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Research article

Activity profiling reveals changes in the diversity and activity of proteins in Arabidopsis roots in response to nematode infection

Marion Hütten [a], Melanie Geukes [a], Johana C. Misas-Villamil [b, c], Renier A.L. van der Hoorn [b, d], Florian M.W. Grundler [a], Shahid Siddique [a, *]

[a] Rheinische Friedrich-Wilhelms-University of Bonn, INRES – Molecular Phytomedicine, Karlrobert-Kreiten-Straße 13, 53115 Bonn, Germany
[b] Plant Chemetics Lab, Max-Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany
[c] Botanical Institute and Cluster of Excellence on Plant Sciences, University of Cologne, 50674 Cologne, Germany
[d] Plant Chemetics Lab, Department of Plant Sciences, University of Oxford, South Parks Road, OX1 3UB Oxford, UK

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ABSTRACT

Cyst nematodes are obligate, sedentary endoparasites with a highly specialised biology and a huge economic impact in agriculture. Successful parasitism involves morphological and physiological modifications of the host cells which lead to the formation of specialised syncytial feeding structures in roots. The development of the syncytium is aided by a cocktail of nematode effectors that manipulate the host plant activities in a complex network of interactions through post-translational modifications. Traditional transcriptomic and proteomic approaches cannot display this functional proteomic information. Activity-based protein profiling (ABPP) is a powerful technology that can be used to investigate the activity of the proteome through activity-based probes. To better understand the functional proteomics of syncytium, ABPP was conducted on syncytia induced by the beet cyst nematode *Heterodera schachtii* in Arabidopsis roots. Our results demonstrated that the activity of several enzymes is differentially regulated in the syncytium compared to the control roots. Among those specifically activated in the syncytium are a putative S-formyl-glutathione hydrolase (SFGH), a putative methylesterase (MES) and two unidentified enzymes. In contrast, the activities of vacuolar processing enzymes (VPEs) are specifically suppressed in the syncytium. Competition labelling, quantitative gene expression and T-DNA knock-out mutants were used to further characterise the roles of the differentially regulated enzymes during plant–nematode interaction. In conclusion, our study will open the door to generate a comprehensive and integrated view of the host-pathogen warfare that results in the formation of long-term feeding sites for pathogens.

1. Introduction

Biotrophic plant parasites have developed lifestyles that allow them to penetrate and establish specific structures for nutrient uptake within the host while avoiding the activation of defence responses. The sugar beet cyst nematode *Heterodera schachtii* Schmidt is a biotrophic endoparasite with a highly specialised biology. This parasite induces modifications in the root system that impede the nutrient and water supply of the host plant, leading to substantial yield losses (Sasser and Freckman, 1986). Due to their wide range of hosts, these nematodes are able to infect different crops within the families Chenopodiaceae and Brassicaceae, including the plant *Arabidopsis thaliana*, which has been established as a model organism for analysing the molecular aspects of the plant–nematode interaction (Sijmons et al., 1991).

The infective stage juveniles (J2) of *H. schachtii* hatch from eggs that are stored in the cyst, the modified dead body of the females. The J2 worms invade the host roots near the tip and move intracellularly towards the central cylinder. During penetration, the nematodes pierce single cells with their stylets, resulting in the spontaneous collapse of the cytoplasm of these cells; therefore, the paths of the invading J2 are delineated by necrotic cells. Having reached the vascular cylinder, they probe the individual cells by gentle stylet stabbing. In cases of cell collapse, they continue

* Corresponding author.
E-mail addresses: mhuetten@uni-bonn.de (M. Hütten), mel-ge@freenet.de (M. Geukes), misas@mpipz.mpg.de (J.C. Misas-Villamil), renier.vanderhoorn@plants.ox.ac.uk (R.A.L. van der Hoorn), grundler@uni-bonn.de (F.M.W. Grundler), siddique@uni-bonn.de (S. Siddique).

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moving until they succeed in inducing an initial syncytial cell (ISC) (Sobczak et al., 1997; Wyss and Grundler, 1992). Within 24 h after selection, the ISC fuses with adjacent cells by local dissolution of cell walls, and the formed syncytium hypertrophies. Two days after selection of the ISC, the cells incorporated into the syncytium are enlarged and exhibit features of a typical syncytium. The cytoplasm is condensed and enriched with ribosomes, endoplasmic reticulum, mitochondria and plastids. Additionally, the nuclei are enlarged, cytoskeleton is rearranged, and the central vacuole is replaced by several smaller vacuoles in the syncytium (Golinowski et al., 1996; Kyndt et al., 2013). Solutes are withdrawn by the nematode throughout its parasitic life stages, and the syncytium induces a strong sink for assimilates in the plant. The development of the syncytium is accompanied by massive transcriptomic and metabolic changes in the infected tissue, and these changes have been studied in detail in our previous works (Hofmann et al., 2010; Siddique et al., 2009; Szakasits et al., 2009; Wieczorek et al., 2006). During the following two weeks, the nematodes continue to draw nutrients from the roots and develop into males and females after moulting three times (J3, J4 and adult). A female-associated syncytium is composed of approximately two hundred cells and reaches its maximum size approximately 10 days after infection. Syncytia of females remain functional for several weeks until egg production is completed, the females die afterwards and transform into typical brown cysts, which contain several hundred eggs. Syncytia of males are much smaller and short living (Sobczak et al., 1997). After the third developmental stage male juveniles stop feeding, their syncytia degenerate, and the animals become vermiform. Adult males hatch from the juvenile cuticle and migrate in search of adult females for copulation.

The whole process of penetration, migration and feeding site establishment is aided by secretions, which act on the host plant as effectors (reviewed by Mitchum et al., 2013). The identification of these effectors has been significantly facilitated by the development of new sequencing technologies in recent years. However, it remains largely unknown how these nematode effectors induce and orchestrate the massive physiological and structural changes in the plants. Proteomic studies concerning the host side of the plant–nematode interaction are rare. In 1995, the protein composition of the feeding sites of *H. schachtii* in *A. thaliana* was studied (Schmidt, 1995). There was a significant increase in the abundance of the protein encoded by the myrosinase gene *PYK10* around the syncytia compared to the non-infected roots. Similarly, a root proteomic study was performed by analysing nematode resistant and susceptible cotton (*Gossypium hirsutum* L.) cultivars infected with the root-knot nematode *Meloidogyne incognita* (Callahan et al., 1997). Several polypeptides were found to be regulated differentially as a result of the infection; for example, a novel 14 kDa polypeptide was expressed at higher levels in young galls of the resistant isoline at 8 dpi. In a similar study, the roots of nematode-resistant genotypes of cotton (*Gossypium hirsutum* L.) and coffee (*Coffea canephora*) infected with *M. incognita* and *Meloidogyne paranaensis* were compared to their corresponding non-infected roots using two-dimensional gel electrophoresis, and this analysis led to the identification of several differentially regulated proteins (Franco et al., 2010).

Conventional transcriptomic and proteomic analyses do not cover the complete cellular regulatory mechanism, which also includes posttranslational modifications. The activities and functions of proteins are not only determined by phosphorylation, but also by other post-translational modification, such as glycosylation, acetylation, carbonylation, and certainly in the case of disease these modifications are known to play an important role (Huber and Hardin, 2004; Pastore and Piemonte, 2013). Therefore, the amount of cellular mRNA does not necessarily result in a higher level of corresponding functional protein. A recently developed method, which has turned out to be highly useful for the identification and annotation of enzyme activities, is “Activity-Based Protein Profiling” (ABPP). Pioneered by Cravatt, Bogyo and co-workers (Cravatt et al., 2008; Kato et al., 2005; Verhelst and Bogyo, 2005), it has evolved into an effective tool for the identification and functional characterisation of proteins in extracts and living cells (Edgington et al., 2009; Gu et al., 2010; Hang et al., 2006; Nodwell and Sieber, 2012; Uttamchandani et al., 2008; van der Hoorn et al., 2004; van der Hoorn and Kaiser, 2012; Weerapana et al., 2010, 2011). ABPP is based on the design of biotinylated or fluorescent active-site-directed small molecules (probes) that irreversibly bind to the active side residues of enzymes in complex proteomes; thus, this method gathers information on the functional state of the enzymes rather than on their abundance. Most activity-based probes (ABPs) target a large, but manageable, fraction of the proteome with shared catalytic features by achieving a desired level of intraclass coverage and minimal extra-class cross-reactivity (Cravatt et al., 2008). The labelling is covalent and irreversible, facilitating the imaging of the labelled enzymes on protein gels by fluorescent scanning and the identification of labelled proteins by affinity capture and mass spectrometry (MS) (Gu et al., 2010; Nodwell and Sieber, 2012; van der Hoorn et al., 2004; Kolodziejek and van der Hoorn, 2010). Van der Hoorn et al. (van der Hoorn et al., 2004) introduced DCG-04 to plant science, which is an activity-based probe for papain-like cysteine proteases, and this probe illustrated the potential of ABPP as it has been used to reveal senescence-induced protease activities (Martinez et al., 2007), defence-related protease activation (Gilroy et al., 2007) and various pathogen-derived inhibitors that target tomato proteases (Rooney et al., 2005; Song et al., 2009; Tian et al., 2007; van Esse et al., 2008).

In this study, we used two different probes to determine the differential activities of vacuolar processing enzymes (VPEs) and serine hydrolases (SHs) in root tissues upon infection with *H. schachtii*. These enzyme families (VPEs and SHs) were chosen for analyses because of their previously described role in plant–pathogen interactions and availability of reliable probes to perform ABPP (Rojo et al., 2004; Misas-Villamil, 2010; Liu et al., 1999). Furthermore, gene expression analysis was performed for the differentially activated enzymes to generate an integrated view of pre- and posttranslational regulation events in the syncytia. T-DNA loss-of-function mutants were used to study the importance of these differentially regulated enzymes during nematode and syncytium development. In this way, we were able to show that the activity of the various enzymes was differentially regulated in female-associated syncytia compared to the control roots. Furthermore, our results revealed that the functional activity of these enzymes did not necessarily correlate with their gene expression.

2. Material and methods

2.1. Plant and nematode culture

Seeds of *A. thaliana* ecotype Columbia were surface-sterilised for 5 min in 0.6% sodium hypochlorite, then incubated for 3 min in 70% ethanol and subsequently rinsed four times with sterile water. Knop medium was prepared as previously described (Siddique et al., 2009). Five seeds for each treatment were transferred onto an agar layer in 9 cm Petri dishes and grown at 25 °C with a photoperiod of 16 h at 700 μE m^2 s^{-1} for 12 days. The quadruple mutant genotype *qvpe* (Gruis et al., 2002) that lacks all known VPEs in *A. thaliana* (At2g25940, At1g62710, At4g32940 and At3g20210) has been previously described.

H. schachtii was cultivated in vitro on mustard (*Sinapsis alba* cv.

Albatros) roots growing in Knop medium supplemented with 2% sucrose (Sijmons et al., 1991). Second-stage juveniles (J2) of *H. schachtii* were hatched in a funnel containing 3 mM zinc chloride. After surface sterilisation with 0.05% $HgCl_2$ and four washes with sterile water, they were transferred in a water suspension to the roots of the grown Arabidopsis plants. For optimal development of the nematodes and a sufficient infection rate, each plant was inoculated with 60–70 nematodes and was stored under the same light conditions for more than 10 days. Sex and stage of the developing nematodes was determined under a dissecting microscope on a ground glass screen. Females usually were in the third developmental stage when syncytia were sampled at 10 dpi.

Afterwards, the female-associated syncytia were cut under a microscope considering that the females were carefully removed from syncytia and were immediately frozen in liquid nitrogen. Corresponding root sections of the non-infected Arabidopsis plants served as a reference (Fig. 1). All experiments were replicated three times.

2.2. Activity-based protein profiling

Proteins from the root samples were extracted by grinding the roots in an Eppendorf tube and were quantitatively analysed by photometric measurement using the RC DC™ Protein Assay (Pharmacia LKB Ultraspec III Spetrophotometer) at 750 nm to ensure equal amounts of proteins in each sample during subsequent steps.

The probes used for ABPP were provided by the van der Hoorn lab at the Max-Planck-Institute for Plant Breeding Research (Cologne). Labelling was usually performed by incubating the extracted proteins in 50 µl containing 125 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) buffer (pH 7.5), 2 mM dithiothreitol (DTT) and 2 µM of a rhodamine-tagged fluorophosphonate probe (RhFP) for 1–2 h at room temperature in the dark. The samples labelled with 2 µM AMS101 were incubated in 125 mM sodium acetate (NaAc, pH 5.5) buffer containing 2 mM DTT and were labelled for 3 h under the same conditions as the RhFP probes. In the case of competition labelling, the samples were pre-incubated with the corresponding inhibitors at 50 µM for 30 min prior to labelling with the probe. Phenylmethanesulfonylfluoride (PMSF) prevents the subsequent labelling of RhFP, and TYR-VAL-ALA-ASP-chloromethylketone (YVAD-cmk) competes for the same targets as AMS101. The same volume of dimethyl sulfoxide (DMSO) was used as a non-probe control.

After incubation, the labelled proteins were separated on 12% sodium dodecyl sulfate (SDS) gels and visualised by in-gel fluorescence scanning using a Typhoon FLA 9000 scanner. Fluorescence intensity was measured using the ImageQuant TL software (GE Healthcare Life Sciences, http://www.gelifesciences.com).

2.3. Quantitative real-time PCR

RNA was extracted from the syncytial and control root material using a Nucleospin RNA Xs (Macherey–Nagel, Germany) kit according to the manufacturer's instructions and was transcribed into cDNA using random primers and a High Capacity cDNA Reverse Transcription Kit (Life-technologies catalogue number, 4368814). 18S rRNA (3 biological replicates) and Actin (1 biological replicate) were used as an internal reference, as previously described (Hofmann et al., 2010). The samples were analysed using quantitative real-time PCR in 20 µl reactions containing 10 µl of Fast SYBR Green Master Mix (Applied BioSystems), 2 mM $MgCl_2$, 0.5 µl each of forward and reverse primers (10 µM), 2 µl of complementary DNA (cDNA), and water in 20 µl total reaction volume. For the internal reference, the cDNA was diluted 1:100. qRT-PCR was carried out at 95 °C for 20 s, followed by 40 cycles each with 95 °C for 3 s and 60 °C for 30 s. The melting curve analysis was conducted at 95 °C for 15 s, 60 °C for 1 min with increments of 0.3 °C every 15 s up to 95 °C. The expression of 18S and Actin was used to analyse the changes in transcript levels using the formula $(1 + E)^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.4. Nematode infection assay

Plants were grown and inoculated as described above. The number of nematodes was counted at 14 dpi. The sizes of the females and their associated syncytium were measured using the Leica Application Suite (4.3.0) software (LAS: Leica Microsystems, http://www.leica-microsystems.com). All experiments were repeated three times.

3. Results

We used ABPP to analyse the changes in the active proteome of

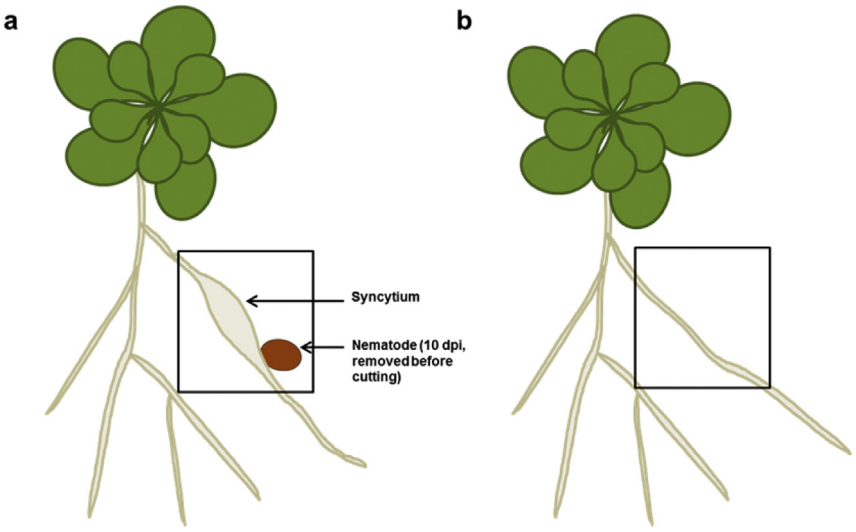

Fig. 1. Scheme of root sampling for ABPP analysis. (a) Syncytium samples were cut from the infected roots after carefully removing the nematodes. (b) Root sections from the uninfected roots were used as control.

roots after infection with *H. schachtii*. Root sections containing female- associated syncytium were collected at 10 days post infection (dpi), as described in the methods section. Corresponding root sections from uninfected plants were used as controls (Fig. 1). For conduction of ABPP labelling a total of 100 μg of protein was required.

3.1. Vacuolar processing enzymes (VPEs)

Vacuolar processing enzymes (VPEs) are cysteine proteases that are classified in the legumain family C13 (clan CD). In Arabidopsis, a total of four VPE-encoding genes (αVPE, βVPE, γVPE and δVPE) are known and are subdivided into seed-type and vegetative-type VPEs (Nakaune et al., 2005; Yamada et al., 2005). Seed-type βVPE is responsible for the maturation of the seed storage proteins and the activation of antimicrobial peptides, whereas the vegetative-type αVPE and γVPE play pivotal roles during stress and senescence conditions (Hara-Nishimura et al., 1998, 2005). δVPE is specifically expressed in the seed coat and regulates cell death (Nakaune et al., 2005).

The fluorescent activity-based probe AMS101 is potent and highly specific for all four VPEs (Misas-Villamil et al., 2013). This probe contains an aza-epoxide reactive group and a Bodipy fluorescent tag. Labelling with AMS101 resulted in a strong signal at 43 kDa and two weak signals at 40 kDa and 38 kDa in control roots (Fig. 2a). Compared to the control, the intensity of the signal at 43 kDa was weaker in the female-associated syncytia, while the other two signals remained unchanged. Surprisingly, an additional weak fluorescent band at 37 kDa was present only in the syncytium samples (Fig. 2a). We further validated these observations by quantifying the fluorescence intensity from the protein gels (Fig. 2b).

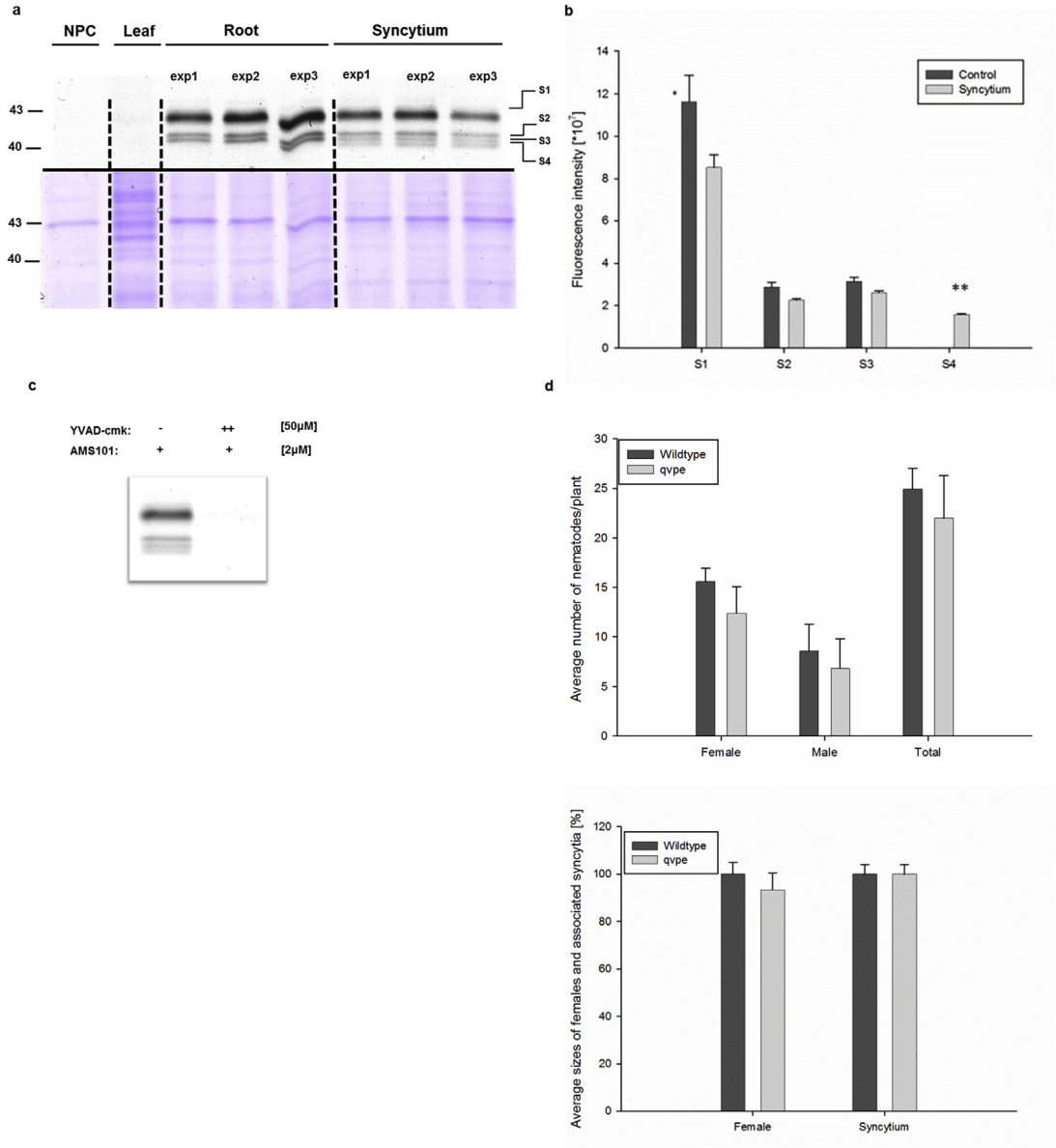

Fig. 2. AMS101 labelling of the syncytium, root and leaf. (a) Comparative labelling of VPEs in leaf, syncytium and non-infected root material with AMS101. Experiment was repeated in three biological replicates (exp1, exp2 and exp3) and blue colour gel shows staining of total protein with coomassie blue. NPC, non-probe control. (b) Fluorescence intensity of the gel signals from Fig. 2a. Asteriks indicate significant difference to control (t-test; $p < 0.05$). (c) Competition labelling of VPEs with inhibitor YVAD-cmk. (d) Nematode infection assay using the quadruple knockout mutant (qvpe), which shows the number and sizes of the nematodes and associated syncytia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Caspase-1 or VPE-specific inhibitors bind to both intermediate and mature forms of VPE (Hara-Nishimura et al., 2005; Hatsugai et al., 2004). Therefore, we performed a competition labelling with the covalent, irreversible chlorometylketone-based caspase-1, 4 and 5 inhibitor YVAD-cmk (Fig. 2c). The assay confirmed the specificity and performance of the AMS101 labelling as pre-incubation with the inhibitor prevented labelling with the probe.

Next, we investigated the changes at the transcript level for the genes encoding all four VPEs in Arabidopsis after nematode infection. A recent transcriptome analysis by Szakasits et al. (Szakasits et al., 2009) showed that αVPE and γVPE transcripts were significantly reduced in the syncytia compared to the non-infected roots, whereas βVPE transcripts were significantly upregulated. To confirm the reliability of the microarray analysis, we performed quantitative real-time-PCR (qPCR) with root sections that were cut from infected roots at 10 dpi. The results obtained from the qPCR analysis are in line with the gene chip analysis (Table 1).

We further characterised the role of VPEs in nematode and syncytium development by performing infection assays with *H. schachtii* using a quadruple knock-out mutant of VPE (qvpe) (Gruis et al., 2002). The numbers of females and males were counted at 14 dpi. Furthermore, the average sizes of the females and corresponding syncytia were measured. We expected that the roots of the *A. thaliana* knock-out mutants would exhibit a higher infection rate compared to the wild-type plants. However, knocking out the VPE-encoding genes did not result in significant changes in the infection rate or nematode development (Fig. 2d).

3.2. Serine hydrolases (SHs)

Serine hydrolases comprise a large collection of enzymes from different structural classes and are known to be involved in numerous physiological and pathological processes (Nodwell and Sieber, 2012; van der Hoorn and Kaiser, 2012; Liu et al., 1999). To study the role of serine hydrolases in the plant–nematode interaction, we labelled total protein extracted from infected and uninfected roots at 10 dpi using a fluorophosphonate (FP)-based probe with a rhodamine (Rh) reporter tag (RhFP, (Liu et al., 1999)). This probe was previously used to identify the activities of over 50 serine hydrolases in Arabidopsis leaf extracts (Kaschani et al., 2009). After labelling and separating on a protein gel, nine fluorescent signals were detected by scanning (Fig. 3a). Four fluorescent signals (s_4, s_6, s_8 and s_9) exhibited an increased protein activity in the syncytia compared to the non-infected roots. Of these signals, s_4 and s_8 had not been previously described. However, s_6 is a putative S-formylglutathione hydrolase (SFGH), and s_9 is a putative methylesterase (Kaschani et al., 2009). Similarly, there were five signals that remained unaffected (s_1, s_2, s_3, s_5 and s_7) in the syncytium. Of these five signals, s_1, s_2, s_3 and s_5 were previously identified as tripeptidyl peptidase-II (TPP2), prolyl oligopeptidases-like proteins (POPL), serine carboxypeptidase-like proteins (SCPL) and carboxylesterase-like proteins (CXE), respectively. The identity of s_7 is currently unknown. The measurements of the fluorescence intensity confirmed our observations. In particular, the methylesterase (s_9) showed a significant increase in activity (Fig. 3b). Preincubation with Ser protease inhibitor PMSF suppresses RhFP labelling of some of these proteins (Fig. 3c). This selective suppression is consistent with the selectivity of PMSF and consistent with previous findings (Kaschani et al., 2012).

To generate an integrated view of the SH activities in the syncytium, we looked at the transcriptome data (Szakasits et al., 2009) for the genes encoding the SHs detected in this experiment. Of the signals that showed increased activity, SFGH (s_6) is encoded by a single gene in Arabidopsis (At2g41530), and this enzyme catalyses the last step in the detoxification of formaldehyde by hydrolysing S-formylglutathione to formic acid and glutathione (Kordic et al., 2002). Transcriptome data revealed that there was also an upregulation in the expression of SFGH mRNA in the syncytium compared to the control roots (Supplementary Table ST1). Similarly, Kaschani et al. (Kaschani et al., 2009) identified methylesterase (s_9) as a product of the MES2 (At2g23600) and MES3 (At2g23610) genes. MES hydrolyses methylated phytohormones, such as indoleacetic acid, salicylic acid and jasmonic acid. Transcriptome analysis data revealed that, while MES3 is upregulated in the syncytium compared to the control roots, probe sets for MES2 were not specific; therefore, these data were not included in the analysis (Szakasits et al., 2009). In contrast, no significant differences were observed in transcript activity between the syncytia and control roots for TPP2 (At4g20850). For POPL (s_2), SCPL (s_3) and CXE (s_5), different isoforms have been simultaneously detected in previous studies (Kaschani et al., 2009), which makes it difficult to identify the gene/s encoding the detected SHs. Nonetheless, a look at the expression of all the genes encoding the previously detected SCPL, CXE, and POPL (Kaschani et al., 2009) did not reveal significant changes at the transcript level (Supplementary Table ST1).

Further characterisation of the detected SHs using knockout mutants was not performed in this study due to a lack of prior identification, the unavailability of homozygous T-DNA mutants, and the functional redundancy among the multigene SH families.

4. Discussion

The cyst nematode *H. schachtii* induces metabolically active syncytial feeding sites in roots. These syncytia are the sole source of nutrients for the nematodes throughout their lives. In this paper, we studied the functional proteomics of the syncytium induced by *H. schachtii* in Arabidopsis roots using Activity-based Protein Profiling (ABPP).

4.1. Activities of vacuolar processing enzymes are reduced in the syncytium

Vacuolar processing enzymes (VPEs) are cysteine proteases that were originally found to be the processing enzymes responsible for the maturation of seed storage proteins (Hara-Nishimura et al., 1991). In Arabidopsis, four VPEs have been identified, and these VPEs have been subdivided into seed-type (βVPE, At1g62710) and vegetative-type VPEs (αVPE, At2g25940 and γVPE, At4g32940). δVPE (At3g20210) was found in Arabidopsis (36) and belongs to neither of these two groups. It is considered an uncharacterised-

Table 1
Gene expression of the VPEs in 5- and 15-dpi syncytia analysed by Szakasits et al. (2009) and confirmed by qRT-PCR. Statistically significant fold changes in syncytia compared to non-infected control roots are indicated by stars (Fisher's t-test and Bonferroni correction, q < 5%). ∞ indicate that signal was below level of detection.

Affimetrix chip (Szakasits et al., 2009)					qRT-PCR	
Name	Locus	Gene symbol	M value (log2)	Fold change	ddCt value	Fold change
α	At2g25940	VPE	−2.4	−5.28*	−1.95	−3.86
β	At1g62710	VPE	2	4.00*	1.0	2.0
γ	At4g32940	VPE	−1.3	−2.46*	∞	∞
δ	At3g20210	VPE	0.1	0.93	−0.77	−1.7

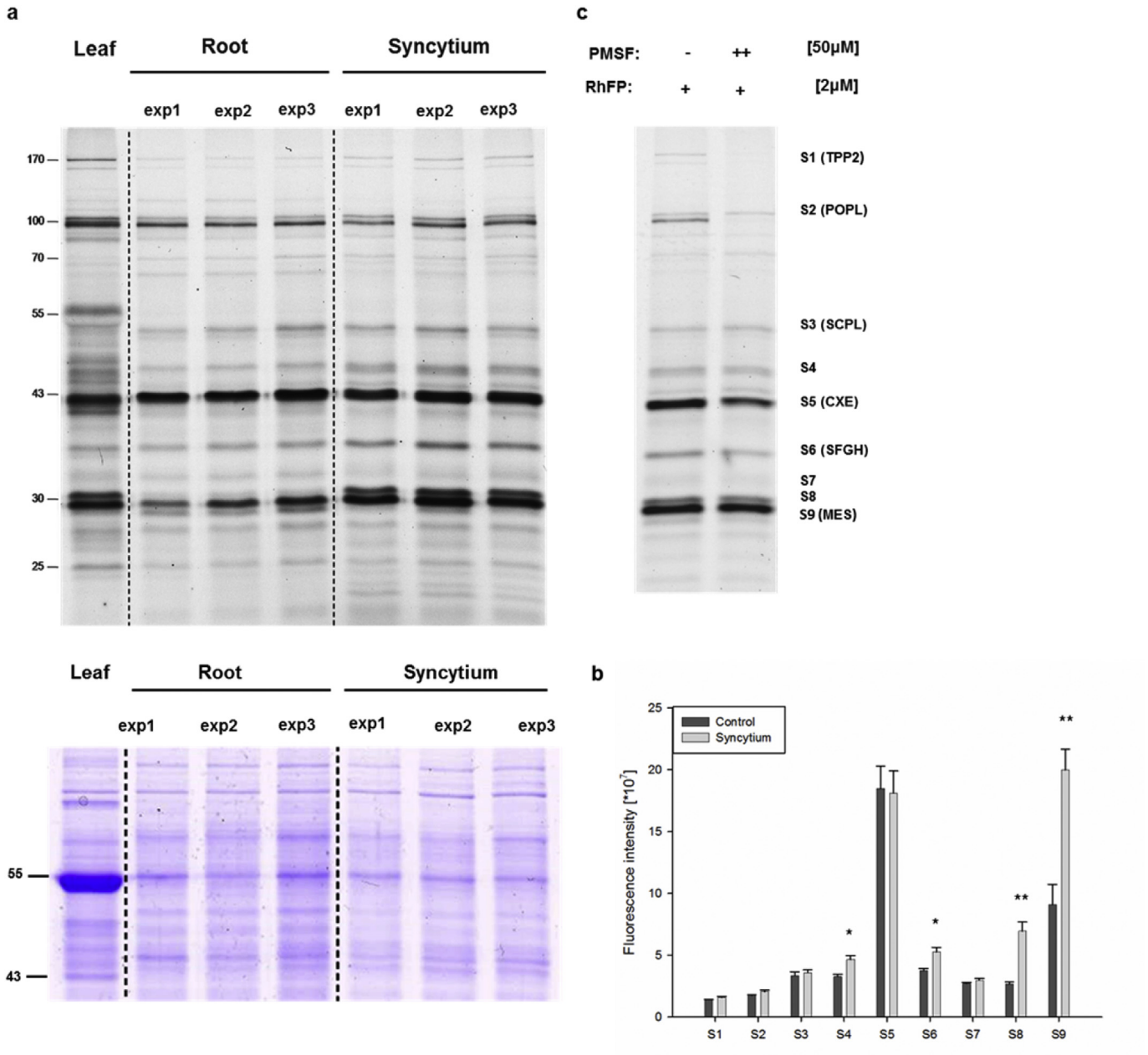

Fig. 3. RhFP labelling of the syncytium, root and leaf. (a) Comparative labelling of the SHs in leaf, syncytium and non-infected root material with RhFP. Experiment was repeated in three biological replicates (exp1, exp2 and exp3) and blue colour gel shows staining of total protein with coomassie blue. (b) Fluorescence intensity of the gel signals from 3a. Asteriks indicate significant difference to control (t-test; $p < 0.05$). (c) Competition labelling with the inhibitor PMSF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

type VPE. Vegetative-type VPEs have been shown to be upregulated in vegetative organs under stress conditions and during senescence (Hara-Nishimura et al., 1998). The plant Hypersensitive response is an efficient defence tool that leads to well-organised programmed cell death (PCD). In animals, PCD is mediated by caspases, which are cysteine proteases. VPE was the first described proteinase in plants to exhibit caspase-like activity and has been shown to be involved in vacuole-mediated hypersensitive cell death in TMV-infected tobacco leaves (*Nicotiana benthamiana*) (Rojo et al., 2004; Hatsugai et al., 2004). Similarly, it was recently shown that colonisation-associated cell death in Arabidopsis roots caused by the mutualistic fungus *Piriformospora indica* is mediated by VPEs (Qiang et al., 2012).

Because plant-parasitic nematodes rely on living plant tissues for parasitism, they need to avoid the activation of the plant cell death machinery. Indeed, after labelling with AMS101, we observed a significant decrease in the activity of the VPEs in the syncytia compared to the control roots (Fig. 2a and b). These results suggested that nematode might be able to overcome the VPE-mediated defence responses by injecting inhibitor proteins into the host cells. Interestingly, knocking out the VPEs did not affect the susceptibility of the plants to nematodes (Fig. 2d). At least two hypotheses could account for this result. First was already made in previous study (Gruis et al., 2002). Gruis et al. (Gruis et al., 2002) did not observe any phenotype or accumulation of seed proteins after knocking out β- and δ-VPE probably due to functionally redundant proteolytic enzymes other than VPE homologs. Although disputed in the literature, support for these proteolytic enzymes has been shown in

soybean. Scott et al. isolated a protein from seeds capable of processing legumin, whose molecular mass was distinctly different than VPE (Scott et al., 1992). Second hypothesis might suggest that the special and temporal suppression of VPEs by the nematodes is essential and therefore close to complete, so that any further decrease in activity does not result in any change in plant susceptibility. For future work, however, it would be interesting to study the effect of overexpression lines on the plant–nematode interaction.

4.2. Selective activation of serine hydrolases in the syncytium

The serine hydrolase (SH) family is one of the largest and most diverse classes of enzymes found in nature, and these proteins are involved in a wide range of physiological processes, including metabolism, development, and immunity (Nodwell and Sieber, 2012; Liu et al., 1999; Kaschani et al., 2012). All SHs feature an active site that contains an activated serine residue, which performs nucleophilic attack on the substrate, resulting in a covalent intermediate. The Arabidopsis genome encodes hundreds of SHs that belong to a dozen of large multigene families, such as proteases, lipases, transferases and esterases (Kaul et al., 2000). We applied the fluorophosphonate probe (RhFP) to the syncytium protein extracts to profile the activities of the SHs. After labelling, we observed significant changes in the activities of four different SHs: SFGH (s_6), MES (s_9), and two unidentified proteins (s_4 and s_8) (Fig. 3a and c). Kaschani et al. (Kaschani et al., 2009) observed the increased activity of SFGH in Arabidopsis leaves after infection with the fungal pathogen *Botrytis cinerea* in *pad3* mutants compared to Col-0. *pad3* plants are deficient in camalexin production and exhibit enhanced susceptibility to *B. cinerea*. The increased activity of SFGH in the syncytium, as well as in the leaves of infected *pad3* mutants, suggests that SFGH might play an important but as yet unknown role in plant–pathogen interactions. Similarly, the activity of a methylesterase (s_9) was increased in the female-associated syncytium compared to the control roots. MES hydrolyses methylated phytohormones, such as indoleacetic acid, salicylic acid and jasmonic acid; therefore, it is possible that increased activity is important for the maintenance of syncytial functions. Unfortunately, loss of function homozygous mutants for SFGH and MES3 were not available and, therefore, could not be used in this study.

ABPP with FP-probes identified the differential activities of SHs in the root and syncytium proteomes. These enzymes represent diverse families of enzymes, as previously shown (Kaschani et al., 2009). However, not all SHs were detected in our analysis. This could be due to several reasons. First, many Arabidopsis genes are not expressed in the tissues and conditions tested. Second, the abundance of some enzymes may be under the detection limit and third, some enzymes might not be active under the tested conditions. For example, it has been shown that RhFP labelling is strongly influenced by pH (Kaschani et al., 2009). Finally, RhFP may not react with every serine hydrolase. Nevertheless, the differential activities of the detected enzymes suggest changes in a variety of biochemical pathways in the syncytium. Unfortunately, the biological functions and significance of a majority of the enzymes are unknown. Accordingly, annotation of their biological and biochemical functions would require functional characterisation using reverse genetic approaches. This may not be an easy task, considering that the majority of SHs belong to large gene families that may have redundant members.

5. Conclusions

Sedentary parasitic nematodes manipulate plant functions to induce and maintain a highly active nurse cell system in the roots. This manipulation leads to changes in the abundance as well as activity of several proteins, such as serine hydrolases and vacuolar processing enzymes in the nematode feeding site that may not be detected by traditional transcriptomic or proteomic approaches. In this study, we have shown the proof-of-concept for the utility of ABPP-method to display the differential activities of various enzymes upon nematode infection. Our results hinted towards the existence of nematode effectors that may inhibit or activate enzyme function at post-translational level. Future work will aim to characterize the identity and functions of the differentially activated host as well as nematode proteins, which may provide new exciting insights into the plant–nematode interactions. Considering that there is not much known about functions of nematode effector proteins in host, application of ABPP in future will provide a powerful tool to characterize functions of such effectors within the host. This in turn will help generating an integrate picture of changes during plant–nematode interaction at pre- and post-translational level. To start with, more probes should be used to study interactions involving different hosts and nematodes species at various time-points of infection process. For example, it will be interesting to investigate and compare functional proteomics using ABPP during a compatible and incompatible plant-interaction.

Contribution

Marion Hütten and Melanie Geukes performed the experiments. Johana C. Misas-Villamil supervised ABPP experiments. Renier van der Hoorn and Florian M. W. Grundler critically reviewed the manuscript. Shahid Siddique and Marion Hütten wrote the manuscript. Shahid Siddique designed the research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.09.008.

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