Differential protein expression in *Colletotrichum acutatum*: changes associated with reactive oxygen species and nitrogen starvation implicated in pathogenicity on strawberry

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SUMMARY

The cellular outcome of changes in nitrogen availability in the context of development and early stages of pathogenicity was studied by quantitative analysis of two-dimensional gel electrophoresis of *Colletotrichum acutatum* infecting strawberry. Significant alterations occurred in the abundance of proteins synthesized during appressorium formation under nitrogen-limiting conditions compared with a complete nutrient supply. Proteins that were up- or down-regulated were involved in energy metabolism, nitrogen and amino acid metabolism, protein synthesis and degradation, response to stress and reactive oxygen scavenging. Members belonging to the reactive oxygen species (ROS) scavenger machinery, superoxide dismutase and glutathione peroxidase, were up-regulated at the appressorium formation stage, as well as under nitrogen-limiting conditions relative to growth with a complete nutrient supply, whereas abundance of bifunctional catalase was up-regulated predominantly at the appressorium formation stage. Fungal ROS were detected within germinating conidia during host pre-penetration, penetration and colonization stages, accompanied by plant ROS, which were abundant in the apoplastic space. Application of exogenous antioxidants quenched ROS production and reduced the frequency of appressorium formation. Up-regulation in metabolic activity was detected during appressorium formation and nutrient deficiency compared with growth under complete nutrient supply. Enhanced levels of proteins related to the glyoxylate cycle and lipid metabolism (malate dehydrogenase, formate dehydrogenase and acetyl-CoA acetyltransferase) were observed at the appressorium formation stage,

in contrast to down-regulation of isocitrate dehydrogenase. The present study demonstrates that appressoria formation processes, occurring under nutritional deprivation, are accompanied by metabolic shifts, and that ROS production is an early fungal response that may modulate initial stages of pathogen development.

INTRODUCTION

Colletotrichum acutatum Simmonds, with its broad geographical host range, is the primary causal agent of strawberry anthracnose, causing petiole, stolon, crown fruit and root infections (Freeman and Katan, 1997; Kenneth *et al.*, 2002). Under nutritional limitation, conidia germinate on the plant surface and develop an adjacent appressorium, breach host tissue via an emerging penetration peg, and enter the cuticle, cell wall and cell, where an infection vesicle forms. Penetrating mycelia originating from the infection vesicle grow and acquire nutrients from the host (Leandro *et al.*, 2001; O'Connell *et al.*, 2000). Although *C. acutatum* is a generalist invader, the fungus possesses a very brief (less than 12 h) symptomless biotrophic phase before entering the extended necrotrophic phase, which involves major nutritional changes (Horowitz *et al.*, 2002; Kenneth *et al.*, 2002). The switch in fungal lifestyle is attributed to lack of nutrients, one of the signals controlling the expression of genes involved in pathogenicity. Recent studies on *C. acutatum* infecting strawberry have indicated that a defect in the *nir1*-like transcription factor involved in nitrogen metabolism confers morphological abnormalities and lack of pathogenicity (Horowitz *et al.*, 2006). This study also determined that the effect of nitrogen starvation on virulence is stage-specific and that nitrogen availability is a dominant factor in directing fungal morphogenesis. Nitrogen utilization in fungi is a tightly regulated process that confers the ability to take up nitrogen from a variety of sources when preferred substrates are lacking,

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while efficient regulation of this process most likely allows fungi to colonize the infected host plant (Pellier *et al.*, 2003). For example, in *Colletotrichum lindemuthianum*, targeted gene disruption of CLNR1, the AREA-NIT2-like global nitrogen regulator, results in reduced pathogenicity (Pellier *et al.*, 2003). Moreover, nitrogen starvation induces fungal gene expression during plant infection in a variety of pathogens (Divon *et al.*, 2005; Divon and Fluhr, 2007; Donofrio *et al.*, 2006; Snoeijsers *et al.*, 2000). Results obtained with *Fusarium oxysporum* indicate that the global nitrogen regulator FNR1 regulates fungal nutrition genes and fitness during pathogenesis on tomato (Divon *et al.*, 2006).

The ability to adapt to changes in nutrient availability is an essential attribute of many successful pathogens. Protein analysis has been successfully used for the study of various developmental trends in filamentous fungi of economic interest, such as *Aspergillus oryzae* (Nandakumar *et al.*, 2002), *Trichoderma reesi* (Lim *et al.*, 2001), *Erysiphe pisi* (Curto *et al.*, 2006) and *Botrytis cinerea* (Fernandez-Acero *et al.*, 2006). Similar studies in *Magnaporthe grisea* have provided information on proteins associated with the development and generation of appressoria (Kim *et al.*, 2004). Even though several examples link pathogenicity to nutrient regulation, its contribution to the virulence of *Colletotrichum* spp. remains largely unexplored.

In the present study, the effect of nitrogen availability on development and early stages of pathogenicity was further studied through two-dimensional gel electrophoresis (2-DE) of *C. acutatum* infecting strawberry. Quantitative analysis of proteins synthesized during appressorium formation compared with growth under nitrogen-limiting conditions or in the presence of a complete nutrient supply revealed significant alterations in the abundance of different proteins. The functional implications of the identified proteins, with special emphasis on the essential role of reactive oxygen species (ROS) as an early response to nitrogen deficiency, were studied. A combination of pharmacological and microscopic approaches were taken to substantiate the possibility that the accumulation of ROS is part of the signalling mechanism involved in governing the early stages of pathogen development in the presence of the host.

RESULTS

2-DE, spot analysis and identification of differentially expressed proteins

Nine 2-DE gels, three gels per treatment covering the pH range 4–7, were resolved. Representative gels are presented (Fig. 1). Examination of gels by means of Delta2D and Z3 software followed by visual confirmation revealed 36 spots that showed a significant decrease or increase in staining intensity. Spot differences were only considered if a relative fold-change of > 2 or < 0.5 was measured among the treatments and was consistently mani-

fested in all replicates. Relative quantification was achieved by computer-based comparison of gel images, which matches and compares protein-spot intensities between gels. The sum of each spot represents the relative quantity of corresponding protein, relative to other spots on the gel. The average relative quantity of each spot and the fold-change, calculated as an expression ratio, are presented in Table 1.

As indicated, analyses of spots were focused on those whose relative quantity ratio differed between the treatments by at least two-fold. All of the 36 differentially regulated proteins were analysed by LC-MS/MS and the most accurate database match for each protein is listed in Table 2. Good agreement was observed between the theoretical molecular mass of the identified proteins and their location in the 2-DE gels (Fig. 1, Table 2). Most of the assemblies were derived from proteins of other fungi such as *Aspergillus* spp., *Neurospora crassa*, *Magnaporthe oryzae*, *Colletotrichum* spp. and *Gibberella zeae*. These results suggest that there are many peptide sequences in common between proteins in *C. acutatum* and those in other ascomycetous fungi. In general, variably expressed proteins belonged to the following main functional categories: (1) ROS scavenger machinery, (2) carbohydrate and lipid metabolism, (3) nitrogen metabolism and (4) protein synthesis and degradation.

In the case of the appressorial protein maps (Fig. 1), 18 differentially expressed protein spots were induced during the appressorium formation stage (App) compared with growth under nitrogen-limiting (MM) and complete nutrient (Reg) conditions, and 11 spots were common for mycelial growth under nitrogen-limiting conditions and during the appressorium formation stage, compared with growth under complete nutrient supply (Tables 1 and 2). Their functional significance in relation to nitrogen-starvation perception, development and pathogenicity is discussed below.

Abundance of oxidative stress-related factor

Enzymes associated with the ROS scavenger machinery, Cu/ZnSOD and glutathione peroxidase (GPX) (spots 4, 14, Fig. 1A,B, respectively), were up-regulated during the appressorium-formation (App) stage (5.9-fold and 2.3-fold change, respectively), compared with growth with a complete nutrient (Reg) supply, but were not significantly different from the corresponding proteins synthesized under nitrogen-limiting (MM) conditions (Table 1). The bifunctional catalase peroxidase (CAT2) (spot 60, Fig. 1B) was significantly up-regulated during appressorium formation compared with mycelia grown under nitrogen limitation or a complete nutrient supply (5.2- and 2-fold change, respectively).

QRT-PCR was used to analyse transcriptional levels of the corresponding genes. During nitrogen limitation and the appressorium formation stage, ten-fold higher expression was detected for *sod1* compared with that in dormant conidia and growth with

Fig. 1 Representative 2-DE gels of *Colletotrichum acutatum* from: (A) MM, mycelia grown in minimal medium; (B) App, appressorium formation stage; (C) Reg, mycelia grown in complete nutrient supply. Proteins (500 µg) were resolved in first-dimension strips, pH 4–7 linear gradient, and second-dimension gels (12% SDS-PAGE). Proteins were detected using colloidal CBB G-250 staining. Molecular mass (Mr) in kDa is denoted on the left, while the pI (IEF) is given at the top of the figure. Protein spots marked with arrows, which were significantly differentially expressed between treatments, were further characterized and identified (see Table 1).

Table 1 Detection and quantification of differentially expressed protein spots in *Colletotrichum acutatum* under various conditions*

Spots marked in images	Spot no.	Spot property†			Spots marked in images	Spot no.	Spot property		
		App relative quantity	MM relative quantity	Expression ratio‡			App relative quantity	Reg relative quantity	Expression ratio
MM	1	0.206	1.984	0.104	MM	1	0.206	0.901	0.229
	2	nd§	0.81	—		2	nd	nd	—
	4	1.068	1.253	0.852		4	1.068	0.179	5.966
	5	0.008	0.358	0.022		5	0.008	0.172	0.047
	20	0.12	0.279	0.430		20	0.12	0.315	0.381
	22	0.055	0.11	0.500		22	0.125	0.08	1.5
	33	0.228	0.308	0.740		33	0.228	0.491	0.464
	34	0.004	0.175	0.023		34	0.004	0.009	0.444
	37	0.334	0.8	0.418		37	0.334	0.78	0.428
	39	nd§	0.037	—		39	nd	0.627	—
	40	0.812	1.63	0.498		40	0.812	0.58	1.400
	41	0.136	0.872	0.156		41	0.136	0.653	0.208
	42	0.387	0.82	0.472		42	0.387	0.92	0.421
	57	0.101	0.369	0.274		57	0.101	0.53	0.191
	58	0.02	0.018	1.111		58	0.129	0.16	0.806
App	7	1.547	0.245	6.314	App	7	1.547	0.729	2.122
	14	0.706	0.437	1.616		14	0.706	0.31	2.277
	15	0.312	0.015	20.800		15	0.312	0.007	44.571
	16	0.383	0.42	0.912		16	0.383	0.12	3.192
	26	0.421	0.25	1.684		26	0.421	0.202	2.084
	28	1.426	0.124	11.500		28	1.426	0.054	26.407
	32	0.368	0.09	4.089		32	0.368	0.23	1.600
	36	0.816	0.25	3.264		36	0.816	0.174	4.690
	43	0.343	0.122	2.811		43	0.343	0.18	1.906
	44	0.905	0.377	2.401		44	0.905	0.202	4.480
	46	0.271	0.008	33.875		46	0.271	0.09	3.011
	48	0.163	0.075	2.173		48	0.163	0.062	2.629
	49	0.499	0.436	1.144		49	0.499	0.489	1.020
	51	0.407	0.15	2.713		51	0.407	0.023	17.696
	52	0.906	0.636	1.425		52	0.906	0.203	4.463
	53	0.778	0.476	1.634		53	0.778	0.372	2.091
Reg	55	0.625	0.041	15.244	Reg	55	0.625	0.068	9.191
	56	0.462	0.445	1.038		56	0.462	0.007	66.000
	60	0.617	0.119	5.185		60	0.617	0.298	2.070
	8	nd	0.988	—		8	nd	1.9	—
	38	0.053	0.106	0.500		38	0.192	0.422	0.455

*Comparison of the relative quantities of *C. acutatum* protein spots among the treatments, generated from Fig. 1: appressorium formation stage (App) compared with w.t. grown in minimal medium (MM) and w.t. grown under complete nutrient supply (Reg). Proteins were analysed by the Delta2D software (version 3.2) (Decodon, Greifswald, Germany). Three images for each treatment were grouped to calculate the average relative quantity of each individual protein spot.

†Relative quantity of each spot was quantified by setting total spot quantity on a gel to 100%.

‡The numerical expression ratio is the mean of the relative quantity of the 'sample spot' divided by the mean of the relative quantity of the 'master spot', calculated for each gel and spot. The numerical expression ratio was calculated by determining local background regions of 17; average spot size is 5, weak spot sensitivity is 20% and noise cut-off is 40%. Proteins were considered differentially expressed when a relative fold-change of > 2 or < 0.5 was measured.

§nd, not detectable.

a complete nutrient supply (Fig. 2A). SOD activity was assessed by gel-activity assays (Fig. 2B). The two detected putative SOD enzymes corresponded to MnSOD (upper band) and Cu/ZnSOD (lower band), based on a previous report (Dolashka-Angelova

et al., 1999). Cu/ZnSOD activity was markedly enhanced by nitrogen starvation and at the appressorium formation stage, compared with activity observed in the presence of complete nutrient supply and in dormant conidia (Fig. 2B). Transcript levels of *cat2*

Table 2 Proteins identified by LC-MS/MS from spots that differed in their expression ratio among growth of wild type (w.t.) of *Colletotrichum acutatum* under nitrogen-limiting conditions (mm), w.t. growth under complete nutrient supply (reg) and during the appressorium formation stage (app). Proteins were classified according to functional categories

Function*	Spot†	GenBank accession no.‡	Best database match§	Mol. mass;pI/¶experimental/theoretical	Coverage (%)††	Total score‡‡	App Reg MM regulation§§
ROS scavenging and stress response	4	AAD19338	Cu-Zn superoxide dismutase (<i>Glomerella cingulata</i>)	20.3;6.3/16;5.9	29(8)	90	+ – +
	14	EAA74714	Hypothetical protein, similar to glutathione peroxidase (<i>Gibberella zeae</i>)	18.8;6.5/19.1;6.3	15(5)	93.3	+ – +
	15	EAA57651	Hypothetical protein, CD: pfam00012 Hsp70 protein (<i>Aspergillus nidulans</i>)	26.3;5.8/72.3;5.7	8(4)	90	+ – –
	33	EAA77628	Hypothetical protein, similar to putative stress response protein (<i>Gibberella zeae</i>)	20.6;4.7/28.4;4.33	9(2)	82	– + +
	40	XP366662	Hypothetical protein, similar to cell death-related protein (<i>Magnaporthe grisea</i>)	15.9;5.1/15;5.3	8(1)	93	– – +
	60	AP007175.1	Hypothetical protein, similar to bifunctional catalase peroxidase (Cat2) (<i>Aspergillus oryzae</i>)	80;6.2/82;5.8	4(4)	91	+ – –
Main carbohydrate, glyoxylate and lipid metabolism	1	EAA59740	Hypothetical protein, CD: Cytochrome c oxidase subunit pfam02284 (<i>Aspergillus nidulans</i>)	14.8;4.8/17.8.5;5.6	10(2)	93	– + +
	8	CAF31997	Putative isocitrate dehydrogenase (<i>Aspergillus fumigatus</i>)	40.4;4.1/41.7;8.7	10(2)	97	ndi + –
	20	CAA82232	Enolase 2 (<i>Ricinus communis</i>)	25.9;6.14/47.9;5.6	13(3)	90.6	– + +
	36	XP680863	Hypothetical protein, glutathione-dependent formaldehyde activating (<i>Aspergillus nidulans</i>)	14;6.7/14.7;6.03	17(5)	92	+ – –
	37	P54117	Glyceraldehyde 3-phosphate dehydrogenase (<i>Glomerella lindemuthiana</i>)	24.18;6.6/36.3;6.54	12(4)	85	– + +
	44	EAA69530	Hypothetical protein, CD: pfam00171 aldehyde dehydrogenase family (<i>Gibberella zeae</i>)	53.6;6.18/53.4;5.4	9(4)	92	+ – –
	52	EAA75069	Hypothetical protein, CD: Formate dehydrogenase NAD-dependent (<i>Gibberella zeae</i>)	38.6;6.2/40;6.1	16(8)	94	+ – –

Table 2 *continued.*

Function*	Spot†	GenBank accession no.‡	Best database match§	Mol. mass; pI/¶experimental/theoretical	Coverage (%)††	Total score‡‡	App Reg MM regulation§§
Cellular, signal transduction and co-factor metabolism	53	EAA68236	Hypothetical protein, CD: Malate dehydrogenase (<i>Gibberella zeae</i>)	23.7;6.5/34;6.4	25(8)	93	+ --
	56	EAA73569	Hypothetical protein, CD: Acetyl-CoA acetyltransferase (<i>Gibberella zeae</i>)	49.6;6.6/43.6;7.5	6(3)	83	+ -- +
	58	EAA77220	Hypothetical protein, similar to succinate dehydrogenase (<i>Gibberella zeae</i>)	196.7;6/50.2;5.8	1(1)	92	---
	2	Q7RVL0	GTP-binding nuclear protein GSP1/Ran (<i>Neurospora crassa</i>)	14.8;5/24.2;6.4	21(5)	93	nd nd +
	7	XP457640	Hypothetical protein, similar to CipC protein of <i>Emericella nidulans</i> (<i>Debaryomyces hansenii</i>)	14;5.9/14.9;5.3	14(4)	86	+ --
	26	XP328263	Hypothetical protein, similar to protoporphyrinogen oxidase [Coenzyme metabolism] (<i>Neurospora crassa</i>)	60;6.17/57.9;5.8	8(3)	83	+ --
	28	S30118	Orotate phosphoribosyl transferase (<i>Colletotrichum graminicola</i>)	28.7;6.7/25.2;5.9	11(2)	89	+ --
	42	XP326472	Hypothetical protein, CD: Calmodulin-related Ca ²⁺ -binding protein (<i>Neurospora crassa</i>)	13.6;4.3/16.3;4.5	18(2)		- + +
	48	EAA74730	Hypothetical protein, similar to secretory pathway gdp dissociation inhibitor CD: pfam00996 (<i>Gibberella zeae</i>)	54.5;5.6/51.4;5.37	9(4)	94	+ --
	49	CAD60606	Hypothetical protein, CD: Thiosulfate sulfur transferase (<i>Podospira anserine</i>)	35.6;6/38.4;6.7	10(3)	91	+ -- +
	55	EAA76084	Hypothetical protein, CD: pfam00890 FAD binding domain, Cytochrome b5-like heme/steroid binding domain (<i>Gibberella zeae</i>)	70.2;6.6/67.9;6	16(13)	88	+ --
	57	XP323373	β -tubulin chain (<i>Neurospora crassa</i>)	38.5;5.2/49.4;4.84	8(3)	85	- + +
Nitrogen and amino acid metabolism. Protein synthesis, transcription and degradation	5	XP322910	Ketol-acid reductoisomerase precursor (<i>Neurospora crassa</i>)	29.2;6.5/44.6;8.5	6(2)	95	-- +

Table 2 *continued.*

Function*	Spot†	GenBank accession no.‡	Best database match§	Mol. mass; pI/¶experimental/theoretical	Coverage (%)††	Total score‡‡	App Reg MM regulation§§
	16	AAB00322	Glutamine synthetase (<i>Glomerella cingulata</i>)	39.9; 5.7/39.9; 5.7	23(11)	89	+ – +
	22	CAD70393	Conserved hypothetical protein, similar to 20S proteasome subunit alpha type 6 (<i>Neurospora crassa</i>)	29.5; 6.2/27.7; 5.6	13(4)	90	– – +
	32	BAE62886	Hypothetical protein, similar to 26S proteasome regulatory subunit mts4 (<i>Aspergillus oryzae</i>)	41.4; 6.7/100; 4.89	1(1)	89	+ + –
	34	AAF82115	Cobalamin-independent methionine synthase (<i>Aspergillus nidulans</i>)	14.9; 5.9/86.8; 6.36	3(3)	93	– – +
	39	EAA77131	EF2_NEUCR elongation factor 2 (EF-2) (<i>Gibberella zeae</i>)	41.5; 6.2/91.6; 6.45	6(4)	95	+ – +
	41	CAC28704	Hypothetical protein, similar to ubiquitin-conjugating enzyme ubcP3 (<i>Neurospora crassa</i>)	16.8; 5.2/18.5; 5	5(1)	93	– + +
	43	EAA68262	Hypothetical protein, similar to prolidase (<i>Gibberella zeae</i>)	21.4; 6.2/26.1; 5.8	6(2)	86	+ – –
	46	CAF05873	Glycine hydroxymethyl transferase cytosolic (<i>Neurospora crassa</i>)	23.7; 6.5/52.9; 6.93	6(2)	82	+ – –
	51	XP_682383	Hypothetical protein, similar to threonine dehydrogenase and related Zn-dependent dehydrogenases (<i>Aspergillus nidulans</i>)	40.7; 6.2/42.5; 5.3	8(3)	89	+ – –
Unknown function	38	XP328441	Hypothetical protein (<i>Neurospora crassa</i>)	26.5; 6.3/36.2; 6.3	8(2)	90	nd – +

*Main functional categories.

†Assigned spot number as indicated in Fig. 1.

‡Accession numbers in the NCBI databases.

§Best database match according to NR-NCBI or specific databases; in the case of hypothetical proteins, putative functions were found by using the on-line conserved domains (CD) search in the NCBI database. The CD with the highest score was listed as the CD for the respective hypothetical protein. The corresponding organism from which the identified protein originates is denoted in parentheses.

¶Experimental and theoretical mass (kDa) and pI of identified protein. Experimental values were calculated with PD-Quest software and standard molecular-weight markers. Theoretical values were retrieved from the protein database.

††Percentage sequence coverage and number of peptides identified in parentheses.

‡‡Total peptide score: a peptide was considered to be of high quality if its Pep-Miner identification score was greater than 80 and had a sequest Xcore of 1.5 for single-charged peptides, 2.5 for double-charged peptides and 3 for triple-charged peptides.

§§Significantly up- or down-regulated (+/–, respectively, among treatments).

¶¶nd, not detectable.

Fig. 2 (A) Quantitative real-time PCR (QRT-PCR) analysis of expression of *sod1*, *cat2*, *hsp70* and a proteasome of *Colletotrichum acutatum* during the appressorium formation stage (APP), in dormant conidia (CONIDIA), growth under complete nutrient supply (REG) and nitrogen-limiting conditions (MM). Values were calculated as fold-change of expression of the corresponding gene among treatments relative to that at the appressorium formation stage. All samples were analysed in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template). Averaged C_T values, the cycle at which the amplification curve reached threshold fluorescence, were then normalized to the endogenous β -tubulin control gene. Fold-changes in target genes were determined by the $2^{-\Delta\Delta C_T}$ method, where $-\Delta\Delta C_T = (C_{T\text{Target}} - C_{T\beta\text{-tubulin}})_{\text{treatment}} - (C_{T\text{Target}} - C_{T\beta\text{-tubulin}})_{\text{control}}$. The appressorium-extracted sample was used as the control treatment. Mean comparisons of the $2^{-\Delta\Delta C_T}$ values among treatments were calculated using LSD, according to the Tukey–Kramer multiple comparison test (treatments with different letters are significant at $P < 0.05$). (B) Relative superoxide dismutase (SOD) activity assay. SOD activity was determined by staining the native polyacrylamide gel with nitro blue tetrazolium and riboflavin. Protein samples (20 μ g) were extracted from dormant conidia (CONIDIA), the appressorium formation stage (APP), growth under a complete nutrient supply (REG) and from mycelia of *C. acutatum* grown under nitrogen-limiting conditions (MM). The two apparent SODs, MnSOD and Cu/ZnSOD, are denoted by arrows.

increased during the appressorium formation stage in comparison with mycelia grown under the different nutritional conditions, albeit by only 2.5-fold. Expression of *cat2* in dormant conidia was four-fold higher than in mycelia grown under different nutritional conditions (Fig. 2A). Additional proteins associated with stress and defence were identified: spot 15, which designates a protein similar to Hsp70 from *Aspergillus nidulans*, was up-regulated at the appressorium formation stage, compared with growth under nitrogen limitation or under complete nutrient supply (21- and 44.5-fold change, respectively; Fig. 1B).

Expression of the corresponding *hsp70*, analysed by QRT-PCR, during the appressorial formation stage and in dormant conidia, was ten-fold higher than that measured in mycelial samples from cultures grown under different nutritional conditions (Fig. 2A). An additional hypothetical protein, similar to a stress-response protein from *G. zeae* (spot 33, Fig. 1A), and a protein similar to a cell death-related protein from *M. oryzae* (spot 40, Fig. 1A) were up-regulated under nitrogen-limiting conditions.

Fungal ROS production in pre-penetration and penetration structures during nitrogen deprivation and host invasion

The detection of increased levels of ROS scavengers during the appressorium formation stage and under nitrogen limitation prompted us to investigate the distribution of ROS products *in vitro* with different supplied nutrients, and *in vivo* during pathogenesis. Generation of intracellular ROS was investigated using H_2DCFDA as a probe to detect and quantify intracellularly produced H_2O_2 . ROS generation was observed within 3 h of conidial germination in water but was not detected in dormant conidia (Fig. 3A–C). ROS were observed in germinating conidia on a hard surface in the presence of water and accumulated in the subsequent appressorium structure (as indicated by the arrows in Fig. 3D–F), indicating changes in ROS synthesis during development. ROS was barely detectable in mycelia growing in the presence of a complete nutrient supply (Fig. 3G,H), as opposed to the enhanced levels that accumulated under nitrogen-limiting conditions (Fig. 3I,K). ROS intensity decreased when preferred nitrogen sources such as ammonia (Fig. 3I) were available, in contrast to nitrate (Fig. 3L), a less preferred source (arrows indicate an individual conidium with accumulated ROS). Likewise, there was a significant increase in ROS accumulation within 3 h, as measured by fluorescence values, in conidia germinating in the presence of water, as compared with germination under complete nutrient supply or dormant conidia. In addition, ROS decreased with the addition of preferred nitrogen sources such as ammonia, but increased with less preferred nitrogen sources such as nitrate (Fig. 4). Following strawberry inoculation, rapid accumulation of ROS was mainly observed beneath the appressorium structures and appeared to be highly localized in the host apoplast at the host–appressorium interface (Fig. 5A,B). Halo formation was frequently observed near the appressorial structures, possibly due to a decrease in host ROS accumulation (Fig. 5C). Generation and accumulation of ROS was observed within the appressorium structures, the emerging penetration peg and infection vesicles, 16 h post-inoculation (as shown by the arrows in Fig. 5E–G, respectively) (Fig. 5D–G). ROS was observed in the intercellular and intracellular penetration hyphae as indicated by arrows (Fig. 5H–J), and accompanied all host-colonization stages up to 4 days post-inoculation (Fig. 5K,L).

Fig. 3 Assessment of intracellular reactive oxygen species (ROS) production under different nutritional conditions and morphologies of *Colletotrichum acutatum*. *In vitro* ROS production in fungal cells was determined using the H_2DCFDA probe, and visualized with a laser-scanning confocal microscope. Rapid generation of ROS was detected within 3 h after germination (A,B), and 12 h after germination at the appressorium formation stage (C). ROS levels were barely detected in mycelia grown with a complete nutrient supply (D). ROS levels were lower in conidia germinating in the presence of ammonium as sole nitrogen source (E), while enhanced levels of ROS were detected in fungal hyphae grown under nitrogen-limiting conditions (F). Enhanced levels of ROS were also detected in germinating conidia in the presence of nitrate as sole nitrogen source (G). Micrographs A, D, G and J were analysed by fluorescence microscopy and corresponding images B, E, H and K are light micrographs as viewed under differential interference contrast (DIC) microscopy. Micrographs C, F, I and L were analysed through superposition of DIC and fluorescence microscopy. Scale bars = 16 μm .

Fig. 4 Quantitative relative fluorescence measurements of *Colletotrichum acutatum* conidia germinating under different nutritional conditions: M3S-rich medium, dormant conidia (dormant), minimal medium without nitrogen (mm), minimal medium with ammonium (mm+a), minimal medium with nitrate (mm+n), water without a nitrogen source (water), water with ammonium (water+a) and water with nitrate (water+n) as sole nitrogen sources. After incubation, conidia were immersed in 80 μ L of 10 μ M H₂DCFDA for 1 h. Relative fluorescence of the H₂DCFDA probe was measured within 10 h. Mean comparisons of fluorescence values were calculated using LSD, according to the Tukey–Kramer multiple comparison test at $P < 0.05$. Fluorescence values for treatments with different letters are significant.

Effect of antioxidant application on appressorium development during nutritional limitation

The role played by ROS in cell differentiation in *C. acutatum* under nitrogen-limiting conditions was tested using proline, *N*-acetyl-L-cysteine (NAC) and mannitol, which act as potent antioxidants and inhibitors of ROS production. Unlike proline, addition of NAC (Fig. 6A) and mannitol (Fig. 6B) quenched ROS levels, as determined by fluorescence, characteristic of non-enzymatic defence against oxidative stress. A significant decrease in ROS accumulation was correlated with a significant decrease in the percentage of appressorium formation with applications of threshold concentrations of 0.025 mM NAC or 250 mM mannitol, respectively (Fig. 6A,B).

Proteins associated with energy and lipid metabolism

Several of the differentially regulated proteins detected in the 2-DE gels appeared to be involved in energy metabolism. Isocitrate dehydrogenase (IDH), a typical tricarboxylic acid cycle protein, was markedly up-regulated (spot 8, Fig. 1D; Tables 1 and 2) under growth in the presence of a complete nutrient supply, although it was down-regulated under nitrogen-limiting conditions, and absent during the appressorium formation stage. Enzymes involved in the glyoxylate cycle (formate dehydrogenase and malate dehydrogenase) and in fatty acid metabolism (aldehyde dehydrogenase and acetyl-CoA acetyltransferase (protein spots 44, 52, 53 and 56, respectively) were up-regulated (4.5-, 4.46-, 2-, and 65.7-fold, respectively) during the appressorium formation stage, compared with growth with a complete nutrient supply (Table 1, Fig. 1B,D). Protein spot 2, corresponding to a GTP-binding nuclear protein GSP1/Ran belonging to the Rab GTPases, was up-regulated under nitrogen limitation but was not detected during the appressorium formation stage or under complete nutrient supply (Fig. 1A, Tables 1 and 2). In relation to increased catabolism, also worth noting was the identification of different key enzymes of the glycolysis/gluconeogenesis pathways, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (protein spot 37) and glutathione-dependent formaldehyde (protein spot 36), which were differentially expressed among treatments (Fig. 1). This response suggests a shift in *C. acutatum* metabolism required for compensation during increased stress.

Occurrence and distribution of lipid bodies during nutritional deprivation

To study further the potential role of lipid metabolism under nutrient deprivation and during appressorium function, the distribution of lipid bodies was examined under different growth conditions and developmental stages by Nile Red staining. Confocal analysis of germinating conidia in water showed movement of lipid-droplet reserves, mobilized from the conidium into the germ-tube apex and to the incipient appressorium, within 12 h (as indicated by the arrows in Fig. 7A,F). Lipid bodies appeared inside secondary conidia at the initial stages of production (arrows in Fig. 7. C,D). The appearance of lipid droplets was essentially varied for conidia germinated in the presence of a complete nutrient supply or in water. Under glucose or nitrogen deficiency, large lipid deposits were observed before being taken up into large vacuoles (Fig. 7G–I). Lipid bodies observed during growth in the presence of glucose and nitrate (Fig. 7J,K) or under complete nutrient supply (Fig. 7L,M) were disseminated along the hyphae and were generally smaller and homogeneous in size compared with growth under nutritional limitation (Fig. 7E–I). These observations suggest that fatty acid metabolism is increased under the nutritional deprivation that occurs during appressorium formation.

Proteins associated with nitrogen metabolism, protein synthesis and degradation

Up-regulation of proteins involved in nitrogen metabolism, such as glutamine synthetase (GS) (spot 16, Fig. 1B), and enzymes involved in the biosynthesis of different amino acids or protein synthesis such as glycine hydroxymethyltransferase (spot 46, Fig. 1B), ketol acid reductoisomerase (spot 5, Fig. 1A), cobalamin-

Fig. 5 Assessment of *in vivo* fungal reactive oxygen species (ROS) production during infection of strawberry by *Colletotrichum acutatum*. Cross-sections of infected tissues were prepared 12 and 72 h post-inoculation. Section samples were incubated with H_2DCFDA for 1 h and examined by confocal microscopy. Superposition of differential interference contrast (DIC) and fluorescence images revealed a rapid response of ROS accumulation beneath the appressorium depression, as indicates by the arrows (A,B), and directly adjacent to the host cell. Local accumulation of plant ROS response and halo formation was observed close to the appressorium formation (C). Penetration of an appressorium germ tube accompanied by fungal ROS production (D, light micrograph), with the right corresponding image (E) and F analysed through superposition of DIC and fluorescence microscopy. Formation of an infection vesicle and hyphae growing in the intercellular spaces of host cells accompanied by ROS production (G–J). Colonization, 72 h post-inoculation, of inner-layer cells was accompanied by fungal ROS production as analysed by DIC and fluorescence microscopy (K) and L the corresponding image analysed by fluorescent microscopy. Scale bars = 10 μm .

Fig. 6 Effect of *N*-acetyl-L-cysteine (NAC) and mannitol on appressorium formation and intracellular ROS production of *Colletotrichum acutatum*. Conidia were germinated in water in the presence of 0.005–1 mM NAC or 1–1000 mM mannitol for 12 h before measuring ROS (Flu) and percentage appressorium formation (App). Mean comparisons from three independent experiments of germination and appressorium formation were calculated using LSD, according to the Tukey–Kramer multiple comparison test at $P < 0.05$. Mean values with different letters (Flu, upper case; App, lower case) are significant.

independent methionine synthase (spot 34, Fig. 1A), threonine dehydrogenase (spot 51, Fig. 1B) and prolidase (spot 43, Fig. 1B), were differentially expressed among treatments (Fig. 1, Tables 1 and 2).

Proteins involved in degradation via the proteasome were detected in profiles from the appressorium-formation stage and nitrogen limitation. The 20S proteasome subunit alpha type 6 and the 26S proteasome regulatory subunit mts4 (spots 22 and 32, respectively) were up-regulated during nitrogen limitation. Expression analysis of the 20S proteasome subunit alpha type 6 revealed a relative increase in proteasome expression during appressorium formation compared with dormant conidia or mycelial growth under different conditions (Fig. 2A). Up-regulation of these proteins may demonstrate a general activation of the degradation apparatus, but may also indicate specific functions in appressorium formation.

DISCUSSION

The present study focuses on quantitative changes in differentially regulated proteins which may play a role under the nitrogen

deprivation that occurs during the development and pathogenicity of *Colletotrichum acutatum* in strawberry. The hypothesis that plant-pathogenic fungi experience nitrogen nutrient limitation during *in planta* growth is based on our previous data in *C. acutatum*, which showed that *nir1* is required for transcription of nitrate reductase (NR) necessary for nitrate assimilation, which is also involved in additional processes related to appressorium formation (Horowitz *et al.*, 2006). Results presented in the present study demonstrate that nutrition deprivation conditions, which occur during appressoria formation, increase the demand for energy and metabolite precursors and require precise regulation. Likewise, nitrogen deprivation and early pre-penetration stages are accompanied by an intricate balance between host and pathogen ROS accumulation. Linkage between nutritional regulation, development and ROS generation was proposed recently in *Colletotrichum trifolii*, whereby Cdc42 (a highly conserved small GTP-binding protein) together with Ras (a small monomeric GTP binding protein) are critical for proper growth and development, especially hyphal morphology, conidiation and appressorium differentiation (Chen *et al.*, 2006; Ha *et al.*, 2003). Expression of dominant active Cdc42 or Ras in *C. trifolii* induces

Fig. 7 Cellular distribution of lipid droplets under nutrient deficiency compared with growth with a complete nutrient supply in germinated conidia and mycelia of *Colletotrichum acutatum*. Images A, C, E, G, I, J and L were analysed by fluorescence microscopy while images B, D, F, H, K and M are superpositions of differential interference contrast and fluorescence images. Conidia were allowed to germinate for 12 h in water drops (A–F) or in minimal media (MM) in the presence of nitrate and absence of glucose (G,H), in MM in the presence of glucose and absence of nitrate (I), MM in the presence of glucose and nitrate (J,K), or complete nutrient supply (G) and were stained for lipid bodies using Nile Red. Numerous small lipid droplets were observed under growth with a complete nutrient supply (indicated by arrows), whereas under nitrogen- or glucose-limiting conditions, larger lipid droplets were observed coalescing into vacuolar structures (indicated by arrows). Scale bars = 10 μ m.

large amounts of ROS under nutrient deprivation conditions, which was quenched by proline (Chen *et al.*, 2006). Similar to the fungal response, in plants, ROS, notably hydrogen peroxide, mediate an early root response to nutrient deficiency, suggesting a link between ROS generation and nutrient deprivation (Shin *et al.*, 2004). These reports together with findings presented in this study suggest that ROS generation is involved in a nutrient signalling cascade that may regulate fungal development under nitrogen deficiency occurring during the early stages of infection.

Antioxidant enzymes as indicators of ROS during appressorium morphogenesis

ROS accumulation is balanced via a cellular antioxidant system, some major players of which are catalase, SOD and components of the ascorbate–glutathione cycle (Aguirre *et al.*, 2005; Chen and Dickman, 2005; Rodriguez and Redman, 2005). Up-regulation of SOD, GPX and CAT2 during the appressorium formation phase and differential regulation under nitrogen limitation prompted an examination of the spatial and temporal distribution of ROS *in vitro*, under different morphologies and growth conditions, and *in vivo* during strawberry colonization. ROS, in particular the superoxide anion (O_2^-), its conjugated acid the perhydroxyl radical (HO_2) and their dismutation product hydrogen peroxide (H_2O_2), have been regarded as inevitable harmful by-products of aerobic metabolism. However, there is increasing evidence supporting an alternative view, in which specific enzymes that produce ROS regulate different cellular functions including cell proliferation, cell differentiation, signal transduction and ion transport. Several reports in different eukaryotic systems have shown a correlation between developmental processes and the up-regulation of specific antioxidant enzymes, such as SODs, catalases, catalase peroxidases and peroxiredoxins (Aguirre *et al.*, 2005). For example, an MnSOD gene is induced during conidial development in *Colletotrichum graminicola* (Fang *et al.*, 2002), and one of the developmental pathways in the slime mould *Physarum polycephalum* is accompanied by strong induction of SOD activity (Aguirre *et al.*, 2005). SOD is essential for stationary-phase survival in *Saccharomyces cerevisiae* (Longo *et al.*, 1996), and in *Candida albicans* a cytoplasmic MnSOD is expressed upon initiation of the stationary phase (Lamarre *et al.*, 2001). In *Magnaporthe oryzae*, a catalase (catB) mutant phenotype displayed reduced sporulation, conidia and appressoria more prone to collapse under hyperosmotic stress. It is suggested that the large subunit catalase (CatB) in *M. oryzae* is involved in the formation or maintenance of a robust fungal cell wall (Skamnioti *et al.*, 2007). Induction of antioxidant enzymes in structures that are undergoing development and the association of these enzymes with fully differentiated structures suggest that ROS are produced initially and during appressorium differentiation in *C. acutatum*. Furthermore, application of NAC and mannitol quenched ROS

levels and decreased appressorium formation (Fig. 6). The ability of these antioxidants to inhibit appressorium formation is consistent with the idea that ROS are required for the initiation of developmental processes. Similar results were obtained with *M. oryzae*, where scavenging of the oxygen radicals by exogenous antioxidants such as ascorbic acid or MnTMPyp significantly delayed the development of appressoria and altered their morphology. Moreover, mutants disrupted in two superoxide-generating NADPH oxidase encoding genes, *Nox1* and *Nox2*, are incapable of causing disease owing to a lack of appressorium-mediated cuticle penetration (Egan *et al.*, 2007).

ROS perception and antioxidant responses in *C. acutatum*

A successful pathogen must be able to overcome or suppress the complex array of ROS-mediated host defences. Microbial suppression of ROS-mediated defences by secretion of ROS-scavenging enzymes such as SOD and catalase, which convert ROS into less reactive species, has been documented in plant and animal pathogens (Jennings *et al.*, 1998). As is well known, H_2O_2 generation plays a role in restricting fungal penetration and inhibiting fungal invasion, leading to the hypersensitive response and triggering rapid necrosis at infection sites, or activating defence-related genes (Apel and Hirt, 2004; Divon *et al.*, 2006; Garre *et al.*, 1998). Rapid accumulation of ROS by the host, which was observed beneath the appressorial structures, is apparently caused by local mechanical pressure generated by the developing appressoria. In *C. lindemuthianum*, it was shown that appressorium maturation, but not function, is sufficient to induce most plant defence responses (Veneault-Fourrey *et al.*, 2005). The presence of abundant ROS produced by the host to account for penetration failure has also been observed for *Colletotrichum coccodes* causing anthracnose disease in tomato. Furthermore, enzymatic removal of H_2O_2 resulted in increased penetration success (Mellersh *et al.*, 2002). In some of the observed penetration events, the plant ROS response was not observed, suggesting that *C. acutatum* evades recognition by the host, detoxifies the compounds (as indicated by halo formations near the appressoria) or is able to suppress other key defences capable of generating ROS. Up-regulation of fungal ROS scavengers during the appressorium formation stage could be an indication of the involvement of enzymatic ROS scavenging in protecting the fungus from the general stress generated by host defence mechanisms. This is in agreement with the requirement of Cu/ZnSOD for the protection of *Candida albicans* against oxidative stresses and full expression of its virulence (Hwang *et al.*, 2002). Various findings demonstrated that loss of catalase CATB function for *M. oryzae* resulted in compromised pathogen fitness but did not influence the accumulation or detoxification of the host-imposed oxidative burst; however, CATB appeared to play a part in strengthening the fungal wall

prior to host penetration (Skamnioti *et al.*, 2007). These results suggest alternative and additional roles of these enzymes in pathogenesis. Despite the evidence indicating that ROS are related to, and probably required for, cell differentiation in eukaryotic cells, the sources of these ROS and their contribution to development remain to be determined. Recent research has demonstrated a novel role for the *noxA* NADPH oxidase-encoding gene, the primary source of ROS, in regulating the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloë festucae*, and its grass host, *Lolium perenne* (Tanaka *et al.*, 2006). These results suggest that fungal ROS production is critical in maintaining this mutualistic fungal–plant interaction. Results presented here suggest that ROS and the ROS scavenger machinery play important roles in mechanisms related to regulation of the morphology and host–pathogen interaction of the *C. acutatum* pathosystem under nitrogen deprivation.

A metabolic shift is mediated by coordinated regulation of the glyoxylate cycle

Induction of the glyoxylate cycle indicates that the cell is utilizing lipid metabolism as its predominant source for ATP generation, involving β -oxidation of fatty acids and the production of acetyl-CoA (Wang *et al.*, 2003, 2005). Lipid-droplet deposits observed during nutrition deprivation indicate that they are utilized during appressorium development (Fig. 7). IDH, an important branch-point between catabolic and anabolic processes in the cell, was clearly up-regulated under growth with a complete nutrient supply (Fig. 1). Thus, the catabolic process occurring during appressorium formation in *C. acutatum* induced the synthesis of a series of proteins involved in the glyoxylate cycle (formate dehydrogenase and malate dehydrogenase), or in fatty acid metabolism (aldehyde dehydrogenase and acetyl-CoA acetyltransferase). Two key studies examining the role of the glyoxylate cycle in the fungal phytopathogens *M. oryzae* and *Leptosphaeria maculans* have demonstrated that mutants lacking ICL activity, a key enzyme of the pathway, are unable to germinate and cause disease (Idnurm *et al.*, 2002; Solomon *et al.*, 2004). Recent work in *C. lagenarium* suggests that peroxisomal metabolic pathways play functional roles in appressorial and subsequent host-invasion steps (Asakura *et al.*, 2006). Similarly, in the present study, it is suggested that *C. acutatum* uses lipid metabolism extensively during appressorium formation and under conditions of nitrogen deprivation (Fig. 7).

Enhancement of nitrogen and protein metabolism during early developmental events

Increased abundance of GS, which allows assimilation of nitrogen and biosynthesis of glutamine when preferential primary nitrogen sources are lacking, is consistent with previous findings in *C. acutatum* that nitrogen starvation prevails during appres-

sorium formation (Horowitz *et al.*, 2006). Similar enhanced expression of GS has been documented during pathogenesis of *Colletotrichum gloeosporioides* on *Stylosanthes guianensis* (Stephenson *et al.*, 1997). In *Gibberella fujikuroi* it was shown that GS has a significant impact on transcriptional control of primary and secondary metabolism (Teichert *et al.*, 2004), and thus its expression might contribute to the metabolic shift that occurs during appressorium formation and under nitrogen starvation. Consequently, an increase in differentially regulated proteins during appressorium formation and under nitrogen-limiting conditions appears to be represented by proteins involved in amino acid metabolism, and protein synthesis and degradation. Kim *et al.* (2004) have demonstrated up-regulation of the mts4 subunit of the proteasome 26S during appressoria formation previously in *M. oryzae*, with 20S proteasome subunits, which take part in the degradation of targeted proteins. Furthermore, Kim *et al.* (2004) suggested that the proteasome might be involved in mobilizing storage proteins during appressorium formation. Therefore, the function of the proteasome subunit in appressorium formation deserves further study.

In conclusion, this study reports on an initial approach for proteome analyses of *C. acutatum* infecting strawberry. Here we propose that ROS and nitrogen deficiency play an important role in the developmental programme occurring in germinating conidia under nutritional deprivation. Activation of the glyoxylate bypass, and generation and elimination of ROS may be fundamental in permitting pathogenicity to proceed before host invasion. To our knowledge, no comparable study on nutrient deprivation has demonstrated a link between ROS, nitrogen deficiency and development. These results should deepen our understanding of *Colletotrichum* biology, as well as the biology of other hemibiotrophs, in relation to nutritional adaptation during the early phase of infection, possibly providing new potential targets for disease prevention.

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

Colletotrichum acutatum wild type (w.t.) C.a 149 (IMI391664) (Horowitz *et al.*, 2006) was used throughout this study. The w.t. strain was maintained at 25 °C on modified Mathur's medium (M3S) (Freeman *et al.*, 1993) or on regeneration (Reg) medium [145.7 g/L mannitol (Sigma Aldrich, Steinheim, Germany), 4 g/L yeast extract (Difco Laboratories, Detroit, MI), 1 g/L soluble starch, 50 ml/L pea juice (Robinson and Sharon, 1999)]. Prior to seedling inoculation or fungal culturing, conidia were isolated by flooding 5- to 6-day-old cultures with distilled water, and adjusted to a final concentration of 10^5 conidia/mL with a haemocytometer (Brand, Wertheim, Germany). For induction of nitrogen-limiting conditions, the w.t. strain was cultured in flasks

containing M3S liquid medium and incubated at 25 °C with horizontal shaking at 150 r.p.m. for 2 days. Cultures were then filtered through Whatman no. 54 filter paper and subcultured in flasks with minimal medium (MM), 20 g/L D-glucose, 1 g/L KH_2PO_4 , 0.5 g/L MgSO_4 , 0.5 g/L KCl, 0.15 g/L $\text{CaCl}_2(2\text{H}_2\text{O})$, 3 mg/L $\text{FeSO}_4(7\text{H}_2\text{O})$, 3 mg/L $\text{ZnSO}_4(7\text{H}_2\text{O})$, 1.25 mg/L $\text{CuSO}_4(5\text{H}_2\text{O})$, 350 µg/L $\text{MnSO}_4(\text{H}_2\text{O})$, 250 µg/L $\text{Na}_2\text{MoO}_4(2\text{H}_2\text{O})$, 6.25×10^{-6} µg/L biotin and 1.25 mg/L thiamine for 6 h. For culturing in complete growth medium, mycelia were subcultured on Reg medium for 6 h. Mycelia cultured in MM and Reg media were then harvested by filtering through Whatman no. 54 filter paper, lyophilized, frozen in liquid nitrogen and stored at -80 °C, prior to RNA or protein extractions. For appressoria induction, conidia of *C. acutatum* (10^6 conidia/mL) were spread onto glass Petri dishes (13 cm diameter) containing 10 mL of water and were incubated for 12 h in the dark at 25 °C. Appressoria formation was confirmed by microscopy and the germinated conidia were harvested by scraping them off the Petri dishes using a rubber policeman. The appressoria were then harvested by collecting on 0.45-µm sterile nylon membrane filters (Milipore, Billerica, MA), lyophilized and subjected to RNA or protein extraction.

***In vitro* and *in vivo* intracellular ROS and lipid-droplet detection**

Intracellular levels of ROS in *C. acutatum* were monitored with the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA) (Molecular Probes, Leiden, The Netherlands), an established compound used to detect and quantify intracellularly produced H_2O_2 . H_2DCFDA (10 µM) in 50 mM MES buffer pH 6.2 (Duchefa Biochemie, Haarlem, The Netherlands) was used to detect ROS production. For measurement of ROS upon nutritional induction, 80 µL conidia (10^5 conidia/mL) were resuspended in Reg medium or in water containing 5 mM of one of the various nitrogen-containing compounds tested (glutamine, urea, nitrate, nitrite or ammonium sulphate), and allowed to germinate in Microtest plates (Sarstedt, Inc., Newton, NC) for various times within a 16-h period, before staining for 1 h with the H_2DCFDA probe. Fluorescence levels were then measured by fluorometry (Microplate Fluorescence Reader FL600; BIO-TEK, Winooski, VT). Fluorescence levels were corrected by subtracting the fluorescence background of each medium used and dividing by ROS levels measured with the water treatment to obtain relative values. Following ROS production *in planta*, slices were prepared from inoculated strawberry stolons and leaves 12–72 h post-inoculation (p.i.) and subjected to the fluorescent dye for 1 h before confocal microscopic observation. Lipid droplets were visualized in germinating conidia and appressoria by staining with a Nile Red solution (Sigma Aldrich) as previously described (Thines *et al.*, 2000). Cytological analysis was performed with freshly harvested conidia incubated under different nutritional conditions. Suitable

material was mounted directly in 5 µg/mL Nile Red in glycerol solution. This solution was prepared from an initial solution of 1 mg/mL Nile Red in acetone. Within a few seconds, lipid droplets began to fluoresce.

Antioxidant pharmacological treatments

For all pharmacological treatments, conidia were resuspended with antioxidant solutions at various concentrations: *N*-acetyl-L-cysteine (0.005–1 mM), L-proline (0.5–4 mM) and mannitol (1–1000 mM) (Sigma Aldrich), and allowed to germinate for 3–12 h in Microtest plates for fluorescent measurements, or on Glasstic (HYCOR Bio-medical Inc., Garden Grove, CA) slides for determining percentage appressorium formation. Induction of appressorium formation and germination upon antioxidant treatments was determined by placing 20 µL of a 5×10^5 conidia/mL suspension of w.t. isolate into three of the Glasstic slides. Conidial germination and appressorial development were observed in three microscopic fields of each of the three cells per slide. Three germination and appressorium formation phenotypes were assessed: conidial germination and immediate appressorium formation, germ-tube formation ($> 2 \times$ the conidial length) and subsequent appressorium formation, and conidial germination without appressorium formation. The described phenotypes were calculated for each strain and treatment, based on respective percentages of the germinating conidia. The mean and standard deviation were calculated for three independent experiments. Mean comparisons of germination and appressorium formation were calculated using LSD, according to the Tukey–Kramer multiple comparison test at $P < 0.05$.

Protein extraction

Protein extraction from *C. acutatum* mycelia and appressoria was carried out according to Hurkman and Tanaka (1986), with minor modifications. Dry mycelia, conidia or appressorium-forming structures (0.25 g) were ground and homogenized to a very fine powder in a chilled mortar and pestle with liquid nitrogen in 5 mL extraction buffer (0.1 M Tris, pH 8.0, 5% w/v sucrose, 2% w/v SDS, 50 mM DTT, 2 mM PMSF) and 100 µL of Complete Inhibitor [one tablet of complete protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in 1 mL ddH₂O]. The homogenates were kept on ice for 10 min and centrifuged at 4000 *g* for 30 min at 4 °C. The supernatant fraction was collected and transferred to a new tube and 5 mL of water-saturated phenol solution was added. The mixture was kept on ice with shaking for 10 min. The phenol fraction was collected and transferred to a new tube containing 5 mL of extraction buffer. The tube was kept on ice, shaken for 10 min, re-centrifuged at 4000 *g* for 30 min at 4 °C, and the phenol fraction was again transferred to a new tube. The proteins were precipitated overnight with 25 mL of 0.1 M ammonium acetate in cold methanol at -20 °C, followed by centrifugation at

4000 g for 30 min at 4 °C. The pellet was washed three times with 15 mL of 0.1 M ammonium acetate in cold methanol, and again with 15 mL of cold acetone. The air-dried pellet was dissolved in 600 µL of rehydration solution containing 9 M urea, 3% (w/v) CHAPS, 0.5% (w/v) Triton X-100, 2% (v/v) IPG (immobilized Ph gradient) buffer, 0.3% (w/v) DTT and 0.002% (w/v) bromophenol blue. The protein content was measured with the bicinchoninic acid protein assay kit (Sigma, St Louis, MO) according to the manufacturer's instructions.

2-DE, image acquisition and mass-spectrometry analysis of proteins

2-DE was performed with immobilized pH gradients according to the manufacturer's recommendations (Amersham Pharmacia Biotech, Amersham, UK), with minor modifications. For analytical and preparative gels, 13-cm IPG strips (pH 4–7) (Amersham Pharmacia Biotech) were rehydrated overnight with 250 µL of rehydration solution containing 50 µg protein at room temperature. The isoelectric focusing step was conducted at 18 °C with a Multiphor II apparatus (Amersham Biosciences) under the following conditions: 300 V for 15 min, 500 V for 15 min, 1000 V for 15 min, 1500 V for 15 min, 2000 V for 15 min, 2500 V for 15 min, 3000 V for 15 min and 3500 V for 4 h. The focused strips were equilibrated twice for 15 min in 10 mL of equilibration solution; the first equilibration solution contained 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, 0.002% bromophenol blue and 2 mM tributylphosphine (TBP); the second equilibration solution was the same except that TBP was replaced with 2.5% (w/v) iodoacetamide. The second dimension was SDS-PAGE in a vertical slab of 12.5% acrylamide with an SE 600 Series Vertical Slab Gel Unit (Hoeffer Scientific Instruments, San Francisco, CA). The protein spots were visualized by staining with colloidal Coomassie Blue G-250. 2-DE was conducted three times with each protein sample, while accurate and reproducible protein patterns were produced among technical replicates from independent extractions. Image analysis software Delta2D, version 3.2 (Decodon, Greifswald, Germany) as well as Z3 software (Compugen Inc., Nes Ziona, Israel) were used for gel-to-gel matching and spot quantification. The data were obtained from three independent experiments, and gels with best resolution were digitized and compared using the Delta2D software. To ascertain quantitative changes in proteomic maps of each treatment, comparisons of the absolute quantities of each spot among the treatments were generated by the Delta2D software (version 3.2). Relative quantity of the computed spot was calculated by setting total spot quantity on a gel to 100%, excluding the background. Three images for each treatment were grouped to calculate the average quantities of all individual spots. The numerical expression ratio that determines the fold-change is the mean of the relative quantity of the 'sample spot' divided by the mean relative quantity of

the 'master spot', calculated for each spot. These values were used to designate the significantly differentially expressed spots. We focused our analyses of spots on those whose relative quantity ratio differed between the treatments by at least two-fold, while all other proteins were classified as 'not regulated'. The analysis was re-evaluated by visual inspection, focusing on those spots that were most dramatically altered between treatments, and consistent in all replicates. Spots showing consistent differential expression patterns between treatments were excised from the gels, digested and subjected to LC-MS/MS analysis to determine protein identity. For in-gel proteolysis, proteins were reduced with 10 mM DTT (60 °C for 30 min) and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate (room temperature for 30 min). The gel pieces were dehydrated with acetonitrile and rehydrated with 10% acetonitrile in 10 mM ammonium bicarbonate containing trypsin (modified trypsin; Promega, Madison, WI) at a 1 : 100 enzyme-to-substrate ratio. The gel pieces were incubated overnight at 37 °C and the resulting peptides were recovered and analysed. Electrospray-ion-trap analysis of proteins was performed at the Smoler Proteomics Center (Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel). The tryptic peptides were resolved by reversed-phase chromatography on 0.1 × 200-mm fused silica capillaries (J&W, 100 micrometer ID) packed with Everest reversed-phase material (Grace Vydac, Hesperia, CA). The peptides were eluted with linear 50-min gradients of 5–95% acetonitrile with 0.1% formic acid in water at flow rates of 0.4 µL/min.

Mass spectrometry (MS) was performed by an ion-trap mass spectrometer (LCQ-DecaXP, Finnigan, San Jose, CA) in positive mode, using repetitive full MS scans followed by collision-induced dissociation (CID) of the three most dominant ions, selected from the first MS scan. The MS data were clustered and analysed using the Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose, CA) or Pep-Miner (Beer *et al.*, 2004), searching the NR-NCBI or specific databases. In addition, a trained operator assessed the accuracy of identification of individual peptides visually. A peptide was considered to be of high quality if its Pep-Miner identification score was greater than 80 and it had a Sequest Xcore of 1.5 for single-charged peptides, 2.5 for double-charged peptides and 3.0 for triple-charged peptides. To determine the possible functions and classifications of hypothetical proteins, the sequence information was used to search for conserved domains (CDs) in an on-line CD search in the NCBI database. The CD with the highest score was listed as the CD for the respective hypothetical protein.

Detection of SOD activity

Negative staining (Hwang *et al.*, 2002), detected the activities of two types of SOD (Cu/ZnSOD and MnSOD) on a non-denaturing polyacrylamide gel. The two bands of SOD activity were identified

Gene Name	Product	Primer sequence (5'–3')
Sod.dg.F	Cu/Zn superoxide dismutase	CTTTGAGCAGGAGTCCGAGTC
Sod.dg.R	Cu/Zn superoxide dismutase	ACCCCTTGCCRAGRTCGTCRGTRCC
Hsp.dg.F	Heat-shock protein 70	AAGATGAAGGAGACCGCYGAG
Hsp.dg.R	Heat-shock protein 70	ACAGCCTCATCGGGGTTGACGGAC
Proteasome.dg.F	Proteasome 26S subunit	CAAGTTGARTAYGCYTTCAAGG
Proteasome.dg.R	Proteasome 26S subunit	GTGTARACYTGGCTGATGTTKGC
Catalase.dg.F	Bifunctional catalase	GCHTGGTTCAAGCTC
Catalase.dg.R	Bifunctional catalase	ACRAAGAAGTCGTTSGTCA
SOD.qrt.F	Cu/Zn superoxide dismutase	TTGTGGGGATTGAAGTGAGG
SOD.qrt.R	Cu/Zn superoxide dismutase	CCACCATTACCTGGGACATC
HSP.qrt.F	Heat-shock protein 70	AAGTCCTCACCACCAAGTG
HSP.qrt.R	Heat-shock protein 70	AGAAGGAGGAGGACCGTGTT
Proteasome.qrt.F	Proteasome 26S subunit	CAAGTTGARTAYGCYTTCAAGG
Proteasome.qrt.R	Proteasome 26S subunit	GTGTARACYTGGCTGATGTTKGC
Catalase.qrt.F	Bifunctional catalase	GTCGCCTGCTCTCAAGAAGG
Catalase.qrt.R	Bifunctional catalase	CCCAGGCAGTAGAGATGAGCTT
β -tubulin.qrt.F	Tubulin β -chain	TACCGACAAAGGTGGAGGAC
β -tubulin.qrt.R	Tubulin β -chain	AGGATGTCGAGGACCAGATG

The suffix dg refers to degenerate primers while qrt refers to primers used in quantitative real-time (QRT)-PCR experiments (F indicates forward primer; R indicates reverse primer).

Table 3 Primers used throughout this study.

according to Dolashka-Angelova *et al.* (1999). The gel was incubated with gentle shaking in 50 mM phosphate buffer (pH 7.8) for 10 min, in nitro-blue-tetrazolium solution (1 mg/mL) for 10 min and then in 50 mM phosphate buffer (pH 7.8) containing 0.01 mg/mL riboflavin (Sigma Aldrich) and 3.25 mg/mL N,N',N',N'-tetramethylethylenediamine (TEMED) for 10 min at room temperature. Areas of SOD activity remained clear when the gel was exposed to the light. The gel was scanned immediately after the photochemical reaction and the data were quantified using the NIH Image program (Macintosh, North Mathews, Urbana, IL).

Isolation and analysis of nucleic acids

Plasmid DNA, propagated in *Escherichia coli* strain DH5 α , was isolated using the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). Total RNA from *C. acutatum* mycelia, conidia and the appressorium formation stage was extracted using Tri-reagent according to the manufacturer's instructions (Sigma-Aldrich).

Gene expression analysis by quantitative real-time (QRT) PCR

Regions of the genes encoding *C. acutatum* SOD (*sod1*), bifunctional catalase (*cat2*), heat-shock protein (*hsp70*) and proteasome subunit alpha type 6 were cloned by PCR amplification on cDNA templates with degenerate primers based on peptide sequences. Mycelia grown under nitrogen-limiting conditions, a complete nutrient supply and at the appressorium formation

stage were separated from the medium by filtration, and total RNA was isolated using Tri-reagent. Reverse transcription was carried out on 1 μ g total RNA treated with RQ1 DNase (Promega) using Reverse-iTTM RTase Blend (ABgene, Epsom, UK) with an anchored oligo-dT primer. The resultant cDNA was subjected to PCR amplification in 50- μ L reactions with the following components: 4 μ L cDNA, 5 U/ μ L *Taq* DNA polymerase, 1 $\times$ *Taq* polymerase buffer, 0.2 mM dNTP and the upstream and downstream primers listed in Table 3 to final concentrations of 0.2 μ M. Samples were tested for the presence of genomic DNA contamination by using extracted treated RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. RT-PCR products were resolved in 0.8% agarose gels and cloned. For QRT-PCR, cDNA samples were diluted 1 : 10 to a final template concentration with primers (330 nM) which were designed based on the sequenced cDNA clones listed in Table 3. Real-time detection was performed with Absolute SYBR green ROX mix (ABgene) in a Rotor-Gene 3000 machine (Corbett Research, Sydney, Australia) and results were analysed with the Rotor-Gene 6 software. The β -tubulin gene served as an endogenous control while the appressorium-extracted sample was used as the calibrator (Yarden and Katan, 1993). A mixture of all cDNAs was used as a template for calibration curves designed for each pair of primers, for all the treatments. Relative quantification was calculated based on $\Delta\Delta C_T$ values. The ΔC_T value was determined by subtracting the C_T results for the target gene from those of the endogenous control gene and then normalizing, as suggested by Rotor Gene ($\Delta\Delta C_T$). The final relative quantification value is

$2^{-\Delta\Delta C_T}$, which represents the level of expression of the gene in relation to the appressorium-stage treatment (relative quantification). Each experiment was conducted four times with similar results, and results from one representative experiment are presented. Mean values of $2^{-\Delta\Delta C_T} \pm$ SD of the same cDNA measurements of each treatment were used for mean comparisons by LSD, according to the Tukey–Kramer multiple comparison test at $P < 0.05$.

Infection assays, light and confocal microscopy

For infection assays, stolons and leaves of 2-week-old strawberry daughter plants cv. Malach, an anthracnose-susceptible strawberry cultivar, were infected with a suspension of *C. acutatum* conidia at a concentration of 10^5 conidia/mL. Individual 10- μ L drops of inoculum suspension were placed on strawberry stolons and leaves, incubated for 12–72 h at 25 °C in a moist chamber, and samples were removed at 12-, 24-, 48- and 72-h intervals for observation. Tissue slices were sampled from beneath the site at which inoculum droplets were applied and mounted directly in H₂DCFDA probe for microscopic examination of ROS. Alternatively, *in vitro* germinating conidia exposed to different nutritional treatments were examined for ROS production or lipid-body detection by microscopic observation. All microscopic observations and image acquisitions were performed with an Olympus IX-81 FluoView FV500 confocal microscope (Olympus Optical Co., Tokyo, Japan) equipped with a 488-nm argon-ion laser. Transmitted light images were obtained using Nomarski differential interference contrast microscopy. For ROS detection, samples were excited by 488-nm light and the emission was collected through a BA 505–525 filter. A BA 660 IF emission filter was used to detect autofluorescence. For lipid-body detection, samples were excited by a 543-nm HeNe laser and image acquisition was accomplished using an E600LP (long pass) emission filter.

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