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Proteomic assessment of host-associated microevolution in the fungus *Thielaviopsis basicola*

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Summary

***Thielaviopsis basicola*, a soil-borne pathogen with a broad host range and a cosmopolitan distribution, is emerging as a major risk to sustainable cotton production in Australia. Previous studies suggested that host specialization has occurred making *T. basicola* an ideal model for a comparative proteomic analysis of strains isolated from different hosts. Elucidation of the genomic diversity and investigation of the functional differences in the Australian population could provide valuable information towards disease control. In this study, isolates of *T. basicola* were investigated for genomic (internal transcribed spacers region), proteomic and cotton virulence level variations. Internal transcribed spacers sequence analysis revealed that isolates are grouped based on host of origin irrespective of geographical origin. At the proteome level a degree of diversity was apparent and hierarchical clustering analysis of the data also demonstrated a close correlation between the proteome and the host of origin. LC-MS/MS analysis and identification using cross-species similarity searching and *de novo* sequencing of host-specific differentially expressed proteins and the virulence-correlated proteome allowed successful identification of 43 spots. The majority were found to be involved in metabolism. Spots that were correlated with host and virulence differences included a hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain, glyceraldehyde-3-phosphate dehydrogenase, arginase and tetrahydroxynaphthalene reductase.**

Introduction

The soil-borne plant-pathogenic fungus, *Thielaviopsis basicola* (Berk. et Br.) Ferr., is commonly found in cultivated and non-cultivated soils (Yarwood, 1981). This pathogen causes the disease black root rot in a broad range of plant species and significantly impacts on production of crops as diverse as cotton, carrot, lettuce, tobacco, soybeans and pansy (Johnson, 1916; Otani, 1962). Infected plants appear stunted and chlorotic, and their roots have black lesions with numerous fungal spores (King and Presley, 1942). Two types of spores are produced by *T. basicola:* hyaline, cylindrical phialospores (endoconidia) and thick, dark-walled chlamydospores, both of which are used for identification (Nag Raj and Kendrick, 1975). Analysis of rDNA indicates that *Thielaviopsis* belongs to the teleomorphic genus *Ceratocystis*, although the teleomorphic state has not been observed (Paulin-Mahady *et al*., 2002). *T. basicola* is a hemibiotroph and, although it can be cultured on agar media, in the soil environment it is effectively an obligate parasite of plants (Nan *et al*., 1992; Hood and Shew, 1997). Despite its poor saprophytic ability in soil, *T. basicola* is difficult to control as a result of the persistent nature of the chlamydospores in soil.

In culture, isolates grown on agar medium differ in colony appearance and spore morphology. In 1999, Punja and Sun carried out a morphological and genetic study on 50 isolates recovered from different geographic origins and hosts. They showed a good correlation between morphologically similar isolates from common geographic regions or hosts and random amplified polymorphic DNA banding patterns. However, exceptions to

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this correlation were also observed suggesting that isolate variation may also result from the adaptation to a specific host. When a sample of 27 isolates from the previous study was assessed for virulence on bean leaves, a good correlation between levels of virulence, morphology and genetic similarities within these groups was also revealed (Punja, 2004). Unfortunately, the physiological and biochemical basis of this virulence diversity and its correlation with pathogenicity on roots were not investigated. Geldenhuis and colleagues (2006) used microsatellite markers to study populations of *T. basicola* from chicory and groundnut in South Africa. They found that populations on each host were genetically uniform, irrespective of geographic origin, and that the two populations were distinct from each other. They interpreted their results as indicating clonal spread of host-specialized lineages from a source population, probably in Europe.

Thielaviopsis basicola has been present on a diverse range of crops including legumes, tobacco and ornamentals in Australia since at least the early 20th century (Allen, 1990). In recent decades there have been two reports of new introductions of the pathogen into cropping systems. The fungus was first reported on cotton in 1989 (Allen, 1990) and has since spread throughout the cotton-growing areas (Nehl *et al*., 2004). The cotton strains were apparently not pathogenic to tobacco in the field, suggesting the introduction of a new genotype (Nehl *et al*., 2004). At about the same time *T. basicola* became established on lettuce crops in Queensland (O'Brien and Davis, 1994). Lettuce isolates were not pathogenic to cotton, and it was suggested that they had been introduced on imported peat moss (O'Brien and Davis, 1994). As in South Africa, *T. basicola* in Australia may consist of populations specialized for particular hosts.

To date, evidence for the role of host in microevolution of *T. basicola* has relied on selectively neutral DNA markers. Proteomic comparison of *T. basicola* isolates has the potential to highlight some important phenotypic differences that could be related to pathogenic specialization. We hypothesized that isolates of *T. basicola* from different hosts belong to genetically distinct groups; that these groups will show consistent differences in their proteome; and that at least some of these differences will be explicable in terms of their effects on pathogenicity. In this study we compared isolates from the putatively newly introduced lineages on cotton and lettuce with those on carrot (Fig. 1, Table 1). Internal transcribed spacers (ITS) sequence analysis was used to assess their genomic differences, while two-dimensional electrophoresis (2-DE) and mass spectrometry were used to evaluate their proteomic phenotypic differences.

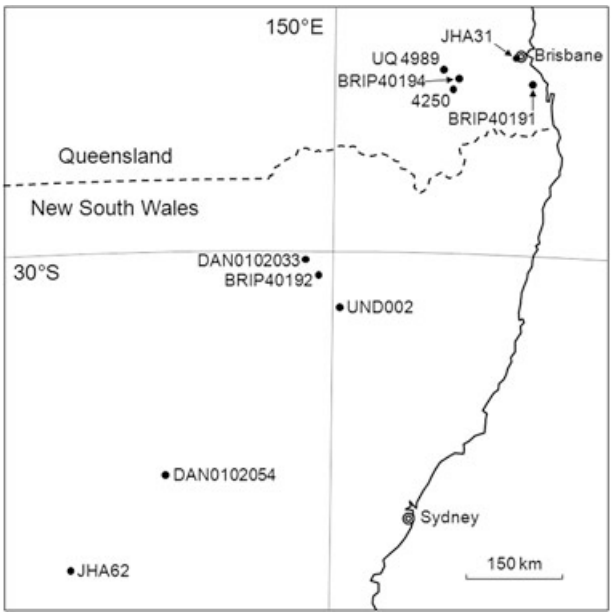

Fig. 1. Map showing sampling areas of the *T. basicola* isolates in eastern Australia.

Results

ITS sequence analysis

All isolates from a particular host (cotton, lettuce and carrot) had identical ITS sequences, which differed between hosts. Among all available full-length ITS sequences from *T. basicola*, polymorphisms were detected at seven base positions, and six unique haplotypes were identified (Fig. 2). The cotton strains shared a haplotype with database sequences from tobacco and an unknown host (Fig. 2). The haplotype of the lettuce strains was most similar to that of the cotton strains. The carrot strains belonged to the haplotype with the most members and included database sequences from carrot, *Primula* and *Betula* and from soil. The remaining three haplotypes were each represented by a single strain from the databases. The distances among isolates of *T. basicola* were small relative to the distance between *T. basicola* isolates and the out-group, *Thielaviopsis thielavioides* (Fig. 2).

Comparison of 2-DE protein maps from T. basicola *isolates*

Visual comparison of the 2-DE gels revealed a similar protein pattern for the 12 isolates and, more interestingly, specific features common to *T. basicola* isolates originating from the same host. As shown in Fig. 3, isolates recovered from cotton tended to have a simpler proteome with an average number of 650 spots detected while the proteome of isolates originating from lettuce and carrot were more complex with an average of 715 and 750 spots

Table 1. Isolates of *T. basicola* used in this study.k

Isolate	Host of origin	Geographic origin	Source
BRIP40192	Cotton	Narrabri, NSW	Jan Dean, Queensland DPI
DAN0102033	Cotton	Togo Farm field 56, GPS coordinate, S30 06.1843′ E149 33.7854′, NSW	John Harvey, UQ
UND002	Cotton	Undoolya Farm Field 02, GPS coordinate S-30 46.5860′ E150 07.3340′, NSW	John Harvey, UQ
DAN0102054	Cotton	Whitegates Farm Field 8, GPS coordinate S 33 06.0805′ E 147 15.8266′, NSW	John Harvey, UQ
JHA62	Cotton	'Gundaline field b3' Twynam farm east of Hay, NSW	John Harvey, UQ
BRIP40191	Lettuce	Rochedale, Brisbane, QLD	Jan Dean, Queensland DPI
UQ 4989	Lettuce	Darling Down, QLD	John Harvey, UQ
BRIP40194	Lettuce	Toowoomba, QLD	Jan Dean, Queensland DPI
4250	Lettuce	Cambooya, QLD	John Harvey, UQ
5247-6	Carrot	Western Australia	John Harvey, UQ
JHA31	Carrot	Commercial carrot-supermarket Brisbane, QLD	John Harvey, UQ
WAC10715	Carrot	Myalup, Western Australia	John Harvey, UQ

detected respectively. Moreover, some proteins seemed to be specific to or expressed at a higher level in isolates from a single host. In order to substantiate these observations, gels from isolates recovered from the same host were matched and reference gels representing all spots from these isolates were created. These reference gels were then matched in a high level experiment. To see if a relationship could be established between the proteome of the different isolates, a hierarchical clustering analysis was performed (Fig. 4). Three distinct clusters could be identified. The first cluster grouped isolates recovered from cotton, the second from lettuce and the last one from carrot. Based on the above results, an additional analysis of the data indicated that the 12 *T. basicola* isolates had a common protein pattern of 264 spots and that this number increased dramatically when isolates from a common host were considered, with cotton isolates sharing 477 spots, lettuce 450 spots and carrot 607 spots.

In order to gain further insight into the biochemical differences between these isolates, we used the PDQuest software to find unique and differentially expressed proteins (Table 2).

Protein spots only detected in isolates originating from the same host. We were able to establish that: (i) based on their electrophoretic mobility 17 spots were only detected in cotton isolates, (ii) 10 spots present in each lettuce isolate did not have any corresponding match in gels from cotton and carrot isolates and (iii) 48 spots were only found in carrot isolates (Table 2).

Analysis of these protein spots by MS/MS combined with MS/MS ion searched database and *de novo*

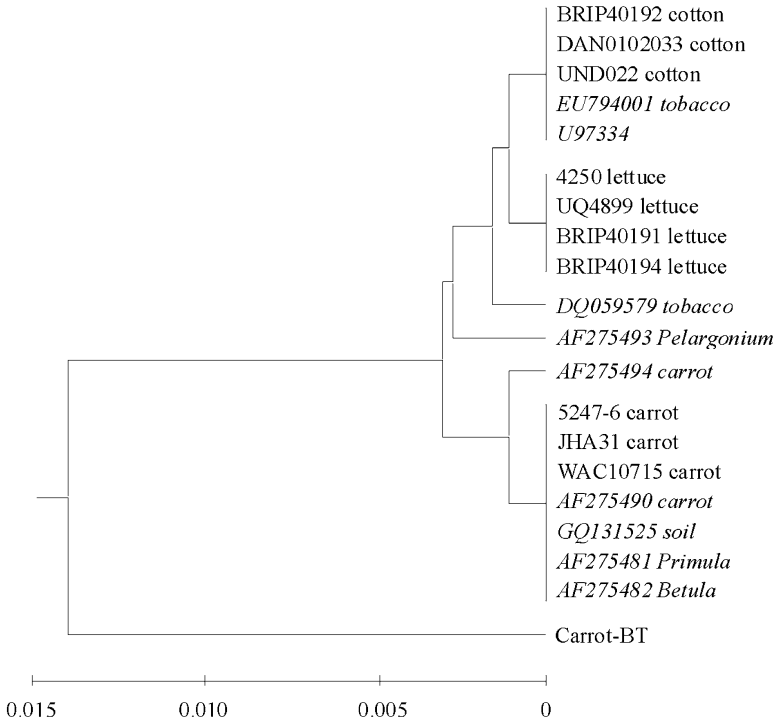

Fig. 2. UPGMA distance tree of *T. basicola* isolates based on ITS sequences. Host or substrate of origin is shown where known. Names in italics represent sequences obtained from databases. The tree was rooted with the sequence from an isolate of *T. thielavioides* (Carrot-BT).

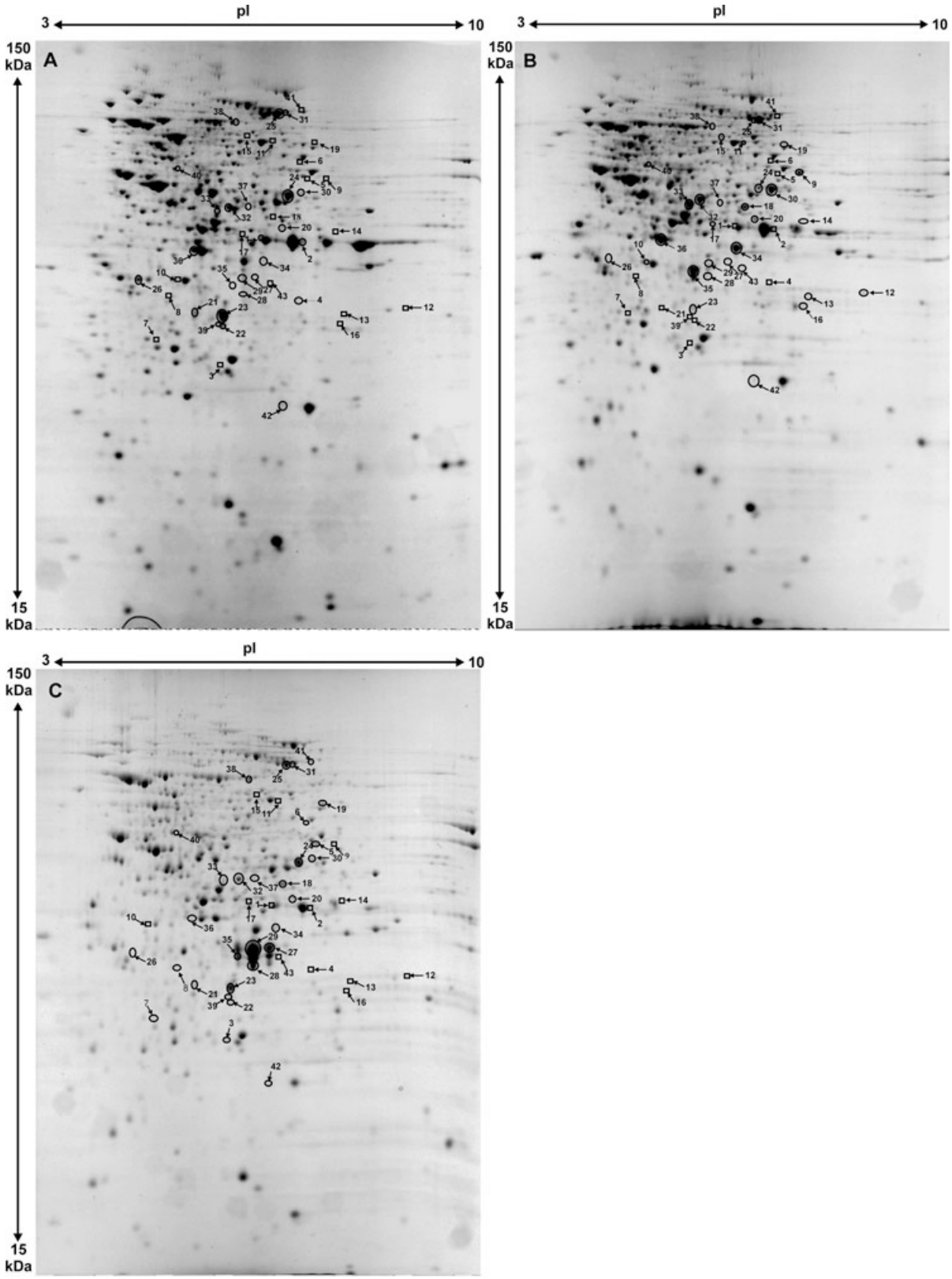

Fig. 3. Typical Blue silver stain 2-DE gels of *T. basicola* soluble proteins from cotton (A), carrot (B) and lettuce (C) isolates. Arrows refer to spots analysed by LC-MS/MS. □ indicates the localization of spots from other isolates and not visible on that gel.

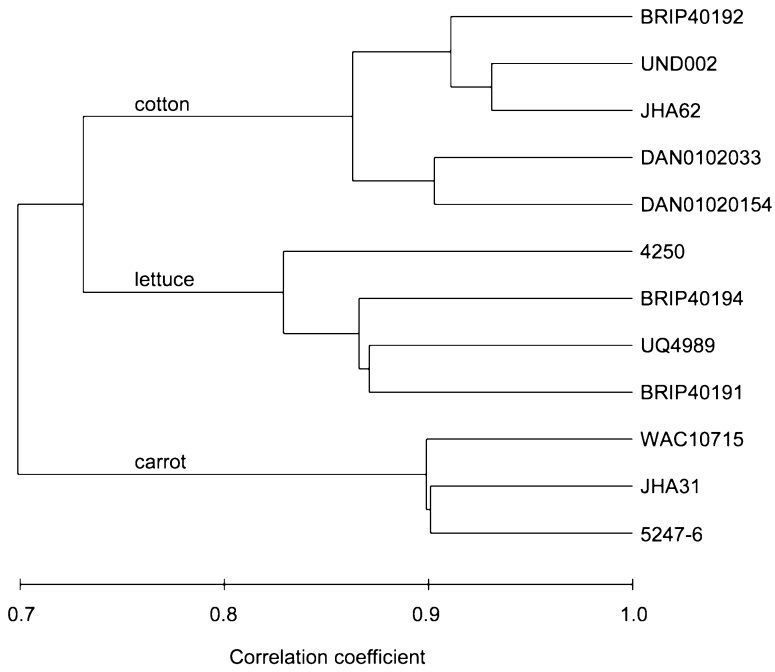

Fig. 4. Classification of the proteomes from the 12 *T. basicola* isolates. Hierarchical clustering was performed using the nearest neighbour method with the Pearson correlation as a similarity measure.

sequencing led to the identification of three spots in cotton isolates, six spots in lettuce isolates and ten spots in carrot isolates (Fig. 3, Table 3, Table S1 and Appendix S1). The remaining spots could be neither confidently manually cut nor identified by cross-species identification as the *T. basicola* genome has not been sequenced. Eight of the proteins that could be identified corresponded to hypothetical proteins. To assign a possible function to these proteins, a BLAST search was performed to identify putative conserved domains and the Kyoto Encyclopedia of Genes and Genomes was used to attribute a possible cellular role. We found that the majority of these proteins were involved in different metabolic pathways such as carbohydrate metabolism (spots 1, 2 and 16), amino acid metabolism (spots 4, 8, 9, 10 and 43), protein biosynthesis (spot 5), nucleotide metabolism (spots 3, 6 and 15), energy metabolism (spot 13) and metabolism of cofactors and vitamins (spot 16), while the others corresponded to proteins with possible roles in the structure and/or function of the cytoskeleton (spots 11, and 14), an antiproliferative protein (spot 12) and to a putative coat protein of *Magnaporthe oryzae* virus 1 (spot 41). In two cases, two proteins per spot were identified (spots 7, and 17). Co-migration of multiple proteins is possible and has previously been reported by Lim and colleagues (2003).

Less abundant protein spots. Further analysis of the gel images revealed that 27 protein spots were detected in both lettuce and carrot isolates but not in the cotton isolate, 27 were detected in carrot and cotton isolates but not in the lettuce isolate while 58 protein spots were found in cotton and lettuce isolates but not in the carrot isolates (Table 2). We also found protein spots from a common host with at least a twofold decreased expression level by comparison with isolates from the other two hosts: one in the cotton isolates and six in the carrot isolates (Table 2).

Table 2. Proteome variability between *T. basicola* isolates originating from different hosts.

Host	Protein spots detected in only one group[a]	Protein spots not detected in one group[b]	Protein spots differentially expressed[c]		Protein spots preferentially expressed[d]	
			Higher	Lower	Higher	Lower
Cotton	17	27	15	5	2	1
Carrot	48	58	185	10	37	6
Lettuce	10	27	17	5	5	0

a. Protein spots found in all isolates originating from one host but absent in all other isolates.
b. Protein spots not detected in any isolates from one host but present in all isolates originating from the two other hosts.
c. Protein spots significantly ($P < 0.05$) over- or under-expressed in isolates originating from one host.
d. Subset of the differentially expressed protein spots in isolates originating from the same host. The expression of these protein spots was at least twofold changed in all the isolates within the host group when compared with the two other host groups.

Table 3. List of identified proteins.

Expression[a]	Spot No	Virulence (Spearman's correlation coefficient)[b]	Protein to which peptide sequence is assigned	Putative conserved domain	EC number[c]	Predicted Molecular function
OD in cotton	1		Glyceraldehyde 3-phosphate dehydrogenase		1.2.1.12	Carbohydrate metabolism: Glycolysis and glyconeogenesis
OD in cotton	2	0.67*	Glyceraldehyde 3-phosphate dehydrogenase		1.2.1.12	Carbohydrate metabolism: Glycolysis and glyconeogenesis
OD in cotton	4		Hypothetical protein MGG_06530	Aspartate aminotransferase	2.6.1.1	Amino acid metabolism
OD in lettuce	5	−0.703*	Hypothetical protein SS1G_08631	tRNA_bind_EMAP-II_like		Protein biosynthesist RNA-binding
OD in lettuce	6	−0.62*	Inosine-5′-monophosphate Dehydrogenase		1.1.1.205	Nucleotide metabolism
OD in lettuce	7	−0.645*	Triosephosphate isomerase ran/spi1 binding protein		5.3.1.1	Carbohydrate metabolism: Glycolysis and glyconeogenesis Intracellular transport
OD in lettuce	8		Acetolactate synthase small subunit, mitochondrial precursor		2.2.1.6	Amino acid metabolism
OD in lettuce	3	−0.603*	Adenylyl-sulfate kinase		2.7.1.25	Nucleotide metabolism
OD in lettuce	41		Putative coat protein of *Magnaporthe oryzae* virus 1			
OD in carrot	9		Hypothetical protein FG06751.1	4-aminobutyrate aminotransferase and related aminotransferases		Amino acid metabolism
OD in carrot	10		Acetolactate synthase small subunit, mitochondrial precursor		2.2.1.6	Amino acid metabolism
OD in carrot	11		Hypothetical protein BC1G_12988	WD40		Wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly
OD in carrot	12		Prohibitin			Antiproliferative protein
OD in carrot	13		NADH-ubiquinone oxidoreductase 304 kDa subunit precursor		1.6.5.3 1.6.99.3	Energy metabolism: Oxidative phosphorylation
OD in carrot	14		Septin			Cytoskeleton organization
OD in carrot	15		CTP synthase		6.3.4.2	Nucleotide metabolism (pyrimidine)
OD in carrot	16		Hypothetical protein FG08971.1	Formyltetrahydrofolate deformylase	3.5.1.10	Carbohydrate Metabolism: Glyoxylate and dicarboxylate Metabolism of Cofactors and Vitamins
OD in carrot	17		Glyceraldehyde 3-phosphate dehydrogenase Methylenetetrahydrofolate dehydrogenase		1.2.1.12 1.5.1.15	Carbohydrate metabolism: Glycolysis and glyconeogenesis Metabolism of Cofactors and Vitamins
OD in carrot	43		Pyrroline-5-carboxylate reductase		1.5.1.2	Amino acid metabolism
ND in cotton	18		Hypothetical protein	K homology RNA-binding Domain		Nucleic acid binding
ND in cotton	19	−0.58*	Hypothetical protein FG08266.1	Acetyl-CoA hydrolase		Carbohydrate metabolism: Pyruvate metabolism
cotton ↓	20		Glyceraldehyde 3-phosphate		1.2.1.12	Carbohydrate metabolism: Glycolysis and dehydrogenase glyconeogenesis
ND in carrot	21		Tetrahydroxynaphthalene reductase			Melanin Biosynthesis
ND in carrot	22		Tetrahydroxynaphthalene reductase			Melanin Biosynthesis

Table 3. *cont.*

Expression[a]	Spot No	Virulence (Spearman's correlation coefficient)[b]	Protein to which peptide sequence is assigned	Putative conserved domain	EC number[c]	Predicted Molecular function
Carrot ↓	23		Tetrahydroxynaphthalene Reductase			Melanin Biosynthesis
Carrot ↓	24		Citrate synthase, mitochondrial precursor		2.3.3.1	Carbohydrate metabolism: Citrate cycle, Glyoxylate and dicarboxylate metabolism
Carrot ↓	25		Aconitate hydratase, mitochondrial precursor		4.2.1.3	Carbohydrate metabolism: Citrate cycle
Cotton ↑	26	0.594*	Arginase		3.5.3.1	Biosynthesis of small molecules
Lettuce ↑	27	−0.746**	Hypothetical protein An07g00400	Rossmann-fold NAD(P)(+)-binding proteins		Secondary metabolites biosynthesis, transport and catabolism (General function prediction only)
Lettuce ↑	28	−0.734**	Hypothetical protein An07g00400	Rossmann-fold NAD(P)(+)-binding proteins		Secondary metabolites biosynthesis, transport and catabolism (General function prediction only)
Lettuce ↑	29	−0.674*	Hypothetical protein An07g00400	Rossmann-fold NAD(P)(+)-binding proteins		Secondary metabolites biosynthesis, transport and catabolism (General function prediction only)
Lettuce ↑	42		HEX1			Major component of the Woronin body
Carrot ↑	30		Citrate synthase, mitochondrial precursor		2.3.3.1	Carbohydrate metabolism: Citrate cycle, Glyoxylate and dicarboxylate metabolism
Carrot ↑	31		Aconitate hydratase		4.2.1.3	Carbohydrate metabolism: Citrate cycle
Carrot ↑	32		Phosphoglycerate kinase		2.7.2.3	Carbohydrate metabolism: Glycolysis and Glyconeogenesis
Carrot ↑	33		S-adenosylmethionine synthetase		2.5.1.6	Amino acid metabolism
			Proteasome regulatory particle subunit Rpt4			Protein folding, sorting and degradation
			Hypothetical protein FG09321.1	Acetyl-CoA acetyltransferase	2.3.1.9	Carbohydrate metabolism: Pyruvate metabolism
Carrot ↑	34		Guanine nucleotide-binding protein beta subunit			
Carrot ↑	35		Hypothetical protein FG03206.1	Rossmann-fold NAD(P)(+)-binding proteins		Secondary metabolites biosynthesis, transport and catabolism (General function prediction only)
Carrot ↑	36		Sec 14 cytosolic factor			
	37	−0.799**	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase		2.5.1.54	Amino acid transport and metabolism
	38	−0.79**	Arginyl-tRNA synthetase		6.1.1.19	Amino acid metabolism
			Hypothetical protein CHGG_03266	Glycyl-tRNA synthetase	6.1.1.14	Amino acid metabolism
	39	−0.72**	Tetrahydroxynaphthalene Reductase			Melanin Biosynthesis
	40	0.657*	Rab GDP-dissociation inhibitor			

a. 'OD' stand for 'only detected' and 'ND' stand for 'not detected'.
b. This column shows the result of Spearman's rank correlation between the protein expression of each isolate and their average virulence level. Spearman's correlation coefficient
* significant at 0.05 and ** significant at 0.01.
c. http://www.expazy.ch/enzyme

Of these proteins, 13 were selected for MS/MS analysis, and following a MASCOT MS/MS ion search, we were able to identify eight of them. Three spots, which were less abundant in carrot isolates, corresponded to tetrahydroxynaphthalene reductase (spots 21, 22 and 23), an enzyme involved in melanin biosynthesis, four spots represented proteins associated with carbohydrate metabolism (two were less abundant in cotton isolates: spots 19, 20, and two were less abundant in carrot isolates spots 24, 25) while one spot not detected in cotton isolates possessed a K homology RNA-binding domain (spot 18) (Fig. 3, Table 3, Table S1 and Appendix S1).

More abundant protein spots. Comparison of the 2-D gels images further revealed that some protein spots were markedly more abundant (from twofold to 50-fold) in isolates from a common host when compared with isolates from the other two hosts (Table 2). We found that two protein spots were increased in all cotton isolates. *De novo* analysis of the MS/MS file for one of these spots found a confident match for arginase (spot 26). Five protein spots were more abundant in all lettuce isolates compare with the other isolates. Four of these spots were analysed by MS/MS. Three of them were similar to a hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain (spots 27, 28 and 29) while the last one (spot 42) corresponded to a HEX1 protein, which is the major component of the Woronin body in filamentous fungi. Finally, 37 spots were found to be more abundant in the carrot isolates. LC MS/MS analysis of seven of these spots led to the identification of; three proteins involved in carbohydrate metabolism (spots 30, 31 and 32), one corresponding to a guanine nucleotide-binding protein beta subunit-like protein (spot 34), one (spot 35) hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain, a Sec 14 cytosolic factor (spot 36) and two possible protein identifications were found in spot 33 (Fig. 3, Table 3, Table S1 and Appendix S1).

Comparison of T. basicola *isolates based on the function of the proteins identified*

Of 43 spots identified, we could distinguish different expression characteristics, functional clusters and roles. First, we found that both qualitative and quantitative differences can be observed for the same protein. This is the case for spots 1, 2 and 20 identified as a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and for spots 21, 22 and 23 identified as tetrahydroxynaphthalene reductases that were either only detected within isolates from one host or less abundant within isolates from one host. This could indicate specific post-translational modifications and/or the existence of isoforms for these two proteins and suggests that the expression of protein variants could be host-dependent. A second observation was that a given protein could be highly expressed in isolates originating from a common host and expressed at much lower level in isolates from other hosts. A notable example is provided by protein spots 27, 28 and 29, which correspond to a hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain. These spots represented up to 1.8% of the total protein expression level on gels containing protein extracts from lettuce isolates, but only 0.5% in carrot and less than 0.1% in cotton isolates. Such a large difference in expression level suggests important functional variation between isolates originating from a particular host.

Identification of proteins that are correlated with isolate virulence levels

We postulated that the fungus has adapted its proteome to maximally benefit from the host plant and therefore assure its survival. Based on this hypothesis, we explored the possibility that the expression level of specific protein spots in different isolates would correlate with the level of virulence against cotton. As shown in Fig. 5, the isolates possessed different levels of virulence. As a group, the isolates originating from cotton and carrot were highly significantly ($P < 0.01$) more virulent to cotton than the isolates recovered from lettuce. The cotton and carrot groups were not significantly different to each other. There were also significant ($P < 0.05$) differences in virulence between isolates within host groups. A Spearman's rank correlation performed across the 12 isolates between protein expression of each isolate and the average virulence level for each of these isolates revealed that 52 protein spots were negatively correlated with the level of virulence (nine protein spots differed significantly at the $P = 0.01$ level and 43 proteins spots differed significantly at the $P = 0.05$ level) while 30 protein spots were positively correlated with the level of virulence (five proteins spots differed significantly at the $P = 0.01$ level and 25 proteins spots differed significantly at the $P = 0.05$ level). Of the spots that could be manually cut and analysed by MS/MS, 14 were identified (reported in Table 3 with their respective correlation coefficients). Some of these corresponded to hypothetical proteins and their possible function was therefore assigned based on their putative conserved domains. Two protein spots were associated with carbohydrate metabolism (spots 2, and 19), three corresponded to a hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain (spots 27, 28 and 29) while the others may be involved in protein biosynthesis (spot 5), amino acid metabolism (spots 37, and 38), biosynthesis of small molecules (spot 26), nucleotide metabolism (spots 3, and 6), melanin biosynthesis (spot 39) and Rab GDP-

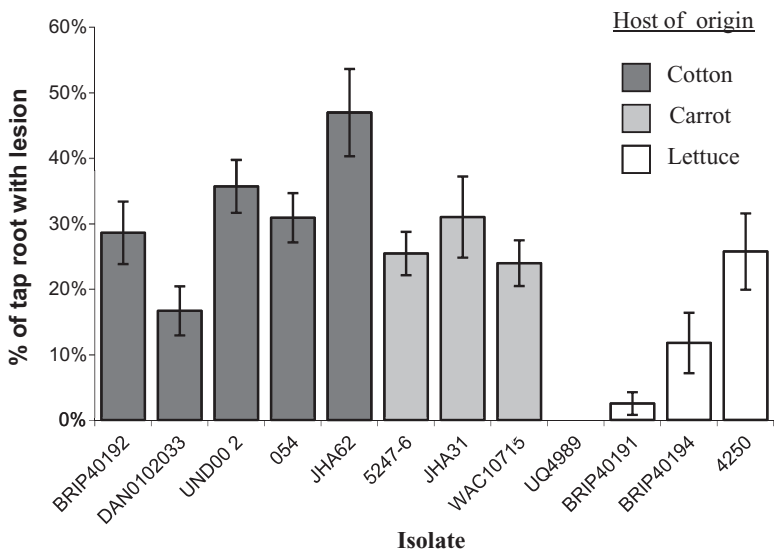

Fig. 5. Pathogenicity to cotton of *T. basicola* isolates varied according to the host of origin. Vertical lines denote standard errors of the mean from three combined experiments ($n = 15$).

dissociation inhibitor (spot 40). Finally, the last spot resulted in more than one protein identification (spot 7).

Discussion

Proteome analysis of *T. basicola* isolates reveals evidence of substantial differences that are likely to be due to host adaptation. In terms of protein expression patterns, our results demonstrate qualitative and quantitative flexibility of the *T. basicola* proteome. Our hierarchical clustering analysis of the proteome also reveals a correlation between the proteome and the host of origin. We should emphasize here that this classification relies on both quantitative (protein expression level) and qualitative (protein presence or not detected) data that cannot be deduced from genetic analysis alone. Therefore, proteome analysis as an approach to characterize *T. basicola* biodiversity confers a unique advantage over genetic studies and will reveal major functional differences that are likely to improve our understanding of this fungal plant pathogen. This data may, in future, allow development of better methods of pathogen control.

Some of these host-specific proteomic variations were analysed by identifying protein spots with unique electrophoretic mobility, proteins that varied in abundance in isolates from different hosts and proteins that correlated with virulence against cotton. More than 50% of the protein spots that were identified corresponded to hypothetical proteins. Based on the identification of their putative conserved domains, we established that the majority were involved in primary and secondary metabolism. While *T. basicola,* as a soil inhabitant, can survive extended periods of time in the absence of a host, the survival of this hemibiotrophic fungus (Hood and Shew, 1997) also depends on its close association with its host. Therefore, some of these proteins may be important in plant disease establishment.

Interestingly, two unique protein spots (16 and 17) in carrot-derived isolates are involved in the one carbon pool metabolism involving folate. Carrots are known to be a source of folate (Freisleben *et al*., 2003) and therefore differential expression of these proteins may reflect an adaptation of *T. basicola* to its host.

A few protein spots corresponded to GAPDH. This protein has previously been observed as multiple spots in fungi (Hernandez *et al*., 2004; Fernandez-Acero *et al*., 2006), which may correspond either to isoforms or different post-translational modifications. While the role of GAPDH in glycolysis is well-characterized, activities influencing many other cellular processes have also been reported (Sirover, 2005). Interestingly, recent studies have shown the importance of GAPDH as a virulence factor in several microbial pathogens (Alderete *et al*., 2001). Moreover, in *Candida albicans* (Gozalbo *et al*., 1998) and *Paracoccidioides brasiliensis* (Barbosa *et al*., 2006), it was characterized as an adhesion, involved in binding to host extracellular matrix components to establish infection.

Spot 26, which corresponds to an arginase, had a higher expression level in cotton and was positively correlated with level of virulence to cotton. This enzyme catalyses the first step of arginine degradation in the urea cycle. Besides being a substrate for arginases, arginine is also a substrate for nitric oxide (NO) synthase. In the bacterium *H. pylori,* arginase inhibits NO production by eukaryotic cells, therefore protecting the bacteria and contributing to successful host infection (Gobert *et al*., 2001). Induction of arginase has also been reported in transcriptome analysis of the pathogenic fungus *Histoplasma capsulatum* exposed to reactive nitrogen species and was implicated in reducing NO production by the host thereby facilitating

persistent macrophage infections (Nittler *et al*., 2005). In plants NO has an immune protective role, mediating plant defence against pathogens and serving as a signal in hormonal responses (Durner and Klessig, 1999; McDowell and Dangl, 2000; Wendehenne *et al*., 2001). It is therefore tempting to speculate that the higher expression of arginase in cotton isolates may be involved in the reduction of NO production by the host plant.

Mascot analysis of the MS/MS spectra of protein spots 21, 22 and 23 identified tetrahydroxynaphthalene reductase. This enzyme operates in the fungal melanin biosynthesis pathway and, together with trihydroxynaphthalene reductase, catalyses the reduction of tetrahydroxynaphthalene to scytalone (Thompson *et al*., 2000). Punja and Sun (1999) reported variation in colony pigmentation between isolates of *T. basicola* that correlated with the level of virulence to bean leaves (Punja, 2004). Highly virulent isolates were darkly pigmented while weakly virulent isolates were less so. Lindermann and Toussoun (1966) reported that albino chlamydospores of *T. basicola* germinated differently to normal chlamydospores and that they were less resistant to hydrolytic enzymes. This evidence suggests that alterations to pigmentation are accompanied by variations in cell wall composition that might account for virulence differences between *T. basicola* isolates. Recently in *Verticillium dahliae*, tetrahydroxynaphthalene reductase has been implicated as a possible pathogenicity factors facilitating colonization and microsclerotia production (El-Bebany *et al*., 2010). In the plant pathogens, *Magnaporthe grisea, Colletotrichum* spp., *Gaeumannomyces graminis* and *Venturia inaequalis* melanin play an essential role in maintaining turgor pressure that enables plant penetration. In other pathogens such as *Sclerotinia*, melanin aids survival of the pathogen in adverse conditions [for review see (Butler *et al*., 2001)].

Our analysis of the ITS region revealed that all isolates used in this study were nested among those of *T. basicola* reported in other published work. The level of intraspecific variation in the ITS was within the typical range for fungal species (Nilsson *et al*., 2008) and there was a clear discontinuity in ITS sequences between *T. basicola* and its closest relative *T. thielavioides* (Paulin-Mahady *et al*., 2002). This evidence suggests that cotton, lettuce and carrot strains all belong to the same species and phenotypic differences between them therefore represent intraspecific variation.

Based on analysis of simple sequence repeat markers, Geldenhuis and colleagues (2006) suggested that *T. basicola* originated in Europe and was introduced into South Africa and other parts of the world where it has propagated as clonal lineages. The population structure of *T. basicola* in North America determined from random amplified polymorphic DNA markers is also consistent with this model (Punja and Sun, 1999). The three groups of strains in this study, cotton, lettuce and carrot, could represent the clonal descendents of the introduction into Australia of a single strain or group of closely related strains associated with each host. This would explain why there was no variation in ITS sequences between isolates from the same host, but considerable variation within the species as a whole. Existence of pathotypes in which specific ITS sequences are correlated with host-associated lineages has been reported in other fungi such as *Ceratocystis fimbriata* (Baker *et al*., 2003) and recently *Corynespora cassiicola* (Dixon *et al*., 2009).

In summary, our genomic and proteomic analysis provides evidence that in Australia, *T. basicola* originated from descendents of single strains or groups of closely related strains associated with specific hosts. MASCOT MS/MS ion searching, *de novo* sequencing and sequence similarity searches identified host-specific differentially expressed proteins and proteins with expression levels that could be correlated with virulence against cotton. These proteins included a hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain, a GAPDH, an arginase and a tetrahydroxynaphthalene reductase, providing insight into the biochemical diversity of *T. basicola*. This work has laid the foundation for further studies to characterize the virulence-related proteomic differences within the *T. basicola* cotton-adapted strains and may help in the design of better strategies for the control of black root rot in cotton.

Experimental procedures

Fungal isolates

Table 1 and Fig. 1 report the host origin and the geographic location of the 12 isolates of *T. basicola* used in this study. All the isolates were grown on potato dextrose agar [19.5 g potato dextrose agar (Oxoid), 14.5 g Bacto-agar, distilled water 1 l] at 25°C.

DNA extraction and ITS sequence analysis

Endoconidia and mycelia from the surface of a 5-day-old plate were used to inoculate 50 ml of potato dextrose broth and cultured with rotary shaking at 25°C for 5 days. Mycelia, that were recovered by filtration, washed with MilliQ water, dried between sheets of blotting paper and lyophilized, were ground to a fine powder in a mortar and pestle. Genomic DNA extraction was performed according to Andrianopoulos and Hynes (1988) with the exception of the extraction buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 2% SDS).

The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS and 5.8S RNA regions of the nuclear ribosomal RNA gene using TopTaq Master Mix (QIAGEN, Melbourne, Australia) (White *et al*., 1990). Both strands of the ITS region were sequenced using the PCR primers, at the

Australian Genome Research Facility, Brisbane, Australia. Each unique sequence was deposited in GenBank as HM031125 (cotton), HM031128 (carrot), HM031126 (lettuce).

Sequences were edited and aligned using the ClustalW procedure in MEGA 4 software (Tamura *et al*., 2007). All full-length ITS sequences of *T. basicola* available on public databases were also imported and included in the alignment, as well as the sequence from an isolate of *T. thielavioides* (HM031127) from carrot. To assess similarities between sequences from different isolates, sequences were clustered in MEGA 4 using the UPGMA method, with the number of differences as the distance matrix. Insertions or deletions of single bases were coded as a new character state (González *et al*., 2006).

Protein extraction and quantification

Endoconidia (2.5×10^6 spores) from the surface of a 5-day-old plate were used to inoculate 50 ml of potato dextrose broth and cultured with rotary shaking at 25°C for 24 h. Proteins from mycelia (50 mg) were extracted with a Mini Beadbeater (four times, 30 s, glass beads) in a boiling solution of 0.3% SDS, 40 mM Tris-HCl pH 8 and 3% DTT. After extraction, samples were heated (100°C, 5 min) cooled to ambient temperature, diluted in the IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG-buffer pH 3–10: Amersham Biosciences) and centrifuged at 15 000 g (15 min, 20°C). Protein concentration of the supernatant was determined using the 2-D quant kit from Amersham Biosciences.

2-DE

Rehydrated IPG strips (Görg *et al*., 2000) were cup-loaded with protein (250 μg for analytical gels or 500 μg for preparative gels) and IEF was carried out on the IPGphor II (Amersham Biosciences) at 20°C with a current limit of 50 μA/strip to a total volt-hour-product of 30 kVh (analytical gels) or 45 kVh (preparative gels). Before second dimension analysis, the strips were equilibrated according to Görg and colleagues (2000). Second dimension separation was achieved on a PROTEAN II system (Bio-Rad) with 1.5 mm SDS polyacrylamide gels (12%) at 10 mA/gel until the bromophenol blue dye front reached the anodic end of the SDS-gel.

Protein visualization, gel imaging and data analysis

Proteins were visualized by blue silver staining (Candiano *et al*., 2004), recorded for image analysis and analysed with PDQuest advanced 2-D analysis software (Bio-Rad) as previously described (Coumans *et al*., 2009). Following a procedure adapted from Xu and colleagues (Xu *et al*., 2005) 2-D gel images from the different isolates were grouped according to their host of origin and three lower match sets were created. These lower match sets compiled all protein spots detected on the 2-D gel images within the group and for quantitative comparison of protein spot expression levels between the three different hosts, gel normalization was performed using the local regression method of the PDQuest software. The three lower match sets were then combined and matched in a high-level experiment. Statistical evaluation on the expression levels of all the protein spots from all the isolates was performed using SPSS software. Hierarchical clustering (SPSS software) was conducted using the nearest neighbour method with the Pearson correlation used as a measure of similarity. One-way ANOVA followed by Tukey's method were used to identify differentially expressed protein spots. Differences were considered significant at a probability (P) value less than 0.05. 'Preferentially' expressed proteins were defined as a protein spots with at least a twofold change in all the isolates within a host group when compared with the two other host groups. To assess if a twofold change within a host group was sufficient to consider a protein spot 'preferentially' expressed between hosts, we evaluated the extent of differential expression between four biological replicates for each of the following isolates BRIP40192 (cotton), 524-6 (carrot) and UQ4989 (lettuce). We found that the average coefficient of variation (CV%) within an isolate for the identified spots was 28.2%, which corresponds to less than a 1.3-fold change.

Protein identification and database search

Relevant protein spots from preparative gels were manually excised, destained and digested as previously described (Coumans *et al*., 2009). Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford, MA). Samples (1–5 μl) were concentrated and desalted onto a micro C18 precolumn (500 μm × 2 mm, Michrom Bioresources, Auburn, CA) with $H_2O : CH_3CN$ (98:2, 0.05% HFBA) at 15 μl min^{-1}. After a 4 min wash the precolumn was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 μm × ~12 cm) containing Magic C18 (~10 cm, 200 Å, Michrom) manufactured according to Gatlin and colleagues (1998). Peptides were eluted using a linear gradient of $H_2O : CH_3CN$ (98:2, 0.1% formic acid) to $H_2O : CH_3CN$ (55:45, 0.1% formic acid) at ~300 nl min^{-1} over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 μ) to a low volume tee (Upchurch Scientific) where HV (2400 V) was applied and the column tip positioned ~1 cm from the Z-spray inlet of an QTof Ultima API hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTof operated in data-dependent acquisition mode. A Tof MS survey scan was acquired (m/z 350–1700, 1 s) and the two largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (m/z 50–2000). Peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Measure program. Protein identification was achieved using the $NCBI_{nr}$ database (20091128) by conventional search (MASCOT version 2.1 or 2.2, Matrix Science, London, England) and automated *de novo* sequencing using PEAKS Studio 4.5 (Bioinformatics Solutions, Waterloo, Ontario, Canada) as previously described (Coumans *et al*., 2010). Possible cellular roles for the proteins identified were determined using the Kyoto Encyclopedia of Genes and Genomes.

Determination of the virulence levels of T. basicola *isolates against cotton*

A chlamydospore suspension was obtained from 14-day-old carrot agar cultures [4.5% UHT carrot juice (Harvey Fresh, Harvey Western Australia, 1.1% agar) based on the filtration and settling methods developed by Meyer and Shew (1991) and Hawthorne and Tsao (1970) respectively. Briefly, a spore/hyphal suspension was filtered three times through 25 mm syringe mounted filter holder (Sartorius AG, Goettingen, Germany) with 0.034 mm aperture woven stainless steel mesh (Metal Mesh, Sydney, Australia). Disruption of chlamydospore clumps and hyphae was achieved by sonication at 25 W for 1 min 30 s using a VC50T Model Vibra-Cell sonicator (Sonics and Materials, Newtown, USA). Two final filtrations were performed as previously, followed by a 20 s sonication to disperse spore clumping. Finally, chlamydospore settling took place for 20 min in sterile water, after which the majority of the supernatant (containing endoconidia and hyphal fragments) was removed and the remaining chlamydospores resuspended in sterile water to a final concentration of 5000 chlamydospores ml^{-1}.

Pathogenicity assays were performed in bioassay chambers constructed using 100 mm × 100 mm square plastic Petri dishes (Labserve, Auckland, New Zealand) fitted with a wet (30 ml, distilled water) paper wrapped sponge piece. Five surface sterilized cotton seeds (Sicot 189) (Coumans *et al*., 2009) were placed for germination and incubated at 25°C for 1 day and then at 23/18°C (day/night). After 3 days, cotton seedlings were inoculated by depositing 2 µl of chlamydospore suspension above the root hair zone of each seedling. Following inoculation, the chambers were incubated at 23/18°C (day/night) with a 12 h light/12 h dark cycle. After 10 days, roots were examined for lesions (extent of progression of the fungus as indicated by the presence of chlamydospores that are produced rapidly as the fungus is established and able to produce reproductive hyphae (Christou, 1962).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Peptide sequence of the identified proteins.
Appendix S1. Mass spectrum of single peptide identifications.

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