Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Kathryn Hatch April 10, 2018

Double transformation of *Medicago truncatula* and analysis of biotin ligase production in root cells: towards the creation of a mycorrhiza-induced INTACT system

by

Kathryn Hatch

Roger Deal
Adviser

Biology Department

Roger Deal

Adviser

David Lynn

Committee Member

Amanda Starnes

Committee Member

2018

Double transformation of *Medicago truncatula* and analysis of biotin ligase production in root cells: towards the creation of a mycorrhiza-induced INTACT system

By

Kathryn Hatch

Roger Deal

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology Department

2018

Abstract

Double transformation of *Medicago truncatula* and analysis of biotin ligase production in root cells: towards the creation of a mycorrhiza-induced INTACT system

By Kathryn Hatch

Symbiosis between plants' root systems and mycorrhizal fungi is known to be very beneficial for both organisms. Mycorrhizae tend to benefit from a reliable source of carbon, while plants benefit with increased growth rates and resistance to many types of stressors. The most common type of mycorrhizae is arbuscular mycorrhizae (AM), which form structures within as well as around cells, and so make changes to the plants at a cellular level. One way to study these changes would be to use the Isolation of Nuclei TAgged in specific Cell Types (INTACT) method to isolate nuclei from the cells interacting with AM fungi. In this method, there are two main transgenes: *NTF,* which binds GFP and a biotin ligase recognition peptide (BLRP) to the nuclear envelope, and *BirA* which codes for biotin ligase production. When both are expressed, biotin ligase biotinylates the NTF protein, allowing for the isolation of nuclei with streptavidin-coated magnetic beads. Usually in INTACT, *NTF* is controlled by a cell type specific promotor while *BirA* is constitutive, allowing for the isolation of the nuclei of the cells in which the promotor is active. However, to study mycorrhizal colonization of roots and isolate colonized specific cell types, *BirA* would need to be controlled by a promotor induced by mycorrhizal infection. To test whether the biotinylation of nuclei is quick enough for this system to be effective, the production of inducible *BirA* needs to be studied in plant roots. This study chose to make *BirA* estrogen-inducible, as estrogen is not produced normally in plants, so the exposure could be controlled. This paper focuses on the creation of the estrogen-inducible *BirA* construct and the creation of doubly transgenic plants with constitutive *NTF* and estrogen-inducible *BirA* that can be used to test the viability of INTACT with two selective promotors. This work serves as a foundation for developing a method to allow detailed studies of gene expression and chromatin changes that take place as plant cells interact with AM fungi.

Double transformation of *Medicago truncatula* and analysis of biotin ligase production in root cells: towards the creation of a mycorrhiza-induced INTACT system

By

Kathryn Hatch

Roger Deal

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology Department

2018

Acknowledgements

Firstly, I would like to thank Roger Deal and all of the Deal lab at Emory University for their encouragement and help in brainstorming and troubleshooting, especially Marko Bajic, Paja Sijacic, and Kelsey Maher. This project would not have progressed so far without them.

Also, I would like to thank the Queitsch Lab at the University of Washington for sending us their pGreen plasmid with the constitutive NTF that we needed.

Finally, I would like to thank my friends and family for all of their encouragement and support during this whole process.

Table of Contents

List of Tables and Figures

Introduction

Mycorrhizae are a type of fungus that associate with the roots of plants and form symbiotic relationships with these plants. In this symbiosis, the fungi invade the plant roots, often benefiting the plants with increased nutrient uptake and accepting needed nutrients in return. The mycorrhizae provide a further reach than the roots of the plant, allowing for the uptake of nutrients normally not available for the plants and packaging them in a way that plants can use. In return, plants often provide organic carbon for the mycorrhizae (Smith & Read, 2008). There are two types of fungi that form this symbiosis with plants. Ectomycorrhizae tend to form associations with many types of trees and shrubs and integrate into the roots around and between cells. Arbuscular mycorrhizae (AM) associate with a wider range of species including many crop plants, inserting hyphae between cells but also penetrating root cells to form branched, tree-like arbuscular structures, for which the mycorrhizae are named, that grow within cells (Bücking, Liepold, & Ambilwade). As AM fungi are the ones most often associated with crop plants, the benefits that they provide plants are varied and often help to increase plant tolerance to stress.

Mycorrhizal colonization of the underground plant structures is an ancient symbiosis, that includes the colonization of mycorrhizae on roots of seed plants, and the underground structures of mosses and ferns (Smith & Read, 2008). There is evidence of this association between plants and fungi dating back to the early Devonian era, around 400 million years ago. Plant fossils found in the Rhynie chert deposit in Scotland from this time period show remains of arbuscular structures consistent with mycorrhizal infection (Remy et al, 1994). This fossil evidence for ancient symbiosis is also supported by ribosomal DNA sequencing of arbuscular mycorrhizae. Phylogenetic analysis suggests the origin of arbuscular mycorrhizae around 300-400 million years ago based on the divergence of arbuscular mycorrhizae from ectomycorrhizae, and the divergence arbuscular mycorrhizae into distinct groups (Simon et

al, 1993). This indicates even earlier associations between phototrophs and fungi. Evidence of ancient mycorrhizal colonization, as well as logical arguments of the stressful living conditions on land have led to the hypothesis that fungi may have helped phototrophs transition to terrestrial life (Selosse & Tacon, 1998). This idea is supported by the observation that symbiosis with fungi mitigates stress in plants in many ways.

Benefits of an Arbuscular Mycorrhizal Network

Mycorrhizae confer many benefits to plants that may help increase growth. This is so well documented that there are companies that provide seeds that come pre-coated with mycorrhizae. Companies, like *MycoGold*, sell mycorrhizal inoculums, with specific blends of fungal species for different crops (*Mycorrhizal Fungi Biological Inoculants for Farmers*, 2014). A quick search on *Amazon.com* pulls up hundreds of options for buying mycorrhizae to supplement one's soil ("Results: Mycorrhizae", accessed April 2018). There is research that supports this industry and documents some of the various ways that mycorrhizae can help plants.

In the 1970s, researchers, using onions as their plant host and the AM *Endogone* as the model fungus, preformed a simple test that displayed greater growth in plants associated with mycorrhizae than in plants with no mycorrhizal colonization (Mosse & Hayman, 1971). Although they did not go into deeper detail of how the mycorrhizae were helping the plants grow, this paper showed that this symbiotic relationship was beneficial for at least one crop plant. Other studies also supported this trend of greater growth in plants associated with mycorrhizae (Gerdemann, 1968). There are many ways that mycorrhizae could increase the growth of plants. As stated before, mycorrhizae trade nutrients with plants for carbon. One important nutrient that AM fungi help plants obtain is phosphorus. In fact, researchers found that AM fungi on the roots of wheat provide most of the phosphorus for plants' needs. Interestingly, even when there is phosphorus in the soil that is easily available for the plant to

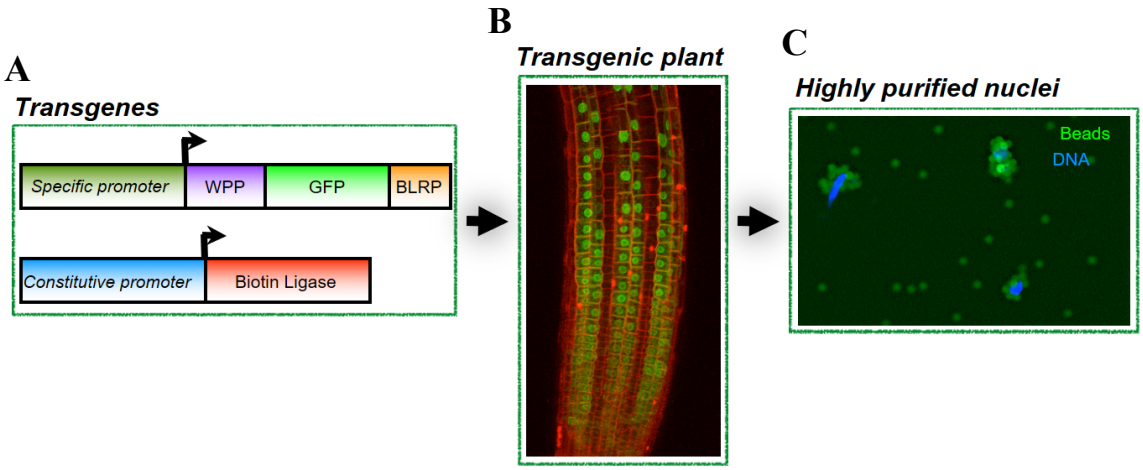
directly take up, the AM fungi still provides similar percentages of the total phosphorus uptake as when there is little available phosphorus, indicating that plants preferentially take up phosphorus from mycorrhizae (Li et al., 2006). Mycorrhizae also increase sulfur and nitrogen uptake into plants in deficient conditions, accessing sources that plants are unable to convert into usable forms (Allen & Shachar-Hill, 2009; Atul-Nayyar et al., 2009).

Arbuscular mycorrhizae can also mediate the effects that many different types of stressors have on plants. AM fungi can alleviate stress from harmful heavy metals like cadmium. Plants associated with mycorrhizae showed reduced decreases in biomass and lower amounts of cadmium-induced root isoflavonoids when compared to plants that did not have mycorrhizal colonization (Aloui et al., 2012; Schützendübel & Polle, 2002). Mycorrhizae also help protect plants against drought, which is a major stressor for many crops. Studies have found that the nutrient benefits due to mycorrhizal colonization allow plants to have decreased responses to drought stress, showing decreased loss of biomass (Tuo et al., 2017; Subramanian et al., 2005). Overall, many studies have shown the importance of the microbiome growing on the roots of plants. However, there have been few studies of the overall epigenetic and transcriptional changes in the root cells when they are invaded by the arbuscular structures of mycorrhizae. Knowing the extent of the changes in chromatin and gene expression of crop plants undergoing AM colonization will aid understanding the establishment of this symbiosis and may lead to the improvement of crop plant responses to various stressors.

Methods of Isolating Nuclei from Specific Plant Cell Types

In order to identify changes in gene expression during symbiosis, we must first be able to isolate the nuclei of the cells that are targeted by mycorrhizal infection. Isolation of Nuclei TAgged in Specific Cell Types (INTACT) is a relatively new method of cleanly isolating nuclei of specific cell types. INTACT allows for the isolation of cell type-specific

nuclei in transformed plants. There are two transgenes used for INTACT: the *Nuclear Tagging Fusion* (*NTF*) gene driven by a cell type-specific promotor, and the *BirA* gene. The NTF protein contains a nuclear envelope-targeting sequence (WPP domain of RANGAP1), green fluorescent protein (GFP), and a biotin ligase recognition peptide (BLRP) such that expression of the gene from a cell type-specific promotor results in the tagging of the nuclear envelope exclusively in that cell type. *BirA* is constitutively expressed in all cells, such that *BirA* will add a biotin molecule to NTF in the cells where they are co-expressed. The biotinylated nuclei can then be isolated by first introducing streptavidin-coated magnetic beads which bind to the biotin, then capturing the beads and biotinylated nuclei on a magnet (Deal & Henikoff, 2010).



From Wang and Deal (2015) *Methods in Molecular Biology*. 1287

Figure 1. A. Transgenes used in INTACT are a cell type-specifically expressed NTF and constitutively expressed biotin ligase introduced on one plasmid. B. *Arabidopsis thaliana* root, producing NTF under control of the cell type-specific promotor *GLABRA2* (shown in green) in the nuclei of cells non-hair epidermal cells. C. Isolated non-hair nuclei bonded to streptavidin coated beads.

However, INTACT only uses one specific promotor, for *NTF,* along with constitutively expressed *BirA*. In order to find the specific changes that occur in chromatin of certain cell types when they are infected by mycorrhizae, both NTF and biotin ligase would need specific promoters: *NTF* with the cell type-specific promotor and *BirA* with a promotor

that responds to mycorrhizal infection. This would allow the isolation of cells of a specific type that were also infected by mycorrhizae, as only one cell type would be expressing the NTF protein, while a subset of that cell type that is also infected by mycorrhizae, would be able to produce biotin ligase, allowing for the use of INTACT to isolate those very specific nuclei with streptavidin-coated beads. Purification of nuclei using of anti-GFP beads would also isolate all nuclei expressing NTF for comparison to those purified with streptavidin.

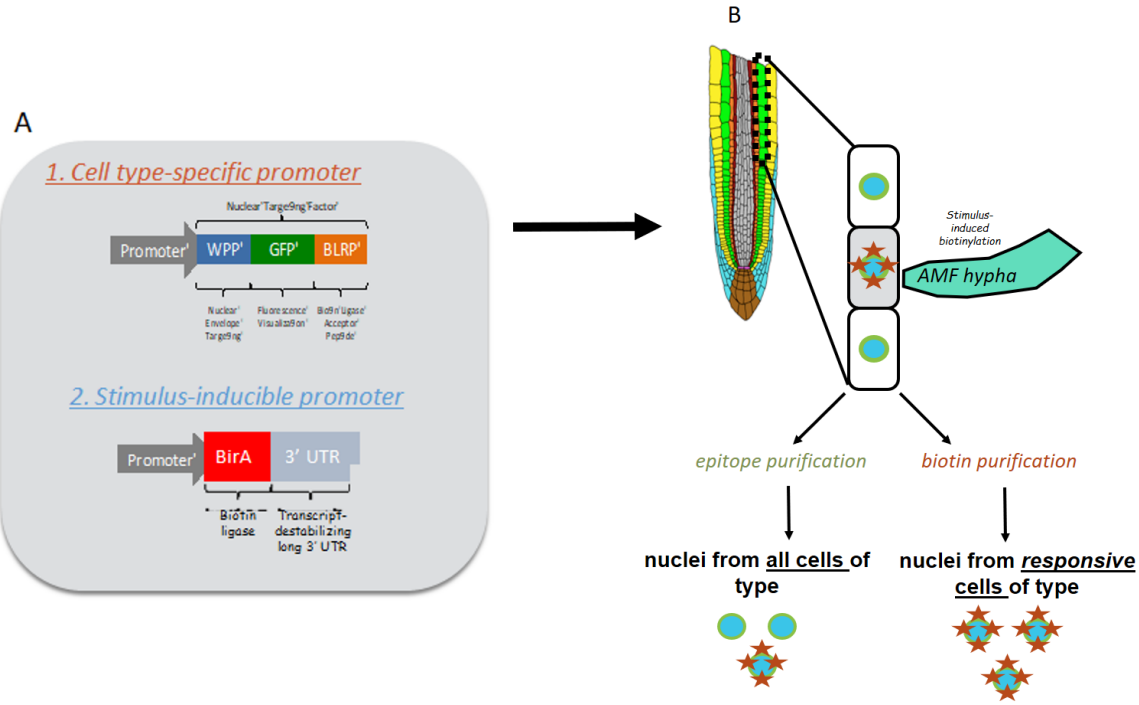


Figure 2. Isolation of nuclei using INTACT with cell type-specific promotor for *NTF* and mycorrhiza-induced promotor for *BirA*. A. Both *NTF* and *BirA* have specific promotors. B. The hyphae of AM fungi would interact with specific cells, inducing the production of biotin ligase, allowing for the biotinylation of nuclei and binding of streptavidin coated magnetic beads and the isolation of those nuclei.

Before creating this system, it was necessary to ensure that the inducible production of biotin ligase is quick enough for INTACT to be useful in identifying the first changes in chromatin and gene expression when cells are invaded by arbuscular mycorrhizae. However, as these constructs were not already made, plasmids with both genes were needed to produce double transgenic plants containing *NTF* and inducible *BirA* to test the kinetics of biotin ligase production and biotinylation of NTF-tagged nuclei.

Aims

The goal of this project is to create transgenic *Medicago truncatula*, a model organism for legumes also known as barrel clover, through a double transformation with constructs that will allow for a time course analysis of the production of biotin ligase and biotinylation of nuclei. In order to test the viability of using a stimulus-inducible *BirA* in INTACT, we will need to perform a time course analysis on the production of biotin ligase. Thus, we will first need to create a construct with estrogen-inducible *BirA* to test the production rates of biotin ligase. We chose to make *BirA* estrogen inducible for the time course analysis study, as estrogen is not produced in plants but is easy to introduce to induce biotin ligase production. As we wanted to study the kinetics of biotin ligase production and its biotinylation of NTF, we used a separate plasmid to insert a constitutively expressed *NTF* gene.

Methods

Although we did not have a plasmid containing estrogen-inducible *BirA*, we did have a plasmid, pB7WG with the *BirA* sequence, which needed to be amplified and transferred first to an entry vector, pENTR, and finally to the destination vector, pMDC7 which had an estrogen inducible promotor. Then this plasmid could be taken up by competent *Agrobacterium rhizogenes,* a tumor inducing bacteria capable of transferring plasmid DNA to plant genomes and also inducing the production of new roots containing these transgenes.

Creating an Estrogen-inducible BirA plasmid

To create a plasmid with estrogen-inducible *BirA*, the *BirA* gene sequence was amplified first from two different plasmids (New Intact and pB7WG), as one did not seem to be the correct length initially, using the Q5® High-Fidelity DNA Polymerase (M0491). Primers included the specific CCAC overhang to match the overhangs on the TOPO plasmid

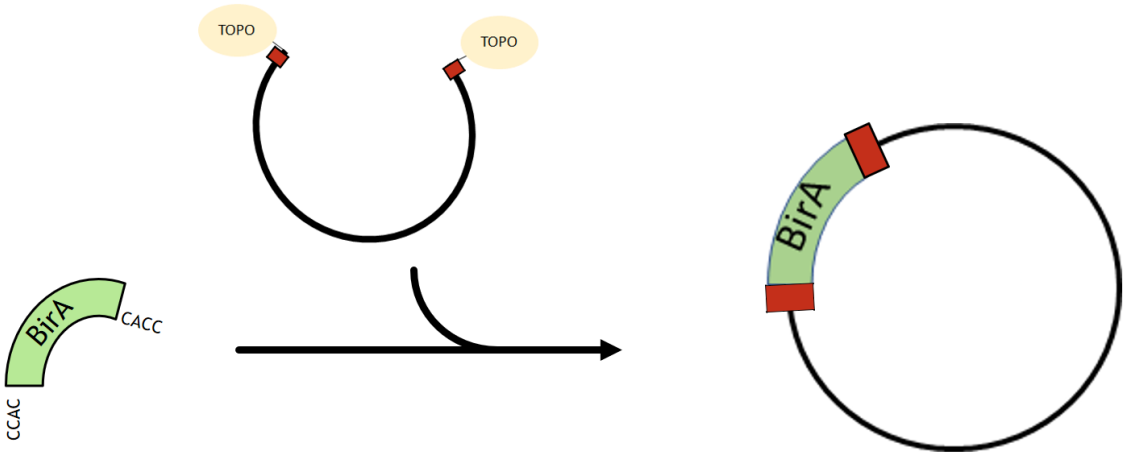


Figure 3. The amplified *BirA* with the CCAC overhang which allowed for correct orientation during the insertion into the entry vector pENTR. When the *BirA* sequence was mixed with pENTR, the resulting plasmid contained *BirA* between LR recombination sites, primed for a recombination reaction between the entry vector and the destination vector, pMDC7.

pENTR. Gel Electrophoresis was used to confirm the presence of the *BirA* PCR product. The PCR product was then mixed with the plasmid pENTR to create plasmids primed for LR

cloning, which would allow for the recombination of the *BirA* gene into the plasmid with an estrogen inducible promotor, pMDC7.

The resulting plasmid was introduced into heat shock competent DH5α *E. coli* and grown on 50 µg/ml kanamycin (Kan50) plates for selection. Resistant colonies were grown overnight, and an isolated colony was grown the next night in liquid culture with selection at 37ºC. The bacteria were lysed, and the plasmid was isolated. A restriction digest was completed with the restriction enzymes XhoI and NheI and run on a gel to ensure the correct orientation of *BirA* in the pENTR vector. Once the insertion of *BirA* into pENTR with the correct orientation was confirmed through gel electrophoresis and sequencing, an LR recombination reaction was run to recombine the *BirA* gene from pENTR into the destination vector pMDC7, placing it under the control of the estrogen-inducible promotor. pENTR, containing the correct *BirA* sequence was mixed with pMDC7, a plasmid with an estrogen inducible promotor in front of the lethal ccdb gene within the LR recombination sites, and the Invitrogen Gateway LR clonase 11 Enzyme Mix. The mix was incubated overnight, and

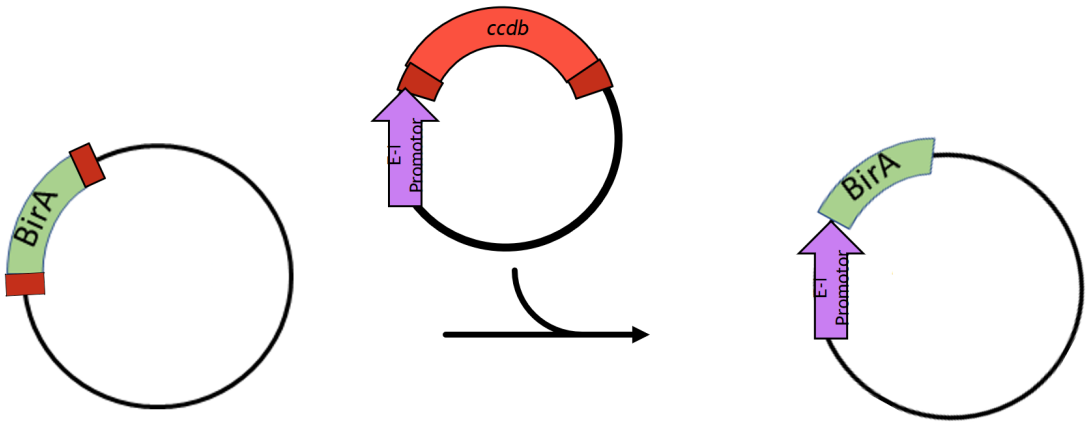


Figure 4. The entry vector pENTR-BirA and the destination vector pMDC7 were combined with LR clonase enzyme mix, to allow for the recombination of the *BirA* gene into pMDC7. This resulted in plasmids with estrogen inducible *BirA,* pMDC7-BirA

Proteinase K subsequently was added to destroy the enzyme mix. This produced a plasmid with an estrogen-inducible *BirA* sequence, which was again introduced into heat shock competent DH5α *E. coli* and grown with 100 µg/ml spectinomycin (Spec100) to select for bacteria containing the correct plasmid. The plasmid was isolated from a single colony of bacteria using the QIAprep® Miniprep Kit and sent for sequencing to ensure the correct orientation and sequence of *BirA*.

Using Electroporation to Introduce the pMDC7-BirA and UBQp:NTF Plasmids into Competent Bacteria

About 1 ng of the pMDC7-BirA plasmid was added to electro-competent K599 *Agrobacterium rhizogenes* which were electroporated with parameters of 2.5 kV at 25 µF and 200 Ω. The *Agrobacterium* were grown on LB plates with Spec100 selection for one day. We obtained the Ubiquitin promoter-driven *NTF* construct (*UBQp;NTF)* from the Queitsch lab at the University of Washington as all of our plasmids containing *NTF* also had a gene for the production of biotin ligase. As this plasmid (pGreen) cannot replicate without the co-plasmid, pSoup, which carries the *RepA* gene needed for plasmid replication, pSoup was introduced into heat shock competent DH5α *E. coli* and grown on agar with tetracycline at 5 µg/ml concentration for selection. pSoup was then isolated from the *E. coli* again using the QIAprep® Spin Miniprep Kit. About 1 ng of each plasmid, pSoup and pGreen, were combined with electro-competent K599 *Agrobacterium rhizogenes* and electroporated with the same parameters as before. The bacteria were plated on LB agar with Kan50 selection and grown for two to three days. Tetracycline selection was not necessary as the bacteria were not be able to grow without pSoup also present in the bacteria, allowing replication.

Starting Medicago truncatula Seedlings

Between 500 and 600 *Medicago truncatula* wild type A17 seeds were prepared by being gently shaken in sulfuric acid, which removed the seed coat, and then gently bleached

to sterilize. The seeds were placed on wet sterile filter paper in plates and wrapped in aluminum foil to prevent light exposure. The seeds were then left to germinate and grow for three days.

Growing isolated colonies of Agrobacterium rhizogenes strain K599 in Liquid Culture

In order to grow a large number of bacteria, an isolated colony of K599 *Agrobacterium rhizogenes* containing the pMDC7-BirA plasmid was added to 5 ml of liquid LB with Spec100 and shaken overnight at 180 rpm and 28°C. Then 1 ml of this culture was added into three 50 ml tubes containing 35 ml of LB and Spec100 which were shaken overnight with the same conditions. A similar procedure was used initially with the K599 *Agrobacterium* containing the pGreen and pSoup plasmids, but with Kan50 instead of Spec100. However, growth was extremely slow, taking several days and creating abnormal growths, so YEB media was used in a second attempt. Growth was still slow, taking about two days for each step.

Injecting the Seedlings and Selecting for Transformed Roots

The optical density (OD) of the *A. rhizogenes* liquid culture was found before the bacteria were spun down into a pellet, and the LB broth was removed. Then Injection Media was added until the OD equaled one. The liquid cultures of bacteria, one containing the pMDC7-BirA and the other containing pGreen with the ubiquitin promotor-driven *NTF*, were then poured into petri dishes in a 1:1 ratio. Seedlings were soaked in the solution and stabbed with a small needle about ten times to ensure that the *Agrobacterium rhizogenes* entered the roots. The 1 cm tips of the roots were cut to promote lateral root growth. The seedlings were placed on modified Fahraeus media (FM) agar plates and grown for three days, before they were transferred to FM agar plates with kanamycin and hygromycin at a concentration of 5 μg/ml for selection for doubly transformed roots, as pMDC7-BirA contained the resistance

gene for hygromycin resistance when present in plant tissue, while the pGreen (*UBQp:NTF*) expressed resistance to kanamycin when in plants.

Results

After cloning *BirA*, four of the PCR products, P1, P2, NI1, and NI2, showed promise of having the correct *BirA* sequence and were selected to be introduced into the TOPO plasmid pENTR (Figure 5). P1 and P2 were products from the pB7WG plasmid (P), while NI1 and NI2 were products of a New Intact pB7WG (NI). Although all products were

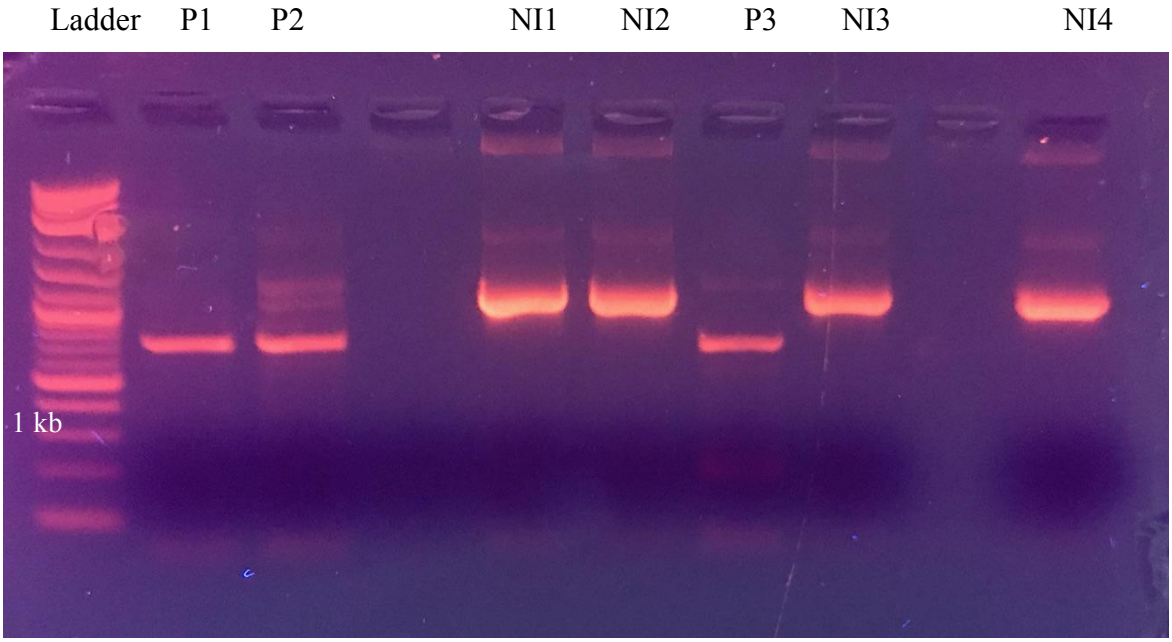


Figure 5. Results of High Fidelity PCR cloning of the *BirA* gene from plasmids pB7WG designated reactions P1, P2, and P3 and New Intact designated reactions NI1, NI2, NI3, and NI4 on a 1.5 Agarose gel.

sequences of *BirA* the products from the New Intact plasmid were bigger, most likely because of the addition of the *MYC* recognition sequence to the *BirA* sequence of the New Intact plasmid. The *MYC* sequence is a recognition site for MYC antibodies, so that Western blots can use MYC antibodies to probe for biotin ligase.

After adding *BirA* from P1, P2, NI2 and NI2 to pENTR and obtaining individual colonies, a restriction digest was run on the plasmids from each colony. The resulting fragments were expected to be 2.2 kb, 1.0 kb, and 0.2 kb if *BirA* was inserted in the correct orientation, and 3.0 kb, 0.3 kb, and 0.2 kb if insert in the incorrect orientation.

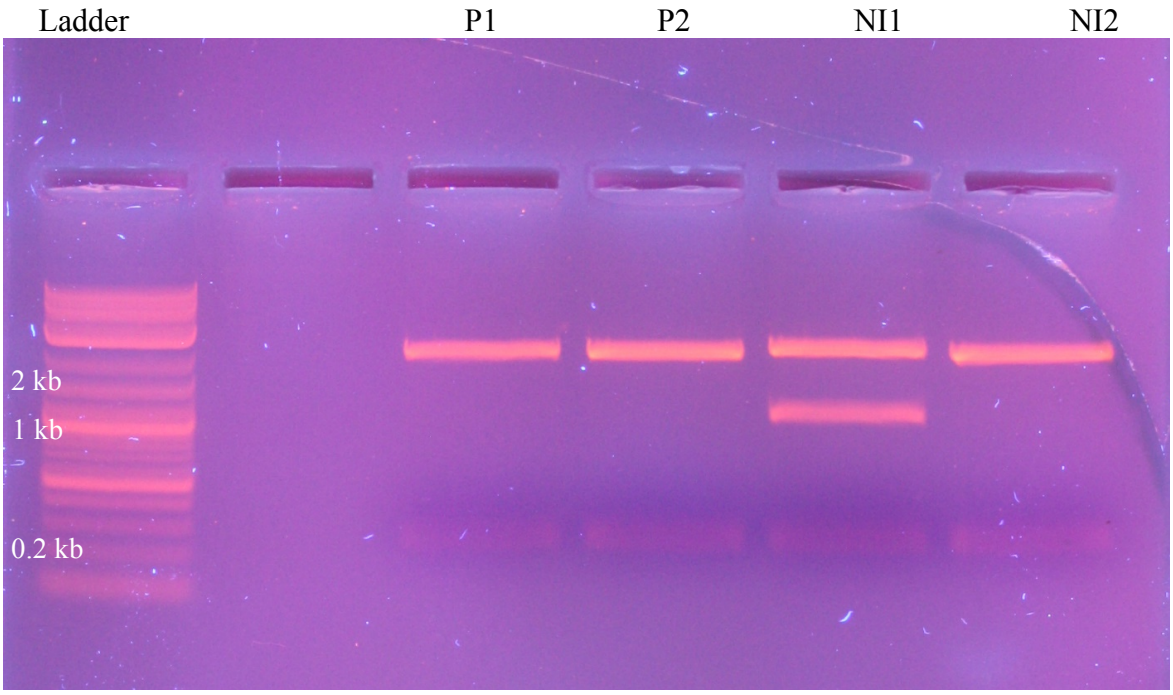


Figure 6. Results of the digest of four TOPO clone plasmids from *BirA* samples P1, P2, NI1, and NI2 on a 1.5% agarose gel.

Only NI1 showed the proper number of fragments of the proper size, indicating the correct insertion of *BirA* (Figure 6). This was confirmed with Sanger sequencing. After the products from the LR recombination reaction with NI1 and pMDC7 were confirmed through Sanger sequencing, the resulting pMDC7-BirA plasmid was used to transform *Medicago truncatula* seedlings.

Some of the plants did show phenotypic evidence of a double transformation with the potential for more transformed roots growing.

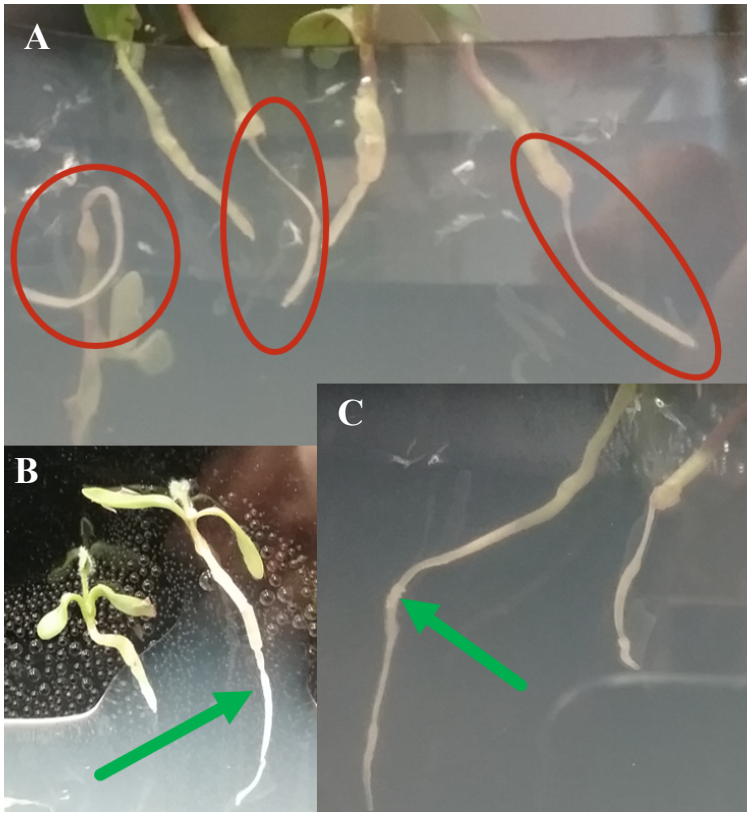


Figure 7. *Medicago truncatula* roots 10 days after injection and 7 days after being placed on selection. A. Roots circled in red look unhealthy, indicating they are not resistant to the selection, and thus are not transformed. B&C. Roots highlighted by the green arrows show more promise as they are relatively healthy, indicating resistance to the selection, but do not show the hairy root phenotypes expected when roots are transformed by *A. rhizogenes*.

Some *Medicago truncatula* seedlings had roots that were still growing and seemed relatively healthy even though they were still not showing growth indicative of transformed roots, such as the phenotype of hairy roots or lateral root growth (Figure 7 B and C). Although the roots seemed healthy, they had not been on selection long and the lack of specific phenotypes made definitive conclusion of transformation much harder. This indicated the potential for more transformed root growth given more time on the selective media, but did not show enough evidence of transformation.

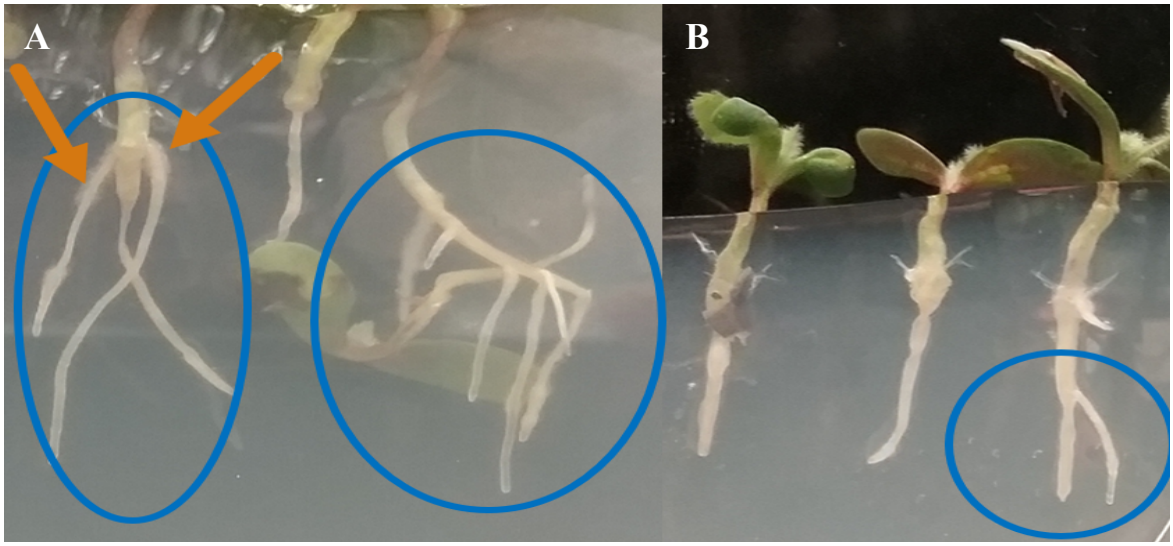


Figure 8. Roots 10 days after injection and 7 days after being placed on selection. Blue circles indicate lateral root growth onto the agar, indicative of transformation. A. Orange arrows also show hairy root phenotype expected of *A. rhizogenes*-transformed roots.

Lateral root growth expressing the hairy root phenotype, growth indicated by the orange arrows in Figure 8, causes the roots to appear fuzzy and indicated successful transformation of root tissue. New root growth that was able to grow robustly on selection also supported the identification of truly transformed roots. There were a few roots that did show this phenotype (Figure 8) implying double transformation of the roots. These roots should have copies of the genes from both plasmids in all cells of the new root growth, allowing them to be used for the time course analysis of *BirA* induction and NTF biotinylation upon estrogen induction.

Discussion

There was the potential for several of the plants being doubly transformed. A few seedlings displayed the hairy root phenotype on their lateral root growths, and many more showed the potential for more root growth, as the procedure for transforming roots dictates that the seedlings be left on selection for one to two weeks, and the data came from seedlings after only one week on selection. Also, many of these new roots were growing into the agar which had both Kan50 and Hygro50 for selection, suggesting that the growths within the agar contained the genes from both plasmids. Overall this method did produce roots that were potentially doubly transformed and could be used to study the kinetics of biotin ligase production, and to test whether INTACT would be a potential method of studying the effects of mycorrhizal infection of *Medicago truncatula* roots. As hairy roots have the ability to grow without the rest of the plant, even one transformed root could grow enough on plates to provide tissue for the next steps of this experiment. Because several roots showed promising hairy root phenotypes on their lateral root growths, the next steps of the study can proceed once enough roots grow to provide enough tissue.

The next steps of this project are to check the transformed roots for the production of the NTF protein which would indicated that they are at least single transformants. We can then place the transformed seedlings on estrogen-containing plates for specific amounts of time, and then use the transformed roots for two separate Western Blots, one checking for the production kinetics of biotin ligase and one checking the biotinylation of NTF. We could also perform INTACT purification of nuclei to determine the fraction of nuclei that can be purified at each time point. These experiments will allow us to determine if this approach will ultimately be useful for the purification of nuclei from cells invaded by mycorrhizae.

As the transformed roots have a ubiquitously-expressed *NTF* gene, GFP will be produced in every cell of the transformed roots, so the production of GFP can be checked at

any time, with a fluorescence microscope. To ensure that the *BirA* gene was integrated into the roots though, the *Medicago* roots must first be placed on plates where estrogen is present to induce the production of biotin ligase. The time course analysis will involve leaving the roots on 5 μM β-Estradiol plates for 0 minutes, 20 minutes, 40 minutes, 80 minutes, and 160 minutes, as protein production is expected to be relatively rapid. The root tissue would be frozen as soon as it was removed from the plates at each time point.

Once the root tissue is frozen, it can be homogenized and used for several different procedures. One procedure, to show that biotin ligase is being produced, would be to complete a Western Blot using antibodies for the *MYC* tag, which is included in the *BirA* sequence of pMDC7-BirA. Comparing the amount of biotin ligase for each time point would simply show how quickly biotin ligase is being produced in the transformed root cells when estrogen is introduced. The other western would use streptavidin instead of antibodies. This blot would be used to test whether the production of biotin ligase would correlate well with the timeframe with which the biotin ligase would biotinylate the biotin ligase recognition peptide (BRLP) domain of the NTF protein. As biotin ligase must biotinylate the NTF protein on the nuclei for INTACT, this blot would be a strong indicator for the viability of this system in roots infected by mycorrhizae.

However, performing INTACT on the transformed roots would be the best indicator of the viability of this system. The rest of the root tissue not used in the western blots would then be used to attempt INTACT. The same time points would be used to pinpoint when biotin ligase production has progressed enough to give the best yield of nuclei, and whether it would be efficient enough to use when studying mycorrhizae-root cell interactions.

Lastly, this method should be tested in a second type of plant. Although *Arabidopsis thaliana* roots cannot be infected by mycorrhizae, they are good candidates to test the estrogen-inducible *BirA* system. *Arabidopsis* plants would also need to be transformed before

testing out the inducible *BirA* INTACT with similar methods. Showing that this system is also viable in another model organism would indicate that this dual specific-promotor INTACT system could be used for broader research purposes with other organisms.

Although in this experiment there was only phenotypic evidence of dual transformation, there is potential for the completion of these tests which would create a new way for people to study genomes under specific inducible conditions. Further work is needed, but using INTACT with two specific promotors could also help researchers study rapid changes that only affect subsets of cells of a specific type, allowing for more selective isolation of nuclei.

Eventually, this system will be used to isolate nuclei from select cells of specific types that have been invaded by mycorrhizae. In this way, the differences between the genomes can be analyzed to identify the epigenetic changes that potentially lead to the changes in phenotype allowing plants to survive stressors more easily when colonized by mycorrhizae. Identifying the epigenetic changes in roots from mycorrhizal colonization is the first step in creating plants better able to survive natural stressors. As arbuscular mycorrhizae are most often the colonizers of crop plants, there is the potential for creating more hardy crop plants from the information gained from a mycorrhizae-induced INTACT system.

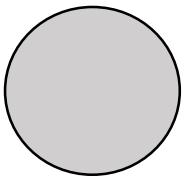
Appendix

Methods and Procedures

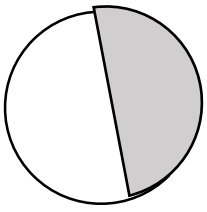
Modified Fahraeus Media

O.5 $MgSO_4$	1 ml
0.7 M KH_2PO_4	1ml
0.4 M $NaHPO_4$	2ml
0.5 M NH_4NO_3	1 ml
20 µM FeEDTA	2.5 ml
1 g/L Micronutrients	100 µl
H_2O	to 1 L
Phytoblend™ agar	8 g

Autoclaved, then1 ml of 1 M CaCl was added. For plates with no selection or plates with 5 µl of 1 mM β-Estradiol, full plates were made:



For plates with selection, 100 µl each of Kan50 and Hygro50 was added. Poured to make half plates:



LB Media

Tryptone	2 g
Yeast Extract	1 g
NaCl	2 g
H_2O	to 200 ml

When made for plates, 3 g of bacteriological agar were also added before autoclaving.

YEB Media

Tryptone	1 g
Yeast Extract	0.2 g
LB Nutrient Broth	1 g
Sucrose	1 g
$MgSO_4$	0.098 g
H_2O	to 200 ml

Injection Media

10x PBS	7 ml
1 mM Acetosyringone	700 µl
Silwet L-77	7 µl
H_2O	to 70 ml

Heat-Shock Procedure

About 1 µl of plasmid was added to DH5α *Escherichia coli* at an OD of 1 and incubated on ice for half an hour. The bacteria were then heat shocked by being placed in 42ºC water for 30 seconds. LB was immediately added, and the bacteria were shaken for 1

hour at 37ºC. Then 100 µl of the bacteria were plated onto LB agar plates with selection. The bacteria were left overnight at 37ºC.

Electroporation Procedure

About 1 ng of plasmid was added to K599 *Agrobacteria rhizogenes* at an OD of 1. The bacteria were then shocked with 2.5 kV at 25 µF and 200 Ω and quickly recovered with 200 µL of LB. The bacteria were shaken at 28ºC for one and a half hours before being plated on LB plates with selection. They were left overnight at 28ºC.

Seedling Germination

Medicago truncatula seeds were placed in 5 ml of concentrated sulfuric acid and shaken for 8 minutes to remove the shell of the seeds and allow for germination. The sulfuric acid was removed, and the seeds were washed with cold water three times while being stirred to prevent clumping and to remove traces acid. The seeds were then shaken for 5 minutes in 12 ml of 30% bleach for sterilization. They were then washed three times with autoclaved deionized water. The seeds were then pipetted onto sterile wet filter paper.

Reference

Allen, J.T. and Y. Shachar-Hill. "Sulfur Transfer through an Arbuscular Mycorrhiza". *Plant Physiology,* vol. 149, pp. 549-560. 2009. DOI: 170.140.142.252

Aloui, A., E. Dumas-Gaudot, Z. Daher, D. van Tuinen, S. Aschi-Smit, and D. Morandi. "Influence of arbuscular mycorrhizal colonisation on cadmium induced *Medicago truncatula* root isoflavonoid accumulation". *Plant Physiology and Biochemistry*, 60. pp 233-239. 2012.

Atul-Nayyar, A., C. Hamel, K. Hanson, and J. Germida. "The arbuscular mycorrhizal symbiosis links N mineralization to plant demand". *Mycorrhiza*, vol. 19. pp. 239-246. 2009.

Bücking, H., E. Liepold, and P. Ambilwade. "The Role of the Mycorrhizal Symbiosis in Nutrient Uptake of Plants and the Regulatory Mechanisms Underlying These Transport Processes" *Plant Science*, Dr. Nabin Kumar Dhal (Ed.), InTech, DOI: 10.5772/52570. 2012.

Deal, R. and S. Henikoff. "A Simple Method for Gene Expression and Chromatin Profiling of Individual Cell Types within a Tissue". *Developmental Cell*, vol. 18, 2010. pp 1030-1040.

Gerdemann, J.W. "Vesicular-Arbscular Mycorrhiza and Plant Growth". *Annual Review of Phytopathology*, vol. 61. pp. 397-418. 1968.

Li. H., S.E. Smith, R.E. Holloway, Y. Zhu, and F.A. Smith. "Arbuscular mycorrhizal fungi contribute to phosphorus uptake by wheat grown in a phosphorus-fixing soil even in the absence of positive growth responses". *New Phytologist*, vol. 172. pp. 536-543. 2006.

Mosse, B., and D.S. Hayman. "Plant Growth Responses to Vesicular-Arbuscular Mycorrhiza". *New Phytologist*, vol. 70. pp 29-34. 1971.

Remy, W., T. Taylor, H. Hass, and H. Kerp. “Four hundred-million-year-old vesicular arbuscular mycorrhizae”. *Proceedings of National Academy of Sciences of the United States of America*, vol. 91. pp 11841-11843. 1994.

Schützendübel, A., and A. Polle. “Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization”. *Journal of Experimental Botany*, vol. 53. pp. 1351-1365. 2002.

Selosse, M-A, and F. Le Tacon. “The land flora: a phototroph-fungus partnership?”. *Trend in Ecology & Evolution*, vol. 13. pp. 15-20. 1998.

Simon L., J. Bousquet, R.C. Lévesque, and M. Lalonde. “Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants”. *Nature*, vol. 363. pp. 67-69. 1993.

Smith, S. E. & D.J. Read, *Mycorrhizal Symbiosis*. Third Edition. Elsevier, 2008, New York, NY.

Subramanian, K.S., P. Santhanakrishnan, and P. Balasubramanian. “Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress”. *Scientia Horticulturae*, vol. 107. pp. 245-253. 2006.

Tuo X.Q., L. He, & Y.N. Zou. “Alleviation of Drought Stress in White Clover after Inoculation with Arbuscular Mycorrhizal Fungi”. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* vol.45(1), pp.220-224. 2017. DOI: 10.15835/nbha45110709

Wang, D. and R. Deal. “Epigenome profiling of specific plant cell types using a streamlined INTACT protocol and ChIP-seq”. *Methods in Molecular Biology*, vol. 1284, pp 3-25. 2015. DOI: 10.1007/978-1-4939-2444-8_1

Electronic Sources

Mycorrhizal Fungi Biological Inoculants for Farmers. MycoGold: Achieve Golden Yields, 2014. [Webpage online] Accessed 1 April 2018. Available from www.mycogold.com/; Internet

"Results: Mycorrhizae". *Amazon.com*. [Database online] Accessed 1 April 2018. Available from www.amazon.com/s/ref=nb_sb_noss_1?url=search-alias%3Daps&field-keywords=mycorrhizae; Internet