

# A defect in *nir1*, a *nirA*-like transcription factor, confers morphological abnormalities and loss of pathogenicity in *Colletotrichum acutatum*

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## SUMMARY

A non-pathogenic mutant of *Colletotrichum acutatum*, designated Ca5, exhibited epiphytic hyphal growth and did not cause lesions on strawberry plants but grew necrotrophically when inoculated directly onto wounded stolons. In the absence of an external nitrogen source, the mutant exhibited extended germ-tube growth prior to appressorium formation. The deduced product of the impaired gene (*nir1*) is similar to NirA, an *Aspergillus nidulans* transcriptional regulator of nitrogen metabolism. Inoculation of leaves with wild-type or Ca5 conidia in the presence of a preferred nitrogen source resulted in massive epiphytic hyphal production, appressorium formation and rapid symptom development. Expression of *C. acutatum* wild-type nitrate reductase (*nit1*) and glutamine synthetase (*gln1*) was induced by nitrate but only *nit1* expression was repressed in a rich medium. *nit1* transcription increased during the appressorium-production stage, indicating that nitrogen starvation constitutes a cue for the regulation of appressorium development. The presence of *nit1* transcript during various phases of infection is indicative of partial nitrogen starvation *in planta*. cAMP-dependent protein kinase A (PKA) was determined to be a negative regulator of immediate post-germination appressoria formation in the wild-type. As inhibition of PKA activity in the *nir1* mutant did not affect appressoria formation, we suggest that NIR1 acts either in parallel or downstream of the PKA pathway. Our results show that *nir1* is a pathogenicity determinant and a regulator of pre-infection development under nitrogen-starvation conditions and that nitrogen availability is a significant factor in the pre-penetration phase.

## INTRODUCTION

*Colletotrichum acutatum* is the causal agent of anthracnose on strawberry (*Fragariae* × *ananassa* Duch.) (Howard *et al.*, 1992). This pathogen's ability to attack different plant parts adds to the complex nature of strawberry anthracnose (Freeman and Katan, 1997). Early stages of invasion include adhesion to the host surface, germination, host-cuticle penetration via appressoria, and plant colonization (O'Connell *et al.*, 2000). Recent ultrastructural studies have shown that *C. acutatum*, although a generalist invader, undergoes a very brief biotrophic stage before commencing the extended necrotrophic phase. During the latter phase, the fungus differentiates secondary hyphae, which are thinner than the primary hyphae and grow extensively, leading to disorganization and mortality of the infected host cell (Curry *et al.*, 2002; Horowitz *et al.*, 2002). Several studies have suggested that lack of nutrients is one of the signals controlling the expression of genes involved in the pathogenicity of various microbial plant pathogens (Pelletier *et al.*, 2003). Major nutritional changes are believed to occur during: (i) the early pre-penetration phase, during which the fungus is dependent mainly on preacquired nutrient supplies; (ii) the onset of biotrophy, during which the fungus presumably acquires nutrients adjacent to living host cells; and (iii) the cessation of biotrophy and the initiation of the necrotrophic phase. The infection process requires the spatially and temporally regulated development of appropriate infection structures, suggesting the occurrence of tightly regulated fungal development *in planta*. Carbon and/or nitrogen starvation have been reported to induce or increase the expression of a number of *in planta*-induced genes of several fungal plant pathogens (Snoeijs *et al.*, 2000). For example, the *mpg1* gene of the hemibiotrophic rice blast fungus *Magnaporthe grisea* encodes a hydrophobin-like protein involved in appressorium formation that is necessary for pathogenicity and is expressed *in vitro* under conditions of carbon and nitrogen starvation (Talbot *et al.*, 1993). Similarly, the expression of the avirulence gene *avr9* of the biotrophic fungal tomato pathogen *Cladosporium fulvum* is

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Nucleotide sequence data reported here are available in the GenBank database under accession numbers DQ192665 (*nir1*), DQ192666 (*nit1*) and DQ192667 (*gln1*).

induced under nitrogen, but not carbon, starvation (Perez-Garcia *et al.*, 2001; Van den Ackerveken *et al.*, 1994), yet nitrogen starvation does not control some other effector genes that are required for full virulence of *Cl. fulvum* (Thomma *et al.*, 2006). The CgGS gene and the CgDN3 gene, which encode a glutamine synthetase (GS) and a product of unknown function, respectively, in *Colletotrichum gloeosporioides*, the hemibiotrophic fungal pathogen of tropical legumes, were obtained through a screen for genes that are specifically induced *in vitro* under nitrogen starvation (Stephenson *et al.*, 1997, 2000). These genes have been shown to be expressed *in planta* during the early stages of the *C. gloeosporioides* infection process. In *C. lindemuthianum*, the nitrogen regulator 1 (an AREA/NIT2-like global nitrogen regulator) is required for the infection cycle (Pellier *et al.*, 2003). Thus, it is frequently assumed that biotrophic and hemibiotrophic fungal pathogens encounter nitrogen-limiting conditions at the onset of the infection process and that nitrogen starvation constitutes one of the signals involved in the regulation of genes that are induced *in planta* (Snoeijs *et al.*, 2000). One of the additional better-studied components of environmental perception is the cAMP-dependent protein kinase A (PKA) signalling pathway. PKA has been shown to play an important role in nutrient sensing in the saprophytic yeast *Saccharomyces cerevisiae*. Likewise, PKA appears to play an integral role in surface sensing, vegetative growth and appressoria formation in species of the filamentous phytopathogen *Colletotrichum* (Lee *et al.*, 2003) and *Magnaporthe grisea* (Adachi and Hamer, 1998).

Fungi are able to utilize a wide array of nitrogen-containing compounds but some nitrogen compounds are more preferentially used than others. In the saprophytic filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, *areA* and *nit2*, which interact with the pathway-specific regulators NirA and Nit4, respectively, act to facilitate the expression of genes involved in metabolizing alternative nitrogen sources (Marzluf, 1997).

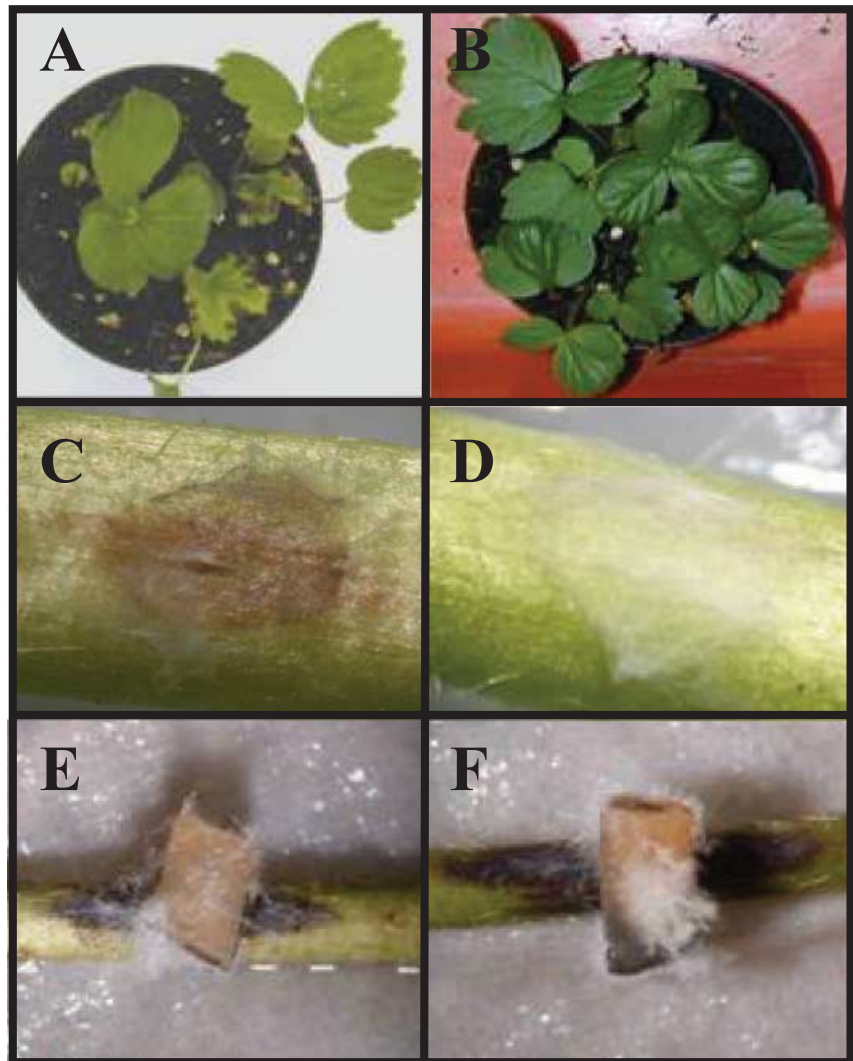
Previously, we developed a large-scale forward genetic approach to identify pathogenicity factors of the fungus *C. acutatum* (Horowitz *et al.*, 2004). One of the reduced pathogenicity mutants, designated Ca5, was shown to be affected at a key step in the infection process. In this study, molecular analysis of the Ca5 mutant allowed us to identify the first pathogenicity gene *nir1*, isolated from strawberry-infecting *C. acutatum*, which encodes a protein involved in nitrogen metabolism. These findings, and the fact that the Ca5 mutant is blocked at a very early phase of the infection cycle, led us to examine the effect of nitrogen availability on fungal morphogenesis and pathogenicity. Our results demonstrate that a common regulator of nitrogen metabolism is required for this fungus' growth and development during the biotrophic phase of its life cycle.

## RESULTS

### Characterization of the Ca5 mutant phenotype

The Ca5 REMI (restriction-enzyme-mediated integration) mutant, impaired in its pathogenicity on strawberry, did not exhibit any significant differences in radial growth or conidiation compared with the wild-type, when cultured on modified Mathur's medium (M3S), considered to be a rich growth medium. Unlike the wild-type, which produced symptoms within 4–5 days on strawberry seedlings, the mutant strain was unable to produce symptoms 2 weeks following spray inoculation (Fig. 1A,B). Inoculation of stolons with the Ca5 strain produced epiphytic hyphae, in contrast to the necrotic lesions formed by the wild-type, 1 week after inoculation (Fig. 1C,D). The Ca5 strain was able to grow necrotrophically and conidiate on leaves and stolons when conidia were inoculated directly, by drop inoculation, onto wounded sites. Furthermore, inoculation with M3S medium plugs of wild-type and the Ca5 strain supported massive epiphytic growth which culminated with entry to the plant tissue and development of necrotrophic symptoms (Fig. 1E,F). When placed on a plastic surface or strawberry leaves, most of the germinating conidia produced appressoria in close proximity to the conidia within 12 h (Fig. 2A,C). By contrast, the Ca5 mutant formed elongated germ tubes and underwent branching prior to appressorium formation on plastic and strawberry leaf surfaces (Fig. 2B,D). At all times tested between 1 and 14 days post-inoculation, the Ca5 mutant was recovered from strawberry tissues on selective media containing hygromycin B, indicating that it can survive on the host without causing plant necrotrophy and wilting. Microscopic observation of strawberry inoculated with the Ca5 mutant revealed that fungal hyphae were restricted to the host cuticle, the epidermis and subepidermal layers. The Ca5 mutant was unaffected in its germination rates on rich media (mean  $\pm$  SD,  $95.3 \pm 3.4\%$ ) compared with the wild-type ( $92.5 \pm 2.5\%$ ), or on minimal medium (MM) supplemented with urea ( $92.2 \pm 5.4\%$  for the wild-type and  $90.5 \pm 4.3\%$  for the Ca5 mutant). In both strains, presence of preferred nitrogen sources increased conidial germination (characterized by the formation of long germ tubes and branching) compared with that observed in water (Fig. 2E,F).

The ability of Ca5 and the wild-type to form appressoria was further examined on a plastic surface (in the presence of water). Conidia of Ca5 germinated normally in water but formed appressoria only at a low frequency (Fig. 2B,D): 88% ( $\pm 6.6$ ) of the germinating Ca5 conidia germinated without appressorium formation, whereas 6.5% ( $\pm 4.1$ ) germinated and formed elongated germ tubes with subsequent appressorium formation. This is in significant contrast to the wild-type strain, where 94.9% ( $\pm 4.1$ ) of the germinating conidia produced adjacent appressoria and only 2.6% ( $\pm 0.9$ ) germinated with subsequent appressorium formation.



**Fig. 1** Pathogenicity phenotypes of wild-type (A) and non-pathogenic mutant Ca5 (B) strains of *Colletotrichum acutatum* on a susceptible strawberry cultivar, 2 weeks after spray inoculation of non-wounded plants with  $10^5$  conidia/mL. Drop inoculation of non-wounded strawberry stolons with conidia of wild-type (C) and Ca5 mutant (D), and M3S-medium plugs of wild-type (E) and Ca5 mutant (F) on non-wounded stolons, 1 week after inoculation.

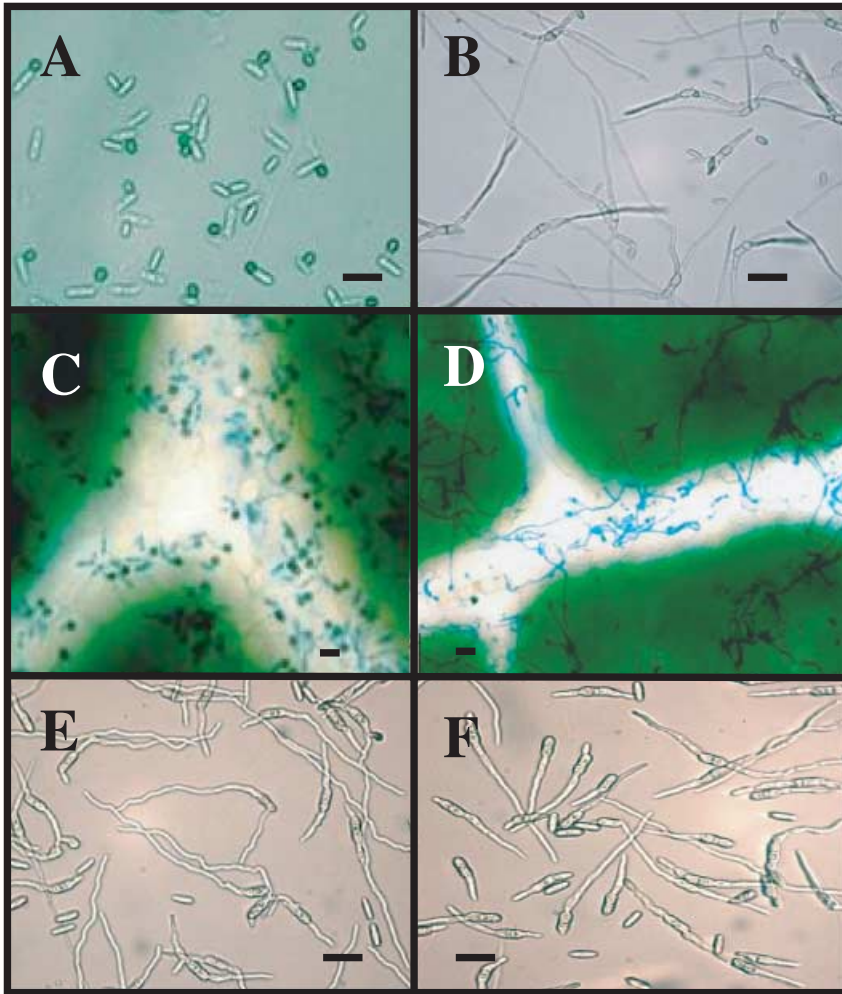
Similar results were also observed when conidial suspensions of the wild-type and Ca5 strains were placed on strawberry plants (Fig. 2C,D).

#### **Ca5 is affected in a gene encoding a putative Zn(II)<sub>2</sub>Cys<sub>6</sub> transcriptional activator**

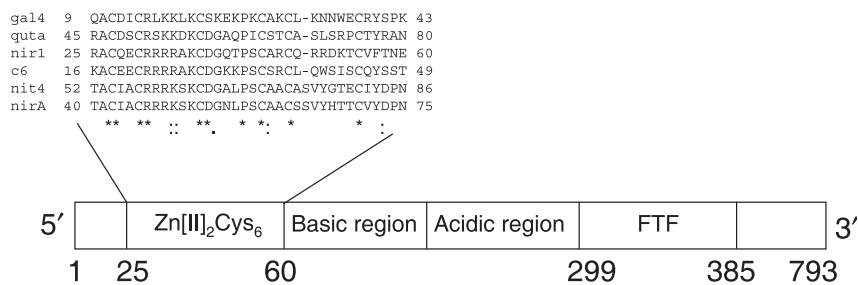
To determine the genetic nature of the mutant strain, Ca5 genomic DNA was digested with *Xba*I (the enzyme used for linearization of the REMI vector was *Hind*III) and subjected to Southern analysis. The presence of one hybridizing band (~7.2 kb) indicated the occurrence of a single-copy insertion event (data not shown). Isolation of flanking genomic DNA was conducted by inverse PCR with inverted primers originating from the *hph* gene cassette. The 2.5-kb PCR product was cloned, sequenced and used to design primers for walking downstream along the genomic DNA. The entire gene (~3.8 kb in length) was cloned and

fully sequenced. Sequencing indicated that the REMI vector was inserted in the Ca5 mutant at nucleotide 1156. Hybridization patterns of genomic DNA digested with several restriction enzymes consistently indicated the presence of a single copy of this sequence in the *C. acutatum* genome.

The genomic DNA sequence indicated that the gene contains seven putative introns (based on conserved splice signals) and encodes a protein of 793 amino acids (Fig. 3). The deduced protein displays the characteristics of a transcriptional activator belonging to the zinc cluster family. The first domain is a typical zinc binuclear cluster (Zn(II)<sub>2</sub>Cys<sub>6</sub>) comprising six cysteine residues located at the N terminus of the protein followed by a basic and acidic region. Downstream of the zinc cluster, a fungal transcriptional activator motif is present, starting at position 299. The zinc cluster domain is highly similar to the *Aspergillus fumigatus* putative C6 transcriptional activator (60% identity within the zinc cluster domain), indicating that the *C. acutatum* protein is a



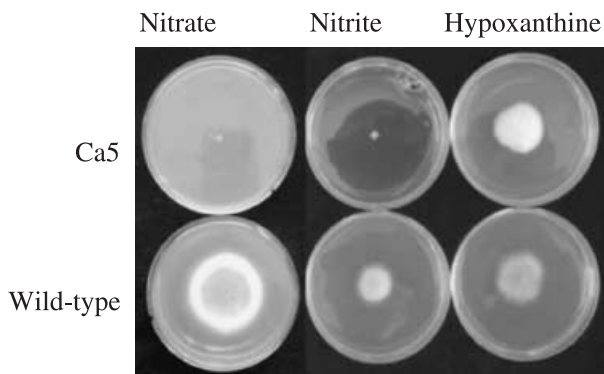
**Fig. 2** Germination phenotype of the wild-type and non-pathogenic mutant Ca5 strains of *Colletotrichum acutatum* conidia resuspended in minimal medium lacking a nitrogen source. (A,B) Wild-type and Ca5, respectively, on a plastic surface. (C,D) Wild-type and Ca5, respectively, on strawberry leaves, 12 h after drop inoculation. (E,F) Wild-type and Ca5, respectively, on a plastic surface, in the presence of urea. Bar = 20 µm.



**Fig. 3** The predicted NIR1 protein of *Colletotrichum acutatum* harbours a zinc finger cluster domain followed by a basic and acidic region. At position 299 a fungal transcription factor (FTF) motif is present. Sequence alignment of Zn(II)<sub>2</sub>Cys<sub>6</sub> domains from NIR1 and comparable subdomains (35 residues in total) from other fungal zinc cluster transcriptional activators were analysed by a CLUSTALW program. Sequences shown are from: NIRA, *Aspergillus nidulans*; NIT4, *Neurospora crassa*; QUTA, *Aspergillus nidulans*; Gal4, *Saccharomyces cerevisiae*; C6, *Aspergillus fumigatus*; and NIR1, *Colletotrichum acutatum*. Identical amino acids are marked by asterisks and similar residues indicated by dots.

putative transcriptional activator belonging to the fungal zinc cluster family. Moreover, the predicted protein had high sequence similarity to specific fungal transcription factors analysed in *N. crassa* (NIT4; Fu *et al.*, 1989) and *A. nidulans* (NIRA; Burger

*et al.*, 1991) with 48% and 51% identity, respectively, and may be involved in nitrogen metabolism (Fig. 3). We thus designated the *C. acutatum* gene *nir1*. To assess whether the mutation in *C. acutatum nir1* affects nitrogen metabolism in this fungus, the



**Fig. 4** Growth phenotype of wild-type of *Colletotrichum acutatum* and non-pathogenic mutant Ca5 strains on minimal media containing nitrate, nitrite or hypoxanthine as sole nitrogen sources.

mutant was cultured on minimal media containing nitrate, nitrite or hypoxanthine as the sole nitrogen source. The Ca5 mutant grew as well as the wild-type on hypoxanthine but failed to grow on nitrite or nitrate, in contrast to the wild-type, which developed normally under the latter conditions (Fig. 4).

**Expression analysis of nir1 in the wild-type and mutant strains**

To assess the involvement of *nir1* in nitrogen metabolism, we followed its expression in cultures grown in minimal medium supplemented with different nitrogen sources. Quantitative RT-PCR (QRT-PCR) experiments with primers C6RT1qrt listed in Table 1 demonstrated that *nir1* is expressed at low levels (as indicated from later cycle threshold values (C<sub>T5</sub>). *nir1* expression increased in the presence of available glutamine, or nitrate, as the sole nitrogen source. *nir1* expression levels measured after prolonged growth in the presence of nitrate as the sole nitrogen source were significantly ( $P < 0.05$ ) higher than those detected in cultures grown in M3S or Reg-rich medium or *nir1* transcript levels measured in dormant conidia (Table 2). Expression of *nir1* at the appressorium-formation stage increased by 12-fold, relative to expression in dormant conidia (Table 2). Under similar experimental conditions, *in planta nir1* transcript levels were undetectable. To determine whether *nir1* was at all expressed in the Ca5 mutant, we performed RT-PCR analysis (utilizing primers CaRTfor and CaRTrev; Table 1). The results (Fig. 5B) indicated that the gene is expressed but the transcript size (500 bp) is smaller than that of the wild-type (720 bp). Thus, the insertion event of vector pGH-1 in Ca5 altered expression of the *nir1* gene, but did not totally abolish it. In addition, *nit1* transcript was not detected in the Ca5 strain (unlike in the wild-type cultured under the same conditions), suggesting insufficient activity of the Nir1 transcriptional activator in the mutant. In all cases, expression of *gln1* was evident (Fig. 5B).

**Table 1** Primers used throughout this study.

Primer	Sequence (5' to 3')
tub2B	GACRTCCTTCATRGGCA
genec	GAGGAATCCAGACCGTATGATG
T7BgIII	TAATACAGATCTCACTATAGGG
Tubfor-qrt	AGGATGTCGAGGACCAGATG
Tubrev-qrt	TACCGACAAAGGTGGAGGAC
SP6Kpnl	CCAAGCTATTTAGGTACCACTA
149WT4	TCGATCAGAACTTCTCGACAGA
149Hpal2B	GGAAACGTCCACTCCAAGACA
Inhph/for	TCGATCAGAACTTCTCGACAGA
Inhph/rev	GTAICTCGCCGATAGTGAAAC
CaRTfor	GTAAGTGGTGCAGCTCCCTGAC
CaRTcDNA	CGTAGGTACCTCAGTGGTGGGC
CaRTrev	CGACGGCGGCTTACAGCACGGC
C6RT1.for.qrt	GTTCCAAGAATCGACCTCCA
C6RT2.rev.qrt	TAATAGGCGGTTAGCCATGC
MgNR1	AAGGGYTTCTCHTGGGGWGCY
MgNR3	ACCAWGGGTRTRTCATCATT
Moco	GAATGATGAAYAACCCWTGGT
Nadr	GTDATNCCNGTACCACRCADATCAT
CaNRfor	GGAGGAAACCTGGCCAACGGA
CaNRrev	CGATTTACCGCAGATCATGAC
GSfor	CCCAACATTCTCGTCTCTCC
Gsrev	CTCGAAGTAACCGTAGCCCT
Nit1 qrt-for	TCCTTAGACAGCGAAA
Nit1 qrt-rev	AGGATCTTTGGGCTGCTGA
Gln1 qrt-for	ATGAGTGCGCCAAATTGATG
Gln1 qrt-rev	ACTCCTGCTCGAGACCGAAC
Catf2	ACAAACGGTCTACTTACATTGTA
Ca5(1A)	TCATGTGCTCTGTACCCT
hphf	CTGTGCGAAGTTTCTGATCG

**Effect of nitrogen source on morphology and pathogenicity of C. acutatum**

Wild-type conidia resuspended in water or minimal medium lacking a nitrogen source tended to form appressoria adjacent to the conidia on strawberry leaves within 12 h, followed by the appearance of typical anthracnose symptoms (Figs 2C and 6A). However, inoculation with the Ca5 mutant under the same conditions did not result in lesion development (Fig. 6B). By contrast, addition of organic or inorganic nitrogen supplements to minimal medium inhibited the characteristic immediate appressorium formation and promoted extended germ-tube growth. Addition of external nitrogen sources such as glutamine or urea to the wild-type strain and the Ca5 mutant induced massive epiphytic hyphal growth and subsequent formation of appressoria, followed by rapid symptom development (Fig. 6C,D). Thus, exogenous supplements of appropriate nitrogen sources complemented the non-pathogenic phenotype of the *nir1* mutant. The influence of different nitrogen sources on efficacy at inciting disease on strawberry was determined. Significant differences were observed

**Table 2** Changes in gene expression levels of fungal *nit1*, *gln1* and *nir1*, relative to the  $\beta$ -tubulin reference gene *in planta* and in culture (under different nutritional conditions) as determined by QRT-PCR.

Gene treatment	Fold change ( $2^{-\Delta\Delta C_t}$ )*		
	<i>nit1</i>	<i>gln1</i>	<i>nir1</i>
<i>In planta</i>			
Non-inoculated plants	nd†	nd	nd
2 days p.i.‡	1	1	nd
3 days p.i.	1.27 ± 1	0.54 ± 0.07	nd
4 days p.i.	2.08 ± 0.54	2.51 ± 0.72	nd
<i>In culture</i> §			
Reg	0.36 ± 0.03	1.13 ± 0.04	0.09 ± 0.04
M3S	0.29 ± 0.1	5.9 ± 0.8	0.12 ± 0.02
Nitrate	2.7 ± 0.22	4.32 ± 0.09	13 ± 4.02
Glutamine	2.6 ± 0.4	0.47 ± 0.16	2.49 ± 0.1
Nitrate 24 h	7.4 ± 2.3	13.7 ± 0.52	1.11 ± 0.2
Appressoria	1	1	1
Conidia	0.12 ± 0.03	6.3 ± 0.7	0.08 ± 0.2

\*cDNA was synthesized from 1  $\mu$ g total RNA. All samples were analysed in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template).  $C_t$ , the cycle at which the amplification curve reaches threshold fluorescence, was used to determine the relative amount of transcript. Averaged  $C_t$  values were then normalized (to adjust for different amounts of cDNA within each reaction) to the endogenous control gene,  $\beta$ -tubulin. Fold change in target genes was 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 appressoria-extracted sample was used as the control in experiments where nitrogen availability was monitored or extracts from seedlings at the 2-day post-inoculation phase in experiments involving *in planta* expression analysis. Mean values of  $2^{-\Delta\Delta C_t} \pm$ SD of the differences of three independent cDNA measurements from the same RNA samples from the same treatments are presented. †nd = not detected.

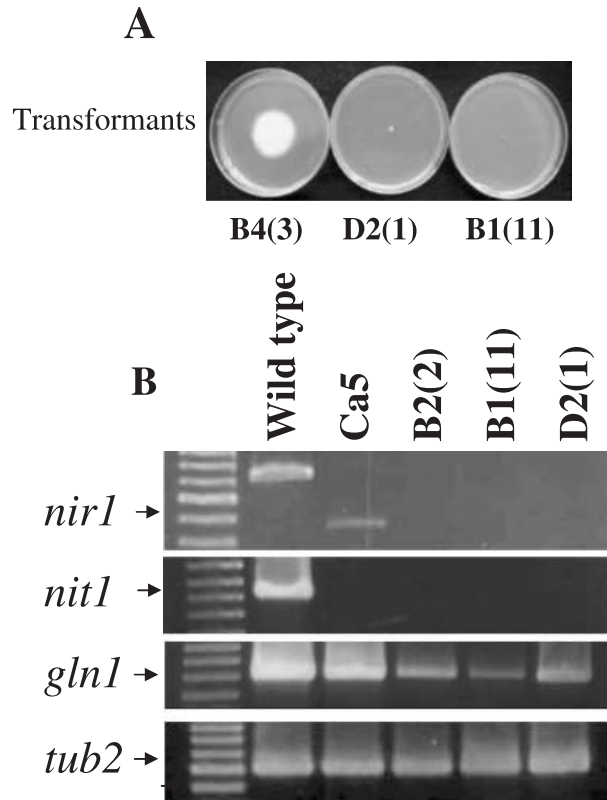
‡p.i. = post-inoculation.

§Unless stated otherwise, RNA was extracted from 5-day-old cultures. Appressoria were collected from 16-h-old conidial germlings forming appressoria on glass plates.

in the time required to reach 50% mortality of seedlings inoculated with wild-type conidia resuspended in ammonium sulfate ( $5.2 \pm 1.2$  days) and glutamine ( $4.1 \pm 1.5$  days), compared with wild-type conidia resuspended in nitrate ( $10.5 \pm 2$  days) or water ( $11.2 \pm 1.2$  days).

### Expression profile of nitrogen-metabolism-related genes

To assess the availability of nitrogen source during strawberry colonization, we tested the expression of nitrate reductase and glutamine synthetase (designated *nit1* and *gln1*, respectively) by QRT-PCR. Regions of the genes encoding *C. acutatum nit1* and *gln1* were cloned from *C. acutatum* cDNA by PCR amplification with degenerate primers (Table 1). On the basis of the sequenced cDNA clones, primers for relative RT-PCR and QRT-PCR with



**Fig. 5** (A) Growth of *nir1* gene-disruption mutants D2(1) and B1(11) compared with a non-disrupted strain B4(3), on minimal medium containing nitrate. (B) Expression of *nit1* and *gln1* in the Ca5 mutant and gene-disrupted mutants compared with the wild-type strain. Cultures were initially grown in M3S rich medium and subsequently transferred to minimal medium supplemented with 5 mM glutamine for 2 days, prior to RNA extraction. Primers used for the specific amplification are listed in Table 1.

*C. acutatum nit1* and *gln1* were designed (Table 1). Transcriptional regulation of *C. acutatum nit1* and *gln1* in response to different nitrogen sources and in the M3S and Reg-rich growth medium was examined. *nit1* expression was down-regulated in M3S and Reg-rich medium and up-regulated in minimal medium containing nitrate (Table 2). Expression of *gln1* was highest within 24 h after addition of nitrate and decreased after 5 days of growth in the presence of nitrate. Expression of *gln1* was not significantly altered after prolonged growth in minimal medium or rich medium. In addition, the presence of glutamine in the medium repressed *gln1* expression. To investigate further the availability of nitrogen sources in conidia during appressorium formation and host colonization, expression levels of fungal *nit1* and *gln1* were monitored. Transcript levels of *nit1* increased eight-fold during the appressorium-formation stage in comparison with dormant conidia, as opposed to *gln1* expression which was six-fold higher in dormant conidia when compared with the appressorium-formation stage (Table 2). Expression of fungal *nit1* and *gln1* relative to fungal  $\beta$ -tubulin *in planta* was determined

from 2 days after colonization until the onset of the symptomatic phase. The relative transcript abundance of both *nit1* and *gln1* was similar during all phases of plant colonization (Table 2).

### Nitrate-non-utilizing mutants of *C. acutatum* and pathogenicity

To examine further whether the loss of pathogenicity and the developmental defect (impaired appressorium formation) were linked to impaired nitrogen utilization, *C. acutatum* nitrate-non-utilizing mutants (*nit*) were produced. Mutant phenotyping was assessed on the basis of growth on nitrate-, nitrite- and hypoxanthine-supplemented media. The largest group consisted of *nit1* mutants (in which mutations occurred within the nitrate reductase structural locus), and one group of *nitM* mutants (affected at the molybdenum co-factor loci). Pathogenicity of all the isolated mutants on strawberry seedlings and stolons was significantly reduced compared with the wild-type (data not shown). However, in contrast to the Ca5 mutant, all the tested *nit* mutants produced appressoria in a manner similar to that of the wild-type.

### Targeted disruption of *nir1* by homologous recombination

To inactivate *nir1* by homologous gene replacement, a linear *nir1*-disruption construct, designated *nir1:hyg3*, was obtained by introducing the hygromycin B resistance expression cassette (*hph*), including the promoter and terminator sequences of the *trpC* gene of *A. nidulans*. This cassette was flanked by *nir1* genomic DNA (positions 1–2751 and 2968–3753, for the 5' and 3' flanks, respectively; Fig. 7A). A double-recombination event between the homologous regions of the *nir1:hyg3* vector and the *nir1* gene would be expected to replace ~300 nt of the endogenous *nir1* gene with the *hph* gene cassette. Several putative transformants were selected after plating on a medium containing hygromycin. Analysis of gene-disruption events was first performed with primer set 149wt4 and 149Hpal2B (Table 1), designed on the basis of regions flanking the *hph* cassette. In the case of an ectopic integration, two amplicons (~2500 and 565 bp) were produced, whereas in the case of homologous recombination the shorter amplicon was no longer evident (Fig. 7). These results were confirmed with an additional set of primers—*hphf*, and *Catf2* (Table 1). Among the 120 hygromycin B-resistant transformants generated with *nir1:hyg3*, 11% harbored gene-replacement events.

### Disruption of *nir1* affects appressorium-formation rates and abolishes pathogenicity

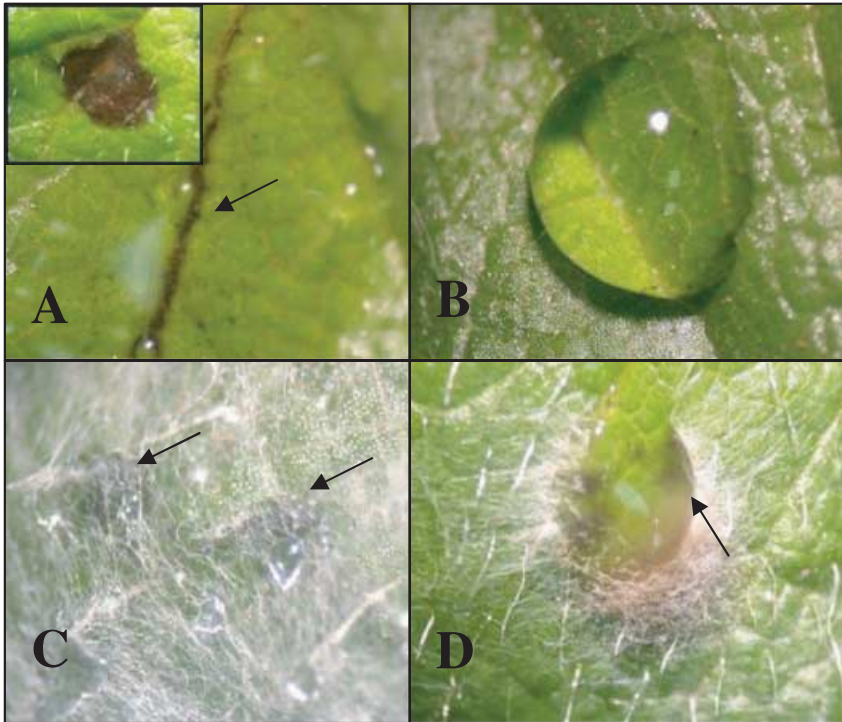
Radial growth rate on solid M3S agar plates indicated that the hyphal extension rates of the *nir1* disruption mutants were equal

to those of the wild-type culture. The cultures of the *nir1*-disrupted mutants [B1(11), D2(1) and B2(2)] conidiated normally. When conidial suspensions were applied to glass slides they adhered, germinated and then produced elongated germ tubes, a morphological feature distinct from the wild-type, which immediately produced melanized appressoria. In additional experiments performed on leaf and plastic surfaces, significant differences existed in the extent of appressorium formation by the gene-replacement mutants compared with that of the wild-type. Thus, 7.2% ( $\pm 1.2$ ) of the germinating conidia of B1(11) produced appressoria adjacent to conidia and 75% ( $\pm 3.5$ ) germinated without forming an appressorium. Conidia of the gene replacement mutant D2(1) produced 9.5% ( $\pm 2.4$ ) appressoria adjacent to conidia and 66.8% ( $\pm 5.4$ ) germinated without forming an appressorium, compared with approximately 95% production of appressoria by the germinating wild-type conidia (as described earlier, in the comparison with the Ca5 strain). All gene-replacement mutants were tested for their ability to cause disease on strawberry compared with the wild-type and Ca5 mutant strains. Within 2 weeks of inoculating host leaves with conidial suspension droplets, typical disease symptoms were produced by the wild-type isolate and non-disrupted transformants [B2(1) and B4(3)], with large, clearly visible, necrotic lesions at the sites of inoculation (Fig. 8). By contrast, leaves inoculated with the *nir1*-disrupted mutants [D2(1), B1(11) and B2(2)] remained healthy and were symptomless. When analysed *in vitro*, growth of the gene-disrupted mutants on minimal medium containing nitrate as the sole nitrogen source was restricted relative to the profuse growth of the wild-type strain (Fig. 4), as expected of strains lacking NIR1 function.

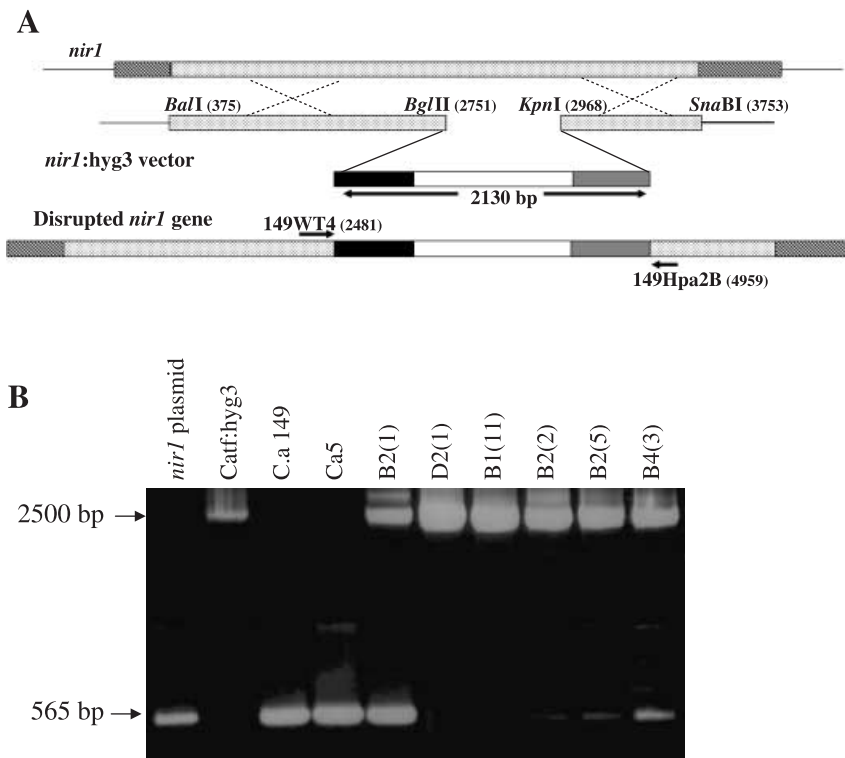
Expression of *nir1* was no longer evident in the gene replacement mutants, unlike in the Ca5 strain, in which an insertion event of vector pGH-1 altered expression of the *nir1* gene. Expression of the structural gene *nit1* was no longer evident in all gene knockout mutants (Fig. 5).

### Factors affecting PKA activity alter appressorium formation patterns

The impaired appressorium formation pattern in the Ca5 strain along with the fact that PKA has been shown to be involved in appressorium formation in various *Colletotrichum* species such as *C. lagenarium* and *C. trifolii* led us to examine whether *nir1* and PKA signalling are linked. For this purpose, we utilized a pharmacologically based approach, taking advantage of documented effects of cAMP, caffeine (Rollins and Dickman, 1998) and the PKA specific inhibitor KT5720 on PKA-regulated fungal development. Amending the conidial suspension with the KT5720 (5–25  $\mu\text{M}$ ) antagonist did not significantly affect the immediate appressoria formation phenotype characteristic of the wild-type strain. By contrast, application of the PKA agonists

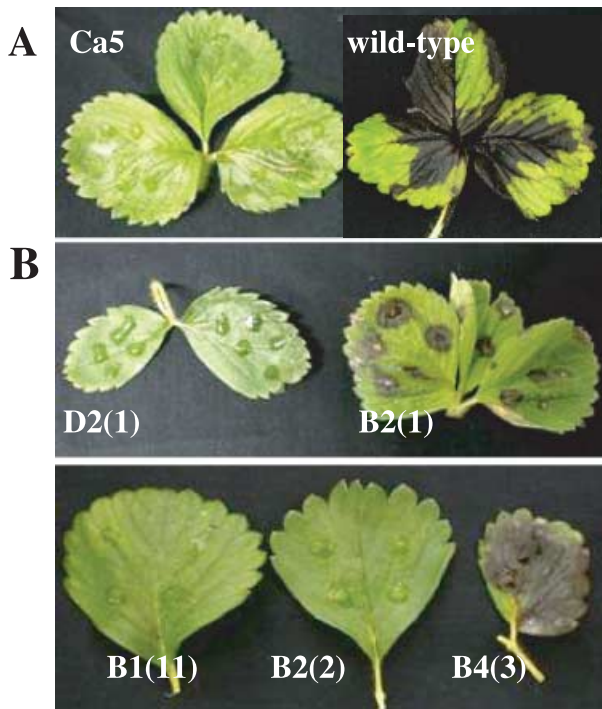


**Fig. 6** Germination and appressorium formation of *Colletotrichum acutatum* wild-type and Ca5 mutant strains on strawberry leaves, 4 days post-inoculation. (A) Drop inoculation with conidia of wild-type resulted in massive appressoria formation adjacent to strawberry leaf cells, leading to necrotic lesions (further magnified at upper left). (B) Drop inoculation with the Ca5 strain was not accompanied by appressorial formation. Epiphytic germination of wild-type (C) and Ca5 strain (D) conidia, resuspended in minimal medium supplemented with urea.



**Fig. 7** (A) Diagrammatic representation of homologous recombination events associated with replacement of part of the *nir1* gene of *Colletotrichum acutatum* with the hygromycin resistance (HygR) gene cassette by the disruption vector *nir1:hyg3*. *nir1:hyg3* contains a heterologous *hph* gene cassette flanked by 5' and 3' *nir1* genomic fragments. The *hph* gene cassette comprised the promoter and terminator of the *trpC* gene of *Aspergillus nidulans* with the coding region of the *hph* gene of *Escherichia coli* (HygR). Positions of primers 149WT4 and 149Hpa2B used for gene-disruption evaluation are shown. (B) PCR analysis for potential gene-disruption mutants of wild-type *Colletotrichum acutatum* with primers 149WT4 and 149Hpa2B, flanking the *hph* gene cassette. The 565-bp fragment indicates ectopic integration of the *nir1:hyg3* construct. Absence of the 565-bp fragment and appearance of only a 2500-bp fragment is an indication of a homologous recombination event.





**Fig. 8** Pathogenicity of different *Colletotrichum acutatum* strains on strawberry leaves, as determined by the drop inoculation method. (A) The Ca5 mutant and wild-type strains. (B) Gene-disruption mutants D2(1), B1(1), B2(2) compared with non-disrupted mutants B2(1) and B4(3).

cAMP (5–25 mM) or caffeine (2.5–50 mM) reduced normal appressorium formation by approximately 75%. These results suggest that PKA is a negative regulator of immediate appressoria formation. When applied to the Ca5 strain, which does not produce appressoria after germination, neither the agonists nor the inhibitor had a significant effect on development (Fig. 9). Lack of a phenotypic response of both the Ca5 strain and the D2(1) *nir1* gene replacement mutant (data not shown) to the various PKA effectors suggests that both *nir1* and PKA are involved in the normal appressorium formation process. However, whether they function in parallel or *nir1* is downstream of PKA has yet to be determined.

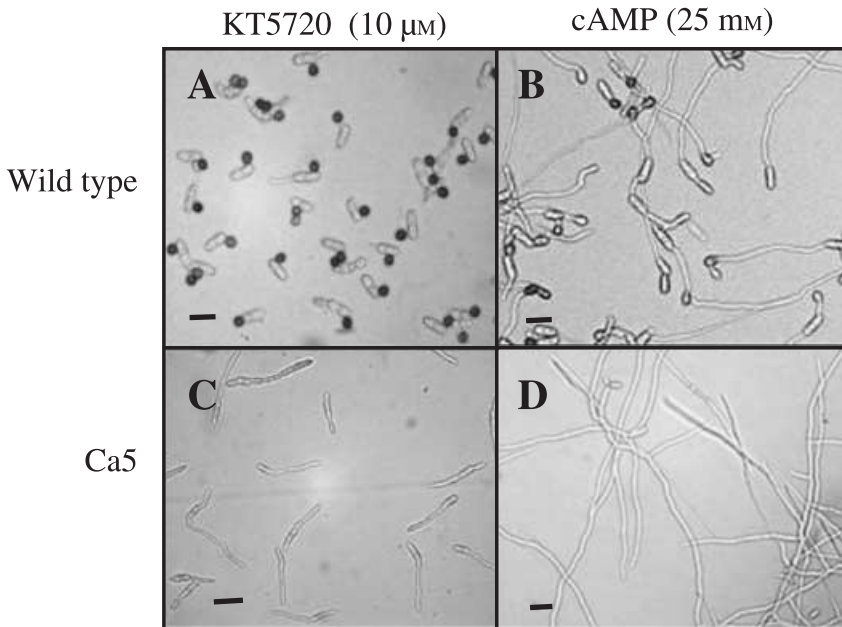
## DISCUSSION

In this study, we characterized a non-pathogenic mutant of *C. acutatum*, designated Ca5. Contrary to the wild-type, which forms appressoria adjacent to conidia, this non-pathogenic mutant germinates to form an elongated germ tube in the absence of any supplemental nitrogen sources. An interesting aspect of the phenotype of this mutant is its ability to form necrotrophic lesions and conidiate when preferred nitrogen sources are available. Even though Ca5 was non-pathogenic, it could be re-isolated from strawberry plants up to 2 weeks after inoculation. This

observation is consistent with previous reports on survival and re-isolation of *C. acutatum* from non-host plants (Freeman *et al.*, 2001).

Several lines of evidence suggest that *nir1*, the gene which was mutated in the Ca5 strain, is a nitrogen regulatory gene of *C. acutatum*. First, *nir1* shares homology with the reported, positively acting nitrogen regulatory proteins NIRA in *A. nidulans* (25%) (Burger *et al.*, 1991) and NIT4 in *N. crassa* (22%) (Fu *et al.*, 1989) (Fig. 3). Secondly, the Ca5 mutant is defective in its ability to utilize nitrate and nitrite. Similarly, *nirA* loss-of-function mutations of *A. nidulans* lead to non-inducibility and consequent inability to utilize nitrate or nitrite (Burger *et al.*, 1991). Gene-disruption experiments demonstrated that inactivation of *nir1* results in non-pathogenic phenotypes similar to the mutant strain Ca5, proving that *nir1* is required for pathogenicity. Expression of *nir1* increased when the wild-type was cultured in either minimal medium containing nitrate and glutamine or at the appressorium stage, as compared with growth in rich medium and dormant conidia. Many of the fungal transcriptional activators belonging to the zinc cluster family control catabolic pathways: NIT4 in *N. crassa* mediates nitrate induction (Yuan *et al.*, 1991), FACB in *A. nidulans* controls the expression of acetate utilization (Todd *et al.*, 1997), UaY in *A. nidulans* regulates purine utilization (Suarez *et al.*, 1995) and QUTA in *A. fumigatus* regulates the utilization of quinic acid (Pain *et al.*, 2004). Other examples of transcription factors containing a Zn(II)<sub>2</sub>Cys<sub>6</sub> finger protein that are involved in the regulation of pathogenicity have been found in *Fusarium solani* f. sp. *pisi* (CTF1, which is involved in the transcriptional activation of a cutinase-encoding gene; Li and Kolattukudy, 1997) and in *Colletotrichum lagenarium*, where CMR1 has been shown to be involved in the expression of the melanin biosynthesis genes *scd1* and *thr1* (Tsuji *et al.*, 2000). These findings, together with ours regarding the Ca5 mutant phenotype which is impaired in the regulatory factor that affects appressorium formation, suggest the presence of a common mechanism that regulates transcription of appressorium-forming genes in a manner related to both development and nitrogen metabolism.

Formation of appressoria adjacent to conidia on strawberry leaves occurs in a nitrogen-limiting environment, a condition that prevails during this early infection stage. In the case of *C. acutatum* infection of strawberry, the biotrophic phase, in which the fungus maintains a symptomless existence within its host cells while removing nutrients from them, is followed by a transition to a necrotrophic phase, which involves major nutritional changes. Therefore, a mutation in the catabolic pathways required to utilize plant constituents could abolish or reduce pathogenicity. In *C. lindemuthianum*, targeted gene disruption of CLNR1, the AREA/NIT2-like global nitrogen regulator, resulted in reduced pathogenicity (Pelletier *et al.*, 2003). By contrast, disruption of the *M. grisea* NUT1 gene, a homologue of *areA* and *nit2*,



**Fig. 9** Effect of cyclic AMP (cAMP), caffeine and the PKA inhibitor KT5720 on the conidial germination phenotype of *Colletotrichum acutatum*. (A,B) Wild-type conidia in the presence of 10  $\mu\text{M}$  KT5720 or 25 mM cAMP, respectively. (C,D) Germination of the Ca5 conidia in the presence of 10  $\mu\text{M}$  KT5720 or 25 mM cAMP, respectively. Bar = 20  $\mu\text{m}$ .

had only a slight effect on infection efficiency (Froeliger and Carpenter, 1996). Gene disruption of *areA*-GF of *Gibberella fujikuroi*, a homologue of the major nitrogen regulatory gene *areA*, conferred a significant reduction in gibberelin production (Tudzynski *et al.*, 1999). But examination of the transcriptional adaptation of *Fusarium oxysporum* f. sp. *lycopersici* (the causal agent of vascular wilt in tomato) during nutritional stress and plant colonization indicated that nitrogen starvation partially mimics *in planta* growth conditions (Dixon *et al.*, 2005).

Nitrogen starvation was assessed by following expression of nitrogen metabolism genes during different phases of fungal development and plant colonization. Unlike *nit1*, expression of *gln1* was not modulated by availability of nitrogen sources *in vitro* and thus could not be used as an indicator for nitrogen starvation. By contrast, *C. gloeosporioides* glutamine synthetase transcription is induced by nitrogen starvation in culture and its expression is elevated during pathogenesis on *Stylosanthes guianensis* (Stephenson *et al.*, 1997). Our results demonstrate that *nit1* expression is induced by contact with hard surfaces during appressorium formation and during colonization of the plant. The reduction in pathogenicity of *C. acutatum nit* mutants and expression of *nit1* in all the infection stages supports the notion that partial nitrogen-starvation conditions prevail during colonization.

Our findings demonstrate that the effect of nitrogen availability on virulence is stage specific. The early stages of infection are biotrophic in nature and are accompanied by nitrogen availability limitations. Later stages, which are necrotrophic in nature, include hyphal biomass accumulation and subsequent appressoria produced from mature hyphae. As the *nir1* mutant does not have the ability to react to the lack of nitrogen that is characteristic of the

early infection stages, the morphogenetic response is that of hyphal formation. However, lack of the nitrogen source does not enable the mutant to accumulate biomass (unless an exogenous nitrogen source is supplied). Thus, in the wild-type, at the early stage, nitrogen limitation induces immediate appressoria formation (required for survival and plant penetration) and at the later, necrotrophic stage, nitrogen is no longer a limiting factor. The early developmental block imposed by the lack of NIR1 in the mutant renders it incapable of completing the biotrophic phase required for virulence. However, addition of external glutamine or urea complemented the non-pathogenic Ca5 phenotype.

Our results demonstrate that PKA signalling plays a role in the germination phenotype of *C. acutatum*. As PKA has been shown to be involved in pathogenicity of other *Colletotrichum* species (Lee *et al.*, 2003), it is conceivable that this is also the case for *C. acutatum*. Yang and Dickman (1999) as well as Yamauchi *et al.* (2004) demonstrated that inactivation of PKA did not abolish appressoria formation. Our suggestion that PKA is a negative regulator of appressorium formation is in agreement with the mentioned observations. Thus, increasing, rather than inhibiting, PKA activity results in impaired appressoria formation. We demonstrated that exposing the *nir1* mutant to PKA effectors did not suppress the non-appressorium-forming phenotype. This observation indicates that both *nir1* and PKA are involved in the normal appressorium formation process. However, whether they function in parallel or *nir1* is downstream of PKA has yet to be determined. The report of the negative cAMP-dependent expression regulation of a GATA-binding protein in rat (Zhang *et al.*, 2002) may support the possibility of a similar hierarchal positioning of PKA and NIR1 in *C. acutatum*. Nonetheless, it is also possible that cAMP may directly affect additional, alternative,

cellular components (Kopperud *et al.*, 2003), which may prove to be involved in NIR1 function.

Based on our results, the *C. acutatum nir1* transcription factor is required for induction of appressorium formation under conditions of nitrogen starvation. We conclude that the C6 transcription factor may play a role in appressorium development under nitrogen starvation, and may act in concert with PKA. Our results also suggest that *nir1* is not essential for the necrotrophic phase of pathogen development in the host and supports the hypothesis that limitation of preferred nitrogen sources is stage specific and may lead to the induction of specific pathogenicity genes, resulting in disease development.

## EXPERIMENTAL PROCEDURES

### Fungal strains and culture conditions

*C. acutatum* wild-type isolate C.a. 149 (IMI391664), pathogenic on strawberry, was used in REMI assays (Horowitz *et al.*, 2004). All *C. acutatum* strains, wild-type and transformants, were grown at 25 °C on modified Mathur's medium (M3S) as previously described (Freeman *et al.*, 1993) or regeneration medium (Horowitz *et al.*, 2004). Prior to seedling inoculation, conidia were isolated by flooding 5- to 6-day-old cultures with distilled water and adjusted to a final concentration of  $10^5$  with a haemocytometer (Brand, Wertheim, Germany). For appressorium-RNA preparation, conidia of *C. acutatum* were spread onto Petri dishes ( $10^6$  conidia per dish) containing 10 mL of water and were incubated for 12 h. The conidia were then harvested by scraping them off the Petri dishes and centrifuging at 12 000 *g* for 15 min. The pellet containing conidia and appressoria was lyophilized and subjected to RNA extraction. For nitrogen growth property assays, the wild-type and mutant Ca5 strains were cultured at 25 °C on M3S and then subcultured on minimal medium containing 20 g/L D-glucose, 1 g/L  $\text{KH}_2\text{PO}_4$ , 0.5 g/L  $\text{MgSO}_4$ , 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  $\text{Na}_2\text{MoO}_4(2\text{H}_2\text{O})$ ,  $6.25 \times 10^{-6}$  µg/L biotin and 1.25 mg/L thiamine, supplemented with one of the various nitrogen-containing compounds (glutamine, urea, nitrate, nitrite or ammonium sulfate) as the sole nitrogen source at a final concentration of 5 mM. Mycelia were grown in flasks containing 100 mL M3S or MM. For both genomic DNA and total RNA extractions, mycelium was collected at various incubation times at 25 °C. Mycelium was frozen in liquid nitrogen and stored at -80 °C prior to nucleic-acid extraction.

### Infection assays, light microscopy and induction of appressorium formation

The screening procedure for identification of the altered-pathogenicity mutants and the pathogenicity tests were

performed with seedlings of strawberry cv. Malach, an anthracnose-susceptible strawberry cultivar, as previously described (Horowitz *et al.*, 2004). Additional pathogenicity tests with the non-pathogenic strain Ca5, the gene-disrupted mutants, nitrate-non-utilizing mutants and the wild-type were performed by either spray inoculation, foliar dip inoculation or a droplet leaf inoculation method using a conidial suspension ( $10^5$  conidia/mL). Spray and drop inoculations were performed on 15-week-old seedlings or 6- to 8-week-old strawberry daughter plants, or detached leaves from daughter plants. Foliar dip inoculation was performed as previously described (Horowitz *et al.*, 2004). Briefly, 12-week-old seedlings were inverted and inserted (excluding the roots) into a conidial suspension for 1 min. After inoculation (regardless of the method used) seedlings or detached leaves were transferred immediately into moist chambers and incubated for 20 days at 25 °C. To assess the contribution of external nitrogen sources to disease development, either glutamine, ammonium chloride, nitrate (10 mM, each) or water were added to the conidial suspensions used in the foliar-dip inoculation method.

For observations using Nomarski differential interference contrast microscopy, strawberry stolon and leaf segments were inoculated with a conidial suspension of the wild-type or Ca5 mutant strain at a concentration of  $10^5$  conidia/mL. Strips of epidermal tissue were sampled from beneath the site at which inoculation droplets were applied and directly mounted, in distilled water, for microscopic examination. Assays for induction of appressorium formation and germination by the *C. acutatum* nitrate-non-utilizing mutants, gene-disrupted mutants, Ca5 mutant and wild-type strains were observed on glass slides (HYCOR; Biomedical Inc., Garden Grove, CA). Briefly, 20 µL of a  $5 \times 10^5$  conidia/mL suspension of each isolate was placed into three of the glass cells. After 12 h of incubation at 25 °C, conidial germination and appressorial development were observed in three microscopic fields in 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 phenotype was calculated for each strain and treatment, on the basis of respective percentage of the germinating conidia. The mean and standard deviation were calculated from three independent experiments. Mean comparisons of germination and appressorium formation were calculated using the least significant difference (LSD), according to the Tukey-Kramer multiple comparison test at  $P < 0.05$ .

### Fungal transformation and plasmid rescue

Electroporation of germinating conidia was performed according to Horowitz *et al.* (2002). To recover pgH-1 (Yakoby *et al.*, 2001) and flanking fungal genomic DNA from the integration site,

genomic DNA of REMI mutant Ca5 was digested with *Xba*I. The digested products were ethanol-precipitated (0.1 M NaCl, 2 $\times$ , v/v, 95% ethanol) and subsequently subjected to ligation (T4 DNA ligase) in a 100- $\mu$ L reaction volume. The ligated product was used as a template for inverse PCR (inPCR) with a pair of primers, inH-PHfor and inHPHrev (Table 2), designed according to the flanking regions of the *hph* cassette. The inPCR was performed in 50- $\mu$ L mixtures, with the Expand long template PCR system (Roche, Mannheim, Germany). Amplification conditions were as follows: template denaturation for 2 min at 94 °C, ten cycles of 10 s denaturation at 94 °C, 30 s annealing at 65 °C and 30 s elongation at 68 °C, and 20 cycles of 10 s denaturation at 94 °C, 30 s annealing at 65 °C and 30 s elongation at 68 °C. The elongation phase was extended by 20 s in each cycle and the final elongation phase was 7 min at 68 °C. The ~2.5-kb PCR product was cloned into a pGEM-T vector (Promega, Madison, WI) and sequenced.

### Isolation and analysis of nucleic acids

Plasmid DNA, propagated in *Escherichia coli* strain DH5 $\alpha$ , was isolated using the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). DNA was extracted from *C. acutatum* as described by Rodriguez (1993). For Southern hybridization, 10  $\mu$ g of digested genomic DNA were subjected to electrophoresis on 1% agarose gels, and transferred to nylon membranes (Hybond N+ Amersham, Little Chalfont, UK). ECL-based Southern hybridizations were performed overnight according to the manufacturer's directions (Amersham). Total RNA from fungal mycelia, appressoria and strawberry plants was extracted using Tri-reagent (Sigma-Aldrich, Steinheim, Germany).

### Gene expression analysis by QRT-PCR and relative RT-PCR

Total RNA was extracted from cultured mycelia, conidia, the appressorium-formation stage or infected seedlings at 24-h intervals from 1 to 6 days after inoculation and from control non-inoculated plants. QRT-PCR of inoculated and non-inoculated seedlings was performed with poly(A)<sup>+</sup> RNA (Oligotex mRNA mini kit, Qiagen). Reverse transcription was carried out on 1  $\mu$ g total RNA treated by RQ1 DNASE (Promega) using Reverse-IT<sup>TM</sup> RTase Blend (ABgene, Epsom, UK) with an anchored oligo-dT primer. Reverse-transcribed RNA (cDNA) was stored at -20 °C. For relative RT-PCR experiments amplification was performed in 50- $\mu$ L reactions with the following components: 4  $\mu$ L cDNA, 5 U Taq DNA polymerase, 1 $\times$  Taq polymerase buffer, 0.2 mM dNTP and upstream and downstream primers to final concentrations of 0.2  $\mu$ M. RNA 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 on 1.4% agarose gels, recorded and saved

in tagged information format files for quantification using the NIH Image program. The genes encoding *C. acutatum* nitrate reductase (NR) (*nit1*) and glutamine synthetase (GS) (*gln1*) were cloned by PCR, using primers (MgNR1/MgNR3 and Moco/Nadr for *nit1* and GSfor/GSrev for *gln1*; Table 1) designed on the basis of conserved sequences. For RT-PCR-based analysis of *nit1* and *gln1* expression levels, we used primers CanRfor/CanRev and GSfor/GSrev, respectively (Table 1). A fragment of the *C. acutatum*  $\beta$ -tubulin-encoding gene (*tub2*) was amplified and cloned using primers tub2b and geneC (Yarden and Katan, 1993; Table 1).

For QRT-PCR, reverse transcription was carried out as described for the relative RT-PCR procedure. cDNA samples were diluted 1 : 10 to the final template concentration for real-time PCR. Real-time detection was performed with ABsolute SYBR green ROX mix (ABgene) in a Rotor-Gene 3000 machine (Corbett Research, Sydney, Australia) and results analysed with the rotor-gene 6 software. The endogenous control was the  $\beta$ -tubulin gene. The calibrator used was either the appressoria-extracted sample in experiments where nitrogen availability was monitored or extracts from seedlings at the 2-day post-inoculation phase in experiments involving *in planta* expression. Primers (200  $\mu$ M) used for real-time experiments are listed in Table 1. Primer efficacy was examined by running reactions with different concentrations of template and verifying a slope of  $-3.2 \pm 0.1$  for  $\beta$ -tubulin,  $-3.5 \pm 0.4$  for *nit1*,  $-3.4 \pm 0.2$  for *gln1* and  $-5.7 \pm 0.4$  for *nir1* for a curve of cycle threshold ( $C_T$ ) values vs. the log values of template concentrations. A mixture of all cDNAs used for all the treatments was used as template for calibration curves. Relative quantification was calculated on the basis of  $\Delta\Delta C_T$ . The  $\Delta C_T$  value was determined by subtracting the  $C_T$  results for the target gene from that for 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 control (appressoria stage or inoculated seedlings 2 days post-inoculation) treatment (relative quantification). Each experiment was repeated three times, with similar results, and results for one experiment are presented. The averages and the standard deviation presented are the results of three independent cDNA measurements from the same RNA samples from the same treatments. Mean comparisons of  $2^{-\Delta\Delta C_T}$  values were calculated using LSD, according to the Tukey-Kramer multiple comparison test at  $P < 0.05$ .

### Disruption of *nir1*

The fungal transformation vector pHA-1.3 (Redman and Rodriguez, 1994) was used as a source for the *E. coli* hygromycin phosphotransferase (*hph*) gene regulated by promoter and terminator elements from the *trpC* gene of *A. nidulans*. The *hph* gene was excised from pHA-1.3 by digestion with *Sal*I, and subcloned into corresponding sites of pBluescript II KS (Stratagene, La

Jolla, CA) to increase the number of available restriction sites. The resulting vector was named ks:hyg and used as a PCR template to introduce new *Bgl*II and *Kpn*I sites (for primers used, see Table 1). The product was cloned into a pGEM-T vector (Promega) to form pGEM:hyg. The *hph* gene was then excised with *Bgl*II and *Kpn*I and subcloned into the corresponding sites in the *nir1* plasmid to produce *nir1*:hyg3. In the replacement vector *nir1*:hyg3, the *hph* cassette is flanked on the 5' and 3' ends by 2.7 and 0.9 kb, respectively, of sequences homologous to the *nir1* locus. The *nir1*:hyg3 vector was digested with *Bal*I and *Sna*BI at positions 375 and 5666 bp, respectively, and the resulting 5.3-kb linear fragment was used to transform germinated conidia of *C. acutatum*. The *nir1*:hyg3 vector is shown diagrammatically in Fig. 8.

### Molecular analysis of targeted gene-disruption mutants

Inactivation of *nir1* was performed by one-step gene replacement. To screen for the replacement of *nir1*, genomic DNA of 120 independent *C. acutatum* transformants was analysed by PCR. Two pairs of primers were used to evaluate gene-disruption events or ectopic integration. The first set consisted of the *hphf*, based on the *hph* sequence (within the hyg cassette), and *Catf2* based on the endogenous *nir1* sequence which is not present in the gene-replacement construct. An event of homologous recombination occurred when a band of 2.8 kb appeared. A second pair of primers was designed, flanking the *hph* cassette (149WT4 at position 2481 and 149HpaI2B at position 4959; Table 2 and Fig. 7). In the case of ectopic integration, two bands were expected, at ~2500 and 565 bp, whereas homologous recombination occurred when the small band was no longer evident.

Subsequently, the *nir1*-replaced strains were tested for pathogenicity and growth properties on minimal medium supplemented with nitrate as the sole nitrogen source.

### Production of nitrate-non-utilizing mutants

Chlorate medium, based on minimal medium emended with 15 g/L KClO<sub>3</sub>, was used to generate *nit* mutants (Correll *et al.*, 1987). Puhalla's minimal nitrate agar (MM) (Puhalla, 1985), a sucrose-salt medium containing nitrate as the nitrogen source, was used to recognize *nit* mutants and for complementation (heterokaryon) tests. Mycelial plugs were placed aseptically in the middle of plates containing chlorate media and incubated at 25 °C. Fast-growing sectors emerging from the restricted colonies were transferred to MM plates and examined after a 4-day incubation period. Colonies with a thin expansive mycelium that were unable to utilize nitrate as a sole nitrogen source and did not produce (consequently grew as thin expansive colonies with no) aerial hyphae (mycelium) on MM were considered to be *nit* mutants. Nitrate, nitrite and hypoxanthine media were used for partial

phenotyping of *nit1* or *nitM*. Complementation between *nit* mutants was tested on MM and heterokaryons were usually evident within 10 days. Mutants capable of forming prototrophic heterokaryons were classified as harbouring mutations at different loci.

### Assay of conidial development on plastic artificial surface following application of cAMP, caffeine or the PKA inhibitor KT5720

cAMP was purchased from Calbiochem, caffeine and KT5720 from Sigma. Stock solutions of cAMP and caffeine were prepared in dH<sub>2</sub>O whereas stocks of KT5720 were prepared in dimethyl sulfoxide. Assays for induction of appressorium formation and germination by Ca5 mutant and wild-type strains in the presence of PKA agonists and the inhibitor were performed on glass slides, as described above.

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