Transient Expression of the Rabies Glycoprotein in Soybean

By

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A Thesis Submitted to the Faculty of the Graduate School of Western Carolina University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology.

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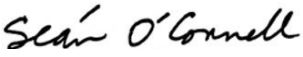
Committee Member: Seán O'Connell Signature: 

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TRANSIENT EXPRESSION OF THE RABIES GLYCOPROTEIN IN SOYBEAN

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ABSTRACT

TRANSIENT EXPRESSION OF THE RABIES GLYCOPROTEIN IN SOYBEAN

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Rabies is a highly preventable disease that is still claiming thousands of lives every year. An edible rabies vaccine for animals would reduce the cost and resources needed for the vaccine to reach less developed regions by simplifying administration of the vaccine and removing the need for cold-chain transport. When expressed on its own, the glycoprotein of rabies has been shown to form virus-like particles (VLPs), which structurally resemble the native virus without any chance of being infectious. VLPs of other viruses produced in plants have been shown to produce varying levels of immunity in animal studies, and some virus VLPs are used today in FDA approved vaccines. The promise of VLP vaccines coupled with soybean as a promising system due to its scalable production, protein rich seeds, and extensive leaf material, makes an edible vaccine possible. This research presents the optimization of a successful agroinfiltration method for soybean leaf tissue, as well as production of a plasmid containing a GUS (β-glucuronidase) reporter gene and the rabies Glycoprotein (G protein) containing a His-tag and ER (endoplasmic reticulum) retention signal sequences for future work towards the transient and transgenic expression of the G protein in soybean. A syringe-mediated injection agroinfiltration method for effective transient transformation of soybean leaf tissue has been developed. The new plasmid will allow for simple detection of transformed soybean tissue using GUS, and isolation and detection of the G protein in the transformed samples using the His-tag. The findings of this

project provide protocols and products developed to work toward a successful edible rabies vaccine for animals. While this research lays the foundation for producing an edible vaccine for animals, this is a steppingstone toward a much greater goal. Edible vaccines for humans would overcome the limitations that hold traditional vaccines back from reaching many underdeveloped regions, where people are still dying from preventable disease.

CHAPTER ONE: INTRODUCTION

This research demonstrated the development of agroinfiltration methods to produce transient soybean leaf tissue, as well as produced a plasmid containing GUS (β-glucuronidase) and the rabies Glycoprotein (G protein) containing a His-tag and an ER retention signal sequences for future work towards the transient and transgenic expression of the G protein in soybean. When expressed on its own, the G protein has been shown to form virus-like particles (VLPs), which structurally resemble the native virus without any chance of being infectious. This coupled with soybean being a common agricultural crop that offers protein rich seeds and extensive leaf material, make an edible vaccine possible. The findings of this project provide protocols and products developed to work toward a successful edible rabies vaccine for animals. While this research lays the foundation for producing an edible vaccine for animals, this is a steppingstone toward a much greater goal. Edible vaccines for humans would overcome the limitations that hold traditional vaccines back from reaching many underdeveloped regions, where people are still dying from preventable disease.

According to the World Health Organization, infectious diseases were one of the greatest causes of death in 2019.[1] From plagues to the yearly flu outbreaks, infectious diseases posed a major threat in the past and, while mortality due to infectious diseases has significantly decreased, they remain one of the top causes of death today.[1] Rabies is a well-known disease that is fairly rare in well developed countries where the vaccine is easily accessible. Rabies is caused by the virus *Rabies lyssavirus*, a neurotropic virus that belongs to the family Rhabdoviridae. Rhabdoviruses are characterized by their bullet shape and their negative-sense, single-stranded RNA genome. This genome encodes five proteins: the nucleoprotein, phosphoprotein, matrix

protein, polymerase, and glycoprotein (G protein). The virus is composed of a helical ribonucleoprotein core with a surrounding envelope. One of the main components of this envelope and focus in this research is the G protein. The G protein is responsible for the formation of trimeric spikes on the surface of the virus and is a recognizable antigen.[2]

The rabies virus infects cells via adsorption by binding to cellular receptors and consequently releasing their ribonucleoprotein core into the cell, where the rabies-encoded polymerase begins transcription of its genome in the cell. The proteins are assembled to form new virions, and these are released from the cell through budding at the plasma membrane. As a neurotropic virus, the rabies virus infects nerve cells and is known to follow neural pathways from the peripheral nervous system to the central nervous system. Its mechanism of travel along neural pathways is not well understood currently, but it is hypothesized that the phosphoprotein plays an important role in its movement.[3] After peripheral infection, the rabies virus must travel to the brain to cause symptoms. The pathogenic mechanism of the virus is still not clear, although impairment of neuronal functions is the leading hypothesis, as the original hypotheses of apoptosis and histopathological lesions have not been shown to occur in mice models.[4] The incubation period of rabies can vary from weeks to months in individuals and is possibly influenced by the site of infection and thus the distance the virus must travel. Symptoms begin with fatigue, fever, headache, and general flu-like symptoms, and progress to more severe symptoms like cerebral dysfunction, confusion, delirium, hallucinations, and insomnia within two to ten days. Unfortunately, once clinical symptoms appear within a patient the disease is nearly always fatal. Currently, there are only 20 recorded cases of patients surviving rabies.[2] Therefore, the only effective treatment of rabies is prevention via the rabies vaccine and immediate treatment by post-exposure prophylaxis (PEP).

In situations where an individual is likely to come into continuous contact with the virus, it is recommended that these individuals receive continuous vaccine boosters to maintain a safe serum titer every 6 months. For the general population where exposure is rare, no vaccination is necessary.[2] There are currently a few main vaccines available for human vaccination and post-exposure immunization through PEP. The human diploid rabies vaccine (HDCV) and the purified chicken embryo cell vaccines (PCECV) are the current vaccines recommended by the CDC.[5] These vaccines utilize inactivated virus to stimulate an immune response and produce immunity in patients without the risk of infection. Immunization is the most effective method of prevention of the rabies disease, but rapid treatment after exposure with PEP is also very effective. A study by Quaiambao et al. involving the efficacy of PEP reported that all infected patients treated in the study were still alive one year later.[6] PEP usually involves multiple doses of a rabies vaccine along with human rabies immunoglobulin (HRIG) administered on the day of the rabies exposure.[2]

Although in the United States rabies disease kills only one or two people a year, worldwide this disease is much more deadly, causing roughly 59,000 deaths, according to the CDC.[7] The CDC also presents evidence that rabid dogs account for 99% of the rabies deaths in humans worldwide. Animal vaccination programs using traditional inactivated vaccine have been shown to reduce the incidence of human infections, but these programs are not accessible or feasible in many areas of the world.[7] Effective rabies treatments exist for those that are able to rapidly access them, but for populations without this infrastructure, treatment is not always available. Areas such as Asia and Africa contain high stray dog populations living within dense human populations.[8] This environment is optimal for the infection and spread of rabies within the dog population and humans.

Vaccines remain effective treatments for rabies both preventatively and for postexposure prophylaxis, but they present limitations that prevent their worldwide accessibility and usage. Human and animal rabies vaccines require refrigeration at 2° to 8° Celsius and cannot be frozen.[9] While this limitation impacts the cost in developed countries, it is still easily transported and readily available. However, in less developed countries this requirement presents a challenge for its transportation and limits its accessibility in many regions of the world. The vaccines also require intramuscular administration, requiring syringes and trained individuals, again adding to the cost and limits of the vaccine.

Vaccine research and development is a dynamic and rapidly growing field, producing many possible solutions to the limitations presented. Edible vaccines present a solution to many of the challenges and the high cost of traditional vaccines, allowing them to reach areas of the world not feasible for traditional vaccines. Edible vaccines are simply vaccines that are administered orally. When consumed orally, these vaccines elicit an immune response similar to that of traditional vaccines to produce protective immunity in an individual.[10] Edible vaccines offer advantages over traditional vaccines in many ways, from the production of the antigen to the administration of the vaccine.

The knowledge and tools are already available to produce edible vaccines. One method of production is to utilize *Agrobacterium tumefaciens* in a process called agroinfiltration to express the antigen in plants. *A. tumefaciens* is a bacterium capable of inserting a desired genetic sequence into a host plant's genome. It does this by infecting a cell and inserting a region of the genome called T-DNA (Transfer DNA) into the host cell's genome. This biological process can be utilized to insert a sequence of desired DNA into a host cell by transforming the *A. tumefaciens* with a Ti plasmid containing a T-DNA with a gene of interest. Once a cell is

infected and the T-DNA has integrated into the host's genome, the gene can be expressed and the desired protein produced. Depending on what type of cells are infected, agroinfiltration can be used to produce either transient or transgenic transformants. Transient expression involves inserting and expressing a gene in non-stem cells and does not result in the offspring of the plant possessing that gene. Transgenic expression involves the integration of the gene into the genome of stem cells that can be induced to develop into a complete plant; this would cause all the cells in the plant and the plant's offspring to also contain that gene in their genome. It should be noted that agroinfiltration is only one of the methods of transforming plant cells and is less effective against some plant species than others. Bioballistics and viral infiltration are also methods capable of transforming plant cells, but these methods are more costly and require equipment that is not always readily available.

There have been numerous successful demonstrations of antigen expression in transient plant tissue or transgenic plants such as the expression of a hepatitis B surface antigen in tobacco cells using agroinfiltration.[11] While transient and transgenic expression is mostly studied in tobacco, other plants have also demonstrated the expression of proteins, such as lettuce, eggplant, tomato, soybean, and others.[12] By expressing the G protein to form a VLP in plants, a person could be exposed to the antigen in order to produce antibodies, and thus immunity, without risk of infection.

A VLP is a multiprotein structure that mimics the native conformation of a virus, but does not contain a viral genome, and thus cannot be infectious. Some viral structural proteins, like the G protein, will self-assemble forming VLPs. An ER retention signal and ER signal peptide have been shown to improve VLP formation, immunogenicity, and expression.[13, 14] Vaccines based on VLPs are effective, safe, and generally less expensive than other vaccine

types. Today, there are multiple VLP vaccines approved by the FDA and used worldwide, including hepatitis B and human papillomavirus vaccines, with even more VLP vaccines in clinical trials, such as an influenza virus vaccine.[15]

For this research project I sought to develop agroinfiltration methods for the transient expression of the G protein in soybean by optimizing agroinfiltration protocols using the reporter gene GUS, as well as produce a plasmid for future agroinfiltration containing the GUS gene and the G protein with a His-tag and ER retention signal. Agroinfiltration offers a simple, cost effective method for transforming plants and suits the needs of this research well. Soybean as a plant host for this experiment offers many advantages. Soybean has been shown to be resistant to agroinfiltration, but this project has shown success and has improved the process. Despite its difficulty, soybean is still the most effective and pragmatic plant host due to a variety of reasons. Soybean seeds provide native stabilizing and storage proteins to improve the stability and longevity of the antigen produced, high levels of protein levels are possible in the seeds, and refrigeration of the seeds is not required.[16] These benefits along with the scalability due to in-place infrastructure make it an ideal plant host for this research.

This project provides data and products necessary for transient expression of the G protein in soybean, with the end goal of transgenic expression in mind. This research presents the successful agroinfiltration of soybean leaves, as detected by the reporter gene GUS, and the production of a plasmid containing the G protein with a His-tag and ER retention signal to continue work into expression of the G protein in soybean by replicating these protocols. This work developed a syringe-mediated injection agroinfiltration method for effective transient transformation of soybean leaf tissue. Sterile soybean seeds made by plants grown in sterile, autoclaved soil were also produced and stored for future work. With these optimized protocols

and the constructed plasmid, a foundation has been laid for continued work towards G protein transient expression and eventual transgenic expression in soybean.

CHAPTER TWO: MATERIALS AND METHODS

Growth Conditions

Agrobacterium tumefaciens liquid cultures were grown in autoclaved 30g/L YEP broth (20g/L peptone, 10g/L yeast extract) containing filter sterilized 50mg/ml gentamycin and 50mg/ml kanamycin and incubated 2 nights at 27°C with shaking at 200rpm. *A. tumefaciens* solid media cultures were grown on 30g/L YEP, 1.5% agar plates containing 50mg/ml gentamycin and 50mg/ml kanamycin for selection at 27°C for 2 nights.

E. coli liquid cultures were grown in autoclaved 25g/L LB broth (10g/L tryptone, 10g/L NaCl, 5g/L yeast extract) containing filter sterilized 50mg/ml kanamycin at 37°C overnight with 200rpm shaking. *E. coli* solid media cultures were grown on 25g/L LB, 1.5% agar plates containing kanamycin at 37°C overnight.

Soybean seeds were spread in a tray of soil and lightly covered with soil. The tray was then placed in a clear trash bag to maintain moisture and was placed in the growth chamber. The growth chamber maintained a 16-hour day and 8-hour night cycle, and 23°C temperature. Soybeans were also planted in pots of soil under the same conditions.

Sterile soybean plants were grown in the growth chamber under 16-hour day and 8-hour night cycles, at 23°C. These plants were grown from seeds sterilized in 10% bleach for 5-10 minutes and placed in soil that was autoclaved one time. Once primary leaves formed on the seedlings, they were transferred to the WCU greenhouse where they grew until they produced sterile seeds. Plants were watered until soil was dripping 2-3 times a week, and plants were fertilized using a general plant fertilizer every 2-3 weeks.

Site Directed Mutagenesis for Insertion of His-tag and SEKDEL Sequences

Site directed mutagenesis was used to insert a His-tag and an ER retention signal SEKDEL to the C terminus end of the rabies G protein sequence (AGN94258.1). Primers SDM1F and SDM1R (sequences provided in Table 2 in the Appendix) were designed with an overlapping region that incorporates the insertion His-tag sequence. Another set of primers SDM2F and SDM2R (sequences provided in Table 2 in the Appendix) were also similarly designed but with the SEKDEL ER retention sequence.

First, PCR was done using the pORE-E4-GPRO plasmid containing the G protein as the template (plasmid information provided in Table 4 in the Appendix) and primers SDM1F and SDM1R to add a His-tag to the C terminus of the G protein. The PCR reaction contained 2ul (50ng) of pORE-E4 template, 1.25ul (125ng) of each primer, 25ul of Phusion Hot Start Master Mix, 1.5ul of DMSO, and 19ul of water for a total reaction volume of 50ul. The thermocycler parameters were set at: 98°C for 30s, (98°C for 30s, 55°C for 30s, 72°C for 4min) x18 cycles, 72°C for 20 minutes. A gradient PCR was done prior to this in order to determine the optimal annealing temperature of 55°C for the reaction. The 50ul reaction was equally aliquoted into 5 PCR tubes and the PCR reaction was done at the following annealing temperatures: 70.0°C, 62°C, 57.5°C, 53.8°C, and 50.0°C. Successful insertion of the His-tag was tested using a *DpnI* digest of the PCR product followed by gel electrophoresis, and sequencing. The *DpnI* digest contained 0.2ul of *DpnI* to 7ul of PCR product and digested for 15 minutes at 37°C. All the following *DpnI* digests used identical concentrations. The digest product was run a 1% agarose gel at 100V for 30 minutes and all gels following were run at these conditions. All sequencing samples were prepared by transforming DH5α *E. coli* competent cells with the digested PCR product, growing colonies on selection media and purifying plasmids using an alkaline lysis

miniprep technique. Plasmid samples were adjusted to a concentration of 150ng/ul in 20ul and sent to Euofins Genomics for sequencing. The plasmid pORE-E4-GPRO with the addition of the His-tag was named pRAB-HIS.

Following the His-tag insertion, a PCR reaction using newly constructed pRAB-HIS (plasmid information provided in Table 4 in the Appendix) and primers SDM2F and SDM2R was done to insert the SEKDEL sequence at the C terminus of the G protein. The PCR reaction contained 2ul (10ng) of pRAB-HIS template, 1.25ul of each primer, 25ul of Phusion Hot Start Master Mix, 1.5ul of DMSO, and 19ul of water for a total reaction volume of 50ul. Again, a gradient PCR with the 50ul reaction equally aliquoted into 5 separate PCR tubes was done with annealing temperatures at 50°C, 55°C, 60°C, 65°C, and 70°C to determine the optimal annealing temperature of 55°C for the reaction. The PCR reaction parameters were set at: 98°C for 30s, (98°C for 30s, 55°C for 30s, 72°C for 4min) x18 cycles, 72°C for 20 minutes. The insertion of SEKDEL was confirmed using a *DpnI* digest and gel electrophoresis as previously described, along with sequencing. The newly created plasmid (pRAB-HIS with SEKDEL added) was named pRAB-HIS-ER.

SLiCE (Seamless Ligation Cloning Extract) for Substitution of Signal Peptide

SLiCE was used to attempt to substitute a plant ER signal peptide sequence for the native rabies signal peptide at the N terminus of the G protein. The ER signal peptide sequence "MKTNLFLFLIFSLLLSLSSA" from the *Arabidopsis* protein chitinase was amplified out of the *Arabidopsis* genome using PCR with primers SLICE1F and SLICE1R (sequences provided in Table 2 in the Appendix) to use as the insert sequence for the SLiCE reaction.

Genomic *Arabidopsis* DNA was isolated using the following protocol. Two to three leaves were ground in a microcentrifuge tube with a disposable pestle, and 400ul of Extraction

Buffer (50mM MOPS (3-(N-morpholino)propanesulfonic acid), 5mM EDTA, 2mM DTT), was added and mixed. The mixture was spun down and 300ul of the supernatant was collected and transferred to a fresh tube. A 300ul aliquot of isopropyl alcohol was added and the sample was incubated at room temperature for 2 minutes. The sample was centrifuged for 5 minutes at top speed, and the supernatant was discarded. The pelleted DNA was rinsed with 300ul of ethanol and spun again. The ethanol was removed, and the pellet air dried for 15 minutes. The pellet was then resuspended in 25ul of water.

A PCR reaction using isolated genomic *Arabidopsis* DNA as the template and the primers SLICE1F and SLICE1R was done. The PCR reaction contained 3ul (225ng) of template, 3ul of each primer, 1.5ul of DMSO, 25ul of Phusion Hot Start Master Mix, and 14.5ul of water for a total reaction volume of 50ul. The thermocycler parameters were as follows: 98°C for 30s, (98°C for 30s, 52.9°C for 30s, 72°C for 15s) x39 cycles, 72°C for 20 minutes. Gel electrophoresis was used to confirm the PCR reaction worked. The insert was then gel purified using an Invitrogen Gel Purification Kit.

The plasmid pRAB-HIS-ER was linearized using a restriction digest with *EcoRI* to produce the linear vector for the SLiCE reaction. The digestion reaction contained 1000ng of pRAB-HIS-ER, 2ul of 10x FastDigest buffer, 2ul of *EcoRI*, and water to a volume of 20ul. The reaction was incubated for 20 minutes at 37°C. After digestion, the linearized vector was gel purified using Invitrogen Gel Purification Kit.

To produce SLiCE *E. coli* extract, Ken Motohashi's SLiCE extract protocol was followed.[17] In short, *E. coli* was cultured to an OD600 of 2.0-3.0 and were then harvested by centrifugation. Cells were washed using sterile cold water and were resuspended in BugBuster lysis reagent and incubated 10 minutes at room temperature. The lysate was then centrifuged,

and the supernatant was combined with an equal volume of 80% glycerol to be snap frozen with liquid nitrogen and stored at -80°C.

The standard SLiCE reaction contained 10ng of linearized vector, a range of 1:1 to 1:10 molar ratio of linear vector to insert, 1ul of 10x SLiCE Buffer (500mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 10mM ATP, and 10mM dithiothreitol) and 1ul of SLiCE extract, for a total volume of 10ul. The reaction was incubated at 37°C for 30 minutes and 5ul of SLiCE product was used to transform 100ul of competent *E. coli* cells, as described in the "*E. coli* and *A. tumefaciens* Transformation" section.

Transformed *E. coli* cells were plated on LB-Kan selection plates. Growth on selection plates suggested kanamycin resistance is present and colonies were grown in 3ml LB broth with 50mg/ml kanamycin, then miniprepped to obtain the plasmid present. The plasmid identity was tested using restriction digest with *SmaI*. The *SmaI* digest contained 2ul of FastDigest buffer, 5ul of plasmid, 1ul *SmaI*, and water to 20ul. The digest product was run on an agarose gel under previously described conditions.

Construction of pRAB-GUS

Traditional restriction digest cloning methods were used to produce pRAB-GUS from pGFPGUSplus. Primers GPRO1F and GPRO1R (sequences provided in Table 2 in the Appendix) were designed to replicate the G protein and NOS terminator out of pRAB-HIS-ER, as well as insert an *XbaI* restriction cut site at the 5' end, and an *EcoRI* restriction cut site at the 3' end of the insert.

A PCR reaction was done using pRAB-HIS-ER as the template in order to amplify out the G protein and produce the insert with *XbaI* and *EcoRI* cut sites on either end. The PCR reaction contained 1ul (6ng) of template, 3ul of each primer, 25ul of Phusion Hot Start Master

Mix, 1.5ul of DMSO, and 16.5ul of water for a 50ul reaction volume. The digest was then incubated at 37°C for 5 minutes. The PCR parameters were set at: 98°C for 30s, (98°C for 30s, 53°C for 30s, 72°C for 15s) x49 cycles, 72°C for 20 minutes. The PCR reaction was confirmed to work by gel electrophoresis of the PCR product. The PCR product was gel purified using Invitrogen Gel Purification Kit.

A digestion of pGFPGUSplus using *EcoRI* and *XbaI* linearized the plasmid and cut out the GFP gene and NOS terminator following it. The G protein insert was also digested using *EcoRI* and *XbaI* to produce sticky ends for ligation. The *EcoRI* and *XbaI* double digest reaction for the plasmid contained 2ul (1000ng) of pGFPGUSplus, 2ul of 10x FastDigest buffer, 1ul of *EcoRI*, 1ul of *XbaI*, and 14ul of water to a total volume of 20ul. For the insert, the double digest reaction contained 10ul of the PCR product, 3ul of 10x FastDigest buffer, 1ul of *EcoRI*, 1ul of *XbaI*, and 15ul of water to a total volume of 30ul. The digest product was gel purified using Invitrogen Gel Purification Kit.

A ligation to combine the linearized pGFPGUSplus vector with the G protein insert was done using the ThermoScientific Rapid Ligation Kit. The ligation reaction contained 20, 50, or 100ng of linearized vector, a range of 3:1 to 5:1 molar ratio of insert to vector, 4ul of rapid ligation buffer, 1ul of T4 DNA ligase, and water to a volume of 20ul. The reaction was briefly vortexed and incubated at 22°C for 5 minutes. 2-5ul of ligation mixture was used to transform competent *E. coli* cells. Transformed *E. coli* cells were plated on LB-Kan selection plates. Isolated colonies grown on selection plates were transferred to 3ml of LB broth with 50mg/ml kanamycin. *E. coli* was then miniprepped and the resulting plasmid identity was tested using *StuI* digests, *EcoRI* and *XbaI* double digests, PCR using primers to produce a known sequence length, and sequencing.

The *StuI* digest contained 1000ng of the plasmid miniprepped from transformed *E. coli* colonies, 5ul of 10x CutSmart buffer, and water to a volume of 50ul, and was incubated at 37°C for 5-15 minutes. The *EcoRI* and *XbaI* double digest was done using the same reaction as the previous double digest. The PCR reaction to test for G protein insertion was done using primers GPRO1F and GPRO1R. The PCR reaction contained 10ng of the ligated plasmid, 3ul of each primer, 25ul of Phusion Hot Start Master Mix, 1.5ul of DMSO, and water to a 50ul reaction volume. The PCR parameters were set at: 98°C for 30s, (98°C for 30s, 53°C for 30s, 72°C for 15s) x49 cycles, 72°C for 20 minutes. Another PCR reaction was also done with the ligated plasmid, but primers GPRO1F and SDM1R were used. The parameters were the same, but with a gradient of annealing temperatures at 53°C, 54.3°C, 57.3°C, and 60°C. Presence of PCR products were tested using gel electrophoresis at the same standard conditions as previous gels.

E. coli and *A. tumefaciens* Transformation

E. coli was transformed using the following protocol. 100 ul of competent DH5α cells were thawed on ice from -80°C storage, and plasmid DNA was added directly to the cells. Cells were gently mixed and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 90 seconds and then immediately returned to ice for 2 minutes. A 400ul aliquot of SOC media was added to 100ul of cells, and cells were incubated at 37°C for 45-60 minutes with shaking. Following this, cells were centrifuged, and the supernatant was removed. Cells were resuspended in the remaining supernatant and spread on plates to incubate at 37°C for one day.

A. tumefaciens was transformed using the following protocol. Competent cells were thawed on ice from -80°C storage. Plasmid DNA was added directly to thawed cells, and then incubated on ice for 5 minutes. Cells were submersed in liquid nitrogen for 5 minutes, and then transferred to a 37.5°C water bath for 5 minutes. A 1ml aliquot of SOC media was then added to

the cells and incubated at 28°C for 3 hours with shaking. Cells were spun down, resuspended in supernatant, and spread on plates to incubate at 28°C for two days.

Leaf Disc Vacuum Syringe Agroinfiltration

This protocol was based on the Matsuo, et al. leaf disc agroinfiltration protocol for tobacco.[18] Agrobacteria containing pGFPGUSplus or pRAB-HIS-ER was cultured in 250ml of YEP broth containing gentamycin and kanamycin for two nights, cells were spun down, and resuspended in Infiltration Buffer (10mM MES-KOH pH 5.7, 150uM acetosyringone, 10mM $MgCl_2$, 0.5mM DTT, and immediately before use 0.01% Tween 20 was added) to an OD600 of 0.8. The resuspension sat at room temperature for one hour before use.

Soybean leaves were sterilized by soaking in a 10% bleach solution with 0.01% Tween for 5-10 minutes and were then rinsed with sterile water 3-4 times. The leaf edges were trimmed off, and squares of ~8.5mm width were cut using a sterile razor from the leaves so all sides of the squares were cut. Using a 30ml plastic syringe, the plunger was removed, and leaf discs were inserted into the syringe. The plunger was replaced and about 10ml of resuspend agrobacteria solution was drawn into the syringe. The syringe was inverted, air was pressed out, and the tip was sealed using parafilm firmly pressed to it. The plunger was pulled to create a vacuum, the syringe was shaken to dislodge any stuck or clumped leaves, and the plunger was rapidly released. This was repeated three times.

Leaf pieces were removed from the syringe and excess agrobacteria was washed away using sterile water. The leaf squares were then placed adaxial side down on MS plates (4.5g/L MS basal salts, 3% sucrose, and 0.8% agar) to incubate at 23°C in 16h day and 8h night cycles for 3 days.

Negative control samples underwent an identical protocol but were infiltrated with an Infiltration Buffer without agrobacteria.

Whole Seedling Sonication and Vacuum Agroinfiltration

This protocol was based on the King et al. protocol for intact soybean seedling agroinfiltration.[19] Agrobacteria was cultured in 500ml of YEP broth containing gentamycin and kanamycin for two nights. Cells were spun down and resuspended in Infiltration Buffer to an OD600 between 0.6 and 0.8. Tween 20 was added immediately before use to a concentration of 0.01% final v/v.

Soybean seedlings were gathered after their primary leaves emerged, typically around 14 days of growth time. Seedlings were submerged in a beaker of agrobacteria resuspension and sonicated at a frequency of 42kHz by emersion in a Bransonic sonicator bath for 30-40 seconds. The seedlings were then placed in a large beaker and submerged in agrobacteria resuspension, and the beaker was placed inside a plastic vacuum chamber. The in-house vacuum system was used to produce vacuum pressure. Submerged seedlings were put under vacuum pressure for three 5-minute periods, with complete release of pressure between each period. Seedlings were then replanted in soil and incubated in the growth chamber for 2 days.

Syringe Injection Agroinfiltration

This protocol was based on the Zhao et al. *Arabidopsis* agroinfiltration protocol.[20] Agrobacteria was cultured in 100ml of YEP broth with kanamycin and gentamycin for one night. Cells were spun down and resuspended in Infiltration Buffer to an OD600 of 0.3. A 1ml syringe with the needle removed was used to draw up about 1ml of the agrobacteria resuspension. The syringe was then pressed firmly to the abaxial underside of the intact leaf still attached to the plant, and the plunger was depressed to inject about 0.1ml of the agrobacteria resuspension. This

was done 2-5 times per leaf in different areas of the leaf. Mature leaves were used in this protocol. The leaf was then left to incubate on the plant for 3 days in the growth chamber.

Histochemical GUS Staining

GUS staining of transformed tissues was done using a GUS staining solution (0.5mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt), 5ul/ml DMSO, 50mM $NaPO_4$). Agroinfiltrated leaves were submersed and incubated in GUS staining solution for one day. If blue staining was difficult to see on leaves, they were then transferred to 70% ethanol to clarify leaves by removing chlorophyll for as many nights as necessary with ethanol changes daily. Photographs were then taken to document blue GUS staining.

His-tag Nickel Affinity Pulldown Assay

Leaf tissue (about 5 leaf discs or 2 intact leaves) was ground to a paste using a mortar and pestle with Binding Buffer (20mM sodium phosphate, 300mM sodium chloride, and 10mM imidazole). The paste was then transferred to a tube and centrifuged at top speed for 5 minutes. The supernatant was transferred to a new tube and 100ul nickel beads were added per 600ul of supernatant, the tube was then incubated with mixing in the cold room at 4°C for 1.5 hours. The sample was centrifuged at top speed for 1 minute and the supernatant was discarded. 500ul of Binding Buffer was added per 100ul of beads to the tube and mixed. The sample was centrifuged again at top speed for one minute. The supernatant was again discarded. 500ul Wash Buffer (25mM imidazole in PBS) was added, mixed, centrifuged, and the supernatant was discarded, and this was repeated 4 times. Elution Buffer (250mM imidazole in PBS) was then added, and the mixture sat at room temperature for 3 minutes. Again, the sample was centrifuged at top speed for one minute and the supernatant was removed and saved.

SDS-PAGE and Western Blot Analysis

A 10% Tris-glycine reducing SDS-PAGE gel was used with 10ul of sample loaded. The gel was run in Running Buffer (3g/L Tris base, 14.4g/L glycine, 1g/L SDS) for 45 minutes at 200 volts.

Sponges, paper, and nitrocellulose membrane were soaked in Transfer Buffer (25mM Tris base, 192mM glycine, 15% ethanol) for a few minutes and a sandwich was then made in the following order: sponge, paper, protein gel, nitrocellulose membrane, paper, sponge. The sandwich was properly aligned with the membrane toward the positive terminal, and it was submersed and run in Transfer Buffer for 60 minutes at 100 volts. An icepack and stir bar were added to the Transfer Buffer to keep the solution cool.

The membrane was removed from the sandwich and placed in a Blocking Buffer (5% BSA in PBS-T) for 60 minutes with shaking. The membrane was washed three times for 5 minutes in PBS-T. For the His-tag probe, the membrane was soaked with shaking in 20ml PBS-T with 1:5000 His-tag-HRP probe for 60 minutes. For the G protein antibody, a 1:5000 dilution for the primary antibody (rabbit anti-G protein antibody) and a 1:20000 dilution for the secondary antibody (mouse anti-rabbit secondary with horseradish peroxidase) was used, and the membrane was soaked with shaking for 60 minutes. The membrane was imaged using a Bio-Rad ChemiDoc to detect for HRP light emission.

Miniprep Protocol

E. coli minipreps used the following protocol: cells were spun down, supernatant removed, and resuspended in the remaining supernatant. Miniprep Solution 1 (100ul of 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0) and 1ul of RNase A was added and mixed. Miniprep Solution 2 (200ul of 1% SDS, 0.2M NaOH) was added, and the samples were

incubated on ice for 2 minutes. Miniprep Solution 3 (150ul of 3.0M potassium acetate, pH 5.5) was added, and the samples were incubated on ice for an additional 2 minutes. Samples were spun down at top speed for 5 minutes, and 450ul of the supernatant was transferred to a fresh tube. An equal volume of isopropyl alcohol was then added, and the mixture sat at room temperature for 2 minutes. The mixture was spun at top speed for 5 minutes, and the supernatant was removed and discarded. 200ul of ethanol was then added, and then spun again at top speed for 3 minutes. The ethanol was removed as much as possible using a micropipette and the tube was then inverted and allowed to dry until no traces of ethanol could be detected. The sample was then resuspended in 25ul of water.

A. tumefaciens minipreps were done using the following protocol. Cells were spun down, supernatant removed, and resuspended in 100ul of ice cold Miniprep Solution 1 with 4mg/ml lysozyme. Cells were vortexed for 10 seconds and incubated at room temperature for 30 minutes. Miniprep Solution 2 (200ul) was added and mixed and incubated for 30 minutes at room temperature. Ice cold Miniprep Solution 3 (150ul) was added and mixed and incubated on ice for 5 minutes. Samples were spun down at top speed for 5 minutes, and 450ul of the supernatant was transferred to a fresh tube. An equal volume of isopropyl alcohol was then added, and the mixture sat at room temperature for 2 minutes. The mixture was spun at top speed for 5 minutes, and the supernatant was removed and discarded. Ethanol (200ul) was then added, and then spun again at top speed for 3 minutes. The ethanol was removed as much as possible using a micropipette and the tube was then inverted and allowed to dry until no traces of ethanol could be detected. The sample was then resuspended in 25ul of water.

CHAPTER THREE: RESULTS

Insertion of His-tag and ER Retention Signal using Site Directed Mutagenesis

A His-tag was added to the G protein in order to allow for isolation and detection of the G protein in agroinfiltrated, transformed sample tissues. An ER retention signal SEKDEL was added to increase accumulation and protein levels of the G protein in the transformed tissue. Both sequences were added to the C terminus, as shown in Figure 1, using site directed mutagenesis.

Figure 1. Rabies G protein after SEKDEL and His-tag insertion, resulting in pRAB-HIS-ER.

A gradient PCR was used to determine the optimal annealing temperature of the His-tag insertion primers SDM1F and SDM1R. The PCR reaction used pORE-E4-GPRO as the template and primers SDM1F and SDM1R and was a 50ul reaction equally aliquoted into five separate PCR tubes. As seen in Figure 2A, multiple annealing temperatures gave positive PCR replication at the expected band size of 9.5kb. A band present at the expected size suggests the primers effectively bound and replicated the target sequence. An annealing temperature of 55°C was chosen as the optimal temperature. Miniprepped template plasmid was included as a control to ensure bands present in PCR products were not due to the template.

Following this, a PCR reaction using template pORE-E4-GPRO and primers SDM1F and SDM1R was done using the previously established annealing temperature of 55°C. A *DpnI* digest was done on the PCR product to remove template DNA and confirm the PCR reaction occurred; a band present after digestion at 9.5kb suggests the PCR reaction occurred. The agarose gel of the *DpnI* digest of the PCR product is shown in Figure 2B. The expected band size

of 9.5kb is present. With *DpnI* digest removing template DNA, it can be concluded that the band present is a result of the PCR reaction, suggesting the plasmid has the His-tag inserted. His-tag insertion was also confirmed using sequencing of newly produced plasmid "pRAB-HIS", shown in Table 5 in the Appendix.

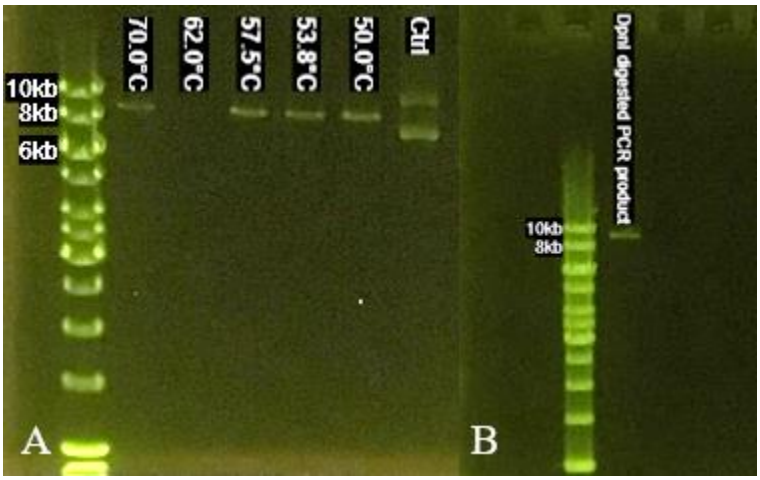

Figure 2. A. Agarose gel of a gradient PCR using template pORE-E4-GPRO and primers SDM1F and SDM1R with an annealing temperature range from 50°C to 70°C. Unaltered template plasmid was included as a positive control labeled "Ctrl". B. Agarose gel of the PCR product produced using template pORE-E4-GPRO and primers SDM1F and SDM1R and digested using *DpnI*.

A PCR reaction using the same gradient annealing temperatures was done using template pRAB-HIS and primers SDM2F and SDM2R, in order to determine an optimal annealing temperature. An annealing temperature of 55°C was optimal and used in a following PCR reaction with the same template and primers. Insertion of the SEKDEL ER retention signal sequence was confirmed through sequencing, shown in Table 5 in the Appendix, producing the new plasmid "pRAB-HIS-ER".

Substitution of Signal Peptide using SLiCE

Substitution of the native signal peptide with a plant ER signal peptide to the N terminus of the G protein was attempted using SLiCE. While a native signal peptide is present on the G

protein, substitution of a plant specific signal peptide was thought to increase expression. The signal peptide from *Arabidopsis* chitinase was used based on its predicted cleavage site from SignalIP. An insert sequence containing the signal peptide and a linearized pRAB-HIS-ER vector was needed for the SLiCE reaction.

Creation of the insert sequence was done using a PCR using genomic *Arabidopsis* DNA and primers SLICE1F and SLICE1R. The PCR product was loaded on an agarose gel into a large well to be gel purified. The signal peptide concentration and purity were measured after gel purification with a NanoDrop resulting in a concentration of 9.9ng/ul and a 260/280 ratio of 1.30. Figure 3 shows the expected band size of 118bp for the signal peptide.

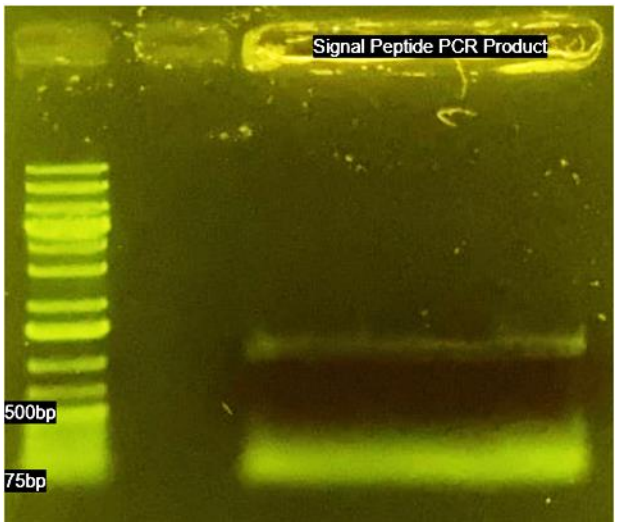

Figure 3. Agarose gel of signal peptide PCR product using genomic *Arabidopsis* DNA as template and primers SLICE1F and SLICE1R. A large well was created to gel purify the signal peptide insert.

The plasmid pRAB-HIS-ER was used as the vector in the SLiCE reaction and was linearized using an *EcoRI* digest. Figure 4 shows the digested plasmid and undigested plasmid, a band size of 9.5kb was expected for the digested plasmid. The linear vector was gel purified and measured using a NanoDrop giving a concentration of 11.5ng/ul and a 260/280 ratio of 1.18.

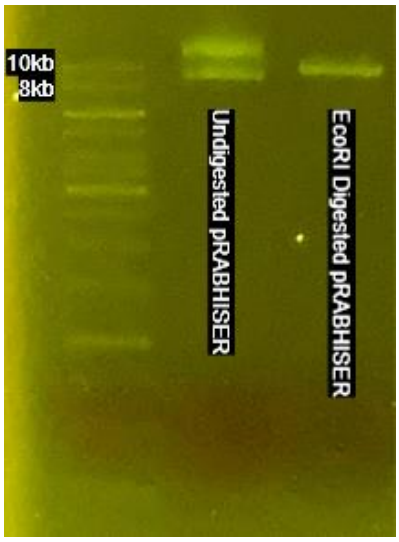

Figure 4. Agarose gel of pRAB-HIS-ER digested with *EcoRI* in order to create a linear vector. A band size of 9.5kb was expected for the digest product. Undigested plasmid was included as a control.

A standard SLiCE reaction using a molar ratio of insert to vector of 3:1 was used to transform competent *E. coli* cells that were then plated on LB-Kan selection plates. The transformation resulted in two small colonies. This SLiCE reaction is labeled "SLiCE-1". A PCR using primers SLICE1F and SLICE1R was done using miniprepped plasmid from the two colonies as the template, and a negative control using the original pRAB-HIS-ER template. A band of 118bp for colony A and C was expected if the signal peptide is present in the plasmid. The negative control was expected to show no bands. A band was present in all three samples under the 250bp ladder mark at about 200bp, as shown in Figure 5, indicating the three bands are likely due to primers. Miniprepped plasmid from the transformed colonies was sequenced as well, and sequencing showed the signal peptide was not present.

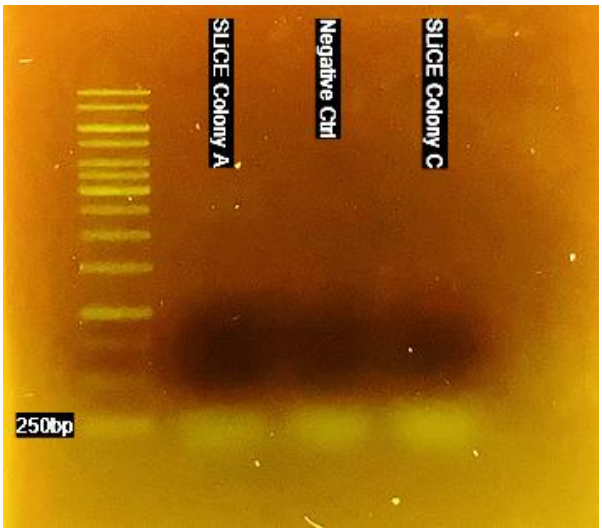

Figure 5. Agarose gel of PCR products from plasmids isolated from *E. coli* colonies transformed with the SLiCE product and grown on LB-Kan selection plates. A negative control was included using the original pRAB-HIS-ER plasmid and was expected to show no band.

A total of sixteen SLiCE reactions were done using a range of molar ratios, fresh insert, linear vector, and SLiCE extracts. Aside from the two colonies previously mentioned, no other *E. coli* transformations with SLiCE products showed growth with kanamycin selection. Each SLiCE reaction is listed in Table 1.

Table 1. Each SLiCE reaction performed, and their concentrations and components.

SLiCE Reaction	Molar Ratio (Insert:Vector)	Vector Amount per Reaction Volume	Fresh SLiCE Extract
SLiCE-1	3:1	23ng/20ul	No
SLiCE-2	2:1	20ng/20ul	No
SLiCE-3	2:1	50ng/50ul	No
SLiCE-4	3:1	50ng/50ul	Yes
SLiCE-5a	5:1	25ng/25ul	Yes
SLiCE-5b	5:1	25ng/25ul	No
SLiCE-6a	10:1	20ng/20ul	Yes
SLiCE-6b	10:1	20ng/20ul	No
SLiCE-7a	3:1	20ng/20ul	Yes
SLiCE-7b	3:1	20ng/20ul	No
SLiCE-8a	5:1	20ng/20ul	Yes
SLiCE-8b	5:1	20ng/20ul	No
SLiCE-9a	7:1	20ng/20ul	Yes
SLiCE-9b	7:1	20ng/20ul	No
SLiCE-10a	10:1	20ng/20ul	Yes
SLiCE-10b	10:1	20ng/20ul	No

Agroinfiltration using pRAB-HIS-ER

Following the production of the pRAB-HIS-ER plasmid, the leaf disc vacuum syringe agroinfiltration protocol was performed using *A. tumefaciens* transformed with pRAB-HIS-ER. Soybean leaf discs agroinfiltrated using pRAB-HIS-ER were first tested using SDS-PAGE and Western blots with a His-tag probe in order to detect the presence of the His-tagged G protein. As shown in Figure 6A, the His-tag probe blot showed significant nonspecific binding indicated by the bands present in the negative control sample. The negative control sample underwent an identical protocol as the transformed samples but was infiltrated using only infiltration buffer without *A. tumefaciens*. Because bands of similar size to the transformed samples were present in the negative control sample, the blot could not be used to confidently determine if the G protein was present. Faint bands are present around the 55kDa ladder mark in the two transformed samples, and the expected band size of the G protein is 58kDa.

Following the inconclusive results when attempting to detect the protein His-tag, a G protein antibody was ordered and used in place of the His-tag probe. Western blots using a G protein antibody also showed nonspecific binding in untransformed control samples. As shown in Figure 6B, the negative control sample and the pRAB-HIS-ER transformed leaf tissue showed bands around 55kDa. The expected size of the G protein is 58kDa, but due to the nonspecific binding, a confident conclusion cannot be drawn.

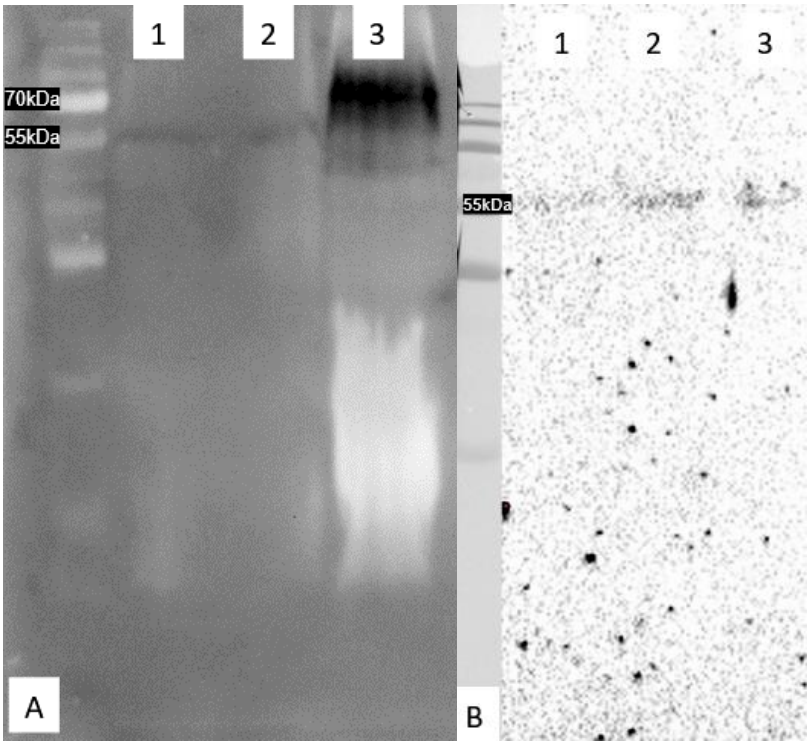

Figure 6. In both blots lanes 1 and 2 are transformed leaf disc samples, 5 leaf discs were randomly chosen from the transformed batch. Lane 3 was a negative control of non-transformed leaf disc tissue. A. Western blot using His-tag probe to test for the presence of the G protein in pRAB-HIS-ER transformed leaf disc tissue. 10ul was loaded for each sample. B. Western blot using a G protein antibody to test for the presence of the G protein in pRAB-HIS-ER transformed leaf disc tissue. The expected size of the G protein is 58kDa.

A nickel bead pulldown assay was also done on the pRAB-HIS-ER transformed leaf disc tissue to attempt to isolate the His-tagged G protein. The pulldown elution was tested for the presence of the G protein using an SDS-PAGE gel and Western blot using a His-tag probe, as shown in Figure 7. Again, a band size of 58kDa was expected if the G protein was present. Both transformed samples in lane B and C had bands of around the correct size, but a negative control wasn't included in the blot, and whether the present protein is the G protein or not is not conclusive.

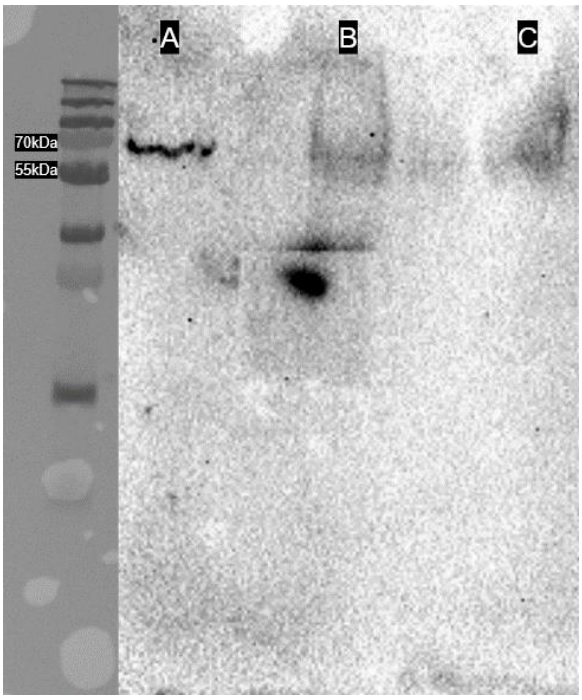

Figure 7. Western blot of the nickel bead pulldown elution of leaf discs transformed with pRAB-HIS-ER. Lane A is the His-tag positive control, B is Nickel Bead Pulldown Elution 1, and C is Nickel Bead Pulldown Elution 2. The expected band size for the G protein 58kDa.

The plasmid pRAB-HIS-ER was also used in whole seedling sonication and vacuum agroinfiltration protocols. Soybean whole seedlings were transformed using *A. tumefaciens* containing pRAB-HIS-ER. Leaves were harvested from the transformed seedlings and the tissue was ground to a paste with extraction buffer. The presence of the G protein in these samples was tested using Western blots with His-tag probes. Again, Western blots using the His-tag probe showed significant nonspecific binding in the negative control untransformed seedling tissue, as shown in Figure 8. The negative control untransformed seedling tissue underwent the same protocol as the transformed tissue, but with the absence of the *A. tumefaciens*. The four transformed seedling tissue samples in lanes A through D contained 2-3 leaves selected at random from the transformed seedlings. The His-tag positive control is a protein with a known His-tag present used to ensure the Western blot worked.

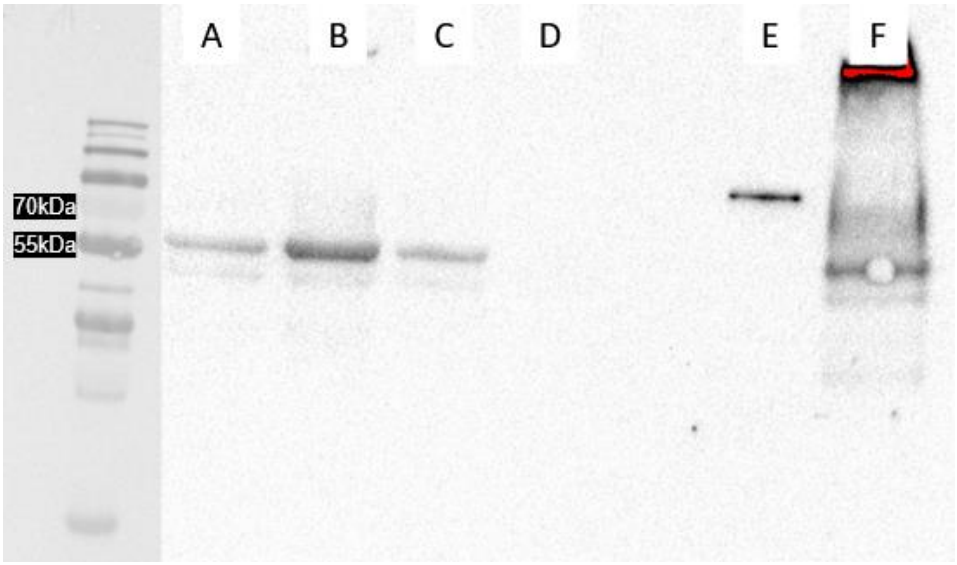

Figure 8. Western blot using a His-tag probe to test for the presence of the G protein in pRAB-HIS-ER transformed seedling tissue. Lanes A, B, C, and D are four transformed tissue samples containing 2-3 leaves selected at random from the transformed seedlings. Lane E was a His-tag positive control, and F was an untransformed seedling tissue negative control. Three of the four samples show a band slightly above the 55kDa ladder mark. The untransformed seedling tissue negative control sample shows bands at the same locations at the transformed samples.

A Western blot using a G protein antibody was also done on the seedlings transformed with pRAB-HIS-ER. Faint bands were present in all three seedling tissue samples around the expected size of 58kDa, but very faint bands of the same size in the negative control sample also appear to be present, shown in Figure 9. Again, the untransformed negative control tissue underwent the same protocol, but with the absence of *A. tumefaciens*, and the three transformed seedling tissue samples contained 2-3 leaves selected at random from the transformed seedlings.

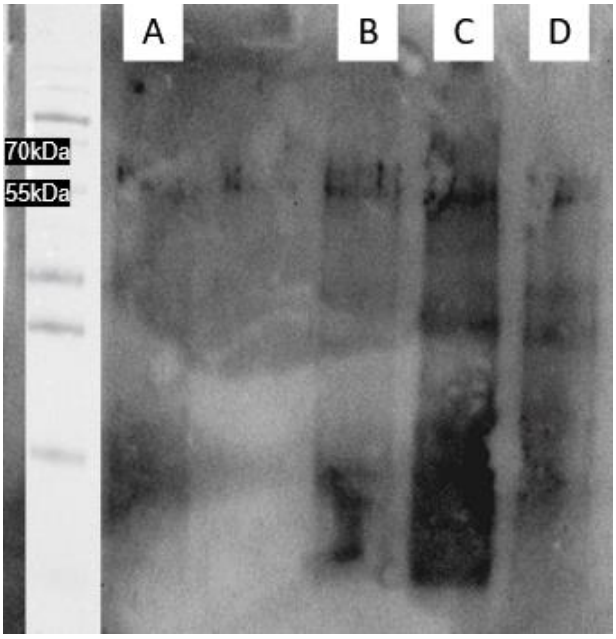

Figure 9. Western blot using a G protein antibody to test for the presence of G protein in pRAB-HIS-ER transformed seedling tissue. Lane A is the Untransformed Seedling Tissue Control, and lanes B, C, and D are all three transformed tissue samples contain 2-3 leaves selected at random from transformed seedlings. The untransformed sample underwent the same protocol as the transformed samples, but with the absence of *A. tumefaciens*. Bands around the size of 58kDa appear to be present in the transformed samples, but a very faint band also appears to be present at that size in the negative control sample.

Agroinfiltration using pGFPGUSplus

Due to the inconclusive results obtained from attempting to detect expression by detecting the presence of the G protein in samples using a His-tag or G protein antibody, a different detection method was needed. A new plasmid, pGFPGUSplus, was decided on due to its two reporter genes, GFP and GUS.

A. tumefaciens was transformed with pGFPGUSplus to be used in agroinfiltration protocols. To confirm transformation with the correct plasmid, transformed *A. tumefaciens* was miniprepped to isolate the plasmid and the resulting plasmid was digested with *NdeI* to confirm it was the pGFPGUSplus. The plasmid pGFPGUSplus was expected to produce three bands at about 8.2kb, 3kb, and 2.5kb. The expected bands were present, as shown in Figure 10.

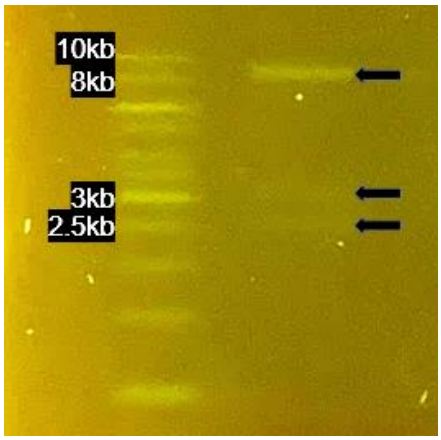

Figure 10. *NdeI* digest of plasmid miniprepped from *A. tumefaciens* transformed with pGFPGUSplus. Three bands at 8.2kb, 3kb, and 2.5kb were expected.

Soybean and *Arabidopsis* leaf discs were transformed with *A. tumefaciens* containing pGFPGUSplus. These transformations were tested using histochemical GUS staining, a process where a substrate, X-gluc, is added to tissue to test for the presence of β-glucuronidase, produced by the GUS gene, resulting in blue staining of tissues if transformation and reporter gene expression is successful. As shown in Figure 11, this leaf disc agroinfiltration protocol produced very little transformed soybean leaf tissue. There are two small regions on two leaf discs that present GUS staining, out of the around fifty leaf discs that underwent the protocol. The protocol produced no transformed tissue in *Arabidopsis* leaf disks.

Figure 11. Soybean leaf discs transformed with pGFPGUSplus. Arrows mark small regions of GUS staining, indicating transformed leaf tissue.

The syringe injection protocol, injecting *A. tumefaciens* suspension into the abaxial side of the leaf, was also used on soybean and *Arabidopsis* leaves using *A. tumefaciens* transformed with pGFPGUSplus. The presence of transformed tissue was tested using histochemical GUS

staining and observing blue-dyed tissue. *Arabidopsis* leaves transformed using this protocol showed GUS staining in the leaf, as shown in Figure 12.

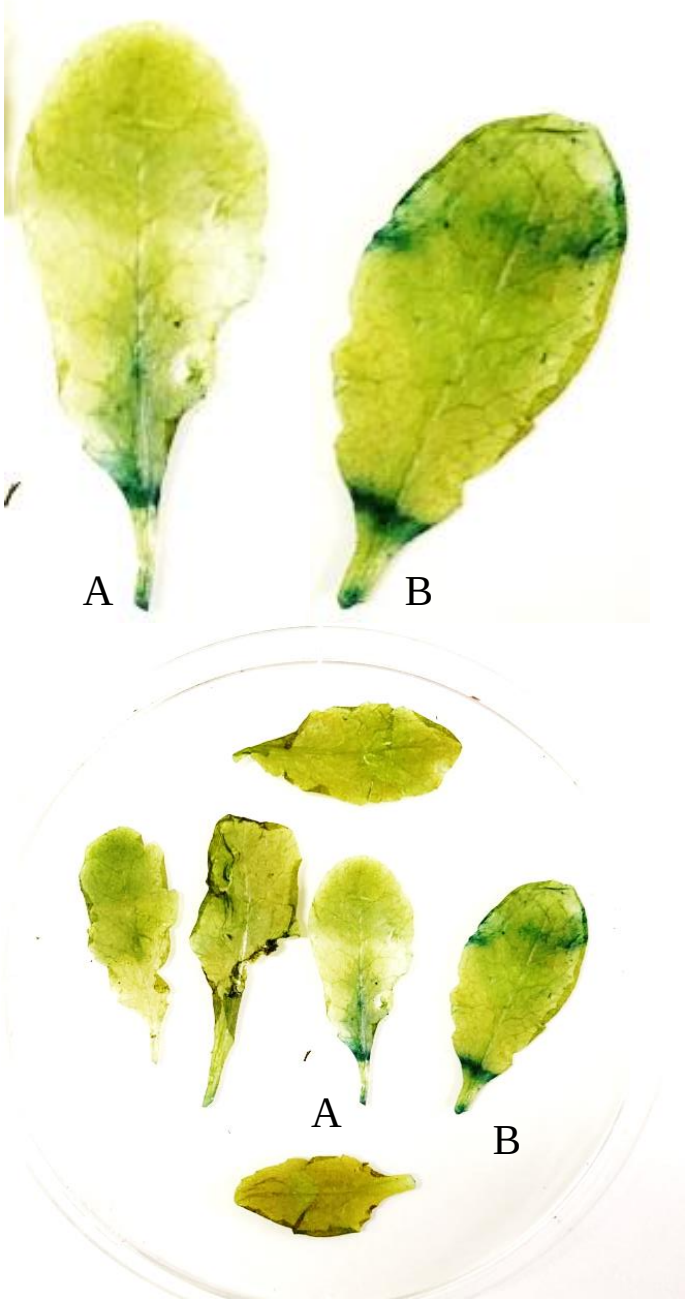

Figure 12. *Arabidopsis* leaves transformed with pGFPGUSplus. Blue stained regions indicate transformed tissue expressing the GUS gene.

Soybean leaves transformed using this protocol also showed GUS staining around the injection sites, indicated by blue stain, as shown in Figure 13. Out of the total eighteen leaves

agroinfiltrated, nine showed at least some area of GUS staining. GFP imaging was attempted using a UV light box on *Arabidopsis* and soybean leaves transformed with pGFPGUSplus, but fluorescence was unable to be detected.

Figure 13. Soybean leaves transformed with pGFPGUSplus. Blue stained regions indicated tissue that was transformed and expressing the GUS gene.

Construction of pRAB-GUS

With an effective agroinfiltration protocol identified, the next step of the project was to produce a plasmid containing the G protein and replicate those protocols. It was decided that using the pGFPGUSplus plasmid used in the successful agroinfiltration protocols would be the best option, as it also provides a reporter gene to aid in detecting successful transformation. The G protein was to be inserted in place of the GFP gene in pGFPGUSplus. To do this, traditional

cloning methods and ligations were used. An insert sequence containing the G protein with sticky ends, and a linearized vector made from pGFPGUSplus with the GFP gene removed and with sticky ends was needed.

The G protein insert was created using PCR with the pRAB-HIS-ER plasmid as the template and primers GPRO1F and GPRO1R. The PCR products G1 and G2, which were identical PCR reactions, were run on an agarose gel and produced bands at the expected size of 1882bp, shown in Figure 14. The smaller bands are likely due to primers binding a non-targeted sequence and replicating.

The double-digested vector used in the ligations was produced by digesting pGFPGUSplus with *EcoRI* and *XbaI* in order to cut out the GFP gene and linearize the plasmid. The double-digested pGFPGUSplus was also run on an agarose gel and produced a band of the expected size of around 12.5kb, as shown in Figure 14.

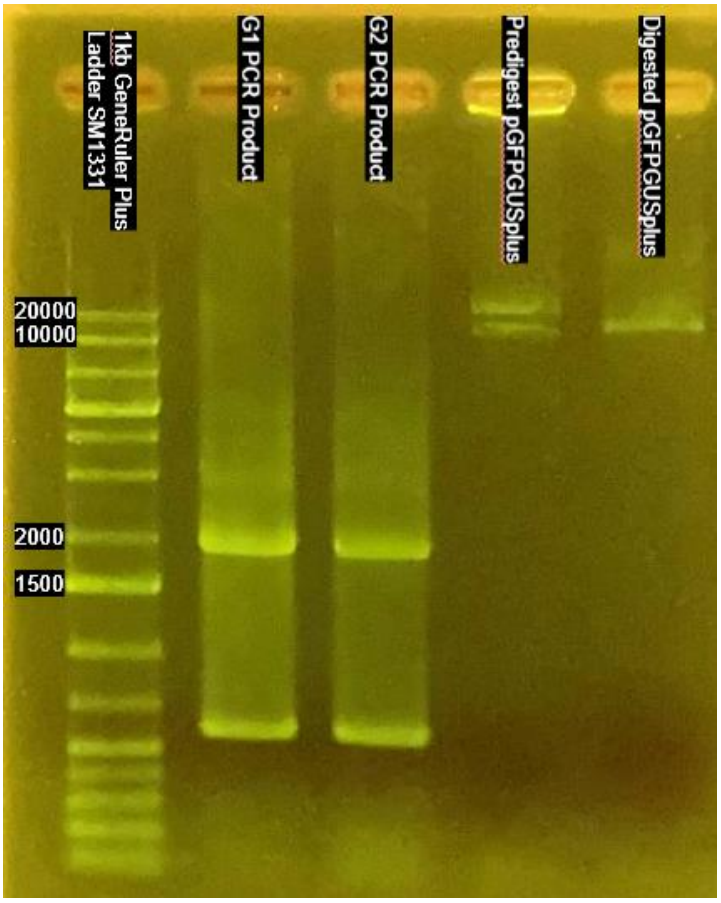

Figure 14. G1 and G2 PCR products produced the expected band size of around 1882bp, suggesting it replicated the targeted G protein sequence. Digested pGFPGUSplus produced a single band at the expected size around 12.5kb, suggesting the digest worked as expected. The smaller bands present in G1 and G2 PCR products are likely due to nonspecific primer bindings and replicating a different portion of the template.

The previously made vector and insert were ligated using ThermoScientific Rapid Ligation Kit. The ligation products were then transformed into competent *E. coli* cells and plated on LB-Kan selection plates. Colonies that grew on the selection plates were miniprepped, and the plasmid identity was confirmed by PCR and restriction digests. Ligation "LA" when transformed into *E. coli* resulted in two colonies on the LB-Kan selection plates, these colonies are denoted "LA-1" and "LA-2". Ligation "LA" was done with an insert to vector molar ratio of 5:1 and 50ng of template. Colonies LA-1 and LA-2 were miniprepped and the resulting plasmid digested with *StuI*. With *StuI* having one cut site within the G protein gene sequence but none in the GFP

gene, and one within the template plasmid. A digest of a plasmid containing the G protein was expected to produce two bands at about 11kb and 3.4kb while one band of 13kb is expected if the G protein is absent. As shown in Figure 15, one miniprepped plasmid, derived from the colony LA-1, produced the expected digestion result of two bands at the expected sizes, while colony LA-2 showed the result to be expected if the G protein was absent from the plasmid. Sequencing was also done to confirm the presence of the G protein in the plasmid pRAB-GUS, as shown in Table 5 in the Appendix.

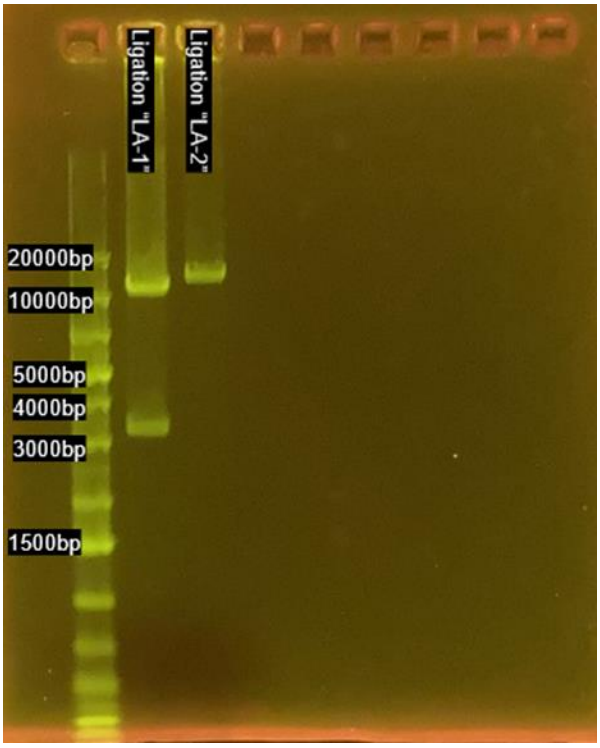

Figure 15. Agarose gel of *StuI* digests of two colonies transformed with the ligation product from ligation "LA". LA-1 and LA-2 denote two isolated colonies formed on LB-Kan selection plates from *E. coli* transformed with ligation LA product. LA-1 produced the expected result of two bands at 11kb and 3.4kb, suggesting the G protein is present in the plasmid.

Production of Sterile Soybean Seeds

Previous work on this project attempted transgenic transformation of half seed explants and cotyledons but were unable to complete the experiments due to excessive fungal

contamination of explants. It was found that sterile soybean seeds resulted in less fungal contamination. In preparation for future work on this project, sterile soybean seeds were grown in the WCU greenhouse in autoclaved, sterile soil. The plants grew until they produced seeds, and the seeds were harvested, dried, and stored for future work in transgenic transformation and expression.

CHAPTER FOUR: DISCUSSION

This research has worked to lay the groundwork and produce products and protocols necessary for future work towards transient and transgenic expression of the rabies G protein in soybean. The rabies G protein, when expressed on its own in cells, forms a VLP, which would allow the G protein to be used as an antigen to illicit an immune response and produce immunity in an animal without any risk of actual infection or symptoms. Soybean offers many advantages as a system for G protein production and generating an edible vaccine. It is a common crop with vast infrastructure, and offers cellular advantages, such as high protein levels and stability in seeds, and extensive leaf material. Combining the G protein and soybean allows for a promising possibility of an edible vaccine, where the G protein is expressed in soybean leaf or seed tissue and the ingestion of that tissue produces an immune response.

As this project has progressed with research and experiments, many difficulties arose, and the aims and goals of the project have shifted. The first goal of the project was the addition of a His-tag and ER retention signal onto the C terminus of the G protein by site directed mutagenesis and the substitution of a plant ER signal peptide by SLiCE. The His-tag was added to allow detection and isolation of the G protein from transformed leaf samples using His-tag probes in Western blots and Nickel bead pulldown assays. The addition of the ER retention signal and substitution of the ER signal peptide in the G protein were both done to improve accumulation of the G protein in the ER. The His-tag and the ER retention signal were both successfully added, ultimately confirmed by sequencing, as shown in Table 5 in the Appendix.

One particular difficulty that presented itself was the apparent non-specific binding of the His-tag probe to untransformed soybean tissue. At first, the His-tag was the preferred method of

detection by Western blots and isolation by nickel bead pulldowns. However, with the His-tag probe consistently producing bands in untransformed soybean tissue used as negative controls, as shown in Figures 6 and 8, the plasmid being used at the time, pORE-E4-GPRO, was sequenced and it was determined a His-tag was not present on the plasmid.

A G protein antibody was then used in Western blots for detection of the protein in samples, but also gave mostly inconclusive results as non-specific binding still appeared to be present. One Western blot, shown in Figure 9, using the G protein antibody produced distinct bands in the transformed soybean samples, but with a possible faint band still present in the negative control, the blot could not be interpreted with confidence.

These limitations in detecting the presence of the G protein in soybean tissue forced the project to shift to a new approach. A His-tag was added to the G protein, and optimization of the agroinfiltration protocols using a new plasmid, pGFPGUSplus, containing two reporter genes was decided to be the best course. With pGFPGUSplus, two new methods of detecting successful transformation of soybean tissue were now available. Detecting GFP fluorescence was not successful with a UV light box, likely due to the low levels of expression present in transformed regions. However, GUS staining proved to be an effective method of detecting transformed tissue and became the preferred method of testing transformed leaf tissue.

With an effective method of testing for successful transformation, the agroinfiltration protocols were optimized and first performed on *Arabidopsis* leaves. The leaf disc vacuum syringe method was attempted on *Arabidopsis* leaves, but resulted in dead leaves unable to survive the stress of the protocol. This is likely due to *Arabidopsis* leaves being more fragile relative to soybean or tobacco leaves, with which the protocol is more commonly used. The leaf disc method was also used on soybean using the pGFPGUSplus plasmid but resulted in only two

of the around fifty leaf discs agroinfiltrated showing any GUS staining. The whole seedling sonication and vacuum agroinfiltration method was also tested on soybean using pGFPGUSplus and produced no detectable transformed tissue.

The syringe injection method proved to be more effective for *Arabidopsis* and seemed to be an effective method of transformation. Of the eighteen soybean leaves that underwent syringe injection agroinfiltration, nine showed at least some region of blue GUS staining, indicating those regions had been successfully transformed with and expressing the GUS gene, as shown in Figure 13.

Based on the data collected in this project, the syringe injection agroinfiltration method is the most effective method of those tested. This is surprising given that the method is not common in soybean transformation literature. It should be noted that, unlike injection into *Arabidopsis* leaves, the injection into the abaxial side of soybean leaves did not result in a spreading of the injected agrobacteria suspension fluid into surrounding tissues, as observed during injection. Despite this, GUS staining was still detected in and around the injection site on soybean leaves and didn't appear to be limited by the spread of the injected fluid.

The pGFPGUSplus plasmid had been shown to be effective in transforming soybean leaf tissue, and it was decided that this plasmid would be used moving forward with G protein transformations. The GFP gene was removed and in its place the G protein with a His-tag and SEKDEL ER retention signal was added, producing pRAB-GUS. This new plasmid contains the GUS gene for effective and easy detection of transformed regions of leaves and contains the His-tagged G protein gene with an ER retention signal under the control of a CaMV 35S promoter, a constitutive over-expression promoter. The G protein is expected to be expressed in the ER.

Using this plasmid, transformed regions can be identified by GUS staining, and further experiments can be done to determine the presence of the G protein.

One of the main over-arching goals of this project was to demonstrate transient expression of the G protein in soybean tissue. With the newly constructed plasmid pRAB-GUS and an effective agroinfiltration method identified, a foundation for future work into this project has been laid. The plasmid pRAB-GUS provides the benefits of the reporter gene GUS in detecting transformed tissue, while also containing the G protein gene to detect and isolate using the added His-tag. With the new syringe injection protocol, transformation of leaf tissue requires much less time and resources, and this project's data has shown it to be more effective than the leaf disc syringe vacuum or whole seedling sonication and vacuum protocols.

The future of this project involves transgenic expression of the G protein and the production of a transgenic line of soybean plants, with eventual plans to produce an edible vaccine. The pRAB-GUS plasmid will likely be effective in other agroinfiltration methods targeted at transgenic expression. Sterile soybean seeds have been produced by growing plants in autoclaved, sterile soil and harvesting the seeds, and are stored for future work. Immunogenicity of the transient leaf tissue expressing the G protein is also planned to be tested by oral ingestion of the tissue in mice to confirm if an immune response occurs.

While an edible rabies vaccine is the ultimate goal, the research and work from this research project are also applicable to other edible vaccines such as distemper, another common viral disease in animals. Edible vaccines offer many advantages over traditional injected vaccines, such as not requiring a freezing storage temperature and not requiring trained individuals to administer a shot. The limitations of traditional injected vaccines have been brought to the public eye during the COVID pandemic, where we witnessed the difficulties of

transporting and storing vaccines requiring -80°C or -20°C freezer temperatures, and this being a limiting factor to access for less developed regions. The difficulties of having enough trained personnel in some regions of the world to administer the vaccine was also witnessed. These limitations affect the cost of transporting and administering vaccines, and greatly affect equitable access to vaccines worldwide.

Many infectious diseases are preventable today by vaccines, but despite this, deaths from these preventable diseases persist. Edible vaccines offer the potential to ameliorate some of the challenges and limitations that prevent traditional vaccines from reaching all regions of the world equally. The results from this research contribute towards this goal by providing products and protocols for effective transient transformation of soybean to express the G protein. This will allow future work into transgenic transformation, immunogenicity testing using orally fed mice, and eventual animal trials of an edible rabies vaccine.

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APPENDIX

Table 2. Primers

Name	Description	Sequence (5’ to 3’)
SDM1F	Forward primer for site directed mutagenesis insertion of His-tag to pORE-E4-GPRO, producing pRAB-HIS. Overlapping region is italicized.	*GCTCGGTACCTTTTACCATC*ACCATCACCATCACTAGTGATATCCCTGTGTGAAATTG
SDM1R	Reverse primer for site directed mutagenesis insertion of His-tag to pORE-E4-GPRO, producing pRAB-HIS. Overlapping region is italicized.	*GATGGTAAAAGGTACCGAGC*TCAAGTCTAGTCTCACCACCGGAC
SDM2F	Forward primer for site directed mutagenesis insertion of ER retention signal to pRAB-HIS, producing pRAB-HIS-ER. Overlapping region is italicized.	*GGTGGTGAGACTAGACTTTCTG*AAAAGGATGAACTTGAGCTCGGTACCTTTTAC
SDM2R	Reverse primer for site directed mutagenesis insertion of ER retention signal to pRAB-HIS, producing pRAB-HIS-ER. Overlapping region is italicized.	*CAGAAAGTCTAGTCTCACCACC*GGACTTGTGAGACTCCCATGAAGAGATG
SLICE1F	PCR amplification of *Arabidopsis* chitinase signal peptide for SLiCE. Forward primer.	CTTGGATCCACCCGGGAATGGTTTTGC
SLICE1R	PCR amplification of *Arabidopsis* chitinase signal peptide for SLiCE. Reverse primer.	GTGTAGATGGGGAACTTTGCATTTGCTGG
GPRO1F	PCR amplification of G protein out of pRAB-HIS-ER. Forward primer.	GCATCTAGAATGGTGCCACAGGCA
GPRO1R	PCR amplification of G protein out of pRAB-HIS-ER. Reverse primer.	CGCGAATTCGATCTAGTAACATAGATGACAC

Table 3. Bacterial Strains

Strain	Description	Selection Markers	Source
GV3101	*Agrobacterium tumefaciens* used for agroinfiltration of plant tissues.	Gentamycin	Storm Lab
DH5α	*E. coli* used for plasmid replication, isolation, and storage.	None	Storm Lab

Table 4. Plasmids

Name	Description	Selection Markers	Source
pORE-E4-GPRO	Plasmid containing the G protein.	Kanamycin	Storm Lab
pRAB-HIS	pORE-E4 plasmid containing the G protein with an inserted His-tag.	Kanamycin	Constructed
pRAB-HIS-ER	pORE-E4 plasmid containing the G protein with an inserted His-tag and SEKDEL sequence.	Kanamycin	Constructed
pGFPGUSplus	Plasmid containing reporter genes GUS and GFP.	Kanamycin	Addgene.com
pRAB-GUS	Constructed plasmid containing GUS and G protein.	Kanamycin	Constructed

Table 5. Sequencing Results

Plasmid	Sequencing Results (sequence additions are denoted by italics)
pRAB-HIS	NNNNNNNNNNCTGNNGNCGCAGGGTTAACAGATCTGAGCCAACTCA GCACAACCTCAGAGGAACTGGNNNGGAAGTTTCTGTGACTCCACAGT CCGGCAAGATCATCTCTTCATGGGAGTCTCACAAGTCCGGTGGTGAG ACTAGACTTGAGCTCGGTACCTTTTAC*CATCACCATCACCATCAC*TAGT GATATCCCTGTGTGAAATTGTTATCCGCTACGCGTGATCGTTCAAACA TTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGA TGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTA ACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAG TCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGC GCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGA TCCCATGGGAAGTTCCTATTCCGAAGTTCCTATTCTCTGAAAAGTATA GGAACTTCAGCGATCGCAGACGTCAACGTGGATACTTGGCAGTGGTT ACTTGGCTTTTCCTTTATTTTCTTTTGGACGGAAGCGGTGGTTACTTTG TCACACATTTAAAAAAACACGTGTTTCTCACTTTTTTCTATTCCCGTC ACAAACAATTTTAAGAAAGATCCATCTATCGTGATCTTTCTATCAAAC AAAAGAAAAAAGGTCTTCATAGTAACGCTACAACATCAAATATGTGG TTGCTCTGACATCAGTCGGGAAAATAAGGATATGGCGGCATTGGCCA CATCTATTGGGGTCCCAACTTCCTTTCACAAAAAAATTAAATTGGGTG TCCCAACTTTTATCTTTGATATAGTGACATGAGTATCGGGAGCATNGG ANATGGATAAAATGNNAACTANNNNATTCTGGTTATTTTTGATNNTN NTNNTNNNAAG
pRAB-HIS-ER	GCCCNNCAAGTCCGGNCCTNNNAAAGGATTNNNNTTCANAAAGTTGT GCCAGGATTCGAAAGGCCTACACTTTCTTCAACAAGACCCTCANGAA GCTGACGCCCACTACAAGTCTGTTAGGACCTGGAACGAGATCCTGCC ATCTAAGGGTTGCCTTAGAGTTGGTGGAAGGTGCCATCCACATGTGA ACGGTGTTTTCTTCAACGGCATCATCCTCGGACCAGATGGAAACGTG TTGATCCCAGAGATGCAGTCCTCTTTGCTTCAGCAGCACATGGAATTG

	CTCGAGAGCAGCGTTATCCCACTTGTTCACCCACTTGCTGATCCCTCT ACCGTGTTCAAGGATGGTGATGAGGCTGAGGATTTCGTCGAGGTTCA CCTTCCAGACGTGCACAATCAAGTGTCCGGTGTGGATCTTGGACTTCC AAACTGGGGAAAGTACGTGCTCCTTTCTGCTGGTGCTCTTACCGCCTT GATGCTGATCATTTTCCTCATGACCTGCTGCCGCAGGGTTAACAGATC TGAGCCAACTCAGCACAACCTCAGAGGAACTGGAAGGGAAGTTTCTG TGACTCCACAGTCCGGCAAGATCATCTCTTCATGGGAGTCTCACAAG TCCGGTGGTGAGACTAGACTT*TCTGAAAAGGATGAACTT*GAGCTCGGTA CCTTTTACCATCACCATCACCATCACTAGTGATATCCCTGTGTGAAAT TGTTATCCGCTACGCGTGATCGTTCAAACATTTGGCAATAAAGTTTCT TAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTC TGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACG TTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATT TAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAAT TATCGCGCGCGGTGTCATCTATGTTACTAGATCCCATGGGAAGTTCCT ATCCGAAGTNNNNNTNNNNNNNNNNNNNNNNNNN
pRAB-GUS	NNNNAAGGCAGTGCCAAGCTTGCATGCCTGCAGGTCCCCAGATTAGC CTTTTCAATTTCAGAAAGAATGCTAACCCACAGATGGTTAGAGAGGC TTACGCAGCAGGTCTCATCAAGACGATCTACCCGAGCAATAATCTCC AGGAAATCAAATACCTTCCCAAGAAGGTTAAAGATGCAGTCAAAAG ATTCAGGACTAACTGCATCAAGAACACAGAGAAAGATATATTTCTCA AGATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTCAC AAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTTCC CACTGAATCAAGGGCCATGGAGTCAAAGATTCAAATAGAGGACCTA ACAGAACTCGCCGTAAAGACTGGCGAACAGTTCATACAGAGTCTCTT ACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCAC GACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGA CCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACC TCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAG TGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGG AAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATG GACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAAC CACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAA GGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATAT AAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGAATGG TGCCACAGGCACTTCTCTTCGTGCCACTTCTTGAATTCCCACTCTGCT TCGGAAAGTTCCCCATCTACACCATTCCAGATAAGCTCGGACCTGGG AGCCAATCGAAATTCATCATCTCAGGCGCCCCACAANTTCCTGGTAG AAAAAGAAGATGCACCACCCCTCNGGNNTTCCGACATGANCTAAAG GCGGGGAANTACTCCGCNCCANATGA

Table 6. Media and Buffers

a. YEP

Component	Concentration

Peptone	20g/L
Yeast Extract	10g/L

b. Luria Broth (LB)

Component	Concentration
Tryptone	10g/L
NaCl	10g/L
Yeast extract	5g/L

c. 10x SLiCE Buffer

Component	Concentration
Tris-HCl, pH 7.5	500mM
$MgCl_2$	100mM
ATP	10mM
Dithiothreitol (DTT)	10mM

d. SOC Media

Component	Concentration
Tryptone	2%
Yeast extract	0.5%
NaCl	10mM
KCl	2.5mM
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose	20mM

e. Murashige and Skoog (MS) Media

Component	Concentration
MS Basal Salts	4.5g/L
Sucrose	3%
Agar (solid media)	0.8%

f. Infiltration Buffer

Component	Concentration
MES-KOH pH 5.7	10mM
Acetosyringone	150uM
$MgCl_2$	10mM
Dithiothreitol (DTT)	0.5mM
Tween 20 immediately before use	0.01% final v/v

g. GUS Staining Solution

Component	Concentration
X-gluc	0.5mg/ml
DMSO	5ul/ml
$NaPO_4$	50mM

h. Extraction Buffer

Component	Concentration
3-(N-morpholino)propanesulfonic acid (MOPS)	50mM
Ethylenediaminetetraacetic acid (EDTA)	5mM
Dithiothreitol (DTT)	2mM

i. Binding Buffer

Component	Concentration
Sodium phosphate	20mM
Sodium chloride	300mM
Imidazole	10mM

j. Wash Buffer

Component	Concentration
Imidazole	25mM
Phosphate-buffered saline (PBS)	To volume

k. Elution Buffer

Component	Concentration
Imidazole	250mM
Phosphate-buffered saline (PBS)	To volume

l. Running Buffer

Component	Concentration
Tris base	3g/L
Glycine	14.4g/L
Sodium dodecyl sulfate (SDS)	1g/L

m. Transfer Buffer

Component	Concentration
Tris base	25mM
Glycine	192mM
Ethanol	15%

n. Blocking Buffer

Component	Concentration
Bovine serum albumin (BSA)	5%
Phosphate-buffered saline with 0.05% Tween 20 (PBS-T)	To volume

o. Miniprep Solution 1

Component	Concentration
Glucose	50mM
Ethylenediaminetetraacetic acid (EDTA)	10mM
Tris-HCl pH 8.0	25mM

p. Miniprep Solution 2

Component	Concentration
Sodium dodecyl sulfate (SDS)	1%
NaOH	0.2M

q. Miniprep Solution 3

Component	Concentration
Potassium acetate, pH 5.5	3.0M