Proteomic identification of extracellular proteins regulated by the Gna1 Gα subunit in *Stagonospora nodorum*

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Abstract

The fungus *Stagonospora nodorum* is the causal agent of stagonospora nodorum blotch (syn. leaf and glume blotch) disease of wheat. The *Gna1*-encoded Gα protein is an important signal transduction component in the fungus, which is required for full pathogenicity, sporulation and extracellular depolymerase production. In this study, we sought to gain a better understanding of defects associated with the *gna1* mutant by using two-dimensional gel electrophoresis to analyse the extracellular proteome for differences to the wildtype. Mass spectrometry analysis of altered abundant protein spots and peptide matching to the *Stagonospora nodorum* genome database have led to the identification of genes implicated in cell wall degradation, proteolysis, RNA hydrolysis and aromatic compound metabolism. In addition, quantitative RT-PCR has demonstrated that some of the encoding genes showed differential expression throughout host infection. Implications of these proteins and their corresponding genes in fungal virulence are discussed.

Keywords: Fungus, extracellular, Galpha, proteomics, Gna1, signal transduction, *Stagonospora nodorum*

Introduction

The heterotrimeric G protein signalling pathway in phytopathogenic fungi is well-characterised for its role in development and pathogenesis (Bolker 1998; Lee et al. 2003). The heterotrimer consists of α, β, and γ subunits that are coupled to the cytoplasmic side of a membrane-bound G protein coupled receptor. The heterotrimer is activated by interaction of an extracellular ligand with the receptor. This causes the dissociation of the Gα subunit from the βγ dimer and hence allows interaction with intracellular targets (Borkovich 1996). Perturbation of the pathway often results in severe phenotypic defects and compromised pathogenicity (Bolker 1998; Lee et al. 2003). Because of the importance of heterotrimeric G protein signalling in phytopathogenesis, transcriptomics (Eichhorn et al. 2006; Gronover et al. 2004) and proteomics (Tan et al. 2008) have recently been used to identify genes and proteins that are affected by this pathway. This has led to the identification of key genes that play critical roles phytopathogenicity (Eichhorn et al. 2006; Siewers et al. 2005; Tan et al. 2008). In addition, mounting evidence indicates that the production of fungal extracellular proteins is affected by heterotrimeric G protein signalling (Girard et al. 2004; Gronover et al. 2001; Prados-Rosales et al. 2006; Solomon et al. 2004). However, a detailed analysis of extracellular proteome regulation by heterotrimeric G protein signalling is lacking.

The fungal extracellular proteome is of significant interest as an increasing number of studies have led to the identification of secreted proteins that function as key determinants of the outcome of plant infection (Ciuffetti et al. 1997; Ellis et al. 2007; Friesen et al. 2006; Lauge and De Wit 1998). Two-dimensional gel electrophoresis (2-DE) proteomics is rapidly becoming the method of choice to dissect fungal extracellular proteomes (Abbas et al. 2005; Grinyer et al. 2005; Medina et al. 2005; Medina et al. 2004). The recent sequencing of plant pathogenic fungal genomes (Cuomo et al. 2007; Dean et al. 2005; Hane et al. 2007; Kamper et al. 2006) have now allowed for a more thorough analyses of the fungal proteome (Bohmer et al. 2007; Paper et al. 2007; Tan et al. 2008) using high resolution mass spectrometry for protein-to-gene assignment (Andersen and Mann 2000).

Stagonospora nodorum is a major fungal pathogen of wheat (Solomon et al. 2006a). The role of heterotrimeric G protein signalling in the pathogenicity of *S. nodorum* has been investigated through the disruption of the *Gna1* Gα subunit gene (Solomon et al. 2004). Mutants carrying the Gna1 impairment were reduced in their ability to colonise the host and showed developmental abnormalities such as abolished sporulation and an albino phenotype. Two-dimensional gel electrophoresis was recently used to compare the intracellular proteome of the *S. nodorum* wildtype and a *gna1* mutant for altered abundant proteins. This study identified several Gna1-regulated proteins including a positively regulated short-chain dehydrogenase (Sch1). Genetic dissection of *Sch1* revealed a required role in asexual development, an important asset of the *S. nodorum* pathogenic life cycle (Tan et al. 2008). Biochemical analysis of the *gna1* mutant has also indicated that the production of extracellular proteases and cell wall degrading enzymes was affected (Solomon et al. 2004). In this study, 2-DE was used to compare extracellular proteome of the *S. nodorum* wildtype and a *gna1* mutant in an attempt to identify individual protein components that are affected by *Gna1* disruption. Subsequently, these proteins were subjected to mass spectrometry analysis and gene identification was facilitated with mass spectra matching through a theoretical protein dataset derived from the sequenced *S. nodorum* genome (Hane et al. 2007). In addition, the expression profile of genes that encoded the target proteins was analysed via quantitative real time (RT)-PCR during host infection.

Materials and methods

Gene nomenclature

The nomenclature of all *S. nodorum* genes mentioned in this study are denoted by the prefix 'SNOG' used in conjunction with the designated gene number. Details of the version 1 annotated sequenced genome can be found at the Joint Genome Institute (California) website;

http://genome.jgi-psf.org/Stano1/Stano1.home.html

Growth and maintenance of *Stagonospora nodorum*

S. nodorum SN15 wildtype (Department of Agriculture, Western Australia) and the *gna1-35* strain carrying a disruption in *Gna1* (SNOG_10086) were used in this study and were maintained on complex media as described (Solomon et al. 2004). For the analysis of the extracellular proteome, 150 mg of fungal mycelia were grown in minimal medium broth (pH 6.0) which consisted of 10 mM sodium glutamate as a carbon source. The fungus was grown to a vegetative phase by incubation at 22^{o}C shaking at 150 rpm for three days.

Growth and maintenance of wheat

Growth of *Triticum aestivum* (cv. Amery) and wheat infections were performed as previously described (Solomon et al. 2004).

Protein extraction

Cell-free extract supernatant was obtained by filtering the fungal broth through cheese cloth and again through a Millipore 0.2 μm filter. Proteins in the filtered supernatant were precipitated with 5% (w/v) trichloroacetic acid, pelleted by centrifugation at 4,000 *g* for 15 min, washed with ice-cold 90% acetone and aspirated prior to solubilisation in multiple surfactant solution (MSS) as described by Tan et al. (2008).

Two-dimensional gel electrophoresis (2-DE)

Protein concentration was estimated with a Bio-Rad *RC-DC* protein assay kit. For isoelectric focusing, Bio-Rad 7 cm pH 3-10 IPG strips were actively rehydrated with MSS containing the protein sample (200 μg). Rehydration, isoelectric focussing, SDS polyacrylamide gel

electrophoresis and protein staining techniques were carried out in accordance with Tan et al. (2008). Images of protein gels were captured using the ProXPRESS scanner (Perkin Elmer). All gels were performed in biological triplicates. Spot detection and gel analyses were performed with the ProGENESIS Workstation 2005 software (Linear Dynamics) under default settings. Protein spots were considered differentially abundant if $p<0.05$ (unpaired t-test) and $\geq$two-fold difference in the normalised densitometry value of matching spots between the average gels (Supplementary Data A). These spots were excised from gels and the proteins trypsin digested (Taylor et al. 2005).

Mass spectrometry analysis and database searching

Tryptic digested peptides were analysed on an Agilent 1100 series capillary LC system coupled to an Applied Biosystems QSTAR Pulsar i LC-MS/MS system as previously described (Tan et al. 2008). Mass spectra searches were performed with the Mascot search engine version 2.1.04 (Matrix Science) against the Broad-predicted protein set derived from the genome of *S. nodorum* (16,597 sequences; 6,455,598 residues).

Enzymatic assay for mannitol dehydrogenase

Protein fractions were assayed for the cytoplasmic enzyme marker mannitol dehydrogenase (Solomon et al. 2005).

Signal peptide analysis

Polypeptide sequences were analysed for N-terminal signal peptides with the SignalP 3.0 (Bendtsen et al. 2004) and PrediSi (Hiller et al. 2004) softwares under default settings. Signal peptides were considered present if predictions were positive in SignalP and PrediSi.

RNA isolation and RT-PCR

RNA isolation was carried out with the TRIzol reagent (Invitrogen). The extracted RNA was DNase-treated with DNA-*free* reagent (Ambion). First strand cDNA and gene transcript abundance was analysed via RT-PCR as previously described (Tan et al. 2008). All reactions were performed in technical duplicates from pooled biological triplicates. Intron-spanning primers designed to

amplify actin (*Act1*; SNOG_01139) were used to check all cDNA samples were free of genomic DNA via PCR.

Genes coding for *Act1* and elongation factor 1α (*Ef1α*; SNOG_11663) were used as constitutive expressed controls for transcript abundance normalisation as previously described (Tan et al. 2008). All primer sequences can be found in Supplementary Data B

Statistical analysis of transcript abundance

The expression of each gene was normalised and correlated with protein abundance data to identify possible correlations using cDNAs derived from the *in vitro* growth condition. Gene expression between SN15 and *gna1-35* were deemed differentially abundant under the criteria that $p<0.05$ in an unpaired t-test and $\geq$two-fold difference in the normalised transcript abundance. Gene expression during SN15 infection on wheat was analysed with ANOVA set for Tukey-Kramer test in conjunction with a Dunnett's control. Gene expression was deemed significantly different if $p<0.05$ and $\geq$two-fold difference in the normalised transcript abundance relative to the Dunnett's control.

Genbank accession numbers

Genes analysed in this study are available in the Genbank/EMBL databases under the accession numbers; EAT92151 (SNOG_00656), EAT92343 (SNOG_00848), EAT91218 (SNOG_01569), EAT86982 (SNOG_05918), EAT87038 (SNOG_05974), EAT86011 (SNOG_06180), EAT86322 (SNOG_06491), EAT86323 (SNOG_06492), EAT84328 (SNOG_08052), EAT82079 (SNOG_10685), EAT81577 (SNOG_11078), EAT80028 (SNOG_12730), EAT78951 (SNOG_13504), EAT78241 (SNOG_14370), EAT77116 (SNOG_15451) and EAT76642 (SNOG_16063).

Results

Comparative proteomic analysis and the identification of genes that correspond to the differentially abundant proteins

The extracellular proteomes of SN15 and *gna1-35* were analysed by 2-DE (Fig. 1). A total of 189 unique protein spots were identified in the SN15 and *gna1-35* samples. Of these, 17 were reduced in abundance in *gna1-35* whilst eight showed an increase. LC-MS/MS was used to obtain peptide spectra of these spots and the resulting data were matched against the *S. nodorum* predicted protein set to find the matching genes (Table 1 and Supplementary Data C). Sixteen genes were identified from the 25 spots. Protein spots that were less abundant in *gna1-35* matched to genes that code for a putative subtilisin-like protease (*Snp3*: SNOG_06492), serine protease (SNOG_06491), malate dehydrogenase (SNOG_05974), acetylxylan esterase (SNOG_15451), cell wall glucanase (SNOG_01569), vacuolar targeting protein (SNOG_00656) and proteins of unknown function (SNOG_06180, 11078 and 12730). Protein spots that were more abundant in *gna1-35* matched to genes that code for a putative peptidylprolyl isomerase (SNOG_00848), RNase (SNOG_16063), tyrosinase (SNOG_14370) and proteins that showed significant BlastP matches to the hypothetical predictions in the NCBI dataset (SNOG_05918, 06180, 11078, 12730 and 13504). Two genes (SNOG_08052 and SNOG_10685) have no apparent Genbank orthologs and hence, were considered unique to *S. nodorum*. Occasionally, more than one protein was identified per spot (E7, E8, E14, E16 and E17). Mascot was unable to assign a significant gene match to the mass spectrum generated from protein spot E12.

Intracellular protein contamination was determined by assaying the marker enzyme mannitol dehydrogenase. No activity was detected in the extracellular extract whilst significant activity was observed in all intracellular protein fractions of SN15 and *gna1-35*. This indicates that cytoplasmic contamination of the extracellular proteome was below detectable levels (data not shown).

N-terminal signal peptide analysis

A bioinformatic approach was used to validate the subcellular localisation of all putative signalling target proteins through the analysis for N-terminal signal peptides via SignalP. N-terminal signal peptides were predicted for 12 of the 16 extracellular proteins (Table 1). SignalP predictions were essentially confirmed by PrediSi with the exception of the SNOG_00848

peptidylprolyl isomerase polypeptide which returned a negative signal peptide prediction with the latter.

Transcriptional analysis of genes that corresponded to differentially abundant protein spots

The expression of genes that code for proteins of altered abundance was examined by RT-PCR to determine the level of correlation between protein spot intensity and transcript abundances. To demonstrate that *Act1* is a suitable constitutively expressed housekeeping gene in both strains, expression was normalised against *Ef1α* transcript abundance. The normalised *Act1*/*Ef1α* expression profile of both strains was similar and hence indicates constitutive expression (Fig. 2A). Of the 16 genes examined, 10 showed a positive correlation between protein and transcript abundance (Fig. 2B). Eight of these genes showed transcriptional down regulation whereas two were up-regulated in *gna1-35*. Six genes showed no correlation between protein and transcript abundances (Fig. 2C).

Quantitative RT-PCR was also used to determine the expression profile of these genes in SN15 during infection on wheat. Sampling time points were one, three, five and eight days post infection which coincided with host penetration, proliferation, onset and late pycnidiation, respectively (Solomon et al. 2006b). Constitutive expression of *Ef1a* was demonstrated at different infection time points when normalised to *Act1* transcript abundance (Fig. 2A). For genes that showed correlation between protein and transcript abundances *in vitro*, seven showed differential expression during infection. Two genes (SNOG_00656 and SNOG_12730) showed a biphasic expression pattern whereby maximal transcript abundance was observed during early and late infection. Two genes (SNOG_06180 and SNOG_16063) showed increased expression during late infection coinciding with pycnidiation. Two genes (SNOG_06492 and SNOG_15451) showed greater transcription at one day post infection. Only SNOG_11078 demonstrated maximal expression during the mid-infection period whereas no expression was observed for three genes (SNOG_01569, SNOG_06491 and SNOG_10685) during infection.

In planta expression analysis was also carried out for genes that showed no correlation between protein and transcript abundances. Four genes showed differential transcript abundance during the infection period (SNOG_00848, SNOG_08052, SNOG_14370 and SNOG_13504) whereas one gene showed constitutive expression (SNOG_05974). Expression was not observed for one gene (SNOG_05918) during infection (Fig. 2C).

Discussion

In this study, we have shown that *Gna1* disruption has resulted in extensive changes in the extracellular proteome of *S. nodorum*. To our knowledge, this is the first report that provides a detailed analysis of direct and indirect regulation of the extracellular proteome by a signalling component in a plant pathogenic fungus. In other studies, Gα subunit inactivation resulted in a reduced accumulation of cognate partners (Ivey et al. 1999; Kasahara and Nuss 1997; Parsley et al. 2003; Yang et al. 2002). Hence, it is possible that proteome changes observed in this study were a result of perturbation in other parts of the heterotrimeric G protein pathway such as the Gβγ dimer rather than Gna1 alone. In addition, proteome changes may also be a consequence of the fungus attempting to cope with the loss of Gna1 function and thus resulting in indirect regulation.

***gna1-35* showed extensive alterations of the extracellular proteome**

The disruption of *Gna1* resulted in alterations of 13.2% of detectable extracellular protein spots and thus indicating a major regulatory role for it in defining the *S. nodorum* extracellular proteome. Ten genes affected by Gna1 inactivation showed correlation between protein and transcript abundances. Majority of these genes affected by *Gna1* disruption showed differential expression throughout the infection period, thus suggestive of stage-specific roles during infection. It was also noted that protein and transcript abundances of some genes did not correlate *in vitro*. Similar observations were previously made from studies of other biological systems using both proteomics and transcriptomics to analyse gene expression (Fessler et al. 2002; Gygi et al. 1999). This could be due to post-translational modifications or protein/RNA stability (Kozak 2005; Mann and Jensen 2003). This highlights the importance of proteomics as a tool that can be used to look for direct and indirect effects of signalling perturbation at the protein level.

Gα subunit inactivation alters the expression of extracellular depolymerases

It is widely considered that necrotrophic fungi utilise a large array of cell wall degrading enzymes to break through the host cell wall barrier (Walton 1994). Cell wall degrading enzyme and proteolytic activities were significantly reduced in *gna1-35* (Solomon et al. 2004). The use of 2-DE

allowed a detailed insight into individual depolymerases that are altered in *gna1-35*. For instance, a reduction in the abundance of a putative cell wall degrading enzyme acetylxylan esterase (SNOG_15451) was observed in *gna1-35*. Transcript abundance of the putative acetylxylan esterase gene was maximal in SN15 during one day post infection indicating that the gene product may function to break down the host epidermal cell wall to facilitate entry. We have also identified two putative proteases that showed reduced abundance in *gna1-35*. *Snp3* encodes a previously identified alkaline subtilisin-like protease (Bindschedler et al. 2003) and showed differential expression *in planta* suggesting a possible role in infection. Another reduced abundant putative protease gene (SNOG_06491), orthologous to *Prt1* of *Fusarium oxysporum* (Di Pietro et al. 2001) was identified. Its lack of expression *in planta* suggests that it is not required for pathogenicity. It was noted that both proteases differed in the predicted and observed relative molecular mass. It is known that proteases may be subjected to partial proteolysis prior to activation (Newport and Agabian 1997). Evidence of extracellular protease regulation by heterotrimeric G protein signalling in fungi has been previously reported (Gronover et al. 2001; Prados-Rosales et al. 2006). However, the role of fungal extracellular proteases during infection is yet to be fully defined.

A putative extracellular RNase (SNOG_16063) represented in spot E20 suggests that extracellular nucleic acid metabolism may be subjected to regulation by heterotrimeric G protein signalling. *S. nodorum* is a necrotrophic pathogen and consequently the degradation of host tissue would result in the release of nucleic acids. Increased RNase expression during infection indicates nucleotide degradation from the host plant may play a role during the later stages of infection when plant tissue has undergone extensive necrosis. Interestingly, we have observed that it is often difficult to extract good quality intact RNA from heavily infected leaf material (data not shown). Hence, the extracellular RNase may participate in RNA degradation to produce basic nutrients for assimilation during infection (Lindberg and Drucker 1984).

Aromatic compound metabolism

The role of melanin in fungal pathogenicity is well-defined (Kawamura et al. 1997; Salas et al. 1996). It was previously hypothesised that *S. nodorum* may utilise dihydroxyphenylalanine (DOPA), a derivative of aromatic amino acids, as a precursor to melanin biosynthesis and the disruption of *Gna1* may have perturbed the process (Solomon et al. 2004). This was supported by a number of experimental observations that include albino mutants, massive extracellular accumulation of tyrosine and DOPA (Solomon et al. 2004) and up-regulation of 3-dehydroquinate dehydratase in *gna1-35* (Tan et al. 2008). The latter is an enzyme that is associated with intermediates of the aromatic amino acid biosynthesis and quinate catabolism pathways. This study has identified a putative tyrosinase that was up-regulated in *gna1-35*. A function of tyrosinase is to convert tyrosine into DOPA-based compounds for melanin production (Lerch 1981). It could be speculated that tyrosinase is up-regulated as a response to tyrosine and DOPA accumulation. We hypothesise that *Gna1* disruption caused an uncoupling of melanisation that may have subsequently led to the accumulation of the aromatic compounds. Investigations to test this hypothesis are currently underway.

Identification of a putative extracellular malate dehydrogenase

A putative malate dehydrogenase protein (SNOG_05974) was identified in spot E7 and E8. It was unexpected to find malate dehydrogenase secreted knowing its role as a key intracellular citric acid cycle enzyme (Gietl 1992). Orthologs of the enzyme were found in the secretome of *Aspergillus flavus*, the causal agent of aspergillosis, during growth on rutin (Medina et al. 2005) and the head blight fungus *Fusarium graminearum* isolated from infected wheat heads (Paper et al. 2007). The role of extracellular malate dehydrogenases is unknown.

The putative malate dehydrogenase protein lacked a predicted N-terminal signal peptide sequence as with three other extracellular proteins identified in this study. Signal sequences play a role in post-translational protein localisation to subcellular compartments (Martoglio and Dobberstein 1998). Classical protein secretion to the extracellular environment initiates upon protein migration to the endoplasmic reticulum which requires an N-terminal signal peptide

(Conesa et al. 2001). It is possible that these extracellular proteins does not possess a classical N-terminal signal peptide signature or may be exported through an alternative secretory mechanism (Cleves et al. 1996).

Conclusion

This study has demonstrated that 2-DE is an effective method for identifying extracellular proteins that are altered in abundance in a signalling mutant. This study has revealed that *Snp3* and a putative acetylxylan esterase are major Gna1-controlled depolymerases that are expressed during wheat infection. Furthermore, the identification of a putative extracellular tyrosinase that accumulated in *gna1-35* further support a previous hypothesis that *Gna1* disruption perturbed melanisation in *S. nodorum*. Genes encoding these proteins are now key targets for further analysis via reverse genetic techniques as we seek to validate their functional roles and to determine the connection with the *gna1-35* phenotype. These functional studies will lead to a greater understanding of signalling in phytopathogenic fungi.

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Table 1. Identification of differentially abundant proteins with LC-MS/MS and Mascot.

Spot	Fold diff. a)	Broad ID (SNOG)	Description	Observed/ *predicted* pI	Observed/ *predicted* M_r (kDa)	*MOWSE*; peptide number (% coverage)	Signal peptide b)	Transcript correlation c)
E1	-4.4	00656	Vacuolar targeting protein	5.65; *6.14*	33.1; *36.7*	*219*; 6 (25)	Y	Y
E2	--	11078	Unknown	6.73; *6.06*	38.0; *37.8*	*843*; 20 (52)	N	Y
E3	--	11078	Unknown	6.90; *6.06*	37.6; *37.8*	*78*; 19 (50)	N	Y
E4	--	11078	Unknown	7.09; *6.06*	37.4; *37.8*	*902*; 21 (56)	N	Y
E5	-2.7	15451	Acetylxylan esterase	5.80; *5.48*	29.3; *33.2*	*128*; 4 (18)	Y	Y
E6	+3.3	14370	Tyrosinase	6.64; *5.09*	26.3; *45.8*	*414*; 12 (26)	Y	N
E7-1	-2.4	06492	Subtilisin-like protease	7.14; *7.71*	30.2; *40.6*	*213*; 4 (17)	Y	Y
E7-2	-2.4	05974	Malate dehydrogenase	7.14; *9.33*	30.2; *35.8*	*176*; 5 (16)	N	N
E8-1	-6.7	06492	Subtilisin-like protease	7.68; *7.71*	29.8; *40.6*	*250*; 5 (15)	Y	Y
E8-2	-6.7	05974	Malate dehydrogenase	7.68; *9.33*	29.8; *35.8*	*68*; 3 (10)	N	N
E9	--	06492	Subtilisin-like protease	8.39; *7.71*	29.8; *40.6*	*367*; 7 (27)	Y	Y
E10	-8.5	06492	Subtilisin-like protease	8.25; *7.71*	26.7; *40.6*	*457*; 10 (33)	Y	Y
E11	-17.0	06492	Subtilisin-like protease	7.70; *7.71*	26.8; *40.6*	*286*; 6 (23)	Y	Y
E12	--	Not ID		4.81;	28.5;			
E13	--	12730	Unknown	4.98; *4.64*	28.6; *41.2*	*259*; 7 (22)	Y	Y
E14-1	--	12730	Unknown	5.15; *4.64*	28.0; *41.2*	*198*; 6 (19)	Y	Y
E14-2	--	01569	Cell wall glucanase	5.15; *4.79*	28.0; *49.4*	*132*; 4 (7)	Y	Y
E15	--	06180	Unknown	4.55; *4.74*	27.7; *22.4*	*57*; 1 (4)	Y	Y
E16-1	++	05918	Unknown	4.73; *5.01*	23.6; *24.4*	*168*; 4 (21)	Y	N
E16-2	++	08052	No Genback matches	4.73; *4.69*	23.6; *26.5*	*143*; 4 (15)	Y	N
E17-1	++	05918	Unknown	5.10; *5.01*	23.9; *24.4*	*161*; 5 (25)	Y	N
E17-2	++	08052	No Genbank matches	5.10; *4.69*	23.9; *26.5*	*77*; 3 (12)	Y	N
E18	++	05918	Unknown	5.28; *5.01*	24.0; *24.4*	*210*; 5 (25)	Y	N
E19	+5.6	13504	Unknown	6.47; *6.27*	18.0; *19.7*	*297*; 8 (34)	Y	N
E20	++	16063	RNase T1	7.73; *6.28*	<15.0; *18.0*	*97*; 3 (21)	N	Y
E21	+3.2	00848	Peptidylprolyl isomerase	4.97; *4.89*	<15.0; *13.9*	*326*; 8 (43)	N	N
E22	++	10685	No Genbank matches	5.21; *5.41*	<15.0; *33.6*	*100*; 3 (18)	Y	Y
E23	--	06491	Serine protease	6.83; *5.13*	<15.0; *37.6*	*315*; 7 (17)	Y	Y
E24	--	06491	Serine protease	7.52; *5.13*	<15.0; *37.6*	*306*; 6 (16)	Y	Y
E25	--	06491	Serine protease	7.78; *5.13*	<15.0; *37.6*	*295*; 6 (17)	Y	Y

[a)]Fold difference of matching protein spots is calculated from normalised spot value of SN15 relative to *gna1-35*. Fold difference designated by (--) indicates protein spots that were not observed in the *gna1-35* 2-DE gels whereas (++) designate spots that were not observed in all SN15 2-DE gels.

[b)]N-terminal signal peptides were predicted for 13 of the 16 extracellular proteins using SignalP. Predictions were essentially confirmed by PrediSi with the exception of the SNOG_00848 polypeptide which returned a negative signal peptide prediction.

[c)]Refer to Fig. 2.

Fig. 1. Representative 2-DE gels of (A) SN15 and (B) *gna1-35* showing differentially abundant proteins in the extracellular proteome. Biological replicate gels are presented in Supplementary Data D.

Fig. 2. Protein spot/transcript abundance graphs for each of the targets identified via 2-DE. The transcript profiling of each gene is comprised of two panels. The panel on the left is a comparison of relative protein (white bars) and transcript (black bars) levels for each of the targets *in vitro*. Asterisks located on top of bar graphs signify significant differences in protein and transcript abundances. 'S' and 'G' on the *x*-axis denote SN15 and *gna1-35*, respectively. The panels on the right (line graphs) depict gene expression *in planta* for each target gene. Numbers on the *x*-axis are days post infection and * denotes differential gene expression relative to the Dunnett's control group 'D'. SE bars are shown. Section (A) shows transcript expression normalisation of *Act1* and *Ef1*α housekeeping genes whereas (B) and (C) indicates correlation and non-correlation between protein spot and transcription abundances, respectively.