Differential protein expression in response to the phytoalexin brassinin allows the identification of molecular targets in the phytopathogenic fungus *Alternaria brassicicola*

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SUMMARY

The effects of the cruciferous phytoalexin brassinin on the protein expression patterns of the phytopathogenic fungus *Alternaria brassicicola* were investigated. Cell-free protein extracts of mycelia of *A. brassicicola* induced with brassinin at 0.50 and 0.10 mM were fractionated, and the proteins in soluble fractions were separated by two-dimensional electrophoresis. Spots corresponding to differentially expressed proteins were digested and analysed by liquid chromatography-electrospray ionization-mass spectrometry. The number of differentially expressed proteins was significantly higher in mycelia treated with brassinin at 0.50 mM (96 protein spots) than in mycelia treated with brassinin at 0.10 mM (18 protein spots). The majority of differentially expressed proteins included proteins involved in metabolism, processing, synthesis and several heat shock proteins (HSPs). Brassinin concentrations below 0.30 mM induced HSP90, a protein involved in the regulation of morphogenetic signalling in fungi, suggesting that 0.30 mM is a minimal concentration of brassinin necessary for the protection of brassicas against *A. brassicicola*. These results reveal that HSP90 is a potential target for inhibition in stressed *A. brassicicola* and confirm that brassinin has strong detrimental effects on *A. brassicicola*, suggesting that its detoxification by the fungus suppresses an important defence layer of the plant.

INTRODUCTION

The safety and security of staple crops are under challenges and threats posed by fungi and other pests on a daily basis (Strange and Scott, 2005). Despite preventative measures and the consistent use of fungicides, plant fungal diseases continue to cause major damage to crops, amounting to huge yield losses worldwide. Although serious public concerns regarding the environmental and health issues caused by the use of pesticides are expressed regularly, their consumption over the past few years has increased in a number of countries (Euromonitor, 2008). To decrease signifi-

cantly the use of pesticides, different pest management practices must be adopted across countries and continents; however, before current practices can be transformed, a much better understanding of the interactions between plants and their pests is essential. Towards this goal, we have investigated the interactions of the plant pathogen *Alternaria brassicicola* (Schwein.) Wiltshire with economically important *Brassica* species (Pedras *et al.*, 2009a). *Alternaria brassicicola* causes Alternaria black spot, a plant disease affecting numerous economically important vegetables (e.g. cauliflower, broccoli, cabbage, rutabaga) and oilseeds (canola, rapeseed, mustard) (Rimmer and Buchwaldt, 1995; Thomma, 2003). Importantly, we have established that *A. brassicicola* detoxifies the phytoalexin brassinin (Pedras *et al.*, 2009a), one of the most important plant defence metabolites, as a result of its dual role as a biosynthetic precursor of other phytoalexins and selective antifungal activity. Phytoalexins are antimicrobial secondary metabolites produced *de novo* by plants in response to different forms of stress, including microbial attack, UV radiation and heavy metal salts (Bailey and Mansfield, 1982). In general, and also in the particular case of *Brassica* spp. (Brassicaceae family, common name 'crucifer'), in response to pathogen attack, plants from any given family biosynthesize phytoalexins of related chemical structures (Pedras *et al.*, 2011). In a counter-attack, fungal pathogens produce enzymes that detoxify phytoalexins by mostly oxidative or hydrolytic reactions (Pedras and Ahiaonu, 2005). These detoxification processes favour the pathogen and are highly detrimental to the plant.

The antifungal activity of brassinin is partly a result of its dithiocarbamate group, a toxophore present in broad-spectrum agrochemicals used to control fungi and weeds (Szolar, 2007). However, *A. brassicicola* produces the inducible brassinin hydrolase BHAb, which detoxifies brassinin to 3-indolylmethanamine and displays remarkable substrate specificity (Fig. 1) (Pedras *et al.*, 2009b). Other pathogens of *Brassica* spp., such as, for example, *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] and *Sclerotinia sclerotiorum* de Bary, detoxify brassinin also using specific enzymes, i.e. brassinin oxidase (Pedras *et al.*, 2008) and brassinin glucosyl transferase (Sexton *et al.*, 2009), respectively. The fungal detoxification of brassinin is strongly detrimental to the plant as its absence prevents the biosynthesis of all derived chemical defences (e.g.

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Fig. 1 Detoxification of brassinin to 3-indolylmethanamine by *Alternaria brassicicola* (using hydrolase BHAb), and chemical structure of camalexin.

cyclobassinin, brassilexin, spirobrassinin), causing the plant to become more vulnerable to pathogens. For this reason, it has been proposed that plant infections caused by selective plant fungal pathogens, such as *A. brassicicola* and *L. maculans*, might be controlled with inhibitors of phytoalexin-detoxifying enzymes, coined 'paldoxins' (Pedras, 2008; Pedras *et al.*, 2008, 2009c, 2010). The important advantage of paldoxins relative to fungicides is their greater selectivity towards specific targets and the lack of fungicidal activity.

It has been shown that phytoalexins, such as camalexin, a cruciferous phytoalexin produced by wild species (e.g. *Camelina sativa* and *Arabidopsis thaliana*), induce the activity of superoxide dismutase (SOD) and brassinin-detoxifying enzymes in mycelial cultures of *L. maculans* (Pedras *et al.*, 2008) and *A. brassicicola* (Pedras *et al.*, 2009a), respectively, and also inhibit protein synthesis (Pedras *et al.*, 2009c). The treatment of cultures of *L. maculans* with an analogue of brassinin led to the detection of differentially expressed genes using a cDNA-amplified fragment length polymorphism (AFLP) method (Gellatly *et al.*, 2001). In addition, cultures of *A. brassicicola* induced with camalexin have been shown to differentially express genes related to cell protection against oxidative damage. The activated genes appeared to be involved in sterol, sphingolipid and melanin biosynthesis, and drug efflux (Sellam *et al.*, 2007). More recently, these results have been confirmed and expanded, as camalexin was found to activate two mitogen-activated protein (MAP) kinases in *A. brassicicola*: AbHog1 and AbSlit2. Mutant strains of *A. brassicicola* lacking these functional MAP kinases showed hypersensitivity to both brassinin and camalexin. Consequently, these phytoalexins could activate fungal MAP kinases potentially involved in the protection of the fungus (Joubert *et al.*, 2011). Nonetheless, although it is clear that phytoalexins affect several metabolic processes occurring in plant fungal pathogens, the knowledge of these processes is rather incomplete. Yet, this knowledge could lead to the discov-

ery of new molecular targets, crucial to the development of reasonable strategies to control plant fungal diseases. Proteomics techniques are valuable in the identification of targets that could be useful to control fungi, whether plant or human pathogens (Fernández Acero *et al.*, 2011). However, to the best of our knowledge, the investigation of fungal proteomes induced differentially by phytoalexins has not been reported.

To understand the interaction between *A. brassicicola* and *Brassica* spp., the phytoalexin brassinin was used as a surrogate of brassica defence metabolites. Proteomics techniques were applied to detect and identify the proteins potentially involved in the protection of *A. brassicicola* from stress caused by brassinin. Using two-dimensional electrophoresis (2-DE), followed by the identification of peptides (liquid chromatography-electrospray ionization-mass spectrometry, LC-ESI-MS) obtained from tryptic digests of the differentially expressed proteins, we have discovered that higher concentrations of brassinin (0.50 mM) affect a substantially larger number of proteins (96 proteins) than brassinin at lower concentration (0.10 mM) (18 proteins). Among the down-regulated proteins, several were involved in metabolism, including the biosynthesis of melanin and cryptic metabolites yet to be uncovered in *A. brassicicola*. Altogether, our results indicate that brassinin is able to arrest or slow down fungal cell growth, a process that could protect plants from fungal invasion, if its concentration remained at effective levels during the early stages of fungal infection.

RESULTS

2-DE, LC-ESI-MS analysis and protein identification

To investigate the effect of brassinin on protein expression in *A. brassicicola*, mycelial cultures were treated with brassinin at two concentrations (0.50 and 0.10 mM) and incubated for 20 h. The concentrations were chosen on the basis of the rates of transformation of brassinin by *A. brassicicola* (complete transformation in 20 h at 0.10 mM vs. <10% transformation at 0.50 mM) and mycelial growth inhibition on solid media (Pedras *et al.*, 2009a). Proteins were extracted from mycelia and the soluble fractions of treated mycelia and control mycelia (treated with dimethylsulphoxide, DMSO) were analysed by 2-DE. Figure 2 shows representative 2-D gels of fraction S obtained for mycelia treated with brassinin at 0.50 mM and control mycelia (for treatments with brassinin at 0.10 mM, see Fig. S2 in Supporting Information). In total, 78 of 96 proteins differentially expressed on treatment with brassinin at 0.50 mM were successfully identified by capillary LC-ESI-MS (18 spots could not be identified). Among the 78 spots identified, 13 protein spots shared the same identity with different pI values in the 2-D gels. Therefore, 65 different proteins were identified, 37 of which were up-regulated and 28 of which were down-regulated relative to control 2-D gels. The list of

Fig. 2 Two-dimensional (2-D) gel analysis of proteins in fraction S from control cultures of *Alternaria brassicicola* and cultures treated with 0.50 mM brassinin. Total loading protein, 1 mg. Proteins were resolved on a gel using isoelectric focusing gradient 3–10 NL strips in the first dimension, and 10% polyacrylamide SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis) gels in the second dimension. Protein spots were visualized by Coomassie blue staining of the gels. Each result is a compilation of three independent experiments and differentially expressed spots are labelled as 'U' for up-regulated proteins (44 spots) and 'D' for down-regulated proteins (34 spots) relative to control. Seventy eight differentially expressed protein spots marked with arrows were identified using capillary liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (Tables S1 and S3).

up-regulated and down-regulated proteins induced by brassinin at 0.50 mM is presented in Tables S1 and S3 (see Supporting Information). On treatment of mycelia with brassinin at 0.10 mM, 16 of 18 differentially expressed protein spots were successfully identi-

fied by capillary LC-ESI-MS. Among these, 14 were up-regulated and two were down-regulated relative to control gels (Tables S2 and S4, see Supporting Information). That is, the number of differentially expressed proteins (96 protein spots) was

significantly higher in mycelia treated with brassinin at 0.50 mM than in mycelia treated with brassinin at 0.10 mM (18 protein spots).

All spots of interest were picked and digested with trypsin, and the subsequent fragments were analysed by capillary LC-ESI-MS. Using the MASCOT search engine, none of these peptide sequences was identified as belonging to *A. brassicicola*, indicating that Swiss-Prot and National Center for Biotechnology Information nonredundant (NCBI nr) databases used by MASCOT have not yet integrated peptide sequences from *A. brassicicola*. However, the majority of the peptide sequences from *A. brassicicola* generated in this work were identified in related species and, especially, in *Pyrenophora tritici-repentis*, *Phaeosphaeria nodorum* and *L. maculans*. These similarities are not surprising as these fungal species and *A. brassicicola* are phylogenetically related (Dothideomycetes) (Schoch *et al.*, 2009). Finally, when peptides for each previously identified protein (Tables S3 and S4) were submitted to the Joint Genome Institute (JGI) database, the corresponding proteins from *A. brassicicola* were successfully identified (Tables S1 and S2). In addition, the corresponding predicted M_r and pI values for the majority of the identified proteins were similar to those experimentally determined by 2-DE in this work (Tables S1 and S2).

Functional classification and cellular localization

On the basis of gene ontology classification, Kyoto Encyclopedia of Genes and Genomes (KEGG) protein interaction pathways and Pathway Tools, the functional categories and number of differentially expressed proteins present in fraction S, in response to treatment with brassinin at 0.50 and 0.10 mM, are shown in Fig. 3. In mycelia treated with brassinin at 0.50 mM, the number of differentially expressed proteins identified (78) was substantially higher than in mycelia treated with brassinin at 0.10 mM (16). A substantial number (55%) of these identified proteins were implicated in metabolism in both 0.50 and 0.10 mM brassinin-treated mycelia (Fig. 4). The analysis of proteins in fraction S resulting from treatment with brassinin at 0.50 mM demonstrated that enzymes of glycolysis, the pentose phosphate (PP) pathway, amino acid metabolism, polyamine metabolism, glycogen and melanin biosynthesis were differentially expressed. By contrast, treatment of mycelia with brassinin at 0.10 mM affected a smaller number of proteins, namely enzymes of the tricarboxylic acid (TCA) and PP pathways. It is noteworthy that brassinin at 0.50 mM increased the expression levels of enzymes related to polyamine biosynthesis (glutamate dehydrogenase, arginase and spermidine synthase), but decreased the expression levels of enzymes involved in glycogen [phosphoglucosylase, uridine diphosphate (UDP)-glucose pyrophosphorylase and 1,4- α -glucan branching enzyme] and melanin (1,3,6,8-tetrahydroxynaphthalene reductase, scytalone dehydratase and 1,3,6-trihydroxynaphthalene reductase) biosynthesis (Fig. S3). Because a number of functions have been attributed to

Fig. 3 Classification of up- and down-regulated proteins according to their function. Functional classes were deduced for the differentially expressed proteins identified in fraction S on treatment with brassinin at 0.50 and 0.10 mM, and are listed in Tables S1 and S2, respectively.

polyamines, including growth factors, second messengers, antioxidants, nutrients, stabilizers of DNA, RNA and membranes, the up-regulation of related metabolic enzymes is not surprising (Thomas and Thomas, 2001). However, glycogen and melanin biosynthesis require energy-rich molecules, such as UDP-glucose and NADH (nicotinamide adenine dinucleotide, reduced form), and therefore the down-regulation of their biosynthetic pathways suggests that energy-rich molecules are diverted to pathways involved in defence, such as the production of stress response proteins.

Among the 65 differentially expressed proteins identified in *A. brassicicola* by treatment with brassinin at 0.50 mM, seven up-regulated proteins and one down-regulated protein were associated with protein processing (Tables S1 and S3, Figs 3 and 4). These proteins included aspartyl proteinase, mitochondrial-processing peptidase subunit, Xaa-Pro dipeptidase, putative dipeptidyl-peptidase 3, putative aminopeptidase, proteasome subunit β type-1 precursor, putative subtilase-type proteinase and thimet oligopeptidase. In addition, one up-regulated proteasome

Fig. 4 Distribution (%) of differentially expressed proteins by functional category families estimated from the data in Tables S1 and S2. Only those families with abundances higher than 3% are shown; the remainder are grouped in the slice under other function.

subunit α type-4 was identified after treatment of mycelia with brassinin at 0.10 mM. Post-translational processing of proteins by proteases is an important molecular response involved in adaptation to stress (Jenal and Hengge-Aronis, 2003). This processing may lead to the activation and inhibition of the activity of proteins. In addition to protein degradation associated with regulatory processes, transcriptional control is an important regulatory factor associated with stress responses (Missall *et al.*, 2006). Four proteins were identified to be involved in translational control, including two up-regulated proteins (similar to elongation factor 1- γ and putative polyadenylate-binding protein) and two down-regulated proteins (elongation factor 3 and similar to elongation factor 2).

Three heat shock proteins (HSPs) were up-regulated on treatment with brassinin at 0.50 mM, and included HSPSSB, HSP70 (mitochondrial precursor) and HSP60 (mitochondrial precursor). HSP70 of the mitochondrial matrix is essential for the translocation of cytosolic precursor proteins across two mitochondrial membranes (Voos and Röttgers, 2002). HSP60 plays an important role in protein folding and, consequently, provides a protected environment for the adenosine triphosphate (ATP)-dependent folding of unfolded or newly synthesized single proteins or protein domains. Similarly, when mycelial cultures were treated with brassinin at 0.10 mM, three HSPs were up-regulated; in addition to HSP70, two other up-regulated HSPs were identified: HSP90 and a putative HSP90-binding co-chaperone (Tables S2 and S4).

Other differentially expressed proteins in mycelia treated with brassinin at 0.50 mM included two proteins of cell division (putative proliferating cell nuclear antigen and cell division control protein CDC48), one of cell structure and integrity (similar to actin-related protein 2/3 complex subunit 1A), two of redox homeostasis (similar to glutathione reductase and glyoxal oxidase), two of transport (Ran-specific guanosine triphosphatase-activating

Fig. 5 Prediction of the subcellular localization of identified and differentially expressed proteins in fraction S on treatment with brassinin at 0.50 and 0.10 mM using the YLoc web server.

protein 1 and voltage-gated potassium channel subunit β -1) and five proteins with unknown function. Among the 65 and 16 proteins identified on treatment with brassinin at 0.50 and 0.10 mM, respectively, only three proteins shared identity: transaldolase, HSP70 and 2-epi-5-epivaliolone synthase.

To obtain additional information on the cellular components affected by treatment of mycelia with brassinin, the proteins identified through MASCOT were further analysed to predict their subcellular localization (Fig. 5). The majority of differentially expressed proteins were localized in the cytoplasm and mitochondrion; however, the relative amount of mitochondrial proteins was higher in mycelia treated with brassinin at 0.10 mM.

Confocal microscopy

Because brassinin affected the differential expression of mitochondrial proteins, it was pertinent to investigate the potential inhibition of mitochondrial function in cultures of *A. brassicicola* treated with brassinin. In order to verify the effect of brassinin on the mitochondrial membrane potential, mycelial cells were incubated for 20 h with brassinin (0.10 and 0.50 mM) and then treated with rhodamine 123 and 3,3'-diethylxoxycarbocyanine iodide [DIOC6(3)]. The incubation of mycelia with brassinin decreased the fluorescence of the potentiometric probe rhodamine 123 and DIOC6(3) at the hyphal region (Fig. S4, see Supporting Information), where mitochondria are more active. Hence, these results suggest that brassinin inhibits the mitochondrial respiratory chain of *A. brassicicola*. Similar results were obtained using confocal microscopic examination of *Puccinia recondite*, *Erysiphe graminis* and *Botrytis cinerea* after treatment with the fungicide

Fig. 6 Western blot analyses of the cell-free extracts (supernatant S) obtained from mycelial cultures treated for 20 h with brassinin (0.10–0.50 mM) and of control cell-free extract.

azoxystrobin (Swift *et al.*, 2001), also an inhibitor of the fungal mitochondrial respiratory chain.

Western blot analysis of HSP90 expression

To confirm the quantitative changes in HSP90 on treatment of mycelia with brassinin at 0.10 mM, but not at 0.50 mM, mycelial cultures of *A. brassicicola* were incubated without and with different concentrations of brassinin (0.10, 0.20, 0.30, 0.40 and 0.50 mM) for 20 h. The cultures were filtered, the mycelia were extracted with extraction buffer (see Experimental procedures) and the resulting cell-free extracts (Fig. S1, see Supporting Information) were examined by Western blot analysis. The liquid broth was extracted with ethyl acetate and the organic extract was analysed by HPLC to determine the amounts of brassinin remaining in culture after 20 h of incubation. No brassinin was detected in cultures treated with 0.10 mM, whereas cultures treated with 0.50 mM contained c. 90% of brassinin.

For one-dimensional Western blotting, proteins (35 µg) derived from each sample were loaded into each lane, resolved via 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The Western blot results in Fig. 6 demonstrate that the treatment of mycelia with 0.10 and 0.20 mM of brassinin generated a strong band with a molecular mass of c. 88 kDa that corresponded to HSP90 obtained from 2-DE (about 86 kDa). One band of approximately 75 kDa was considered to be noise, probably generated from the proteolytic degradation of HSP90. The fact that HSP90 was detected by Western blot analyses of protein extracts from mycelia treated with 0.10 and 0.20 mM brassinin, but not in control mycelia or in mycelia treated with 0.50 mM brassinin, showed that Western blot data were consistent with the proteomics data (Tables S1 and S2).

DISCUSSION

Differential protein expression profiles were observed by 2-DE after treatment of mycelial cultures of *A. brassicicola* with the crucifer-

ous phytoalexin brassinin at two different concentrations (0.50 and 0.10 mM). Brassinin at 0.50 mM up-regulated enzymes of the PP pathway (glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase and glucose-6-phosphate isomerase) and down-regulated enzymes of the pyruvate pathway (pyruvate dehydrogenase, pyruvate decarboxylase and pyruvate carboxylase) (Fig. S1). The PP pathway is a major pathway for the recycling of NADP⁺ to NADPH (nicotinamide adenine dinucleotide phosphate, oxidized and reduced forms) and for the production of ribose-5-phosphate; NADPH is important for the protection of cells against oxidants (Chiapello *et al.*, 2010; Ralser *et al.*, 2007, 2009). In addition, it has been suggested that glucose-6-phosphate dehydrogenase levels might be particularly important during plant infection by the rice blast fungus (Wilson *et al.*, 2010). Furthermore, brassinin at 0.10 mM induced, among others, three proteins involved in the TCA cycle (aconitate hydratase, ATP citrate synthase and malate dehydrogenase). That is, our proteomics data indicate that many affected proteins are involved in primary metabolism, particularly in the TCA cycle and PP pathway, both important for the production of energy-rich molecules, such as NADPH and ATP. We suggest that cells of *A. brassicicola* under stress caused by brassinin redirect metabolic fluxes to mount effective defence mechanisms. In addition, results obtained with the mitochondrial potentiometric assays, rhodamine 123 and DiOC6(3), strongly suggest that some molecular targets of brassinin are involved in mitochondrial function. Importantly, CDC48 was down-regulated in mycelia treated with brassinin at 0.50 mM. CDC48, a member of the AAA (ATPases associated with various cellular activities) protein family conserved in eukaryotes, is involved in different cellular processes ranging from the degradation of endoplasmic reticulum proteins to cell-cycle regulation and organelle biogenesis. In *Caenorhabditis elegans*, CDC48 has been shown to promote the re-formation of the nucleus (Ramadan *et al.*, 2007). Down-regulation of this protein suggests that brassinin at higher concentration can reduce cell proliferation through the down-regulation of CDC48. In short, brassinin appears to decrease or arrest cell growth and disturb the energetic status of fungal cells. *In planta*, if the brassinin concentration remains at effective levels, such effects could protect cells during the initial fungal attack.

Contrary to the phytoalexin camalexin (Sellam *et al.*, 2007), our proteomics data indicate that brassinin does not affect the membrane integrity of fungal cells. This result is not surprising because the chemical structures of these phytoalexins are quite different. Although the indole moiety is present in both brassinin and camalexin, the substituents at carbon-3 are clearly different, i.e. a methylene group (CH₂) attached to a methyl dithiocarbamate vs. a thiazolyl ring. Therefore, the mechanism of action of each phytoalexin, although probably multi-site, is expected to bind different targets. Indeed, the inhibitory activity of brassinin against *A. brassicicola* is substantially lower than that of camalexin (c. 40% vs. 100% at 0.50 mM).

Recently, proteomics studies of a few phytopathogenic fungi have shown that, during fungal infection of plant cells, the secreted and intracellular proteins are cell wall-degrading enzymes (cellulases, glucanotransferases, endoglucanases, phospholipases, proteinases and chitinases) implicated in plant cellulose, hemicellulose, pectin and starch catabolism (Mehta *et al.*, 2008; Tan *et al.*, 2009). Our proteomics data revealed that proteins predicted to be secretory proteins using YLoc software do not belong to the group of cell wall-degrading enzymes (nine predicted proteins). Hence, the phytoalexin brassinin does not appear to induce enzymes involved in plant cell wall catabolism, although, in general, these are secretory enzymes that might have been diluted in the culture broth and presented in undetectable amounts in the mycelial extracts.

The up-regulation of different HSPs triggered by brassinin indicates that stress levels in *A. brassicicola* correlate with brassinin treatments. Our results are consistent with other work showing that stress can affect the differential expression of various metabolic enzymes (Chiapello *et al.*, 2010; Wilson *et al.*, 2010). In general, HSPs, which are among the most conserved proteins, are important regulators of diverse constitutive metabolic processes and are markedly up-regulated during stress. It has been suggested that HSPs are recruited to reorganize fungal cells under stress conditions (Guimaraes *et al.*, 2011). There are two major cytoplasmic isoforms of HSP90 (α and β) (Chen *et al.*, 2005; Csermely *et al.*, 1998); although HSP90 β is expressed constitutively, HSP90 α is generally stress inducible and is expressed in most tissues. HSP90 α is important for long-term cellular adaptation, differentiation and evolution, and may therefore be a more cytoprotective form of HSP90 (Sreedhar *et al.*, 2004). Interestingly, the control of HSP90 is viewed as a potential strategy to treat human fungal diseases because it enhances the efficacy of existing antifungals, blocks the emergence of drug resistance and exerts broad-spectrum activity against diverse fungal pathogens (Cowen *et al.*, 2009). For example, in *Candida albicans*, a serious fungal pathogen of humans, HSP90 regulates the morphogenetic transition from yeast to filamentous forms required for virulence (Shapiro and Cowen, 2010). Our results show that concentrations of brassinin of 0.30 mM or greater do not induce HSP90, suggesting that this is a minimal concentration for the protection of brassicas against *A. brassicicola*. Nonetheless, because brassinin is transformed by BHAb, the plant cannot maintain an effective concentration during the early stages of the infection (Pedras *et al.*, 2009b). We have established previously that leaves of *B. juncea* infected with *A. brassicicola* contain mainly the product of detoxification of brassinin (*N*-acetyl-3-indolylmethanamine, c. 10 nmol/g of fresh weight), together with other phytoalexins. Hence, these results indicate that HSP90 is a potential target of inhibition in stressed *A. brassicicola*; inhibitors of brassinin degradation

(paldoxins against BHAb) could prevent indirectly the synthesis of fungal HSP90 in infected plants by increasing the brassinin concentration. If such inhibitors had synergistic activity with brassinin and other phytoalexins produced during plant infection, the effective concentrations necessary to treat brassicas could be decreased. Although BHAb is an enzyme inducible by brassinin, it was not detected in cultures treated with brassinin, probably as a result of the extremely small amounts produced (Pedras *et al.*, 2009b). However, the strong up-regulation of salicylaldehyde dehydrogenase by brassinin suggests that the oxidation of an aromatic aldehyde(s) was taking place in cultures stressed with brassinin at 0.50 mM; however, indolyl-3-carboxaldehyde is a product of brassinin metabolism in *L. maculans*, but does not appear to be a product in *A. brassicicola*. Thus, the role of this dehydrogenase in cultures requires further clarification.

Brassinin appears to down-regulate two enzymes potentially involved in secondary metabolite pathways, namely 2-epi-5-epivaliolone synthase, a shikimate metabolite, and versicolorin reductase, a polyketide. Both enzymes suggest the presence of cryptic metabolites yet to be discovered in *A. brassicicola*. Although no versicolorin-type metabolites have been reported in *A. brassicicola*, several polyketides have been found (Pedras *et al.*, 2009a). Valiolone-type metabolites have not been reported so far and will be rather difficult to isolate from liquid cultures because of their high polarity (Mahmud, 2009). In this context, it is worth mentioning that brassinin has been found to inhibit the biosynthesis of secondary metabolites (sirodesmins) produced by *L. maculans* (Pedras *et al.*, 1993).

In summary, our results further confirm that brassinin has strong detrimental effects on *A. brassicicola*, suggesting that its detoxification by the fungus suppresses an important defence layer of the plant, and emphasizing that the detoxification of brassinin can be very damaging to the plant. For these reasons, plant treatments that include inhibitors of brassinin-detoxifying enzymes are likely to bring stronger benefits to crop protection (paldoxins and other type of inhibitors) than those previously foreseen. Further proteomics work will examine the differential expression of insoluble proteins present in mycelial pellets (PP and MP, Fig. S1) of *A. brassicicola* treated with brassinin.

EXPERIMENTAL PROCEDURES

Reagents, chemicals and antibodies

2-DE materials [immobilized pH gradient (IPG) buffer, strips, etc.] were obtained from GE Healthcare (Baie-D'Urfe, QC, Canada). Unless indicated, all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). Brassinin was synthesized and purified as reported previously (Pedras and Jha, 2006).

Fungal cultures and brassinin treatments

Alternaria brassicicola isolate ATCC 96866 was subcultured on V8-agar plates under constant light at 23 ± 1 °C for 10 days (Pedras *et al.*, 2009a). Cultures of *A. brassicicola* were initiated by inoculating spores (10^6 spores per flask) in 250-mL Erlenmeyer flasks containing 100 mL of potato dextrose broth (PDB) medium, and incubated on a shaker at 120 rpm at 23 ± 1 °C. After 48 h, brassinin dissolved in DMSO (100 µL) was added to each flask and cultures were incubated for 20 h (final concentrations of brassinin in culture, 0.10 and 0.50 mM); a similar amount of DMSO was added to control cultures. The mycelia were separated from the broth using gravity filtration through cheese cloth and frozen immediately.

Protein extraction and fractionation

The frozen mycelia (c. 2 g) were shaved over ice-cold extraction buffer A (4 mL) and ground (mortar) for 10 min. Buffer A contained 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM ascorbic acid and 1 : 200 (v/v) protease inhibitor cocktail (P-8215; Sigma-Aldrich). The ground extract was centrifuged for 15 min at 15 000 *g* to yield a supernatant and a pellet (PP). The supernatant was then centrifuged for 60 min at 130 000 *g* to generate the supernatant (SP) and the pellet (MP). The soluble protein fraction from the supernatant was dialysed three times for 2 h against water in the presence of 1 : 5000 (v/v) protease inhibitor cocktail and then lyophilized. The lyophilized proteins were dissolved in DeStreak rehydration solution (1.5 mL) and the suspension was centrifuged for 10 min at 30 000 *g*. The resulting supernatant was used for proteomics analyses (fraction S) (Fig. S1) (Bridge *et al.*, 2003).

2-DE and image analysis

One of the problems confronting proteomics analyses of fungi is the complexity of their proteomes and the separation of the crude protein mixtures. Subcellular fractionation can reduce the complexity of the sample for analysis. In this work, proteins were fractionated into three subcellular fractions (Fig. S1), and the soluble fractions S obtained from the treatment of cultures with brassinin at 0.50 and 0.10 mM, and controls (identical conditions without brassinin), were used for 2-DE (c. 1 mg of protein) and protein identification. For each experimental condition, three biological replicates were analysed. The molecular mass of the proteins separated on the 2-DE polyacrylamide gels ranged from 10 to 150 kDa and the *pI* value from 3 to 10.

Immobiline DryStrip pH 3–10 NL, 24 cm, were rehydrated for 10 h at 20 °C in 450 µL of rehydration solution with IPG buffer (0.5%) containing *A. brassicicola* extracted proteins (fraction S, approximately 1 mg of total protein determined by the Bradford method; Bradford, 1976). The protein samples were prepared from 12 independent biological experiments, giving a total of 12 samples (first set of six samples includes three from control and three from mycelia treated with brassinin at 0.50 mM; second set of six samples includes three from control and three from mycelia treated with brassinin at 0.10 mM). Isoelectric focusing (IEF) was carried out using the Ettan IPGphor 3 IEF System (GE Healthcare) under the following conditions: step 1, 500 V for 1 h; step 2, 2000 V for 3 h; step 3, 3500 V for 10 h; step 4, 8000 V for 4 h. Focused IPG strips were equili-

brated in 15 mL of equilibration buffer A [6 M urea, 2% SDS (w/v), 50 mM Tris-HCl, pH 8.8, 30% glycerol (v/v) and 130 mM dithiothreitol (DTT)] for 15 min each, and then incubated in equilibration buffer B [6 M urea, 2% SDS (w/v), 50 mM Tris-HCl, pH 8.8, 30% glycerol (v/v) and 135 mM iodoacetamide] for 15 min each. The second equilibration of IPG strips was performed first with buffer A for 15 min and then with buffer B for 15 min. Equilibrated IPG strips were then placed and fixed using 0.5% of hot agarose (w/v) on the top of the gel plate. The second dimension SDS-PAGE was performed in an Ettan DALT six Large Vertical System (GE Healthcare) using 10% polyacrylamide with the standard marker proteins (molecular mass range, 10–225 kDa; V8491; Promega, Madison, WI, USA). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

The gels were scanned using a GS-800 densitometer (Bio-Rad, Mississauga, ON, Canada) and analysed using PDQuest 2-D analysis software version 8.0.1 (Bio-Rad). 2-DE virtual gels were generated from 12 independent protein samples, as reported above. The spot patterns of all the gels were matched to each other and the quantity of each spot was normalized using a local regression model available in PDQuest. The average intensities of resolved spots were compared using quantitative, qualitative and statistical functions within the PDQuest software. The spot boundary tool was applied to detect large, saturated and irregularly shaped spots. The intensity of the spot was measured by summing the pixels within each spot boundary (spot volume) and is defined as [optical density (OD) of the spot] $\times$ (area occupied by the spot). The spots that were significantly different in median quantity (Student's *t*-test; $P = 0.05$ or less) were considered to be significantly different. A minimum of a 2.0-fold change was considered for the up-regulated proteins and 0.5-fold for the down-regulated proteins. A total of about 800–1000 protein spots was detected on all 12 2-D gels. Poorly defined spots and spots with normalized volumes of <9 ($V = OD \times \text{area}$) were excluded from further analysis. Ninety-six spots corresponding to significant differences in protein expression levels were observed on 2-D gels of mycelia treated with brassinin at 0.50 mM relative to 2-D gels of control mycelia ($P < 0.05$). To estimate the reproducibility of our 2-D gel images, the coefficients of variation were calculated for each analysed protein spot. Of the total 184 protein spots analysed, 160 (87%) showed a coefficient of variation of $<50\%$, whereas only nine protein spots (5%) showed a coefficient of variation of $>70\%$. Thus, the proteomics data presented herein are accurate and reproducible.

Trypsin digestion

The spots of interest were excised manually, washed with NH_4HCO_3 , reduced with tris(2-carboxyethyl)phosphine (TCEP), alkylated with iodoacetamide and dehydrated in acetonitrile following a standard protocol from Agilent (5188-2749 Protein In-Gel Tryptic Digestion Kit, Mississauga, ON, Canada). All steps were performed in a Thermomixer R (VWR International, 21516-166, Mississauga, ON, Canada) with constant shaking at 500 rpm. Digestions were carried out for 4 h at 37 °C using modified trypsin. Trypsin-generated peptides were submitted for capillary LC-ESI-MS analysis.

LC-ESI-MS analysis and protein identification

LC-ESI-MS was performed with an Agilent 1200 capillary high performance liquid chromatograph (HPLC) equipped with an enrichment column

(Zorbax SB-C18 capillary reversed phase column; 0.5 mm × 35 mm, 5 µm particle size) and an analytical column (Zorbax 300 SB-C18, 0.3 mm × 150 mm, 5 µm particle size); a gradient elution of 3%–80% solution B (0.1% formic acid in 90 : 10, v/v, acetonitrile–water) in buffer A (0.1% formic acid in water), operated at a flow rate of 5 µL/min, was used to elute 5 µL of the tryptic peptide solution. The Agilent MSD Trap XCT detector was operated in the unique peptide scan auto-MS/MS mode. The ionization mode was positive electrospray with an Agilent orthogonal source. The electrospray source was operated at 350 °C and the nebulizer gas pressure and the drying gas flow rate were set at 70 psi and 12 L/min, respectively. MS analysis was performed in positive-ion mode and with a maximum accumulation time of 200 ms; the smart target was 100 000 and the MS scan range was *m/z* 100–2000. Automatic MS/MS was performed in auto MS mode with the number of parents 2, a fragmentation amplitude of 1.0 V and scan *m/z* 50–2200. The complete system was fully controlled by Agilent ChemStation software. Mascot software from Matrix Science Ltd. (Boston, MA, USA) was used for protein database searching.

The LC-ESI-MS spectral data obtained as Mascot generic files (mgf) were exported to the MASCOT search engine (http://www.matrixscience.com/search_form_select.html) and screened against the Swissprot and NCBI nr database. The parameters used for protein identification included: the carbamidomethylation of cysteine as a fixed modification and the oxidation of methionine as a variable modification; peptide tolerance, 1.2 Da; MS tolerance, 0.6 Da; peptide charge, +2. One miss cleavage was allowed and identification was considered to be significant when the proteins were identified with at least two different tryptic peptides as first candidate.

Bioinformatic analysis

To obtain information about the putative functional identity of the proteins, the sequence identification number (GI, GenInfo Identifier; <http://www.ncbi.nlm.nih.gov/Sitemap/sequenceIDs.html>) of the protein, obtained by MASCOT search of the LC-ESI-MS data, was used to verify the functional assignment of proteins in the NCBI Entrez Protein database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein>). In addition, all sequences of the identified proteins were compared with the NCBI nr protein database using BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To identify the corresponding proteins in *A. brassicicola*, the peptide matches to protein hits obtained by MASCOT analyses were subjected to BLAST analysis against the JGI database (<http://genome.jgi-psf.org/Altbr1/Altbr1.home.html>).

Proteins were assigned to molecular functional categories on the basis of the gene ontology classification (<http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Altbr1>), the KEGG database (<http://www.genome.jp/kegg/kegg1.html>) or Pathway Tools (<http://bioinformatics.ai.sri.com/ptools/>; <http://metacyc.org/>). Subcellular localization was predicted using YLoc (<http://www.bs.informatik.uni-tuebingen.de/Services/YLoc/webloc.cgi>) (Briesemeister *et al.*, 2010).

Confocal microscopy

The fluorescent probes DIOC6(3) and rhodamine 123 were used for confocal microscopy analysis of the mitochondrial membrane potential. Rhod-

amine 123 was dissolved in double-distilled water to 1.0 mM. Mycelia obtained without (control) and with pre-incubation with brassinin 0.10 or 0.50 mM (at 23 °C for 20 h) were placed on 35-mm round glass-bottomed microwell dishes (MatTek Corporation, Ashland, MA, USA) containing PDB medium (2 mL) only (control) and brassinin at 0.50 or 0.10 mM. The dishes were then incubated with rhodamine 123 at 20 µM (50× dilution of stock) for 30 min at 37 °C. The glass dishes were then rinsed three times with 2 mL of medium (5 min per rinse), followed by further washes in Tris-buffered saline (TBS), and visualized under a laser scanning confocal microscope (Zeiss LSM 510 microscope; Oberkochen, Germany). Images (optical sections) were obtained from the cells of interest using a 40× oil-immersion objective. The same protocol was used for the examination of DIOC6(3), but with a concentration of 10 µM. Images with rhodamine 123- and DIOC6(3)-stained cells were obtained using green excitation at 488 nm with an emission filter at 522 nm.

Western blot analyses and antibodies

Cultures of *A. brassicicola* were grown as described above and treated with brassinin at various concentrations (0.10–0.50 mM). Mycelia were separated from the broth by filtration, and protein extraction and fractionation were carried out as reported above. The culture broth was extracted twice with ethyl acetate, the solvent was concentrated in a rotary evaporator and the residue was analysed by HPLC to quantify brassinin, as reported previously (Pedras *et al.*, 2009b).

Two peptides were selected to prepare antibodies against HSP90 (FLRELISNASDALDKIRYEALSDPSKLDGSKDLRI and YMSSKKTFEISPKSAI-IKELKRKVEADGEDDRTVK) based on the sequence of the HSP90 protein from *P. tritici-repentis* Pt-1C-BFP. These two peptides were chosen on the basis of their close location to the N and C termini on HSP90, sequence homology to minimize cross-reactivity with other proteins (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), hydrophobicity analyses (<http://web.expasy.org/protscale/>) and prediction of secondary structures (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html). These peptides were synthesized at the Vaccine and Infectious Disease Organization (VIDO), Saskatoon, SK, Canada, as described by Fields and Noble (1990), and the purity of each peptide was determined by HPLC. Synthetic peptides were coupled to keyhole limpet haemocyanin (KLH) or to chicken egg white albumin (CEWA) using a protocol described previously (Grabarek and Gergely, 1990; Staros *et al.*, 1986). Rabbits were primed (first injection) with peptides conjugated to KLH. The first boost was with peptides conjugated to CEWA, the next boost was with peptides conjugated to KLH and the last boost was with peptides conjugated to CEWA (Fernando *et al.*, 2008). Rabbits were immunized using protocols that were approved by the University of Saskatchewan Committee on Animal Use and Supply and followed the guidelines established by the Canadian Council for Animal Care (N°19940212, investigator D. Wilson).

Proteins were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose sheets (0.2 µm, N°162-0150, Bio-Rad) for immunoblot analysis and stained with Ponceau S (Sigma) in 1% acetic acid to verify equal loading in each lane. After destaining in water, the blots were washed twice in TBS containing 0.05% (v/v) Tween-20 and incubated for 3 h in the same buffer plus 5% (w/v) defatted milk powder. The blots were then incubated with rabbit serum (1 : 50 dilution) overnight at 4 °C in milk buffer. Following two washes in TBS with 0.05% (v/v) Tween-20, the blots

were incubated with 1 : 2000 diluted alkaline phosphatase-conjugated mouse anti-rabbit IgG (Fc) immunoglobulin (N₀ sc-2358, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in milk buffer for 1.5 h at room temperature. After three washes with TBS, a standard nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate revelation was made (AP conjugate substrate kit, N0170-6432, Bio-Rad).

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SUPPORTING INFORMATION

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

Fig. S1 Fractionation of proteins in mycelia from the fungus *Alternaria brassicicola*.

Fig. S2 Two-dimensional electrophoresis (2-DE) analyses of proteins in fraction S from control cultures of *Alternaria brassicicola* (A) and cultures treated with 0.10 mM brassinin (B). Total loading protein, 1 mg. Proteins were resolved on a gel using isoelectric

focusing gradient 3–10 NL strips in the first dimension, and 10% polyacrylamide SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis) gels in the second dimension. Protein spots were visualized by Coomassie blue staining of the gels. Each result is a compilation of three independent experiments and differentially expressed spots are labelled as 'U' for up-regulated proteins and 'D' for down-regulated proteins relative to control. Sixteen differentially expressed protein spots marked with arrows were identified using capillary liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (Tables S2 and S4).

Fig. S3 Summary of metabolic pathways of *Alternaria brassicicola* affected by brassinin at 0.50 and 0.10 mM (Tables S1 and S2). Enzymes identified in this investigation are boxed: green represents up-regulated and yellow represents down-regulated proteins (red type, from 0.50 mM brassinin; blue type, from 0.10 mM brassinin; black underline, present in both 0.50 and 0.10 mM brassinin). P, phospho or phosphate; PP, diphospho or diphosphate.

Fig. S4 Confocal laser scanning microscopy images of mycelial cells from *Alternaria brassicicola* treated with rhodamine 123 and 3,3'-dihexyloxacarbocyanine iodide [DIOC6(3)]. Cells were incubated for 30 min in potato dextrose broth (PDB) medium containing 20 mM rhodamine 123 without (A) or with 0.10 mM brassinin (B) or 0.50 mM brassinin (C). Hyphae stained with 10 mM DIOC6(3) without (D) or with 0.10 mM brassinin (E) or 0.50 mM brassinin (F). Scale bar, 10 µm.

Table S1 Proteins differentially expressed in cultures of *Alternaria brassicicola* (controls) and cultures induced with brassinin at 0.50 mM. Proteins were identified using capillary liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS).

Table S2 Proteins differentially expressed in cultures of *Alternaria brassicicola* (controls) and cultures induced with brassinin at 0.10 mM. Proteins were identified using capillary liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS).

Table S3 Induction with 0.50 mM brassinin.

Table S4 Induction with 0.10 mM brassinin.

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