Identification of Novel Proteins Involved in the Arbuscular Mycorrhizal Symbiosis

Bridget Price-Roberts

Thesis submitted to the University of Ottawa

in partial Fulfilment of the requirements for the

Masters of Science in Biology

Department of Biology

Faculty of Science

University of Ottawa

Abstract

Arbuscular mycorrhizal fungi (AMF) form a mutually beneficial symbiotic relationship with a majority of land plants through an exchange of nutrients. Despite the importance of AM symbiosis in agricultural and ecological settings, relatively little is known about how the fungal symbiont actively promotes symbiosis. To overcome a host's immune response, plant pathogens secrete effector proteins that modify a host to suppress an immune response. Few effectors have been identified in AMF, as bioinformatics methods have failed to accurately predict their sequences. To successfully colonize a plant, AMF form structures called arbuscules within plant root cortical cells. Arbuscules are a primary site of nutrient exchange during AMF symbiosis. This work is built on the hypothesis that AMF produce effector proteins to promote symbiosis, and that arbuscules are a site of effector secretion. Using *Rhizophagus irregularis, Glomus versiforme* and *Medicago truncatula* this work applies a proteomics-based approach using a new biotechnology to identify fungal proteins secreted by AMF. This novel approach using proteomics and proximity labelling to identify proteins by mass spectrometry is the first time this system has been used to study the plant-AMF relationship. In this work, mass spectrometry identifies a total of 24 *R. irregularis* proteins and two *G. versiforme* proteins that are candidate effectors involved in the plant-AMF symbiosis.

Résumé

Les champignons mycorhiziens arbusculaires (CMA) forment une relation symbiotique mutuellement bénéfique avec une majorité de plantes terrestres grâce à un échange de nutriments. Malgré l'importance de la symbiose AM dans les milieux agricoles et écologiques, on sait relativement peu de choses sur la façon dont le symbiote fongique favorise activement la symbiose. Pour surmonter la réponse immunitaire d'un hôte, les agents pathogènes des plantes sécrètent des protéines effectrices qui modifient l'hôte pour supprimer la réponse immunitaire. Peu d'effecteurs ont été identifiés chez les CMA, car les méthodes bioinformatiques n'ont pas permis de prédire avec précision leurs séquences. Pour réussir à coloniser une plante, les CMA forment des structures appelées arbuscules à l'intérieur des cellules corticales des racines. Les arbuscules sont un site primaire d'échange de nutriments au cours de la symbiose des CMA. Ce projet est basé sur l'hypothèse que les CMA produisent des protéines effectrices pour promouvoir la symbiose, et que les arbuscules sont un site de sécrétion des effecteurs. En utilisant *Rhizophagus irregularis*, *Glomus versiforme* et *Medicago truncatula*, nous appliquons une approche protéomique utilisant une nouvelle biotechnologie pour identifier les protéines fongiques sécrétées par les CMA. Dane ce project, la spectrométrie de masse identifie un total de 24 protéines R. irregularis et deux protéines G. versiforme qui sont des effecteurs candidats impliqués dans la symbiose plante-CMA.

Acknowledgements

Foremost, I would like to thank my supervisor, Dr. Allyson MacLean for her guidance, patience and immense knowledge shared with me over the past three years. And most importantly, for buying a brand-new set of pipettes when I started working in the lab, I would not have been able to accomplish this work without them.

I wish to show my deep gratitude to Dr. Glen Uhrig for performing the mass spectrometry work and troubleshooting its use in these experiments.

I would also like to thank my committee members, Dr. Marina Cvetkovska and Dr. Owen Rowland for their advice throughout this degree.

A huge thank you to all past and present members of the MacLean lab for creating a welcoming and friendly environment. Thank you to Mina Nasr, for taking care of the lab, and helping with my experiments. A huge thank you to Dane Goh for endless hours of entertainment while working in the lab, it's been a gas. I very much appreciate Dr. Maryam Nourimand and Dr. Mina Ghahremani for their helpful advice. Thank you to Dominique Daniels, Mairead Green and Monique Power for your assistance in the lab, pushing through to wash every last dish and not rolling your eyes when I added to the pile. Thank you to Daniel Gariano and Téa Backlund for caring for my plants when I was unable to.

I would also like to thank my former advisor, Dr. Rees Kassen for introducing me to microbial research and giving an inexperienced student their first shot in science. I am extremely grateful to my mentor and close friend, Dr. Sonal Shewaramani – thank you for always being there through the ups and downs and training me in the lab. Thank you to all members of the Kassen lab for their friendship, I am especially grateful to Noah Houpt for his valuable comments on this thesis.

I want to acknowledge the encouragement and support from my parents, Suzanne Price and Nigel Roberts. Without my unique upbringing, I wouldn't be where I am today. Many thanks to my siblings and friends for their emotional support and comradery. A special thanks to Haytham Saleh for his motivation and confidence in me throughout this degree.

I would like to acknowledge and thank Henrietta Lacks; her contribution has been instrumental to this work and scientific research globally.

During my research, I received financial support through the University of Ottawa and NSERC.

Unless otherwise noted, all figures here were created with BioRender.com.

Table of Contents

List of Figures

List of Tables

Abbreviations

AM – Arbuscular mycorrhizal

AMF – Arbuscular mycorrhizal fungi

CRN - Crinkler

CSSP – Common symbiosis signalling pathway

DMSO – Dimethyl sulfoxide

FDR – False discovery rate

GFP – Green flourescent protein

LB – Lysogeny broth

LCOs – Lipooligosaccharides

MAMPs – Microbe-associated molecular patterns

Myc – Mycorhizzal

NCBI – National Centre for Biotechnology Information

NES – Nuclear exclusion signal

NLS – Nuclear localization signal

Nod – Nodulation

PAMPs – Pathogen-associated molecular patterns

PSM – Peptide spectrum matches

YFP – Yellow flourescent protein

Chapter 1: Overview of Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) are a phylum of fungi capable of forming beneficial symbiotic relationships with 80 % of vascular plants (B. Wang & Qiu, 2006). Symbiotic structures from these ancient microorganisms have been identified in fossils dating back 450 million years (Remy et al., 1994). It is hypothesized that AMF helped plants transition from aquatic environments to land (Rich et al., 2021) by helping anchor the plants in soil and providing nutrients that plants were otherwise unable to access. The broad range of plants that AMF colonize, in combination with the significant nutrients with which AMF supply plants, makes the plant-AMF symbiosis a globally significant beneficial interaction (Harrison, 2005).

Host plants engage in a beneficial symbiosis with AMF when in nutrient-limiting conditions. AMF provide nutrients from the surrounding environment, in particular phosphorus and nitrogen (Javot, Pumplin, et al., 2007) that host plants are otherwise unable to access and are limiting to plant growth. In exchange, host plants supply AMF with carbon in the form of sugar (Doidy et al., 2012) and lipids (Bravo et al., 2017; Luginbuehl et al., 2017; Rich et al., 2021). When plants are engaged in symbiosis with AMF, up to 20 % of a host's fixed carbon is transferred to AMF (Bago et al., 2000). Given that plants sacrifice such a substantial proportion of their energy to AMF under certain conditions, this interaction plays an important role in the ecosystems in which it occurs. In addition to providing host plants with essential nutrients, AMF increase water uptake, drought and salinity tolerance and disease resistance (Augé et al., 2015; Pozo & Azcón-Aguilar, 2007; Smith & Read, 2008).

I. Colonization by Arbuscular Mycorrhiza Fungi

Host plants deprived of phosphorous secrete chemical signals into the rhizosphere in the form of strigolactones (Besserer et al., 2006). AMF spores exist in the soil, which grow hyphae in search of an appropriate plant host (Giovannetti et al., 2010). Upon recognition of strigolactones, AMF spores use up their limited carbon reserve and their energy comes from the breakdown of triacylglycerol (Bago et al., 2000). At this stage AMF spores commit themselves to symbiosis through the activation of oxidative metabolism (Besserer et al., 2008). AMF hyphae must successfully locate a host, establish symbiosis, and receive carbon from a host plant before using up its carbon reserve; these fungi are obligate symbionts and require carbon from a host plant to survive and complete their life cycle. If a spore does not reach a plant root and has not yet committed itself to the symbiosis, it will return to its dormant state (Bago et al., 2000).

AMF secrete their own signalling molecules; specifically, mycorrhizal (myc) factors that trigger the initiation of the plant's common symbiosis signalling pathway (CSSP) (J. Sun et al., 2015). The CSSP is a genetic pathway required for the formation of a successful symbiosis with both AMF and rhizobia (Oldroyd, 2013). Rhizobia are nitrogen-fixing bacteria that form a beneficial symbiosis with leguminous plants. Genes involved in the plant-rhizobia symbiosis have been studied extensively and many of the genes essential to this interaction are also required in the AMF symbiosis; together these fundamental genes form the CSSP (Oldroyd, 2013; Parniske, 2008). To initiate symbiosis, a host plant must recognize a beneficial symbiont, through either myc factors from AMF or nodulation (nod) factors from rhizobia. Myc factors were discovered on the assumption that they act similarly to nod factors. There is speculation that other myc factors may be involved in the symbiosis (MacLean et al., 2017). Two important myc factors are lipochitooligosaccharides (LCOs) and chitin, which promote root growth and branching (Maillet et al., 2011). Myc factors are perceived by a variety of cell receptors, one being CERK1, a lysin motif (LysM) pattern recognition receptor that recognizes chitin and elicits an immune response, while simultaneously activating the CSSP, enabling the symbiosis (Feng et al., 2019; Miyata et al., 2014). Other cell receptors important in the perception of myc factors are DMI1, DMI2, and DMI3 (Genre et al., 2013). Together, these cause nuclear calcium spiking which is an essential component of the CSSP as it activates transcription factors that signal genes involved in the symbiosis (Oldroyd & Downie, 2006).

Once fungal hyphae are in contact with host plant roots, AMF form a hyphopodium along the host's root epidermal cells. The host plant in turn forms a pre-penetration apparatus that guides fungal growth and reorganizes its plant cells to accommodate the fungal symbiont (Genre et al., 2005). Morphological changes occur inside the plant cell, including migration of the nucleus, and the reorganization of cytoskeletal elements and endoplasmic reticulum components (Genre et al., 2008, 2005). Once inside the innermost layer of cells, the cortical cells, AMF form branched structures called arbuscules, which are the site of nutrient exchange and are critical to the symbiosis (Luginbuehl & Oldroyd, 2017). Arbuscules are surrounded by a plant derived membrane called the periarbuscular membrane that contains transporters that act to transfer nutrients between symbionts (Breuillin-Sessoms et al., 2015; Javot, Pumplin, et al., 2007; Krajinski et al., 2014). The establishment of arbuscules in the plant cell creates a gap between the plant and fungal walls called the periarbuscular space, an acidic environment where nutrients must cross to be exchanged between the plant and fungus (Javot, Penmetsa, et al., 2007). Arbuscules survive for seven to ten days after which they collapse and degrade. The cortical cell then returns to its pre-colonization state (Pumplin & Harrison, 2009) or it may be re-colonized anew. A schematic of this colonization process is depicted in Figure 1.

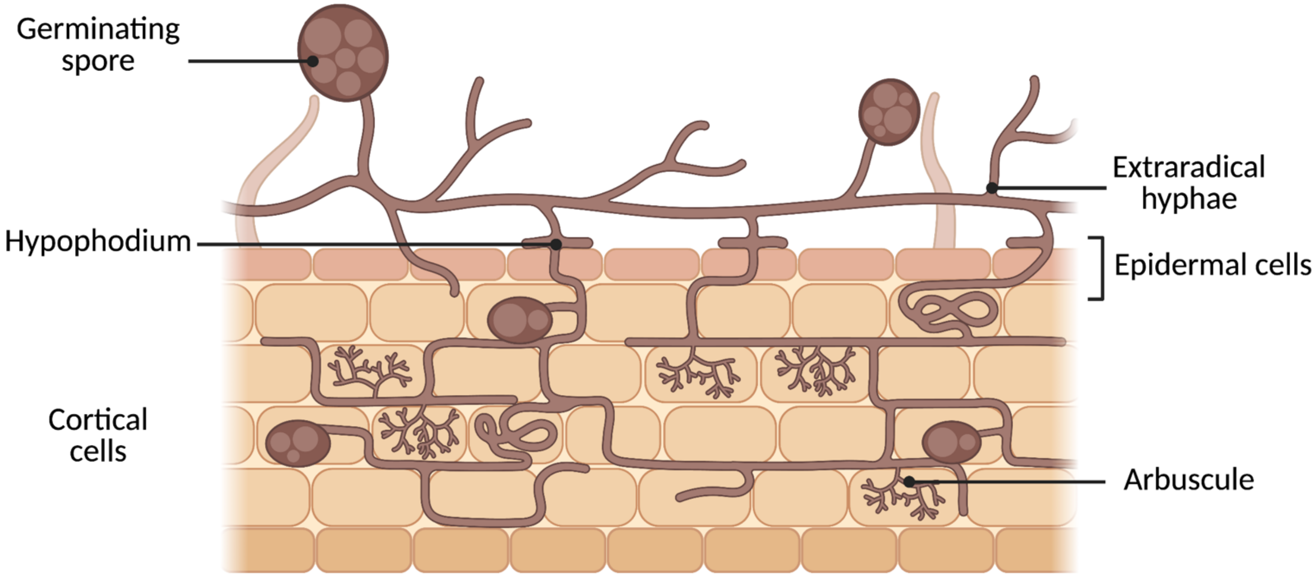


Figure 1. Colonization of a plant root by arbuscular mycorrhizal fungi. Arbuscular mycorrhizal fungal spores exist in the soil. Emerging hyphae from germinating spores migrate towards a host plant root, guided by chemical signals that are diffused by host plant roots when deprived of phosphorus. Once in contact with a host plant root, fungal hyphae penetrate through the outermost layer of epidermal cells, growing through the cell layers until reaching the cortical cells, where they form arbuscules. Arbuscules are the primary site of nutrient exchange between the two organisms.

II. Nutrient Exchange in Arbuscular Mycorrhizal Fungi

Both nitrogen and phosphorous are often limiting to plant growth. While phosphorus is one of the most important nutrients that AMF provides plants, AMF and plants only absorb soluble inorganic orthophosphate (Bucher, 2007; Koide & Kabir, 2000; Schachtman et al., 1998). Though phosphorus exists in the ecosystem in many forms, most phosphorus exists in an insoluble form due to immobilization and precipitation with other soil minerals (Rengel & Marschner, 2005). AMF can increase phosphorus supply to the host plant through two mechanisms. First, they can expand further into the soil through extraradical hyphae and access nutrients that plants are unable to. Second, AMF can form a beneficial association with phosphatase secreting bacteria (X. X. Wang et al., 2017), allowing them to access a soluble form of phosphorous (Ezawa & Saito, 2018; Sato et al., 2019).

To support maximal growth of crops, farmers frequently add commercial fertilizers to nutrient-depleted soils, which includes both supplemental phosphorus and nitrogen. Only a small portion of the added nutrients are absorbed by the crops. Most of the nutrients from the fertilizer runoff into the surrounding environment (Beman et al., 2005) or become insoluble forms that are unusable to plants (Vance et al., 2003). Leading to negative effects on the ecosystem such as eutrophication. Eutrophication is a process wherein an imbalance of nutrients in aquatic ecosystems leads to a 'bloom' of cyanobacteria. These cyanobacteria secrete hazardous compounds and decrease dissolved oxygen concentrations in their environment, causing the death of numerous species of wildlife. To solve this issue, biofertilizers comprised of AMF and/or rhizobia are currently being studied (Haldar & Sengupta, 2016). AMF can help plants uptake the nutrients that already exist in an environment, providing a more sustainable alternative to commercial fertilizers. This work is a key step to better understand how the plant-AMF symbiosis is maintained and may allow for the replacement of chemical fertilizers with AMF in agriculture.

III. Effectors

Host plants regulate the extent of AMF colonization to ensure reciprocal nutrient exchange with their symbionts (C. Wang et al., 2018). Plants not only manage beneficial microbes, but they also must defend themselves against pathogenic ones. To distinguish between these two groups and respond accordingly, plants recognize microbe associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs), which activates MAMP-triggered immunity or PAMP-triggered immunity, respectively. Plants recognize both MAMPs and PAMPs using pattern recognition receptors that respond to the cues elicited by non-self-organisms. When activated, pattern recognition receptors initiate intracellular signaling cascades to respond to invaders. Some of these responses include the expression of defence-related genes, secretion of chitinases, and generation of reactive oxygen species (Bozsoki et al., 2017; Miya et al., 2007; Shimizu et al., 2010). To evade these immune responses, symbionts have evolved effectors (note that the term symbiont refers to both pathogenic and beneficial plant-microbe associations). Effector proteins act to promote colonization and subsequent dispersal of the symbiont by suppressing host immunity, and manipulating host cell physiology (De Jonge et al., 2011; De Wit et al., 2009; Giraldo & Valent, 2013; Ökmen & Doehlemann, 2014; Zuccaro et al., 2014).

Effector proteins employed by plant pathogenic bacteria, oomycetes and fungi act to promote host colonization. AMF can engage in symbiosis with a broad range of plants, yet despite their global significance, much remains unknown regarding the maintenance of this symbiotic interaction (Thoms et al., 2021), particularly with respect to the role of fungal proteins and genes. In the AMF plant symbiosis, only three candidate effector proteins have been identified to date, including SP7 (Kloppholz et al., 2011), RiCRN1 (Voß et al., 2018), and RiSLM (Zeng et al., 2019). In contrast, many other plant-associated fungi secrete a much greater number of effectors (Stergiopoulos & de Wit, 2009). There is a growing understanding on how the three identified AMF effector proteins help maintain a symbiotic relationship with their host in unique ways and it is speculated that more effectors are involved in this symbiosis (Kloppholz et al., 2011; Zeng et al., 2019).

The first identified effector protein, SP7, is localized to the plant nucleus where it interacts with the transcription factor ERF19. ERF19 controls the expression of plant defense proteins (Kloppholz et al., 2011). By counteracting the expression of ERF19, SP7 can promote the AMF symbiosis. The ectomycorrhizal fungi *Laccaria bicolor* forms a beneficial symbiotic relationship with plants and secretes the effector MiSSP7. This effector from *L. bicolor* is essential to the formation of the Hartig net, a fungal structure that facilitates nutrient exchange between *L.*

bicolor and its host, similar to arbuscules produced by AMF (Plett et al., 2011). MiSSP7 enters the nucleus of its host plant's cells where it impairs jasmonic acid signaling by interacting with the negative regulatory factor PtJAZ6 (Plett et al., 2014). PtJAZ6 is a transcriptional repressor protein, controlling plant defense-related genes. Upon expression of jasmonic acid, PtJAZ6 is degraded, and host defense genes are expressed. MiSSP7 protects PtJAZ6 from degradation and as a result PtJAZ6 continues to repress plant-defense related genes, and the symbiosis is promoted. Upon perception of a symbiont, jasmonic acid signals host defense genes. To counteract these, both beneficial and pathogenic symbionts have evolved effectors to manipulate normal jasmonic acid signalling (Gimenez-Ibanez et al., 2014; He et al., 2004; Plett et al., 2014), promoting the symbiosis.

The second effector, RiCRN1, is a crinkler (CRN) effector that is involved with proper development of the symbiosis (Voß et al., 2018). CRN effectors are mostly found in plant-pathogen oomycetes, where they were originally classified to be involved in processes controlling cell death and necrosis (Adhikari et al., 2013) within the host nucleus (Schornack et al., 2010). All CRNs contain a characteristic N-terminal motif (LXLFLAK) essential to the effector's intracellular localization (Schornack et al., 2010). Recent research has identified a CRN effector that displays kinase activity, suggesting that CRNs have other roles in symbiosis such as interference with signalling cascades during infection (Scott et al., 2010; van Damme et al., 2012). RiCRN1 from *R. irregularis* (formerly known as *Glomus intraradices*) accumulates in parallel with the plant phosphate transporter, *MtPT4,* a marker of AMF symbiosis (Javot, Pumplin, et al., 2007). Similar to other CRNs, RiCRN1 was visually confirmed to localize to the host plant nucleus (Voß et al., 2018). Gene silencing of RiCRN1 resulted in much smaller arbuscules, diminishing the symbiosis (Voß et al., 2018). This work demonstrates that the expression of RiCRN1 facilitates the AM symbiosis.

RiSLM, the third AMF effector protein, promotes symbiosis by binding chitin and interfering with chitin-regulated responses (Zeng et al., 2019). Chitin is a key component of the fungal cell wall, and to overcome an immune response both beneficial and harmful fungal symbionts secrete LysM effectors (Sánchez-Vallet et al., 2015; Zeng et al., 2019). RiSLM is a LysM effector secreted by the AMF *R. irregularis* that binds chitin and chitosan (Schmitz et al., 2019). Chitin is recognized by pattern recognition receptors as a MAMP and trigger immune responses including defense-gene induction, secretion of chitinases and generation of reactive oxygen species (Bozsoki et al., 2017; Miya et al., 2007; Shimizu et al., 2010). To prevent these defense-related responses, LysM effectors protect fungal hyphae by binding chitooligosaccharides, chitooligosaccharides are produced when chitin is broken down. Homologs to this LysM effector have been identified in five other AMF species and in plant pathogens, where they interfere with chitin-triggered immunity (Jonge et al., 2010; Kombrink & Thomma, 2013; Lee et al., 2014; Marshall et al., 2011; Mentlak et al., 2012; Takahara et al., 2016).

In summary, previously described AMF effector proteins have been shown to facilitate the symbiosis by inhibiting the transcription of plant defence proteins, plant cell death proteins and plant immune response proteins to chitin and its derivatives. These methods are similar to the ways by which pathogenic symbionts modify a host.

Classically, bioinformatics has been used to predict candidate microbial effectors (Kamel et al., 2017; Zeng et al., 2018). While this method has been successful in predicting fungal effectors in select pathogenic fungi (Dong et al., 2015), this assumption has yet to be experimentally verified for AMF. Moreover, there is increasing evidence that an unconventional protein secretion system may play an important role in plant-microbe interactions (Kuo et al., 2014; Tisserant et al., 2012). The objective of this research project is to utilize a proteomic-based approach to identify AMF effectors that is not biased on the assumption that these fungal effectors contain a signal peptide.

IV. BioID and Proximity labelling

To identify effectors involved in the AMF symbiosis, this work utilizes BioID. BioID is a form of proximity labelling that labels proteins *in vivo* (Roux et al., 2012), revealing various protein networks and their involvement in cellular processes. Proximity labelling takes advantage of BirA, a 35 kDa DNA-binding biotin protein ligase from the bacterium *Escherichia coli* that uses biotin as a transcriptional repressor by binding an acetyl-CoA carboxylase (Chapman-Smith & Cronan, 1999). Biotin and ATP combine to form biotinyl-AMP. Biotinyl-AMP remains bound by BirA until recognition of an extremely specific lysine residue within a biotin acceptor tag sequence. Upon recognition of its substrate, BirA releases biotinyl-AMP which covalently binds to the lysine residue (Figure 2A). Researchers identified a version of BirA with a single site amino acid substitution that has a lower affinity for biotinyl-AMP. This promiscuous biotin ligase is called BirA* whose biotinyl-AMP is released prematurely by BirA* and binds to close-by primary amines covalently and non-discriminately on lysine residues. As a result, proteins within a 10 nm distance of BirA* are tagged with biotin (Kim et al., 2014). BirA* has been used in mammalian systems to identify interacting proteins of the nuclear pore complex (Kim et al., 2014), whole organellar proteomes, (Hung et al., 2016) local proteomes inside cilia (Mick et al., 2015), at synaptic clefts (Loh et al., 2016), mitochondria (Han et al., 2017), and stress granules (Markmiller et al., 2018). BioID has been used in plant systems to identify proteins that interact with the effector AvrPto from the bacterial plant pathogen, *Pseudomonas syringae* (Conlan et al., 2018) and identify where in host plants effectors are secreted by *Ustilago maydis* (Lo Presti et al., 2017). It has also been used in *Arabidopsis thaliana* to understand the pathway involved with nuclear membrane protein degradation (Huang et al., 2020).

A major disadvantage of BirA* is that it has an optimal activity at 37°C and may require up to 24 hours (h) of incubation with exogenous biotin to sufficiently label nearby proteins with biotin. Through directed evolution, more efficient variants of BirA* called TurboID (35 kDa) and miniTurbo (28 kDa) (Figure 2B) were created (Branon et al., 2018). These biotin ligases are more efficient in plant systems than their previous BirA* isoform (Mair et al., 2019). Experiments using BioID and proximity labelling techniques with TurboID and miniTurboID discovered that these isoforms function similarly to BirA*, but at lower temperatures and with reduced time requirements (Figure 2C) (Branon et al., 2018; Mair et al., 2019). TurboID and miniTurboID have been tested in plant systems and can successfully identify interacting proteins (Mair et al., 2019; Y. Zhang et al., 2019). Mair et al. (2019) showed that TurboID is best at tagging proteins that are in low abundance. Given the success of the BioID system, along with the increased efficiency of TurboID at appropriate temperatures and timeframes, this work uses TurboID to identify AMF proteins that have been secreted into the root cortical cells of colonized plants during symbiosis.

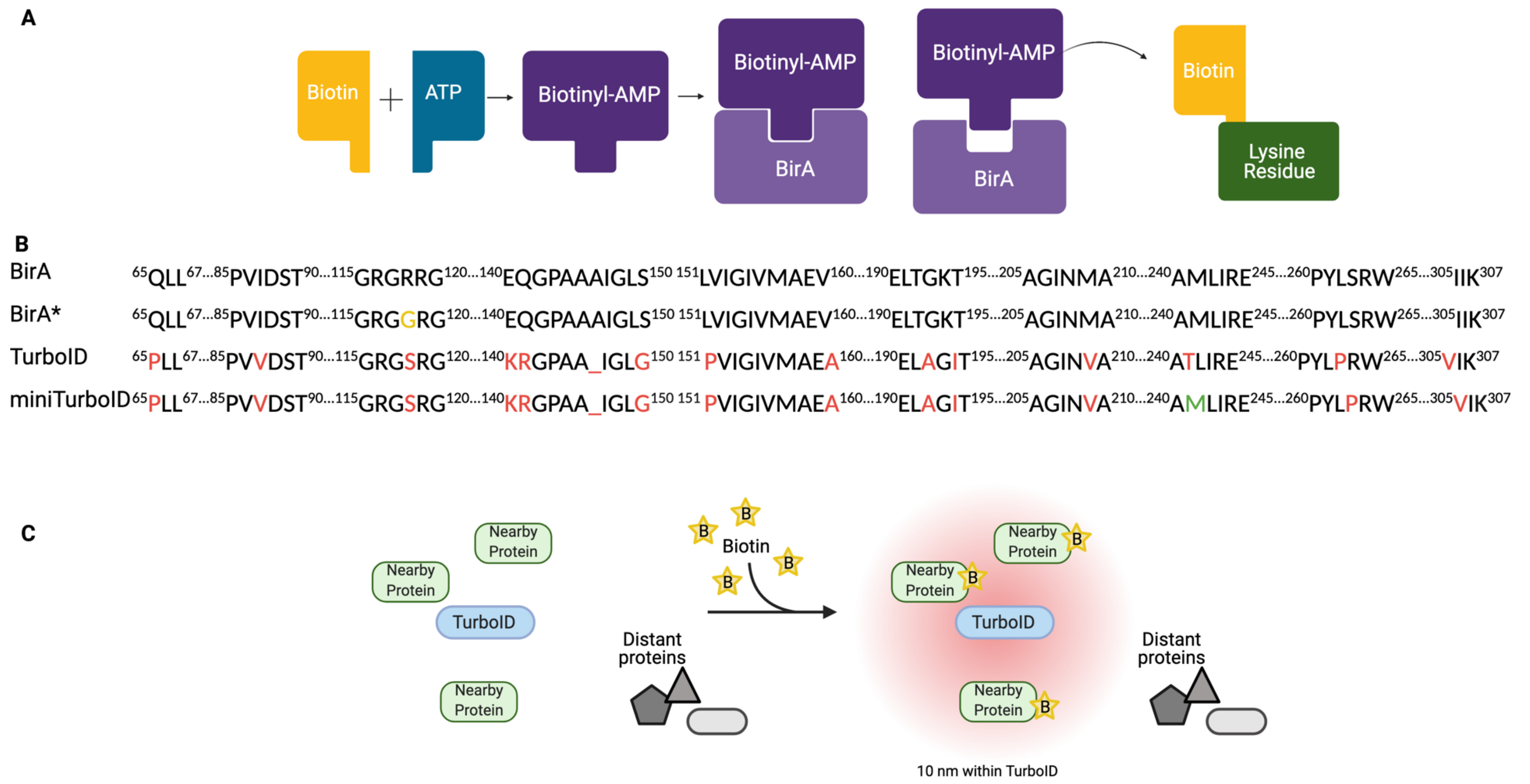


Figure 2. BioID activity. (A) BirA is an enzyme that catalyzes a two-step reaction, first, biotin and ATP must combine to form biotinyl-AMP. Biotinyl-AMP remains bound by BirA until BirA recognizes a specific lysine residue within a biotin acceptor tag sequence, in which the biotinyl-AMP is released, and the nearby lysine residue is tagged with biotin. (B) Substitutions in amino acid sequences of BirA*, TurboID and miniTurboID. BirA* has one amino acid substitution, TurboID has 15 substitutions and miniTurboID has 13 substitutions relative to the original BirA sequence (Branon et al., 2018). Red font highlights differences from the original BirA sequence, yellow font

highlights the single mutation in the BirA* sequence, and green font highlights a difference between the TurboID and miniTurboID sequence. Numbers indicate the position with respect to the BirA amino acid sequence. Underscore indicates a deletion in the sequence. (C) Proteins within a 10 nm distance of TurboID are tagged with biotin, assuming there is available ATP, biotin, and the temperature is within 22°C – 37°C.

Chapter 2: Identifying Effectors in Arbuscular Mycorrhizal Fungi

Plant-AMF symbioses are extremely important for plants in both natural and agricultural systems, but the biochemical mechanisms driving this interaction are poorly understood. I sought to identify the effector proteins that underpin the symbiotic relationship between AMF and plants using the BioID proximity labelling system. This research is based on the hypothesis that i) AMF produce effector proteins, and ii) arbuscules are a site of effector secretion into host plant cells. These hypotheses are rooted in previous literature relevant to other plant-microbe symbioses, such as haustoria-producing fungal and oomycete pathogens. Haustoria are feeding structures that absorb water and nutrients from host plants and are structurally analogous to arbuscules in AM symbiosis. Because haustoria from the flax pathogen *Melampsora lini* are rich in small-secreted proteins (Catanzariti et al., 2006), it was hypothesized that they are the site of effector exchange (De Jonge et al., 2011; Dodds et al., 2009). Subsequent research confirmed that haustoria are a site of effector exchange in the oomycete pathogen *Phytophthora infestans* (Whisson et al., 2007) as well as the fungal pathogen *M. lini* (Rafiqi et al., 2017). This work with pathogenic symbionts suggests that arbuscules may likewise be the site of effector protein exchange in AMF. Until recently, it was unknown whether AMF secreted effector proteins (Kloppholz et al., 2011; Voß et al., 2018; Zeng et al., 2019). To date, only three putative AMF effector proteins have been identified, meanwhile pathogenic symbionts secrete a large number of effectors. This work takes a proteomics approach to identify fungal effectors inside colonized plant cells with fully formed arbuscules.

I. Experimental Design

Previous work using bioinformatics to predict AMF effectors has failed to identify *bona fide* effector proteins, and we choose to adopt a proteomics approach to identify fungal effectors instead. Based on evidence in other plant-microbe interactions where structures such as haustoria are a site of effector secretion, the framework of this research is based on the hypothesis that arbuscules are the site of effector secretion in AMF. The goal of my research is to identify fungal effectors that are secreted into root cortical cells via arbuscules via the BioID proximity labelling system. To express TurboID exclusively within the colonized cells, we employed an expression vector in which a symbiosis-responsive promoter (*MtPT4p*) drives the expression of TurboID. *MtPT4p* is exclusively expressed in colonized cells with fully formed arbuscules (Ivanov & Harrison, 2014; Javot, Penmetsa, et al., 2007) (Figure 3). Because TurboID will only be expressed inside the plant cell (and not within the fungal symbiont), any fungal proteins that are tagged and identified by this approach must have been secreted from the symbiont and have crossed the periarbuscular membrane.

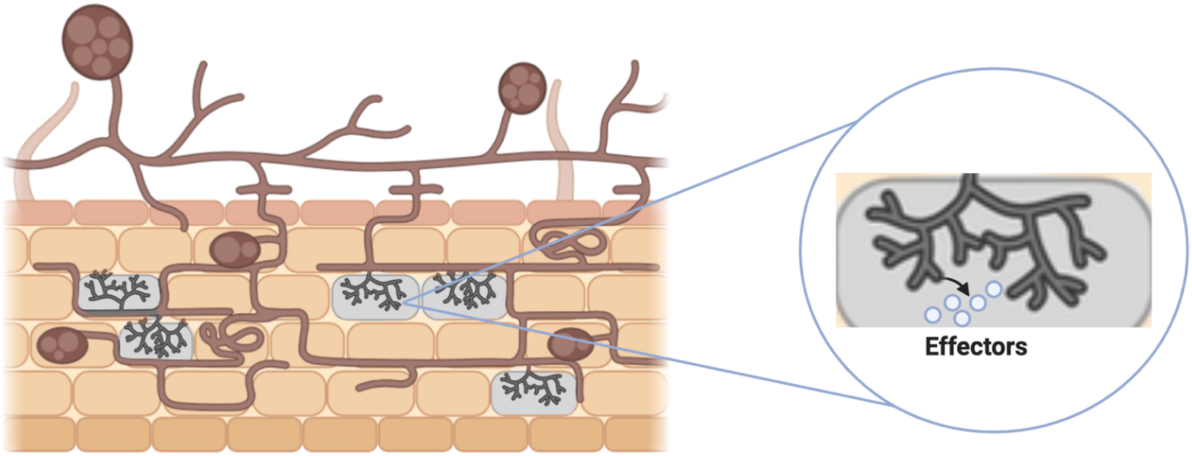


Figure 3. Expression of *MtPT4p*, occurs exclusively in root cortical cells with fully formed arbuscules. Using *Agrobacterium rhizogenes* to transform plant roots to express TurboID controlled by *MtPT4p.* we can ensure TurboID is exclusively found in cells with fully formed arbuscules.

Many effector proteins that have been identified contain a nuclear localization signal (NLS) (Deslandes & Rivas, 2011; Sugio et al., 2014). Within the nucleus, effectors are able to directly manipulate the host cellular machinery. In the case of fungal plant pathogens, the rusts, powdery mildews and *Magnaporthe oryzae,* effectors have been shown to interact with cytosolic or nuclear proteins (Stergiopoulos & de Wit, 2009). My goal was to identify AMF effectors that are targeted either to the nucleus or cytosol of host plant cells. To localize TurboID in the nucleus, I have used a TurboID-YFP-NLS fusion and that has been demonstrated to localize to the nucleus when expressed in plants (Mair et al., 2019). To localize TurboID within the cytoplasm, I have used a TurboID-NES-YFP fusion that has been shown to be excluded from the plant cell nucleus (Mair et al., 2019). By targeting both the nucleus and cytoplasm, we can gain insight into possible effector function, based on where the identified proteins are targeted within a plant cell (Figure 4).

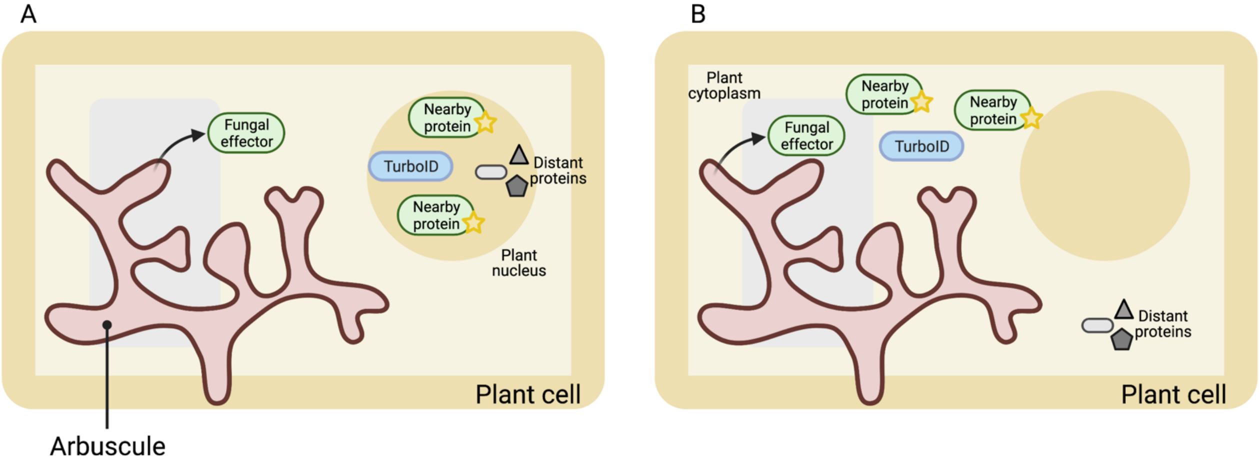


Figure 4. TurboID localization to the nucleus and cytosol. Using *Agrobacterium rhizogenes* plant roots are genetically transformed to contain *MtPT4p*-TurboID-YFP-NLS or *MtPT4p*-TurboID-NES-YFP. The *MtPT4p* promoter is exclusively expressed in cells with fully formed arbuscules. (A) By localizing TurboID to the plant nucleus, we can identify proteins primarily localized to the nucleus of colonized plant cells, including any proteins that may have moved from the arbuscule into the plant. (B) Localization of TurboID within the plant cell cytoplasm.

Initial experiments tested the *pMtPT4*-TurboID-YFP-NLS-nosT in pkm43GW-RR construct alongside the pkm43GW-RR empty vector (EV) as a negative control in plants inoculated or not with *R. irregularis*. Once experimental troubleshooting was completed, this construct and AMF species were used to perform most of the experiments described in this thesis. Due to the success of the original experiment, we chose to perform three other experiments using the same promoter, terminator, and destination vector. The first two experiments used the TurboID-YFP fusion targeted to either the plant cytoplasm or nucleus and colonized by *Glomus versiforme* (now known as *Diversispora epigaea)*. The final experiment used *R. irregularis* with the TurboID-YFP fusion localized within the plant cytoplasm. This final experiment provides valuable information, but its data is not included in this thesis.

II. Materials and Methods

i. Plasmid construction

A list of all plasmids used in this study can be found in Table 1. Gateway cloning allows for the directional entry of a DNA fragment into a destination vector. Using Gateway®Cloning Dr. Mina Ghahremani (University of Ottawa, Ottawa, Ontario) created the constructs used in these experiments. Beginning with the *MtPT4p* promoter in pDONR4,1 (provided by Dr. Maria Harrison, Boyce Thompson Institute, Cornell, NY) followed by TurboID-YFP-NLS in pDONR1,2 (Addgene #127361) or TurboID-NES-YFP in pDONR1,2 (Addgene #127358) and ending with nosT in pDONR2,3 (provided by Dr. Maria Harrison, Boyce Thompson Institute, Cornell, NY) into the pkm43GWRR (provided by Dr. Maria Harrison, Boyce Thompson Institute, Cornell, NY) destination vector. See supplementary material for plasmid maps.

For each plasmid (*MtPT4p* promoter in pDONR4,1, TurboID-YFP-NLS in pDONR1,2 or TurboID-NES-YFP, nosT in pDONR2,3 and pkm43GW-RR), 1 µL was added to a reaction mixture with 1 µL of Gateway™ LR clonase™ enzyme mix and incubated at 25°C overnight. The reaction was terminated by adding 1 µL of Proteinase K and incubated at 37°C for 15 minutes. This reaction was then stored at -20°C.

To transform *E. coli* DH5α chemically competent cells, 1 µL of this reaction was added to 100 µL of competent cells and left on ice for 20 minutes. To heat shock cells, they were placed in a water bath at 42°C for 45 seconds. The cells were placed back on ice for two minutes and 900 µL of LB was added, the mixture was incubated at 37°C shaking for one h before being plated on Lysogeny broth (LB) agar plates supplemented with 50 mg/L spectinomycin. After overnight growth, a single colony was picked, and successful transformations were confirmed by PCR. A glycerol stock and purified plasmid were created.

Table 1. Plasmids used in root transformation.

Construct	Description
MtPT4p-TurboID-YFP-NLS-nosT in pkm43GWRR	Localize TurboID in the nucleus of cells with arbuscules
MtPT4p-TurboID-NES-YFP-nosT in pkm43GWRR	Localize TurboID in the cytoplasm of cells with arbuscules
pkm43GWRR	Empty vector negative control

ii. *Plasmid purification*

To create a purified plasmid, the GenElute™ Plasmid miniprep kit (Sigma-Aldrich) was used following the manufacturer's instructions. Briefly, a 5 mL culture was grown from a single colony overnight in LB with appropriate antibiotics at 37°C and a pellet was collected by centrifuging at 13,000 rpm for one minute. The pellet was resuspended in 200 µL of resuspension solution and mixed by pipetting. 200 µL of lysis solution was added and the mixture was gently inverted. 350 µL of neutralization solution was added and mixed by inverting. The cell debris was pelleted by centrifuging at 12,000 g for 10 minutes. To prepare the column, 500 µL of column preparation solution was added and centrifuged at 12,000 rpm for one minute, discarding the flow-through. The supernatant was loaded into the column and centrifuged at 12,000 rpm for 1 minute, discarding the flow-through. 750 µL of wash solution was added and centrifuged at 12,000 rpm for 1 minute. Flow-through was discarded and the tube was centrifuged again to remove any remaining ethanol. The column was then transferred to a new tube and 50 µL of elution buffer was added. The tubes were centrifuged at 12,000 rpm for 1 minute, with the flow-through containing the plasmid. Plasmid concentration was measured using a NanoDrop™ (Thermo Scientific) and the plasmid DNA was stored at -20°C.

iii. *Competent cell preparation and transformation*

To create chemically competent *E. coli* DH5 alpha cells, the following protocol was performed. An *E. coli* DH5 alpha glycerol stock was streaked onto LB agar and grown overnight at 37°C. A single colony was picked and grown in 5 mL LB shaking at 37°C overnight. The 5 mL culture was suspended in 500 mL LB and left to grow at 37°C shaking at 200 rpm. Once the culture reached an optical density measured at 600 nm (OD_{600}) of 0.4, the culture was centrifuged at 4000 rpm at 4°C for 10 minutes. Next, the pellet was resuspended with 200 mL of 0.1 M $CaCl_2$ and centrifuged at 4000 rpm at 4°C for 10 minutes. Cells were then resuspended in 10 mL of 100 mM $CaCl_2$ with 15% (v/v) glycerol. 100 µL aliquots were made and flash-frozen in liquid nitrogen. Cells were stored at -80°C.

To create electrocompetent *Agrobacterium rhizogenes* ARqua1 competent cells the following protocol was performed. *A. rhizogenes* ARqua1 were grown on LB plates containing 100 mg/mL spectinomycin for three days at 28°C. A single colony was picked and grown in 5 mL

LB broth with 0.05 mg/mL spectinomycin overnight at 28°C shaking. The following day, the 5 mL culture was inoculated into 50 mL LB with 0.05 mg/mL spectinomycin shaking at 28°C for 12 h. 2 mL from the 50 mL culture was resuspended into 500 mL of LB with 0.05 mg/mL spectinomycin and left shaking at 28°C until the culture reached an OD_{600} of 0.6. Once the culture reached an OD_{600} of 0.6, the culture was placed on ice until being centrifuged at 4000 g at 4°C for 15 minutes. After centrifugation, the supernatant was removed, and cells were resuspended in enough cold, sterile nanopure water to cover cells. After the pellet was fully resuspended, the bottles were filled with 500 mL cold water and vortexed. Centrifugation and cell resuspension was repeated as previously described twice. On the second resuspension rather than using cold water, cold 10 % glycerol was used instead. Following centrifugation, the supernatant was removed, and cells were finally resuspended in cold 10 % (v/v) glycerol, with just enough to cover the cells. Cells were aliquoted into microcentrifuge tubes, flash-frozen in liquid nitrogen and stored at -80°C.

To transform *A. rhizogenes* ARqua1 electrocompetent cells, 1 µL of the desired plasmid was added to 100 µL of competent cells and the cells were shocked using the ECL setting. 900 µL of LB was added and cells were grown for two h rotating at 28°C. 100 µL of this suspension was plated on LB agar with appropriate antibiotics (50 mg/L spectinomycin) and grown for three days at 28°. A single isolated colony was resuspended in 100 µL of water then plated on YEP agar (10 g bacto peptone, 5 g NaCl, 10 g yeast extract in 1 L water) with appropriate antibiotics (50 mg/L spectinomycin) and left to grow for two days at 28°C.

iv. *Medicago truncatula root transformation*

To insert the genes of interest, *M. truncatula* A17 plant roots were transformed through hairy-root transformation with *A. rhizogenes* strain ARqua1 following the protocol described in (Floss et al., 2013) and is described below.

Briefly, *M. truncatula* seeds were removed from seed pods and surface sterilized in sulfuric acid for nine minutes. Next, seeds were washed with sterile water four times and then with a 10% (v/v) bleach, 0.1% (v/v) tween 20 solution shaking for 10 minutes. The 10% bleach/0.1% tween 20 solution was washed away with sterile water four times and seeds were resuspended in sterile water and left shaking at room temperature in the dark for three hours. Seeds were then incubated in sterile water at 4°C overnight in the dark. Finally, seeds were placed in sterile glass Petri plates held to the bottom of the plate through surface tension and flipped upside down to allow roots to grow vertically overnight at 28°C in the dark.

Seed coats were removed, and the root tip was cut on an angle and dipped in the appropriate transformed *A. rhizogenes* strain. The seedling was then placed in Fähraeus medium (0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 20 µM KH_2PO_4, 10 µM Na_2HPO_4, 20 µM ferric citrate, 1.0 mM NH_4NO_3, 33 µg/L $MnCl_2$, 33 µg/L $CuSO_4$, 7 µg/L ZnSO4·7H2O, 100 µg/L H_3BO_3, 33 µg/L Na_2MoO_4, 218 mg/L MES free acid monohydrate, 2.5 g/L Gelzan™) with 25 mg/L kanamycin in low light conditions for five days at 18°C with 16 µM/m^2/s of light on a 16h light / 8h dark cycle. Plants were then grown for three to four weeks at 45 µM/m^2/s at 25°C / 16h light and 22°C / 8h dark. Using ZEISS Axio Zoom.V16 fluorescent microscope transformed plants were identified through the red root system at a wavelength of 550-590 nm (Figure 5C).

After successfully transformed plants were identified and marked, plates containing plants were laid horizontally and water was added with the lids slightly removed to allow plants to adjust to lower humidity levels. The plants were incubated for two days at 45 µM/m^2/s at 25°C / 16h light and 22°C / 8h dark before moving them into cones. For an overview of this process, refer to Figure 5.

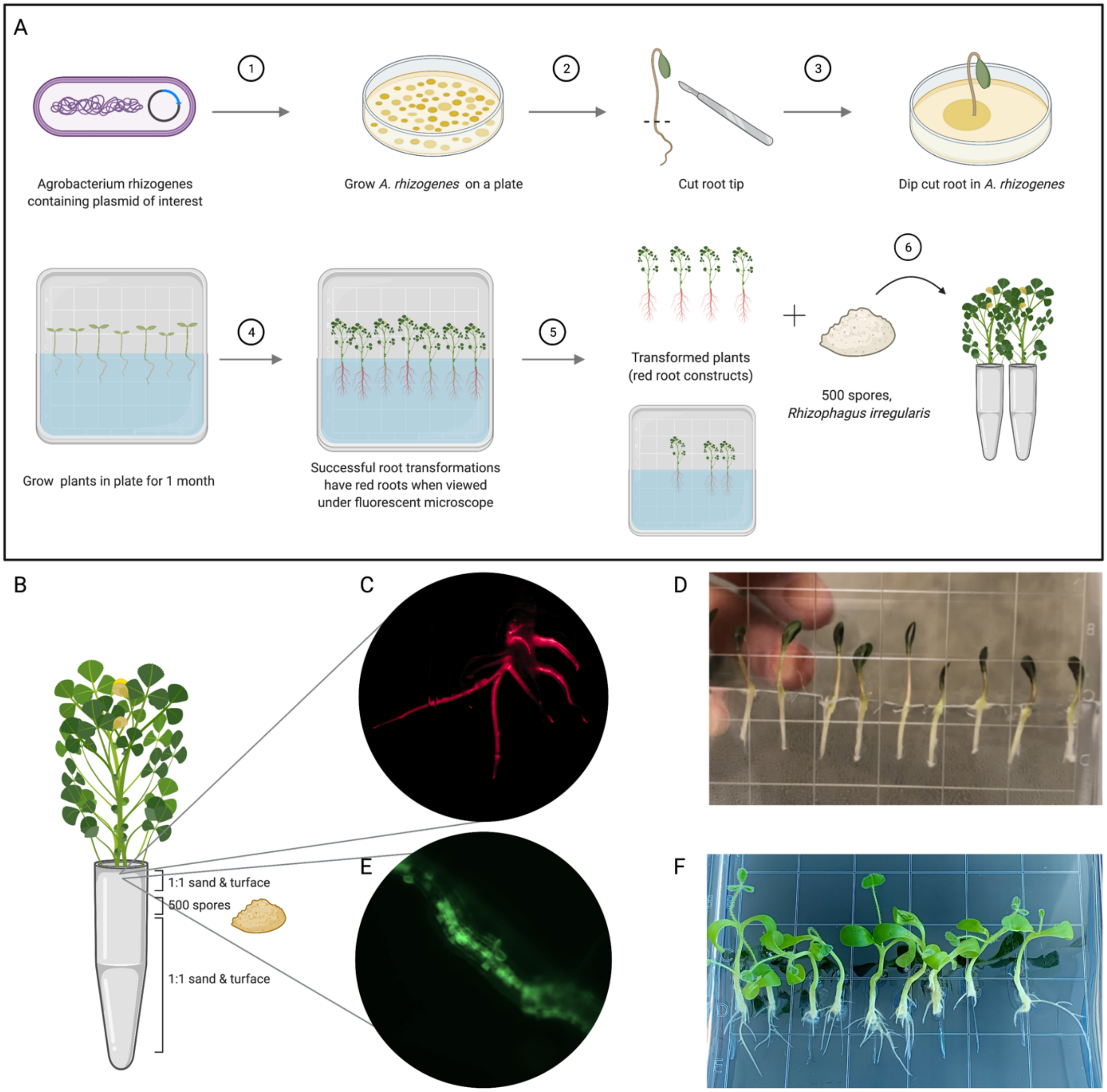


Figure 5. The various steps involved with hairy root transformation. (A) Overview of the Ri-T-DNA root transformation protocol to insert genes of interest into a plant. (B) Cone set up (C) Successfully transformed red roots visualized under a fluorescent microscope at a wavelength of 550-590 nm. (D) Plants directly after inoculation with *Agrobacterium* in Fähraeus medium. (E) A colonized plant root by AMF, visualized with a fluorescent microscope at a wavelength of 500-550 nm. Photograph by Mina Nasr. (F) Plants 2-3 weeks after transformation with *Agrobacterium* and have developed hairy roots. Photograph by Allyson MacLean.

v. *Growth conditions of transformed plants*

Successfully transformed plants were moved into cones containing 500 spores of *R. irregularis* (DAOM 197198) with two plants/cone. Cones contained a 1:1 mixture of sterile sand and turface. Fungal spores were located about 1 inch below the surface (Figure 5B).

Once plants were in cones the plants were grown in a growth chamber at 200 µM/m^2/s on a 16 h light / 25°C and 8h dark / 22°C cycle with 60 % humidity. Fertilization began one week after plants had been in cones and from then on plants were fertilized twice a week with half-strength Hoagland's containing 20 µM potassium phosphate (Hoagland & Arnon, 1940). Plants were watered with sterile Milli-Q water 3-4 times/week or as plants required.

vi. *Root Harvest*

To assess root colonization two cones were randomly selected from the experiment (one of each construct). The shoot of the plant was removed along with approximately one inch from the top and bottom of plant roots. As arbuscular mycorrhizal fungi are incapable of colonizing these parts of the plant. The remaining root was rinsed in water. Roots were then placed in a 50% (v/v) ethanol solution for 15 minutes. Next, roots were cleared using a 20% (v/v) potassium hydroxide solution overnight at 65°C. Roots were then washed three times for ten minutes shaking in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4). Finally, 0.2 ng/mL of a wheat germ agglutinin Alexa Fluor 488 conjugate was added to roots in PBS solution. Samples were then visualized or stored in the dark at 4°C and assessed at a later date. Using a ZEISS Axio Zoom.V16 fluorescent microscope at a wavelength of 500-550 nm the gridline intersect method was used to quantify the percentage of colonized roots in each plant (Figure 5E) (McGonigle et al., 1990). Plant roots were harvested after they had been in cones for about five weeks, once the plants were 30-40% colonized.

Plants were 'watered' with biotin (200 mM stock in DMSO) diluted in water at 25°C to obtain a final concentration of 500 µM biotin (Figure 8). Alongside biotin-treated plants a DMSO control was present. To water plants, the biotin solution was poured over the plants, which were left to sit in the biotin solution for five minutes. Plants were then removed from the biotin solution to allow it to drip out from the containers. Plants were incubated like this for 10 h at 25°C. Then, plants were removed by gently pouring out the potting mix and rinsing the roots in cold water to stop the reaction (See Figure 8). The shoot of the plant was removed along with approximately one inch from the top of the root system. The roots were flash-frozen in liquid nitrogen and stored at -80°C until further use.

vii. Crude protein extraction

To confirm that the addition of exogenous biotin was sufficient for TurboID to successfully label nearby proteins, a crude protein extraction was performed. Root samples were crushed using a liquid nitrogen-cooled mortar and pestle to create a fine powder and stored at -80°C. 200 mg of each sample was aliquoted into a 2 mL Eppendorf and a 1:1 ratio of protein extraction buffer (150 mM Tris-HCl, 150 mM NaCl, 10% (v/v) glycerol, 10 mM EDTA, 10 mM DTT, 0.5% (w/v) PVP, 0.1% (v/v) Tween 20) was added to samples and mixed thoroughly. Next, samples were centrifuged at 4°C for 15 min at 13,000 rpm. The supernatant was transferred to a new tube and combined with Laemmli sample buffer (277.8 mM Tris-HCl pH 6.8, 44.4% (v/v) glycerol, 4.4% LDS, 0.02% bromophenol blue, 355 mM 2-mercaptoethanol). Samples were then boiled at 95°C for 5 minutes, prior to being stored at -20°C or run on a gel.

viii. Western blot

Proteins were separated by 10% SDS-PAGE at 150 volts for 70 minutes and transferred to activated Immuno-Blot® PVDF membranes (BioRad) using a Trans-Blot Semi-Dry transfer cell system (BioRad) with a 10-minute run at 1.3 A and 25 volts. The membrane was blocked using 5% (w/v) bovine serum albumin when using a streptavidin antibody, or 5% (w/v) skim milk powder when using a GFP antibody (that also binds YFP). Blocking solution was made in tris buffered saline solution (50 mM Tris, 150 mM NaCl, pH 7.6) (TBS) and contained 1% (v/v) tween 20. The membrane was probed with antibodies diluted in their appropriate blocking buffer. The membrane was then probed with either a 1 / 5,000 dilution of streptavidin-HRP antibody (S911, Thermo Fisher Scientific) or a 1 / 3,000 dilution of anti-GFP (GF28R, Invitrogen) and left overnight at 4°C. When using the anti-GFP antibody, the following day, the membrane was washed four times, for ten minutes each, with blocking buffer. Following the washes, a 1 / 6,667 dilution of secondary anti-mouse antibody (G-21040, Invitrogen) was left for one h shaking at room temperature. When the streptavidin antibody was used, it was not necessary to add a secondary antibody, as the streptavidin antibody is fused to horseradish peroxidase. Therefore, following incubation, antibodies were removed by washing the membrane four times with 1X TBS. To develop the membrane Pierce™ ECL Western Blotting substrate (Thermo Scientific) was applied according to the manufacturer's instruction and proteins were visualized with a ChemiDoc MP Imaging System (BioRad). Alongside protein samples, the Precision Plus Protein™ All Blue Prestained Protein Standards (#1610373, BioRad) protein marker was used.

A Coomassie Brilliant Blue stain was done to ensure equal loading of proteins. Proteins were separated by a 10 % SDS-PAGE gel and staining was done following the Cold Spring Harbour protocol (Simpson, 2003). Briefly, the gel was submerged in reagent A (0.5 g CBR-250, 25 % (v/v) isopropanol, 10 % (v/v) acetic acid) and microwaved for one minute or until boiling. The gel was cooled for 5 minutes at room temperature shaking, reagent A was removed, and the gel was rinsed with water. This was repeated with reagent B (0.05 g CBR-250, 10 % (v/v) isopropanol, 10 % (v/v) acetic acid) and reagent C (0.02 g CBR-250, 10% (v/v) acetic acid). Finally, reagent D (10 % (v/v) acetic acid) was added and microwaved for one minute and the gel was cooled at room temperature by shaking for 15 minutes with a laboratory tissue to absorb excess dye.

ix. Purification of biotinylated proteins

To purify biotinylated proteins, we slightly modified the protocol described under 'Affinity purification of biotinylated proteins' by Mair et al (2019), briefly, 6 mL of tissue was resuspended in 6 mL of extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton-X-100, 0.5% Na-deoxycholate, 1 mM EDTA, 1 mM DTT, 1X complete, 1 mM PMSF) and incubated at 4°C shaking for 10 minutes. 1 µL of Lysonase (Millipore) was added and the sample incubated an additional 15 minutes at 4°C shaking. Extracts were then distributed into 2 mL Eppendorfs and sonicated in an ice bath four times for 30 seconds on a high setting, with 90 seconds breaks on ice. The suspension was then centrifuged at 15,000 rpm for 15 minutes at 4°C. Next, the supernatant was transferred into a PD-10 desalting column (GE healthcare) to remove excess free biotin following the manufacture's gravity protocol. Then, 20 µL of the protein extract was diluted 1:5 to avoid interference of buffer components and a Bradford assay was performed. A 150 µL slurry of High Capacity Magne™ Streptavidin Beads (Promega) was pre-washed with extraction buffer and added to samples which were incubated overnight shaking at 4°C. The next day, beads were separated from the protein extract using a magnetic rack and washed with 1 mL of the following solutions while incubating on a rotor wheel for 8 minutes. Twice with cold extraction buffer, once with cold 1 M KCl, once with cold 100 mM Na_2CO_3, once with room temperature 2 M Urea in 10 mM Tris pH 8, and twice with cold equilibration buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton-X-100, 0.5% Na-deoxycholate, 1 mM EDTA, 1 mM DTT). The remaining equilibration buffer was removed from the beads and beads were frozen at -80°C until further processing.

The following step was only done on a small portion of the beads to run a western blot and confirm that biotinylated proteins were washed appropriately before sending the beads for mass spectrometry. Beads were resuspended in 100 µL of equilibration buffer and 20 µL of beads were removed and resuspended in 20 µL of 4X Laemmli buffer supplemented with 10 mM DTT and 2 mM biotin. This portion of beads were run on a streptavidin immunoblot to ensure successful protein purification.

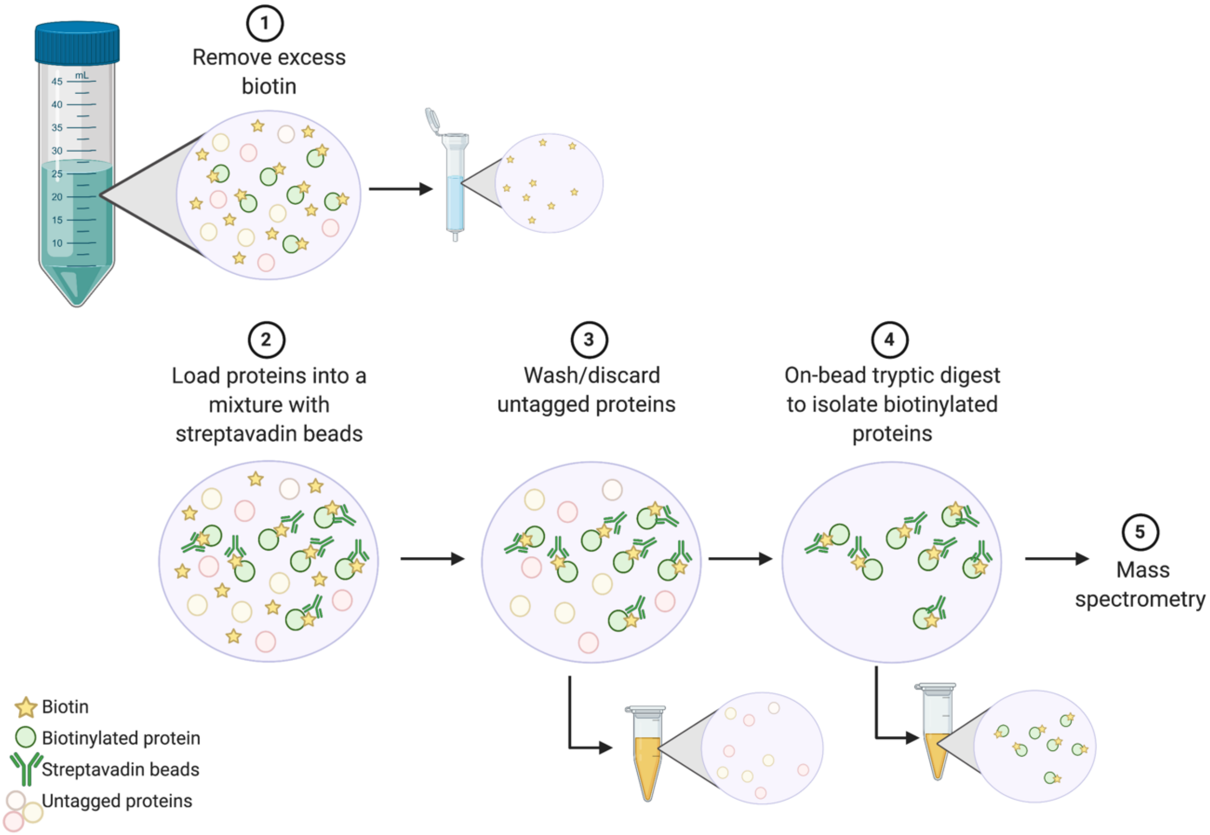


Figure 6. Purification of biotinylated proteins using magnetic streptavidin beads. Total protein was extracted and loaded into a PD-10 desalting column (GE healthcare) to remove excessive biotin; a critical step shown by Mair et al. (2019). After removing excessive biotin, streptavidin beads are added to the protein mixture, and these are left shaking overnight at 4°C. Unbound proteins are removed by a series of washes (refer to materials and methods). Bound proteins are removed through an on-bead tryptic digest and then mass spectrometry is performed.

x. Mass Spectrometry

Mass spectrometry was performed by Richard Glen Uhrig (University of Alberta, Edmonton, Alberta). Beads were washed in a 2 M Urea and 50 mM Tris-HCl pH 8.0 solution followed by three 50 mM TEAB washes. Next, an on-bead tryptic digest was done by adding trypsin to the beads. Data was acquired by data dependent acquisition / shotgun using a label-free quantification approach on a Thermo LUMOS device. Next, MaxQuant was run for quantification under default settings with protein, peptide and peptide spectrum matches (PSM) false-discovery rate (FDR) of 0.01 (Tyanova et al., 2016). Separate searchers were performed for *M. truncatula* and *R. irregularis* genomes downloaded from DOEJGI (Chen et al., 2018; Tang et al., 2014; Tisserant et al., 2013; Young et al., 2012) using custom made REV_decoy database.

xi. Data Analysis

SignalP 5.0 (using Eukarya setting) and SecretomeP 2.0 (using mammalian setting) were used to predict the likelihood of protein secretion of the identified proteins (Almagro Armenteros et al., 2019; Bendtsen et al., 2004).

We used cNLS Mapper to determine if identified proteins contain a predictable nuclear localization signal (NLS) (Kosugi et al., 2009). A score of 8, 9 or 10 is exclusively localized to the nucleus. A score of 7 or 8 is partially localized to the nucleus. A score of 3, 4, or 5 is localized to both the nucleus and cytoplasm. And a score of 1 or 2 is localized to the cytoplasm.

Chapter 3: Results

I. Exploiting Proximity-Based Labeling to Study the AM Symbiosis

TurboID has been employed in model organisms *Arabidopsis thaliana* and *Nicotiana benthamiana* (Branon et al., 2018; Mair et al., 2019). This is the first time TurboID has been applied in *M. truncatula* to study the plant-AMF symbiosis. As a result, we have had to establish an appropriate protocol, which we based on the research performed by Mair et al. (2019). To ensure optimal labelling activity, a source of exogenous biotin should be provided to the plant, so as to ensure labeling is not limited by endogenous biotin levels. We assessed various methods of treating mycorrhizal roots with exogenous biotin, including varying the incubation time, and exploring the best method to supply exogenous biotin. To treat plants with exogenous biotin Mair et al. (2019) investigated vacuum infiltration of plant tissue with a biotin solution and, submerging plant tissues in a biotin solution for one hour. Their work demonstrated that vacuum infiltration of a biotin solution is not necessary to achieve maximal labelling (Mair et al., 2019). A primary issue we identified at the outset of this research was a concern that removal of plants from their containers prior to labelling would inevitably rip and damage plant roots and extra-radical (external) hyphae, disrupting the interactions between symbionts and potentially causing a leakage of fungal proteins into the surrounding environment within root cells, leading to inaccurate results (ie., false positives). Our objective was to establish (if possible) a protocol that would enable efficient labelling of proteins while not disrupting the mycorrhizal roots and extra-radical hyphal networks.

i. Exogenous biotin is required for TurboID to efficiently label proteins

Instead of removing plants from their containers to submerge the root systems into a biotin solution, we decided to trial a less invasive approach in which we applied exogenous biotin by ‘soaking’ root systems via submerging the entire container into a beaker containing a biotin solution (or DMSO control). In other words, the plants (and root systems) remain undisturbed in their original container. The beaker and container would then be returned to their growth chamber to incubate for a set amount of time.

An unintended advantage of the TurboID labelling system is that TurboID self-biotinylates, which can be used as a proxy to assess extent of labelling and expression of this biotin ligase. The TurboID-YFP-NLS fusion used in these experiments is a known size (65 kDa) and by performing streptavidin and anti-GFP immunoblots (using an anti-GFP antibody that binds YFP) we can identify TurboID to ensure that it is successfully being expressed in this system.

In our first experiment we investigate two parameters: First, whether TurboID is being expressed in our transformed roots, and whether we can detect it via western blotting. And second, whether the 'soaking' treatment is an effective method to add exogenous biotin and enables effective labelling of proteins. Our experiment confirmed that TurboID is expressed in this system and that the addition of exogenous biotin by 'soaking' results in more biotinylated proteins (Figure 7) than no exogenous biotin (ie., DMSO control as biotin is typically dissolved in DMSO). This experiment used roots transformed with the pkm43GWRR empty vector (both uncolonized and colonized by *R. irregularis*), and TurboID-YFP-NLS and TurboID-YFP-NES expressing roots colonized by *R. irregularis*, comparing plants that were treated with either 250 µM biotin or water. To soak plants, containers were submerged into their designated solution for two hours at 25°C. The immunoblot (Figure 7) demonstrates that, as expected, both the nuclear and cytoplasm localized TurboID is expressed within this system. And soaking plants with biotin is an effective treatment to enable labeling of proteins within transformed roots. Though not conclusive, we were able to visually validate that when TurboID is localized to the host nucleus, it labels different proteins than when it's localized to the host cytosol (Figure 7). Many proteins are detected in both the pkm43GWRR (empty vector) and TurboID samples when treated with or without exogenous biotin and these likely correspond to naturally biotinylated proteins or proteins that are otherwise bound by streptavidin antibody.

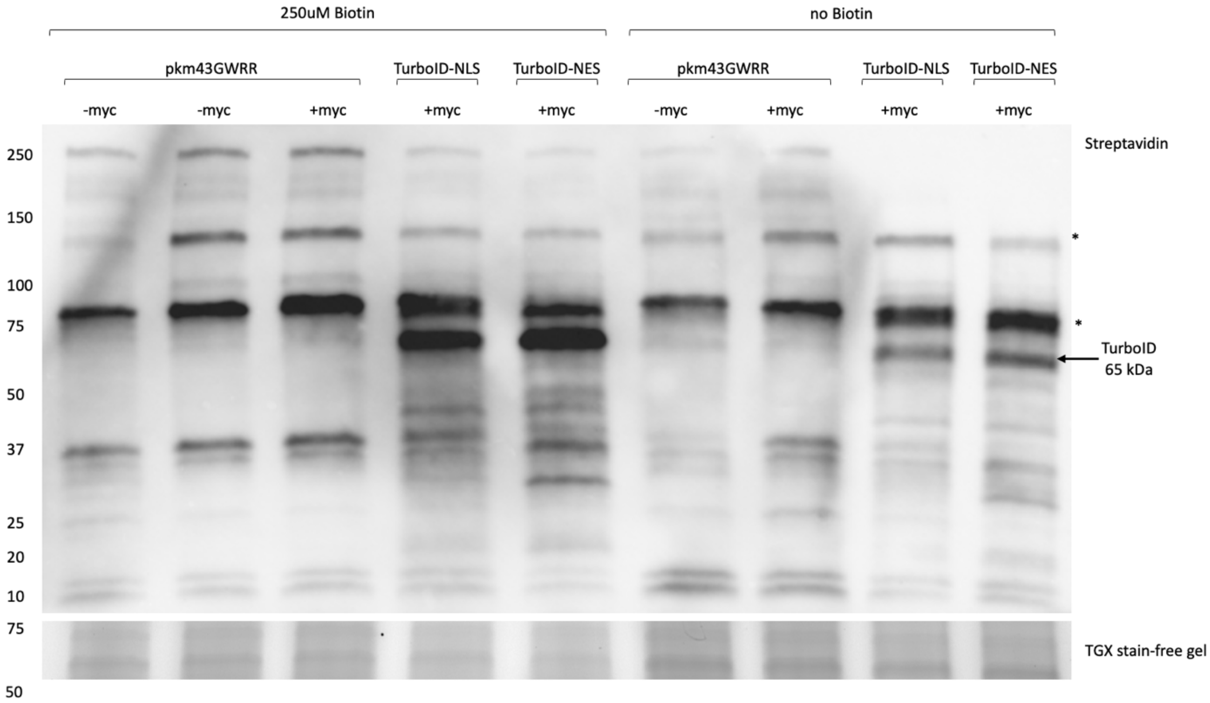


Figure 7. Different proteins are identified when TurboID is localized to the nucleus versus the cytoplasm and, 'soaking' is an effective method to add exogenous biotin. Streptavidin immunoblot from crude protein extraction of plant roots expressing TurboID-YFP localized to either the nucleus or cytoplasm and colonized by *R. irregularis* treated both with and without biotin by 'soaking'. pkm43GWRR is present as an empty vector negative control. +Myc indicates 200 spores of *R. irregularis* present in sample, or -Myc indicates no spores present in the sample. TGX stain-free gel present as a loading control. Asterix indicates naturally biotinylated proteins.

ii. Watering Plants with Exogenous Biotin is Suitable

After performing this initial experiment, we decided to test a higher concentration of biotin and compare treating the plants through two different techniques we called ‘watering’ and ‘soaking’ (Figure 8). Note, in both ‘soaking’ and ‘watering’, plants remain in their original containers, solutions are adjusted to 25°C, and incubations occurred inside the growth chambers in which the plants had been maintained for around eight weeks prior to the experiment.

Soaking: Submerge plants in a 500 µM biotin solution for 3 h.

Watering: Submerge plants in a 500 µM biotin solution and pour solution over plants. Leave the plants submerged for 5 minutes and then remove plants from solution. Ensure that plants have adequate drainage, and the biotin solution can drip out from the bottom of the container for at least 5 h. We tested ‘watering’ for both 5 h and 10 h.

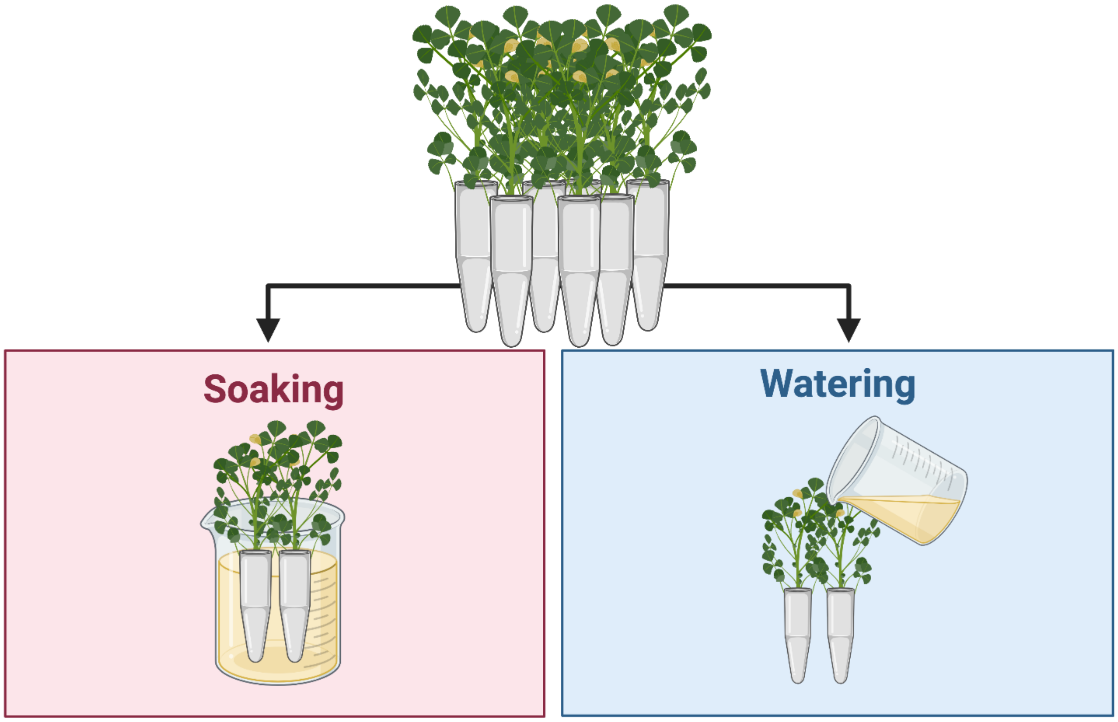


Figure 8. Addition of exogenous biotin to plant roots through two methods termed ‘soaking’ and ‘watering’. To ‘soak’ plants, the containers are submerged into a 500 µM biotin solution and left to incubate for 3 h. To ‘water’ plants, a 500 µM biotin solution or was poured over the plants and left to drip out for 5 h before harvesting the plants.

Plants expressing TurboID-YFP-NLS and the pkm43GWRR empty vector colonized by *R. irregularis* were treated by either watering or soaking with 500 µM biotin or 500 µM DMSO. We wanted to figure out if the 'watering' technique could be used to label proteins just as well as the 'soaking' technique. After treating plants by either 'watering' or 'soaking' their streptavidin immunoblot demonstrates that 'watering' is just as effective as the 'soaking' technique (Figure 9). We were satisfied with this result, as the 'watering' technique is more natural for the plants, whereas the submerging of root systems into a biotin solution for several hours may induce a stress response that could alter both the plant and fungal proteomes.

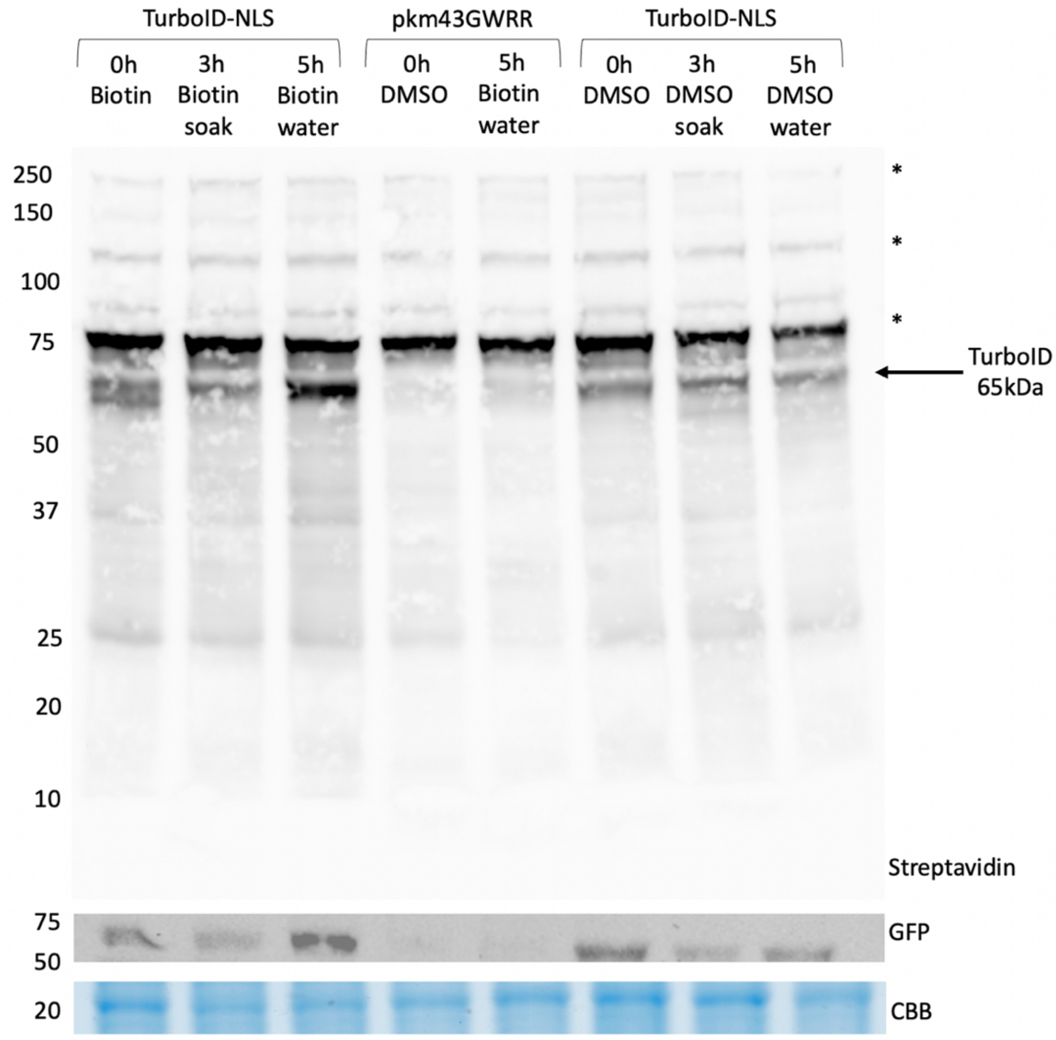


Figure 9. Immunoblots to assess proximity-based labelling in plant roots colonized by *R. irregularis*, treated with biotin solution via 'watering' and 'soaking'. A TurboID-YFP-NLS fusion was expressed in plant roots, under control of the *pMtPT4* promoter. *M. truncatula* plants transformed with an empty vector, pkm43GWRR, were included as a negative control. Plants were inoculated with 500 spores of *R. irregularis* and were harvested once colonization reached 30 - 40 % root length. Exogenous biotin was added by either 'soaking' or 'watering' the plants (See Figure 8). Coomassie brilliant blue (CBB) stain present as a loading control. Asterix indicates naturally biotinylated proteins.

iii. Watering plants for 10 h with exogenous biotin is optimal

Next, we decided to assess the effect of increasing incubation time of ‘watering’ roots with exogenous biotin. Using the watering technique, exogenous biotin was applied to plants, and they were left to incubate for both 5 h and 10 h at 25°C in a growth chamber. Plants expressing TurboID-YFP-NLS and colonized by *R. irregularis* were used in this experiment to compare treatments. These experiments demonstrated that the ‘watering’ treatment for 10 h results in increased labelling of proteins compared to plants treated with biotin after 0 h, 5 h, and 10 h incubation (Figure 10). From this experiment we concluded that ‘watering’ with biotin followed by a 10 h incubation period is appropriate. After optimizing these methods, all further experiments were performed using the ‘watering’ technique for 10 h with exogenous biotin to identify both plant and fungal proteins.

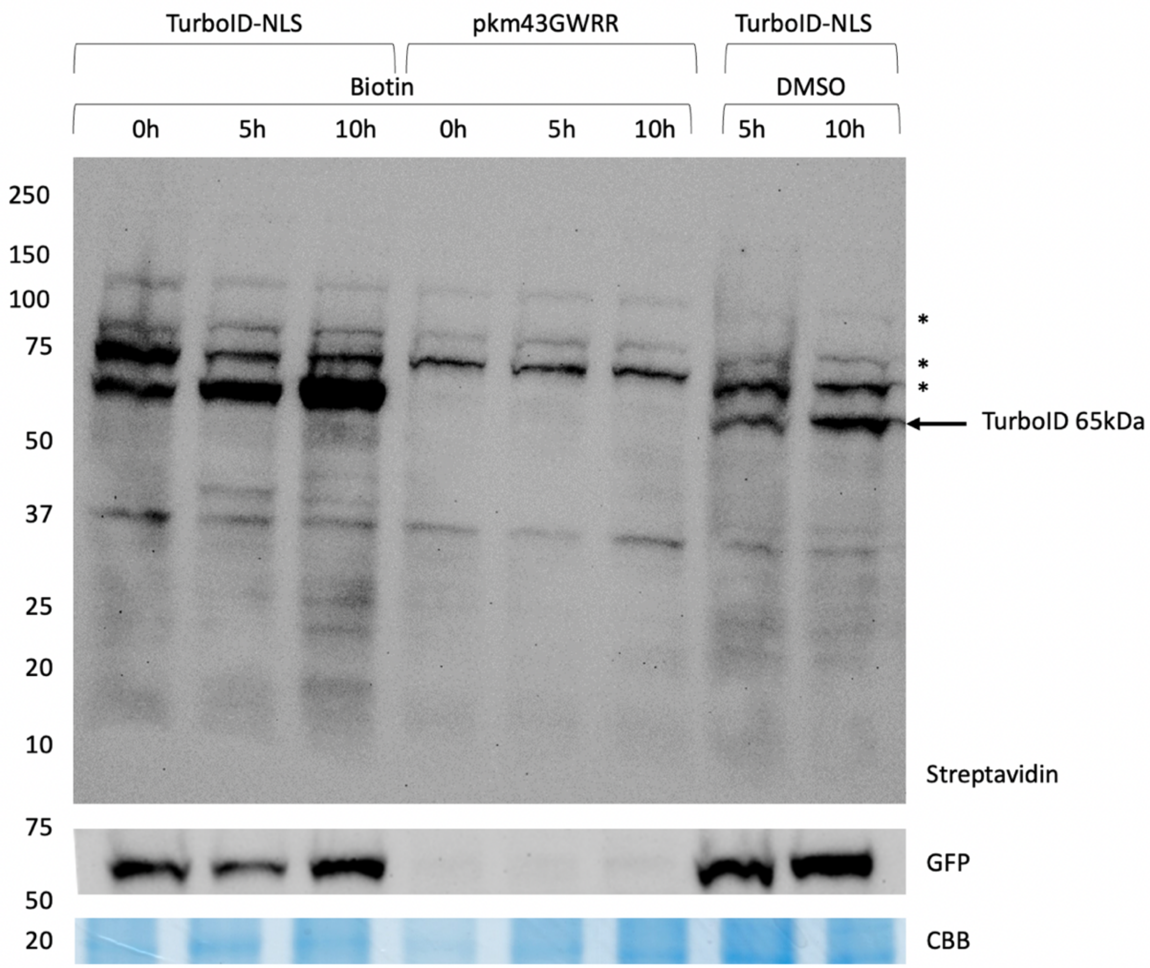


Figure 10. Immunoblots to assess proximity-based labeling in root systems colonized by *R. irregularis,* treated with an exogenous biotin solution via 'watering'. Plants expressing *pMtPT4*-TurboID-YFP-NLS-nosT or pkm43GWRR that were 40% colonized by *R. irregularis* were harvested after 'watering' and incubation for 5 h and 10 h with either 500 µM biotin or 500 µM DMSO at 25°C. Activity of TurboID was visually determined using a streptavidin immunoblot. Anti-GFP antibody present to show expression of TurboID. Coomassie brilliant blue (CBB) stain present as a loading control. Asterix indicates naturally biotinylated proteins.

After developing appropriate conditions to apply exogenous biotin, we performed the subsequent experiments by ‘watering’ plants with exogenous biotin for 10 h incubation at 25°C in the growth chamber where the plants were previously growing. All plants were transformed to express the *pMtPt4* promoter followed by TurboID-YFP-NLS or TurboID-NES-YFP alongside the pkm43GWRR empty vector negative control. Plants were then colonized by either *R. irregularis* or *G. versiforme*. To identify labelled proteins, we purified biotinylated proteins (both plant and fungal) from ground root samples and employed mass spectrometry.

II. Identification of *Rhizophagus irregularis* Proteins

Using mass spectrometry to identify proteins, the plants expressing TurboID-YFP-NLS and pkm43GWRR empty vector colonized by *R. irregularis* identified a total of 68 *R. irregularis* proteins. Of the 68 proteins, only 24 proteins were found exclusively within the plants expressing TurboID-YFP-NLS (Table 2, Figure 7). The remaining 44 proteins were either found in both the TurboID-YFP-NLS sample and empty vector or exclusively the empty vector samples.

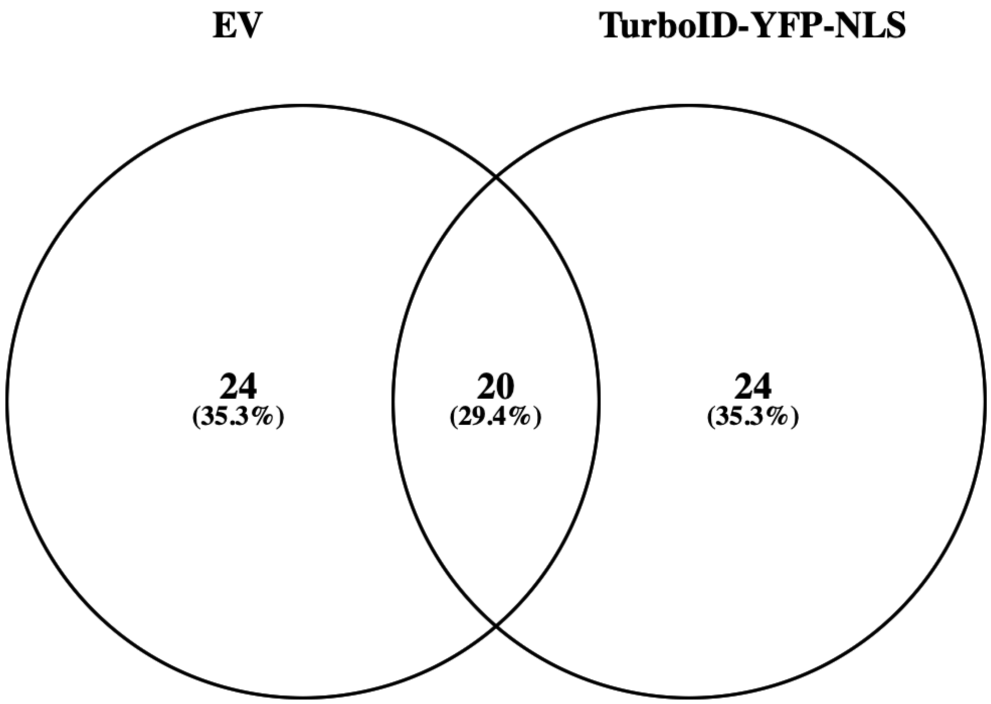


Figure 11. ***R. irregularis*** **proteins identified by mass spectrometry in plants expressing either the pkm43GWRR empty vector (EV) or, TurboID-YFP-NLS and colonized by** ***R. irregularis.*** This image was created with Venny 2.0 (Oliveros, n.d.).

Table 2. *R. irregularis* proteins identified within the TurboID-YFP-NLS sample. Annotations were accessed from DOEJI *R. irregularis* DAOM 197198 v2.0 (Chen et al., 2018; Tisserant et al., 2013). SecretomeP and SignalP websites were used to predict protein secretion. cNLS Mapper predicts the probability of a nuclear localization signal present on the identified protein.

Protein ID	Annotation	SecretomeP	SignalP	cNLS Mapper
1457184	60S ribosomal protein L38 Ribosomal L38e protein family	0.681	0.0016	Bipartite 6.3, 7.3
1476267	Unknown	0.545	0.14	Monopartite 6
1480749	Ran GTPase-activating protein Leucine rich repeat	0.183	0.0007	Bipartite 6
1490821	Unknown	0.472	0.11	Monopartite 5 Bipartite 6, 5.2
1498834	Serine/threonine protein kinase Protein kinase domain	0.836	0.26	no
1505880	Vacuolar sorting protein VPS24	0.699	0.0002	no
1520404	Membrane trafficking and cell signalling protein HRS Contains VHS and FYVE domains FYVE zinc finger	0.38	0.001	Bipartite 6.6, 5.2
1603201	Chitin synthase/hyaluronan synthase (glycosyltransferases) Chitin synthase Cytochrome b-5 like heme/steroid binding domain Glycosyl transferase Family 2	0.644	0.0012	Bipartite 5.2
1604807	Serine/threonine protein kinase Dual-specificity kinase Protein kinase domain Mg^{2+}, Co^{2+} transporter, MgtE	0.424	0.0009	no
1623510	DNA binding RNA polymerase	0.204	0.0012	Monopartite 5.5, 6.5, 6
1654193	Phospholipase D1, D Phospholipase D active site motif PLD-like domain	0.325	0.001	no
1658574	Unknown	0.784	0.0007	no
1669455	Ribosomal protein S6 kinase Non-specific serine/threonine protein kinase Protein kinase domain Mg^{2+}, Co^{2+} transporter, MgE	0.43	0.001	no
1769015	Monodehydroascorbate/ ferredoxin reductase Monodehydroascorbate reductase (NADH) Pyridine nucleotide-disulphide oxidoreductase Rieske [2Fe-2S] Reductase C-terminal	0.343	0.005	no
1769456	Predicted helicase DEAD-box superfamily Helicase conserved C-terminal domain DEAD/DEAH box helicase	0.263	0.0005	Monopartite 7.5 Bipartite 5.2, 5, 5.6, 5.7
1774368	Unknown	0.396	0.0005	no
1777450	HMG (high mobility group) box	0.168	0.0004	Bipartite 5.6, 5.7
1788523	F box like	0.206	0.0058	no
1793246	Unknown	0.663	0.0002	Bipartite 7, 7
1810914	Unknown	0.478	0.04	Bipartite 6
1835445	Unknown	0.401	0.0035	Bipartite 5.4, 5.6
1847553	Predicted AAA-ATPase	0.536	0.0006	no

1881816	Cytochrome P450/CYP11/CYP12/CYP24/CYP27 subfamilies Unspecific monooxygenase	0.345	0.0005	Monopartite 5.5, 7.5
247758	Helicases RNA helicase AAA domain	0.201	0.0033	Monopartite 5.5 Bipartite 5,6.7,6.6,6.4, 6.5,5.3,5.3,6.5,6.6

III. Identification of *Glomus versiforme* proteins

The following table (Table 3) includes all the proteins identified exclusively within the TurboID-YFP-NLS and TurboID-NES-YFP samples, colonized by *G. versiforme*.

Table 3. ***G. versiforme*** **proteins identified by mass spectrometry in TurboID-YFP-NLS and/or TurboID-NES-YFP samples and absent in the pkm43GWRR empty vector control.** Annotations accessed from NCBI using GenBank numbers (X. Sun et al., 2019). SecretomeP and SignalP predict protein secretion. cNLS Mapper predicts the probability of a nuclear localization signal present on the identified protein.

						Detected in	
Hypothetical protein	**GenBank**	**Annotation**	**SecretomeP**	**SignalP**	**NLS mapper**	**NLS (3 samples)**	**NES (4 samples)**
Glove_332g35 OR Glove_508g12	RHZ63154.1 OR RHZ49998.1	RHZ63154.1 is RabSF1-4, G1-5 box, GTP/MG2+ binding site [chemical binding] Switch I & II region Putative GEF interaction site [polypeptide binding] Putative effector interaction site, interaction site, RabF1-5	0.369 OR .375	0.0059 OR 0.0026	none for both	3	3
Glove_296g24	RHZ68265.1	Calmodulin	0.675	0.0004	none	3	4
Glove_283g97	RHZ69489.1	GluZincin Zinc binding site [ion binding]	.5	0.0008	none	0	3
Glove_8g25	RHZ90115.1	Rab GTPase family 6 G1-5 box GTP/MG2+ binding site Switch I & II region Putative GEF interaction site [polypeptide binding] Putative effector interaction site Putative GDI interaction site RabF1-5, RabSF1-4	0.649	0.0006	none	2	2
Glove_229g108	RHZ73731.1	TPR repeat [signal transduction mechanisms] and [structural motif] SLR repeat [structural motif] Sel1 Pkc like ATP binding site	.398	0.0023	none	1	2
Glove_199g195	RHZ76318.1	Unknown	.556	0.0025	none	0	2
Glove_71g180	RHZ85125.1	Unknown	.859	0.0016	none	1	2
Glove_58g5	RHZ85891.1 RHZ85890.1	Both unknown	.751 for both	0.0045 for both	none for both	0	2
Glove_271g28	RHZ70502.1	Tolloid (TLD)	0.398	0.0003	none	2	0
Glove_8g16	RHZ90072.1	Unknown	.329	0.0047	Bipartite 6.9,5	1	1
Glove_375g14	RHZ58182.1	PIN domain Putative active site	0.389	0.0021	none	2	1
Glove_271g18	RHZ70432.1	Unknown	.536	0.0003	Bipartite 6.8, 5	0	1

Identified proteins do not contain a predictable nuclear localization signal

The presence of a predicted nuclear localization signal is not always consistent with the results of our experiment. None but one of ten *G. versiforme* proteins contains a predicted nuclear localization signal with a prediction made using NLS Mapper. Meanwhile, our experiment demonstrates that the protein is localized to the nucleus. In the case of another protein, there is a predicted nuclear localization signal, however our experiment localizes the protein exclusively within the plant cytosol.

Chapter 4: Discussion

I. *In silico* Methods are Unsuccessful to Predict AMF Effectors

Previous *in silico* attempts to predict AMF effectors have been unsuccessful (Kamel et al., 2017; Sędzielewska Toro & Brachmann, 2016). The main goal of these experiments was to identify fungal effectors secreted by AMF to promote symbiosis using a proteomics approach. In the past, bioinformatics has been used to successfully predict effectors in bacterial, oomycete and fungal plant pathogens, however, when applied to AMF genomic sequences these methods have failed to discover a single *bona fide* effector protein. As a general approach to predict microbial effectors *in silico* researchers use bioinformatics to identify proteins that encode an N-terminal secretion signal (signal peptide) that indicates a given protein is likely to be secreted from the microbe. This conventional N-terminal secretion signal is 16 - 30 amino acids in length and acts to direct the protein to the endoplasmic reticulum, where the secretion signal is cleaved to generate a mature protein. As discussed earlier, bioinformatics methods have been unsuccessful in identifying effector proteins in AMF, yet research has also shown that unconventional methods may be used to secrete effector proteins (T. Liu et al., 2014; Ridout et al., 2006) that cannot yet be predicted using bioinformatics. With a growing body of evidence that bioinformatics is unsuccessful to predict AMF effectors, this points towards a different secretion method used by AMF effectors.

Predicting the subcellular localization of microbial effectors may be similarly challenging when analyses are restricted to *in silico* methods only. Caillaud et al. (2012) studied effectors from the oomycete pathogen, *Hyaloperonospora arabidopsis*, that form haustoria (structurally analogous to arbuscules in AMF) within host plant cells. They found that *in silico* predictions were only about 20 % accurate to determine the localization of the effector proteins within the plant host (Caillaud et al., 2012). Compared to bacterial effector proteins which can reliably use bioinformatics to predict nuclear localization, they predict that oomycete effectors may have evolved other ways to target the host nucleus, such as associating with endogenous host proteins (Caillaud et al., 2012). Despite the lack of a predictable nuclear localization signal, a number of fungal effectors have been characterized to localize to the host nucleus (Djamei et al., 2011; Plett et al., 2011).

The primary goal of this research project is to identify secreted AM fungal effector proteins involved in promoting the AM symbiosis. To accomplish this objective, we expressed a nuclear- and cytoplasm-localized version of TurboID in *M. truncatula* roots that were colonized by either *R. irregularis* or *G. versiforme*. Biotinylated proteins were isolated and mass spectrometry identified both fungal and plant proteins that were expressed during AM symbiosis. TurboID is expressed only within the plant cells, so any fungal proteins that are detected must have been secreted from the fungus into the plant host. Our most notable finding is the identification of two potential *G. versiforme* effector proteins.

II. Experimental Design

When designing this experiment, we had to consider that AMF are obligate biotrophs that need a host plant to survive. This caveat makes it more difficult to study the AMF symbiosis, as it requires two organisms. *R. irregularis* is a commonly used model species of AMF; it was the first AMF species to be cultured *in vitro* and its fungal spores can be kept as a powder in the fridge (Chabot et al., 1992; St-Arnaud et al., 1996). Since we use mass spectrometry to identify proteins, it is essential that we have access to high quality genome sequences of the organisms used in these experiments. Both the *R. irregularis* and *G. versiforme* genomes have been sequenced (Chen et al., 2018; X. Sun et al., 2019; Tisserant et al., 2013). After selecting these fungal species to study, we chose to use *M. truncatula* to perform this experiment. *M. truncatula* is a model legume frequently employed to study plant-microbe interactions (Barker et al., 1990; Cook, 1999) due to its ability to form root symbioses with both rhizobia and AMF. It is a self-pollinating legume that produces a lot of seed and is closely related to many crop and pasture legumes (Choi et al., 2004). It also has a small, diploid genome of approximately 500 Mbp (Tang et al., 2014; Young et al., 2012) and is relatively easy transform genetically (Floss et al., 2013; Samac & Austin-Phillips, 2006). *Agrobacterium rhizogenes* is a species of bacteria that is commonly used in plant biology to transform the roots of legumes including *M. truncatula* by inserting the genes of interest (in this experiment the gene encoding TurboID) into a plant's genome through hairy root disease (Ono & Tian, 2011). While the plant’s shoots remain unaltered, the roots systems are co-transformed with the genes of interest and a root-inducing plasmid. The root-inducing plasmid is responsible for initiating the proliferation of plant roots. Plant roots are transformed within three weeks of being infected with the *A. rhizogenes.* An advantage of transforming roots via *A. rhizogenes* versus creating stably transformed plants is that the latter process requires up to a year to produce seed from transgenic lines (Chabaud et al., 2007).

To identify roots that have been transformed to express the genes of interest, we have employed the destination vector, pkm43GWRR plasmid. This plasmid contains a dsRED gene under the control of a plant ubiquitin promoter, *AtUBQp*, ensuring uniform expression of dsRED across all plant cells. Thus, *M. truncatula* roots that have been transformed emit a fluorescent signal when visualized using a fluorescent microscope at a wavelength of 550-590 nm (Limpens et al., 2004) (Figure 5C). *M. truncatula* roots that have successfully been transformed could therefore be visually identified using a microscope. The pkm43GWRR plasmid also contains a kanamycin resistance gene, allowing for selection of transformed roots upon media supplemented with kanamycin. These characteristics make it easy to distinguish between root systems that have been successfully transformed and those that have not.

When designing this experiment, we had the option of selecting the *MtPT4p* promoter or a blue copper binding protein (*MtBCPp)* promoter to regulate the expression of TurboID. The *MtPT4p* promoter expresses gene expression specifically within the root cortex of colonized cells (Pumplin & Harrison, 2009). *MtBCPp* is expressed in cortical cells containing fungal hyphae and in neighbouring non-colonized cells. Using the *MtBCPp* promoter to direct the expression of TurboID would provide information regarding proteins expressed at different stages of arbuscule development and degradation. Instead, we chose to use the *MtPT4p* promoter, tagging all proteins involved in the symbiosis when the arbuscule is fully formed and operating. It would be interesting to compare the proteins we have identified in this work with proteins labelled in which the *MtBCPp* promoter was used to control expression of TurboID. A future labelling experiment performed using the *MtBCPp* promoter to regulate the expression of TurboID may provide further information relevant to identifying potential fungal effectors and would also capture plant proteins that are expressed at different stages of arbuscule development.

We selected TurboID as the most suitable BirA* variant to perform these experiments based on several of its defining characteristics, including the observation that it is highly active and thus has a better ability to tag low abundance proteins that are less prominent in a cell (Mair et al., 2019). In contrast, miniTurbo has a lower level of activity that is better suited to the investigation of protein-protein interactions. Because we anticipate fungal effectors to be in low abundance in plant cells, we hypothesize TurboID is the most suitable BirA* variant for this experiment.

After deciding which BirA* variant to use in the experiments, we determined where to localize the biotin ligase within the host plant cell. We were interested in targeting the nucleus since effectors within the host nucleus can directly manipulate the host cellular machinery (Poueymiro & Genin, 2009). This work suggests that effector localization within the nucleus is key to a successful symbiosis (Caillaud et al., 2012). In addition to targeting the nucleus, we were also interested in targeting the host cytoplasm, because majority of fungal effectors are secreted here (Khan et al., 2018).

III. Identified Proteins of Interest

The proteins of interest identified from experiments using plants transformed with TurboID-YFP-NLS and TurboID-NES-YFP colonized with *G. versiforme* are annotated as calmodulin (RHZ68265.1 or Glove_296g24), and a gluzincin, zinc-binding protein (RHZ69489.1 or Glove_283g97).

i. Protein of Interest: calmodulin

Calmodulin is a calcium sensor protein and has four highly conserved domains containing structural motif E-F hands. These E-F hands can also be referred to as a helix-loop-helix structure and each E-F hand bind a single calcium ion (Strynadka & James, 1989). The characteristic E-F hand domain is an essential component to calmodulin and its ability to bind calcium. The E-F hand domain is highly conserved across all eukaryotes and in the presence of calcium, calmodulin binds and undergo a conformational change, revealing two hydrophobic domains (Chin & Means, 2000). The appearance of these hydrophobic domains allow calmodulin to act as a second messenger and interact with calmodulin binding proteins to control a number of cellular processes (Berridge et al., 2000; Popescu et al., 2007). For example, calcineurin is a calmodulin binding protein and its enzymatic activity is controlled by calmodulin. When bound to calmodulin calcineurin dephosphorylates its targets (Rusnak & Mertz, 2021).

Calcium is a universal messenger and calcium signalling is used across all eukaryotic organisms to regulate cellular process. In the context of plants, calcium signalling plays a major role in plant immunity where pathogen-derived molecules effect calcium levels within a cell (Dodd et al., 2010). Other external factors including light, temperature, drought, and salt stress affect calcium levels within a plant cell too. Calmodulin has been identified to work in combination with effector proteins in other symbiotic relationships for example, the bacterial effector, HopE1 from *P. syringae* uses calmodulin as a cofactor to reduce the secretion of a plant-immunity related protein, promoting the infection of the pathogen (Guo et al., 2016). Additionally, calmodulin is essential to the role of the SF15 effector from *P. infestans.* SF15 in combination with calmodulin supresses host immunity by interfering with signalling components. Not only does the calmodulin-effector binding interfere with host immunity, but they found that binding of SF15 to calmodulin is essential to the localization of the effector within the host plant cell (Zheng et al., 2018).

Calmodulin was detected in both TurboID samples localized to the host nucleus and cytoplasm in *M. truncatula* plants colonized by *G. versiforme* and not in any of the pkm43GWRR empty vector samples*.* Within the nucleus, calmodulin may bind to another effector and together they interfere with immunity signalling pathways. This action would be similar to the HopE1 effector from *P. syringae*, or SF15 from *P. infestans*. Alternatively, calmodulin could act within the nucleus as a cofactor to facilitate the expression of genes involved in promoting the AM symbiosis. Within the cytosol, calmodulin may act as a second messenger where after binding calcium it interacts with other proteins to interfere with signalling pathways related to host-immunity. The activity of some cytosolic host proteins involved in the AM symbiosis may rely on the calcium-sensing calmodulin protein. Transporters related to the symbiosis may change their conformation when calmodulin is bound and thus, function differently. Alternatively, calmodulin may interact with other proteins to decrease host immunity upon perception of a foreign invader.

ii. Protein of Interest: gluZincin

Our second protein of interest is annotated as gluZincin, or protein containing a zinc binding site (RHZ69489.1). Zinc is an important cofactor of intracellular proteins, serving as an essential structure or filling a catalytic role. The gluzincin family contain the zinc-binding XEXXH motif that simultaneously binds water and a zinc ion, forming an active catalyst.

In response to effectors, plants have nucleotide binding and leucine-rich repeat (NBLRR) proteins that can recognize effector proteins. Some NBLRR proteins contain a plant homeodomain that bind zinc (X. Zhang et al., 2018, 2017). Zinc-finger proteins can act as transcription factors in a plant cell (W. X. Liu et al., 2013), these proteins have a characteristic arrangement of cysteine and histidine residues that have a role in binding zinc. The plant pathogen *P. aeruginosa* has a type VI secretion system where the effector Vgr2b C-terminal domain is a member of the zinc-metallopeptidase family. The Vgr2b effector disrupts the host cell membrane, having a similar effect of beta-lactam antibiotics (Wood et al., 2019). The bacterial pathogen, *Yersinia pseudotuberculosis* secretes a protein through a type VI secretion system that binds zinc (T. Wang et al., 2015). By interfering with zinc transportation, this effector manipulates host immunity. Based on these previous studies, it is possible that the zinc-binding protein identified here might have a role in interfering with plant immunity-related pathways.

Gluzincin was detected in the TurboID samples localized to the host cytoplasm in *M. truncatula* plants colonized by *G. versiforme,* and not detected in the TurboID samples localized to the host nucleus. In addition to transporting the macronutrients phosphorus and nitrogen, AMF can also transfer zinc, a micronutrient into host plant cells (Cardini et al., 2021; Kothari et al., 1991). The level of zinc in a cell must be tightly regulated and excessive levels of zinc can be toxic (Sinclair & Krämer, 2012). It’s possible that arbuscules transfer zinc bound to a zinc binding protein into a host plant. Monitoring the zinc levels is crucial and it’s possible that AMF secrete a zinc-binding protein bound to zinc to facilitate the transfer to the plant without effecting free zinc levels within the host plant cell.

Altogether, both calmodulin and gluzincin have the potential to be involved in the AMF symbiosis as effectors and future work will investigate these proteins and their role in the symbiosis.

iii. Proteins of Interest from R. irregularis

We identified 24 proteins within the TurboID-YFP-NLS plants colonized by *R. irregularis* that were not detected in the empty vector negative control samples (Table 2). These 24 proteins are potential AM effectors that have a role in mediating the AM symbiosis. We do not focus as closely upon these proteins as with *G. versiforme* proteins of interest because these initial experiments with *R. irregularis* did not include technical replicates (discussed below).

Of the 24 identified proteins present exclusively within the plants expressing TurboID-YFP-NLS colonized by *R. irregularis* few similar molecular functions were identified. A majority of the protein (7) annotations consist of unknown proteins.

Two of the three previously predicted effectors from *R. irregularis* (SP7 and RiCRN1) are predicted to localize to the plant nucleus. However, our proteomics approach using TurboID-YFP-NLS transformed plants colonized by *R. irregularis* did not identify these AMF effectors. It's possible that these proteins are only present in cells without arbuscules, and that arbuscules are not a site of effector secretion. Or, alternatively, because fungal proteins are in such a low abundance relative to plant proteins within this system, these effectors may have been missed in this work. These studies also used a different approach to identify the effector localization than the approach we took in this work. To determine the localization of SP7, researchers fused a GFP tag to the protein and expressed it within the filamentous fungus *Aspergillus nidulans.* Scientists were visually able to confirm that this protein localizes to the host plant nucleus. Next, they tested whether this protein could localize within a plant cell nucleus by expressing the SP7-GFP fusion in *N. benthamiana* leaves and *M. truncatula* hairy roots. Again, scientists were able to visually confirm that the SP7-GFP fusion localizes to the plant nucleus (Kloppholz et al., 2011). To determine the localization of RiCRN1 scientists fused GFP to the protein of interest and expressed this protein fusion in *N. benthamiana* leaves (Voß et al., 2018). Their work fuses GFP to a fungal protein and expresses the fungal protein-GFP fusion within a plant cell. Meanwhile, the work described in this thesis identifies fungal proteins that are secreted within the plant host nucleus, mimicking how this interaction occurs naturally. Further work is required to verify localization and identification of the previously described effector proteins from *R. irregularis.*

The initial set of samples using *M. truncatula* plant roots expressing TurboID-YFP-NLS and colonized by *R. irregularis* were used as a trial run to test how to properly prepare the samples for mass spectrometry. Having established optimal conditions for a mass spectrometry run, we then submitted a second set of samples of *M. truncatula* plants expressing TurboID localized to either the nucleus or cytosol and colonized by *G. versiforme* with technical replicates. This second set of data that includes technical replicates allows us to extract meaningful data in terms of reproducibility of replicates. Whereas we do not have that same flexibility with the initial set of samples with *M. truncatula* plants colonized by *R. irregularis*. As a result, we do not have a couple proteins that we can focus on rather we have several interesting proteins that are likely involved in the symbiosis.

i. Identified fungal proteins do not contain a conventional N-terminal secretion signal

A majority of the fungal proteins that we identified by mass spectrometry as being present in host cells are not predicted to contain a conventional secretion signal. In total, only three AMF proteins that we identified via mass spectrometry correspond to proteins that score a low probability of encoding a signal peptide based on SignalP predictions. In contrast to SignalP, the online prediction tool SecretomeP uses common features identified in proteins that are unconventionally secreted (ie., do not contain a signal peptide). When applied to our list of proteins identified via mass spectrometry (Table 2, Table 3) this method identified six secreted proteins in the *R. irregularis* samples. Of the identified *G. versiforme* proteins, five achieved scores consistent with secretion when using SecretomeP, while none of the identified *G. versiforme* proteins were predicted to be secreted via SignalP. Altogether, these results indicate AMF may employ an unconventional secretion system to release effectors into host cells, a hypothesis that should be further investigated in future studies.

ii. Identified fungal proteins do not contain a conventional, predictable nuclear localization signal

As a means of determining whether our fungal proteins of interest encode conventional nuclear localization signals, we employed the online tool 'NLS Mapper'. Previous research has demonstrated that bioinformatics-based methods do not always successfully predict nuclear localization signals in microbial effector proteins (Caillaud et al., 2012). Furthermore, *in silico* methods may falsely identify effectors as being nuclear localized when experimental data indicates otherwise. For example, *in silico* analysis predicted a nuclear localization signal on three effectors from the bacterial plant-pathogen *Ralstonia solanacaerum* RipAD, RipD and RipL. Researchers fused YFP to each of the effectors and in *vivo* experiments revealed that rather than localizing to the nucleus, all three of the effectors were visually identified within other subcellular components (Jeon et al., 2020). Although bioinformatics has been used to predict secretion and subcellular localization of fungal effectors in pathogenic relationships, it is not always reliable. Of the fungal proteins we identified by mass spectrometry to be localized to the host nucleus, only 15 of 32 proteins possess a predicted nuclear localization signal.

Identification of proteins via mass spectrometry relies on having a high-quality genome sequence available with accurately annotated proteins. However, it is important to consider that even the best *in silico* methods may fail to identify protein-coding sequences. For this reason, the samples that we analyzed may have contained proteins that were detected by mass spectrometry but did not correspond to genome sequence data and thus were not identified. With further research this data may change, and more proteins may be identified. As a result, the samples that we analyzed may have proteins present that are not detected by mass spectrometry.

IV. Plant Proteins Involved in the AM Symbiosis

Secondary to the fungal proteins identified in this work, we have also mapped the plant proteome of *M. truncatula* cortical cells that contain mature arbuscules. Between the two experiments performed using plants expressing TurboID-YFP-NLS colonized by either *R. irregularis* or *G. versiforme* we have identified a total of nine *M. truncatula* proteins that were found in common to be expressed in both experiments exclusively within the TurboID samples and not detected in the empty vector negative control. Note that these results are limited to only TurboID-YFP-NLS transformed plants (and do not include the TurboID-NES-YFP transformed plants). Though nine proteins is a low number, most of the previously identified proteins involved in symbiosis are not localized to the nucleus (MacLean et al., 2017; Pumplin et al., 2010). The *M. truncatula* proteins identified exclusively in the TurboID-YFP-NLS sample have a role in several cellular processes. We do not yet have the data from the TurboID-NES-YFP experiment with plants colonized by *R. irregularis*. It is worth performing symbiosis assays to see if overexpressing these proteins in plant roots positively impacts the symbiosis.

Table 4. Nine *M. truncatula* proteins were identified in both experiments using TurboID plants colonized by either *R. irregularis* or *G. versiforme*. Protein name corresponds with NCBI's annotation under 'note' for the protein.

Protein Name	**Protein function**
Medtr1g089850	Putative BSD domain-containing protein (Synapse-associated protein, putative)
Medtr3g463530	Actin filament bundling protein P-115-ABP
Medtr2g103730	Phospholipase D (EC 3.1.4.4)
Medtr5g088850	Late embryogenesis abundant protein (Putative Late embryogenesis abundant protein, LEA-14)
Medtr1g100627	Uncharacterized protein
Medtr4g084250	Calcyclin-binding protein
Medtr4g086510	CXE carboxylesterase (Putative carboxylesterase) (EC 3.1.1.1)
Medtr7g096350	ABA-inducible bHLH-type transcription factor (Putative transcription factor bHLH family)
Medtr3g104850	Carbon-nitrogen family hydrolase (Putative omega-amidase) (EC 3.5.1.3)

Mass spectrometry identified 330 *M. truncatula* proteins expressed during colonization by *R. irregularis* and 345 when colonized by *G. versiforme*. 108 *M. truncatula* proteins were found exclusively within the TurboID-YFP-NLS sample in plants colonized by *R. irregularis.* While 44 *M. truncatula* proteins within TurboID-YFP-NLS or TurboID-NES-YFP when colonized by *G. versiforme.* Over half of the *M. truncatula* identified proteins were identified in both the empty vector control and TurboID-YFP-NLS samples and are likely proteins that are naturally biotinylated.

IV. Future Work

The next step of this research project is to investigate the potential roles of the fungal proteins identified in this work, based on a hypothesis that these are fungal effectors that act to promote colonization (Table 3). To further elucidate their role in mediating the AMF symbiosis, one could overexpress the proteins of interest in transformed *M. truncatula* roots and assess AMF colonization levels. By overexpressing a protein *in planta* that acts to promote colonization, we would hypothesize that higher root colonization levels might be observed in symbiosis assays. Other experiments should investigate the subcellular localization of these proteins in host root cells. By creating a recombinant fusion of YFP or GFP to the protein of interest and expressing the protein fusion in *N. benthamiana* or *M. truncatula cells*, we may gain insight into its biological role (Dixit et al., 2006).

In addition to identifying fungal proteins, this work suggests that arbuscules may be a site of effector secretion into root cortical cells. Further research must be done to investigate this possibility. It has already been established that penetration structures such as haustoria from plant pathogenic oomycetes are the site of effector secretion (S. Wang et al., 2017). Though there are clear conceptual similarities between haustoria and arbuscules, we have no prior evidence that arbuscules are a site of effector release into host cells. My research suggests that arbuscules may indeed be a site of effector secretion in AMF. In addition, it would be interesting to determine if the proteins identified in this work are also found in adjacent cells to those with arbuscules. To identify proteins in neighbouring cells one could use the *MtBCPp* promoter (explained above) rather than the *pMtPT4* promoter described in these experiments.

This work identified numerous *M. truncatula* proteins that are expressed in root cortical cells during symbiosis. It would be interesting to apply this technology to identify which plant proteins are expressed in cells with arbuscules compared to cells in a colonized mutant plant that lacks arbuscules – would the mutant that lacks arbuscules express the same proteins as an uncolonized plant? The biggest advantage of this system is the ability to target specific cells by employing a symbiosis specific promoter. By targeting specific cells, we have a higher chance of detecting interesting and symbiotically-relevant proteins that are in low abundance in the host plant cortical cells. Previous proteomics and transcriptomics work to understand the symbiosis typically involve harvesting whole root systems, which consist of both uncolonized and colonized roots. On the contrary, this is an accurate method that can pinpoint specific cells that we are interested in and provide insight regarding the AM symbiosis.

TurboID has the potential to advance the field of plant biology by identifying essential proteins involved with various cellular processes. TurboID can be used to study the proteome of organelles at various stages during colonization. Not only is this applicable to plant systems, but it also has tremendous potential for use in symbiotic relationships which can be more difficult to study, especially when organisms are obligate biotrophs and scientists have yet to figure out how to genetically manipulate these unique organisms.

V. Conclusion

We have developed a non-invasive methodology to treat mycorrhizal roots with exogenous biotin as a means of enabling proximity-based labelling experiments in plants. This approach has successfully identified candidate fungal effectors that would not have been predicted using the current technology and bioinformatics-based approaches. Moreover, our data suggests that arbuscules may be a site of effector secretion by AMF during symbiosis, however further work is required to support these hypotheses. In summary, we conclude that proximity-based labelling is a promising new technology to further identify and characterize novel proteins that play important roles in the AM symbiosis.

Literature cited

Adhikari, B. N., Hamilton, J. P., Zerillo, M. M., Tisserat, N., Lévesque, C. A., & Buell, C. R. (2013). Comparative Genomics Reveals Insight into Virulence Strategies of Plant Pathogenic Oomycetes. *PLoS ONE*, *8*(10), 1–15. https://doi.org/10.1371/journal.pone.0075072

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., … Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology*, *37*(4), 420–423. https://doi.org/10.1038/s41587-019-0036-z

Augé, R. M., Toler, H. D., & Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza*, *25*(1), 13–24. https://doi.org/10.1007/s00572-014-0585-4

Bago, B., Pfeffer, P., & Shachar-hill, Y. (2000). Carbon metabolism and transport in Arbuscular Mycorrhizas. *Plant Physiology*, *124*, 949–957.

Beman, J. M., Arrigo, K. R., & Matson, P. A. (2005). Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. *Nature*, *434*(7030), 211–214. https://doi.org/10.1038/nature03370

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., & Brunak, S. (2004). Feature-based prediction of non-classical and leaderless protein secretion. *Protein Engineering, Design and Selection*, *17*(4), 349–356. https://doi.org/10.1093/protein/gzh037

Berridge, M. J., Lipp, P., & Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nature Reviews*, *1*, 11–21.

Besserer, A., Bécard, G., Jauneau, A., Roux, C., & Séjalon-Delmas, N. (2008). GR24, a Synthetic Analog of Strigolactones, Stimulates the Mitosis and Growth of the Arbuscular Mycorrhizal Fungus Gigaspora rosea by Boosting Its Energy Metabolism. *Plant Physiology*, *148*, 402–413. https://doi.org/10.1104/pp.108.121400

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., … Séjalon-Delmas, N. (2006). Strigolactones Stimulate Arbuscular Mycorrhizal Fungi by Activating Mitochondria. *PLoS Biology*, *4*(7), 1239–1247. https://doi.org/10.1371/journal.pbio.0040226

Bozsoki, Z., Cheng, J., Feng, F., Gysel, K., Vinther, M., Andersen, K. R., … Stougaard, J. (2017). Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception. *PNAS*, *114*(38), 8118–8127. https://doi.org/10.1073/pnas.1706795114

Branon, T. C., Bosch, J. A., Sanchez, A. D., Udeshi, N. D., Svinkina, T., Carr, S. A., … Ting, A. Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nature Biotechnology*, *36*(9), 880–898. https://doi.org/10.1038/nbt.4201

Bravo, A., Brands, M., Wewer, V., Dörmann, P., & Harrison, M. J. (2017). Arbuscular mycorrhiza-specific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. *New Phytologist*, *214*(4), 1631–1645. https://doi.org/10.1111/nph.14533

Breuillin-Sessoms, F., Floss, D. S., Karen Gomez, S., Pumplin, N., Ding, Y., Levesque-Tremblay, V., … Harrison, M. J. (2015). Suppression of arbuscule degeneration in Medicago truncatula phosphate transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3. *Plant Cell*, *27*(4), 1352–1366. https://doi.org/10.1105/tpc.114.131144

Bucher, M. (2007). Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. *New Phytologist*, *173*(1), 11–26. https://doi.org/10.1111/j.1469-8137.2006.01935.x

Caillaud, M. C., Piquerez, S. J. M., Fabro, G., Steinbrenner, J., Ishaque, N., Beynon, J., & Jones, J. D. G. (2012). Subcellular localization of the Hpa RxLR effector repertoire identifies a tonoplast-associated protein HaRxL17 that confers enhanced plant susceptibility. *Plant Journal*, *69*(2), 252–265. https://doi.org/10.1111/j.1365-313X.2011.04787.x

Cardini, A., Pellegrino, E., Declerck, S., Calonne-Salmon, M., Mazzolai, B., & Ercoli, L. (2021). Direct transfer of zinc between plants is channelled by common mycorrhizal network of arbuscular mycorrhizal fungi and evidenced by changes in expression of zinc transporter genes in fungus and plant. *Environmental Microbiology*, *00*. https://doi.org/10.1111/1462-2920.15542

Catanzariti, A. M., Dodds, P. N., Lawrence, G. J., Ayliffe, M. A., & Ellis, J. G. (2006). Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell*, *18*(1), 243–256. https://doi.org/10.1105/tpc.105.035980

Chabaud, M., Ratet, P., Araújo, S. D. S., Sofia, A., Lopes, R., Duque, A., … Barker, D. G. (2007). Agrobacterium tumefaciens-mediated transformation and in vitro plant regeneration of M. truncatula. In *Medicago truncatula handbook* (pp. 1–34).

Chabot, S., Bécard, G., & Piché, Y. (1992). Life Cycle of Glomus Intraradix in Root Organ Culture . *Mycologia*, *84*(3), 315–321. https://doi.org/10.1080/00275514.1992.12026144

Chapman-Smith, A., & Cronan, J. E. (1999). Molecular biology of biotin attachment to proteins. *Journal of Nutrition*, *129*, 477–484. https://doi.org/10.1093/jn/129.2.477s

Chen, E. C. H., Morin, E., Beaudet, D., Noel, J., Yildirir, G., Ndikumana, S., … Corradi, N. (2018). High intraspecific genome diversity in the model arbuscular mycorrhizal symbiont Rhizophagus irregularis. *New Phytologist*, *220*(4), 1161–1171. https://doi.org/10.1111/nph.14989

Chin, D., & Means, A. R. (2000). Calmodulin: A prototypical calcium sensor. *Trends in Cell Biology*, *10*(8), 322–328. https://doi.org/10.1016/S0962-8924(00)01800-6

Choi, H. K., Kim, D., Uhm, T., Limpens, E., Lim, H., Mun, J. H., … Cook, D. R. (2004). A Sequence-Based Genetic Map of Medicago truncatula and Comparison of Marker Colinearity with M. sativa. *Genetics*, *166*(3), 1463–1502. https://doi.org/10.1534/genetics.166.3.1463

Conlan, B., Stoll, T., Gorman, J. J., Saur, I., & Rathjen, J. P. (2018). Development of a Rapid in planta BioID System as a Probe for Plasma Membrane-Associated Immunity Proteins. *Frontiers in Plant Science*, *9*, 1–13. https://doi.org/10.3389/fpls.2018.01882

De Jonge, R., Bolton, M. D., & Thomma, B. P. H. J. (2011). How filamentous pathogens co-opt plants: The ins and outs of fungal effectors. *Current Opinion in Plant Biology*, *14*(4), 400–406. https://doi.org/10.1016/j.pbi.2011.03.005

De Wit, P. J. G. M., Mehrabi, R., Van Den Burg, H. A., & Stergiopoulos, I. (2009). Fungal effector proteins: Past, present and future: Review. *Molecular Plant Pathology*, *10*(6), 735–747. https://doi.org/10.1111/j.1364-3703.2009.00591.x

Deslandes, L., & Rivas, S. (2011). The plant cell nucleus. *Plant Signaling & Behavior*, *6*(1), 42–48. https://doi.org/10.4161/psb.6.1.13978

Dixit, R., Cyr, R., & Gilroy, S. (2006). Using intrinsically fluorescent proteins for plant cell imaging. *Plant Journal*, *45*(4), 599–615. https://doi.org/10.1111/j.1365-313X.2006.02658.x

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., … Kahmann, R. (2011). Metabolic priming by a secreted fungal effector. *Nature*, *478*(7369), 395–398. https://doi.org/10.1038/nature10454

Dodd, A. N., Kudla, J., & Sanders, D. (2010). The language of calcium signaling. *Annual Review of Plant Biology*, *61*, 593–620. https://doi.org/10.1146/annurev-arplant-070109-104628

Dodds, P. N., Rafiqi, M., Gan, P. H. P., Hardham, A. R., Jones, D. A., & Ellis, J. G. (2009). Effectors of biotrophic fungi and oomycetes: Pathogenicity factors and triggers of host resistance. *New Phytologist*, *183*(4), 993–1000. https://doi.org/10.1111/j.1469-8137.2009.02922.x

Doidy, J., Grace, E., Kühn, C., Simon-Plas, F., Casieri, L., & Wipf, D. (2012). Sugar transporters in plants and in their interactions with fungi. *Trends in Plant Science*, *17*(7), 413–422. https://doi.org/10.1016/j.tplants.2012.03.009

Dong, Y., Li, Y., Zhao, M., Jing, M., Liu, X., Liu, M., … Zhang, Z. (2015). Global Genome and Transcriptome Analyses of Magnaporthe oryzae Epidemic Isolate 98-06 Uncover Novel Effectors and Pathogenicity-Related Genes, Revealing Gene Gain and Lose Dynamics in Genome Evolution. *PLoS Pathogens*, *11*(4), 1–30. https://doi.org/10.1371/journal.ppat.1004801

Ezawa, T., & Saito, K. (2018). How do arbuscular mycorrhizal fungi handle phosphate? New insight into fine-tuning of phosphate metabolism. *New Phytologist*, *220*(4), 1116–1121. https://doi.org/10.1111/nph.15187

Feng, F., Sun, J., Radhakrishnan, G. V., Lee, T., Bozsóki, Z., Fort, S., … Oldroyd, G. E. D. (2019). A combination of chitooligosaccharide and lipochitooligosaccharide recognition promotes arbuscular mycorrhizal associations in Medicago truncatula. *Nature Communications*, *10*, 1–12. https://doi.org/10.1038/s41467-019-12999-5

Floss, D. S., Schmitz, A. M., Starker, C. G., Stephen Gantt, J., & Harrison, M. J. (2013). Gene

Silencing in Medicago truncatula roots using RNAi. *Methods in Molecular Biology*, *1069*, 163–177. https://doi.org/10.1007/978-1-62703-613-9_12

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., … Barker, D. G. (2013). Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in Medicago truncatula roots and their production is enhanced by strigolactone. *New Phytologist*, *198*(1), 190–202. https://doi.org/10.1111/nph.12146

Genre, A., Chabaud, M., Faccio, A., Barker, D. G., & Bonfante, P. (2008). Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both Medicago truncatula and Daucus carota. *Plant Cell*, *20*(5), 1407–1420. https://doi.org/10.1105/tpc.108.059014

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., & Barker, D. G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in Medicago truncatula root epidermal cells before infection. *Plant Cell*, *17*(12), 3489–3499. https://doi.org/10.1105/tpc.105.035410

Gimenez-Ibanez, S., Boter, M., Fernández-Barbero, G., Chini, A., Rathjen, J. P., & Solano, R. (2014). The Bacterial Effector HopX1 Targets JAZ Transcriptional Repressors to Activate Jasmonate Signaling and Promote Infection in Arabidopsis. *PLoS Biology*, *12*(2). https://doi.org/10.1371/journal.pbio.1001792

Giovannetti, M., Avio, L., & Sbrana, C. (2010). Fungal Spore Germination and Pre-symbiotic Mycelial Growth - Physiological and Genetic Aspects. In H. Koltai & Y. Kapulnik (Eds.), *Arbuscular Mycorrhizas: Physiology and Function* (pp. 3–32). https://doi.org/10.1007/978-90-481-9489-6_1

Giraldo, M. C., & Valent, B. (2013). Filamentous plant pathogen effectors in action. *Nature Reviews Microbiology*, *11*, 800–814. https://doi.org/10.1038/nrmicro3119

Guo, M., Kim, P., Li, G., Elowsky, C. G., & Alfano, J. R. (2016). A Bacterial Effector Co-opts Calmodulin to Target the Plant Microtubule Network. *Cell Host and Microbe*, *19*(1), 67–78. https://doi.org/10.1016/j.chom.2015.12.007

Haldar, S., & Sengupta, S. (2016). Microbial Ecoloogy at Rhizosphere: Bioengineering and Future Prospective. In D. K. Choudhary, A. Varma, & N. Tuteja (Eds.), *Plant-Microbe Interaction: An Approach to Sustainable Agriculture*. https://doi.org/10.1007/978-981-10-2854-0

Han, S., Udeshi, N. D., Deerinck, T. J., Svinkina, T., Ellisman, M. H., Carr, S. A., & Ting, A. Y. (2017). Proximity Biotinylation as a Method for Mapping Proteins Associated with mtDNA in Living Cells. *Cell Chemical Biology*, *24*(3), 404–414. https://doi.org/10.1016/j.chembiol.2017.02.002

Harrison, M. J. (2005). Signaling in the Arbuscular Mycorrhizal Symbiosis. *The Annual Review of Microbiology*, *59*, 19–42. https://doi.org/10.1146/annurev.micro.58.030603.123749

He, P., Chintamanani, S., Chen, Z., Zhu, L., Kunkel, B. N., Alfano, J. R., … Zhou, J. M. (2004). Activation of a COI1-dependent pathway in Arabidopsis by Pseudomonas syringae type III effectors and coronatine. *Plant Journal*, *37*, 589–602. https://doi.org/10.1111/j.1365-313X.2003.01986.x

Hoagland, D., & Arnon, D. . (1940). Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. *Soil Science*, (50), 463–483.

Huang, A., Tang, Y., Shi, X., Jia, M., Zhu, J., Yan, X., … Gu, Y. (2020). Proximity labeling proteomics reveals critical regulators for inner nuclear membrane protein degradation in plants. *Nature Communications*, *11*(1), 1–10. https://doi.org/10.1038/s41467-020-16744-1

Hung, V., Udeshi, N. D., Lam, S. S., Loh, K. H., Cox, K. J., Pedram, K., … Ting, A. Y. (2016). Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nature Protocols*, *11*(3), 456–475. https://doi.org/10.1038/nprot.2016.018

Ivanov, S., & Harrison, M. J. (2014). A set of fluorescent protein-based markers expressed from constitutive and arbuscular mycorrhiza-inducible promoters to label organelles, membranes and cytoskeletal elements in Medicago truncatula. *Plant Journal*, *80*(6), 1151–1163. https://doi.org/10.1111/tpj.12706

Javot, H., Penmetsa, R. V., Terzaghi, N., Cook, D. R., & Harrison, M. J. (2007). A Medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *PNAS*, *104*(5), 1720–1725. https://doi.org/10.1073/pnas.0608136104

Javot, H., Pumplin, N., & Harrison, M. J. (2007). Phosphate in the arbuscular mycorrhizal symbiosis: Transport properties and regulatory roles. *Plant, Cell and Environment*, *30*(3), 310–322. https://doi.org/10.1111/j.1365-3040.2006.01617.x

Jeon, H., Kim, W., Kim, B., Lee, S., Jayaraman, J., Jung, G., … Segonzac, C. (2020). Ralstonia solanacearum Type III effectors with predicted nuclear localization signal localize to various cell compartments and modulate immune responses in Nicotiana spp. *Plant Pathology Journal*, *36*(1), 45–53. https://doi.org/10.5423/PPJ.ER.08.2019.0227

Jonge, R. De, Esse, H. P. Van, Kombrink, A., Shinya, T., Desaki, Y., Bours, R., … Thomma, B. P. H. J. (2010). Conserved Fungal LysM Effector Ecp6 Prevents Chitin-Triggered Immunity in Plants. *Science*, *329*, 953–955.

Kamel, L., Tang, N., Malbreil, M., San Clemente, H., Le Marquer, M., Roux, C., & dit Frey, N. F. (2017). The comparison of expressed candidate secreted proteins from two arbuscular mycorrhizal fungi unravels common and specific molecular tools to invade different host plants. *Frontiers in Plant Science*, *8*, 1–18. https://doi.org/10.3389/fpls.2017.00124

Khan, M., Seto, D., Subramaniam, R., & Desveaux, D. (2018). Oh, the places they'll go! A survey of phytopathogen effectors and their host targets. *Plant Journal*, *93*(4), 651–663. https://doi.org/10.1111/tpj.13780

Kim, D. I., Birendra, K. C., Zhu, W., Motamedchaboki, K., Doye, V., & Roux, K. J. (2014). Probing nuclear pore complex architecture with proximity-dependent biotinylation. *PNAS*, *111*(24), 2453–2461. https://doi.org/10.1073/pnas.1406459111

Kloppholz, S., Kuhn, H., & Requena, N. (2011). A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Current Biology*, *21*(14), 1204–1209. https://doi.org/10.1016/j.cub.2011.06.044

Koide, R. T., & Kabir, Z. (2000). Extraradical hyphae of the mycorrhizal fungus Glomus intraradices can hydrolyse organic phosphate. *New Phytologist*, *148*(3), 511–517. https://doi.org/10.1046/j.1469-8137.2000.00776.x

Kombrink, A., & Thomma, B. P. H. J. (2013). LysM Effectors: Secreted Proteins Supporting Fungal Life. *PLoS Pathogens*, *9*(12), 1–4. https://doi.org/10.1371/journal.ppat.1003769

Kosugi, S., Hasebe, M., Tomita, M., & Yanagawa, H. (2009). Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(25), 10171–10176. https://doi.org/10.1073/pnas.0900604106

Kothari, S. K., Marschner, H., & Römheld, V. (1991). Contribution of the VA mycorrhizal hyphae in acquisition of phosphorus and zinc by maize grown in a calcareous soil. *Plant and Soil*, *131*(2), 177–185. https://doi.org/10.1007/BF00009447

Krajinski, F., Courty, P. E., Sieh, D., Franken, P., Zhang, H., Bucher, M., … Hause, B. (2014). The H+-ATPase HA1 of Medicago truncatula is essential for phosphate transport and plant growth during arbuscular mycorrhizal symbiosis. *Plant Cell*, *26*(4), 1808–1817. https://doi.org/10.1105/tpc.113.120436

Kuo, A., Kohler, A., Martin, F. M., & Grigoriev, I. V. (2014). Expanding genomics of mycorrhizal symbiosis. *Frontiers in Microbiology*, *5*, 1–7. https://doi.org/10.3389/fmicb.2014.00582

Lee, W. S., Rudd, J. J., Hammond-Kosack, K. E., & Kanyuka, K. (2014). Mycosphaerella graminicola lysm effector-mediated stealth pathogenesis subverts recognition through both cerk1 and cebip homologues in wheat. *Molecular Plant-Microbe Interactions*, *27*(3), 236–243. https://doi.org/10.1094/MPMI-07-13-0201-R

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., … Geurts, R. (2004). RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula. *Journal of Experimental Botany*, *55*(399), 983–992. https://doi.org/10.1093/jxb/erh122

Liu, T., Song, T., Zhang, X., Yuan, H., Su, L., Li, W., … Dou, D. (2014). Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5686

Liu, W. X., Zhang, F. C., Zhang, W. Z., Song, L. F., Wu, W. H., & Chen, Y. F. (2013). Arabidopsis Di19 functions as a transcription factor and modulates PR1, PR2, and PR5 expression in response to drought stress. *Molecular Plant*, *6*(5), 1487–1502. https://doi.org/10.1093/mp/sst031

Lo Presti, L., Zechmann, B., Kumlehn, J., Liang, L., Lanver, D., Tanaka, S., … Kahmann, R. (2017). An assay for entry of secreted fungal effectors into plant cells. *New Phytologist*, *213*(2), 956–964. https://doi.org/10.1111/nph.14188

Loh, K. H., Stawski, P. S., Draycott, A. S., Udeshi, N. D., Lehrman, E. K., Wilton, D. K., … Ting, A. Y. (2016). Proteomic Analysis of Unbounded Cellular Compartments: Synaptic Clefts. *Cell Press*, *166*, 1295–1307. https://doi.org/10.1016/j.cell.2016.07.041

Luginbuehl, L. H., Menard, G. N., Kurup, S., Van Erp, H., Radhakrishnan, G. V., Breakspear, A., … Eastmond, P. J. (2017). Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. *Science*, *356*(6343), 1175–1178. https://doi.org/10.1126/science.aan0081

Luginbuehl, L. H., & Oldroyd, G. E. D. (2017). Understanding the arbuscule at the heart of endomycorrhizal symbioses in plants. *Current Biology*, *27*(17), 952–963. https://doi.org/10.1016/j.cub.2017.06.042

MacLean, A. M., Bravo, A., & Harrison, M. J. (2017). Plant signaling and metabolic pathways enabling arbuscular mycorrhizal symbiosis. *Plant Cell*, *29*(10), 2319–2335. https://doi.org/10.1105/tpc.17.00555

Maillet, F., Poinsot, V., André, O., Puech-Pagés, V., Haouy, A., Gueunier, M., … Dénarié, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature*, *469*(7328), 58–64. https://doi.org/10.1038/nature09622

Mair, A., Xu, S., Branon, T. C., Ting, A. Y., & Bergmann, D. C. (2019). Proximity labeling of protein complexes and cell type-specific organellar proteomes in Arabidopsis enabled by TurboID. *ELife*, 1–45. https://doi.org/10.1101/629675

Markmiller, S., Soltanieh, S., Server, K. L., Mak, R., Jin, W., Fang, M. Y., … Yeo, G. W. (2018). Context-Dependent and Disease-Specific Diversity in Protein Interactions within Stress Granules. *Cell*, *172*(3), 590-604.e13. https://doi.org/10.1016/j.cell.2017.12.032

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., … Rudd, J. J. (2011). Analysis of two in planta expressed LysM effector homologs from the fungus mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, *156*(2), 756–769. https://doi.org/10.1104/pp.111.176347

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., & Swan, J. A. (1990). A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. *New Phytologist*, *115*(3), 495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., … Talbot, N. J. (2012). Effector-mediated suppression of chitin-triggered immunity by Magnaporthe oryzae is necessary for rice blast disease. *Plant Cell*, *24*, 322–335. https://doi.org/10.1105/tpc.111.092957

Mick, D. U., Rodrigues, R. B., Leib, R. D., Adams, C. M., Chien, A. S., Gygi, S. P., & Nachury, M. V. (2015). Proteomics of Primary Cilia by Proximity Labeling. *Developmental Cell*, *35*(4), 497–512. https://doi.org/10.1016/j.devcel.2015.10.015

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., … Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *PNAS*, *104*(49), 19613–19618. https://doi.org/10.1073/pnas.0705147104

Miyata, K., Kozaki, T., Kouzai, Y., Ozawa, K., Ishii, K., Asamizu, E., … Nakagawa, T. (2014). The bifunctional plant receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. *Plant and Cell Physiology*, *55*(11), 1864–1872. https://doi.org/10.1093/pcp/pcu129

Ökmen, B., & Doehlemann, G. (2014). Inside plant: Biotrophic strategies to modulate host immunity and metabolism. *Current Opinion in Plant Biology*, *20*, 19–25. https://doi.org/10.1016/j.pbi.2014.03.011

Oldroyd, G. E. D. (2013). Speak, friend, and enter: Signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology*, *11*(4), 252–263. https://doi.org/10.1038/nrmicro2990

Oldroyd, G. E. D., & Downie, J. A. (2006). Nuclear calcium changes at the core of symbiosis signalling. *Current Opinion in Plant Biology*, *9*(4), 351–357. https://doi.org/10.1016/j.pbi.2006.05.003

Oliveros, J. C. (n.d.). Venny. An interactive tool for comparing lists with Venn’s diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html.

Ono, N. N., & Tian, L. (2011). The multiplicity of hairy root cultures: Prolific possibilities. *Plant Science*, *180*(3), 439–446. https://doi.org/10.1016/j.plantsci.2010.11.012

Parniske, M. (2008). Arbuscular mycorrhiza: The mother of plant root endosymbioses. *Nature Reviews Microbiology*, *6*(10), 763–775. https://doi.org/10.1038/nrmicro1987

Plett, J. M., Daguerre, Y., Wittulsky, S., Vayssieres, A., Deveau, A., Melton, S. J., … Martin, F. (2014). Effector MiSSP7 of the mutualistic fungus Laccaria bicolor stabilizes the Populus JAZ6 protein and represses jasmonic acid (JA) responsive genes. *PNAS*, *111*(22), 8299–8304. https://doi.org/10.1073/pnas.1322671111

Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legué, V., Brun, A., … Martin, F. (2011). A secreted effector protein of laccaria bicolor is required for symbiosis development. *Current Biology*, *21*(14), 1197–1203. https://doi.org/10.1016/j.cub.2011.05.033

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Seay, M., Gerstein, M., … Dinesh-Kumar, S. P. (2007). Differential binding of calmodulin-related proteins to their targets revealed through high-density Arabidopsis protein microarrays. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(11), 4730–4735. https://doi.org/10.1073/pnas.0611615104

Poueymiro, M., & Genin, S. (2009). Secreted proteins from Ralstonia solanacearum: a hundred tricks to kill a plant. *Current Opinion in Microbiology*, *12*(1), 44–52. https://doi.org/10.1016/j.mib.2008.11.008

Pozo, M. J., & Azcón-Aguilar, C. (2007). Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology*, *10*(4), 393–398. https://doi.org/10.1016/j.pbi.2007.05.004

Pumplin, N., & Harrison, M. J. (2009). Live-cell imaging reveals periarbuscular membrane domains and organelle location in Medicago truncatula roots during arbuscular mycorrhizal symbiosis. *Plant Physiology*, *151*, 809–819. https://doi.org/10.1104/pp.109.141879

Pumplin, N., Mondo, S. J., Topp, S., Starker, C. G., Gantt, J. S., & Harrison, M. J. (2010). Medicago truncatula Vapyrin is a novel protein required for arbuscular mycorrhizal symbiosis. *Plant Journal*, *61*(3), 482–494. https://doi.org/10.1111/j.1365-313X.2009.04072.x

Rafiqi, M., Gan, P. H. P., Ravensdale, M., Lawrence, G. J., Ellis, J. G., Jones, D. A., … Dodds, P. N. (2017). Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell*, *22*(6), 2017–2032. https://doi.org/10.1105/tpc.109.072983

Remy, W., Taylor, T. N., Hass, H., & Kerp, H. (1994). Four hundred-million-year-old vesicular arbuscular mycorrhizae. *PNAS*, *91*, 11841–11843.

Rengel, Z., & Marschner, P. (2005). Nutrient availability and management in the rhizosphere: Exploiting genotypic differences. *New Phytologist*, *168*, 305–312. https://doi.org/10.1111/j.1469-8137.2005.01558.x

Rich, M. K., Vigneron, N., Libourel, V., Keller, J., Xue, L., Hajheidari, M., … Delaux, P.-M. (2021). Lipid exchanges drove the evolution of mutualism during plant terrestrialization. *Science*, (372), 864–868.

Ridout, C. J., Skamnioti, P., Porritt, O., Sacristan, S., Jones, J. D. G., & Brown, J. K. M. (2006). Multiple avirulence paralogues in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. *Plant Cell*, *18*(9), 2402–2414. https://doi.org/10.1105/tpc.106.043307

Rusnak, F., & Mertz, P. (2021). *Calcineurin : Form and Function*. *80*(4), 1483–1521.

Samac, D., & Austin-Phillips, S. (2006). Alfalfa (Medicago sativa L.) in: Wang K (ed). In *Agrobacterium Protocols* (Vol. 1). Retrieved from http://books.google.com/books?id=XvVgaENhs0UC&pgis=1

Sánchez-Vallet, A., Mesters, J. R., & Thomma, B. P. H. J. (2015). The battle for chitin recognition in plant-microbe interactions. *FEMS Microbiology Reviews*, *39*(2), 171–183. https://doi.org/10.1093/femsre/fuu003

Sato, T., Hachiya, S., Inamura, N., Ezawa, T., Cheng, W., & Tawaraya, K. (2019). Secretion of acid phosphatase from extraradical hyphae of the arbuscular mycorrhizal fungus Rhizophagus clarus is regulated in response to phosphate availability. *Mycorrhiza*, *29*, 599–605. https://doi.org/10.1007/s00572-019-00923-0

Schachtman, D. P., Reid, R. J., & Ayling, S. M. (1998). Phosphorus Uptake by Plants: From Soil to

Cell. *Plant Physiology*, *116*, 447–453. https://doi.org/10.1104/pp.116.2.447

Schmitz, A. A. M., Pawlowska, T. E., & Harrison, M. J. (2019). A short LysM protein with high molecular diversity from an arbuscular mycorrhizal fungus , Rhizophagus irregularis. *Mycoscience*, *60*, 63–70.

Schornack, S., Van Damme, M., Bozkurt, T. O., Cano, L. M., Smoker, M., Thines, M., … Huitema, E. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(40), 17421–17426. https://doi.org/10.1073/pnas.1008491107

Scott, M. S., Boisvert, F. M., McDowall, M. D., Lamond, A. I., & Barton, G. J. (2010). Characterization and prediction of protein nucleolar localization sequences. *Nucleic Acids Research*, *38*(21), 7388–7399. https://doi.org/10.1093/nar/gkq653

Sędzielewska Toro, K., & Brachmann, A. (2016). The effector candidate repertoire of the arbuscular mycorrhizal fungus Rhizophagus clarus. *BMC Genomics*, *17*(1). https://doi.org/10.1186/s12864-016-2422-y

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., … Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant Journal*, *64*, 204–214. https://doi.org/10.1111/j.1365-313X.2010.04324.x

Simpson, R. J. (2003). Rapid Coomassie blue staining of protein gels. *Cold Spring Harbor Protocols*, *2010*(4), 1–3. https://doi.org/10.1101/pdb.prot5413

Sinclair, S. A., & Krämer, U. (2012). The zinc homeostasis network of land plants. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1823*(9), 1553–1567. https://doi.org/10.1016/j.bbamcr.2012.05.016

Smith, S., & Read, D. (2008). *Mycorrhizal Symbiosis, 3rd Edn*. London, UK: Academic Press.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., & Fortin, J. A. (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus Glomus intraradices in an in vitro system in the absence of host roots. *Mycological Research*, *100*(3), 328–332. https://doi.org/10.1016/S0953-7562(96)80164-X

Stergiopoulos, I., & de Wit, P. J. G. M. (2009). Fungal Effector Proteins. *Annual Review of Phytopathology*, *47*(1), 233–263. https://doi.org/10.1146/annurev.phyto.112408.132637

Strynadka, N. C. ., & James, M. N. G. (1989). Crystal structures of the helix-loop-helix calcium-binding proteins. *Annual Review of Biochemistry*, *58*, 951–998. https://doi.org/10.1016/b0-08-043152-6/00344-2

Sugio, A., MacLean, A. M., & Hogenhout, S. A. (2014). The small phytoplasma virulence effector SAP11 contains distinct domains required for nuclear targeting and CIN-TCP binding and destabilization. *New Phytologist*, *202*(3), 838–848. https://doi.org/10.1111/nph.12721

Sun, J., Miller, J. B., Granqvist, E., Wiley-Kalil, A., Gobbato, E., Maillet, F., … Oldroyd, G. E. D.

(2015). Activation of Symbiosis Signaling by Arbuscular Mycorrhizal Fungi in Legumes and Rice. *Plant Cell*, *27*(3), 823–838. https://doi.org/10.1105/tpc.114.131326

Sun, X., Chen, W., Ivanov, S., MacLean, A. M., Wight, H., Ramaraj, T., … Fei, Z. (2019). Genome and evolution of the arbuscular mycorrhizal fungus Diversispora epigaea (formerly Glomus versiforme) and its bacterial endosymbionts. *New Phytologist*, *221*(3), 1556–1573. https://doi.org/10.1111/nph.15472

Takahara, H., Hacquard, S., Kombrink, A., Hughes, H. B., Halder, V., Robin, G. P., … O'Connell, R. J. (2016). Colletotrichum higginsianum extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity. *The New Phytologist*, *211*, 1323–1337. https://doi.org/10.1111/nph.13994

Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S., … Town, C. D. (2014). An improved genome release (version Mt4.0) for the model legume Medicago truncatula. *BMC Genomics*, *15*(1), 1–14. https://doi.org/10.1186/1471-2164-15-312

Thoms, D., Liang, Y., & Haney, C. (2021). Maintaining symbiotic homeostasis: how do plants engage with beneficial microorganisms while at the same time restricting pathogens? *Molecular Plant-Microbe Interactions*, 1–8. https://doi.org/10.1094/mpmi-11-20-0318-fi

Tisserant, E., Kohler, A., Dozolme-Seddas, P., Balestrini, R., Benabdellah, K., Colard, A., … Franken, P. (2012). The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. *New Phytol*, *193*, 755–769. https://doi.org/10.1080/16506079209455913

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., … Martin, F. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *PNAS*, *110*(50), 20117–20122. https://doi.org/10.1073/pnas.1322630111

Tyanova, S., Temu, T., & Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nature Protocols*, *11*(12), 2301–2319. https://doi.org/10.1038/nprot.2016.136

van Damme, M., Bozkurt, T. O., Cakir, C., Schornack, S., Sklenar, J., Jones, A. M. E., & Kamoun, S. (2012). The Irish Potato Famine Pathogen Phytophthora infestans Translocates the CRN8 Kinase into Host Plant Cells. *PLoS Pathogens*, *8*(8). https://doi.org/10.1371/journal.ppat.1002875

Vance, C. P., Uhde-Stone, C., & Allan, D. L. (2003). Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. *New Phytologist*, *157*(3), 423–447. https://doi.org/10.1046/j.1469-8137.2003.00695.x

Voß, S., Betz, R., Heidt, S., Corradi, N., & Requena, N. (2018). RiCRN1, a crinkler effector from the arbuscular mycorrhizal fungus rhizophagus irregularis, functions in arbuscule development. *Frontiers in Microbiology*, *9*, 1–18. https://doi.org/10.3389/fmicb.2018.02068

Wang, B., & Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, *16*(5), 299–363. https://doi.org/10.1007/s00572-005-0033-6

Wang, C., Reid, J. B., & Foo, E. (2018). The art of self-control – Autoregulation of plant–microbe symbioses. *Frontiers in Plant Science*, *9*, 1–8. https://doi.org/10.3389/fpls.2018.00988

Wang, S., Boevink, P. C., Welsh, L., Zhang, R., Whisson, S. C., & Birch, P. R. J. (2017). Delivery of cytoplasmic and apoplastic effectors from Phytophthora infestans haustoria by distinct secretion pathways. *New Phytologist*, 205–215. https://doi.org/10.1111/nph.14696

Wang, T., Si, M., Song, Y., Zhu, W., Gao, F., Wang, Y., … Shen, X. (2015). Type VI Secretion System Transports Zn2+ to Combat Multiple Stresses and Host Immunity. *PLoS Pathogens*, *11*(7), 1–25. https://doi.org/10.1371/journal.ppat.1005020

Wang, X. X., Hoffland, E., Feng, G., & Kuyper, T. W. (2017). Phosphate uptake from phytate due to hyphae-mediated phytase activity by arbuscular mycorrhizal maize. *Frontiers in Plant Science*, *8*, 1–8. https://doi.org/10.3389/fpls.2017.00684

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., … Birch, P. R. J. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature*, *450*(7166), 115–118. https://doi.org/10.1038/nature06203

Wood, T. E., Howard, S. A., Förster, A., Nolan, L. M., Manoli, E., Bullen, N. P., … Filloux, A. (2019). The Pseudomonas aeruginosa T6SS Delivers a Periplasmic Toxin that Disrupts Bacterial Cell Morphology. *Cell Reports*, *29*(1), 187-201.e7. https://doi.org/10.1016/j.celrep.2019.08.094

Young, N. D., Debellé, F., Oldroyd, G. E. D., Geurts, R., Steven, B., Udvardi, M. K., … Mayer, K. F. X. (2012). *The Medicago Genome Provides Insight into the Evolution of Rhizobial Symbioses*. *480*(7378), 520–524. https://doi.org/10.1038/nature10625.The

Zeng, T., Holmer, R., Hontelez, J., te Lintel-Hekkert, B., Marufu, L., de Zeeuw, T., … Limpens, E. (2018). Host- and stage-dependent secretome of the arbuscular mycorrhizal fungus Rhizophagus irregularis. *Plant Journal*, *94*(3), 411–425. https://doi.org/10.1111/tpj.13908

Zeng, T., Rodriguez-Moreno, L., Mansurkhodzaev, A., Wang, P., van den Berg, W., Gasciolli, V., … Limpens, E. (2019). A lysin motif effector subverts chitin-triggered immunity to facilitate arbuscular mycorrhizal symbiosis. *New Phytologist*, *225*(1), 448–460. https://doi.org/10.1111/nph.16245

Zhang, X., Farah, N., Rolston, L., Ericsson, D. J., Catanzariti, A. M., Bernoux, M., … Kobe, B. (2018). Crystal structure of the Melampsora lini effector AvrP reveals insights into a possible nuclear function and recognition by the flax disease resistance protein P. *Molecular Plant Pathology*, *19*(5), 1196–1209. https://doi.org/10.1111/mpp.12597

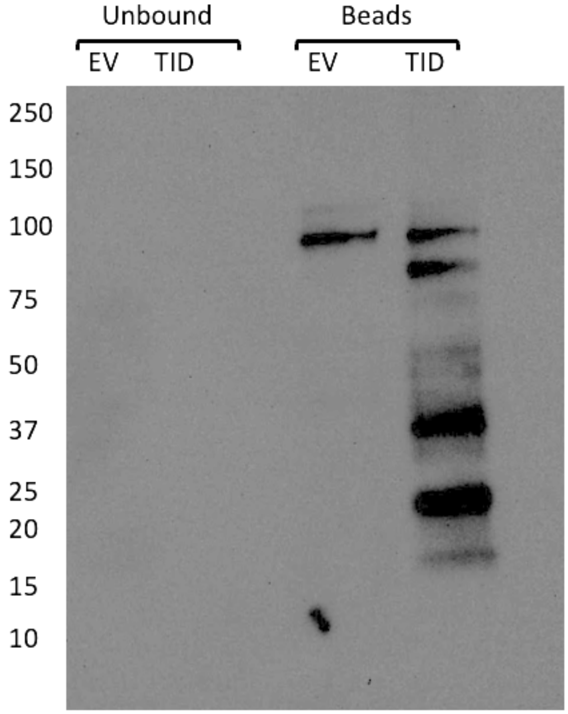
Zhang, X., Nguyen, N., Breen, S., Outram, M. A., Dodds, P. N., Kobe, B., … Williams, S. J. (2017). Production of small cysteine-rich effector proteins in Escherichia coli for structural and functional studies. *Molecular Plant Pathology*, *18*(1), 141–151. https://doi.org/10.1111/mpp.12385

Zhang, Y., Song, G., Lal, N. K., Nagalakshmi, U., Li, Y., Zheng, W., … Dinesh-Kumar, S. P. (2019). TurboID-based proximity labeling reveals that UBR7 is a regulator of N NLR immune receptor-mediated immunity. *Nature Communications*, *10*, 1–17. https://doi.org/10.1038/s41467-019-11202-z

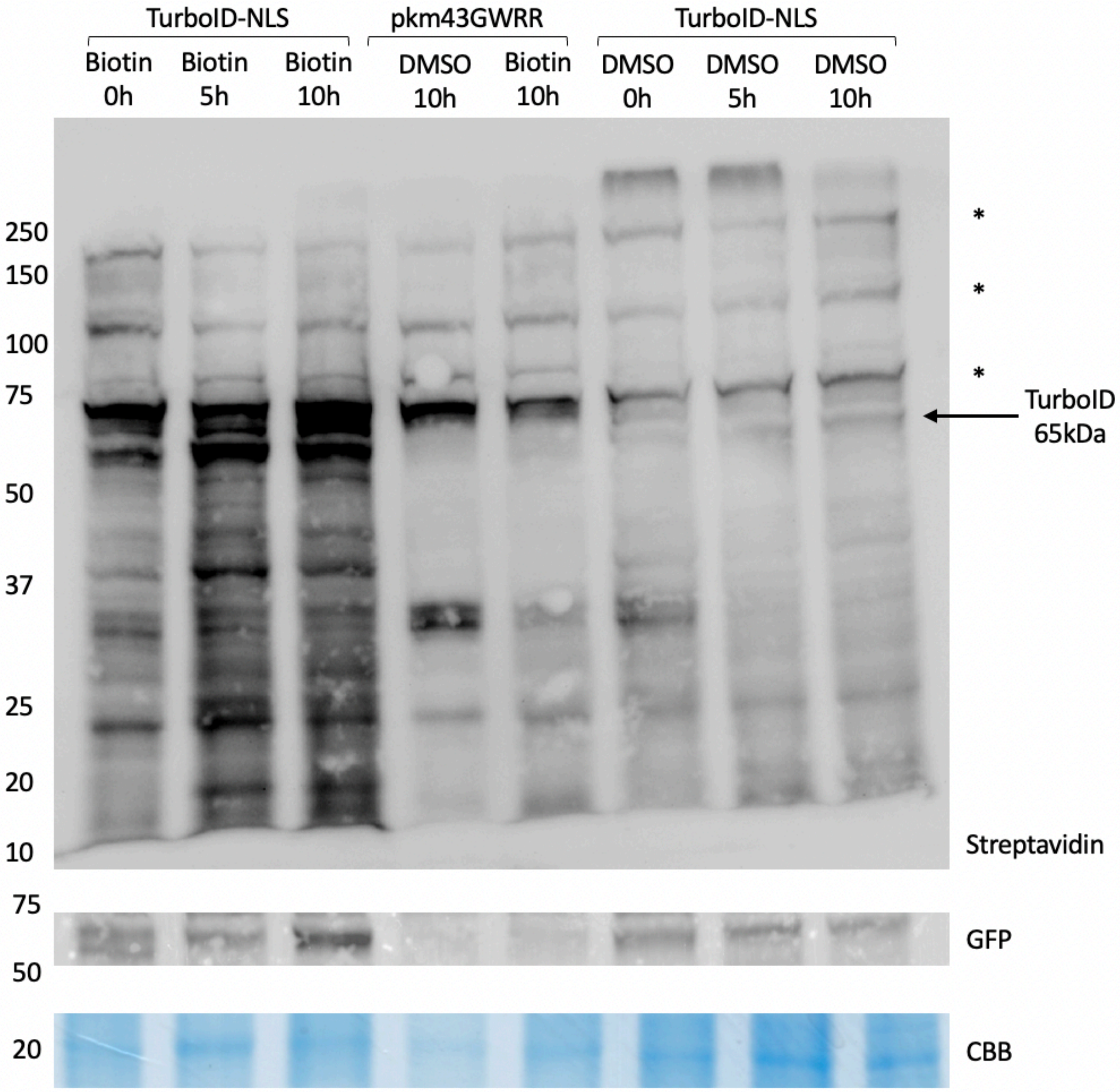
Zheng, X., Wagener, N., McLellan, H., Boevink, P. C., Hua, C., Birch, P. R. J., & Brunner, F. (2018). Phytophthora infestans RXLR effector SFI5 requires association with calmodulin for PTI/MTI suppressing activity. *New Phytologist*, *219*(4), 1433–1446. https://doi.org/10.1111/nph.15250

Zuccaro, A., Lahrmann, U., & Langen, G. (2014). Broad compatibility in fungal root symbioses. *Current Opinion in Plant Biology*, *20*, 135–145. https://doi.org/10.1016/j.pbi.2014.05.013

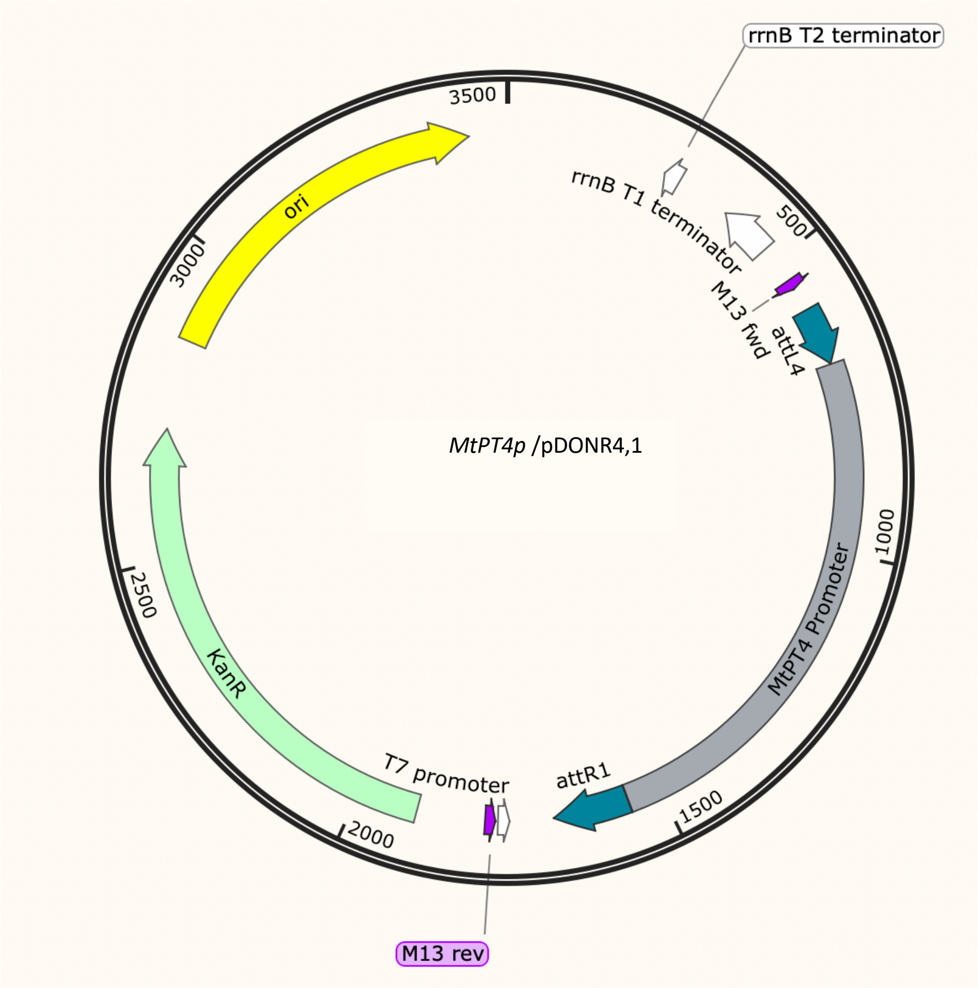
Supplementary Information



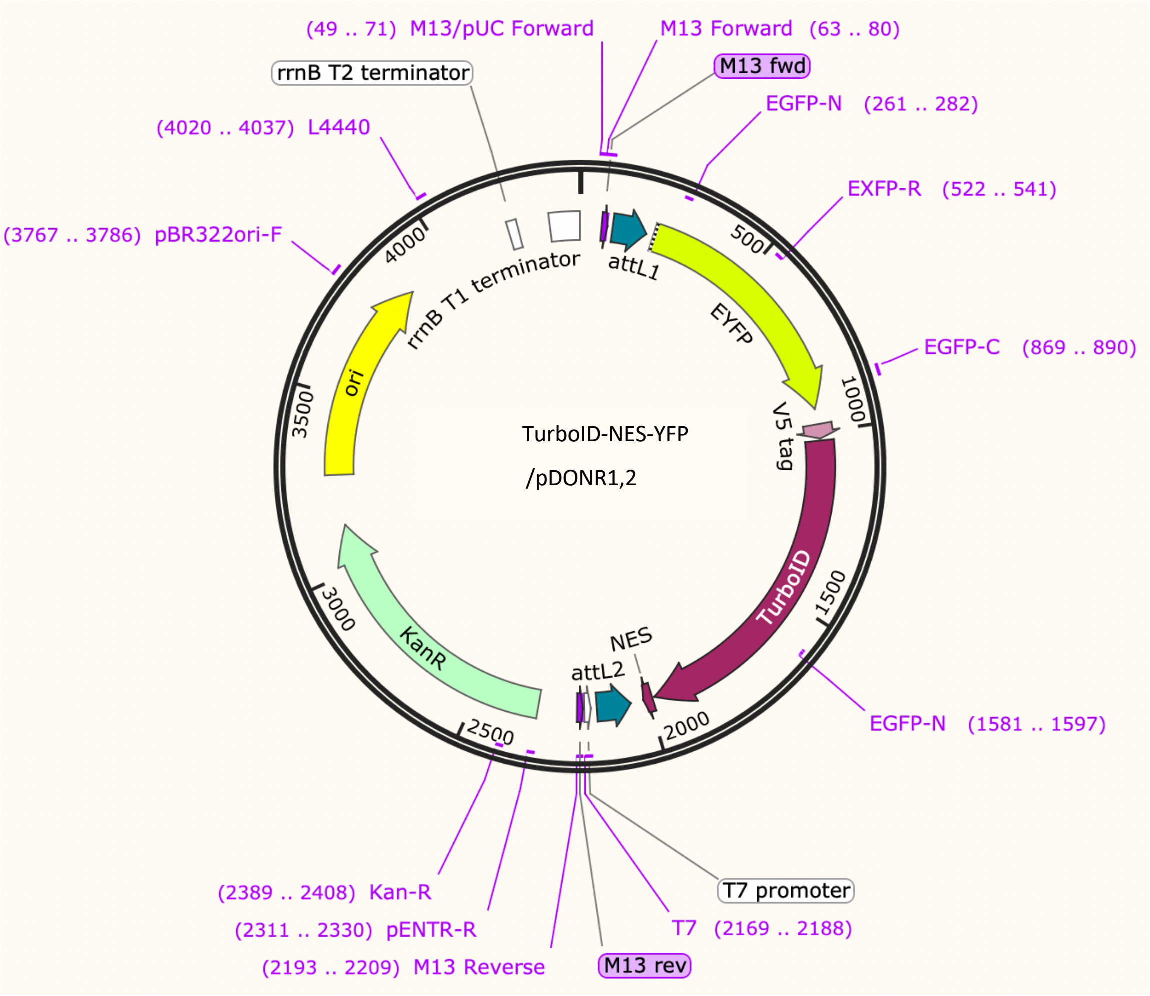
Supplementary Figure 1. Successful protein purification of biotinylated proteins within TurboID expressing plants. The plants used in this experiment were harvested after 'watering' for 5 h incubation with exogenous biotin. See materials and methods "purification of biotinylated proteins". "EV" refers to the pkm43GWRR empty vector negative control and "TID" refers to TurboID localized to the host nucleus.



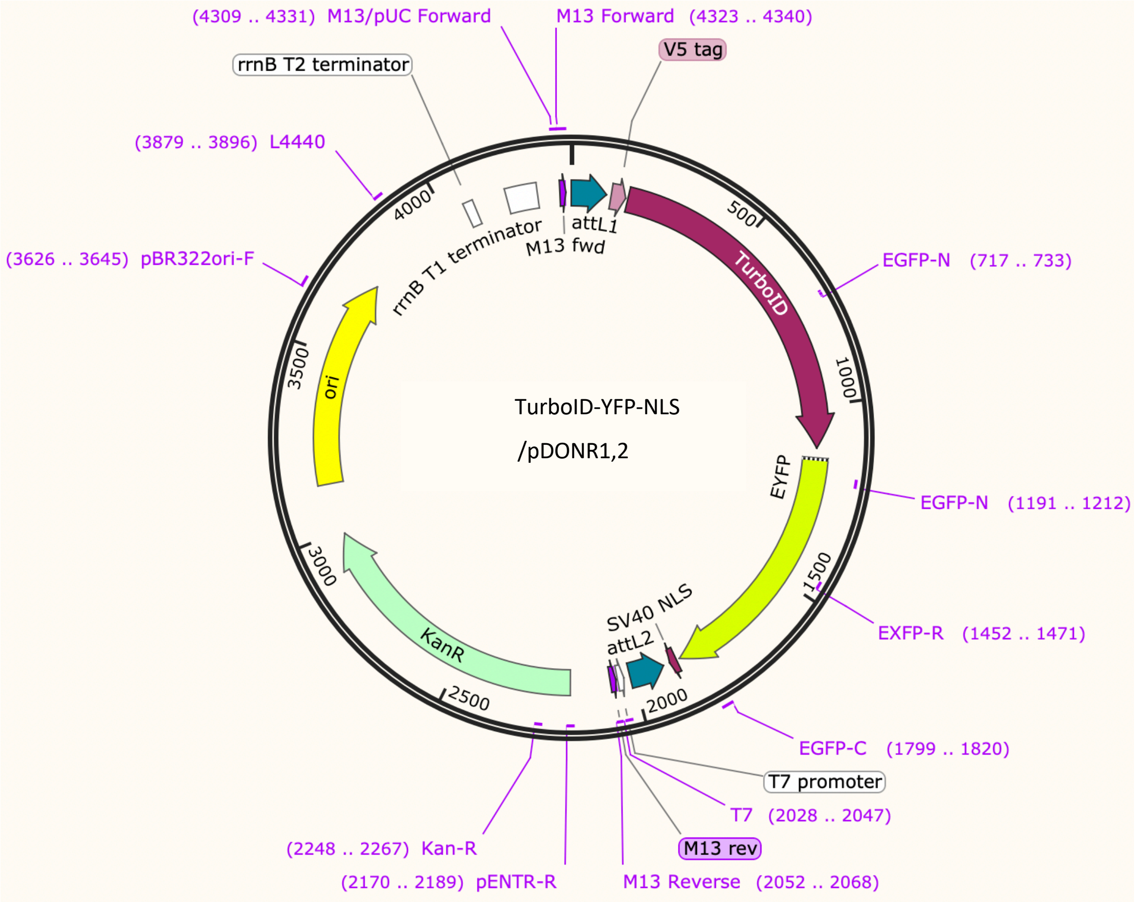
Supplementary Figure 2. Streptavidin blot of samples sent for mass spectrometry. Plants colonized by 500 spores of *R. irregularis* and transformed to express either TurboID-YFP-NLS or pkm43GWRR (empty vector). Samples were harvested using the watering technique with 500 µM biotin solution or 500 µM DMSO. Asterix indicates naturally biotinylated proteins. Anti-GFP antibody used to confirm the expression of TurboID. Coomassie brilliant blue (CBB) stain present as loading control.



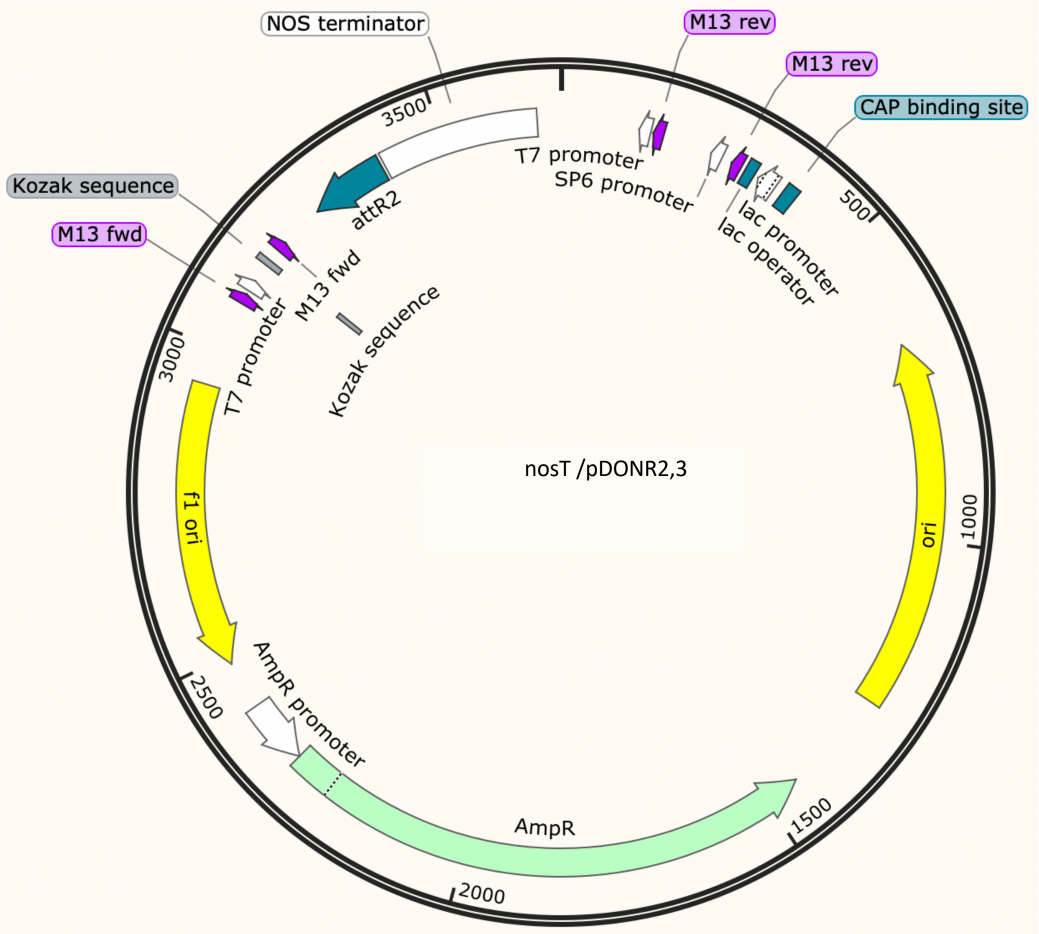
Supplementary Figure 3. Plasmid map for *MtPT4p* promoter within the pDONR4,1 plasmid.



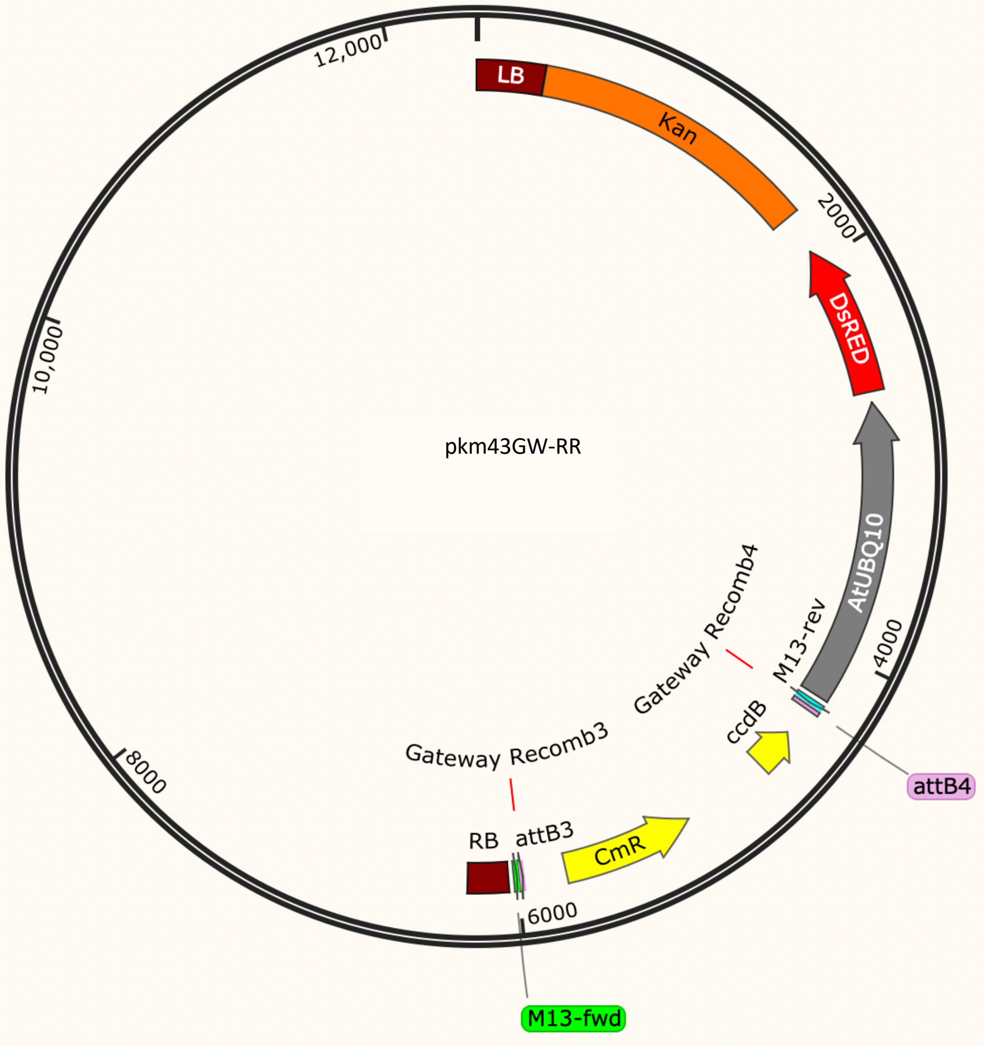
Supplementary Figure 4. Plasmid map for TurboID localized to the host cytoplasm within the pDONR1,2 plasmid.



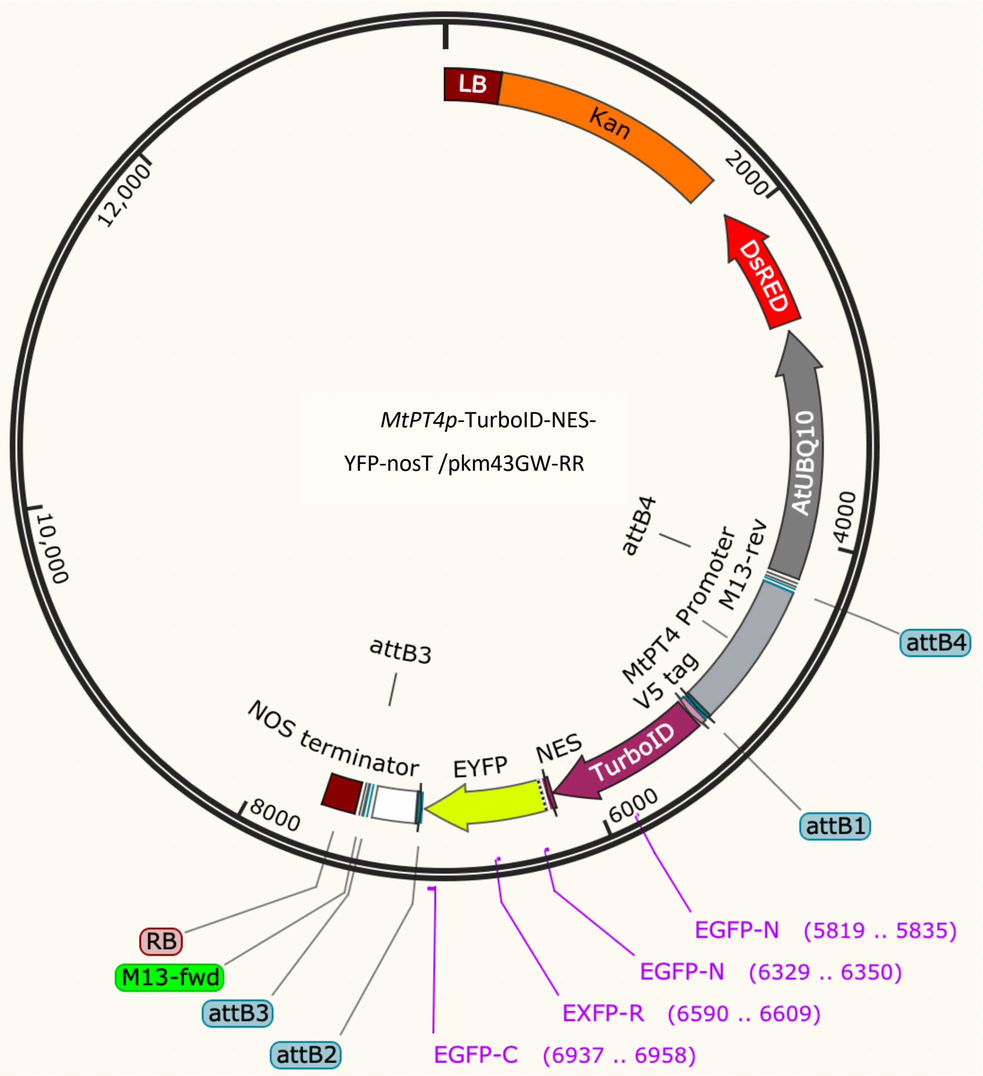
Supplementary Figure 5. Plasmid map for TurboID localized to the nucleus within the pDONR1,2 plasmid.



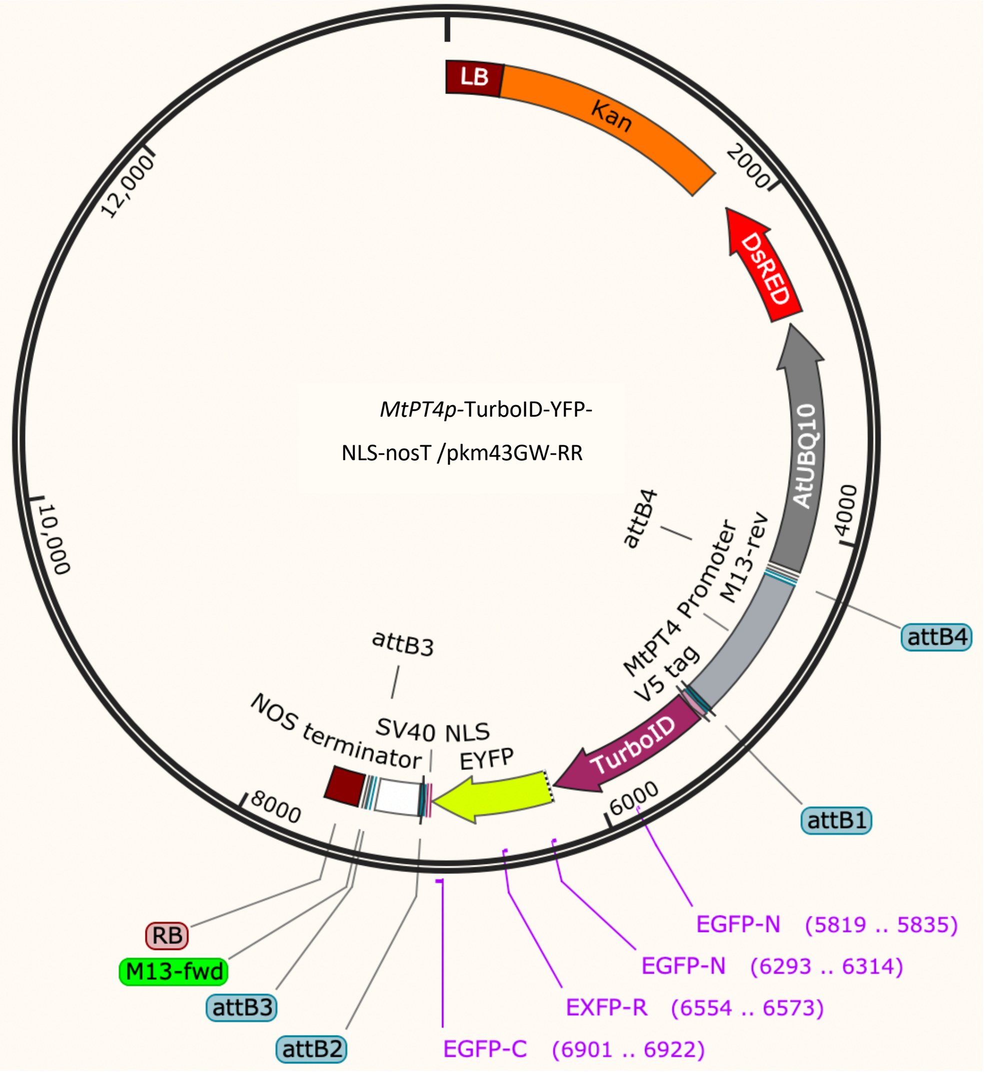
Supplementary Figure 6. Plasmid map for the nosT transcriptional terminator within the pDONR2,3 plasmid.



Supplementary Figure 7. Plasmid map for pkm43GW-RR destination vector. This plasmid was used as a negative control in experiments described in this thesis.



Supplementary Figure 8. Plasmid map for TurboID-NES-YFP and with the pkm43GW-RR destination vector and under control of the *MtPT4p* promoter.



Supplementary Figure 9. Plasmid map for the TurboID-YFP-NLS construct under control of the *MtPT4p* promoter and with the pkm43GW-RR destination vector.