Induction of Distinct Defense-Associated Protein Patterns in *Aphanomyces euteiches* (Oomycota)–Elicited and –Inoculated *Medicago truncatula* Cell-Suspension Cultures: A Proteome and Phosphoproteome Approach

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A comprehensive proteomic approach was applied to investigate molecular events occurring upon inoculation of *Medicago truncatula* cell-suspension cultures with the oomycete root pathogen *Aphanomyces euteiches*. Establishment of an inoculation assay in the cell cultures allowed a direct comparison between proteins induced by elicitation with a crude culture extract of the oomycete and by inoculation with *A. euteiches* zoospores representing the natural infection carrier. Oxidative burst assays revealed responsiveness of the cell cultures for perception of elicitation and inoculation signals. The plant “elicitation proteome” resembles the “inoculation proteome” in early incubation stages and includes proteins induced following initial oxidative burst and defense reactions, but also proteins involved in the antioxidative system. However, approximately 2 days after incubation, the inoculation proteome differs drastically from the proteome of elicited cultures, where a cessation of responses assignable to *A. euteiches* elicitation occurred. The specific protein induction patterns of zoospore-inoculated cells appeared consistent with the protein induction identified in recent studies for an *A. euteiches* infection in planta and consist of three functional groups: i) pathogenesis-related proteins, ii) proteins associated with secondary phenylpropanoid or phytoalexin metabolism, and, particularly, iii) proteins assigned to carbohydrate metabolism and energy-related cellular processes. Phosphoproteomic analyses revealed consistent and specific activation of these defense-related pathways already at very early timepoints of inoculation, providing evidence that the identified protein profiles are representative for an established *A. euteiches* infection of *M. truncatula*.

The oomycete root pathogen *Aphanomyces euteiches* is accountable for a severe root rot disease in several legume crop plants, including pea, where it is considered to be the most destructive disease in areas with temperate or humid climate (Gaulin et al. 2007; Hagedorn 1989; Levenfors et al. 2003). The typical process of the disease is characterized by discolored honey-brown lesions at the host root tissue and subsequently appearing symptoms of root rotting accompanied by significant reductions of root mass. For the parasitic interac-

tion formed with the model legume *Medicago truncatula*, functional genomic analyses, mainly proteomic approaches but also an RNA interference (RNAi)-mediated gene-silencing approach on a group of defense-related proteins (PR-10), have been performed in order to characterize the molecular mechanisms underlying host cellular defense responses (Colditz et al. 2004, 2005, 2007; Nyamsuren et al. 2003).

In general, defense responses of plants against infection by invading pathogens are initiated by pathogen recognition via sensitive perception mechanisms. Concerning the molecular biology of plant pathogenesis, this initial process can be mediated either by a gene-for-gene interaction or by association of nonhost-specific microbial signal molecules, termed general elicitors or pathogen-associated molecular patterns (PAMPs) (Nürnberger et al. 2004; Zhang et al. 2000). Cultivar-specific resistance response occurs when a plant possesses a dominant resistance (*R*) gene and the pathogen expresses the complementary avirulence (*AVR*) proteins (Hammond-Kosack and Jones 1996). Usually, a so-called hypersensitive response (HR) will be induced: a rapid and localized cell death of the challenged host tissue to inhibit the spread of the pathogen (Dixon and Harrison 1994). By contrast, PAMP-mediated resistance represents a more adaptive defense system that adjusts the responses to the severity of infection and does not involve recombinatory events at the genetic level in general (Tellström et al. 2007). Thus, activation of a relatively vigorous response like the HR is not obligatorily because a variety of other defense responses are possible: induction of an oxidative burst; expression of defense-related genes and their products such as chitinases, glucanases, and other pathogenesis-related (PR) proteins; restructuring and reinforcement of the plant cell wall; and the production of antimicrobial secondary compounds like isoflavones, phytoalexins, and other phenolic depositions (Felix et al. 1991; Hammond-Kosack and Jones 1996; Peck et al. 2001).

Unlike the products of avirulence genes, general elicitors are expressed by a broad range of microorganisms and usually are indispensable for their biological fitness. Several of these elicitor molecules have been identified and characterized, including the hepta- β -glucoside elicitor of the oomycete *Phytophthora sojae/megasperma* (Sharp et al. 1984), chitin derivatives from crude cell-wall extracts of higher fungi (Ren and West 1992), yeast invertase (Basse et al. 1992) or yeast extract-derived elicitors (YE), glycoproteins like the *Phytophthora* CBEL (Gaulin et al. 2006) or even smaller peptide fragments (Pep-13) (Halim et al. 2004), and flagellin (flg22) representing the first isolated

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general elicitor from gram-negative bacteria (Felix et al. 1999). These elicitors have been applied frequently to cultured plant cells for the elucidation of defense responses. Effects of elicitation on primary as well as secondary metabolite accumulation have been characterized in several plant species at the transcript (Batz et al. 1998; Tellström et al. 2007), proteome, and metabolome level (Broeckling et al. 2005; Farag et al. 2008; Felix et al. 1991; Peck et al. 2001).

In addition to proteomic approaches, several investigations have implicated involvement of protein phosphorylation and dephosphorylation in the regulation of elicitor-stimulated defense responses since treatments of plant cells with pathogen-derived elicitors revealed rapid changes of the phosphoprotein profiles ("phosphoproteomics") (Dietrich et al. 1990; Felix et al. 1991; Lecourieux-Ouaked et al. 2000; Peck et al. 2001; Viard et al. 1994). By contrast, inhibition of protein phosphorylation results in the cessation of diverse defense-related pathways such as medium alkalization, generation of reactive oxygen species (ROS), induction of defense gene expression, phytoalexin synthesis, and the HR-like cell death (Grosskopf et al. 1990; Suzuki et al. 1995, 1999). Hence, proteins differentially phosphorylated in response to microbial elicitation are very likely components of signal transduction pathways following microbe and elicitor perception.

At this toehold, cell cultures of plants represent an appropriate and convenient medium for applied proteomic studies. A two-dimensional gel electrophoresis (2-DE) approach was car-

ried out to identify upstream phosphorylation pathways in tobacco cells after exposure to the oomycete *Phytophthora* spp. elicitor cryptogein (Lecourieux-Ouaked et al. 2000). Peck and associates (2001) established a comprehensive phosphoproteome map for *Arabidopsis* cell cultures in response to bacterial and fungal elicitors. For *M. truncatula*, proteome reference maps for cell suspension cultures have been established (Imin et al. 2004; Lei et al. 2005; Watson et al. 2003).

In the studies presented here, we investigated the parasitic interaction formed between the oomycete root pathogen *A. euteiches* and *M. truncatula* using cell-suspension cultures deriving from *M. truncatula* A17 root cells. By inoculating them with an *A. euteiches* zoospore solution on the one hand, which mediates the initial infection processes in the soil, and applying a crude *A. euteiches* elicitor extract obtained from liquid cultures of the oomycete on the other hand, we were able to distinguish between host molecular defense responses of the cells' "inoculation proteome" and the "elicitation proteome". Therefore, we established *A. euteiches* infection-like stages in the zoospore-inoculated *M. truncatula* cell cultures. Elicitor accumulation was achieved by collecting the culture medium, wherein the pathogen grew during a 10-day period. Proteomics was combined with phosphoproteomic analyses in order to comprehensively characterize infection-specific protein patterns of early defense-related signaling in *M. truncatula*.

RESULTS

Experimental design.

To investigate the pathogenic interaction formed between *M. truncatula* and the oomycete root pathogen *A. euteiches*, liquid suspension cell cultures generated from *M. truncatula* 'Jemalong A17' roots were exposed independently to two different fractions deriving from *A. euteiches* cultures. i) Cell cultures were inoculated with a zoospore inoculum of *A. euteiches*. ii) Cell cultures were elicited with a crude elicitor obtained from *A. euteiches* culture medium (maltose-peptone [MPB]), wherein the pathogen was cultured over a 10-day-period. At this time-point, *A. euteiches* cultures showed a linear growth (days 8 to 10) after a period of marked increases of fresh weights between days 5 and 8 (Fig. 1A). From day 5 on, maturing of zoosporangia is initiated (Fig. 1A, asterisk) but release of zoospores has not been induced (discussed below). At day 10, cultures were filtered to remove *A. euteiches* mycelia before usage as crude elicitor extract. Additionally, 10-min-boiled culture medium was used for culture elicitation in a separate experiment. To control cultures, lake water that did not contain zoospores (control to i) and pure MPB growth medium in absence of *A. euteiches* cultures (control to ii) were added to the culture flasks. All fractions tested were applied in equal volumes (1 ml) to the cell cultures.

Cell viability (Vb), as determined using the trypan blue exclusion method, significantly decreased in the cultures treated with the zoospores inoculum compared with the controls (Fig. 1B).

For molecular analyses, biological samples of four independent culture flasks were harvested from both control and *A. euteiches* suspended cell cultures at different timepoints: 0.25, 1, 2, 3, and 4 days post treatments. For the analysis of protein phosphorylation, additional samples were collected at 1.5 h following treatments. Cells were subsequently extracted from the cell culture medium.

Measurement of ROS

in *M. truncatula* cell suspension cultures after inoculation with an *A. euteiches* zoospore suspension and elicitation with *A. euteiches* culture medium.

For verification of the responsiveness of *M. truncatula* cell cultures to *A. euteiches*, oxidative burst assays were performed

Fig. 1. A, Growth curve of *Aphanomyces euteiches* in liquid maltose-peptone (MPB) medium and **B,** *Medicago truncatula* cell viability (Vb) after *A. euteiches* inoculation and elicitation. **A,** Increases in fresh weights (Δ mg) of *A. euteiches* mycelia cultured in liquid MPB medium. Fresh weights of 10 independent cultures were measured for 10 days at 24-h intervals and increases to the fresh weights of the initial inoculums were determined. Formation of sporangia was initiated at day 5 of cultivation (marked with an asterisk). **B,** *M. truncatula* cell viability as determined via the trypan blue (TB) exclusion test. After different treatments, Vb (%: ratio of viable cells to TB-stained dead cells) was calculated by counting 500 cells. Treatments: *A. euteiches* zoospore inoculum (white triangles), *A. euteiches*-filtered culture medium (white diamonds), heat-treated *A. euteiches*-filtered culture medium (gray diamonds), negative controls (lake water [black triangles] and MPB medium [black diamonds]). The data shown are the results of four biological replicates.

and established as a test system to evaluate signal perception deriving from *A. euteiches* cultures (500 µl of lake water containing 125,000 zoospores per culture flask and MPB culture medium in equal volume, respectively). Additionally, heat-treated *A. euteiches* culture medium was applied to the cell cultures.

All fractions were added independently to discrete cell cultures and a luminol-based chemiluminescence assay was performed to determine the production of hydrogen peroxide (H_2O_2) during a period of 1 h after application. Again, four biological replicates were tested. As positive control, the well-established plant defense reaction elicitor invertase was added to the cultures, applied in a final concentration of 50 µg ml⁻¹. As negative controls, lake water that did not contain zoospores and pure MPB medium in the absence of *A. euteiches* cultures was used.

Addition of invertase led to the highest measured values for the production of H_2O_2 (Fig. 2), with an average maximal concentration of 4.22 (±0.23) µM at 10 min after stimulation. Compared with the positive controls, *A. euteiches* zoospore inoculation and elicitation with culture medium both induced moderate oxidative burst reactions, reaching approximately half of the maxima of the positive controls at 15 min post treatments; maximal averaged values were 2.27 (±0.16) µM H_2O_2 for the zoospores and 1.88 (±0.14) µM H_2O_2 for *A. euteiches* culture medium, respectively. After attaining the peak of maximal H_2O_2 production, values for the oxidative burst decreased continuously but were measurable up to 55 min after treatment. Interestingly, even the heat-treated medium in which *A. euteiches* had been cultured induced an oxidative burst reaction, reaching 62% of the maxima of the non-heated culture medium (1.17 [±0.14] µM H_2O_2 at 15 min). By contrast, lake water and MPB culture medium did not stimulate an increased H_2O_2 production in any of the measurements carried out. In addition, application of 3- and 5-day-old culture filtrate of *A. euteiches* did not result in a measurable H_2O_2 production (data not shown).

Microscopic analysis of *A. euteiches* inoculation in *M. truncatula* cell-suspension cultures.

In order to evaluate germination and growth of *A. euteiches* in the inoculated cell-suspension cultures as well as any association established between both organisms, microscopic studies of the cell cultures at different timepoints after inoculation with the zoospores were performed.

Microscopic pictures from *A. euteiches*-inoculated *M. truncatula* cell cultures are shown in Figure 3. After inoculation, cell cultures were allowed to stand for 1.5 h in the dark in order to enable an active movement of zoospores toward the plant cells. During this initial period, mobile (biflagellate) zoospores (Fig. 3B and C) released from zoosporangia (Fig. 3A) were detectable in proximity to the plant cells (Fig. 3C). To avoid effects of oxygen deficiency, plant cultures then were carefully transferred on a swiveling table to gently pivot them during another incubation of 3.5 h in the dark. Within this time period, encysting and germination of cysts was initiated (Fig. 3D). Associations, whether between germinated cysts and plant cells or direct contacts of the germ tube tips to the plant cells, were observed frequently (Fig. 3D and E). At this stage of development, cell cultures were replaced on the culture shaker in the dark. Further microscopic studies (>1.5 days postinoculation [dpi]) revealed that hyphae growth had continued, building on a ramified mesh of absorptive mycelia that encompass the plant cells (Fig. 3D). Thus, an associative cell-to-cell network was formed where, especially, aggregates of plant cells were incorporated. From 3 dpi on, plant cells appeared significantly challenged from pathogen inoculation, showing brown discolorations and cell death at 4 to 5 dpi

(shown also in Fig. 1B). In contrast, cells of cultures elicited with the crude elicitor exhibited no obvious differences compared with the mock-inoculated cells (shown also in Fig. 1B).

Thus, inoculation of *M. truncatula* cell suspension cultures with *A. euteiches* zoospores led to infection-like profiles, as are known for plant-pathogenic root-infecting oomycetes (Birch et al. 2006; Gaulin et al. 2007; Kamoun 2006). However, no formation of appressoria and haustoria could be observed in the cells of the suspension cultures.

2-DE analysis of *M. truncatula* cell suspension cultures elicited and inoculated with *A. euteiches*.

For the monitoring and identification of differences between protein profiles of *A. euteiches*-elicited and -inoculated *M. truncatula* cell suspension cultures and those of control cultures, proteome maps of extracted culture material harvested at the timepoints 0.25, 1, 2, 3, and 4 days after incubation were produced using two-dimensional (2-D) gel electrophoresis. Therefore, material from four independent biological experiments was taken into account.

2-DE revealed consistent and reproducible resolution of protein patterns comprising approximately 1,000 prominent protein spots per gel in the cell suspension cultures according to the electrophoresis parameters applied (isoelectric focusing [IEF] range of pI 3 to 10 and M_r range of 10 to 100 kDa). The proteomic profiling led to the identification of protein patterns appearing consistently at early stages of an *A. euteiches* inoculation (0.25 to 1 dpi) that differ from protein patterns appearing consistently at late inoculation stages (3 to 4 dpi). The 2-dpi timepoint represented a transitional period between early and late inoculation stages, where proteins of both groups could be found induced.

Proteome maps show *M. truncatula* cell-suspension cultures noninoculated and inoculated with *A. euteiches* zoospores at an early inoculation stage (Fig. 4A, 0.25 dpi) and a late inoculation stage (Fig. 4B, 3 dpi) compared with 2-D maps of cell cultures nonelicited and elicited with *A. euteiches* culture medium at equal timepoints after treatments (Fig. 4C and D, respectively). Evaluation of gels for both early and later inoculation stages revealed independent induction patterns of 19 proteins to be differentially abundant in each time period (Fig. 4A,

Fig. 2. Induction of oxidative burst in *Medicago truncatula* suspension-cultured cells after treatment with invertase and two *Aphanomyces euteiches* fractions. Cells were treated with invertase (gray squares) at 50 µg ml⁻¹, with equal volumes of *A. euteiches* zoospores in lake water (125,000 zoospores; white triangles), *A. euteiches*-filtered culture medium (white diamonds), heat-treated *A. euteiches*-filtered culture medium (gray diamonds), or lake water representing the negative control to the zoospores treatment (black triangles) and maltose-peptone (MPB) medium as negative control to the *A. euteiches* culture medium (black diamonds), respectively. Production of H_2O_2 was determined utilizing the luminol-based chemiluminescence assay. The shown data are the results of four biological replicates.

spots 1 through 19, and B, spots 20 through 38). Moreover, protein spots were found induced compared with the corresponding protein spots of mock-inoculated control gels.

After elicitation of cells with *A. euteiches* culture medium, 10 protein spots were found to be differentially induced in gels of initial elicitation stages (Fig. 4C) and 6 proteins for subsequent elicitation stages (Fig. 4D). Interestingly, only three proteins were found to be de novo induced during early elicitation compared with proteins induced after inoculation with *A. euteiches* zoospores (Fig. 4C, spots 39 through 41). The same was the case for later elicitation events (Fig. 4D, spots 42 through 44). After elicitation with the heat-treated *A. euteiches* culture medium, a reduced number of protein spots was found induced: seven protein spots at 0.25 days (spots 3, 4, 6, 10, 11, 40, and 41) and four protein spots at 3 days (spots 3, 41, 43, and 44) post treatments (Fig. 4C and D; corresponding spots are labeled with asterisks).

In total, 44 different protein spots were found induced after treatments with *A. euteiches*, whereof 38 proteins were induced after inoculation with the zoospore inoculum while only 16 proteins were induced after elicitation with culture medium, of which 11 proteins also appeared after application of the heat-treated elicitor. In gels of mock-inoculated controls, two proteins could be detected showing constantly increased abundances compared with gels of *A. euteiches*-inoculated cells (Fig. 3, M1 and M2).

Mass spectrometry-based identification of proteins induced after elicitation and inoculation with *A. euteiches*.

For identification of proteins that were induced after *A. euteiches* inoculation and elicitor treatment as labeled in Figure 4,

tryptic in-gel digestion and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analyses were carried out. Subsequent alignment of the resulting peptide mass fingerprints (PMF) to the *M. truncatula* expressed sequence tag (EST) sequence database (*Medicago truncatula* Gene Index [MtGI]) led to the identification of all 44 proteins that were found induced in *M. truncatula* cell suspension cultures after *A. euteiches* inoculation. By contrast, additional blast analysis of all sequences to the “Aphano DB” EST database (Madoui et al. 2007) resulted in no matches found for any sequence analyzed. Hence, all proteins identified were regarded as being of plant origin.

In Table 1, the identified proteins are listed according to their specific induction, displaying the timepoints of their occurrence protein induction at early stages of *A. euteiches* zoospore inoculation (spots 1 to 19) and protein induction at later inoculation stages (spots 20 to 38) (both stages are shaded dark gray), protein induction at the transitional 2-dpi timepoint (shaded in light gray) of all 38 proteins, additional induction of proteins after *A. euteiches* elicitor treatment (spots 39 to 44), and protein induction of mock inoculation (M1 and M2).

The identified induction pattern for early stages of *A. euteiches* inoculation comprises proteins involved in pathogen recognition and initial defense responses, including those related to ion homeostasis, production of ROS, signal transduction, cell wall restructuring, and redox reactions: spot 2, with sequence similarities to a plasma membrane-type ATPase 9; superoxide dismutase precursor (spot 6); In2-1 protein/glutathione S-transferase (GST) (spot 3); phospholipid hydroperoxide glutathione peroxidase (spot 7); two voltage-dependent anion channel porins (VDAC), 1.3 and 1.1 (spots 10 and 11); and a

Fig. 3. A through D, Microscopic analyses of *Aphanomyces euteiches* zoospore-inoculated *Medicago truncatula* cell cultures. A, *A. euteiches* zoosporangia (zs). B, Mobile (biflagellate) zoospore (flagella which are easily identified in the microscope are not visible here). C, Zoospore (zs) appearing in proximity of plant cell (pc). D, Germinating cyst (gc) forming a germ tube; contact zone (cz) between germ tube and plant cell. E, Germinating cyst in contact with plant cell. F, Formation of a ramified mesh of absorptive mycelia that encompass the plant cells. *A. euteiches* hyphae (h) are partially covered by plant cells as marked in gray.

Fig. 4. Proteome maps of *Medicago truncatula* A17 cell suspension cultures, mock inoculated (controls) and incubated with *Aphanomyces euteiches* zoospores and culture medium. Gels for **A** and **C**, an early timepoint (0.25 days) and **B** and **D**, a late timepoint (3 days) post treatments are shown. **A** and **B**, Two-dimensional (2-D) gels of cell cultures inoculated with lake water and with *A. euteiches* zoospores at **A**, 0.25 and **B**, 3 days postinoculation (dpi). Differentially induced proteins that were identified by mass spectrometry are numbered 1 to 38, M1, and M2. **C** and **D**, 2-D gels of cell cultures elicited with maltose-peptone (MPB) culture medium and *A. euteiches* MPB cultures (filtered) at **C**, 0.25 and **D**, 3 dpi. Differentially induced and identified proteins are numbered. Protein spots labeled with asterisks were found induced after treatment with heat-treated *A. euteiches* culture medium. All gels are stained with Coomassie Brilliant Blue.

Table 1. Analysis of the inoculation proteome and elicitation proteome of *Medicago truncatula* cell-suspension cultures inoculated with *Aphanomyces euteiches* (0.25 to 4 days; 1.5 h) with protein identification for induced proteins after early and late *A. euteiches* cell inoculation stages, cell elicitation, and mock inoculation (1–44, M1, and M2)

Prot. ^b	TC no. ^c	Annot. ^d	Protein name	MW/pI	Cov. (%) / pep ^e	Days ^a				
						0.25	1	2	3	4
Early										
01	94217	P14710	Disease resistance response protein pi49 (PR10)	16.9/4.76	26/5	+++	++	+	–	–
02	96074	P19456	Theoretical protein similar to a plasma membrane ATPase 2, partial	17.8/5.70	39/4	++	++	–	–	–
03	100553	Q9FQ95	In2-1 protein/glutathione <i>S</i> -transferase	29.4/4.90	29/7	+++	++	+	+	+
04	EST334563	Q7XJJ7	<i>N</i> -acyl ethanolamine amidohydrolase, partial (39%)	29.7/5.14	27/4	+++	++	++	+	+
05	104406	–	Hypothetical protein	28.5/5.65	34/4	+	+	–	–	–
06	107039	P27084	Superoxide dismutase (Mn) precursor—garden pea	29.1/8.59	28/6	+++	+++	+	–	–
07	106328	Q8W259	Phospholipid hydroperoxide glutathione peroxidase	22.6/9.00	52/11	++	++	–	–	–
08	106859	Q9FVL0	Non-symbiotic hemoglobin 1 (MEDsa GLB1)	18.0/9.51	51/9	+++	+++	+	–	–
09	108603	P83291	NADH-cytochrome b5 reductase-like protein (B5R), partial (85%) (E.C. 1.6.2.2)	34.0/8.29	24/7	+++	+++	++	+	–
10	100708	Q6W2J3	Voltage-dependent anion channel porin (VDAC) 1.3	36.3/8.95	33/11	+++	++	+	–	–
11	100732	Q6W2J5	Voltage-dependent anion channel porin (VDAC) 1.1	36.2/9.02	28/10	+++	+++	++	–	–
12	100663	P12886	Alcohol dehydrogenase 1, partial (82%) (E.C. 1.1.1.1)	32.7/6.06	33/8	+++	+++	++	–	–
13	100593	Q93VR3	GDP-mannose 3,5-epimerase	43.2/5.88	25/8	++	++	+	+	–
14	94406	BAD12188	12-oxophytodienoic acid 10, 11-reductase (OPR)	45.5/6.33	19/7	++	+	+	–	–
15	106554	P30075	Chalcone synthase 4 (Naringenin-CHS 4) (CHS12-1)	39.7/8.57	21/6	+	+	+	+	–
16	100663	P12886	Alcohol dehydrogenase 1, partial (82%) (E.C. 1.1.1.1)	32.7/6.06	29/7	+++	++	++	–	–
17	106580	P46257	Fructose-bisphosphate aldolase, cyt. isozyme 2	43.7/7.21	30/7	+++	++	++	–	–
18	106518	P34922	Glyceraldehyde-3-phosphate dehydrogenase, cyt.	39.5/7.23	39/9	+++	++	++	+	–
19	98412	P48786	Pathogenesis-related homeodomain protein (PRHP), partial (4%)	37.7/9.57	25/6	++	++	+	–	–
Late										
20	106355	Q8L6K8	Pprg2 protein/PR-10-like protein	16.9/4.45	32/6	+	++	++	+++	+++
21	111449	AAR28024	TAF7, partial (69%)	15.8/4.76	26/4	–	–	–	+	+
22	100230	AAA34121	Pentameric polyubiquitin, partial (28%)	12.1/9.26	36/5	–	–	+	+	++
23	109209	P54152	Peptide methionine sulfoxide reductase (Protein-methionine-S-oxide reductase, Peptide Met(O) reductase, Fruit-ripening protein E4)	22.3/5.89	36/6	–	+	++	++	+++
24	94274	O81926	Thaumatococin-like protein PR-5b precursor (Fragment)	24.5/6.82	38/6	–	–	–	++	+++
25	101874	Q84ZV0	R 14 protein, partial (19%) (TIR-NBS-LRR class)	20.9/7.09	19/4	–	+	+	+	++
26	98399	P27484	Glycine-rich protein 2, partial (45%)	20.7/7.30	66/6	–	+	+	++	++
27	98399	P27484	Glycine-rich protein 2, partial (45%)	20.7/7.30	58/5	–	+	++	++	++
28	94281	Q9LK16	Isoflavone reductase (IFR) (2'-hydroxyisoflavone red.)	35.6/5.40	24/10	–	–	–	+	++
29	93954	Q9FT25	Probable pyridoxin biosynthesis protein PDX1 (pVPDX1)	36.0/5.63	23/6	–	–	–	+	++
30	108147	Q9LFG2	Diaminopimelate epimerase-like protein, partial (81%)	38.3/5.72	24/6	–	–	+	++	++
31	102424	BAB09856	2-Nitropropane dioxygenase-like protein	35.7/6.11	32/9	–	–	+	+	++
32	106842	P93327	Class I chitinase (EC 3.2.1.14)	35.9/7.85	25/6	–	+	++	+	+
33	109785	Q84T14	Vacuolar ATPase subunit E (fragment), partial (54%)	27.5/8.46	30/5	–	–	–	+	+
34	100429	O48905	Malate dehydrogenase cytoplasmic	39.5/7.25	24/8	–	+	++	++	+++
35	94614	Q8W4G8	Fructose-bisphosphate aldolase-like protein	42.7/8.34	34/8	–	++	++	++	++
36	103200	P93033	Fumarate hydratase 1 mitoch. prec. (fumarase 1)	56.6/8.84	23/7	–	+	+	+	++
37	107373	Q43621	Glutathione reductase cyt. (GR) (GRase) (GOR2)	62.0/8.51	24/8	–	+	+	++	+
38	101549	Q9SZX3	Argininosuccinate synthase, chloroplast precursor (citrulline–aspartate ligase), partial (51%)	60.9/6.34	37/9	–	+	++	++	++
Elicited										
39	106425	A45116	L-Ascorbate peroxidase cytosolic	27.1/6.72	60/13	++	++	++	+	–
40	102424	BAB09856	2-Nitropropane dioxygenase-like protein	36.0/5.98	34/8	+++	++	+	–	–
41	107346	Q6K9N6	Putative succinyl-CoA ligase (GDP-forming) β-chain, mitoch.	45.0/5.18	22/5	+	+	++	+	–
42	94259	O49235	2,4-D inducible glutathione <i>S</i> -transferase (E.C. 2.5.1.18)	25.6/6.24	45/11	–	+++	++	++	–
43	94796	O23960	Biotin carboxylase precursor (acetyl-CoA carboxylase)	60.0/7.59	22/12	–	+	++	+	–
44	100988	P25890	Cat2 catalase (E.C. 1.11.1.6)	60.7/8.72	25/13	–	+++	++	–	–
Mock										
M1	106899	Q9STY6	40 s ribosomal protein S20-like protein	13.9/9.36	44/6	+	+	–	–	–
M2	106692	P30707	60 s ribosomal protein L9 (gibberellin-regulated protein GA)	27.8/9.32	41/7	+	+	–	–	–

^a Protein induction index, representing relative in-gel protein abundances (always referring to the correspondent abundances in the control gels): – = no detectable differences in abundance or even absence of protein; + = slightly increased abundance, 1.5- to 2-fold increased; ++ = clearly increased abundance, 2- to 3-fold increased; +++ = highly increased abundance, >3-fold increased. Induction periods are highlighted additionally with gray-shaded boxes; dark gray indicates protein induction at early and later inoculation stages (spots 20 to 38) and light gray indicates protein induction at the transitional 2-dpi timepoint.

^b Protein numbers. Early = proteins induced at early inoculation stages, Late = proteins induced at late inoculation stages, Elic. = additional proteins induced specifically upon elicitation, and Mock = proteins induced in mock-inoculated controls:

^c Tentative consensus sequence (TC) or accession number.

^d Annotation number.

^e Percent covered and peptide number.

12-oxophytodienoic acid 10-11 reductase (OPR) (spot 14) of the octadecanoid pathway. Additionally, PR protein induction (spots 1, 8, and 19) was initiated as well as the induction of proteins associated with secondary phenylpropanoid metabolism involved in phytoalexins synthesis (spots 9 and 15).

Among the proteins induced at later *A. euteiches* inoculation stages, PR proteins became predominant: a Pprg 2/PR10-like protein (spot 20), a thaumatin-like protein PR-5b precursor (spot 24), and a class I chitinase (spot 32). Interestingly, with protein spot 25 (R14 protein), a Toll/Interleukin1 receptor nucleotide-binding site leucine-rich repeat (TIR-NBS-LRR)-type resistance (R-) protein could be identified. Moreover, enzymes involved in amino acid biosynthesis were found upregulated (spots 30 and 38) as well as another enzyme involved in secondary phenylpropanoid metabolism (isoflavone reductase [IFR], spot 28).

Further, a significant protein pattern was found to be particularly induced in cell cultures inoculated with the *A. euteiches* zoospores inoculums at both early and late inoculation stages, comprising enzymes of carbohydrate metabolism involved in energy-related cellular processes. Located in a specific gel area (32 to 43 kDa molecular weight, pI range of 6 to 8), it revealed inoculation-related induction together with spots 14 to 16, abundant in close proximity to this pattern. This functional cluster represents spots 12 and 16 (two isoforms of an alcohol dehydrogenase 1), spots 17 and 35 (fructose-bisphosphate aldolase-like protein), spot 18 (glyceraldehyde-3-phosphate dehydrogenase), spot 34 (malate dehydrogenase), and spot 36 (fumarate hydratase 1).

At early cell elicitation stages, clearly fewer proteins are found compared with early *A. euteiches* zoospore inoculation stages (Fig. 4A compared with C). The analyses revealed similar induction of protein spots 2 to 6, 10, and 11 that already were found induced at an early *A. euteiches* inoculation stage, including the superoxide dismutase precursor (spot 6) as putative key enzyme of early defense response, as well as the two VDAC porin-related proteins (spots 10 and 11). Interestingly, the mentioned proteins were also found induced after elicitation with the heat-treated medium. Additional induced proteins of cell elicitation are L-ascorbate peroxidase (spot 39) and a Cat2 catalase (spot 44) involved in detoxification processes of ROS, a 2,4-D-inducible glutathione S-transferase (spot 42), and two enzymes of carbohydrate metabolism (spots 40 and 41). In contrast to an *A. euteiches* zoospores inoculation, PR proteins as well as proteins associated with secondary phenylpropanoid or phytoalexin metabolism failed to appear consequently in the protein patterns of elicited *M. truncatula* cell cultures. Moreover, the already described protein cluster involved in carbohydrate metabolism and energy-related cellular processes (spots 12, 16 to 18, and 34 to 36) was continuously absent in all approaches carried out with elicited cell culture tissue.

In gels of mock-inoculated control cultures, two ribosomal proteins (spots M1 and M2) were found induced compared with *A. euteiches*-inoculated cell cultures.

Phosphoproteomic analysis of *M. truncatula* cell suspension cultures elicited and inoculated with *A. euteiches* using specific antibodies for detection of peptide phosphorylation.

In order to identify proteins involved in early defense-related signaling processes after *A. euteiches* inoculation, proteomic analyses combined with subsequent immunoblotting experiments using antibodies targeted against serine-, tyrosine-, and threonine-phosphorylated peptides were performed for an initial timepoint of 1.5 h post treatments.

Immunoblotted membranes for corresponding gels of *A. euteiches*-inoculated *M. truncatula* cell cultures (Fig. 5A, anti-

phosphoserine, -phosphotyrosine, and -phosphothreonine) and *A. euteiches*-elicited cells (Fig. 5B, anti-phosphoserine) were compared with those of the mock-treated controls (anti-phosphoserine). The membranes for *A. euteiches*-elicited cultures immunoblotted with anti-phosphoserine covered all labeled spots that appeared also on anti-phosphotyrosine and -phosphothreonine immunoblots of the corresponding cultures; thus, only anti-phosphoserine membranes are shown in Figure 5B identifying the three types of phosphorylations—also in the controls.

Obviously, the vast majority of phosphorylated protein spots could be detected in gels of *A. euteiches* zoospore-inoculated cell cultures: 41 different protein spots (P1 to P41) and an additional 6 protein spots that had been already found induced in 2-D gels of *A. euteiches*-inoculated cells (spots 20, 32, and 34 to 37) could be identified to be phosphorylated, representing 35 different *M. truncatula* proteins in total (Table 2). In comparison, analyses of *A. euteiches*-elicited cell cultures revealed detection of 13 phosphorylated protein spots representing 10 different *M. truncatula* proteins. Moreover, all these proteins were identified as being phosphorylated after *A. euteiches* inoculation. In both mock-inoculated controls, the same number of phosphorylated proteins was detected in the immunoassays: eight and seven protein spots representing six and five different proteins, respectively.

As can be assumed for peptide phosphorylation events in plant tissue, the vast majority of phosphorylated amino acids were serine residues: 41 of 47 phosphorylated protein spots exhibited phosphorylations of serine (Table 2). Nine serine phosphorylated protein spots also revealed tyrosine phosphorylations and seven were found to be secondary phosphorylated at threonine residues. Only six protein spots revealed specific threonine phosphorylations (Table 2).

Mass spectrometry-based identification of proteins detected in phosphoproteomic analyses.

A phosphoproteomic analysis using tryptic in-gel digestion and mass spectrometry-based protein analysis (MUGI) led to the identification of all phosphorylated proteins detected after *A. euteiches* inoculation. Again, no significant scores were found in additional sequence blasts performed with the “Aphano DB” EST database (Madoui et al. 2007).

Consistent with the results obtained from the 2-DE analyses, protein phosphorylation in cell cultures after *A. euteiches* zoospore inoculation appeared to be different from that of *A. euteiches*-elicited culture tissue in terms of i) the specific detection of phosphorylation events among PR proteins, ii) proteins associated with secondary phenylpropanoid or phytoalexin metabolism, and iii) proteins assigned to carbohydrate metabolism and energy-related cellular processes (Table 2).

Specific phosphorylation of proteins following *A. euteiches* zoospore inoculation of cell cultures was observed for a PR-10-like protein (spot 20) and a class I chitinase (spot 32) representing PR proteins, an NADH-cytochrome b5 reductase-like protein (P13), and three isoforms of an IFR (P15, P40, and P41) as members of secondary phenylpropanoid metabolism, and for several enzymes of carbohydrate metabolism such as alcohol dehydrogenase-like proteins (P9, P17, and P18), pyruvate dehydrogenase E1 (P14), fructose-bisphosphate aldolase-like protein (P19, P23, and spot 35), glyceraldehyde-3-phosphate dehydrogenase (P21 and P22), ENO 2 enolase (P25 to P29), formate-tetrahydrofolate ligase (P32), aconitate hydratase (P36), putative mitochondrial ATP synthase (P38), malate dehydrogenase (spot 34), and fumarate hydratase 1 (spot 36). Thus, compared with the 2-DE analyses of *A. euteiches*-inoculated cells, an exceeding number of protein spots related to carbohydrate metabolism was detected specifically phosphorylated after *A. euteiches* inoculation, located in the already mentioned gel area (32- to 43-kDa

A

A. euteiches infected
(Anti phosphoserine)

1.5 hpi

Mock (noninfected)
(Anti phosphoserine)

1.5 hours

A. euteiches infected
(Anti phosphotyrosine)

1.5 hpi

A. euteiches infected
(Anti phosphothreonine)

1.5 hpi

B

A. euteiches elicited (MPB)
(Anti phosphoserine)

1.5 hours

A. euteiches nonelicited
(Anti phosphoserine)

1.5 hours

Fig. 5. Immunoblot analysis of two-dimensional (2-D) gels from *Aphanomyces euteiches*-inoculated and -elicited cell cultures using antibodies against serine, tyrosine, and threonine phosphorylated peptides (1.5 h post treatments). **A.** Immunoblots of 2-D gels from *A. euteiches*-inoculated and mock-inoculated (control) cell cultures for anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine. Labeled protein spots identified by mass spectrometry are numbered: P1 to P41. **B.** Immunoblots of 2-D gels from *A. euteiches*-elicited and maltose-peptone (MPB) medium (control)-treated cell cultures for anti-phosphoserine. Labeled protein spots identified by mass spectrometry are numbered.

molecular weight, pI range of 6 to 8). Again, this phosphorylation pattern remained continuously absent in *A. euteiches*-elicited cultures as well as in all controls.

In addition, several spots representing the same proteins appeared phosphorylated: the ENO 2 enolase (five isoforms, represented by tentative consensus sequences (TC) 100309 and 105276), GST (four isoforms, represented by TC 94259 and 107452), L-ascorbate peroxidase (three isoforms TC 106425), and alcohol dehydrogenase (three isoforms, TC 103798 and 100664) (Table 2).

DISCUSSION

In the studies presented here, we expanded the investigations on elicitor-stimulated molecular defense responses to an analysis of inoculation-triggered defense reactions by applying infectious mobile zoospores from the oomycete plant pathogen *A. euteiches* to *M. truncatula* cell-suspension cultures. This approach allowed a direct comparison of protein patterns induced by *A. euteiches* inoculation with those induced by elicitation. In addition, comparison with protein profiles induced by in planta infections became possible. Although cultured cells may never be representative of all aspects of parasitic interaction, the cell cultures were shown to be competent for perception of the diffusible signals released by *A. euteiches*. Significant decreases in cell viability only occur after inoculation of *M. truncatula* cells with *A. euteiches* zoospores and indicate a fully compatible interaction. Thus, the *M. truncatula* cell-suspension cultures represent a simplified system suitable for monitoring and dissecting molecular events of early and late *A. euteiches* inoculation stages.

M. truncatula cell suspension cultures exhibit responsiveness for *A. euteiches* inoculation and elicitation signals.

An oxidative burst assay was applied to prove the responsiveness of the cell cultures for perception of *A. euteiches* inoculation and elicitation signals. Production of H₂O₂ was slightly higher after inoculation than after elicitation but relatively moderate compared with a treatment with invertase. The heat-treated *A. euteiches* elicitor exhibited induction of approximately 60% of the H₂O₂ maxima of the corresponding nonheated fraction. This indicates a heterogeneous composition of the crude elicitor extract containing heat-stable constituents, putatively cell wall oligomers of the oomycete, as well as heat-sensitive molecules (e.g., proteins that become inactivated by the heat-treatment). Elicitor samples harvested at earlier timepoints (2 to 5 days) did not induce any oxidative burst reaction when applied to the cell cultures. Because *A. euteiches* cultures reveal a period of rapid increases in fresh weights between days 5 and 8 of culturing, autolysis of the cultures and the release of proteolytic enzymes additionally acting as plant elicitors is highly unlikely. The potential of the elicitor (and even that of the heat-treated elicitor) to induce an oxidative burst that is comparable with the oxidative burst reaction of the zoospore fraction indicates that plant defense suppressors are not abundant in the elicitor fraction.

Microscopic analysis of *A. euteiches* zoospore-treated *M. truncatula* cell cultures revealed a compatible interaction under the conditions used. Therefore, an elaborated method for culturing the plant cells was established that allowed cell-to-cell association with the pathogen but minimized effects of oxygen deficiency. After germination of cysts, hyphae growth was observed to veer toward the plant cells and direct contact between germ tubes and plant cells was achieved. At later inoculation timepoints (>2 dpi), hyphae appeared in intimate contact with the plant cells by forming a cell-to-cell network. These findings correlate with the significant decrease of viable plant cells in the cultures. Because the association during infection requires living

host cells to complete the infection process (Kamoun 2006), this association resembles the intercellular hyphae network formed after *A. euteiches* infections in planta, although no appressoria or haustoria formation could be observed in the cultured cells.

However, the microscopic and oxidative burst assays provided the basis for a comprehensive analysis of the molecular events induced by inoculation compared with a sole elicitation under almost uniform and constant experimental standards.

Proteomic analysis reveals induction of distinct defense-associated protein patterns in *A. euteiches*-elicited and -inoculated *M. truncatula* cell cultures.

Although earlier studies on the pathogenic interaction formed between *M. truncatula* and *A. euteiches* identified a PR-10-like protein group involved in tolerance toward *A. euteiches* infection (Colditz et al. 2005, 2007), the present proteomic analysis allowed identification of proteins which are induced after inoculation but not after elicitation. Because no matches were found in the "Aphano DB" EST database (Madoui et al. 2007) for any sequence analyzed, all proteins identified were regarded as being of plant origin.

The proteomic differences found suggest distinct molecular defense responses in *M. truncatula* initiated after inoculation with the zoospores compared with an elicitation of the cells. A first hint is given by a significantly increased number of induced proteins after inoculation with the zoospores: whereas 38 proteins were found induced in gels of *A. euteiches*-inoculated cell cultures, only 16 proteins revealed induction after *A. euteiches* elicitation. Especially at later timepoints, protein induction in inoculated cell cultures was clearly enhanced compared with elicited cells (19/6 induced proteins). Furthermore, protein induction in elicited *M. truncatula* cell cultures has a more transient character and appears to be confined to 3 days. Thus, after initial stimulation of defense responses due to elicitation, the cell cultures were not further challenged. By contrast, protein abundances for the induced proteins at later *A. euteiches* inoculation timepoints (2 to 4dpi) showed slight but steady increases.

Protein induction at early *A. euteiches* inoculation and elicitation timepoints.

Qualitative evaluation of protein induction after *A. euteiches* incubation shows a set of seven proteins (numbers 2 to 6, 10, and 11) to be induced upon both applications (inoculation and elicitation) at early timepoints. These candidates are most likely involved in initial elicitor recognition and oxidative burst reaction. The other proteins (numbers 1, 7, 8, 9, and 12 to 19) were found specifically induced in the zoospore-inoculated cultures. They were abundant already at early timepoints but revealed their induction peaks at later inoculation timepoints and will be discussed below.

The production of ROS represents the major functional element of the oxidative burst reaction involved in primary pathogen defense, membrane restructuring, and defense gene expression as well as programmed cell death and senescence (Jones 2001; Teixeira et al. 2005). A complex antioxidant metabolic system comprising enzymes such as superoxide dismutases (protein 6), ascorbate peroxidases (protein spots 39, P11, P12, and P39), catalases (proteins 44 and P30), various members of GST (proteins 3, 42, P2, P3, P6, and P7, appearing as several isoforms), glutathione reductases (GR; proteins 37 and P30), and glutathione phospholipid hydroperoxide peroxidases (PhGPx; proteins 7 and P4) was found to be induced in the *M. truncatula* cell cultures to maintain cell redox homeostasis and to protect cells against potentially hazardous effects of ROS.

Protein no. 4 reveals similarities to *N*-acylethanolamine (NAE) amidohydrolases known as endogenous lipid- and mem-

brane-bound constituents of plant and animal tissues. In tobacco, they function as endogenous modulators of pathogen elicitor signaling (Tripathy et al. 2003). An elicitor-activated increase of the NAE level induced systemic-acting defense gene expression (e.g., phenylalanine ammonia-lyase [PAL]) within hours after treatment. High-affinity NAE-binding proteins were identified from *Arabidopsis thaliana* and *M. truncatula* tissue that were assumed to mediate the NAE-dependent PAL and defense gene activation (Tripathy et al. 2003). Putatively, the protein identified here is involved in a vegetative NAE-signaling pathway participating in elicitor perception and defense gene activation.

Proteins 10 and 11 were identified as voltage-dependent anion channel porins, VDAC1.3 and VDAC1.1. VDAC are generally considered to conduct the major metabolite transport across the mitochondrial outer membrane (OMM) (Heins et al. 1994; Lee et al. 1998; Wandrey et al. 2004). In plants, VDAC might also be located in the boundary membrane of glyoxisomes (Corpas et al. 2000). Analyses of the subcellular localization of *Lotus japonicus* LjVDAC1.1 and LjVDAC1.3 re-

vealed their presence at vesicular and plasma-membrane-associated nonmitochondrial structures (Wandrey et al. 2004). These findings indicate that VDAC might be related to secretory pathways also in vegetative tissues because VDAC fusion proteins were found to be associated to secretory vesicles in animals (Buettner et al. 2000). Interestingly, proteomic studies on isolated symbiosome membranes from legume nodules elucidated accessory involvement of VDAC in symbiotic metabolite exchange between plants and interacting microbes (Wienkoop and Saalbach 2003). Hence, in addition to their ubiquitous targeting to the OMM, a participation of VDAC in plant interactions with microbes and functional relations to the secretome might be possible.

Protein induction

at late *Aphanomyces euteiches* inoculation timepoints.

Protein induction at late *A. euteiches* inoculation timepoints (2 to 4 dpi) is almost entirely confined to *M. truncatula* cell cultures inoculated with zoospores. Here, protein patterns were induced that are specifically related to a sustained defense re-

Table 2. Analysis of the inoculation proteome, elicitation proteome, and phosphoproteome of *Medicago truncatula* cell suspension cultures inoculated with *Aphanomyces euteiches* (0.25 to 4 days; 1.5 h) with protein phosphorylation at serine (ser), tyrosine (tyr), and threonine (threo) residues (1.5 h)

Protein ^b	TC no. ^c	Annot. ^d	Protein name	MW/pI	Cov. (%) / pep ^e	Phosphorylation ^a		
						Ser	Tyr	Threo
Inoculated								
P1	108781	PO9886	Small heat-shock protein chloroplast precursor	18.4/4.43	19/4	+	+	–
P2	94259	O49235	2,4-D inducible glutathione <i>S</i> -transferase (EC 2.5.1.18 EC 2.5.1.18)	25.6/5.10	28/8	+	–	+
P3	94259	O49235	2,4-D inducible glutathione <i>S</i> -transferase	25.6/6.24	39/10	+	–	+
P4	94412	O48646	Probable phospholipid hydroperoxide glutathione peroxidase mitoch. prec. (PHGPx), partial (74%)	19.3/7.37	26/7	+	–	–
P5	106697	BAA12982	Nucleoside diphosphate kinase I (<i>Pisum</i>) (PNDKN1)	18.6/5.98	20/5	+	+	–
P6	94259	O49235	2,4-D inducible glutathione <i>S</i> -transferase	25.6/7.30	34/9	+	+	+
P7	107452	Q948X4	Glutathione <i>S</i> -transferase	24.3/8.12	26/7	+	+	+
P8	106311	Q41119	Cyclophilin (EC 5.2.1.8)	18.4/9.36	34/6	+	–	–
P9	103798	Q9LW35	Alcohol dehydrogenase-like protein, partial (36%)	24.6/5.57	34/5	+	–	–
P10	106494	Q9M611	Soluble inorganic pyrophosphatase, partial (90%)	24.7/6.23	35/8	+	+	+
P11	106425	A45116	L-ascorbate peroxidase cytosolic - garden pea	27.1/6.28	40/8	–	–	+
P12	106425	A45116	L-ascorbate peroxidase cytosolic - garden pea	27.1/6.28	34/7	–	–	+
P13	108603	P83291	NADH-cytochrome b5 reductase-like protein (B5R)	34.0/8.29	20/8	+	–	–
P14	94528	P52904	Pyruvate dehydrogenase E1 component β subunit, mitoch. prec. (PDHE1-B)	35.1/8.32	19/5	+	–	–
P15	94281	Q9LKI6	Isoflavone reductase (IFR) (2'-hydroxyisoflavone red.)	35.6/5.40	30/11	+	–	–
P16	94406	BAD12188	12-oxophytodienoic acid 10, 11-reductase (OPR)	45.5/6.08	22/7	+	–	–
P17	100664	P12886	Alcohol dehydrogenase 1, partial (46%) (E.C. 1.1.1.1)	41.1/6.25	46/7	+	+	–
P18	100663	P12886	Alcohol dehydrogenase 1, partial (82%) (E.C. 1.1.1.1)	41.1/6.33	38/6	+	–	–
P19	106580	P46257	Fructose-bisphosphate aldolase, cyt. isozyme 2	43.7/7.41	31/7	+	–	–
P20	100539	O24076	Guanine nucleotide-binding protein β subunit	38.2/7.21	50/15	+	–	–
P21	106518	P34922	Glyceraldehyde-3-phosphate dehydrogenase, cyt.	39.6/7.51	30/7	+	–	–
P22	106518	P34922	Glyceraldehyde-3-phosphate dehydrogenase, cyt.	40.0/7.55	35/8	+	–	–
P23	94614	Q8W4G8	Fructose-bisphosphate aldolase-like protein	41.7/7.55	34/12	+	–	–
P24	106831	P29677	Mitochondrial processing peptidase α subunit	55.1/5.04	19/7	+	–	–
P25	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.10	22/10	+	+	–
P26	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.54	41/15	+	+	–
P27	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/6.34	37/14	+	–	–
P28	105276	Q7XBE4	ENO 2 Enolase (E.C. 4.2.1.11)	52.6/8.03	45/11	+	–	–
P29	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	54.5/8.20	26/11	+	–	–
P30	107373	Q43621	Glutathione reductase cytosolic (GR) (GRase)	57.0/9.34	19/7	+	–	–
P31	100988	P25890	Catalase (EC 1.11.1.6 EC 1.11.1.6 EC 1.11.1.6)	62.9/8.51	22/12	+	–	–
P32	107891	A43350	Formate-tetrahydrofolate ligase (E.C. 6.3.4.3)	67.9/8.25	25/15	+	–	–
P33	100696	Q9LKJ4	Alanine aminotransferase, partial (71%) (E.C. 2.2.6.1.2)	52.7/6.03	19/6	+	–	–
P34	100696	Q9LKJ4	Alanine aminotransferase, partial (71%) (E.C. 2.2.6.1.2)	52.7/6.20	23/7	+	–	+

(continued on next page)

(continued on next page)

^a Marked with + after *A. euteiches* cell inoculation, cell elicitation, and mock inoculation (P1–P41); – = no phosphorylation event detected.

^b Protein numbers. Inoc. = proteins phosphorylated in inoculated cells, Iden. = phosphorylated proteins already identified at early and late inoculation stages, Elicit. = proteins phosphorylated upon elicitation, Mock = proteins phosphorylated in Mock-inoculated controls, and MPB = proteins phosphorylated in maltose-peptone (MPB)-incubated controls.

^c Tentative consensus sequence (TC) or accession number.

^d Annotation number.

^e Percent covered and peptide number.

sponse. Parallel to a sustained defense response at the protein level in the zoospore-inoculated cells, significantly decreasing rates of viable cells were observed for later inoculation time-points. As already mentioned, their induction was indicated at early inoculation timepoints via identification of proteins 1, 8, 9, and 12 to 19 that appeared absent in elicited cells.

In *A. euteiches*-inoculated cell cultures (2 to 4 dpi), proteins from three functional groups were induced: i) PR proteins, ii) proteins associated with secondary phenylpropanoid or phytoalexin metabolism, and iii) proteins assigned to carbohydrate metabolism and energy-related cellular processes. Among the PR proteins, two members of PR10-like proteins (TC 94217 [protein 1] and TC 106355 [protein 20]), one PR-5b thaumatin-like protein (protein 24), and a class I chitinase (protein 32) were found to be induced. These results are coincident with the detected PR protein induction in *A. euteiches*-inoculated *M. truncatula* roots as reported previously (Colditz et al. 2004, 2005, 2007), indicating similar molecular responses to an *A. euteiches* inoculation in planta as in the cell cultures. Interestingly, RNAi-mediated gene silencing of an *MtPr10-1*

isoform (TC 109466) (Colditz et al. 2007) revealed the strongest silencing effects at the protein level for these particular two PR-10 forms, TC 94217 and TC 106355. Thaumatin-like PR-5b was found with antagonistic induction pattern to PR-10 in the *MtPr10* RNAi lines; here, it reveals induction peaks at later inoculation timepoints (protein 24, 3 and 4 dpi) when at least one of the PR10-like proteins (protein 1, TC 94217) is clearly reduced in abundance.

In addition to the PR protein induction, an R- protein was detected by identifying the R14 protein (TC101874). The *R14* gene is a member of a resistance gene cluster in soybean providing resistance to powdery mildew and Phytophthora stem and root rot (Graham et al. 2002). Because the R14 protein was specifically identified to be induced in *A. euteiches* zoospore-inoculated cell cultures but not in elicited cells, it might be involved in *A. euteiches* recognition and response, specifically during a physical interaction between both partners. Moreover, the R14 protein was induced at later inoculation timepoints, where an associative network between plant cells and pathogen has been established.

Table 2. (continued from preceding page)

Protein ^b	TC no. ^c	Annot. ^d	Protein name	MW/pI	Cov. (%) / pep ^e	Phosphorylation ^a		
						Ser	Tyr	Threo
P35	102206	Q9STH1	Stress-induced protein sti1-like protein, partial (94%)	58.5/6.64	20/8	+	–	+
P36	94680	P49608	Aconitate hydratase (Citrate hydrolyase)	109.4/7.39	23/20	+	–	–
P37	100552	Q8GUR9	Thioredoxin h	12.8/5.10	33/6	+	+	–
P38	106863	Q6L460	Putative mitochondrial ATP synthase	21.4/5.20	42/12	–	–	+
P39	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/5.52	40/8	–	–	+
P40	100786	Q9SDZ1	Isoflavone reductase homolog 1	33.8/5.58	39/10	–	–	+
P41	100786	Q9SDZ1	Isoflavone reductase homolog 1	33.8/6.18	35/9	–	–	+
Identified								
20	106355	Q8L6K8	Pprg2 protein/PR-10-like protein	16.9/4.45	32/6	+	–	–
32	106842	P93327	Class I chitinase (EC 3.2.1.14)	35.9/7.85	25/6	+	–	–
34	100429	O48905	Malate dehydrogenase cytoplasmic	39.5/7.25	24/8	+	–	–
35	94614	Q8W4G8	Fructose-bisphosphate aldolase-like protein	42.7/8.34	34/8	+	–	–
36	103200	P93033	Fumarate hydratase 1 mitoch. prec. (Fumarase 1)	56.6/8.84	23/7	+	–	–
37	107373	Q43621	Glutathione reductase cyt. (GR) (GRase) (GOR2)	62.0/8.51	24/8	+	–	–
Elicited								
P5	106697	BAA12982	Nucleoside diphosphate kinase I (Pisum) (PNDKN1)	18.6/5.98	20/5	+	+	–
P6	94259	O49235	2,4-D inducible glutathione S-transferase	25.6/7.30	34/9	+	+	+
P10	106494	Q9M611	Soluble inorganic pyrophosphatase, partial (90%)	24.7/6.23	35/8	+	+	+
P11	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	40/8	–	–	+
P12	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	34/7	–	–	+
P17	100664	P12886	Alcohol dehydrogenase 1, partial (46%) (E.C. 1.1.1.1)	41.1/6.25	46/7	+	+	–
P25	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.10	22/10	+	+	–
P26	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.54	41/15	+	+	–
P32	107891	A43350	Formate-tetrahydrofolate ligase (E.C. 6.3.4.3)	67.9/8.25	25/15	+	–	–
P33	100696	Q9LKJ4	Alanine aminotransferase, partial (71%) (E.C. 2.2.6.1.2)	52.7/6.03	19/6	+	–	–
P34	100696	Q9LKJ4	Alanine aminotransferase, partial (71%) (E.C. 2.2.6.1.2)	52.7/6.20	23/7	+	–	–
P35	102206	Q9STH1	Stress-induced protein sti1-like protein, partial (94%)	58.5/6.64	20/8	+	–	+
37	107373	Q43621	Glutathione reductase cyt. (GR) (GRase) (GOR2)	62.0/8.51	24/8	+	–	–
Mock								
P1	108781	PO9886	Small heat-shock protein chloroplast precursor	18.4/4.43	19/4	+	+	–
P5	106697	BAA12982	Nucleoside diphosphate kinase I (Pisum) (PNDKN1)	18.6/5.98	20/5	+	+	–
P6	94259	O49235	2,4-D inducible glutathione S-transferase	25.6/7.30	34/9	+	+	+
P10	106494	Q9M611	Soluble inorganic pyrophosphatase, partial (90%)	24.7/6.23	35/8	+	+	+
P11	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	40/8	–	–	+
P12	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	34/7	–	–	+
P17	100664	P12886	Alcohol dehydrogenase 1, partial (46%) (E.C. 1.1.1.1)	41.1/6.25	46/7	+	+	–
P25	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.10	22/10	+	+	–
P26	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.54	41/15	+	+	–
P31	100988	P25890	Catalase (EC 1.11.1.6 EC 1.11.1.6 EC 1.11.1.6)	62.9/8.51	22/12	+	–	–
MPB								
P5	106697	BAA12982	Nucleoside diphosphate kinase I (Pisum) (PNDKN1)	18.6/5.98	20/5	+	+	–
P6	94259	O49235	2,4-D inducible glutathione S-transferase	25.6/7.30	34/9	+	+	+
P11	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	40/8	–	–	+
P12	106425	A45116	L-ascorbate peroxidase cytosolic—garden pea	27.1/6.28	34/7	–	–	+
P17	100664	P12886	Alcohol dehydrogenase 1, partial (46%) (E.C. 1.1.1.1)	41.1/6.25	46/7	+	+	–
P25	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.10	22/10	+	+	–
P26	100309	Q6RIB7	ENO 2 Enolase (E.C. 2.3.1.1.6)	53.8/5.54	41/15	+	+	–

The B5R (protein 9), naringenin-CHS4 (protein 15), and IFR (protein 28) proteins are associated with secondary phenylpropanoid and isoflavonoid metabolism involved in the synthesis of *M. truncatula* pterocarpan phytoalexin medicarpin, the major phytoalexin in *Medicago* spp. (Higgins 1972). Phenylpropanoid-derived isoflavonoids of legumes generally exhibit a substantial impact on mutualism as well as plant disease or defense, serving as key signaling molecules and antimicrobial compounds of primary defense (Farag et al. 2008). The initial biochemical step of isoflavone synthesis is the derivatization from the flavones naringenin and liquiritigenin to 2-hydroxyisoflavone. This complex reaction is catalyzed by an isoflavone synthase (IFS) and a cytochrome P450 (Farag et al. 2008). The P450 enzymatic activity is enhanced by a specific B5R (protein 9), presumably by acting as an alternative electron donor to NADPH (Winkel-Shirley 2001). In our studies, the B5R was detected to be additionally phosphorylated in *A. euteiches*-inoculated cell cultures (P13), indicating activation of an IFS-cyt. P450-B5R functional multi-enzyme complex, as was proposed by several authors (Burbulis and Winkel-Shirley 1999; Hrazdina and Wagner 1985; Stafford 1974). One step prior, naringenin-CHS4 (protein 15) catalyzes the synthesis of naringenin-chalcone which isomerizes under specific conditions into naringenin. The third enzyme found to be induced is embedded at a later downstream position in the isoflavone/pterocarpan metabolism; the IFR (protein 28) catalyzes the reaction of 2'-hydroxyformononetin to vestinone (Guo et al. 1994). Coincidentally, the IFR appeared to be induced primarily at 3 and 4 dpi whereas the naringenin-CHS4 as well as the B5R revealed induction already at early inoculation timepoints. Because vestinone represents the precursor for medicarpin, it is evident that synthesis of medicarpin is induced in the *M. truncatula* cell cultures after *A. euteiches* inoculation. Moreover, three different protein spots representing two IFR were found to be specifically phosphorylated after *A. euteiches* inoculation. By contrast, none of these enzymes were detected with increased abundances or phosphorylation in gels of cell cultures treated with the *A. euteiches* elicitation medium. The absence of protein patterns from the isoflavonoid metabolism in the elicited cell cultures was also reported for the infection proteome of the *A. euteiches*-tolerant *M. truncatula* line F83.005-9. By contrast, the susceptible F83.005-5 line exhibited significant induction of an isoliquiritigenin 2-*O*-methyltransferase, IFR1, and a chalcone reductase (Colditz et al. 2005). Thus, induction of medicarpin synthesis in *M. truncatula* cell cultures indicates a fully compatible interaction formed with *A. euteiches*.

Numerous proteins were identified to be specifically induced in *A. euteiches*-inoculated cell cultures that are involved in primary metabolic and energy-related cellular processes, including carbohydrate metabolism (glycolysis, tricarboxylic-acid [TCA] cycle, and oxidative phosphorylation) as well as the synthesis of amino acids. In addition, an exceedingly large number of enzymes related to carbohydrate metabolism was detected specifically phosphorylated after *A. euteiches* inoculation. Among the 10 enzymatic steps in the glycolytic pathway, four were identified as being differentially regulated after *A. euteiches* inoculation (and additionally phosphorylated [P]): fructose-bisphosphate aldolase (proteins 17, 35 [also phosphorylated], P19, and P23), glyceraldehyde-3-phosphate dehydrogenase (proteins 18, P21, and P22), enolase (P25–P29), and malate dehydrogenase (34, also phosphorylated). The mitochondrial pyruvate dehydrogenase complex (detected phosphorylated: P14) catalyzes the key regulatory step of carbohydrate metabolism, the oxidative decarboxylation of pyruvate to acetyl CoA, CO₂, and NADH, linking glycolysis to the TCA cycle. Increased abundances for an alcohol dehydrogenase (16, P17,

and P18) is likely the result of increased rates of respiratory CO₂ evolution, particularly through glycolysis and oxidative pentose phosphate pathways, as described to occur in plant cell cultures after elicitation with oomycete stimulators (Broeckling et al. 2005; Norman et al. 1994). TCA cycle-induced proteins in *A. euteiches*-inoculated cell cultures include an aconitate hydratase (P36) converting citrate to isocitrate, a succinyl-CoA ligase (41, specifically induced in elicited cell cultures), fumarate 1 (36, also phosphorylated), and alanine aminotransferase (P33 and P34), which converts pyruvate to alanine. Two enzymes involved in oxidative phosphorylation and ATP synthesis were found with increased phosphorylation after inoculation: the general mitochondrial processing peptidase forming part of complex III in plants (cyt. bc₁; P24) and a putative mitochondrial ATP synthase (P38).

Induced protein phosphorylation patterns confirm the qualitative molecular defense-response of *M. truncatula* cell cultures after *A. euteiches* inoculation as detected via 2-DE.

Phosphorylation and dephosphorylation play a pivotal role in perception and transduction of signals in plant defense responses as documented by treatment of plant cell cultures with (fungal) elicitors (Dietrich et al. 1990; Felix et al. 1991, 1994; Lecourieux-Ouaked et al. 2000; Peck et al. 2001; Viard et al. 1994). However, evaluation of distinct phosphorylation events of not only elicited but also inoculated cell cultures has not yet been described, to our knowledge.

Comparison of protein phosphorylation patterns for *A. euteiches*-elicited and -inoculated cells revealed 13 proteins being phosphorylated which can be assigned to antioxidative and energy-related processes. Subtraction of seven proteins that showed also phosphorylation after incubation in the absence of *A. euteiches* leads to five proteins (P32 to P35 and 37) representing a formate-tetrahydrofolate ligase, two alanine aminotransferases, a stress-induced sti1-like protein, and a GR.

Several proteins involved in antioxidant metabolism and cell redox homeostasis were found to be induced at early inoculation timepoints and are also phosphorylated at an initial inoculation stage (1.5 hours postinoculation [hpi]). GST represent the most redundant protein (four isoforms: P2, P3, P6, and P7) of this group. Moreover, one PhGPx (P4) and two GRs (37 and P30) were also detected phosphorylated, next to three isoforms of an L-ascorbate peroxidase (P11, P12, and P39), a catalase (P31), and a thioredoxin h (P37). Interestingly, proteins that were found to be specifically induced in *A. euteiches*-inoculated cell cultures at later inoculation timepoints, including i) PR proteins and proteins of ii) secondary phenylpropanoid and iii) carbohydrate metabolism, were already detected phosphorylated at this initial timepoint of 1.5 hpi, indicating an early activation of the mentioned defense-related metabolic pathways. Thus, the PR-10-like protein (20, i), a class I chitinase (32, i), B5R (P13, ii), IFR (P15, ii), alcohol dehydrogenases (P9, P17, and P18, iii), pyruvate dehydrogenase (P14, iii), fructose-bisphosphate aldolases (P19, P23, and 35, iii), glyceraldehydes-3-phosphate dehydrogenases (P21 and P22, iii), a mitochondrial processing peptidase (P24, iii), enolase 2 (P25 to P29, iii), alanine aminotransferases (P33 and P34, iii), citrate hydrolase (P36, iii), ATP synthase (P38, iii), and a fumarase (36, iii) were identified as being phosphorylated.

Modulation of carbon metabolism—an outlook.

The protein induction and phosphorylation profiles of *A. euteiches* zoospore-inoculated cell cultures indicate the particular and substantial activation of proteins involved in carbohydrate metabolism (20 enzymes among the related pathways). One aspect of this altered metabolic setup might be a pathogen-

induced reprogramming of cellular carbohydrate metabolism toward the synthesis of carbon sources required by the pathogen; another is the activation of secondary isoflavone metabolism that requires altered primary metabolism.

Referring to the latter aspect, constitutively accumulated glucosides are generally regarded as stable, soluble storage forms of isoflavanoids typically localized in the cell's vacuole (Mackenbrock and Barz 1991). Simultaneous accumulation of proteins from carbohydrate and phenylpropanoid metabolism is supported by an earlier study for an *A. euteiches*-tolerant *M. truncatula* line, where five members of carbohydrate metabolism were induced together with enzymes of the phenylpropanoid metabolism (Colditz et al. 2005). Thus, induction of both mentioned enzymatic classes maintains marker character for an established and sustained *A. euteiches* infection in *M. truncatula*.

Very recently, Farag and associates (2008) elucidated novel pathways in phenylpropanoid and isoflavanoid biosynthesis of elicitor-specific responses in *M. truncatula* cell cultures; according to the elicitor applied, three isoflavones (afformosin, alfalone, and irisolidone) were identified for the first time in *M. truncatula*. The biosynthetic origin of these compounds is still a matter of uncertainty (Deavours et al. 2006); pulse labeling studies using exogenous substances now suggested naringenin, precursor for 5-hydroxyisoflavones in legumes, as point of origin (Farag et al. 2008). Occurrence of the CHS4 indicates synthesis of naringenin in *A. euteiches*-inoculated cell cultures. However, in response to fungal yeast elicitor, the phytoalexin end product medicarpin is mainly increased in *M. truncatula* cell cultures instead of the newly identified isoflavones (Farag et al. 2008). Our results suggest a coincident biosynthesis of medicarpin in the cell cultures after *A. euteiches* inoculation, because the inoculation-induced IFR is embedded at a later downstream position in the pterocarpan metabolism leading to synthesis of vestinone, the precursor for medicarpin. Further studies may elucidate whether the mentioned alternative pathways in isoflavonoid biosynthesis are also activated in *A. euteiches*-inoculated *M. truncatula* cell cultures.

MATERIALS AND METHODS

Cell cultures and inoculation with *A. euteiches*.

M. truncatula ('Jemalong A17') cell suspension cultures were obtained by transferring sterile root explants of 1-week-old seedlings on sterile Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with the phytohormones 2,4-dichlorophenoxyacetic acid (1 mg liter⁻¹) and kinetin (0.1 mg liter⁻¹), as described previously by Baier and associates (1999) and Scheidle and associates (2005). During 8 weeks, growth of callus tissue was induced and non-necrotic calli were subcultivated to obtain callus cell cultures of root tissue. For establishment of *M. truncatula* cell suspension cultures, small amounts of callus tissue were transferred into liquid MS medium. Cell cultures were kept at 25°C in the dark while shaking at 130 rpm. Cell cultures consisting of single cells and small cell aggregates were used for subcultivation, which was carried out regularly at intervals of 8 to 10 days into 15 ml of fresh MS medium in 125-ml culture flasks (Schott Duran Group, Mainz, Germany). Inoculation experiments were performed 2 to 3 days after cell transfer to fresh medium.

Exposure to *A. euteiches* (Drechsler), strain ATCC 201684 (kindly provided by S. Rosendahl, Department of Mycology, Botanical Institute, University of Copenhagen, Denmark), was carried out by prudently adding 1 ml of *A. euteiches* zoospores in lake water (250,000 zoospores per culture flask) or a crude *A. euteiches* elicitor obtained from culture medium (MPB: 3%

[wt/vol] maltose and 1% [wt/vol] peptone), wherein the oomycete was cultured for 10 days. Therefore, 10 agar plugs (1 cm²) were cut out from *A. euteiches* vegetative mycelium growing on corn meal agar plates and were transferred to 20 ml of autoclaved MPB. Culture flasks were incubated for 10 days at 20°C in the dark without agitation. To initiate the release of zoospores, the MPB was removed and the mycelia were washed with autoclaved lake water taken from a lake in the royal gardens of Herrenhausen, Hannover, Germany (Colditz et al. 2004, 2005; Nyamsuren et al. 2003). The remaining MPB medium was filtered using 595 ½ S & S folded filters (Schleicher & Schuell, Dassel, Germany) to remove mycelia and the filtrate was subsequently used for elicitation of cell cultures as mentioned above. In further experiments, 1 ml of heat-treated MPB (boiled for 10 min after filtration) was added to *M. truncatula* cell cultures. Control root cultures were mock inoculated with the same volume of autoclaved lake water or culture medium (MPB).

After inoculation with the zoospore inoculum, cell cultures were allowed to stand for 1.5 h in the dark in order to enable movement of zoospores toward the plant cells. Cell cultures were subsequently gently pivoted on a swiveling table for 3.5 hours in the dark in order to initiate encysting of spores and to minimize effects of oxygen deficiency of plant cells. Afterward, the cultures were replaced on the culture shaker in the dark.

Biological samples of four different experiments were harvested from independent culture flasks for both control and *A. euteiches*-treated cell cultures at different timepoints: 0.25, 1, 2, 3, and 4 days. For the analysis of protein phosphorylation, additional samples were collected at 1.5 h. Therefore, separated culture flasks were transferred to the shaker 30 min after inoculation. For harvesting, plant cells were extracted from the cell culture medium, washed briefly with protein extraction buffer, and transferred to liquid nitrogen for further molecular analyses.

A. euteiches growth curve determination.

For determination of *A. euteiches* mycelium growth in liquid-grown MPB cultures, fresh weights of 10 independent cultures were measured for 10 days at 24-h intervals and fresh weights of the initial inoculum (mean of 10 agar plugs [10 × 1 cm²] in 20 ml of MPB) were subtracted to obtain a growth curve. Culture flasks were incubated at 20°C in the dark without agitation.

Determination of *M. truncatula* cell viability via the trypan blue exclusion test.

For assessment of *M. truncatula* cell viability in the cultures inoculated as described above, the trypan blue (TB) exclusion assay was carried out following a modified protocol for *Medicago* cell-suspension cultures (Steward et al. 1999). Therefore, a 300-μl aliquot of fresh cells of each treatment was diluted with the same volume of culture medium to avoid osmotic stress and stained with 200 μl of a 0.4% (wt/vol) TB aqueous solution for 30 min. The cells were rinsed two times with 300 μl of culture medium. Cell viability expressed as a percentage of living cells was calculated by microscopic evaluation of 500 cells on each slide (Nikon Eclipse 80i). Cells were counted as alive when cytoplasm was clear (not TB stained). Conversely, cells that had incorporated the TB dye were counted as dead. This assay was carried out for each culture and treatment for 10 days at 24-h intervals.

Oxidative burst measurements.

Oxidative burst measurements were performed using the H₂O₂-dependent chemiluminescence assay according to Warm

and Laties (1982). Therefore, 2 g of cells was resuspended in 8.5 ml of MS medium for 3 h. Then, cell cultures were subjected to different experimental treatments: yeast invertase (Sigma-Aldrich, Seelze, Germany) at 50 µg ml⁻¹ as positive control, sterile lake water or culture medium (MPB) as negative controls, and *A. euteiches* zoospores in lake water (125,000 zoospores/culture) and *A. euteiches*-filtered culture media as described above. All fractions were added in equal volumes of 500 µl to the cell cultures, maintaining equal ratio of inoculums to cells as used for the proteomic analyses. Subsequently, oxidative burst measurements were started by transferring 200 µl of suspension-cultured cells to 700 µl of 50 mM phosphate buffer (pH 7.9) and 100 µl of 1.2 mM luminol (Sigma-Aldrich). Chemiluminescence assay was initiated by adding 100 µl of potassium-hexacyanate (Fluka, Seelze, Germany). Luminescence was determined with a Sirius luminometer (Berthold Technologies, Bad Wildbach, Germany).

Microscopic analysis of *A. euteiches* inoculation.

For identification of *A. euteiches* structures in zoospore-inoculated *M. truncatula* cell cultures, microscopic studies were carried out with a Nikon Eclipse 80i microscope combined with a Nikon Digital-Sight DS-2MV digital camera (Nikon, Düsseldorf, Germany).

Phenol extraction of proteins.

Phenol extraction of total protein from the cells was carried out as described previously (Colditz et al. 2004, 2005). For IEF, 1 mg of protein was diluted with 350 µl of rehydration buffer, consisting of 8 M urea, 2% (wt/vol) CHAPS, 100 mM dithiothreitol, 0.5% (vol/vol) IPG buffer for correspondent pI range (pH 3–10 nonlinear [NL]; Amersham Pharmacia Biotech, Uppsala, Sweden), and a trace of bromophenol blue.

2-DE.

2-DE was performed for four independent biological experiments at each mentioned timepoint of harvesting, by combining IEF (pI 3–10 NL) using an IPGphor system (Amersham Pharmacia Biotech) with a sodium dodecyl sulfate–tricine gel electrophoresis (Protean II XL, 20 by 20 cm; BioRad, Richmond, CA, U.S.A.) as already described (Colditz et al. 2005). Gels were stained with 0.1% (wt/vol) Coomassie Brilliant Blue (BioRad) overnight and scanned on a UMAX Power Look III Scanner (UMAX Technologies, Fremont, CA, U.S.A.).

For analysis of in-gel protein abundance, each gel prepared was additionally scanned on a Fujifilm FLA-3000 Fluorescence Laser Imager (Fujifilm Medical Systems USA, Stamford, CT, U.S.A.). The relative spot intensities of selected proteins were measured as light absorption units (internal densitometric unit) by using the AIDA Image Analyser (v3.20) evaluation software (Raytest USA, Wilmington, NC, U.S.A.). Therefore, nearby background absorption values were subtracted. For each protein spot analyzed, the corresponding absorption units of four different experiments were measured and averaged. The absorption coefficient was determined for each selected protein spot by comparison of the corresponding in-gel intensities between gels of *A. euteiches*-inoculated cells and gels of controls. These coefficients are displayed as a protein induction index in Table 1.

Immunoblotting experiments.

For immunoblotting experiments, gels were blotted onto Immobilon-polyvinylidene fluoride membranes (Millipore, Schwalbach/Ts., Germany) in transfer buffer (20 mM Tris base, 20% [vol/vol] methanol, and 150 mM glycine). Blots were incubated overnight with antibodies directed against

phosphorylated serine (Monoclonal Anti-Phosphoserine clone PSR-45; Sigma-Aldrich), tyrosine (Monoclonal Anti-Phosphotyrosine clone PT-66; Sigma-Aldrich), or threonine (Monoclonal Anti-Phosphothreonine clone PTR-8; Sigma-Aldrich) amino acid residues in incubation buffer (100 mM Tris-HCl [pH 7.4], 0.1% Tween 20, and 150 mM NaCl). Immune-positive protein spots were stained using the Vectastain ABC-Kit (Vector Laboratories/Axxora, Lörrach, Germany) according to the manufacturer's protocol.

Tryptic in-gel digestion of protein spots, MALDI-TOF/mass spectrometry-based protein identification.

For tryptic digestion, protein spots of interest were excised manually from the gels. In-gel digestion with trypsin (Sequencing Grade Modified trypsin; Promega Corp., Madison, WI, U.S.A.) was carried out for 24 h at 37°C. Mass spectrometry measurements were performed at an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described previously (Colditz et al. 2007).

Protein annotation.

PMF obtained in Mascot database were further analyzed using the *M. truncatula* EST database (MtGI; The Institute for Genome Research, Rockville, MD, U.S.A.). In case of successful sequence matching in MtGI, sequences were verified by performing BlastP protein annotation of the in silico translation products at the United States National Center for Biotechnology Information. Theoretical peptide mass and pI of the polypeptides were evaluated at EXPASY for final confirmation according to their positions in the 2-DE gel map. Additionally, blast analysis of all sequences to the "Aphano DB" EST database (Madoui et al. 2007) was carried out.

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