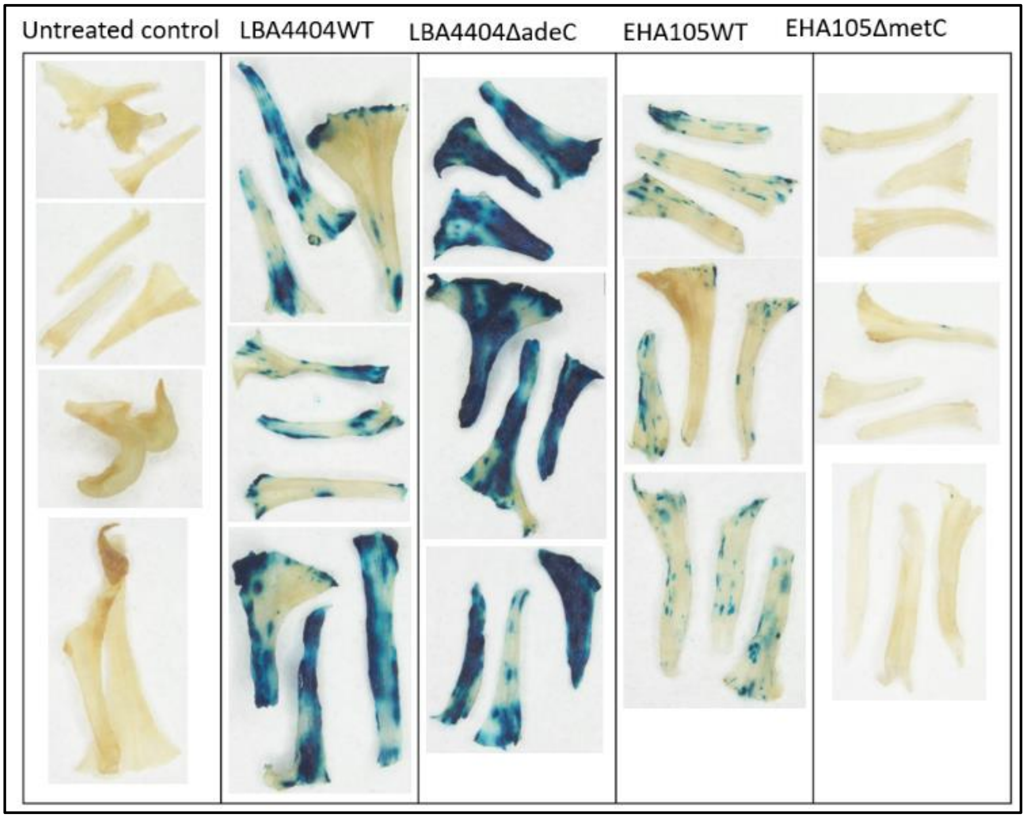

Figure 4.1: The first replication of petioles transformed with the different strains of *Agrobacterium.*

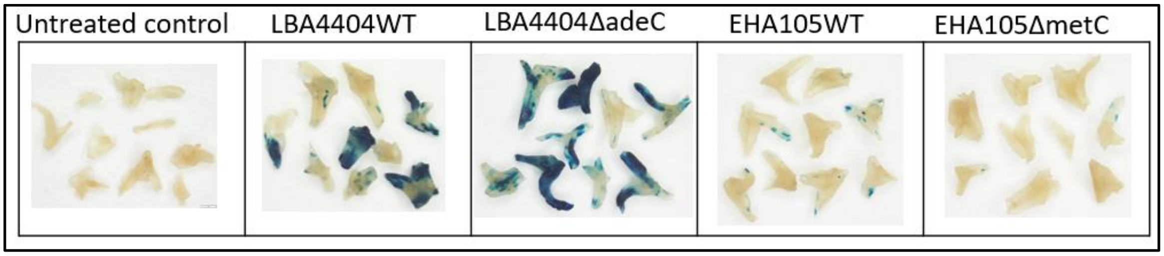

Figure 4.2: The first replication of nodes, which were cut in half, transformed with the different strains of *Agrobacterium*.

Table 4.1: Mean squares and respective significance levels of the analysis of variance.

	DF	Mean Sq
Replication	2	0.00280^{ns}
Strains	3	0.50783^{**}
Tissue	1	0.06277^{**}
Strain:Tissue	3	0.00216^{ns}
Residuals	14	0.00538

** significant at 1% probability by *F* test.

Table 4.2: Ratio area, in average, of tissue of *Mimulus* line DUN transformed by four strains of *Agrobacterium.*

Strain	Ratio average (%)	Tukey *
LBA4404ΔAdeC#2	23.92	A
LBA4404WT	21.76	A
EHA105WT	1.33	B
EHA105ΔMet#5	0.03	C

*Different letters mean significant differences at 0.05 level

Second experiment – Materials and Methods

The experiment was performed using one prototrophic (LBA404WT), two mutated (LBA404ΔAdeC#2, LBA404ΔAdeC#3) strains, and one auxotrophic (LBA404ΔMet#5) strain of *Agrobacterium*. The numbers in the strain names indicate the colony selected at the end of the process (Timothy Chappell, personal communication). The vector utilized the CsVMV promoter (Samac, Tesfaye et al. 2004), the *NEGAN* gene for anthocyanin production (Yuan, Sagawa et al. 2014), followed by a spectinomycin resistance cassette, and the Rubisco terminator (Samac, Tesfaye et al. 2004), assembled as described in Chapter 3. The strains were used to infect petiole explant tissues from *Mimulus* line CAC22.

The experiment was organized in completely random design and was conducted in two replications, each one consisting of nine explants. A negative control was also included.

To obtain sterile plants for the experiment, the seeds were sterilized using a protocol developed by Rachel Kerwin (personal communication). The sterilization was performed by placing ~25 seeds in a 1.7-ml microcentrifuge tube with 1.2% sodium hypochlorite and 0.05% detergent solution. This was followed by 10 minutes of inversion and four washes with sterile water. Then, the seeds were suspended in sterile water and vernalized (4 °C) for seven days. The sterile seeds were germinated in 23 °C with a 16/8 hour light cycle using Halco brand T8 fluorescent bulbs. Seeds were kept on 100x20 mm Petri plates for six weeks containing medium consisting of ½-strength Murashige and Skoog (MS) plus 10 g L^{-1} sucrose and 3.0 g L^{-1} Gelzan^{TM} at pH 6.0.

Bacterial cultures were grown overnight in 2 mL liquid Ym medium containing antibiotic selection and incubated at 28°C for 24 hours while rotating at 180 rpm. The following day, cultures were transferred to 50 mL liquid Ym medium containing antibiotics and incubated at 28°C while rotating at 180 rpm for an additional 24 hours. All bacterial media used with auxotrophic strains contained 0.77 g L^{-1} Ura DO supplement (Takara Bio, Mountain View, CA, USA). Bacterial cultures were then subjected to centrifugation for 15 minutes at 4500 rpm in order to obtain a pellet. The supernatant was then removed, followed by resuspension in plant inoculation medium containing MS salts and 20 g L^{-1} sucrose at pH 5.5, supplemented with 100 μM acetosyringone (AS) at an OD_{600} between 0.1-0.2.

Petiole explants were excised from 6-week-old plants and placed in sterile Petri plates containing the *Agrobacterium* suspension. These explants were incubated for ten minutes then blotted briefly onto sterile paper to dry. Following the drying, they were transferred to 100x15 mm Petri plates containing *Mimulus* co-cultivation medium, which contained MS salts plus 20 g L^{-1} sucrose, 5.37 µM NAA, 4.44 µM BAP, 200 µM AS and 3 g L^{-1} Gelzan^{TM}.

Following three days on co-cultivation medium, explants were transferred to 100x15 mm Petri plates containing resting medium. The resting medium was the same as the co-cultivation medium except for the addition 10 mg L^{-1} meropenem.

Following this period, images were taken using Olympus DP27 microscope and Olympus CellSens software (**Figure 4.3**). The purple area and total area of the treatments were obtained using the software ImageJ (Abramoff 2004). The ANOVA was performed using RStudio (RStudio Team, 2020). The ratio for area of transformation was calculated as follows:

$$Ratio\ for\ area\ of\ transformation\ =\ purple\ area\ /\ total\ area$$

Second experiment - Results

No purple area was detected in the negative control, suggesting no contamination. The analysis of variance showed a significant effect on transformation for the strains of *Agrobacterium* used (**Table 4.3**). The Tukey test proved that the only significant difference among the strains is with LBA404ΔMet#5, which had a lower efficiency (**Table 4.4**). This experiment also showed that petiole explants of *Mimulus* line CAC22 can be transformed by *Agrobacterium* using the *NEGAN* gene as the reporter. Although we were able to obtain transformed tissue, the overall efficiency was low, ranging from 0.014% to 4.027% (**Figure 4.3**). This may be due to the particular genotype, as this experiment was carried out before the genotype screen.

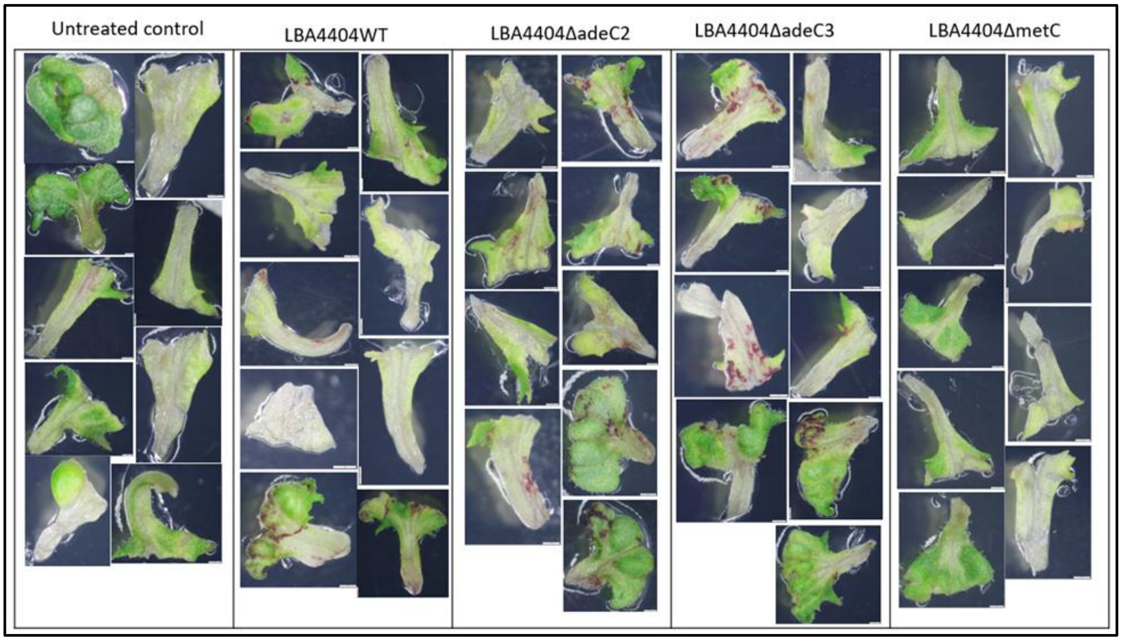

Figure 4.3: One replication of petioles from *Mimulus* line CAC22 transformed with four strains of *Agrobacterium*.

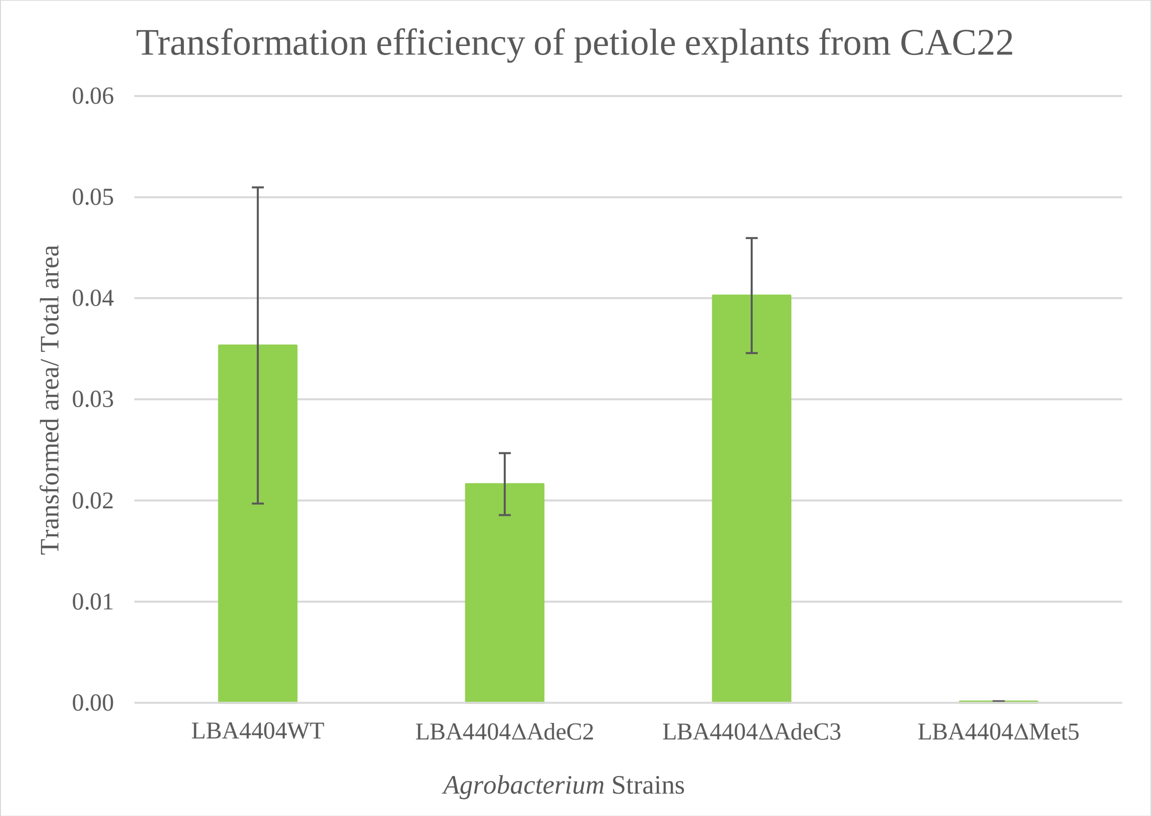

Figure 4.4: Average transformation efficiency of petiole explants using *Mimulus* line CAC22 with four strains of *Agrobacterium*.

Table 4.3. Mean Squares and respective significance levels of the analysis of variance.

Source of Variation	**DF**	**Mean Square**	**Pr(>F)**
Strains	3	$4.9203e^{-11}$	$5.543e^{-8}$
Residuals	3	$6.3464e^{-07}$	

Table 4.4: Ratio area, in average, of *Mimulus* line CAC22 tissue transformed with four strains of *Agrobacterium*.

Strain	**Ratio average (%)**	**Tukey**
LBA404WT	3.533	A
LBA404ΔAdeC#2	2.163	A
LBA404ΔAdeC#3	4.027	A
LBA404ΔMet#5	0.014	B

Discussion

Because of the low transformation efficiency with both EHA105 *Agrobacterium* strains, these strains were excluded from further experimentation. In the second preliminary experiment, LBA4404ΔMet#5 was the only strain which produced significantly less transformed area. It was later found that LBA4404ΔAdeC#3 is a wild-type strain and not a mutated or auxotrophic strain. This explains why it was not found to be significantly different from the wild-type strain in terms of transformation efficiency. Because of this, LBA4404ΔAdeC#3 was excluded from any more experiments. Although LBA4404ΔMet#5 performed significantly poorer than the other strains, it is still desirable enough to test in other genotypes given that the advantages to using auxotrophic strains of *Agrobacterium*.

CHAPTER 5

CONCLUSIONS

The genotype screen was valuable in understanding the performance of 15 selected genotypes from the yellow-flowered clade of *Mimulus*. This experiment was necessary for the contribution to the *Mimulus* tissue culture pipeline because of the highly differential performance of these genotypes in tissue culture. This collection of genotypes was able to be narrowed down to a diverse set which were then implemented in an experiment involving *Agrobacterium*-mediated transformation. The portion of the experiment utilizing indirect regeneration with petiole explants found that genotypes SF, CAC22, CAC27, CAC9 and CAC415 performed best. The nodal explant portion of the genotype screen found genotypes SF, CAC9, CAC27, and MED102 to be the best. SF, CAC22, CAC27, CAC415, an MED102 were tested in the experiments involving transformation.

While LBA4404ΔMet#5 produced low *NEGAN* expression in the second preliminary experiment, this strain was tested again following the genotype screen. This was because of the utility of auxotrophic strains when dealing with plants that are highly sensitive to *Agrobacterium*, such as *Mimulus*. It was worthwhile to see if it would be compatible with the genotypes that were established as best from the genotype screen. Because it produced significantly lower transformation efficiency compared to LBA4404 WT and LBA4404ΔAde#2, this strain is not a good candidate for transformation of the genotypes it was tested on. This includes, CAC22, CAC27, CAC415, MED102, and SF.

It is interesting to note the low *NEGAN* expression in the second preliminary experiment when compared to GUS expression in the first. This could be due to bleed-over of GUS into neighboring cells. It could also be due to the use of the cassava mosaic virus (CsVMV) promoter as opposed to the cauliflower mosaic virus (CaMV35S) promoter. While both are strong constitutive promoters, literature on *Mimulus* transformation utilizes the CaMV35S promoter.

The chapter 3 experiment to test transformation parameters showed there exists a significant difference in days of cocultivation and genotype with the use of petiole explants. This was not the case with nodal explants as only *Agrobacterium* strain had a significant effect. Based on the outcome of significant differences using multiple days of cocultivation with petiole explants, I would recommend using five days as the appropriate period of time. If one were to choose to reduce the size of the experiment, the top two performers in terms of rate of transformation were CAC27 and CAC415. This is ideal given that CAC27 is *M. nasutus* and CAC415 is *M. guttatus*.

A follow-up to this study should continue testing the parameters of *Agrobacterium*-mediated transformation. It would be useful to evaluate dark versus light conditions in cocultivation. It would also be advantageous to utilize the *NEGAN* construct with the 35S promoter as opposed to the current CsVMV promoter.

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