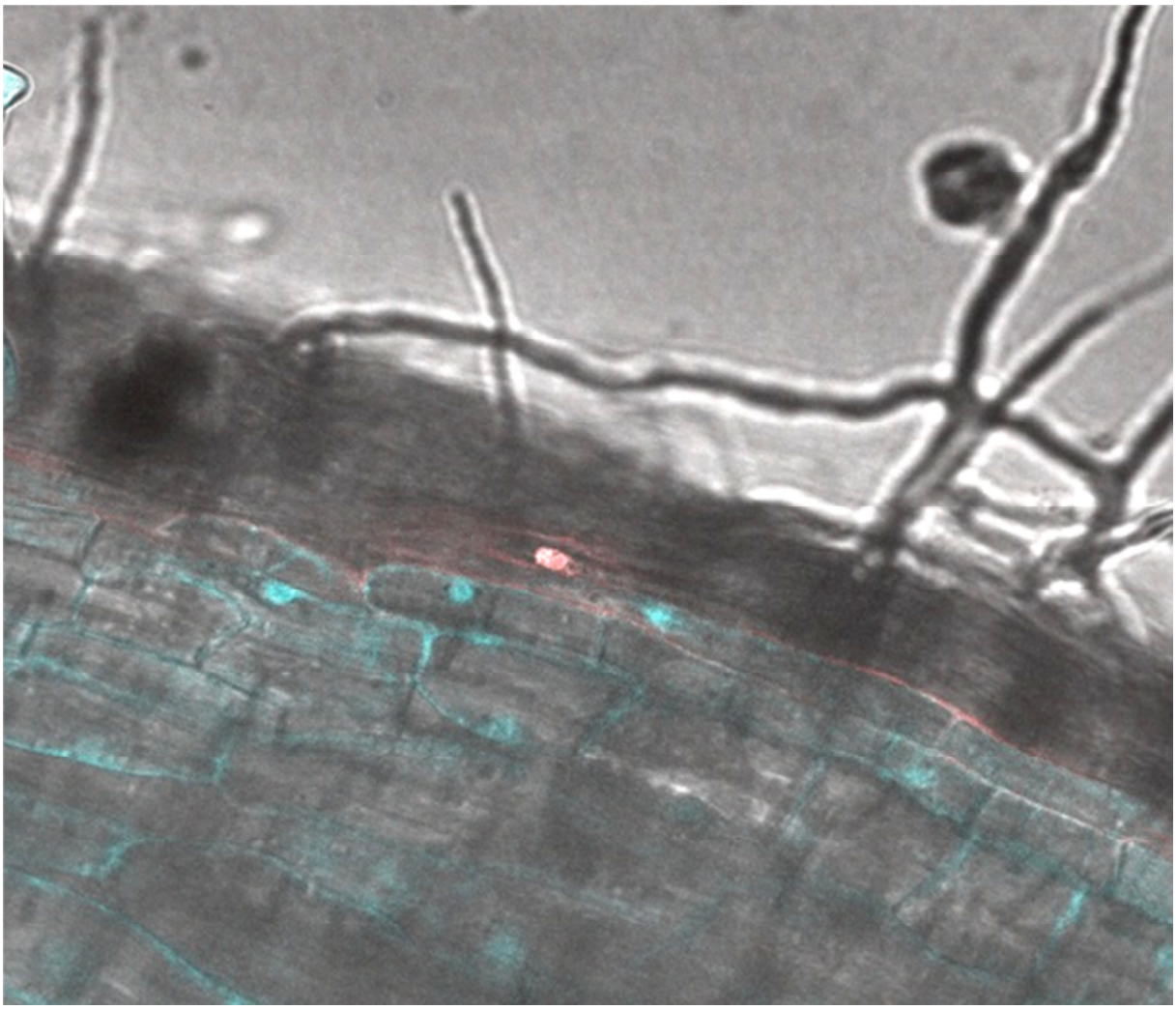
Identification and characterization of *Medicago truncatula* marker genes for recognition of fungal signals in the arbuscular mycorrhiza symbiosis



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Karlsruhe, den 30. 05. 2011

Hannah Kuhn

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Directory

Abbreviations

AFB	Auxin F Box
AM	Arbuscular mycorrhiza
BAK1	BRI1 associated kinase
BAP	6-Benzylaminopurine
BR	Brassinosteroid
BRI1	Brassinosteroid insensitive 1
CaMV	Cauliflower mosaic virus
CamR	Chloramphenicol resistance
CCamK	Calcium/calmodulin-dependent kinase
ccdB	control of cell division or death B
CFP	Cyan fluorescent protein, GFP derivative
4-Cl-IAA	4-Chloroindole-3-acetic acid
Cyp51	cytochrome P450 enzymes of the 51 family
Cyp51G	cytochrome P450 enzymes of the 51 family in green plants
Dap1	Damage associated protein 1
DMI	Does not make infection
DsRED	*Discosoma spec*. red fluorescent protein
ENOD	Early nodulin
EST	Expressed sequence tag
2,4-D	2,4-Dichlorophenoxyacetic acid
2-FAA	2-furylacrylic acid
GFP	Green fluorescent protein of *Aequorea victoria*
GUS	β-glucuronidase
HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Insig	Insulin induced gene
IPD3	Interacting protein of DMI3
KanR	Kanamycin resistance
LCOs	Lipo-chitooligosaccharides
LRF	Lateral root formation
LYK	Lysine motif receptor-like kinase
LysM	Lysin motif
MAMP	Microbe-associated molecular pattern
MSBP1	Membrane bound steroid binding protein 1

1-NAA	1-Naphthaleneacetic acid
NF	Nod factor
NFP	Nod factor Perception
NFR	Nod-factor receptor
NORK	Nodulation Receptor Kinase
NPA	naphthylphthalamic acid
NSP	Nodulation signaling pathway
P35S	35S promoter of cauliflower mosaic virus
PAA	2-Phenylacetic acid
PGRMC	Progesterone receptor membrane component
Pubq10	Ubiquitin 10 promoter of *A. thaliana*
PIN	PIN-FORMED
PPA	Pre penetration apparatus
RAM	Reduced arbuscular mycorrhiza
Ri-plasmid	Root -inducing plasmid
RNAi	RNA interference
Rol	Root locus
rpm	Rotations per minute
SCAP	SREBP cleavage-activating protein
SCF	Skp1-Cul1-F-box-protein
SERK	somatic embryogenesis receptor kinase
SmR	Streptomycin resistance
SP	Signal peptide
SpR	Spectinomycin resistance
SREBP	Sterol regulatory element binding proteins
SSH	Suppressive substractive hybridization
STI	Soybean trypsin inhibitor
SymRK	Symbiosis Receptor Kinase
TC	Tentative consensus sequence
T35S	35S terminator of cauliflower mosaic virus
T-DNA	Transfer DNA
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TEF1α	Translation elongation factor 1 alpha
TIBA	Tri-iodo-benzoic acid
TIR1	Transport Inhibitor Resistant 1
Tnos	Nopalin synthetase terminator of *Agraobacterium tumefaciens*
wt	Wild type
Y2H	Yeast Two-Hybrid

Abstract

In the complex community of soil organisms communication it is of major importance to distinguish between friend and foe. Arbuscular mycorrhizal fungi have since a long time been one of the oldest and most reliable allies of plants on this planet. The great success of this intimate symbiosis between plant roots and glomeromycotan fungi bases on the reciprocal exchange of nutrients and the resulting benefits for both partners. The hyphal network connected to the roots tremendously increases the amount of nutrients accessible to the plant. *Vice versa*, photosynthates delivered to the obligate biotrophic fungus, enable the growth of the hyphae and the completion of the fungal life cycle. However to undergo this mutualistic interaction, bilateral recognition is a key process necessary to induce the symbiotic program in both partners. Plants are able to perceive and decipher millions of chemical signals and interpret them into the respective cellular responses. Consequently, changes in gene expression are necessary to induce the answers to these stimuli. In the case of arbuscular mycorrhizal symbiosis recognition of the fungal partner was shown to occur already prior to physical contact due to the perception of diffusible fungal signal compounds.

The identification and characterization of plant marker genes upregulated in response to fungal signals will on one hand help to understand the initiated cellular program necessary for establishment of the symbiosis and accommodation of the fungal partner. On the other hand, plant marker genes can serve as a tool to characterize and finally identify fungal signal molecules. This study describes the identification and transcriptional characterization of five *Medicago truncatula* marker genes induced after recognition of *Glomus intraradices* during and prior to appressoria formation. Expression studies of those genes revealed the existence of parallel signal transduction necessary to establish a response to fungal stimuli, which initializes the symbiotic program of the plant. Moreover, the differential expression of the markers shows that distinct fungal signal molecules are secreted during consecutive steps of recognition in the crosstalk of both partners. An investigation of the involvement of auxin signaling in the plant response highlights a particular role for phenylacetic acid during fungal recognition. Linked to this finding, a suppression of conventional auxin and gibberellic acid signaling was found to be necessary in order to drive the expression of the marker genes. The further characterization of one of the examined genes, *MtMSBP1* for the first time emphasizes the necessity for fine-tuning the plant sterol metabolism in order to allow intracellular accommodation of fungal hyphae and consequently to establish a functioning symbiosis.

Zusammenfassung

Kommunikation ist eine Grundvoraussetzung für die Unterscheidung von Freund und Feind in der komplexen Gemeinschaft bodenlebender Organismen. Die arbuskulären Mykorrhiza-Pilze gehören seit jeher zu den ältesten und verlässlichsten Verbündeten der Landpflanzen auf dieser Erde. Der große evolutionäre Erfolg dieser Symbiose begründet sich auf dem gegenseitigen Austausch essentieller Nährstoffe und der sich daraus ergebenden Vorteile für beide Partner. Einerseits vergrößert das mit der Wurzel assoziierte Hyphennetzwerk die Menge der, für die Pflanze verfügbaren Nährstoffe um ein Vielfaches. Im Gegenzug erhält der obligat biotrophe Pilz Photosynthese-Assimilate von der Pflanze, die ihm Hyphenwachstum und die Bildung neuer Sporen ermöglichen. Grundvoraussetzung für das Zustandekommen dieser mutualistischen Interaktion ist die gegenseitige Erkennung und die daraus folgende Aktivierung des symbiontischen Programms beider Partner. Pflanzen sind in der Lage zwischen Millionen chemischer Signale zu differenzieren und entsprechende zelluläre Antworten einzuleiten. Die Perzeption und Weiterleitung von Stimuli resultiert dabei in vielen Fällen in einer veränderten Transkription von Schlüsselgenen. In der arbuskulären Mykorrhiza Symbiose findet eine solche Erkennung des Pilzes durch die Pflanze bereits vor einem eigentlichen direkten Kontakt durch die Perzeption diffusibler pilzlicher Signale statt.
Die Identifikation und Charakterisierung von pflanzlichen Markergenen, die in Folge der Erkennung pilzlicher Botenstoffe exprimiert sind kann sehr zum Verständnis des zellulären Programms beitragen, das für die Etablierung der Symbiose notwendig ist. Zusätzlich könnten solche Markergene eine Identifizierung der perzipierten Signalmoleküle ermöglichen. Diese Arbeit beschreibt die Identifizierung und transkriptionelle Charakterisierung von fünf *Medicago truncatula* Genen die in Folge der Erkennung von *Glomus intraradices* induziert sind. Eine Induktion der Transkription dieser Gene erfolgt hierbei bereits vor der Bildung von Appressorien durch Erkennung chemischer Botenstoffe. Die detaillierte Analyse der Expressionsmuster der Gene macht deutlich, dass unterschiedliche Signalwege bei der Transduktion der Signale und Einleitung der entsprechenden Antwort beteiligt sind. Des Weiteren konnte gezeigt werden, dass im Zuge des Signalaustausches zwischen Pflanze und Pilz unterschiedliche pilzliche Moleküle einerseits konstitutiv und andererseits nach vorhergehender Stimulation durch Wirtswurzeln sekretiert werden. Eine besondere Rolle von Phenylessigsäure sowie die Notwendigkeit die konventionelle Auxin- und Gibberellin-Signalgebung zu unterdrücken, zeigte sich bei der Untersuchung des Einflusses von Auxinen auf die AM-induzierte Expression der Markergene. Die detailliertere Charakterisierung von *MtMSBP1*, eines der Markergene, lieferte außerdem erstmals einen Nachweis über die Beteiligung von Steroiden in der intrazellulären Etablierung des Pilzes.

1 Introduction

1.1 Life in the underground

The world of the underground is a fascinating place, and there is a lot going on below the earth's surface. In many cases, it is indeed a very crowded environment, busy like the subway of Tokyo or the streets of New York, with bitter competition for limited space and food. Already a handful of soil can contain more organisms than people live on earth. In one single cubic decimeter of good quality soil, 10 billions of soil inhabitants might be present (Bardgett, 2005). Accordingly, in a balanced soil, plants grow in an active and lively environment. The mineral content of the soil and its physical structure are important for their prosperity, but it is the life in the substrate that powers its cycles and provides its fertility. Without the activities of soil organisms, organic material would accumulate and in consequence, there would be no food for plants. The soil biota includes, besides animals of a huge size range, also microbes like amoebas, algae, bacteria and fungi. Of these, bacteria and fungi play key roles in maintaining a healthy soil. They act on one hand as decomposers that break down organic materials to extract soluble nutrients from organic matter. On the other hand they interact with other soil inhabitants in either pathogenic or mutualistic associations. Plants that are dependent on soil as a substrate have to cope with the environment that it offers to them. In contrast to us humans, they are not able to escape to another place that offers better circumstances when water and food are in short supply, or when an enemy is lurking. They are literally rooted in the ground. Nevertheless, plants are no passive and insensitive organisms as often considered. In contrast they are able to take advantage of many effective ways to communicate, albeit in a chemical rather than in an oral way. Thus, plants are in constant communication with a plethora of diverse organisms in their rhizosphere (Estabrook & Yoder, 1998). In such a way, they establish relationships of various characters such as mutualistic and pathogenic ones. It is also not uncommon for a symbiotic relationship to take place right next to a hostile one. Importantly, the plant must be able to differentiate between friend or foe, between beneficial microorganisms and pathogenic ones. To fulfill this task, plants actively affect the structure of rhizosphere communities by releasing attractants and repellents from their roots, and vice versa are able to perceive and discriminate various compounds form bacteria and fungi. One of the major topics of this work is the aspect of plant recognition of arbuscular mycorrhizal fungi that are important interaction partners for the majority of the plants on our planet. An association with those unique organisms assists the plant in many different aspects of coping with the environment that the soil offers. Thus, the arbuscular mycorrhiza symbiosis does not only improve plant

growth, but also protects plants from various abiotic and biotic stresses such as drought or high soil salinity and attack by pathogens. One of the major goals of current plant science is to improve the agricultural properties and stress adaptabilities of plants, and the arbuscular mycorrhiza symbioses is one of the possibilities to achieve this aims. Moreover, the fact that global reserves of cheap mineral phosphorous, which is needed in high amounts in fertilizers, will be depleted within 50 to 100 years (Cordell et al., 2009) requires new solutions for conventional agriculture. Phosphorous is one of life's fundamental elements and the main nutrient provided by arbuscular mycorrhiza fungi to plants. The understanding of arbuscular mycorrhiza signaling and establishment is one approach to make use of this interaction in many aspects of a sustainable agriculture.

1.2 The arbuscular mycorrhiza symbiosis – an old story

The arbuscular mycorrhiza (AM) is an almost ubiquitous symbiosis that on our world is the rule rather than the exception. Actually more than 200000 species of terrestrial plants, including members of the bryophytes, pteridophytes, gymnosperms and about 80 percent of all angiosperms are able to undergo this interaction. Moreover, the theory exists that the development of arbuscular mycorrhiza was one of the critical steps that enabled the conquest of the landmass by plants. Indeed the fungal partners in this symbiosis were shown to originate already in the Ordovician, which is indicated by fossil spores and hyphae that were aged to over 460 million years (Redecker *et al.,* 2000). The discovery of 400 million years old colonized fossils (Gray *et al*., 1982; Remy *et al*., 1994), together with the fact that recent liverworts are also able to interact with arbuscular mycorrhizal fungi (Duckett *et al*., 2006) led to the theory of coevolution of glomeromycotan fungi with land plants (Figure 1.1; Pirozynski & Malloch, 1975; Simon *et al*., 1993). As the existence of roots in the Devonian has been preceded by these very old interactions, the association with fungal hyphae was of outstanding importance for the first land plants to access the scarce nutrients in the substrate. This most probably led to an evolutionary advantage for those plants and to the fact that today the majority of land plants undergo this kind of interaction. Noticeable, plants, not able to enter an AM, as members of the Brassicaceae (including *Arabidopsis thaliana*), Caryophyllaceae, Chenopodiaceae, and Uricaceae (Vierheilig *et al*., 1996; Smith & Read, 1997), emerged much later in history, approximately 100 million years ago (Brundrett, 2002).

Besides the nutritional aspect of this symbiosis which mainly relies on the supply of phosphorus but also on nitrogen copper and zinc (Ezawa et al., 2002; Jin et al., 2005) and the resulting positive effect on plant growth, AM plants are also supported by the symbiont in their ability to cope with biotic and abiotic stresses. Mycorrhizal plants were shown to be more resistant to drought and increased humidity, salt stress, strong soil acidity as well as

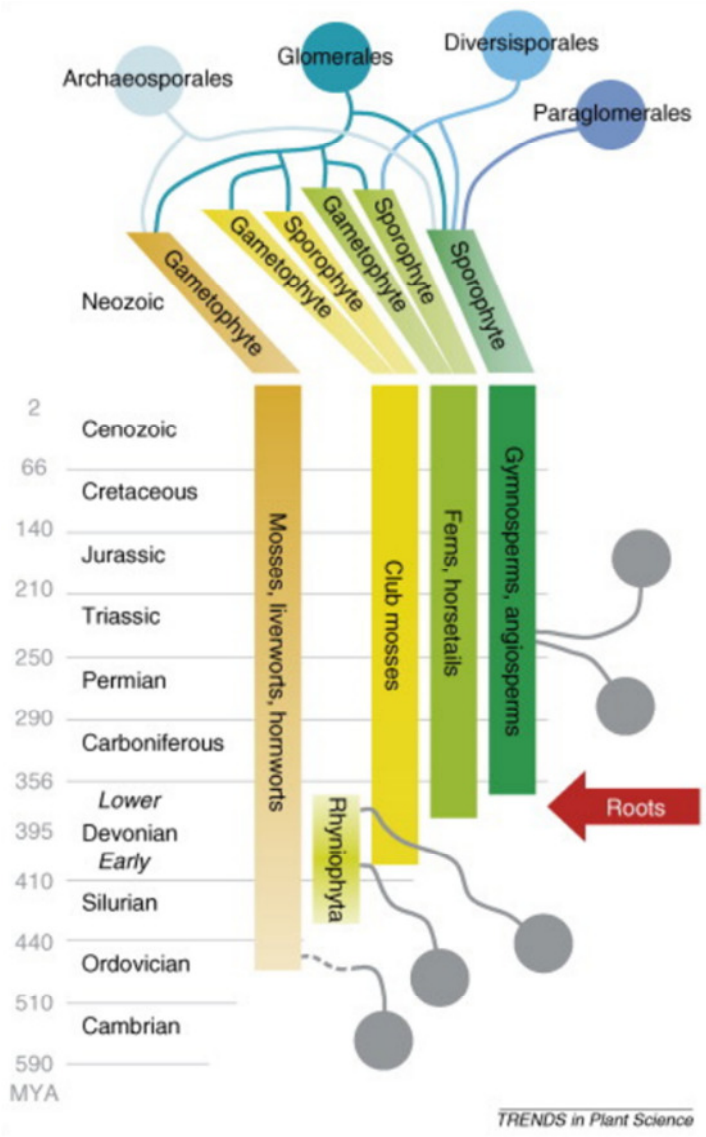


Figure 1.1 Summary of AM fungal distribution throughout geological periods and plant taxa.
Bonfante & Genre (2008). The chart localizes the currently known AM fossils on a time scale, side by side with plant evolution. Fossil of early mycorrhizal associations have been dated back to the Ordovician (dashed line), preceding the emergence of roots (red arrow). MYA = million years ago.

heavy metal exposure (Augé, 2001; Jacquot-Plumey *et al.*, 2003; S. Gianinazzi *et al.*, 2005). An increased resistance to pathogenic attacks relies on one hand on the improved nutrient supply of the plant symbiont but is also due to priming of the defense response by AM fungi (Colditz *et al.*, 2005; Liu *et al.*, 2007). In return the fungal symbiont that depends on this association to fulfill its lifecycle, receives support in the form of carbon hydrates and of a place to live providing protection from other soil organisms. As an endomycorrhizal association, the arbuscular mycorrhiza symbiosis is defined by the entrance of the fungal hyphae into the cortex cells of the plant roots and the organogenesis of the arbuscules. Those tree-like structures that gave the name to the symbiosis are the organs of nutrition exchange and thus are regarded as the beating heart of the symbiosis.

1.3 Excerpts from the life of an arbuscular mycorrhizal fungus

All fungi that undergo an arbuscular mycorrhizal symbiosis belong to the clade of the Glomeromycota, one of the main phyla in the fungal kingdom. Being obligate biotrophs, for those fungi it is of great advantage to have no host specifity. Thus, they can enter a symbiosis with almost every plant that is able to form an arbuscular mycorrhiza. The Glomeromycota consist of 239 species of the orders Glomerales, Diversisporales, Archaeosporales and Paraglomerales that can be discriminated on behalf of their life cycle, sporulation, morphology and molecular characteristics (Schüßler & Walker, 2010). Besides morphological differences, those fungi are coenocytic with non-septated and branched hyphae. The relative big spores can reach sizes of up to 0.5 mm in the case of *Gigaspora gigantea.* They are multinucleated and can contain up to 2000 nuclei, depending on the species (Bécard & Pfeffer, 1993). Despite the worldwide distribution and ecologic importance of AM fungi, our knowledge of those microorganisms is limited. This is on one hand because the fungi cannot be grown in axenic culture due to their obligate biotrophy. On the other hand, genetic manipulation of the glomeromycotan fungi is difficult as no sexual cycle is known and only one report of successful but only transient transformation exists (Helber & Natalia Requena, 2008).

The lifecycle of AM fungi is divided into two different phases of growth (Figure 1.2 and Figure 1.3): In the pre symbiotic phase, the mature spores residing in the soil germinate after a period of dormancy. Germination is influenced by abiotic circumstances such as humidity, pH and temperature of the soil (Green *et al.*, 1976) and promoted by biotic factors such as root exudates of suitable host plants (Bécard & Piché, 1989; Gianinazzi-Pearson *et al.,* 1990; Nair *et al.,* 1991). The non-symbiotic growth of the hyphae relies on the mobilization of triacyl glycerol and glycogen storage and is consequently limited in time. If the fungal mycelium does not succeed in finding and colonizing a suitable host root within approximately four weeks, a new phase of dormancy is induced. This is initiated by retraction of the cytoplasm into the spore and followed by the successive septation of the empty hyphal compartments. In such a way, one single spore is able to germinate up to ten times and stays viable for months (Koske, 1981). In the case that the mycelium finally succeeds in reaching the rhizosphere of a compatible host root, it perceives plant signals, which led to an induction of growth as well as to hyphal branching (Giovannetti *et al.,* 1993; Buee *et al.,* 2000). This phenomenon is thought to increase the probability of the hyphae to contact the root and relies on a plant derived ‘branching factor’ which has been characterized as strigolactone 5-deoxystrigol (Akiyama *et al.,* 2005). The contact of the hyphae with the root surface initiates the symbiotic growth phase of the fungus. As a result, the mycelium forms thickened hyphae, which are termed hyphopodia or appressoria. These structures serve as penetration organs

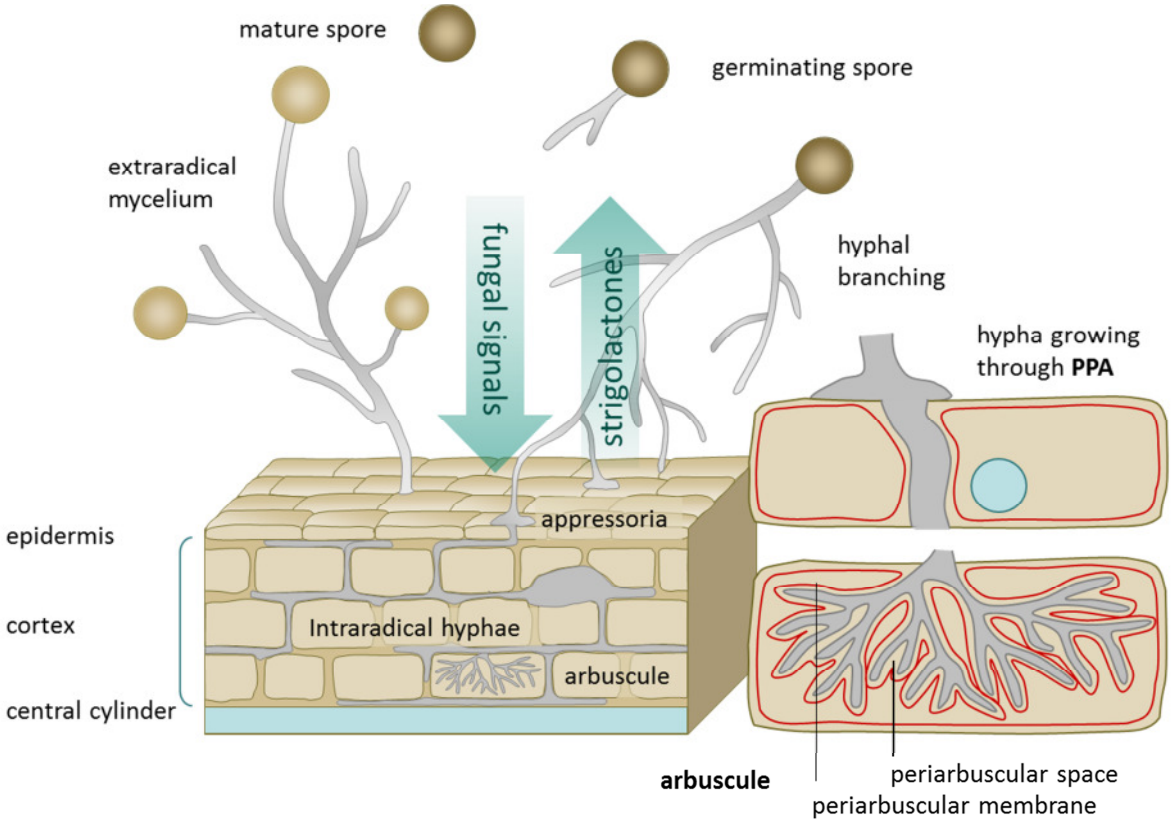


Figure 1.2 Lifecycle of the arbuscular mycorrhizal fungus *Glomus intraradices*
Mature spores germinate and undergo intensive hyphal branching after perception of plant strigolactones in the rhizosphere of suitable host plants. On the surface of the root, the hyphae thicken and build hyphopodia-like appressoria, which function as penetration structures. The epidermis cells facilitate the entry of the fungus via formation of a prepenetration apparatus (PPA). After entrance into the host root, the fungus quickly spreads in the apoplast via growth of intraradical hyphae. Finally, the hyphae enter plant cells of the root cortex via invagination of the plant plasma membrane. By dichotomous branching, they build tree-like structures named arbuscules, which are thought to be the main organs of nutrition exchange in the symbiosis. The cavity of the periarbuscular space, surrounded by the fungal membrane and the periarbuscular membrane of the plant forms the symbiotic interface. The lifecycle is fulfilled by the growth of new extraradical mycelium and the emergence of new spores either inside or outside of the root.

and mediate the entry of the fungus into the root. This step is tightly regulated and fine-tuned in accordance with the underlying plant cells, which allow and facilitate the entrance of the hyphae. The morphology and abundance of appressoria depends on the combination of both symbiotic partners. *G. intraradices* for example usually forms very few and unobtrusive appressoria on epidermal cells of *M. truncatula* plants. The penetration of the root surface occurs either between two epidermal cells (Demchenko *et al.,* 2004) or via intracellular transition of a single epidermal cell. In the second case of a direct penetration, the underlying cell forms a canal-like pre penetration apparatus (PPA) that leads the hypha through the cytoplasm (Genre *et al.,* 2005). This passage is initiated by the migration of the plant nucleus from the site of contact towards the opposed side of the cell. Afterwards this is followed by the aggregation of microtubules, actin filaments as well as ER cisternae and finally results in the invagination of the cytoplasmic membrane alongside the migration path. Once in the apoplast of the host root, the fungus quickly spreads via the formation of intraradical hyphae

that colonize the root cortex. Here they enter the plant cells a second time to form the main organs of nutrition exchange, the arbuscules. After invagination of the cytoplasmic membrane of the cell, the hyphae undergo an intense dichotomous branching leading to the formation of a tree-like structure. The establishment of the arbuscule inside of the plant cell coincides with dramatic changes of the cellular organization and architecture. Thus, the big central vacuole is fragmented, the nucleus migrates to a central position, the cytoskeleton is rearranged and a network of plastids, mitochondria and ER surrounding the arbuscule is installed (Scannerini & Bonfante-Fasolo, 1983; Bonfante & Perotto, 1995; Gianinazzi-Pearson *et al.*, 1996; Genre & Bonfante, 1997; Genre & Bonfante, 1998; Harrison, 1999; Blancaflor *et al.*, 2001; Fester *et al.*, 2001; Lohse *et al.*, 2005; Genre *et al.*, 2008). The periarbuscular plant membrane surrounding the arbuscule forms, in cooperation with the hyphal membrane, a new apoplastic symbiotic compartment. This compartment is called the periarbuscular space and defined as symbiotic interface (Bonfante & Perotto, 1995). The plant, as well as the fungus induces the expression of specific transporter proteins that mediate the exchange of nutrients into and out of the periarbuscular space. The energy, necessary for the transport processes is provided by the activity of plant and fungal proton ATPases that lead to the installation of an electrochemical gradient (Gianinazzi-Pearson *et al*, 1990; Gianinazzi-Pearson *et al.*, 2000; Ferrol *et al.*, 2002; Krajinski *et al.*, 2002; Requena *et al.*, 2003). The best-characterized plant transporter situated in the periarbuscular membrane (Pumplin & Harrison, 2009) is the *M. truncatula* Phosphate transporter 4 (MtPT4). This protein is present exclusively in arbuscule-containing cells where it promotes the uptake of phosphate from the periarbuscular space into the plant cell (Harrison *et al.*, 2002; Javot *et al.*, 2007). After a few days of activity, the arbuscules collapse, are recycled and leave an intact cell behind that can be colonized another time (Dickson & Smith, 2001). Besides the arbuscules, some AM fungi, such as *G. intraradices*, additionally form lipid rich vesicles inside of the plant root, which are thought to be storage organs (Cooper & Lösel, 1978; Nemec, 1981). The last phase of the fungal lifecycle starts with the growth of new extraradical mycelium. Those hyphae absorb nutrients out of the soil and deliver them to the intraradical mycelium. Additionally, new spores emerge on the extraradical mycelium. In the case of *G. intraradices* the ability to form new spores is not restricted to the extraradical hyphae as also intraradical spores are formed. The colonization of additional roots can be performed either directly by the extraradical hyphae or by the germination of novel spores. Noteworthy, extraradical hyphae can also colonize roots of different plants and plant species resulting in the formation of a belowground hyphal network connecting many different plants within an ecosystem. It is of special importance for the laboratory work with arbuscular mycorrhizal fungi that different phases of the fungal lifecycle occur parallel, so that a proper

separation and isolation of different hyphal structures is often very difficult. However, this is only another challenge in the work with those unique and fascinating organisms.

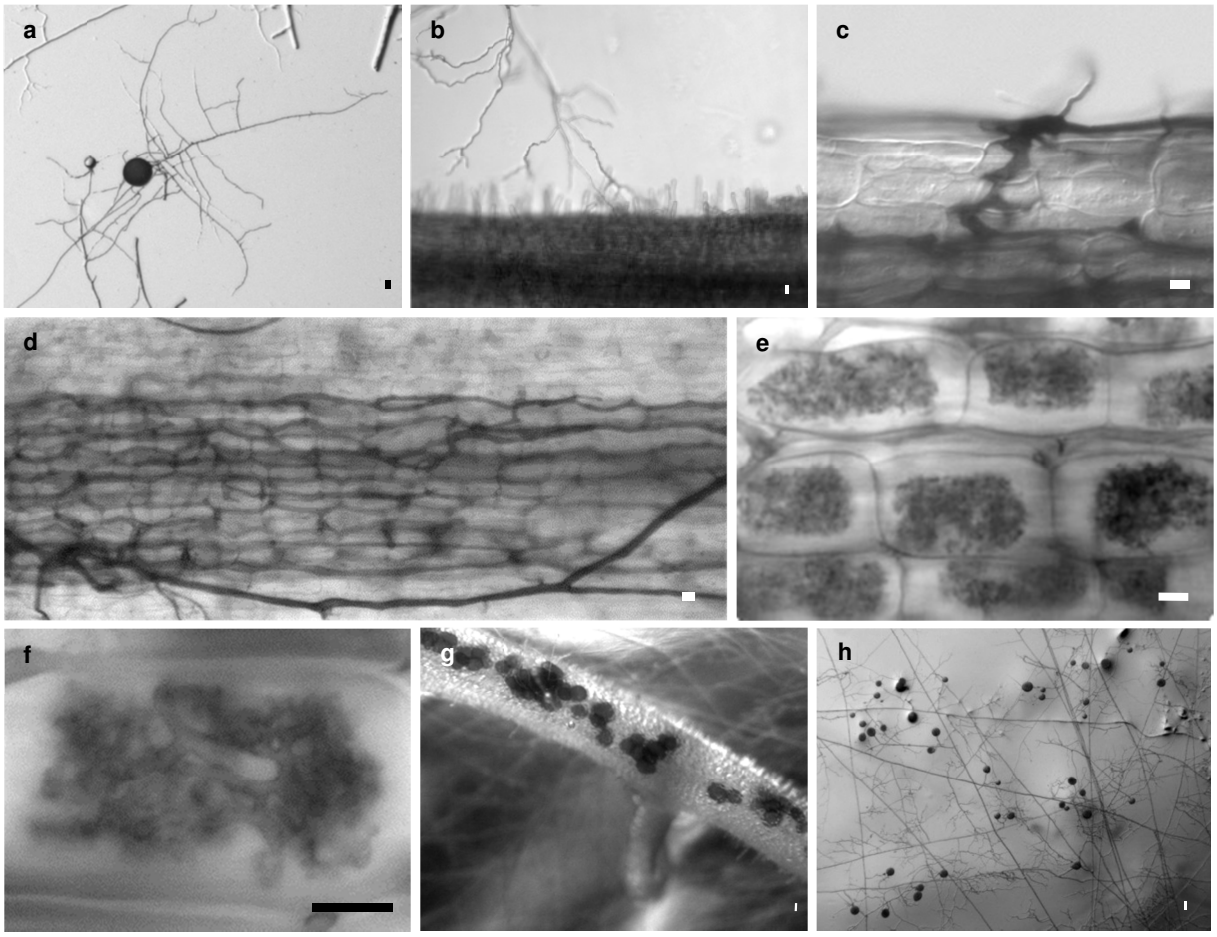


Figure 1.3 Stages of the lifecycle of *G. intraradices*
Fungal structures inside of *Medicago truncatula* roots were stained with black ink. (a) Germinated spore, (b) branching hypha approaching a host root, (c) appressorium, (d) intraradical hyphae network, (e) and (f) arbuscules, (g) spores inside of the root, (h) extraradical mycelium with spores. Bar = 10 µm.

1.4 *Medicago truncatula* and *Glomus intraradices* as model organisms

M. truncatula and *G. intraradices* have been proposed as model organisms for research on arbuscular mycorrhiza within the scope of the DFG priority program SPP 1084 „Molecular Basics of Mycorrhizal Symbioses“. This decision was based on several advantages that both organisms possess in comparison with other plants and AM fungi:

Being a member of the Papilionoideae sub-family of the legumes *M. truncatula* is able to undergo symbiosis with Rhizobia as well as with arbuscular mycorrhizal fungi. As both symbioses share components of a common symbiosis (SYM) pathway, an investigation of both interactions in the same plant is of special interest (Duc *et al.*, 1989; Gianinazzi-Pearson *et al.*, 1991; Sagan *et al.*, 1995; Marsh & Schultze, 2001; Lum & Hirsch, 2003; Limpens & Bisseling, 2003; Parniske, 2004). Unfortunately, *Arabidopsis thaliana*, the best-established

model organism for plants is one of the rare vascular plants that is not able to interact with arbuscular mycorrhizal fungi. *M. truncatula* is closely related to several crop and pasture legumes, as for example *Medicago sativa*. *M. sativa* is not only the most important forage legume in the USA (Choi *et al*., 2004) but is also widely used as forage plant in Australia and Mediterranean Europe. Favorably, *M. truncatula* has a relatively small, diploid genome (haploid size approximately 550 Mbp), which is currently being sequenced by an international cooperation, making it useful for genetics and genomics (Young *et al*., 2005). Another advantage of *M. truncatula* is its self-fertility and the production of a large number of seeds on a relatively small plant, which makes it amenable to high-density culture (Barker *et al*., 1990). Finally, it is relatively easy to transform with Agrobacteria. Since its adoption by the international community as a model species, a number of useful tools and resources have been developed for *M. truncatula*. Those include for example genome sequencing resources (http://www.jcvi.org/cgi-bin/medicago/overview.cgi), an index of *M. truncatula* expressed sequence tags (EST) (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago), a collection of mutant populations (Tadege *et al*., 2005) as well as tools and protocols for transcriptome, proteome, and metabolome analysis (Watson *et al*., 2003; Gallardo *et al*., 2003; Manthey *et al*., 2004; Barnett *et al*., 2004; Broeckling *et al*., 2005; Benedito *et al*., 2008) and the availability of an oligonucleotide microarray based on 36000 tentative consensus sequences of the available ESTs (Küster *et al*., 2007) as well as an Affymetrix Gene Chip. Additionally, a great advance for the analysis of *M. truncatula* genes is the *Medicago* Gene Expression Atlas (http://mtgea.noble.org/v2/), which provides quantitative gene expression data for many genes during plant development and in response to biotic and abiotic stimuli (Benedito *et al*., 2008).

The arbuscular mycorrhizal fungus *G. intraradices* is a member of the Glomerales included in the phylum of the Glomeromycota. The fungus is widespread in the world, present in many different ecosystems and able to colonize many plant species including agriculturally important ones (Smith & Read, 2008). *G. intraradices* is one of the most studied AM fungi as it colonizes host plants very rapidly. The work with AM fungi is difficult, as being obligate biotrophs, those fungi can only be propagated in association with a suitable host root. Nevertheless, *G. intraradices*, in contrast to other AM fungi can be grown *in vitro* monaxenic culture with *Agrobacterium rhizogenes*-transformed carrot roots (Bécard & Fortin, 1988). Additionally, it is the only species whose spores are available commercially in pure form in large quantities. Unfortunately, transformation of the fungus was achieved only transiently so far (Helber & Requena, 2008) as successful selection of positive stable transformants is prevented by the coenocytic state of the hyphae. *G. intraradices* was chosen as a candidate for the first whole genome sequencing project on an AM fungus. However, assembly of the genome revealed problems, probably due to the existence of multiple copies of most genes.

This indicates a high level of polymorphism within *G. intraradices*, possibly caused by the presence of heterokaryontic nuclei, each containing approximately 15 Mbp of DNA (Hijri & Sanders, 2004; Martin *et al*., 2008). Additionally, recent approaches revealed that the fungus exhibits biparental inheritance. However it was not possible to determine whether a simple mixing of nuclei or also nuclear fusion and meiosis occurs in *G. intraradices* although evidence for recombination was found (Croll & Sanders, 2009; Sanders & Croll, 2010).

1.5 Exchange of signals during establishment of the AM symbiosis

The establishment of the arbuscular mycorrhiza symbiosis coincides with tremendous changes in the cellular morphology and physiology of both partners. To initiate and control these alterations, an extensive exchange of signals between both partners is necessary during the different stages of the symbiosis. The translation of signals into physiological changes requires regulation of gene expression and protein activity. As in many other symbiotic interactions, also in the arbuscular mycorrhiza, it becomes more and more apparent that an exchange of signals already starts prior to physical contact (Kosuta *et al*., 2003; Weidmann *et al*., 2004; Oláh *et al*., 2005; Navazio *et al*., 2007; Kosuta *et al*., 2008; Gutjahr *et al*., 2009; Chabaud *et al*., 2011; Kuhn *et al*., 2010; Maillet *et al*., 2011). Likewise, such an exchange was shown to be essential for the establishment of the well-studied association of legumes with Rhizobia (Lerouge *et al*., 1990). For the root nodule symbiosis, it is described that perception of plant flavonoids induces the biosynthesis and release of bacterial lipo-chitooligosaccharides designated as Nod factors (Dénarié *et al*., 1996; Long, 1996; Spaink, 2000). These signal molecules are perceived by Nod factor receptors of the plant root hairs and initiate the symbiotic program in the host roots (Radutoiu *et al*., 2007). During arbuscular mycorrhizal symbiosis signal exchange occurs in a similar way. Already since several years now, a compound released by host roots has been characterized as strigolactone 5-deoxystrigol (Akiyama *et al*., 2005). This molecule was designated as branching factor as it induces extensive branching in surrounding hyphae. The receptor for this molecule has not yet been identified, nevertheless it could be shown that hyphal branching is preceded by alterations in the transcription of mitochondrial genes, followed by an increased consumption of oxygen as well as changes in the shape and density of mitochondria (Tamasloukht *et al*., 2003; Besserer *et al*., 2006). An induction by strigolactone however is not sufficient for initiation of penetration structures by the fungus (Nagahashi & Douds, 1997). Actually, it was shown that neither branching of the hyphae nor the presence of root exudates or living plant cells are necessary to induce appressoria formation (Nagahashi & Douds, 1997). Artificial surfaces are however not sufficient to induce appressoria development even if root exudates are added. This observation indicates that

structural properties of the epidermis are probably needed for the formation of those penetration structures.

Since a long time now, evidence is accumulating for the existence of a fungal signal that prepares the plant host for the establishment of the symbiosis (Kosuta *et al*., 2003; Weidmann *et al*., 2004; Oláh *et al*., 2005; Navazio *et al*., 2007; Kosuta *et al*., 2008; Gutjahr *et al*., 2009; Chabaud *et al*., 2011; Kuhn *et al*., 2010; Maillet *et al*., 2011). In analogy to the Nod factors, this compound has been designated as Myc factor and was for a long time one of the most wanted molecules in arbuscular mycorrhizal research. Already in 2003, Kosuta and colleagues were able to show that *G. intraradices* hyphae secrete factors that are able to induce the expression of *MtENOD11* in the zone of emerging root hairs of host roots. This induction also occurred if fungus and root were separated by a cellophane membrane. The existence of diffusible fungal signal molecules was confirmed by Oláh *et al.* (2005) who reported an increased lateral root formation (LRF) in response to signals released by germinated spores of different AM fungi. In 2007, Navazio *et al*. observed a transient cytosolic Ca^{2+} response in *Glycine max* cells induced by supernatant of germinated spores of *G. intraradices*. Remarkably, this compound was found to be produced also by germinated spores that had not been induced by host roots or treated with strigolactones, indicating that AM fungi, in contrast to Rhizobia release signal molecules constitutively. Equally prepared supernatant of germinated spores was able to induce an accumulation of starch in roots of *Lotus japonicus* (Gutjahr *et al*., 2009). In contrast, the need for an induction of fungal hyphae to induce a cytosolic Ca^{2+} oscillation similar to the Ca^{2+} spiking observed during Nod factor recognition, was shown by Kosuta *et al.* (2008). Performing fluorescence microscopy with a cameleon calcium reporter, they observed that only fungal hyphae that had undergone branching were able to induce an oscillatory calcium signature in root hair cells of *M. truncatula*. Finally, a nuclear Ca^{2+} spiking of similar signature was shown to be induced by either appressoria or germinated spores' exudates in *L. japonicus* atrichoblasts (Chabaud *et al*., 2011). Recently it turned out that the structures of the molecules inducing those responses in the root are similar to Nod factors. Thus, in a direct approach, searching for fungal lipo-chitooligosaccharides (LCOs), Maillet and colleagues (2011) indeed identified fungal signal molecules, designated as Myc-LCOs, which were able to induce typical responses in *M. truncatula* roots.

1.6 The symbiosis pathway

The existence of diffusible signal molecules is however not the only overlap of AM and rhizobial symbiosis. Analyses of legume mutants impaired in nodule and AM formation have identified proteins as important components of a common symbiosis pathway, the so-called

SYM pathway (Figure 1.4; reviewed by Parniske, 2008). This signal transduction pathway is thought to originate in the more ancient arbuscular mycorrhiza symbiosis and to have been adopted by the rhizobial interaction (Gerard Duc *et al*., 1989; La Rue & Weeden, 1994; Kistner & Parniske, 2002). The core of this pathway is formed by three proteins designated as DMI2, DMI1 and DMI3 (does not make infection). These represent a membrane-spanning leucine-rich repeat containing receptor kinase (MtDMI2 - SYMRK/NORK in *L. japonicus*; Endre *et al*., 2002; Stracke *et al*., 2002), a potassium-permeable channel (MtDMI1 - Pollux and Castor in *L. japonicus*; Ané *et al*., 2004; Imaizumi-Anraku *et al*., 2005; Charpentier *et al*., 2008) and a calcium-calmodulin-dependent kinase (MtDMI3 - CCamK in *L. japonicus*; Lévy *et al*., 2004; Mitra *et al*., 2004). Perception of Nod factors is mediated by the LysM motif-containing Nod factor receptors MtLYK3/LjNFR1 and MtNFP/LjNFR5 (Radutoiu *et al*., 2007), while no receptor has yet been identified responsible for AM signal perception. Chitin derivatives, such as Nod factors, are usually recognized by LysM motif-containing receptors Kaku *et al.,* 2006), indicating that also fungal lipo-chitooligosaccharides should be recognized by proteins similar to the NF receptors. Indeed, only very recently, the only LysM motif receptor present in the non-legume *Parasponia andersonii* was identified due to its similarity to MtNFP/LjNFR5. This receptor kinase of *P. andersonii* was shown be critical for both, successful mycorrhization and an atypical association with rhizobia similar to nodulation (Op den Camp *et al*., 2011). After perception of diffusible molecules, the signal is transmitted to DMI2 and DMI1, both acting upstream of Nod- and Myc factor-induced calcium oscillations. Calcium channels involved in generation of this calcium signals have however not been identified and the role of DMI1 and DMI2 in transduction of the signals and production of the Ca^{2+} oscillations is still in the dark. Additional to DMI1 and 2, the nuclear pore associated proteins NUP85 and NUP133 and NENA (all identified in *L. japonicus*; Kanamori *et al*., 2006; Saito *et al*., 2007; Groth *et al*., 2010) are required for calcium spiking. Although their mode of involvement is also unknown, it is speculated that unidentified downstream events depending on the nucleoporins might contribute to membrane potential alterations at the nuclear envelope involving the ion channel DMI1. DMI3 is subsequently thought to act as decoder of the Ca^{2+} signal in cooperation with the interacting nuclear protein IPD3/CYCLOPS (Messinese *et al*., 2007; Yano *et al*., 2008). For rhizobial interaction, it is known that a complex of DMI3 and IPD3 regulates downstream activation of the transcription factors NSP1, NSP2, ERN1, ERN2 and ERN3. (Kaló *et al*., 2005; Smit *et al*., 2005; Heckmann *et al*., 2006; Andriankaja *et al*., 2007; Murakami *et al*., 2007; Middleton *et al*., 2007). These have been shown to bind to cis-regulatory elements of Nod factor-responsive genes such as *ENOD11*, and thus to regulate target gene expression (Andriankaja *et al*., 2007; Hirsch *et al*., 2009). Recently, first indications for a transcriptional regulator involved in Myc factor perception were found. The *ram1* mutant (reduced arbuscular mycorrhiza) was identified in a

screen for mycorrhiza-defective *M. truncatula* plants (personal communication with Giles Oldroyd, John Innes Centre, Norwich, UK). The corresponding gene encodes a GRAS type transcription factor that is indispensable for mycorrhizal colonization and Myc factor-induced response. Interestingly, a constitutively active version of the DMI3 protein triggers spontaneous nodule formation in the absence of rhizobia (Gleason *et al.*, 2006; Tirichine *et al.*, 2006), indicating that the activity of this protein is sufficient to trigger the organogenesis program. In this context, it has to be asked why no spontaneous nodule formation occurs in response to Myc factor or AM fungal contact-elicited activation of DMI3. The absence of spontaneous nodulation might be due to the existence of a negative regulatory mechanism concerning nodule formation occurring during AM. Interestingly also the calcium responses elicited by Nod factors are clearly distinguishable of the signature induced by AMF with the single spikes being less frequent and more irregular (Kosuta *et al.*, 2008). In which way those different signatures are decoded by DMI3 and transmitted into the individual transcriptional responses is not known and an interesting topic of research for the future.

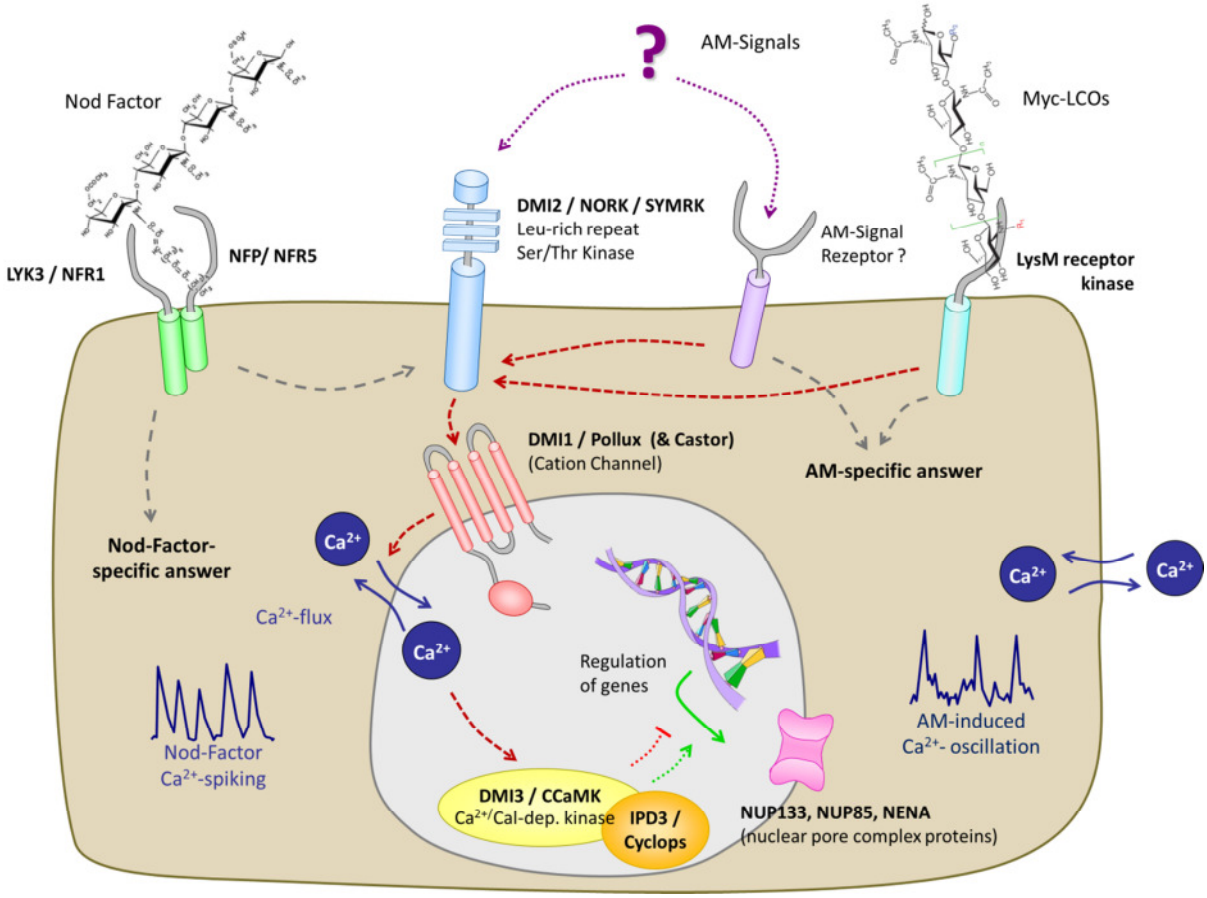


Figure 1.4 model of the SYM pathway common for AM and root nodule symbiosis
Perception of AM fungal or rhizobia-derived signals by specific receptors triggers early signal transduction. The receptor kinase DMI2 and the potassium channel DMI1 act upstream of the Nod- and Myc factor-induced calcium signatures. The putative nucleoporins NUP85, NUP133 and NENA are required for calcium spiking, although their mode of involvement is currently unknown. The calcium–calmodulin-dependent protein kinase DMI3 interacts with IPD3 within the nucleus and thus probably decodes the ca^{2+} signatures and mediates the activity of transcription factors, specific for both symbioses. *M. truncatula* as well as *L. japonicus* protein nomenclatures are given.

1.7 Differential gene expression in *Medicago truncatula* during establishment of arbuscular mycorrhiza symbiosis.

Several comparative studies were performed concerning the mycorrhiza-induced expression of genes (Journet *et al*., 2002; Wulf *et al*., 2003; Liu *et al*., 2003, 2004; Weidmann *et al*., 2004; Küster *et al*., 2004; Grunwald *et al*., 2004; Manthey *et al*., 2004; Maldonado-Mendoza *et al*., 2005; Hohnjec *et al*., 2005; Güimil *et al*., 2005; Franken *et al*., 2007; Gutjahr *et al*., 2008). However, most of the genes identified in those studies are AM-induced but not mycorrhiza-specifically expressed. A major step forward towards the identification of mycorrhiza-specifically expressed genes was the approach of Liu *et al*. (2007) who compared the expression patterns of *M. truncatula* genes during association with three different AM fungi. This led to the identification of a core set of 39 mycorrhizal-induced genes, which were further examined according to their local or systemic expression and induction in a split root system. In this approach, the authors were able to describe ten genes that were locally expressed in mycorrhizal roots in response to *G. intraradices*, *Glomus versiforme* and *Gigaspora gigantea* and not detected in comparable control roots. However, the induction of those genes was only tested during a mature symbiosis, while the expression over proceeding colonization was not assessed. In 2008, Gutjahr and colleagues examined the expression of 18 *Oryza sativa* genes induced during AM development (Güimil *et al*., 2005). Among those, they found three genes to be upregulated during early interaction with *G. intraradices* and two of them, a putative peroxidase (*AM1*) and a protease inhibitor (*AM2*), expressed only locally in a split root system. However, *in situ* hybridization with *AM1* revealed transcript localization in developing arbuscules and in cortex cells flanking intercellularly growing hyphae pointing to an expression at later stages. Despite the importance of the initial phase of recognition and appressoria formation during the interaction for the symbiosis, still little is known about mycorrhiza-specific induction of genes prior to or during appressoria formation:

A slight and transient induction of plant defense genes in response to contact with arbuscular mycorrhizal fungi was reported by several authors (Bonanomi *et al*., 2001, Blilou *et al.,* 2000; Ruíz-Lozano *et al*., 1999, Siciliano *et al*., 2007). Expression of those genes was however not mycorrhiza-specific but was also induced during pathogen attack. Weidman *et al*. (2004) reported an induction of genes prior to physical contact between AM fungus and root. The authors identified 11 genes expressed during and prior to appressoria formation. The corresponding proteins were suggested to play roles during cell wall modification, translation and signal transduction. An expansin was found to be induced during PPA formation in epidermal cells by Siciliano *et al*. (2007). Expansins are involved in cell wall loosening and modification and have previously been reported to be upregulated after infection of plants by

AM fungi (Journet *et al*., 2002; Liu *et al*., 2003; Weidmann *et al*., 2004). In addition, expansins were observed to localize along the walls of mycorrhizal cells and in the matrix of the periarbuscular space (Balestrini *et al*., 2005). An increased plasticity of the cell wall is probably needed for penetration of cells to allow entry of hyphae into epidermal and cortex cells as well as in the periarbuscular apoplast where they might contribute to the loosely connected state of the cell wall components. Additionally, it has been shown that expansins also play a role during root nodule symbiosis as well as during infection by nematodes (Giordano & Hirsch, 2004; Gal *et al*., 2006). Besides the expansin, Siciliano and associates identified in a suppressive subtractive hybridization-based quantitative PCR analysis eight genes solely induced during PPA formation. A different approach was chosen by Liu *et al*. (2003) who examined the expression of genes during appressoria formation by *G. versiforme* with the help of cDNA macroarrays. They likewise found several genes induced by the fungus. Nevertheless, almost none of the genes from the mentioned reports were shown to be mycorrhiza-specifically expressed and early induced. Mycorrhiza specifically induced genes are of special interest as they could serve as marker genes for an early time-point of mycorrhizal colonization. However, the only report describing early mycorrhiza-induced and specifically expressed genes was published by Brechenmacher *et al*. (2004) who could identify a *M. truncatula* germin-like protein, already described by Doll *et al*. (2003) as mycorrhiza specifically expressed during appressoria formation by *Glomus mossae*.

1.8 Involvement of hormones in the arbuscular mycorrhizal symbiosis

Arbuscular mycorrhiza symbiosis has substantial effects on the host physiology in terms of nutrition, growth, stress and pathogen resistance. Plant hormones are key regulators for virtually every aspect of plant physiology, affecting gene expression and transcription levels, cellular division, and growth. They have also been reported to act as signals during the interaction of plants with microbes (Pozo & Azcón-Aguilar, 2007; Bari & Jones, 2009; Verhage *et al*., 2010; Spaepen & Vanderleyden, 2011). For establishment and functioning of the AM symbiosis, it is necessary to fine-tune the interaction in order to coordinate plant and fungus. Regarding this, plant hormones are believed to be involved in regulating the molecular interchange that orchestrates the complex symbiotic program in the host plant (Bettina Hause *et al*., 2007; López-Ráez *et al*., 2010; Ludwig-Müller, 2010).

Most of the plant hormones described are known to play roles during the AM symbiosis (Hause *et al*., 2007) and subsequently some of the changes in the host roots are related to changes in the relative abundance of plant hormones. Advances in research on hormone involvement in AM mostly originate from the analysis of several mutants and transgenic plants disturbed in hormonal balance. Among phytohormones, ethylene, salicylic acid,

abscisic acid, and jasmonic acid are known to be key players in regulation of plant defense response to microbes (reviewed by Pieterse *et al*., 2009). In arbuscular mycorrhiza symbiosis an inverse correlation between root colonization and the levels of ethylene and salicylic acid has been reported (Blilou *et al*., 1999; Herrera Medina *et al*., 2003; Riedel *et al*., 2008). In contrast, abscisic acid and jasmonic acid were shown to have positive impact on arbuscule formation (Isayenkov *et al*., 2005; Herrera-Medina *et al*., 2007). Jasmonic acid and other jasmonates seem to play a major role during arbuscular mycorrhiza symbiosis (Hause et al., 2007). Experimental data gained from treatment of mycorrhizal plants with jasmonic acid revealed contrasting results as colonization was either promoted or inhibited by the addition of the hormone (Regvar *et al*., 1996; Ludwig-Müller *et al*., 2002). Interestingly, the effect strongly depended on the concentration of jasmonates applied to the plants, suggesting that the homeostasis of jasmonic acid within the plant may control the mycorrhization. Colonization of roots by arbuscular mycorrhizal fungi revealed increased levels of jasmonic acid in several plant species including *M. truncatula* (Vierheilig & Piché, 2002; Hause *et al*., 2002; Stumpe *et al*., 2005; Meixner *et al*., 2005), additionally indicating a role for this hormone in the symbiosis.

Cytokinin receptors are thought to be essential for the establishment of different symbioses of plants with beneficial microorganisms. In the case of arbuscular mycorrhiza, it was shown that infected plants accumulate cytokinins in both shoots and roots in comparison to non-colonized ones (Allen *et al*., 1980; Drüge & Schönbeck, 1992; van Rhijn *et al*., 1997). However, the levels increased only during a late phase of mycorrhization (Danneberg *et al*., 1992). Nevertheless, during establishment of the symbiosis cytokinins are supposed to be involved in suppression of defense responses, as a decreased expression of two pathogenesis-related genes was detected in mycorrhizal roots accompanied by increased levels of cytokinins (Ginzberg *et al*., 1998; Shaul *et al*., 1999). It is not certain, however, whether altered cytokinin abundance is due to plant or fungal production, as arbuscular mycorrhizal fungi have been shown to synthesize cytokinin-like substances as well (Barea & Azcón-Aguilar, 1982).

Auxin involvement during AM interaction has been proposed due to the fact that mycorrhizal roots exhibit morphological characteristics linked to auxin signaling, as for example an increased abundance of lateral roots (Ludwig-Müller, 2000). Several reports on altered levels of different auxin derivatives exist for mycorrhizal plants (Ludwig-Müller *et al*., 1997; Kaldorf & Ludwig-Müller, 2000; Torelli *et al*., 2000; Yao *et al*., 2005; Meixner *et al*., 2005; Fitze *et al*., 2005; Campanella *et al*., 2007; Jentschel *et al*., 2007; Ludwig-Müller & Güther, 2007; Meixner *et al*., 2007). Interestingly, different auxin derivatives seem to have differential impact in regulation of arbuscular mycorrhizal development as their level change in

dependence of progression of the colonization (Jentschel *et al*., 2007). An involvement of auxins in the early establishment of the symbiosis was proposed by Oláh *et al,* (2005) who observed a stimulation of lateral root formation in *Medicago truncatula* after treatment of roots with a diffusible factor from different arbuscular mycorrhizal fungi. This finding points to a role of auxin signaling during the early steps of symbiotic recognition. Recently, Hanlon & Coenen (2010) confirmed the observation that auxin signaling within host roots is required for early stages of AM formation, including presymbiotic stage. Thus, root branching in tomato mutant root organ cultures with hyperactive polar auxin transport was increased in response to inoculation with *G. intraradices*.

No reports have been found describing an involvement of brassinosteroids in arbuscular mycorrhizal development. However, brassinosteroids are interacting with multiple plant hormones (Zhang *et al*., 2009). Especially brassinosteroid and auxin signaling are closely linked, as a normal auxin response depends on an intact BR pathway (Vert *et al*., 2008). Accordingly, both hormones are acting synergistically to promote cell elongation, and mutants in either pathway show similar phenotypes, including dramatic growth defects (reviewed by Halliday, 2004).

Only few data have been published by now about a possible involvement of gibberellic acid (GA) in AM interaction. On one hand, a decrease of gibberellic acid in roots of mycorrhizal plants is described (Allen *et al*., 1982) but in contrast, also increased levels of GA were reported (Clapperton *et al*., 1985; Shaul-Keinan *et al*., 2002). The treatment of *Pisum sativum* with gibberellic acid, however led to a strong inhibition of arbuscular mycorrhiza formation (El Ghachtouli *et al*., 1996) and in agreement with this, treatment of *Citrus macrophylla* roots with the GA biosynthesis inhibitor paclobutrazol increased the AM colonization (Michelini *et al*., 1989).

1.9 *MtMSBP1* – a gene induced during early AM contact

During my Diploma Thesis, I described the identification and initiated the characterization of *MtMSBP1* a plant gene, which is induced during early interaction of *M. truncatula* and *G. intraradices*. The gene was found to be upregulated in a microarray comparison of root fragments in contact with appressoria forming *G. intraradices* with non-inoculated tissue. This induction was verified by real-time quantitative PCR and microscopical observations using promoter reporter constructs (Figure 1.5). Even more interesting, carrying out time-lapse microscopy, I was able to show that the expression of *MtMSBP1* is induced even prior to physical contact between the two partners as a result of perception of diffusible fungal signals by the plant (Figure 1.5; Figure 4.7; Kuhn *et al*., 2010).

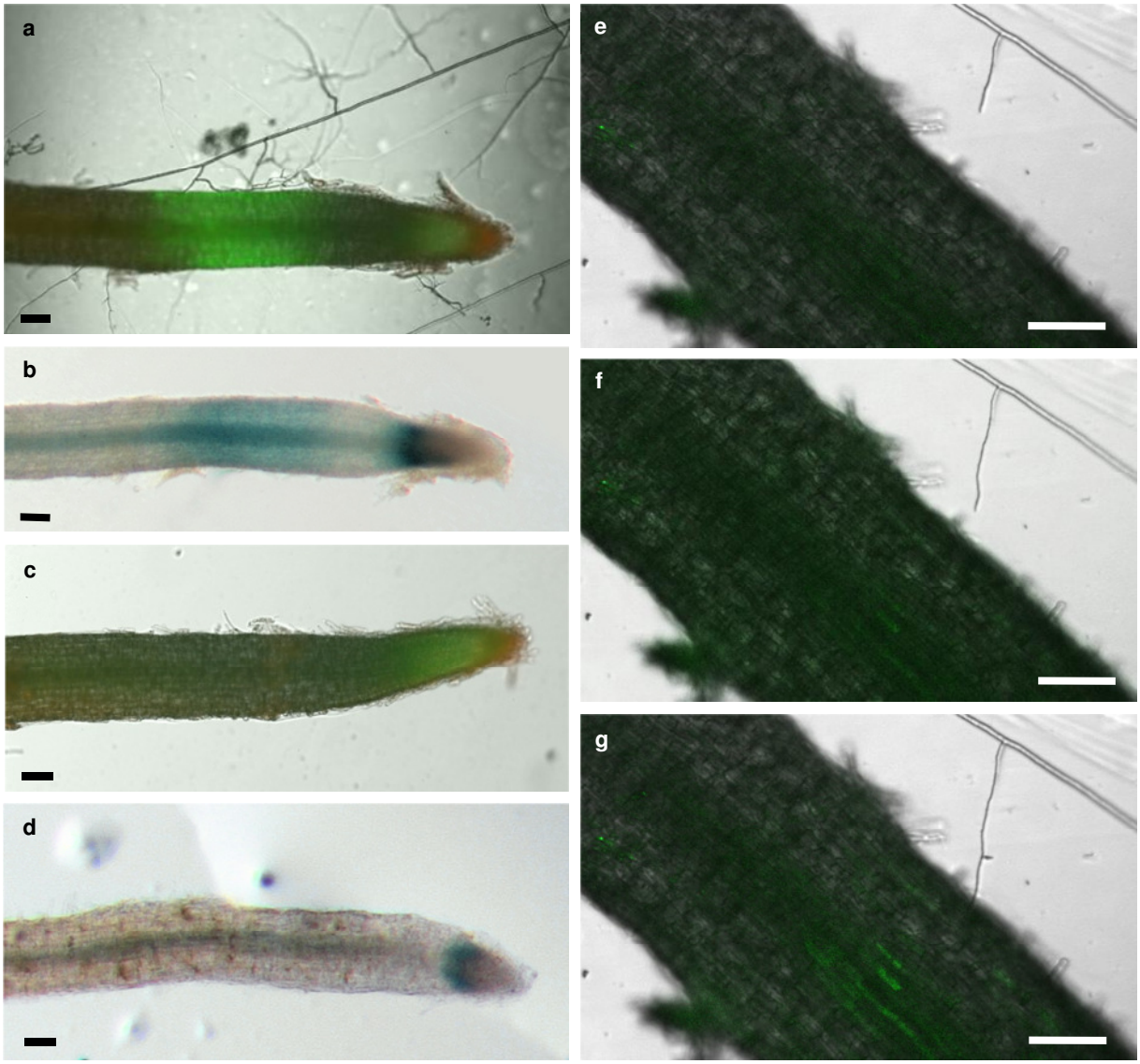


Figure 1.5 Analysis of P*MtMSBP1*$_{1518bp}$-GFPGUS promoter reporter
(a) To (g) Expression of the P*MtMSBP1*$_{1518bp}$-GFPGUS fusion in hairy root explants visualized by GFP-fluorescence ((a), (c), (e) – (g)) or histochemical staining for GUS-activity. (b), (d). (a), (b), (e) - (g) Expression of the P*MtMSBP1-GFP-GUS* construct in hairy roots mycorrhized *in vitro*. (a) Promoter activity indicated by GFP fluorescence in roots in contact with highly branched hyphae. (b) The same root as in (a) was stained for β-glucuronidase activity. (c) GFP-fluorescence and (d) GUS staining of a corresponding non-mycorrhizal control hairy root. (e) – (g) Time-lapse GFP expression study of *MtMSBP1* expression during early stages of mycorrhiza formation. Expression of the P*MtMSBP1*$_{1518bp}$-GFPGUS construct in *M. truncatula* hairy root explants inoculated with *G. intraradices*. *MtMSBP1* promoter activity, indicated by green GFP fluorescence, rises as branched hyphae, originating from a runner hypha, approach the root surface. Relative time is given in hours and minutes. Bar = 100 µm.

The *MtMSBP1* gene encodes a member of a protein family described as membrane bound steroid binding proteins with members in most eukaryotes. One of the plant homologues is the well-characterized *Arabidopsis thaliana* MSBP1 protein, which has 59 % identity to MtMSBP1 and was shown to bind several sterols including progesterone, stigmasterol and the brassinosteroid 24-epi-brassinolide (Yang *et al.*, 2005). AtMSBP1 was described to be involved in inhibition of cell elongation in the hypocotyl, stimulation of gravitropism by

regulating vesicle trafficking and auxin redistribution (Yang *et al*., 2008). An additional function assigned to AtMSBP1 is negative regulation of brassinosteroid (BR) signaling by enhancing the endocytosis of the BR receptor component BAK1 (Song *et al*., 2009). While the *A. thaliana* protein localizes at the cytoplasmic membrane (Yang *et al*., 2005), I found first indication for the *M. truncatula* homologue MtMSBP1 to be targeted to the ER membrane. This observation is consistent with the ER localization of animal and fungal homologues of the protein (Min *et al*., 2005), which was described for instance for humans, rats and *S. cerevisiae* (Nölte *et al*., 2000; Hand & Craven, 2003; Min *et al*., 2005). Nevertheless, the microscopic studies of MtMSBP1 localization fused to GFP were done with conventional light microscopy and will be repeated in this work using a confocal laser scanning microscope.

1.10 Aim of this work

Aim of this work is the identification of early mycorrhiza induced genes of *M. truncatula*. Candidate genes, upregulated during appressoria formation by *G. intraradices* will be tested for their mycorrhizal specifity and induction in response to diffusible fungal signals. The expression patterns of the genes during arbuscular mycorrhizal development will be analyzed. Moreover, induction of the genes in dependence of the SYM pathway and in response to different stimuli such as plant hormones will be investigated. Finally, promoter reporter constructs will be employed to examine the suitability of interesting candidate genes for use as marker genes for perception of fungal signals. Additionally, *MtMSBP1* a gene identified during my Diploma Thesis will be further characterized in its expression pattern and examined according to its function during arbuscular mycorrhiza development.

2 Material

2.1 Organisms

2.1.1 *Escherichia coli* strains

Standard culture methods were used.

DB3.1

Genotype:	F$^-$ gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1
Properties:	Streptomycin resistant (50 mg/l). A mutation in the gyrase (gyrA462) enables the cloning of plasmids containing the *ccdB*-gene.

Top 10 (Invitrogen C4040-10, C4040-03, C4040-06)

Genotype:	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str$_R$) *end*A1 *nup*G
Properties:	Streptomycin resistant (50 mg/l). Suitable for blue-white screening.

DH5α

Genotype:	F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–
Properties:	Suitable for blue-white screening.

XL1-Blue

Genotype:	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]
Properties:	Tetracyclin resistant (25 mg/l). Suitable for blue-white screening.

2.1.2 *Agrobacterium rhizogenes*

Agrobacteria were grown under constant shaking in TY- or LB media at 28°C with appropriate antibiotics.

ARqua1	Quandt, 1993 Agropine-type pRiA4b (pA4-type) (250 kb)
Ri plasmid:	pRiA4b, wt
Genotype:	Onc^{+}, Sm^{R}, biotype II (Keane *et al.*, 1970)
Phenotype:	Streptomycin resistant (600 mg/l) derivative of the wt strain R1000 (White *et al.*, 1985), derivative of the strain A4T (Moore *et al.*, 1979).
Properties:	Induces the growth of transgenic roots, which resemble wt roots in their morphology.

2.1.3 *Sinorhizobium meliloti*

Sinorhizobia were grown under constant shaking in TY or LB medium at 28°C with appropriate antibiotics.

Rm2011	J. Dénarié, INRA CNRS (Toulouse, France)
Genotype:	wt, Sm^{R}, plasmids pSymA (1.35 Mb) and pSymB (1.68 Mb)
Phenotype:	prototroph, symbiotic effective
Properties:	Streptomycin resistant (300 mg/l) derivative of the wt strain SU47.

2.1.4 *Daucus carota*

Hairy roots of *D. carot*a were grown on M medium (Bécard & Fortin, 1988).

2.1.5 *Medicago truncatula*

Jemalong A 17	Cultivar from Australia
Genotype:	Wt, Myc^{+}, Nod^{+}, Fix^{+}
	DMI1 (883 amino acids), DMI2 (925 amino acids), DMI3 (523 amino acids)
	(Penmetsa & Cook, 1997)
C71 (domi)	*dmi1-1*
	EMS Nod^{-}, Myc^{-} mutant of Jemalong A17. G1264A point mutation at

	the 5' splice site of the third intron. Causes a missplicing event. (Penmetsa & Cook 1997; Ané *et al.*, 2004)
TR25	*dmi2-1* γ-ray Nod$^-$, Myc$^-$ mutant of Jemalong A17. One-base pair deletion leading to a frame shift and a premature translational termination in the extracellular domain. predicted truncated protein of 356 amino acids. (Sagan *et al.*, 1995; Endre *et al.*, 2002)
TRV25	*dmi3-1* γ-ray Nod$^-$, Myc$^-$ mutant of Jemalong A17. Fourteen-base pair deletion leading to predicted truncated protein of 209 amino acids. (Sagan *et al.*, 1998, Lévy *et al.*, 2004)

2.1.6 *Glomus intraradices*

G. intraradices was grown in monaxenic culture together with *Daucus carota* hairy roots on M medium (Bécard & Fortin, 1988).

DAOM 181602	Initially isolated in Florida, USA (Schenck & Smith, 1982).
Genotype:	wt

2.1.7 *Colletotrichum trifolii*

C. trifolii race 2 MUT3930 (Ameline-Torregrosa *et al.*, 2008) was kindly provided by Richard O'Connell Max Planck Institute for Plant Breeding Research, Cologne. The fungus was grown on complete medium.

2.1.8 *Saccharomyces cerevisiae*

Yeast strain AH109 was designed for detecting protein interactions during a two-hybrid screen. It contains *ADE2*, *HIS3*, *lacZ,* and *MEL1* reporter constructs that are only expressed in the presence of GAL4-based protein interactions, when full-length GAL4 binds the corresponding upstream activating sequence. The genes are also expressed when an *in-vivo* two-hybrid interaction unites the GAL4 DNA-binding and activation domains. *S.cerevisiae* is grown in YPDA medium.

AH109 Clontech Laboratories, Inc. (USA): 630448.

Genotype: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, g*al80*Δ, *LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ, MEL1*wt

2.2 Chemicals

Chemicals were purchased from AppliChem GmbH (Darmstadt), Fluka Chemie GmbH (Taufkirchen), Merck KgaA (Darmstadt), Carl Roth GmbH & Co. (Karlsruhe) and Sigma Aldrich Chemie GmbH (Taufkirchen).

2.2.1 Organic chemicals

Table 2.1 Organic chemicals

chemical	provider: product number
2,4,5-T, 2,4,5-trichlorophenoxyacetate acid	(Duchefa Biochemie B.V., Haarlem, NLT0915.0025)
2,4-D, 2,4-dichlorophenoxyacetic acid	(Duchefa Biochemie B.V., Haarlem, NL: D0911.0100)
2-Log DNA Ladder	(New England Biolabs GmbH, Frankfurt a.M.: N3200)
Agar-Agar (Kobe I pulv.)	(Carl Roth GmbH & Co., Karlsruhe: 5210.2)
Agarose Electrophoresis Grade	(Invitrogen GmbH, Karlsruhe: 15510-027)
Ampicillin sodium salt	(Carl Roth GmbH & Co., Karlsruhe: K029.1)
Augmentin (Amoclav® 2,2 g dry powder)	(Hexal AG, Holzkirchen: Zul. Nr. 54801.0100)
Bromphenol blue sodium salt	(SERVA Electrophoresis GmbH, Heidelberg: 15375.01)
Chloramphenicol	(Duchefa Biochemie B.V., Haarlem, NL: C0113)
Chitin from shrimp shell	(Sigma Aldrich Chemie GmbH, Taufkirchen: C9752)
DEPC, Diethyl pyrocarbonate	(Carl Roth GmbH & Co., Karlsruhe: K028.2)
dNTPs (peqGOLD dNTP-Set 4 x 25 µmol)	(peqLab Biotechnologie GmbH, Erlangen: 20-2010)
EDTA disodium salt dihydrate	(Carl Roth GmbH & Co., Karlsruhe: 8043.2)
Ethidium bromide solution 1%	(Carl Roth GmbH & Co., Karlsruhe: 2218.2)
Formaldehyde 37%	(Carl Roth GmbH & Co., Karlsruhe: 7389.1)
Formamide	(Carl Roth GmbH & Co., Karlsruhe: P040.1)
GA3, Gibberellic acid	(Sigma Aldrich Chemie GmbH, Taufkirchen: G7645)
Glucanex	(Novozymes, Sigma Aldrich Chemie GmbH: L1412)
Glycin	(Carl Roth GmbH & Co., Karlsruhe: 3908.3)
IAA, indole-3-acetic acid	(Duchefa Biochemie B.V., Haarlem, I0901.0005)
IBA, indole-3-butyric acid	(Duchefa Biochemie B.V., Haarlem, I0902.0005)
Kanamycin monosulfate	(Duchefa Biochemie B.V., Haarlem, NL: K0126)
Myo-Inositol	(Sigma Aldrich Chemie GmbH, Taufkirchen: 5125)
1-NAA, 1-naphthalene acetic acid	(Duchefa Biochemie B.V., Haarlem, NL:N0903.0025)
Nicotinic acid	(Sigma Aldrich Chemie GmbH, Taufkirchen: N-4126)
PAA, Phenylacetic acid	(Sigma Aldrich Chemie GmbH, Taufkirchen: P1662-1)
Pelikan ink 4001, black	(Pelikan Vertriebsgesellschaft mbH & Co. KG., Hannover)

chemical	provider: product number
Phytagel (Gellam gum)	(Sigma Aldrich Chemie GmbH, Taufkirchen: P-8169)
Progesterone	(Sigma Aldrich Chemie GmbH, Taufkirchen: P878)
Pyridoxine hydrochloride	(Carl Roth GmbH & Co., Karlsruhe: T914.2)
RNAlater RNA Stabilization Reagent	(Qiagen GmbH, Hilden: 76104)
SDS /Sodium dodecyl lsulfate	(Carl Roth GmbH & Co., Karlsruhe: 4360.2)
Spectinomycin	(Duchefa Biochemie B.V., Haarlem, NL: S0188.0005)
Streptomycin sulfate	(Sigma Aldrich Chemie GmbH, Taufkirchen: S-6501)
Tetracyclin hydrochloride	(Carl Roth GmbH & Co., Karlsruhe: 0327.1)
Trypan blue	(Merck KgaA, Darmstadt: 1.11732.0025)
Trypton/Pepton	(Carl Roth GmbH & Co., Karlsruhe: 8952.2)
Tween®20	(AppliChem GmbH, Darmstadt: A1389.0500))
X-GlcA sodium salt	(Apollo Scientific Ltd, Bredbury, GB: BIMB1121)
Yeast extract	(Carl Roth GmbH & Co., Karlsruhe: 2363.2)

2.2.2 Enzymes

- Antarctic Phosphatase (New England Biolabs GmbH, Frankfurt a.M.: M0289 S)
- DNase I (Amplification Grade) (Invitrogen GmbH, Karlsruhe: 18047-019)
- Gateway® LR Clonase® II Enzyme Mix (Invitrogen GmbH, Karlsruhe: 11791020)
- MESA GREEN qPCR MasterMix Plus (Eurogentec GmbH, Köln, Germany: RT-SY2X-06+WOUFL)
- Phusion™ High-Fidelity DNA Polymerase (New England Biolabs GmbH, Frankfurt a.M.: F-530S)
- Proteinase K (Invitrogen GmbH, Karlsruhe)
- Restriction endonucleases (New England Biolabs GmbH, Frankfurt a.M.) (Fermentas, Burlington, Kanada) (Boehringer, Mannheim) (Amersham Biosciences, Piscataway, USA)
- SAP (Shrimp Alkaline Phosphatase) (Amersham Biosciences, Piscataway, USA: 70173)
- SuperScript™ II Reverse Transcriptase (Invitrogen GmbH, Karlsruhe: 18064-022)
- T4 DNA Ligase (Promega GmbH, Madison, USA: M1801)

2.2.3 Kits

- DNeasy Plant Mini Kit (Qiagen GmbH, Hilden: 69104)
- pENTR/D-TOPO® Cloning Kit (Invitrogen GmbH, Karlsruhe: K2400-20)
- innuPREP Plant RNA Kit (Analytik Jena AG, Jena: 845-KS-2060250)
- TOPO TA Cloning® Kit (with pCR®2.1-TOPO® vector) (Invitrogen GmbH, Karlsruhe: K4500-01)
- Zymoclean™ Gel DNA Recovery Kit (Hiss Diagnostics GmbH, Freiburg: D4002)

2.2.4 Oligonucleotides

Oligonucleotides were purchased form Eurofins MWG Operon GmbH. Primers for Real-time PCR were designed with the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Table 2.2 oligonucleotides

description	Sequence (5' – 3')	lenght	T_m
PSMBP10-F	**CAC C**GA AGA GGT CAT GAG CAT AT	23 bp	60,6℃ (52,4℃)
PSMBP10-R	GGT TTT TGA GGG TTG GTG GAG A	22 bp	60,3℃
MtMSBP1-F2	**CAC C**AT GGC ACT TCA ACT ATG GTC	24 bp	57.9℃ (53.7℃)
MtMSBP1-R3	TTC CTC CTC TTT ATC CGC ATC AAC	24 bp	57.9℃
UbiDsRED-F	CTA CAT TCT GAG CCT CTT TCC TTC	24 bp	61,0℃
UbiDsRED-R_{KpnI}	GTA TGA TAA TTC GAG **GGT ACC$_{(KpnI)}$** CG	23 bp	60,6℃
MtMSBP1-RNAi-F2	**CAC C**AA CCC TCA AAA ACC ATG	21 bp	57.9℃ (47.9 ℃)
MtMSBP1-RNAi-R2	CTC CAT TTC AGC AGC AAA GTC	21 bp	57.9℃
PTC106-F1	**CAC C**AA GTG TAG TAA GAG AGC GAC	24 bp	62.7 ℃ (55.3 ℃)
PTC106-F2	**CAC C**AG GAT GAA CCA ATA ATT CCA AAG	27 bp	61.9 ℃ (55.3 ℃)
PTC106-R1	GGT TTA TTA TTT GTT AGA TGT AAC TTG	27 bp	55.9 ℃
TC106noSP-Asc1-F	TAA **GGC GCG CC**A TGC AGT TTG TCT TGG ACA CAG TC	35 bp	73.0 ℃ (57.9 ℃)
TC106noSTOPAscR	TAT **GGC GCG CC**T TCT TTC TGG AAA ACA AGA GGG A	34 bp	70.7 ℃ (57.1 ℃)
PTC107-F1	**CAC C**CT CTT TGC AAT CAC CAG AGG	24 bp	64.4 ℃ (57.3 ℃)
P1074T-F1	**CAC C**AA TAA AAA TGA ATT TAG CTT GAC	24 bp	57.4 ℃ (50 ℃)
PTC107-R1	CTT AAG AAT CAA ATT AAA GCC TTA ATG	27 bp	55.9 ° C
TC107noSP-Asc1-F	ATA **GGC GCG CC**A TGA GAA AAG ATC CAG GAA CAC ACT	36 bp	71.7 ℃ (56.5 ℃)
TC107noSTOPAscR	ATT **GGC GCG CC**T GCA TCA TAT TTA GCG ACA CTT G	33 bp	70.7 ℃ (57.1 ℃)
SMBP1-NdeI_fw	ATA **CAT ATG** CTC TTC GGT TCC TCA GAT ACC	30 bp	65.4 ℃ (59.8 ℃)

SMBP1-BamH1_rev	ATA **GGA TCC** TTC CTC CTC TTT ATC CGC	27 bp	65.0 °C (53.7 °C)
MtSERK2-Nde1_fw	GCG **CAT ATG** ATG GAA CAA GTG ACT TCA	27 bp	63.4 °C (49.1 °C)
MtSERK2-BamH1_rv	TAT **GGA TCC** AGC TCC AGT ATT ACT ACC	27 bp	63.4 °C (51.4 °C)
MTCyp51G1-Nde1_fw	GTA **CAT ATG** TTC ATA ATA CCC AAA TCC	30 bp	58.9 °C (46.9 °C)
MTCyp51G1-BamH_rv	TAT **GGA TCC** CTA TTG ATT AAC AGA AAG	27 bp	58.9 °C 44.6 °C)

Real-time PCR Primer:			
MtTEF-qPCR-F3	TAC TCT TGG AGT GAA GCA GAT G	22 bp	58.4 °C
MtTEF-qPCR-R2	GTC AAG AGC CTC AAG GAG AG	20 bp	59.4 °C
GiTEF_F1	TGT TGC TTT CGT CCC AAT ATC	24 bp	55.9 °C
GiTEF_R1	GGT TTA TCG GTA GGT CGA G	22 bp	56.7 °C
MtDella3-qPCR-F1	TTT GGC TGA AGG AGT TGA AAG	24 bp	55.9 °C
MtDella3-qPCR-R1	TCC ATC CAA CAA GAA GAC AAT G	25 bp	56.5 °C
MtGA2Ox-qPCR-F1	CAA AGA GTT CAC GTG GTT GG	23 bp	57.3 °C
MtGA2Ox-qPCR-R1	GAA AGG CCA AAT ATC ATG TGC	24 bp	55.9 °C
MtPT4-qPCR-F1	GTG CGT TCG GGA TAC AAT ACT	21 bp	57.9 °C
MtPT4-qPCR-R1	GAG CCC TGT CAT TTG GTG TT	20 bp	57.3 °C
MtMSBP1-qPCR-F3	CTG ATA AGC CTC TTC TTA TGG C	22 bp	58:4.1 °C
TC77110-rev	TCC AAC CTT AAC GTA CTT TCC C	22 bp	58.4 °C
TC95008-qPCR-F1	CAA CGA TCA TGA TAA ACC CTT G	22 bp	56.5 °C
TC95008-qPCR-R1	GAT TCA GCT TCA GTA GCT TCC	21 bp	57.9 °C
TC100804-qPCR-F3	GAG AAA GAT GCA ACG AGT GG	20 bp	57.3°C
TC100804-qPCR-R3	TCA TTC TTT CTG GAA CAC AAC TG	23 bp	57.1°C
TC106351-qPCR-F	TCA AAT TCA CAC CAT TTG CTC	21 bp	54.0°C
TC106351-qPCR-R	CCT TCT TCC ACT CTT GGT GTC	21 bp	59.8°C
TC107197-qPCR-F1	TGC TTT TTG AAT AAT TGT ATG TGA TG	26 bp	57.6°C
TC107197-qPCR-R1	TGC ATA AGT TAC CTT GTG AAA TCC	24 bp	55.3°C
TC112474-qPCR-F1	AGT GTG TTG GTG AAG TTT GTT TTT	24 bp	55.9°C
TC112474-qPCR-R1	TCA ATC GAA TAG AAT ACA TGT CTC G	25 bp	58.1°C
MtERF19_qpcrf1	TAA TCC TCC ACC GAT TCG AC	20 bp	57.3 °C
MtERF19_qpcrr1	TCC ATC ACA GAA AAA CCA ACC T	22 bp	56.5 °C
MtAP2-qPCR-F1	TTA TGC TAC ACC GGA AGC TG	20 bp	57.3 °C
MtAP2-qPCR-R1	TCT GGC AAA GTT GTT GCT TG	20 bp	55.3 °C

Restriction endonuclease recognition sites and CACC sequence for ligation into pENTR/D-TOPO are highlighted in bold. Melting temperatures of the oligonucleotide without CACC are given in parentheses.

2.3 Plasmids

2.3.1.1 pENTR/D-TOPO (Figure 2.1 a)

Properties: Plasmid for directional blunt end TOPO cloning of PCR-fragments. Entry vector for Gateway technology.

Selection: Kanamycin resistance (50 mg/l) in *E. coli*.

Origin: Invitrogen GmbH, Karlsruhe

2.3.1.2 pCR2.1/TOPO (Figure 2.1 b)

Properties: Plasmid for TOPO TA cloning of PCR-fragments with 3'-protruding A.

Selection: Blue-white screen, ampicillin- (100 mg/l) and kanamycin resistance (50 mg/l) in *E. coli.*

Origin: Invitrogen GmbH, Karlsruhe

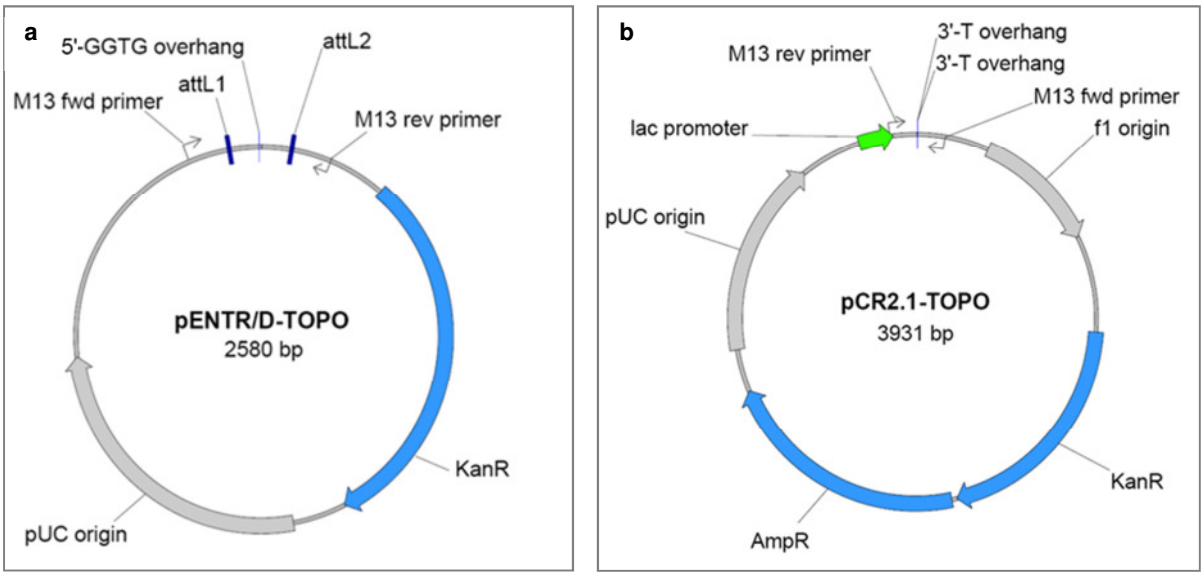


Figure 2.1 TOPO Vectors pENTR/D-TOPO and pCR2.1-TOPO
(a) pENTR/DTOPO for directional TOPO cloning. (b) pCR2.1-TOPO for TOPO TA cloning.

2.3.1.3 pKGWFS7,0 (Figure 2.2 a)

Properties: Binary Gateway destination plasmid for *Agrobacterium*-mediated transformation of plants. Promoter reporter vector with eGFP and GUS as reporter genes.

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1

Origin: (Karimi *et al.*, 2002)

University of Gent, Belgium, Flanders Institute for Biotechnology, Department of Plant Systems Biology, http://www.psb.ugent.be/gateway/index.php?NAME=pKGWFS7&_app=vector&_act=construct_show&

2.3.1.4 pPGFPGUS-RedRoot (Figure 2.2 b)

Properties: Binary Gateway destination plasmid for *Agrobacterium*-mediated transformation of plants. Promoter reporter vector with eGFP and GUS as reporter genes. Contains DsRED cassette for selection of transformed roots.

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1

Origin: pKGWFS7,0 (Karimi *et al.*, 2002) was modified to contain DsRED cassette from Limpens *et al.* (2005).

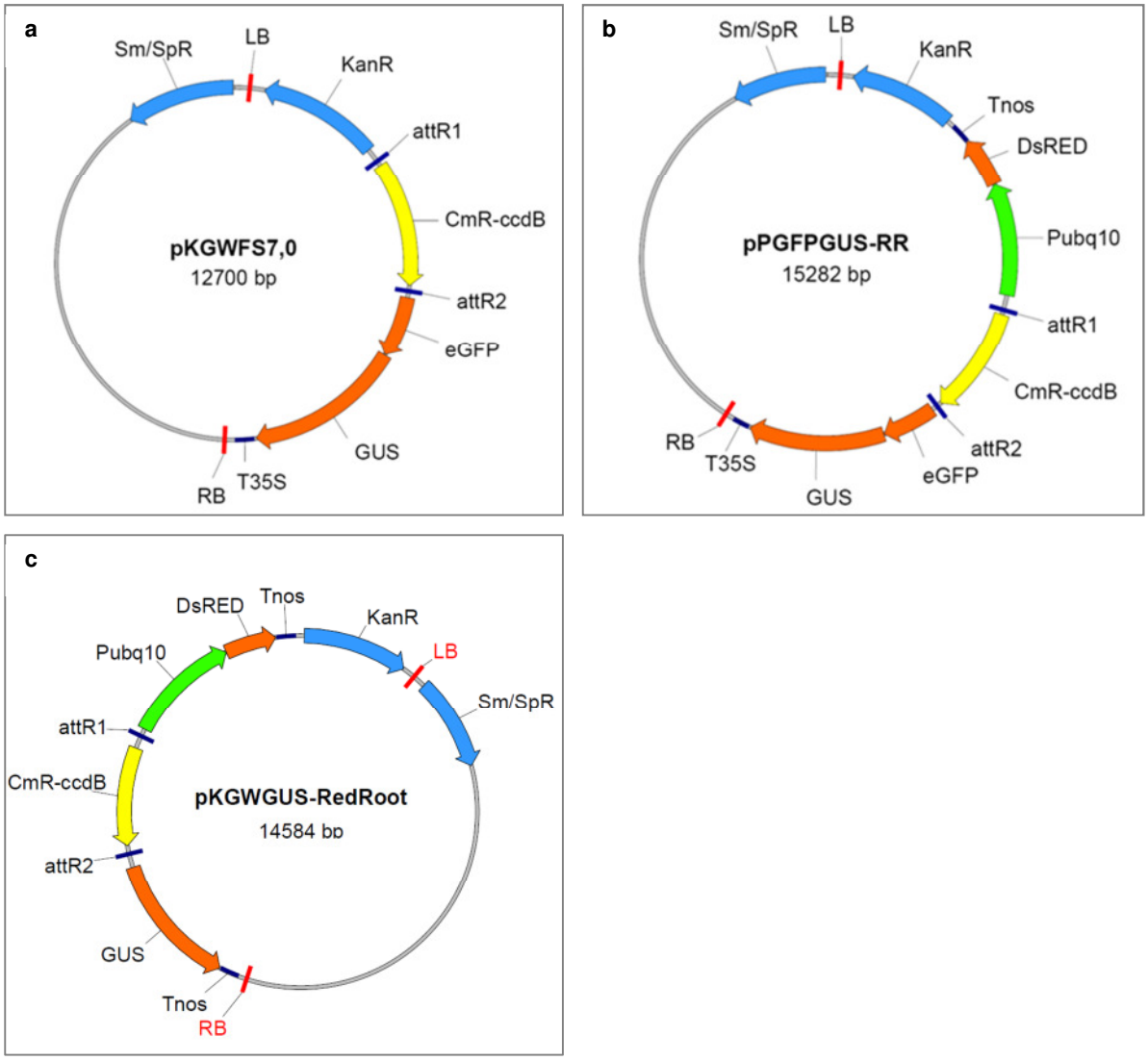


Figure 2.2 Promoter reporter plasmids pKGWFS7,0, pPGFPGUS-RR and pKGWGUS-RR
Gateway vectors for promoter reporter constructs with eGFP and GUS as reporter genes. (a) pKGWFS7,0. (b) pKGWFS7,0 modified to contain DsRED cassette. (c) pKGW,0 modified to contain DsRED cassette and GUS from pLP100.

2.3.1.5 *pKGWGUS-RR* (Figure 2.2 c)

Properties: Binary Gateway destination plasmid for *Agrobacterium*-mediated transformation of plants. Promoter reporter vector with GUS as reporter gene. Contains DsRED cassette for selection of transformed roots.

Selection: Spectinomycin (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli.*

Origin: Kindly provided by Cristina Albarran-Nogales: Modified pKGW,0 (Karimi *et al.*, 2002) to contain GUS cassette from pLP100 (Szabados *et al.*, 1995).

2.3.1.6 *pHKRedNuc* (Figure 2.3 a)

Properties: Binary Gateway destination plasmid for *Agrobacterium*-mediated transformation of plants. Vector for C terminal fusion of DsRED with nuclear localization signal of the *Aspergillus nidulans* stuA gene (Cloned over SpeI).

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1.

Origin: Modified pKGW,0 (Karimi *et al.*, 2002)

2.3.1.7 *pHKRedNuc-BlueRoot* (Figure 2.3 b)

Properties: Binary Gateway destination plasmid for *Agrobacterium*-mediated transformation of plants. Vector for C terminal fusion of DsRED with nuclear localization signal of the *Aspergillus nidulans* stuA gene (Cloned over SpeI). Contains CFP cassette for selection of transformed roots (Cloned over KpnI).

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1.

Origin: Modified pKGW,0 (Karimi *et al.*, 2002)

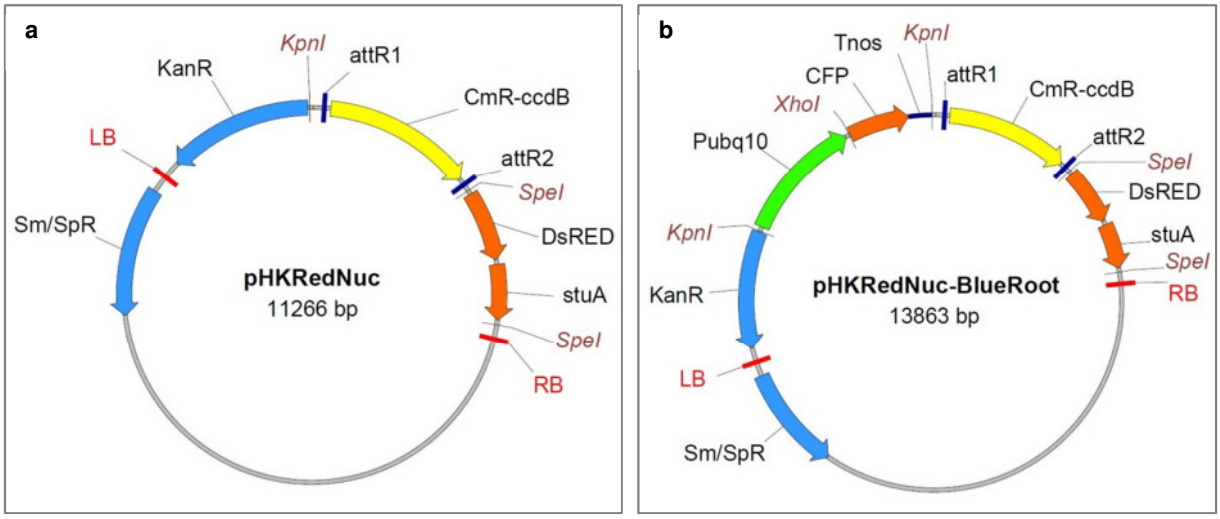


Figure 2.3 Plasmids pHKRedNuc and pHKRedNuc-BlueRoot
Gateway vectors for C terminal fusion of DsRED-stuA (a). pHKRedNuc. (b) pHKRedNuc was modified to contain the CFP cassette.

2.3.1.8 pK7FWG2,0 (Figure 2.4 a)

Properties: Binary Gateway destination plasmid for Agrobacterium-mediated transformation of plants. Vector for C terminal fusion of GFP.

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1

Origin: Kamiri *et al.*, 2002
University of Gent, Belgium, Flanders Institute for Biotechnology, Department of Plant Systems Biology,
http://www.psb.ugent.be/gateway/index.php?NAME=pKGWFS7&_app=vector&_act=construct_show&

2.3.1.9 pCGFP-RedRoot (Figure 2.4 b)

Properties: Binary Gateway destination plasmid for Agrobacterium-mediated transformation of plants. Vector for C terminal fusion of GFP. Contains DsRED cassette for selection of transformed roots.

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1.

Origin: Modified pK7FWG2,0 (Karimi *et al.*, 2002) to contain DsRED cassette from Limpens *et al.* (2005).

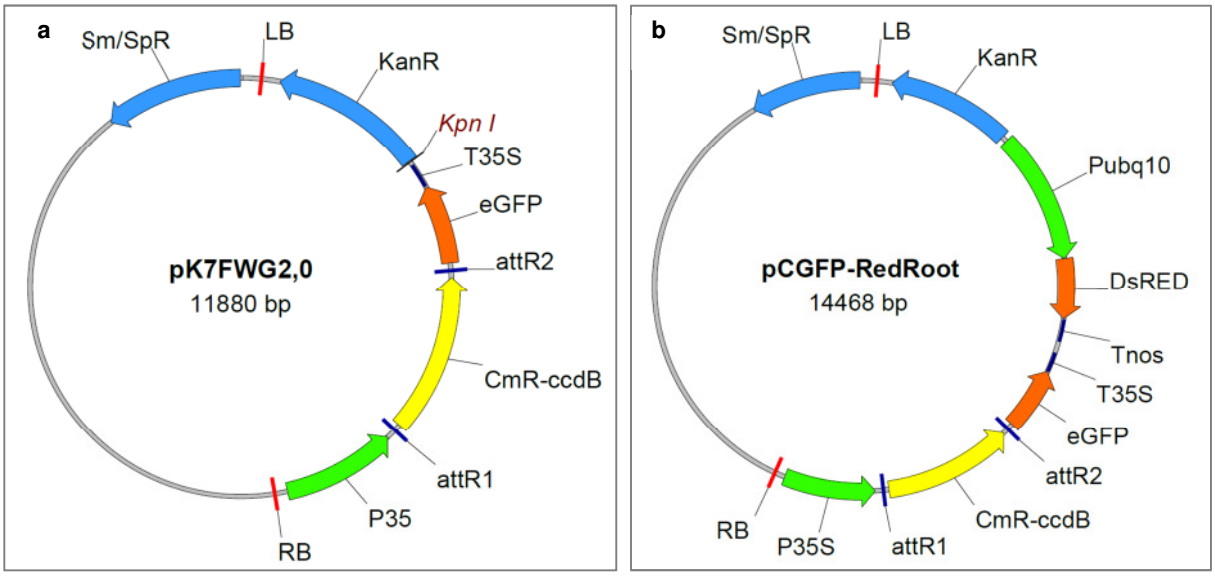


Figure 2.4 Plasmids pK7FWG2,0 and pCGFP-RedRoot
Gateway vectors for C terminal fusion of eGFP (a). pK7FWG2,0. (b) pK7FWG2,0 was modified to contain the DsRED cassette

2.3.1.10 pK7GWIWG2DII (Figure. 2.5)

Properties: Binary Gateway destination plasmid for Agrobacterium-mediated transformation of plants. Vector for expression of hairpin RNAs for gene silencing.

Selection: Spectinomycin resistance (50 mg/l) and Chloramphenicol resistance (34 mg/l) in *E. coli* DB3.1.

Origin: (Kamiri *et al.*, 2002)
University of Gent, Belgium, Flanders Institute for Biotechnology, Department of Plant Systems Biology,
http://www.psb.ugent.be/gateway/index.php?NAME=pKGWFS7&_app=vector&_act=construct_show&

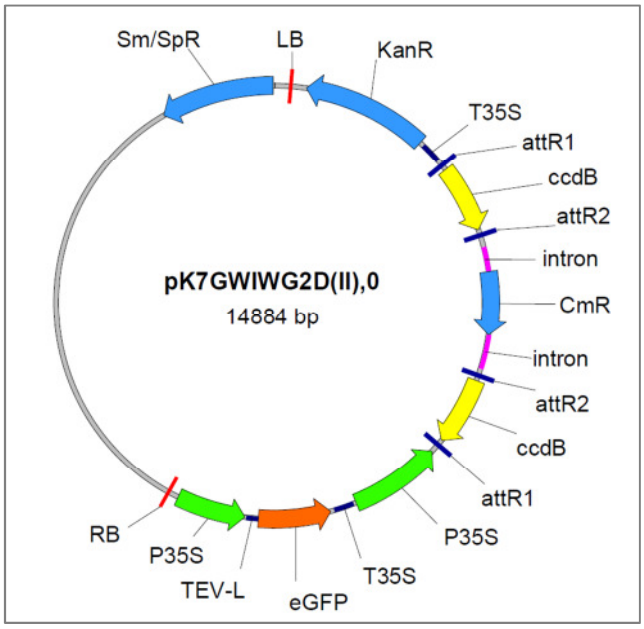


Figure 2.5 Plasmid pKGWIWG2D(II),0
Gateway vectors for construction of RNAi silencing constructs.

2.3.1.11 pGADT7-Rec (Figure 2.6 a)

Properties: Plasmid for yeast two hybrid analysis. Vector for N terminal fusion of GAL4 activation domain.

Selection: Ampicilin resistance (100 mg/l) in *E. coli*.

Origin: Clontech Laboratories, Inc. (Louret *et al.*, 1997).

2.3.1.12 pGBKT7 (Figure 2.6 b)

Properties: Plasmid for yeast two-hybrid analysis. Vector for N terminal fusion of GAL4 DNA binding domain.

Selection: Kanamycin resistance (50 mg/l) in *E. coli*.

Origin: Clontech Laboratories, Inc. (Louret *et al.*, 1997).

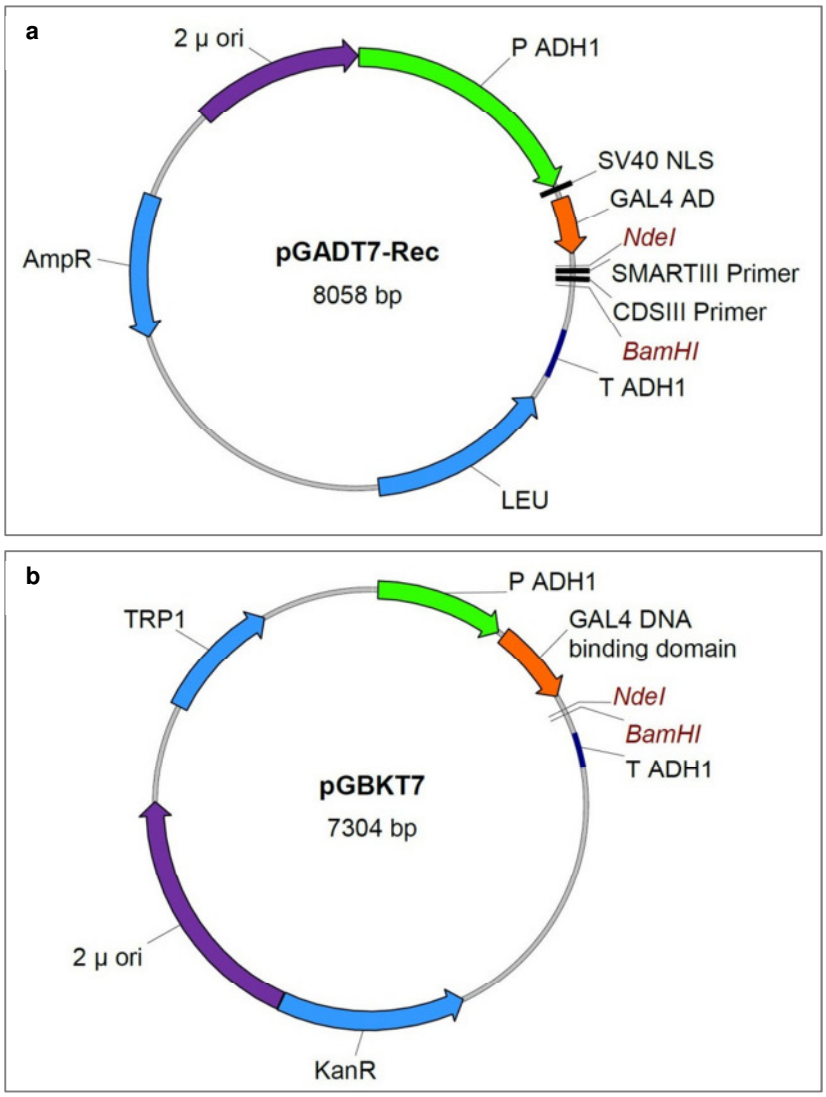


Figure 2.6 Vectors for yeast two hybrid analysis
Vectors for N terminal fusion of the GAL4 activation domain (a). or DNA binding domain (b). The plasmids contain the 2 µ origin for extra chromosomal replication in *S. cerevisiae* and leucin or tryptophan as auxotrophic markers for selection.

2.4 Solutions and buffers

All buffers and solutions were prepared with distilled water if not described otherwise.

2.4.1 Chitin

Chitin stock was prepared as described in Millet *et al*. (2010): 250 mg Chitin was resuspended in 25 ml H_2O and the suspension was autoclaved. The supernatant was used as stock solution and stored at - 21°C.

2.4.2 Hormone solutions

Progesterone, IAA, IBA, 1-NAA, 2,4-D, 2,4,5-T, 4-Cl-IAA, PAA and Picloram were solved in 100 % Ethanol, prepared as 1000 x stock solutions, and stored at - 21 °C.

2.4.3 Citric acid / sodium citrate buffer

Used for isolation of *G. intraradices* spores out of M medium. 17 ml of 0.1 M citric acid and 83 ml of 0.1 M sodium citrate were mixed and dH_2O was added to give 1 l. pH was set to 6.0 with NaOH.

2.4.4 GUS Stain

GUS buffer:	50 mM NaH_2PO_4 pH 7, 1 mM EDTA
GUS prefix buffer:	50 mM NaH_2PO_4 pH 7, 1 mM EDTA, 0.3 % Formaldehyde
0,1 M X-GlcA stock solution	22.22 mg X-GlcA in 500 µl N,N-dimethylformamide.

2.4.5 Plasmid miniprep:

I. 50 mM TrisHCl pH 7.5, 10 mM EDTA, 100 µg/ml RNAse A
II. Lysis Puffer, 0,2 M NaOH, 1 % SDS
III. 0.9 M KAc (75 ml stock solution, 175 ml H_2O (stock: 60 ml 5 M KAc, 11.5 ml concentrated acetic acid, 28.5 ml H_2O)

2.4.6 10x TAE buffer (pH 8,5)

Used for agarose gel electrophoresis of nucleic acids. 242 g tris(hydroxymethyl) aminomethane and 37.2 g Na_2 EDTA dihydrate were dissolved in 4.5 l water. 57.1 ml of concentrated acetic acid were added and filled up with water to give 5 l. Buffer was used in 0.5 x concentration.

2.4.7 TB buffer (500 ml)

Used for preparation of chemically competent *E. coli*. 1.512 g of pipes was dissolved in 450 ml of water and pH was set to 6.7. 9.32 g of KCl were added and buffer was sterilized by autoclaving. Afterwards 27.5 ml of sterile 1M MnCl and 7.5 ml of sterile 1 M $CaCl_2$ were added. The buffer was prechilled before usage.

2.4.8 TE buffer (10x)

10 ml tris buffer pH 8 and 20 ml 0.5 Na_2EDTA pH 8 were added to 800 ml water. Water was added to give 1 l and buffer was autoclaved. The buffer was used in 1 x concentration.

2.4.9 Polyethylene glycol (PEG 50 %)

50 g of PEG MW 3350 was soluted in ddH_2O to give 100 ml. The solution was autoclaved and stored in a tightly capped bottle to prevent evaporation of water and a subsequent increase in PEG concentration.

2.4.10 1 M Lithium acetate

The LiAc solution was prepared in ddH_2O and autoclaved. The final pH was be between 8.4 - 8.9.

2.4.11 1 M Tris buffer (1 l)

121.14 g tris(hydroxymethyl)aminomethane were added to 800 ml of water and the desired pH was adjusted with HCl. Was filled up to 1 l with water and autoclaved.

2.5 Media

All media were prepared with distilled water and autoclaved at 121 °C for 20 min. 1.5 % agar-agar was used for solid media if not described otherwise. Antibiotics were added after sterilization and cooling down to ca. 55°C.

2.5.1 LB medium

Used for cultivation of *E. coli*.

10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract. pH 7.

2.5.2 SOC medium

Used for transformation of bacteria.

5 g/l yeast extract, 20 g/l tryptone, 0.584 g/l NaCl, 0.186 g/ KCl, 2.4 g/l $MgSO_4$,. pH 7.5. 20 ml of sterile 20% (w/v) glucose were added after autoclaving.

2.5.3 SOB medium

5 g/l yeast extract, 20 g/l tryptone, 0.584 g/l NaCl, 0.186 g/l KCl, add 10 ml 1 M $MgCl_2$ after autoclaving. pH 7.5.

2.5.4 TY medium

Used for cultivation of *Agrobacterium rhizogenes* and *Sinorhizobium meliloti*.

5 g/l tryptone, 3 g/l yeast extract, 0.9 g/l $CaCl_2 \cdot 2\ H_2O$. pH 6.8.

2.5.5 Hewitt medium (Hewitt, 1966)

Used for watering of plants inoculated with *S. meliloti*.

Table 2.3 Hewitt medium

substance	Stock solution	Final concentration	per l of medium
$MgSO_4 \cdot 7\ H_2O$	18.4 g/l	18.4 mg/l	**1 ml**
Na-FeEDTA	2.45 g/l	24.5 mg/l	**10 ml**
$MnSO_4 \cdot 4\ H_2O$	2.23 g/l	2.23 mg/l	**1 ml**
H_3BO_3	18 g/l	3.06 mg/l	**0.17 ml**
$ZnSO_4 \cdot 7\ H_2O$	2.9 g/l	0.29 mg/l	**0.1 ml**
$CuSO_4 \cdot 5\ H_2O$	2.4 g/l	0.24 mg/l	**0.1 ml**
NaCl	5.85 g/ll	5.58 mg/l	**1 ml**
$CoSO_4 \cdot 6\ H_2O$	52.6 g/l	52.6 mg/l	**1 ml**
$K_2SO_4 \cdot 7\ H_2O$	2.9 g/l	33.35 mg/l	**11.5 ml**
$CaCl_2$	4.75 g/l	58.9 mg/l	**12.4 ml**
$NaH_2PO_4 \cdot 2\ H_2O$	20.8 g/l	208 mg/l	**10 ml**

The pH was set to 7.2 – 7.3.

2.5.6 YPDA medium

Used for cultivation of *S. cerevisiae.*

20 g/l Bacto peptone, 10 g/l Yeast extract, 0.003% adenine hemisulfate (15 ml of a 0.2% solution). pH 6.5. Add 2 % glucose (50 ml of a sterile 40% stock solution) after autolaving.

2.5.7 Drop out medium

Used for selection of *S. cerevisiae* transformants.

6.7 g/l Yeast Nitrogen Base (w/o amino acids), 20 g/l glucose (add 50 ml of a sterile 40 % solution after autoclaving), 100 ml drop out amino acid mix, 1.5 – 2 % Bacto Agar
pH to 5.6 or 6 – 6.5

drop out amino acid mix:

Prepared without amino acids used for selection. L-amino acids were used.

200 mg/l Adenine hemisulfate
200 mg/l Arginine HCl
200 mg/l Histidine HCl monohydrate
300 mg/l Isoleucine
1000 mg/l Leucine
300 mg/l Lysine HCl
200 mg/l Methionine
500 mg/l Phenylalanine
2000 mg/l Threonine
200 mg/l Tryptophan
300 mg/l Tyrosine
200 mg/l Uracil
1500 mg/l Valine

2.5.8 H_2O agar

Used for germination of seeds. 0.8% (w/v) agar-agar in distilled water.

2.5.9 Complete medium (1l)

Used for cultivation of *C. trifolii*.

50 ml 20 x nitrate salts
(120 g/l $NaNO_3$, 10.4 g/l KCl, 10.4 g/l $MgSO_4$ x 7 H_2O, 30.4 g/l KH_2PO_4)
1 ml 1000 x trace elements
(22 g/l $ZnSO_4$ x 7 H_2O, 11 g/l H_3BO_3, 5 g/l $MnCl_2$ x 4 H_2O, 5 g/l $FeSO_4$ x 7 H_2O, 1.6 g/l $CoCl_2$ x 5 H_2O, 1.6 g/l $CuSO_4$ x 5 H_2O, 1.1 g/l $(NH_4)_6Mo_7O_{24}$ x 4 H_2O, 50 g/l Na_4EDTA, pH 6.5-6.8 (KOH))
10 g glucose
2 g peptone
1 g yeast extract
1 g casaminoacids
1 ml vitamin solution
(0.1 g/l D-biotin, 0.1 g/l pyridoxine HCL, 0.1 g/l thiamine HCL, 0.1 g/l riboflavin, 0.1 g/l p-aminobenzoic acid, 0.1 g/l nicotine)
pH 6.5

2.5.10 M medium (Bécard & Fortin, 1988)

Used for cultivation of transgenic roots.

Table 2.4 M medium

substance	Stock solution	Final concentration	per l of medium
Macro elements			
KNO_3 [1]	3.2 g/l	80 mg/l	
$MgSO_4 \cdot 7\ H_2O$	29.24 g/l	731 mg/l	**25 ml**
KCl	2.6 g/l	65 mg/l	
KH_2PO_4 [1]	48 mg/l	4.8 mg/l	**100 ml**
$Ca(NO_3)_2$	115.2 g/l	288 mg/l	**2.5 ml**
Na-FeEDTA	3.2 g/l	8 mg/l	**2.5 ml**
Micro elements			
$MnCl_2 \cdot 4\ H_2O$	6 g/l	6 mg/l	**1 ml**
H_3BO_3	1.5 g/l	1.5 mg/l	
$ZnSO_4 \cdot 7\ H_2O$	2.65 g/l	2.65 mg/l	
other micro elements			
$Na_2MoO_4 \cdot 2\ H_2O$	0.0024 g/100 ml	0.0024 mg /l	**100 µl**
$CuSO_4 \cdot 5\ H_2O$	0.13 g/100 ml	0.13 mg/l	
Vitamins			
Glycine	0.3 g/l	3 mg/l	
myo-Inositol	5 g/l	50 mg/l	**10 ml**
Nicotinic acid	50 mg/l	0.5 mg/l	
Piridoxine HCl	10 mg/l	0.1 mg/l	
Thiamine HCL	10 mg/l	0.1 mg/l	

[1] Used in different concentrations in liquid medium

The pH was set to 5.5. 10 g/l D-Sucrose were added for *in vitro* culture of roots. 0.3 % (w/v) phytagel were added to solidify.

2.5.11 Modified Fåhraeus medium (Fåhraeus, 1957)

Used for transformation of *M. truncatula* roots.

Table 2.5 modified Fåhraeus medium

substance	Stock solution	Final concentration	per l of medium
Macro elements			
$CaCl_2$	0.9 M	0.9 mM	1 ml
$MgSO_4$	0.5 M	0.5 mM	1 ml
KH_2PO_4	0.7 M	0.7 mM	1 ml
Na_2HPO_4	0.4 M	0.8 mM	2 ml
Ferric Citrate	20 mM	20 µM	1 ml
NH_4NO_3	1 M	0.5 mM	0.5 ml
Micro elements			
$MnCl_2$	1 mg / ml	100 µg / L	100 µl
$CuSO_4$	1 mg / ml	100 µg / L	100 µl
$ZnCl_2$	1 mg / ml	100 µg / L	100 µl
H_3BO_3	1 mg / ml	100 µg / L	100 µl
Na_2MoO_4	1 mg / ml	100 µg / L	100 µl

The pH was set to 6.5. 0.9 % of agar-agar were added to solidify the medium. The medium was poured as slanted agar.

2.6 Special equipment

Table 2.6 special equipment

equipment	product	manufacturer
Real-time Cycler	iCycler MyIQ	BioRad Laboratories, München
Epifluorescence microscopes and cameras	Axio Imager. Z1 with Axiocam	Zeiss, Göttingen
	SteREO Lumivar.V12 with Axiocam	Zeiss, Göttingen
confocal laser scanning microscope	Leica TSP SP5	Leica Microsystems GmbH, Wetzlar

3 Methods

3.1 *In vitro* culture

3.1.1 Isolation of *G. intraradices* spores

Medium from hyphal compartment of bicompartemental plates was solved in three volumes of citric acid / sodium citrate buffer by stirring for ca. three hours. The spore suspension was washed with tap water through three sieves with pore sizes of 1000, 125, and 40 µm respectively. Spores were collected form the 40 µm sieve, dried on filter paper and frozen in liquid nitrogen.

3.1.2 Germination of *M. truncatula* seeds

Germination was conducted as described in Boisson-Dernier *et al*. (2001). In short, *M. truncatula* seeds were treated with concentrated sulfuric acid to sterilize the seed surface and scarify the water impermeable coat. The seeds were then washed with sterile water and placed onto water agar plates. Those were incubated at 4°C overnight in the dark to synchronize germination and afterwards germinated at 27°C for another night. For preparation of plants seedling were placed on modified Fåhraeus slanted agar in such a way that the roots pointed to the thicker side of the agar (Figure 3.1 a). The plates were sealed with parafilm and the roots covered with aluminum foil (Figure 3.1 b). After incubation in a 45° angle for one week at 25 °C with a cycle of 16 h of light (62,5 µE/sm^2) and 8 h of darkness the plates were incubated vertically further on.

3.1.3 Treatment of *M. truncatula* plants with ground spores of *G. intraradices*

Frozen spores isolated from 80 bipartite plates were ground in liquid nitrogen. M medium without sucrose was added to give a final volume of 50 ml. 2 ml of the spore suspension were added to respectively four *M. truncatula* plantlets in 2 ml reaction vessels. The plants were incubated in small greenhouses at 25 °C in a plant incubator with a cycle of 16 h of light (62,5 µE/sm^2) and 8 h of darkness. In case of incubation over 6 hours, the plants were kept constantly in light. After treatment, the roots were cut and RNA was extracted.

3.1.4 Treatment of *M. truncatula* plants with hormones or chitin

Three to four ten days old *M. truncatula* plants were transferred into 2 ml reaction vessels containing M medium without sucrose with 20 µM of phosphate supplemented with the substances that were to be tested. The cups were placed into small greenhouses that had

been sprayed with water to provide enough humidity. The greenhouses were incubated at 25 °C in a plant incubator with a cycle of 16 h of light (62,5 µE/sm^2) and 8 h of darkness. In case of an incubation over 6 hours the plants were kept constantly in light. After treatment, the roots were cut and RNA was extracted.

3.1.5 Preparation of transgenic *M. truncatula* hairy roots (Figure 3.1)

The Transformation *of M. truncatula* roots was done under sterile conditions after a modified protocol as described in Boisson-Dernier *et al.* (2001). Shortly 50 to 100 µl of an *A. rhizogenes* overnight culture with the respective binary plasmid were plated on TY agar and grown for 2 days at 28 °C. The root tip of *M. truncatula* seedlings was cut and the remaining root dipped into the bacterial lawn. Several treated seedling were placed on modified Fåhraeus slanted agar. The plates were sealed with parafilm and the roots covered with aluminum foil. After incubation in a 45° angle for one week at 21 °C with a cycle of 16 h of light (62,5 µE/sm^2) and 8 h of darkness, the plates were incubated vertically for up to three more weeks at 25 °C. Positively transformed roots were selected either by antibiotics resistance or by green or red fluorescence depending on the transformed T-DNA.

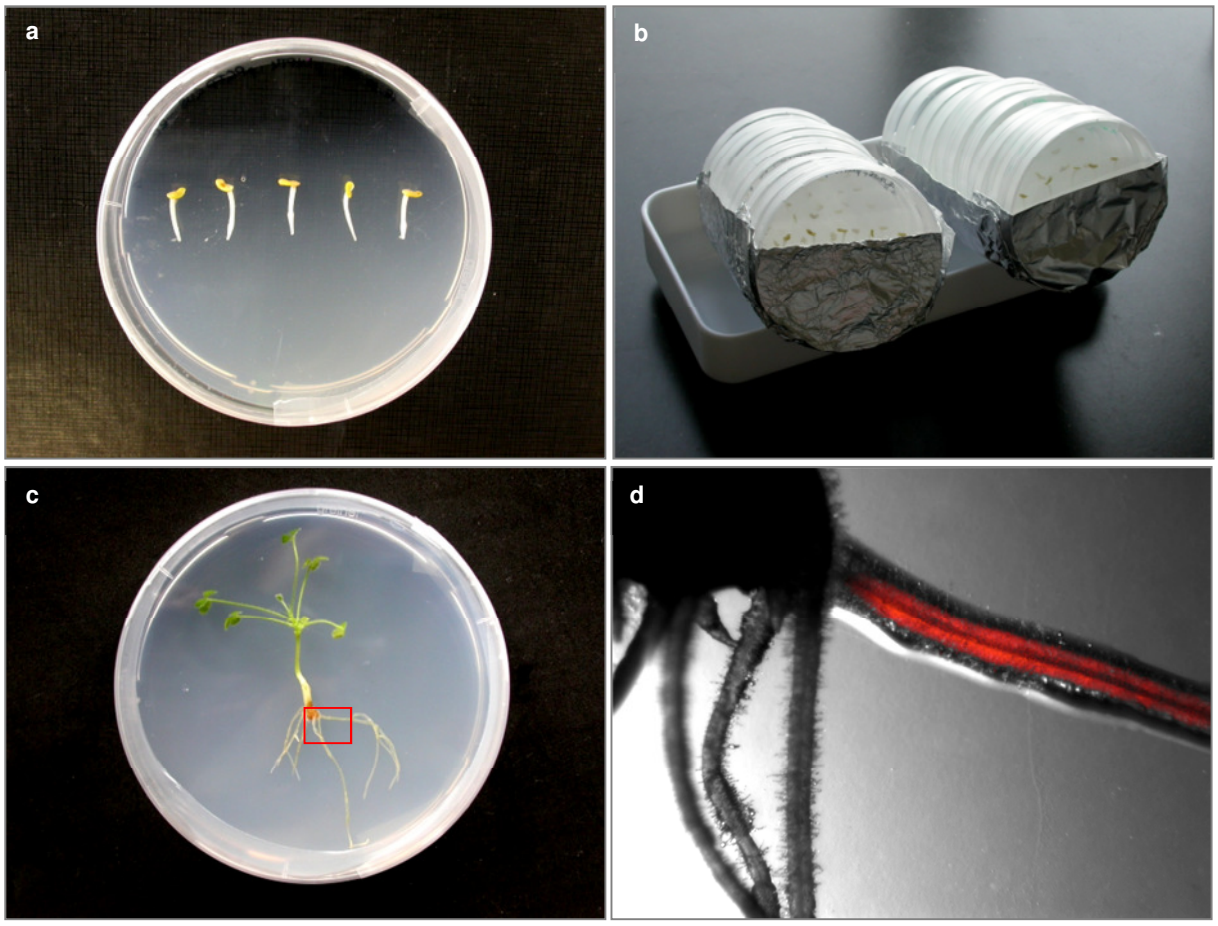


Figure 3.1 Transformation of *M. truncatula* roots (a) Agrobacterium treated seedlings of *M. truncatula* on modified Fåhraeus slanted agar. (b) The roots w covered with aluminum foil to protect them from light. (c) and (d) composite *M. truncatula* plant four weeks after transformation. Transgenic hairy roots are red fluorescent.

3.1.6 Explantation and *in vitro* culture of transgenic *M. truncatula* hairy roots (Figure 3.2)

To explant transgenic hairy roots of *M. truncatula* the roots were cut from composite plants under sterile conditions and propagated on M medium at 27 °C in the dark. To remove *A. rhizogenes,* the roots were subcultured on medium containing decreasing concentrations of augmentin (400, 200 and 100 and 0 mg/l).

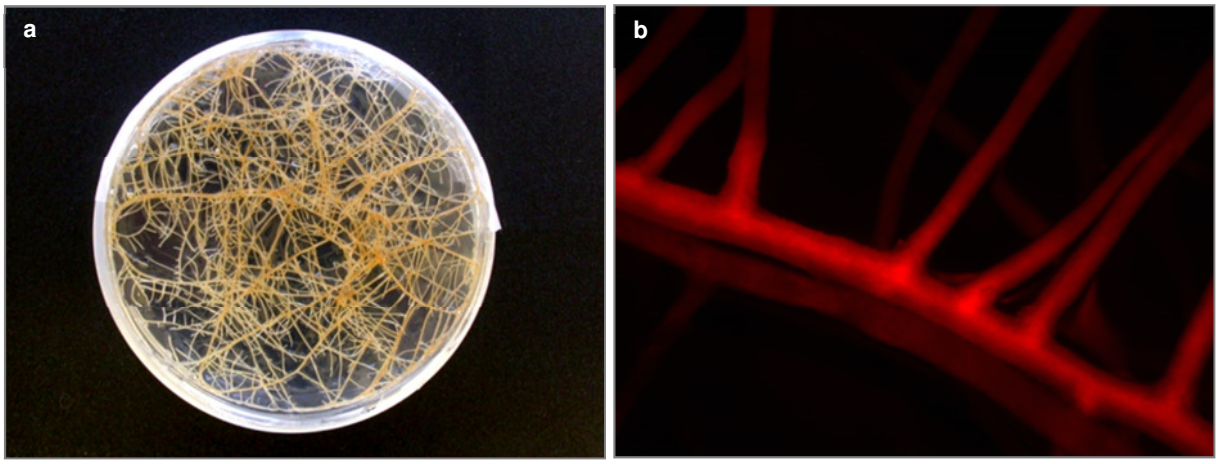


Figure 3.2 *in vitro* culture of transgenic *M. truncatula hairy roots*
(a) The roots are grown on solid M medium. (b) DsRED fluorescence of transgenic roots.

3.1.7 *In vitro* culture of mycorrhizal *Daucus carota* hairy roots

As an axenic culture of AM fungi is not possible, *G. intraradices* was grown in monaxenic culture together with *D. carota* hairy roots as host. The transgenic mycorrhizal roots were propagated as described for *M. truncatula* hairy roots. One to three month old roots were used as inoculum for new plates or for mycorrhization of other roots.

3.1.8 Mycorrhization of *M. truncatula* roots (Figure 3.3)

For colonization of other roots, *D. carota* hairy root – *G. intraradices* cultures were grown in the distal part of bipartite plates as described (St-Arnaud *et al*., 1996; Benedito *et al*., 2008; Takeda *et al.,* 2009). The proximal compartment contained M medium without sucrose, which allows the hyphae to propagate but prevents the roots from growing. When the ERM was fully developed (after 1 – 2 month), the medium of the hyphal compartment was removed and whole *M. truncatula* plants or hairy root explants were mycorrhized in this system either in liquid or solid M medium, respectively.

For colonization of entire plants, fresh extraradical mycelium was grown in 2 x liquid M medium without sucrose but with 20 µM of Phosphate in the proximal compartment as described in Helber & Requena (2009). After 10 d, three to five seedlings were planted into

openings on the plate lid close to the border of the bipartite plate. Plants were incubated at 25°C with a photoperiod of 16 h of light. The distal part of the plate was covered with black paper to protect the inoculum from light.

Hairy root explants were colonized on solid M medium containing 20 µM Phosphate. To prepare colonization, extraradical hyphae were allowed to grow on a cellophane membrane on top of the M medium. Hyphal passage to the distal compartment was facilitated by a swell (a ramp or step from the distal compartment to the border with the proximal compartment) and a thin layer of M medium poured on top of the cellophane membrane. To synchronize the colonization of hairy root explants, extraradical hyphae were grown ca. 7 d until the fungal compartment was covered with hyphae. Hairy root explants were then added and covered with some water. A second cellophane membrane or, in case of experiments that required microscopic observations, a bio foil (bioFoil50 (hydrophilic)) was set on top to maximize the contact area between fungus and root. The plates were incubated at 27 °C in the dark. Under these conditions, extraradical hyphae crossed the compartment border after two to three days and started to colonize the new roots.

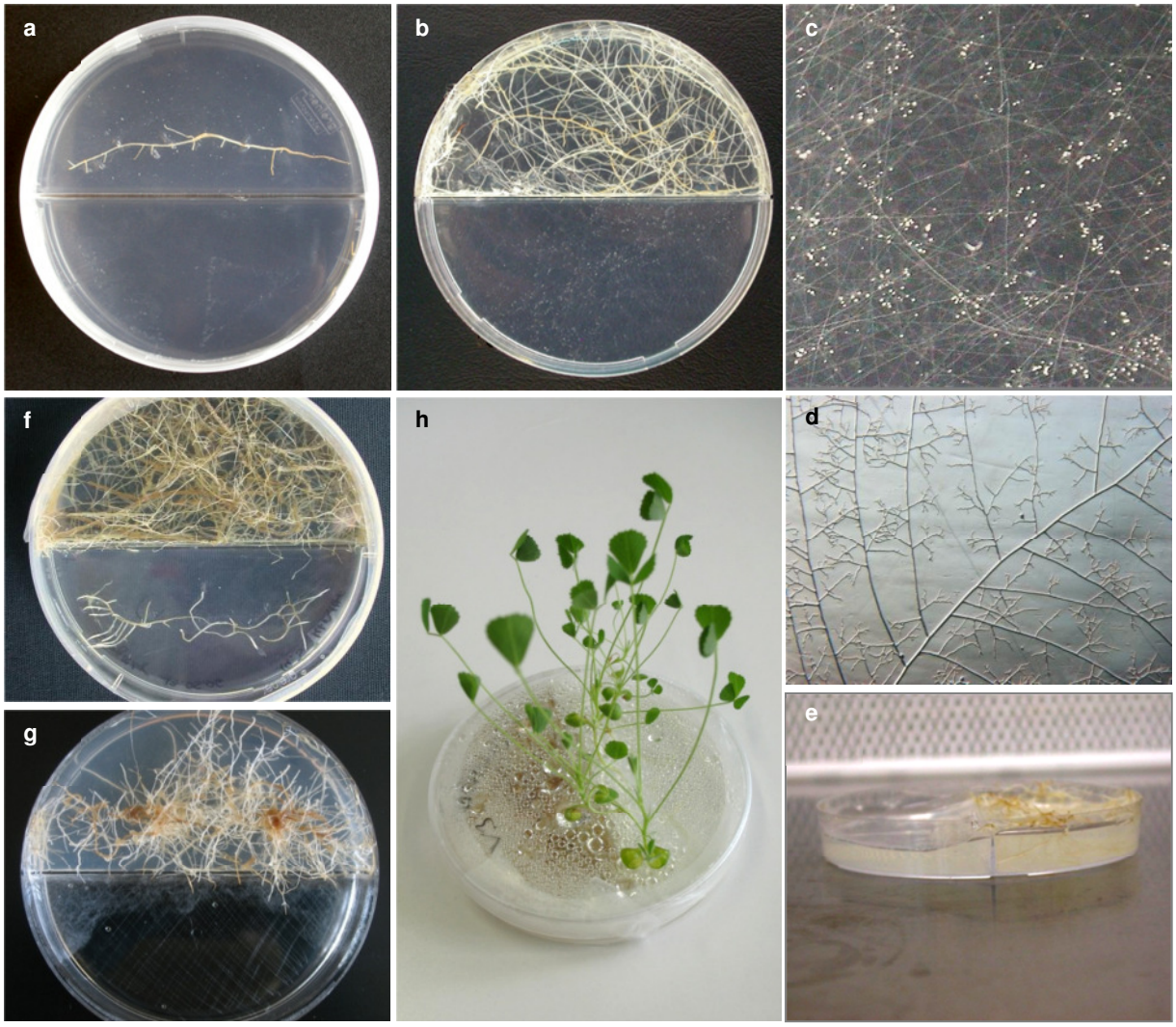


Figure 3.3 *in vitro* System for colonization of *M. truncatula* roots
In vitro system for mycorrhization of seedlings and hairy root explants. (a) Bi-partite plate freshly inoculated with *D. carota* roots. (b) Bi-partite plate after 6 weeks of growth of *D. carota* roots. (c and d) Hyphae and spores of *G. intraradices* growing in solid M medium, originating from *D. carota* mycorrhizal root cultures located in the distal compartment. (e) Bi-partite Petri dish prepared for inoculation of hairy root explants. The medium swell next to the compartment border facilitates colonization of the empty compartment by *G. intraradices hyphae*. (f) *M. truncatula* hairy root explants are colonized by *G. intraradices* hyphae originating from *D. carota roots*. (g) Bi-partite Petri dish for liquid inoculation of hairy root explants (h) *M. truncatula* seedlings, planted into liquid medium for inoculation with *G. intraradices* hyphae. The openings in the lid of the petri dish are sealed with sterile silicon fat.

3.1.9 Enrichment of appressoria-containing root fragments

To collect root fragments that were enriched in appressoria abundance, colonization of hairy roots was set up as described above. The growth of hyphae was observed every day with the help of a stereomicroscope. Contact sites between fungal hyphae and plant roots were cut and stored in RNAlater solution at 4 °C before being frozen in liquid nitrogen until further processing. Control fragments which were not in contact with hyphae were cut in parallel from plates without fungal inoculum.

3.1.10 Quantification of mycorrhizal colonization

Quantification of mycorrhizal colonization was done as described by (Trouvelot *et al.*, 1986) using the program 'Mycocalc' (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). In addition to the values described by Trouvelot *et al.* (1986) abundance of intraradical hyphae in the mycorrhizal part of the root system (i %) was determined as described for arbuscule abundance and calculated equally.

3.1.11 Inoculation of *M. truncatula* composite plants with *S. meliloti*

Composite *M. truncatula* plants with transgenic roots transformed with the promoter reporter construct P*MtMSBP1*$_{1518bp}$-GFPGUS were planted into 300 ml pots into calcinated clay soaked with Hewitt nutrient solution (Hewitt, 1966). Plants were inoculated with 5 ml of an overnight culture of *S. meliloti* RM2011 in TY medium, that was previously washed with 0.85 % NaCl solution. Plants were grown at 25 °C with a photoperiod of 16 hours of light and 8 hours of darkness, and watered with sterile water. After three weeks, plants were removed from the pots and nodules of transformed roots were stained for GUS activity.

3.1.12 Inoculation of *M. truncatula* hairy roots with *C. trifolii*

An equally amount of *C. trifolii* spores was inoculated on solid M medium with sucrose and grown for one week at 25 °C. *M. truncatula* hairy root explants were transferred to the plates either with or without separation by a cellophane membrane. Co-incubation was done for one day.

3.1.13 Treatment of *M. truncatula* hairy roots with sterols and lipids

1 ml of M medium with sucrose was pipetted into each well of sterile 12 well pates. The medium was pre-mixed with either 5 µl/ml of lipids or sterol preparation (Dissertation of Esther Serrano Padial: Biochemical characterization of the self-splicing GIN1 protein in arbuscular mycorrhizal fungi, Tübingen, 2005), 1 µl/ml of 1 mM progesterone solution (in EtOH) or the corresponding amounts of solvents. 3-5 transgenic hairy roots of *M. truncatula* were put into one well of sterile 12 well plates. The plates were incubated in the dark at RT for 24 h.

3.2 Histology

3.2.1 Staining for β-Glucuronidase (GUS) activity

Staining for β-Glucuronidase activity was done as described by (Fester *et al.*, 2002). Roots were incubated in 50 mM sodium dihydrogen phosphate (pH 7), 1 mM EDTA, 0.3%

formaldehyde for 30 min at room temperature. Then the roots were washed several times in 50 mM sodium dihydrogen phosphate (pH 7.0), 1 mM EDTA and stained for 2 h or up to overnight at 37°C in 1 - 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA) in the same buffer. After staining, the roots were washed with water.

3.2.2 Ink and vinegar stain of fungal structures in plant root tissue (Vierheilig *et al.*, 1998)

The staining procedure was carried out in small glass petri dishes on a hot plate. The roots were heated with 10 % KOH to 80° C for ca. 30 min until they appeared to be transparent. After thoroughly washing with water, the staining was conducted for 3 min with 5 % black Pelikan ink 4001 in household vinegar at RT. The roots were then washed several times with 10 % (v/v) vinegar in water and left in the same solution overnight for destaining. Stained roots were stored at RT in glycerol.

3.3 Microscopy

Microscopy was done either with the conventional epifluorescence microscope Zeiss AxioImager Z1 or the Laser Scanning microscope Leica TCS SP5 with long working distance water immersion objective HCX APO L U-V-I 63.0x0.90 WATER UV for higher magnifications. Roots were prepared on microscope slides or directly on solid medium and covered with water and bio foil for observation. eGFP was excited with an argon laser of 488 nm wavelength and emitted light was detected from 496 nm to 536 nm. DsRED1 was excited with a HeNe laser of 561 nm and emitted light was detected from 568 nm to 645 nm. For detection of more than one fluorescence sequential scanning was applied.

3.4 *In silico* Analyses

M. truncatula TC sequences were identified in the DFCI Medicago Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago) and checked for their expression in the Medicago gene expression Atlas (http://mtgea.noble.org). Genomic sequences were obtained by aligning TCs against the available genome data (http://medicago.org/genome/cvit_blast.php). Homologous proteins were identified via NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments were done with Jalview (http://www.jalview.org/download.html) using the Clustal W web sevice. The identification of conserved domains was done with ExPASy ScanProsite (http://www.expasy.org/tools/scanprosite/), Pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml) and EMBL-EBI InterProScan Sequence Search (http://www.ebi.ac.uk/InterProScan/). Protein localization was predicted by CBS TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) and

WoLF PSORT (http://wolfpsort.org/). Transmembrane helices and signal peptides were forecast with the help of CBS TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/).

3.5 Molecular biology techniques

3.5.1 Preparation of chemically competent *E. coli* (Inoue *et al.*, 1990)

Chemically competent *E. coli* were prepared on ice under sterile conditions with a protocol modified after the simple and efficient method described by Inoue *et al*. (1990). One colony of *E. coli* was inoculated in 50 ml of LB medium with appropriate antibiotics and grown over night at 37 °C under continuous shaking. The next morning 500 ml of SOB medium were inoculated with the overnight culture to give an $O.D._{600}$ of 0.2. The culture was grown at RT under continuous shaking until it reached an $O.D._{600}$ of 0.6. After 10 min on ice the bacteria were pelleted for 10 min at 2500 rpm and 4 °C in a GSA rotor. The pellet was resuspended in 200 ml of TB Buffer and left for 10 min on ice. Afterwards the centrifugation was repeated as described above. The pellet was resuspended in 10 ml of TB Buffer and 700 µl of DMSO were added. Bacteria were frozen in liquid nitrogen as aliquots of 100 µl.

3.5.2 Transformation of plasmids into chemically competent *E. coli*

Chemically competent cells were thawed on ice. The plasmid DNA was added and mixed by carefully stirring with the pipet tip. After 30 min of incubation on ice the bacteria were heat shocked for one minute at 42 °C. 500 µl of SOC medium were added and the cells were shaken for one hour at 37 °C before being plated on LB agar with appropriate antibiotics.

3.5.3 Preparation of electrocompetent agrobacteria

Electrocompetent agrobacteria were prepared under sterile conditions on ice. One colony of *A. rhizogenes* or was inoculated into 3 ml of LB medium with appropriate antibiotics and grown over night at 28 °C under continuous shaking at 250 rpm. 2 ml of the overnight culture were added to 500 ml of LB medium and grown under the same conditions until an $O.D._{600}$ of 0.5 – 0.6. The bacteria were then kept on ice for 15 min and afterwards centrifuged at 4 °C and 5000 rpm for 10 min in a GSA rotor. The pellet was resuspended in 500 ml of bidistilled water and centrifuged once more as described above. The washing step was repeated as before and the pellet subsequently resuspended in 10 ml of 10 % glycerol. After an additional centrifugation step, the cells were finally resuspended in 3 ml of 10 % glycerol and the bacteria were frozen in liquid nitrogen as 50 µl aliquots.

3.5.4 Transformation of plasmids into electrocompetent agrobacteria

Electrocompetent cells were thawed on ice. The plasmid DNA was added and the suspension was pipetted into a pre-chilled 4 mm gap electroporation cuvette. A current of 2 kV with 200 Ω and 25 µF was applied for ca. 5 msec. Afterwards 500 µl of SOC medium was added immediately. The bacteria were allowed to regenerate for 2 – 3 h under continuous shaking at 28 °C before they were plated on TY medium with appropriate antibiotics.

3.5.5 Transformation of *S. cerevisiae* (Gietz & Woods, 2002)

The yeast strain was inoculated the into 5 ml of liquid 2x YPDA medium and incubated overnight on a rotary shaker at 200 rpm and 30°C. A bottle of 2x YPDA and a 250 ml culture flask were prewarmed in the same incubator.

The titer of the yeast culture was determined by pipetting 10 µl of the culture into 990 µl of water in a spectrophotometer cuvette and measuring the OD at 600 nm. It was assumed that a suspension containing 10^6 cells/ml gives an OD_{600} of 0.1. 50 ml of the pre-warmed 2x YPDA were added to the pre-warmed culture flask and inoculated with the overnight culture to give 5 x 10^6 cells/ml. The flask was incubated at 30°C and 200 rpm. When the cell titer was at least 2 x 10^7 cells/ml (OD_{600} = 0.02 in a 1:100 dilution), which took about 4 hours, the cells were harvested by centrifugation at 5000 rpm for 5 min in a 50 ml tube centrifuge, washed in 25 ml of sterile water and resuspended in 1 ml of sterile water.

Salmon sperm carrier DNA was boiled for 5 min and chilled in an ice/water bath while harvesting the cells. The cell suspension was transferred to a 1.5 ml microcentrifuge tube, centrifuged for 30 sec and the supernatant was discarded. Water was added to a final volume of 1.0 ml and the suspension was vortexed vigorously to resuspend the cells. 100 µl of the cells were pipetted into 1.5 ml tubes, one for each transformation, centrifuged at maximum speed for 30 sec and the supernatant was removed. Sufficient transformation mix for the planned number of transformations plus one extra was prepared and kept on ice:

	number of transformations		
Reagent	**1**	**5 (6X)**	**10 (11X)**
PEG 3500 50% w/v	240 µl	1440 µl	2640 µl
LiAc 1.0 M	36 µl	216 µl	396 µl
Boiled SS-carrier DNA	50 µl	300 µl	550 µl
Plasmid DNA plus water	34 µl	204 µl	374 µl
Total	360 µl	2160 µl	3960 µl

360 µl of the transformation mix were added to each transformation tube and the cells were resuspended by vortex mixing vigorously. The tubes were incubated in a 42°C water bath for 40 min and afterwards centrifuged at maximal speed for 30 sec. The transformation mix was removed with a pipette. 1.0 ml of sterile water was added to each tube, the pellet was stirred with a pipette tip and gently vortexed.
10 µl of the suspension were diluted in 1.0 ml of water and 200 µl samples were plated onto SC selection medium plates. The yeast inoculum was spread onto the plate by gently distributing the fluid completely with a sterile glass rod with a minimum of strokes. The fluid was allowed to be taken up by the plate prior to incubation. The plates were incubated at 30°C for 3 to 4 days.
In case of transformation of pGADT7-Rec and pGBKT7 drop out medium plates minus LW without leucine, tryptophan (for selection of plasmids) and minus LWHA additionally without histidine and adenine (for selection of positive interactions) were used.

3.5.6 Isolation of bacterial plasmid DNA from *E. coli* (Miniprep)

A 3ml culture of the plasmid containing cells was grown overnight. The next morning 2 ml were pelleted at max rpm in a microcentrifuge. The cell pellet was resuspended in 200 µl of solution I. Afterwards 200 µl of solution II were added and mixed by inverting the tube. After addition of 200 µl of solution III the precipitated proteins and cell debris were pelleted by centrifugation at maximal rpm for 15 min. Subsequently the supernatant was decanted into 600 µl of isopropanol, mixed, incubated for 2 min at RT and the DNA was pelleted by centrifugation at maximal rpm for 15 min. 500 µl of 70 % EtOH were added to the emerging pellet and another 5 min centrifugation was done. The supernatant was discarded and the DNA-Pellet dried at 68 °C. The plasmid DNA was eluted in 50 µl of TE buffer for 10 min at 68 °C. The DNA was stored at -21 °C.

3.5.7 Isolation of plasmid DNA from *S. cerevisiae*

3 ml of 2x YPDA or drop out medium were inoculated with the yeast colony and grown for two days at 30°C and 200 rpm. 2 ml of the culture were centrifuged for 30 sec at maximal speed. The supernatant was discarded and the cells resuspended in 500 µl of freshly prepared glucanex solution (120 mg/l glucanex, 60 mg/l BSA). The suspension was incubated for 1 h at 30 °C and afterwards centrifuged for 1 min at 7900 g. The pellet was resuspended in in Solution I that was also used for preparation of plasmids out of *E. coli*. After 5 min of incubation at RT 50 µl 10 % SDS were added followed by 30 min of incubation in a 68 °C water bath. Next 200 µl of 5 M KAc were added and mixed by inverting the tube. After 1 h of incubation on ice, the suspension was centrifuged for 5 min at 13000 rpm and the supernatant transferred to 750 µl of isopropanol. The DNA was precipitated for 10 min at

13000 rpm and afterwards washed with 500 µl of 70 % EtOH. After a last centrifugation step the pellet was dried at RT and solved in 25 µl of dH_2O. 5 µl of the preparation were transformed into electrocompetent *E. coli*.

3.5.8 Isolation of genomic DNA from *M. truncatula*

Sterile grown plants of *M. truncatula* were ground in liquid nitrogen. Genomic DNA was extracted with the Qiagen DNeasy Plant Mini Kit according to the manufacturer's protocol 'Purification of Total DNA from Plant Tissue (Mini Protocol)'. The DNA was eluted in 50 µl. The quality of the DNA was assessed by agarose gel electrophoresis and spectrophotometric measurement. The genomic DNA was stored at 4 °C.

3.5.9 Determination of DNA and RNA concentration and quality

The concentration of nucleic acids was calculated in respect to the absorption of the bases at 260 nm according to the following formula:

$C\ [\mu g/ml] = A_{260} \times V \times F$

Where A_{260} represents the absorption at 260 nm wavelength, V the dilution coefficient of the sample and F represents a multiplication factor, specific for each kind of nucleic acid (DNA: 50, RNA: 40). The contamination of nucleic acids with proteins was calculated by taking the quotient of A_{260} and A_{280}, which should give a value of 1.8 for DNA and 2.0 in the case of RNA. Other organic components as e.g. phenols or polysaccharides that absorb at 230 nm were detected by calculating the quotient of A_{260} and A_{230}, which should be higher than 1.8.

3.5.10 Agarose gel electrophoresis of nucleic acids

DNA or RNA fragments were separated in 0.8 % - 2 % agarose gels in 0.5 x TAE buffer depending on the size of the fragments. 2-Log DNA Ladder was used as a standard marker.

3.5.11 Isolation of DNA out of agarose gels

Isolation of DNA out of agarose gels was done with the Zymoclean™ Gel DNA Recovery Kit according to the manufacturer's protocol.

3.5.12 Precipitation of DNA

One tenth of volume of 3 M sodium acetate pH 5.2 and 2.5 x volume of ice cold >99 % EtOH were added to at least 100 µl of DNA solution. The DNA was precipitated for 30 min at – 80 °C or overnight at – 21 °C and afterwards pelleted by centrifugation at 4 °C and maximal rpm for 30 min. 500 µl of 70 % EtOH were added to the pellet and the centrifugation was

repeated for 10 min. Subsequently the DNA was dried at room temperature and eluted in distilled water or TE buffer for 10 min at 68 °C.

3.5.13 Restriction enzyme digestion of DNA

DNA was cut with restriction enzymes in the appropriate buffers according to the manufacturer's protocol. To prevent re-ligation of the fragments, DNA ends were dephosphorylated with Antarctic Phosphatase. The enzyme was added directly to the restriction reaction. Preparative digestions were set up in a minimal volume of 50 µl.

3.5.14 Ligation of DNA-fragments

DNA fragments were ligated into a vector backbone using a molar proportion of 1:3. 1µl of T4 DNA Ligase (1-3 U/µl) was used in a standard setup of 20 µl volume. The reaction was incubated at 16°C over night and directly used to transform competent bacteria.

3.5.15 Amplification of defined DNA-fragments via PCR with Taq polymerase

A PCR reaction was in most cases set up in 25 µl volume. As template 1 µl of a 1:100 – 1:1000 dilution of plasmid DNA or a 1:10 dilution of cDNA or genomic DNA was used. Additional reaction components were: 2.5 µl 10x PCR buffer, 1 µl (50 µM) $MgCl_2$, 1 µl 10 µM forward and reverse primer, 0.25 – 0.5 µl polymerase and DEPC-treated water.

The reaction conditions were the following for standard applications:

5 min at 94°C
30 sec at 94°C
30 sec at the T_m of primers
1 min each kb at 72°C
10 min at 72°C

Step 2 – 4 were repeated for 25 – 35 cycles depending on the kind of application.

3.5.16 Amplification of defined DNA-fragments via PCR with Phusion polymerase

PCR was performed as described in the manufacturer's protocol.

3.5.17 Cloning of PCR fragments into pCR 2.1/TOPO

PCR fragments with 3' A overhangs were treated as described in the manufacturer's protocol with the exception that only 0.5 µl of the vector was used in one reaction and the incubation time was extended to 30 min. When blunt ended PCR fragments were to be used, 0.25 µl of

Taq polymerase were added after the PCR and another 10 min Elongation step was performed prior to the TOPO cloning.

3.5.18 Directional cloning of PCR fragments into pENTR / D-TOPO

The reaction was set up as described in the manufacturer's protocol with blunt ended PCR fragments. Instead of 1 µl of vector only 0.5 µl were used and the incubation time was extended to 30 min.

3.5.19 L/R Recombination of entry and destination clone

L/R recombination was done with Gateway® LR Clonase® II Enzyme Mix as described in the manufacturer's protocol.

3.5.20 Isolation of total RNA from plant tissue

To isolate total RNA from plant tissue the samples were ground in liquid nitrogen either with a mortar and pestle or in a pre-chilled mixer mill with steel beads of 5 mm diameter. The RNA was extracted from the tissue powder by the use of the innuPREP Plant RNA Kit (Analytik Jena) according to the manufacturer's protocol. The elution was done in 25 – 30 µl of RNase free water and the sample was supplemented with 0.1 µl of RNAse Out (Invitrogen) per 10 µl of RNA. The integrity, quality and concentration of the extracted RNA was assessed by gel electrophoresis (1.2 % agarose) and spectrophotometrical measurement. Total RNA was stored at – 80 °C.

3.5.21 Synthesis of single stranded cDNA

If available, 1 µg of total RNA was digested with DNase I (Amplification grade; Invitrogen) for 15 min at RT in a 10 µl reaction with the appropriate buffer. The DNase digested RNA was tested for residual genomic DNA by amplification of a 241 bp fragment of MtTEF1α. In case of complete DNase digestion reverse transcription was performed in a 20 µl reaction using the SuperScript II reverse transcriptase and Oligo(dT)$_{(12\text{-}18)}$ primer (Invitrogen). Therefore 1 µl of 25 mM EDTA was added to the DNase treated RNA and the reaction was stopped by incubation at 65 °C for 10 min. Afterwards 1 µl of Oligo(dT)$_{(12\text{-}18)}$ primer (0.5 µg/µl) and 1 µl of 10 µM dNTPs were added and the reaction incubated for 5 min at 65 °C. Subsequently the reaction mixture was supplemented with a master mix consisting of 4 µl of 5x first strand buffer, 1 µl of SuperScript II RT (200 units), 1 µl 0.1M DTT, 1 µl RNaseOUT (40 units/µl) and the reverse transcription performed for 1 h at 42 °C. After 10 min at 70 °C to stop the reaction, the cDNA was stored at -20 °C.

3.5.22 Quantitative Real-time PCR

Transcript levels of genes were determined on a Bio-Rad iCycler MyIQ using MESA Green qPCR MasterMix Plus (Eurogentec) and a primer concentration of 0.2 pmol/µl in 25 µl reactions. A 1 µl sample of a 1:2.5, 1:5 or 1:10 dilution of single-stranded cDNA was used as a template. For each primer set (Table 2.2) gradient PCR was performed to determine the optimal annealing temperature. Final PCR conditions were selected as follows: 3 min at 95 °C (no cycling); 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C (40 cycles). Every real-time PCR run was done a in three biological replicates with three technical replicates per reaction. Melt curve data was collected after each run. Tenfold dilution standard curves were taken for each primer set to determine the efficiency of amplification. Threshold cycle location as well as further data analysis was performed with the IQ5 optical system software version 2.0 using the ΔΔCt method based on (Vandesompele *et al.*, 2002). Transcript levels were normalized to the constitutively expressed translation elongation factor 1-alpha MtTEF1a (TC106470, DFCI *M. truncatula* gene index) (Hohnjec *et al.*, 2003; Wulf *et al.*, 2003; Nyamsuren *et al.*, 2007) as follows:

$$= 2^{(\bar{x}\ \mathrm{Ct\ control} - \bar{x}\ \mathrm{Ct\ treatment})\ \mathrm{gene\ x} - (\bar{x}\ \mathrm{Ct\ control} - \bar{x}\ \mathrm{Ct\ treatment})\ \mathrm{MtTEF1\alpha}}$$

Relative expression values for individual treatments were calculated as follows:

$$= 2^{\bar{x}\ \mathrm{Ct}\ MtTEF1\alpha - \bar{x}\ \mathrm{Ct\ gene\ x}}$$

Standard deviation of relative expression:

$$= \sqrt{\sigma\ \mathrm{Ct\ gene\ x}^2 + \sigma\ \mathrm{Ct}\ MtTEF1\alpha^2} \times \ln 2 \times \mathrm{relative\ expression\ gene\ x}$$

4 Results

4.1 *M. truncatula* genes involved in early AM recognition

4.1.1 Identification of *M. truncatula* genes involved in early recognition processes during appressoria formation

Plants are able to perceive arbuscular mycorrhizal fungi at very early stages of the symbiosis even before physical contact (Kosuta *et al*., 2003; Weidmann *et al*., 2004 Cahbaud *et al*., 2011; Navazio *et al*., 2007; Kosuta *et al*., 2008; Gutjahr *et al*., 2009; Oláh *et al*., 2005; Maillet *et al*., 2011; Op den Camp *et al*., 2011). Among other reactions, this perception is manifested in changes in gene expression that prepare the plant for the accommodation of the fungal partner. In order to identify novel marker genes of an early interaction, *M. truncatula* seedlings were mycorrhized in vitro using a bi-compartmental plate system with liquid medium (Figure 3.3), in a previous work. Eleven days post inoculation, corresponding to the time-point of maximal appressoria abundance, plants were collected and analyzed under the stereomicroscope. Root segments harboring contact points were harvested and RNA was extracted. Transcriptional analysis, performed using the *M. truncatula* 16kOLI1 microarray (Hohnjec *et al*., 2005), yielded ca. 500 genes regulated *versus* non-colonized root segments (Kuhn *et al*., 2010).

Table 4.1 Results of the microarray analysis of selected early-induced genes

	name	*p*-value	M	A	n	putative annotation
TC106351	MT006798	0.400099	0.507442	8.380705	2	Kunitz type protease inhibitor STI-like
TC112474	MT015000	NA	0.951782	6.935900	1	Kunitz type protease inhibitor STI-like
TC106971	MT000362	0.036063	1.250916	10.894818	4	Steroid membrane binding protein
TC107197	MT002169	0.327960	1.472291	7.962890	2	Specific tissue protein
TC100804	MT015421	NA	5.228080	6.598630	1	Kunitz type protease inhibitor STI-like

M: log2-ratio of expression ratio. A: log2-ratio of signal intensities.

Several candidate genes upregulated during appressoria stage, with induction levels between 0.5 and 5 (Table 4.1) were checked for their *in silico* transcriptional profile using the *M. truncatula Gene* Index MtGI (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago and the *Medicago* gene expression Atlas (http://mtgea.noble.org; Figure 4.1) and validated by real-time PCR. Four genes were further verified in different biological replicates of appressoria-enriched hairy root explants (Figure 4.2). Three of those genes were previously identified as mycorrhiza-specific but their induction was only studied at later stages (*TC106351*, *TC112474*, *TC107197*; Wulf *et al*., 2003; Liu *et al*., 2007; Grunwald *et al*.,

2004). Two of those genes encoded Kunitz type soybean trypsin protease inhibitors while the third one was a protein of unknown function designated as Specific tissue protein. A family of putative trypsin inhibitors from the Kunitz type was previously found to be induced in *M. truncatula* four weeks after inoculation with *Glomus mossae* (Grunwald *et al.*, 2004). One of those genes designated as *MtTi1* corresponds to *TC10635*. Promoter reporter fusions made by the authors showed a strong promoter activity at arbuscules three weeks post infection. The fourth candidate gene, which was not previously reported as mycorrhiza-induced (*TC106972*), showed similarity to a family of membrane bound steroid binding proteins conserved among eukaryotes and was subsequently designated as *MtMSBP1*. This gene is not mycorrhiza specifically expressed but also responses to a couple of other stimuli such as hormone treatment or inoculation with *Phymatotrichopsis omnivora* (Uppalapati *et al.,* 2009). The observed induction of this gene during the appressoria stage in this work points to an interesting role of *MtMSBP1* also during symbiosis. As this was the first report of a mycorrhiza-dependent induction of this gene, the analysis of *MtMSBP1* was initiated during my Diploma Thesis and was followed up during this study. In further analysis the fifth gene, also encoding a Kunitz type protease inhibitor that had also been reported to be mycorrhizal specific induced was included in the experiments (*TC100804*, Liu *et al.*, 2007).

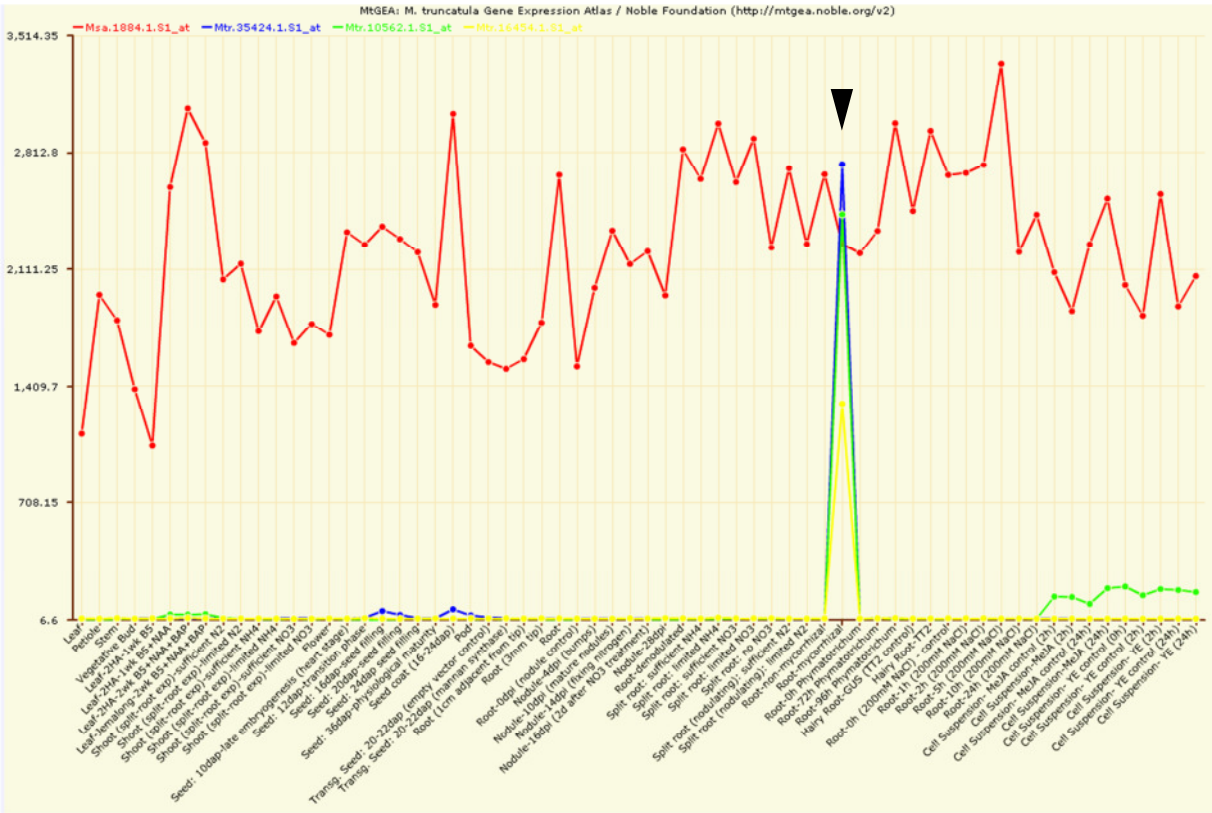


Figure 4.1 Expression pattern of selected genes in the *Medicago* gene expression Atlas
Expression of candidate genes in samples of the gene expression Atlas *TC100804* (Mtr.35424.1.S1_at; blue), *TC106351* (Mtr.16454.1.S1_at; yellow), *TC107197* (Mtr.10562.1.S1_at; green) are mycorrhiza specifically expressed (arrowhead; the mycorrhizal sample bases on roots, 21 days post infection) except for a weak basal expression. TC106971 (Mtr.1884.1.S1_at/*MtMSBP1;* red) is higher expressed but not induced in a fully developed symbiosis. No identical BLAST hit was found for *TC112474*.

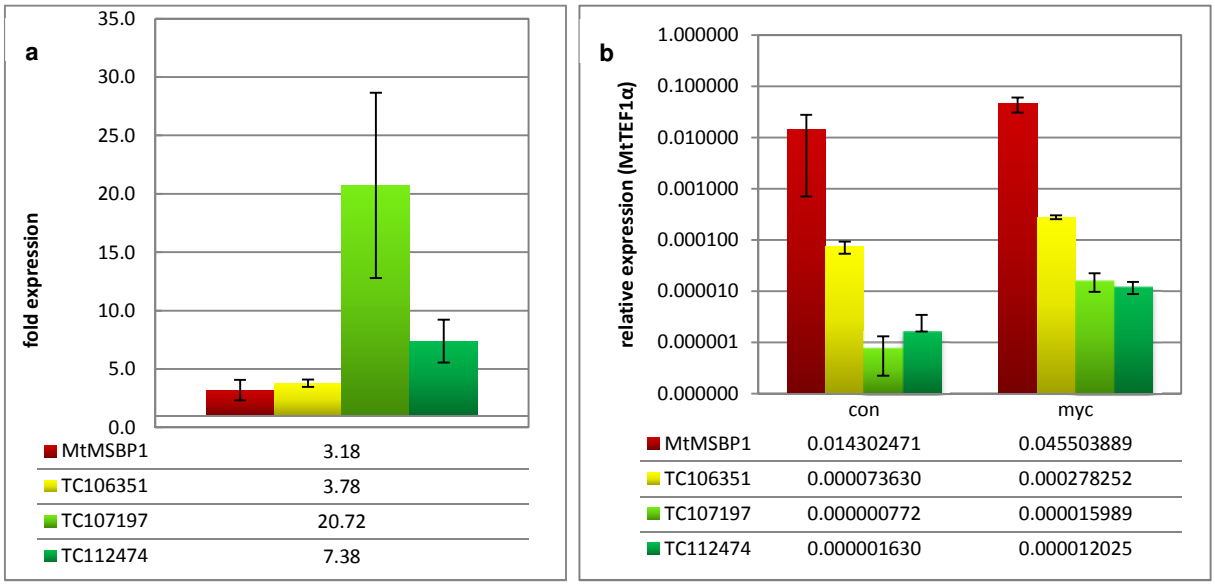


Figure 4.2 Gene expression analysis of early-induced genes in appressoria-enriched root fragments
Expression of selected early-induced genes in appressoria-enriched root fragments in comparison to non-colonized fragments. The transcript accumulation is given as fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of three replicates are given.

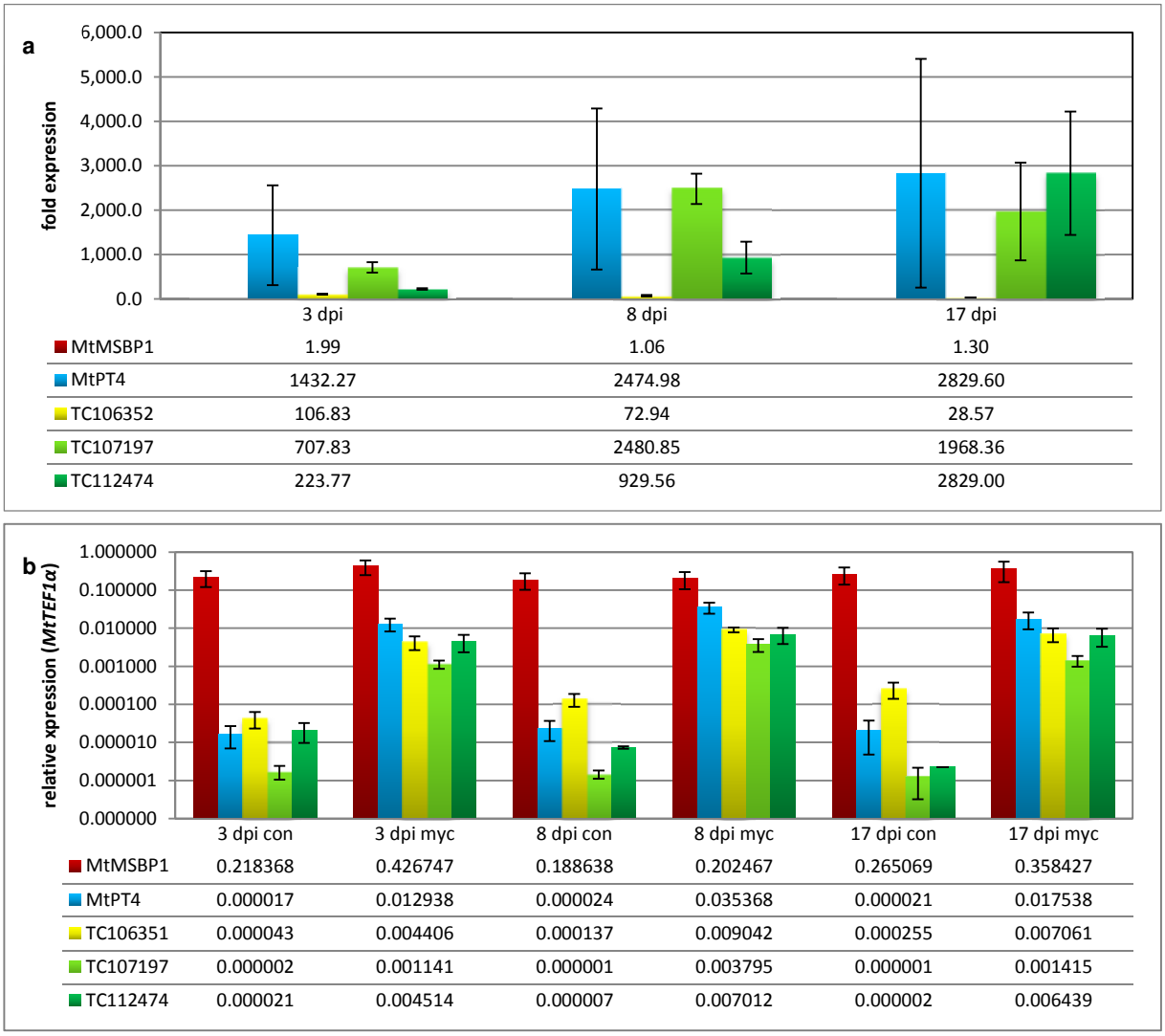
4.1.2 Time course analysis of mycorrhizal-induced genes

In order to study the regulation of the identified early-induced genes in more detail, their expression pattern was analyzed at different time-points during colonization of *M. truncatula* roots by *G. intraradices*. The plant phosphate transporter PT4 was included in the analysis as a control for the progression of the colonization process. MtPT4 is restricted to arbuscule containing cells (Harrison *et al.,* 2002; Javot *et al.,* 2007) and thus can serve as an indicator of a functional symbiosis. The expression of the genes of interest was tested in both, transgenic hairy roots as well as in full plants. While in hairy roots *MtMSBP1* and *TC106351* showed the highest expression in the earliest samples at an appressoria-rich stage (3 dpi), this was not the case for the expression in plant roots. In this experiment, the expression of most of the tested genes rose during proceeding colonization. This was not true for *MtMSBP1*, which showed no induction in the experiment with entire plants at any of the tested time-points.

An increased transcript accumulation during proceeding colonization could be observed for *TC100804*, which was only tested in the plant samples. While *TC107197* and *TC112474* showed in both experiments the highest transcript abundance during later colonization (Figure 4.3 and Figure 4.4), those genes were already induced at 3 dpi in the hairy root explants (Figure 4.3). This was however not true for the earliest time-point tested when using entire plants (1 dpi; Figure 4.4). In this experiment, a significant induction could only be

observed after 12 dpi for *TC112474* and 25 dpi for *TC107197*. This inconsistency could be due to the fact that the colonization proceeded much faster in the hairy root samples as in roots of entire plants, which is confirmed by the early induction of *MtPT4*: While in the hairy root samples a significant induction of *MtPT4* could already be observed at 3 dpi, the expression started to rise not before 12 dpi in the experiment with entire plants. In the latter samples, expression of *MtPT4* correlated with induction of *TC112474*. In addition, *TC106351* that is already induced after 3 dpi in hairy root explants is expressed later in the experiment using entire plant roots. However, TC107197 is neither induced at 12 dpi during colonization of entire plant roots indicating that also additional differences in the experiments, besides a faster colonization might contribute to the alteration of the expression patterns.

To examine whether a perception of the fungus had already taken place after 1 dpi in the experiment with roots of entire plants, expression of a defense-related transcription factor

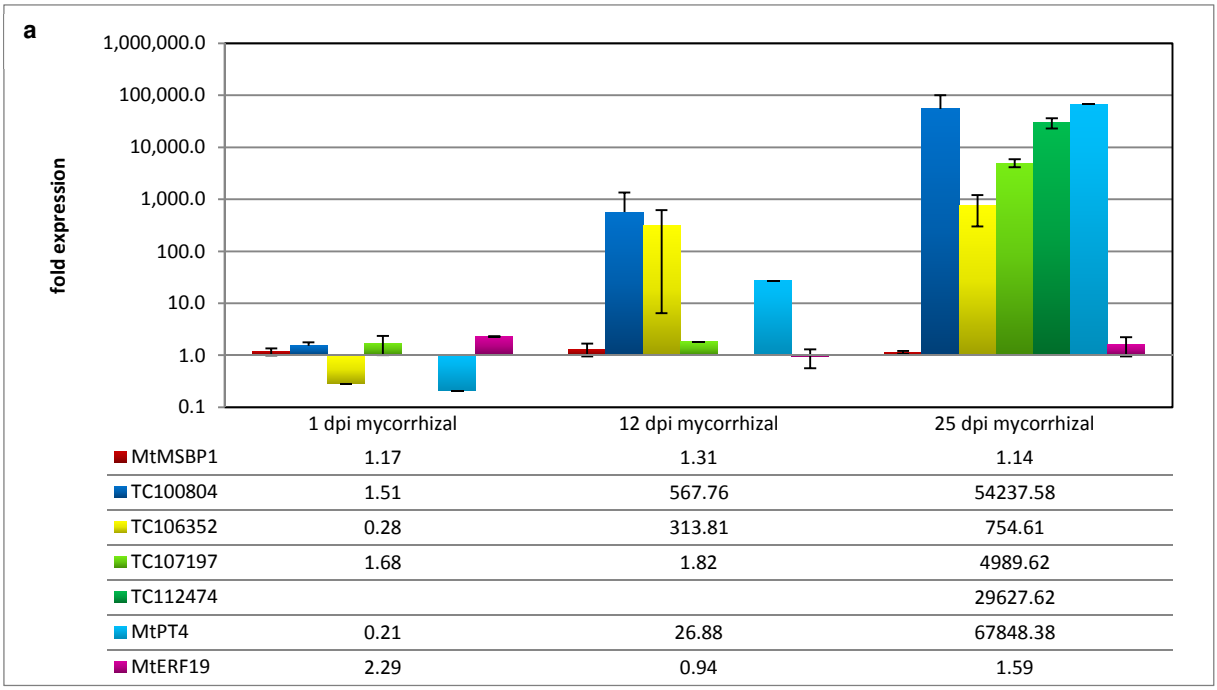


	3 dpi	8 dpi	17 dpi
MtMSBP1	1.99	1.06	1.30
MtPT4	1432.27	2474.98	2829.60
TC106352	106.83	72.94	28.57
TC107197	707.83	2480.85	1968.36
TC112474	223.77	929.56	2829.00

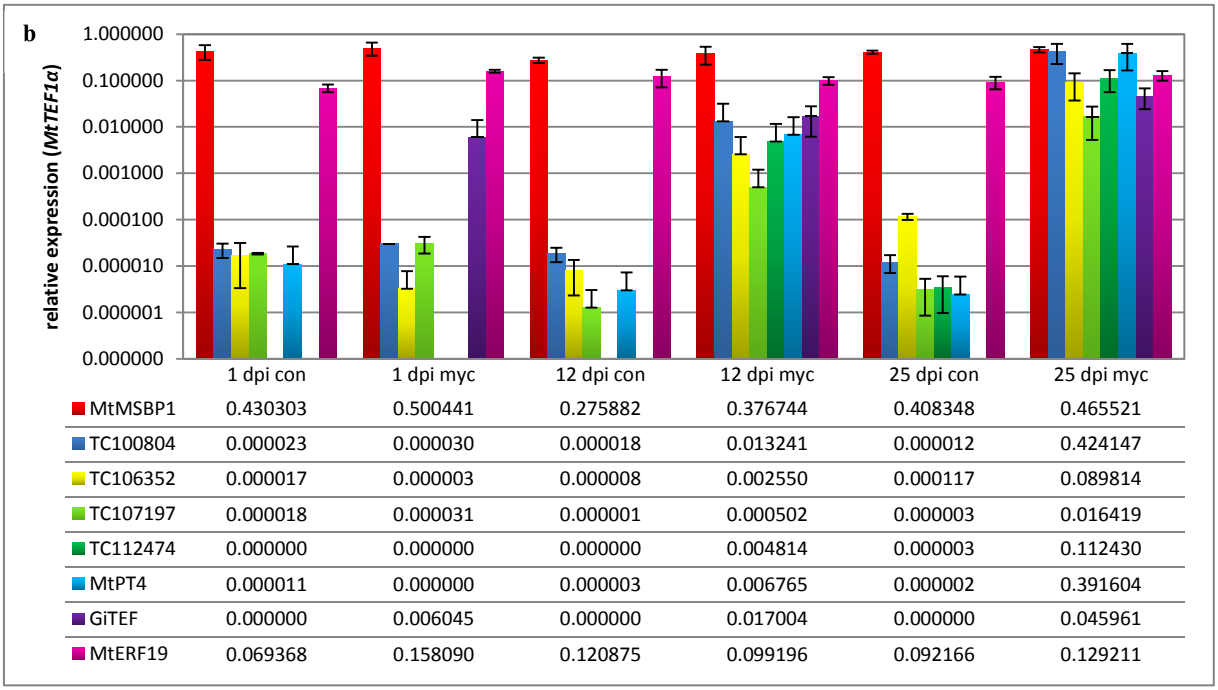
	3 dpi con	3 dpi myc	8 dpi con	8 dpi myc	17 dpi con	17 dpi myc
MtMSBP1	0.218368	0.426747	0.188638	0.202467	0.265069	0.358427
MtPT4	0.000017	0.012938	0.000024	0.035368	0.000021	0.017538
TC106351	0.000043	0.004406	0.000137	0.009042	0.000255	0.007061
TC107197	0.000002	0.001141	0.000001	0.003795	0.000001	0.001415
TC112474	0.000021	0.004514	0.000007	0.007012	0.000002	0.006439

Figure 4.3 Time-course expression analysis during hairy root colonization with *Glomus intraradices*. Roots were sampled after 3, 8, and 17 days post infection (dpi) on solid medium. The expression is compared with equally treated non-colonized roots and expressed as fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of two biological replicates are given.

was measured in those samples. A transient and mild plant defense response was shown to occur during early AM interaction by Liu and colleague (2003). It was found that expression of *MtERF19* (Kloppholz, Kuhn & Requena in review) was induced at 1 dpi but not at later time-points. This transcription factor is a member of the ERBEP family. The closest homolog of this gene in *Lotus japonicus* (Asamizu *et al.,* 2008) belongs to the family of ERF



	1 dpi mycorrhizal	12 dpi mycorrhizal	25 dpi mycorrhizal
MtMSBP1	1.17	1.31	1.14
TC100804	1.51	567.76	54237.58
TC106352	0.28	313.81	754.61
TC107197	1.68	1.82	4989.62
TC112474			29627.62
MtPT4	0.21	26.88	67848.38
MtERF19	2.29	0.94	1.59



	1 dpi con	1 dpi myc	12 dpi con	12 dpi myc	25 dpi con	25 dpi myc
MtMSBP1	0.430303	0.500441	0.275882	0.376744	0.408348	0.465521
TC100804	0.000023	0.000030	0.000018	0.013241	0.000012	0.424147
TC106352	0.000017	0.000003	0.000008	0.002550	0.000117	0.089814
TC107197	0.000018	0.000031	0.000001	0.000502	0.000003	0.016419
TC112474	0.000000	0.000000	0.000000	0.004814	0.000003	0.112430
MtPT4	0.000011	0.000000	0.000003	0.006765	0.000002	0.391604
GiTEF	0.000000	0.006045	0.000000	0.017004	0.000000	0.045961
MtERF19	0.069368	0.158090	0.120875	0.099196	0.092166	0.129211

Figure 4.4 Time-course expression analysis during plant colonization with *Glomus intraradices*
Roots of entire plants were sampled after 1, 12 and 25 days post infection (dpi) in liquid medium. The expression is compared with equally treated non-colonized roots and expressed as a fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of three biological replicates are given.

transcription factors (Tournier *et al.,* 2003). Members of the same sub family have been shown to be induced in response to pathogen infection, mechanical wounding, abiotic stresses as well as specifically by ethylene, jasmonic acid, and salicylic acid. They are described as activators of expression of target defense proteins. The induction of this gene indicates indeed the presence of a transient defense response during the early steps of AM interaction and confirms that the plant roots have already recognized the presence of the fungus and initiated a corresponding cellular response after 1 dpi.

4.1.3 Expression of AM-induced genes in wild type and symbiotic mutants hairy root explants during early contact with hyphae.

As observed during transcript analysis of hairy root colonization in a time-dependent manner, the expression of the identified genes rose already at a very early time-point (3dpi). However, this observation could not be confirmed in the earliest inoculation samples of entire plants. This might be due to the different time-points analyzed (3dpi in hairy roots and 1 dpi in full plants, respectively). To test the expression of the identified genes in hairy roots also after one day of contact with *G. intraradices* hyphae, additional samples were prepared.

At 1 dpi in wild type (wt) hairy roots *TC106351*, *TC107197* and *TC112474* were induced while *MtMSBP1* and *TC100804* were not significantly changed in expression (Figure 4.5). The differences observed between inoculation of hairy roots and full plants can subsequently not be attributed to discrepancies in the analyzed time-points but probably originate in the different experimental setup such as the growth of the extraradical mycelium in liquid *versus* solid medium or in the *Agrobacterium rhizogenes*-mediated transformation used to generate the hairy roots. During *A. rhizogenes*-mediated transformation the natural T-DNA of the bacterial root-inducing Ri-plasmid is transferred to the host plant cells and integrated into the plant genome (Chilton *et al*., 1982; Moore *et al*., 1979; White & Nester, 1980). Besides the root locus (*rol*) genes, which are necessary and sufficient to induce hairy roots (Boulanger *et al*., 1986; Cardarelli *et al*., 1987; Spena *et al*., 1987) the T-DNA also encodes for *aux1* and *aux2* genes, which are involved in auxin biosynthesis (Huffman *et al*., 1984). As hairy roots generated by *A. rhizogenes* can grow in phytohormone-free medium because of the integration and expression of the T-DNA genes, those are supposed to alter auxin responsiveness. Changed auxin sensitivity might therefore be responsible for the altered expression of the marker genes in hairy roots compared to roots of entire plants. However the low-virulent *A. rhizogenes* strain ARqua1 (Quandt *et al*., 1993) used in this study was shown to induce hairy roots that phenotypically behave similarly to wild type roots (Quandt *et al*., 1993). Hairy roots produced by transformation with this strain performed normal during nodulation as well as in mycorrhizal colonization (Boisson-Dernier *et al*., 2001), indicating

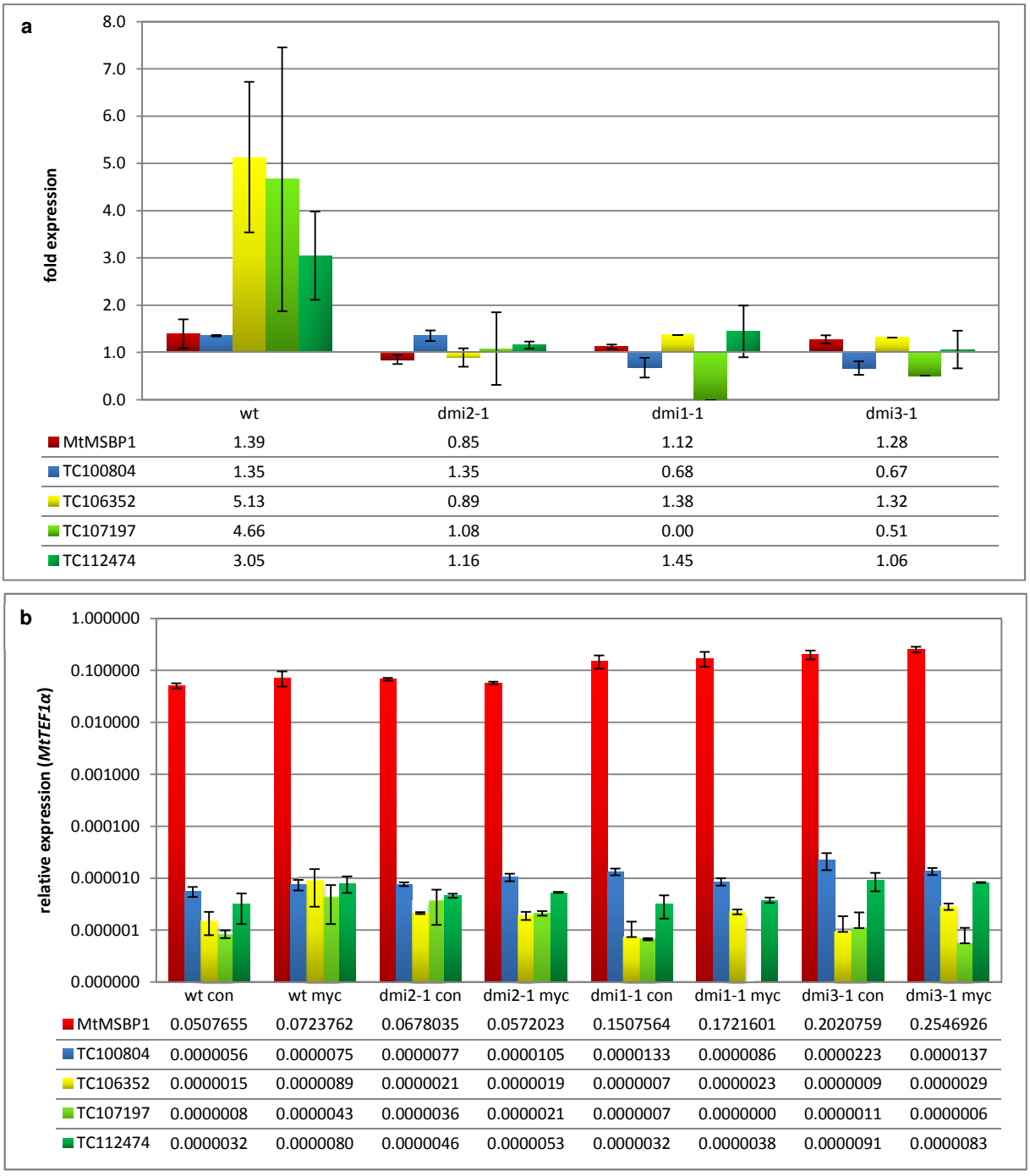


Figure 4.5 Expression of marker genes in wt and mutant hairy root explants after 1 d of contact with extraradical hyphae.
Analysis of transcript abundance after one day of contact between hairy roots and extraradical hyphae of *Glomus intraradices*. The expression is compared with equally treated non-inoculated roots and expressed as fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of two different biological replicates are given.

that plant hormones produced by the hairy root cultures do not disturb the development of the symbioses. As proteins of the SYM pathway were shown to be essential for the early interaction of the both symbiotic partners, the expression of the identified genes was additionally tested in hairy root lines of symbiotic (SYM) mutants of the key players in this

pathway. In the *dmi1-1*, *dmi2-1*, and *dmi3-1* mutant backgrounds no upregulation of *TC106351*, *TC107197* and *TC112474* could be observed. This indicates that those three genes, upon contact, are induced in dependence of the SYM pathway underlining their importance for the symbiosis.

4.1.4 Diffusible fungal signals induce the expression of AM-induced genes in partial dependence of the SYM pathway.

As described in the paragraphs before, several of the identified genes were induced at a very early time-point during the interaction of *M. truncatula* roots and *G. intraradices* extraradical hyphae. Our particularly interest involves identification of plant genes that could serve as marker genes for perception of diffusible fungal signals. Therefore, in a next step it should be tested whether some of those genes are likewise induced without physical contact of hyphae and root. To elucidate this, fungus and hairy roots were separated by a cellophane membrane, which allowed diffusion of soluble compounds but prevented direct contact (Figure 4.6). An obvious induction of expression prior to physical contact in wt background was observed for, *TC106351* and *TC107197*, whereas *TC112474* was only slightly upregulated (Figure 4.7). Interestingly a slight induction was also observed for *MtMSBP1*, which appeared to be increased if compared to induction after direct contact with hyphae. This might be due to an upregulation of the gene in all cells responsive to fungal signal molecules, while a more restricted induction of the gene in the area in contact with hyphae resulted in a dilutional effect. No increase in expression was detected for *TC100804*. In the *dmi* mutants, the overall expression of the genes was lower, excluding *MtMSBP1*.

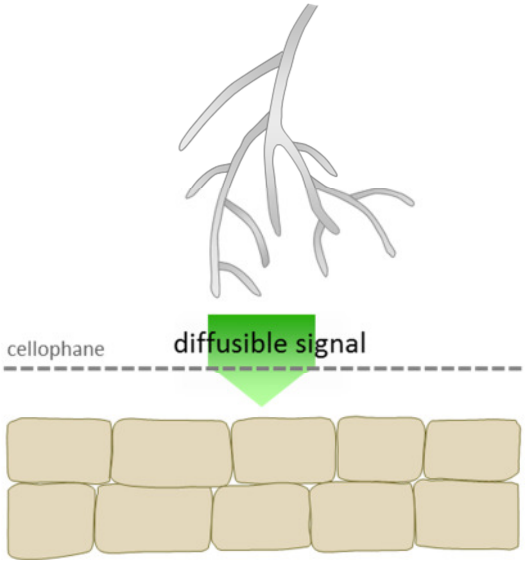


Figure 4.6 Schematic experimental setup
To examine the induction of genes prior to physical contact, hairy root explants were separated by the host roots with a cellophane membrane.

Additionally, the induction for most of the genes was abolished, except for *TC106351,* in the *dmi2-1* mutant roots as well as for *TC112474* in *dmi3-1* background. The upregulation of those genes independent of the DMI1 and DMI3 proteins indicates the existence of a parallel pathway that is involved in regulation of signal transduction after perception of diffusible signals. Nevertheless, the overall expression level seems still to be dependent on the SYM pathway.

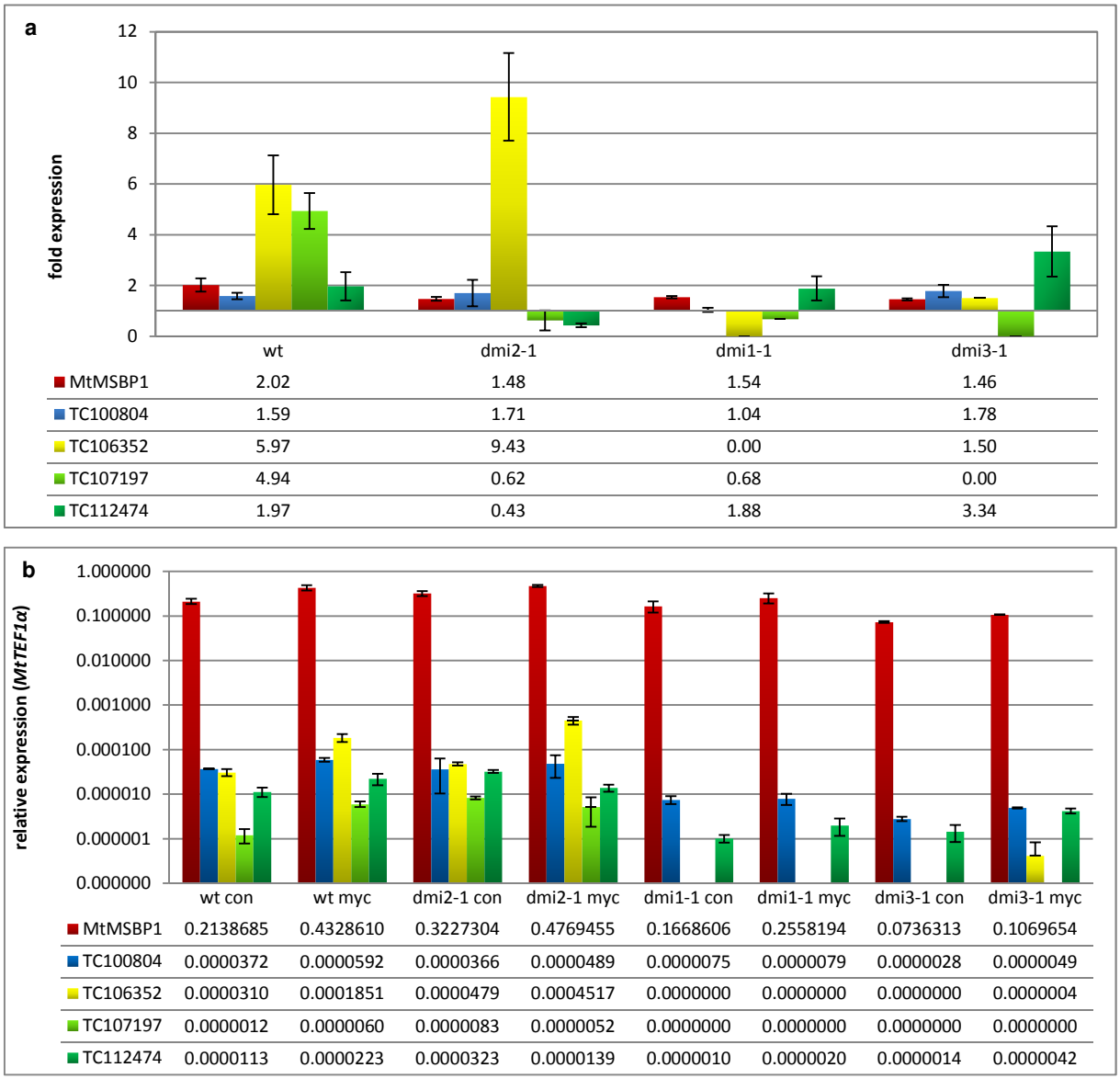
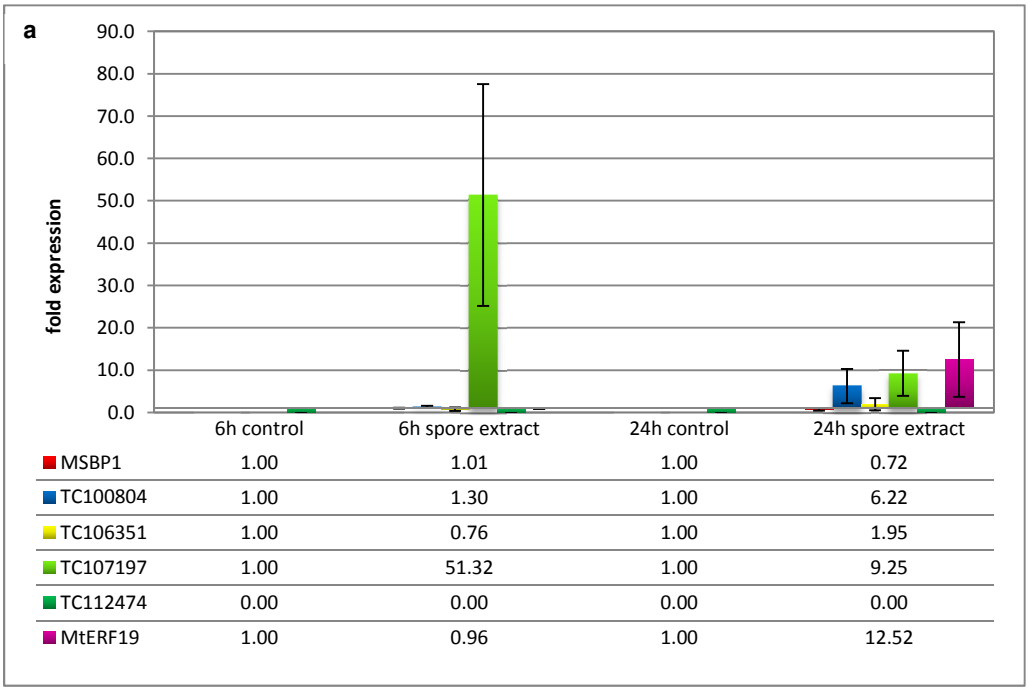


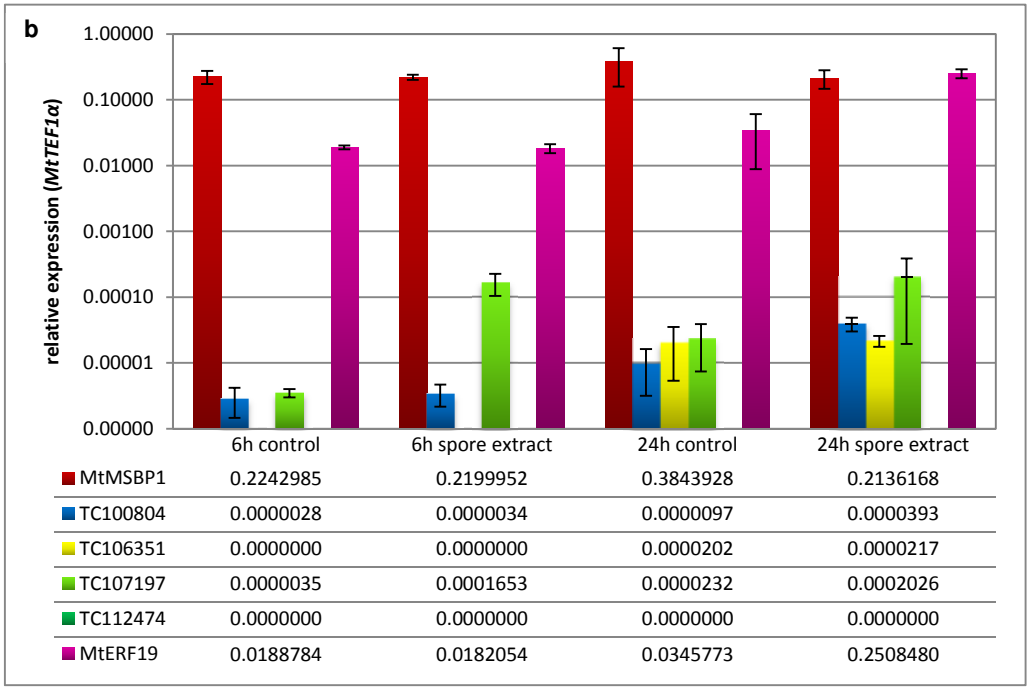
Figure 4.7 expression of marker genes after contact with diffusible fungal signals.
M. truncatula hairy root explants were co-cultivated with extraradical fungal hyphae separated by a cellophane membrane. The expression is compared with equally treated non-inoculated roots and expressed as a fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of three different biological replicates are given.

4.1.5 Non-germinated *G. intraradices* spores contain active fungal signal molecules

As described above, diffusible fungal signals were able to induce the expression of *TC106351* and *TC107197*. The extraradical hyphae that were used in this co-incubation experiment were induced by plant signals as the roots were merely separated by a cellophane membrane allowing the diffusion of plant-derived signals. Based on this



	6h control	6h spore extract	24h control	24h spore extract
MSBP1	1.00	1.01	1.00	0.72
TC100804	1.00	1.30	1.00	6.22
TC106351	1.00	0.76	1.00	1.95
TC107197	1.00	51.32	1.00	9.25
TC112474	0.00	0.00	0.00	0.00
MtERF19	1.00	0.96	1.00	12.52



	6h control	6h spore extract	24h control	24h spore extract
MtMSBP1	0.2242985	0.2199952	0.3843928	0.2136168
TC100804	0.0000028	0.0000034	0.0000097	0.0000393
TC106351	0.0000000	0.0000000	0.0000202	0.0000217
TC107197	0.0000035	0.0001653	0.0000232	0.0002026
TC112474	0.0000000	0.0000000	0.0000000	0.0000000
MtERF19	0.0188784	0.0182054	0.0345773	0.2508480

Figure 4.8 Expression analysis of early-induced genes in response to ground *G. intraradices* spores
M. truncatula plantlets were treated for 6 h or 24 h with ground spores of *G. intraradices*. RNA was extracted out of roots. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

experimental setup, it was not able to differentiate whether the induction of the plant genes occurred due to a constitutively produced fungal signal or if the production of the responsive signaling molecule was induced after perception of plant factors. An induction of gene expression due to a constitutively produced signal secreted by germinated spores was already reported by Weidman *et al.,* (2004). In this direction a transient elevation of intracellular Ca^{2+} in soybean (*Glycine max*) cell cultures (Navazio *et al.,* 2007) as well an accumulation of starch in roots of *Lotus japonicus* (Gutjahr *et al.,* 2009) have also been observed after application of geminating spores supernatant.

To elucidate whether spores without prior plant contact contain the signal molecules that induce the expression of early marker genes, *G. intraradices* spores were collected during dormancy, ground in liquid nitrogen and applied to plant roots for either 6 or 24 hours.Treatment of plant roots for 6 h revealed an induction of *TC107197* (Figure 4.8). This observation is consistent with the existence of a constitutive signal present in dormant spores prior to germination. The fact that *TC106351* was not induced by the ground spores in addition confirms the assumption that the signal necessary for induced expression of this gene is *de novo* produced by the fungus after perception of plant signals. The factor prompting the expression of *TC106351* must therefore be either distinct form the one that induces *TC107197* or the same molecule is produced in higher amount after induction of the hyphae by the plant and subsequently is able to induce both genes. Interestingly after 24 hours of treatment, the expression of *TC100804* and *MtERF19* increased while the induction of *TC107197* diminished. The delayed upregulation of *TC100804* could be a hint for the involvement of an additional signal that might be produced only after processing of a fungal molecule. Another possibility is the existence of a second signal present in lower concentration that therefore requires more time to induce a response. Finally, also the existence of primary and secondary activation loops influencing the expression of different genes might be an explanation for the delayed induction. The activation of *MtERF19* is possibly due to the onset of a defense response as a result of recognition of common fungal MAMPs (microbe-associated molecular patterns), that has already been observed during the early time-point of the interaction (paragraph 4.1.2; Kloppholz, Kuhn & Requena in review).

4.1.6 *Colletotrichum trifolii* is able to induce the expression of *MtMSBP1* but not of the mycorrhiza-specific genes

Plants possess an innate immune system that is able to detect conserved molecules of pathogenic and beneficial microbes called microbe associated molecular patterns (MAMPs) (reviewed by Zhang & Zhou, 2010). Recognition of those conserved microbial epitopes by plant receptors induces the first line of plant defenses. Only a few MAMPs have been described so far for fungi, including chitin, ergosterol and N-glycosylated yeast peptides

(Basse & Boller, 1992; Georg Felix *et al*., 1993; Kuchitsu *et al*., 1993; Granado *et al*., 1995). AM fungi do not possess ergosterol but campesterol, a sterol that is also found in plants (Fontaine *et al*., 2001) and thus will not be recognized as a MAMP. However, chitin is a major component present in all fungal cell walls (Bonfante-Fasolo *et al.,* 1990), and evidence exists that chitin-related elicitors could be responsible for the activation of defense responses of plants during AM (Salzer & Boller, 2000; Salzer *et al*., 2000; Bonanomi *et al*., 2001).

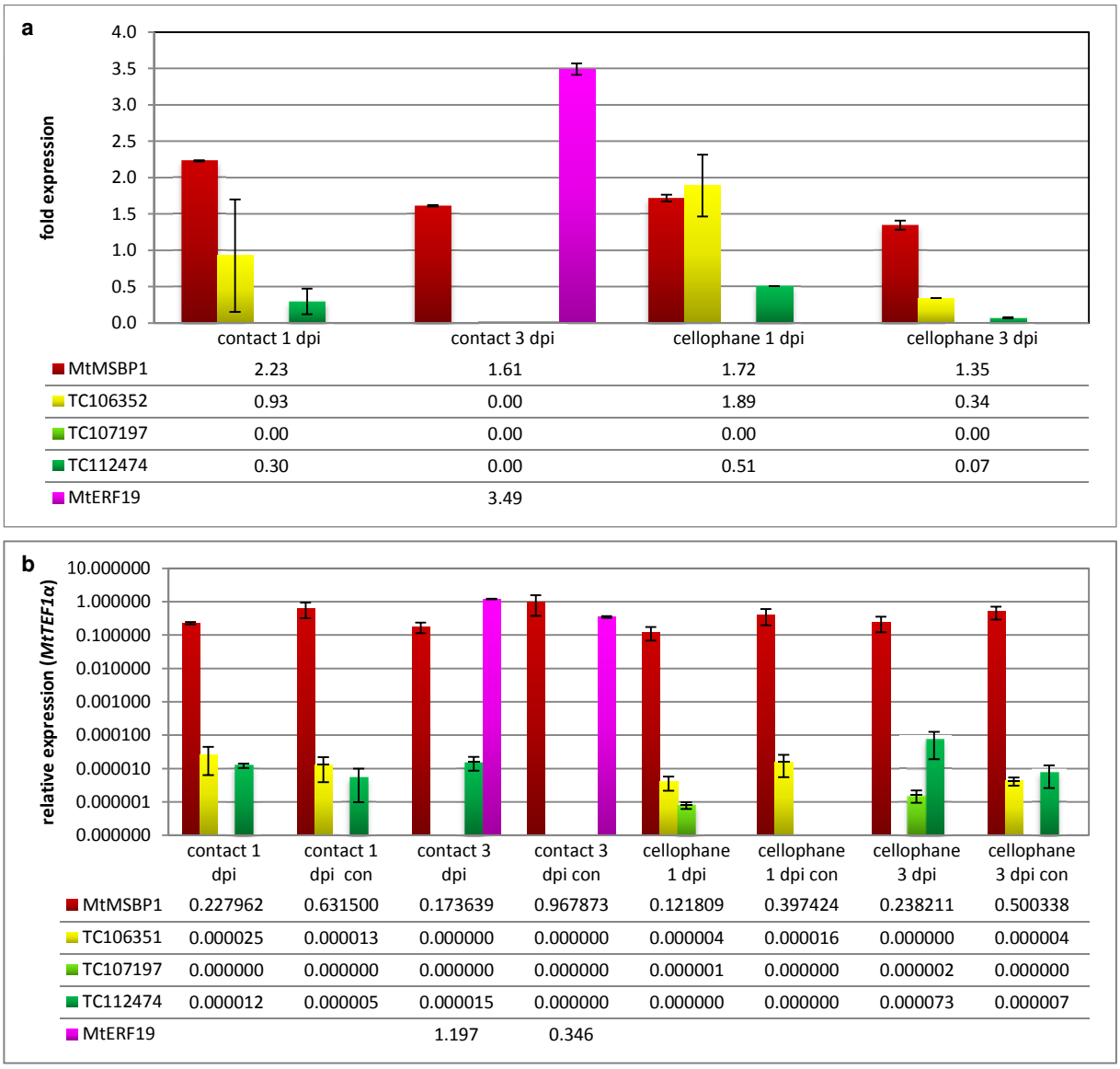


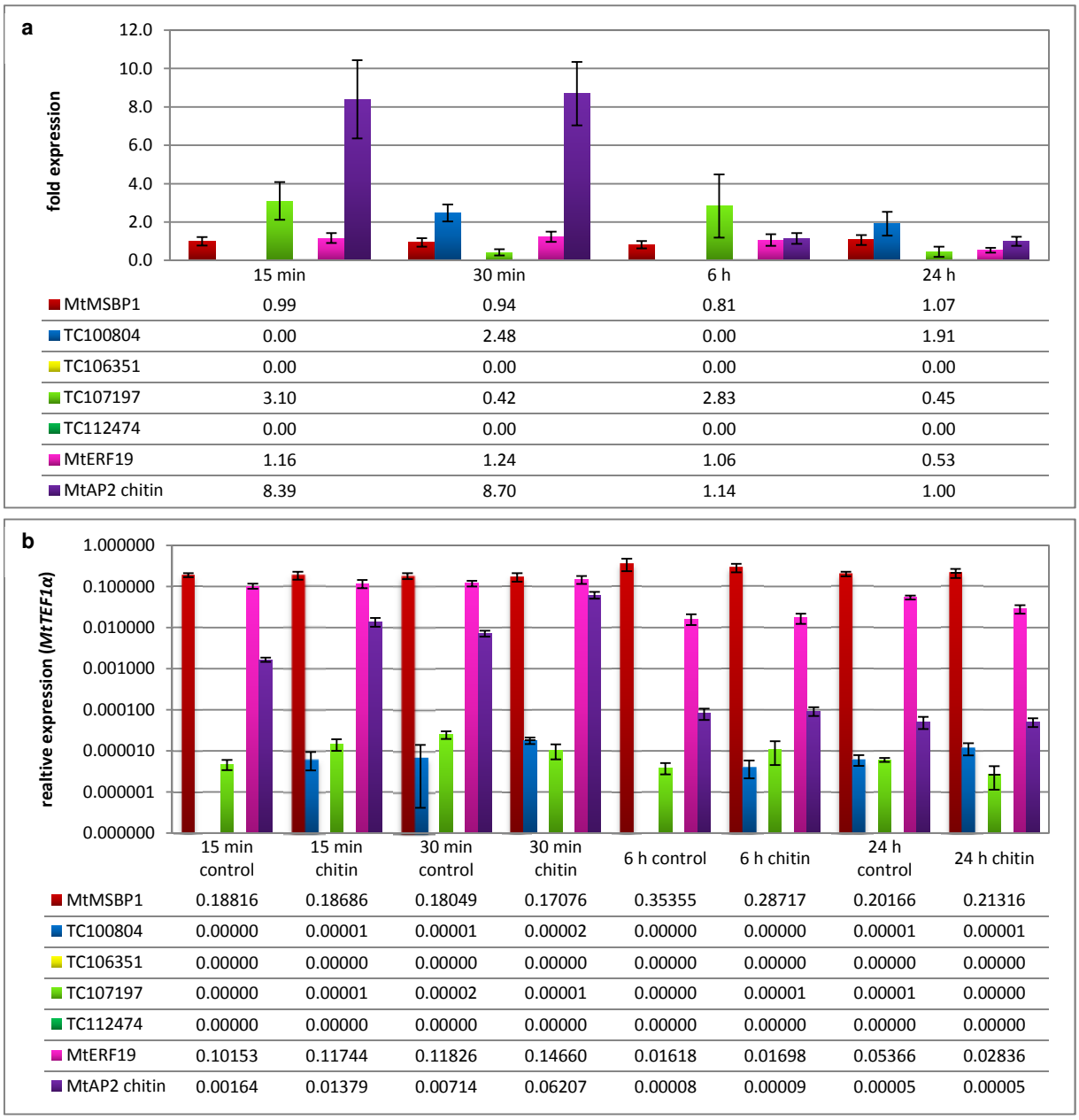
Figure 4.9 expression of marker genes after inoculation of hairy roots with *C. trifolii*.
M. truncatula hairy root explants were co-cultivated with C. trifolii in contact or separated by a cellophane membrane. The expression is compared with equally treated non-inoculated roots and expressed as a fold of expression relative to control (a) or expression relative to *MtTEF1α* (b). Standard deviations of the average of two different biological replicates are given.

To investigate a possible induction of the identified genes in response to recognition of general fungal MAMPs, *M. truncatula* hairy root explants were inoculated with the legume pathogen *C. trifolii*. This was done either with or without the addition of a cellophane membrane. In none of the different setups, a significant induction of any of the tested genes

was observed except for *MtMSBP1* that was transiently induced (Figure 4.9). This coincides with the response to *Phymatotrichopsis omnivora* reported in the *Medicago* gene expression Atlas. In contrast, the results for the other genes indicate that the activation of those is due to a factor specifically present in arbuscular mycorrhizal fungi.

4.1.7 Some of the AM-induced genes are responsive to chitin

To confirm the results obtained for the infection with *C. trifolii,* chitin was tested as a fungal MAMP against the expression of the marker genes. The application of 100 µg/ml of



	15 min	30 min	6 h	24 h
MtMSBP1	0.99	0.94	0.81	1.07
TC100804	0.00	2.48	0.00	1.91
TC106351	0.00	0.00	0.00	0.00
TC107197	3.10	0.42	2.83	0.45
TC112474	0.00	0.00	0.00	0.00
MtERF19	1.16	1.24	1.06	0.53
MtAP2 chitin	8.39	8.70	1.14	1.00

	15 min control	15 min chitin	30 min control	30 min chitin	6 h control	6 h chitin	24 h control	24 h chitin
MtMSBP1	0.18816	0.18686	0.18049	0.17076	0.35355	0.28717	0.20166	0.21316
TC100804	0.00000	0.00001	0.00001	0.00002	0.00000	0.00000	0.00001	0.00001
TC106351	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
TC107197	0.00000	0.00001	0.00002	0.00001	0.00000	0.00001	0.00001	0.00000
TC112474	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
MtERF19	0.10153	0.11744	0.11826	0.14660	0.01618	0.01698	0.05366	0.02836
MtAP2 chitin	0.00164	0.01379	0.00714	0.06207	0.00008	0.00009	0.00005	0.00005

Figure 4.10 Expression analysis of early-induced genes in response to chitin
M. truncatula plantlets were treated for 15 or 30 min, 6 or 24 h with 100 µg/ml shrimp shell chitin . RNA was extracted out of roots. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of two different biological replicates are given

shrimp shell chitin to *M. truncatula* plant roots resulted in no induction in most of the genes (Figure 4.10). Nevertheless, a slight but only transient upregulation of *TC107197* and *TC100804* after 15 and 30 min of treatment was observed, correlating with the induction of the closest *M. truncatula* homologue of a chitin-induced AP2 transcription factor of *A. thaliana* (AT1G22810; Libault *et al.,* 2007). However, the missing induction of *MtERF19* in all of the treatments might indicate that shrimp shell chitin is not able to facilitate the expression of this gene and subsequently an ERF19-mediated defense response might not be induced. Nevertheless, the upregulation of *TC107197* and *TC100804* was not expected but might be of interest in the context of the recently published description of a chitin derived mycorrhizal fungal signal molecule (Maillet *et al.,* 2011). Those Lipo-chitooligosaccharides designated as Myc-LCOs are based on a chitin backbone with a structure similar to the well-described Nod factors (reviewed in D'Haeze & Holsters, 2002). Both are recognized by LysM receptors and in the non-legume *Parasponia andersonii* it has even been shown that, a common receptor is required for both, successful mycorrhization and nodulation (Op den Camp *et al.,* 2011). In this context, it might be speculated whether the induction of *TC100804* and *TC107197* relates to an unspecific recognition of chitin derivatives present in the shrimp shell preparation.

4.2 Suitability of the identified genes as early AM markers in promoter reporter constructs

The possibility to use promoter fusions in reporter constructs is a valuable tool in the examination of the arbuscular mycorrhiza symbiosis. In *M. truncatula,* MtPT4 and the blue-copper binding protein MtBcp1 that are expressed in arbuscule containing cells have been identified and widely used as markers for a functional symbiosis and formation of arbuscules (Harrison *et al.*, 2002; Hohnjec *et al.*, 2005; Javot *et al.*, 2007). However arbuscules are formed during a mature symbiosis and marker genes for an early arbuscular mycorrhizal colonization or perception of fungal signals are rare. *MtENOD11*, which has been used as an early marker gene for perception of diffusible fungal signals is induced after Nod factor perception as well (Charron *et al.*, 2004). Consequently, this gene cannot be used to distinguish between fungal and Rhizobial diffusible signal molecules and additional marker gene promoters are needed to fulfill this task.

4.2.1 Promoters of *TC106351* and *TC107197* are deregulated if taken out of the genomic context.

The previous experiments showed that *TC106351* and *TC107197* are mycorrhiza-specific induced genes that are upregulated upon perception of diffusible fungal signals. In the case of *TC107197* this induction occurs in fully dependency of the SYM pathway while *TC106351* seems to be regulated partially SYM-independent. In order to study these genes in more detail and use them as marker genes for perception of fungal signals in *M. truncatula* ca. 1000 bp of each promoter including the 5' untranslated region (PTC106-F1 and R1; PTC107-F1 and R1) were fused to GFP-GUS in the binary vector pPGFPGUS-RR and introduced into *M. truncatula* hairy roots. Despite the mycorrhiza-dependency observed in the transcript analysis for both genes, the transgenic roots showed constitutive β-glucuronidase (GUS) activity (Figure 4.11 a). To exclude the possibility that important regulatory motifs of the promoters were excluded from the promoter reporter constructs, longer regions, including 4 kb upstream of the ATG were also analyzed (PTC1074T-F1 and R1; PTC106-F2 and R1). The staining of the generated hairy roots revealed the same result as already observed with the shorter construct. The activity of both promoters was always strong, independent of the mycorrhizal status of the roots (Figure 4.11 b). Further on, we hypothesized that this deregulation could be due to the high stability of the GFP-GUS transcript that led to a strong GUS signal. A regulation of transcript abundance of *TC106351* and *TC107197* could occur independently of the activity of both promoters by siRNA-mediated inactivation of the mRNAs. To analyze the hypothesis whether *TC107197* is regulated via its transcript stability, the open reading frame of the gene was fused to the β-glucuronidase gene with the help of

the binary vector pKGWGUS-RR and expressed under the control of the 4 kb promoter construct (TC107noSP-Asc1-F and TC107noSTOPAscR). Examination of the corresponding transgenic hairy roots revealed that also in this construct constitutive GUS activity was present although to a lower extent (Figure 4.11 c).

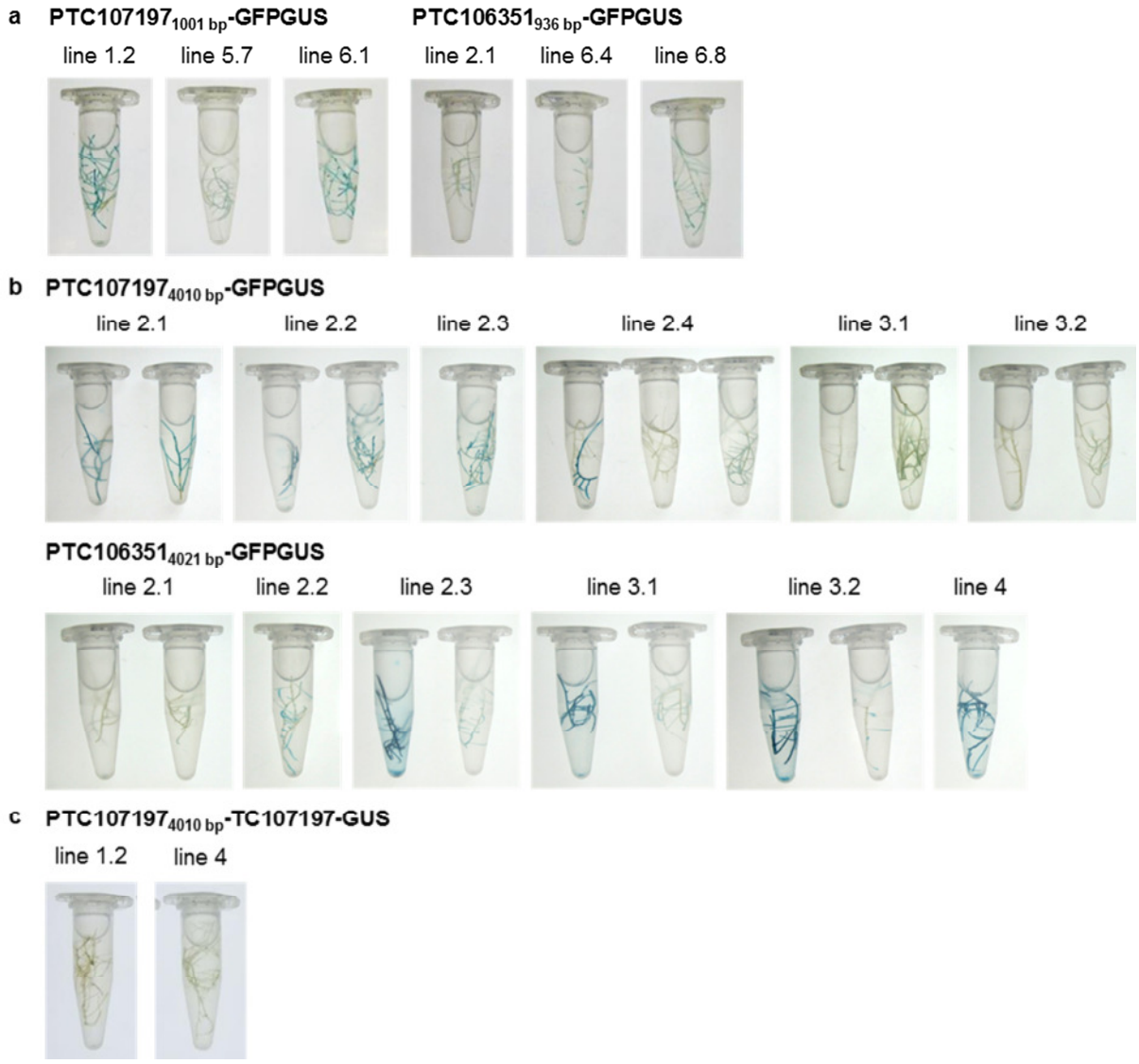


Figure 4.11 Stain for β-glucuronidase activity in hairy roots transformed with *TC106351* and *TC107197* promoter reporter constructs
One (a) or four (b) kb of the promoters including 5' UTR upstream of the ATG were fused to GFPGUS in a promoter reporter construct by the use of pPGFPGUS-RR. (c) The ORF of *TC107197* was fused to GUS in pKGWGUS-RR and expressed under the control of the four kb promoter. All the roots showed blue color when tested for GUS activity.

4.2.2 *MtMSBP1* as early arbuscular mycorrhiza-induced marker gene

MtMSBP1 was the only gene induced during early arbuscular mycorrhizal contact in the oligo array analysis that has not been described before. Moreover, induction of the gene was found to be restricted to early time-points of AM interaction. According to this, the gene was selected for further analysis. During my Diploma Thesis, I analyzed the expression of a 1518 bp promoter fragment of *MtMSBP1* during early interaction of *M. truncatula* roots with *G. intraradices*. Using GUS and GFP in a promoter reporter construct, I was able to show that a local induction of the gene occurs after contact of hyphae with host roots (Figure 1.5, Kuhn *et al.,* 2010). Even more, by performing time-lapse microscopy, an increased promoter activity could be observed prior to physical contact in response to fungal molecules secreted by branching hyphae in the vicinity of the root. This observation confirms the results of the expression analysis of the gene (Figure 4.7) and makes the promoter reporter a candidate for analysis of perception of diffusible fungal signals by the plant.

4.2.3 A *MtMSBP1* truncated promoter is sufficient for expression after hyphal contact

Up to now, no specific conserved response elements have been described in promoters of early mycorrhiza-induced genes. In contrast, the induction of genes during nodulation and AM has been linked to the existence of an AT-rich motif in the promoter of the early nodulin gene *MtENOD11*. *In silico* analysis of the region upstream of the ATG in *MtMSBP1* revealed the presence of an AT-rich motif TTATTTTTTCTTTAATAA at -275bp (consensus TTATT(N)$_{7\text{-}12}$AATAA) (Figure 4.12). A second AT-rich motif, not matching completely the consensus TATT (N)$_5$-AATAA, was found at -176 bp. This AT-rich motif is present in a number of early

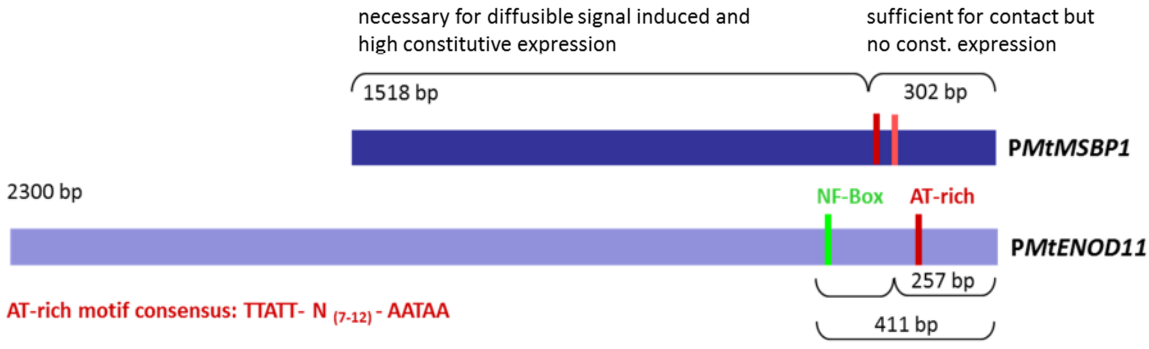


Figure 4.12 Comparison of *MtMSBP1* and *MtENOD11* promoters
The promoters of *MtMSBP1* and *MtENOD11* both contain AT-rich motifs whereas an NF-box is only present in the *MtENOD11* promoter. One of the motifs of *MtMSBP1* is fully consistent with the consensus sequence (-275 bp) while the other (-176 bp) lacks 2 bases between the repetitive elements. For the *MtENOD11* promoter it is known that the 2300 bp fragment is sufficient for AM-fungi diffusible signal and contact elicited expression (DMI independent). The 257 bp fragment is sufficient for infection correlated early and late expression during AM and *S. meliloti* colonization while it is not sufficient for NF elicited expression. The AT-rich motif is required for high-level expression during AM and Nodulation (DMI dependent). The NF-Box is sufficient for NF-elicited expression (DMI dependent).

nodulin promoters and its deletion has been associated to a decrease of expression of the corresponding genes in nodules (Vijn *et al*., 1995; Hansen *et al*., 1999; Rodriguez-Llorente *et al*., 2003). For the *MtENOD11* promoter it is known that a 2300 bp promoter fragment upstream of the ATG is sufficient for SYM-dependent, AM-fungi diffusible signal and contact-elicited expression (Figure 4.12; Kosuta *et al.,* 2003; Boisson-Dernier *et al.,* 2005). A shorter 257 bp fragment is sufficient for infection-related early and late expression both during AM and *S. meliloti* colonization. However, it is not sufficient for Nod factor (NF)-elicited expression (Boisson-Dernier *et al.,* 2005). The AT-rich motif itself has been shown to be required for SYM-dependent high-level expression during AM and nodulation (Boisson-Dernier *et al.,* 2005). Andriankaja *et al.* (2007) showed that an NF-Box present in a 411 bp promoter fragment is sufficient for NF-elicited SYM-dependent expression of *MtENOD11* in root hairs and essential for the binding of root hair–specific nuclear factors (AP2/ERF transcription factors ERN1, ERN2 and ERN3).

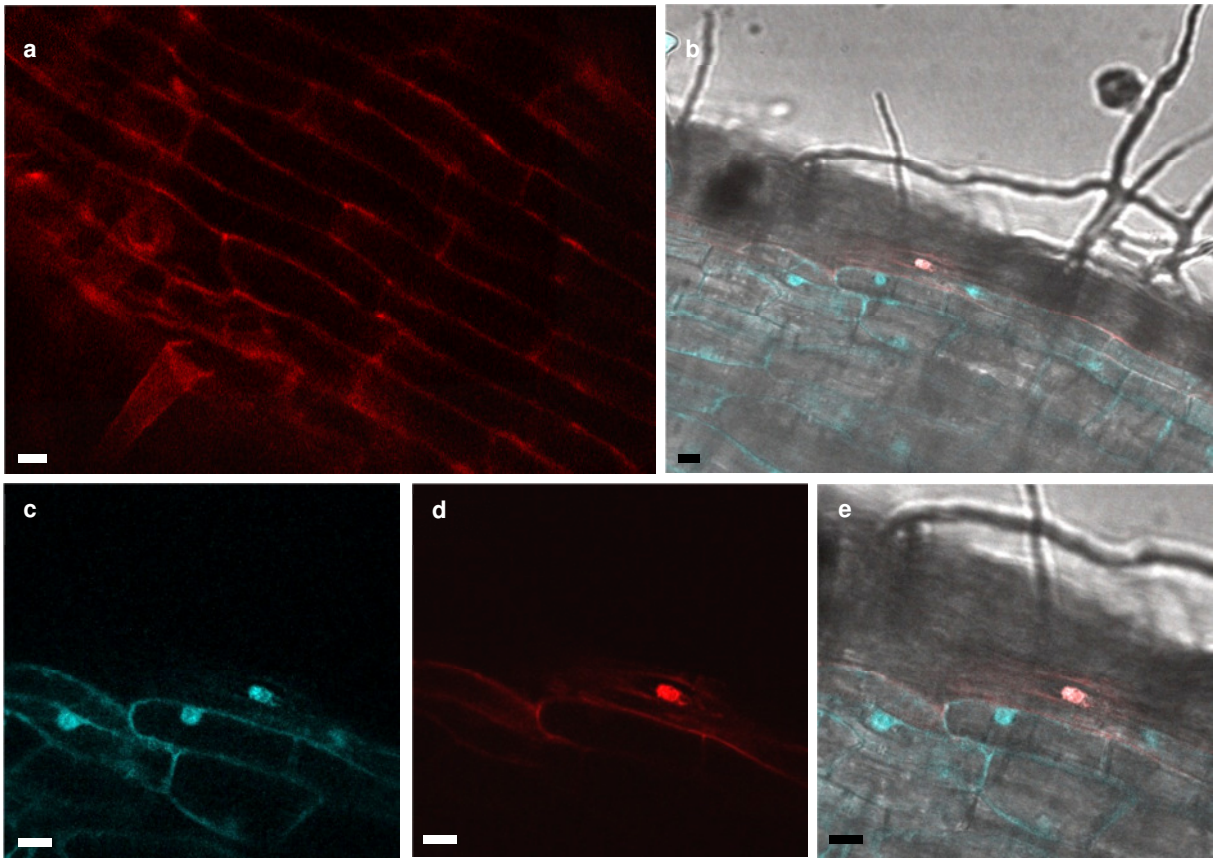


Figure 4.13 A proximal region of the *MtMSBP1* promoter is sufficient to induce expression after hyphal contact
A truncated promoter (302 bp) was fused to the reporter gene DsRED fused the nuclear localization signal of stuA from *A. nidulans* and transformed into *M. truncatula* hairy roots. (a) Non-mycorrhizal control root. (b) Overview of contacting hyphae in the rhizodermis. The nucleus of one single cell in the vicinity of contacting hyphae shows red fluorescence, whereas constitutive blue CFP fluorescence, indicating root transformation, can be observed in the nucleus and cytoplasm of all cells. (c) to (d) Detailed magnification of the same root. Bar = 10 µm**.**

To investigate whether a shorter promoter of *MtMSBP1* containing the AT-rich motifs would be sufficient to drive mycorrhiza-induced expression, a promoter deletion (302 bp upstream from the ATG) comprising both AT-rich motifs was fused to the DsRED reporter gene in the binary vector pHKRedNuc-BR. To facilitate visualization, the nuclear localization signal of the *stuA* gene from the filamentous fungus *Aspergillus nidulans* was fused to DsRED. Confocal visualization of transgenic roots expressing this construct showed no asymbiotic expression of the reporter gene (Figure 4.13 a). In contrast, when transgenic roots were mycorrhized, nuclear localization of DsRED could be observed only in cells in contact with *G. intraradices* hyphae while constitutive CFP fluorescence was observed in all cells (Figure 4.13 b - e). This result indicates that this truncated promoter is sufficient to trigger mycorrhiza-specific gene induction. However, time-lapse observation of roots harboring this construct showed that promoter induction only took place after direct contact and not by the diffusible signal secreted by branching hyphae. This data leads to the hypothesis that one or both AT-rich motifs might be responsible for a contact-mediated promoter activity. In contrast, constitutive expression as well as an activation in response to diffusible factors requires promoter elements more upstream. In future investigations, promoter fragments depleted in one or both AT-rich motifs should be employed to confirm the involvement of those motifs in the regulation of the gene and examine their role in more detail.

4.2.4 *MtMSBP1* is induced in nodules

Albeit the existing differences of the mutualistic interactions in function and morphology arbuscular mycorrhiza and root nodule symbiosis share common features in the signaling that leads to establishment of the interaction. The well-described SYM pathway comprises proteins that have been shown to be essential for both symbiosis and mutations in those proteins lead to an aborted infection in either case. (reviewed by Parniske 2008) There have been nine proteins described so far that are of major importance in both interactions and further mutants are still being discovered.

As a function in both interactions has been assigned for several proteins, it was tested whether *MtMSBP1* expression might be induced in nodule symbiosis as well. Therefore, composite plants with P*MtMSBP*$_{11518\ bp}$-GFPGUS promoter reporter construct transformed hairy roots were generated and inoculated with *Sinorhizobium meliloti*.

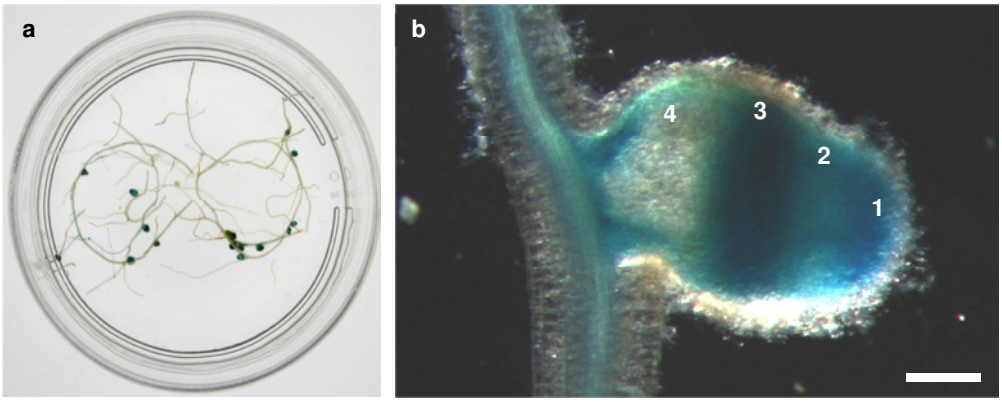


Figure 4.14 Activity of PMtMSBP$_{11518\ bp}$-GUS promoter reporter construct in root nodules
MtMSBP1 is expressed in mature root nodules as indicated by GUS activity. (a) overview of hairy roots witch show blue staining in all mature nodules. (b) Detailed view on a nodule consisting of meristematic tissue (1), pre-fixing zone (2), interzone (3) and nitrogen fixation zone (4). Bar = 250 µm.

An activation of the promoter reporter fusion could be observed in mature root nodules with a strongest GUS activity mainly in the meristem, pre-fixing zone, interzone and steele (Figure 4.14). Due to this expression pattern, it can be stated that MtMSBP1 seems to play a role not only in arbuscular mycorrhiza but also during nodule symbiosis of *M. truncatula* with *S. meliloti*. To test the suitability of the gene as a marker gene for perception of fungal signal molecules, it should be analyzed in the future whether an induction of P*MtMSBP*$_{11518\ bp}$-GFPGUS occurs already during an early interaction of *M. truncatula* roots with *S. meliloti*. Personal communication with Giles Oldroyd (John Innes Centre, Norwich, UK) however indicated that *MtMSBP1* is not induced after perception of Nod factors but specifically upregulated by AM fungal signals.

4.3 Functional analysis of MtMSBP1

4.3.1 MtMSBP1 localizes at the endoplasmic reticulum.

The expression pattern of *MtMSBP1* observed in real-time PCR investigation and observation of promoter reporter fusions pointed to a role of the protein during early arbuscular mycorrhizal colonization. To further characterize MtMSBP1 in more detail and investigate its subcellular localization, the protein was analyzed *in silico*. MtMSBP1 was predicted to contain a signal anchor and an N terminal transmembrane region to target the protein to a membrane (Figure 4.15). Furthermore, the WoLF PSORT program (http:// wolfpsort.org) identified a C terminal endoplasmic reticulum (ER) membrane retention signal, ADKE (amino acid code), indicating that this protein could localize to the ER. This postulation is consistent with the localization of animal and fungal homologues of MtMSBP1 to this organelle (Meyer *et al.,* 1996; Nölte *et al.,* 2000; Hand & Craven, 2003; Min *et al.,* 2005). On the other hand, it contradicts the cytoplasmic membrane localization reported for AtMSBP1, the *A. thaliana* homologue of MtMSBP1 with 59 % amino acid identity (Yang *et al.,* 2005).

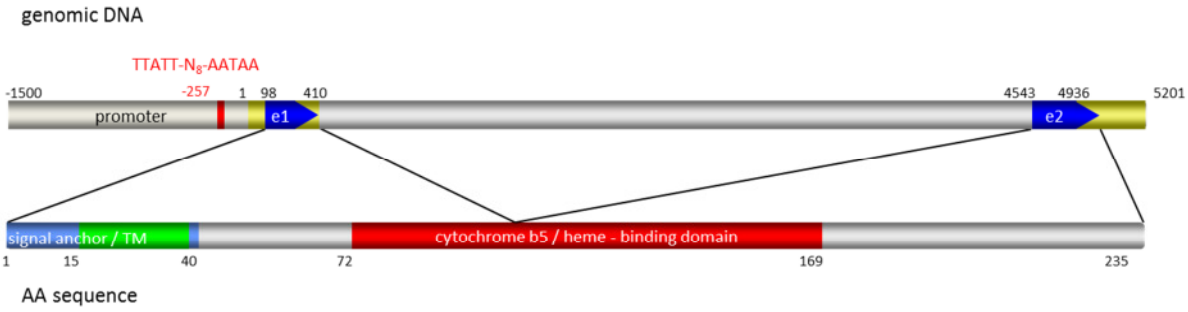
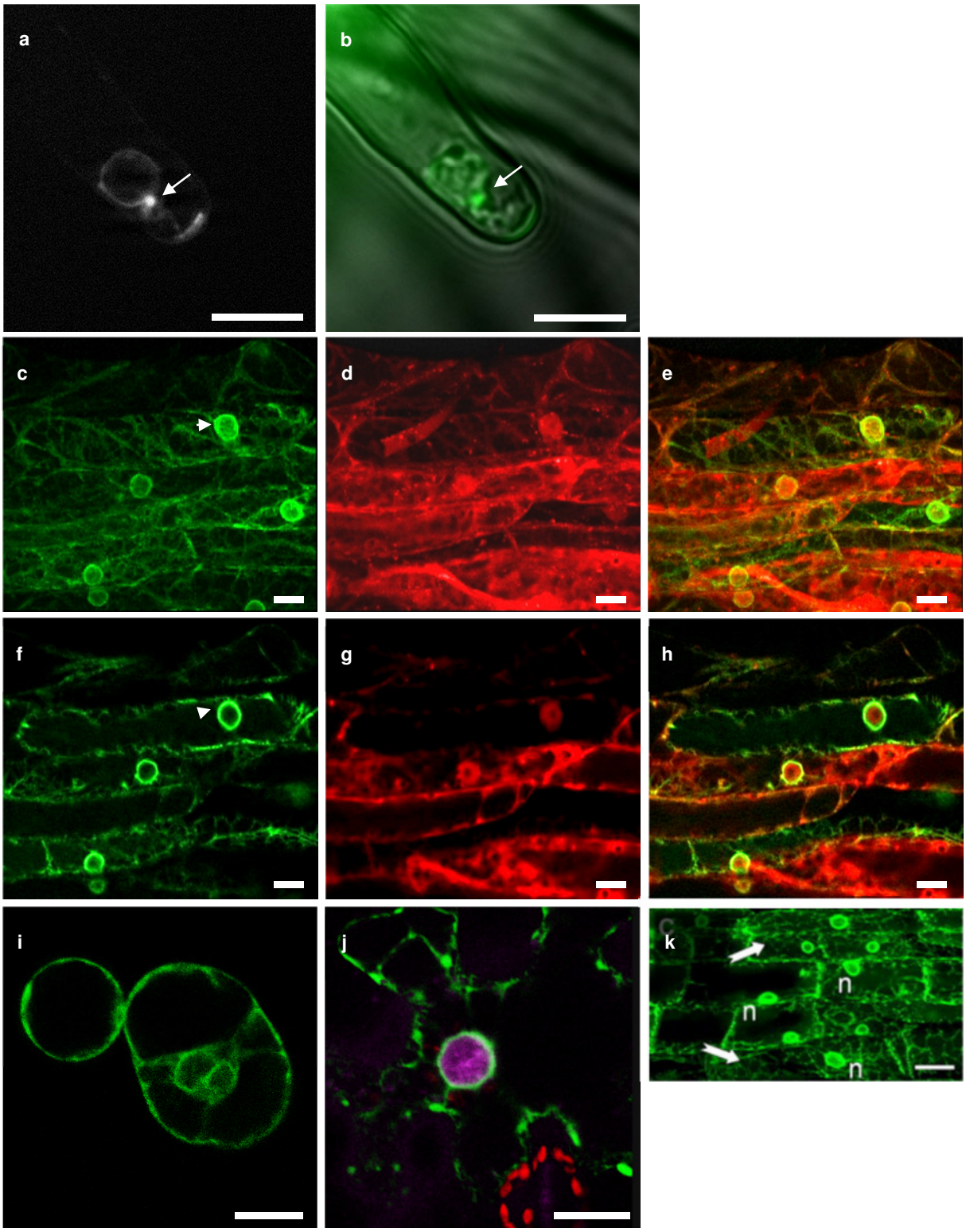


Figure 4.15 genomic situation and protein domains of MtMSBP1

In order to observe the localization of MtMSBP1 *in vivo*, the open reading frame was PCR-amplified (MtMSBP1-F2 / MtMSBP1-R3) and cloned into the binary vector pCGFP-RR resulting in a C terminally GFP-tagged version of the protein under the control of the CMV 35S constitutive promoter. The same T-DNA contained an Ubiquitin 10 promoter driven DsRED construct, which was used to select successfully *A. rhizogenes* transformed *M. truncatula* roots and as a fluorescent marker for cytoplasm and nuclei of transformed cells (Figure 4.16. d, e, g, h). In young root hairs, the GFP signal was observed at perinuclear localization as well as in strands projecting from the nucleus towards the root hair tip. Frequently MtMSBP1-GFP accumulated as a bright spot close to the nuclei (Figure 4.16 a, b). Confocal microscopy of root cortex cells showed that the GFP signal localized at the nuclear and cortical portions of the ER as well as at the cytoplasmic connections linking the



both populations. In contrast to the DsRED signal, which was observed within the nucleus the GFP fluorescence localized to the nuclear envelope but could not be detected inside of the nucleus (Figure 4.16 c to h). The localization of MtMSBP1-GFP at the ER strongly resembles the localization of the construct P35S:GFP-HDEL, containing the HDEL ER retention signal, shown by Genre *et al.,* (2005) (Figure 4.16 k). To test whether the ER

Figure 4.16 Subcellular localization of MtMSBP1-GFP.
(a) to **(j)** Expression of *MtMSBP1-GFP* under control of the CMV 35S promoter in *M. truncatula* hairy root explants. **(a)** and **(b)** Localization of MtMSBP1-GFP in root hairs. GFP fluorescence can be observed in the ER surrounding the nucleus (arrow) and in the cytoplasmic ER projections connecting to cortical ER of the root hair tip. **(c)** to **(h)** Localization of MtMSBP1-GFP in cortical root cells observed under the confocal microscope in a single z layer **(c)** to **(e)** or in a maximum projection of several layers **(f)** to **(h)**. Green fluorescence **(c, f)** corresponds to MtMSBP1-GFP, while red fluorescence **(d, g)** shows the reporter DsRED under the control of the *ubiquitin10* promoter as a control**. (e, h)** represent the overlay of the two fluorescence channels. MtMSBP1-GFP can be observed at the ER network as well as in the perinuclear ER (arrowhead) but never inside the nucleus. In contrast, DsRED localizes in the cytoplasm and inside the nucleus while absent in the nucleolus. **(i)** and **(j)** Expression of the fusion protein MtMSBP1-GFP in heterologous systems. (i) in *A. thaliana* protoplasts and **(j)** in *N. benthamiana* epidermal cells. GFP fluorescence corresponding to MtMSBP1 localizes in both cases to the ER. In epidermal cells of *N. benthamiana* red fluorescence from chloroplast of the stomata guard cells and the cell nucleus stained with DAPI in blue can also be observed. **(k)** Figure from Genre *et al.,* (2005) showing ER labeled by P35S-SP-GFP-HDEL construct in *M. truncatula* hairy roots. N = nucleus, arrows indicate ER network. Bars = 10 µm (a – j) and 20 µm (k).

localization pattern of MtMSBP1 was *M. truncatula* specific, protoplasts of *A. thaliana* and epidermal cells of *Nicotiana benthamiana* were transiently transformed with the same construct (this experiment was done and the pictures were kindly provided by Melanie Krebs at the ZMBP, University of Tübingen). In both cases MtMSBP1-GFP showed ER localization, including perinuclear localization (Figure 4.16 i, j).

4.3.2 Inactivation of MtMSBP1 results in a mycorrhiza-defective phenotype.

To test the significance of MtMSBP1 for the symbiosis, the expression of the gene was knocked down with the help of an RNAi construct (Figure 4.17). Thus a 200 bp fragment of the gene including the nucleotides -14 to +185 with respect to the A of ATG was PCR-amplified (MtMSBP1-RNAi-F2 / MtMSBP1-RNAi-R2) and cloned into the binary vector pK7GWIWG2DII where it integrated twice: First in forward and second in reverse orientation. Expression of the construct under control of the 35S promoter led to the formation of a RNA hairpin structure and the subsequent destruction of the complementary RNA. To prevent the downregulation of the second *M. truncatula* MSBP2 the construct was designed in a region of relatively low similarity between both genes (Figure 4.17).

After transformation of *M. truncatula,* several stable transgenic hairy root explant lines with different levels of gene downregulation were obtained. All lines grew similar to the control lines and showed no developmental defects under non-symbiotic conditions. Five hairy root lines were analyzed by PCR, and used for mycorrhizal analyses. The expression levels of *MtMSBP1* in the five RNAi hairy root lines analyzed were strongly reduced with respect to two control hairy root lines (transformed only with *A. rhizogenes* (line1) or with control plasmid (line2)) (Figure 4.18 a, b). The expression of *MtMSBP2* (*TC95008*) coding for the second isozyme was checked in all lines and it was not significantly affected. Transgenic RNAi hairy root lines were mycorrhized *in vitro* and analyzed at 12 dpi after ink staining of

Figure 4.17 Alignment of *MtMSBP1* and *MtMSBP2* transcripts.
The available cDNAs of both genes were aligned to each other using ClustalW. The start and stop codons are marked by red and black boxes respectively and the RNAi construct which was used to silence *MtMSBP1* is indicated with a green bar.

fungal structures. The results showed that the frequency of mycorrhizal colonization in RNAi lines was similar to that from control lines (Figure 4.18 c). However, several important phenotypic differences were observed. Appressoria from RNAi lines often developed clumps of highly septated hyphae that ended in aborted infections (Figure 4.19). Nevertheless, infection succeeded in other areas of RNAi lines and then the fungus developed intracellular hyphae, arbuscules and vesicles. Nevertheless, the number of arbuscules was strongly reduced in RNAi lines (ca. 80% reduction, Figure 4.18 c). Even more significant was that the abundance of collapsing arbuscules and septated hyphae was strongly increased with respect to the control lines (Figure 4.19).

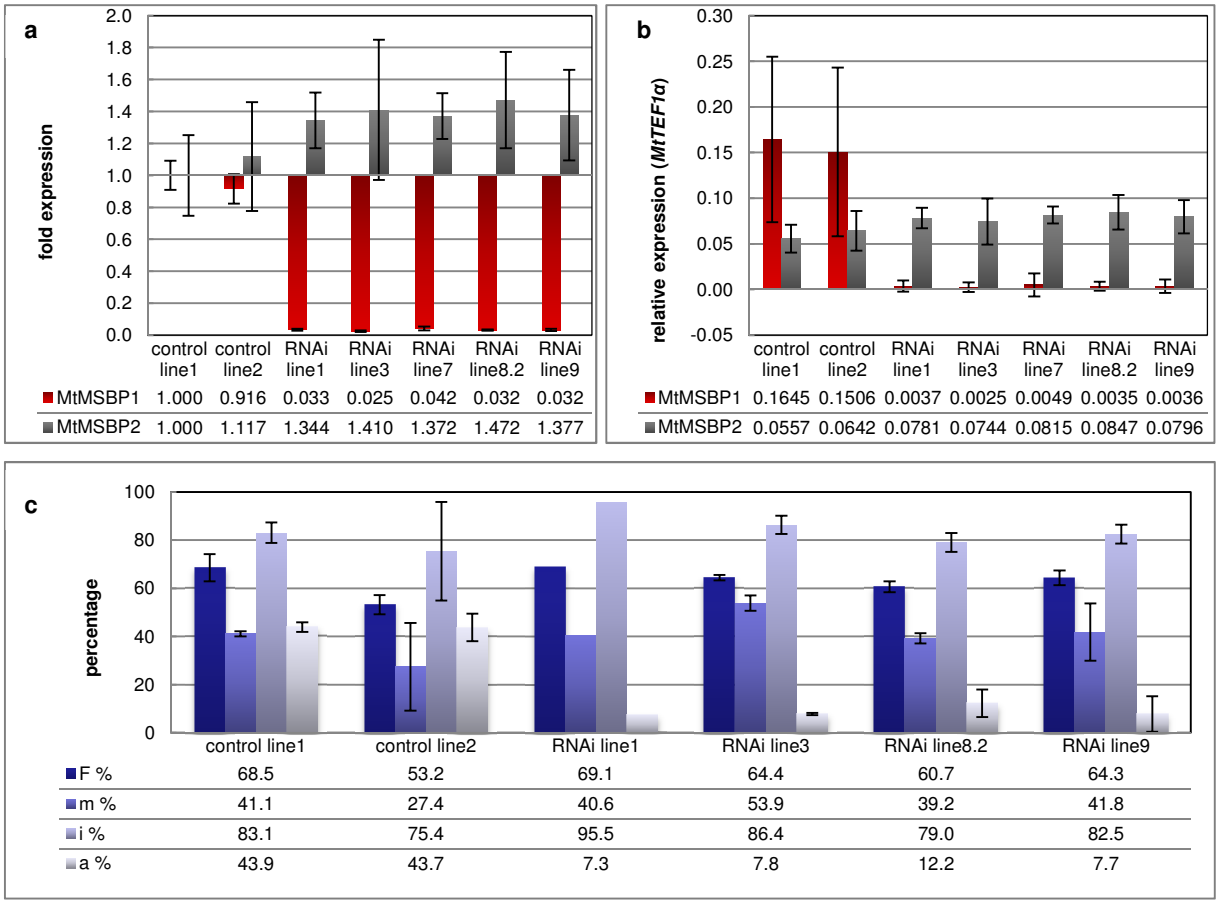


Figure 4.18 RNAi silencing of *MtMSBP1* expression.
Deficiency of MtMSBP1 in stable transgenic hairy root explants harboring P*35S-MtMSBP1*-RNAi constructs. (a, b) Fold expression of *MtMSBP1* and *MtMSBP2* was analyzed by quantitative Real-time PCR in control hairy root lines 1 (transformed merely with *A. rhizogenes*) and 2 (transformed with control vector) and five independent transgenic lines indicating that *MtMSBP1* RNA interference was successful whereas the similar *MtMSBP2* transcript was not degraded. (c) Colonization level was analyzed according to Trouvelot *et al.,* (1986) after ink and vinegar staining of roots 12 dpi with *G. intraradices*. In addition to the parameters F % (frequency of mycorrhiza in the root system), m % (intensity of the mycorrhizal colonization in mycorrhizal parts of the root system), a % (arbuscule abundance in the mycorrhizal parts of the root system) the value for i % (intraradical hyphae abundance in mycorrhizal parts of the root system) was determined using mycocalc software. Note that a% is reduced in most RNAi lines compared to control lines

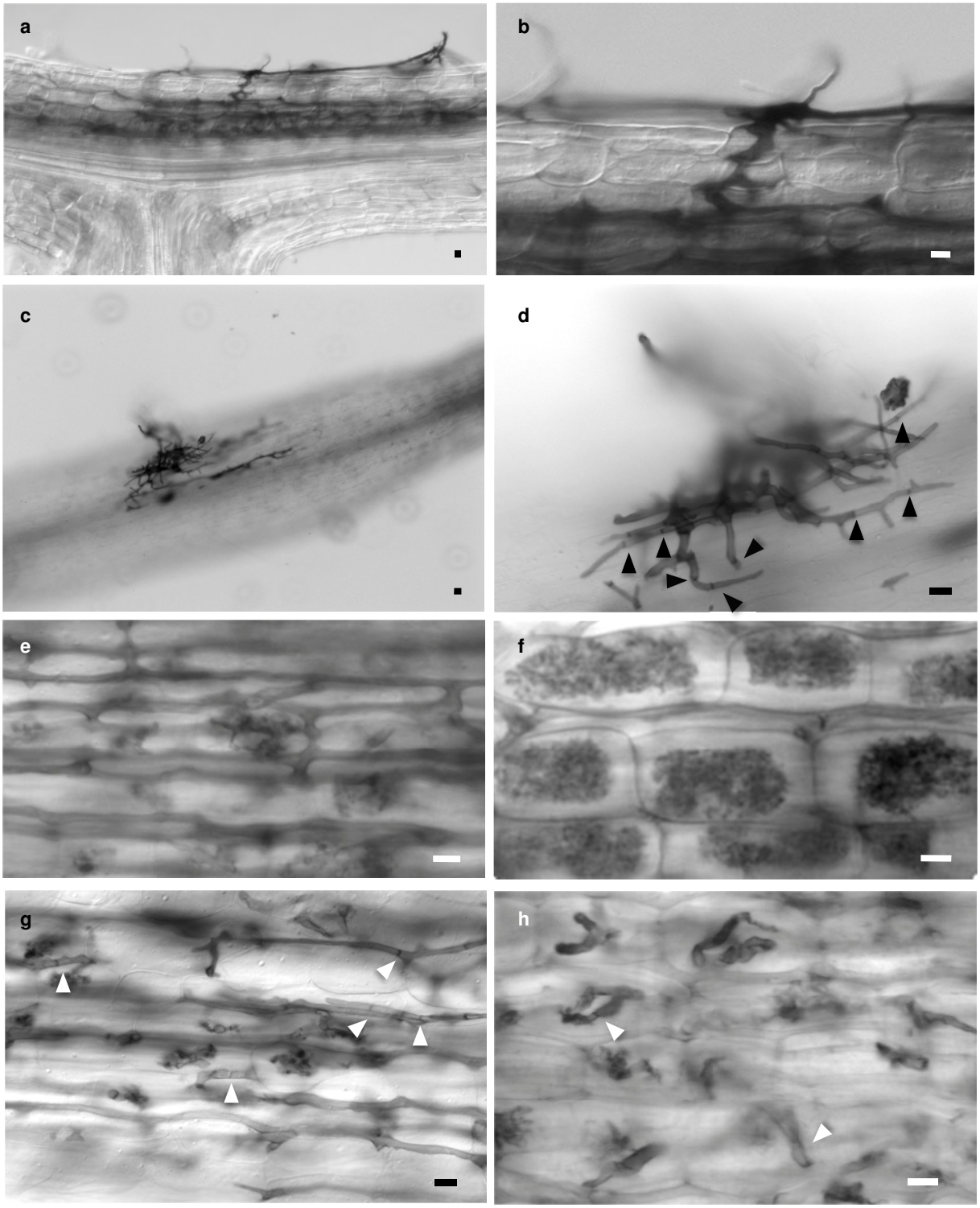


Figure 4.19 Ink stain of mycorrhizal roots with reduced *MtMSBP1* expression.
Ink stain of mycorrhizal roots often revealed an abnormal appressoria and arbuscule morphology in *MtMSBP1* RNAi lines (c, d, g and h) compared to control lines (a, b, e and f) hairy roots. Increased septation was frequently observed in hyphae developing in RNAi transgenic lines (arrowheads) (bar = 10µm).

4.3.3 A cue that MSBP1 has impact on abundance of free sterols in *M. truncatula roots*

Concerning the role of MtMSBP1, membrane bound steroid binding proteins are a family of proteins first described in animals and later found in plants and fungi (Falkenstein *et al.,* 1996; Hand & Craven, 2003; Iino *et al.,* 2007). Recent work with the mammalian and yeast/fission yeast homologues have shown that this family of proteins might have a conserved role in the control of the sterol biosynthesis by binding and regulating cytochrome P450 enzymes such as the yeast Cyp51 (Mallory *et al.,* 2005; Hughes *et al.,* 2007). In this respect, Yang *et al.,* (2005) also noted that in *A. thaliana*, from the 115 induced genes in transgenic lines over-expressing *AtMSBP1*, 13% were related to the steroid/sterol metabolism and signaling. From experiments using sterol biosynthesis inhibitor fungicides, it is known that those molecules affect the development of fungal structures in arbuscular mycorrhiza. For instance root colonization, spore production and mycelium architecture, including arbuscules are affected (Zocco *et al.,* 2008; Campagnac *et al.,* 2008; Campagnac *et al.,* 2009; Oger *et al.,* 2009; Campagnac *et al.,* 2010). A direct impact, not only on the AM fungal growth but also on the physiology and metabolic activities of the AM fungus was shown by Zocco *et al.,* (2010). Particularly application of fenpropimorph to extraradical hyphae resulted in a decrease in fungal alkaline phosphatase (ALP) and succinate dehydrogenase (SDH) activities associated with the extraradical mycelium. Moreover, *MtPT4* expression level was reduced and consequently phosphorous transport affected. Taken

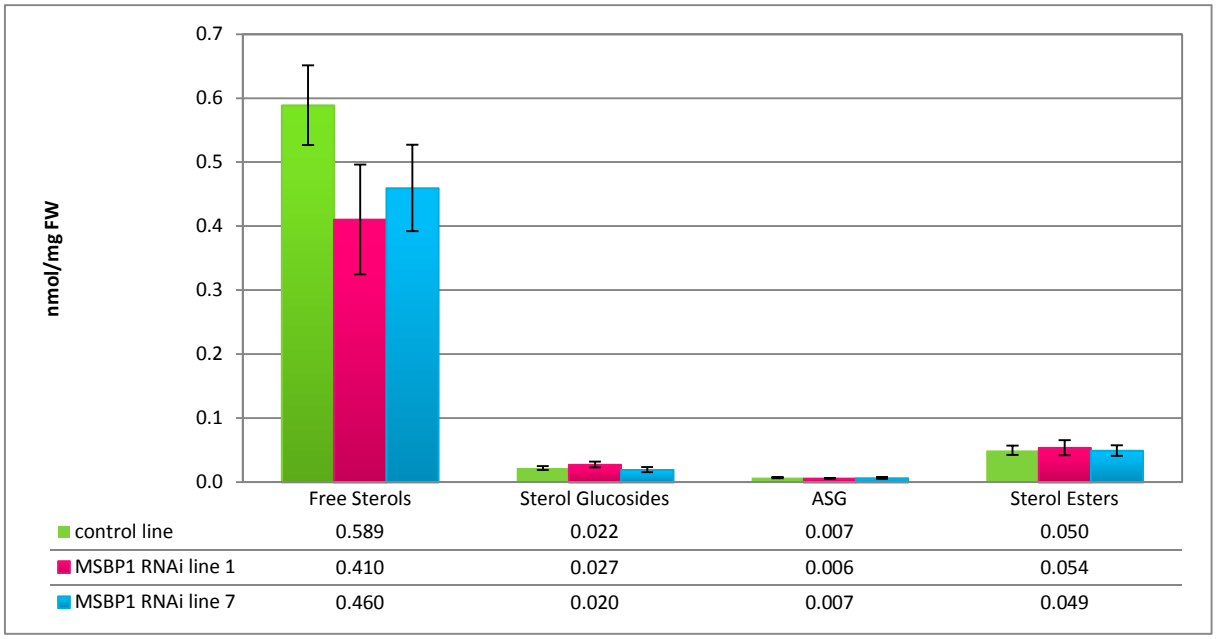


Figure 4.20 Sterol composition of MtMSBP1 RNAi lines and control line
Abundance of free sterols, sterol glycosides, acylated sterol glycosides and sterol esters were analyzed in *M. truncatula* hairy root empty vector control and two RNAi lines of MtMSBP1. Sterol amounts are given with respect to fresh weight (FW) of roots. Standard deviations of the average of 5 different biological replicates are given.

together these data show that plant and fungal sterols might play an important role in regulating arbuscular mycorrhizal functioning inside the root.

To test the influence of MtMSBP1 on sterol composition in the root empty vector control and RNAi line 1 of *MtMSBP1* was analyzed according to Wewer *et al.* (2011) in cooperation with Vera Wewer and Prof. Dr. Peter Dörmann at the department of molecular physiology and biotechnology of plants (IMBIO) at the Rheinische Friedrich-Wilhelms-University in Bonn. Albeit only little differences were detected in RNAi roots in comparison with the empty vector control line, a decreased abundance of free sterols was measured in response to reduced

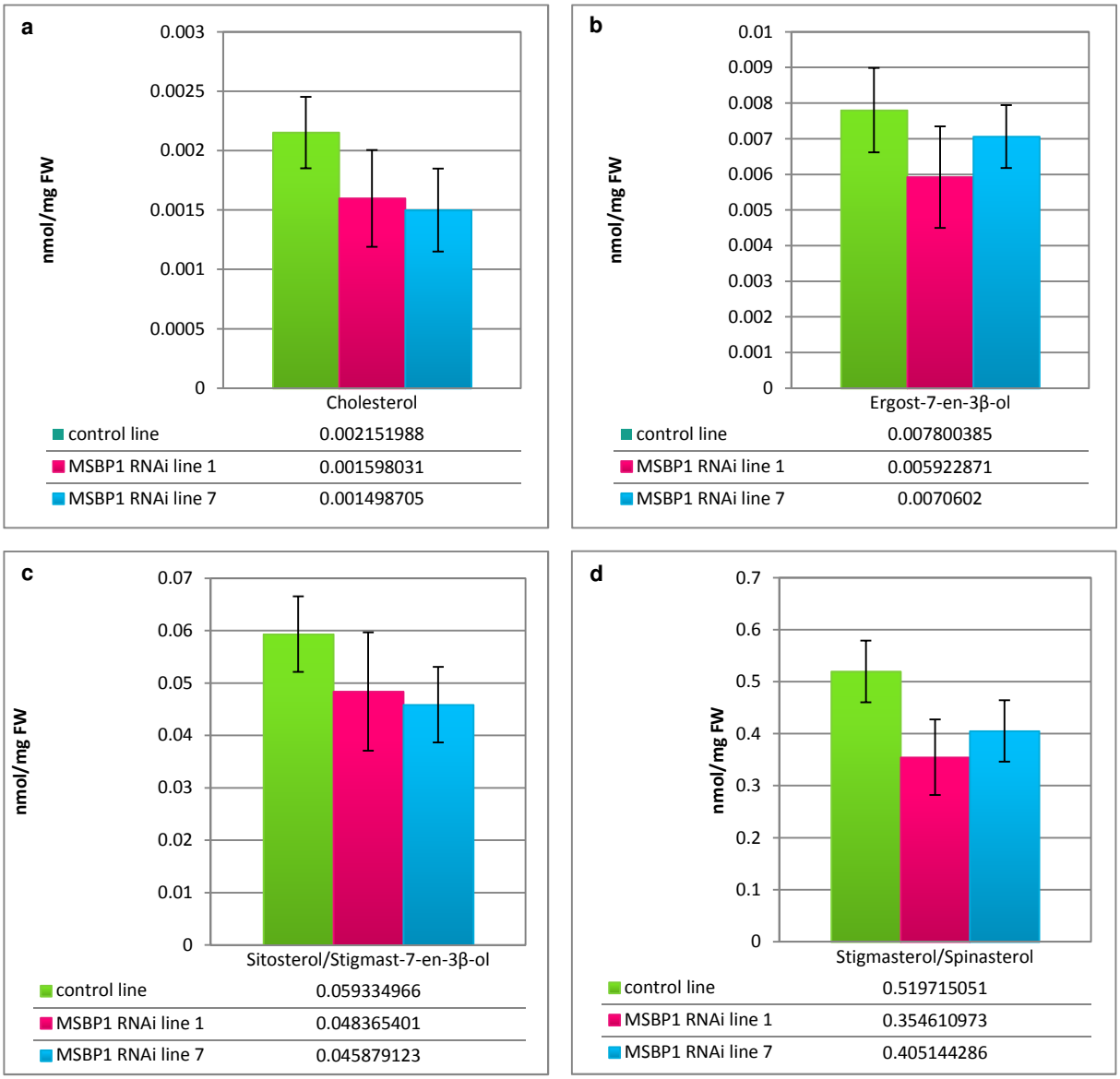


Figure 4.21 Composition of free sterols of MtMSBP1 RNAi lines and control line
Abundance of the free sterols cholesterol, ergost-7-en-3β-ol, sitosterol/stigmast-7-en3β-ol and stigmasterol/spinasterol were analyzed in *M. truncatula* hairy root empty vector control and two RNAi lines of MtMSBP1. Sterol amounts are given with respect to fresh weight (FW) of roots. Standard deviations of the average of 5 different biological replicates are given.

MtMSBP1 expression (Figure 4.20). This observation is contrasted by the measurements of sterol glycosides, acylated sterol glycosides and sterol esters, which did not change in correlation with MtMSBP1 abundance. In a more detailed characterization of the free sterols it was observed that all measured compounds (cholesterol, ergost-7-en-3β-ol, mixture of sitosterol/stigmast-7-en3β-ol and mixture of stigmasterol/spinasterol declined in the RNAi lines in comparison to the control line (Figure 4.21). The single components of the mixtures could not be distinguished because of same masses (Lefebvre *et al.,* 2007)). Although the standard deviations of the measurements showed no significant differences, a clear trend of decreased free sterol abundance can be depicted.

4.3.4 Activation of MtMSBP1 and the early induced genes is independent of progesterone and AM fungal sterols

Homologous proteins of MtMSBP1 are described to be involved in regulation of sterol metabolism. Furthermore, the homologous protein of MtMSBP1 in *A. thaliana* has been shown to have steroid binding activity (an ability that gave the name to this protein; Yang *et al.,* 2005) and is postulated to be involved in perception of sterols. A competitive binding assay revealed different binding affinities for several sterols including progesterone for which the lowest dissociation constant of 30 mM was measured. In addition, suppressed expression of *AtMSBP1* resulted in hypersensitivity to progesterone, while enhanced expression led to a reduced response to progesterone. In mammals, it was shown that progesterone is able to trigger expression of *PGRMC1* (a rat homologue of *MSBP1*) (Labombarda *et al.,* 2003), which has not been shown for any of the plant homologues of this

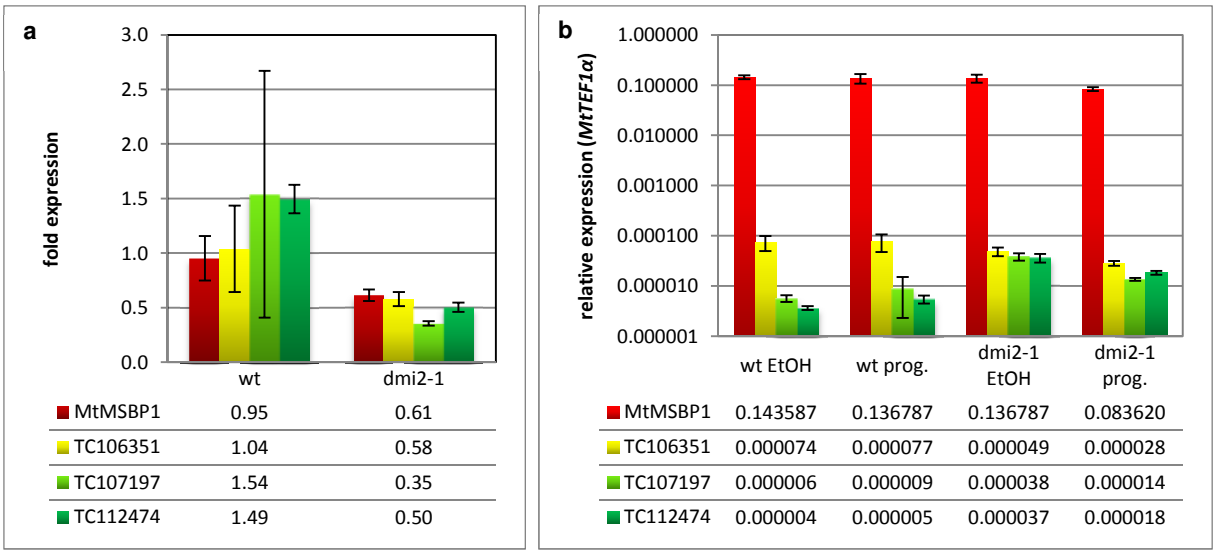


Figure 4.22 Application of progesterone does not activate the early-induced genes
M. truncatula hairy root explants with wt and dmi2-1 background were treated for 24 h with 1 µM progesterone. RNA was extracted out of roots. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of two different biological replicates are given.

gene. It is therefore tempting to speculate whether progesterone binding could influence the expression of the gene itself in *M. truncatula* as well.

To address this question *M. truncatula* hairy roots explants were treated with 1 µM of progesterone for 24 hours and the expression of *MtMSBP1* and the other early-induced genes was tested. However, no induction of any of the tested genes was observed (Figure 4.22). This was true for hairy roots with wt background as well as for *dmi2*-1 mutant roots. Progesterone itself can therefore be excluded as an activator of gene expression during arbuscular mycorrhiza symbiosis. Nevertheless, this result was not completely unexpected as many different steroids exist in plants and fungi and if an activation of gene expression is due to the perception of a steroid by MtMSBP1 this must not necessarily be progesterone. In contrast, recently AtMSBP1 has been shown to be also involved in perception of 24-epibrassinolide (24-eBL) by specifically interacting with the extracellular domain of BAK1 (BRI1 Associated receptor Kinase 1) that mediates brassinosteroid (BR) perception in

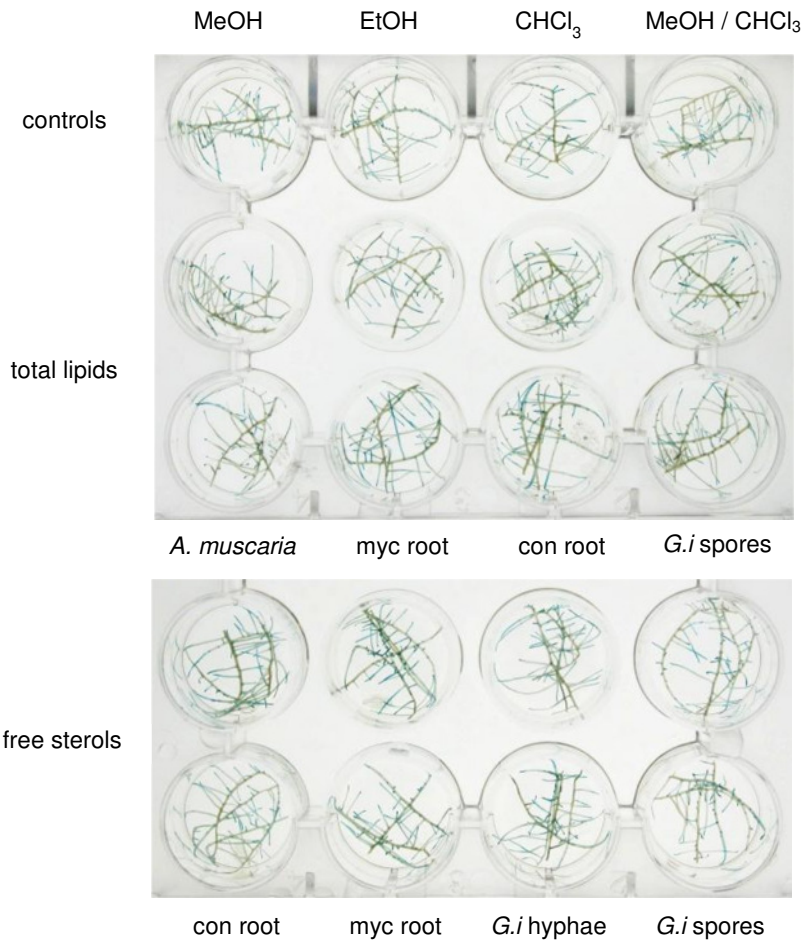


Figure 4.23 Application of fungal lipids to PMtMSBP$_{1518\ bp}$-GUS hairy root explants
M. truncatula hairy root explants transformed with a MtMSBP1 promoter reporter construct were incubated over night with 5 x 10^{-3} dilutions of total lipids or free sterols extracted from *G. intraradices* hyphae or spores, mycorrhizal carrot roots, non-colonized carrot roots and *Amanita muscaria* mycelium. Appropriate control treatments were applied as well. Roots were stained for GUS activity.

combined action with the BR receptor BRI1 (brassinosteroid-insensitive 1). Additionally beneath the binding to progesterone, AtMSBP1 was also shown to bind 24-eBL as well as to 5-dihydrotestosterone and stigmasterol.

To further test the possibility whether any sterols derived from arbuscular mycorrhizal fungi would be able to activate the expression of *MtMSBP1* itself, different sterol preparations of *G. intraradices* were tested for their ability to induce expression in a *MtMSBP1* promoter reporter assay. Therefore a 1518 bp promoter fragment upstream of the ATG including the 5' UTR of *MtMSBP1* (PSMBP10-F / PSMBP10-R) was fused to ß-glucuronidase in the binary vector pPGFPGUS-RR and stably transformed into *M. truncatula* hairy roots. Nevertheless, after application of different extractions of sterols and total lipids no changes in GUS activity could be observed in comparison to control roots, which were treated with the respective solvents (Figure 4.23). Thus, it can be stated that neither progesterone nor other AM fungal sterols are able to drive the expression of *MtMSBP1*. Whether *G. intraradices* sterols might be able to induce the transcription of one of the other early-induced genes remains to be tested.

4.3.5 MtMSBP1 does not interact with MtCyp51 or MtSERK2 in yeast

Several interaction studies have been carried out for MtMSBP1 homologous proteins in different organisms including mammals, fungi and plants and interaction partners have been found to be mainly involved in sterol metabolism or steroid signaling. It is described that in unicellular eukaryotes and mammals, MtMSBP1 homologues interact with P450 proteins participating in sterol synthesis (Hand *et al.,* 2003; Mallory *et al.,* 2005; Craven *et al.,* 2007; Hughes *et al.,* 2007; Thompson *et al.,* 2007). In yeast for instance Dap1 (damage associated protein 1) is described to interact with Cyp51/lanosterol demethylase and the mammalian PGRMC1 binds to Cyp51, Cyp3A4, Cyp7A1/cholesterol 7α-hydroxylase and Cyp21A2/21-hydroxylase in humans (Hughes *et al.,* 2007).

An interaction with PGRMC1 has additionally been demonstrated for Insig-1 (insulin-induced gene-1) and SCAP (SREBP cleavage-activating protein) in mammalian cell culture (Suchanek *et al.,* 2005). These two proteins are involved in induction of proteins related to cholesterol synthesis (Goldstein *et al.,* 2002; Horton *et al.,* 2002; Yang *et al.,* 2002; Sun *et al.,* 2005; Goldstein *et al.,* 2006; Gong *et al.,* 2006). This regulation might not be conserved in *M. truncatula* as no similar genes exist in the genome project, or those have not yet been sequenced. In other plants no proteins with unambiguous homology to Insig-1 are found whereas the sterol-sensing domain of SCAP seems to be partially conserved and present in proteins of mostly unknown function. In plants, only direct interactions of MSBP1 have been tested with the *A. thaliana* homologue of the protein. As already mentioned above, Song et

al. (2009) showed that AtMSBP1 interacts with the extracellular domain of BAK1, which is involved in BR perception in concerted action with the BR receptor BRI1. Noteworthy, in plants, the Cyp51G1 14α-demethylase catalyzes a step in biosynthesis of brassinosteroids.

To analyze the described interactions of MtMSBP1 homologous proteins in *M. truncatula*, the ORF of the gene was fused without signal peptide (SP) and transmembrane helix to the GAL4 activation domain in the yeast two-hybrid vector pGADT7-Rec (SMBP1-NdeI_fw/SMBP1-BamH1_rev). The *M. truncatula* homologues for Cyp51 (MtCYP51G1 without SP (DQ335779.1)), and BAK1 (MtSERK2 somatic embryogenesis receptor kinase 2) (HM640001.1) extracellular domain) were integrated in the corresponding vector pGBKT7, where they were fused to the GAL4 DNA binding domain (MtSERK2-Nde1_fw/MtSERK2-BamH1_rv; MTCyb51G1-Nde1_fw/MTCyb51G1-BamH_rv). The ability of the proteins to interact was tested Additionally the opposite combinations of vectors and proteins were also included in the analysis.

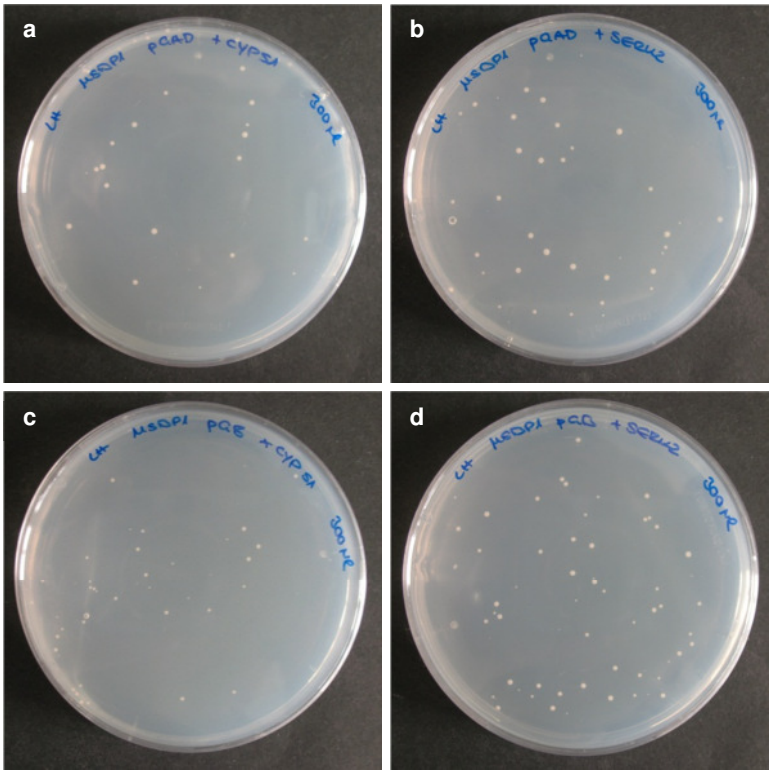


Figure 4.24 Co-transformation of yeast two hybrid plasmids
Positive co-transfomants of MtMSBP1 in pADT7-Rec with CYP52G1 (a) or SERK2 (b) in pGBKT7 or vice versa MtMSBP1 in pGBKT7 with CYP52G1 (c) or SERK2 (d) in pADT7-Rec.

The co-transformation of MtMSBP1 in pADT7-Rec with CYP52G1 or SERK2 in pGBKT7 or vice versa MtMSBP1 in pGBKT7 with CYP52G1 or SERK2 in pADT7-Rec yielded few colonies on plates for selection of positive co-transformants (drop out medium without leucine and tryptophan (LW); Figure 4.24). However, no colonies grew on plates used for

selection of positive interactions (drop out medium without leucine, tryptophan, histidine and adenine (LWHA)). To be sure about the negative result 20 colonies of each co-transformation were inoculated on drop out medium without LWHA (Figure 4.25 a-d). One colony of each co-transformation was additionally inoculated together with a positive and negative control on drop out medium without LW and LWHA. The positive control consisted of murine p53 in pGBKT7 and simian virus 40 (SV40) large T-antigen in pGADT7-Rec, which were shown to interact in a yeast two-hybrid assay (Iwabuchi *et al.,* 1993; Li & Fields, 1993). Only the positive control grew on plates without LWHA indicating that MtMSBP1 did interact neither with MtCyp51G1 nor with MtSERK2. However, this data is preliminary at is has not been tested yet whether the protein fusions are properly produced by the yeast strain. This should be tested in the future to verify the results.

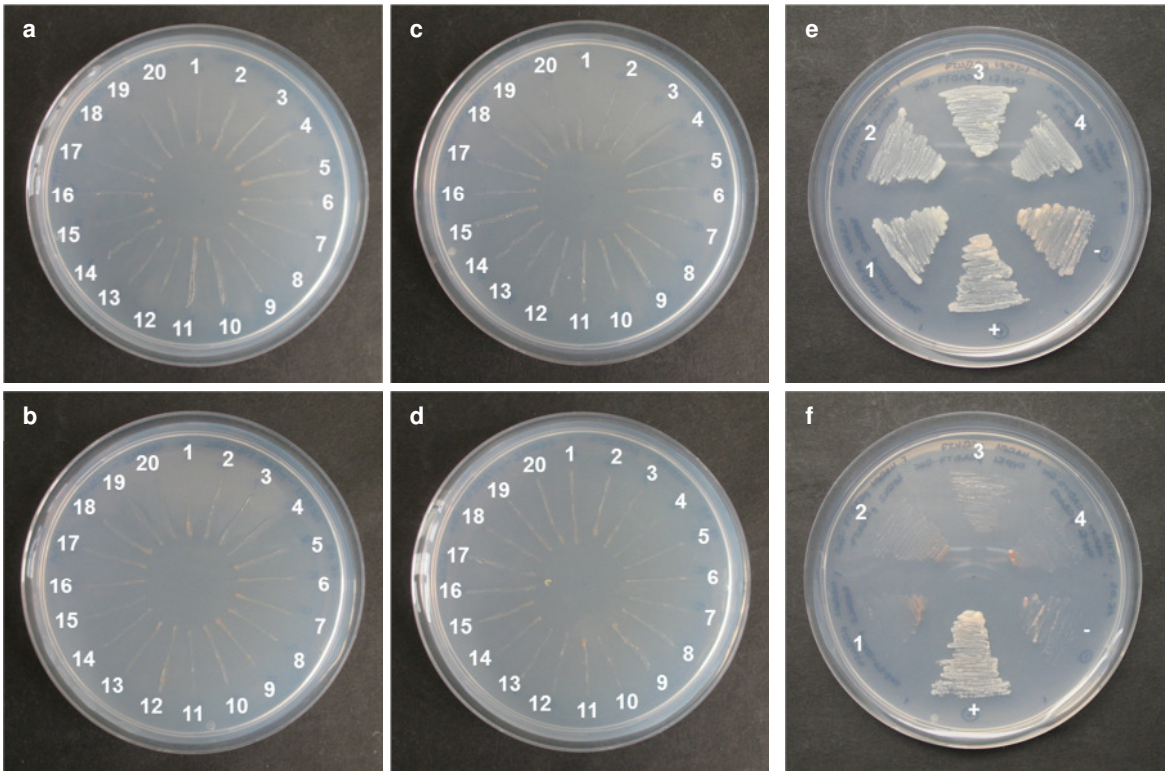


Figure 4.25 Growth of obtained Y2H clones on selection media
(a) to (d) Twenty colonies of each co-transformation inoculated on drop out medium without leucine (L), histidine (H), tryptophan (W) and adenine (A): (a) MtMSBP1-pGADT7-Rec + MtCyp51G1-pGBKT7 (b) MtMSBP1-pGADT7-Rec + MtSERK2-pGBKT7 (c) MtMSBP1-pGBKT7 + MtCyp51G1-pGADT7-Rec (d) MtMSBP1-pGBKT7 + MtSERK2-pGADT7-Rec (e) and (f) One colony of each co-transformation inoculated together with positive and negative control on drop out medium without LW (e) or without LWHA (f). 1: MtMSBP1-pGBKT7 + MtSERK2-pGADT7-Rec; 2: MtMSBP1-pGADT7-Rec + MtSERK2-pGBKT7; 3: MtMSBP1-pGBKT7 + MtCyp51G1-pGADT7-Rec; 4: MtMSBP1-pGADT7-Rec + MtCyp51G1-pGBKT7; +: p53- pGBKT7 + SV40 large T-antigen- pGADT7-Rec; -: Two non-interacting proteins.

4.4 Involvement of the plant hormones auxin and gibberellic acid in regulation of early mycorrhiza-induced genes

4.4.1 PAA treatment induces the expression of *TC107197* and *TC100804*

There are several evidences indicating an involvement of auxins in the regulation of arbuscular mycorrhiza symbiosis (Gunze & Hennessy, 1980; Xie *et al.,* 1998; Müller, 1999; Hanlon & Coenen, 2010). The levels of several auxins have been shown to change during fungal colonization in different plants (Ludwig-Müller *et al.,* 1997; Kaldorf & Ludwig-Müller 2000, Ludwig-Müller & Cohen, 2002, Fitze *et al.,* 2005, Meixner *et al.,* 2005, 2007, Jentschel *et al.,* 2007, Campanella *et al.,* 2007). The patterns of abundance observed in those studies indicate that probably distinct auxins become important during different stages of the symbiosis. This is an interesting idea as several different molecules with auxin activity such as IAA, IBA, 4-Cl-IAA and PAA are naturally occurring in plants but their distinct roles in plant development hast not been examined satisfactorily (Figure 4.26). To address the question if auxins could have an impact on the expression of the early-induced genes *M. truncatula* plants were treated with different available naturally occurring and synthetic (1-NAA, 2,4-D, 2,4,5-T, picloram) auxin derivatives (Figure 4.26).

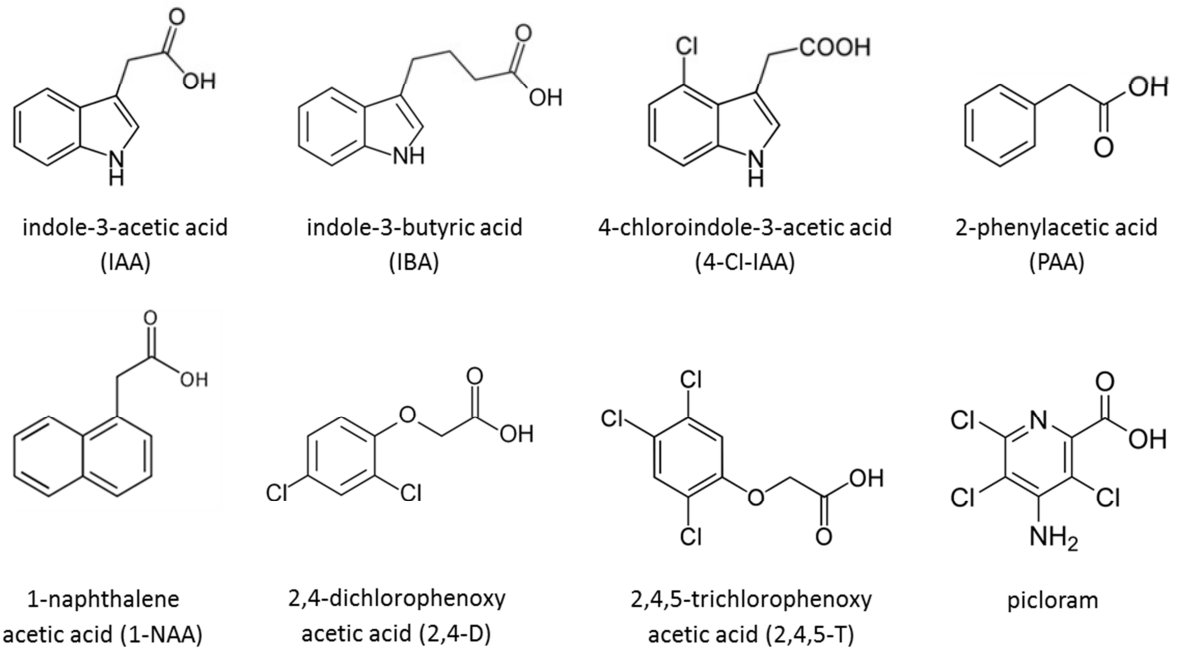


Figure 4.26 Naturally-occurring and synthetic auxins used in this study

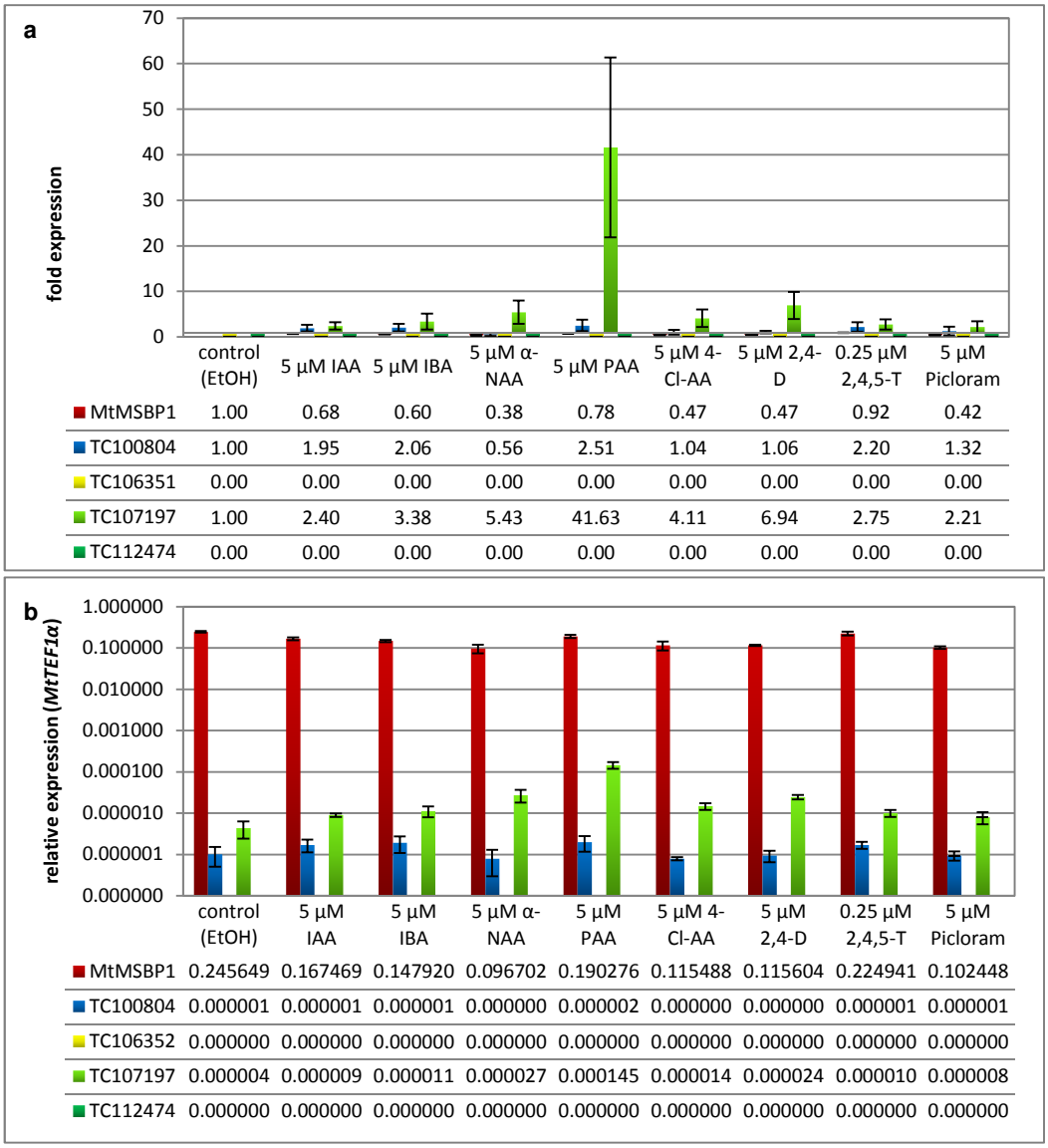


Figure 4.27 Expression of marker genes after treatment with different auxins.
M. truncatula plantlets were treated for 6 h with the given concentration of different auxins or with the corresponding concentration of the ethanol, which was used as a solvent. RNA was extracted out of roots. PAA was able to induce the expression of *TC107197* as well as *TC100804*, whilst having no unambiguous influence on the expression of the other genes tested. *TC107197* was induced by the other auxins as well, but to a much lower level. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

Remarkably, after 6h of incubation PAA had strongly induced the expression of *TC107197* in roots but also slightly raised *TC100804* promoter activity (Figure 4.27). Additionally *TC107197* and *TC100804* were upregulated by several other auxins but to a much lesser extent, while *MtMSBP1*, *TC106351* and *TC112474* were not induced by any of the compounds (Figure 4.27). Interestingly, the level of free PAA was reported to rise during an early interaction of *T. majus* and *G. intraradices* (Jentschel *et al.,* 2007) indicating a special role of this naturally-occurring auxin during the onset of the symbiosis. Despite the even higher induction of the genes that was observed during later colonization (Figure 4.3, Figure

4.4), none of the auxins reported to increase in level in this stages (mainly IAA and IBA) was able to elevate the expression level of *TC107197* to a similar high degree as observed with PAA (Figure 4.27).

4.4.2 PAA-induced *TC107197* expression depends on DMI1 and DMI2 but not on DMI3

As PAA induced the expression of *TC107197* in wt roots, it was to be tested in a next step if this induction was dependent on the SYM pathway, which would strengthen a possible role for this auxin during arbuscular mycorrhizal signaling. To elucidate this issue *dmi1-1*, *dmi2-1* and *dmi3-1* plant roots were treated for 6 h with PAA, as well as with IAA and 1-NAA. While

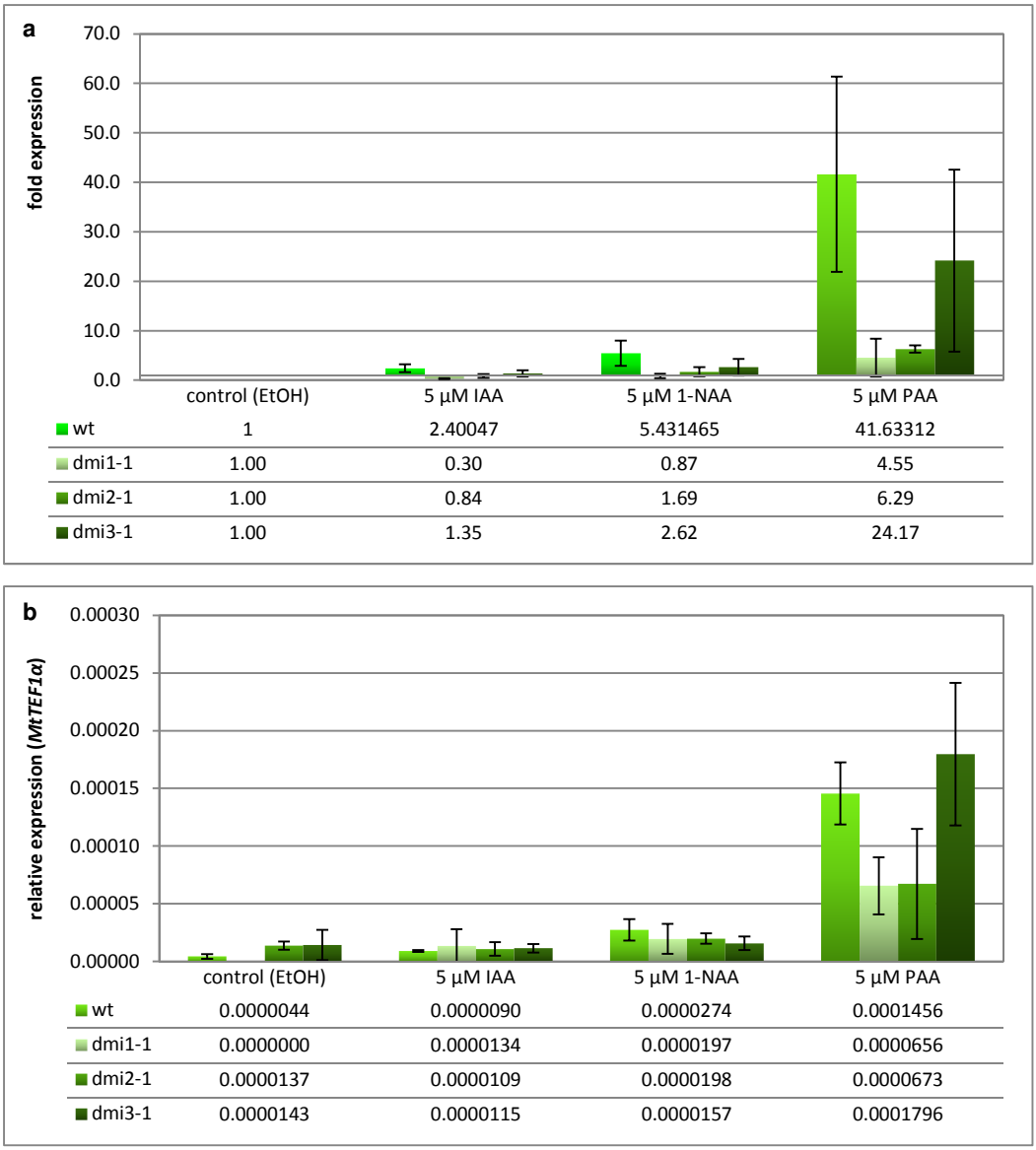


Figure 4.28 Expression of *TC107197* in wt and mutant plants after treatment with different auxins.
M. truncatula plantlets were treated for 6 h with the given concentration of different auxins. PAA induced the expression of *TC107197* in wt and dmi3-1 mutants to a similar level while level of induction was strongly reduced in dmi1-1 and dmi2-1 plants. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviation of the average of three different biological replicates is given.

the expression level of *TC107197* in the *dmi3-1* roots was comparable with the level obtained in wt, the other mutants showed a reduced transcript abundance of the gene (Figure 4.28). The same observation was made also for the slighter induction occurring after 1-NAA treatment, which was abolished in *dmi1-1* and *dmi2-1* mutant plants but less reduced in *dmi3-1*. The dependency of the auxin-mediated induction of *TC107197* on the two components of the SYM pathway reinforces the role of these substances during arbuscular mycorrhizal symbiosis. Additionally, the fact that the Ca^{2+}/calmodulin-dependent kinase DMI3 is not necessary to switch on *TC107197* expression confirms the idea of an existence of a parallel pathway playing a role during early AM signaling.

4.4.3 The PAA-mediated induction of *TC107197* depends on PAA concentration.

Auxins are described to have differential effects if abundant in different concentration. It is long known, that the concentration of IAA is very important in determining the nature of the growth response and the optimum auxin concentration differs for different organs

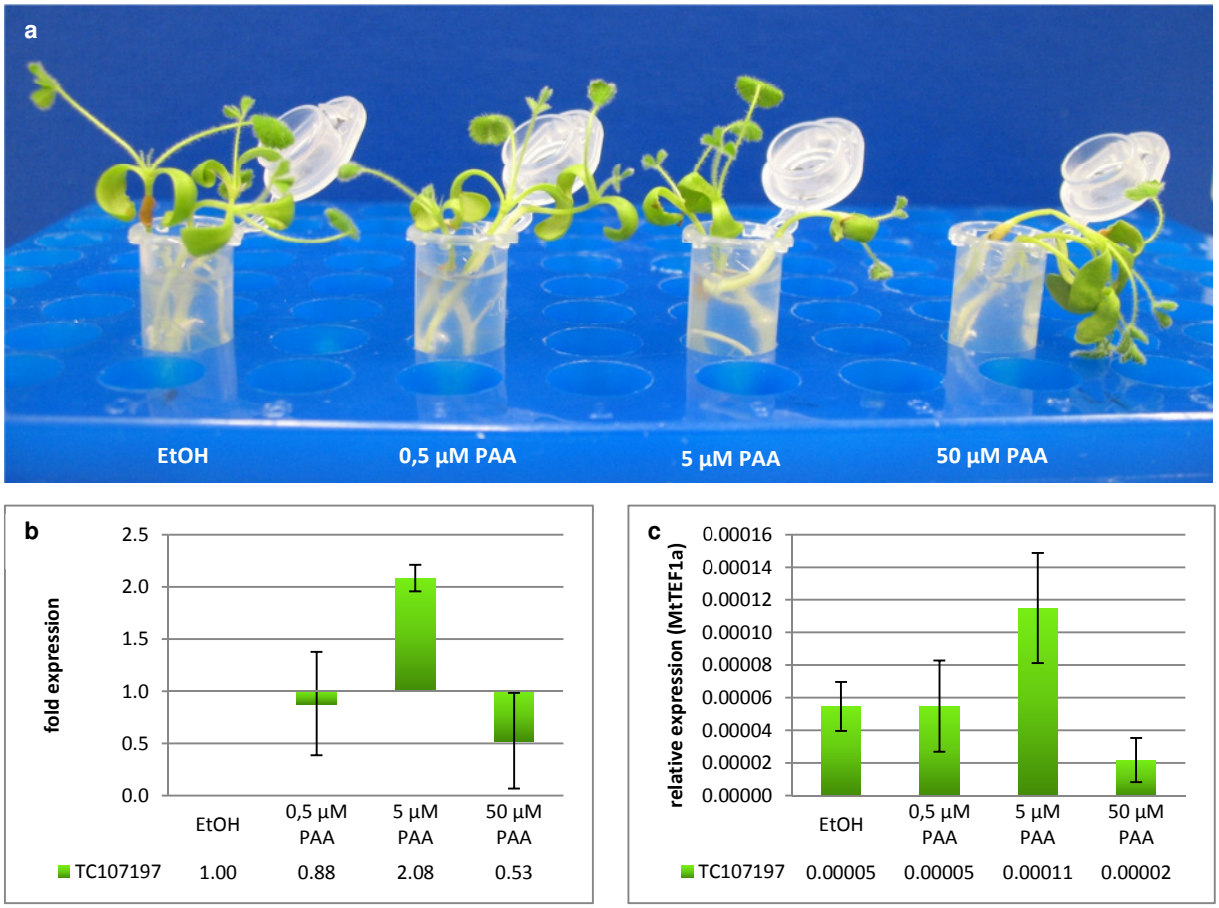


Figure 4.29 Application of different PAA concentrations to *M. truncatula* plants.
(a) *M. truncatula* plantlets were treated for 6 h with 0.5, 5, or 50 µM of PAA. An induction of *TC107197* expression could be only observed in those roots incubated with 5 µM of the auxin. The expression was even reduced by the addition of 50 µM of PAA that could correlate with the bad overall constitution of the plants in those samples (b) Fold expression normalized to *MtTEF1α* and relative to the control sample. (c) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

(Went & Thimann, 1937). In roots, the application of low concentrations of IAA leads to stimulation of growth whereas higher concentrations inhibit the elongation. To test whether the induction of *TC107197* depends on the PAA concentration applied to the plants, plant roots were incubated for 6 h with 0, 0.5, 5, or 50 µM of PAA and tested for *TC107197* expression. After the treatment it already became apparent that high PAA concentrations (50 µM) were generally detrimental for plant health (Figure 4.29 a). Interestingly, an induction of *TC107197* was only observed in roots treated with 5 µM PAA (Figure 4.29 b, c) indicating that only a narrow range of PAA concentrations is able to activate the expression. Application of 50 µM PAA even led to a decreased transcript abundance of *TC107197*, which was most probably the result of the poor health of the plants.

4.4.4 Application of the auxin signaling inhibitor 2-furylacrylic acid promotes the response of *TC107197* to different stimuli.

The previous experiments have shown that the auxin PAA is involved in induction of *TC107197* and that this auxin signal is linked to arbuscular mycorrhizal signaling. In order to further investigate the mechanisms of auxin-mediated induction of *TC107197*, an inhibitor of auxin signaling was applied. 2-furylacrylic acid (2-FAA, Figure 4.30) is a small molecule that has been described to inhibit auxin-mediated (IAA, 1-NAA and 2,4-D) responses in *A. thaliana* without interfering with auxin transport (Armstrong *et al*., 2004; Sungur *et al*., 2007). The structural requirements for natural and synthetic auxins have been determined to the existence of a carboxylic acid moiety separated by approximately 0.5 nm from an aromatic ring in the proper orientation (Porter & Thimann, 1965). Although 2-FAA satisfies the structural requirements of an auxin (Figure 4.30), it has distinct anti-auxin effects including blockage of auxin-induced gene expression, interference with auxin-regulated proteolysis of an AUX/IAA transcription factor and attenuation of root elongation. Furthermore, although 2-FAA does block primary root growth, it does not induce the expression of synthetic or auxin-regulated genes (Sungur *et al*., 2007). However, the cellular target of 2-FAA has not yet been identified.

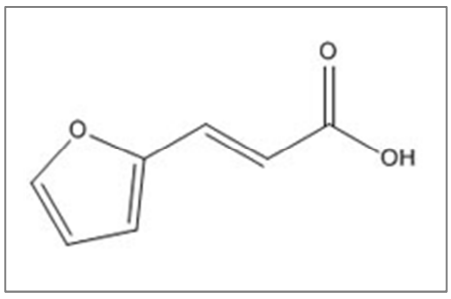


Figure 4.30 2-furylacrylic acid (2-FAA)

To examine the influence of an inhibitor of auxin signaling on the expression of *TC107197*, roots of *M. truncatula* plants were treated with 2-FAA alone or in combination with either PAA or an extract of supernatant from *G. intraradices* germinating spores. This ethyl acetate extract was shown to contain compounds that are able to induce the expression of *TC107197* (Albarran Nogales, Kuhn & Requena *et al.* unpublished results). As the spores used to produce this extract were germinated in absence of plant roots, it can be stated that this induction was due to a constitutively produced molecule released by germinating hyphae of *G. intraradices*. Interestingly this constitutive signal was able to rise the expression of *TC107197* but not *TC106351* indicating the existence of different signals that are important for early recognition processes. In contrast to what was expected, the addition of 2-FAA resulted in an even more increased expression of *TC107197* (Figure 4.31). This was true for PAA as well as for extract-dependent induction of the gene. Furthermore, 2-FAA alone was also able to induce the expression of *TC107197*. If 2-FAA is not able to inhibit the effect of PAA on *TC107197* expression, it seems that its described inhibitory influence on signaling of other auxin derivatives positively influences PAA signal transduction.

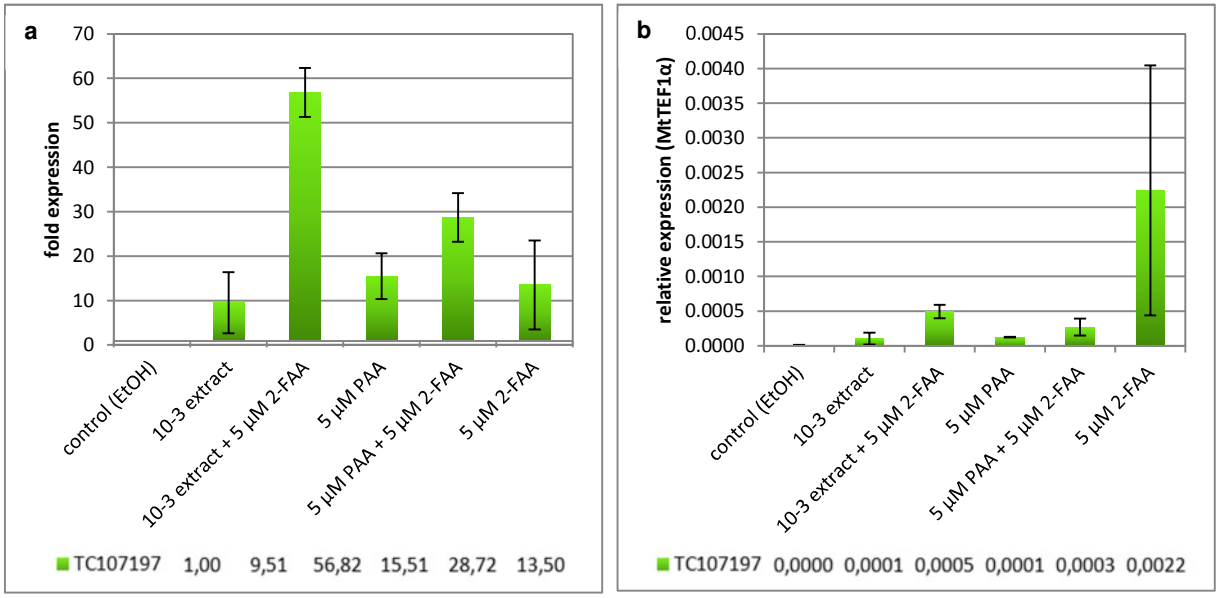


Figure 4.31 expression of *TC107197* after application of 2-FAA.
M. truncatula plantlets were treated for 6 h with 5 µM of 2-FAA alone and in combination with either 5 µM PAA or fungal spore supernatant extract. While *TC107197* expression was already induced by PAA and the fungal extract the application of 2-FAA additionally increased this response. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

4.4.5 Inactivation of MtMSBP1 influences the PAA-mediated induction of *TC107197*

With respect to auxin signaling, Yang *et al*., (2008) showed that an *A. thaliana* homologue of MtMSBP1 is involved in regulation of auxin signal transduction and redistribution of auxin by stimulating the cycling and influencing the distribution of the PIN2 auxin transporter under gravi-stimulation. Additionally the same group showed that application of IAA revealed an enhancement of inhibitory effects of auxin on root growth in *MSBP1*-overexpressing seedlings. Vice versa, this effect was suppressed in MSBP1-deficient plantlets. To test whether MtMSBP1 has an influence on the PAA dependent induction of *TC107197*, RNAi hairy root lines of *MtMSBP1* were treated with 5 µM of PAA and tested for their expression of *TC107197*.

The analysis of *TC107197* transcript abundance revealed that the overall expression of *TC107197* was generally higher in *MtMSBP1* RNAi roots than in hairy roots transformed with

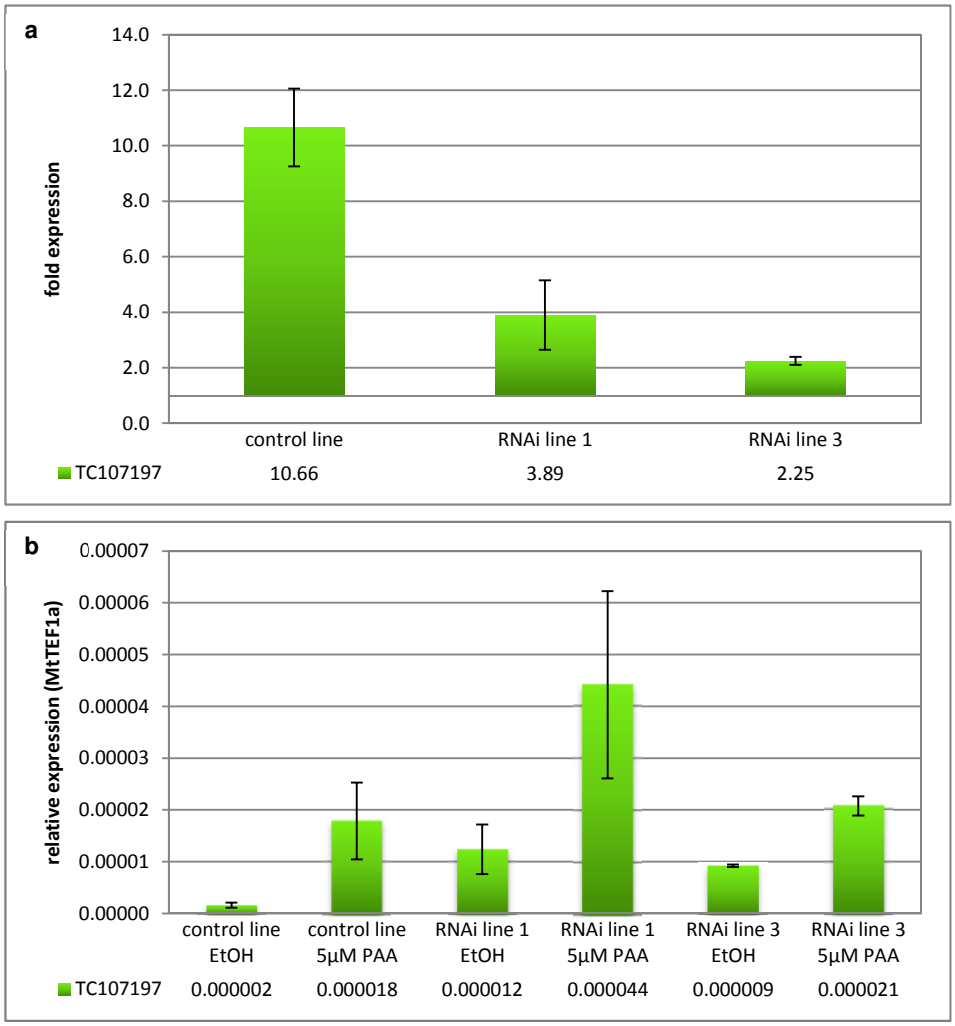


Figure 4.32 Transcript abundance of *TC107197* in *MtMSBP1* RNAI hairy roots after PAA treatment.
M. truncatula hairy roots transformed with the *MtMSBP1* RNAi construct were treated for 6 h with 5 µM of PAA. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

an empty vector control. The fold induction of *TC107197* after PAA treatment was decreased in RNAi roots if compared to empty vector control lines but this was probably due to the higher basal expression level of the RNAi roots. Thus, it is difficult to conclude whether silencing of *MtMSBP1* has a negative effect on PAA signaling or a positive effect on *TC107197* basal expression. To confirm one of both hypotheses the experiment should be repeated with additional hairy root control lines.

4.4.6 Gibberellic acid negatively influences marker gene expression in hairy root explants.

Another hormone that has been shown to be involved in regulation of arbuscular mycorrhiza symbiosis is gibberellic acid (GA). Exogenous GA has been reported to have strong inhibitory effect on symbiosis formation in pea (El Ghachtouli *et al*., 1996) leading to increasing suppression of AM formation if applied in rising concentrations to plants. Vice versa, treatment of *Citrus macrophylla* roots with the GA biosynthesis inhibitor paclobutrazol increased the AM colonization (Michelini *et al*. 1989).

Moreover, gibberellic acid is also closely linked to auxin signaling (reviewed by Chandler, 2009). For instance a cross talk of both hormones induces lateral root formation (LRF) (Berova & Zlatev, 2000; Busov *et a*l., 2006; Gou *et a*l., 2010). Likewise, increased LRF is reported to occur in response to AM fungal signals released by germinated spores if co -

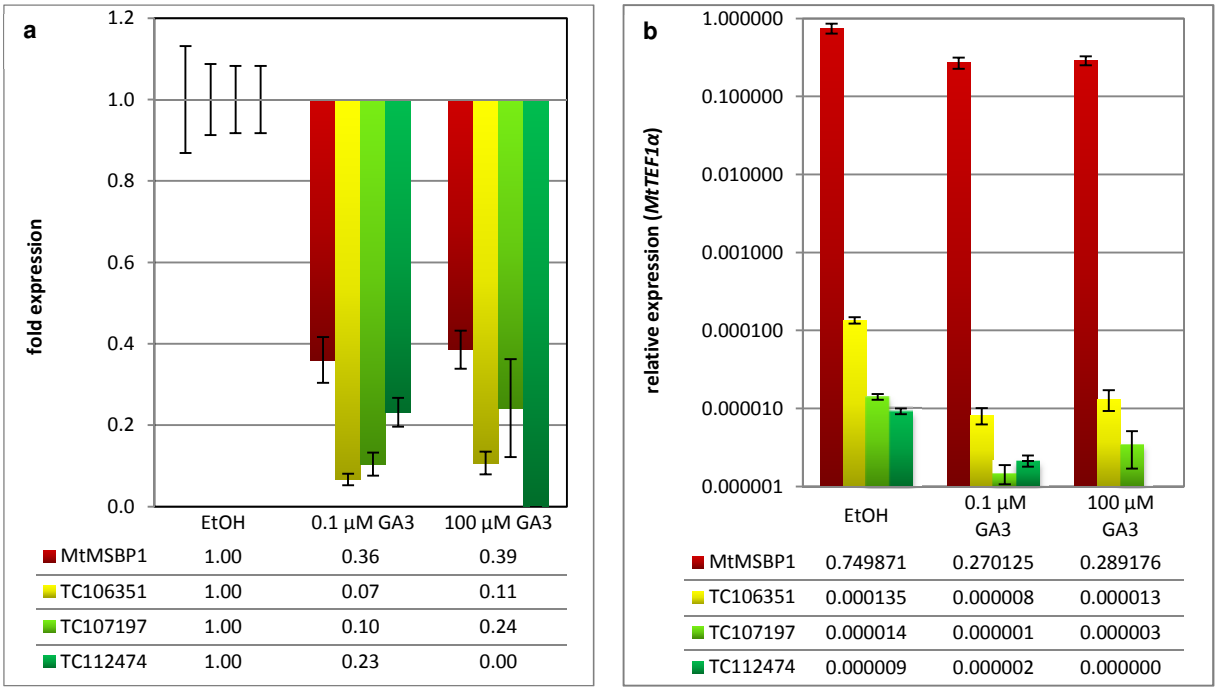


Figure 4.33 Expression analysis after GA treatment of hairy root explants.
M. truncatula hairy roots transformed with the *MtMSBP1* RNAi construct were treated for 6 h with 0.1 or 100 µM of GA solved in EtOH. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of two different biological replicates are given.

cultivated with host roots (Oláh *et al*., 2005) or AM fungal lipo-chitooligosaccharides (Myc-LCOs) (Maillet *et al*., 2011). On the other hand, gibberellic acid has been shown to induce the expression of protease inhibitors (Cercós *et a*l., 1999). As *TC106351* and *TC107197* are annotated as Kunitz type protease inhibitors, we hypothesized that the induction of those genes during arbuscular mycorrhiza symbiosis could involve GA signaling.
To test the influence of gibberellic acid on the expression of the candidate genes, hairy root explants were incubated for 6 hours with 0.1 or 100 µM of GA respectively. After GA treatment for 6 hours a strong downregulation of the expression could be observed for all of the tested genes (*MtMSBP1*, *TC106351*, *TC107197* and *TC112474*) and both concentrations applied (Figure 4.33), indicating a negative role of GA signaling also during the early stage of the symbiosis.

4.4.7 Gibberellic acid signaling is suppressed already during initial contact

As gibberellic acid negatively influences the development of the arbuscular mycorrhiza symbiosis (El Ghachtouli *et al*., 1996; Michelini *et al*., 1989), a successful colonization and arbuscule formation relies on a downregulation of the gibberellic signaling pathway. This can be achieved through different mechanisms. First GA can be degraded directly by the activity of GA2 oxidases (Hedden & Phillips, 2000) and/or the suppression of transcription of GA responsive genes is mediated by DELLA proteins (Silverstone *et al*., 2001; A Dill *et al*., 2001).

In the *M. truncatula* gene expression Atlas we identified three different DELLA proteins that were specifically induced during a fully developed AM symbiosis showing only a weak basal expression and one GA2 oxidase (GA2ox) that was slightly upregulated (Figure 4.34). The elevated expression of those genes confirms the necessity to repress GA signaling during arbuscular mycorrhiza. As the data in the gene expression Atlas contains only information about a late stage of the symbiosis (21 dpi), all the four genes were tested for their expression after one day of contact of *M. truncatula* hairy root explants and *G. intraradices* extraradical hyphae (Figure 4.35). At this early interaction only one DELLA protein (subsequently called DELLA3) and the GA2 oxidase showed a significant upregulation while the other two DELLAs were not even expressed. This data supports the idea that a need exists to suppress GA signaling during the initiation of the symbiosis. Additionally the outcome of the experiment suggests that MtDELLA3 is responsible for suppression of GA mediated response during early interactions of *G. intraradices* and *M. truncatula* and in this task is supported by the GA2 oxidase that keeps the level of GA low.

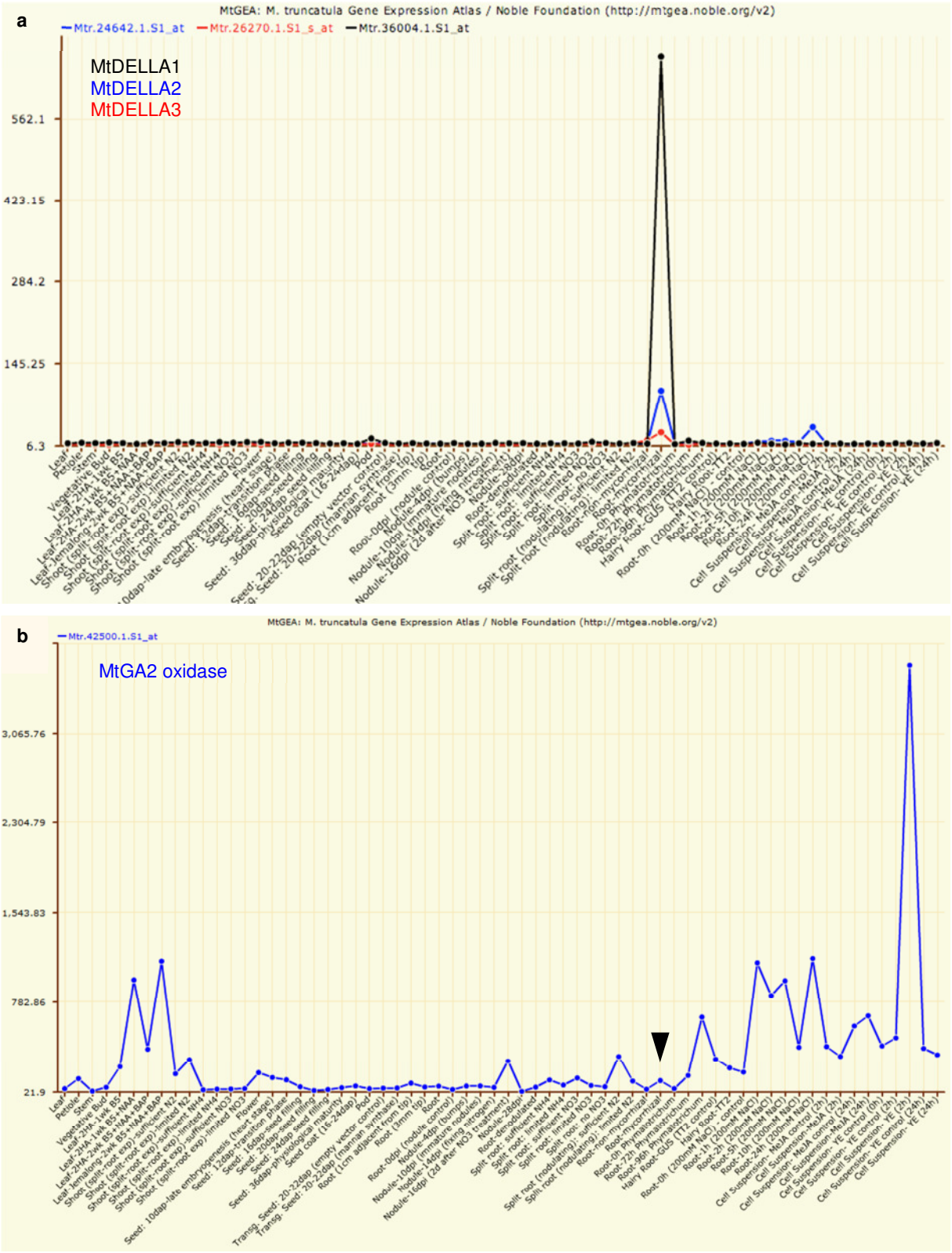


Figure 4.34 Expression pattern of selected DELLA proteins and GA2 oxidase in the *M. truncatula* gene expression Atlas
(a) The DELLA proteins are almost exclusively expressed in the mycorrhizal roots 30 days post inoculation with *G. intraradices* (DELLA1 shows a slight induction in pods and DELLA2 is weakly expressed in roots treated with 200 mM NaCl). (b) The Ga2 oxidase is expressed in several samples and treatments but also induced during AM symbiosis. Arrowhead indicates mycorrhizal sample.

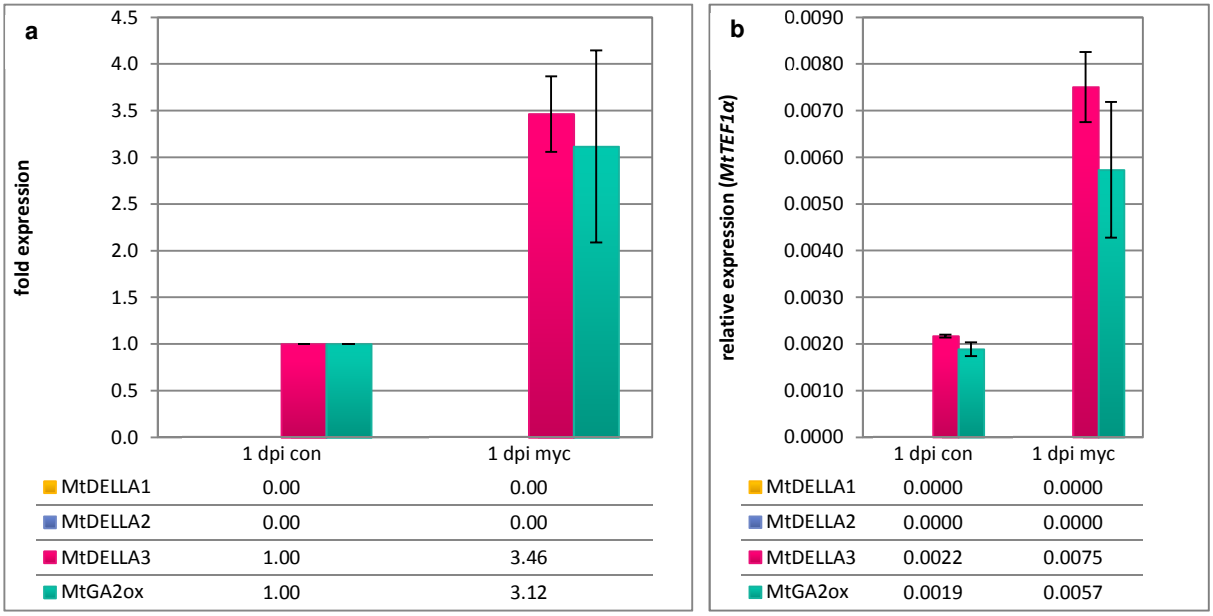


Figure 4.35 Analysis of transcript abundance of mycorrhiza-induced DELLA and GA2 oxidase proteins. *M. truncatula* hairy roots were co-cultivated for one day with *G. intraradices* extraradical hyphae. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of two different biological replicates are given.

4.4.8 MtDELLA3 and MtGA2ox are downregulated in response to auxins except PAA

As already stated, gibberellic acid and auxin signaling are known to interact during regulation of various processes and through different mechanisms (reviewed in Chandler 2009). In this context it has been reported that auxin influences GA biosynthesis and degradation via the regulation of several GA oxidase genes (Wolbang & Ross, 2001; O'Neill & Ross, 2002; Ozga *et al.*, 2003; Wolbang *et al.*, 2004; Frigerio *et al.*, 2006; Serrani *et al.*, 2008). In barley and tomato application of exogenous IAA or 2,4-D has been shown to suppress the expression of GA2 oxidase leading to an increased degradation of GA. Nevertheless, a crosstalk of auxin and gibberellic acid does not only occur at the level of biosynthesis regulation. Transcription factors DELLA proteins are likely targets of hormones and indeed they have been shown to be affected by multiple hormones besides GA (Fu & Harberd, 2003; Achard *et al.*, 2003). In the case of auxins, it has been shown that attenuation of auxin transport or signaling by application of the auxin transport inhibitor NPA delays the GA-induced disappearance of DELLA proteins from root cell nuclei (Fu & Harberd, 2003). Consequently, it can be concluded that normal auxin signaling promotes DELLA degradation.

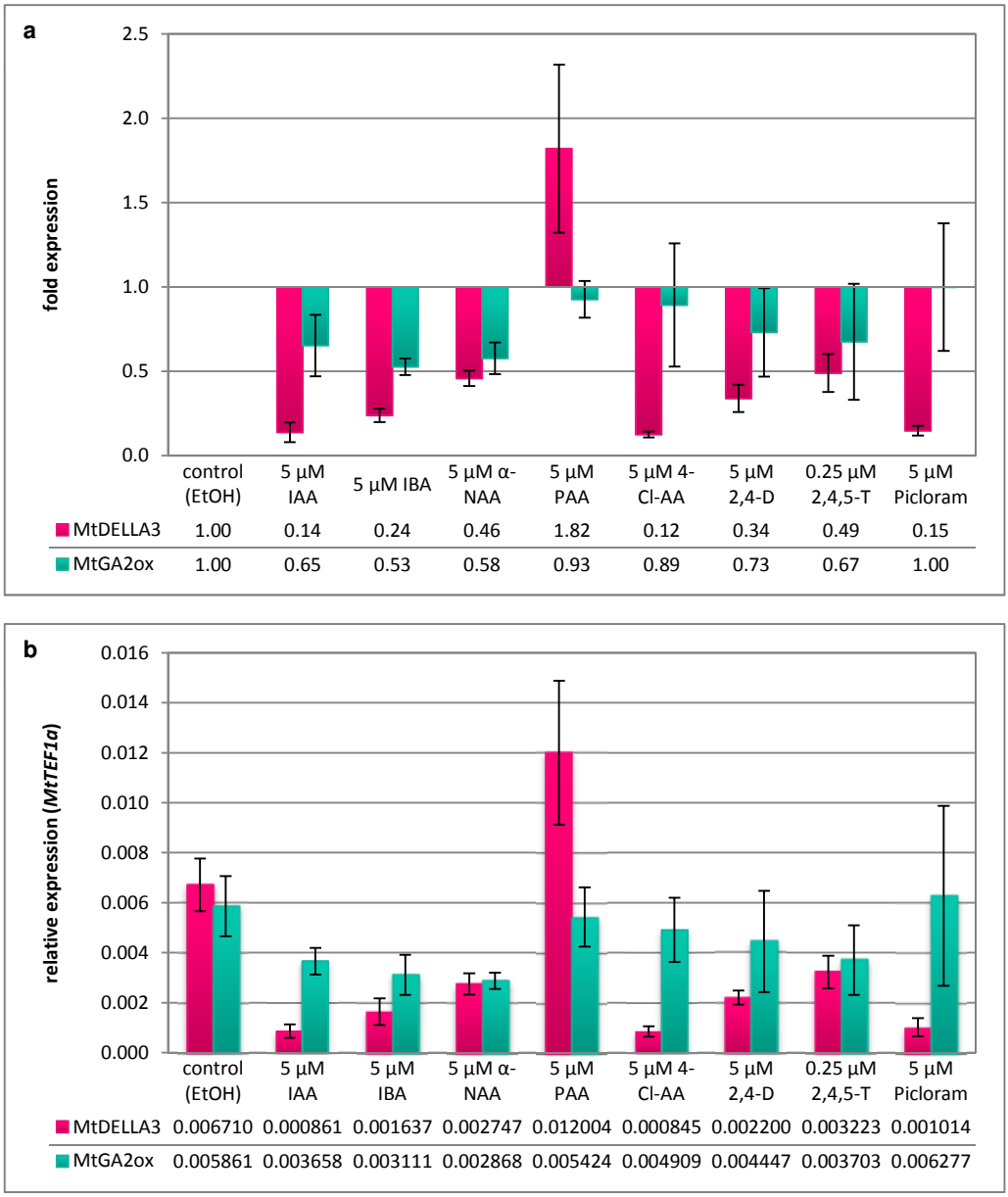


Figure 4.36 Expression analysis of MtDELLA3 and MtGA2ox in roots treated with different auxins
M. truncatula plantlets were treated for 6 h with the given concentration of different auxins or with the corresponding concentration of the ethanol, which was used as a solvent. RNA was extracted out of roots. (a) Fold expression normalized to *MtTEF1α* and relative to the control sample. (b) Expression level relative to *MtTEF1α*. Standard deviations of the average of three different biological replicates are given.

To investigate a possible role of auxins in the regulation of *MtDELLA3* and *MtGA2ox* the expression of those genes was tested in the *M. truncatula* roots treated for 6 hours with different naturally occurring and synthetic auxins. Analysis of transcript abundance revealed that most of the applied auxins led to a downregulation of the transcription of both genes (Figure 4.36). In contrast, the treatment with PAA that had already been shown to induce the expression of *TC107197* in the same samples did not alter the expression level of *MtDELLA3* nor *MtGA2ox* significantly.

The finding that PAA induces the expression of one of the early induced genes as well as the fact that it does not contribute to enhanced GA signaling clearly separates its effect in arbuscular mycorrhiza from those of the other auxins.

To summarize the results concerning the involvement of auxin and GA signaling in expression of the marker genes it can be stated that PAA seems to play a special role in initiation of the symbiosis. Furthermore a suppression of GA as well as conventional auxin signal transduction seems to be necessary during onset of the interaction contributing to the induction of *TC107197*. Moreover, a possible involvement of MtMSBP1 in the PAA-dependent expression of TC107197 can be hypothesized and should be investigated in more detail in future.

5 Discussion

The success of the arbuscular mycorrhiza symbiosis is based on the reciprocal recognition of the interaction partners. The possibility for the plant to distinguish between beneficial and pathogenic microorganisms enables the entrance of the fungus into the root, the establishment of the interaction and the maintenance of biotrophy. Defense responses occurring in reaction to pathogenic fungi are only transiently induced in arbuscular mycorrhiza and rapidly suppressed after successful recognition of the symbiotic partner. Intense studies of signal exchange in this interaction have revealed that a molecular communication occurs already before physical contact in both directions. Thus, strigolactones released from host roots have been shown to induce hyphal branching in AM fungi (Akiyama *et al*., 2005) and a diffusible signal from the fungus elicits responses such as calcium spiking, lateral root formation and symbiosis-related gene expression in host plants (Navazio et al., 2007; Kosuta *et al*., 2003; Weidmann *et al*., 2004, Oláh *et al*., 2005). The fungal signal, recognized by the plant that has been a mystery over a long time was recently characterized as a lipo-chitooligosaccharide (Maillet *et al*., 2011). However, there is evidence accumulating for the existence of more than one fungal signal molecule. In this work further evidence for the existence of several signals, acting through different signaling pathways is provided. Furthermore, *MtMSBP1,* an early mycorrhiza-induced gene was characterized in its expression pattern and function during arbuscular mycorrhiza symbiosis and the results point to an involvement of sterols during arbuscular mycorrhiza establishment and maintenance. Moreover, a connection of mycorrhiza-related regulation and gene expression to auxin and gibberellic acid signaling is drawn indicating a specialized role for PAA during early mycorrhiza recognition processes.

5.1 Different fungal signals are involved in early recognition processes

In an initial microarray experiment in which the transcript abundance of appressoria-enriched root fragments was compared to non-colonized root pieces, several genes were identified as induced during this important stage of the symbiosis. Some of those genes were already described as being mycorrhiza-specifically induced in *M. truncatula* roots by Wulf *et al*. (2003), Grunwald *et al*. (2004) and Liu *et al*. (2007). Liu and colleagues observed an upregulation of *TC100804*, *TC106351*, *TC107197* and *TC112474* together with a core set of genes, which were induced in response to inoculation with three different mycorrhizal fungi after 22 or 28 dpi (*Glomus intraradices*, *Gigaspora gigantea* and *Glomus versiforme*). The fifth gene that was identified in the oligo array but was not mycorrhiza-specific was designated as *MtMSBP1* due to its similarity to *A. thaliana MSBP1* (Yang *et al*., 2005). Analysis of the gene expression patterns in the *Medicago* gene expression Atlas confirmed the high specifity for mycorrhiza-dependent expression for *TC106351*. However, it also showed an induction unrelated to mycorrhizal colonization in the expression of *TC100804* in seed coats and filling (Benedito *et al*., 2008; Pang *et al*., 2008) and for *TC107197* in leaves after NAA or NAA/BAP treatment (Imin *et al*., 2008) as well as in suspension culture cells treated and non-treated with methyl jasmonates and yeast elicitors (Naoumkina *et al*., 2007). *MtMSBP1* was not found to be mycorrhiza-specifically expressed but to respond to a couple of biotic and abiotic stimuli such as for instance NAA/BAP treatment (Imin *et al*., 2008) or infection with *Phymatotrichopsis omnivora* (Uppalapati *et al*., 2009).

The expression patterns of all of those genes during progression of mycorrhizal colonization revealed that an induction occurred already at a very early stage of the interaction of *M. truncatula* and *G. intraradices* (1 dpi). Although the expression of *TC100804*, *TC106351*, *TC107197* and *TC112474* increased during proceeding colonization, this early upregulation points to a role of the genes already during fungal recognition and/or accommodation. *MtMSBP1* is the only tested candidate gene that was merely induced at the earliest time-points analyzed, which indicates a special need for this protein during the early interaction. The temporary and comparably weak induction of *MtMSBP1* might also be the reason that it has not been described as mycorrhiza-induced before in other microarray experiments that aimed the identification of AM-induced genes (Manthey *et al*., 2004; Hohnjec *et al*., 2005; Liu *et al*., 2007; Deguchi *et al*., 2007; Fiorilli *et al*., 2009; Gomez *et al*., 2009; Güther *et al*., 2009). As *MtMSBP1* is highly expressed also in non-mycorrhizal samples, it will not be able to serve as an unambiguous marker gene for early recognition. In comparison to the other genes, the induction of *MtMSBP1* in all experiments was very weak, which is most probably due to the high expression of the gene and the locally restricted induction observed with promoter reporter constructs (Diploma Thesis, Kuhn *et al*., 2010). Consistent with this

assumption is the fact that the strongest upregulation of *MtMSBP1* was measured in the appressoria-enriched tissue where areas responding to early contact stimuli are more abundant compared to entire normal roots in which a dilutional effect is expected to occur. The induction of *MtMSBP1* observed in response to diffusible fungal signals is in agreement with the gene as specific marker for Myc factor perception (Giles Oldroyd, John Innes Centre, Norwich, UK, personal communication).

The results of the expression analyses of *TC106351, TC107197, TC112474* and *TC100804* are summarized in Figure 5.1. The gene responsive to the most treatments was *TC107197*. While the induction of *TC106351* required either a complex interaction with the growing fungus (diffusible or non-diffusible compounds from induced hyphae) or the presence of a complex extract from germinated spores, *TC107197* was additionally upregulated by non-germinated spores and single stimuli such as PAA and chitin. As an interpretation of those results, it can be stated that *TC106351* seems to require different and/or additional signals than *TC107197*. This assumption of the involvement of distinct signals in gene induction is further confirmed by the fact that after germination of *G. intraradices* spores, a signal was secreted that was able to induce *TC106351* and *TC107197* expression while the release of compounds that are contained within non-germinated spores of *G. intraradices* led to a

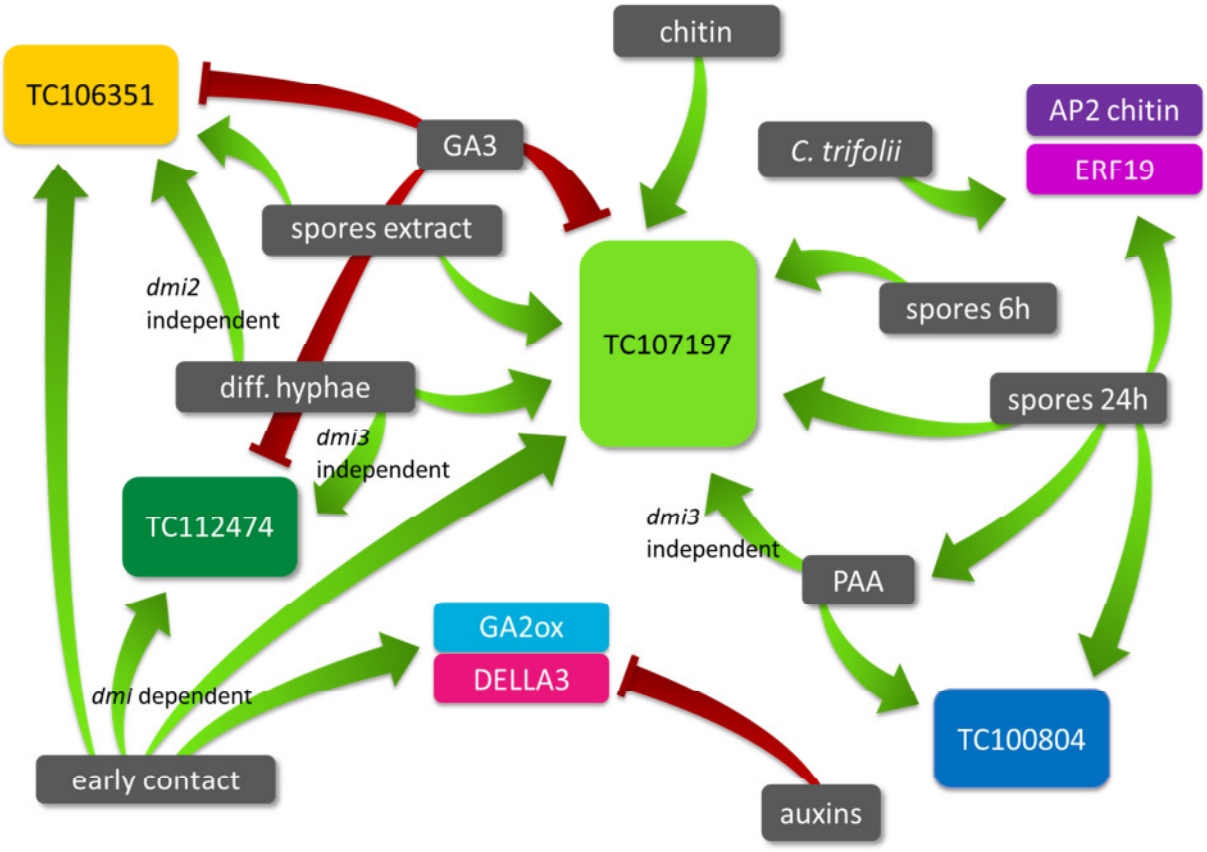


Figure 5.1 Overview of expression analyzes of mycorrhiza-specific induced genes

restricted activation of *TC107197* and *TC100804*. If it would be the same signal molecule that induced the expression of *TC107197* previous to and after germination of spores, a release of the compound should have also activated *TC106351*. Thus, it can be concluded that at least two distinct signals induce the expression of the both genes. The different nature of the molecules required for the activation of *TC106351*, *TC107197* and *TC112474* becomes even more apparent if the SYM-dependency of the expression is taken into consideration. While the activation of *TC107197* after early contact with *G. intraradices* hyphae required the presence of all three DMI proteins, *TC106351* was upregulated independently of DMI2 and *TC112474* independently of DMI3. This does not only indicate the need for several signals for a proper recognition of the fungus but additionally points to the existence of a second pathway parallel to the common SYM signaling. Interestingly, after 24 hours of treatment with ground *G. intraradices* spores, the expression of *TC100804* and *MtERF19* rose while the induction of *TC107197* decreased. The delayed upregulation of *TC100804* could be a hint for the involvement of an additional signal that requires processing by plant proteins such as chitin mono- or oligomers that act as elicitors of defense responses once they are released by the activity of plant chitinases (van Esse *et al*., 2007; Bolton *et al*., 2008; de Jonge *et al*., 2010). As an alternative, it is possible that some of the genes are induced in a secondary activation loop that has to be preceded by activation of other genes. A weak activation of the defense-related transcription factor *MtERF19* (Kloppholz, Kuhn & Requena in review) was observed at the onset of the mycorrhizal interaction, while treatment with ground spores or inoculation of roots with *C. trifolii* led to a high level of induction. The induction in response to *G. intraradices* inoculation occurred only temporary and is consistent with a transient defense response, described at the onset of arbuscular mycorrhizal interactions (Lambais & Mehdy, 1995; Gianinazzi-Pearson *et al*., 1996; Bonanomi *et al*., 2001; Liu *et al*., 2003). The high expression of *MtERF19* in response to *G. intraradices* ground spores, in contrast, might be due to the perception of fungal MAMPs by the plant innate immune system. In this regard, Salzer and Boller (2000) suggested that AM fungi release chitin elicitors, which could induce a defense response. Similarly, an elicitor-derived from an extract of extraradical mycelium of *Glomus intraradices* was able to induce phytoalexin synthesis in soybean cotyledons (Lambais, 2000). Additionally it has been shown that upon early contact with roots, *G. intraradices* induces the expression of a chalcone synthase, the first enzyme in the phenyl propanoid pathway in *M. truncatula* (Bonanomi *et al*., 2001).

The fact that the application of chitin from shrimp cells did not elicit the expression of *MtERF19* could be due to the reason that this chitin might not be recognized as a MAMP and thus does not induce the expression of defense genes. Anyway, the induction of a *M. truncatula* homologue to a chitin-induced AP2 transcription factor of *A. thaliana*

(AT1G22810; Libault *et al*., 2007) indicates that recognition of chitin subunits has occurred but the signal probably is not transmitted into defense gene induction. In addition, a slight and transient upregulation of *TC107197* and *TC100804* after 15 and 30 min of chitin treatment was observed. This activation is of special interest in the context of the recently published isolation of chitin-derived AM fungal signal molecules designated as Myc-LCOs (Maillet *et al*., 2011). Those lipo-chitooligosaccharides based on a chitin backbone resemble in their structure the Nod factors secreted by rhizobia. Both compounds are recognized by LysM motif-containing receptors and the presence of a common receptor for both molecules in the non-legume nodulating plant *Parasponia andersonii* (Op den Camp *et al*., 2011) indicates that also other chitin-derived molecules with similar structure might be unspecifically recognized if abundant in high amounts.

AtMSBP1 is described to bind several sterols and among those progesterone with the highest affinity. Ergosterol is known to be recognized as a MAMP by plants and other fungal sterols could work as signaling molecules in a similar way. However, neither progesterone nor mycorrhizal fungal lipid extracts or free sterol preparations were able to drive the expression of the early marker genes or to induce the activity of the P*MtMSBP1*$_{1518bp}$GFPGUS promoter reporter construct in hairy roots. However, the AM fungal lipids and sterols have not been tested for the induction of the other marker genes, and additional sterols such as 24-eBL as well as 5-dihydrotestosterone and stigmasterol, which have also been shown to be bound by AtMSBP1 should be tested for a potential function as signal molecules. In my previous work, I showed that a 1518 bp promoter fragment of *MtMSBP1* is induced after hyphal contact and that this increase in expression also occurs prior to physical contact in response to branching fungal hyphae in the vicinity of the root (Kuhn, Diploma Thesis and Kuhn *et al*., 2010). The induction of the gene in response to hyphal branching correlates with the observation that *MtENOD11* is induced in root areas underneath branched hyphae as well (Kosuta *et al*., 2003). These findings indicate that not all hyphae are able to produce the signal/s inducing expression of these two genes. Therefore, it is likely, that pre-stimulation of branching by plant strigolactones does not only increase the probability of a hypha to contact the root but also makes AM fungal hyphae competent for symbiosis.

The use of a shorter *MtMSBP1* promoter fragment of 302 bp in this work revealed that this truncated promoter is sufficient for induction of the gene after hyphal contact while neither constitutive expression nor induction in response to diffusible signals was observed. In this regard, it can be stated that the 1213 bp missing in the truncated promoter are necessary for diffusible signal-induced and high constitutive expression of *MSBP1*. Interestingly, the truncated 302 bp promoter fragment includes two AT-rich motifs, one of them perfectly matching the published consensus sequence (Boisson-Dernier *et al*., 2005). This AT-rich

motif is present in a 257 bp promoter fragment that is sufficient for infection-related early and late expression of the early nodulin *MtENOD11* both during AM and *S. meliloti* colonization (Boisson-Dernier *et al*., 2005). In accordance with the expression pattern observed for *MtMSBP1*, also in *MtENOD11* a longer promoter is needed for expression elicited after perception of diffusible signals. Thus, it is described that a NF-box in a 411 bp fragment of the *ENOD11* promoter is required for induction of the gene in response to NF (Andriankaja *et al*., 2007). Taken together this suggests that the AT-rich region might be responsible for the mycorrhiza-specific induction of *MtMSBP1* and *MtENOD11* after physical contact, while more upstream regions can mediate induction by earlier diffusible signals from AM fungi or Nod factors from rhizobia. Together with the fact that *MtMSBP1* is induced in response to appressoria formation and to diffusible molecules released by induced *G. intraradices* hyphae co-cultivated with *M. truncatula* roots, these findings led to the following model (Figure 5.2): Hyphae of *G. intraradices* perceive information on the availability of a host root possibly in the form of secreted strigolactones. The perception of those branching factors induces an intense hyphal branching and metabolic changes that lead to the secretion of diffusible fungal signals. These signals are perceived by plant roots and result in the induction of the *MtMSBP1* promoter via elements upstream of the – 302 bp region. After further approaching of the hyphae, physical contact is initiated during which a second signal

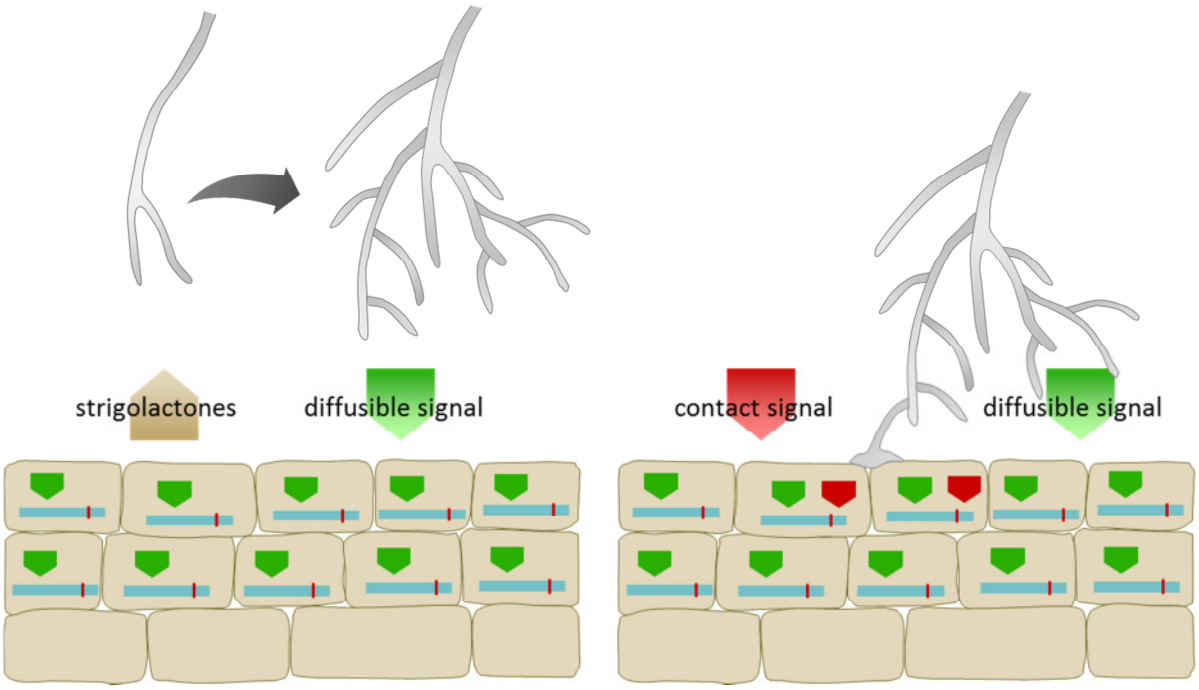


Figure 5.2 Functional model of MtMSBP1 promoter activation by AM fungal signals
Branched hyphae are formed by runner hyphae after perception of plant signals like strigolactones. This makes fungal hyphae competent for the production of a diffusible signal that reaches the rhizodermis and subepidermis and induces expression of *MtMSBP1* acting on the distal part of the promoter. After hyphal contact/appressoria formation a second fungal signal also triggers induction of *MtMSBP1* but acting on the proximal part of the promoter. This induction only takes place in cells in direct contact or very close to the contacting hypha.

is perceived by the plant. This additionally triggers induction of *MtMSBP1* acting on the proximal part of the promoter including the AT-rich motifs. That induction only takes place in cells in direct contact or very close to the contacting hypha. Nevertheless, the possibility remains that not two distinct signals lead to this differential activation of the promoter regions but that one signal in rising concentrations, correlating with increasing vicinity of the hyphae might be responsible. Besides the common features of both promoters, *MtENOD11* and *MtMSBP1*, *MtENOD11* also shows an interesting expression pattern in the use of promoter reporter constructs. Thus, Chabaud *et al*. (2002), using transgenic *M. truncatula* roots, showed that *MtENOD11* was induced in epidermal cells in the vicinity of appressoria in dependence of an intact SYM cascade. However, Kosuta *et al*., (2003) reported that if fungus and plant were separated by a cellophane membrane, induction of *MtENOD11* was not limited to cells in contact with the fungus. Interestingly, and in contradiction to the previous finding, this activation occurred independently of the SYM pathway. These observations resemble on one hand the expression patterns observed for the different promoter constructs of *MtMSBP1* thus a relatively broad induction takes place in response to diffusible signals. However in the case of contact, *MtMSBP1* promoter undergoes an additional activation whereas the *MtENOD11* promoter seems to lose the primary activation and is afterwards restricted to cells that are in direct contact with the hyphae. Nevertheless, if the differential induction of the both *MtMSBP1* promoter fragments occurs in response to two distinct signals those might possibly be acting through different signaling pathways which would also explain the contradictory results published for the *MtENOD11* activation

The need for a plant-mediated hyphal induction to become competent for the release/production of additional signals was also reported by Kosuta *et al*., (2008). They showed that calcium oscillations in host roots prior to hyphal contact were only detected if branching hyphae were in the vicinity of responsive areas. No calcium response was observed if runner hyphae that had not undergone branching were approaching. Different areas of the root responding to distinct fungal stimuli were additionally observed by Chabaud *et al*. (2011). They reported the occurrence of a calcium signature in cells directly contacted by AM hyphopodia, and in a small percentage of the surrounding epidermal cells (one to two cells distant from ramifying hyphae and hyphopodia). Nevertheless, a 10 fold concentrated fungal exudate applied to the roots induced Ca^{2+} oscillations in most epidermal cells from a broader region corresponding to the developing and mature root hair zone, which is the primary target for AM root infection (Chabaud *et al*., 2002; Genre *et al*., 2005, 2008).

5.2 Existence of a second pathway parallel to the SYM signaling cascade

The common SYM pathway has long been accepted as a paradigm for signaling events in arbuscular mycorrhiza as well as in rhizobia symbiosis (Catoira *et al*., 2000). Meanwhile evidence accumulates that alternative signaling routes exist, complementing SYM-dependent signal transmission (Figure 5.3). First indications for the existence of an alternative to the SYM signaling in the AM symbiosis came up very soon after the first finding of the existence of a diffusible fungal signaling molecule. While *MtENOD11* was shown to be induced in dependence of DMI2 in response to hyphal contact (Chabaud *et al*., 2002), activation occurred independently of the SYM pathway after perception of a diffusible signal (Kosuta *et al*., 2003). In 2005, Oláh *et al*. observed that lateral root formation induced prior to contact with germinated spores was DMI1 and DMI2-dependent but independent of DMI3. Further evidence for SYM-independent gene expression was provided by Kistner and colleagues (2005), who measured changes in AM-induced gene expression in wt and mutants of the SYM pathway in *L. japonicus*. Additional information related to transcriptional responses confirming the presence of an alternative pathway was provided by Gutjahr *et al*. (2008). The authors reported an expression of the arbuscular mycorrhiza-induced genes AM1 and AM2 independent of the SYM genes *CASTOR*, *POLLUX*, *CCAMK*, and *CYCLOPS* in rice. In this work here, also several cues pointed to the involvement of other signaling components that are not related to any of the DMI proteins. While during early contact, all of the tested genes were SYM-dependently induced, after induction with diffusible fungal signals *TC106351* and *TC112474* were upregulated independently of DMI2 and DMI3 respectively. Noteworthy, the overall expression level of the genes seemed still to be dependent on the SYM pathway. Additional indication for the existence of a parallel pathway was provided by the fact that the PAA-mediated response of *TC107197* is sustained in *dmi3-1* mutant whereas the expression measured in the *dmi1-1* and *dmi2-1* mutants was decreased in comparison to the wt.

Contradictory results concerning the involvement of proteins in the SYM pathway recently arose when the fungal factor elicitating root branching was characterized as a lipo-chitooligosaccharide (Maillet *et al*., 2011). Chitin oligomers are recognized by plants in pathogenic as well as in mutualistic interactions via receptors containing LysM motifs in their extracellular domains. (Radutoiu *et al*., 2003; Limpens *et al*., 2003; Arrighi *et al*., 2006; Smit *et al*., 2007; Miya *et al*., 2007; Shimizu *et al*., 2010). The *M. truncatula* Nod factor receptors MtLYK3 and MtNFP have been described to be essential for NF elicited signaling but dispensable for recognition of AM fungal-derived signals and arbuscular mycorrhizal colonization (Wegel *et al*., 1998; Catoira *et al*., 2000; Arrighi *et al*., 2006; Smit *et al*., 2007). It was therefore expected that additional receptors must exist that are responsible for perception of fungal signals. In contrast to what was published before, Maillet and colleagues

(2011) observed an involvement of NFP in the perception of Myc-LCOs. This correlates with the results observed in the non-legume *P. andersonii* that is able to undergo a symbiosis with rhizobia different from conventional nodulation as well as with AM fungi. This plant possesses one single LysM motif-containing receptor related to NFP, and silencing of this protein via RNAi revealed a need for the receptor in both symbioses (Op den Camp *et al.*, 2011). A similar observation was made for NSP2, one of the transcription factors that are important in signal transduction in response to Nod factors but were thought to be dispensable during arbuscular mycorrhizal interactions (Catoira *et al.*, 2000). However, the results of Maillet *et al.*, (2011) showed that lateral root formation in response to synthetic Myc-LCOs was impaired in *nsp2* mutant roots compared to wt *M. truncatula* roots. Furthermore the lateral root formation elicited after Myc-LCO treatment was observed to be dependent on all three DMI proteins (Maillet *et al.*, 2011) while the same response induced by germinating AM fungal spores was previously shown to occur independently of DMI3 (Oláh *et al.*, 2005).

All these findings, together with the results from this work, strengthen the assumption that additional signaling exists parallel to the described SYM pathway. As evidence exists that different signals are secreted by AM fungi, additionally to the now identified Myc-LCOs (see above), a recognition by distinct receptors seems to be involved. Furthermore, the available

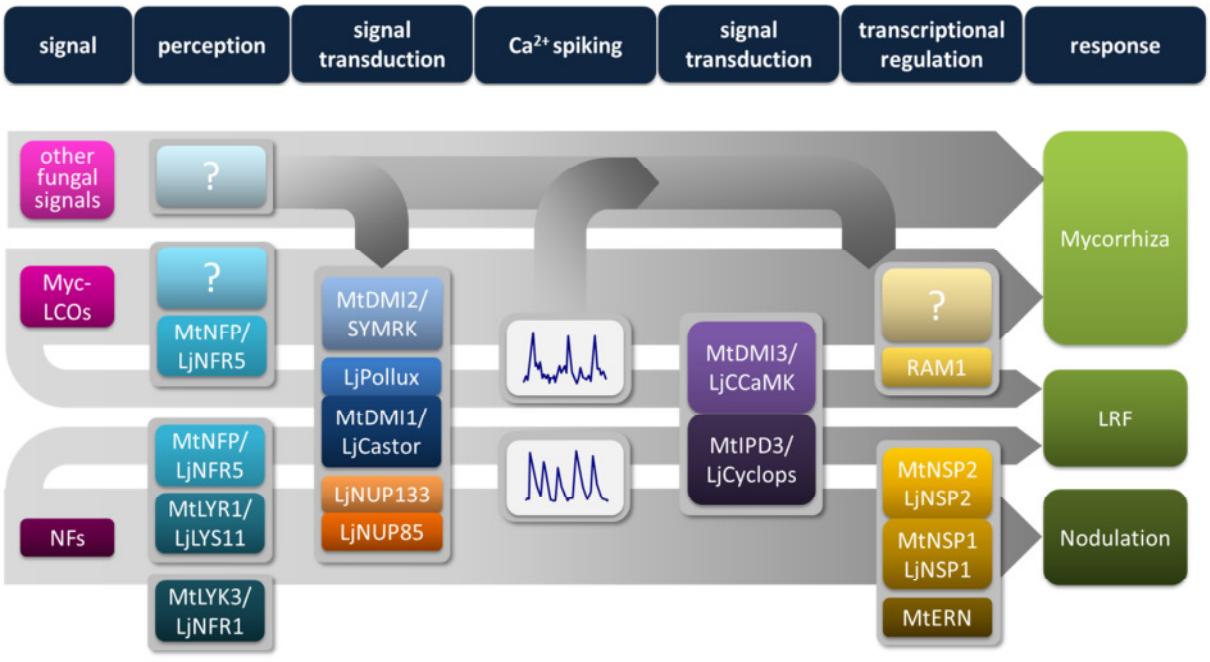


Figure 5.3 Symbiosis-related signaling transduction in Rhizobial and arbuscular mycorrhizal symbiosis
Current model of the common SYM pathway with additional information for parallel signaling that might occur in AM symbiosis. This representation implies all signals required for Ca^{2+} spiking. The proteins names are given for *M. truncatula* and *L. japonicus*. The mentioned proteins are important for all pathways that are in contact with the corresponding grey boxes LRF = lateral root formation.

data suggests an intense crosstalk between the different pathways, as some responses are only partially SYM-dependent or independent as for example the expression of *TC106351* and *TC112474* after induction with diffusible fungal molecules.

5.3 *TC106351* and *TC107197* promoters are deregulated in promoter reporter constructs

The original idea of the initial microarray experiment on which this work bases was the identification of plant genes that could serve as marker genes for early arbuscular mycorrhizal recognition processes. This aim was achieved by the identification of the mycorrhiza-specific expressed genes *TC100804*, *TC106351*, *TC112474*, *TC107197*. Those were shown to be induced after 1 day of contact with extraradical hyphae as well as upon perception of diffusible fungal signals. In this work, measurement of the transcript abundance of those genes already served as a marker for perception of signal molecules related to arbuscular mycorrhizal signaling. To develop a cheaper and faster assay for identification of signaling molecules that drive the expression of those genes promoter reporters should be constructed. Fusion of the genes' promoters to reporter genes such as β-glucuronidase or GFP would provide valuable tools to quickly screen for substances active during mycorrhizal signaling. As the sequencing and assembly of the *M. truncatula* genome is still in progress, genomic data was only available for *TC106351* and *TC107197*. In this work, I fused 1 kb and 4 kb promoter fragments of both markers to the GUS reporter gene and tested the expression in transgenic hairy root lines. Unfortunately, all transformed lines showed GUS activity also in a non-mycorrhizal context. This was not expected as both genes show only a very low basal expression as detectable only by real-time PCR analysis. Apparently, the natural regulation of the genes was altered when the promoters were taken out of the genomic context. This was especially unexpected for *TC106351* as the use of a 979 bp promoter GUS reporter construct was successfully reported for this gene (Grunwald *et al.*, 2004). However, the authors did not show any non-mycorrhizal control roots in their work. Nevertheless, after obtaining those results we speculated that the strong GUS staining could be due to a high stability of the transcript that led to a strong activity despite a very weak promoter activity. An involvement of small RNAs in the regulation of those genes is possible as it is described that microRNAs and siRNAs are involved in many regulatory mechanisms including regulation of mRNA stability, or repression of translation (Brodersen *et al.*, 2008; Lanet *et al.*, 2009). To test this hypothesis, the open reading frame of *TC107197* was fused in front of the GUS reporter and the entire construct was expressed under control of the 4 kb promoter fragment. Unfortunately this also wasn't able to diminish the unspecific expression observed with this promoter reporter. Regarding those results, it has to be assumed that

other regulating mechanisms take influence either on the activity of the promoters or on the stability of the RNA or protein. This could be for instance due to a methylation pattern occurring in the genome that is lost after PCR amplification and cloning of the promoter. Another possibility would be a changed accessibility of the genomic region into which the construct was integrated after *A. rhizogenes*-mediated transformation. Regarding to the use of a *TC106351* promoter reporter by Grunwald and colleagues it has to be asked whether also the use of different promoter reporter vectors could lead to this kind of misregulation. However, the binary plasmids used in this study (pPGFPGUS-RR and pKGWGUS-RR) are commercially available and were solely modified to contain a cassette for constitutive DsRED expression that was used to select positively transformed roots.

5.4 Plant hormones as regulators of early AM development

Plant hormones are essential factors for many developmental processes of plants. They also play important roles during interaction of plants with microbes. Since a long time involvement of plant hormones has been studied during arbuscular mycorrhiza development (reviewed by Ludwig-Müller, 2010; Gutjahr & Paszkowski, 2009). Here in this work the contribution of auxins and gibberellic acid to early arbuscular mycorrhiza signaling has been examined in more detail (Figure 5.4).

The application of several naturally-occurring (IAA, IBA, 4-Cl-IAA and PAA) and synthetic (1-NAA, 2,4-D, 2,4,5-D, picloram) auxins to *M. truncatula* plant roots revealed a special role for phenylacetic acid in regulation of early mycorrhizal-induced genes. Among all tested auxin derivatives PAA had the strongest influence on the expression of *TC107197* and *TC100804*. Tests showed that an induction of *TC107197* depends on the PAA concentration applied to the roots as only 5 µM PAA was able to activate the expression. Among the four endogenous auxins PAA is the only phenyl-derivative. The presence of PAA was confirmed for various plant species and tissues (Wightman & Lighty, 1982) During arbuscular mycorrhizal interaction elevated levels of free PAA have been demonstrated for roots of *T. majus* (Jentschel *et al*., 2007). An increased abundance of this compound was found especially during the early interaction of *T. majus* and *G. intraradices,* which corroborates the finding that PAA induces the expression of early-induced genes such as *TC107197*. Remarkably, PAA has been shown to be of importance also during actinorhizobial interaction of *Alnus glutinosa* with *Frankia* species where it is released by the bacteria. Furthermore, exogenous PAA added to *A. glutinosa* roots, resulted in the formation of thick, short lateral roots, which resembled actinorhizal nodules (Hammad *et al*., 2003). With respect to the fact that also other root symbiotic bacteria produce PAA (Slininger *et al*., 2004; Somers *et al*., 2005) it can be speculated that PAA preferentially plays a role in plant root interactions with soil

microorganisms (Simon & Petrásek, 2011). The dependence of PAA-induced expression of *TC107197* on DMI1 and DMI2 additionally confirms the involvement of this compound in early AM signaling. Regarding PAA mode of action it is known that it usually exhibits weaker auxin effects in plants in comparison with IAA (Small & Morris, 1990) and acts as an inhibitor of carrier-mediated efflux and polar transport of IAA in pea (Johnson & Morris, 1987; Morris & Johnson, 1987). This indicates a special role for PAA in control of auxin transport during root–microbe interactions.

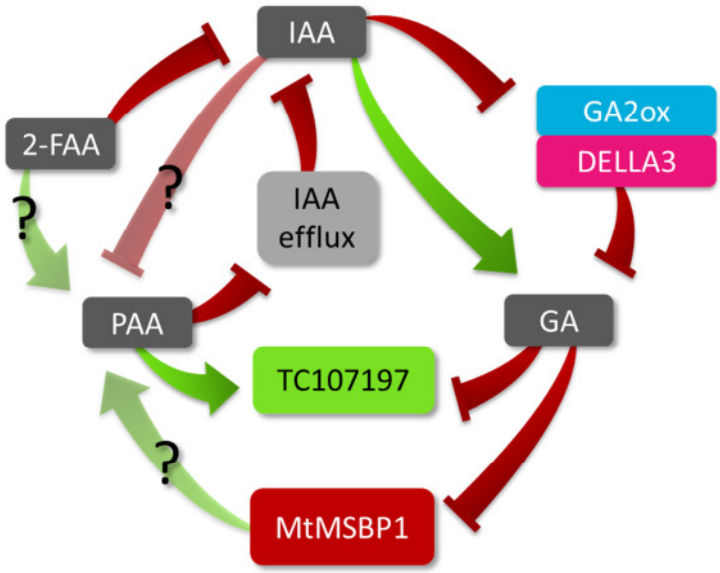


Figure 5.4 Involvement of auxins and GA in regulation marker gene expression
The model combines data obtained in this work and pre-published findings. Pastel arrows indicate unproven hypothesis. 2-FAA is known to inhibit IAA signaling which usually promotes GA signal transduction in roots by additionally stimulating DELLA degradation and repressing GA2ox transcription. Repression of GA has positive effect on expression of the early marker genes. MtMSBP1 might subsequently promote PAA signaling, which would then further induce TC107197 expression, inhibit IAA efflux and disturb IAA signaling.

Still, it has to be asked whether the positive effect of PAA on expression of *TC107197* relies on a signaling effect of the molecule itself or on the inhibitory effect of this molecule regarding auxin efflux. Although PAA is described to induce auxin-related responses such as elongation of *Phaseolus vulgaris* internode segments by the same mechanisms than IAA (Small & Morris, 1990), data for the involvement of $SCF^{TIR1/AFB}$ ubiquitin ligases (one of the main actors in auxin signaling) in PAA signal transduction are completely lacking (Simon & Petrášek, 2011). The fact that also other auxins (including IAA) besides PAA induced *TC107197* expression, although to a much lesser extent, suggests a role of PAA as signal molecule itself. Nevertheless, if IAA accumulated in cells due to inhibition of efflux caused by PAA an addition of this compound would increase natural auxin signaling. As a contradiction to that hypothesis, Small and Morris (1990) reported that the elongation of *P. vulgaris* internode segments induced by PAA was greater than the elongation induced by the auxin efflux inhibitor NPA. This would indicate that PAA does not cause growth by preventing the

loss of endogenous auxin. However, to rule out the possibility that merely inhibition of IAA efflux by PAA induces *TC107197* expression it would be interesting to see the effect of NPA or TIBA, two inhibitors of auxin efflux, on *TC107197* expression. As in contrast to IAA, PAA transport across membrane seems not to be mediated by any carrier (Johnson & Morris, 1987) NPA addition should merely affect IAA efflux. Remarkably, after addition of the auxin signaling inhibitor 2-FAA the answer to PAA application was even increased. This effect is quite puzzling as it was expected that 2-FAA, that has been described as an inhibitor for IAA, 1-NAA and 2,4-D-triggered signal transduction, would suppress the PAA-induced response as well (Armstrong *et al.*, 2004; Sungur *et al.*, 2007). As the opposite was the case, it has to be assumed that inhibition of canonical auxin (IAA) signaling has a positive or additive effect on PAA signal transduction, opposing the assumption that inhibition of IAA efflux promotes *TC107197* expression. As a conclusion this implies that IAA signaling normally inhibits PAA-induced expression of *TC107197* and that inhibition of IAA transport by PAA leads to a disturbance of IAA signaling related to symbiosis rather than to a stimulation.

Unfortunately, the data obtained for involvement of MtMSBP1 in PAA-mediated *TC107197* expression is equivocal and can be interpreted in different ways. Whereas the basal expression of *TC107197* was generally higher in *MtMSBP1* RNAi roots than in control roots, the fold induction of *TC107197* after PAA treatment was decreased in RNAi roots within each line in comparison to vector control. Accordingly, either MtMSBP1 negatively influences *TC107197* expression or it inhibits PAA-mediated induction of the gene. Nevertheless, as a

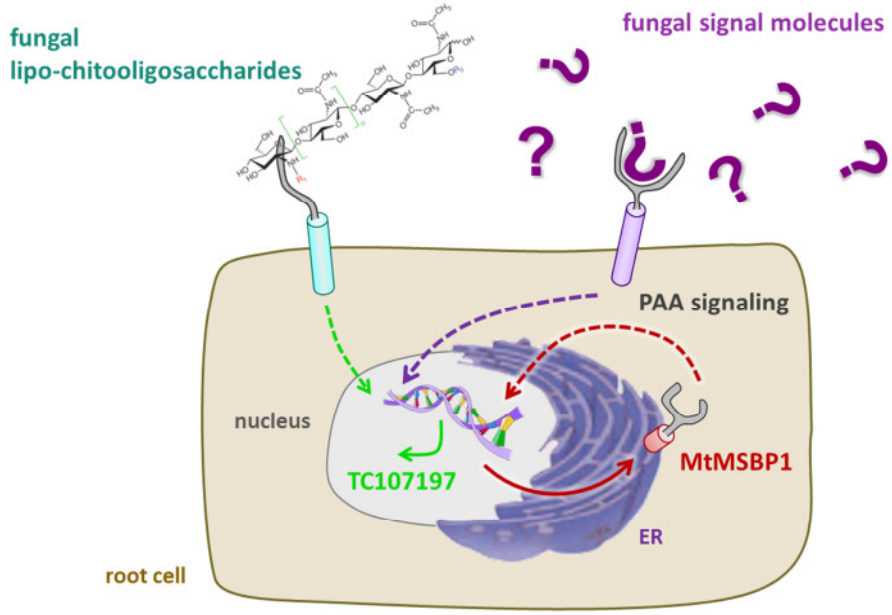


Figure 5.5 Model of MtMSBP1 action on PAA-mediated TC107197 expression
Hypothesis: The perception of fungal lipo-chitooligosaccharides and/or other signal molecules by plant receptors induces the expression of *MtMSBP1* and TC107197. MtMSBP1, located in the ER influences the expression of TC107197 by taking positive influence on PAA signaling.

parallel induction of both genes occurs during initial interaction in AM, a repressive impact of MtMSBP1 on *TC107197* is not very plausible. More probable is the second hypothesis that MtMSBP1 positively influences the PAA-mediated induction of *TC107197* (Figure 5.5).

Despite intense research on hormonal involvement in arbuscular mycorrhiza regulation relatively less is known about the function of gibberellic acid. Nevertheless, more and more evidence accumulates pointing to a suppressive role of GA on development of the symbiosis (El Ghachtouli *et al.,* 1996; Michelini *et al*., 1989). Therefore, it is interesting to take a closer look in GA signaling related to AM especially as gibberellic acid signaling is also closely linked to auxin-mediated signal transduction (reviewed by Chandler, 2009). For roots, for instance it has been reported that auxin promotes the degradation of DELLA proteins (Fu & Harberd, 2003). Additionally, auxin influences gibberellic acid levels as it prevents the degradation of GA by suppressing the expression of GA2 oxidase in barley and tomato (*Hordeum vulgare* and *Solanum lycopersicum)* (Wolbang *et al*., 2004; Serrani *et al*., 2008). In this work, I was able to confirm the proposed negative influence of GA also on early arbuscular mycorrhizal plant gene expression, as GA treatment suppressed the expression of *TC107197*, *TC106351, TC112474* and *MtMSBP1*. Moreover, investigation of transcript abundance of three *DELLA* transcription factors and one *GA2 oxidase* revealed an upregulation of one of the *DELLA* genes designated as *MtDELLA3* as well as *MtGA2ox* during early arbuscular mycorrhiza symbiosis. Both results confirm a need for suppression of gibberellic acid signaling during early arbuscular mycorrhizal symbiosis, which is mediated by both, suppression of GA signal transmission as well as a reduced GA abundance. Measurement of the expression of *MtDELLA3* and *MtGA2ox* after treatment with auxins revealed an involvement of gibberellic acid and auxin crosstalk in regulation of those genes. Remarkably all the applied auxin derivatives led to a repression of *MtDELLA3* and *MtGA2ox* expression except PAA, which had no apparent negative influence.

All together, the observations made in this work concerning auxin and GA effects led to the conclusion that canonical auxin signaling as well as GA signal transduction are probably altered during initial interaction *of M. truncatula* and *G. intraradices.* A suppression of auxin is suggested to be linked to an inhibitory effect of PAA on auxin efflux (Johnson & Morris, 1987), which might in consequence diminish the positive effect of IAA on GA signaling (Chandler, 2009; Fu & Harberd, 2003). The findings can be combined to the following working hypothesis (Figure 5.4 and Figure 5.5): Perception of fungal signals leads to the expression of *MtMSBP1* and other early-induced genes. MtMSBP1 subsequently promotes PAA signaling which leads to a disturbance of IAA signal transmission by inhibiting IAA efflux. As a consequence of missing IAA activity, GA signaling might be suppressed and transcription of the early marker genes would be further elevated.

Long time ago, (Barea & Azcón-Aguilar, 1980) reported the production of substances that behaved like auxins in specific bioassays by germinated *G. intraradices* spores. This finding was confirmed by Ludwig-Müller *et al*., (1997) who found that *G. intraradices* produces small amounts of IAA but not IBA whereas no auxin was detected in hyphae grown in the vicinity of a host plant (Jentschel *et al*., 2007). In another publication, (Azcón-Aguilar & Barea, 1981) described that at least two gibberellin-like substances were synthesized by the fungus. However whether any of the hormones that are involved in early arbuscular mycorrhizal signaling might be of fungal origin is at the moment too speculative.

5.5 TC107197 might be involved in cell elongation

TC107197 was shown to be a gene already early induced during arbuscular mycorrhiza formation as well even prior to physical contact and in response to other stimuli such as PAA. The expression pattern of the gene during progression of mycorrhizal colonization points to a role of this protein during initial and later interaction of the both partners. However, no putative function was described for similar proteins and there are no previously described domains detected in the amino acid sequence of the predicted protein. The only characteristic identified is the existence of an N terminal signal peptide while the rest of the protein consists of repeats. Proteins of similar structure were found to be tissue-specifically expressed in different plants and several organs as for example in epicotyl of *Cicer arietinum* (Muñoz *et al.,* 1997). Interestingly, similar genes are mostly found to be expressed in organs whose cells undergo elongation processes such as epicotyl, mesocotyl, root and stem tissues (de Vries *et al*., 1985; Williams *et al*., 1990; Muñoz *et al*., 1997), indicating a possible role for this kind of proteins in growth. As this protein was shown to be induced by the auxin PAA during this study, a function in cell elongation seems to be plausible. Regarding this assumption, the function of TC107197 protein during arbuscular mycorrhiza might be linked to alteration in root morphology following AM colonization, such as increased lateral root formation and root elongation (Fitter, 1985; Hetrick *et al*., 1988; Price *et al*., 1989; Schellenbaum *et al*., 1991; Berta *et al*., 1995). Alternatively, TC107197 might be involved in cell wall modification during fungal entry, as cell elongation also requires modification of the plant cell wall (Darley *et al*., 2001). However, without any additional functional data about the protein a probable function for example in lateral root formation, which is increased already prior to fungal contact, remains speculative.

5.6 Possible roles of protease inhibitors during AM symbiosis

Three potentially secreted Kunitz protease inhibitor of the STI type were found to be induced during early interaction of *M. truncatula* and *G. intraradices* in this study. The upregulation of several of these genes suggests a special role for this family of protease inhibitors in controlling arbuscular mycorrhiza symbiosis. In 2009, Takeda *et al.* described four subtilases induced during arbuscular mycorrhizal colonization of *L. japonicus*. Two of them were induced already four days after infection and showed promoter activity adjacent to intraradical fungal hyphae or in cells harboring them. RNAi silencing of both genes led to a decrease in intraradical hyphae and arbuscule abundance. This suggests a role for these subtilases during the fungal infection process and particularly in arbuscule development. A possible task for protease inhibitors during symbiosis could be related to the regulation of such proteases in controlling the development of arbuscules or other fungal structures inside of the plant root. *TC106351*/MtTi1 was previously described by Grunwald *et al.* (2004) as one of several trypsin inhibitors induced during arbuscular mycorrhiza symbiosis. Different expression patterns of those genes led to the suggestion of distinct roles for the different proteins during symbiotic development, which might be mediated through differences in their enzymatic characteristics. Trypsin inhibitors were additionally described as having antifungal activity (Chiang & Hadwiger, 1991; Blee, 1998; Giudici *et al*., 2000). Thus, they could be involved in the control of fungal growth. As a trypsin protease has been shown to accumulate during mycorrhization in pea (Slezack *et al.,* 1999), it is possible that a balanced expression of both the protease and its corresponding inhibitor is necessary to control the development of the fungus inside of the root as in the case of arbuscule and PPA formation and degradation.

Furthermore, proteolytic activity has been shown to be involved in induction of plant defense. Processing of the systemin precursor into a mature plant peptide hormone was reported to be necessary for activation of systemic defense responses to wounding by insects (Schaller & Ryan, 1996). Likewise, processing of precursors into active proteins is a conserved mechanism of regulation of many proteins as for example hormones, growth factors, receptors, adhesion proteins as well as bacterial and viral proteins (Barr *et al*., 1991; Nakayama, 1997; Berger & Altmann, 2000; Tanaka *et al*., 2001). Accordingly, in signaling processes, protease inhibitors could be involved in hindering proteases from processing precursors of signal molecules and thus preventing the perception of signals. In arbuscular mycorrhiza, a possible role could be the suppression of defense responses elicited by perception of fungal MAMPs or plant signal proteins.

5.7 The role of MtMSBP1 during AM symbiosis

The work with MtMSBP1 was initiated during my Diploma Thesis where I analyzed the expression of the gene via promoter reporter fusions. This gave a first idea of the involvement of the protein during early arbuscular mycorrhizal interaction. Data obtained during the current work confirmed this assumption. Further characterization of the protein provided a more complete picture of the function of MtMSBP1 during arbuscular mycorrhizal and rhizobial symbiosis.

Reducing the abundance of this protein by RNAi revealed that MtMSBP1 depletion has severe impact on the functioning of the symbiosis not only during the early interaction as the expression pattern would suggest. Mycorrhizal RNAi hairy root explants showed impairments in appressoria formation as many aborted entry structures were observed. Despite the hyphae often failed in penetrating the plant root, the occurrence of successful entry points led to a frequency of mycorrhizal colonization that was comparable to the vector control roots. This implicates that while MtMSBP1 is important for proper appressoria formation and entry of the hyphae, once the fungus has entered the root it has no influence on the colonization of the apoplast. The observation that the overall colonization of the root is not impaired in *MtMSBP1* RNAi roots might also be due to the fact that *M. truncatula* and *G. intraradices* form an interaction of the Arum type. There, a few entry points are sufficient to colonize the whole root system. Whether a complete knockout of the gene rather than a maybe leaky silencing would have a stronger effect on penetration, and as a result also on colonization, is an important question. Since several mutant collections are now available for *M. truncatula* (http://bioinfo4.noble.org/mutant/index.php; http://195.220.91.17/legumbase/) the examination of such a mutant would be a suitable experiment for the future. Additionally to the impact on the early interaction, the abundance and development of the arbuscules were severely disturbed. Often, arbuscules observed in RNAi roots had an abnormal morphology and were either not properly developed or quickly degraded. Obviously, MtMSBP1 function seems to be important especially during phases of the symbiosis where the fungus enters plant cells. This is the case during penetration that often occurs by intracellular transition of epidermal cells through the formation of a prepenetration apparatus, and during the arbuscule formation in the cortex cells. Noticeably, both cell colonization events require reorganization of the host plasma membrane. There are several hints that suggest an involvement of MtMSBP1 in sterol metabolism and homeostasis (see below). A negative influence of *MtMSBP1* RNAi on membrane functioning is therefore easily conceivable. The phenotype of *MtMSBP1* RNAi lines could accordingly be due to a disturbed sterol metabolism and the consequential disorder of the membrane function needed for the entry of the fungus (Figure 5.7).

Mammalian homologues of MtMSBP1 have shown to be involved in regulation of sterol metabolism by interacting with P450 proteins important during sterol biosynthesis such as Cyp51 that catalyzes demethylation of lanosterol. In plants, Cyp51G1 demethylates obtusifoliol during sterol metabolism (Figure 5.6, Boutté & Grebe, 2009) but an interaction of MtMSBP1 with Cyp51G1 could not be confirmed for *M. truncatula*. A second association of significance during regulation of sterol metabolism is the interaction of the MSBP1 homologue PGRMC1 with mammalian Insig-1 (insulin-induced gene-1) and SCAP (SREBP cleavage-activating protein; (Suchanek *et al.,* 2005), two proteins involved in induction of proteins related to cholesterol synthesis (Goldstein *et al*., 2002; Horton *et al*., 2002; Yang *et al*., 2002; Sun *et al*., 2005; Goldstein *et al*., 2006; Gong *et al*., 2006). Insig-1 has been shown to interact and regulate HMG-CoA reductase (HMGR) which catalyzes the rate-limiting step from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid (Burg *et al*., 2008) during sterol biosynthesis. Interestingly HMGR of *M. truncatula* has been shown to be induced at early stages of mycorrhiza symbiosis (Liu *et al*., 2003) and to interact with the DMI2 protein during nodulation and arbuscular mycorrhiza formation (Kevei *et al*., 2007); Rieger & Requena, unpublished). However, connection between MSBP1 and HMGR is either not conserved in *M. truncatula* (no similar genes exist in the genome project) or mediated by other proteins.

An involvement of MtMSBP1 in sterol biosynthesis is further confirmed by the ER localization of the protein demonstrated in this work. Plant sterol biosynthesis is known to take place at the endoplasmic reticulum as demonstrated by biochemical fractionation studies (Hartmann & Benveniste, 1987; Moreau *et al*., 1998) and by subcellular localization of sterol biosynthesis enzymes (Leivar *et al*., 2005; Busquets *et al*., 2008; Men *et al*., 2008). Regarding to the impact of MtMSBP1 on sterol metabolism, I was able to show in cooperation with Vera Wewer and Prof. Dr. Peter Dörmann (IMBIO) that the abundance of free sterols is decreased in *MtMSBP1* RNAi lines. LeFebvre *et al.* reported in 2010 that spinasterol is the major neutral lipid of *M. truncatula* root plasma membrane followed by ergost-7-en-3β-ol, stigmast-7-en-3β-ol, and Δ7-avenasterol. Among those, spinasterol, ergost-7-en-3β-ol and stigmast-7-en-3β-ol were found to be reduced in MtMSBP1 RNAi roots while Δ7-avenasterol was not measured. This finding indicates that although the interaction of MSBP1 with Cyp51 is not conserved in *M. truncatula,* the protein nevertheless influences sterol metabolism probably by interacting with other P450 proteins. As MtMSBP1 seems to be involved in regulating the biosynthesis of major sterols *of M. truncatula* plasma membrane, a disturbance of membrane functioning is very likely to occur when MtMSBP1 is impaired. The importance of sterol homeostasis for the establishment of AM is supported by the results of experiments using sterol biosynthesis inhibitor fungicides (Campagnac *et al*., 2008). Thus, application of fenpropimorph was shown to dramatically affect the pattern of

root sterols and this, in turn, impeded arbuscular mycorrhizal symbiosis. Fenhexamide, another sterol biosynthesis inhibitor fungicide, which did not induce major changes in the sterol profile of the root, nor modified the total colonization by *G. intraradices*, led however to a strong reduction of arbuscule frequency, which mimics the effect of *MtMSBP1* silencing.

Apart from their role as components of biological membranes, sterols serve as precursors for the biosynthesis of steroid hormones such as brassinosteroids in plants (Figure 5.6; (Benveniste, 2004; Suzuki & Muranaka, 2007; Boutté & Grebe, 2009)). As *A. thaliana* MSBP1 has been shown to bind the BR 24-eBL (Yang *et al.*, 2005), as well as to interact

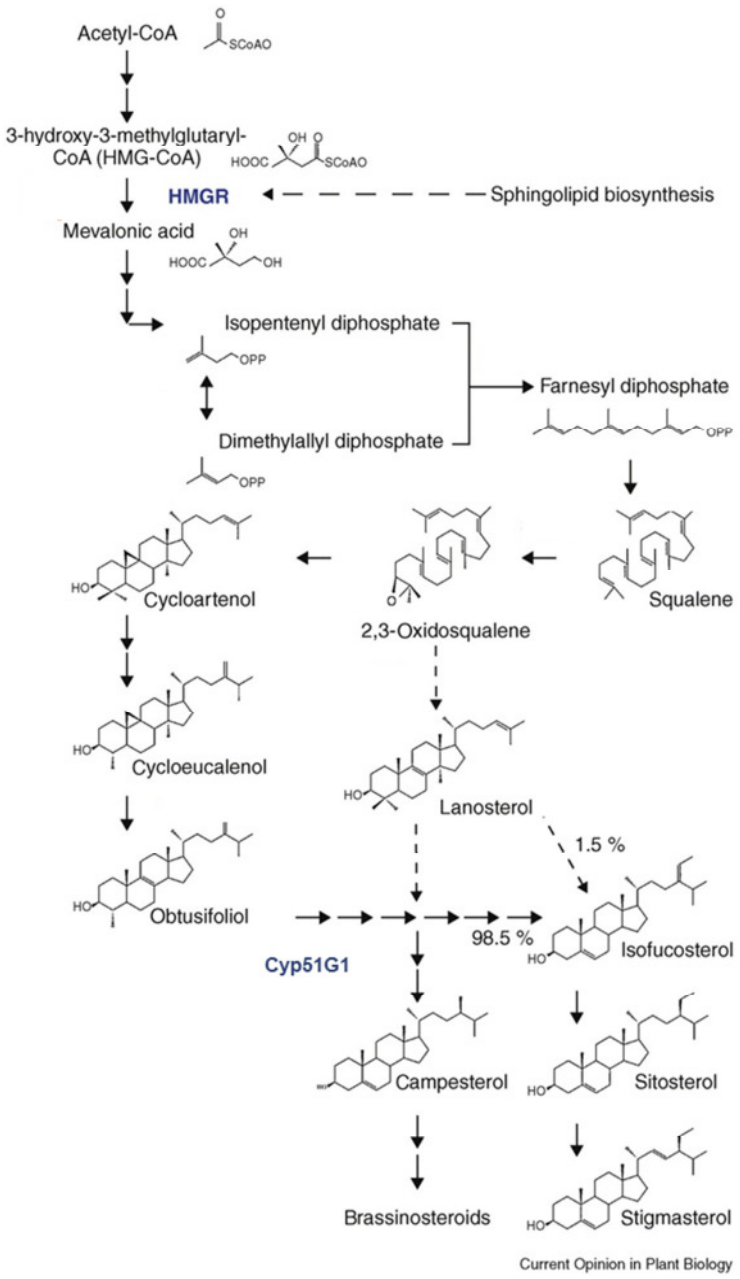


Figure 5.6 Sterol biosynthesis in *A. thaliana*
Selected steps of the sterol biosynthesis. Modified after Boutté & Grebe (2009). HMGR and Cyp51G1 that are probably regulated by MSBP1 are highlighted in blue.

with, and enhance the endocytosis of BAK1 (Song *et al.*, 2009), an involvement in negative regulation of brassinosteroid synthesis and function might also be possible for MtMSBP1. However, an interaction with BAK1 as shown for AtMSBP1 could not be confirmed for MtMSBP1, as the extracellular domain of MtSERK2, the closest BAK1 homologue in *M. truncatula* did not interact with MtMSBP1 in a Y2H assay. However, although SERK2 is the most similar protein of *M. truncatula* to BAK1 of *A. thaliana* (full aa sequence (83 % identity) or only extracellular domain (78 % identity)), it is not described to be the BAK1 orthologue. In total, six SERK proteins are described for *M. truncatula* (Nolan *et al.*, 2011) and those should be tested for a possible interaction with MtMSBP1 as well.

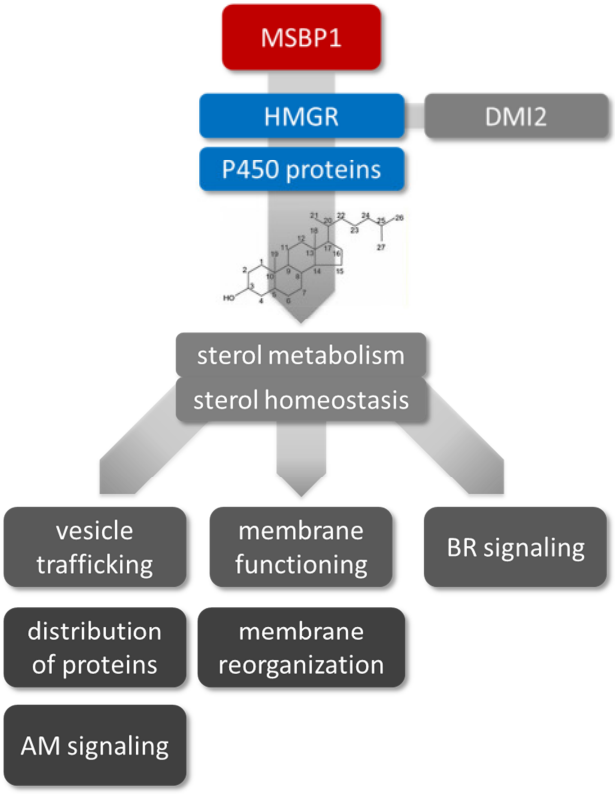


Figure 5.7 Model of MSBP1 function
MSBP1 regulates sterol metabolism and homeostasis through direct and indirect interaction with HMGR and P450 proteins. Subsequently vesicle trafficking, membrane functioning and BR sinaling are affected and take influence on distribution of proteins (e.g. involved in signaling) and membrane reorganization during AM symbiosis.

Analysis of PAA-dependent TC107197 expression in *MtMSBP1* RNAi roots suggested that MtMSBP1 might be involved in regulation of phenylacetic acid-mediated signaling (see above). Additional indication for a connection of MSBP1 to auxin was provided by Yang *et al.* (2008) who showed that AtMSBP1 participates in control of auxin transport by regulation of the distribution of the auxin efflux carrier PIN2. Enhanced vesicle trafficking was observed in the case of *AtMSBP1* overexpression. Therefore, the authors suggested, MSBP1 might

increase the cycling of PIN2-containing vesicles and as a consequence enhance the asymmetric PIN2 redistribution. Concerning PIN2 cycling, it has been shown that an altered sterol composition interferes with PIN2 endocytosis and auxin inhibition of PIN2 endocytosis requires sterol function (Men *et al*., 2008). Thus, the influence of MSBP1 on PIN distribution and auxin singal transduction might be related to regulation of sterol metabolism as well. Studies carried out on sterol-deficient mutants of *A. thaliana* have detected defects in the polar localization of PIN and AUX1 proteins, indicating that the polar delivery of cargos in plants also depends on the sterol composition of the plasma membrane (Souter *et al*., 2002; Willemsen *et al*., 2003; Grebe *et al*., 2003; Kleine-Vehn *et al*., 2006). However, a general growth defect could not be observed in *MtMSBP1* RNAi roots indicating that the overall auxin distribution might not be disturbed. Possibly a decreased abundance of MtMSBP1 influences the fine-tuning of auxin signaling that is needed during arbuscular mycorrhizal development but the remaining protein is sufficient to maintain general auxin transport.

Besides the role of MtMSBP1 in AM development, promoter activity of the gene was also observed in mature nodules. This result additionally points to a function of the protein during rhizobial symbiosis. GUS staining showed that MtMSBP1 was highest expressed in the zones of the nodule not involved in nitrogen fixation, including meristematic tissue, pre-fixing zone and interzone. Thus it can be assumed that MtMSBP1 might play a role in initiation of the interaction rather than during the mature symbiosis.

6 Conclusions and perspectives

These are exciting days for research in arbuscular mycorrhiza. Since almost ten years evidence is accumulating that arbuscular mycorrhizal fungi secrete a diffusible signal that triggers a recognition process in suitable host roots. This signaling compound has been termed as the Myc factor referring to the Nod factor secreted by rhizobia. A long time this Myc factor has been the most wanted molecule in mycorrhizal research until it was finally identified as lipo-chitooligosaccharide mixture in January this year (Maillet *et al.*, 2011). Indeed, not only the name is analogous between Nod and Myc factors but also the structure of both compounds is very similar. One could expect that now, once that the Myc factor is published the interest in fungal signal molecules would decline. But this is not the case as this study together with other publications presents new pieces of evidence for the existence of additional fungal signaling compounds. Moreover, it was also shown in this work that while some signal molecules are secreted constitutively by the fungus others exist that require stimulation by host plant factors in order to be produced/secreted. The identification and analysis of novel marker genes for the perception of fungal signals will help not only to identify such molecules but also to unravel the recognition process between the both symbiotic partners. Currently, efforts are undertaken to establish a biochemical bioassay for identification of additional fungal signal molecules with the aid of the marker genes described in this study (Albarran Nogales, Kuhn, Requena, unpublished).

Closely linked to the expression analysis of early induced genes it was further possible to demonstrate the existence of signal transduction alternative to and independent of the SYM pathway that has long been regarded as a dogma for symbiotic signaling. Furthermore one of the identified marker genes has already helped to gain new insights into the involvement of plant hormones in early arbuscular mycorrhizal recognition processes. It was demonstrated with the help of *TC107197* expression that a need to suppress gibberellic acid signaling exists during symbiosis initiation and furthermore it was possible to link this downregulation to an alteration of auxin signaling.

The dependency of PAA-mediated expression of *TC107197* on MtMSBP1 drew the attention to this interesting protein playing a role during arbuscular mycorrhiza as well as rhizobial symbiosis. The characterization of MtMSBP1 revealed a dependency of proper mycorrhizal development on sterol metabolism and identified one of the proteins that might play a major role in regulation of sterol homeostasis during arbuscular mycorrhizal symbiosis. In the future closer examination of MtMSBP1 will help to specify the role of this protein as well as of sterol functioning during signaling and intracellular accommodation of the fungus.

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KONFERENZTEILNAHMEN

09. 2009	9. VAAM Symposium „Molecular Biology of fungi“ in Münster mit Vortrag: **Kuhn, H., Vogt, C., Albarran, C., Requena, N.** *MtMSBP1*, Induced by a Diffusible Fungal Signal through the SYM Pathway, is Critical for Mycorrhization in *Medicago truncatula*
04. 2008	“European conference on fungal genetics” in Edinburgh mit Posterpräsentation: **Kuhn, H., Requena, N.** A secreted arbuscular mycorrhizal fungal effector induces the plant gene *MtMSBP1*
09. 2007	7. VAAM Symposium „Molecular Biology of fungi“ in Hamburg mit Posterpräsentation: **Kuhn, H., Requena, N.** *MtMSBP1* – A *Medicago truncatula* marker gene to screen for fungal signals during early arbuscular mycorrhizal colonization by *Glomus intraradices*
10. 2006	DFG Kolloquium “Molecular Basics of Mycorrhizal Symbiosis” in Tutzing mit Vortrag: **Kuhn, H., Müller, C., Kleber, R., Requena, N.** Plant markers of early mycorrhizal colonization
03. 2006	Jahrestagung der VAAM in Jena mit Vortrag: **Kuhn, H., Requena, N.** A plant bioassay to fish for early mycorrhizal signal molecules

PUBLIKATIONEN

07. 2011	**Kloppholz, S., Kuhn, H., Requena, N.** (2011) A secreted fungal effector of *Glomus intraradices* promotes symbiotic biothrophy. *Current Biology*. **21**: 1204-1209.
02. 2010	**Kuhn H., Küster H., Requena N.** (2010) Membrane Steroid Binding Protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*. *New Phytologist*. **185**: 716-733.
01. 2010	**Heupel S., Roser B., Kuhn H., Lebrun M-H., Villalba F., Requena N.** (2010) Erl1 a novel ERA-like GTPase from *Magnaporthe oryzae* is required for full root virulence and is conserved in the mutualistic symbiont *Glomus intraradices*. Molecular Plant Microbe Interactions **23**: 67-81.