Gene induction during plant-microbe interactions: The role of chitinases during fungal infection and the investigation of mycorrhiza-induced genes in the model plant *M. truncatula*.

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Summary

In this thesis, the model legume *Medicago truncatula* was used for research on plant-microbe interactions. Unlike most other plants, legumes are able to form two distinct root symbioses. Together with soil-borne fungi of the Glomeromycota, they form the arbuscular mycorrhiza and with rhizobial bacteria, they form nitrogen fixing root nodules. Here, plant responses to these symbiotic microbes were investigated, and compared to the plant's defence response against antagonistic microbes.
Plant chitinases have an important role in the interplay between plants and microbes; they have been shown to act as defence-related antifungal enzymes, but they seem to be involved in symbiotic processes as well. In previous work, genes encoding eight different chitinases were identified in *M. truncatula*. The main part of this thesis is dedicated to an in-depth study of these genes with regard to their regulation and function. Additionally, the expression patterns of genes that are related to the arbuscular mycorrhizal interaction of *M. truncatula* were analysed in order to further explore this important symbiosis.

In roots of the *M. truncatula* ecotype R108-1, expression of the gene *Mtchit5*, encoding a class V chitinase, was induced during nodule formation after infection with wild type rhizobia, but not in response to pathogenic fungi or arbuscular mycorrhizal fungi. *Mtchit5* transcripts were first detectable in roots forming nodule primordial and accumulated during nodule ripening. The gene was induced in response to purified Nod factors and also in ineffective white nodules formed by a mutant rhizobial strain. Phylogenetic analysis of the deduced amino acid sequence revealed that the putative Mtchit5 chitinase forms a separate clade within class V chitinases of plants. These results, together with the additional finding that Mtchit5 expression is high in flowers, indicate that Mtchit5 is a putative early nodulin that is specifically induced by rhizobia in roots and may have a function in plant developmental processes.

The gene *Mtchit4*, encoding a class IV chitinase, is induced during infection by pathogenic fungi in roots of *M. truncatula* but not during mycorrhiza formation. During nodule formation, *Mtchit4* was strongly induced only in the *M. truncatula* ecotype Jemalong A17 after infection with wild-type rhizobium strains. Its expression was elevated in nodules formed with a K-antigen deficient rhizobium mutant, but not in response to purified Nod factors. The putative Mtchit4 chitinase is closely related to pathogenesis-related class IV chitinases from other plants, and it is assumed that Mtchit4 is a pathogenesis related protein. This is supported by an additional study that revealed a low overall expression of Mtchit4 throughout the plant, independent of the plant's symbiotic status, and an in silico analysis of the Mtchit4 promoter sequence, which contains a variety of putative cis-elements related to plant defence.

The expression of two genes encoding class I chitinases, *Mtchit1a* and *Mtchit1c*, were compared to the expression of Mtchit4 in leaves, roots and flowers and after infection with a mycorrhizal fungus. In contrast to the constitutively expressed Mtchit1c, the expression of Mtchit1a was similar in leaves or roots but low in flowers. Both chitinase genes were not affected by mycorrhizal infection. The differential expression patterns, together with sequence data and in silico promoter analyses, suggest that these genes encode pathogenesis related chitinases, that are specifically regulated in response to infection by pathogenic fungi.

Mtchit3-3 is a class III chitinase gene that was specifically induced in mycorrhizal roots. The Mtchit3-3 promoter directs reporter gene expression to arbuscule containing cells, which is consistent with mycorrhiza-related elements found in the promoter sequence. Disruption of the Mtchit3-3 gene expression in root organ cultures stimulated spore germination of mycorrhizal fungi and in one fungal strain resulted in a higher probability of root colonization and spore formation. No effect on the abundance of arbuscules within colonized roots became apparent. Mtchit3-3-GFP fusion constructs revealed that the putative signal peptide could direct the Mtchit3-3 protein to the apoplast. It is suggested that the chitinase Mtchit3-3

is enzymatically active and might act on chitin in the fungal cell wall or fungal chitin-related signals during the symbiosis and it may be involved in communication processes between plant and AM.

The class III chitinase genes *Mtchit3-1* and *Mtchit3-4* are induced in response to infection by pathogenic fungi in roots of *M. truncatula*. *Mtchit3-4,* but not *Mtchit3-1,* was also slightly induced during mycorrhiza formation. According to their gene and protein structures in comparison to chitinases in other plant species and additional in silico promoter analyses, it is proposed that Mtchit3-1 is a pathogenesis related chitinase while Mtchit3-4 may be related in a general way to fungal infections.

The functionality of the arbuscular mycorrhizal symbiosis was measured by comparing the plant's nutritional status and growth response of three mycorrhizal fungi from two different phylogenetic taxa, namely *Glomus intraradices*, *Glomus mosseae* and *Scutellospora castanea.* Mycorrhiza formation enhanced biomass accumulation and nutritional status of the plants in each case, although the response was not related to the colonization degree.
To supplement the expression data of chitinase genes in relation to different fungal infections, the expression was measured in the roots of *M. truncatula* plants colonized with the three AMF. In addition, a selected set of other symbiosis related genes were tested that responded differently to the AMF colonization. It can be concluded that a subset of the genes that respond to colonization by the two *Glomus* species also responded to at least one fungus from the *Gigasporaceae*. These data indicate that different genes showing arbuscule-specific gene expression in colonized roots are regulated by different mechanisms, depending on the fungal partner.

Chapter 1

General introduction

List of figures and tables:

Abbreviations:

AM	arbuscular mycorrhiza
AMF	arbuscular mycorrhizal fungi
CCaMK	calcium calmodulin dependent protein kinase
ENOD	early nodulin gene
GH	glycosyl hydrolase
HR	hypersensitive reaction
LRR	leucine rich repeat
MAPK	mitogen-activated protein kinase
myc	mycorrhiza-related
NAD(P)H	nicotinamide-adenine-dinucleotide (phosphate), reduced form of $NAD(P)^+$
NFB	Nod factor binding
Nod	nodulation, nodule-related
NFR	Nod factor receptor
NFP	Nod factor perception
PR	pathogenesis-related
RLK	receptor like kinase
ROS	reactive oxygen species
sym	symbiotic, symbiosis-related

Scope of the thesis

Interactions of plants with microorganisms have become an increasingly important topic in plant science. One particular question is how plants are able to perceive and distinguish different microbes; how they are able to defend themselves successfully against antagonistic microbes while at the same time conferring an advantage to mutualistic ones. This field of science has made an extremely good progress in the last decade, and several important problems have been solved, not only because of the technical improvements of molecular biology. And still, for every question that has been answered, new questions arise.

Chitinases are hydrolysing enzymes that are produced ubiquitously by plants. Chitin, the substrate of chitinases, has not been found in plants but is formed by plant-associated organisms, in particular as component in the cell wall of true fungi. There are countless examples of studies showing that plant chitinases are induced in relation to stress factors and attack by antagonistic fungal pathogens. These studies indicate that chitinases are an important part of the plant's defence machinery. However, some chitinases are not induced by pathogens but in response to symbiotic interactions. Indeed, the basis for this thesis was a study of eight chitinase genes in *Medicago truncatula* roots that were found to be differentially induced upon challenges with different microbes (Salzer et al, 2000). These eight chitinases belong to five known classes, based on their amino acid sequences. As expected from previous work, most of these chitinase genes were induced in response to pathogens and two of the described genes were induced exclusively after contact of the roots with symbiotic organisms. The question arose, whether these particular chitinases have a role in symbiosis. To answer this question, the work of this thesis started with in vivo and in silico studies of the genes encoding these chitinases and of their regulation during infection with microbes.
The functional symbiosis of plants with arbuscular mycorrhizal fungi is based on the specific regulation of a large number of genes, in addition to chitinase genes. Therefore, to supplement the chitinase studies, the transcriptional changes of different mycorrhiza-specific genes in response to selected arbuscular mycorrhizal fungi were investigated.

This thesis starts with a general introduction to summarize current knowledge on plant-microbe interactions and the model systems that were used here. The subsequent chapters describe experimental results, followed by a general discussion. Each chapter starts with a short introduction to point out the most important aspects of the respective topic.

Naturally, the author of this thesis has not performed all of the work by herself, as indicated by the author lists at the beginning of each chapter. However, the author was substantially involved in the experimental setup, the performance and evaluation of all the results presented here. The use of the results for this thesis was co-ordinated with co-authors of the publications, to whom the author is truly grateful.

1.1. Interaction of plants with microbes

Plants are autotrophic organisms that are surrounded by heterotrophic microorganisms that may exploit them to acquire nutrients for their proliferation. Plants need to be able to distinguish between two kinds of microbial approaches: in unfriendly approaches, generally defined as antagonistic interactions, the microbes benefit at the expense of the plant and sometimes cause disease. In contrast, in friendly approaches, generally defined as mutualistic interactions, the plant benefits from the microbes and vice versa. The borders between antagonistic and mutualistic symbiosis are however not always clear. A given interaction may have both antagonistic and mutualistic aspects, depending on the environmental conditions or the fitness of the organisms.

1.1.1. Antagonistic interactions

1.1.1.1. Plant diseases

Many plant diseases arise through the attack by antagonistic microbes. These diseases comprise a variety of symptoms ranging from small local lesions to the death of the whole plant. However, severe plant diseases are not very common, due to the plant's efficient defence systems. Only if a microbe overcomes these defences, it enters a *compatible interaction* and it can multiply and exploit the plant. This leads in most cases to distinct disease symptoms (Jones & Takemoto, 2004). In contrast, when the microbe is warded off by the plant defence system, the interaction is *incompatible* and no disease symptoms occur.
Many pathogens are restricted in their host range, while others are more generalistic. Some pathogens with a small host range are specialized on various crop plants and cause problems for agriculture. To counter this threat, efficient fungicides and other antimicrobial agents are developed and this forms a big part of the agrochemical research and industry.

1.1.1.2. Preformed defence, passive resistance

Antagonistic microorganisms use a variety of strategies to attack plants. They all weaken, inactivate or circumvent the plant's defence mechanisms. This can be seen, for example, in the interaction of *Pseudomonas* with *Arabidopsis thaliana* (Hauck et al, 2003) and of *Cladosporium* with tomato (van den Burg et al, 2003).
Being constantly endangered by microbes in the environment, plants use a preformed defence as first protection. Preformed defence mechanisms include lignified cell walls and waxy surfaces to block an invader at the outside of the plant. Once a pathogen has penetrated these first physical barriers, it may be confronted with preformed toxic substances, including protease inhibitors or antimicrobial enzymes like chitinases and beta-1,3-glucanases (Arlorio et al, 1992; Sela-Buurlage et al, 1993).

1.1.1.3. Recognition

Elicitors
Recognition of an antagonistic microbe is a prerequisite for initiation of defence reactions (Boller, 1995; Jones & Takemoto, 2004). *Elicitors* are microbial substances characteristic for whole classes of microbes that induce defence reactions in plants. But also plant molecules generated specifically upon pathogen attack elicit such defence reactions (e.g. Mauch et al, 1988a; Ren & West, 1992; Felix et al, 1993, 1999). Abiotic elicitors exist as well; wounding, wind, flooding and touching may cause the induction of a series of defence reactions in plants (e.g. Hedrick et al, 1988; Stintzi et al,

1993). Examples for microbial elicitors are the pathogen associated molecular patterns, *PAMPs*. The PAMPs are “general elicitors”, i.e. proteins or small molecules that are highly characteristic and conserved among certain classes of microbes. They directly induce defence reactions through interaction with specific receptors on the plant cell surface (Asai et al, 2001; Gomez-Gomez & Boller, 2002). One such PAMP is chitin, an important element of the cell wall of fungi. Chitin fragments induce strong defence reactions in plants (Ren & West, 1992; Ramonell et al, 2005).

Gene-for-gene resistance
The concept of the gene-for-gene based resistance implies a highly refined form of recognition of specific pathogen products. These are encoded by microbial *avirulence genes* and are recognized by the gene products of their counterparts in incompatible host plants, the *resistance genes* (Martin et al, 1993; Jones et al, 1994; Tang et al, 1996; Parniske et al, 1997). Resistance gene products are receptors that perceive the avirulence gene products directly or indirectly. Recognition triggers the induction of defence related signalling cascades and confers a strong and specific resistance response including local necrosis, known as the *hypersensisitve response*, HR.
Resistance genes are members of large gene families that are under increased diversification pressure, as they confer specific recognition of pathogens and are therefore targets of selection in the coevolution of plants and microbes (Bishop, 2000; Jones & Dangl, 2006).

1.1.1.4. Local defence responses

Typically, addition of elicitors such as microbial fragments or chitin oligomers induces defence reactions (Felix et al, 1993, 1999; Ramonell et al, 2005). Certain reactions can be elicited also by jasmonic acid and ethylene, two plant hormones that are part of the defence machinery itself, showing that these reactions are part of an elaborated system to act in a fast and efficient way upon an attack. Global analyses of gene expression have shown that several hundreds of genes are up-regulated in response to elicitors; among them are previously known defence related genes, but also protein kinases and phosphatases, WRKY transcription factors and others (Ramonell & Somerville, 2002).

The hypersensitive response
One reaction to pathogen infection is the hypersensitive response, *HR*. The HR is characterized by rapid, localized biochemical defence, such as the production of ROS, reorganization of the cytoplasm, apposition of material to the cell walls in the proximity of the invading pathogen and the synthesis of phytoalexins and pathogenesis-related (*PR*) proteins. The HR results in the death of cells around the infection site, which prevents the infection to spread through the tissue (Lamb & Dixon, 1997; Kombrink & Schmelzer, 2001).

Reactive oxygen species
An important fast effect often associated with defence responses is the oxidative burst, in which *reactive oxygen species*, ROS, are rapidly formed. ROS can be triggered both after general elicitor or PAMP perception and during pathogen invasion. Predominant forms of ROS are superoxide and hydrogen peroxide, that are produced through the action of membrane anchored NAD(P)H-dependent oxidases and peroxidases (Lamb & Dixon, 1997; Bolwell et al, 2002). The oxidative burst goes together with transmemebrane ion fluxes, extracellular alkalinisation and protein phosphorylation (Felix et al, 1993; Peck et al, 2001; Dat et al, 2002). ROS are signal compounds that may also be produced in several internal sources like chloroplasts or peroxisomes in relation to abiotic stresses (Dat et al, 2002).
Depending on the location and dose, ROS can activate diverse signalling cascades or act directly as toxic compounds. They also cause modification of apoplastic structures and are furthermore known to be involved in senescence and cell death.

Transcriptional activation induces synthesis of phytoalexins
The range of pathogen induced defence reactions includes the production of phytolalexins, a group of low molecular substances that inhibit microbes at the site of pathogen contact. The activation of diverse phytoalexin synthesis pathways occurs within a short time after elicitor treatment or infection with pathogenic fungi (Ebel & Grisebach, 1988). Some of the phytoalexins are sesquiterpenoids, polyacetylenes, stilbenoids and isoflavonoids such as glyceollin in soybean (Ebel & Grisebach, 1988).

1.1.1.5. Systemic defence responses

Local infection by a pathogen may sometimes confer a certain resistance against infection or stimuli not only at the infection site, but also in distal tissues. There are two forms of this phenomenon: The *systemic acquired resistance*, SAR, which is established after a pathogen infection (Grant & Lamb, 2006) and the *induced systemic resistance*, ISR, which is conferred by non pathogenic, plant growth promoting bacteria (Sticher et al, 1997; Pieterse et al, 2003).

Systemic acquired resistance
SAR occurs in certain plants that have been infected with a pathogen at one site and there develop a HR. These plants develop resistance in distal organs against the same or even different pathogens. The SAR is mediated through salicylic acid signalling pathways (Heath, 2000; Grant & Lamb, 2006) and is generally associated with the systemic induction of pathogenesis related (PR) proteins such as chitinases or beta-1,3-glucanases and thaumatin-like substances (Kombrink & Schmelzer, 2001). The effect of SAR can last up to weeks or even months (Sticher et al, 1997).

Induced systemic resistances
The ISR confers resistance to above-ground plant parts after infection of roots with rhizobacteria, such as certain *Pseudomonas* strains. ISR acts against a broad spectrum of pathogenic organisms, including fungi, bacteria and viruses.
The ISR is induced through a different signalling pathway than the SAR and requires jasmonic acid and ethylene as signalling compounds. These phytohormones are acting synergistically, together with other factors such as bacterial siderophores and *O*-antigen or nitric oxide (e.g. Heath, 2000; Grant & Lamb, 2006). In contrast to SAR, the ISR does not involve enhanced formation of PR proteins, but the two mechanisms of resistance are compatible and additive when induced simultaneously.

1.1.1.6. PR proteins

Pathogen infection leads to local and often systemic de novo formation of many proteins, such as chalcone isomerase and other enzymes of the phenylpropanoid pathway leading to the production of phytoalexins and lignins and enzymes that are part of the biosynthesis pathways of defence-related phytohormones (Gianinazzi-Pearson et al, 1996; Kapulnik, 1996). A special class among these newly formed proteins are the pathogenesis-related proteins, the *PR* proteins (van Loon, 1985). They include chitinases and beta-1,3-glucanases that are evidently directed against the main components of fungal cell walls (Mauch et al, 1988b; Sela-Buurlage et al, 1993).
The expression of PR proteins is developmentally, tissue or organ specifically regulated, and generally tightly organized. They are accumulating in the intercellular spaces as well as vacuoles of various plant cells during interaction with pathogenic microorganisms (Neuhaus et al, 1991). Typically, they are resistant to proteolytic degradation or acidic pH. PR proteins are induced upon different stimuli from viruses, bacteria or fungi and elicitor treatment (e.g. Boller et al, 1983; Stintzi et al, 1993), but in some cases, they can also be induced by abiotic stress, such as drought, salinity, wounding or heavy metals (e.g. Lawton & Lamb, 1987; Stintzi et al, 1993).

1.1.2. Mutualistic interactions of plants with fungi: the arbuscular mycorrhiza as an example

The mutualistic symbiosis between fungi and plants was named mycorrhiza, after the Greek words for *myces* and *rhiza*, standing for *Fungus* and *Root* respectively. Since then, a series of definitions were given, using amongst others the words mutualistic, symbiotic, beneficial, supportive to describe the mycorrhizal association. Brundrett summarized previous attempts to categorise the mycorrhiza, and compared the different types of mycorrhizae, separating it from other plant-fungal interactions. "*A mycorrhiza is: a symbiotic association essential for one or both partners, between a fungus (specialised for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer. Mycorrhizas occur in a specialised plant organ where intimate contact results from synchronised plant-fungus development."* (Brundrett, 2004). Most plants form mycorrhiza, but there are some exceptions. For example the members of the *Brassicaceae* and *Chenopodiaceae* families are unable to enter any mutualistic fungal symbiosis.
There are several forms of mycorrhizae, which are to a certain extent dependent on the host-fungus species compatibility. The *outer* or *ecto*-mycorrhiza is mainly formed between trees and fungi of the *Basidiomycetes* or *Ascomycetes*. The shortened and thickened plant roots host a hyphal network in the outer root layers forming the Hartig net as nutrient exchange unit. The fungus also forms an extraradical mycelium to acquire mineral nutrients in the soil (Tagu & Martin, 1996). Morphologically, there is a transition with unclear borders from *ecto*- to *ectendo*- to the *inner* or *endo*-mycorrhiza. The endomycorrhiza is characterized by the intracellular localization of the nutrient exchange unit, often in the form of a tree-like structure, the *arbuscule*, in the root cortex of mainly herbous plant species. Therefore this endomycorrhiza is also termed the *arbuscular mycorrhiza* (AM) or *vesicular arbuscular mycorrhiza* (VAM).

1.1.2.1. Arbuscular mycorrhiza

The symbiosis of plants with arbuscular mycorrhizal fungi is one of the most widespread and oldest symbiotic life forms, and is thought to date back to the Ordovician, 460 Mio years ago (Remy et al, 1994; Redecker et al, 2000). The fungi in these mutualistic associations are members of the fungal phylum *Glomeromycota* that are able to colonize over 80% of all plant species (Schüssler et al, 2001).

Fungal importance and biodiversity
The fungi are completely dependent in their nutrition and reproduction on their host plants. The plants, in return, benefit from the fungi: it has been shown that they become more resistant to drought, phosphate- and nitrogen starvation (St Arnaud et al, 1995; Gianinazzi-Pearson, 1996). Especially in nutrient poor soils, the fungi with their small and flexible hyphae are able to reach water and nutrient reservoirs where plants cannot reach them. These goods are transported through the fungal network into the plant roots, where they are exchanged for plant derived sugars, the photosynthetic products. A healthy AM community with intact soil structures is of big agricultural importance, as symbiotic plants achieve higher biomass and crop yields particularly in nutrient poor soils. Increased overall plant fitness and resistance against nematodes or pathogens can, to a certain extent, prevent high input of agrochemicals.
It is also clear that the mycorrhizal symbiosis cannot be seen only as a relation between a plant and its fungus, but that it is embedded into a soil community, which is referred to as the rhizosphere (Rambelli, 1973). In this, the mycorrhizal roots form the largest root fraction and AMF networks are ubiquitous, and it might therefore be referred to as the mycorrhizosphere (Johansson et al, 2004). AMF provide a key component of the microbial population and most interactions are depending on their presence. This means that the fungal symbiosis is not only of importance for the fitness of single plants but it is crucial for the maintenance of biodiversity in plant and soil communities. Increased diversity of AMF causes an increase in plant biodiversity (Van der Heijden et al, 1998) as well as in microbial diversity (Johansson et al, 2004).

Fungal classification
Arbuscular mycorrhizal fungi are asexual, multinucleate soil living fungi. They do not form fruit bodies but small, multinucleate spores with layered walls. These spores are able to survive for a long time, and over periods of dryness and heat. The phylum *Glomeromycota* is a sister clade of Basidiomycota and Ascomycota, and was only recently defined (Schüssler et al, 2001). It currently comprises approximately 200 described species distributed among ten genera, most of which were defined primarily by spore morphology. But lately, also DNA sequences have been used to describe AMF taxa and their phylogenetic relationship (Figure 1).
While the major concepts of mycorrhizal functioning, the exchange of nutrients and metabolites, were proposed in the 1960s, their verification at the molecular level only started approximately 10 years ago (reviewed by Koide & Mosse, 2004).

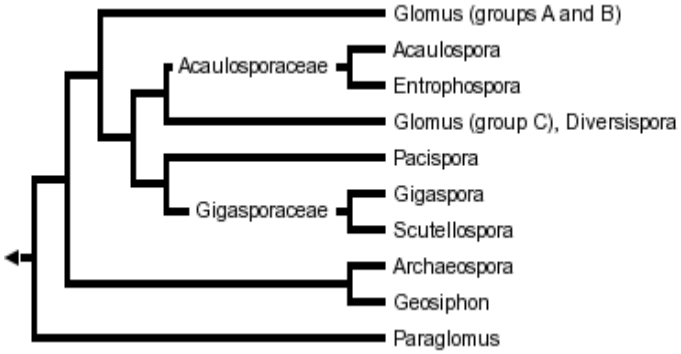

Figure 1 Phylogenetic tree based on analyses of ribosomal small subunit sequences. *Glomus* subgroups as defined by (Schwarzott et al, 2001). Tree taken from http://www.tolweb.org/tree?group=Glomeromycota, visited August 2006.

Classification of AMF is done via morphological characteristics. Spores are analyzed under the microscope, with different staining methods that make spore walls distinguishable. The number and ornamentation of the outer or inner cell walls are characteristic for each species. Although these characteristics are specific, they can be different dependent on the status of the spores; aged, dry or decaying spores may look different, even if they belong to the same species. Spores that were isolated from different soils can again look different. However, to distinguish and classify spores by morphological methods is quite difficult and needs expert knowledge.
Nowadays, molecular tools are established for AMF phylogenetic analyses. These tools comprise amplification of fungal DNA with specific primers, and the analysis and comparison of the amplified DNA sequences (e.g. Redecker, 2000). DNA sequences are independent of age and status of the spores, and can be amplified directly out of root material, independent from spores. Therefore, the phylogenetic relations of the AM fungi are being reorganized as the tools of molecular biology deliver new results.

1.1.2.2. The AMF life cycle

Contact and attachment to the roots
The AMF colonization process begins with fungal spore germination (Figure 2 A, B). Spore germination, first hyphal growth and branching are stimulated by root exudates from potential host plants, whereas non host plants have no effect on the hyphal growth or even are inhibitory (e.g. Buee et al, 2000; Gianinazzi-Pearson,1996). These preinfection processes are stimulated by signal molecules, synthesized and secreted by the roots as root exudates, and volatiles such as CO_2 (Gadkar et al, 2001; Bécard & Piché, 1989). Spores are also able to germinate, and hyphae grow without the presence of roots. However, in the absence of possible host roots after some time the germinating hyphae cease and decay. Branching factor, an only recently identified sequiterpene

(Akiyama et al, 2005; Parniske, 2005), causes the germinating hyphae to grow in a fine and highly branched mycelium. Branching is thought to enhance the chances for the hyphae to reach and contact a root (Figure 1 C).
Similar to the branching factor, which is produced by plants as compatibility signal to the fungus, the branched fungal hyphae seem to secrete a diffusible signal to the roots. This signal leads to a controlled induction of the symbiosis program in root areas that are in actual contact with the fungus, including the expression of symbiosis-related genes like ENOD11 (Chabaud et al, 2002; Kosuta et al, 2003).

Appressorium formation, entry into the root
When hyphae get into contact with a host plant root hyphal tips swell and form appressoria at the root epidermis (Figures 2 D). Appressoria are attachment structures formed on the root surface, from which the fungi can enter into the roots (Figure 3), which are triggered from topological signals on the cell wall surface. It was shown that appressorium structures can be formed on isolated root epidermal cell walls, but not on cortical or vascular cells (Gadkar et al, 2001). On these cells no penetration was observed. Many *myc*$^-$ mutants are blocked at this stage (e.g. Gao et al, 2004; Gianinazzi-Pearson, 1996; David-Schwartz et al, 2003; Paszkowski et al, 2006) and non host plants do not support appressorium formation.
Appressorium formation is followed by penetration of the upper cell layers of the root and proliferation of intraradical hyphae (Figure 2 E). Penetration into roots is achieved by wall-degrading hydrolytic enzymes by the fungus, and the hydrostatic pressure of the hyphal tip. The entry of the fungus through the epidermis into the outermost cell layers is assisted by the plant by forming an opening a cleft at the middle lamella (Demchenko et al, 2004) and the establishment of a special cytoskeletal arrangement, the prepenetration apparatus (Genre et al, 2005).

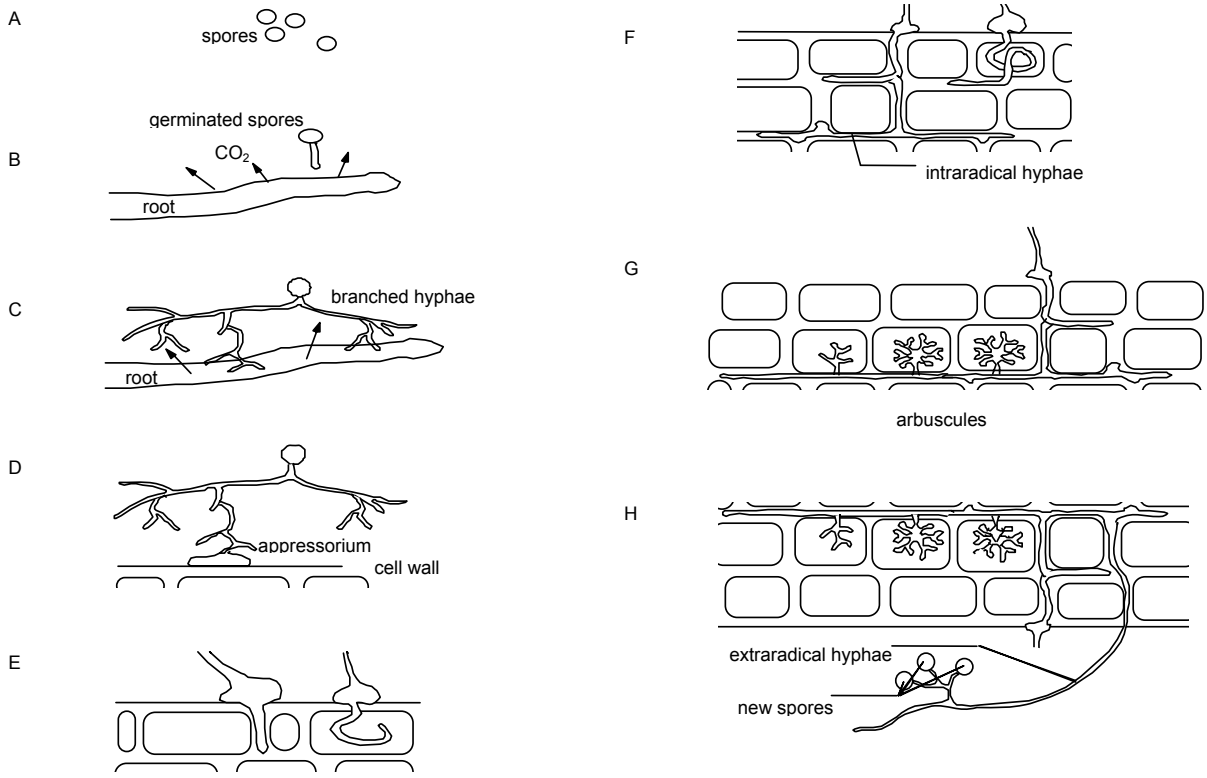

Figure 2 Life cycle of an AMF. The entry into host roots, intraradical growth and generation of offspring spores. Scheme originally designed by P. Salzer (unpublished).

Arbuscule formation and completion of the fungal life cycle

After entry into the root, fungal hyphae grow intercellularly through the root until they reach the inner cortical cell layers (Figure 2 F). The intraradical hyphae ultimately start to penetrate cortical cell walls and form intracellular hyphal structures, the arbuscules. Depending on the type of mycorrhiza, heavily curled (*Paris* type) or highly branched (*Arum* type; Figure 3) haustorial structures (Smith & Smith, 1997) are developed, generally in neighbouring cells, forming patches of arbusculate cells (Figure 2 G). However, at no time point, the fungus enters the plant cytoplasm. In the arbuscule-containing cells a periarbuscular membrane is formed that surrounds the arbuscule with an extremely large contact surface between the two symbionts. This is the place where nutrients are transferred between the plant and the fungus, mediated through a series of specific proteins (e.g. Liu et al, 2003; Wulf et al, 2003). Despite their central role in nutrient exchange, arbuscules have a relatively short life, already after a few days or weeks they collapse and become degraded. Aging and collapse of arbuscules goes together with the formation of intraradical septae (Dickson & Smith, 2001) and the decrease of phosphate transporters that are involved in nutrient exchange (Harrison et al, 2002). The former arbuscule-containing plant cell is not damaged by the arbuscule degradation and can host further arbuscules. Colonization of the plant root spans in some cases large areas of the roots, often with hyphal bridges from one cell layer to the other. Apart from arbuscules, some AMF develop vesicles, thin-walled, lipid-filled structures, thought to serve for lipid storage. To finish its life cycle the fungus exits the root again followed by extensive growth of external hyphae and sporulates in the soil (Figure 2 H).

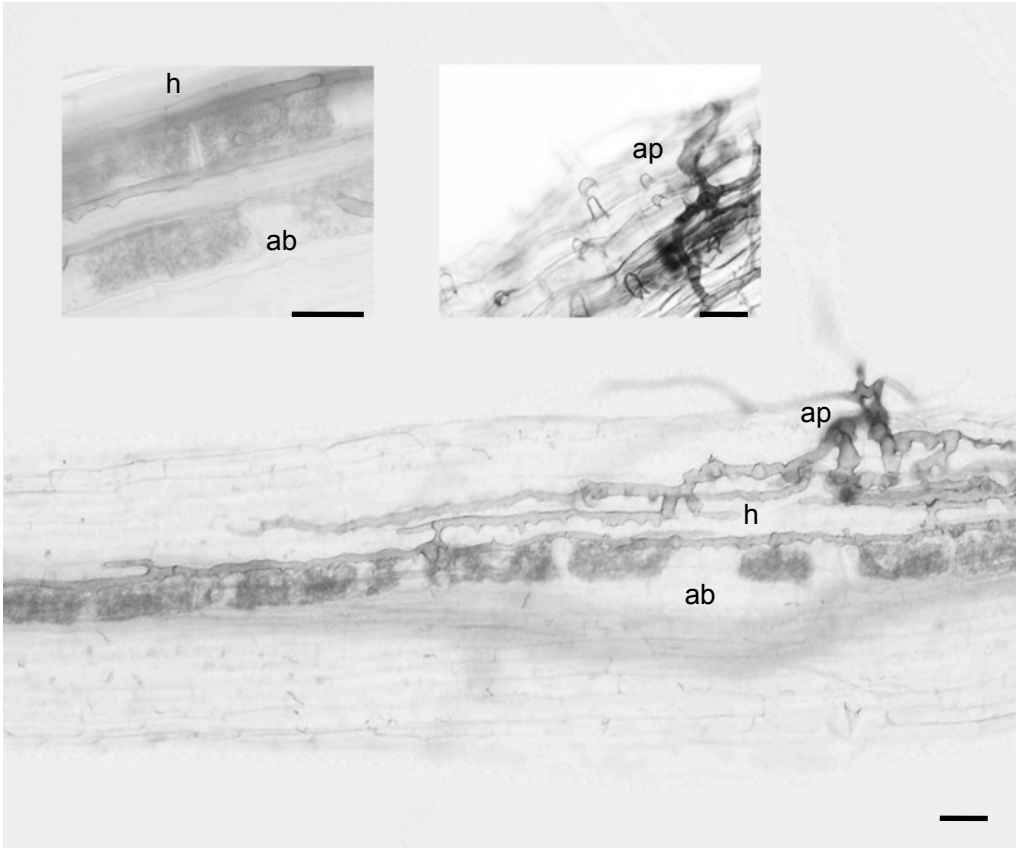

Figure 3 Pictures of appressoria (ap), arbuscules (ab) and intraradical hyphae (h) of strawberry roots colonized by *G. mosseae*. Bars equal 30 µm . Pictures by N. Feddermann.

1.1.2.3. Metabolite exchange

Plant roots are constantly expanding, growing into new areas of the soil and using the nutrients and water therein. During root growth the younger parts of the root grow into regions of the soil that are rich in nutrients. These younger parts of the root and root hairs are able to take up phosphate, nitrate and other nutrients directly from the soil. The older root parts are in a nutrient poor zone, in particular in phosphorus, which is quickly depleted and moves very slowly through diffusion. Furthermore, nutrients are heterogeneously distributed in the soil, which makes it also difficult for the plants to reach nutrients. Thus, mainly older root parts are dependent on mycorrhiza for supply of nutrients, as AMF are able to reach into the zones that are not depleted yet.

Mineral nutrient uptake in arbuscular cells through specific membrane transporters
Phosphate is one of the most important mineral nutrients for all living organisms. It is needed as structural component in nucleic acids and proteins as well as in energy turnover, protein regulation and other functions. Most of the phosphate in soil is inorganic and fixed in practically insoluble minerals. A small fraction is present in organic compounds, such as plant-derived phytate. Thus, phosphate is present in soils in relatively high amounts, but most of it is not freely accessible for plants. In many soils phosphorus is a growth limiting factor for plants.
Plants secrete phosphatases into the rhizosphere to hydrolyze phosphate from organic compounds (Joner et al, 2000). Specialized high affinity phosphate transporters, MtPt1 and MtPt2, which are highly induced in phosphate starved plants, are located at the root epidermis. These specialized transporters are responsible for uptake of solubilised phosphate via a *direct pathway*, directly from the soil into the roots (Liu et al, 1998).

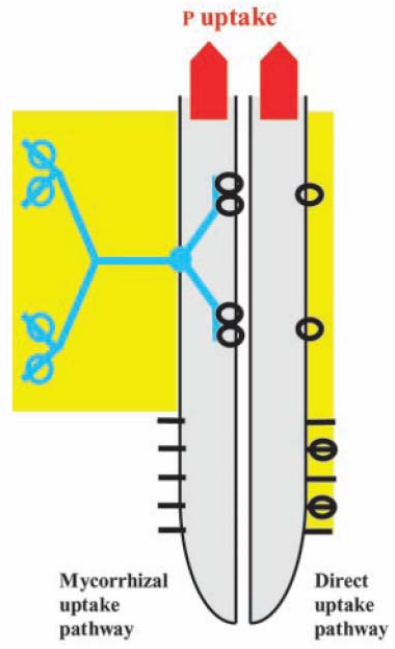

Figure 4 `Diagrammatic representation (not to scale) of direct and mycorrhizal uptake pathways into plant roots. In the direct pathway, high-affinity plant P transporters (black circles) located in the epidermis and root hairs are involved in uptake of orthophosphate from the soil solution directly into plant cells. If the rate of uptake exceeds the rate of diffusion of P in the soil solution, the concentration of P is reduced leading to 1- to 2-mm zones of depletion (narrow yellow band) close to the root surfaces, which limit the rate of uptake. The mycorrhizal pathway involves uptake of P from the soil solution by AM fungal transporters (blue circles) located in external hyphae. P is then translocated rapidly over considerable distances (1-15 cm) and is delivered to fungus-plant interfaces in the root cortex. Plant P transporters located at these interfaces (black circles) absorb P into root cortical cells.´ Picture and text from (Smith et al, 2003, Figure 1)

As depicted in Figure 4, the *indirect pathway* delivers phosphate into the root cortex through fungal hyphae, while epidermal phosphate transporters are downregulated during the AM. This seems to be the dominant way in most plants and probably accounts for most if not all of the inorganic phosphate (Pi) acquisition (Pearson & Jacobsen 1993; Smith et al, 2003).
Phosphate is taken up from the soil by AMF through fungal phosphate transporters, of which the ones from *G. versiforme*, *G. intraradices* and *G. mosseae* have been characterized (Harrison & van Buuren, 1995; Maldonado-Mendoza et al, 2001; Benedetto et al, 2005). Within the extraradical fungal

hyphae phosphate is condensed to polyphosphate, and transported into the intraradical hyphae. At the intraradical hyphae, more exactly in the arbuscules, polyphosphate is hydrolysed by enhanced phosphatase activity and released into the periarbuscular space (Solaiman & Saito, 2001; Ohtomo & Saito, 2005). Phosphate is then taken up and incorporated into the plant cortex cells through a low affinity transmembrane transporter, such as the *Medicago truncatula* MtPt4, which is present in the periarbuscular membrane (Harrison et al, 2002). Of the phosphate transporter gene family, *MtPt4* is the only one that is strongly upregulated during symbiosis, and corresponding genes have been identified and characterized in potato, *StPt3*, and tomato, *LePt1*, (Rausch et al, 2001). Functional transporter genes have also been identified in rice, maize and wheat (Paszkowski et al, 2002; Guimil et al, 2005; Glassop et al, 2005), although in these species they are not orthologous to MtPt4 (Paszkowski et al, 2002). Expression of *MtPt4* was found to be correlated with the degree of mycorrhizal colonization (Isayenkov et al, 2004) and thus can be used as mycorrhizal marker.
The mycorrhizal symbiosis is also important with respect to another nutrient, nitrogen. Inorganic nitrogen is taken up by the fungus in the extraradical hyphae, and incorporated into arginine (Jin et al, 2005), transported to the intraradical structures, where it is released as ammonia after arginine breakdown and then translocated into the plant cortex cells via ammonia channels (Govindarajulu et al, 2005). Furthermore, several nitrate, zink or copper transporters of the plant have been shown to be expressed in high amounts in mycorrhizal roots (e.g. Hohnjec et al, 2005; Frenzel et al, 2005; Burleigh et al, 2003). It is interesting, that members of the nitrate and zink transporter gene families are differentially regulated, like the members of the phosphate transporter gene family, showing upregulation of several genes, while others are reduced in mycorrhizal roots (Liu et al, 2003; Hohnjec et al, 2005).
The uptake of phosphate and other nutrients by arbuscular plant cells may be linked to a high plant and fungal H^+-ATPase activity observed at the periarbuscular membrane (Gianinazzi-Pearson et al, 2000; Krajinski et al, 2002; Requena et al, 2003). H^+-ATPases are assumed to drive the transmembrane proton gradient that is required for some of the transmembrane transporter activities and are responsible for an acidic pH in the periarbuscular space. Other genes induced in the arbuscular cells are for instance several multifunctional aquaporins with yet unknown functions that are probably involved in signalling (Küster et al, 2004), as well as cytochromes (Hohnjec et al, 2005), or glutathione-S-transferase, which are involved in energy acquisition (Wulf et al, 2003).
Although most of the nutrient transfer between the two symbiotic partners takes place in the arbuscules, the arbuscules do not seem to be the sole place of nutrient exchange. It is almost certain that nutrient transfer may additionally occur at the intracellular hyphae (Gianinazzi-Pearson et al, 1991).

Plant derived carbon as energy source for AMF
The completion of the AMF life cycle is entirely dependent on the plant symbiosis. Fungal growth, spore production and mycelial transport activities require a high amount of energy. Energy in form of organic carbon is obtained from plant derived sugars, in some cases a large fraction of the photosynthesis products are delivered to the fungus (Wright et al, 1998). For the symbiotic fungus 100% of all carbohydrates are supplied by the plant, as extraradical hyphae, in contrast to intraradical hyphae, do not absorb and utilize sugars like glucose and fructose (Solaiman & Saito, 1997; Bago et al, 1999; Pfeffer et al, 1999). It has to be noted that the intraradical and extraradical mycelium together with the spores form one continuum, in which anastomoses may connect the plasma of converging extraradical hyphae (Giovannetti et al, 2001). Yet, an intraradical and an extraradical hyphal carbon metabolism can be separated.
The major storage forms of carbon in spores and hyphae are lipids, trehalose and glycogen (Bécard et al, 1991; Bonfante et al, 1994; Shachar-Hill et al, 1995; Bago et al, 1999, 2003; Pfeffer et al 1999). Of these, lipids appear to be the main storage and transport form in the extraradical hyphae, but also carbohydrates may also be transported, although this might be less favourable because of osmotic effects (Shachar-Hill et al, 1995; Solaiman & Saito, 1997). Photosynthetic assimilates are supplied to the root cells in the form of sucrose. Most likely, in the arbuscules, assimilate transfer includes export across the plant plasma membrane and active uptake across the fungal plasma membrane, driven by an increased H^+-ATPase activity at the arbuscular membrane (Gianinazzi-Pearson et al, 1991,

2000). In the fungal cytosol the assimilates are then converted into triacylglycerides, amino acids or incorporated into glycogen pools.

Mycorrhizal roots are sink tissues
Utilization of sucrose as a source of carbon and energy by the fungus at the plant-fungus interface depends on its cleavage into hexoses. On the plant side this is catalysed by differentially expressed acid invertases, alkaline invertases and sucrose synthases that are found in arbuscule containing cells and are regarded as key enzymes for sucrose turnover in these cells (Blee & Anderson, 1998, 2002; Hohnjec et al, 2003; Ravnskov et al, 2003). As the action of invertases is irreversible, hydrolysis from sucrose into glucose and fructose is responsible for creation of a sucrose gradient between source and sink tissues and by cleavage of sucrose the osmotic pressure is increased (Blee & Anderson, 1998; Hohnjec et al, 2003; Ravnskov et al, 2003). One of the plant hexose transporters, MtSt1, (Harrison, 1996) could be involved in the regulation of periarbuscular sugar content in these cells, and so regulate the activity of the sucrose turnover in cortical mycorrhizal cells.
Plants seem to control their sugar supply to the fungus carefully, in order to keep their costs to benefit ratio equilibrated. Higher demand of sugar in the roots causes higher production demands on fixed carbon from photosynthesis, mainly sucrose, and thus increases the amount of photosynthetically active organs (Wright et al, 1998). This is also evident from plants grown under limiting light conditions; they develop less AMF colonization. On the other hand, plants that are phosphate or nitrogen starved allow a higher degree of colonization in order to increase their nutrition status via the fungal supply. Nevertheless, the concept of functional diversity among fungal and plant partners implies that nutrient acquisition is not necessarily a determining factor for the host compatibility and colonization ability. The strength of the sugar sink in arbuscular roots is dependent on the physiological requirements of the partners and not only on their genotypic compatibility (Burleigh et al, 2002; Smith et al, 2003). In fact, the plant phosphate uptake from the fungus is not directly influenced by the phosphate supply by the fungus. But in contrast, the phosphate uptake of fungi from the soil and translocation into the intraradical compartment is determined by the sugar supply from the plant (Bücking & Shachar-Hill, 2005).

1.1.3. Mutualistic interactions of plants with bacteria: the nodule symbiosis as an example

Nitrogen is an essential nutrient for all living organisms. It is incorporated into biological compounds such as nucleic acids and proteins. Although nitrogen is present in the atmosphere in vast amounts, the nitrogen pool is not available for most organisms because of the inert chemical nature of dinitrogen, its main atmospheric form. The only organisms that are capable of incorporating nitrogen into organic compounds, under high costs of energy, are diazotrophic bacteria. A large fraction of these bacteria live in soils, of which two groups establish symbiosis with plants: *Frankia* establish symbioses with a wide range of host plants such as *Alnus*, *Casuarina*, *Hippophae* and *Myrica*. The *Rhizobia* (Figure 5) induce nodules in roots of legumes and will be considered in detail below.
The symbiotic fixation of nitrogen is an important contribution to the atmospheric nitrogen turnover. It is also of special interest to agriculture, as nitrogen is a limiting nutrient for plants and has to be provided as fertilizer in many systems. In contrast, symbiotically fixed nitrogen is a natural and cheap source for agronomical important plants. The soils are enriched in nitrogen via the symbiotic activity and thus the careful use of legumes in farming, as e.g. in crop rotations, is advantageous for soils and sustainable environment.

1.1.3.1. Legumes establish a mutualistic symbiosis with rhizobia

Almost all legumes enter symbioses with gram-negative soil bacteria of the family *Rhizobiaceae* with many members (Figure 5). The name *Rhizobium* comes from the Greek, meaning *root living*. The

nodulating genera of the *Rhizobiaceae* are the *Sinorhizobium* (*Rhizobium*), *Mesorhizobium, Bradyrhizobium, Allorhizobium* and *Azorhizobium*, which are generally termed *Rhizobia*. Rhizobia differ in their host specificity. The host range can be relatively narrow (e.g. *S. meliloti*) and comprises in some cases only one plant species (Perret et al, 2000).

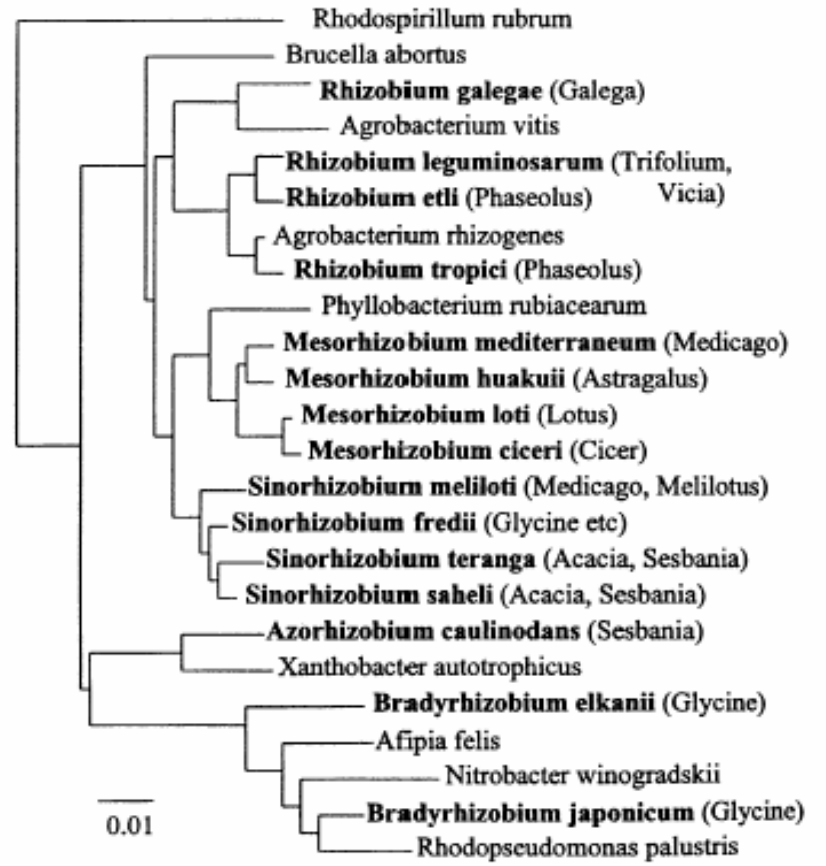

Figure 5 16S rDNA phylogenetic tree of rhizobia (bold letters) and related bacteria. The plant hosts are indicated in brackets: rhizobia of different phylogenetic branches are able to nodulate the same plant species. Picture taken and text modified after Debellé et al (2001).

1.1.3.2. Rhizobial life cycle and nitrogen fixation

Plant rhizobium contact; bacterial Nod factor structure is crucial for host compatibility
Plant-secreted secondary metabolites, mainly flavonoids, trigger the activation of bacterial *nodulation genes (nodA, nodB, nodC etc.*) which results in the biosynthesis and secretion of *Nod factors*. Nod factors are lipo-chitooligosaccharide based bacterial signal molecules that are essential for rhizobial infection. The Nod factor backbones are synthesized by the *nodA, nodB, nodC* genes, which are essential for Nod factor synthesis and common to all rhizobial strains. The *nodD* gene codes for a transcriptional activator that induces the transcription of the *nodABC* operon in a host specific manner (Honma et al, 1990). Other *nod* genes are species specific and determine host specificity by adding a variety of decorations to the Nod factors (reviewed by Perret et al, 2000) and so, each bacterial strain produces a spectrum of Nod factors (see also Figure 10). Nod factors are also perceived by non host plant species and to a certain degree cause specific reactions (Staehelin et al, 1994; Müller et al, 2000; Yokoyama et al, 2000). It could be shown that the Nod factor structure is not correlated with the taxonomic position of the bacterial species but rather with its *nod* gene sequence. Mutations in these genes result in alterations in host specificity by alterations in Nod factor features

(Roche et al, 1999; Debellé et al, 2001). The compatibility of host plant and bacterial strain is strongly dependent on the structure of Nod factors but for the full host establishment of the symbiosis, additional factors are required, such as the combination of polysaccharides on the bacterial cell surface (Schultze & Kondorosi, 1998).
The perception of Nod factors by host legumes triggers many of the early developmental steps in the bacteria-plant symbiosis, even in the absence of bacteria. This makes the Nod factors unique and a perfect tool for the communication between the chitin-compound producing bacteria and the chitin-compound receiving plants. Nod factors alone are sufficient to activate a majority of the early responses that are normally induced during the interaction with bacteria (e.g. Denarié et al, 1996). A reaction to Nod factors in plant root hairs occurs within minutes and in nano- or picomolar concentrations (Shaw & Long, 2003).
In the root hair cells Nod factors induce rapid ion fluxes (Ca^+, K^+, H^+, Cl^-) across the plasma membrane causing membrane depolarization (Ehrhardt et al, 1992; Gehring et al, 1997; Felle et al, 1995). By rearrangements of the actin cytoskeleton root hairs start to swell and curl, and undergo reinitiation of tip growth and deformation towards the site of Nod factor origin (Catoira et al, 2001; Esseling et al, 2003) (Figure 6 A). In the region around the nucleus calcium spiking, rhythmic Ca^+ fluctuations, occur, which are involved in signal transduction inducing downstream reactions. Early nodulins, Nod factor induced genes, are synthesized in the root hair and in the dividing cells of the root cortex cell division begins, which leads to the formation of nodule primordia.

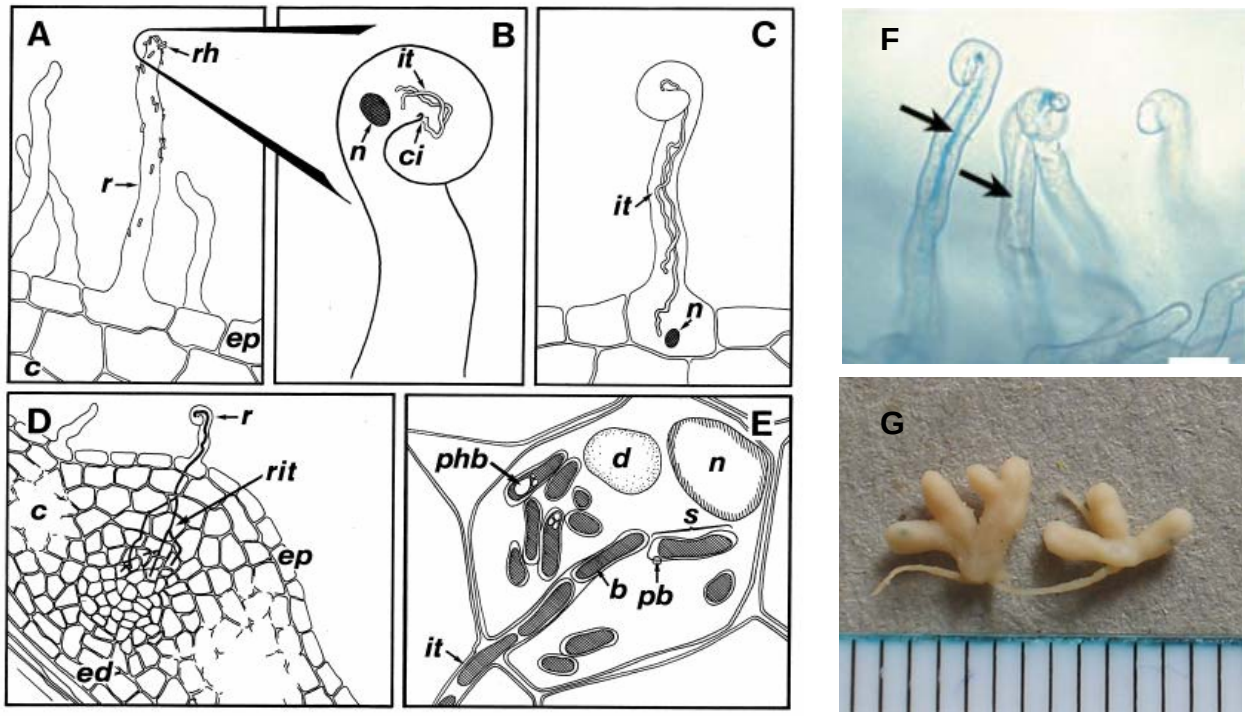

Figure 6 The formation of root nodules. A - E: Invasion of legume root hairs by *Rhizobium*. rh, rhizobia; r, root hairs; *ep*, epidermis; ci, center of infection; n, plant nucleus; it, infection thread(s); rit, ramifying infection thread; c, cortex; *ed*, endodermis; b, bacteroids; s, symbiosomes; phb, poly-β-hydroxybutarate; pb, peribacteroid membrane; *d*, digestive vacuole. Picture and legend from Perret et al (2000, Figure 1). F: Curled root hairs and infection threads (arrows) in root hairs, bar equals 50 µm. (picture from Stracke et al, 2002). G: Nodules of *M. truncatula* inoculated with *R. meliloti*, next to a 1 mm ruler. Picture by N. Feddermann.

Bacterial entrance and differentiation

Nodule development is initiated by the approaching of rhizobia towards young roots of the host plant by chemotaxis (Figure 6 A). Bacteria assemble at the tip of root hairs and are encaved by the curling root hair tip (Figure 6 B). *Infection threads*, specialized tubular structures, are subsequently formed at the root hair tip by plasma membrane invaginations (Figure 6 C). The infection thread grows towards the nodule primordium guided by the root hair structure. In certain plants, a preformed cortex structure, the preinfection thread, is established, which joins the infection thread in the root cortex (Kannenberg et al, 1994). The infection thread then ramifies (Figure 6 D) and forms a network in the underlying root cortex, through which bacteria are transported and enter the cortex cells by endocytosis. They differentiate into bacteroids, a specialized symbiotic form of the bacteria (Figure 6 E). The bacteroids, which proliferate by cell division, reside in the cytoplasm of the plant cells but are enclosed by a plant derived membrane, the peribacteroid membrane and its own bacteroid membrane (Day et al, 2000): these nitrogen-fixing organelles are called symbiosomes. Each symbiotic plant cell may contain several hundreds of symbiosomes. After infection of the primordium cells the formation of a new organ, the root nodule, is initiated.

Nodule formation

Nodules of two types are observed in legumes, which differ in their morphology and life span. Determinate nodules are formed in e.g. *Lotus japonicus* or *Glycine max*. In contrast to determinate nodules, the indeterminate nodules have a persistent meristem and are formed for example in *Medicago truncatula*, *Pisum sativum*, *Vicia faba* or *Trifolium repens* (Pawlowski & Bisseling, 1996; Müller et al, 2001). The formation of indeterminate nodules in *Medicago truncatula* is well known; cells from the nodule primordium grow as an apical meristem, adding cells to the inner nodule tissues, while the nodule lengthens. As a result, a developmental gradient of infected cells is established along the nodule axis. Infected cells are located in the inner nodule zones, where cells do not divide. The zone below the meristem is the pre-infection zone, the middle and biggest zone is specified by the nitrogen-fixing zone containing bacteriods and at the distal zone, rhizobia are released from the infection thread and start to differentiate. These different nodule tissues do not only differ in their local and functional properties, but also the expression of nodulation genes is different (Pawlowski & Bisseling, 1996). The meristematic activity in determinate nodules ceases early and the nodules expand spherically by expansion of the inner nodule tissue. A radial gradient is developed in these nodules, where the oldest part is in the innermost zone.

The bacteria of both types of nodules are able to revert to free living bacteria after nodule senescence (Müller et al, 2001). Mature nodules have a stem like organization with a vascular tissue and may have adopted their developmental programme from root development. Plants can form spontaneous nodules without the presence of Nod factors or rhizobial infection, but they do not exhibit the infection pattern with infection thread formation and the typical gene expression and therefore resemble the ineffective white nodules with defective or mutant rhizobial strains (Tirichine et al, 2006a).

Nitrogen fixation by nitrogenase

Bacteroids in the nitrogen-fixing zone of the nodules reduce atmospheric nitrogen to ammonia, which is used by the plant in exchange for organic carbon that serves as energy source for the bacteria (Udvardi & Day, 1997; Day et al, 2000). Nitrogenases are protein complexes consisting of dinitrogenase and dinitrogenase reductase that contain iron/molybdenum cofactors for activity. Dinitrogenase is encoded by the bacterial gene family *nifD*, *nifK* and *nifH*, where *nif* stands for nitrogen fixation. In a reducing reaction, nitrogen is converted into ammonia, a form that can be assimilated by plants.

Although the nitrogen fixation as a whole usually requires oxygen for respiration, nodules require a low-oxygen environment for optimal activity of bacterial nitrogenase, which is very oxygen labile. This *oxygen dilemma* is overcome by a compartmented cell structure and high levels of oxygen-binding leghaemoglobins (Appleby, 1994). These plant encoded, symbiosis specific proteins provide an efficient system to keep oxygen away from the nitrogenase, having an extremely fast oxygen

association rate and a slow dissociation rate (Delgado et al, 1998). Because this leads to low levels of free oxygen in the respiratory chain, the bacteroids need a specific oxygenase, encoded by the bacterial *fixNOQP* operon, with an extremely high affinity for oxygen (Preisig et al, 1993; Delgado et al, 1998).

Nutrient transfer
Root nodules represent strong sink tissues for the plant, not only because growth and reorganisation processes need a high amount of energy but also because the bacterial symbionts require photosynthetic assimilates to fuel the nitrogen fixation. In the symbiosomes, nutrients are exchanged between the partners via the symbiotic interface, the peribacteroid membrane. Bacteroids deliver ammonia or amino acids via transmembrane channels into the peribacteroid space. These nitrogen compounds are taken up by the plant cells under action of plant derived H^+ATPases (Udvardi & Day, 1997; Day et al, 2000).
In plant cells the fixed nitrogen compounds are incorporated into glutamine that is then further promoted into other metabolites. In exchange for nitrogen the bacteria are provided with carbon compounds that have been generated by photosynthetic activities from the plant. Sucrose is transported into the infected zone of the nodules, processed into hexoses by sucrose synthases, members of the nodule essential SucS family, and invertases (Küster et al, 1993; Hohnjec et al, 2003; Day et al, 2000). Hexoses are then metabolized into organic acids, mainly malate and succinate. These are translocated via the peribacteroid membrane into the bacteroids and then incorporated into the bacterial metabolism (Day et al, 2000).

1.1.4. Recognition and signal perception in plant-microbe interactions involve transmembrane receptor-like kinases

1.1.4.1. Early steps in antagonistic plant-microbe interactions

Receptor like kinases are involved in recognition of PAMPs
In order to generate a defence reaction, plants need to chemically identify an approaching pathogen. Fungal or bacterial signal molecules are recognized by specific intracellular or membrane bound receptors that mediate a localized and rapid defence response in the respective cells (e.g. Jones & Takemoto, 2004). Important in the recognition of pathogens are the transmembrane *receptor-like kinases*, RLK, that have a *leucine rich repeat*, LRR, extracellular domain connected to a catalytic intracellular kinase domain (Asai et al, 2001; Gomez-Gomez & Boller, 2002). Certain PAMPs are perceived by the possibly dimeric LRR RLKs, and the signals are mediated via a phosphorylation regulated MAP kinase signalling cascade (Peck et al, 2001; Nühse et al, 2003).
Receptor-like kinases are diverse in their functions and are highly conserved. Plant LRR RLKs share structural homology with animal Toll-like receptors (TLR). But although for example the animal TLR5 is able to recognize the same stimulus as the FLS2 (*flagellin sensitive 2*) in *Arabidopsis thaliana*, namely bacterial flagellin (Felix et al, 1999; Hayashi et al, 2001), it recognizes a different epitope than the plant receptor, which indicates an independent evolution of the two receptor kinases (Kistner & Parniske, 2002; Jones & Takemoto, 2004).

Downstream reactions in plant defence
Within minutes after elicitor recognition, the first symptoms are efflux of Cl^- and K^+, together with influx of Ca^+ and protons. These ion fluxes lead to membrane depolarization and alkalinization of the extracellular medium (Felix et al, 1993; Nürnberger et al, 1994). The increased concentrations of cytoplasmic calcium ions are a necessary part of elicitor sensing pathways that lead to defence reactions not only in antagonistic interactions (Romeis et al, 2001; del Pozo et al, 2004). However, it

seems that plants use different cytosolic calcium elevation signatures in order to differentiate between several elicitors, including abiotic factors.
Following the rapid ion fluxes, activation and deactivation of various proteins by phosphorylation indicate the activation of certain MAPK pathways (Zhang & Klessig, 2001; Asai et al, 2001; Peck et al, 2001). It is interesting that the signals from several LRR RLKs that recognize different stimuli can be fed into the same MAPK pathway (Jones & Takemoto, 2004). In some cases, elicitor perception induces a hypersensitive response and production of reactive oxygen species, although the HR is not necessarily required for resistance (Kombrink & Schmelzer, 2001). Ultimately, elicitor perception leads to downstream reactions such as activation of phytoalexins and local expression of PR genes as well as the systemic activation of resistance-related gene expression.

1.1.4.2. Common early steps in the nodulation and formation of arbuscular mycorrhiza

It is a well accepted hypothesis that symbiotic mechanisms in nodule formation evolved at least in part from the older arbuscular mycorrhiza. Indeed, parts of the *symbiotic programs* are shared among both interactions, especially some of the early signalling events. Thus, plant mutants defective in nodulation are often also defective in the AM symbiosis, and these mutants reveal common genetic factors for the symbiosis (Kistner & Parniske, 2002).
Mutants are useful tools to genetically dissect the development of both symbioses, although the discovery of non-nodulating, *nod*$^-$, mutants is naturally easier and less labour intense as the search for non-mycorrhiza forming, *myc*$^-$, mutants. Collections of nodulation mutants are available for *Lotus japonicus*, *Pisum sativum* and *M. truncatula*. *M. truncatula* is also the main object for AM research together with tobacco, maize and tomato, in which symbiosis mutants are described (e.g. Bonfante et al, 2000; Marsh & Schultze, 2001; David-Schwartz et al, 2001; Paszkowski et al, 2006).

Nod factor receptors
Several *receptor-like kinases*, RLKs, are implicated in the recognition of symbiotic microbes. *Nod factor receptors*, NFR, most probably recognize Nod factors (Madsen et al, 2003; Radutoiu et al, 2003), but a receptor that would take part in the perception of a putative Myc factor has not been identified yet. The Nod factor-related RLKs have a weak overall similarity with the receptors implied with the defence generating RLKs and have similarities in their reaction machinery (Gomez-Gomez & Boller, 2002; Kaku et al, 2006).
Nod factors are thought to be perceived by two transmembrane RLKs forming a heterodimeric receptor complex. Their extracellular LysM (*lysin motif*) domain is believed to be responsible for the binding of the chitin-oligomeric part of the Nod factors by comparing analogies in their amino acid sequences to chitin binding proteins (Madsen et al, 2003; Radutoiu et al, 2003). In *M. truncatula* the LysM RLK gene family includes the *LysM domain-containing receptor-like kinases* LYK3 and LYK4 and *Nod factor perception* NFP (Limpens et al, 2003; Arrighi et al, 2006). The corresponding RLKs in *Lotus japonicus* are the *Nod factor receptors* NFR1 and NFR5, encoded by *SYM1* and *SYM5*, and SYM2 and SYM10 in pea (Limpens et al, 2003; Madsen et al, 2003; Radutoiu et al, 2003). Nodulation mutants of these genes are not blocked in the formation of the mycorrhizal symbiosis (Ben Amor et al, 2003). Mutant analyses indicate that the LysM receptor-like kinases are not involved in the formation of AM, suggesting that the receptors for mycorrhizal signals, the putative Myc factors, are different (Cullimore & Denarié, 2003; Mulder et al, 2006).

Early events in nodulation and mycorrhiza formation
In nodule formation, the earliest reactions associated with Nod factor perception are calcium influx together with a chloride efflux from root hair cells that cause membrane depolarization about 1 minute after Nod factor addition. Membrane repolarisation is achieved by a potassium efflux shortly after (Ehrhardt et al, 1992; Felle et al, 1995). The rapid opening of plasma membrane ion channels in response to Nod factor perception is believed to be implicated in the reorientation of root hair tip

growth (Charron et al, 2004). There is also evidence for signaling and very early reactions in the mycorrhizal symbiosis (Chabaud et al, 2002; Kosuta et al, 2003).
Recent genetic studies have identified common elements in these early reactions. The *DMI* genes, named after *Does Not Make Infection*, belong to the *nod*$^-$ and *myc*$^-$ mutants (Catoira et al, 2000). With respect to nodulation, the *dmi* mutants are blocked at different early steps, as they are partially defective in root hair deformation, calcium oscillation and gene expression (Wais et al, 2002; Catoira et al, 2000). With respect to AM formation, the *dmi* mutants are not able to form the prepenetration apparatus to allow AMF entry into the roots (Genre et al, 2005).

Downstream reactions of Nod factor binding
After perception of Nod factors by LysM RLKs, the signal is probably transmitted to a LRR RLK, which also acts early in perception of the putative Myc factor and is probably the converging step in signaling shared with the Nod factor induced signalling cascade (Figure 7) (Yoshida & Parniske, 2005). This LRR RLK is the *M. truncatula* DMI2, which corresponds to SYM19 in pea, NORK in *Medicago sativa,* and SYMRK in *Lotus japonicus* (Stracke et al, 2002; Endre et al, 2002; Geurts & Bisseling, 2002).
DMI1, SYM8 in pea, acts downstream of DMI2 and is a putative ligand-gated cation channel and possibly involved in both calcium flux and calcium spiking (Ané et al, 2004). *Castor* and *Pollux,* the *DMI1* homologues in *Lotus japonicus* (encoded by *SYM4*), have been shown to code for plastid located ion channels, although plastids are unlikely to be internal calcium stores (Bonfante et al, 2000; Imaizumi-Anraku et al, 2005). The exact function of these proteins is still unknown. Furthermore, calcium spiking was shown to depend on the function of NUP133, a gene encoding a nucleoporin specifically expressed in root hairs that is essential for the establishment of functional nodules (Kanamori et al, 2006).
The stimulus generated by Nod factors and possibly also by AMF most likely induces phospholipid signalling cascades through the activation of RLKs, and processing by small G-proteins, associated with the G-protein coupled receptors and increased levels of secondary messenger molecules (Figure 7) (Pingret et al, 1998; den Hartog et al, 2001; Charron et al, 2004). These messengers activate in turn downstream elements, including *DMI3,* SYM9 in pea. *DMI3* encodes a chimeric calcium/calmodulin-dependent kinase, CCaMK (Lévy et al, 2004; Mitra et al, 2004).

Calcium oscillations
The rhythmic, rapid efflux and subsequent influx of calcium ions from the endoplasmatic reticulum, called calcium spiking, is one of the early events in the Nod factor transduction events and takes place within 10 to 30 min after Nod factor perception (e.g. Wais et al, 2000). These oscillations in cytosolic calcium act as secondary messenger in the nodulation signalling pathway and the mycorrhizal signalling pathway as depicted in Figure 7 (e.g. Riely et al, 2004; Oldroyd & Downie, 2006).
DMI3 of *M. truncatula* encodes a calcium/calmodulin-dependent protein kinase, CCaMK, which is required for the transduction of calcium oscillations (Lévy et al, 2004; Mitra et al, 2004). CCaMK could be the calcium sensor required to translate Nod factor elicited calcium oscillations into a specific downstream signalling cascade, and it also has an important regulatory function in later nodule stages and expression of early nodulin genes, *ENODs* (Tirichine et al, 2006b). DMI3 has a calcium dependent autophosphorylation activity by which the activity of its kinase domain towards its substrate is regulated. This in turn controls the formation of nodules. Abolishing the autoregulatory function results in nodule formation without bacterial stimulation or Nod factor treatment (Gleason et al, 2006). This shows that calcium oscillations and calmodulin binding are central to the nodulin gene expression and nodule formation, and can even be restored in mutant legumes by a CCaMK from rice (Godfroy et al, 2005). In the case of mycorrhiza formation, the DMI3 CCaMK is also required for signal perception, but it is fed into a different downstream cascade, which is not activated by Nod factors. Therefore the CCamK step is the most downstream common event of the two symbioses (Weidman et al, 2004).

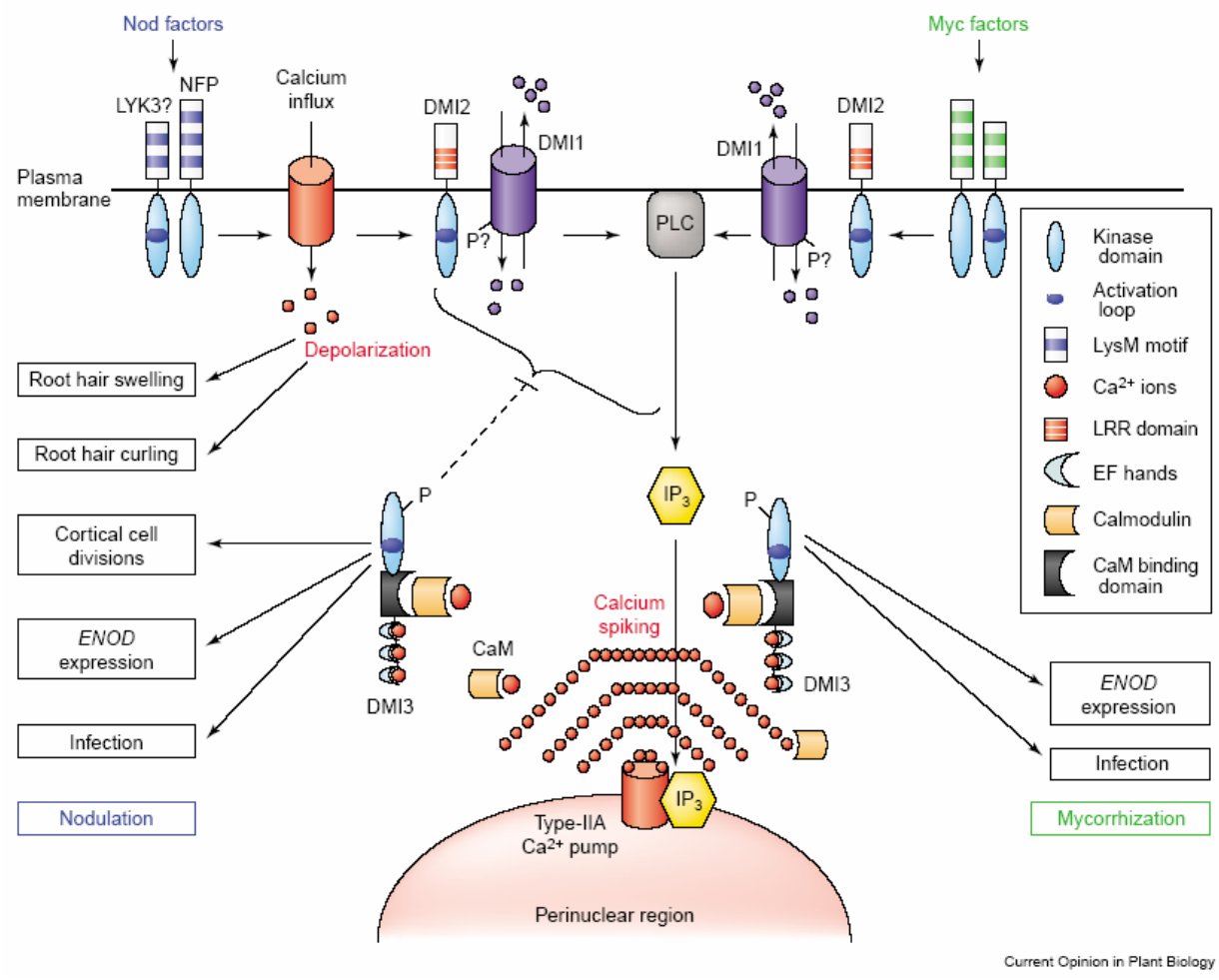

Figure 7 Proposed model of the Nod-factor signaling pathway.
Picture from Riely et al, 2004 (not including the nucleoporin NUP133).

1.1.4.3. Different plant signalling pathways are activated by different microorganisms

The perception of e.g. chitin oligomeric signalling compounds is a crucial step in the interaction of plants with microbes. In order to generate appropriate reactions, such as root hair curling prior to nodulation, plants need to discriminate between different signals. As the partly divergent signalling pathways of the mycorrhizal and rhizobial symbioses indicate, plants seem to be able to distinguish between certain signalling molecules. A chitin oligomer binding protein was recently identified, that seems to be related to plant defence and it was therefore suggested that binding of chitin molecules is involved in recognition of microbial signals (Kaku et al, 2006).
Indeed, plants seem to be able to distinguish between different classes of fungi. For example, certain symbiosis-defective *Lotus japonicus* mutants are not defective for the infection with rust fungi. Furthermore, *Arabidopsis thaliana*, which is readily infected by rust fungi but not by AMF, does not have clear orthologues of these *sym* genes. This shows that the same genes are not required for infection with an antagonistic fungus, and that symbiotic and pathogenic fungi use at least in part different entry strategies into the plant (Mellersh & Parniske, 2006).

1.1.5. Common elements in the symbiosis of plant with arbuscular mycorrhizal fungi and rhizobia

The AM and rhizobial symbioses both represent an invasion of the plant by microorganisms. Plants have evolved a tightly regulated recognition system to prevent abuse of the symbiotic system by antagonistic microorganisms. It is also important in mutualistic interactions that the rate of symbiotic structures is regulated to avoid exhaustion of the plant. It was shown that plants are able to control the amount of colonization in the AM interaction (e.g. Gao et al, 2004) and the number of nodules (e.g. Sun et al, 2006) by a mechanism that is termed the autoregulation of nodulation and mycorrhization. For example, nodulation does not take place when sufficient nitrogen is available to the plant. The autoregulation of nodulation involves a systemic signal (van Brussel et al, 2002) and this control is regulated by the shoots, which is not surprising, as shoots are the source of carbon that is delivered into the bacteroids (Nishimura et al, 2002; Krusell et al, 2002; Searle et al, 2003).

1.1.5.1. Endosymbiotic microbes trigger plant defence reactions

Mutualistic microbial entry and proliferation within the roots trigger at least some of the reactions that go together with other fungal or bacterial infections. A microbial infection is prevented for example in incompatible rhizobial strains and in those having deficiencies in their surface polysaccharide patterns, indicating that a distinct recognition pattern is required for symbiotically active microorganisms. Weak transient defence responses are observed when AMF enter host roots. Flavonoid biosynthesis (Shaw et al, 2006) and the production of ROS, reactive oxygen species (Salzer et al, 1999; Fester & Hause, 2005), are observed as well as the increased presence of general phytoalexins (Gianinazzi-Pearson, 1996). However, there is evidence that the defence mechanism of the plant is indeed actively repressed in initial stages of a symbiotic infection (Volpin et al, 1995; Bartsev et al, 2004; Demchenko et al, 2004; Guenoune et al, 2005).
ROS are produced in root cortical cells associated with intraradical hyphae but also with arbuscules in correlation with arbuscule degradation (Salzer et al, 1999; Fester & Hause, 2005). ROS are important messenger molecules generally produced after infection with fungi or bacteria. The ROS produced by senescing arbuscules lead to several reactions, for example the production of jasmonates (Hause et al, 2002) which in turn induce carotenoid synthesis and could be involved in the increased resistance against invading pathogens, as it is observed sometimes in AM colonized roots (Fester et al, 2002; Hause & Fester, 2005).
As another important group of signalling molecules, flavonoids are crucial for symbiosis establishment. In the rhizobial symbiosis, they act directly on the bacteria and stimulate bacterial gene expression by interaction with e.g. the *nodD* transcriptional regulator that induces *nodABC* expression (Broughton et al, 2000). In AMF they stimulate spore germination, but are not necessary for successful symbiosis formation (Bécard et al, 1995). A related signalling factor, the *branching factor*, has been chemically identified (Akiyama et al, 2005; Besserer et al, 2006). The presence of compatible Nod factors, and not necessarily infection by bacteria, but the accumulation of flavonoids enhance the colonization with AMF (e.g. Xie et al, 1995). On the other hand, certain flavonoids, such as medicarpin in *Medicago* and glyceollin in soybean (Ebel & Grisebach, 1988), are antimicrobial phytoalexins, secreted to prevent an attack by pathogenic microbes. This again displays the dual function of signalling molecules used by plants in reaction to their environment in very distinct ways.

1.1.5.2. Early nodulins

Calcium spiking and the action of CCamK in the plant-rhizobium symbiosis appear to activate transcription factors, which in turn seem to have substantial roles in symbiosis. In nodule development the transcription factor NIN, for *nodule inception* in *Lotus japonicus* (Schauser et al, 1999), and the GRAS transcription factors NSP1 and NSP2 in *M. truncatula* (Kalo et al, 2005; Smit et al, 2005) are the next steps downstream of the calcium spiking that lead to activation of early nodulin genes, *ENODs*, during early stages of the nodule formation and in the nodule primordium stage.

ENODs are also expressed during AMF infection. The spatial and temporal expression pattern is characteristic for each *ENOD* gene and different stage of the symbiosis development. The most prominent genes are the early nodulin genes *ENOD11* and *ENOD12* (Journet et al, 2001), homologues produced by the plant not only during preinfection and infection stages with rhizobia, but also in inner cortical cells containing recently formed arbuscules (Journet et al, 1994, 2001). *ENOD11* expression is also linked to the presence of exudates from mycorrhizal hyphae, although *ENOD11* activation was independent of DMI1, DMI2 and DMI3. Upon direct fungal contact to the roots, however, the signal is controlled by a cell specific regulation process and the *ENOD11* expression is circumvented in the DMI2 mutant by addition of Nod factors (Chabaud et al, 2002; Kosuta et al, 2003). This indicates that the signalling cascade of the Nod and putative Myc factors are differently integrated by the plant.
Subsequent to the induction of *ENOD11*, *ENOD12*, *ENOD2*, *ENOD40* and other *ENOD* gene families, a battery of genes are induced that seem to be required for infection thread growth, nodule formation and cell cycle control, entrance of bacteria and nitrogen fixation, for instance leghaemoglobins that are relatively early induced during nodule formation, as well as chitinases or phenylalanine ammonia lyase. Late nodulin genes are, in contrast to *ENODs*, involved in the setup of nodules and expressed in order to fulfil nodule functions. Some of the *ENODs* and late nodulins are required for both the mycorrhiza and the bacterial symbiosis, linking the two interactions again, on a different level of regulation. For example, *ENOD40,* which is encoded by two genes in *M. truncatula*, differing in their timing during nodule formation, is important for functioning nodules and is also involved in arbuscular formation in the root cortex cells (Staehelin et al, 2001; Kumagai et al, 2006).
Interestingly, the presence of *ENOD* genes in rice, and the observation that transgenic rice is able to activate the expression of an *ENOD* gene by a Nod factor dependent mechanism (Reddy et al, 1999) suggests that at least a part of the pathways for symbiotic interactions are conserved in legume and non-legume plants.

1.2. Chitin and chitinases

1.2.1. Chitin: structure, occurrence and function

Chitin, the polymer of β-1-4-linked N-acetyl-D-glucosamines (Figure 8 a), which is very insoluble to most solutes. It is said to be the second most abundant natural polysaccharide after cellulose. About one in six aminosaccharide residues is devoid of its acetyl group; chitosan is the deacetylation product of chitin, poly-(1-4)-2-amino-2-deoxy-β-D-glucose (Figure 8 b).
Chitin exists in two crystalline polymorphic forms. Commercially available chitin preparations are made from shrimp or crab shell or are obtained from the squid pen, which has a different chemical arrangement (a-form and b-form respectively).

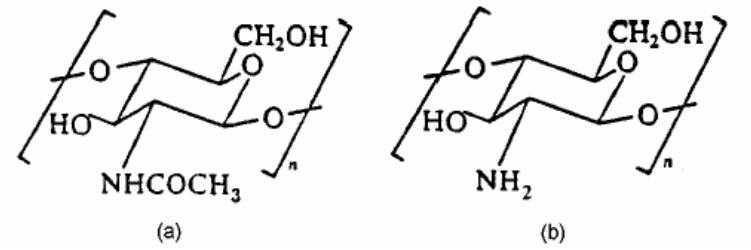

Figure 8 The chemical structures of monomers of chitin (a) and chitosan (b).

Chitin is the major component of the exoskeleton of arthropods and nematodes. It is a major cell wall component of bacteria, algae and most higher fungi, such as *Basidiomycetes*, *Ascomycetes* and *Deuteromycetes*. Up to 60% of their cell wall can be consisting of chitin.
The word 'chitin' comes from the Greek word *chiton*, meaning *coat of mail* or *tunic*, a certain piece of clothing. Chitin was first identified in mushrooms, under the name of fungine, and later in the shell of crustaceans, under the name of chitin. Like cellulose in plants, chitin acts as a stabilizing material for cell walls in fungi, molds and yeasts.

1.2.2. Chitinase characterization

Chitinases are poly-β-1,4-(2-acetamido-2-deoxy-D-glucoside) glycanohydrolases that form the EC 3.2.1.14 and are grouped, depending on their mode of action, into exo- and endo chitinases (Neuhaus et al, 1996). Endochitinases cleave chitin polymers randomly at the internal glycosidic bonds and generate chitin oligomers, such as chitotriose, chitotetraose or chitopentaose. Exochitinases have a preference for the nonreducing end of chitin chains, and create *N*-acetyl monomers. The most obvious plant chitinase substrate is microbial polymeric chitin, but they are also able to bind to and hydrolyse chitin fragments and similar molecules, for example decorated chitin oligomers and chitosan oligomers.

Plant chitinases are classified into different classes and catalytic families
Plants possess different classes of chitinases, which can be distinguished by their primary protein structure, substrate specificity, mechanisms of catalysis and sensitivity to inhibitors (Shinshi et al, 1990 and Collinge et al, 1993; Neuhaus et al, 1996). Class I chitinases (Figure 9) are mostly vacuolar basic chitinases. They possess an N-terminal cysteine rich chitin-binding domain, the CRD, which is connected to a hinge region. This cysteine rich domain and its spacer are to a certain degree involved in regulation of the catalytic activity and substrate binding (Iseli et al, 1993; Suarez et al, 2001). Together with the CRD domain, a large C-terminal catalytic domain is present in class I chitinases. The class II chitinases are distinguished from class I chitinases by their acidic pI and lack of the chitin binding and hinge region (Figure 9). Otherwise the two classes are structurally similar. The class III chitinases (Figure 9) do not possess any sequence homology to class I or II chitinases, unlike class IV chitinases which have evolved from class I chitinases (Araki & Torikata, 1995). These contain a cysteine rich domain together with the hinge region and are generally shorter in length (Figure 9). The class V chitinases again do not have similarities to the other chitinase classes (Figure 9), but show a weak similarity to bacterial exochitinases (Melchers et al, 1994).

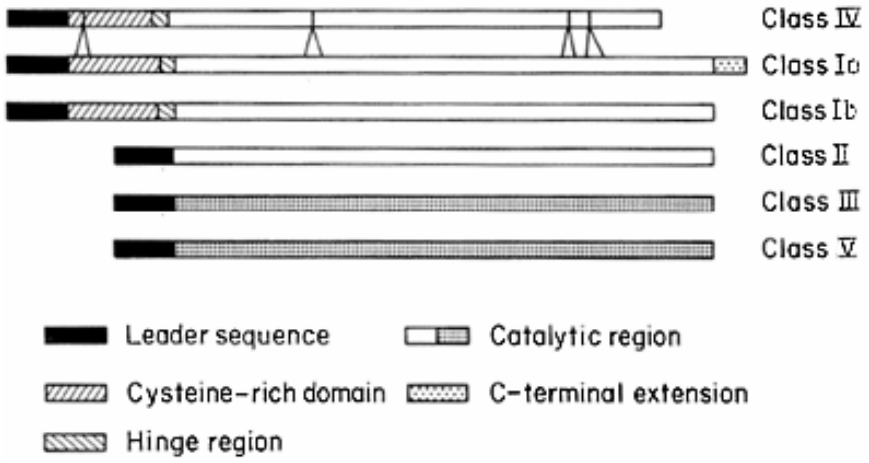

Figure 9 Chitinase structures. Picture adapted from Collinge et al (1993; Figure 1).

Plant chitinases are members of two separate glycosyl hydrolase families with different hydrolytic mechanisms

Chitinases produced by plants are divided into two families of *glycosyl hydrolases* (GH): Class III and V chitinases belong to the GH family 18, known from all kingdoms, whereas the class I, II and IV chitinases, which comprise the largest part of plant chitinases, belong to the GH family 19. These two groups of GH have different hydrolytic mechanisms (Henrissat, 1991). In addition, plant chitinases with lysozyme (EC 3.2.1.17) activity have bacterial peptidoglycan (b-1,4-linked GlcNac and N-acetyl muramic acid) as alternative substrate.

Chitinases of the GH family 18 use a substrate assisted catalysis by a similar mechanism that is also known for bacterial chitinases or avian egg white lysozyme (e.g. Makino et al, 2006). In this two step mechanism, the anomeric configuration of the substrate residue is retained, i.e. the alpha or beta configuration of the substrate is retained in the product. This *retaining cleavage* mechanism has been studied best in hevamine, a chitinase from rubber tree (Terwisscha van Scheltinga et al, 1995; Brameld et al, 1998; Fukamizo, 2000; Bokma et al, 2002). In contrast to the retaining cleavage mechanism, chitinases of GH family 19 catalyze the hydrolysis of chitooligosaccharides with a single step, the *inversion cleavage* mechanism (Iseli et al, 1996; Schultze et al, 1998), i. e. the hydrolysis of a beta-glycosidic bond creates a product with the alpha configuration and vice versa (Verburg et al, 1992; Fukamizo, 2000).

A speciality of some chitinases is their chitin binding domain at the N-terminal end of the protein chain (Suarez et al, 2001). This domain is characterized by a high number of cysteine residues, present at homologous positions in class I and IV chitinases, that stabilize the protein structure. Similarities of this domain to chitin-binding lectins such as wheat germ agglutinin and hevein maybe due to fusion of diverse ancestor domains to new proteins during evolution (Shinshi et al, 1990; Collinge et al, 1993).

The primary sequences of the GH family 19 chitinases are not homologous to animal or phage lysozymes, but based on structural analogies in the active site the two enzyme groups are expected to have the same cleavage mechanism (Schultze et al, 1998; Monzingo et al, 1996; Brameld et al, 1998). Therefore, some of the so-called plant lysozymes also show chitinase activity and are members of the class I, II or IV chitinases (Brunner et al, 1998; Bishop et al, 2000).

Based on the divergence of their hydrolytic mechanisms, it was speculated that the plant specific chitinases of the GH family 19 have evolved independently from the GH 18 chitinases. However, they may have evolved from a common chitin binding ancestor in order to fulfil specific requirements and the lysozyme activity of some isoforms was adopted later in evolution (Shinshi et al, 1990; Iseli et al, 1996).

1.2.3. Chitinases have diverse functions

Chitinases are found in all kingdoms. Depending on the organism, chitinases have different functions. Bacterial chitinases for instance, are involved in nutritional processes, using chitin for delivery of carbon and nitrogen (e.g. Fukamizo, 2000). In yeasts and other fungi, they can be part of the cell wall restructuring processes and daughter cell separation (e.g. Kuranda & Robbins, 1991). Chitinolytic activity was found in viruses, mammals and plants, despite the fact that chitin is not present in these organisms. Plant chitinases have been thoroughly investigated and have been found in a variety of tissues (Table 1). Since many chitinases are strongly induces in response to pathogen infection, they are thought to be primarily involved in defence against fungi with chitin-containing cell walls. The lysozyme activity that was in some cases found along with chitinase activity has been interpreted as plant defence mechanism against possible bacterial attacks.

However, chitinases in certain plant organs can also be developmentally regulated. They have been found to be induced in leaf senescence (Hanfrey et al, 1996) and flower development (Leung, 1992; Lotan et al, 1989), seed germination (Petruzzelli, et al 1999; Wu et al, 2001) or embryogenesis (de Jong et al, 1992; van Hengel et al, 1998; Helleboid et al, 2000). Possibly, they are involved in plant organ growth and development by generating or degrading signal molecules, as in spruce embryo

development (Wiweger et al, 2003). They are also induced in nodulation (Staehelin et al, 1995) and in ectomycorrhiza formation (Salzer et al, 1997a, b). On top, plant chitinases may have functions unrelated to chitin degradation. For instance, two chitinases class I and class II, accumulating in the aerial parts of winter rye during cold adaptation, have been shown to confer cold protection by acting as ice-binding antifreeze proteins (Pihakaski-Maunsbach et al, 2001).

Process / Function	Chitinase type	Plant species	Reference
Pathogenesis	Intracellular, class IV	*N. tabacum*	Melchers et al 1994
	Basic, class I and acidic, class II	*A. thaliana*	Thomma et al 1998, Samac & Shah 1991, Bishop et al 2000
	Class IV	*A. thaliana*	de A Gerhardt et al 1997
	Intracellular endochitinase	*P. vulgaris*	Boller et al 1983, Meier et al 1993
	Class IV	*V. vinifera*	Robinson 1997
Nodulation	Acidic, class III	*S. rostrata*	Goormachtig et al 1998
	Acidic	*M. sativa*	Minic et al 1998
	Class V	*M. truncatula*	Salzer et al 2000
	Extracellular	*P. sativum*	Staehelin et al 1994
Mycorrhiza	Basic, extracellular, class I	*P. abies*	Salzer et al 1997
Growth process	Chitinase-like (related to class II)	*A. thaliana*	Zhong et al 2002
Embryogenesis	Acidic, extracellular, class IV	*D. carota*	De Jong et al 1992, van Hengel et al 1998
	Extracellular	*Cichorium*	Helleboid et al 2000
	Basic, class IV	*P. glauca*	Dong & Dunstan 1997
Chilling/frost resistance	Extracellular, class I and class II	*S. cereale*	Yeh et al 2000
	Intra- and extracellular class II	*L. esculentum*	Ding et al 2002
	Intra- and extracellular class II	*Cynodon sp.*	de los Reyes et al 2001
Programmed cell death	Class IV	*B. napus*	Hanfrey et al 1996
	Class IV	*A. thaliana*	Passarinho et al 2001
Storage protein	Chitinase-like (class III homolog)	*Musa spp.*	Peumans et al 2002
Inhibitor	Basic, extracellular endochitinase	*S. tuberosum*	Guevara et al 1999
	Basic, extracellular endochitinase	*C.lachrymajobi*	Collinge et al 1993

Table 1 Putative functions of plant chitinases of different origin and classes. Table (not complete) after Kasprzewska and references therein (2003).

1.2.4. Chitinases involved in plant defence

Induction of defence-related chitinases is generally accompanied by the induction of beta-1,3-glucanases (EC 3.2.1.39). Chitinases and beta-1,3-glucanases are amongst the most studied and well characterized PR proteins (Kombrink et al, 1988; Stintzi et al, 1993; Melchers et al, 1994): beta-1,3-glucanases are placed in the PR-2 class, chitinases class I, II and IV belong to PR-3, chitinases of the class III belong to PR-8, and chitinases of class V belong to PR-11 (Melchers et al, 1984). An increased interest in plant defence mechanisms also increased the interest in plant chitinases, since they were the first PR proteins whose function was identified.

Pathogen-related chitinases are involved in plant defence

Generally, the pathogenesis-related chitinases are detected at low level in all plant parts, but as typical PR genes, plant chitinases, together with beta-1,3-glucanases, are induced after fungal (e.g. Hedrick et al, 1988; Büchter et al, 1997) or viral (e.g. Lawton et al, 1992; Heitz et al, 1994) infections. Chitinases are also induced in response to fungal elicitors (e.g. Mauch et al, 1988a) or plant hormones, such as the defence-related ethylene or salicylic acid (Boller et al, 1983; Stintzi et al, 1993; Broglie et al, 1986). In addition, abiotic stresses like ozone treatment, wounding and osmotic stress, can cause induction of chitinases (Schraudner et al, 1992; Hamel & Bellemare, 1995; Arie et al, 2000). On the other hand, a constitutively high level of chitinase activity is thought to be a protection against fungal or bacterial attack in tissues that are likely to be attacked, like in grape fruits or pineapple (Pocock et al, 2000; Taira et al, 2005a).

The expression of chitinases is often tightly regulated, as shown for example in bean leaves, where the transcription and enzymatic activity of chitinases after *Pseudomonas syringae* infection is temporally regulated and race specific (Voisey & Slusarenko, 1989). In pepper leaves, chitinase and beta-1,3-glucanase were differentially induced following the infection by *Xanthomonas campestris* (Hong et al, 2000). Furthermore, Salzer et al. (2000) reported differential accumulation of chitinase gene transcripts of several classes during infection with different pathogenic fungi, but also during nodule formation and AM formation in roots of *M. truncatula*.

Chitinases and beta-1,3-glucanases show antimicrobial activities in experimental systems

Chitinases are not only induced by several plants on pathogenic stimuli, but these PR proteins also act directly antifungal, as initially shown in bean leaf preparations (Schlumbaum et al, 1986). However, the antifungal chitinase activity was greatly enhanced when chitinase and beta-1,3-glucanase were combined (Mauch et al, 1988b). Similar findings have been reported subsequently (e.g. Sela-Buurlage et al, 1993; Melchers et al, 1994; Brunner et al, 1998) and it could be shown that pathogen induced chitinases exhibited specific activity against the hyphal tips of pathogenic fungi like *Fusarium* species (Benhamou et al, 1990; Arlorio et al, 1992; Sela-Buurlage et al, 1993). Furthermore, studies with transgenic plants showed that overexpression of some chitinases, alone or together in combination with other antifungal proteins lead to enhanced resistance against infection with pathogenic fungi (e.g. Broglie et al, 1991; Vierheilig, 1993; Zhu et al, 1994; Jach et al, 1995).

The role of antimicrobial chitinases in planta

There is no doubt that chitinases play a major role in plant defence, which is shown by the numerous examples of investigated chitinases. It seems that they have a double function against pathogenic colonization (Kasprzewska, 2003; Taira et al, 2005b): Apoplastic chitinases play a role in the early stage of pathogenesis. They cause dissolution of fungal cell walls and thereby release chitin oligomers that subsequently act as elicitor molecules. These fragments are recognized by specific PAMP receptors and general defence reactions are induced. Pathogenesis-related induced chitinases secondly play a role in the active defence mechanism. These are synthezised in order to hydrolyse the invading pathogen after disruption of the cells and vacuoles by the fungus or during the HR.

1.2.5. Chitinases in symbiosis

Because plant chitinases are typically induced in plant-pathogen interactions, they are primarily considered as PR proteins with a role in defence against chitin-containing organisms. In addition to their role as PR proteins, chitinases were shown to have diverse functions in tissues that are not under attack and may have specific functions there. It seems that non-pathogenic induced chitinases have a more subtle function and that in some cases their presence was not detected by common methods. However, their functions in developmental or symbiotic interactions are less well understood.
Of particular interest is the induction of specific isoforms of chitinase in symbiosis (Salzer et al, 2000). Chitinases obviously exhibit differential activity against different groups of colonizing fungi. For instance, tobacco plants, overexpressing defence-related chitinases, did not prevent a symbiotic fungal colonization by AMF, while they were clearly more resistant to the pathogenic fungus *Rhizoctonia solani* (Vierheilig et al, 1995). This suggests that the AMF are not affected by the defence-related chitinases. The same is true for nodule inducing rhizobia that cause an increase in chitinase expression during nodule formation but are not prevented to enter the roots (Goormachtig et al, 1998; Xie et al, 1999).

1.2.5.1. Elicitor cleavage

On the role of mycorrhiza-related chitinases only little is known. Certain chitinases were found to be exclusively induced in roots of AM colonized *M. truncatula* but not in response to pathogen attack (Salzer et al, 2000). Similarly, AM-related chitinases were expressed transiently in alfalfa, pea or *M. truncatula* during and before the actual colonization (e.g. Volpin et al, 1994; 1995; Slezack et al, 2001; Bestel-Corre et al, 2002).
In spruce cell cultures, elevated chitinase activity was induced by its ectomycorrhizal symbiont *Amanita muscaria* (Sauter & Hager, 1998). In a subsequent study, these chitinases were shown to inactivate the ectomycorrhizal fungal elicitors, which occurred obviously through cleavage of the elicitor-active chitin oligomers into small inactivate fragments (Salzer et al, 1997a). Inactivation was required to achieve a compatible interaction between plant and fungus and thereby reducing some general defence reactions. Furthermore, the chitinases had no inhibitory effects on the growth of the fungus *Suillus variegatus* (Salzer et al, 1997b). Inactivation of fungal elicitors may lead to enhancement of compatibility between the two partners and a role of symbiosis-induced chitinases was proposed as defence-reducing agents in ectomycorrhizal symbioses. Certain cellular and molecular properties of the ectomycorrhizal and endomycorrhizal symbioses are similar, making ectomycorrhiza a potential model for endomycorrhizae. Therefore, a similar role for some chitinases in AMF was proposed, based on the findings in ectomycorrhizal systems (Salzer et al, 2000; Salzer & Boller, 2001).
Interestingly, in eucalyptus, the extent of chitinase induction was correlated to the colonization ability, that is the aggressiveness, of the fungus. This indicated, too, that the induced chitinase activities were at least not inhibiting for the ectomycorrhiza formation, and perhaps had a positive effect on the establishment of the symbiosis (Albrecht et al, 1994). Despite these indications that certain chitinase activities may be helpful or even required for a successful mycorrhiza development, the functions of plant chitinases in mycorrhiza formation remain generally unknown.

1.2.5.2. Nod factor cleavage

Nod factors, produced by rhizobia in response to plant secreted flavonoids, are required for successful nodulation. The bacterial signal molecules trigger a series of events in the host root hairs, such as rapid ion fluxes across the plasma membrane and calcium spiking. These events also lead to the induction of nodulin genes.
Structurally, Nod factors are tetra- or pentamers of β-1,4-linked *N*-acetylglucosamines that are *N*-acylated by specific fatty acids at the non reducing end and modified by additional substituents on

the reducing and non reducing end (Figure 10). Their biological activity is determined by the length of the chitin backbone, the structure of the *N*-linked fatty acid, and the substitutions on the oligosaccharide backbone. Both the chitin backbone and the *N*-linked fatty acid are required for binding to root hair receptors and the activation of some parts of the following signalling cascade. The specific decorations at the reducing and non reducing end of the backbone might specify the host compatibility and seem to be involved in the infection process and regulation of the number of nodules (Perret et al, 2000). Undecorated chito-oligomers are not able to confer any of the typical nodulation reactions on certain host plants, which shows the importance of the diverse decorations.

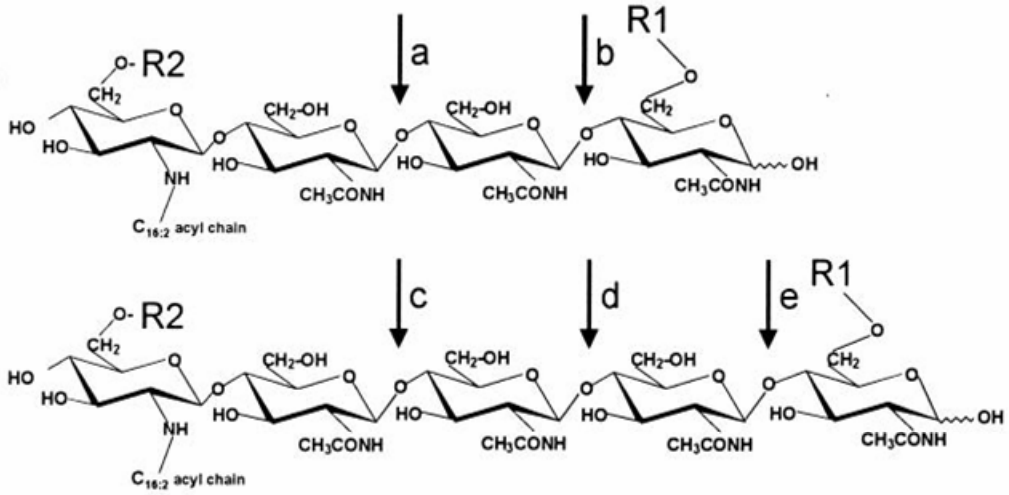

Figure 10 Sites of hydrolytic cleavage (a-e) of Nod factors of *Sinorhizobium meliloti* by enzymes isolated from the roots of *Medicago sativa*, R1, sulphatyl; R2, acetyl. Picture from Perret et al (2000; table 7).

Nod factors elicit their own degradation in the rhizosphere by induction of secreted hydrolases in their host plants (Staehelin et al, 1995; Ovtsyna et al, 2005). Rhizobia produce Nod factors that are *N*-acylated mostly with fatty acids that vary in their length and the degree of unsaturation. These *N*-acyl chains and the modifications of the chitin backbone influence the stability of Nod factors against degradation (Staehelin et al, 1994, 2000; Ovtsyna et al, 2000). Responsible for the degradation of Nod factors are chitinases and other hydrolases, which cleave the chitin backbone in accordance to its length and substitutions (Staehelin et al, 1994; Schultze et al, 1998; Ovtsyna et al, 2000 and for review see Perret, 2000).
The Nod factor decorations confer specificity and so induce responses on host plants but also protect the Nod factors against hydrolysis. In fact, cleavage leads to inactivation and thus reduces Nod factor signalling (Staehelin et al, 1994). This could be an important tool for the plant to control the action of Nod factors and reduce the number of infection sites, being part of the autoregulation of nodulation.

1.3. *Medicago truncatula*, a model legume

1.3.1. Legume research and model plants

Basic science is nowadays done mostly on model organisms, not only in plant sciences. Baker's yeast (*Saccharomyces cerevisiae*) or *Ashbya gossypii* are models for fungal research; *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish, rabbit and mouse are the models for animal and human research. In plant science the model plants are *Arabidopsis thaliana*, tobacco, rice, maize and

many others. The most prominent one, *Arabidopsis thaliana* from the *Brassicaceae*, is very well suited to study almost all kinds of fundamental questions. However, a major disadvantage of this plant is that it does not entertain any mutualistic symbioses with soil living organisms. To study symbiotic interactions, model plants within the family of *Leguminosae*/*Fabaceae* have been selected, because of their ability to establish two types of symbioses, the nitrogen fixing symbiosis with rhizobia and the AM.

The family of *Leguminosae,* with 20'000 single species in three subfamilies (Caesalpinieae, Mimosoideae, Papilionoideae) and more than 700 genera, is the one of the most species-rich families of plants. Legumes provide some of the most important food crops and are therefore important for the world's livelihood and economy. They can be grown in most habitats and provide a wide range of natural products, such as flavours, drugs, poisons and dyes. Grain legumes deliver a big portion of the human and animal food protein and vegetable oil, examples for this are alfalfa, soybean and peanut.

The special importance of legumes in terrestrial nitrogen cycling is their ability to fix atmospheric nitrogen together with their bacterial symbiotic partner.

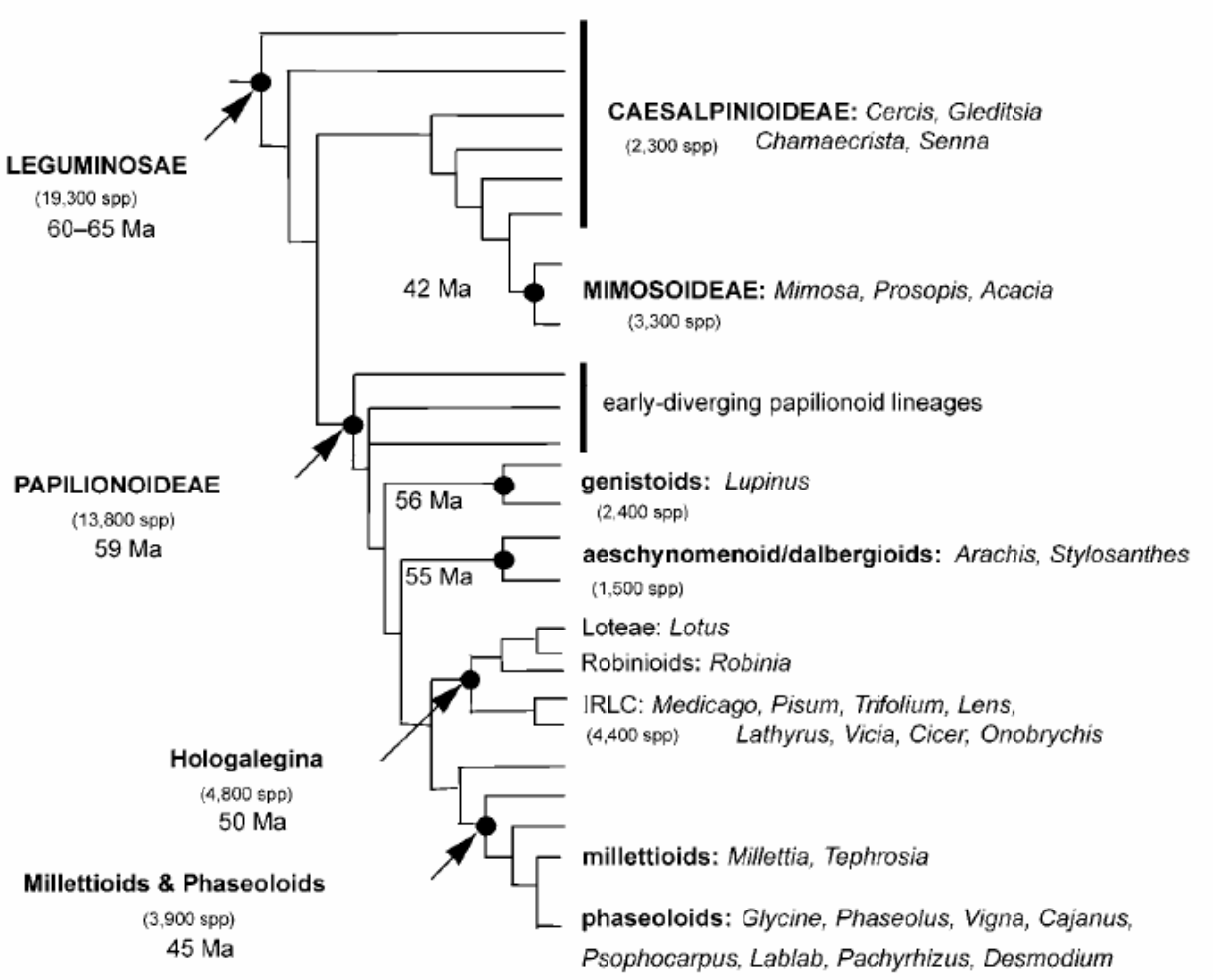

Figure 11 'Simplified schematic tree of legume family (modified from Doyle and Luckow, 2003). The three subfamilies (Caesalpinioideae, Mimosoideae, and Papilionoideae) and major subclades identified by recent molecular phylogenetic studies are shown in boldface (Kajita et al, 2001; Wojciechowski et al, 2004) and their positions are indicated by black circles (estimated number of taxa from Lewis et al, 2005, and ages [in millions of years] from Lavin et al, 2005).' Picture and text taken from Gepts et al. (2005; and references therein).

1.3.2. *Medicago truncatula*, the barrel medic

The forage plant *M. truncatula* was proposed as a model plant only recently (Barker et al, 1990) and there is great acceptance in the legume community (Cook, 1999); hence, the *Medicago Steering committee* was founded in 2001 (see: http://www.medicago.org). Along with the other model legumes *Lotus japonicus* and soybean, *M. truncatula* has advanced to one of the most studied legumes. Results from biochemical and genetic studies, especially on the processes related to the nitrogen fixing bacterial symbiosis, are accumulating in these plants. This knowledge can, to a certain extent, be translated onto other plants with genomic approaches.

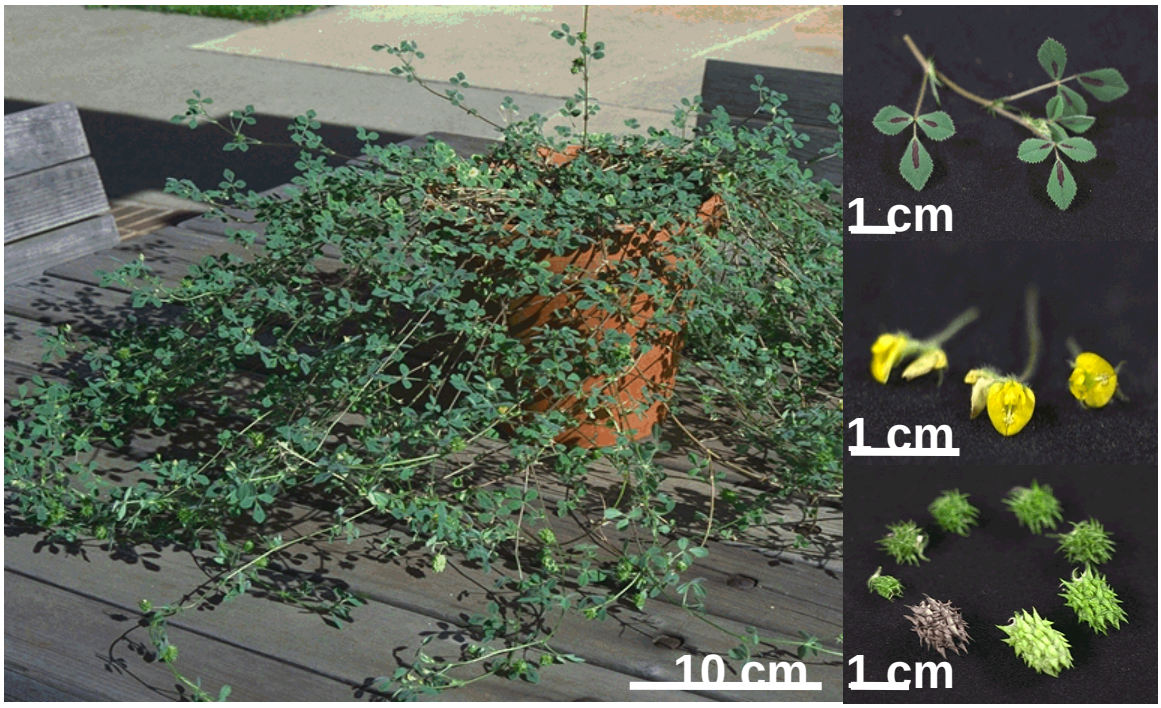

Figure 12 *Medicago truncatula* in different growth stages, flowers and developing seed pods. Bars equal 10 cm and 1 cm. Pictures adapted from http://www.ccrc.uga.edu/personnel/hahn/mtimages.htm (September, 2006).

The genome sequencing program

The reasons to choose *Medicago truncatula* as model system are that is has, unlike most other legumes, a relatively small genome, simple genetics and a short generation time of three to four month. A broad set of resources is available, amongst them a newly developed Affymetrix gene array that contains approximately 52,700 probe sets representing genes in *M. truncatula* and *M. sativa* (Tesfaye et al, 2006). A collection of mutants and ecotypes is built up and made open to the public by the steering committee. In Table 2 are some of the features listed, that are currently available from the website of the *M. truncatula* sequencing consortium.

The *M. truncatula* genome is closely related to pea and alfalfa, but less related to soybean. A project was launched to sequence the genomes of the most frequently used model legumes; the *M. truncatula* complete physical and genomic map will be publicly available within the next 1 - 2 years. Gene sequencing centres and Universities within America and Europe, e.g. Sanger (JIC), TIGR, Genescope (INRA) are working together in this costly project. The genetic informations will be extremely valuable for genomic comparisons with other plant genomes, and as a foundation for improving the knowledge of crop and forage legumes.

In 2001 a new nomenclature of genes identified by sequence or mutation in this species, as well as genes identified through phenotypic variation in natural populations, was presented (VandenBosch & Frugoli, 2001). These nomenclature guidelines were adapted from the ones that have previously presented for Arabidopsis (Meinke and Koornneef, 1997, to find in http://www.arabidopsis.org, May, 2006).

Genome size	180 Mbp
Chromosomes	8
GC content coding region	41.10 %
GC content noncoding region	30.26 %
projected BAC's	2070
sequenced BAC's	1839
Sequencing Redundancy	16.69 %
estimated Genes	20'000 – 28'000
avr. Gene size	1600 – 2200
Gene density	4.3 – 5.5 kb/gene
ESTs	226'923
EST coverage (gen. matches)	52.4 % (18'612)

Table 2 *M. truncatula* statistics (May 2006), after www.medicago.org (May, 2006)

Root Organ Culture (ROCs) and composite plants as transformed versions of M. truncatula
Unfortunely, *M. truncatula* is a model plant that is not as readily transformed as other model plants, like *Arabidopsis thaliana* or tobacco. The expression of transgenes in Medicago plants is up to now a rather time consuming and labour intense work. With the exception of a few species, e.g. alfalfa, legumes seem to be particularly recalcitrant to genetic transformation by *Agrobacterium tumefaciens*. Alternatively, a technique using *Agrobacterium rhizogenes* as a vector to introduce transgenes into the root system, producing *composite plants*, has been established and is now well accepted in the legume research community.
Hairy roots are obtained from plantlets that are transformed with *Agrobacteria*: The lower part of the developing root is injured and the lesion site is infected with the transgene delivering *Agrobacterium rhizogenes*. After successful infection, the plant develops new roots from the infected site, and some of these roots are stably transformed. The plant can then be planted in soil, to result in *composite plants* that are transgenic in the belowground tissues. These can then be studied as the plants develop normally. The disadvantage of this system is that the shoot is not transgenic so that only a vegetative propagation of the plant is possible.
If transgenic roots are separated from the shoot, they can be cultivated and develop into a *hairy root culture*, or *root organ culture*. These roots are stably transformed and grow clonally under auxotrophic conditions. The advantages of this system are its small storage size in Petri dishes, its clonal growth and sterility. The hairy root transformation method was developed long time ago on carrot roots (Bécard & Fortin, 1988), and is now a standard method in many laboratories (Boisson-Dernier et al, 2001). A major breakthrough was the discovery that these transgenic roots, initially from carrot, could be colonized by AMF (Bécard & Fortin, 1988). Production and maintenance of such symbiotic tissue is now a standard method to study enzymatic in vivo processes in model plants. This is especially in important in *M. truncatula* where we do not have appropriate methods to

produce transgenic offspring. Approaches to obtain stably transformed plants have been published, but up to now could not be reproduced (Trieu & Harrison, 1996).

1.4. Model fungi

The use of AMF as research instruments lead to the isolation and propagation of several commonly accepted model isolates. The species *G. intraradices* seems to be the most used AMF species in publications, but also *G. mosseae* is rather frequently used.
The two species belong to the same *Glomus* group. Both fungi are found in similar habitats, and colonize a similar host range. However, some differences appear in the timing of colonization, the spatial distribution in the root zone, and in their behaviour during competitive colonization (Alkan et al, 2006).

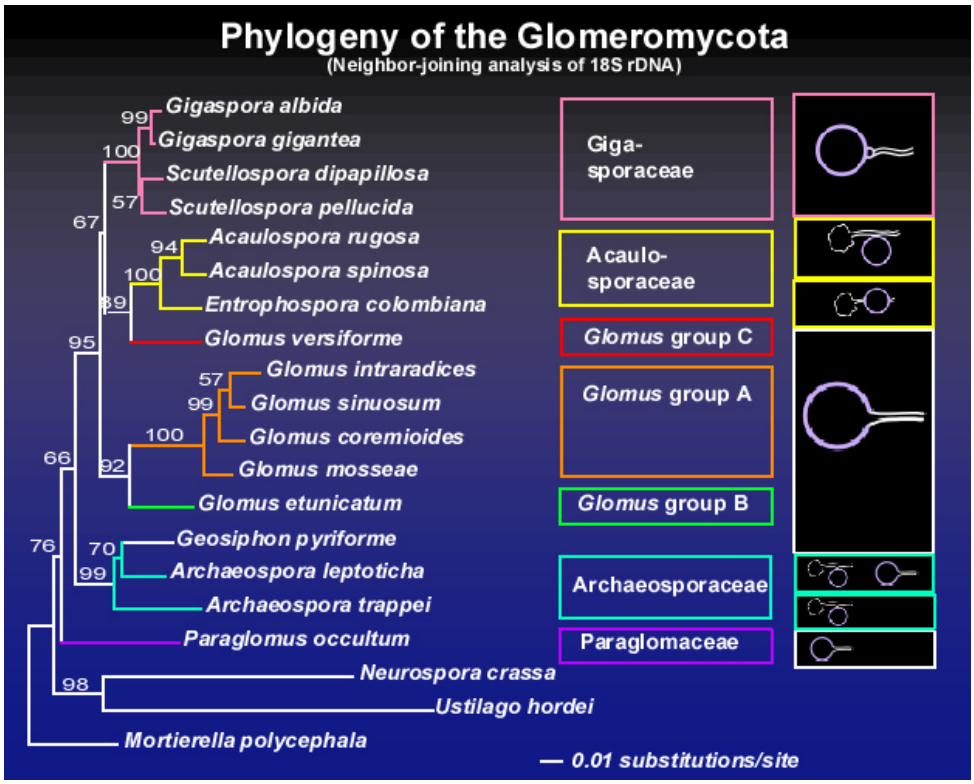

Figure 13 Phylogenetic tree for overview over the different *Glomus* groups. Groups are shown in color, together with the corresponding spore morphology. Picture by courtesy of D. Redecker.

G. intraradices is widely used as a model fungus in hairy root cultures (e.g. Journet et al, 2001; Chabaud et al, 2002). Several fungal cultivars have been isolated from *G. intraradices*, and are available for research. The fungi are generally cultivated in *Daucus carota* (carrot) hairy root cultures to yield a relatively high number of offspring spores in a short generation time, until they are transferred to *M. truncatula* cultures. However, due to low infection frequencies, lack of synchronization and other reasons, it seems not possible to cultivate most of the known AMF by this method. This is the case for example for *G. mosseae*, another widely used AMF, which does not readily colonize carrot roots.

1.5. Identification of symbiosis related genes

During the the establishment of the AM symbiosis, the cells of the root cortex are heavily reorganized and also the fungus undergoes important structural changes. Plant cells hosting arbuscules undergo cell wall degradation and reconstruction processes that change their morphology. Energy has to be provided for these processes as well as for the acquisition and the transport of nutrients over long distances in both partners and across the periarbuscular membranes. Specific regulaton of a high number of genes is involved in such processes on both sides. This is also true during the establishment of the nitrogen fixing nodule symbiosis, where new, bacteria hosting organs are formed in the roots of legumes. The processes involved in the formation of functional nodules are clearly different from the establishment of a functional AM symbiosis. One of the keys to successful symbiosis formation seems to be the coordinated up-and down regulation of genes needed for plant microbe interaction. It remains a great challenge to understand what is involved in the formation of symbiotic structures, and the physiological changes that both partners have to make, not to mention the delicate interdependence of the two organisms.

The tools, nowadays used to monitor the genetic processes involved in symbiosis establishment, are for example large-scale gene expression studies and EST sequencing, using methods such as suppression subtractive hybridisation (e.g. Brechenmacher et al, 2004; Grunwald et al, 2004; Frenzel et al, 2005) and micro – or macro gene array experiments (e.g. Liu et al, 2003; Hohnjec et al, 2005; El Yahyaoui et al, 2004). With these methods, a large number of symbiosis-specifically regulated genes have been identified, with overlapping results between the different studies. However, the plant-microbe combinations are varying. In order to get an overview of the results of such studies, a system of functional categories was created, which proved to be useful for the classification of the vast number of genes (Journet et al, 2002).

Several interesting genes that are regulated in the mycorrhizal symbiosis have been identified from large scale studies, and subsequently studied intensely by genetic and biochemical methods. Such candidate genes are for example the germin-like *MtGlp1* (Doll et al, 2003), the glutathione S-transferase *MtGst1* (Wulf et al, 2003) and the Ser carboxypeptidase *MtScp1* (Liu et al, 2003). Nevertheless, data from large scale approaches are, despite the high number of discoveries, not fully comprehensive and need to be evaluated carefully (e.g. Breuninger & Requéna, 2004). One example is the weakly expressed chitinase gene *Mtchit3-3* (Salzer et al, 2000) that was detected by quantitative PCR and has so far only been found in one other study (Guimil et al, 2005). The same sort of approach has been taken to identify genes on the side of the AMF partner (Delp et al, 2003; Requena et al, 2003).

Interestingly, comparative studies show that different programs are activated during infection with *Rhizobia* and AMF than with only one of the microbes alone (e.g. Journet et al, 2002; El Yahyaoui et al, 2004; Küster et al, 2004). It will be one of the next challenges of research to explore the relationships between these two symbioses.

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Chapter 2

Sinorhizobium meliloti-induced chitinase gene expression in *Medicago truncatula* ecotype R108-1: a comparison between symbiosis-specific class V and defense-related class IV chitinases

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* Mtch1 was later renamed: Mtchit1a
** Mtchit2 was later renamed: Mtchit1c

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Abbreviations:

AM	Arbuscular mycorrhiza(l)
AMF	Arbuscular mycorrhizal fungus
Mt	Medicago truncatula
BA	Bacterial Artificial Chromosome
bp	Base Pairs
RT-PCR	Polymerase Chain Reaction after Reverse Transcription

2.1. Abstract

The *Medicago truncatula* (Gaertn.) ecotypes Jemalong A17 and R108-1 differ in *Sinorhizobium meliloti*-induced chitinase gene expression. The pathogen-inducible class IV chitinase gene, *Mtchit4*, was strongly induced during nodule formation of the ecotype Jemalong A17 with the *S. meliloti* wild-type strain 1021. In the ecotype R108-1, the *S. meliloti* wild types Sm1021 and Sm41 did not induce *Mtchit4* expression. On the other hand, expression of the putative class V chitinase gene, *Mtchit5*, was found in roots of *M. truncatula* cv. R108-1 nodulated with either of the rhizobial strains. *Mtchit5* expression was specific for interactions with rhizobia. It was not induced in response to fungal pathogen attack, and not induced in roots colonized with arbuscular mycorrhizal (AM) fungi. Elevated *Mtchit5* gene expression was first detectable in roots forming nodule primordia. In contrast to *Mtchit4*, expression of *Mtchit5* was stimulated by purified Nod factors. Conversely, *Mtchit4* expression was strongly elevated in nodules formed with the K-antigen-deficient mutant PP699. Expression levels of *Mtchit5* were similarly increased in nodules formed with PP699 and its parental wild-type strain Sm41. Phylogenetic analysis of the deduced amino acid sequences of *Mtchit5* (calculated molecular weight = 41,810 Da, isoelectric point pH 7.7) and *Mtchit4* (calculated molecular weight 30,527 Da, isoelectric point pH 4.9) revealed that the putative Mtchit5 chitinase forms a separate clade within class V chitinases of plants, whereas the Mtchit4 chitinase clusters with pathogen-induced class IV chitinases from other plants. These findings demonstrate that: (i) *Rhizobium*-induced chitinase gene expression in *M. truncatula* occurs in a plant ecotype-specific manner, (ii) *Mtchit5* is a putative chitinase gene that is specifically induced by rhizobia, and (iii) rhizobia-specific and defense-related chitinase genes are differentially influenced by rhizobial Nod factors and K antigens.

2.2. Introduction

Under conditions of nitrogen limitation, legumes establish a symbiosis with nitrogen-fixing soil bacteria belonging to the family of *Rhizobiaceae*. Once the rhizobia have entered the root hairs, infection threads are formed, and some cortical root cells become meristematic resulting in the development of a highly specialized symbiotic organ, the nodule. Formation of nodules is the result of a molecular dialogue between rhizobia and the host plant. The plants secrete flavonoids into the rhizosphere, where they stimulate the rhizobia to synthesize Nod factors (lipo-chitooligosaccharide signal molecules). The Nod factors induce various responses in the host plant, including root hair deformation and cortical cell division (Perret et al, 2000). In addition to Nod factors, specific rhizobial surface compounds, exopolysaccharides (EPS), lipopolysaccharides (LPS), cyclic β-glucans and K antigens (also named capsular polysaccharides) are required for establishing a symbiosis. Increasing data suggest that certain forms of surface polysaccharides interact with components of the host plant at different stages of the symbiosis (e.g. Pellock et al, 2000), thereby avoiding or suppressing inducible plant defence reactions (Niehaus et al, 1993; Albus et al, 2001; Mithöfer et al, 2002). Whether K antigens have an effect on genes induced during nodulation has not been studied yet. K antigens of *Sinorhizobium meliloti* are analogous to the group II K antigens of *Escherichia coli*. They adhere tightly to the cell surface of the rhizobia and usually contain a high proportion of Kdo (3-deoxy-D-manno-2-octulosonic acid; Petrovics et al, 1993; Reuhs et al, 1993; Kannenberg et al, 1998).

Nodulins are plant proteins that are specifically induced during the life cycle of a nodule. According to the timing of their synthesis during nodule development, they are divided into early and late Nodulins. Early Nodulins are synthesized during root hair deformation, infection thread formation and nodule morphogenesis. Late Nodulins appear first in nodules after bacterial endocytosis. Most early Nodulins seem to be involved in symbiosis-related alterations of the plant cell wall. Some of them exhibit similarities to pathogenesis-related proteins (Gamas et al, 1998; Goormachtig et al, 1998). A number of Nodulins are also expressed in roots colonized by mycorrhizal fungi (Wyss et al, 1990; Hirsch and Kapulnik, 1998), indicating a common role in arbuscular mycorrhizal (AM) and rhizobial symbiosis. For instance, *Medicago truncatula* transformants constitutively expressing the *enod40* gene exhibited an accelerated development of nodules (Charon et al, 1999) and also an increased colonization with AM fungi (Staehelin et al, 2001).
Chitinases are frequently regarded as pathogenesis-related proteins. They hydrolyse chitin (poly β-1,4-*N*-acetylglucosamine), which is a major component of most higher fungi and arthropods. Usually, plants possess a set of different chitinases, which belong to the glycosyl hydrolase families 18 or 19 and are subdivided into different classes (Melchers et al, 1994; Neuhaus et al, 1996; Hamel et al, 1997). Synthesis of chitinases is often induced in response to pathogen attack. Chitinases have antifungal activity, particularly in combination with β-1,3-glucanase (e.g. Mauch et al, 1988). When overexpressed in transgenic plants, certain chitinases enhance resistance against fungal pathogen attack (e.g. Broglie et al, 1991). Some chitinases also display lysozyme activity and hydrolyse β-1,4-linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycanes of bacterial cell walls (e.g. Brunner et al, 1998).

Apart from their role in pathogen defense, chitinases also have a role in symbiotic interactions. However, their function in symbioses is less well understood. Certain chitinase isoenzymes are specifically induced in soybean nodules (Staehelin et al, 1992; Xie et al, 1999) and near aborted infection threads in the interaction between alfalfa and *Sinorhizobium meliloti* (Vasse et al, 1993). In *Sesbania rostrata* plants, a chitinase (Goormachtig et al, 1998) and a chitinase-related gene (Goormachtig et al, 2001) are strongly induced during the early stages of stem nodule development. Chitinases of legumes

have received particular attention because certain rhizobial Nod factors are substrates for chitinases (reviewed by Perret et al, 2000). The degradation products formed are only weakly active on their respective host plants (Heidstra et al, 1994; Staehelin et al, 1994). Thus, it has been proposed that cleavage of Nod factors is necessary to limit the amount of active Nod factors after their perception by the host plant (Staehelin et al, 1995; Goormachtig et al, 1998). Chitinases can also be directly involved in processes related to plant development by modifying extracellular *N*-acetylglucosamine-containing arabinogalactan proteins (De Jong et al, 1992; van Hengel et al, 2001). In addition to these enzymatically active chitinases, a putative receptor kinase with a C-terminal class V chitinase domain (Kim et al, 2000) and chitinase homologs without catalytic activity have been identified (Perlick et al, 1996; Goormachtig et al, 2001).

Medicago truncatula (= barrel medic) is widely used as a model plant that allows studies on symbiotic plant–microbe interactions (Cook et al, 1997). *M. truncatula* exists in many ecotypes. The ecotype Jemalong was selected for the ongoing American and European *M. truncatula* genome sequencing and gene expression projects (van den Bosch and Stacey, 2003). The ecotype R108-1 is known for its superior in vitro regeneration and transformation properties (Hoffmann et al, 1997). In our previous work we isolated partial chitinase DNA sequences from an *M. truncatula* bacterial artificial chromosome (BAC) library and determined transcript levels by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) in various pathogenic and symbiotic interactions (Salzer et al, 2000). Transcripts of the class IV chitinase gene, *Mtchit4*, continuously accumulated in the interaction of *M. truncatula* Jemalong A17 with *S. meliloti* 1021. In our previous work (Salzer et al, 2000), however, we were not able to identify a chitinase gene that is specifically induced during interactions with *S. meliloti*. In order to learn more about chitinase gene expression during nodule formation and about the effects of rhizobial signal molecules, we extended our studies on the *M. truncatula* ecotype R108-1. The comparison of chitinase gene expression patterns between the ecotypes Jemalong A17 and R108-1 drew our attention to the class IV chitinase gene, *Mtchit4*, and the class V chitinase gene, *Mtchit5*. In this work we present a comparative, real-time RT-PCR-based investigation of *Mtchit4* and *Mtchit5* gene expression and introduce *Mtchit5* as a new rhizobia-specific induced gene that is inversely influenced by Nod factors and K antigen compared to the defense-related *Mtchit4* gene.

2.3. Materials and methods

2.3.1. Treatments and culture of plants, bacteria, and fungi

Medicago truncatula (Gaertn.) ecotype R108-1 was kindly provided by E. Kondorosi (CNRS, Gif-sur-Yvette, France), ecotype Jemalong A17 by T. Huguet (CNRS-INRA Castanet-Tolosan, France). Seeds were freshly released from seed pods, treated with concentrated sulphuric acid for 4 min, rinsed 8 to 10 times with autoclaved water and germinated in Petri dishes containing 1% water agar. Germination and growth occurred in a Sanyo Versatile Environmental Test Chamber (Sanyo Electric, Japan) with 18-h day (140 µmol photons m^{-2} s^{-1}, 22°C), 6-h (18°C) night cycles. When used for spraying experiments, seeds were germinated in vertical orientation with roots shaded by aluminium foil. For all other experiments, seeds were germinated in horizontal orientation and planted into Magenta jars containing Terra Green (Oil Dri type III R; Lobbe Umwelttechnik, Iserlohn, Germany) after the primary leaves had started to expand. B&D nutrient solution (Broughton and John, 1979) with 2 mM KNO_3 and 0.25 mM KH_2PO_4 was continuously supplied from a reservoir, which was connected by a wick with the Terra Green.
The following *Sinorhizobium meliloti* strains and mutants were used in this work: *S. meliloti* 1021 wild type; *S. meliloti* Sm41 wild type and its K-antigen-deficient derivative PP699, which is mutated in the *rkp-1* (formerly *fix-23*) region (Putnoky et al, 1990; Petrovics et al, 1993; Kiss et al, 1997); AK631/*psZ*::Tn5 with a mutated *lpsZ* gene (Reuhs et al, 1995); and PP666h, which is AK631 mutated in the *rkp-1* (formerly *fix-23*) region (Putnoky et al, 1990). The bacteria were grown in GTS medium (Kiss et al, 1979) on a rotatory shaker (150 rpm) at 27°C. For inoculation, 1 ml of the bacterial solution (OD_{600} about 1.0) was either sprayed directly on the roots or pipetted to the rhizosphere. In control experiments, 1 ml GTS medium was applied. The plant material was harvested after the indicated times, frozen in liquid nitrogen and stored at -80°C until extraction of RNA.
Nod factors were purified from *S. meliloti* strain 1021(pEK327) (Schultze et al, 1992). Supernatants of bacterial cultures were extracted with *n*-butanol and fractionated by reverse-phase HPLC (Waters C18 column), using 35% acetonitrile/water, 40 mM ammonium acetate as the mobile phase. The fractions containing NodSm-IV($C_{16:2}$, S) were collected and desalted as described by Staehelin et al. (2000). For treatment of roots, 1 ml of 0.5 mM NodSm-IV($C_{16:2}$, S), was sprayed on vertically grown roots of young plants, which had reached the stage of expanded cotyledons. In control experiments, 1 ml water was sprayed.
Culture and inoculation with the AM fungus *Glomus intraradices* was performed as previously described (Salzer et al, 2000) with about 400 spores, which had been isolated from *Daucus carota*/*Glomus intraradices* in vitro cultures (Bécard and Fortin, 1988). In control experiments, an equivalent amount of water was added. The degree of mycorrhization was determined by the gridline intersection method (Giovanetti and Mosse, 1980).
Culture and inoculation with *Fusarium solani* f. sp. *phaseoli* strain W-8 (kindly provided by the Novartis fungal pathogen collection) was performed as previously described (Salzer et al, 2000). For mock inoculation, V8 agar plugs were placed on the root surface.

2.3.2. Cloning, sequencing and sequence analysis

Cloning and sequencing of Mtchit5
An adapter-ligated library was constructed with the Universal Genome Walker kit (Clontech, Palo Alto, CA, USA) and the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany). To this purpose, genomic DNA was isolated from roots of axenically grown *M. truncatula* R108-1 plants using the Nucleon Phyto Pure DNA extraction kit (Amersham Biosciences) followed by three extractions with phenol: chloroform (1:1). Aliquots

of the purified DNA were digested with *Dra*I, *Eco*RV, *Pvu*II or *Stu*I and adapters were ligated to both blunt cut ends of the obtained DNA fragments according to the protocol of the Genome Walker kit. First PCR was performed with an aliquot of the adapter-ligated DNA fragments with the adapter-specific primers, Adaptor 1 (Genome Walker kit) and *Mtchit5*-specific primers GSP1 or GSP3 (Table 1) followed by a second, nested PCR with 1:1,000-diluted aliquots of the first PCR using the nested adapter-specific primers, Adaptor 2 (Genome Walker kit) and nested *Mtchit5*-specific primers GSP2 and GSP4 (Table 1). PCR was performed with the Long Template PCR System according to the manufacturer's recommendations (Roche Diagnostics) using buffer 3. Thermal cycling was performed with a Techne Progene cycler (Techne, Duxford, Cambridge, UK). The cycling program for both PCRs was 1 cycle: 2 min at 92°C, 10 cycles: 10 s at 92°C, 30 s at 60°C and 90 s at 68°C, 20 cycles: 10 s at 92°C, 30 s at 60°C, and 90 s at 68°C with an increased extension per cycle of 20 s at 68°C. PCR products obtained after the second PCR were purified with the High Pure PCR purification kit (Roche Diagnostics) and cloned into pGEMT-easy using the pGEMT-easy Vector System (Promega, Madison, WI, USA). DNA sequencing was performed with the ABI Prism Big Dye Terminator Sequencing Reaction kit and an ABI 310 sequencer following the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). The sequences obtained from the adapter-ligated library were assembled and primers were designed (Mtchit5 start-f, Mtchit5 stop-b) that covered the 5' and 3' ends of the assembled sequences. The primers used for cloning and sequencing reactions in forward and backward direction are listed in Table 1.

Cloning and sequencing of Mtchit4

A database comparison in TIGR (http://www.tigr.org/tdb/tgi/mtgi/) with the partial *Mtchit4* sequence (accession number AF167328) obtained from the *M. truncatula* Jemalong A17 BAC clone 46B22 (Salzer et al, 2000) resulted in a 100% match with the tentative class IV chitinase consensus sequence TC60709. Primers Mtchit4 start-f and Mtchit4 stop-b comprising the start and the stop codon of TC60709 were designed (Table 1). Using the Expand PCR System, *Mtchit4* was synthesized using genomic DNA from *M. truncatula* cv. R108-1 as template, cloned into pGEMT-easy, and sequenced.

Sequence analysis

Sequences were analysed using EditView 1.0 (Perkin Elmer). Sequence alignments (BestFit, PileUp), translation of the nucleotide acid sequences (Translate), database searches (BLASTP, BLASTN), determination of the molecular weight (Map) and isoelectric point (Isoelectric), analysis of known protein motives (HmmerPfam), and analysis of signal peptide (SPScan) were performed with the Accelrys SeqWeb Version 2.1 Web-based Sequence Analysis. In an alternative approach, analysis of signal peptides was also performed with PSORT (old version for bacterial/plant sequences, http://psort.nibb.ac.jp/form.html). For phylogenetic analysis, deduced amino acid sequences were aligned with Clustal W and distance matrices were calculated (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Unrooted phylogenetic trees were constructed with Phylip version 3.6 (http://evolution.genetics.washington.edu/phylip.html) applying the Neighbor-Joining method 3.6a3. All other molecular techniques were performed according to Sambrook et al. (1989).

2.3.3. Semi quantitative and quantitative RT-PCR

Semi quantitative RT–PCR was performed entirely as previously described (Salzer et al, 2000), except that 31 PCR cycles were performed.
For real-time RT–PCR, RNA was isolated from roots, leaves and flowers of *M. truncatula* R108-1, treated with DNAse I and reverse-transcribed as previously described (Salzer et al, 2000), except that 10 units RNase-free DNase I and reaction buffer from MBI Fermentas (Vilnius, Lithunia) were used.
Real-time PCR was performed with a Gene Amp 5700 Sequence Detection System (Applied Biosystems) with the default thermal cycling program for Sybr Green analysis. The thermal profile was, 1 cycle: 50°C, 2 min, 1 cycle: 95°C, 10 min, 40 cycles: 95°C, 15 s, 60°C, 1 min. A 25- µl reaction volume consisted of 1 µl of cDNA, 12.5 µl Sybr Green PCR 2× reaction mix, 5.5 µl water, 3 µl of 2 µM gene-specific forward primers, and 3 µl of 2 µM gene-specific backward primers (Table 1). Gene-specific primers for *Mtchit5*, and *Mtchit4* were designed based on sequence information of the full-length coding region. Gene specificity of the primers was controlled by comparison in pairs (BestFit) with partial chitinase sequences of *Mtchit1**, *Mtchit2***, and *Mtchit3-2* (Salzer et al, 2000), and full-length sequences of *Mtchit3-3* (AY238969), *Mtchit3-4* (AY238970), and *Mtchit3-1* (AY294484). Calibration was performed with a dilution series of 6.4 ng, 3.2 ng, 1.6 ng, 0.8 ng and 0.4 ng genomic DNA from *M. truncatula* cv. R108-1 corresponding to 22,122, 13,061, 6,530, 3,265, and 1,632 copies of *Mtchit5*, *Mtchit4* and ubiquitin. Copy numbers were calculated based on a haploid genome weight of 0.49 pg (Blondon et al, 1994) under the assumption of *Mtchit5* and *Mtchit4* being single-copy genes (Salzer et al, 2000). DNA was isolated from axenically grown seedlings with DNeasy (Qiagen) and subsequently treated with RNase following the manufacturer's instructions. Ubiquitin (*ubi*) was measured as internal standard using an *M. truncatula*-specific primer combination (Salzer et al, 2000). For calculation of copy numbers from amplification plots, a threshold of 0.1 was used. All samples were measured in triplicate. Standardized expression levels are given as *Mtchit*/*ubi* ratios. They were calculated from the average copy numbers of the chitinase and the *M. truncatula*-specific ubiquitin transcripts

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Table 1 Primers used for construction of the adaptor-ligated library, cloning and sequencing of *Mtchit5* and *Mtchit4*, and primers used for real-time PCR. The following primers have already been described elsewhere: Adaptor primer 1, and nested adaptor primer 2 (Universal Genome Walker kit user manual, Clontech, http://www.clontech.com), sequencing primers M13 forward and M13 backward (pGEMT and pGEMT-easy Vector Systems, http://www.promega.com), Mtchit 3-3f, Mtchit 3-3b, Mtubi-f, Mtubi-b (Salzer et al, 2000). *Tm* Annealing temperature.

Name	Tm (°C)	Sequence	Comment
Cloning primers			
GSP1	67.1	CCGATCCGGTCTCCTTGTCATACACC	Gene-specific *Mtchit5* forward, first PCR
GSP2	67.2	CCCGATTCCATGCACATTCGGATCCTG	Gene-specific *Mtchit5* forward, nested PCR
GSP3	67.1	CAGGATCCGAATGTGCATGGAATCGGG	Gene-specific *Mtchit5* backward, first PCR
GSP4	67.1	GGTGTATGACAAGGAGACCGGATCGG	Gene-specific *Mtchit5* backward, nested PCR
Mtchit5 start-f	63.1	GTGACATGCCACTTAAGAATGTTGAACAC	5' end of Mtchit5, full-length clone forward
Mtchit5 stop-b	63.8	GTCACAAATTATCACACACTTGGTTTGTAAACC	5' end of Mtchit5, full-length clone backward
Mtchit4 start-f	67.7	ATGACTATGATGGGAAACAAATCACTAAGCATATGTATG	5' end of Mtchit4, full-length clone forward
Mtchit4 stop-b	66.2	TAAGCAAGTAAGGTTATCACCAGGAGCAACAC	3' end of Mtchit4, full-length clone backward
Sequencing primers			
Dra I-specific 1	64.4	GTGATGTATCTATGGAAGCTGGAGGG	For internal regions of Dra I fragment, forward
Dra I-specific 2	65.3	GCCTTTCACACGTGTAGTTGATGATGGTG	For internal regions of Dra I fragment, backward
Mtchit4 seq1	64.4	GCTAATGTGGCTGACATCCTTACACAAG	For internal regions *Mtchit4*, forward
Mtchit4 seq1b	64.4	CTTGTGTAAGGATGTCAGCCACATTAGC	For internal regions *Mtchit4*, backward
Mtchit4 seq2	64.2	GAACTACAACTATGGACCAGCTGGAAG	For internal regions *Mtchit4*, forward
Mtchit4 seq2b	64.2	CTTCCAGCTGGTCCATAGTTGTAGTTC	For internal regions *Mtchit4*, backward
Primers for real-time PCR			
Chit5 rt-f	59	GGGTTGATGGTGGAATGGCG	Gene-specific *Mtchit5*, forward
Chit5 rt-b	60.5	GATCCGGTCTCCTTGTCATAC	Gene-specific *Mtchit5*, backward
Chit4 rt-f	66.6	GGTGATGCATATTGTGGCACAGGG	Gene-specific *Mtchit4*, forward
Chit4 rt-b	67.4	GCAGCAGCAACCTCACGTTTGGAG	Gene-specific *Mtchit4*, backward

2.4. Results

2.4.1. Differential expression of chitinase genes in the *M. truncatula* ecotypes Jemalong A17 and R108-1

To extend our knowledge about chitinase gene expression in the model legume *M. truncatula*, we compared the expression patterns of *Mtchit1**, *Mtchit2***, *Mtchit3-1*, *Mtchit3-2*, *Mtchit3-3*, *Mtchit3-4*, *Mtchit4*, and *Mtchit5* (Salzer et al, 2000) between the ecotypes Jemalong A17 and R108-1. Applying conventional semi-quantitative RT-PCR for these eight genes, we found two remarkable differences between chitinase gene expression in nodulated roots of the two *M. truncatula* ecotypes. (i) *Mtchit4* transcripts, which continuously accumulated during nodule formation of *S. meliloti* 1021 on the ecotype Jemalong A17 (Salzer et al, 2000), was not elevated above its basic expression level in nodules formed with the *S. meliloti* wild-type strains 1021 and Sm41 in the ecotype R108-1 (Figure 1). (ii) *Mtchit5* expression, which was previously not detected in roots of *M. truncatula* cv. Jemalong A17 nodulated with *S. meliloti* Sm1021 (Salzer et al, 2000), could be detected in roots of the ecotype R108-1 that were nodulated with 1021 or Sm41 (Figure 1). By performing a higher number of PCR cycles, 31 instead of 28 (Salzer et al, 2000), *Mtchit5* expression could also be observed in nodulated roots of *M. truncatula* Jemalong A17. However, the signal was so weak, that it could hardly be seen (Figure 1).

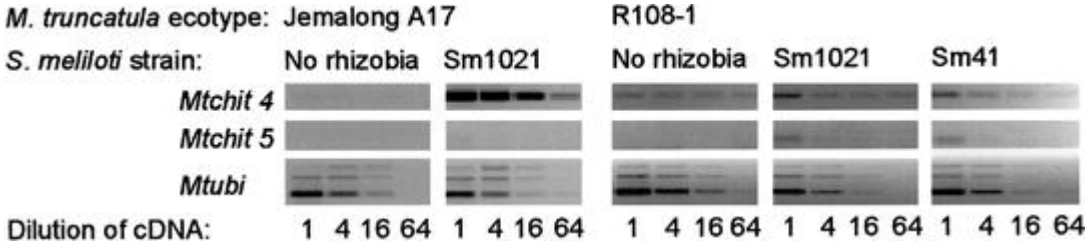

Figure 1 Differential expression of chitinase genes in various *Medicago truncatula* ecotypes. The plants were inoculated with the *Sinorhizobium meliloti* wild-type strains 1021 or Sm41, or were mock-inoculated with GTS medium (no rhizobia). RNA was isolated from nodulated roots 5 weeks post inoculation. cDNA obtained by reverse transcription has been diluted 1:1, 1:4, 1:16, and 1:64 and was subsequently subjected to 31 PCR cycles. The picture shows the ethidium bromide-stained PCR products. Strong expression of *Mtchit4* is apparent in the ecotype Jemalong A17. Expression of *Mtchit5* is obvious in the ecotype R108-1 but hardly detectable in the ecotype Jemalong A17. *M. truncatula*-specific ubiquitin (*Mtubi*) served as cDNA loading control. The picture shows the result, as it was obtained in two independent experiments

2.4.2. Cloning and sequencing of *Mtchit4*

A database search in the TIGR *M. truncatula* database (http://www.tigr.org/tdb/tgi/mtgi/) resulted in a 100% match between the partial *Mtchit4* sequence (AF167328, Salzer et al, 2000) and the tentative consensus sequence TC60709 of *M. truncatula* cv. Jemalong A17. Primers “Mtchit4 start-f” beginning with the start codon and “Mtchit4 stop-b” beginning with the stop codon of TC60709 were designed (Table 1) and used for PCR with genomic DNA of *M. truncatula* cv. R108-1. A single 1,245-bp PCR product was obtained comprising the full-length coding region of *Mtchit4*. The coding region was interrupted by a 396-bp intron located

at the conserved position of introns of class IV chitinase genes in angiosperms (Wiweger et al, 2003). Sequence data of *Mtchit4* are deposited at GenBank and are accessible under AY490790. Sequence comparison between putative *Mtchit4* and TC60709 gene products revealed >98% identity. The putative gene product of *Mtchit4* has a calculated molecular weight of 30,447 Da, an isoelectric point at pH 4.86, and possesses a secretion signal peptide for apoplastic targeting.

2.4.3. Cloning and sequencing of *Mtchit5*

From an adapter-ligated library, constructed on the basis of genomic DNA from *M. truncatula* R108-1, a 993-bp *Dra*I, a 356-bp *Pvu*I, and a 321-bp *Eco*RV fragment were amplified by PCR. Sequencing of the cloned fragments showed that they could be assembled to a full-length gene sequence. With the primers Mtchit5 start-f and Mtchit5 stop-b (Table 1), we directly obtained a 1,345-bp PCR product, which contained the full-length *Mtchit5* coding region. Sequence analysis revealed an open reading frame of 1,149 bp without introns. The ATG start codon was positioned 51 bp downstream of the putative TATA box, CTATATACA, and 24 bp downstream of a TAA stop codon. No polyadenylation signal was found within the 56 bp of the cloned 3' untranslated region. The *Mtchit5* sequence data are deposited at the EMBL Nucleotide Sequence Database and are accessible under AJ515476. The putative *Mtchit5* gene product is comprised of 383 amino acids, has a calculated molecular weight of 41,810 Da, and an isoelectric point at pH 7.66.

2.4.4. Sinorhizobium-specific induction of *Mtchit5* gene expression

To quantify *Mtchit5* and *Mtchit4* gene expression, primers were designed that met the requirements of real-time PCR (Table 1). Transcript expression levels of *Mtchit5* and *Mtchit4* were related to the expression levels of the *M. truncatula*-specific ubiquitin gene (*ubi*) that served as an internal standard for plant-derived mRNA (Salzer et al, 2000). We therefore expressed the standardized transcript expression levels as *Mtchit5*/*ubi* and *Mtchit4*/*ubi* ratios. In non-inoculated roots and leaves of *M. truncatula* R108-1, *Mtchit5*/*ubi* ratios were very low, amounting to ca. 0.002. In flowers, *Mtchit5*/*ubi* ratios were above the basal expression levels in roots but still in a range below 0.05 (Figure 2 a). In roots of *M. truncatula* R108-1 nodulated with *S. meliloti* wild-type Sm41, the *Mtchit5*/*ubi* ratios were more than 100-fold elevated above the basic expression levels in non-nodulated roots (Figure 2 a). Specificity of *Mtchit5* induction for interactions with rhizobia became apparent by measuring its expression in other types of plant-microbe interactions. In roots colonized by the arbuscular mycorrhizal fungus *Glomus intraradices*, the *Mtchit5*/*ubi* ratios remained low (Figure 2 a). In highly mycorrhized roots (58% of the root length contained fungal structures, i.e. intraradical hyphae, arbuscules or vesicles), the *Mtchit5*/*ubi* ratio reached a value of only 0.021 compared to 0.276 in nodulated roots. The *Mtchit5*/*ubi* ratio remained low also in roots challenged with the pathogenic fungus *Fusarium solani* f. sp. *phaseoli* (Figure 2 a) that caused visible browning around the sites of inoculation.
Mtchit4 expression levels remained low in roots of *M. truncatula* R108-1 plants nodulated with *S. meliloti* Sm41 (Figures 2 b, 3 b), whereas inoculation of R108-1 plantlets with *Fusarium solani* f. sp. *phaseoli* resulted in elevated expression of *Mtchit4*, 8 days post inoculation (Figure 2 b). Mycorrhiza formation with *Glomus intraradices* did not raise *Mtchit4* expression levels (Figure 2 b). Responsiveness of the *M. truncatula* cv. R108-1 roots to AM fungal colonization was demonstrated by induction of the mycorrhiza-inducible class III chitinase gene, *Mtchit3-3* (Salzer et al, 2000). At 58% root colonization, an *Mtchit3-3*/*ubi* ratio of 0.2 was attained.

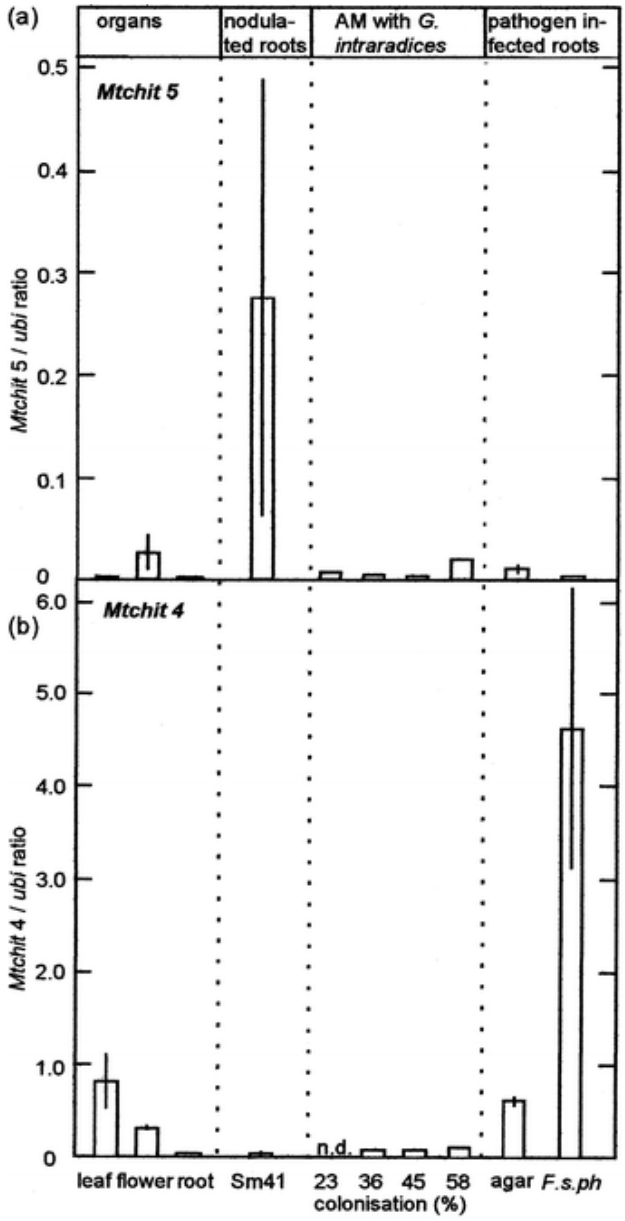

Figure 2 Specific induction of *Mtchit5* in nodulated roots of *M. truncatula* R108-1. Expression values of *Mtchit5* (a) and *Mtchit4* (b) were determined with real-time RT-PCR. RNA was analyzed from leaves (*leaf*), and flowers (*flower*), and roots (*root*) of 7-week-old plants, from nodulated roots inoculated with *S. meliloti* wild type strain Sm41 (*Sm41*), and from roots colonized with the AM fungus *Glomus intraradices*. The percentage of the root length containing AM fungal structures (*colonization*) is given for the individual plants 5 weeks post inoculation. From pathogen-challenged roots, RNA was analyzed 8 days post inoculation (*F.s.ph. = Fusarium solani* f. sp. *phaseoli*; *agar* = mock inoculation). The gene expression values of *Mtchit5* and *Mtchit4* were related to the expression values of *M. truncatula*-specific ubiquitin and are given as *Mtchit5*/*ubi* or *Mtchit4*/*ubi* ratios. The columns represent the mean values (±SE) of three independent experiments, except for mycorrhizal roots, where single values of plants with a specific degree of colonization are shown. All copy numbers were measured in triplicate

2.4.5. *Mtchit5* expression correlates with nodule development

Time-course experiments were performed to learn at which stage of nodule formation the expression of *Mtchit5* becomes elevated (Figure 3 a). Early time points (up to 3 days post inoculation) were analyzed on *M. truncatula* cv. R108-1 seedlings that had been grown on agar plates for 7 days, and then directly sprayed with a suspension of *S. meliloti* wild-type strain Sm41. At these early time points, which corresponded to the stage of root hair deformation and bacterial invasion, no increased expression of *Mtchit5* was measured in roots. Later time points (8, 14 and 35 days post inoculation) were measured in plants that had been transferred to Magenta jars and were subsequently inoculated with a rhizobial suspension. In roots of these plants (harvested 8 days post inoculation), levels of *Mtchit5* transcripts were about 4-fold higher compared to mock-inoculated control plants. At this stage of the symbiosis, the first nodule primordia started breaking through the root cortex. During the further ontogeny of the nodules, expression levels of *Mtchit5* steadily increased and reached more than 100-fold-elevated *Mtchit5*/*ubi* ratios 5 weeks post inoculation (Figure 3 a). For comparison, *Mtchit4* expression levels were measured in the same *M. truncatula* plants. As shown in Figure 3 b, *Mtchit4*/*ubi* ratios decreased over the time of the experiment, both in roots inoculated with *S. meliloti* Sm41 and in the mock-inoculated roots.

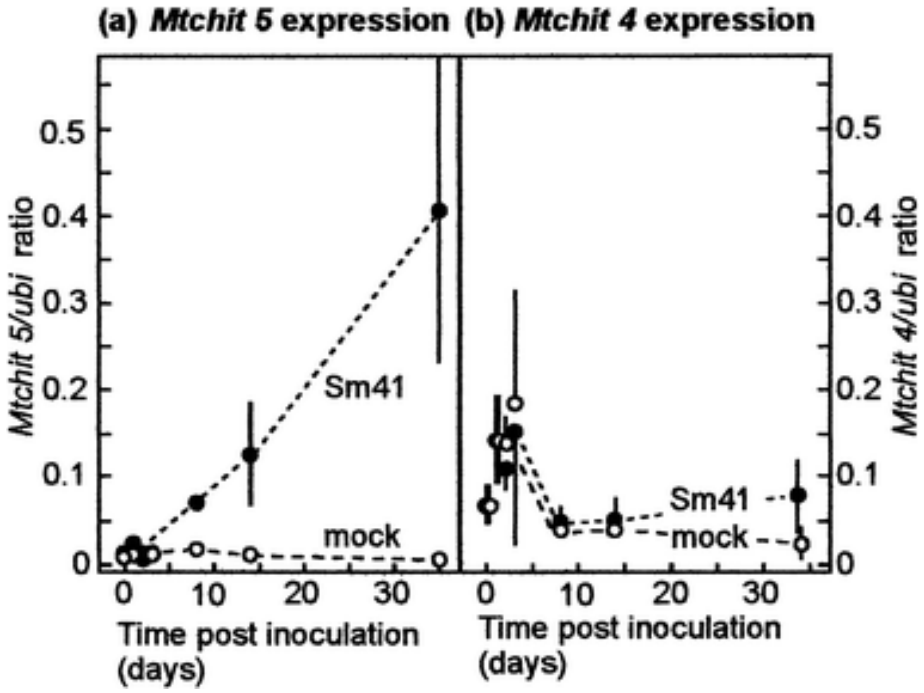

Figure 3 Time-dependent increase of *Mtchit5* expression during nodule formation. Time courses of *Mtchit5* (a) and *Mtchit4* (b) expression values are depicted after inoculation with *S. meliloti* wild-type strain Sm41. *M. truncatula* R108-1 plants were grown on agar for 7 days, then sprayed with bacteria (*Sm41*) or GTS medium (*mock*) and harvested 0, 1, 2, and 3 days later. To measure later stages of the symbiosis (8, 14, and 35 days post inoculation), 7-day-old seedlings were planted into Magenta jars and were inoculated with a bacterial suspension or GTS medium as a control. The *Mtchit5*/*ubi* and *Mtchit4*/*ubi* ratios were determined by real-time RT-PCR. The data shown are the means (±SE) of two independent experiments with copy numbers determined in triplicate per experiment

In an additional experiment, *M. truncatula* R108-1 plants were inoculated with mutants that are unable to establish an effective symbiosis. The *S. meliloti* strain AK631/*psZ*::Tn5 did not form nodules (Figure 4 a), whereas PP666h induced a number of small white nodule primordia (Figure 4 b). Plants inoculated with these strains remained small and produced purple leaves under nitrogen-limiting conditions (Figure 4 f). While *Mtchit5* expression remained at its very low basic level in roots inoculated with AK631/*psZ*::Tn5, considerable increases in *Mtchit5*/*ubi* ratios were found in roots infected with PP666h (Figure 5 a). This indicates that *Mtchit5* expression correlates with development of nodule primordia. For comparison, the expression levels were also determined for *Mtchit4*. The tested strains did not induce significant differences in expression of this gene (Figure 5 b).

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Figure 4 Symbiotic interactions between *M. truncatula* R108-1 and different *S. meliloti* mutants (5 weeks post inoculation). a Roots inoculated with AK631/*psZ*::Tn5. b Roots inoculated with PP666h. *White arrowheads* indicate nodule primordia that did not develop to nodules. c Roots nodulated with Sm41. d Nodules induced by PP699 (Sm41 mutated in the *rkp-1* region). e Non-inoculated roots of plants germinated at the same time as the inoculated ones (*no Sm*). f Phenotype of plants at the time of harvest. A plant growth-promoting effect is seen for the effective symbioses with PP699 and Sm41. Bars = 1 cm (a-e), 5 cm (f)


Roots infected with:
a
AK631 lpsZ::Tn5
b
PP666h
c
Sm41
d
PP699
e
no Sm
Plants cultured with:
f
no Sm
AK631 lpsZ::Tn5
PP666h
PP699
Sm41

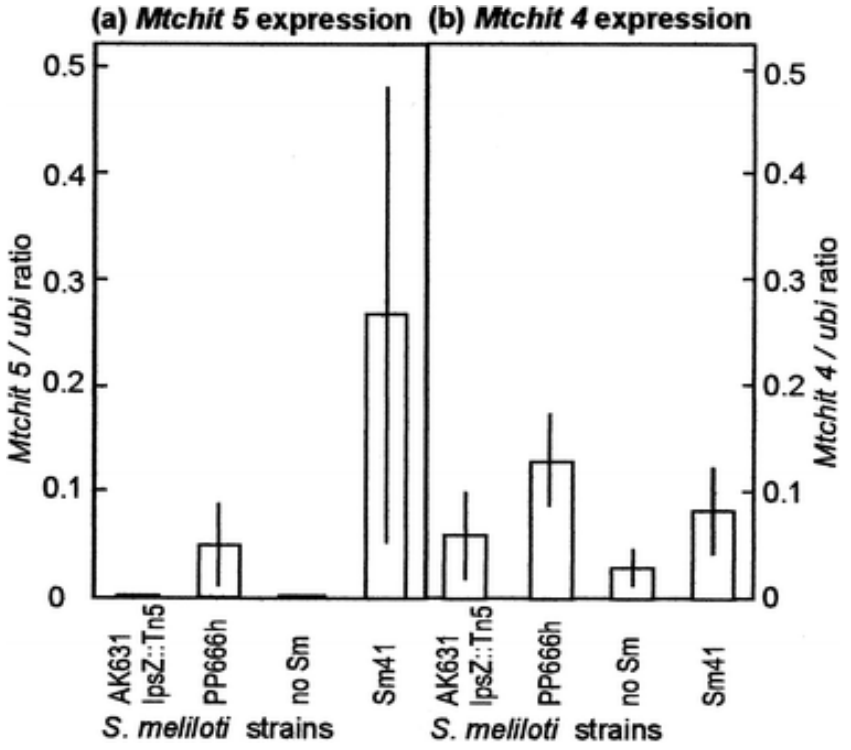


Figure 5 Expression of chitinases in *M. truncatula* R108-1 roots during ineffective symbioses. Plants were inoculated with *S. meliloti* AK631*lpsZ*::Tn5, PP666h or for comparison with Sm41 wild type. Five weeks later, *Mtchit5* (a) and *Mtchit4* (b) expression levels were determined by real-time RT-PCR. Chitinase gene expression levels are shown as *Mtchit5*/*ubi* or *Mtchit4*/*ubi* ratios. Elevated *Mtchit5*/*ubi* ratios were found in roots with ineffective nodule primordia induced by PP666h as well as in plants forming effective nodules with Sm41. Data indicate means (±SE) of three independent experimental series

2.4.6. Differential influence of Nod factors on expression of *Mtchit5* and *Mtchit4*

To study whether application of purified Nod factors elicited expression of *Mtchit5* and *Mtchit4*, 1 ml of the tetrameric Nod factor, NodSm-IV($C_{16:2}$, S), was sprayed on roots at a concentration of 0.5 µM. Control plants were mock-treated with water. Within 48 h, *Mtchit5*/*ubi* ratios were significantly elevated in the Nod factor-treated compared to water-treated roots (Figure 6 a). *Mtchit4* expression, in contrast, steadily decreased, both in Nod factor-treated and water-treated control roots (Figure 6 b).

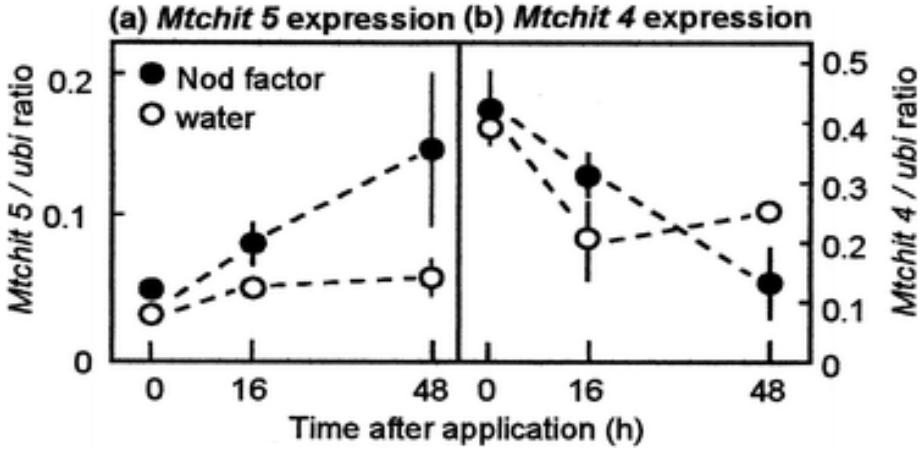


Figure 6 Effect of Nod factors on gene expression of *Mtchit5* and *Mtchit4*. Roots of 1-week-old *M. truncatula* R108-1 plantlets were sprayed with 1 ml 0.5 µM NodSm-IV($C_{16:2}$, S) or water. *Mtchit5* (a) and *Mtchit4* (b) expression levels were measured after indicated time points by real-time RT–PCR. The chitinase expression levels are given as *Mtchit*/*ubi* ratios. Data shown are means (±SE) of two independent experimental series with expression values determined in triplicate

2.4.7. Differential influence of K antigen on expression of *Mtchit5* and *Mtchit4*

A possible effect of K antigen on chitinase gene expression was investigated by a mutant-based approach. R108-1 plants were inoculated either with *S. meliloti* Sm41 or its mutant PP699 (Putnoky et al, 1990). PP699 is mutated in the *rkp-1* region and thus deficient in K antigen synthesis (Petrovics et al, 1993; Kiss et al, 1997). Plants inoculated with PP699 formed pink nitrogen-fixing (fix^+) nodules (Figure 4 d) that promoted plant growth under nitrogen-limiting conditions (Figure 4 f). Roots infected with the parent *S. meliloti* strain Sm41 (Figure 4 c) developed nodules slightly faster than PP699, suggesting a symbiosis-promoting effect of K antigen in this interaction. Compared to mock-inoculated roots, *Mtchit5*/*ubi* ratios (5 weeks post inoculation) were more than 100-fold increased in roots nodulated with either Sm41 or PP699. The degree of *Mtchit5* induction did not show any significant difference between the two strains (Figure 7 a). The *Mtchit4* gene, in contrast, showed a different induction profile between Sm41 and PP699. Compared to *S. meliloti* wild type strain Sm41, roots infected with the K-antigen-deficient mutant PP699 exhibited on average 4.5-fold higher *Mtchit4*/*ubi* ratios (Figure 7 b).

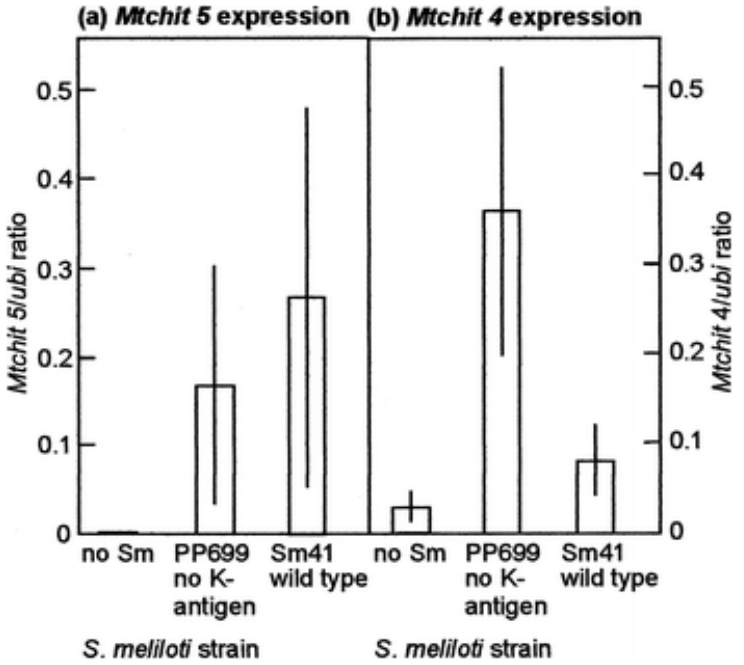


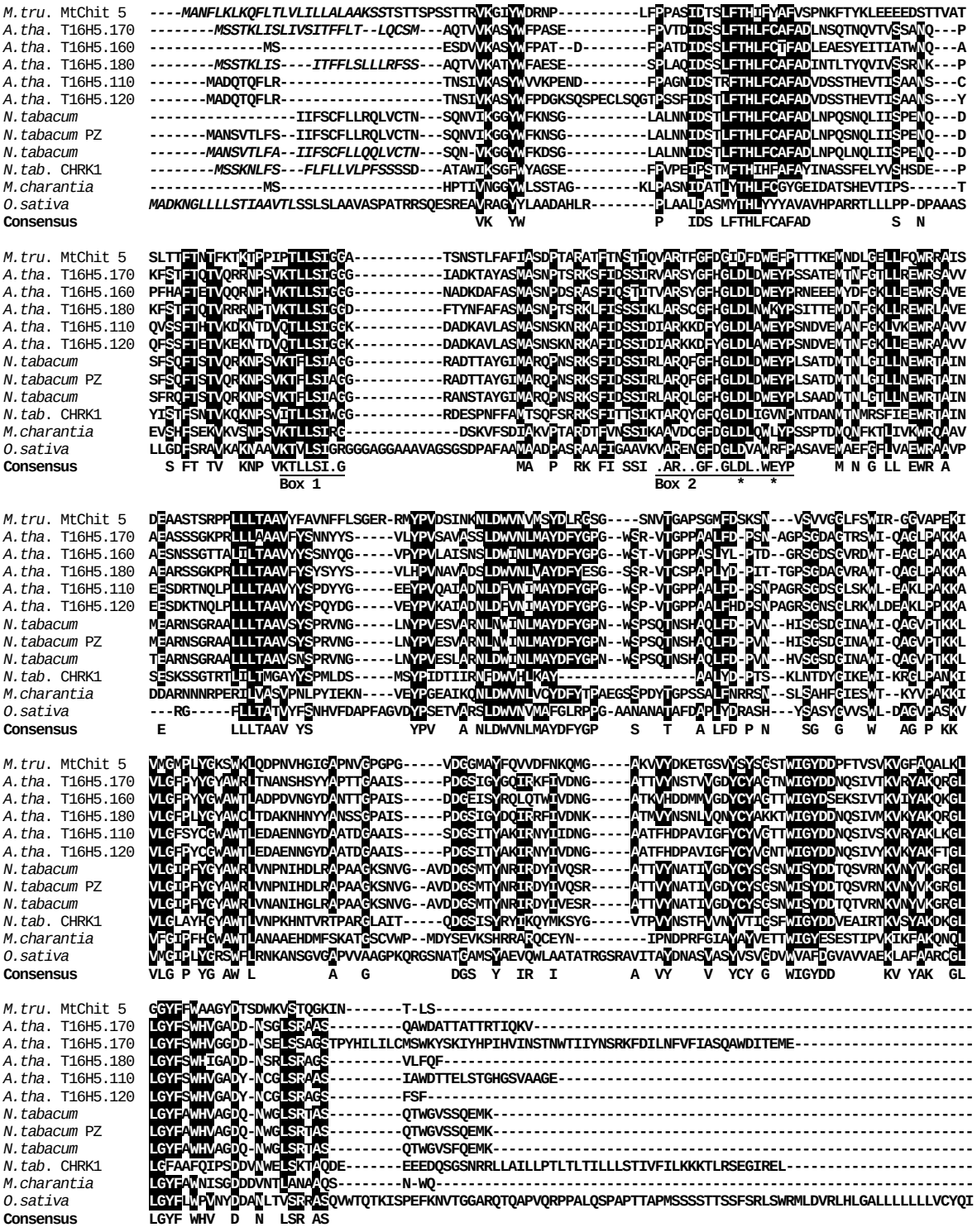
Figure 7 Expression of *Mtchit5* and *Mtchit4* in the interaction between *M. truncatula* R108-1 and *S. meliloti* PP699, a K-antigen-deficient mutant of Sm41. Plants were inoculated with Sm41 and PP699, which is mutated in the *rkp-1* region. Control plants were inoculated with GTS medium (*no Sm*). Five weeks later, nodulated roots were harvested and *Mtchit5* (a) and *Mtchit4* (b) expression levels were measured with real-time RT-PCR. Columns show mean values (±SE) of *Mtchit5*/*ubi* and *Mtchit4*/*ubi* ratios from three independent experiments with copy numbers measured in triplicate per experiment

2.4.8. Putative properties of the deduced *Mtchit5* polypeptide

The HmmerPfam analysis of the deduced amino acid sequence revealed that the putative *Mtchit5* gene product belongs to glycosyl hydrolase family 18. The two consensus motifs of class V chitinases, box 1 within the substrate-binding cleft, and box 2 within the catalytic centre contained the enzymatically active Asp (D) and Glu (E), respectively (Watanabe et al, 1993; Kim et al, 2002; Figure 8). This suggests that the putative Mtchit5 protein is an enzymatically active class V chitinase. The localization of the mature protein could not be clearly predicted. PSORT predicted cleavage of a signal peptide at position 26, while SPScan revealed no presence of a secretory signal peptide.

next page:

Figure 8 Alignment of plant class V chitinases. Deduced amino acid sequences of chitinases from *M. truncatula* (*M. tru.*), *Arabidopsis thaliana* (*A. tha.*), *Nicotiana tabacum*, *Momordica charantia* (bitter melon), and *Oryza sativa* were aligned. Amino acid residues identical to the consensus sequence are labeled in *black*. The consensus sequence for plant class V chitinases (*consensus*) was established under the criterion of 67% amino acid identity per residue. Putative secretion signal peptides are typed in *italics*. The conserved regions of the substrate binding cleft, Box 1, and the catalytic centre, Box 2, are *underlined*. The enzymatically essential amino acids D and E are indicated by *stars* (*). Gaps that have been introduced to fit the alignment are indicated by *hyphens* (-)


M.tru. MtChit 5
A.tha. T16H5.170
A.tha. T16H5.160
A.tha. T16H5.180
A.tha. T16H5.110
A.tha. T16H5.120
N.tabacum
N.tabacum PZ
N.tabacum
N.tab. CHRK1
M.charantia
O.sativa
Consensus
Box 1
Box 2

2.4.9. Phylogenetic analysis of class V and IV chitinases

For phylogenetic analysis, BLASTP searches in the EMBL, PIR and SwissProt databases against the putative *Mtchit5* and *Mtchit4* gene products were performed. In the case of *Mtchit5*, the 24 top-scored matches of class V chitinases, in the case of *Mtchit4* the 19 top-scored matches with class IV chitinases were selected. The entire amino acid sequences were used for the construction of unrooted phylogenetic trees. As expected, there was a large phylogenetic distance between the class IV and the class V chitinases. Within the class V chitinases, the putative *Mtchit5* gene product formed an individual clade that was distant from other class V chitinases of plants, animals and *Homo sapiens* (Figure 9 a). Within the phylogenetic tree, class V chitinases from *Nicotiana tabacum* grouped together with chitinases and further members of the glycosyl hydrolase family 18 of *Arabidopsis thaliana*. The deduced *Mtchit5* gene product had the highest similarities to the *A. thaliana* chitinase homolog T16H5.170 (53% similarity, 36% identity), to the tobacco pz chitinase (52% similarity, 33% identity; Heitz et al, 1994) and to the tobacco Chi-V (52% similarity, 33% identity; Melchers et al, 1994).
The putative *Mtchit4* gene product was closely related to other class IV chitinases from legumes. The chitinases most related to Mtchit4 were either pathogen induced or expressed in nodulated roots. As with the class V chitinases, the class IV chitinases exhibited a strict species-related grouping (Figure 9 b). The *Mtchit4* gene product has the highest similarities to the endochitinase PR4 precursor of *Phaseolus vulgaris* (82% similarity, 75% identity; Lange et al, 1996; accession number: P27054), the class IV chitinase of *Galega orientalis* (81% similarity, 73% identity; accession number: AAP03085) and the class IV chitinase of *Vigna unguiculata* (79% similarity, 71% identity; accession number: CAA61281).

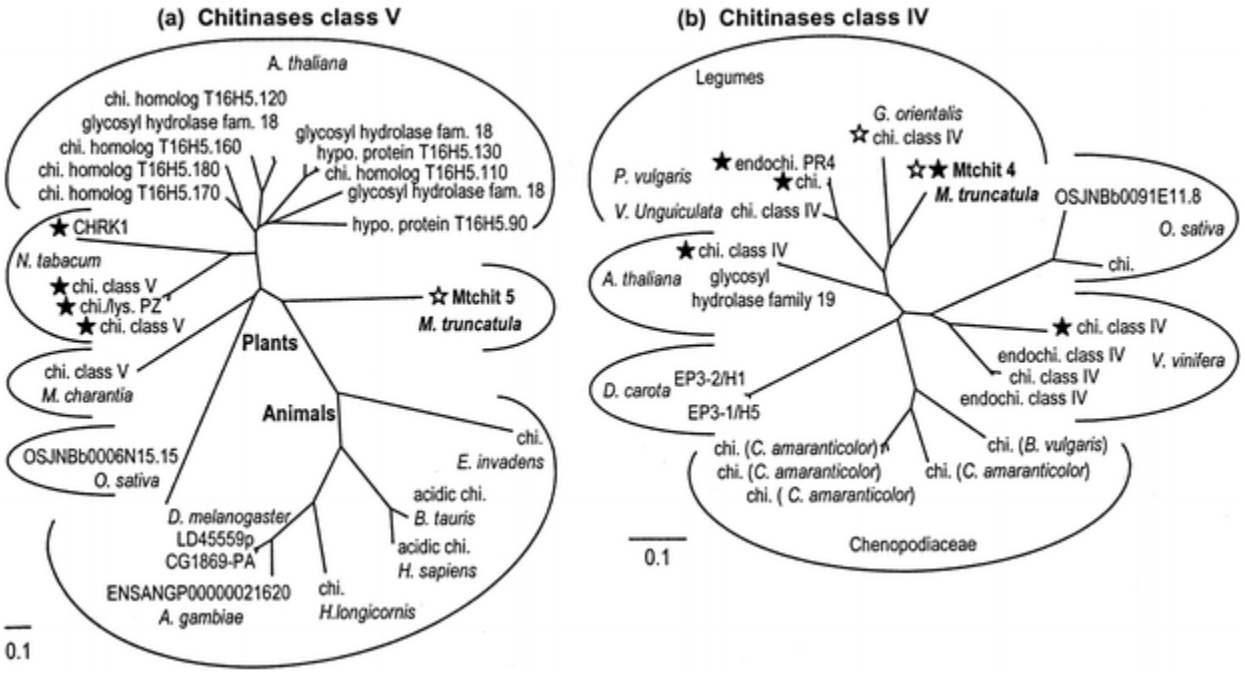


Figure 9 Unrooted phylogenetic trees of class V (a) and class IV (b) chitinases. A BLASTP search was performed with the full-length amino acid sequences of the putative *Mtchit5* and *Mtchit4* gene products. Twenty-four class V amino acid sequences were obtained with a bit score >125, and were used to construct a phylogenetic tree. These sequences originate from plants (*M. truncatula*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa*, *Momordica charantia*), insects (*Drosophila melanogaster*, *Anopheles gambiae*), mammals (*Bos taurus*, *Homo sapiens*), the tick *Haemaphysalis longicornis*, and the protist *Entamoeba invadens*. In the case of class IV chitinases, 20 amino acid sequences with a bit score >300 were obtained. The sequences stem from rice (*Oryza sativa*), wine (*Vitis vinifera*), sugar beet (*Beta vulgaris*), pigweed (*Chenopodium amaranticolor*), carrot (*Daucus carota*), thale cress (*Arabidopsis thaliana*), cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris*), and goat's rue (*Galega orientalis*). *Black stars* indicate chitinases that are pathogen induced; *white stars* indicate chitinases that are *Sinorhizobium* induced. No information was available about induction of chitinases without labeling. The bars give 0.1 amino acid exchanges per residue. Note the different scales applied for class V and class IV chitinases

2. 5. Discussion

Ecotype-specific variation in nodule formation is known from natural *M. truncatula* populations. For example, the Algerian *M. truncatula* cultivar DZA315.16 is able to form nitrogen-fixing nodules with the *S. meliloti* strain A145, whereas the Australian cultivar Jemalong 6 formed only small ineffective nodules with this strain (Tirichine et al, 2000). In our studies, comparison of chitinase gene expression in nodulated roots revealed a qualitative difference between the *M. truncatula* ecotypes R108-1 and Jemalong A17. Nodule formation with *S. meliloti* 1021 resulted in transcript accumulation of *Mtchit4* in Jemalong A17, whereas expression of this chitinase gene was not elevated above its basic expression level in R108-1 (Figure 1). In contrast to *Mtchit4*, expression of *Mtchit5* was induced in both ecotypes. Compared to R108-1, expression in Jemalong A17 was rather low, however (Figure 1). A quantitative difference in chitinase gene expression was also found for *Mtchit3-3*, a chitinase gene of *M. truncatula* that is specifically induced in response to AM formation (Salzer et al, 2000). AM-induced transcript levels of *Mtchit3-3* were about 5-fold lower in R108-1 than in Jemalong A17 (our unpublished data).

We consider *Mtchit4* to be a chitinase gene that is related to a general plant defense response for the following reasons: (i) The gene was induced upon infection with the pathogenic fungus *Fusarium solani* f. sp. *phaseoli* in *M. truncatula* R108-1 (Figure 2) and Jemalong A17 (Salzer et al, 2000). (ii) *Mtchit4* is closely related to pathogen- and elicitor-induced class IV chitinases of legumes, wine, and the non-symbiotic *Arabidopsis* (Figure 9). (iii) *Mtchit4* was not induced by rhizobial Nod factors (Figure 6 b) or by symbiotic AM fungi (Figure 2 b). Considering *Mtchit4* induction as a part of a general defense response, low transcript levels in the interaction between *M. truncatula* R108-1 and *S. meliloti* Sm41 would indicate an optimal compatibility between the two symbiotic partners. In this view, enhanced accumulation of *Mtchit4* transcripts induced by the K-antigen-deficient mutant PP699 is remarkable. We suggest therefore that K antigens are involved in suppression of plant defense reactions directed against the invading bacteria. This is reminiscent of the proposed function of other surface polysaccharides (Mithöfer, 2002). Future work is required to demonstrate a direct link between K antigen and suppression of inducible plant defense reactions.

Real-time RT-PCR enabled us to quantitatively analyze *Mtchit5* expression, even though transcripts accumulated only at low abundance. Using this approach, we found that transcription of *Mtchit5* is specifically induced in the symbiosis with *S. meliloti*. Fungal pathogens and mycorrhizal fungi did not induce *Mtchit5* in roots of R108-1 (Figure 2 a). Thus, *Mtchit5* can be considered as a putative chitinase gene that is specific for nodules in *M. truncatula* ecotype R108-1. *Mtchit5* belongs to the class V chitinases and forms a separate clade within the phylogenetic tree of class V chitinases of plants and animals (Figure 9). *Mtchit3-3*, which is specifically expressed in mycorrhizal roots of both *M. truncatula* ecotypes (Salzer et al, 2000; Bonanomi et al, 2001; chapter 3), belongs to the class III chitinases and is structurally different from class V chitinases. This indicates that *M. truncatula* evolved two types of chitinase, which are independently activated in response to nodule formation and mycorrhiza formation. In the tripartite symbiosis with *S. meliloti* and AM fungi, expression of both genes is induced (data not shown).

Mtchit5 expression had already occurred early during nodule development. Although this gene is also expressed in flowers, we consider *Mtchit5* as an early nodulin-like gene. This is reminiscent of the *Srchi13* chitinase gene from *Sesbania rostrata*, whose transcripts transiently accumulated during nodule formation (Goormachtig et al, 1998). *Mtchit5* and *Srchi13* are members of the glycosyl hydrolase family 18 (Henrissat et al, 1991), but they do

not belong to the same class of chitinases. The *Srchi13* gene codes for a class III chitinase, whereas *Mtchit5* codes for a putative class V chitinase.

In contrast to *Mtchit4*, *Mtchit5* seems not to be part of a plant defense reaction. There are several arguments in favor of this assumption: (i) The *Mtchit5* gene is activated during nodulation, but not when roots are challenged with a fungal pathogen. (ii) *Mtchit5* is expressed in low abundance. This is not typical of defense-related genes, which are usually strongly induced. (iii) In response to Nod factors, transcripts of *Mtchit5* accumulated to levels that are far above those induced after inoculation with *S. meliloti* Sm41 at the same time (compare Figures 3 a and 6 a). (iv) Expression patterns of *Mtchit5* differed from those of the defense-related *Mtchit4* (e.g. see Figure 7).

In fact, we can only speculate about the function of *Mtchit5*. Its symbiosis-specific induction and its responsiveness to Nod factors suggest a function during establishment of the nodule symbiosis. A role of chitinases in developmental processes is known from somatic embryo formation of carrot cells (de Jong et al, 1992). Recent findings indicate that *N*-acetylglucosamine-containing arabinogalactan proteins of carrot cells are sensitive to chitinase cleavage and that the chitinase-modified proteins reinitiate cell division of non-dividing protoplasts (van Hengel et al, 2001). Although functionality and subcellular localization of the putative Mtchit5 chitinase have not been studied experimentally, it is tempting to speculate that Mtchit5 could modify arabinogalactan proteins in the dividing cells of the nodule meristem. Targets of Mtchit5 enzymatic activity might be early Nodulins, such as PsENOD5 (Scheres et al, 1990), MtENOD16 and MtENOD20 (Greene et al, 1998), which are predicted to be possibly *O*-glycosylated with arabinogalactans.

In conclusion, this comparative study on expression of two *M. truncatula* chitinases opens a first insight into the differences between regulation of symbiosis-specific and pathogen-related chitinase genes during nodule formation and in response to rhizobial signal molecules. The identification of *Mtchit5* as an early nodulin-like gene, suggests a function that is different from plant defense. Future in situ mRNA hybridization and antibody-based localization studies as well as the analysis of the enzymatic properties of the purified *Mtchit5* proteins are required to better understand the role of this chitinase during nodulation.

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Appendix 1

Comparative study of *M. truncatula* chitinases of the GH family 19

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List of figures and tables:

Key words:
chitinase; defence; promoter, gene, protein structure; expression pattern

Abbreviations:

CRD	cystein-rich chitin-binding domain
EST	expressed sequence tag
Glycosyl Hydrolase	GH
Mtchit, chit	chitinase
PR	pathogenesis-related
PCR	polymerase chain reaction

A1.1. Abstract

The chitinases of the glycosyl hydrolase (GH) family 19 are traditionally considered as pathogen-related (PR) proteins and respond to fungal pathogenic stimuli. Several genes encoding chitinases of GH family 19 have been identified in the model legume *Medicago truncatula*. Their expression was regulated differentially in response to pathogenic and mycorrhizal fungal infection (Salzer et al, 2000). Chitinases of GH family 19 can be subdivided further into the classes I, II and IV on the basis of their amino acid sequences. They all have a common primary structure with respect to the catalytic domain. Chitinases of class I and IV possess, in addition, an N-terminal cysteine rich chitin binding domain.
In this study, four different genes encoding chitinases of GH family 19 in *Medicago truncatula* are presented. Expression studies were done for three of them, together with an *in silico* analysis of their promoter sequences:
Mtchit1a was previously identified as a chitinase class II (*Mtchitinase II*, Salzer et al, 2000), but to our current knowledge it is a class I chitinase with an N-terminal cystein-rich binding domain. *Mtchit1b*, a gene that had escaped the previous analysis, represents a chitinase very similar to the product of the *Mtchit1a* gene. It is obviously expressed since it is represented in the EST library, but it is not studied further in the present work. *Mtchit1c* corresponds to a previously identified class I chitinase (*Mtchitinase I*, Salzer et al, 2000); we rename it here to avoid confusions. *Mtchit4* is a member of class IV chitinases and was formerly designated *Mtchitinase IV* (Salzer et al, 2000).
In a gene expression study, the expression rates of the three chitinases *Mtchit1a*, *Mtchit1c*, and *Mtchit4* were measured in leaves, roots and flowers of *M. truncatula*. *Mtchit4* had a significantly higher expression rate in leaves than in roots or flowers, and transcript levels were slightly reduced in roots colonized with the mycorrhizal fungus *Glomus intraradices*. In contrast, expression of *Mtchit1a* was not significantly altered in leaves or roots but significantly lower in flowers. Expression of *Mtchit1a* was not affected by infection with mycorrhizal fungi. *Mtchit1c* was constitutively expressed in leaves and roots and not altered in response to mycorrhizal infection. These distinct expression patterns, together with the sequence data, suggest a specific regulation of synergistically acting chitinases, allowing a fine-tuning of defense-response reactions in different tissues.

A1.2. Introduction

Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of the β-1,4-N-acetyl-D-glucosamine linkages in chitin polymers, the main structural component of fungal cell walls. There are seven classes of chitinases, class I-VII, that belong to the glycosyl hydrolase (GH) family 18 or 19, depending on the primary structure and enzymatic mode of action (Shinshi et al, 1990; Collinge et al, 1993; Neuhaus et al, 1996). The GH family 19 chitinases, classes I, II, and IV, are found primarily in plants, but some genes have been identified in bacteria. Chitinases of these three classes share homologies in their catalytic domains but are distinguished from each other by different domain compositions. In contrast to chitinases of the class II, the class I chitinases posses a cysteine rich hevein domain and a shorter hinge region at the N-terminus. These enzymes use the same *inversion cleavage* mechanism as lysozyme (Iseli et al, 1996) and they often have some lysozyme activity. The similarity of the three classes of enzymes with respect to the enzymatic mechanism and the primary amino acid sequence indicates that they developed from a common ancestral glycohydrolase (Shinshi et al, 1990; Iseli et al, 1996).
Infection of plants by pathogenic fungi triggers plant defense reactions and leads to the expression of PR proteins. PR proteins are diverse in their functions and structures, and one important class are the chitinases (e.g. Benhamou et al, 1990; Broglie et al, 1991). Together with β-1,3-glucanases, chitinases have been shown to inhibit fungal growth in vivo and in vitro (e.g. Mauch et al, 1988; Broglie et al, 1991). Chitinase expression has been reported from grapes, bean, pea, tobacco or rice in response to various kinds of biotic and abiotic stresses (e.g. Voisey and Slusarenko, 1989; Hamel and Bellemare, 1995; Taira et al, 2005).
In a previous study, expression patterns of different chitinase genes have been studied in the roots of the model legume *Medicago truncatula* after infection with pathogenic fungi (Salzer et al, 2000). All investigated GH family 19 chitinases were strongly induced by *Fusarium solani f. sp. phaseoli* and also by *Phytophthora megasperma f. sp. medicaginis*. During the interaction with the mutualistic symbiotic arbuscular mycorrhizal fungus *G. intraradices*, the three chitinases were differently induced (Salzer et al, 2000).
This suggested that in *M. truncatula* roots chitinases are induced in response to a fungal infection, although it seems that the transcription rate is individually influenced by the nature of fungal signaling and cell wall composition or other species-specific components.

To get more information on the regulation of fungal responsive chitinases, a comparison was done on the GH family 19 chitinase genes and their deduced protein sequences, including phylogenetic protein analyses, expression patterns during presence of mycorrhizal fungi and analysis of promoter regulating elements.

A1.3. Materials and Methods:

A1.3. 1. Gene isolation, cloning and sequencing

Mtchit1a was amplified from genomic DNA from the *M. truncatula* Jemalong A17 ecotype by PCR with the forward primer 5'-CGACACACCAAACTGAATTAACACTCTTC-3', and the reverse primer 5'-CAATCCAAGTTATCACCAGGACTCAC-3' (Tm = 60° C). Isolation of the genes, cloning and sequencing was performed as described in Salzer et al (2004; chapter 2) for *Mtchit4*. *Mtchit1b* was found during a computational analysis of the putative promoter sequence of *Mtchit1a*, and has not been cloned and sequenced. The *Mtchit1c* gene was partially sequenced (Salzer, unpublished), and manually compiled.

A1.3.2. Gene expression measurements by real-time RT-PCR

RNA isolation, RT reaction and real-time PCR protocols are described in chapter 4. The primers that were used to perform real-time PCR are equal to the primers that were reported in Salzer et al (2000).

A1.3.3. Biocomputational sequence analyses

Genomic sequences were compiled and analyzed as described in Salzer et al (2004), where also the phylogenetic analysis is described. Protein sequence alignments were done with the help of the multiple sequence alignment programs (http://www.ebi.ac.uk/clustalw and http://align.genome.jp/), the neighbour-joining trees were contructed with PAUP 4.0.
pSort (http://psort.hgc.jp/form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP) were used to predict signal peptides and protein targeting. The gene coding sequences were used to screen the gene databases in www.tigr.org/tdb/tgi/mtgi or http://www.ncbi.nlm.nih.gov. Sequences that were spanning the coding region were compared and searched for sufficiently long 5' upstream sequences. The same done to obtain the promoter sequence of a beta-glucanase, which has similarity to the *Medicago sativa* acidic glucanase AAV66071. Newly identified putative promoter sequences were analysed in the programs of the Softberry-sequence analysis suite (www.softberry.com). For obtaining putative transcription factor binding sequences, the promoters were analyzed in http://www.dna.affrc.go.jp/PLACE/signalscan.html and further manually processed.

A1.4. Results

A1.4.1. Gene and protein structures

Here, four genes of family GH 19 chitinases are presented that have been isolated and sequenced in the genome of *M. truncatula*, three of which have already been partially sequenced before (Salzer et al, 2000). A sequence alignment of the deduced amino acid sequences corresponding to these four genes is presented in Figure 1. The amino acid sequences have relatively high homology to each other, and to chitinases of their respective classes. Mtchit4 has 36% identity and 50% similarity with Mtchit1a and 33% identity and 46% similarity with Mtchit1c, whereas Mtchit1a and Mtchit1c share 55% identity and 67% similarity. Mtchit1a and Mtchit1b are highly similar, with 83 % identity and 88 % similarity.

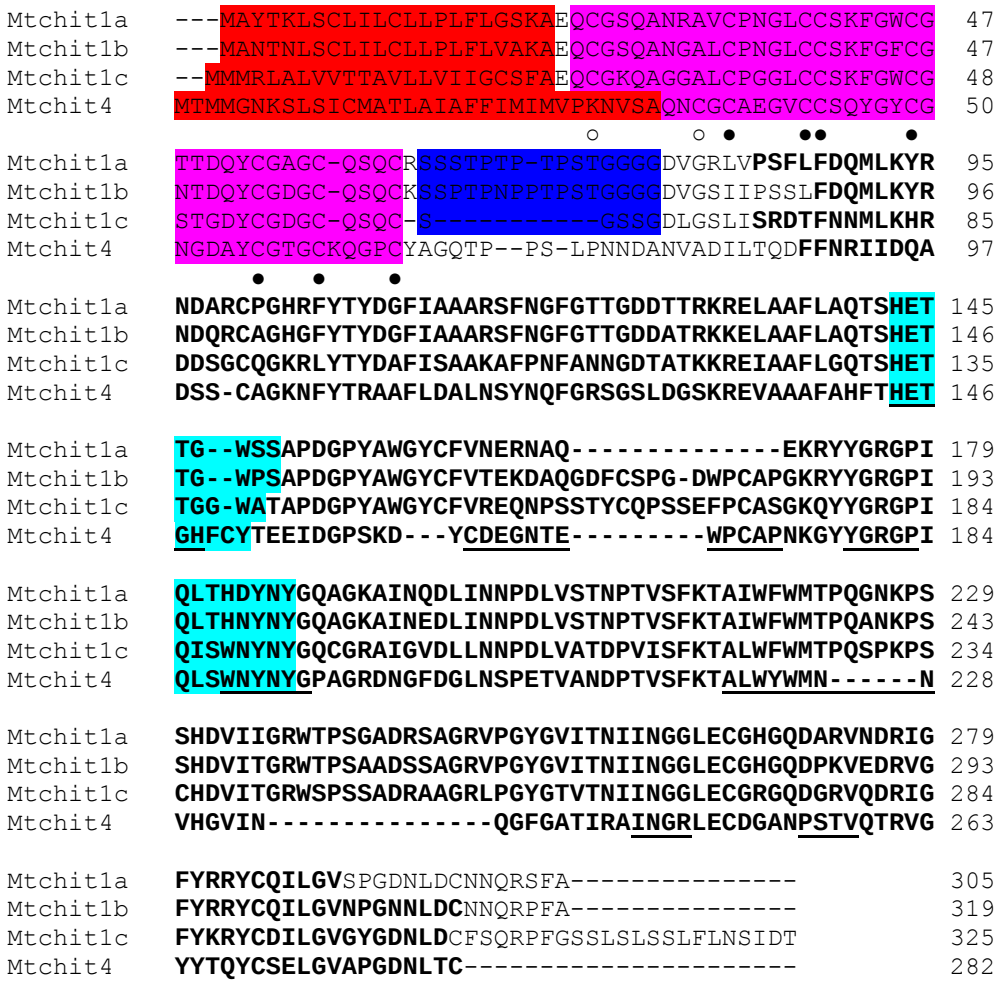

Figure 1 Alignment of GH family 19 chitinases. Indicated are signal peptides in red, chitin binding domains in pink, glycine rich hinge regions (for close-up see also Figure 3) in dark blue and the catalytic domains in bold case. The positions of the intron boundaries are indicated in turquoise. Circles indicate conserved cysteine residues, the open circles represent positions typical for class I or IV, respectively. Underlined are residues that may be catalytic or involved in active site architecture. None of the sequences has a vacuolar targeting signal at its C-terminus.

Mtchit1a has initially been described as a class II chitinase (*Mtchitinase II*; AF167323 in Salzer et al, 2000), but after evaluation of its complete sequence it was classified as a class I chitinase and therefore renamed. In the TIGR *M. truncatula* database the TC106402 sequence derived from expressed sequence tags was found identical to the predicted coding sequence and the gene sequence is identical to a region on BAC mth2-29o24. The *Mtchit1a* gene contains a 1983 bp open reading frame, comprising two introns of 911 and 151 bp length. A putative polyadenylation signal was found 25 bp downstream of the stop codon. The deduced preprotein contains a cleavable signal peptide of 22 amino acids (red in Figure 1), indicating that it enters the secretory pathway, as is typical for class I chitinases. The mature protein comprises an N-terminal chitin binding domain characteristic for class I chitinases, the so called hevein domain (pink), with a hinge region (dark blue) and a catalytic domain (bold case). The pI of the mature protein is 8.05 with a molecular mass of 31 kDa. A C-terminal vacuolar targeting signal is not present (Neuhaus et al, 1991), and an extracellular protein localization is therefore suggested.

A second chitinase class I open reading frame was found on BAC mth2-29o24 at -5013 bases upstream of the *Mtchit1a* gene and was named *Mtchit1b*. The sequence of *Mtchit1b* comprises 2885 bp, containing two introns. A putative CAAT box (CCAATT at -87 bp), a TATA box (CTATAAATT at -27 bp) and a polyadenylation site (+91 downstream of the stop codon) were identified. This novel gene has not been reported in Salzer et al (2000), instead, the expressed sequence tag TC106349 (TIGR *M. truncatula* database) was found to be identical to the predicted coding region. Together with the regulatory elements, it can be assumed that this gene is expressed and regulated by unknown stimuli. In the deduced protein sequence the same domains are present as in Mtchit1a (Figure 1), including the cleavable signal peptide of 22 amino acids. The predicted mature protein has a pI of 6.37 and a mass of 31.9 kDa.

Mtchit1c has previously been named *Mtchitinase I* (AF167322) by Salzer et al (2000), but was renamed to avoid confusion. Its sequence revealed a 975 bp open reading frame that corresponded to TC106842, derived from expressed sequence tags. The same open reading frame sequence was also found on BAC mth1-8p19. A polyadenylation signal lies +27 downstream of the stop codon. Intron boundaries are present within the conserved GH family 19 motifs, shown in turquoise in the protein sequence (Figure 1). The deduced sequence of the protein contains a cleavable signal peptide of 23 amino acids (red). The predicted mature protein has a basic pI of 7.05 and a molecular weight of 32.5 kDa. It is characterized by a GH19 domain. Computational targeting proposed that it is a type III membrane protein in the plasma membrane or the ER membrane. An extension at the C-terminus is present, which has no similarities with the necessary vacuolar targeting signal of some chitinases (Neuhaus et al, 1991).

The *Mtchit4* gene has a length of 1245 bp and has been described in (Salzer et al, 2004); chapter 2 in this thesis). The 3' UTR region of the *Mtchit4* gene contained polyadenylation enhancing sequences just downstream of the stop codon (stop:A/GTT/AT/AAATATTAG), and (TATAAA/TA/TGTG:aataaa:AA/GA/TTAT/AAAT/AT) but not the actual aataaa-site. The *Mtchit4* deduced protein (Figure 1) contains a cleavable signal peptide (red), a chitin binding domain (pink), and the catalytic domain (bold). One intron is positioned between HETG and DFCY (turquoise), and the boundary sequences of the second intron (turquoise) are present, although the nucleotide sequence did not show an additional intron at this position. The predicted mature protein has a pI of 4.86 and a mass of 30.4 kDa.

A1.4.2. Regulating elements in the chitinases promoters

In the putative promoter sequence pMtchit1a*,* the 5' upstream region of *Mtchit1a,* a putative CAAT box at -124 bp and at -74 a putative TATA box were identified (Figure 2), together with the transcription start point 49 bp upstream of the start codon, which could not be confirmed experimentally. The sequences upstream as well as downstream of the two chitinases Mtchit1a and Mtchit1b were not similar, indicating differences in gene regulation. A TATA box at -69 bp with a transcription start point at -36 bp from the start codon were found, but no CAAT box could be identified in the putative promoter sequence of *Mtchit1c,* pMtchit1c (Figure 2). In the putative Mtchit4 promoter, CAAT and TATA boxes were found at positions -70 and at -148, respectively (Figure 2).

The three putative promoter sequences were analysed by several promoter prediction programs and manual compilation. The putative promoters of all three chitinases, ca. 2 kb in length, contained three clusters of enhanced *cis* regulatory element presence of approximately 250 - 350 bp length each: in addition to a putative proximal promoter region just upstream of the transcription start site, each sequence had two *cis*-clusters further upstream and close to each other (Figure 2). The most interesting features are depicted in Figure 2.

In all three chitinase promoter sequences, many potential Dof-binding sites (AAAG-containing sites) were found (not shown). Dof binding sites are implied in tissue specific regulation or signaling. However, the recognition sequence is short and occurs rather frequently by random distribution. The same is true for possible WRKY box sequences (TTGACC, encoded in blue in Figure 2), which occured frequently as well. WRKY transcription factor binding elements could be involved in gene regulation in response to several stress factors and often are associated with certain other motifs. What was interesting and made the three promoter sequences different from other chitinase promoters was a high frequency of stress and hormone (mainly gibberellic acid and jasmonic acid) responsive elements, that were also found in the glucanase promoter. All three promoters contained elements known to be pathogen or defence related, which is consistent with the proposed functions of the corresponding genes. Furthermore, many elements that are root, seed or flower-related were also found, and these might confer tissue dependent regulation.

pMtcht1a was the only sequence that contained a unique sugar related element (ACGTA box) and a high number of putative hormone responsive elements of which two (TGTCTC; ACGTSSSC) were found in the promoter sequence of the glucanase. The elements in pMtchit1c were mainly related to biotic and abiotic stress such as one element that may be associated with heat shock (CCACGTCA) and was not shared with other sequences. The sequence of pMtchit4 contained one possible defence-related element (GTTAGTT) shared with glucanase as well as several possible auxin-related elements (KGTCCCAT; GGTCCCAT; CACGCAAT) that were not shared with the promoter sequences of other chitinases or the glucanase. pMtchit4 also contained elements that may be specifically involved in elicitor or light activation of genes (CCGTCC). One such element was shared among pMtchit4 and pMtchit1a, namely the WRKY box (TTGACC, encoded in blue in Figure 2). These two promoters also share an interesting putative MYB binding site (MACCWAMC, encoded in green in Figure 2), that is found in phenylpropanoid biosynthetic genes, typically induced in plant defence reactions (Figure 2). On the other hand, the two class I chitinase promoter sequences shared two elements possibly involved in salicylic acid and stress relation (TCATCTTCTT, encoded in yellow in Figure 2) as well as a pathogenesis related element (YTGTCWC, encoded in red in Figure 2), which is also found in promoters of Mtchit3-1 and the glucanase. Only one single motif was found only in promoters of chitinases of the GH family 19 (TTTTTTCC).

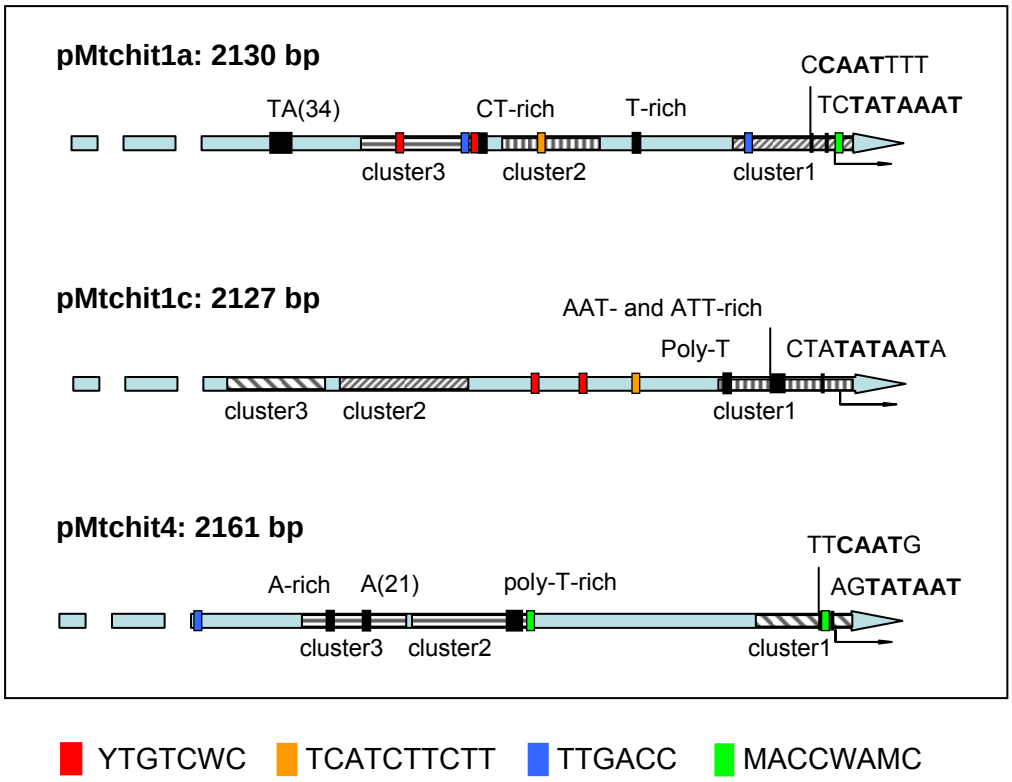

Figure 2 Features of the putative promoter sequences of GH family 19 chitinases. Arrangement of features distributed in the three chitinases of GH19 family chitinases. The clusters of the three promoters were not similar to each other, and the closest relations were not within the same promoter, as the color code indicates: similar clusters contain similar colors. However, the overall similarity between the sequences is very low. The arrows indicate the transcriptional start. Y: C or T, W: A or T, M: A or C.

A1.4.3 Phylogeny of GH family 19 chitinases

As shown in Figure 3, a phylogenetic analysis was performed with the 14 most closely related chitinases in the database to the Mtchit1a protein sequence, using a BlastP search. This analysis confirmed that Mtchit1a is a class I chitinase. The lowest sequence similarity of 65% and identity of 76% to Mtchit1a has the *N. tabacum* class I chitinase (AAB23374) and the chitinase Go-cht1b of *G. orientalis* (AAP03087) has the highest similarity of 88% and an identity of 83%. The phylogram (Figure 3) shows one large group of chitinases around Mtchit1a, that contains only legume encoded chitinases, each of which are inducible by rhizobia or found in root nodules. One separate clade represents chitinases from Solanaceae that have been characterized previously as pathogen inducible, or having antifungal activity (Shinshi et al, 1990; Neuhaus et al, 1991; van Buuren et al, 1992; Suarez et al, 2001). When Mtchit1c was submitted to BlastP, a different group of class I chitinases was returned than with Mtchit1a. Mtchit1c has a similarity of 82% to 94% and an identity of 72% up to 94% to *M. acuminata* (*Zingiberales*) and the *M. sativa* (AAB41325) chitinase, respectively. In the phylogram of these chitinases (Figure 4), the largest and closest group again consists of chitinases of legume origin. Only limited information was available on the induction in response to pathogen or rhizobia, however, it seems that the legume chitinase induction is, amongst others, controlled by microbial stimulation. A phylogenetic analysis of the most closely related chitinases of Mtchit4 is presented in Salzer et al, 2004 (see chapter 2). An unrooted phylogram is shown here again, for a comparative overview (Figure 5).
In general, it can be stated that the GH family 19 chitinases are rather closely related to their counterparts from other plant species, showing a remarkably high similarity within the different classes.
Interestingly, when comparing protein primary sequences, the hinge region that connects the CRD (cystein-rich chitin-binding domain) to the catalytic domain is different in the two class I chitinases. In the group of Mtchit1a, a longer, TP-rich region is found with high variability among the sequences of this group (Figure 6). This hinge region is similar to the corresponding sequence of the CHNA chitinase of tobacco (Suarez et al, 2001) although in the Solanaceae group a pattern is visible that distinguishes the group from the legumes. In the group of Mtchit1c, the hinge region is GS-rich, shorter and less variable. Some of the proteins show little homology of this region to the other two variants, in this case, the hinge region is characterized by a PAP-containing sequence. In two of the chitinases of class IV a glycine rich region was seen, which seems not to be a general feature of this chitinase class.

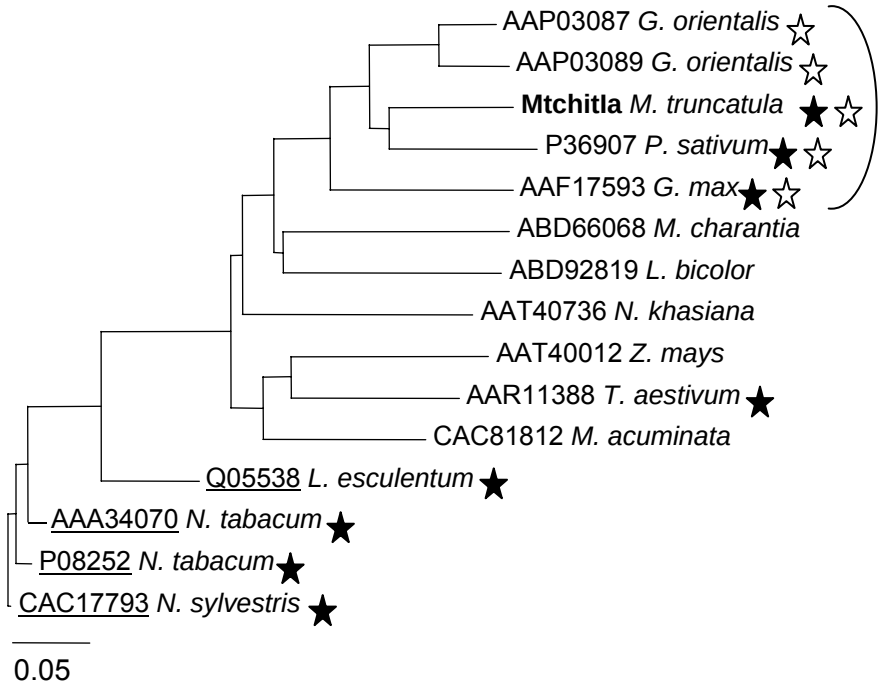

Figure 3 Unrooted phylogram of chitinases similar to Mtchit1a. The group of chitinases from leguminous species is indicated by a bracket. Filled stars indicate antifungal or pathogen inducible chitinases, white stars indicate rhizobium inducible chitinases. Underlined sequences seem to have a vacuolar targeting signal by sequence comparison (Neuhaus et al, 1991). The bar indicates 0.05 amino acid exchanges per residue.
Abbreviations and references, if available, are G; *Galega*, M; *Medicago*, P; *Pisum* (Vad et al, 1993), G; *Glycine* (Gijzen et al, 2001), M; *Momordica*, L; *Limonium*, N; *Nepenthes* (Eilenberg et al, 2006), Z; *Zea* (Tiffin, 2004), T; *Triticum*, M; *Musa*, L; *Lycopersicum* (Danhash et al, 1993; Robertson et al, 1997), N; *Nicotiana* (Shinshi et al, 1990; Neuhaus et al, 1991; van Buuren et al, 1992; Suarez et al, 2001).

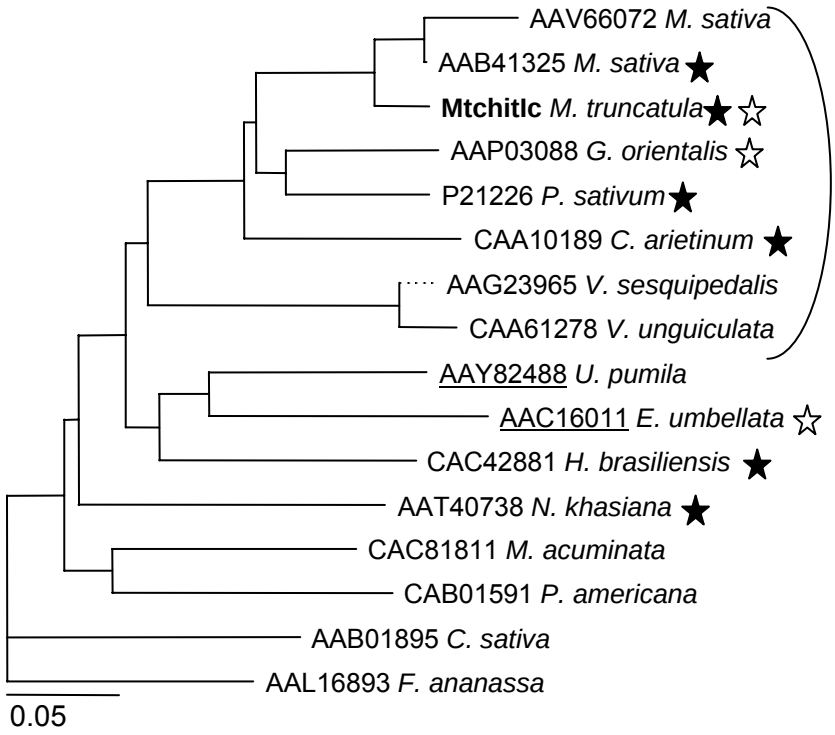

Figure 4 Unrooted phylogram of chitinases similar to Mtchit1c. The group of chitinases from leguminous species is indicated by a bracket. Filled stars indicate antifungal or pathogen inducible chitinases, white stars indicate rhizobium inducible chitinases. The bar corresponds to 0.05 amino acid exchanges per residue. Underlined sequences seem to have a vacuolar targeting signal that is similar to the tobacco signal (Neuhaus et al, 1991).
Abbreviations and references, if available: M; *Medicago*, G; *Galega*, P; *Pisum* (Chang et al, 1995; Vad et al, 1991) C; *Cicer*, V; *Vigna*, U; *Ulmus*, E; *Elaeagnus* (Kim & An 2002), H; *Hevea* (O'Riordain et al, 2002), N; *Nepenthes* (Eilenberg et al, 2006), M; *Musa*, P; *Persea* (Sowka et al, 1998), C; *Castanea*, F; *Fragaria.*

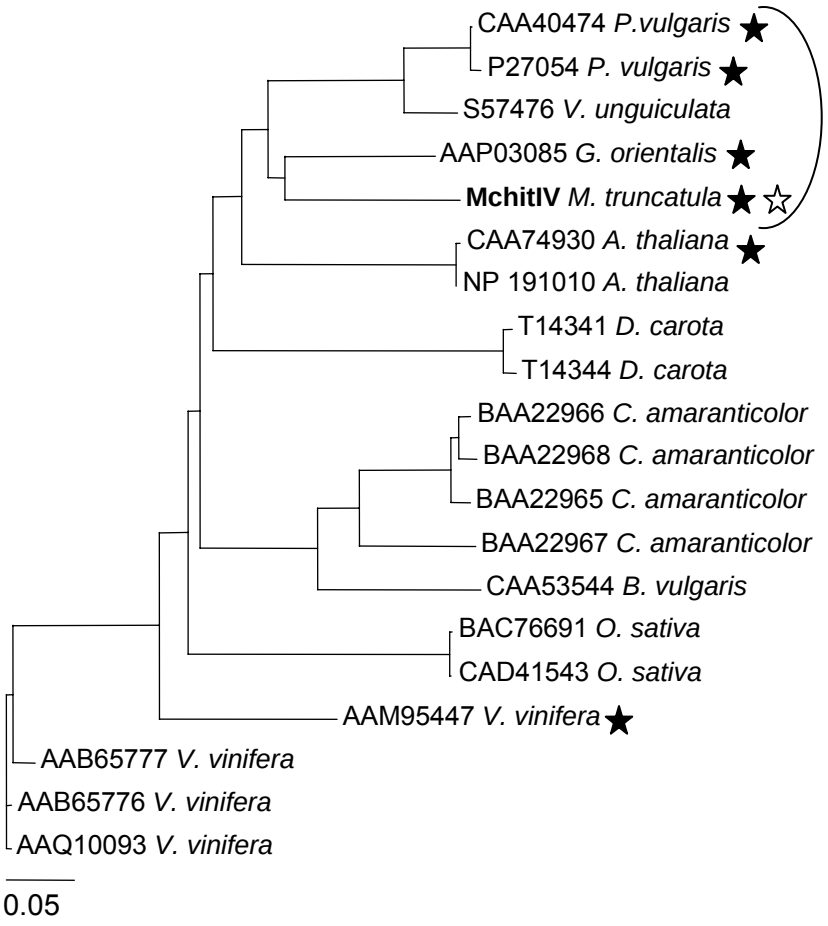

Figure 5 Unrooted phylogram of chitinases similar to Mtchit4. The group of chitinases from leguminous species is indicated by a bracket. Filled stars indicate antifungal or pathogen inducible chitinases, white stars indicate rhizobium inducible chitinases. None of the chitinases had a vacuolar targeting sequence. Tree modified after (Salzer et al, 2004, chapter 2). The bar corresponds to 0.05 amino acid exchanges per residue.
Abbreviations used are: P; *Phaseolus*, V; *Vigna*, G; *Galega*, M; *Medicago*, A; *Arabidopsis*, D; *Daucus*, C; *Chenopodium*, B; *Beta*, O; *Oryza*, V, *Vitis*.

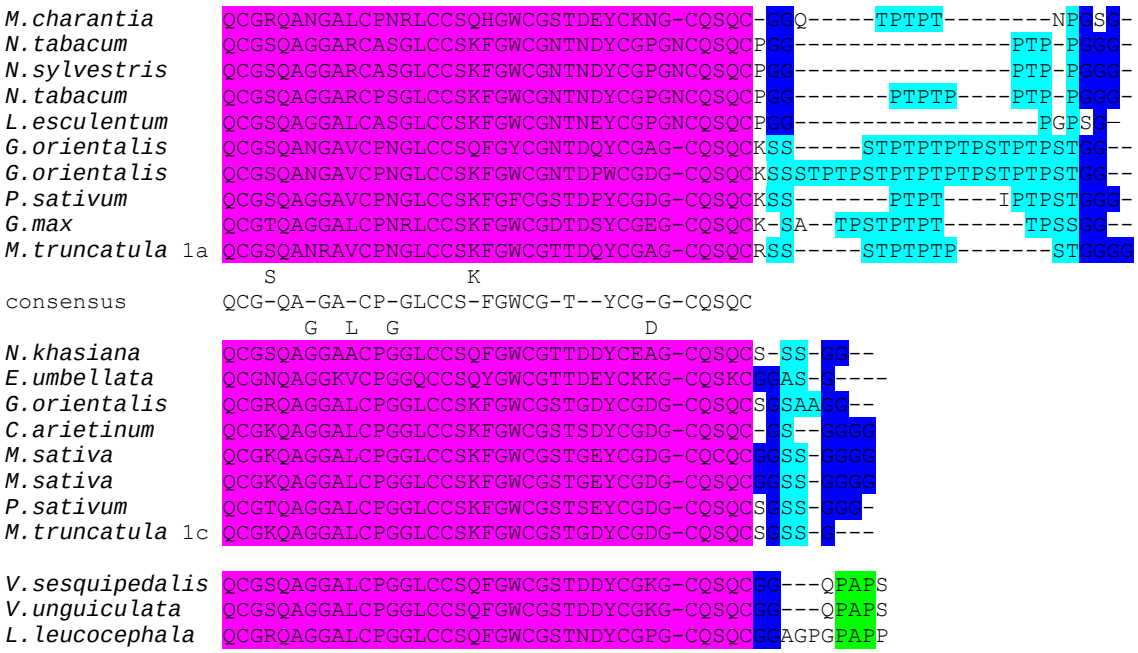

Figure 6 The different forms of CRD and hinge regions of chitinases class I. The two classes of CRD and hinge regions in the Mtchit1a and Mtchit1c chitinases in *M. truncatula* have the highest differences in the hinge regions, and slight differences in the CRD, which is indicated in the letters beside the consensus sequence. One group of hinge sequences is characterized by a TP-rich sequence, while the other one has a GS-rich sequence. A third group of proteins have a third sequence, that is characterized by a PAP containing sequence, L; Leucaena (Kaomek et al, 2003) and for other abbreviations and references, see figures 3-5.

A1.4.4 Expression of genes encoding chitinases of GH family 19 in different tissues

The transcript levels for chitinases GH family 19 were measured in roots, leaves and flowers of mature plants by quantitative qPCR. Expression of *Mtchit1a* was highest in leaves, insignificantly lower in roots and significantly lower in flowers (Figure 7 a). Transcript levels of *Mtchit1c* in leaves, roots and flowers were similar (Figure 7 b). Indeed, this gene seems to be constitutively expressed in these tissues. As in the case of *Mtchit1a*, there was a statistically significantly higher expression of *Mtchit4* in leaves, compared to roots or flowers (Figure 7 c).

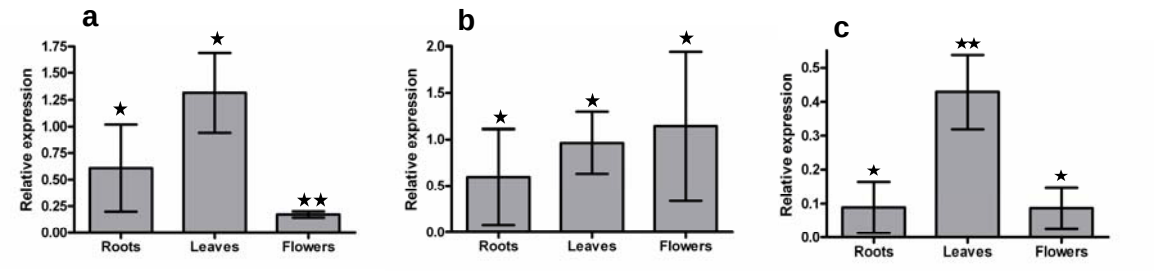

Figure 7 Chitinase expression patterns of *Mtchit1a, Mtchit1c* and *Mtchit4*. Gene expression levels were normalized to the expression of the *M. truncatula* ubiquitin *Mtubi*. The difference of bars with different numbers of stars is statistically significant according to the Mann-Whitney U test.

A1.4.5 Expression of genes encoding chitinases of GH family 19 in mycorrhizal and non-mycorrhizal roots

Transcript levels for chitinases GH family 19 were measured by quantitative qPCR in non-mycorrhizal roots and in roots infected by the mycorrhizal fungus *Glomus intraradices*.
Mtchit1a and *Mtchit1c* did not exhibit significantly different transcript levels in mycorrhizal and nonmycorrhizal roots (Figure 8 a, b). In contrast, *Mtchit4* transcripts showed a significant decrease in mycorrhizal roots when compared to non-inoculated control roots (Figure 8 c).

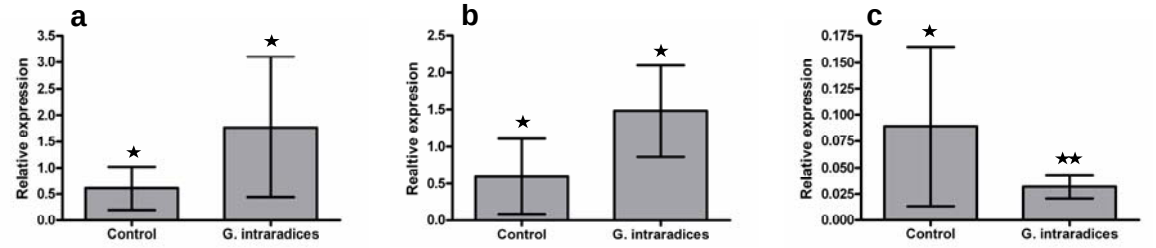

Figure 8 *Mtchit1a, Mtchit1c* and *Mtchit4* expression in mycorrhizal plants. Stars refer to statistical significance, measured by the Mann-Whitney U test.

A1.5. Discussion

Several factors are known that trigger the induction of defence-related proteins such as chitinases. Most importantly, they are induced in response to pathogen attack. In addition, they can also be induced by elicitors and by plant hormones such as ethylene and jasmonate (Boller et al, 1983; Stintzi et al, 1993), or by abiotic stresses such as drought, salinity, wounding, cold, UV light and heavy metal addition (e.g. Hamel & Bellemare, 1995; Arie, 2000; Schraudner, 1992). The glycosyl hydrolase (GH) family 19 chitinases are commonly considered as PR proteins that are inducible in response to several kinds of biotic and abiotic stresses.
Chitinases of class I have antifungal activity (Schlumbaum et al, 1986). However, the antifungal activity is considerably enhanced by the combined application of chitinase of class I and β-1,3-glucanase (Mauch et al, 1988). Interestingly, a tobacco chitinase of class I exhibited clear antifungal activity against *Fusarium*, whereas a tobacco chitinase of class II did not affect fungal growth (Sela-Buurlage et al, 1993), indicating that a difference in protein domain composition can influence the antifungal activity.
The *M. truncatula* chitinase class IV, Mtchit4, was strongly induced in response to pathogenic attack, and therefore appears to have a clear role in the plant defense machinery as a PR protein (Salzer et al, 2000; Salzer et al, 2004, chapter 2). Mtchit1a and Mtchit1c are induced in response to the pathogen *F. solani* f. sp. *phaseoli* (Salzer et al, 2000) and thus seem to belong to the classical PR proteins as well (Neuhaus et al, 1996). However, this study is not comprehensive and as the example of Mtchit1b shows, there are several other members of the GH family 19 chitinases in the genome of *M. truncatula*, which are not treated here. In the near future the complete genomic sequence of *M. truncatula* will be available, and it will certainly be interesting to identify and discuss a complete set of chitinases in this legume.

The differences in gene regulation suggest that the protein function is different among the three GH family 19 chitinases investigated here, despite a common predicted reaction mechanism (Collinge et al, 1993; Bishop et al, 2000). Because the protein primary structures of the three chitinases are rather closely related (Figure 1), and the putative active centers share common residues with related chitinases from other plants, a similar enzymatic mechanism is most probable. Interestingly, the CRD and hinge regions differ considerably among the two class I chitinases. The CRD in the GH family 19 class of enzymes are not part of their active chitin cleavage domain. A TP-rich variant of the hinge region has been shown not to change the antifungal activity, but the substrate specificity (Suarez et al, 2001). In vivo, the proline residues in this hinge region are often converted into hydroxyproline (Sticher et al, 1992) and the presence of fully or partially hydroxylated proline residues in the hinge region might influence the subcellular targeting. Most probably the hydroxyproline status also influences the substrate binding (Sticher et al, 1992). Hence, the altered CRD and hinge regions of *Mtchit1a* and *Mtchit1c* might influence their substrate binding specificities.
The C-terminal composition of amino acids was shown to influence the subcellular targeting of chitinases and other proteins. While class II and IV chitinases generally are secreted to the outside of plant cells, class I chitinase have been found secreted or located intracellularly, in the vacuoles (Figures 3 - 5). This localization is determined by a C-terminal extension called the vacuolar targeting signal (Neuhaus et al, 1991). By comparison, such a C-terminal extension can clearly be excluded in Mtchit1a, as in most of the chitinases of this group (Figure 3), and therefore we assume that it is secreted to the apoplast.
In Mtchit1c, a C-terminal extension was found, which is distinct from the sequence that was identified to direct tobacco chitinases to the vacuole (GLLVDTM; Neuhaus et al, 1991). However, analyses on C-terminal protein extensions showed that it is not one specific consensus sequence that is responsible for vacuolar targeting, but certain regularities were

found in vacuolar sorting sequences. These include in most cases the presence of small uncharged amino acids, hydrophobic amino acids and the presence of an isoleucine, leucine or valine in a certain order (Neuhaus & Rogers, 1998; Matsuoka, 2000). The composition of the Mtchit1c C-terminal extension, with its row of serines and leucines together with the short sequence NSIDT at the very C-terminus, meets these requirements and thus may represent a vacuolar targeting sequence.

A difference in gene regulation of *Mtchit1a*, *Mtchit1c* and *Mtchit4* was found in relation to pathogenic fungi, but also to mycorrhizal fungi (Salzer et al, 2000; Figure 8). It was hypothesized that the regulation is based on the difference in the promoter regions of the three genes. Indeed, several potential regulatory elements were found, that could be involved in the regulation in response to stress, pathogenesis, or hormones. The putative promoters display a low sequence similarity to each other and the combination of transcription factor binding sites is distinct for each of them (Figure 2). It seems that it is the combination of regulatory motifs that confers the differences in expression patterns. The promoter sequences were compared to an acidic glucanase promoter sequence of the same plant which proved useful as reference, as in general, beta-1,3-glucanases show similar regulation on stress responses as the PR genes.
The phylogenetic analysis demonstrates that the chitinases are rather closely related to each other and to the respective counterparts in other plants (Figures 3-5). Nevertheless, in all three trees the legume chitinases are grouped together, and seem to be influenced by similar factors within the groups. It is easy to imagine that chitinases act synergistically, depending on the specific requirements of the plant. An interesting example of the combined action of two chitinases from different but related groups is proposed for chitinases of the carnivorous *Nepenthes* (Figures 3, 4). These chitinases show different inducibility upon chitin injection into the closed trap pods, mimicking the presence of chitin exoskeletons of intruding prey. The combined forces of chitinases first lead to degradation of long polymers to release glucosamine oligomers from the prey, which then are digested by other chitinases (Eilenberg et al, 2006).

An important aspect is that legumes entertain two different kinds of symbioses, unlike most other plants (Harrison, 2005). The symbiotic life style might be the reason why legumes have a different chitinase repertoire, and may have a different regulation mechanism than other plants. However, each chitinase as part of the plant-microbe communication machinery has at least one expressed homologue in *A. thaliana* that does not form symbioses with rhizobia or arbuscular mycorrhizal fungi. However, these may not be closely related on the gene sequence level and not necessarily induced by the same stimuli as their relatives in *M. truncatula* (Passarinho & de Vries, 2002; Feddermann, unpublished). This might point to a difference in the legume specific use of chitinases. A more thorough investigation using genomic as well as expressed sequence tag databases combined with experimental data on chitinase genes in the different plants could certainly give more information on the chitinase regulation system that is used by legumes compared to non-legumes.

It seems that the induction of root chitinase of the GH family 19 is stimulated by the fungal presence but differentially influenced depending on the nature of the fungal cell wall composition (Salzer et al, 2000). The expression studies in relation to mycorrhizal symbiosis clearly showed that none of the three chitinases is induced by mycorrhiza formation. Reduced defense reactions may occur in the mycorrhizal symbiosis (e.g. Gianinazzi-Pearson et al, 1996) and indeed, we found a slight reduction of *Mtchit4* in mycorrhizal roots (Figure 8 and Salzer et al, 2004, chapter 2). However, in relation to infection with two additional AM fungal species the levels were not significantly different from the control (Feddermann et al, 2006, chapter 4), indicating that Mtchit4 is not relevant for the symbiosis.

In conclusion, the *M. truncatula* GH family 19 chitinases show distinct expression patterns in response to plant-microbe interactions. Similar patterns are also found in other plant species. Similarities in structure and regulation of chitinases indicate specific functions for the different chitinase classes, which may serve characteristic purposes in plant-microbe interactions. The orchestration of expression of the different chitinases seems crucial for plant defense, nodule development and mycorrhizal fungal infection. However, a more intense investigation, especially on the enzymatic activities of the GH family 19 chitinases in *M. truncatula*, could help to understand the specific roles of Mtchit1a, Mtchit1c and Mtchit4. For this, certain factors remain to be determined, such as their glycosylation status in vivo, the intracellular localization and protein substrate specificity (e.g. Schultze et al, 1998), which certainly will be subject to future studies.

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Chapter 3

Ectopic expression of the mycorrhiza-specific chitinase gene *Mtchit3-3* in *Medicago truncatula* root-organ cultures stimulates spore germination of glomalean fungi

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Glomus intraradices
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Abbreviations:

AM	arbuscular mycorrhiza
AMF	arbuscular mycorrhizal fungi
Mt	*Medicago truncatula*
chit	chitinase
PCR	polymerase chain reaction
bp	base pair
ROC	root organ culture
ubi	ubiquitin
GUS	β-glucuronidase

3.1. Abstract

Expression of *Mtchit3-3*, a class III chitinase gene, is specifically induced by arbuscular mycorrhizal (AM) fungi in roots of the model legume *Medicago truncatula* and its transcripts accumulate in cells containing arbuscules.

Agrobacterium rhizogenes-transformed roots and root-organ cultures of *M. truncatula* were used to study effects of *Mtchit3-3* on AM fungi.

This work provides evidence for enzymatic activity of the *Mtchit3-3* gene product and shows with promoter:*gus* fusions that a 2 kb fragment located 5' upstream from the translational start codon of *Mtchit3-3* is sufficient to confer arbuscule-dependent gene expression. By fusing the *Mtchit3-3* coding region to the CaMV *35S* promoter the expression pattern was disrupted. Surprisingly, disruption stimulated spore germination of *Glomus intraradices* and *Glomus constrictum*, and in the case of *G. intraradices* resulted in a higher probability of root colonization and spore formation. However, no effect on the abundance of arbuscules within colonized roots became apparent.

These observations demonstrate that disruption of the tight arbuscule-dependent expression pattern of *Mtchit3-3* has effects on the early interaction between roots and AM fungi.

3. 2. Introduction

The majority of all vascular plants live in symbiosis with microorganisms. The most abundant type of symbiosis, namely the arbuscular mycorrhiza (AM), is formed with fungi from the order Glomeromycota. Fossil records suggest that the AM symbiosis is evolutionary ancient, dating back to the plants colonization of land, about 430 million years ago (Redecker et al, 2000). Today, the glomalean fungi are obligate biotrophs and depend on their host plants for nutrition and completion of their life cycle. The plants benefit from AM fungi by an improved mineral acquisition. In the so-called arbuscules, which are highly branched fungal structures that penetrate cortical root cells, phosphate especially is translocated from the fungus to the plant (Rausch & Bucher, 2002). Because of its mutualistic character the AM symbiosis is not only advantageous for the individual plant and its fungal partner, but as a whole for productivity and biodiversity of natural ecosystems (van der Heijden et al, 1998).

The AM fungal colonization of the host roots involves a series of events that are tightly regulated and controlled by both partners. Before infection, AM fungi recognize and respond to their potential hosts. Compounds secreted by the roots of host plants stimulate branching of hyphae from germinating AM fungal spores (Giovannetti et al, 1993; Giovannetti et al, 1996; Pinior et al, 1999; Tamasloukht et al, 2003). Throughout a successful colonization of the root cortex and the subsequent formation of new fungal spores, signals must be exchanged between the symbiotic partners, inducing developmental stage-specific patterns of gene expression in the host roots and the AM fungi (Liu et al, 1998; Delp et al, 2003; Wulf et al, 2003). Characterization of the DMI1, DMI2 and DMI3 mutants in *Medicago truncatula* that do not make infections with AM fungi and rhizobia demonstrated that proper signal transduction in plants is essential for successful AM formation (Ané et al, 2004; Lévy et al, 2004). An example of a gene that is induced early during AM formation and nodulation of M. *truncatula* is *enod40*. Constitutive expression of this gene caused accelerated colonization and formation of more arbuscules by the AM fungus *Glomus intraradices* (Staehelin et al, 2001*)*. We identified the class III chitinase gene *Mtchit3-3* in an expression study of eight *M. truncatula* chitinase genes in interactions with symbiotic and pathogenic microorganisms as being expressed in a mycorrhiza specific manner (Salzer et al, 2000). Thus, *Mtchit3-3* is not induced during nodulation or pathogen attack but is induced in a strict mycorrhiza-dependent manner. In addition, *in situ* analysis showed that transcripts of *Mtchit3-3* specifically accumulated in plant cells harboring arbuscules (Bonanomi et al, 2001).

To obtain more information about this specifically mycorrhiza induced chitinase gene, we cloned the *Mtchit3-3* gene and its promoter. Exploiting *M. truncatula* root-organ cultures (ROCs) as an AM model system, we show here that ectopic expression of *Mtchit3-3* stimulates spore germination of different glomalean fungi but has no obvious effect on the abundance of arbuscules within colonized roots.

3.3. Materials and Methods

3.3.1. Biological materials

Medicago truncatula cv. Jemalong A17 plants, *in vitro* cultures of *Glomus intraradices* Schenck & Smith (DAOM 197198) on *Daucus carota* (carrot) roots, *Fusarium solani* f. sp. *phaseoli* strain W-8, and *Phytophthora megasperma* f. sp. *medicaginis* strain 652 were grown as previously described (Salzer et al, 2000). *Glomus constrictum* (ISCB 133; Oehl et al, 2003) was propagated in pot cultures with *Plantago lanceolatum* serving as host plant. Axenic *M. truncatula* ROCs were grown on M-medium (Becàrd & Fortin, 1988) with 1% sucrose if not stated otherwise. All *M. truncatula* ROCs were kept at 22°C in the dark at ambient CO_2 atmosphere and were subcultured after 6 wk. All manipulations on ROCs were performed under sterile conditions.

To examine spore germination, three approx. 5-cm long root pieces were transferred to fresh M-medium (Becàrd & Fortin, 1988). In the case of *G. intraradices* about 20 single spores were directly removed from the Gellan Gum of 4- to 6-month-old carrot/*G. intraradices in vitro* cultures and placed on M-medium in direct contact with the transferred root pieces. In the case of *Glomus constrictum*, spores were isolated from pot cultures by sieving. A 125-µm sieve fraction was collected in a centrifuge tube and covered with a 70% sucrose solution. After centrifugation for 2 min at 700*g*, spores floating on top of the sucrose solution were collected and extensively washed with tap water. Collected spores, free of soil particles, were subsequently surface sterilized in 2% (w : v) chloramine T, 200 µg ml^{-1} streptomycin, 100 µg ml^{-1} gentomycin, and finally washed with sterile water. To determine spore germination in absence of ROCs, spores were directly placed on M-medium. The positions of the spores were labeled with a circle of 0.5 cm in diameter on the bottom of the Petri dishes.

To establish AM in transformed *M. truncatula* ROCs, spores from 4- to 6-month-old carrot/*G. intraradices in vitro* cultures were used. To obtain a homogeneous inoculum within one experiment, Gellan Gum containing a counted number of spores was sampled and chopped with a scalpel. About 500 µl of the chopped Gellan Gum, containing about 150 spores were spread on the M-medium in the center of a 9 cm Petri dish. Root pieces from 4- to 5-wk-old *M. truncatula* ROCs were placed on top of the spores. The area with the inoculated spores was labeled on the bottom of the Petri dishes. *M. truncatula* ROCs were subcultured twice, namely 6 and 12 wk after inoculation with *G. intraradices*. For subculturing, root pieces were transferred together with extraradical hyphae and newly formed spores.

To establish ROC pathosystems, 1 cm diameter agar plugs were taken from 2-wk-old cultures of *F. solani* or *P. megasperma* and placed on top of newly subcultured *M. truncatula* ROCs. Empty agar plugs were used for mock inoculation.

To demonstrate *Mtchit3-3* promoter activity *A. rhizogenes* transformed *M. truncatula* composite plants were inoculated with about 400 spores of *G. intraradices* that have been isolated from 4- to 6-month-old *D. carota/G. intraradices in vitro* cultures and grown as described for nontransformed plants (Salzer et al, 2004, chapter 2). Plantlets were inoculated 2 wk after transfer from Petri dishes to Magenta jars. For mock inoculation, 3 ml of sterilized water was added.

3.3.2. Cloning and molecular analyses

cDNA of *Mtchit3-1*, *Mtchit3-3*, and *Mtchit3-4* was synthesized from RNA (5'/3'-RACE Kit; Boehringer, Mannheim, Germany). To obtain the full-length genomic clones, proof-reading polymerase chain reaction (PCR) was performed with BAC 72H13 serving as template for *Mtchit3-3*, BAC 80G10 (Salzer et al, 2000) serving as template for *Mtchit3-4*, and genomic DNA serving as template for *Mtchit3-1*. The putative promoter region of *Mtchit3-3* was cloned from adapter ligated DNA synthesized from BAC 72H13 using the Universal Genome Walker

Kit (Clontech, BD Biosciences, Erembodegem, Belgium). First PCR was performed with adapter-ligated DNA as template and an adapter-specific (Adapter 1, Genome Walker Kit) and *Mtchit3-3*-specific primer combination (Table 1). Nested PCR was performed with a 1:1000 dilution of the products of the first PCR, and the primer combination 'Adapter 2' (Genome Walker Kit) and '*Mtchit3-3* nested' (Table 1). For cloning purposes PCR reactions were performed with the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany) applying following cycling conditions: 1 cycle of 2 min at 92°C, 10 cycles of 10 s at 92°C, 30 s at 60°C and 90 s at 68°C, and 20 cycles of 10 s at 92°C, 30 s at 60°C, 90 s at 68°C increasing the incubation time for 20 s per cycle at 68°C. The PCR products were purified and cloned into pGEMT-easy (Promega, Madison, WI, USA). Inserted DNA was sequenced with the ABI Prism Big Dye Terminator Sequencing Reaction Kit and an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences are listed in Table 1. Sequence analyses were performed with the Accelrys SEQWEB Version 2.1 Web-based Sequence Analysis. Presence of signal peptides was analyzed with PSORT (old version for bacterial/plant sequences, http://psort.nibb.ac.jp/form.html). The unrooted phylogram was constructed with PAUP (Phylogenetic Analysis Using Parsimony for Mac version 4.0 beta 10, D. L. Swafford, 2002; Sinauer Associates, Sutherland, MA, USA, http://paup.csit.fsu.edu). Sequences were obtained by BLASTN searches in the *Medicago truncatula* TIGR database, and BLASTX searches in GenBank and Swissprot databases. Nucleotide sequences of *Mtchit3-3*, *Mtchit3-4*, and *Mtchit3-1* were deposited at GenBank. The accession number of *Mtchit3-3* is AY238969, of *Mtchit3-4* AY238970, of *Mtchit3-1* AY294484.

3.3.3. Vector construction for plant transformation

Construction of the Mtchit3-3 promoter:gus fusion
Two kilobases of the putative promoter of *Mtchit3-3* were isolated from an adapter ligated *PvuII* fragment, which was obtained by gene walking on BAC 72H13, and cloned into pGEMT-easy (Promega). A *SmaI/NotI* fragment was released from pGEMT-easy and was introduced into the pTA+ vector (EMBL, Heidelberg, Germany). A *ClaI-EcoRI* fragment containing the *Mtchit3-3* promoter region was then introduced into pGreen II 0000 (Hellens et al, 2000) resulting in pGreen.*Mtchit3-3.* To obtain an intron containing *gus* gene a *gus*(INT):*nos* fragment was released (*SalI-EcoRI*) from *35S*:*gus*(INT) (Vancanneyt et al, 1990; Lindroth et al, 1999) and subcloned in pTA+. From pTA+ *gus*(INT):*nos* was released with *BamHI* and *NotI* and introduced into pGreen.*Mtchit3-3* to give pGreen.p*Mtchit3-3:gus*(INT)*:nos*. Finally the *35S:km* cassette of the pGreen system was cloned into the *HpaI* site of pGreen.*Mtchit3-3:gus*(INT):*nos* resulting in pGreen.*Mtchit3-3:gus*(INT)*nos*.*35S:km*.

Construction of CaMV 35S:Mtchit3-3 sense and CaMV 35S:Mtchit3-3 antisense
The *Mtchit3-3* open reading frame was amplified with the Expand Long Template PCR System using '*Ch3-3*start-f' and '*Ch3-3*stop-b' primers (Table 1) and BAC72H13 as template. The amplification product was cloned into pGEMT-easy. The *Mtchit3-3* open reading frame was released from pGEMT-easy with *EcoRI* and introduced into the *EcoRI* cloning site of the CaMV *35S* promoter:terminator cassette (http://www.pgreen.ac.uk/a_cst_fr.htm). Orientation of the insert was determined by restriction analysis with *EcoRV* and *StuI*. Selected *35S* cassettes with the *Mtchit3-3* insert were cut with *EcoRV* and introduced into the *EcoRV* cloning site of pGreen 0229. Integration and orientation of the constructs were analyzed by sequencing. Nucleotide sequences of newly designed primers are shown in Table 1. To generate transformed controls we used *35S*:*gus*(INT) (*35S* vector control) and when applicable pGreen.*Mtchit3-3:gus*(INT)*nos*.*35S:km*.

3.3.4. Raising transgenic *M. truncatula* composite plants and ROCs

Binary vectors were introduced into *Agrobacterium rhizogenes* LBA 9402 by electroporation. Electroporated cells were grown on yeast extract mannitol broth at 28°C for 2 d. CaMV *35S:Mtchit3-3* sense-, antisense- and *Mtchit3-3:gus*(INT).*35S:km*-containing cells were selected on kanamycin (100 mg l^{-1}) plus tetracycline (10 mg l^{-1}). *35S:gus*(INT)-containing cells were selected on kanamycin alone. Presence of the appropriate constructs in the recovered transformants was verified by PCR. Single *A. rhizogenes* colonies were grown into a thick lawn of bacteria and used for plant transformation.
Medicago truncatula plantlets were used for *A. rhizogenes* root transformation (Boisson-Dernier et al, 2001) when the radicle reached a length of about 1 cm. After inoculation, the plantlets were co-cultivated with *A. rhizogenes* on M-medium supplemented with 0.5% sucrose for two days. Explants were then transferred to M-medium containing cefotaxime (200 mg l^{-1}). Kanamycin (50 mg l^{-1}) was included in the M-medium after transformation with *Mtchit3-3:gus*(INT).*35S:km*. After 4 wk culture on M-medium, seedlings were planted into Terra Green and were grown as described for nontransgenic *M. truncatula* plants (Salzer et al, 2004, chapter 2).
To establish *M. truncatula* ROCs, viable roots emerging from the inoculation site after approximately 3 wk were isolated and cultured on M-medium supplemented with cefotaxime. Root organ cultures visually free of microbial contamination were transferred to M-medium without antibiotics and absence of *A. rhizogenes* was verified by PCR with a *Vir D3*-specific primer combination (Lindroth et al, 1999). Presence of the proper constructs was confirmed by PCR (for primer sequences see Table 1). Cultures supporting amplification of the proper constructs, without amplifying the 800 bp *Vir D3* fragment were selected. Cycling conditions were: 1 cycle of 1 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 57°C, and 90 s at 72°C, with a final extension for 10 min at 72°C.

3.3.5. Analysis of mycorrhiza formation, gene expression and β-glucuronidase (GUS) activity

Germination of spores in *M. truncatula* ROCs was analyzed with a binocular. Spores were placed on M-medium and the inoculation sites were encircled with a marker (0.5 cm diameter) on the bottom of the Petri dishes. Spores that formed germinating hyphae crossing the labeled inoculation site were regarded as germinated. The germination frequency was calculated per Petri dish as the number of germinated spores related to the total number of inoculated spores.
The degree of total colonization of ROCs within one Petri dish and the amount of arbuscules were analyzed on 30 randomly sampled root pieces about 1 cm long after clearing with KOH and staining with Trypan blue (Salzer et al, 2000). Microscopic analysis of mycorrhizal structures was performed with a Zeiss Axioplan microscope (Zeiss, Jena, Germany). The whole length of the root pieces was scanned with 10 × 20-fold magnification. If a single mycorrhizal structure (i.e. an intraradical hyphae, an arbuscule or a vesicle) was present within the optical field, the corresponding root sector was regarded as mycorrhizal. If one arbuscule was present, the root sector was regarded as having arbuscules. If one highly branched arbuscule was present, the root sector was regarded as having highly branched arbuscules. Arbuscules were regarded as highly branched when at least half the volume of the root cell was filled with arbuscular structures. The degree of colonization was calculated by relating the number of optical fields with mycorrhizal structures to the total number of optical fields examined. Relative amounts of arbuscules were calculated by relating the number of optical fields with arbuscules to the number of optical fields with mycorrhizal structures. The relative amount of highly branched arbuscules was calculated by relating the

number of optical fields with highly branched arbuscules to the number of optical fields with arbuscules.
The number of newly formed spores was determined with the binocular microscope by counting the spores outside the labeled inoculation site.
Real-time PCR after reverse transcription (RT-PCR) was performed exactly as previously described using chitinase primer combinations that have been tested for their gene specificity, and ubiquitin primer combinations that specifically bind to DNA of plant origin (Salzer et al, 2000; Salzer et al, 2004, chapter 2). Calibration of the Gene Amp 5700 Sequence Detection System (Applied Biosystems) was performed with a dilution series of genomic DNA from *M. truncatula* and copy numbers were calculated on the basis of a haploid genome weight of 0.49 pg (Blondon et al, 1994) under the assumption that *Mtchit3-3* and *Mtchit4* are single-copy genes (Salzer et al, 2000). Each cDNA preparation was measured in triplicate. Standardized expression levels were calculated from the average copy numbers of the chitinase and the plant ubiquitin transcripts. All expression levels are therefore given as *Mtchit3-3*/*ubi* or *Mtchit4*/*ubi* ratios.
The GUS activity was visualized (Stomp, 1992) after a 16-24 h enzymatic reaction at 37°C and subsequent incubation in ethanol. Segments of *G. intraradices* inoculated roots showing GUS activity were cleared in KOH and stained with Trypan blue to examine mycorrhizal structures.
Extraction of proteins and analysis of chitinase activity and isoforms in gels after nondenaturing polyacrylamide gel electrophoresis (PAGE; Davis system) was performed according to Trudel & Asselin (1989). The concentration of proteins was determined according to Bradford (1976) using bovine serum albumin as standard.

Primer name	Sequence (5'−3')
Cloning primers	
Mtchit3-1 3' RACE 1	CCCTGGTGCTTGTAACTTTGTTTC
Mtchit3-1 3' RACE 2	CATGGAGCCAGTGGATCAATTC
MtChit3-1 specific-RT	GGTGAAGGCTTAACAATAGGCAGC
Mtchit3-1 5' RACE 1	CCTGTGCTTCCACAAAACCACC
Mtchit3-1 5' RACE 2	GATGAGCTAGCAGGAAGTCC
Mtchit3-3 3' RACE 1	CCTTGTCAATACAATCCTGGTG
Mtchit3-3 3' RACE 2	CCTTGAAGATGCATGGAAGCAGTGG
Mtchit3-3 5' RACE 1	GCACAGTGGAAGTAAGATCAGTAGC
Mtchit3-3 5' RACE 2	CCACTGCCTGCAGCCTCTGG
MtChit3-3 specific-RT	GCAGAACCTTTAATAGCTGG
Mtchit3-4 3' RACE 1	CCCTGATGCATTTATGAAC
Mtchit3-4 3' RACE 2	CCAACGGCTGCAGGAAGCGG
MtChit3-4 specific-RT	CCGTCTTTGGAAGAACCTTTAATAAC
Mtchit3-4 5' RACE 1	CCGCTTCCTGCAGCCGTTGG
Mtchit3-4 5' RACE 2	GTTCATAAATGCATCAGGG
Ch 3-3 start-f	CATGAAAATGGCGTTGAAATCAACTATCTCATTC
Ch 3-3 stop-b	TCACACATGGCTCTTGATGGAGGAGC
pMtchit3-3 specific	AAGGAAGTATTCTCGCAACGAACCGTCAA
pMtchit3–3 nested	TCTCGCAACGAACCGTCAATGAAACTAAC
Control and sequencing primers	
35S forward	CAGCCCAAGCTTTCTAGAGG
35S Term reverse	GAATTCGGTACGGCTGAAATCACC
Mtchit3-3 forward	CTTGGTCCTGCTGTTCTTGACG
Mtchit3-3 reverse	CGTCAAGAACAGCAGGACCAA
35S Term *Eco*RI reverse	CCCTTATCGGGAAACTACTCAC
*35S Eco*RI forward	CAAGTGGATTGATGTGACATCTCC
Left border *Eco*RV reverse	CAAGCTCGGAATTAACCCTCAC
Right border *Eco*RV forward	GGCCAGTGAATTGTAATACGACTC
gus reverse	AGTTTAGGCGTTGCTTCCGCCAGT
RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.	

Table 1 Primer sequences used for polymerase chain reaction

3.4. Results

3.4.1. Gene structure and phylogeny

Performing 3' and 5' rapid amplification of cDNA ends (RACE) on RNA from mycorrhizal *M. truncatula* roots, *Mtchit3-3* was cloned and sequenced. Sequence analysis revealed an open reading frame of 930 bp. The predicted gene product had a calculated molecular mass of 31 745 Da, an isoelectric point at pH 5.4, and a secretion signal peptide with a putative cleavage site between residues 22 and 23 of the preprotein. The predicted *Mtchit3-3* protein possesses the catalytically essential amino acids Asp (D) and Glu (E) at conserved positions in the catalytic domain (Figure 1 a) and contains the consensus sequence of the substrate binding cleft (Watanabe et al, 1993; Kim et al, 2002). Catalytic activity of the *Mtchit3-3* gene product against colloidal chitin was studied by *35S*-driven expression in roots of *M. truncatula* composite plants having the *Mtchit3-3* sense construct incorporated. Roots from plants transformed with *35S:Mtchit3-3* antisense or *35S* vector control constructs were used as controls. Real-time RT-PCR revealed elevated *Mtchit3-3* transcript levels (*Mtchit3-3/ubi* ratios) of roots having incorporated the *Mtchit3-3* sense construct (Figure 2 a). All *Mtchit3-3* copy numbers are related to ubiquitin (*ubi*) and are expressed as *Mtchit3-3/ubi* ratios. The expression levels of *Mtchit4*, which is a class IV chitinase gene of *M. truncatula* that is induced by pathogenic fungi and rhizobia (Salzer et al, 2004, chapter 2), were measured as an additional reference. *Mtchit4*/*ubi* ratios attained similar low values in all roots. Roots with elevated *Mtchit3-3*/*ubi* ratios exhibited increased chitinase activity. While control roots (*35S*:*Mtchit3-3* antisense and *35S* control vector) exhibited a distinct pattern of four major chitinase isoforms after nondenaturing PAGE of protein extracts and subsequent in-gel activity assays, in *Mtchit3-3* overexpressing roots intense chitinolytic activity with poor resolution into distinct bands was observed. Dilution series revealed that this intense chitinase activity resulted from a chitinase isoform that was different from the four major chitinase isoforms of the control roots (Figure 2 b). Without dilution, activity of this new chitinase isoform almost completely superimposes on the activity of the four major isoforms that are still active.

Phylogenetic analysis showed that *Mtchit3-3* was most related to *Mtchit3-4* (Figure 1 b), a chitinase gene that is induced in *M. truncatula* roots during symbiotic interactions with rhizobia and AM fungi (Salzer et al, 2000). Other putative class III chitinases of *M. truncatula* including, *Mtchit3-1*, a class III chitinase gene of *M. truncatula*, which is induced in response to pathogen attack, are phylogenetically distant from *Mtchit3-3* and *Mtchit3-4*. *Mtchit3-3* is also distant from class III chitinases from *Brassicaceae* and *Chenopodiaceae*, which cannot undergo symbiotic associations with AM fungi and rhizobia.

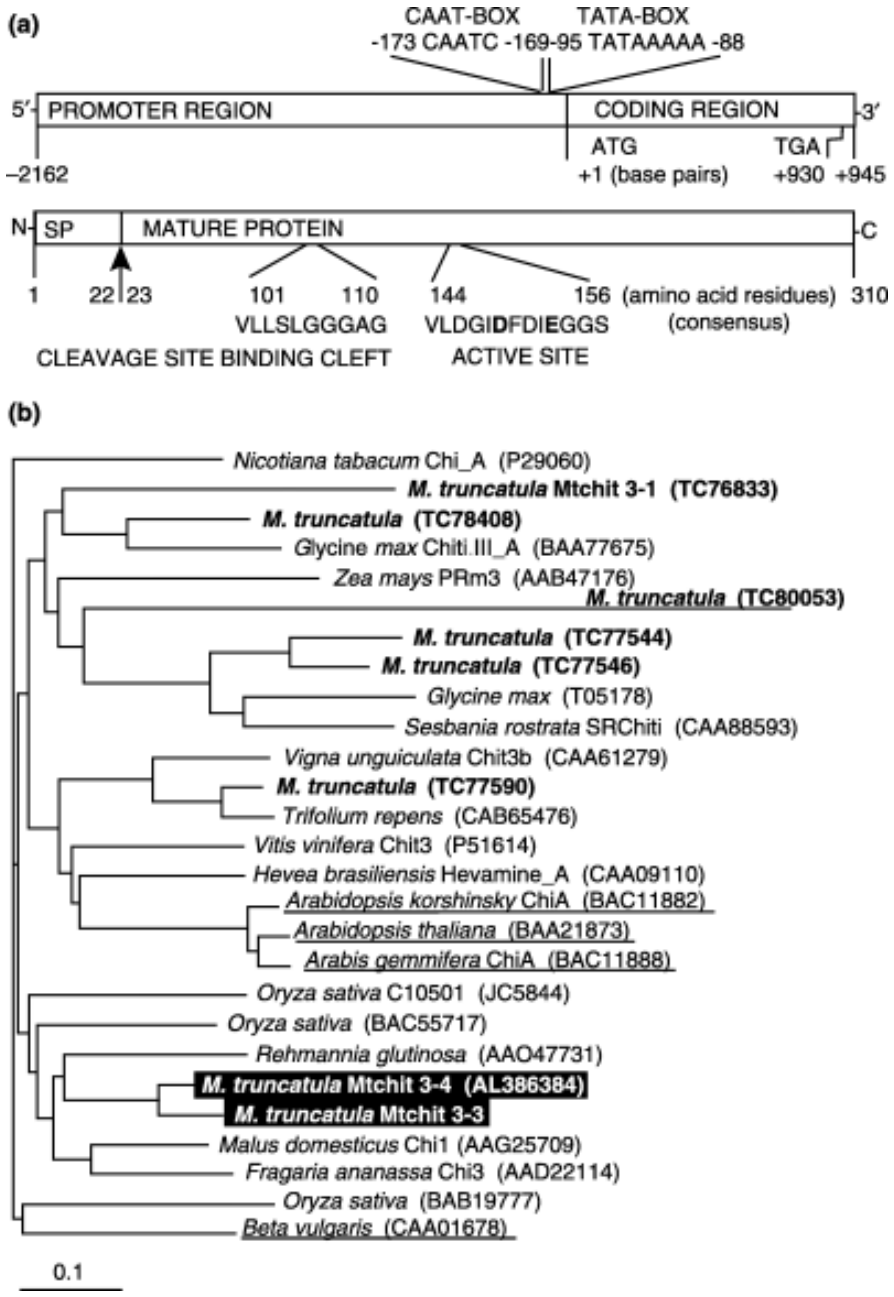

Figure 1 The *Mtchit3-3* gene, its gene product, and phylogenetic relationship. (a) Map of *Mtchit3-3* gene (top) and its putative gene product (bottom). SP, signal peptide; the signal peptide cleavage site between residue 22 and 23 is indicated by arrow. For catalytic activity essential amino acids D and E are printed bold within the consensus sequence of the active site of chitinases. (b) Unrooted phylogram of plant class III chitinases based on deduced full-length amino acid sequences. Chitinases from *Medicago truncatula* are printed bold, mycorrhiza-induced chitinases white, chitinases from plants that cannot form arbuscular mycorrhiza (AM) are underlined. Accession or TC numbers are given in brackets.

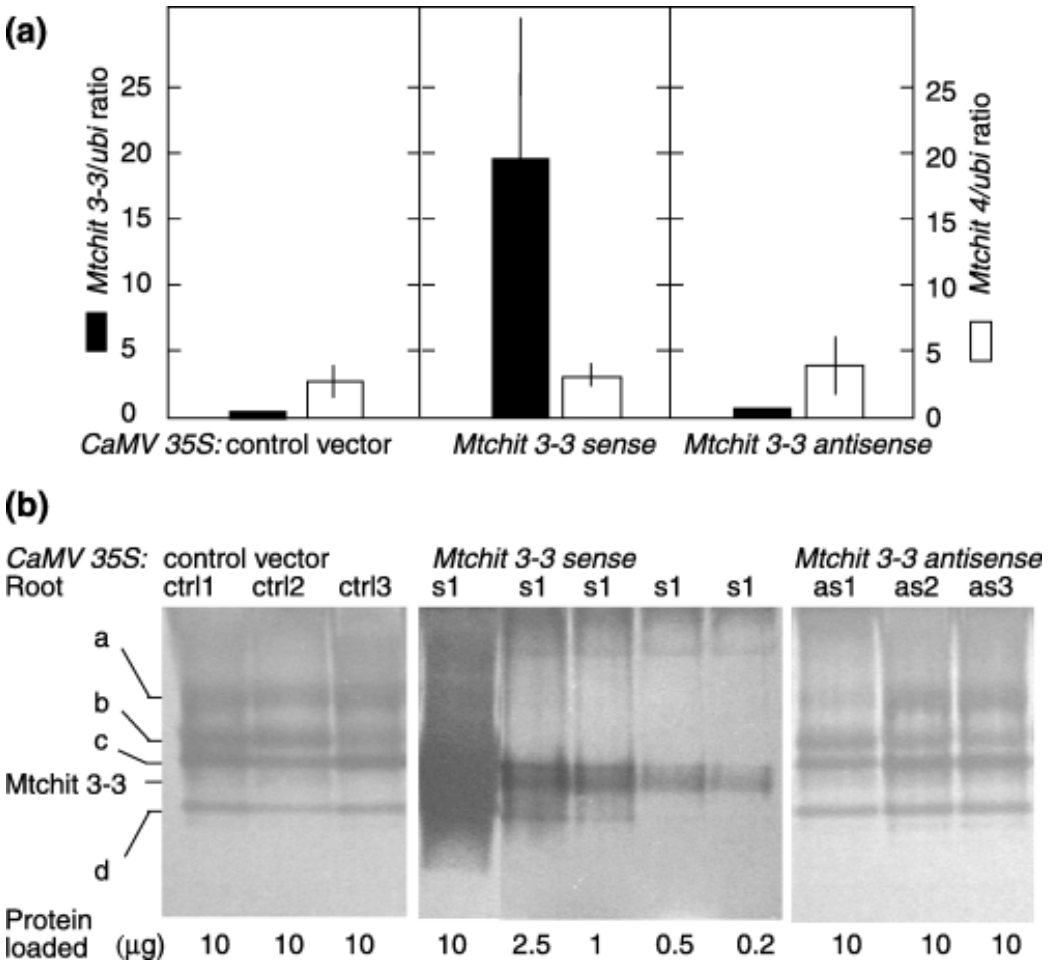

Figure 2 *Mtchit3-3* gene expression and chitinase activity after native polyacrylamide gel electrophoresis (PAGE). (a) *Mtchit3-3* and *Mtchit4* transcript levels in roots of *Medicago truncatula* composite plants were determined by real time reverse transcriptase polymerase chain reaction. The diagram shows *Mtchit3-3*/*ubi* ratios (closed columns) and *Mtchit4*/*ubi* ratios (open columns) obtained in plant roots having incorporated the *35S* control vector, *35S*:*Mtchit3-3* sense or *35S*:*Mtchit3-3* antisense constructs. One column represents the means (± SE) of three different roots. (b) Chitinase activity in *M. truncatula* composite plants visualized in gels after nondenaturing PAGE. Proteins were extracted from roots having incorporated the *35S* control vector, the *35S:Mtchit3-3* sense or the *35S:Mtchit3-3* antisense construct. Extracts (10 µg protein per lane) from roots of three different plants (ctrl1, ctrl2 and ctrl3) having incorporated the control vector and extracts from roots of three different plants (as1, as2 and as3) having incorporated the antisense construct were loaded on the gel. For the selected *Mtchit3-3* sense-transformed plant (s1) a dilution series of the protein extract is shown. 'a', 'b', 'c' and 'd' indicate the four major chitinase isoforms present in control roots, 'Mtchit3-3' indicates the position of the chitinase isoform that becomes apparent in the dilution series prepared from the *Mtchit3-3* overexpressing roots.

3.4.2. Promoter activity of *Mtchit3-3* in mycorrhizal roots of *M. truncatula* composite plants

To demonstrate and localize promoter activity, additional 2 kb of the 5'-region upstream the translational start codon of *Mtchit3-3* were cloned and sequenced. The first putative TATA box and CAAT box sequences were found at positions -88 and -169, respectively (Figure 1 a). Approximately 2 kb of the 5' upstream region of *Mtchit3-3* were fused to the *gus* reporter gene, and *Agrobacterium rhizogenes* transformed composite plants were initiated. The GUS activity became apparent in roots in mycorrhizal association with *G. intraradices* and was not observed in roots without AM fungi (Figure 3 a, b). Obvious GUS activity was limited to arbuscule-containing cells in the inner root cortex. *Mtchit3-3* promoter-driven GUS activity and presence of fungal structures within the same plant cell were confirmed by subsequent staining of fungal structures with Trypan blue (Figure 3 c, d). This pattern of *Mtchit3-3* promoter activity corroborates our previous *in situ* hybridization data on specific accumulation of *Mtchit3-3* transcripts in arbuscule containing cells (Bonanomi et al, 2001).

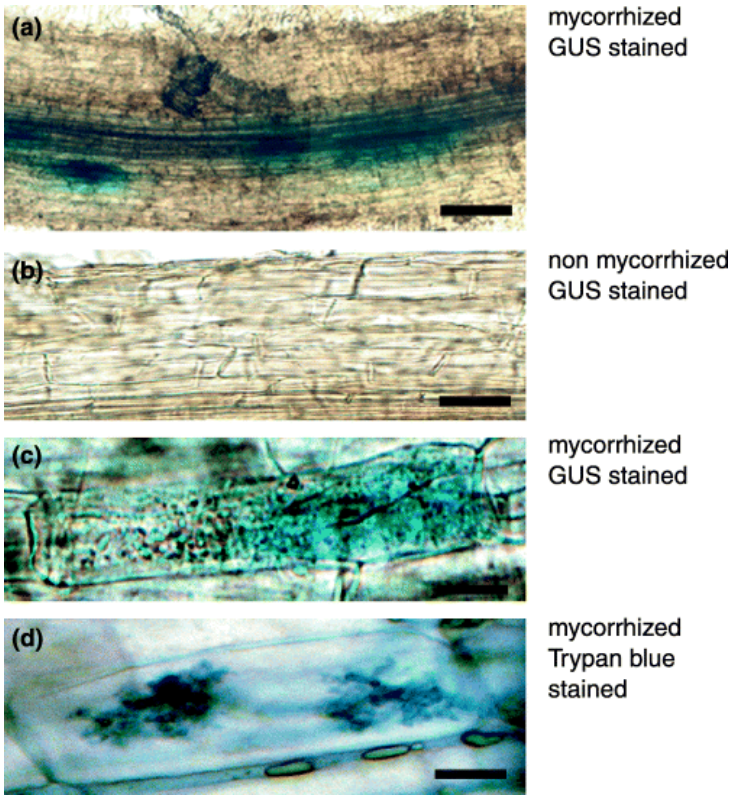

Figure 3 Arbuscule-dependent activation of the *Mtchit3-3* promoter. Localization of the *Mtchit3-3* promoter activity was performed in *Mtchit3-3*:*gus* transformed composite *Medicago truncatula* plants 6 wk post-inoculation with *Glomus intraradices* or mock inoculation with water. (a) *Mtchit3-3* promoter activity visualized by GUS staining (blue) in the inner cortex of a root colonized by *G. intraradices*. (b) No GUS staining can be detected in nonmycorrhizal roots of p*Mtchit3-3*:*gus*-transformed plants. (c) GUS activity (blue) in a cortical root cell containing an arbuscule. (d) Subsequent staining with Trypan blue of the same cell as shown in (c) reveals arbuscular structures inside the cell. Bars, (a, b) 400 µm, (c, d) 80 µm.

3.4.3. Initiation of transgenic *M. truncatula* ROCs constitutively expressing *Mtchit3-3*

To study effects of constitutive *Mtchit3-3* expression upon glomalean fungal colonization, young seedling roots were transformed with *A. rhizogenes 35S*:*Mtchit3-3* sense or *35S*:*Mtchit3-3* antisense constructs and vector constructs serving as transformed controls. The ROC lines were established from individual transformed roots, isolated from different composite plantlets. About 20 ROCs with integrated constructs, but free of *Agrobacterium* contamination after cefotaxime treatment, were selected and low copy number insertion in the selected lines was confirmed by DNA blot analysis (not shown). We selected the lines A3, B2, and C5, transformed with *35S*:*Mtchit3-3* sense, N2, N3 and N5, transformed with *35S*:*Mtchit3-3* antisense, and O2, O5, Q1, P3, P7 and P8 as transformed controls. Real-time RT-PCR revealed average basic *Mtchit3-3* expression levels (*Mtchit3-3*/*ubi* ratios) of 0.02 in ROCs having incorporated the control vectors. The cultures transformed with the antisense construct showed slightly reduced *Mtchit3-3*/*ubi* ratios (Figure 4). The ROCs having incorporated the *Mtchit3-3* sense construct (lines A3, B2 and C5) showed *Mtchit3-3*/*ubi* ratios four to six times above those of the control cultures (Figure 4). The basic *Mtchit3-3*/*ubi* ratios found in control ROCs were in the same range as those found in roots of whole nonmycorrhizal, nontransformed *M. truncatula* plants.

The *35S* promoter was active in younger and older parts of the ROC. Within roots, *35S* promoter activity was detected in the entire root and in all tissues including cortex and epidermis. The strongest activity was found in the cambium (not shown).

The morphology of the constitutively *Mtchit3-3*-expressing ROCs was indistinguishable from that of the *35S*:*Mtchit3-3* antisense ROCs and the transformed controls in terms of growth rate and branching pattern (not shown).

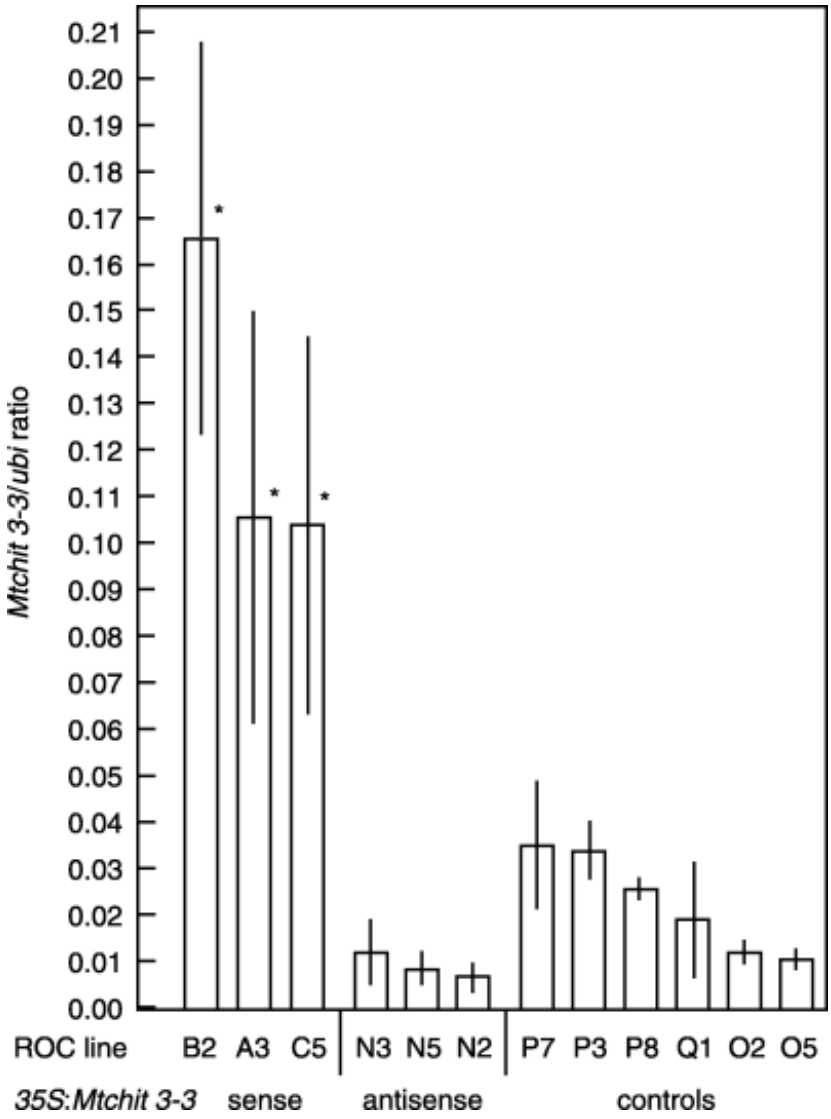

Figure 4 *Mtchit3-3* expression in transformed *Medicago truncatula* root organ cultures (ROCs). *Mtchit3-3*/*ubi* ratios were measured by real-time reverse transcriptase polymerase chain reaction in axenic ROCs 4 wk after transfer to fresh M-medium. The diagram shows the *Mtchit3-3*/*ubi* ratios of the ROCs B2, A3, C5 (*35S:Mtchit3-3* sense), N3, N5, N2 (*35S:Mtchit3-3* antisense), K7, D3, K8, Q1, O2 and O5 (transformed controls). The values are the means (± SE) from two independent RNA extractions and reverse transcriptase reactions with polymerase chain reactions performed in triplicate. Stars indicate expression levels that are significantly different from the controls (P = 0.001, Mann-Whitney U-test).

3.4.4. Stimulation of *G. intraradices* and *G. constrictum* spore germination by *35S*-driven *Mtchit3-3* expression in ROCs

To study whether constitutive *35S*-driven expression of *Mtchit3-3* in ROCs affects AM fungi during the preinfection stage, spore germination of two glomalean species was studied. In the case of *G. intraradices* the *M. truncatula* ROC lines A3, B2, C5 that are constitutively expressing *Mtchit3-3* and the transformed controls Q1, P7, P8 and P3 were used. The number of germinated spores was analyzed 7 d and 14 d post-inoculation. For comparison, *G. intraradices* spore germination was also studied in presence of carrot Ri T-DNA-transformed ROCs that are the standard host system for culturing *G. intraradices in vitro* (Becàrd & Fortin, 1988). Basic spore germination frequency of *G. intraradices* spores on M-medium in absence of ROCs was rather low 1 wk post inoculation but reached a value of about 10% after 2 wk (Figure 5). The germination frequency of the spores in presence of ROCs having incorporated a control vector reached values of about 30% 7 d post inoculation (Figure 5 a). In *M. truncatula* ROCs constitutively expressing *Mtchit3-3* about 70% of the *G. intraradices* spores formed germination hyphae within 7 d. At 2 wk post inoculation, on average, 80% of the spores were germinated in presence of constitutively *Mtchit3-3* expressing ROCs, whereas spore germination in presence of *M. truncatula* ROCs having incorporated a control vector or in presence of *D. carota* ROCs was about 50% (Figure 5 b). The *Mtchit3-3* expression levels were verified 2 wk post inoculation and were found to be in the range depicted in Figure 4. Statistical analyses showed that *35S*-driven expression of *Mtchit3-3* is associated with stimulation of spore germination; the germination frequency was significantly higher in ROCs with *35S*-driven *Mtchit3-3* expression compared with the controls (P = 0.0001, Mann-Whitney U-test).

In the case of *Glomus constrictum* time-course experiments were performed using *M. truncatula* ROC lines B2 (*35S*:*Mtchit3-3* sense transformed), Q1 (transformed control) and N5 (*35S*:*Mtchit3-3* antisense transformed). For comparison, *G. constrictum* spore germination was also studied in absence of ROCs. All three ROC lines stimulated spore germination compared with the germination on M-medium without roots (Figure 6). Similar stimulation was found in transformed controls and in ROCs having incorporated the *35S*:*Mtchit3-3* antisense construct, reaching average spore germination frequencies of 10% 6 wk post inoculation. Significantly higher germination frequencies (P < 0.001 Mann-Whitney U-test) were observed in the presence of ROCs with *35S*-driven expression of *Mtchit3-3* (Figure 6). On average, 70% of the spores had germinated within 6 wk.

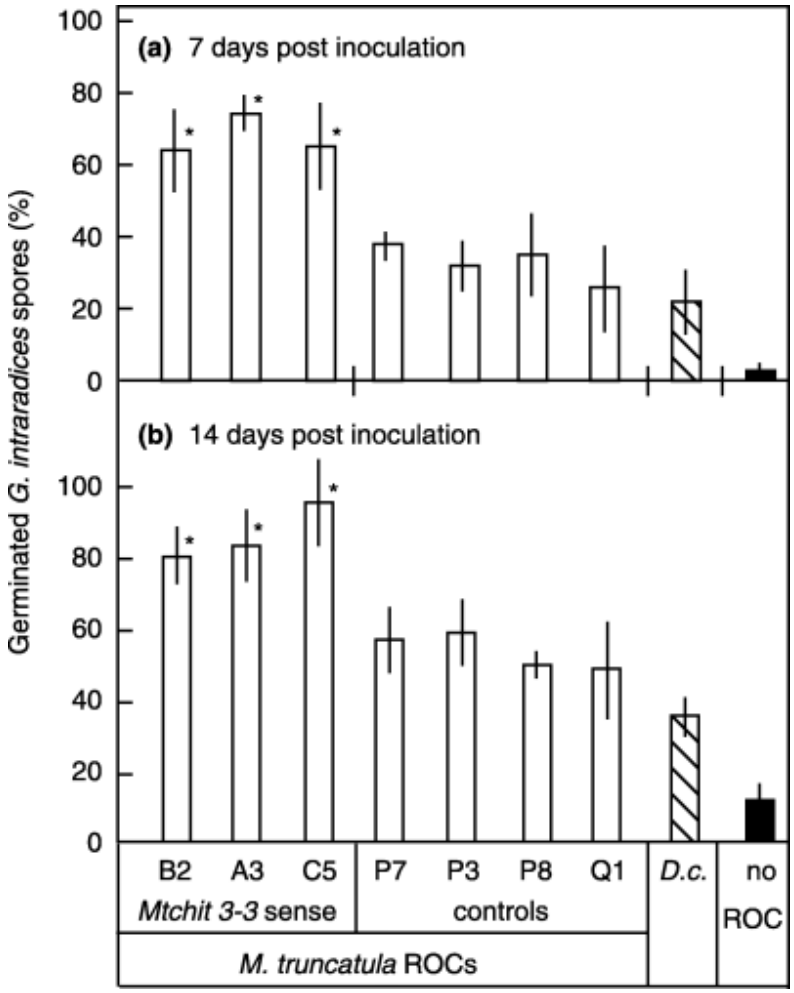

Figure 5 Effect of constitutive *Mtchit3-3* expression on *Glomus intraradices* spore germination. Twenty single spores were put on M-medium in presence of *Medicago truncatula* ROCs constitutively expressing *Mtchit3-3* (B2, A3 and C5) or transformed control root organ cultures (ROCs) (P7, P3, P8 and Q1). In addition, 20 spores were put on M-medium in presence of *Daucus carota* ROCs (*D.c*) or on M-medium in absence of ROCs (no ROC). The columns are means (± SD) of the germination frequencies determined in six individual Petri dishes per ROC line and the M-medium (a) 7 d and (b) 14 d post inoculation. Stars indicate germination frequencies that are significantly above those in presence of control ROCs ($P < 0.0001$, Mann-Whitney *U*-test). The experiment was repeated twice with similar results

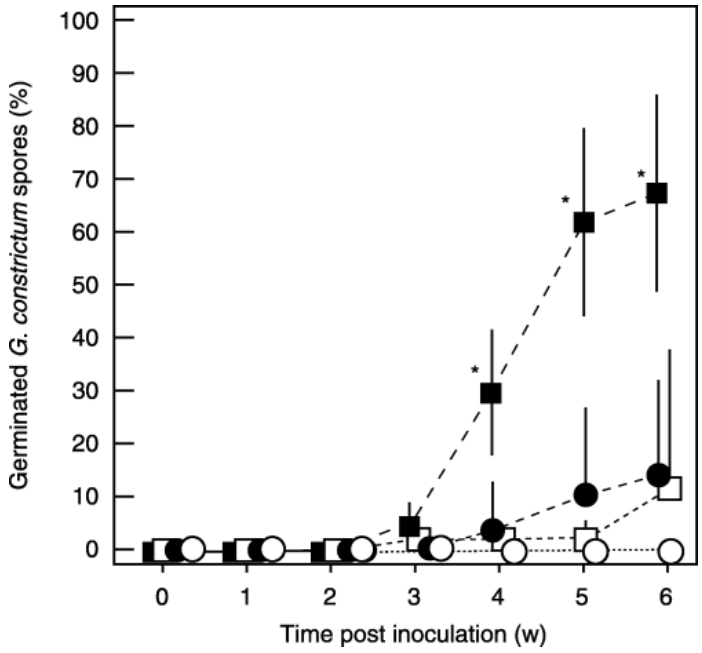

Figure 6 Effect of constitutive *Mtchit3-3* expression in root organ cultures (ROCs) on *Glomus constrictum* spore germination. Fifty single spores of *G. constrictum* were put on M-medium in presence of the *Medicago truncatula* ROCs B2 (*35S*:*Mtchit3-3* sense, closed squares), Q1 (transformed control, closed circles) or N5 (*35S*:*Mtchit3-3* antisense, open squares). For comparison, spores were put on M-medium without ROCs (open circles). Germination frequency was determined weekly in 12 Petri dishes established per ROC line. Data points represent the average germination frequency (± SD) determined on 12 × 50 spores per ROC line. Stars indicate data points with germination frequencies significantly above those observed in the control ROCs ($P < 0.001$, Mann-Whitney *U*-test). The experiment was repeated twice with similar results.

3.4.5. Colonization of ROCs, sporulation and reinfection

We performed long-term experiments to verify that *Glomus intraradices* could complete its life cycle and produce infective spores in *M. truncatula* ROCs with disrupted *Mtchit3-3* expression. For this experiments the ROC lines A3, C5, B2 (*35S:Mtchit3-3* sense), N2, N3 and N5 (*35S*:*Mtchit3-3* antisense), and O2 and Q1 (transformed control) were selected and inoculated with *G. intraradices*. Colonization and spore formation were measured after the second subculturing of the inoculated ROCs. With all different constructs, *M. truncatula* ROCs were able to form AM with *G. intraradices in vitro*. However, the probability of establishing a propagating AM association differed in the different ROCs. Mycorrhizal structures were found in 17 out of 26 Petri dishes established with ROCs having incorporated the *35S*:*Mtchit3-3* sense construct after two rounds of subculturing, but these structures were found in only 10 out of 21 Petri dishes with ROC lines having incorporated the *35S*:*Mtchit3-3* antisense construct and in only two out of 12 Petri dishes with transformed control ROCs (Table 2). An average colonization of 22% was found in ROCs having incorporated the *35S*:*Mtchit3-3* sense, and an average of 7% in ROCs having incorporated the *35S*:*Mtchit3-3* antisense construct, and 0.3% in the transformed controls. The highest

degree of colonization, up to 80%, was found in ROCs constitutively expressing *Mtchit3-3*. The Mann-Whitney signed-rank test indicated that there is a higher probability that ROCs constitutively expressing *Mtchit3-3* will be colonized than the transformed controls or the *35S*:*Mtchit3-3* antisense transformed cultures (T_1 =-3.11 (P = 0.002) and T_1 = -2.09 (P = 0.022), respectively).

We found no difference in the relative amount of arbuscules formed within mycorrhizal areas of the ROCs having integrated the *Mtchit3-3* sense or antisense construct. In ROCs having incorporated the *Mtchit3-3* sense construct on average in 47% (± 12.5% SD) of the optical fields analysed arbuscules were found; in ROCs with the antisense construct on average in 43% (± 14.2% SD) of the optical fields. There was no difference in the ratio of highly branched to clumped arbuscules. In ROCs with the *Mtchit3-3* sense construct, on average, 48% (± 20% SD) of the optical fields showing arbuscules also contained highly branched arbuscules; in ROCs with the *Mtchit3-3* antisense construct 49% (± 22% SD). In transformed control ROCs, the degree of colonization was too low to determine reliably the relative amount of arbuscules within the colonized area of the root. Despite the high number of optical fields containing arbuscules, the total number of arbuscules in ROCs is much lower than in mycorrhizal roots of a whole plant. In the ROCs analyzed in our experiments, intraradical hyphae are the predominant intraradical structures (Figure 7).

Glomus intraradices sporulated in both of the two subsequent subcultures of *M. truncatula* ROCs. Five weeks after the second subculturing, new spores were formed in 17 out of 26 Petri dishes with ROC lines having incorporated the *35S*:*Mtchit3-3* sense construct. In only 10 out of 21 Petri dishes with ROC lines having incorporated the *35S*:*Mtchit3-3* antisense construct and in two out of 12 Petri dishes with control ROCs spores were found. The Mann-Whitney signed rank test indicates that significantly more often spores were formed in *35S*:*Mtchit3-3* sense ROCs than in the *35S*:*Mtchit3-3* antisense ones (T_1 = -3.11; P = 0.002; Table 2).

Newly formed spores isolated from Gellan Gum of *M. truncatula 35S:Mtchit3-3* sense transformed ROCs were able to germinate, infect and establish new AM in ROCs of *D. carota* as well as *M. truncatula*, indicating that *G. intraradices* can complete its life cycle in ectopically *Mtchit3-3*-expressing ROCs and form infective spores. *Glomus constrictum* was unable to complete its life cycle in ROCs of *M. truncatula* or *D. carota*. Only in some rare cases formation of appressoria and intraradical hyphae was observed in *M. truncatula* ROCs but never formation of arbuscules, vesicles or newly formed spores.

The *Mtchit3-3* expression levels were verified at the set-up of the experiments, and values were within the range depicted in Fig. 4. Analysis of *Mtchit3-3* expression levels in ROCs colonized with *G. intraradices Mtchit3-3* revealed no mycorrhiza-dependent induction of *Mtchit3-3* gene expression. For example, ROCs with 29%, 31%, 47%, 55%, or 66% colonized root length exhibited *Mtchit3-3* expression levels in the same range as ROCs without fungal structures.

3.4.6. Induction of *Mtchit4* expression in ROCs by pathogens is independent from ectopic expression of *Mtchit3-3*

Phytophthora megasperma f. sp. *medicaginis* and *F. solani* f. sp. *phaseoli* are pathogens that infect roots of whole *M. truncatula* plants and cause severe disease symptoms (Salzer et al, 2000). Among the pathogen-induced chitinase genes of *M. truncatula*, *Mtchit4*, a class IV chitinase gene, is induced strongest in plant roots being challenged with either of the pathogens (Salzer et al, 2000). To study whether constitutive *Mtchit3-3* expression in ROCs has an influence on elicitation of *Mtchit4*, *M. truncatula* ROC pathosystems were established with *P. megasperma* f. sp. *medicaginis* and *F. solani* f. sp. *phaseoli* on ROC lines A3, C5, B2 (*35S*:*Mtchit3-3* sense) and the transformed control lines O2, O5, and Q1. In all ROC lines, *F.*

solani and *P. megasperma* caused an elevation of the *Mtchit4*/*ubi* ratio. In constitutively *Mtchit3-3*-expressing ROCs, the induced *Mtchit4* expression levels were equal to or even above the average expression levels measured in transformed controls; however, this increase was statistically not significant. This indicates that disruption of the mycorrhiza-dependent *Mtchit3-3* expression pattern does not impair elicitation of the defense-related chitinase gene *Mtchit4*.

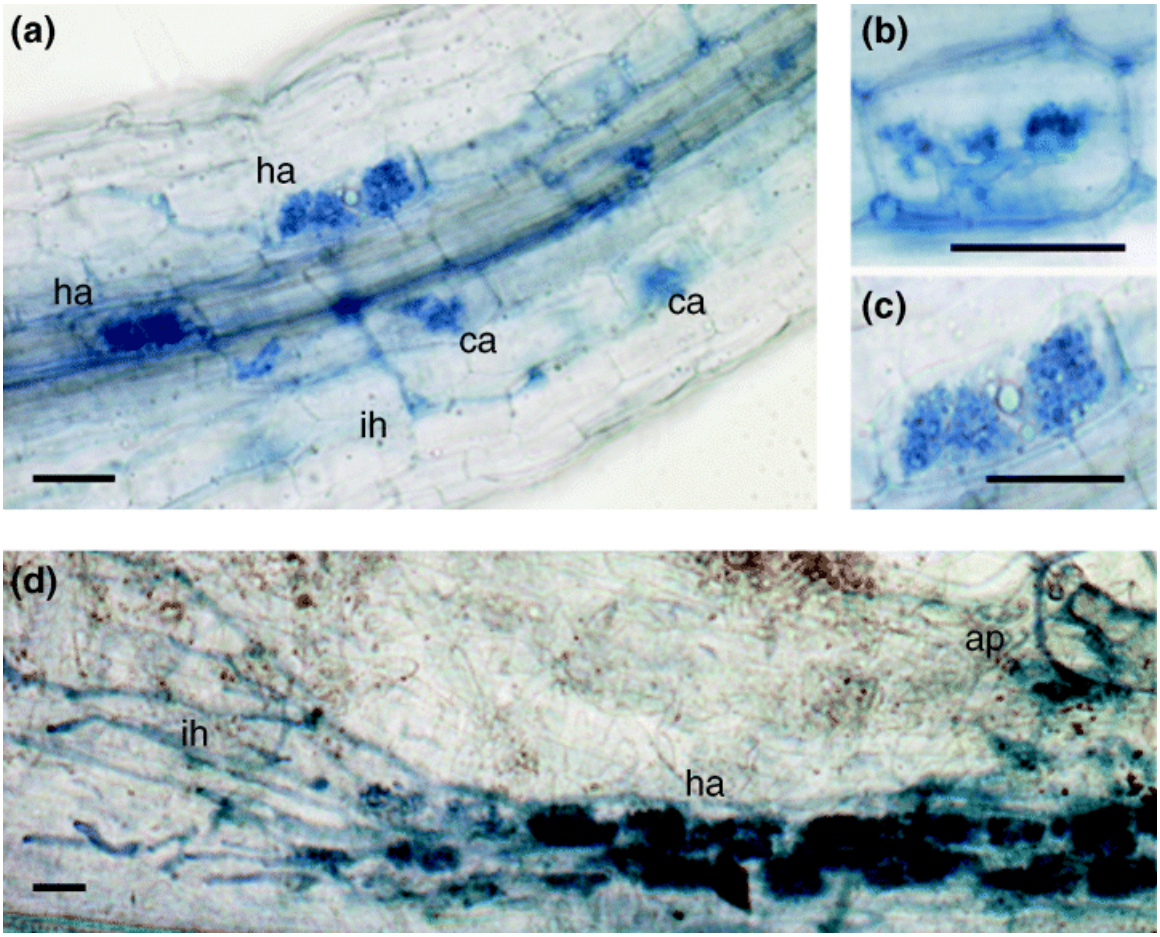

Figure 7 Intraradical structures formed by *Glomus intraradices* in *Agrobacterium rhizogenes*-transformed *Medicago truncatula* ROCs and composite plants. (a) Trypan blue-stained root area showing the colonization patterns as typically found in ROCs. Intraradical hyphae (ih) are the predominant fungal structures accompanied by scattered highly branched arbuscules (ha) and clumped arbuscules (ca). (b) Magnification of a clumped arbuscule. (c) Magnification of a highly branched arbuscule. (d) Arbuscular mycorrhizal structures as typically found in *A. rhizogenes*-transformed composite *M. truncatula* plants. Numerous intraradical hyphae are growing at the front of the extending fungus followed by young and densely packed mature arbuscules. The oldest part of the mycorrhiza is indicated by the position of the appressorium (ap). Bars, 100 µm.

	Root colonization in replicate Petri dishes[b] (% root length with fungal structures)			Spore formation in replicate Petri dishes[b] (number of new spores per Petri dish)		
	Sense	Antisense	Empty vector	Sense	Antisense	Empty vector
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	3	0	0	10	0	0
	4	0	1	15	0	14
	4	1	3	22	0	24
	12	2		45	0	
	12	3		51	0	
	27	4		58	0	
	28	4		80	0	
	29	10		104	0	
	31	20		190	0	
	38	36		247	24	
	38	36		250	182	
	42	36		339	186	
	47			348		
	55			368		
	60			500		
	66			510		
	80			650		
Means	22	7	0	146	19	3

Table 2 *Medicago truncatula* root organ cultures (ROCs) that have incorporated the *Mtchit3-3* sense construct show a higher probability of being colonized and forming new spores compared to controls or antisense ROCs.

[a]Colonization in *Mtchit3-3* sense ROCs was compared with controls or antisense ROCs ($T_1 = -3{,}11$, $P = 0.002$, and $T_1 = -2{,}09$, $P = 0.022$, respectively) using the Mann–Whitney signed-rank test. Spores were more often found in sense than in antisense transformed ROCs ($T_1 = -3.34$, $P = 0.002$, Mann-Whitney signed-rank test).

[b]Spore formation and intraradical colonization were measured 5 wk after the second subculture. Results from individual Petri dishes obtained in two independent experimental series are given. The ROC lines having incorporated *35S*:*Mtchit3-3* sense (sense), *35S*:*Mtchit3-3* antisense (antisense) and *35S* control vector constructs (empty vector) were inoculated and subcultured twice.

3.5. Discussion

We have cloned the specific arbuscular mycorrhiza induced class III chitinase gene, *Mtchit3-3*, of *M. truncatula* and provide evidence that the predicted gene product is an active chitinase. Whether its enzymatic properties are different from other class III chitinases of *M. truncatula* cannot be predicted from the sequence data. We have no direct proof that Mtchit3-4, another symbiosis-related chitinase of *M. truncatula*, and Mtchit3-1, a pathogen-related chitinase of this plant that have been cloned, possess chitinase activity. The activation of the *Mtchit3-3* promoter and accumulation of the *Mtchit3-3* transcripts (Bonanomi et al, 2001) are tightly regulated during AM formation. In *M. truncatula*, activation of two other mycorrhiza specific promoters has been described, namely *MtGst1* (Wulf et al, 2003) and *MtPT4* (Harrison et al, 2002). By pairwise comparisons we found no apparent similarities in the promoter organization of *Mtchit3-3* and *MtGst1* and *MtPT4*. However, some sugar responsive elements are common to all of the three AM-specific inducible promoters in *M. truncatula*.

It is generally known that plant roots influence the presymbiotic development and metabolism of glomalean fungi (Pinior et al, 1999; Buee et al, 2000; Tamasloukht et al, 2003). Root exudates, for example, trigger the presymbiotic hyphal branching (Buee et al, 2000) or stimulate expression of fungal mitochondrial genes during spore germination (Tamasloukht et al, 2003). Similarly, we found that presence of *D. carota* ROCs and *M. truncatula* ROCs having integrated the control vectors stimulated AM spore germination *in vitro* (Figures 5 and 6). Rather unexpectedly, *35S*-driven expression of *Mtchit3-3* in ROCs accelerated germination of spores of arbuscular mycorrhizal fungi and resulted in a higher number of spores that have developed germination hyphae. We think that this stimulation could be the result of the mycorrhiza independent *35S*-driven expression of *Mtchit3-3* leading to a release of the ectopically expressed *Mtchit3-3* gene product to the apoplast. In the apoplast, the *Mtchit3-3* gene product might influence the molecular crosstalk between the fungus and the root. It might act on target molecules of the plant leading to the formation of an active signal. In addition, when reaching the medium the *Mtchit3-3* gene product might directly act on the walls of the AM fungal spores. We have no information on the mechanism by which the *Mtchit3-3* gene product accomplishes stimulation of spore germination. However, we expect that the mechanism is dependent on mycorrhiza specific properties inherent in the *Mtchit3-3* chitinase. Commercially available chitinase, for example that of *Streptomyces griseus* had no stimulating effect on spore germination (M. Elfstrand, unpublished). We obtained no evidence that the stimulating effect of *Mtchit3-3* results from differences in metabolic activities that might have been introduced into the ROCs by the different vector constructs. The growth rate of all ROC lines was similar and all ROCs grew equally well on different carbohydrate sources. The stimulating influence on spore germination is reminiscent of the M161 myc mutant of tomato, which is still able to elevate the low germination frequency of *G. intraradices* from 7% in absence of roots to 38% in presence of M161 plants. However, M161 plants are less stimulating than wild-type plants, which induce 69% spore germination (David-Schwartz et al, 2001). To ascribe some ectopically expressed chitinases a stimulatory effect on spore germination is not necessarily a contradiction to experiments that demonstrated antifungal activity of chitinases against pathogenic and AM fungi *in vitro* (Mauch et al, 1988; Arlorio et al, 1992; Vierheilig et al, 2001). Effects of constitutive expression of chitinase genes *in planta* can differ from their effects exerted after addition of the purified protein to the hyphae. For example, constitutive expression of several chitinase genes in tobacco roots did not adversely affect colonization by *Glomus mossae* despite proven antifungal activity *in vitro* and significantly increased chitinase activities *in planta* (Vierheilig et al, 1995). Moreover, enzymatic properties of the *Mtchit3-3* gene product are expected to be different from those of chitinases that exhibited antifungal activity. Symbiosis-related properties of chitinases are known from interactions of plants with rhizobia. Schultze

and coworkers (Schultze et al, 1998) reported that certain Nod factors can only be cleaved by specific chitinases. Similarly, one could expect that the *Mtchit3-3* gene product could also specifically act on fungus-derived signal molecules.

We assume in the case of *G. intraradices* that the higher probability of AM fungal colonization upon *35S*-driven expression of *Mtchit3-3* directly reflects the stimulating effect on spore germination. The variability of spore formation of *G. intraradices* within different Petri dishes established with the same ROC line was high, a phenomenon also known from other *in vitro* culture systems (Declerck et al, 2001). Although no quantitative conclusions can be drawn from our spore production experiments, the most important observation is that *35S:Mtchit3-3*-transformed *M. truncatula* ROCs not only stimulate *G. intraradices* spore germination but allow the AM fungus to colonize the roots and complete its life cycle by formation of infective spores. That *G. constrictum* failed to establish an arbuscular mycorrhizal association with *M. truncatula* ROCs despite intensively stimulated spore germination points to fungus species-specific differences in the interaction with ROCs. In the case of *G. constrictum*, processes occurring after the stimulation of spore germination seem to have failed and impaired *M. truncatula* ROCs in forming a stable arbuscular mycorrhizal association with this fungus.

That constitutive expression of *Mtchit3-3* in ROCs had no effect on the abundance and the ratio of highly branched to clumped arbuscules was unexpected because in roots colonized with *G. intraradices* transcripts accumulated in cells containing arbuscules. Absence of effects in ROCs is probably due to the only moderate elevation of the *Mtchit3-3* expression levels achieved by the *35S* promoter. Despite four- to six-fold elevation above the expression levels of the controls, *35S*-driven *Mtchit3-3* expression in ROCs reached values of maximally 10% of those induced in highly mycorrhizal roots of whole plants. That no induction of *Mtchit3-3* expression was detected upon colonization of ROCs with *G. intraradices* was probably due to the low number of arbuscules formed in *M. truncatula* ROCs. In this context, it should be mentioned that despite low phosphate content of the M-medium the association of *M. truncatula* ROCs with *G. intraradices* is not necessarily mutualistic. The ROCs without AM fungus grew as vigorously as ROCs in presence of *G. intraradices*. Vigorous growth of *P. megasperma* and *F. solani* on M-medium might also be the reason that no differences in susceptibility of ROCs upon *35S*-driven *Mtchit3-3* expression became apparent.

In conclusion, by fusing the *Mtchit3-3* coding region to the CaMV *35S* promoter, *Mtchit3-3* was constitutively expressed in all parts of the ROCs independent from the AM fungus. The resulting transgenic ROC phenotype stimulated AM spore germination but caused no changes in the intraradical colonization pattern. These results demonstrate that disrupting the tight regulation of an arbuscule-dependent expressed gene can influence the balance between host roots and its AM partner. In the case of *Mtchit3-3*, an early step of interaction, namely stimulation of spore germination by the host roots was affected.

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Appendix 2

Localization of the plant chitinase Mtchit3-3 via GFP fusion

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Key words:
Arbuscular mycorrhizal fungi
Mycorrhiza-specific
Chitinase
Mtchit3-3
Localization via GFP

Abbreviations:

Mtchit	Chitinase
GFP	Green fluorescent protein
AMF	Arbuscular mycorrhiza(l) (fungus)
LB	Luria Bertani medium
LB, RB	left border, right border (transformation vector)

A2. 1. Abstract

Arbuscular mycorrhiza is arguably the most important symbiosis between plants and fungi. During this symbiotic interaction, a series of events trigger mycorrhiza specific expression of plant genes. In the model legume *Medicago truncatula*, one of the genes expressed in a mycorrhiza-specific way is *Mtchit3-3*, encoding a class III chitinase. Its transcripts have been localized in cells that contain arbuscules and ectopic expression of *Mtchit3-3* in root organ cultures enhanced spore germination and mycorrhiza formation.

In an attempt to localize the *Mtchit3-3* gene product at the subcellular level, to test the functionality of its putative signal peptide, translational fusions of *Mtchit3-3* to the green fluorescent protein (GFP) were constructed and tested in epidermal cells of onion and *M. truncatula*. Onion epidermis cells bombarded with the construct showed an extracellular localization of green fluorescence in plasmolysed cells, indicating that the fusion product was secreted. GFP fluorescence was also observed in *M. truncatula* leaves, but it could not be localized to a cellular compartment in plant culture.

These results give evidence that the signal peptide of Mtchit3-3 is functional, directing the protein to the secretory pathway.

A2. 2. Introduction

Arbuscular mycorrhiza (AM) is arguably the most widespread symbiotic interaction of plants with fungi from the genus Glomeromycota (Schüssler et al, 2001). Plants and fungi benefit form the symbiosis by exchange of plant derived photosynthetic products, sugars, against phosphate and nitrate that the fungi are able to take up from the soil. Arbuscular mycorrhizal fungi (AM) are obligate biotrophs that are dependent on the plant for completion of their life cycle.

The formation of a functional AM is tightly controlled. Following the first contact, the fungus forms appressoria on the surface of the host roots which are the place of fungal root epidermis penetration (Bonfante & Perroto, 1995). The fungus enters the root with the help of rearrangements in the plant cells cytoskeleton (Genre et al, 2005) and growths into the root cortex, where it spreads and enters plant cells. Within root cortex cells, hyphae differentiate into specialized structures, arbuscules, the place of nutrient exchange between plant and fungus. Many plant derived enzymes have been identified in the periarbuscular space (e.g. Harrison et al, 2002) and numerous efforts have been and are being undertaken to identify the players that are involved in this symbiotic interaction and shed light into the cellular processes involved in AM.

Chitinases are part of the recognition machinery of plants and microbes, as shown in the bacterial nitrogen-fixing symbiosis (e.g. Staehelin et al, 2000). The model legume *Medicago truncatula* forms two different root endosymbioses. Within a group of microbial regulated chitinases (Salzer et al, 2000) two were found to be symbiosis-related: the nodule-specific class V chitinase Mtchit5 (Salzer et al, 2004; chapter 2) and the mycorrhiza specific Mtchit3-3. In a subsequent study, the importance of the class III chitinase Mtchit3-3 in the formation and establishment of the AM was investigated (Elfstrand et al, 2005; chapter 3). In roots with a disrupted ectopical *Mtchit3-3* expression, the influence of root exudates on AM spore germination and mycorrhiza formation was tested. The germination rate of *G. intraradices* and *G. constrictum* spores was increased and in the case of *G. intraradices* the probability of colonization was increased in roots with higher *Mtchit3-3* expression when compared to control roots. Furthermore the *Mtchit3-3* promoter was primarily active in cells harboring arbuscules. This corroborated an earlier in situ approach, which located gene transcripts to cells that host mature arbuscules (Bonanomi et al, 2001).

To obtain information on the chitinase protein that is so closely related with AM, its reading frame was fused to the GFP reporter gene and introduced into plant expression systems. Transient transformation of onion and *M. truncatula* leaves showed that the fusion protein was expressed and secreted into the extracellular expression space. Unfortunately, stably transformed *M. truncatula* hairy roots were not useful for localization of the respective gene product. Nevertheless, these data stongly indicate that the *Mtchit3-3* gene product enters the secretory pathway and is secreted to the apoplast of root cells, or even more probably into the periarbuscular space to act directly on the AMF.

A2.3. Materials and Methods

A2.3.1. Vectors and materials

pGEM-Teasy was purchased from Promega (Madison, WI, USA), and used according to the manufacturers instructions. The NSPAX vector, harbouring *eGFP* was kindly provided by M. Heinlein from the University of Basel. pGreenII and the 35S cassettes, as well as pSoup were obtained from JIC, as described on the pGreen website (www.pgreen.ac.uk). pGreen.km was constructed by inserting a 35S::km cassette into the *HpaI* site close to the LB, within the t-DNA region.
All mentioned plasmids were cloned into *E. coli* Top10 cells, and selected on LB-medium containing ampicillin or kanamycin. Minipreps were prepared with the Machery & Nagel DNA extraction kit (Machery & Nagel, Oensingen, Switzerland). Sequence identity of all PCR-amplified fragments was verified by sequencing.

A2.3.2. Plasmid construction

Mtchit3-3 coding region was amplified by PCR from BAC 72H13 (D. Cook, Davies University, California, US; Elfstrand et al, 2005). Primers used for *Mtchit3-3* amplification were 3-3-for (introducing a *SacI* site at the 5′ end) and 3-3-rev, see list of primers below. The PCR was performed under the following cycling conditions with the High fidelity PCR kit (Roche, Basel, Switzerland): 1 cycle: 94° C, 2 min; 10 cycles: 94° C 15 sec, 60° C 30 sec, 72° C 90 sec, 20 cycles: 94° C 15 sec, 60° C 30 sec, 72° C 90 sec with a 10 sec increase at each cycle, 1 cycle: 72° C 7 min. The PCR product was subcloned into the pGEM-Teasy vector and the plasmid was opened by restriction digestion with *SpeI* and *SalI*. GFP had been released from its vector by restriction digestion with *BlnII* (*AvrII*) and *XhoI* and was subsequently ligated to the open sides of the pGEM-Teasy vector. From this resulting pGEMTeasy::Mtchit3-3::GFP vector, the fusion product was released by the flanking *SacI* sites, and inserted into the *SacI* digested 35S cassette, which gave 35S::Mtchi3-3::GFP. PCR screening was performed to find forward insertions.
For control experiments, GFP was amplified from NSPAX using the GFP-for (introducing a *SacI* site and a start ATG) and GFP-rev (introducing a *SacI* site at the 3′ end) primers. PCR was performed with the High fidelity PCR kit with the following cycling conditions: 1 cycle: 94° C, 2 min; 1 cycle: 94° C 15 sec, 55° C 30 sec, 72° C 90 sec; 10 cycles: 94° C 15 sec, 61° C 30 sec, 72° C 90 sec, 20 cycles: 94° C 15 sec, 61° C 30 sec, 72° C 90 sec with a 10 sec increase each cycle, 1 cycle: 72° C 7 min. The PCR product was subcloned into the pGEM-Teasy vector. GFP was released by *SacI* and introduced into the *SacI* site in 35S cassette, to yield 35S::GFP.
The 35S::Mtchi3-3::GFP and 35S::GFP promoter cassettes were released from the vector by restriction digestion with *BsrBI* and introduced into pGreen.km, which had been blunt ended opened by *EcoRV*, which yielded pGreen.km/35S::Mtchi3-3::GFP and pGreen.km/35S::GFP.

Primers

3-3-for:	5′-AGGCAACACGAGCTCATGAAAATGGCGTTGAAATC-3′
3-3-rev:	5′-CATGGCTCTTGATGGAGGAGCTA-3′
GFP-for	5′-GATTCCCCGGAGCTCGACATGCG-3′
GFP-rev	5′-TGACCTCGAGCTCTGCAACTTGAGGTAGT-3′

A2.3.3. Transient transformation using surface bombardment

5 to 10 µg of the DNA was coated onto gold particles (microcarrier; Biorad; Reinach, BL, Switzerland) that have been stored in glycerol at 4° C. DNA-coated gold particles were bombarded into the surface of the plant material using a Biorad PDS-1000/He Biolistic Particle Delivery System at a pressure of 650 psi.

Onion
Onion (*Alium cepa*; Supermarket) bulb segments were bombarded and incubated at room temperature in the dark, on a piece of wet paper towel. On the following day, the epidermis was peeled off for observation.

M. truncatula
M. truncatula leaves were cut from young Jemalong A17 plants and surface sterilized with 12% hypochlorite for 1 min and the leaves were treated with 12% mannitol for 5 min. After bombardment the leaves were placed on MS-medium in Petri dishes, and incubated in the dark at room temperature over night.

Onion epidermis and *M. truncatula* leaves were observed using a Zeiss Axioplan microscope (Zeiss, Jena, Germany), and GFP was excited by using blue laser light at 488 nm.

A2.3.4. Transformation of *M. truncatula* hairy roots

Transformation
Together with pSoup, pGreen.km/35S::Mtchit3-3::GFP was electroporated into *A. rhizogenes* LBA9402 and bacteria were selected on YEB with kanamycin/tetracycline/rifampicin. Single *A. rhizogenes* colonies were selected and grown into a thick lawn of bacteria, used for plant transformation.
The transformation and generation of transgenic root cultures was essentially performed as in Elfstrand et al. 2005. *M. truncatula* seedlings were grown for three days on water agar. When the radicle reached a length of about 1 cm, the root tip was cut off and the cut edge was inoculated with bacteria. After inoculation, the plantlets were cultivated on M-medium for two days. Plantlets were then transferred to M-medium with cefotaxime/kanamycin for selection and transferred to new plates every week. Roots emerging from the inoculation site after several weeks of growth were isolated and cultured on M-medium with cefotaxime/kanamycin. Growth of the root organ cultures was strongly interfered by the heavy growth of the *Agrobacteria*. After selection with cefotaxime, cultures were chosen that had been optically free of bacteria and subcultured to M-medium without antibiotics, and absence of *A. rhizogenes* was verified by PCR with a Vir D3 specific primer combination (Lindroth et al, 1999). Some roots showed presence of Vir D3 and visible contamination and were further cultured on M-medium with antibiotics until no more contamination was detected. Cultures supporting amplification of the proper constructs, without amplifying the 800 bp Vir D3 fragment, were selected for subculturing.

Inoculation
Root cultures were inoculated with *G. intraradices* Schenk and Smith (DAOM 197198) that had been propagated with carrot roots as described in Elfstrand et al (2005).

A2.4. Results

A2.4.1. Localization of fluorescence in epidermis cells transiently expressing Mtchit3-3:GFP

Mtchit3-3::GFP constructs were introduced via transient transformation into epidermal cells of *M. truncatula* leaves. After appropriate incubation, several cells displayed green fluorescence under the fluorescence microscope, showing that this construct is functional, (Figure 1).
As control, the GFP gene was fused to the CaMV 35S promoter, and the construct was introduced in *M. truncatula* leaves (data not shown) and onion epidermis (Figure 2). Cells with GFP activity showed bright green fluorescence, proving that the construct was functional in plant cells. In the case of onion cells, the fluorescence was clearly visible inside the cytoplasm; bright green was visible around the vacuole with the highlighted nucleus and cytoplasmic strands, visible as green stretches crossing the cell body (Figure 2 a). When these cells were plasmolysed the fluorescence retracted within the cell body (Figure 2 b). The same patterns have been published elsewhere (e.g. Burleigh et al, 2003).
For localization of the Mtchit3-3::GFP, the construct was introduced into epidermal cells of onion bulbs. Expression of the fusion protein was shown by the appearance of several green fluorescing cells. The cells that were monitored showed green fluorescence in a sharp edge around the cells that was restricted to single cells (Figure 3). The pattern of nucleus and cytoplasmic strands was absent here, and the cell body was evenly dark. When the cells were plasmolysed, the green fluorescence did not retract together with the cell body, but stayed in the space between cell wall and plasma-membrane (Figure 4). This strongly indicated that the Mtchit3-3::GFP fusion protein had an extracellular localization.

A2.4.2. Localization of GFP in root organ cultures

The observation of green fluorescence in transformed *M. truncatula* roots was difficult because of their strong yellow autofluorescence. Observation of green fluorescence was possible in the fluorescence microscope, but it was rather weak against the background and difficult to see. It was stronger in younger parts of the roots and meristem buds, where single cells were possible to see (Figure 5 a). Regions and root pieces have been observed that developed higher abundance of green fluorescence (Figure 5 b), but also in these regions, a clear localization of the level of single cells was not possible.
Infection with *G. intraradices* revealed slightly higher green fluorescence around infection sites. Light spots of the size of single cells have been observed, which could have been due to fluorescence in arbuscules (data not shown, due to low picture quality). These spots were clearly located in the cortex cells but masked by the surrounding cell layers. Hence, with a standard fluorescence microscope, a clear subcellular localization was not possible because of the root thickness and high background fluorescence of the roots.

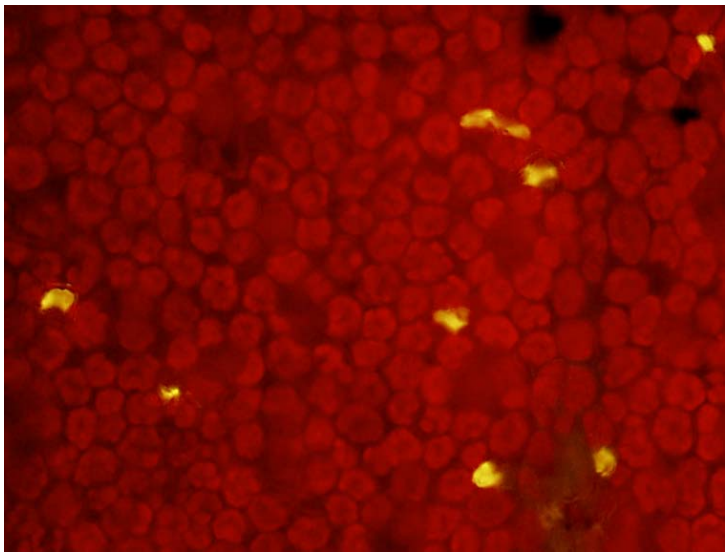

Figure 1 Transient expression of the Mtchit3-3:GFP vector in the *M. truncatula* leave surface. Expression of the green-yellow fluorescent protein was observed in the fluorescence microscope on the surface of young leaves. Red fluorescence originates from the chlorophyll in the leaves.

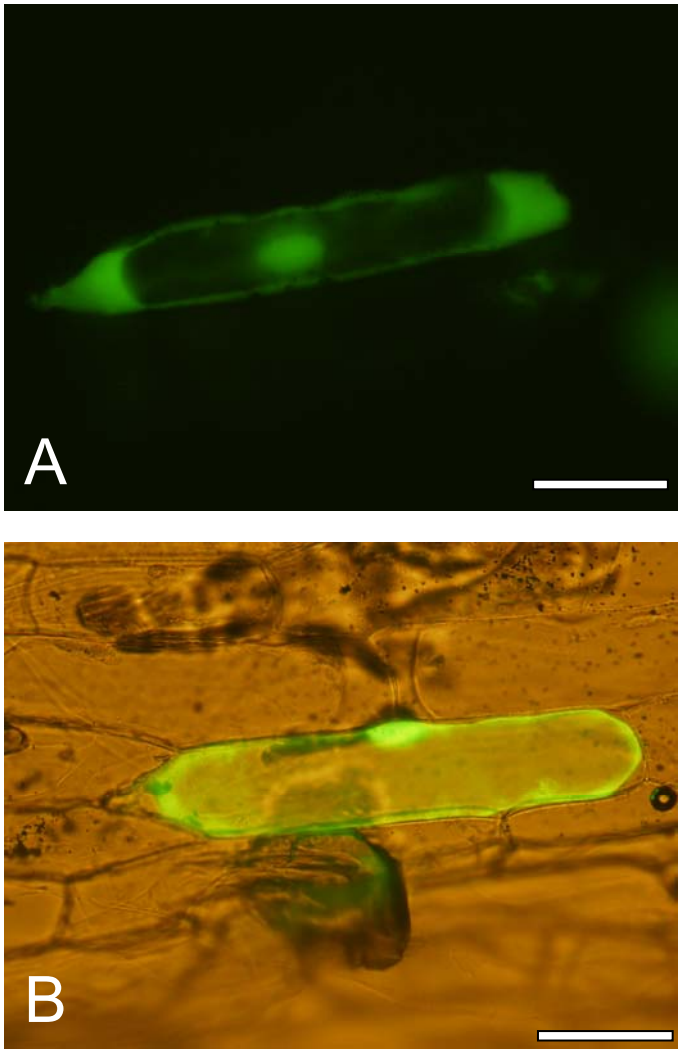

Figure 2 As positive control, GFP was expressed in onion epidermis cells with intracellular localization Bars equal 40 µm.
A. Fluorescence microscopy of an onion cell expressing GFP under the 35S promoter: green Fluorescence is visible in the cytoplasm round the vacuole and close to the nucleus (bright spot).
B. Overlay of bright field and fluorescence microscopy of a plasmolysed onion cell: the dark brown cell wall is visible and the retracting protoplast of the plasmolysed cell. Green fluorescence, representing GFP, is located inside the cytoplasm.

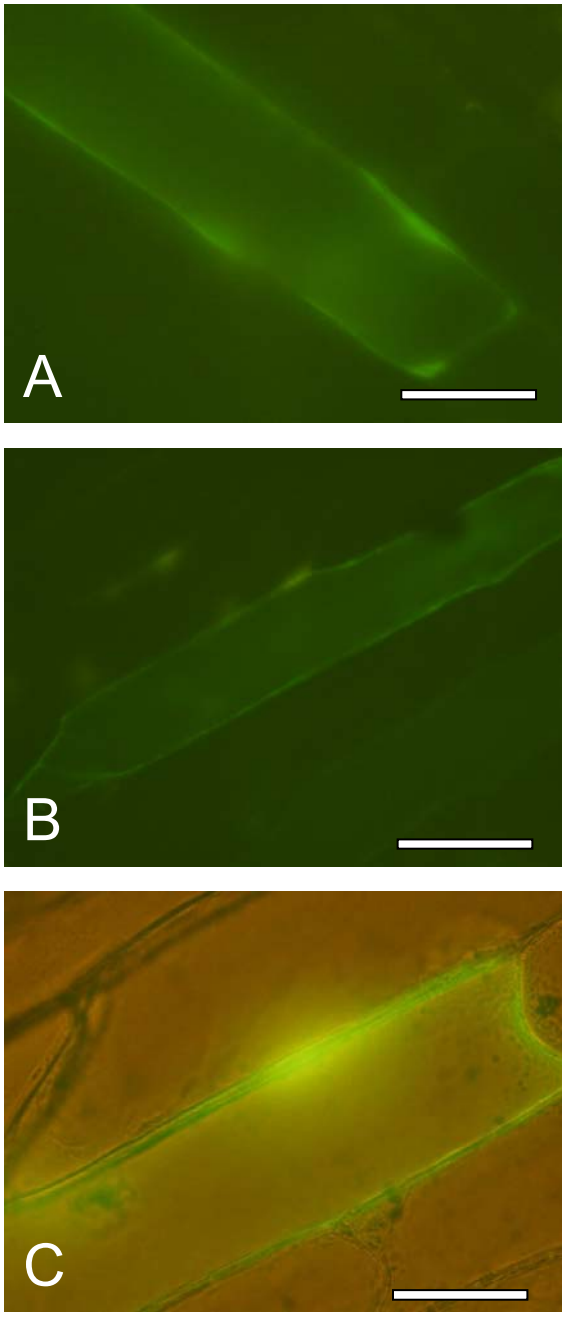

Figure 3 Fluorescence microscopy of onion cells transiently expressing the class III chitinase Mtchit3-3 fused to GFP. Bars equal 40 µm and 20 µm.
A. and B. Green fluorescence is visible at the edges of the cell.
C. Overlay of bright field and fluorescence microscopy: The cell wall is visible dark brown, co-located with it is GFP as a green band around the cell body. (The bright yellow spot is beginning autofluorescence of the onion cells).

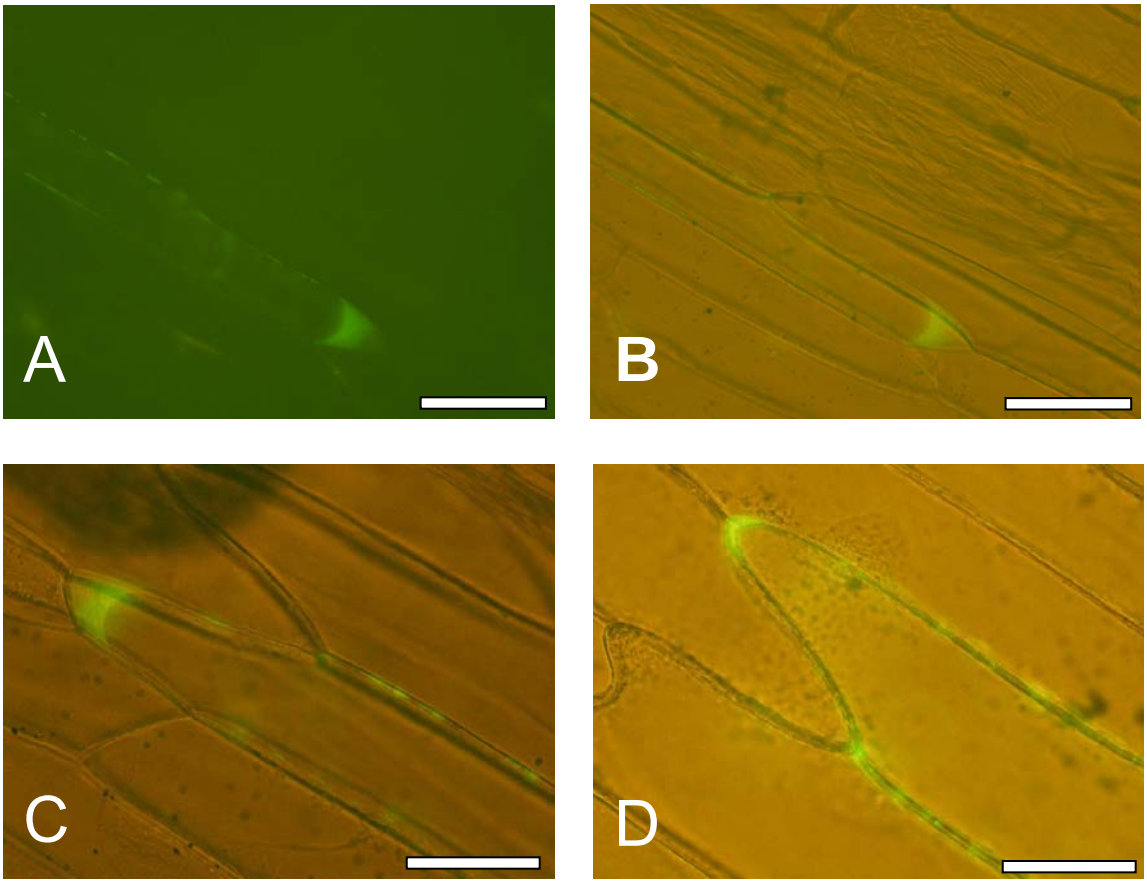

Figure 4 Mtchit3-3-GFP fluorescence was found outside of plasmolysed onion cells. Bars equal 40 µm and 20 µm.
Green fluorescence is not retracting with the protoplast, when the cells are plasmolysed, it stays in the interspace between the cell membrane and the cell wall. A and B. The same cell shown as fluorescence photograph (left) and as an overlay of bright field and fluorescence on the right. C and D. Overlay of bright field and fluorescence microscopy of plasmolysed onion cells.

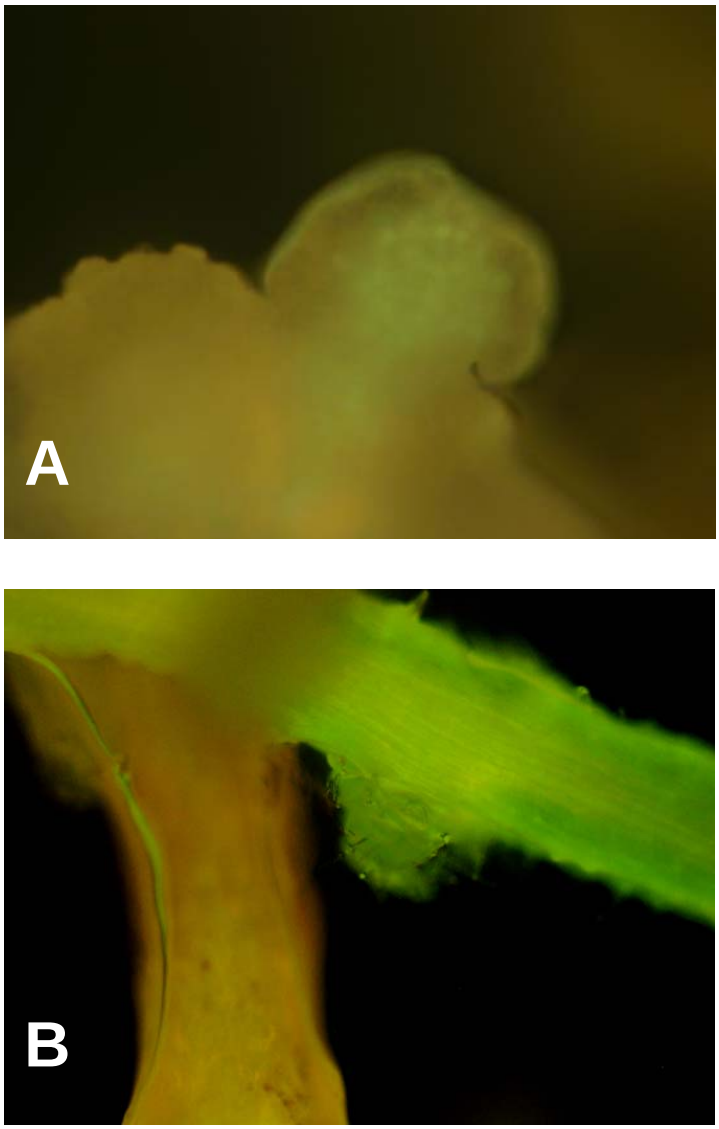

Figure 5 *Agrobacterium* transformed *M. truncatula* roots showing green fluorescence.
A. Light green fluorescence is visible in young and meristematic tissues, where the auto fluorescence is not as strong as in the root corpus. Single cells are visible as green spots that lie in the meristem bud.
B. Regions of high green fluorescence were visible, however, a clear structure was not evident due to surrounding tissue obscuring the fluorescence.

A2.5. Discussion

The chitinase gene *Mtchit3-3* was previously identified and characterized as a mycorrhiza dependent gene (Salzer et al, 2000). Overexpression of its gene product had an enhancing effect on arbuscular mycorrhizal spore germination, mycorrhizal colonization and offspring spore formation (Elfstrand et al, 2005, chapter 3). To gain information about the localization of this protein, the gene was fused to GFP (green fluorescence protein) and its expression was studied in several plant systems.

Mycorrhiza formation triggers a series of events in the plant as well as in the fungus. These events include information exchange between the two partners, attachment and entry of the fungus into the host root, and finally the establishment of arbuscules. Arbuscules are finely branched and exhibit therefore a large surface area, separated from the plant cell by the periarbuscular space. Periarbuscular transmembrane transporters were identified that serve as translocators for nutrients, mainly phosphate, nitrogen and sugar, from one partner to the other (e.g. Harrison & Dewbre, 2002; Liu et al, 2003; Hohnjec et al, 2005). The best studied so far is the *M. truncatula* phosphate transporter MtPt4 that has clearly been localized to the periarbuscular space, and was confirmed to be involved in phosphate transport (Harrison et al, 2002). In addition to phosphate-, sugar- and nitrate transporters, a number of other genes have been identified, some with yet unknown function. Among these are H^+ATPases (Liu et al, 2003), MtGst1 (Wulf et al, 2003) or cytochromes (Hohnjec et al, 2005). These are presumably involved in energy acquisition and turnover. Other proteins, like MtCEL1 (Liu et al, 2003), several endoglucanases (Hohnjec et al, 2005) or lectins (Frenzel et al, 2005) are most probably situated at the periarbuscular membrane and have direct contact to the fungus. Under symbiotic conditions, Mtchit3-3 is most probably active in arbuscule containing cells. Transcripts have been found exclusively in these cells and the *Mtchit3-3* promoter directed reporter gene expression to arbuscule containing cells (Bonanomi et al, 2001 and Elfstrand et al, 2005, chapter 3). It was first suggested that Mtchit3-3 reduces defence reactions by elicitor cleavage or other signalling compounds. This was based on the findings that transcripts are accumulating around arbuscules, but not close to other intraradical structures, and also on the fact that there are chitinases identified that have supportive activity in a symbiotic context (Salzer et al, 1997). However, Elfstrand et al (2005, chapter 3) showed that under ectopic expression it also may act in the surrounding medium directly on fungal spores, and this has not been reported for other arbuscule specific genes. In this case the mode of action should therefore not only be located in the arbusculated cells.
The *Mtchit3-3* gene was sequenced and the deduced protein sequence revealed a predicted cleavable signal peptide of 22 amino acids length, one possible glycosylation site and no transmembrane domains, as is characteristic for class III chitinases; a computer-based prediction of its structure revealed conserved helix, coil and beta strand regions, when compared to other class III chitinases (Feddermann, unpublished), which makes Mtchit3-3 a typical chitinase of the class III. Its predicted acidic pI corresponds to the pH in the periarbuscular space (Smith et al, 2001), although the pH optimum of the Mtchit3-3 chitinase could not be determined (Feddermann, unpublished) and does not necessarily correspond to the periarbuscular pI. Together, a periarbuscular localization of the protein is expected. The extraradical function found in Elfstrand et al (2005, chapter 3) could be due to apoplastic targeting of the protein through the signal peptide and its transport to the outer root tissues and the surrounding medium. However, with the transient expression system used in this study, it could be shown that the signal peptide is indeed directing the protein out of the cells.

GFP reporter genes are used nowadays almost in all living organs, which shows the utility of the reporter enzyme. Fusion proteins with GFP have been used to explore targeting to a variety of subcellular locations (e.g. Haseloff et al, 1997). The GFP, however, is a large protein attachment and is likely to influence the function of its attached protein, or even

abolish it. In mice, for instance, homozygotic expression is lethal (M. Peppelenbosch, personal communication), due to the toxicity of the protein. In plants there is a high probability that GFP has certain functions that are interfering with the plants function.
Elfstrand et al (2005, chapter 3) reported that the gene product of *Mtchit3-3* is functional in transgenic mycorrhizal roots when the gene was introduced into the roots. From the experiments presented here, it can be concluded that the fusion protein is translated correctly, since GFP fluorescence could be observed. This strongly indicates that the unmodified Mtchit3-3 is also translated correctly and transported correctly in the secretory pathway.
The enzymatic function of the chitinase might be disturbed by fusion to other proteins. Indeed, all attempts to produce and isolate the Mtchit3-3 protein with help of purification tags failed. Although a high expression rate was measured, the purified products did not display any chitinase activity. This makes it doubtful that the GFP fusion product is enzymatically functional. However, this does not affect our analysis of the protein localization. The approach taken in this study is well suited to study the functionality of the signal peptide and the localization of the protein.

Conclusion
Summarizing, it can be said that the data provide good evidence that chitinase Mtchit3-3 is located in the apoplast. Fluorescence arising from the fusion protein in plasmolysed onion cells was clearly visible in the space outside of the cell body. GFP Fluorescence was also observed in transiently transformed *M. truncatula* leaves. Although the localization was not as clear, this proved correct expression of the transgene in *M. truncatula.* Thus, Mtchit3-3 is probably secreted into the apoplast or, even more probably, into the periarbuscular space.

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Chapter 4

Medicago truncatula shows distinct patterns of mycorrhiza related gene expression after inoculation with three different arbuscular mycorrhizal fungi

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real-time PCR
Scutellospora
symbiosis-induced

Abbreviations:

AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
Mt	Medicago truncatula
Ubi	ubiquitin
MCA	malonyl-Co-enzyme A :acyl carier protein transacylase
Pyr DH	pyruvate dehydrogenase E1 alpha subunit
Bcp1	blue copper protein
Pt4	phosphate transporter
Gst1	glutathione S-tranferase
St1	sucrose transporter
CCA	cinnamoyl-Co-enzyme A reductase
SucS	sucrose synthase
Chit	chitinase
FK	fructokinase

4.1. Abstract

In a given plant, colonization by different arbuscular mycorrhizal fungal (AMF) species alter the plant nutrition and growth differently. We have set up an experiment to compare the functionality of AMF of different phylogenetic taxa in *M. truncatula* on the level of nutrition and gene expression. For the analysis of transcript levels of selected symbiosis related genes in the mycorrhizal roots, we made use of quantitative PCR

Mycorrhiza-formation enhanced biomass accumulation and nutritional status of the plants, although not in relation to the colonization degree. Seventeen genes were responding differently to the fungal colonization, and 11 were regulated by the AMF presence. Only the mycorrhiza-specific chitinase Mtchit3-3 was reduced in flowers of mycorrhizal plants.

Genes that have been shown to respond to colonization by fungi of the genus *Glomus* also respond to at least one fungus from the *Gigasporaceae*. Our data also indicate that different genes with arbuscule-specific gene expression in colonized roots are regulated by different mechanisms.

4.2. Introduction

Arbuscular mycorrhiza (AM) is a symbiotic association that is formed between fungi of Glomeromycota (Schüssler et al, 2001) with more than 80% of the terrestrial plants. Arbuscular mycorrhizal fungal (AMF) colonization improves the plant nutrition and especially provides the plant with phosphate from the soil. This symbiosis is an important component of many terrestrial ecosystems and it enhances growth and survival of numerous plant species (Smith & Read, 1997; van Der Heijden et al, 1998). Before and during the colonization of plant roots by AMF, a complex network of signal perception, amplification, and transduction in the host plant is brought into play (Harrison, 1999). The most prominent feature of this symbiosis are highly branched structures, arbuscules, that are formed inside root cortical cells. Within the plant root the development of the symbiotic interface is associated with AM-specific gene expression in the root cortex. Non-targeted gene isolation approaches and micro array experiments have identified several previously unknown AM-specific genes (Liu et al, 2003; Wulf et al; 2003; Grunwald et al, 2004; Hohnjec et al, 2005). The common basis of many recent studies has been the model plant species *M. truncatula* but the AMF species have varied between the studies. Salzer et al (2000) and Wulf et al (2003) among others used *G. intraradices*; Liu et al (2003), Harrison (1996) and Chiou et al (2001) used *G. versiforme* and Grunwald et al (2004) have used *G. mosseae*.

There is interest in the way how different AMF species interact with a given host plant. One set of studies on different plant/fungus combinations showed that in the majority of cases the fungal P uptake and translocation by the plant make important contributions to the interaction (Burleigh et al, 2002; Smith et al, 2004). However, the fungal P contribution was not related to colonisation or to growth and not even to the plants P responses (Burleigh et al, 2002; Smith et al, 2004). Burleigh et al (2002) also showed that different AMF species affect plant gene expression differently; known P starvation genes (MtPT2 and Mt4) were highly induced in the non-mycorrhizal control under a P-starvation regime, but the expression of these genes varied in response to AMF colonization. Plants inoculated with *Glomus mosseae* showed the lowest transcript level while *Gigaspora rosea* colonized plants had transcript levels similar to in the non-mycorrhizal control plants. This indicates that different AMF species affect both the plant's nutrition and growth but also the expression rate of certain genes in different ways (Burleigh et al, 2002; Smith et al, 2004).

Several studies lead to the concept that there is a functional diversity among AMF (Burleigh et al, 2002; Gao et al, 2001, Hart & Reader, 2002, Smith et al, 2004). However, the question raises if there is such a functional diversity with regard to AM-induced genes. Furthermore, there is some overlap in the AM-associated gene expression profiles in *M. truncatula* roots colonized by *G. mosseae* and *G. intraradices* (Hohnjec et al, 2005), but does that also apply to interactions with AMF taxa outside the *Glomaceae*? To address these questions, we set up an experiment to compare the expression of 17 previously identified AM-specific or AM-regulated genes. These genes were tested in interactions between *M. truncatula* and three selected fungal species with different taxonomic status; *Glomus intraradices* and *Glomus mosseae* belong to *Glomus* group A, while *Scutellospora castanea* belongs to the *Gigasporaceae* (Redecker, 2002; and figure 13 in chapter 1).

We also asked whether belowground fungal activities influenced the expression of symbiosis-related chitinase genes in aerial parts of the plants. For instance, transcripts of the symbiosis-related gene Mtchit5 can be detected not only in roots, but also in other tissues like flowers and leaves (Salzer et al, 2004, chapter 2). Mtchit3-3, another chitinase gene previously detected to be arbuscule specific in roots, was detected in aerial organs, unlike for instance the strictly root specific genes MtPt4 or MtGst1 (Wulf et al, 2003). In addition, the promoter sequences of four genes were compared to find out whether there are elements that are important for mycorrhiza-specific gene induction.

4.3. Material and Methods

4.3.1. Plant growth conditions and sampling

M. truncatula cv. Jemalong A17 seeds were released from their seed pods and stratified for 8 min in concentrated sulphuric acid. After germination for one week in water agar, seedlings were transferred in pots filled with autoclaved TerraGreen (Lobbe Umwelttechnik, Iserlohn, Germany) and grown in 65% humidity, with 18 hours daytime under 80000 LUX, 22° C at day, and 18° C at night.
After five weeks, plantlets were transferred into pots that had been inoculated with ca. 10 mL soil inoculum of *G. mosseae* ISCB13 (Oehl et al, 2003), *G. intraradices* Schenk & Smith (DAOM 197198) or *S. castanea* BEG01. One portion of the plants was inoculated with a 1:1:1 autoclaved mixture of the inoculum. Plants were monitored during growth and watered once a week and fertilized with B&D (Broughton & John, 1979) once a week. There were five or six pots for each of the three combinations of *M. truncatula* with different AMF fungi, representing biological replicates that were harvested and processed separately.
Flower samples were taken whenever available, flash frozen and stored at –80° C until RNA extraction. Ten weeks after inoculation leaf samples were taken and frozen in liquid nitrogen. Flowers and leaves were chosen randomly of all stages, from very young to fully-grown. Root samples were taken from plants that were removed from their pots, rinsed and freed from the surrounding Terra Green and dried slightly with paper towel. Roots were then cut into 1 cm pieces with a scalpel blade and mixed to avoid sampling artefacts. Material was frozen in liquid nitrogen immediately after mixing.
For determination of dry weight and micronutrient measurements, portions of the plant material were dried in paper bags for three days at 80° C in a drying oven.
AMF colonization and the internal structures were determined after Trypan blue staining of root segments using a variation of the gridline intersection method as described in Salzer et al (2000) and Elfstrand et al (2005, chapter 3).

4.3.2. Phosphorus, carbon and nitrogen content and determination of physiological parameters

Samples of dried plant material were ground at half power with a Ball mill (Retsch, MM 2224; Haan, Germany) in steel containers for 45 sec. For carbon and nitrogen measurements 2 mg of the ground material were weighed into tin capsules and measured in an automated nitrogen and carbon analyzer mass spectrometer (http://www.sercongroup.com/index.htm). Measurements were done in triplicate.
Phosphate content was determined essentially as described Onishi & Gall (1978) and Watanabe & Olsen (1965) but with some modifications; 100 mg ground dry plant material were ashed at 600° C for 12 hours, redissolved in 0.5 M NaOH and neutralized with 0.5 M HCl. Colorimetric measurements were done in a Spectrophotometer (Anthos reader 2001, Anthos Labtec Instruments; Salzburg, Austria) with 1.6% polyvinylpyrrolidon, 2.64 mM Na_2EDTA, 60 mM hydroxylamine, 20 mM H_2SO_4 and 0.88% ammonium-heptamolybdate. The results were analysed using the Mann-Whitney U test and linear regression analysis.

4.3.3. RNA preparation, reverse transcription and quantitative PCR

Total RNA was extracted from 100 mg frozen material with the NucleoSpin RNA Plant kit (Machery & Nagel, Oensingen, Switzerland) according to the manufacturers' instructions and concentrated by precipitation with ethanol and sodium acetate. 1 µg of RNA was used to prepare cDNA, essentially as described in Salzer et al, 2004 (chapter 2), but after the reverse transcription samples were diluted with 75 µl water and stored at 4° C until

use in real time PCR. Primers for real time PCR were created by hand and are listed in Table 1.
M. truncatula DNA was extracted by the Quiagen DNAeasy Plant extraction kit (Basel, Switzerland) according to the manufacturers' instructions, and diluted to a standard curve that served for the quantitative PCR. Real-time PCR was performed on a Gene Amp 5700 Sequence Detection System with Power SYBR Green PCR Mastermix (both Applied Biosystems) as described in Salzer et al (2004, chapter2) and Elfstrand et al (2005, chapter 3). The results were analysed using the Mann-Whitney U test and linear regression analysis.

4.3.3. Computational analysis of the chitinase promoter sequence

Promoter sequences were obtained after sequencing, as described in Elfstrand et al (2005, chapter 3) for the Mtchit3-3 promoter. Expression data of expressed sequence tags were compiled after extensive search through the databases (www.tigr.org/tdb/tgi/mtgi or http://www.ncbi.nlm.nih.gov). Promoter sequences were submitted to programs of the softberry prediction suite (www.softberry.com), the PLACE signal scan search program (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and FOOTPRINTER 2.0 (http://wingless.cs.washington.edu/htbin-post/unrestricted/FootPrinterWeb/FootPrinterInput2.pl).

Primers used for real time RT-PCR:

Name:	Forward primer:	Reverse primer:
MtUbi	GTGAAGACCTTGACCGGCAAAAC	GGTGAAGCGTGGACTCTTTCTGG
MtMCA	TGGAAGCCAAAGCAAAGTCT	TCCAGGTCCCAATTCATAGC
MtPyr DH	AAGGTCAGAGAAGTGGCGAA	TGGGATCCCTACCAGCATAG
MtBcp1	GCAAGGCACAATGTTTTCAA	TTGCCATGACAACTCCAAAC
MtPt4	ACGTTCTTGGTGACGGAAAC	AGTTCTTGAGTCCTGGCGAA
MtGst1	GGAGACAATGTGGTTGTTTTGG	GGTTGGAAGACCAAACCTGA
MtNarbonin	TGCCATTGCGATGACATAAT	GGAGCAATGGACACCAGTTT
MtSt1	CATATGCCAGGTTGCAGTAGC	GAGCTGCAGAACGAATCTCC
MtGH3	GAAATGGACCGTCGTCAGTT	GACGTGCCACTAACCCACTT
MtCCA	AGGCTGTGCCGGTGTTATAC	CTGCTTCCTTTGCAACCTTC
MtSucS1	CACGTAACGGGGAGCTGTAT	AGGACCACCATGGCAAGTAG
MtHATPase	ACATGGATGCTGCCGGTAAT	ATCTGCTCTGGTGCACCTTT
MtFK	TCCAAGTGCTGACATGCTTC	AGGGTAGGCGAAGGTTAGGA
Mtchit3-3	CCTTGTCAATACAATCCTGGTG	GCAGAACCTTTAATAGCTGG
Mtchi3-4	CCCTGATGCATTTATGAAC	CATATTTGGAAGAACCTTTAATAACTGG
Mtchit3-1	CCTGGTGCTTGTAACTTTGTTTC	GGTGAAGGCTTAACAATAGGCAGC
Mtchit4	GGTGATGCATATTGTGGCACAGGG	GCAGCAGCAACCTCACGTTTGGAG
Mtchit5	GGGTTGATGGTGGAATGGCG	GATCCGGTCTCCTTGTCATAC

Table 1 Primers used for real – time PCR. Primers of MtUbi and chitinase genes have been used in Salzer et al, 2000 (Salzer et al, 2000).
Abbreviations: MtUbi, ubiquitin; MCA, malonyl-Co-enzyme A:acyl carier protein transacylase; Pyr DH, pyruvate dehydrogenase E1 alpha subunit; MtBcp1, blue copper protein; MtPt4, phosphate transporter; MtGst1, glutathione S-tranferase; MtSt1, sucrose transporter; CCA, cinnamoyl-Co-enzyme A reductase; MtSucS1, sucrose synthase; Mtchit, chitinase; FK, fructokinase.

4.4. Results

4.4.1. Physiological data

The degree of AMF colonization after ten weeks was on average 58.2% after inoculation with *G. intraradices*, 23.2% after *G. mosseae* inoculation and 10.4% after inoculation with *S. castanea* (Figure 1). The colonization of the three fungi all differed significantly ($p < 0.01$).
The three fungal species had developed their typical colonization patterns. The number of appressoria per successful entry into the roots was different in the three fungi. *G. intraradices* seemed to be the most successful in entering the roots (Figure 1 a, d), in the case of *G. mosseae* we found more appressoria that did not lead to intraradical infections (Figure 1, b and e). In *S. castanea*, which developed the smallest degree of colonization, unsuccessful appressoria (Figure 1 c and f) were much more common than in *G. intraradices* ($p = 0.0173$) and *G. mosseae* ($p = 0.026$). The progression of the fungus within the cortex of the roots differed as well: the two *Glomus* species developed a similar pattern, with *G. intraradices* showing many arbuscules, groups of vesicles and thin intraradical hyphae (Figure 1 a). *G. mosseae* developed less arbuscules and somewhat thicker hyphae but had only rarely vesicles (Figure 1 b). As expected, there were considerable differences in the colonization patterns of *S. castanea* compared to the two *Glomus* species. *S. castanea* developed highly coiled and thick hyphal structures, which were accompanied by arbuscules (Figure 1 c).
Plants that had not been inoculated by AMF grew slower and remained smaller, compared to inoculated plants. They developed less secondary and tertiary branches and leaves of smaller area (data not shown). Consequently these plants developed less biomass, as measured by plant dry weight (Figure 2). Non-inoculated plants also had less flowers and seed pods compared to the AMF inoculated plants, in some cases the plant did not flower at all during the experiment. AMF colonization also increased phosphorus (P) and nitrogen (N) contents of the shoots in comparison to the non inoculated plants (Figure 2), but irrespective of the degree of colonization; *S. castanea* inoculated plants, with the lowest degree of colonization, produced almost as much biomass as the highly colonized *G. intraradices* plants.

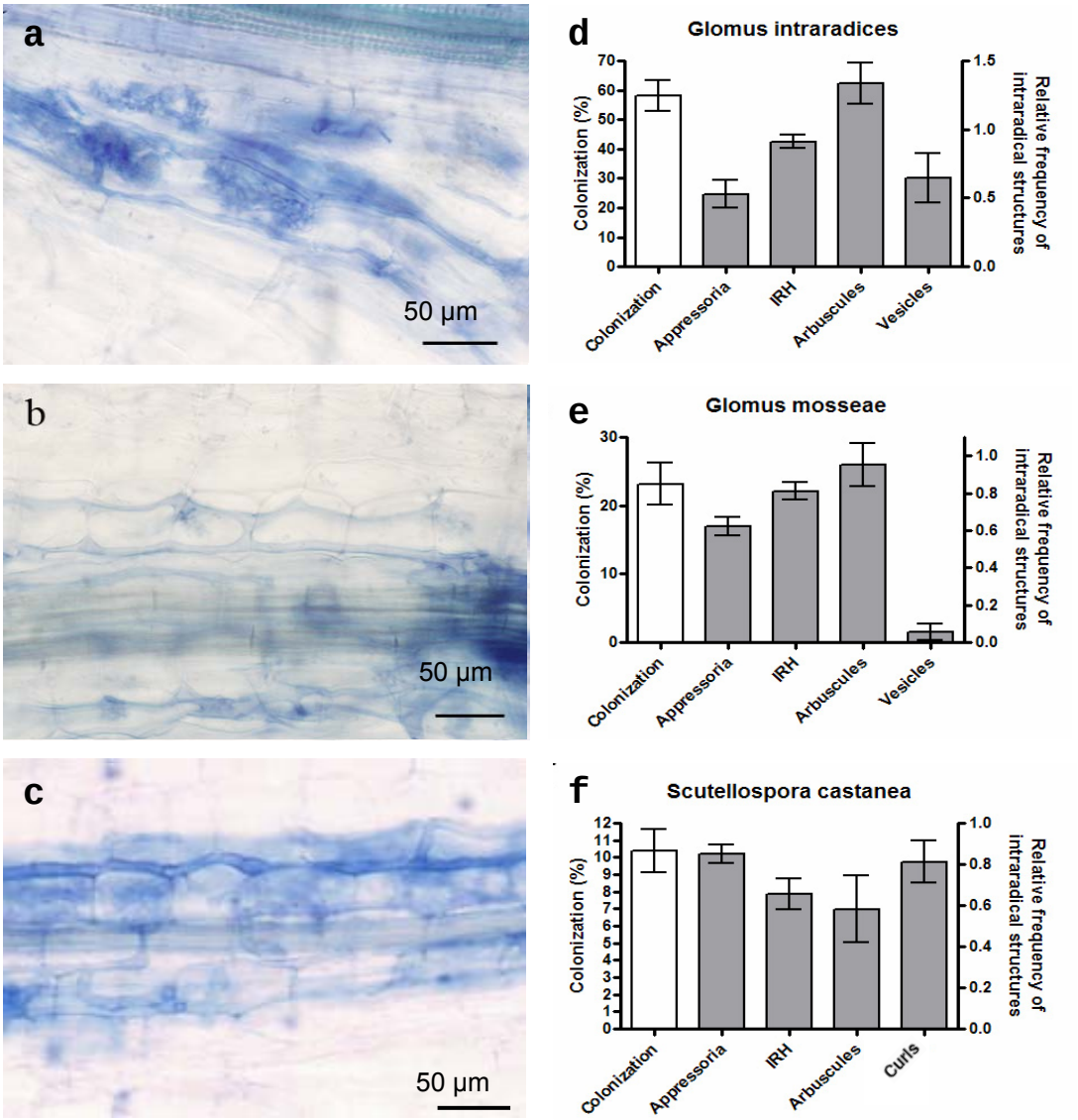


Figure 1 Mycorrhizal structures in roots of *M. truncatula* after 10 wk of symbiotic growth. (a-c) Typical views of root samples after staining with Trypan Blue; fungal structures appear in dark blue.
(a) *G. intraradices* typically grows with thin and straight hyphae in the root cortex. Arbuscules are penetrating into neighboring host cells. (b) *G. mosseae* exhibits the same basic growth pattern as *G. intraradices*, but with slightly thicker hyphae. (c) *S. castanea* typically grows with thick and short, curved hyphae along the innermost layers of cortical root cells. Hyphal coils and arbuscules are formed in the subtending cells.
(d-f) Degree of colonization (white bar, scale on the right-hand side) and relative frequency of intraradical structures (grey bars, scale on the right-hand side), as determined by the gridline intersection method. The comparison of the mycorrhizal patterns was based on intersects that contained fungal structures of any kind.

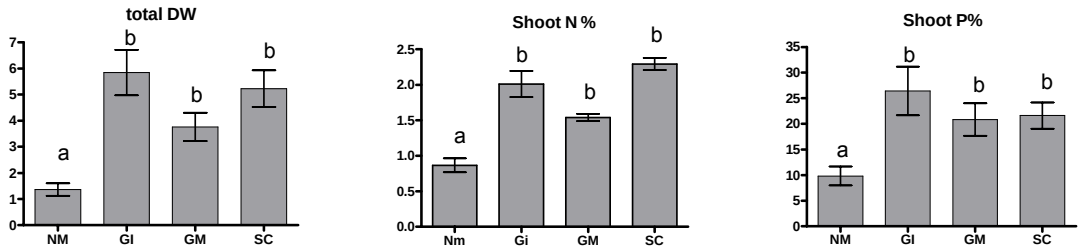


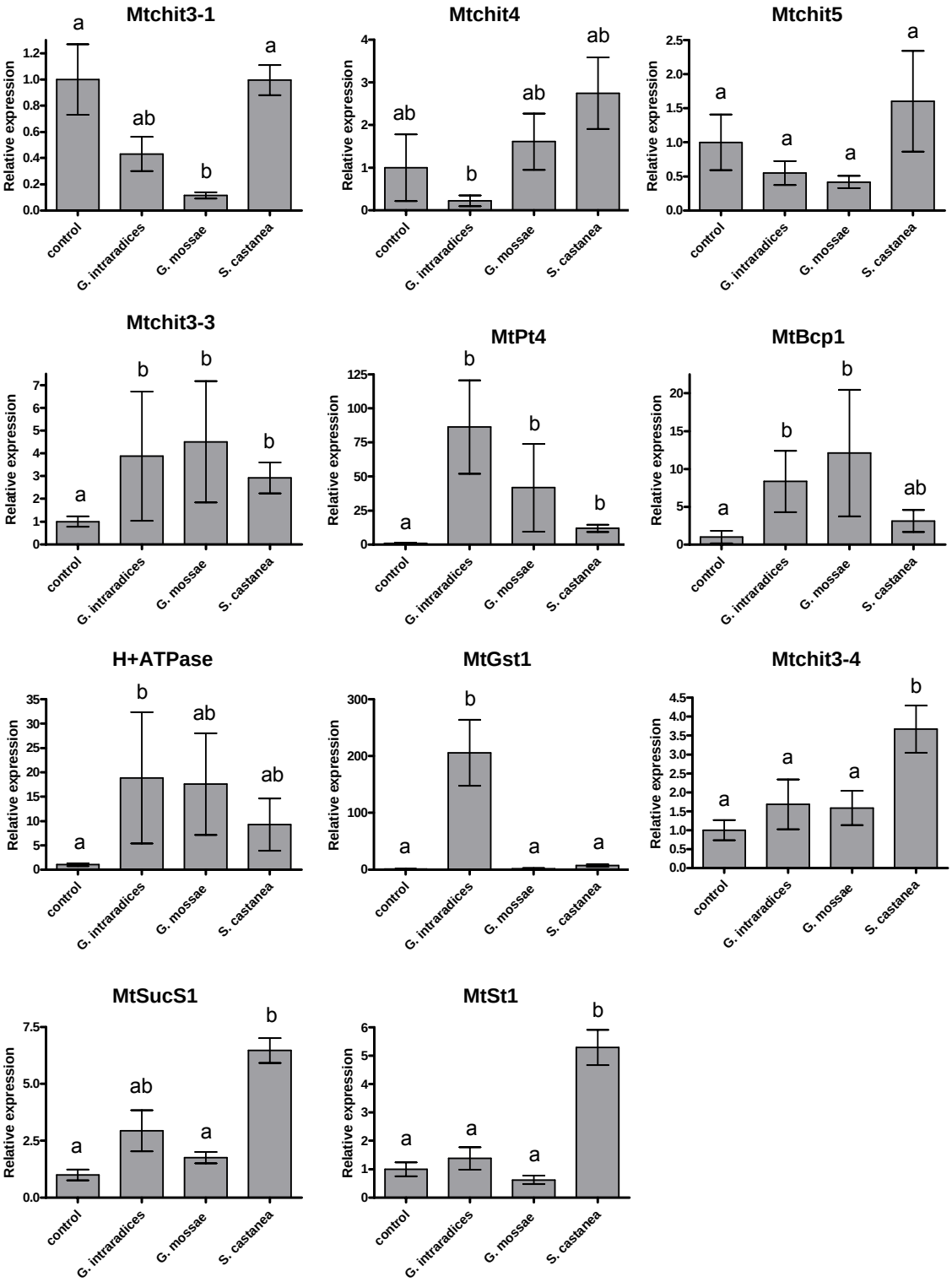
Figure 2 Biomass (a), shoot N content (b) and shoot P content (c) in plants colonized by different mycorrhizal fungi, as compared to non-mycorrhizal control plants. The bars in the figures represent the standard deviations. Letters in the graphs indicate statistically significant differences by the Mann-Whitney U test.

4.4.2. Real-time PCR data

The response to AMF colonization varied among the analyzed genes. MtBcp1 (Figure 3 f), Mtchit3-3 (Figure 3 d) and MtPt4 (Figure 3 e) were significantly induced by all three AMF. H^+-ATPase (Figure 3 g) was also induced by all three AMF but the induction was only significant after *G. intraradices* colonization. Surprisingly, MtGst1 (Figure 3 h) showed strong and significant induction only after *G. intraradices* colonization. The expression of MtPt4, MtBcp1 and MtGst1 correlated to the degree of root colonization (Table 2). Apart from being related to the root colonization, the expression of these genes was also associated with the shoot phosphate content (Table 2). A relatively large group of genes, including MtSucS1 (Figure 3 j), MtSt1, narbonin, pyruvate dehydrogenase E1 alpha subunit and cinnamoyl Co-enzyme A reductase (Table 2), responded to AMF colonization but the induction of these genes were statistically significant in roots of *S. castanea* colonized plants only. The expression levels of these genes did not show any correlation to the degree of AMF colonization. However, the expression levels of some genes correlated to shoot nutrient contents. Mtchit3-4 correlated to the shoot phosphate content (Table 2), cinnamoyl Co-enzyme A reductase, MtSucS1, MtSt1, and narbonin correlated to shoot nitrogen content (Table 2). Transcripts of the early Nodulin gene Mtchit5 (Figure 3 c) were not significantly induced after inoculation with any of the AMF, neither were GH3 (Table 2), Mtchit4 (Figure 3b) or malonyl Co-enzyme A:acyl carrier protein transacylase (Table 2). The transcript levels of the class III chitinase gene Mtchit3-1 (Figure 3 a) and fructokinase (Table 2) were significantly reduced in roots colonized with *G. mosseae* but unaffected in root colonized with either *G. intraradices* or *S. castanea*.

Next page:

Figure 3 Expression patterns of representative genes in roots after AMF colonization. (a) Mtchit3-1 (b) Mtchit4 and (c) Mtchit5 are examples of genes that are non-induced in our experiment, Mtchit4 and Mtchit5 are also our controls for pathogen- and rhizobium-induced gene expression respectively. (d) Mtchit3-3, (e) MtPt4, (f) MtBcp1, (g) H^+-ATPase and (h) MtGst1 are the members of the group of genes that show arbuscule-specific induction. (i) Mtchit3-4, (j) MtSucS1 and (k) MtSt1 are examples of the symbiosis-induced group of genes that are only significantly induced by *S. castanea*. The bars in the graphs represent the standard deviation. Letters in the graphs indicate statistically significant differences by the Mann-Whitney U test.


Mtchit3-1
Mtchit4
Mtchit5
Mtchit3-3
MtPt4
MtBcp1
H+ATPase
MtGst1
Mtchit3-4
MtSucS1
MtSt1
Relative expression
control
G. intraradices
G. mossae
S. castanea

Gene name	Predicted expression patten	Relative expression *G. intraradices*	*G. mossae*	*S.castanea*
Arbuscle specific genes				
MtGst1[b,c, e]	Arbuscle specific	205.7[a]	1.8	7.0
MtPt4[b,c, e]	Arbuscle specific	86.4[a]	41.7[a]	11.9[a]
MtBcp1[b,c, e]	Arbuscle specific	8.3[a]	12.1[a]	3.1 [a]
H+-ATPase	Arbuscle specific	18.9[a]	17.6	9.3
MtChit3-3[c, e]	Arbuscle specific	3.9[a]	4.5[a]	2.9 [a]
Symbiosis induced genes				
Cinnamyl Co-enzyme A[c, d, e]		3.5	1.7	3.1[a]
MtSucS1[d]	Symbiosis induced	2.9	1.8	6.5[a]
MtChit3-4[c, e]	Symbiosis induced	1.7	1.6	3.7[a]
MtSt1[d, e]	Arbuscle specific	1.4	0.6	5.3[a]
Pyruvate dehydrogenase E1 alpha subunit		1.2	0.4	2.4[a]
Narbonin[d, e]	Symbiosis induced	1.1	1.6	7.4[a]
Non-regulated genes				
Malonyl Co-enzymeA		2.2	0.2	4.7
GH3	Auxin responsive	1.0	1.1	4.0
MtChit5	Early nodulin	0.6	0.4	1.6
MtChit3-1	Pathogen induced	0.4	0.1	1.0
MtChit4	Pathogen induced	0.2	1.6	2.7
Fructokinase		0.3	0.1	0.8

[a] significantly induced $p < 0.05$ (Mann-Whitney U test) compared to the non inoculated material
[b] significant correlation $p < 0.05$ to the degree of root colonization (Spearmans rank correlation coefficient)
[c] significant correlation $p < 0.05$ to shoot phosphate content (Spearmans rank correlation coefficient)
[d] significant correlation $p < 0.05$ to shoot nitrogen content (Spearmans rank correlation coefficient)
[e] significant correlation $p < 0.05$ to shoot carbon content (Spearmans rank correlation coefficient)

Table 2 Expression levels of the analyzed genes in *M. truncatula* roots colonized by the AMF normalized to the basal gene expression in nonmycorrhizal roots.

4.4.3. Chitinase expression in aerial tissues

We analyzed the expression of the *M. truncatula* chitinase genes in leaves and flowers. Expression of all five genes could be detected in these organs. There were no significant differences in expression levels among non-inoculated plants and AMF colonized plants. However, Mtchit5, Mtchit3-3 and Mtchit3-4 were relatively highly expressed in leaf tissue compared to in roots (15-, 5- and 5 times higher respectively) while Mtchit4 and Mtchit3-1 were expressed at similar levels through out the plant. In flowers, all three genes showed similar basal expression levels as in leaves; however, there was a difference in their regulation: Mtchit3-3 showed significant down-regulation in plants colonized with all three AMF, while Mtchit3-4, Mtchit3-1, Mtchit4 and Mtchit5 were not regulated after AMF colonization.
To better understand the group of *S. castanea* associated genes we also analyzed the gene expression of these genes in leaves. In some cases we found the transcripts to be quite abundant, this was the case with for instance MtSt1 and Mtchit3-4. MtSucS1 could be detected but the expression was very low compared to in roots, whereas the pyruvate dehydrogenase E1 alpha subunit gene showed similar expression levels as in roots. None of the genes showed any statistically significant regulation in leaves after AMF colonization. For comparison we also tested MtPt4 and MtBcp1 with cDNA from leaves and flowers without obtaining any substantial amplification of the targets.

4.4.4. Promoter sequences of mycorrhiza regulated genes

The two chitinase genes Mtchit3-3 and Mtchit3-4 were relatively highly expressed in aerial tissues, and interestingly, Mtchit3-3, but not Mtchit3-4, showed a difference in gene expression in flowers in respect to mycorrhizal colonization. In order to learn more about these differences the promoters of the two genes were compared to the strictly root and mycorrhiza-dependent MtPt4 and MtGst1. The promoter sequences were submitted to the TSSP program from the softberry sequence analysis tools website. This software recognizes regions in the putative plant promoters that contain a high density of putative *cis* regulatory elements, so called clusters, forming one promoter unit. Our searches revealed that in all four analyzed promoter sequences a putative core promoter was present, containing the transcriptional start site, a TATA box and a CAAT box. In all four sequences a putative proximal promoter region was predicted (*prox*, Figure 4 a, b), the region of 200-300 bp upstream of the transcriptional start site (Shahmuradov et al, 2005). Within the published 1 kb promoter sequence of MtPt4, that is necessary and sufficient to deliver arbuscule specificity (Karandashow, 2003), an additional, more distal cluster was predicted (*dist*, Figure 4 a, b). Similarly, the 1.7 kb promoter region of MtGst1 had two predicted plant promoter regions (*prox* and *dist,* Figure 4 a, b), but the first 0.9 kb was sufficient to confer arbuscule dependency (Wulf et al, 2003). When the clusters of all four promoter sequences were compared, a relatively low sequence similarity was observed, and the most closely related regions were the putative pMtchit3-3, and the pMtchit3-4 promoter units (Figure 4 b). The PLACE signal scan search finds known motifs of putative transcription factor binding sites in a given sequence, and returned a large number of such signal sequences for each investigated promoter (Figure 4 c). Use of the NSITEM-PL program of the softberry program suite, together with FOOTPRINTER 2.0, revealed that six additional sequence motifs are present in all four promoter sequences. All motifs vary in their abundance and combination within the different promoters (Figure 4 d), and no correlation was obvious.

The promoter sequence of Mtchit3-3, pMtchit3-3 with the putative transcription start point at -59 bp, has been identified previously (Elfstrand et al, 2005, chapter 3). The sequenced 2 kb Mtchit3-3 promoter is known to direct arbuscule specific expression. The strict arbuscule specificity was related to a region further upstream of approximately 900 bp of the start codon via promoter-GUS fusions of truncated promoters (Elfstrand, personal communication). pMtchit3-3 contains certain sugar and hormone responsive elements, but also light responsive elements and root specific elements were found (data not shown). However, most of the motifs were not unique to pMtchit3-3 (Figure 4 c, d). A rather high number of dehydration and water stress responsible elements were predicted in pMtchit3-3, other than in the two other arbuscule-specific promoters. One element that was found to be absent in pMtchit3-4, but is present in all three arbuscule-specific promoters (GNATATNC; the P1BS-element), is an element found in promoters of phosphate starvation responsive genes in several plant species. This element in rice and barley phosphate transporter promoters is required for gene induction in responses to phosphate deprivation (Schünman et al, 2004), and the cis element might therefore have a connection to the phosphate status of mycorrhizal cells. An interesting feature in the Mtchit3-3 promoter is a sequence that corresponds to an expressed sequence tag sequence. The deduced 53 amino acids peptide has weak similarities to transcription factors, but in roots is not correlated to the expression rate of Mtchit3-3 (Salzer, Elfstrand, Feddermann, unpublished data). The Mtchit3-4 promoter was obtained after comparison of the gene sequence with a database sequence, but it was not experimentally confirmed to be responsible for Mtchit3-4 regulation. In the promoter region, a relatively long proximal promoter (Figure 4) was predicted. Within this cluster, a TATA box (CTTATAAA) was found 95 bp upstream of the start ATG, and a CAAT box (CCAATGT) at -159 bp. The putative transcription start point is 65 bp upstream of the start codon. pMtchit3-4 shares a small number of putative regulatory motifs with the three other tested promoter sequences, and contains root specific elements, hormone responsive elements, light- and cotyledon responsive motifs and seed specific elements (data not shown).

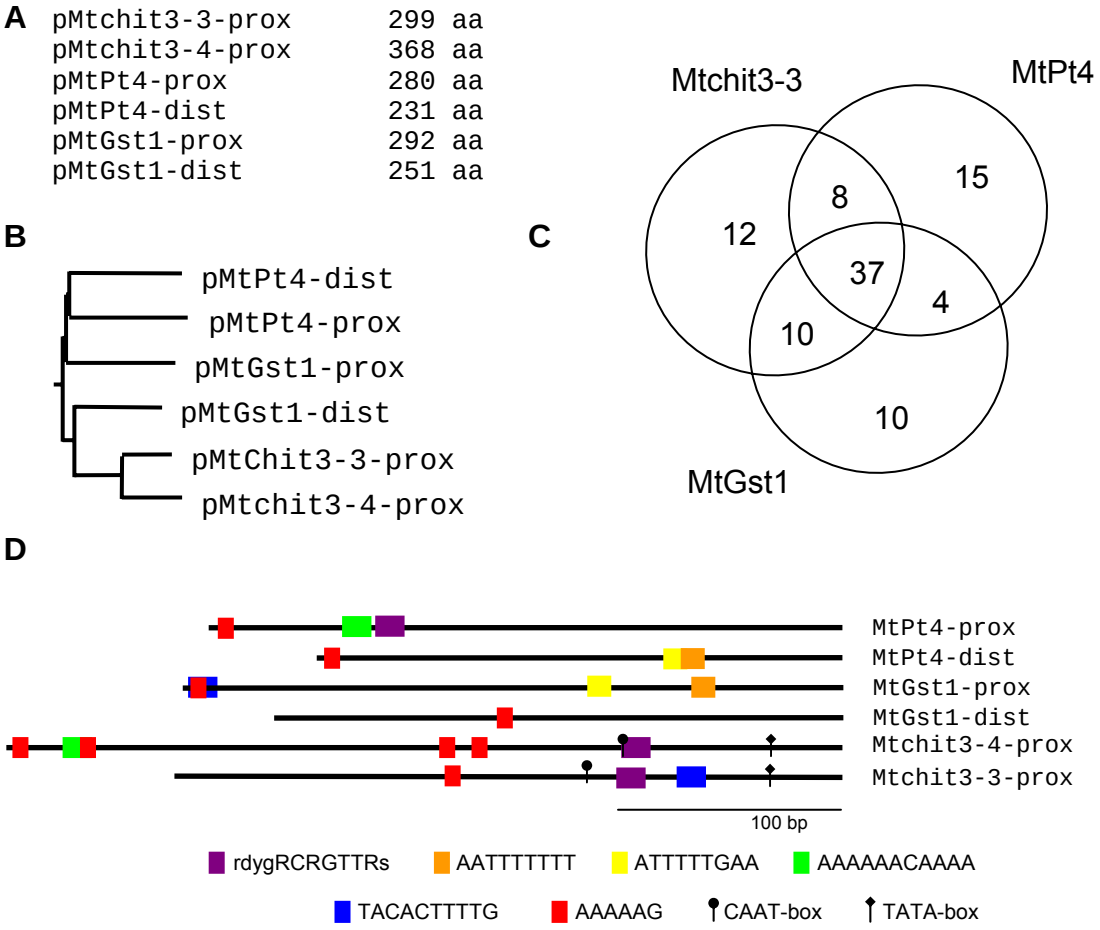


Figure 4 Predicted promoter regions and putative regulatory motifs in mycorrhiza-regulated promoters.
A: Length of the predicted promoter regions, *prox* refers to proximal promoters, and *dist* refers to distal promoter regions. B: Arbitrary distance tree of the single cis-element clusters. The two predicted chitinase proximal promoter sequences are the closest related promoter regions. C: The three tested whole length arbuscule-specific promoter sequences were submitted to PLACE signal scan search, and putative cis regulatory elements were counted. Most motifs are shared between the three promoters and only few elements are shared between only two sequences. The total number of motifs were 96. In contrast, the Mtchit3-4 promoter sequence did not share 9 motifs with any of the three arbuscule specific promoters. 31 motifs of a total of 105 motifs were predicted in all four sequences. D: Distribution of six motifs within the predicted promoter regions found via phylogenetic footprinting, the predicted TATA and CAAT boxes for the chitinase promoters are shown. D: A or G or T, R: A or G, Y: C or T.

4.5. Discussion

In this study, we present an analysis of *M. truncatula* plants that have been inoculated with *G. intraradices*, *G. mosseae* and *S. castanea*. We have analysed the effects that three different fungal species of two different taxa have on the physiological status of the plant and shoot tissues.
The colonization patterns of the three individual fungi that we found in our plants were anticipated from other studies (Burleigh et al, 2002, Smith et al, 2004). We found a small amount of vesicles formed by *G. mosseae*, this is probably due to our decision to harvest the material at ten weeks post inoculation. *G. mosseae* and *G. intraradices* are known to be able to form vesicles (e.g. INVAM database), unlike the fungi of Gigasporaceae.
The shoot dry weight of mycorrhizal *M. truncatula* was on average 4 times that of non-mycorrhizal control plants. This growth response agrees with what has been reported by Burleigh and co-authors (Burleigh et al, 2002). Also the nitrogen and phosphorus content of the shoots increased, together with an increased biomass, on average 2 and 3 times respectively in AMF colonized plants. The shoot phosphate gain is lower than what has been found in comparable experiments (Burleigh et al, 2002). However, it is remarkable that the growth response and phosphate gain were similar in mycorrhizal plants irrespective of the colonizing AMF species, given that the degree of colonization varied so much between the species. We found that the nutrient gain was about equal in *G. intraradices* and *G. mosseae* colonized plants, despite a lower degree of root length colonization and a lower abundance of arbuscules in *G. mosseae* colonized roots. *G. intraradices* developed a higher presence of arbuscules and more hyphae per colonized root length compared to *G. mosseae* (Figure 1, b and d).
AMF with a high degree of root colonization such as *G. intraradices* are not necessarily better at providing their host plants with phosphate than other AMF species (Burleigh, 2001). It has been shown that AMF species, for instance species belonging to the Gigasporaceae, with a relatively low degree of colonization are able to support the plant in a similar efficiency than a high colonization species (Burleigh et al, 2002) and (Smith et al, 2004). Also, in our study *S. castanea* was almost equally as good as *G. intraradices* in providing mineral nutrients to its host plant although it had a much lower degree of root colonization. One reason for this might be that many species of the Gigasporaceae are known to form a large soil mycelium, something that members of the *Glomaceae* do not. It is still unclear whether the number of arbuscules or the absorptive mycelium is a more limiting factor for host nutrient acquisition (Hart and Reader, 2002), but our results clearly show that both should be taken into consideration.

We tested whether some of the genes are regulated in either leaves or flowers in response to root colonization. As control genes, we used MtBcp1 and MtPt4 that are known not to be induced in any other tissues than arbusculate roots (Wulf et al, 2003). The expression of these two genes was at the lower level of detection and we therefore conclude that these two control genes are not expressed in flowers or leaves. None of the tested genes showed a significant change in expression, and are therefore not influenced by the infection status of the plant belowground tissue. This is an interesting factor, as the above ground tissue of the AM plants profited greatly from the colonization with e.g. increased biomass (Figure 2). None of the genes seemed to be influenced in leaf or flower tissue by the enhanced nutrient uptake or the increased biomass production, especially the genes that have metabolic related functions.
The presence of chitinase genes was examined also in flowers and Mtchit3-3 was the only gene of the ones we tested that showed any significant changes, namely, a significantly down regulation in response to all three mycorrhizal treatments. The induction of Mtchit3-3 in roots is known to be arbuscule specific (Bonanomi et al, 2001, Elfstrand et al, 2005, chapter 3) and a possible function of the gene product is discussed in Elfstrand (2005, chapter 3). We also know that the closely related class III chitinase Mtchit3-4 and Mtchit3-1 genes (Elfstrand et al, 2005, chapter 3) are to a certain extent induced upon pathogenic or mycorrhizal infection. In the present study they respond differently to the individual fungi; Mtchit3-3 is induced in a mycorrhiza-specific way,

whereas Mtchit3-4 is upregulated only in *S. castanea* colonized roots and Mtchit3-1 shows no regulation after AMF colonization. Despite a relatively high sequence similarity of the coding regions, the promoter sequences of these genes are, like the differences in gene expression, different from each other.
Expression of Mtchit3-4 was induced, like Mtchit3-3, after infection with mycorrhizal fungi of the Glomeromycota (this study) but also, unlike Mtchit3-3, in response to pathogens and rhizobial infection (Salzer et al, 2000). The regulation of these two genes differs substantially from each other in roots, leaf and flowers, considering their high gene sequence similarity (Elfstrand et al, 2005). Probably, these chitinases have originated from gene duplication, followed by considerable genetic changes in their noncoding promoter regions. Indeed, an analysis of the two putative promoters showed that they are rather distinct (Figure 4). However, no obvious pattern of regulatory motifs could be found in either of the promoter sequences, especially if compared to the promoter sequences of strictly arbuscule specific promoters. In fact, the putative promoters of the two genetically related chitinase genes are still more similar to each other than in relation to pMtGst1 and pMtPt4, that are physiologically more similar to Mtchit3-3 (Figure 4).
Since only few regulatory motifs are common among the arbuscular-regulated promoters, we propose that there are several distinct mechanisms that induce arbuscule-specific expression in roots. This is in agreement with the finding that the MtGst1 promoter is specific to arbuscule cells in *M. truncatula*, but the corresponding promoter in potato also responds to pathogen interactions (Strittmacher et al, 1996). However, at the moment, the number of available AM-induced promoters is too small to draw any firm conclusions from this investigation.

The chitinase gene Mtchit4 which can be regarded as a marker gene for the induction of plant defence responses, and Mtchit5 which is a nodule specific gene (Salzer et al, 2004, chapter 2), were not induced in any AMF colonized roots or any other tissues of AMF colonized plants. Actually, the genes were expressed at very low levels, suggesting that, although it is likely that other micro-organisms were present in the in the system after ten weeks of cultivation, they did not greatly influence the gene expression patterns in our material. Mtchit5 transcription was found to be activated in early nodule formation and therefore classified as an early Nodulin (Salzer et al, 2004, chapter 2). It was proposed to be involved in nodule formation. This relatively high presence in flowers, but not in leaves, had also been found previously (Salzer et al, 2004, chapter 2). It could indicate a function of the Mtchit5 gene product in meristem formation or organization, as is also proposed for other chitinases (e.g. De Jong et al, 1992).

We examined gene expression of a number of other genes in addition to chitinases. Among them were genes that have specified functions at defined steps of mycorrhiza formation are assumed to be influenced during AM formation in a similar way, like for instance the arbuscule dependent genes MtPt4, or MtGst1, that have been shown to correlate to the amount of arbuscules in the roots (Harrison et al, 2002; Isayenkov et al, 2004). We also analysed genes that have been shown to be associated with mycorrhiza but of which the functions are not clearly assigned to intraradical structures, like narbonin, a gene involved in storage functions. Most of the genes that we tested were induced to some degree after ten weeks of symbiotic growth, and we could separate these induced genes into two groups.
The first group of genes consists of known arbuscule-specific genes that show relatively high induction by the two *Glomus* species. MtBcp1 is a mycorrhiza-specific gene (Küster et al, 2004), and Hohnjec et al (2005) report that the gene is induced in *G. intraradices*- and *G. mosseae* colonized roots. We found that MtBcp1 is significantly induced also in *S. castanea* colonized roots but the relative expression level is lower than in *G. intraradices*- and *G. mosseae* colonized roots. MtPt4 (Harrison et al, 2002) and Mtchit3-3 (Bonanomi et al, 2001; Elfstrand et al, 2005, chapter 3) show similar induction patterns as MtBcp1. They are significantly induced in interactions with all AMF but the relative expression level is higher after colonization with *Glomus* species than with *S. castanea*. The fact that we find more arbuscules, as well as intraradical hyphae in roots colonized by the two

Glomus species, supports the idea that the expression of these genes is associated with presence of fungal structures.
A member of H^+-ATPases is induced after colonization with all three AMF but the induction is only significant after *G. intraradices* colonization. Also the induction pattern of MtGst1 (Wulf et al, 2003) is remarkable in our study as it is highly and specifically induced in *G. intraradices* colonized roots. This gene has previously been found to be highly induced in *M. truncatula* roots colonized by *G. intraradices* as well as *G. mosseae* (Hohnjec et al, 2005). The expression of MtGst1 is not directly associated with the frequency of arbuscules in the material as the *G. mosseae* colonized roots actually show a higher relative frequency of arbuscules than *G. intraradices* colonized roots. It is possible that the root colonization needs to reach a certain threshold before this gene will be highly induced.
A second group of genes was induced marginally by the two *Glomus* species, but much more strongly by *S. castanea*. This group is dominated by genes that are known to be symbiosis-induced, which also includes a nodulation related induction. MtSucS1 (Hohnjec et al, 1999; Hohnjec et al, 2003), narbonin (Hohnjec et al, 2005) and Mtchit3-4 (Salzer et al, 2000). But the group also includes MtSt1, a gene that is well known to be induced in cells that harbour arbuscules (Harrison, 1996). MtSt1 was previously known to be induced by *G. intraradices*, *G. versiforme* and *G. mosseae* colonization (Harrison, 1996; Hohnjec et al, 2003). Several genes in this group are involved directly in carbohydrate sensing or metabolism; the sucrose synthase gene MtSucS1 is known to be expressed in sink tissues (Hohnjec et al, 2003), but its expression is also associated to sink tissues such as the root tip and arbuscules (Harrison, 1996). The sugar transporter gene MtSt1 and also the sucrose synthase gene MtSucS1 are more situated at the periphery of the cell sugar turnover. But in this group we find also the pyruvate dehydrogenase alpha subunit gene, which is involved in the principal energy acquisition pathways of the cell. Some of the genes in this group are involved in secondary metabolism. Narbonin is a storage protein (Hohnjec et al, 2005), cinnamoyl Co-enzyme A reductase is necessary for lignin production in plants (Boerjan et al, 2003). It is possible that these genes respond to changes in the root metabolism that is caused by the presence of the AMF. Expression of genes associated with the secondary metabolism is sometimes sensitive to the ageing of the tissue and the fact that we harvested our material a month later than Hohnjec and co-workers (Hohnjec et al, 2005) could account for some of the differences in gene regulation patterns between our studies. For instance the above mentioned gene cinnamoyl Co-enzyme A reductase was down regulated in the study by Hohnjec and co-workers (Hohnjec et al, 2005). The discrepancy to our results could, in part, be explained by the differences in age and consequently in secondary wall formation.
A third group of genes comprised those that were not significantly influenced by any of the three AMF species. Some of these genes show either up regulation, such as GH3, or down regulation, for instance malonyl Co-enzyme A:acyl carrier protein transacylase (Hohnjec et al, 2005). We were unable to find any statistically significant changes in the expression of the fructokinase gene, in contrast to an earlier study. Perhaps, this gene shows age-dependent regulation. The same might be true for the auxin inducible GH3 which was previously found to be up regulated in mycorrhiza (Hohnjec et al, 2005).

The relation between gene expression and physiological parameters in the group of symbiosis induced genes is less clear than in the group of arbuscule specific genes. The expression of the majority of the symbiosis-induced genes are also related to improved mineral nutrition, but within this group the relations to shoot nitrogen content seem to be more pronounced than in the arbuscule specific group. This indicates that genes within this group respond to different stimuli than the arbuscule specific genes do.
The genes belonging to the arbuscule specific group generally showed significant correlations to the degree of mycorrhization and to the shoot phosphorus content but not to the root phosphorus content. It has been shown that the expression of MtPt4 in AMF colonized roots is more influenced by the Pi status of the shoot than by the external or local Pi concentration in the root (Burleigh & Harrison, 1999). It is possible that this may

be the case for the other genes as well. It is also possible that the morphological differences in the AM that *M. truncatula* forms with *G. intraradices* and *S. castanea* explain, at least in part, the difference between the arbuscule-specific genes and the group dominated by symbiosis induced genes.

In conclusion we find that genes that have been shown to respond to colonization by fungi of the Glomeraceae also respond to *S. castanea* from the Gigasporaceae. Surprisingly, *S. castanea* induces a different set of mycorrhiza-associated genes as the two Glomus species *G. intraradices* and *G. mosseae*. Taken together with previously published data our data may suggest that different genes with arbuscule-specific gene expression in colonized roots are regulated by different mechanisms and possibly by different signal transduction pathways.

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Chapter 5

General Discussion

General Discussion

Plants have evolved several sensory capacities to perceive diverse environmental stimuli such as light, gravity, temperature, drought and touch. In addition, they have the ability to recognize microbes by way of chemical and mechanical cues. They are constantly targets of a large number of pathogenic microorganisms that are for example using the plant's metabolic products as nutrient resources. Plants need to defend themselves against these antagonists that are causing plant disease or death. On the other hand, plants can recruit certain bacteria and fungi for improved nutrient acquisition via mutualistic symbiosis. A central theme of this thesis was to investigate how plants are able to successfully defend themselves against antagonistic microbes, and while at the same time they are able to promote colonization by symbiotic microbes, with a particular focus on the regulation of chitinase gene expression.

5.1. Chitinases in plants serve diverse biological functions

During plant-microbe interactions plants produce a multitude of chitinases, and it seems that these chitinases have distinct functions in different interactions. The fact that chitinases have different catalytic activities, such as the hydrolysis of fungal cell wall components, elicitors and other microbial signal molecules, but also bacterial cell walls, make them versatile and necessary tools for the interactions with microbes. The introductory part of this thesis (Table 1) gives an overview over some chitinases and their functions in plant-microbe interactions.
Chitinases are divided into five classes according to their amino acid sequence and protein structure. However, this classification does not necessarily correlate with chitinase functions; enzymes of the same classes can serve different functions and vice versa. For example, structurally similar chitinases of the class III are induced either in response to pathogen attack (Métraux et al, 1989; Lawton et al, 1992), or during mycorrhizal infection (Salzer et al, 2000), or during root nodule formation (Goormachtig et al, 1998). It seems that the temporal and spatial coordination of the activities of several chitinases is characteristic for certain microbial interactions and developmental stages in each plant species (e.g. Zhong et al, 2002). Therefore, a complex regulation of the chitinase gene expression is crucial for a functional plant system.
As far as is known, plant chitinases are not subject to major natural posttranscriptional gene regulation. It may therefore be reasonable to assume that induction or repression of a given chitinase gene leads to a similar induction or repression of the corresponding enzyme activity. Therefore studies of chitinase gene expression may give indications of their enzymatic functions, as has been also assumed by other authors (Wu et al, 1994; Salzer et al, 2000; Kim & An, 2002).

5.2. Plant chitinases exhibit multiple substrate specificities

Antimicrobial activity
The most important antagonistic interactions of plants with microorganisms are arguably the interactions with necrotrophic or biotrophic pathogenic fungi. These fungi contain chitin in their cell walls, which is the apparent target of some plant chitinases. Chitinases, especially the ones that are induced after pathogen infection as part of the plant defence, possess antifungal activity and inhibit fungal growth by weakening or degrading the fungal cell walls (Mauch et al, 1988; Arlorio et al, 1992; Sela-Buurlage et al, 1993).
Being antifungal, chitinases are a priori suited as agricultural pest control agents. Indeed, transgenic plants expressing chitinases constitutively showed a higher resistance towards

pathogens (Broglie et al, 1991; Zhu et al, 1994) and it seemed that chitinases have a potential in genetic engineering (Tesfaye et al, 2005; Vellicce et al, 2006). In most instances, resistance was tested under laboratory or greenhouse conditions, but two reports on field trials have also been published (Grison et al, 1996; Pasonen et al, 2004). Several reports point out that applications of plant or bacterial chitinases in pathogenic fungal and insect control in crops are especially successful when chitinases are combined with other agents, for example beta-1,3-glucanases or bacterial toxins (e.g. Jach et al, 1995).

The complex structure of the fungal cell walls might be a reason why the antifungal activity of chitinases is enhanced by glucanase activity (Mauch et al, 1988). Fungal cell walls are mainly composed of chitin and linear and branched glucan chains that are associated with proteins and other compounds. There is a high variation of the relative amounts of these components between different groups of fungi. During growth of filamentous fungi, the cell wall components are first produced at the growing tip and then crosslinked, and it was suggested that the growing tip is particularly susceptible to chitinase activity. Indeed, the tips of some fungi were shown to swell and burst after chitinase treatment (Mauch et al, 1988; Arlorio et al, 1992). Some chitinases are also able to hydrolyse bacterial cell walls due to an additional lysozyme activity, which can in some cases be dominating over the chitin cleavage activity (Stintzi et al, 1993; Brunner et al, 1998). Bacterial cells walls consist of peptidoglycan, a network of peptides and long-chain glycans with a backbone of alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid moieties. The ability of chitinases to lyse bacterial test solutions is often used for experimental characterization of purified enzymes (e.g. Brunner et al, 1998; Suarez et al, 2001). It remains an open question to what extent this antibacterial activity is also relevant in resistance to bacterial disease resistance.

Interestingly, in an experiment with transgenic tobacco expressing various chitinases with antifungal activity against pathogenic fungi, the same chitinases did not inhibit the proliferation of mutualistic symbiotic mycorrhizal fungi (Vierheilig et al, 1995). It was even proposed that a certain hydrolysing activity towards AMF cell walls may enhance the symbiotic interaction; during arbuscule formation, a chitinase may help to act on the fungal cells walls in order to allow ramification of the hyphae. Plant chitinases could also be involved in the degradation of the arbuscules after their life span is over (Slezack et al, 2001).

Obviously, different fungi differ in their sensitivity to chitin. One reason for this could be that the accessibility of the chitin in the fungal cell walls to the chitinase differs between the fungal species during an infection (Spanu et al, 1989; Vierheilig et al, 1995). Furthermore, the chitin of different fungi has different degrees of deacetylation. Different chitinases differ in their activity on de-acetylated chitin (e.g. Brunner et al, 1998). Most probably small differences in the enzyme's active site, that have been acquired during the coevolution of plant chitinases and their fungal substrates, are responsible for these specificities among chitinases.

Chitin fragments

While hydrolyzing fungal cell walls during an infection, chitinases produce soluble chitin fragments of varying chain lengths. These chitin fragments are subsequently recognized by the plant as elicitors by way of specific receptors. Recognition of elicitors triggers defence reactions in the plant via signaling pathways that are initiated through the action of the receptor kinases, including the induction of defence related chitinases and other defence mechanisms (Hamel et al, 1997; Kasprzewska, 2003). Elicitors are also produced from symbiotic fungi (Salzer et al, 1997), which are supposedly generated during an initial defence reaction towards the fungus (Gianinazzi-Pearson, 1996), and are part of the infection process.

In many plants, only chitin fragments of a certain minimal chain length are recognized as elicitors. The monomer, *N*-acetyl glucosamine, and the dimer, chitobiose, are usually inactive in triggering a defence reaction. Depending on their preferences for chitin oligomers,

chitinases can therefore either produce or cleave elicitor-active fragments. Most chitinases are also able to cleave longer-chain chitin oligomers as well as chitosan oligomers. However, they differ in the relative activity on these substrates (e.g. Brunner et al, 1998). The model was proposed that certain plant chitinases produce the elicitor active chitin oligomers from the attacking fungi and complementary acting chitinases degrade them into smaller inactive fragments (Brunner et al, 1998). It could be that the elicitor activity is regulated by the presence of several enzymes and their specific activities at the infection site. Thereby the intensity of plant reactions is temporally and locally regulated at the infection site by the turnover of active elicitors.
The chitinase regulation is especially important in the interaction of plants with their symbiotic partners. In these interactions, at an initial phase, defence reactions are sometimes observed that are similar to the reactions induced during pathogen infection, including a hypersensitive response. These defence reactions are thought to be induced by fungal elicitors, possibly produced by chitinases during the early stages of the symbiosis (Gianinazzi-Pearson, 1996). Chitinases that are found at this stage of interaction resemble the chitinases that are induced in pathogen interactions, including similar substrate specificities (Slezack et al, 2001). However, elicitors of symbiotic fungi may then be degraded and inactivated by the action of induced chitinases in order to achieve a functional symbiosis. A successful infection of the plant by the compatible mutualistic fungi is obviously only achieved in the absence of a resistance response (e.g. Volpin et al, 1994; Xie et al, 1999). Chitinase regulation during AM formation has been studied in leek, *M. sativa* and bean (Spanu et al, 1989; Lambais & Mehdy, 1998).

Nod factor cleavage
A scientifically and economically important plant-microbe interaction is the mutualistic symbiosis of legumes with rhizobia. The formation of root nodules is initiated by recognition of the appropriate bacterial signals, the Nod factors. Nod factors essentially are chitin fragments of chain length of four or five units, decorated with a fatty acid on the non-reducing end and various side groups at the reducing and non-reducing end, and thus, can be potentially cleaved by chitinases. Chitinases of all classes have been tested for their activity to degrade natural and artificial Nod factors of diverse length and decorations. Cleavage of Nod factors and their derivatives indeed occurred with these chitinases, and the activity and cleavage position often depended on the number of *N*-acetyl glucosamine monomers and the decorations (Schultze et al, 1998; Staehelin et al, 2000; Ovtsyna et al, 2000). Interestingly, chitinases from non legume plants were also able to degrade Nod factors, showing a basic ability of plant chitinases to act towards these bacterial signal molecules (Schultze et al, 1998; Ovtsyna et al, 2000); different chitinases were found to have different substrate cleavage preferences (Schultze et al, 1998), similar to what was observed for chitinases that were tested for chitin or chitosan oligomer cleavage (Brunner et al, 1998). It was speculated that variability in their natural substrate could be a reason for this, i.e. modifications in fungal or bacterial derived substrates (Schultze et al, 1998).
The fact that chitinases have differential activities with respect to elicitor formation and inactivation or Nod factor cleavage may be important for their evolution. Indeed, analysis of different related chitinases indicate that evolutionary change occurs predominantly at amino acid positions that have direct contact to the substrate (Bishop et al, 2000). This indicates a scenario of co-evolution of chitinases and their microbial substrates.

Plant endogenous substrates
Chitinases have not only been found at the initiation of nodulation, but also in fully developed nodules. It is likely that they are not involved in Nod factor cleavage at this stage of the symbiosis (e.g. Staehelin et al, 1992; Xie et al, 1999; Goormachtig et al, 2001). In addition,

chitinases have also been found in several plant tissues that were not subject of microbial attack, or abiotic stress. They were found for instance in flowers, seeds and embryos and ripening fruits (e.g. Robinson et al, 1997).
It was assumed that chitinases, expressed in a tissue-specific manner and not induced by pathogens or stress, could have roles in normal plant growth and development. These chitinases have possibly plant endogenous molecules as substrates, which may act as signalling molecules in processes like organogenesis. Indeed, glycolipids, endoglycans or *N*-acetyl glucosamines that are found in secondary cell walls, were speculated to be endogenous substrates for chitinases (Benhamou & Asselin, 1989). Furthermore, endogenous molecules could serve as substrates for chitinases involved in embryo development in spruce (Kragh et al, 1996; Wiweger et al, 2003), wheat (Benhamou & Asselin, 1989), carrot (De Jong et al, 1992; van Hengel et al, 1998) and *Arabidopsis* (Zhong et al, 2002).

5.3. Possible functions of *M. truncatula* chitinases

5.3.1. Mtchit1a and Mtchit1c

General properties of class I chitinases
Chitinases of the class I are typically vacuolar or secreted chitinases, thought to be major components of the plant defence against invading fungi. As typical PR proteins they are strongly induced and highly active after pathogen infection in several plant species such as tobacco, and have been thoroughly investigated (e.g. Shinshi et al, 1990; Collinge et al, 1993). Typically, class I chitinases are also induced after treatments with elicitors or the stress hormone ethylene (Kasprzewska, 2003). However, in one instance, a class I chitinase was also induced in mycorrhizal roots (Slezack et al, 2001).
Following a pathogen attack, class I chitinases and class II chitinases are often induced in parallel. In several cases it was found that the class I chitinases exhibited a stronger direct antifungal activity against the fungal cell walls than the closely related members of the class II chitinases that are also induced after infection (Sela-Buurlage et al, 1993; Brunner et al, 1998). It was proposed that the different chitinase classes might act synergistically in defence reactions (Wu et al, 1994; Brunner et al, 1998).
Interestingly, in the fully sequenced *Arabidopsis thaliana* genome only one chitinase of the class I was found along with several class II and class IV chitinases. These seem to have certain roles in plant defence but only a fraction of the genes were found to be transcribed (Passarinho & de Vries, 2002). The class I chitinase has been identified as an ethylene inducible, and likely vacuolar enzyme, that is regulated in a tissue specific and age dependent manner; it does not seem to have a predominant role in a general stress response (Samac, 1990). A*rabidopsis thaliana* possesses in addition to the GH family 19 chitinases several expressed class V chitinases that are involved in defence related functions, and possibly complement the defence apparatus (Passarinho & de Vries, 2002).
There are class I chitinases that are most abundant in seeds and fruits. Some of these were identified as a major allergen in uncooked food, e.g. in bananas, avocado and chestnut. Allergenicity to these products results from sensitization to the chitin-binding domain of chitinases and other proteins containing the hevein domain. Nevertheless, next to their functions in plant defence, in some plants the class I chitinases have important functions in organs that are not under pathogen attack. For example, the pitchers of the pitcher plant, *Nepenthes*, contain class I chitinases that most probably are involved in the degradation of the trapped insects (Eilenberg et al, 2006).

Expression patterns of Mtchit1a and Mtchit1c
The expression levels of two *M. truncatula* class I chitinase genes, *Mtchit1a* and *Mtchit1c*, have been measured after fungal infection. Both chitinases were induced upon infection with the pathogens *Fusarium* or *Phytophthora*, both in plant roots (Salzer et al, 2000) and in root organ cultures (Salzer & Feddermann, unpublished). After infection with a mycorrhizal fungus both genes showed slightly, but not significantly, elevated expression levels (appendix 1). Concluding from these results, it seems that Mtchit1a and Mtchit1c are actively transcribed in response to pathogen infection and that both chitinases belong to the PR protein group of chitinases.
However, *Mtchit1a* and *Mtchit1c* differed in their basic expression levels throughout the plant tissues. Whereas *Mtchit1c* showed similar expression levels in all parts of the plant, *Mtchit1a* expression was found to be higher in leaves than in roots and significantly lower in flowers (appendix 1). Their basic expression levels in roots were similarly low but approximately six times higher compared to the defence related chitinase Mtchit4 (chapter 2).

Possible functions of Mtchit1a and Mtchit1c
A relatively low expression level in healthy tissues, and a strong local induction following pathogen attack but not after symbiotic microbial infection is not unusual for PR proteins (e.g. Shinshi et al, 1990). In the case of both Mtchit1a and Mtchit1c, this typical pattern was observed, and both class I chitinases seem to belong to the PR proteins (Salzer et al, 2000; appendix 1). They are most probably regulated independently from each other and furthermore, they might be located in different cellular compartments and differ in their substrate specificity (appendix 1). The differentially regulated transcription rates, together with the characteristics of their gene and protein structures, suggest that these chitinases act synergistically upon a pathogen attack in *M. truncatula* roots (appendix 1).
A differential chitinase expression pattern was observed for instance in maize, where several chitinases of the class I are present, but each is differentially induced by stimuli like infection, ethylene and wounding (Wu et al, 1994). Similar to the findings in *M. truncatula*, differences in the hinge region and the C-terminal extension were found, but only one of the two described chitinases was strictly wound- and infection inducible (Wu et al, 1994). Nevertheless, in this report, a similar concept is proposed as discussed in appendix 1; the differential regulation and possible enzyme targeting matches the different requirements of the plant during a fungal attack, which is also in accordance to other reports (e.g. Brunner et al, 1998).
It has to be noted however, that in the genome of *M. truncatula* several other chitinases of the GH family 19 are present, as represented by Mtchit1b in appendix 1, the expression of which has not been analyzed in this thesis. Therefore, our expression measurements of *Mtchit1a* and *Mtchit1c* only provides an incomplete picture of chitinase I regulation. Once the genome sequence of *M. truncatula* will be available, it will be interesting analyze its complete set of chitinases and their expression.

5.3.2. Mtchit4

General properties of class IV chitinases
As in the case of chitinases of the class I, several class IV chitinases have been found to be involved in plant defence. As class IV chitinases are GH family 19 chitinases, they share a common hydrolytic domain with the class I and II chitinases, and therefore probably have a similar reaction mechanism. Hence, some class IV chitinases have been found to be associated with similar functions. A class IV chitinase for example has been identified to be part of a preformed plant defence in grape berries (Robinson et al, 1997), while inducible chitinases of the classes I and III were found in leaves (Busam et al, 1997). Pathogen inducible chitinases of the class IV have been found in bean, rape or sugar beet (Margis-

Pinheiro et al, 1992; Rasmussen et al, 1992; Nielsen et al, 1994). On the other hand, chitinases of the class IV have also been found to target plant endogenous substrates in carrot (de Jong et al, 1992) and take part in embryogenesis in spruce (Wiweger et al, 2003).
The common ancestor of the phylogenetically related class I and class II chitinases was a basic chitinase of the class II type (Shinshi et al, 1990). The class IV chitinases have subsequently evolved from class I chitinases, and are therefore the most recent class (Hamel et al, 1997; Bishop et al, 2000). This evolution of several, rather similar chitinases, may mirror a certain co-evolutionary adaptation of plant chitinases to the substrates targeted in their microbial partners.

Expression pattern of Mtchit4
Mtchit4 expression in *M. truncatula* was found to be at a relatively low level in flowers, leaves and roots (chapter 4) of healthy tissues, while it was induced upon fungal infection in roots (Salzer et al, 2000; chapter 2). It was found that Mtchit4 transcripts accumulated in roots challenged with the pathogen *Fusarium solani* but not with mycorrhizal fungi, where its expression levels were unaffected or even slightly reduced (chapter 2 and 4, appendix 1). This expression pattern is consistent with the pattern that is found for other PR proteins, and indicates a defence related function for Mtchit4.
Differences in the induction of *Mtchit4* were observed when rhizobium strains were compared; after infection with a K-antigen deficient strain an enhanced Mtchit4 transcription was found, while in the wild-type inoculated roots the expression level remained at its low basic level. Mtchit4 expression levels were therefore not influenced by the rhizobial infection and the subsequent nodule formation, but by the presence of the bacterial surface polysaccharides (chapter 2). Similarly, its expression was not affected by purified Nod factors. These findings indicate that Mtchit4 induction could be influenced by a certain plant-rhizobium compatibility (chapter 2).

Possible functions of Mtchit4
Mtchit4 seems to be involved in a general defence reaction. This is supported by the specific increased expression in response to pathogenic fungi and incompatible rhizobia (Salzer et al, 2000; chapter 2) and the same induction pattern after infection within both investigated plant species (chapter 2). However, the possibly suppressed expression of Mtchit4 in symbiotic interactions with mycorrhizal fungi or compatible rhizobia as well as the addition of purified Nod factors could indicate that plants indeed discriminate very distinctly between the different microbes. A defence reaction towards these microbes is restricted to certain incompatible interactions, and might be reduced in response to compatible interactions. A reduction of defence related genes was observed for general defence related genes as well as for chitinases and beta-1,3-glucanases (Gianinazzi-Pearson, 1996). A similar function has been assigned to the pathogen specific extracellular class IV chitinase in bean (Margis-Pinheiro et al, 1991).
The phylogenetic analysis of class IV chitinases suggested a close relation of Mtchit4 chitinase to other pathogen inducible chitinases, not only of the legumes, but also of other plant species, as has been previously found for this class of chitinases (Hamel et al, 1997). In *Arabidopsis thaliana* only five out of eight chitinase class IV genes have been found to be actively expressed and have an assigned function in the plant. Interestingly, the genes that are not expressed code for chitinases that do also not seem to be functional enzymes due to substitutions in their amino acid sequences (Passarinho & de Vries, 2002). In analogy to this situation, and to the fact, that active class IV chitinases have been identified in pathogen infected tissues in several plant species, it can be assumed that Mtchit4 is an active chitinases that is involved in defence reactions toward pathogenic or incompatible microbes in *M. truncatula*.

5.3.3. Mtchit5

General properties of class V chitinases
Class V chitinases are rather distinct from the other chitinases, and are the least known class of chitinases, however, these enzymes of the GH family 18 are most interesting. The *M. truncatula* Mtchit5 belongs to this class of enzymes and from the presence of a conserved glutamic acid in its active site, it can be assumed that it is an active enzyme (Watanabe et al, 1993). Members of the GH family 18 group comprise not only chitinase subclasses but also xylanase inhibitors, narbonins and hevamines or a receptor kinase, forming a structurally and functionally unrelated group of proteins (Durand et al, 2005). An example for a chitinase of this group that may function in recognition events is the tobacco CHRK1, which is a protein kinase predicted to possess an extracellular chitinase-like domain. This domain is speculated to be involved in chitin-oligomer binding or recognition events at the cell surface rather than in chitin cleavage as it shows no chitin cleavage activity (Kim et al, 2000).

Expression pattern of Mtchit5
Mtchit5 gene transcripts were initially only detectable after polymerase chain reaction with a high number of amplification cycles (chapter 2). Therefore this gene is generally expressed at a low level. Transcription of the *Mtchit5* gene after infection with rhizobia correlated particularly with the development of nodule primordia; the transcripts constantly accumulated during nodule ripening (chapter 2) but also, to a lower extent, in ineffective nodule primordia. Therefore *Mtchit5* might be associated with the development of these newly formed root organs. On the other hand, *Mtchit5* transcripts were also found in high amounts in flowers of symbiotic and non symbiotic *M. truncatula* plants (chapter 4). These findings suggest that it is involved in organ developmental processes, as has been reported for other chitinases (De Jong et al, 1992; van Hengel et al, 1998).

Possible functions of Mtchit5
The Mtchit5 expression pattern in nodulated roots suggest that it is an ENOD, involved in plant endogenous processes during root nodule growth (chapter 2). Its relatively high presence in aerial tissues indicates that it also could have a function in other tissues than in root nodules.
The Mtchit5 expression pattern has several similarities to expression patterns of other nodulin genes that are involved in plant endogenous functions. Several of these nodulin genes that are found in root nodules were found to be expressed in non-symbiotic tissues. Based on these findings, it was speculated that some of them have been recruited during evolution from these tissues to serve functions in the symbiotic organs (Szczyglowski & Amyot, 2003). For example in flowers, chitinases are supposed to have functions in sexual reproduction, in pollen recognition or germination. In tobacco flowers, a chitinase was found in ovaries (Lotan et al, 1989) and chitinase activity was found in petunia flowers (Leung, 1992). The activity was located mainly in the petals and was especially high in the stigma but absent from the style. The style however is the region of pollen tube growth, while the stigma is involved in pollen recognition (Leung, 1992) and the chitinase activity could be involved in this process. The relatively high presence of Mtchit5 in flowers could indicate that in *M. truncatula* this obviously developmentally regulated chitinase is involved in similar functions. It might be interesting to find out, in the future, whether Mtchit5 expression is associated with specific flower organs.
Elongation of the infection thread initiated by rhizobial infection in the root hairs resembles the polar tip growth of the pollen tube in flowers towards the ovules (Szczyglowski & Amyot, 2003). Similar to the perception of Nod factors in root hairs, the floral pollen tube is only initiated after compatible interactions that require transmembrane receptor kinases (e.g. Li & Gray, 1997). The directional tube tip growth is accompanied by cytoplasmic guidance from the surrounding tissues (Brewin, 1991), involvement of calcium spiking and small G-proteins

that are found in flowers as well as in nodules (Borg et al, 1997). MADS box genes, that play a crucial role in flower development, are also found to be implicated in nodule formation (Zucchero et al, 2001).
There are striking parallels in gene expression between flowers and root nodules, and it is therefore reasonable to expect similar functions of the associated genes in these organs; some of the *ENOD* genes expressed during formation of nodules are also strongly expressed in flowers (Szczyglowski & Amyot, 2003). The *Mtchit5* expression pattern shows similarities to these ENOD expression patterns. Additionally, as has been shown for other chitinases, plant endogenous substrates are likely to be target of the Mtchit5 enzyme. However, any enzymatic substrate preferences could not be determined. Its appearance at the nodule primordium and accumulation during nodule growth suggest an important role in the nodule development. It is therefore most probably involved in plant processes that belong to development or organogenesis.

5.3.4. Mtchit3-3, Mtchit3-4 and Mtchit3-1

General properties of class III chitinases
M. truncatula produces several, highly similar chitinases of the class III (chapter 3). Class III chitinases belong to the GH family 18, and have a different protein structure as the above mentioned chitinases of the GH family 19. Despite a different hydrolytic mechanism some class III chitinases have also been found to be defence-related and inducible by similar stimuli, such as virus infection or elicitor treatment in cucumber (Métraux et al, 1989), tobacco (Lawton et al, 1992), or chickpea (Vogelsang & Barz, 1993). They are an important part of the defence machinery, as for example in grape leaves, a chitinase class III is associated with systemic acquired resistance and has been found together with a class I chitinase in response to pathogenic fungal infection (Busam et al, 1997). A constitutively expressed class III chitinase was found in the vascular bundles, hydathodes and guard cells in *Arabidopsis thaliana*, which was ethylene inducible and might be involved in plant aging (Samac et al, 1990). These examples show that class III chitinases have versatile functions similar to chitinases of other classes.
Chitinases of class III may also be important in the mycorrhizal symbiosis, as shown in this thesis. It may be that the chitinase activity induced in later stages of the mycorrhiza formation corresponds to a class III chitinase (e.g. Xie et al, 1999). Evidence for involvement in symbiotic functions of class III chitinases was given in *S. rostrata* stem root nodules. One of the two identified chitinases exhibited cleavage activity against lipochitooligomers in vitro, while a second chitinase did not act hydrolytic because of an amino acid replacement in the active site, but probably had binding capacity. It was suggested that these class III chitinases are involved in certain stages of the symbiosis, and may even play a crucial role in signalling (Goormachtig et al, 1998, 2001).

Expression pattern of Mtchit3-3
The class III chitinase Mtchit3-3 in *M. truncatula* roots is de novo expressed in a mycorrhiza specific manner (Salzer et al, 2000; chapter 3 and chapter 4). Expression data together with results of mRNA in situ hybridization studies indicated that it was induced in arbuscular cells and had a function in arbuscules (Bonanomi et al, 2001). Indeed, promoter fusion experiments revealed that the Mtchit3-3 promoter directed GUS reporter activity to arbuscule containing root cortex cells (chapter 3), and within this promoter sequence certain motifs were identified that could be involved in the specific regulation of the Mtchit3-3 gene in mycorrhizal roots (chapter 4). Ectopic expression of *Mtchit3-3* in *Agrobacterium rhizogenes* transformed composite plants resulted in an enhanced chitinase activity and strongly supported the idea that Mtchit3-3 is an active enzyme (chapter 3).

Possible functions of Mtchit3-3

The localization of Mtchit3-3 in arbuscular cells indicates an active role in root colonization. This chitinase may act directly on the fungus by degrading chitin in the cell wall of the arbuscule or indirectly by releasing chitin fragments. To test whether a constitutive chitinase expression influences the arbuscular mycorrhizal symbiosis, transgenic root organ cultures were produced that express Mtchit3-3 under a constitutive promoter. Using *Agrobacterium* transformed *M. truncatula* root cultures and an in vitro culture system of *G. intraradices*, the advantages of a model legume were combined with the convenience of an axenic fungal inoculum to study the effects of the constitutively expressed arbuscule specific gene in the AM. This work provided evidence for chitinase activity of the *Mtchit3-3* gene product. Moreover, expression of this chitinase had an effect on the mycorrhiza, compared to control cultures; the time frame of mycorrhiza formation was altered in the root cultures that exhibited a disrupted Mtchit3-3 pattern (chapter 3).

Ectopic expression of Mtchit3-3 influenced the spore germination and growth rate of the hyphae, but not apparently their physiology. Although this chitinase is expected to be secreted, it does not exhibit substantial antifungal activity against growing AMF hyphae or pathogenic fungi (chapter 3; Salzer & Feddermann, unpublished). Unfortunately, heterologous expression of the gene and purification of the corresponding Mtchit3-3 protein could not be achieved, and it is not possible to predict its enzymatic activity.

Arbuscule formation in the root cortex was not influenced by constitutive expression of Mtchit3-3, although the main activity of the naturally expressed Mtchit3-3 was expected in the periarbuscular cells (Bonanomi et al, 2001; chapter 3). Furthermore, these transgenic roots exhibited a colonization pattern by *G. constrictum*, *G. intraradices* (chapter 3) and *G. castanea* (Feddermann, unpublished) that is similar to the wild type-like colonization pattern. Since the abundance of arbuscules within colonized roots was not altered, it became apparent that Mtchit3-3 was not involved in the regulation of arbuscule formation (chapter 3). In addition, Mtchit3-3 expression seemingly does not affect the transcription rate of the set of mycorrhiza-induced genes presented in chapter 4; in the presence of constitutively expressed Mtchit3-3, no significant difference in gene expression was observed from any of the genes in comparison to control roots (Feddermann & Elfstrand, unpublished). However, it can be assumed that the Mtchit3-3 is active in the arbuscule containing zone. When the reporter gene GFP was fused to the Mtchit3-3 reading frame and this construct was introduced in root cultures by in *Agrobacterium* mediated transformation, there was no clear fluorescence signal. Therefore, it remains open whether this chitinase comes into contact with the cell wall of the fungal arbuscule (appendix 2).

It could be speculated, that Mtchit3-3 is involved in the formation of the arbuscular mycorrhiza in arbuscule containing cells and that the physiological properties of these cells are a trigger for the expression of the gene. For instance, arbuscule containing cells are tissues with high local sink strength, which could be involved in a local induction of Mtchit3-3. However, it is unlikely that alone the sink strength is responsible for the induced expression of Mtchit3-3 for several reasons. An enhanced Mtchit3-3 expression was not observed in other tissues than colonized root areas, considering that e.g. the growth zone of root tips is also characterized by a high sink activity. Also flowers are in general strong sink tissues, and a local increased Mtchit3-3 transcription should be expected from a strictly sink dependent gene regulation. But in fact, down regulation of the Mtchit3-3 expression has been found in flowers of the well nourished symbiotic plants (chapter 4). Therefore, other factors as alone the sink strength of arbuscule containing cells seem to be required for the induction of the *Mtchit3-3* gene. Such a factor could be a certain concentration of hexoses and thereby Mtchit3-3 may be connected to the activity of arbuscule-specific invertases. However, further investigation will be needed to test this hypothesis.

Expression pattern of Mtchit3-1 and Mtchit3-4

Several class III chitinases have previously been identified as fungal inducible genes with different expression patterns in roots of *M. truncatula* (Salzer et al, 2000). In contrast to the mycorrhiza specific Mtchit3-3, transcripts of Mtchit3-4 were not only found in mycorrhizal roots, but also after rhizobial infection and upon compatible pathogenic fungal interactions (Salzer et al, 2000). However, its expression pattern differed clearly from the one of other chitinases (chapter 4), as it was found to be slightly elevated in mycorrhizal roots, but only significantly induced after infection with a fungus from the Gigasporaceae, that exhibited a low degree of colonization. Its transcription level was, similar to Mtchit3-3 levels, elevated in leaves compared to in roots, but not regulated by the mycorrhizal status of the plant (chapter 4). A third interesting class III chitinase, Mtchit3-1, showed increased expression in roots in response to pathogenic fungi (Salzer et al, 2000). Rather low Mtchit3-1 expression was measured in all plant parts, irrespective of the symbiotic status of the plants (chapter 4), while a reduced expression was found in mycorrhizal roots infected with fungi of the Glomaceae.

Together with the composition of the Mtchit3-4 and Mtchit3-1 promoter regulatory units (chapter 4), the responses to fungal infection of both chitinases resemble the responses of defence-related class III chitinases. Mtchit3-1 indeed had an expression pattern in roots and aerial tissues that was in general comparable to the one of the class I chitinases (Salzer et al, 2000; chapter 2 and 4). In analogy to the regulation of the GH family 19 chitinases, these differences in expression patterns indicate a differential regulation of the class III chitinases in response to different fungi.

Possible functions of Mtchit3-4

Mtchit3-4 has a very high protein homology to the mycorrhiza specific Mtchit3-3, 95% at the amino acid level, which could suggest a similar enzymatic function, despite obviously different roles in plant-microbe interactions (chapter 3 and 4). It is therefore hypothesized that the regulation of these two closely related genes is more important than the enzymatic activity for their prospective role in *M. truncatula* roots. Mtchit3-4 seems to be inducible by signals associated with several classes of fungi, in contrast to all other here investigated chitinases. In homology with Mtchit3-3 and other class III plant chitinases (Métraux et al, 1989; Samac et al, 1990; Lawton et al, 1992), an apoplastic protein targeting is most probable for this chitinase, but it could not be experimentally verified. It can therefore be assumed, that Mtchit3-4 might have direct contact to an infecting fungus within the root and most probably also in leaves. Interestingly, it is also found in rhizobium infected roots as well as in certain interactions with mycorrhizal fungi, and it might also be involved in symbiotic plant-bacterial interactions. Therefore Mtchit3-4 might be induced as a general plant response to microbial infection.

In bean roots infected by *G. intraradices,* it was speculated that enhanced chitinase activity was involved in fungal growth control under high phosphate conditions, which generally are restrictive to AM formation (Lambais & Mehdy, 1998). Similarly, a chitinase has been found in aborted rhizobial infection threads probably involved in the autoregulation of nodulation (Vasse et al, 1993). In parallel to these chitinases, Mtchit3-4 could be implicated in such processes during microbial colonization. Additionally, considering the differences in gene induction after colonization with different fungal species (chapter 4), where it was highly induced in a low colonizing interaction, Mtchit3-4 might be an indicator for the compatibility of a certain plant-microbe combination and it could be reasonable to speculate that it functions in combination with glucanases or other defence-related- and symbiosis-induced proteins (Hohnjec et al, 2005).

Possible functions of Mtchit3-1

Mtchit3-1 seems to have a rather different regulation from the other class III chitinases investigated in this work, and resembles the one of the plant defence involved chitinases in

leaves and roots (chapter 4). From this responsiveness towards pathogenic fungi, it could be assumed that Mtchit3-1 is involved in pathogen interactions (Salzer et al, 2000) and has a role in plant defence. By comparison of its protein sequence, it is more closely related to a group of chitinases that may be defence-related than to sequences of a group of putative symbiosis-involved sequences around Mtchit3-3 (chapter 3). Within these class III chitinases, Mtchit3-1 has one rare amino acid substitution in its active site, and contains a valine (Val147) instead of an isoleucine, that is found in most other chitinases at this place (chapter 3). It is not known whether Mtchit3-1 is an active hydrolase, although the valine is not actively taking part in the hydrolysis (Terwisscha van Scheltinga et al, 1995), and it is unlikely that the cleavage process is influenced by this amino acid substitution.
It seems that the *M. truncatula* Mtchit3-1 is a class III chitinase that has a role in plant defence, and it is most probably important in the interactions with pathogenic fungi. A possible defence related chitinase has also been found in *Arabidopsis*, where only one single chitinase in this class has been identified (Passarinho & de Vries, 2002). The Mtchit3-1 regulation shows similarities to the one of defence related chitinases and could be implicated in similar functions. However, Mtchit3-1 belongs to another group of glycosyl hydrolases and might act as a supplementary chitinase. Therefore, considering the proposed synergistic activities of different chitinases (appendix1), Mtchit3-1 could be an additional element of an inducible plant defence reaction (Salzer et al, 2000; chapter 4 and appendix 1).

5.4 Concluding remarks

In conclusion, plants have developed systems to recognize chemical signals that are produced by microbes and recognized by plants in a specific way in order to create a specific reaction towards the microbe. Among these signals are chitin derived molecules. Chitinases are important in the generation, inactivation and interpretation of these signals:
In antagonistic interactions with pathogenic fungi, chitinases are implicated in the direct hydrolysis of the chitin in fungal cell walls, leading to reduced fungal activity. The chitin in the cell walls of these fungi also provides elicitors that induce defence activity in plants. Plants may be able to distinguish differently decorated chitin fragments released by the action of different forms of chitinases. It is likely that upon pathogen attack, a distinct pattern of chitinases is produced in order to optimally react to the fungal attack.
The establishment of both bacterial and fungal symbioses require the coordinated regulation of genes that are involved in the structural, metabolic and defence-related organisation of the symbiosis. Specific induction of chitinases occurs in certain stages of a symbiosis. The chitinases that are produced during later stages in both symbioses are probably involved in regulatory or developmental functions, rather than in the direct interaction with the microbes. It is possible that the plant has endogenous substrates for these chitinases, but the substrate specificity of these symbiosis-related chitinases remains to be determined.

The three most interesting *M. truncatula* chitinase genes, *Mtchit4*, *Mtchit5* and *Mtchit3-3*, belong to the genes that are directly related to plant-microbial interactions, and may be considered as markers for plant defence, nodulation and arbuscular mycorrhiza formation, respectively.

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