RESEARCH ARTICLE

Tricholoma vaccinum host communication during ectomycorrhiza formation

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One sentence summary: The genome and secretome of Tricholoma vaccinum reveal insights into its early signaling potential with its host spruce before physical contact, including regulation of small secreted proteins and cellulolytic enzymes.

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ABSTRACT

The genome sequence of Tricholoma vaccinum was obtained to predict its secretome in order to elucidate communication of T. vaccinum with its host tree spruce (Picea abies) in interkingdom signaling. The most prominent protein domains within the 206 predicted secreted proteins belong to energy and nutrition (52%), cell wall degradation (19%) and mycorrhiza establishment (9%). Additionally, we found small secreted proteins that show typical features of effectors potentially involved in host communication. From the secretome, 22 proteins could be identified, two of which showed higher protein abundances after spruce root exudate exposure, while five were downregulated in this treatment. The changes in T. vaccinum protein excretion with first recognition of the partner were used to identify small secreted proteins with the potential to act as effectors in the mutually beneficial symbiosis. Our observations support the hypothesis of a complex communication network including a cocktail of communication molecules induced long before physical contact of the partners.

Keywords: genome sequence; proteome; secretome; ectomycorrhiza; Tricholoma; spruce

INTRODUCTION

The ectomycorrhizal symbiosis between fungi and fine roots of perennial plants, often forest trees, is in existence for more than 120 Mio years, and is of high importance for the stability of forest ecosystems in the boreal climate zones (Hibbett and Matheny 2009). Due to the fact that mycorrhiza is well suited to increase stress tolerance in the tree symbionts, we studied the interaction between Norway spruce (Picea abies) commonly used in forestry (Jansson et al. 2013) and its fungal symbiont, Tricholoma vaccinum. This widespread basidiomycete grows slowly as late stage ectomycorrhizal fungus with high host specificity for Picea abies and low compatibility to Pinus sylvestris (Deacon, Donaldson and Last 1983; Molina, Massicotte and Trappe 1993).

To establish a mutual symbiosis, the fungal mycelium develops a mantle around the root, and the hyphae grow in the apoplast between the root cells to form a Hartig’ net, essential
for the exchange of nutrients between the partners (Krause and Kothe 2006). The different developmental stages in mycorrhiza are dependent on a complex communication between both organisms (Barker, Tagu and Delp 1998; Felten, Martin and Legué 2012; Krause et al. 2013; Raudaskoski and Kothe 2015). Recognition of a potential host for mycorrhiza formation can be achieved through specific or unspecific signals in the plant root exudate, which consists of carbohydrates, amino and organic acids (Buee et al. 2000; Zeng, Mallik and Setliiff 2003; de la Peña, Badri and Loyola-Vargas 2012). In response, small molecules including oligopeptides and small proteins are released by the fungus (De-la-Peña et al. 2008; Hempel et al. 2009). Among these secreted proteins, some were found to play a role in detoxification, e.g. by acting as metal chelators (Kothe, Müller and Krause 2002; Bellion et al. 2006; Asimwe et al. 2012; Gherghe and Krause 2012).

Typical secreted fungal proteins are hydrophobins, which are relevant for fruiting body development and mycorrhiza establishment (Mankel, Krause and Kothe 2002; Plett et al. 2012). These small cell wall proteins, which self-assemble to build an interface between hydrophobic and hydrophilic surfaces, can play a role in host specificity (Wösten 2001; Mankel, Krause and Kothe 2002). Hydrophobin expression has been studied in Laccaria bicolor, where a strong regulation of hydrophobins in different mycorrhizal stages and with different hosts could be shown (Plett et al. 2012).

The finding of secreted proteins of bacteria, oomycetes and fungi affecting plant development culminated in the detection of phytopathogen-produced effector proteins, which are taken up into plant cells where they can alter plant physiology (Hempel et al. 2009; Kamoun 2009; Brown, Antoniw and Hammond-Kosack 2012; Rafiqi et al. 2013). Effector proteins carry special features: they are small, share a special amino acid composition, do not contain Pfam domains and include repetitive motifs (Saunders et al. 2012; Rafiqi et al. 2013; Plett and Martin 2015). The first effectors from a basidiomycete were found for the smut disease causing fungus Ustilago maydis (Müller et al. 2013). Pit2 and Pep1 are proteins interfering with the host response of maize, connected to salicylic acid signaling and peroxidase activity. In search of new effector proteins, mycorrhizal signaling proved to be rather complex (Plett and Martin 2015).

The first secretome for an ectomycorrhizal fungus was published for L. bicolor (Vincent et al. 2012), with the small effector protein MiSSP7 involved in ectomycorrhiza establishment (Plett et al. 2011).

In order to allow for prediction of potential effectors and other secreted proteins, genome information of ectomycorrhiza fungi will be helpful. The late sequencing of many more ectomycorrhizal fungi revealed vast differences in genome size and complexity. The ectomycorrhizal ascomycete Tuber melanosporum has the largest fungal genome so far, with a size of 125 Mbp. Its proteome differs widely from that of L. bicolor, and no secretion of effectors is known from Tuber (Martin et al. 2010). In comparative studies, up to 37% of the upregulated genes coding for secreted proteins were unique, and small secreted proteins included some with effector potential, indicating a very specialized evolution of this host–symbiont communication (Kohler et al. 2015).

In genome and transcriptome analyses of ectomycorrhizal fungi, it was speculated that in addition to effectors, a lower number of cellulytic enzymes might be present as compared to wood decay fungi (Kohler et al. 2015). In the present study, we sequenced the genome of T. vaccinum and specifically addressed secreted proteins to find evidence for host interactions. We searched for differentially regulated proteins after addition of spruce root exudates, such as to allow for host signals to change T. vaccinum protein secretion.

MATERIALS AND METHODS

Culture conditions and exudate sampling

Tricholoma vaccinum CKB6154 (FSU: 4731 Jena Microbial Resource Collection, Jena, Germany) was cultivated in 50 ml liquid MMNb media as described previously (Kotike et al. 1987). To investigate the early signaling response of T. vaccinum to spruce root exudates, spruce seedlings (Landesforst Mecklenburg-Vorpommern, Germany) weregerminated and grown according to (Krause and Kothe 2006). For spruce root exudates, 20 replicates of 8-week-old seedlings were transferred to a sterile pipette tip box with 100 ml of a growth liquid (80 ml A. dest., 20 ml MMN) in a moisture chamber. After 1 week, the exudates containing media were filtered and 2-week-old T. vaccinum liquid cultures were supplied with 2 ml of the exudates and incubated for another 2 weeks to achieve high protein yields with this slow growing fungus. A total of 24 replicates were used for control and 24 replicates for exudates treatment.

Genome sequencing

Total DNA from T. vaccinum liquid cultures was isolated (Krause and Kothe 2006) and sequenced (GATC Biotech, Konstanz, Germany) for 100 bp single read whole genome sequencing (Illumina Hiseq 2000). Adapter clipping (Martin 2011), quality trimming (ConDeTri; Zerbin and Birney 2008; Smeds and Künstner 2011) and de novo assembly were constructed using Velvet 1.2.08 and VelvetOptimizer 2.2.5 (Zerbin and Birney 2008; http://bioinformatics.net.au/software.velvetoptimiser.shtml).

Prediction of protein coding genes was performed with the ab initio gene prediction tool AUGUSTUS based on hidden Markov model (Stanke et al. 2008). SCIPiO (Hatje et al. 2011) was used to map 160 L. bicolor proteins to the new genome assembly. The structure of these genes was split for use as test (30 genes) and training (130 genes) sets. As additional training genes, 275 genes from CEGMA (Parra, Bradnam and Korf 2007), a database for core proteins, were mapped into the assembly. Finally, we tested pre-compiled hidden Markov model parameters for L. bicolor on the T. vaccinum genome. Training and validation was performed following AUGUSTUS instructions (http://www.molecularrevolution.org/molevolfiles/exercises/augustus/training.html). Best prediction accuracy was achieved with pre-compiled hidden Markov model parameters for L. bicolor. INPARANOID (Remm, Storm and Sonnhammer 2001) was used to identify orthologs between predicted T. vaccinum proteins and proteins of L. bicolor, Coprinopsis cinerea, Amanita muscaria, Paxillus involutus, Pisolithus tinctorius, Schizophyllum commune and CEGMA (Remm, Storm and Sonnhammer 2001). For the identified orthologs between T. vaccinum and L. bicolor, functional annotation of L. bicolor by KEGG pathways, Gene Ontology (GO) and Signal P (SigP) were transferred to T. vaccinum. The sequence was uploaded at genome.jgi-psf.org, JGI IMG/ER (ID 59348). For Venn diagrams, the online tool gene list venn diagram (http://genevenn.sourceforge.net/) was used. To control the performance of AUGUSTUS, we downloaded predicted secretomes from the Fungal Secretome Knowledge Base (http://proteomics.ysu.edu/secretomes/fungi.php) for the species L. bicolor, Magnaporthe grisea, Moniliophthora perniciosa, C. cinerea and U. maydes and compared the results using Scipio.
Protein isolation and secretome analysis

Three replicates of T. vaccinum control and exudate treated cultures, each consisting of eight replicates, were harvested. The supernatant was filtered through Miracloth (Calbiochem, EMD Millipore, Billerica, MA, USA) and extraction was performed (Kim et al. 2009). Proteins were rehydrated in rehydration buffer (8 M urea, 2 M thio urea, 4% CHAPS). Protein concentration was determined using the Bradford assay (Bradford 1976).

The two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) was performed in triplicates with IPG-IEF (24 cm 3–11NL, Immobiline DryStrip, GE Healthcare) following SDS-PAGE (12%). Approximately 250 μg protein was used in a total volume of 450 μl rehydration buffer containing 2% (vol/vol) IPG-buffer (3-11NL, GE healthcare) and DeStreak reagent (12 μl/ml, GE healthcare). The strips were focused in an isoelectric focusing unit (Ettan IPGphor 2, GE healthcare) using 3 h 300 V, 4 h 600 V, 4 h 1000 V, 4 h 8000 V, 24000 Vh, 800 V step. SDS-PAGE was performed with 12 W per gel in an electrophoresis unit. Gels were stained with Comassie Brilliant Blue G-250 followed by destaining (10% acetic acid, 20% ethanol).

Spot patterns of the gels were analyzed with the Delta2D program version 4.3 (Decodon, Greifswald, Germany). Significantly different spot abundances (P < 0.05) were manually selected for matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF/TOF). Excised spots were digested with 20 μg/ml trypsin (Promega, Mannheim, Germany) and extracted with 0.1% trifluoroacetic acid (1:1 v/v water:acetoniitrole) for 1 h at room temperature. Approximately 2 μl peptide solution was mixed 1:1 with NTA matrix (10 mg ml−1 a-cyano-3-hydroxycinnamic acid in 50% acetoniitrole, 50% water, 0.1% trifluoroacetic acid, 7 mM nitrilotriacetic acid) and transferred on a MTP AnchorChip-target 800/384 (Bruker Daltonics, Germany).

Peptides were analyzed on ultraflexXtreme MALDI-TOF/TOF (Bruker Daltonics, Germany) using a flex control software 3.3 for data collection (Bruker Compass 1.3) and flexAnalyse 3.3 for spectra analysis/peak list generation (Bruker Compass 1.3). Pulsed laser ionization was performed with an Nd-YAG laser (wavelength: 335 nm) and start of time measurement. Acceleration was achieved by electrodes in field-free drift region. Laser induced dissociation was used to get post-source decay fragments and ion selection via the precursor ion selector. Separation of fragments and precursor was achieved by the LIFT-unit. Ions were separation by their m/z. MS was performed in reflection mode and post-decay analyses. Peptide mass fingerprints and peptide fragmentation fingerprints were used for data analyses.

Database search and bioinformatics pipeline

The amino acid sequences for the query were analyzed using the new database resulting from the T. vaccinum genome. Results from ProteinScape 3.0 (Protagen) coupled to MASCOT 2.3 (Matrix Sciences, UK) were obtained with following parameters: fixed modification for carbamidomethyl derivates, variable for the oxidation of methionine, mass values monoisotopic, a peptide mass tolerance of 100 ppm and a maximum of one missed trypsin cleavage. A Mascot score higher than 60 was needed for a significant protein identification.

The genome of T. vaccinum was conceptually translated to yield a protein database. The identified proteins were checked for secretory signal peptides (Emanuelsson et al. 2007) with TargetP 1.1, SecretomeP 2.0 and SignalP 4.1. Further analyses with the server TMHMM 2.0 for transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/), ProtComp 8.0 using LocDB and PotLocDB and (http://linux1.softberry.com/berry.phtml?topic=protcompa&group=help&subgroup=proloc), WolfPSort 0.2 (http://www.genscript.com/psort/wolf.psort.html) for protein localization were used according to an established bioinformatics pipeline (Brown, Antoniw and Hammond-Kosack 2012). Pfam domains of proteins were found using Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and analyses of the Pfam domains were performed with the interpro database (http://www.ebi.ac.uk/interpro).

The amino acid content of proteins was measured with the online tool ProtParam (http://web.expasy.org/protparam/) and internal repeat-rich sequences with T-REKS (Jorda and Kajava 2009). The search for conserved motifs was performed via protein pattern search by gene infinity (http://www.geneinfinity.org/sms/sms_protpatterns.html). Nuclear localization signals (cNSL mapper, http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and nuclear export signals (NetNES 1.1, http://www.cbs.dtu.dk/services/NetNES-1.1/) were identified. The search of carbohydrate active enzymes was performed with the CAZYmes analysis toolkit (CAT, http://mothra.ornl.gov/cgi-bin/cat/cat.cgi).

Laccase and cellulase detection

T. vaccinum liquid cultures were incubated for 4 weeks before treatment with 500 μl spruce exudates or MMNa in A. dest. (1:5; v/v) as control. Cultures were incubated for 24 h. We used 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS) to measure the enzyme activity of laccases photometrically at 420 nm (Madhavan et al. 2014). As a negative control, heat inactivated samples (10 min, 90 °C) were used. Cellulases in culture supernatants were detected using carboxymethylcellulose in 96 well plates (Johnsen and Krause 2014).

Immunofluorescence staining and microscopy

Mycorrhizal cocultures (Kottek et al. 1987; Asiimwe et al. 2010) were fixed for 1 day (50 mM PIPES pH 6.7, 25 mM EGTA pH 8.0, 5 mM MgSO₄, 4% formaldehyde), frozen in tissue freezing medium (Leica, Wetzlar, Germany) and cryocuttings (35–40 μm thick sections, cryostat CM 1510; Leica, Wetzlar, Germany) were examined (Zeiss Axiopt, Carl Zeiss, Jena, Germany) and documented with a CCD camera (Junior Spot; Visitron, Munich, Germany) using filters 24 (FITC: fluorescein isothiocyanate fluorescence), 02 (DAPI: 4′,6-diamidino-2-phenylindole staining) and 10 (auto-fluorescence). Fungal and plant nuclei were stained with DAPI after mounting for microscopy in 0.1 M Tris-HCl (pH 8.0)-50% glycerol and 1 mg ml⁻¹ phenylenediamine.

Immunofluorescence staining was performed using anti-hydrophobin S. commune Sc3 first antibody (rabbit, 1:2000) and FITC conjugated, mouse-anti rabbit antibody (1:100; Sigma-Aldrich, Steinheim, Germany) as described (Fischer and Timberlake 1995).

RESULTS

Genome sequence of T. vaccinum

Two biological replicates used for genome sequencing of T. vaccinum yielded 64 122 102 and 79 361 873 reads with an average GC content of 47%. The complete genome size was 44 192 576 bp (Table 1) and the achieved coverage was over 325-fold. Other fungi like Neurospora crassa or A. thiersi have a comparable
Table 1. Comparison of secretomes.

<table>
<thead>
<tr>
<th>Lifestyle</th>
<th>Ectomycorrhizal</th>
<th>Arbuscular mycorrhizal</th>
<th>Phytopathogenic</th>
<th>Mycotrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Tricholoma vaccinum</td>
<td>Laccaria bicolor⁴</td>
<td>Rhizophagus irregularis⁵</td>
<td>Ustilago maydis⁵</td>
</tr>
<tr>
<td>Strain</td>
<td>Staude GK6514</td>
<td>S238N-H82</td>
<td>DAOM-197198</td>
<td>521</td>
</tr>
<tr>
<td>Genome size (Mb)</td>
<td>44.2</td>
<td>64.9</td>
<td>135</td>
<td>19.7</td>
</tr>
<tr>
<td>Number of genes</td>
<td>11 981</td>
<td>20 614</td>
<td>28 232</td>
<td>6522</td>
</tr>
<tr>
<td>Average gene length (bp)</td>
<td>1666</td>
<td>1533</td>
<td>933</td>
<td>1935</td>
</tr>
<tr>
<td>Average exon length (bp)</td>
<td>218</td>
<td>210.1</td>
<td>151</td>
<td>1051</td>
</tr>
<tr>
<td>Average intron length (bp)</td>
<td>75.8</td>
<td>92.7</td>
<td>81</td>
<td>127</td>
</tr>
<tr>
<td>Predicted secreted proteins</td>
<td>206</td>
<td>2931</td>
<td>376</td>
<td>574</td>
</tr>
<tr>
<td>Secreted proteins from total gene repertoire (%)</td>
<td>1.72</td>
<td>14.22</td>
<td>1.33</td>
<td>6.53</td>
</tr>
<tr>
<td>Interesting small secreted proteins</td>
<td>g8303; g3988</td>
<td>MISSP7</td>
<td>SP7</td>
<td>Pit2</td>
</tr>
<tr>
<td>Target</td>
<td>Unknown</td>
<td>Plant cell nucleus</td>
<td>Plant cell nucleus</td>
<td>Plant apoplast</td>
</tr>
</tbody>
</table>

Footnotes:

Figure 1. Function of predicted T. vaccinum proteins based on L. bicolor orthologs by KEGG annotation.

Conceptually translated proteome and ortholog prediction

For functional annotation, 353 proteins could be assigned to a GO annotation and 1440 via KEGG, both based on the L. bicolor orthologs (Fig. 1). Of the 2324 L. bicolor proteins with a KEGG annotation, 1501 (65%) have an ortholog in T. vaccinum (Fig.S1, Supporting Information). Those without ortholog mainly belong to unknown pathways, carboxylases and protein kinases (Fig. 2). Lower numbers of genes were present for phosphotransferases with an alcohol as acceptor, unspecific monooxygenases, glycogen biosynthesis pathway or glucan-1,3-beta-glucanases and -glucosidases. However, all proteins of L. bicolor without ortholog coverage with 98-fold (Galagan et al. 2003) and 45-fold (Hess et al. 2014), respectively. The N₅₀ value conclude that 1395 sequences made up more than half of the total sequence length, equal or longer than 6134 bp.

The T. vaccinum genome included 11 981 protein coding genes, consisting of 70 392 exons (2–5565 bp lengths), with an average length of 218 bp. The exome is 15 403 810 bp and represents about 34% of the genome. With a total of 59 348 introns (on average four per gene and a minimum of one intron per gene), the maximum intron number was found for gene g139 with 54 introns. The average intron length remains 75.8 bp, the lowest 6 bp appearing in 12 genes and the longest 4946 bp in gene g6962.
in *T. vaccinum* are present with other genes with similar KEGG annotations. Thus, the *T. vaccinum* genome provides a complete set of metabolic core enzymes, while secondary metabolite proteins and other genes are present in reduced numbers, in line with the smaller genome size. To test for the fungal core genome, we searched for *L. bicolor*, other ectomycorrhizal fungi or saprotrophic *S. commune* and *C. cinerea* orthologs (Fig. S2, Table S1, Supporting Information). We could identify only 42% of *T. vaccinum* genes in all tested fungi.

**Secretome prediction**

We followed a bioinformatics pipeline to predict the secretome of *T. vaccinum* leading into a pool of potential communication signals (Fig. 3). Of the proteome, 8499 proteins were predicted with localization in the cytosol, peroxisome or nucleus; 1714 of the sequences were predicted to be secreted, 1768 contained secretion and mitochondrial import sequences. Of the secreted proteins, 573 have a typical N-terminal secretion sequence, 401 of which do not carry a transmembrane domain (108 have one and 64 have two or more transmembrane domains and, consequently, belong to the class of membrane proteins). Of these, 302 proteins were classified extracellular, with five sequences lacking a methionine start amino acid. Of the remaining 297, extracellular location was highly probable (score ≥ 17) for 206, while 91 had probable (score 1–17) secretome association. Thus, 1.7% of the genomic proteins are part of the predicted secretome of *T. vaccinum*. Interestingly, the comparison of secreted proteins by *T. vaccinum* with the secretomes of *L. bicolor*, *M. grisea*, *M. perniciosa*, *C. cinerea* and *U. maydis* indicated that *T. vaccinum* has mostly unique postulated secreted proteins without orthologs in the secretomes of other fungi (Table S2, Supporting Information). Only some additional hits to the fungal secreted proteins appeared searching in the genome sequence of *T. vaccinum*. Thus, some were not postulated as being secreted in the *T. vaccinum* genome.

**Tricholoma vaccinum secretome analysis by 2D-SDS-PAGE**

The identification of secreted proteins of *T. vaccinum* revealed information on proteins excreted during cultivation under laboratory conditions. Out of 75 proteins extracted from 2D gels, 55 were identified with a Mascot score over 60 (Table S3, Supporting Information). They belong to 22 different proteins including five small, secreted proteins with less than 300 amino acid residues (Tables S4 and S5, Supporting Information). Of these 22 proteins, 12 showed a typical secretion signal at their N-terminus. Five additional proteins were predicted to be excreted via a non-classical pathway, and five proteins were not predicted as secreted proteins, with one of them putatively located in mitochondria and four in the cytosol, peroxisome or nucleus and are, thus, arisen from cell lysis.

**Exudates modify the excreted protein composition of the secretome**

To evaluate the immediate early response without direct contact and to identify proteins specifically produced before and during partner recognition, we added spruce root exudates (Figs S3–S8, Supporting Information). The changed secretome revealed seven proteins regulated before any first physical contact (Table 2). Two proteins were upregulated. Protein g1377 showed 3-fold regulation and shared similarity with a hypothetical protein of *L. bicolor* and a peptidase lacking a Pfam domain. A cytosolic localization was predicted for this protein because of a
Figure 3. Bioinformatics pipeline for T. vaccinum proteins.

Table 2. Differentially regulated secreted proteins after spruce exudate application.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Pfam domain</th>
<th>Sequence similarity</th>
<th>Ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1377</td>
<td></td>
<td>(a) Hypothetical protein Laccaria bicolor 88% (XP 001876777.1)</td>
<td>2.995</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Peptidase (zinc peptidase like family) Coprinopsis cinerea 85% (XP 001837336.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g8303</td>
<td>PF 11937</td>
<td>Malate dehydrogenase Gloeophyllum trabeum 52% (EPQ52251.1)</td>
<td>2.066</td>
<td>0.002</td>
</tr>
<tr>
<td>g8018</td>
<td></td>
<td>Not identified</td>
<td>0.169</td>
<td>0.034</td>
</tr>
<tr>
<td>g3925</td>
<td>PF 12708</td>
<td>Pectate lyase superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(a) Hypothetical protein Agaricus bisporus 64% (EKM82792.1)</td>
<td>0.235</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Exo-beta-1,3-glucanase Gloeophyllum trabeum 63% (EPQ57185.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g3988</td>
<td></td>
<td>Hypothetical protein Serpula lacrymans 38% (EGN99237.1)</td>
<td>0.242</td>
<td>0.000</td>
</tr>
<tr>
<td>g3925</td>
<td>PF 12708</td>
<td>Pectate lyase superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(a) Hypothetical protein Agaricus bisporus 64% (EKM82792.1)</td>
<td>0.437</td>
<td>0.026</td>
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<tr>
<td></td>
<td></td>
<td>(b) Exo-beta-1,3-glucanase Gloeophyllum trabeum 63% (EPQ57185.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

missing secretion signal, stressing the necessity to compare experimental and in silico analyses. The second, 2-fold upregulated, Pfam domain including protein g8303 (PF11937) shared similarity with a malate dehydrogenase. Five proteins were repressed after root exudate addition with a cut-off of 2-fold regulation. All five carried classical N-terminal secretion signals. The downregulated protein g8018 (0.17-fold) possibly is a cellulose-degrading enzyme. Two hits were similar to a pectate lyase homolog, protein g3925 for plant cell wall degradation and protein g3988 without Pfam domain.
Figure 4. In silico analyses of the Pfam domains of potentially secreted proteins.

**Predicted functions for secreted proteins using Pfam domains**

Pfam analyses showed conserved domains for half of the 206 likely secreted proteins \( (n = 102) \) with 67 different functional domains (Fig. 4). Most prominent are proteins with a function in nutrition and energy metabolism, which constitutes 52% of the secretome and 26% of the predicted proteome. Domain PF00704, belonging to glycosyl hydrolysis family 18, is relevant in carbohydrate metabolism. Proteins with this domain appeared with six hits. The next group included 19% of proteins with Pfam domains typical for cell wall degrading functions (Table 3). Other proteins related to mycorrhiza establishment (9%), including fungal hydrophobins and two proteins that might be involved in signaling. They shared similarities with plant pathogens: g4012 with PF00188 from cysteine-rich secretory proteins found in pathogenicity-related proteins (Milne et al. 2003), and g7831 with PF10342, known from a small secreted protein in L. bicolor (http://www.uniprot.org/uniprot/B0CXV6, Martin et al. 2008) and reported to be involved in pathogenicity (Saunders et al. 2012). Although the number of proteins included in potential mycorrhiza functions is low, the most abundant domain with seven hits was PF01185, belonging to fungal hydrophobins. Additionally, three hits for PF05730 CFEM domain consisting of an eight-cysteine motif known to be relevant in pathogenesis were found (Kulkarni et al. 2005).

Identified proteins from the 2D-SDS-PAGE analyses belong mainly to nutrition and energy metabolism as well (Fig. 5). Five proteins might be relevant for cell wall degradation and two for detoxification, while no proteins were found with a putative role in mycorrhiza, which is completely in line with the axenic cultivation during these experiments. From the identified proteins, only two had been predicted from the in silico analysis. First, g1288 with a Pfam domain for the glycosyl hydrolase family 20 and a GO annotation for carbohydrate metabolism (0005975), and second g1345 with a Pfam NAD(P)-binding Rossmann-like domain. Blast analyses revealed 54% amino acid similarity to a FAD/NAD(P)-binding domain-containing protein from Stereum hirsutum FP-91666 SS1 associated with flavin containing amine oxidoreductases involved in ammonium nutrition.

**The role of CAZy enzymes connected with plant cell wall degradation**

Prediction for plant cell wall degrading enzymes was also performed with the CAZy grouping where we can conclude that from the 11 981 proteins predicted in the genome, 82 copies exist for CAZy enzymes involved in plant cell wall degradation (<1%, Table 4). Of the 206 potentially secreted proteins, around 6% were associated with cell wall lysis, less than predicted with the Pfam domain search, which could be related to non-classical secretion pathways (see Table 4).

Using the CAZy annotation, 14 proteins are carbohydrate active enzymes, but only one is associated with families directly connected to cell wall lysis (g8018; CAZy: GH5, PFAM00150). As this protein was less abundant after exudate exposure, this might be a relevant finding. The difference between predicted secretome and 2D-SDS-PAGE analyses indicated that many of the plant cell wall degrading enzymes were not expressed in T. vaccinum. This is different from the Pfam domain analyses, where most of the secreted proteins have a possible connection to plant cell wall lysis.

Since both, 2D-SDS-PAGE and in silico prediction, revealed three different laccases in the fungal genome, we verified laccase excretion. Indeed, T. vaccinum was able to excrete laccases into the supernatant, and addition of spruce exudates slightly increased laccase activity but not significantly, as was expected from 2D-SDS-PAGE evaluation (Fig. 6B).

In addition, cellulose excretion was verified (Fig. 6A). Application of spruce exudates reduced cellulase activity in accordance with our findings on lower cellulase abundance in 2D-SDS-PAGE.

**Small secreted proteins as potential effectors**

Potential effectors were predicted with further candidates from 2D-SDS-PAGE being included in this analysis. In total, 113 proteins were smaller than 300 aa, and 11 less than 100 aa; 104 proteins did not show Pfam domains, making those interesting aspirants for effectors. Half of these proteins (46) showed an alanine content over 10%, and 8 even over 15%. For serine, 43 over 10%, 5 over 15% were scored and 8 proteins were
Table 3. Pfam domains of secreted proteins relevant for plant cell wall degradation.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Secretome</th>
<th>Regulation by exudates</th>
<th>Target</th>
<th>Pfam domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>g6909</td>
<td>Predicted</td>
<td>Downregulated</td>
<td>Cellulose</td>
<td>PF 01822 WSC domain</td>
</tr>
<tr>
<td>g1845;</td>
<td>Predicted; excreted</td>
<td></td>
<td>Cellulose</td>
<td>PF 00150 Cellulases</td>
</tr>
<tr>
<td>g2770;</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g3225;</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g8018</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g2947;</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g5504;</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g4632;</td>
<td>Excreted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g4736</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g921</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g5142</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g4409</td>
<td>Excreted</td>
<td>Not regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g1288</td>
<td>Excreted</td>
<td>Not regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g7250;</td>
<td>Excreted</td>
<td>Not regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g9668</td>
<td>Excreted</td>
<td>Not regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g9068</td>
<td>Excreted</td>
<td>Not regulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g5183</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g3925</td>
<td>Predicted; excreted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g7461;</td>
<td>Predicted; excreted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g10353</td>
<td>Predicted; excreted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g7714</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. In silico analyses of the Pfam domains of proteins detected by 2D-SDS-PAGE.

enriched in cysteine residues (>5%, Table 5). Of the proteins without Pfam domains, a high number carried the motifs [L/I]xAR or [Y/F/W]xC, whereas other motifs like the high conserved DELD motif known from small secreted proteins in Piriformospora indica were not found in T. vaccinum (Table S6, Supporting Information). Except for priA (g319) and hydrophobin (g5362), all others are hypothetical proteins with no known function.

For the hydrophobin, a localization verified its secretion as a coating of aerial hyphae in the mantle of T. mycorrhiza (Fig. 7). Four proteins (g1981, g2490, g7859, g8660; Table 5) share feature...
**DISCUSSION**

The potential of secreted proteins to function as communication signals in early ectomycorrhizal interaction was analyzed using the fungal genome and secretome of *T. vaccinum* and exudates of its host, spruce. The genome size of *T. vaccinum* with 44.2 Mb is relatively low, but lies within the range of mycorrhizal fungi between 38 and 125 Mb (Kohler et al. 2015). The ectomycorrhizal fungi *A. muscaria* (40 Mb) and *Sebacina vermisfera* (38 Mb) have even lower genome sizes (Kohler et al. 2015). The predicted protein number (approximately 12 000) is lower than in other mycorrhizal fungi (15 000–23 000; Kohler et al. 2015). The repertoire of signaling peptides was shown in silico analyses with 1.7% of encoded genes belonging to the predicted secretome. In comparison, the wheat pathogen *Fusarium graminearum* has a higher percentage (4.2%; Brown, Antoniw and Hammond-Kosack 2012) of secreted proteins, as was the case for the ectomycorrhizal basidiomycete *L. bicolor* (14.2%; Martin et al. 2008) and other phytopathogenic and mycotrophic fungi except the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (1.33%; Tisserant et al. 2013, compare Table 1). The GC content of both ectomycorrhizal fungi is nearly identical (46.7% in *L. bicolor*), but a lower average intron length in *T. vaccinum* genes was observed (92.7 bp in *L. bicolor*; Martin et al. 2008).

All secreted proteins in *T. vaccinum* grouped into different functional categories where communication is needed, namely adhesion, cell wall lysis, pathogen protection and nutrition specifically during the saprophytic stage (Vincent et al. 2012). The by far most prominent Pfam domain carrying protein family in *T. vaccinum* are hydrophobins, necessary for adhesion (Mankel, Krause and Kothe 2002). Here, we showed the coating of hyphal tips with hydrophobins verifying the predicted excretion. Previously, it was shown that the nine hydrophobins (Hyd1–Hyd9) of *T. vaccinum* are differently expressed during development (D. Sammer, pers. comm.). For example, Hyd8 is covering the hyphal cell wall to lower surface tension for the development of aerial mycelium. Interestingly, an upregulation of hyd4 and hyd5 transcripts was shown during spruce root exudates and volatile exposure (D. Sammer, pers. comm.). In addition, cerato-platanin-like proteins are known to self-assemble like hydrophobins and could be necessary for adhesion in *T. vaccinum* and *L. bicolor* (Vincent et al. 2012). This domain is also present in the necrosis inducing Snodprot1 protein from the phytopathogenic ascomycete *Ceratocystis fimbriata* f. sp. *platani* that causes canker stain (Pazzagli et al. 1999). Our data support the notion that the mutualistic symbiosis partner *T. vaccinum* excretes proteins also known from phytopathogens in typical plant–fungus interactions.

In addition, two subtilisins were identified which have been described to be regulated during mycorrhiza formation in *L. bicolor* (Martin et al. 2008). Important for pathogen protection
Table 5. Selected small secreted proteins from *T. vaccinum* with features known from fungal effectors.

<table>
<thead>
<tr>
<th>Protein (ID)</th>
<th>Found in</th>
<th>Size (AA)</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>Pfam domain</th>
<th>Protein homology/organism/sequence identity/accession number</th>
<th>Amino acid composition (%)</th>
<th>Repeat-rich</th>
<th>Motif</th>
<th>NLS: score</th>
</tr>
</thead>
<tbody>
<tr>
<td>g319</td>
<td>Prediction</td>
<td>235</td>
<td>24.6</td>
<td>5.95</td>
<td>No</td>
<td>priA protein/ Coprinopsis cinerea okayama/73%/ XP.001828720.2</td>
<td>cys: 7.7</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g450</td>
<td>Prediction</td>
<td>239</td>
<td>25.8</td>
<td>7.84</td>
<td>No</td>
<td>Predicted protein/ Laccaria bicolor/42%/ XP.001879573.1</td>
<td>cys: 7.5</td>
<td>Yes</td>
<td>No</td>
<td>b: 2.5; m: 5.7, 2</td>
</tr>
<tr>
<td>g3238</td>
<td>Prediction</td>
<td>112</td>
<td>12.0</td>
<td>4.92</td>
<td>No</td>
<td>Hypothetical protein/ Moniliophthora roreri/65%/ ESK95615.1</td>
<td>cys: 7.1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g3839</td>
<td>Prediction</td>
<td>321</td>
<td>33.3</td>
<td>8.09</td>
<td>No</td>
<td>Hypothetical protein/ Punctularia strigosaxonata/49%/ EIN12496.1</td>
<td>cys: 8.1</td>
<td>Yes</td>
<td>No</td>
<td>b: 2.4</td>
</tr>
<tr>
<td>g5362</td>
<td>Prediction</td>
<td>122</td>
<td>12.0</td>
<td>4.56</td>
<td>No</td>
<td>Hydrophobin 2/Heterobasidion annosum/38%/ ABA46362.1</td>
<td>cys: 6.6; gly: 10.2</td>
<td>No</td>
<td>No</td>
<td>b: 2.4</td>
</tr>
<tr>
<td>g7333</td>
<td>Prediction</td>
<td>261</td>
<td>28.1</td>
<td>6.38</td>
<td>No</td>
<td>Predicted protein/ Laccaria bicolor/45%/ XP.001885972.1</td>
<td>cys: 8</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g7559</td>
<td>Prediction</td>
<td>140</td>
<td>14.7</td>
<td>4.63</td>
<td>No</td>
<td>Hypothetical protein/ Trichoderma atroviride/45%/ EHK39458.1</td>
<td>cys: 5.7; gly: 15</td>
<td>No</td>
<td>[Y/F/W]xC</td>
<td>No</td>
</tr>
<tr>
<td>g9002</td>
<td>Prediction</td>
<td>115</td>
<td>11.7</td>
<td>4.35</td>
<td>No</td>
<td>Protein of unknown function/ Pyronema omphalodes/32%/ CXX11522.1</td>
<td>cys:7; gly: 11.3</td>
<td>No</td>
<td>[Y/F/W]xC</td>
<td>No</td>
</tr>
<tr>
<td>g11648</td>
<td>Prediction</td>
<td>160</td>
<td>17.7</td>
<td>9.21</td>
<td>No</td>
<td>Hypothetical protein/ Legionella pneumophila/29%/ YP.007566639.1</td>
<td>cys: 5</td>
<td>No</td>
<td>No</td>
<td>b: 2.2</td>
</tr>
<tr>
<td>g1981</td>
<td>Prediction</td>
<td>74</td>
<td>8.0</td>
<td>5.50</td>
<td>No</td>
<td>Glutamyl-tRNA reductase/ Corynebacterium massiliense/53%</td>
<td>cys: 0; Ala: 16.2; Leu: 18.9</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g2490</td>
<td>Prediction</td>
<td>100</td>
<td>10.9</td>
<td>7.85</td>
<td>No</td>
<td>Hypothetical protein CTHT0024890/Chaetomium thermophilum var. thermophilum DSM 1495/36%/EGS20653.1</td>
<td>cys: 1; Gly: 13; Val: 13</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g7859</td>
<td>Prediction</td>
<td>89</td>
<td>9.5</td>
<td>6.21</td>
<td>No</td>
<td>Hypothetical protein/ Lactobacillus mucosae/33%/ WP.006501054.1</td>
<td>cys: 0; Ala: 11.2; Leu: 11.2</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g8660</td>
<td>Prediction</td>
<td>97</td>
<td>10.6</td>
<td>6.89</td>
<td>No</td>
<td>Casein kinase I isoform delta-like/ Cucumis sativus/30%/ XP.00134054.1</td>
<td>cys: 0; Ala: 10.3; His: 13.4</td>
<td>No</td>
<td>No</td>
<td>b: 2.4; 2.1</td>
</tr>
<tr>
<td>g8303</td>
<td>2D-SDS-PAGE</td>
<td>259</td>
<td>27.6</td>
<td>8.80</td>
<td>PF11937</td>
<td>Malate dehydrogenase/ Gloeophyllum trabeum/52%/EPQS2251.1</td>
<td>cys: 1.5</td>
<td>No</td>
<td>[Y/F/W]xC</td>
<td>b: 2.3</td>
</tr>
<tr>
<td>g3988</td>
<td>2D-SDS-PAGE</td>
<td>154</td>
<td>16.4</td>
<td>4.90</td>
<td>No</td>
<td>Hypothetical protein SERLA73DRAFT182140/ Serpula lacrymans var. lacrymans S7.3/38%/WP.00111510.1</td>
<td>cys: 1.9; Ser: 11.7; Thr: 10.4</td>
<td>No</td>
<td>[Y/F/W]xC</td>
<td>b: 3.6</td>
</tr>
<tr>
<td>g5791</td>
<td>2D-SDS-PAGE</td>
<td>132</td>
<td>14.3</td>
<td>5.42</td>
<td>PF12681</td>
<td>Glyoxalase/ Labrenzia aggregata/47%/ WP.006935470.1</td>
<td>cys: 0.0; Ala: 12.9</td>
<td>No</td>
<td>No</td>
<td>m: 2.5</td>
</tr>
<tr>
<td>g6089</td>
<td>2D-SDS-PAGE</td>
<td>232</td>
<td>24.7</td>
<td>5.57</td>
<td>PF07731</td>
<td>Laccase 2/ Coprinopsis cinerea/68%/ XP.001831045.2</td>
<td>cys: 0.9; Pro: 10.8</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>g10866</td>
<td>2D-SDS-PAGE</td>
<td>225</td>
<td>24.6</td>
<td>6.29</td>
<td>PF07732</td>
<td>Laccase/ Laccaria bicolor/73%/ XP.001874989.1</td>
<td>cys: 1.8</td>
<td>No</td>
<td>No</td>
<td>b: 2.6; 3.5; 2.5</td>
</tr>
</tbody>
</table>

MW: molecular weight; PI: pH of uncharged protein; NLS: nuclear localization signal; b: bibartaite; m: monopartaite.
Figure 7. Hydrophobin in *Tricholoma* hyphae. Hyphae of the hyphal mantle in a mycorrhized root (A) show hydrophobin after immunofluorescent labeling with Sc3 antibodies (B). DAPI stained nuclei (C) and auto-fluorescence (D) of the mycorrhized root are shown for comparison. Bars (A) 20 μm, (B) 8 μm.

Figure 8. Upregulated proteins after spruce exudate exposure. Predicted amino acid sequences are shown for proteins g3988 (A) and g8303 (B). Straight underlined: secretion signal, dotted underlined: nuclear exportation signal, bold: nuclear localization signal, double underlined: Pfam domain, in brackets: motif.

might be an enzyme including the domain PF05426 for an alginate lyase, destroying alginate from algal and bacterial biofilms (Rehm and Valla 1997). Two other proteins (g1075, g6995) might be involved in bacterial antibiotics with a peptidoglycan binding LysM domain (PF01476) and thus may contribute to spruce health (Cairney and Burke 1994). Hence, *T. vaccinum* could reduce biofilm production, e.g. of soil pseudomonads on the rhizoplane. This is in accordance with *L. bicolor* showing antibacterial features like lysozyme production (Vincent et al. 2012).

In *F. graminearum*, 70% of the proteins with Pfam domains are involved in plant cell wall degradation, whereas in *T. vaccinum* these enzymes encompass about 19% (Brown, Antoniw and Hammond-Kosack 2012). Together with other potentially excreted peptidases and lipases, these enzymes might support growth in the apoplast during Hartig’ net formation or during saprophytic life on dead plant material (Cairney and Burke 1994). *L. bicolor* also secretes numerous proteases, like aspartic peptidase A1 with 82% amino acid similarity to an aspartic peptidase (g621) of *T. vaccinum* (Vincent et al. 2012).

Cell wall degrading enzymes included three laccases, cellulases and pectinases. In the secretome of *L. bicolor*, only a pectinesterase was found to be present among plant cell wall degrading enzymes (Vincent et al. 2012). The ability of *Tricholoma* species to produce ligninolytic and cellulolytic enzymes has been reported earlier (Norkrans 1950; Haselwandter, Bobleter and Read 1990; Bending and Read 1997). We confirmed laccase and cellulase excretion by *T. vaccinum*. In mycorrhized roots of *P. abies* and *Larix decidua* with *L. amethystea*, a higher laccase activity was found compared to non-mycorrhizal roots (Münzenberger et al. 1997). The regulation of cellulolytic activity in the *T. vaccinum* supernatant indicates that the early response to plant root exudates may be relevant for this late stage mycorrhizal fungus. Comparing Pfam domains and the CAZy enzymes between the potential and experimentally proven secreted proteins, a strong decrease in plant cell wall lytic proteins indicated an adaptation to the host and differences between the genomic toolbox and steady-state excretion. Interestingly, the identified cell wall degrading enzymes like g8018, a cellulase, were even less abundant during exudate exposure. Downregulation of these proteins minimizes the risk of inducing plant defense. On average, ectomycorrhizal fungi have 62 copies of CAZys and 120 saprotrophs; however, *T. vaccinum* with 82 has a stronger cell wall degrading capacity compared with other ectomycorrhizal fungi (Kohler et al. 2015).
Interestingly, *T. vaccinum* has 21 copies of enzymes belonging to the GH3 family, including β-1,4-glucosidases for cellulose and β-1,4-xylosidases for xylan degradation, a higher number than all ectomycorrhizal fungi studied previously, and also more than some saprotrophs, like the white rot causing *Punctaria strigosozonata* with the highest number (14) found so far (Kohler et al. 2015). None of the identified GH3 enzymes has a secretion signal, thus they were not found in the secretome and may be excreted under some specific conditions via a non-classical pathway, or else never be secreted.

In search for potential effectors, we checked for feature similarity to known fungal small secreted proteins. The [L/I]xAR motif, found in many postulated small secreted proteins in *T. vaccinum*, is known from effectors of the rice blast pathogen M. oryzae (Yoshida et al. 2009). The [Y/F/W]xWC motif was found in haustoria-forming pathogenic fungi (Godfrey et al. 2010). Both motifs might act to aid effector translocation to manipulate plant response, e.g. reduce defense reactions with plant pathogens.

The regulated small protein g8303 might constitute a very early secreted effector. The lack of host specificity is consistent with an unspecified upregulation of the mutualistic effector MiSSP7 in *L. bicolor* in the presence of host and non-host root exudates (Plett et al. 2011). Different from g8303 and MiSSP7, transcripts of another mycorrhiza specific effector, Sp7 of *R. irregularis* and Glomus intraradices, accumulates after physical contact (Kloppholz, Kuhn and Requena 2011). Likely, Sp7 as well as MiSSP7 are needed to manipulate plant response, which is a possible function also for g8303. Both known effectors target the plant cell nucleus and thereby achieve fungal colonization. *L. bicolor* MiSSP7 interferes with a negative regulator of jasmonic acid and blocks this phytohormone's action, which, in turn, enhances mycorrhization (Plett et al. 2014). In contrast to MiSSP7, Sp7 has a nuclear localization signal-like pathogenic effector, also found in g8303 (Kloppholz, Kuhn and Requena 2011; Rovenich, Boshoven and Thomma 2014). Protein g8303 also carries a nuclear export signal not reported for the other two effectors, but known from p25 of the beet necrotic yellow vein virus of sugar beet, for which the fungus *Polymyxa beta* is a vector. This protein can switch localization between nucleus and cytoplasm (Vetter et al. 2004). A second putative effector downregulated during exudate exposure is g3988 with structural similarities to g8303. It also carries the [Y/F/W]xWC motif and a nuclear localization signal. Both features hint to a potential role in early signaling, targeting plant defense.

Here, we used the genome and proteomic studies of an ectomycorrhizal fungus to investigate immediate early response of the fungus to its host plant long before physical contact ensues. The application of root exudates exerted a change in the secretome of the fungus. However, this was likely in an unspecified manner. Regulation of small excreted proteins was observed, most of which with unknown function, like the small secreted proteins g8303 and g3988, making them good candidates for future analysis.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online.

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We thank Maria Pötsch for protein analyses, and Joseph G. H. Wessels for the antisera against *S. commune* hydrophobins.

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**Conflict of interest**. None declared.

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