Identification of Genes that Regulate Phosphate Acquisition and Plant Performance during Arbuscular Mycorrhizal Symbiosis in *Medicago truncatula* and *Brachypodium distachyon*

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Introduction

Most vascular flowering plants, including all proposed biofeedstock species, are able to develop symbiotic associations with arbuscular mycorrhizal (AM) fungi. The symbiosis develops in the roots and has a profound effect on plant productivity, largely through improvements in plant phosphorus nutrition. The overall objectives of the work were to identify and characterize plant genes involved in the regulation and functioning of the AM symbiosis in *Medicago truncatula* and *Brachypodium distachyon.*

In the Harrison lab, we have worked with *M. truncatula* for many years and we have been responsible for the development of several AM symbiosis resources in this system. In this project, we leveraged resources developed previously for *M. truncatula* to identify plant transcription factors that regulate development of the AM symbiosis and symbiotic phosphate (Pi) transfer. In parallel, we started to develop *B. distachyon* as a model system for the analysis of AM symbiosis in grasses. Our long term goal is understand the molecular basis of functional compatibility in the AM symbiosis.

Arbuscular mycorrhizal symbiosis in *Brachypodium distachyon*

Research on AM symbiosis in *B. distachyon* was an entirely new undertaking so our initial efforts focused on developing the conditions appropriate for studying the symbiosis. We established growth conditions, that enable development of AM symbiosis and reveal functionality of the symbiosis. As our overall objective is to dissect the molecular basis of functional compatibility in the AM symbiosis, the first step was to identify *B. distachyon* AM symbioses that differed in their functionality. We evaluated five different species of AM fungi and assessed their potential to promote significant increases in growth of *B. distachyon* under low Pi conditions. The species selected for evaluation included *Glomus intraradices*, *Glomus versiforme,* and *Gigaspora gigantea*, which have been shown to promote growth in many plant species. Additionally, we acquired *Glomus white* (INVAM NC172) and *Gigaspora decipiens* from Dr. J Bever, University of Indiana.

All five AM fungi colonized *B. distachyon* roots and established the structures typical of the AM symbiosis. Based on colonization levels, it was not possible to predict whether or not one AM symbiosis would be more effective (for the plant) than another. However, at 60 days post-inoculation, there were clear differences in the response of *B. distachyon* in the different symbioses. The variation in growth and Pi content reflects a phenomenon called functional compatibility. This work was published (Hong et al., 2012) and we continued by analyzing the root and shoot transcriptomes of 3 symbioses via RNA-seq. For each symbiosis and control, three biological replicate RNA-seq libraries were prepared from roots and shoots at each timepoint and sequenced on an Illumina Hiseq. The sequencing was deep and included paired-end reads (100bp) from the roots so that transcriptional information about the fungal symbionts could also be obtained. 3350 Brachypodium genes that show a fold-change of ≥ 2 (Benjamini-Hochberg adjusted p-value <0.01) were identified including many symbiosis-induced transporters and transcription factors not reported previously. The Brachypodium data are considerably more comprehensive than the *M. truncatula* data where the gene chip covers only 2/3 of the genome, or the rice data, where only 228 induced genes were reported. These data were obtained as a collaboration between the Harrison and

Hudson labs. A comprehensive analysis of the dataset is ongoing. The follow paragraph provides one or two brief highlights.

Ammonium, phosphate and nitrate transporters are among the most highly induced and/or highly expressed genes in Brachypodium mycorrhizal roots. Two genes (Bradi2g56290 and Bradi2g56300) predicted to encode ammonium transporters, show between 1166 - 2000 fold induction in Brachypodium roots during symbiosis. The Brachypodium ortholog (Bradi 2g45520) of the *M. truncatula* MtPT4 and rice OsPT11 symbiotic phosphate transporters is induced over 300-fold with read counts of >25000 reads per million (RPM), however, in Brachypodium, there are 4 additional symbiosis-induced Pi transporters that do not exist in the *M. truncatula* or rice genomes but are present in other grass genomes, including sorghum. We generated Brachypodium transgenic plants expressing Bradi2g45520 and Bradi1g52590 protein-GFP fusions. Characterization of the transgenic plants indicates that transporters are located in the periarbuscular membrane. Functional analyses of the phosphate and ammonium transporters has been initiated and a manuscript describing the RNA-seq data is in preparation.

AM symbiosis in *Brachypodium distachyon* stimulates tiller production.

The experiments outlined above also led to some unexpected findings of that may be of particular significance to the DOE mission. Surprisingly, following colonization with *G. intraradices* or *G. white*, *B. distachyon* shows a dramatic increase in the number of tillers. At harvest (9 weeks post inoculation), the mock-inoculated plants showed an average tiller number of 3.6 tillers while the plants inoculated with *G. intraradices* showed an average of 13.2. This 4-fold increase in tiller number was surprising. During these experiments the plants are grown under low-Pi conditions, but the increase in tiller number does not seem to be simply the result of increase Pi in the colonized plants. We cannot stimulate such an increase in tiller number in non-inoculated plant simply by the application of higher Pi fertilizer. It is possible that in the absence of a fungal symbiont, *B. distachyon* cannot access the additional Pi (we still have to check Pi levels). Alternatively, it is possible that the AM symbiosis also alters the hormone levels in the plant, and this promotes tiller formation. As tiller number clearly impacts the final biomass levels, this may be a significant finding and we will investigate this aspect further. Possibly related to this, we have shown that in *M. truncatula*, development of the symbiosis results in increases in GA_3 levels in the roots and shoots.

REGULATION OF AM SYMBIOSIS IN *M. TRUNCATULA*

Analysis of transcription factors in AM symbiosis.

Development of the AM symbiosis is accompanied by significant changes in plant gene expression, not only locally in cells colonized by the AM fungus but also systemically throughout the plant. In *M. truncatula* array-based transcript profiling has been used to document these changes on a genome wide scale. These experiments identified an AM symbiosis-induced gene sets and also a smaller AM symbiosis-specific gene set.

Quantitative RT-PCR to profile expression of 1000 transcription factors in mycorrhizal roots of *M. truncatula*

To begin to identify the transcriptional regulators responsible for the control of gene expression in AM symbiosis, we undertook profiling for transcription factors in mycorrhizal roots of *M. truncatula*. Through a collaboration with Dr. M. Udvardi's group at the Noble Foundation, we used a quantitative RT-PCR transcription factor profiling platform to identify transcription factors induced in AM symbiosis. The platform has unique primer sets for 1000 transcriptional regulators predicted from the EST datasets and the partially completed *M. truncatula* genome and is predicted to represent approximately half of the transcription factors in the genome. Using this system, we compared transcription factor gene expression in control and mycorrhizal roots and identified 119 transcription factor genes that were up-regulated in *M.*

truncatula/G. intraradices samples compared to non-mycorrhizal *M. truncatula* samples. 77 TF genes were down-regulated in mycorrhizal samples (criteria: E ≥ 1.75, expression ratio ≥ 1.5 for up-regulation and ≤ 0.68 for down-regulation, and coefficient of variation (CV) ≤ 50%).
Additionally, 4 TF genes were not expressed at a detectable level in non-mycorrhizal *M. truncatula* samples, and could represent potential mycorrhiza-specific TF genes (1092.m00033, 1185.m00003, 1145.m00006, 1524.m00004). Interestingly, one of them is a GRAS family member (1524.m00004). This class of transcription factors includes well known regulators of root development and of nodulation.
The most highly induced (≥ 10-fold) TF genes in mycorrhizal samples were an AP2 domain-containing protein (1679.m00002), an AP2/EREBP (1260.m00022), and a SBP family member (988.m00017). Interestingly, there were novel TFs and transcriptional regulators among mycorrhiza-induced genes such as members of the RR; CCHC (Zn) and DHHC (Zn); FIS and AraC; and BD families.

To determine function of the transcription factors we obtained Tnt1 insertion lines in a selection of these transcription factors. A GRAS factor (named Reduced Arbuscular Mycorrhiza 1 (RAM1) was of particular interest as analysis of RAM1 promoter-GUS fusions indicated that it is expressed exclusively in the root inner cortex cells, specifically in the regions of the roots colonized by AM fungi. To address the role of TF69 during symbiosis, we obtained a *M. truncatula* line (NF5445) with a Tnt1 insertion in *TF69* at nucleotide 930 downstream from the first ATG. Analysis of the mutant indicated that as it is essential for the branching stage of arbuscule development. In *ram1-3*, the fungus forms appressoria and enters the root successfully; however, arbuscule formation is seriously impaired and densely branched arbuscules are not formed (Fig. 2). Through analyses of the *ram1-3* mutant and through analyses of roots overexpressing *RAM1*, we were able to determine that RAM1 regulates two genes that are essential for arbuscule branching, *EXO70I* and *STR* and this provides a partial explanation for the phenotype. These data were published in Plant Physiology (Park et al., 2015). The data indicate that RAM1 is a key regulator of development of the symbiotic interface essential for nutrient exchange in the symbiosis. In addition to this GRAS factor, functional analyses of a second GRAS factor, RAD1, reveals that it also plays a role in development of a wild-type symbiosis, and analysis of a Myb factor is in progress.

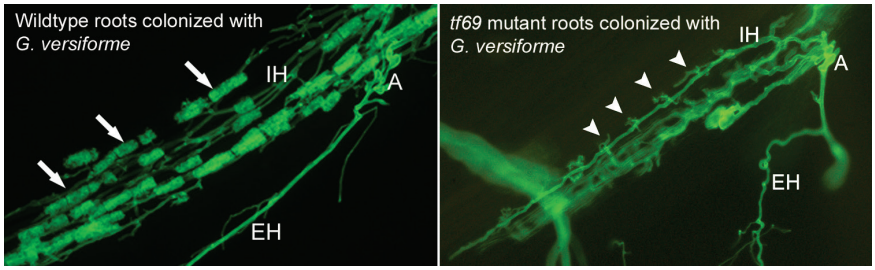

Fig. 2. Arrows indicate arbuscules and arrowheads indicate to hyphal branches that have entered a cell but fail to develop into arbuscules (A, appressorium, IH, intercellular hyphae, EH, extraradical hyphae).

The knowledge gained in *M. truncatula* can be leveraged to provide insight into symbiosis in the grasses. The Brachypodium ortholog of RAM1 shows 278-fold, 258-fold and 85-fold increases in expression during symbiosis with *G. versiforme*, *Gi. gigantea* and *G. intraradices* respectively. We anticipate that Brachypodium *RAM1* will likewise control development of the symbiotic interface and experiment to evaluate this are in progress.

Hong JJ, Park YS, Bravo A, Bhattarai KK, Daniels DA, Harrison MJ (2012) Diversity of morphology and function in arbuscular mycorrhizal symbioses in Brachypodium distachyon. Planta **236:** 851-865

Park H, Floss DS, Levesque-Tremblay V, Bravo A, Harrison M J (2015) Hyphal branching during arbuscule development requires Reduced Arbuscular Mycorrhiza 1. Plant Physiology **doi:10.1104/pp.15.01155**