Analyses of transcriptional alterations during the mutualistic interaction between the model legume *Medicago truncatula* and the arbuscular mycorrhiza fungus *Glomus intraradices*, and characterisation of mycorrhiza-specific lectins

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Abbreviations

- AM — arbuscular mycorrhiza
- AP — alkaline phosphatase
- APS — ammoniumperoxidisulfate
- bp — base pair
- BSA — bovine serum albumin
- BCIP — 5-brome-4-chlor-3-indolylphosphate, toluidinesalt
- bZip — basic leucine zipper
- CDP-Star — Disodium 2chloro-5-(4-methoxyspiro{1,2-dioxetane 3,2'-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate
- CLSM — confocal laser scanning microscopy
- ddUTP — didesoxy- Uraciltriphoshate
- DEPC — Diethylpyrocarbonat
- DIG — digoxigenin
- dmi — does not make infection
- dpi — days past inoculation
- DTT — Dithiotreitol
- EDTA — Ethylendiamine- N-, N-, N-, N- tetraacetate
- EST — expressed sequence tag
- GUS — β-glucuronidase
- Hepes — N-(2-Hydroxyethyl)-piperazine-N'2' ethanesulfonic acid
- IgG — Immunoglobulin G
- kDa — kilo Dalton

- kV — kilo Volt
- Mb — Mega base pairs
- min — minute
- MtGI — *Medicago truncatula* gene index
- NBT — nitro-blue-tretrazoliumchloride
- O. D. — optical density
- ORF — open reading frame
- p*MtLec*5 / p*MtLec*7 — promoter of the *MtLec*5 or *MtLec*7 gene
- PMSF — Phenylmethansulfonylfluoride
- RACE — Rapid amplification of cDNA ends
- RT — room temperature
- SDS — sodiumdodecylsulfate
- SMART — switching mechanism at 5' end of RNA transcript
- TBE — Tris / Borate / EDTA
- TC — tentative consensus sequence
- TEMED — N, N, N', N'-tetramethylenediamine
- Tris — Tris-hydroxymethylaminomethane
- Tween20 — Polyoxyethylensorbitanmonolaurate
- UTR — untranslated region

Abstract

In the endomycorrhizal symbiosis, which most plants are able to form, host interacts with fungi of the phylum *Glomeromycota* developing the arbuscular mycorrhiza symbiosis. This is characterised by the exchange of phosphate from the fungus to the plant and carbohydrates vice versa. The broad distribution of the symbiosis, also in many economic crops, underlines the importance of this plant-microorganism mechanism.

In order to analyse these transcriptional changes, which occur before a physical contact, a 16k microarray has been hybridised with probes deriving from mycorrhizal roots and roots of *Medicago truncatula* in an early-mycorrhizal phase. Analyses revealed several genes to be differentially expressed. Amongst these one putative calmodulin-regulated transporters has been identified. The putative importance of these genes for the initiation of the symbiosis is underlined by the hypothetical calcium spiking event in this phase.

In silico transcriptional analyses revealed 33 mycorrhiza induced genes. Amongst these four lectin-like sequences have been identified. Additionally, in former experiments three genes of this family have been identified to be specifically expressed. These seven AM-regulated lectin-like genes represent a distinct branch of all *M. truncatula* lectins. Deletion analyses showed that the promoters of these two genes trigger arbuscule specific transcription, when using approximately 300 bp of the 5' upstream region, respectively. In electrophoretic mobility shift assays AM-specific expressed proteins bound to 90 bp of the promoter of *MtLec*5*,* assuming potential *cis*-regulatory elements in this region. Confocal laser scanning microscopy was used to localise the MtLEC5 protein. Transport of this protein into the vacuole suggests a function as storage protein.

Keywords: arbuscular mycorrhiza, lectin, promoter

Zusammenfassung

Die meisten Landpflanzen, unter ihnen auch viele landwirtschaftlich bedeutende, sind in der Lage, mit Pilzen der Ordnung *Glomeromycota* die sogenannte arbuskuläre Mykorrhiza Symbiose einzugehen. Dabei kommt es hauptsächlich zum Austausch von Phosphat vom Pilz zu der Pflanze und Kohlenhydrate von der Pflanze zum Pilz.

In Microarray Analysen von symbiotischen und früh-symbiotischen *Medicago truncatula* Pflanzen wurden mehrere differentiell exprimierte Gene identifiziert. Eines dieser Gene kodiert für einen Calmodulin abhängigen Kationen Transporter. Während der Initiation der Mykorrhiza-Symbiose wird eine sogenannte Calcium-Oszillation im Zytoplasma postuliert, was eine mögliche Bedeutung dieser Transporter unterstreicht.

Durch *in silico* Transkriptionsanalysen konnten 33 Mykorrhiza-spezifisch induzierte TCs identifiziert werden. Unter diesen befanden sich vier Gene, die hohe Ähnlichkeit zu Lektinen besitzen. Zusammen mit anderen Studien wurden somit insgesamt sieben dieser Proteine als Mykorrhiza-spezifisch identifiziert. In phylogenetischen Studien aller bekannter Lektine aus *M. truncatula* bilden diese einen eigenen Zweig. Promotor-Analysen von zwei dieser Gene zeigten Arbuskel-spezifische Aktivierung auf. Reportergen Expression konnte beobachtet werden, wenn mindestens 300 bp des jeweiligen Promotors verwendet wurden. In EMSA Analysen konnte die Bindung AM-spezifischer Proteine an 90 bp des *MtLec*5 Promotors gezeigt werden, was in diesem Bereich *cis*-regulatorische Elemente vermuten lässt. Da Lektine unterschiedliche Funktionen besitzen, wurde konfokale Laserscanning Mikroskopie eingesetzt, um MtLEC5 innerhalb der Zellen zu lokalisieren. Der Transport dieses Proteins in Vakuolen, lässt vermuten, dass es sich um ein Speicherprotein handelt.

Schlagwörter: arbuskuläre Mykorrhiza, Lektin, Promotor

1. Introduction

Soil provides a huge habitat for different types of organisms, which interact with each other. Most terrestrial plants are anchored through their roots in the soil, therefore representing one of the partners in these diverse interactions. Consequently, from the perspective of the plant, some kind of ”communication“ occurs through secretion of exudates from the plant or direct interaction with soil-inhabiting microorganisms. Most bacteria and fungi of the rhizosphere are highly dependent on the association with plants (Bais *et al.*, 2004), and in this environment microorganisms can cumulate up to 10^{10} to 10^{12} organisms g^{-1} soil (Foster, 1998). Depending on the kind of interaction, a parasitism or mutualism is formed. Several different gram-negative (*Rhizobium, Agrobacterium*) and gram-positive (*Frankia*) bacteria participate in these associations, as well as oomycetes (*Phytium, Phytophtora*) and fungi of the classes of the Glomeromycetes (*Glomales*), Ascomycetes (*Erisyphe, Tuber*) and Basidiomycetes (*Ustilago, Laccaria*). Beside several root-pathogen interactions, for example the root rot caused by *Phytophtora* and *Phytium*, two symbioses exist: The root nodule symbiosis is formed between gram-negative bacteria of the genera *Rhizobium* and *Bradyrhizobium* and plants from the order *Leguminosae*. The mycorrhiza symbiosis is formed between fungi of the phylum *Glomales* and most plants. The term *mycorrhiza* has been developed from the Greek *fungus-root*. This study will focus especially on the mutualistic interaction between fungi, which form a so called arbuscular mycorrhiza (AM), and the model-legume *Medicago truncatula.*

1.1 The model plant *Medicago truncatula*

Model organisms provide a tool to study principles of physiological and molecular processes. The first and most popular model organism is the bacterium *Echerichia coli*, which has been the source of extensive genetic studies. *Drosophila melanogaster* and *Caenorhabditis elegans* are still widely used to unravel developmental or cell biological questions in eukaryotes. *Mus musculus* is one model organism, which is investigated to answer vertebrate-specific aspects.

In plants such kinds of studies have been done using the *Brassicacea Arabidopsis thaliana*. Sequencing of the whole genome of this model plant by the *Arabidopsis* Genome Initiative in the year 2000 (TAIR, http://www.arabidopsis.org/) represents a milestone for plant genetics and revealed deep insights into the molecular biology of plants.

Although *Arabidopsis* provides a powerful tool to investigate several processes in plants, it is not able to form the two major root symbioses: As it belongs to the *Brassicaceae*, it is not able to form a root nodule symbiosis with *Rhizobia*. Additionally it belongs to the minority of plants, that can not be colonised by AM fungi. Legumes, in contrast, are capable of entering both symbioses, so that a member of this group is preferably used as a model plant.

The *Leguminosae* (*Fabaceae*) consist of more than 18,000 species, which are classified into 650 genera, representing the third largest family of higher plants (Polhill and Raven, 1981, Doyle, 2001). The legumes play an important role in the nutrition of human beings and animals, and represent the second major nutritional source after the grasses (www.legumes.org). As mentioned above, they are able to enter the mutualistic interaction with the gram-negative soil bacteria of the genus *Rhizobium*, in which they are enabled to fix atmospheric nitrogen to ammonia, as well as the mycorrhiza symbiosis. During the last years the mediterranean legume *Medicago truncatula* has developed to function as a model plant (Barker *et al.*, 1990, Cook, 1999, Oldroyd and Geurts, 2001). In brief, *M. truncatula* has a

small genome compared to other legumes of about 500 Mb (Blondon *et al.*, 1994), only 5 times larger than this of *Arabidopsis thaliana*. In comparison, the genome of pea is with about 4300 Mb 40 times larger. Additionally, *M. truncatula* possesses a diploid genome and a relative short generation time of only 3 months. The availability of approximately 300,000 EST (expressed sequence tags) sequences in the TIGR MtGI 8.0 (*Medicago truncatula* gene index) provides a powerful tool for comparative transcriptional analyses, such as the construction and hybridisation of microarrays. These allow the investigation of transcription profiles of several thousand genes in parallel in one experiment, as more than 5,000 different DNA-samples can be spotted per cm². Until now, three different microarrays are available inhabiting *M. truncatula* samples: The Mt6k-RIT microarray (Küster *et al.*, 2004) consists of 6,048 PCR amplified cDNA probes derived from a EST sequencing project from uninfected and mycorrhizal roots and from young root nodules (Journet *et al.*, 2002). The sequence information of Mt16kOLI1, a further development of the Mt6k-RIT, originates from the TIGR MtGI version 5. All available 16,086 ESTs of this version of the gene index have been printed on the chip as 70mer oligonucleotides (Hohnjec *et al*., 2005). The company Affymetrix (Santa Clara, CA, USA) offers a commercially available system using its GeneChip® technology. This array is composed of over 61,000 probes from *Medicago truncatula, Medicago sativa* and additionally *Sinorhizobium meliloti*. The first two mentioned have been used to investigate the expression patterns of mycorrhizal plants and root nodules (Küster *et al.*, 2004, El Yahyaoui, 2004) as well as of mycorrhizal plants, inoculated with two different AM fungi (Hohnjec *et al*., 2005).

Finally, most of the important crop plants within the legumes are found in the *Papilionideae* subfamily, representing the *phaseolid* legumes. *M. truncatula* is likewise a member of this group (Young *et al.*, 2003) and therefore a suitable object for comparative studies. Summarising, all the properties mentioned above as there are the ability to form the root

symbioses, the relatively short generation time and the small genome, the availability of an efficient and rapid transformation protocol (Wang *et al.*, 1996) and of extensive sequence data, as well as the phylogenetic close relationship to important crop plants turns *M. truncatula* into a perfect model organism.

1.2 The arbuscular mycorrhiza

Plant growth depends on the availability of nutrients. Therefore the enhancement of uptake of these means a huge growth-advantage for plants, if they are able to form a root symbiosis, in which they benefit from the exchange of nutrients. One major characteristic of the mycorrhizal symbiosis is the uptake of carbohydrates by the fungus from the plant and of phosphate vice versa. Two different types of mycorrhizal symbioses can be distinguished, ecto and endomycorrhiza. The first described ectomycorrhiza is characteristically formed between trees and a fungal partner (Frank, 1885). Plant hosts include members of *Pinaceae*, *Fagaceae*, *Betulacea*, *Myrtaceae* as well as some monocytoledons and ferns (Wilcox, 1968), the microsymbionts originate from different genera of Basidiomycetes, some Ascomycetes and two members of the Zygomycetes (Isaac, 1992).

In comparison, more than 80 % of all terrestrial plants are capable of developing an arbuscular mycorrhizal interaction with the members of the Zygomycota, which can be classified into one order, the Glomales, which is subdivided into six genera and 149 species (Bentivenga and Morton, 1994, Morton and Benny, 1990). This symbiosis is also referred to as endomycorrhiza as the fungi invade the root cortex and colonise root cells. Fossil findings from the Devonian era showed association of these plants with arbuscular mycorrhiza fungi since they first colonised land (Pirozynski and Malloch, 1975, Remy *et al.*, 1994) so that this interaction may be therefore several hundred million years old.

One major common characteristic of these fungi is their obligate biotrophic nature, i. e. their growth depends on the interaction with a herbal partner. However, germination of the AM fungal spores and initial growth can occur in the absence of plant roots, but root exudates are able to stimulate these processes (Balaji *et al.*, 1995, Bécard and Piché, 1989). The active components of the exudates are so far unknown, but some evidence exists, suggesting an exchange of signals prior to physical contact (Buee *et al.*, 2000, Giovanetti *et al.*, 1993). Initiation of the symbiosis occurs when fungal hyphae reach the root surface. When contacting the outer cell layers, a signal from the epidermal cells induces broadening and differentiation of the hyphae to form appressoria (Tester *et al*., 1987). After contact between host and symbiont a penetration hypha is developed that penetrates the root. In some species hyphae enter the root cortex between two epidermal cells *(Arum* type)*,* whereas in others the hyphae break through the cell wall of an epidermal cell or a root hair (*Paris* type) and grows subsequently through the cell (Bonfante-Fasolo, 1984). The molecular mechanisms of these events are so far unknown, but AM fungi are known to produce different enzymes, which seem to enable them to disintegrate the cell walls of the host plant (García-Garrido *et al.*, 1992, Garcia-Romera *et al.*, 1991, Rejon-Palomares *et al.*, 1996). After entering the root cortex the fungus starts spreading. Depending on the host plant the fungus may show different growth patterns (Gerdemann, 1965). *Arum* and *Paris* type have been named after the species these growth patterns first have been observed at (Smith and Smith, 1997). As the former one is more widespread, most analyses have been focused on this type. During colonisation according to this pattern, hyphae grow intercellular, and rarely penetrate the exodermis to form hyphal coils in the exodermous cells, which represents the *Paris* type of colonisation (Bonfante-Fasolo and Perotto, 1984). When the fungus reaches the inner cortex cell layer, it penetrates the cell and starts branching at the tip. This leads to highly dichotomous branched structures, referred to as arbuscules from the latin *arbuscula*, which means little tree, as these

structures appear under the microscope. Therefore, these typical structures are the eponyms for the AM. Although the fungus has penetrated the cell wall, the arbuscule remains separated from the cytoplasm of the root cell via the cell membrane and remains in the apoplast. On the other side the membrane extends to completely surround the arbuscule. The fungal cell wall becomes thinner and the arbuscules grow, coming in close contact with the host cell. Both are separated only by the narrow apoplast, which is derived from the plant (Bonfante-Fasolo, 1992, Smith and Gianinazzi-Pearson, 1988). This interface is supposed to be the main locus for the transposition of phosphate and possibly carbohydrates, as there exists some information for carbohydrate uptake from the intercellular hyphae (Smith and Smith, 1989, Smith, 1993). After approximately ten days the arbuscule begins to olden, and subsequently collapses and decays. The host cell remains undamaged and is able to take up a new arbuscule (Alexander *et al.*, 1988).

The plant cell responds specifically to the penetration of its cell wall and the invasion of the fungus with fragmentation of the vacuole, migration of the nucleus to a central position in the cell and proliferation of the organelles (Balestrini *et al.*, 1992, Bonfante and Perotto, 1995, Carling and Brown, 1982). The plasma membrane extends to surround the arbuscule to more than four-fold of its surface size and forms the periarbuscular membrane (Alexander *et al.*, 1989).

Some fungal species are able to form vesicles, which are filled with lipids and assumed to function as a storage reserve (Smith and Gianinazzi-Pearson,1988). After colonisation of the plant the external mycelium starts to grow extensively in the soil. There it is – among others – responsible for the uptake of mineral nutrients and their transport to the plant. Besides, it is able to colonise additional roots and often to produce spores, which completes the life cycle of the fungus.

1.3 Transcriptional analyses to identify AM-related genes

In order to gain insight into cellular and molecular processes leading to alterations during several biological processes, diverse different approaches have been used: Studies of these plant-microorganism mutualistic interactions revealed extensive alterations during the formation of nodules or the mycorrhiza. These extensive changes are of morphological as well as physiological nature, as new structures are formed for the special requirements of the interaction between plant and microsymbiont. Therefore it seems quite obvious that these changes are caused by alterations in the transcription-level of several genes involved in signal transduction, protein synthesis and several other functional classes.

In the past different approaches have been used to identify these transcriptional alterations: In firstly used forward genetic approaches, mutants have been identified and characterised, which were defective in developing this symbiosis (Duc *et al*., 1989, Gianinazzi-Pearson *et al.*, 1991). Subsequently mutants unable to form an AM have been detected in *Medicago truncatula* and *Lotus japonicus*, which are additionally unable to enter a root nodule symbiosis. In these mutants of *M. truncatula*, named *dmi1, dmi2* and *dmi3* (does not make infection), fungal colonisation is arrested at the epidermal cell layer (Blaylock *et al.*, 1997, Calantzis *et al.*, 2001, Catoira *et al.*, 2000, Sagan e*t al.,* 1995). These findings suggest, that both symbioses, the AM and the root-nodules, share common signal transduction pathways during their initiation.

As one major feature of the AM is the exchange of nutrients, for example the reception of phosphate from the fungus by the plant, approaches mainly focused on the isolation of responsible genes. This resulted in the identification of the corresponding AM-specific phosphate transporters in *M. truncatula* (Harrison *et al.*, 2002) potato (Rausch *et al*., 2001) and tomato (Nagy *et al.*, 2005), which have been found to be exclusively expressed in mycorrhizal roots. In contrast, the constitutively expressed phosphate transporters have been

found to be down-regulated in *M. truncatula* (Liu *et al.*, 1998). Several other mycorrhiza-induced genes have been identified using classical cloning techniques: a tonoplast intrinsic protein from parsley (Roussel *et al.,* 1997), an aquaporin from *M. truncatula* (Krajinski *et al.*, 2000), H^+-ATPases in arbuscule containing cells of tobacco (Gianinazzi-Pearson *et al.*, 1991a), tomato (Ferrol *et al.*, 2002) and *M. truncatula* (Krajinski *et al.*, 2002) and a class III chitinase gene (Bonanomi *et al.*, 2001).

With the establishment of automated high-throughput sequencing the era of plant genomics provided more comprehensive sequence data, which has intensively been used for the identification of AM-specific genes: High-throughput transcriptomic (VandenBosch and Stacey, 2003) and proteomic (Bestel-Corre *et al.*, 2002, Watson *et al.*, 2003) approaches enabled profiling of gene expression during symbiosis. Several different expressed sequence tags (EST) sequencing projects were accomplished resulting in up to just now 230,000 EST sequences deposited in the public available TIGR *Medicago truncatula* gene index (MtGI, http://www.tigr.org/tdb/tgi/mtgi, Quackenbusch et al., 2000). Additionally, Journet and associates (2002) accomplished large-scale EST sequencing and analyses to characterise root symbiotic interactions. This rising amount of sequence data provides a source of global analyses to access transcriptional changes during the development of the AM. Subtractive suppressive hybridisation (SSH) libraries have been constructed, in which AM-induced genes have been enriched (Wulf *et al.*, 2003, Grunwald *et al.*, 2004). Subsequently, cDNA and oligonucleotide microarrays have been developed, which have been used to investigate the AM and nodule symbioses (Küster *et al.*, 2004, Manthey *et al.*, 2004), early and late stages of the AM (Brechenmacher *et al.*, 2004) and the mycorrhizal symbiosis in dependence on different fungal partners involved in this interaction (Hohnjec *et al.*, 2005). During these studies, different AM-specific transcripts have been identified: Using a SSH approach (Wulf *et al.*, 2003) for example a glutathione S-transferase, one germin-like protein and a miraculin-

like protein have been isolated, which have been characterised in more detail (Doll *et al., 2003,* Grunwald *et al.,* 2004). Microarray analysis led to the perception, that *MtBcp*1, a member of the blue copper binding proteins, is specifically upregulated in arbuscule-containing regions of AM-fungus containing regions of the roots (Hohnjec *et al*., 2005).

In contrast, few studies dealt with genes that are down-regulated in respect to non-mycorrhizal roots. For example, the not specifically induced phosphate transporters *MtPT*1 and *MtPT*2 (Liu *et al*., 1998, Versaw *et al*., 2002) and *psam*2 from pea (Krajinski *et al*., 1998) are known to be repressed during the AM.

1.4 Objectives of this work

Genes, which are exclusively AM induced or whose transcription level is changed due to the AM, are of special interest, as they may provide some information about the fact, that 80 % of all terrestrial plants are able to form the AM and only 20 % are disabled. As it is assumed that colonisation of the mainland by plants only became possible through interaction with symbiotic fungi and the formation of mycorrhiza related structures, it appears obvious that the ability to form an AM has been lost by the latter ones during evolution.

On this account the identification of transcriptional alterations during the development of the AM symbiosis delineates the first step to analyse this mutualistic interaction. Many different directed and non-directed approaches have been used to look for such AM-related genes. Most studies have been focused on a completely developed symbiosis, representing all developmental stages within the plant, from appressoria to intraradical hyphae and arbuscules, which interact with the host root. One aim of this thesis is the identification of new, so far unknown mycorrhiza specific genes. Therefore, a huge amount of available sequence data

from different sources is used: Electronic Northern blot analysis is used to extract genes from the MtGI, which are only abundant in EST libraries from mycorrhizal roots. Signalling between plant and fungus is predicted to occur in the early-symbiotic state, before a physical contact between the both partners has taken place. Consequently, these genes are of high interest, that are differentially expressed in this particular state. Therefore, comparative microarray experiments are performed from early-mycorrhizal roots and a symbiosis being composed of appressoria as well as intra- and extraradical hyphae and arbuscules.

Promoter deletion analyses provide a powerful tool to investigate the promoters of differentially expressed genes, and therefore unravel the reason for this specific regulation. Promoters of selected AM-specific genes are analysed to localise potential *cis*-regulatory elements, which mediate AM-specific expression. Last, functional analyses are undertaken to provide a perception of the role and the importance of these proteins during the AM.

2 Material and Methods

2.1 General Methods

2.1.1 Plant growth and inoculations

Plants were grown and inoculated with mycorrhiza fungus *Glomus intraradices* as described at Wulf *et al.* (2003). In brief, *Medicago truncatula* cv. Jemalong A17 seeds were sterilised for 10 min in 86 % sulfuric acid. After extensive washing with water they were treated for 5 min with 6 % NaOCl. To remove dispensable NaOCl several additional washing steps have been performed. Seeds were vernalised over night at 4°C and germinated at room temperature (RT) for 2 days in the dark and additional 2 days at light. Germinated plants were transferred into open pots inhabiting a mixture of vermiculite and a commercially available *G. intraradices* inoculum (Biorize Sarl, Dijon, France). Plants were grown in greenhouse with light/dark cycles 16h/8h and harvested after 6 or 21 days. Control plants were treated in parallel without mycorrhiza inoculum. During plant growth plants were watered twice a week with one-half strength Hoagland solution (Arnon and Hoagland, 1940).

After harvesting plants were immediately frozen in liquid nitrogen. Randomly selected roots were used to stain fungal structures with ink (Vierheilig *et al.*, 1998) or acid fuchsin (Gerdemann, 1965) and mycorrhizal colonisation was calculated according to Trouvelot *et al.* (1986).

2.1.2 Staining of fungal structures

Two methods have been used to stain fungal structures: Staining with ink (Vierheilig *et al.*,

1998) was started by incubation of the roots in 10 % (w/v) KOH at 90°C for 10 – 20 min depending on the age of the plants. After extensive washing with water to remove KOH staining was performed using 5 % (v/v) black ink in 8 % (v/v) acetic acid for 10 – 15 min at 90°C. Background staining was removed by extensive washing with water. Roots were cut and incorporated in glycerol on glass slides.

Acid fuchsin staining was done according to Gerdemann (1965): Roots were incubated in at 90°C in 10 % (v/v) KOH for 10 – 15 min. After extensive washing roots were rinsed for 4 min at 90°C in 1 % (v/v) HCl. Staining occurred for 15 min at 90°C in acid fuchsin solution (87.5 % (v/v) lactic acid, 6.25 % (v/v) glycerol, 6.25 % (v/v) H_2O, 0.1 % (w/v) acid fuchsin). Destaining was done over night in destaining solution (87.5 % (v/v) lactic acid, 6.25 % (v/v) glycerol, 6.25 % (v/v) H_2O) and roots were embedded on glass slides in glycerol.

2.1.3 Protein extraction for EMSA analysis

Crude protein extract was produced by grinding 5 g of frozen root material under liquid nitrogen. Powder was suspended in 20 ml extraction buffer (17.2 % (v/v) glycerol, 20 mM Tris-HCl, pH 7.9, 0.1 mM DTT, 2 mM EDTA, 0.1 mM PMSF and 0.01 mM Leupeptin) and incubated for 20 min on ice. After 10 min centrifugation at 15,000 x g at 4°C crude extract was concentrated using Vivaspin 20 ml concentrator (Vivascience, Hannover, Germany) columns with a size exclusion of 30 kDa. Protein concentration was measured using Bradford assay.

2.1.4 Measurement of protein concentration according to Bradford

Protein concentration was measured according to Bradford (1976). Therefore a standard was

measured using different concentrated solutions of BSA (bovine serum albumin, MBI Fermentas, Vilnius, Lithuania). Afterwards protein crude extract was measured and concentration read off the standard line. 100 µl of each 1:10 diluted protein solution was diluted in 4.9 ml Bradford solution (2.25 µg/ml Coomassie Brilliant Blue G250, 16 % (v/v) ethanol, 8 % (v/v) o-phosphoric acid). Absorption was measured at 560 nm using a Photometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.1.5 DNA-isolation from *Medicago truncatula*

For DNA isolation 1 g of plant material was ground under liquid nitrogen and 15 ml extraction buffer (0.1 M Tris-HCl, pH 7.0, 0.05 M EDTA, 0.5 M NaCl), 1 ml 20 % (w/v) SDS and 10 µl RNase A (10 mg/ml) were added. Suspension was incubated at 65°C for 20 min, 5 ml 5 M K-acetate was added and solution was chilled on ice for 20 min. Afterwards cell debris was removed by centrifugation for 20 min at 5,400 x g. First precipitation occurred via addition of ½ volume Isopropanol, incubation for 30 min at -20°C and centrifugation for 15 min at 5,400 x g. Pellet was dissolved in 700 µl H_2O and final precipitation step was performed by adding 0.1 volume of 3 M Na-acetate and 1 volume isopropanol. After washing with 70 % (v/v) ethanol DNA was suspended in 50 µl H_2O.

2.1.6 RNA isolation

Total RNA was extracted using a LiCl method (Franken and Gnädinger, 1994). In brief, frozen plant material was ground under liquid nitrogen and 800 µl NTES-buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 9.0, 5 % (v/v) SDS, 1.7 % (v/v) β-Mercaptoethanol) and phenol : chloroform : isoamylalcohol mixture (P:C:I, 25 : 24 : 1) were

added. Samples were incubated for 5 min at room temperature (RT) and centrifuged for 5 min at 13,000 x g. Upper phase was removed and phenol step was repeated three times. RNA was precipitated over night by adding 40 µl acetic acid and 560 µl ethanol at -20°C. After centrifugation pellet was suspended in 200 µl DEPC containing H_2O (H_2O_{DEPC},1 %, v/v), 200 µl 4 M LiCl was added and incubation was performed for 4 h at 4°C. Repeating the precipitation by centrifugation a third precipitation occurred over night at -20°C by adding 200 µl DEPC treated H_2O, 300 µl ethanol and 20 µl 3 M Na-acetate, pH 4.8. After final centrifugation, RNA was suspended in 100 µl H_2O_{DEPC}. Concentration was measured at a wavelength of 260 nm using a spectral photometer (Amersham Parmacia Biotech).

For microarray hybridisations obtained RNA was concentrated to 1.25 µg/µl using Microcon-30 columns (Millipore, Schwalbach, Germany) and stored at -80°C until use.

2.1.7 Reverse transcription for cDNA synthesis

Standard cDNA synthesis was done using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) . Therefore 2 µg of total RNA was added to 4 µl 5 x reaction buffer, 2 µl 10 mM dNTP-mix, 1 µl enzyme (200 U/µl) and the according amount of DEPC treated H_2O to a final volume of 20 µl. Reactions were carried out for 1 h at 42°C.

2.1.8 Polymerase chain reactions

Standard PCR-reactions were prepared using volumes of 50 µl. Therefore 5 µl of corresponding 10 x reaction buffer, 1 µl 10 mM dNTP-mix, 10 µM primer 1 and 2, respectively, according volume of template and 1 µl of Advantage II polymerase mix (Clontech, Mountain View, CA, USA). Reactions were carried out in Biometra TRIO

Thermoblock (Biometra, Göttingen, Germany). Programs varied depending on the annealing temperatures of the primers.

2.1.9 Restriction digests

Restriction digests have been done using enzymes from MBI Fermentas company (MBI Fermentas, Vilnius, Lithuania). Reactions were carried out according to the manufacturer.

2.1.10 Ethanol purification of DNA

To purify ribonucleic-acids and remove small DNA-fragments same volume of ice-cold isopropanol and 1/10 volume 3 M Na-acetate was added to the particular sample. After incubation at -20°C for 30 min DNA was precipitated by centrifugation at 13,000 x g. After washing with 70 % (v/v) ethanol DNA was suspended in H_2O.

2.1.11 Cloning of PCR fragments

PCR fragments were cloned into the desired plasmids using standard T4 ligase (MBI Fermentas, Vilnius, Lithuania). Therefore, plasmid and ethanol purified insert were combined in a 3 : 1 proportion. 2 µl of 10 x buffer and 1 U ligase were added and the reaction was incubated for 2 h at 22°C and over night at 4°C. Afterwards 5 µl of the reaction was used for the transformation of *E. coli.*

For ligation in the pGemT® easy vector, pGemT® easy – vector system I (Promega, Madison, WI, USA) kit has been used according to manufacturers protocol.

Gateway® technology (Invitrogen, Carlsbad, CA, USA) has been used to clone promoter fragments and the open reading frame of *MtLec*5 into the particular binary vectors. Therefore, 300 ng of purified PCR fragment, 300 ng of pDONR207 vector (Invitrogen, Carlsbad, CA, USA), 4 µl 5 x reaction buffer and 4 µl BP-Clonase™ II enzyme-mix were added. Reactions were carried out over night at 25°C. After transformation in *E. coli* and confirmation of successful cloning pDONR207, inhabiting the fragment of interest, were isolated. A second reaction was used to transfer the fragment into the destination vector: 300 ng of pDONR207 inhabiting the insert was added to 300 ng of destination vector, 4 µl 5 x reaction buffer and 4 µl LR-Clonase™ II enzyme-mix. Reactions were carried out over night at 25°C; 5 µl of the reaction batch were used to transform *E. coli*.

2.2 Microbiological Methods

2.2.1 Preparation of competent *Escherichia coli* cells

Heat shock competent cells were prepared according to Tang *et al.* (1994) and Nakata *et al.* (1997). Therefore, bacteria were grown in LB medium (0.5 % (w/v) Yeast extract, 1 % (w/v) Tryptone, 1 % (w/v) NaCl) to an O. D. (optical density) at 600 nm of 0.4. Cells were precipitated at 4°C and 4000 x g and suspended in ½ volume 50 mM $CaCl_2$. After incubation on ice for 20 min, cells were centrifuged again and 1/10 volume 50 mM $CaCl_2$ containing 15 % (v/v) Glycerol was added. Cells were either used directly for transformation or frozen at -80°C till use.

2.2.2 Transformation of *Escherichia coli*

E. coli strain TOP10 F' (Invitrogen, Carlsbad, CA, USA) was used to clone desired fragments. After ligation 5 µl of the reaction batch was used to transform *E. coli* via heat shock. Cells were incubated with the batch for 20 min on ice. After 45 sec heat shock at 42°C and additionally 2 min chilling on ice, 1 ml of SOC medium (10 mM NaCl, 0,5 % (w/v) Yeast extract, 2 % (w/v) Tryptone, 2,5 mM KCl, pH 7,0; 12,5 µl 2 M $MgCl_2$, 50 µl 1 M Glucose) was added and regeneration occurred by incubation at 37°C with extensive shaking. Finally cells were spread on LB-agar plates (0.5 % (w/v) Yeast extract, 1 % (w/v) Tryptone, 1 % (w/v) NaCl, 1.5 % (w/v) Agar Agar, pH 7.0) with the according antibiotics (100 µg/ml ampicillin or 50 µg/ml kanamycin, depending on the vector used).

Genotype of *E. coli* Top10 F':

F$^-$ *mcr*A (*mrr-hsd*RMS-*mcr*BC) Δ80 lacZ M15 lacX 74 *deo* R*rec*A1 *ara*D139 (*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

2.2.3 Plasmid isolation

Plasmids were isolated using alkaline lysis according to Sambrock and associates (1989) with slight modifications. In brief, 4 ml over night culture was centrifuged for 1 min at 13,000 x g. Cells were suspended in 100 µl solution I (50 mM Glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) and incubated for 5 min on ice. Afterwards 200 µl solution II was added (0.2 M NaOH, 1 % (w/v) SDS) and another chilling step was performed. Solution II was added (3 M K-acetate, 12 % (v/v) acetic acid) and after a third incubation on ice, cell debris was removed by centrifugation for 10 min at 13,000 x g. Plasmids were purified by alcohol precipitation. Therefore 1 volume isopropanol and 1/10 volume 3 M Na-acetate, pH 4.8 were

added and DNA was spun down for 30 min at 13,000 x g. After washing with 70 % (v/v) ethanol DNA was suspended in 50 µl H_2O and stored at -20°C.

2.2.4 Sequencing

All cloned DNA fragments have been verified via sequencing of the inserts. If not mentioned otherwise, sequencing was performed by the MWG Biotech AG company (Ebersberg, Germany).

2.2.5 Preparation of competent *Agrobacterium rhizogenes* cells

Over night culture of bacteria in LB medium (0.5 % (w/v) Yeast extract, 1 % (w/v) Tryptone, 1 % (w/v) NaCl) containing 600 µg/ml streptomycin was centrifuged for 10 min at 4°C and 4000 x g. Cells were suspended in 1/10 volume 10 % (v/v) Glycerol and additionally centrifuged. Centrifugation / suspending were repeated three times and cells were finally used directly for transformation or stored at -80°C.

2.2.6 Transformation of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes strain ARqua 1 (Quandt *et al.*, 1993) was transformed with the binary plasmids via electroporation. Therefore 2 µg plasmid and 40 µl of electrocompetent cells were transferred into a electroporation cuvette (BioRad Gene pulser cuvette, 0.4 cm electrode gap, BioRad, Hercules, CA, USA). Afterwards a pulse of 2.5 kV was applied for 0.5 ms at 2.5 µF. 500 µl SOC (10 mM NaCl, 0,5 % (w/v) Yeast extract, 2 % (w/v) Tryptone, 2,5 mM KCl, pH 7,0; 6.25 µl 2 M $MgCl_2$, 25 µl 1 M Glucose) was added and cells were

incubated for 30 min on ice. Regeneration occurred for 3 h at 28°C with rigorous shaking. Cells were spread on LB agar (0.5 % (w/v) Yeast extract, 1 % (w/v) Tryptone, 1 % (w/v) NaCl, 1.5 % (w/v) Agar Agar, pH 7.0) containing petri-dishes with the according antibiotics (50 µg /ml kanamycin, 600 µg/ml streptomycin) and transformants have been obtained after 2 d growth at 28°C.

2.3 Tissue culturing methods

2.3.1 *Agrobacterium rhizogenes*-mediated transformation of *M. truncatula*

M. truncatula roots were transformed according to a modified protocol of Vieweg and associates (2004) with slightly modifications. Therefore, *Medicago truncatula* cv Jemalong A17 seeds were sterilised and germinated for two days in the dark on Farhaeus Medium (0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 20 µM Fe-citrate, 0.5 mM NH_4NO_3 plus 100 µg/l (w/v) $MnCl_2$, $CuSO_4$, $ZnCl_2$, H_3BO_3, Na_2MoO_4, respectively; Boisson-Dernier *et al*., 2001) and 24h at light. Bacteria, grown in liquid culture, were injected 3 times at the hook of the hypocotyl using a syringe. Transformants were incubated for 3 weeks at 24°C and a 16h/8h light/dark period vertically on Farhaeus medium to induce hairy roots. After cutting the main root of the promoter-GUS containing transformants to promote growth of lateral roots the plants were inoculated with AM fungus for another 21d. At least a histochemical staining for β-glucuronidase-activity was performed to investigate activation of the *uidA* gene.

2.3.2 *Agrobacterium rhizogenes*-mediated transformation of *Nicotiana tabacum*

Nicotiana tabacum cv Samsun roots were transformed using the same protocol as for *M.*

truncatula.

2.3.3 Histochemical analysis of transgenic roots

Histochemical assays for β-glucuronidase activity were performed as described by Jefferson and associates (1997). Roots were incubated in the staining solutions in the dark at 37°C for 1 to 2 h and subsequently cleared in 70% ethanol for 1 h.

2.4 Microarray Analyses

2.4.1 Scope and layout of the microarray

Mt16kOLI1 microarray contains 16,086 probes consisting of 70mer oligonucleotides representing all TCs of the TIGR *M. truncatula* gene index 5 (http://www.tigr.org/tdb/mtgi) as well as different truncated GAPDH controls to analyse hybridisation efficiency (Küster *et al.*, 2004). Background and unspecific hybridisation assessments were performed using 226 probes containing only spotting buffer, and twelve probes served as negative controls. Mt16kOLI1 consists of 48 grids arranged in 12 metarows and 4 metacolumns. Each grid contains 702 spots in 27 rows and 26 columns with 24 columns carrying 27 probed and 2 columns carrying 20 probes. Each probe exists twice in the same grids throughout the arrays.

2.4.2 Cy-labelling of hybridisation probes for microarray analysis

Cy3 and Cy5 labelling was done according to Küster *et al.* (2004). Therefore 20 µg RNA, 2.5 µg double-anchored oligo$(dT)_{15}$VN primers and 5 µg of random hexamers were used. Labelled cDNA was purified using CyScribe GFX Columns (Amersham Biosciences,

Freiburg, Germany) and labelling efficiency was checked. The Mt16kOLI1 microarray contains 16,086 70mer oligonucleotide probes (Qiagen) representing all TCs of the TIGR *M. truncatula* Gene Index 5 (http://www.tigr.org/tdb/mtgi) and additional GAPDH controls. Microarrays were prepared and hybridised as described by Hohnjec *et al.* (2005).

2.4.3 Analysis of microarray image data

Image processing was performed using the ImaGene 5.5 software (BioDiscovery, Los Angeles). Data files were imported into the EMMA2 array analysis software (Dondrup *et al.*, 2003). Data were Lowess normalised with a floor value of 20 and a *t*-statistic was used to identify regulated genes. Genes were significantly expressed if $P \leq 0.5$ and $M \leq -1$ or $M \geq 1$, where M depicts the expression ratio.

2.5 *In silico* transcriptional analyses

2.5.1 cDNA libraries and sequencing

The MtGim SSH library was generated as described earlier (Wulf *et al.,* 2003). The MtAmp library was generated by MediGenomix (Martinsried, Germany) from mycorrhizal roots of *M. truncatula* using a directional cloning strategy with a cutoff of 500 bp. cDNA was prepared by reverse transcription of poly A+ enriched RNA. The cDNA was directionally ligated into the pGEM-T® vector (Promega, Madison, WI, USA) using GCA TGC GGC CGA GGC GGC CGA CAT G and CTG CAG GCC ATT ATG GCC GGG adaptors. Plasmids containing cDNA inserts were propagated in *Escherichia coli* DH10B cells. cDNA sequences were obtained using standard protocols on the MegaBace 1000 sequencer (Amersham Biosciences, Freiburg,

Germany).

Genotype of *E. coli* DH10B:

F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L (Str^R) *nup*G

2.5.2 Sequence processing, annotation and clustering

To cluster and annotate the ESTs obtained, BioMake software package was used. BioMake processes sequence data as follows: beginning with the raw EST trace files, a normalisation step using phred (Ewing *et al.,* 1998) was performed. Afterwards, low-quality regions were removed according to the phred 13 quality definition (Ewing and Greene, 1998). Finally, the vector content needed to be clipped, leading to EST reads in fasta file format. Clustering of the EST reads also was performed by the BioMake software package according to the TIGR clustering algorithm (Liang *et al*., 2000). The sequence data obtained consist of TCs and single (unclustered) EST reads, called singletons. Comparisons to the TIGR MtGI were done in order to identify identical TCs. BLAST comparisons against the GenBank non-redundant database and annotations also were done using the BioMake software. Blastx searches were carried out in order to identify homologous genes in other organisms (Altschul *et al.,* 1997).

2.5.3 *In silico* analysis of gene expression

To identify TCs representing putative mycorrhiza-specific genes, an electronic Northern (eNorthern) algorithm was developed by comparing MtGim and MtAmp EST clusters via a BLAST (Altschul *et al.,* 1997) homology search against TIGR (Liang *et al.,* 2000) using an e-

value cut-off of $1e^{-10}$ and sequence identity of at least 95 %. Those clusters that hit only the MtAmp or MtGim library were assumed to represent novel mycorrhiza-specific genes of *M. truncatula*.

2.5.4 Quantitative real-time RT-PCR

Primer design and quantitative RT-PCR was carried out according to Wulf and associates (2003). All quantitative realtime RT-PCR experiments were repeated (technical control) in order to verify RNA-accumulation values. Primer sequences used for RT-PCR are shown in Table 1.

MtGI-TC[a]	Left primer (5´-3´)	Right primer (5´-3´)
TC64428	tacgaaaatcaaacatttcac	gcagagatgatgacaataaga
TC66505	atctctttattgtttccaagg	aggatagaggatcaaattcaa
TC74096	aattcaacctttatcaccatt	attatcttgctcgatcttctc
TC74325	aataagatggatagtggcatt	aattgtaaacgttcataagca
TC76060	ttcacaagttctctcattcag	agaacactttcaattttaggg
TC71483	tcctatcttcccaagtaagag	aattgctgttaagcaacataa
TC65799	tgggataacctctaaagaaag	ttttgttggtatcaaagagtg
TC67209	accagaatcagaatatccaac	ttgcagtagagcttttacaga
TC74500	gtagtcacttctatcagccgt	catctatgatttcaagtttcg
TC67079	aacttcctttagcaatttcag	gtttcttgtagctcatgattg
TC68426	aaaaatggttcaactggttat	tgtcgatgagatacttgatgt
TC66580	atgaattcttaaccaaccaat	tttccgtaactattgatgcta
TC75569	gaaccagaaagacataaggag	aatgcttattgcatcaaagta
TC71486	gctttaatatttggaaagagc	gtatacgtttggtgctgataa
TC60540	agaagtgtccacacacaagta	caacactttatggaaaatgaa
TC68166	agtgaaagaccatgaaagaat	ataagttataatcctgcctcg
TC69333	caatgtgaggatattgagatg	tcttttatcgtgaacacaaac
TC75254	gatactgacatggcaacttta	aactggttgaaacacatgata
TC75360	ctatcaaattcaacaaggaca	aactcaaacggtagacaagat
TC73332	accatttgaagttcacctaat	ctacaacgttttaatgacgag
TC65476	gaacacaacagtcaaaacaac	atagtccagccattattcagt
TC63901	aaataaaaattttggtggtgt	gacataccaataagcacttga
TC66155	tgatagcagctgattttctta	atctaccaaaattgaattgga

MtGI-TC[a]	Left primer (5´-3´)	Right primer (5´-3´)
TC70909	caaaattgtagtgttggagaa	ataaatgcatattgtgttgga
TC72502	attctattgcatcattcatca	ggatcttgtcttgtctaaacc
TC76132	tcttctcccataggttgtaat	ttttgtagttggagatgaaaa
TC67410	tccctcctcatcaatactact	cagagtgtgtttatgcaagtt
TC76092	gattaacgacaaaaaggatct	gcttgaaataactgtcatcaa
TC59827	atgtcttggtaggtttgactt	gtaaattatcaaagggaggtg
TC60541	ttgctagcacattgtttaact	agttacatcaaccatttcaca
TC63954	agctctttcacttttagctgt	gcaaagactggagttactctt
TC64306	cataaccatcttcaacaagaa	actcagttacagagcacaatg
TC67060	tactttcactgtctctgatcg	ctggttatgacgattaacaaa

Table 1: Primer sequences used for quantitative real-time reverse-transcription PCR

[a] MtGI: *Medicago truncatula* gene index; TC = tentative consensus sequence

2.5.5 Sequence Analysis

Sequences have been analysed concerning conserved short protein patterns using the PROSITE (Gattiker *et al.,* 2002; Sigrist *et al.,* 2002) database at ExPASy. Searches for sequences similarities were done using Blast2X (Altschul *et al.,* 1997). *M. truncatula* genomic sequence data were obtained from the *M. truncatula* Genome Sequencing Project at the Advanced Center for Genome Technology of the University of Oklahoma, (Norman, OK, USA) and the Noble Foundation (Ardmore, OK, USA).

2.6 Analyses of AM-specific lectin-like genes

2.6.1 Cloning of whole cDNA sequences

SMART™ technology (Clontech, Mountain View, CA, USA) was used to clone whole cDNA sequences. Therefore total RNA was extracted from mycorrhizal roots and reverse transcribed

into SMART II cDNA according to the manufacturers protocols. 3' and 5' RACEs were done for each gene using one gene specific primer and either an oligo d(T) or the SMART UNP (universal nested primer: AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG). PCR fragments were cloned into the pGEM®-Teasy vector (Promega, Madison; WI, USA). Plasmids containing cDNA inserts were propagated in *Escherichia coli* TOP10 F' strain and insert was sequenced after reisolation.

2.6.1.1 Cloning of genomic sequences

Lectin sequences were amplified from genomic DNA using following primer pairs:

Gene	primer 1 (5'-3')	primer 2 (5'-3')
*MtLec*6	TTT CAT TAC TTT CCT CTT CC	CAA CAA TTC ACA ACA AAG
*MtLec*8	CAC AAC ACT ACA ATG G	CAT AAC ATA TTC ATA TCA TCC
*MtLec*9	AAT GGC TCT TTC TTC AGC	GTT TAT CAA TTA TCA CAT TG
*MtLec*10	GGG ACC ACT ACA TTT TGA CG	CAT AAT GAG TGA ATT TGC

Lectin genes were amplified using standard PCR (chapter 2.1.8) and cloned into pGemT® easy vector (chapter 2.1.9). After transformation and isolation of plasmids containing cloned fragments, inserts were sequenced.

2.6.2 Sequence Analyses

ClustalW analysis (Jeanmougin *et al.,* 1998) and multiple alignment were done according to Thompson *et al.* (1997). Standard computational DNA modifications were performed using the Vector NTI software (Invitrogen, Carlsbad, CA, USA). TargetP tool was used to predict subcellular location of the lectins (Emanuelsson *et al.*, 2000) and cleavage sites of putative signal peptides (Nielsen *et al.*, 1997).

2.6.3 Construction of GFP fusion proteins

MtLec5 open reading frame was amplified using modified specific primers to add attB sites for cloning (5' to 3': GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG CCA ACT CCA TAC CAA AAC TCC as forward primer and GGG ACC ACT TTG TAC AAG AAA GCT GGG TCA AGT TTG AAA CAA AGG ACC AAG AAA as reverse primer). 1 ng genomic DNA was used in PCR as template. The resulting PCR fragment was cloned to the N- terminal end of a *gfp6* gene into pMDC 84 vector (Curtis and Grossniklaus, 2003) using Clonase technique. For deletion of the probable presequence similar cloning technique was used. Forward primer (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG ACT CAT TAT CTT TCA ACT TCC) was designed to introduce a start codon (underlined) into the truncated protein.

For control plasmid pCAMBIA 1302 (Hajdukiewicz *et al.*, 1994) was used that contains an *mgfp5* gene under control of an 35S promoter.

2.6.4 GFP detection using confocal laser scanning micropscopy

Transgenic roots were examined using a Leica MZ FL III binocular with GFP filter. Confocal laser scanning microscopy was done using Carl Zeiss Laser Scanning Systems LSM510 with filters giving an excitation spectrum between 505 and 530 nm and 560 nm. Processing of images was done using Zeiss LSM Image Examiner Version 3.2.0.70.

2.7 Promoter analysis of AM-specific lectin-like genes

2.7.1 Isolation of lectin promoters from BAC sequences and by inverse PCR

Isolation of the lectin promoter sequences have been done as described by Frenzel *et al.*

(2005). For amplification of the *MtLec*5 promoter, 1 ng of genomic DNA was amplified with specific primers (5′ to 3′: GAA TTC GGG AAA ATT GAC TAA CGT TGT GAA A and GGA TTC GGT ATT ATG TTG TGT ATG ATG C) deduced from the corresponding BAC sequence. The PCR product was cut with *Eco*RI and *Bam*HI and cloned into the corresponding sites into the pLP100 vector (Szabados *et al.,* 1995). For the inverse PCR to amplify the *MtLec*7 promoter region, 2 µg of genomic *M. truncatula* DNA was cut with the restriction enzymes *Eco*RI, *Xba*I, *Xho*I, *Pvu*I, *Pae*I, or *Hind*III. Circularisation of 50 ng of digested DNA was carried out in 30 µl using 0.6 U of T4-DNA ligase at 16°C. The flanking regions were amplified using 10 ng of circularised DNA and *MtLec*7-specific outward orientated primers (5′ to 3′: GAG ATG GAA TTA CCT TCT TCA and GAT GGT AAT GAA AAG GAT TTG AGT CC). Of the resulting PCR fragment, a 1,503 bp *MtLec*7 promoter region was amplified using specific primers (5′ to 3′: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC ACC GCG CAT AAT GAT TGA GG and GGG ACC ACT TTG TAC AAG AAA GCT GGG TGA ATC CGC GAT GGT TCT AAA TTA GTG) and 1 ng of genomic DNA as template. The resulting PCR product was cloned into the pMDC163 vector (Curtis and Grossniklaus, 2003).

2.7.2 Promoter Deletion Analyses

*MtLec*5 promoter sequences were amplified using specific primers (5' to 3': GAA TTC CCT TAG GTC TAT ATT AAA TGA ACT for 300bp of the promoter (p*MtLec*5(-301/+23)), GAA TTC TTT ATT TAC TGT TAA AAA TTA CC for p*MtLec*5(-151/+23), GAA TTC ATT GGT AAT TAT TTT TCG AAA ACA TCC for 75bp of the promoter and GGA TCC GGT ATT ATG TTG TGT ATG ATG C as reverse primer, respectively). PCR products were restricted with *Eco*RI and *Bam*HI and cloned into the corresponding sites of the pLP100 vector. p*MtLec*7(-304/+52), p*MtLec*7(-150/+52), p*MtLec*7(-75/+52) were cloned with the same strategy using

specific primers (GAA TTC CAT AAG TAC TTA CAA AAT GTT TAG G for the 304bp fragment, GAA TTC ATT AAA ATC GTT CAA AAT CGA CTA G for the 150bp fragment, GAA TTC GCC TGC CTT TGT TCA ACT TTT TTT for the 75bp fragment and GGA TTC GGT TGT AAA TTA GAA AAT TTT GG as reverse primer, respectively). Cloning of the p*MtLec*7(-986/+52) fragment was done using the Gateway® technology (Invitrogen, Carlsbad, CA, USA). The promoter region was amplified using specific primers modified with attB sites (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TAG CTA AGA GAC TAA ATT GG and GGG ACC ACT TTG TAC AAG AAA GCT GGG TGA ATC CGC GAT GGT TCT AAA TTA GTG) and subsequently cloned into vector pMDC163.

2.7.3 Electrophoretic Mobility Shift Assay

2.7.3.1 3' end labelling of DNA

Electrophoretic mobility shift assay (EMSA) was done using DIG Gel Shift Kit, 2nd Generation (Roche, Mannheim, Germany). Therefore, oligonucleotides were 3' end labelled with DIG-11-ddUTP. Oligonucelotides were annealed by adding 100 pmol of complementary DNA fragments, respectively, heating to 95°C and cooling down to 15°C at 0.2°C decrease per second. 4pmol of annealed primers were used for 3' end labelling: 4 µl 5 x labelling buffer, 1 µl 1 mM DIG ddUTP solution, 4 µl 25 mM $CoCl_2$ solution and 400 U terminal transferase were added and incubated for 15 min at 37°C. Reaction was stopped by adding 2 µl 0.2 M EDTA, pH 8.0.

Primer pairs used for EMSA analysis:

Fragment	forward (5´-3´)	reverse (5´-3´)
p*MtLec*5		
(-301/-271)	CCT TAG GTC TAT ATC TAA ATG AAC TAA TTG	CAA TTA GTT CAT TTA GAT ATA GAC CTA AGG
(-281/-251)	GAA CTA ATT GTG CTT TAA ACT TTT AGT TCA	TGA ACT AAA AGT TTA AAG CAC AAT TAG TTC
(-261/-231)	TTT TAG TTC ATT TTA TTA GTT GAT AAA ATC	GAT TTT ATC AAC TAA TAA AAT GAA CTA AAA
(-241/-211)	TGA TAA AAT CTA TGA ACA TTT TGT TTA CTT	AAG TAA ACA AAA TGT TCA TAG ATT TTA TCA
(-221/-191)	TTG TTT ACT TGG TCC ATG CTA CTC ATA ATT	AAT TAT GAG TAG CAT GGA CCA AGT AAA CAA
(-201/-171)	ACT CAT AAT TTA TCA CTC AAA TTA GCT GTA	TAC AGC TAA TTT GAG TGA TAA ATT ATG AGT
(-181/-151)	ATT AGC TGT ACT TCA TTG TAA ATA TCA TCC	GGA TGA TAT TTA CAA TGA AGT ACA GCT AAT

2.7.3.2 Dot blot to check labelling efficiency

Labelling efficiency was tested using dot blot. DIG labelled DNA were diluted 1:10 and 1:100. One microlitre of each dilution were dotted on a nitrocellulose membrane. DNA was fixed for 30 min on an UV-table. Membrane was blocked for 30 min in blocking solution (0.5 % (w/v) blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5)). Anti-DIG-AP (alkaline phosphatase) antibody was added for 1 h diluted 1:5000 in blocking solution. After two times washing with maleic acid buffer with 0.3 % (v/v) Tween20 detection of the signal occurred in substrate buffer (100 mM Tris-HCl, 0.5 mM $MgCl_2$, ph 9.5) containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml).

2.7.3.3 Electrophoretic mobility shift assay

Binding reactions were carried out in binding buffer (20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2 % (v/v) Tween 20, 30 mM KCl) using 1.5 µg poly [d(A-T)] (1 µg/ml), 0.8 ng DIG labelled oligonucleotides and 5 µg crude protein extract from mycorrhizal and non-mycorrhizal roots or without any protein. Binding reaction was carried

out for 20 minutes at room temperature. Reactions were stopped by adding 5 µl loading buffer (0.25 x TBE buffer (35.6 mM Tris, 35.6 mM boric acid, 0.8 mM EDTA, pH 8.0), 60 % glycerol, 40 % (w/v) bromphenol blue). Samples were applied to an 8 % non-denaturing polyacrylamide gel (15 % (v/v) glycerol, 0.25 x TBE buffer, 8 % (w/v) acrylamide/bisacrylamide (37.5 : 1), 0.002 % (v/v) TEMED, 0.0005 % (w/v) APS). After running of the gel the DNA was electro-blotted on a nitrocellulose membrane using a electro blotting apparatus (Trans Blot SD, Semi Dry Transfer Cell, BioRad, Hercules, CA, USA). Transfer occurred at 20 V for 1 h and a limit of 0.77 A using 0.25 x TBE buffer. After transfer DNA was fixed on the membrane via cross-linking on a UV-light emitting table for 30 min.
Detection of the DIG-labelled DNA was performed according to the manufacturers ”DIG Application Manual for Filter Hybridization“ protocol (Roche, Mannheim, Germany), similar to the detection procedure for the dot blot (chapter 2.7.3.2) with following differences: After blocking of the membrane antibody was added in a 1:15,000 dilution. Signals were detected on Lumi-film (Chemiluminiscent Detection Film, Roche, Mannheim, Germany) and in substrate buffer 1:100 diluted CDP-Star® (Roche, Mannheim, Germany) as substrate for the AP (alkaline phosphatase).

2.7.4 Computational analyses

Promoter sequences were analysed using the Wise2 algorithm of the PromoterWise tool (Birney *et al.*, 2004) and MatInspector, to identify common promoter motives (Quandt *et al.*, 1995).

2.8 Chemicals and solutions

All utilised chemicals accorded high analytical grade and, if not mentioned otherwise, were obtained from Applichem (Darmstadt, Germany), Clontech (Mountain View, CA, USA), Duchefa (Haarlem, Netherlands), Fluka/Sigma-Aldrich (Taufkirchen, Germany), Invitrogen (Carlsbad, CA, USA), MBI Fermentas (Vilnius, Lithuania), Roche (Mannheim, Germany) or Roth (Karlsruhe, Germany).

All solutions and media were sterilised by either autoclaving or filtration using a 22 µm micro filter.

3 Results

3.1 Transcriptional analyses using Microarray hybridisation

3.1.1 *M. truncatula* is 6 dpi with *G. intraradices* in an early-mycorrhizal phase

In order to analyse transcriptional changes during the development of the mycorrhiza symbiosis *Medicago truncatula* plants were inoculated for two different time periods, 6 and 21 days, with *Glomus intraradices*. After harvesting, colonisation frequency was measured and finally calculated by staining of the fungal structures and subsequent counting of arbuscules and hyphae. After inoculation for six days no mycorrhizal structures could be observed in the inner parts of the roots and no appressoria have been developed in this system referring the early-symbiotic phase of the symbiosis (data not shown). Inoculation with the AM fungus for 21 days resulted in a frequency of inoculation (F) of nearly 100 %, a colonisation intensity (M) of 81 % and a relative arbuscule frequency (a) of 81 %. All developmental stages of the AM from appressoria and arbuscules to intra and extra-radical hyphae and vesicles were abundant, so that a complete developed symbiosis has evolved (data not shown).

3.1.2 Microarray hybridisation

After determination of the mycorrhization efficiency RNA was isolated from plants inoculated with AM fungus and from non mycorrhizal roots. RNA was reverse transcribed and labelled with cy dyes. Two different experiments were carried out: Firstly, Mt16k-OLI1 microarray was hybridised with probes from 21 dpi mycorrhizal and non-mycorrhizal roots. In a second experiment oligonucleotide chips were hybridised with probes from early-symbiotic and non-symbiotic roots. Therefore, it was possible to identify transcriptionally regulated genes, which

are differentially expressed in the completely developed mycorrhiza or in the early-symbiotic phase in comparison to non-mycorrhizal roots. After hybridisation data were statistically evaluated and transferred into a spreadsheat program allowing sorting of expression values of both experiments in parallel. Genes, showing at least two fold regulation with a probability of more than 95 %, were denoted to be specific up or down regulated. Finally these genes were sorted into three different classes: I, regulated exclusively 21 dpi, II, regulated exclusively in the early-symbiotic phase and III, regulated in both stages (data not shown).

3.1.3 Microarray data are concordant with former studies

After statistical evaluation and sorting, genes were clustered into different functional classes according to Journet *et al.* (2002). In order to check parameters used for the analysis of the transcription data, these were compared to data obtained in different former studies: *MtPT*4*,* the mycorrhiza specific phosphate transporter is known to be highly induced in arbuscule containing cells (Harrison *et al.*, 2002). Hybridisation of the arrays showed 32 fold induction of the *MtPT*4 gene after 21 days of mycorrhization but not in the early-symbiotic phase (table 2), where no arbuscules were present and no phosphate exchange takes place. In contrast *MtPT*1, a non-regulated phosphate transporter, which is known to be repressed during the AM symbiosis is more than 2 fold significantly repressed 21 dpi and even 6 dpi. Another already described mycorrhiza specific gene, *MtGst*1*,* is 28 fold induced 21 dpi. As it has also been identified using a proteomic approach (Bestel-Corre *et al.*, 2002) and is additionally known to be arbuscule specific transcribed (Wulf *et al.*, 2003), it is obvious that it appears not to be regulated in plants 6 dpi. *VfLb*29, a mycorrhiza and nodule specific leghemoglobin (Vieweg *et al.*, 2004), is upregulated 3 fold after 21 days but remarkably more than 3 times down-regulated 6 dpi. In summary, several genes, which have been shown to be AM-specific induced, have been identified in the microarray experiment to be induced in mycorrhizal

roots.

Oligo ID	TIGR ID	Annotation	expression level M
MT009707	TC85743	Phosphate transporter, *MtPT*4	5
MT014321	TC76987	Leghemoglobin, *VfLb*29	1.49
MT00901	TC85868	Glutathione S-transferase, *MtGst*1	5.31
MT009186	TC85933	Phosphate transporter, *MtPT*1	-1.28

Table 2: Expression levels of former described genes

Several genes, which have been identified in former studies, showed in accordance specific expression in dependence on mycorrhization.

Oligo ID depicts the location of the probe on the microarray. TC values give the identifier in the TIGR gene index and M the expression level in regard to non-mycorrhizal roots in logarithmic scale. The negative value stands for a down-regulation.

3.1.4 Up and down-regulated genes are distributed equally during a completely developed AM

763 genes could be identified, which were differentially regulated 21 days after mycorrhization. 407 of these were upregulated, representing about 53 % of the regulated genes, whereas 356 were down-regulated (47 %). Independently from the large amount of genes of unknown function, without homology to known genes or genes which are known to have miscellaneous functions a high amount of genes, representing 11 % of all regulated genes, involved in protein synthesis and processing are down regulated (figure 1). Additionally, genes involved in defence and stress or DNA metabolism appear to be more down regulated, whereas genes involved in the transcription of genes, secondary metabolism or membrane transport are more upregulated. For example the well studied mycorrhiza specific phosphate transporter *MtPT*4 (Harrison *et al.*, 2002) is 32 fold upregulated 21 days after mycorrhization in comparison to non-mycorrhized roots but is not regulated after 6 days. Additionally, a zinc transporter, an ammonium transporter and two amino acid transporters

could be identified to be up to 4 fold upregulated.

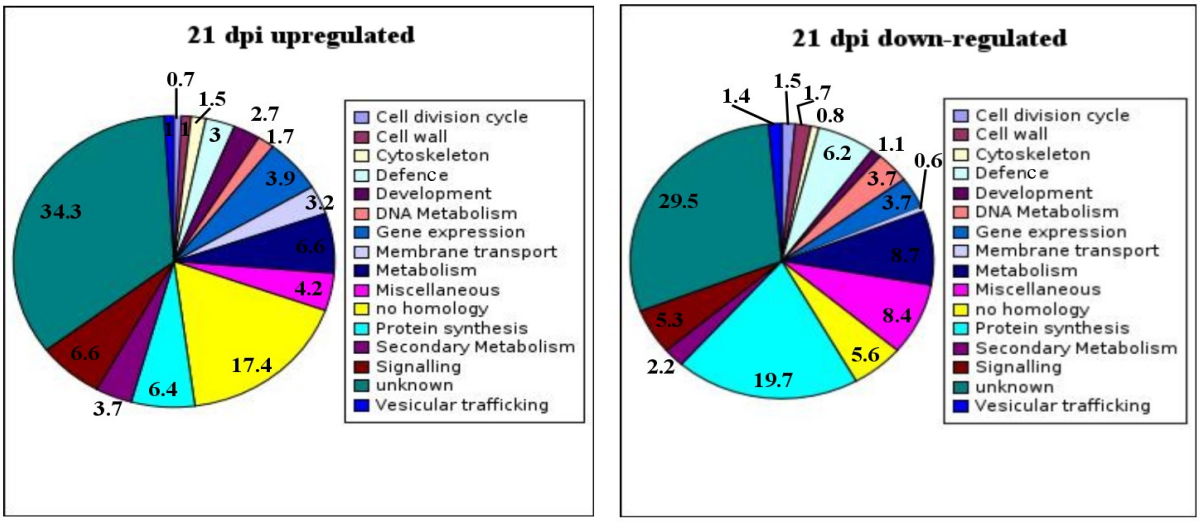

Figure 1: Regulation of *M. truncatula* genes 21 dpi with *Glomus intraradices*:

In total 763 plant genes, which were at least 2 fold down- (356) or upregulated (407) in *M. truncatula* roots, that were inoculated with AM fungus for 21 days have been classified. Proteins were grouped into functional categories according to Journet *et al.* (2002). Numbers depict the rate of the particular functional class in percent.

3.1.5 Six day early-mycorrhizal roots show transcriptional decrease of defence-involved genes

In the early-mycorrhizal phase only 167 genes appear to be differentially regulated. In comparison to the completely developed symbiosis after 21 days, where up and down-regulated genes were roughly distributed equally, 86 % (142 genes) were at least 2-fold down-regulated and only 23 (14 %) were found to be upregulated. Several of these genes have no clearly determinable function. Genes showing similarities to genes of known functions are involved in defence, development, gene expression, membrane transport, metabolism, secondary metabolism and signalling (figure 2). Down-regulated genes were distributed through all functional classes, but with high contents involved in defence and stress

responses, gene expression and signalling.

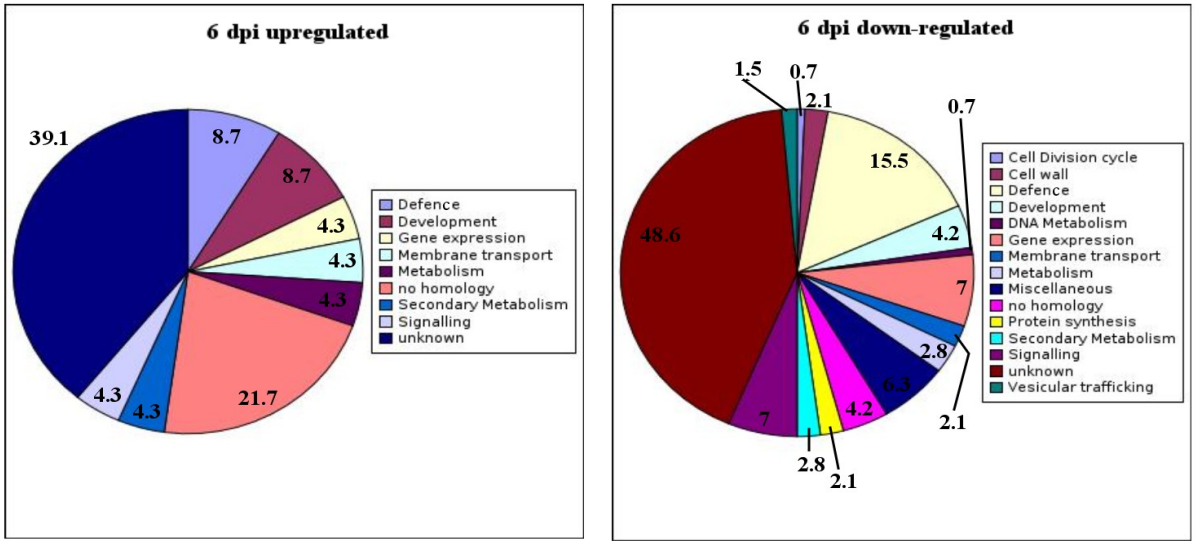

Figure 2: Regulation of *M. truncatula* genes, 6 dpi with *G. intraradices*

Classification of 165 differentially expressed genes, 6 days after inoculation with *Glomus intraradices.* In total 142 genes were down regulated at least 2 fold and 23 genes are upregulated representing approximately 14 % of all regulated genes. Numbers depict the rate of the particular functional class in percent.

3.1.6 Thirty-six genes show transcriptional alteration 6 and 21 dpi

Thirty-six genes could be identified, that were specifically expressed at both analysed points of time. A large amount of these genes belongs to the functional classes of unknown or miscellaneous function and genes involved in defence and stress mechanisms (figure 3). All of the latter ones appear to be down-regulated at both time points.

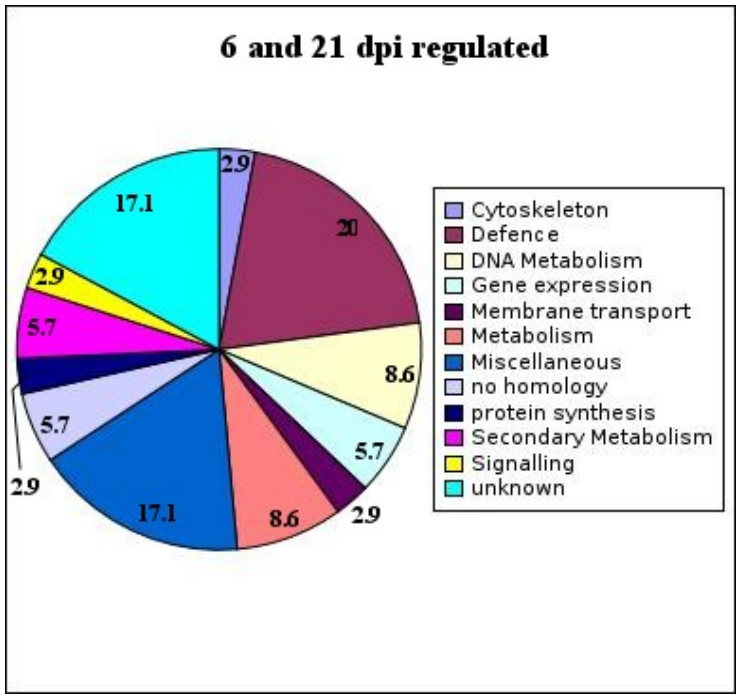

Figure 3: 6 dpi and 21 dpi regulated genes

Thirty-six genes were at least 2 fold induced or repressed after 6 and 21 dpi and clustered into functional classes. Approximately 17 % were represented by genes of unknown or miscellaneous function and 20 % represent genes assumed to be involved in defence mechanisms. Numbers depict the rate of the particular functional class in percent.

Most of the regulated genes seem to be comparably regulated in both points of time, up or down-regulated when compared to non-mycorrhizal roots (table 3) with only few exceptions: For example *VfLb*29, a leghemoglobin which is known to be specifically induced during mycorrhiza and the root-nodule symbiosis (Vieweg *et al.*, 2004) is about 3 times down-regulated after 6 days but 3 times upregulated after 21 days.

Oligo ID	TIGR ID	Annotation	M 6dpi	M 21dpi	Functional Class
MT005563	TC92278	Kinesin heavy chain	1,01	3,37	Cytoskeleton
MT007216	TC76803	Protein kinase MMK4	-2,08	-1,53	Defence
MT014167	TC76638	Pprg2 protein	-1,04	-1,31	Defence
MT015581	TC77728	Putative resistance protein	-1,41	-1,3	Defence
MT015842	TC86227	Functional candidate resistance protein	-2,83	-1,66	Defence
MT016002	TC92835	Functional candidate resistance protein	-1,93	-1,64	Defence
MT001587	TC78664	Syringolide-induced protein	-2,14	-2,08	Defence
MT002265	TC79210	Isoflavone-7-O-methyltransferase	-1,12	-1,21	Defence
MT005557	TC93233	Topoisomerase IA	1,17	2,36	DNA Metabolism
MT014572	TC76927	Histone H3 variant H3.3	-1	-1,23	DNA Metabolism
MT015593	TC86259	Histone H4	-1,05	-1,35	DNA Metabolism
MT009061	TC88075	ZPT2-14	-1,75	-1,54	Gene expression
MT009187	TC88516	WRKY DNA-binding protein	-2,1	-1,12	Gene expression
MT008865	TC88171	Chloride channel	-1,54	-1,25	Membrane transport
MT003613	TC89431	Photosystem II reaction center M protein	-1,2	1,48	Metabolism
MT004055	TC90579	NADH dehydrogenase	-1,29	1,02	Metabolism
MT004368	TC91563	Gamma-glutamytransferase homolog	1,41	1,65	Metabolism
MT006554	TC85022	N8 protein	-1,46	1,68	Miscellaneous
MT007911	TC86706	Cytochrome c	-1,01	-1,2	Miscellaneous
MT014321	TC76987	Leghemoglobin 29 (VfLb29)	-1,84	1,49	Miscellaneous
MT015531	TC78125	Cyclase	-1,05	-1,1	Miscellaneous
MT001306	TC78397	Galactose binding protein	-1,35	1,01	Miscellaneous
MT03313	TC80887	FKBP lke protein	1,35	1,91	Miscellaneous
MT004861	TC90451	Hypothtical protein	1,2	2,06	no homology
MT005763	TC92391	Hypothtical protein	1,01	-2,54	no homology
MT012331	TC91847	Translation initiation factor	2,71	4,22	Protein synthesis
MT000172	TC85552	3-hydroxy-3-methylglutaryl-coenzyme A	-1,63	1,77	SecondaryMetabolism
MT014273	TC85552	3-hydroxy-3-methylglutaryl-coenzyme A	-1,19	1,7	SecondaryMetabolism
MT015393	TC77353	Serpentine resceptor class d	-3,4	1,36	Signalling
MT004784	TC82756	Similar to GP|17381278|gb|AAL3605,1	-1,61	1,21	unknown
MT008323	TC87140	GP|16648734|gb|AAL25559,1	1,01	1,17	unknown
MT009554	TC79058	Unknown protein	-1,84	-2,01	unknown
MT014641	TC86580	Unknown protein	1,29	1,75	unknown
MT015582	TC77730	Unknown	-1,7	-1,38	unknown
MT015661	TC87804	Unknown protein	-2,15	1,16	unknown

Table 3: Differentially expressed genes 6 and 21 dpi

Differentially expressed genes, that are regulated at least 2-fold, are grouped into functional clusters. Oligo ID depicts the location of the oligonucleotide representing a special gene on the Mt16k microarray. The TC values give the identifier in the TIGR gene index. M-values denote the expression value in regard to non-mycorrhizal roots in logarithmic scale. Negative values stand for down-regulated genes.

3.2 Transcriptional analyses using electronic Northern approach

3.2.1 Generation of EST-clusters from two *M. truncatula-G. intraradices* AM cDNA libraries

Two different cDNA-libraries were constructed and used for EST-sequencing. The first one designated MtAmp was generated from *M. truncatula* roots 5 weeks after inoculation with *Glomus intraradices*. At this point of time, all developmental stages of an AM (arbuscules, inter- and intracellular hyphae, appressoria and vesicles) were present. Mycorrhiza parameters (Trouvelot *et al.,* 1986) were same as described in chapter 3.1. From this library, a total of 3,805 ESTs was generated, with an average length of 479 bp. All ESTs with a length of at least 100 bp were submitted to the EMBL database under accession numbers AJ500944-AJ504393. The second library, MtGim, was generated by suppression subtractive hybridisation (SSH) and 1,841 EST with an average length of 416 bp were obtained. An initial characterisation of 34 cDNAs of the MtGim library revealed the presence of different mycorrhiza-induced genes in this library (Wulf *et al.,* 2003). So far, the remaining cDNAs have not been characterised in detail. All MtGim-ESTs that were at least 50 bp in length were submitted to the EMBL database and can be accessed under AJ499169-AJ500943 (Frenzel *et al*., 2005).

Clustering of the MtAmp ESTs revealed 534 tentative consensus (TC) sequences and 1,857 singletons, whereas clustering of MtGim ESTs resulted in the identification of 297 TCs and 611 singletons. Of the 534 MtAmp-TCs, only 4.9 % were novel in comparison to all other libraries in the TIGR *M. truncatula* Gene Index (MtGI). In contrast, a notably higher number of the 297 MtGim-TCs encoded novel genes, since 34.7 % of the MtGim-TCs did not match any other TIGR MtGI TC. To analyse all TCs derived from both libraries in a single step, all 5,646 MtGim- and MtAmp-ESTs were clustered to one population of 833 TC and 2,349

singletons. Since clustering protocol was identical to that used by TIGR (Quackenbusch *et al.,* 2000) and in order to ease cross-referencing of the data presented here, TIGR MtGI TC numbers were used as references for MtAmp/MtGim TCs.

3.2.2 *In silico* screening for novel AM-specific transcripts

In silico expression analysis was applied to identify those TCs that are composed of ESTs exclusively derived of the MtGim- or MtAmp-libraries, but not from any other cDNA-library of the TIGR MtGI. This search revealed 115 MtAmp/MTGim-specific TCs. These TCs, likely to represent novel mycorrhiza-specific or at least mycorrhiza-related genes, were annotated and grouped into functional categories according to Journet *et al.* (2002). After blastx searches, 10 TCs showed highest homologies to fungal genes, indicating that these might represent *G. intraradices* genes (table 4). A large group of 52 TC sequences did not reveal homologies to any other sequence and also did not contain significant matches to the PROSITE database (table 5).

MtGI-TC	bp	Annotation	GenBank Entry	E-value	N_{MtAmp}	N_{MtGim}
TC64085	559	mRNA for histone H4 (*A.bisporus*)	X94189	$7e^{-32}$	--	4 / 4
TC68248	362	isolate RWM_243 28S ribosomal RNA (*Glomus mosseae*)	AF396793	e^{-173}	--	5 / 5
TC68246	561	isolate RWC_658 28S ribosomal RNA (*Glomus caledonium*)	AF396794	e^{-115}	--	45 / 45
TC75488	641	strain DAOM212595 small subunit ribosomal RNA gene (*Glomus mosseae*)	U96143	$9e^{-41}$	--	2 / 2
TC67625	669	cytochrome P-450 cyp509A1 (*Cunninghamella elegans*)	Q9P493	$5e^{-41}$	--	2/2
TC59129	487	Glo8 isolate M3 small subunit ribosomal RNA gene (*Glomus sp.*)	AY129636	0.0	--	2 / 2
TC75848	458	mRNA for hypothetical protein (*Glomus mosseae*)	AJ242917	0.0	--	2 / 2

MtGI-TC	bp	Annotation	GenBank Entry	E-value	N_{MtAmp}	N_{MtGim}
TC74547	717	histone H3 (*Mortierella alpina*)	Q9HDN1	$6e^{-67}$	1/2	1/2
TC68247	390	isolate SI_141 28S ribosomal RNA (*Glomus intraradices*)	AF396797	e^{-177}	--	11 / 11
TC75784	596	vesicular transport protein sec18 homolog (*Schizosaccharomyces pombe*)	T50122	$1e^{-12}$	--	2/2

Table 4: MtGim-/MtAmp-specific TCs with highest homologies to sequences of fungal origin

(N: number of EST in a library); MtGI depicts the *Medicago truncatula* gene index, N the number of the expressed sequence tags in a library.

MTGI-TC	bp	N_{MtAmp}	N_{MtGim}
TC63455	921	1/5	4/5
TC71585	649	1/5	4/5
TC71978	460	-	5/5
TC72388	610	1/4	3/4
TC72546	296	-	4/4
TC71536	575	-	5/5
TC72266	343	-	4/4
TC74813	280	-	2/2
TC64951	675	3/3	-
TC73661	617	1/3	2/3
TC73656	615	-	3/3
TC64827	350	-	3/3
TC65527	202	-	3/3
TC73785	147	-	3/3
TC73242	387	-	3/3
TC64807	632	-	3/3
TC64793	611	1/3	2/3
TC74270	300	-	2/2
TC65745	463	-	2/2
TC67236	385	-	2/2
TC74867	221	-	2/2
TC66251	251	-	2/2
TC67552	434	-	2/2
TC67547	611	-	2/2
TC67067	371	-	2/2

MTGI-TC	bp	N_{MtAmp}	N_{MtGim}
TC67791	408	-	2/2
TC65735	411	-	2/2
TC75233	517	2/2	-
TC67349	377	-	2/2
TC75302	497	-	2/2
TC66690	570	-	2/2
TC65891	633	-	2/2
TC75970	428	-	2/2
TC74472	655	-	2/2
TC74247	460	-	2/2
TC67260	586	-	2/2
TC75969	802	-	2/2
TC74463	296	-	2/2
TC74186	487	1/2	1/2
TC65823	493	-	2/2
TC73954	304	-	2/3
TC67344	582	-	2/2
TC65995	541	-	2/2
TC74126	418	-	2/2
TC75555	594	-	2/2
TC67674	631	-	2/2
TC65854	374	-	2/2
TC70769	667	-	7/7
TC75097	612	1/2	1/2
TC75784	428	2/2	-
TC66878	601	-	2/2
TC75914	368	-	2/2

Table 5: MtGim-/MtAmp-specific TCs without significant homologies

MtGI depicts the *Medicago truncatula* gene index, N the number of the expressed sequence tags in a library.

The remaining group of 53 TCs displayed significant homologies to plant genes (Table 6). Twenty of these were homologous to proteins of unknown function of other plants. Thus, these proteins probably encode *M. truncatula* proteins but predictions of their putative

functions are not possible. Finally 33 revealed highest homologies to plant genes with assigned functions. Of those, 5 encoded putative transporter proteins, four encoded lectin-like proteins and 6 gene products involved in signal transduction.

MtGI-TC	bp	Annotation	GenBank Entry	E-value	N_{MtAm}	N_{MtGim}
signal transduction						
TC64428	494	NBS-LRR type disease resistance protein (*Phaseolus vulgaris*)	Q93W58	$3e^{-17}$	-	3/3
TC66505	491	lectin-like protein kinase (*Arabidopsis thaliana*)	Q9FG33	9e-^{42}	-	2/2
TC74096	615	receptor like protein (*A. thaliana*)	T49121	$6e^{-56}$	2/2	-
TC74325	1046	seven transmembrane protein MLO2 (*Oryza sativa*)	Q93XC8	e^{-123}	1/2	1/2
TC76060	625	serine protein kinase-like (*A. thaliana*)	T47560	$6e^{-20}$	-	2/2
TC71483	1236	protein kinase (*A. thaliana*)	G96593	$2e^{-74}$	-	5/5
metabolism						
TC65799	909	dihydrodipicolinate synthase (*Glycine max*)	S50750	$2e^{-94}$	1/2	1/2
TC67209	591	phosphoenolpyruvate carboxylase (*A. thaliana*)	Q9CA39	$4e^{-76}$	-	2/2
TC74500	206	beta-galactosidase (Lactase) (*F. ananassa*)	Q93X57	$3e^{-19}$	-	2/2
TC67079	555	epoxide hydrolase homolog (*G. max*)	Q39856	$2e^{-72}$	-	2/2
TC76132	438	blue copper-binding protein (*O. sativa*)	AAO37971	$1e^{-21}$	-	2/2
TC60541	960	germin-like protein (*Medicago truncatula*)	AAO32795	$1e^{-72}$	-	4/4
TC63954	473	germin-like protein (*A. thaliana*)	Q9FMA8	$1e^{-32}$	-	4/4
post-transcriptional modification/ protein processing						
TC68426	519	serin carboxypeptidase-like protein (*A. thaliana*)	T49188	$7e^{-43}$	-	2/2
TC66580	596	cysteine proteinase precursor (*Astragalus sinicus*)	Q9FS02	$7e^{-71}$	-	2/2
TC75569	526	prolyl endopeptidase (*A. thaliana*)	Q8RXQ7	$1e^{-55}$	-	2/2
TC71486	643	phosphoprotein phosphatase (*Medicago sativa*)	T09544	$1e^{-86}$	-	4/5
TC60540	665	ribonuclease T2 (*Cicer arietinum*)	Q9ZQX1	$2e^{-13}$	-	2/2
cell structure and extracellular space						
TC68166	1373	agglutinin II precursor (*C. lutea*)	Q39529	$4e^{-54}$	-	29/29
TC69333	1033	bark lectin II precursor (*Sophora japonica*)	P93536	$5e^{-49}$	-	6/6
TC75254	606	agglutinin II precursor (*C. lutea*)	Q39529	$3e^{-23}$	-	2/2
TC75360	599	lectin (*S. japonica*)	P93536	$8e^{-37}$	-	2/2
transport						
TC70909	672	MDR-like ABC transporter (*O. sativa*)	Q8GU68	$7e^{-46}$	-	7/7
TC63901	950	copper transporter (*A. thaliana*)	AAM67571	$1e^{-17}$	-	4/4
TC66155	242	Zn transporter ZNT4 (*T. caerulescens*)	Q9FPW7	$4e^{-15}$	-	2/2
TC73332	405	ammonium transporter (*O. sativa*)	BAC65232	$1e^{-12}$	-	3/3
TC65476	552	sugar transporter (*A. thaliana*)	AAM78192	$1e^{-65}$	-	3/3

MtGI-TC	bp	Annotation	GenBank Entry	E-value	N_{MtAm}	N_{MtGim}
unclassified, function unknown						
TC72502	439	nodulin-like protein (*A. thaliana*)	Q9FHJ9	$1e^{-18}$	-	4/4
TC67410	536	RING zinc finger protein (*A. thaliana*)	Q9M8K4	$3e^{-21}$	2/2	-
TC76092	337	myotubularin (*A. thaliana*)	Q9CAF1	$3e^{-20}$	2/2	-
TC59827	722	cytokinin-specific binding protein (*Vigna radiata*)	Q9ZWP8	$1e^{-31}$	1/6	5/6
TC64306	963	vesicle-associated membrane protein (*A. thaliana*)	O23429	e^{-109}	1/3	2/3
TC67060	708	mitochondrial inner membrane protein (*A. thaliana*)	Q9S724	$1e^{-52}$	2/2	-
hypothetical, unknown proteins						
TC71232	497	hypothetical protein (*O. sativa)*	AAP03413	$2e^{-43}$	-	6/6
TC59539	958	hypothetical protein (*O. sativa)*	AAO39879	6e-36	-	7/7
TC63326	715	hypothetical protein OSJNBb0016H12.7 (*O. sativa)*	AAP03413	$7e^{-66}$	-	5/5
TC63653	614	hypothetical protein (*O. sativa)*	Q8H7L0	$3e^{-32}$	-	4/4
TC73773	539	hypothetical protein (*Plasmodium falciparum*)	Q8IEM0	$4e^{-15}$	-	3/3
TC64871	646	hypothetical protein (*A. thaliana*)	Q8LCS0	$9e^{-35}$	3/3	-
TC73566	658	hypothetical protein F12B17.70 (*A. thaliana*)	F12B17.70	$3e^{-75}$	3/3	-
TC64610	298	hypothetical protein (*A. thaliana*)	T20K12.120	$6e^{-11}$	-	3/3
TC73173	672	hypothetical protein [imported] (*A. thaliana*)	At2g34680	$8e^{-56}$	-	3/3
TC64788	515	OSJNBa0014K08.26 protein (*O. sativa*)	Q8LIY8	$2e^{-40}$	-	2/2
TC67169	629	hypothetical protein (*A. thaliana*)	At1g10490	$6e^{-26}$	-	2/2
TC66818	655	hypothetical protein F9N12.8 (*A. thaliana*)	F9N12.8	$2e^{-36}$	-	2/2
TC65882	504	hypothetical protein (*A. thaliana*)	T16L1.40	$5e^{-19}$	-	2/2
TC63150	497	hypothetical protein (*A. thaliana*)	F14L2.70	$3e^{-19}$	2/2	-
TC66584	516	hypotetical P0501G01.24 protein (*O. sativa)*	Q9AX93	$2e^{-59}$	-	2/2
TC67556	725	Gb|AAC80617.1 (*A. thaliana*)	Q9FFD5	$7e^{-16}$	2/2	-
TC75304	645	unknown protein (*A. thaliana*)	AY088569.1	$2e^{-90}$	2/2	-
TC66240	450	hypothetical protein (*A. thaliana*)	At2g38010	$3e^{-39}$	-	2/2
TC66672	627	hypothetical 71.0 kDa protein	Q8W4D0	$1e^{-53}$	-	2/2
TC75639	723	hypothetical protein (*A. thaliana*)	At2g46080	$1e^{-71}$	2/2	-

Table 6: MtGim-/MtAmp-specific TCs with homologies to plant sequences

MtGI depicts the *Medicago truncatula* gene index, N the number of the expressed sequence tags in a library.

3.2.3 RNA accumulation studies

All 33 TC sequences with significant similarities to plant genes were selected for experimental expression profiling using quantitative real-time RT-PCR. For this approach, *M. truncatula* plants were inoculated with *Glomus intraradices*. Two independent sets of plants were grown and used as biological replicates in RNA accumulation analyses. Mycorrhiza parameters of this plant sets were as follows: for set 1: F: 100 %; M: 34.73 %, m: 34.73 %, a: 64.42 %, A: 23.42 % and for set 2: F: 77.78 %; M: 40.56 %, m: 52.14 %; a: 61.92 %, A: 25.11 %.

The *MtEf-1α* gene encoding the constitutively expressed translation elongation factor 1 alpha was chosen for normalisation. Specific primer pairs were designed for each gene and the selected primer sequences were compared to the TIGR *Medicago truncatula* Gene Index to verify specificity. All primers were used first to amplify genomic DNA of *M. truncatula*. Amplification products were obtained for all genes except TC60540 and TC67079 (data not shown), proving that at least the remaining 31 genes originated from *M. truncatula* and not from the microsymbiont. Prior to RNA-accumulation analyses, all RNA preparations were checked for DNA contaminations by PCR using primer combinations that span intron sequences of known gene sequences (data not shown).

MtEf-1α was found to be the most abundant gene showing a constant expression in control and mycorrhizal roots. After RT-PCR, melting curves were analysed and in all reactions, single amplification products were present. Gene induction levels calculated on the basis of real-time RT-PCR are shown in table 6. Of the 33 TCs analysed for AM-induced expression, 18 were verified to be at least three fold induced in both samples of mycorrhizal roots. Remarkably, the AM-specific phosphate transporter gene *MtPT*4 was more than 10^4 fold induced in both sets of mycorrhizal roots. This corresponds well to the detection of 53 *MtPT*4 ESTs in the MtGim library, in contrast to only 6 *MtPT*4 ESTs identified in the random cDNA

libraries MtBC and MHAM (TIGR MtGI) and indicates that the MtGim cDNA collection represents a resource for novel mycorrhiza-induced genes that are summarised in table 7.

MtGI-TC	Induction level biological replicate 1	Induction level biological replicate 2	Annotation[a]
signal transduction			
TC64428	10.7	59.2	NBS-LRR type disease resistance protein
TC66505	3.1	15.8	lectin like protein kinase
TC74096	1.1	3.1	receptor like protein
TC74325	3.6	6.1	seven transmembrane protein MLO2[a]
TC76060	n.d.	0.4	serine protein kinase like
TC71483	n.d.	267.9	probable protein kinase
metabolism			
TC65799	12.4	21.0	dihydrodipicolinate synthase
TC67209	0.6	2.0	phosphoenolpyruvatcarboxylase
TC74500	3.9	18.8	beta-galactosidase
TC67079	5.3	18.7	epoxide hydrolase homolgue
TC76132	24505.2	69515.6	blue copper binding protein
post transcriptional modification/ protein processing			
TC68426	42.5	485.0	serin carboxypeptidase like protein[3]
TC66580	9.1	1681.3	cystein proteinase precursor
TC75569	∞	n.d.	prolyl endopeptidase
TC71486	0.6	1.8	phosphoprotein phosphatase
cell wall and extracellular space			
TC75254	∞	∞	agglutinin II precursor[2]
TC68166	∞	1586.2	agglutinin II precursor[2]
TC75360	139.8	114.6	lectin[2]
TC69333	7482.2	7477.6	bark lectin II precursor[2]
TC60541	5971.8	8699.7	germin-like protein[2, 4]
TC63954	80.6	115.2	germin-like protein[2, 4]
transport			
TC70909	n.d.	46.1	MDR-like ABC-transporter
TC63901	56.3	164.4	copper transporter
TC66155	0.4	n.d.	zinc transporter
TC73332	130.3	450.0	ammonium transporter
TC65476	5.4	16.3	sugar transporter
unclassified/ unknown function			
TC72502	1.1	7.0	nodulin-like protein
TC67410	0.6	2.4	RING zinc finger protein

MtGI-TC	Induction level biological replicate 1	Induction level biological replicate 2	Annotation[a]
TC76092	1.5	2.4	myotubularin
TC59827	1.0	2.9	cytokinin-specific binding protein
TC64306	0.8	4.0	vesicle-associated membrane protein
TC67060	1.3	1.7	mitochondrial inner membrane protein

Table 7: Induction levels of genes represented by MtGim-/MtAmp-specific TCs

MtGI depicts the *Medicago truncatula* gene index, N the number of the expressed sequence tags in a library. n.d.: no products detected using cDNA of non-mycorrhizal or mycorrhizal roots

∞: no PCR products detectable after amplification of cDNA deriving from non-mycorrhiza roots, PCR products were obtained after amplification of cDNA of mycorrhizal roots

[a]Similarities to previously described arbuscular mycorrhizal-induced genes: [1]Manthey *et al.*, 2004, [2]Wulf *et al.*, 2003; [3]Liu *et al.*, 2003, [4]Doll *et al.*, 2003

3.2.4 A family of *M. truncatula* lectin genes is induced during arbuscular mycorrhiza

Notably, four novel lectin-like sequences, also referred to as agglutinins, were strongly induced in mycorrhizal roots (table 6). Previous reports on *M. truncatula* lectin genes (*MtLec*1-4) were focused on genes induced during root nodule development and nitrogen fixation (Bauchrowitz *et al.* 1996; Mitra and Long 2004). During the initial characterisation of the MtGim-library (Wulf *et al.* 2003), three AM-induced lectins represented by TIGR TC69503 (*MtLec*5), 69334 (*MtLec*6) and 69628 (*MtLec*7) were identified. In the approach presented here, four novel AM-specific lectin-like sequences were found, represented by TIGR TC68166 (*MtLec*8), 69333 (*MtLec*9), 75254 (*MtLec*10) and 75360 (*MtLec*11). Hence, seven different AM-induced lectin-sequences have been identified in *M. truncatula* up to now.

3.3 Sequence analyses of the AM-specific lectins

Rapid amplification of cDNA ends (RACEs) were done in order to identify the full size cDNA sequences of these seven genes. Resulting sequences were between 1027 and 1190 bp long. Nucleotide sequences of the *MtLec*5-11 genes have been submitted to the GenBank database with the consecutive accession numbers DQ314207 to DQ314213. Largest open reading frames indicate proteins lengths between 267 and 281 amino acids, except for the *MtLec*10 sequence. For this gene, a cDNA sequence of 1125 bp was cloned, but the predictable open reading frame consists only of 339 bp and aborts due to two directly consecutive stop codons (figure 4). Translation of this open reading frame would consequently lead to a truncated protein of 113 amino acids. ClustalW analysis of the known *M. truncatula* lectins reveals that MtLEC10 aligns to the C-terminal ends of other known lectins indicating that the N-terminal part of *MtLec*10 appears to be missing.

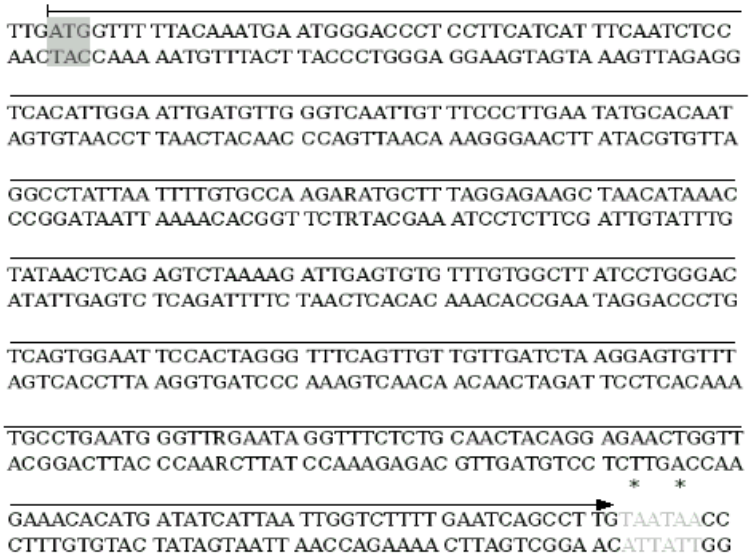

Figure 4: Section of *MtLec*10 cDNA sequence

*MtLec*10 consists of 1125 bp cDNA sequence. Longest open reading frame of 339 bp is indicated by an arrow. Putative start codon is indicated by grey box. ORF is aborted by two consecutive stop codons, indicated by asterisks and highlighted letters.

In Clustal W analysis the remaining six lectin-like proteins showed similarities from 38 % (*MtLec*7 / *MtLec*11) to 92 % (*MtLec*6 / *MtLec*9) to each other (figure 5).

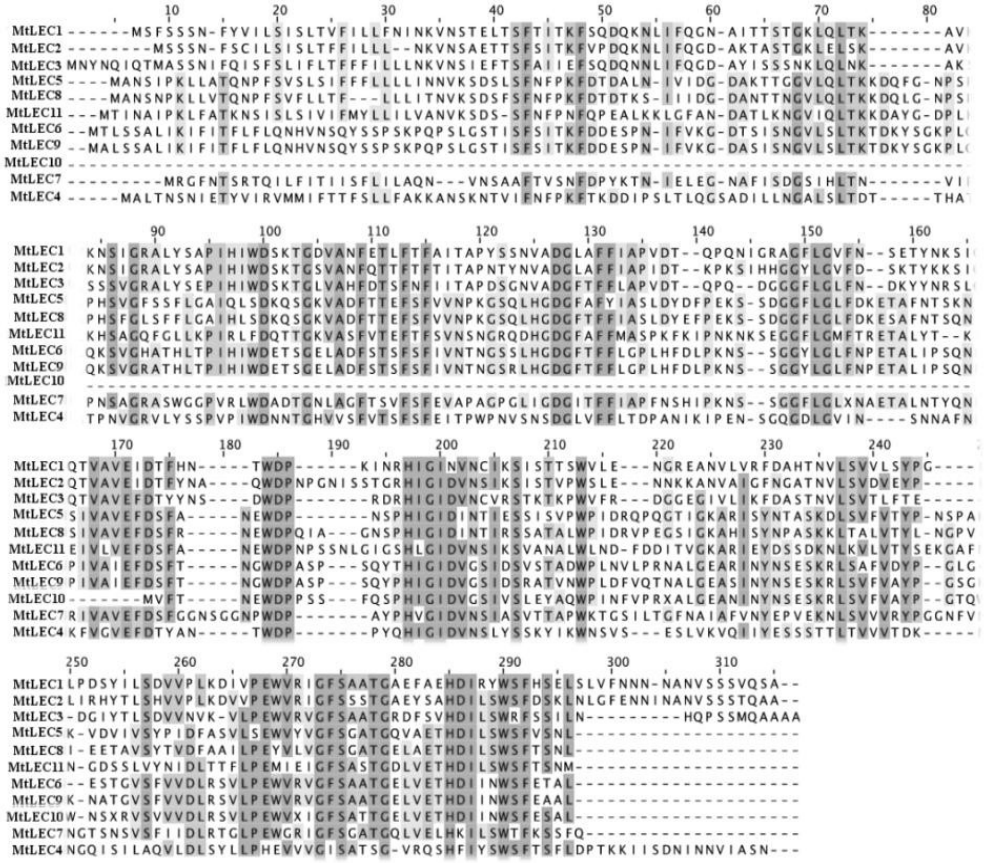

Figure 5: Multiple alignment of *M. truncatula* lectins

Boxes indicate conserved positions amongst the sequences (at least 6 sequences show identical amino acids at corresponding positions). The alignment of the deduced *M. truncatula* lectin protein sequences reveals that MtLEC10*,* consisting of only 113 amino acids, corresponds to the C-terminal ends of the other lectins. This is quite obvious the region of high similarity between all sequences. The N-terminal part of MtLEC10 seems to be missing as its first methionine, encoded by the ATG start codon, aligns to the 172nd amino acid of the deduced consensus sequence. The remaining six mycorrhiza-specific lectins show identities between 38 % and 92 % to each other. Numbers indicate positions to a putative consensus sequence.

Multiple alignment was used to draw a phylogenetic tree. Additionally an another nodule specific lectin-like sequence (TC78397) identified from the TIGR MtGI by *in silico* expression profiling also was also included in this analysis. This revealed that AM-induced lectin-sequences group in one branch when compared with the lectin-like proteins induced during the root nodule symbiosis (figure 6).

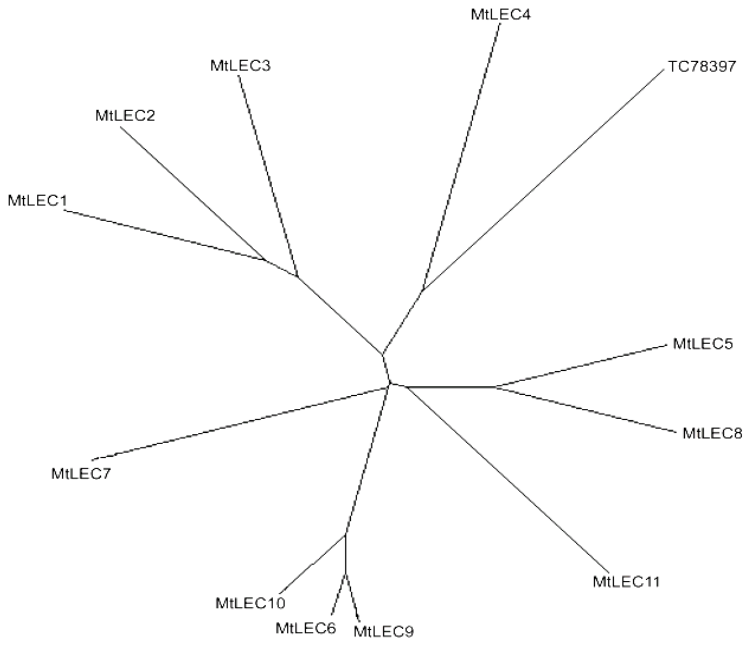

Figure 6: Unrooted tree based on ClustalW alignment

The following sequences were included, if available GenBank or otherwise MtGI accession numbers are given in brackets: *MtLec*1 (X60386), *MtLec*2 (X60387), *MtLec*3 (X82216), *MtLec*4 (TC77066), *MtLec5* (DQ314207), *MtLec*6 (DQ314208), *MtLec*7 (DQ314209), *MtLec*8 (DQ314210), *MtLec*9 (DQ314211), *MtLec*10 (DQ314212), *MtLec*11 (DQ314213) and the nodule-specific lectin-like sequence encoded by TC78397; AM-specific lectins form a unique branch of the phylogenetic tree, whereas MtLEC4, which is known to be regulated during root-nodule symbiosis (Mitra and Long, 2004), shows highest similarity to TC78397, which codes for another nodule specific lectin-like sequence.

In order to identify the genomic structure of these genes, specific primer pairs for *MtLec*6,

*MtLec*8, *MtLec*9 and *MtLec*10 were deduced, that span the whole cDNA sequence of the corresponding genes. Cloning of the sequences of the AM-specific lectin-like protein genes from genomic DNA and sequencing revealed that none of these genes contains an intron. At the time oint of this analysis, genomic sequences of *MtLec*5, *MtLec*7 and *MtLec*11 have been already deposited in GenBank database and contained also no introns.

3.4 Targeting of AM-specific MtLEC5 protein

3.4.1 MtLEC5 is predicted to be targeted at the vacuole

Up to now, nothing is known about the function of the identified mycorrhiza-specific lectin genes. Therefore targeting of the mycorrhiza specific lectins was investigated. The targetP software tool is able to predict putative targeting of a protein sequence and provides an additional module for the calculation of possible signal peptides. Using this tool, an N-terminal signal peptide of 34 amino acids was predicted for MtLEC5 with a reliability of over 90 % (table 8, reliability class 1). This leader peptide is likely to be cleaved and the mature protein is targeted into the secretory pathway. Similar results were obtained for all other lectins, except for MtLEC10, which consists of a truncated open reading frame only.

Name	Len	cTP	mTP	SP	other	Loc	RC	TPlen
MtLEC1	277	0.047	0.011	0.983	0.024	S	1	30
MtLEC2	280	0.104	0.002	0.985	0.030	S	1	26
MtLEC3	273	0.010	0.036	0.921	0.150	S	2	37
MtLEC4	278	0.007	0.055	0.666	0.427	S	4	32
MtLEC5	274	0.045	0.025	0.919	0.076	S	1	34
MtLEC6	278	0.335	0.066	0.697	0.019	S	4	23

Name	Len	cTP	mTP	SP	other	Loc	RC	TPlen
MtLEC7	267	0.004	0.080	0.970	0.031	S	1	28
MtLEC8	275	0.004	0.138	0.916	0.120	S	2	31
MtLEC9	279	0.356	0.059	0.686	0.019	S	4	23
MtLEC10	113	0.218	0.131	0.035	0.774	-	3	-
MtLEC11	281	0.017	0.040	0.956	0.093	S	1	35

Table 8: TargetP results of MtLEC1 - MtLEC11

TargetP computational analysis indicates for all analysed lectin amino acid sequences, except the truncated MtLEC10 sequence, a probable location in the secretory pathway (SP, Loc). Putative cleavage sites for the signal peptides are given (TPlen). Len = length of the protein, cTP = score of a localisation in the chloroplast, mTP = score of a mitochondrial localisation, SP = score of a targeting to the secretory pathway, other = score of an other localisation, Loc = prediction of localisation, RC = reliability class, TPlen = predicted presequence length

3.4.2 Subcellular localisation of MtLEC5 using confocal laser scanning microscopy

To prove the *in silico* analyses, confocal laser scanning microscopy (CLSM) was used. For this purpose different fusions with a gene coding for a green fluorescent protein (*mgfp6,* Schuldt *et al.*, 1998) were constructed (figure 7 a): The complete open reading frame of *MtLec*5 was cloned in an N-terminal position in frame to *mgfp6* under the control of either a double 35S promoter or the formerly described native *MtLec*5 promoter (Frenzel *et al*., 2005, chapter 3.5). TargetP software predicted a putative signal peptide of 34 amino acids at the N-terminal site. To analyse if this presequence influences the targeting of the fusion-protein, a deletion construct was used with a truncated *MtLec*5 open reading frame missing the first 34 amino acids. As a cytoplasmatic control a fusion-less GFP5 under the control of a double 35S promoter was used. Constructs were transferred to *Agrobacterium rhizogenes* strain *ARqua1*

and *M. truncatula* roots were transformed producing transgenic hairy roots. Plants containing the fusion-protein under the control of the native *MtLec*5 promoter were mycorrhized as described for three weeks.

Expression of the GFP fusion proteins was first visualised using a binocular featuring a special GFP-Filter. Expression efficiency – estimated by fluorescing lateral roots – varied between 10-30 % of all lateral roots. Successful transformed roots exhibited a strong fluorescence in the transgenic roots (Figure 7 b). These roots were cut and used for confocal laser scanning microscopy. When investigating the subcellular localisation, the cytoplasmatic localised GFP appeared as a fluorescing seam surrounding the cell lumen, whereas inside the cells no GFP-expression could be observed (figure 7 b, I). In contrast, in roots transformed with the MtLEC5-GFP fusion constructs fluorescence occurs in the inner part of the cell (figure 7 b, II). Remarkably, the promoter used for the expression of the respective construct had no impact on the targeting of the fusion protein, as fluorescence appeared in the same pattern independently if expresson was controlled by the 35S or the native *MtLec*5 promoter (figure 7 b, III). When deleting the predicted signal peptide the expression pattern changed to the one of the cytoplasmatic targeted phenotype (figure 7b, IV).

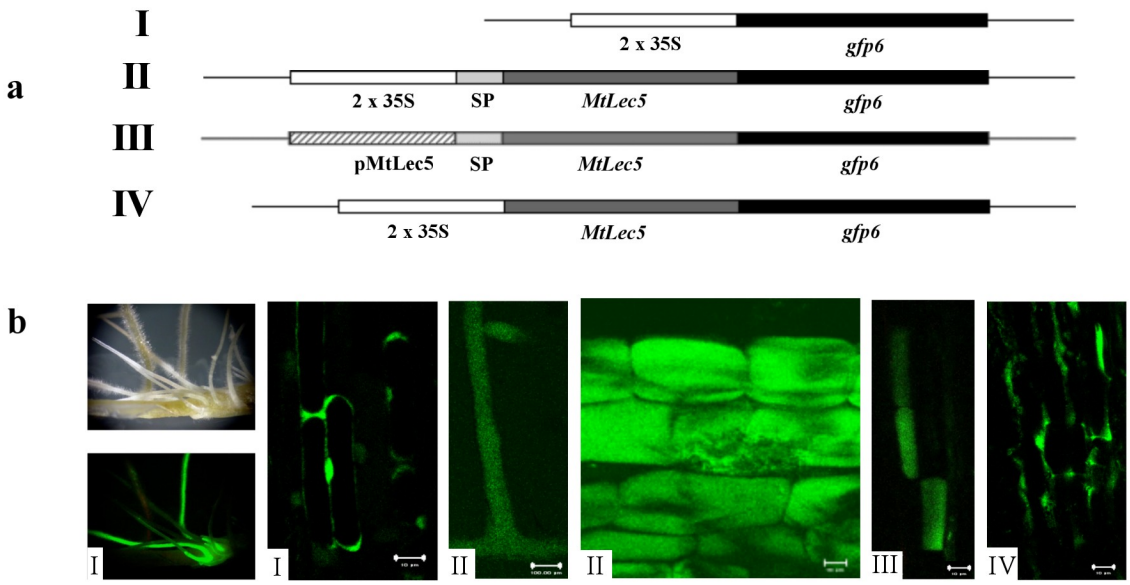

Figure 7: Localisation of MtLEC5 in transformed *M. truncatula* hairy roots

a: Schematical representation of vector constructs used for MtLEC5 subcellular localisation. Four constructs were used to investigate subcellular localisation of *MtLec*5: white box, double 35S promoter, light grey, predicted signal peptide (SP), dark grey, *MtLec5* gene without predicted leader sequence, black, *gfp6* gene, striped, p*MtLec*5(-1037/+23)

b: Localisation studies in transgenic roots.

I, left: Bright field image of *M. truncatula* hairy roots transformed with the double 35S::GFP construct (upper micrograph) and fluorescence image of the same root (lower micrograph). Transgenic roots carrying the vector construct show a bright fluorescence. I, right: CLSM images of roots expressing the 2x 35S::GFP construct. Expression of the GFP is mostly abundant in the periphery of the cells. II, left: root hair, and right: root cortex cells expressing the MtLEC5::GFP fusion protein. Fluorescence can be observed inside the cell. III: CLSM image of roots, transformed with a MtLEC5::GFP fusion construct under the control of the *MtLec*5 promoter after mycorrhization. Mycorrhization does not affect the targeting of this fusion-protein as fluorescence is visible in the same pattern as in II. VI: Root cortex cells expressing the truncated *MtLec*5 protein without the predicted leader peptide fused to GFP. Expression of the chimeric protein appears in the cytoplasm.

Scale bars indicate 10 µm for I right picture, II right picture, III and IV, and 100 µm for II left picture

3.5 Promoter analyses

3.5.1 Analysis of mycorrhiza-specific transcriptional regulation

Reporter gene anlayses have widely been used to unravel specific transcription activation. They provide the first step to identify *cis*-regulatory elements in the promoters and subsequently the corresponding transcription factors. The specificity of the transcriptional activation of several genes during the AM leads to the hypothesis of mycorrhiza specific regulatory elements and putative transcription factors. Few AM-specific genes have been used for promoter analysis. Therefore additional data can lead to the identification of these elements.

The 5' upstream region of *MtLec*5 (*pMtLec*5) has been isolated from BAC clone mth2-20e4 (GenBank accession no AC126010). For p*MtLec*7 inverse PCR technique was used. Cursory sequence analysis revealed in the *MtLec*5 promoter a consensus sequence to a TATA-box from -33 to -26 bp from the transcription start (TATATAT). No TATA-box sequence could be identified in the *MtLec*7 promoter. In initial analyses 1,060 bp of p*MtLec*5 (p*MtLec*5(-1037/+23), in respect to the transcription start) and 1,503 bp of p*MtLec*7 (p*MtLec*7(-1451/+52)) were fused to a *gus* reporter gene. Thereby the 5' untranslated regions (UTR) were included in the analyses. Binary vectors were transferred to *Agrobacterium rhizogenes* and *M. truncatula* transgenic roots were induced by transformation. After transgenic roots formation plants were inoculated as described and expression of the β-glucuronidase was visualised by histochemical staining. Finally, fungal structures were stained. No reporter gene activity could be observed in roots of non-mycorrhizal plants (data not shown). However both promoters showed strong activity in transgenic roots colonised by the AM fungus *G. intraradices* (figure 8).

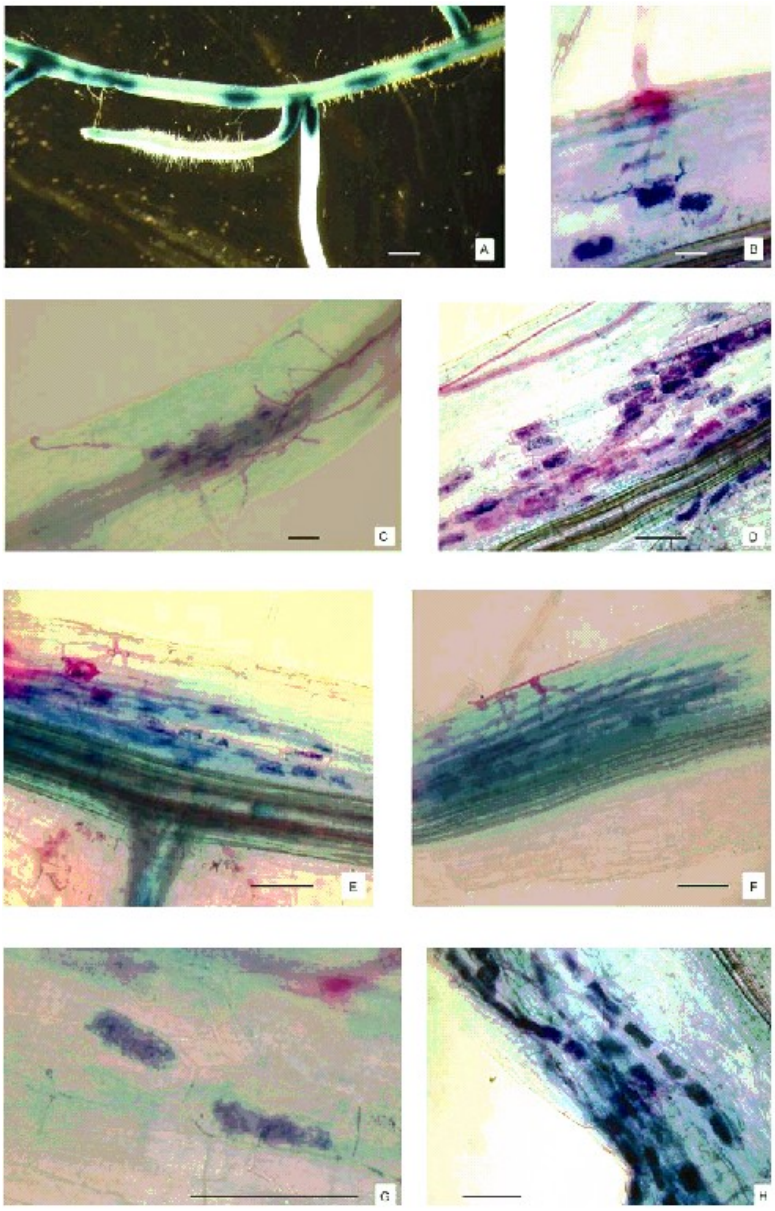

Figure 8: Promoter activities in *Glomus intraradices*-colonised roots

Histochemical analysis of transgenic *Medicago truncatula* roots carrying A, B, C and D the p*MtLec*5 GUS or E, F, G and H p*MtLec*7 GUS fusions. *M. truncatula* plants with *Agrobacterium rhizogenes* transformed hairy roots were colonised by *G. intraradices* and stained for GUS activity after 3 weeks of inoculation. B through H, double stainings for GUS activity and subsequent acid fuchsin staining to detect the fungus. A, Intense *MtLec*5 promoter activity is detectable in parts colonised by the AM fungus (magnification bar: 2mm). B and D, arbuscules showing intense *MtLec*5 promoter acitivity (magnification bars: 10 and 25 µm, respectively). C, p*MtLec*5 activity is localised in inner cortical cell layers where arbuscules have been formed; no GUS activity can be detected at the root surface after contact

with *G. intraradices* hyphae (magnification bar: 20 µm). No p*MtLec*7 activity was observed in the vicinity of vesicles (E, magnification bar: 25 µm) or appressoria (F, magnification bar: 50 µm); in contrast, p*MtLec*7 is highly active in arbuscule containing cells (G and H, magnification bars: 25 µm).

3.5.2 Analysis of mycorrhiza-specific transcriptional regulation: promoter-deletion analyses

Initial analyses of the upstream sequences of p*MtLec*5 and p*MtLec*7 showed arbuscule specific reporter gene expression when using large promoter fragments of 1000 bp and more (Frenzel *et al.*, 2005). Therefore the promoters of these two genes were selected for more detailed promoter analyses. Successive shortening of the promoter fragments should lead to an alteration of transcription pattern of the reporter gene when a *cis*-regulatory element is deleted.

In order to identify the minimum promoter, which is sufficient to mediate the mycorrhiza specific expression, different *MtLec*5 and *MtLec*7 promoter-*uidA* fusions were constructed: Approximately 300 bp, 150 bp and 75 bp of the *MtLec*5 promoter sequences were used in the promoter deletion analyses. For *MtLec*7, beside p*MtLec*7(-304/+52), p*MtLec*7(-150/+52) and p*MtLec*7(-75/+52) an additional p*MtLec*7(986/+52) construct was analysed in these gain of function analyses (figure 9 b).

After induction of *M. truncatula* hairy roots via *A. rhizogenes* mediated transformation and mycorrhization, 301 bp of p*MtLec*5 were able to activate transcription of the reporter gene, visualised by β-glucuronidase activity. Similar expression pattern of the GUS gene was observed when compared to the approximately 1 kb fragment (figure 9, c I, d I). When using 150 or 75 bp of p*MtLec*5 promoter activity breaks down to a zero level. No activation of the promoter could be observed in non-mycorrhizal transgenic roots at all (data not shown).

Therefore, assumed regulatory promoter motifs appear to be located in the region between -300 and -150 bp from the transcription start.

In β-glucuronidase assays of transgenic roots 986 and 304 bp of the *MtLec*7 promoter were sufficient to enable a strong reporter gene expression (figure 9 c II). Activity appeared specifically in arbuscule containing cells as revealed by subsequent double staining with acid fuchsin (figure 9 d II). In *MtLec*7 promoter-reporter gene containing roots GUS expression appears to be restricted to the arbuscule containing cells, as was observed identically using the previously described long promoter fragments of 1kb and more. No reporter gene expression could be observed using the 150 bp and 75 bp promoter-*uidA* containing transgenic roots or in non-mycorrhizal roots (data not shown). Consequently it appears, that the promoter of *MtLec*7 seems to contain AM-responsive elements located in the p*MtLec*7(-304/-150) area (figure 9a).

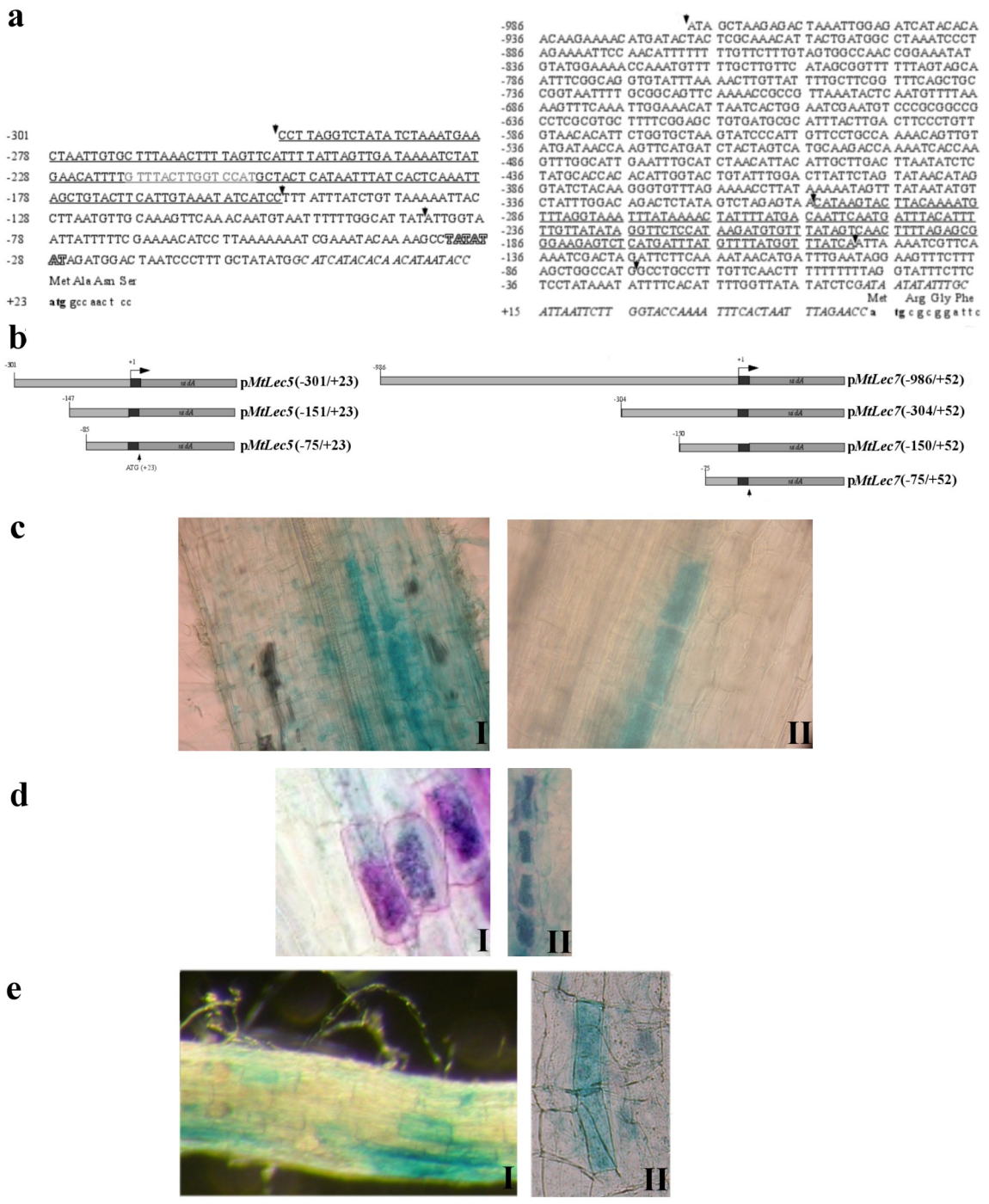

Figure 9: Promoter-reporter gene analyses in transgenic *M. truncatula* roots

a: Nucleotide sequences of the *MtLec*5 and *MtLec*7 promoter regions. Indicated positions are related to the transcription start (+1). Arrows indicate sites of the deletions used in reporter gene analyses. TATA-box consensus sequence of the *MtLec*5 promoter is given in contour, 5' untranslated regions (5' UTR) are in italics. Putative AM-specific responsive elements containing sequences are underlined.

b: Diagram of various promoter deletion fragments used in deletion analyses. Promoter lengths are given in distance from the transcription start (position +1). Black boxes indicate 5' UTRs.

c: *M. truncatula* roots were transformed and subsequently inoculated with *Glomus intraradices*. Three weeks after inoculation GUS staining was performed.

I: transgenic roots transformed with a construct of 301 bp of the *MtLec*5 promoter fused to a *uidA* gene. II p*MtLec*7(-304/+52) fused to the *uidA* gene. *MtLec*5 promoter activity is detectable in cells in the inner cortical cell layer colonised by the fungus. Weak GUS staining can be observed in cells adjacent to arbuscule-containing cells. No reporter gene expression appears in the outer cell layers.

d: Staining of fungal structures with acid fuchsin shows that p*MtLec*5 (I) and p*MtLec*7 (II) are mainly active in arbuscule containing cells. (Indicated by the purple colour, which originates from the overlay of the light-blue GUS and the pink acid fuchsin staining.)

e: Hairy roots of *N. tabacum* cv. Samsun carrying the heterologous p*MtLec*5(-1039/+23) promoter-GUS construct. I: Reporter gene expression appears in regions of mycorrhizal colonisation. II: Arbuscule-containing cells are displaying GUS-activity.

3.5.3 p*MtLec*5(-1037/+23) is also active in the non legume *Nicotiana tabacum*

Most terrestrial plants are known to be able to form the AM-symbiosis. As endomycorrhizal structures have been observed in fossils of the earliest plants, it is assumed that signalling events, which lead to the specific transcription of genes may be conserved. Consequently promoters of AM-specific genes would be able to mediate likewise specific activation of reporter genes in only remotely related species.

The arbuscule specific expression of the analysed *MtLec*5-promoter in *M. truncatula* leads to the question whether this transcription activation can also be observed in non-legume plants. Therefore *Nicotiana tabacum* was transformed with *Agrobacterium rhizogenes* producing transgenic roots containing p*MtLec*5(-1037/+23) fused to the *uidA* gene. Composite plants

containing transgenic roots were inoculated with *Glomus intraradices.* In subsequent β-glucuronidase activity stainings, a reporter gene activation pattern identical to *M. truncatula* was observed. Reporter gene expression was restricted to parts of the roots with fungal colonisation (figure 9 e I), and in particular to discrete cells harbouring arbuscules (figure 9 e II). This result indicates, that the observed mechanism of mycorrhiza-specific transcription activation is also conserved in the non-leguminous plant *N. tabacum* and suggests that the notional positive regulator, which mediates this specificity in mycorrhizal roots, represents a conserved phenomenon.

3.5.4 Electrophoretic mobility shift assay reveals potential DNA-binding proteins motives

To specify the binding site of the postulated positive regulator element, the protein binding sites of p*MtLec*5 were analysed by Electrophoretic Mobility Shift Assays (EMSA). As 301 bp of the *MtLec*5 5' upstream region provide AM specific expression of the *uidA* reporter gene and the 151 bp region does not lead to an activation, it is likely that potential transcription-factor binding sites, which are responsible for the specific expression pattern, are located in the *pMtLec*5(-301/-151) region. Therefore, this region was studied in detail and divided into 30 bp fragments with 10 bp overlaps each (figure 10 a). The fragments were labelled and incubated with crude protein extracts from mycorrhizal or non-mycorrhizal *M. truncatula*. A second control binding reaction was performed without any protein to exclude artificial shifts of the gel composition. Figure 10 b shows specific alteration in running of the fragments -301 /-271, -281/-251, -261/-231 and -241/-211. These four fragments show a gel shift after incubation with protein extract from mycorrhizal roots. Remarkably, no specific shifts could be observed in the control experiments after incubation with proteins from non-mycorrhizal

roots, indicating that the observed shifts might result from binding of putative mycorrhiza specific regulators to the analysed promoter fragments.

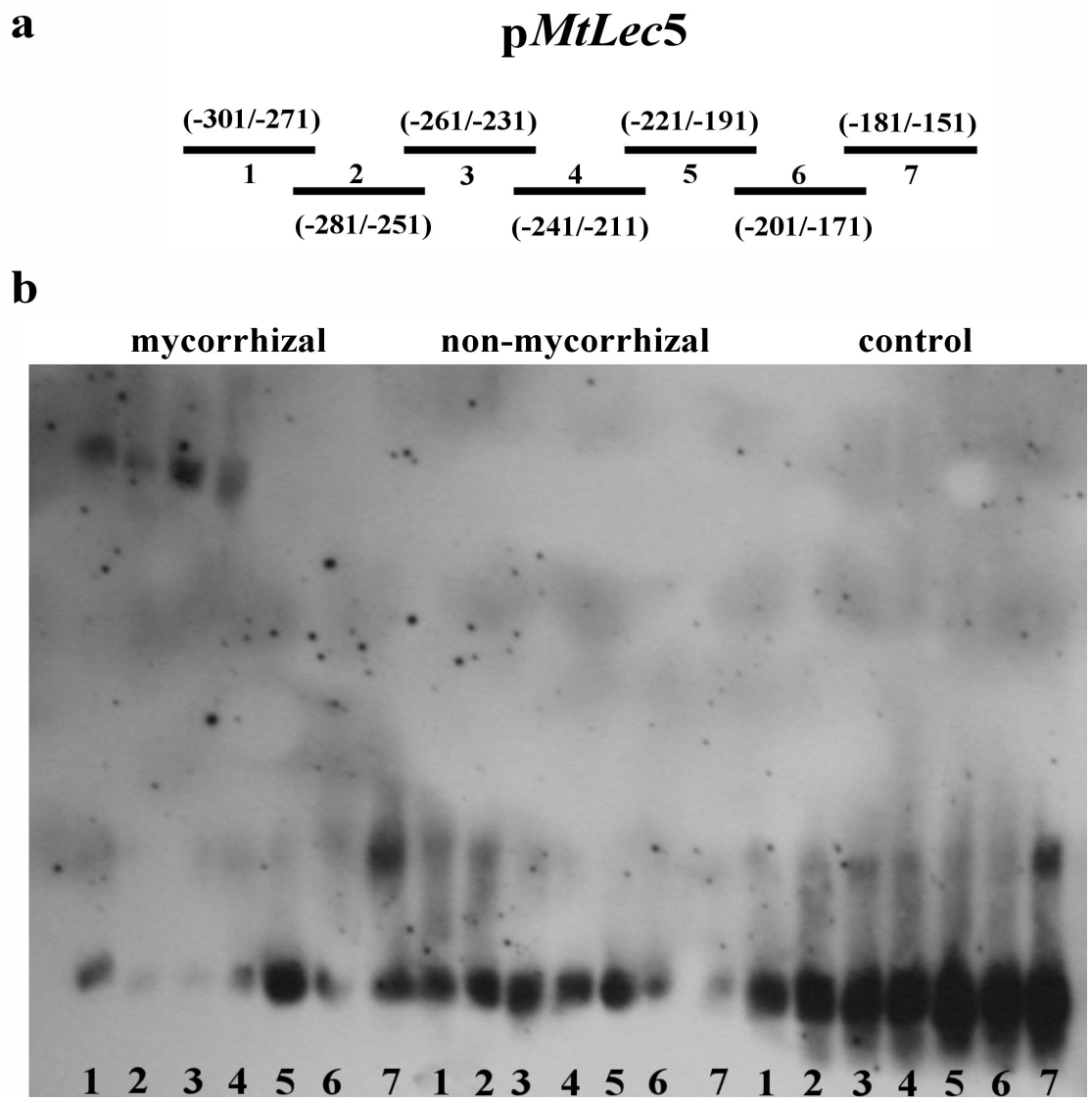

Figure 10: Electrophoretic mobility shift assay (EMSA) of p*MtLec5*(-301/-151)

a. Schematic overview of the partitioning of p*MtLec*5(-301/-151) into 7 segments, that have been tested in EMSA analysis for binding of mycorrhiza specific proteins.

b: p*MtLec*5(-301/-151bp) was divided into seven 30 bp fragments with 10 bp overlaps. EMSA was carried out with protein extract from mycorrhizal or non-mycorrhizal roots and without proteins (control). Fragments 1 to 4 (in total -301 to -211 bp upstream of the transcription start) show a specific shift after incubation with protein extract from mycorrhizal roots. No shifts were detected after incubation with proteins of non-mycorrhizal roots or in the water control experiment. Positions of the analysed promoter fragments in relation to the

transcription start: **1**, -301 to -271bp, **2**, -281 to -251bp, **3**, -261 to -231bp, **4**, -241 to -211bp, **5**, -221 to -191, **6**, -201 to -171bp, **7**, -181 to -151bp

3.5.5 p*MtLec*5 and p*MtLec*7 share common sequence motifs

Similar expression pattern due to the arbuscule specific activity of p*MtLec*5 and p*MtLec*7 suggests that both promoters might share at least one common sequence motif, which might be recognised by the postulated regulatory proteins. First, computational comparison of both sequences was done in order to identify conserved motifs within the promoter fragments. The PromoterWise tool compares two DNA sequences and identifies inversions and translocations using the Wise2 algorithm, because potential promoter motifs may be located in both directions on the upstream regions.

When comparing p*MtLec*5(-301/-211), showing binding of AM specific proteins in EMSA analysis and p*MtLec*7(-304/-150), which mediates arbuscule specific expression in reporter gene fusions, a common 8 bp motif was found. This sequence motif (AAC ATT TT) is located on the plus strand of p*MtLec*5(-230/-223) and on the minus strand of p*MtLec*7(-291/-284). This common sequence shows no homology to any other known regulatory promoter element and may represent a new mycorrhiza specific potential transcription factor binding site.

Comparison of promoter sequences to a database containing already described motifs of plant promoters, the MatInspector tool detects in each promoter a so called circadian clock associated module. A third common promoter motif, a nodulin consensus sequence is abundant twice in p*MtLec*7(-304/-150) and once in p*MtLec*5(-301/-151) with high matches. A summary of the analysed sequences and detected motifs is shown in figure 11.

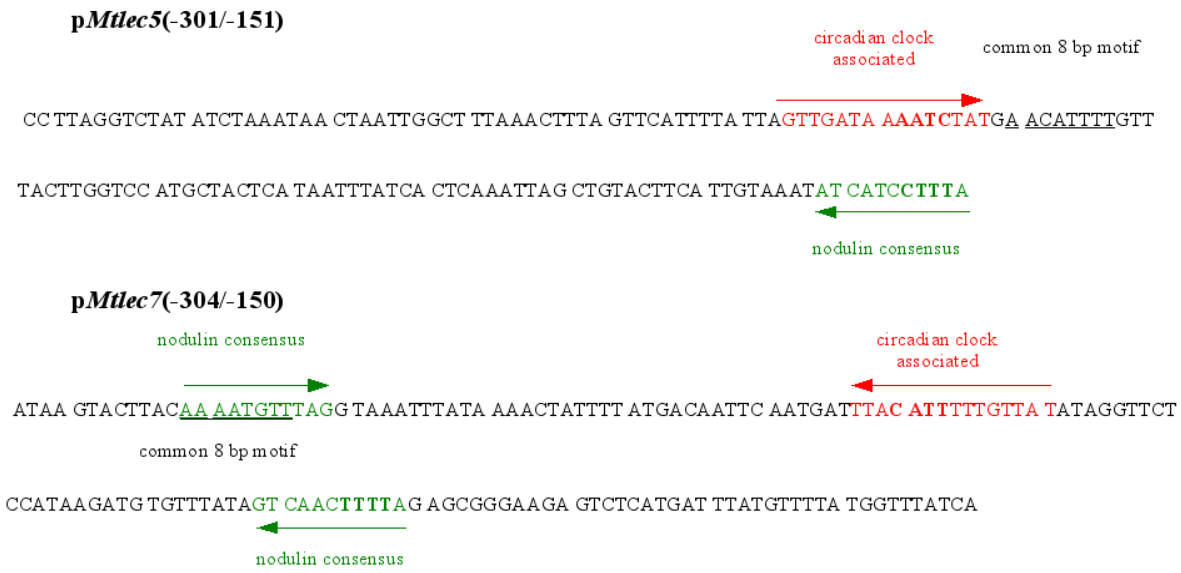

Figure 11: Comparative analyses of p*MtLec*5(-301/-151) and p*MtLec*7(-304/-150) sequences

The promoter regions, identified to mediate the AM-specific transcription, share three common motifs. MatInspector identifies one circadian clock associated module (marked in red) in each of the fragments and one or two nodulin consensus sequences (green) in p*MtLec*5(-301/-151) and p*MtLec*7(-304/-150), respectively. Additionally one common 8bp motif (underlined) is abundant in both sequences which so far has not been described before.

4 Discussion

More than 80 % of all terrestrial plants are able to form a symbiosis with arbuscular mycorrhizal fungi, including several important crops. This symbiosis is characterised by the exchange of carbohydrates from the plant to the fungus, whereas mainly phosphate and other mineral nutrients are transported the other way around. Therefore both partners benefit from this interaction. Additionally, mycorrhizal plants are known to possess higher resistance against several plant pathogens, as well as higher tolerance against heavy metals and increased salt concentrations when associated with additional soil microorganisms such as bacteria (Vivas *et al.*, 2003, Rabie *et al.*, 2005). Consequently, analyses leading to deeper insights of this mutualistic interaction are of high interest.

4.1 Microarray analysis provides the basis for a discussion about initiation of the AM

The identification of novel mycorrhiza-specific genes in host plants represents the first step in understanding the molecular processes during symbiosis. Two different methods have been used to analyse transcriptional changes. Firstly, hybridisation of a cDNA-based microarray was used to identify differences in the transcriptional pattern of a completely developed symbiosis and the early-symbiotic phase. The latter one was characterised by lack of a physical contact in form of appressoria. Expression pattern of several genes in mycorrhizal roots coincides with former analysis, indicating that the correct parameters have been chosen to evaluate the microarray raw data. For example the AM-specific phosphate transporter *MtPT*4 (TC85743, Harrison *et al.*, 2002) and the glutathione S-transferase *MtGst*1 (TC 85868, Wulf *et al.*, 2003) have been found highly induced, whereas the common phosphate transporter *MtPT*1 (TC77186, Liu *et al.*, 1998) was repressed in comparison to non-inoculated plants. Additionally, a global overview has been achieved of up and down-regulated genes in

the early and late symbiotic phase that may lead to new insights in the global expression of genes and provides the starting point to analyse the development of the AM in more detail: In mycorrhizal roots, 21 dpi with AM fungus, up and down-regulated genes are approximately distributed in equal shares, whereas in early-symbiotic roots most affected genes appear to be down-regulated and only few are found induced.

Interestingly, several genes have been observed to be only regulated exclusively when no appressoria have even developed. As these structures are also abundant 21 dpi, it is assumed that the high colonisation intensity influences the neighbouring parts of the roots, so that specifically regulated transcripts can be observed in the 6 dpi roots. For example, one gene, shows high similarity to a cyclic nucleotide and calmodulin-regulated ion channel (TC79605). Calmodulin is a highly conserved calcium-binding protein, which is involved in many calcium-dependent cellular processes. In plants these include phytochrome-regulated gene expression and chloroplast development (Bowler and Chua, 1994), cell division (Vantard *et al.*, 1985), gravitropism (Stinemetz *et al.*, 1987) and microtubule organisation (Cyr, 1991). Molecular processes of the early steps in the two root symbioses are predicted to be conserved as several genes are known to be induced at this time point (Ane *et al.*, 2004, Calantzis *et al.*, 2001, Sagan *et al.*, 1995).

During initiation of the root nodule symbiosis calcium spiking can be observed. Mutant analyses revealed three genes, that control both types of root symbioses (Duc *et al.*, 1989) named *dmi*1 to 3 (does not make infections). In the rhizobial symbiosis this calcium oscillation is found temporally appearing in between the expression of *dmi*2 and 3 referred to as calcium spiking (Erhardt *et al.,* 1996, figure 12). The latter one encodes a calcium/calmodulin dependent protein kinase (Lévy *et al.*, 2004, Mitra *et al.*, 2004) and might react on this event. Consequently, Ca^{2+} flux is anticipated to occur during the initiation of the AM (figure 12). The abundance of an additional potential symbiotic calmodulin dependent

protein supports this hypothesis. Its predicted function as ion transporters suggests that this protein is probably involved in early signalling events.

The down-regulation of an additional gene, showing high similarity to proteins containing a putative calmodulin-binding domain (TC77353 and TC87357), also supports the hypothesis that calcium plays an important role for the initiation of the symbiosis, suggesting an oscillation of this cation comparable to root nodules. TC77353 shows in blastx analysis high similarities to MLO-like proteins that belong to the group of guanine nucleotide-binding protein-coupled receptors. These proteins are known to function as negative regulators of host defence, because loss of a particular MLO protein leads to resistance of *Hordeum vulgare* to powdery mildew (Büschges *et al.*, 1997). Similar or comparable function of this gene for the AM-symbiosis would open the possibility to fill the gaps of molecular processes during the initiation. Consequently, this may result in the verification or modification of a discussed model for common events during the initiation of the two root symbioses (Figure 12).

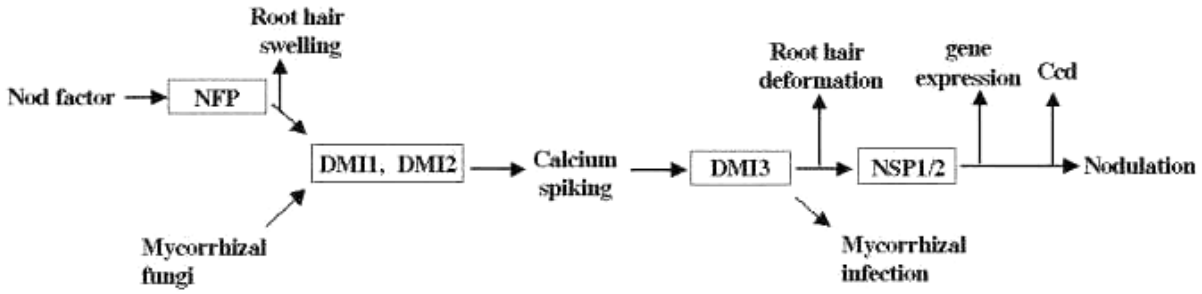

Figure 12: Working model for common signal transduction pathways leading to the root symbioses (Oláh *et al.,* 2005)

DMI1, 2 and 3 are known to be induced during the initiation of the root nodule symbiosis as well as the AM. Calcium spiking has been observed in root nodules between the occurrence of DMI2 and 3 and is therefore predicted to take place also in mycorrhizal roots. Mycorrhizal infection is supposed to proceed until the expression of DMI3. Therefore, the calmodulin regulated genes may be expressed after calcium spiking or the transription of DMI3.

Ccd: cortical cell division

4.1.1 Alterations in transcription pattern of functional diverse genes

During development of the AM several morphological and physiological changes occur in the root of the infected plant. Therefore it appears obvious that these are caused by intense transcriptional alterations of genes belonging to different functional classes. Microarray analyses performed during this thesis revealed transcriptional changes of genes belonging to all of these classes.

AM fungi colonise host roots by penetrating their cell walls and entering the apoplast. The nucleus moves to a central position of the host cell and cell organelles proliferate. This is accompanied by the reorganisation of cell walls and the cytoplasm. These conversions desire a set of proteins that are responsible for cell wall degradation and assembly and alterations in the cytoskeleton (Hohnjec *et al.,* 2005, Manthey *et al.*, 2004). The transcription of several different genes involved in cell wall conversions have been identified to be effected by the AM. For example TC88957, which additionally has been identified by Hohnjec and associates (2005), shows similarities to a polygalacturonate-degrading enzyme and may serve for modifications of the cell wall or extracellular matrix. Similar functions are supposed for *MtCel*1 that has been identified to be induced in *Glomus versiforme* colonised roots of *M. truncatula* (Liu *et al.*, 2003).

Several analyses report an induction of proteins related to protein synthesis and modification and defence (Dumas-Gaudot *et al.*, 1994, Gianinazzi-Pearson, 1996, Salzer *et al.*, 2000). In respect to protein expression different ribosomal proteins have been identified to be induced after 21 days of mycorrhization consistent to the observations of Journet *et al.* (2001). Interestingly, using digital expression profiling Journet reported strong induction of a translation initiation factor (TC91847), that has also been identified in this thesis to be upregulated after 6 and 21 of mycorrhization. Concerning defence, TC86086 was detected to be induced, coding for a probable Kunitz-type protease inhibitor. These proteins are predicted

to fine-tune protease activity during arbuscule degradation or to modulate plant defence responses caused by the fungus (Hohnjec *et al.*, 2005). Therefore exclusive appearance in the completely developed symbiosis fits quite well in this theory as in the early-symbiotic roots no arbuscules have been developed.

In an arbuscular mycorrhiza fungi act as a kind of extension of the roots and enable the plant to thorough exploration of the soil (Smith and Read, 1997, Pfeffer *et al.*, 1999). Beside the mentioned *MtPT*4, several additional transporters have been found to be induced after 21 dpi: TC77463 codes for an ammonium transporter, TC83425 for a proline transporter, TC86374 for an ABC-transporter. Additionally, TC88701 describes the first mycorrhiza induced manganese transporter, MtZIP7, whose transport properties were characterised in yeast expression systems (López-Millán *et al.*, 2004). Identification of the induction of several different putative transporter proteins underlines the importance of the AM for upgrade of the uptake of nutrients. Most recent studies on AM induced transporters were focused on specific phosphate transporters (Harrison *et al*, 2002, Nagy *et al.*, 2005, Rausch *et al.*, 2001), but acquisition of nitrate is also supported by the identification of induced nitrate transporters in different species (Hohnjec *et al.*, 2005, Hildebrandt *et al.*, 2002). The here described ammonium transporter affiliates to these reports.

Sucrose displays the major transport molecule in plants for carbon source to sink transport (Hawker, 1985). TC77988 is annotated to encode a sucrose-phosphate synthase that may be involved in the metabolism of this carbohydrate (Blee and Anderson, 2002, Hohnjec *et al*., 2003, Ravnskov *et al*., 2003). After hydrolysis of sucrose to the particular hexoses, monomers enter glycolysis. A putative glyceraldehyde-3-phosphate dehydrogenase (TC85747) found to be increased in the microarray analysis may participate at this process, although up to now genes of the tricarboxylic acid cycle have not been identified to be AM specific upregulated.

Remarkably, seven genes coding for probable auxin related proteins are found to be highly

induced during mycorrhization (TCs: 79906, 77465, 82150, 82358, 83162, 88776, 88867). This huge amount of upregulated auxin response factors or auxin induced proteins underlines reports of increased auxin biosynthesis in mycorrhizal roots (Kaldorf and Ludwig-Müller, 2000).

Concerning AM related receptors, several receptor-like kinase genes have been found to be induced (TCs: 77660, 78763, 88607, 88669, 89062, 89551, 90272, 92959) or repressed (TCs: 83559, 85563, 87576, 90231, 91705) indicating intense changes of signal transduction pathways during the AM. Noteworthy, as the first signalling events have to be constitutive present in uninoculated roots, no gene of this family could bei identified to be specifically expressed after six days of mycorrhization. TC82283, a putative phytosulfokine LRR-type receptor kinase, induced after 21 dpi appears to be interesting since phytosulfokine constitutes a sulfated intercellular peptide-signal involved in cellular development and proliferation (Matsubayashi *et al.*, 2002). For initialising recognition events between plant and fungus so called myc-factors are postulated to be perceived by the hosts (Cullimore and Dénarié, 2003) but so far no details are known about details of such event. Therefore identification of this myc-factor and the correspondent receptor is one of the major tasks of the researches on the AM.

Colonisation of roots by mycorrhizal fungi lead to intense reorganisation of morphology and physiology of the plant. These modifications imply transcriptional changes that are mediated by specific transcription factors. Several putative transcription factors falling to different classes have been identified to be induced in the AM: Among others, microarray analyses revealed two genes probably coding for basic leucine zipper (bZip) proteins (TC80554, TC86844), one homeobox protein (TC80748) and one Myb-like transcription factor (TC77052). So far, only one Myb-like transcription factor, Mt-pan, has been characterised in *M. truncatula* to be specific in lateral root initials, giant cells induced by nematodes and in

root nodules (Koltai *et al.*, 2001). Homeobox and bZip transcription factors are known to be involved in defence and hormone action (Zhou *et al.*, 2000, Fukazawa *et al.*, 2000). Remarkably, bZIP transcription factors are assumed to induce the expression of glutathione S-transferases (Johnson *et al.*, 2001), of which one member, *MtGst*1, represents a well studied AM-specific gene (Wulf *et al*., 2003). In the early-symbiotic phase no transcription factor like genes have been identified to be specifically induced, indicating that at this point of time signal transduction may not have reached the nucleus. Transcriptional alterations observed in the microarray analysis may be explained by protein modifications, for example the activation of existing G protein-coupled receptors as is assumed for the nodule symbiosis (Pingret *et al.,* 1998).

Centralised, several genes of different functional classes have been identified to be upregulated during the AM. Therefore, microarray analyses provide first insights in regulation of these genes and may give starting points to select genes that might be important for the symbiosis. However, gene annotation was borne on similarities of TCs, that represent only fragments of cDNAs, on characterised proteins. Therefore, gene annotation and function has to be proven and microarrays constitute only introducing analyses.

4.2 Electronic Northern transcriptome profiling identifies novel AM-specific genes

The Mt16kOLI1 microarray contains all known TCs from the TIGR *M. truncatula* gene index, version 5.0. The latest available version is the MtGI 8.0. Consequently, not all current present TCs can be analysed due to their AM-specificity. Additionally, only two states can be observed: mycorrhizal and non-infected roots, but transcription of upregulated genes under different circumstances cannot be excluded. To circumvent these problems, the advantage of the availability of sequence data from different biological sources have been used. In *in silico*

analysis cDNA libraries of different sources have been analysed to identify genes, which are only abundant in libraries originating from mycorrhizal roots. Quantitative RT-PCR verified these findings. Despite few TCs from fungal origin, in total 33 TCs could be identified showing similarities to plant genes of at least a predicted function. Amongst these a copper transporter was found to be specifically induced queuing in a number of AM specific transporters, such as phosphate transporters (Harrison *et al.*, 2002, Paszkowski *et al.,* 2002, Rausch *et al.*, 2001, Nagy *et al.*, 2005), a sugar transporter (Harrison, 1996), a zinc transporter (Wulf *et al.,* 2003) or a nitrate transporter (Hildebrandt *et al.*, 2002). Identification of this high number of different transporters indicate an extensive exchange of nutrients and minerals, not only focused on phosphate.

Additionally, another member of the transmembrane MLO-like proteins has been identified to be AM specific induced, representing the second member of this gene family (chapter 4.1). Several additional AM-induced sequences, identified via the *in silico* approach, belonged to gene families of already reported AM-induced genes, such as germin-like proteins (Doll *et al.*, 2003, Wulf *et al.*, 2003), serine-carboxypeptidases (Liu *et al.*, 2003) or blue copper binding proteins (Wulf *et al.*, 2003). Remarkably, four different lectin-like sequences were strongly induced in AM roots. In former analyses three genes of this family have been identified to be transcribed in the same manner (Wulf *et al.*, 2003), so that lectins form the largest group of AM-induced genes. Consequently, it appears to be quite obvious that these specific lectins have to fulfil an important role during the symbiosis. This is supported by sequence analysis: The phylogenetic tree of all so far known *M. truncatula* lectins reveals that the AM-specific genes represent a distinct branch. High sequence similarities of these lectins may indicate similar function for the symbiosis.

4.3 MtLEC10 appears to represent a pseudogene

Sequence analysis revealed that one of the lectin gene family members, *MtLec*10*,* appears to represent a pseudogene. *MtLec*10 consists of an open reading frame that would lead to a peptide of 113 amino acids. Abortion of a potential translation would occur by two consecutive stop codons. If comparing the remaining part of the cDNA sequence in blastx-analyses against protein databases, no homology to any other plant protein can be observed. Therefore it seems to be likely that this pseudogene has evolved by translocation of the N-terminal part to another position of the genome separating the gene in two parts. The C-terminal part, which aligns to the remaining lectins, shows no striking high similarity to any of the other sequences. Consequently it is assumed that *MtLec*10 appears not to be a result of a duplication event of any other known *M. truncatula* lectin. But the existence of so far unknown members of the lectin multigene family cannot be excluded.

The role of pseudogenes is not well understood. Translation has been shown in human *in vivo* (Brosius *et al.*, 1996, McCarrey and Thomas, 1987), but no expression at protein level has been observed in plants so far. One potential role of pseudogenes was suggested in regulation of gene expression as they may be a source of antisense RNA (McCarrey and Riggs, 1986, Korneev *et al.*, 1999). Although *MtLec*10 is transcriptionally regulated during arbuscular mycorrhiza symbiosis, it has not necessarily has to play an important role for maintaining this interaction. Another lectin *MtLec*2 known to be nodule specific has additionally been shown to be not obligat necessary for maintaining the symbiosis (Bauchrowitz *et al.,* 1996). Anyway, it remains interesting why pseudogenes are specifically regulated if they do not play a role as translated proteins.

4.4 Biological functions of lectins

The detection of a number of AM-specific lectin like genes in *M. truncatula* raised the question about the function of these genes. As lectins are known to fulfil several functions, subcellular localisation should give a first hint about their function. Lectins - also known as agglutinins - in plants are known to have several different functions. They are defined as proteins that specifically bind reversibly to carbohydrates (Peumans and van Damme, 1995) and display a wide heterogeneity with respect to their sequence, sugar-binding specificity and regulation pattern. Until recently, plant lectins were supposed to mediate the binding of foreign glucans and thus mainly participating in recognition and defence responses.

Several plant lectins are known to be involved in the regulation of plant-microbe interactions. Binding to various oligosaccharides of different organisms led to some advice that lectins might play a role in defence against different organisms: Indirect resistance mechanism against bacteria is assumed because binding studies showed interaction of plant lectins with bacterial cell wall peptidoglycans (Broekaert and Peumans, 1986, Ayouba *et al.*, 1994). Hevein, a lectin from the latex of the rubber tree (*Hevea brasiliensis*) has an antifungal activity (van Parijs *et al.*, 1991). Wheat germ agglutinin (WGA) is able to inhibit the development of larvae of the cowpea weevil, the European corn borer and the Southern corn rootworm (Murdock *et al.* 1990, Czapla and Lang, 1990). Some lectins even have effects on higher animals, which for example results in an enlargement and subsequent damage of the pancreas (Pusztai *et al.*, 1990).

Several hints exist that lectins play an important role in the root nodule symbiosis (Diaz *et al.* 1989, Bajaj *et al.* 2001). The *Rhizobium*-legume interaction is known to be very specific and it is assumed that this specificity is achieved in some extent through the action of plant lectins which are localised on the root surface. For these lectins it is hypothesised that they might play a role in very early events of the symbiosis by binding to the surface of the bacteria

(Hamblin and Kent, 1973, Bohlool and Schmidt, 1974). The root nodule symbiosis is highly species-specific between a special host species and its rhizobial partner species. This specificity is supposed to be mediated by the specific binding of the plant-lectins to bacterial structures. These findings have been supported by introducing a lectin from one legume species into another. This enabled formation of nodules with the symbiont of another species (Diaz *et al.*, 1989, van Rhijn *et al.*, 1998). Another root lectin from *Dolichos biflorus,* which is localised on the surface of root hairs, has been assumed to bind to a Nod factor and therefore might represent a receptor or transporter for the initiation of nodule formation (Etzler *et al.*, 1999, Kalsi and Etzler, 2000).

Peanut nodule lectin (PNL) has been localised among others in the large vacuole of nodule parenchyma cells (Vanden Bosch *et al.*, 1994). Other lectins have been found to be targeted in vegetative tissues such as leaves and bark of the leguminous tree *Sophora japonica* (Herman *et al.*, 1988, Baba *et al.*, 1991). Because of annual accumulation and depletion pattern of the bark lectin authors suggest a function as storage protein (Baba *et al.*, 1991) where igenauert serves as nitrogen repository.

However, the recent detection of cytoplasmatic plant lectins indicated a further role of these lectins in endogenous protein-carbohydrate interactions (van Damme *et al.,* 2004). These findings led to the hypothesis that such cytoplasmatic as well as nuclear lectins are involved in cellular regulation and signalling due to the stimulation by biotic or abiotic stimuli (van Damme *et al.*, 2004).

4.5 MtLEC5 appears to be located in the central vacuole

As plant lectins are known to fulfil several different biological functions, the subcellular localisation might give some hints about the functions of the AM-specific ones. Therefore,

confocal laser scanning microscopy has been used to show that MtLEC5 is targeted to the inner parts of the cell. Different constructs have been used to show this: The expression of the MtLEC5-GFP fusion protein has been either under the control of the 35S or the native promoter to minimise the possibility of artificial effects of mis-targeting to false cell compounds when using the constitutive 35S promoter. Expression pattern of the chimeric proteins leads in both cases to strong fluorescence in the inner parts of the cells. Therefore it appears that in this case the constitutive expression of MtLEC5 does not affect its targeting. Additionally it is anticipated that the proteins, which are involved in the transport of this particular protein are not AM-specific expressed, as this should have an impact on the localisation of MtLEC5.

Expression of the ”pure" green fluorescent protein results in strong fluorescence at the edge of the transgenic cells as it should appear in the cytoplasm. In contrast the MtLEC5-fusion protein containing cells show fluorescence in the inner parts of the cells suggesting transport into the central vacuole, when compared to the cytoplasmatic targeted GFP and previously described CLSM studies on *M. truncatula* (Di Sansebastiano *et al.,* 2001, Rodriguez-Llorente *et al*., 2003).

Deletion of the predicted signal peptide leads to a return of the fluorescence in the cytoplasm. However, no conclusions can be drawn about the prediction of its length by the software. The leader sequence may be shorter than the predicted 34 amino acids. In this case, an N-terminal part of *MtLec*5 would have been deleted, which would have no impact on targeting because of the missing leader peptide. On the other hand, it may be longer than 34 amino acids. In this case absence of only an N-terminal fragment of the signal peptide leads to the alteration in targeting. A third fusion of solely the predicted leader peptide with the *gfp* gene would give more insights to this question.

Nevertheless, several lectin-like proteins are known to be transported into the vacuole, in

particular several of the defence related ones. A possible role in defence mechanisms appears conceivable as several plant defence genes are known to be induced during the AM (Gao *et al.*, 2004). A lectin, found in rhizomes and seeds of nettle (*Urtica dioita*, van Parijs *et al.*, 1992) is supposed to be involved in the control of colonisation of rhizomes by endomycorrhizal fungi (Peumans and van Damme, 1995). Certainly, MtLEC5 does not fit into this model, because in the vacuole it is not able to interact with the fungal cell wall. Therefore, such a function appears unlikely as vacuolar proteins may have no impact to an invader but more to a herbivore. Consequently, transport of MtLEC5 to the vacuole assumes that it functions as storage protein, supporting the hypothesis that AM colonisation improves the nitrogen nutrition of plants. For instance, in carob (*Ceratonia siliqua* L.) nitrogen nutrition was improved by the formation of the symbiosis (Cruz *et al.*, 2004).

Storage proteins represent a source for amino acids or exacting nitrogen. In mycorrhizal roots induction of nitrate transporters has been observed (Hildebrandt *et al.*, 2002) supposing that AM fungi are able to promote nitrogen uptake. This enhanced nitrogen content in the cells may be assimilated and built in storage proteins. Most lectins, which are predicted to function as storage proteins, are found in seeds. But some lectins have been found targeted in vegetative tissues such as leaves and bark of the leguminous tree *Sophora japonica* (Herman *et al.*, 1988, Baba *et al.*, 1991), where they are assumed to function as nitrogen storage (Baba *et al.*, 1991).

However, as CLSM analyses reveal some insight into the subcellular localisation of this particular protein, the identity as a lectin has not been proven – its annotation relies on sequence similarities. Several other proteins show similarities to lectins. Therefore, it cannot be excluded, that *MtLec*5 may serve as for example an α-amylase-inhibitor or an arcelin, which also show high similarities to lectins. These proteins are known to be involved in defence mechanisms and may also be targeted to vacuoles (Moreno *et al.*, 1990, Hamelryck *et*

al., 1996).

4.6 Promoter analyses of AM-specific lectins show arbuscule specific expression

Beside their importance for functional studies, AM-specific transcripts represent the starting point to unravel the molecular mechanism of the transcriptional induction upon mycorrhiza development. One important task of analysing specific transcribed genes is the identification of *cis*-regulatory elements and their corresponding tanscription factors. Therefore, intensive investigations have been performed for two of the mycorrhiza-specific lectins. The promoter of *MtLec*5 was deduced from the corresponding *M. truncatula* BAC clone whereas inverse PCR technique was used to clone the promoter of *MtLec*7. Approximately 1,500 bp of the 5' upstream sequence could be isolated. Reporter gene studies showed an arbuscule specific transcription for both members of the AM-specific lectin family, suggesting a role during arbuscule formation and functioning during late stages of the AM-symbiosis. No promoter activities were detected in the vicinity of appressoria which represent early steps of AM-development. Appressoria are only formed at epidermal cells of host plants (Giovanetti *et al.* 1993, Nagahashi and Douds, 1997) indicating a host plant-fungus recognition at this stage. Thus, the two lectins *MtLec*5 and *MtLec*7 do not play a role during recognition at early stages of symbiosis development. As corresponding TCs of these two lectins first appeared in the TIGR gene index version 7, they were not included in the microarray analyses (chapter 4.1) to prove this hypothesis.

In a second step in the characterisation of the promoters the production of deletion constructs has been chosen. Different lengths of the 5' upstream sequences were used for the promoter deletion studies. These confirm the arbuscule-specific activity of p*MtLec*5 and p*MtLec*7: Similar expression patterns have been obtained when using up to approximately 300 bp of the

promoters, respectively. In contrast, when using only approximately 150 bp or 75 bp of the promoters, regarding to the transcription start, no activity could be observed at all.

Activation of transcription depends on the binding of different DNA-binding proteins to special *cis*-regulatory elements. If these promoter elements are missing due to a deletion event no transcription of these genes is able to take place. Consequently it seems reasonable that putative AM-responsive elements in the promoters of these two genes are located in the area between -300 bp and -150 bp.

4.6.1 p*MtLec*5 is also active in the non-legume *Nicotiana tabacum*

As the arbuscular mycorrhiza symbiosis has been observed in fossils of first terrestrial plants and is therefore several hundred million years old, it seems quite obvious that mechanisms leading to the development of this interaction are highly conserved. For instance mycorrhiza specific phosphate transporters have been identified in several organisms. Therefore, an interesting task is to seek out if this holds also for basal events such as the initiation of alteration of transcription. First analyses have been focused on *VfLb*29, which is known to be nodule as well as arbuscule induced (Vieweg *et al.*, 2004). The promoter of this leghemoglobin is also active in *Nicotiana tabacum.* Karandashov and associates (2004) showed activity of the promoters of several AM-specific phosphate transporters in different species.

To investigate, if signal transduction pathways in tobacco lead also to an activation of p*MtLec*5, *N. tabacum* roots were transformed with *A. rhizogenes* carrying p*MtLec*5(-1037/+23) fused to an *uidA* gene. Arbuscule specific expression of the reporter gene indicates that tobacco and *M. truncatula* share additional common or at least similar signal transduction pathways leading to the expression of at least *MtLec*5 assisting the hypothesis that signalling

in AM-forming plants is highly conserved in regard to the symbiosis.

4.6.2 EMSA reveals binding of AM-specific proteins to p*MtLec*5

Promoter deletion analyses showed for p*MtLec*5 an activation of the reporter gene when using 301 bp of the upstream region, but no activation when using 151 bp. Therefore the AM-regulatory elements have to be positioned inside this region. Therefore the corresponding sequence was divided into 30 bp fragments with 10 bp overlap at each site and electrophoretic mobility shift assay has been carried out. In EMSA four fragments show specific binding of proteins derived from mycorrhizal roots, whereas no shifts were detected when using protein extract from control roots. Therefore, the DNA-binding proteins leading to the specific alterations in the movement of the DNA in the gel have to be originated from the differences between the two protein extracts, namely the mycorrhization.

Consequently, location of putative positive regulator binding sites in p*MtLec*5 could be narrowed down to 90 bp of represented by these four overlapping fragments. As these promoter elements are known to consist of only 5 up to 20 bp, it can be anticipated that p*MtLec*5 contains more than one element, which proteins are able to bind to in an AM specific manner. Comparable results have been obtained for *VfLb*29*,* where promoter deletion analysis indicated the occurrence of two different regulatory modules (Vieweg *et al.*, 2005).

4.6.3 p*MtLec*5 and p*MtLec*7 share common motifs

The experimental approaches described in this work lead to the identification of short promoter areas of two co-regulated genes, which potentiate the initiation of transcription. Such promoter sequences of co-regulated genes can be used to identify conserved regulatory

elements by *in silico* approaches. A comparison of the corresponding promoter areas revealed a common 8 bp sequence motif in the promoters of *MtLec*5 and 7*,* which shows no homology to any known regulatory element. This motif represents one candidate for a mycorrhiza specific promoter element. Further analyses of other AM-specific promoter sequences will strengthen this hypothesis. Besides comparing both promoters and searching for conserved motifs, the sequences were scanned for already described plant promoter motifs. Two promoter modules were found to be present in both promoter areas: The nodulin consensus sequence is often found in promoters of nodule specific genes (Sandal *et al*., 1987), e.g. in the promoter of the N23 gene analysed by Jørgensen and associates (1991) or the promoter of *lbc3* (Ramlov *et al.,* 1993). In the *lbc3* promoter this element is part of an organic specific element (OSE), which directs nodule specific expression. In contrast, another OSE of the *lba* promoter is not able to mediate nodule specificity (She *et al.*, 1993). Circadian clock associated motifs, of which one member depicts the other common promoter motif, are found in promoters of genes which enable organisms to coordinate their circadian activities for example with the external dark/light cycles. CCA1 (circadian clock associated) and LHY (late elongated hypocotyl), two Myb-like transcription factors, are able to bind directly to this motif within the *TOC1* promoter (Alabadi *et al.,* 2001, Harmer *et al.*, 2000) to repress its expression and function therefore as negative regulators. Nevertheless, although the detection of these modules in the promoters of two mycorrhiza specific lectins is likely to be not significant, it remains interesting whether such modules also exist in upstream regions of other AM specific genes and whether they exhibit a role during the mycorrhiza symbioses.

In summary, during this thesis two different methods have been used to identify transcriptional changes during the AM symbiosis. Microarray hybridisation revealed genes which are differentially induced or repressed during an early-symbiotic phase as well as in a

fully developed symbiosis. The data obtained provide a starting point to get new insights in the early molecular events during the development of the AM. Electronic Northern analyses have identified four lectin-like sequences, so that in summary with former results seven have been shown to be AM specific, which form a unique branch of *M. truncatula* lectins. Confocal laser scanning microscopy revealed targeting of one of these lectins in the vacuole assuming a putative function as storage protein. Specific promoter activity has been shown in arbuscule containing cells and the search for putative *cis*-regulatory promoter elements revealed 90 bp of p*MtLec*5 showing AM specific binding of putative transcription factors, leading to the assumption, that more than one of these regulatory proteins are involved in the activation of AM specific transcription. Computational analyses revealed that p*MtLec*5 and 7 share in total three common motifs, one of these is so far unknown.

4.7 Outlook

Transcriptional and functional analysis of novel AM-induced genes

The early events during the development of the arbuscular mycorrhizal symbiosis are only fragmentary. Three genes have been identified to be necessary for the initiation of both root-symbioses leading to a model of the molecular events occurring in this phase. Nevertheless, this is only a hypothesis that lacks many intermediate steps. Microarray hybridisation revealed new genes, which are specifically regulated in the presence of AM fungi but before a physical contact has occurred. Some of them are supposed to show calmodulin mediated calcium dependency. Therefore they are interesting candidates which may lead to novel insights about the development of the AM. Detailed transcriptional analyses regarding these genes on *M. truncatula* DMI1 and DMI2 mutants might unravel, if these genes are involved in this signal transduction pathway, and at which time points these genes might interfere.

Subsequently, the working model of the initiation of the root symbioses may be improved.

Detailed promoter analyses to identify AM-specific transcriptions factors and cis-regulatory promoter elements

Additional promoter analysis of all ”early“ and ”late“ mycorrhizal genes may lead to the identification of AM specific *cis*-regulatory elements. Using a large set of genes and promoters, bioinformatic approaches may be developed to look for these elements in so far not analysed promoters. At least the identification of the hypothetical AM specific transcription factors, binding to these promoter elements, using yeast one hybrid screening, DNA footprinting and EMSA for instance would complete these promoter analyses.

Additionally, intense promoter analysis of all AM-specific lectins may unravel the question why these gene family is mycorrhiza specific induced.

Functional analysis of AM-specific lectin genes

The location of MtLEC5 in the vacuole suggests, that this protein may be deposited there as a storage protein for the allocation of nitrogen. Nevertheless, another function cannot be excluded. Further analyses are needed to verify the annotation of this protein. Binding studies may reveal the specific binding of these proteins to different carbohydrates.

Additionally, gene knock-down using posttranscriptional gene silencing would give more insights about the biological function and the role of the lectins during the arbuscular mycorrhiza.

References

- Alabadi D., Oyama T., Yanovski M. J., Harmon F. G., Mas P., Kay S. A. (2001) Reciprocal regulation between TOC1 and LHY/CC1. Science 293: 880-883
- Alexander T., Meier R., Toth R., Weber H. C. (1988) Dynamics of arbuscule development and degeneration in mycorrhizas of *Triticum aestivum* L. and *Avena sativa* L with reference to *Zea mays* L. New Phytol 110: 363-370
- Alexander T., Toth R., Meier R., Weber H. C. (1989) Dynamics of arbuscule development and degeneration in onion, bean and tomato with reference to vesicular-arbuscular mycorrhizae in grasses. Can J Bot 67: 2505-2513
- Altschul S. F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W., Lipman D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402
- Ane J. M., Kiss G. B., Rieley B. K., Penmetsa R. V., Oldroyd G. E., Ayax C., Lévy J., Debellé F., Baek J. M., Kalo P., Rosenberg C., Roe B. A., Long S. R., Dénarié J., Cook D. R. (2004) *Medicago truncatula* DMI1 required for bacterial and fungal symbiosis in legumes. Science 303: 1364-1367
- Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana.* Nature 408: 796-815
- Arnon D. I., Hoagland D. R. (1940) Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Sci 50: 463-483
- Ayouba A., Causse H., van Damme E. J. M., Pneumans W. J., Cambillau C. Rougé P. (1994) Interactions of plant lectins with the components of the bacterial cell wall peptidoglycan. Biochem Syt Ecol, 22: 153-159
- Baba K., Ogawa M., Nagano A., Kuroda H., Sumiya K. (1991) Developmental changes in the bark lectin of *Sophora japonica* L. Planta 183: 462-470
- Bais H. P., Park S. W., Weir T. L., Callaway R. M., Vivanco J. M. (2004) How plants communicate using the underground information superhighway. Trends Plant Sci 9: 26-32.

References

- Bajaj M., Soni G., Singh C. (2001) Interaction of pea (*Pisum sativum* L.) lectins with rhizobial strains. Microbiol Res, 156: 71-74
- Balaji B., Poulin M J., Vierheilig H., Piché Y. (1995) Responses of an arbuscular-mycorrhizal fungus, *Gigaspora margarita*, to exudates and volatiles from the Ri T-DNA-transformed roots of nonmycorrhizal and mycorrhizal mutants of *Pisum sativum* L. sparkle. Exp Mycol 19: 275-283
- Balestrini R., Berta G., Bonfante P. (1992) The plant nucleus in mycorrhizal roots: positional and structural modifications. Biol Cell 75: 235-243
- Barker D. G., Bianci S., Blondon F. (1990) *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. Plant Molecular Biology Reporter 8: 40-49
- Bauchrowitz M. A., Barker D. G., Truchet G. (1996) Lectin genes are expressed throughout root nodule development and during nitrogen-fixation in the *Rhizobium-Medicago* symbiosis. Plant J 9: 31-43
- Bécard G., Piché Y. (1989) Fungal growth stimulation by CO2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55: 2320-2325
- Bentivenga S. P., Morton J. B. (1994) Systematics of Glomalean endomycorrhizal fungi: current views and future direction. In: Mycorrhizae and Plant Health, ed. F. L. Pfleger, RG, Lindermann, pp. 283-308, St. Paul, MN, APS Press
- Bestel-Corre G., Dumas-Gaudot E., Poinsot V., Dieu M., Dierick J. F., van Tuinen D., Remacle J., Gianinazzi-Pearson V, Gianinazzi S. (2002) Proteome analysis and identification of symbiosis-related proteins from *Medicago truncatula* Gaertn. by two-dimensional electrophoresis and mass spectometry. Electrophoresis 23: 122-137
- Birney E., Clamp M., Durbin R., (2004) GeneWise and Genomewise, Genome Res 14: 988-995
- Blaylock L. A., Penmetsa R. V., Cook D. R., Harrison M. J. (1997) Identification of mycorrhizal mutants of *Medicago truncatula*. In Radical Biology: Advances and perspectives on the function of plant roots, ed. H Flores, J. P. Lynch, D. Eisenstat, pp. 500-501, Rockville, MD: Am Soc Plant Physiol
- Blee K. A., Anderson A. J. (2002) Transcripts for genes encoding soluble acid invertase and sucrose synthase accumulate in root tip and cortical cells containing mycorrhizal arbuscules. Plant Mol Biol 50: 197-211

References

- Blondon F, Marie D., Brwon S., Kondorosi A. (1994) Genome size and base composition in *Medicago sativa* and *Medicago truncatula* species. Genome 37: 264-270
- Bohlool B. B., Schmidt E. L. (1974) Lectins: A possible basis for specificity in the *Rhizobuim*-legume root nodule symbiosis. Science, 185: 269-271
- Boisson-Dernier A., Chabaud M., Garcia F., Bécard G., Rosenberg C., Barker D. G. (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant-Microbe Interact 6: 695-700
- Bonanomi A., Wiemken A., Boller T., Salzer P. (2001) Local induction of mycorrhiza-specific class III chitinase genes in cortical root cells of *Medicago truncatula* containing developing or mature arbuscules. Plant Biol 3: 194-200
- Bonfante P, Perotto S (1995) Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol 130. 3-21
- Bonfante-Fasolo P. (1984) Anatomy and morphology of VA mycorrhizae. In: VA Mycorrhizae, ed. C. L. Powell, D. J. Bagyaraj, pp. 5-33, Boca Raton, FL, CRC Press
- Bonfante-Fasolo P., Perotto S. (1992) Plant and endomycorrhizal fungi: the cellular and molecular basis of their interaction. In: Molecular Signals in Plant Microbe Communications, ed. DPS Verma, pp. 445-470, Boca Raton, FL, CRC Press
- Bowler C., Chua N. H. (1994) Emerging themes for plant signal transduction. Plant Cell 6: 1529-1541
- Bradford M. (1976) A rapid and sensitive method for quantitation of microgram quantities utilizing the principles of protein-dye binding. Analyt Biochem 72: 248-254
- Brechenmacher L., Weidmann S., van Tuinen D., Chatagnier O., Gianinazzi S., Franken P., Gianinazzi-Pearson V. (2004) Expression profiling of up-regulated plant and fungal genes in early and late stages of *Medicago truncatula*-*Glomus mossae* interactions. Mycorrhiza 14: 253-262
- Broekaert W. F., Peumans W. F. (1986) Lectin release from seeds of *Datura stramonium* and interference of the *Datura stramonium* lectin with bacterial motility. Im TC Bog-Hansen, E van Driessche, eds, Lectins, Biology Biochemistry, Clinical Biochemistry, Vol 5. Walter de Gruyter, Berlin: 57-65

References

- Brosius J., Tiedge H. (1996) Reverse transcriptase: mediator of genomic plasticity. Virus Genes 11: 163-179
- Buee M. Rossignol M., Jauneau A. Ranjeva R., Becard G. (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant-Microbe Interact 13: 693-698
- Büschges R., Hollricher K., Panstruga R., Simons G., Wolter M., Frijters A., van Daelen R., van der Lee T., Diergarde P., Groenendijk J., Topsch S., Vos P., Salamini F., Schulze-Lefert P. (1997) The barley MLO gene: A novel control element of plant pathogen resistance. Cell 88: 695-705
- Calantzis C., Morandi D., Arnould C., Gianinazzi-Pearson V. (2001) Cellular interaction between *G. mossae* and a Myc$^-$ *dmi2* mutant in *Medicago truncatula*. Symbiosis 30: 97-108
- Carling D. E., Brown M. F. (1982) Anatomy and physiology of vesicular-arbuscular and nonmycorrhizal roots. Phytopathology 72: 1108-1114
- Catoira R., Galera C., de Billy F, Penmetsa R. V., Journet E., Maillet F., Rosenberg C., Cook, D. R., Gough C., Dénarié J. (2000) Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. Plant Cell 12: 1647-1665
- Cook D. R. (1999) *Medicago truncatula* – a model in the making. Current Opinion in Plant Biology 2: 301-304
- Cruz C., Green J. J., Watson C. A., Wilson F., Martins-Loução M. A. (2004) Functional aspects of root architecture and mycorrhizal inoculation with respect to nutrient uptake capacity. Mycorrhiza 14: 177-184
- Cullimore J., Dénarié J. (2003) How legumes select their sweet talking symbionts. Science 302: 575-578
- Curtis M. D., Grossniklaus U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133: 462-469
- Cyr, R. J. (1991) Calcium/calmodulin affects microtubule stability in lysed protoplasts. J Cell Sci 100: 311-317
- Czapla T. H., Lang B. A. (1990) Effect on plant lectins on the larval development of European corn

borer (*Lepidoptera*: *Pyralidae*) and southern corn rootworm (*Coleoptera*: *Chrysolmelidae*). J Econ Entomol, 83:2480-2485

- Di Sansebastiano G. P., Paris N., Marc-Martin S., Neuhaus J. M. (2001) Regeneration of a lytic central vacuole and of neutral peripheral vacuoles can be visualized by green fluorescent proteins targeted to either type of vacuoles. Plant Physiol 126: 78-86
- Diaz C., Melchers L. S., Hooykaas P. J. J., Lugtenberg B. J. J., Kijne J. W. (1989) Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature, 338: 579-581
- Doll J., Hause B., Demchenko K., Pawlowski K., Krajinski F. (2003) A member of the germin-like protein family is a highly conserved mycorrhiza-specific induced gene. Plant Cell Physiol 44: 1208-1214
- Dondrup M., Goesemann A., Bartels D., Kalinowski J., Krause L., Linke B., Rupp O., Szyrba A., Pühler A., Meyer F. (2003) EMMA: a platform for consistent storage and efficient analysis of microarray data. J Biotechnol 106: 135-146
- Doyle J. J. (2001) Leguminosae. In: Brenner S., Miller J. H. (eds.): Encyclopedia of Genetics. Academic Press, San Diego, USA, pp. 1081-1085
- Duc G., Trouvelo A., Gianinazzi-Pearson V. Gianinazzi S. (1989) First report of non-mycorrhizal mutants (Myc-) obtained in pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.). Plant Sci 60: 215-222
- Dumas-Gaudot E., Guillaume P., Tahiri-Alaoui A., Gianinazzi-Pearson V., Gianinazzi S. (1994) Changes in polypeptide patterns in tobacco roots colonized by two *Glomus* species. Mycorrhiza 4: 215-221
- El Yahyaoui F, Küster H, Ben Amor B, Hohnjec N, Pühler A, Becker A, Gouzy J, Vernié T, Gough C, Niebel A, Godiard L, Gamas P (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol 136: 3159-3176
- Emanuelsson O., Nielsen H., Brunak S. von Heijne G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005-1016
- Erhardt D. W., Wais R., Long S. R. (1996) Calcium spiking in plant root hairs responding to rhizobium

nodulation signals. Cell 85: 673-681

- Etzler M. E., Kalsi G., Ewing N. N., Roberts N. J., Day R. B., Murphy J.B. (1999) A Nod factor binding lectin with apyrase activity from legume roots. Proc Natl Acad Sci USA 96: 5856-5861
- Ewing B., Green P. (1998) Base-calling of automated sequencer traces using Phred. II. Error probabilities. Genome Res 8: 186-194
- Ewing B., Hillier D., Wendl M. C., and Green P. (1998) Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. Genome Res 8: 175-185
- Ferrol N., Pozo M. J., Antelo M., Azcón-Aguilar C. (2002) Arbuscular mycorrhizal symbiosis regulates plasma membrane H^+-ATPase gene expression in tomato plants. J Exp Bot 53: 1683-1687
- Foster R. C. (1988) Microenvironments of soil microorganisms. Biol Fertil Soils 6:189-203
- Frank B. (1885) Ueber die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. Ber Dtsch Bot Ges 3: 128-45
- Franken P., Gnädinger F. (1994) Analysis of parsley arbuscular mycorrhiza: infection development and mRNA levels of defense-related genes. Mol Plant Microbe Interact 7: 612-620
- Frenzel A., Manthey K., Perlick A. M., Meyer F., Pühler A., Küster H., Krajinski F. (2005) Combined transcriptome profiling reveals a novel family of arbuscular mycorrhizal-specific *Medicago truncatula* lectin genes. Mol Plant-Microbe Interact 18: 771-782
- Fukazawa J., Sakai T., Ishida S., Yamaguchi I., Takahashi Y (2000) Repression of shoot growth, a bZip transcriptional activator, regulates cell elongation by controlling the level of gibberellins. Plant Cell 12: 901-915
- Gao L. L., Knogge W., Delp G., Smith F. A., Smith S. E. (2004) Expression patterns of defense-related genes in different types of arbuscular mycorrhizal development in wild-type and mycorrhiza defective mutant tomato. Mol Plant-Microbe Interact 17: 1103-1113
- García-Garrido J. M., Cabello M. N., García-Romera I., Ocampo J. A. (1992) Endoglucanase activity in lettuce plants colonized with the vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum*. Soil Biol Biochem 24: 955-959

References

- García-Romera I., García-Garrido J. M., Martinezmolina E., Ocampo J. A. (1991) Production of pectolytic enzymes in lettuce root colonized by *Glomus mossae*. Soil Biol Biochem 23: 597-601
- Gattiker A., Basteiges E., Bairoch A. (2002) ScanProfile: A reference implementation of a PROSITE scanning tool. Appl Bioinformatics 1: 101-108
- Gerdemann J. W. (1965) Vesicular-arbuscular mycorrhizas formed on maize and tulip tree by *Endogone fasciculata*. Mycologia 57: 562-575
- Gianinazzi-Pearson (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8: 1871-1883
- Gianinazzi-Pearson V., Gianinazzi S., Guillemin J. P., Trouvelot A., Duc G. (1991) Genetic and cellular analysis of resistance to vesicular arbuscular (VA) mycorrhizal fungi in pea mutants. In Advances in Molecular Genetics of Plant-Microbe Interactions, ed. H. Hennecke, DPS Verma, pp. 336-342, Netherlands: Kluwer
- Gianinazzi-Pearson V., Smith S. E., Gianinazzi S. Smith F. A. (1991) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas V. Is H^+-ATPase a component of ATP-hydrolising enzyme activities in plant-fungus interfaces? New Phytol 117:61-74
- Giovanetti M., Sbrana C., Avio L., Citernesi A. S., Logi C. (1993) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol 125: 587-593
- Grunwald U., Nyamsuren O., Tamasloukht M., Lapolin L, Becker A., Mann P., Gianinazzi-Pearson V., Krajinski F., Franken P. (2004) Identification of mycorrhiza-regulated genes with arbuscule-related expression profile. Plant Mol Biol 55: 553-566
- Hajdukiewicz P., Svab Z., Maliga P. (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25: 989-994
- Hamblin J., Kent. S.P. (1973) Possible role of phytohemagglutinin in *Phaseolus vulgaris* L. Nat New Biol 245: 28-30
- Hamelryck T. W., Poortmans F., Goosens A., Augenon G., van Montagu M., Wyns L., Loris R. (1996) Crystal structure of arcelin-5, a lectin-like defence protein from *Phaseolus vulgaris*, J Biol Chem 271: 32796-32802

References

- Harmer S. L., Hogenesch L. B., Straume M., Chang H. S., Han B., Zhu T., Wang X., Kreps J. A., Kay S. A. (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290: 2110-2113
- Harrison M. J. (1996) A sugar transporter from *Medicago truncatula*-altered expression pattern in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J 9: 491-503
- Harrison M. J., Dewbre G. R., Liu J. (2002) A phosphate transporter from *Medicago trncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14: 2413-2429
- Hawker J. S. (1985) Sucrose. In P. M. Dey, R. A. Dixon, eds, Biochemistry of storage carbohydrates in green plants. Academic Press, London, pp 1-51
- Herman E. M., Hankins C.N., Shannon L. M. (1988) Bark and leaf lectins of *Sophora japonica* are sequestered in protein-storage vacuoles. Plant Physiol 86: 1027-131
- Hildebrandt U., Schmelzer E., Bothe H. (2002) Expression of nitrate transporter genes in tomato colonized by an arbuscular mycorrhizal fungus. Physiol Plant 115: 125-136
- Hohnjec N., Perlick A. M., Pühler A., Küster H. (2003) The *Medicago truncatula* sucrose synthase gene *MtSucS*1 is activated both in the infected region of root nodules and in the cortex of roots colonized by arbuscular mycorrhizal fungi. Mol Plant-Microbe Interact 16: 903-915
- Hohnjec N., Vieweg M. F., Pühler A., Becker A., Küster H. (2005) Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza. Plant Physiol 137: 1283-1301
- Isaac C. (1992) Fungal-Plant interactions. Chapman & Hall, Cambridge, UK. ISBN 0-412-36470-0
- Jeanmougin F., Thompson J. D., Gouy M., Higgins D. G., Gibson T. J. (1998) Multiple sequence alignment with Custal X. Trends Biochem Sci 23: 403-405
- Jefferson R. A., Kavanagh T. A., Bevan M. V. (1987) GUS fusions: ß-glucuronidases a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-907
- Johnson C., Glover G., Arias J. (2001) Regulation of DNA binding and *trans*-activation by a xenobiotic stress-activated plant transcription factor. J Biol Chem 276: 172-178

References

- Jørgensen J. E., Stougaard J., Marcker K. A., (1991) A two-component nodule-specific enhancer in the soybean N23 gene promoter. Plant Cell 3: 819-827
- Journet E. P., van Tuinen D., Gouzy J., Crespeau H., Carreau V., Farmer M. J., Niebel A., Schiex T., Jaillon O., Chatagnier O., Godiard L., Micheli F., Kahn D., Gianinazzi-Pearson V., Gamas P. (2002) Exploring the root symbiotic programs of the model legume *Medicago truncatula* using EST analysis. Nucleic Acids Res 30, 5579-5592
- Kaldorf M., Ludwig-Müller J. (2000) AM fungi might affect the root morphology of maize by increasing indole-3-butyric acid biosynthesis. Physiol Plant 109: 58-67
- Kalsi G., Etzler M. E. (2000) Localization of a Nod factor binding protein in legume roots and factors influencing its distribution and expression. Plant Physiol 124: 1039-1048
- Karandashov V., Nagy R., Wegmüller S., Amrherin N., Bucher M. (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 101: 6285-6290
- Koltai H., Dhandaydham M., Oppermann C., Thomas J., Bird D. (2001) Overlapping plant signal transduction pathways induced by a parasitic nematode and a rhizobial endosymbiont. Mol Plant-Microbe Interact 14: 1168-1177
- Korneev S. A., Park J. H., O' Shea M. (1999) Neuronal expression of neural nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from NOS pseudogene. J Neurosci 19: 7711-7720
- Krajinski F., Biela A., Schubert D., Gianinazzi-Pearson V., Kaldenhoff R., Franken P. (2000) Arbuscular mycorrhiza development regulates the mRNA abundance of *Mtaqp1* encoding a mercury-insensitive aquaporin of *Medicago truncatula*. Planta 211: 85-90
- Krajinski F., Hause B., Gianinazzi-Pearson V., Franken P. (2002) *Mtha1,* a plasma membrane H^{+}-ATPase gene from *Medicago truncatula*, shows arbuscule-induced expression. Plant Biol 4: 754-761
- Krajinski F., Martin-Laurent F., Gianinazzi S. Gianinazzi-Pearson V., Franken P. (1998) Cloning and analysis of *psam*2, a gene from *Pisum sativum* L. regulated in symbiotic arbuscular mycorrhiza and pathogenic root-fungus interactions. Physiol Mol Plant Pathol 52: 297-307
- Küster H., Hohnjec N., El Yahoui F., Manthey K., Gouzy J., Dondrup M., Meyer F., Kalinowski J.,

References

Brechenmacher L. (2004) Construction and validation of cDNA-based Mt6k-RIT macro- and microarrays to explore root endosymbioses in the model legume *Medicago truncatula*. J Biotechnol 108: 95-113

- Lévy J., Bres C., Guerts R. *et al.* (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303: 1361-1364
- Liang F., Holt I., Pertea G., Karamycheva S., Salzberg S. L., Quackenbusch J. (2000) An optimized protocol for analysis of EST sequences. Nucleic Acids Res 28: 3657-3665
- Liu H., Trieu A. T., Blaylock L. A., Harrison M. J. (1998) Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: Regulation in response to phosphate and colonization by arbuscular mycorrhizal fungi. Mol Plant-Microbe Interact 11: 14-22
- Liu J., Blayllock L. A., Endre G., Cho J., Town C. T., VandenBosch K. A., Harrison M. J. (2003) Transcript profiling coupled with spacial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. Plant Cell 15: 2106-2123
- López-Milán A. F., Ellis D. R., Grusak M. A. (2004) Identification and characterization of several new members of the ZIP family of metal ion transporters in *Medicago truncatula*. Plant Mol Biol 54: 583-596
- Manthey K., Krajinski F., Hohnjec N. Firnhaber C., Pühler A., Perlick A. M., Küster H. (2004) Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies collection of novel genes induced during *Medicago truncatula* root endosymbioses. Mol Plant-Microbe Interact 17: 1063-1077
- Matsubayashi Y., Ogawa M., Morita A., Sakagami Y. (2002) An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. Science 5572: 1470-1472
- McCarrey J. R., Riggs A. D. (1986) Determinator-inhibitor pairs as a mechanism for threshold setting in development: a possible function for pseudogenes. Proc Natl Acad Sci USA 83: 679-683
- McCarrey J. R., Thomas K. (1987) Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. Nature 326: 501-505
- Mitra R. M., Gleason C. A., Edwards A., Hadfield J., Downie J. A., Oldroyd G. E., Long S. R. (2004) A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic for symbiotic nodule development: gene identification by transcript-based cloning. Proc Natl Acad Sci USA 101: 4701-4705

References

- Mitra R. M., Long S. R. (2004) Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the *Medicago truncatula/Sinorhizobium meliloti* symbiosis. Plant Physiol 134: 595-604
- Moreno J., Altabella T., Chrispeels J. (1990) Characterization of α-Amylase-Inhibitor, a lectin-like protein in the seeds of *Phaseolus vulgaris*. Plant Physiol 92: 703-709
- Morton J. B., Benny G. L. (1990) Revised classification of arbuscular mycorrhizal fungi (zygomycetes): a new order, glomales, two new suborders, glomineae and gigasporineae, and two new families, acaulosporaceae and gigasporaceae, with an amendation of glomaceae. Mycotaxon 37: 471-491
- Murdock L. L., Huesing J. E., Nielsen S. S., Pratt R. C., Shade R. E. (1990) Biological effects of plant lectins on the cowpea weevil. Phytochemistry 29: 85-89
- Nagahashi G., Douds D. D. J. (1997) Appressorium formation by AM fungi on isolated cell walls of carrot roots. New Phytol 136: 299-304
- Nagy R., Karadashov V., Chague V., kalinkevich K., Tamasloukht M., Xu G., Jakobsen I., Levy A. A., Amrhein N., Bucher M. (2005) The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. Plant J 42: 236-250
- Nakata Y., Tang X., Yokoyama K. K. (1997) Preparation of competent cells for high efficiency plasmid transformation of *Escherichia coli*. Methods Mol Biol 69: 129-137
- Nielsen H., Engelbrecht J., Brunak S., von Heijne G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10: 1-6
- Oláh B., Brière C., Bécard G., Dénarié J., Gough C. (2005) Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. Plant J 44: 195-207
- Oldroyd G. E., Geurts R. (2001) *Medicago truncatula*, going where no plant has gone before. Trends Plant Sci 6: 552-554
- Paszkowski U., Kroken S., Roux C., Brigg S. P. (2002) Rice phosphate transporters include an evolutionary divergent gene specifically activated in arbuscular mycorrhizal symbiosis. Proc Natl Acad

References

Sci USA 99: 13324-13329

- Peumans W. J., van Damme E. J. M. (1995) Lectins as plant defence proteins. Plant Physiol 109: 347-352
- Pfeffer P. E., Douds D. D., Becard G., Shachar-Hill Y. (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120: 587-598
- Pingret J. L., Journet E. P., Barker D. G. (1998) *Rhizobium* Nod factor signaling: Evidence for G protein-mediated transduction mechanism. Plant Cell 10: 659-671
- Pirozynski K. A., Malloch D. W. (1975) The origin of land plants: a matter of mycotrophism. Biosystems 6: 153-164
- Polhill R. M., Raven P. H. (eds., 1981) Papilionidae. In: Advances in legume systematics, part 1, Royal Botanic Garden, Kew, Richmond, Surrey, UK, pp. 191-208
- Pusztai, A., Ewen, S. W. B., Grant, G., Peumans, W. J., van Damme, E. J. M., Rubio, L., Bardosz, S. (1990) The relationship between survival and binding of plant lectins during small intestine passage and their effectiveness as growth factors. Digestion 46: 308-316
- Quackenbusch J., Cho J., Lee D., Liang F., Holt I., Karamycheva S., Parvici B., Pertea G., Sultana R., White J. (2000) The TIGR gene indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucl Acids Res 29: 159-164
- Quandt H. J, Pühler A., Broer I. (1993) Transgenic root nodules of *Vicia hirsuta*: A fast and efficient system for the study of gene expression in intermediate-type nodules. Mol Plant-Microbe Interact 6: 699-706
- Quandt K., Frech K., Karas H., Wingender E., Werner T. (1995) MatInd and MatInspector - New fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 23: 4878-4884
- Rabie G. H., Almadini A. M. (2005) Role of bioinoculants in development of salt tolerance of *Vicia faba* plants under salinity stress. Afr J Biotechnol 4: 210-222
- Ramlov, K. B., Laursen, N. B., Stougaard, J, Marcker, K. A. (1993) Site-directed mutagenesis of the organ-specific element in the soybean leghemoglobon *lbc3* gene promoter. Plant J 4: 577-580

References

- Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule containing cells in potato. Nature 414: 462-470
- Ravnskov S., Wu Y., Graham J. H. (2003) Arbuscular mycorrhizal fungi differentially affect expression of genes coding for sucrose synthase in maize roots. New Phytol 157: 539-545
- Rejon-Palomares A., García-Garrido J. M., Ocampo J. A., García-Romera I. (1996) Presence of xyloglucan-hydrolizinglucanases (xyloglucanases) in arbuscular mycorrhizal symbiosis. Symbiosis 21: 249-261
- Remy W., Taylor T. N., Hass H., Kerp H. (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91: 11841-11843
- Rodriguez-Llorente I. D., Pérez-Hormaeche J., Dary M., Caviedes M. A., Trinh T. H., Kondorosi A., Ratet P., Palomares A. J. (2003) *MsPG3* polygalacturonase promoter elements necessary for expression during *Sinorhizobium meliloti-Medicago truncatula* interaction. Plant Soil 257: 19-26
- Roussel H., Bruns S., Gianinazzi-Pearson V., Hahlbrock K. Franken P. (1997) Induction of a membrane intrinsic protein-encoding mRNA in arbuscular mycorrhiza and elicitor-stimulated cell suspension cultures of parsley. Plant Scienc 126: 203.2101
- Sagan M., Morandi D., Tarenghi E., Duc G. (1995) Selection of nodulation and mycorrhizal mutants in the model-plant *Medicago truncatula* (Gaertn) after gamma-ray mutagenesis. Plant Sci 111: 63-71
- Salzer P., Bonanomi A., Beyer K., Vögeli-Lange R. Aeschenbacher R. A., Lange J. A. W., Kim D., Cook D. R., Boller T. (2000) Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation and pathogen infection. Mol Plant-Microbe Interact 13: 763-777
- Sambrock J., Fritsch E. F., Maniatis T., (1989) Molecular Cloning: A Laboratory Manual Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY
- Sandal N. N., Bojsen K., Marcker K. A. (1987) A small family of nodule specific genes from soybean. Nucl Acids Res 15: 1507-1519
- Schuldt A. J., Adams J. H. J., Davidson C. M., Micklem D. R., Haseloff J. St Johnston D., Brand A. H. (1998) Miranda mediates assymmetric protein and RNA localization in the developing nervous system. Gene Dev 12: 1847-1857

References

- She Q., Lauridsen P., Stougaard J., Marcker K. A., (1993) Minimal enhancer elements of the leghemoglobin *lba* and *lbc3* gene promoters from *Glycine max* L. have different properties. Plant Mol Biol 22: 945-956
- Sigrist C. J., Cerutti L., Hulo N., Gattiker A., Falquet L., Pagni M., Bairoch A., Bucher P. (2002) PROSITE: a documented database using patterns and profiles as motif descriptors. Brief Bioinform 3: 265-274
- Smith F. A., Smith S. E. (1989) Membrane transport at the biotrophic interface: an overview. Aust J Plant Physiol 16: 33-43
- Smith F. A., Smith S. E. (1997) Structural diversity in (vesicular)-arbuscular mycorrhizal symbioses. New Phytol 137: 373-388
- Smith S. E. (1993) Transport at the mycorrhizal interface. Mycorrhiza News 5: 1-3
- Smith S. E., Gianinazzi-Pearson V. (1988) Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. Annu Rev Plant Physiol Plant Mol Biol 39: 221-244
- Smith S. E., Read D. J. (1997) Mycorrhizal symbiosis. Academic Press, London
- Stinemetz C. L., Kuzmanoff K. M., Evans M. L., Jarret H. W. (1987) Correlation between calmodulin activity and gravitropic sensitivity in primary roots of maize. Plant Physiol 84: 1337-1342
- Szabados L., Charrier B., Konodorosi A., de Bruiln F. J., Ratet P. (1995) New plant promoter and enhancer testing vectors. Mol Breed 1: 419-423
- Tang X., Nakata Y., Li H.-O., Zhang M., Gao H., Fujita A., Sakatsume O., Ohta T.,Yokoyama K. (1994) The optimization of preparation of competent cells for transformation of *E. coli*. Nucleic Acids Res 22: 2857-2858
- Tester M., Smith S. E., Smith F. A. (1987) The phenonemon of ”nonmycorrhizal“ plants. Can J Bot 65: 419-31
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res, 25:4876-4882
- Trouvelot A., Kough J. L., Gianinazzi-Pearson V. (1986) Mesure du taux de mycorhization VA d' un

système radicuaire. Recherche des méthodes d' estimation ayant une signification fonctionelle. Pages 217-221 in Mycorrhizae: Physiology and Genetics. V. Gianinazzi-Pearson and S. Gianinazzi, eds. INRA Press, France

- van Damme E. J. M., Barre A., Rougé P., Peumans P. J. (2004) Cytoplasmatic/nuclear plant lectins: A new story. Trends Plant Sci 9: 484-489
- van Parijs J., Broekaert W. F., Goldstein I. J., Peumans W. J. (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183: 258-262
- van Parijs J., Joosen H. M., Peumans W. J., Geuns J. M., van Laere A. J. (1992) Effect of the lectin UDA (*Urtica dioita* agglutinin) on germination and cell formation on *Phycomyces blacesleeanus* Burgeff. Arch Microbiol 158: 19-25
- van Rhijn P., Goldberg R. B., Hirsch A. M. (1998) *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. Plant Cell 10: 1233-1249
- Vanden Bosch K. and Stacey G. (2003) Advances in legume biology. Plant Physiol 131: 839-839
- VandenBosch K. A., Rodgers L. R., Sherrier D. J., Kishinevsky B. D. (1994) A peanut nodule lectin in infected cells and in vacuoles in the extracellular matrix of nodule parenchyma. Plant Physiol 104: 327-337
- Vantard M., Lambert A. M., Mey J. D., Picquot P., Van Eldik L. J. (1985) Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: localization in the mitotic apparatus. J Cell Biol 101: 488-499
- Versaw W. K., Chiou T. J., Harrison M. J. (2002) Phosphate transporters of *Medicago truncatula* and arbuscular mycorrhizal fungi. Plant Soil 244: 239-245
- Vierheilig H., Coughlan A. P., Wyss U., Piche Y. (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64: 5004-5007
- Vieweg M. F., Frühling M., Quandt H. J., Heim U., Bäumlein H., Pühler A., Küster H., Perlick M. (2004) The promoter of the *Vicia faba* L. leghemoglobin gene *VfLb29* is specifically activated in the infected cells of root nodules and the arbuscule-containing cells of mycorrhizal roots from different legume and non-legume plants. Mol Plant-Microbe Interact 17:62-69

References

- Vieweg M. F., Hohnjec N., Küster H. (2005) Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula.* Planta 220: 757-766
- Vivas A., Vörös I., Biró B., Barea J. M., Ruit-Lozano J. M., Azcón R (2003) Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated Cd-adapted strain of *Brevibacillus sp.* in improving plant tolerance to Cd contamination. Appl Soil Ecol 24: 177-186
- Wang J. H., Rose R. J., Donaldson B. I. (1996) *Agrobacterium* mediated transformation and expression of foreign genes in *Medicago truncatula*. Aust J Plant Physiol 23: 265-270
- Watson B. S., Asirvatham V. S., Wang L., Sumner L. W. (2003) Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiol 131: 1104-1123
- Wilcox H. (1968) Mycorrhizae. In: Plant roots – the hidden half, 2nd edn. Marcel Dekker, New York, pp. 149-174. ISBN 0-8247-6883-3
- Wulf A. Manthey K., Doll J., Perlick A. M., Linke B., Bekel T., Meyer F., Franken P., Küster H., Krajinski F. (2003) Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago trunactula*. Mol Plant-Microbe Interact 16: 306-314
- Young N. D., Mudge J., Ellis T. H. N. (2003) Legume genomes: more than peas in a pod. Curr Opin Plant Biol 6: 199-204
- Zhou J. M., Trifa Y., Silva H., Pontier D., Lam E., Shah J., Klessig D. F. (2000) NPR1 differentially interacts with members of a TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. Mol Plant-Microbe Interact 13: 191-202

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Declaration

I declare that the studies presented here were composed and carried aout by myself, using only the here mentioned materials and methods.

Furthermore, I declare that the work presented in this thesis has not been published elsewhere previously and has not been submitted as another thesis before.

I hereby affirm the truth of this statement with my signature.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Promotionsarbeit selbständig und nur unter der Verwendung der hier genannten Hilfsmittel, Methoden und Quellen verfasst habe.

Desweiteren erkläre ich, dass die vorliegende Arbeit weder als Diplomarbeit noch in anderer Form veröffentlicht wurde.

Die Richtigkeit meiner Angaben bestätige ich mit meiner Unterschrift.

Hannover,