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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-

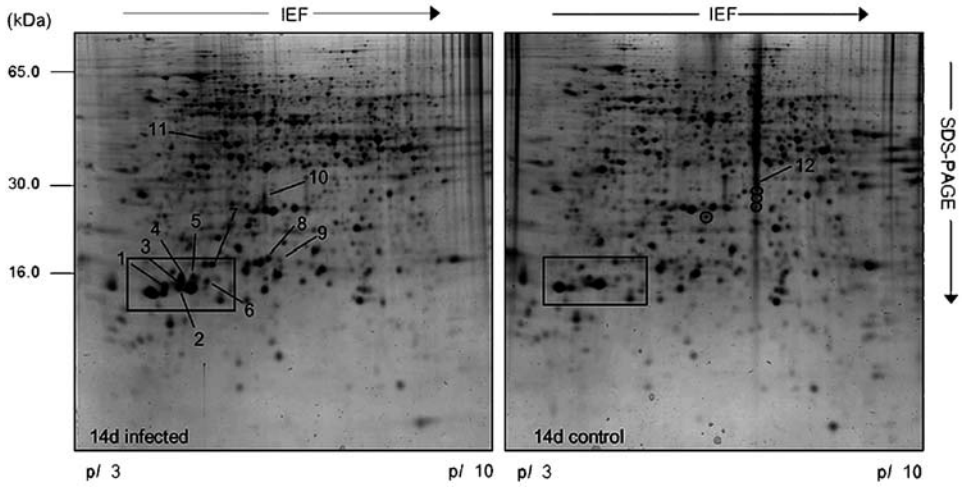

Figure 1. The 2-D resolution of the *M. truncatula* root proteome infected/non-infected with *A. euteiches*, 14 days after inoculation. Differentially expressed proteins that were identified in this study are labelled with arrows and numbered. Four proteins of the control gel which were detected as differentially 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

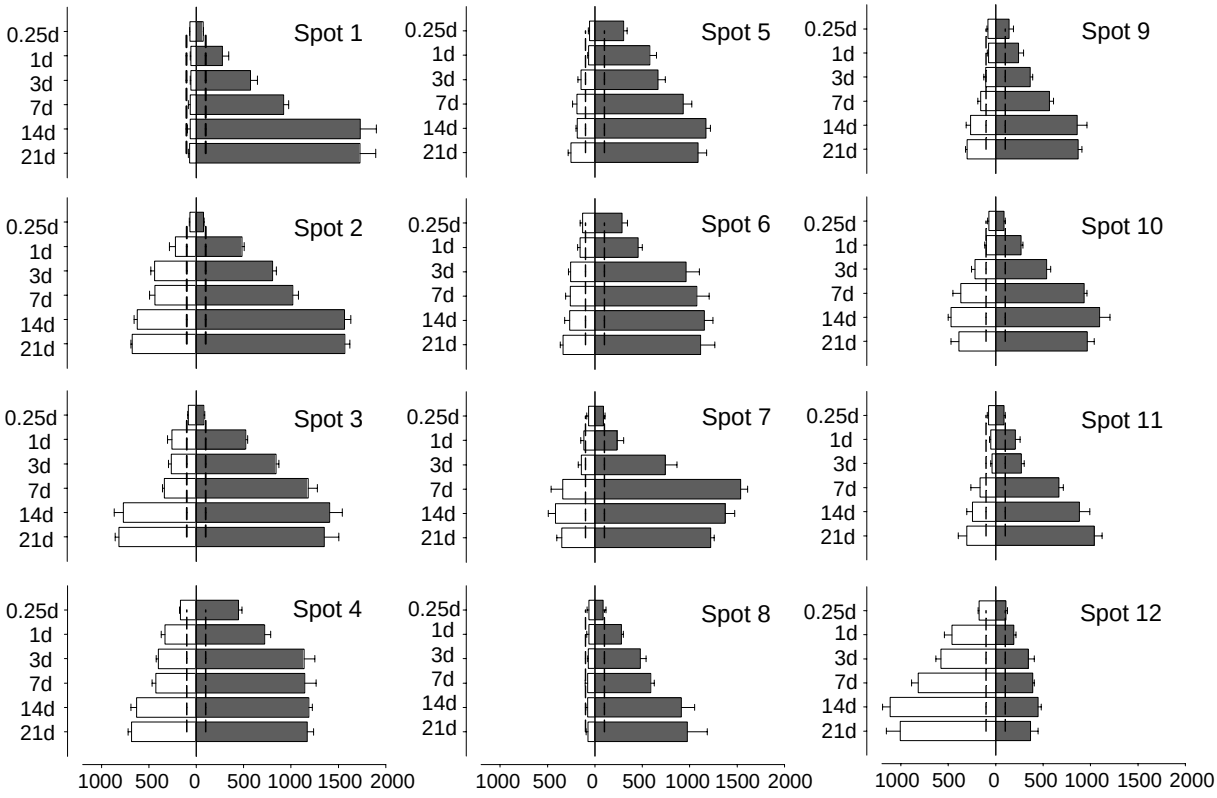

Figure 2. Abundance of proteins 1–12 expressed differentially after infection with *A. euteiches*. Time course of changes in protein abundance for proteins 1–12 after infection of *M. truncatula* roots with *A. euteiches*. Protein abundance was quantified by densitometric units, LAU. Grey columns represent 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

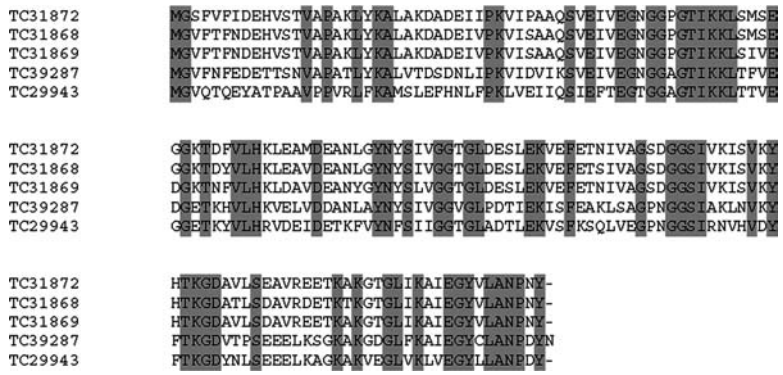

Figure 3. Alignment of deduced amino acid sequences of identified proteins number 1–6. The MtGI-IDs of the corresponding amino-acid sequences are indicated.

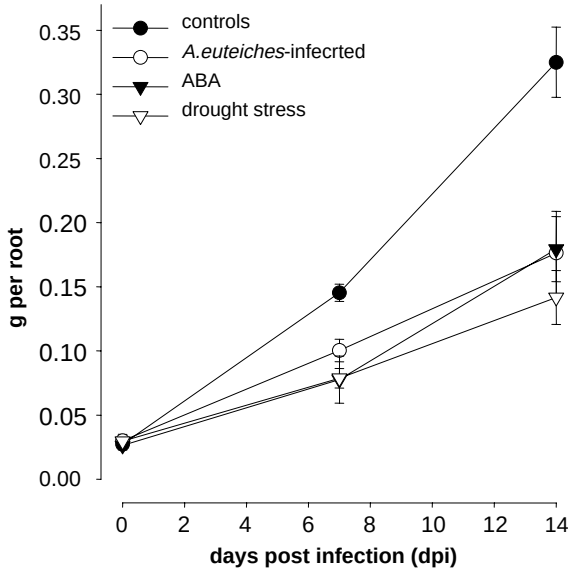

Figure 4. Fresh weight development of roots from control plants, *A. euteiches* infected plants, ABA-treated plants and drought stressed plants.

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

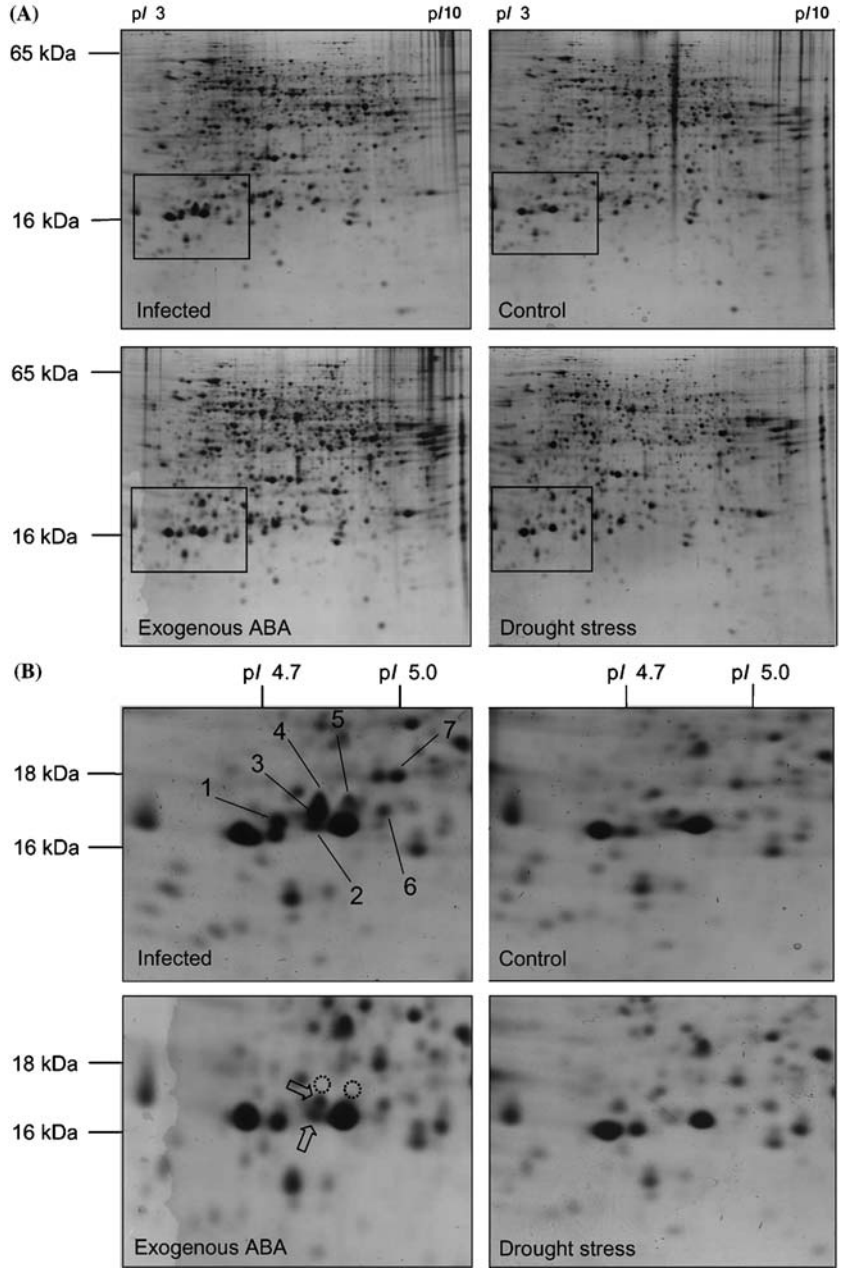

Figure 5. (A) Entire maps, and (B) detailed maps (molecular mass range of 16–18 kDa, p*I*-range of pH 4.7–5.0, boxed in (A) of Coomassie stained 2-D protein profiles of infected and non-infected *M. truncatula* roots after 14 days of infection (infected, control), roots treated with 75 μM exogenous ABA (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.

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