Functional genomic approaches to analyse the parasitic interaction between the model legume *Medicago truncatula* and the oomycete *Aphanomyces euteiches*

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Abstract

The common root rot caused by the oomycete *Aphanomyces euteiches* is a major yield-reducing factor in legume crop production, and it is considered to be the most destructive disease of pea in areas with temperate climates. Disease development with discolored lesions, a watery rotting of root tissue and a significant reduction of root mass are typical symptoms and is well-characterized, but so far very little is known about the molecular mechanisms of the disease and the nature of host cellular responses.

Comparative functional genomic approaches were carried out to systematically identify plant genes and proteins that show altered regulation in the model legume *Medicago truncatula* after *A. euteiches* infection. A SSH-cDNA library was established that revealed 51 cDNAs to be strongly induced in infected roots. In proteomic approaches, 11 proteins were found to be produced *de novo* or strongly induced in 2-D protein maps of infected root tissues. The results obtained from both approaches carried out with the *M. truncatula* line A17 revealed the significant induction of mainly defense-related genes and proteins such as Pathogenesis Related (PR) proteins, cell wall proteins and enzymes of antimicrobial phytoalexin synthesis pathways.

The most prominent changes in the *M. truncatula* gene and protein profiles after *A. euteiches* infection occurred in a set of PR-10 transcripts and proteins that includes ABA-responsive proteins (ABR17). Their role in this interaction was further investigated via 2-DE in two additional *M. truncatula* lines (F83.005-5 and -9) showing different levels of susceptibility to *A. euteiches* infection compared to the moderately infected A17 line. The analysis revealed a strong correlation of PR-10 abundance with the infection level as detected by physiological and histochemical measurements *in planta*. Their ABA-dependent regulation was also demonstrated. Thus, exogenous ABA application led to an enhanced susceptibility to *A. euteiches* infection, while previous inoculation with the mycorrhiza fungus *Glomus interadices* suppressed subsequent root colonization by *A. euteiches*. These results were reflected by clearly increased or decreased abundance of PR-10 proteins. Hence, these proteins indicate the disease severity at a cellular level.

Proteomic analysis of the *M. truncatula* lines showing varying levels of susceptibility to *A. euteiches* led to the identification of an additional 14 up-regulated proteins; among them are two proteasomes subunits that might provide a first hint to the mechanism of plant resistance in this interaction.

Keywords: *Medicago truncatula, Aphanomyces euteiches*, proteomics

Zusammenfassung

Die von dem Oomyceten *Aphanomyces euteiches* verursachte gemeine Wurzelfäule ist einer der größten ertragsreduzierenden Faktoren im Leguminosenanbau und gilt als die bedeutendste Krankheit im Erbsenanbau in gemäßigten Klimaten. Der Krankheitsverlauf, gekennzeichnet durch symptomatische Läsionen und Fäulnis des Wurzelgewebes sowie einer signifikanten Reduktion der Wurzelmasse, ist gut charakterisiert, aber nur wenig ist bisher über seine molekularbiologischen Mechanismen bekannt.

In vergleichenden funktional-genomischen Ansätzen sollten Gene und Proteine der Modell-Leguminose *Medicago truncatula* identifiziert werden, die nach Infektion mit *A. euteiches* differentiell reguliert sind. In einer SSH cDNA-Bank konnten 51 cDNAs aufgefunden werden, die nach Infektion stark induziert waren. Proteomanalysen infizierter Wurzeln führten zunächst zur Detektierung von 11 neu oder stark induzierten Proteinen. Beide für die *M. truncatula* Linie A17 durchgeführten Ansätze wiesen eine Reihe qualitativer Gemeinsamkeiten auf: Die signifikante Induktion vorwiegend abwehrspezifischer Gene und Proteine wie ‚Pathogenesis Related' (PR) Proteine, Zellwandproteine und Enzyme zur Synthese antimikrobiell wirkender Phytoalexine.

Die wesentlichsten Veränderungen in den Gen- und Proteinmustern von *M. truncatula* erfolgten aber innerhalb der PR-10 Gen/Protein-Familie, die auch Abscisinsäure-responsive ABR17 Proteine umfasst. Ihre infektionsabhängige Induktion wurde mittels 2-dimensionaler Gelelektrophorese in der A17 Linie und in zwei zusätzlichen *M. truncatula* Linien (F83.005-5 und -9) mit unterschiedlicher Infektionsempfindlichkeit untersucht. Die Analysen ergaben, dass die Abundanz der PR-10 Proteine stark mit dem in physiologischen und histochemischen Untersuchungen ermittelten Infektionsniveau der Pflanze korreliert. Exogene Applikation von ABA führte zu einer gesteigerten Wurzelinfektion durch *A. euteiches*, während vorherige Inokulierung mit dem Mykorrhizapilz *Glomus interadices* zu einer verminderter Infektion der Wurzeln führte. Beide Effekte gingen mit gesteigerten bzw. erniedrigten PR-10 - Signalen einher, so dass diese Proteine offenbar das Infektionsniveau auf zellulärer Ebene widerspiegeln.

Proteomanalysen der anderen beiden *M. truncatula* Linien führten zur Identifikation 14 weiterer Proteine; unter ihnen 2 Proteasom-Untereinheiten, die einen ersten Hinweis auf eine in der Pflanze ausgeprägte Resistenz bedeuten könnten.

Schlagwörter: *Medicago truncatula*, *Aphanomyces euteiches*, Proteomanalysen

Contents

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Abbreviations

ABA	abscisic acid
ALP	alkaline phosphatase
avr genes	‘avirulence’ genes
BAC	bacterial artificial chromosome
cDNA	complementary DNA
cyt	cytochrome
dsRNA	double-stranded RNA
EST	expressed sequence tag
dpi/hpi	days post inoculation/hours post inoculation
HR	hypersensitive response
kb	kilo bases
kDa	kilo daltons
Mbp	mega base-pairs
MW	molecular mass (in daltons)
NB-LRR	‘nucleotide-binding site plus leucine-rich repeat’
NO	nitric oxide
PMF	peptide mass fingerprinting
p*I*	isoelectric point
PCR	polymerase chain reaction
PR	pathogenesis related
PTGS	post transcriptional gene silencing
R genes	‘resistance’ genes
RNase	ribonuclease
RNAi	RNA-interference
ROS	reactive oxygen species
SSH	‘Suppression Subtractive Hybridization’
TC	tentative consensus sequence
TMV	tobacco mosaic virus
2-DE	two-dimensional gel electrophoresis
WRKY	zinc-finger type transcription factors, defined by the N-terminal conserved amino acid sequence ‘WRKYGQK’

Chapter 1

General Introduction

Functional genomics as a tool to study plant-pathogen interactions

Due to the recent completion of full genome sequencing projects in several model organisms (*Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Mus musculus*, *Arabidopsis thaliana* and others) and also of the human genome project, researches in genomics are now undergoing an expansion from sequencing and mapping of genomes ("structural genomics") towards an emphasis on genome function analysis ("functional genomics"). Functional genomics has the aim to understand the function of genes, and it consists of systematic approaches to study all genes or proteins of an organism or specific tissue under special physiological conditions in parallel (Hieter and Boguski 1997). Thus, functional genomic approaches are integrative gene expression analyses that rely very much on recently devised methodologies for visualization of complex gene and protein expression patterns. This set of methods includes tools of molecular biology and biochemistry, classical genetics and bioinformatics.
Understanding the function of genes plays an essential role in the characterization of disease processes and their development. In principle, basic approaches to characterize the biology of diseases are similar in tissues of animals as well as of plants or other organisms. Functional genomic approaches are well-suited for comparing gene expression between disease situations and controls.

The ability to monitor both mRNA and protein populations qualitatively and quantitatively raises the prospects to link changes in gene expression caused by some exogenous influence to changes in cellular biochemistry, as displayed by the appearance of certain protein patterns. These tools are very appropriate in order to study plant disease development in both resistant and susceptible interactions, including comparable approaches to control references as well as time-course based analysis to detect early and subsequently occurring molecular events.
Transcriptomic approaches allow the analysis of thousands of distinct and well-defined gene products representing a comprehensive coverage of the transcriptome (Alba *et al.* 2004). Beside micro-array hybridization technologies, the approach of Suppression Subtractive

Hybridization (SSH) has emerged as a powerful tool. It allows the detection of differentially expressed and enriched cDNAs out of a library, which are exclusively expressed due to a certain exogenous influence.

In recent years, proteomic tools have emerged as a powerful complement to those on the transcriptome level for studying function and regulation of genes and their products under different biological conditions. The proteome reflects a more direct image of a cell's physiologic state by monitoring the sub-cellular localization of proteins, formation of multi-subunit complexes and abundance as a marker for their activity (Rose *et al.* 2004). The classical two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) functions as the key strategy presented in this work.

Molecular Biology of plant-pathogen interactions

Plant diseases initiated by pathogens (bacteria, viruses, fungi, nematodes), challenges by invertebrates, mechanical wounding or a prolonged exposure to abiotic environmental stresses are unique because of the plant's sessile lifestyle and lack of an immune system. Hence, plants have established a more general resistance response as compared to the immune response of vertebrates, which seldom prevents disease from occurring. On the other hand, there exist also specific responses at the cellular level that generally lead to a reduction of disease extent or severity (Hammerschmidt *et al.* 2001). Moreover, in plant-pathogen interactions most plants exhibit a surprising capability to resist a large range of pathogens (Dangl and Jones 2001).

Beyond a passive or basal protection at the plant surface provided by waxy cuticular layers and anti-microbial compounds, plant resistance to diseases involves a broad array of inducible defense responses: (i) the accumulation of cell wall structural polymers such as callose, lignin or suberin, (ii) the synthesis of various proteins and antimicrobial compounds such as phytoalexins, pathogenesis-related (PR) proteins, special cell wall proteins like proline-rich proteins (*e.g.*), and (iii) hydrolytic enzymes such as chitinases and proteinase inhibitors (Corbin *et al.* 1987; Després *et al.* 1995).

In an immediate plant response to a pathogenic challenge, reactive oxygen species (ROS) and nitric oxide (NO) play an important synergistic role for the rapid activation of a broad repertoire of defense responses. The typical ROS accumulated in stressed plant cells are

super-oxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which may be directly toxic to pathogens, but moreover contribute to structural reinforcement of cell walls by cross-linking various extracellular proteins such as (hydroxyl-)proline-rich glycoproteins to the polysaccharide matrix, by increasing the amount of lignin polymers and by inducing enzyme activities such as those of peroxidases and salicylic acid (SA). On the other hand, NO inhibits enzymes (catalase, peroxidase) that detoxify H_2O_2, but the main contribution of NO to plant defense is that *de novo* synthesized NO plays the key role in pathogen recognition which leads to the induction of several defense and cell protection signaling cascades. (Hammond-Kosack and Jones 2000)

Gene products of *R* (resistance) genes are generally involved in the initial recognition of pathogens. For the activation of *R* genes, various data from molecular analysis of plant-pathogen interactions support the model of a gene-for-gene plant disease resistance. In general, these interactions involve two basic processes: (i) the perception of pathogen attack, followed by (ii) plant responses that limit disease progress (Ellis *et al.* 2000). Hence, the products of *R* genes act as receptors for products of corresponding pathogen avirulence (*avr*) genes. When the corresponding *R* and *avr* genes are present in both host and pathogen, the result is disease resistance (Dangl and Jones 2001). *R* genes encode a diversity of proteins separated into five classes; the majority of these gene products include a nucleotide-binding domain plus leucine-rich repeats (NB-LRR) with functions in DNA-protein or protein-protein interactions (Dangl and Jones 2001). Moreover, there is a link between some of these proteins to the ATP/ubiquitin-mediated proteolysis pathway for cellular protein degradation, mainly represented by proteasomes, which are also involved in the activation of programmed cell death (PCD) as a part of hypersensitive response (HR) (Peart *et al.* 2002; Kim *et al.* 2003; Sullivan *et al.* 2003).

Specific signal molecules derived from pathogens that are recognized by the plant host are commonly termed ‘elicitors’ (eponym: Noel Keen, D. of Plant Pathology, University of California, Riverside, CA). Two types of elicitors are generally recognized: (i) non-specific elicitors such as cell wall glucans, chitin oligomers and glycoproteins, which do not exhibit differences in cultivar responses within host plant species, and (ii) specific elicitors, mainly signal peptides and proteins that are encoded by *avr* genes, which cause specific responses in cultivars carrying the matching *R* genes (Cheng *et al.* 1998).

Subsequent local and systemic plant defense responses are often referred to pathogenesis-related (PR) proteins. The extensively investigated PR proteins are plant-specific proteins;

they are currently classified into 14 families (Van Loon and Van Strien 1999). PR proteins include (i) cell wall-degrading enzymes (glucanases, chitinases), (ii) antimicrobial polypeptides (*e.g.* ribonucleases, peroxidases, defensins, thionins) and (iii) components of signal transduction pathways (*e.g.* lipoxygenase) (Hammond-Kosack and Jones 2000). It is postulated that the induction of PR proteins refers to pathological or related situations that are at least described in two different plant-pathogen interactions and their accumulation occurs not only locally but also systemically (Van Loon and Van Strien 1999). Often, the same proteins are induced not only in incompatible interactions but also in compatible ones but much more weakly and slowly (Hammond-Kosack and Jones 2000).

Plant signal molecules such as salicylic acid (SA), jasmonic acid (JA) and ethylene play an essential role in conducting disease-associated signals from the point of penetration to more distant non-infected plant tissues in order to enhance the plant's defense capacity, a phenomenon commonly described by the term systemically acquired resistance (SAR) (Van Loon and Van Strien 1999; Hammerschmidt *et al.* 2001; Ton and Mauch-Mani 2004). The induced defense reaction is initiated by a transcriptional activation ("priming") of *R* genes encoding the defense-related (PR) proteins mentioned above. JA and ethylene are both required for the activation of proteinase inhibitor genes, chitinase genes and certain PR proteins, while SA contributes many roles not only in plant defense responses (Hammond-Kosack and Jones 2000).

A prominent feature of plant defense response reactions against pathogens within the legumes and some other plant families is the synthesis of flavanoid phytoalexins by the phenylpropanoid pathway. Phytoalexins are low molecular weight antimicrobial active compounds, which are accumulated rapidly at sites of pathogen infection. The synthesis of most phytoalexins requires numerous biosynthetic enzymes that become activated after primary metabolic precursors have entered secondary pathways (Hammond-Kosack and Jones 2000). On the other hand, many of these enzymes are up-regulated after stimulation of pathogen elicitors (Akashi *et al.* 1999). In recent years, many enzymes of this highly branched pathway were investigated from the molecular genetic point of view; however, the genetic proof of phytoalexins in plant defense and in plant-pathogen interactions until now has only been determined for the *Arabidopsis* phytoalexin camalexin, the grapevine phytoalexin resveratol and the *Medicago* phytoalexin medicarpin (Winkel-Shirley 2001).

The above summary of plant responses to pathogen attack represents only a short overview of this large topic. In the remainder of this here presented work, molecular events of the parasitic interaction formed between the model legume *Medicago truncatula* and the oomycete root pathogen *Aphanomyces euteiches (Drechs.)* will be reviewed in detail.

The oomycete *Aphanomyces euteiches* and the Aphanomyces root rot

Oomycetes are a group of diverse fungal-like organisms phylogenetically placed within the *Stramenopiles* kingdom (Leipe et al. 1994). This group, consisting of 70 genera with more than 500 described species, includes terrestrial and aquatic species of filamentous, coenocytic habit forming fungal-like myceliums. Among them are significant pathogens of plants, insects and animals as well as saprophytic species (Judelson 1996). Terrestrial oomycetes are mainly parasites of vascular plants causing serious root rot diseases or foliar blight diseases with important economic and social impact (Bourke 1991). The most prominent example is the late blight disease on potato/tomato caused by *Phytophthora infestans*. Other thoroughly investigated plant-oomycete pathosystems are the interactions formed between *Phytophthora sojae* and soybean, *Bremia lactucae* and lettuce and *Perenospora parasitica* and *Arabidopsis thaliana* (Roetschi *et al.* 2001).

Within the family *Saprolegniaceae*, the genus *Aphanomyces* includes 45 species and appears as an ecologically diverse group of saprophytes or parasites on fish, crayfish and plants (Grünwald 2003). *Aphanomyces* is a diploid organism producing oospores and zoospores. Some species occur only on one host while others have a broader host range.
Aphanomyces euteiches (Drechs.) causes a root rot affecting several legumes including pea, bean, alfalfa, red clover and subterranean clover. The *Aphanomyces* root rot, also called common root rot, is considered to be the most destructive disease of pea in areas with temperate, humid climates occurring in nearly all pea-growing areas of North America, northern Europe, Australia, New Zealand and Japan (Hagedorn 1989; Levenfors *et al.* 2003). It was described for the first time in the 1920s in the state of Wisconsin (USA) by Jones and Drechsler (1925), mainly appearing during humid pea-growing seasons. *A. euteiches* isolates exhibit a distinctive host preference (and different *forma speciales* are recognized, *e.g. A. euteiches f. sp. pisi*), but nearly all isolates are highly pathogenic to pea cultivars even when isolated from a different host (Levenfors *et al.* 2003).

Characteristic symptoms of this root rot disease are honey-brown colored lesions spreading through the root cortex, a watery rotting of root tissue, a significant reduction of root mass and the appearance of *A. euteiches* oospores (Engqvist and Ahvenniemi 1997). *A. euteiches* infection results in stunted plants showing symptoms of drought stress caused by a reduced supply of water and nutrients from the root, or in the complete death of the seedlings (Hagedorn 1989). Furthermore, significant reductions in crop yields and seed yields of pea cultivars as well as decreases in the protein content of seeds were determined in areas where the soils revealed high *A. euteiches* inoculum (Engqvist and Ahvenniemi 1997).

The disease cycle is initiated by the anchorage of mobile vegetative zoospores at the root surface (Figure 1). These amphitrichous (= two-flagellate) zoospores are released from sporangia in high quantity. The zoospores encyst at the root surface and then germinate as hyphae that grow through the host tissue while damaging and disrupting it. After a few days, hyphal growth stagnates and generative thick-walled oospores are formed after fusion of oogonia and antheridia (meiosis). These oospores are capable of persisting for years in a dormant state and they constitute the primary source of inoculum in the soil (Mitchel and Yang 1996). The parasitic phase of *A. euteiches* is supposed to be biotrophic as the pathogen obtains nutrients from living host cells (Kjoller and Rosendahl 1998).

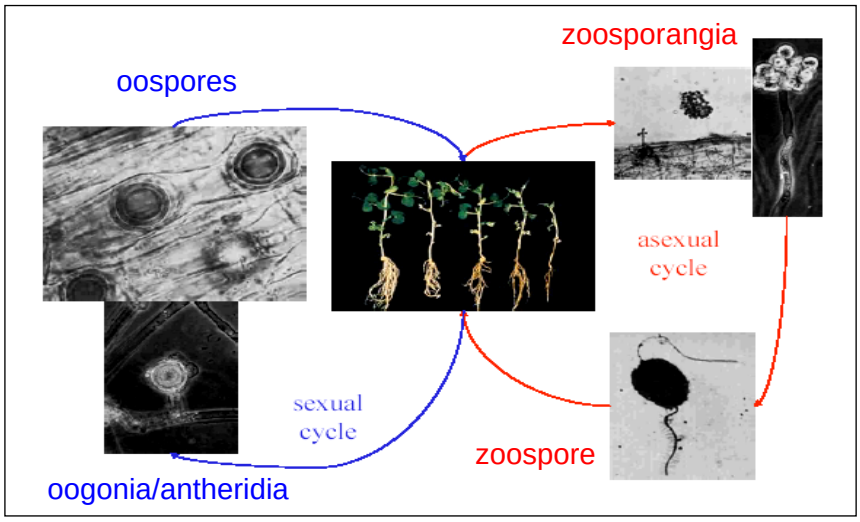

Figure 1. *Simplified life cycle of the oomycete Aphanomyces euteiches* (Modified of N. J. Grünewald 2003)

Due to a lack of knowledge about pathogen variability and mechanisms associated with the *in-planta* expression of partial resistance and the polygenic inheritance of resistance, pea cultivars with acceptable levels of resistance against *A. euteiches* are up to now not available (Pilet-Nayel *et al.* 2004). Moreover, no fungicides or adequate biological control methods are available to reduce disease severity. For *Medicago*, which is supposed to be moderately

infected by *A. euteiches* as compared to *Pisum*, accessions with enhanced levels of resistance were recently described, such as the alfalfa population *WAPH-5* and seven *M. truncatula* accessions with similar resistance levels (Vandemark and Grünwald 2004).

Recent phylogenetic studies based on rRNA sequence comparisons indicate that oomycetes are more closely related to chrysophytes, diatoms and heterokont algae than to all groups of 'real' fungi (Förster *et al.* 1990; Leipe *et al.* 1994). Oomycetes are consequently less well-characterized than any other fungal group (Judelson 1996). A key difference between the genetics of oomycetes and that of true fungi appears in the life cycle that oomycete vegetative structures are diploid, with meiosis occurring in well-differentiated gametangia (Judelson 1996).

The model legume *Medicago truncatula*

With more then 18.000 species classified into 650 genera, legumes (*Leguminosae/Fabaceae*) represent the third largest family of higher plants (Polhill and Raven 1981; Doyle 2001). Producing seeds and fruits that are rich in protein and nitrogen content, legumes are second only to grasses in agricultural importance providing approximately one-third up to two-third of the nutritional requirements for humans and the major source for animal feeding (www.legumes.org). Most cultivated legumes are found within the *Papilionidaea* subfamily representing the 'tropical' or 'phaseolid' legumes (including the genera *Phaseolus*, *Vigna* and *Glycine*) and the 'temperate' or 'galegoid' legumes (including the genera *Melilotus*, *Trifolium*, *Medicago*, *Pisum*, *Vicia*, *Lotus*, *Cicer* and *Lens*) (Young *et al.* 2003).

The legumes unique feature is the capacity to convert atmospheric nitrogen into nitrogenous plant-accessible compounds, mainly ammonia, mediated by a mutualistic interaction to *Rhizobia* soil bacteria. Furthermore, legumes form symbiotic relations to mycorrhizae fungi to enhance their uptake of phosphate, water and other soil nutrients (Gianinazzi-Pearson 1996). Hence, there is the need to understand the molecular aspects of legume-microorganism interactions, but large genome sizes, polyploidy and lack of suitable transformation protocols are retarding progress in molecular legume biology.

During recent years, this gap has been fulfilled by establishing *Medicago truncatula*, commonly known as barrel medic, as a model legume to investigate symbiotic plant-microorganism interactions, but also as a model plant for genomic studies in addition to the

plant model *Arabidopsis thaliana* (www.medicago.org). *M. truncatula* is also a close relative of the world's most important forage legume alfalfa (*Medicago sativa*).
M. truncatula has emerged as widely accepted model plant for the following reasons:

(i) *M. truncatula* exhibits a relative small genome size (~ 470 Mbp), that is only half the size of alfalfa and almost ten times smaller than that of pea (~ 4340 Mbp) (Blondon *et al.* 1994). Furthermore it is diploid (2n = 16) and self-fertile. In contrast, alfalfa is not suitable for genomic researches as it is a tetraploid and obligate out-crossing species.
(ii) *M. truncatula* as part of the *'Galegoid phylum'* is phylogenetically related to the most important legume crops like pea, faba bean, chick pea, alfalfa, lentis and clover. This genetic proximity provides an easy transfer of genomic information obtained for *M. truncatula* to all these other legume crops.
(iii) Large genome sequencing projects have been initiated for *M. truncatula* (*Medicago truncatula* Gene Index, MtGI). They provide a dense physical map, about 150.000 BAC-sequences and 200.000 ESTs available in public databases. These libraries contain not only developmentally regulated genes expressed in different plant organs but also those that are expressed under a range of physiological conditions, stresses and biotic interactions.
(iv) *M. truncatula* is capable of forming complex interactions with microorganisms, which is reflected by the development of numerous biosynthetic pathways impacting both mutualistic and disease interactions. These, such as the isoflavanoid pathway (*e.g.*), are an explicit feature that's missing in *Arabidopsis*, which makes *M. truncatula* a highly important model for higher plants.
(v) *M. truncatula* exhibits a relatively fast generation time and adequate transformation efficiency.

In recent years, a large range of different *M. truncatula* ecotypes has been described and genetically characterized (Jenczewski *et al.* 1997; Prosperi 2000; Prosperi et al. 2001). These derive from different Mediterranean habitats representing a wide adaptation to different living conditions. Due to this natural genetic diversity and the powerful genetic tools now becoming available for *M. truncatula*, this plant has become a popular model for investigating plant-microorganism interactions, including the susceptibility and resistance of legumes to pathogens.

Objectives of this work

The study presented here comprises different functional genomic approaches to analyze changes in gene expression and protein profiles of *Medicago truncatula* roots after infection with the oomycete plant pathogen *Aphanomyces euteiches*. Analysis of changes in gene and protein expression combined with physiological observations should provide important new insights into plant responses occurring at a molecular level during this plant-oomycete interaction. The investigations focus on four major objectives:

(i) Transcriptional profiling of *M. truncatula* roots infected with *A. euteiches* to identify novel genes up-regulated during this pathogenic interaction (chapter 2).

(ii) A proteomic approach to identify *M. truncatula* proteins induced in roots after infection with *A. euteiches* (chapter 3).

(iii) Root proteome profiling of three different *M. truncatula* lines to detect particular proteins involved in altered susceptibility and resistance to *A. euteiches* (chapter 4).

(iv) Proteome analysis of the tripartite interaction between *Medicago truncatula* roots, *Glomus intraradices* and the parasitic oomycete *Aphanomyces euteiches* to detect proteins involved in mechanisms of bioprotection (chapter 5).

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Transcriptional profiling of *Medicago truncatula* roots after infection with *Aphanomyces euteiches* (oomycota) identifies novel genes upregulated during this pathogenic interaction☆

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Abstract

Common root rot of pea caused by *Aphanomyces euteiches* has been the major yield-reducing factor for pea production during the last decades. In this study, a systematic sequencing of expressed sequence tags (ESTs) was chosen to obtain a first global picture of the assembly of genes involved in pathogenesis. For this purpose, a pathosystem between the model legume *Medicago truncatula* and *A. euteiches* was established. Typical symptoms of this disease such as root discoloration and a reduction of root mass were observed in the model legume. Significant transcriptional changes in the host plant occurred already 6 days after inoculation. To identify a large number of plant ESTs, which are induced at this time point, a cDNA-library was established by Suppression Subtractive Hybridization. Five hundred and sixty ESTs have been generated of this library. On the one hand, EST-annotations showed homologies to a number of classical pathogenesis-related (PR) and defense genes. A notable number of the ESTs, however, were derived from novel genes not matching entries of the large-scale *M. truncatula* sequence collection. Hybridization experiments showed that also within these new ESTs, 21% are induced after pathogen infection. Hence, the here presented transcriptomic approach demonstrates that classical pathogenesis mechanisms as well as new specific gene regulations are involved in root rot disease development caused by *A. euteiches*.
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Keywords: *Aphanomyces euteiches*; *Medicago truncatula*; *Pisum sativum*; Expressed sequence tags; Suppression subtractive hybridization; cDNA-AFLP

1. Introduction

Parasitic oomycetes cause severe diseases on many crop plants. *Aphanomyces euteiches* (*Saprolegniaceae*) is a serious pathogen on pea worldwide, but several other legumes as alfalfa, snapbean and red clover are also described to be susceptible [1,2]. *A. euteiches* was suggested to form a biotrophic association with legumes. The disease cycle is initiated by the release of primary zoospores that encyst at the surface of zoosporangia. From here, secondary heterokont zoospores are released which encyst at solid surfaces before germination and infection of the host root. After few days, the pathogen will form thick-walled oospores in the root. These will serve as resting stage and will constitute the primary inoculum source in the soil [3]. *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 live saprophytically within dead plant material [4].

To date, no effective fungicides, resistant pea cultivars or biological control methods are available for the control of

☆ *MtAPHEU*-EST sequences can be found at GenBank as accession numbers AJ547891–AJ548395.

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this pathogen [5–7]. There are reports about the bioprotective effect of arbuscular mycorrhizal fungi [8], 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. However, so far 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–fungi associations cannot be generally transferred to plant–oomycete associations [9]. Transcription profile comparison between *A. euteiches*-infected and control roots provides 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* [10,11]. But so far, the *A. euteiches*–legume association has not been the central aspect of transcriptomic approaches. Initiation and realization of large expressed sequence tags (EST) programmes using model organisms have become very effective and straightforward strategies to obtain global views on transcriptional changes during different plant–microbe interactions [12,13]. Large EST- and genomic sequencing projects have been initiated for *Medicago truncatula* (www.medicago.org) and *Lotus japonicus* [14]. These two legumes possess appropriate genetic features and capacities to form various symbiotic as well as pathogenic associations. The annual medic *M. truncatula* is a diploid and self-fertile plant with a small genome size, fast generation time and high transformation efficiency [15]. *M. truncatula* is phylogenetically related to *Pisum sativum*, which is the most important grain legume in Europe. Both species show a high degree of colinearity and syntheny [16]. This should allow to transfer the results obtained using *M. truncatula* as model system to this important crop legume. The aim of the present study was to analyze transcriptional profiles of *M. truncatula* during infection by *A. euteiches* in order to gain insight into molecular and physiological changes in diseased legume roots.

2. Materials and methods

2.1. Plant material

Seeds of *M. truncatula c.v. Jemalong* A17 were surface-sterilized by 10 min treatment with concentrated sulphuric acid, 5 min incubation in 6% NaClO and three washing steps with distilled water. Seeds were pre-germinated on moist filter paper for 2 days in the dark. Seedlings were transferred into pots containing a 1:2 mixture of sterilized expanded clay and vermiculite. Plants grown under constant conditions in a greenhouse (220 $\mu E\ m^{-2}\ s^{-1}$ for 16 h; 22 °C, 65% humidity) and fertilized with half-strength Hoagland's solution [17].

Seeds of *P. sativum L.* (*c.v. Solara*) were surface-sterilized for 10 min in 6% NaClO and washed several times with distilled water. Seedlings were grown under same conditions as described above.

For inoculation with *A. euteiches Drechs.* (ATCC 201684), 10 disks of 1 cm^2 fungal mycelium grown on corn meal agar were cut out and cultured in 20 ml maltose peptone broth (MPB) for 9 days at room temperature. Zoospore production was induced by washing the mycelium twice in autoclaved lake water. Zoospores were counted and diluted to appropriate concentrations. After 10 days, the plantlets were inoculated by application of zoospore suspensions at the stem basis. Control plants were mock-inoculated with autoclaved lake water.

2.2. Harvest of plants and histochemical staining procedure

Control and inoculated plants were harvested, roots were cut off, washed with sterile water and the fresh weight of each root system was determined.

From each treatment, half of the roots were used for histological analyses: after incubation in 10% (w/v) KOH for 30 min at 90 °C, the roots were stained with trypan blue for detection of oospores. The remaining roots were stained overnight to detect fungal alkaline phosphatase activity as described by Tisserant et al. [18]. ALP-stained roots were cleared in 3% (w/v) NaClO. After staining, roots were cut into pieces of 1 cm length before light microscopy.

For quantification of root infection, 10 root pieces of each fraction and inoculation treatment were placed on microscope slides. The appearance of stained oospores and ALP-stained hyphae were counted.

2.3. RNA extraction

A. euteiches total RNA was isolated from 1-week-old mycelium grown in MPB medium. The RNeasy Plant mini kit from Qiagen (Hilden, Germany) was used for all RNA extractions.

2.4. cDNA-AFLP

M. truncatula total RNA was isolated from infected and non-infected roots 6, 14 and 21 days after infection. cDNA-AFLP method was performed according to modified protocol described earlier by Vos et al. [19] and Bachem et al. [20], using *Apo*I and *Mse*I restriction enzymes. Sequences of adapters and primers used are shown in Table 1. Selective amplification products were separated on 4% denaturing polyacrylamide gels at 45 W for 2 h. The gels were silver stained as described by Bassam et al. [21]. Bands of interest were cut out of gel and incubated in 100 µl TE buffer for 2 h. AFLP fragments were re-amplified, using the same conditions as during pre-amplification. Amplification products were cloned into the pGEM-Teasy vector (Promega, Madison, USA). To confirm that cloned cDNA

Table 1
cDNA-AFLP Primer sequences

Adapters and primers	Sequence (5′–3′)
Apo I adapter	CTC GTA GAC TGC GTA CC CAT CTG ACG CAT GGT TAA
Mse I adapter	GAC GAT GAG TCC TGA GTA CTC AGG ACT CAT
Apo I pre-amplification primer	CTC GTA GAC TGC GTA CCA AT
Mse I pre-amplification primer	GAC GAT GAG TCC TGA GTA A
Apo I selective amplification primer	GAC TGC GTA CCA ATT CNN (NN = GC; AC; CA)
Mse I selective amplification primer	GAT GAG TCC TGA GTA ANN (NN = GC; AC; CA; GA; CT; AG)

fragments correspond to selected AFLP-bands, inserts of 10 colonies of each transformation reaction were sequenced.

2.5. Suppression subtractive hybridization cDNA-library

Suppression subtractive hybridization (SSH) [22] was used to generate a cDNA-library enriched for sequences induced in *M. truncatula* roots infected with *A. euteiches*.

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 subtractive kit (Clontech, Palo Alto, USA). SMART-cDNAs derived from control roots and *A. euteiches* were pooled 5:1 and used as driver population. 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).

2.6. cDNA hybridizations (reverse Northern hybridizations)

Inserts of cDNA clones were amplified, separated on 2% TAE gels [23] and transferred to nylon membranes. Digoxygenin-labeled cDNA probes were synthesized by amplification of SMART-cDNA using the PCR-Digoxygenin-labeling mix (Roche Diagnostics Corporation, Mannheim, Germany). The filters were hybridized and signals were detected according to the protocol of the Digoxygenin supplier (Roche Diagnostics Corporation, Mannheim, Germany).

2.7. EST analysis

Clones of the SSH-library were manually picked and sequenced. The raw sequences were trimmed to eliminate vector, SMART-cDNA-primer and SSH-adaptor sequences. A quality clipping was proceeded according to phred 13 quality [24]. The remaining ESTs were clustered [25] according to TIGR (The Institute for Genome research; http://www.TIGR.org) protocol. Clustering and automatic annotations were done using a BioMake software package (Bekel and Meyer 2003, in press). Comparisons to the TIGR *Medicago truncatula* gene index (MtGI) were done in order to identify identical tentative consensus sequences (TCs). BlastX searches were carried out in order to identify homologous genes in other organisms [26].

3. Results

3.1. Establishment of a Medicago truncatula–Aphanomyces euteiches pathosystem

The typical disease symptoms with yellow-honey brown root discolorations were observed in *M. truncatula* seedlings after inoculation with *A. euteiches*. Zoospores of the pathogen were observed attached to *M. truncatula* root surfaces. After trypan blue staining, oospores were visible in the root cortex (Fig. 1). A similar disease development was observed in pea roots. Ten days after inoculation with either 2.5×10^5 or 1×10^6 zoospores, the root fresh weights of both plant species were clearly reduced as compared to non-inoculated control plants (Fig. 2). Moreover, a rapid increase in percent root lengths with oospores was observed until 10 days after inoculation in both species. At the latest time point analyzed, 21 dpi, oospores were found in more than 60% of the root system of both species (Fig. 3a).

Alkaline phosphatase (ALP)-activity of active *A. euteiches* could be visualized as a black precipitate in hyphae growing through the root cortex. The percentage of root lengths with ALP-active mycelium of the pathogen was also similar in pea and *M. truncatula* roots (Fig. 3b).

3.2. cDNA-AFLP analysis of the interaction time course

A cDNA-AFLP approach was chosen to determine the time point during the disease development, where the transcription profile between infected and control roots showed the highest percentage of differentially expressed cDNAs. Infected 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. The size of AFLP fragments which could be detected after staining ranged from 50 to 900 bp. For each primer combination, 50–80 bands were observed. In total, about 6500 bands were detectable. Comparison of cDNA-AFLP patterns revealed two upregulated and two

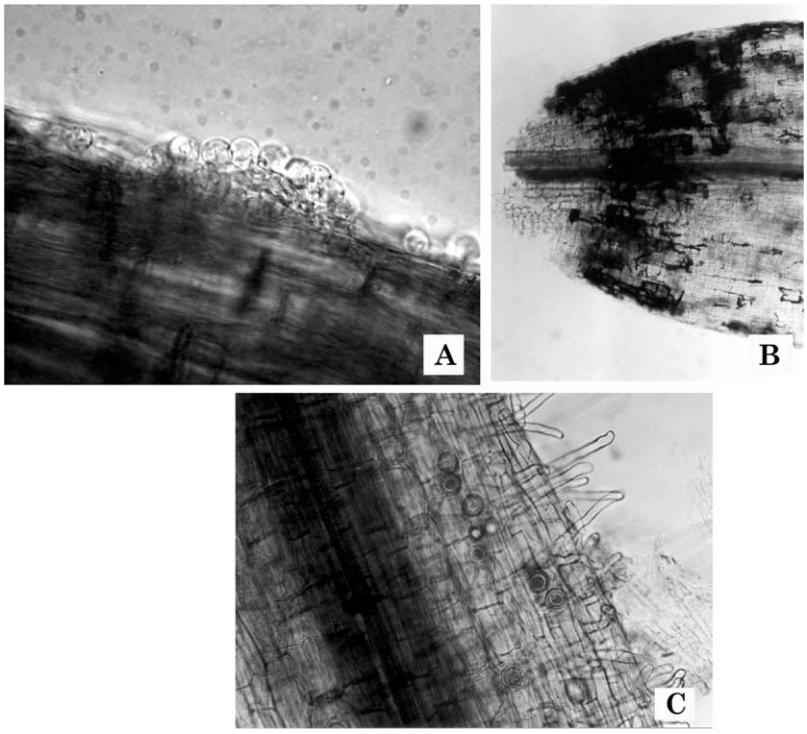

Fig. 1. Light microscopy studies of the *A. euteiches–M. truncatula* interaction. (A) Zoospores of *A. euteiches* attached to *M. truncatula* root surface. (B) Alkaline phosphatase (ALP)-stained hyphae of *A. euteiches* colonizing the root cortex. (C) Oospores of the pathogen within the root cortex of *M. truncatula*.

downregulated fragments in infected roots (Fig. 4). Differential RNA accumulation of these four fragments already occurred after 6 days, the earliest time point analyzed, and remained constant until 21 days after inoculation. To obtain first sequence information on differential expressed genes, the four differentially expressed bands were cloned. After cloning, inserts of 10 colonies of each transformation reaction were sequenced. All sequences obtained after cloning of one selected AFLP fragment were identical indicating that the obtained sequences are not deriving from background fragments but correspond to the differentially expressed AFLP fragments. The sequences were compared to the GenBank database, similarities to already existing plant sequences were found for all four fragments (Table 2).

3.3. Generation and analysis of an SSH–EST library enriched for cDNAs induced in M. truncatula roots 6 days after inoculation with A. euteiches

A cDNA-library was generated by SSH to obtain a large number of ESTs, representing genes upregulated in *A. euteiches*-infected roots of *M. truncatula* 6 days after inoculations. To avoid the enrichment and cloning of cDNAs from *A. euteiches* origin, the RNA of non-infected roots was mixed with RNA extracted from *A. euteiches* mycelium grown in liquid culture. After amplification during SSH-procedure, PCR fragments were cloned and about 2000 clones were obtained. In a first attempt to analyze the SSH-library, 192 clones of this cDNA population were analyzed concerning their RNA accumulation pattern in infected roots by reverse Northern hybridization analysis. Fifty one of these cDNAs were strongly induced in *A. euteiches*-infected roots, whereas after hybridization to the control root cDNA, no or very weak signals were detectable (Fig. 5).

After confirmation that the library contains to a high extent cDNAs from plant genes induced by *A. euteiches*

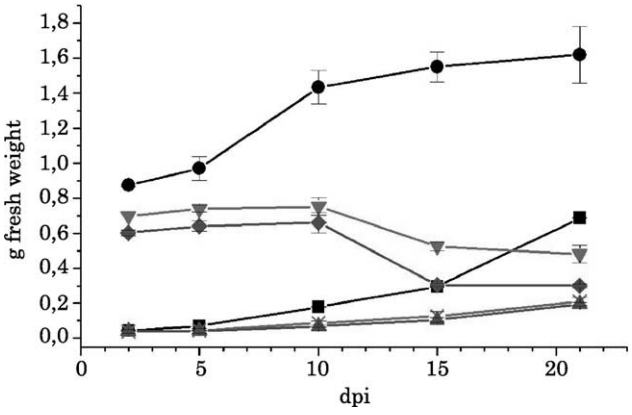

Fig. 2. Root fresh weight development of *P. sativum* and *M. truncatula* plants infected with two different concentrations of *A. euteiches* zoospores. Each point is the mean of two replicates, bars indicate standard errors. Circles: *P. sativum* mock-inoculated control plants; down-triangles: *P. sativum* inoculated with 250.000 zoospores; diamonds: *P. sativum* inoculated with one million zoospores; squares: *M. truncatula* mock-inoculated control plants; up-triangles: *M. truncatula* inoculated with 250.000 zoospores; crosses: *M. truncatula* inoculated with one million zoospores.

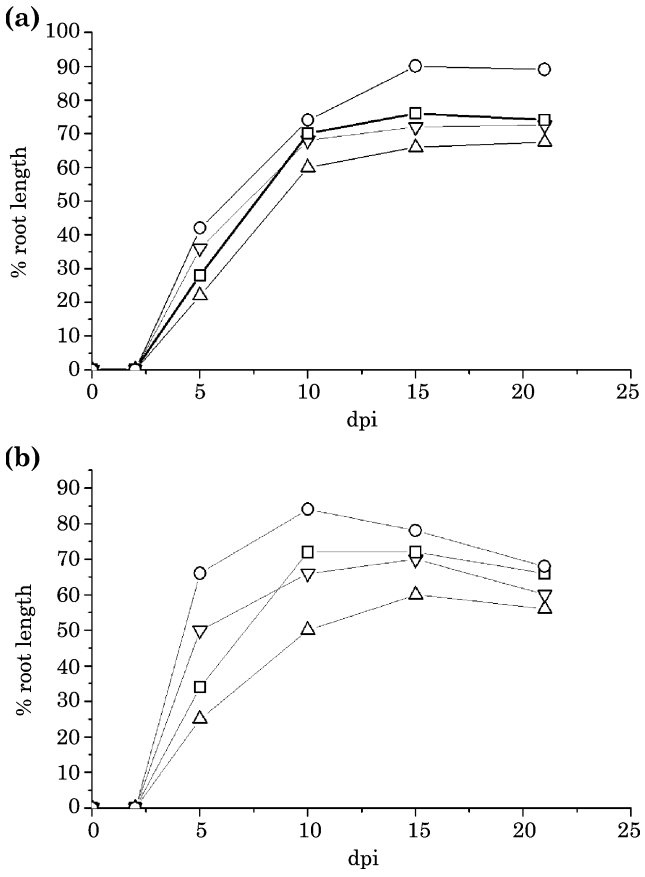

Fig. 3. Development of the *A. euteiches* infection in roots of *P. sativum* and *M. truncatula* inoculated with two different concentrations of *A. euteiches* zoospores. (a) Percent root length with oospores of *A. euteiches*. (b) Percent root length with alkaline phosphatase-active hyphae. Circles: inoculated control plants; up-triangles: *P. sativum* inoculated with 250.000 zoospores; diamonds: *P. sativum* inoculated with one million zoospores; squares: *P. sativum* inoculated with 250.000 zoospores; down triangles: *M. truncatula* inoculated with one million zoospores; up-triangles: *M. truncatula* inoculated with 250.000 zoospores.

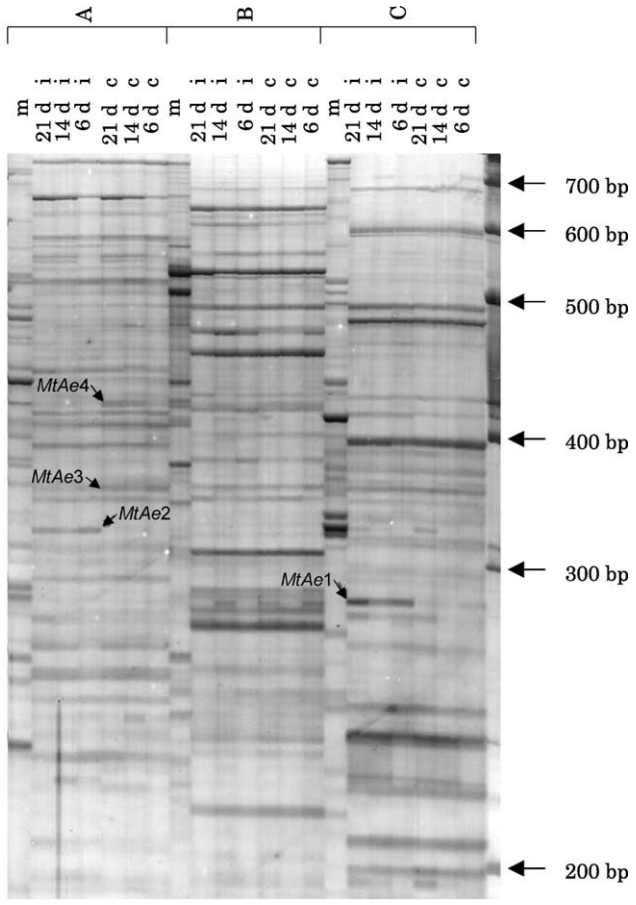

Fig. 4. cDNA-AFLP time-course analysis of the *M. truncatula*–*A. euteiches* interaction. AFLP pattern of cDNAs of control roots (c) and infected roots (i) were generated using different primer combinations: (A) *Apo* I-2/*Mse* I-5; (B) *Apo* I-2/*Mse* I-4; (C) *Apo* I-2/*Mse* I-2. For this experiment RNA was extracted of inoculated and mock-inoculated *M. truncatula* roots at 6, 14, 21 days after inoculation. These time points are indicated. The corresponding cDNA-AFLP pattern of cDNA of *A. euteiches* vegetative mycelium grown in liquid culture are indicated (A.e.). Fragment sizes of the DNA ladder (m) and fragments which were cloned and sequences are indicated.

infection, 560 ESTs were generated. SSH-cDNA sequences were designated as *MtAPHEU* (*Medicago truncatula*–*Aphanomyces euteiches*). *MtAPHEU*-EST with a length of more than 50 bp were submitted to GenBank and sequences can be found as accession numbers AJ547891–AJ548395.

Sequence annotations of differentially expressed cDNAs within the first 196 clones of the library are shown in Table 3. In this population of upregulated cDNAs, two fragments matched a PR4-like sequence encoded by MtGI-TC87236, which was already identified to be strongly induced by cDNA-AFLP. Two fragments showed similarities to class 10 PR proteins. With only one exception, fragment 1b011 which is similar to an acotinate hydratase sequence of the red algae *Gracilaria verrucosa*, all fragments showed highest similarities to plant genes and in most cases to legume sequences.

Clustering of these ESTs according to the TIGR protocol revealed 74 TC sequences and 330 singletons. Singletons with a sequence length less than 100 bp were ignored for further cluster analysis. A TC encoding a class 10 PR protein, assembled from 13 SSH–ESTs was the most redundant sequence among the cDNA population. To get a global view on this EST-population, the whole EST-collection was annotated and classified into 11 broad categories (Fig. 6). Only one functional class was assigned to each cluster. Notably, the cluster of genes corresponding to abiotic stress and development contained six singletons with similarity to abr17, an ABA-responsive protein of *P. sativum*. Most ESTs had to be classified into the 'no function' category and a large number of ESTs had to be assigned to the 'no homology' category.

3.4. Analysis of new EST sequences

Surprisingly, 46 out of the 560 non-clustered ESTs did not match (*e*-value below 1×10^{-10}) any EST deposited so far in the *M. truncatula* gene index (MtGI release 6.0). This indicated that these ESTs correspond to novel, so far unknown

Table 2
Sequence annotation of differentially expressed cDNA-AFLP fragments

Fragment Nr.	Best GenBank database match	RNA accumulation pattern	MtGI match
MtAe1	Germin-like protein, *P. sativum*	Increased	TC87484
MtAe2	Pathogenesis-related protein 4A, *P. sativum*	Increased	TC87236
MtAe3	Serine/threonin protein kinase, *Glycine max*	Decreased	–
MtAe4	UDP-glycose:flavonoid glycosyltransferase, *G. max*	Decreased	TC78509

M. truncatula genes, which have not been identified in previous sequencing projects. After BlastX-search, 25 of these 46 new *M. truncatula* ESTs did not show any significant similarity (*e*-value below 1×10^{-10}) to sequences within the GenBank database.

ESTs without similarities to already existing MtGI-entries were further analyzed concerning their differential expression during *A. euteiches* infection. Of these 46 ESTs, eight sequences (1b11, 1c05, 1c11, 1f09, 1h03, 1h06, 1h09 and 2c11) have already been included within the first pool of 192 clones analyzed by initial reverse Northern screening. Three of these sequences (1b11, 1c05 and 1h03) showed significantly increased signal after hybridization to infected root cDNA. The inserts of the remaining 38 clones were analyzed in a second reverse Northern blot. The house-keeping gene *MtEf1-α*, encoding the translation elongation factor EF-1 alpha, was used as non-regulated control gene. The PR4-gene encoded by MtGI-TC87236, which was already identified by cDNA-AFLP and by screening of the first 192 clones of the SSH-library, was used as an internal positive control. The *MtEf-1α* cDNA showed even and strong hybridization to infected root cDNA and to

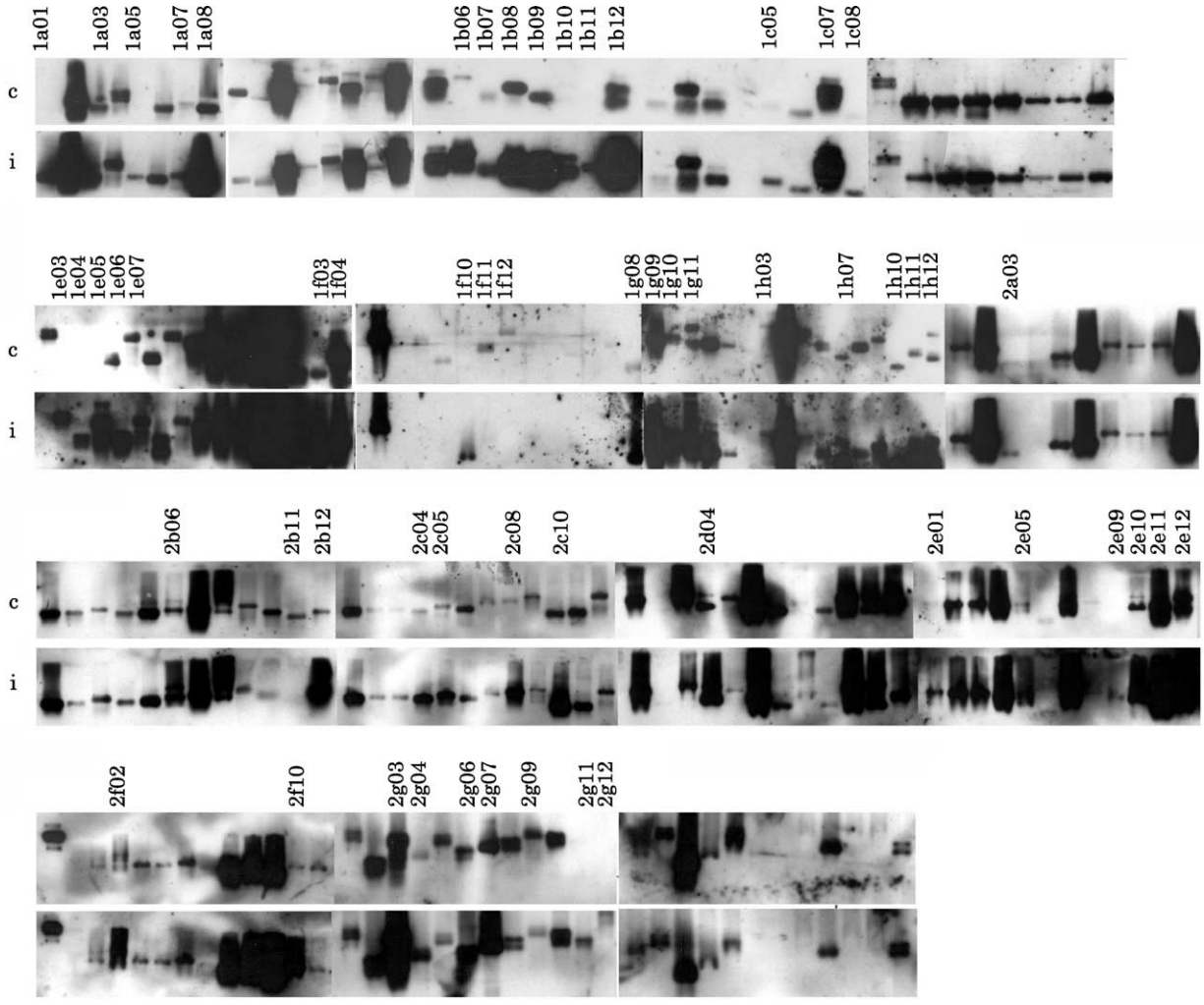

Fig. 5. cDNA-hybridization (reverse Northern hybridization) in order to analyze RNA accumulation of 192 *MtAPHEU*-cDNA clones in control and *A. euteiches* infected roots. The inserts of the clones were amplified, blotted on two nylon membranes and hybridized to labeled cDNA of non-infected (c) and infected (i) roots. *MtAPHEU*-index numbers of the cDNA fragments showing stronger RNA accumulation in infected roots are indicated.

Table 3
Sequence annotations of differentially expressed SSH-cDNAs

Clone ID	Length (bp)	Matching sequence from the Genbank 'nr' data base BlastX)	Origin of matching sequence	MtGI/GenBank identities
Upregulated in infected roots				
1a01	505	ABA-responsive protein abr17	*P. sativum*	TC82086
1a03	529	Albumin precursor 1	*P. sativum*	TC88792
1a05	618	RABA	*L. japonicus*	TC77335
1a07	737	ABA-responsive protein abr17	*P. sativum*	TC82086
1a08	526	Pathogenesis-related protein 4A	*P. sativum*	TC87236
1b06	255	No hits	–	AJ548171
1b07	0	–	–	–
1b08	269	Chitinase		
1b09	256	Proline rich protein auxin-induced	*M. truncatula*	TC85742
1b10	444	No hits below 1×10^{-10}	–	TC80304
1b11	400	Aconitate hydratase precursor	*G. verrucosa*	AJ548017
1b12	404	Disease resistance protein pi49 (pr10)	*P. sativum*	TC76518
1c05	419	No hits below 1×10^{-10}	–	AJ548280
1c07	238	Putative extracellular dermal glycoprotein	*Cicer arietinum*	TC77207
1c08	41	No hits	–	–
1e03	461	No hits	–	AJ547999
1e04	386	Thaumatin-like protein precursor Mdtl1	*Malus domestica*	TC77149
1e05	0	–	–	–
1e06	44	No hits	–	–
1e07	327	Serine palmitoyltransferase	*Solanum tubersosum*	TC86313
1f03	358	No hits below 1×10^{-10}	–	TC76608
1f04	544	Dehydrin related protein	*P. sativum*	TC76699
1f10	198	No hits	–	AJ548106
1g08	7	No hits	–	–
1g09	21	No hits	–	–
1g10	336	No hits	–	TC83308
1g11	599	Putative senescence-associated protein	*P. sativum*	TC76492
1h03	266	No hits	–	AJ547977
1h07	310	Pathogenesis-related protein 4A	*P. sativum*	TC87236
1h10	537	Cytochrome P450	*Glycyrrhiza echinata*	
1h11	324	Polygalacturonase inhibiting protein 1	*Arabidopsis thaliana*	TC78258
1h12	50	No hits	–	–
2a03	337	Probable cinnamoyl-CoA reductase	*A. thaliana*	TC78732
2b06	0	–	–	–
2b11	418	Hypothetical protein F25P12.93	*A. thaliana*	TC91629
2b12	191	No hits	–	TC76608
2c04	307	Pathogenesis-related protein 4A	*P. sativum*	TC81815
2c05	556	No hits	–	TC83308
2c08	159	Tumor-related protein clone NF34	*Nicotiana tabacum*	TC87871
2c10	22	No hits	–	–
2d04	291	ACR4	*A. thaliana*	TC87620
2e01	402	Thaumatin-like protein precursor Mdtl1	*M. domestica*	TC60001
2e05	599	Unknown protein	*A. thaliana*	TC90135
2e09	0	–	–	–
2e10	0	–	–	–
2e11	90	Disease resistance response protein Pi49 (PR19)	*P. sativum*	TC90135
2e12	229	Probable cytochrome P450 monooxygenase	*Zea mays*	TC76394
2f02	501	Putative extracellular dermal glycoprotein	*C. arietinum*	TC77209
2f10	309	Tumor-related protein clone NF34	*N. tabacum*	TC87871
2g03	579	Early nodulin 12B precursor (N-12B)	*Medicago sativa*	TC85247
2g04	395	Beat-1,3-glucanase	*G. max*	TC77400
2g06	270	Pathogenesis-related protein 1	*N. tabacum*	TC86002
2g07	539	ACR4	*A. thaliana*	TC87620
2g11	0	–	–	–
2g12	463	Probable 12-oxophytodienoate reducatase	*Catharanthus roseus*	TC85809
Downregulated in infected roots				
1f11	478	Shaggy-like protein kinase kappa	*A. thaliana*	TC78596
1f12	381	Hypothetical protein F19B15.130	*A. thaliana*	TC79474
1g09	0	–	–	–

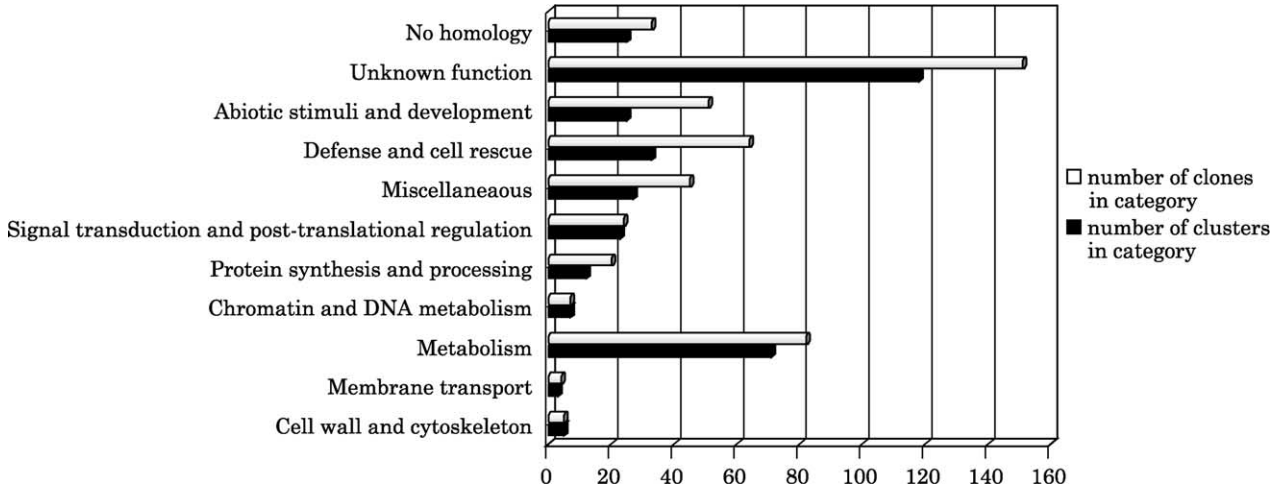

Fig. 6. Distribution of SSH–EST (expressed sequence tag) clones. The 11 broad categories that were used for classification based on data base (BlastX) similarity are indicated.

non-infected root cDNA, respectively. In contrast, the PR4-fragment and seven of the 38 inserts showed clearly increased RNA accumulation after hybridization to the infected cDNA probe as compared to control roots, indicating an induced RNA accumulation of the corresponding genes (Fig. 7). Summarizing the two reverse Northern screenings of this study: EST sequencing revealed 46 sequences which were novel for *M. truncatula*, whereof 10 are clearly induced in *A. euteiches* infected roots. Five of these fragments showed strong signals (1c05, 1h03, 3f08, 7e05) after hybridization to *A. euteiches* infected root cDNA and weaker, but detectable signals after hybridization to control root RNA. This indicates that the corresponding genes are plant genes which are transcribed at basal level in non-infected roots but are significantly upregulated after *A. euteiches* infection. The plant origin of the five induced cDNA-fragments showing only very weak signal intensities after hybridization to control plant RNA (1b11, 5c01, 5c03, 6c10, 6d11) was analyzed using gene specific primers. Amplification of *M. truncatula* and *A. euteiches* genomic DNA showed that all of these fragments except 1b11 which showed highest similarity to a sequence from a red algae, are of plant origin (data not shown). This demonstrates that the population of SSH–ESTs, which seem to encode new *M. truncatula* genes implies roughly the same percentage of *A. euteiches*-induced genes as the total number of analyzed 196 SSH-cDNAs.

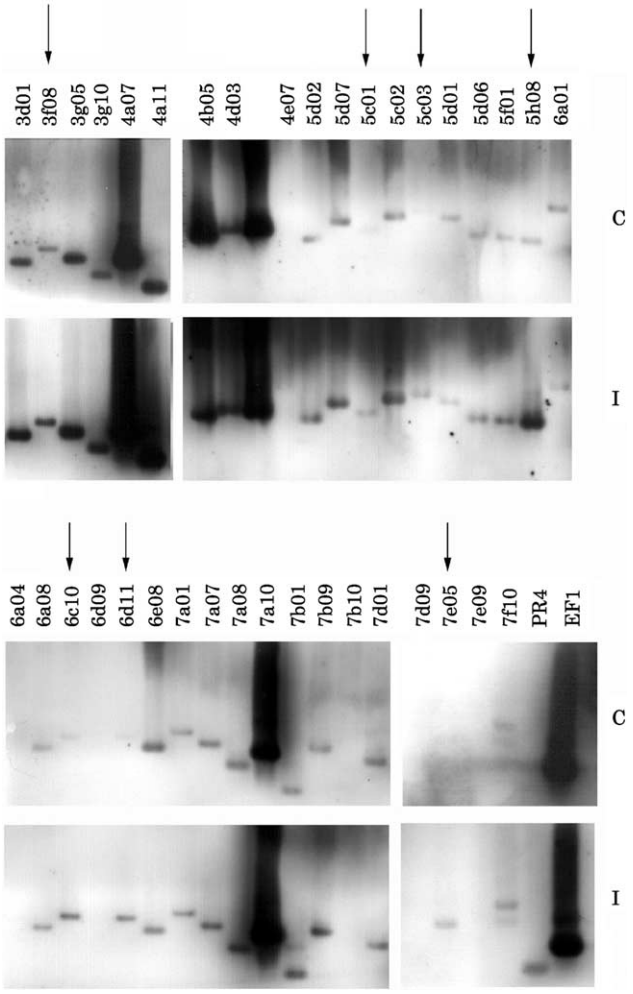

Fig. 7. cDNA-hybridization (reverse Northern hybridization) in order to analyze RNA accumulation of 38 SSH-cDNA clones in control and *A. euteiches* infected roots. The inserts of the clones were amplified, blotted on two nylon membranes and hybridized to labeled cDNA of non-infected (c) and infected (i) roots. *MtAPHEU*-index numbers of the corresponding genes are indicated. Internal controls are marked by: PR4 (positive control) and Ef1 (constitutive control).

4. Discussion

Transcriptome analysis in different model organisms has been applied for several years for the global analysis of plant–microbe interactions [27,28]. In order to study the economically important root rot of legumes caused by *A. euteiches*, *M. 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 *P. 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*. This disease development is similar to what previously has been observed in pea [4]. 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 after infection of a root system, the parasite has been shown to be inactive shortly after first symptoms appear on the host plant [4]. Therefore, early stages of the interaction were chosen for transcription profiling. 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 6 days after inoculation, notable numbers of differentially expressed cDNAs were detectable. These observed differences in the transcription pattern remained until the last time point analyzed, 3 weeks after inoculation. Four genes, which show either an increased or a decreased RNA accumulation during the infection, were cloned and sequenced. One of these genes encoded a typical class 4 PR protein.

Therefore, a cDNA-library which is 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 6 days after inoculation resulted in 26.5% upregulated 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 [29]. 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 PR protein-encoding class. The induction of PR proteins, defined as plant proteins induced specifically in pathological or related situations [30,31] is a result of the plant defensive response and an indication of recognition between host plant and parasite. One of these genes encodes a typical class 4 (PR) protein containing a hevein-domain, structural units that are capable to bind chitin [32]. Another PR protein-encoding cDNA was the most redundant gene in this SSH-library. This 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) [33]. This phytohormone is not only an important signal for the plant physiological and molecular response to a water deficit but it plays also an essential role in triggering gene expression upon wounding and pathogen attack [34]. 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 signaling 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 after pathogen colonization. In addition to observed gene inductions which have been also previously described to occur during biotic or abiotic stress, 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 MtGI. This large-scale EST database contains a collection of over 1.8×10^6 *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 deriving from tissues challenged with different pathogens. Of the 560 *A. euteiches*-induced cDNAs identified in this study, more than 8% 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 cDNA could be of great importance for future research in order to understand the specific mechanism underlying this agronomical important legume root-rot disease.

Acknowledgements

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Proteomic approach: Identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*

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Abstract

The legume root rot disease caused by the oomycete pathogen *Aphanomyces euteiches* is one major yield reducing factor in legume crop production. A comparative proteomic approach was carried out in order to identify proteins of the model legume *Medicago truncatula* which are regulated after an infection with *A. euteiches*. Several proteins were identified by two dimensional gel electrophoresis to be differentially expressed after pathogen challenge. Densitometric evaluation of expression values showed different regulation during the time-course analysed. Proteins regulated during the infection were identified by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Among the differentially expressed proteins, two encoded putative cell wall proteins and two were designated as small heat shock proteins. Furthermore, an isoform of the chalcone-O-methyltransferase was found to be increased in infected roots. The majority of induced proteins belonged to the family of class 10 of pathogenesis related proteins (PR10). Previously, various PR10-like proteins have been shown to be regulated by general stress or abscisic acid (ABA). Therefore, these proteins were further investigated concerning their regulation in response to drought stress and exogenous ABA-application. Complex regulation patterns were identified: three of the *A. euteiches*-induced PR10-like proteins were also induced by exogenous ABA-but none of them is induced after drought stress. In contrast, three of these proteins are down-regulated by drought stress. Hence, the strong expression of different PR10-family members and their regulation profiles indicates that this set of proteins plays a major role during root adaptations to various stress conditions.

Abbreviations: 2-D, 2-dimensional; BLAST, basic local alignment search tool; EST, expressed sequence tag; IEF, isoelectric focussing; MALDI-TOF-MS, matrix assisted laser desorption/ionization-time of flight-mass spectrometry; MtGI, the *Medicago truncatula* gene index; MW, molecular weight; PR, pathogenesis related; PMF, peptide mass fingerprinting; TC, tentative consensus sequence; TIGR, The Institute For Genome Research

Introduction

The plant pathogen *Aphanomyces euteiches* (*Drechs*) causes a root rot disease in several legumes of economic importance, including pea (*Pisum sativum*) (Hagedorn, 1989). This disease can be regarded as one major yield-reducing factor in legume crop production. Characteristic

symptoms of the disease are honey-brown coloured lesions spreading through the root cortex, a watery rotting of root tissue, a reduction of root mass and the appearance of *A. euteiches* – oospores in the root cortex (Engqvist and Ahvenniemi, 1997). These thick-walled oospores serve as a resting stage and constitute the primary inoculum source in the soil (Mitchel and Yang, 1966). After germination of oospores, the new disease cycle is initiated by the release of primary zoospores that encyst at the surface of zoosporangia. From here, secondary zoospores are released which encyst at root surfaces before germinating and infecting host roots. After a few days, the pathogen will form new oospores in the root. A widespread occurrence of this oomycete pathogen in soils of temperate regions has been observed. In alfalfa, the *Aphanomyces* root rot disease is controlled by resistance (Grau, 1990). In contrast, the only strategy to avoid *A. euteiches*-induced losses in pea production is the avoidance of poorly drained and heavily infested soils (Grau, 1990).

Despite the enormous economic impact of this disease, little is known about the molecular background of this legume-parasite interaction and its regulation at a cellular level. The study of interactions formed by leguminous plants as well as a study of general legume biology using agronomical important legume species such as pea or alfalfa is complicated because of their large genome sizes or polyploidy. Moreover, for the crop legume *P. sativum*, efficient transformation protocols are missing.

During the last decade, two model legumes, *Medicago truncatula* (www.medicago.org) and *Lotus japonicus* (Handberg and Stougaard, 1992) have been established mainly in order to get insights into agronomical important legume-microbe interactions due to their suitability for plant genomics. The recent initiation of a whole genome sequencing program and the availability of more than 190 000 expressed sequences tags (ESTs) of *M. truncatula* in public databases (*M. truncatula* gene index; MtGI) now offers possibilities for an efficient analysis of differentially expressed genes and gene products in a legume root after infection with *A. euteiches*.

Since the two model legumes have been established, intensive research approaches were carried out mainly focussed on transcribed sequences (Fedorova *et al*., 2002; Journet *et al*., 2002; Nyamsuren *et al*., 2003; Wulf *et al*., 2003). Recent analysis of yeast transcriptome and proteome profiles have shown that protein amounts are not always correlated to mRNA amounts (Gygi *et al*., 1999) and in contrast to transcriptome analyses, the study of protein populations enables a more direct access to cell processes by monitoring the actual pattern of translated gene products. We therefore initiated a project to investigate the infection process on proteome level. Very recently, 2-D gels of total protein from *M. truncatula* and of proteins extracted from different organs were published, representing the first protein reference maps for this organism (Mathesius *et al*., 2001; Watson *et al*., 2003). Furthermore, symbiosis-related proteins induced by the mycorrhizal fungus *Glomus mosseae* and the nitrogen fixing bacterium *Sinorhizobium meliloti* were monitored by 2-D electrophoresis (Bestel-Corre *et al.,* 2002; Djordjevic *et al*., 2003).

Here we present a comparative proteomic approach with the aim to study differential protein expression in roots of *M. truncatula* after infection with the oomycete pathogen *A. euteiches*.

Materials and methods

Plant material and growth conditions

Seeds of barrel medic (*M. truncatula Gaertn*. cv. Jemalong A17) were sterilised by 5 min treatment with concentrated sulphuric acid, followed by an incubation in a 3% (v/v) sodium hypochlorite solution for 5 min, and intensive washing with distilled water. Seeds were allowed to germinate for 3 days in the dark at room temperature. The germinated seedlings were then planted in pots containing a sterile 2:1 v/v mixture of expanded clay and vermiculite and grown under controlled growth conditions in the greenhouse (22 °C, 65% humidity, 16 h photoperiod). After 3 days, seedlings were fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Fertilization was repeated once at 7 days after inoculation. Zoospores of *A. euteiches* were produced as described previously (Nyamsuren *et al*., 2003). Briefly, *A. euteiches* was cultured in Maltose

Pepton broth for 9 days and zoospores production was induced by washing the mycelium in autoclaved lake water taken from a lake in the gardens of Herrenhausen, Hannover. Seven days after transplanting, seedlings were inoculated with *A. euteiches Drechs.* (ATCC 201684) by pouring 5 ml of the zoospore suspension containing 200 000 zoospores ml^{-1} at the base of the stem. Control plants were mock-inoculated with an equal volume of autoclaved lake water. One day before inoculation, all pots were water saturated. Apart from this flooding step, the plants were watered twice a week. Plants were harvested at several time points from 6 h to 3 weeks after inoculation with zoospores. For protein extraction, the roots were cut off, washed in distilled water to remove soil, frozen in liquid nitrogen and stored at −80 °C.

For plant production under drought stress conditions, the seedlings were not watered at all after the initial fertilisation step. For treatment with phytohormone ABA, 5 ml of 0.5 × Hoagland's solution containing 75 μM ABA (Sigma Chemicals, Sigma-Aldrich Co., Munich, Germany) were poured on the stem base of roots. The ABA-treatment was repeated after 7 days.

Phenolic protein extraction and sample preparation for IEF

Total root protein was extracted and precipitated according to a modified protocol of Hurkman and Tanaka (1986). A 0.5 g of root tissue was ground briefly in liquid nitrogen and homogenized in 750 μl lysis buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCL, 2 mM PMSF and 2% (v/v) β-mercaptoethanol, pH 8.0). An equal volume of saturated phenol was added and the samples were mixed by shaking at 300 rpm for 30 min. After centrifugation, the phenolic phase was diluted with lyses buffer (1:1 v/v). Proteins were precipitated in the phenolic phase after a centrifugation step with 100 mM ammonium acetate in methanol at −20 °C for at least 4 h and centrifuged again. The pellet was washed three times with cold 100 mM ammonium acetate in methanol and once with cold 80% acetone.

The vacuum dried pellet was resuspended in 350 μl rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 100 mM DTT, 0.5% v/v IPG buffer for correspondent pH-values (pH 3–10NL/4–7L, Amersham Pharmacia Biotech, Uppsala, Sweden) and a trace of bromophenol blue.

2-D electrophoresis (isoelectric focussing IEF, SDS-PAGE)

For first dimension isoelectric focussing (IEF), 18 cm immobilised dry strips with pH gradients 3–10 non-linear or 4–7 linear (Amersham Pharmacia Biotech) were rehydrated with 350 μl protein samples in rehydration buffer. Isoelectric focussing was done for 24 h using the IPGphor system (Amersham Pharmacia Biotech) with voltages from 30 to 8000 V at maximum. Strips were equilibrated for 15 min in equilibration solution I (30% v/v glycerol, 50 mM Tris-HCL pH 8.8, 6 M Urea, 2% (w/v) SDS, a trace of bromophenol blue, 0.01 g DTT ml^{-1} w/v) and equilibration solution II (same compounds like equilibration solution I, but DTT substituted by 0.025 g iodoacetamide ml^{-1}).

For 2-D poly-acrylamide-gel-electrophoresis (SDS-PAGE), IPG strips were fixed vertically onto SDS-tricine-polyacrylamide gels of 12 or 14% acrylamide. SDS-PAGE was carried out for 20 h at 30 mA mm^{-1} gel layer. Gels were subsequently Commassie-stained overnight using 0.1% Coomassie brilliant blue G250 (Bio Rad, Richmond, CA, USA) after treatment with the fixing solution (40% v/v methanol, 10% v/v acetate) for at least 2 h.

Scanning and relative expression analysis

Coomassie-stained gels were scanned on an UMAX Power Look III Scanner (UMAX Technologies, Fremont, USA) or on a Fujifilm FLA-3000 Fluorescence Laser Imager (Fujifilm Medical Systems USA, Stamford, CT, USA). To analyse relative expression patterns, protein spots of interest were labelled by the AIDA Image Analyser v3.20 Evaluation software (Raytest USA, Wilmington, NC, USA) and their values of Light Absorption Units (LAU, internal densitometric unit) were determined. The relative absorption units were obtained after subtraction of background absorption which was determined from four different blank parts of the corresponding 2-D gels. All shown values are averages of four technical repetitions. Lines indicate standard deviations.

Trypsin digestion

Protein spots of interest were manually excised from the gels and in-gel digested by trypsin according to a protocol of Williams *et al*. (1997). Gel plugs were treated in three washing steps with initially 250 μl acetonitrile:water (1:1 v/v) for 5 min, followed by 250 μl acetonitrile: ammoniumbicarbonate (50% v/v:50 mM) and finally 250 μl acetonitrile: ammoniumbicarbonate (50% v/v:10 mM), both for 30 min. After washing procedures, gel plugs were dried by vacuum centrifugation and digested for 24 h at 37 °C using 0.1 μg activated trypsin (sequencing grade modified trypsin, Promega Corporation, Madison, WI, USA) per 15 mm^3 gel sizes and covered with 20 μl ammonium hydrogen carbonate. The samples containing the trypsin-digested proteins were mixed at a 1:1 ratio with a solution of water: acetonitrile:TFA (67:33:0.1) saturated with α-cyano-cinnamic acid.

Peptide mass fingerprinting / mass spectrometry

Mass spectra measurements were obtained with a Biflex III matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF/MS, Bruker Daltonics, Bremen, Germany). All measurements were done under following conditions: ionization with a nitrogen laser at 337 nm, 3 Hz repetition rate, positive reflector mode at accelerating potential of 20 000 kV, delayed extraction, output signal digitalized at rates of 1 GHz, internal calibration against peaks of known peptides (AngioII (M + H) + mono at 1046.54 Da, P mono at 1347.74 Da, ACTH (1-17)(M + H) + mono at 2093.09 Da).

The search for PMF matches was performed in the different databases using the following parameters: taxonomy:all entries; enzyme:trypsin; missed cleavages:1; peptide tolerance: 0.08%; mass values: MH^+ and monoisotopic.

Protein annotations / online resources / databases

The peptide mass fingerprints were compared to the *M. truncatula* EST database (Medicao truncatula Gene Index, MtGI, TIGR) (http://www.tigr.org/tdb/mtgi). In case a PMF matched an EST of the MtGI, the EST sequence was used for protein annotation by BlastP search of the *in silico* translation products. If no EST-matches were found in the MtGI, PMF matches were also searched in two alternative databases: SWISS-PROT (http://www.ebi.ac.uk/swissprot/) and TrEMBL (http://www.ebi.ac.uk/trembl/). All BLAST analyses were done at the US National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Theoretical peptide mass and p*I* was determined at Expasy (http://www.expasy.org/tools/pi_tool) to confirm that the MW and the pI match that of the respective protein excised from the gel.

Results

To identify differences between protein profiles of non-infected and *A. euteiches*-infected *M. truncatula* roots, a time course of the *M. truncatula–A. euteiches* interaction was analysed by 2-D gel electrophoresis. For this purpose plants were grown and inoculated with the pathogen in three biological control experiments. After inoculation of the seedlings with *A. euteiches* zoospores, symptoms as described previously (Nyamsuren *et al*., 2003) were observed: (i) light-brown root discoloration appeared on infected plant roots; (ii) after 10 days, root fresh weights of infected plants as compared to non-inoculated plantlets were about 50% decreased; and (iii) oospores and hyphae of the pathogen were detectable in infected roots from day three on after inoculation.

For 2-D gel electrophoresis, total protein of *A. euteiches* infected or non-infected roots from *M. truncatula* was extracted at following time points: 6 h, 1, 3, 7, 14 and 21 days post inoculation. Protein samples of the infection time course were separated by 2-D gel electrophoresis. To verify results, three independent sets of plants were used as biological controls. As technical controls, all protein preparations and subsequent 2-D electrophoreses were repeated 4 times. These biological and technical controls revealed a very high degree of reproducibility, differences in protein abundance could be verified in biological and technical control of each timepoint analysed (data not shown).

About 500 prominent protein spots could be resolved in a p*I*-range of 3–10 and a molecular mass range between 10 and 100 kDa. First differences in protein profiles of non-infected and in-

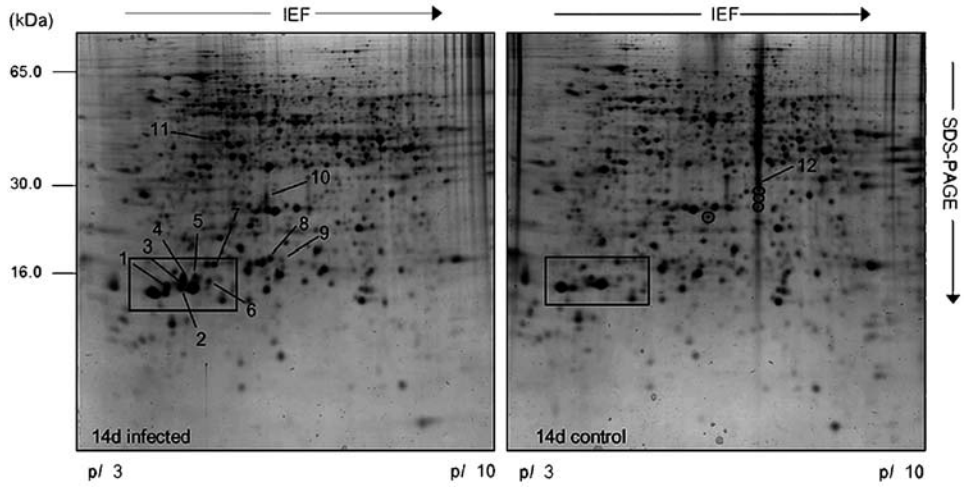

Figure 1. The 2-D resolution of the *M. truncatula* root proteome infected/non-infected with *A. euteiches*, 14 days after inoculation. Differentially expressed proteins that were identified in this study are labelled with arrows and numbered. Four proteins of the control gel which were detected as differentially expressed but could not identified as yet, are marked with circles.

fected roots were detectable at 6 h or 1 d after infection and all observed differences in protein profiles could also be detected in corresponding profiles of subsequent time-points. Proteins which showed different spot intensities in proteome maps of infected roots as compared to control roots were considered to be differentially expressed. Figure 1 shows 2-D maps of non-infected and infected roots at 14 days after inoculation, differentially expressed protein spots are indicated. In response to the *A. euteiches* infection, 16 differentially expressed proteins were detectable during all stages of infection analysed (indicated by arrows, respectively circles in Figure 1), whereof 5 seem to be repressed and 11 proteins show stronger signals in infected roots.

Identification of differentially expressed proteins

To identify the differentially expressed proteins, all 16 differentially expressed protein spots were excised from the gels and analysed by MALDI-TOF mass spectrometry after tryptic in-gel digestion. Comparison of the obtained Peptide Mass Fingerprints (PMFs) was carried out with predicted PMFs of translated sequences of the MtGI at The Institute for Genome Research (TIGR, www.tigr.org). If no matches were found within the MtGI, alternative databases for other plants were used. These searches allowed the identification of 12 of the 16 differentially expressed proteins (Table 1). In case of successful protein identification, more than four peptides matches were found for each PMF. Six of the proteins were either identified as a *pi*49-like protein, pathogenesis-related proteins of class 10 (PR10)-like, or abscisic acid-responsive proteins *ABR*17-like. Despite their different annotations these proteins show a high sequence similarity to each other and belong all to the PR10-like family. For this reason, all these proteins are designed as PR10-like proteins in this work. The remaining upregulated proteins showed similarities to two 18.2 kDA class I heat shock-like proteins (18.2 class I *HSPs*), a prolin-rich protein, a glycine-rich protein, and one isoliquiritigenin 2-0-methyltransferase. A cold acclimation-specific protein *CAS*18 or dehydrin-like proteins could be detected as down-regulated.

Comparison of protein abundance

For a detailed analysis of the protein spots with respect to expression level quantification and the time course of regulation, all 2-D gels were densitometrically analysed.

For this purpose, expression values of the proteins were quantified by their absorption units. A gel to gel normalization was carried out by measuring the absorption units of one protein spot which was apparent with nearly identical signal intensities on each gel. Polypeptides were consid-

Table 1. In response to *A. euteiches* infection differentially expressed proteins of the *M. truncatula* root proteome.

Spot no.[1]	Expression profile[2]	MtGI TC/EST[3]	Best matching gene product[4]	EST sequence coverage[5] (%)	pI/MW[6]	Accession number[7]	Species[8]
1	+ + +	TC 39287	Disease-resistance-response protein pi 49	66	4.76/16.68	P14710	*P. sativum*
2	+ +	TC 31868	ABA-responsive protein ABR17	49	4.87/16.62	Q06931	*P. sativum*
3	+	TC 31872	ABA-responsive protein ABR17	52	4.83/16.90	Q06931	*P. sativum*
4	+	TC 31872	ABA-responsive protein ABR17	54	4.83/16.90	Q06931	*P. sativum*
5	+ +	TC 31869	ABA-responsive protein ABR17	53	4.95/16.48	Q06931	*P. sativum*
6	+ +	TC 29943	Pathogenesis-related protein class 10 [PR10]	68	5.01/17.30	P93333	*M. truncatula*
7	+ +	TC 36563	18.2 kDa class I heat shock protein	84	5.03/18.17	27880	*M. sativa*
8	+ + +	TC 28623	18.2 kDa class I heat shock protein	75	5.81/18.35	27880	*M. sativa*
9	+ +	TC 31880	Proline-rich protein	54	6.15/18.23	Q40376	*M. truncatula*
10	+ +	BG 648786 EST 10405	Partially similar to: glycine-rich cell wall structural protein	86	5.41/23.98	P10495	*Phaseolus vulgaris*
11	+ +	TC 33591	Isoliquiritigenin 2-O-methyltransferase	62	4.90/41.14	P93324	*M. sativa*
12	–	TC39644	Cold acclimation-specific protein CAS18/Dehydrin-like protein	66	6.02/32.24	Q40331	*M. falcata*
				65		Q945Q7	*M. sativa*

[1]Spot numbers correspond to the numbers given in Figure 1.
[2]Expression profile: + + +: induced, no expression in non-infected roots; + +: clearly up-regulated, +: increased expression, less than 2-fold induced; –: down-regulated.
[3]Best matching sequence (TC, EST) of the MtGI.
[4]Protein annotation based on BLASTX search.
[5]TC/EST sequence coverage.
[6]Predicted molecular mass and pI of the protein.
[7]Protein accession number.
[8]Organism of origin.

ered to be induced, when the corresponding spots were apparent in gels of infected tissue but not in gels of corresponding control tissues (spot absorption not higher than background absorption) (Figure 2). Using these restrictions, protein spots 1 and 8 were found to be induced, these two proteins could not be detected in control gels at any timepoint analysed.

If protein abundance was increased over all time-points by at least factor 2 between pathogen treated and control plants, polypeptides were considered to be increased in infected roots. Using these criteria, the abundance of proteins 2, 5, 6, 7, 9, 10 and 11 is increased in *A. euteiches* infected roots. In contrast, the induction of proteins represented by numbers 3 and 4 was only in the range of 1.7 at 14 and 21 days after infection. But at earlier time-points, these two proteins showed a higher induction level and expression levels in non-infected roots increased at 14 and 21 days after inoculation. Protein spot 12 was found to be down-regulated by factor 2.48 at 14 days after inoculation in pathogen treated roots in comparison to control roots.

Changes in expression of all identified proteins became detectable at day 1 after inoculation with

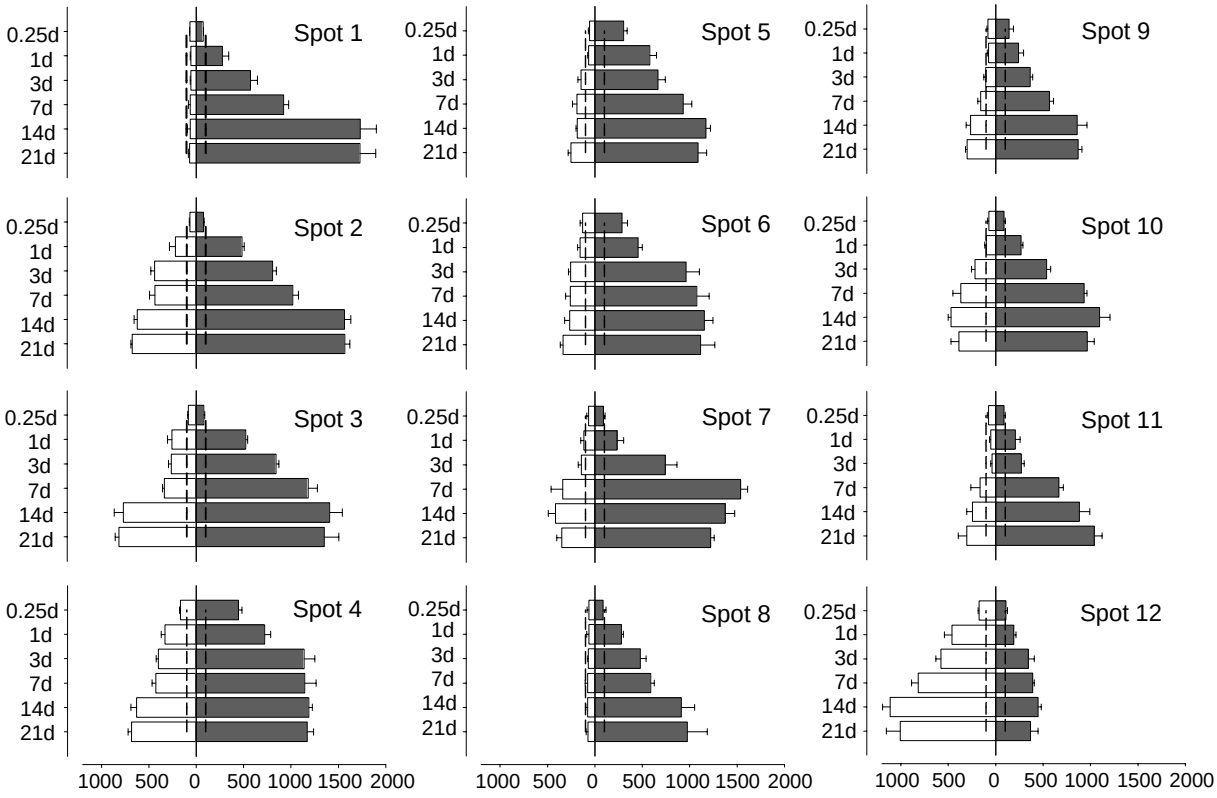

Figure 2. Abundance of proteins 1–12 expressed differentially after infection with *A. euteiches*. Time course of changes in protein abundance for proteins 1–12 after infection of *M. truncatula* roots with *A. euteiches*. Protein abundance was quantified by densitometric units, LAU. Grey columns represent the expression values in infected tissue, white columns show the expression in control roots, and protruding lines indicate standard deviations of 5 measurements. Dotted lines indicate an expression of LAU 100, which corresponds to allocated reference values obtained for blank gel sections without protein content.

A. euteiches, except for proteins 4, 5 and 6, which were induced already after 6 h (0.25 day). During prolonged infection, the expression level increased to values between 870 light absorption units (LAU) (spot 9) and 1733 LAU (spot 1) at maximum until 14 days after inoculation, while spot 7 showed highest expression already after 7 days. For the majority of analysed protein spots, a minor decrease in expression between 14 and 21 days was detectable.

2-D root proteome of plants treated with drought stress or exogenous ABA application

Seven of the twelve characterized differentially expressed proteins were located within a limited area of the proteome maps, ranging between 16 and 18 kDa and pH 4.7–5.0 (boxed in Figure 1). From these seven differentially expressed proteins of this area, six belonged to the PR10-like family. The pathogen-induced members of the PR10-family which are all located in this area show high sequence similarities to each other (identities on amino acid level are between 46 and 91%) (Figure 3) and to previously described ABA-responsive proteins of other plants. To investigate the regulation of these proteins by exogenous ABA or by drought stress conditions, further 2-D electrophoresis analyses were carried out. Plants were cultivated under drought stress condition, a major actuator of ABA- responsive protein expression (Timothy *et al.*, 1989). To prove, whether the protein expression can be induced by exogenous ABA application, plants were treated with 75 μM ABA as described by Luo *et al.* (1992), capable to induce signals on a molecular level. Root fresh weights of plants of all four treatments are shown in Figure 4. Drought stressed, *A. euteiches*-infected and ABA-treated plants showed similar levels of decreased

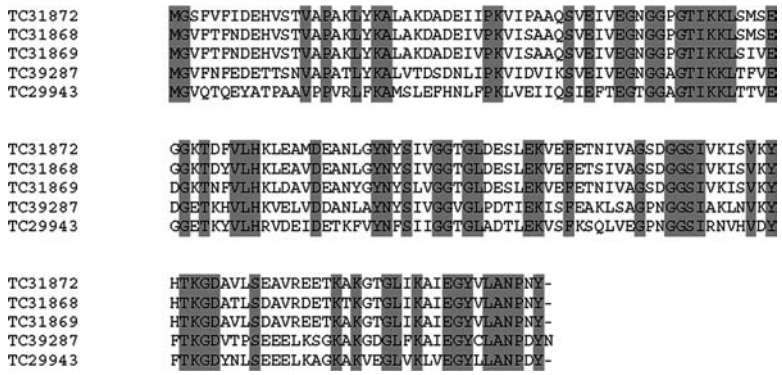

Figure 3. Alignment of deduced amino acid sequences of identified proteins number 1–6. The MtGI-IDs of the corresponding amino-acid sequences are indicated.

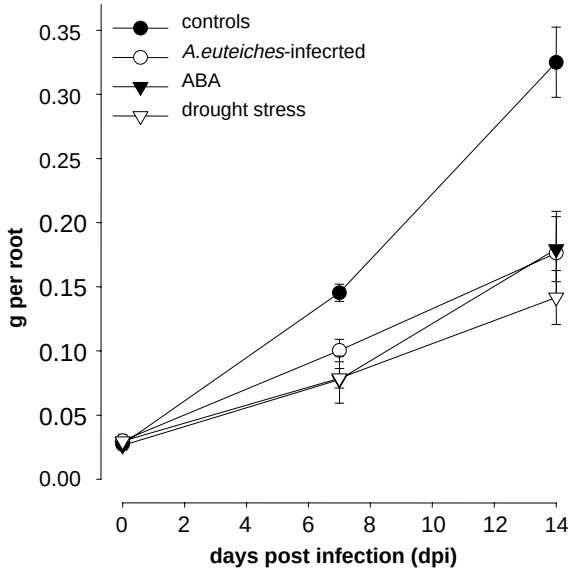

Figure 4. Fresh weight development of roots from control plants, *A. euteiches* infected plants, ABA-treated plants and drought stressed plants.

weight accumulation in comparison to control plants. 2-D analyses of drought stressed and ABA-treated plants were carried out and carefully compared in the gel areas containing the PR10-like proteins, which have been shown to be induced after *A. euteiches* infection. All 2-D analyses of drought stressed and ABA-treated plants were repeated four times for each time-point as it was the case for previous protein profiles.

A complex regulation pattern was identified for these PR10-like proteins during the different conditions analysed (Figure 5). The protein represented by spot 1 which clearly was induced in pathogen infected roots, could neither be induced by ABA nor by drought stress. The expression of the PR10-like proteins represented by spots 4 and 5 that are up-regulated in *A. euteiches* infected roots could also not be induced by either of the two treatments. Hence, the induction or regulation of proteins represented by spot numbers 1, 4 and 5 in pathogen-infected roots can be regarded as specific with respect to the two additional conditions analysed.

The expression of the PR10-like proteins represented by spots 2 and 3 was nearly 2-fold increased in infected roots compared to non-infected control roots. The expression of these proteins was also slightly increased in roots of ABA-treated plants, as compared to non-infected control plants, but expression of these proteins seems to be clearly repressed by drought stress. The protein represented by spot 6, upregulated in infected roots, is also slightly upregulated by ABA but not in drought-stressed roots. This means 3 of the analysed *A. euteiches* induced PR10-like proteins are regulated by exogenous ABA-application but none of them is induced after drought stress. In contrast, 2 of these proteins are significantly down-regulated by drought stress.

Discussion

The root proteome of the model legume *M. truncatula* has been analyzed in previous studies (Mathesius *et al.*, 2001; Bestel-Corre *et al.*, 2002; Watson *et al.*, 2003) and the proteome maps obtained in the here presented work are coincident with these earlier published maps: after Coomassie

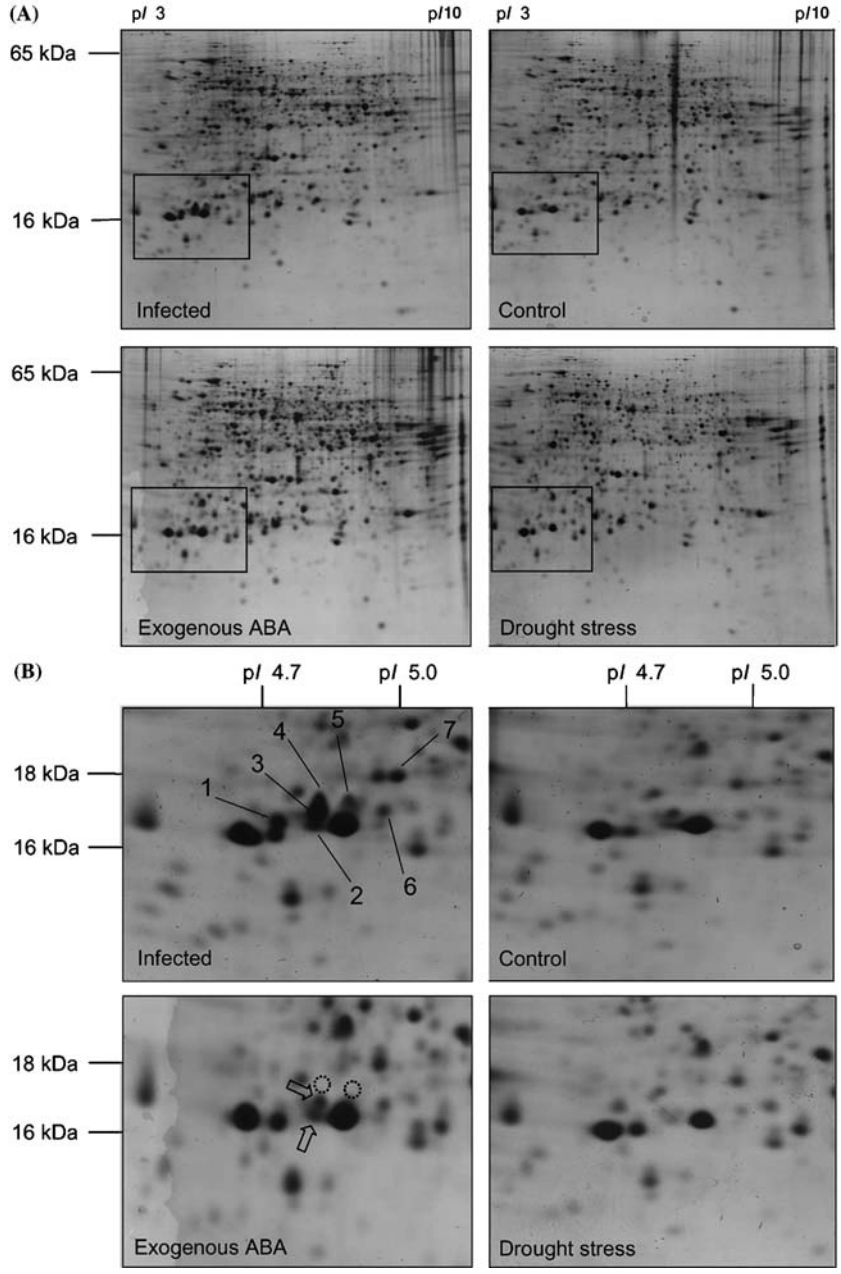

Figure 5. (A) Entire maps, and (B) detailed maps (molecular mass range of 16–18 kDa, p*I*-range of pH 4.7–5.0, boxed in (A) of Coomassie stained 2-D protein profiles of infected and non-infected *M. truncatula* roots after 14 days of infection (infected, control), roots treated with 75 μM exogenous ABA (exogenous ABA) and roots of plants which were grown under drought stress conditions (drought stress). Differentially expressed proteins are numbered and labelled with arrows. Protein spots (spots 2–5), which were apparent in the gels after ABA treatment, are marked with arrows, those, which were not detectable, are labelled with dashed circles at the correspondent gel position.

staining, about 500 well resolved protein spots were detectable. In comparison to transcriptomic approaches where more than 5000 different cDNA sequences of the *M. truncatula*-root transcriptome could be identified in one single experiment (Journet *et al*., 2002), the number of 500 proteins which were detectable on the root proteome maps appears to be rather small. This phenomenon is

apparently due to technical limitations of the experimental system used in this study: (i) current protocols for protein extraction from root tissues do not allow the isolation of proteins with inappropriate biophysical properties; e.g. hydrophobic proteins are often lost during extraction procedures due to their low solubility; (ii) the molecular mass- and p*I*- range used for 2-D separation further limits the number of proteins detectable by the classical 2-D gel electrophoresis and (iii) proteins of low abundance often do not show up on the 2-D gels. Methods for analyzing larger amounts of proteins as MudPIT (multidimensional protein identification technology) have already been successfully applied for plant systems (Koller *et al*., 2002), but a quantification of expression levels is not possible with this technology. Hence, to obtain first information on root proteins which are differentially expressed in response to *A. euteiches*, the classical 2-D gel electrophoresis followed by mass spectrometry has been applied.

Of the 500 protein spots detectable on the 2-D gels, 16 were differentially expressed in response to the pathogen. Twelve of these spots could be assigned to cDNA-sequences after comparison of their PMFs against translated sequences of the MtGI. This resulted in an identification of six different members of the PR10-like protein family, two heat shock proteins, two cell wall proteins, one isoliquiritigenin 2-0-methyltransferase and one protein with similarities to the cold acclimation-specific protein CAS18. The latter protein was found to be downregulated in infected roots, the remaining proteins showed an increased abundance in infected roots. It seems rather surprising, that no proteins of the pathogen *A. euteiches* have been detected. Reference 2-D maps of proteins deriving from *A. euteiches* mycelium grown in liquid culture have been generated and revealed around 450 detectable proteins of the oomycete (data not shown), but *A. euteiches*-proteins have not been detected in the protein maps of infected roots. This phenomenon might reflect the fact that only a restricted number of root cells of a plant are infected by the pathogen. Furthermore, *A. euteiches* shows a restricted growth with a very short metabolic active phase after root colonization (Kjoller and Rosendahl, 1998), which could also be the reason that *A. euteiches* proteins could not be detected in the protein maps.

Within the group of proteins showing an increased abundance in infected roots, two proteins showed similarities to class I heat-shock proteins. The expression pattern of these HSPs in infected roots presumably points out a function in repair and degradation processes during stress-specific plant cell responses (Györgyey *et al*., 1991). Two putative cell wall proteins, which might play a role during architectural modifications after invasion of pathogenic microorganisms in the root tissue, were also found to show an increased abundance in pathogen infected roots. Another induced protein showed similarities to the isoliquiritigenin 2-O-methyltransferase (chalcon OMT), a key enzyme during flavonoid biosynthesis.

The majority of proteins with increased expression in infected roots showed similarities to members of the PR10-family. The precise function of PR10-proteins, which are structurally related to ribonucleases, remains unclear. It was supposed that some PR10-family members are capable of cleaving foreign RNA molecules after pathogen challenge (van Loon and van Strien, 1999). Within the family of PR10-proteins, some members have been shown to be induced by ABA application (Iturriaga *et al*., 1994). This might suggest that ABA is involved in the observed expression regulation of the various members of the PR10-family found in this study. ABA plays a cardinal role in major physiological processes such as (i) embryo morphogenesis and development of seeds; (ii) seed dormancy and germination; (iii) adaptive responses to adverse environmental conditions, mainly water balance and supply; and (iv) plant defence from invading pathogens. One prominent example for the latter ABA-function are tomato mutants with reduced ABA-levels which are much more resistant to *Botrytis cinerea* than wild type plants (Audenaert *et al*., 2002).

Plant proteins designated as ABA- and environmental stress-inducible proteins can be grouped according to their regulation pattern into three major classes: (i) a protein-set inducible by exposure to stress challenge such as pathogen invasion and also by increasing ABA levels; (ii) a protein-set inducible specifically by stress challenge but not by ABA; and (iii) a protein-set inducible by ABA but not by any stress challenge (Luo *et al*., 1992; Rock, 2000). In stressed vegetative tissues, ABA levels rise up to 40-fold within a few hours (Zeevaart, 1999),

therefore the observed protein inductions could be due to increased ABA as a general stress response in pathogen-infected root tissues. For this reason, further 2D-PAGE experiments were carried out using proteins from drought-stressed plants or from plants which were treated with 75 μM ABA in order to investigate whether the observed changes in PR10-expression due to increased ABA-levels or general stress responses. Luo *et al*. (1992) reported that an exogenous exposure of *Medicago sativa* seedlings to ABA at a concentration of 75 μM rapidly induced ABA- and stress-inducible genes. Our results obtained from 2D-PAGE experiments from drought-stresses plants or from ABA-treated plants indicate that only some of the identified PR10-like proteins are inducible by exogenous ABA, but none of the observed protein inductions were observed on roots of drought-stressed plants. In contrast, the expression of two PR10-like proteins was clearly decreased in drought-stressed roots. This result suggests that the induction of several PR10 proteins in *A. euteiches* is not part of the general stress response.

Recently, transcriptional profiling has been carried out in order to identify cDNA sequences which show an increased RNA accumulation in pathogen infected roots (Nyamsuren *et al*., 2003). Consistently to the here presented proteomic approach, a number of sequences coding for PR10- or ABA-responsive proteins have been found to be also strongly induced on transcriptional level.

As mentioned before, suggestions about the function of PR10-proteins are up to now very speculative. The complex regulation pattern of six highly homologous PR10-proteins shown here suggests that these proteins are involved in adaptation processes in response to changing environmental conditions. Moreover, the finding that PR10 proteins are among the strongest expressed proteins in *M. truncatula* root tissue and their complex regulation indicates that this class of proteins plays a key role in root adaptation to different stresses, and further experiments have to be done to understand the biological function of PR10 proteins.

Acknowledgements

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Title:

Comparison of root proteome profiles of different *Medicago truncatula* lines and ABA-treated plants indicates proteins involved in susceptibility and resistance to *Aphanomyces euteiches*

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Abbreviations:

ABA: abscisic acid; dpi: days post infection; ALP: alkaline phosphatase; BLAST: Basic Local Alignment Search Tool; dpi: days post inoculation; CBB: Coomassie Brilliant Blue; EST: expressed sequence tag; ExPASy: Expert Protein Analysis System; IEF: isoelectric focussing; LAU: Light Absorption Units; MALDI-TOF/MS: Matrix-assisted laser desorption/ionization-time of flight/mass spectrometry; MtGI: *Medicago truncatula* Gene Index; PMF: peptide mass fingerprinting; PR proteins: Pathogenesis Related proteins; TC: Tentative consensus sequence; TIGR: The Institute for Genome Research; TrEMBL: Translated EMBL (European Molecular Biology Laboratory) nucleotide sequences; 2-D: two-dimensional.

Keywords: *ABA, Aphanomyces euteiches, Medicago truncatula, PR10, Proteomics*

Abstract

The parasitic interaction formed between the oomycete *Aphanomyces euteiches* and the model legume *Medicago truncatula* was analyzed by a comparative proteomic approach. Investigations on the disease development revealed two *M. truncatula* lines of the French CORE collection, F83.005-5 (R2002) and F83.005-9 (R2002), which showed either increased or decreased susceptibility to *A. euteiches* as compared to the widely adopted line A17. Several proteins were identified by two-dimensional gel electrophoresis to be differentially induced after pathogen challenge in the two *M. truncatula* accessions with altered disease susceptibility. Abundance levels of proteins that show increased signals in infected roots of the susceptible line F83.005-5 as compared to A17 correlate to increased infection levels. In contrast, proteins showing enhanced signals in the more resistant line F83.005-9 could be involved in mechanisms that lead to an improved disease resistance. Among these proteins were two proteasome alpha subunits, which might be involved in a specific defence response. Beside the analysis of different *M. truncatula* lines, the role of abscisic acid (ABA) in the development of the *A. euteiches* root infection was studied. Exogenous application of ABA prior to root infection resulted in an increase of susceptibility of the A17 line. Evaluation of the abundance levels of a group of ABA-responsive proteins of the Pathogenesis Related class 10 (PR-10) like group, which were previously identified to be regulated after *A. euteiches* infection, revealed that the abundance of these proteins is clearly enhanced in the susceptible line as well as in roots of the line A17, which were treated with ABA prior infection.

Introduction

In the recent years, approaches combined under the general term *proteomics* emerged as a powerful tool to analyse the protein status of cell types, organelles, tissues, organs or whole organisms (Schmid 2002). In contrast to mRNA expression profiling, proteomic approaches represent in a more direct way the cellular status as it monitors the actual protein composition of cells of a certain tissue, which are directly influenced by biochemical cellular pathways (Gygi *et al.* 1999). In the last few years, rapidly expanding plant genomic data and expressed sequence tag (EST) databases provide prerequisites for straight-forward identification of plant proteins. Beside the widely used model plant *Arabidopsis thaliana*, several other plant models are currently established to address specific questions in plant biology. The family of legumes is one of the most important groups of plants worldwide, as an important source of protein and because of their capacity to symbiotically fix nitrogen. In response to the need for a relatively simple genetic system in legumes, *Medicago truncatula* was selected as a model species for legume biology (Cook 1999; VandenBosch 2003). Unlike the major crop legumes, *Medicago truncatula* shows suitable features for efficient molecular and genetic analyses. First proteome reference maps of different tissues of *Medicago truncatula* were published recently (Mathesius *et al.* 2001; Watson *et al.* 2003) and symbiosis-related proteins induced by mycorrhizal fungi *Glomus mosseae* and Rhizobia bacteria *Sinorhizobium meliloti* were detected and monitored (Bestel-Corre *et al.* 2002; Djordjevic *et al.* 2003).

In this study, the model legume *Medicago truncatula* was used to analyse the parasitic plant-pathogen interaction formed between legumes and the oomycete *Aphanomyces euteiches* (Drechs.). The *Aphanomyces* root rot caused by this pathogen appears in several legumes of economic importance including pea, where it is considered to be the most destructive disease worldwide (Hagedorn 1989; Levenfors *et al.* 2003). Typical disease symptoms are a watery rotting of root tissue, honey-brown coloured lesions spreading through the root cortex, a significant reduction of root mass and the appearance of thick-walled oospores, which serve as a resting stage in the soil and as primary inoculums source. Due to a lack of knowledge about pathogen variability and mechanisms associated with the *in-planta* expression of partial resistance and the polygenic inheritance of resistance, pea cultivars with acceptable levels of resistance against *A. euteiches* are up to now not available (Pilet-Nayel *et al.* 2004). Although the *A. euteiches*-induced legume root rot is of great economic impact, only very few studies on the molecular background of this disease have been undertaken so far.

For *Medicago truncatula*, a large scale of different ecotypes has been described and genetically characterized (Jenczewski *et al.* 1997; Prospéri 2000; Prospéri *et al.* 2001) originating from different Mediterranean habitats and thus presenting a wide adaptation to different conditions. This high biological variability associated with the development of genomic and genetic data allowed *M. truncatula* to be a suitable model to analyse legume-pathogen interactions (Torregrosa *et al.* 2004). Very recently, seven *M. truncatula* accessions with different resistance levels to *A. euteiches* similar to a resistant alfalfa (*M. sativa*) population were described, while two accessions were detected to show enhanced susceptibility as the alfalfa cultivar *Saranac* (Vandemark and Grünwald 2004).

Here, we present a comparative proteomic analysis of the interaction formed between three different *M. truncatula* lines, the *M. truncatula* A17 and the two French lines F83.005-5 (R2002) and F83.005-9 (R2002) (Prospéri, J.-M., Montpellier, France), and the oomycete *A. euteiches* strain ATCC 201684 (Rosendahl, S., Copenhagen, Denmark). The objective of this study was to evaluate the resistance level of these lines to *A. euteiches* under pot culture conditions. Subsequent proteomic profiling was carried out in order to link the observed differential responses of these *M. truncatula* accessions to detected differences in root proteome profiles.

Results

Evaluation of A. euteiches infection in roots of M. truncatula A17, F83.005-5 (R2002) and F83.005-9 (R2002)

Screening of different *M. truncatula* accessions from the French CORE collection (Prospéri, J. M., INRA-SGAP laboratory, Montpellier, France; Prospéri 2000) using *in vitro* conditions led to the identification of two lines (F83.005-5 and F83.005-9) displaying differential responses following *Aphanomyces euteiches* inoculation (C. Jacquet, unpublished results). To assess their susceptibility and/or resistance levels to the pathogen under pot culture conditions, root fresh weights and development of the pathogen in the host tissue after infection were compared. For the different *M. truncatula* lines (A17, F83.005-5 and F83.005-9) assessed here, an inoculation solution of 0.5 x 10^6 zoospores was applied. This inoculum induced a moderately aggressive infection and allowed a comparison of disease levels between the three lines.

After inoculation of *M. truncatula* seedlings, typical disease symptoms (yellow-brown discolorations or lesions in the root cortex) were observed on roots of the three different *M. truncatula* lines (not shown). To assess the infection rate, two biological repetitions were accomplished. For evaluation of the infection grade, root fresh weights of non-infected and infected plants were compared (Fig. 1, A). Starting from ten days post inoculation until the maximum of infection reached 15 dpi, the three lines displayed clear differences concerning the root development after infection. The *M. truncatula* F83.005-5 line shows the highest reduction of fresh weight in infected roots (maximal ratio: 2.6) while the fresh weight of infected roots in the F83.005-9 line is only 1.75 fold decreased. Regarding this parameter, the *M. truncatula* line A17 maintains an intermediate position between the ecotypes F83.005-9 and F83.005-5.

In-planta histochemical staining of the infected roots was performed in order to determine the percentage of host tissue colonized with alkaline phosphatase (ALP) - active *A. euteiches* hyphaes and the appearance of oospores (Fig. 1, B and C). ALP- active hyphae could be microscopically visualized as black structures on root surfaces and in-between the cortex cells. As documented previously, maximal infection rate of *M. truncatula* with *A. euteiches* is obtained at 15 dpi (Nyamsuren *et al.* 2003; Colditz *et al.* 2004). At this time point, 79% root area of the ecotype F83.005-5 were colonized, whereas only 64% root area of the cultivar A17 and 44% of the ecotype F83.005-9 were colonized. Similar results could be obtained for the presence of *A. euteiches* oospores. These data suggest that the F83.005-9 ecotype has a higher

resistance capability against an infection with *A. euteiches*, while the F83.005-5 ecotype is supposed to be more susceptible to *A. euteiches*.

2-D root proteome maps of infected M. truncatula F83.005-5 and F83.005-9

To identify differences in protein profiles of roots of the three *M. truncatula* lines, root proteome maps of non-infected and infected roots were carried out using 2-D gel electrophoresis. After phenolic protein extraction, about 500 – 700 prominent protein spots could be resolved on 2-D gels in the molecular mass range of 10 to 100 kDa and the IEF range of p*I* 3 to 10. For comparison of proteome profiles, the gels of at least two biological repetitions and six technical controls were consulted. All observed differences in protein profiles and spot intensities mentioned subsequently are the result of the comparison of proteome maps of infected root tissue between the different *M. truncatula* lines, as well as to maps of non-infected control roots. All noted differences were reproducible on every 2-D map carried out. Minor distinctions in protein pattern based on single, non-reproducible events were not further investigated.

Figure 2 shows 2-D maps of non-infected control roots and infected roots of *M. truncatula* F83.005-5 and F83.005-9 at 15 days post inoculation. Differentially displayed protein spots are indicated on gels of infected roots. Among these induced proteins, spots 1 to 6 (framed in a box in Fig. 2) are encoding PR-10 like proteins, which were described previously to be induced upon *A. euteiches* infection (Colditz *et al.* 2004).

Protein spots of the F83.005-5 and F83.005-9 ecotypes, which showed differential abundance levels, are labelled in Figure 2 by R1-R6 and S1-S8. These differences in profiles and spot intensities were manifested in comparison to the other *M. truncatula* line, and all these proteins were found to be up-regulated or *de novo* induced versus proteome maps of control tissue. Maps of the more resistant ecotype F83.005-9 revealed six protein spots differentially regulated after infection with *A. euteiches* (R1-R6) and maps of the more susceptible F83.005-5 line indicated 8 differentially regulated spots (S1-S8).

Identification of differentially regulated proteins by peptide mass fingerprinting (PMF)

For identification of proteins showing differential patterns in gels of infected root tissue of the two ecotypes F83.005-5 and F83.005-9, protein spots shown in Figure 2 were excised from gels and tryptic digested for subsequent MALDI-TOF mass spectrometry analysis. The resulting Peptide Mass Fingerprints (PMFs) were matched confidently against the *M. truncatula* EST sequence database (*M. truncatula* gene Index (MtGI) at www.tigr.org). In the

case that no matches were found in this database, alternative databases for other plants were consulted.

PMF analysis allowed the identification of fourteen proteins, listed in Table 1. All proteins were found on the bases of >5 matching peptides, peptide coverage was always >39% and predicted and observed MWs and p*I*s were nearly identical. The six differentially abundant proteins (R1-R6) detected in the F83.005-9 line were identified as a plasma membrane intrinsic polypeptide, an alpha-soluble NSF attachment protein (Alpha-SNAP2), two proteasome alpha subunits (R3, R4), the alpha 6 subunit (PAF1) and the alpha type 4 subunit C (PSA4), an Initiation factor 5A-2 (elF-5A 2) and a cytochrome-b5 reductase-like protein. Among the eight proteins (S1-S8) found to be induced in the F83.005-5 line were three enzymes involved in the phenylpropanoid pathway (S1-S3), including an isoliquiritigenin 2-O-methyltransferase (cOMT), which previously was found to be regulated upon the infection of A17 with *A. euteiches* (Colditz *et al.* 2004). Furthermore five enzymes of primary metabolisms were identified (S4-S8), two alcohol dehydrogenase 1–like proteins (ADH1) and three cytoplasmic isozymes of a fructose-bisphosphate aldolase 2 (ALF2) (from Pea).

Comparison of abundance of fourteen proteins regulated in the two M. truncatula lines with altered A. euteiches-susceptibility and -resistance

A set of proteins (R1-R6 and S1-S8) was found to be differentially regulated in roots of the two *M. truncatula* lines after infection with *A. euteiches*. For a quantitative analysis of protein regulation upon infection with *A. euteiches*, protein patterns on all 2-D gels were densitometrically analysed. The intensities of protein spots were evaluated by their light absorption units (LAU) after subtraction of nearby background absorption. Additionally, a gel to gel normalization was carried out by adjusting the LAU values of one reference protein spot which showed nearly identical signals on each gel analysed (data not shown). Generally, protein spots were declared to be *de novo* induced in gels of infected tissue, when the measured LAU values for the corresponding area in the control gels were not significantly higher than 100 LAU as obtained as average value for background areas without protein content. Protein spots, which showed at least 2-fold increased LAU values in comparison to the corresponding spots in control gels (LAU >150), were defined to be up-regulated.

Figure 3 displays the light absorption units (LAU) for the induced proteins R1 to R6 and S1 to S8 of the F83.005-9 line and F83.005-5 line after infection with *A. euteiches* in comparison to the A17 line (infected) and to averaged control values. Based on these measurements, regulation levels as mentioned in Table 1 were obtained. Protein spots R1 to R6 were clearly

induced as compared to the corresponding spots in control gels as well as to those in the F83.005-5 gels. Spot numbers R2, R3, and R4 were found to be strongly induced in comparison to the more susceptible F83.005-5 line as here the corresponding LAU values remained at background level. In comparison to the protein spots of gels of infected A17 tissue, only R4 and R6 remain strongly induced in the F83.005-9 line. The other spots were slightly induced with increased LAU values less then 2-fold and the measured absorption for R5 remained similar in the A17 line.

The majority of protein spots S1 to S8 regulated in the more susceptible F83.005-5 line upon infection were clearly induced in this line as compared to the F83.005-9 line and to the A17 line as well. These spots were also clearly induced as compared to the corresponding spots in control gels of non-infected roots. Only spots S5 and S8 were slightly induced with regard to these spots out of the F83.005-9 line. Compared to the absorption intensities of the protein spots among infected A17 gels, S2-S4 and S6 were found to be clearly induced, S5 and S7 showed slightly increased abundance, while S1 and S8 showed a similar induction level.

Evaluation of A. euteiches infection in roots of M. truncatula cv. A17 pre-incubated with exogenous abscisic acid (ABA)

A set of ABA-responsive proteins of the group of PR-10 like proteins shows clear regulation after root infection in all three *M. truncatula* accessions (Fig. 2) (Colditz *et al.* 2004). Therefore, the influence of exogenous ABA application on the infection progress of *A. euteiches* in roots of *M. truncatula* A17 was further investigated regarding the aspect of an increased susceptibility to the pathogen. Seedlings of the A 17-line were treated with 75µM ABA twice before inoculation and two biological repetitions were carried out. This ABA-concentration was found to induce this protein pattern which is entirely induced upon challenge with *A. euteiches* (Colditz *et al.* 2004).

After exogenous ABA application, the root development was clearly reduced as documented by increased ratios of non-treated to treated root fresh weights and inhibited root length growth. This effect was strengthened when the roots were additionally infected with *A. euteiches* (Fig. 4, A and B). To test if this ABA-concentration has an effect on the pathogen, 75µM ABA was applied to CMA plates of *A. euteiches*. The ABA application showed no effect on the growth and development of the oomycete compared to cultures without ABA treatment (data not shown).

To evaluate the infection level in the ABA-treated roots, *in-planta* histochemical straining was performed. In *M. truncatula* A17 roots, which were treated with exogenous ABA,

increased infection parameters as compared to non-treated roots could be detected (Fig. 4, C and D). The infection was clearly enhanced compared to roots without exogenous ABA treatment, indicating that ABA can increase the susceptibility to *A. euteiches*.

Comparison of protein abundance of the six PR10-like proteins previously described in three M. truncatula lines and in roots after exogenous ABA application

Detailed studies on the abundance levels of the PR-10 protein group were carried out in order to investigate their role in susceptibility to *A. euteiches*. Absorption values (LAU) of these six proteins were measured in roots of the two French *M. truncatula* lines and in roots of A17, which were treated with ABA prior to the inoculation with the pathogen.

Figure 5 (A+B) shows the abundance of the six PR-10 like proteins. For a better comparison, two data sets of the A17 line (A17 control, A17 + ABA) were re-evaluated. The measurements showed that the susceptible line F83.005-5 displays highest abundance levels of these protein pattern (in the gels as well as densitometrically measured absorption values), whereas the abundance levels in the resistant line F83.005-9 are clearly below those measured in the A17 line. Interestingly, A17 plants that were treated with ABA prior to an *A. euteiches* infection, show a similar high level of abundance as the susceptible line F83.005-5. The exogenous application of ABA also caused an increase in the abundance of the proteins 2, 3 and 6, indicating that these proteins are mainly induced by ABA, whereas the induction of spots 1, 4 and 5 in infected roots is probably a consequence of the infection itself rather than of increased ABA levels. Hence, this increase in abundance of this PR-10 protein pattern correlates to the increase in susceptibility that was detected after ABA treatment prior root infection.

Discussion

In the recent years, proteomic tools emerged as a powerful complementation to transcriptomic approaches to study function and regulation of genes and their products. We have accomplished a proteomic approach to characterize the parasitic interaction between *M. truncatula* and the oomycete root pathogen *Aphanomyces euteiches* (Drechs.), which supplements a transcriptomic approach previously realized on this particular interaction (Nyamsuren *et al.* 2003). In contrast to our previous study on the root proteome of the widely adopted *M. truncatula* A17 line (Colditz *et al.* 2004), two *M. truncatula* accessions with altered susceptibility were analyzed concerning their response to an *A. euteiches* infection. In the present study, we investigated the infection development in roots of two French *M. truncatula* ecotypes: the F83.005-9 (R2002) line, which was considered to be more resistant and the F83.005-5 (R2002) line, which was supposed to be more susceptible as compared to A17 in response to an infection with *A. euteiches* after *in vitro* screenings (C. Jacquet, unpublished data). After comparison of root fresh weights, hyphal spreading in the roots and *A. euteiches* oospore development in all three *M. truncatula* accessions, indeed a higher level of susceptibility in the F83.005-5 plants and higher resistance ability in the F83.005-9 plants in comparison to the A 17 line could be confirmed. According to these investigations on root infection level, the *M. truncatula* line A17 maintains an intermediate position with regard to *A. euteiches* susceptibility as compared to the two *M. truncatula* accessions of the French ecotype collection. Recent studies on the susceptibility of different *M. truncatula* accessions to *A. euteiches* also support a moderate susceptibility of *M. truncatula* Jemalong, which is closely related to line A17 (Vandemark and Grünwald 2004). The moderate susceptibility of A17 is also mirrored by the abundance levels of one group of ABA-responsive proteins (ABR17) of the PR-10 class. Members of this gene family were described to be induced in several plant-pathogen interactions (Lo *et al.* 1999; Jwa *et al.* 2001; McGee *et al.* 2001) and a group of these proteins is induced in roots of the A17 line after *A. euteiches* infection (Colditz *et al.* 2004). In this study we could show that their abundance is further increased in the more susceptible line, whereas decreased abundances were found in the line showing higher resistance as compared to the intermediate A17 line.

In order to identify proteins, which might be responsible for the observed differences in *A. euteiches* susceptibility, proteome maps of non-infected and infected roots of all three *M. truncatula* accessions were compared. Upon infection with *A. euteiches*, we were able to

detect 14 proteins differentially regulated in the two French *M. truncatula* ecotypes F83.005-5 (sus.) and F83.005-9 (res.) as represented by spots R1-R6 and S1-S8. These proteins were found to be induced in comparison to non-infected root tissue as well as to infected roots of the other ecotype, respectively. Abundance levels of proteins up-regulated in infected roots of the highly susceptible line correlate to the increased infection levels regarding hyphal spreading and oospore development. Thus, their increased expression might be a consequence of faster disease development. Three of the proteins, which are induced in the more susceptible F83.005-5 line, encoding for an isoliquiritigenin (chalcone) 2-O-methyltransferase (cOMT), an isoflavone reductase homolog (IFR1) and a chalcone reductase (CHR), are enzymes of the phenylpropanoid pathway. In *Medicago spp.*, these enzymes are required for the production of medicarpin, the major phytoalexin accumulated in response to fungal pathogens (Dixon *et al.* 1992; Winkel-Shirley 2001). Hence, the increased infection level obtained in the F83.005-5 line might lead to an increased expression of these medicarpin biosynthesis enzymes in response to the *A. euteiches* infection.

Proteins induced after infection in the resistant line are likely to be involved in plant resistance mechanisms. Among the proteins, which were clearly induced in the more resistant F83.005-9 line, we could identify two proteasome alpha subunits (R3, R4). Proteasomes are cytosolic multi-subunit proteases representing the major enzyme complex for protein degradation by the ATP/ubiquitin-mediated proteolysis pathway. In this pathway, ubiquitin is covalently attached to cellular proteins and these ubiquitinated proteins are targeted for degradation by the proteasome complex (Kim *et al.* 2003). Furthermore, ubiquitylation is involved in regulation of plant disease resistance responses, in the manner that specific ubiquitin-associated proteins serve as signalling components required for disease resistance specification by triggering defence response signal transduction cascades (Peart *et al.* 2002). Their induction is effected by either intracellular or extracellular elicitors and could be confirmed for oomycete elicitors of *Phytophthora infestans* (Peart *et al.* 2002). Hence, the induced expression of these two proteasome subunits in the more resistant *M. truncatula* F83.005-9 line marks a first hint to a developed plant resistance response.

In our previous study on proteomic changes in *M. truncatula* A17 roots, a group of ABA-responsive proteins of the PR-10 group were identified which underlie a complex regulation by the *A. euteiches* infection as well as by exogenous ABA (Colditz *et al.* 2004). Therefore, detailed studies on the role of ABA during root infection by *A. euteiches* were carried out in the here presented work. A pre-incubation with exogenous applied ABA to *M. truncatula* A17

roots before inoculation with *A. euteiches* led to a clearly enhanced root colonization mirrored by decreased root fresh weights and lengths, increased hyphal spreading and oospore formation as compared to roots without ABA treatment. This result is coincident with previous findings that ABA is able to increase plant susceptibility to different pathogens (Audenaert *et al.* 2002; Mohr and Cahill 2003). The absorption values of the six proteins were very similar to those measured in gels of the more susceptible F83.005-5 line (infected), which showed also a similar colonization level with *A. euteiches*. This indicates that the ABA-treatment increases the level of *A. euteiches* susceptibility of the A17 line towards the level, which was determined for the highly susceptible line F83.005-5.

Summarizing our results on the ABA-dependent protein expression and the effect of exogenously applied ABA, we hypothesize that ABA plays a crucial role in susceptibility of *M. truncatula* to the root pathogen *A. euteiches* and influences directly the appearance of the ABA-responsive proteins of the PR-10 group.

Materials and Methods

Plant material

Seeds of *Medicago truncatula c.v. Jemalong* A17 and of the French accessions F83.005-5 (R2002) and F83.005-9 (R2002) (Prospérie, J. M., INRA-SGAP, Montpellier, France) were sterilised by 5 min treatment with concentrated sulphuric acid and 5 min incubation in a 3 % (v/v) Sodium Hypochlorite solution. After surface sterilisation, seeds were washed intensively with distilled water and pre-germinated on water agar plates (1.5% (w/v)) for 3 days in the dark at room temperature. Five seedlings were then transferred in pots (Ø 7.5 cm) containing a 1:2 (v/v) mixture of sterile expanded clay and vermiculite. Plants grew under constant growth conditions in the greenhouse (22 °C, 65 % humidity, 16 h photoperiod at 220µE $m^{-2}s^{-1}$). After three days, seedlings were fertilized with half-strength Hoagland`s solution (Hoagland and Arnon 1950) and fertilization was repeated half-weekly during the experiment. Plants were additionally watered twice a week.

Aphanomyces euteiches cultures and plant inoculation

Aphanomyces euteiches Drechs. strain ATCC 201684 (Rosendahl, S., Department of Mycology, Botanical Institute, University of Copenhagen) was cultured in 20 ml Maltose Peptone broth (MPB) (3g L^{-1} maltose, 1g L^{-1} peptone) for 9 days at 24°C in the dark. For infection of plants, a solution of *A. euteiches* zoospores was necessary. Therefore, zoospore production was induced by washing the mycelium in autoclaved lake water, taken from a lake in the royal gardens of Herrenhausen, Hannover, and incubated over night in autoclaved lake water.

M. truncatula plantlets were inoculated seven days after transplanting by applying 5 ml of the zoospore suspension containing 100.000 zoospores ml^{-1} at the base of the stem. Control plants were mock-inoculated with an equal volume of autoclaved lake water. One day before inoculation, all pots were water saturated.

For a pre-incubation treatment with phytohormone *(±)-cis, trans*- abscisic acid (ABA), an equal volume (5 ml) of half-strength Hoagland´s solution containing dissolved *(±)-cis, trans*-ABA (Sigma Chemicals, Sigma-Aldrich Co., Munich, Germany) at 75 µM final concentration out of an methanol stock solution were poured on the stem base of roots. The ABA-treatment was applied during seven days before the inoculation step with *A. euteiches* zoospores in closed plant pots as two separate application steps at day -7 and day -3 before inoculation.

Harvest of plants and histochemical staining procedure

Inoculated plants and control plants were harvested at the time-point of maximal infection 15dpi. Roots were cut off, washed with deionised water to remove soil particles and the roots fresh weight and length was determined. For protein extraction, root samples were frozen in liquid nitrogen directly after harvest and stored at - 80 °C.

Roots collected from each treatment and plant pot were used for histochemical analyses. For the detection of *A. euteiches* oospores in the root tissue, roots were incubated in a 10% (w/v) KOH solution for half an hour at 90°C, washed once with deionised water and then staining procedure with an solution containing 5% (v/v) ink and 8% (v/v) acetic acid was carried out by an incubation for 20 min in the water bath at 90°C. To detect fungal alkaline phosphatase activity (ALP activity) fresh harvested roots were stained overnight as described by Tisserant *et al.* (1993). Stained roots were cleared in 3% (w/v) NaClO for 5 min.

For evaluation of the infection rate, the percentages of apparent oospores and ALP-active mycelium in the stained roots were determined. Therefore, roots were cut into pieces of 1 cm length and 100 of them were picked out randomly and placed on microscope slides. The appearance of stained oospores and hyphae were counted.

Phenolic protein extraction and sample preparation for IEF

Total root protein was extracted and precipitated according to a modified protocol of Hurkman and Tanaka (1986). 0.5 g of root tissue was ground briefly in liquid nitrogen and homogenized in 750 µl lysis buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCL, 2 mM PMSF and 2 % (v/v) ß-mercaptoethanol, pH 8.0). 750 µl saturated phenol was added and the samples were mixed by shaking at 300 rpm for 30 min. After centrifugation (17.000 g, 3 min, 4 °C), the phenolic phase was removed and mixed with an equal volume of lyses buffer. After another centrifugation step, proteins were precipitated in the phenolic phase at - 20 °C for at least 4 h by adding 5 volumes of 100 mM ammonium acetate in methanol. Total protein was pelleted by centrifugation (17.000 g, 3 min, 4 °C) and washed three times with cold 100 mM ammonium acetate in methanol and once with cold 80 % (v/v) acetone.

The vacuum dried pellet was solubilized in 350 µl rehydration buffer (8 M urea, 2 % (w/v) CHAPS, 100 mM DTT, 0.5 % v/v IPG buffer for correspondent pH-values (pH 3-10NL, Amersham Pharmacia Biotech, Uppsala, Sweden), a trace of bromophenol blue).

For first dimension isoelectric focussing (IEF), 18 cm Immobilised Dry Strips with pH gradients 3-10 non-linear (Amersham Pharmacia Biotech) were rehydrated with 350 µl protein samples in rehydration buffer. Isoelectric focussing was carried out for 24 h using the IPGphor system (Amersham Pharmacia Biotech) with voltages from 30 V up to 8000 V at maximum. Strips were equilibrated for 15 min in equilibration solution I (30% v/v glycerol, 50 mM Tris-HCL pH 8.8, 6 M Urea, 2 % (w/v) SDS, a trace of bromophenol blue, 0.01 g DTT ml^{-1} w/v) and equilibration solution II (same compounds like equilibration solution I, but DTT substituted by 0.025 g iodoacetamide ml^{-1}).

For second dimension poly-acrylamide-gel-electrophoresis (SDS-PAGE), IPG strips were fixed vertically onto SDS-tricine-polyacrylamide gels of 12-14 % acrylamide. SDS-PAGE was carried out for 20 h at 30 mA per mm gel layer. Gels were incubated in a fixing solution (40% v/v methanol, 10% v/v acetate) for at least 2 h and Commassie-stained overnight using 0.1 % Coomassie Brilliant Blue CBB-G250 (Bio Rad, Richmond, CA, USA). After staining procedure, gels were washed intensively with deionised water.

Gel scanning and Determination of protein abundance

Coomassie-stained gels were scanned on an UMAX Power Look III Scanner (UMAX Technologies, Fremont, USA) or on a Fujifilm FLA-3000 Fluorescence Laser Imager (Fujifilm Medical Systems USA, Stamford, CT, USA). To analyse the protein abundance, protein spots of interest were labelled by the AIDA Image Analyser v3.20 Evaluation software (Raytest USA, Wilmington, NC, USA) and their values of Light Absorption Units (LAU, internal densitometric unit) were determined. The absorption units were obtained after subtraction of background absorption, which was determined from 5 different blank gel parts without protein content located close to the valuated protein patterns. All measured values are averages of four technical repetitions, and standard deviations were determined.

Trypsin Digestion

Protein spots were excised manually from the gels and in-gel digested with trypsin according to a protocol of Williams *et al.* (1997). Before the tryptic digestion, gel plugs were treated in three washing steps, initially with 250 µl acetonitrile : water (1:1 v/v) for 5 min, followed by 250 µl acetonitrile : ammoniumbicarbonate (50 % v/v: 50 mM) and finally 250 µl acetonitrile : ammoniumbicarbonate (50 % v/v: 10 mM), both for 30 min. After washing procedures, gel plugs were vacuum-dried and digested for 24 h at 37 °C using 0.1 µg activated trypsin (Sequencing Grade Modified trypsin, Promega Corporation, Madison, WI, USA) per 15 mm^3

gel sizes, covered with 20 µl ammonium hydrogen carbonate. The trypsin-digested protein samples were mixed at a 1:1 ratio with a solution of water : acetonitrile : TFA (67:33:0.1) saturated with α-cyano-cinnamic acid.

Peptide mass fingerprinting/ Mass spectrometry

Mass spectra measurements were obtained with a Biflex III Matrix-assisted laser desorption ionization time-of-flight mass Spectrometer (MALDI-TOF/MS, Bruker Daltonics, Bremen, Germany). All measurements were done under following conditions: Ionization with a nitrogen laser at 337 nm, 3 Hz repetition rate, positive reflector mode at accelerating potential of 20.000 kV, delayed extraction, output signal digitalized at rates of 1 GHz, internal calibration against peaks of known peptides (AngioII (M+H)+mono at 1046.54 Da, P mono at 1347.74 Da, ACTH (1-17)(M+H)+ mono at 2093.09 Da).

The search for PMF matches was performed in the different databases using the following parameters: Taxonomy: all entries; Enzyme: Trypsin; Missed cleavages: 1; Peptide tolerance: 0.08%; Mass values: MH^+ and monoisotopic.

Protein annotations/ online resources/ databases

The peptide mass fingerprints were compared to the *M. truncatula* EST database (Medicao truncatula Gene Index, MtGI, TIGR) (http://www.tigr.org/tdb/mtgi). In case a PMF matched an EST of the MtGI, the EST sequence was used for protein annotation by BlastP search of the *in silico* translation products. If no EST-matches were found in the MtGI, PMF matches were also searched in two alternative databases: SWISS-PROT (http://www.ebi.ac.uk/swissprot/) and TrEMBL (http://www.ebi.ac.uk/trembl/). All BLAST analyses were done at the US National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Theoretical peptide mass and p*I* was determined at EXPASy (http://www.expasy.org/tools/pi_tool) to confirm that the MW and the pI match that of the respective protein excised from the gel.

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Figure Legends:

Figure 1:

Infection development determined for the *M. truncatula* lines A17, F83.005-9 and F83.005-5 during infection with *A. euteiches*, from 0dpi to 15dpi: A) Ratios of root fresh weights (non-infected x infected^{-1}) (5dpi to 15dpi). B) Percentages of infected root tissues after *in-planta* evaluation of alkaline phosphatase activity. C) Percentages of infected root tissues after *in-planta* evaluation of oospores formation. A-C) Error bars indicate standard deviations of different measurements (2 biological repetitions).

Figure 2:

Root proteome maps of two *M. truncatula* accessions, the resistant F83.005-9 (res.) and the susceptible F83.005-5 (sus.) non-infected (control)/infected at 15 dpi (CBB-stained). Differentially induced proteins that were identified by MS are numbered: R1-R6 for the F83.005-9 accession, S1-S8 for the F83.005-5 accession.

Figure 3:

In-Gel Abundance of induced proteins after infection with *A. euteiches* at 15 dpi, determined in the three *M. truncatula* lines A17, F83.005-5 (sus.) and F83.005-9 (res.), displayed next to these of controls (averaged values). Light Absorption Units (LAU) measured for the protein spots R1-R6 and S1-S8 as labelled in *Figure 1*. Lines in-between the bars indicate standard deviations of different measurements (2 biological, 6 technical repetitions).

Figure 4:

Comparison of infection development determined for the *M. truncatula* line A17 during infection with *A. euteiches* (from 0dpi to 15dpi) with A17 plants after ABA pre-application (75µM) and with *A. euteiches* infected A17 plants after ABA pre-application (75µM). A) Ratios of root fresh weights (non-treated x treated^{-1}) (5dpi to 15dpi). B) Single root lengths. C) Percentages of infected root tissues after *in-planta* evaluation of alkaline phosphatase activity. D) Percentages of infected root tissues after *in-planta* evaluation of oospores formation. A-D) Error bars indicate standard deviations of different measurements (2 biological repetitions).

Figure 5:

A) Detailed root proteome maps (M_r -range: 16-18 kDa, p*I*-range 4.7-5.0) of *M. truncatula* after different treatments: A17 controls, F83.005-9 infected with *A. euteiches*, A17 with pre-application of ABA (75µM), A17 infected (spot numbers 1-6 labelled in this map refer to abundance values in *Figure 5B*), A17 with pre-application of ABA (75µM) and infected with *A. euteiches*, F83.005-5 infected, (CBB-stained). B) Corresponding In-Gel Abundance of protein spots 1-6 as labelled in *Figure 5A*, measured as Light Absorption Units (LAU) for all treatments displayed in *Figure 5A*. Lines in-between the bars indicate standard deviations of different measurements (2 biological, 6 technical repetitions).

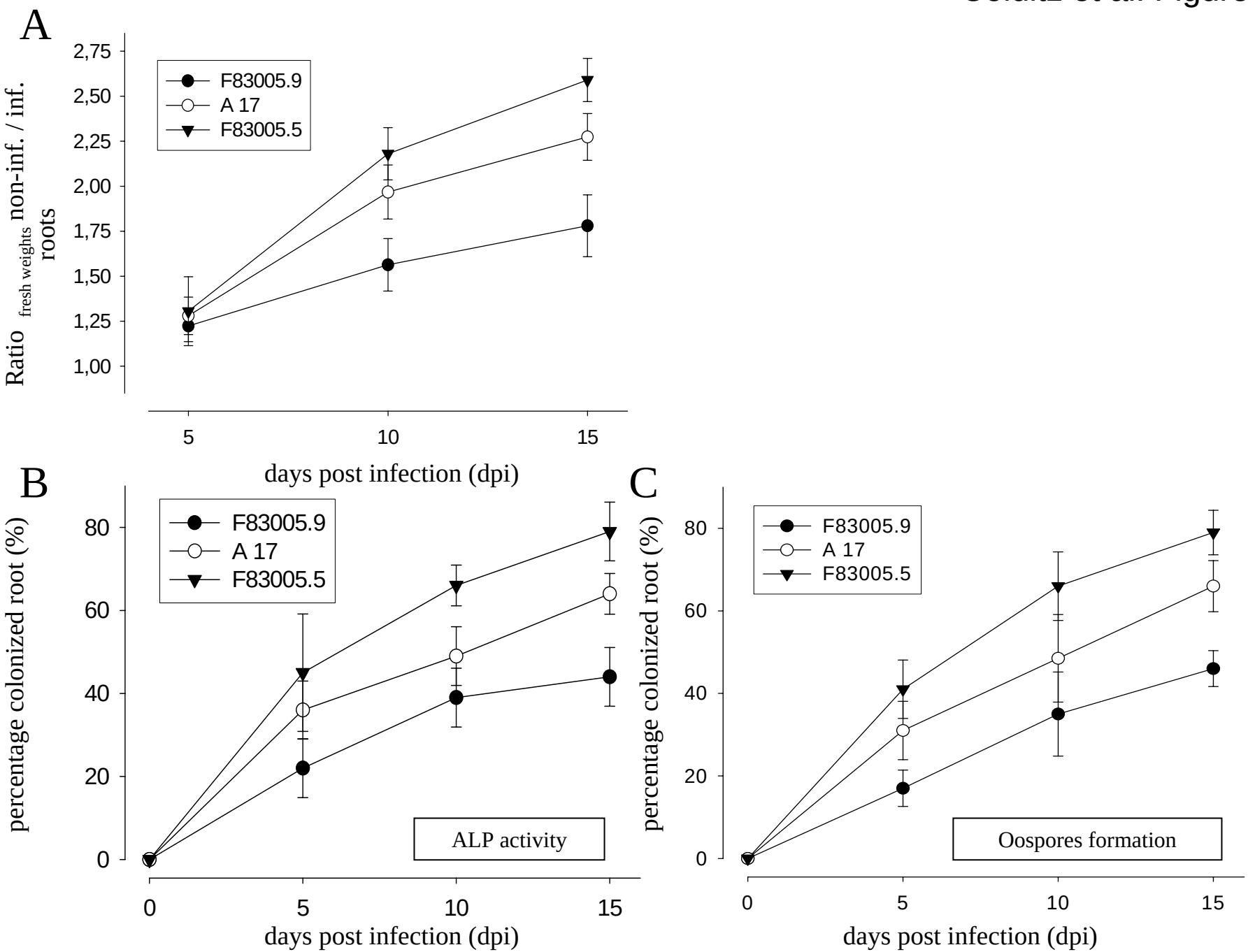
Table:

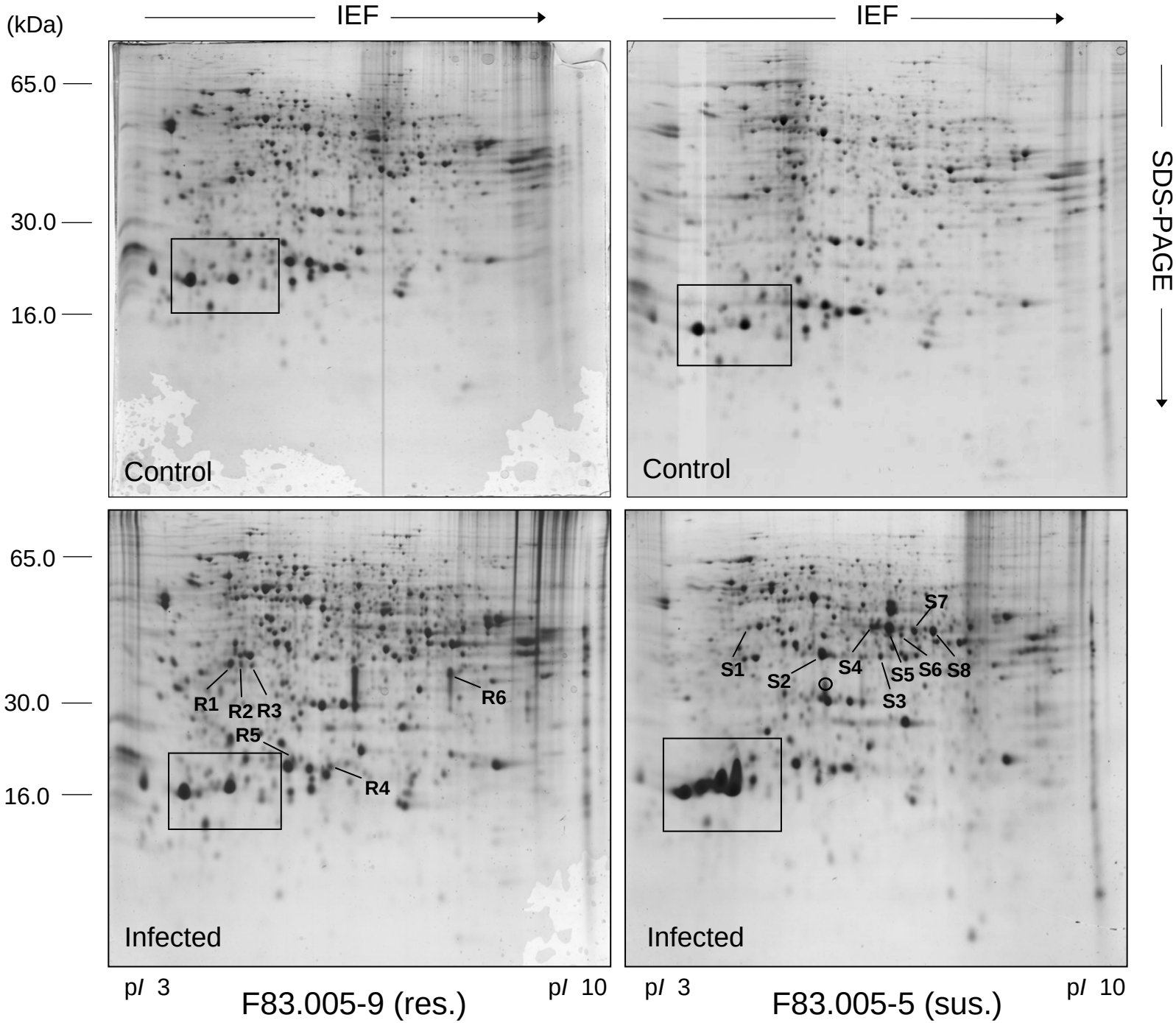
Table 1. Regulated proteins detected in the *M. truncatula* root proteome of the lines F83.005-9 (R1-R6) and F 83.005-5 (S1-S8) in response to infection with *A. euteiches*.

Spot no.[1]	Regulation[2]	MtGI TC[3]	Best matching gene product[4]	Seq. coverage (%)[5]	No. of matched peptides[6]	p*I* / M_r[7]	Access. number[8]	Species[9]
R1	++ (-5, con) + (-17)	TC 76864	Plasma memmbrane intrinsic polypeptide	46	8	4.92/ 34142	Q 9SMK5	*Cicer arietinum*
R2	+++ (-5) ++ (con) + (-17)	TC 77211	Alpha-soluble NSF attachment protein [Alpha-SNAP2]	39	6	4.94/ 33571	Q 9 S PE6	*Arabidopsis thaliana*
R3	+++ (-5) ++ (con) + (-17)	TC 77218	20S proteasome alpha 6 subunit [PAF1]	44	7	5.1/ 34260	Q 8H 1Y 2	*Nicotiana benthamiana*
R4	+++ (-5, -17) ++ (con)	TC 85873	Proteasome subunit alpha type 4 (EC 3.4.25.1) [PSA4], subunit C	57	8	5.9/ 17284	O 8 1148	*Arabidopsis thaliana*
R5	++ (-5, con) = (-17)	BG 455161	Initiation factor 5A-2 (eIF-5A 2)	42	5	6.17/ 20.943	Q 945F4	*Medicago sativa*
R6	++ (-5,-17 ,con)	TC 79175	Cytochrome-b5 reductase-like protein * *	56	7	8.67/ 31194	Q(8LBD3	*Arabidopsis thaliana*
S1	++ (-9, con) = (-17)	TC 33591	Isoliquiritigenin 2-O-methyl-transferase	58	9	4.9/ 41140	P93324	*Medicago sativa*
S2	++ (-9, -17 ,con)	TC 77184	Isoflavone reductase homolog [IFR1]	64	10	5.75/ 34148	Q 9SD Z 1	*Glycine max*
S3	++ (-9, -17 ,con)	TC 39404	Chalcone reductase [CHR]	51	9	6.02/ 34903	Q 4 35 56	*Medicago sativa*
S4	++ (-9, -17 ,con)	TC 39549	ADH1_PEA Alcohol dehydrogenase 1 (EC 1.1.1.1)	46	7	5.95/ 41155	P12886	*Pisum sativum*
S5	++ (con) + (-9, -17)	TC 39549	ADH1_PEA Alcohol dehydrogenase 1 (EC 1.1.1.1)	38	6	6.09/ 41155	P12886	*Pisum sativum*
S6	++ (-9, -17 ,con)	TC 39291	ALF2_PEA Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	53	7	6.15/ 38490	P46257	*Pisum sativum*
S7	++ (-9, con) + (-17)	TC 39291	ALF2_PEA Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	59	8	6.42/ 39530	P46257	*Pisum sativum*
S8	++ (con) + (-9) = (-17)	TC 39291	ALF2_PEA Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	66	11	6.77/ 39530	P46257	*Pisum sativum*

[1] Spot number correspond to numbers given in Figure 2
[2] Regulation level: +++: induced, ++rup-regulated, +: increase abundance (less than 2-fold induced), = : similar abundance. Regulation profiles of each spot in comparison to spot abundance in gels of: (-5): F83.005-5 infected, (-9): F83.005-9 infected, (-17): A17 infected plants, (con): control plants
[3] Best mmatching T TC sequence of the MtGI
[4] Protein annotation based on Mascot blastx search
[5] Sequence coverage of mmatched peptides to the translated TTC (protein) sequence
[6] Nummber of mmatched peptides to the translatd TTC (protein) sequence
[7] Predicted p*I* and mmolecular weight M_r of the protein
[8] Accession nummber of protein
[9] Or r ganism of origin

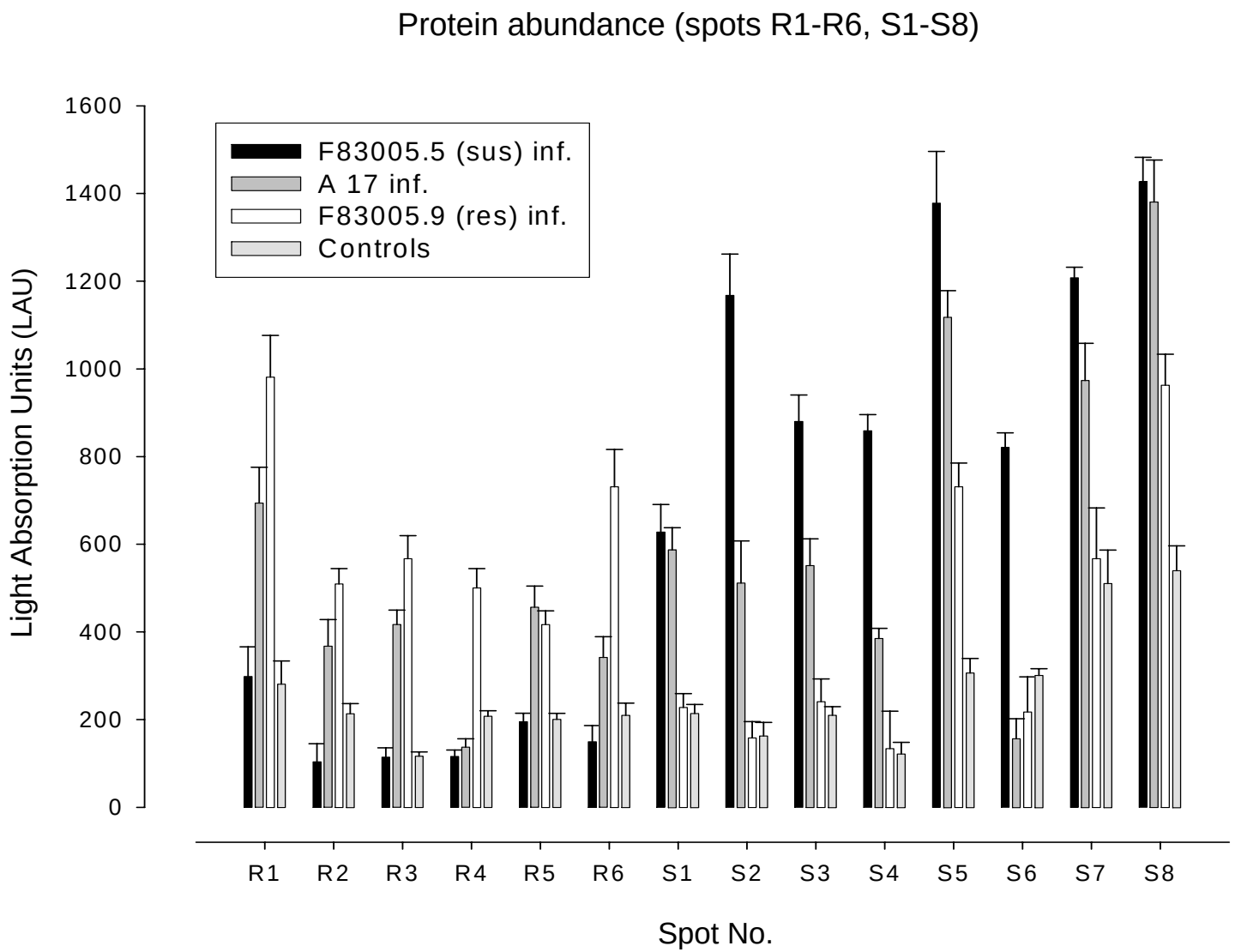
Colditz et al. Figure 1



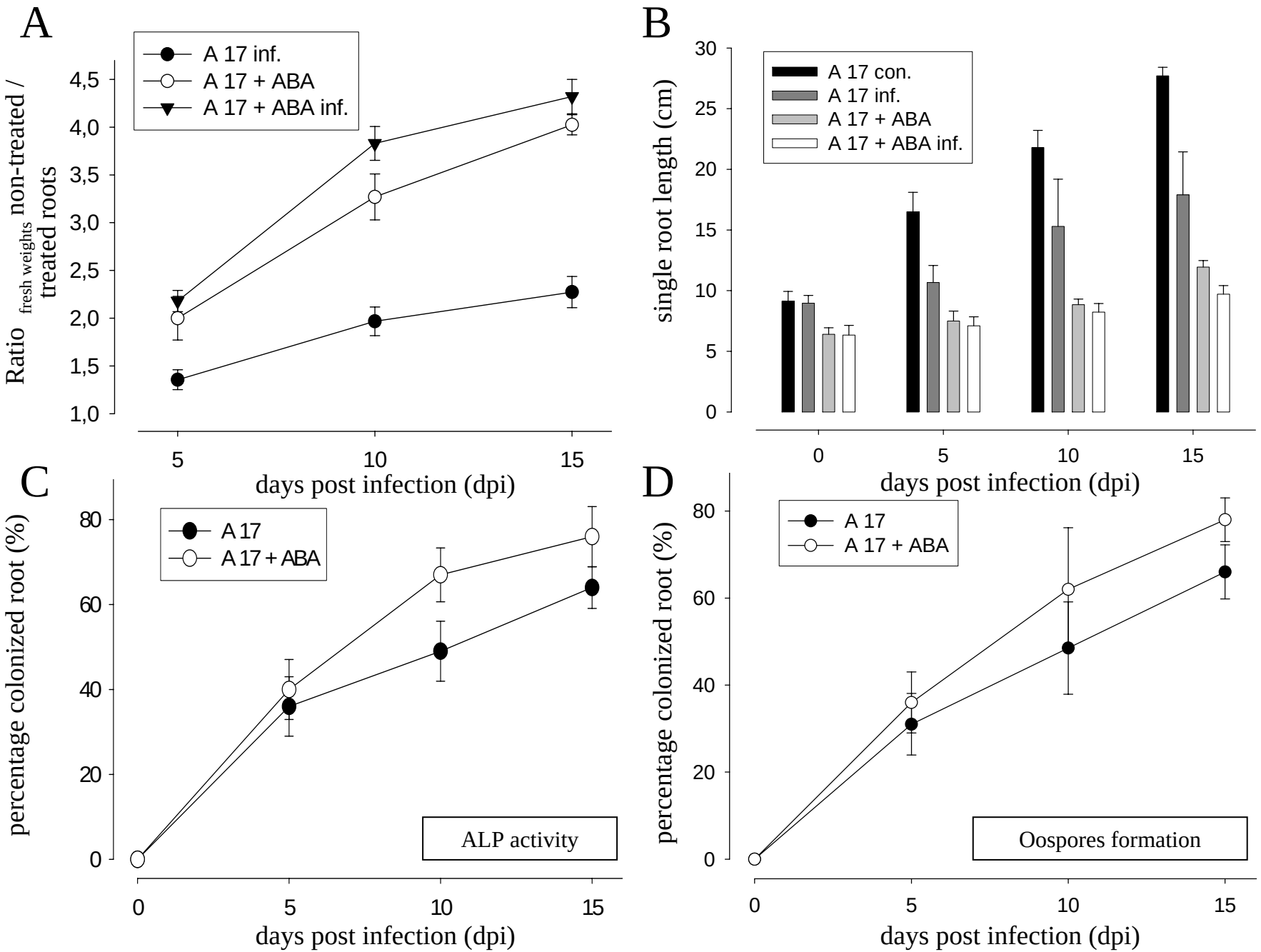


Colditz et al. Figure 2

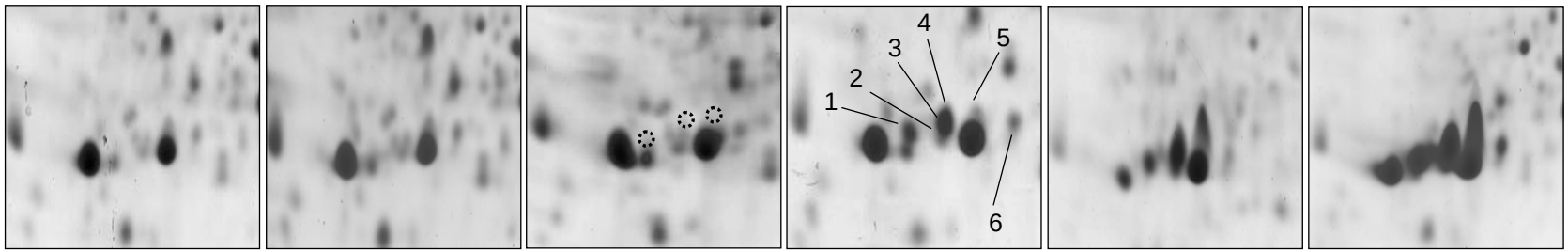
Colditz et al. Figure 3



Colditz et al. Figure 4

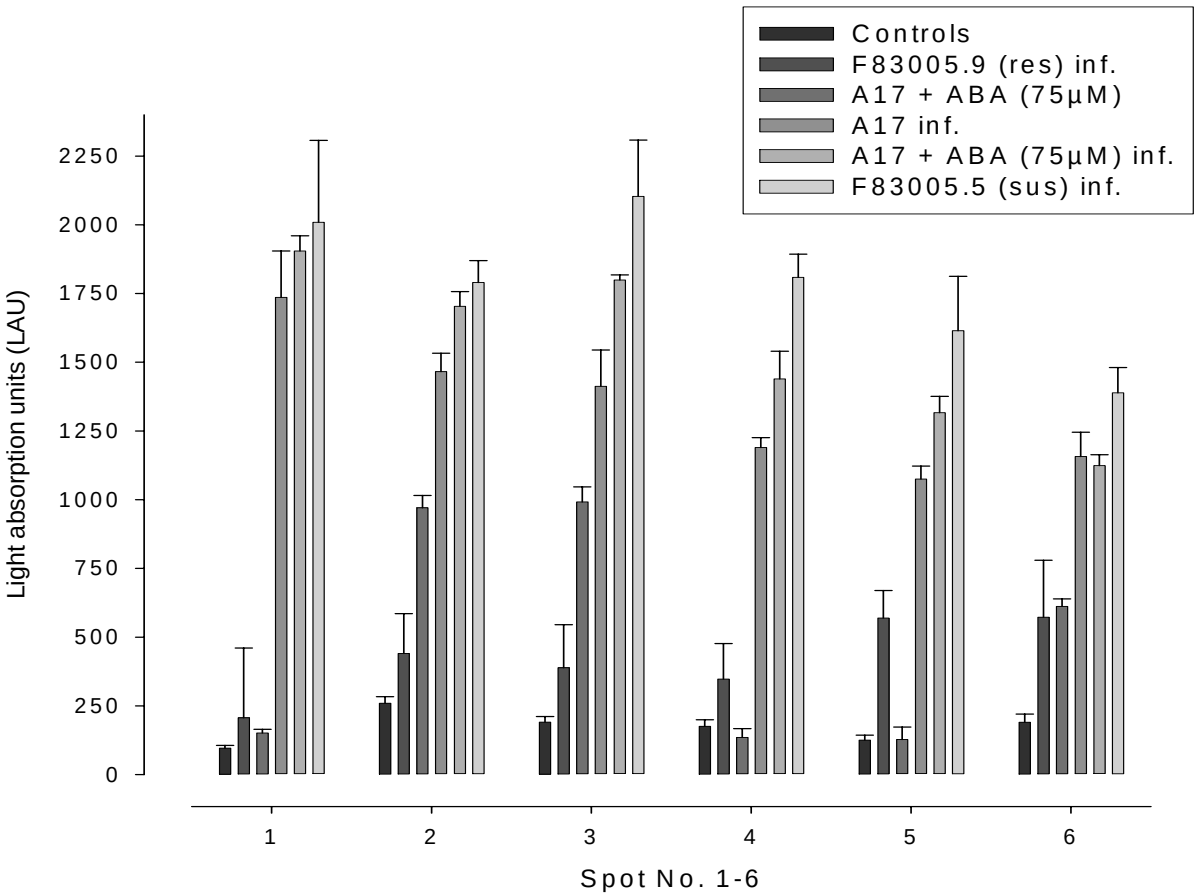


A



A 17 con. | F83.005-9 inf. | A 17+ABA (75µM) | A 17 inf. | A 17+ABA (75µM) inf. | F83.005-5 inf.

B



Colditz et al. Figure 5

Title:

Proteome analysis of the tripartite interaction between *Medicago truncatula* roots, *Glomus intraradices* and the parasitic oomycete *Aphanomyces euteiches* reveals proteins that are correlated to the bioprotective effect

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Proteome analysis of *M. truncatula* roots inoculated with *G. intraradices* and *A. euteiches*

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Abbreviations:

ALP: Alkaline phosphatase; dpi: days post infection; CBB: Coomassie Brilliant Blue; LAU: Light Absorption Units; PMF: Peptide Mass Fingerprinting; PR proteins: Pathogenesis Related proteins; TC: Tentative consensus sequence; 2-D: Two-dimensional.

Keywords: *Aphanomyces euteiches, Glomus intraradices, Medicago truncatula, Mycorrhiza, Proteomics, PR10, Proteasome*

1 Abstract

The effect of an established arbuscular mycorrhiza (AM) symbiosis on a root infection by the parasitic oomycete *Aphanomyces euteiches* was analysed. For this study, the tripartite interaction between the model legume *Medicago truncatula*, the AM-*fungus Glomus intraradices* and *Aphanomyces euteiches* was chosen. Root fresh weights as well as pathogen spreading pattern in the root system indicate that mycorrhizal plants show a clearly increased resistance against *A. euteiches* as compared to non-mycorrhizal plants. To unravel the molecular background of this bioprotective effect, root proteome profiles were analyzed. Previous studies revealed proteins whose expression was correlated to resistance or susceptibility to *A. euteiches* in different *M. truncatula* ecotypes. The expression levels of these proteins were quantified in mycorrhizal roots either infected or not by *A. euteiches.* This analysis showed that one protein with similarities to proteasome subunits shows the highest expression during the tripartite interaction, indicating that this protein might be involved in bioprotection against *A. euteiches* to mycorrhizal plants.

2 Introduction

Root colonization with arbuscular mycorrhiza (AM) fungi has been reported to improve the plants resistance against root pathogens (Dehne 1982; Kjøller and Rosendahl 1996; Slezack *et al.* 1999). For this bioprotective effect, several potential mechanisms have been suggested: (I) an enhanced physical resistance and damage compensation capability of the plant due to an improved nutritional status, (II) changes in the microbial populations of the mycorrhizosphere, (III) competition effects between invading microorganisms (IV), increased production of secondary metabolisms as antimicrobial compounds and (V) the activation of plant defence via accumulation of defence-related proteins. In general, the mutualistic association between AM fungi of the phylum Glomeromycota and plant roots suggests a direct interaction between the microsymbionts and root pathogens that resort similar trophic requirements (Graham 2001).

Aphanomyces euteiches (Drechs.) is a biotrophic pathogen that challenges cortical root tissue mainly of pea but also of several other legumes. This oomycete causes a root rot disease, which is considered to be the most destructive disease in pea growing areas (Levenfors *et al.* 2003). Beside typical disease symptoms as a watery rotting of root tissue and yellow-brown coloured lesions spreading through the root cortex, a significant reduction of root mass is effected due to the pathogen. The appearance of thick-walled oospores, which serve as a resting stage in the soil and as primary inoculums source during many years after soil-inoculation, complicates and limits a successful disease control. Moreover, because of a lack of knowledge about pathogen variability and mechanisms associated with the expression of partial resistance *in-planta* and the polygenic inheritance of resistance, pea cultivars with acceptable resistance levels against *A. euteiches* are up to now not available (Pilet-Nayel 2004).

In this study, we present a proteomic approach combined with an evaluation of physiological data to characterize more precisely the infection development of the *A. euteiches* root rot on *M. truncatula* roots after pre-establishing mycorrhizal symbiosis with *Glomus intraradices*. The up to now published investigations on this particular interaction realised between *A. euteiches* as root pathogen and *Glomales* mycorrhizal fungi (*Glomus intraradices*, *Glomus mosseae*) on legumes (*Pisum*, *Medicago*) were mainly focussed on altered enzymatic activities and variances in balances of energetic reserves such as fatty acids (Kjøller and Rosendahl 1996; Larsen and Bødker 2001). These studies proposed a reduction of root disease severity due to the mycorrhiza symbiosis. But so far only few investigations were carried out that refer to modifications in the plants protein compositions of challenged root cells. Slezack *et al.* (1999) reported an enhanced endoproteolytic activity of 'trypsin-like' serine endoproteases after colonization with both microorganisms, and Bestel-Corre *et al.* (2002) detected proteins that were induced in the *M. truncatula* root proteome after inoculation with *Glomus mosseae* and nitrogen fixing bacteria *Sinorhizobium meliloti*.

After previous investigations on changes in the *M. truncatula* root proteome after infection with *A. euteiches* (Colditz *et al.* 2004, Colditz *et al.* submitted), we broad the *M. truncatula* root proteome profiling in response to biotic interactions by establishing an approach that refers to the colonization with both microorganisms, *G. intraradices* and *A. euteiches*. Thus, we investigated if changes in protein pattern are altered when a mycorrhizal symbiosis was pre-established before infection with *A. euteiches*.

3 Materials and Methods

Plant growth and inoculation

Seeds of *Medicago truncatula c.v. Jemalong* A17 were surface sterilised by 5 min treatment with concentrated sulphuric acid and 5 min incubation in a 3 % (v/v) Sodium Hypochlorite solution. Seeds were washed intensively with distilled water and pre-germination occurred on water agar plates (1.5% w/v) during 3 days at room temperature in the dark. Seedlings then were planted under pot conditions (Ø 7.5 cm) into a 1:2 v/v mixture of sterile expanded clay and vermiculite (controls) and let grow in the greenhouse (22 °C, 65 % humidity, 16 h photoperiod). Seedlings were fertilized with half-strength Hoagland`s solution (Hoagland and Arnon 1950) twice a week and additionally watered several times during the experiment.

Inoculation with *Glomus intraradices* was carried out using a commercially available inoculum (Granular AMF inoculum, BIORIZE, Dijon, France) and granulate was mixed 1:10 with the clay/vermiculite-mixture where the seedlings were potted into (+ myc.). For inoculation with *Aphanomyces euteiches Drechs.*, strain ATCC 201684 (Rosendahl, S., Department of Mycology, Botanical Institute, University of Copenhagen) was cultured in 20 ml Maltose Peptone broth (MPB) for 9 days at 24°C in the dark. Zoospore production was induced by incubation the mycelia in autoclaved lake water (royal gardens of Herrenhausen, Hannover, Germany) after two washing steps. Inoculation of the *M. truncatula* plantlets with *A. euteiches* was carried out 20 days after *G. intraradices* inoculation was carried out by applying 5 ml of the zoospore suspension containing 100.000 zoospores ml^{-1} at the base of the stem (+ *A. e.*). Control plants were mock-inoculated with an equal volume of autoclaved lake water. One day before inoculation, all pots were water saturated.

Harvest of plants, in planta histochemical staining

Control plants and inoculated plants (+ myc. / + myc. + *A. e*) were harvested. Roots were cut off and washed with deionised water to remove soil particles. The roots fresh weight was determined. Root samples were frozen in liquid nitrogen directly after harvest and stored at -80 °C.

For *in planta* detection of *G. intraradices* mycorrhizal structures and *A. euteiches* oospores, fresh harvested roots were incubated in a 10% (w/v) KOH solution for 30min. at 90°C, washed once with deionised water and then stained with 5% (v/v) ink and 8% (v/v) acetic acid during 20min. at 90°C. The intensity of mycorrhizal colonisation in the roots was evaluated using the computer program “Mycocalc” (Trouvelot *et al.* 1986).

To determine *A. euteiches* alkaline phosphatase activity (ALP activity), roots were stained overnight as described by Tisserant *et al.* 1993. Stained roots were cleared in 3% (w/v) NaClO for 5 min. Evaluation of the percentages of oospores and ALP-active *A. euteiches* mycelium was achieved by cutting the roots into pieces of 1 cm length and counting the number of root pieces containing stained oospores and hyphae out of 100 randomly collected root pieces.

Protein extraction

Total root protein was extracted and precipitated following a modified protocol of Hurkman and Tanaka (1986). Therefore, 0.5 g of root tissue was grounded briefly in liquid nitrogen and homogenized in 750 µl lysis buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCL, 2 mM PMSF and 2 % (v/v) ß-mercaptoethanol, pH 8.0). An equal volume of water saturated phenol was added and samples were mixed at 300 rpm for 30 min. After centrifugation (17.000g, 3 min, 4 °C), the phenolic phase was removed and mixed with an equal volume of lyses buffer. The samples were centrifuged again and proteins were precipitated in the phenolic phase at - 20 °C for at least 4 h after adding 5 volumes of 100 mM

ammonium acetate in methanol. Total protein was pelleted by centrifugation (17.000g, 3 min, 4 °C) and washed three times with cold 100 mM ammonium acetate in methanol and once with cold 80 % acetone. The vacuum dried pellet was solubilized in 350 µl rehydration buffer containing 8 M urea, 2 % (w/v) CHAPS, 100 mM DTT, 0.5 % v/v IPG buffer for correspondent pH-values (pH 3-10NL/4-7L, Amersham Pharmacia Biotech, Uppsala, Sweden) and a trace of bromophenol blue.

2-Dimensional Gel Electrophoresis (isoelectric focussing IEF, SDS-PAGE)

First dimension isoelectric focussing (IEF) was carried out by rehydration 18 cm Immobilised Dry Strips with pH gradients 3-10 non-linear (Amersham Pharmacia Biotech) with 350 µl protein samples in rehydration buffer. IEF was performed onto an IPGphor system (Amersham Pharmacia Biotech) for 24h with voltages from 30 V up to maximal 8000 V. Strips were subsequently equilibrated 15min. in equilibration solution I (30% v/v glycerol, 50 mM Tris-HCL pH 8.8, 6 M Urea, 2 % (w/v) SDS, a trace of bromophenol blue, 0.01 g DTT ml^{-1} w/v) and 15min. equilibration solution II (same compounds like equilibration solution I, but DTT substituted by 0.025 g iodoacetamide ml^{-1}).

For second dimension poly-acrylamide-gel-electrophoresis (SDS-PAGE), IPG strips were fixed vertically onto SDS-tricine-polyacrylamide gels of 13 % acrylamide. PAGE was performed during 20 h at 30 mA per mm gel layer. Gels were Commassie-stained overnight using 0.1 % Coomassie Brilliant Blue G250 (Bio Rad, Richmond, CA, USA) after fixation in 40% (v/v) methanol/10% (v/v) acetate for at least 2 h. Before gel scanning, gels were washed intensively with deionised water.

Gel scanning and densitometric evaluation of protein abundance

Coomassie-stained gels were scanned on an UMAX Power Look III Scanner (UMAX Technologies, Fremont, USA) or on a Fujifilm FLA-3000 Fluorescence Laser Imager

(Fujifilm Medical Systems USA, Stamford, CT, USA). For protein abundance analysis, spots of interest were labelled by the AIDA Image Analyser v3.20 Evaluation software (Raytest USA, Wilmington, NC, USA) and densitometric values as Light Absorption Units (LAU) were determined after subtraction of background absorption, which was measured in 5 different blank gel parts without protein content located close to the valuated protein patterns. All measured values are averages of four technical repetitions, and standard deviations were determined.

4 Results

Infection development

To evaluate the *A. euteiches* infection development in *M. truncatula* roots either pre-inoculated or not with *G. intraradices* under pot culture conditions, root fresh weights of non-infected and infected plants were compared. All experiments were repeated 3 times and plants of all three biological repetitions showed similar mycorrhiza parameters: the frequency of infection (F) at 20 dpi was nearly 100%, the intensity of mycorrhization (M) ranged between 68.1 and 74.3% and the frequency of arbuscules was between 47.3 and 53.6%. During the time-course of mycorrhiza development (0 dpi-35 dpi), the root fresh weights of mycorrhizal plants (myc) were clearly increased as compared to the roots of non-mycorrhizal plants: Ratio $R_{\text{fresh weights}}$: (non-myc) x $(\text{myc})^{-1}$ decreased from R=0.9 (5 dpi) to R=0.71 (35 dpi) (Figure 1A). At 20 dpi, half of the mycorrhizal plants and half of the non-mycorrhizal plants were inoculated with *A. euteiches* zoospores by applying 0.5 x 10^6 zoospores at the stem base of each plant. After infection with the pathogen zoospores, non-mycorrhizal plants showed dramatic losses in root fresh weights: R = (non-myc) x $(\text{non-myc} + A.e.)^{-1}$) reached 1.36 at 35 dpi. In contrast, mycorrhizal plants showed significantly lower losses in root fresh weights as compared to non-mycorrhizal plants: R = (myc.) x $(\text{myc} + A.\ e.)^{-1}$ was 1.18 at 35 dpi. Moreover, typical disease symptoms (yellow-brown discolorations or lesions in the root cortex) of the *A. euteiches* infection were reduced in A17 roots of mycorrhizal plants (data not shown).

To compare the pathogen spreading in non-mycorrhizal and mycorrhizal plants, *in planta* histochemical staining of *A. euteiches*-infected roots was carried out in order to detect Alkaline phosphatase (ALP)–active *A. euteiches*-hyphae and the appearence of oospores. As shown in Figure 1B, after inoculation non-mycorrhizal and mycorrhizal roots were colonized by enzymatic active pathogen hyphae, but the percentage of root tissue which was colonized

by pathogen hyphae was clearly reduced in mycorrhizal roots as compared to non-mycorrhizal plants. The same was true for the oospore frequency, which was significantly higher in non-mycorrhizal roots. Hence, a bioprotective effect with regard to root fresh weights and pathogen spreading was observed in roots of mycorrhizal plants.

2-D root proteome maps of M. truncatula inoculated with G. intraradices and/or A. euteiches

To get insights into molecular background of the observed bioprotection in mycorrhizal *M. truncatula* roots with regard to an *A. euteiches* infection, the corresponding root proteome profiles were analysed. Recently, such proteomic approaches revealed the identification of a set of proteins, which were induced in *M. truncatula* roots after *A. euteiches* infection (Colditz et al. 2004) and furthermore, the analysis of two *M. truncatula* accessions with induced or decreased susceptibility to *A. euteiches* revealed differences in the root proteomes of both *M. truncatula* lines after *A. euteiches* infection (Colditz et al. submitted). In the here presented work, proteins, which were identified in these recent studies to be involved in the response to an *A. euteiches* infection, were analyzed concerning their expression levels in roots showing a bioprotection after mycorrhization. The resulting 2-D gels displayed about 500 – 700 prominent protein spots that could be resolved in between the molecular mass range of 10 to 100 kDa and the IEF range of p*I* 3 to 10. To confirm differences detected in the protein pattern, gels of at least two biological replicates and six technical controls were evaluated. Minor distinctions in protein pattern based on single, non-reproducible events were not further investigated.

Figure 2 displays 2-D maps of: non-mycorrhizal control roots (control), *A. euteiches*- infected roots (*A. e.* infected), mycorrhizal roots (Myc) and mycorrhizal roots, which were subsequently infected (at 20 dpi with regard to mycorrhizal treatment) with *A. euteiches* (Myc + *A. e.* infected). All roots were harvested at the same time-point of 35 dpi (with regard to mycorrhizal treatment). Differentially apparent protein spots of the set of previously identified

proteins (1-6, S1-3 or R1-R6) are indicated on the gels. Spots 1 to 6, framed in a box, are encoding pathogenesis related proteins of class 10 (PR-10 like) and were described to be induced after *A. euteiches* infection in *M. truncatula* cv Jemalong (Colditz *et al.* 2004). Further protein spots, labelled with R1-R6, were found to show different abundance in mycorrhizal *A. euteiches* infected roots as compared to the three other conditions analysed. In previous studies, these six proteins have been found to show an increased expression in infected roots of a *M. truncatula* line with decreased susceptibility to *A. euteiches*. Two proteins labelled by S1 and S3, which have been described earlier to show an increased expression in infected roots of a *M. truncatula* line with increased susceptibility, were also found to show differences in their expression level in roots showing the bioprotective effect.

Regulation level of differentially abundant proteins

To quantify differences in expression levels within roots of all four treatments, particular protein spots of all 2-D gels were densitometrically analysed by the measurement of their light absorption units (LAU). From all determined averaged absorption values displayed in Figure 3, nearby background absorption was subtracted and a gel to gel normalization was carried out by adjusting the LAU values of one reference protein spot which showed nearly identical signals on each gel analysed. This evaluation of the absorption levels revealed that the proteins analysed here could be categorized into three regulation classes. One class, including proteins labelled 1-5, contains proteins upregulated in *A. euteiches* infected roots as compared to non-infected roots. Most of these proteins show a slightly decreased expression in *A. euteiches* infected mycorrhizal roots as compared to non pathogen-infected mycorrhizal roots. A second regulation class comprising proteins labelled 6, R1-R3, R5, R6, S1 and S3. These proteins show strongest expression in mycorrhizal roots and reduced expression levels in *A. euteiches* infected roots or *A. euteiches* infected mycorrhizal roots. In non-inoculated control roots, this protein set shows lowest expression values. Protein R4, which was earlier

reported to be induced in infected roots of a line with increased resistance against A. euteiches shows the most interesting expression pattern with regard to the biotrophic effect: it shows the highest expression level in mycorrhizal roots after pathogen inoculation.

5 Discussion

In the recent years, several studies on the influence of mycorrhiza colonization on infection of plants by pathogens have been reported. Root pathogens as , including investigations that were focussed on interactions realized between *Glomus mosseae* and *Glomus intraradices* to oomycete root pathogen *Aphanomyces euteiches* (Kjøller and Rosendahl 1996; Bødker *et al.* 1998; Slezack *et al.* 1999). Beside biochemical analyses on altered enzyme activities and approaches based on genome or transcriptome analyses (Wulf *et al.* 2003), to our knowledge only single proteome analyses on mycorrhiza-specific or symbiosis-related changes in protein pattern as well as on protein modifications due to oomycete pathogens have been realized (Bestel-Corre et al. 2002; Colditz et al. 2004). Proteomic approaches provide the facility for direct protein and regulation studies at a cellular level referred to mutualistic or parasitic plant-microorganisms interactions that contribute an integrated understanding of molecular events involved in these processes.

In this study we present a proteomic approach to investigate the influence of a *Glomus intraradices* mycorrhization of *M. truncatula* A17 to the infection development of the oomycete *A. euteiches*. Evaluation of physiological data revealed that mycorrhizal *M. truncatula* plants exhibit of an enhanced root mass production as compared to non-mycorrhizal control plants, and moreover, that an *A. euteiches* infection is repressed in mycorrhizal plants as documented with a reduced root colonization of the pathogen and also a reduced decrease in root mass production. These data confirm previous findings for pea, that the presence of mycorrhizal fungi indeed reduces *A. euteiches* root rot severity (Kjøller and Rosendahl 1996; Bødker *et al.* 1998). Reasons for this reduction in disease severity are supposed to be (i) a diminution of downward infection development starting at the epicotyl downwards the root system due to a competition between the microorganisms for infection sides and nutrients, (ii) an enhanced physical resistance and damage compensation capability

of the plant due to an improved nutritional status, (iii) a fundamental change in the mycorrhizosphere of microbial populations, and also (iv) the production of antimicrobial compounds and induction of plant defence mechanisms, respectively specific modifications in the profiles of proteins and fatty acids (Kjøller and Rosendahl 1996; Slezack *et al.* 1999; Larsen and Bødker 2001). *In-planta* histochemical staining of *M. truncatula* roots inoculated with both microorganisms revealed that a competition for physical space as for infection sides is presumably the case, which led to an obvious reduction of typical disease symptoms of an *A. euteiches* infection where the root tissue was penetrated by mycorrhizal structures and *A. euteiches* hyphae or oospores were not apparent.

Referring to the plants proteome after inoculation with *G. intraradices* and/or *A. euteiches*, several changes in protein pattern and abundances were detectable. A special role in plant responses to microbe interactions in *M. truncatula* roots is supposed to play a group of Pathogenesis Related class 10 (PR-10) like proteins (spots 1-6), which reveals high homology to abscisic acid responsive proteins ABR17 (Iturriaga *et al.* 1994). These proteins are constitutively abundant in the *M. truncatula* root proteome (Mathesius *et al.* 2001), but found to be specifically induced and accumulated upon microbial attack and especially upon *A. euteiches* infection (Colditz *et al.* 2004). In the present study, these PR-10 like proteins were found to be slightly induced in mycorrhizal plants, which confirms previous findings by Bestel-Correl *et al.* (2002) upon mycorrhization, but much stronger induced upon infection with *A. euteiches*. 2-D root maps of mycorrhizal plants infected with *A. euteiches* revealed a clearly reduced abundance of these proteins as compared to those of *A. euteiches* infected non-mycorrhizal plants. When the abundance levels of this protein set indicate the intensity of microbial infection, than a reduced *A. euteiches* infection in mycorrhizal plants as demonstrated with the physiological data could be confirmed.

A set of proteins (7-12) were found to be clearly induced in mycorrhizal A17 plants, whether infected or not infected with *A. euteiches,* as compared to control plants or plants of A17 only infected with *A. euteiches*. These proteins were found to be induced and identified previously in a French *M. truncatula* accession after infection with *A. euteiches,* which show an enhanced resistance to the challenge by the pathogen (Colditz *et al.* submitted). Thus, these proteins are rather likely involved in an improved defence response. Among these proteins are two proteasome alpha subunits (9, 10). Spot 9 reveals a nearly identical induction level in mycorrhizal A17 plants non-infected and infected with *A. euteiches,* while the abundance of protein spot 10 is clearly enhanced in mycorrhizal plants that were additionally *A. euteiches* infected. Proteasomes are cytosolic multi-subunit proteases representing the major enzyme complex for protein degradation by the ATP/ubiquitin-mediated proteolysis pathway (Kim *et al.* 2003). Moreover, protein ubiquitylation is involved in regulation of plant disease resistance responses as specific ubiquitin-associated proteins function as signalling components required for triggering defence response signal transduction cascades (Peart *et al.* 2002). These results bridge a link to the findings of Slezack *et al.* (1999), who could show an increase in endoproteolytic protease activity during the development of mycorrhizal symbiosis, which moreover was found to be stimulated by additionally infection with *A. euteiches*.

For a protective effect of mycorrhizal symbiosis against plant pathogens, an induction of antimicrobial phytoalexins was proposed. In fact, in *M. truncatula* an increase in phe ammonium lyase (PAL) and chalcone synthase (CHS) was observed after mycorrhization (Harrison and Dixon 1993). Here, we could detect two major enzymes of the phenylpropanoid pathway to be induced due to mycorrhization and/or *A. euteiches* infection; an isoliquiritigenin (chalcone) 2-O-methyltransferase (cOMT) represented by spot 13 and a chalcone reductase (CHR) (spot 14). In *Medicago spp.*, these enzymes are required for the production of medicarpin, the major phytoalexin accumulated in response to fungal pathogens

(Winkel-Shirley 2001). Their abundance was stronger increased in mycorrhizal A17 plants than in plants infected (only or additionally) with *A. euteiches*. Hence, a putative protective effect represented by the detection of these proteins was triggered by mycorrhization of *M. truncatula* roots without the stimuli of the pathogen. The protection against *A. euteiches* might be pre-established by achieving mycorrhizal symbiosis.

5b Acknowledgments

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7 Figure Legends:

Figure 1:

Infection development in *M. truncatula* A17 roots during infection with *Glomus intraradices* (myc.), *A. euteiches* (+ *A. e.*, from 20dpi on), or with both microorganisms (myc. + *A. e.*), from 5dpi to 35dpi: A) Ratio R for root fresh weights (R = (non-myc.) x (infected)$^{-1}$, or R = (myc.) x (myc. + *A. e.*)$^{-1}$, respectively). B) Percentages of *A. euteiches* root colonization after *in planta* evaluation of alkaline phosphatase (ALP) activity and oospores formation (Oosp.) in mycorrhizal plants (+ myc.) and non-mycorrhizal plants (- myc.), from 5dpi (= 25dpi, respectively) to 15dpi (= 35dpi, respectively). Error bars indicate standard deviations of different measurements (2 biological replicates).

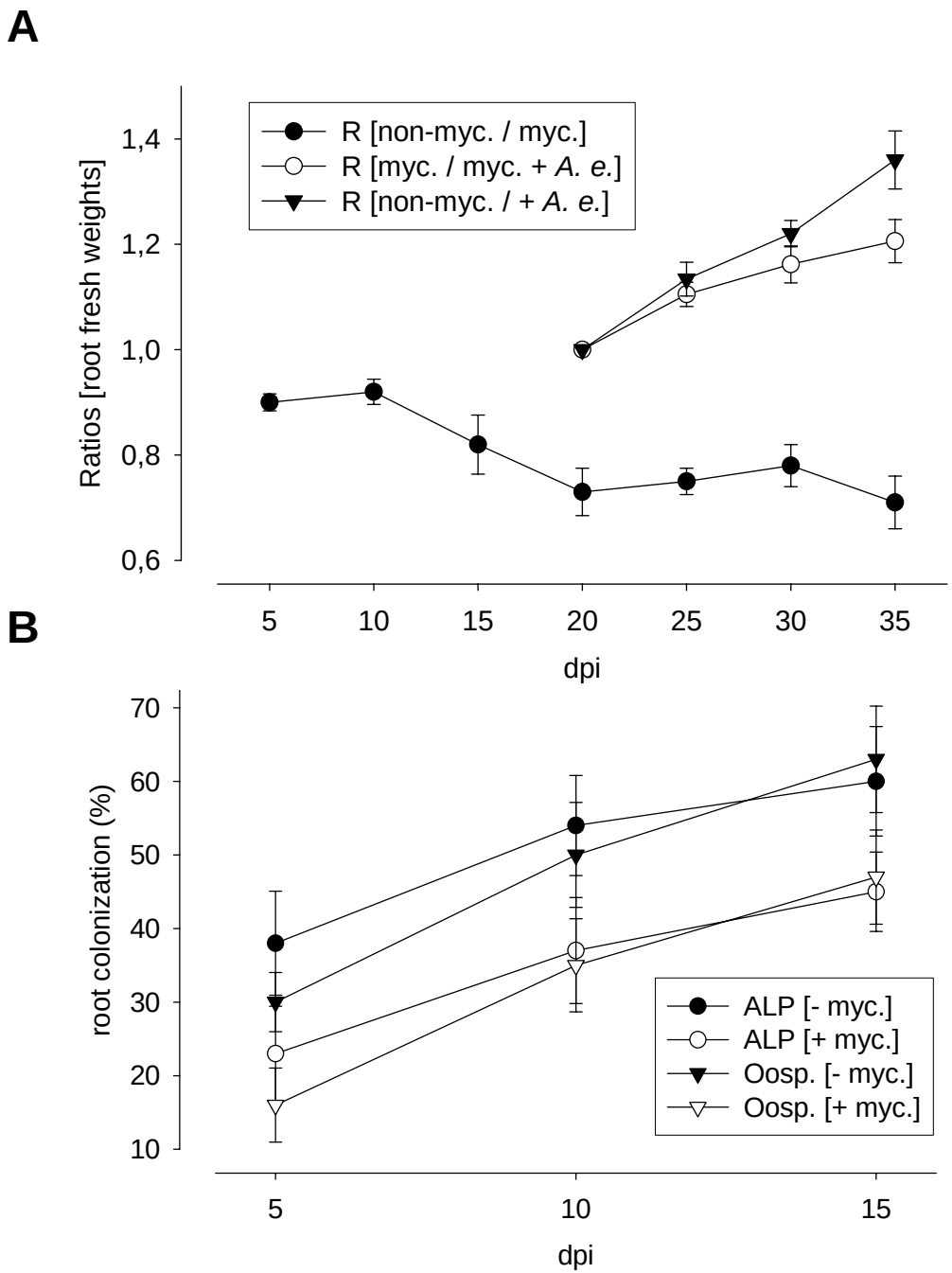
Figure 2:

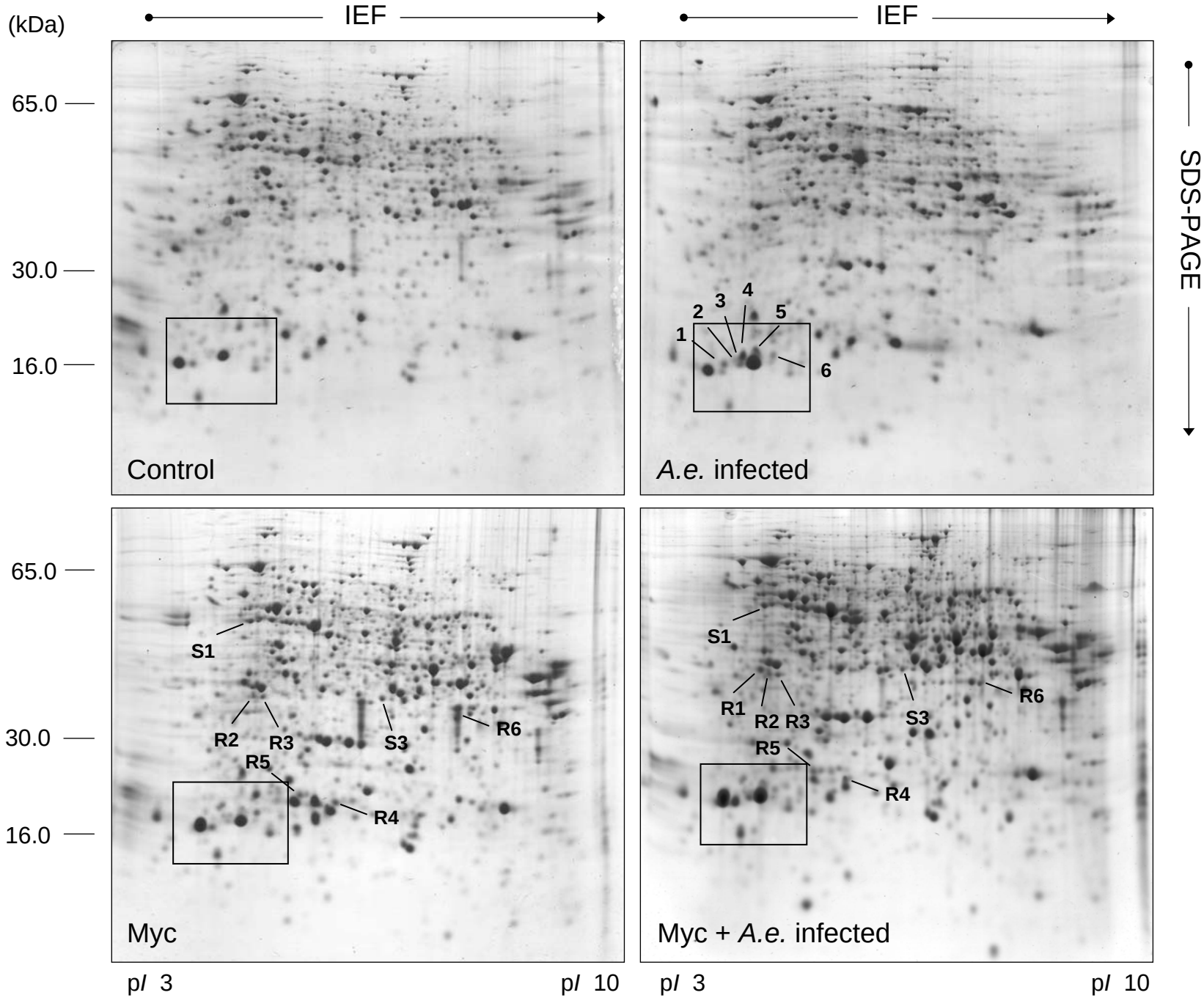
Root proteome maps of *M. truncatula* A17 at 15dpi (= 35dpi, respectively), from non-infected root tissue (control), *A. euteiches* infected tissue (*A. e.* infected), roots inoculated with mycorrhiza fungi *Glomus intraradices* (Myc), and from root tissue inoculated with both microorganisms (Myc. + *A. e.* infected). Differentially induced and identified proteins are numbered: 1 to 6 (additionally framed in a box), 7 to 14. All gels are CBB-stained.

Figure 3:

In-Gel Abundance of induced proteins after infection with *A. euteiches* (*A. e.* infected), with *Glomus intraradices* (myc.) and with both microorganisms (myc. + *A. e.*), at 15 dpi (= 35dpi, respectively), displayed beside these of corresponding spots in gels of non-inoculated root tissue (control). Light Absorption Units (LAU) measured for the protein spots 1 to 14 as labelled in *Figure 2*. Additionally, the protein identification of each spot is displayed. Lines in-between the bars indicate standard deviations of different measurements (2 biological, 6 technical replicates).

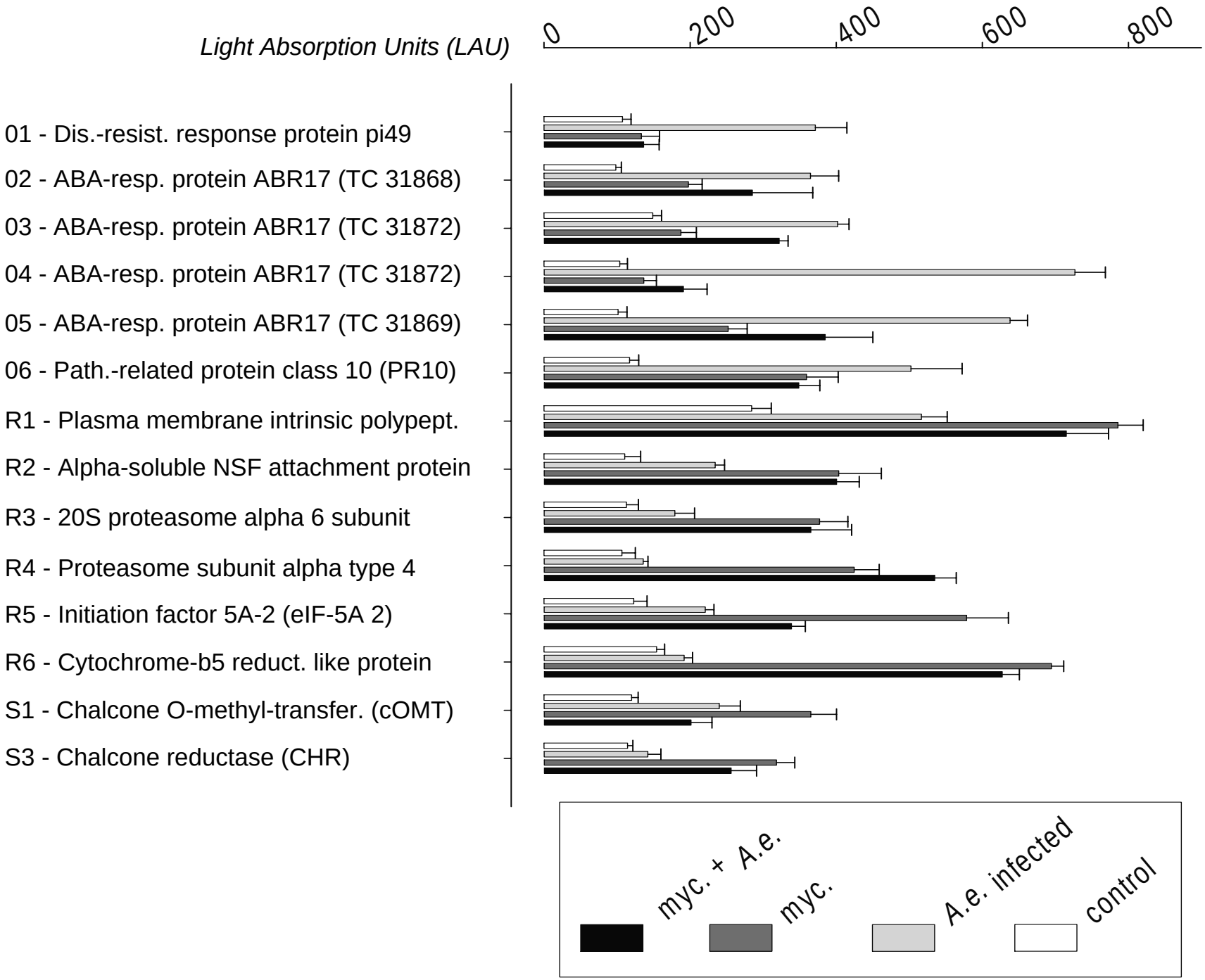
Colditz and Krajinski Figure 1





Colditz and Krajinski Figure 2

Colditz and Krajinski Figure 3



Chapter 6

Conclusions and Outlook

Correlation between detected transcriptomic and proteomic responses of *M. truncatula* upon *A. euteiches* infection

M. truncatula has emerged as widely accepted model plant to study plant and legume genomics as it exhibits a relative small genome size and is phylogenetically related to the most important legume crops like pea, alfalfa and faba bean. Moreover, a large genome sequencing program has been initiated for *M. truncatula* providing approximately 200.000 ESTs available in public databases, which allows a transfer of obtained genomic information for *M. truncatula* to all other mentioned legumes (chapter 1).
Comparison of fresh weight development and root colonization with ALP-active mycelium and oospores from *A. euteiches* revealed a very similar disease development for *Pisum sativum* and *Medicago truncatula* (chapter 2). Therefore, *M. truncatula* is considered to be well- suited to study the molecular mechanisms of this legume root rot disease.

In the present study, comparison of the obtained SSH-cDNA library (chapter 2) and the 2-D analysis (chapter 3 and 4) for *A. euteiches* induced expressional changes revealed several strong qualitative correlations, beside major quantitative differences in the number of detectable cDNA singletons and protein spots.
In both approaches, most prominent changes in *M. truncatula* gene expression and in the abundance of gene products upon *A. euteiches* infection occurred in the group of PR-10 transcripts and proteins. Among the cDNA populations, SSH-ESTs encoding class 10 PR proteins were the most redundant sequences with 13 detected singletons (chapter 2). Moreover, 6 singletons encoding an ABA-responsive protein (ABR17) were found which share high sequence similarities to PR-10 proteins (chapter 3). With 6 different detectable protein spots in the 2-D maps, this group of ABA-inducible PR-10 proteins also represents the most abundant and significant modification in the *M. truncatula* root proteome (chapters 3 and 4). This protein group also includes a disease-resistance-response protein *pi49* from pea, which was found to be up-regulated among the SSH-cDNA populations, as well as being to

be induced *de novo* in 2-D root protein profiles. The *pi49* protein spot showed the strongest induction signal among this particular protein set (chapter 3).

Other findings consistent between both approaches were obtained for the induction of proline-rich proteins (TC 85742; TC 31880), which play a role during architectural reorganization of cell walls upon pathogen attack or wounding. Furthermore, two cytochrome enzymes involved in the isoflavonoid pathway were found to be up-regulated, cyt-P_{450} (two matches in the SSH) and a cyt-b_5 reductase-like protein detected in 2-D maps of the more resistant F83.005-9 line (chapter 4). Both enzymes are biochemically linked to each other because cyt-b_5 reductase is required for maximal activity of petunia cyt-P_{450} as alternative e^- donor to NADPH; cyt-b_5 (= cyt-P_{450}) reductase is generally associated with cyt-P_{450} proteins (Winkel-Shirley 2001). Three further major enzymes of medicarpin phytoalexin synthesis were found to be up-regulated in the proteomic approaches (cOMT, IFR1, CHR; chapter 4). Thus, there is clear indication that this pathway is activated in *M. truncatula* root cells upon *A. euteiches* infection.

With cDNAs encoding for PR-1 and PR-4A, other members of PR protein families were detected in the SSH library. Unfortunately, the corresponding protein spots could not been identified among the differentially regulated proteins in the 2-D maps. Reasons for this are presumably the generally lower sensitivity of the proteomic technique or the putative membrane-associated locations of these proteins that make them difficult to detect in proteomic approaches.

In conclusion, qualitative evaluation of *M. truncatula* SSH-cDNAs and proteins (identified by 2-D PAGE) that are up-regulated upon *A. euteiches* infection revealed several correlations that refer to defense-related plant responses. These include the induction of PR proteins, enzymes of the phenylpropanoid pathway involved in phytoalexin synthesis and of putative cell wall proteins. Moreover, both approaches led to the detection of several up-regulated singletons, for which database analysis did not allow a confident identification and which presumably correspond to novel, so far unknown *M. truncatula* genes and gene products.

The role of Pathogenesis Related class 10 (PR-10) - like proteins in defense reactions of *Medicago truncatula* upon infection with *A. euteiches*

Several changes in protein pattern and abundances were detectable in the *M. truncatula* root proteome after inoculation with *A. euteiches*. Among these modifications in protein profiles, the most prominent changes involved a group of Pathogenesis Related class 10 (PR-10) like

proteins as it exhibits a complex regulation by *A. euteiches* infection as well as by exogenous applied ABA (chapters 3 & 4). This protein group - displayed in 2-D gels in the MW–range of 16 to18 kDa and a p*I*-region of 4.7 to 5.0 - consists of a disease-resistance-response protein pi49, which was found to be induced *de novo*, four protein spots of three ABA-responsive proteins (ABR17) and a PR-10 protein (all found to be up-regulated, chapter 3). Their differential regulation as compared to non-inoculated control root tissue was primarily detectable 6 hpi to 1 dpi and showed maximal expression at 15 dpi with 2- to 4-fold increased abundance (chapter 3). Moreover, upon *A. euteiches* infection this protein set showed a strongly enhanced induction in the highly susceptible *M. truncatula* F83.005-5 accession but a moderate induction level in the A17 line, while its abundance in the more resistant F83.005-9 line was significantly reduced (chapter 4). Hence, the abundance levels of this protein set are positively correlated with the infection level as measured by physiological data, and therefore can be regarded as an indicator for the extent of cellular stress response to the pathogen's challenge. The explicit role of ABA for the induction of these proteins is discussed hereafter.

PR-10 proteins belong to an extensive family of intracellular defense-related plant proteins, which have been shown to be present ubiquitously in the plant kingdom (Sikorski *et al.* 1998; Park *et al.* 2004). Several studies revealed that PR-10 proteins share high amino acid sequence similarities with the major food allergen of celery and with tree pollen allergens, but due to the structural similarity to a ginseng ribonuclease they have been classified as ribonuclease-like PR proteins (Moiseyev *et al.* 1994; Warner *et al.* 1994; Van Loon & Van Strien 1999). PR-10 proteins are suggested to be involved in general plant development programs as they are found to be constitutively abundant and are conspicuous in the *M. truncatula* root proteome, with several different isoforms (Mathesius *et al.* 2001). However, many reports point out a rapid accumulation of *PR-10* transcripts to a much greater extent in incompatible interactions to bacterial or fungal pathogens (Lo *et al.* 1999; Jwa *et al.* 2001; McGee *et al.* 2001). This suggests that PR-10 proteins represent a major component of the active plant defense repertoire.

PR-10 proteins are soluble acidic proteins of 155 to 160 amino acid residues with a striking conserved sequence motif GXGGXG throughout the members of this protein family. This motif is flanked by conserved lysine residues 13 amino acids upstream and 19 amino acids downstream that generally is known as a phosphate-binding loop (P-loop) typical of protein kinases or nucleotide-binding proteins. Presumably, this function as a binding side for an

RNA phosphate group indicates a ribonucleolytic activity. Moreover, the amino acid sequence predicts several phosporylation sites (Park *et al.* 2004).
Further analysis on the nucleotide sequence of potato *PR-10a* gene revealed a 30-bp regulatory promoter sequence (-135 to –105) that is required for transcription activation after elicitation or wounding stimuli. This particular region is recognized by two nuclear binding factors (*PR-10a* Binding Factors PBF-1/PBF-2). The multimeric PBF-1 is positively regulated by phosphorylation and strongly associated with *PR-10a* gene activation. Additionally, a protein kinase is involved in the mechanism that leads to activation of *PR-10a* gene expression (Després *et al.* 1995). Furthermore, two WRKY transcription factors involved in *PR-10* gene regulation identified in parsley are likely to be the counterparts of potato PBF-1/-2 (Eulgem *et al.* 1999).

The precise function of PR-10 proteins remained for a long time unknown and still is under discussion. However, a very recent analysis by Park *et al.* (2004) confirmed the presumed mechanisms. The authors clearly demonstrate that a PR-10 isolated from hot pepper (CaPR-10) with high sequence similarities to the corresponding sequence of *M. truncatula* and *Solanum tuberosum* (potato), exhibits antifungal and antibiotic activity. Furthermore, the presumed RNase was shown to be active against foreign viral or fungal RNA and also against hot pepper or yeast RNA. CaPR-10 is considered to function as a RNase type I, but its molecular mass of 18 kDa is more characteristic for a RNase type II.
Another very significant finding was the detection of a phosphorylated form of CaPR-10 in 1-DE around 19 kDa, which showed a nearly 5-fold enhanced cleavage activity as compared to the non-phosphorylated 18 kDa CaPR-10 form (Park *et al.* 2004). The change in molecular mass seems too large to be explained by phosporylation events, but treatment with alkaline phosphatase led to the decomposition of the 19 kDa protein into the 18 kDa form. This provides evidence that the 19 kDa form really is a phosphorylated CaPR-10.

The 2-D maps as presented in chapters 3, 4 and 5 also display in that previously mentioned gel region (MW –range of 16 to18 kDa, p*I*-region of 4.7 to 5.0) pairs of protein spots among this set of PR-10 proteins, consisting of spots with a slightly higher molecular mass (spots 1, 4 and 5) accompanied by spots below them with lower molecular mass (spots 2, 3, 6 and unlabeled spots of this set, respectively). These spots differ in molecular mass by approximately 0.3 to 0.8 kDa (chapter 3). Taking into account that phosphorylation events will lead to a p*I*-shift on the 2-D maps towards a more acidic p*I* position, then two spots on

top of each other are unlikely to represent non-phosphorylated and phosphorylated forms of one protein. Hence, changes in MW due to phosphorylation events in this particular region of 2-D maps result in smaller distinctions and the phosphorylated form might represent the corresponding non-phosporylated form that is positioned a little beneath and switched towards basic p*I*. The allocation of corresponding proteins among one protein family with a high sequence similarity is extremely limited as protein identification was achieved by PMF analysis and phosphorylation events are believed to be hardly detectable by this technique.
However, further analysis revealed that it was mostly the slightly larger protein species described above that were induced upon *A. euteiches* infection, suggesting that they are detected in a state of active cell defense primarily to cleave extrinsic RNA. On the other hand, the spots of lower molecular mass might represent those MtPR-10 proteins that were found to be constitutively abundant in the *M. truncatula* root proteome (Mathesius *et al.* 2001), although their abundance is also significantly increased upon *A. euteiches* infection (chapter 3). It becomes evident that the activated proteins represented by the spots 1, 4 and 5 are detectable in much higher abundance in the highly susceptible F83.005-5 line as compared to the moderately susceptible A17 line (chapter 4). By contrast, these particular protein spots are barely detectable in 2-D gels of the more resistant F83.005-9 line indicating a low cellular stress status (chapter 4). These findings support the hypothesis that PR-10 proteins represent cytosolic “reporter-proteins” which are considered to indicate the extent of pathogenic penetration. Moreover, these proteins are significantly down-regulated in mycorrhizal plants that were subsequently infected with *A. euteiches*, while they are clearly up-regulated in analogue analysis only infected with *A. euteiches* at the same evaluation time-point (chapter 5). These results may reflect a bioprotective effect of mycorrhization in relation to root pathogens and suggests that PR-10 proteins are involved in these processes.

The role of ABA in regulation of PR-10 proteins

Within the family of PR-10 proteins, some members have been shown to be highly induced not only under stress situations but also by exogenous ABA application (Luo *et al.* 1992; Iturriaga *et al.* 1994). ABA as plant signal molecule has a cardinal role in major physiological processes such as seed development, germination and dormancy, but also in adaptive responses to adverse environmental conditions and stresses (drought, cold and osmotic stresses) or in plant defense from invading pathogens. With regard to ABA- or stress-inducible proteins, generally three major classes can be distinguished: (i) proteins inducible by exposure to stress challenge and also by increased ABA levels, (ii) proteins inducible

specifically by stress but not by ABA, and (iii) proteins inducible only by ABA (Luo *et al.* 1992; chapter 3).

The detected members of PR-10 proteins that were induced upon *A. euteiches* infection were found to be at least partially regulated by exogenous ABA application (chapters 3, 4). These results confirm previous findings, that PR-10 proteins are inducible by plant signal molecules such as ABA, SA, JA or ethylene as well as under stress conditions (Park *et al.* 2004).

Interestingly, the MtPR-10 protein spots of higher molecular mass represented by spots 1, 4 and 5 were found to be barely up-regulated after ABA application; these spots became apparent only after subsequent *A. euteiches* infection (chapter 4). This indicates their specific accumulation or conversion into the active form is due to pathogen-related stimuli. The increased accumulation of the other protein spots (2, 3, 6 and unlabeled spots of this set) clearly demonstrate that several factors including altered ABA levels are involved in the cellular regulation of PR-10 proteins. ABA might function as a "mediator" between a certain stress signal and the plant's response.

Such an intermediate role has been well described by Li *et al.* (2002) for an ABA-activated protein kinase (AAPK) that regulates plasma membrane ion channels. Phosphorylation of this kinase is required for the specific binding of an RNA-binding protein (AAPK-interacting protein 1, AKIP) to messenger RNA encoding for dehydrin, a protein involved in cell protection under stress conditions. Hence, ABA-dependent activation of RNA-binding proteins might represent an effective plant cell regulation mechanism of protein dynamics during rapid sub-nuclear reorganization (Li *et al.* 2002). Presumably there exists an analoguous mechanism for the ABA-dependent activation of PR-10 proteins as the ABA-responsiveness of this protein class was clearly proved.

A proposed model of molecular events involved in PR-10 protein activation upon pathogen infection is displayed in Figure 1.

Model for a PR-10 protein-mediated plant defense response upon pathogen infection

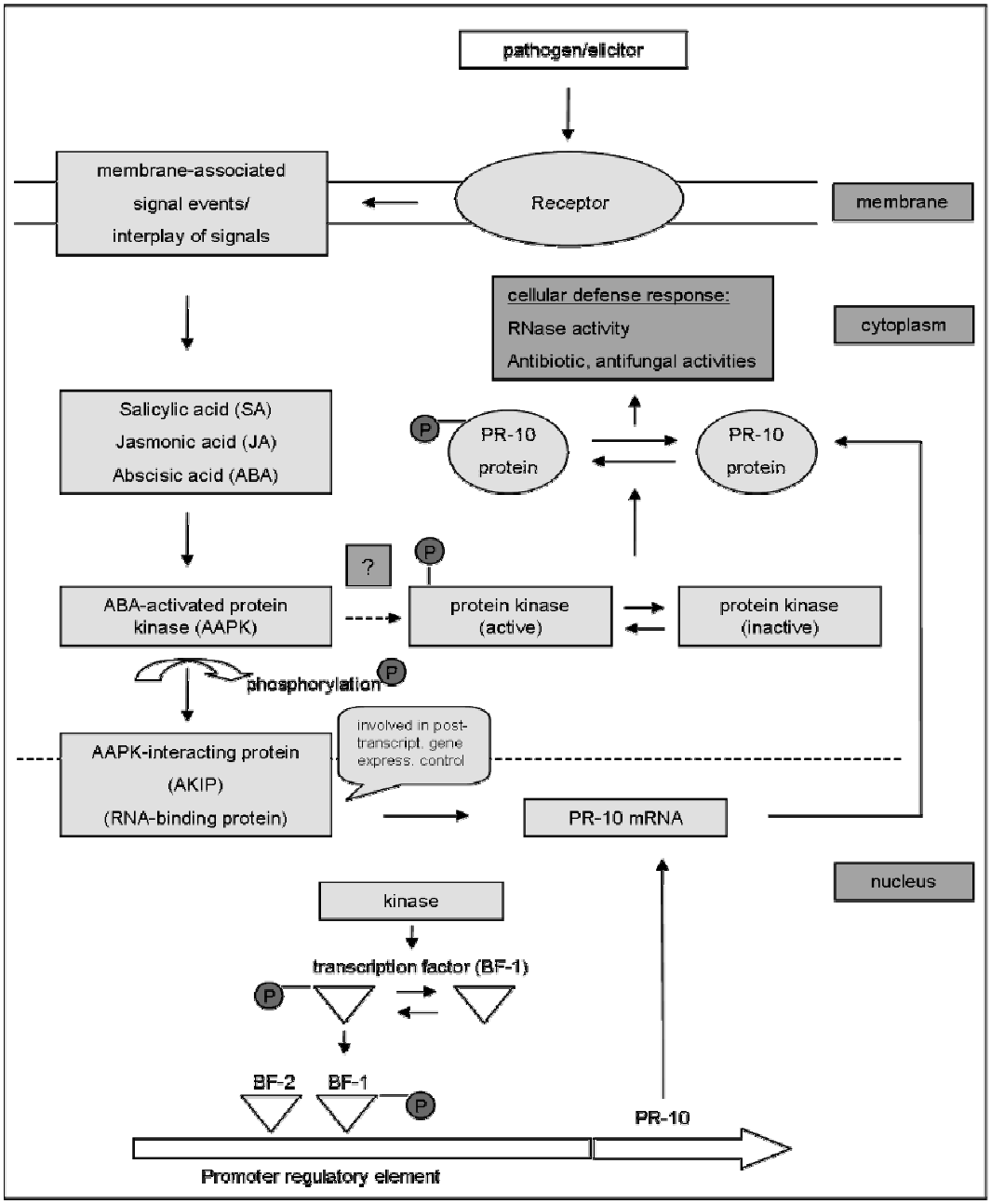


Figure 1. *Proposed molecular events of PR-10 activation* (based on investigations by Després *et al.* 1995; Eulgem *et al.* 1999; Li *et al.* 2002 and Park *et al.* 2004 upon different plant species).

Stress/elicitor stimuli are perceived by the cell that leads to activation of a PR-10 synthesis cascade. Through interplay of kinases and phosporylation events, DNA binding activity of transcription factors (BF-1/2) is effected. BF-1 binding is essential for *PR-10* transcription and subsequent protein synthesis. Cytoplasmic PR-10 regulation occurs by phosphorylation of a protein kinase or the proteins itself. Presumably altered ABA concentrations lead to the activation of a specific ABA-activated protein kinase (AAPK) (or possibly the aforementioned kinase -?-) and -after phosphorylation- of an AAPK-interacting RNA-binding protein (AKIP). Activated AKIP presumably interacts and regulates rapid PR-10 protein dynamics.

Outlook

Applying functional genomic approaches led to the identification of several genes and proteins that were induced in *M. truncatula* root cells upon infection with the oomycete *A. euteiches*. Focusing mainly on the root proteome revealed different regulation patterns of proteins which are involved in general plant defense responses as well as those that are considered to be specifically induced after infection with *A. euteiches*. The latter mentioned changes in protein profiles refer to those proteins that were found to be induced *de novo* or became greatly more abundant in the *M. truncatula* root proteome after pathogen infection. Further investigations should focus on these proteins in order to obtain a more detailed understanding of the molecular mechanisms underlying this plant-oomycete interaction. Some particularly promising areas for future research are discussed below.

Proteins that are involved in the ATP/ubiquitin-mediated proteolysis pathway:
Two proteasome alpha subunits were found to be induced in the more resistant *M. truncatula* accession F83.005-9 upon *A. euteiches* infection (chapter 4) as well as in 2-D gels of mycorrhizal A17 roots (non-infected or infected with the pathogen, chapter 5). Proteasomes represent the major cytosolic enzyme complex for protein degradation (Kim *et al.* 2003). As ubiquitylation of proteins was found to be involved in the defense response signaling of *R* genes, the induction of these proteins provides a first hint to a developed plant resistance or to participate in bioprotective mechanisms (Peart *et al.* 2002; Sullivan *et al.* 2003). The precise role of the proteasome machinery in plant defense could be further investigated within the *M. truncatula - A. euteiches* interaction.

Functional analysis of PR-10 proteins by a RNA-interference (RNAi) approach:
Investigations on the role of PR-10 proteins in the interaction between *M. truncatula* and *A. euteiches* have already been initiated. To determine whether an altered abundance of these proteins leads to significant changes during *A. euteiches* infection, a post-transcriptional gene silencing (PTGS) approach mediated by RNA interference (RNAi) was initiated. Therefore, a transformation vector (pFGC 5941, Invitrogen Carlsbad, CA, USA) capable of producing a *PR-10* dsRNA was constructed. Primers [(1)] were deduced from highly conserved *PR-10* sequence regions, which were identified after alignment of different *M. truncatula* PR-10 sequences (encoded by TCs 29943, 31868, 31869, 31872 and 39287; chapter 3). Amplification products were cloned into the pFGC 5941 vector. The transfer of this vector

construct into *M. truncatula* A17 roots was achieved by *Agrobacterium rhizogenes*-mediated root transformation as described by Boisson-Dernier *et al.* (2001) and transgenic hairy root cultures were obtained (Figure 2B). Control roots were transformed with the empty pFGC 5941 vector. To proof transformation of the root cultures with the PR-10 RNAi construct, DNA was isolated from the root cultures and used as template for PCR using a combination of vector-specific and the *PR-10* primers (Figure 2A). Evaluation via 2-D PAGE of total root protein of the transgenic hairy roots (non-infected and infected with *A. euteiches*) as compared to 2-D maps of control roots will show whether this PTGS approach led to a reduced abundance of PR-10 members.

Hypothetically, such a reduction in *PR-10* expression or protein abundance presumably might change the susceptibility upon *A. euteiches* infection as a result of an altered plant defense response.

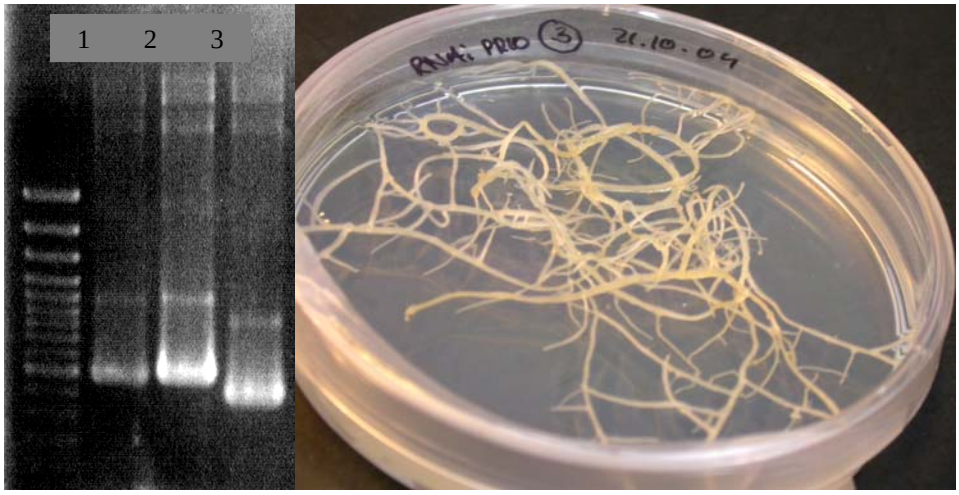


Figure 2. A) PCR-Amplification of PR-10 fragments in sense and anti-sense direction with a combination of (pFGC-5941) vector-specific primers (lanes 1+2) and with a gene-specific primer (lane 3). B) Transgenic hairy root cultures, growing on M-medium.

(1): PR10_for. (5'-3'): -GGG GAC AAG TTT GTA CAA AAA AGC CTC TGT TCC TCC TGT TAG GCT CTT CAA-
PR10_rev. (5'-3'): -GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAC TTA GAT TAT AAT CAC CTT TGG-

Phosphoproteomic approach to detect activated PR-10 proteins:

Park *et al.* (2004) showed that phosphorylation of PR-10 proteins is required to enhance the RNase activity and that the phosporylated proteins appear in higher molecular weight as a 19 kDa fragment. In the studies presented here, mainly PR-10 protein spots with higher MW were found to be induced upon *A. euteiches* infection (spots 1, 4 and 5). Presumably these protein spots represent the active or phosphorylated forms. Further analysis using approaches to detect phosphorylated proteins in 2-D gels (for example by use of phosphoprotein gel staining methods) is now essential to confirm this hypothesis. These data should provide a more detailed understanding of PR-10 expression upon *A. euteiches* infection.

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Appendix

Publications list

Nyamsuren O, **Colditz F**, Rosendahl S, Tamasloukht M, Bekel T, Meyer F, Küster H, Franken P, Krajinski F. 2003. Transcriptional profiling of *Medicago truncatula* roots after infection with *Aphanomyces euteiches* (oomycota) identifies novel genes upregulated during this pathogenic interaction.
Physiological and Molecular Plant Pathology **63:** 17-26.

Colditz F, Nyamsuren O, Niehaus K, Eubel H, Braun H-P, Krajinski F. 2004. Proteomic approach: Identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*.
Plant Molecular Biology **55**: 109-120.

Colditz F, Braun H-P, Jacquet C, Niehaus K, Krajinski F. 2004. Comparison of root proteome profiles of different *Medicago truncatula* lines and ABA-treated plants indicates proteins involved in susceptibility and resistance to *Aphanomyces euteiches.*
Under revision.

Colditz F, Krajinski F. 2004. Proteome analysis of the tripartite interaction between *Medicago truncatula* roots, *Glomus intraradices* and the parasitic oomycete *Aphanomyces euteiches* reveals proteins that are correlated to the bioprotective effect.
In preparation.

Posters and abstracts

Colditz F, Nyamsuren O, Niehaus K, Eubel H, Braun H-P, Krajinski F. 2004. Proteom-analyse zu Charakterisierung der pathogenen Interaktion zwischen dem Oomyceten *Aphanomyces euteiches* und Leguminosen.
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5th European Conference on Grain Legumes, 7-11 June 2004, Dijon, France.

Oral presentations

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Declaration

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

Furthermore, I declare that the work presented in this PhD thesis has not been published elsewhere previously and has not been submitted as a diploma thesis or any other examination thesis.

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

Erklärung

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

Des weiteren versichere ich, dass die vorliegende Promotionsarbeit noch nicht veröffentlicht wurde, weder als Diplomarbeit noch in einer anderen Form als Prüfungs- oder Studienarbeit.

Die Richtigkeit meiner Angaben bestätige ich mit meiner Unterschrift.

Hannover, den