High Resolution Analysis of Fungal Secretomes

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Introduction

Fungi are microbial eukaryotes with major roles in terrestrial ecology, as soil saprobes, beneficial plant symbionts and devastating plant pathogens. Because plant-associated fungi have a common phylogenetic origin and a long history of co-evolution with plants, they likely share ancestral functions involved in interactions with host plants. Studies on genes/functions involved in pathogenicity and symbiosis have highlighted a particular class of effectors corresponding to secreted fungal proteins (SFPs). Preliminary experiments highlighted SFPs as interesting proteins families in *Magnaporthe grisea* (fungal rice leaf pathogen), *Leptosphaeria maculans* (fungal rapeseed leaf/stem pathogen) and *Laccaria bicolor* (fungal pine/poplar root symbiont). Genome-wide transcriptomics experiments showed that *M. grisea* genes specifically expressed during infection mainly encode SFPs. The survey of the genome sequence of *L. bicolor* highlighted a large-scale amplification of genes encoding SFPs. In *L. maculans*, recent genome sequencing showed that genes involved in avirulence encode secreted proteins and are embedded in heterochromatic-like regions as solo genes. Together, these preliminary results strongly suggest that SFPs could play a role in the interactions between *M. grisea*, *L. maculans* and *L. bicolor* and their respective host plants. The systematic study of fungal proteins that are secreted during interactions with host plants should significantly widen our knowledge of molecular players contributing to fungal plant symbiosis or pathogenicity. Additionally, this project should provide an interesting comparison between SFP repertoires from symbiotic and pathogenic fungi, which will be of general interest for genomics and evolutionary scientists.

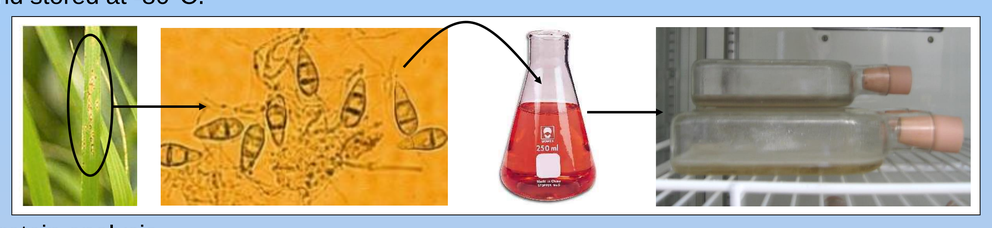
Objective 1: Identification of *M. grisea* secretome

Many secretomic sudies have reported the contamination issue originating from cell lysis releasing cytosolic proteins into the extracellular medium. One goal of the present work was to identify all the secreted proteins that could be resolved by 2-DE and assess the purity of extracellular compartment by comparing it with intracellular proteins found in the mycelium.

Materials and Methods

Growth conditions of *Magnaporthe grisea*

Fungus was initially grown in Erlenmeyer flask containing 100mL TNK-YE (55mM glucose, 23mM NaNO3, 14mM KH_2PO_4, 2mM $MgSO_4$ $7H_2O$, 0.7mM $CaCl_2$ $2H_2O$, 15µM $FeSO_4$ $7H_2O$, 2g/L yeast extract, oligoelements) medium (pH 5.6) for 3 days (110 rpm, 26°C), after which supernatant was discarded and mycelium homogenized by grinding. 1/20 of ground mycelium was then transfered into Roux's bottles containing 100mL TNK-YE medium. pH and growth rate were monitored during the course of the experiment. After 4 and 9 days, mycelium and liquid medium were separated by filtration on Miracloth, immediately frozen and stored at -80°C.

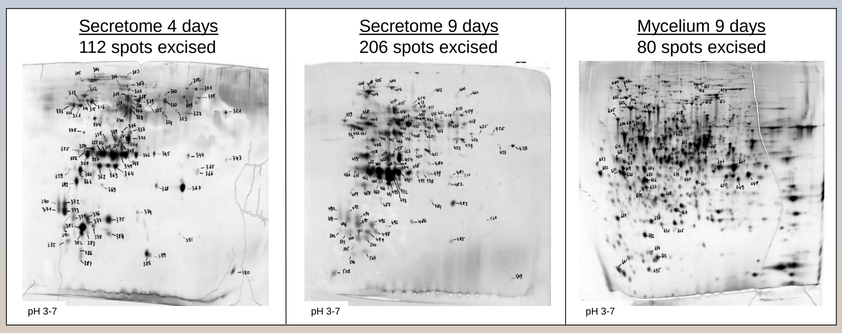


Protein analysis

Mycelium samples were rinsed, dried and lyophilized before grinding. Proteins were recovered using a 10% TCA/acetone precipitation method. 15mL medium samples were further filtered (0.22 µm), dialyzed (MWCO 1000Da) twice for 10h in 5L H_2O at 4°C and lyophilized. Secreted proteins were recovered by resuspending the lyophilized medium in 400µL IEF buffer (urea, thiourea, DTT, CHAPS, CA's). 2-DE conditions were as follows: 20 µg protein load, 18cm IPG strips (pH 3-10NL or 3-7), 10% SDS-PAGE, MS compatible silver nitrate staining.

MS analysis

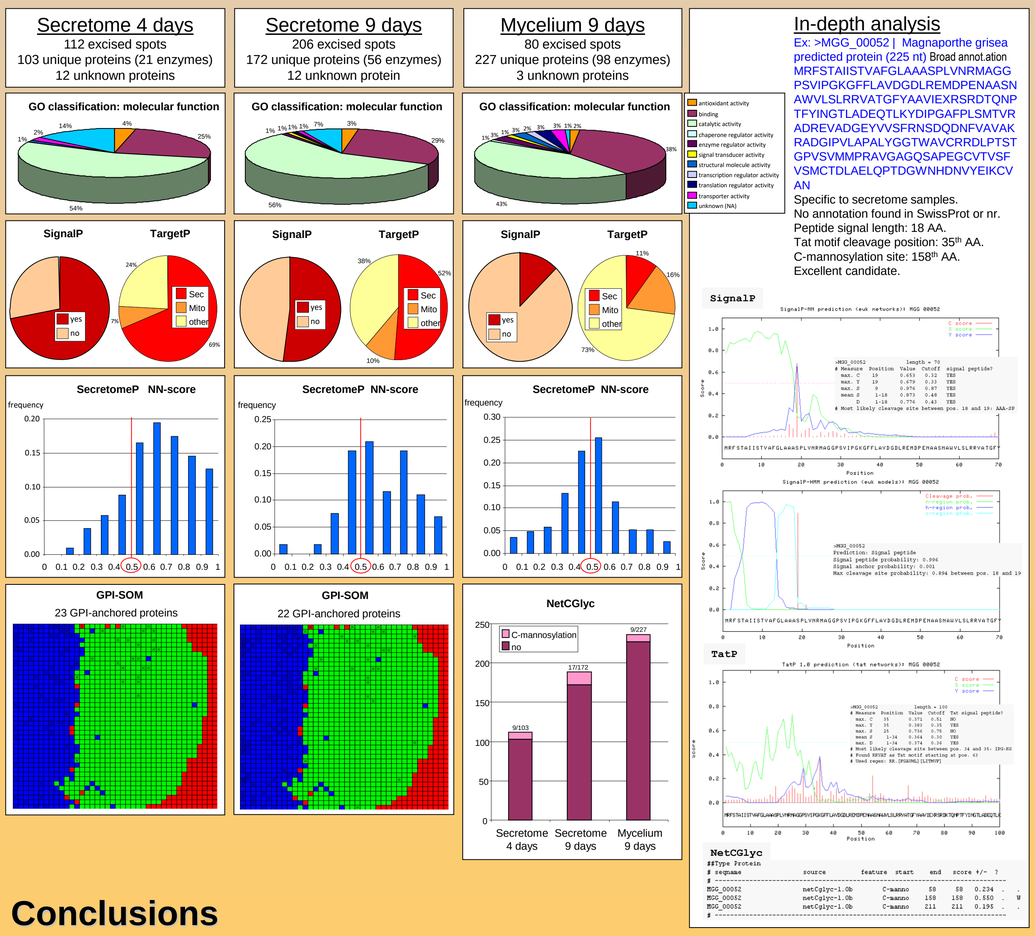
398 spots were manually excised (secretome 4 days 112 spots, secretome 9 days 206 spots and mycelium 80 spots), trypsin-digested and analyzed using nLC-ESI-MS/MS (LCQ ion trap). Peptide lists were searched against the 18031 *M. grisea* protein sequences retrieved at the Broad Institute (http://www.broad.mit.edu).



Bioinformatic analysis

Sequences were re-annotated against SwissProt using Blast2GO (www.blast2go.de); GO terms and E.C numbers were retrieved. Secretion occurrence was investigated using SignalP, TargetP, TatP and SecretomeP (http://www.cbs.dtu.dk/services/), along with the presence of GPI anchors using GPI-SOM (http://gpi.unibe.ch/). Potential C-mannosylation sites were also searched using NetCGly (http://www.cbs.dtu.dk/services/).

Results



In-depth analysis

Ex: >MGG_00052 | Magnaporthe grisea predicted protein (225 nt) Broad annot.ation

MRFSTAIISTVAFGLAAASPLVNRMAGG PSVIPGKGFFLAVDGDLREMDPENAASN AWVLSLRRVATGFYAAVIEXRSRDTQNP TFYINGTLADEQTLKYDIPGAFPLSMTVR ADREVADGEYVVSFRNSDQDNFVAVAK RADGIPVLAPALYGGTWAVCRRDLPTST GPVSVMMPRAVGAGQSAPEGCVTVSF VSMCTDLAELQPTDGWNHDNVYEIKCV AN

Specific to secretome samples.
No annotation found in SwissProt or nr.
Peptide signal length: 18 AA.
Tat motif cleavage position: 35th AA.
C-mannosylation site: 158th AA.
Excellent candidate.

Conclusions

2-DE is a suitable technique to isolate secreted proteins from *in vitro* grown *M. grisea* prior to MS identification. A more refined analysis of the sequences using various on-line bioinformatic tools allowed us to verify the minimal contamination of the extracellular medium, especially during early growing phase (4 days). Most of the secreted proteins presented a signal peptide and were predicted to be secreted, unlike the proteins identified in the mycelium.

Objective 2: Isolation of secreted proteins from *L. maculans* and *L. bicolor*

A thourough survey of the literature pertaining to fungal secretomics revealed how difficult it is to obtain uncontaminated extracellular compartments and recover the highly diluted secreted proteins. The quality of the proteomic results, and electrophoretic patterns in particular, varies greatly from one organism to another. Another issue to overcome lies in the fact that most secreted proteins are mannosylated, thus producing smeared 2-D gels. Satisfactory protein isolation was achieved on *M. grisea* secretome leading to proper electrophoretic separation (see objective 1) while no proteomic result was reported on secreted proteins from *L. maculans* and *L. bicolor* so far. Our second goal was thus to isolate secreted proteins released in liquid media from *in vitro* grown fungi (*L. maculans* and *L. bicolor*) and assess the quality of the extraction through gel-based methods (1- and 2-DE).

Materials and Methods

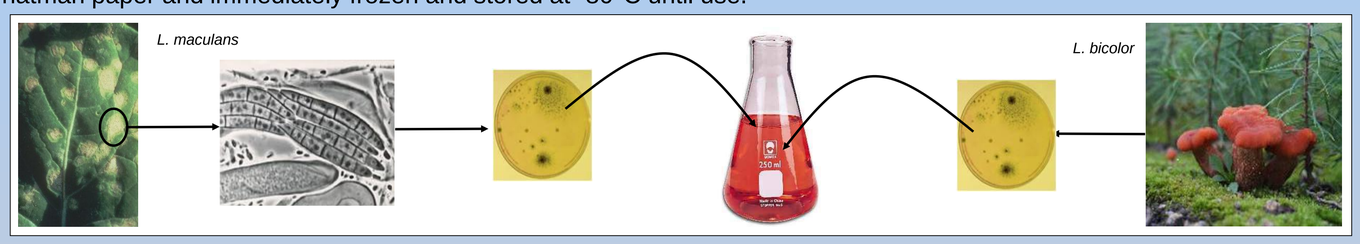
Growth conditions

Leptosphaeria maculans

Spores were isolated from maculated rapeseed leaves, spread on Petri dishes and the mycelium was transfered into Erlenmeyer flask containing 100mL Fries (5g/L YE, 30g/L sucrose, 5g/L tartrate NH_4, 1g/L KH_2PO_4, 0.5g/L $MgSO_4$ 7H2O, 0.13g/L $CaCl_2$, 0.1g/L NaCl, 1g/L NH_4NO_3) medium. After 1 week, mycelium and liquid medium were separated by filtration on Whatman paper and immediately frozen and stored at -80°C until use.

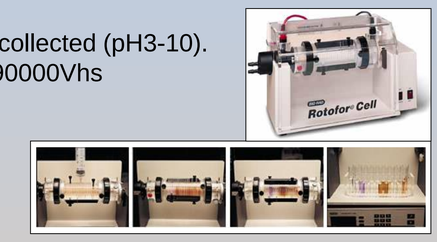
Laccaria bicolor

Spores were isolated from carpophore, spread on Petri dishes and the mycelium was transfered into Erlenmeyer flask containing 100mL P5Lb (0.5g/L di-NH4 tartrate, 1g/L KH2PO4, 0.5g/L MgSO4 7H2O, 5g/L maltose D+, 20g/L glucose D+, 1mL/L thiamine-HCl, 1mL/L 10% Kanieltra) medium. After 5 weeks, mycelium and liquid medium were separated by vacuum-filtration on Whatman paper and immediately frozen and stored at -80°C until use.

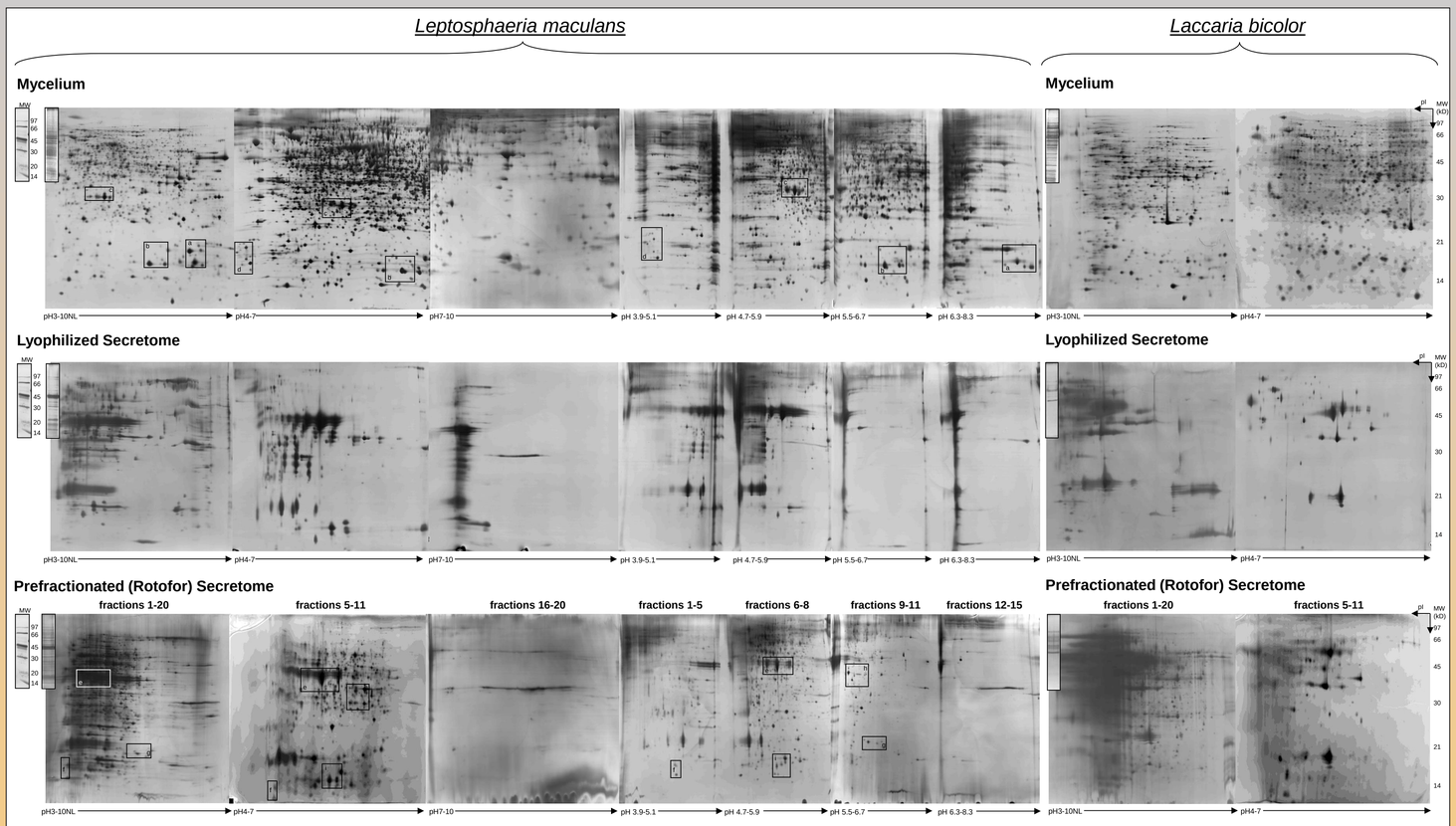


Optimization of protein extraction protocol

Mycelium: ground in liquid N2 and proteins extracted using 10 % TCA/acetone.
Secretome: filtered (0.22 µm), dialyzed (twice in 5L H_2O at 4°C, MWCO 3500Da), 1mM PMSF and 0.05% PVP added.
lyophilization of 15mL medium or pre-fractionation using liquid-phase IEF.
Liquid-phase IEF: Rotofor (Bio-Rad) large chamber using 30mL of medium, 20 fractions collected (pH3-10).
IPG-IEF: 24cm IPG ReadyStrip pH3-11NL, pH4-7, pH7-10 and micro-range (Bio-Rad), 90000Vhs
SDS-PAGE: 20x24cm homecast 12% acrylamide gel, 150V for 11h
Staining: nitrate silver method, MS compatible
Image analysis: Progenesis P240 (Nonlinear)



Results



Leptosphaeria maculans

extraction method	protein load (µg) / medium volume (mL)	pH range	number of spots
mycelium			
TCA/acetone	100 µg	3-10NL	1862
TCA/acetone	100 µg	4-7	3632
TCA/acetone	100 µg	7-10	729
TCA/acetone	4 x 100 µg	3.9-5.1/4.7-5.9/5.5-6.7/6.3-8.3	4260
secretome			
lyophilization	15 mL	3-10NL	852
liquid-phase IEF	30 mL	3-10NL	1740
lyophilization	15 mL	4-7	519
liquid-phase IEF	30 mL	4-7	861
lyophilization	15 mL	7-10	123
liquid-phase IEF	30 mL	7-10	145
lyophilization	15 mL	3.9-5.1/4.7-5.9/5.5-6.7/6.3-8.3	986
liquid-phase IEF	30 mL	3.9-5.1/4.7-5.9/5.5-6.7/6.3-8.3	1941

Laccaria bicolor

extraction method	sample volume	pH range	number of spots
mycelium			
TCA/acetone	100 µg	3-10NL	1935
TCA/acetone	100 µg	4-7	2564
secretome			
lyophilization	15 mL	3-10NL	468
liquid-phase IEF	30 mL	3-10NL	1123
lyophilization	15 mL	4-7	146
liquid-phase IEF	30 mL	4-7	437

Conclusions

Protein assays and electrophoretic patterns indicated that all liquid media contained very few secreted proteins, which were further lost when applying a precipitation method such as TCA/acetone (not shown). Lyophilization followed by resolubilization in IEF buffer limited protein loss, yet highly abundant proteins proved difficult to separate using 2-DE (horizontal streaks). A prefractionation step using liquid-phase IEF reduced the prominence of the most abundant proteins, thus unraveling proteins secreted in lower quantities and considerably increasing the number of spots resolved on 2-D gels. Spots separation was optimized by using IPG strips covering narrower pH ranges. Although secreted alkaline proteins could not be resolved (pH7-10 and 6.3-8.3), acidic to neutral were adequately focused, even the most prominent proteins.