Molecular Analyses of the Pathogenic Interaction Formed Between the Model Legume *Medicago truncatula* and the Oomycete *Aphanomyces euteiches*

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Dedicated to my mother Odsuren Darjaa.

Хайрт ээждээ зориулав.

Common root rot of pea, which is the major yield – reducing factor for pea production world-wide is caused by the oomycete *Aphanomyces euteiches.* The model legume *Medicago truncatula* was chosen to study the molecular mechanisms of this disease. Young seedling of *M. truncatula* were effectively infected with zoospores of *A. euteiches* and similar disease development as in pea was observed. *A. euteiches* colonizes the root system and the disease symptoms occur mainly in the plant root. Therefore the root tissue was used for the analyses. cDNA-AFLP method was used to determine the time point of disease development where the most transcriptional changes occur. The earliest time point determined, was chosen to establish a SSH-cDNA-library enriched for the genes preferentially expressed in plant root after infection with oomycete. In total 560 ESTs from this library were sequenced and annotated. EST-annotations showed homologies to a number of classical pathogenesis related and defence genes. Pre-screening of 192 SSH-cDNA clones revealed 51 ESTs (26.5 %) showing increased mRNA accumulation in the infected plant root. 46 from total of 560 annotated ESTs showed no homology to previously identified *M. truncatula* genes. Moreover 10 of this new *M. truncatula* genes were confirmed to be up-regulated in the roots after infection.

Two genes from the Kunitz-type trypsin inhibitor family were analysed for their structure, expression and function. One of them, *MtMir*-1, is a gene induced in *M. truncatula* roots by *A. euteiches,* as well as by symbiotic AM (arbuscular mycorrhizal) fungi. Another one, *MtMir*-2, is induced in *M. truncatula* roots exclusively during symbiotic and not during pathogenic interaction. Complete cDNA sequences of both genes were determined. Moreover, promoter regions were isolated. Promoter-reporter gene fusions were used for promoter studies.

Using dsRNA *MtMir*-1 gene was silenced in transgenic roots of *M. truncatula*. Changes occurring in global gene expression pattern after silencing of *MtMir*-1 gene were studied using microarray technique.

The transcription profile of *M. truncatula* root 30 minutes and 6 days after infection with *A. euteiches* was also studied using microarrays.

Keywords: *Medicago truncatula, Aphanomyces euteiches*, transcription profiling

Die gemeine Wurzelfäule bei der Erbse, der größte erntereduzierende Faktor der Erbsenproduktion weltweit, wird durch den Oomyzet *Aphanomyces euteiches* verursacht. Die Modell-Leguminose *Medicago truncatula* wurde ausgewählt, um die molekularen Mechanismen dieser Krankheit zu untersuchen. Junge Sämlinge von *M. truncatula* konnten effektiv mit Zoosporen von *A. euteiches* infiziert werden. Dabei wurden ähnliche Befallssymptome wie bei der Erbse beobachtet. *A. euteiches* kolonisiert das Wurzelsystem und Krankheitssymptome treten hauptsächlich dort auf. Deswegen wurde Wurzelgewebe für die Analysen ausgewählt. Die cDNA-AFLP-Methode wurde benutzt, um den Zeitpunkt der Krankheitsentwicklung zu bestimmen, an dem die meisten Veränderungen auf Ebene der Transkription auftreten. Der früheste Zeitpunkt dieser Analyse wurde ausgewählt, um eine SSH-cDNA-Library zu etablieren, die angereichert ist für Gene, welche bevorzugt in der Pflanzenwurzel nach Infektion mit dem Oomyzet exprimiert werden. Insgesamt wurden 560 EST dieser Library sequenziert und annotiert. Die EST-Annotationen zeigten Homologien zu einer Anzahl von klassischen Abwehr- und Pathogen-bezogenen-Genen. Eine Analyse von 192 SSH-cDNA-Klonen ergab 51 ESTs (26,5 %), die eine erhöhte mRNA-Akkumulation in den infizierten Pflanzenwurzeln aufwiesen. 46 der 560 annotierten ESTs zeigten keine Homologie zu vorher identifizierten *M. truncatula* Genen. Darüber hinaus konnte für 10 dieser neuen *M. truncatula* Gene eine Hochregulation in den Wurzeln nach Infektion belegt werden.

Zwei Gene der Kunitz-Typ Trypsin Inhibitoren Familie wurden auf ihre Struktur, Expression und Funktion hin analysiert. Das eine, *MtMir-1*, ist ein Gen, welches in *M. truncatula* –Wurzeln sowohl durch *A. euteiches* als auch durch symbiotische AM (arbuskuläre Mykorrhiza) Pilze induziert wird. Das andere, *MtMir-2*, wird in *M. truncatula*-Wurzeln ausschließlich während der Symbiose und nicht durch die Pathogen-Interaktion induziert. Die kompletten cDNAs beider Gene wurden bestimmt. Darüber hinaus wurden die Promoter-Regionen isoliert. Promoter-Reporter-Gen-Fusionen wurden für Promoter-Analysen eingesetzt.

Mittels dsRNA wurde das *MtMir-1*–Gen in transgenen Wurzeln von *M. truncatula* ausgeschaltet. Veränderungen der globalen Gen-Expressions-Muster durch das ausgeschaltete *MtMir-1*-Gen wurden mittels Mikroarray-Technik analysiert.

Auch die Transkriptionsprofile von *M. truncatula*-Wurzeln 30 Minuten und 6 Tage nach Infektion mit *A. euteiches* wurden mittels Mikroarrays untersucht.

Schlagwörter: *Medicago truncatula, Aphanomyces euteiches*, transkriptions profil

AFLP	=	Amplified fragment length polymorphism
AM	=	Arbuscular Mycorrhiza
Amp	=	Ampicillin
APS	=	Ammoniumperoxidisulfate
bp	=	Base pairs
cDNA	=	Complementary DNA
CI	=	Chloroform isoamylalcohol
CMA	=	Corn meal agar
dATP	=	Deoxy-adenosine-5`-triphosphate
DEPC	=	Diethylpyrocarbonate
DIG	=	Digoxygenin
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTP	=	Deoxy-ribonucleotide-5`-triphosphate
dsRNA	=	Double stranded RNA
EDTA	=	Ethylene-diamine-tetra-acetic acid
EtBr	=	Ethidiumbromide
EtOH	=	Ethanol
EST	=	Expressed sequence tag
for	=	Forward
GUS	=	ß-Glucoronidase
IPTG	=	Isopropyl-ß-D-thiogalactate pyranoside
kb	=	Kilo base
LB	=	Luria-Bertani-medium
Log	=	Logarithm
mg	=	Milligram
min	=	Minutes
mM	=	Millimolar
MOPS	=	3-Morpholino-1-propansulfonic acid
MPB	=	Maltose peptone broth
NADP	=	β- Nicotineamide adenine dinucleotide phosphate
NCBI	=	National Center of Biotechnology Information
nm		Namometer

nt	=	Nucleotide
OD	=	Optical density
ON	=	Over night
PCR	=	Polymerase chain reaction
PCI	=	Phenol chloroform isoamylalcohol
PMS	=	Phenazine methosulfate
RACE	=	Rapid amplification of cDNA ends
rev	=	Reverse
RNA	=	Ribonucleic acid
RNAi	=	RNA interference
RNase	=	Ribonuclease
rpm	=	round per minute
RT	=	Reverse transcription
RT	=	Room temperature
SDS	=	Sodium dodecyl sulfate
SSC	=	Saline sodium citrate
SSH	=	Suppression subtractive hybridization
TAE	=	Tris-acetate-EDTA
Taq	=	*Thermus aquaticus*
TE	=	Tris-EDTA
TBE	=	Tris-boric acid
TEMED	=	N,N,N′,N′-Tetramethylendiamine
TC	=	Tentative concensus
Tris	=	Tris-(hydroxymethyl)-aminomethane
UV	=	ultra violet
v	=	volume
w	=	weight
x-Gal	=	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

During their life cycle plants are exposed to numerous biotic and abiotic environmental stresses. These stresses can result from environmental conditions, injuries caused by insect attack or mechanical wounding, different symbiotic or pathogenic bacteria, fungi, nematodes and oomycetes. Although plants may suffer damage to a lesser or greater extent, many survive these attacks some don't. The speed of a plant's response to infection affects the level of resistance expressed (Lyon *et al.,* 1990).

To initiate defence reaction rapidly, some plants recognise specific substances from pathogens. Even though they lack an immune system, plants are surprisingly resistant to most pathogens. Many plant species react to fungal or bacterial invasion by synthesising polymers such as lignin or callose, which act as physical barriers and inhibit the pathogen from gaining an entrance and spreading through the plant. Some antimicrobal compounds, such as simple phenylpropanoids, are synthesised before pathogen attack (Maher *et al*., 1994). Other antipathogen defences are induced by infection. A further common defence reaction induced after infection is hypersensitive response (HR), in which cells immediately surrounding the infection site die rapidly, depriving the pathogen of nutrients and preventing its spread (Cordelier *et al.,* 2003).

One frequently observed event following infection by pathogen is the transcriptional activation of PR (pathogenesis-related) proteins (van Loon, 1985; Bowles, 1990; Somssich and Hahlbroch, 1998). Currently, there are 17 independent PR protein families. For some of those proteins an antifungal activity has been described (van Loon and van Strien, 1999; Neuhaus, 1999; Christinsen *et al*., 2002).

The production of phytoalexins is a further common response of plants to pathogen. Phytoalexins are a chemically diverse group of secondary metabolites with strong antimicrobal activity that accumulate around the site of infection. Isoflavonoids are common phythoalexins in the legume family. Isoflavanoids are generally undetectable in plant tissues before infection, but they are synthesised rapidly after pathogen attack because of the activation of new biosynthetic pathways.

In addition to localised defences, plants possess various inducible defence mechanisms that establish an enhanced defensive capacity in plant parts distant from the site of primary attack, thereby protecting the plant systemically against subsequent invasion. This phenomenon, called systemic acquired resistance (SAR), develops over a period of several days following initial infection (Ryals *et al.,* 1996).

Recent studies revealed, that plant signal molecules SA (salicylic acid), JA (jasmonic acid) ET (ethylene) play a dominant role in network of interconnecting signalling pathway (Dong, 1998; Reymond and Farmer, 1998; Glasebrook, 1999; Pieterse and van Loon Feys and Parker, 2002).

In the last few years, numerous plant resistance genes known as R gene have been isolated from different plants (Cooley *et al.,* 2000). Most of the R genes are thought to encode receptors that recognize and bind specific molecules originating from pathogens and alert the plant to the pathogens presence. The specific pathogen molecules recognized are referred as elicitors, and include proteins, peptides, lipids or polysaccharide fragments arising from the pathogen cell wall, the outer membrane or a secretion process (Boller 1995). The R gene products themselves are nearly all proteins, with leucine-rich domain that is repeated inexactly several times in the amino acid sequence (Hammond-Kosack and Jones 1997). *Avr* (avirulence) genes are the genes of pathogen, which encodes specific elicitors.

When corresponding R and *avr* genes are present in both host and pathogen, the result is disease resistance. If either is inactive or absent, disease results (Floh 1971).

Most investigations on the molecular basics of plant-pathogen interactions are focused on interactions which cause disease symptoms on the upper part of the plant and little is known about root-pathogen associations. And up to now only very little is known about the molecular basis of root-oomycete associations. Recent studies have shown that insight gained on the molecular regulation of plant-fungus associations cannot be generally transferred to plant-oomycete associations (Judelson, 1997). The position of oomycetes as a unique lineage of stramenopile eukaryotes, unrelated to true fungi but closely related to heterokont algae, has been well established using molecular phylogenetic analyses that are based on ribosomal RNA sequences (Kumar *et al*.,1996; Van de Peer *et al*., 1997; Paquin *et al*., 1997). From these analyses it is evident that oomycetes evolved the ability to infect plants independently of other eukariotic plant pathogens and are likely to have unique mechanism for doing so (Kamoun, 2001). In the present study *Medicago truncatula-Aphanomyces euteiches* pathosystem was chosen to study the molecular basis of plant root-oomycete interaction.

Oomycetes, also known as water molds, are terrestrial and aquatic fungallike organisms in the kingdom Stramenopila. They are filamentous protists which must absorb their food from the surrounding water or soil or may invade the body of

another organisms to feed. The terrestrial oomycetes are mostly parasites of vascular plants, and include several important plant pathogens. Plant diseases caused by oomycetes are known for their important economical and social impact. The most prominent example is *Phytophthora infestans*, the cause of the Irish potato famine. During one week in the summer of 1846, this disease wiped out almost the entire potato crop in Ireland. Nearly a million Irish died in the Great Famine and one-and-a-half million emigrated to other countries (Ristano, 2002).

The oomycete *Aphanomyces euteiches* Drechs*,* in the order *Saprolegniaceae,* causes one of the most destructive diseases of pea (*Pisum sativum* L.), a root rot. It was first reported in the 1920s in United States, and has been widely observed in many pea growing areas in North America, Australia, New Zealand, Japan and Europe (Kraft and Pfleger, 2001)

Several other legumes as alfalfa, snapbean and red clover are also described to be susceptible (Delwiche *et al.,* 1987; Pfender and Hagedorn, 1982; Greenhalgh *et al.,* 1985). Root rot causes early stagnation of root growth and symbiotic nitrogen fixation; it limits the uptake of water and nutrients (Grau, 1990). The typical symptoms of the root rot are water-soaked, honey-brown lesions spreading from the location where the root tip had been inoculated with the pathogen. Infection with *A. euteiches* can result in the death of seedlings, but more often results in stunted, chlorotic plants. Roots and hypocotyls develop light to dark brown lesions, but unlike other root-rot pathogens which cause seedling collapse, hypocotyls infected with *Aphanomyces* tend to remain rigid, resulting in stunted but upright seedlings (Grau, 1990).

The disease cycle is initiated by direct germination of sporangia on the surface of susceptible plants or indirectly by zoospore formation. While both direct germination of sporangia and zoospores can act as inoculum for plant infection, it is thought that the latter are responsible for rapid infections because of their greater numbers and more efficient germination. The underlying mechanism involved in the perception of an environmental signal and the molecular events that lead to the genesis of germ tubes or zoospores are not at all understood. The initial parasitic phase of the pathogen is relatively short. After few days oospores are formed and the fungus enters its resting state. Oospores constitute the primary inoculum source in the soil (Mitchell and Yang, 1966). *A. euteiches* can grow on a variety of carbon sources, but studies of its life cycle in plant roots suggests that the relationship is biotrophic, as

the pathogen mycelium shows activity in living plant tissues only and does not grow saprophytically within dead plant material (Kjøeller and Rosendahl, 1998).

To date, no effective fungicides, resistant pea cultivars or biological control methods are available for the control of this pathogen (Cerenius et al. 1992; Rao et al. 1995). Only avoidance of fields with high disease potential can prevent the problem (Oyarzun, 1993). In contrast, resistant alfalfa cultivars are available (Munkvold *et al*., 2001)

There are reports about the bioprotective effect of arbuscular mycorrhizal fungi (Kjøeller and Rosendahl, 1996; Bodker *et al.,* 1998; Slezack *et al.,* 2000) but development of new strategies to control *A. euteiches* is necessary and will require a detailed knowledge of the molecular mechanisms underlying this oomycete-plant association.

The most thoroughly investigated plant-oomycete pathosystems are the interaction between potato/tomato and *Phytophthora infestance*, lettuce and *Bremia lactucae*, soybean and *Phytophthora sojae* (Judelson, 1996). Many resistance genes have been identified in these plant-oomycete pathosystems (Al-Kherb *et al.,* 1995; Anderson and Buzzell, 1992; Crute and Pink, 1996).

During the last years several attempts have been made to profile the genes whose expression was induced by oomycete-pathogens. Using the potato-*P.infestans* pathosystem Birch *et al.,* (1999) identified several potato genes induced in late-blight-resistant potato cultivar undergoing the hypersensitive response (HR). In later studies identification of 64 transcripts up-regulated in *P. infestans* during early stages of infection has been reported (Avrova *et al.,* 2003). By surveying potato EST libraries from diverse tissues, 1200 sequences potentially involved in the resistance response of potato against of *P. infestans* were sequenced (Ronning *et al.,* 2003). Using hybrid plants derived from late blight resistant and late blight susceptible potato lines several genes were identified potentially playing a role in horizontal resistance (Evers *et al.,* 2003). Studies made by screening of arabidopsis defence response mutants with *P. porri* suggest that resistance of arabidopsis against *P. porri* does not depend on salicylic acid (SA)-, ethylene-, or jasmonate-dependent signalling pathway (Roetschi *et al.,* 2001).

***Medicago truncatula* as a model legume**

Legumes represent the third largest family of flowering plants. Many agronomically important plants are legumes, such as pea, soybean, bean, alfalfa, clover and peanut. These plants have the unique capacity to establish an atmospheric-nitrogen-fixing-symbiosis with soil bacteria collectively named rhizobia, and to form symbiotic root mycorrhizae with soil borne fungi, thus facilitating their uptake of phosphate, water and other soil nutrients (Gianinazzi-Pearson, 1996; Albrecht *et al.,* 1999). However, features such as tetraploidy, large genome and lack of efficient transformation protocols for make them unwieldy and slows the progress on the genetic characterisation of this crops.

Motivated initially by the needs to understand the molecular basis of symbiotic nitrogen fixation, researches selected the barrel medic *Medicago truncatula* as a model system for legume biology (Barker *et al.,* 1990; Cook *et al.,* 1997; Cook 1999; Bell *et al.,* 2000).

M. truncatula, a close realtive of alfalfa, has a genome size of ~500-600 Mbp, only half that of alfalfa, and almost 10 times smaller than that of pea, but ~ 4-times larger than that of *Arabidopsis thaliana* (Blondon *et al.,* 1994). It has a diploid genome with 2n = 16 chromosomes. Its autogamous nature, short generation time, prolific seed production and high transformation and regeneration efficiency have made it the system of choice for many studies of basic and applied legume research.

High level of macro- and microsyntheny of *M. truncatula* to pea, alfalfa and soybean were identified (Thoquet *et al.,* 2002; Gualtieri *et al.,* 2002). This suggests, that the results obtained using *M. truncatula*, could be easily transferred to other important crop legumes.

Initiation and realization of large EST programmes using model organisms have become very effective and straightforward strategies to obtain global views on transcriptional changes during different plant-microbe interactions (Fedorova *et al.,* 2002; Journet *et al.,* 2002). Large EST- and genomic sequencing projects have been initiated for *M. truncatula* (www.medicago.org; www.genome.ou.edu/medicago.html). Up to date, > 204,264 ESTs, generated from more than 30 different cDNA libraries are available in public databases. The libraries not only encompass most major plant tissues, but also incorporate developmental stages and treatments with pathogens,

bacterial and fungal symbionts, insect pests and abiotic stressors (Frigoli and Harris, 2001).

The aim of the present study

The aim of the present study was to analyse the transcription profiles of *Medicago truncatula* during an infection by *Aphanomyces euteiches* in order to gain insight into molecular and physiological changes during disease development.

Information on both the physical and functional annotation of the genome can be gained through transcript profiling (Hughes *et al.,* 2001). In recent years, transcript profiling has become synonymous with gene expression analysis, mostly because of the technical difficulty and greater molecular complexity of proteomics and metabolomics (Smith, 2000).

The modern methods of molecular biology provide us a broad possibility of comparing different gene populations and identifying those genes exclusively expressed in one of them. The methods such as differential display (DD), cDNA-amplified restriction fragment polymorphism (cDNA-AFLP), Suppression Subtractive Hybridization (SSH) and microarray hybridization have been used to reveal organ or tissue specific as well as genes induced or repressed during different biotic and abiotic stresses in broad range of plant species.

A key feature of the SSH method is simultaneous subtraction and normalization that makes it possible to equalize abundance of target cDNAs in the subtracted population. As a result, rare differentially expressed transcripts can be enriched by ~1000-fold (Diatchenko *et al.,* 1996). The major drawback of SSH is the presence of background clones representing non-differentially expressed cDNA species in the subtracted libraries (Rebricov *et al.,* 2000).

Microarray technology allows the measurement of mRNA abundance in cells for thousands of genes in parallel and permits an assay of global gene expression patterns under variety of experimental conditions (Singh *et al.,* 2003).

Considering the advantages and disadvantages of above mentioned techniques, in the present work, cDNA-AFLP method was used to get an overall view of gene expression pattern of *M. truncatula* roots after infection with *A. euteiches* and determine the time point of disease development where the highest number of transcriptional changes occur. SSH method was used to identify large number of differentially expressed genes. Microarray hybridization method was used to

compare the gene expression patterns in the early and later stages of disease development as well as for the study of co-regulated genes. Transcription profile comparison between *A. euteiches*-infected and control roots provide a tool to obtain first views on molecular and physiological processes underlying the disease development. Recent studies have shown that a number of plant genes which are regulated during arbuscular mycorrhiza symbiosis are also regulated after infection by *A. euteiches* (Krajinski *et al*., 1998; Lapopin *et al*., 1999). But so far, the *A. euteiches*-legume association has not been the central aspect of transcriptomic approaches.

2.1 Materials

2.1.1 Chemicals, reagents, kits and equipment

Chemicals reagents, kits and equipment were obtained from following companies:

Amersham Biosciences	Buckinghamshire, England
BioDiscovery Inc.,	Los Angeles, CA, USA
Biometra	Göttingen, Germany
BioRad	Hercules, CA, USA
BIORIZE R&D	Dijon, France
Clontech	Palo Alto, CA, USA
Intas Compact	Göttingen, Germany
Invitek	Berlin-Buch, Germany
Invitrogen	Carlsbad, CA, USA
Kodak	Rochester, NY, USA
Leica	Bensheim, Germany
Macherrey-Nagel	Düren, Germany
MBI Fermentas	St. Leon-Rot, Germany
PerkinElmer	Boston MA, USA
Pharmacia Biothech	Buckinghamshire, England
Promega	Madison, WI, USA
Qiagen	Maryland, MD, USA
Roche Diagnostics	Mannheim, Germany
Sigma-Aldrich	Taufkirchen, Germany
Stratagene	La Jolla, CA, USA

2.1.2 Organisms

Escherichia coli XL1-Blue MRF'	(Stratagene, La Jolla, CA, USA)
Escherichia coli XL1-Blue	(Stratagene, La Jolla, CA, USA)
Escherichia coli TOP 10F'	(Invitrogen, Carlsbad, CA, USA)
Escherichia coli DH5α	(Invitrogen, Carlsbad, CA, USA)
Agrobacterium rhizogenes ARqua I	(Quandt *et al.,* 1993)
Agrobacterium tumefaciens EHA105	(Hood *et al.,* 1993)
Aphanomyces euteiches Drechs. ATCC 201684	(S. Rosendahl, University of Copenhagen)
Glomus intraradices BB-E-Sc-02	(Biorize R&D, Dijon, France)
Medicago truncatula Gaertn. A17	

2.1.3 Vectors

Following vectors were used for this work:

pGEM-T Easy	(Promega, Madison, WI, USA)
TOPO	(Invitrogen Carlsbad, CA, USA)
pLP 100	Szabados *et al.,* 1995
pDONR™ 207	(Invitrogen Carlsbad, CA, USA)
pFGC 5941	(Invitrogen Carlsbad, CA, USA)

2.1.4 Oligonucleotides

Table 1. List of oligonucleotides used in this study

Oligonucleotide	**Sequnce 5´→3´**	**Annealing t (Cº)**	**Reference**
Apo I adaptor	CTC GTA GAC TGC GTA CC CAT CTG ACG CAT GGT TAA		1
Mse I adaptor	GAC GAT GAG TCC TGA G TA CTC AGG ACT CAT		1
ApoI+0 primer	CTC GTA GAC TGC GTA CCA AT	52	1
MseI+0 primer	GAC GAT GAG TCC TGA GTA A	52	1
Apo1 (+GC)	GAC TGC GTA CCA ATT GC	50.4	1
Apo2 (+AC)	GAC TGC GTA CCA ATT AC	50.4	1
Apo3 (+CA)	GAC TGC GTA CCA ATT CA	50.4	1
Apo4 (+GA)	GAC TGC GTA CCA ATT GA	50.4	1
Apo5+CT	GAC TGC GTA CCA ATT CT	50.4	1
Apo6+AG	GAC TGC GTA CCA ATT AG	50.4	1
Mse1 (+GC)	GAT GAG TCC TGA GTA AGC	51.4	1
Mse2 (+AC)	GAT GAG TCC TGA GTA AAC	51.4	1
Mse3 (+CA)	GAT GAG TCC TGA GTA ACA	51.4	1
Mse4 (+GA)	GAT GAG TCC TGA GTA AGA	51.4	1
Mse5 (+CT)	GAT GAG TCC TGA GTA ACT	51.4	1
Mse6 (+AG)	GAT GAG TCC TGA GTA AAG	51.4	1
PR4-for	GTG AGT GGT CAG AGT GCA	56	1
PR4-rev	AGG TGA CCA TTC TGA ACG C	56.7	1
tef1α_for	CAA TGT GAG AGG TGT GGC AAT	60.3	3
tef1α_rev	GGA GTG AAG CAG ATG ATC TGT TG	60.6	3
MtMir1_for	CGG TTC CCA TAT ACA AAT GTG GAC	61	1
MtMir1_rev	AGA GAG GCT TTC ATC AAG CTT GTT G	61.3	1
MtMir1_5‘RACE	ATC AGC TCG GAG TTT CTT GCC	59.8	1
MtMir1_3‘RACE	CAA CAA GCT TGA TGA AAG CCT CTC T	61.3	1
MtMir2_for	TGA TTA TCA CTG GGA CAG ATA	54	1
MtMir2_rev	CAT ATC AAC AGT TCC ACA TTC	54	1
MtMir1_GWI_GSP1	GTA ATT AGC ATC AGC TCG GAG TTT CTT GCC	66.8	1
MtMir1_GWI_GSP2	GGT CCA CAT TTG TAT ATG GGA ACC GGG ATA	66.8	1
MtMir1_GWII_GSP1	GGC ATG TTG TCA CCA AGA GCA CAA ACC CT	68.1	1
MtMir1_GWII_GSP2	GAA TAC AAT TGG CCC TTT GAA GGG TTA TTT CT	64.4	1
MtMir2_GW_GSP1	GAG ACA CAC ATG AGC AAG AAT AAT GAG GGT TC	66.9	1
MtMir2_GW_GSP2	CTT GTA ATG TAA TAA CTA GGC TTA CAC TC	60	1
MtMir1_BamHI-p	GGA TCC CAG CTC GGA GTT TCT TGC	66.1	1
MtMir1_EcoRI-p100	GAA TTC ATG AAA AAC ACA TTG CTA GC	58.5	1
MtMir1_EcoRI-p300	GAA TTC GCC AAT TGT ATT GCA TAT TGG	60.4	1
MtMir1_EcoRIp-900	GAA TTC ACG TTA CTC CCT CCG TCC TA	64.8	1
MtMir2_BamH-p	GGA TCC GTT AGA TGT AAC TTG TAA	57.6	1
MtMir2_EcoRI-p800	GAA TTC CAG CTT ATA CTC TGA TAA G	58.1	1
pLP100_for	GGG GTT CCG CGC ACA TTT CCC CG	60	1
MtMir1_attB1_for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT TCC CAT ATA CAA ATG TGG AC	73.5	1
MtMir1_attB2_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AGA GGC TTT CAT CAA GCT TG	75	1
MtMir2_attB1_for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CCC TCA TTA TTC TTG CTC AT	73.5	1
MtMir2_attB2_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ACT ATC TGT CCC AGT GAT AA	75	1
*Chs*A_front	CCA ATT AAG ATA AAA CGT TGA ATG	54.2	1
*Chs*A_back	CAC TTA CTT ACA CTT GCC TTG GAG	61	1
MtMir1_600_RNAi_for	AGC TGA AGC TTC AAA TGA ACA AGT	57.6	1
MtMir1_600_RNAi_rev	AGC TGA AGC TTC AAA TGA ACA AGT	54.8	1
MtMir1_200_RNAi_for	CTT AAA GCA TAC AGA ACA ATG	52	1
MtMir1_200_RNAi_rev	CAT TTG TAT ATG GGA ACC G	52.4	1
MtMir2_RNAi_for	TAA ACC ATG TCA ATG AGA TTA TC	53.5	1
MtMir2_RNAi_rev	ATC TTA CCA TTC TCA TTC AAC A	52,8	1
MtPT_for	GTC GCC TTG TTT GGA ACA TTC CCC GG	68	3
MtPT_rev	TCA CAT CTT CTC AGT TCT TGA GTC C	61.3	3
SMART II oligonucleotide	AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG	65	2
CDS primer	AAG CAG TGG TAA CAA CGC AGA GTA $CT_{(30)}$ $N_{-1}N$ (N=A, C, G or T; N_{-1}=A, G, or C)	65	2
PCR primer	AAG CAG TGG TAA CAA CGC AGA GT	65	2

Continuation of table 1

Oligonucleotide	**Sequnce 5´→ 3´**	**Annealing t (Cº)**	**Reference**
Nested primer 1	TCG AGC GGC CGC CCG GGC AGG T	73.3	2
Nested primer 2R	AGC GTG GTC GCG GCC GAG GT	67.6	2
DT_{15}VN primer	TTTTTTTTTTTTTTTVN; V=A,G,C; N=A,C,G,T	37	1
1b11_for	GAA TTG GTC ACT GTC TGC T	54.5	1
1b11_rev	CAG GCA AGT TCA AGA AGT C	54.5	1
5c01_for	CTG CAC TGT TTG AAA GTG T	52.4	1
5c01_rev	ACT GTG CTG CTA AGA AAT G	52.4	1
5c03_for	GAA GCA TAA TAG GAT TGG A	50.2	1
5c03_rev	AAC ACT CAG GAA TTT CAA C	50.2	1
6c10_for	TAG TGC ACT TGA CAT CCA	51.4	1
6c10_rev	GTT GCA AAA TCT TCA GGC	51.4	1
6d11_for	TCC CGC AAA CTT GCT CAA	53.7	1
6d11_rev	ACT AAG AGA CCC AAT TGG	51.4	1

1- this work
2- Clontech, Palo Alto, CA, USA
3- Wulf *et al*., 2003

2.1.5 Buffers, solutions, media

Table 2. List of the buffers, solutions and media used in this work.

Name	Content	
TAE buffer:	40 mM	Tris-acetate pH 8.0
	1 mM	EDTA
TBE buffer:	40 mM	Tris-borate pH 8.0
	1mM	EDTA
TE buffer:	10 mM	Tris-HCl pH 8.0
	1 mM	EDTA
10x MOPS buffer	200 mM	MOPS
(RNA gel electrophoresis):	80 mM	Na-acetat
	10 mM	0.5 M EDTA
	pH 7.0	
DNA extraction buffer:	0.1 M	Tris-HCl, pH 7.0
	50 µM	EDTA 0.5 M
	0.15 M	NaCl (3M, pH 4.8)
RNA Sample buffer:	100 µl	Formamyd
	38 µl	Formaldehyd
	20 µl	10 x MOPS buffer
	42 µl	DEPC-H_2O
STEB buffer	20 mM	Tris-HCl
(for protein extraction):	10 mM	$NaHCO_3$
	10 mM	$MgCl_2$
	0.1 mM	Na_2 EDTA (0.5 M)
	10 mM	β-mercaptoethanol
	10 %	Sucrose
	0.1 %	Triton X-100
10 x Electrode buffer	25 mM	Tris-HCl
(native page):	192 mM	Glycine
	pH 8.3	
Staining buffer	22 ml	0.05 M Tris-HCl pH 8.0
(native page):	25 mg	Glucose 6-phosphate (Sigma-Aldrich)
	1 ml	5 mg/ml NADP (Sigma-Aldrich)
	1 ml	5 mg/ml NBT (Sigma-Aldrich)
	1 ml	5 mg/ml PMS (Sigma-Aldrich)
Separation buffer	1.5 M	Tris-HCl pH 8.8
(native page):		
7.5 % Separation gel	2 ml	Acrylamid
(native page):	2 ml	Separation buffer
	400 µl	2 % APS (ammoniumpersulfat)
	600 µl	2 % Triton X-100
	8 µl	TEMED
	3 ml	H_2O
Stacking gel	0.5 ml	Acrylamid (29.2/0.8)
(native page):	1 ml	Stacking buffer
	200 µl	2 % APS
	300 µl	2 % Triton X-100
	4 µl	TEMED
	2 ml	H_2O
Stacking buffer	0.5 M	Tris-HCl pH 6.8
(native page):		

Continuation of table-2

Name	Content	
ALP staining solution (staining of oomycete structures):	*Digestion medium:*	
	0.05 M	Tris-Citrate buffer pH 9.2
	5 %	Sorbitol
	150 u/ml	Cellulase
	15 u/ml	Pectinase
	Staining medium:	
	0.05 M	Tris-Citrate buffer pH 9.2
	1 mg/ml	α-Naphtylphosphat
	1 mg/ml	Fast-Blue
	0.5 mg/ml	$MnCl_2$
	0.5 mg/ml	$MgCl_2$
Trypan-blue staining solution:	10 g	Phenol
	10 ml	H_2O
	10 ml	Glycerol
	10 ml	Lactat
	30 mg	Trypan-blue
GUS staining solution (1L):	13.8 g	NaH_2PO_4
	17.8 g	$NaHPO_4$
	pH 7.0	
	2 ml	EDTA (0.5 M)
	211 mg	$K_4Fe(CN)_6$
	164 mg	$K_3Fe(CN)_6$
	1 mM	X-Gluc (added just before using)
0,5 x Hoagland solution:	2,5 mM	$Ca(NO_3)_2$ x 4 H_2O
	2,5 M	KNO_3
	1 mM	$MgSO_4$ x H_2O
	20 µM	KH_2PO4
	50 µM	NaFe-EDTA
	0,2 µM	Na_2MoO_4 x H_2O
	10 µM	H_2BO_3
	0.2 µM	$NiSO_4$ x 6 H_2O
	1 µM	$ZnSO_4$ x 7 H_2O
	2 µM	$CuSO_4$ x 5 H_2O
	0.5 M	$MnCl_2$ x 4 H_2O
	0.2 µM	$CoCl_2$ x 6 H_2O
DEPC- H_2O:	0.03%	DEPC dissolved in H_2O
Binding-silane solution (AFLP gel):	50 ml	Abs. Ethanol
	1.5 ml	8 % Acetic Acid
	150 µl	Metacryloxypropyltrimethylsilane (Sigma-Aldrich)
Polyacrylamid gel solution (AFLP gel):	4 %	(29 % Acryl : 1 % Bisacryl)
	7.5 M	Urea
	1 x	TBE buffer
Formamid sample buffer (AFLP gel):	98 %	Formamide
	10 mM	EDTA
	0.25 %	(w/v) Bromphenolblau
	0.25 %	(w/v) Xylene-Cyanol FF

Continuation of table 2

Name	Content	
Silver solution	1.5 g	Silver nitrate
(AFLP gel):	1.5 ml	Formaldehyd (37%)
	1 L	H_2O
Developing solution	30 g	Natrium carbonate
(AFLP gel):	1.5 ml	Formaldehyd (37%)
	4 µM	Natriumthiosulfat
	1 L	H_2O
DIG-Denaturation solution	0.5 M	NaOH
(DIG detection system):	1.5 M	NaCl
DIG-Neutralization solution	0.5 M	Tris-HCl, pH 7.5
(DIG detection system):	3 M	NaCl
DIG-Detection solution	100 mM	Tris-HCl, pH 9.5
(DIG detection system):	100 mM	NaCl
Maleic acid buffer (1L)	0.1 M	Maleic acid
(DIG detection system):	150 mM pH 7.5	NaCl
20 x SSC	3 M	NaCl
(DIG detection system):	300 mM	Na-citrate
	pH 7.0	
0.5 x washing buffer	0.5 x	SSC
(DIG detection system):	0.1 %	SDS
2x washing buffer	2x	SSC
(DIG detection system):	0.1 %	SDS
LB medium (1L)	10 g	Trypton
	5 g	Yeast extract
	10 g	NaCl
	pH 7.0	
YEP medium (1L)	10 g	Trypton
	5 g	Yeast extract
	5 g	NaCl
	pH 7.0	
SOC medium (1L)	20 g	Tryptone
	0.58 g	NaCl
	0,185 g	KCl
	2.03 g	$MgCl_2$ x 7 H_2O
	2.46 g	$MgSO_4$ x 7 H_2O
	3.6 g	Glucose
SOB medium	1% (w/v)	Tryptone
	0.5% (w/v)	Yeast extract
	10 mM	NaCl
	2.5 mM	KCl
	after autoclaving sterile 10 mM $MgSO_4$ added	
CMA (1L):	17 g	CMA (Sigma-Aldrich)
MPB (1L):	3 g	Maltose
	1 g	Peptone
Hogness Freeze Medium (HFM):	*Solution I (800 ml):*	
	622 g	87 % (v/v) glycerol (=520 ml)
	4.99 g	trisodiumcitrate-dehydrate
	9 g	(di) ammoniumsulfate
	0.99 g	magnesiumsulfate-heptahydrate

Continuation of table 2

Name	Content
	Solution II (200 ml):
	62.72 g di-kaliumhydrogenphosphate x 3 H_2O
	17.96 g kaliumdihydrogenphosphate
Modified Fahraeus medium:	*Macro elements:*
	0.9 mM $CaCl_2$
	0,5 mM $MgSO_4$
	0.7 mM KH_2PO_4
	0.8 mM Na_2HPO_4
	20 µM Ferric citrate
	0.5 mM NH_4NO_3
	Micro elements:
	100 µg/L $MnCl_2$
	100 µg/L $CuSO_4$
	100 µg/L $ZnCl_2$
	100 µg/L H_3BO_3
	100 µg/L Na_2MoO_4
	15 g/L Agar
M-Medium:	Stock solutions for M-Medium:
	Macroelements-1: (1L; 100 x conc.)
	8 g KNO_3
	73.1 g $MgSO_4$ x H_2O
	6.5 g KCl
	Macroelements-2: (1L; 100 x conc.)
	28 g $Ca(NO_3)_2$ x 4 H_2O
	Microelements-1: (1L; 1000 x conc.)
	4.1 g $MnCl_2$ x 4 H_2O
	1.5 g H_3BO_2
	2.65g $ZnSO_4$ x 7 H_2O
	0.0024 g Na_2MoO_4 x 2 H_2O
	0.13 g $CuSO_4$ x 5 H_2O
	Microelements- 2: (1L; 1000 x conc.)
	0.75 g KJ
	Fe-EDTA (1L; 100 x conc.)
	0.8 g NaFe-EDTA
	Vitamins:
	0.1 g Glycine
	5 g Myoinosin
	0.05 g Nicotin acid
	0.01 g Pyridoxine HCl
	0.01 g Thiamin HCl
	For 1L M-Medium: 10 ml from 100 x concentrated solutions, and 1 ml from 1000 x concentrated solutions are taken.
	10 g Sucrose
	3 g Phytagel
	Autoclaved

2.1.6 Antibiotics and selection substrates

Table 3. A list of antibiotics and selection substrates used.

Name of the substance	Final concentration
Ampicillin	50 µg/ml or 100 µg/ml
Kanamycin	25 µg/ml or 50 µg/ml
Streptomycin	50 µg/ml or 600 µg/ml
Augmentin	400 µg/ml
Gentamycin	40 µg/ml
Rifampicillin	5 µg/ml
IPTG	8 µg/ml
X-Gal	40 µg/ml
PPT	5 µg/ml

2.2 Microorganisms

2.2.1 *Escherichia coli*

In general, cultivation of *Escherichia coli* was done as described by Sambrook *et al.,* (1989). IPTG/X-Gal or different antibiotics were added depending on the experiment. Glycerol stocks of bacterial cultures were stored at -80°C in 15% final concentration of glycerine. Production and transformation of heat shock competent cells were made according to protocol of Sambrook *et al.,* (1989).

2.2.1a Plasmid DNA extraction from *Escherichia coli*

Plasmid DNA extractions from *E. coli* were done using Invisorb® Spin Plasmid Mini Kit (Invitek, Berlin-Buch, Germany).

2.2.2 *Agrobacterium rhizogenes*

A. rhizogenes ARqua I strain (Quandt *et al.,* 1993) was cultivated at 28°C, with agitation in a liquid YEP medium containing 600 µg/ml streptomycin. 50 µl competent cells were transformed with 1 µl plasmid using electroporation method. Transformed cells were plated on YEP-agar medium and grown for 2 days at 28°C.

2.2.3 Cultivation of *Aphanomyces euteiches*

Aphanomyces euteiches Drechs. (ATCC 201684), isolated from infected pea in Denmark, was kindly provided by Prof. Søren Rosendahl, Department of Mycology, University of Copenhagen. *A. euteiches* was grown on corn meal agar (CMA) for 3 days in the dark and stored at 4°C until use. New cultures were made every 2

months. All observations *of A. euteiches* were made using the light microscope (Leica MPS 32, Bensheim, Germany). For zoospore induction, 10 disks of 1 cm^2 fungal mycelium grown on CMA were cut out and cultured in 20 ml maltose peptone broth (MPB) for 9 days at room temperature in the dark. Zoospore production was induced by washing the mycelium twice in an autoclaved lake water and incubating overnight at RT in a dark. Zoospore concentration was determined and diluted to appropriate concentrations.

2.2.4 *Glomus intraradices*

Commercially available inoculum of *G. intraradices* BB-E-Sc-02 (BIORIZE R&D, Dijon, France) was used for inoculations.

2.3 Plants

2.3.1 Cultivation of *Medicago truncatula*

Seeds of *Medicago truncatula c.v. Jemalong* A17 were surface-sterilised by 10 min treatment with concentrated sulphuric acid, 5 min incubation in 6% NaClO and three washing steps with distilled water. Seeds were pregerminated on moist filter paper for 2 days in a dark. Seedlings were transferred into pots containing a 1:2 mixture of sterilised expanded clay and vermiculite. Plants grown under constant conditions in a greenhouse (220 $\mu Em^{-2}s^{-1}$ for 16h; 22°C, 65% humidity) and fertilised with half-strength Hoagland`s solution (Hoagland and Arnon, 1950).

2.3.2 Inoculation of *Medicago truncatula* with *Aphanomyces euteiches*

10 days old seedlings of *M. truncatula* were inoculated by application of zoospore suspensions with appropriate concentration at the stem basis. For inoculation of root cultures, 1 ml of zoospore suspension was added onto root cultures of *M. truncatula* 2 weeks after transferring them to fresh agar plates.

All control plants and root cultures were mock inoculated with autoclaved lake water.

2.3.3 Visualisation of *Aphanomyces euteiches* structures in the plant roots

The method of Kjøller and Rosendahl (1998) was used to visualise active mycelium of *A. euteiches* in *M. truncatula* roots by histochemical staining for alkaline

phosphatase (ALP) activity. The roots were pre-incubated in digestion medium as described by Tisserant et al. (1993), stained ON, cleared in 3 % sodium hypochloride for 20 min. The oospores of *A. euteiches* were visualised by trypan-blue staining as described for determination of arbuscular mycorrhizal (AM) structures.

2.3.4 Inoculation of *Medicago truncatula* with *Glomus intraradices*

Inoculation of *M. truncatula* with *G. intraradices* was carried out using the method of Dumas-Gaudot *et al.,* (1994). Inoculum of *G. intraradices* was mixed 1:1 with sterile vermiculite-expanded clay substrate.

2.3.5 Staining of *Medicago truncatula* roots for arbuscular mycorrhizal (AM) structures

AM structures in *M. truncatula* roots colonised with *G. intraradices* were visualised by trypan-blue staining. Roots were cleared in 2 % KOH for 30 min at 90°C, rinsed 3 times with H_2O, incubated in trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and H_2O) for another 30 min at 90°C and destained with 50 % glycerol.

2.3.6 *Agrobacterium rhizogenes*- mediated transformation of *Medicago truncatula*

Electroporation was used to transform the *A. rhizogenes* strain ARqua I (Quandt *et al.,* 1993) with binary vectors. *M. truncatula* hairy roots were transformed according to the protocol of Boisson-Dernier *et al*., (2001). The *A. rhizogenes* strain containing the binary vector of interest was grown ON on YEP medium containing 50 µg/ml kanamycin (Km) and 600 µg/ml streptomycin (Sm). Radicles of about 30 hours old seedlings were cut under sterile conditions, approximately 3 mm from the root tip. The sectioned surface was coated with *A. rhizogenes* by slightly scraping on the surface of the plate. Seedlings were then placed on a Petri dish containing modified Fahraeus medium and 25 µg/ml Km and incubated at 20°C in a growth chamber. About one week after inoculation, plates were transferred to 25°C growth chamber for another 2-3 weeks.

2.3.7 Histochemical staining of transgenic roots for GUS-gene activity

GUS staining was performed according to Jefferson *et al*. (1987). The roots were incubated in GUS staining solution ON at 37°C, cleared in 70 % ethanol, observed and documented under the light microscope (Leica MPS 32, Bensheim, Germany).

2.4 Molecular biological methods

2.4.1 Genomic DNA extraction

Leaf material of *M. truncatula* was used for the genomic DNA extraction following the method of Dellaporta *et al.,* (1983). Up to 1g leaf material was fine grinded in liquid nitrogen and transferred into 50 ml falcon tubes. For the extraction of *A. euteiches* genomic DNA, one week old mycelium grown in MPB medium was used. After adding 15 ml DNA extraction buffer, 1 ml 20% SDS and 10 µl RNase, probes were incubated at 65°C for 10 min. Then 5 ml K-acetate (5M) were added, the reaction was mixed carefully and centrifuged for 5 min at 5000 x g. After a filtration step, an equal volume of PCI (25:24:1) was added and centrifuged for 5 min at 5000 x g. The upper phase was taken to a new falcon tube, an equal volume of CI was added, carefully mixed and centrifuged for 5 min 5000 x g. The upper phase was transferred to a new tube and 0.1 volume Na-acetate, 0.6 volume isopropanol were added, incubated at -20 °C for one hour, and centrifuged for 20 min at 5000 x g, at 4°C, washed with 75% EtOH and resuspended in H_2O.

2.4.2 RNA extraction

RNeasy Plant Mini Kit (Qiagen, Maryland, MD, USA) was used for all RNA extractions according to suppliers instruction.

2.4.3 Estimation of nucleic acid concentration

DNA and RNA concentrations were measured spectrophotometrically using Ultrospec® 3000 photometer (Pharmacia Biotech., Buckinghamshire, England).
Concentration was measured at λ=260 nm. The correlation between the absorption and DNA (RNA)-concentration was calculated as follows:

$$\text{dsDNA-concentration} = (A_{260\ nm} \times df \times 50)\ \mu g/ml$$

$$\text{RNA-concentration} = (A_{260\ nm} \times df \times 40)\ \mu g/ml$$

(A- absorption; df- dilution factor)

The purity of nucleic acid was checked by determination of the coefficient of 260nm/280nm. For pure DNA this coefficient is > 1,8; for pure RNA it is between 1,9-2,1.

2.4.4 DNA gel electrophoresis

Electrophoresis was carried out in 0.8-2% agarose gels. 0.5 or 1x TAE buffer was used as a gel and as a running buffer (Sambrook *et al.,* 1989). A 1 kb or 100 bp Plus DNA ladder (MBI Fermentas, St. Leon-Rot, Germany) was used for sizing the DNA fragments. Gels were stained in 1 µg/ml ethidium bromide solution. DNA was visualised under UV light at 302 nm and documented with a video printer (Compact Imager, Intas Compact, Göttingen, Germany).

2.4.5 Purification of DNA fragments from agarose gels

Nucleotrap Extraction kit (Macherrey-Nagel, Düren, Germany) or Invisorb Spin DNA Extraction kit (Invitek, Berlin-Buch, Germany) were used for the purification of DNA fragments from agarose gel according to instructions of supplier.

2.4.6 RNA gel electrophoresis

Separations of RNA were carried out in 1,2 % agarose gel under denaturing conditions. All buffers were set with DEPC-H_2O. 1x MOPS buffer was used as a gel and as a running buffer. Same volume of freshly prepared sample buffer was added to the RNA probes, denatured at 65°C for 10 min and cooled on ice. 2 µl 6x loading dye (MBI Fermentas, St. Leon-Rot, Germany) containing 1% ethidium bromide was added to the samples before loading the gel. The gel and the sample buffer contained 3% formaldehyde. RNA was visualised under UV light at 302 nm and documented with a video printer (Compact Imager, Intas Compact, Göttingen, Germany).

2.4.7 Digestion of DNA

DNA was digested in reaction volume of 50 µl with 1 µl (1-20 U) restriction enzyme in the reaction buffer of supplier. Complete digestion of genomic DNA was carried out by incubation ON at 37°C. Plasmid DNA digestions were incubated at 37°C for 1 hour.

2.4.8 Key restriction enzymes used

The following enzymes from MBI-Fermentas (St.Leon-Rot, Germany) were used in this study: *Eco*RI, *Eco*RV, *Bam*HI, *Apo*I, *Mse*I, *Ssp*I, *Ehe*I, *Sma*I, *Xba*I, *Rse*I. Buffers recommended by supplier were used.

2.4.9 Cloning of DNA fragments

Using T/A based cloning system, PCR derived DNA fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) or TOPO ™ TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

T4 DNA Ligase (MBI Fermentas, St.Leon-Rot, Germany) was used for ligation reactions of DNA fragments into the pLP100 vector. GATEWAY™ cloning system was used for construction of vectors used for gene silencing.

2.4.10 Labelling of DNA fragments

DNA fragments were incorporated with Digoxygenin-11-dUTP using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the protocol of supplier.

2.4.11 Polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed in Trio-Thermoblock (Biometra, Göttingen, Germany). In most cases Red *Taq*-polymerase (Sigma-Aldrich, Taufkirchen, Germany) was used. The standard amplification protocol was as follows: initial denaturing at 95°C for 2 min; denaturing at 94°C for 30 sec; annealing temperatures between 55-65°C, depending on the primers used, for 30 sec; elongation at 72°C for 30 sec. 30-35 cycles were performed.

2.4.12 Reverse-transcription based PCR (RT-PCR)

1 µg total RNA was reverse transcribed by MMLV-RT (Promega, Madison, WI, USA) in a total volume of 20 µl. 1 µl of 1:10 diluted cDNA was taken as a template for the RT-PCR. 15-30 standard PCR cycles were performed.

2.4.13 Rapid amplification of cDNA ends (RACE-PCR)

5′- and 3′- RACE techniques were used to obtain the full size cDNA sequences of interesting genes. First strand SMART cDNA was synthesised using SMART® PCR cDNA Synthesis Kit (Clontech, Palo-Alto, CA, USA) and taken as a template for RACE-PCRs. For 5′ end synthesis, SMART II oligonucleotide and gene specific reverse primers were used. For 3′ end synthesis, CDS primer and gene specific forward primers were used.

2.4.14 Native PAGE

A. euteiches-specific glucose-6-phosphate dehydrogenase (Gd) activity was detected in host plants and oomycete, using the method described by Kjøller and Røsendahl (1996).

1 g plant material and 6 day old mycelium of oomycete was grinded in STEB buffer, transferred into eppendorf tubes and centrifuged for 10 min at 4°C at 20000 x g. Supernatant was then taken to another tube and centrifuged for 2 min. 100 µl supernatant was carefully transferred to a new tube and a drop of bromphenole blue was added. 5-20 µl extract was loaded on a gel.

Electrophoresis was carried out in a discontinuous, vertical electrophoretic system. Separation gel contained 7.5 % acrylamid, stacking gel contained 3.75 % acrylamid. Electrophoresis was carried out in 1 x electrode buffer at 400 V/40 mA for 35 min with cooling to 4°C. Gels were stained for glucose-6-phosphate dehydrogenase (Gd) activity as described by Soltis and Soltis (1989). The gels were incubated in a staining buffer for approximately 10 min at 37 °C in a dark and documented with digital camera (Olympus, Tokyo, Japan).

2.4.15 cDNA- Amplified Fragment Length Polymorphism (cDNA- AFLP)

cDNA-AFLP method was performed according to a modified protocol described earlier by Vos *et al.,* (1995) and Bachem *et al.,* (1996), *(fig. 1).*

a. First strand cDNA synthesis

5 µg of total RNA was reverse transcribed by MMLV-RT (50 u/µl) using oligo(dT) primer in final volume of 30 µl.

b. Second strand synthesis

Second strand synthesis was carried out using 5 units of DNA Polymerase I (10000u/ml), in the presence of RNase H (1000 u/ml), in a final volume of 100 µl.

Reaction was incubated at 16°C for 2 hours, stopped by adding 5 µl EDTA (0.5 M). After phenol/chloroform extraction, DNA was ethanol precipitated and dissolved in 10 µl H_2O.

c. Restriction digest

The cDNA was first digested with *Apo*I and subsequently with *Mse*I restriction enzymes, as these enzymes require different incubation temperatures. 10 µl cDNA was digested with 5 units of *Apo*I in reaction volume of 40 µl at 50°C. After 2 hours, 5 units of *Mse*I was added and incubated at 37°C for another 2 hours. It is important that the restriction enzymes are still active during the ligation to ensure complete digestion and to prevent re-annealing.

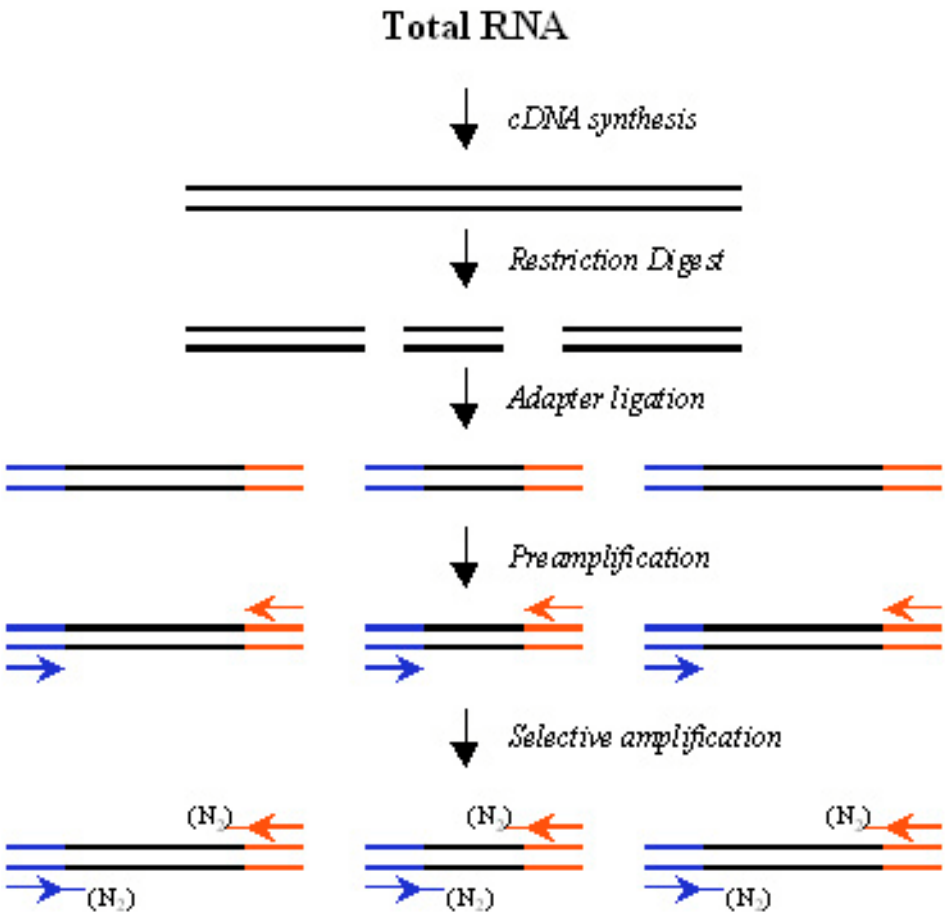

Fig. 1 **Schematic representation of cDNA-AFLP method**. Highly stringent PCR conditions facilitated by adding double-stranded adapters on the ends of restriction fragments which serve as primer binding sites during amplification. Selective fragment amplification is achieved by adding two more bases (N_2) on to the PCR primers which only then be successfully extended if the complementary sequence is present in the fragment flanking the restriction site.

d. Adapter ligation

Directly after digestion adapters are ligated onto sticky ends which act as primers during subsequent PCRs.

ApoI and MseI adapters (50 pmol each) were ligated to the digested DNA, using T4 DNA ligase (5 units) at 37°C for 3 hours.

e. Pre- amplification

This step amplifies all of the cDNA fragments in the primary template as it uses primers with no selective extensions (+0).

The product of the ligation reaction was 1:10 diluted and used as a template for the PCR.

The reaction with a final volume of 50 µl contained 20 µl primary template, 0.5 µl dNTP's (25mM), 1 µl ApoI+0 primer (100ng/µl), 1 µl MseI+0 primer (100ng/µl), 6.5 µl 10 x Red*Taq*-PCR buffer, 19 µl H_2O, 2 µl Red*Taq* polymerase (1U/µl, Sigma-Aldrich, Taufkirchen, Germany). 20 cycles at 94°C for 30 sec, 52°C for 30 sec, 72°C for 60 sec were performed. 3 µl pre amplification product were checked on 1.5 % agarose gel.

f. Selective amplification

Selective amplification was carried out using primers with 2 selective nucleotides in order to amplify subsets of the cDNA fragment population.

The pre-amplification product was diluted 1:10 with H_2O. 1 µl diluted pre-amplification product, 0.8 µl dNTP's (5mM), 2 µl ApoI+NN primer (50ng/µl), 2 µl MseI+NN primer (50ng/µl), 2 µl 10 x Red*Taq*-PCR buffer, 1 µl Red*Taq* polymerase (1U/µl, Sigma-Aldrich, Taufkirchen, Germany) and 11.2 µl H_2O were mixed in 0.2 ml PCR tube. PCR cycles were as follows: 94°C for 30 sec, 65°C for 30 sec, 72°C for 60 sec. In the first 12 cycles, primer binding temperature was lowered by 0.7 °C in each cycle. Annealing temperature of the following 23 cycles was 56°C.

g. Polyacrylamid gel electrophoresis

Electrophoretic separation of cDNA-AFLP fragments was carried out in 4 % denaturing polyacrylamid gels using Sequi Gen Cell sequencing gel apparatus (Bio-Rad, Hercules, CA, USA). One of the glass plates of the gel apparatus was treated with binding silane solution for 5-10 min with the purpose that the gel matrix is fixed on the glass plate for silver staining later. The other glass plate is treated with Acrylease (Stratagene, La Jolla, CA, USA) for 2 min in order to prevent the stacking

of the gel on it. 70 ml of 4 % gel solution was prepared for each gel. Directly before casting the gel, polymerisation reaction was started by adding 70µl of TEMED and 140µl of Ammoniumperoxidisulfat (APS). After about 90 min in RT, the gel was completely polymerised. The equilibration of the gel occurred by running the gel for 20 minutes in advance before loading the samples. 1/5 volume of formamide-sample buffer was added to the samples and denatured at 99°C for 5 min. 5-6 µl sample was loaded on a gel. Duration of electrophoresis depended on the size of interested PCR products.

h. Silver staining of DNA in polyacrylamid gels

DNA fragments separated in polyacrylamid gel were visualised by silver staining according to the protocol of Bassam *et al.,* (1991).

The gel which was fixed on one of the glass plates, was incubated for 20 min in 1 L acetic acid (8 %) and then rinsed with H_2O three times for 2 min. Then it is incubated in 1 L silver solution for 30 min. Before adding, in a 4-10°C cooled developing solution, the gel is rinsed in 1 L H_2O for a maximum of 5 sec. The developing process was stopped by immediate transfer of the gel into an acetic acid (8 %) bath. After 3 min incubation the gel was washed in 1 l H_2O for 5-10 min and finally dried ON in RT.

i. Isolation, re-amplification and cloning of fragments from polyacrylamid gels

Bands of interest were cut out of the gel and incubated in 100 µl TE buffer for 2 hours. AFLP fragments were re-amplified, using the same conditions as during pre-amplification. Amplification products were cloned into the pGEM-Teasy vector (Promega, Madison, WI, USA) following the protocol of supplier.

2.4.16 Suppression Subtractive Hybridisation (SSH)

The SSH experiment was carried out using the PCR select cDNA subtraction kit (Clontech, Palo-Alto, CA, USA).

1 µg total RNA from each sample (control root, infected root, *A. euteiches* mycelium) was used to produce SMART-cDNA using the SMART cDNA synthesis kit (Clontech, Palo Alto, USA). This SMART-cDNA was used to perform a SSH using the PCR select cDNA subtraction kit (Clontech, Palo Alto, USA). SMART-cDNAs derived from control roots and *A. euteiches* were pooled 5:1 and used as a driver. Amplification products were cloned into the pGEM-Teasy vector (Promega,

Madison, USA), and transformed into supercompetent *E.coli* XL1-Blue MRF' cells (Stratagene, La Jolla, CA). Glycerol stocks of bacteria were stored at -80°C in Hogness Freeze Medium (HFM) containing 100 µg/ml ampcillin.
Solution I and solution II were autoclaved separately and mixed afterwards to obtain 1 l of 10 x HFM. The freeze medium was made by mixing 1 vol of 10 x HFM with 9 vol of LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0).

2.4.17 Reverse Northern Blot

To confirm that the subtracted cDNA fragments represent differentially expressed genes, reverse Northern blot analysis was performed.
cDNA fragments were amplified by colony PCR. 5 µl PCR product was separated in parallel on two 1.5 % agarose gels. After denaturation and neutralisation DNA was capillary transferred onto nylon membranes and fixed at 120°C for 30 min.
Tester and driver SMART-cDNAs were DIG labelled by PCR using High Fidelity *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). Two hybridisations were performed in parallel, one with tester SMART-cDNA probe and one with driver SMART-cDNA probe.

2.4.18 Virtual Northern Blot

This method gives similar information as provided by the classical Northern analyses and used to prove the single candidate clones for differential expression.
Tester and driver SMART-cDNAs were amplified by Long distance (LD) PCR. 20 µl from each PCR product were separated on 1.5 % agarose gel, denatured, neutralised, capillary transferred and fixed onto nylon membranes.
Candidate clones deriving from SSH-library were labelled using PCR DIG Probe Synthesis Kit (Roche Diagnostics, Taufkirchen, Germany) and used as a probe for the hybridisations.

2.4.19 Genomic Southern Blot

20 µg genomic DNA were digested ON with 12 units of appropriate restriction enzymes. The digested DNA was separated on 1 % agarose gel ON at 15 V. The gel was incubated 10 min in 0.25 M HCl, 15 min in denaturing solution, 15 min in neutralisation solution and finally rinsed in 2 x SSC. The DNA was capillary transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim,

Germany). DIG nonradioactive system (Roche Diagnostics, Mannheim, Germany) was used for hybridisation and detection.

2.4.20 GenomeWalking

DNA walking is a method for finding unknown genomic DNA sequences adjacent to a known sequence (Siebert *et al.,* 1995).

This method was used in this work to find out the 5′ upstream region of *MtMir*-1 gene, the potential promoter sequence. Universal GenomeWalker ™ Kit (Clontech, Palo-Alto, CA, USA) was used for this purpose, following the suppliers protocol PT3042-1 *(fig. 2).*

For the construction of DNA libraries, four fractions of *M. truncatula* genomic DNA were digested with four different restriction enzymes which leave blunt ends. For each library 2,5 µg genomic DNA was taken and digested with *Sma*I, *Ehe*I, *Eco*RV and *Ssp*I. Digested DNA was purified by the phenol/chloroform method and precipitated with ethanol. Then, GenomeWalker adapters were ligated by T4 DNA ligase at 16°C ON. Two gene specific primers (GSP1 and GSP2) were designed according to the instructions given in suppliers manual. The primary and nested PCRs were done using the Advantage 2 Polymerase mix (Clontech, Palo-Alto, CA, USA).

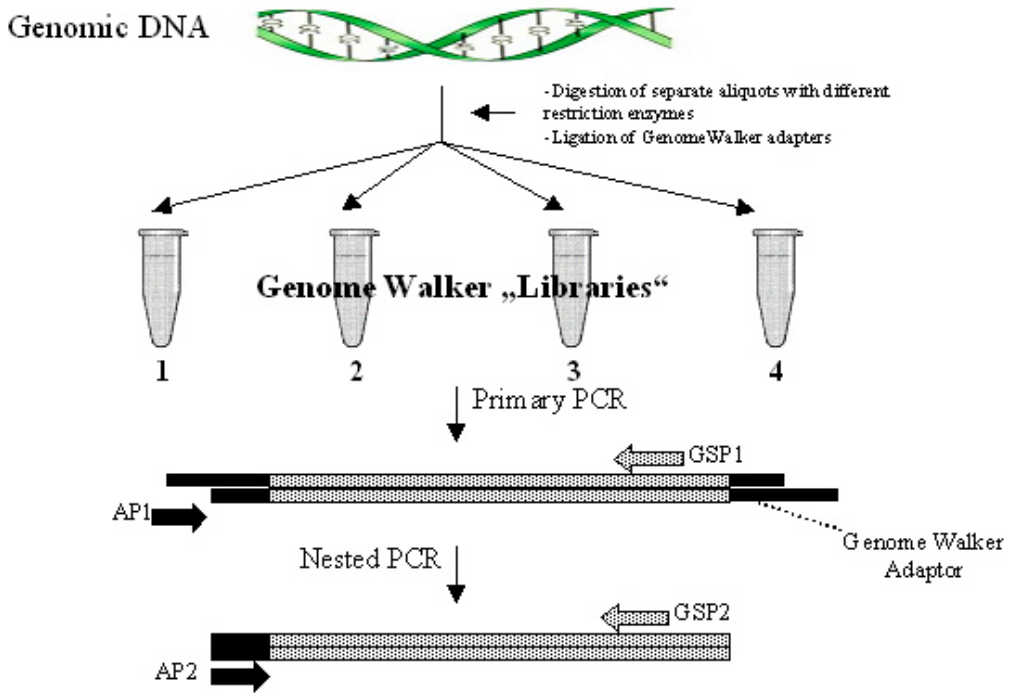

Fig. 2 Schematic representation of Genome Walker method.

PCR products were analysed on a 1.5 % agarose gel. Expected products were cut out of the gel, purified with DNA purification kit (Macherey-Nagel, Düren, Germany), cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced by MWG-biotech (Ebersberg, Germany).

2.4.21 Promoter deletion analysis

The promoter regions of interesting genes were amplified from genomic DNA of *M. truncatula* using promoter sequence specific primers. The promoters contained *Bam*HI and *Eco*RI restriction enzyme recognition sites at their 3´ and 5´ ends. These fragments were introduced into *Bam*HI and *Eco*RI restriction sites of the binary vector pLP100 (Szabados *et al.,* 1995) which encodes β-glucuronidase (GUS) (see appendix B for vector map).

The presence of the promoter fragments in the pLP100 vector were verified by PCR. Plant transformation vectors were electroporated into *A. rhizogenes*. Roots of *M. truncatula* were transformed as described by Boisson-Dernier *et al.,* (2001). The transgenic hairy root selection was taken place on Fåhraeus medium containing 25 μg/ml kanamycin. Approximately 1 cm kanamycin resistant, transgenic root tips were cut and transferred onto Petri dishes containing M-medium. M-medium contained 400 μg/ml augmentin in order to suppress the growth of agrobacteria.

Transgenic roots containing *A. euteiches* inducible promoter fragments were inoculated with zoospores of *A. euteiches*. Roots containing mycorrhiza inducible promoter fragments were inoculated with *G. intraradices*. *A. euteiches* inoculated roots were stained for GUS activity after 6 days of inoculation. *G. intraradices* inoculated roots were stained after 3 weeks.

2.4.22 Generation of the expression clones for gene silencing by RNA interference (RNAi)

The Gateway technology (Invitrogen, Carlsbad, CA, USA) was used to construct the binary vectors used for gene silencing by RNA interference.

It is a modified cloning technology based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989).

*att*B primers were designed following the recommendations of the manufacturer. 506 bp long coding sequence of the *MtMir*-1 gene, located between 200-705 bp, was amplified from cDNA using *att*B1 and *att*B2 primers. These primers have template

specific sequence at their 3´ end and the DNA recombination sequence at their 5´end. PCR products were purified by QIAquick PCR purification kit (Qiagen, Maryland, MD, USA). BP recombination reaction was performed with a*tt*B-PCR products and pDONR 207 donor vector to generate the entry clones. This reaction is catalysed by the BP Clonase enzyme mix. *E. coli* strain TOP 10F´ was transformed with donor vector and colony PCR was made to prove the presence of inserted fragments. Positive colonies were grown ON in LB medium containing 40 µg/ml gentamycin. Plasmids were extracted using Invisorb Spin Plasmid Mini Kit (Invitek, Berlin-Buch, Germany). 300 ng entry clone and 300 ng destination vector pFGC 5941 were used for LR recombination reaction. The pFGC 5941 vector has a kanamycin resistance gene (kan^R) for bacterial selection, a Basta® resistance (bar) gene for plant selection, a CaMV 35S promoter driving dsRNA synthesis and a 1352 bp *Chs*A intron from the petunia chalcone synthase A gene. (see appendix D for vector map). The LR reaction generates expression clones containing the fragments in antisence and sense directions linked with *Chs*A intron. This reaction is catalysed by the LR Clonase enzyme mix. The expression vector was transformed into *E. coli* strain TOP 10F´. Colony PCR was performed using the linker specific forward or reverse (*Chs*A-front, *Chs*A-back) and *att*B1 or *att*B2 primers, to prove the presence of inserted fragments in both directions. Positive colonies were grown ON in LB medium containing 50 µg/ml kanamycin. Plasmids were extracted using Invisorb Spin Plasmid Mini Kit (Invitek, Berlin-Buch, Germany).

When the antisense and sense strains are integrated into the plant genome, it is transcribed into RNA. Since the antisense and sense strains have complementary sequences, after transcription they form a double stranded RNA with a single stranded loop (*fig. 3*). *A. rhizogenes* was used to integrate the sense and antisense strains into the genome of *M. truncatula*.

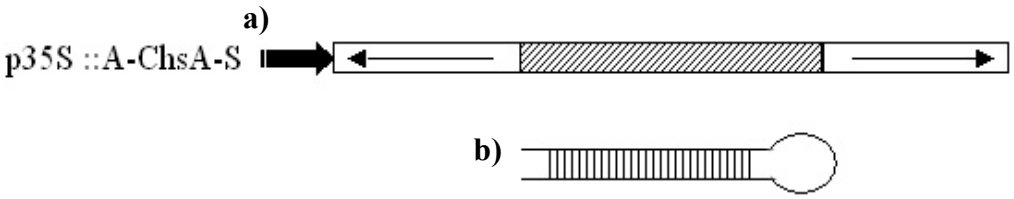

Fig. 3 **P35::A-ChsA-S construct**. a.- Gene specific sequences (open boxes with arrows indicating the orientation) in antisense (A) and sense (S) orientations were linked with a *Chs*A intron (hatched box) and controlled by the 35S promoter (solid arraw). When transcribed, it forms a double stranded RNA stem with a single-stranded loop (b).

A. rhizogenes strain ARqua I was transformed by electroporation, with destination vectors. The colonies were selected on LB plates containing 50 µg/ml kanamycin and 600 µg/ml streptomycin.

M. truncatula hairy roots were transformed as described by Boisson-Dernier *et al*., (2001). Selection of transformed hairy roots was a on modified Fåhraeus medium containing 5 µg/ml PPT. After 2-3 weeks transformed root tips were cut out and replaced on M-medium containing 400 µg/ml augmentin and the remaining plantlets were transferred into substrate (1:2 mixture of sterilised expanded clay and vermiculite), grown for 2 weeks and then inoculated with zoospores of *A. euteiches*. Root cultures were inoculated with zoospores after two weeks. After 6 dpi total RNA was extracted and used for further analyses. The control plants were transformed with *A. rhizogenes* containing an empty pFGC 5941 vector.

2.4.23 Microarray hybridisation

Medicago truncatula Mt8k microarrays were kindly provided by Helge Küster (University of Bielefeld, Germany). Mt8k microarray represents approximately 5,700 root interaction transcripts and 1,700 transcripts from flowers and developing seeds, three replicates of each transcript are present on a single microarray. Microarray hybridisation and data analyses were carried out as described by Küster *et al.,* 2003.

In this work the microarray hybridisation method was used for two purposes: In a first hybridisation experiment, total RNA from control and infected roots of *M. truncatula* after 30 min as well as 6 days after inoculation with *A. euteiches* was used for hybridisation.

a. Synthesis of amino-allyl-labelled first-strand cDNA from total RNA

16 µg total RNA was reverse transcribed by Superscript II reverse transcriptase (Stratagene, La Jolla, CA, USA) using 2 µl of double-anchored oligo $dT_{15}VN$ primer (see table a). Amino-allyl labelled cDNA was cleaned from nucleotides and other low-molecular weight molecules with amino groups, using CyScribe^{TM} GFX purification kit (Amersham Biosciences, Buckinghamshire, England). To 20 µl of cDNA 1 µl of 1M sodium bicarbonate was added. Coupling of Cy3 and Cy5 fluorescent dyes to amino-allyl labelled first strand cDNA was taken place in amber Eppendorf tubes for 1 h at RT. All remaining dyes were blocked with the amino

groups of hydroxylamine by adding 4,5 µl 4 M hydroxylamine and incubating at RT for 15 min. After this step, fluorescently labelled cDNAs of the two populations being compared, were mixed together and cleaned with Qia-quick PCR purification kit (Qiagen, Maryland, MD, USA). 2 µl of the probe were separated on 1 % agarose gel and scanned for Cy3 and Cy5 fluorescence on a Typhoon phosphoimager (Amersham Biosciences, Buckinghamshire, England).

b. PCR labelling of SMART-cDNA with amino-allyl

Alternatively, SMART-cDNA synthesised using the SMART cDNA synthesis kit (Clontech, , Palo-Alto, CA, USA), was amino-allyl labelled by PCR. 1 µg total RNA was reverse transcribed by PowerScript reverse transctiptase (Clontech, , Palo-Alto, CA, USA). 50 x dNTP stock solution including amino-allyl-dUTP (2:1 aadUTP/dTTP mix), SMART PCR primer and Advantage 2 Polymerase mix (SMART cDNA synthesis kit, Clontech, Palo-Alto, CA, USA) was used for amino-allyl labelling of SMART cDNA by LD-PCR. Coupling of Cy3 and Cy5 fluorescent dyes were the same as for RNA samples.

c. Hybridisation of microarrays

Microarrays were pre-hybridised in DIG EasyHyb hybridisation solution (Roche Diagnostics, Mannheim, Germany) containing 5 µg/ml sonicated salmon sperm DNA for 1 h at 42°C. Following pre-hybridisation, microarrays were washed with H_2O for 1 min at RT, shortly rinsed with ethanol and centrifuged for drying. Hybridisation was taken place in an automated slide processor (ASP) (Amersham Biosciences, Buckinhamshire, England). Hybridisation solution was DIG EasyHyb hybridisation solution (Roche Diagnostics, Mannheim, Germany) containing 45 µg/ml sonicated salmon sperm DNA. Samples were denatured at 65°C for 5 min immediately before injection. After 16 h of hybridisation, slides were washed twice in 2 x SSC for 1 min, once with 0,1 x SSC for 1 min at RT and centrifuged for drying. Scanning of slides in the Cy3 and Cy5 channels were carried out with a pixel size of 10 µm using a ScanArray 4000 microarray scanner (Perkin Elmer, Boston, MA, USA).

d. Analyses of microarray hybridisation data

ImaGene 5.0 (BioDiscovery Inc., Los Angeles, CA, USA) software was used for spot identification and quantification. The mean intensities of signal pixels and the mean intensities of local background pixels were calculated for each spot of the microarray images in both Cy3 and Cy5 channels. In case if in both channels, the difference between the mean intensity of the signal pixels and the mean intensity of local background pixels divided by the standard deviation of local background pixels was equal or less than 1, (R<=1) the spot was flagged empty. In addition, manual flags were set for the spots where hybridisation artefacts occurred. After the image processing the raw data was imported into EMMA 1.0 microarray analysis software (Dondrup *et al.,* 2003). During import the flagged spots were removed and the remaining spots were used for normalisation and the calculation of the expression ratios (M-value) and average signal intensities (A-value). The logarithm to the base 2 of the ratio of intensities was computed for each spot using the formula for the ratio $M=\log_2(R/G)$ and the average intensity in both channels was calculated using the formula $A=\log_2(RG)^{0,5}$. Where R and G denote intensities of the red and green channels. $R=I_{ch1}-Bg_{ch1}$ and $G=I_{ch2}-Bg_{ch2}$, where I_{ch1} or I_{ch2} is signal intensities of a spot in channel 1 or 2 and Bg_{ch1} or Bg_{ch2} is the local background intensity of a spot in channel 1 or 2, respectively (Dudoit *et al.,* 2002). To be able to work with the logarithm to the base 2 in case of negative R or G values and to reduce the variation introduced by very low absolute signal intensities, a floor value of 20 was introduced before normalisation. After applying normalisation M vs. A, scatterplots of the data were generated and lists of candidate genes for differential expression were obtained by applying t-test. Additionally, the confidence indicator p is computed for each gene, using Holm's method. Genes were considered differentially expressed if p<=0,1 and M>=1 or p<=0,1 and M>=-1.

3.1 Cultivation of *Aphanomyces euteiches*

The cultivation of the *A. euteiches* was successful under laboratory conditions. All the different stages of the life cycle of this oomycete could be observed.

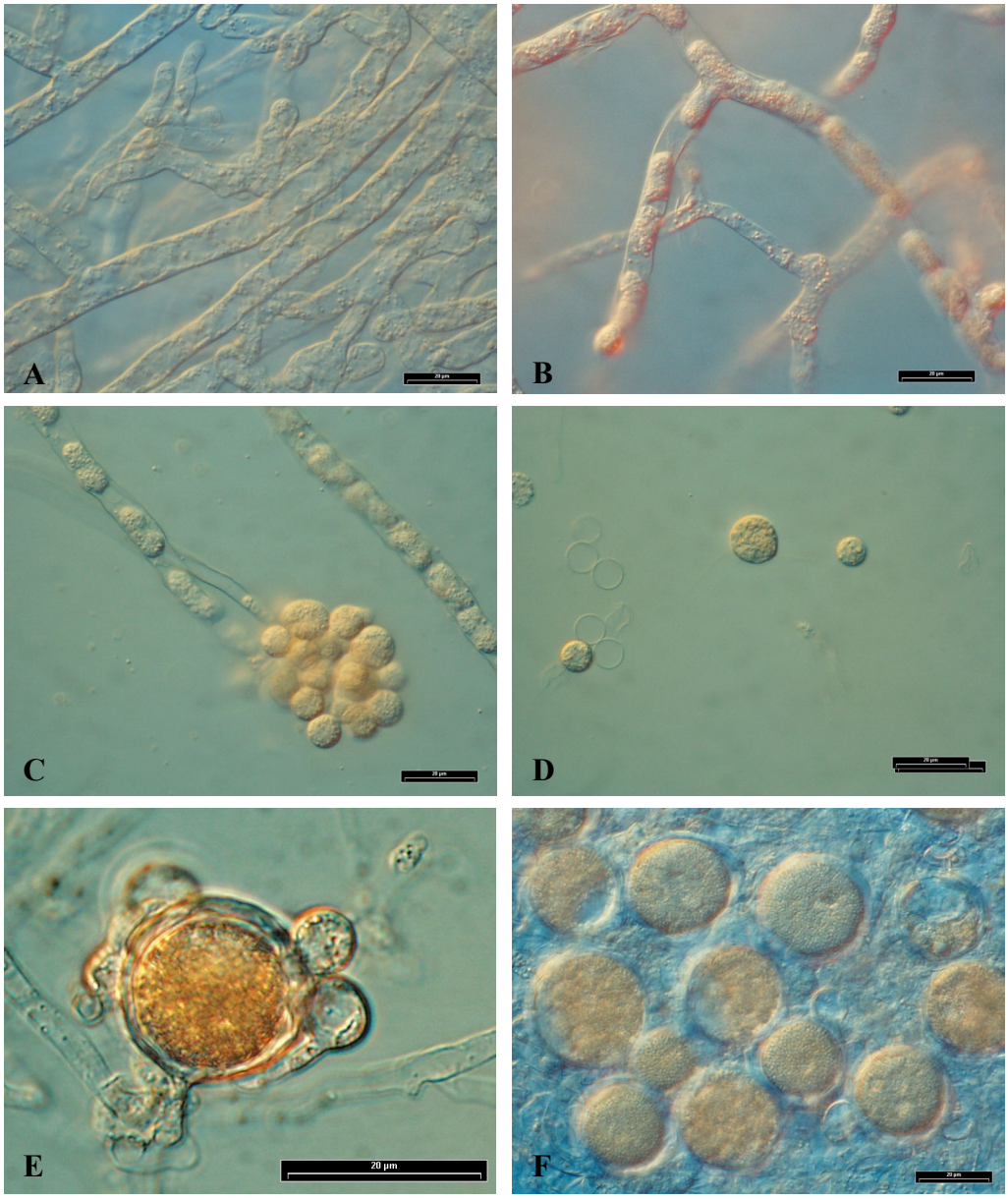

Fig. 4 **Vegetative and reproductive stages of oomycete *Aphanomyces euteiches* life cycle**. **A**. Nonseptate hyphae; **B**. Filamentous sporangium; **C**. Primary zoospores released from the sporangium; **D**. Kidney-shaped zoospore with two flagella; **E**. Anteridium penetrating oogonium; **F**. Mature oospores. The bars represent 20 µm.

The vegetative state of *A. euteiches* is a diploid hyphae which is nonseptate, i.e. lacking in cross walls (*fig. 4A*). On complete media like CMA (corn meal agar) or a liquid MPB (maltose peptone broth) medium, *A. euteiches* shows the typical fungal-like hyphae growth. Asexual reproduction starts by the formation of sporangia. The sporangia of *A. euteiches* are filamentous and resemble vegetative hyphae (*fig. 4B*). Under nutrient-limiting conditions, sporangia germinate indirectly by producing zoospores which are asexual spores that are mobile by means of two flagella (*fig. 4C; D*).

Zoospore formation *in vitro* could be initiated by washing the hyphae in sterile lake water. Using this protocol approximately $2x10^5$ zoospore/ml could be obtained. Sexual reproduction in *A. euteiches* occurs after formation of two dissimilar gametangia: a large round oogonium and a smaller anteridium (*fig. 4E*). Oogonium fertilisation results in a thick-walled zygote called an oospore (*fig. 4F*). Oospores function as resting spores during the disease cycle.

3.2 Colonisation of *Medicago truncatula* root system with *Aphanomyces euteiches*.

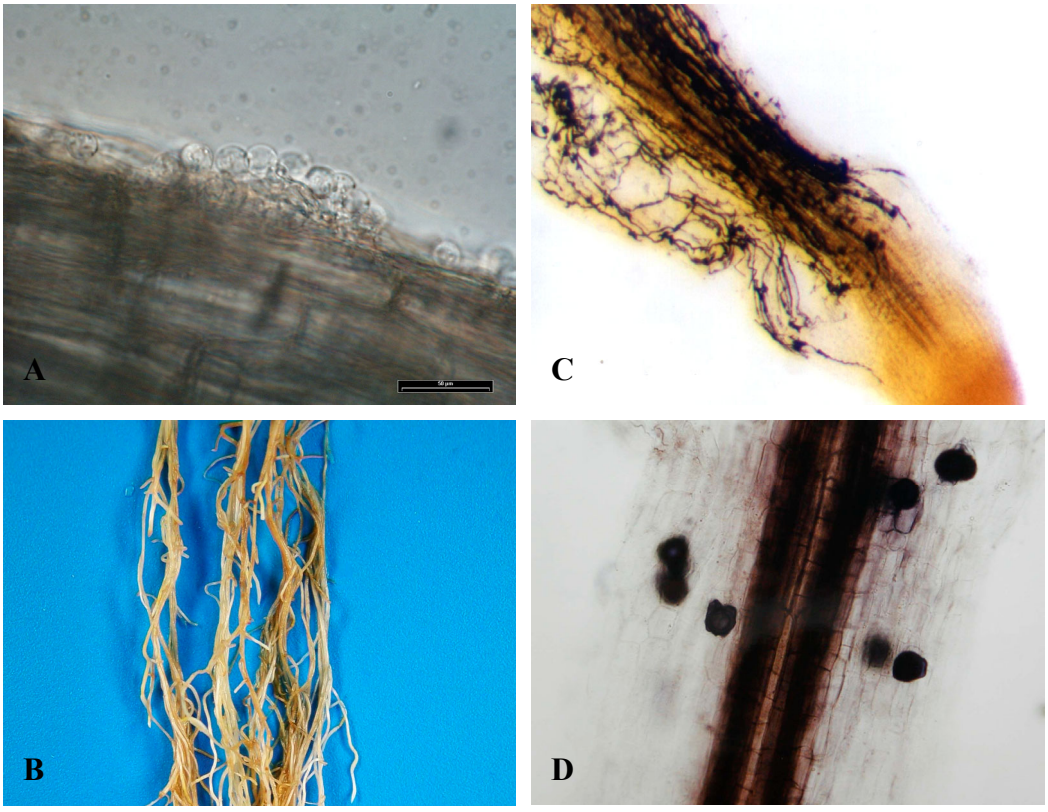

Fig. 5 **Colonisation of *M. truncatula* roots by oomycete *A. euteiches*. A.** Zoospores of pathogen attached to the root. **B.** Sympthoms of root rot. **C.** Oomycete hyphae stained for ALP. **D.** Trypan blue stained oospores of pathogen in the root.

One week old seedlings of *M. truncatula* were inoculated with zoospores of *A. euteiches* (10^6 zoospore/ml). Zoospores of the pathogen were observed attached to *M. truncatula* root surfaces after 6 hours *(fig. 5A)*. The typical disease symptoms with yellow-honey brown root discolorations could be observed after 6 days *(fig. 5B)***.** Active hyphae of *A. euteiches* could be visualised as a black precipitate in the root cortex by alkaline phosphatase staining (ALP) *(fig. 5C)*. Oospores in the root cortex were visible after trypan blue staining *(fig. 5D)*. 5 days after inoculation the percentage of root length colonised with hyphae rapidly increased. After 10 days oospores started to develop. At the latest time point analysed, 21 dpi, oospores were found in more than 60 % of the root system. Pathogen structures have never been observed in control plants mock inoculated with sterile lake water.

3.3 Detection of *A. euteiches* glucose-6-phosphate dehydrogenase (Gd) activity in *M. truncatula* roots

In order to analyse the metabolic stage of *A. euteiches* in infected roots, the activity of *A. euteiches* specific glucose-6-phosphate dehydrogenase (Gd) was analysed in infected root systems.

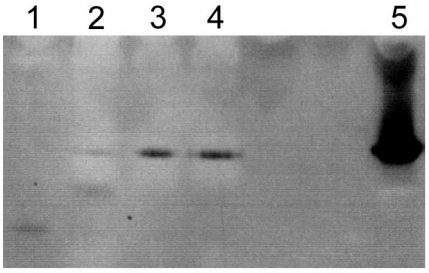

Fig. 6 Glucose-6-phosphate dehydrogenase (Gd) activity of *A. euteiches* in *M. truncatula* roots after 6 days of infection. Lane 1- protein extract of non infected *M. truncatula* root as negative control. Lane 2, 3 and 4- total protein of *M. truncatula* root infected with *A. euteiches* (4 µl, 8 µl and 10 µl). Lane 5- protein extract of *A. euteiches* mycelium as positive control.

Total protein was extracted from *M. truncatula* roots 6 days after infection with *A. euteiches*. *A. euteiches* total protein was extracted from 6 day old mycelium grown in MPB medium and used as a positive control. Total protein of infected roots and mycelium was separated electrophoretically and the Gd activity was detected as described by Soltis and Soltis (1989) (*fig. 6*). This experiment shows that *A. euteiches* shows detectable metabolic activity in *M. truncatula* roots 6 days after inoculation.

Transcription profiling:

3.4 cDNA-AFLP results

Using an amplified restriction fragment polymorphism (AFLP)- derived technique for RNA fingerprinting (cDNA-AFLP) (Bachem *et al.,* 1996), transcriptional changes in *M. truncatula* roots infected with *A. euteiches* have been analysed. The aim of this approach was to compare different time points of the interaction in order to determine an early time-point where highest levels of transcriptional changes occur.

Inoculated and non-inoculated roots were harvested at 6, 14 and 21 days after inoculation and used for RNA extraction. In this small-scale cDNA profile comparison by cDNA-AFLP, 14 primer combinations were performed using three types of *Apo*I and six different *Mse*I selective primers (for primer sequences see table-1). For each primer combination, 50-80 bands were observed. The largest detectable amplification products were about 1000 bp in size and the smallest fragments were approximately 100 bp. Comparison of cDNA-AFLP patterns revealed two up-regulated and two down-regulated fragments in infected roots *(fig. 7).* Differential RNA accumulation of these four fragments already occurred after six days, the earliest time point analysed, and remained constant until 21 days after inoculation. The four differentially expressed bands were re-amplified and cloned. Five clones were randomly picked for each fragment and all clones of one transcript represented the same sequence. This result confirmed that cDNAs which were sequenced correspond to the differentially expressed fragment on the gels. The sequences are shown in *fig. 8*. The sequence analysis showed that all four fragments are highly similar to already existing plant sequences in GenBank database *(table-4).*

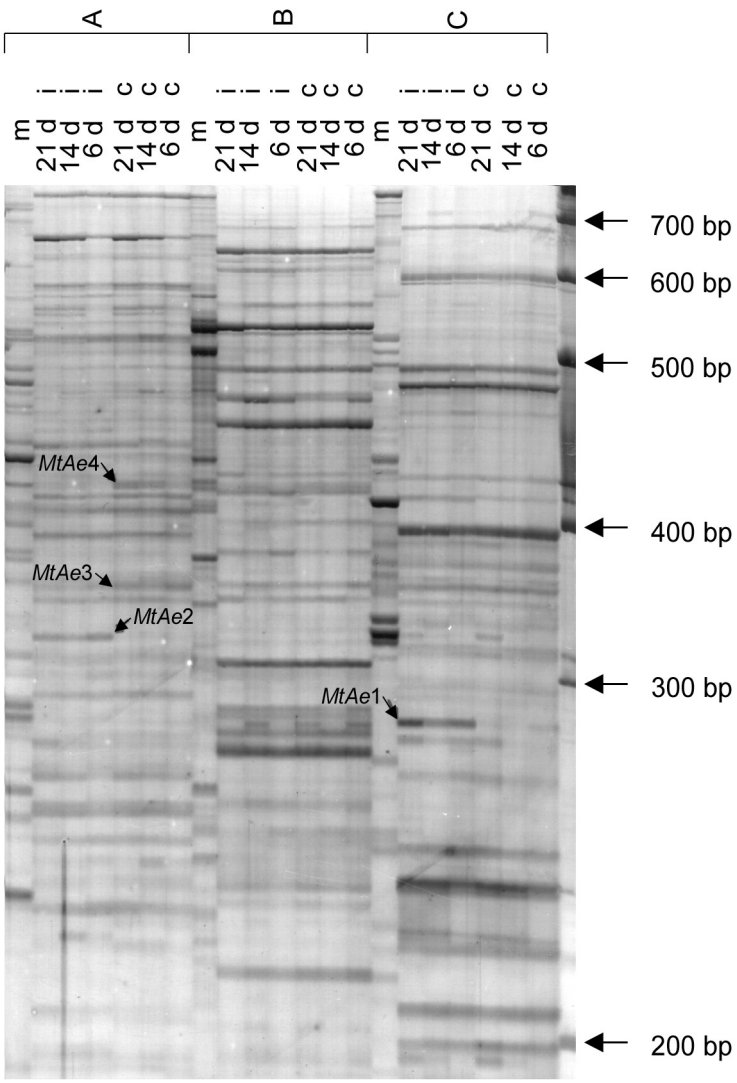

Fig. 7 Example of a cDNA-AFLP gel produced by amplification of templates derived from non-infected (c) and infected (i) roots of *M. truncatula* after 6, 14 and 21 days after infection with *A. euteiches*. *A. euteiches* mycelium derived templates are marked (m). The different primer combinations marked A (Apo2/Mse5), B (Apo2/Mse4) and C (Apo2/Mse2).

***MtAe*1**

GATGAGTCCTGAGTAAACCCACCTCACATTCACCCTAGGGGAACAGAGAT 50
CCTTATAGTACTCGAAGGCACTCTTTATGTTGGATTTGTCACGTCCAATC 100
AAGACAACAATCGTCTCTTCACCAAAGTGCTAAACAAGGGTGATGTGTCT 150
GTGTTCCCAATTGGTCTTATTCATTTTCAACTAAATGTGGGATATGGCAA 200
CGCTGTTGCTATTGCTGGACTTAGCAGTCAAAACCCAGGAGTTATCACCG 250
TTGCAAATGCTTTGTTTACTCAGGACTCATCA 282

***MtAe*2**

GATGAGTCCTGAGTAACTGGGACTACAACACTGCCAGTGTATACTGTGCT 50
ACCTGGGATGCCAACCAGCCCTTGTCATGGCGTAGCAAATATGGTTGGAC 100
TGCCTTTTGTGGACCAGCTGGGCCAACAGGCAGAGATTCTTGCGGCAAAT 150
GCTTGACTGTGACAAATACTGCAACTGGAGCTCAGGTAACAGTGAGAATA 200
GTGGACCAATGCTCCGACGGTGGACTTGACTTAGGTGTGAATGTCTTCAA 250
TCAAATTGATACCAATGGACAGGGCGTTCAGAATGGTCACCTTACAGTTA 300
CTCAGGACTCATCA 314

***MtAe*3**

GATGAGTCCTGAGTAACTGGGCATTGATTAGGTGCAGCACACCACCAGAA 50
GTACCAAGACCAGTGGAAAATGAGCCGCCGCCACCGCCACCACCAATGAA 100
GGTTGGACCACCAGTTGATGCTGTCGGGGTTGGCAGTACTAGTAAGAGGA 150
TGTCGGGGAATAATGAAATGAAGTCTGGGGGTAAATATCTGGATTTTGAG 200
TTCTTTTAGTTTGGATTCCTTGATCTACTTTTTATTGTATTTGCCATGAA 250
TATGATCAGTTATTTTTGCAGCAGCTGCTCAGTATTACCATGTTAGTTCA 300
GCTTTACCTATTTTGTAGTTACTCAGGACTCATCA 335

***MtAe*4**

GATGAGTCCTGAGTAACTAAGTTTGGATGAGTTGTTGCCAGAAGAGTTTT 50
TGGAGAGGACAAAGGAGAAGGGAATGGTTGTTAGAAACTGGGCACCACAA 100
GGTTCAATACTAAGACATAGCTCAGTTGGTGGGTTCGTGACTCATTGTGG 150
ATGGAACTCCGTGTTGGAAGCTATTTGCGAAGGAGTTCCAATGATAACGT 200
GGCCACTTTACGCGGAGCAGAAGATGAATAGATTGATTTTGGTGCAAGAA 250
TGGAAAGTGGCTTTGGAATTGAATGAGTCAAAAGATGGGTTTGTGAGTGA 300
AAATGAGTTGGGGGAGAGAGTTACTCAGGACTCATCA 337

Fig. 8 The nucleotide sequences of the four differentially expressed fragments derived from cDNA-AFLP analysis. The primer sequences are underlined.

Table-4. Sequence annotation of differentially expressed cDNA-AFLP fragments

Fragment nr.	Best GenBank database match	RNA accumulation pattern	MtGI match
*MtAe*1	germin-like protein, *Pisum sativum*	increased	TC87487
*MtAe*2	pathogenesis-related protein 4A, *Pisum sativum*	increased	TC87236
*MtAe*3	serine/threonin protein kinase, *Glycine max*	decreased	-
*MtAe*4	UDP-glycose:flavonoid glycosyltransferase, *Glycine max*	decreased	TC78509

3.5 Confirmation of differential RNA accumulation of PR4 gene

Sequence specific primers were made for one PR4-like gene, corresponding to the MtAe2 fragment (table-4). Semiquantitative RT-PCR showed increased RNA accumulation of the PR4 gene (designated *MtPR4*-1 in this work) in infected roots *(fig. 9)*. For this reason, the *MtPR4*-1 gene was used as internal control marker, representing an *A. euteiches* induced gene, in subsequent analyses.

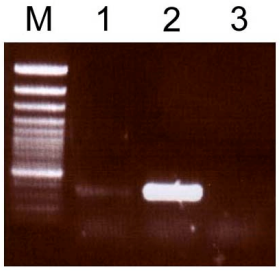

Fig. 9 RT-PCR amplification of PR4 from 1- control roots of *M. truncatula*, 2- infected roots, and 3- *A. euteiches* cDNA. M- 100 bp Plus DNA marker.

Transcription profiling:

3.6 Generation of cDNA library by Suppression Subtractive Hybridisation (SSH)

A cDNA-library was generated by suppression subtractive hybridisation (SSH) to obtain a large number of ESTs, representing genes up-regulated in *A. euteiches*-infected roots of *M. truncatula* 6 days after inoculations. To avoid the enrichment and cloning of cDNAs of *A. euteiches* origin, the RNA of non-infected roots was mixed with RNA extracted from *A. euteiches* mycelium. PCR fragments were cloned and about 2000 clones were obtained. Of this cDNA population, 192 clones were analysed concerning their RNA accumulation pattern in infected roots by reverse Northern hybridisation analyses. 51 of these cDNAs were strongly induced in *A. euteiches* -infected roots, whereas after hybridisation to the probe pooled from control roots and *A. euteiches* mycelium, no or very weak signals were detectable *(fig. 10)*. Sequence annotations of differentially expressed cDNAs are shown in table-8.

Within this up-regulated cDNAs, three fragments encoded the *MtPR4*-1 gene, which was already found by cDNA-AFLP. One fragment encoded a class-10 PR-protein. With only one exception, all fragments showed highest similarities to plant genes and in most cases to legume sequences.

After confirmation that the library contains to a high extent cDNAs from plant genes induced by *A. euteiches* infection, 560 ESTs were generated. SSH-cDNA sequences were designated as *Mt*APHEU (*Medicago truncatula-Aphanomyces euteiches*). 504 *Mt*APHEU-ESTs with a length of more than 100 bp were submitted to GenBank and the sequences can be found under accession numbers AJ547891-AJ548395.

Clustering of these ESTs according to the TIGR protocol revealed 74 tentative consensus (TC) sequences and 269 singletons with a sequence length more than 100 bp. A TC encoding a class 10 pathogenesis-related protein, assembled from 13 SSH-ESTs was the most redundant sequence among the cDNA population.

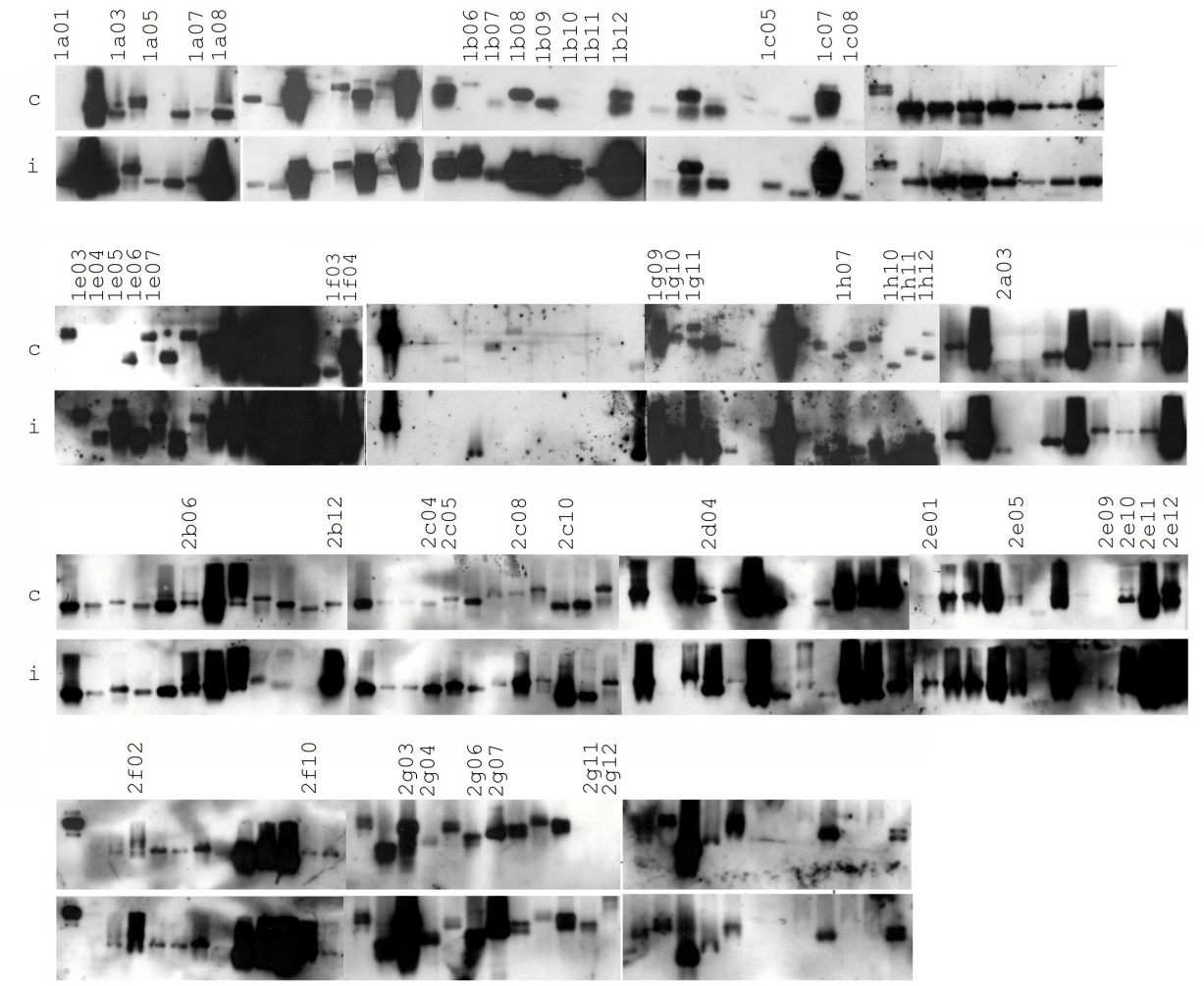

Fig. 10 **Reverse Northern hybridisation**. 192 PCR fragments from the SSH library were PCR amplified and blotted onto two identical nylon membranes. One membrane was hybridised with the digoxigenin labelled cDNA probe derived from control roots (c). The second membrane was hybridised with digoxigenin labelled cDNA probe derived from infected roots (i).

Table 5. Sequence annotation of 51 clones which showed increased mRNA accumulation in *A. euteiches* infected roots of *M. truncatula*.

#	Mtaehac-index	Length (bp)	Matching sequence from the Genbank "nr" data base (blastX)	Origin of matching sequence	MtGI identity
1.	1a01	505	aba responsive protein abr17	*Pisum sativum*	TC76640
2.	1a03	529	albumin precursor 1	*Pisum sativum*	TC88792
3.	1a05	618	small GTPase Rab2	*Nicotiana tabacum*	TC77335
4.	1a07	737	Aba responsive protein abr17	*Pisum sativum*	TC82086
5.	1a08	526	pathogenesis-related protein 4A	*Pisum sativum*	TC87236
6.	1b06	255	no hits	…	…
7.	1b08	269	chitinase	*Medicago truncatula*	TC85742
8.	1b09	256	no hits below $1e^{-10}$	…	TC85238
9.	1b10	444	no hits below $1e^{-10}$	...	TC80304
10.	1b11	400	aconitate hydratase precursor	*Gracilaria verrucosa*	...
11.	1b12	404	class 10 PR protein	*Medicago sativa*	TC76518
12.	1c05	419	no hits below $1e^{-10}$	...	
13.	1c07	238	no hits below $1e^{-10}$	*Medicago truncatula*	TC77207
14.	1c08	41	no hits	...	...
15.	1e03	461	no hits	**I. …**	…
16.	1e04	386	thaumatin-like protein 1 precursor	*Pyrus pyrifolia*	TC77149
17.	1e05	0	...	...	...
18.	1e06	44	no hits	...	...
19.	1e07	327	serine palmitoyltransferase	*Lotus japonicus*	TC86313
20.	1e08	316	no hits below $1e^{-10}$	*Arabidopsis thaliana*	TC79474
21.	1f02	6	no hits	...	...
22.	1f04	544	dehydrin related protein	*Pisum sativum*	TC76699
23.	1g09	21	no hits	...	...
24.	1g10	336	no hits	...	TC83308
25.	1g11	599	putative senescence-associated protein	...	TC76492
26.	1h07	310	pathogenesis-related protein 4A	...	TC87236
27.	1h10	537	cytochrome P450	*Pisum sativum*	TC81652
28.	1h11	324	polygalacturonase inhibitor protein	*Pisum sativum*	TC78258
29.	1h12	50	no hits	*Arabidopsis thaliana*	TC85744
30.	2a03	337	probable cinnamoyl-CoA reductase	*Arabidopsis thaliana*	TC78732
31.	2b06	0	...	...	...
32.	2b12	191	no hits	*Brassica napus*	TC76608
33.	2c04	307	pathogenesis-related protein 4A	*Pisum sativum*	TC81815
34.	2c05	556	no hits	...	TC83308
35.	2c08	159	no hits	...	...
36.	2c10	22	no hits	...	...
37.	2d04	291	protein F4N2.2	*Arabidopsis thaliana*	TC87620
38.	2e01	402	thaumatin-like protein 1 precursor	*Pyrus pyrifolia*	TC77149
39.	2e05	599	no hits	...	TC90135
40.	2e09	0	...	...	...
41.	2e10	0	...	...	...
42.	2e11	90	no hits	...	TC76518
43.	2e12	229	no hits below $1e^{-10}$	...	TC76394
44.	2f02	501	putative extracellular dermal glycoprotein	*Cicer arietinum*	TC77209
45.	2f10	309	putative lemir (miraculin) protein	*Arabidopsis thaliana*	TC87871
46.	2g03	579	putative ripening related protein.	*Cicer arietinum*	TC85249
47.	2g04	395	glucan-endo-1,3-beta-glucosidase precursor	*Cicer arietinum*	TC77400
48.	2g06	270	Pathogenesis related protein 1 precursor	*Arabidopsis thaliana*	TC86002
49.	2g07	539	hypothetical protein	*Arabidopsis thaliana*	TC87620
50.	2g11	0	...	...	...
51.	2g12	463	probable 12-oxophytodienoate reductase	*Vigna unguiculata*	TC85807

To get a global view on this EST population, the whole non clustered EST-collection was annotated and classified into 11 broad categories *(fig.11)*. Only one functional class was assigned to each cluster. Notably, the cluster of genes corresponding to abiotic stress and development contained six singletons with

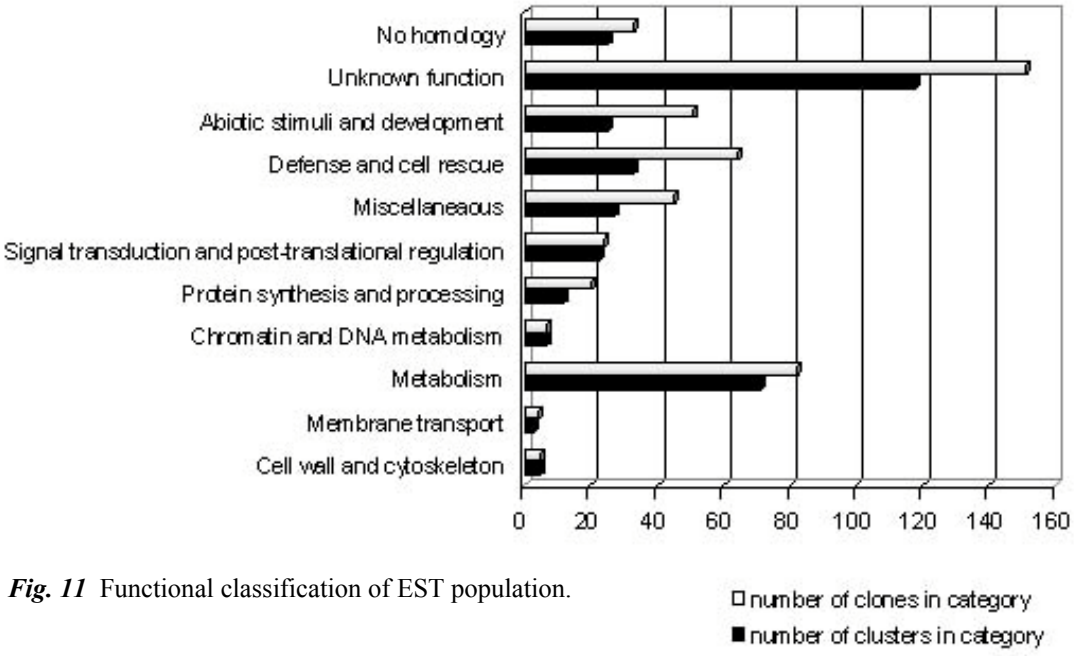

Fig. 11 Functional classification of EST population.

similarity to abr 17, an ABA-responsive protein of *P. sativum*. Most of ESTs had to be classified into the "unknown function" category and a large number of ESTs had to be assigned to the "no homology" category.

Surprisingly, 46 out of the 560 non-clustered ESTs did not match any EST deposited in the *Medicago truncatula* gene index (MtGI version 5.0). This indicates that these ESTs correspond to new unknown *M. truncatula* genes, which have not been identified in previous sequencing projects. Among these 46 ESTs, 25 did not show any significant similarity (e-value below $1e^{-10}$) after blastx - search of deposited GenBank database. One gene (1b11) is found to be of *A. euteiches* origin.

ESTs without similarities to already existing MtGI (version 5.0) entries were further analysed concerning their differential expression during *A. euteiches* infection. Of these 46 ESTs, eight (1b11, 1c05, 1c11, 1f09, 1h03, 1h06, 1h09 and 2c06) were already analysed by reverse Northern within the first pool of 192 clones. Three of these sequences (1b11, 1c05 and 1h03) showed significantly increased signals after hybridisation to infected root cDNA. The inserts of the remaining 38 clones were

analysed in a second reverse Northern blot. The housekeeping gene *MtEf1*-α was used as non-regulated control gene. The *MtPR4*-1 was used as an internal positive control. The *MtEf1*-α showed even and strong signal to infected as well as to non-infected root cDNAs. In contrast, compared to the control root cDNA, *MtPR4*-1 and seven of the 38 genes, showed stronger signals to the infected root cDNA *(fig. 12)*. Together with the three ESTs detected from the first reverse Northern hybridisation, 10 of 46 ESTs showed increased RNA accumulation in the roots infected with *A. euteiches.*

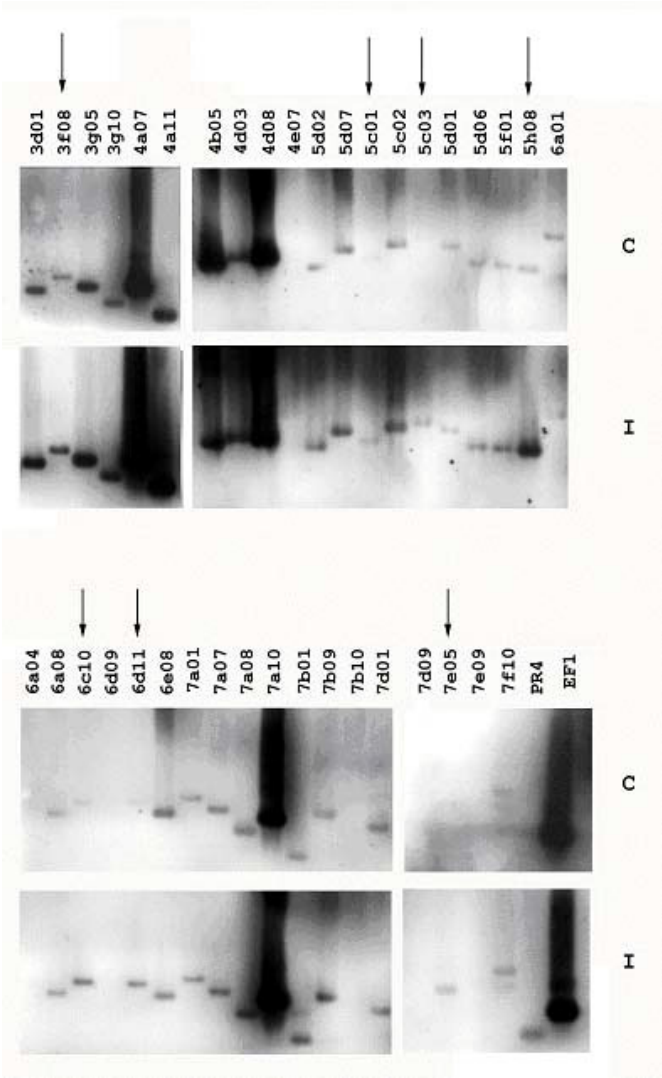

Fig. 12 In this reverse Northern hybridisation, 38 out of 46 new *M. truncatula* genes from SSH-cDNA bank were analysed for their RNA accumulation in control and *A. euteiches* infected roots. The inserts of the clones were amplified, blotted on two nylon membranes and hybridised to labelled cDNA of non-infected (c) and infected (i) roots. *MtAPHEU*-index numbers of corresponding clones are indicated. Internal controls are marked by: PR4 (inducible possible control) and Ef1 (constitutively expressed control).

Five of these ESTs (1c05, 1h03, 3f08, 5h08 and 7e05) showed strong signals after hybridisation to *A. euteiches* infected root cDNA, and weaker, but detectable signals after hybridisation to control root cDNA. This indicates that the corresponding genes are plant genes which are transcribed at a basal level in non-infected roots but are significantly up-regulated after *A. euteiches* infection.

3.7 Confirmation of plant origin of the differentially expressed cDNA-fragments

The plant origin of the remaining five cDNA fragments (1b11, 5c01, 5c03, 6c10 and 6d11), showing only weak signals after hybridisation to the control root cDNA, were analysed using gene specific primers. Amplification of these fragments from *M. truncatula* and *A. euteiches* genomic DNA showed that all the fragments except the fragment 1b11are of plant origin *(fig. 13)*.

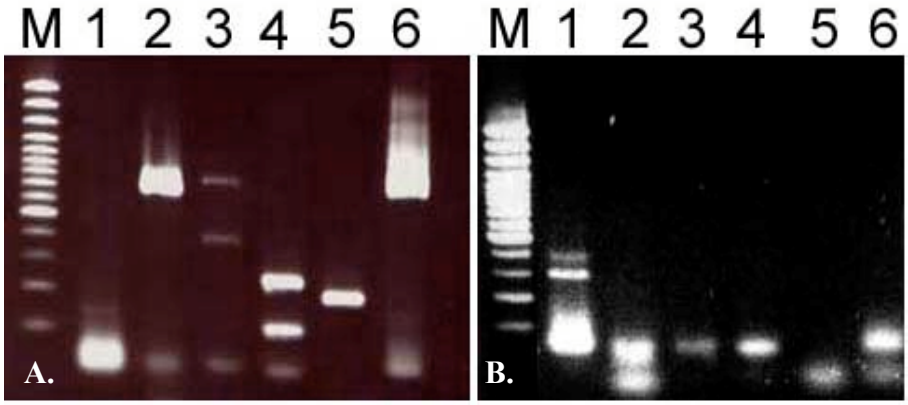

Fig. 13 Amplification of five cDNA fragments from **A.** *M. truncatula* and **B.** *A. euteiches* genomic DNA.
M- 100 bp Plus DNA marker
1- 1b11
2- 5c01
3- 5c03
4- 6c10
5- 6d11
6- *MtEf1*-α plant control gene

3.8 Analyses of the *MtMir*-1 gene

From the SSH library, TC 0072 (647 bp) was chosen for further analyses. The *MtMir*-1 cDNA was assembled from seven different ESTs (mtaehac7h07; 4f09; 5g10; 4e01; 2f10; 7a12; 06f01) (*fig. 14A*).

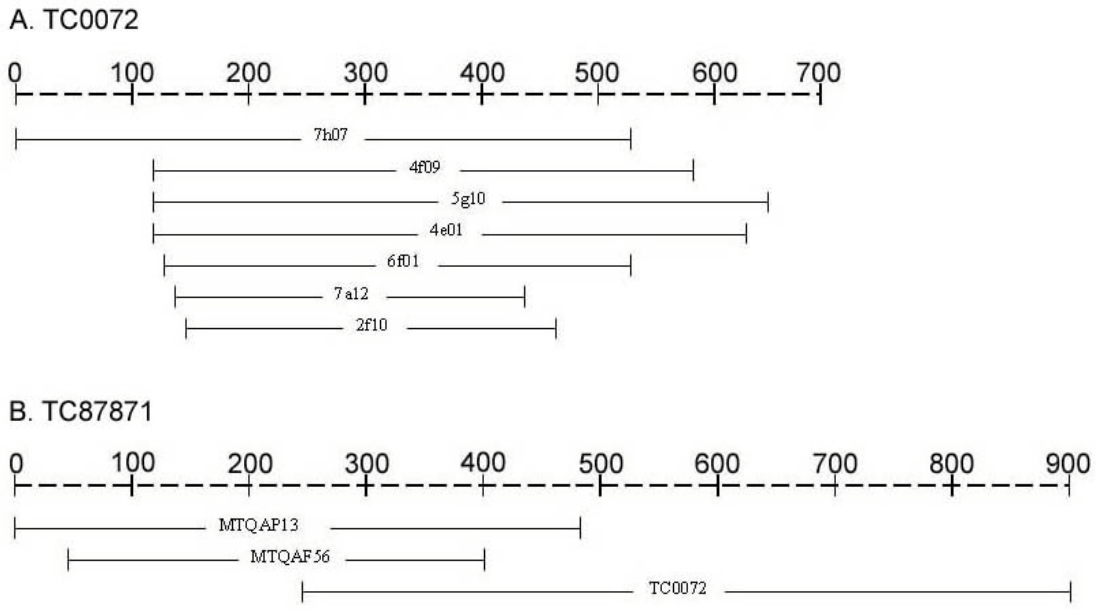

Fig. 14 **A**. TC0072 (647 bp) from the SSH-cDNA bank, is assembled from seven different ESTs. **B**. MtGI TC87871 is assembled from TC0072 and two ESTs (MTQAP13 and MTQAF56) derived from nematode infected roots.

Two ESTs of the TIGR *M. truncatula* EST database (MtGI version 5.0), deriving from nematode infected *M. truncatula* roots, were found to be homologous to TC0072. These sequences could be assembled with TC0072 *(fig. 14B)*. The corresponding 905 bp sequence is submitted in the TIGR *Medicago truncatula* Gene Index (MtGI version 7.0) under TC number 87871.

Blastn search of the NCBI (http://www.ncbi.nlm.nih.gov/) databank revealed highest homologies to a tumor-related protein sequence NF34 of tobacco (*Nicotiana tabacum*), a miraculin like protein *LeMir* from tomato (*Lycopersicon esculentum)*, tumor-related protein from interspecific hybrids between *Nicotiana glauca* and *Nicotiana langsdorffi.* Therefore, the *A. euteiches* induced gene found of this work was designated *MtMir*-1 (*Medicago truncatula*-miracalin like protein 1) according to the proposed *M. truncatula* nomenclature (VandenBosh and Frugoli, 2001).

3.8.1 RNA accumulation studies of *MtMir*-1 gene

Sequence specific primers were constructed for *MtMir*-1. RT-PCR result shows strong RNA-accumulation of *MtMir*-1 in the roots infected with *A. euteiches (fig. 15)*. No PCR product could be amplified from non-infected roots or from leaves of non infected and infected plants *(fig. 16)*. No amplification products were obtained using *A. euteiches* mycelium. This result implies, that the gene is transcribed exclusively in infected roots of *M. truncatula*.

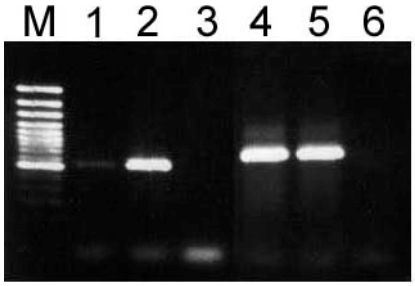

Fig. 15 **RT-PCR**. Sequence specific primers of *MtMir*1 were used to amplify the fragments from cDNAs derived from control (1) and infected roots (2) (6 days after inoculation) of *M. truncatula* as well as from cDNA derived from *A. euteiches mycelium* (3). The *MtEF1α* (*M. truncatula* transcription elongation factor 1α) gene was used as constitutively expressed control (4;5;6). M- 100 bp Plus DNA marker.

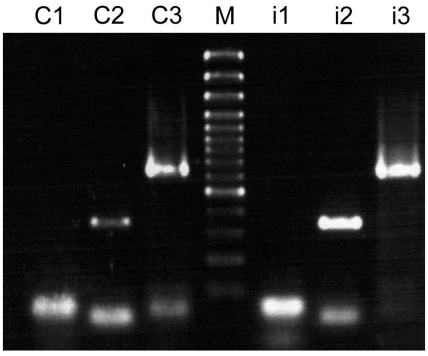

Fig. 16 **RT-PCR.** *MtMir*1 amplification from leaves of non infected and infected plants (c1; i1). The internal control genes were used. c2, i2- MtPR4-1. c3, i3- tef1α (transcription elongation factor 1α) as a constitutively expressed control gene.

3.8.2 Differential hybridisation analyses of *MtMir*-1

The results of reverse northern blot (see *fig. 10*, clone 2f10) and RT-PCR analysis could be confirmed by Virtual Northern Blot hybridisation *(fig. 17)*. *MtMir*-1 mRNA could be detected only in *M. truncatula* roots after *A. euteiches* infection.

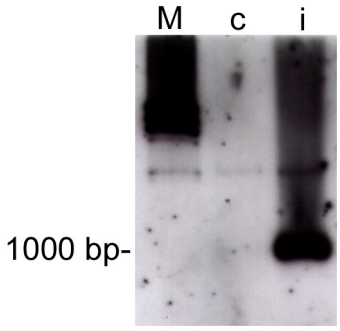

Fig. 17 **Virtual Northern Blot hybridisation.** The same amount of cDNA of control (c) and infected (i) roots were blotted on nylon membrane and hybridised against *MtMir*1 gene specific sequence labelled with digoxigenin (DIG). M-DIG-labelled marker.

3.8.3 Analysis of the *MtMir*-1 cDNA sequence

3' and 5' RACE-PCR techniques were performed to obtain the complete cDNA sequence of the *MtMir*-1 gene. First strand SMART-cDNA was amplified from total RNA of infected roots using SMART® PCR cDNA Synthesis Kit (Clontech, Palo-Alto, CA, USA) and was used as a template for RACE-PCRs. 5' end of the cDNA was amplified using a gene specific reverse primer (MtMir1_5'RACE) and a SMART II Oligonucleotide. For the amplification of the 3' end, gene specific forward primer (MtMir1_3'RACE) and CDS primers were used (see table-1 for primer sequences). About 300 bp fragment of the 5' end and about 250 bp of the 3' end of the cDNA were amplified, cloned and sequenced.

These fragments could be aligned with the already known sequence of the gene and *MtMir*-1 could be extended up to 862 bp *(fig. 18)*.

3.8.4 Sequence analyses of *MtMir*-1 gene

In the 862 bp *MtMir*-1 cDNA sequence the first ATG triplet is located 175 bp downstream of the 5´cDNA end.

ATCCATCACAAACCAAACTTAATTACAAACTASTTAAAGCATACAGAACA 50
ATGAAAAACACATTGCTAGCATTTTTCTTTCTCTTTACCTTCTTAAGCTC 100
ACAACCACTTCTTGGAGCAGCTGAAGCTTCAAATGAACAAGTGGTTGACA 150
CATTAGGCAAGAAACTCCGAGCTG**ATGCTAATYACTAGTRTTATCCCGGT** 200
TCCCATATACAAATGTGGACCTTATGGTAAATGTAGAAGCAGTGGTTCAA 250
GTCTTGCCCTAGCAAGTAATGGAAAAACTTGCCCTCTTGATGTTGTGGTT **300**
GTTGATAGATATCAAGCTTTGCCACTTACTTTTATCCCTGTTAACCCTAA 350
GAAGGGTGTTATTCGTGTGTCTACTGATTTARACATCAAATTCTCATCTC 400
GTGCTACTTGTCTACACCATTCCATGGTGTGGAAGCTTGATCGTTTTAAT 450
GTATCTAAGAGACAATGGTTTATTACTATTGGTGGTGTTGCTGGAAATCC 500
GGGATGGGAAACCATTAATAATTGGTTCAAGATTGAGAAGTATGGTGATG 550
CTTATAAGTTGGTGTTTTGCCCTAGTGTGGTGCAGTCTTTCAAGCATATG 600
TGTAAGGATGTTGGAGTATTTGTTGATGAAAATGGAAATAAGCGTTTGGC 650
TTTAAGCGATGTTCCCCTTAAAGTTAAATTTCAACAAGCTTGATGAAAGC 700
CTCTCTAATATAATGCATAAATAGATATGAAAACATGCATTTCTCTTTCA 750
ATAATGTTTATTGGGATCCTCAAAGTTAGGCTTCCTTATTACAAACAAAT 800
AAAATAAATAAACTTATTAATGTTTATCATCCTAAAAAAAAAAAAAAAAA 850
AAAAAAAAAAAA-3' 862

Fig. 18 **The complete cDNA sequence of *MtMir*-1**. ORF is indicated in boldface type. 5´end is underlined.

The 516 bp long open reading frame encodes a protein of 172 amino acids. The 5' untranslated region consists of 174 bp and 3' untranslated region of 172 bp. Amino acid similarity search revealed strongest homology to a tumor-related proteins and miraculin-like proteins *(table-6)*.

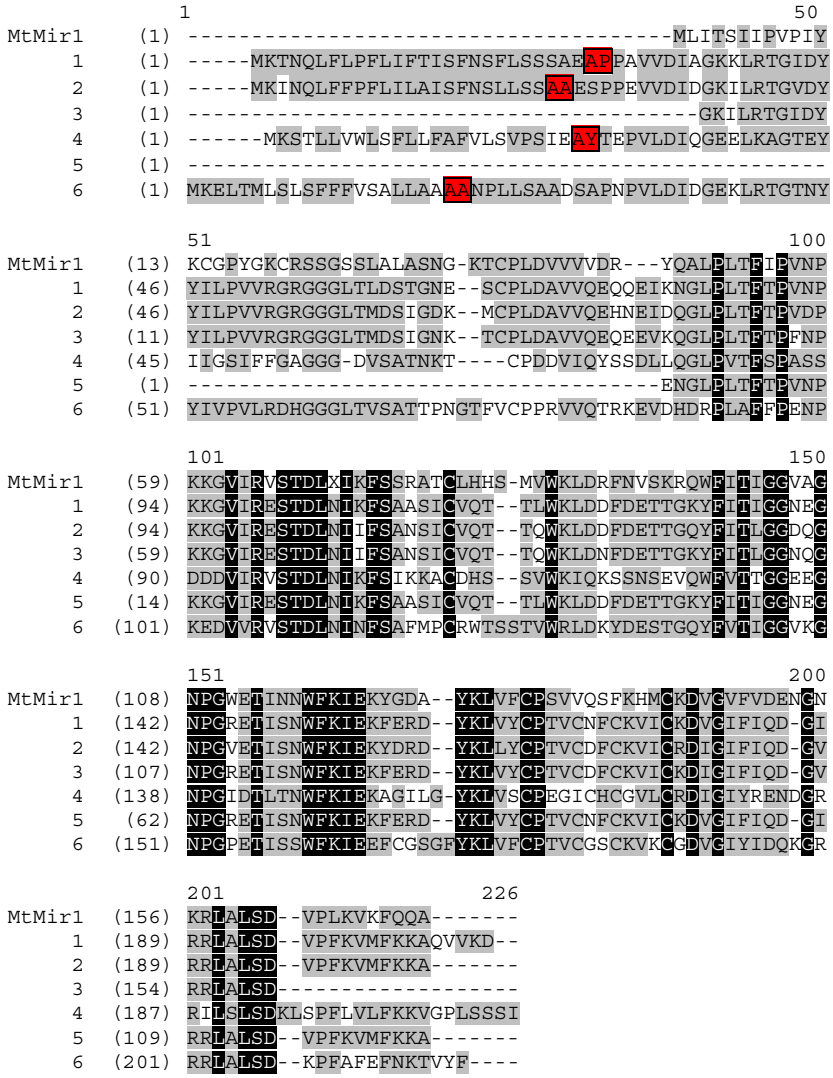

Fig. 19 Alignment of *MtMir*-1 with homologous MLPs from different organisms. Identical sequences are shadowed black, sequences with similarities are shadowed grey. Signal peptide cleavage sites are shadowed red.
1-tumor-related protein from tobacco
2- LeMir from tomato
3- Miraculin-like protein from aubergine
4- Miraculin-like protein from poplar
5- Tumor-related protein from hybrid tobacco
6- Miraculin from sweet berry

Table 6. *At the amino acid sequence level MtMir-1 shows high identity to different plant miraculin-like proteins.*

Organism	Protein	GeneBank accession number	% identity to *MtMir*-1	e-value
Nicotiana tabacum	tumor-related protein	U66263	57%	1e-38
Lecopersicum esculentum	*LeMir*	U70076	56%	2e-37
Nicotiana glauca and *Nicotiana langsdorffi*	tumor-related protein	D26457	61%	7e-36
Solanum melongena	miraculin-homologue	AB23651	56%	2e-34
Richadella delcifica	miraculin	D38598	53%	7e-31
Populus balsamifera subsp. *trichocarpa* x *Populus deltoides*	Kunitz-trypsin inhibitor-4	AY378089	49%	6e-28

Alignment of the amino acid sequences of different plant miraculin-like proteins revealed conserved domains (*fig. 19*).

SignalP V1.1 - signal peptide location and cleavage site prediction program (Nielsen *et al.,* 1997) were used to analyse different miraculin-like protein (MLP) sequences for presence of signal peptides on the N-terminus of the amino acid sequence. Signal peptides were predicted for MLPs from tobacco, tomato, poplar and the miraculin from sweet berry. *MtMir*-1 as well as MLP from aubergine and hybrid tobacco seems not to have a signal peptide *(fig. 19).*

Comparison of *MtMir*-1 cDNA sequence with the genomic DNA sequence obtained from *M. truncatula* genome shotgun sequencing project at the University of Oklahoma (www.genome.ou.edu/medicago.html) shows that the *MtMir*-1 has no introns and consists of only one exon.

3.8.5 Genomic Southern blot analysis of *MtMir*-1 gene

Genomic Southern blot analysis was performed to identify the number of copies of the *MtMir*-1 gene in the *M. truncatula* genome. As it is seen on *fig. 20* there are two bands in each lane. This result shows that the *MtMir*-1 gene could belong to a multigene family.

Fig. 20 **Genomic Southern blot of *MtMir*-1**. 20 µg genomic DNA of *M. truncatula* was digested with *Eco*RV (lane 1) and *Xba*I (lane 2) and blotted onto nylon membrane. An 600 bp fragment of *MtMir*-1 gene was labelled with digoxigenin and used as probe in the hybridisation experiment.

3.9 Analyses of *MtMir*-2 gene

A gene with sequence similarity to *MtMir*-1 was identified in an EST-project of mycorrhized roots. The corresponding fragment *MtMyc-8* was found to be up-regulated in the *M. truncatula* roots colonised by mycorrhizal fungi (Wulf *et al.,* 2003). The complete cDNA sequence of *MtMir*-2 gene *(fig. 21)* was provided by J. Doll (University of Hannover, Germany).

The arbuscular mycorrhiza (AM) induced gene *MtMyk*-8 was renamed in this work to *MtMir*-2 (*Medicago truncatula*-miraculin like protein 2) according to the proposed *M. truncatula* nomenclature (VandenBosh and Frugoli, 2001).

Amino acid sequence alignment of *MtMir*-1 and *MtMir*-2 showed 19.5 % identity (*fig. 22*).

AAAGCCATTGAGTGTAAGCCTAGTTATTACATTACAAGTTACATCTAACA 50

AATAATAAACC**ATGTCAATGAGATTATCTATTAGAACCCTCATTATTCTT** 100

GCTCATGTGTGTCTCTTTATAACGACAACAACAATAGCTCAGTTTGTCTT 150

GGACACAGTCGGAGAACCCGTTGAAGGCGACGAAGAATACTTCATCCGTC 200

CAGTTATCACAAACAAAGGAGGACGTTCCACTATGGTCAGCAGAAATGAA 250

TCATGCCCTTTACATGTTGGTCTTGAGCTCACTGGCTTAGGACGCGGGCT 300

GGTTGTCAAATTCACACCATTTGCTCCCCATCATGACTTCGACGATGTTA 350

GGGTTAACAGAGACTTGAGAATAACATTCCAAGCTTCATCAAGTTGTGTA 400

CAATCAACAGAATGGAGATTAGGTGAGAAAGACACCAAGAGTGGAAGAAG 450

GTTGATTATCACTGGGACAGATAGTGCTACCAATGGATCATATGGTAACT 500

TCTTTAGGATTGTAGAGACCCCACTTGAAGGTATGTATAATATACAATGG 550

TGTCCTACAGAGGTATGTCCAAGTTGTAAATTTGAATGTGGAACTGTTGA 600

TATGTTGAATGAGAATGGTAAGATTTTGTTGGCCCTAGATGGTGGTCCCC 650

TCCCTCTTGTTTTTCAGAAAGAATAATTTTTAATTTATAGGTACCCTTGT 700

TTTTATTTACAATGGAGCTATATATTGTTCATTTCAGTTAATAAAATTGG 750

AAGAGATATTACATGTACTTTGTTCTTGATTCATATAATAAAGCTCTAAA 800

AGTTTGTGTAGAACGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3' 846

Figure-21. **Complete cDNA sequence of *MtMir*-2**. ORF is indicated in boldface type.

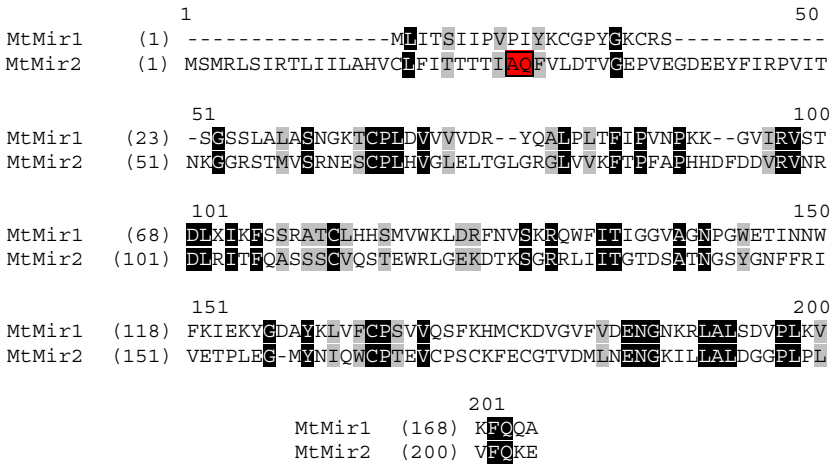

Fig. 22 Alignment of amino acid sequences of *MtMir*-1 and *MtMir*-2 genes. The identical amino acids are shadowed black and similar ones shadowed grey. Signal peptide cleavage site of *MtMir*-2 is shadowed red.

3.10 mRNA-accumulation studies of *MtMir*-1 and *MtMir*-2

The RT-PCR result shows that the *MtMir*-2 gene is up-regulated only in the mycorrhized roots and not in the roots infected with *A. euteiches (fig. 23)*. It turned out that *MtMir*-1 is also transcribed in mycorrhized roots of *M. truncatula (fig. 23)*.

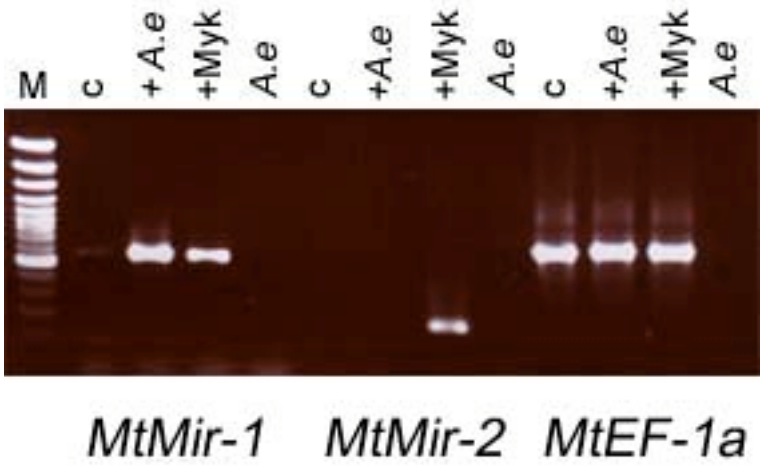

Figure-23. **RT-PCR**. cDNA derived from sterile roots (c) of *M. truncatula*, roots infected with A. euteiches *(+A.e)*, mycorrhized roots (+Myk) and *A. euteiches* mycelium (*A.e*) were used to amplify *MtMir*1 and *MtMir*2 genes. tef 1α gene was used as a constitutively experessed control. M- 100 bp Plus DNA marker.

The *MtMir*-2 genomic DNA sequence was obtained from genome shotgun sequencing project at the University of Oklahoma (www.genome.ou.edu/medicago.html). *MtMir*-2 has no introns at the genomic DNA as it was the case for *MtMir*-1.

In the 846 bp *MtMir*-2 transcript sequence the first ATG triplet is located 62 bp downstream of the 5′ cDNA end. The 615 bp long open reading frame codes a protein of 205 amino acids. The 5' untranslated region consists of 61 bp and 3' untranslated region of 170 bp *(fig. 21)*. In contrast to *MtMir*-1, the SignalP software predicted a 26 amino acid long signal peptide in *MtMir*-2 protein sequence *(fig. 22)*.

3.11 Promoter isolation of *MtMir*-1 gene

Since at the time point of starting this promoter analyses, the *M. truncatula* genomic sequence was not yet available, the promoter region of the *MtMir*-1 gene was isolated using the Universal GenomeWalker^{TM} Kit (Clontech, Palo-Alto, CA, USA). Four libraries were constructed after digestion of genomic DNA with *Sma*I, *Ehe*I, *EcoR*V or *Ssp*I respectively. Adapters were ligated to the ends of digested DNA. Gene specific primers located at 5' end of the cDNA and directed upstream of the gene (MtMir1_GWI_GSP1; MtMir1_GWI_GSP2) were constructed. In the first genome walker experiment an 450 bp fragment was amplified from the *Sma*I-library and an 250 bp fragment was amplified from *Eco*RV-library. *(fig. 24A)*. These fragments were cut out from the gel, purified, cloned and sequenced.

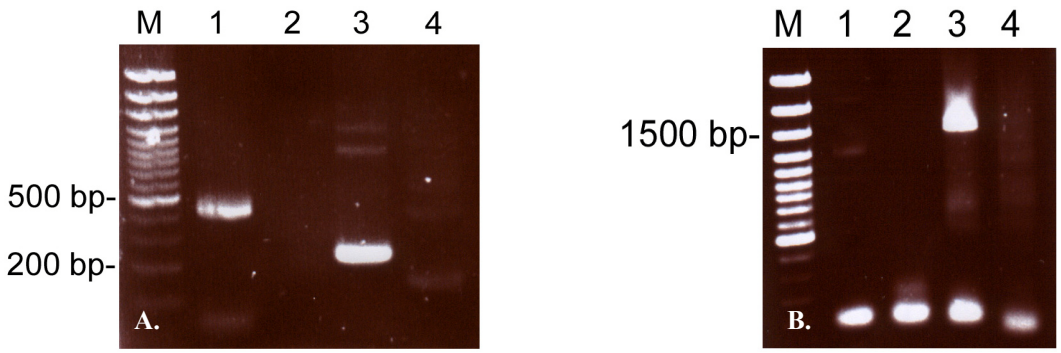

Fig. 24 **A**. Nested PCR from first genome walker experiment. **B**. Nested PCR from second genome walker experiment. M- 100 bp Plus DNA marker; 1-*Sma*I, 2-*Ehe*I, 3-*Eco*RV, 4- *Ssp*I digested DNA library

Both fragments could be aligned with the known sequence of the *MtMir*-1 gene. Therefore, the 5' end of *MtMir*-1 gene was extended by 243 bp. Since this could not be the entire promoter sequence, further genome walking steps were performed using new primers (MtMir1_GWII_GSP1; MtMir1_GWII_GSP2). This time, of the *Eco*RV-library, approximately 1500 bp long fragment could be amplified *(fig. 24B)*. After cloning, sequencing and aligning of this fragment, the 5' end of *MtMir*-1 gene could be extended by another 612 bp. Together with the 243 bp obtained from the first experiment, an 855 bp long promoter region of *MtMir*-1 gene was identified by genome walking *(fig. 25)*. The predicted TATA box of the promoter is located 29 bp upstream of the cDNA start of the *MtMir*-1 gene.

```
TACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTATCATTTAAGTAGACATAGTTTA      60
ATTTTGTGTGAATCAATTAGTGAGTTGAATTATTTGATTTTATTTGTGTTATCTACACGT     120
CGTTTAATTAAACGTTACTCCCTCCGTCCTAAATTGTATGATGTTTTGGGCATTTCACAC     180
ATATTAAGAAATGCAATTAATATTGTGTGGAAAAAAGATATTATGAGTTGTTTTACAAAA     240
TTGTCCTTAATGAATAATTGGGAAAGATAAATGAAAGAATTGAAAGAAGAGAAAGTAATA     300
AATAATTAAGGTTATAATAGGAAAAGTAACATTAATGTTGCATTGGTATTTTAAAGCGAC     360
ATATAATTTGGGACAGATTTTTTTCCTTAAAACGACATACAATTTGGGACGGAGGGAGTA     420
GTTTTTCTTTTCAGCGGTTTAAGCATCTTGTAATCCAAAAAAGAAGAAAAAAACTTTCAA     480
ATGTACTTGCTTGTTTACTAGATACTTAGACCGTTTAATAAGTCTGGCTATTATTCCTTT     540
GTTTAGTTTTCTTCAAAATCAATATACTGCATTCATATCAATTATATTATATTCAAAATA     600
GACTCTCCAAATATTATAATTAATTGAAAGTCATCAAAGGMTAATAATATAAAGAAATAA     660
CCCTTCAAAGGGCCAATTGTATTCATATCAAAAGGGTTTGTGCTCTTGGTGACAACATGC     720
CAATTGTATTGCATATTGGTCAAAAGTTTGGCCTTAATTAAAAGTGTGGAAATTGTTTAG     780
GTTACCCATAGATAAGAAAATGTAAGTACGTACTTCCCTCTATAAATTGATATTCTTCAA     840
ATTGCCATAAACTTCATCCATCACAAACCAAACTTAATTACAAATTACTTAAAGCATACA     900
GAACAATGAAAAACACATTGCTAGCATTTTTCTTTCTCTTTACCTTCTTAAGCTCACAAC     960
CACTTCTTGGAGCAGCTGAAGCTTCAAATGAACAAGTGGTTGACACATTAGGCAAGAAAC    1020
TCCGAGCTGATG
```

Fig. 25 **855 bp sequence upstream from the 5´ cDNA end of *MtMir*-1 gene (putative promoter region).** TATA box located 31 bp upstream 5´cDNA end is indicated by the box around the sequence. 5´end of the cDNA is unterlined. Translation start is indicated in boldface type.

3.12 Promoter isolation of the *MtMir*-2 gene

Identification of *MtMir*-2 promoter was carried out as described for the *MtMir*-1. PCR revealed several bands over 500 bp in all DNA libraries except the library digested with *Ssp*I *(fig. 26)*. *MtMir-2* gene was extended upstream by 2152 bp after aligning the longest fragment, obtained from genome walking, with the known sequence *(fig. 27)*. The predicted TATA box is localised 27 bp upstream the 5´ cDNA end.

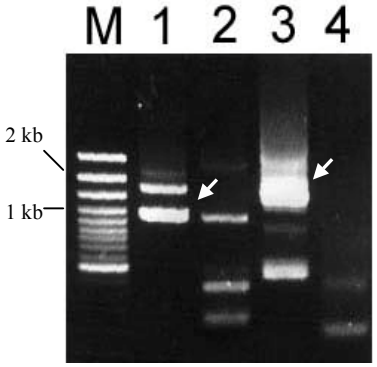

Fig. 26 Nested PCR from genome walker experiment. 1-*Sma*I, 2-*Ehe*I, 3-*Eco*RV, 4- *Ssp*I digested DNA library. M- 100 bp Plus DNA marker;
The bands indicated by arrows were cut out from the gel, purified and sequenced.

TAATTTTAATCATATTTAATTGTCTATTTTTTATGCAAAACTGATTGTCCACATATTATATTTATATGAT 70
ACCACTCGAATCGCAAGACAGGAAGTTTGATTAATAATATGGATAGCTAGCTATGATACTAGCATATGCT 140
CCTATGGGAAGAAATAACACATCAGGATACTCTTGTTGGTAGCTAAATAAATTCTTAACTAATAAAGGGA 210
ATTACTATATGCTTGTCGAGAAGACAAGTTTCAGTAGGCTAATTCTACCTTATATACTTGACTATTAGTT 280
AGTAGGATGGTAAAACAAACATATTTCCTAACTAACAAGATATTTATAAAAGTAGAGATCAAGCTTTATC 350
GATACTAGAACTTGGTACATTAGCATGTGTTGAATGAATCTGACATGGACTTTCTAGCAATTTGGATTTA 420
CTGATCATGATCTGCCTTCCTATAATTTCTCTTAGTACATCATTTAAAGATAATCATAGTATACTCACAA 490
AAAAGGACAAGATTATACTTTCAATTACACCATACTATGTTTCTAGGCAAACTCAACGCAATCTTAAAAG 560
TTCATGACTTTTGTGCAGTTTTAAAATATGTACCTGATTCCTTATGGATGATTTCAAATGTATGAAAGTT 630
AAAACTTACGGTGAAGTCGATCATACCATGCTCTNTTAGTTTCTGTAGCAGCCAAAATGAGTTGATACGT 700
CAACGCCTTAAAATCAGCATATCGATGCTATCAGTCGCCCTGGCTTGTCATCTTGATTCCTCCTCCAAAC 770
TTTTTCGCCCTTTAACTTGTTTATCAATAGCATCCTATACCCATGCTTTCTTTATTCTAACCTTTCTTCG 840
TGGTACAGCCTATATTGGTCTATTTTACATAGCTATTTCCTAACGAACCAAGAGGGGTATCTGTTCGCCT 910
AATTGAAAACATATCTCGATCGCAGCTTCCACAACAAAGGTATTTATAGCCTTCTATGGACTTGGGTTTC 980
GGTTGCTTAGTCTCCAAGTAATAGGGGTATTTATGTATTTAGGGGCGGTTTCTAGAAGATTCTAGAGATT 1050
ATTGAGGCGGCGCCATGGATAATGCGTGCCCGGGGCCCTTGGGCTTCTTTTAAAGGTATTAAGGCCTTCT 1120
TGAGGGGTCTAAAAGCCCTTTTGGAGGTCTTTCAGGTCCTTAGGAATATTATGACAGTCCAAGACTAAAA 1190
GAGCTGAAGAGTTTAAAGTTCAGAATTTAAATACTGACAATGAGAAAAATACTAACATACTAACATTTGT 1260
CATTTAAATAGAGAAAAAAGTGTAGTAAGAGAGCGACAAACTTTTTTAAAATTTTTTTTATGTAAAAAGA 1330
GTGTGACAACATGTGAAGAACATGTGTTAAAAAAAATATAGAACACAAACAATGTCTTTTCTTTTATTCA 1400
ATTCAGCTTATACTCTGATAAGAATATTCGAATATGGTTCTACAGAACGGATTAATTCTACATATTTTAG 1470
GGCACTATTTCTATGCAAGAAACACCATTTTTGTCAATCTAATTTTTTCGATAGAAGGGAGGGAAATGAA 1540
GAAGACAAAAGTGTTTGTCTGTTTCATTGGACGATTCATACTAGGGAAAATTCGGCATTGTCTCTTGTCC 1610
ATATTTCCATCAAAAATCCGTAATCCAAACAAAGTAATTAACAAGATACTTATTCTTGACTTAGTTAATA 1680
TCGATGGCATTGTTTGACCCTAAAAGGTACTCTCTTGGTTTCAAAACATGTTTTGTTTTATATTCAATAT 1750
GTCTCATGATAAATATCACATTTATAAAAATAAATTATCTCAAAAGAGTGTCACTTTCAAATGAGTAAAC 1820
AACATTATATAGACAATGGGAGTAAAAAACATTTCTCAAAATTTGTGTAGTTTTATATTTTGAATATAAC 1890
ATTAGTTACTTTATTCAAATTATATCTCAAATCAATATTGTTTCTATATCTTAAAAAAGATAATATAAAA 1960
TTCTTATGATTATTATCATAAATTTAAGTATTTAACAACTACAATACAATTTTGTTAAAATTTTACAATA 2030
ATTATTTTAGAACAATCATTGATTTATATTATAATTACACTTTTCGGTCTATATAAAAATTACAAACTTT 2100
TAAGTGCATGTATCACAGTTCTATAAATACTTAGCCTAGTAACAAAACCCATAAACCCATAAAGCCATTG 2152
AGTGTAAGCCTAGTTATTACATTACAAGTTACATCTAACAAATAATAAACC**ATG**

Fig. 27 2152 bp sequence upstream *MtMir*-2 gene. TATA box localised 24 bp upstream of the 5´cDNA end is indicated by the box around the sequence. 5´end of the cDNA is underlined. Translation start is indicated in boldface type.

3.13 *MtMir*-1 promoter deletion analysis

For the *MtMir*-1 promoter-reporter gene studies approximately 100 bp, 300 bp or 900 bp fragments of potential promoter region of *MtMir*-1 gene, including 177 bp sequence of 5' region upstream the translation start, were transformed into roots of *M. truncatula (fig. 28)*. The promoter-GUS constructs (pLP100-p100; pLP100-p300; pLP100-p900) of *MtMir*-1 gene were prepared as described in Materials and Methods.

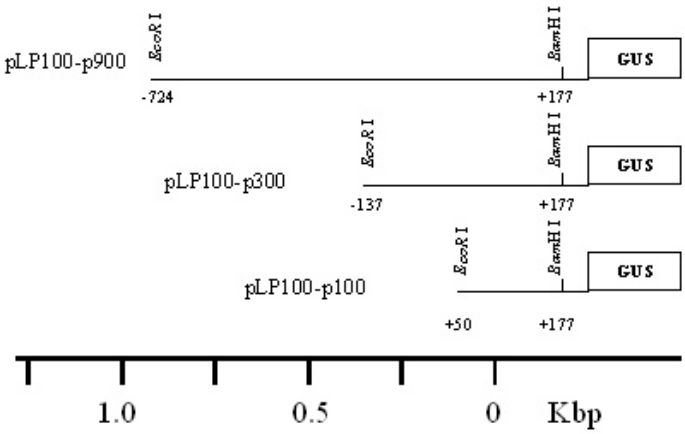

***Fig. 28 MtMir*-1 promoter deletions**. The three constructs used in this study are shown. PLP100-p100 contains sequences from +177 to +50 (127bp); pLP100-p300 contains sequences from +177 to –137 (315 bp); pLP100-p900 contains sequences from +177 to –724 (901 bp). The numbers represent the nucleotide numbers relative to the 5´cDNA end.

The presence of transgenes in each transgenic root culture were confirmed by PCR analysis. Using vector specific forward (pLP100_for) and *Eco*RI reverse (*Eco*RI-p100, *Eco*RI-p300, *Eco*RI-p900) primers constructs were amplified from the genomic DNA isolated from transgenic roots *(fig. 29)*.

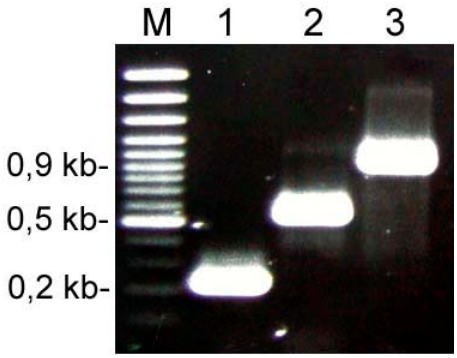

Fig. 29 **PCR amplification of promoter deletion fragments from genomic DNA.** Fragments were amplified from genomic DNA isolated from hairy root cultures containing: 1- pLP100-p100; 2- pLP100-p300; 3- pLP100-p900 constructs. Fragment sizes correspond to the promoter fragment length plus vector sequence. M- 100 bp Plus DNA marker.

Transgenic hairy roots containing the pLP100-p100 and pLP100-p300 constructs did not show any GUS-gene activity as well as the non-infected control roots (data not shown).

In contrast, transgenic hairy roots transformed with the pLP100-p900 construct, showed strong GUS-gene expression 6 days after inoculation with *A. euteiches (fig. 30)*. Hence, the pathogen-responsive element necessary for a induction of *MtMir*-1 transcription is located in the area between –300 and –900 bp upstream of the transcription start.

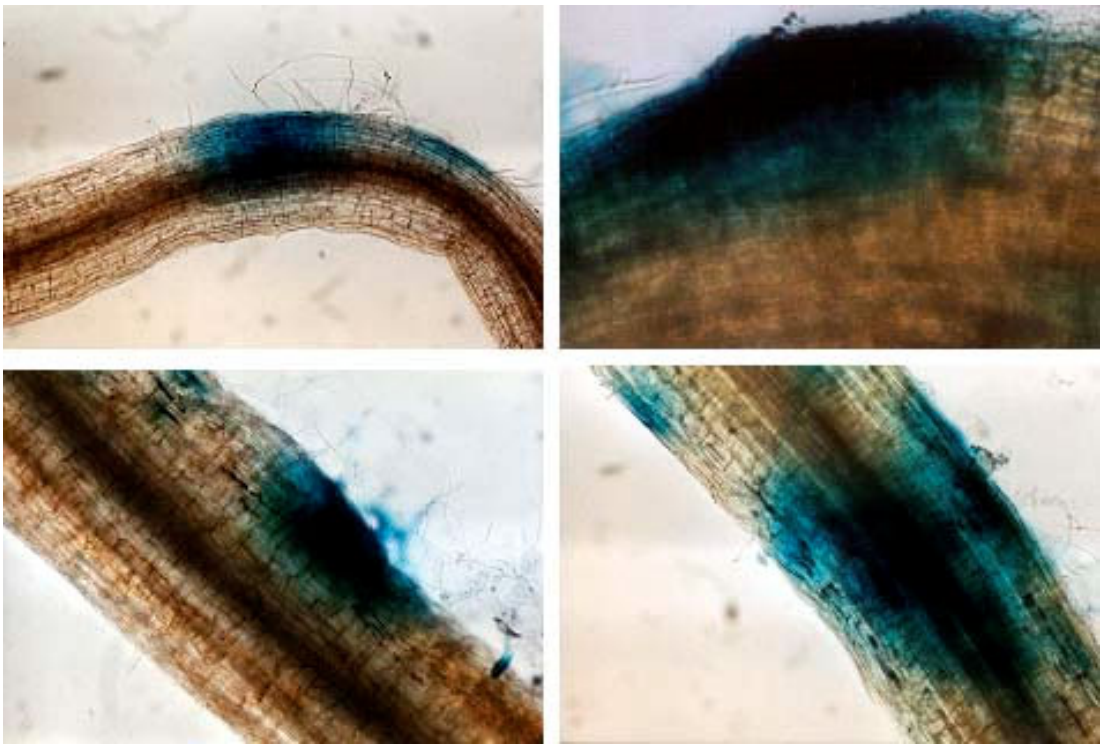

Fig. 30 Histochemical staining of transgenic hairy roots for GUS gene activity.

Cortical cells of transgenic roots which showed zoospore attachment as well as the root parts colonised by the *A. euteiches* hyphae showed promoter activity.

3.14 Promoter analysis of *MtMir*-2

To analyse the promoter activity of the *MtMir*-2 gene, an 797 bp fragment of the promoter region (between +49 and –748) of *MtMir*-2 gene was introduced into binary vector pLP-100, in front of the promoterless GUS-gene. The transgenic hairy roots were obtained by *A. rhizogenes* mediated transformation of *M. truncatula*. The presence of the transgene was confirmed by PCR using DNA of hairy root cultures (*fig. 31).*

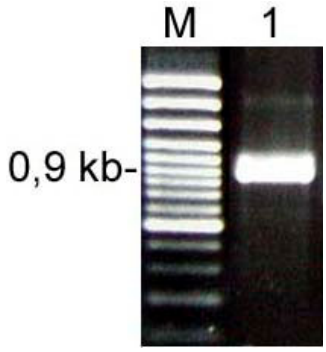

Fig. 31 **PCR amplification of promoter deletion fragment from genomic DNA.** Fragments were amplified from the genomic DNA isolated from hairy root cultures containing: 1- pLP100-p797 construct. The fragment is bigger in a size because they contain vector sequence. M- 100 bp Plus DNA marker.

Composite plants which developed transgenic hairy roots were inoculated by the AM fungi *G. intraradices*. Three weeks after inoculation with *G. intraradices*, transgenic roots were evenly mycorrhized. Different stages *of G. intraradices* colonisation could be seen in the roots *(fig. 32 A; B).* The histochemical staining of mycorrhized transgenic roots for GUS gene activity showed that the promoter gene was active in root parts harbouring AM structures *(fig. 32 C; D).* In non-inoculated control roots neither fungal structures nor GUS gene activity were detectable.

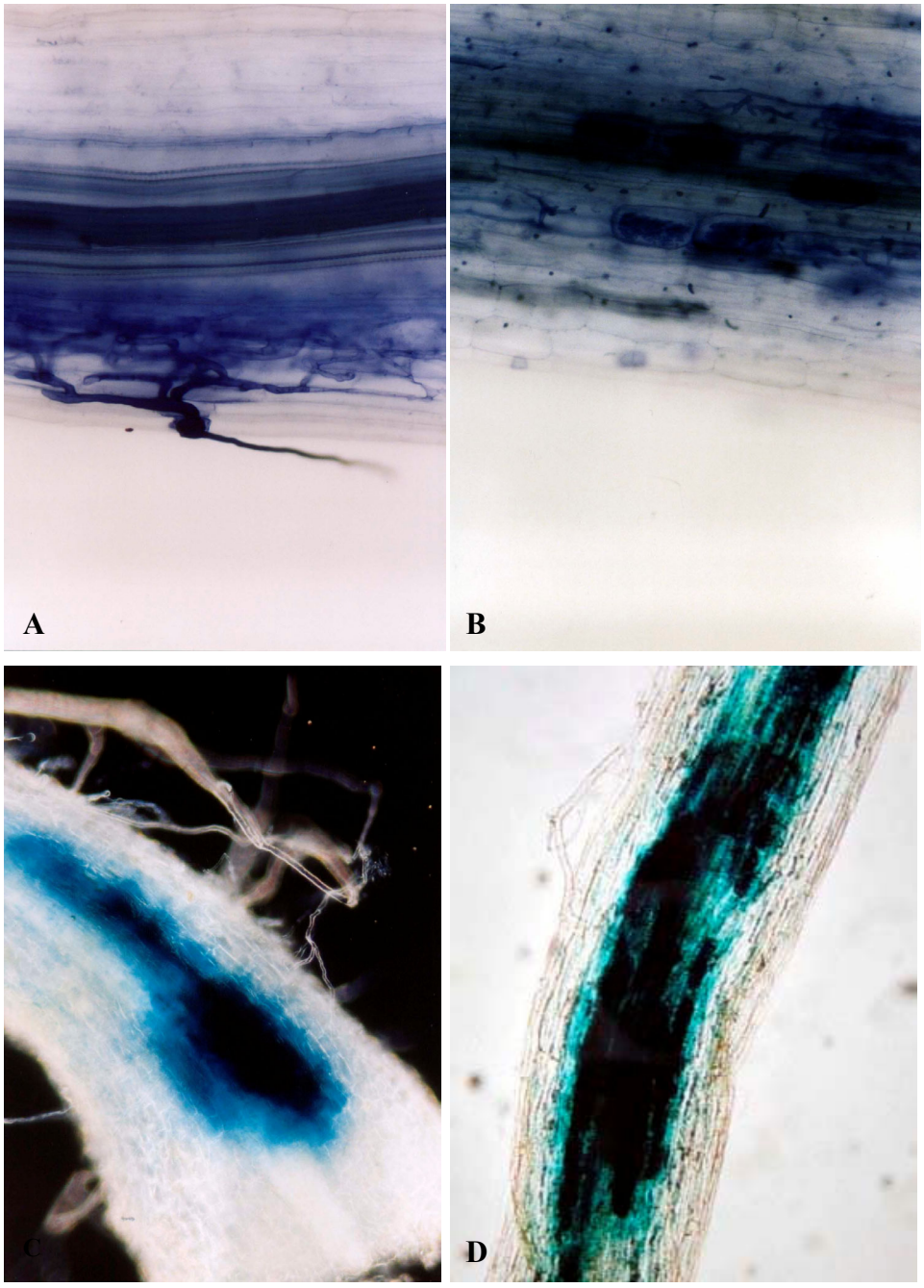

Fig. 32 **Analyses of transgenic *MtMir*-2 promoter-GUS hairy roots**. Visualisation of fungal structures in mycorrhized roots by trypan blue staining: A- appressorium of *G. intraradices*; B- arbuscules of *G. intraradices*. B, C- GUS-activity in transgenic hairy root parts colonised by *G. intraradices*.

Functional analysis:

3.15 Silencing of *MtMir*-1 gene

An post transcriptional gene silencing (PTGS) approach by RNA-interference (RNAi) was used to get first insights into the function of *MtMir*-1.

Transformation vectors that produce RNAs capable of duplex formation (pFGC 5149), were constructed as described in 2.4.22. RNAi constructs were transferred into the roots of *M. truncatula* plantlets and transgenic hairy root cultures were obtained *(fig. 33 A; B)* as described by Boisson-Dernier *et al.,* (2001). Control roots were transformed with an empty pFGC 5149 vector.

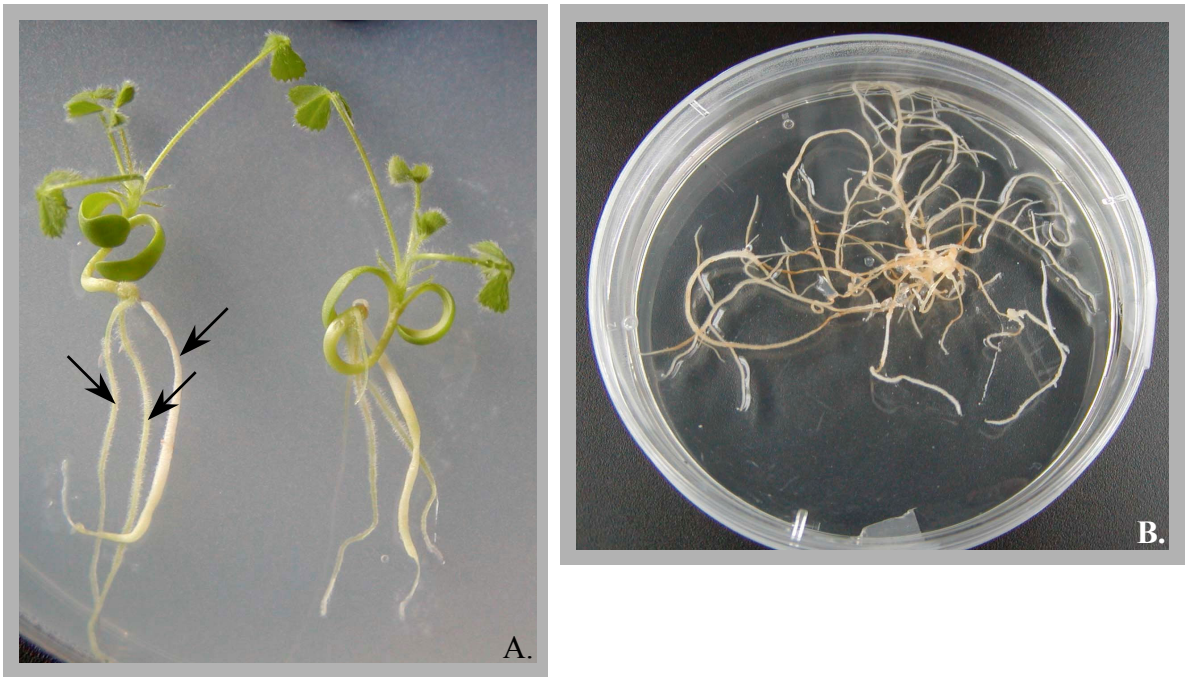

Fig. 33 **A.** Transgenic hairy roots (indicated with arrows) of *M. truncatula* growing on Fahraeus Medium (25 mg/l kanamycin). **B.** Root cultures on M-Medium (400 mg/l augmentin).

Two lines of root cultures transformed with the empty vector (pFGC-1 and pFGC-2) and two lines transformed with constructs providing PTGS of *MtMir*-1 gene (MtMir-1-PTGS-1 and MtMir-1-PTGS-2) were chosen for further analyses.

Consequently, transgenic roots were infected with zoospores of *A. euteiches*. Histochemical staining of roots for ALP-activity was done 6 days after inoculation. There were no visible phenotype difference between hairy roots carrying PTGS-constructs, vector control lines and wild type roots. All roots inoculated with zoospores were colonised with *A. euteiches* mycelium to the same extend (data not shown).

RNA-accumulation studies were carried out in order to proof the silencing of *MtMir*-1-expression in root cultures carrying the PTGS-constructs. Specific primers were designed which bind near to 5´end of *MtMir*-1 cDNA. These primers amplify a fragment of the gene which was not used for the construction of PTGS-vectors (*fig. 34*).

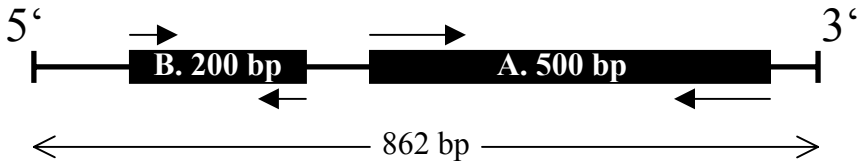

Fig. 34 **A.** *MtMir*-1 cDNA fragment used for the PTGS-vector construction. **B.** cDNA fragment used for RT-PCR and virtual Northern analyses.

This 200 bp fragment could be amplified by RT-PCR only from cDNAs derived from MtMir-1-PTGS containing roots infected with *A. euteiches*. It was not possible to amplify this fragment from cDNAs derived from the empty pFGC vector-containing roots before and after induction of the gene with pathogen (*fig*. 35).

RNA-accumulation studies were repeated by virtual Northern hybridisation analyses (*fig. 36A*). Using a labelled probe of *MtMir*-1 gene, a fragment with about 1 kb size could be detected in the control roots infected with *A. euteiches*. No bands were detected in the MtMir-1-PTGS roots infected with *A. euteiches*.

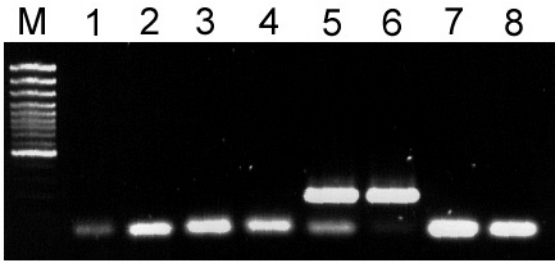

Fig. 35 **RT-PCR**. In order to amplify only the endogenous mRNA of *MtMir*-1 gene and not the PTGS-construct, two primers (MtMir1_200_RNAi_for/rev) were constructed. These primers bind to the cDNA region of *MtMir*-1 gene which is located adjacent to the sequence taken for PTGS-construct. Therefore only cDNAs derived from the whole length mRNA should be a target for amplificaiton. cDNAs derived from two independent root cultures transformed with empty vector, and two independent lines of roots transformed with vector containing a coding region of *MtMir*-1, were taken for analysis.
Lane-1 and 2: pFGC-1 and 2 - non infected;
lane 3 and 4: MtMir-1-PTGS-1 and 2 –non infected;
lane 5 and 6: : pFGC-1 and 2 – infected with *A.e*;
lane 7 and 8: MtMir-1-PTGS-1 and 2 – infected with *A.e*;
M- 100 bp Plus DNA marker.

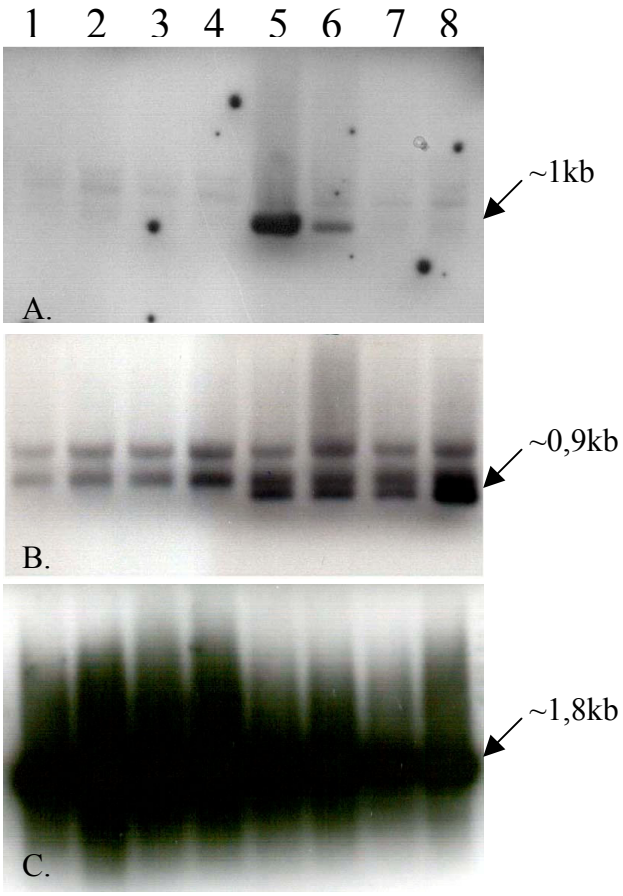

Fig. 36 **Virtual Northern Blot**. 15 µl SMART-cDNA amplified by LD-PCR was blotted onto nylon membranes.
A. 200 bp fragment of *MtMir*1-cDNA not including the sequence used for PTGS construct was labelled with digoxigenin and used as a probe for hybridization.
B. Stress inducible MtPR4-1 gene was used as a positive control for infection. This MtPR4-1-probe detects 2 other bands in each cDNA unspecifically.
C. *MtEf*-1α gene was used as constitutive expressed positive control.
The membranes were washed under stringent conditions. Approximate size of the bands are indicated.

Transcription profiling:

3.16 Gene expression analyses of *M. truncatula* roots 30 min and 6 days after inoculation with *A. euteiches* using microarray technology

Total RNA from control and infected roots of *M. truncatula* after 30 min of inoculation with *A. euteiches* (30min) as well as total RNA from control and infected roots after 6 days (6d) of inoculation were labelled with fluorescent dye as described in 2.4.23a and used as a probe for hybridization. For each experimental condition, two biological controls were analysed (i.e. RNAs were extracted from two independently inoculated plants). For each biological control two technical controls (i.e. two aliquots were made from each biological control RNA and treated throughout all the experimental steps separately) were analysed (*table-7*).

Table-7. Experimental design of first microarray hybridisation experiment.

RNA probes used for array analyses		cDNA labelling with Cy dyes	Probe composition	Name of the hybridised slides	Name of the experiment
30'-Bc-1	30'**c-1**	30'c-1-**t-1**	30'**c**-1-**t-1**	30'Bc-1-t-1	**30'-Bc-1**
		30'c-1-**t-2**	30'**i**-1-**t-1**		
	30'**i-1**	30'i-1-**t-1**	30'**c**-1-**t-2**	30'Bc-1-t-2	
		30'i-1-**t-2**	30'**i**-1-**t-2**		
30'-Bc-2	30'**c-2**	30'c-2-**t-1**	30'**c**-2-**t-1**	30'Bc-2-t-1	**30'-Bc-2**
		30'c-2-**t-2**	30'**i**-2-**t-1**		
	30'**i-2**	30'i-2-**t-1**	30'**c**-2-**t-2**	30'Bc-2-t-2	
		30'i-2-**t-2**	30'**i**-2-**t-2**		
6d-Bc-1	6d-**c-1**	6d-c-1-**t-1**	6d-**c**-1-**t-1**	6dBc-1-t-1	**6d-Bc-1**
		6d-c-1-**t-2**	6d-**i**-1-**t-1**		
	6d-**i-1**	6d-i-1-**t-1**	6d-**c**-1-**t-2**	6dBc-1-t-2	
		6d-i-1-**t-2**	6d-i-1-**t-2**		
6d-Bc-2	6d-**c-2**	6d-c-2-**t-1**	6d-**c**-2-**t-1**	6d-Bc-2-t-1	**6d-Bc-2**
		6d-c-2-**t-2**	6d-**i**-2-**t-1**		
	6d-**i-2**	6d-i-2-**t-1**	6d-**c**-2-**t-2**	6d-Bc-2-t-2	
		6d-i-2-**t-2**	6d-**i**-2-**t-2**		

30'- *root material 30 minutes after inoculation*
6d- *root material 6 days after inoculation*
Bc- *biological controls of experiments*
c- *control roots mock inoculated with sterile lake water*
i- *roots infected with A. euteiches*
t- *technical control*

Original hybridization data are shown in *fig. 37*. After hybridization image processing software ImaGene 5.0 (BioDiscovery Inc., Los Angeles, CA, USA), was applied to detect the spots and quantify the signals. Because the quality of technical controls were similar, replicate arrays of technical controls were grouped. The raw data were imported into EMMA 1.0 microarray analysis software (Dondrup *et al.,* 2003).

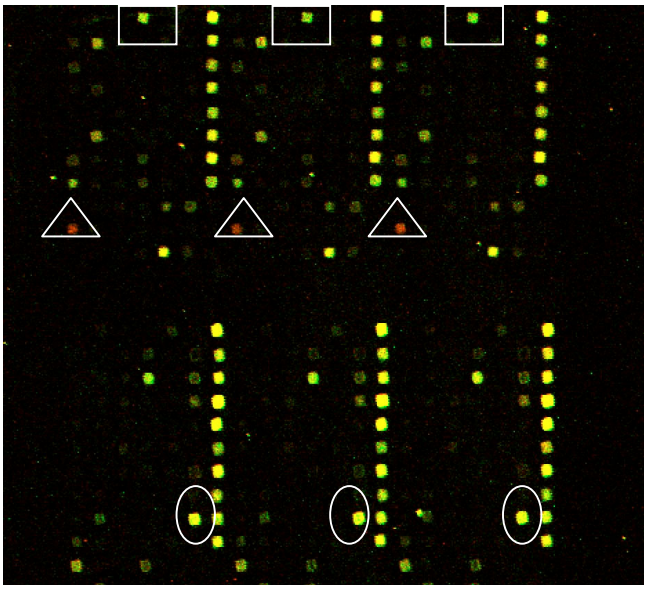

Fig. 37 A cut from original data obtained by hybridizing Mt8k microarray. Examples of up- and down-regulated genes as well as constitutively expressed gene are marked by triangles, rectangular and circles correspondingly. Every gene is present in three copies on the microarray.

After applying normalization, M vs. A scatterplots were generated for each biological control *(fig. 38)*.

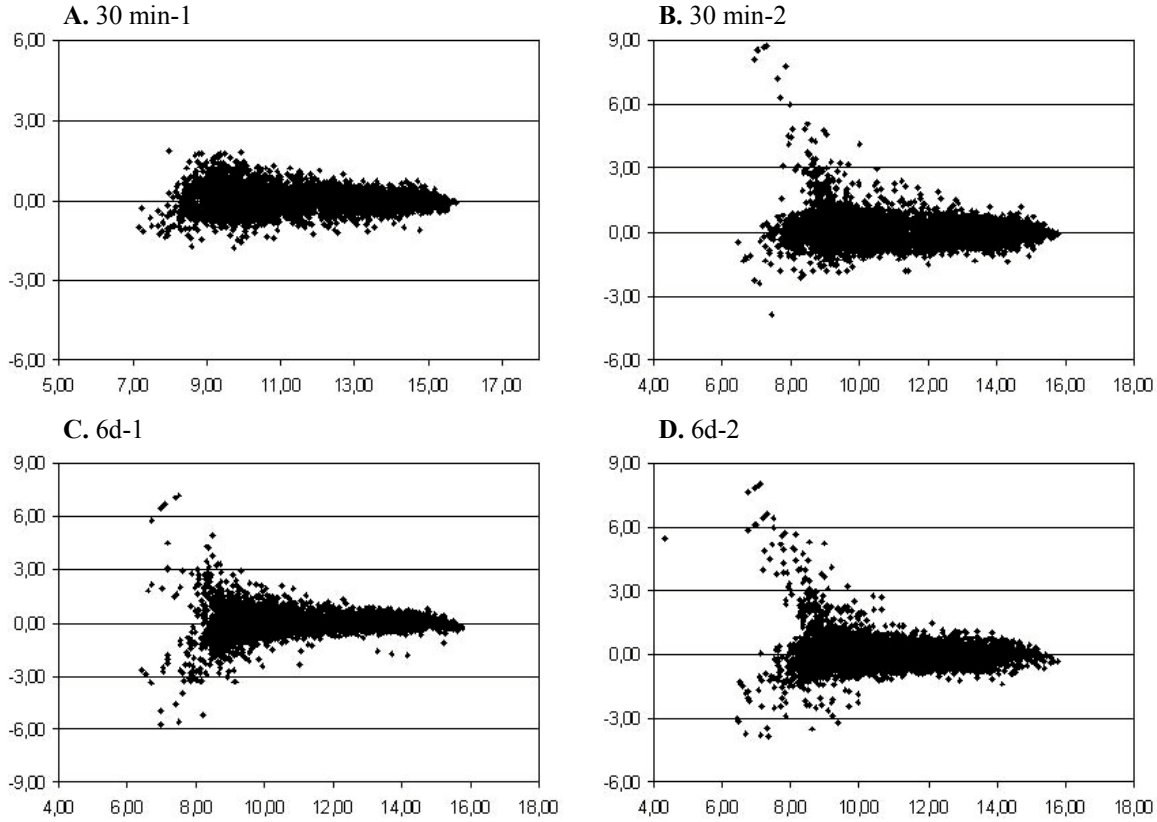

Fig. 38 Scatterplots showing M vs. A plot of all biological control slides after lowess normalization. X-value A. Y-value M.

In order to show the variation between the biological control experiments M vs. M scatter plots were generated *(fig. 39)*.

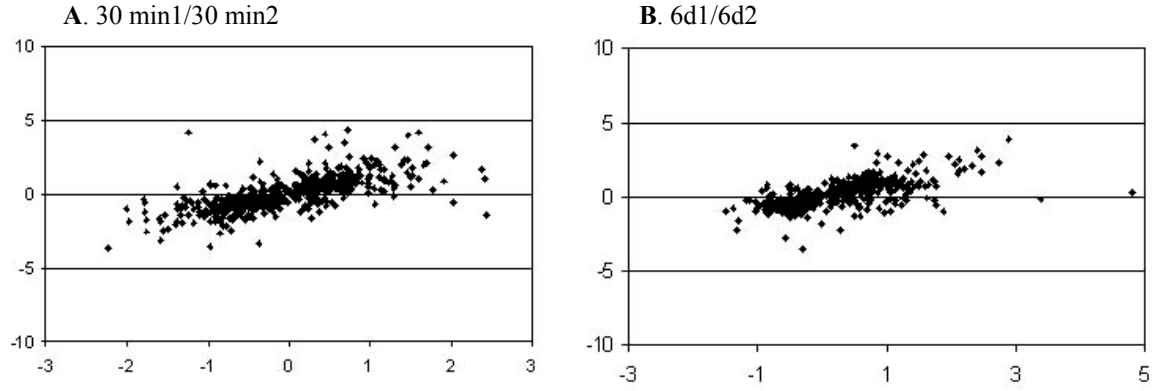

Fig. 39 M vs. M scatterplots showing variations between biological control expreriments.

Lists of candidate genes for differential expression were obtained by applying t-test. Those spots which were found in both biological controls and at least in one of them two-fold up- or down-regulated (M>or=1 or M<or=-1) and characterized by p-value of p<0.1, were selected. It resulted in the detection of 138 genes regulated after 30 min *(table-8a; b),* 207 genes 6 days after inoculation *(table-9a; b)*. 35 genes were found to be regulated in both experimental conditions in the same *(fig. 40)*.

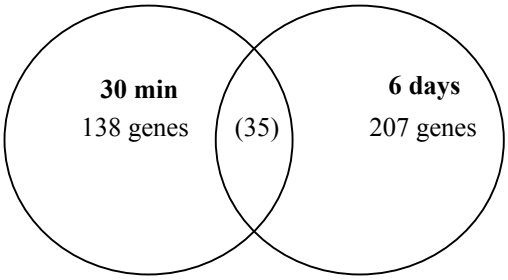

Fig. 40 Venn diagram showing an overview of *A. euteiches* induced genes 30 minutes and 6 days after inoculation.

Table-8a. The list of genes up-regulated 30 min after infection. Gene identity, annotation and functional class of each gene is shown according to MENS (Medicago EST Navigation System, http://medicago.toulouse.inra.fr/Mt/EST). M-value of biological control 1 and 2 (bc1; bc2) are shown. Genes showing regulation after 30 min as well as after 6 days are indicated in bold face.

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
1.	DSLC131	Ribulose bisphosphate carboxylase	-	1,53/1,02
2.	MtC00798	Arabinogalactan-protein precursor	I, Cell Wall	1,39/1,03
3.	MtC00485,1	Arabinogalactan-protein precursor	I, Cell Wall	0,9/1,61
4.	**MtC90836**	**Myosin**	**II, Cytoskeleton**	**1,34/0,69**
5.	MtC91062	Putative cytoskeletal protein-like protein	II, Cytoskeleton	0,92/1,27
6.	MtD22686	Cation diffusion facilitator 2	III, Membrane transport	0,61/1,10
7.	MtC00257	Adp-ribosylation factor	IV, Vesicular trafficking secretion and protein sorting	1,24/0,80
8.	**MtC00207**	**Ras-related protein rab7**	**IV, Vesicular trafficking secretion and protein sorting**	**1,05/1,22**
9.	MtC00121	S-adenosyl-l-homocysteinase	IV, Vesicular trafficking secretion and protein sorting	0,95/1,18
10.	**MtC00117,1**	**Polyubiquitin**	**IX, Protein synthesis and processing**	**1,8/1,99**
11.	MtC00096	40s ribosomal protein s6	IX, Protein synthesis and processing	1,38/0,59
12.	MtBA56C06	Elongation factor 1-alpha	IX, Protein synthesis and processing	1,24/1,03
13.	MtC00017,2	40s ribosomal protein s9	IX, Protein synthesis and processing	1,21/0,98
14.	**MtC93002**	**Ubiquitin-conjugating enzyme e2 (3'utr)**	**IX, Protein synthesis and processing**	**1,12/1,02**
15.	**MtC00325**	**60s ribosomal protein l15**	**IX, Protein synthesis and processing**	**1/1,16**
16.	MtC00047	40s ribosomal protein s24 (s19)	IX, Protein synthesis and processing	0,79/1,43
17.	MtC90530	Upp synthetase	IX, Protein synthesis and processing	0,78/1,08
18.	MtC90428	30s ribosomal protein s16	IX, Protein synthesis and processing	0,74/1,15
19.	MtC00324	60s ribosomal protein l35	IX, Protein synthesis and processing	0,69/1,11
20.	MtC93222	Serine carboxypeptidase ii	IX, Protein synthesis and processing	0,69/1,06
21.	**MtC00002,1**	**Elongation factor 1-alpha**	**IX, Protein synthesis and processing**	**0,23/1,17**
22.	MtC00028	Serine proteinase inhibitor	IX, Protein synthesis and processing	0,23/1,09
23.	MtC00046	S-adenosylmethionine synthetase	V, Primary metabolism	2,11/2,50
24.	MtC90961	Phenylalanine ammonia-lyase	V, Primary metabolism	1,42/2,12
25.	**MtC10397**	**Chloroplast cytochrome b6**	**V, Primary metabolism**	**1,31/0,82**
26.	MtC10333	Cysteine synthase	V, Primary metabolism	1,09/0,96
27.	MtD22714	Phosphoglycerate kinase	V, Primary metabolism	0,72/1,08
28.	MtC00218,2	Malate dehydrogenase	V, Primary metabolism	0,71/1,15
29.	MtC30578	Blue copper-binding protein	V, Primary metabolism	0,7/1,37
30.	MtC20048	Nadh-ubiquinone oxidoreductase	V, Primary metabolism	0,6/1,04
31.	**MtC00030**	**Glyceraldehyde 3-phosphate dehydrogenase**	**V, Primary metabolism**	**0,51/1,25**
32.	**MtC20054,2**	**Phospho-2-dehydro-3-deoxyheptonate aldolase precursor**	**V, Primary metabolism**	**0,5/1,33**
33.	MtC10255	Lipase class 3	V, Primary metabolism	0,19/1,04
34.	MtC10863,2	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	2,12/ 1,73
35.	MtC20397,4	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	1,97/2,71
36.	MtC10863,3	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	1,52/1,82
37.	MtC20397,1	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	1,43/1,71
38.	**MtC10863,1**	**Chalcone synthase**	**VI, Secondary metabolism and hormone metabolism**	**1,37/2,12**
39.	MtC20397,5	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	1,05/1,01
40.	MtC30184	S-adenosyl-methionine-sterol-c- methyltransferase	VI, Secondary metabolism and hormone metabolism	0,85/1,21
41.	MtC93164	Coproporphyrinogen iii oxidase precursor	VI, Secondary metabolism and hormone metabolism	0,76/1,03
42.	**MtC45216**	**Udp-glycose:flavonoid glycosyltransferase**	**VI, Secondary metabolism and hormone metabolism**	**0,63/1,11**
43.	**MtC50948,1**	**Hmg-y related protein**	**VII, Chromatin and DNA metabolism**	**0,74/1,53**
44.	MtC10493	Wrky-type DNA-binding protein	VIII, Gene expression and RNA metabolism	2,09/1,52
45.	MtC10374	Zinc finger c2h2 containing protein	VIII, Gene expression and RNA metabolism	1,47/0,77
46.	MtC10310	Kruppel-like zinc finger protein	VIII, Gene expression and RNA metabolism	1,19/2,29
47.	MtC91811	B-zip transcription factor	VIII, Gene expression and RNA metabolism	1,12/1,45
48.	**MtC00402**	**Transcription factor**	**VIII, Gene expression and RNA metabolism**	**1,07/1,15**
49.	MtC10507	Rna-binding protein precursor	VIII, Gene expression and RNA metabolism	0,69/1,54
50.	MtC93018	PR- transcriptional factor and erf	VIII, Gene expression and RNA metabolism	0,51/1,17
51.	MtC10888	Ef-hand calcium-binding domain	X, Signal transduction	1,58/2,75

Continuation of table 8a

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
52.	MtC20118	Mitogen-activated protein kinase	X, Signal transduction and post-translational regulation	2,21/1,84
53.	MtC00346	Calmodulin	X, Signal transduction and post-translational regulation	1,19/1,14
54.	**MtC45594**	**Rac-like GTP binding protein rho**	**X, Signal transduction and post-translational regulation**	**1,14/0,56**
55.	MtC93201	Ser/thr protein phosphatase	X, Signal transduction and post-translational regulation	1,02/0,99
56.	MtC00050	His-containing phosphotransfer protein	X, Signal transduction and post-translational regulation	0,63/1,06
57.	MtC10643	Calcineurin b-like protein	X, Signal transduction and post-translational regulation	0,42/1,32
58.	MtD22717	Cdc2-related protein kinase	XI, Cell division cycle	1,2/0,84
59.	MtC00405	Ef-hand ca2+-binding domain	XII, Miscellaneous	2,06/2,19
60.	MtC00020	14-3-3 protein	XII, Miscellaneous	1,52/0,37
61.	**MtC00084,2**	**PR10**	**XII, Miscellaneous**	**1,05/0,88**
62.	MtC10823	Zinc finger protein	XII, Miscellaneous	1,01/2,65
63.	MtC00067	Flavodoxin	XII, Miscellaneous	0,77/1,10
64.	MtC20238	Class III endochitinase	XII,A, Defense and cell rescue	2,32/2,02
65.	**MtC00168**	**PR10**	**XII,A, Defense and cell rescue**	**1,2/0,81**
66.	MtC90574	Abc transporter	XII,A, Defense and cell rescue	1,15/0,83
67.	MtC00309,1	Metallothionein	XII,A, Defense and cell rescue	1,03/0,17
68.	MtC91700	Beta-hexosaminidase precursor	XII,A, Defense and cell rescue	0,85/2,93
69.	MtC90151	Harpin induced protein-like protein	XII,A, Defense and cell rescue	0,56/1,08
70.	MtC91472	Ripening-related protein	XII,B, Abiotic stimuli and development	0,7/1,28
71.	MtC91091	-	XII,C, Unknown function	1,7/2,03
72.	MtC92233	-	XII,C, Unknown function	1,5/0,56
73.	MtC91404	-	XII,C, Unknown function	1,48/0,79
74.	**MtC00475**	**No homology**	**XII,C, Unknown function**	**1,41/0,89**
75.	**MtC00174,2**	**4f5rel-like protein**	**XII,C, Unknown function**	**1,37/1,14**
76.	MtC91587	Apg proline-rich protein	XII,C, Unknown function	1,24/1,21
77.	**MtC60493**	**2s albumin precursor**	**XII,C, Unknown function**	**1,11/1,24**
78.	**MtD05702**	**Unknown function**	**XII,C, Unknown function**	**1,11/0,47**
79.	**MtC00174,1**	**4f5rel-like protein**	**XII,C, Unknown function**	**1,1/0,48**
80.	MtC63223	Unknown function	XII,C, Unknown function	1,1/0,48
81.	MtC10026	-	XII,C, Unknown function	1,08/1,31
82.	MtC90247	Glu/gln rich protein	XII,C, Unknown function	1,05/0,73
83.	MtD20153	Esterase	XII,C, Unknown function	1,04/1,00
84.	MtC93327	-	XII,C, Unknown function	1,03/1,16
85.	**MtC00726**	**Uncharacterized cys-rich domain**	**XII,C, Unknown function**	**1,02/1,39**
86.	MtC90357	-	XII,C, Unknown function	0,99/1,85
87.	**MtD22621**	**Unknown function**	**XII,C, Unknown function**	**0,93/1,00**
88.	MtC00196,2	ABA-inducible protein	XII,C, Unknown function	0,82/1,40
89.	MtC10950	Zn-finger ran-binding	XII,C, Unknown function	0,78/1,61
90.	MtC91460	Zinc finger	XII,C, Unknown function	0,77/1,04
91.	MtD01700	Pap fibrillin	XII,C, Unknown function	0,76/1,09
92.	MtD22773	Early nodule-specific protein-like	XII,C, Unknown function	0,71/1,29
93.	MtD22673	Unknown function	XII,C, Unknown function	0,65/1,32
94.	MtC91423	Gf14 phi multigene family	XII,C, Unknown function	0,62/1,39
95.	MtC91858	Zn-finger ring	XII,C, Unknown function	0,54/1,27
96.	MtD02855	No homology	XIII, No homology	1,92/2,93
97.	**MtD22562**	**No homology**	**XIII, No homology**	**1,26/1,95**
98.	**MtD22795**	**No homology**	**XIII, No homology**	**1,08/1,39**
99.	MtD04283	No homology	XIII, No homology	0,85/1,12
100.	MtC91516	-	XIII, No homology	0,79/1,35
101.	mt--abc955104f11	No homology	XIII, No homology	0,73/1,25
102.	MtD22715	No homology	XIII, No homology	0,73/1,09
103.	MtC10410	-	XIII, No homology	0,52/1,33
104.	MtC91979	-	XIII, No homology	0,46/1,39
105.	**HLG200**	**Hlg 1,0 well 200**	**XIV CONTROL NO FUNCTIONAL CLASS**	**1,16/0,80**

Table-8b. The list of genes down-regulated 30 min after infection. Genes showing regulation after 30 min as well as after 6 days of infection are indicated in bold face.

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
1.	MtC91831	Oxidoreductase	V, Primary metabolism	-0,57/-1,13
2.	MtGmLS-31	Oxalate oxidase	V, Primary metabolism	-0,66/-1,09
3.	MtC20123	Beta-ketoacyl-acp synthase	V, Primary metabolism	-1,5/-1,06
4.	MtC10626	Nadh-cytochrome b5 reductase	V, Primary metabolism	-0,53/-1,07
5.	**MtC60370**	**Photosystem ii protein x precursor**	**V, Primary metabolism**	**-0,38/-1,13**
6.	MtC00497	[Nodulin] oxidosqualene cyclase ([nodulin] mtn18)	VI, Secondary metabolism and hormone metabolism	-0,31/-1,19
7.	MtC10148,10	Cytochrome p450 + 26s rrna	VI, Secondary metabolism and hormone metabolism	-1,74/-1,14
8.	**MtC10079**	**Chalcone--flavonone isomerase**	**VI, Secondary metabolism and hormone metabolism**	**-0,83/-1,24**
9.	**MtC10148,2**	**Cytochrome p450 + 26s rrna**	**VI, Secondary metabolism and hormone metabolism**	**-1,27/-0,60**
10.	**MtC93035**	**Nucleoid chloroplast dna-binding protein**	**VII, Chromatin and DNA metabolism**	**-0,52/-1,20**
11.	**MtC91628**	**Splicing factor**	**VIII, Gene expression and RNA metabolism**	**-1,03/-1,01**
12.	MtC10166,1	40s ribosomal protein s4	IX, Protein synthesis and processing	-1,19/-0,26
13.	MtC50936	30s ribosomal protein s5	IX, Protein synthesis and processing	-1/-0,83
14.	MtC91485	Ribosomal protein s13	IX, Protein synthesis and processing	-0,78/-1,04
15.	MtC30475	Prolyl 4-hydroxylase alpha subunit precursor	IX, Protein synthesis and processing	-0,63/-1,11
16.	**MtC30427**	**Calcium dependent protein kinase**	**X, Signal transduction and post-translational regulation**	**-0,4/-1,15**
17.	MtC90054	-	XII,C, Unknown function	-2,61/-1,23
18.	MtAe5	-	XII,C, Unknown function	-2,13/-1,42
19.	**MtC91489**	**-**	**XII,C, Unknown function**	**-1,64/-1,16**
20.	MtC00617	-	XII,C, Unknown function	-1,57/-0,69
21.	MtC40023,1	Peroxidase	XII, Miscellaneous	-1,2/-0,77
22.	MtD22875	Oleosin	XII, Miscellaneous	-0,91/-1,38
23.	MtC50752	Lectin	XII, Miscellaneous	-0,87/-1,04
24.	MtC50092	Unknown function	XII,C, Unknown function	-0,33/-1,15
25.	MtC10690	[Nodulin] mtn13	XII,A, Defense and cell rescue	-1,04/-0,46
26.	**MtC00704**	**Beta-ig-h3/fasciclin domain proline-rich region**	**XII,C, Unknown function**	**-0,57/-1,04**
27.	**MtC00044,1**	**Dehydrin**	**XII,B, Abiotic stimuli and development**	**-0,56/-2,79**
28.	MtC90541	Cation efflux protein	XII,C, Unknown function	-1,02/-0,39
29.	MtAe14	-	XII,C, Unknown function	-1,79/-1,15
30.	mt--abc955113f02	No homology	XIII, No homology	-1,23/-0,86
31.	MtD22691	No homology	XIII, No homology	-0,3/-1,41
32.	MtD00053	No homology	XIII, No homology	-1,09/-1,00
33.	MtC50719	-	XIII, No homology	-2,34/-1,14

Table-9a. List of genes up-regulated 6 days after infection. Genes showing regulation after 6 days as well as after 30 min of infection are indicated in bold face.

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
1.	MtC10021	Cell wall protein	I, Cell Wall	1,78/0,28
2.	MtC30178,2	Extensin	I, Cell Wall	0,96/2,17
3.	MtC00003	Repetitive proline-rich mtprp1-like protein	I, Cell Wall	0,92/2,42
4.	MtC90474	(Ser-pro)x-rich cell wall protein	I, Cell Wall	0,67/1,44
5.	MtC00116	Repetitive proline-rich mtprp2-like protein	I, Cell Wall	0,6/1,78
6.	MtC10315	Cellulose synthase catalytic subunit	I, Cell Wall	0,28/-1,57
7.	MtC00320	Caffeic acid 3-o-methyltransferase	I, Cell Wall	0,17-1,03
8.	**MtC90836**	**Myosin**	**II, Cytoskeleton**	**0,49/1,82**
9.	MtC50332	2-oxoglutarate/malate translocator precursor	III, Membrane transport	1,18/1,03
10.	MtD00065	Bile symporter; bile acid transporter	III, Membrane transport	0,83/1,02
11.	MtC45640	Sucrose transport protein	III, Membrane transport	0,65/1,32
12.	MtC60917	Pd002542, tonb dependent receptor protein signature containing protein	III, Membrane transport	0,51/1,54
13.	MtC00195	Dnaj domain containing protein	IV, Vesicular trafficking secretion and protein sorting	1,1/2,20
14.	**MtC00207**	**Ras-related protein rab7**	**IV, Vesicular trafficking secretion and protein sorting**	**0,68/1,04**
15.	**MtC93002**	**Ubiquitin-conjugating enzyme e2 (3'utr)**	**IX, Protein synthesis and processing**	**1,17/1,67**

Continuation of table 9a

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
16.	MtC00092,1	Cysteine proteinase precursor	IX, Protein synthesis and processing	1,06/2,17
17.	**MtC00117,1**	**Polyubiquitin**	**IX, Protein synthesis and processing**	**0,91/2,41**
18.	MtC00036,2	Protein translation factor	IX, Protein synthesis and processing	0,58/1,27
19.	MtC00321	Skp1-like protein	IX, Protein synthesis and processing	0,5/1,41
20.	MtC30310	Ubiquitin-conjugating enzyme e2	IX, Protein synthesis and processing	0,27/3,16
21.	MtC00104	Cysteine proteinase	IX, Protein synthesis and processing	0,18/1,30
22.	MtC00065,1	Acid phosphatase	V, Primary metabolism	1,17/0,51
23.	MtC20039	Dihydrolipoamide s-acetyltransferase component (e2) of pyruvate dehydrogenase complex	V, Primary metabolism	1,13/1,33
24.	**MtC10397**	**Chloroplast cytochrome b6**	**V, Primary metabolism**	**1,11/1,99**
25.	**MtC20054,2**	**Phospho-2-dehydro-3-deoxyheptonate aldolase precursor**	**V, Primary metabolism**	**0,96/1,14**
26.	MtC10322	Glutenin	V, Primary metabolism	0,87/1,73
27.	MtC00326	6-phosphogluconate dehydrogenase	V, Primary metabolism	0,74/1,64
28.	MtC00057	Esterase	V, Primary metabolism	0,74/1,55
29.	MtC20175	Alanine--glyoxylate aminotransferase 2 precursor	V, Primary metabolism	0,55/1,38
30.	MtC00025	Udp-galactose-4-epimerase	V, Primary metabolism	0,49/3,14
31.	MtC00119	Chlorophyll a/b binding protein	V, Primary metabolism	0,44/1,04
32.	MtC10159	Fructokinase	V, Primary metabolism	0,29/1,19
33.	MtC50790	Glutamine synthetase precursor	V, Primary metabolism	0,15/-1,17
34.	**MtC45216**	**Udp-glycose:flavonoid glycosyltransferase**	**VI, Secondary metabolism and hormone metabolism**	**0,79/1,46**
35.	**MtC10863,1**	**Chalcone synthase**	**VI, Secondary metabolism and hormone metabolism**	**0,73/1,18**
36.	MtC10756	Profucosidase precursor	VI, Secondary metabolism and hormone metabolism	0,7/1,34
37.	**MtC50948,1**	**Hmg-y related protein**	**VII, Chromatin and DNA metabolism**	**1,04/1,98**
38.	MtC10249	DNA topoisomerase ii signature containing protein	VII, Chromatin and DNA metabolism	0,62/1,04
39.	MtC10139	Rna binding protein	VIII, Gene expression and RNA metabolism	1,11/1,17
40.	MtC10102	High mobility group protein	VIII, Gene expression and RNA metabolism	1,05/0,88
41.	MtC00793	Rna-binding domain containing protein	VIII, Gene expression and RNA metabolism	0,86/1,54
42.	MtC45374	Zinc finger protein	VIII, Gene expression and RNA metabolism	0,82/1,14
43.	MtC50402	RNA helicase	VIII, Gene expression and RNA metabolism	0,72/1,36
44.	MtC00232	Myb transcription factor	VIII, Gene expression and RNA metabolism	0,64/1,16
45.	MtC90627	Forkhead-related transcription factor	VIII, Gene expression and RNA metabolism	0,63/1,01
46.	MtC00106	High mobility group protein	VIII, Gene expression and RNA metabolism	0,62/1,16
47.	MtC10711	Homeodomain-leucine zipper protein	VIII, Gene expression and RNA metabolism	0,51/1,44
48.	**MtC00402**	**Transcription factor**	**VIII, Gene expression and RNA metabolism**	**0,33/1,12**
49.	MtC10758,1	Ser/thr protein kinase	X, Signal transduction and post-translational regulation	1,53/1,81
50.	MtC50031	Protein kinase	X, Signal transduction and post-translational regulation	1,17/1,13
51.	MtC00080	Calmodulin	X, Signal transduction and post-translational regulation	0,86/1,79
52.	**MtC45594**	**Rac-like GTP binding protein rho**	**X, Signal transduction and post-translational regulation**	**0,24/1,62**
53.	MtC10112	Peroxidase precursor	XII, Miscellaneous	1,6/1,70
54.	MtC00421	Peroxidase	XII, Miscellaneous	0,84/1,08
55.	MtC30528,1	C3hc4-type ring zinc finger protein	XII, Miscellaneous	0,83/1,27
56.	MtC20068	Mtd2 ring zinc-finger protein	XII, Miscellaneous	0,48/1,43
57.	MtC10070	Lipoxygenase	XII, Miscellaneous	0,48/1,06
58.	**MtC00084,2**	**PR10 pathogenesis-related / leginsulin chimer**	**XII, Miscellaneous**	**0,46/1,80**
59.	MtC00041,1	Metallothionein	XII,A, Defense and cell rescue	1,06/-0,68
60.	**MtC00168**	**PR10**	**XII,A, Defense and cell rescue**	**0,87/1,62**
61.	MtC00663	PR-1 pathogenesis-related protein -like	XII,A, Defense and cell rescue	0,73/2,08
62.	MtC30019,1	Nematode resistance (hs1pro-1)-like protein	XII,A, Defense and cell rescue	0,24/2,17
63.	MtC00640,1	Germin-like protein (oxalate oxidase)	XII,B, Abiotic stimuli and development	2,41/1,08
64.	MtC10438	Auxin-induced protein	XII,B, Abiotic stimuli and development	1,46/1,48
65.	MtD22746	Ripening related protein	XII,B, Abiotic stimuli and development	1,23/0,79
66.	MtC00172	Germin-like protein (oxalate oxidase)	XII,B, Abiotic stimuli and development	1,12/-0,74
67.	MtC00056	Cold acclimation responsive protein budcar	XII,B, Abiotic stimuli and development	1,01/2,47
68.	MtC00667	Aluminum-induced auxin-repressed protein	XII,B, Abiotic stimuli and development	0,29/1,12
69.	**MtC00044,1**	**Dehydrin**	**XII,B, Abiotic stimuli and development**	**0,09/1,57**

Continuation of table 9a

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
70.	**MtC00174,1**	**4f5rel-like protein**	**XII,C, Unknown function**	**1,41/1,49**
71.	MtC40018	Putative methyltransferase duf248	XII,C, Unknown function	1,32/2,15
72.	MtC00499,2	-	XII,C, Unknown function	1,28/1,08
73.	MtC93068	[Nodulin] mtn21-like protein	XII,C, Unknown function	1,18/1,69
74.	**MtC00475**	**No homology**	**XII,C, Unknown function**	**1,1/0,85**
75.	KV2-5M17	[Nodulin] mtn93	XII,C, Unknown function	1,09/2,28
76.	MtC10446,1	Transmembrane protein	XII,C, Unknown function	0,92/-1,04
77.	MtC30401	Skp1 component	XII,C, Unknown function	0,88/1,27
78.	MtC10380	Epsin n-terminal homology	XII,C, Unknown function	0,85/1,36
79.	MtC91376	-	XII,C, Unknown function	0,78/1,35
80.	MtC10886	Plastocyanin-like proline-rich region	XII,C, Unknown function	0,77/1,39
81.	MtC90498,1	Protein kinase leucine-rich repeat plant specific	XII,C, Unknown function	0,76/1,00
82.	MtC90989	Proline-rich region	XII,C, Unknown function	0,72/1,16
83.	**MtC60493**	**2s albumin precursor**	**XII,C, Unknown function**	**0,71/1,00**
84.	**MtD22621**	**Unknown function**	**XII,C, Unknown function**	**0,7/1,01**
85.	MtC40184	Ring-h2 finger protein	XII,C, Unknown function	0,64/1,93
86.	**MtC00174,2**	**4f5rel-like protein**	**XII,C, Unknown function**	**0,61/1,15**
87.	**MtD05702**	**Unknown function**	**XII,C, Unknown function**	**0,6/1,12**
88.	MtD22890	Unknown function	XII,C, Unknown function	0,58/1,01
89.	MtC30346	Oxidoreductase fad/nad (p)-binding	XII,C, Unknown function	0,51/1,04
90.	MtC90044	Curculin-like \(mannose-binding\) lectin	XII,C, Unknown function	0,45/4,09
91.	MtC30562	Gly-rich protein	XII,C, Unknown function	0,44/1,39
92.	**MtC00726**	**Uncharacterized cys-rich domain**	**XII,C, Unknown function**	**0,44/1,03**
93.	MtC00633	Selenoprotein-like protein	XII,C, Unknown function	0,44/1,18
94.	MtC10873	-	XII,C, Unknown function	0,42/1,16
95.	MtC40196	Zn-finger in ran bp domain containing protein	XII,C, Unknown function	0,37/1,27
96.	MtC30154,1	Wound-induced protein like protein	XII,C, Unknown function	0,31/1,08
97.	MtC10028	Unknown function	XII,C, Unknown function	0,27/2,52
98.	MtC00499,1	No homology	XII,C, Unknown function	0,14/2,84
99.	MtC10038,2	No homology	XIII, No homology	2,2/1,41
100.	**MtD22562**	**No homology**	**XIII, No homology**	**1,83/1,91**
101.	MtC00720,2	Multispecific proteasome protease	XIII, No homology	1,6/1,08
102.	MtC91486	-	XIII, No homology	1,19/1,04
103.	MtC90830	Gelsolin sec23/sec24 helical domain	XIII, No homology	1,13/1,71
104.	**MtD22795**	**No homology**	**XIII, No homology**	**0,94/1,33**
105.	MtC92064	-	XIII, No homology	0,86/1,08
106.	MtC91972	-	XIII, No homology	0,76/2,38
107.	MtBC32E12	Na	XIII, No homology	0,74/1,20
108.	MtC91880	-	XIII, No homology	0,7/1,14
109.	MtC90850	-	XIII, No homology	0,64/1,19
110.	MtC30453,1	-	XIII, No homology	0,47/3,07
111.	MtC93053	No homology	XIII, No homology	0,12/-1,04
112.	**HLG200**	**Hlg 1,0 well 200**	**XIV CONTROL NO FUNCTIONAL CLASS**	**0,62/1,28**

Table-9b. List of genes down-regulated 6 days after infection.

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
1.	MtC10969	Udpglucose:protein transglucosylase	I, Cell Wall	-0,95/-1,19
2.	MtC00010	Membrane intrinsic protein	III, Membrane transport	-0,33/-1,48
3.	MtC00001	Aquaporin	III, Membrane transport	-0,66/-1,38
4.	JVCPG19	Tm protein	III, Membrane transport	-0,9/-1,83
5.	MtC10612,1	Adp,atp carrier protein precursor	III, Membrane transport	-1,41/-1,99
6.	MtC00027	Membrane channel protein	III, Membrane transport	-1,58/-2,78
7.	MtC00109,2	Adpribosylation factor	IV, Vesicular trafficking secretion and protein sorting	-0,54/-1,19
8.	MtC00150	60s ribosomal protein l32	IX, Protein synthesis and processing	-0,3/-1,02
9.	MtC00550,1	Endoplasmic reticulum hsc70cognate binding protein precursor	IX, Protein synthesis and processing	-0,32/-2,37
10.	MtC00066	60s ribosomal protein l21	IX, Protein synthesis and processing	-0,37/-1,27
11.	MtC00108	60s ribosomal protein l26	IX, Protein synthesis and processing	-0,42/-1,42
12.	MtC00083,1	Nascent polypeptide associated complex alpha chain	IX, Protein synthesis and processing	-0,48/-1,50
13.	MtC00015	Peptidylprolyl cistrans isomerase (cyclophilin)	IX, Protein synthesis and processing	-0,51/-1,42
14.	MtC00167	40s ribosomal protein s11	IX, Protein synthesis and processing	-0,52/-1,17
15.	MtC00550,2	Endoplasmic reticulum hsc70cognate binding protein precursor (bip)	IX, Protein synthesis and processing	-0,65/-1,18
16.	MtC00163	60s acidic ribosomal protein p1	IX, Protein synthesis and processing	-0,65/-1,05
17.	MtC00239	Heat shock cognate 70 kda protein	IX, Protein synthesis and processing	-0,69/-1,04
18.	MtC00786	Heat shock cognate protein	IX, Protein synthesis and processing	-0,71/-2,39
19.	MtC00038	60s ribosomal protein l35	IX, Protein synthesis and processing	-0,82/-1,17
20.	MtC00073	60s ribosomal protein l11	IX, Protein synthesis and processing	-0,83/-1,57
21.	MtC50070	Aspartyl proteases active site containing protein	IX, Protein synthesis and processing	-0,85/-1,04
22.	MtC00134	60s ribosomal protein l28	IX, Protein synthesis and processing	-0,86/-1,03
23.	MtC00118,1	40s ribosomal protein s23	IX, Protein synthesis and processing	-0,86/-1,28
24.	MtC10071	40s ribosomal protein s3a	IX, Protein synthesis and processing	-0,87/-1,70
25.	MtC00011	60s ribosomal protein l9	IX, Protein synthesis and processing	-0,89/-1,27
26.	MtC01542	60s ribosomal protein l3	IX, Protein synthesis and processing	-0,91/-1,08
27.	MtC00048	40s ribosomal protein s21	IX, Protein synthesis and processing	-0,97/-1,12
28.	MtC00586	60s ribosomal protein l31	IX, Protein synthesis and processing	-1,04/-1,35
29.	MtC00516	40s ribosomal protein s13	IX, Protein synthesis and processing	-1,04/-0,76
30.	MtC00138	40s ribosomal protein s17	IX, Protein synthesis and processing	-1,06/-0,47
31.	MtC00052	60s ribosomal protein l41	IX, Protein synthesis and processing	-1,1/-1,19
32.	MtC00141	40s ribosomal protein s29	IX, Protein synthesis and processing	-1,18/-0,82
33.	KV118A23	Efc4	IX, Protein synthesis and processing	-1,18/-0,95
34.	MtC00135	40s ribosomal protein s8	IX, Protein synthesis and processing	-1,19/-1,21
35.	MtC00077	40s ribosomal protein s25	IX, Protein synthesis and processing	-1,2 /-1,13
36.	MtC00142	40s ribosomal protein s5	IX, Protein synthesis and processing	-1,21/-0,60
37.	**MtC00325**	**60s ribosomal protein l15**	**IX, Protein synthesis and processing**	**-1,23/-0,47**
38.	MtC00414	60s ribosomal protein l26	IX, Protein synthesis and processing	-1,29/-1,47
39.	MtC00176	60s ribosomal protein l13	IX, Protein synthesis and processing	-1,31/-1,42
40.	MtC00539	60s ribosomal protein l21	IX, Protein synthesis and processing	-1,39/-1,26
41.	MtC00749	60s ribosomal protein l7a	IX, Protein synthesis and processing	-1,38/-0,97
42.	MtC00089	60s acidic ribosomal protein po	IX, Protein synthesis and processing	-1,39/-0,68
43.	**MtC00002,1**	**Elongation factor 1alpha**	**IX, Protein synthesis and processing**	**-1,53/-1,39**
44.	MtC00318	40s ribosomal protein s3	IX, Protein synthesis and processing	-1,6/-1,68
45.	MtC10175	40s ribosomal protein s12	IX, Protein synthesis and processing	-1,79/-0,38
46.	MtC93403	Dihydrofolate reductase	V, Primary metabolism	-0,44/-1,13
47.	**MtC60370**	**Photosystem ii protein x precursor**	**V, Primary metabolism**	**-0,5/-1,05**
48.	MtC10064	Adenosine kinase	V, Primary metabolism	-0,76/-2,10
49.	MtC00577	Acyl carrier protein (nadhubiquinone oxidoreductase)	V, Primary metabolism	-0,92/-1,06
50.	MtC00018	5methyltetrahydropteroyltriglutamatehomocysteine methyltransferase	V, Primary metabolism	-0,92/-1,06
51.	MtC00016	Nucleoside diphosphate kinase	V, Primary metabolism	-1,03/-1,37
52.	MtC00098,1	Acyl carrier protein	V, Primary metabolism	-1,11/-0,60
53.	MtC00772	Serine hydroxymethyltransferase	V, Primary metabolism	-1,28/-0,89

Continuation of table 9b

#	Gene ID	Annotation	Functional class	M-value (bc1/bc2)
54.	MtC00415	Cytochrome c	V, Primary metabolism	-1,34/-1,01
55.	**MtC00030**	**Glyceraldehyde 3phosphate dehydrogenase**	**V, Primary metabolism**	**-1,39/-1,86**
56.	MtC10068	Enolase	V, Primary metabolism	-1,55/-2,42
57.	**MtC10079**	**Chalconeflavonone isomerase**	**VI, Secondary metabolism and hormone metabolism**	**-0,29/-1,24**
58.	**MtC10148,2**	**Cytochrome p450 + 26s rrna**	**VI, Secondary metabolism and hormone metabolism**	**-0,7/-1,52**
59.	MtC10121,1	Cytochrome p450 72a1	VI, Secondary metabolism and hormone metabolism	-0,86/-2,63
60.	MtC40004,1	Cytochrome p450	VI, Secondary metabolism and hormone metabolism	-1,5/-2,35
61.	**MtC93035**	**Nucleoid chloroplast dnabinding protein like**	**VII, Chromatin and DNA metabolism**	**-0,6/-1,17**
62.	MtC10749	Histone h4	VII, Chromatin and DNA metabolism	-1/-0,29
63.	MtC30166	Nucleolar histone deacetylase	VII, Chromatin and DNA metabolism	-1,32/-0,76
64.	**MtC91628**	**Splicing factor**	**VIII, Gene expression and RNA metabolism**	**-0,53/-1,66**
65.	MtC30209	Polyadenylatebinding protein	VIII, Gene expression and RNA metabolism	-0,75/-1,07
66.	MtC91092	Helix loop helix containing protein	VIII, Gene expression and RNA metabolism	-1,37/-1,01
67.	**MtC30427**	**Calcium dependent protein kinase**	**X, Signal transduction and posttranslational regulation**	**-0,6/-1,04**
68.	MtC60790	Oleosin	XII, Miscellaneous	-0,57/-1,17
69.	MtC45080	Betaglucosidase	XII, Miscellaneous	-0,65/-1,07
70.	MtC40041	Calreticulin precursor	XII, Miscellaneous	-1,31/-1,89
71.	MtC10085,1	Glycosyl hydrolase	XII, Miscellaneous	-1,34/-1,43
72.	MtD22921	Gamma thionin; knottin	XII,A, Defense and cell rescue	-0,38/1,09
73.	MtD22777	Auxin responsive saur protein	XII,B, Abiotic stimuli and development	-0,74/1,08
74.	MtC20177	Ghmp kinase	XII,C, Unknown function	-0,35/2,24
75.	MtC50530	-	XII,C, Unknown function	-0,42/1,05
76.	**MtC00704**	**Betaigh3/fasciclin domain prolinerich region**	**XII,C, Unknown function**	**-0,46/1,51**
77.	MtC30550,1	-	XII,C, Unknown function	-0,47/1,01
78.	MtAe88	-	XII,C, Unknown function	-0,54/1,07
79.	MtD22941	Unknown function	XII,C, Unknown function	-0,61/1,03
80.	**MtC91489**	**-**	**XII,C, Unknown function**	**-0,62/1,35**
81.	MtC00393	Prorich protein	XII,C, Unknown function	-0,72/1,60
82.	MtC10504,1	-	XII,C, Unknown function	-0,89/1,07
83.	MtC90145	-	XII,C, Unknown function	-1/0,42
84.	MtC10344	-	XII,C, Unknown function	-1/0,39
85.	MtC00604	Unknown function	XII,C, Unknown function	-1/0,43
86.	MtC20007	Cell elongation protein diminuto	XII,C, Unknown function	-1,02/ 0,54
87.	MtC40074	-	XII,C, Unknown function	-1,04/ 0,67
88.	MtGmLS333	-	XII,C, Unknown function	-1,1/0,47
89.	MtC00130	Glu rich protein	XII,C, Unknown function	-1,24/1,43
90.	MtC00107	Plant lipid transfer signature containing protein	XII,C, Unknown function	-1,58/1,81
91.	mtabc955116f10	No homology	XIII, No homology	-0,13/1,26
92.	MtC90482	Transketolase c terminal transketolase central region	XIII, No homology	-0,64/1,09
93.	MtC91127	-	XIII, No homology	-1,46/0,60
94.	GAPDHCentralOligo	Gapdh central 69mer oligo	XIV CONTROL NO FUNCTIONAL CLASS	-1,03/1,05
95.	GAPDH5Oligo	Gapdh 5' 69mer oligo	XIV CONTROL NO FUNCTIONAL CLASS	-1,1/1,38

3.17 Microarray study of global gene expression pattern changes affected by silencing of *MtMir*-1 gene.

The aim of this experiment was to study the influence of the *MtMir*-1 gene on the global gene expression pattern during *A. euteiches*-induced disease development. The transgenic root cultures carrying empty pFGC 5149 vector and MtMir-1-PTGS constructs were used for microarray analyses (*table-10*).

Table-10. Experimental design of the second microarray hybridisation experiment.

RNA probes used for array analyses		cDNA labelling with Cy dyes	Probe composition	Name of the hybridised slides	Name of the experiment
pFGC-1 Bc-1	**c-1**	c-1-**t-1**	**c**-1-**t-1**	Bc-1-t-1	**PFGC-Bc-1**
		c-1-**t-2**	**i**-1-**t-1**		
	i-1	i-1-**t-1**	**c**-1-**t-2**	Bc-1-t-2	
		i-1-**t-2**	**i**-1-**t-2**		
PFGC-2 Bc-2	**c-2**	c-2-**t-1**	**c**-2-**t-1**	Bc-2-t-1	**PFGC-Bc-2**
		c-2-**t-2**	**i**-2-**t-1**		
	i-2	i-2-**t-1**	**c**-2-**t-2**	Bc-2-t-2	
		i-2-**t-2**	**i**-2-**t-2**		
MtMir-1-PTGS-1 Bc-1	**c-1**	**c-1**	**c**-1	Bc-1	**MtMir-1-PTGS Bc-1**
	i-1	**i-1**	**i**-1		
MtMir-1-PTGS-2 Bc-2	**c-2**	**c-2**	**c**-2	Bc-2	**MtMir-1-PTGSBc-2**
	i-2	**i-2**	**i**-2		

pFGC –1 and 2 *roots not silenced for MtMir-1 gene*
MtMir-1-PTGS-1 and 2 *roots silenced for MtMir-1 gene*
Bc- *biological controls of experiments*
c- *control roots mock inoculated with sterile lake water*
i- *roots infected with A. euteiches*
t- *technical control*

M vs. A scatterplots of all biological control experiments *(fig. 41)* and M vs. M scatterplots showing variations between biological control experiments *(fig. 42)* are generated.

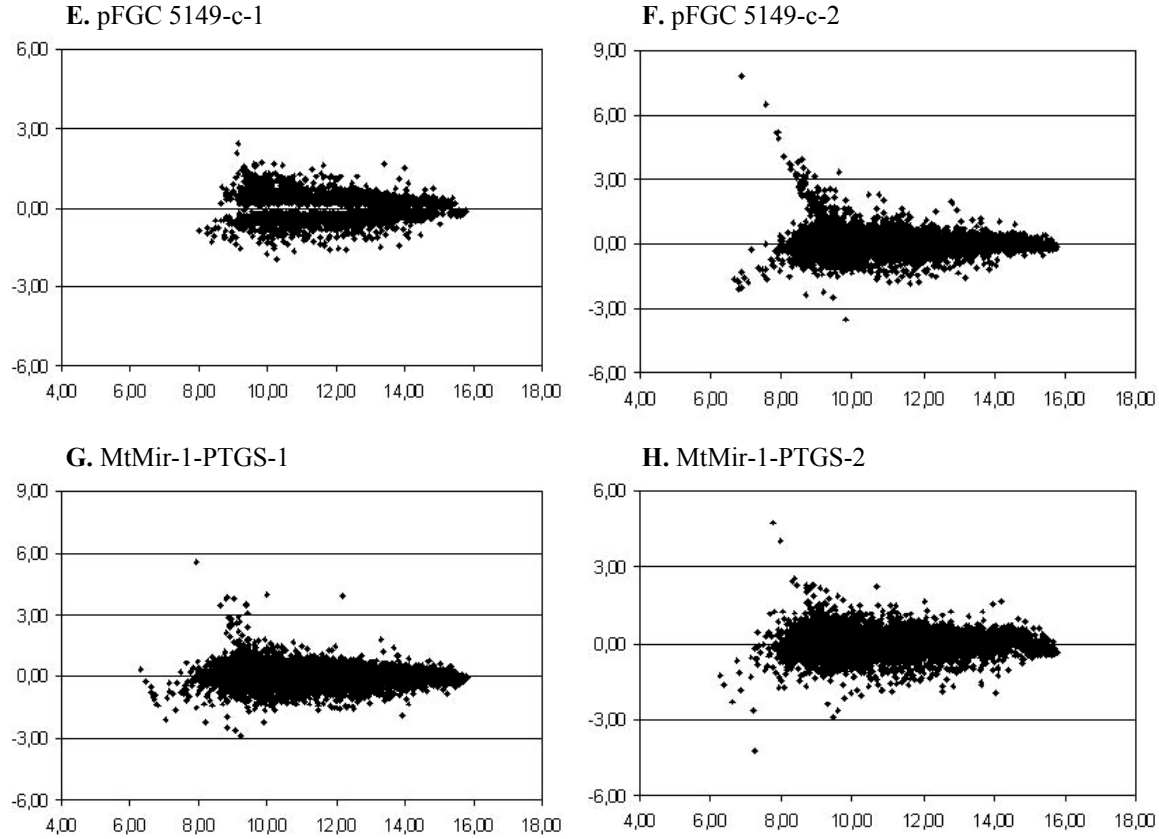

Fig. 41 Scatterplots showing M vs. A plot of all biological control slides after lowess normalization. X-value A. Y-value M.

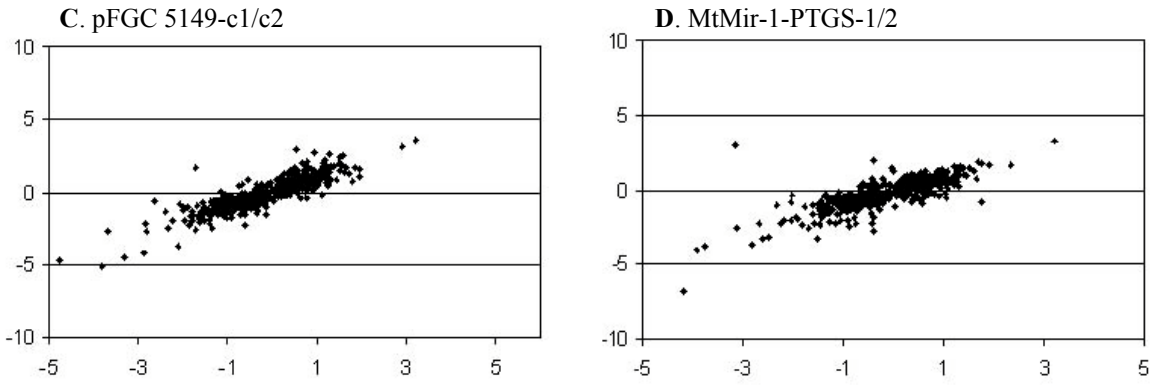

Fig. 42 M vs. M scatterplots showing variations between biological control expreriments.

The microarray hybridization experiment revealed 299 genes which exibited altered expression *(table-12).* These genes could be grouped into three clusters *(table-11)*. The first cluster contains 108 genes which were regulated (> or = 2 fold up or down) in both non silenced (pFGC-1 and 2) and silenced (*MtMir*-1-PTGS-1 and 2) roots lines. A second cluster contains 72 genes which were regulated in an empty pFGC carrying roots, but not found to be regulated in the MtMir-1-PTGS construct carrying roots. Cluster III contains 119 genes which were not regulated in an empty pFGC carrying roots, but regulated in roots carrying the MtMir-1-PTGS contstruct.

Table 11. The overview of regulated genes identified by the microarray study. Genes whose expression were not affected by silencing of *MtMir*-1 gene are in cluster I. Genes whose expression were affected by silencing of *MtMir*-1 gene are in cluster II and III.

Cluster #	Description	Number of up-regulated genes	Number of down-regulated genes	Total number of genes
Cluster I	(pFGC 5149 and *MtMir*-1-PTGS)	35	73	108
Cluster II	(pFGC 5149)	31	41	72
Cluster III	(*MtMir*-1-PTGS)	71	48	119

Table 12a. The list of genes up-regulated in both pFGC 5149 and the *MtMir*-1-PTGS construct carrying roots after infection with *A. euteiches*.. Gene identity, annotation and functional class of each gene is shown according to MENS (Medicago EST Navigation System, http://medicago.toulouse.inra.fr/Mt/EST). M-value of each gene in pFGC 5149 and MtMir-1-PTGS experiments are also shown.

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	MtC10480,1	Caffeic acid 3-o-methyltransferase	I, Cell Wall	1,23	1,25
2.	MtC00798	Arabinogalactan-protein precursor	I, Cell Wall	1,03	1,07
3.	MtC93333	Cysteine proteinase precursor	IX, Protein synthesis and processing	1,31	1,66
4.	MtC10148,3	18s ribosomal rna gene	IX, Protein synthesis and processing	1,19	1,26
5.	MtC91090	4-hydroxyphenylpyruvate dioxygenase	V, Primary metabolism	1,28	1,5
6.	MtC00025	Udp-galactose-4-epimerase	V, Primary metabolism	1,23	1,96
7.	MtC20026	3-ketoacyl-coa thiolase peroxisomal	V, Primary metabolism	1,01	1,69
8.	MtC20397,4	Chalcone synthase	VI, Secondary metabolism and hormone metabolism	1,01	1,06
9.	MtC10199	Late embryogenesis abundant protein	XII, Miscellaneous	1,22	1,12
10.	MtC90030	Chitin-binding protein	XII,A, Defense and cell rescue	1,68	1,17
11.	MtC10312	Chitinase	XII,A, Defense and cell rescue	1,22	1,06
12.	MtC00666	Wound-induced protein-like	XII,A, Defense and cell rescue	1,05	1,16
13.	MtC10317,1	Osmotin-like protein	XII,B, Abiotic stimuli and development	3,2	3,24
14.	MtC10364	-	XII,C, Unknown function	1,71	1,11
15.	MtC00752	Unknown function	XII,C, Unknown function	1,56	1,57
16.	MtC10348,1	Glyoxalase/bleomycin resistance protein/dioxygenase domain	XII,C, Unknown function	1,53	1,65
17.	MtAe14	-	XII,C, Unknown function	1,5	1,16
18.	MtC10409	Octicosapeptide/phox/bem1p proline-rich region	XII,C, Unknown function	1,46	1,05
19.	MtC30334	Lys/ser rich protein	XII,C, Unknown function	1,42	1,08
20.	MtC93138	Transmembrane protein	XII,C, Unknown function	1,36	1,35
21.	MtC00203	Unknown function	XII,C, Unknown function	1,35	1,03
22.	MtC00370	-	XII,C, Unknown function	1,33	1,25
23.	MtC40184	Ring-h2 finger protein	XII,C, Unknown function	1,3	1,07
24.	MtC30370	-	XII,C, Unknown function	1,3	1,1
25.	MtC91002	-	XII,C, Unknown function	1,29	1,49
26.	MtC10326	Cwf15/cwc15 cell cycle control protein	XII,C, Unknown function	1,29	1,23
27.	MtC00642	Protein of unknown function duf588	XII,C, Unknown function	1,28	1,17
28.	MtC10058	Usp domain	XII,C, Unknown function	1,2	1
29.	MtC30129	Mtn21-like/2 duf6 domains containing protein	XII,C, Unknown function	1,06	1,22
30.	MtC20211	Unknown function	XII,C, Unknown function	1,04	1,66
31.	MtC00068	[Nodulin] mtn1 precursor	XII,C, Unknown function	1,03	1,62
32.	MtC45077	-	XII,C, Unknown function	1,03	1,17
33.	MtC10299	-	XIII, No homology	1,79	1,29
34.	MtD02831	No homology	XIII, No homology	1,18	1,3
35.	MtC10217	-	XIII, No homology	1,01	1,11

Table 12b. The list of genes down-regulated in both pFGC 5149 and the *MtMir*-1-PTGS construct carrying roots after infection with *A. euteiches*.

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	JVCPG-39	[Nodulin] mtn12	I, Cell Wall	-1,08	-1,05
2.	MtC10315	Cellulose synthase catalytic subunit	I, Cell Wall	-1,29	-1,11
3.	MtC00797	Pectinesterase	I, Cell Wall	-1,41	-1,15
4.	MtC10024	Pro rich protein	I, Cell Wall	-1,44	-1,59
5.	MtC10084	Proline-rich protein	I, Cell Wall	-1,46	-1,01
6.	MtC10945	Caffeoyl-coa o-methyltransferase	I, Cell Wall	-1,59	-1,09
7.	MtC10148,9	Pro rich protein	I, Cell Wall	-1,66	-1,29
8.	MtC00548,1	Endoxyloglucan transferase	I, Cell Wall	-2,42	-1,62
9.	MtC10122	Proline-rich protein	I, Cell Wall	-4,18	-3,31
10.	MtC00223	Ran-binding protein	III, Membrane transport	-1,21	-1,26
11.	MtC20057,1	Inorganic phosphate transporter	III, Membrane transport	-1,23	-1,32

Continuation of table12b

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
12.	MtC10060	Chloride intracellular channel protein	III, Membrane transport	-1,36	-1,29
13.	MtC00001	Aquaporin	III, Membrane transport	-2,03	-1,98
14.	MtC00218,1	Malate dehydrogenase	V, Primary metabolism	-1,06	-1,18
15.	MtC00030	Glyceraldehyde 3-phosphate dehydrogenase	V, Primary metabolism	-1,32	-1,34
16.	MtC30223	Iron/ascorbate-dependent oxidoreductase	V, Primary metabolism	-1,4	-1,53
17.	MtC10068	Enolase	V, Primary metabolism	-1,45	-1,65
18.	MtC10491,1	2og-fe (II) oxygenase superfamily	V, Primary metabolism	-1,5	-1,18
19.	MtC00065,1	Acid phosphatase	V, Primary metabolism	-2,22	-1,84
20.	MtC10905	Squalene synthase	VI, Secondary metabolism and hormone metabolism	-1,47	-1,39
21.	MtC40169	Gibberellin 20-oxidase ((gibberellin 3-beta-hydroxylase)	VI, Secondary metabolism and hormone metabolism	-1,47	-1,03
22.	MtC40004,1	Cytochrome p450	VI, Secondary metabolism and hormone metabolism	-1,7	-2,23
23.	MtC00296,1	Flavonol 3-o-glucosyltransferase	VI, Secondary metabolism and hormone metabolism	-2,01	-1,51
24.	MtC10121,1	Cytochrome p450 72a1	VI, Secondary metabolism and hormone metabolism	-2,05	-1,84
25.	MtC30450	Istone h1	VII, Chromatin and DNA metabolism	-1,17	-1,04
26.	MtC00165	Histone h3	VII, Chromatin and DNA metabolism	-1,29	-1,41
27.	MtC00730	Dna binding protein s1fa	VIII, Gene expression and RNA metabolism	-1,18	-1,06
28.	MtC90003	Ser/thr protein kinase	X, Signal transduction and post-translational regulation	-1,36	-1,1
29.	MtC30440,1	Protein kinase domain containing protein	X, Signal transduction and post-translational regulation	-1,9	-1,44
30.	MtC00087,3	GTP-binding nucleocytoplasmic transport ran-like protein	XI, Cell division cycle	-1,25	-1,15
31.	MtC50295,1	Cullin	XI, Cell division cycle	-1,49	-1,09
32.	MtC10055	Lectin	XII, Miscellaneous	-1,04	-1,16
33.	MtC00274	Lipid-transfer protein	XII, Miscellaneous	-1,04	-1,56
34.	MtC91313	Zinc-finger	XII, Miscellaneous	-1,06	-1,06
35.	MtC00437	Non-specific lipid transfer-like protein	XII, Miscellaneous	-1,12	-1,44
36.	MtC90512	Zinc finger b box domain containing protein	XII, Miscellaneous	-1,12	-1,13
37.	MtC10911	Peroxidase	XII, Miscellaneous	-1,2	-1,34
38.	MtC10297	Nonspecific lipid-transfer protein	XII, Miscellaneous	-1,22	-1,12
39.	MtC40023,1	Peroxidase	XII, Miscellaneous	-1,26	-1,02
40.	MtC00012	Peroxidase	XII, Miscellaneous	-1,47	-1,55
41.	MtC10085,1	Glycosyl hydrolase	XII, Miscellaneous	-2,44	-1,94
42.	MtC10039	Endochitinase	XII,A, Defense and cell rescue	-1,04	-1,04
43.	MtC40027	S-adenosyl-l-methionine:salicylic acid carboxyl methyltransferase	XII,A, Defense and cell rescue	-1,05	-1,52
44.	MtC00123	Pathogenesis-related protein	XII,A, Defense and cell rescue	-1,17	-1,27
45.	MtC10717	Peroxidase precursor	XII,A, Defense and cell rescue	-1,63	-1,56
46.	MtC10018	Glutathione s-transferase	XII,A, Defense and cell rescue	-1,82	-1,71
47.	MtC00172	Germin-like protein (oxalate oxidase)	XII,B, Abiotic stimuli and development	-1,6	-1,49
48.	MtC92094	Purple acid phosphatase-like protein	XII,B, Abiotic stimuli and development	-2,61	-3,67
49.	MtC00013	Albumin / leginsulin precursor	XII,C, Unknown function	-1,01	-1,32
50.	MtC00033	Pollen specific protein-like protein	XII,C, Unknown function	-1,03	-1,04
51.	MtC90852	-	XII,C, Unknown function	-1,04	-1,15
52.	MtC40005	Glycosyl transferase family 2	XII,C, Unknown function	-1,14	-1,05
53.	MtC00063	No homology	XII,C, Unknown function	-1,18	-1,28
54.	MtC30395	Leginsulin / albumin	XII,C, Unknown function	-1,19	-1,22
55.	MtC00343,1	Fruit-induced csf-2 protein - like	XII,C, Unknown function	-1,24	-1,2
56.	MtC40162	[Nodulin] mtn3	XII,C, Unknown function	-1,26	-1,31
57.	MtC00107	Plant lipid transfer signature containing	XII,C, Unknown function	-1,27	-1,58
58.	JVCPG-31	[Nodulin] mtn3	XII,C, Unknown function	-1,27	-1,28
59.	MtC00620	E1 protein and def2/der2 allergen	XII,C, Unknown function	-1,38	-1,42
60.	MtC00393	Pro-rich protein	XII,C, Unknown function	-1,41	-1,18
61.	MtC00513	Leginsulin / albumin	XII,C, Unknown function	-1,41	-1,37
62.	MtC00472	No homology	XII,C, Unknown function	-1,43	-1,99
63.	MtC00343,2	-	XII,C, Unknown function	-1,49	-1,09
64.	MtC90145	-	XII,C, Unknown function	-1,49	-1,56
65.	MtC00688	Fruit-induced csf-2 protein - like	XII,C, Unknown function	-1,51	-1,25

Continuation of table 12b

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
66.	MtC00014	Pro/his-rich protein	XII,C, Unknown function	-1,59	-1,37
67.	MtC45044	-	XII,C, Unknown function	-2,91	-1,02
68.	MtC91512	-	XIII, No homology	-1,03	-1
69.	MtD22551	No homology	XIII, No homology	-1,13	-1,22
70.	MtC90482	Transketolase c terminal transketolase central region	XIII, No homology	-1,21	-1,29
71.	MtC93033	No homology	XIII, No homology	-1,24	-1,16
72.	MtC93053	No homology	XIII, No homology	-1,3	-1,44
73.	GAPDH5Oligo	Gapdh 5' 69-mer oligo	XIV CONTROL NO FUNCTIONAL CLASS	-1,33	-1,01

Table 12c. List of genes up-regulated only in pFGC 5149 carrying roots after infection with *A. euteiches.*.

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	MtC10943	Translation initiation factor 5	IX, Protein synthesis and processing	1,31	-
2.	MtC00469	40s ribosomal protein s6	IX, Protein synthesis and processing	1,29	-
3.	MtC40182	Ubiquitin-like protein activating enzyme	IX, Protein synthesis and processing	1,13	-
4.	MtC20220	26s proteasome regulatory subunit	IX, Protein synthesis and processing	1,08	-
5.	MtC00790	40s ribosomal protein s17	IX, Protein synthesis and processing	1,02	-
6.	MtC00083,1	Nascent polypeptide associated complex alpha chain	IX, Protein synthesis and processing	1,01	-
7.	MtC93002	Ubiquitin-conjugating enzyme e2 (3'utr)	IX, Protein synthesis and processing	1	-
8.	MtC50754	Glycosyltransferase	V, Primary metabolism	1,14	-
9.	MtC10479	Acid phosphatase	V, Primary metabolism	1,02	-
10.	MtC10572	Rna lariat debranching enzyme	VIII, Gene expression and RNA metabolism	1,02	-
11.	MtC10888	Ef-hand calcium-binding domain	X, Signal transduction	1,77	-
12.	MtC45277	Casein kinase / dual specificity kinase	X, Signal transduction and post-translational regulation	1,17	-
13.	MtD01663	Glycoside hydrolase, family 18	XII,A, Defense and cell rescue	1,27	-
14.	MtC10834	Disease resistance response protein 206 - like	XII,A, Defense and cell rescue	1,03	-
15.	MtC30064	Auxin-induced protein-like protein	XII,B, Abiotic stimuli and development	1,02	-
16.	MtC00174,1	4f5rel-like protein	XII,C, Unknown function	1,47	-
17.	MtC90020	-	XII,C, Unknown function	1,43	-
18.	MtC40073	Nodulin-like protein	XII,C, Unknown function	1,42	-
19.	MtD05388	Unknown function	XII,C, Unknown function	1,3	-
20.	JVCPG-29	[Nodulin] mtn1	XII,C, Unknown function	1,15	-
21.	MtC30550,1	-	XII,C, Unknown function	1,12	-
22.	MtC30272	Cyclin-like f-box	XII,C, Unknown function	1,11	-
23.	MtC30084	-	XII,C, Unknown function	1,09	-
24.	MtC91496	-	XII,C, Unknown function	1,09	-
25.	MtC00070	Unknown function	XII,C, Unknown function	1,08	-
26.	MtC30562	Gly-rich protein	XII,C, Unknown function	1,03	-
27.	MtC93327	-	XII,C, Unknown function	1	-
28.	MtD22795	No homology	XIII, No homology	1,4	-
29.	MtD00053	No homology	XIII, No homology	1,19	-
30.	MtD09036	No homology	XIII, No homology	1,08	-
31.	MtC91341	-	XIII, No homology	1	-

Table 12d. List of genes up-regulated only in MtMir-1-PTGS carrying roots after infection with *A. euteiches*..

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	MtC10607	Caffeic acid o-methyltransferase	I, Cell Wall	-	1,48
2.	MtC91309	1 4-benzoquinone reductase	I, Cell Wall	-	1,21
3.	MtC92155	Non intermediate filament ifa binding protein	II, Cytoskeleton	-	1,05
4.	MtC10629	Synaptobrevin-like / vesicle-associated membrane protein	IV, Vesicular trafficking secretion and protein sorting	-	1,28
5.	MtC30066	Synaptosomal associated protein 25	IV, Vesicular trafficking secretion and protein sorting	-	1,03
6.	MtC90330	Cysteine proteinase	IX, Protein synthesis and processing	-	1,6
7.	MtC90342	Myrosinase-associated protein	IX, Protein synthesis and processing	-	1,46
8.	MtC30310	Ubiquitin-conjugating enzyme	IX, Protein synthesis and processing	-	1,29
9.	MtC00690,1	Proteasome regulatory subunit	IX, Protein synthesis and processing	-	1,19
10.	MtC90590	Elongation factor 1-alpha	IX, Protein synthesis and processing	-	1,15
11.	MtC00117,1	Polyubiquitin	IX, Protein synthesis and processing	-	1,13
12.	MtC20151,1	Translation initiation factor 6 (eif-6)	IX, Protein synthesis and processing	-	1,03
13.	MtC91778	Aminopeptidase 2 autointerpro: cytosol aminopeptidase	IX, Protein synthesis and processing	-	1,01
14.	MtC45211,1	S-adenosylmethionine decarboxylase proenzyme	V, Primary metabolism	-	1,42
15.	MtC60381	Pyridine nucleotide-disulphide oxidoreductase dimerisation domain	V, Primary metabolism	-	1,26
16.	MtC00430,1	Cysteine synthase	V, Primary metabolism	-	1,15
17.	MtC10397	Chloroplast cytochrome b6	V, Primary metabolism	-	1,05
18.	MtC91781	Glutathione s-transferase	VI, Secondary metabolism and hormone metabolism	-	1,25
19.	MtC10198	Cytochrome p450 83	VI, Secondary metabolism and hormone metabolism	-	1,17
20.	MtC10249	Dna topoisomerase II signature containing protein	VII, Chromatin and DNA metabolism	-	1,15
21.	MtC10688	Myb-related protein	VIII, Gene expression and RNA metabolism	-	1,71
22.	MtC10374	Zinc finger c2h2 containing protein	VIII, Gene expression and RNA metabolism	-	1,12
23.	MtC10881	Transcription factor vsf-1	VIII, Gene expression and RNA metabolism	-	1,07
24.	MtC10978	Transcription initiation factor IIe beta subunit	VIII, Gene expression and RNA metabolism	-	1,03
25.	MtC10758,1	Ser/thr protein kinase	X, Signal transduction and post-translational regulation	-	1,48
26.	MtC10763	Annexin	X, Signal transduction and post-translational regulation	-	1,42
27.	MtC30317	Polyphosphoinositide binding protein ssh2p,	X, Signal transduction and post-translational regulation	-	1,27
28.	MtC30141	Ser/thr protein phosphatase	X, Signal transduction and post-translational regulation	-	1,17
29.	MtC10148,5	18s ribosomal rna	XII, Miscellaneous	-	1,73
30.	MtC30293	Zinc finger protein	XII, Miscellaneous	-	1,33
31.	MtC93156	Atp/GTP-binding site motif a (p-loop) and ring finger - containing protein	XII, Miscellaneous	-	1,19
32.	MtC30389	Peroxidase precursor	XII, Miscellaneous	-	1,13
33.	MtC60434	Mtd1	XII, Miscellaneous	-	1,06
34.	MtC00405	Ef-hand ca2+-binding domain containing protein	XII, Miscellaneous	-	1,04
35.	MtC20238	Class III endochitinase	XII,A, Defense and cell rescue	-	1,16
36.	MtC30019,1	Nematode resistance (hs1pro-1)-like protein	XII,A, Defense and cell rescue	-	1,13
37.	MtC00285	Cysteine-rich antifungal protein -like	XII,A, Defense and cell rescue	-	1,02
38.	MtC00044,1	Dehydrin	XII,B, Abiotic stimuli and development	-	1,22
39.	MtC00681	In2-1 protein	XII,B, Abiotic stimuli and development	-	1,11
40.	MtC00337	(Auxin, heat, ethylene and wounding-induced) arg 2 - like protein	XII,B, Abiotic stimuli and development	-	1,06
41.	MtC30250	Stress related protein	XII,B, Abiotic stimuli and development	-	1,03
42.	MtC45422	Transmembrane protein	XII,C, Unknown function	-	2,12
43.	MtC90044	Curculin-like \(mannose-binding\) lectin	XII,C, Unknown function	-	1,52
44.	MtC45388	-	XII,C, Unknown function	-	1,46
45.	MtC20096	-	XII,C, Unknown function	-	1,43
46.	MtC20055	Tms membrane protein/tumour differentially expressed protein	XII,C, Unknown function	-	1,33
47.	MtC00407	Unknown function	XII,C, Unknown function	-	1,3
48.	MtC10363	-	XII,C, Unknown function	-	1,29
49.	MtC30535,1	Lim domain containing protein	XII,C, Unknown function	-	1,29
50.	MtC00130	Glu rich protein	XII,C, Unknown function	-	1,27
51.	MtC10357	-	XII,C, Unknown function	-	1,27
52.	MtC90647	-	XII,C, Unknown function	-	1,25

Continuation of table12d

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
53.	MtC90571	[Nodulin] mtn19	XII,C, Unknown function	-	1,24
54.	MtC30353	-	XII,C, Unknown function	-	1,22
55.	MtC45659	Hrs domain containing protein	XII,C, Unknown function	-	1,2
56.	MtC45368	-	XII,C, Unknown function	-	1,18
57.	MtD05702	Unknown function	XII,C, Unknown function	-	1,16
58.	JVCPG-46	[Nodulin] mtn19	XII,C, Unknown function	-	1,16
59.	KV2-5M17	[Nodulin] mtn93	XII,C, Unknown function	-	1,16
60.	MtC10007	Fruit-induced csf-2 protein - like	XII,C, Unknown function	-	1,13
61.	MtC00372,1	-	XII,C, Unknown function	-	1,11
62.	MtC90247	Glu/gln rich protein	XII,C, Unknown function	-	1,1
63.	MtC30279	Putative auxin-repressed protein	XII,C, Unknown function	-	1,1
64.	MtC10384	Band 7 protein	XII,C, Unknown function	-	1,06
65.	MtC10709	-	XII,C, Unknown function	-	1,04
66.	MtC91825	-	XII,C, Unknown function	-	1,04
67.	MtC10960	-	XII,C, Unknown function	-	1,03
68.	MtD22059	Thioredoxin domain 2; glutaredoxin-related protein	XII,C, Unknown function	-	1,02
69.	MtC40060	Mouse obesity tubby-like protein	XII,C, Unknown function	-	1,02
70.	MtC91985	-	XIII, No homology	-	1,64
71.	MtC10038,2	No homology	XIII, No homology	-	1,09

Table 12e. List of genes down-regulated only in the pFGC 5149 carrying roots after infection with *A. euteiches.*

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	MtC00004,1	[Nodulin] mtn12 prolin-rich protein	I, Cell Wall	-1,05	-
2.	GVSN-24D7	Prp4	I, Cell Wall	-1,07	-
3.	MtC00055	Actin depolymerizing factor	II, Cytoskeleton	-1,25	-
4.	MtC00027	Membrane channel protein	III, Membrane transport	-1,02	-
5.	MtC10996	Outer mitochondrial membrane porin	III, Membrane transport	-1,19	-
6.	MtC00709	Phosphatidylethanolamine-binding	III, Membrane transport	-1,31	-
7.	MtC00028	Serine proteinase inhibitor	IX, Protein synthesis and processing	-1,08	-
8.	MtC00016	Nucleoside diphosphate kinase	V, Primary metabolism	-1,06	-
9.	MtC00331	Acid phosphatase	V, Primary metabolism	-1,06	-
10.	MtC00680,1	Narbonin	V, Primary metabolism	-1,15	-
11.	MtC00241	Asparagine synthetase [glutamine-hydrolyzing]	V, Primary metabolism	-1,17	-
12.	MtC00463	Pyruvate kinase, cytosolic isozyme	V, Primary metabolism	-1,27	-
13.	MtC00374,1	Soluble inorganic pyrophosphatase	V, Primary metabolism	-1,31	-
14.	MtC00333	Thiazole biosynthetic enzyme precursor	V, Primary metabolism	-1,32	-
15.	MtC10185,1	Cytochrome p450	VI, Secondary metabolism and hormone metabolism	-1,73	-
16.	MtC00346	Calmodulin	X, Signal transduction and post-translational regulation	-1,27	-
17.	MtC10136,1	Peroxidase	XII, Miscellaneous	-1,04	-
18.	MtC40012	Lipoxygenase	XII, Miscellaneous	-1,15	-
19.	MtC00084,1	Leginsulin	XII, Miscellaneous	-1,37	-
20.	MtC10144	Phosphatase	XII, Miscellaneous	-1,43	-
21.	MtC20059	Monodehydroascorbate reductase	XII,A, Defense and cell rescue	-1	-
22.	MtC00306	Glutathione peroxidase	XII,A, Defense and cell rescue	-1,03	-
23.	MtC10383	Phytochelatin synthetase	XII,A, Defense and cell rescue	-1,04	-
24.	MtC00224	Metallothionein-like protein type 3	XII,A, Defense and cell rescue	-1,08	-
25.	MtC00309,1	Metallothionein	XII,A, Defense and cell rescue	-1,18	-
26.	MtC92175	Germin-like protein (oxalate oxidase	XII,B, Abiotic stimuli and development	-1,09	-
27.	MtC20178	Hormone-regulated protein	XII,B, Abiotic stimuli and development	-1,1	-
28.	MtC00358	Transmembrane low temperature and salt responsive protein homolog	XII,B, Abiotic stimuli and development	-1,43	-
29.	MtC40145,1	Unknown function	XII,C, Unknown function	-1,01	-
30.	MtC30081	Glycerophosphoryl diester phosphodiesterase	XII,C, Unknown function	-1,01	-

Continuation of table 12e

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
31.	MtC10424	[Nodulin] mtn3-like protein	XII,C, Unknown function	-1,06	-
32.	MtC10662	Lipoxygenase lh2 domain	XII,C, Unknown function	-1,14	-
33.	MtC00245	Fruit-induced csf-2 protein - like	XII,C, Unknown function	-1,15	-
34.	MtC00741	Protein of unknown function upf0185	XII,C, Unknown function	-1,22	-
35.	MtC10446,1	Transmembrane protein	XII,C, Unknown function	-1,46	-
36.	MtC93288	Plasma membrane protein	XII,C, Unknown function	-1,84	-
37.	MtC00704	Beta-ig-h3/fasciclin domain proline-rich region	XII,C, Unknown function	-2,27	-
38.	MtC92160	-	XIII, No homology	-1,02	-
39.	mt--abc955106g01	No homology	XIII, No homology	-1,04	-
40.	MtC45184,2	-	XIII, No homology	-1,08	-
41.	MtD22569	No homology	XIII, No homology	-1,14	-

Table 12f. List of genes down-regulated only in MtMir-1-PTGS construct carrying roots after infection with *A. euteiches*.

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
1.	MtC10021	Cell wall protein	I, Cell Wall	-	-1,79
2.	MtC00471	Tubulin beta chain	II, Cytoskeleton	-	-1,15
3.	MtC00356,2	Tubulin beta chain	II, Cytoskeleton	-	-1,22
4.	MtC10204	Outer plastidial membrane protein porin	III, Membrane transport	-	-1,12
5.	MtC10612,1	Adp,atp carrier protein precursor	III, Membrane transport	-	-1,26
6.	MtC10259	2-oxoglutarate/malate translocator	III, Membrane transport	-	-1,68
7.	MtC00118,1	40s ribosomal protein s23	IX, Protein synthesis and processing	-	-1,01
8.	MtC45369	40s ribosomal protein sa (p40)	IX, Protein synthesis and processing	-	-1,05
9.	MtC00148	40s ribosomal protein s7	IX, Protein synthesis and processing	-	-1,1
10.	MtC20091,1	Proteasome regulatory subunit s12	IX, Protein synthesis and processing	-	-1,13
11.	MtC20366,1	Cysteine proteinase	IX, Protein synthesis and processing	-	-1,17
12.	MtC00089	60s acidic ribosomal protein po	IX, Protein synthesis and processing	-	-1,2
13.	MtC00318	40s ribosomal protein s3	IX, Protein synthesis and processing	-	-1,29
14.	MtC20369	N-carbamoyl-l-amino acid amidohydrolase	IX, Protein synthesis and processing	-	-1,45
15.	MtC00046	S-adenosylmethionine synthetase	V, Primary metabolism	-	-1,01
16.	MtC10318	Atp synthase d chain, mitochondrial	V, Primary metabolism	-	-1,04
17.	MtC90195	Carbonyl reductase [nadph]	V, Primary metabolism	-	-1,06
18.	MtC00592,1	Udp-glucose glucosyltransferase	V, Primary metabolism	-	-1,13
19.	MtC30078	Seed storage protein	V, Primary metabolism	-	-1,18
20.	MtC00018	Vitamin-b12-independent methionine synthase isozyme	V, Primary metabolism	-	-1,18
21.	MtC00180	Basic blue copper protein	V, Primary metabolism	-	-1,41
22.	MtC10064	Adenosine kinase	V, Primary metabolism	-	-1,46
23.	MtC00698	Malate dehydrogenase, cytoplasmic	V, Primary metabolism	-	-1,54
24.	MtC10119,1	Farnesyl pyrophosphate synthetase	VI, Secondary metabolism and hormone metabolism	-	-1,19
25.	MtC00510	Chalcone--flavonone isomerase	VI, Secondary metabolism and hormone metabolism	-	-1,22
26.	MtC00085,1	Histone h3,2	VII, Chromatin and DNA metabolism	-	-1,06
27.	MtC00632	Histone h4	VII, Chromatin and DNA metabolism	-	-1,1
28.	MtC10735	Plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor	XII, Miscellaneous	-	-1,01
29.	MtC10827,2	Basic 7s globulin /extracellular dermal glycoprotein	XII, Miscellaneous	-	-1,05
30.	MtC00303	Non-specific lipid transfer-like protein	XII, Miscellaneous	-	-1,07
31.	MtC60108	Cupin; cupin domain	XII, Miscellaneous	-	-1,07
32.	MtC30379,1	Basic 7s globulin /extracellular dermal glycoprotein	XII, Miscellaneous	-	-1,09
33.	MtC40041	Calreticulin precursor	XII, Miscellaneous	-	-1,38
34.	MtC10070	Lipoxygenase	XII, Miscellaneous	-	-1,62
35.	MtC20391	Hypersensitive reaction induced hsr201 protein -like	XII,A, Defense and cell rescue	-	-1,11
36.	MtC00045	Auxin-down regulated adr6-like protein	XII,B, Abiotic stimuli and development	-	-1,09

Continuation of table 12f

#	Gene ID	Annotation	Functional class	pFGC 5149	MtMir-1-PTGS
37.	MtC00276	Germin-like protein (oxalate oxidase)	XII,B, Abiotic stimuli and development	-	-1,18
38.	MtC00060,1	[Nodulin] mtn5 (non specific lipid transfer protein)	XII,C, Unknown function	-	-1,01
39.	MtC40209	-	XII,C, Unknown function	-	-1,02
40.	MtC30434	Burp domain	XII,C, Unknown function	-	-1,08
41.	JVCPG-33	[Nodulin] mtn5	XII,C, Unknown function	-	-1,09
42.	MtC93004	Leucine-rich repeat proline-rich reach, plant specific	XII,C, Unknown function	-	-1,1
43.	MtC10504,1	-	XII,C, Unknown function	-	-1,18
44.	MtC00378	Leginsulin / albumin	XII,C, Unknown function	-	-1,22
45.	MtC40018	Putative methyltransferase duf248; generic methyltransferase	XII,C, Unknown function	-	-1,23
46.	MtC10203	Phosphate-induced protein-like protein	XII,C, Unknown function	-	-1,26
47.	MtC00074	Fruit-induced csf-2 protein - like	XII,C, Unknown function	-	-1,29
48.	MtC45436	-	XII,C, Unknown function	-	-1,45

4.1 *Medicago truncatula-Aphanomyces euteiches* pathosistem is an appropriate model to study molecular interaction between legume and oomycete

Transcriptome analysis in different model organisms has been applied for several years for a comprehensive analysis of plant-microbe interactions (Colebatch *et al.,* 2002; Journet *et al.,* 2002; Fedorova *et al.,* 2002; Wulf *et al.,* 2003). In order to study the economically important root rot of legumes caused by *Aphanomyces euteiches*, *Medicago truncatula* was chosen. *M. truncatula* is subject to large scale EST analysis (www.medicago.org) and is phylogenetically closely related to the important crop legume *Pisum sativum.*

Comparison of fresh weight development, oospore development and ALP-activities of the pathogen suggest a similar disease development in *P. sativum* and *M. truncatula* (Nyamsuren *et al.,* 2003). This disease development is similar to what previously has been observed in pea (Kjøeller and Rosendahl 1998). This suggests that *M. truncatula* can be used as a model plant to study the molecular background of the *A. euteiches*- induced root rot disease in pea. *A. euteiches* has been shown to have a short active phase in the root system after infecting it, the pathogen has been shown to be inactive shortly after first symptoms appear on the host plant (Kjøeller and Rosendahl 1988). Therefore, early stages of the interaction were selected for transcription profiling.

4.2 First view of transcriptional changes occuring in *M. truncatula* roots after infection of *A. euteiches* was obtained by cDNA-AFLP

To identify the optimal time point for a large-scale transcription profiling, a cDNA-AFLP experiment was carried out. This experiment has shown that at six days after inoculation, notable numbers of differentially expressed cDNAs were detectable. These observed differences in the transcription pattern remained until the last time point analysed, namely three weeks after inoculation. Four genes, which show either an increased or a decreased RNA accumulation during the infection, were cloned and sequenced. One of the difficult procedures in RNA fingerprinting is to verify that the band isolated and analysed further is the same as the one visualised in the original amplification (Bachem *et al.,* 1996). Therefore, after re-amplification and cloning,

for each of the four fragments five randomly picked clones were selected for sequencing. One of the cDNAs, which showed higher RNA accumulation in *A. euteiches* infected roots, encoded a class 4 pathogenesis-related protein (PR-4). The PR-4 family of PR proteins consists of class I and class II chitinases (Bravo *et al.,* 2003). At present, chitinases are grouped into seven different classes (I-VII) depending on their structure and functional properties (Neuhaus, 1999). Because the chitinase substrate chitin is the main component of many fungal walls and expression of many chitinase genes is induced by pathogens, chitinases have long been proposed to play roles in the active defence response of plants. Antifungal activities have been described for some of the proteins belonging to the PR-4 family (Hejgaard *et al.,* 1992; Ponstein *et al.,* 1994; Caruso *et al.,* 1996).

Another cDNA, which also showed increased RNA accumulation in infected roots, encoded a germin-like protein. Germin has been discussed as an antifungal protein since the discovery of its pathogen-induced accumulation in leaves of wheat and barley (Dumas *et al.,* 1995; Hurkman and Tanaka, 1996). Since germin is identified as functional oxalate oxidase, it is proposed that the germin might be involved in defence reactions such as degradation of oxalate as a fungal toxin (Thompson *et al.,* 1995). It is also discussed that the germins and germin-like proteins with no oxalate oxidase activity may play a structural role in cell-wall re-enforcement during pathogen attack (Schweizer *et al.,* 1999).

4.3 SSH reveals a high number of *A. euteiches* induced *M. truncatula* genes

A cDNA library, enriched for *A. euteiches*-induced genes, was established by SSH from RNA at the earliest time point. A pre-screening of 192 SSH-cDNA clones for increased RNA-accumulation at six days after inoculation resulted in 26.5% up-regulated genes in this SSH-cDNA population. Levels of differentially expressed cDNAs in SSH-pools have been reported to range within less than 10% and to up to 95 % and to depend mainly on the biological material (Desai *et al.,* 2000). The background of non-differentially expressed cDNAs in this SSH-library is most probably due to dilution effects resulting from non-infected tissue areas within the infected-root systems.

Among the genes, which were identified as being induced by the root-pathogen, two belonged to the pathogenesis-related (PR) protein-encoding class. The induction of PR-proteins, defined as plant proteins induced specifically in pathological or related

situations (van Loon *et al.,*1994; van Loon and van Strien, 1999) is a result of the plant defensive response and an indication of recognition between the host plant and the pathogen. One of these genes encodes a typical class 4 (PR) protein containing a hevein-domain, structural units that are capable to bind chitin (van Damme *et al.,* 1999). Another PR-protein encoding cDNA was the most redundant gene in this SSH-library. The TC encoding a class 10 PR protein could be assembled from 13 singletons. For PR-10 protein-like genes it could be demonstrated that they can be transcriptionally activated by abscisic acid (ABA) (Iturriaga *et al.,* 1994). This phytohormone is not only an important signal for the plant physiological and molecular response to a water deficit, it plays also an essential role in triggering gene expression upon wounding and pathogen attack (Moons *et al.,* 1995). Beside the PR-10 encoding sequence, the SSH-library contained six further cDNAs encoding ABA-responsive proteins. This highly redundant presence of PR-10 and ABA-responsive proteins indicated that ABA-mediated signalling is involved in the interaction between the plant and the pathogen. The induction of various ABA-responsive genes after a *A. euteiches* infection could be a direct effect of the plant response. Alternatively, the ABA-content of the host plant could be increased as a consequence to desiccation and senescence of the root system after pathogen colonization. The presence of a dehydrin related protein among the up-regulated genes further indicate the involvement of drought stress. The synthesis of dehydrins is a common response to drought in plants and its RNA accumulation is affected by ABA (Giordani *et al.,* 1999).

Cinnamoyl-CoA reductase and cytochrom p450 are the enzymes involved in the biosynthesis of isoflavonoids. Isoflavonoids comprise a large group of secondary metabolites involved in plant-microbe interaction. These compounds include the pterocarpans, such as the fungicides medicarpin from alfalfa (*Medicago sativa*) and pisatin from pea (*Pisum sativum*). The pathway that leads to isoflavonoid synthesis is a branch of the general phenylpropanoid pathway that exists in all higher plants. It is known that genes encoding enzymes of this pathway are developmentally and tissue-specifically induced by different stresses. Induction of these genes in response *to A. euteiches* indicates the induction of the isoflavonoid biosynthesis in *M. truncatula* after infection with pathogen.

One of the interesting genes found in the SSH-cDNA library was serine palmitoyltransferase-like sequence (SPT), confirmed to be up-regulated in responce

to *A. euteiches*. Birch *et al.,* (1998) have reported an involvement of SPT early in the potato hypersensitive response (HR) to *Phythophtora infestans* and suggested that sphingolipid signalling in a plant-pathogen interaction leads to apoptosis. SPT catalyses the first step in the synthesis of sphingolipids, an important class of second messengers involved in the regulation of cell death and proliferation in animals (reviewed in Merrill *et al.,* 1997).

In addition to the observed gene induction which have been also previously described to occur during biotic or abiotic stresses, a number of cDNA sequences which have not been described to be involved in pathogen response were found to be induced after *A. euteiches* infection in this study. The most unexpected output of this SSH-approach is probably the significant number of *A. euteiches* induced cDNAs which do not match entries of the *M. truncatula* Gene Index (MtGI). This large-scale EST database contains a collection of over $1.8x10^5$ *M. truncatula* cDNA sequences (MtGI-release 6.0-december 2002) originating from various tissues and physiological conditions. A significant number of entries of this collection are derived from tissues challenged with different pathogens. Of the 560 *A. euteiches* induced cDNAs identified in this study, more than eight percent seem to be new *M. truncatula*-sequences, since they have not been identified in previous sequencing projects, which are deposited in the MtGI-collection. The finding of large number of *A. euteiches* induced new cDNAs, indicates that these gene products or regulation mechanisms play a rather specific role during pathogenic root-oomycete interactions and might not be involved in common stress responses. Hence, these cDNAs could be of great importance for future research in order to understand the specific mechanism underlying this agricultural important legume root-rot disease.

4.4 *MtMir*-1- is a pathogen induced *M. truncatula* gene

One *A. euteiches* induced cDNA, clone 2f10, encoded the partial sequence of a protein with similarity to a protein called miraculin, isolated from the miracle berry (*Richadella dulcifica*), a west African shrub. Because of its sequence similarity to miraculin, the *M. truncatula* gene was named *MtMir*-1 (*Medicago truncatula* miraculin). cDNAs encoding *MtMir*-1 gene were one of the most abundant sequences in the SSH-cDNA library. The *MtMir*-1 sequence was assembled from seven different ESTs from this SSH-library. Two ESTs of this gene were found in

TIGR *Medicago truncatula* Gene Index (MtGI version 5.0), deriving from a cDNA-library constructed from *M. truncatula* roots after infection with the nemathode *Meloidogyne incognita*.

A full-length cDNA sequence of *MtMir*-1 (862bp) was identified by RACE-PCR. The 516 bp ORF encodes a protein of 173 amino acids. A database similarity search revealed that the deduced amino acid sequence has similarities to several cloned plant genes of unknown function. The highest similarity 61 % at the amino acid level is to a deduced protein from a cDNA, TID91 (D26457), representing a gene expressed in stress-induced, genetic tumor tissues found in interspecific hybrids between *Nicotiana glauca* and *Nicotiana langsdorffii* (Fujita *et al.,* 1994). TID91 was demonstrated to be strongly expressed in tobacco callus tissue but absent in leaves and stems. Expression in roots was not examined (Fujita *et al.,* 1994). Other related sequences of unknown functions include a tumor-related protein, clone NF34, from tomato (57 % identity) which was discussed to be a potential elicitor of hypersensitive response (HR) (Karrer *et al.,* 1998). *LeMir*, a protein that is induced early after infection of tomato with the root-knot nematode *Meloidogyne javanica* shows 56 % identity to *MtMir*-1. *LeMir*-mRNA was detecetd in roots, hypocotyl, and flower tissues, with highest expression in the root (Brenner *et al.,* 1998).

Sequence analysis indicates that *MtMir*-1 and miraculin belong to the soybean trypsin-inhibitor family. Members of this family are characterized by the presence of an N-terminal signature sequence. Many but not all members have shown to have inhibitory activity against a range of serine proteinases (Laskowski and Kato, 1980). The highest similarity of *MtMir*-1 to a known enzymatic inhibitor is to a Kunitz trypsin inhibitor-4 (accession number AAQ84217), found in hybrids between *Populus balsamifera* subsp. *trichocarpa* and *Populus deltoides* (Miranda *et al.,* unpublished).

Kunitz-type trypsin inhibitors represent one group of Kunitz-type proteinase inhibitors and are divided into three groups based on their abilities to inhibit chymotrypsin, trypsin and tissue-type plasminogen activator (tPA) (Song and Suh 1998). Kuniz-type trypsin inhibitors are more specific towards trypsin than chemotrypsin and they do not inhibit tPA (Song and Suh 1998).

Different members of Kunitz-type proteinase inhibitors are described to be induced by different biotic and abiotic environmental stresses (Bryant *et al.,*1976; Graham *et al.,*1986; Ryan *et al.,* 1987; Kang *et al.,* 2002).

In pea (*Pisum sativum*), the major seed trypsin/chemotrypsin inhibitors have been characterized and genetically mapped (Domoney *et al.,* 1994, 1995) and have been implicated in plant response to water-deficit stress (Welham and Domoney, 2000). Three Kunitz trypsin inhibitor genes isolated from trembling aspen (*Populus tremuloides*) found to be induced by wounding and herbivory and the overexpressed product of PtTI2 showed *in vitro* inhibition of bovine trypsin (Haruta *et al.,* 2001).
This suggests that *MtMir*-1 could possibly have an inhibitory function against fungal proteinases. As it was mentioned before, the roots colonized by *A. euteiches* are likely to undergo water stress because of the damaged root system. Therefore, it could be hypothesised that *MtMir*-1 is induced as a reaction to water-deficit stress, as in the case of pea trypsin inhibitors. On the other hand, the RT-PCR results showed that the *MtMir*-1 gene was induced not only in parasitic oomycete colonized roots, but also in mycorrhized roots. This implies that the induction of *MtMir*-1 might not be directly influenced by water-deficit, but it is a part of plant response or recognition mechanism against root interacting microorganisms.
It is known that the Kunitz-type trypsin inhibitor gene family represents a distinct group of proteins. Some members are present in the plant genome as a multigene family and some contain few genes (Gruden *et al.,* 1997; Domoney *et al.,* 2002). Genomic Southern blot analysis revealed that *MtMir*-1 probe binds to two different fragments of *M. truncatula* genome. Genomic DNA sequence shows that a *MtMir*-1 posses no introns. It seems, that this phenomenon is not rare among the representatives of the trypsin inhibitor gene family, since five proteinase inhibitor genes isolated from potato, ten trypsin inhibitor genes from pea and the *LeMir* gene from tomato were described to carry no introns (Gruden *et al.,* 1997; Page *et al.,* 2002; Brenner *et al.,* 1998).
RT-PCR analysis showed that the *MtMir*-1 gene was not present and not induced in leaves of *M. truncatula* after infection with *A. euteiches*. This suggests that *MtMir*-1 is specifically induced in the root as a response to invading microorganisms.

4.5 *MtMir*-2- is a mycorrhiza induced *M. truncatula* gene

Another gene, which showed increased mRNA accumulation, was identified in mycorrhized roots of *M. truncatula* (Wulf *et al.,* 2003). The partial sequence of this gene showed high homology to miraculin and similarity to *MtMir*-1. Therefore, the gene was named *MtMir*-2 and further analysed in the present work. The

corresponding TC (78015) was found in MtGI 7.0, which consists of 14 ESTs, exclusively from the cDNA libraries of mycorrized *M. truncatula* roots. In contrast to *MtMir*-1, *MtMir*-2 was not regulated in responce to *A. euteiches*. The expression pattern indicates that *MtMir*-2 is specifically induced in response to mycorrhizal fungi.

During the colonization of plant roots with mycorrhizal fungi, genes of plant defence mechanisms are also regulated (Gianinazzi-Pearson, 1996). Since *MtMir*-2 is not regulated by the pathogenic oomycete, it is difficult to speculate about its possible role in plant defence against fungal invaders. The role of this protein in a mycorrhizal symbiosis is still unclear.

4.6 Inducible promoters of *MtMir*-1 and *MtMir*-2 genes

For the further gene regulation analysis of *MtMir*-1 and *MtMir*-2 the corresponding promoter regions were isolated. One trypsin inhibitor promoter was investigated through reporter-gene studies and has been shown to be induced as a response to water-stress in some but not all organs of *Pisum sativum* (Welham and Domoney, 2000). In the current work, DNA walking method was used for amplifying the promoter regions. *MtMir*-1 and *MtMir*-2 are both belonging to Kunitz-trypsin inhibitor gene family and showing different expression patterns. This indicates that they may have different transcription factors activating these genes in different situations. But on the other hand, the evidence that *MtMir*-1 is also activated in response to mycorrhizal fungi suggests that it could share common regulatory mechanisms.

In order to identify the shortest promoter region, responsible for the activation of the gene, promoter deletion fragments analysed by the fusion to the GUS-reporter gene and used for *M. truncatula* hairy root transformation.

Transgenic roots containing the longest (~800 bp) promoter::GUS fusion fragment showed reporter gene activity in the cortical cells, showing attachment of zoospores, as well as in the root segments colonised with *A. euteiches* hyphae. In contrast, in roots carrying shorter fragments, the GUS-reporter gene was not active. These results suggest that important motifs for transcriptional activation of *MtMir*-1 gene are located in an area between –800 and –300 on its promoter. However, additional work

is necessary to determine the exact regulation motifs and the transcription factors itself, which specifically regulates the transcription of *MtMir*-1 gene.

In case of *MtMir*-2, an 800 bp promoter fragment was shown to activate the transcription in the mycorrhized root tissue. No reporter gene activity was observed in non-mycorrhized roots. A similar promoter activity was reported previously. Promoter studies of AM-regulated gene *MtGst*1 showed specific activity of this gene in arbuscule containing cells of mycorrhized root, as well as in the direct vicinity of cells containing fungal hyphae (Wulf *et al.,* 2003). Additional work is required to verify whether the *MtMir*-2 gene is coregulated with *MtGst*1.

4.7 Transgenic root cultures of *M. truncatula* completely silenced for *MtMir*-1 gene

To study the function of *MtMir*-1, transgenic roots were produced carrying a construct to provide post-transcriptional gene silencing.

Regulation of eukaryotic gene expression occurs at different stages of protein synthesis: at the transcriptional, RNA processing and translational levels and during protein maturation. The postranscriptional levels started to attract the most attention since in 1998 Andrew Fire *et al.,* have demonstrated in the worm *Caenorhabditis elegans* that dsRNA (double-stranded RNA) may specifically and selectively inhibit the gene expression in an extremely efficient manner. This phenomenon was called RNA interference (RNAi). RNAi is a post transcriptional gene silencing process (PTGS) in which dsRNA induces the degradation of homologous RNA sequence. Soon after this discovery, it became clear that the earlier reports about PTGS in plants (Napoli *et al.,* 1990), gene quelling in fungi (Cogni *et al.,* 1996) and gene silencing with antisense RNA (Fire *et al.,* 1991) describe diverse variants of RNAi.

Gene silencing was perceived initially as an unpredictable and inconvenient side effect of introducing transgenes into plants. It now seems that it is the consequence of accidentally triggering the plants adaptive defence mechanism against viruses and transposable elements (Waterhouse *et al.,* 2001).

Since the discovery of RNAi, it was described in diverse organisms: in protozoa (Ngo *et al.,* 1998), hydra (Lohmann *et al.,* 1999), fruit fly (Kennerdell and Carthew, 1998), zebrafish (Wargelius *et al.,* 1999), frog (Oelgeschlager *et al.,* 2000), mammals (Wianny and Zernicka-Goetz, 2000), fungi (Cogni *et al.,* 1996) and different plants (Waterhouse *et al.,* 1998).

Although the mechanism of RNAi is not well understood, it seems to provide an effective way to unravel the gene function (Montgomery *et al.,* 1998; Fire, 1999; Sharp, 1999). RNAi has revolutionized the study of genes in *C. elegans*. Over 4000 genes on chromosome I and III of *C. elegans* are being analysed systematically (Fraser *et al.,* 2000; Gonczy *et al.,* 2000). For the organisms such as *M. truncatula*, whose whole genome is being completely sequenced, the next challenge would be to understand the functions and interplay of genes in that organism. For this purpose RNAi could be the method of choice. In the present work RNAi method was used to study the function of *MtMir*-1 and *MtMir*-2.

Using a construct providing the production of dsRNAs, transgenic roots were obtained *in vitro*. In these roots the *MtMir*-1 transcripts were completely undetectable. However, the silencing through dsRNA has been shown to be more efficient as by the antisense or sense RNA (Levin *et al.,* 2000), in most cases reported, the gene activation was reduced less than 100 %. In case of the *MtMir*-1 gene, it seems that dsRNA-mediated genetic interference has completely down-regulated the expression of this gene. Even after PCR-amplification no transcript could be detected in *MtMir*-1-PTGS carrying roots. This result could also be confirmed by virtual Northern blot analysis. One explanation of complete down-regulation of the *MtMir*-1 could be that this gene is an inducible and not a housekeeping gene. Roots containing PTGS constructs showed no detectable phenotype changes after infection with *A. euteiches*. This could be because of the unspecific response of *MtMir*-1 against *A. euteiches* or it could also be that *A. euteiches* is able to inhibit the products of *MtMir*-1.

The results of this study suggest that *A. rhizogenes* mediated transformation provides a fast, convenient and efficient method to introduce dsRNA-expressing constructs into the roots of *M. truncatula*. Therefore, use of this *in vitro* method is highly recommended for the study of root biology such as root branching, rhizobium symbiosis or root-pathogen interaction.

4.8 Microarray – is a powerful technique to study global gene expression pattern

In this study, DNA microarray technology was utilized for two specific objectives: In one experiment, to obtain transcription profiles of the *M. truncatula* root 30 minutes and 6 days after inoculation with *A. euteiches*. In a second experiment, the influence of *MtMir*-1-PTGS on transcription profiles of other genes was analysed.

These microarray experiments resulted in determination of an enormous data set.

From a set of 8000 *M. truncatula* genes on the 8kMt microarray, 138 genes were identified to be regulated after 30 min and 207 genes after 6 days of inoculation. 35 of the genes which were regulated after 30 min were also regulated in the same manner after 6 days. Among these 35 genes, plant stress responsive genes such as PR10 and chalcone syntethase were found to be up-regulated. The presence of these genes indicates that the plant defence system is activated already 30 minutes after contact with the pathogen. Interestingly, a dehydrin gene was found to be down-regulated (M=-2,79) 30 min after infection, but found to be up-regulated (M=1,57) after 6 days. In fact, up-regulation of dehydrin after 6 dpi was already detected in current work by SSH method. There are also some genes that were induced or repressed at one time point, while oppositely regulated at another time point. As it was expected, there were different transcription factors, such as mitogen-activated protein kinase (MAPKinase), several zinc-finger proteins, b-Zip transcription factor and PR-transcription factor, were found to be up-regulated after 30 min, indicating a rapid recognition of the pathogen by the plant and activation of its signalling pathways leading to defence response.

Beside several genes previously described to be regulated during biotic and abiotic stress situations, this study revealed a large number of genes, with no homology to known genes (29) or genes with unknown function (78). Further investigation of these genes will permit development of a hypothesis to explain the plant root – oomycete interaction.

The experimental evidence, that the set of genes regulated after 30 min of infection differs from those regulated 6 days after, suggests that the plant responds to the different stages of disease development with specific reactions.

An evidence that the genes previously identified by cDNA-AFLP or SSH methods have been found in the microarray results, indicates the reliability of the arrays.

However, confirmatory approaches such as Northern Blots, qRT-PCR and *in situ* hybridization are essential in supporting of the array data.
Although the Mt8k microarray contains a significant number of *M. truncatula* genes, it does not contain the comprehensive set of all *M. truncatula* genes. Hence, for the identification of new genes the methods like cDNA-AFLP or SSH are preferable.

The second objective, for which microarrays were used in this work, was the study of genes influenced by *MtMir*-1 silencing. The global gene expression pattern of *M. truncatula* roots infected with *A. euteiches* was studied in control and *MtMir*-1 gene silenced roots. A similar experiment was done on human cells transformed with GFP gene from jellyfish (*Aequoria vicroria*). This gene was then silenced by siRNAs and the impact on global gene expression pattern was studied using microarrays. Results of this experiment revealed that the siRNA-mediated gene silencing has exquisite sequence specificity for the target mRNA and does not induce detectable secondary changes in the global gene expression pattern (Chi *et al.,* 2003). The gene which was silenced in the above mentioned experiment was an exogenous gene. In case of *MtMir*-1, an endogenous gene was silenced and it might be possible that this silencing influence the expression of other genes.
299 genes showing changed expression were identified to show altered expression and were clustered into three groups *(table-11).* Genes belonging to the first group seem not be affected by the *MtMir*-1 silencing. But genes from cluster II and III seem to be affected by the *MtMir*-1 silencing. The genes in cluster II are showing up- or down-regulation in the control roots but not in the *MtMir*-1 silenced roots. The genes in cluster III are not showing changed expression in control roots, but they are up- or down-regulated in the *MtMir*-1 silenced roots. Hence, this experiment was barely a beginning but a set of genes with significantly altered expression pattern correlated with silencing of *MtMir*-1 gene could be identified.
A microarray is effectively equivalent to 8 000 simultaneous Northern blots and thus provides information on how the expression pattern of the gene relates to many others (Duffield, 2003). Up to now there is no report on using microarrays for the study of global gene expression pattern changes affected by silencing a particular endogenous gene by RNAi. Therefore, this was the first attempt made. The results are encouraging, however, optimization of experimental materials and conditions are required. The plant materials used in this study were transformed hairy root cultures.

Even small differences in cell passage or media metabolism can lead to differences in global gene expression pattern (Chi *et al.,* 2003). Therefore, for instance, roots of complete transformed plants silenced for the *MtMir*-1 gene need to be developed to avoid any influence which could affect the global gene expression pattern. It is not to exclude that even the empty pFGC 5941 vector might have changed the expression pattern of the roots which were used as a control in this experiment.

4.9 Outlook

Using the modern molecular biological methods enormous number of *A. euteiches* induced *M. truncatula* genes were obtained in this work. Beside the genes involved in common plant response against different environmental stresses, there are new genes identified not previously described to be involved in plant-pathogen interactions. Further investigations will show whether these genes are specifically induced by *A. euteiches*.

The data available from the whole genome sequencing project of *M. truncatula* will make the cloning of the promoter regions of different genes much easier and faster. Promoter studies of *A. euteiches* induced genes will give important information about the genes expression regulations during plant-oomycete interaction.

First results of gene silencing by RNAi obtained in this work encourages us to use this method for studying the function of *A. euteiches* induced genes.

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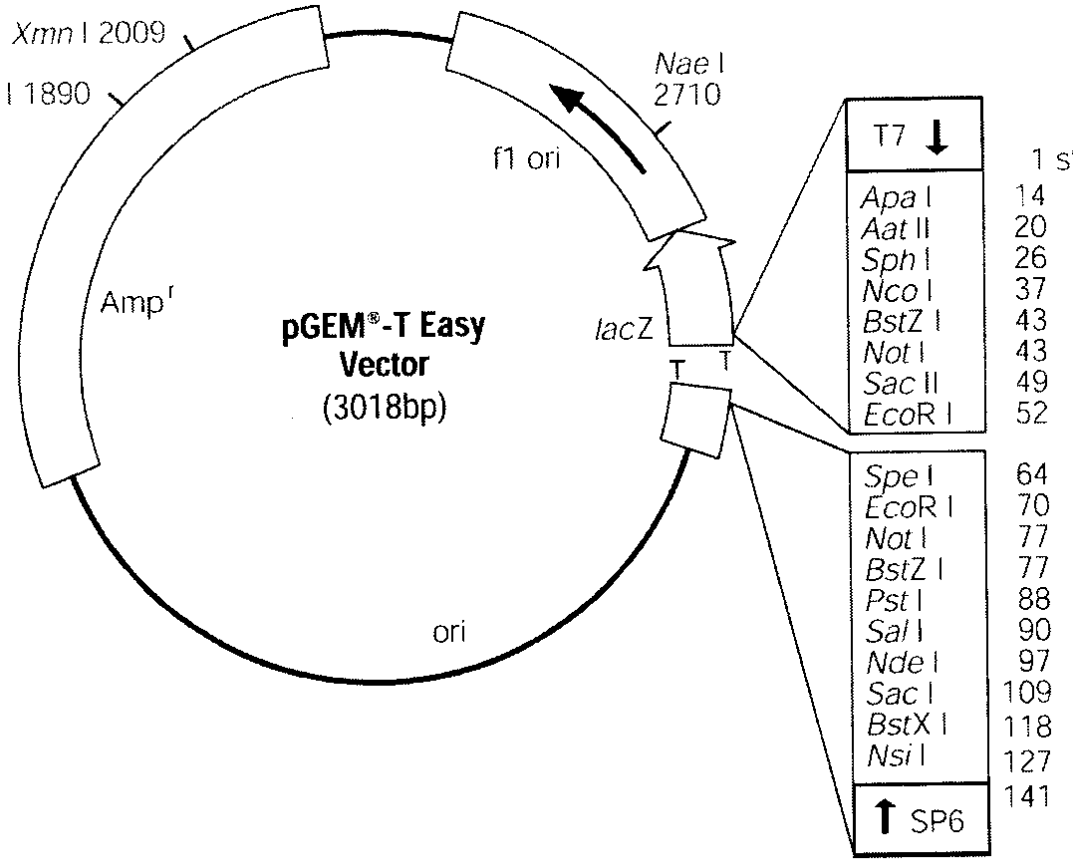
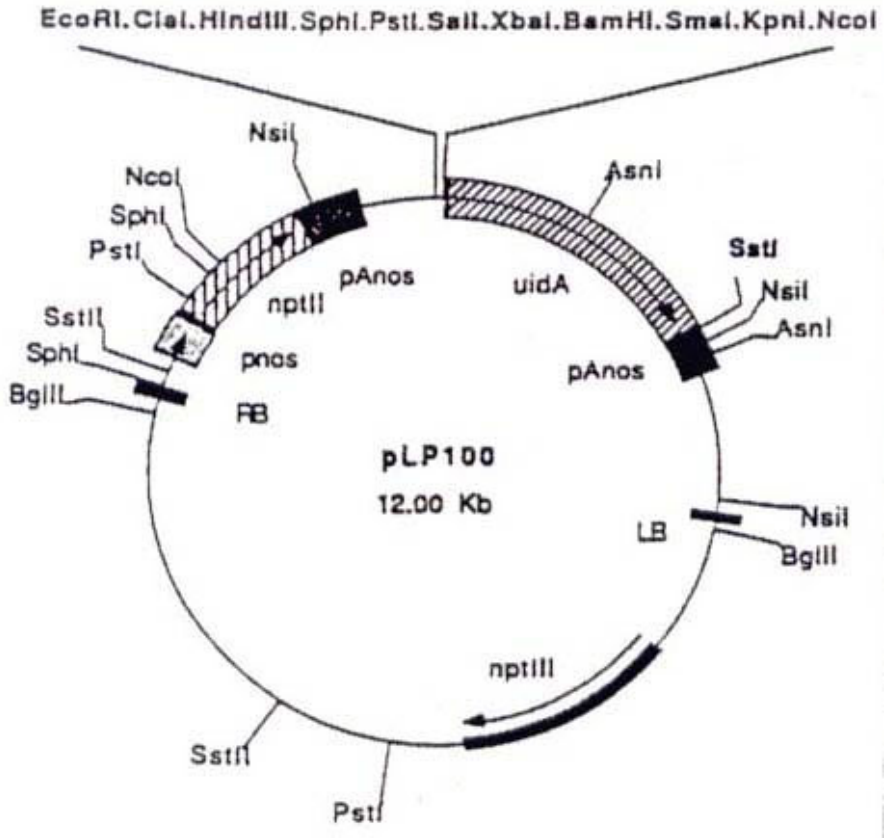
Appendix A. pGEM®-T Easy vector (Promega, Madison, WI, USA) circle map and sequence reference points:



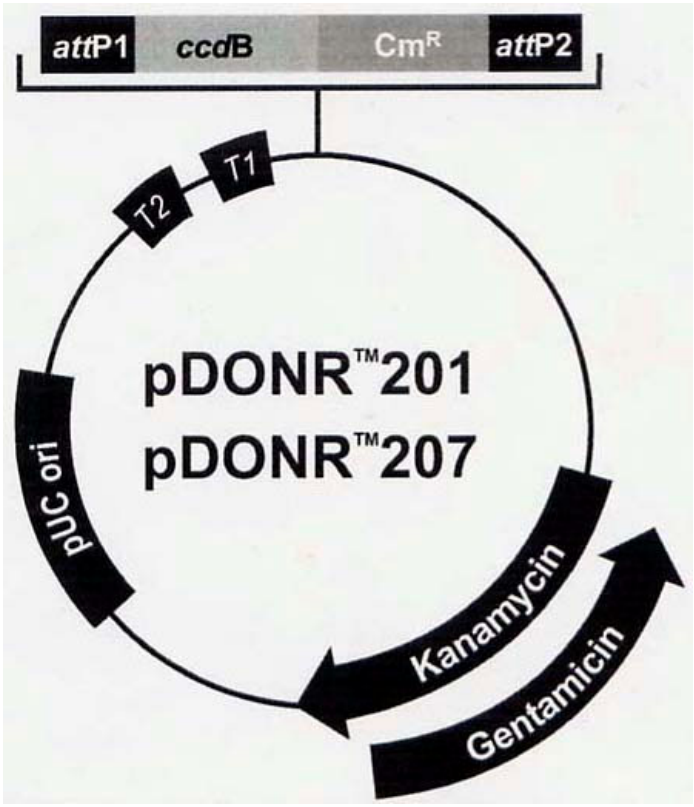
pGEM®-T Easy Vector sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter (-17 to +3)	2999-3
SP6 RNA Polymerase promoter (-17-+3)	139-158
Multiple cloning region	10-128
*lac*Z start codon	180
lac operon sequence	2836-2996, 166-395
lac operator	200-216
ß-galactamase coding region	1337-2197
phage f1 region	2380-2835
binding site of pUC/M13 Forward Sequencing Primer	2956-2972
binding site of pUC/M13 Reverse Sequencing Primer	176-192

Appendix B. Map of pLP100 vector (Szabados *et al.,* 1995).



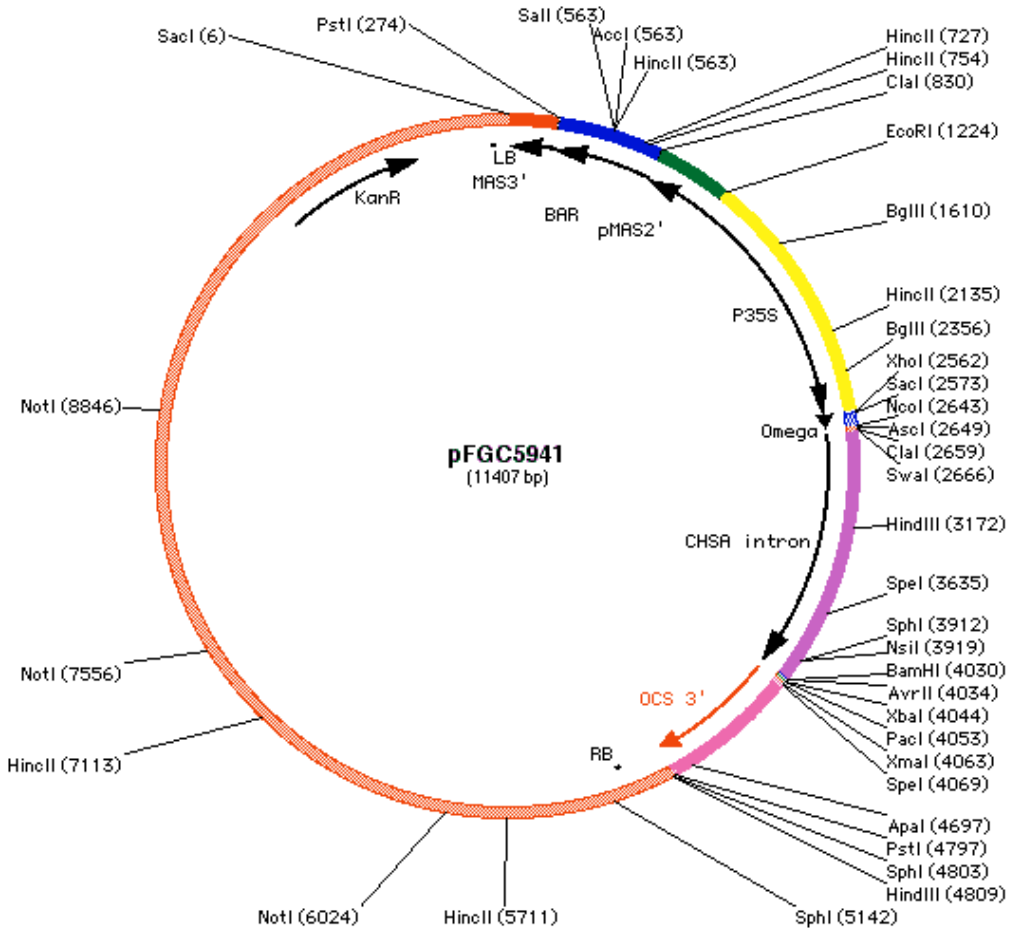
Appendix C. Map of pDONR 207 vector (5585 nucleotide) (Invitrogen, Carlsbad, CA, USA).



*rrn*B T2 transcription termination sequence (c):	73-100
*rrn*B T1 transcription termination sequence (c):	232-275
Recommended forward priming site:	300-324
*att*P1:	332-563
*ccd*B gene (c):	959-1264
Chloramphenicol resistance gene (c):	1606-2265
*att*P2 (c):	2513-2744
Recommended reverse priming site:	2769-2792
Gentamycin resistance gene:	3528-4061
pUC origin:	4909-5582

(c)= complementary strand

Appendix D. Map of pFGC 5941 vector (ChromDB, Arizona, AZ, USA).



Km Bacterial kanamycin resistance gene (also called kanR).
Cm Bacterial chloramphenicol resistance gene.
Hyg Plant hygromycin resistance gene.
Bar Gene encoding resistance to the herbicide BASTA.
OCS 3' Poly adenylation signal sequence from *Agrobacterium tumefaciens*.
MAS 3' Poly adenylation signal sequence from *Agrobacterium tumefaciens*.
MAS 1' Plant promoter from *Agrobacterium tumefaciens*.
CaMV 34S Viral promoter; this constructs have the omega leader of TMV (Gene 1987;60(2-3):217-25) at the end of the promoter sequence; this sequence is thought to increase translation efficiency, but it is not expected to influence RNAi.
LB T-DNA left border.
RB T-DNA right border.
GUS 360 base pair fragment from the GUS (*Escherichia coli* beta-glucuronidase gene).
ChsA intron 1,353 bp fragment from the petunia Chalcone synthase A gene.

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