An exploration of *Medicago truncatula* as a platform for the production of heterologous proteins

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ABSTRACT

Plant molecular farming refers to the process of utilising plants as bioreactors to produce high value proteins, including enzymes, antibodies, and other therapeutic molecules. Almost all molecular farming initiatives employ *Nicotiana benthamiana* as a host plant. Herein, we investigated the potential of a model legume, *Medicago truncatula*, as an alternative platform for synthesis of heterologous protein. We exploited well established methodologies developed for the study of root endosymbioses to genetically transform *M. truncatula* root systems via *Agrobacterium rhizogenes*. The suitability of *M. truncatula* as a platform for the production of heterologous proteins was then examined through the cultivation of composite plants and immortalised root organ cultures (ROCs) that accumulate recombinant Green fluorescent protein (GFP). Our results demonstrate that recombinant GFP accumulates at high levels in composite *M. truncatula* plants up to 15 weeks post-transformation. Immortalised ROC lines continue to accumulate recombinant GFP beyond 57 weeks post-transformation. Purification of recombinant GFP from ROCs resulted in yields of 780 mg/kg and 194 mg/kg of root tissue, respectively. Sustained accumulation of high levels of recombinant GFP accordingly demonstrate the potential of *M. truncatula* as a viable alternative platform for heterologous protein production.

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ABBREVIATIONS

AMF: Arbuscular mycorrhizal fungi

CBB: Coomassie Brilliant Blue

FPLC: Fast liquid protein chromatography

GFP: Green fluorescence protein

HIS: Histidine

MRC: *Medicago* root culture

MtBCP1: *Medicago* blue copper protein

ROC: Root organ culture

T4SS: Type IV secretion system

CHAPTER 1: INTRODUCTION

Plant molecular farming

Plant molecular farming or biopharming is a term that refers to the use of plants to synthesise products of pharmaceutical relevance (Ahmad et al., 2012). Compared to traditional expression systems such as chicken eggs or bacterial cell cultures, biopharming is considered to be an economically viable, environmentally friendly, efficient, and easily scalable approach to produce safe vaccines (Benvenuto et al., 2023). Additionally, plants can produce complex properly folded proteins with eukaryotic post-translational modifications that not all traditional systems can achieve (Shanmugaraj, Bulaon, & Phoolcharoen, 2020).

Biopharming first emerged in the 1980s with plant based pharmaceutical products entering the market shortly thereafter (Düring et al., 1990; Horn, Woodard, & Howard, 2004; Lerouge et al., 1998). The first product produced via plant molecular farming was a human growth hormone chimeric gene in tobacco and sunflower callus tissue (Barta et al., 1986). Since then, significant developments have been made in the field including the production of HIV, hepatitis B, and anthrax antigens in plants (Shanmugaraj et al., 2020). In addition to antigens, antibodies can also be produced *in planta*. Notably, monoclonal antibodies against the Ebola virus, (EBOV), can be effectively produced in *Nicotiana benthamiana* leaves via agroinfiltration (Tripathy et al., 2021). *N. benthamiana* has become the plant of choice for most biopharming approaches, with *N. tabacum*, a fellow member of the *Nicotiana* genus used frequently as well. However, a broad range of plants including *Arabidopsis thaliana*, *Medicago truncatula*, *Medicago sativa* (alfalfa), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Lactuca sativa* (lettuce), *Arachis hypogea* L. (peanut), *Zea mays* (corn), *Oryza sativa* (rice), and even carnivorous plants *Drosera capensis* and *Nepenthes alata* have been assessed as biopharming candidates, with antigens, antibodies, or recombinant proteins successfully produced in all

(Miguel et al., 2019; Shanmugaraj et al., 2020; Tripathy et al., 2021). Plants are also capable of producing other therapeutic products such as enzymes. In 2012, Elelyso, a treatment for Gaucher disease became the first plant derived therapeutic approved by the FDA. Elelyso is made from taliglycerase alfa produced in a transgenic carrot cell suspension (Leblanc, Waterhouse, & Bally, 2021).

With the onset of the SARS-CoV-2 pandemic in December 2019 came pressing need to rapidly deploy vaccines to prevent the spread and mitigate the symptoms of COVID-19. This led to developments in the biopharming field with multiple plant-based vaccines entering production (Maharjan & Choe, 2021). Biopharming is a significant advancement in infectious disease control as it can act to bridge the gap between wealthy countries with easy access to vaccines and poorer countries that have been left behind during past pandemics (Ma et al., 2013). It may also allow for more rapid deployment of vaccines in response to future pandemics and rapidly evolving viruses such as influenza. The SARS-CoV-2 pandemic highlighted a global need for increased vaccine production capacity, and biopharming has already begun to bridge this gap (Margolin et al., 2020). The SARS-CoV-2 viral antigen has been expressed *in planta* by many different researchers (Demone et al., 2022; El Jaddaoui et al., 2022; Funk, Laferrière, & Ardakani, 2021; Margolin et al., 2023). Notably, SARS-CoV-2 vaccine Covifenz® manufactured by Canadian company Medicago Inc. using *N. benthamiana* as a host for antigen production was approved for use by Health Canada, pushing plant molecular farming as a method for vaccine production into the realm of public awareness (Benvenuto et al., 2023). Although the vaccine project was cancelled due to withdrawing of shareholder investment, the development and approval for use of a Canadian made plant-based vaccine was a significant step forward in the Canadian biopharming landscape.

While multiple advances have been made in the biopharming field in the past four decades, there are many potential approaches that have yet to be explored. To date, most biopharming strategies employ plant leaves that are transiently transformed to express heterologous genes that encode products of interest (Shanmugaraj et al., 2020). Callus tissue cultures, or suspended cell cultures are employed less often, and plant roots are very rarely utilized. Production of heterologous proteins in the roots of *N. benthamiana* and *N. tabacum* has been attempted successfully, however leaf tissue is considered to be a preferable platform for both species, as *Nicotiana* roots can be tedious and relatively inefficient to transform. (Aragão et al., 2023; Biswas et al., 2023).

Heterologous proteins

Heterologous proteins are defined as proteins produced in a host that are not endogenous to that host. This requires that exogenous DNA be introduced to the host organism, which then uses its endogenous machinery to synthesize the protein encoded by the exogenous DNA (Desai, Shrivastava, & Padh, 2010). In this way, a host organism can be effectively exploited to act as a production platform. Heterologous proteins such as antibodies and antigens are vital to support biomedical research, which includes drug and vaccine development. (Burnett & Burnett, 2020). Heterologous proteins include recombinant proteins, which are by definition encoded by recombinant DNA via assembly of two or more DNA segments (Overton, 2014).

Traditional heterologous protein production systems

Antigens are a vital component of vaccines as these contain the epitopes that are recognized by the immune system, driving the production of antibodies that will act to combat future infection. Typically, antigen production employs cell-based production platforms

consisting of mammalian, insect, bacterial, or yeast cells, or embryonated chicken eggs (Burnett & Burnett, 2020; Rajaram et al., 2020). These platforms often require cultivation in chambers called bioreactors under strict biosafety containment regulations. The protein of interest is then harvested from the cell cultures and is purified for formulation as a vaccine (Rajaram et al., 2020).

Traditional methods to produce recombinant protein are safe and successful but are not without room for improvement (Leblanc et al., 2021). Bacterial cells are the most frequently used production platform, and carry the benefit of being low cost, quick growing, and frequently yielding high quantities of protein (Burnett & Burnett, 2020). However, prokaryotes such as bacteria lack the ability to perform post-translational modifications that are specific to eukaryotic cells, which can have significant impacts on the activity, and stability (half-life and clearance) of the products created. Bacteria are also limited in the size of proteins they can produce and may experience difficulty in correctly folding proteins above 30kDa (Burnett & Burnett, 2020). Insect cells have the ability to perform many post-translational modifications, but some of these modifications are undesirable (Hong et al., 2022; Jarvis, 2003). Glycosylation is a post-translational modification through which a complex carbohydrate known as a glycan is covalently attached to a protein or lipid (Webster & Thomas, 2012). Glycosylation in insect cells may incorporate immunogenic sugars that can elicit non-targeted immune responses if not removed before clinical use (Hong et al., 2022; Jarvis, 2003). This same issue is of concern in heterologous proteins generated in yeast cultures (De Wachter, Van Landuyt, & Callewaert, 2021). Mammalian cells can perform post-translational modifications and glycosylation that more closely resembles human glycosylation profiles; however, this system is expensive due to complex growth requirements and long production time. There is also higher risk of human

pathogen contamination in mammalian cell cultures (O'Flaherty et al., 2020). Chicken eggs are another common strategy employed in the production of therapeutics. Scalability of this method is limited, due to high demand for chicken eggs, and limited resources can also negatively impact the timely production of vaccines (Rajaram et al., 2020). During avian flu epidemics, large numbers of domestic birds may be culled, leading to a reduced egg supply in a time of high demand. Additionally, there are concerns regarding allergic reactions to ovalbumin (Rajaram et al., 2020)

Plants as heterologous protein production systems

The use of plants as biological systems for heterologous protein production is a complementary approach that has the potential to mitigate some of the challenges associated with more commonly used expression systems. Plants have the added benefit of being able to perform post-translational modifications that bacterial cells are unable to, including glycosylation. While it is important to note that there are differences in human pattern glycosylation and the glycosylation performed by plants, plant-based glycosylation is not associated with the incorporation of immunogenic sugars, as is a concern in insect cells (Burnett & Burnett, 2020). There is also no reasonable risk of contamination with human viruses or prions when heterologous protein is manufactured in plants, and concerns regarding ovalbumin allergies are eliminated (Horn et al., 2004; Rajaram et al., 2020). Additionally, plants have simple, low-cost growing requirements that are relatively environmentally friendly.

***Medicago truncatula*, a model legume and potential heterologous protein production system**

Medicago truncatula (**Figure 1.**) is a legume that is native to the Mediterranean basin, and a relative of *M. sativa*, more commonly known as alfalfa. A member of the *Fabaceae* family,

M. truncatula has the ability to engage in beneficial symbioses with nitrogen fixing soil bacteria, referred to as rhizobia, and arbuscular mycorrhizal fungi (AMF). Accordingly, *M. truncatula* has an extensive history of careful study within the context of root endosymbiosis (Barker et al., 1990; Floss et al., 2013). The ability of *M. truncatula* to engage in ecologically and agriculturally relevant symbioses has been a driver of its elevation to model legume status. The small size, short lifecycle, and sequenced diploid genome of *M. truncatula*, along with its ability to self-fertilize, all contribute to make this species an optimal model (Von Wettberg at al., 2019; Krishnakumar et al., 2015). *M. truncatula* has a generation time of 3-6 months, with flowering typically occurring 10-12 weeks after seed germination (Küster, 2013). Importantly, *M. truncatula* is amenable to genetic transformation with *Agrobacteria* species.

Within a biopharming context, *M. truncatula* leaves have been assessed as a platform for the expression of heterologous protein with promising results, including a report of greater accumulation of heterologous protein compared against *N. tabacum* (Abranches et al., 2005). *M. truncatula* plant cell suspension cultures have also been assessed as a platform for recombinant protein production, with successful production and secretion of the SARS-CoV-2 Spike protein and the Receptor Binding Domain of the Spike protein reported (Rebelo et al., 2022). Additionally, production and secretion of recombinant human erythropoietin, a glycosylated hormone, in *M. truncatula* cell culture has been achieved (Pires et al., 2012). Interestingly, there is evidence that *M. truncatula* cell suspension cultures contain a reduced amount of proteases compared to the better-established tobacco bright yellow 2 (BY-2) system (Santos et al., 2018). Compared to *N. benthamiana* and *N. tabacum*, *M. truncatula* produces thick, robust radicles upon germination that are readily amenable to *Agrobacterium*-mediated genetic transformation (Floss et al., 2013). Of note, successful expression of the SARS-CoV-2 Spike protein was

demonstrated in the roots of composite *M. truncatula* plants during the course of an undergraduate honours thesis project (Miles & MacLean, 2022). The well-studied, robust root system of *M. truncatula* may thus represent a viable candidate for a novel, root-based biopharming system.

A possible issue that limits the full potential of heterologous protein accumulation *in planta* is the silencing of transgenes by the host plant (Iida et al., 2023). Gene silencing involves the production of RNA interference (RNAi) that acts to target exogenous genes either at the transcriptional or post-transcriptional level. In nature, this serves as a mechanism of defence against pathogens. RNAi begins with the formation of hairpin, or double strand, RNAs, followed by cleavage of these RNAs by DICER-like proteins creating small interfering RNAs (siRNAs) (El-Sappah et al., 2021). siRNAs are incorporated into the RNA induced silencing complex which initiates the activation of the complex. The complex will then recognize the complementary mRNA sequence for degradation (El-Sappah et al., 2021). To prevent gene silencing, expression vectors used in biopharming approaches commonly include RNA silencing suppressor proteins, such as P19 and NSs (Vargason et al., 2003).

Agrobacterium-mediated genetic transformation

A. rhizogenes (*Rhizobium rhizogenes*) is a Gram-negative species of phytopathogenic bacteria naturally present in soil, which encode a root inducing (Ri) plasmid. The Ri plasmid contains a transfer-DNA 'T-DNA' region that is excised and transferred into host cells via a Type IV secretion system (T4SS), and genes that confer virulence and direct plant cells to synthesise opines and auxin (Mauro, Costantino, & Bettini, 2017). This pathway also involves a two-component sensory-signal transduction system induced by phenolic signal molecules, with

*vir*A as the phenolic receptor, *vir*G as the signal receiver, and other *vir* genes playing accessory roles (Bahramnejad et al., 2019).

'*Agrobacterium* mediated transformation' refers to the introduction of foreign genetic material into a host cell via bacterial infection with agrobacteria. *A. rhizogenes* induces hairy root disease in susceptible plants upon infection, whereas *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) induces crown gall disease (Krenek et al., 2015). Hairy root disease is characterised by uncontrolled root growth with multiple fine, "hairy" roots emerging from the site of infection. By introducing suitable plasmid vector into *A. rhizogenes*, which is then inoculated upon the root system of a plant host, it is possible to induce the formation of transgenic hairy roots that express heterologous genes of interest (Gelvin, 2014). This process is a 'transient' method of genetic transformation, as only the root systems of host plants are modified, and any progeny that derive from the 'transformed' plant will be wild-type.

The ARqua1 strain is an engineered strain of *A. rhizogenes* that has a well-documented ability to mediate the genetic transformation of *M. truncatula* roots, including ecotypes A17 and R108 (Floss et al., 2013; Garmier et al., 2017; Quandt, Puhler, & Broer, 1993). ARqua1 carries the Ri mega plasmid pRiA4B (Quandt et al., 1993). The T-DNA region of the pRiA4B plasmid is non-contiguous, meaning the T-DNA is separated into TL-DNA and TR-DNA, with a non-transferred spacer sequence in between (White et al., 1985). Responsible for the hairy root phenotype, the *rol* oncogenes are found within the TL-DNA (Ozyigit, Dogan, & Artam Tarhan, 2013). *rol*A, B, C, and D are associated with rapid root growth upon regeneration, stimulating increased branching. *rol* gene products act to perturb plant physiology by altering plant hormone perception (Mauro et al., 2017). Throughout colonization, opine synthesis yields compounds which are catabolized by the infecting bacterial cells as a source of energy, carbon, and nitrogen.

Agrobacterium strains can be classified based on the type of opine synthesised, with *A. rhizogenes* being classified as agropine type (Bahramnejad et al., 2019). On the pRiA4B plasmid of ARqua1, *mas*1, *mas*2, and *ags*1 genes are expressed to promote the biosynthesis of opines in transformed plant tissue. Encoded by the *mas*2 gene is a conjugase which functions to catalyze the formation of deoxy-fructosyl-glutamine from glucose and glutamine. The *mas*1 gene encodes the reductase which reduces deoxy-fructosyl-glutamine to mannopine. *ags*1 mediates the cyclization of mannopine to agropine via agropine synthesis (Bouchez & Tourneur, 1991). *aux*1 and *aux*2 are auxin biosynthesis genes important for the induction of *rol* oncogenes that cause hairy root growth as well as the production of auxin needed to support this growth. (Bahramnejad et al., 2019; Ozyigit et al., 2013).

A binary vector system composed of the ARqua1 Ri plasmid, and recombinant pHREAC backbone plasmid (Peyret, Brown, & Lomonossoff, 2019) encoding the sequence corresponding to our protein of interest was used to transform *M. truncatula* roots as depicted in **Figure 2.**. Upon infection of tissues in the radicle, T-DNA from the pHREAC backbone plasmid is transferred to *M. truncatula* root cells, with the *vir*, *rol*, *aux*, *mas*, and *ags* genes on the Ri plasmid driving the establishment of transgenic roots expressing our genes of interest (Bahramnejad et al., 2019). The pHREAC plasmid is designed to drive high levels of heterologous gene expression in plants, with successful production of multiple recombinant proteins demonstrated in model plant *N. benthamiana* (Peyret et al., 2019). The pHREAC plasmid contains a double CaMV *35S* promoter, an NSs suppressor of silencing, and conveys kanamycin resistance to both transformed bacteria and plant tissues (Peyret et al., 2019).

Infection of plant roots is initiated by creating a wound through which *Agrobacterium* can infect the plant as described in (Floss et al., 2013). Upon entry into the plant, *A. rhizogenes*

comes in contact with plant phenolic and sugar molecules, triggering the excision and transfer of T-DNA into plant cells via the T4SS of the *Agrobacterium* (Gelvin, 2014). When T-DNA is incorporated into the plant genome, insertions are thought to occur at random locations, may occur in multiple different places in the genome, may be the product of partial (incomplete) T-DNA transfer, or may integrate into inactive regions of the DNA such as telomeres (Gelvin, 2021). Hairy root transformation results in the accumulation of heterologous proteins in root cells only, creating composite *M. truncatula* plants that contain wild-type (non-transformed) shoots and germ tissues. As the genetic transformation is not inherited in progeny, such hairy root transformations are referred to as 'transient' transformations, even though the transferred genes and resulting root systems can be maintained for multiple years.

Root organ cultures as a long-term method of heterologous protein production

ROCs are plant tissue cultures generated from roots propagated upon solid media with no shoots attached. These cultures can be easily and efficiently generated from transformed *M. truncatula* root tissue (Hause & Yadav, 2020). Introduction of *rol* oncogenes during agroinfection results in transformed root tissue with unlimited regeneration potential, creating immortalised root lines (Mauro et al., 2017). By subculturing ROCs regularly to fresh media, transformed root tissue will continue to grow and significant amounts of tissue can be generated. ROCs may be beneficial in that they can continue to grow and produce heterologous proteins long past the natural lifecycle of *M. truncatula.* If ROCs are not subcultured regularly, resource limitation will result in a reduction and eventual cessation of growth. However, root tissue will begin to regenerate once again when introduced to fresh media. *N. tabacum* ROCs have been previously explored as a production system for monoclonal antibodies with significant successes documented (Häkkinen et al., 2014; Lonoce et al., 2016; Sharp & Doran, 2001).

ROCs are commonly used in AMF research, as AMF are obligate biotrophs and require symbiosis with a living plant host for survival and spore production. While *M. truncatula* roots can be successfully used to support the production of AMF spores, *Daucus carota* (carrot), is almost invariably the plant host of choice (Goh et al., 2022). ROCs generated from transformed *M. truncatula* root tissue offer many potential benefits as a system for the production of heterologous proteins, including minimal care requirements, simple growth conditions, efficient use of space, and maximum allocation of resources to transformed root tissue. The creation of ROCs begins with the genetic transformation of *M. truncatula* radicles as outlined in **Figure 2.**, but instead of transferring seedlings to pots three weeks post-transformation, transgenic root tissue is excised and plated on *Medicago* root culture (MRC) media as depicted in **Figure 3.**. As ROCs do not produce leaves or aerial tissues of any kind, they cannot perform photosynthesis and are no longer autotrophic. MRC media contains sucrose to provide a necessary carbon and energy source, in addition to standard micro- and macronutrients required to sustain plant growth.

Rol oncogenes transferred to *M. truncatula* from the ARqua1 Ri plasmid during infection confer unlimited regeneration capabilities to transgenic tissue, meaning that root tissue is effectively immortalised and will continue to grow as resources allow. The genetic stability of hairy roots allows for the preservation of transgenic root lines that accumulate high levels of relevant proteins, which can be sub-cultured to produce large amounts of transgenic tissue when needed. Notably, Häkkinen et al. showed that 16 years after generation and throughout multiple subcultures, *Hyoscyamus muticus* (Egyptian henbane) hairy roots showed no significant loss in metabolic activity (Häkkinen et al., 2016). Sharp & Doran documented levels of mouse IgG in transgenic *N. tabacum* root tissue cultures over the course of 3 years and found *N. tabacum* roots

to be morphologically stable and levels of antibody accumulation to be "essentially unvarying" (Sharp & Doran, 2001). The option to create long-term ROCs is an advantage unique to working with plant roots.

Rhizosecretion as a potential route of heterologous protein collection

Rhizosecretion refers to the secretion of proteins, metabolites, and other compounds from the plant into the rhizosphere via roots (Gaume et al., 2003). Protein extraction directly from plant tissue requires that proteins be liberated and then separated from tissue, cell debris, and other compounds and secondary metabolites. Protein collection via rhizosecretion into hydroponic media could greatly facilitate protein isolation from unwanted biological materials, or at the very least minimise the amount of time, steps, and effort taken to purify protein (Gaume et al., 2003). Fewer purification steps may also mean less protein is lost during purification, rendering the process more efficient. Rhizosecretion of proteins can be achieved by addition of a signal peptide that directs proteins for secretion to the apoplast, which is sufficient direction to result in rhizosecretion when the relevant gene is expressed in the roots (Borisjuk et al., 1999; Finer, 1999). In *M. truncatula*, proteins may be directed for rhizosecretion via fusion with the *Medicago* blue copper protein (MtBCP1) signal peptide (Ivanov & Harrison, 2014). MtBCP1 was first discovered as a protein that is enriched in colonised *M. truncatula* roots during symbiosis with AMF (Valot et al., 2006). Subsequent research demonstrated that the attachment of the MtBCP1 signal peptide to a fluorescent protein is sufficient to target the recombinant protein for secretion to the apoplast (Ivanov & Harrison, 2014; Pumplin & Harrison, 2009). Rhizosecretion of various products including antibodies and recombinant proteins, has been attempted in *N. tabacum* and *N. benthamiana* roots, as well as in *M. truncatula* roots with

varying degrees of success documented in all 3 species (Bocciarelli, 2014; Makhzoum et al., 2013; Medina-Bolívar & Cramer, 2004).

Green fluorescent protein as a visual marker of transformation

GFP exhibits fluorescence when excited by light in the blue to ultraviolet light wavelength range, roughly 380 nm to 475 nm (Zimmer, 2002). Recombinant GFP tagged with a translational fusion to an octo-histidine (8xHIS) at the C-terminus was selected as our protein of interest for this project. This choice was made due to its ability to act as a visual marker of genetic transformation in *M. truncatula* roots, while also permitting observation of changes in protein accumulation in roots over time in living tissues. It also permits facile evaluation of whether individual transformation events generate uniformly transformed root systems or if patches of root tissue remain non-transformed.

Research Objectives

Herein, we strove to explore the potential of *M. truncatula* roots as an expression platform for the production heterologous proteins both in composite plants and root organ cultures. We aimed to assess protein accumulation over time in composite plants to optimise harvest timepoints, and to assess ROCs as a long-term, stable, method of protein production.

This study demonstrates that *M. truncatula* is an appropriate production platform for heterologous proteins that can be collected and purified. Using both composite plants and ROCs, the accumulation of recombinant GFP was assessed overtime, yielding promising results. Heterologous protein accumulation was observed at high levels in composite plants up to 15 weeks post-transformation, and beyond 57 weeks post-transformation in seven independent ROC lines. Purification of heterologous protein from ROC tissue resulted in yields of 780 mg/kg and

194 mg/kg of fresh tissue, respectively, yields comparable to those obtained from industry standard *N. benthamiana*.

Figure 1. 10-week-old *M. truncatula*. (A) whole plant, (B) leaf, (C) flower, (D) seed pod.

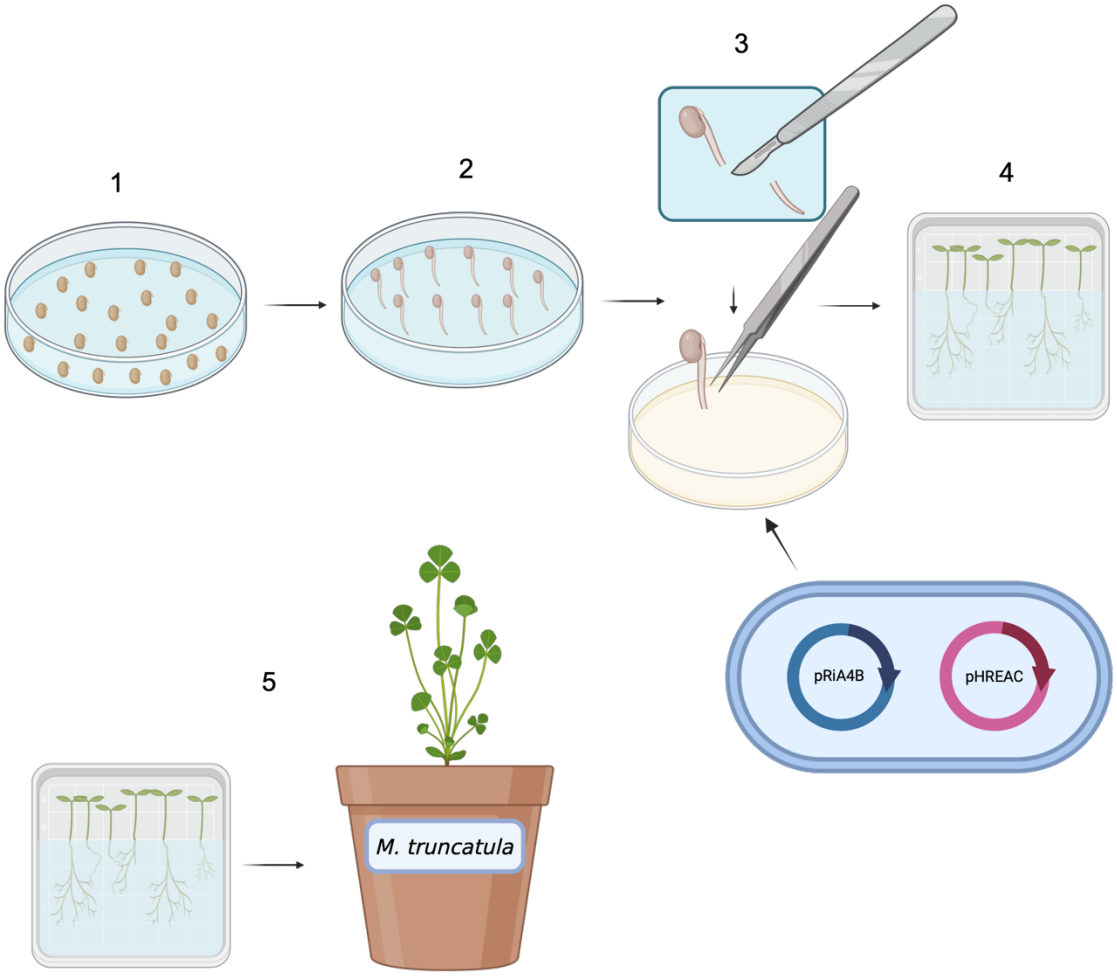


Figure 2. Illustration of the process of *Agrobacterium*-mediated transformation of *M. truncatula* roots for the creation of composite plants. 1. This process begins with the sterilisation and subsequent plating of *M. truncatula* seeds for germination on glass petri plates. 2. Seeds are germinated overnight. 3. After germination, the tip of the radicle is excised with a scalpel prior to dipping in *A. rhizogenes* containing both the pRiA4B and pHREAC plasmids. 4. Seedling is transferred to Fahraeus media to allow root regeneration and seedling growth. 5. Transformed seedlings with regenerated roots are transferred to pots 3 weeks later. Image created with BioRender.

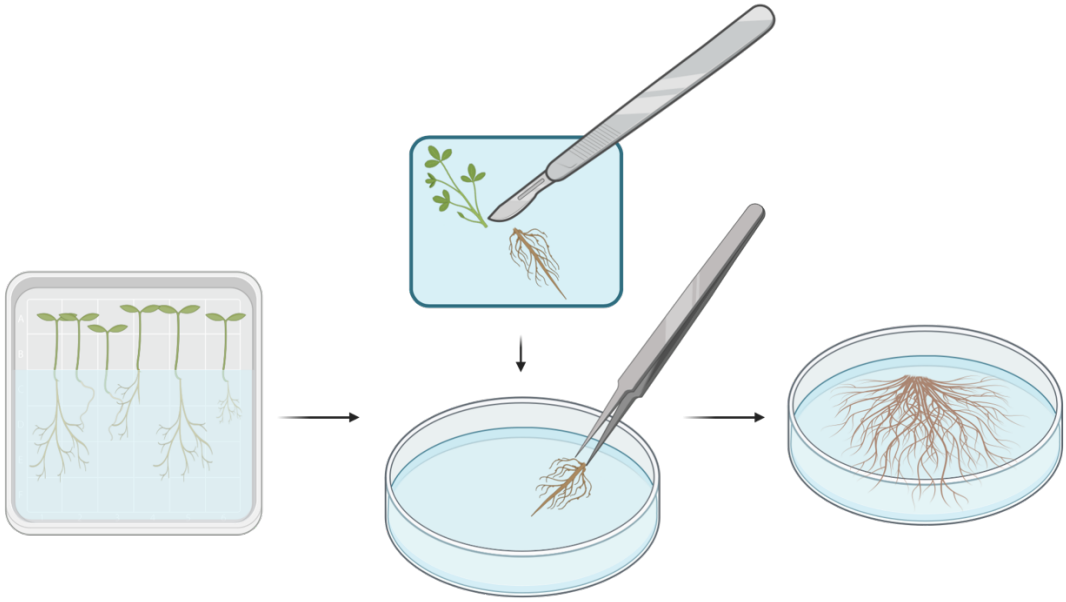


Figure 3. The creation of ROCs from *M. truncatula* roots. After transformation according to protocol outlined in **Figure 2.**, transformed roots are excised and plated on MRC media. Image created with BioRender.

CHAPTER 2: METHODS

Preparation of plasmid constructs

The plasmid construct containing the sequence encoding recombinant 8xHIS tagged eGFP was designed and cloned by Dr. Jordan Demone (**Supp. Figure 1.**). This plasmid is based upon a pHREAC backbone (Peyret et al., 2019) that includes kanamycin resistance (KanR) genes to allow for selectivity of transformed bacteria and plant roots. The plasmid construct was designed with the 8xHIS tag on the C-terminus of eGFP to facilitate detection and purification. An empty vector (EV) pHREAC plasmid was used throughout this project as a negative control (**Supp. Figure 2.**). Plasmids were transformed into chemically competent *E. coli* strain DH5α (Invitrogen) to create glycerol stocks for long-term storage at -80C. To prepare plasmids for use, glycerol stocks were streaked onto LB agar plates supplemented with 50mg/L kanamycin. Plates were incubated at 37℃ overnight and the following day 5mL overnight cultures supplemented with 50mg/L kanamycin were inoculated with individual *E. coli* colonies. Cultures were incubated overnight at 37℃ and then plasmid was prepared using a Sigma-Aldrich GenElute™ Plasmid Mini Prep Kit according to manufacturer instructions. All plasmid stocks were diluted to a final concentration of 20ng/uL using an elution buffer prior to electroporation.

Preparation of electrocompetent Agrobacteria

A. rhizogenes strain ARqua1 cells from a glycerol stock were streaked onto an LB agar plate containing 100 mg/L streptomycin before being incubated at 28℃ for 3 days. Following incubation, one ARqua1 colony was selected and transferred to a 5mL liquid LB media culture supplemented with 100 mg/L streptomycin and subsequently incubated at 28℃ overnight. 500mL of liquid LB media with 100 mg/L streptomycin was inoculated with 500uL of the aforementioned 5mL liquid culture and incubated while shaking at 28℃ overnight. The culture was incubated to reach an optical density at 600 nm (OD600) of 0.5-1.0, as determined via

spectrophotometer (Thermo Scientific BioMate 3). The culture was cooled on ice for 15 minutes before centrifugation (Beckman Coulter Avanti J-E centrifuge) at 5000 rpm (rotor 16.25) for 15 minutes at 4°C. After the supernatant was discarded, the bacterial pellet was resuspended in autoclaved cold water prior to centrifugation again according to the above specifications. The supernatant was again discarded, with resuspension and centrifugation steps repeated twice more for a total of 3 cycles. The pellet was then resuspended into 10mL of 10% glycerol and centrifuged a final time. The supernatant was discarded, and the pellet was resuspended in 10mL of 10% glycerol. 40uL aliquots were flash frozen for storage at -80°C.

A. tumefaciens strain AGL1 cells were prepared according to the same methods outlined for *A. rhizogenes* strain ARqua1 cells. All solid media was supplemented with ampicillin 100mg/L and liquid media with 50mg/L ampicillin in lieu of streptomycin.

Bacterial transformation

Electrocompetent *A. rhizogenes* ARqua1 or *A. tumefaciens* AGL1 cells (40uL) were transformed with 40ng of plasmid DNA via electroporation (BIO-RAD MicroPulser ™) with settings suitable to the electroporation of bacterial cells. Cells were then incubated at 28°C in 1mL of LB broth for 1.5 hours to allow phenotypic expression of antibiotic resistance encoded by the plasmid. 100uL of the liquid culture was then plated on LB agar plates supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin to select for successfully transformed ARqua1 cells, or 100 mg/L ampicillin and 50 mg/L kanamycin to select for successfully transformed AGL1 cells. The plates were then incubated at 28°C for 3 days.

Cultivation of *N. benthamiana*

N. benthamiana was grown in a greenhouse under natural lighting that was supplemented with high pressure sodium lights set to a photoperiod of 20h light, 4h dark at 25°C. Wild type *N. benthamiana* seeds were sown upon Pro-Mix General Purpose soil that had been soaked with 1x Miracle Gro fertiliser (Water Soluble All Purpose Plant Food, 24:8:16) prior to transfer to individual pots after 2 weeks. Growing plants were fertilized with 100mL of 1.1g/L 1x Miracle Gro fertiliser (Water Soluble All Purpose Plant Food, 24:8:16) once per week and watered with tap water as needed until agroinfiltration was performed at 5 weeks post germination.

Cultivation of *M. truncatula*

M. truncatula A17 ecotype plants were used for this project. After manually removing seeds from seed pods, seeds were treated with 98% H_2SO_4 for 9 minutes to remove the seed coat, and then sterilised in a 10% bleach, 0.1% Tween® 20 solution for 10 minutes. Seeds were then transferred to damp autoclaved filter paper in a sterile glass petri plate, wrapped in tin foil, and incubated at 4°C for 2 days. After 2 days, the petri plate was transferred to a drawer to incubate at room temperature for one day while maintaining dark conditions. Germinated seeds were then exposed to light at room temperature for two more days before seedlings were transferred to pre-washed and autoclaved turface purchased from Turface Athletics. Plants were grown in a growth chamber with a photoperiod of 16h light at 25°C, 8h dark at 22°C with a light intensity of 160 uM/m^2s. Each pot was fertilized with 40mL of 1x Miracle Gro fertiliser (Water Soluble All Purpose Plant Food, 24:8:16) once per week and watered with tap water as needed. After two weeks of growth in turface, *M. truncatula* plants were transplanted in a 1:1 playsand: turface blend to grow under the same conditions until seed pods were produced. Seeds pods were allowed to dry on the plant and were only collected when they readily fell off the plant with little

force applied. The seed pods were then incubated at 28℃ for a week before use, as we have observed that drying the seeds improves germination.

M. truncatula seed sterilisation and germination

M. truncatula A17 ecotype seeds were sterilised and germinated according to the Floss et al. (Floss et al., 2013). Seed sterilisation and germination occurred in parallel with TY plate preparation and incubation, with seeds being sterilised the same day bacterial colonies were plated. After removing seeds from seed pods, seeds were treated with 98% H_2SO_4 for 9 minutes to remove the seed coat, and then sterilised in a 10% bleach, 0.1% Tween® 20 solution for 10 minutes. Seeds were then allowed to imbibe autoclaved water overnight at 4℃. Imbibed seeds were plated on sterile glass petri plates which were wrapped in foil, flipped upside down, and incubated at 28℃ overnight to germinate with roots growing downwards.

M. truncatula root transformation

A. rhizogenes strain ARqua1 was used to transform *M. truncatula* roots according to the established protocol by Floss et al. with slight adjustments, as fluorescence from eGFP allowed for additional screening throughout (Floss et al., 2013). ARqua1 readily infects and induces hairy roots in *M. truncatula* A17, making it an appropriate strain for this project. To provide a point of entry, root tips of germinated seedlings were cut off at an angle with a sterile scalpel before being dipped into transformed ARqua1 that was previously cultured upon TY agar at 28℃ for 2 days. The seed coats were also gently removed with sterile forceps, prior to dipping into bacterial culture. The seedlings were then placed into Fahraeus media (F) plates supplemented with 25 mg/L of kanamycin to select for transformed roots (**Supp. Table 1.**) (Floss et al., 2013). A subset of roots transformed with empty vector plasmid were included as a negative control. F plates

were sealed with parafilm that was slit several centimeters near the top to permit gas exchange. Plates were placed in a growth chamber kept at 18°C with a photoperiod of 8h light, 16h dark and a light intensity of 16 uM/m^2s for 5 days to promote agroinfection. After 5 days, the plates were transferred to a second growth chamber with a photoperiod of 16h light (25°C), 8h dark (22°C) with a light intensity of 45 uM/m^2s where they were kept for 16 days.

M. truncatula root screening and cultivation of composite plants

Three weeks post-transformation, roots were screened for the first time to identify transformants that express *eGFP*. Fluorescence was visualized via fluorescence microscopy using a Zeiss AxioZoom.V16; excitation at 470nm, emission at 525nm). Seedlings that exhibited fluorescence in newly emergent roots were selected for transplanting. These seedlings were hardened overnight at room temperature by removal of the F plate lid, to allow for seedling acclimation to a lower humidity environment. Seedlings were next transplanted individually into a 1:1 playsand: turface blend in pots (12.7cm diameter, 8.89cm height, 766.55mL total volume). Turface and play sand were washed and autoclaved before use. The pots were transferred to a growth chamber set to a photoperiod of 16h light at 25°C, 8h dark at 22°C with a light intensity of 160 uM/m^2s until harvest. Each pot was fertilized with 40mL of 1x Miracle Gro fertiliser (Water Soluble All Purpose Plant Food, 24:8:16) once per week and watered with tap water as needed. Pots were covered with domes during the first week of growth to maintain higher humidity.

M. truncatula root harvest from composite plants

At 5, 6, 7, 11, 13, and 15-weeks post-transformation, the entire root system was removed from pots, rinsed in tap water, blotted with paper towel, screened for fluorescence as described

above, and weighed. Roots were flash frozen in liquid nitrogen before being stored at -80℃. Plant shoots, which are not transformed, were discarded.

ROC generation and maintenance

Transformed *M. truncatula* A17 roots that express *eGFP* were generated and screened according to the methods outlined above. However, as a means of creating ROCs, transformed roots that exhibited fluorescence were excised with a sterile scalpel and forceps, and were plated onto MRC media with 200 mg/L cefotaxime to eliminate residual ARqua1 agrobacteria (**Supp. Table 2.**). ROCs were maintained at room temperature (18-20℃) in a single closed drawer in the lab over the course of this research. Two weeks after initial plating, ROCs were screened once again for fluorescence using fluorescence microscopy (Zeiss AxioZoom.V16; excitation 470nm, emission at 525nm). Root segments that exhibited strong, uniform fluorescence were excised and replated onto fresh MRC media. This process was repeated until seven ROC lines originating from seven independent transformation events were established, each exhibiting bright, uniform fluorescence when examined under microscope. Fluorescence in ROCs was observed over time and cultures were replated and subcultured to promote growth when fluorescence levels began to diminish, at least once per month.

Agroinfiltration of *N. benthamiana* leaves

A single AGL1 colony carrying the plasmid of interest was inoculated into a 5mL liquid LB media culture with 50mg/L ampicillin and 50mg/L kanamycin and incubated at 28℃ overnight. The following afternoon, 500uL of the overnight culture was sub-cultured into 500mL (1:1000 dilution) of liquid LB media with 50mg/L ampicillin and 50mg/L kanamycin, which was

incubated while shaking at 28℃ overnight. The culture was incubated until an optical density at 600 nm (OD600) of 0.25 was measured using a spectrophotometer.

After incubation, the 500mL culture was centrifuged (Beckman Coulter Avanti J-E centrifuge) at 5000 rpm (rotor 16.25) for 15 minutes at 4℃. The pellet was resuspended in 500mL of infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone, pH 5.6) and incubated at room temperature for 1 hour before infiltration. Spot infiltration of the abaxial side of leaves that had not yet fully expanded was conducted with a flat tipped 1mL syringe. Infiltrated plants were returned to the greenhouse until leaves were harvested 5 days post infiltration and flash frozen in liquid nitrogen.

Protein extraction and purification

Protein extraction and purification was conducted according to a protocol that was optimized for protein purification from *N. benthamiana* leaves (Demone et al., 2022) with some changes made to the extraction buffer. For ROCs, root tissue was gently removed from MRC plates immediately before protein extraction. Plant tissues were ground in liquid nitrogen with a mortar and pestle, and ground tissues were resuspended in an extraction buffer (10mM imidazole in PBS with 2% Tween® 20). Cellulase enzyme blend (Sigma, SAE0020) and Viscozyme® (Sigma, V2010) were added to the buffer, and the cell lysate was sonicated (Kontes Micro Ultrasonic Cell Disruptor) in 4 bursts of 30 seconds each at 65% of total output. The lysate was then centrifuged (Beckman Coulter Avanti J-E centrifuge) at 13000 rpm (rotor 16.25) for 45 minutes at 4℃ and filtered through four layers of cheesecloth. A final filtration through a 0.20um pore membrane was conducted using vacuum filtration. The lysate was then passed through a 5mL HisPur Ni-NTA resin column using the AKTA Pure Protein Purification System.

Protein was eluted into 5mL fractions, using 500mM imidazole in 1xPBS. Protein concentration was determined via Bradford method, following the manufacturer's instructions.

Sample analysis

Protein samples were analysed using Coomassie Brilliant Blue (CBB) stained 15% SDS-PAGE gels and Western blots. Frozen plant tissues were ground in liquid nitrogen before adding an equivalent volume of 1x PBS (pH 7.4) to sample weight (1:1 vol/wt.). 2x Laemmli sample buffer with 5% 2-mercaptoethanol was added to the correct dilution based on PBS volume. Samples were boiled for 10 minutes, centrifuged (Eppendorf Centrifuge 5424 R) at 15000rpm for 5 minutes, and 10-15uL was loaded on to 15% SDS PAGE gels electrophoresed at 170V (constant voltage). Protein transfer to PVDF membranes (BIO-RAD Immun-Blot ® PVDF Membranes for Protein Blotting, 0.2um) was achieved using the Bio-Rad Trans-Blot Turbo Transfer System (25V, 1.0A, 30 minutes). PVDF membranes were blocked using a 5% w/v milk blocker solution with 1% Tween® 20. Membranes were then incubated with primary conjugated anti-HIS (Sigma Life Science Monoclonal Anti-6x His Tag, 1:2000, SAB2702219) or anti-GFP (Invitrogen GFP Monoclonal Antibody, 1:3000, GF28R) antibody at room temperature for 2 hours. GFP previously purified from *N. benthamiana* was diluted to 500ng and 750ng and frequently included as a positive control.

CHAPTER 3: RESULTS

Heterologous protein accumulation in composite *M. truncatula* plants

Expression of *eGFP* in transformed, hairy roots was assessed as a proof-in-principle throughout the lifecycle of *M. truncatula* composite plants with the initial intention of determining whether there is an optimal harvest time point for plants that produce the encoded protein. Optimal time point for tissue collection may vary between different heterologous proteins and is defined here as the point in time post-transformation at which root biomass is maximal and heterologous protein accumulation is relatively high. It was anticipated that heterologous protein levels may decrease over the life cycle of the plant. However, after detecting high levels of recombinant eGFP in root tissues collected at 5-, 6-, and 7-weeks post-transformation, we extended our analyses to assess eGFP accumulation in *M. truncatula* roots up to 15-weeks post-transformation (**Figure 4.**).

Analyses of root tissue harvested from composite plants at 5, 6, 7, 11, 13, and 15-weeks post-transformation show sustained accumulation of recombinant eGFP throughout the *M. truncatula* lifecycle, and up to 15 weeks post-transformation, as evident in **Figure 4**. Fluorescence was observed at the first screening time point 3 weeks post-transformation and continued to be observed throughout the 12-week experimental period. Western blotting performed with anti-HIS and anti-GFP antibodies further indicated the presence of recombinant eGFP over the course of the 12-week experiment. Furthermore, the accumulation of high levels of recombinant eGFP was reflected by the appearance of a visible band of approximately 28 kDa on CBB-stained SDS-PAGE gels, in samples obtained from *eGFP*-expressing roots.

As shown in **Figure 4.**, accumulation of recombinant GFP was consistently detected in *M. truncatula* roots at relatively high levels throughout the lifecycle of *M. truncatula* up to 15 weeks post-transformation. This is apparent when observing the comparative intensity of the

recombinant GFP band that appears on the CBB stained SDS-PAGE gels against the intensity of the purified recombinant GFP positive control bands (500 ng and 750 ng), both of which appear at 28kDa. The loading of known amounts of purified recombinant GFP allows for a qualitative (more than or less than) assessment of the amount of recombinant GFP in each *M. truncatula* root sample loaded on the gel. It is important to note that this does not allow us to conduct a quantitative assessment of the amount of recombinant GFP in each root sample, but rather enables us to estimate if the amount of recombinant GFP in each root sample is more than or less than the loaded positive controls. Note in particular a relative high intensity of the recombinant GFP band in all samples on the CBB stained SDS-PAGE gels at 5 and 6-weeks post-transformation, and the samples in lanes 2,3,5 and 6 at 7 weeks post-transformation. In each of the aforementioned samples, the intensity of the recombinant GFP band is greater than that of the 750ng of purified recombinant GFP loaded as a positive control, suggesting that the amount of recombinant GFP in the sample loaded is greater than 750ng.

Figure 4. illustrates mixed results regarding changes in protein accumulation levels over time, the most striking being the decline in fluorescence in the fluorescence microscopy images over the course of the experiment after 11 weeks post-transformation. Note that **Figure 4.** includes results obtained from 30 individual composite plants and therefore does not display the accumulation of recombinant GFP over time in the same five samples. The 5,6,7, and 11 weeks post-transformation time points all exhibit strong fluorescence observed under a dissecting microscope, however the 13- and 15-week time points show a significant decrease in fluorescence when compared to previous time points, with the 15-week time point showing the weakest fluorescence of all. The CBB stained SDS-PAGE gels also reflect this trend to a similar degree. High intensity bands of recombinant GFP at 28kDa are visible at 5,6-, and 7-weeks post-

transformation, but not in the later time points. The recombinant GFP band at 28kDa is visible in 2 samples at 15 weeks post-transformation, albeit at a lower intensity than that first 3 time points. This band is either non or barely visible for all samples at 11- and 13-weeks post-transformation. Data from the CBB stained SDS-PAGE gels and fluorescence microscopy imaging suggests a decline in the accumulation of recombinant GFP over time, however this trend is not as clearly reflected in the Western blots of the same samples.

Given that fluorescence microscopy images show that fluorescence diminishes over time, and that CBB stained SDS-PAGE gels display lower relative levels of recombinant GFP at later time points, we would expect to see lower levels of recombinant GFP detected during Western blotting as the experiment progressed. Contrarily, **Figure 4.** illustrates that high levels of recombinant GFP that appear to be comparable to the levels detected at earlier time points, when probed with anti-HIS antibody and anti-GFP antibody. It is important to note that through multiple repetitions of both CBB staining of SDS-PAGE gels and Western blotting with the two respective antibodies these trends were consistently observed.

Data obtained from the assessment of 30 independently transformed composite plants indicates that GFP accumulation occurred as early as 5 weeks post-transformation, and that the proportion of GFP in relation to endogenous protein is only likely to decrease as plants age. In consideration of these observations, purification from transformed *M. truncatula* roots harvested at 4 weeks post-transformation was attempted. Purification of recombinant 8xHIS tagged GFP was conducted using the AKTA Pure Protein Purification System with a HisPur Ni-NTA resin column, resulting in a yield of 62 mg GFP per kg of fresh weight of root tissue. This was a surprisingly low yield given the levels of recombinant 8xHIS tagged GFP detected via Western blotting in previous experiments (**Figure 4.**) although preliminary Western blots were not

performed on the plants that were collected for protein purification. Moreover, as the protein extraction protocol is optimised for extraction from *N. benthamiana* leaves, modifications to the protocol may have been required to obtain an increased amount of purified protein.

In summary, promising results pertaining to stable recombinant GFP accumulation in composite *M. truncatula* plant roots were obtained. Recombinant GFP was detected via Western blotting, CBB stained SDS-PAGE gels, and fluorescence microscopy in root tissue samples up to 15 weeks post-transformation, with high levels of accumulation confirmed via Western blotting throughout the experiment. However, one round of purification of recombinant 8xHIS tagged GFP from transgenic *M. truncatula* root tissue resulted in low yields. Further work is required to optimize the isolation of heterologous protein from *M. truncatula* roots of composite plants.

Heterologous protein accumulation in *M. truncatula* ROCs

ROC lines that were genetically transformed to express the same *eGFP* construct as composite *M. truncatula* plants were generated in parallel to assess the pros and cons of each system. To investigate the rate at which ROCs generate biomass (i.e., growth rate), established ROCs were excised, weighed, and plated on fresh media, a process that was repeated every 10 days. Removal of ROCs from their sterile growing environment for weighing introduced the risk of microbial contamination, thus MRC plates used in this experiment were supplemented with 200 mg/L of cefotaxime. The transfer of ROCs to fresh media (as opposed to returning them to their original plates) better reflects the weekly fertilisation regime of plants that are cultivated in a greenhouse and helps to ensure that growth rate is not limited by nutrient availability. As reflected in **Figure 5.**, ROCs displayed the greatest percentage mass increase from 30-40 days with an average increase in mass of 155% over this period. The second greatest mass increase

occurred from 0-10 days, with an average increase in mass of 119% within the first measurement period.

As shown in **Figure 6.**, the use of ROCs also enabled observation of (changes in) location and intensity of fluorescence over time. **Figure 6.** exhibits the relative changes in localization of fluorescence in the same ROC captured via fluorescence microscopy at eight time points from 5 to 14 weeks post-transformation. The highest levels of fluorescence are in regions of new and active growth, where higher levels of transcriptional activity might reasonably be expected to lead to higher levels of GFP accumulation. In older segments of the ROC, fluorescence fades over time, possibly due to accumulation of lignin as in composite plants, and/or due to decreased cellular activity in older tissues. It is important to note that the ROC in **Figure 6.** was not transferred to fresh medium throughout the period of observation, meaning that no new resources were available to the tissues during this time. This is relevant because we have observed that reducing the amount of time between the re-plating of transformed roots contributes positively to the maintenance of strong GFP fluorescence. This is not surprising given that as resources become limited, tissue growth slows and eventually ceases. When ROCs are re-plated regularly, i.e., once every 2 months, fluorescence does not diminish even in the oldest regions of ROCs; regular replating of ROCs is therefore crucial for maintenance.

Transformed root tissues from seven independent ROC lines, each resulting from an independent transformation event, were sampled to assess eGFP accumulation via SDS-PAGE and Western blotting, at 45 weeks post-transformation. All seven lines were found to exhibit high levels of accumulation of recombinant GFP relative to other proteins in root tissue, as displayed in **Figure 7**. While a direct comparison should be interpreted with caution, we note that samples of crude lysate obtained from the ROCs (**Figure 7** panel A; CBB stained

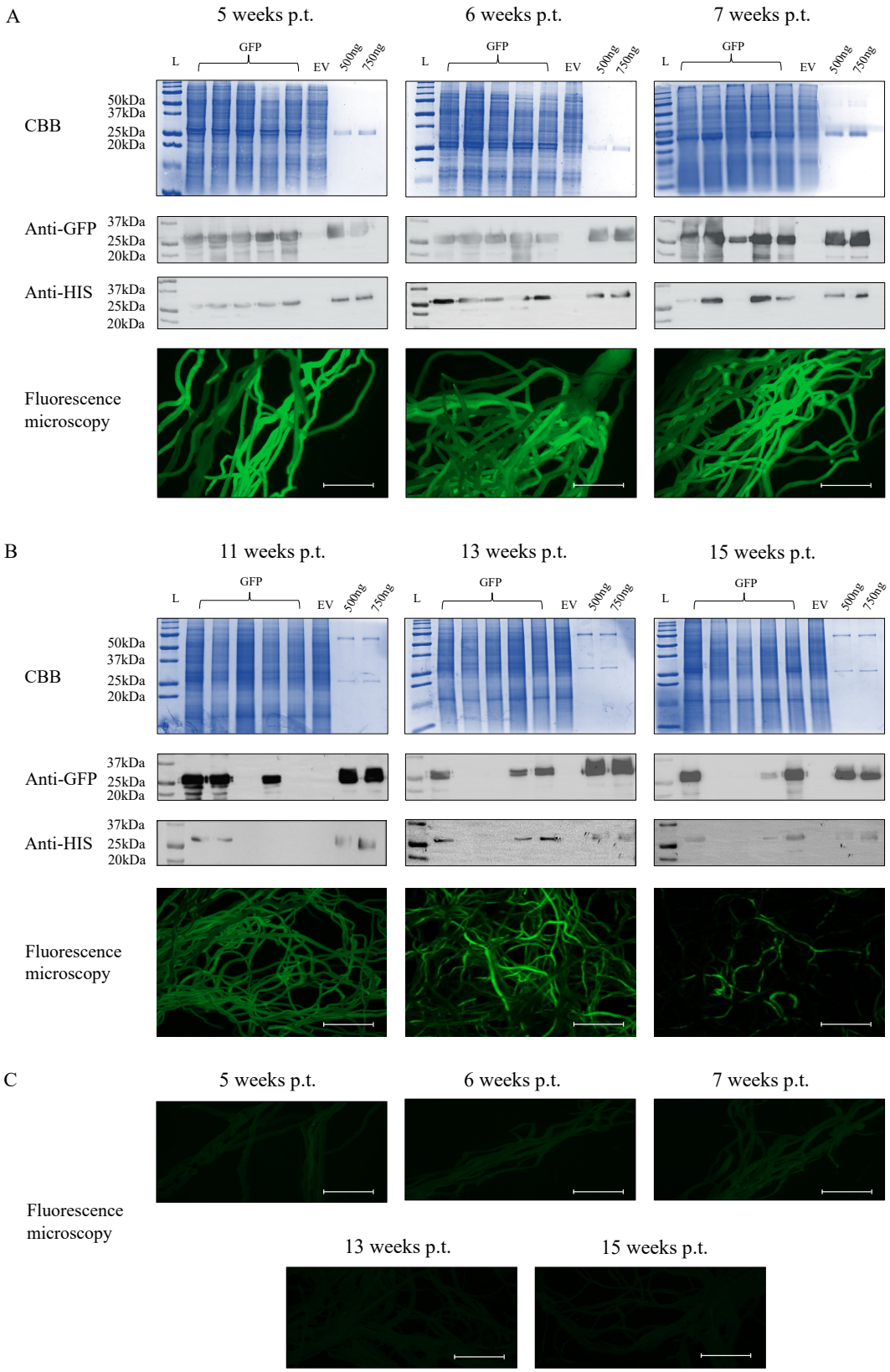
acrylamide gel) indicate that a greater proportion of target protein is produced in these lines, relative to endogenous proteins, when compared against similar data generated using composite plants (**Figure 4.**).

Purification of protein from *M. truncatula* ROC tissue

Purification of recombinant 8xHIS tagged GFP from *M. truncatula* ROC tissue was performed based upon the protocol used to purify GFP from composite *M. truncatula* plants. However, due to the low recovery of purified protein from composite *M. truncatula* plant root systems, some alterations were made to the protocol. Tween® 20 was added to the extraction buffer to a concentration of 2%, and cellulase and Viscozyme® were also added to assist in the extraction of recombinant GFP from plant cells. As before, purification was conducted using the AKTA Pure Protein Purification System with a HisPur Ni-NTA resin column. Recombinant 8xHIS tagged GFP was purified from root tissue harvested from all seven independent root lines which were selected and subcultured to achieve the highest level of GFP fluorescence possible. As discussed, **Figure 7.** documents the high levels of accumulation of recombinant GFP in all seven root lines relative to endogenous *M. truncatula* proteins. Leading up to purification, ROCs were split in two and were re-plated on a weekly basis to rapidly propagate root tissues. During the first attempts at purification from composite *M. truncatula* plant root systems, it was noted that the root lysate obtained from the extraction process was very viscose in comparison to *N. benthamiana* leaf lysate. Therefore, roughly 10g of transgenic *M. truncatula* root tissue was collected for each purification. In parallel, *N. benthamiana* leaves were agroinfiltrated with AGL1 (OD600 0.25) carrying the same pHREAC backbone 8xHIS GFP construct. GFP was then purified from 30g of leaf tissue collected 5 days post infiltration. Purification of recombinant 8xHIS tagged GFP from *M. truncatula* ROCs resulted in yields of 780 mg/kg and 194 mg/kg,

respectively. These numbers are comparable to yields of recombinant 8xHIS GFP purified from *N. benthamiana*, which were 920 mg/kg and 450 mg/kg, respectively. To assess protein purity and investigate the effectiveness of the purification, samples of crude lysate, column flowthrough, and eluted protein were analyzed via CBB SDS-PAGE and Western blotting (**Figure 8.**). Detection of recombinant GFP in the flowthrough when probed with anti-GFP antibody but not when probed with anti-HIS antibody indicates that a portion of recombinant GFP that accumulated in both *N. benthamiana* leaves and *M. truncatula* roots does not have an 8xHIS tag attached. This is an issue of concern, as if lacking the appropriate affinity tag, recombinant protein will not bind to the Ni column and therefore will not be separated from the rest of the crude lysate. This phenomenon has been previously observed when attempting to purify 6xHIS tagged SARS-CoV-2 RBD from transgenic tobacco BY-2 and *M. truncatula* cell suspension cultures as well as from *N. benthamiana* leaves (Rebelo et al., 2022; Shin et al., 2021).

Although the absence of the 8xHIS tag was observed in samples obtained from both *N. benthamiana* leaves and *M. truncatula* ROCs, it appears to be more prevalent in the ROCs. **Figure 9.** displays recombinant GFP purified from *N. benthamiana* leaves and recombinant GFP purified from *M. truncatula* ROCs each of which had been aliquoted, flash frozen, and then thawed prior to electrophoresis. Note that the recombinant GFP purified from *M. truncatula* roots was detected by the anti-GFP antibody and is visible on the CBB stained SDS-PAGE gel but was not detected by the anti-HIS antibody. This most likely indicates that although recombinant GFP is present, the protein lacks an 8xHIS tag. It is important to note that **Figure 9.** only encompasses results from a single purification attempt from each plant species, and repeat purifications are necessary to determine if this is a reproducible phenomenon.


A
5 weeks p.t.
6 weeks p.t.
7 weeks p.t.
L
GFP
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500ng
750ng
CBB
50kDa
37kDa
25kDa
20kDa
Anti-GFP
37kDa
25kDa
20kDa
Anti-HIS
37kDa
25kDa
20kDa
Fluorescence microscopy
B
11 weeks p.t.
13 weeks p.t.
15 weeks p.t.
L
GFP
EV
500ng
750ng
CBB
50kDa
37kDa
25kDa
20kDa
Anti-GFP
37kDa
25kDa
20kDa
Anti-HIS
37kDa
25kDa
20kDa
Fluorescence microscopy
C
5 weeks p.t.
6 weeks p.t.
7 weeks p.t.
Fluorescence microscopy
13 weeks p.t.
15 weeks p.t.

Figure 4. Accumulation of recombinant eGFP in roots throughout the life cycle of *M. truncatula*. Entire root systems of composite plants were harvested at 5,6,7,11,13, and 15-weeks post-transformation (p.t.). Panel A displays samples harvested at 5-, 6-, and 7-weeks post-transformation. Panel B displays samples harvested at 11-, 13-, and 15-weeks post-transformation. Panel C displays fluorescence microscopy images of *M. truncatula* roots transformed with an empty vector (EV) plasmid as a negative control. Samples were run on 15% SDS-PAGE gels and stained with CBB. Recombinant 8xHIS tagged GFP (28kDa) was detected via Western blot using anti-HIS or anti-GFP antibodies, as indicated beside respective blots. Presence of recombinant eGFP was also confirmed using fluorescence microscopy imaging at each timepoint. Root systems of composite plants transformed with an EV plasmid were used as a negative control. For SDS-PAGE and Western blots, 500 ng and 750 ng of purified recombinant eGFP were loaded as positive controls. Scale bars on all microscopy images represent 4000um.

A

B

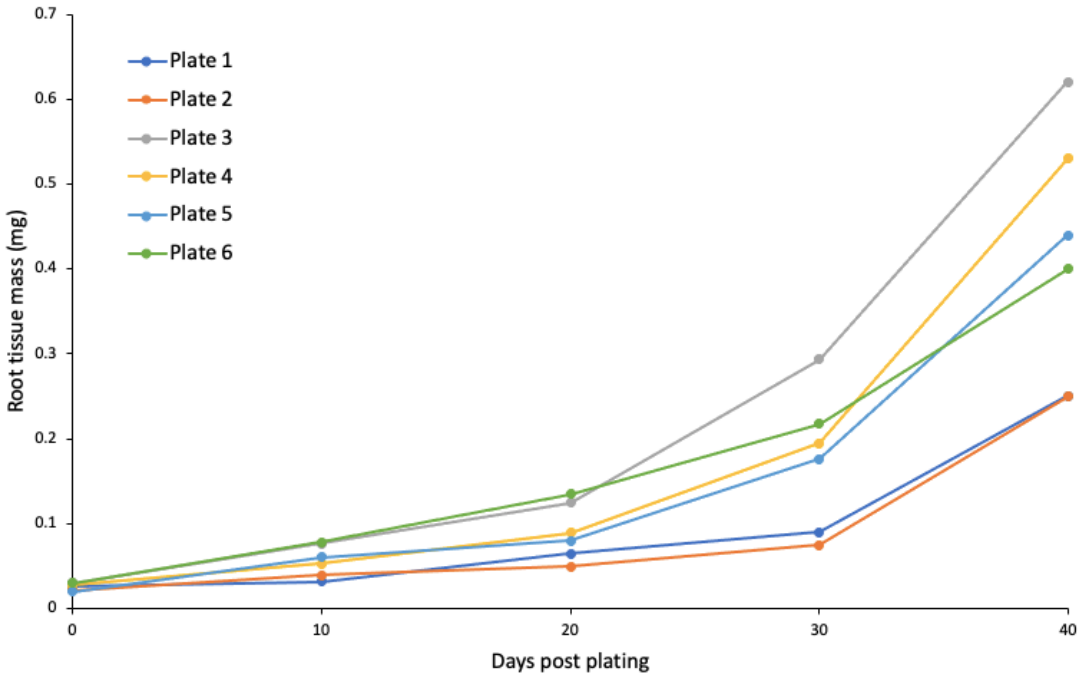

Figure 5. Panel A: *M. truncatula* ROC 61 weeks post transformation. Panel B: Growth rate of *M. truncatula* ROCs accumulating recombinant GFP from 0-40 days post plating. In this assay, ROCs were removed from MRC media, weighed, and placed on to fresh media at each time point.

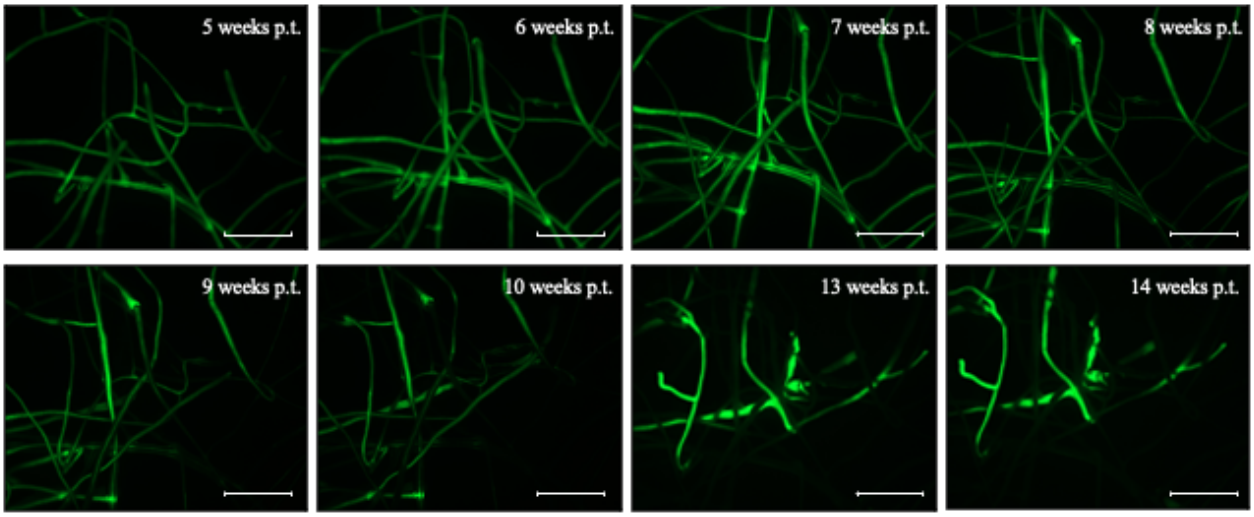

Figure 6. A plate of *M. truncatula* roots exhibiting relative distribution of GFP fluorescence over time. Three weeks after radicle transformation, the shoots were removed from seedlings and segments of transformed roots were placed upon solid growth media to generate ROCs. ROCs were stored at room temperature in the absence of light, and images were obtained at 5 to 14-weeks post-transformation (p.t.), as indicated. Images were taken of the plate positioned in the same way at each time point, to capture changes in relative location and intensity of GFP fluorescence over time. Scale bars on all microscopy images represent 4000um.

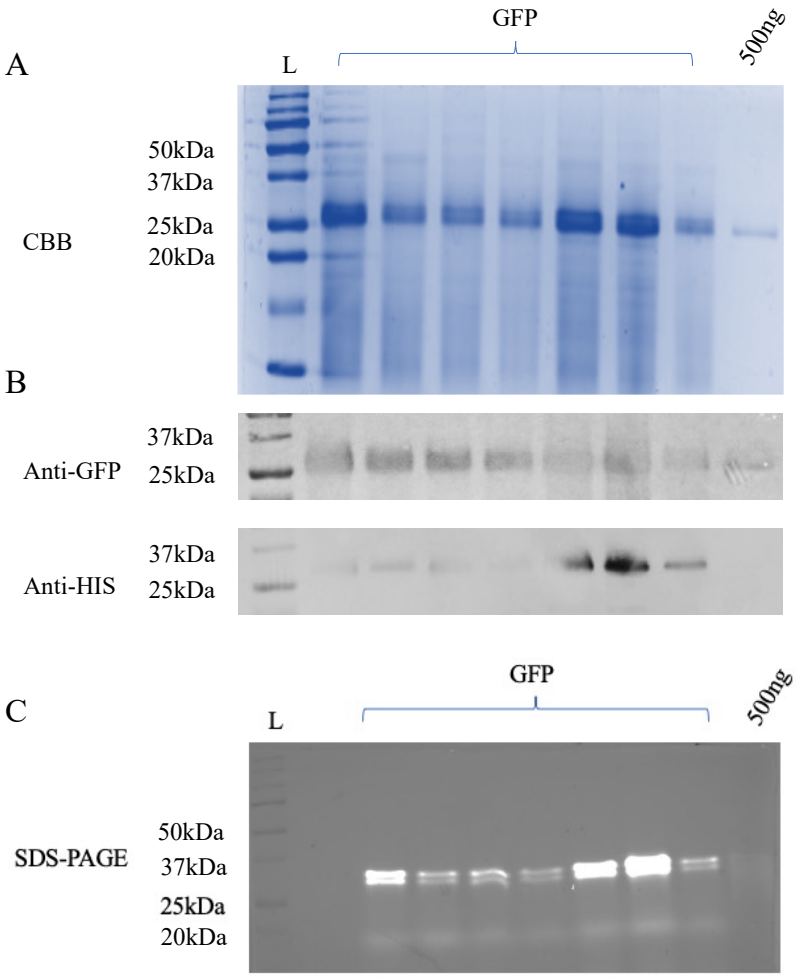

Figure 7. Recombinant GFP accumulation in ROCs. Tissue was collected from seven ROC lines 45 weeks post-transformation, each derived from an independent transformation event, prior to pooling for purification. Panel A: Samples were electrophoresed on 15% SDS-PAGE gels that were subsequently stained with CBB. Panel B: Recombinant 8xHIS tagged GFP (28kDa) was detected via Western blot using anti-GFP and anti-HIS antibodies. 500ng load of purified recombinant GFP was used as a positive control. Panel C: In gel fluorescence of recombinant GFP was imaged under UV light using the BioRad ChemiDoc Imaging System.

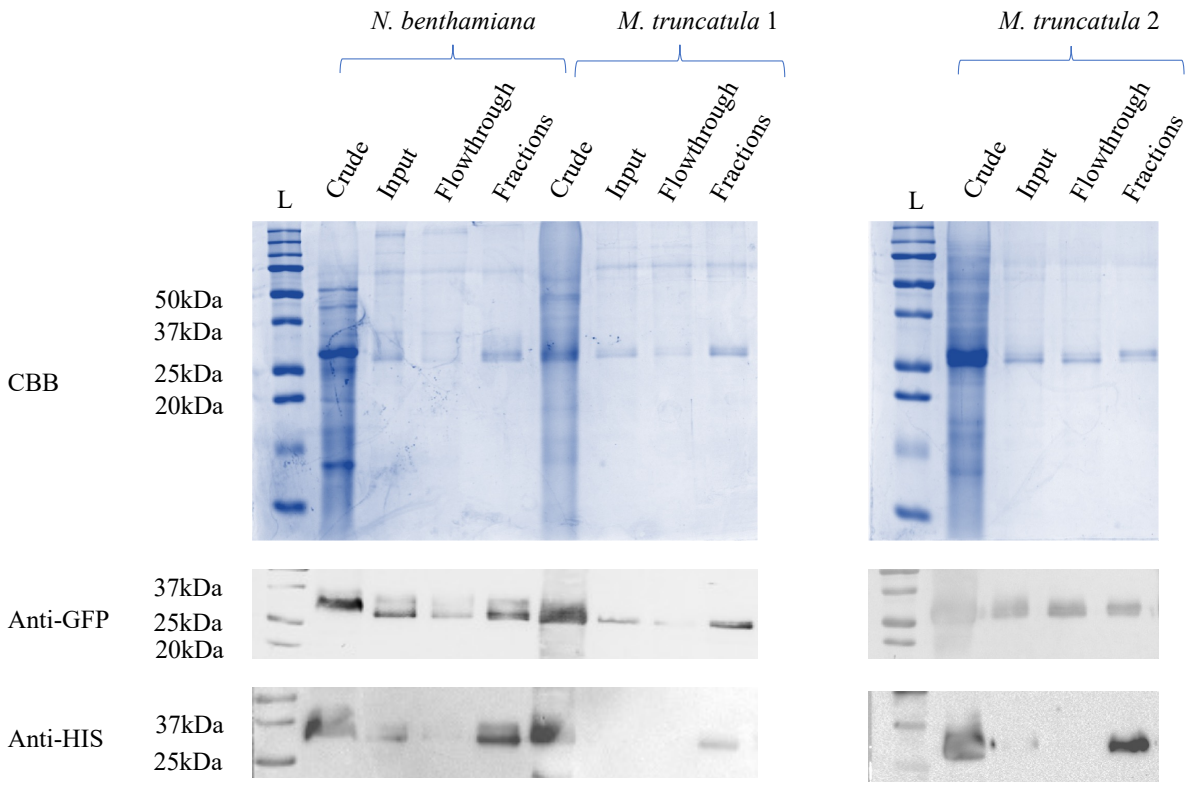

Figure 8. Purification of recombinant 8xHIS tagged GFP from *M. truncatula* ROC tissue. Purification was conducted using the AKTA Pure Protein Purification System with a HisPur Ni-NTA resin column. Purification from *N. benthamiana* leaves resulted in a yield of 450 mg/kg and purification from *M. truncatula* roots resulted in yields of 194 mg/kg (*M. truncatula* 1), and 780 mg/kg (*M. truncatula* 2). Samples were run on 15% SDS-PAGE gels and stained with CBB. Recombinant 8xHIS tagged GFP (28kDa) was detected via Western blot using anti-HIS or anti-GFP antibodies.

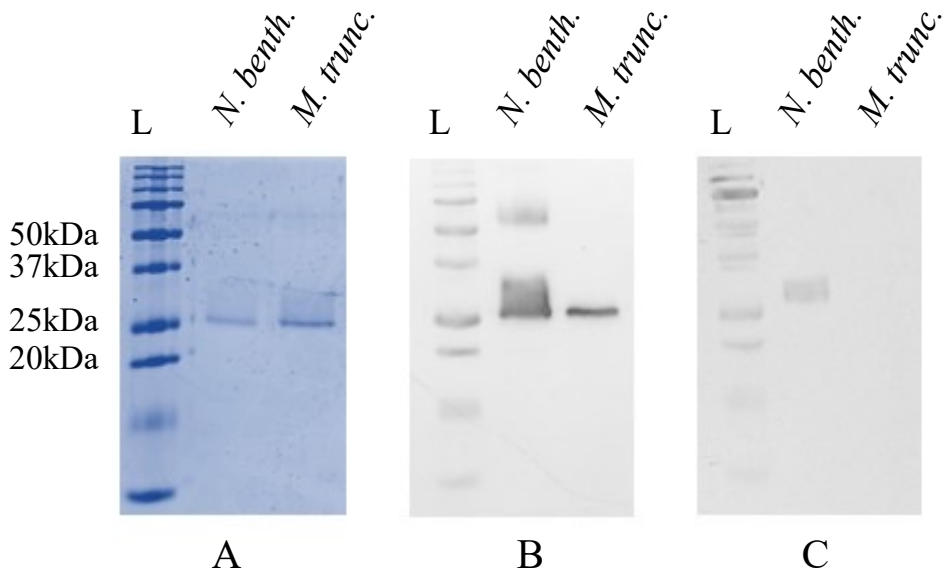

Figure 9. HIS tag loss in recombinant GFP purified from *M. truncatula* ROC tissue. 1ug of purified recombinant GFP was loaded. Panel A: Samples were run on 15% SDS-PAGE gels and stained with CBB. Recombinant 8xHIS tagged GFP (28kDa) was detected via Western blot using (Panel B) anti-GFP and (Panel C) anti-HIS antibodies.

CHAPTER 4: DISCUSSION

M. truncatula as a potential platform for the production of heterologous proteins

The purpose of this project was to assess the potential of *M. truncatula* as a platform for heterologous protein production. We achieved our objective by monitoring the accumulation of recombinant GFP in composite *M. truncatula* plants over time, by generating ROCs that accumulated recombinant GFP at high levels, and through the purification of GFP from both systems.

The accumulation of recombinant GFP was assessed in three different ways: Western blotting, CBB stained SDS-PAGE gels, and fluorescence microscopy. Western blotting with anti-GFP and anti-HIS antibodies was found to be a reliable method to detect the presence of recombinant GFP throughout the lifecycle of composite *M. truncatula* plants and in ROCs. The use of two different antibodies offered additional reliability in protein detection. In addition to detecting the protein of interest, the use of the anti-HIS antibody offered insight into the amount of recombinant GFP that was being produced with the 8xHIS tag attached, knowledge that would become relevant in later purification steps. The use of both antibodies should allow for the detection of truncated or degraded recombinant GFP, however the anti-GFP antibody offers the benefit of identifying recombinant GFP that may lack an 8xHIS tag. CBB stained SDS-PAGE gels served to visually assess the proportion of recombinant GFP present in each *M. truncatula* root sample relative to other native *M. truncatula* proteins, which was beneficial in addressing whether the relative accumulation of recombinant GFP in *M. truncatula* roots changed over time. CBB stained 15% SDS-PAGE gels were additionally employed as loading controls for Western blots. Fluorescence microscopy was used to visually monitor the accumulation of recombinant GFP in living, intact *M. truncatula* roots without the need to grind and process tissue samples.

The long-term accumulation of recombinant GFP in *M. truncatula* roots is important to assess for multiple reasons. Allowing composite *M. truncatula* plants to grow for a longer period of time prior to harvest allows more transgenic root tissue to be generated, theoretically resulting in increased protein yield. This should also mean more recombinant GFP can be purified from *M. truncatula* root tissue relative to the amount of time and labour required to generate said root tissue, i.e., a greater payoff for effort input as most of the labour associated with the creation of composite plants lies upfront in the transformation process. For reference, accumulation of heterologous proteins in *N. benthamiana* typically peaks within a week to ten-days, post-agroinfiltration. As discussed, prior, accumulation of heterologous proteins over an extended period in plants can be difficult to achieve due to the occurrence of gene silencing (El-Sappah et al., 2021). Therefore, accumulation of heterologous protein *in planta* over as long a period as described in this study is worthy of note. The lack of gene silencing is almost certainly due to the inclusion of the NSs silencing suppressor that is encoded in pHREAC (Peyret et al., 2019). However, our data also indicate that *M. truncatula* root systems can tolerate the expression and accumulation of high levels of heterologous protein (in the form of GFP) throughout the plant's entire life cycle. It is important to highlight that **Figure 4.** displays results from 30 unique transgenic *M. truncatula* root samples, each resulting from an independent transformation event. Therefore, accumulation is not tracked in the same composite *M. truncatula* plants over time, but rather was assessed by harvesting the entire root systems of unique composite plants at the 6 different time points. Fluorescence was first observed in *M. truncatula* roots at 3 weeks post-transformation, and accumulation was continuously detected in *M. truncatula* roots via CBB stained SDS-PAGE gels, Western blotting, and fluorescence microscopy at 5, 6, 7, 11, 13, and 15-weeks post-transformation. Throughout this experiment,

composite *M. truncatula* plants grew from seedlings with cotyledons to full size plants, flowered, and even produced seed pods, effectively completing an entire lifecycle. In addition to completing growth and reproductive cycles, composite *M. truncatula* plants continued to accumulate recombinant GFP with no apparent detriment to plant health and no obvious signs of reduced growth rate or seed set.

This time point harvest experiment was conducted not only to determine whether long-term accumulation of recombinant GFP occurs in *M. truncatula* roots, but also to assess whether the accumulation of recombinant GFP changed with time. This was relevant information to collect as a decrease in the accumulation of recombinant GFP over time would mean that at a certain point, the benefit associated with the additional transgenic root biomass generated during longer growth periods would be eclipsed by diminishing recombinant GFP levels.

eGFP accumulation was monitored in composite plants via the application of molecular techniques (SDS-PAGE, Western blotting) and microscopy (visualization of fluorescence). While all methods demonstrated that eGFP readily accumulates in *M. truncatula* roots, we observed that fluorescence noticeably decreased with plant age, a trend that was less obvious in CBB stained gels, and not evident in Western blots. One explanation for the discrepancy between the Western blots and the microscopy data could be the increased lignification of *M. truncatula* roots over time. Lignification describes the process by which the creation and accumulation of lignin occurs. A main component of the secondary plant cell wall, lignin is a biopolymer made up of monomers synthesised in the cytoplasm and deposited in the secondary cell wall of vascular plants, including in plant roots (Liu, Luo, & Zheng, 2018; Yadav & Chattopadhyay, 2023). These monomers are subsequently polymerized, creating lignin (Yadav & Chattopadhyay, 2023). Lignin provides structural support to vascular plants, offers protection

against biotic and abiotic stressors, and assists in the transport of water and minerals through the vascular bundles within plants (Liu et al., 2018; Yadav & Chattopadhyay, 2023). Notably, lignin is hydrophobic and helps to prevent water loss, a property that is particularly relevant in plant roots (Yadav & Chattopadhyay, 2023). Lignin deposition begins after the formation of the primary cell wall, and continues throughout plant growth and development, thus accumulating throughout the plant life cycle leading to increased amounts of lignin in mature plant roots (Liu et al., 2018).

Higher levels of lignin are expected to fortify *M. truncatula* roots as plant growth and development progress and have the potential to interfere with the detection of fluorescence in intact (i.e., non-dissected) roots. Lignin has a refractive index (RI) of 1.61, the highest of all plant tissues, (compared to air pockets at 1.00 and water at 1.31). Differences in RI throughout plant tissue can cause photons to deviate from their trajectory resulting in light scattering. Thicker tissue samples also increase light scattering. When photons deviate too far from their straight trajectory it can result in a loss of signal, resulting in a signal that is undetectable via fluorescence microscopy (Hériché et al., 2022). It is possible that increased lignin accumulation in *M. truncatula* roots at later stages in the *M. truncatula* lifecycle interferes with fluorescence detection by creating a large RI differential which causes photons to scatter. The thickening of roots with age may also contribute to this interference. These changes might mean that even if GFP accumulated to high levels inside roots, fluorescence may not be detected from intact root systems. Fluorescence microscopy alone is therefore not an accurate assessment of recombinant GFP accumulation levels in *M. truncatula* roots, especially at later stages in the *M. truncatula* lifecycle. Although lignification limits the ability to accurately screen *M. truncatula* roots for fluorescence as development progresses, fluorescence microscopy was effective enough at all

time points for a qualitative confirmation of the presence of fluorescence prior to harvest and is therefore still a highly useful non-destructive method of root screening.

Lignin confers structural integrity and protection to plant cells by enforcing the cell wall, however due to this reinforcement heterologous proteins may be effectively trapped within root tissue. Therefore, when considering an optimal harvest time point, lignin accumulation over time may be a more important limiting factor than any other age-related decline in protein accumulation. For example, it is possible that the fortification of *M. truncatula* roots with lignin interferes with the extraction of heterologous proteins and may be the reason we obtained an unexpectedly low yield of purified protein from composite plants. However, we consider it unlikely that lignin would have accumulated to high levels in 4-week-old plants. Purification from even younger, smaller plants is less desirable due to the number of individual plants that would be required to amass a substantial amount of root tissue. Modifications to the extraction protocol such as the inclusion of ligninolytic enzymes should be explored, and further optimization for the purification of protein from *M. truncatula* roots is needed.

A second explanation for the conflicting results depicted in **Figure 4.** pertaining to recombinant GFP accumulation over time may be due to the presence of increased amounts of non-transformed root tissue as development progresses. At each time point throughout the experiment, the entire root system of each composite *M. truncatula* plant was collected. During the generation of hairy root systems, it is rare for the entire root system to uniformly express the genes of interest. Non-transformed adventitious roots can emerge from above the point of transformation, resulting in the growth of non-transformed tissue. It is also possible that not all root tissue that develops from the transformed tip of the seedling radicle expresses the genes of interest encoded by the transferred T-DNA. This can result in sections of the composite plant

root system being phenotypically 'non-transformed', and in this case, contributing to root biomass that does not accumulate recombinant GFP. *M. truncatula* roots were not pre-screened for fluorescence using fluorescence microscopy prior to harvest, meaning that sections of the composite plant root system that were non-transformed, and/or were not accumulating recombinant GFP, were collected along with transformed sections of the root system. This was done intentionally, as our goal was to evaluate *M. truncatula* as a potential expression platform of therapeutic proteins such as antibodies, enzymes, and recombinant proteins for subunit vaccines. Such molecules are not fluorescent and cannot be tracked visually as easily as GFP. However, as a result, it is possible that transformed tissue was increasingly diluted by the growth of non-transformed tissue over time, resulting in seemingly diminishing levels of relative recombinant GFP accumulation, despite the capacity of the transformed sections of the root system to accumulate recombinant GFP remaining unchanged. The collection of only transformed tissues (confirmed via pre-screening with fluorescence microscopy) is an option to provide a more accurate assessment of recombinant GFP accumulation in transformed root systems over time.

Purification of recombinant GFP from *M. truncatula* ROCs resulted in yields comparable to those achieved when recombinant GFP was purified from *N. benthamiana*. This exciting result speaks to the promise of *M. truncatula* ROCs as a system to produce heterologous proteins. We speculate that the humid and sterile growing environment of ROCs (maintained in sealed, sterile Petri plates) may have led to reduced lignification of ROC tissues. If this is the case, it might have facilitated the extraction of recombinant GFP from *M. truncatula* root tissue. Optimization of the extraction and purification protocols for *M. truncatula* roots may further increase purification yields.

The ability to selectively propagate ROCs with high levels of protein accumulation, and lower levels of lignin accumulation in *M. truncatula* ROCs compared to composite *M. truncatula* plant root systems are benefits specific to ROCs. When considering the advantage of tracking fluorescence and the fact that most proteins of therapeutic interest are not fluorescent, it may be beneficial to employ an expression vector that encodes GFP, DsRed, or RUBY that would allow for visual tracking of transformed tissues in future experiments with non-fluorescent proteins. 8xHIS tag loss after purification from *M. truncatula* roots is a potential cause for concern that must be further investigated.

All benefits observed in composite *M. truncatula* plants, including long-term accumulation and high relative levels of recombinant GFP, were also observed in ROCs. One of the most valuable benefits to be gleaned from the use of ROCs in this experiment was the possibility to selectively propagate transformed tissue that expresses high levels of our protein of interest, using fluorescence microscopy. The benefit of this becomes apparent in consideration of **Figure 7.**, where the band of recombinant GFP at 28kDa displays a significantly greater intensity relative to banding that is attributable to endogenous *M. truncatula* proteins. **Figure 7.** can also be compared to the CBB stained SDS-PAGE gels presented in **Figure 4**. Note the increase in relative intensity of the recombinant GFP band in **Figure 7**. Whereas **Figure 4.** displays root tissue samples taken from the entire root system of composite plants, including non-transformed tissue, **Figure 7.** displays samples taken from ROC lines that were subcultured to propagate lines that exhibited the strongest levels of fluorescence observed. While it would be feasible to collect only the most fluorescent regions of composite plant roots via screening with fluorescence microscopy, cultivation of ROCs is more efficient (and less tedious) as large amounts of transgenic tissue do not need to be separated from an entangled root system.

ROCs are generally considered to be immortalised tissue lines and are expected to continue to grow and accumulate heterologous protein long past the natural life cycle of *M. truncatula* plants. Recombinant GFP was detected via fluorescence microscopy up to 57 weeks post-transformation, at which point experimentation for this project was stopped. Not only does this immortalization allow for the long-term preservation of ROC lines that accumulate heterologous proteins of interest, it also means that the amount of transgenic tissue generated from a single transformation event is potentially unlimited. This significantly reduces the time and labour dedicated to the actual transformation step of creating ROCs. For these reasons, we conclude that ROCs offer a greater potential as a platform for the production of recombinant proteins, compared to composite plants. Nevertheless, it is worth considering that composite *M. truncatula* plants offer the benefits of non-sterile growing conditions and may be a more suitable platform in certain instances, depending upon user needs and resources available.

M. truncatula **compared to industry heavyweight,** ***N. benthamiana***

A full analysis of *M. truncatula* as a potential system for the production of heterologous proteins must include comparison to the current standard in plant molecular farming, *N. benthamiana* leaves. *N. benthamiana* quickly emerged as the system of choice for the production of heterologous proteins in plants given its elevated susceptibility to pathogens which makes it highly amenable to transformation, high product yields upon purification, and short production timeline (Leblanc et al., 2021). Additionally, agroinfiltration, the method by which *N. benthamiana* leaves are transformed, is relatively conducive to automation, granting industry appeal to *N. benthamiana*. Many biotech companies including Kentucky BioProcessing, PlantForm, CapeBio, and in Canada, the former Medicago Inc., adopted *N. benthamiana* as their

primary plant host for the production of heterologous proteins and products (Leblanc et al., 2021).

The levels of recombinant GFP accumulation assessed in both composite plants and ROCs, as well as the long-term sustained accumulation observed in both root systems, speaks to the promise of *M. truncatula* roots as a biopharming system. Our observations suggest that heterologous protein production in the roots of composite plants is efficient, given the high proportion of heterologous protein produced relative to endogenous proteins. Long-term, sustainable expression also enables the proliferation of more root biomass per transformation event, maximising the return on labour input. However, if heterologous protein cannot be easily extracted from composite plants, this system will have limited appeal compared to *N. benthamiana*.

ROCs on the other hand resulted in high purification yields that were comparable to those of *N. benthamiana*. The ability to screen for GFP fluorescence enabled the selective propagation of root lines with very high levels of recombinant GFP, which again speaks to the efficiency of *M. truncatula* roots as a system for heterologous protein synthesis. Sustained accumulation of recombinant GFP in ROCs up to 57 weeks post-transformation is an exciting demonstration of transient expression that is nonetheless stable, and in addition to being novel, is promising in that it allows for the preservation of ROC lines that accumulate proteins of interest. This implies that a single successful transformation event can yield transgenic root tissue that can be propagated over many months, eliminating the need for repeated transformation steps when additional transgenic biomass is required. There is excellent potential for the adoption of ROCs in research settings where the development of multiple ROC lines of interest could feasibly support the creation of a transgenic root tissue bank from which samples can be obtained, as needed. Again,

ROCs require minimal care on a week-to-week basis, take up little space, and their only requirements for growth are a low-cost sucrose-based media, and room temperature conditions. However, it must be noted that ROCs require maintenance under sterile conditions for optimal results.

Future directions

Rhizosecretion of proteins of interest, including heterologous proteins, from transgenic roots into liquid media can be achieved by using a signal peptide to direct the secretion of proteins to the plant apoplast. In *M. truncatula*, an MtBCP1 signal peptide is likely to be suitable (Ivanov & Harrison, 2014). The benefits of rhizosecretion as a method for heterologous protein collection include the ability to sustainably collect heterologous protein throughout the plant life cycle, and the elimination of the need for extraction of proteins from plant tissue. As discussed, accumulation of recombinant GFP was detected at multiple time points between 3 weeks post-transformation and 15 weeks post-transformation in composite *M. truncatula* plants, offering a large time frame during which heterologous protein could feasibly be collected. Secretion of heterologous protein from *M. truncatula* roots would eliminate the concerns associated with the accumulation of lignin over time, thus making composite plants a viable system for production of heterologous proteins. This would be greatly beneficial, as using composite plants would circumvent the need for the sterile growth conditions required by ROCs. Rhizosecretion would also be possible from ROCs, but it may be challenging to maintain the necessary level of sterility required for the collection of protein over an extended period.

Lacking shoots and leaves, ROCs are no longer autotrophic and require an external carbon source. This is primarily what drives the requirement for sterility, as the inclusion of sucrose in the MRC medium makes it an excellent environment to promote bacterial growth.

The secretion of proteins into a liquid media containing sucrose would require a sterile environment that may limit the widespread adoption and scaling up of this system. It may be feasible to collect protein from secreting ROCs by immersing these tissues in a solution that lacks sucrose, but eventually ROCs will require a carbon source to continue protein production. Adding antibiotics to liquid media may also help mitigate contamination.

Accordingly, composite *M. truncatula* plants may be best suited for the collection of heterologous proteins via rhizosecretion. If successfully implemented, this method of protein production and collection would likely be the most amenable to industry. The secretion of protein into liquid media opens the possibility for protein purification to be automated, whereas the elimination of a need for sterile cultivation renders the process cost effective, and the ability to collect protein throughout the entire lifecycle of *M. truncatula* would likely constitute an efficient use of biomass. Rhizosecretion also allows for non-destructive measurements of protein accumulation over time via Bradford assay, or in the case of recombinant GFP, a fluorometric assay.

Concluding remarks

We have demonstrated that stable, high-level accumulation of recombinant GFP in composite *M. truncatula* plants is readily achievable throughout their life cycle. However, purification of heterologous proteins from composite plants indicates a need for improved yield. The most promising results came from *M. truncatula* ROCs, which achieved protein purification yields comparable to industry-standard *N. benthamiana*. Notably, these yields were obtained from transgenic root tissue 55 weeks post-transformation, highlighting the potential for long-term cultivation and storage of elite ROCs in tissue culture banks. Based on these findings, *M.*

truncatula, particularly in the form of ROCs, shows strong potential as a biopharming platform for producing heterologous proteins and warrants further evaluation.

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APPENDIX

Supplementary Table 1. Fahraeus media.

Chemicals or Stock	**Per Litre of Fahraeus media**
$CaCl_2$ (0.9M)	1ml
$MgSO_4$ (0.5M)	1ml
Ferric citrate (20mM)	1ml
NH_4NO_3 (0.5M)	2ml
KH_2PO_4 (0.35M)	58ul
Na_2HPO_4 (0.2M)	50ul
H_3BO_3 (1mg/ml)	100ul
Micronutrients stock	33ul
Na_2MoO_4 (1mg/ml)	33ul
MES	0.218g
pH	7.4
Gelrite	2.5g

Supplementary Table 2. MRC media.

Chemicals or Stock	**Per Litre of MRC**
N6 Major Salts	50.0ml
SH Minor Salts	0.5ml
SH Vitamins	0.5ml
EDFS Stock	10.0ml
Myo-Inositol	50.0mg
Sucrose	10.0g
pH	5.8
Kalys Agar	5.0g

N6 Major Salts

Chemical	Per Litre
$MgSO_4x7H_2O$	1.85g
KNO_3	1.415g
$(NH4)_2SO_4$	4.63g
$CaCl_2x2H_2O$	1.66g
KH_2PO_4	0.1g

SH Minor Salts

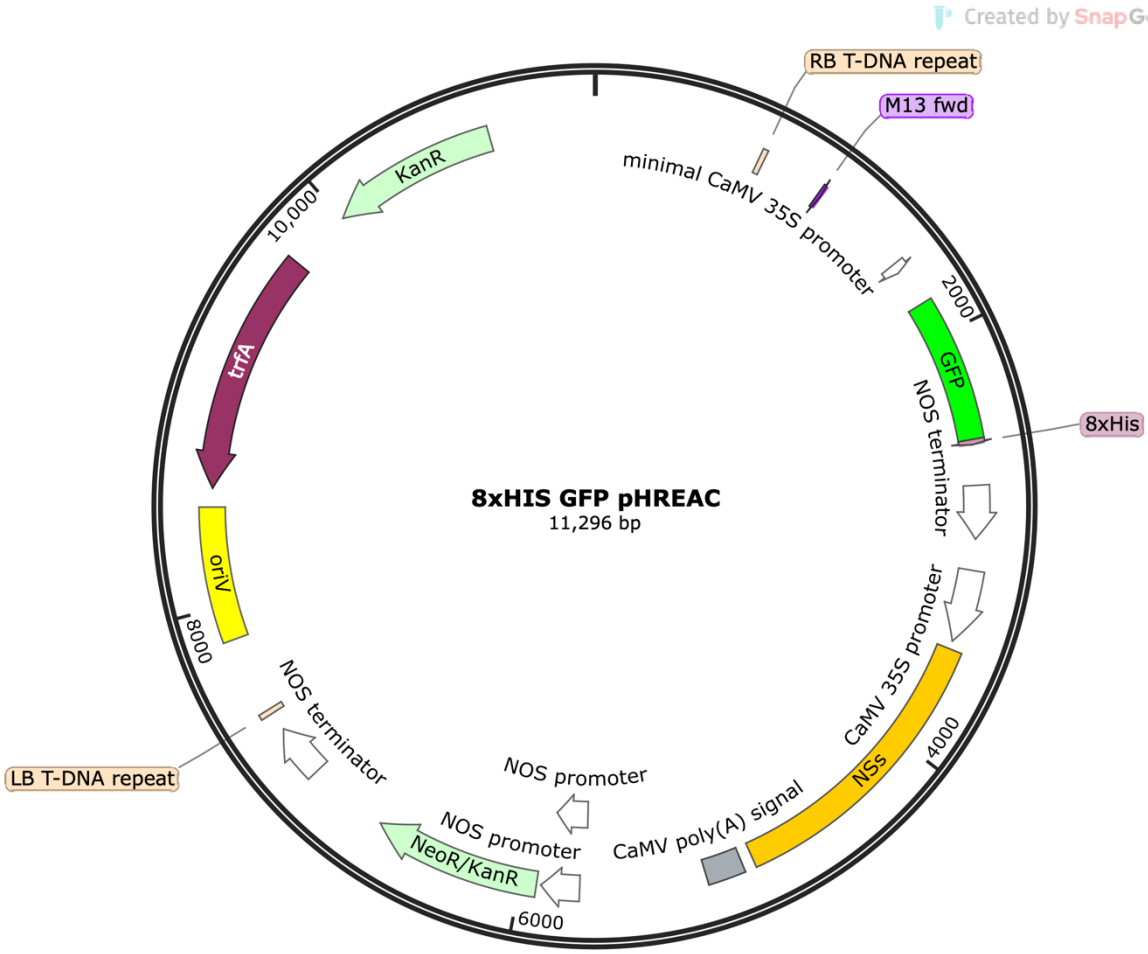
Chemical	Per 100ml
$MnSO_4xH_2O$	1.0g
H_3BO_3	500mg
$ZnSO_4$ x $7H_2O$	100mg
KI	100mg
$Na_2MoO_4x2H_2O$	10mg
$CuSO_4$	12.8mg
$CoCl_2x6H_2O$	10.0mg

SH Vitamins

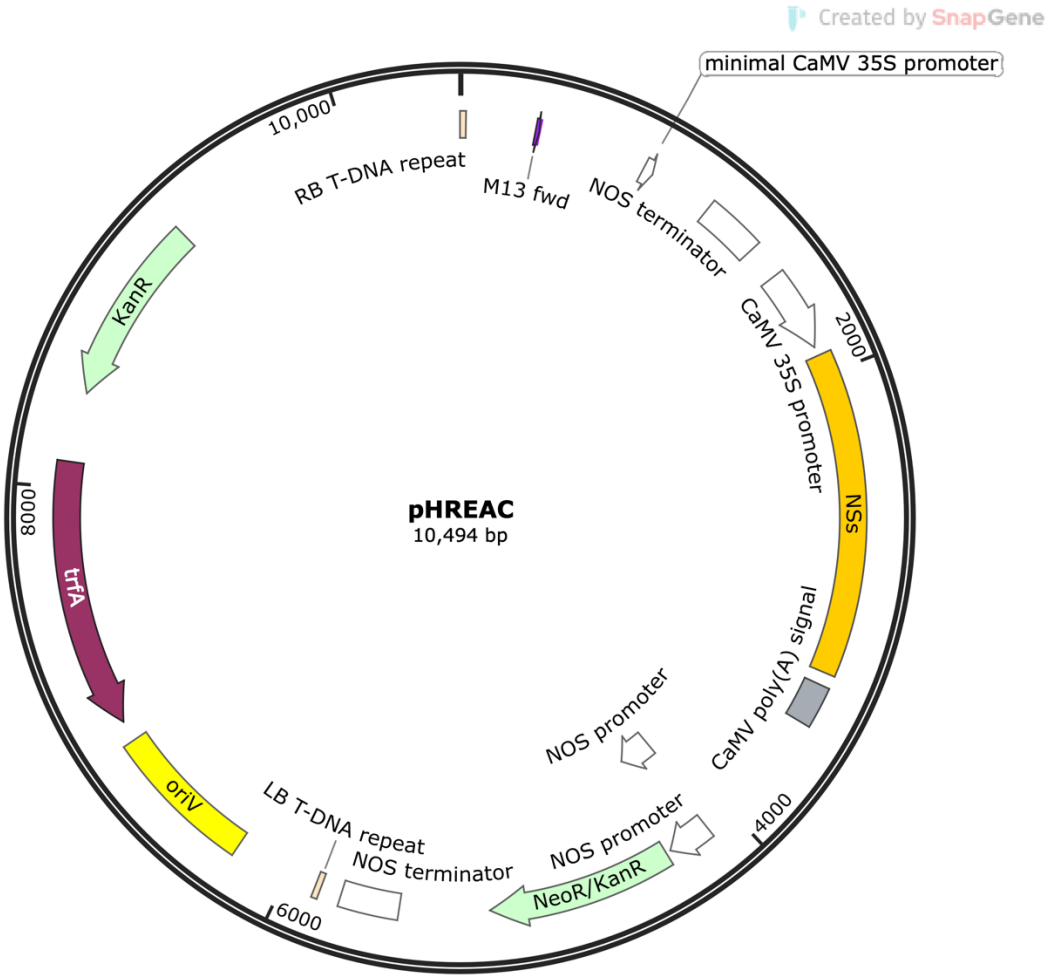
Chemical	Per 100ml
Nicotinic acid	500mg
Thiamine HCl	500mg
Pyridoxine HCl	500mg

Stock Solutions

EDFS (Ferric-EDTA) 697.4mg per 100ml



Supplementary Figure 1. Map of pHREAC plasmid encoding 8xHIS GFP. Created with SnapGene.



Supplementary Figure 2. Map of empty vector pHREAC plasmid (Addgene Plasmid #134908). Created with SnapGene.