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Transcriptional profiling and functional analysis of heterokaryon incompatibility in *Neurospora crassa* reveals that reactive oxygen species, but not metacaspases, are associated with programmed cell death

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Heterokaryon incompatibility (HI) is a nonself recognition phenomenon occurring in filamentous fungi that is important for limiting resource plundering and restricting viral transfer between strains. Nonself recognition and HI occurs during hyphal fusion between strains that differ at *het* loci. If two strains undergo hyphal fusion, but differ in allelic specificity at a *het* locus, the fusion cell is compartmentalized and undergoes a rapid programmed cell death (PCD). Incompatible heterokaryons show a macroscopic phenotype of slow growth and diminished conidiation, and a microscopic phenotype of hyphal compartmentation and cell death. To understand processes associated with HI and PCD, we used whole-genome microarrays for *Neurospora crassa* to assess transcriptional differences associated with induction of HI mediated by differences in *het-c pin-c* haplotype. Our data show that HI is a dynamic and transcriptionally active process. The production of reactive oxygen species is implicated in the execution of HI and PCD in *N. crassa*, as are several genes involved in phosphatidylinositol and calcium signalling pathways. However, genes encoding mammalian homologues of caspases or apoptosis-inducing factor (AIF) are not required for HI or programmed cell death. These data indicate that PCD during HI occurs via a novel and possibly fungal-specific mechanism, making this pathway an attractive drug target for control of fungal infections.

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INTRODUCTION

The hallmark growth habit of filamentous fungi is the interconnected mycelial network that makes up a fungal colony (Glass *et al.*, 2004). Hyphal fusion within a single colony is required to form the mycelial network, which

permits transport of cytoplasm, organelles and nutrients. Hyphal fusion events can also occur between colonies that are genetically different (nonself fusion). The stability of heterokaryons formed by nonself fusion events is dependent on allelic specificity at nonself recognition loci termed *het* (for heterokaryon) or *vcg* (for vegetative compatibility group) (Garnjobst & Wilson, 1956; Glass & Dementhon, 2006; Leslie & Zeller, 1996; Saupé, 2000). If individuals that differ in *het* allelic specificity undergo hyphal fusion, the fusion cell is rapidly compartmentalized by septal plugging and dies (Biella *et al.*, 2002; Jacobson *et al.*, 1998; Marek *et al.*, 2003). Heterokaryon incompatibility (HI) is associated with restriction of viral transfer between fungal individuals and prevention of resource plundering (Biella *et al.*, 2002; Debets & Griffiths, 1998; Debets *et al.*, 1994; van Diepeningen *et al.*, 1997). Hyphal death during HI shows some features that are similar to apoptosis in mammalian cells, including shrinkage of plasma membrane, membrane-bound vesicle formation, DNA condensation and TUNEL-positive nuclei (Biella *et al.*, 2002; Jacobson *et al.*, 1998; Leslie & Zeller, 1996; Marek *et al.*, 2003). Markers

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Abbreviations: BAGEL, Bayesian analysis of gene expression levels; DCF, dichlorofluorescein; DIC, differential interference contrast; GPI, glycosylphosphatidylinositol; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HI, heterokaryon incompatibility; IP3, inositol 1,4,5-triphosphate; MAPK, mitogen-activated protein kinase; PCD, programmed cell death; ROS, reactive oxygen species; PHS, phyto-sphingosine; *TS inc*, temperature-sensitive incompatible.

The expression data associated with this paper have been deposited at the *Neurospora* functional genomics database (<http://www.yale.edu/townsend/Links/ffdatabase/introduction.html>) (Experiment ID# 50).

Four supplementary tables and a supplementary figure are available with the online version of this paper.

associated with apoptosis have also been reported upon exposure of various filamentous fungi to treatment with H₂O₂, acetic acid, farnesol, sphingolipids and amphotericin B (Castro *et al.*, 2008; Chen & Dickman, 2005; Cheng *et al.*, 2003; Leiter *et al.*, 2005; Mousavi & Robson, 2004; Phillips *et al.*, 2003; Semighini *et al.*, 2006).

One of the best-studied HI systems is the *Neurospora crassa* *het-c pin-c* (Glass & Kaneko, 2003; Glass & Dementhon, 2006). The *het-c* locus, which encodes a glycine-rich plasma-membrane protein (Sarkar *et al.*, 2002), has three specificities, *het-c1*, *het-c2* and *het-c3* (Kaneko *et al.*, 2006; Saupe & Glass, 1997; Wu *et al.*, 1998; Wu & Glass, 2001). The *pin-c* locus encodes a protein with a conserved, filamentous-fungal-specific domain termed HET (Kaneko *et al.*, 2006), which is found in many genes involved in HI (Fedorova *et al.*, 2005; Glass & Dementhon, 2006). Similar to *N. crassa het-c*, three allelic variants also occur at *pin-c* (Kaneko *et al.*, 2006) (Fig. 1). Alleles at *het-c* and *pin-c*

show severe linkage disequilibrium, such that isolates can be classified into one of three *het-c pin-c* haplotypes: *het-c1 pin-c1*, *het-c2 pin-c2* and *het-c3 pin-c3*. Alleles at *het-c* have previously been shown to be under balancing selection (Wu *et al.*, 1998), consistent with the role of the *het-c pin-c* haplotypes in mediating nonself recognition. HI requires genetic interactions between alternate *het-c* and *pin-c* alleles (i.e. *het-c1* and *pin-c2*) and interactions between alternate *het-c* alleles (i.e. *het-c1* and *het-c2*) (Fig. 1) (Glass & Dementhon, 2006; Kaneko *et al.*, 2006; Sarkar *et al.*, 2002). Although *het* loci have been cloned and characterized in both *N. crassa* and *Podospira anserina* (Glass & Dementhon, 2006; Saupe, 2000), relatively little is known about how interactions between alternate incompatibility proteins translate into the phenotype of HI and cell death.

In this study, we exploited the fact that the *het-c2/pin-c1* interaction in laboratory strains is naturally temperature sensitive. Thus, a heterokaryon between a *het-c1 pin-c1*

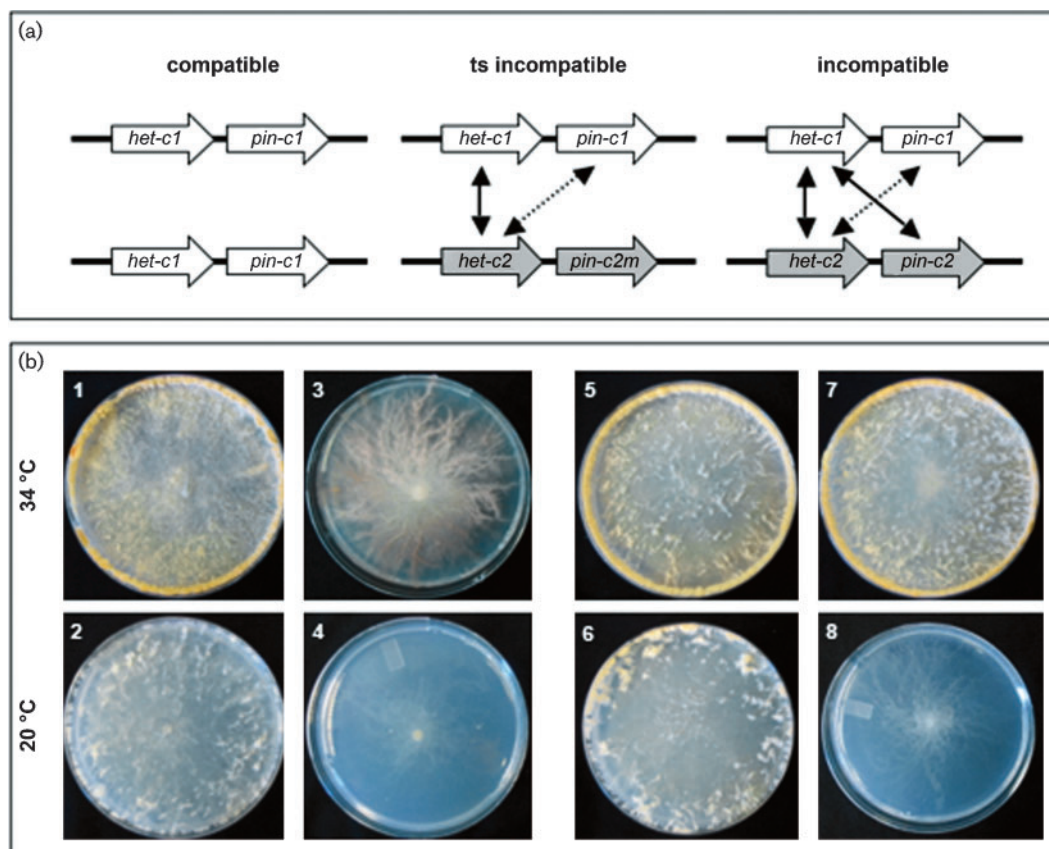


Fig. 1. *het-c* and *pin-c* genetic interactions during HI. (a) Interactions between *het-c* and *pin-c* are required for nonself recognition (arrows), while interactions between *het-c1* and *het-c2* contribute to the severity of the HI phenotype (Kaneko *et al.*, 2006). The *het-c2-pin-c1* interaction is naturally temperature-sensitive (shown by dotted arrows). (b) Heterokaryons carrying identical alleles at *het-c* and *pin-c* show no genetic interactions and are fully compatible at all temperatures (plates 1 and 2; FGSC 4564 + FGSC 6103). Heterokaryons of alternate *het-c pin-c* haplotype (*het-c1 pin-c1* + *het-c2 pin-c2*) are incompatible at all temperatures (plates 3 and 4; FGSC 4564 + JH1). The temperature-sensitive incompatible (*TS inc*) heterokaryon (*het-c1 pin-c1* + *het-c2 pin-c2^m*) is fully compatible at 34 °C, but is incompatible at 20 °C (plates 7 and 8; XK81 + FGSC 456). Plates 5 and 6 contain a compatible heterokaryon (*het-c2 pin-c2* + *het-c2 pin-c2^m*; XK81 + Xa-3).

strain and a *pin-c2* loss-of-function mutant strain (*het-c2 pin-c2^m*) is compatible and shows reduced cell death when grown at 34 °C. When transferred to 20 °C, this strain rapidly induces HI and high levels of cell death (Kaneko *et al.*, 2006). We utilized this temperature-sensitive incompatible heterokaryon to evaluate morphological and transcriptional alterations associated with HI as a measure of how the physiological status of the colony was altered by the induction of HI, using oligonucleotide arrays representing all annotated genes for *N. crassa* (Kasuga *et al.*, 2005; Tian *et al.*, 2007). From expression profiling data, we predicted and showed that reactive oxygen species (ROS), phosphatidylinositol and calcium signalling pathways play a role early during HI and programmed cell death (PCD). However, homologues to genes involved in apoptosis in higher eukaryotic species, such as caspases (metacaspases) and apoptosis-including factor (AIF), were not required for HI in *N. crassa*.

METHODS

Strains and growth conditions. All strains (see Supplementary Table S1, available with the online version of this paper) were grown on Vogel's minimal medium (Vogel, 1956) or Bird's minimal medium (BMM) (Metzenberg, 2004) with supplements as required, and crossed on Westergaard's medium (Westergaard & Mitchell, 1947). Heterokaryons with the helper strain (FGSC 4564) were used as females in crosses (Perkins, 1984). Transformations were performed as described by Margolin *et al.* (1997). Heterokaryons were forced by co-inoculation of conidial suspensions of each pair of isolates onto minimal medium. For profiling experiments, two BMM plates per time point were overlaid with sterilized cellophane circles and inoculated with 8 mm plugs of hyphae. Heterokaryons were grown in constant light for 16 h at 34 °C prior to transfer to 20 °C. Plates were sampled at each time point by peeling the cellophane/hyphae and freezing immediately in liquid N₂. The transcriptional profiling experiment was repeated twice in its entirety.

Microarray construction and hybridization. Microarrays were constructed and performed as described by Tian *et al.* (2007). Briefly, RNA was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturer's protocol and cleaned using the RNeasy miniprep protocol (Qiagen). cDNA was prepared using the ChipShot Indirect cDNA Synthesis and labelling protocol from Promega/Corning (Promega) according to the manufacturer's protocol with the following exceptions: dried cDNA was resuspended in 9 µl 0.06 M sodium bicarbonate, and quenching was accomplished by the addition of 4.5 µl 4 M hydroxylamine. Hybridizations were performed using Pronto kits (Promega) according to the manufacturer's protocol and as described by Tian *et al.* (2007). Images were acquired with an Axon GenePix 4000B scanner and GenePix Pro 6 software (Molecular Devices); each slide was also examined manually.

Data analysis. Spots with intensities greater than the background plus 3 standard deviations and less than 0.02 % of pixels saturated were selected for further analysis. We used a closed-circuit design for microarray analysis (Townsend & Hartl, 2002; Townsend, 2003) (see Fig. 3a), and determined relative gene expression levels using Bayesian Analysis of Gene Expression Levels (BAGEL) (Townsend & Hartl, 2002; Townsend, 2004). All expression data are deposited at the *Neurospora* functional genomics database (<http://www.yale.edu/townsend/Links/ffdatabase/introduction.html>).

Genes were clustered based on their differential expression in the incompatible heterokaryon relative to the compatible, accounting for confidence intervals (Eisen *et al.*, 1998). Comparability of the two replicate experiments was assessed in two ways. Firstly, cDNA from the same conditions at the same time point in the two experiments was co-hybridized to a single slide and the raw data were visualized as a scatter plot. Secondly, correlations were calculated using Microsoft Excel for the normalized BAGEL data at time 0 for the two experiments, comparing compatible heterokaryons in the two experiments, incompatible heterokaryons in the two experiments, and compatible vs incompatible heterokaryons for the combined experimental data.

Methylene blue and DCF staining. Segments of cellophane and mycelium were laid on slides and stained with a 0.003 % (w/v) solution of the vital dye methylene blue (Suzuki *et al.*, 2000). For ROS detection, compatible and incompatible heterokaryons were grown on sterile cellophane laid over BMM plates. Sections of cellophane with hyphae from the edges of the colony were cut, laid on a microscope slide and treated with 10 µM 2',7'-dihydrochlorofluorescein diacetate (H2DCFDA), which is oxidized by ROS to dichlorofluorescein (DCF). Heterokaryons were incubated with the dye solution for 1–5 min prior to microscopic examination. Images were acquired under differential interference contrast (DIC) and under fluorescence with a Zeiss Axioskop II fluorescence microscope using OpenLab 4.0.3 software. Images were resized in Adobe Photoshop CS. The proportion of dead (methylene blue) or fluorescent (DCF) compartments was counted as a percentage of the total number of compartments in an image at each time point. Calculations were replicated 10–20 times per sample and per time point, and the mean taken.

Construction of strains. The $\Delta pp-1$ and $\Delta mak-2$ strains are ascospore lethal, so we used the *Diploid* (*Dip*) mutant strain (FGSC 9537) for strain construction. In *Dip* crosses, about 2/3 of the ascospores are large and diploid. Streaking *Dip* progeny onto sorbose plates result in restoration of haploid strains. First, *Dip* was crossed with a *his-3* strain (R15-07) to obtain a *his-3; Dip* strain (R23-20; Supplementary Table S1). This strain was crossed to $\Delta pp-1$ and $\Delta mak-2$ strains to construct *his-3; \Delta pp-1* and *his-3; \Delta mak-2* strains (Supplementary Table S1). Strain genotype was verified by PCR. Other deletion strains were crossed with *het-c1 pin-c1* strains and *het-c2 pin-c2* strains with auxotrophic markers to construct strains for heterokaryon tests (Supplementary Table S1).

RESULTS

Transfer of the temperature-sensitive incompatible (*het-c1 pin-c1+het-c2 pin-c2^m*) heterokaryon to permissive temperature results in rapid induction of cell death

Under laboratory conditions, strains of alternate *het* specificity can be forced to grow as heterokaryons by plating individuals with complementary nutritional requirements onto minimal medium; growth is extremely slow, conidiation is suppressed (Fig. 1) and hyphal compartmentation and death occurs in ~30–35 % of the hyphal compartments (Kaneko *et al.*, 2006; Perkins, 1988b; Xiang & Glass, 2002). Forced heterokaryons of identical *het* specificity are indistinguishable from a wild-type strain (Fig. 1). When grown at 34 °C, the macroscopic phenotype of the temperature-sensitive (*het-c1 pin-c1+het-c2 pin-c2^m*) (*TS inc*) heterokaryon is also indistinguishable

from a wild-type heterokaryon (Kaneko *et al.*, 2006), but shows a typical HI phenotype upon transfer to 20 °C (Kaneko *et al.*, 2006) (Fig. 1). To evaluate the rapidity of induction of HI and PCD in the *TS inc* heterokaryon, we assessed both the macroscopic and microscopic events associated with the induction of HI over an 8 h time-course. As a control, we used a heterokaryon between the *het-c2 pin-c2^m* mutant strain and a strain of identical *het-c2 pin-c* haplotype (*het-c2 pin-c2 arg-5; pan-2 A*; Supplementary Table S1). The macroscopic phenotype of this heterokaryon was identical to that of a wild-type strain at all temperatures.

The *het-c2 pin-c2* compatible and *TS inc* heterokaryons were grown for 16 h at 34 °C and then transferred to 20 °C. Prior to transfer, the compatible and the *TS inc* heterokaryons showed a similar macroscopic and microscopic phenotype (Fig. 2, time 0; Table 1). Although few macroscopic differences are apparent between the compatible and the *TS inc* heterokaryons following transfer to 20 °C (Fig. 2a), many differences are evident at a microscopic level (Fig. 2b). Hyphal compartments in the *TS inc* heterokaryon became highly vacuolated and exhibited a granular appearance (Fig. 2b, row I). Using the vital dye methylene blue, many compartmentalized and

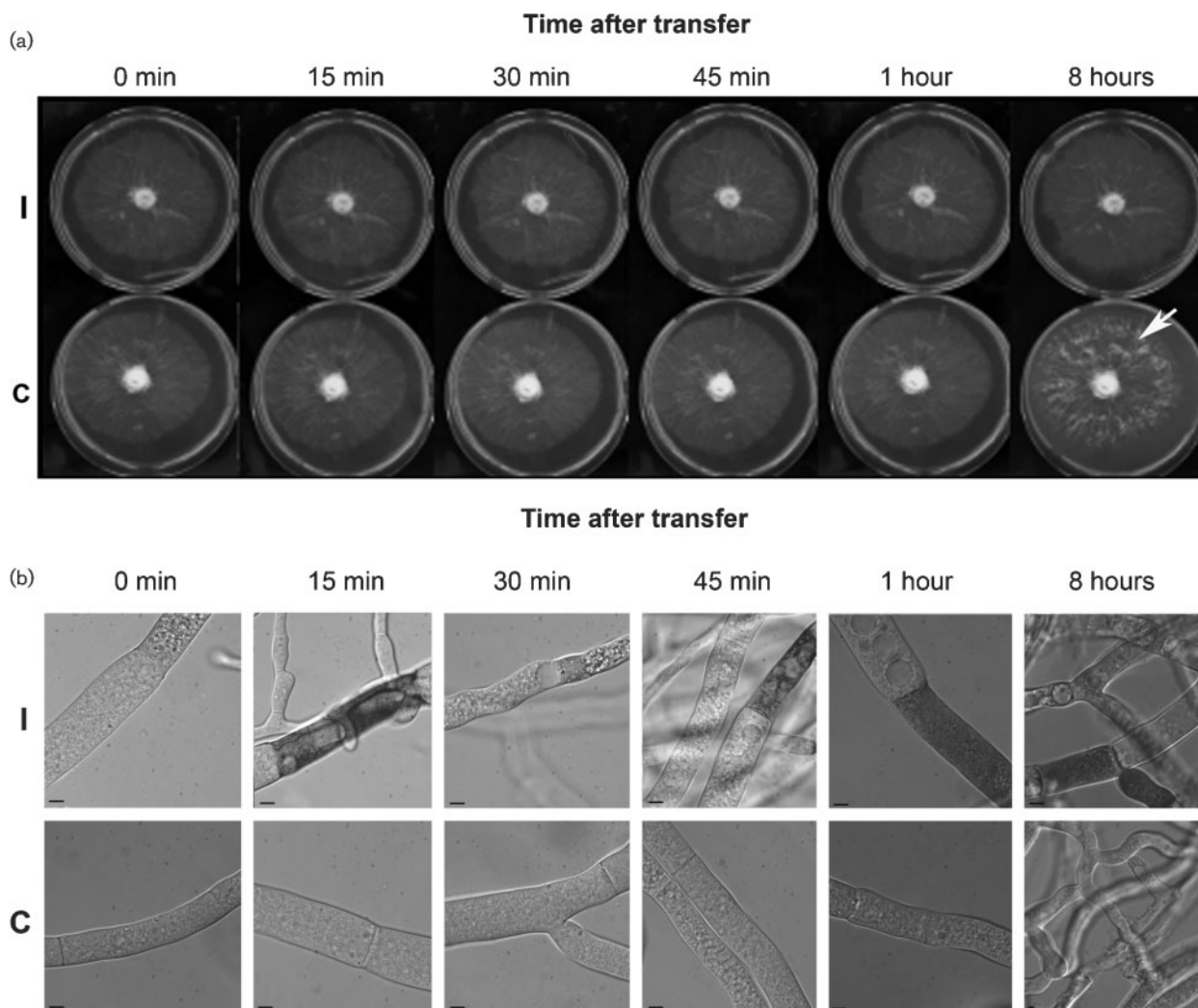


Fig. 2. Macroscopic and microscopic characteristics of HI. (a) Growth of a heterokaryon between XK81 and FGSC 456 (temperature-sensitive incompatible, *TS inc*, row I) compared to growth of a heterokaryon between XK81 and Xa-3 (compatible; row C) grown at permissive temperature for HI (20 °C). Both heterokaryons were grown at 34 °C for 16 h (0 min) and then transferred to 20 °C, and photographs of heterokaryons growing on Petri dishes were taken at the time points indicated following shift to 20 °C. The arrow shows conidiation observed in the compatible heterokaryon after 8 h, while the *TS inc* heterokaryon shows no growth or conidiation during the same time period. (b) DIC micrographs of hyphae from the *TS inc* (row I) and compatible (row C) heterokaryons stained with the vital dye methylene blue and photographed prior to transfer (0 min) and at the indicated time points after transfer to 20 °C.

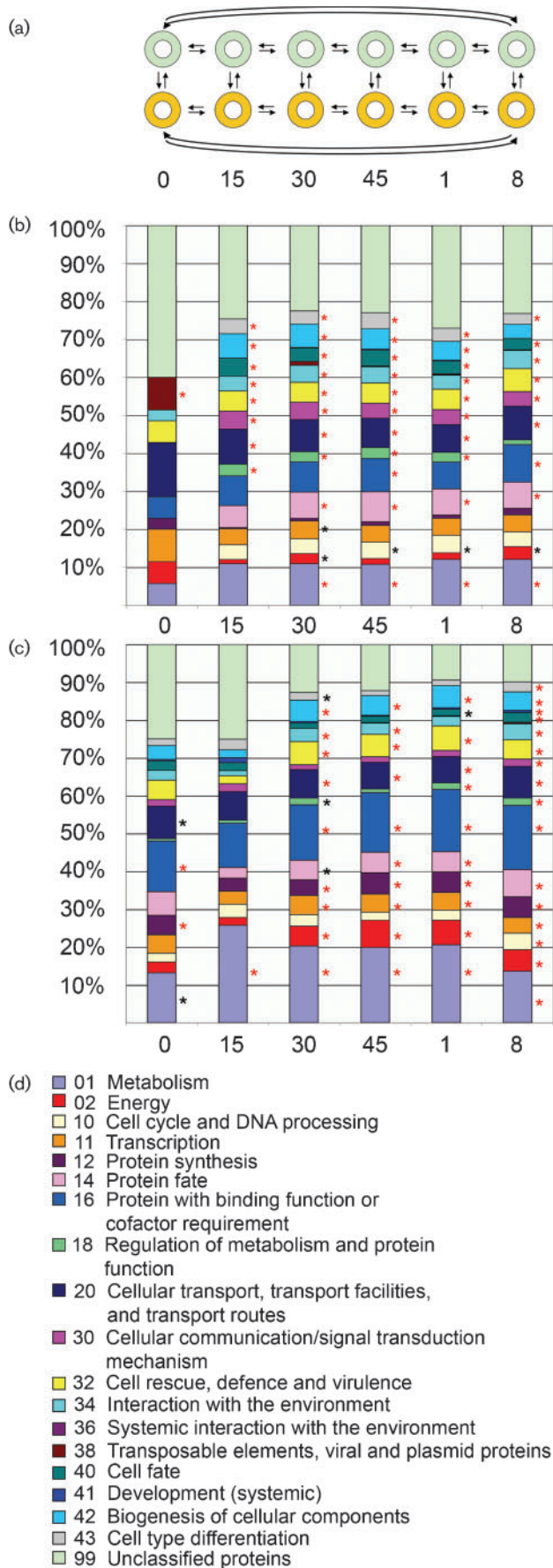


Fig. 3. Functional categories of up- and downregulated genes during HI. (a) Closed-circuit experimental design used for microarray analysis. Doughnuts represent sampled cultures; arrows represent hybridizations, where the arrowhead points to the sample labelled with Cy5 and the tail points to the sample labelled with Cy3. Green, compatible heterokaryon (XK81 + Xa-3); orange, *TS inc* heterokaryon (XK81 + FGSC 456). Time points of analysis are indicated below. (b) Functional categories of genes that were upregulated in the *TS inc* heterokaryon at time points before and after transfer to 20 °C. (c) Functional categories of genes that were downregulated in the *TS inc* heterokaryon at time points before and after transfer to 20 °C. Asterisks indicate statistically over-represented functional categories (red asterisks indicate $P < 0.01$, black asterisks indicate $P < 0.05$). (d) Key for functional categories shown in (b) and (c).

dead hyphal segments were observed in the *TS inc* heterokaryon (Fig. 2b, row I), which were evident as early as 15 min after transfer. The percentage of dead hyphal compartments greatly increased in the *TS inc* heterokaryon, reaching a peak of ~37% at 1 h after transfer (Table 1). Macroscopic morphological differences between the compatible and the *TS inc* heterokaryons were apparent after ~8 h of incubation at permissive temperature. As shown in Fig. 2(a), the compatible heterokaryon grew to the edge of the plate and produced aerial hyphae and conidia (row C), while the *TS inc* heterokaryon showed little growth and remained aconidial (row I).

Transcriptional profiling reveals that the expression of a large number of genes is elevated during induction of HI

The rapid induction of HI and PCD upon transfer of the *TS inc* heterokaryon to permissive temperature makes it an attractive model for assessing physiological responses associated with HI. Although growth rate is severely reduced (from 7 cm day⁻¹ to 1–2 cm day⁻¹), only ~30% of the hyphal compartments are dead, even when all nuclei carry an incompatible *het-c pin-c* haplotype (Glass & Kaneko, 2003; Mylyk, 1975; Perkins, 1988a). Thus, we anticipated that transcriptional profiling would reveal physiological processes associated both with cell death and with cell survival.

We sampled RNA from the *het-c2 pin-c2* compatible and the *TS inc* heterokaryons prior to transfer (0 min) and at 15 min, 30 min, 45 min, 60 min and 8 h post-transfer to permissive temperature. Expression levels of a number of candidate genes were verified using quantitative RT-PCR (Supplementary Fig. S1). Strong statistical support was obtained for relative expression levels of 3447 genes (Supplementary Table S2). To identify variation in expression profiles at each time point between the compatible heterokaryon and the *TS inc* heterokaryon, the data were grouped into clusters of genes whose expression levels significantly increased or decreased in the *TS inc* heterokaryon relative to the compatible

Table 1. Percentage of dead hyphal compartments in the compatible (Xa-3+XK81) and TS incompatible (XK81+FGSC 456) heterokaryons

| Time point | Compatible heterokaryon | TS incompatible heterokaryon |
|------------|-------------------------|------------------------------|
| 0 min | 8.4 % | 11.6 % |
| 15 min | 15.4 % | 20.7 % |
| 30 min | 17.1 % | 32.9 % |
| 45 min | 14.0 % | 31.5 % |
| 1 h | 10.0 % | 37.1 % |
| 8 h | 7.8 % | 22.4 % |

heterokaryon at each time point. Thus, for each time point, two clusters ('UP' and 'DOWN') for a total of 12 clusters were generated (Supplementary Table S2). Genes that showed a statistically significant difference in expression levels for each cluster were evaluated by functional category analysis using FunCat (<http://mips.gsf.de/projects/funcat>) (Ruepp *et al.*, 2004) (Fig. 3b–d). Fig. 3(b) shows the fraction of genes in each category in each 'UP' cluster, while Fig. 3(c) shows the fraction of genes in each category in the 'DOWN' clusters. Asterisks indicate functional categories that were statistically over-represented in each cluster.

Prior to transfer, the compatible and *TS inc* heterokaryons were similar in expression patterns, although a number of genes in specific pathways showed transcriptional differences even at this time point (Fig. 3b, c; time 0). The functional category 'Transposable elements, viral and plasmid proteins' was enriched in the UP category (27 genes) in the *TS inc* heterokaryon at time 0, the only time point during the entire time-course that showed enrichment for this category. There were more genes in the DOWN category at time 0 (218 genes), showing enrichment of functional categories 'Protein synthesis' and 'Metabolism'. These differences could possibly be due to the fact that the *TS inc* and compatible heterokaryons are not completely isogenic. However, upon transfer, expression profiling revealed a rapid and diverse transcriptional response to HI, with the numbers of both down- and upregulated genes increasing to ~500–700.

At all time points after transfer (15 min to 8 h), genes in the functional categories 'Cellular transport, transport facilities and transport routes' ($P < 0.001$), 'Cellular communication/signal transduction mechanism' ($P < 0.0001$), 'Cell rescue, defence and virulence' ($P < 0.01$), 'Interaction with the environment' ($P < 0.05$), 'Cell fate' ($P < 0.0001$) and 'Cell type differentiation' ($P < 0.001$) increased in relative expression level in the *TS inc* heterokaryon (Fig. 3b). Within the 'Cellular communication/signal transduction mechanism' functional category, genes involved in enzyme-mediated signal transduction (e.g. kinases, GTPases, heterotrimeric G proteins), second messenger signal transduction and transmembrane signal transduction pathways showed a significant increase in

expression level (Supplementary Table S2). In addition, a number of genes involved in phosphatidylinositol, Ca^{2+} and cAMP signalling pathways also showed increased expression levels at the early time points, including the metacaspase-2 homologue NCU09882.

The functional category 'Metabolism' was enriched at all time points in the DOWN categories (Fig. 3c). In the remaining time points, genes within functional categories 'Energy' ($P < 1.5 \times 10^{-13}$), 'Transcription' ($P < 0.01$), 'Protein synthesis' ($P < 0.0005$), 'Protein fate' ($P < 0.05$), 'Protein with binding function or cofactor requirement' ($P < 0.0005$), 'Regulation of metabolism and protein function' ($P < 0.05$), 'Cellular transport, transport facilities and transport routes' ($P < 0.05$), 'Cell rescue, defence and virulence' ($P < 5 \times 10^{-9}$) and 'Biogenesis of cellular components' ($P < 5 \times 10^{-8}$) were all enriched. The reduction in the expression in genes within these functional categories is consistent with the severe reduction in growth rate associated with HI.

It is possible that the induction of HI is associated with stress responses, ultimately resulting in cell death. We therefore evaluated whether the transcriptional changes in the *TS inc* heterokaryon were similar to other stress-response profiles reported in *N. crassa* (Tian *et al.*, 2007; Videira *et al.*, 2009). We first compared the HI transcriptional profile (30 min time point) to the transcriptional profile of *N. crassa* treated with the cell-death-inducing reagent phytospingosine (PHS) (Videira *et al.*, 2009); treatment of germinated conidia with $10 \mu\text{g PHS ml}^{-1}$ resulted in ~30% cell death as assayed via vital dye staining. Of the 386 genes induced during HI, 147 overlapped with the 659 genes induced during PHS treatment, a significant enrichment ($P < 0.005$) (Supplementary Table S3). Functional categories enriched in the overlapping dataset included 'Cellular communication/signal transduction' ($P < 6 \times 10^{-7}$), 'Cell fate' ($P < 2 \times 10^{-6}$), 'Cell rescue, defence and virulence' ($P < 1 \times 10^{-4}$) and 'Protein fate' ($P < 2 \times 10^{-4}$). Enriched subcategories included many signal transduction categories such as protein kinases, modification by phosphorylation, calcium binding, second messenger signalling and regulation of protein activity. In particular, three genes that showed very high expression levels during HI – NCU04554 (putative endochitinase), NCU05309 (putative RNase) and NCU05693 (putative GTPase) (Table 2; asterisks) – were also upregulated in the PHS dataset.

To determine whether the overlap dataset between HI- and PHS-induced genes represented a general stress response, we also compared the transcriptional profile of *N. crassa* undergoing HI to the profile of *N. crassa* undergoing amino acid starvation (Tian *et al.*, 2007). Unlike the HI/PHS comparison, only 15 genes in the HI dataset (336 genes) overlapped with the amino acid starvation response (334 genes), which was not significant. However, of the 491 genes that showed reduced expression level in the *TS inc* heterokaryon, 35 genes overlapped the set of genes that

Table 2. Genes tested for function in HI

| Gene name/ID† | MIPS-defined function | Fold induction at 30 min |
|---|--|--------------------------|
| Phosphatidylinositol-related genes | | |
| NCU01047 | Related to inositol polyphosphate 5-phosphatase OCRL-1 | 3.23 |
| NCU04379* | Probable regulator of phosphatidylinositol-4-OH kinase protein | 6.92 |
| NCU06245* | Related to 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase (phospholipase C) | 2.67 |
| NCU06877 | Related to phosphatidylinositol transfer protein | 2.22 |
| NCU10397 | Related to phosphatidylinositol 4-kinase | - |
| Calcium-related genes | | |
| NCU06177 | Related to calcium/calmodulin-dependent protein kinase C | 3.33 |
| NCU06366* | Related to vacuolar Ca ²⁺ /H ⁺ antiporter | 2.03 |
| Highly upregulated genes | | |
| NCU04554* | Probable endochitinase class V precursor | 34 |
| NCU05309* | Related to Cut9 interacting protein Scn1 | 62 |
| NCU05693* | Related to RBTMx2 protein | 36 |

*Genes upregulated following exposure to PHS (Videira *et al.*, 2009).

†Gene name/ID from Borkovich *et al.* (2004) and the Broad Institute (<http://www.broad.mit.edu/annotation/genome/neurospora/Home.html>).

showed reduced expression during amino acid starvation ($P < 0.005$). Sixteen of these genes in the HI dataset also overlapped with the 103 genes downregulated during PHS treatment. Within this overlap dataset, almost half of the genes are predicted to be involved in metabolism or protein synthesis. Thus, downregulation of genes involved in both metabolism and protein synthesis may be a part of a general stress response in *N. crassa*.

Screening mutants in HI-induced genes for effects on cell death

We constructed homozygous deletion strains (Dunlap *et al.*, 2007) (Supplementary Table S1) of alternate *het-c pin-c* haplotype (*het-c* pin-c**) via crosses to determine whether deletions of selected genes affected HI and/or cell death. We first evaluated strains containing deletions of three genes that showed a significant increase in expression level in both the HI and PHS datasets (Table 2). The NCU04554 gene encodes an endochitinase (*gh18-5*), NCU05309 encodes a hypothetical protein similar to the *Schizosaccharomyces pombe* gene *scn3*, which has an RNase domain (Kimata & Yanagida, 2004), and NCU05693 contains a dynamin domain and is homologous to an Mx (myxovirus resistance) GTPase in mouse and human (Sadler & Williams, 2008). NCU05693 was also recently identified as a dsRNA-activated gene in *N. crassa* (Choudhary *et al.*, 2007).

Heterokaryons of alternate *het-c pin-c* haplotype (*het-c* pin-c**) with homozygous deletions of *gh18-5* (NCU04554) or NCU05309 showed an identical incompatibility and cell death phenotype to that of a wild-type incompatible heterokaryon (Fig. 4a). A heterokaryon of *het-c* pin-c** with homozygous deletions in NCU05693 had a similar macroscopic phenotype to a wild-type incompatible

heterokaryon, but showed significantly more cell death (~35 %; Fig. 4a). These data suggest that NCU05693 may function to promote cell survival in an incompatible heterokaryon.

The functional category of cellular communication and signal transduction was enriched in the UP category at almost all of the time points in the *TS inc* heterokaryon (Fig. 3b), including many genes in the phosphatidylinositol signalling pathways (Supplementary Table S4; FunCat 30.01.09.11, $P < 0.05$). Phosphatidylinositol is found in the membranes of all eukaryotic species, and derivatives of this molecule function in diverse cellular processes, including cell signalling, cell motility, GPI protein anchoring, vesicular trafficking, cytoskeleton remodelling and cytoprotection (Michell, 2008; Strahl & Thorner, 2007). Deletion strains were available for predicted phosphatidylinositol signalling genes including NCU01047 (inositol polyphosphate 5-phosphatase), NCU04379 (regulator of phosphatidylinositol-4-OH kinase), NCU06245 (*plc-1*; phospholipase C), NCU06877 (phosphatidylinositol transfer protein) and NCU10397 (phosphatidylinositol 4-kinase) (Table 2). NCU04379 and NCU06245 also showed high relative expression levels during PHS-induced cell death (Videira *et al.*, 2009). However, heterokaryons of alternate *het-c pin-c* haplotype and carrying homozygous deletions of NCU01047, NCU04379, NCU06245, NCU06877 or NCU10397 (Supplementary Table S1) showed a typical HI phenotype, with cell death percentages comparable to a wild-type incompatible heterokaryon (Fig. 4).

In addition to the phosphatidylinositol signalling pathway, enrichment for genes involved in calcium signalling, homeostasis, transport and binding was also observed in the *TS inc* heterokaryon (Supplementary Table S4; FunCat 30.01.09.03, $P < 0.05$). Calcium is involved in multiple

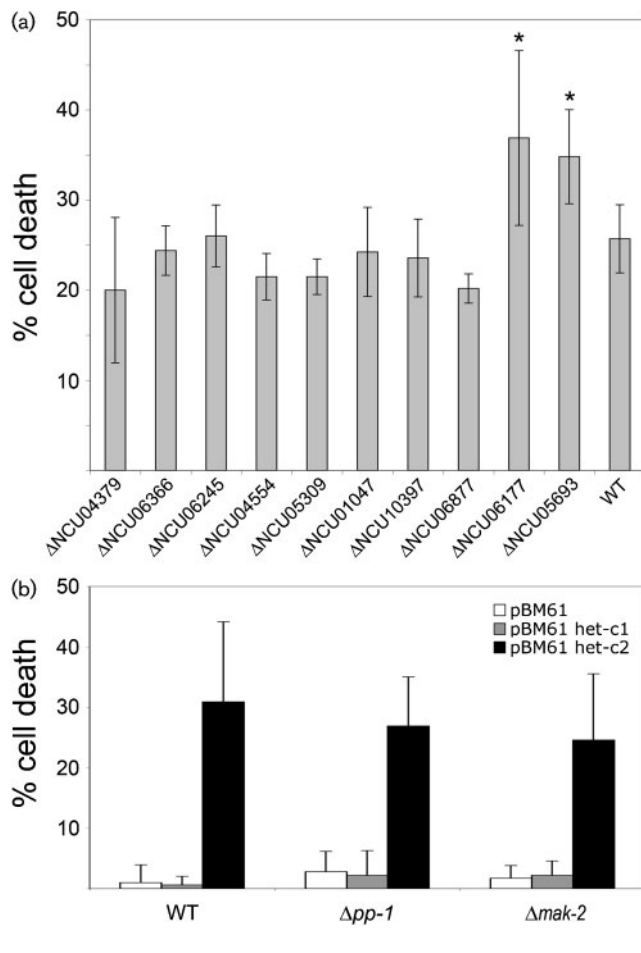


Fig. 4. Effect of deletion mutations on the induction and phenotype of HI. (a) Cell death percentages in heterokaryons carrying homozygous deletions of the indicated genes, but of alternate *het-c* *pin-c* haplotype compared to cell death in a wild-type incompatible heterokaryon. (b) Cell death percentages in wild-type (FGSC 6103), $\Delta pp-1$ and $\Delta mak-2$ transformants bearing either *het-c1*, *het-c2* or pBM61 integrated at the *his-3* locus. Numbers are means \pm SEM of 10 plates, and for each plate 50–100 cells were counted.

aspects of growth and development in filamentous fungi, including circadian rhythms and pathogenesis (Borkovich *et al.*, 2004). One of the canonical Ca^{2+} signalling pathways involves the hydrolysis of phosphatidylinositol 4,5-diphosphate by phospholipase C into 1,2-diacylglycerol and inositol 1,4,5-triphosphate (IP3), resulting in protein kinase C activation and Ca^{2+} release (Strahl & Thorner, 2007). We therefore constructed strains of alternate *het-c** *pin-c** haplotype carrying deletions of NCU06177, encoding calcium/calmodulin-dependent protein kinase C (PKC) and NCU06366, which encodes a protein related to vacuolar Ca^{2+}/H^+ antiporter; NCU06366 also showed increased expression levels during PHS-induced cell death (Videira *et al.*, 2009). The phenotype of the homozygous $\Delta NCU06366$ *het-c** *pin-c** heterokaryons was indistin-

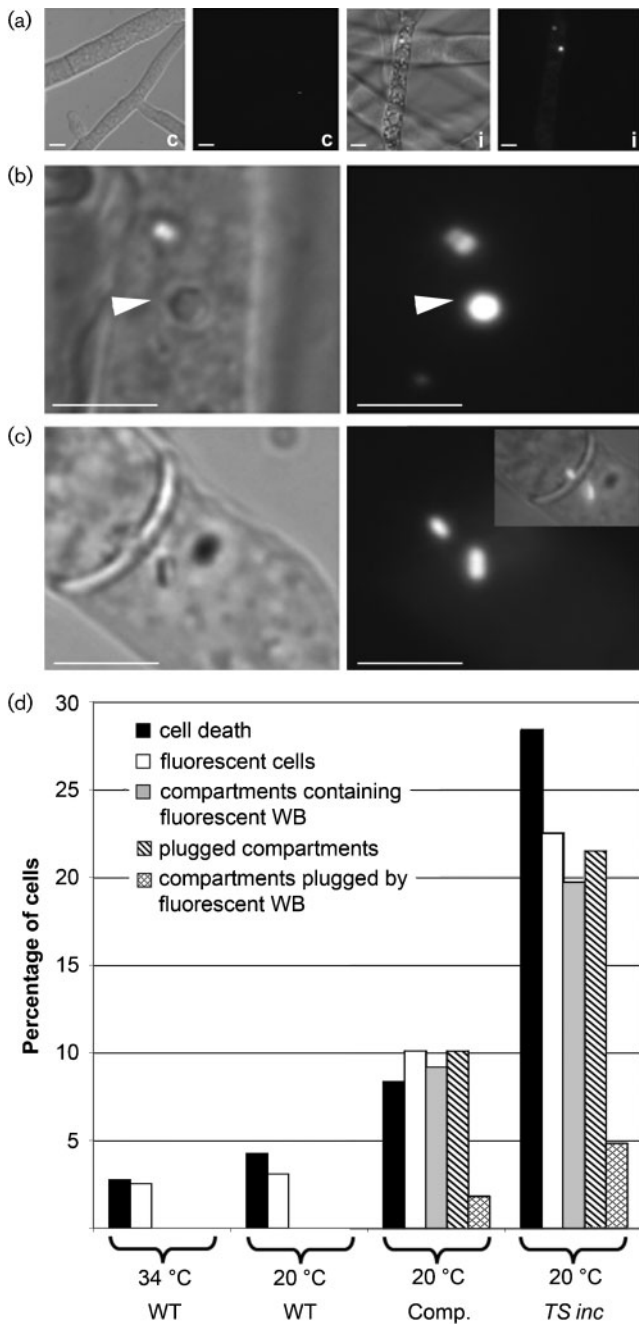
guishable from that of a wild-type incompatible heterokaryon. Although the $\Delta NCU06177$ *het-c** *pin-c** heterokaryon was macroscopically similar to an incompatible heterokaryon, it exhibited a statistically significant increase in cell death (Fig. 4a). *N. crassa* contains two PKC homologues; NCU06544 (*pkc*) has been shown to be involved in regulating the *N. crassa* circadian clock via modification of the transcription factor White Collar-1 (Arpaia *et al.*, 1999), while the function of NCU06177 has not been characterized.

Among the enriched subcategories within the ‘Cellular communication/signal transduction mechanism’ functional category were genes encoding protein kinases. Two well-characterized members of a MAPK pathway in *N. crassa* are *pp-1* and *mak-2*. The *pp-1* gene (NCU00340) encodes a transcription factor homologous to *Saccharomyces cerevisiae* *STE12*, while *mak-2* encodes a homologue of *FUS3* (Li *et al.*, 2005); *STE12* and *FUS3* constitute part of the pheromone response signal transduction pathway. In *S. cerevisiae*, inappropriate stimulation of this pathway results in cell death that exhibits apoptotic features (Severin & Hyman, 2002; Zhang *et al.*, 2006). The $\Delta pp-1$ and $\Delta mak-2$ mutants are also defective in hyphal fusion (Fleissner *et al.*, 2008), suggesting the possibility of an interaction between the cell death and cell fusion pathways.

Because both $\Delta pp-1$ and $\Delta mak-2$ mutants fail to form heterokaryons, we evaluated the effect of these mutations on HI by a transformation protocol (Sarkar *et al.*, 2002; Saupe *et al.*, 1996; Saupe & Glass, 1997; Wu & Glass, 2001). We constructed *his-3*; *het-c1 pin-c1*; $\Delta pp-1$ and *his-3*; *het-c1 pin-c1*; $\Delta mak-2$ strains using a *Dip* (Diploid) strain because mutations in both $\Delta pp-1$ and $\Delta mak-2$ result in ascospore lethality (see Methods; Supplementary Table S1). Wild-type, $\Delta pp-1$ and $\Delta mak-2$ strains were transformed with pBM61-*het-c1* or pBM61-*het-c2* (Margolin *et al.*, 1997). The *het-c1 pin-c1*; $\Delta pp-1$ and *het-c1 pin-c1*; $\Delta mak-2$ transformants bearing the *het-c1* allele showed an identical phenotype to the untransformed and pBM61 vector control strains (growth rate ~ 1.8 cm day $^{-1}$ and ~ 1.4 cm day $^{-1}$, respectively). The *het-c1 pin-c1*; $\Delta pp-1$ and *het-c1 pin-c1*; $\Delta mak-2$ transformants bearing the *het-c2* allele showed a reduced growth rate (0.8 cm day $^{-1}$ and 0.7 cm day $^{-1}$, respectively) and wild-type levels of cell death (Fig. 4b).

ROS are induced during HI

Many proteins play a role in the reaction to oxidative stress, either in the reduction of oxygen radicals or in the induction of other genes that respond to oxidative stress. A subset of these genes showed increased expression levels in the *TS inc* heterokaryon, including genes for generation of ROS (NADPH oxidase *nox-1*; NCU02110) and ROS scavengers (glutaredoxin, NCU01219; *cyc-1* cytochrome *c*, NCU01808) (Supplementary Table S2). ROS and oxidative stress have been implicated in apoptosis in mammals, *S.*



cerevisiae and other filamentous fungi (Chandra *et al.*, 2000; Chen & Dickman, 2005; Ludovico *et al.*, 2005; Madeo *et al.*, 1999; Semighini *et al.*, 2006).

The compatible and *TS inc* heterokaryons were evaluated for the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) to dichlorofluorescein (DCF), an indicator dye for ROS (LeBel *et al.*, 1992). As shown in Fig. 5, ROS were generated in the cytoplasm of *TS inc* heterokaryon within 5–15 min post-transfer to permissive conditions, but not in the compatible heterokaryon. The percentage of ROS-producing compartments in the *TS inc* heterokaryon was similar to the percentage of dead

Fig. 5. ROS are produced during HI and are associated with Woronin bodies. (a) As assessed by DCF fluorescence, the presence of ROS was infrequent in a compatible heterokaryon (c; XK81 + Xa-3). DCF fluorescence was observed when HI was induced in the *TS inc* heterokaryon (i; XK81 + FGSC 456). DCF fluorescence was within discrete hyphal compartments, indicating that the septal pores were plugged in these compartments. (b) Bright dots of fluorescence co-localized with Woronin bodies (WBs) (arrowheads). WB are visible in the DIC images (distinguished by their hexagonal shape) and fluoresced brightly when stained with DCF. (c) Fluorescent WBs were only occasionally associated with plugged septa (merged image in inset). (d) Cell death and DCF fluorescence was quantified in strains Xa-3 (WT) and XK-81 (data not shown) at 34 °C and 20 °C. Data for XK-81 did not differ statistically from those for Xa-3. No fluorescent WBs were observed. Cell death and DCF fluorescence were quantified in the compatible heterokaryon (Xa-3 + XK81) and the *TS inc* heterokaryon (XK81 + FGSC 456) at 20 °C. The number of dead hyphal compartments with fluorescent WB and how often fluorescent WB were associated with septa was quantified in the compatible (Xa-3 + XK81) and *TS inc* (XK81 + FGSC 456) heterokaryons. DCF staining indicated an almost complete overlap between compartments that were dead, fluorescent, contained fluorescent WB, and were plugged. However, less than 5% of the plugged compartments showed fluorescent WB at the septa.

compartments (Fig. 5d). The compatible heterokaryon (Xa-3 + XK81) showed a slight elevation in the percentage of dead/fluorescent cells as compared to parental strains (Fig. 5d), most likely due to strain differences.

In the *TS inc* heterokaryon, DCF fluorescence was concentrated at organelles that resembled the peroxisome-like Woronin body (Fig. 5b). Woronin bodies are membrane-bound modified peroxisomes found in the cytoplasm of filamentous ascomycete species and which plug the septal pore following catastrophic injury (Jedd & Chua, 2000; Markham & Collinge, 1987; Tenney *et al.*, 2000); previous studies have associated an unknown peroxidase/catalase activity with Woronin bodies (Schliebs *et al.*, 2006; Tenney *et al.*, 2000). In HI, septal plugging is a rapid response during *het-c pin-c* incompatibility that occurs less than 5 min post-fusion (Glass & Kaneko, 2003). DCF fluorescence in hyphal compartments in the *TS inc* heterokaryon was always associated with Woronin bodies, although these were only occasionally observed at septa (Fig. 5c, d). These data indicate that although ROS were associated with Woronin bodies during HI, septal plugging during HI is independent of the Woronin body.

Homologues of caspases or apoptosis-inducing factor (AIF1) are not required for HI or PCD in *N. crassa*

The induction of PCD in a number of systems includes activation of a MAPK cascade, accumulation of ROS, release of cytochrome *c* from mitochondria and activation

of caspases (Chandra *et al.*, 2000; Stridh *et al.*, 1998) (reviewed by Adams, 2003). A survey of mammalian and fungal proteins known to play a role in PCD revealed that ~42 genes are conserved in filamentous fungal genomes (Fedorova *et al.*, 2005). The 'Cell death and apoptosis' functional category of genes was enriched at time points 15 min to 1 h (FunCat 40.10.02, $P < 0.05$). Of the 42 *N. crassa* cell death homologues, 11 increased in expression level in the *TS inc* heterokaryon upon induction of HI (Table 3), including genes encoding metacaspase (NCU09882 and NCU02400), cytochrome *c* (NCU01808) and heat-shock protein HSP70 (NCU09602). We chose a subset of these genes to evaluate for HI phenotypes.

Caspases are important executors of the apoptotic pathway in metazoan animals and are present in the cell as inactive zymogens. Upon exposure to an apoptotic signal they are activated via cleavage (executioner caspases) or oligomerization (initiator caspases) (Adams, 2003). In *S. cerevisiae*, the single metacaspase gene, *YCA1*, has been implicated in cell death pathways in response to a variety of chemicals and stresses (Guaragnella *et al.*, 2006; Herker *et al.*, 2004; Madeo *et al.*, 2002; Mazzoni & Falcone, 2008). We constructed strains of alternate *het-c** *pin-c** haplotype containing deletions of predicted metacaspase genes (Δ NCU02400 and Δ NCU09882) and strains of alternate *het-c** *pin-c** haplotype containing deletions of both predicted metacaspase genes (Δ NCU02400 Δ NCU09882) (Supplementary Table S1). However, all the *het-c** *pin-c** Δ NCU09882, Δ NCU02400 and Δ NCU09882 Δ NCU02400 heterokaryons showed a typical HI phenotype, with cell death percentages similar to a wild-type incompatible heterokaryon (Fig. 6). These data indicate that metacaspases are not required for PCD during HI in *N. crassa*.

We also tested whether deletions of the apoptosis-inducing factor (AIF) homologue in *N. crassa* (NCU05850) affected HI or cell death. In metazoans, AIF1 is released from mitochondria during apoptosis, and is predicted to act in

the caspase-mediated apoptotic pathway (Eisenberg *et al.*, 2007; Modjtahedi *et al.*, 2006). Mutations in NCU05850 (Δ *aif-1*) result in strains that show resistance to PHS-induced cell death (Castro *et al.*, 2008), while *aif-1* deletion strains in *Aspergillus nidulans* are more sensitive to farnesol-induced cell death (Savoldi *et al.*, 2008). Heterokaryons carrying homozygous Δ *aif-1* deletions, but of alternate *het-c* *pin-c* haplotype, showed an identical phenotype to a wild-type incompatible heterokaryon (Fig. 6). These data indicate that major components of the apoptotic pathway are not required for cell death during HI, implying that a novel and perhaps fungal-specific PCD pathway is functioning during nonself recognition and HI in filamentous ascomycete fungi.

DISCUSSION

In this study, we assessed transcriptional alterations associated with HI and PCD in *N. crassa* and from these data evaluated the role of various genes/processes implicated in HI and PCD. Analysis of the induction of HI in *N. crassa* has a number of advantages over studies of PCD in other organisms. First of all, the induction of HI and PCD is a very rapid response (Biella *et al.*, 2002; Garnjobst & Wilson, 1956; Glass & Kaneko, 2003; Sbrana *et al.*, 2007). For both metazoan apoptosis and plant-induced PCD, a significant time lag occurs between induction and cell death (Adams, 2003; Reape *et al.*, 2008; Reed, 2000). Second, PCD induced during HI does not require the application of exogenous substances. Thus, studies on HI are in contrast to many studies of apoptosis in *S. cerevisiae* and other filamentous fungi where responses to treatment with toxic metabolites are evaluated (for reviews, see Jin & Reed, 2002; Lu, 2006; Ramsdale, 2008). In the literature, controversy exists over whether cell death in *S. cerevisiae* is a process with real similarities to apoptosis in metazoan cells (Modjtahedi *et al.*, 2006; Vachova & Palkova, 2007;

Table 3. Predicted homologues of apoptotic genes that increase in relative expression level during HI

| Protein† | Name/ID‡ | MIPS-defined function | Largest fold induction (time) |
|------------|--------------------|---|-------------------------------|
| Cyc1p | NCU01808 | Cytochrome <i>c</i> | 2.4 (8 h) |
| Yca1p | NCU09882; NCU02400 | Related to metacaspase 2; probable caspase | 6.3 (30 min); 4.0 (45 min) |
| Hel10p | NCU04928 | Conserved hypothetical protein | 2.9 (1 h) |
| Uth1p | NCU02668 | Related to cell cycle regulation and ageing protein | 2.1 (45 min) |
| FadA/GpaA | NCU06493 | GNA-1 G protein alpha chain | 4.4 (1 h) |
| Ste4p/CGB1 | NCU00440 | Gnb-1 G protein beta subunit | 2.3 (45 min) |
| Lag1p | NCU00008 | Probable longevity-assurance protein LAG1 | 2.9 (1 h) |
| Ppa1p | NCU09747 | Probable vacuolar ATP synthase 22 kDa proteolipid subunit | 2.2 (1 h) |
| TEL1 | NCU00247 | Hypothetical protein | 3.1 (45 min) |
| HSP70 | NCU09602 | HSP70 heat-shock protein 70 (hsp70) | 2.1 (45 min) |

†Predicted apoptotic and PCD homologues according to Fedorova *et al.* (2005).

‡Gene name/ID from Borkovich *et al.* (2004) and the Broad Institute (<http://www.broad.mit.edu/annotation/genome/neurospora/Home.html>).

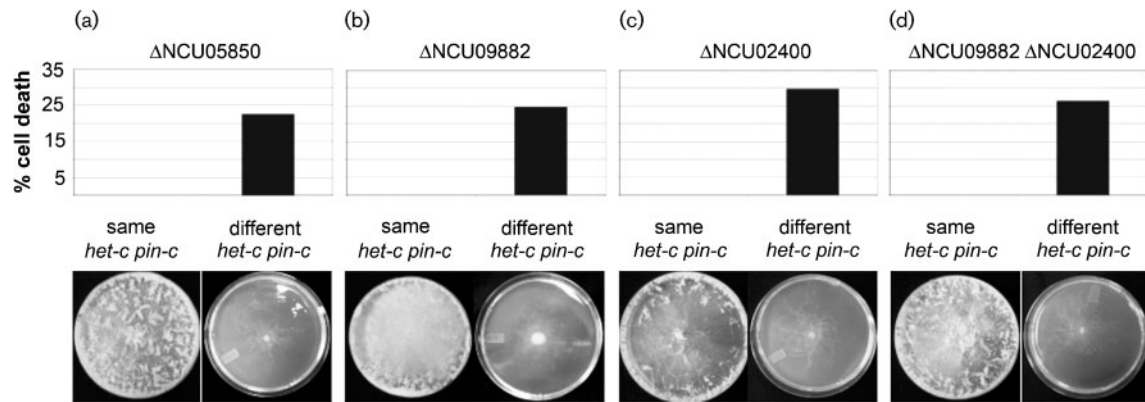


Fig. 6. Effect of the deletion of predicted cell death genes on HI. (a) Heterokaryons carrying homozygous deletions of $\Delta aif-1$ (NCU05850) but of alternate *het-c pin-c* haplotype showed a typical macroscopic HI phenotype and WT levels of cell death. (b, c) Heterokaryons carrying homozygous deletions of predicted metacaspase genes (NCU09882 or NCU02400) but of alternate *het-c pin-c* haplotype show a macroscopic and microscopic phenotype indistinguishable from a WT incompatible heterokaryon. (d) A heterokaryon between two strains carrying homozygous deletions of both predicted metacaspase genes (NCU09882 and NCU02400), but of alternate *het-c pin-c* haplotype, show a typical HI phenotype and cell death percentages.

Vercammen *et al.*, 2007), primarily because of the difficulty of assessing mechanisms of cell death following exposure to toxic compounds and because of secondary effects of mutations in genes that affect the physiology of cells responding to exposure to death-inducing agents.

Mutations in the *S. cerevisiae* metacaspase gene *YCA1* result in strains that are resistant to cell death induced by a variety of stresses/chemicals (Guaragnella *et al.*, 2006; Herker *et al.*, 2004; Khan *et al.*, 2005; Madeo *et al.*, 2002; Mazzoni & Falcone, 2008). However, strains containing mutations in the two predicted metacaspase genes (Δ NCU02400, Δ NCU09882 and Δ NCU02400 Δ NCU09882) and an AIF1 (Δ NCU05850) homologue did not affect any phenotypic aspects associated with HI in *N. crassa*. Although HI displays some downstream characteristics of apoptosis observed in metazoans (Jacobson *et al.*, 1998; Leslie & Zeller, 1996; Marek *et al.*, 2003), the induction of death via nonself recognition and HI may occur by a filamentous-fungal-specific process. Many genes required for HI in filamentous fungi encode proteins containing a HET domain (PF06985), including PIN-C (Kaneko *et al.*, 2006), and which are ubiquitous in the genomes of filamentous ascomycete species, but are notably absent from all other eukaryotic and prokaryotic species. Importantly, the overexpression of just the HET domain is sufficient to induce an HI-like phenotype in *P. anserina* (Paoletti & Clave, 2007). Thus, the filamentous-ascomycete-specific HI pathway is an attractive system to consider for development of novel fungal-specific drugs for plant and human filamentous ascomycete pathogens.

Expression profiling provided insight not only into the physiological processes occurring during the induction of cell death, but also into the cellular response in hyphal compartments of the colony that survive. The transcrip-

tional response to HI induction overlapped significantly with the transcriptional profile for PHS-induced cell death for both up- and downregulated gene sets, suggesting a common cellular response to the induction of cell death/cell survival. However, both the HI and PHS datasets overlapped significantly with the downregulated gene set identified during amino acid starvation, which likely represents a general response to a broad range of stresses in *N. crassa*. The upregulation of genes for the synthesis and signalling pathways of phosphatidylinositol as well as the Ca^{2+} signalling pathways indicate that these two processes may be coordinately regulated during HI. Little is known about phosphatidylinositol signalling in *N. crassa*, although there is evidence for IP₃-activated Ca^{2+} release from vacuoles (Cornelius *et al.*, 1989; Silverman-Gavrila & Lew, 2002). We hypothesize that *N. crassa* responds to HI by increasing phosphatidylinositol synthesis and modification that eventually promotes Ca^{2+} release from the vacuole, which further stimulates the activity of Ca^{2+} -binding proteins and other Ca^{2+} -dependent processes involved in incompatibility. In other systems, IP₃-dependent Ca^{2+} signalling has been implicated in both cell survival and cell death pathways (Rong & Distelhorst, 2008).

Septal plugging is associated with HI, occurring within 5 min of fusion between incompatible strains (Biella *et al.*, 2002; Garnjobst & Wilson, 1956; Glass & Kaneko, 2003; Newhouse & MacDonald, 1991; Sbrana *et al.*, 2007). Catastrophic injury of hyphae in filamentous ascomycete species results in the rapid plugging of septa by the Woronin body; strains containing mutations in the gene, *hex-1*, encoding the crystalline body core of the Woronin body, show extensive cytoplasmic bleeding (Jedd & Chua, 2000; Markham & Collinge, 1987; Tenney *et al.*, 2000). In this study, we observed DCF fluorescence associated with Woronin bodies during HI. Woronin bodies are a modified

peroxisome (Jedd & Chua, 2000; Liu *et al.*, 2008; Tenney *et al.*, 2000) and ROS production has been associated with peroxisomes in a wide variety of organisms (Schrader & Fahimi, 2006). In this study, genes encoding NADPH oxidase, glutaredoxin and cytochrome *c* also increased in relative expression during HI induction. These enzymes are typically involved in ROS responses within the cell (Eisenberg *et al.*, 2007; Takemoto *et al.*, 2007). These results suggest that ROS are generated during induction of HI and may be a downstream effector of PCD.

It is likely that upon nonself recognition and HI, activation of multiple cell death and cell survival pathways is induced. In this case, it is unlikely that most single gene deletions would have a large effect on the HI phenotype. However, mutations in two genes, NCU06177 (encoding a PKC homologue) and NCU05693 (encoding a GTPase), significantly increased cell death in *het-c* pin-c** heterokaryons. It is likely that these genes may be involved in promoting cell survival in cells adjacent to the dying compartments during HI. In *P. anserina*, genes encoding proteins involved in autophagy were identified as being preferentially expressed during HI (Pinan-Lucarre *et al.*, 2003). Similar to the Δ NCU06177 and Δ NCU05693 mutants, mutations in genes essential for autophagy in *P. anserina* resulted in strains showing accelerated cell death during HI (Pinan-Lucarre *et al.*, 2005). Future experiments will delineate the roles of these pathways on cell death and cell survival during HI in *N. crassa*.

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