Functional analysis of arbuscular mycorrhiza-related GRAS transcription factor genes of *Medicago truncatula*

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Rico Martin Hartmann, M. Sc.

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ZUSAMMENFASSUNG

Die Arbuskuläre Mykorrhiza (AM)-Symbiose bezeichnet eine Vergesellschaftung von Pflanzen und Mykorrhizapilzen, die bei etwa 80 % aller Landpflanzen anzutreffen ist und die Nährstoffversorgung der Pflanze, insbesondere mit Phosphat, wesentlich verbessert.

Die Besiedelung von Wurzeln durch AM-Pilze und die Ausbildung einer Schnittstelle für den Nährstoffaustausch wird von einer transkriptionellen Umprogrammierung der Wirtszellen dirigiert. Diverse Studien über die transkriptionellen Veränderungen während der AM-Symbiose identifizierten eine Vielzahl von Genen, die hierbei aktiviert werden. Jedoch lieferten bisherige Studien lediglich Momentaufnahmen des transkriptionellen Status der Wurzel während der Symbioseentwicklung. Um diese Lücke zu schließen, wird in dieser Arbeit der zeitliche Verlauf der Aktivierung von Genen aufgezeigt, die an der AM-Symbioseentwicklung beteiligt sind. Durch Verwendung von Affymetrix *Medicago* GeneChips wurde die Genexpression während des Prozesses der Wurzelbesiedelung durch AM-Pilze zwischen 0 und 42 Tagen ermittelt, wodurch spezifische Muster identifiziert wurden. Die Gene, die in dieser Zeitspanne aktiviert wurden, können in verschiedene funktionelle Klassen unterteilt werden, die auf verschiedene Weise mit der AM-Entwicklung assoziiert sind. Unter diesen wurden 12 Transkriptionsfaktoren (TF) der GRAS-Familie identifiziert, deren Gene anhand ihres zeitlichen Expressionsverlaufs in verschiedene Kategorien unterteilt werden können.

Mithilfe des *Yeast Two-Hybrid*-Systems wurden Protein-Protein-Interaktionen zwischen den GRAS TFs untersucht, wobei sich Interaktionen von MtGras1 mit MtGras4, sowie MtGras4 mit MtRad1 zeigten. Weiterhin scheint MtGras1 einen Homodimer zu bilden.

Um die während des Verlaufs der AM-Symbiose aktivierten *MtGras*-Gene funktionell zu charakterisieren, wurden Promotor-*gusA*int-Analysen in mykorrhizierten Wildtyp-Wurzeln sowie in *MtRam1*- und *MtPt4*-Mutanten untersucht, welche Einschränkungen bezüglich der Arbuskelverzweigung und des funktionierenden Phosphattransports aufweisen. Hierbei zeigten sich Abhängigkeiten von diesen Genen bzw. von den korrespondierenden Entwicklungsstadien. Während *MtRad1*, *MtGras6* und *MtGras4* in AM-Wurzeln unabhängig von MtRam1 und MtPt4 exprimiert werden, sind *MtGras1* und *MtGras7* in hohem Maße abhängig von MtRam1 und MtPt4, was eine Beteiligung dieser Gene an den späten Phasen der Arbuskelentwicklung nahelegt. Ein *Knockdown* von *MtGras1* führte zu einer verminderten Expression mehrerer *MtGras*-Gene, sowie weiterer AM-induzierter Gene. Die Tatsache, dass somit ein GRAS TF identifiziert wurde, welcher in späteren Phasen der Arbuskelentwicklung aktiv ist, aber Elemente der früheren Entwicklung beeinflusst, führte zu dem Modell einer regulatorischen Rückkopplung. Hierbei fungiert MtGras1 als Kontrollpunkt für die korrekte AM-Signalkaskade und wirkt durch die Rückkopplung fördernd auf die Arbuskelentwicklung. *MtGras7*, eines der von MtGras1 regulierten Gene, ist ebenfalls in den späteren Phasen der Arbuskelbildung aktiv. Untersuchungen an einer *Tnt1*-Mutante zeigten, dass *MtGras7* außerdem von MtGras4 reguliert wird. Die Voraussetzungen für die *MtGras7*-Expression würden somit durch MtGras4 in früheren Stadien der Entwicklung geschaffen und in späteren Stadien durch MtGras1 beschleunigt. Aus diesen Erkenntnissen wurde das Modell eines regulatorischen Netzwerks von GRAS TFs erstellt, welches unidirektionale Wirkketten und regulatorische Rückkopplungen integriert und so letztlich die Arbuskelentwicklung moduliert.

Schlagworte: Arbuskuläre Mykorrhiza-Symbiose, zeitliche Expressionsprofile, GRAS-Transkriptionsfaktoren, *Knockdown*-Wurzeln, *Yeast Two-Hybrid*-System

ABSTRACT

The arbuscular mycorrhizal (AM) symbiosis is a widespread beneficial association of vascular plants with different mycorrhizal fungal species. It can be found in ~ 80 % of recent land plants and improves the plant's nutrient supply, in particular with respect to phosphorus.

The colonization of roots by AM fungi and the establishment of a nutrient exchange interface is conducted by a significant transcriptional reprogramming of the host cells. In the last years, transcriptomic studies on mycorrhizal roots revealed insights into this reprogramming and delivered substantial numbers of genes upregulated in AMF colonized roots. However, these studies provided only snapshots of the transcriptional program at a distinct time point in the development of the AM symbiosis. To overcome this drawback, this thesis obtained insights into the chronological development of AM-induced gene expression patterns. By using Affymetrix *Medicago* GeneChips, gene expression levels were monitored during the ongoing process of AMF colonization, revealing distinct gene expression patterns that reflect transcriptional regulations from 0 to 42 days post infection. Different functional classes relevant for AM function were found upregulated in the AM time course, including 12 genes encoding transcription factors (TFs) of the GRAS family. Based on their chronologic expression patterns, these GRAS TF genes were grouped in two major expression categories.

Yeast Two-Hybrid approaches were used to identify protein-protein interactions of GRAS TFs with other AM-related proteins and also amongst the GRAS TFs themselves. These studies revealed interactions between MtGras1 and MtGras4, as well as MtGras4 and MtRad1. Furthermore, MtGras1 appeared to form homodimers.

To functionally characterize *MtGras* genes strongly upregulated during the time course of AM development, promoter-*gusA*int studies were performed in transgenic roots of mycorrhizal wild type plants as well as *MtRam1* and *MtPt4* knockout mutants, showing defects in arbuscule branching and the formation of active, P-transporting arbuscules, respectively. These experiments revealed differential dependencies amongst these genes or from the developmental stages of AM formation in which they are active. While *MtRad1*, *MtGras6*, and *MtGras4* are expressed independently of MtRam1 and MtPt4 in AMF-colonized roots, *MtGras1* and *MtGras7* are highly depending on MtRam1 and MtPt4, suggesting a role in later stages of arbuscule development. Knockdown of *MtGras1* led to a downregulation of several *MtGras* genes as well as other genes correlated with AM formation. The identification of a GRAS TF acting in later stages of arbuscule development that nevertheless also regulates elements of the earlier stages of arbuscule formation led to the establishment of a model proposing a regulatory feedback loop. In this scenario, MtGras1 acts as a checkpoint for proper AM-related signaling, possibly supporting and accelerating arbuscule development via feedback regulation.

One of the *MtGras* genes regulated by MtGras1 is *MtGras7*, being active in the later stages of arbuscule development. *MtGras7* was further shown to be regulated by MtGras4 via analysis of an *MtGras4* knockout line. The prerequisite for *MtGras7* expression would thus be set by an MtGras4 action in the earlier stages und would be even accelerated by MtGras1 upon progression of arbuscule development. Taken together, a regulatory network of GRAS TFs is proposed, integrating different unidirectional as well as feedback regulatory mechanisms that together modulate arbuscule development.

Keywords: arbuscular mycorrhizal symbiosis, time course expression profiling, GRAS transcription factors, knockdown roots, Yeast Two-Hybrid system

CONTENTS

GENERAL INTRODUCTION

The term “symbiosis” was first established ~ 140 years ago by the German scientist Anton de Bary as an association or socialization of two or more organisms (de Bary, 1879). In these first exemplifications, de Bary included both parasitic and mutualistic communities, recognizing that a distinct discrimination between these categories was ambitious. Though some of his statements were hardly accepted by coeval scientists, the field of symbiotic studies arose in the following centuries, and the term symbiosis was further refined. From the 1950s on, the concept symbiosis was generally accepted and was now restricted to mutualistic associations.

In 1951, Anton Quispel postulated various parameters that influenced symbiotic developments, e. g. environmental, nutritional, competitive, and antibiotic factors. He came to the conclusion that “a symbiosis may come into existence because the absence of antibiotic influences makes it possible and the presence of one or more symbiotic influences makes it necessary“ (Quispel, 1951), depicting symbioses as an inevitable aspect of natural life forms.

Two major plant-microbe interactions are extensively studied today: The root nodule symbiosis, where nitrogen-fixing rhizobial bacteria induce and colonize a novel organ, the root nodule, on the roots of legume plants, and the mycorrhiza symbiosis between plant and soil-born beneficial fungi. It is generally assumed that the root nodule symbiosis in roots recruited ancient signaling pathways that were originally established to form the more widespread mycorrhiza symbiosis (Kistner and Parniske, 2002; Oldroyd and Downie, 2006; Parniske, 2008).

Properties of mycorrhizal symbioses

Mycorrhizal symbioses can be divided into endo- and ectomyocorrhizal forms. Ectomycorrhizal characteristics include the establishment of a maze-like hyphal growth through the intercellulars primarily of the outer root cortex, building the so-called „Hartig net“ (Figure 1 A). Transiting into the extraradical mycelium, the Hartig net significantly extends the plant-soil interface to allow an active transport of soil nutrients to the root system (Smith *et al.*, 1994).

In contrast to the situation in ectomycorrhiza, endomycorrhizal fungi are able to colonize the root cortex not only intercellularly, but also intracellularly, ultimately establishing a highly branched plant-fungal interface within cells of the inner cortex (Figure 1 B).

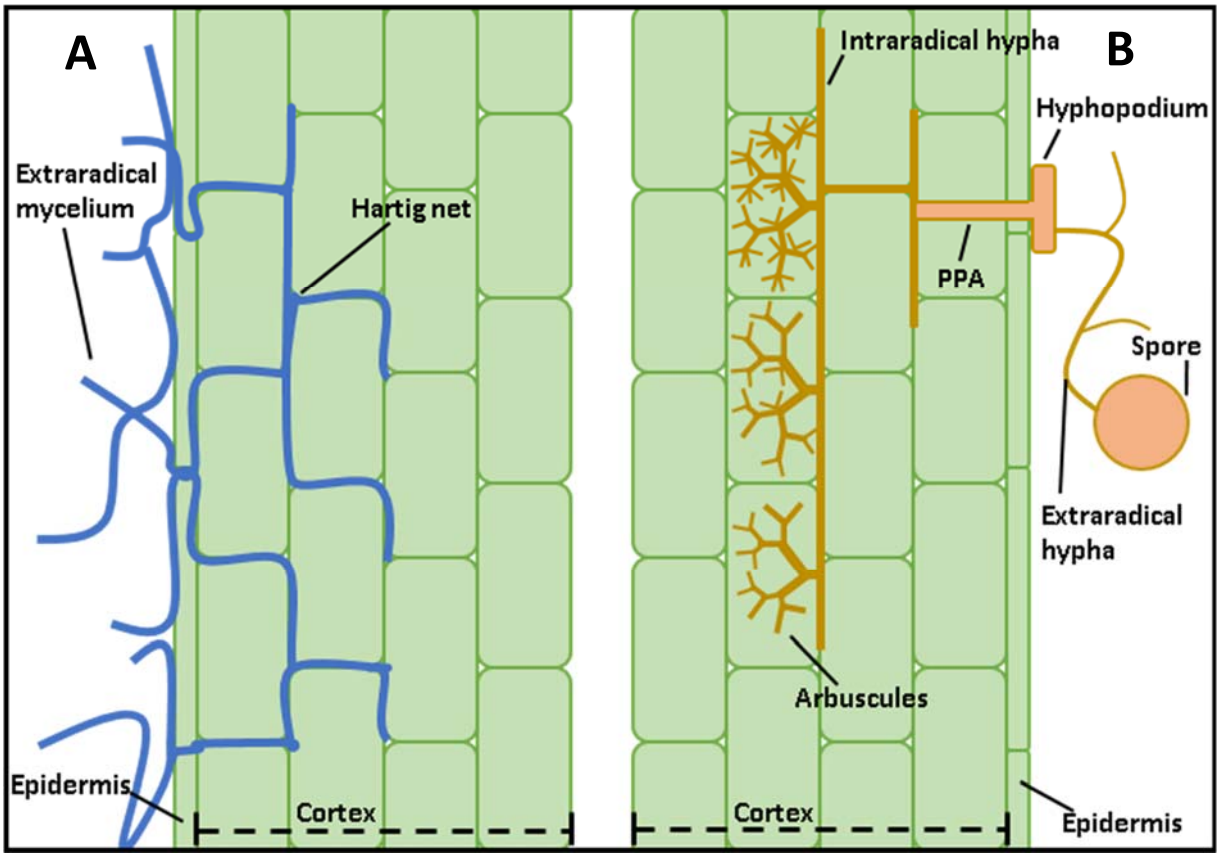

Figure 1: Scheme of the two major types of mycorrhizal symbiotic associations.
A Ectomycorrhizal colonization of a plant root. Ectomycorrhizal fungal hyphae (blue) form a maze-like Hartig net within the intercellular space of the outer cortex. The extraradical mycelium enlarges the contact zone between root, fungus, and the surrounding rhizosphere. **B** Endomycorrhizal [here: arbuscular mycorrhizal (AM)] colonization of a plant root. Emerging from a germinating spore, AM fungal hyphae attach to the epidermis and form a hyphopodium, being the origin for intracellular penetration of the outer cortex via a prepenetration apparatus (PPA) established by the plant. Subsequent intercellular hyphal growth finally leads to the establishment of arbuscules in cells of the inner cortex.

The arbuscular mycorrhizal (AM) symbiosis (from the Latin arbor = tree; Greek mycos = fungus; Greek rhiza = root) is the major form of such an endomycorrhizal interaction. Approximately 80 % of all land plants are able to enter the AM relationship with fungi of the phylum *Glomeromycota* (Smith and Read, 1994; Schüssler *et al.*, 2001; Smith and Read, 2008). It is thought that the colonization of land by early plants ~ 400 mio years ago was supported by fungal-derived nutrients, in particular phosphorus that is limited in the immediate root vicinity due to the slow diffusion of phosphate (Heckmann *et al.*, 2001; Smith and Read, 2008).

Emerging from a germinating spore, AM fungal hyphae are stimulated by plant-exudates (e. g. strigolactones) to branch and grow towards the root surface (Giovanetti *et al.*, 1994; Akiyama *et al.*, 2005). Simultaneously, the AM fungus (AMF) secretes short chain chitin oligosaccharides (Myc-COs) and lipochitooligosaccharides (Myc-LCOs) that trigger cellular responses in preparation for the approaching fungal hyphae, including an oscillation of

cytoplasmic calcium levels designated “calcium spiking” (Oldroyd *et al.*, 2004; Oldroyd and Downie, 2006; Gutjahr *et al.*, 2009; Maillet *et al.*, 2011; Chabaud *et al.*, 2011; Genre *et al.*, 2013;) and the activation of specific pre-contact responses in gene expression (Czaja *et al.*, 2012; Hohnjec *et al.*, 2015).

Upon contact, AM fungal hyphae attach to the root surface and start to establish a thickened structure, the so-called hyphopodium, which induces the formation of an intracellular prepenetration apparatus (PPA) by invagination into the epidermal and subsequent cortical cell layers (Nagahashi and Douds, 1997; Novero *et al.*, 2002; Genre *et al.*, 2005; Bonfante *et al.*, 2010). Reaching the inner cortex, intercellular hyphal colonization takes place. The AM symbiosis is characterized by highly specific intracellular structures, emerging from the intraradical mycelium (Figure 2). Via the plant-supported invagination into cells of the inner cortex, fungal hyphal tips branch to form tree-like dendritic structures, the so-called arbuscules (Harrison *et al.*, 1999; Demchenko *et al.*, 2004).

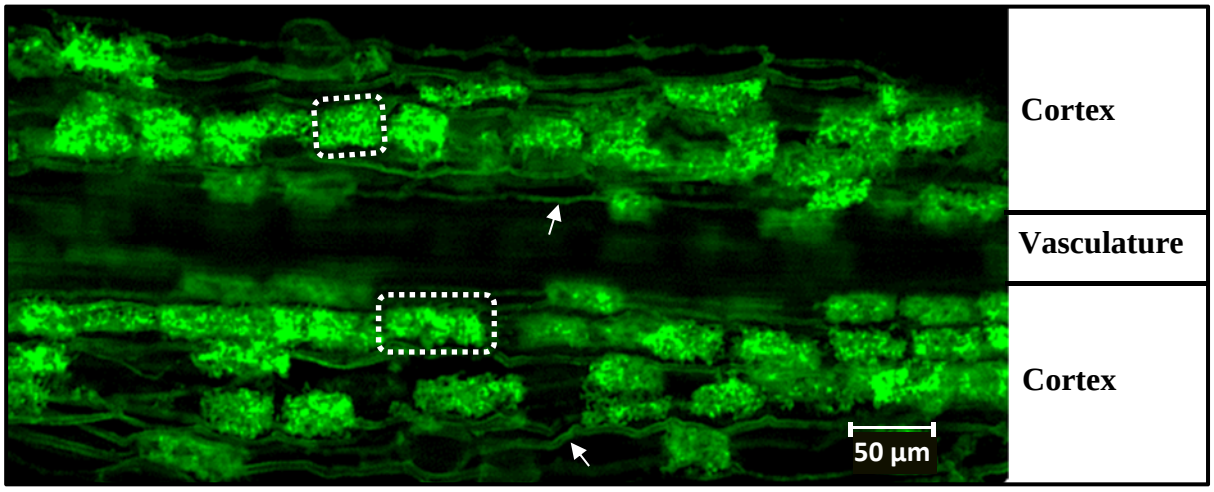

Figure 2: Confocal fluorescence image of arbuscules within roots of *Medicago truncatula*.
Mycorrhizal roots colonized with *Rhizophagus irregularis* were stained with Alexa Fluor488 WGA. Selected arbuscules are indicated by white boxes. Arrows indicate intraradical hyphae.

Arbuscules are surrounded by a plant-originated periarbuscular membrane (PAM), being the central interface for plant-fungal nutrient exchange (Strack *et al.*, 2001, Harrison *et al.*, 2002). The PAM contains a set of various and more or less specific transporters (Figure 3), mainly for phosphate but also for nitrate, ammonium, peptides, and other nutrients, including different minerals and water (Javot *et al.*, 2007; Martin and Nehls, 2009; Bonfante *et al.*, 2010; Giovanetti *et al.*, 2012).

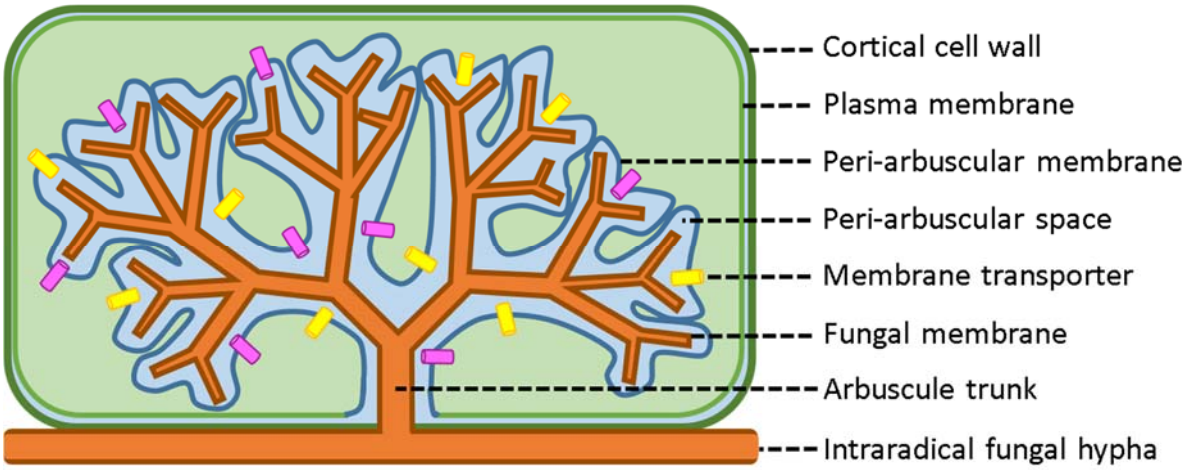

Figure 3: Scheme of an arbuscule-containing cortical cell.
The plant plasma membrane (light green) is extended by the periarbuscular membrane (PAM; dark blue). Several transporters are located within the PAM, generally distinguished in plant-derived nutrient (e. g. phosphate, nitrate, ammonium, peptides, minerals, and water) importers (yellow) and sugar or C16 fatty acid exporters (pink), together mediating the symbiotic exchange between plant and fungus. The PAM encloses the periarbuscular space (PAS; light blue) that forms the apoplastic compartment in direct contact to the fungal membrane (dark brown).

Whereas nutrients are delivered to the plant via PAM-located importers, the plant cell provides the fungal symbiont with organic carbon sources, including hexoses (e. g. glucose), but also fatty acids (Solaiman *et al.*, 1997; Pfeffer *et al.*, 1999; Hohnjec *et al.*, 2003; Zhang *et al.*, 2010; Gutjahr *et al.*, 2012). Fatty acids are particularly important for AM fungi, since these are auxotrophic with regard to fatty acid biosynthesis (Rich *et al.*, 2017) and thus rely on supplementation of C16 fatty acids from the host plant (Luginbuehl *et al.*, 2017). This dependency on the host might be a reason for the biotrophy of AM fungi.

Reprogramming of root cortical cells in AM symbioses

The establishment of a symbiotic interface via the formation of arbuscules within inner cortical cells relies on a fundamental transcriptional reprogramming, mediated by the activation of transcription factors (TFs) belonging to different classes (Hohnjec *et al.*, 2005; Guether *et al.*, 2009; Hogekamp *et al.*, 2011; Devers *et al.*, 2013; Hogekamp and Küster, 2013; Luginbuehl *et al.*, 2017; Gutjahr *et al.*, 2017; Floss *et al.*, 2017; Pimprikar *et al.*, 2018).

TFs can be found in all eukaryotic organisms. Functioning as regulators of gene expression that interact with enhancer regions of promoters to induce or repress transcription of target genes, they control both plant development and thus the reaction to external abiotic and biotic stimuli. The higher number of plant TFs in comparison to animals implies an involvement in the continuous adaption of plants to the environment, which cannot be avoided due to their sessile nature (Riechmann *et al.*, 2000; Shiu *et al.*, 2005).

As mentioned above, one of the first steps in plant cellular responses upon recognition of fungal Myc-COs and Myc-LCOs by LysM receptor kinases such as LYK3 (LysM domain containing receptor-like Kinase), NFP (Nod Factor Perception) and the LRR receptor kinase DMI2 (Doesn't Make Infection) (Wais *et al.*, 2000; Limpens *et al.*, 2003; Oláh *et al.*, 2005; Arrighi *et al.*, 2006; Czaja *et al.*, 2012) is a rhythmic nuclear calcium spiking. Upon signal transduction via unknown second messengers, the nuclear calcium level oscillations are deciphered by the calcium/calmodulin-dependent protein kinase CCAMK (DMI3) that binds and activates IPD3 (Interacting Protein of DMI3) (Oldroyd *et al.*, 2004; Yano *et al.*, 2008; Miller *et al.*, 2013). In addition, recent studies suggest additional, yet unknown regulatory pathways downstream of DMI3 that bypass IPD3 (Jin *et al.*, 2018).

IPD3 itself was found to be a transcription factor that induces the transcription of the *RAM1* (Required for Arbuscular Mycorrhization) gene, encoding a transcription factor that was shown to be a central regulator for pre-contact signaling (Gobbato *et al.*, 2012; Hohnjec *et al.*, 2015), but in particular also for arbuscule branching (Park *et al.*, 2015; Rich *et al.*, 2015; Pimprikar *et al.*, 2016; Luginbuehl *et al.*, 2017).

GRAS transcription factors controlling symbiotic interactions

RAM1 is a member of the GRAS TF family. These TFs form a subgroup of plant TFs, belonging to the GIBBERELLIN-INSENSITIVE (GAI), REPRESSOR of gal1-3 (RGA), or SCARECROW (SCR) subtypes (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Di Laurenzio *et al.*, 1996).

All GRAS proteins share a common conserved GRAS domain in their C-terminal region, consisting of two leucine heptad repeats (LHR), a VHIID, a SAW, and a PFYRE motif (Figure 4; Pysh *et al.*, 1999). The LHR-flanked VHIID domain appears to be crucial for DNA binding of some GRAS TFs (Hirsch *et al.*, 2009). The N-terminus of GRAS proteins is variable as well as intrinsically disordered (Sun *et al.*, 2011) and can also include other motifs, e. g. DELLA, which is known to modulate binding properties with other proteins (Day *et al.*, 2004).

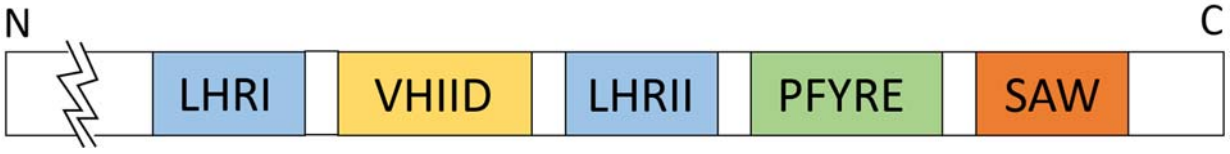

Figure 4: Schematic presentation of GRAS domains in the C-terminal region of GRAS proteins.
In the C-terminus, two leucine heptad repeats (LHRI and LHRII) are flanking the VHIID motif, followed by PFYRE and SAW motifs. The longer and variable N-terminus is not completely represented. Figure not to scale and according to Pysh *et al.*, 1999.

GRAS TFs can be found in a various number of plants, e. g. tomato, rice, grapevine, and *Arabidopsis thaliana* (Huang *et al.*, 2015; Niu *et al.*, 2017). Several physiological reactions are modulated by GRAS TFs, including gibberellic acid (GA) signaling (Peng *et al.*, 1997; Silverstone *et al.*, 1998), root development (Di Laurenzio *et al.*, 1996), and stress response (Huang *et al.*, 2015; Grimplet *et al.*, 2016).

Prominent GRAS TFs have been shown to play a vital role in symbiotic signaling in general (Bucher *et al.*, 2014), e. g. NSP1 and NSP2 (NODULATION SIGNALING PATHWAY) that play a key role in the early transduction of signals during rhizobial and mycorrhizal symbioses (Maillet *et al.*, 2011; Czaja *et al.*, 2012; Hohnjec *et al.*, 2015). In response to the elicitation by rhizobial Nod-factors (NFs), NSP1 and NSP2 form a heterodimer that binds to *cis*-regulatory elements in the promoter of the *ENOD11* gene to induce plant symbiotic signaling responses (Hirsch *et al.*, 2009; Liu *et al.*, 2011). Interestingly, a distortion of the NSP1-NSP2 complex by mutation or knockout of one or both components leads to a significantly reduced arbuscular mycorrhizal colonization in *M. truncatula* (Maillet *et al.*, 2011; Liu *et al.*, 2011) as well, indicating common signaling during AM and root nodule symbioses.

A central component for early AM signaling and AM development is the above-mentioned GRAS TF RAM1 (Required for Arbuscular Mycorrhization) that was first described to be essential for proper AMF colonization in *M. truncatula* (Gobbato *et al.*, 2012). Further studies revealed that *RAM1* mutants are not able to develop highly fine-branched arbuscules in *M. truncatula* and *Petunia hybrida* (Park *et al.*, 2015; Rich *et al.*, 2015; Xue *et al.*, 2015; Pimprikar *et al.*, 2016). RNAseq studies revealed several hundred genes being dependent on *RAM1* (Luginbuehl *et al*., 2017), including genes involved in PAM biogenesis, fatty acid biosynthesis, and several PAM-located transporters that were not expressed in *RAM1* mutants, indicating a central position of *RAM1* in the signaling cascade (Park *et al.*, 2015; Rich *et al.*, 2015; Pimprikar *et al.*, 2016).

Target genes induced by RAM1 include fatty acid biosynthesis and export genes, e. g. *RAM2* (glycerol-3-phosphate acyltransferase) and *STR1/2* (ABC-transporters), encoding proteins required to synthesize and transport C16 fatty acids to the AMF (Zhang *et al.*, 2010; Gobbato *et al.*, 2012; Gutjahr *et al.*, 2012). Another RAM1-target is *EXO70i*, encoding an EXOCYST component shown to be involved in the establishment of the periarbuscular membrane by supporting exocytosis towards the growing hyphal tips, a process that is severely affected in *RAM1* mutants (Pumplin *et al.*, 2010; Zhang *et al.*, 2010; Gutjahr *et al.*, 2012; Gobbato *et al.*, 2012; Park *et al.*, 2015; Rich *et al.*, 2015; Zhang *et al.*, 2015; Xue *et al.*, 2015; Luginbuehl *et al.*, 2017). The linkage of RAM1 with the fatty acid metabolism and its role as a key regulator

in the development of PAMs and thus the establishment of the central plant-fungus symbiotic interface itself highlights the importance of a further assessment of the RAM1-correlated transcriptional regulation during AM. Interestingly, RAM1-dependent gene expression was differentially affected in mutant lines of *Lotus japonicus* in comparison to *M. truncatula* and *P. hybrida*, suggesting variable regulation networks in different AM host species (Pimprikar *et al.*, 2016; Pimprikar *et al.*, 2018). This difference is also striking, when compared to mutants of the *RAM1* homologue *RAD1* (Required for Arbuscule Development) that show an affected arbuscule formation in *L. japonicus*, but normal arbuscule phenotype in *M. truncatula* (Xue *et al.*, 2015; Park *et al.*, 2015).

Although these GRAS TFs were already shown to be specifically integrated into the regulatory network that controls AMF colonization and arbuscule formation, transcriptomic studies revealed that several other GRAS TF genes are activated during AM symbioses (Hogekamp *et al.*, 2011; Gaude *et al*., 2012; Hogekamp and Küster, 2013), indicating that additional and yet uncharacterized GRAS TFs modulate the development of AM symbioses.

Medicago truncatula and *Rhizophagus irregularis* as models for the functional genomics of arbuscular mycorrhiza

Almost all land plants are able to form mycorrhizal symbioses. Unfortunately, *Arabidopsis thaliana*, which is a general model plant for genetic research, is one of the few plants unable to undergo such an association. For this reason, the legume *M. truncatula* was established as a model organism to study symbiotic interactions with AMF, in addition to its initial application to investigate the root nodule symbiosis with soil-borne rhizobial bacteria (Barker *et al.*, 1990; Cook *et al.*, 1999; Rose *et al.*, 2008).

M. truncatula exhibits several advantages that make it suitable as a model organism, such as a small and diploid genome (~ 500 Mb; 2n = 2x = 16; Agarwal and Gupta, 1983), a short generation cycle, and the ability for regeneration from protoplasts or via somatic embryogenesis (Barker *et al.*, 1990). *M. truncatula* is also suitable for *Agrobacterium*-mediated transformation and most importantly for the induction of transgenic hairy roots, enabling the transient expression of genetic tools, e. g. knockdown constructs or reporter gene fusions, for the investigation of gene functions (Boisson-Dernier *et al.*, 2001; Vieweg *et al.*, 2004).

Over the years, different sequencing projects led to the identification of more than 94 % of the *M. truncatula* gene space. The most recent release contains > 50 000 annotated gene products (http://www.medicagogenome.org; Young *et al.*, 2011; Krishnakumar *et al.*, 2015; Tang *et al.*, 2014). Functional analyses in *M. truncatula* are supported by the existence of a database of

mutant lines established by fast neutron bombardment (FNB) or *Tnt1* (Transposable element of *Nicotiana tabacum*) insertions (Tadege *et al.*, 2008).

On the transcriptional level, comprehensive transcript sequencing efforts led to the establishment of expressed sequence tag (EST) based cDNA arrays that provided the first possibility for high-throughput analyses of the transcriptional reprograming in *M. truncatula* (Liu *et al.*, 2003; Küster *et al.*, 2004). Relying on these EST-based as well as initial genomic sequences, the establishment of a first generation of Affymetrix *Medicago* GeneChips enabled standardized genome-wide transcriptomic analyses, including the setup of a comprehensive database for storing, mining and reviewing *Medicago* GeneChip data (https://mtgea.noble.org; Benedito *et al.*, 2008; He *et al*., 2009).

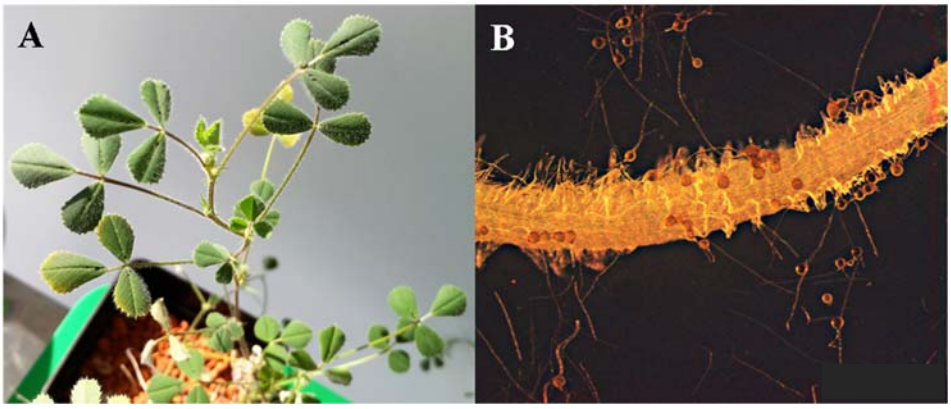

Figure 5: The model legume *Medicago truncatula* and the model AM fungus *Rhizophagus irregularis*.
A Habitus of *M. truncatula* **B** *R. irregularis* hyphae emerging from germinating spores in close vicinity of a single *M. truncatula* root.

With respect to the AM microsymbiont, *Rhizophagus irregularis* (formerly known as *Glomus intraradices*) was used in this study. AM fungi of the Glomeromycota are obligate biotrophs and can thus not be cultivated without host roots, which they depend on to complete their life cycle (Fortin *et al.*, 2002). Efforts to study the genetic repertoire of *R. irregularis* aimed at the transcriptome (Tisserant *et al.*, 2011) and the genome level (Tisserant *et al.*, 2013), and in total revealed more than 28 000 gene models of the 153 Mb *R. irregularis* genome that can be accessed from the *Rhizophagus irregularis* database (http://mycor.nancy.inra.fr).

AIMS OF THIS THESIS

To contribute to the understanding of the transcriptional reprogramming that leads to the formation of arbuscules, the following approaches are purposed. Each of these will be the topic of one of the following three chapters.

1. Identification of candidates for AM-relevant GRAS TFs via the genome-wide monitoring of gene expression in *Medicago truncatula* roots during a time course of colonization with *Rhizophagus irregularis*.
2. Performing protein-protein interaction studies on selected AM-related GRAS TFs using the Yeast Two-Hybrid system.
3. Functional analyses of selected AM-related GRAS TFs in mutant lines or knockdown roots to define and relate regulatory modules that control arbuscule development.

CHAPTER I:

Gene expression during a time course of AM development

Scope:

The major aim of this chapter is the analysis of arbuscular mycorrhiza-related gene expression in a time course of plant roots under progressing fungal colonization. Genome-wide transcription changes in the mycorrhizal roots are detected, using samples from different time points of arbuscular mycorrhizal fungal root colonization for GeneChip hybridizations. The specific expression profiles obtained provide a global view of the transcriptional reprogramming during the development of AM symbioses and can be used to assess the impact of distinct genes at different stages of AM formation. Furthermore, candidate genes for the regulation of AM development are identified for further functional studies.

Contributor roles:

R. M. Hartmann carried out the plant experiments, RNA extractions, and data analyses

N. Hohnjec provided reference GeneChip hybridizations for filtering AM-induced genes

H. Küster performed initial evaluations of GeneChips hybridizations

ABSTRACT

Genome-wide studies of gene expression during an AM time course revealed insights into a substantial reprogramming of roots during the process of AMF colonization. This approach fills a gap in all former studies that monitored transcriptional changes in AM symbiosis at a single time point of colonization. Well-known key factors in AM development were identified and their expression over time was monitored during the process of continuing AMF colonization. In addition, other yet uncharacterized genes were found to follow the ongoing AM establishment. With respect to transcriptional regulators, several families of transcription factor genes were found to be upregulated along the time course of fungal colonization, including several GRAS TF genes that deserve particular attention. Further characterization of GRAS TF gene expression over time revealed two major transcription profile categories that might reflect general biological impacts of the encoded TF on AM development. Finally, the chronological patterns of gene expression during AM-induced transcriptional reprogramming presented in this chapter contributes to the understanding of the impact of specific *M. truncatula* genes on different stages of AM development.

INTRODUCTION

The arbuscular mycorrhizal (AM) symbiosis is a widespread beneficial association of vascular plants with different mycorrhizal fungal species that can be found in ~ 80 % of recent land plants (Smith and Read, 1997; Schüssler *et al.*, 2001; Smith and Read, 2008). The formation of a symbiotic interface between plant and AM fungi (AMF) is manifested as intracellular dendritic fungal structures called arbuscules (Harrison *et al.*, 1999; Demchenko *et al.*, 2004).

In the past 15 years, several studies were aiming at the identification of transcriptional changes during the development of AM symbioses. Initially, expressed sequence tag (EST) based cDNA- and oligonucleotide arrays provided the first possibility for high-throughput analyses of the transcriptional reprograming in *M. truncatula* (Liu *et al.*, 2003; Küster *et al.*, 2004; Küster *et al.*, 2007) that revealed differentially expressed genes in different stages of AM development (Liu *et al.*, 2003), but also in association with different AM (Hohnjec *et al.*, 2005). Further studies demonstrated the differentiation of transcriptional profiles even between distinct early stages of AM formation (Brechenmacher *et al.*, 2004; Weidmann *et al.*, 2004).

Whole-genome transcriptome profiling was improved, when Affymetrix *Medicago* GeneChips were introduced (Benedito *et al.*, 2008; https://mtgea.noble.org). The latest GeneChip versions contain 3.6 million probes, representing ~ 50 000 genes (Affymetrix Inc., 2015), basing on a recent *M. truncatula* genome release which covers more than 94 % of the gene space

(http://www.*Medicago*genome.org; Krishnakumar *et al.*, 2015; Tang *et al.*, 2014). Using the current Affymetrix *Medicago* GeneChips, analyses of (almost) the entire transcriptome of *M. truncatula* thus allow a genome-wide view on transcriptional changes in a multitude of conditions.

GeneChip experiments revealed comprehensive patterns of gene expression in AM symbiotic tissues, including several TF genes (Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013; Gaude *et al.*, 2012). The high number of AM-induced TF genes appears plausible, since the establishment of an AM symbiotic association is accompanied and governed by a fundamental transcriptional reprograming (Hohnjec *et al.*, 2005; Gaude *et al.*, 2012; Bucher *et al.*, 2014).
The inherent drawback in the usage of AM roots containing a mixture of different cell types was overcome, when laser microdissection was used to specifically analyze the transcriptome of arbuscule-containing and the surrounding cortical cells (Gomez *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2012). In addition, early steps of the interaction were studied by including areas connected to hyphopodia (Hogekamp and Küster, 2013).
While these technical advantages allowed the isolation of distinct cell types and thus provided a better spatial resolution of gene expression, genome-wide studies of the transcriptional changes during a time course of AM formation were missing. To achieve a global view on the transcriptional reprograming during the ongoing mycorrhizal colonization from early to late stages, gene expression was investigated in a mycorrhizal time course from 0 to 42 dpi, using the most recent version of *Medicago* GeneChips. This experiment revealed a substantial number of genes from different functional categories including 40 transcriptional regulators that show expression patterns following the ongoing process of mycorrhizal colonization. These genes are thus of special interest for an identification of potential key players in the development of AM symbioses.

MATERIAL AND METHODS

Plant growth, inoculation with AM fungi, and staining of fungal structures

Medicago truncatula Gaertn cv Jemalong genotype A17 seeds were surface-sterilized and scarified as reported by Hohnjec *et al.* (2005). Plants were grown in the climate chamber (relative humidity: 60 %; photosynthetic photon flux: 50 µmol m^{-2} s^{-1}), using a 16 h light (22 °C) and 8 h dark (18 °C) regime

After four weeks of growth, each plantlet was mycorrhized by inoculation in a small amount of water with 2 000 germinating *Glomus intraradices* Schenck and Smith DAOM197198 spores

(Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada), having been reassigned to *Rhizophagus irregularis* (Błaszk., Wubet, Renker, and Buscot) C. Walker & A. Schüßler comb. nov. (Stockinger *et al.*, 2014). After 3-4 hours of inoculation, plantlets were potted into 8x7x7 cm (height x width x depth) pots filled with sterile Seramis® (Seramis GmbH, Mogendorf, Germany). Each pot contained two plants, and remaining spore solution was directly pipetted onto the root surface while potting. Plants were fertilized with half-strength Hoagland`s solution (Arnon *et al.*, 1940) containing 20 µM phosphate. The solution was prepared with deionized water, pH was adjusted to 6.4 with KOH.

To visualize fungal colonization, roots were incubated in 10 % (w/v) KOH at 95 °C for 7 min, repeatedly rinsed with water and incubated in ink staining solution (5 % (v/v) black ink (Sheaffer, Connecticut, USA) in 0.8 % (v/v) acetic acid) at 95 °C for 3 min. Samples were rinsed with 0.8 % (v/v) acetic acid and incubated in 0.8 % acetic acid for 20 min at room temperature. Photo documentation was performed using a Leica MZ 10F stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with an Olympus XC50 camera (Olympus, Hamburg, Germany). Plant roots were harvested at 0, 7, 10, 14, 21, 28, 35, and 42 days post inoculation (dpi) with *R. irregularis*. Plants at 0 days post inoculation were harvested after 3 h of inoculation with *R. irregularis* spores. At each time point, three biological replicates were harvested, each consisting of six pooled root systems.

RNA isolation

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Tissue disruption was carried out via FastPrep®-24 (MP Biomedicals, Santa Ana, USA). RNA was checked using a Bioanalyzer (Agilent, Waldbronn, Germany) to exhibit a high RNA integrity (RIN > 8.5).

Hybridization and data evaluation of GeneChip *Medicago* Transcriptome Assays

Biotinylated aRNA obtained from 100 ng of total RNA for each sample was fragmented as recommended (GeneChip *Medicago* Transcriptome Assay, ThermoFisher Scientific, Schwerte, Germany). The size distribution of the fragmented aRNA was assessed via an Agilent bioanalyzer (Agilent Technologies, Böblingen, Germany) using an RNA 6000 assay. Standard hybridization, post-hybridization wash and double-staining as well as scanning was done as specified for GeneChip *Medicago* Transcriptome Assays (ThermoFisher Scientific, Schwerte, Germany).

Cel files were analyzed using the Expression Console and Transcriptome Analysis Console software (both ThermoFisher Scientific, Schwerte, Germany). Normalization was performed

via the Robust Multichip Average algorithm, intensity values for each probe set were log2-transformed and averaged across the three biological replicates using the Tukey's Bi-weight average algorithm, and expression ratios were evaluated statistically via tools of the Transcriptome Analysis Console (ThermoFisher Scientific, Schwerte, Germany).

Original annotations of the genes represented on the recently developed GeneChip *Medicago* Transcriptome Assays (hereinafter for simplicity called GeneChips) were updated by annotations from the *M. truncatula* genome version 4.0 (Tang *et al.*, 2014), and mapped to probe sets from the previous versions of *Medicago* GeneChips (*Medicago* genome arrays) that were used to construct the *Medicago* Gene Expression Atlas (https://mtgea.noble.org ; Benedito *et al.*, 2008, He *et al*., 2009) as well as to the UniProt (The UniProt Consortium, 2017) database.

Analysis of GeneChip *Medicago* Transcriptome Assays

Probes not mapped to Mt 4.0 (Probes designated TSUnmapped and TSUnmappedRC) were eliminated from the GeneChip Assay data set. The remaining probes (TC and TCscaffold) were searched for duplicates. Duplicates were manually validated and scored basing on sequence alignment data (E-value, sequence identity, hit length) from the Mt 4.0 mapping. Duplicates with lowest alignment scores were eliminated. For duplicates with similar alignment scores, probes showing lower signal intensities on GeneChip hybridizations were discarded. This procedure resulted in a non-redundant set of probes representing all genes of the *M. truncatula* genome release 4.0.

Data sets derived from the mycorrhization time course were filtered for at least 2-fold ($p < 0.05$) AM-induced gene expression using GeneChip hybridization data from *R. irregularis* mycorrhizal roots at 28 dpi vs. non-mycorrhizal roots of comparable age (Hogekamp *et al.*, 2011; Natalija Hohnjec, Leibniz Universität Hannover, personal communication).

A functional classification of AM-upregulated genes was performed by mapping transcripts to the *A. thaliana* proteome and visualization using the MapMan software (Usadel *et al.*, 2005). Genes that could not be mapped to the *A. thaliana* genome by MapMan were manually classified basing on their *M. truncatula* genome version 4.0 (Tang *et al.*, 2014) annotation. Venn diagrams were drawn using the VENNY software (Oliveros, 2017).

RESULTS AND DISCUSSION

Arbuscular mycorrhiza marker gene expression reflects the process of fungal colonization

To monitor the ongoing AMF colonization, ink stainings were performed and evaluated regarding the presence of distinct fungal structures as shown in Figure I_1. In the first seven days after inoculation with *R. irregularis*, fungal structures are limited to germinating spores

and the extraradical mycelium attached to the root surface. First infection units with intraradical hyphae and the first emerging arbuscules were observed at 10 dpi. Intraradical growth and fungal colonization proceeds substantially towards the later time points.

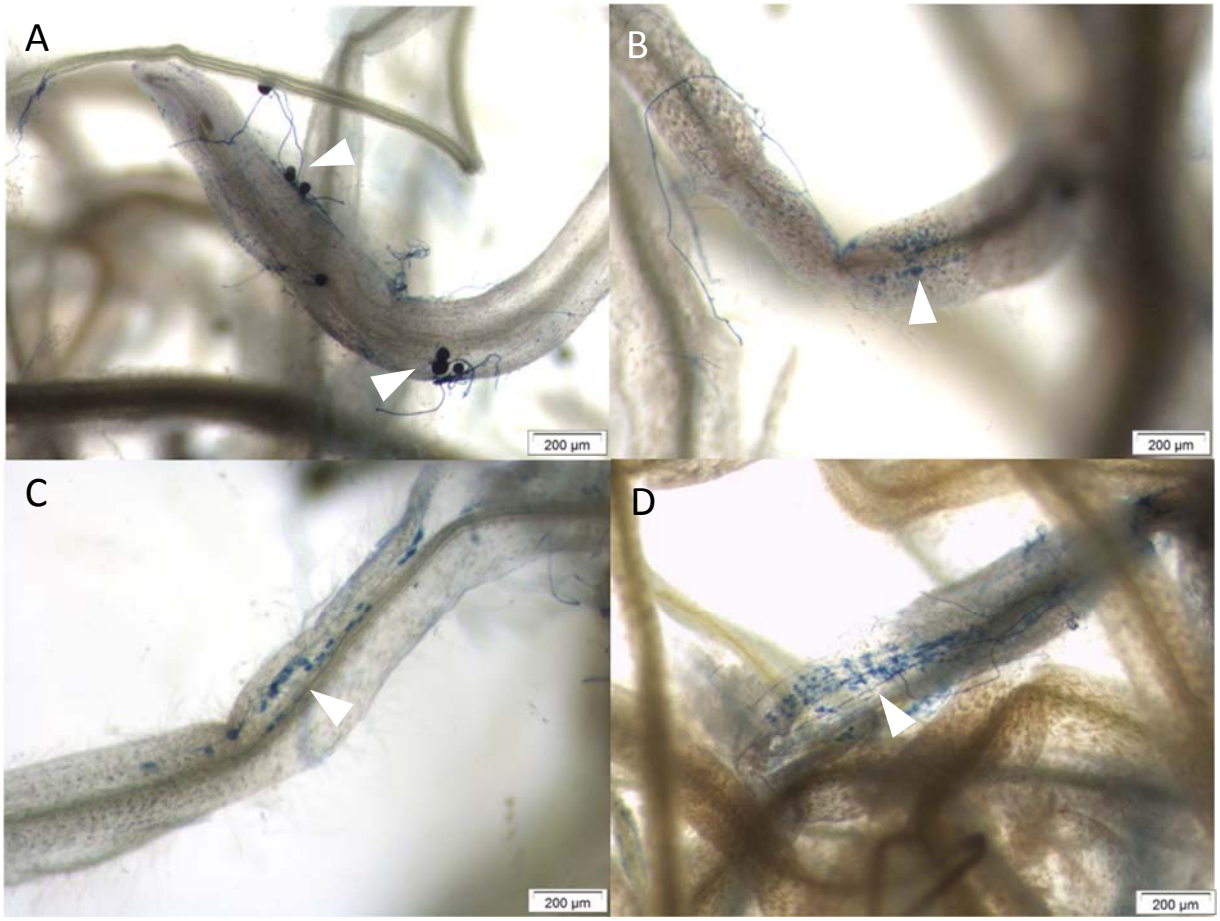

Figure I_1: Ink stainings of *M. truncatula* roots at different time points post inoculation with *R. irregularis*. White arrows indicate relevant structures: **A** Germinating spores and extraradical hyphae attached to the root surface (7 dpi). **B** Initial infection units with first visible arbuscules (10 dpi). **C** Evolving infection unit, extending along the longitudinal root axis (14 dpi). **D** Highly developed infection unit (21 dpi). Abbreviation: dpi, days post inoculation.

To examine transcription in *M. truncatula* roots during the ongoing mycorrhizal colonization, whole-genome GeneChip hybridizations were performed. At each of eight time points of mycorrhizal colonization (0, 7, 10, 14, 21, 28, 35, and 42 dpi), three biological replicates of root material were harvested and used for the isolation of total RNA, which was used for hybridization of *Medicago* GeneChips. Gene-specific signal intensities were plotted to visualize transcriptional regulation during the ongoing AMF colonization. To assess the process of colonization of *M. truncatula* roots with *R. irregularis*, housekeeping genes of *M. truncatula* (*MtTefalpha*) and *R. irregularis* (*GiTefalpha*) were visualized in Figure I_2. While *MtTefalpha* expression remained at a constantly high level during the entire time course, *GiTefalpha* signal intensities are rising during ongoing colonization reflecting the propagating fungal biomass. To

further evaluate the establishment of functional arbuscules, expression of the strongly expressed and arbuscule-specific phosphate transporter gene *MtPt4* (Harrison *et al.*, 2002) was also included. The inclination of *MtPt4* expression between 7 and 28 dpi is higher than the *GiTefalpha* inclination, indicating a superpropotional *MtPt4* activation in comparison to the presence of fungal biomass. *MtPt4* and *GiTefalpha* gene expression start an approximatively log-linear increase between 7 dpi and 42 dpi, fitting to the observation of first intraradical fungal growth between 7 and 10 dpi, as monitored by ink staining (Figure 1). Since signal intensities are shown as log2-values, the log-linear increase of *GiTefalpha* expression is equivalent to an approximately exponential increase of *GiTefalpha* transcript amounts on a linear scale.

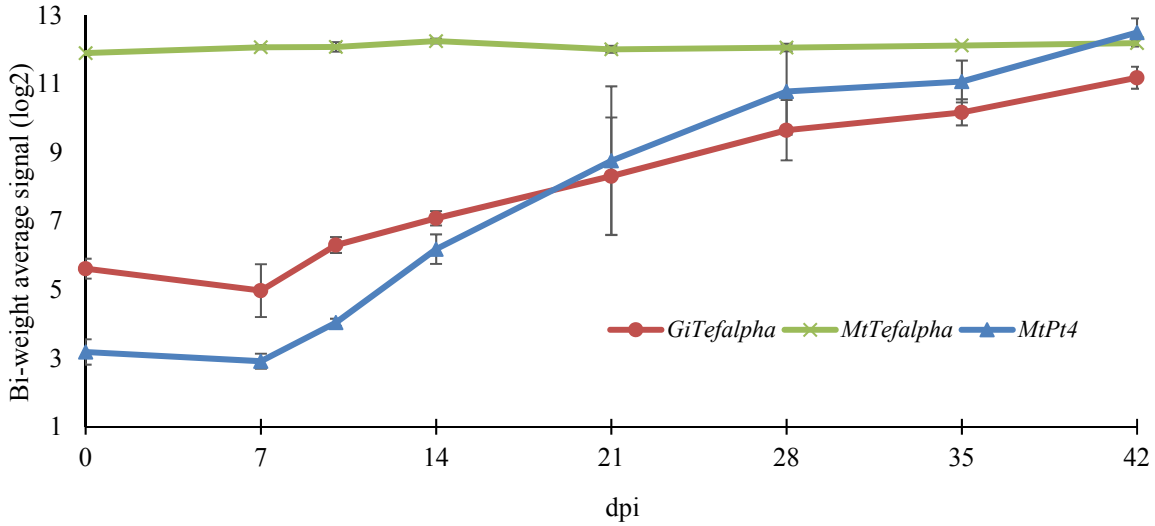

Figure I_2: Time course analysis of marker gene expression in *M. truncatula* roots at different time points (0, 7, 10, 14, 21, 28, 35, and 42 days) post inoculation with *R. irregularis*.
Expression of housekeeping genes *of M. truncatula* (*MtTefalpha*; green daggers) and *R. irregularis* (*GiTefalpha*; red circles) as well as the AM-specific phosphate transporter gene *MtPt4* (blue triangles) was plotted, using signal intensities from Affymetrix GeneChip hybridizations. Standard deviations are indicated as error bars. Highest standard deviations occur at 21 dpi and 28 dpi for *GiTefalpha* and *MtPt4*, while *MtTefalpha* standard deviations remain on a low stable level across the entire time course. Linear regression of *GiTefalpha* signal intensities is shown as dotted line, the approximate polynomial function is presented in red. N = 3 biological replicates, each consisting of a pool of 6 individual root systems per time point. Abbreviation: dpi, days post inoculation.

The superpropotional increase of *MtPt4* transcription from 7 to 28 dpi appears plausible when viewed in the light of the localization of phosphate transporters within the periarbuscular membrane (Figure 3). While exponential growth is one of the most common and widely occurring functions in biophysical systems and thus demonstrates a proper colonization of plant roots by the fungal symbiont, the development of the periarbuscular membrane is correlated superpropotionally. This phenomenon was demonstrated by Dickson and Kolesik (1999) by measuring surface-area-to-volume ratios of highly branched arbuscules in comparison to intracellular hyphal coils. This study demonstrated a ratio of surface area [μm^2] to volume [μm^3] of ~2.5 μm^{-1} for branched arbuscules in contrast to ~0.9 μm^{-1} for intracellular coils. When

considering the development of the symbiotic interface, manifested in the periarbuscular membrane that surrounds arbuscule branches, it becomes evident that the surface area is increasing disproportionally faster than the fungal biomass itself. Since the MtPt4 transporter is located within this fast-growing surface of the PAM, *MtPt4* expression is necessarily higher to maintain the development of the nutritional interface.

It can therefore be concluded that the expression profiles of *GiTefalpha* and *MtPt4* exhibit a pattern that reflects an efficient fungal colonization of the host root as well as the establishment of a functional phosphate-transport interaction. Hence, transcriptome changes along the time course are valid with respect to a proper development of the AM symbiosis.

AM marker gene expression shows a constant increase during the AM time course

To further validate the transcriptional data obtained from the time course of mycorrhizal roots, the expression of prominent AM marker genes, namely *MtPt4* (Harrison *et al.*, 2002; Liu *et al.*, 2003), *MtBcp1* (Hohnjec *et al.*, 2005), *MtScp1* (Liu *et al.*, 2003), *MtLec7* (Frenzel *et al.*, 2005), *MtGlp1* (Doll *et al.*, 2003), *MtHa1* (Krajinski *et al.*, 2002), *MtSbtM1* (Takeda *et al.*, 2011), *MtRam1* (Gobbato *et al.*, 2012), *MtStr2* (Zhang *et al.*, 2010), *MtMyb1* (Liu *et al.*, 2003), and *MtErf1* (Devers *et al.*, 2013) were analyzed.

Table I_1: Overview of AM marker genes selected to evaluate gene expression during a time course of mycorrhizal colonization.

Gene	Annotation	Function according to literature	References
MtPt4	Inorganic phosphate transporter	Essential for transport of phosphate through the periarbuscular membrane towards the plant cytoplasm	Javot *et al.*, 2007
MtScp1	Serine carboxypeptidase	Possibly involved in ligand-processing and mycorrhiza-signaling	Liu *et al.*, 2003
MtGlp1	Germin-like protein	Probably own subgroup of GLPs, specific for AM host plants, unknown function	Doll *et al.*, 2003
MtStr2	ABC transporter	Essential for arbuscule development, located in distal ends of arbuscule branches, elusive substrate	Zhang *et al.*, 2010 Gutjahr *et al.*, 2012
MtBcp1	Plastocyanin-like Blue copper protein	Located in trunk area of arbuscule containing and plasma membrane of surrounding cortical cells, unknown function	Hohnjec *et al.*, 2005 Pumplin *et al.*, 2009 Ivanov *et al.*, 2014
MtLec7	Legume lectin	Possibly binding of carbohydrates on fungal surface	Frenzel *et al.*, 2005
MtHa1	Plasma membrane H+ATPase	Essential for arbuscule branching, probably by acidifying the periarbuscular space	Krajinski *et al.*, 2002 Zhang, 2014
MtSbtM1	Subtilisin-like serine protease	Required for AM fungal colonization, involved in gibberellic acid signaling	Takeda *et al.*, 2009 Takeda *et al.*, 2015
MtRAM1	Reduced AM GRAS TF	Essential for the branching of arbuscule tips, major regulator of arbuscule development	Gobbato *et al.*, 2012 Park *et al.*, 2015
MtMyb1	Myb-like TF	Induces an arbuscule-specific senescence program controlling arbuscule life span and degeneration	Floss *et al.*, 2013 Floss *et al.*, 2017
MtErf1	AP2 domain TF	Required for proper arbuscule development	Devers *et al.*, 2013

Annotations are given based on the *M. truncatula* genome 4.0 as well as on published functions. Acronymogenic parts of gene annotations are underlined.

An overview of the marker genes analyzed including their proposed or putative impact on AM interactions is presented in Table I_1. Since many of these marker genes were reported to be upregulated during the AM symbiosis and in some cases were shown to play vital roles, they represent additional markers for checking the integrity and validity of the AM time course transcription data.

Table I_2: Comparison of selected AM marker gene expression at different time points of mycorrhizal colonization.

Gene	Mt genome 4.0 ID	Expression level (log2) at time points (dpi)								Linear FC	Cell types
		0	7	10	14	21	28	35	42		
MtPt4	Medtr1g028600.1	3,2	2,9	4,0	6,2	8,8	10,8	11,1	12,5	568	ARB* C+ARB**
MtScp1	Medtr3g079590.1	3,0	4,6	6,3	7,4	8,9	10,1	10,2	11,2	73	UBI* C+ARB**
MtGlp1	Medtr4g052770.1	3,4	4,3	4,7	6,5	8,7	10,3	10,5	11,6	129	UBI* C+ARB**
MtStr2	Medtr5g030910.1	3,7	4,4	4,3	4,1	5,2	6,6	7,5	9,0	49	ARB+EPI* n.d.**
MtBcp1	Medtr7g086190.1	2,7	3,0	3,2	3,8	4,9	6,0	6,2	8,0	29	ARB* C+ARB**
MtLec7	Medtr8g068030.1	2,5	3,0	3,7	4,9	6,7	8,5	8,9	10,1	168	ARB*/**
MtHa1	Medtr8g006790.1	3,0	3,2	3,4	3,8	5,5	7,5	7,9	9,7	91	UBI* C+ARB**
MtSbtM1	Medtr5g011320.1	2,8	3,2	3,7	4,1	5,9	7,7	8,2	9,6	113	n. d.* n. d**
MtRAM1	Medtr7g027190.1	2,7	2,9	3,1	3,2	4,9	6,2	7,1	8,5	48	n. r.*/**
MtMyb1	Medtr7g068600.1	2,8	2,7	2,9	3,2	5,8	8,2	8,6	9,8	234	UBI* C+ARB**
MtErf1	Medtr7g009410.1	2,8	2,9	2,8	2,9	3,4	5,1	6,0	7,5	27	ARB* n.d.**

Prominent AM marker genes were selected to prove a proper AMF root colonization and the validity of the obtained transcriptional data. Common gene names and corresponding *M. truncatula* genome 4.0 identifiers are presented next to the log2 average signal intensities of the corresponding GeneChip probe sets between 0 and 42 dpi after inoculation with *R. irregularis*. Columns are colored basing on the log2 expression values (yellow for low values, red for high values, same scale for all columns). Linear transcriptional fold-change (FC) values of mycorrhizal and non-mycorrhizal roots at 28 dpi (Hogekamp *et al.*, 2011; Natalija Hohnjec, Leibniz Universität Hannover, personal communication) are indicated to demonstrate AM-induced gene expression. Cell-specific localizations shown in previous studies (*Hogekamp *et al.*, 2013; **Gaude *et al.*, 2012) distinguish transcriptional induction in arbuscule-containing cortical cells (ARB), non-arbuscule containing cortical cells of mycorrhizal roots (C), epidermal cells of mycorrhizal roots (EPI), and all cell types of mycorrhizal roots (UBI). Genes that could not be detected as expressed are designated as n. d., genes that are not represented on the GeneChip are designated as n. r.. Abbreviations: dpi, days post inoculation; FC, fold change.

All AM marker genes show a continuous transcriptional upregulation in the ongoing mycorrhization time course. Whereas *MtScp1* and *MtGlp1* show the strongest increase of signal intensities already in the early time points, *MtBcp1*, *MtHa1*, *MtSbtM1*, *MtRam1*, *MtStr2*, *MtMyb1*, and *MtErf1* show a similar inclination, with a significant increase between 14 and 21 dpi.

Considering the transcription profiles of AM marker genes (Table I_2), the expression of the fungal housekeeping gene *GiTefalpha* (Figure I_2) as well as the histochemical monitoring of mycorrhizal root material (Figure I_1), the obtained transcriptional data appear valid and can thus be used for further analyses of AM-induced gene transcription profiles.

Transcriptional reprogramming during the ongoing colonization of arbuscular mycorrhizal roots

To mine the time course GeneChip data for mycorrhiza-correlated transcription, gene expression levels were compared between 42 dpi and 0 dpi. Based on the linear expression values, fold-changes in gene expression were calculated along the entire course of mycorrhizal colonization. Fold changes of at least 2-fold and $p < 0.05$ between 42 and 0 dpi were considered to represent a significant transcriptional induction. However, 0 dpi expression values tend to be disproportionally high in comparison to values at 7 or 10 dpi. One explanation for this phenomenon might be the higher stress conditions due to the root harvesting and incubation with AM fungal spore solution just prior to sampling in case of the 0 dpi samples. Thus, 0 dpi expression values might be biased. To overcome this issue, fold-changes were also calculated for 42 dpi in comparison to 7 dpi, the earliest assumed time point of initial arbuscule formation. This procedure resulted in two sets of genes activated at least 2-fold ($p < 0.05$) at 42 dpi vs. 7 dpi and 0 dpi, respectively (Supplemental table S_1).

Since time course expression data not only reflect gene expressions that are responding to AMF colonization but also to the ongoing root development, the 42 vs. 0 dpi and 42 vs. 7 dpi data sets were filtered for AM induction via GeneChip data from 28 dpi mycorrhizal vs. corresponding non-mycorrhizal roots (Hogekamp *et al.*, 2011; Natalija Hohnjec, Leibniz Universität Hannover, personal communication). The resulting sets of AM-induced genes activated over the time of AM fungal colonization were compared and visualized in Figure I_3.

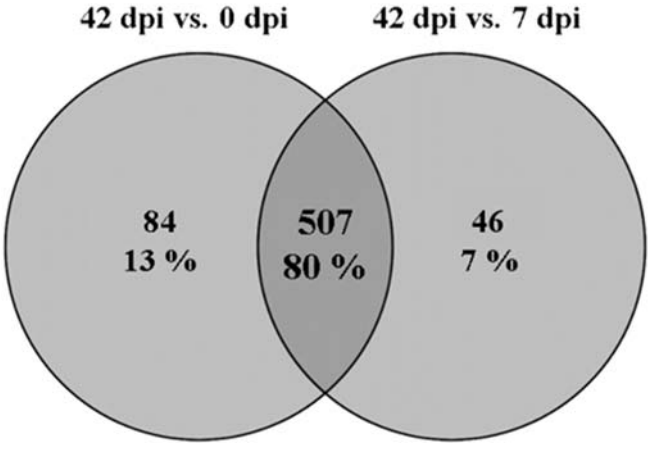

Figure I_3: VENN diagram of genes upregulated at different time points of AM fungal colonization.
AM-induced genes with ≥ 2-fold ($p < 0.05$) expression changes at 42 dpi vs 0 dpi and 42 dpi vs 7 dpi are shown. The complete list of genes is shown in Supplemental Table S_1.

A total of 637 AM-induced genes were found to be upregulated during the time course of fungal colonization. This set of genes that correlate to AM development over time will be subject of functional investigations.

Interestingly, 535 (84 %) of the 637 AM-related genes were also shown to be dependent on the GRAS TF MtRam1 in an experiment studying the transcription of mycorrhizal MtRam1 mutant roots (see Chapter III). This indicates a strong correlation of most of the time course induced genes with arbuscule development, which is highly dependent on *MtRam1* (Park *et al.*, 2015).

Mycorrhizal colonization-related genes reflect the process of AM fungal colonization

To focus on the impact of genes that are required for the established AM symbioses, the 637 genes upregulated during the time course of AM development were further addressed. Genes upregulated during fungal colonization were attributed to functional categories, using the MapMan software to detect major changes in cellular functions (Supplemental Figure S_1) as well as transcriptional reprogramming during the establishment of the plant-fungal interface.

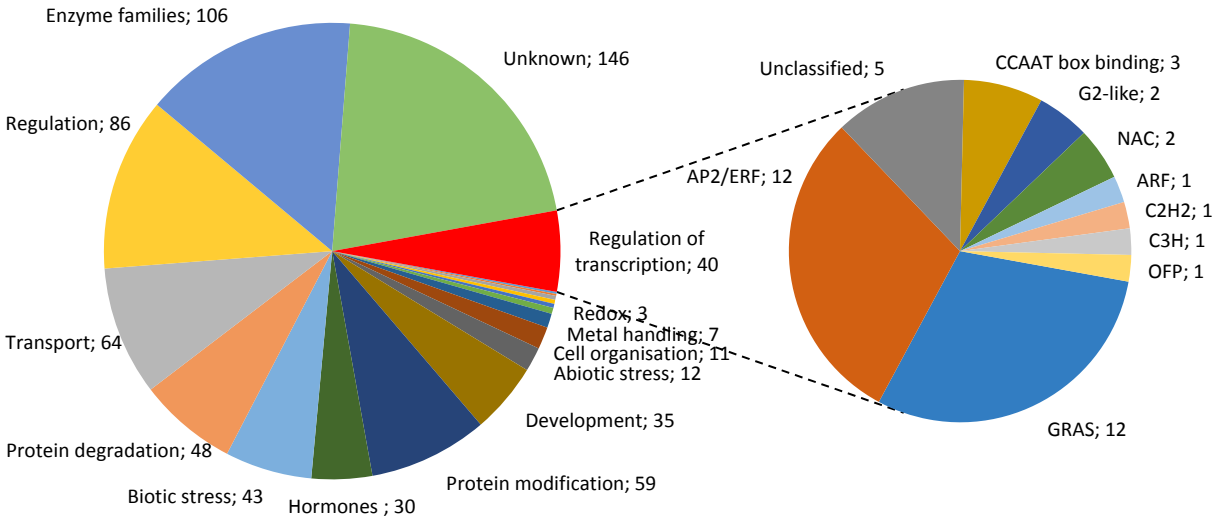

Figure I_4: Functional categories of genes upregulated during colonization with *R. irregularis*.
The 637 genes upregulated during fungal colonization from both 0 to 42 dpi and 7 to 42 dpi were mapped into functional categories using the MapMan software that bases on functional pathways of the *A. thaliana* proteome. Unclassified genes showing an unambiguous annotation in the *M. truncatula* 4.0 genome were manually assigned to the matching category. Members of the transcriptional regulation category (red) were further mapped into major categories of transcriptional regulators, including several transcription factor families. A full list of the categorized genes is shown in Supplemental Table S_2. The original MapMan visualization is shown in Supplemental Figure S_1.

As visualized in Figure I_4, AM-induced gene expression during AM-fungal root colonization reveals substantial numbers of genes involved in regulation, transport, protein degradation/modification, biotic stress, hormones, and development (Supplemental Table S_2)

as being upregulated upon mycorrhizal infection, similar to previous studies using whole-genome microarrays or GeneChips (Küster *et al.*, 2004; Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013; Hohnjec *et al.*, 2015).

Genes mapped into the category "Regulation" are predominantly represented by receptor kinases and genes involved in calcium-signaling (Supplemental Table S_2). Several receptor kinases are known to be involved in AM signaling: NFP (Nod factor perception), LYK3 (LysM domain receptor kinase 3), and DMI2 (Doesn't make infection 2) were initially identified to be involved in signaling induced by rhizobial infection (Endre *et al.*, 2002; Amor *et al.*, 2003; Limpens *et al.*, 2003), but turned out to be also involved in mycorrhizal signaling by perceiving Myc-LCOs (Oláh *et al.*, 2005; Czaja *et al.*, 2012; Zhang *et al.*, 2015). Additionally, it is not surprising that calcium signaling related genes are induced in mycorrhizal roots, since calcium oscillations have been shown to be a key signal in establishing symbiotic interactions including AM (Navazio and Mariani, 2008; Yano *et al.*, 2008; Kosuta *et al.*, 2008).

Transcriptional induction of genes involved in transport mechanisms (Supplemental Table S_2) reflects the central function of mycorrhizal symbioses, i.e. the exchange of nutrients and carbon sources. Many examples for genes encoding plant nutrient importers, predominantly located in the PAM, were identified that were characterized before, including transporters for phosphate (Harrison *et al.*, 2002; Paszkowski *et al.*, 2002), ammonium (Guether *et al.*, 2009), and sulfate (Giovanetti *et al.*, 2014), but also zinc, copper, and iron (Gomez *et al.*, 2009; Kobae *et al.*, 2014). With respect to the export to the AMF, the *MtStr2* gene was found, thought to encode an ABC transporter exporting C16 fatty acids to the microsymbiont (Zhang *et al.*, 2010; Keymer *et al.*, 2017).

Biotic stress response is a key factor of AMF colonization, since it was shown that modulation in biotic defense reactions also affects the compatibility with AM symbiotic fungi (Allen *et al.*, 1989; Gollotte *et al.*, 1993). In this context, several defense-related genes were found upregulated during the time course, including the *MtDefMd1* gene that encodes a defensin acting during later stages in arbuscule containing cells (Uhe *et al.*, 2018; Supplemental Table S_1). Biotic stress responses also include regulation of plant hormone levels like jasmonates and salicylic acid that trigger pathogen responses (Ton *et al.*, 2002). The AM- induced biotic stress response is considered to mediate higher resistance to pathogens ("bioprotection") by priming pathogen responsive reactions (Pozo and Ascón-Aguilar, 2007). A control of biotic stress-related genes was also shown to be involved in restriction of AMF colonization by the

plant itself, e. g. by inducing catalase expression in roots with high phosphate supply (Lambais and Mehdy, 1995; Breuillin *et al.*, 2010). In this way, the nutritional status of the host plant triggers a tight control of the AMF colonization, a process that is called autoregulation (Vierheilig *et al.*, 2004, Vierheilig *et al*., 2008).

Another commonly known plant hormone, gibberellic acid (GA) is generally considered to mediate cross talks between biotic and abiotic conditions, influencing AM-induced signaling (Floss *et al.*, 2013). GA was shown to be involved in regulatory mechanisms in early stages of arbuscule development by destabilizing the CCAMK-CYCLOPS-DELLA complex that is crucial for *RAM1* expression and essential for arbuscule branching (Pimprikar *et al.*, 2016).

Taken together, transcriptional changes in genes involved in biotic stress and hormone metabolism are essential for and characteristic of AM symbiotic roots. However, it is important to consider that the MapMan functional categorization basing on *A. thaliana* functional annotations might not reflect the exact stress-responding situation in *M. truncatula*, since *A. thaliana* is not able to form AM symbioses.

Mycorrhizal colonization-related genes encoding transcriptional regulators

The 40 genes attributed to transcriptional regulation represent 6 % of all genes upregulated during the course of AM development. They were further classified in different transcriptional regulation categories, revealing the highest numbers for AP2/ERF and GRAS TFs. Whereas AP2/ERF TFs are part of gene regulatory networks and integrate metabolic, hormonal, and environmental signals in stress acclimation and signaling (Dietz *et al.*, 2010), several GRAS TFs were already shown to play a role in root symbioses (Bucher *et al.*, 2014, Gobbato *et al.*, 2012, Park *et al.*, 2015; Pimprikar *et al.*, 2016; Pimprikar *et al.*, 2018).

In addition to these, members of several other TF categories are upregulated during the AM time course, including e. g. the two CCAAT box binding factors MtCbf1 and MtCbf2 (Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013.) as well as MtMyb1 (Liu *et al.*, 2003), a regulator of arbuscule degeneration (Floss *et al.*, 2017).

<u>AP2/ERF TF genes upregulated during AM fungal colonization</u>

Overall, 113 AP2/ERF TFs were found to be represented on the *Medicago* GeneChip basing on Mt 4.0 annotations as well as MapMan classifications. From these genes, 12 were found to be upregulated during the time course of AM colonization (Figure I_5), including the prominent TF encoding genes *MtErn2* and *MtErf1*.

Together with MtErn1, MtErn2 was shown to induce *ENOD11* transcription by binding to the *ENOD11* promoter in root nodule symbiosis under negative regulation by MtErn3 (Andriankaja

et al., 2007). However, no AM induced transcriptional regulation could be detected for *MtErn1* (Medtr7g085810.1) or *MtErn3* (Medtr8g085960.1) here. Although the *MrErn2* induction over time appears subtle, these findings suggest a role of *MtErn2*, but not *MtErn1* and *MtErn3* during AMF colonization.

Since MtErf1 is essential for arbuscule branching, knockout of *MtErf1* results in collapsed arbuscules (Devers *et al.*, 2013). In contrast to *MtErn2*, and similar to *MtErf1*, several AP2/ERF TF encoding genes show a significant upregulation during AM colonization, especially Medtr8g468920.1, Medtr6g011490.1, Medtr2g460730.1, and Medtr4g130270.1. The gene expression profiles during the time course of AMF colonization thus reveal new candidates in the search for key regulators of transcriptional reprogramming.

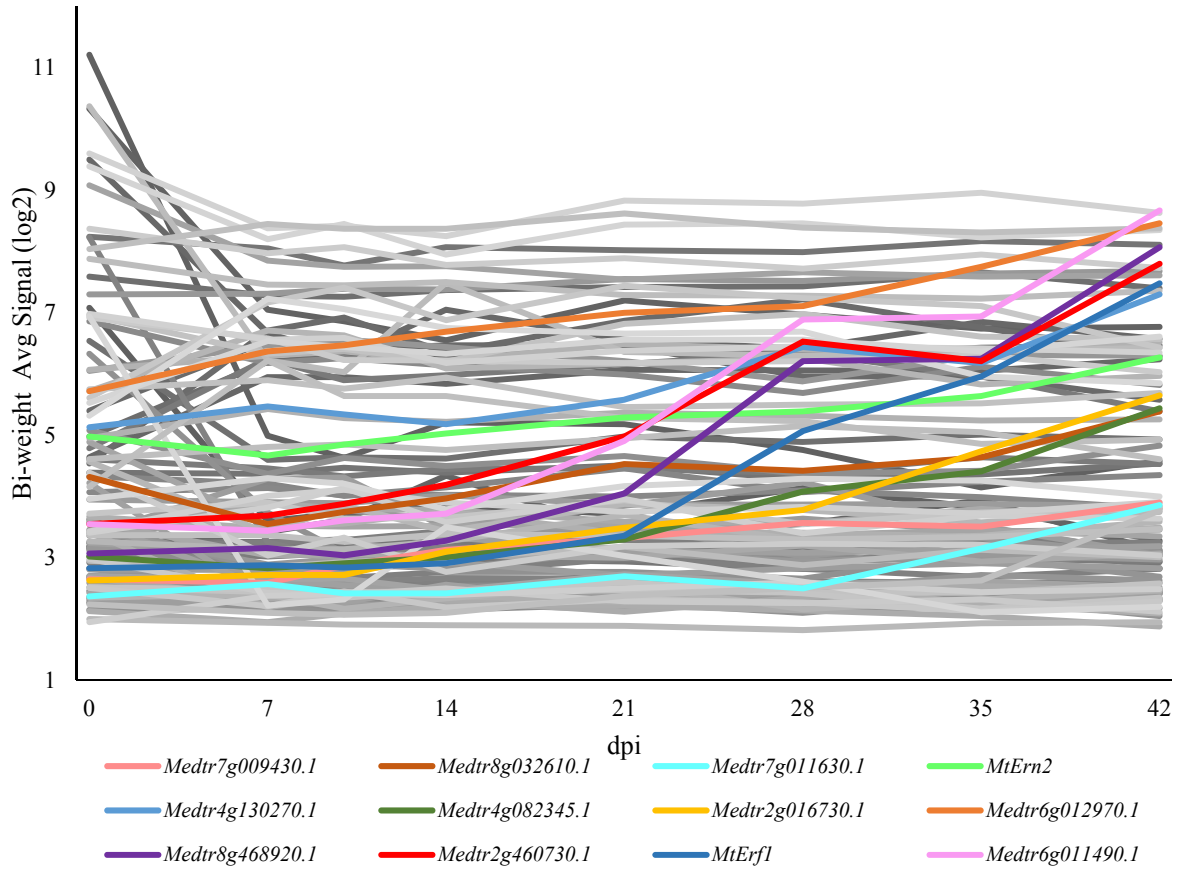

Figure I_5: Time course analysis of AP2/ERF transcription factor gene expression in *M. truncatula* roots at different time points (0, 7, 10, 14, 21, 28, 35, and 42 days) post inoculation with *R. irregularis*.
Genes upregulated at least 2-fold are colored. Gene expressions were plotted using signal intensities measured via Affymetrix GeneChip hybridizations. *M. truncatula* genome v.4.0 (Tang *et al.*, 2014) annotations and MapMan classifications were used to identify AP2/ERF TF annotated probes. N = 3 biological replicates, each consisting of a pool of 6 individual root systems per time point. Abbreviation: dpi, days post inoculation.

GRAS TF genes upregulated during AM fungal colonization

The second prominent group of genes encoding transcriptional regulators found to be upregulated upon AMF colonization is represented by GRAS TF genes (Figure I_6). Several GRAS TF genes were previously shown to be highly AM-induced and involved in AM-signaling as well as the regulation of arbuscule formation (Hogekamp *et al.*, 2011; Gaude *et al.*, 2012; Gobbato *et al.*, 2012; Hogekamp and Küster, 2013; Bucher *et al.*, 2014, Park *et al.*, 2015; Pimprikar *et al.*, 2016; Pimprikar *et al.*, 2018).

In total, 68 GRAS TF-encoding genes are represented on the *Medicago* GeneChip basing on the *M. truncatula* genome version 4.0 (Tang *et al.*, 2014). From these, 11 genes were identified as upregulated during the time course of AMF colonization.
Although *MtMIG1* is not induced in mycorrhizal roots at 28 dpi (Hogekamp *et al.*, 2011), literature data suggest an activation in *M. truncatula* after 25 dpi of inoculation with *R. irregularis* (Heck *et al.*, 2016). Furthermore, time course expression data reveal a significant increase between 0 and 42 as well as between 7 and 42 dpi for *MtMIG1*. This gene was therefore added to the set of AM-induced, colonization-correlated GRAS TF genes (Supp. Table S_1).

The expression levels of all GRAS TF genes represented on the *Medicago* GeneChips along the time course is shown in Figure I_6. Genes upregulated from 0 to 42 or 7 to 42 dpi are highlighted. Among all GRAS TFs represented on the GeneChip, 12 genes found to be upregulated during AM colonization, namely *MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, *MtGras7*, *MtRad1*, *MtRam1*, *MtTF124*, *MtMIG1*, *MtMIG2*, *MtMIG3*, and *MtNsp1* as listed in Table I_3.

Table I_3: Genes encoding GRAS TFs found to be upregulated during the mycorrhizal time course

Gene	Mt 4.0 identifier	MtGEA ID	References
MtGras1	Medtr3g022830.1	Mtr.7264.1.S1_at	Hogekamp *et al.*, 2011 Hogekamp and Küster, 2013
MtGras4	Medtr7g109580.1	Mtr.1484.1.S1_at	
MtGras5	Medtr1g069725.1	Mtr.31955.1.S1_at	
MtGras6	Medtr2g089100.1	Mtr.47463.1.S1_at	
MtGras7	Medtr1g086970.1	Mtr.24642.1.S1_at	
MtRad1	Medtr4g104020.1	Mtr.36004.1.S1_at	Xue *et al.*, 2015 Rey *et al.*, 2017
MtRam1	Medtr7g027190.1	Not represented	Gobbato *et al.*, 2012
TF124	Medtr8g442410.1	Mtr.10469.1.S1_at	Park *et al.*, 2015
MtMIG1	Medtr2g034280.1	Mtr.26270.1.S1_s_at	Heck *et al.*, 2016
MtMIG2	Medtr2g034260.1	Mtr.18636.1.S1_at	
MtMIG3	Medtr2g034250.1	Mtr.50733.1.S1_at	
MtNsp1	Medtr8g020840.1	Mtr.39863.1.S1_at	Liu *et al.*, 2011 Maillet *et al.*, 2011

Gene names, database identifiers and references are listed.

Some of the encoded GRAS TF genes have been shown to play an essential role in AMF colonization and arbuscule development, especially MtNsp1 (Maillet *et al.*, 2011; Liu *et al.*, 2011), MtMIG1 (Heck *et al.*, 2016), MtRad1 (Rey *et al.*, 2017), and MtRam1 (Park *et al.*, 2015). In addition, TF124 was identified as a close homologue of MtRam1 and MtRad1 (Park *et al.*, 2015).

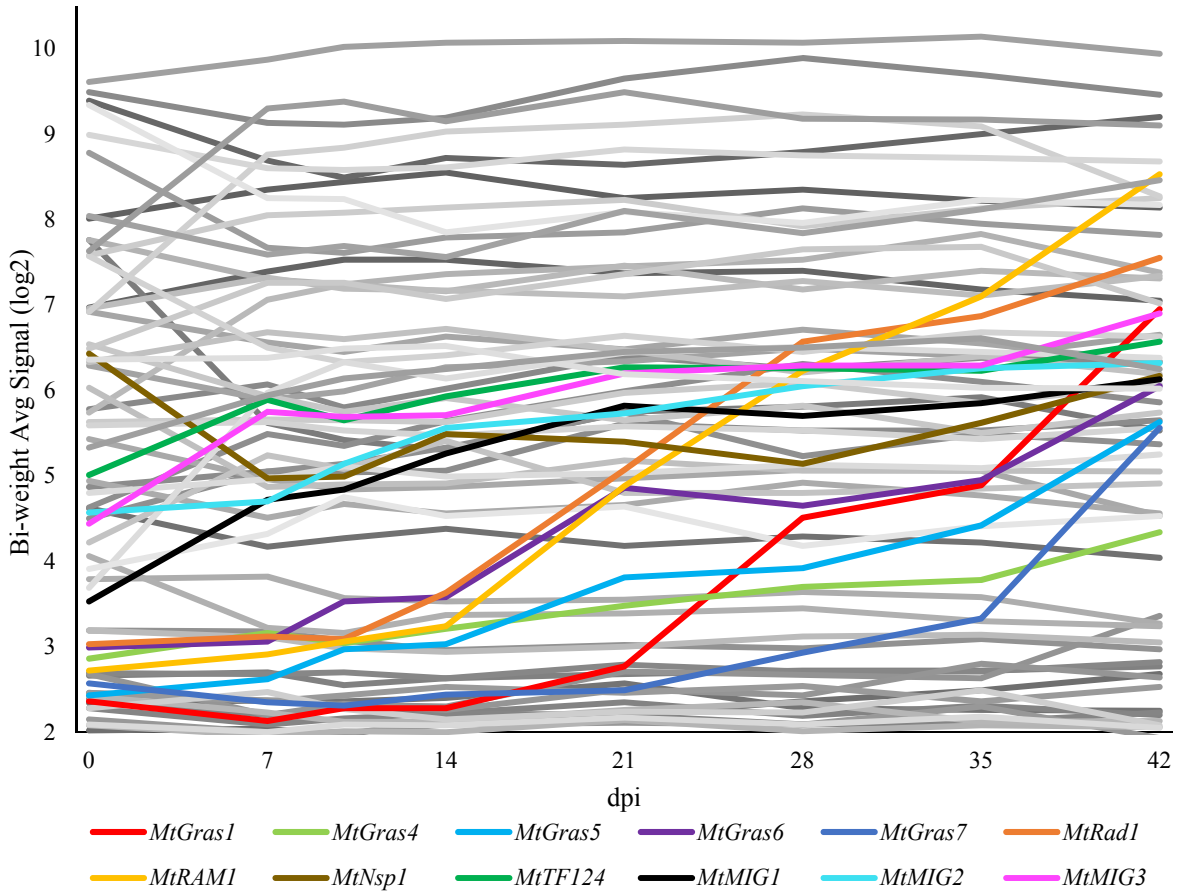

Figure I_6: Time course analysis of GRAS transcription factor gene expression in *M. truncatula* roots at different time points (0, 7, 10, 14, 21, 28, 35, and 42 days) post inoculation with *R. irregularis*.
Genes upregulated at least 2-fold are colored. Gene expressions were plotted, using signal intensities from Affymetrix GeneChip hybridizations. *M. truncatula* genome v.4.0 (Tang *et al.*, 2014) annotations and MapMan classifications were used to identify GRAS TF annotated probes. N = 3 biological replicates, each consisting of a pool of 6 individual root systems per time point. Abbreviation: dpi, days post inoculation.

After the identification of 12 GRAS TF genes activated during the time course of AM fungal root colonization, Euclidean distance based clustering was performed to differentiate their expression profiles based on the signal intensity patterns over time.

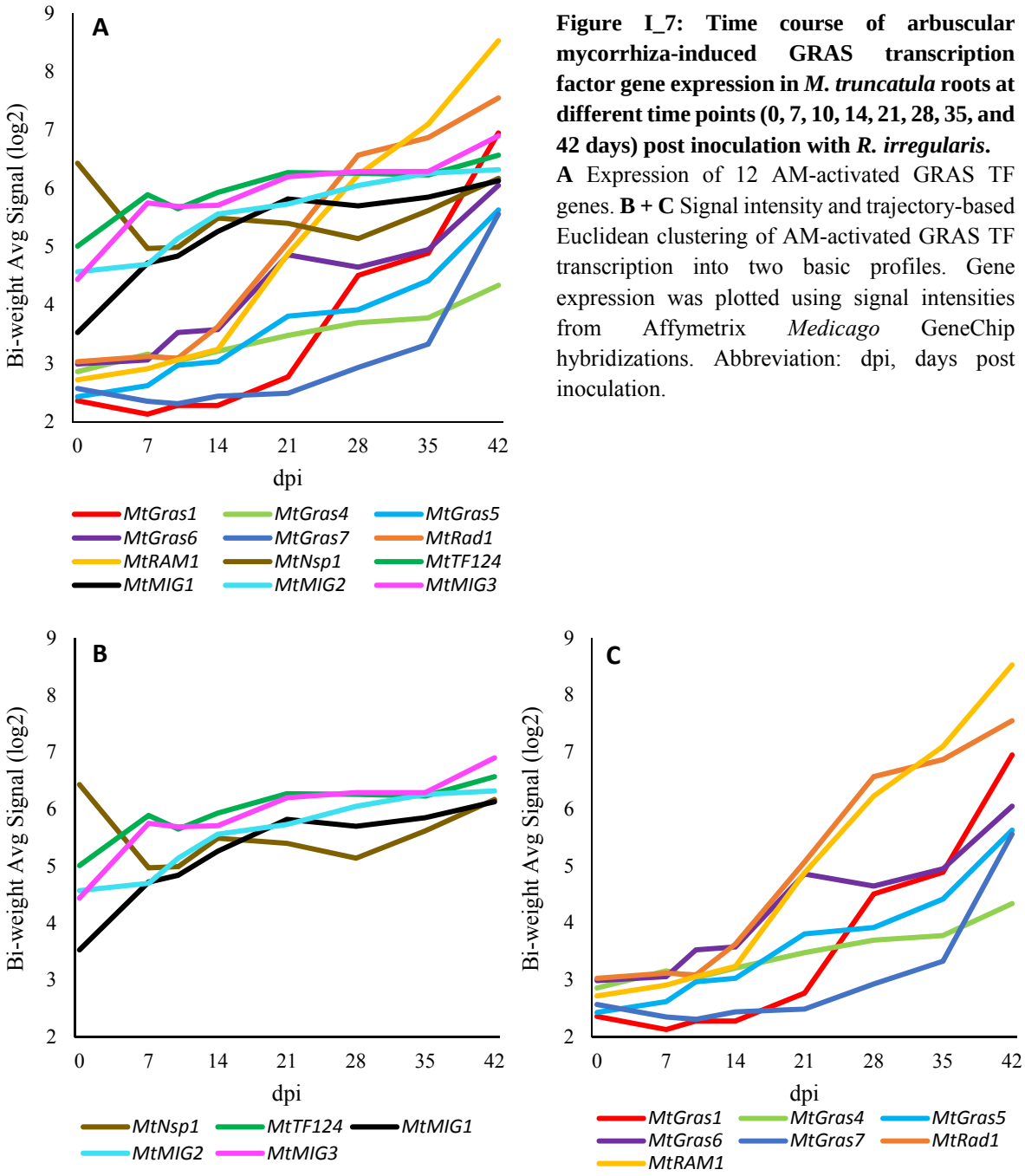

Figure I_7: Time course of arbuscular mycorrhiza-induced GRAS transcription factor gene expression in *M. truncatula* roots at different time points (0, 7, 10, 14, 21, 28, 35, and 42 days) post inoculation with *R. irregularis*.
A Expression of 12 AM-activated GRAS TF genes. **B + C** Signal intensity and trajectory-based Euclidean clustering of AM-activated GRAS TF transcription into two basic profiles. Gene expression was plotted using signal intensities from Affymetrix *Medicago* GeneChip hybridizations. Abbreviation: dpi, days post inoculation.

Interestingly, Euclidean clustering revealed two defined expression clusters of AM-induced GRAS TF genes (Fig I_7 B and C) that reflect previous classifications. The first cluster contains *MtMIG1*, *MtMIG2*, and *MtMIG3* (Figure I_7 B), which also shows an increased expression upon *R. irregularis* spore extract treatment within 24 hours (Heck *et al.*, 2016). Heck *et al.* also postulated the presence of a protein complex of MtMIG1 and MtNSP1. It is thus plausible that this interdependency is reflected on the transcriptional level and that MtMIG1 and MtNsp1 protein interaction partners show a similar gene expression profile over time.

In contrast to the *MtMIG* cluster, GRAS TF genes from the second cluster (Figure I_7), namely *MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, *MtGras7*, *MtRad1*, and *MtRam1* appeared to correlate with fungal colonization, in line with a previous time course analysis using a different fungal inoculum (Hartmann, 2013). Whether or not the inclination of gene expression over time reflects a correlation to more general responses to AM fungal presence or specifically to the ongoing establishment of arbuscules, cannot be finally concluded here.

In general, *MtRam1* and *MtRad1* as well as *MtGras1* show an expression curve with the strongest inclination, resembling the profile of *MtPt4* (Figure I_2), and suggesting an expression-dependency on arbuscule build-up. These findings are in accordance with the arbuscule-specific induction that was found for *MtRad1* via promoter-*gusA*int studies (Hartmann, 2013; Figure III_4). In contrast, histochemical analyses of the p*MtRam1* activation pattern in *M. truncatula* roots colonized with *G. versiforme* revealed an *MtRam1* induction in colonized root sections without a specific upregulation in arbuscule containing cells (Park *et al.*, 2015). At this stage, it is thus not clear, in which cell types the activation reported in Figure I_7 C takes place.

Whereas the expression patterns of *MtGras4* and *MtGras5* tend to follow the overall expression of AM-related GRAS TFs, an increasing expression activation can be observed for *MtGras6* (Figure I_7 C). This increase resembles the *MtRam1* upregulation, but a decrease in transcriptional induction at 21 dpi might imply a more complex regulation. In comparison to *MtRam1* and *MtRad1*, the *MtGras7* induction appears to be delayed (Figure I_7 C) which might be a hint for a function of *MtGras7* either in later stages of colonization in general or in later stages of arbuscule development in particular.

CONCLUSION

The time course of AM-related gene expression revealed insights into the ongoing process of the substantial reprogramming of roots during fungal colonization. The data obtained provide a valuable resource for future studies to functionally characterize genes involved in a variety of cellular functions. Among the genes activated over time, key GRAS transcription factor genes of AM development such as *MtRam1* (Park *et al.*, 2015) and *MtRad1* (Rey *et al.*, 2017) were identified. In addition, other yet uncharacterized GRAS TF genes were found to follow the ongoing AM establishment. Functional analyses of these genes of interest in Chapter III will include promoter-*gusA*int analyses to assess whether or not transcriptional profiles during the AM time course can be correlated to arbuscule-specific or colonization-associated promoter activities.

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CHAPTER II:

Identification of protein-protein interactions of AM-related GRAS transcription factors

Scope:

The aim of this chapter is to unravel protein-protein interactions among GRAS transcription factors, using the Yeast Two-Hybrid technology. AM-related GRAS TFs identified via expression of the corresponding genes in AM roots in Chapter I are tested for interaction both against each other in direct matings and in global screenings, using a library of candidate proteins found in different symbiotic tissues. Together, these approaches aim to identify interconnections within the GRAS regulatory network controlling AM formation.

Contributor roles:

R. M. Hartmann generated a Y2H prey library from symbiotic tissues, performed the cloning of vectors for matings, and executed Y2H experiments

N. Hohnjec provided the total RNA from different defined symbiotic tissues that was used for the generation of the Y2H prey library

ABSTRACT

Arbuscular mycorrhizal (AM) symbioses are widespread beneficial associations among terrestrial vascular plants. Development of a functional plant-fungal interface is governed by the expression of several transcription factors (TFs), including multiple GRAS TFs. While differential transcriptional activity reveals a tight interconnection of the AM-related GRAS TF genes studied here, investigations on the distinct function of the encoded proteins are lacking. To identify interactions among GRAS TFs and proteins from a comprehensive prey library of *Medicago truncatula* roots representing various symbiotic conditions including AM, a Yeast Two-Hybrid approach was used. Whereas library screenings revealed that AM-related GRAS TFs potentially interact with other transcriptional regulators, a pairwise mating approach, combining specific GRAS TF interaction candidates, identified two novel interaction couples. The observed interaction of MtGras4 with both MtGras1 and MtRad1 contributes to a model of both MtGras1- and MtGras4-dependent transcriptional regulation and allowed to place MtGras4 and MtRad1 in the regulatory network controlling arbuscule formation.

INTRODUCTION

In the past decades, several genome-sequencing projects gathered large datasets of genomic sequences and identified thousands of protein-encoding genes (e. g. Young *et al.*, 2011; The Arabidopsis Genome Initiative, 2000; Krishnakumar *et al.*, 2015). Analysis of differential gene expression by transcriptomics is a powerful tool to investigate gene functions. However, considering the huge amount of protein-encoding genes, not only genomic and transcriptomic studies but also investigations of protein-protein interactions (PPI) are necessary to understand the regulatory networks that control cellular processes. PPIs are essential for cellular functions and even slight shifts in PPI networks bear the potential to cause fundamental changes in developmental, life-cycle control, or regulation (Jeong *et al.*, 2001). Several global plant interactome studies were performed, demonstrating complex linkages and regulational interconnections within PPI networks (Uhrig *et al.*, 2006; Morsy *et al.*, 2008; Ding *et al.*, 2009). PPI networks were also shown to influence pathogen recognition in vascular plants (Bogdanove *et al.*, 2002). A bridge to AM developmental regulation was built when heterologous systems were used to identify interactions between several AM-induced key regulatory proteins e. g. NSP1-NSP2 (Hirsch *et al.*, 2009), RAM1-NSP2 (Gobbato *et al.*, 2012), or CCAMK-IPD3 (Yano *et al.*, 2008). In recent studies, more and more AM-related PPIs are discovered, especially in the field of transcriptional regulators (reviewed in Pimprikar *et al.*, 2018).

Several techniques are commonly used for the identification of PPIs, e. g. tandem affinity purification for isolation of protein complexes (Xu *et al.*, 2010), Yeast Two-Hybrid (Y2H; Fields and Song, 1989) and split-ubiquitin approaches (Grefen *et al.*, 2009), or bimolecular fluorescent complementation (BiFC; Kerppola, 2006).

The Yeast Two-Hybrid principle and its application to screen for protein-protein interactions

The Yeast Two-Hybrid (Y2H) technique, first developed and published by Fields and Song (1989), enables the identification of interacting proteins in *Saccharomyces cerevisiae*. Protein interaction studies are performed by fusing each protein of interest to one component of the GAL4 transcriptional activator, creating two hybrid proteins eponymous for the established two-hybrid system.

GAL4 is a yeast transcription factor involved in the galactose metabolism (Kew and Douglas, 1976). Forming a homodimer, GAL4 binds to a specific consensus sequence within the UAS and regulates gene transcription (Carey *et al.*, 1989).

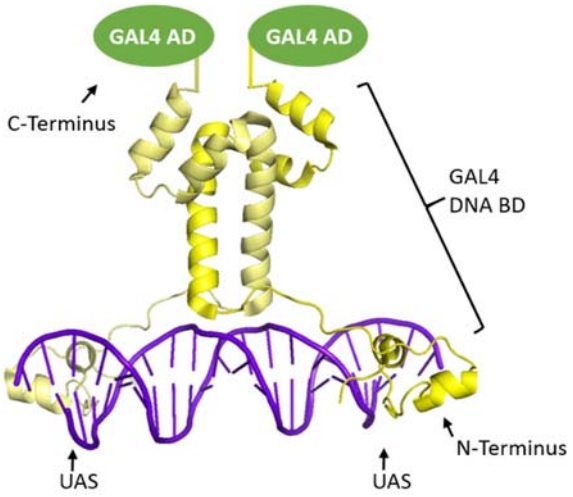

Figure II_1. Structure of the GAL4 TF.
A homodimer is formed by two monomers (yellow and light yellow), each containing a DNA recognition element and a dimerization domain. Each monomer contains also a C-terminal activation domain (green). Structural data were obtained from Hong *et al.*, (2008) and modelled in PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.).

For Yeast Two-hybrid screenings, the GAL4 activation domain (AD) and DNA binding domain (BD) are expressed separately and connected via fused interacting proteins. Thus, the expressed subunits form a multimer consisting of two GAL4 AD fused to the prey protein and two GAL4 BD fused to the bait protein, restoring a functional GAL4 TF (Hong *et al.*, 2008).

The GAL4 protein is reconditioned and functionally active, once the fused proteins of interest are interacting. In this case, the reconditioned GAL4 transcription factor induces expression of marker genes in transgenic yeast cells that can be detected (e. g. by auxotrophy, galactosidase activity, or antibiotic resistance). The two-hybrid principle is visualized in Figure II_2.

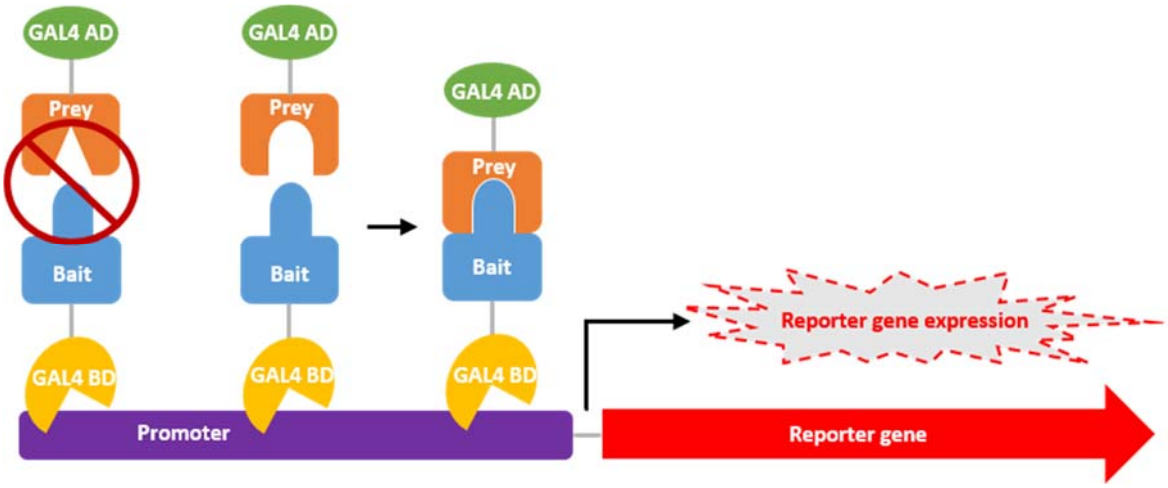

Figure II_2: The Two-Hybrid principle.
Two translational fusions are expressed, one bait protein fused to the GAL4 DNA binding domain (BD), and one prey protein fused to the GAL4 activation domain (AD). Reporter gene expression is induced upon interaction of bait and prey, which enables the reconstitution of a functional GAL4 transcription factor and the association of the GAL4 AD with the reporter gene DNA sequence and subsequent recruitment of proteins of the transcriptional machinery (not shown). This simplified scheme does not include GAL4 dimerization that is explained above.

There are two general methods for performing Y2H assays. A pairwise matrix, combining each candidate with any other can be used to investigate specific PPIs. The second option is to create a library of prey fusion-proteins from any source, tissue or collection to screen for any possible interaction between the bait and the components of the library.

In a direct mating approach, a set of bait proteins is tested against a set of prey proteins in a defined pairwise matrix. Bait- and prey fusions need to be expressed in different *S. cerevisiae* mating type strains (in this case Y2HGold and Y187, representing strains of the mating types "α" and "a" (Herskowitz, 1988)). Matrix based screenings allow the rapid identification of PPIs without the need of further identification (e. g. sequencing) of the interaction partners. However, the system is limited to a defined set of bait and prey constructs that need to be cloned and transformed into yeast cells separately. Hence, matrix based direct matings are disadvantageous when it comes to screenings of many possible interaction partners.

In contrast to direct matings, library screenings allow the identification of PPI between a distinct bait with any protein represented in a cDNA-derived library. A single bait is mated with the entire prey pool and streaked out on selective growth media to obtain single colonies that can be screened for reporter gene expression. Another major difference to direct mating is the need

to identify the gene encoding the interacting protein from a reporter expressing yeast colony. Therefore, each positively screened colony is used in a colony PCR to amplify the ORF contained in the prey construct. The amplified part of the plasmid is sequenced and blasted against a genome/transcriptome database to identify the interactor encoding gene. Once an interaction candidate of putative importance is identified, its interaction with the bait protein has to be confirmed by cloning a full-length version of the prey protein and testing for the interaction with the bait in yeast in a direct approach. This procedure is necessary, since prey constructs from the cDNA library often contain only parts of the CDS or show a frameshift to the fused GAL4 AD domain, possibly leading to false positive results (Koegl and Uetz, 2008).

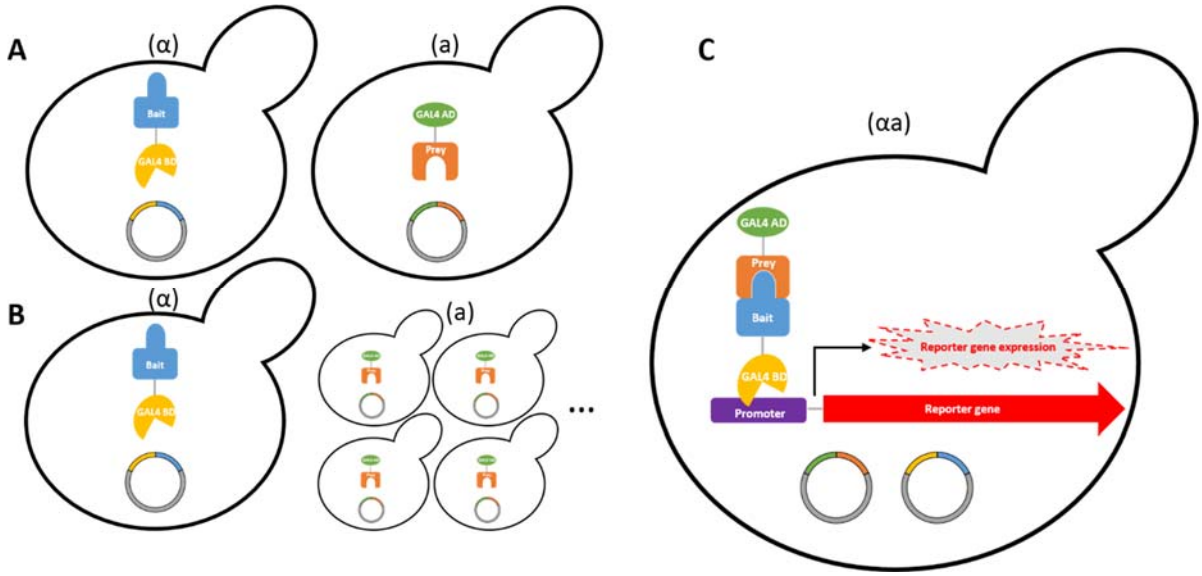

Figure II_3: Yeast Two-Hybrid methods. Haploid cells of mating type (α) are transformed with the bait construct and mated with mating type (a) cells containing the prey construct. **A** Mating of bait and prey strains pairwise. **B** mating of the bait strain with the prey library pool. **C** Diploid cell expressing interacting bait and prey fusion proteins, inducing reporter gene expression.

Limitations of the Yeast Two-Hybrid system: false-positive and false-negative protein-protein interactions

"False positives" designate prey proteins that are screened positive on selective media (and therefore indicate a positive interaction with the bait protein), but are considered not to interact with the bait protein *in vivo*. Occurrence of false positives is a common issue in two-hybrid approaches (Bartel *et al.*, 1993; Serebriiskii *et al.*, 2000) and their abundancy in large-scale approaches was estimated to be roughly 50 % of all interactions found (Von Mering *et al.*, 2002). This phenomenon can be caused by several factors. As stated above, many clones in a prey library consist of partial cDNA sequences, possibly leading to altered folding and exposure of different binding sites in comparison to the full-length clone (Koegl and Uetz, 2008). Altered

folding of prey proteins can also occur due to a different physical and physiological environment in yeast in comparison to the originating plant cell. Apart from technical sources, biological causes for false positive interactions have to be considered. Since the Y2H system relies on the reconstitution and functionality of the GAL4 transcriptional regulator, the interaction needs to occur in the nucleus (Silver *et al*., 1984). Therefore, both bait and prey fusions contain a nuclear localization signal, guiding all hybrid proteins to the nucleoplasm. Thus, it is possible to detect interactions among proteins that do not share a common vicinity *in vivo*, e. g. being nuclear-, cytosolic-, or chloroplast-derived or even of microbial source. These false-positives can be detected by separating the ORF sequences based on their predicted protein localization (Deng *et al*., 2003).

Prey libraries contain virtually all proteins expressed in the source tissue, including a high number of transcriptional regulators (Chevray *et al.*, 1992). Since these proteins are able to induce gene expression, some of them might induce the reporter gene expression by binding to the UAS or in another part of the promotor on their own, also leading to false-positive screening results (Serebriiskii *et al*., 2000). One approach to tackle this issue is the usage of different promotor-reporter gene combinations, filtering proteins that interact with unrelated sequences within the promotor and inducing reporter gene expression without PPI (James *et al.*, 1996). Autoactivation of bait-fusions can also occur when the fused bait protein resembles the function of the GAL4 AD domain, e. g. recruitment of polymerases or adapter proteins to the TF-BD-DNA complex (Melcher *et al.*, 1995; Wu *et al.*, 1996; Bryant and Ptashne, 2003).

Proteins that interact *in vivo* but are failing to produce a detectable interaction in a Y2H approach are called false negatives (Hart *et al.*, 2006). A major cause for the occurrence of false negatives is the choice of a high stringency that only allows the detection of strong PPIs. On the other hand, low stringency drastically increases the abundancy of false positives up to 70 % (Chen *et al.*, 2010), creating a two-edged problem inherent to the Y2H system. In addition, false-negative interactions can theoretically occur due to the incorrect folding of the fusion proteins or a steric hindering of the PPI because of the tagged GAL4 AD and BD domains (Millson *et al*., 2003). Steric hindering might also prevent the GAL4 DNA binding domain from binding to the promoter, repressing reporter gene expression even if the GAL4 protein is completely reconstituted due to PPI.

Application of the Yeast Two-Hybrid system to identify interactors of AM-related GRAS TFs

In the last years, several studies revealed PPIs between AM-related GRAS TFs, e. g. MtNsp1-MtNsp2 (Hirsch *et al.*, 2009), MtRam1-MtGras1 (Park *et al.*, 2015), MtRam1-MtRad1 (Park *et al.*, 2015), MtRam1-MtNsp2 (Gobbato *et al.*, 2012), and MtRad1-MtNsp2 (Heck *et al.*, 2016). With respect to the AM symbiosis, identification of novel interacting GRAS proteins bears the potential to complement the protein-protein interactome of GRAS TFs, in addition to the transcriptional activation studies presented in Chapter I. Moreover, the identification of potential binding partners of known function might help to understand the role of individual GRAS TFs in AM signaling that were not functionally studied so far. Finally, a redundancy of different GRAS TF functions can be identified by checking for all their possible PPI partners and target overlaps in the candidate TFs interactomes.

To investigate possible interplays between the AM-activated GRAS TF genes of *M. truncatula* (*MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, *MtGras7*, *MtRad1*, and *MtRam1*) (Figure I_7) and other proteins, Y2H screenings were performed in global library screenings and direct mating approaches. It will be shown that, in addition to finding putative interactions of GRAS TFs, an interaction between MtGras1, MtGras4 and MtRad1 was found, thus allowing to refine a part of the AM-related MtGras interactome.

MATERIAL AND METHODS

Table II_1: Yeast growth media components and supplements.

Component	Distributor
Minimal Synthetically Defined Base Medium	Clontech Laboratories, Fitchburg, USA
Minimal Synthetically Defined Agar Base Medium	Clontech Laboratories, Fitchburg, USA
-Trp Dropout Supplement	Clontech Laboratories, Fitchburg, USA
-Leu Dropout Supplement	Clontech Laboratories, Fitchburg, USA
-Leu/-Trp Double Dropout Supplement	Clontech Laboratories, Fitchburg, USA
-Ade/-His/-Leu/-Trp Quadruple dropout Supplement	Clontech Laboratories, Fitchburg, USA
Water, double distilled (H_2O)	Carl Roth, Karlsruhe
Aureobasidin A	Clontech Laboratories, Fitchburg, USA
X-α-Gal	Gold Biotechnology, Olivette, USA
Glycerol	AppliChem, Darmstadt
YPDA Rich Medium	Clontech Laboratories, Fitchburg, USA
YPDA Rich Agar Medium	Clontech Laboratories, Fitchburg, USA
ß-mercaptoethanol	SigmaAldrich, St. Louis, USA

Media for yeast culture

YPDA Medium

Add 50 g of YPDA Medium or 70 g of YPDA Agar Medium to 1 l of double distilled water. For 2× or 0.5× YPDA Medium, double or half the amount of medium per liter respectively. Adjust pH to 6.5. Autoclave at 121°C for 15 min. Store at room temperature in the dark.

Dropout Media

Add 26.7 g SD Base Medium or 46.7 g SD Agar Base Medium to 1 l of double distilled water. Additionally, add Dropout Supplements as following: -Trp DO: 0.74 g; -Leu DO 0.69 g; -Leu/-Trp DDO: 0.64 g; -Ade/-His/-Leu/-Trp QDO: 0.6 g. Adjust pH to 5.8. Autoclave at 121 °C for 15 min. Store at room temperature in the dark.

Freezing Medium

Add 30 % sterile glycerol to YPDA or appropriate dropout medium.

Aureobasidin A

Add 20 ml of absolute ethanol to 10 mg of Aureobasidin A to obtain a stock solution of 500 µg/ml. Store at 4 °C. Add 400 µl of the stock solution to 1 l dropout medium after cooling to ~ 55-60 to obtain a final concentration of 200 ng/ml.

X-α-Gal

Freshly prepare 20 mg/ml X-α-Gal in dimethylformamide. Add 2 ml stock solution in 1 l dropout medium after cooling to ~ 55-60 °C.

Kanamycin

Unless stated otherwise, add 1 ml of 50 mg/ml kanamycin (solution in H_2O) to 1 l of yeast medium to obtain a final concentration of 50 µg/ml to inhibit bacterial contamination.

Table II_2: Yeast selective media used in the Yeast Two-Hybrid system.

Acronym	Media formulation	Selection for
SDO/-Trp	SD/-Trp/Kan^{50}	Yeast expressing bait
SDO/-Leu	SD/-Trp/Kan^{50}	Yeast expressing prey
DDO	SD/-Trp/-Leu/Kan^{50}	Yeast expressing bait and prey
DDO/X/A	SD/-Trp/-Leu/ X-α-Gal/AbA/Kan^{50}	Yeast expressing interacting bait and prey (low stringency)
QDO/X/A	SD/-Trp/-Leu/-His/-Ade/X-α-Gal/AbA/Kan^{50}	Yeast expressing interacting bait and prey (very high stringency)

General yeast cultivation

For yeast cultivation in liquid medium (YPDA or SD), use baffled flasks. Incubate at 30 °C / 150 rpm in subdued light. Kanamycin should be added to all cultures at 50 µg / ml to prevent bacterial contamination.

Yeast transformation

The following protocol describes a faster and more efficient transformation method (Daniela Floss, Cornell University, NY, USA, personal communication), modified and adapted for usage of Yeastmaker™ Yeast Transformation System 2 (Clontech Laboratories, Fitchburg, USA) components.

This protocol is suitable for 10 yeast transformations and can be scaled up or down. Bait constructs were transformed into Y2HGold and prey constructs into Y187.

Material

- Selective SDO/-Trp and SDO/-Leu plates
- YPDA plates
- sterilized double distilled water (H_2O)
- 50 % PEG 3350 solution
- 1 M Lithium Acetate
- ß-mercaptoethanol
- Carrier (salmon sperm) DNA, 10 mg/ml
- Yeast host strains (Y2HGold/Y187)

Preparation of competent yeast cells

- Streak yeast strain (Y2H Gold/Y187) on YPDA plates and incubate at 30 °C for 3 days
- Inoculate 10 ml of YPDA with a single yeast colony (diameter 2-3 mm). Place the cultivation tube in the incubator shaker in a ~ 45° angle. For larger volumes use a 250 ml baffled flask. Incubate at 30 °C, 200 rpm overnight.
- Spin down at 1000 × g for 5 min, discard supernatant
- Add sterilized H_2O (same volume like overnight culture), vortex to resuspend.
- Spin down at 1000 × g for 5 min, discard supernatant
- Add sterilized H_2O (same volume like overnight culture), vortex to resuspend.
- Aliquot 1 ml in 1.5 ml tubes.
- Spin down at 3500 × g for 5 min, remove supernatant by pipetting.
- Resuspend in 100 µl freshly prepared buffer (800 µl PEG 3350 [50 %], 200 µl LiAc [1 M], 1.5 µl ß-mercaptoethanol), vortex to resuspend.
- Store competent cells on ice, use within few hours.

Transformation of competent yeast cells

- Boil carrier DNA at 99 °C for 5 min, chill on ice immediately.
- Add 6µl carrier DNA to an aliquot of competent cells.
- Add vector DNA (100-200 ng), mix by tapping.
- Incubate at 45 °C for 30 min, vortex every 10 min.
- Spread 30 µl on selective plates, incubate at 30 °C for 3-5 days.

Autoactivation test

Bait constructs were tested for autonomous activation of yeast reporter gene expression in absence of an interacting prey before they were used in two-hybrid analyses. While weak autoactivation can be compensated by higher stringency levels in the two-hybrid screenings, strong autoactivation disqualifies the bait construct from usage for interaction studies.
Detection of autoactivation at different stringencies was performed by plating transformed yeast cells on DDO (control for co-expression of bait and prey fusion), DDO/X (moderate stringency: α-galactosidase activity), DDO/X/A (high stringency: α-galactosidase activity and Aureobasidin A resistance) plates.

Bait and prey construction

Bait and single prey constructs were cloned using primers and cloning strategies stated in Table II_5. For most constructs, conventional restriction based cloning was performed. In some cases, constructs were generated using the In-Fusion® HD Cloning Kit (Clontech Laboratories, Fitchburg, USA) by creating 15 bp overlaps between linearized vector and PCR amplicon that are recognized and fused by the In-Fusion enzyme. A modified downscaled reaction setup for the In-Fusion reaction was established.

<u>In-Fusion reaction setup</u>

- 1 µl 5× In-Fusion HD Enzyme Premix
- 50-100 ng purified PCR fragment
- 50-100 ng linearized vector
- H_2O to 5 µl

<u>In-Fusion reaction</u>

- Incubate reaction for 15 min at 50 °C
- Transform into competent *E. coli* cells
- Select on appropriate selective plates (LB/Kan^{50} for bait; PA/Amp^{100} for prey)

Prey library construction

Instead of creating single distinct prey constructs, a cDNA based prey library was constructed to test interactions among baits and virtually every possible protein being abundant in symbiotic and non-symbiotic root tissue. A mixture of RNA derived from mycorrhizal, nodulated, Nod-Factor treated, and uninfected roots (shown in Figure II_5) was used to create a cDNA library using Oligo-dT Primer to specifically transcribe mRNA. RNA was checked using a Bioanalyzer (Agilent, Waldbronn, Germany) to exhibit a high RNA integrity (RIN 9.4-10). The library construction was performed according to the "Make Your Own Mate & Plate Library System" user manual (Protocol No. PT4085-1, Clontech, Fitchburg, USA).

Library transformation

The generated cDNA library was transformed into competent yeast cells of the strain Y187 according to the "Yeastmaker Yeast Transformation System 2" user manual derived protocol for library-scale transformations (Protocol No. PT1172-1, Clontech Laboratories, Fitchburg, USA). The cDNA fragments were automatically integrated into the yeast cells genome via endogenous homologous recombination, generating a yeast prey library suitable for two-hybrid screenings. Selection took place on selective SD/-Leu agar plates. Transformation efficiency was evaluated to determine the number of independent clones represented in the library. The grown yeast cells transformed with the prey library were detached from the selective agar plates, resuspended in freezing medium and split into 1 ml aliquots for storage at -80 °C.

Yeast direct mating

For direct mating approaches, Y2HGold cells containing a DBD-Fusion (bait construct) and Y187 cells expressing the prey constructs were mated according to the following protocol:

- Streak yeast and prey expressing cells in appropriate selective dropout medium. Inoculate at 30 °C for 3 days.
- Use an inoculation loop to detach a single colony (diameter 2-3 mm)
- Resuspend in 700 µl YPDA
- Combine 20 µl resuspended bait with 20 µl resuspended prey cell suspension in a tube or 96 well microplate (Greiner Bio-One, Kremsmünster, Österreich)
- Incubate at 30 °C / 200 rpm for 24 h
- Drop 15 µl on selective media (DDO; DDO/X/A; QDO/X/A)
- Incubate at 30 °C for 3-5 days.

Plating on DDO reveals expression of both bait and prey fusions in the mated yeast cells. Selection for α-Galactosidase activity, Aureobasidin A resistance and higher auxotrophy levels (QDO) enable identification of interacting proteins.

Yeast library screening

For a library-wide screening for interacting proteins, yeast cells expressing the bait fusion construct were mated against the cDNA derived prey library. Mating was performed according to the "Matchmaker® Gold Yeast Two-Hybrid System" user manual (Protocol No. PT4084-1). In short, a bait liquid culture was combined with a 1 ml aliquot of the prey library and mated for 20 hours. Cells were streaked on DDO/X/A plates to detect basal interaction. Colonies

screened positive were patched on maximum stringency medium (QDO/X/A) to confirm positive interactions.

Colonies confirmed to exhibit a high stringency PPI were used in a colony PCR to amplify the interacting protein encoding ORF within the prey vector pGADT7. Colony PCR was performed according to the "Matchmaker™ Insert Check PCR Mix 2" user manual. Amplicons were sequenced via Sanger sequencing (Microsynth Seqlab, Göttingen) using a T7 sequencing primer.

Table II_3: Promoter-reporter gene sequence combinations and methods for detection of reporter gene expression in the Yeast Two-Hybrid system used.

UAS	Reporter gene	Gene product	Detection method
GAL1	*HIS3*	Imidazoleglycerol-phosphate dehydratase, catalyzes histidine biosynthesis	Plating on minimal SD medium lacking histidine (SD/-His)
GAL2	*ADE2*	Phosphoribosylaminoimidazole carboxylase, catalyzed purine synthesis	Plating on minimal SD medium lacking adenine (SD/-Ade)
MEL1	*AUR1-C*	Inositol phosphorylceramide synthase, mediates Aureobasidin A resistance	Plating on minimal SD medium containing Aureobasidin (SD/AbA)
MEl1	*MEL1*	α-galactosidase 1, hydrolyses X-α-Gal	Plating on minimal SD medium containing X-α-Gal (SD/ X-α-Gal)

Table II_4: Nomenclature and identifier of *MtGras* genes used in Yeast Two-Hybrid experiments.

Gene	Accession number/Identifier
MtGras1	Medtr3g022830.1 (Mt 4.0)
MtGras4	Medtr7g109580.1 (Mt 4.0)
MtGras5	Medtr1g069725.1 (Mt 4.0)
MtGras6	Medtr2g089100.1 (Mt 4.0)
MtGras7	Medtr1g086970.1 (Mt 4.0)
MtRad1	Medtr4g104020.1 (Mt 4.0)
MtRam1	Medtr7g027190.1 (Mt 4.0)
MtNsp1	Medtr8g020840.1 (Mt 4.0)
MtNsp2	Medtr3g072710.1 (Mt 4.0)
MtDella1	Medtr3g065980.1 (Mt 4.0)
MtDella2	contig_55897 (Mt 3.5)
MtDella3	contig_55897 (Mt 3.5)

Table II_5: Empty vectors, control vectors as well as bait- and prey constructs used in Yeast Two-Hybrid experiments.

Vector	Description	References
pGBKT7 BD	Y2H bait vector, containing GAL4 binding domain	Clontech Laboratories
PGADT7 AD	Y2H library vector containing GAL4 activation domain	Clontech Laboratories
pGBKT7-53	Y2H positive control plasmid	Clontech Laboratories
pGBKT7-Lam	Y2H negative control plasmid	Clontech Laboratories
pGADT7-T	Y2H positive control plasmid	Clontech Laboratories
pGBKT7:MtGras1	ORF of MtGras1, BamHI/EcoRI In-Fusion Cloning	This work
pGBKT7:MtGras4	ORF of MtGras4, BamHI/EcoRI In-Fusion Cloning	This work
pGBKT7:MtGras5	ORF of MtGras5, SmaI	This work
pGBKT7:MtGras6	ORF of MtGras6 , BamHI/EcoRI In-Fusion Cloning	This work
pGBKT7:MtGras7	ORF of MtGras7, SmaI	This work
pGBKT7:MtDella1	ORF of MtDella1, SmaI	This work
pGBKT7:MtDella2	ORF of MtDella2, SmaI	This work
pGBKT7:MtDella3	ORF of Della3, BamHI/EcoRI In-Fusion Cloning	This work
pGBKT7:MtNsp1	ORF of MtGras1, SmaI	This work
pGBKT7:MtNsp2	ORF of NSP2, SmaI	This work
pGBKT7:MtRam1	ORF of MtRam1, SmaI	This work
pGADT7:MtGras1	ORF of MtGras1, SmaI	This work
pGADT7:MtGras4	ORF of MtGras4, SmaI	This work
pGADT7:MtGras5	ORF of MtGras5, SmaI	This work
pGADT7:MtGras6	ORF of MtGras6, BamHI/EcoRI In-Fusion Cloning	This work
pGADT7:MtGras7	ORF of MtGras7, SmaI	This work
pGADT7:MtRad1	ORF of MtRad1, BamHI/EcoRI In-Fusion Cloning	This work
pGADT7:MtDella1	ORF of MtDella1, SmaI	This work
pGADT7:MtDella2	ORF of MtDella2, SmaI	This work
pGADT7:MtDella3	ORF of MtDella3, SmaI	This work
pGADT7:MtNsp1	ORF of NSP1, BamHI/EcoRI In-Fusion Cloning	This work
pGADT7:MtNsp2	ORF of MtNsp2, SmaI	This work
pGADT7:MtRam1	ORF of MtRam1, SmaI	This work

RESULTS

A substantial number of AM-related GRAS TFs exhibits autonomous reporter gene induction in *S. cerevisiae*

Bait constructs for the following AM-related GRAS genes were transformed into *S. cerevisiae* Y2HGold: *MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, and *MtGras7* (Figure I_7; Table II_4; Table II_5). In addition, *MtNsp1* (Liu *et al.*, 2011; Maillet *et al.*, 2011), *MtNsp2* (Maillet *et al.*, 2011), *MtDella1*, *MtDella2*, *MtDella3* (Floss *et al.*, 2013), *MtRad1* (Xue *et al.*, 2015; Rey *et al.*, 2017*)*, and *MtRam1* (Gobbato *et al.*, 2012; Park *et al.*, 2015) were used, since interactions were reported among some of these genes (Hirsch *et al.*, 2009; Gobbato *et al.*, 2012; Park *et al.*, 2015; Heck *et al.*, 2016). Although there were also other interactions reported that were found by Co-Immunoprecipitation (CoIP), pull down assays, and bimolecular fluorescence complementation (BiFC), the interactions mentioned above were the only ones found via Y2H assays.

Detection of bait autoactivation at different stringencies was performed by plating transformed yeast cells on DDO (double dropout medium), DDO/X (double dropout medium containing X-α-Gal), and DDO/X/A (double dropout medium containing X-α-Gal and Aureobasidin A) plates, allowing to detect autonomous induction of the Y2H reporter genes that are designated to detect protein-protein interactions. These reporter genes induce α-Galactosidase activity and resistance to Aureobasidin A (see Table II_3).

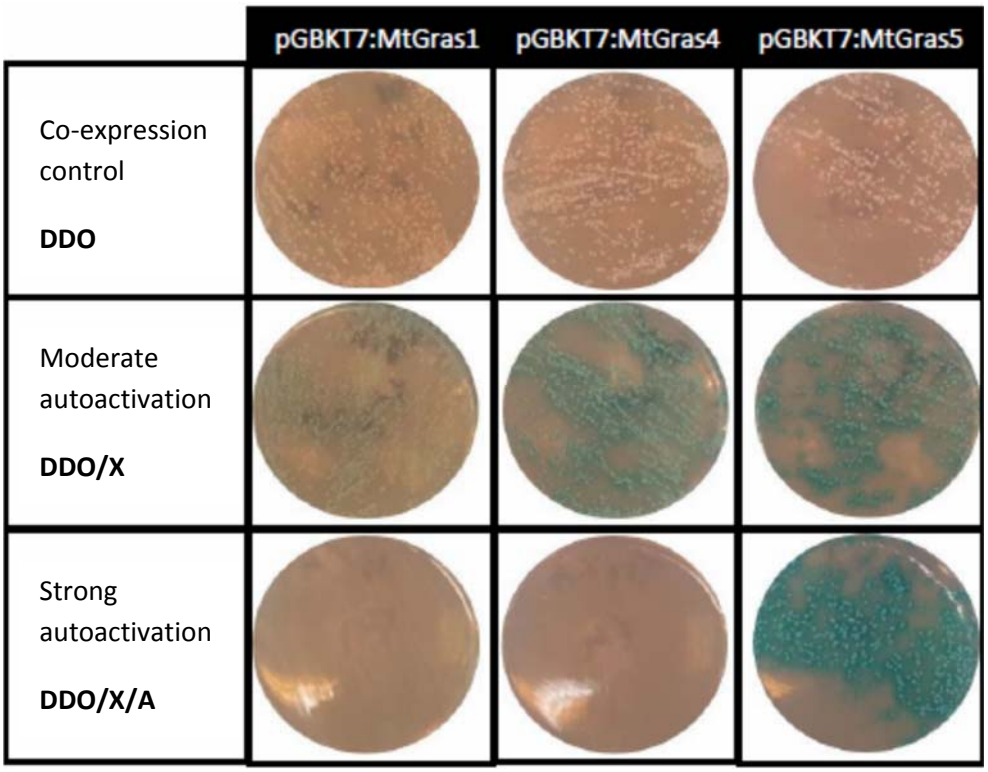

Figure II_4. Representative autoactivation test of selected bait constructs.
Bait constructs were transformed into *S. cerevisiae* Y2HGold and plated on DDO, DDO/X, and DDO/X/A plates allowing to detect Aureobasidin A (A) resistance and α-Galactosidase (X) activity respectively. Cell growth and colorization were monitored after incubation for three days at 30 °C. Autoactivation is indicated by degree of blue coloration on DDO/X and DDO/X/A as well as growth on the high stringency medium DDO/X/A.

Colonies grown on different autoactivation test media showed diverse intensities of coloring and growth. The results for all generated bait constructs are summarized in Table II_6.
In general, the higher the stringency level on which the autoactivation occurs, the more will subsequent Y2H experiments be affected. For DDO/X/A, being the basal stringency level in Y2H screenings, several bait constructs show a significant autoactivation and can therefore not be used at this stringency level.

Table II_6: Autoactivation test results.

Vector	DDO/X	DDO/X/A	QDO/X/A
pGBKT7 BD (EV)	Pale	-	n. t.
pGBKT7-53 (+)	White	-	n. t.
pGBKT7-Lam (-)	Pale	-	n. t.
pGBKT7:MtGras1	Pale	-	n. t.
pGBKT7:MtGras4	Pale	-	n. t.
pGBKT7:MtGras5	Blue	+	-
pGBKT7:MtGras6	Pale	-	n. t.
pGBKT7:MtGras7	Pale	-	n. t.
pGBKT7:MtDella1	Blue	+	-
pGBKT7:MtDella2	Blue	+	-
pGBKT7:MtDella3	Blue	+	-
pGBKT7:MtNsp1	Pale	-	n. t.
pGBKT7:MtNsp2	Blue	+	-
pGBKT7:MtRam1	Blue	+	-

ORFs were subcloned as bait into pGBKT7-T and transformed into *S. cerevisiae* Y2HGold. Autoactivation of PPI indicating reporter gene expression was detected by plating transformed cells on DDO/X, DDO/X/A, and QDO/X/A yeast media plates. Cell growth and colorization were monitored after incubation for three days at 30 °C. Empty vector control (EV) as well as construct serving as positive (+) or negative (-) control for interaction with pGADT7-T are indicated. Abbreviation: n. t., not tested.

Bait-containing yeast strains exhibiting autoactivation on DDO/X/A (*MtGras5*, *MtDella1*, *MtDella2*, *MtDella3*, *MtNsp2*, and *MtRam1*) were also plated on QDO/X/A (quadruple dropout) media, additionally lacking histidine and adenine and representing the highest stringency of selection for reporter gene expression in the Y2H system. No growth of colonies on this maximum stringency level was detected, possibly allowing these bait constructs to be used in mating experiments at least on QDO/X/A medium.

Construction of a Yeast Two-Hybrid prey library from *M. truncatula* root tissues

Total RNA from different symbiotic tissues with RIN values ≥ 9, including *R. irregularis*-colonized arbuscular mycorrhizal roots (21 dpi), root nodules (28 dpi), Nod-Factor treated roots (24 h), and uninfected roots (Hohnjec *et al.*, 2005; Natalija Hohnjec, Leibniz Universität Hannover, Hanover, Germany, personal communication; Figure II_5) was used in the "Make Your Own Mate & Plate Library" System (Clontech Laboratories, Fitchburg, USA) to generate cDNA.

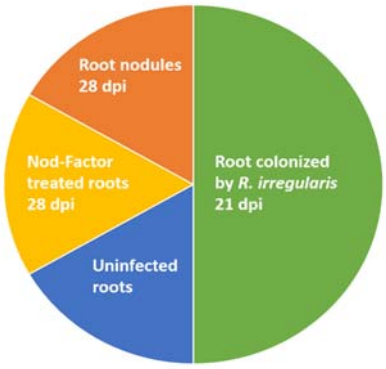

Figure II_5. Distribution of *M. truncatula* total RNA used for the construction of a prey library for Yeast Two-Hybrid screenings.
RNA samples were checked to exhibit a RIN-value > 9 before use.

Oligo dT-primer were used in the first-strand synthesis. First-strand cDNA was amplified using the "Advantage 2 Polymerase Kit" (Clontech Laboratories, Fitchburg, USA). The overall yield of generated ds cDNA was 3.75 µg, which is within the expected range of 2-5 µg. The PCR-generated ds cDNA is shown in Figure II_6. The amplified cDNA length ranged from ~ 0.2 kb to > 6 kb, with a highest concentration at ~ 1 kb. Appearance of the cDNA was as expected, according to the manufacturer's manual (Clontech Laboratories, Fitchburg, USA).

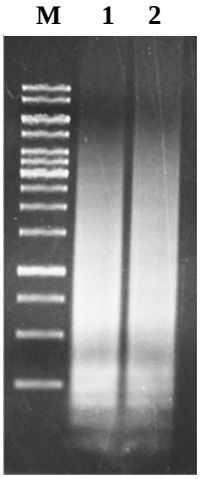

Figure II_6. cDNA generated using SMART cDNA synthesis.
cDNA was generated using oligo dT-primers in the "Make Your Own Mate & Plate Library" System (Clontech Laboratories, Fitchburg, USA). Synthesis was carried out with 1 µg total RNA from different symbiotic conditions as described in Figure II_5. Long Distance PCR was performed using the "Advantage 2 Polymerase Kit" (Clontech Laboratories, Fitchburg, USA) with 22 PCR cycles. Two independent PCR reactions were analyzed on a 1 % agarose gel. **M** 1 kb ladder **1** cDNA amplification #1 **2** cDNA amplification #2.

After PCR amplification, ds cDNA was purified using the CHROMA SPIN+TE-400 Columns (Clontech Laboratories, Fitchburg, USA) and transformed into *S. cerevisia*e strain Y187 according to the library scale yeast transformation protocol. Counting of colonies grown on SD/-Leu media revealed a sufficient library complexity of ~ 3 million independent clones, which is well above the guidelines of > 1 million independent clones (Clontech Laboratories, Fitchburg, USA).

Screening of a Yeast Two-Hybrid prey library from *M. truncatula* root tissues

Library screenings with the *MtGras1*, *MtGras4*, *MtGras6*, and *MtGras7* baits that displayed no autoactivation on DDO/X/A (Table II_6) revealed a high number of yeast colonies expressing reporter genes on medium stringency (DDO/X/A) level (growth and blue coloration). These high numbers of positive clones necessitated a more stringent selection to reduce the number of interacting candidates and to eliminate reporter gene expression due to weak PPIs. Therefore, colonies were transferred to the high stringency media (QDO/X/A). Colonies screened positive on high stringency level were used in a direct PCR to amplify the prey encoding ORF. Amplicons were sequenced and blasted against the *Medicago* genome 4.0 (Young *et al.*, 2011; Krishnakumar *et al.*, 2015; Tang *et al.*, 2014) to identify interacting candidates. The results of all screenings performed are presented in Table II_7. Genes identified were classified according to their putative impact on AM related processes and their AM-related transcription profiles.

Table II_7: Summarized Y2H library screening results.

Bait	Candidates	Nuclear	AM-induced	Mt 4.0 ID	Mt 4.0 annotation
MtGras1	14	2	-	Medtr4g064150.1	GRAS family TF: MtGRAS35*
				Medtr7g062120.1	GRAS family TF: MtGRAS60*
MtGras4	26	3	**2**	**Medtr0055s0120.1**	**senescence-associated protein**
				Medtr6g087760.1	**Domain of unknown function 4378 protein**
				Medtr3g081090.1	development and cell death domain protein
MtGras6	8	4	-	Medtr8g017210.1	C2H2-like zinc finger protein, putative
				Medtr4g103790.1	NOP56 pre RNA processing ribonucleoprotein
				Medtr3g102040.1	polyadenylate-binding protein
				Medtr1g115920.1	U4/U6 small nuclear Prp3 ribonucleoprotein
MtGras7	24	8	-	Medtr2g027860.1	myb-like transcription factor family protein
				Medtr5g016510.1	myb transcription factor
				Medtr3g005240.1	B-block-binding subunit of TFIIC protein
				Medtr3g102840.1	transcription initiation factor
				Medtr5g010620.1	transcription initiation factor TFIIE, beta subunit
				Medtr7g102410.1	PWWP domain protein
				Medtr8g076020.1	calcium-dependent kinase CPK1 adapter protein
				Medtr4g134630.1	survival of motor neuron-related-splicing factor

Candidates from the corresponding library screening were sequentially filtered for nuclear localization and AM-induced gene expression. Remaining interacting candidates were evaluated regarding their biological relevance and possible impact and grouped into three categories: red = No impact on transcription; yellow = Impact on transcription but low AM relevance; green = Evident or putative impact on transcription and possible AM relevance. Genes being upregulated in the AM symbiosis are marked in bold letters. *Nomenclature according to Song *et al.*, 2017

The expression of genes with a putative function in arbuscule development and an upregulation during AM was studied in the time course of AMF colonization (Chapter I).

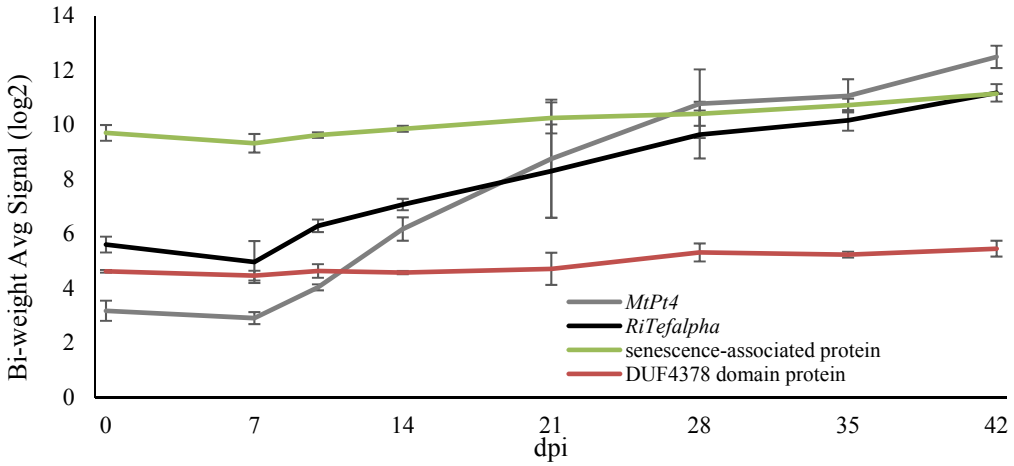

Figure II_7: Expression of the two AM-induced MtGras4 interaction candidates in *M. truncatula* roots at different time points (0, 7, 10, 14, 21, 28, 35, and 42 days) post inoculation with *R. irregularis*.
Gene expression levels were plotted using signal intensities from Affymetrix GeneChip hybridizations. Standard deviations are indicated as error bars. N = 3 biological replicates, each consisting of a pool of 6 individual root systems per time point. Abbreviation: dpi, days post inoculation.

The two genes that were found to possibly interact with MtGras4 do not show a strong induction along the AM time course. Therefore, further experiments aimed at the identification of interaction via pairwise GRAS TF combinations in direct mating approaches.

Identification of GRAS TF interactions via direct mating reveals MtGras1, MtGras4, and MtRad1 interactions

Matrix based direct matings of bait and prey fusion expressing yeast strains were limited due to a high number of baits showing autoactivation of the reporter genes on high stringency media. A representative image of a direct mating experiment is shown in Figure II_8. Growth on DDO indicates successful mating, while growth on DDO/X/A and QDO/X/A indicate PPI on different stringency levels. All clones screened positive on reporter gene expression in the assay shown are considered to be false-positive due to autoactivation, as reported above (see Table II_6).

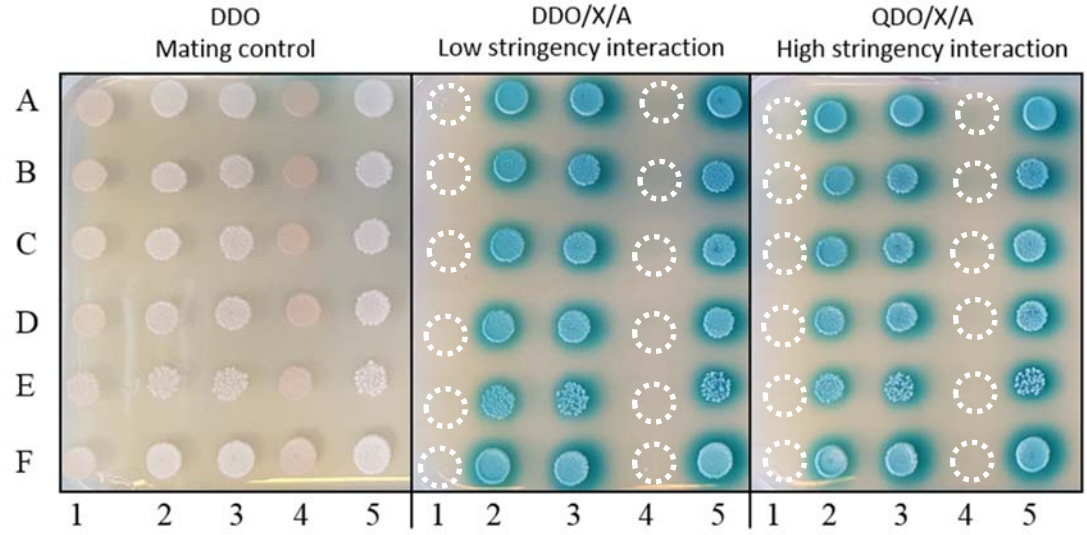

Figure II_8: **Representative image of a matrix-based direct mating experiment.** Different bait fusions are indicated by numbers (1-5), prey fusions by letters (A-F). **DDO** Double dropout medium (SD/-Trp/-Leu). **DDO/X/A** Double dropout low stringency medium (SD/-Trp/-Leu/X-α-Gal/Aureobasidin A). **QDO/X/A** Quadruple dropout high stringency medium (SD-Trp/-Leu/-Ade/-His/X-α-Gal/Aureobasidin A). Cells were mated in 200 µl YPDA medium for 24 h. 15 µl were dropped for each spot and incubated at 30 °C for 3 days. Spots that do not show any growth on DDO/X/A or QDO/X/A are indicated by a white circle.

Although autoactivation was affecting these studies, most bait-prey couples could be tested in at least one combination (e. g. bait-x vs. prey-y / bait-y vs. prey-x).

A summary of the direct mating results are presented in Table II_8. The positive control (pGBKT7-53 vs. pGADT7-T) was found on all stringency levels, while the negative control (pGBKT7-Lam vs. pGADT7-T) did not grow on low or high stringency media. Weak growth and coloration could be observed for Bait-MtGras4 vs. Prey-MtGras1, Bait-MtGras4 vs. Prey-MtRad1, and Bait-MtGras1 vs. Prey-MtGras1 on both stringency levels (Figure II_8)

Table II_8: Yeast Two-Hybrid direct mating matrix of AM-related GRAS TFs.

pGADT7 (Prey) \ pGBKT7 (Bait)	-Lam	53	MtGras1	MtGras4	MtGras5	MtGras6	MtGras7	MtDella1	MtDella2	MtDella3	MtNsp1	MtNsp2	MtRam1
-T													
MtGras1													
MtGras4													
MtGras5													
MtGras6													
MtGras7													
MtDella1													
MtDella2													
MtDella3													
MtNsp1													
MtNsp2													
MtRam1													
MtRad1													

Bait constructs are ordered horizontally, prey constructs vertically. White coloration indicates no reporter gene activation. Dark blue indicates high stringency reporter gene activity, light blue indicates weak activity. Grey coloration indicates a high stringency reporter gene activation that is considered to be caused by the autoactivation of several GRAS TFs (see Table II_6)

The protein-protein interactions of AM-related GRAS TFs identified by Yeast Two-Hybrid direct mating experiments are shown in Figure II_9.

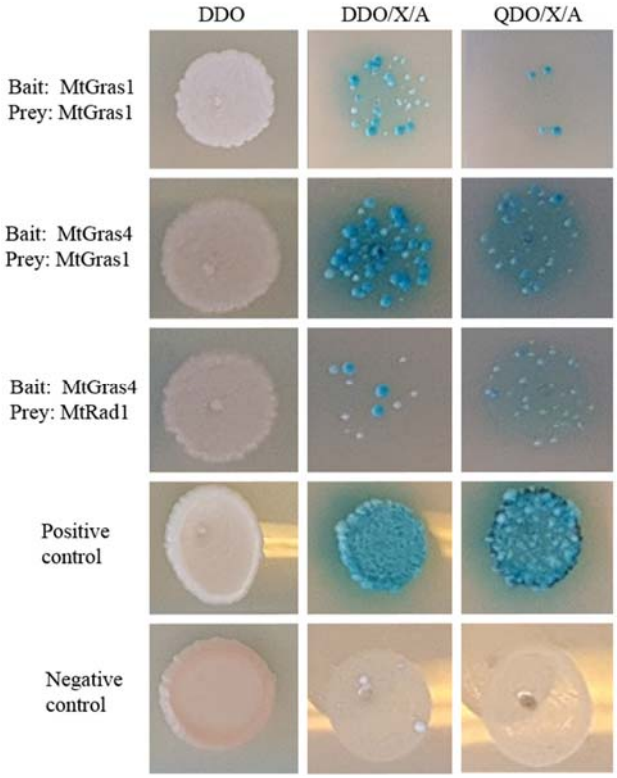

Figure II_9: Direct mating of AM-related GRAS TFs on different media.
DDO Double dropout medium (SD/-Trp/-Leu). **DDO/X/A** Double dropout low stringency medium (SD/-Trp/-Leu/X-α-Gal/Aureobasidin A). **QDO/X/A** Quadruple dropout high stringency medium (SD-Trp/-Leu/-Ade/-His/X-α-Gal/Aureobasidin A). Cells were mated in 200 µl YPDA medium for 24 h. 15 µl were dropped for each spot and incubated at 30 °C for 3 days. Bait and prey constructs expressed in the corresponding yeast clones are indicated.

Based on the results from direct mating, an interaction between MtGras1 and MtGras4, MtGras4 and MtRad1, and MtGras1 with itself can be proposed.

DISCUSSION

Screening for interactors of two AM-related GRAS TFs reveals candidates associated with different cellular processes

Considering the diversity of PPI candidates found in the screening of a prey library with *MtGras1*, *MtGras4*, *MtGras6*, and *MtGras7* baits (Table II_7), putative interaction partners were categorized based on their biological impact and their potential to induce false-positive signals.

Within the first group of interactors, NOP56 is known to be localized in the nucleus and to process RNA in a complex with other processing proteins (Gautier *et al.*, 1997), thus containing protein binding sites. However, the role of NOP56 in RNA processing makes it unlikely to be directly involved in transcriptional activation. A similar conclusion can be drawn for the polyadenylate-binding protein, the U4/U4 small nuclear Prp3-like ribonucleoprotein, and the survival of motor-neuron-related splicing factor. Although polyadenylate binding proteins are involved in gene expression, they are exclusively associated with the posttranscriptional stage (Goss *et al.*, 2013). Prp3 is involved in the formation of a spliceosomal protein complex in yeast (Anthony *et al.*, 1997), suggesting a similar role for the plant orthologue identified. Similarly, the survival of motor-neuron-related splicing factor was found to be a constituent of the spliceosomal complex in humans (Liu *et al.*, 1997). In conclusion, although all of these proteins were described to interact with other proteins, including some connections to RNA modification, there is no clear functional association for an interaction with MtGras TFs.

The second group of PPI candidates includes proteins that are likely to interact with the corresponding bait transcription factor but show a low relevance in terms of mycorrhiza-specific induction (Table II_7). Three transcription initiation factors (TFII) or subunits (Medtr3g005240.3, Medtr3g102840.1, Medtr5g010620.1) were identified to interact with MtGras7 (Table II_7). Transcription initiation factors form a pre-initiation complex, recruiting RNA Polymerase II to induce gene expression (Ranish and Hahn, 1996). Building of the pre-initiation complex and initiation of transcription commonly also includes other transcription factors (Choy and Green, 1993), making an interaction with MtGras7 possible. However, building of the transcription initiation complex is a basal mechanism in gene expression and therefore does not harbor the potential to reveal AM-specific candidates. Whereas the role of TFII subunits as potential PPI candidates might not contribute to a search for the GRAS TF regulatory function, their interaction fits in the picture of autoactivation activity for several bait constructs, as discussed later.

The subgroup member showing a higher presumptive impact on AM is annotated as a (putative) C2H2-like zinc finger protein (Medtr8g017210.1). Zinc finger proteins exhibit DNA-binding properties and are generally associated with transcriptional regulation (Berg, 1990). In this respect, Medtr8g017210.1 might be of biological and functional relevance, but since it is not induced in AM, it is unlikely to contribute to arbuscule formation.
Another candidate is Medtr7g102410.1, encoding a PWWP domain protein (Table II_7). PWWP domain-containing proteins were shown to bind DNA and histones (Maurer-Stroh *et al.*, 2003; Dhayalan *et al.*, 2010) and play a role in the regulation of developmental processes in plants (Hohenstatt *et al.*, 2018). PWWP domains also exhibit PPI properties (Stec *et al.*, 2000), thus making an interaction with a GRAS TF possible. However, these studies indicate that PWWP domain proteins are predominantly influencing gene expression by general nucleosome remodeling and not by directly inducing transcription. Therefore, Medtr7g102410.1 is not a PPI candidate of direct relevance for an MtGras interaction.
Medtr8g076020.1, annotated as a CPK1 adapter protein (Table II_7), belongs to a group of proteins interacting with and/or being phosphorylated by CPK1 (calcium dependent protein kinase 1), mediating nuclear-cytoplasmic translocation and vesicle trafficking (Patharkar and Cushman, 2006; Chehab *et al.*, 2007), but also phosphorylation of transcriptional regulators like the WRKY TFs that in *A. thaliana* respond to pathogen infection (Gao *et al.*, 2013). However, no AM-induced expression was found for this candidate, making a function during arbuscule development unlikely.

A third group of PPI candidates includes proteins with known or putative transcriptional activity and/or a higher relevance for AM symbioses (Table II_7). In this group, a MYB and a MYB-like TF were identified. MYB TFs are of particular interest since with MtMyb1, a member of the MYB family was shown to be involved in the regulation and induction of a transcriptional program for arbuscule degeneration (Floss *et al.*, 2017). Aside this implication in mycorrhiza-specific functions, MYB TFs were described to be involved in biotic stress responses (Mengiste *et al.*, 2003), phosphate deficiency (Rubio *et al.*, 2001), and gibberellic acid signaling (Gubler *et al.*, 1995). All these fields can be associated with the establishment of AM symbioses. Thus, MYB TFs interacting with GRAS TFs might be integrating biotic, nutritional, or hormonal signals into AM-related transcriptional pathways. The MYB and MYB-like TFs that were found in the Y2H screenings (Medtr2g027860.1, Medtr5g016510.1), however, showed no AM-induced expression. In case these TFs are crosslinking the AM-signaling with other pathways, an AM-related function is nevertheless possible.

Another interesting PPI candidate identified to possibly interact with MtGras4 was Medtr3g081090.1, a development and cell death (DCD) domain protein (Table II_7). DCD domains are conserved in a variety of land plants, presumably being involved in developmental processes, differentiation and programmed cell death (Tenhaken *et al.*, 2005). The study mentioned also proposes a role for DCD domains in protein-protein interactions. This PPI candidate would therefore be interesting, since establishment and maturation of the arbuscule interface is a crucial developmental process. In the same manner, a function in senescence processes is likely and would be even more remarkable in the context of MtGras4 as a member of an early signaling cascade, being linked to MtGras7 in the later stages of the arbuscule development (see General Discussion). If this DCD protein is involved in MtGras4-driven transcription, it might fit into the developmental processes that finally control arbuscule development. However, Medtr3g081090.1 is not upregulated during the AM symbiosis. Similar to the situation for MYB TFs, this does not necessarily exclude a function during AM, but would require a similar crosstalk with AM-related processes.
Interestingly, with Medtr4g064150.1 and Medtr7g062120.1, two GRAS family TFs were identified to possibly interact with MtGras1 (Table II_7). According to Song *et al.* (2017), these TFs were designated MtGRAS35 and MtGRAS60. In the AM symbiosis, an interaction between different GRAS TFs has been described, e. g. NSP1-NSP2 (Hirsch *et al.*, 2009), RAM1-RAD1 (Xue *et al.*, 2015), GRAS1-RAM1 (Park *et al.*, 2015) and RAD1-TF124 (Park *et al*., 2015). Therefore, possible interactions between MtGras1, MtGRAS35, and MtGRAS60 might be of interest to reveal PPI networks and regulational interdependencies in the AM symbiosis. However, neither MtGRAS35 nor MtGRAS60 shows AM-induced transcription.

The only AM-induced candidates identified in the Y2H library screening are Medtr005s0120.1 and Medtr6g087760.1, being annotated as a senescence-associated protein and “domain of unknown function” (DUF4378) protein (Table II_7). The DUF4378 protein could neither be identified nor categorized, since all related sequences belong to uncharacterized proteins. The senescence-associated protein, encoded by Medtr005s0120.1, appears to be nuclear-localized, but no further information on the localization or function could be obtained. Both proteins show contradictory transcription patterns in the MtGEA gene expression atlas (Benedito *et al.*, 2008; He *et al*., 2009), with AM-specific upregulation in some experiments and no induction in others. Hence, the time course based expression analysis during ongoing mycorrhization was examined to determine AM-dependent expression of the two candidate genes, as shown in Figure II_7. While the AM marker genes *GiTefa* and *MtPt4* show a strong increase in the course of

mycorrhization, Medtr005s0120.1 and Medtr6g087760.1 do hardly show any AM-related transcriptional activation.
Although no significant upregulation in the course of ongoing mycorrhization was detected for Medtr005s0120.1 and Medtr6g087760.1 in the GeneChip data, a mycorrhiza-related function cannot be excluded. Genes showing a weak transcriptional upregulation in mycorrhizal roots might not be able to be detected in AM whole-root extracts due to a specific expression in specific cell types, an observation also made in cell-specific gene expression studies (Gaude *et al.*, 2012; Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013).

Finally, the question arises why MtGras-interactors reported in the literature (MtRam1 and MtRad1; Park *et al.*, 2015) were not identified, although these genes should be represented in the Y2H library (Figure II_5). Due to the fact that the library complexity as well as the transformation efficiency were satisfying and the library was successfully used for the identification of several CCAAT-box binding factor interactors in other experiments (Stephanie Lauhoff, Leibniz Universität Hannover, Hanover, Germany, personal communication), other reasons have to be considered that will be discussed below.

Autoactivation of *MtGras* bait constructs limits the application of the Yeast Two-Hybrid system for screening GRAS TF interactions

Autoactivation of several *MtGras* bait constructs interfered with Yeast Two-Hybrid experiments (see table II_6). The question whether or not this phenomenon is a common feature for GRAS TFs was addressed in several recent studies. Latest reports suggest that GRAS TFs in general do not exhibit DNA-binding properties but rather bind other DNA-binding TFs (Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014; Hirano *et al.*, 2017; Pimprikar *et al.*, 2018). The observed DNA binding properties of NSP2 (Hirsch *et al.*, 2009) and RAM1 (Xue *et al.*, 2015) might therefore be an exception.

On this basis, the question arises why so many GRAS TFs show autoactivation when used as a bait fusion. The assumption that the fused GRAS TFs are inducing the yeast reporter gene expression by binding to the corresponding yeast promoter turned out not to be the only possible explanation. In a bait fusion, the GRAS TF of interest is fused to the GAL4 DNA BD (Figure II_2). In theory, the activity of this domain needs to be complemented by the GAL AD that binds and recruits other proteins to the promoter. However, the GAL4 AD was found to bind a subunit of TFIID, a multiprotein complex necessary for the recruitment of RNA Polymerase II (Dynlacht *et al.*, 1991; Vashee and Kodadek, 1995). In Eukaryotes, recruitment of RNA polymerase II includes many different subunits to build the transcription preinitiation

complex including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Albright and Tjian, 2000). Interestingly, TFIIE and TFIIC subunits were found to be potential PPI candidates for MtGras7 in the Y2H library screening (see Table II_7). This direct interconnection with the preinitiation complex machinery, resembling and possibly bridging the function of the GAL4 AD is a hint for properties of GRAS TFs in general and might restore the GAL4 function in bait fusions where the GAL4 AD domain is missing. In this case, autoactivation would not occur due to GRAS TF DNA binding but due to a complementation of the GAL4 AD by the GRAS TF bait itself.

While autoactivation caused by TF binding to yeast promotor sequences can be avoided by inserting mutations in the DNA binding region of the VHIID domain in GRAS proteins (Hirsch *et al.*, 2011), the AD complementing functions of several fused GRAS proteins cannot be avoided, since influencing the PPI properties would leave the Y2H system useless.

In concordance with recent studies on GRAS TF PPIs, the high number of bait constructs showing autoactivation can thus be explained by an association of GRAS TFs with the basal transcription machinery, which interferes with the Y2H system.

Direct Y2H mating experiments reveal potential interaction candidates for MtGras1, MtGras4, and MtRad1

Although the autoactivation of many GRAS TFs was an experimental drawback, direct matings revealed potential interactions between MtGras1 and MtGras4 as well as MtGras4 and MtRad1. Furthermore, MtGras1 was shown to interact with itself, potentially forming a homodimer (Figure II_9). Although these interactions were confirmed on the highest possible stringency medium, interactions between MtGras1 and MtRam1 as well as MtGras1 and MtRad1, as described by Park *et al.* (2015), could not be confirmed here. Similarly, other interactions that were described in the literature were not verified in the direct matings, including RAM1-NSP2 (Gobbato *et al.*, 2012), RAM1-RAD1 (Xue *et al.*, 2015), and NSP1-NSP2 (Hirsch *et al.*, 2009). While the autoactivation of individual GRAS TFs found in this study and the higher stringency level used here in comparison to literature studies on MtRam1 interactions (Park *et al.*, 2015) disabled analyses of the bait constructs for MtNsp2 and MtRam1, the lacking interaction between MtNsp1 and MtNsp2 remains surprising. The most reasonable explanation is again the high stringency of the Y2H screening system used here, eliminating many false-positives but in the same way also many true but weak interactions. In addition, several studies showed that repeating Y2H library screenings in different Y2H systems with the same set of genes display little overlap in the interactions that can be found and that each Y2H approach was not able to

detect more than 25 - 36 % of all known interactions (Rajagopala *et al*., 2009; Chen *et al.*, 2010), leading to the conclusion that Y2H experiments are not highly reproducible. On this basis, the interactions found here (MtGras1-MtGras1, MtGras4-MtGras1, and MtGras4-MtRad1) appear even more promising, since the highest possible stringency level set in this experiment suggests a strong interaction between these GRAS TFs. Unfortunately, the interaction between MtGras4-BD vs. MtRad1-AD could not be tested in this orientation of BD and AD fusion, since no MtRad1-AD prey construct was available. Also, the MtGras1-MtGras4 interaction was only found for the MtGras1-BD vs. MtGras4-AD combination. The corresponding MtGras4-VD vs. MtGras1-AD mating did not confirm this result, which calls for additional confirmation using alternative methods, e. g. BiFC.

In conclusion, it appears that the AM-related MtGras TFs MtGras1, MtGras4, and MtRad1 are connected on both the transcriptional level via co-expression and on the protein level via PPI. It will nevertheless be essential to further investigate interdependencies of the corresponding genes, using functional studies in plant mutants and knockdown roots, as reported in Chapter III.

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CHAPTER III:

A regulatory circuit of GRAS transcription factors revolves arbuscule development

Scope:

In this Chapter, functional analyses of AM-related GRAS TF genes identified during a time course of AM development (Chapter I) and via protein-protein interactions (Chapter II) were performed, using different functional approaches. The dependency of the GRAS TF genes on key genes controlling arbuscule branching (*MtRam1*) or function (*MtPt4*) is used to develop a picture for the regulatory network of GRAS TF, controlling arbuscule development. This chapter was submitted for publication in BMC Plant Biology.

Contributor roles:

R. M. Hartmann did *in silico* analyses, performed gene expression studies, carried out RNAi and overexpression experiments, phenotyped transgenic RNAi and insertion mutant roots, and drafted the manuscript this chapter is based on

S. Schaepe cloned an *MtGras1* RNAi construct and demonstrated its knockdown efficiency

R. M. Hartmann, D. Nübel, A. Petersen, and M. Bertolini cloned promoter-reporter gene fusions of *MtGras* genes and performed histochemical studies in transgenic roots

J. Vasilev participated in real-time RT-PCR experiments

N. Hohnjec and H. Küster participated in the interpretation of experimental results, reviewed and edited the manuscript this chapter is based on

ABSTRACT

About 80 % of all land plants establish an arbuscular mycorrhizal (AM) symbiosis with *Glomeromycota* fungi, in order to improve their access to phosphorus and other limiting nutrients from soils. Colonization of roots by AM fungi involves a fundamental transcriptional reprogramming, ultimately leading to the establishment of arbuscules, a highly branched nutrient exchange interface between plant cells and fungal hyphae. Arbuscule development is governed by several AM-activated regulators, amongst them many GRAS transcription factors. We show here that AM-related GRAS transcription factors, belonging to different subgroups, are differentially upregulated during mycorrhization. Based on expression studies in mutants defective in arbuscule branching (*ram1-1*, with a deleted MtRam1 GRAS transcription factor) and the formation of functional arbuscules (*pt4-2*, a mutant in the AM-specific phosphate transporter MtPt4), we demonstrate that the five AM-related GRAS transcription factor genes *MtGras1*, *MtGras4*, *MtGras6*, *MtGras7*, and *MtRad1* can be differentiated by their dependency on MtRam1 and MtPt4, indicating that the network of AM-related GRAS transcription factors consists of different regulatory modules. One module is controlled by the MtRam1- and MtPt4-independent GRAS transcription factor MtGras4 that activates the *MtGras7* gene. Another module is controlled by the MtRam1- and MtPt4-dependent GRAS transcription factor MtGras1. Although a knockdown of *MtGras1* reduces transcription of AM-related GRAS transcription factor genes, including *MtRam1* and *MtGras7*, overexpression of *MtGras1* alone is not sufficient to activate *MtGras* expression. The genome-wide transcription profile of mycorrhized *MtGras1* knockdown roots differs substantially from that of mycorrhized *ram1-1* roots, indicating that MtGras1 is not essential for the activation of marker genes characteristic of functional arbuscules. *MtGras1* knockdown roots display normal fungal colonization, but display a trend towards the formation of smaller arbuscules.

The network of AM-activated GRAS transcription factors consists of separate interconnected modules. We propose that this regulatory circuit provides a flexible response of the symbiotic interface to external (e. g. nutrient availability) and internal (e. g. plant hormones) stimuli, in order to regulate and control the arbuscule life cycle under changing conditions.

INTRODUCTION

Plants are able to form mutualistic associations with microbial soil organisms to their own benefit. The symbiosis with arbuscular mycorrhizal (AM) fungi of the *Glomeromycota* (Schüssler *et al.*, 2001) can be found in more than 80 % of all land plants (Smith and Read, 1997). While the fungal symbiont is supplied with photosynthetically fixed carbon, its widespread mycelial network expands the phosphate depletion zone of the rhizosphere and actively provides phosphorus, but also other nutrients, to the host plant (Smith and Smith, 2011).

Emerging from the hyphopodium that extraradical hyphae form on the root surface, a structure called prepenetration apparatus (PPA, (Genre *et al.*, 2005)) is established in epidermal cells. The PPA guides hyphal growth towards the inner cortex, where fungal hyphae proliferate (Parniske, 2008), ultimately leading to the formation of tree-like intracellular arbuscules (Harrison, 1999; Pumplin *et al.*, 2009). These symbiotic structures are regarded as the central place of nutrient transfer between plant cells and AM fungi (Cox and Tinker, 1976; Harrison *et al.*, 2002). In return for the supply of photosynthetically fixed carbon to the microsymbiont (Bonfante and Genre, 2010; Baier *et al.*, 2010), the AM fungus primarily delivers phosphorus, but also nitrogen compounds, minerals, and water across the periarbuscular membrane to the host plant (Garcia *et al.*, 2016; Lanfranco *et al.*, 2016). This plant-derived, highly specialized interface is thus equipped with a specific composition of transporters and other membrane-associated proteins (Bonfante and Genre, 2010).

Arbuscules are transient structures that only operate for a couple of days (Harrison *et al.*, 2005), and a suite of AM-activated transcriptional regulators belonging to different classes (Devers *et al.*, 2013; Luginbuehl *et al.*, 2017; Gutjahr *et al.*, 2017; Floss *et al.*, 2017; Pimprikar *et al.*, 2018) controls their development, functionality, and degradation. The regular turnover of arbuscules is initiated by a MtMyb1-induced senescence program (Floss *et al.*, 2013) that involves the activation of genes encoding hydrolases and defense-related proteins, together supporting the cellular restructuring of arbuscule-containing cells (Uhe *et al.*, 2018).

Transcription factors (TFs) can be found in all eukaryotic organisms. Functioning as regulators of gene expression that interact with enhancer regions of promoters to induce or repress transcription of target genes, they control both plant development and its reactions to external abiotic and biotic stimuli. The higher number of plant TFs in comparison to animals implies an involvement in the continuous adaption of plants to the environment, which cannot be avoided due to their sessile nature (Riechmann *et al.*, 2000; Shiu *et al.*, 2005).

GRAS transcription factors belonging to the GIBBERELLIN-INSENSITIVE (GAI; (Peng *et al.*, 1997)), REPRESSOR of gal1-3 (RGA; (Silverstone *et al.*, 1998)), or SCARECROW (SCR; (Di Laurenzio *et al.*, 1996)) families form a subgroup of plant TFs. Based on their specific domains, 59 (Zhang *et al.*, 2017) to 68 (Song *et al.*, 2017) members of the GRAS TF family were predicted in *M. truncatula*. Prominent GRAS TFs have been shown to play a vital role in symbiotic signaling (Bucher *et al.*, 2014), e. g. NSP1 and NSP2 that have a key role in the early transduction of signals during rhizobial and mycorrhizal symbioses. In response to the elicitation by Nod-factors (NFs), NSP1 and NSP2 form a heterodimer that binds to *cis*-regulatory elements in the promoter of the *ENOD11* gene (Hirsch *et al.*, 2009). NSP1 and NSP2 also mediate other early Nod- and Myc-factor induced responses, a process that also incorporates the GRAS TF RAM1 (Gobbato *et al.*, 2012; Gobbato *et al.*, 2013). RAM1 was initially shown to be required for early mycorrhizal signaling, but is now known to control arbuscule branching in *Medicago truncatula*, *Lotus japonicus*, and *Petunia hybrida* (Park *et al.*, 2015; Pimprikar *et al*., 2016; Rich *et al.*, 2015; Xue *et al.*, 2015). Transcriptional profiling of *ram1-1* mutants in pre-symbiotic signaling (Hohnjec *et al.*, 2015) and in AM roots (Luginbuehl *et al.*, 2017) revealed several hundred potential targets of RAM1, including many members of the carbohydrate and lipid metabolism (Luginbuehl *et al.*, 2017). A major task of this GRAS TF is the induction of the *RAM2* gene, encoding a glycerol-3-phosphate acyl transferase involved in the production of cutin monomers and fatty acid precursors (Wang *et al.*, 2012; Rich *et al.*, 2017) required for the formation of the periarbuscular membrane (Bravo *et al.*, 2017). In addition, RAM1 is vital for the expression of *MtPt4* and genes encoding other membrane transporters and membrane proteins essential for arbuscule function (Park *et al.*, 2015).

The activation of *RAM1* transcription (Pimprikar *et al.*, 2016) is controlled by DELLA proteins, which are a subgroup of GRAS TFs. DELLA proteins, being inactivated at high GA levels, were thus shown to link the level of plant hormones with arbuscule formation (Floss *et al.*, 2013; Foo *et al.*, 2013).

RAD1, a second prominent member of the AM-related GRAS TF family, was in addition to RAM1 shown to be required for arbuscule development in *M. truncatula* (Rey *et al.*, 2017) and *L. japonicus* (Xue *et al.*, 2015). Interestingly, RAD1 was also shown to physically interact with RAM1 (Xue *et al.*, 2015) as well as TF80 and TF124, two additional AM-related GRAS TFs (Park *et al.*, 2015), suggesting that these regulators interact to control arbuscule development (Park *et al.*, 2015; Xue *et al.*, 2015).

Based on genome-wide expression profiling of mycorrhizal and non-mycorrhizal tissues (Hohnjec *et al.*, 2005; Küster *et al.*, 2007; Gomez *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2011; Hogekamp *et al.*, 2013), several *M. truncatula* genes encoding GRAS TFs (*MtGras* genes) were found to be upregulated in the AM symbiosis, including the *MtRad1* (Rey *et al.*, 2017) and *TF80* gene (Park *et al.*, 2015) mentioned above. To shed light on the contribution of AM-activated *MtGras* genes to the development of AM symbioses and arbuscule formation, we performed comparative gene expression studies and *in situ* localizations of promoter activities in *ram1-1* and *pt4-2* mutants, encoding a key transcriptional regulator of arbuscule branching (Park *et al.*, 2015) and a mycorrhiza-specific phosphate transporter required for the formation of active, phosphate-transporting arbuscules (Javot *et al.*, 2007), respectively. Together with functional studies in *Tnt1* mutants and RNAi-mediated knockdown roots, we position five AM-related GRAS TFs relative to the well-studied AM-related regulator MtRam1 in the regulatory circuit that controls arbuscule development. With MtGras1, we provide evidence that an AM-related GRAS TF is part of a feedback loop with MtRam1 to sustain arbuscule formation.

MATERIAL AND METHODS

Cloning of promoter-*gusA*int fusions and histological analyses

Promoter sequences of *MtGras* genes were amplified from genomic DNA of *M. truncatula* using oligonucleotides specified in Table III_1. PCR-fragments were cloned into pGUS-INT (Küster *et al.*, 1995), in front of the *gusA*int reporter gene cassette. The resulting transcriptional fusions were released using *Spe*I and subcloned into the *Sma*I-digested binary vector pRedRoot (Limpens *et al.*, 2004), after fill-in of 5' overhangs using the Klenow fragment.

GUS assays were performed by incubating roots in GUS staining buffer (Küster *et al.*, 1995) for 4 to 8 hours at 37 °C, if not stated differently. Counterstaining of fungal material was performed using Alexa WGA Fluor 488 (Thermo Fisher Scientific, Langenselbold, Germany), as described above.

Cloning of knockdown and overexpression constructs

An RNAi construct for *MtGras1* was generated by amplification and recombination of a 379 bp long fragment of the *MtGras1* coding region into pDONR^{TM}221 (Gateway®-System, Invitrogen, Karlsruhe, Germany) via the BP-, and subsequently into the binary vector pK7GWIWG2(II)-*Q10*:DsRED (Limpens *et al.*, 2005) via the LR-reaction.

MtGras1 overexpression constructs were generated by PCR-amplification and cloning of the *MtGras1* coding sequence into the vectors 315p9RFP-Pt4-Expr and 917p9RFP-ubi3-Expr (Devers *et al.*, 2013), containing either the *M. truncatula MtPt4* (Harrison *et al.*, 2002) or the *A. thaliana ubiquitin 3* promoter (*AtUbq3*, (Norris *et al.*, 1993)), respectively.

Plant growth, inoculation with AM fungi, and staining of fungal structures

Medicago truncatula Gaertn cv Jemalong genotype A17 seeds were surface-sterilized and scarified as reported (Hohnjec *et al.*, 2003). Plants were grown in the climate chamber (relative humidity: 60 %; photosynthetic photon flux: 50 µmol m^{-2} s^{-1}), using a 16 h light (22 °C) and 8 h dark (18 °C) regime. *M. truncatula* R108 plants harboring a *Tnt1* insertion and corresponding control plants were surface-sterilized and scarified as described above and grown in a phytocabinet (Klimaschrank KPS 1700 Weisshaar, Bad Salzuflen, Germany), using a 16 h light (22 °C) and 8 h dark (22 °C) regime (Osram FLUORA neon tubes, Osram, München, Germany; photosynthetic photon flux: 100 µmol m^{-2} s^{-1}) and a relative humidity of 60 %.

Transgenic roots were obtained by *Agrobacterium rhizogenes* ARqua1 mediated transformation of *M. truncatula* seedlings according to (Vieweg *et al.*, 2004). Bacteria were grown for two days at 30 °C on selective TY (0.5 g/l tryptone; 0.3 g/l yeast extract; 0.07 g/l $CaCl_2x2H_2O$) agar plates. Cells were resuspended in 10 ml PS buffer (40 mM $Na_2HPO_4x2H_2O$, 85 mM NaCl, 17 mM KH_2PO_4; pH 7). The *Agrobacterium* solution was injected into the hypocotyl using a syringe. Seedlings were planted into sterile Seramis® (Seramis GmbH, Mogendorf, Germany), incubated at 18°C in the dark for 16 hours and were then transferred into a phytochamber. To detect transgenic roots, plants were screened after 4 weeks for dsRed expression using a stereomicroscope (Leica MZ 10F, Leica Microsystems, Wetzlar, Germany).

After four weeks of growth (or four weeks after induction of transgenic roots), each plantlet or composite plant was mycorrhized by inoculation in a small amount of water with 2000 spores of germinating *Glomus intraradices* Schenck and Smith DAOM197198 spores (Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada), having been reassigned to *Rhizophagus irregularis* (Błaszk., Wubet, Renker, and Buscot) C. Walker & A. Schüßler comb. nov. (Stockinger *et al.*, 2014). After 3-4 hours of inoculation, plantlets were potted into 8x7x7 cm (height × width × depth) pots filled with sterile Seramis® (Seramis GmbH, Mogendorf, Germany). Each pot contained two plants, and remaining spore solution was directly pipetted onto the root surface while potting. Mycorrhizal and non-mycorrhizal plants were fertilized with half-strength Hoagland`s solution (Arnon *et al.*, 1940) containing 20 µM phosphate. The solution was prepared with deionized water, pH was adjusted to 6.4 with KOH.

To visualize fungal colonization, roots were incubated in 10 % (w/v) KOH at 95 °C for 7 min, repeatedly rinsed with water and incubated in 1x PBS buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM $Na_2HPO_4x2H_2O$, 1.8 mM KH_2PO_4; pH 7.3) containing 20µg/ml Alexa WGA Fluor™ 488 (Thermo Fisher Scientific, Langenselbold, Germany) conjugate overnight. Photo documentation was performed using a Leica MZ 10F stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with an Olympus XC50 camera (Olympus, Hamburg, Germany), a Zeiss Axio Observer Z1 microscope equipped with an AxioCam ICc1 (Carl Zeiss AG, Oberkochen, Germany), and a confocal microscope (Leica TCS SP8 MP, Sohns, Germany). Quantification of fungal colonization was performed using the gridline intersection method (Brundrett *et al.*, 1996). For arbuscule size determination, confocal images of arbuscules were analyzed using the Fiji software (Schindelin *et al.*, 2012).

To study gene expression in transgenic *M. truncatula* A17 roots expressing an *MtGras1* RNAi construct (RNAi:*MtGras1* roots) in comparison to a *gusA*int gene (RNAi:*gusA*int control roots), composite plants were mycorrhized with *R. irregularis* spores as described above and harvested after 54 dpi. To analyze gene expression in *M. truncatula ram1-1* roots in comparison to *M. truncatula* A17 control roots, roots were mycorrhized with *R. irregularis* spores as described above and harvested after 35 dpi. In all experiments, harvesting time points were selected depending on the mycorrhization rate in the different mutants, in order to obtain sufficient overall colonization levels.

Analysis of the *Tnt1* insertion line NF4813

The *Tnt1* (Tadege *et al.*, 2008) insertion line NF4813 (based on *M. truncatula* R108), harboring a *Tnt1* insertion in the coding sequence of *MtGras4* after position +1428, was obtained from the Noble Research Institute (Ardmore, Oklahoma, USA). No stable homozygous *Tnt1* lines could be obtained for other candidate genes. Plants were screened for the *Tnt1* insertion via direct PCR from leaf discs using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Langenselbold, Germany). The PCR was performed using a *Tnt1* binding (*Tnt1*-F) and two gene-specific primers (NF4813_16_for, NF4813_16_rev). Homozygous plants were selected, selfed, and used for seed propagation. Segregating plants being wild type with respect to the *MtGras4* locus were propagated to obtain control plants.

RNA isolation and real-time RT-PCR

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Tissue disruption was carried out via FastPrep®-24 (MP Biomedicals, Santa Ana, USA). Real-time RT-PCR analyses were performed using the SensiFAST™ SYBR® No-ROX One-Step Kit

(Qiagen, Hilden, Germany), using primers listed in Table III_2. Primers were tested for specificity before use. 5 ng of total RNA were used as a template in a 20 µl reaction. RT-PCR reactions followed a three-step cycling program: Reverse transcription at 45 °C for 10 min; polymerase activation at 95 °C for 2 min; PCR amplification with 40 cycles at 95 °C for 5 sec, 55 °C for 10 sec, and 72 °C for 8 sec. The housekeeping gene *MtTefa* (Medtr6g021805.1 in the *M. truncatula* genome; (Tang *et al.*, 2014)) encoding a translation elongation factor was used for normalization. Each biological replicate was measured in three technical replicates. Average values were used to calculate gene expression levels via the $2^{-\Delta CT}$ method with $\Delta CT = CT_{gene} - CT_{MtTefa}$. Statistical significances were calculated using a two-tailed Student's t test in MS Excel 2016 (Microsoft Corp., Redmond, Washington, USA).

Hybridization and data evaluation of GeneChip *Medicago* Transcriptome Assays

Biotinylated aRNA obtained from 100 ng of total RNA for each sample was fragmented as recommended (GeneChip Medicago Transcriptome Assay, ThermoFisher Scientific, Schwerte, Germany). The size distribution of the fragmented aRNA was assessed via an Agilent bioanalyzer (Agilent Technologies, Böblingen, Germany) using an RNA 6000 assay. Standard hybridization, post-hybridization wash and double-staining as well as scanning was done as specified for GeneChip Medicago Transcriptome Assays (ThermoFisher Scientific, Schwerte, Germany).

Cel-files were analyzed using the Expression Console and Transcriptome Analysis Console software (both ThermoFisher Scientific, Schwerte, Germany). Normalization was performed via the Robust Multichip Average algorithm, intensity values for each probe set were log2-transformed and averaged across the three biological replicates using the Tukey's Bi-weight average algorithm, and expression ratios were evaluated statistically via tools of the Transcriptome Analysis Console (ThermoFisher Scientific, Schwerte, Germany).

Original annotations of the genes represented on the GeneChip *Medicago* Transcriptome Assays were updated by annotations from the *M. truncatula* genome version 4.0 (Tang *et al.*, 2014), and mapped to probe sets from the GeneChip *Medicago* genome arrays that were used to construct the *Medicago* Gene Expression Atlas (Benedito *et al.*, 2008; He *et al*., 2009) as well as to UniProt (The UniProt Consortium, 2017). Venn diagrams were drawn using the VENNY software (Oliveros, 2017).

Table III_1: Primers used for generation of gene fusions, overexpression, and RNAi constructs as well as *Tnt1* genotyping.

Reporter gene fusions	
pMtGras1_for	AAAGCATGCAAGCGATGAAAGGGTGTTGAA
pMtGras1_rev	AAACCCGGGGGTGATTTATCTTGAAGAATT
pMtGras4_for	AAAGAATTCCTTGAACCATTATTGGATTA
pMtGras4_rev	AAAAAGCTTATGACGGATTACAAGAGAAA
pMtGras6_for	AAAGCATGCCATTGTACGTAGAGTTCTGA
pMtGras6_rev	AAAAAGCTTGGTTGAAGGAGATTGGAAAA
pMtGras7_XhoI_for	AAACTCGAGCGAATATGCAAAATTGATCC
pMtGras7_HindIII_rev	AAAAGCTTGTTGTTATTGCAAAGAAACTG
pMtRad1_for	AAAGAATTCTCATGAAATTGTTATACAAA
pMtRad1_rev	AAAAAGCTTTGACATATTAATTTCTCAATT
MtGras1 overexpression	
MtGras1oex_for	AAAACCCGGGGATGGATTCAGGTTCGCCATAT
MtGras1oex_rev	AAAACCCGGGTCACCTAAATTTCCAGGCCGA
MtGras1 RNA interference	
RNAi_Gras1_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTGAGGGAATTGAGATATG
RNAi_Gras1_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAATAATATGCACCACCT
Tnt1 genotyping	
NF4813_16_for	AAGTGGGGTCCAGTGCATAG
NF4813_16_rev	TACATTGGAGTCCCCTTCCA
Tnt1-F	GCATTCAAACTAGAAGACAGTGCTACC

Table III_2: Primers used in real-time RT-PCR experiments.

Primer	**NCBI accession**	**Sequence**
MtGras1_for	XM_003598826	TTTAGGCCCTTACAAGATTAC
MtGras1_rev		CTTCACTGAAATAAGTGACCA
MtGras1_oex_for	XM_003598826	TGGATGTAATTTAACGGAGAG
MtGras1_oex_rev		CTTCACTGAAATAAGTGACCA
MtGras4_for	XM_003625927	AGGGTTAAGAAATATTGTTGC
MtGras4_rev		TAAACACATGCCTCACTAAAC
MtGras4_Tnt1_3'_for	XM_003625927	ATAGAATGGTGGAAACTAGGT
MtGras4_Tnt1_3'_rev		CCAAGCTGAGATTGAATACAT
MtGras6_for	XM_003597004	AAGCTTTGGTTATAAATTGCC
MtGras6_rev		GTCCTATGTCAGATTCAAACT
MtGras7_for	XM_003591347	AAACCATAATTCACCTTTGTT
MtGras7_rev		CAGATAATATTGCTTCCATCA
MtRad1_for	XM_003608835	GTGAGGTAGATGAAAATCCAT
MtRad1_rev		GGATAGTTACGGTTGATCTTT
MtMyb1_for	XM_01359369	TACTGCCAAATTTCTGTTCTA
MtMyb1_rev		GGATTGTGTTTTAAAGGATTC
MtPt4_for	AY116211	TCGCGCGCCATGTTTGTTGT
MtPt4_rev		GCGAAGAAGAATGTTAGCCC
GiTefα_for	XM_003588773	ATCCCAAATTTGTTAAGTCTG
GiTefα_rev		ACAGCAACAGTCTGTCTCATA
Giα-Tub_for	GW088233	TGTCCAACCGGTTTTAAAGT
Giα-Tub_rev		AAAGCACGTTTGGCGTACAT
MtRam1_for	XM_003622047	CATTACTACTCCGCAATTTTC
MtRam1_rev		CAACAAACAACCTTTATCCTC

RESULTS

AM-related GRAS TFs of *Medicago truncatula* belong to different subgroups

Based on genome-wide expression profiles recorded by GeneChip hybridizations (Chapter I and Hogekamp *et al.*, 2011) and the Y2H studies presented in Chapter II, a core set of AM-related GRAS TF genes, namely *MtGras1* (designated *TF80* in (Park *et al.*, 2015)), *MtGras4*, *MtGras6*, *MtGras7*, and *MtRad1* (Rey *et al.*, 2017) was selected. In addition, the *MtRam1* gene, encoding a GRAS TF that controls arbuscule branching (Park *et al.*, 2015), was included. The corresponding identifiers from the *Medicago truncatula* genome (Tang *et al.*, 2014), the *Medicago* Gene Expression Atlas (Benedito *et al.*, 2008), and the literature are listed in Table III_3.

Table III_3: Nomenclature of AM-related genes encoding GRAS TFs.

Gene	Genome ID	MtGEA ID	GRAS subgroup	Alternative IDs
MtGras1	Medtr3g022830	Mtr.7264.1.S1_at	GRASM1*/SCL3**	TF80***/MtGRAS25**
MtGras4	Medtr7g109580	Mtr.1484.1.S1_at	GRASM6*/MIG1****	MtGRAS59**
MtGras6	Medtr2g089100	Mtr.47463.1.S1_at	SHR**/****	MtGRAS12**
MtGras7	Medtr1g086970	Mtr.24642.1.S1_at	GRASM6*/MIG1/SCR**	MtGRAS4**
MtRad1	Medtr4g104020	Mtr.36004.1.S1_at	GRASM5*/Os4****/DELLA**	MtSymSCl3/MtGRAS43**
MtRam1	Medtr7g027190	Not represented	GRASM5*/DELLA**	MtGRAS54**

Identifiers (IDs) in the *M. truncatula* genome (Tang *et al.*, 2014), the *Medicago* Gene Expression Atlas (MtGEA; (Benedito *et al.*, 2008)), as well as subgroups and alternative identifiers from the literature are listed (*(Song *et al.*, 2017); **(Zhang *et al.*, 2017); ***(Park *et al.*, 2015); ****(Heck *et al.*, 2016)).

In a phylogenetic tree of the deduced amino acid sequences of these and selected other symbiotic GRAS TF genes, specific groupings were evident (Figure III_1). Interestingly, MtGras4 and MtGras7, two GRAS TFs belonging to the MIG1 family defined by (Heck *et al.*, 2016) share the highest sequence similarities, suggesting a functional relationship. In addition, the close relation of MtRam1 and MtRad1 is of special interest, since these TFs control arbuscule branching as well as arbuscule development and since mutual protein-protein interactions were reported (Park *et al.*, 2015; Xue *et al.*, 2015).

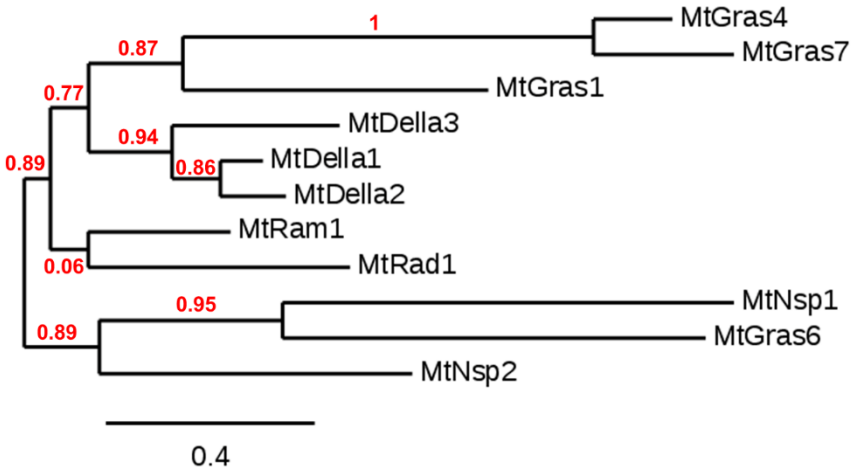

Figure III_1: Phylogenetic analysis of selected GRAS TF sequences.
Analyses were performed on the phylogeny.fr platform (Dereeper *et al.*, 2008; Dereeper *et al.*, 2011). Sequences were aligned using MUSCLE (v3.8.31) (Edgar *et al.*, 2004), gaps were removed by Gblocks (v0.91b) (Castresana *et al.*, 2000)), and trees were constructed using the maximum likelihood method in PhyML (v3.1/3.0 aLRT) (Guindon *et al.*, 2010). Bootstrap values are indicated. Except of the MtGras proteins listed in table 1, the GRAS TF sequences included MtRam1 (Park *et al.*, 2015), MtRad1 (Rey *et al.*, 2017), MtNsp1 (Delaux *et al.*, 2013), MtNsp2 (Maillet *et al.*, 2011) and the three MtDella TFs MtDella1, MtDella2, and MtDella3 (Floss *et al.*, 2013).

AM-related GRAS TF genes are differentially upregulated in the course of mycorrhization

To reveal the timing of GRAS TF action during the development of an AM symbiosis and to validate the time course gene expression data from Chapter I, a real-time RT-PCR-based gene expression study was performed (Figure III_2), using the same RNA material as for the GeneChip analyses presented in Chapter I. In general, these experiments revealed a good correlation between the time course gene expression data from *Medicago* GeneChips (Figure I_7) and from real-time RT-PCR, with a better resolution in the early time points, due to the higher dynamic range of RT-PCR measurements and the lack of background signals (Figure III_2).

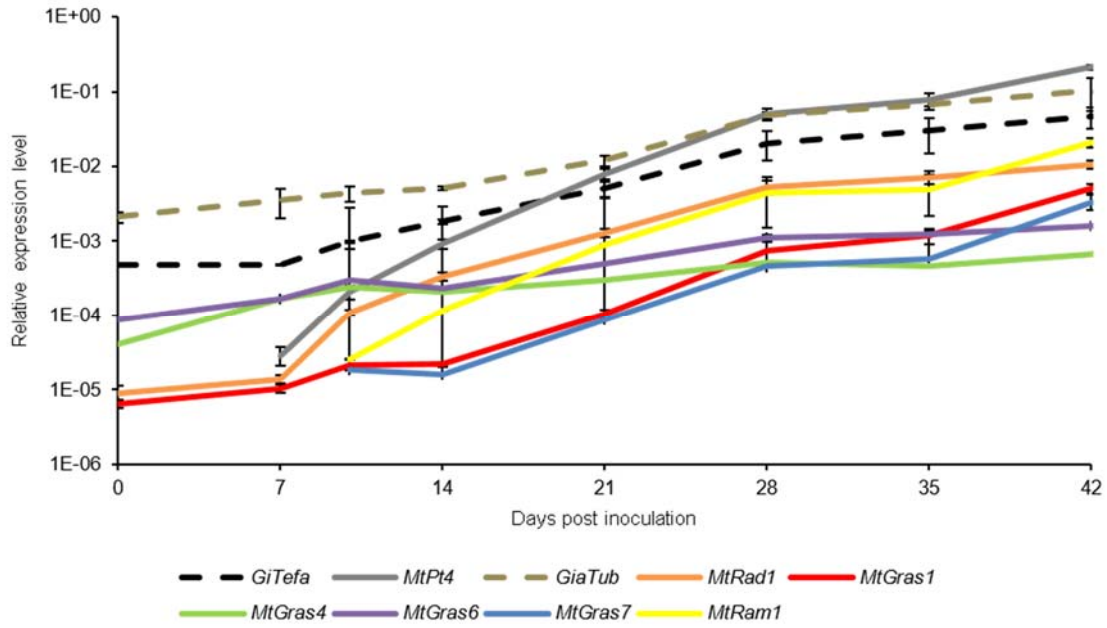

Figure III_2: Time course of AM-responsive gene expression.
28 days after germination, plants were mycorrhized and harvested at the time points indicated. Plants at 0 days post inoculation were harvested after 3 h of inoculation with *R. irregularis* spores. At each time point, three biological replicates were harvested, each consisting of 6 pooled root systems. Relative gene expression levels were determined by real-time RT-PCR. The standard error of the mean is indicated.

Histological analyses of the harvested mycorrhizal roots indicated the predominant presence of *R. irregularis* spores and extraradical hyphae until 7 days post inoculation (dpi), while significant intraradical colonization and arbuscule development started from 10 dpi (Figure I_1). Since root material at 0 dpi already contained germinated *R. irregularis* spores, the AM fungal marker genes *GiTefa* (encoding a translation elongation factor alpha) and *GiaTub* (encoding an *a*-tubulin) are already expressed, whereas transcription of *MtPt4*, encoding an arbuscule-specific phosphate transporter (Harrison *et al.*, 2002; Javot *et al.*, 2007), is only detected upon arbuscule presence. While the expression of *GiTefa* and *GiaTub* showed a log-linear and therefore exponential increase over time, *MtPt4* activation rose even stronger, mirroring the quick and ongoing process of arbuscule build-up.

During mycorrhization, the expression of *MtGras4* and *MtGras6* resembled that of *GiaTub* and *GiTefa*, whereas *MtGras1*, *MtGras7*, *MtRad1*, and *MtRam1* followed the strong rise of *MtPt4* expression. These patterns indicate two different types of activation, with *MtGras4* and *MtGras6* being already markedly expressed in response to fungal presence, while the *MtGras1*, *MtGras7*, *MtRad1*, and *MtRam1* upregulation follows the ongoing build-up of functional arbuscules in *MtPt4*-expressing cells.

AM-related GRAS TF genes differ in their dependency on the GRAS TF MtRam1

To study the dependency of AM-related GRAS TF gene expression on MtRam1, a GRAS TF required for arbuscule branching (Park *et al.*, 2015), comparative gene expression analyses were carried out in *R. irregularis*-mycorrhized roots of wild type plants and *ram1-1* mutants.

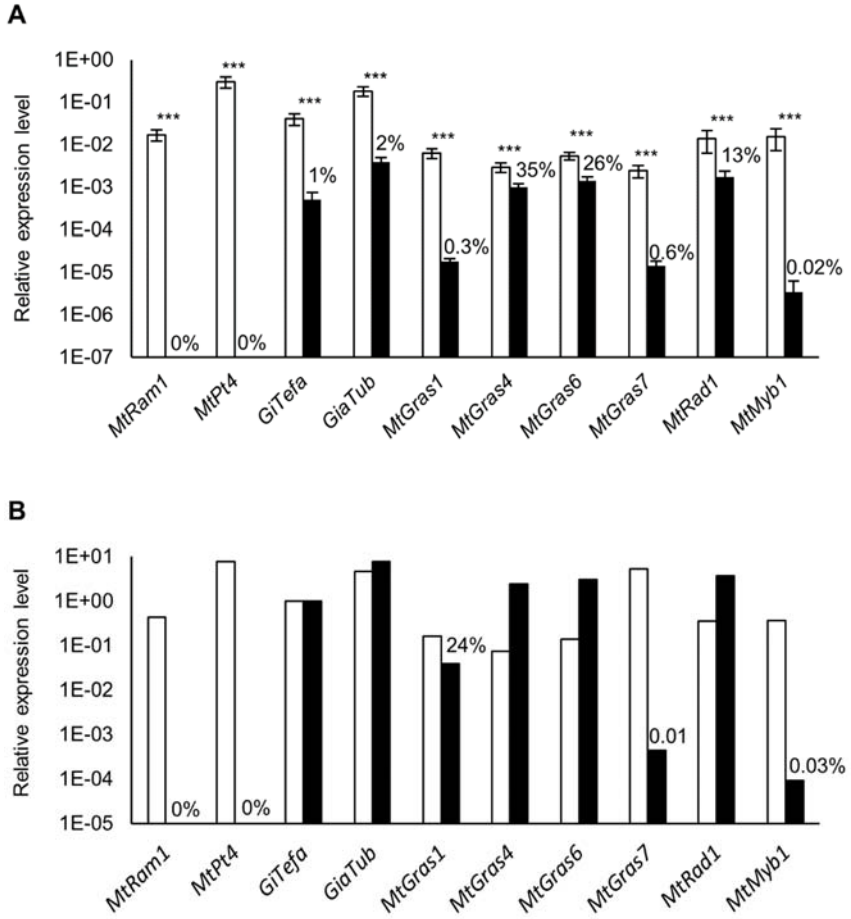

Figure III_3: Relative expression of selected *MtGras* and AM marker genes in mycorrhized *M. truncatula* A17 wild type (white) and *ram1-1* (black) roots.

Transcript amounts are shown relative to *MtTefα* (**A**) and were additionally normalized to the amount of fungal matter by building a ratio with *GiTefa* expression (**B**). Roots were harvested at 36 days post inoculation with *R. irregularis*. n=8 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to the wild type. The following genes were analyzed in addition to the *MtGras* genes listed in table 1: *MtRam1* (Park *et al.*, 2015), *MtPt4* (Harrison *et al.*, 2002), GiTefα (Sokolski *et al.*, 2010), *GiαTub* (Liu *et al.*, 2007), *MtRad1* (Rey *et al.*, 2017), and *MtMyb1* (Floss *et al.*, 2017).

Whereas real-time RT-PCR measurements (Figure III_3) of mycorrhizal roots showed a strong decrease in fungal gene expression (*GiTefa*, *GiaTub*; down to 1-2 %) as well as a complete lack of *MtRam1* and *MtPt4* transcription in *ram1-1* mutants, indicating the absence of highly branched, symbiotically active arbuscules, the reduction of GRAS TF gene transcription appeared diverging. While expression of *MtGras1* and *MtGras7* strongly decreased to 0.3-0.6 % of the level in wild type roots, transcription of *MtRad1*, *MtGras4*, and *MtGras6* was less affected (reduction to 13-35 %). A strongly decreased expression in *ram1-1* mutants

was also observed for the AM-specific *MtMyb1* gene, encoding a key regulator of arbuscule degradation (Floss *et al.*, 2017).

Since fungal colonization is impaired in *ram1-1* mutants and since expression of AM-induced marker genes as well as *MtGras1* and *MtRad1* was lower in *ram1-1* mutants in response to colonization by *Glomus versiforme* (Park *et al.*, 2015), a reduced expression of the AM-induced GRAS TF genes studied here was expected. Thus, expression levels of genes of interest were normalized against *GiTefa* transcription in order to adjust gene expression to the amount of fungal matter (Figure III_3 B). After normalization against *GiTefa*, lower transcription levels of *MtGras1*, *MtGras7*, and *MtMyb1* were still evident in *R. irregularis* colonized roots, indicating that expression of these genes are not just reduced in *ram1-1* mutants as a consequence of lower fungal colonization. GRAS TF gene expression appearing increased in *ram1-1* after *GiTefa* normalization can be explained by regulatory effects or by a lack of absolute correlation between AM fungal gene expression and the amount of fungal structures (Figure III_2).

To achieve a cellular resolution of GRAS TF gene expression, their promoter regions were fused to the *gusA*int reporter gene, and the resulting transcriptional fusions were expressed in transgenic *M. truncatula* roots. These studies demonstrated a clearly AM-induced promotor activity for *MtGras1*, *MtGras4*, *MtGras6*, *MtGras7*, and *MtRad1* in wild type roots, with a predominant or exclusive activation in the arbuscule-containing cells (Figure III_4).

In *ram1-1* mutants, promotor activities of *MtGras1* and *MtGras7* were completely abolished, even after prolonged staining. In contrast, *MtGras4*, *MtGras6*, and *MtRad1* promoters are still AM-induced in the *ram1-1* mutant background. These findings were in line with our gene expression studies (Figure III_3) and suggested a position of MtGras1 and MtGras7 downstream of MtRam1 in the regulatory cascade leading to arbuscule formation, while MtGras4, MtGras6, and MtRad1 have to be placed either upstream or parallel to MtRam1.

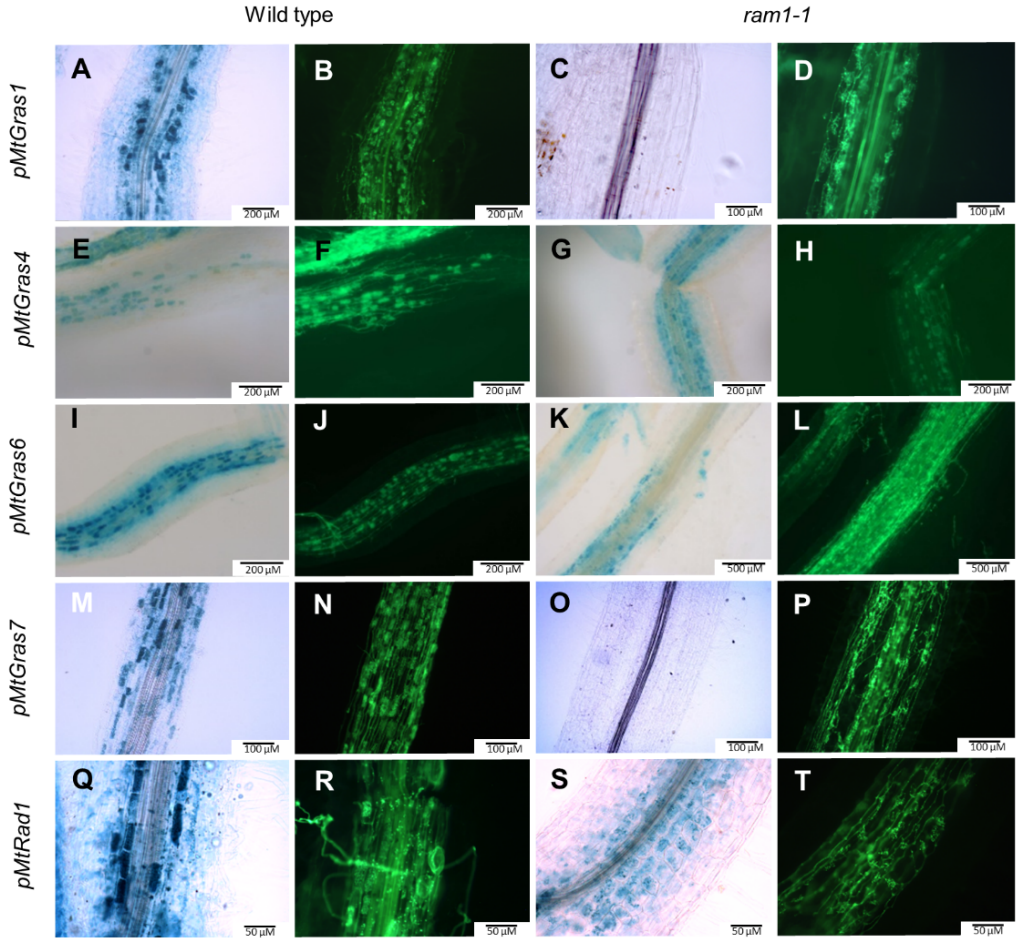

Figure III_4: Histochemical localization of the promoter activities of selected *MtGras* genes in *ram1-1*.
The promoter activities of *MtGras1* (A-D), *MtGras4* (E-H), *MtGras6* (I-L), *MtGras7* (M-P), and *MtRad1* (Q-T) were recorded in transgenic, mycorrhized roots of *M. truncatula* A17 wild type (A, B, E, F, I, J, M, N, Q, and R) and *ram1-1* roots (C, D, G, H, K, L, O, P, S, and T). GUS stainings were performed for 4-8 hours. Alexa WGA Fluor 488 stainings are shown to visualize fungal colonization.

Expression of AM-related GRAS TF genes differs in the dependency on morphologically fully developed arbuscules.

To study the dependency of GRAS TF gene expression on the presence of functional, phosphate-transporting arbuscules, comparative gene expression analyses were carried out in *R. irregularis*-mycorrhized roots of wild type plants and *pt4-2* mutants.

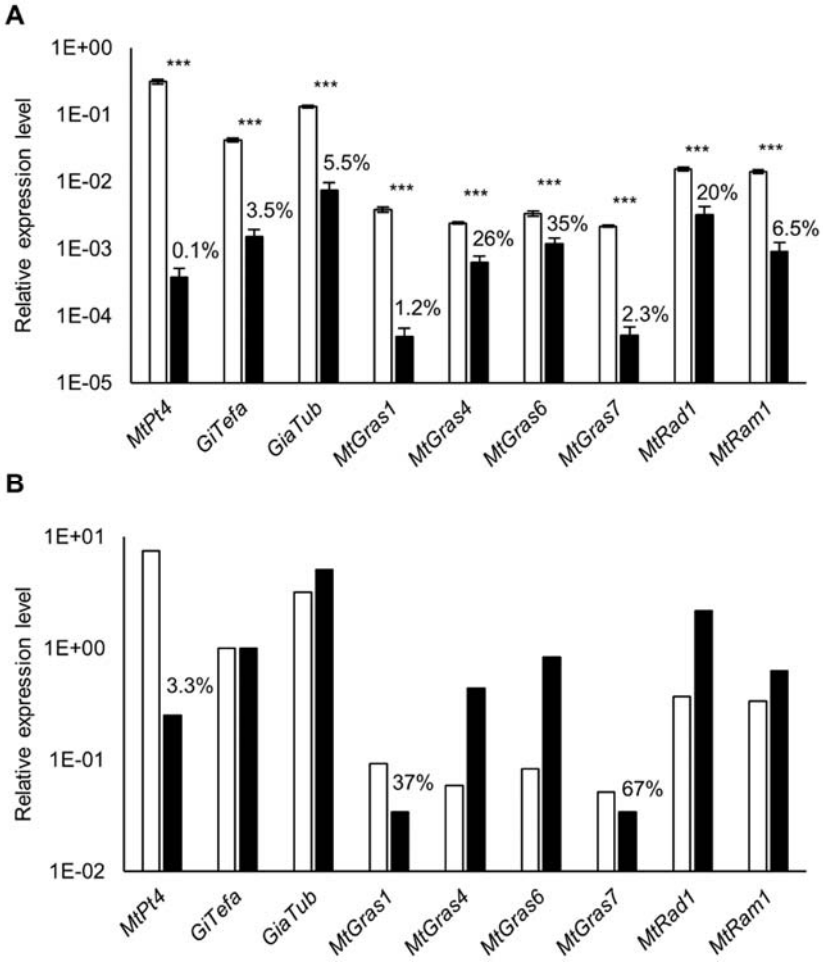

Figure III_5: Relative expression of selected *MtGras* and AM marker genes in mycorrhized *M. truncatula* A17 wild type (white) and *pt4-2* (black) roots.
Transcript amounts are shown relative to *MtTefα* (**A**) and were additionally normalized to the amount of fungal matter by building a ratio with *GiTefa* expression (**B**). Roots were harvested at 36 days post inoculation with *R. irregularis*. n=8 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to the wild type. Genes shown are as defined in the Figure III_3 legend.

Real-time RT-PCR analyses of *pt4-2* mutants (Figure III_5) revealed a regulation of the GRAS TF genes investigated similar to the situation in *ram1-1* mutants (Figure III_3). In addition to *MtPt4*, the *MtGras1* and *MtGras7* genes are most strongly repressed in the *pt4-2* background (to 1.2-2.3 % of wild type expression), whereas other GRAS TF genes were less strongly affected. After normalization to *GiTefa* expression, only *MtGras1* and *MtGras7* were still downregulated to 37-67 %, indicating that their reduced gene expression is not just reflecting a lower degree of root colonization in *pt4-2* roots. Similar to the results for *ram1-1* mutants (Figure III_4), the promoters of *MtGras1* and *MtGras7* are inactive in *pt4-2* roots, while the *MtRad1*, *MtGras4*, and *MtGras6* promoters are still functional in root areas containing the typical prematurely degrading, stunted arbuscules that were regularly observed for *pt4-2* mutants ((Javot *et al.*, 2007); Figure III_6).

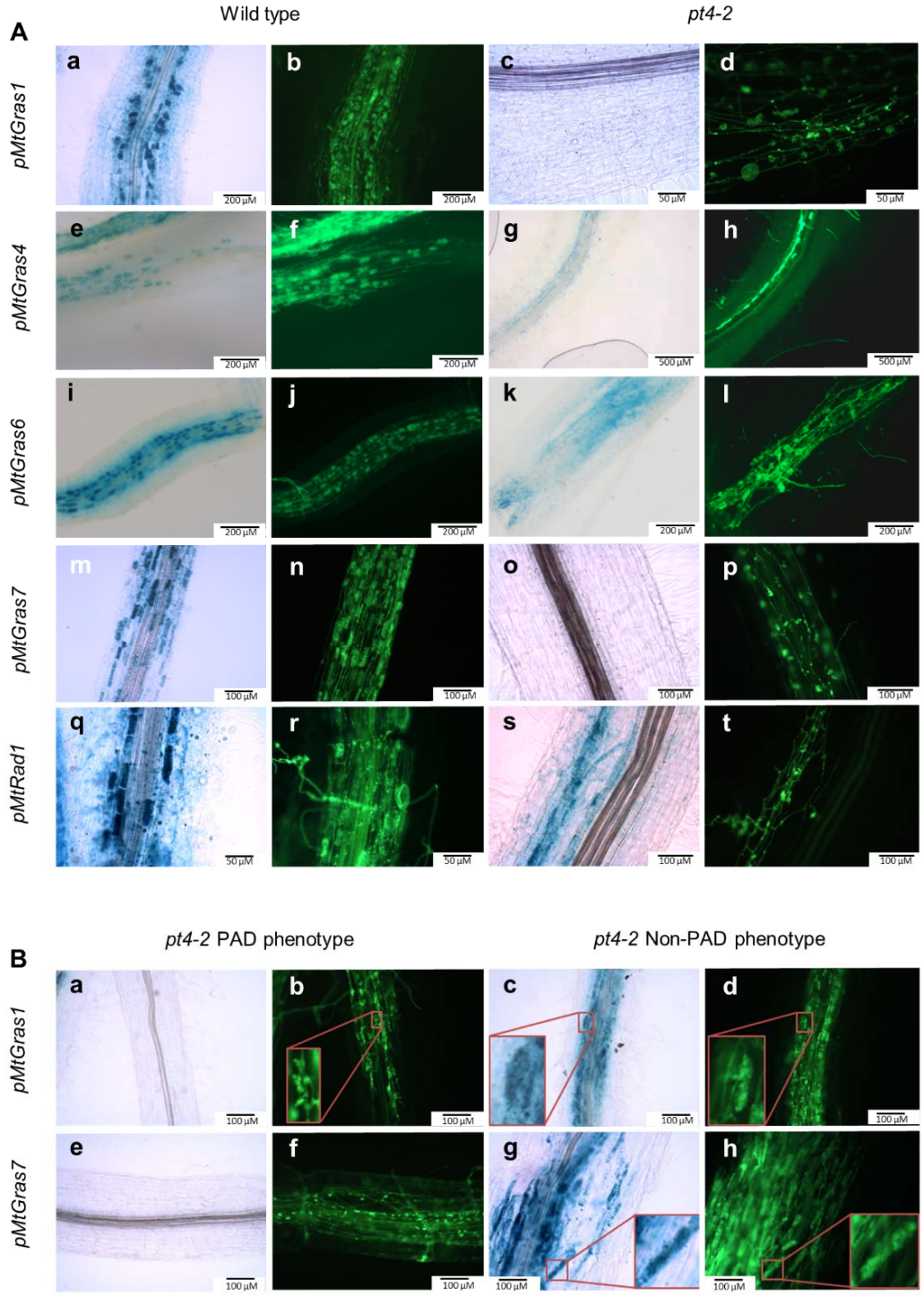

Figure III_6: Histochemical localization of the promoter activities of selected *MtGras* genes in *pt4-2*.
The promoter activities of *MtGras1* (A: a-s), *MtGras4* (A: e-h), *MtGras6* (A: i-l), *MtGras7* (A :m-p), and *MtRad1* (A: q-t) were recorded in transgenic, mycorrhized roots of *M. truncatula* A17 wild type (A: a, b, e, f, i, j, m, n, q, and r) and *pt4-2* roots (c, d, g, h, k, l, o, p, s, and t). The promoter activities of *MtGras1* (B: a-d) and *MtGras7* (B: e-h) were furthermore recorded in transgenic, mycorrhized *M. truncatula pt4-2* roots, showing a premature arbuscule degeneration (PAD; B: a, b, e, f) or Non-PAD phenotype (B: c, d, g, h). GUS stainings were performed for 4-8 hours. Alexa WGA Fluor 488 stainings are shown to visualize fungal colonization. Close-up views of PAD and Non-PAD arbuscules are shown inside red angles.

On the other hand, since the *pt4-2* stunted arbuscule phenotype was not absolutely stable in our growth conditions, some infection units from *pt4-2* roots, that in other areas showed typical premature arbuscule degeneration, occasionally developed WT-like arbuscules. Interestingly, normal *MtGras1* and *MtGras7* promotor activities could now be observed (Figure III_6 B: a-h). This local phenomenon suggests that *MtGras1* and *MtGras7* activation is dependent on a particular stage of arbuscule development that, when modulated by endogenous or exogenous conditions, as e. g. demonstrated for N-starvation (Javot *et al.*, 2011); can alleviate the *pt4-2* phenotype of premature arbuscule degeneration. It thus appears that *MtGras1* and *MtGras7* activation does not simply depend on the *pt4-2* genotype, but on the existence of a symbiotic interface being beneficial for the plant, enabling the development of mature arbuscules. A summary of *MtGras* activity in *ram1-1* and *pt4-2* mutants is presented in Figure III_7.

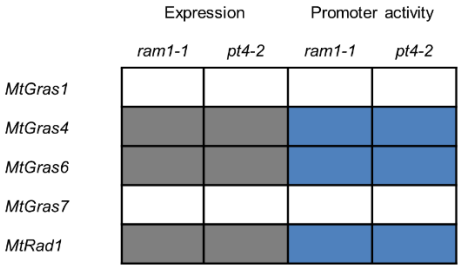

Figure III_7: Summarized data on gene expression and promoter activities of GRAS TF genes in *M. truncatula ram1-1* and *pt4-2* mutants.
Significantly reduced (white) or unaffected (grey) transcript levels after *GiTefa*-normalization (Figure III_3 and Figure III_5) as well as absent (white) or still detectable (blue) promoter activities (Figure III_4 and Figure III_6) in *ram1-1* and *pt4-2* roots displaying a premature arbuscule degeneration phenotype.

In both mutants, *MtGras1* and *MtGras7* expression is hardly detectable by real-time RT-PCR experiments or histological studies of promoter activity, while *MtGras4*, *MtGras6*, and *MtRad1* expression comparable to mycorrhizal wild type roots is observed. With respect to the results from our mutant studies, AM-related GRAS TF genes can thus be divided into two groups, being either MtRam1- and MtPt4-dependent or -independent.

MtGras4 and MtGras7 form a regulatory module within the network of AM-related GRAS TFs

As one representative of an MtRam1- and MtPt4-independent GRAS TF gene, *MtGras4* was further characterized. To understand its role in AM formation, the *Tnt1* transposon insertion line NF4813 was identified in the *Medicago truncatula* mutant database ((Tadege *et al.*, 2008); Figure III_8 A). Plants were inbred to generate a homozygous knockout line, which was tested for the position of the *Tnt1* insertion using genomic PCR (Figure III_8 B). Real-time RT-PCR measurements revealed strongly reduced levels of the *MtGras4* 5' and virtually no remaining *MtGras4* 3' transcript region up- or downstream of the *Tnt1* insertion site, respectively (Figure III_8 C, D).

Among all tested GRAS TF and AM marker genes, the *MtGras4* knockout line showed a reduced expression of *MtGras7*, both on the transcript level (to 22 %, Figure III_8 E) and the activity of the promoter (Figure III_8 I), whereas transcription of all other AM-related GRAS TF genes was unchanged. On the phenotypical level, both the mycorrhization rate and the arbuscule size distribution are unchanged in *MtGras4* knockout mutants (Figure III_8 F, G). Complementation of the *M. truncatula* R108-based *Tnt1* line with the *M. truncatula* A17 *MtGras4* gene led to a restoration of *MtGras7* expression (Figure III_8 H), confirming that *MtGras7*, being strongly upregulated during later stages of mycorrhization (Figure III_2), is a direct or indirect target of MtGras4. Since *MtGras4* expression was independent of MtRam1 and MtPt4, we conclude that the MtGras4/MtGras7 regulatory module operates parallel to the formation of highly branched, functional arbuscules.

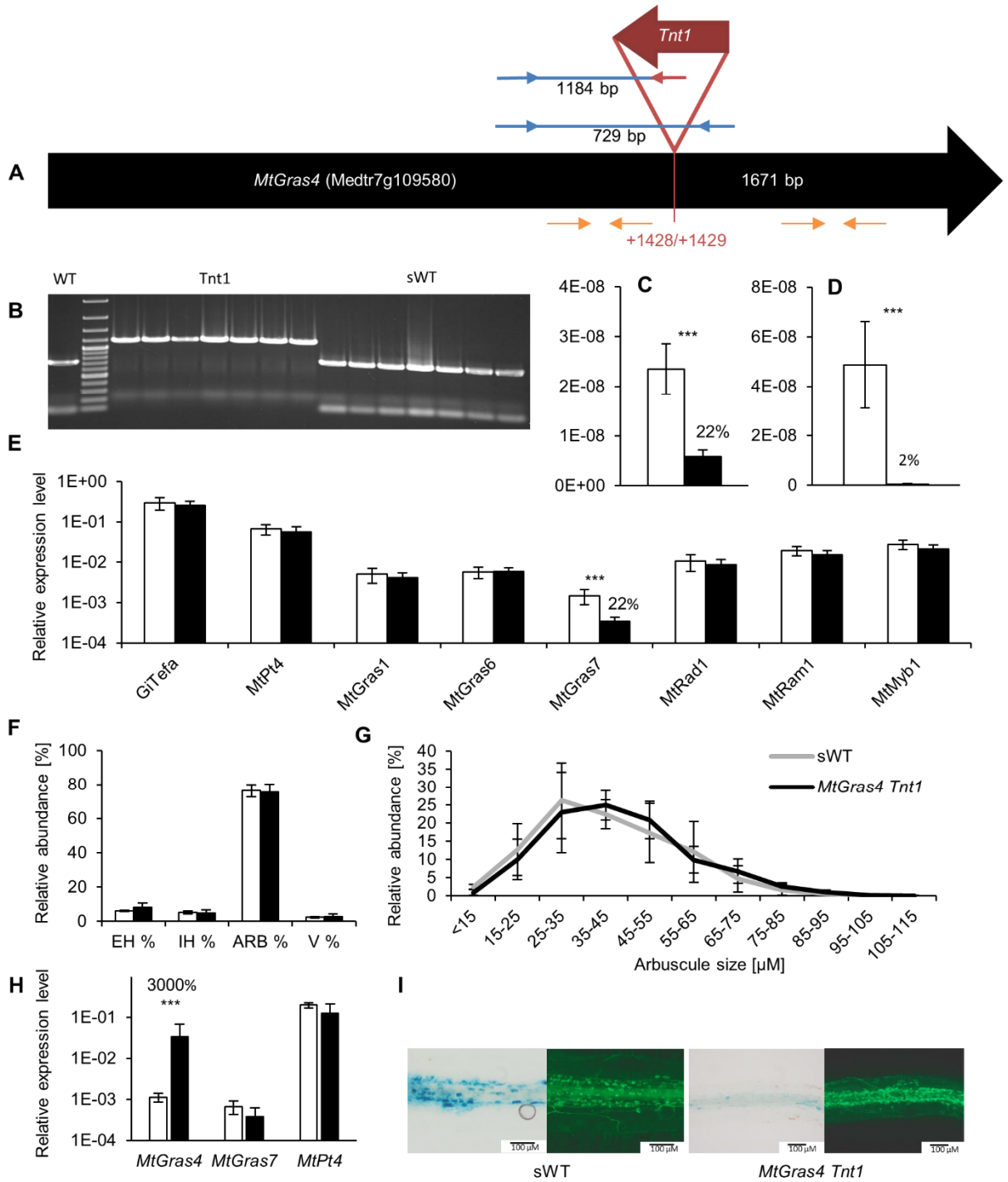

Figure III_8: Molecular and phenotypical analysis of the *MtGras4 Tnt1* insertion-carrying line NF4813.
A Schematic illustration of the *Tnt1* insertion site (red), the primer binding sites for genomic PCR-amplification (blue and red arrows), and the position of real-time RT-PCR primers (orange) in the coding region of *MtGras4* (black). **B** Leaf disc multiplex PCR-based identification of homozygous, *MtGras4 Tnt1* insertion-carrying NF4813 (*Tnt1*), *MtGras4*-segregating NF4813 wild type (sWT), and R108 wild type (WT) plants. A GeneRuler™ 50bp DNA ladder shown as standard. **C** and **D** Relative amount of *MtGras4* transcript regions located 5‘ (**C**) and 3‘ (**D**) of the *Tnt1* insertion. **E** Relative expression of selected marker genes in homozygous, *MtGras4 Tnt1* insertion-carrying plants (black) and segregating wild type plants (white). Transcript amounts are shown relative to *MtTefα* expression. Roots were harvested at 35 days post inoculation with *R. irregularis*. n=8 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to the control roots. **F** Quantification of fungal structures in homozygous, *MtGras4 Tnt1* insertion-carrying plants (black) and segregating wild type plants (white). Roots were harvested at 42 days after inoculation with *R. irregularis*. Root systems were pooled into four pools with four roots each. Standard deviations are indicated as error bars. EH =

External hyphae only; IH = Internal Hyphae only; Arb = arbuscules; V= Vesicles (no arbuscules). **G** Distribution of arbuscule sizes in mycorrhized homozygous, *MtGras4 Tnt1* insertion-carrying and segregating wild type plants. Sizes were measured from 1696 arbuscules in *MtGras4 Tnt1* insertion-carrying plants (black) and 1207 arbuscules in segregating wild type plants (grey). Roots were harvested at 42 days post inoculation with *R. irregularis*. Root systems were pooled into four pools with four roots each. Standard deviations are indicated as error bars. **H** Quantification of *MtGras4*, *MtGras7*, and *MtPt4* in homozygous, *MtGras4 Tnt1* insertion-carrying plants complemented with a native -1206/+2086 genomic region of the *M. truncatula* A17 *MtGras4* gene (black) and empty vector control roots (white). N = 6 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to the control roots. **I** Comparison of pMtGras7-*gusA*int activity in mycorrhizal roots of homozygous, *MtGras4 Tnt1* insertion-carrying and the corresponding segregating wild type (sWT) plants. GUS stainings were performed for 8 hours. Alexa WGA Fluor 488 stainings visualize AM fungal colonization.

MtGras1 knockdown affects the expression of other AM-related GRAS TF genes

As an example for a GRAS TF gene dependent on MtRam1 and MtPt4, *MtGras1* was functionally studied in transgenic RNAi roots exhibiting an *MtGras1* knockdown. Since preliminary RT-PCR measurements revealed multiple effects of an *MtGras1* knockdown on AM-related gene expression, a broad transcriptomics approach was pursued.

A comparative genome-wide transcriptomics study of *R. irregularis*-mycorrhized RNAi:*MtGras1* and RNAi:*gusA*int control roots identified 1020 genes that were at least 2-fold ($p < 0.05$) downregulated in *MtGras1* knockdown roots, indicating the potential of MtGras1 for participating in the regulation of gene expression in AM (Supplemental Table S_4).

A selection of *MtGras* genes differentially expressed in the *MtGras1* knockdown roots is shown in Figure III_9.

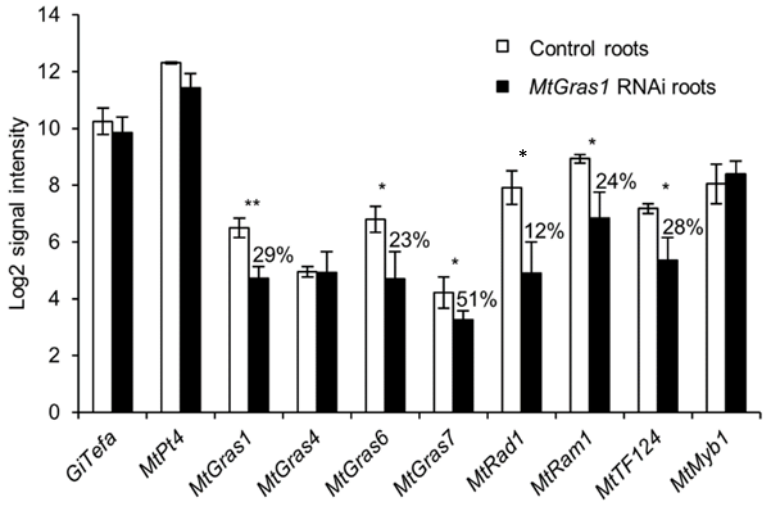

Figure III_9: Expression of *MtGras1* and selected AM marker genes in transgenic, mycorrhized RNAi:*MtGras1* and RNAi:*gusA*int control roots.

Roots were harvested at 35 days post inoculation with *R. irregularis*. *Medicago* Transcriptome Assay hybridizations were performed, using three biological replicates per experimental group. The standard error of the mean is shown. Percental values shown are based on the calculated linear signal intensities. Genes shown are as defined in the Figure II_3 legend.

In line with initial real-time RT-PCR measurements, the RNA interference construct led to a reduction of *MtGras1* expression to 29 % of the wild type level. Interestingly, the *MtGras1* knockdown also reduced transcription of the AM-related GRAS TF genes *MtGras6*, *MtGras7*, *MtRad1* (Xue *et al.*, 2015), *MtRam1* (Park *et al.*, 2015), and also *MtTF124* (Park *et al.*, 2015), while expression of the fungal marker gene *GiTefa* as well as the *M. truncatula MtGras4*, *MtPt4*, and *MtMyb1* genes were not significantly affected (Figure III_9).

The only slight, non-significantly (p=0.11; Figure III_9, Supplemental Table S_3) reduced expression of *MtPt4* in *MtGras1* knockdown roots indicates that the formation of active, phosphate-transporting arbuscules is not impaired by a reduced *MtGras1* expression. Similarly, transcription of *MtMyb1*, encoding a transcription factor controlling arbuscule degeneration (Floss *et al.*, 2017), is not reduced in *MtGras1* knockdown roots (Figure III_9), indicating that MtGras1 does not participate in the initiation of arbuscule degradation, e. g. by activating *MtMyb1*.

Since MtRam1 was shown to be required for *MtGras1* activation ((Park *et al.*, 2015); Figure III_3 and Figure III_4), it is intriguing that *MtRam1* expression is downregulated to 24 % in mycorrhized *MtGras1* knockdown roots (Figure III_9). This finding suggests that MtRam1 and MtGras1 are part of a regulatory feedback loop that maximizes *MtRam1* transcription and thus might enhance AM-correlated gene expression.

The transcription profile of *MtGras1* knockdown roots differs substantially from that of *ram1-1* mutants

Facing the large number of genes downregulated in *MtGras1* knockdown roots (Supplemental Table S_3), the question arose to what extent these patterns of transcriptional changes resemble those in an *MtRam1* knockout. To study this, genome-wide expression was recorded in wild type vs. *ram1-1* roots. This experiment revealed that the expression of 836 genes were at least 2-fold ($p < 0.05$) lower in *ram1-1* knockout roots, including several marker genes for arbuscule function (e. g. *MtPt4*, being transcribed at a 689-fold lower level, Supplemental Table S_4 and S_5). Although a limited set of 250 genes is at least 2-fold lower expressed ($p < 0.05$) in RNAi:*MtGras1* as well as in *ram1-1* roots (Figure III_10 A), the overwhelming pattern of gene expression regulation is characteristic of either the *MtGras1* knockdown or the *ram1-1* knockout.

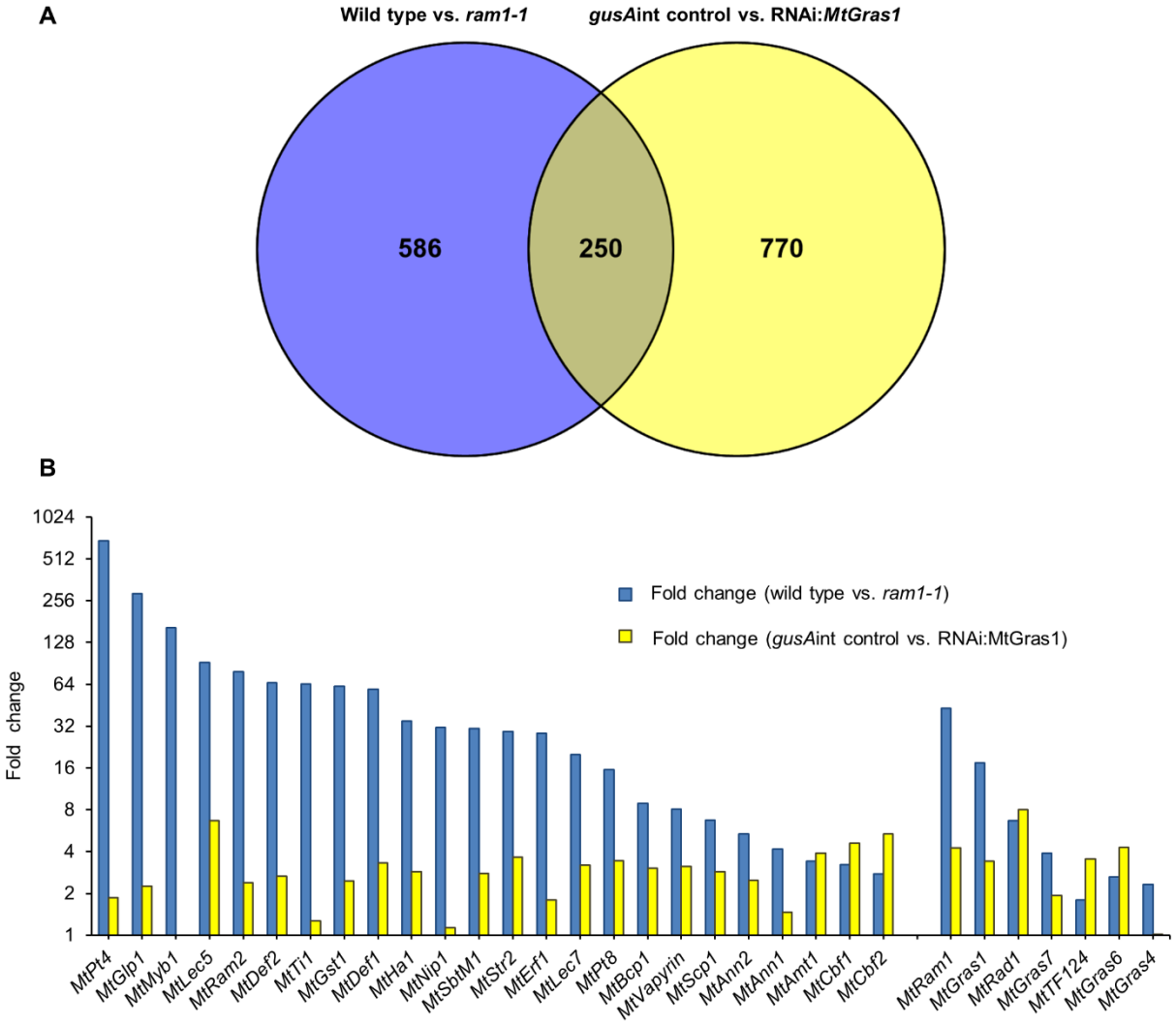

Figure III_10: Transcriptional response of *M. truncatula ram1-1* and *MtGras1* RNAi roots to colonization with *R. irregularis*.
Comparison of gene expression in *M. truncatula ram1-1* mutant as well as *MtGras1* RNAi roots in relation to control roots. Numbers indicate genes downregulated at least 2-fold ($p<0.05$) in the *ram1-1* mutant line or the *MtGras1* RNAi knockdown roots. **B** Comparative visualization of fold changes in *ram1-1* (blue) and *MtGras1* RNAi (yellow) roots in comparison to the corresponding control roots. A selection of 31 well-characterized AM-induced marker genes, downregulated at least 2-fold ($p<0.05$) in relation to control roots were compared in both mutants. The expression data and identifiers corresponding to the genes studied are listed in Supplemental Table S_5.

Specifically, when looking into the detailed pattern of regulation of 31 well-defined AM marker genes (Supplemental Table S_5, Figure III_10B), it is evident that most of these are either only (e. g. *MtMyb1*) or much stronger (e. g. *MtPt4*) downregulated in *ram1-1* mutants, indicating that the core gene expression program activated in arbuscule-containing cells is strongly affected in *ram1-1*, but not or only moderately in *MtGras1* knockdown roots. These findings suggest that although *MtGras1* expression depends on MtRam1 ((Park *et al.*, 2015); Figure III_3 and Figure III_4), MtGras1 is not simply a direct target of MtRam1 to activate downstream genes related to arbuscule formation and function.

MtGras1 overexpression does not activate other AM-related GRAS TF genes

The identification of massive transcriptional effects resulting from an *MtGras1* knockdown prompted us to investigate the effect of *MtGras1* overexpression in transgenic *M. truncatula* roots. In these experiments, the arbuscule-specific *MtPt4* and the general *ubiquitin3* promoters were used to drive *MtGras1* expression in mycorrhized and uninfected roots, respectively. Although a 7.4- and 1523-fold *MtGras1*-overexpression was achieved for mycorrhized and uninfected roots, respectively, leading to comparable amounts of *MtGras1* transcripts in mycorrhizal and uninfected roots (Figure III_11), no activation of other GRAS TF or AM marker genes such as *MtPt4* was detected (Figure III_4), suggesting that MtGras1 is not at the terminal position of a regulatory cascade or requires co-expressed interaction partners to activate transcription of target genes.

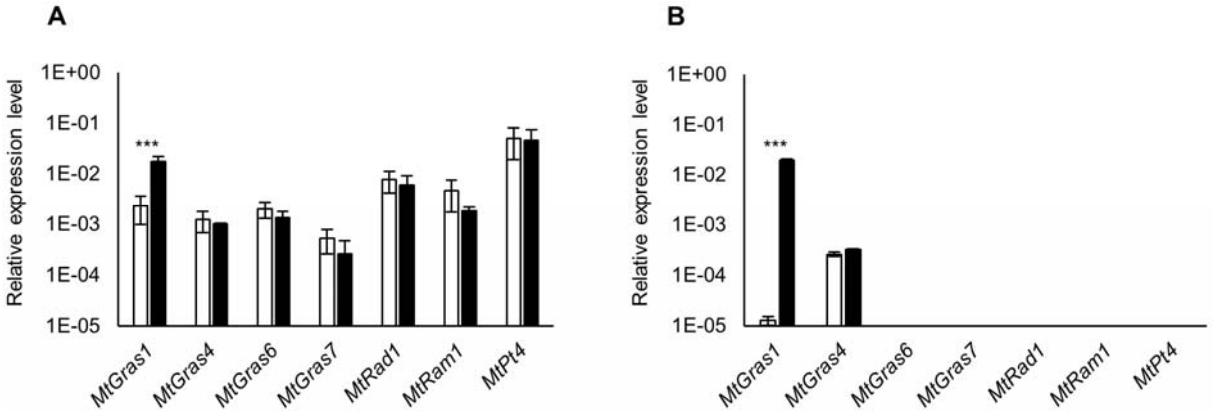

Figure III_11: Relative expression of *MtGras1* and selected AM marker genes in *MtGras1* overexpression roots.

A Mycorrhizal (35 dpi) pPt4:*MtGras1* (black) vs. pPt4:*gusA*int (white) roots. **B** Uninoculated pUbi3:*MtGras1* (black) vs. pUbi3:*gusA*int (white) roots. Transcript amounts are shown relative to *MtTefα*. Roots were harvested at 35 days post inoculation with *R. irregularis*. n=4 biological replicates, error bars represent standard deviations.

MtGras1 knockdown roots show a trend towards the development of smaller arbuscules

To address the question, whether an *MtGras1* knockdown influences the fungal colonization of roots or the maturation of arbuscules, phenotypical studies were performed in comparison to control roots.

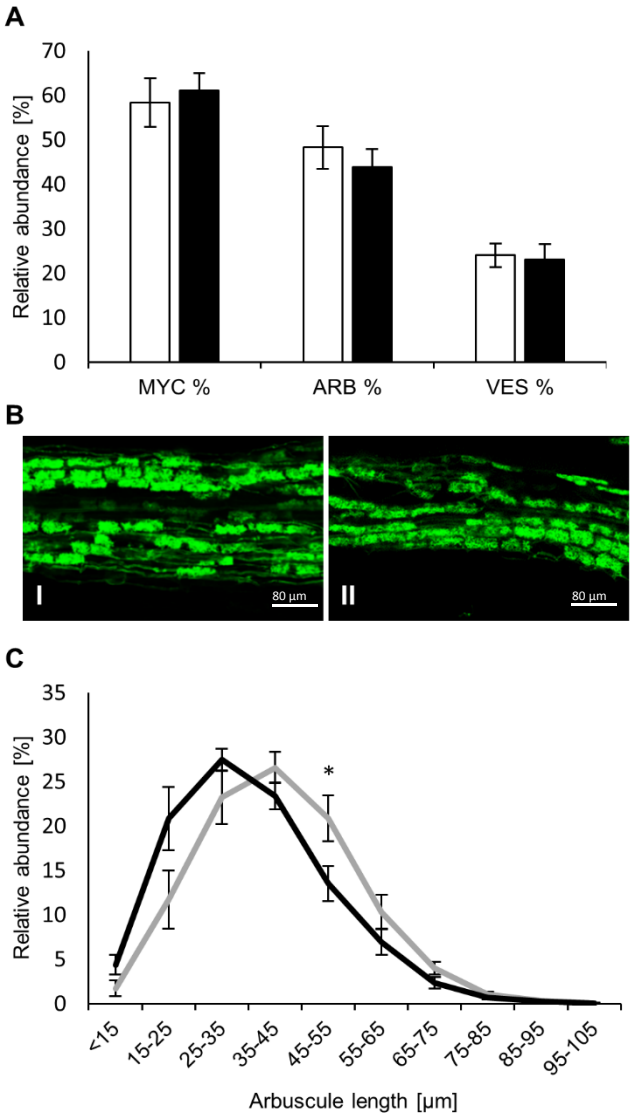

Figure III_9: Morphological analyses of *MtGras1* RNAi mycorrhizal roots in comparison to *gusA*int RNAi control roots.
A Quantification of fungal structures in *MtGras1* RNAi (black) and control roots (white). Roots were harvested at 35 days after inoculation with *R. irregularis*. Standard errors are indicated as error bars. MYC, colonized root fragments; ARB, arbuscules; VES, vesicles. **B** Alexa WGA Fluor 488 stained mycorrhizal *MtGras1* RNAi (I) and control roots (II) **C** Distribution of arbuscule sizes in *MtGras1* RNAi (black) and control roots (grey). Sizes were measured from 9 independent *MtGras1* RNAi and 9 independent control root systems (appr. 3000 arbuscules in total for each group). Roots were harvested at 35 days post inoculation with *R. irregularis*. Bars represent standard errors. Asterisk indicates significance with $p<0.05$.

While no changes in the mycorrhization rate or the frequencies of arbuscules and vesicles were observed (Figure III_12 A) and the arbuscules in *MtGras1* knockdown roots did not show symptoms of premature degeneration (Figure III_12 B), arbuscule length measurements of mycorrhizal RNAi:*MtGras1* in comparison to RNAi:*gusA*int control roots indicated a shift in the distribution of arbuscule sizes (Figure III_12 C). Specifically, *MtGras1* knockdown roots tend to contain a higher proportion of smaller and a lower proportion of large arbuscules, suggesting a delayed or less sustained arbuscule development. In line with the fact that the expression of most marker genes for arbuscule formation and function is not markedly affected in mycorrhized *MtGras1* knockdown roots (Figure III_10), this effect appears subtle and is probably part of a fine-tuning of the arbuscule life-cycle.

DISCUSSION

Colonization of roots by AM fungi ultimately leads to the formation of intracellular arbuscules, functioning as a nutrient exchange interface between plant cells and fungal hyphae. Arbuscule development requires a fundamental transcriptional reprogramming of root cortical cells (Hohnjec *et al.*, 2005; Küster *et al.*, 2007; Gomez *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2012; Hogekamp *et al.*, 2013; Guether *et al.*, 2009), being governed by a suite of AM-activated regulators, including several GRAS TFs (Guether *et al.*, 2009; Hogekamp *et al.*, 2011; Bucher *et al.*, 2014; Park *et al.*, 2015; Xue *et al.*, 2015; Pimprikar *et al.*, 2016; Rey *et al.*, 2017; Rich *et al.*, 2017).

We show here that the five AM-related GRAS TF genes *MtGras1*, *MtGras4*, *MtGras6*, *MtGras7*, and *MtRad1* (Rey *et al.*, 2017) can be classified based on their dependencies on MtRam1 (Park *et al.*, 2015), a key transcription factor controlling arbuscule branching, and MtPt4 (Harrison *et al.*, 2002; Javot *et al.*, 2007), the major AM-specific phosphate transporter. While *MtGras4*, *MtGras6*, and *MtRad1* are still expressed in the absence of *MtRam1* and *MtPt4*, *MtGras1* and *MtGras7* transcription is abolished in the *ram1-1* and *pt4-2* mutants (Figure III_3, Figure III_4, Figure III_5, and Figure III_6). In our growth conditions, *pt4-2* mutants occasionally developed not only prematurely decaying but also vital and fully developed arbuscules (Figure III_6 B: a-h). This phenomenon demonstrates the dependency of *MtGras1* and *MtGras7* transcription not on the genotype, but on the presence of fully developed arbuscules. Obviously, prematurely decaying arbuscules do not reach the phase of *MtGras1* and *MtGras7* expression, while fully developed arbuscules do. This indicates that with respect to transcriptional control, MtGras1 and MtGras7 are placed downstream of MtRam1 action and are thus related to rather late stages of arbuscule formation. In contrast, *MtGras4*, *MtGras6*, and *MtRad1* expression is not abolished in *ram1-1* and *pt4-2* mutants (Figure III_7), allowing to conclude that the encoded GRAS TFs are related to earlier developmental stages. These findings were included in a model, where the AM related GRAS TFs are positioned relative to the establishment of mature arbuscules (Figure III_13).

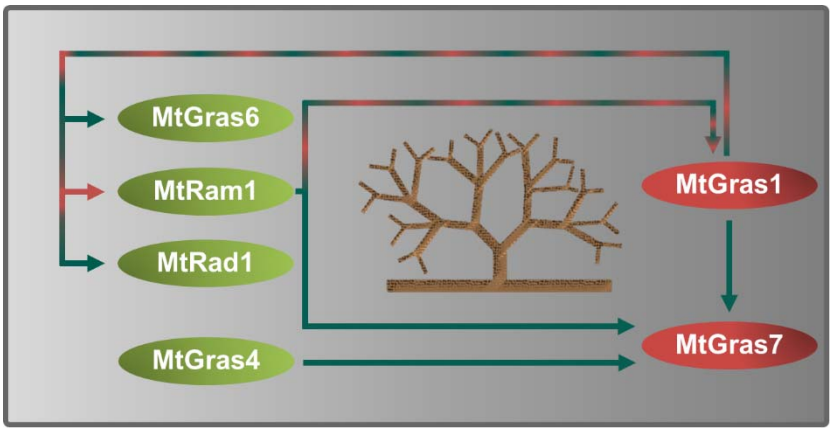

Figure III_13: Model of the regulatory circuit of AM-related GRAS TFs revolving arbuscule development. Components of the network are divided into MtRam1- and MtPt4-independet (MtGras4, MtGras6, and MtRad1) as well as -dependent (MtGras1, MtGras7) GRAS TFs. Whereas green arrows visualize transcriptional activation, the feedback regulation between MtRam1 and MtGras1 is indicated by red/green coloration.

To study the function of an MtRam1- and MtPt4-independent GRAS TF gene, an *MtGras4* insertion mutant in *M. truncatula* R108 was analyzed. Although the *MtGras4* knockout downregulates *MtGras7*, which in contrast to MtGras4 is MtRam1- and MtPt4-dependent, it does not affect fungal colonization or the arbuscule size distribution (Figure III_8). The *MtGras4* knockout, inducing a reduced *MtGras7* transcription in the *M. truncatula* R108 *MtGras4* mutants, was successfully complemented by expressing an *M. truncatula* A17 *MtGras4* gene, indicating a conserved regulation in *M. truncatula* R108 and Jemalong A17 regarding these *MtGras* genes. Interestingly, analyses of *MtGras7* and *MtGras4* upregulation during the AM time course revealed different patterns (Figure III_2). While *MtGras7* appeared to be expressed in an arbuscule-correlated manner that follows *MtPt4* activation, *MtGras4* expression was already detectable from the onset of fungal colonization. In line with the MtRam1- and MtPt4-dependency of *MtGras7* activation (Figure III_3 and Figure III_4), this further confirms that MtGras7 functions in later stages of arbuscule development. In this respect, the dependency of *MtGras7* on the MtRam1- and MtPt4-independent GRAS TF MtGras4 is even more remarkable, since it demonstrated a connection between early, MtRam1-independent (*MtGras4*) and later, MtRam1-dependent stages (*MtGras7*) of arbuscule formation. The somewhat outstanding position of the MtGras4/MtGras7 module within the GRAS TF network is also reflected on the sequence level, where MtGras4 and MtGras7 share a common clade, being isolated from the rest of the GRAS TFs investigated (Figure III_1).

In spite of its dependency on MtRam1 and MtPt4 (Figure III_7), MtGras1 was also found to regulate components placed upstream of arbuscule development, namely *MtGras6*, *MtRad1*, and remarkably also *MtRam1* (Figure III_9). In addition, the AM-related GRAS TF gene and *MtRam1*-homologue *MtTf124* (Park *et al.*, 2015) appears downregulated in *MtGras1*

knockdown mutants. Due to the fact that MtGras1 regulates *MtRam1* expression (Figure III_9), while MtRam1 on the other hand is required for *MtGras1* activation (Figure III_3 and III_4), we propose the existence of a regulatory feedback loop, where MtGras1 enhances *MtRam1* transcription, thus stimulating its own activation as well as the expression of MtRam1-regulated target genes that ultimately lead to the establishment of a functional, highly branched arbuscule (Figure III_13). Since it was demonstrated that MtRam1 is not able to directly stimulate *MtGras1* expression (Park *et al.*, 2015), the activation of *MtGras1* has to be dependent on additional, MtRam1-dependent regulators.

Interestingly, *MtGras1* expression was downregulated in *pt4-2* mutants colonized by *Rhizophagus irregularis* (Figure III_5, Figure III_6) and in *pt4-1* mutants mycorrhized with *Gigaspora gigantea* (Floss *et al.*, 2017), indicating that the encoded GRAS TF acts downstream of the formation of functional arbuscules. It is thus tempting to hypothesize that MtGras1 acts as a checkpoint at a certain point of the rather later stages of the arbuscule life cycle, where the initial signaling for arbuscule development and branching via MtRam1 is completed, and the morphological expansion of the functional, phosphate-transporting fungal interface needs to be accelerated or sustained. The slight shift towards the formation of smaller arbuscules observed in *MtGras1* RNAi roots (Figure III_12) might thus be caused by the lack of MtGras1-activated *MtRam1* transcription (Figure III_9), which would lead to a slower or less sustained, MtRam1-controlled, arbuscule branching. The effect on the arbuscule size distribution is nevertheless small and possibly also suffers from the non-synchronized mycorrhization process, which masks subtle differences in arbuscules sizes.

On the protein level, MtGras1 was shown to interact with MtRam1 in Yeast Two-Hybrid assays (Park *et al.*, 2015), which indicates a similar function of either transcription factor. However, we were able to show that an RNAi-mediated knockdown of *MtGras1* leads to a different molecular phenotype than a knockout of *MtRam1*. Although there are overlaps in the genes regulated by MtGras1 and MtRam1, *ram1-1* mutants show a much stronger downregulation of AM-induced genes characteristic of the presence of functional arbuscules and a strong effect on arbuscule branching (Park *et al.*, 2015), which is not the case for *MtGras1* RNAi roots (Figure III_10). The clearly different transcription pattern of mycorrhized *MtGras1* RNAi roots and mycorrhized *ram1-1* mutants is thus in line with a modifying function of MtGras1 during the establishment of a functional arbuscule interface. An additional hint that MtGras1 alone is unable to activate AM-related genes derives from our overexpression experiments, where an upregulated *MtGras1* transcription had no effect on selected target genes, probably since MtGras1 requires an interaction partner such as MtRam1 (Park *et al.*, 2015).

In addition to the more upstream components regulating arbuscule formation, MtGras1 also regulates *MtGras7* (Figure III_9), which is also MtRam1- and MtPt4-dependent and is thus located at a similar position relative to the formation of mature arbuscules. Incidentally, the downregulation of *MtGras7* in *MtGras1* knockdown roots might explain why *MtGras7* expression is reduced but still detectable in the *MtGras4* insertion mutant (that still expresses *MtGras1*, Figure III_8).

Finally, the effect of MtGras1 on *MtGras7* transcription implies that *MtGras7* can be activated by both MtGras4 and MtGras1 in an independent manner, suggesting that MtRam1-/MtPt4-dependent (MtGras1 and its targets) and -independent regulatory modules (MtGras4 and its target *MtGras7*) are connected via MtGras7.

CONCLUSION

Relying on gene expression and histological studies in symbiotic mutants defective in arbuscule branching or in the formation of phosphate-transporting arbuscules as well as on functional analyses in transgenic knockdown roots or knockout lines, we provide evidence that the network of AM-activated *M. truncatula* GRAS TFs consists of interconnected modules, including an MtRam1-MtGras1 regulatory feedback loop. *MtGras1* knockdown roots displayed normal colonization by AM fungi, but a trend towards the formation of smaller arbuscules was apparent. Although mutants in the AM-specific GRAS TFs RAM1 and RAD1 display more severe AM-related phenotypes in *M. truncatula* and *L. japonicus* (Park *et al.*, 2015; Xue *et al.*, 2015; Pimprikar *et al.*, 2016; Rey *et al.*, 2017), our investigation on additional AM-activated *MtGras* genes provides evidence that a regulatory circuit of multiple GRAS TFs, showing differential dependencies on arbuscule branching and the formation of phosphate-transporting arbuscules, governs and sustains arbuscule development. We propose that this regulatory circuit allows a flexible response of the symbiotic interface towards the external (e. g. nutrient availability; (Javot *et al.*, 2007; Javot *et al.*, 2011) and internal (e. g. plant hormones; (Floss *et al.*, 2013; Foo *et al.*, 2013)) stimuli that together influence and adapt the arbuscule life cycle under changing conditions.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article and its supplementary information files. In addition, the GeneChip *Medicago* Transcriptome Assay data (https://www.ncbi.nlm.nih.gov/geo/, accession number GSE108867) were stored in the Gene Expression Omnibus.

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GENERAL DISCUSSION

In the last years, several attempts have been made to unravel the transcriptional program induced by and being involved in the formation of AM associations. Root material colonized by AMF was compared with non-infected roots to reveal transcriptional regulation. Relying on transcript sequencing and hybridization-based techniques, comprehensive transcriptional data have been gathered, delivering insights into the transcriptional reprogramming in mycorrhizal roots and suggesting candidate genes for functional investigations. However, an inherent drawback is hidden in the fact that previous genome-wide transcriptional studies are based on mycorrhizal material of one single time point of mycorrhization. Thus, all transcriptomic profiles that have been gathered provide only snapshots of the transcriptional program at one stage of AM development. To fully understand the transcriptional basis of AM formation, it is necessary to monitor the chronological changes during the mycorrhization process over time, to assess the impact of different genes at different stages of the colonization process. To achieve this goal, an AM time course, coupled with transcriptomic analyses via hybridization of *Medicago* GeneChips in the most advanced version, formed the basis of this work.

An expression time course reveals insights into the chronological transcriptional reprogramming in roots colonized by AM fungi

Proper colonization of host roots used for time course studies was confirmed by histological staining of fungal structures and by monitoring the process of AM marker gene expression (Figure I_1; Figure I_2; Table I_2). These marker genes include the fungal housekeeping gene *GiTefalpha*, being an indicator for the presence of metabolically active fungal biomass, as well as *MtPt4*, which encodes an AM-specific phosphate transporter, representing the formation of functional nutrient exchange interfaces and thus a functional AM symbiosis. Overall fungal growth turned out to show an exponential increase, while arbuscule interfaces were built even faster, supporting the picture of a continuous and efficient AMF colonization as well as a proper development of the symbiosis in the tissues used for transcriptome profiling.

GeneChip hybridizations at different time points of AMF colonization revealed a substantial number of *M. truncatula* genes being upregulated at least 2-fold between the two earliest time points (0 and 7 dpi) and the latest time point (42 dpi) (Figure I_3; Supplemental Table S_1). Eliminating root development-induced genes using reference GeneChip hybridizations of 28 dpi mycorrhizal vs. corresponding non-mycorrhizal roots revealed the time-related activation of 637 *M. truncatula* genes that show a significant increase during the course of AMF colonization. Upregulated genes included typical AM markers (Table I_1) and were categorized

based on their cellular function (Figure I_4). Many of these genes were classified as involved in processes related to regulation, transport, protein degradation, biotic stress, development, protein modification, and hormone signaling.

It was shown that arbuscule formation requires a massive reprogramming of cellular functions, especially in redirecting the trafficking of synthesized membrane components (Pumplin *et al.*, 2012) and the regulation of exocytotic pathways (Genre *et al*., 2012; Ivanov *et al.*, 2012; Zhang *et al.*, 2015). Due to these rearrangements, virtually all plasma membrane targeting proteins that are expressed during the formation of an arbuscule are redirected to the PAM (Pumplin *et al.*, 2012), reflecting the complete reprogramming of the cell machinery focusing on arbuscule development. It is thus no surprise, and in line with previous studies (Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013), that many genes involved in transport and regulation processes are upregulated in the course of AM development.

A complex network of defense reactions including hormone homeostasis and signaling is part of the cellular program that is responsible for both arbuscule formation and their subsequent degradation (Allen *et al.*, 1989; Gollotte *et al.*, 1993; Lambais, 2000; Vierheilig *et al.*, 2008; Breuillin *et al.*, 2010). These processes are reflected by the activation of genes associated with biotic stress and hormonal functions, e. g. jasmonate, salicylic acid, auxin, and gibberellic acid synthesis or signaling, together controlling maintenance or suppression of mycorrhization (Meixner *et al*., 2005; Foo *et al*., 2013).

The high total number of genes upregulated over time during the AM symbiosis spotlights the importance of genes associated with the regulation of transcription. Facing the massive reprogramming necessary for arbuscule formation, the transcriptional regulators identified to be upregulated in the proceeding mycorrhization deserve particular attention. In total, 40 such genes related to the regulation of transcription were identified (Figure I_4). Further classification identified two major groups of the encoded transcriptional regulators, namely 12 AP2/ERF and 12 GRAS TFs. Focusing on the GRAS TF genes, some known regulators of AM signaling were found, including *MtNsp1* (Liu *et al.*, 2011), *MtMIG1* (Heck *et al.*, 2016), *MtRad1* (Xue *et al.*, 2015; Rey *et al.*, 2017), and *MtRam1* (Gobbato *et al.*, 2012). In addition, some GRAS TF genes that were reported to be induced in the AM symbiosis in previous studies using different transcriptomic platforms (Hogekamp *et al.*, 2011; Hogekamp and Küster, 2013), were found to be upregulated over time as well (Table I_3).

Together, these findings suggest that the AM expression time course is a powerful tool to gain insights into the impact of novel AM-related GRAS TF genes in general and in distinct stages of AMF colonization in particular.

Expression patterns of GRAS TF genes during the AM time course reveal regulatory dependencies

A clustering of *MtGras* gene expression according to their chronological transcription patterns revealed two clusters (Figure I_7). The first cluster contains *MtNsp1*, *MtMIG1*, *MtMIG2*, and *MtMIG3*; the second cluster *MtRam1* and *MtRad1* as well as the novel GRAS TF candidate genes *MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, and *MtGras7*. The two clusters differ regarding the median signal intensity of the included genes as well as the inclination of gene expression during AMF colonization. While genes from the first cluster start at a higher transcription level in comparison to the second cluster, their inclination is much weaker. The strong inclination of the *MtGras* genes from the second cluster can be considered as an indicator for the gene's regulatory impact during the process of AMF root colonization and the development of AM symbioses. Correlations between fungal colonization and gene expression have previously been described for *MtRam1* (Park *et al.*, 2015) and *MtRad1* (Hartmann, 2013). Furthermore, these genes have been shown to be essential regulators in AM developmental signaling (Gobbato *et al*., 2012; Park *et al*., 2015; Xue *et al.*, 2015), and their central role might also be reflected by the fact that these two genes show the strongest induction over the time course of AMF colonization.

Considering the important role of MtRam1 (Gobbato *et al*., 2012; Park *et al*., 2015) and MtRad1 (Xue *et al.*, 2015), it is tempting to speculate that a gene's impact on symbiosis-related signaling is reflected by either its expression level or the inclination of gene expression over time. Functional analyses of the novel GRAS TF genes *MtGras1*, *MtGras4*, *MtGras6*, and *MtGras7* suggest a role of at least MtGras1 in perceiving and accelerating AM induced transcription (Chapter III). In the AMF time course, this position might also be reflected by the chronological increase of *MtGras1* expression (Figure I_7 C). Interestingly, MtGras1 appears to control *MtGras7* together with MtGras4 (Figure III_13), since an *MtGras7* activation was shown to be reduced in *MtGras1* RNAi (Figure III_9) as well as in MtGras4 *Tnt1* lines (Figure III_8). Furthermore, Y2H experiments (see below) indicated a direct interaction between MtGras1 and MtGras4 (Figure II_9), suggesting a common function that might explain the dependency of *MtGras7* expression on both MtGras1 and MtGras4. This co-dependency is also reflected in

the AMF time course, since *MtGras7* activation appears to follow *MtGras1* and *MtGras4* upregulation (Figure I_7 C).

Based on these findings, *MtGras7* is thought to be involved in later stages of arbuscule development. This conclusion is further supported by an arbuscule-specific promoter activity being dependent on the establishment of a functional plant-fungal interface (Figure III_4 and Figure III_6) and by the delayed increase of *MtGras7* expression in the AM time course (Figure I_7 C).

Studies in mutant lines allow to position MtGras TFs within the regulatory network controlling arbuscule formation

To study the dependency of AM-related *MtGras* gene expression on MtRam1, a GRAS TF required for arbuscule branching (Park *et al.*, 2015), as well as on the presence of functional phosphate-transporting arbuscules, gene expression analyses were carried out in *R. irregularis*-mycorrhized roots of *ram1-1* and *pt4-2* mutants, in comparison to wild type roots (Chapter III). Whereas the expression analyses revealed a dependency of *MtGras1* and *MtGras7* transcription on both MtRam1 and MtPt4, gene expression of *MtGras4*, *MtGras6*, and *MtRad1* was less affected (Figure III_3 – III_6). An overview of the arbuscule life cycle in WT as well as in *ram1-1* and *pt4-2* mutants, integrating gene expressions observed in functional studies in these lines (Figure III_7) is presented in Figure 6. To understand the impact of these findings, the mechanisms underlying the observed phenotypes in *ram1-1* and *pt4-2* mutants have to be further addressed.

In a wild type situation, arbuscule development follows a characteristic pattern that includes distinct stages, each exhibiting its own set of transcriptional regulation and cellular processes (Gutjahr and Parniske, 2013). Upon the initial invagination of fungal hypha into the plant cell membrane, building the so-called trunk, first hyphal spreading occurs that results in the formation of an initially branched hypha resembling a birdsfoot (Gutjahr and Parniske 2013). These first few branches are the basis for a subsequent fine branching of the intracellular hypha. It was shown that MtRam1 plays a crucial role in the transition of the birdsfoot-stage to branching arbuscules by regulating a set of essential genes necessary in this stage of arbuscule development. One of these is *MtRam2*, encoding a glycerol-3-phosphate acyl transferase required for the synthesis of C16 fatty acids, being necessary for lipid supply to the periarbuscular membrane (PAM) (Gobbato *et al.*, 2012; Bravo *et al.*, 2017).

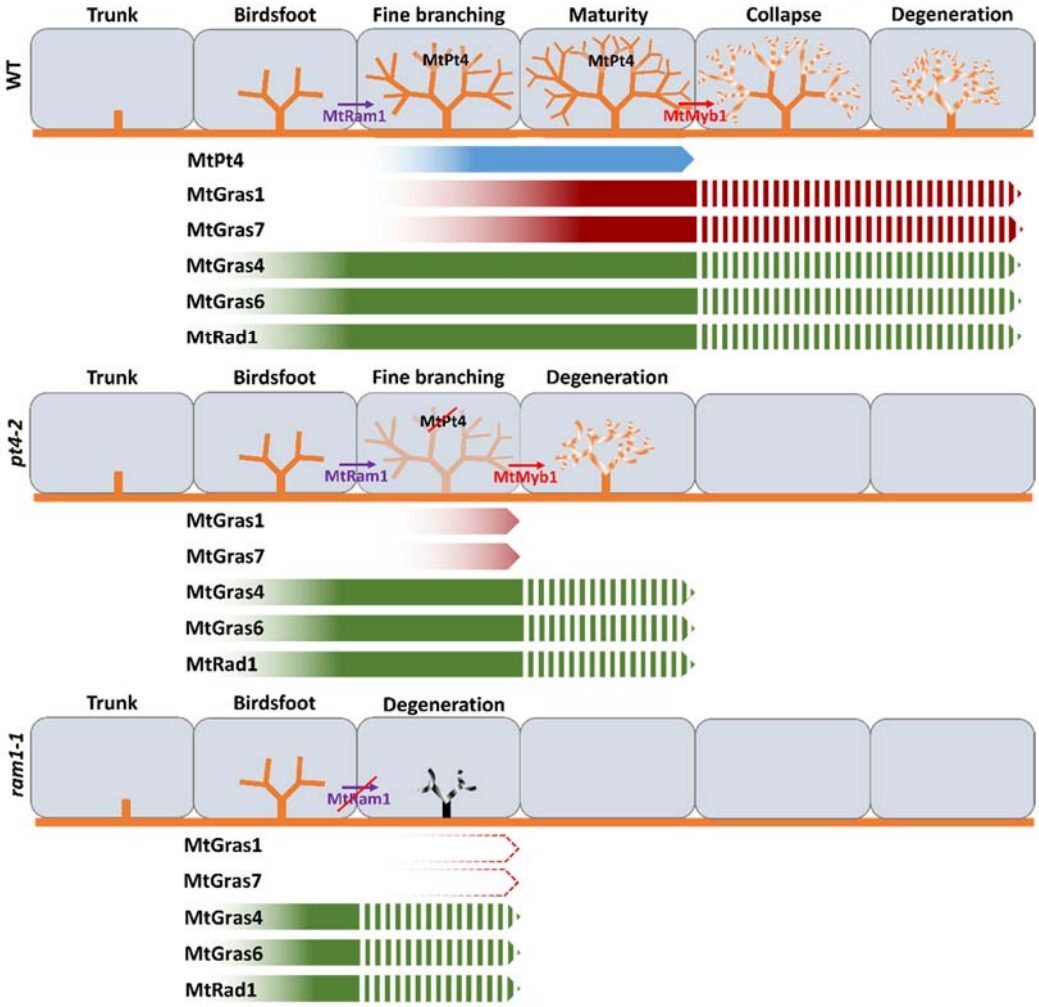

Figure 6: Visualization of AM-related *MtGras* gene expression during different stages of the arbuscule life span in WT and prematurely degrading arbuscules of mutant lines.

Gene expression is modeled via integration of promoter-*gusA*int studies as well as transcriptional analyses of knockout mutants (Figures III_3 - III_7). White stripes indicate elusive expression in later stages. Red arrows indicate gene expression dependent on MtRam1 and MtPt4 (*MtGras1* and *MtGras7*), green arrows indicate genes expression independent of MtRam1 and MtPt4 (*MtGras4*, *MtRad1*, *MtGras6*). The temporally variable branching stage in *pt4-2* mutants is indicated by a shaded coloration of arbuscule branches. Absent expression in *ram1-1* mutants is indicated by dotted lines. Altered arbuscule degeneration in *ram1-1* mutants is represented by a grey arbuscule. The MtRam1-dependent transition from birdsfoot to branching arbuscules is presented in lilac. Induction of degeneration by MtMyb1 is presented in light red. Positioning of MtPt4, MtMyb1, and MtRam1 is based on Javot *et al*. (2007), Floss *et al*. (2013), and Park *et al*. (2015), respectively.

Another important resident of the PAM is the phosphate transporter PT4. The corresponding *MtPt4* gene is directly induced by RAM1 as well and is expressed during the phase of PAM-establishment. It is thus considered to be a marker for the functionality of the plant-fungus nutrient exchange interface (Harrison *et al.*, 2002; Javot *et al.*, 2007; Rich *et al.*, 2017). In *pt4-2*

mutants, arbuscules are no longer functional with respect to phosphate-transport, due to the absence of MtPt4 (Figure III_5) at the plant-fungal interface.

Arbuscules are transient structures, being active just a couple of days before a regular degeneration occurs (Alexander *et al.*, 1988; Harrison *et al.*, 2005). A key factor of native arbuscule degeneration is the MYB TF MtMyb1 that was shown to induce the expression of degradation-related components, e. g. hydrolases, cysteine proteases, and chitinases (Floss *et al.*, 2017), together mediating the regular turnover of arbuscules. In *pt4-2* mutants, exhibiting a non-beneficial nutritional situation, perceived by a missing signal transmitted by the phosphate ion itself (Yang and Paszkowski, 2011), MtMyb1 was shown to be also responsible for the targeted, non-regular arbuscule degeneration by triggering the so-called premature arbuscule degeneration (PAD) (Javot *et al.*, 2007; Floss *et al.*, 2017). Interestingly, premature degeneration in *pt4-2* mutants can be complemented by N-starvation, indicating a regulatory network that integrates various other nutritional conditions and also assesses the cost-benefit ratio of the AM symbiosis (Javot *et al.*, 2011).

Experiments performed here using *pt4-2* mutants (Chapter III) were influenced by an instable PAD phenotype that in our conditions was absent, from some colonized root areas, leading to phenotypically regular arbuscules (Figure III_6), although no severe N-starvation was induced. While no distinct reason for this phenomenon could be spotted, the *pt4-2* phenotype seemed to be modulated by general growth conditions, probably including the light intensity and spectrum, the temperature as well as the relative air humidity. Together, these factors might lead to abiotic signals modulating the assessment of the cost-benefit ratio mentioned above and thus might influence the process leading to the Myb1-induced PAD phenotype. While regular arbuscule degeneration in the WT as well as premature arbuscule degeneration in *pt4-2* is induced by MtMyb1, the degeneration of arrested arbuscules in *ram1-1* must be controlled by alternative mechanisms, since MtMyb1 is not expressed in *ram1-1* mutants (Floss *et al.*, 2017).

While it is assumed that arbuscule degeneration in *pt4-2* mutant roots is a cell-specific process, inducing degeneration of each non-functional arbuscule separately (Javot *et al.*, 2007; Javot *et al.*, 2011), no clear evidence is provided for the timespan of arbuscules in the fine-branching stage and whether this stage is systemically shortened. Kobae and Harta (2010) could show that

under certain conditions, arbuscule degeneration is systemically synchronized, accelerating the turnover of younger arbuscules and leading to the degradation of arbuscules of all ages within a single infection unit at the same time. It is thus possible that the stage of fine-branching might be temporally variable in *pt4-2* mutants, due to a systemic induction and acceleration of

arbuscule degeneration. Expression of *MtGras1* and *MtGras4* appeared to react to the presence or absence of the *pt4-2* specific PAD phenotype, thus being connected to the stage of fine branching in mature arbuscules as well. Whether the expression of *MtGras1* and *MtGras7* observed in some parts of *pt4-2* roots (Figure 6; Figure III_6) is only caused by a compensation of the PAD phenotype or if MtGras1 and MtGras7 are partially active in the fine branching stage that in some cases might be longer or shorter, cannot be finally proven at this point.

MtGras4, *MtGras6*, and *MtRad1* expression was thus dependent on the PAD phenotype, indicating an earlier induction of transcription (Figure 6; Figure III_6). Whether or not these genes are also expressed in cells containing collapsing or degenerating arbuscules, could not be assessed.

In *ram1-1* mutants, MtRam1 and also MtRam1-targets such as MtRam2 and MtPt4 are missing. Consequently, the development of arbuscule formation is arrested in the birdsfoot stage. In mycorrhizal *ram1-1* roots, *MtGras1* and *MtGras7* expression were hardly detectable, while again *MtGras4*, *MtGras6*, and *MtRad1* were less or not at all affected.

Yeast Two-Hybrid studies reveal possible interactions between MtGras1, MtGras4, and MtRad1

To study protein-protein interactions, coding regions of the candidate GRAS TF genes *MtGras1*, *MtGras4*, *MtGras5*, *MtGras6*, *MtGras7*, *MtRam1*, *MtRad1*, and others, were subcloned into Y2H vectors containing a GAL4 DNA-binding domain. Many of the obtained constructs exhibited a strong autoactivation, meaning autonomous induction of reporter genes that should indicate protein-protein interactions in *S. cerevisiae*. Whether this autoactivation was induced by a direct induction of the corresponding reporter gene expression by the GRAS TF itself could not be finally proven here. However, recent findings that DNA binding properties are not a characteristic feature of many GRAS TFs (Hirano *et al.*, 2017; Fukazawa *et al.*, 2014; Yoshida *et al.*, 2014) support the hypothesis that autoactivation of GRAS baits in yeast occurs due to similarities of these TFs to the activation domain of GAL4 (see Chapter II). Furthermore, several PPIs that were described in the literature could not be confirmed here, e. g. MtNsp1-MtNsp2 (Hirsch *et al.*, 2011) or MtGras1-MtRam1 (Park *et al.*, 2015). Major differences to these studies were the choice of Y2H vectors, promoter-reporter combinations and especially the stringencies in filtering for false-positives. It is likely that the stringencies that were used in the Y2H system used in this study might ignore some weak PPIs (Caufield *et al.*, 2012). Due to these limitations, protein-protein interaction studies in the literature are generally aiming at the use of different approaches like BiFC and CoIP (a review about all

known interactions is given in Pimprikar *et al.*, 2018). Although the initial idea of identifying PPI networks of GRAS TFs by Y2H has been implemented in other studies (Park *et al.*, 2015; Xue *et al.*, 2015), the choice of the GRAS TFs tested appears to be critical.
Nevertheless, the identification of MtGras1-MtGras4, MtGras4-MtRad1 as well as MtGras1-MtGras1 interaction by Y2H approaches extends our knowledge on the interaction of several AM-related GRAS TFs.

A model for the regulatory circuit of transcription factors controlling arbuscule development

Integrating all transcriptional, functional and interaction studies on the AM-related GRAS TF genes *MtGras1*, *MtGras4*, *MtGras6*, *MtGras7*, *MtRad1*, and *MtRam1* obtained in this thesis, a model was proposed, depicturing the network of GRAS TFs revolving arbuscule development (Figure III_13).
In Figure 7, this model is extended by results from protein-protein interaction studies that revealed possible interactions of MtGras4 with MtGras1 and MtRad1, respectively. These interactions have not been reported in the literature yet. Interconnections between MtGras1 and MtGras4 are of special interest, since functional analyses showed a transcriptional dependency of *MtGras7* on both MtGras1 and MtGras4 (Figure III_8; Figure III_9). Integrating these results, the interaction between MtGras1 and MtGras4 strengthens the picture of two regulatory modules, acting earlier or later in arbuscule formation, being linked via direct or indirect expression regulation and GRAS TF interactions (Figure II_9). Together, these findings suggest a regulation of *MtGras7* expression by a TF complex including MtGras1 and MtGras4. While *MtGras7* activity was already found to be influenced by MtGras1 and MtGras4 in transcriptomics studies (Figures III_5 and III_7), this Y2H interaction study delivered first hints for a new TF complex, connecting different termini of the regulatory network of AM-related GRAS TFs.

In addition to the MtGras4-MtGras1 interaction, the binding of MtGras4 to MtRad1 might crosslink the MtGras4-MtGras1 complex to the central part of the signaling network controlling arbuscule development (Figure 7).

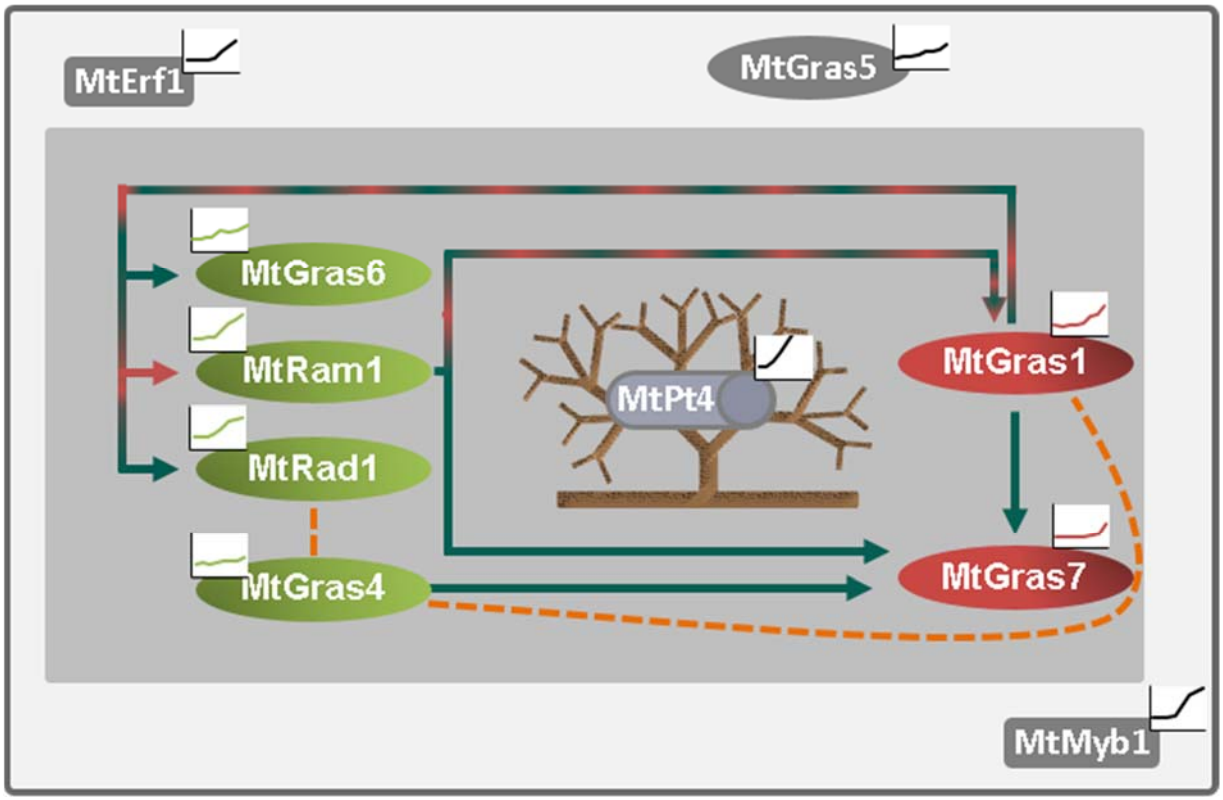

Figure 7: Model of the regulatory circuit of AM-related TFs revolving arbuscule development.
Based on transcriptomic studies (Figure III_5 and III_7), components of the network are divided into MtRam1- and MtPt4-independet (green: MtGras4, MtGras6, and MtRad1) as well as -dependent (red: MtGras1, MtGras7) GRAS TFs acting early or late, respectively. The MtGras network revolves the formation of a functional arbuscule that requires the phosphate transporter MtPt4. Whereas green arrows visualize transcriptional activation, the feedback regulation between MtRam1 and MtGras1 is indicated by red/green coloration. Interactions found in Y2H approaches are shown as dotted orange lines. Expression profiles over time between 0 and 42 dpi of inoculation with *R. irregularis* are indicated for each component (see Chapter I). Additional AM-related transcriptional regulators are roughly placed in vicinity of the MtGras network, based on their presumed stage of action (MtErf1 [Devers *et al*., 2013] and MtMyb1 [Floss *et al*., 2017]) or expression profiles in *ram1-1* mutants (MtGras5; see Supplemental Table S_4).

If the MtGras1-MtGras4 interaction can be confirmed in further experiments, the conclusion would be that the prerequisites for the expression of *MtGras7* in the later arbuscule stages are already set in earlier stages of arbuscule development by activating *MtGras4*. Strong induction of *MtGras7* would then be further triggered by MtGras1, which is associated with later stages (Figure 7).

Based on the model presented in Figure 7, the MtGras7 function can be predicted to be located in the later stages of arbuscule development, possibly accumulating here due to an MtGras1 and MtGras4 controlled expression. Revealing the function of MtGras7 in further approaches might help to understand the meaning of this regulatory mechanism, possibly by finding additional pathways leading to the last stage in the natural life span of arbuscules, namely their degradation during regular turnover (Gutjahr and Parniske, 2013).

To complement the model shown in Figure 7, two regulators of arbuscule development from the literature were incorporated, namely MtErf1, required for arbuscule branching in early stages (Devers *et al*., 2013) and MtMyb1, controlling arbuscule degeneration in later stages (Floss *et al*., 2017).

As described above, arbuscule degeneration is regulated by MtMyb1 (Floss *et al*., 2017). *MtMyb1* expression shows a strong inclination over time during AMF-colonization (Table I_1; Figure 7) which is in accordance to its role in controlling arbuscule degradation. In addition, MtMyb1 activity depends on post-translational regulation through an interaction with MtNsp1 and MtDella (Floss *et al.*, 2017), revealing an additional level of control. The involvement of MtNsp1 and MtDella is of particular interest, since NSP1 and DELLA were shown to be key factors of early arbuscule development (Pimprikar *et al.*, 2016; Hirsch *et al.*, 2009; Floss *et al.*, 2013). This phenomenon is a further hint that the interconnection of MtGras TFs over the distance from earlier to later stages of arbuscule development (Figure 7) is only one of several examples for such an overarching regulatory network.

In conclusion, the model presented in Figure 7 suggests that unidirectional and feedback pathways are part of a complex network of interconnected GRAS and other TFs. It becomes more and more evident that signaling correlated with arbuscule development includes several adjustable regulatory modules, allowing a dynamic control of arbuscule formation and an integration of both external signals as well as internal ones from the plant's metabolism. Furthermore, this model delivers starting points for future studies, in order to reveal novel regulatory aspects of this complex machinery.

FUTURE PERSPECTIVES

The model of an interaction network of GRAS TFs that regulate arbuscule development is not complete. Several questions arise from the assessment of the results obtained:

What are the regulatory dependencies and functions of MtGras7?

MtGras7 expression is strongly induced in the very late stages of AM colonization. It is thus tempting to speculate that MtGras7 is involved in later stages of arbuscule development, possibly in the induction of arbuscule degeneration. A knockdown or knockout of *MtGras7* expression would thus be desirable to unravel the MtGras7 function. The transcriptional dependency of *MtGras7* on both MtGras4 and MtGras1 can be further addressed by expressing MtGras1:RNAi fusions in transgenic roots of the *MtGras4* Tnt1 insertion line NF4813, to achieve a knockdown of *MtGras1* in *MtGras4* knockout roots, potentially leading to a reduction in *MtGras7* expression that is strong enough to enforce a molecular or physiological phenotype.

How is the GRAS TF network regulated on the protein-protein interaction level?

Recent publications on GRAS TFs confirm that PPI studies are of great interest. However, future studies of GRAS TFs should be performed with different approaches. Since Y2H prey library screenings are impaired for many GRAS TFs due to autoactivation, future experiments should focus on direct matings. A promising technology for this purpose is BiFC, especially when performed not in *N. benthamiana* leaves, but in *M. truncatula* roots, since this approach would provide a native environment for translation, folding, translocation and function of the candidate proteins.

What other factors are interacting with the GRAS TF network?

It would be tempting to investigate possible crosstalk between Myb and GRAS TFs. Not only that MYB TFs have been found to interact with GRAS TFs in the Y2H experiments, it is further possible that the GRAS network presented is crosslinked with arbuscule degeneration, e. g. via the induction of MtMyb1 by one component of the GRAS network or via direct interaction with MtMyb1. The most likely candidate for such an association or relation would be MtGras7. Transcriptional analyses of potential *MtGras7* knockdown or knockout lines as well as future protein-protein interaction studies should therefore also focus on AM-related MYB TFs, especially MtMyb1.

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ABBREVIATIONS

AbA	Aureobasidin A
AD	GAL4 activation domain
Ade	Adenine
AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungus
Amp	Ampicillin
ARB	Arbuscule containing cortical cells
BD	GAL4 DNA binding domain
BiFC	Bimolecular fluorescence complementation
BLAST	Basic local alignment search tool
bp	Base pairs
CBF	CAAT-box binding factor
cDNA	Copy DNA
cv.	Cultivar
DCD	Development and cell death
DDO	Double dropout medium
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
ds	Double stranded
e. g.	Exempli gratia
EPI	Epidermal cells
ERF	Ethylene-responsive factor
EST	Expressed sequence tag
et al.	At alia
GEA	GeneExpressionAtlas
GO	Gene Ontology
GUS	Beta-glucuronidase
*gusA*int	*gusA*int gene
His	Histidine
Kan	Kanamycin
KOH	Potassium hydroxide
LCO	Lipochitooligosaccharide
Leu	Leucine
LiAc	Lithium acetate
LMPC	Laser microdissect and pressure catapulting
min	Minutes
ml	Milliliter
mM	Millimolar
mRNA	Messenger
Mt	*Medicago truncatula*
ng	Nanogram
ORF	Open reading frame
PAM	Peri-arbuscular membrane
PAS	Peri-arbuscular space
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PPA	Pre-penetration apparatus
PPI	Protein-protein interaction
QDO	Quadruple dropout medium
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAi	RNA-interference
RT-PCR	Reverse transcription polymerase chain reaction
SD	Synthetically defined medium
SDO	Single dropout medium
TF	Transcription factor
Trp	Tryptophan
UBI	Ubiquitous cell types of mycorrhizal roots
UTR	Untranslated region
WGA	Wheat germ agglutinin
Y2H	Yeast two-hybrid
YPDA	Yeast peptone dextrose adenine medium
Mg	Microgram
µl	Microliter
µm	Micrometer
CO	Chitin oligosaccharide
LCO	Lipochitooligosaccharide
GA	Gibberellic acid
Mb	Megabase
FST	Fast neutron bombardment
CoIP	Co-immuno precipitation

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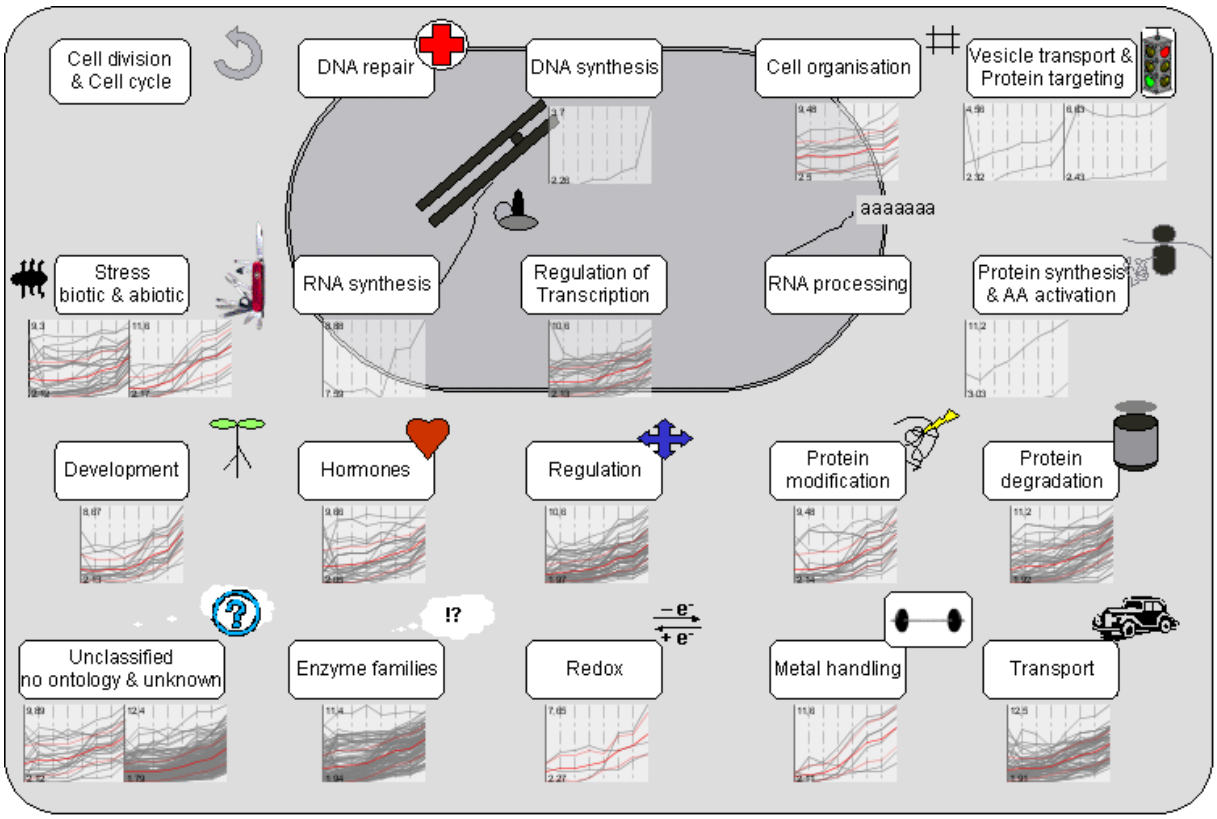
Supplemental Figure



Supplemental Figure S_1: MapMan pathway analysis of genes upregulated during colonization of *M. truncatula* roots with *R. irregularis*.

The 637 genes upregulated during fungal colonization from either 0 to 42 dpi or 7 to 42 dpi (Figure I_3) were mapped into functional categories using the MapMan software that bases on functional pathways of the *A. thaliana* proteome. A full list of the categorized genes is shown in Supplemental Table S_I_2. Note that genes that were manually assigned to specific categories later on (see Figure I_4) are not changed in this view. Gene expression levels are shown basing on log2 signal intensities from GeneChip hybridizations. Scales have been automatically adjusted for each category. Red lines represent the empirical means.

Supplemental Tables

Supplemental Tables as listed below can be found on the Data CD attached.

Supplemental Table S_1
Expression of genes upregulated during the time course of AMF colonization in *M. truncatula* A17 roots at 0, 7, 10, 14, 21, 28, 35, and 42 dpi.

Supplemental Table S_2
Mapman categorization of 637 genes upregulated during the time course of AMF colonization from 0 to 42 dpi.

Supplemental Table S_3 (.xlsx):
Comparison of gene expression in mycorrhizal *M. truncatula* A17 roots expressing an *MtGras1* RNAi construct (RNAi:*MtGras1* roots) or a *gusA*int gene (RNAi:*gusA*int control roots).

Supplemental Table S_4 (.xlsx):
Gene expression in mycorrhizal *M. truncatula ram1-1* mutant roots in comparison to *M. truncatula* A17 control roots.

Supplemental Table S_5 (.xlsx):
Expression of selected AM marker genes in mycorrhizal *M. truncatula ram1-1* mutant or *M. truncatula* A17 RNAi:*MtGras1* roots in comparison to the corresponding control roots.

CURRICULUM VITAE

Name: **Rico Martin Hartmann**

Date of birth: 15th February 1988

Place of birth: Hildesheim

Nationality: German

Academic studies

Since 10/2013	Doctoral researcher at the Institute of Plant Genetics, Unit IV- Plant Genomics at Leibniz Universität Hannover, Germany,
10/2010 - 09/2013	Master of Science in "Biology of Plants" at Leibniz Universität Hannover, Graduation with Master's degree in the Institute of Plant Genetics, Unit IV- Plant Genomics
10/2007 - 10/2010	Bachelor of Science "Plant Biotechnology" at Leibniz Universität Hannover Graduation with Bachelor's degree in the Institute of Ornamental and Woody Plant Science in the Department of Molecular Developmental Physiology

Schooling

2007	Graduation with general qualification for university entrance (Abitur)
2000 - 2007	Secondary school Goethegymnasium in Hildesheim
1998 - 2000	Middle school Orientierungsstufe Stadtmitte in Hildesheim
1994 - 1998	Primary school Grundschule Pfaffenstieg in Hildesheim

CONFERENCE POSTER CONTRIBUTIONS

2016 Conference oral contribution "Functional analysis of mycorrhiza-related GRAS transcription factor genes in *Medicago truncatula*", 3rd Doctoral Researchers Conference in GRK1798, Bad Salzdetfurth

2015 "Functional analysis of mycorrhiza-related GRAS transcription factor genes in *Medicago truncatula*", 2nd Doctoral Researchers Conference of GRK1798, Goslar, Germany

2015 "Functional analysis of mycorrhiza-related GRAS transcription factor genes in *Medicago truncatula*", 36th New Phytologist Symposium, Munich, Germany

2015 "Functional analysis of mycorrhiza-related GRAS transcription factor genes in *Medicago truncatula*", 2nd International Molecular Mycorrhiza Meeting, Cambridge, UK

PUBLICATIONS

2018 **M. Uhe, C. Hogekamp, R.M. Hartmann, N. Hohnjec, H. Küster (2018):** The mycorrhiza-dependent defensin MtDefMd1 of Medicago truncatula acts during the late restructuring stages of arbuscule-containing cells, PLoS ONE 13: e0191841

2018 **R.M. Hartmann, S. Schaepe, D. Nübel, A.C. Petersen, M. Bertolini, J. Vasilev, H. Küster, N. Hohnjec (2018):** A regulatory circuit of GRAS transcription factors revolves arbuscule development, submitted for publication.

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