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Evaluation of the *csr1-2* allele from *Arabidopsis thaliana* CS3102 as an imazapyr herbicide resistance marker for biolistic transformation of the shoot apical meristem

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EVALUATION OF THE *CSR1-2* ALLELE FROM *ARABIDOPSIS THALIANA* CS3102

AS AN IMAZAPYR HERBICIDE RESISTANCE MARKER FOR

BIOLISTIC TRANSFORMATION OF THE

SHOOT APICAL MERISTEM

An Abstract of a Thesis

Submitted

in Partial Fulfillment

of the Requirements for the Degree

Master of Science

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ABSTRACT

Plant genetic engineering requires a marker gene to differentiate transformed cells. To test the *csr1-2* allele as a selectable marker for the shoot apical meristem (SAM), we cloned a genomic fragment from *Arabidopsis thaliana* mutant CS3102 which contained the coding sequence and promoter. We then evaluated the utility of this allele on two model systems, *Arabidopsis* and tobacco, with soybean as the positive control. Initial dose-response experiments were conducted using a seedling plate assay to determine the lethal dose of imazapyr. For the mutant *Arabidopsis thaliana* CS3102, a concentration of 500 μM imazapyr was required to overcome the resistance phenotype and kill 100% of the seedlings. At the lower dose of 50 μM, the herbicide killed all of the tobacco and soybean seedlings. Wildtype (WT) *Arabidopsis thaliana* Columbia seedlings were all killed at an even lower dose of 5 μM. Next, we used biolistics to target the SAM. Experiments using the visible marker gene β-glucuronidase showed transient expression in the epidermal cells of the soybean SAM, indicating our methods were capable of delivering transgenes. Experiments with *csr1-2* did not identify any transformed plants, suggesting this allele is of limited utility for SAM biolistics of *Arabidopsis* and tobacco. Selection with imazapyr did not produce transformants after testing 305 soybean seedlings, 670 tobacco seedlings, and 1,000 *Arabidopsis thaliana* Columbia seedlings. Future research to replace the native *Arabidopsis thaliana* promoter with a constitutive promoter could improve the utility of *csr1-2* as a selectable marker by increasing expression of the resistance gene.

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has been approved as meeting the thesis requirement for the

Degree of Master of Science

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Date   Dr. Jennifer Waldron, Dean of the Graduate College

DEDICATION

To my husband Michael Stump, and to my parents, James and Marianne McMillan, for their unwavering support.

A special dedication to the doctors, nurses, and staff at the University of Toledo ER and Neurosurgery ICU for keeping my brain intact after an accident which allowed me to pursue this degree.

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CHAPTER 1

INTRODUCTION

Plant Biotechnology

Historically, genetic studies were limited to controlled crosses and the observation of phenotypes to infer how genes act to produce variation. Gregor Mendel's famous experiments with pea plants displayed how inheritance of traits functioned through dominant and recessive genes. However, these techniques were limited to experimentation within a single species, as technology to allow for genetic crosses between species had not yet been developed. Modern protocols have made it possible to move genes between species, even between species in separate domains of life. Such transformations have been used to unravel how evolution works at the genetic level and have become an essential tool of plant biotechnology. It is now possible to sequence entire genomes and to create targeted mutations throughout the genome.

In the future, we would like to use these tools to study *Streptanthus polygaloides.* This species has evolved flowers with bilateral symmetry **(Figure 1)** and the ability to hyperaccumulate nickel to deter insect herbivory. To study the genetic pathways for these two traits, it would be useful to transform large DNA fragments containing multiple genes from *Streptanthus polygaloides* into model plant species. For the evolution of flowers, *Arabidopsis* has the ancestral condition of radial flower symmetry **(Figure 1)**. For the hyperaccumulation of nickel, tobacco is a model system that could be engineered for bioremediation of soils.

Figure 1: Evolution of flower symmetry in the Brassicaceae. A: The model plant *Arabidopsis thaliana* produces flowers with radial symmetry which is the ancestral condition. **B:** The related species *Streptanthus polygaloides* has evolved modified sepals that become pigmented over the course of their development, and **C:** *Streptanthus polygaloides* has evolved flowers with bilateral symmetry. Images taken by Dr. Marek Sliwinski and used with permission.

In plants, transformation techniques include *Agrobacterium*-mediated transformation and biolistics, both of which require marker genes for differentiation of transformed cells. A modification of biolistics, which targets the shoot apical meristem (SAM) of seedlings (Aragão et al. 2000), provides a quicker transformation method that does not require the time-consuming steps of growing plants in tissue culture. This method requires a marker gene that targets only the shoot apical meristem (SAM).

In the *Arabidopsis thaliana* genome, the *csr1* gene (**Figure 2**) is on chromosome three and codes for an enzyme involved in amino acid biosynthesis. A mutant *Arabidopsis* allele of this gene which confers resistance to the imidazolinone class of herbicides was discovered in the early 1990s (Sathasivan et al. 1991). Other naturally occurring alleles for imidazolinone resistance have been found in commercially important crops such as wheat, rice, sunflowers, and lentils, leading to the production of

Clearfield® seed marketed by BASF (BASF 2017, BASF 2019, Pfennig et al. 2008). For

plant biotechnology, one of the *Arabidopsis thaliana* mutant alleles of this gene, *csr1-2,*

was used to transform crops using the quicker transformation method of SAM biolistics

(Aragão et al. 2000; Rech et al. 2008).

Figure 2: Genomic map of the *Arabidopsis thaliana csr1* gene. The *csr1* gene (TAIR database entry AT3G48560.1) is shown with upstream genes (TAIR database entry AT3G48570.1 and a small portion of AT3G00840.1). The *csr1* coding sequence does not contain introns, and the *csr1* 5' UTR (sequence in red upstream of the start codon ATG) is predicted to be within 100 bp of the adjacent upstream gene.

<u>History of *Agrobacterium*-mediated Plant Transformation</u>

Agrobacterium has been implemented in plant biotechnology since the mid-1970s (Chilton et al. 1977; Sacristan and Melchers 1977) and is still the most widely used method of plant transformation (Rivera et al. 2012). This method of transformation involves using an attenuated strain of *Agrobacterium tumefaciens* to create transgenic organisms (Hernalsteens et al. 1980). *Agrobacterium tumefaciens* is a gram-negative bacterium (Smith and Townsend 1907) indigenous to soil which enters plant tissue through lesions in the roots or stems and causes cells to grow in an unorganized and unchecked swelling called a gall (Van Larebeke et al. 1974), similar to a cancerous mass in animals. The bacteria transfers a copy of tumor-inducing DNA to plant cells in a manner similar to conjugation (Chilton et al. 1977), and regulated by signaling molecules (Piper et al. 1993). The ability of *Agrobacterium* to introduce its DNA into host plant cells has been exploited for genetic modification of plants in biotechnology (Gelvin 2003), while the harmful properties have been removed (Piper et al. 1993).

Plasmid cloning vectors (small, circular strands of extrachromosomal double-stranded DNA that replicate autonomously) (Sambrook and Russell 2001) are designed with a promoter and coding sequence to express a gene chosen by the researchers. In plasmids, the gene or DNA of choice is called the insert. The plasmid backbone also includes selectable marker regions, which code for antibiotic resistance in bacteria (Arias et al. 2006; Herrera-Estrella et al. 1983; van den Elzen et al. 1985; Wilmink and Dons 1993) or herbicide resistance in plants (Wilmink and Dons 1993). This allows for the quick and efficient selection of genetically transformed plants. Plasmids are introduced to

and taken up by competent *Agrobacterium* cells (Rivera et al. 2012) and the

Agrobacterium is then placed directly on plant tissue which when infected will become a

callus (Heinz and Mee 1969; Saunders and Bingham 1972), an unorganized mass of plant

cells that is actively dividing. A common method of transformation uses tissue culture

(typically sterilized discs of leaf tissue or isolated cells) (American Association for the

Advancement of Science, 1985; Bhojwani and Razdan 1986; Horsch et al., 1989). The

transgenic tissue is induced to grow shoots first, then later roots using specific media and

hormones including auxin and cytokinin (Morel and Wetmore 1951; Walker et al. 1979).

The use of callus tissue can be a slow process, as multiple steps are needed before a full

transgenic plant is grown and transgenic seeds harvested. Often a first generation (T1)

must be grown to observe if transgenes were successfully passed on to the offspring.

A modification of *Agrobacterium* transformation that works best in *Arabidopsis* is

the floral dip method. *Agrobacterium* is combined with optimal concentrations of sucrose

and surfactant in which developing flower buds are immersed (Bent 2006; Clough and

Bent 1998; Curtis and Nam 2001). The *Agrobacterium* is then taken up in actively

dividing floral meristems (Desfeux et al. 2000). Plants with immature floral buds are

preferentially used so the highest chance of transformation of subsequent generations is

achieved (Clough and Bent 1998). A modification of the floral dip method is floral drip,

which also utilizes the mixture of *Agrobacterium,* sucrose, and surfactant (Chen et al.

2010; Wylie et al. 2003). Instead of dipping the entire floral bud, the transformation

media is placed on selected flower buds with a pipette (Zhang and Chen 2012). Both

methods yield transgenic seeds more quickly than callus tissue culture, as the floral

meristems (which will produce flowers and eventually seeds) are targeted directly and no induction of shoot or root is needed. Because floral tissue is targeted, contamination risk during tissue culture is significantly reduced. However, a second (T1) generation must still be grown to observe if desired genotypes have been conferred and whether the plant is a chimera—with both cells containing the transgene and cells that retain wildtype (WT) genotypes (Birch 1997; Lineberger 2020).

Certain plants can be recalcitrant to transformation by *Agrobacterium*, and alternate methods of transformation must be considered (Gregory 2018). In previous research performed at the University of Northern Iowa (Stump and Sliwinski, unpublished data), it was found that the *Agrobacterium* method of transformation was unsuccessful for *Streptanthus polygaloides,* a California wildflower important in studies of nickel accumulation (Meindl and Ashman 2014). The floral drip method was performed on *Streptanthus polygaloides* floral meristems to confer resistance to the herbicide glufosinate ($C_5H_{15}N_2O_4P$). Glufosinate inhibits the synthesis of the amino acid glutamine, which is essential for nitrogen metabolism, and will lead to complete plant death in several weeks (Vencil 2002). For *Streptanthus polygaloides*, the rate of transformation was too low to detect transgenic plants after screening ten thousand seeds. The positive control for the experiment, *Arabidopsis thaliana,* showed a transformation rate of 1% (Stump and Sliwinski, unpublished data). The published rate of transformation for *Arabidopsis thaliana* ranges from 0.5 to 3% (Bent 2006; Clough and Bent 1998).

Another problem with the *Agrobacterium* method is the inability to transform plants using larger DNA fragments (Hamilton et al. 1996). Transformation with DNA

fragments larger than 100 kilobase pairs (kb) is unreliable and unstable, and the method is wholly unsuitable for transformation using complete chromosomes. Chang et al. (2011) showed that transformation of tobacco with *Agrobacterium* carrying a large insert (83 kb) is very unstable compared with transformation using biolistics. The authors found that none of the seven randomly-selected clones used in *Agrobacterium* transformation were maintained. However, the first-generation of tobacco plants and the subsequent second-generation transgenic plants (eight total) maintained the large DNA insert as well as the marker gene conferring kanamycin resistance. Biolistics provides an alternate means of transformation for plants recalcitrant to *Agrobacterium* or for the transformation of large pieces of DNA.

<u>History of Biolistic Plant Transformation</u>

Biolistics, sometimes referred to as the "gene gun" method of transformation, was originally developed in the 1970s as a vehicle of transformation for economically important monocots and cereals that are resistant to *Agrobacterium* transformation, including wheat and corn (Kikkert et al. 2005; Klein et al. 1987; Sanford 1990). A traditional biolistics method uses a shock wave to deliver microparticles coated with DNA fragments into target cells (Rivera et al. 2012). The inert microparticles either embed or pass through the tissue while leaving the desired DNA behind, which is then incorporated into the plant genome. Tissues targeted for biolistics include leaf discs that will become callus tissue, immature leaves, or embryos (Kikkert et al. 2005). Biolistics can be performed with a hand-held gene gun (which is ideal for field work) or in a stationary vacuum chamber (Helenius et al. 2000; Klein et al. 1987). Both the vacuum

chamber and the handheld gene gun methods use small carrier particles (commonly gold or another inert metal) which are coated with a solution containing the gene of choice. Because of the high acceleration of the particles (even in the method that does not use a shockwave), not all plant tissue bombarded will withstand biolistic assault. Only approximately 50% of plant tissue that is involved directly in the biolistic delivery process survives (Russell et al. 1992). Surviving tissue located in the meristematic regions or that located in undifferentiated callus tissue will undergo cellular division and impart any foreign DNA into the new plant cells. Although biolistics can transform larger pieces of DNA, the requirement for the intermediate callus step for selection of transformed cells makes the procedure very slow.

A modification of the standard biolistics procedure is bombardment of embryonic shoot apical meristems as the target and regenerating plants without the callus step (Aragão et al. 2000; Rech et al. 2008). Because the actively growing meristems are used, a plant can be grown directly from the initial biolistic target without using multiple media transfers or hormone treatments. This approach alleviates the problem of possible contamination when transferring callus tissues between media, the need to grow shoots and roots, and significantly reduces the time investment to grow a full plant.

For SAM biolistics, the depth that microparticles reach is critical for transformation to occur. The SAM is dome-shaped and contains the tunica and the corpus. The tunica is the outer portion of the SAM and contains the L1 and L2 layers (**Figure 3, A & B**) that always divide in an anticlinal manner (Brukhin and Morozova 2015). The corpus is the inner portion of the SAM and is also called the L3 layer (**Figure**

3, C), which divides in a periclinal and anticlinal manner. Within the corpus, the cells divide in all planes (Brukhin and Morozova 2015). The central zone (**Figure 3, circle**) is where stem cells reside, and is thus the target for biolistic transformation. The peripheral zone (**Figure 3, P**) will give rise to leaves, floral meristems, and other plant organs and has a higher rate of cellular division than the central zone (Esau 1977). The rib zone (**Figure 3, R**), directly below the central zone, also has a higher rate of cellular division than the slowly dividing central zone and will give rise to the stem. Leaf primordium (**Figure 3, asterisks**) are extensions of the SAM and will become leaves. Because all stem and primary tissues arise from these tissue layers, transformation focused in this region can impact the growing plant quickly and easily. Cells in the SAM central zone region are largely undifferentiated and can be destined to be a variety of plant tissues (Sinnot 1939; White 1939). Through biolistic transformation, DNA can be imparted in SAM cells and can be passed on to new cells through division. For embryonic SAM biolistics, microparticles do not need to reach further than the L3 layer for transformation to be successful. When using an imazapyr resistance marker gene, transformed cells with DNA that confers resistance will grow readily in media containing herbicide, while those meristematic cells that were not transformed will die when subjected to herbicide challenge. Other labs achieved a high rate of transformation by combining SAM, biolistics and an imazapyr resistance marker to generate transgenic plants of soybean, common bean, and cotton (Aragão et al. 2000; Rech et al. 2008).
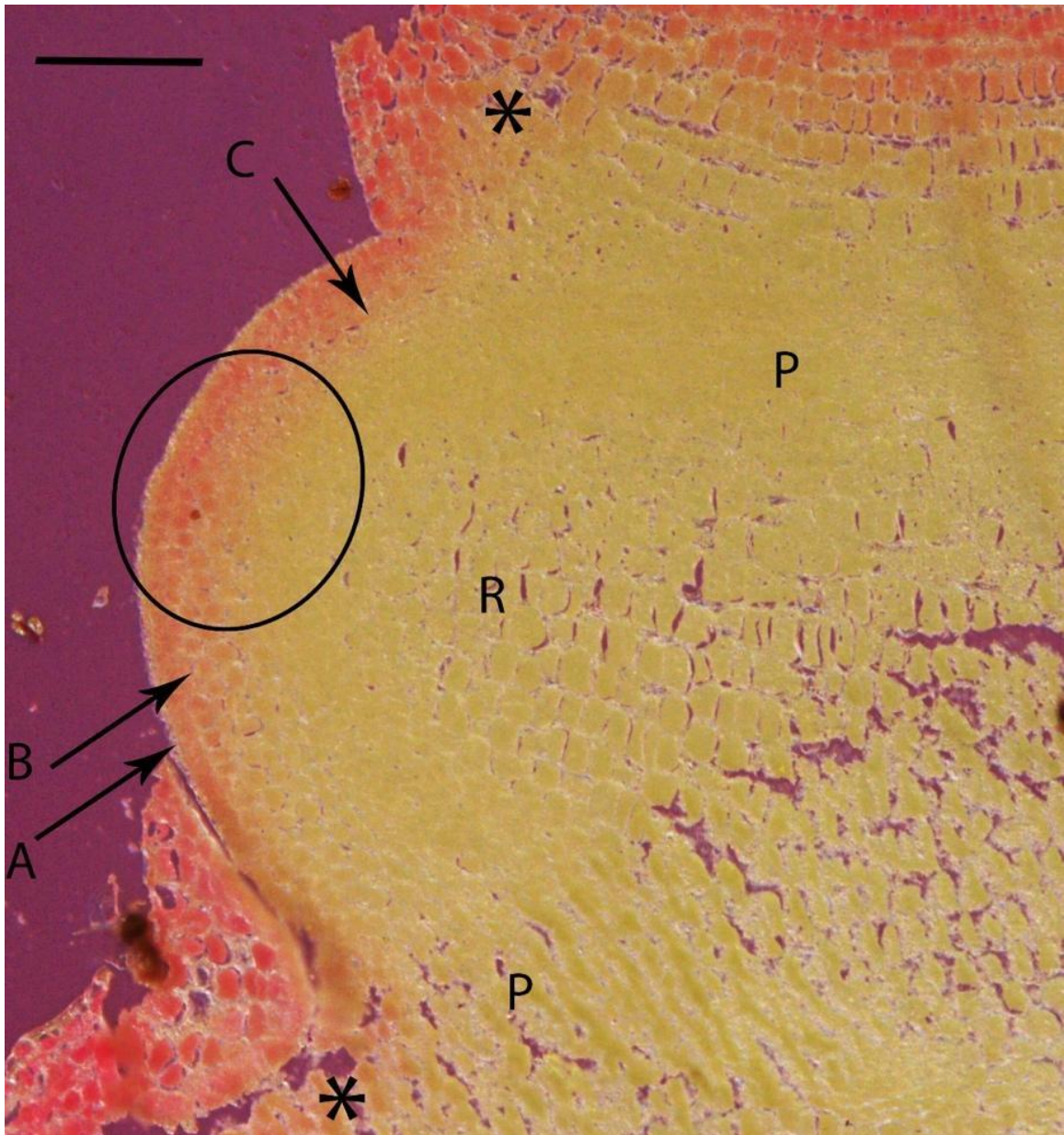
Figure 3: Shoot apical meristem of *Glycine max* embryonic axis. This SAM was sectioned at 25 µm and stained with eosin. **A:** L1 layer; **B:** L2 layer; **C:** L3 layer; **P:** peripheral zones; **R:** rib zone. Circle denotes central zone. **Asterisks:** leaf primordium. Scale bar: 1.0 mm.

Glycine max

Soybean is a legume family member of economic importance and has been genetically modified since the 1980s (Christou et al. 1988; Hinchee et al. 1988; McCabe et al. 1988). The United States is one of the largest producers of soybeans in the world, for both human consumption and animal feed applications, and many of the cultivars used have been genetically modified for herbicide resistance (Guttieri et al. 1996; Padgette et al. 1995; Sebastian et al. 1989), oil production (Cahoon 2003), or other desirable traits. *Glycine max* seeds can be geminated and grown quickly under ideal conditions (moist but not saturated soil and temperatures of 27-29°C) (Purcell et al. 2014), which can be easily emulated in the laboratory. The genome of *Glycine max* was sequenced in 2010 (Schmutz and Jackson, 2010), making it an ideal plant for genetic studies. Transformation of *Glycine max* has been successfully accomplished using *Agrobacterium* since the late 1980s (Chee and Slinghtom 1989; Hinchee et al. 1988). During the same period, transformation with biolistics was also achieved (McCabe et al. 1988).

Nicotiana tabacum

Nicotiana tabacum or cultivated tobacco of the nightshade family (Solanaceae), is commonly used in genetic experiments and is often used in tissue culture applications. Like soybean, tobacco was first genetically modified in the 1980s (An 1985; Barton et al. 1983). It is an ideal research subject due to its relatively fast germination (approximately seven to ten days) and ready growth habit under normal greenhouse or laboratory conditions (21-27°C, with moist but not wet soil and approximately 80% humidity). An

annual plant and native North American crop, *Nicotiana tabacum* has been studied

extensively since the 1800s, mainly due to the tobacco mosaic virus that infects plants

and threatens cash crops (including tomatoes and peppers, as well as tobacco) (Zaitlin

1998). Work with tobacco cultivars has been widespread due to the ability to be infected

by a wide variety of plant viruses and diseases (Goodin et al. 2014). The genome of

Nicotiana tabacum has been sequenced (Sierro et al. 2014). The genome of *Nicotiana*

sylvestris (woodland tobacco, a close ornamental relative of *Nicotiana tabacum*),

commonly used in terpenoid studies (Tissier et al. 2013), and stress tolerance experiments

(Galle et al. 2010; Genschik et al. 1992), had been sequenced a year earlier (Sierro et al.

2013)*.* Tobacco is commonly transformed using *Agrobacterium*. It is also amenable to

biolistic transformation (leaves or leaf discs are the primary targets). *Nicotiana tabacum*

seeds are easily and inexpensively available commercially, which provides a ready

supply for biolistic bombardment. The seeds are significantly smaller than those of

Glycine max at approximately 0.5 - 1 mm diameter*,* and the seeds must be grown to the

true leaf stage for biolistic use targeting the shoot apical meristem region. Given the

diminutive size, more embryos can be targeted to account for the expected tissue loss. As

whole seeds are utilized, they can be grown directly in Petri dishes with standard plant

media in a "lawn" of an appropriate shape to serve as a biolistic target. Those seedlings

that are transformed via biolistics will survive and grow on the same media with the

challenge herbicide added. This single plate approach eliminates transfer shock and

reduces possible contamination.

Arabidopsis thaliana

Arabidopsis thaliana is a compact flowering plant in the Brassicaceae family, which is native to Europe and Asia, as well as parts of North America. Though it is a widespread perennial herb that is considered a weed by some gardeners, *Arabidopsis thaliana* is most commonly known as a genetic model species. Scientific work with the plant began in the early 20th century, long before the advent of genetic transformation and sequencing (Sommerville and Koornneef 2002). Several characteristics of *Arabidopsis thaliana* make it an ideal plant for lab use: its small size, quick maturation and production of seeds, and rapid growth in artificial greenhouse settings. The entire genome of *Arabidopsis thaliana* was sequenced in 2000; it was the first plant genome to be completely sequenced (Arabidopsis Genome Initiative 2000). Since the initial sequencing, much work has been done to identify gene function and expression in *Arabidopsis thaliana* (Bevan and Walsh 2005), and many mutant seeds lines can be purchased from the Arabidopsis Biological Resource Center at the Ohio State University. Because of the quick growth and rapid production of seeds, abundant stocks of *Arabidopsis thaliana* are also readily available for use. As with *Nicotiana tabacum,* the seeds are quite tiny in size compared to *Glycine max* seeds. Biolistics has been successfully used to transform mature leaves of *Arabidopsis thaliana* (Ueki et al. 2009), which suggests SAM biolistics can be used to transform *Arabidopsis* shoot apical stem cells.

Imazapyr and the *csr1-2* Resistance Marker

Imazapyr is an herbicide of the imidazolinone class whose mechanism of action causes death at only actively growing regions of the plant, such as shoot and root meristems (Lee et al. 1999; MacBean 2012). By using *csr1-2* as an imazapyr resistance marker and targeting cells in the shoot apical meristem, a new plant can grow from the transformed stem cell(s) at the tip of the seedling. This method of transformation eliminates the need to grow a subsequent T1 generation. The gene has been shown to work well as a screen for transgenic seeds in Petri dishes (Manabe et al. 2007) and to screen adult plants by spray application (Das et al. 2010). Corresponding mutations have been found to naturally occur in cultivars of cotton, canola, tobacco, corn, and yeast (Hartnett et al. 1990; Manabe et al. 2007; Rajasekaran et al. 1996; Roux and Reboud 2005; Roux et al. 2005; Subramanian et al. 1990; Zhang et al. 2017). Commercial crops under the name Clearfield® utilize natural mutations similar to *csr1-2* for herbicide resistance, without requiring genetic transformation in a laboratory (Pfennig et al. 2008). Imidazolinone herbicides are broad-spectrum herbicides that are absorbed through leaves and roots (Lee et al. 1999; Pfennig et al., 2008) and transferred quickly to growing meristematic regions via xylem and phloem (Yu and Powles 2014). Imazapyr is one herbicide in the imidazolinone class and has been widely studied since its development in the 1970s. Imazapyr specifically targets the enzyme acetohydroxyacid synthase (AHAS, also known as acetolactase synthase [ALS]) through non-competitive inhibition by binding to a site near the active site and blocking substrate access (Rajasekaran et al. 1996; Yu and Powles 2014). AHAS catalyzes a reaction with pyruvate and either

ketobutyrate or ketopyruvate, and results in either the precursor of valine and leucine or the precursor of isoleucine (Lee et al. 1999). Inhibition of AHAS does not allow the plant to produce the branched chain amino acids leucine, valine, and isoleucine (Hartnett et al. 1990; Lonhienne et al. 2018; Rajasekaran et al. 1996; Subramanian et al. 1990). The herbicide targets and kills actively growing meristems, so the plant cannot continue to grow as it is deprived of essential amino acids (Vencil 2002). Susceptible plants will show complete chlorosis or a purple hue, followed by stunted growth and eventual necrosis and death. The combination of imazapyr targeting only meristematic cells and SAM biolistics for transformation limits the formation of chimeric tissue and allows for the growth of genetically modified meristem cells into plants composed wholly of transgenic cells.

For this study, we will use two model plant species for initial testing, tobacco and *Arabidopsis*. We will use soybean as a positive control for SAM biolistics as it had the highest transformation frequency reported in the literature at 9%, calculated as "the total number of fertile transgenic plants divided by the total number of bombarded embryonic axes" compared to 2.7% for *Phaseolus vulgaris* (common bean) and 0.5% for *Gossypium spp.* (cotton) (Rech et al. 2008).

Thesis Aims

1. Conduct dose-response experiments with the herbicide imazapyr on mutant *Arabidopsis thaliana* Columbia CS3102 to quantify the level of resistance generated by *csr1-2* expressed with its native promoter.

2. Conduct dose-response experiments with imazapyr to quantify the appropriate concentrations needed to select transformants in *Glycine max* (soybean)*, Nicotiana tabacum* (tobacco), and *Arabidopsis thaliana* Columbia.

3. Create a plasmid containing the *csr1-2* coding sequence and promoter to test the utility of this allele as a selectable marker using the herbicide imazapyr.

4. Measure the transformation efficiency of *csr1-2* using shoot apical meristem biolistics on tobacco and *Arabidopsis thaliana* Columbia.

CHAPTER 2

METHODS

<u>Dose-Response Screening of Imazapyr Herbicide</u>

To confirm that the CS3102 germplasm of *Arabidopsis thaliana* possesses

resistance to the imidazole class herbicide imazapyr, foliar spray experiments were

performed. CS3102 and WT Columbia seeds of *Arabidopsis thaliana* were obtained from

the Arabidopsis Biological Resource Center (ABRC.osu.edu, The Ohio State University,

Columbus, OH, USA) and planted in separate four-inch square plastic pots using sterile

potting mix (Sun Gro Metro Mix 360). Seeds were subjected to 16-hour photoperiods and

approximately 21°C temperatures. A minimum of two pots of each cultivar were grown

simultaneously for biological replicates. Plants were grown to the stage in which the

second set of true leaves were present, and leaves were thinned so no overlapping leaves

were touching, to ensure herbicide and/or surfactant penetration on all leaf surfaces.

Experimental plants were sprayed with a solution of imazapyr (Nufarm Polaris

Herbicide), surfactant (Sylgard 309, Dow Corning), and deionized water (lab sourced)

using a commercial spray bottle on "mist" setting. Control plants were sprayed with a

mixture of surfactant and deionized water only. The field application concentration of

NuFarm Polaris herbicide (27.7% active ingredient [a.i.] by weight) was used: 0.5%, or

0.47 mL / 100 mL deionized water. Sylgard 309 was utilized as a surfactant at the

recommended dose for use with herbicides: 1 – 3 pints per 100 gallons (or 0.13 mL per

100 mL distilled water; 0.13%). Sprayed plants were segregated by control and

experimental pots in separate watering trays under identical greenhouse conditions to

ensure no herbicide was taken up by the roots of the control plants through irrigation. Plants were observed for one to two weeks for signs of chlorosis and necrosis, and results were recorded.

The herbicide dose-response experiments were conducted by growing seedlings of each species on culture plates. For *Arabidopsis thaliana* and *Nicotiana tabacum,* plastic Petri plates contained 2% technical agar, 0.25 g/L fertilizer (#77860, Jack's Professional Peat Lite 20-10-20, JR Peters, Inc, Allentown, PA, USA), and varying concentrations of imazapyr (collectively called the selection medium). Imazapyr stock solutions at various concentrations (**Table 1**) were made by diluting measured volumes of NuFarm Polaris herbicide (containing 0.87 M imazapyr) with 10 mL of sterile distilled water in sterile 15 mL plastic Falcon tubes. Stock concentrations below 0.01 mM imazapyr were made by serial dilution. Each stock was a 20x concentrate relative to the final working concentration of the herbicide in the selection medium. The stock solutions were covered to protect from light, refrigerated at 4ºC, and stored for up to three months. Imazapyr was applied to culture plates after the agar had set by adding the appropriate imazapyr stock solution directly onto the surface of the media with an adjustable-volume micropipette. The imazapyr solution was spread evenly over the entire plate using sterile glass beads (4 mm in diameter) and allowed to soak in. The addition of 1000 µL of imazapyr stock solution to 100 mm Petri plates (containing approximately 20 mL of media) and 500 µL of stock solution to 60 mm plates (containing approximately 10 mL of media) created a 20-fold dilution to the working concentration. Plates were labeled with the concentration of the imazapyr stock solution added, rather than the final working

concentration, to prevent confusion during sample preparation. The concentrations of

imazapyr stock solutions and the final working concentration created by each stock

solution used for *Arabidopsis thaliana* CS3102, *Arabidopsis thaliana* Columbia, and

Nicotiana tabacum can be found in **Table 1**. Control plates used only fertilizer and

technical agar.

Table 1: Imazapyr concentrations of stock solutions and selection media. Culture media covering a range of imazapyr concentrations were generated by the addition of different 20x stock solutions to the agar. Concentrations used for moderate selection (50% seedling death) and stringent selection (100% seedling death) are indicated. Concentrations shown on culture plate photos, but not included in bar graphs are indicated with an asterisk (*). The selection concentration used by Rech et al. (2008) is also indicated.

	Stock solutions (20x) Imazapyr concentration (mM)		Selection media Imazapyr concentration (µM)		
Arabidopsis thaliana CS3102		0.1		5	
		0.5		25	
		0.7		35	
		1		50	
		2		100	
		5		250	
		10		500	
Arabidopsis thaliana Columbia		0.01		0.5	50%
		0.07		3.5	
		0.1		5	100%
Nicotiana tabacum	*	0.1 nM	*	0.005 nM	
		0.001		0.05	
		0.0025		0.13	50%
		0.005		0.25	
		0.01		0.5	
	*	0.1	*	5	
	*	0.5	*	25	
	*	0.7	*	35	
	*	1	*	50	100%
	*	2	*	100	
Glycine max		0.001		0.05	
		0.0025		0.13	
		0.005		0.25	50%
		0.01		0.5	(Rech)
		0.1		5	
		1		50	100%
	*	2	*	100	

Arabidopsis thaliana and *Nicotiana tabacum* (Smoking Tobacco Seed, All Good Things Seeds, Ojai, CA, USA) seeds were surface sterilized using 70% ethanol for one to two minutes, followed by 1% bleach in sterile water for 20 – 45 minutes, and finally triple rinsed with sterile water. In a laminar flow hood, seeds were placed on Petri plates containing the selection medium and the plates were sealed with micropore tape (#S6 1530-0, 3M). All plates were refrigerated at 4°C for two days, then placed under grow lights with 24-hour photoperiods at room temperature (21 – 24°C). To ensure random placement under light conditions and to account for microclimates, all plates were numbered, and a random number generator (random.org, Trinity College, Dublin, Ireland) was used to place plates under lights. Plates were removed from light when the first true leaves appeared in control plates (8 – 14 days). Petri plates were divided into quadrants randomly and numbered seedlings were selected from random quadrants to obtain fresh weight. For each *Arabidopsis* cultivar, five seedlings were pooled and weighed together. This was replicated ten times for statistical analysis. For *Nicotiana tabacum*, three seedlings were pooled and weighed together, with a total of ten separate weights. The weight from each set of measurements was divided by the number of seedlings to determine the average weight of seedlings. The single seedling weights were then averaged to obtain a single average weight for one seedling listed in the bar graphs in the Results. Fresh weight data was used to generate an ANOVA single-factor analysis to determine significance, and a Student's t-test with Bonferroni post-hoc correction was used to obtain significance between treatments. Statistical analyses were performed using Microsoft Office Excel.

Before herbicide screening of *Glycine max*, a germination rate experiment was performed using the FS HiSOY® seeds (#HS 10A80, courtesy of Debra DeVries, Cedar Falls Equestrian Center, Cedar Falls, IA, USA). Seeds were grown in sterile potting mix (Sun Gro Metro Mix 360) in four-inch plastic pots under 16-hour photoperiods at room temperature (21 – 24°C) and irrigated when soil appeared dry. The germination rate was determined to be 80%, or 20 / 25 seeds germinated.

For herbicide challenge experiments, embryos excised from *Glycine max* seeds (HiSOY® cultivar) were grown in sterile Petri dishes containing quarter-strength (1.08 g/L) Murashige and Skoog (MS) basal salts (#092623120, MP Biomedical, Santa Ana, CA, USA), 2% micropropagation agar (#A038-2.5KG, Caisson Labs, Smithfield, UT, USA), 3% sucrose, and varying concentrations of imazapyr. After dissolving the MS basal salts in about 90% of the final media volume, the pH of the solution was tested and adjusted as necessary with 0.1 N HCl to pH 5.7. Subsequently, the sucrose and agar were added and the media was autoclaved. Imazapyr was added to the culture plates after the agar cooled using the appropriate stock solutions, as described previously for *Arabidopsis thaliana* and *Nicotiana tabacum*. The concentrations of imazapyr stock solutions and the final working concentration created by each stock solution used for *Glycine max* can be found in **Table 1**. Control plates consisted of the same media without herbicide.

Before excising *Glycine max* embryos for culture, whole seeds were surface sterilized with 70% ethanol for 2 – 5 minutes, triple rinsed with sterile water, soaked in 1% bleach solution (Regular Bleach with CLOROMAX, Clorox Company) for 20 minutes, triple rinsed with sterile water, and soaked in sterile water for 1 – 16 hours. The

seeds were then placed in a laminar flow hood and the seed coat was removed from the seed using sterile gloved hands. The embryo axis was excised from the cotyledon and the plumules were removed under a dissecting microscope using sterile forceps (**Figure 4**). The embryos were then dipped in a 1% bleach and sterile water solution to prevent contamination, and placed in Petri dishes with the selection medium at a depth of 1 mm. The dishes were sealed with parafilm, placed in a refrigerator at 4°C for two days, then transferred to 24-hour lights at room temperature (21 – 24°C) until they reached the true leaf stage. When true leaves appeared in the control plate, the plantlets were weighed individually.
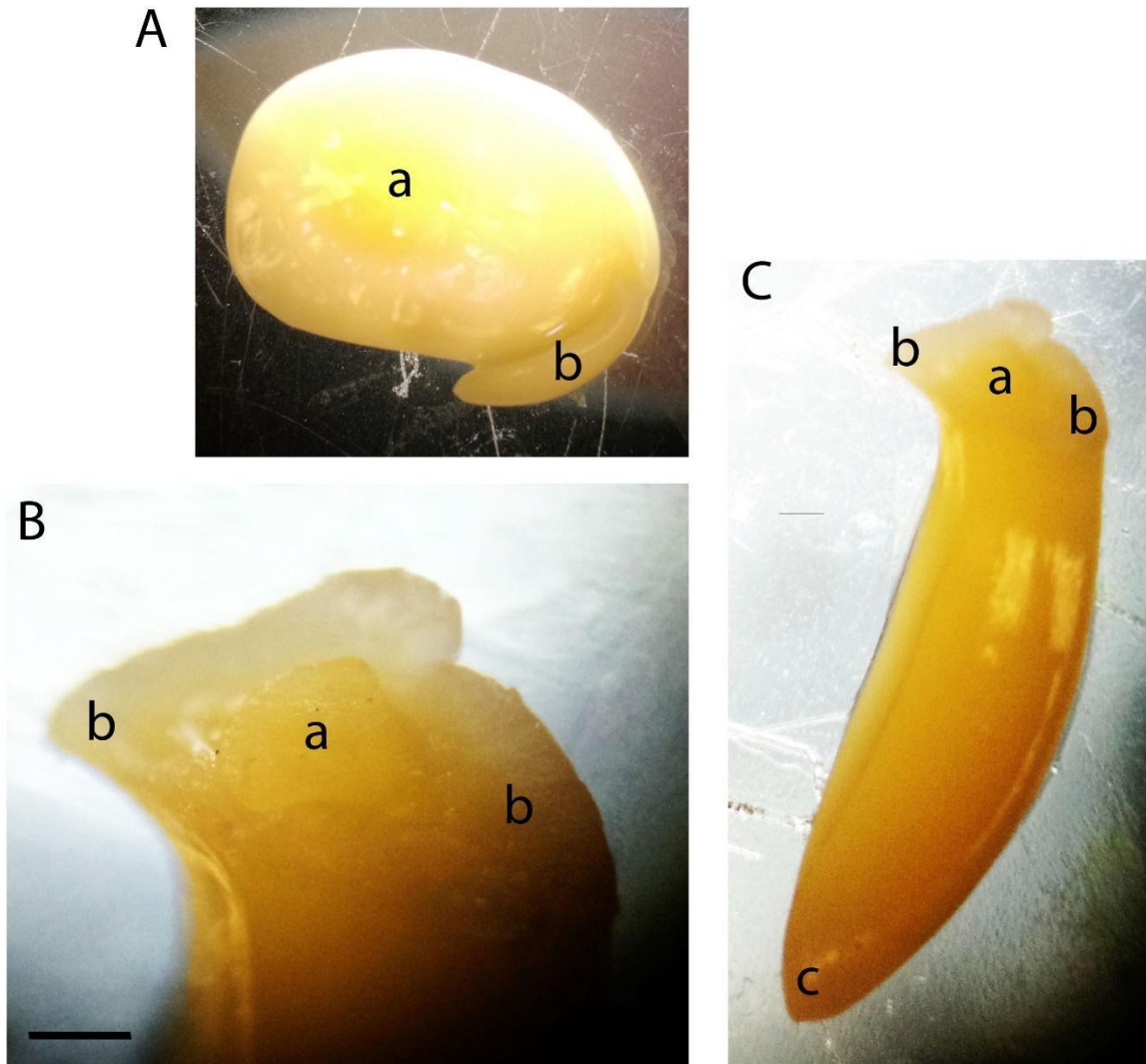
Figure 4: *Glycine max* **seed and embryo. A:** *Glycine max* seed with the seed coat removed. **a:** cotyledon; **b:** embryo axis. **B:** excised embryonic axis with shoot apical meristem (**a**) at the center (the target of biolistic bombardment). Leaf primordium have been excised at locations denoted by **b**. Scale bar: 1.2 mm. **C**: The excised embryonic axis. The shoot apical meristem is denoted by **a**; **b** denotes excised leaf primordium, and **c** denotes root apical meristem.

Cloning the *csr1-2* Allele

Arabidopsis thaliana CS3102 DNA was extracted using the Illustra Nucleon

Phytopure Genomic DNA Extraction kit (GE Healthcare) according to the manufacturer's

directions, starting with 0.1 g fresh plant tissue that was ground in liquid nitrogen using a

sterile mortar and pestle until it became a free-flowing powder. DNA primers were

ordered from Integrated DNA Technologies (Coralville, IA, USA). Primer sequences for

controls were: Actin forward (5' GACTCAAATCATGTTTGAGAC 3') and Actin

reverse (5' CATTTTCTGTGAACGATT 3'). For PCR, a master mix was used, then 25

µL aliquots were placed into PCR tubes. One to two drops of mineral oil were added to

the top. A hot start PCR was used, and the primers were then added to the PCR tubes.

PCR Parameters: GoTaq master mix, 25 mL/reaction: 12.5 mL GoTaq Green,

(Promega, WI, USA), 1 µL forward primer, 1 µL reverse primer, 1 µL DNA template (at

a 1:10 dilution from DNA extraction), 9.5 µL nuclease-free water. Denaturation initial

step: 95°C for two minutes at thirty cycles. Subsequent denaturation step: 95°C for 30

seconds. Annealing step: 45°C for one minute. Extension step: one minute per kilobase

(2.5 minutes total), 72°C for two minutes, 72°C for four minutes, 12°C hold for final step

(BioRad MyCycler). The amplified products were checked with agarose gel

electrophoresis and then cloned using the TOPO TA cloning kit (Invitrogen, USA)

according to the manufacturer's instructions. The resulting plasmid vector **(Figure 5)** was

sent to the DNA Facility at Iowa State University (Ames, IA, USA) for sequencing to

verify that the correct mutation was present.
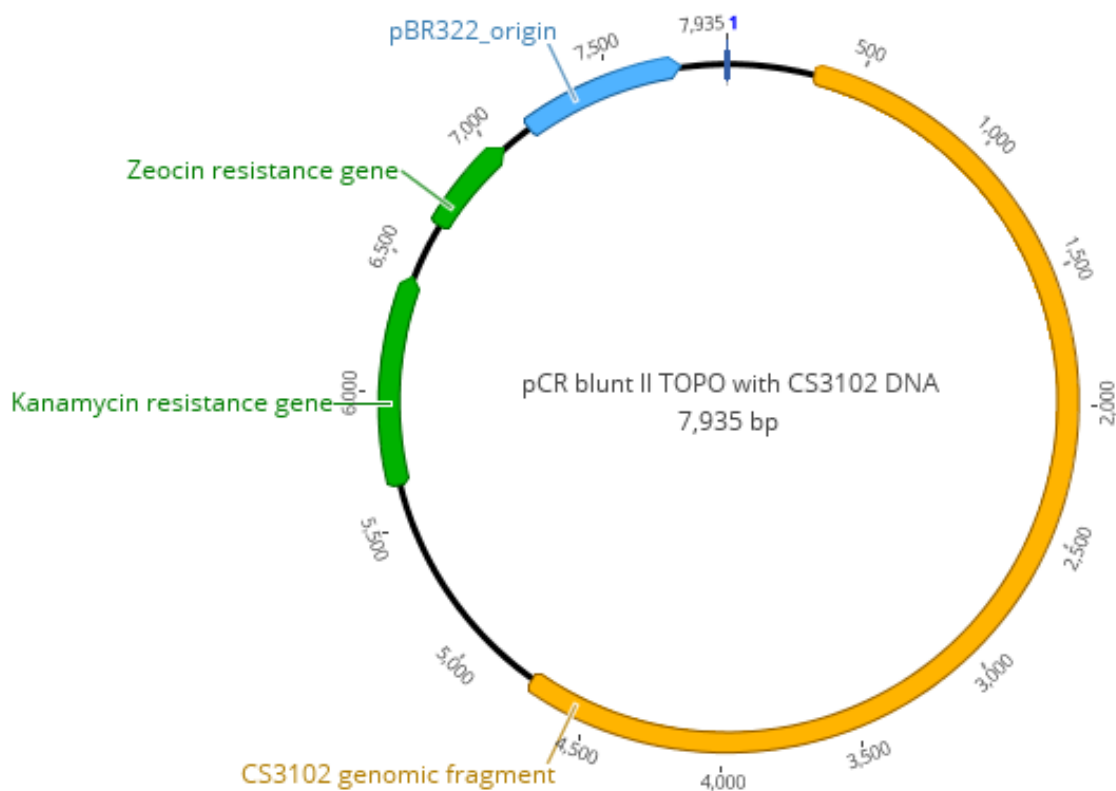
Figure 5: Plasmid map. A genomic fragment containing the *csr1-2* coding sequence and promoter from *Arabidopsis thaliana* CS3102 was cloned into the vector pCR blunt II TOPO.

Biolistic Bombardment

For biolistics, the protocol outlined in Rech et al. (2008) was followed. Prior to biolistics, all surfaces and materials were sterilized using 70% ethanol. When possible, procedures were carried out in a laminar flow hood to prevent contamination. Microcarriers (1000 nm and 1600 nm beads, Seashell Technologies, San Diego, CA, USA) were coated with the *csr1-2* plasmid and pipetted onto macrocarrier discs (#165-2257, Bio-Rad, Hercules, CA, USA) with 100% cold ethanol. Control microcarriers contained no DNA. The PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA,

USA) was set to the following parameters: a Petri dish with bombardment targets (embryos or seedlings) was placed 80 mm below the stopping screen (#165-2257, Bio-Rad, Hercules, CA, USA), and the macrocarrier disc with DNA-coated microcarriers was set at 13 mm above the stopping screen. The rupture disc (#165-2257, 900 psi; #165-2257, 1000 psi; or #165-2257, 1350 psi, Bio-Rad, Hercules, CA, USA) was set 8 mm above the macrocarrier disc. The microcarriers used for bombardment differed slightly from those used in Rech et al. (2008). We used gold particles instead of tungsten particles. Gold is less toxic than tungsten (Russell et al. 1992).

In preparation for biolistics, *Glycine max* (FS HiSOY® cultivar) embryo axes were excised from hydrated seeds in a laminar flow hood using sterile forceps as described earlier. The exposed embryos were kept in sterile water (lab sourced) until ready for biolistics, whereupon they were positioned for bombardment in a ring on the medium in a Petri dish. The shoot apical meristem portion of each embryo was pointed toward the center of the Petri dish at a position meant to optimize the number of microparticles from bombardment that hit the meristem region, while minimizing the risk of tissue damage due to the bombardment shockwave (**Figure 6**).

Figure 6: *Glycine max* embryos placed for biolistic bombardment on a Petri plate.
Shoot apical meristems are positioned toward the center of the plate. Petri dish diameter:
60 mm.

Excised embryos were bombarded using 1000-psi rupture disks, with the actual rupture occurring at approximately 900-1000 psi, and 1000 nm diameter microcarriers. For each biolistics experiment, a minimum of seven embryos were placed on 2% micropropagation agar containing quarter-strength (1.08 g/L) MS basal salts and 3% sucrose. Three plates with embryos received microparticles without DNA and three additional plates with embryos received microparticles with DNA coating. After biolistics, the embryos were not moved on the plate to prevent further shock. Three additional plates with embryos received no biolistics. At random, one plate from each treatment was designated as a control, another received 0.25 μM imazapyr (moderate selection), and the last plate from each biolistic treatment received 50 μM final concentration (stringent selection). Plates not receiving imazapyr received an equal amount of sterile water (lab sourced). Plates were wrapped with parafilm and placed randomly under 24-hour lights. Plates were observed and removed from lights when full necrosis was noted in the no-bombardment or no-DNA control plates containing imazapyr. At that time, any green living tissue arising from the shoot apical meristem area was removed and processed for DNA extraction to determine if the *csr1-2* gene was present. Of the *Glycine max* embryos that underwent bombardment with *csr1-2* DNA, 144 embryos were screened with 0.25 μM imazapyr, 161 embryos were screened with 50 μM imazapyr, and 112 embryos were used as control and were not exposed to the herbicide.

Arabidopsis thaliana Columbia and *Nicotiana tabacum* seeds were surface sterilized with 70% ethanol for 1 – 2 minutes, then placed in 60 mm diameter Petri dishes

containing 2% technical agar and fertilizer (**Figure 7**). For each species, at random, three

plates were designated as controls and did not receive any biolistics, another three plates

received biolistic bombardment without DNA, and the final three plates received

biolistics with DNA-coated microparticles. Parameters used for *Glycine max* were used

for bombardment (**Figure 8**). One plate from each biolistic treatment was designated at

random as a control with no imazapyr, one was given enough imazapyr for moderate

selection, and the final plate received enough imazapyr for stringent selection. Seedlings

were not moved on the plate following biolistics to decrease shock. Plates were wrapped

with micropore tape and numbered. Plates were placed under 24-hour lights and the age

of the seedlings used was noted (it was ensured all seedlings from each biolistic trial were

of the same age). Seedlings were allowed to sit undisturbed until total necrosis was noted

in control plates with imazapyr. Any green plant material arising from the shoot apical

meristem region of the seedlings that received biolistics with DNA was collected and

processed for DNA extraction. Of the *Arabidopsis thaliana* Columbia seedlings that

underwent bombardment with *csr1-2* DNA, approximately 1,000 seedlings were screened

by each imazapyr treatment (moderate or stringent selection) and 500 were used as

controls with no imazapyr selection. Of the *Nicotiana tabacum* seedlings that were

bombarded with *csr1-2*, 670 seedlings were screened under each imazapyr treatment

(moderate or stringent selection) and 300 were used as controls with no imazapyr

selection.
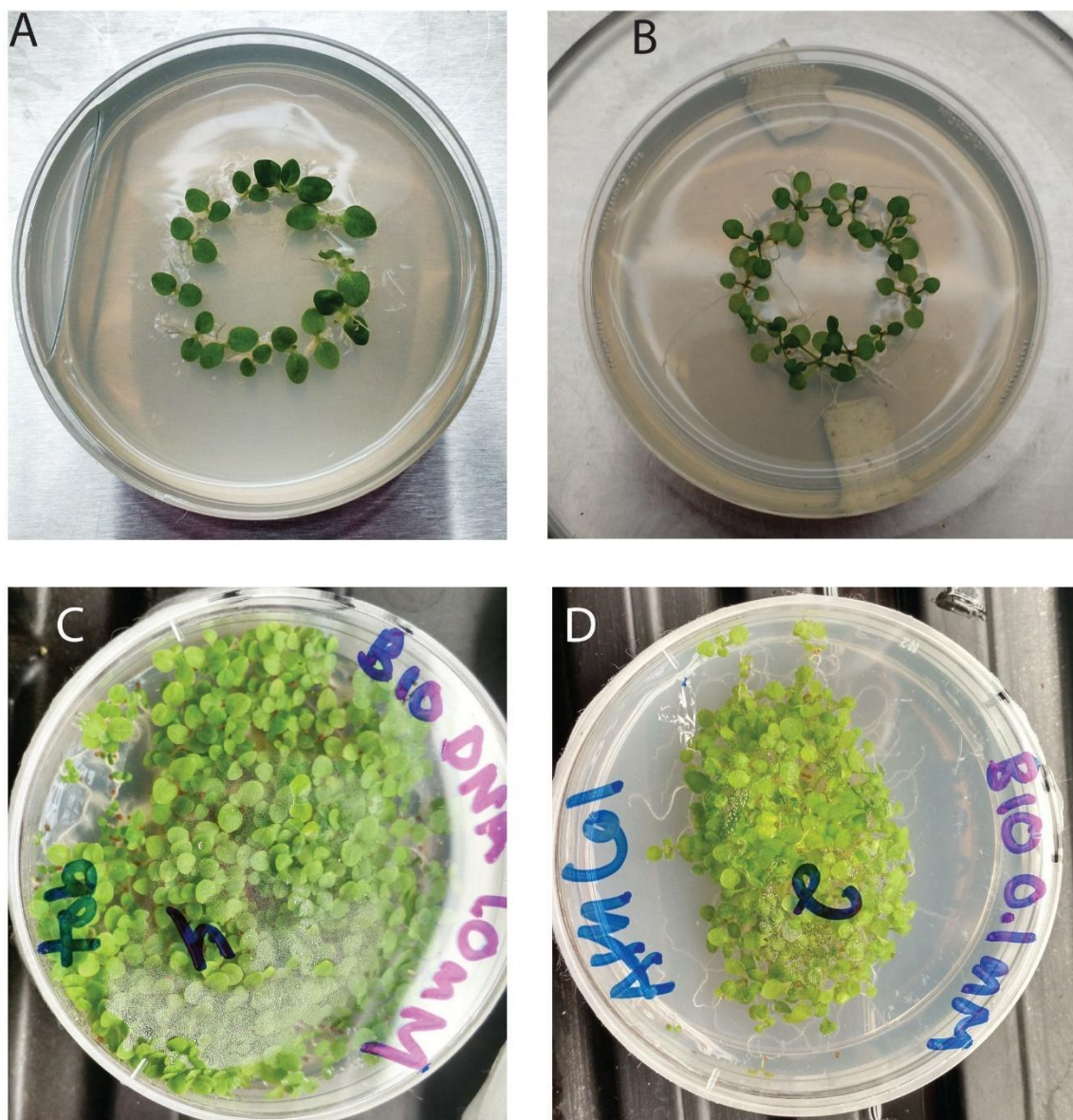
Figure 7: Placement of seedlings in Petri dishes in preparation for biolistics. In initial experiments, *Nicotiana tabacum* (**A**) and *Arabidopsis thaliana* (**B**) were grown in Petri dishes in lawns, then moved to a new Petri dish with growth media and placed in a circle for biolistics. To optimize biolistic penetration and prevent transplant shock, seedlings were grown in lawns (**C:** *Nicotiana tabacum;* **D:** *Arabidopsis thaliana*) and bombarded *in situ*.

Figure 8: Positioning of *Arabidopsis thaliana* Columbia in the biolistics chamber. A: Location of rupture disc at the end of the helium acceleration tube. **B:** Location of microcarrier screen, microcarriers coated with DNA solution, and stopping screen. Seedlings sit 80 mm below the stopping screen within the chamber with no barriers between.

β-glucuronidase Marker Gene

The GUS (β-glucuronidase) reporter gene was used for testing biolistics with *Glycine max*. Gold particles were coated with the plasmid pGFPGUSPlus (Addgene #64401) for biolistic bombardment as described previously. *Glycine max* embryos of the Hutcheson cultivar (Seed World USA, Odessa, FL, USA) were prepared as described earlier. After biolistics, the embryos were removed from the biolistics chamber and stained to determine if any cells expressed the GUS gene. The staining buffer was a mixture of 1 mL 0.5 M sodium phosphate buffer, pH 7.2; 0.2 mL 10% Triton-X; 0.2 mL 100 mM potassium ferrocyanide ($K_4Fe(CN)_6$); 0.2 mL 100 mM potassium ferricyanide ($K_3Fe(CN)_6$); 0.2 mL 10 mM X-Gluc (#G1281C, Gold Biotechnology, St. Louis, MO, USA) dissolved in DMSO and the volume was brought to 10 ml with sterile deionized water. Bombarded embryos were placed in the X-Gluc buffer overnight at 37°C. After staining, embryos were rinsed with 70% ethanol for five minutes. Embryos were then examined under a dissecting microscope for the presence of blue spots, which indicated transient expression. A total of 102 embryos were stained.

Testing Putative Transgenic Plants After Biolistics

Plant DNA from putative transformants was extracted after > 30 days with either the Illustra Nucleon Phytopure Genomic DNA Extraction kit or with the CTAB method as follows. Regenerated plant tissue (100-200 mg of leaf tissue from *Glycine max* or *Nicotiana tabacum*) was ground to a fine paste using a pestle (#FB970M, Thermo Fisher Scientific, Waltham, MA, USA) in 500 μL CTAB buffer (2 g CTAB [hexadecyl trimethyl-ammonium bromide] and 10 mL TRIS buffer, pH 8) in a 2 mL microfuge tube

and incubated at 55°C for 15 minutes (Incu Block, Denville Scientific Inc., Holliston, MA, USA). The mixture was centrifuged for five minutes at 12,000 rcf (Centrifuge 5430, Eppendorf, Hamburg, Germany). The supernatant was transferred to a new microfuge tube with 250 µL of phenol : chloroform : isoamyl alcohol at a ratio of 25:24:1 and mixed by inversion. The mixture was then centrifuged at 13,000 rcf for one minute. The upper aqueous phase was transferred by pipette to a new microfuge tube with 50 µL 1 M sodium acetate and 500 µL cold (-20 ℃) absolute ethanol. The tube was inverted and placed in a -20°C freezer for 12 – 24 hours to facilitate DNA precipitation. The DNA was precipitated by centrifugation, the supernatant was removed by pipette and the pellet was washed with cold (-20℃) 70% ethanol and centrifuged at 13,000 rcf for one minute. This step was repeated twice, and the pellet was dried in a chemical fume hood for approximately 15 minutes, and then resuspended in sterile nuclease-free water. The DNA solution was stored at -20°C and thawed at room temperature for use.

Following extraction, agarose gel electrophoresis was performed to confirm DNA quality. PCR was performed using species-specific actin forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA) to serve as a baseline and confirm the extracted DNA was of sufficient quality. The *Glycine max* primers (Actin forward: 5'GGCTGTGTTTCCCAGCATTC 3'; Actin reverse: 5'CATATCAGACCAGACAGCAAGT 3') and *Nicotiana tabacum* primers (Actin forward: 5'TCAGTCAAGTCACGACCAGC 3'; Actin reverse: 5'GTCTCATATCGGCCGTAGA 3') were used with the thermo-cycler settings as described previously. In the case of *Arabidopsis*, which contains the *csr1* gene in

addition to the *csr1-2* transgene, a restriction enzyme (FD0384, Eco-88I, Thermo Fisher

Scientific, Waltham, MA, USA) was used to determine if the *csr1-2* allele was present.

This restriction enzyme recognition site overlaps the key serine to asparagine point

mutation at position 653 on the *csr1-2* allele in *Arabidopsis thaliana*. If the wildtype *csr1*

gene was present, the restriction enzyme would cut the DNA into two shorter fragments.

If the *csr1-2* allele was present, the DNA would not be cut and the full-length PCR

product would be visible.

For *Glycine max*, multiplex PCR was performed on post-biolistic seedlings to

determine the presence or absence of the *csr1-2* transgene. DNA extraction was

performed on bombarded *Glycine max* embryos using the Illustra Nucleon Phytopure

Genomic DNA Extraction kit using the directions from the kit. DNA was then amplified

using multiplex PCR with soy-specific actin and *csr1-2*-specific primers (*csr1-2* short

forward and *csr1-2* reverse). Multiplex PCR included multiple pairs of primers that

produce bands of different sizes that can be distinguished by gel electrophoresis. In this

case, the actin primers and *csr1-2* primers were run together in a single PCR reaction and

then the presence of one or two bands was determined.

CHAPTER 3

RESULTS

AIM 1: Determine the Herbicide Dose-Response for the Resistant *Arabidopsis* Mutant

Initial experiments were conducted with foliar spraying, following the typical agricultural application of imazapyr, to determine the level of resistance conferred by the native promoter of the *csr1-2* allele in the mutant *Arabidopsis thaliana* CS3102 to compare to the wildtype (WT) *csr1* allele. Foliar spray experiments with a 0.5% imazapyr solution (percent volume of herbicide) on WT *Arabidopsis thaliana* Columbia and CS3102 cultivars found that all plants showed signs of chlorosis and/or necrosis. However, the mutant CS3102 plants were able to survive and produce new leaves from the shoot apical meristem, while WT plants did not progress past this initial stress and became chlorotic before death (see **Figure 9**).
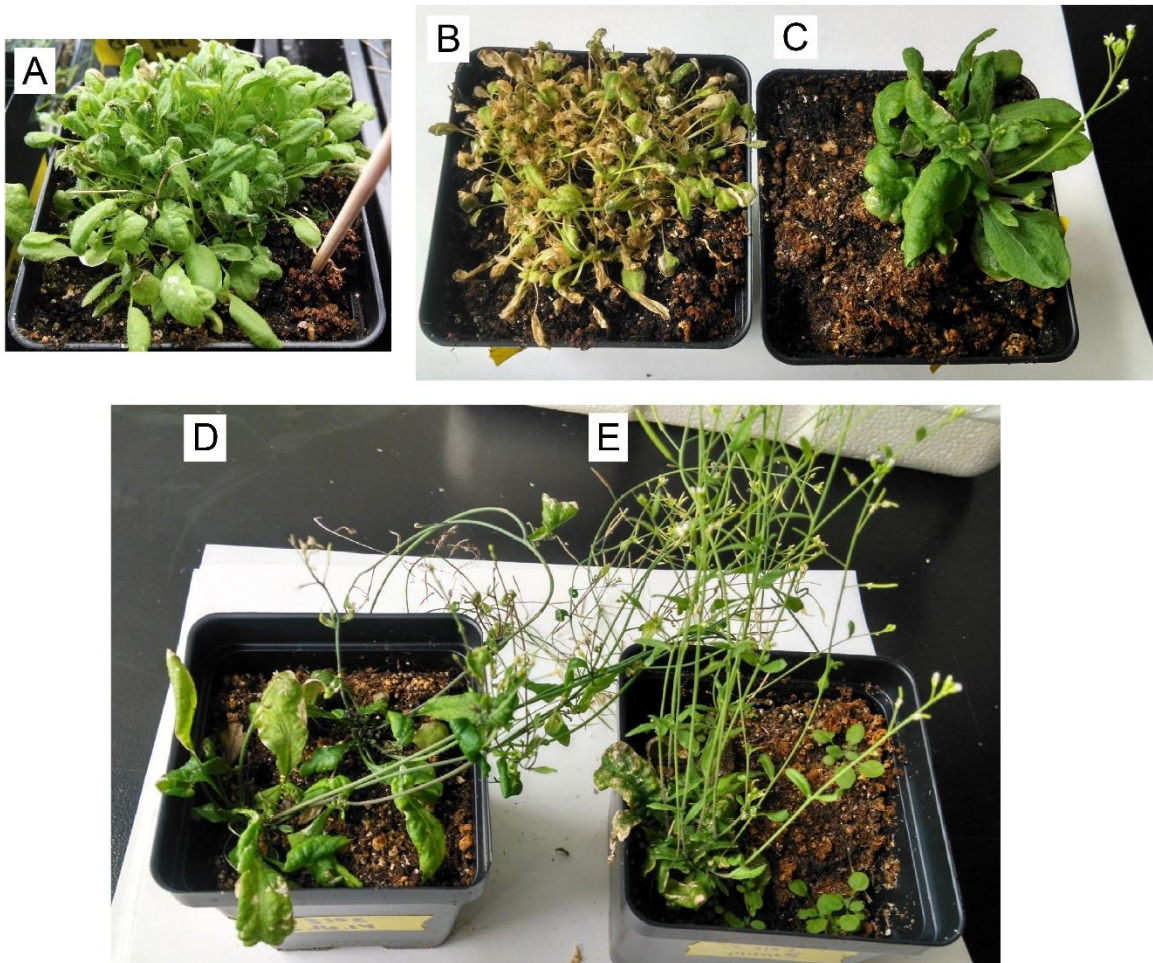
Figure 9: Foliar spray results with wildtype *Arabidopsis thaliana* Columbia and mutant CS3102. A: WT *Arabidopsis thaliana* Columbia several weeks after a control treatment of surfactant only. **B:** WT plants several weeks after application of 0.5% imazapyr and surfactant showed signs of necrosis and stopped growing. **C:** Mutant CS3102 plants several weeks after application of 0.5% imazapyr and surfactant began producing flowers. **D:** Mutant CS3102 plants (treated with 0.5% imazapyr and surfactant) continued growing and produced seeds. **E:** Mutant CS3102 plants several weeks after application of a control treatment of surfactant only. Mutant plants showed similar levels of chlorosis with surfactant only or surfactant plus 0.5% imazapyr, suggesting the surfactant can cause some yellowing of the leaves.

The foliar spray test with imazapyr was repeated five times. Each test showed a similar pattern of early stress on all plants, followed by growth of the resistant germplasm and death of the WT plants within two to four weeks. This confirmed that the *Arabidopsis thaliana* CS3102 mutant expresses resistance to the imazapyr-containing herbicide, whereas the WT plant has no notable resistance.

Next, a seedling assay on Petri plates was used with varying concentrations of imazapyr to determine the level of resistance caused by the *csr1-2* allele in the mutant *Arabidopsis thaliana* CS3102. Seedlings were grown until the first true leaves appeared in the corresponding control plants (8 – 14 days) and then the batch was sacrificed for testing, as described in the Methods section. ANOVA analysis revealed $p=2.02 \times 10^{-10}$ for between group analysis. The values for each treatment were calculated as follows: average weight of seedlings in milligrams (from 10 random groups of 5 seedlings weighted together) ± the standard error of the mean; p values calculated through a 2-tailed paired Student's t-test and Bonferroni post-hoc analysis (p value divided by the number of groups); significance accepted as $p \leq 0.05$ (**Figure 10**). Visual inspection of the selection plates showed that the resistance was partially overcome at 100 µM imazapyr (**Figure 11**) and all mutant seedlings died at 500 µM imazapyr. With the upper limits of *csr1-2* resistance defined by this experiment, we commenced to find appropriate imazapyr dosages for selection of transformants in *Arabidopsis thaliana, Glycine max,* and *Nicotiana tabacum.*
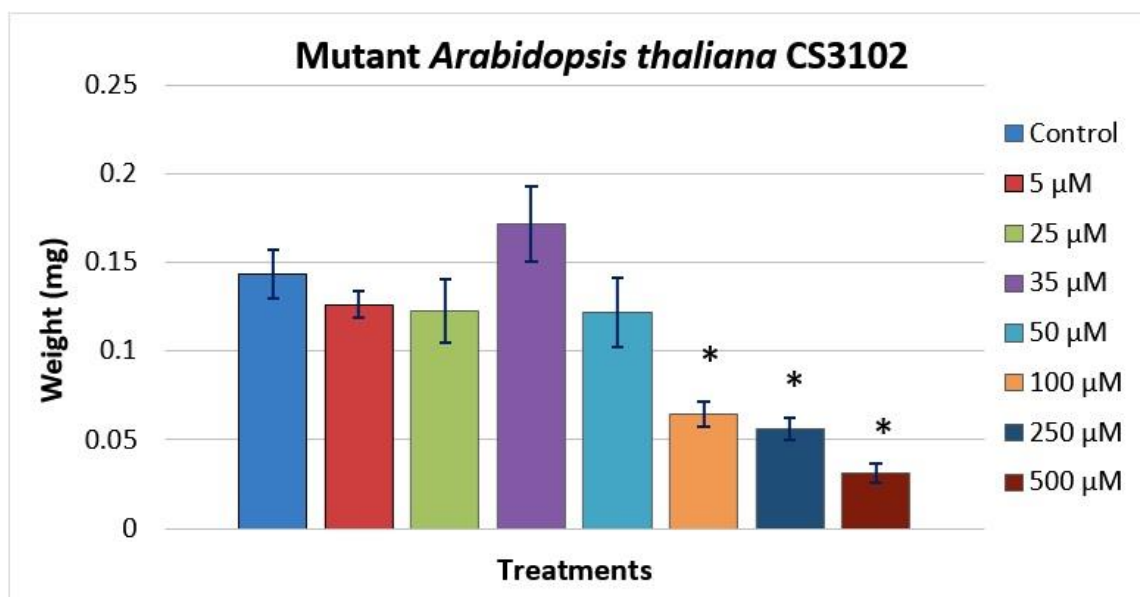
Figure 10: Fresh weights of mutant *Arabidopsis thaliana* CS3102 in dose-response experiments. A significant difference (*) was seen between controls and 100 μM, 250 μM, and 500 μM concentrations ± standard error. The spike in the weight of seedlings treated with 35 μM imazapyr is thought to have been from an error in herbicide application or due to microclimates in the growth area.

Figure 11: Mutant *Arabidopsis thaliana* CS3102 on imazapyr selection plates. *Arabidopsis thaliana* CS3102 were grown on different herbicide concentrations. **A:** Control plate, not subjected to herbicide challenge. No noticeable chlorosis or necrosis. **B:** 5 μM imazapyr. No noticeable effects. **C:** 100 μM imazapyr. This concentration partially overwhelms the herbicide resistance of the seedlings.

AIM 2: Determine the Herbicide Dose-Response for the Non-Resistant Plants

A variety of imazapyr concentrations were tested with seedlings on selection plates to determine the minimal concentrations required to select transformants. For each species, seedlings were grown until the first true leaves appeared in the corresponding control plants (8 – 14 days) and then the batch was sacrificed for testing, as described in detail in the Methods section. The impact of the imazapyr herbicide was assessed by fresh weight of seedlings and by visual inspection of the seedlings.

As expected, wildtype *Arabidopsis thaliana* Columbia seedlings showed necrosis at much lower imazapyr concentrations relative to the herbicide-resistant CS3102 mutant. ANOVA analysis of wildtype *Arabidopsis thaliana* Columbia (**Figure 12**) revealed $p=1.37 \times 10^{-5}$ for between group analysis. The values for each treatment were calculated as follows: average weight of a seedling in milligrams (from 10 random groups of 5 seedlings weighted together) $\pm$ the standard error of the mean; p values calculated through a 2-tailed paired Student's t-test and Bonferroni post-hoc analysis (p value divided by the number of groups); significance accepted as $p \le 0.05$. A control plate with no herbicide treatment was also used which showed no chlorosis or necrosis and ensured seeds were viable. The concentrations of imazapyr chosen for transformant selection experiments were 0.5 μM for moderate selection (~50% seedling death in WT) and 5 μM for stringent selection (lowest concentration with 100% seedling death in WT), as determined by visual inspection of seedlings grown on selection plates. While growth on 5 μM imazapyr had no noticeable effects on the imazapyr-resistant *Arabidopsis thaliana* CS3102 seedlings (**Figure 11 B**), exposure of the WT seedlings to the same herbicide

dose caused complete necrosis (**Figure 13 B**). The imazapyr concentration for stringent selection of *Arabidopsis thaliana* Columbia seedlings (5 μM) is two orders of magnitude less to than the dose needed for stringent selection of *Arabidopsis thaliana* CS3102 (500 μM) due to the presence of the *csr1-2* mutant allele.

Figure 12: Fresh weights of *Arabidopsis thaliana* Columbia in dose-response experiments. A statistically significant difference (*) was seen between the control and either 3.5 μM or 5 μM imazapyr ± standard error.
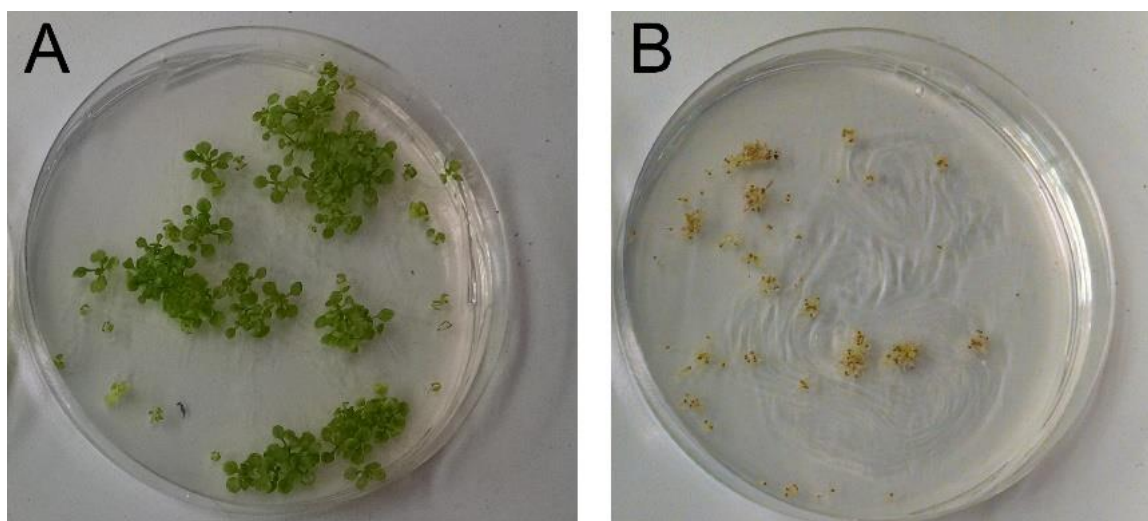
Figure 13: *Arabidopsis thaliana* **Columbia (WT) on imazapyr selection plates. A:** Control plate, not subjected to herbicide challenge. No significant chlorosis or necrosis. **B:** 5 μM imazapyr. Complete necrosis has occurred.

The dose response behavior of *Nicotiana tabacum* showed a greater concentration range between ~50% seedling death (0.13 μM imazapyr) and the lowest working concentration for 100% seedling death (50 μM imazapyr). A control plate with no herbicide treatment was also used to test seed viability. ANOVA analysis revealed $p=6.05 \times 10^{-18}$ for between group analysis. The values for each treatment were calculated as follows: average weight of a seedling in milligrams (from 10 random groups of 3 seedlings weighted together) ± the standard error of the mean; p values calculated through a 2-tailed paired Student's t-test and Bonferroni post-hoc analysis (p value divided by the number of groups); significance accepted as $p \leq 0.05$. (**Figure 14 &**Figure 15). The results for plant growth in 50 μM imazapyr were part of a separate trial and are not included with the results shown in **Figure 14**, although the dose was found to cause full necrosis and death. Thus, the concentrations of imazapyr chosen for transformant

selection experiments were 0.13 μM for moderate selection (~50% seedling death) and 50

μM for stringent selection (lowest concentration with 100% seedling death).
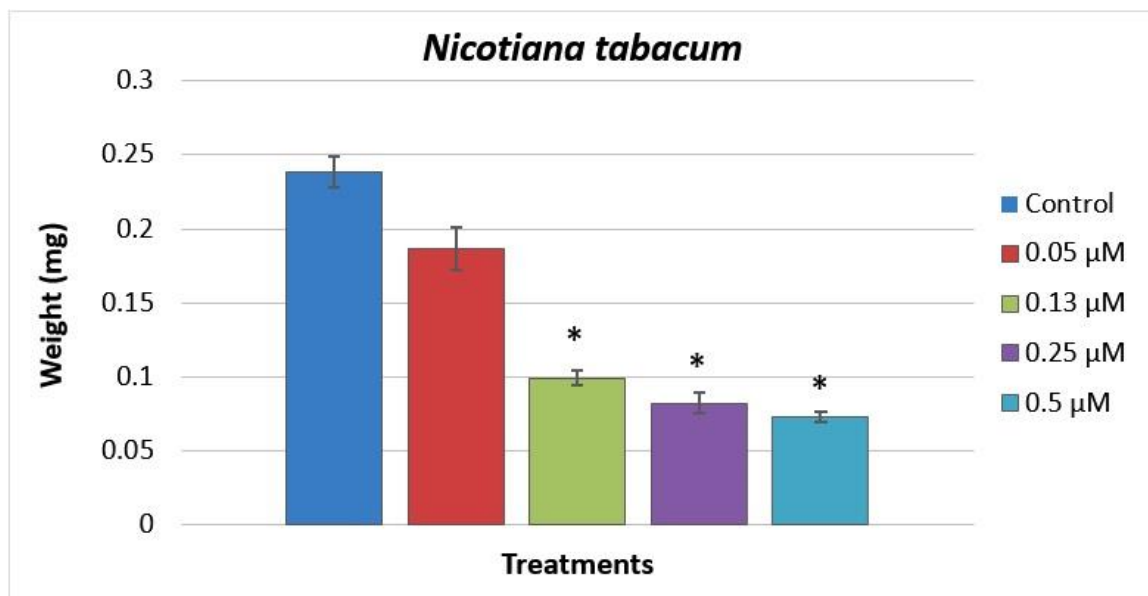
Figure 14: Fresh weights of *Nicotiana tabacum* in dose-response experiments.
Statistically significant differences (*) were seen between the control and either 0.13 μM,
0.25 μM, or 0.5 μM imazapyr ± standard error.
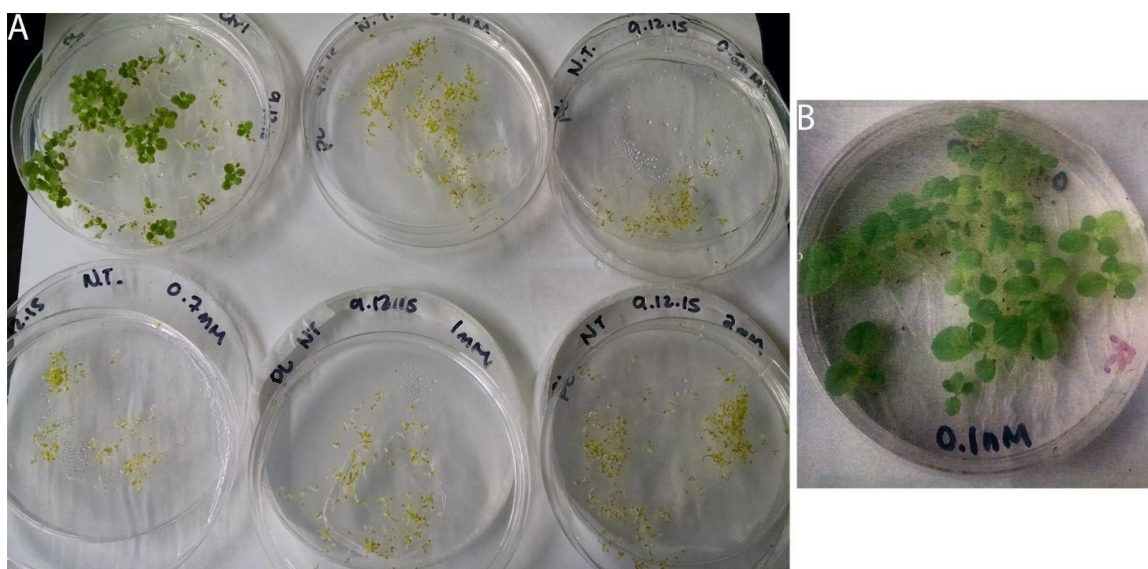
Figure 15: *Nicotiana tabacum* **on imazapyr selection plates. A:** *Nicotiana tabacum* seedlings from an initial set of herbicide dose-response experiments (at concentrations higher than those used for the plant weight statistical analysis in **Figure 14**). From left to right and top to bottom: control, 5 µM, 25 µM, 35 µM, 50 µM, and 100 µM imazapyr. **B:** In a separate trial, a much lower imazapyr concentration of 0.005 nM did not produce much chlorosis in the seedlings.

Glycine max cultures were found to have ~50% embryo death at 0.25 µM imazapyr and 100% embryo death starting at a concentration of 50 µM imazapyr, creating a concentration range very similar to the results observed for *Nicotiana tabacum.* Unlike the other plant species used for this study, *Glycine max* seedlings were grown starting with excised embryos instead of whole seeds. A plate with no herbicide treatment was used as a control for embryo viability. ANOVA analysis revealed $p=4.01 \times 10^{-11}$ for between group analysis. The values for each treatment were calculated as follows: average weight of a seedling in milligrams (from 10 random seedlings weighted individually) ± the standard error of the mean; p values calculated through a 2-tailed paired Student's t-test and Bonferroni post-hoc analysis (p value divided by the number of groups); significance accepted as $p \leq 0.05$. (**Figure 16 & Figure** 17). The concentrations of imazapyr chosen for transformant selection experiments using *Glycine max* were 0.25 µM for moderate selection (closest concentration for ~50% embryo death) and 50 µM for stringent selection (lowest concentration with 100% embryo death).
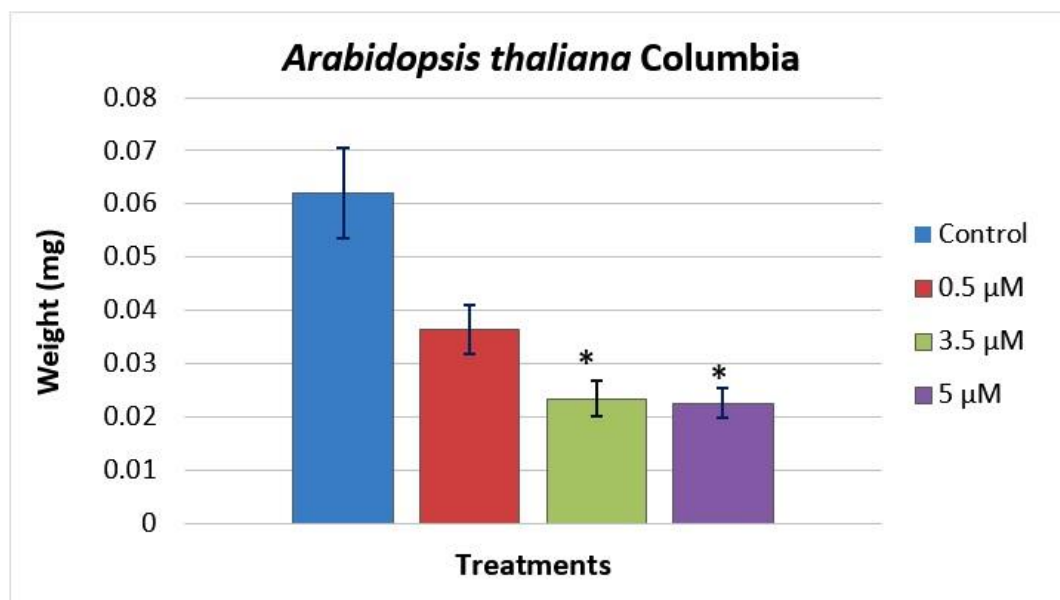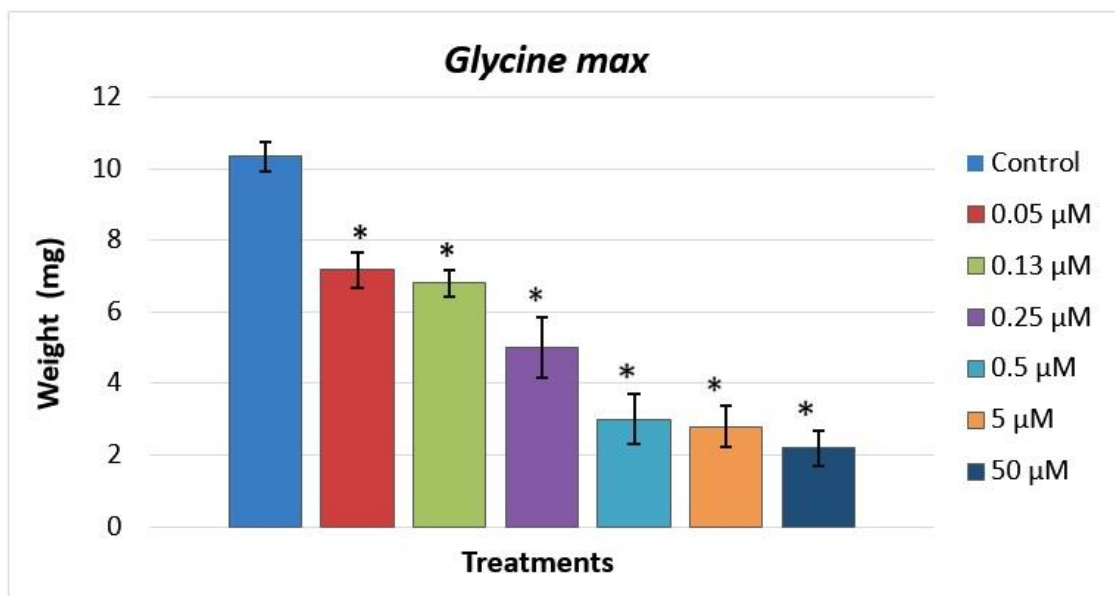
Figure 16: Fresh weights of *Glycine max* in dose-response experiments. Statistically significant differences (*) were seen between the control and each treatment concentration of imazapyr ± standard error.
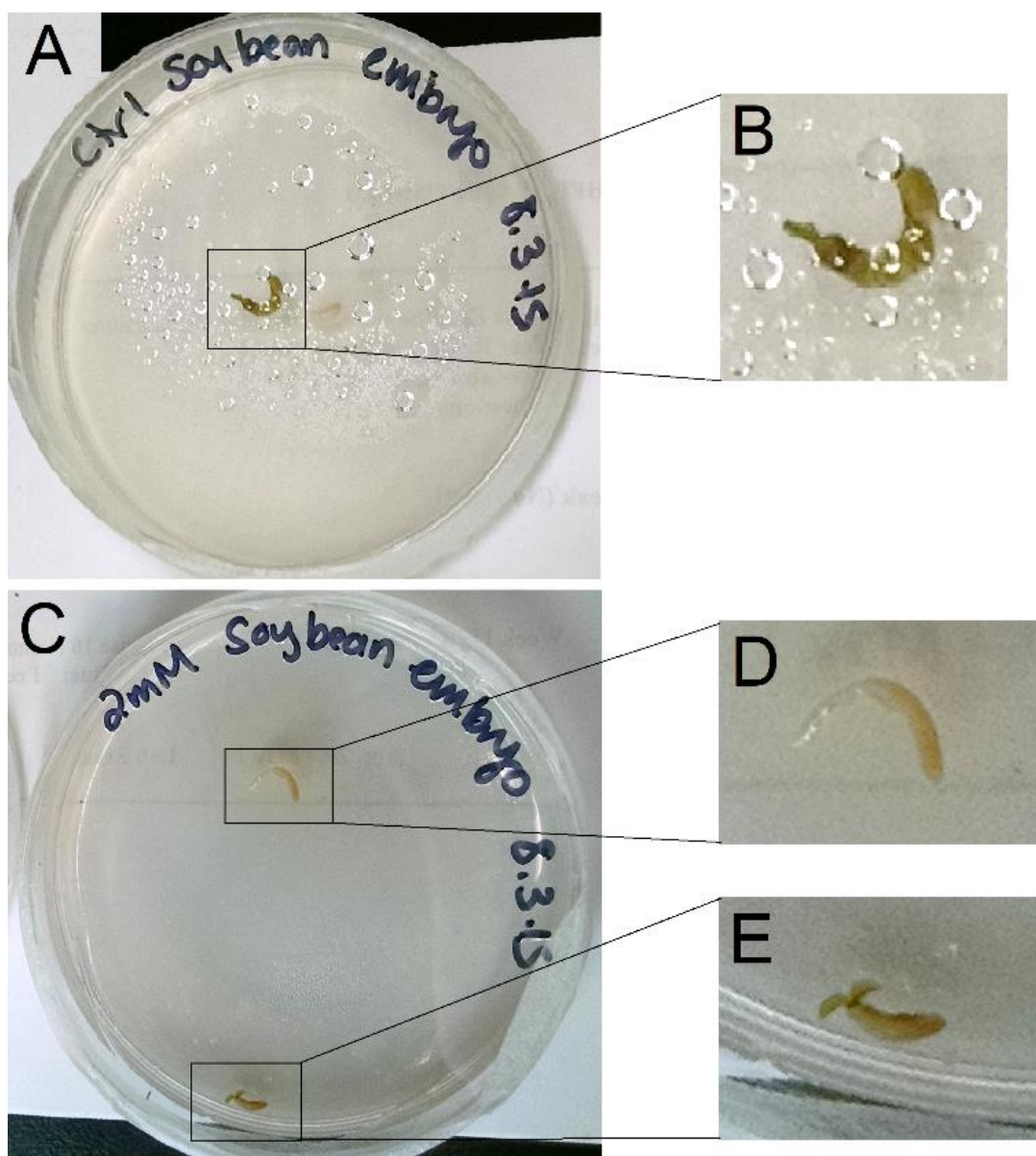
Figure 17: *Glycine max* **embryos in presence of imazapyr. A, B:** Embryonic axis in the control. Small, green, first true leaves can be observed, and the axis shows a lack of chlorosis (**B** is a 3x enlargement of image of **A**). **C**, **D**, **E**: embryos from 100 μM imazapyr herbicide challenge plate. Initial growth in both embryos was followed by complete necrosis. (**D** & **E** are 3x enlargements of image **C**). The results for 50 μM imazapyr (not shown) were similar to those for 100 μM imazapyr, so the lesser concentration was used for stringent selection.

<u>AIM 3: Construct a Vector Containing the Herbicide Resistance Gene</u>

After it was established that the CS3102 germplasm of *Arabidopsis thaliana* showed resistance to imazapyr using the foliar spray test, the surviving plants were used for DNA extraction to ensure that the *csr1-2* allele would be cloned and not the wildtype allele. DNA was extracted from fresh tissue and a genomic fragment was then PCR amplified from this template using the 5' F and 3' R primers shown on the genomic map in **Figure 18.** The amplified product was checked with agarose gel electrophoresis to ensure that a single band was present and then it was ligated into the plasmid pCR 2.1-TOPO TA using the Invitrogen TOPO TA cloning kit. Two of the resulting *E. coli* clones were cultured and the plasmid DNA was isolated using a miniprep kit. The purified plasmid DNA was then sent to the ISU DNA Facility (Iowa State University, Ames, IA, USA) for sequencing to verify that the correct mutation was present at the codon highlighted in red on the genomic map (**Figure 18)**. The sequencing results showed that both clones harbored plasmids with the expected mutation as indicated by the arrow in **Figure 19**. The first clone was used in subsequent experiments.

Codon for susceptibility to imazapyr herbicide

ATGC = coding sequences

ATG = Start/Stop codons

atgc = untranslated regions (UTRs)

atgc = introns

PCR 5' F and 3' R Primers

Sequencing Primer Site

```
5'
aatgtcaaaaataaaacccaattttcatccttaaacgaacctgctgaaaccctaatttcgattaccaattccgatctaaaaagaagtcATGGAAGCCATT
GATTCCGCAATCGATCCTCTCAGAGATTTCGCTAAGAGCAGTGTTCGTCTCGTCCAGCGCTGTCACAAACCCGATCGCAAGGgtaacgccttttctcaaa
aaaatctcatttccgatttttgatctgtagattagggttttctgaaattttgatatcatttgtaattgaattggttatcagGTTTACCGACGTCCGT      5' F primer
GCGTACGGCGATTGGATTTGTGGTGATGGGATTCGTTGGATTCTTCGTGAAGCTCGTTTTCATCCCAATCAACAACATCATCGTTGGATCTTCTTAGtgt
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tcacaagtctcttcttcttctctgtttctctatctcttgctcatctttctcctgaaccATGGCGGCGGCAACAACAACAACAACAACAACATCTTCTTCGATC
TCCTTCTCCACCAAACCATCTCCTTCCTCCTCCAAATCACCATTACCAATCTCCAGATTCTCCCTCCCATTCTCCCTAAACCCCAACAAATCATCCTCCT
CCTCCCGCCGCCGCGGTATCAAATCCAGCTCTCCCTCCTCCATCTCCGGCGTTGCTCAACACAACCACCAATGTCACAACCACTCCCTCTCCAACCAAACC
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GTATTCGCTTACCCTGGAGGTGCATCAATGGAGATTCACCAAGCCTTAACCCGCTCTTCCTCAATCCGTAACGTCCTTCCTCGTCACGAACAAGGAGGTG
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GACCTGTTTTGGTTGATGTTCCTAAAGATATTCAACAACAGCTTGCGATTCCTAATTGGGAACAGGCTATGAGATTACCTGGTTATATGTCTAGGATGCC
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TGCCGATGATCCCGAGTGGTGGCACTTTCAACGATGTCATAACGGAAGGAGATGGCCGGATTAAATACTGAgagatgaaaccggtgattatcagaacctt   Seq primer site
ttatggtctttgtatgcatatggtaaaaaaaacttagtttgcaatttcctgtttgtttttggtaatttgagtttctttttagttgttgatctgcctgcttttt
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attagcaatttacacttccaaaagactatgtaagttgtaaatatgatgcgttgatctcttcatcattcaatggttagtcaaaaaaataaaagcttaacta
gtaaactaaagtagtcaaaaattgtactttagtttaaaatattacatgaataatccaaaacgacatttatgtgaaacaaaaacaatatctagaaaattac   3' R primer
atgctctttttttcttcttctttttgacaaaaaaaaacatctttaattccgatatattgtaaactatatgctgttttttgttttcatcatttcattcttttt
tgttttcatcaatggatttcattgatatgttaaacatgagttacacaacagaccacgtaacaccaataaagattgagacttcttcggaaactaattaaag
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3'
```
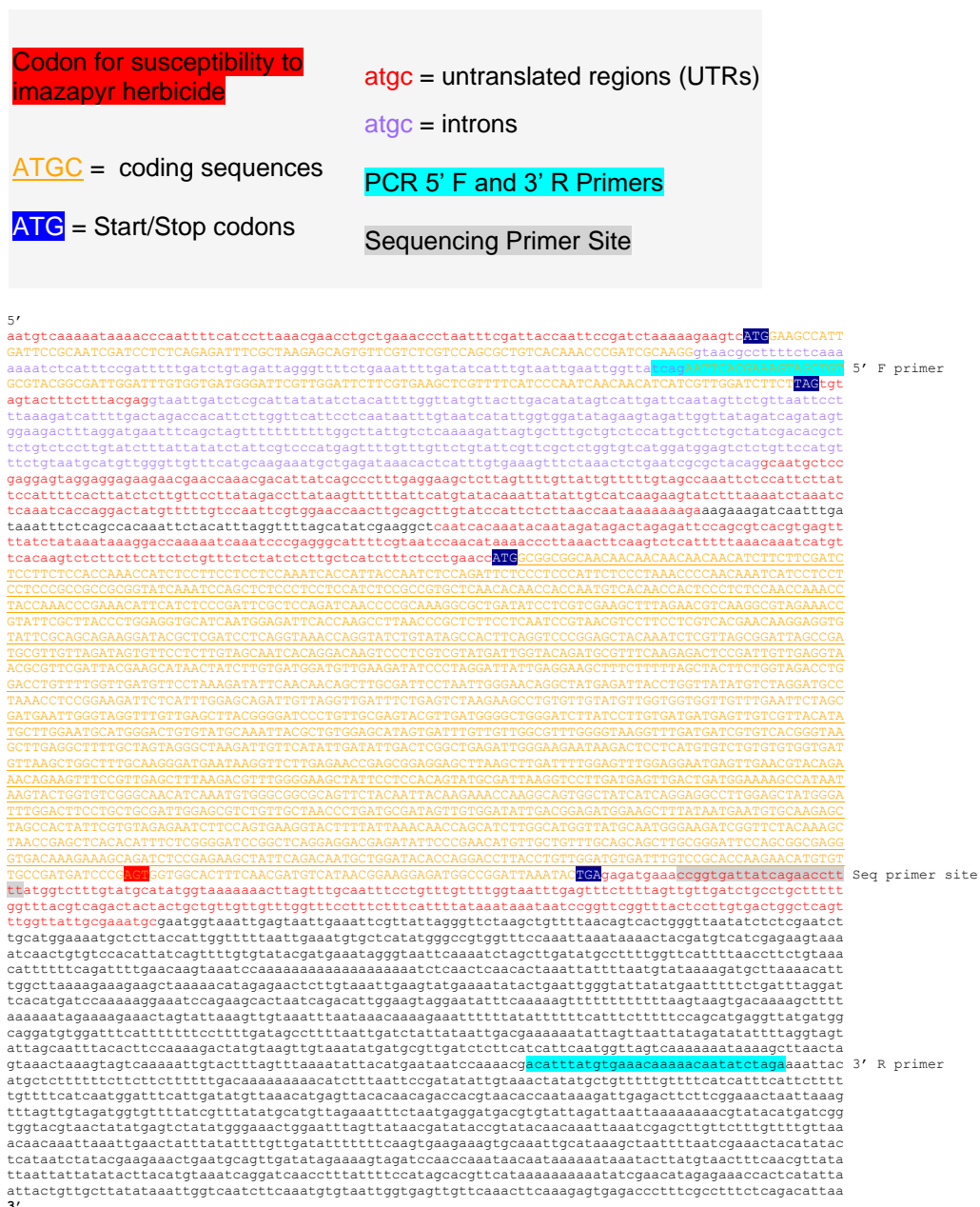
Figure 18: Primer locations on the genomic map of the *csr1* locus. The WT sequence is shown with regions of interest annotated (100 nucleotides per line).
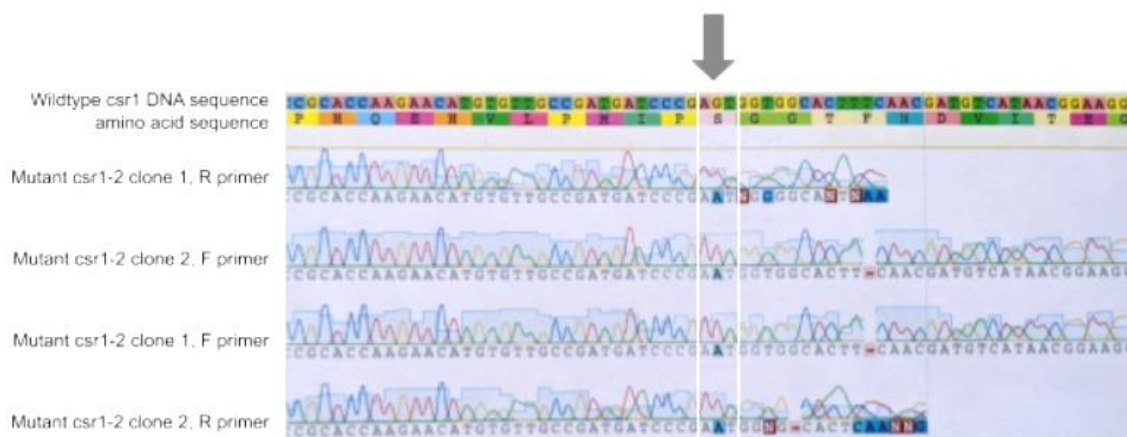
Figure 19: Sequencing results for plasmid vector clones 1 and 2. Sequencing results for two vectors containing the mutant allele *csr1-2* which was PCR amplified and cloned from CS3102 genomic DNA. The arrow indicates the location of the codon of interest, which is AGT (serine, S) in the WT plant and AAT (asparagine, N) on the mutant allele. The point mutation conferring imazapyr resistance was confirmed in both vectors.

AIM 4: Transformation Using Biolistics Targeting the SAM

To confirm that our materials and methods successfully target the SAM, biolistics was conducted with *Glycine max* embryonic axes using the visible marker gene β-glucuronidase. Staining with X-Gluc produced a blue color in transformed cells, indicating the transgene was expressed in the cell (Jefferson et al. 1987). Visual inspection of 102 bombarded *Glycine max* seedlings revealed 13 embryos with transgenic epidermal cells in the SAM. Additional embryos displayed transformed cells outside of the target area of the SAM (**Figure 20**). These experiments confirm that our biolistic parameters were properly set to target the SAM.
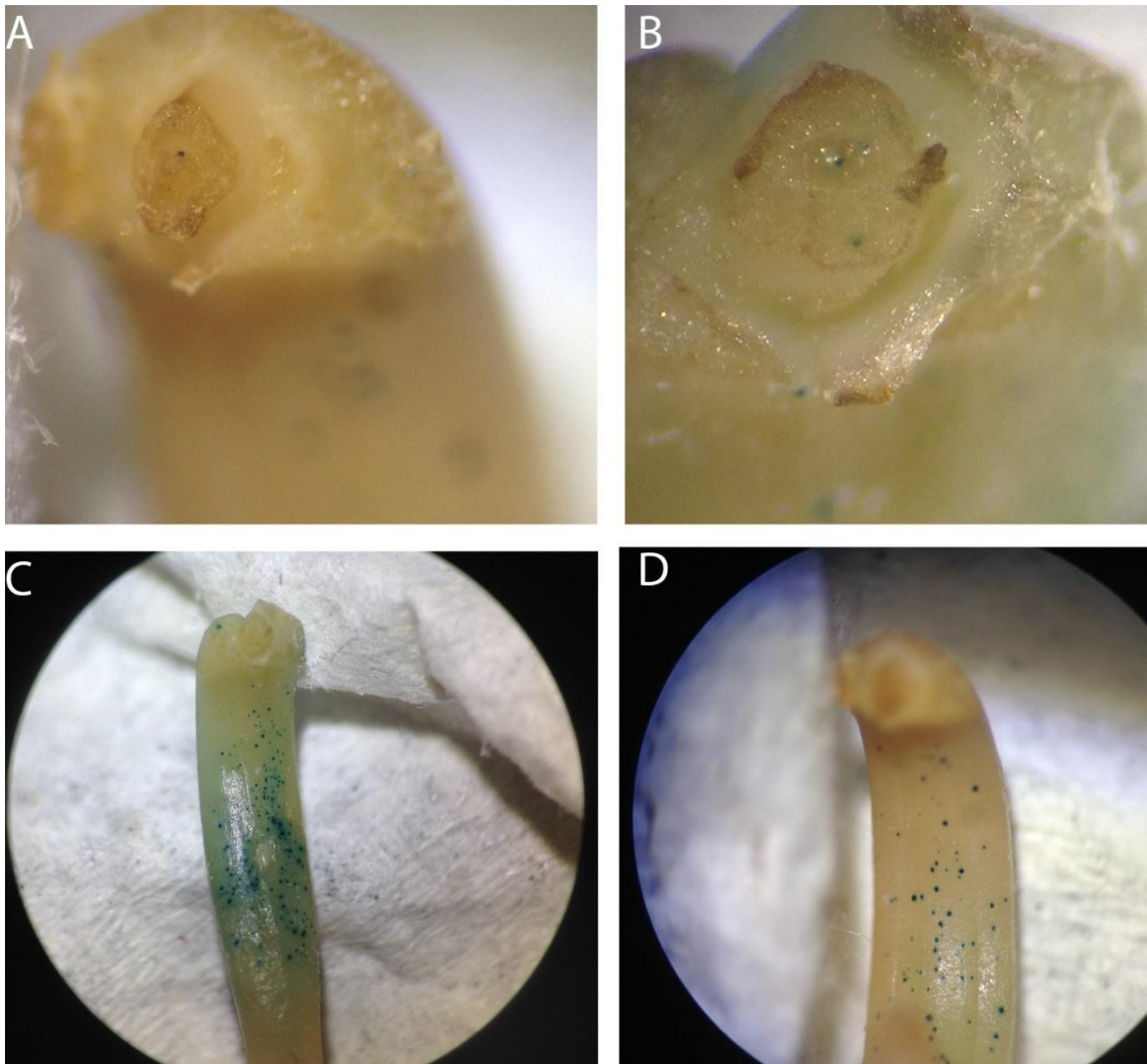
Figure 20: Transient expression of GUS in the shoot apical meristem of *Glycine max* following biolistics. A & B display examples of GUS-expressing cells present in the shoot apical meristem of excised soybean embryos. Transformed cells are stained blue. **C** is a less magnified view of **A**, and **D** is a less magnified view of **B**. As biolistics targets a large area, additional examples of transformed cells can be seen along the embryonic axis outside of the SAM in **C & D**.

The plasmid containing the cloned *csr1-2* allele was used for SAM biolistic experiments with *Glycine max* seedlings and the seedlings were then screened on medium containing imazapyr at a concentration intended for moderate selection (~50% expected death) or stringent selection (100% expected death). Out of 305 embryos screened, no transgenic plants were recovered. Therefore, the transformation rate was less than 0.3%. Three plants (two soybean and one tobacco) showed early promise, with growth persisting through herbicide screening. **Figure 21** shows the three plants that were subjected to SAM biolistics and persisted after herbicide screening. The *Glycine max* embryo in **Figure 21 A** was only grown on selection medium, whereas the seedling in **Figure 21 B** was transferred to sterile soil after several weeks in imazapyr media and allowed to grow through flower and seed stages. No adverse effects were observed, aside from the minor chlorosis that can be seen in **Figure 21 B**. Leaf tissue was collected from each plant and DNA was extracted to test for the presence of the *csr1-2* allele.
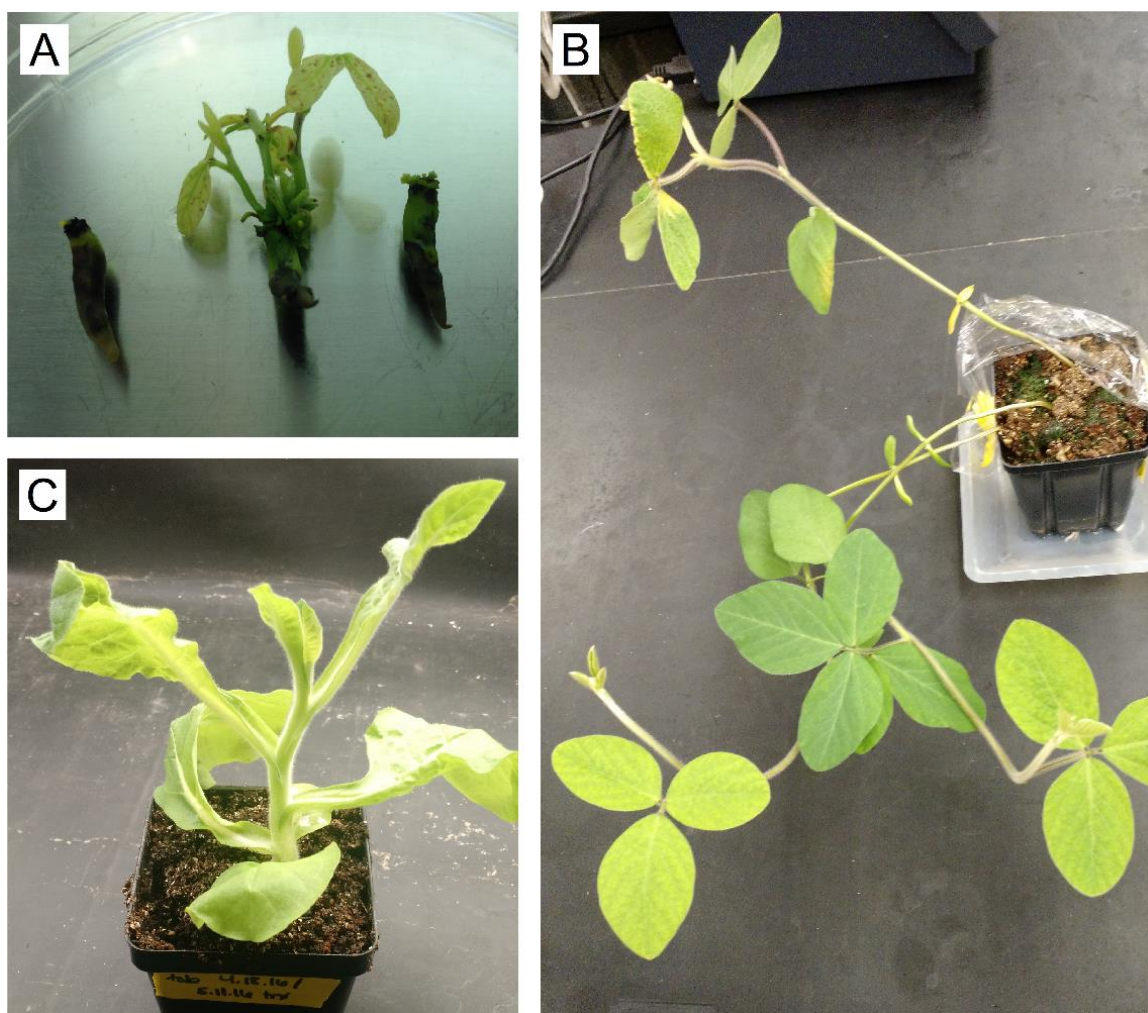
Figure 21: Putative transgenic plants displaying growth through herbicide challenge with imazapyr. A: Soybean embryo that produced leaves while growing on media containing imazapyr. The other embryos only show growth of the hypocotyl. **B:** A second soybean plant grown though herbicide screening was transplanted into soil and later produced seed pods. **C:** Tobacco seedling transplanted to soil after growing on media containing imazapyr. The presence of the transgene could not be confirmed using PCR in any of these plants suggesting they are escapes.

A multiplex PCR was run using primers for soybean actin, as PCR positive control, and primers for the *csr1-2* gene, as a test for genetic transformation (**Figure 22**). A negative control sample consisted of DNA extracted from an untransformed soybean, which does not harbor the *csr1-2* allele. A positive control sample consisted of DNA extracted from *Arabidopsis thaliana* CS3102, which does contain the *csr1-2* allele. Gel electrophoresis showed amplification of soybean actin in both the transformed and untransformed *Glycine max* plants, showing that the DNA template was of sufficient quality for PCR. The *Arabidopsis thaliana* CS3102 DNA used as a positive control showed a band for *csr1-2*, indicating the *csr1-2* primers were working correctly. However, there was no band corresponding to the *csr1-2* gene in either soybean sample, indicating the persistent seedling shown growing on herbicide in **Figure 21 A** was a false positive. No biolistic transformation had occurred. Similar negative results were obtained for the plant in **Figure 21 B**.
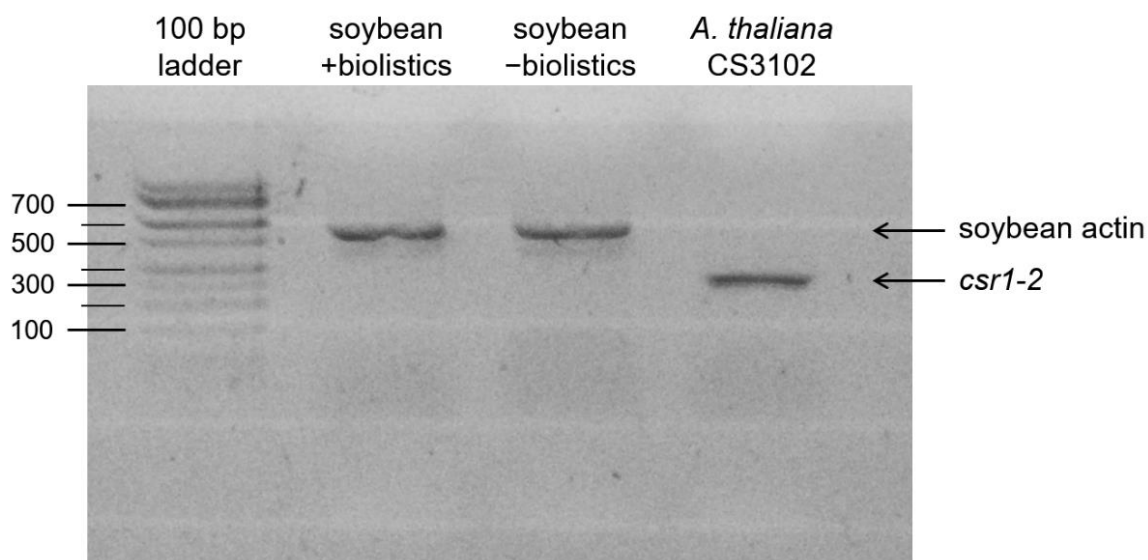
Figure 22: DNA testing of putative *Glycine max* transformant for the *csr1-2* transgene. The multiplex PCR results indicate the *Glycine max* seedling (shown in **Figure 21 A**) does not contain the transgene. Left to right: 100 bp ladder; DNA from false positive *Glycine max* subjected to biolistics and selection on imazapyr, displaying only a single band at 533 bp (soybean actin) and no second band at 352 bp for the *csr1-2* allele; negative control DNA from *Glycine max* not subjected to biolistics, with a single band at 533 bp (soybean actin); positive control DNA from *Arabidopsis thaliana* CS3102, displaying a band at 352 bp, as expected for the *csr1-2* allele. The expected result for a transformed soybean plant would be a pair of bands, one for soybean actin and a second for *csr1-2*.

Similarly, no transformed plants were detected in experiments with the other two plant species tested. Approximately 1,000 *Arabidopsis thaliana* Columbia seedlings were bombarded and screened at the two herbicide concentrations for moderate and stringent selection. Another 500 seedlings served as negative controls, receiving biolistics with no associated DNA coating (gold microparticles only). Approximately 670 *Nicotiana tabacum* seedlings were bombarded and screened at the two herbicide concentrations for moderate and stringent selection. Another 300 seedlings served as negative controls, receiving SAM biolistics with no associated DNA coating (gold microparticles only). No resistant plants were detected out of all the specimens bombarded.

CHAPTER 4

DISCUSSION

<u>Herbicide Dose-Response Experiments</u>

The mutant *Arabidopsis thaliana* CS3102, which carries a resistance allele to imazapyr, *csr1-2*, did not suffer any lasting harmful effects from foliar spray of imazapyr. Initial patchy chlorosis and necrosis was seen in mutant plants after surfactant and imazapyr application, however these were transient, and plants recovered and grew through flowering and seed set. This minor foliar irritation may have been from the surfactant used (similar results were seen in mutant plants sprayed with surfactant only) and did not prevent the formation of seeds. The surfactant used, Sylgard 309, is a nonionic surfactant commonly used in herbicide application. Nonionic surfactants are usually not harmful and have no deleterious effects when applied to plants. However, it has been noted that care must be taken during application, as a high volume of nonionic surfactant can cause plant injury (The University of Georgia, 2020). The dose suggested for field applications of surfactant (0.125% [v/v] in distilled water) may have been too high for greenhouse or laboratory plants, which tend to differ slightly from field-grown plants in both root and shoot structure (Poorter et al. 2016).

Next, dose-response experiments were performed to determine appropriate imazapyr concentrations for selection of *Nicotiana tabacum, Arabidopsis thaliana,* and *Glycine max.* In our seedling plate assay for *Glycine max,* the concentrations of imazapyr that caused ~50% seedling death (moderate selection, 0.25 μM) and 100% seedling death (stringent selection, 50 μM) were found to bracket the 0.5 μM imazapyr concentration

used for selection in the Nature Protocols paper published by Rech et al. (2008). For

Arabidopsis, initial experimental concentrations were based on the "ideal" concentration

of imazapyr found in the Manabe et al. (2007) study of 100 µg/L (~0.4 μM), at which all

wildtype plants died and all *csr1-2* mutants survived. *Arabidopsis thaliana* CS3102 (*csr1-2* mutant) was found to require 100 times more herbicide than wildtype to cause ~50%

and 100% death. The experimental values of effective imazapyr concentrations for

Arabidopsis thaliana Columbia and the *Arabidopsis thaliana csr1-2* mutant are in line

with those observed in a study by Roux and Reboud (2005). In *Nicotiana tabacum,* a

study using *Agrobacterium* tested a wide range of concentrations of imazapyr, from 100

nM to 1 mM (Sathasivan et al. 1991). For the present experiment, the concentration that

caused ~50% seedling death was 0.13 μM imazapyr, which aligns well with the

concentration of imazapyr used previously with *Nicotiana tabacum* (Al-Ahmad et al.

2004). Ultimately, 50 μM imazapyr was used as the upper concentration for both

Nicotiana tabacum and *Glycine max* experiments to ensure complete death as based on

both the experiments described herein, and the values published in the literature.

Certainly, the foliar spray and herbicide dose-response experiments showed the herbicide

was effective.

<div align="center">Cloning the Resistance Gene from Arabidopsis thaliana CS3102</div>

Previous work showed that the native *Arabidopsis* promoter could express

imazapyr resistance in other species such as soybean, common bean, and cotton (Rech et

al. 2008). They cloned a 5718 base pair genomic fragment which contained the imazapyr

resistance gene and its native promoter from *Arabidopsis thaliana* mutant GH90 into a

pBluescriptSK+ plasmid that included the ampicillin resistance gene (*bla*). Attempts were made to acquire their proprietary plasmid, but these attempts were unsuccessful. We therefore PCR amplified and cloned our own genomic fragment of the same size into a pCR-2.1 TOPO plasmid from Invitrogen.

The template DNA for PCR amplification of *csr1-2* was from plants subjected to herbicide foliar spray and which showed resistance to the herbicide imazapyr. This was used as a redundant check to ensure resistance was present. The cloned DNA sequence was also verified through Sanger sequencing to ensure the point mutation was present. In addition to the *csr1-2* allele, our cloned sequence also contained a portion of the upstream gene, Sec 61 subunit gamma 3 (SEC61G3), which is involved in protein transport in the endoplasmic reticulum.

Transformation Efficiency Using Biolistics

To measure the utility of the *csr1-2* allele as a selectable marker gene, we used the PDS-1000/He Particle Delivery System to transform SAM (Rech et al. 2008). In brief, the plasmid vector containing the marker gene was grown in *E. coli*, purified using a miniprep kit, and coated onto microparticles for SAM biolistics. After the coated microparticles were propelled into excised embryos, transgenic SAM cells were selected with imazapyr to grow plants using shoot-inducing hormones.

A variation in this thesis is the use of gold microparticles instead of tungsten. One reason for this is that the tungsten used in Rech et al. (2008) is listed as "submicron" size. This vague specification is not critical when working with larger soy embryos, but can possibly be deleterious when working with diminutive seedling targets. If varying particle

sizes were used as microcarriers, results could vary widely—both in the success of

biolistic transformation and plant survival. *Arabidopsis thaliana* also has smaller cells

than *Nicotiana tabacum* and *Glycine max* (Appendix 1), and larger particles could

potentially destroy more cells than could survive the assault. To reduce variability,

standard size gold microcarriers were selected at both 1600 μm and 1000 μm diameters.

In addition, we selected gold, an inert metal, because tungsten can cause toxicity in plant

tissues and contribute to necrosis and lower transformation rates (Russell et al. 1992).

Gold particles were found to increase transformation rates four-fold (Russell et al. 1992).

 Glycine max embryo transformation has been used since the late 1990s (Yamada

and Isimoto 2012), and Rech et al. (2008) were one of the first to use embryos for

transformation using biolistics with an imidazolinone herbicide resistance gene as a

selection marker (Aragão et al. 2000; Rech et al. 2008; Yamada and Isimoto 2012). This

technique uses a unique combination of biolistics which targets the SAM and an

herbicide that targets actively growing meristems. Other methods and herbicides which

do not target the SAM would lead to higher rates of chimeric expression of the transgene

(Juturu et al. 2015).

 To confirm that our materials and methods were capable of SAM biolistics, we

conducted preliminary experiments using the GUS marker gene with *Glycine max*

embryos. For initial experiments, 900-psi, 1100-psi, and 1350-psi rupture discs were used

on soybean embryos. The Rech et al. (2008) protocol used discs which ruptured at 1200

psi. 1350-psi rupture discs (actual rupture occurring at 1300 psi) tended to blow the

embryos out of the Petri dish. This could lead to contamination and extensive damage to

the *Glycine max* embryos. Embryos were embedded in agar media in Petri dishes to alleviate this problem. However, 1350-psi rupture still tended to blow embryos out of the Petri dish and was deemed too high a pressure for biolistics experiments. 900-psi rupture discs were tested, but ultimately 1100-psi rupture discs were used for our experiments, as it was the closest pressure to what is reported in the literature (Rech et al. 2008). 1100-psi rupture discs did not blow embryos out of the dish and also did not damage plant tissue extensively in *Arabidopsis thaliana* and *Nicotiana tabacum* seedlings (Appendix). Initial *Glycine max* experiments displayed mold contamination, which was eliminated in further experiments by performing all pre-biolistic embryo excisions in a sterilized laminar flow hood. As the experiment from Rech et al. (2008) was performed in Brazil, it proved impossible to use the same cultivars of *Glycine max* in the United States (all attempts were made to procure the same cultivars, but due to international agriculture laws these attempts were not successful). Those used in the literature include BR-16, BR-91, Celeste, Conquista, Doko RC, Nina, Indiana, and Itapu from Embrapa Soybean, Londrina, PR, Brazil (Rech et al. 2008). We used two soybean cultivars, Hutcheson and FS HiSOY® (HS26A32, lot SFS64011). Hutcheson is an older, conventional cultivar registered in 1988 (Buss et al. 1988). HiSOY® is a modern cultivar which is genetically modified to be "Roundup Ready". It contains resistance to glyphosate, a broad-spectrum herbicide in a different class from imazapyr. Both resistance genes can work in the same plant, as evidenced by the doubly-resistant canola cultivar that is available (Simard et al. 2005). The results of our GUS biolistics experiments yielded transient expression in the epidermal cells of soybean SAMs.

Our experiments selecting transformants with *csr1-2* as a marker gene found no transgenic plants for *Glycine max*, *Nicotiana tabacum*, or *Arabidopsis thaliana* Columbia, indicating that the transformation efficiency in our hands is too low to be useful. For *Arabidopsis thaliana,* this may be due to the tightly enclosed apical meristem (Appendix) which may block microparticle bombardment of the target cells. Another problem may be that the native *Arabidopsis* promoter does not generate enough gene expression in other plant species such as our varieties of *Glycine max* and *Nicotiana tabacum*, even though the native promoter was reported to be sufficient in the soybeans tested by Rech et al. Of the crops they tested, soybeans had the highest rate of transformation – soybean at 9%, common bean at 2.7% (*Phaseolus vulgaris)*, and cotton at 0.5% (*Gossypium spp*) (Rech et al. 2008).

<u>Future Directions</u>

To check the depth of microparticle penetration in future SAM biolistic experiments, the GUS marker gene could be used and the SAM sectioned after biolistics to observe the location of the blue transformed cells. If the transgene is not penetrating past the L1 layer, we could modify the protocol by using rupture discs with a higher psi, or in the case of *Arabidopsis thaliana,* we could remove the cotyledons to expose the SAM for biolistics.

We could also change the promoter in our plasmid vector. Expressing *csr1-2* with a constitutive promoter, such as the 35S over-expression promoter, may improve transformation efficiency. There are a number of 35S expression vectors used in plant biotechnology that are available for sub-cloning our *csr1-2* construct.

Finally, we could try a different transformation technique. A recent advance in plant transformation is nanotubule-mediated delivery of transgenes (Landry and Mitter 2019). This method uses sub-micrometer carbon nanotubes which are commercially available. Previous literature successfully used carbon nanotubules to transform both monocotyledon and dicotyledon plants of multiple species including *Nicotiana benthamian*a (a model plant species which is related to tobacco), *Eruca sativa* (arugula), *Triticum aestivum* (wheat), and *Gossypium hirsutum* (cotton) (Demirer et al. 2019). In brief, commercial carbon nanotubules were complexed with a cationic polymer and then incubated with negatively-charged plasmid DNA containing the genes of choice. Mature true leaves of target plants were punctured with a pipette tip and a solution of the DNA and carbon nanotubules was applied to the puncture until absorption. Carbon nanotubules

can transverse the plant cell wall, which can also be achieved through biolistics. However, as previous literature shows, it is difficult to target biolistics microparticles to the stem cells that lie beneath the cortex layer in meristems (Juturu et al. 2015) and biolistics can be damaging, especially to diminutive seedlings like *Arabidopsis* and tobacco used in this thesis. Using carbon nanotubule delivery with the *csr1-2* herbicide resistance gene may result in a higher transformation frequency than seen in this thesis, but that remains to be seen.

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APPENDIX

<u>Sectioning, Staining, and Cellular Surface Area Determination</u>

To determine target cell sizes, several day-old root tip cells were observed microscopically from *Arabidopsis thaliana* Columbia, *Arabidopsis thaliana* CS3102, and *Nicotiana tabacum*. Seeds were surface sterilized as previously described and grown in Petri plates with 2% technical agar and 0.25 g/L fertilizer. *Glycine max* seeds were surface sterilized, soaked in sterile water (lab sourced) for at least 20 minutes, and then dissected to remove the embryonic axes, as previously described. The embryos were then grown on 2% micropropagation agar containing quarter-strength (1.08 g/L) MS basal salts and 3% sucrose until roots were apparent. For all plants, thin sections were made using a scalpel blade, placed on a microscope slide, and stained using a methylene blue dip (#48504, Ricca Chemical Company, Arlington, TX, USA). Slides were observed under a binocular laboratory microscope (Olympus CH-2, Shinjuku, Tokyo, Japan) and the cellular surface areas were calculated.

<u>Sectioning</u>

Seeds were grown in Petri plates as above (see Sectioning, Staining, and Surface Area Determination). Several days post-germination, *Arabidopsis thaliana* Columbia, *Arabidopsis thaliana* CS3102, and *Nicotiana tabacum* seedlings were harvested for sectioning and staining. *Glycine max* seeds were surface sterilized and the embryos excised for sectioning.

Preparation for sectioning protocol:

1. Plant tissue was collected and placed in formalin-acetic acid ethanol (FAA) (#112016, Ricca Chemical Company, Arlington, TX, USA) in a Falcon tube for a minimum of 12 – 16 hours.

2. FAA was removed from the Falcon tube via micropipette and discarded. Plant tissue was placed in small cassettes (#27159-1, Ted Pella Inc., Redding, CA, USA) in a Coplin staining glass jar (#E94, Thermo Fisher Scientific, Waltham, MA, USA) and the following steps were followed for dehydration:

   a. **TBA 1:** Mix: 40% of 95% ethanol (#2716G, Decon Laboratories, Inc., King of Prussia, PA, USA (absolute ethanol, all other concentrations lab sourced and diluted with distilled water)), 10% tert-butyl alcohol (TBA) (#A401-500, Thermo Fisher Scientific, Waltham, MA, USA, appropriate concentrations lab sourced), and 50% distilled water (lab sourced). Immerse cassettes and allow to sit at room temperature (~23°C) for 30 minutes.

   b. **TBA 2:** Mix 50% of 95% ethanol, 20% TBA, and 30% distilled water. Immerse cassettes and allow to sit at room temperature for one hour.

   c. **TBA 3:** Mix 50% of 95% ethanol, 35% TBA, and 15% distilled water. Immerse cassettes and allow to sit at room temperature for 30 minutes.

   d. **TBA 4:** Mix 40% of 95% ethanol, 55% TBA, and 5% distilled water. Immerse cassettes and allow to sit at room temperature for 30 minutes.

   e. **TBA 5:** Mix 25% of 95% ethanol and 75% TBA. Immerse cassettes and allow to sit at room temperature for 30 minutes.

f. **TBA 6:** Immerse cassettes in absolute TBA and allow to sit at room temperature for one hour.

g. **TBA 7:** Immerse cassettes in absolute TBA and add several grains of Eosin (donated/historic stock, Central Scientific, Chicago, IL, USA) and allowed to sit at room temperature for 30 minutes.

h. Mix 50:50 absolute TBA and liquid paraffin (#39503002, Paraplast Plus Tissue Embedding Medium, McCormick Scientific/Leica, Wetzlar, Germany) and immerse cassettes. Allow to sit at room temperature in a chemical hood until the TBA evaporates (up to three days).

i. Immerse cassettes in 100% liquid paraffin and place in an oven overnight at 65ºC

j. Discard liquid paraffin and replace with fresh 100% liquid paraffin. Allow to sit overnight in oven at 65ºC

3. **Embedding:** Cassettes with plant tissue were removed from the paraffin jar and placed in the holding chamber of liquid paraffin in a histoembedder (#MES 1609, Leica, Wetzlar, Germany). Paraffin block molds (#189851, Polysciences, Inc., Warrington, PA, USA) were placed under the paraffin dispenser of the histoembedder and hot paraffin (65-70ºC) was poured in the molds to slightly over-full to prevent significant cooling depressions. The molds were moved to the frozen side of the histoembedder and plant tissue samples were placed in the paraffin using hot forceps. Samples were allowed to sit until cool, then placed in a freezer at -20°C until sectioning was performed.

4. **Sectioning:** Water bath (XH-1003, Premiere, C&A Scientific, Manassas, VA, USA) and slide warmer (XH-2003, Premiere, C&A Scientific, Manassas, VA, USA) were set to temperature (38.0°C for water bath, 37.0°C for warmer). Sample paraffin blocks were removed from freezer and trimmed using a regular razor blade. Blocks were positioned in the microtome (#HM 315 Microm, Thermo Fisher Scientific, Waltham, MA, USA) holder and sectioned using a low-profile blade (#3051835, Thermo Fisher Scientific, Waltham, MA, USA). Resulting paraffin ribbons were transferred to the water bath using a dry regular paintbrush and allowed to sit until expanded and wrinkles flattened. Paraffin ribbons were then placed on glass slides and set on the slide warmer for a minimum of 12.0 hours.

5. **De-paraffining slides:** Slides were placed in a Coplin staining jar in a chemical hood. Xylene (#X3S-4, Thermo Fisher Scientific, Waltham, MA, USA) was poured to cover the slides and the jar was covered. Slides were allowed to sit for five to ten minutes after which they were removed and rinsed using distilled water (lab sourced) and air dried.

Sectioning and Staining

Samples of biolistically-bombarded tissue were sectioned and viewed to determine cellular and nuclear size, and to attempt to view gold microparticles in the plant tissue. Varying rupture disks were used to determine at what pressure microparticles would be propelled into apical meristematic regions with minimal tissue damage. 900 psi rupture disks allowed very few microparticles into plant tissue, especially in *Glycine max* embryos which have a thick, waxy outer coating. The optimal rupture disk for all species was found to be 1100 psi (with rupture actually occurring at approximately 1000 psi). This rupture disk pressure did not destroy excessive tissue in *Nicotiana tabacum,* (**Figure**) which has an exposed apical meristem (**Figure 23**) and was adequate to propel particles into the meristem of *Arabidopsis thaliana,* which has an apical meristem protected by a rosette of leaves (**Figure 24 & Figure**). Initially *Nicotiana tabacum* seedlings and *Arabidopsis thaliana* seedlings were placed in a pattern similar to that of the *Glycine max* embryos in a circle to utilize the optimal placement for microparticle bombardment with approximately 20-25 seedlings on each plate. In subsequent experiments, it was determined that more seedlings on a single Petri plate would yield more transformed seedlings. A 60 mm Petri dish was covered in surface sterilized seeds and they were allowed to grow to at least the cotyledon stage prior to being subjected to biolistic bombardment. By doing this, the number of cells that received microparticles was increased as well as the rate of transformation. Because the embryos have to be dissected out of the *Glycine max* seeds before biolistics, it requires a

significant time investment which is not necessary when growing smaller seeds on the

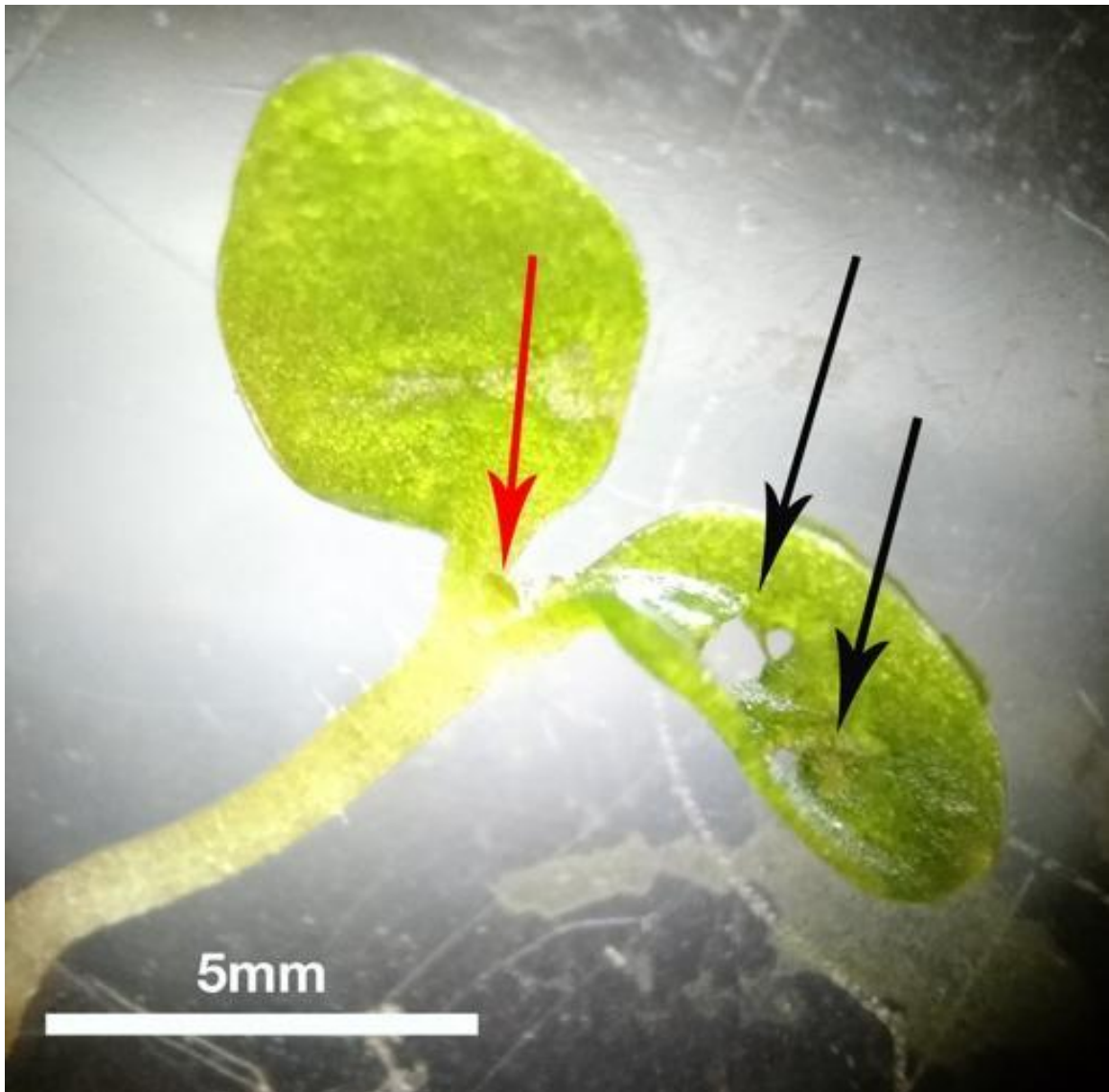media in which they will receive microparticle bombardment.

Figure A1: *Nicotiana tabacum* **seedling after biolistics bombardment.** This image was taken with 10x magnification after biolistics with a 1100 psi rupture disc and 1000 nm diameter gold microcarriers. Black arrows indicate tissue damage from biolistics. Red arrow indicates the exposed apical meristem.
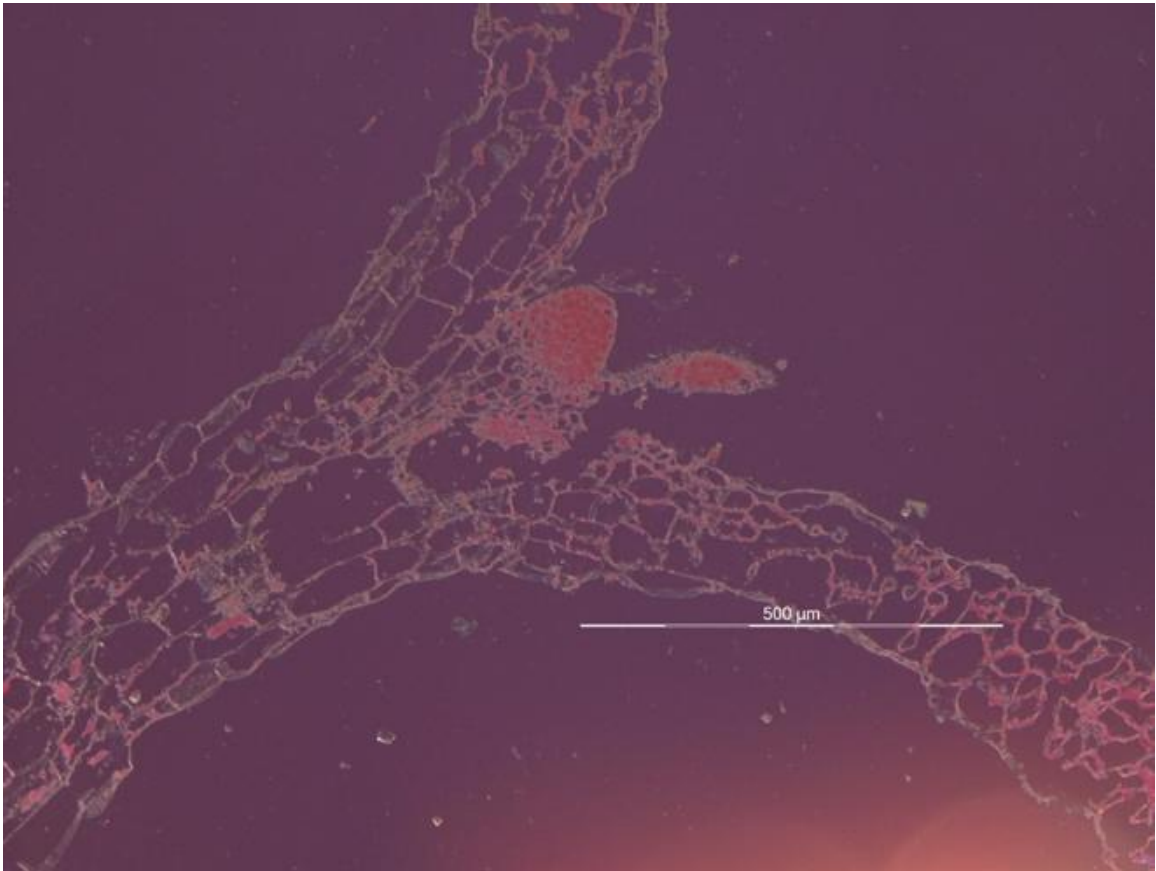
Figure 23: *Nicotiana tabacum* **seedling section.** This image shows a 25 µm section of an eosin-stained tobacco seedling depicting the exposed shoot apical meristem (central, red-stained region).
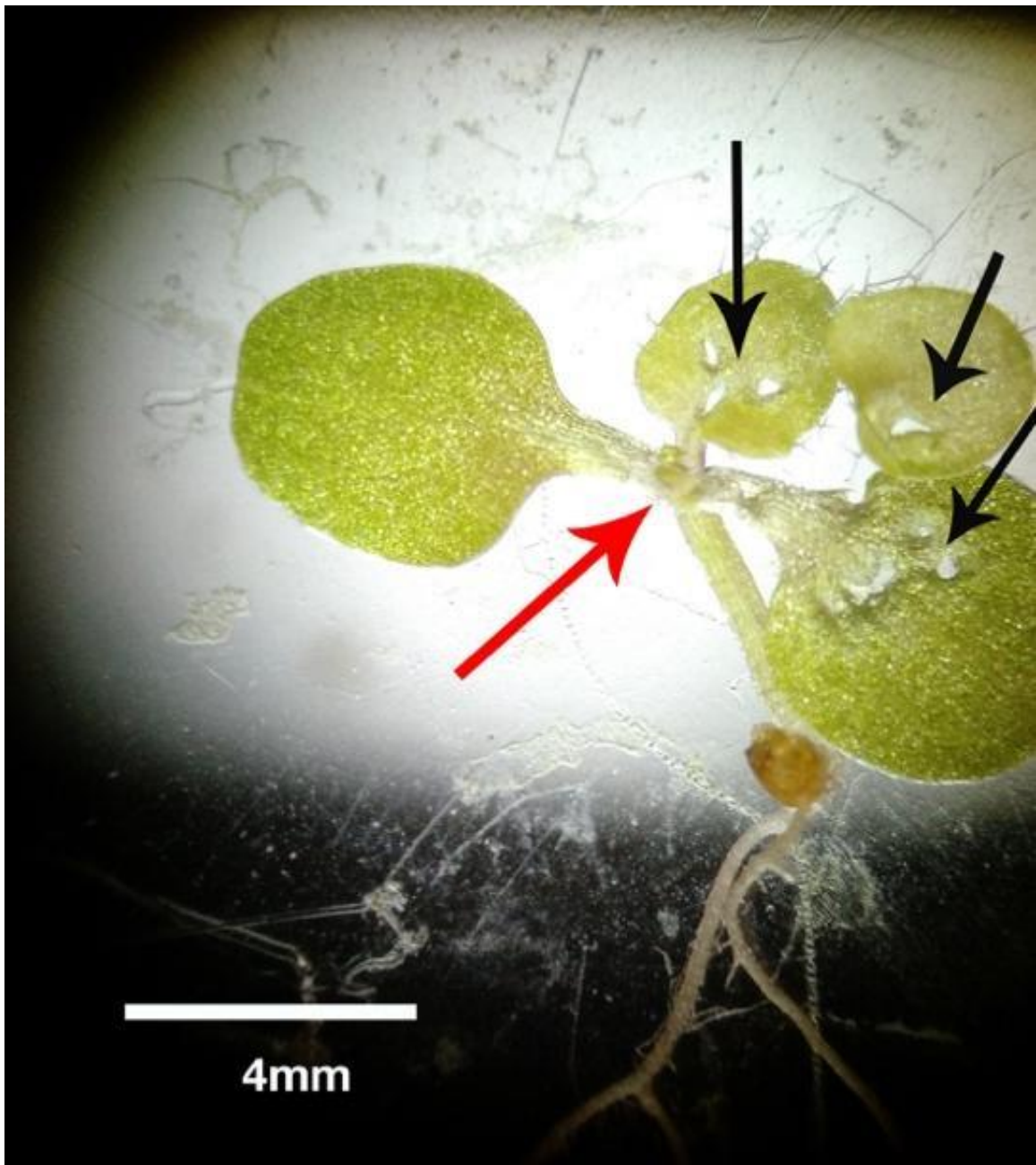
Figure 24: *Arabidopsis thaliana* **Columbia after biolistic bombardment.** This image was taken with 10x magnification after biolistics with a 1100 psi rupture disc and 1000 nm diameter microcarriers. The black arrows indicate tissue damage and the red arrow indicates the shoot apical meristem.

Figure A4: *Arabidopsis thaliana* **Columbia seedling.** This image shows a 25 µm section of an eosin stained *Arabidopsis* seedling. The black arrow indicates the tightly-enclosed apical meristem.

Cellular Surface Area

Cellular and nuclear surface areas were estimated from sections viewed under a microscope. *Arabidopsis thaliana* was found to have an estimated cellular surface area of 309 µm^2 for a meristematic cell and an estimated nuclear surface area of 73.8 µm^2. *Nicotiana tabacum* had an estimated cellular surface area of 417 µm^2 meristematic cell, and an estimated nuclear surface area of 27.1 µm^2. *Glycine max* had an estimated cellular surface area of 2110 µm^2 and a nuclear surface area of 274 µm^2. The gold microparticles have an average diameter of 1.0 µm (1000 nm) and 1.6 µm (1600 nm), so they would be able to pass through the plant cells and into nuclear regions with minimal tissue damage to deliver DNA into the nucleus for genetic transformation.