Functional characterization of the three mitogen-activated protein kinase kinases (MAP2Ks) present in the *Cryphonectria parasitica* genome reveals the necessity of Cpkk1 and Cpkk2, but not Cpkk3, for pathogenesis on chestnut (*Castanea* spp.)

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SUMMARY

The biological function(s) of the *cpkk1*, *cpkk2* and *cpkk3* genes, encoding the three mitogen-activated protein kinase kinases (MAP2Ks) of *Cryphonectria parasitica*, the causal agent of chestnut blight, were examined through knockout strains. Cpkk1, the Mkk1 orthologue, acts in a phosphorylation cascade essential for cell integrity; Cpkk2 is the Ste7 orthologue involved in the pheromone response pathway; Cpkk3 is the Pbs2 orthologue, the MAP2K activated during the high-osmolarity response. Our analysis confirmed the role of each MAP2K in its respective signalling cascade with some peculiarities: abnormal hyphae with a reduced number of septa and thinner cell walls were observed in $\Delta cpkk1$ mutants, and a strong growth defect on solid media was evident in $\Delta cpkk2$ mutants, when compared with the controls. Virulence on chestnut was affected in both the $\Delta cpkk1$ and $\Delta cpkk2$ strains, which were also unable to complete the developmental steps essential for mating. No alterations were reported in $\Delta cpkk3$, except under hyperosmotic conditions and in the presence of fludioxonil. $\Delta cpkk2$ mutants, however, showed higher sensitivity during growth in medium containing the antibiotic G418 (Geneticin).

Keywords: chestnut blight, *Cryphonectria parasitica*, growth, MAP kinase, mating, pathogenesis.

INTRODUCTION

The filamentous ascomycete *Cryphonectria parasitica* is the causal agent of chestnut blight, which has devastated the chestnut forestry of North America since the early 20th century (Dawe and Nuss, 2013). Infection of the fungus with specific hypoviruses (*Hypoviridae* family) is known to cause hypovirulence, resulting in biological control (Milgroom and Cortesi, 2004). Strains containing *Cryphonectria hypovirus 1* (CHV1), the best-characterized

hypovirus type member, display characteristic symptoms, such as reduced or altered sporulation, laccase production, pigmentation, oxalate accumulation, virulence expression and interference with the secretory pathway (Dawe and Nuss, 2013; Turina and Rostagno, 2007). As these phenotypic changes are pleiotropic and take place in a coordinated and specific manner, it has been suggested that hypoviruses perturb one or more regulatory pathways (Nuss, 2005). Preliminary studies have suggested that signal transduction components may be targets for CHV1 in inducing hypovirulence. Previous reports have elucidated the roles of elements belonging to the heterotrimeric G-protein and mitogen-activated protein kinase (MAPK) families (Nuss, 2010).

In microbial and higher eukaryotes, MAPK pathways play pivotal roles in intracellular and intercellular signalling. Development, growth and adaptation to the environment are regulated physiological processes orchestrated by MAPKs, which are composed of three functionally interlinked and successive protein kinases: MAP3K (MAP kinase kinase kinase), MAP2K (MAP kinase kinase) and MAPK. MAPK pathways are the basic modules used to transduce signals through a stepwise phosphorylation relay (Imajo *et al.*, 2006), and the presence of MAPK phosphatases modulates MAPK activity by dephosphorylation (Bhalla *et al.*, 2002). In filamentous fungi, MAPK pathways have established functions in three main processes: (i) the mating pheromone response, characterized by the key function of the Fus3 homologue regulating the expression of genes involved in sporulation, mating and the sexual cycle; (ii) the maintenance of cell wall integrity, centred on the activity of the Slt2 homologue, a regulator of expression of genes related to cell wall biogenesis and structure, adaptation to stress and toxic compounds; and (iii) the response to changes in osmolarity and nutrient sensing, in which the MAPK Hog1 plays a fundamental role in the activation of defence genes in response to oxidative stress, osmotic shock and threats to water homeostasis (Hamel *et al.*, 2012; Rispail *et al.*, 2009). In pathogenic fungi, MAPKs are also involved in virulence and correlated phenomena, such as appressoria formation, host penetration and tissue colonization (Zhao *et al.*, 2007).

The aim of the present study was to perform a functional characterization of the three MAP2Ks encoded by the *C. parasitica* genome: Cpkk1, Cpkk2 and Cpkk3. Recent proposed nomencla-

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ture that takes into account the yeast orthologue would denominate them Cpa-MKK1, Cpa-Ste7 and Cpa-Pbs2, respectively (Hamel *et al.*, 2012). Detailed phenotypic analyses of MAP2K mutants allowed us to assess the commonalities and specificities of the role of each MAP2K in comparison with those already studied in other filamentous fungi and phylogenetically related ascomycete species.

RESULTS

Construction of MAP2K mutants

To analyse the functions of the *cpkk1*, *cpkk2* and *cpkk3* genes in *C. parasitica*, mutants were generated by site-directed double homologous recombination during integrative transformation. To this end, a different linearized plasmid for each transformation (see Experimental procedures), containing a gene cassette conferring hygromycin resistance (*hph*) and flanked by MAP2K-specific sequences, was introduced into strain $\Delta cpku80$, a *C. parasitica* isolate highly efficient at homologous recombination (Lan *et al.*, 2008) (Fig. S1, see Supporting Information). Polymerase chain reaction (PCR) screening of transformants with *hph* and locus-specific primers (data not shown) and Southern blot analyses confirmed the gene replacements of the wild-type allele with the disrupted allele containing the *hph* fragment (Fig. S1). Western blot experiments using previously prepared specific Cpkk1 and Cpkk2 polyclonal antibodies (Turina *et al.*, 2006) detected no protein products in the respective null mutants at 24 h post-inoculation (Fig. 1). In order to avoid the possibility of random mutations occurring ectopically in the genome, two distinct knock-out strains originating from different independent transformation events were analysed for each MAP2K, and all experiments aimed at verifying phenotypic changes were performed on at least two single-spore isolates from each of the two independently obtained knockout strains. We documented and discussed as phenotypic changes caused by specific gene knockout only those common to both independently obtained strains.

Phenotypic characterization of MAP2K knockout strains

Different growth alterations were observed in MAP2K-null mutants when compared with the wild-type strain ($\Delta cpku80$ was used as the control). Under standard growth conditions on PDAMB medium [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L), 25 °C, 12 h/12 h light/dark], both $\Delta cpkk1$ and $\Delta cpkk2$ strains resulted in impaired growth with the absence of aerial hyphae (Fig. 2). Moreover, the growth of $\Delta cpkk2$ always ceased before the colony reached the edge of the plate. On the contrary, in *Endothia parasitica* (EP) complete liquid medium, only the $\Delta cpkk1$ strain exhibited a slower growth rate than the $\Delta cpku80$ isolate, resulting in a smaller amount of mycelium

Fig. 1 Western blot analyses on mitogen-activated protein kinase kinase (MAP2K)-null mutants using Cpkk1 (A) and Cpkk2 (B) polyclonal antibodies. The developed films show the absence of signal in the $\Delta cpkk1$ and $\Delta cpkk2$ strains using the respective anti-MAP2K polyclonal antibody. Equal loading of protein samples was confirmed in a parallel gel that was stained only with Coomassie blue (bottom panels in A and B).

(Table 1). The growth of $\Delta cpkk3$ was unaffected in either solid or liquid medium.

The *cpkk2*-null mutant strain also displayed a strong reduction in vegetative growth on malt agar substrate compared with growth on PDAMB. On the contrary, the other strains, including the control, were unaffected by malt agar (Fig. S2, see Supporting Information).

Changes in colour pigmentation were observed as a result of the loss of either *cpkk1* or *cpkk2* function. In comparison with $\Delta cpku80$, the $\Delta cpkk1$ strain appeared completely white, with extremely hyaline hyphae on the edge of the colony. In contrast, the *cpkk2*-null mutant showed light yellowish mycelium. No colour alterations were observed in the $\Delta cpkk3$ mutant (Fig. 2).

Growth defects in the $\Delta cpkk3$ knockout strain were only noted under acute hyperosmotic conditions. Specifically, on PDAMB supplemented with 2 M sorbitol or 4% NaCl, the $\Delta cpkk3$ strain was completely unable to grow, whereas the other mutants were compromised to a lesser extent in their growth capabilities (Table 1, Figs S3 and S4, see Supporting Information).

Laccase-type phenol oxidase activity was estimated by the colour of the reaction assay on plates containing tannic acid. A strong dark-brown halo was detected around $\Delta cpkk2$ colonies, suggesting an increase in phenol oxidase activity (Fig. 3), but such

Fig. 2 Fungal colony morphology on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] after 6 days. Knockout strains $\Delta cpkk1$, $\Delta cpkk2$, $\Delta cpkk3$ and wild-type $\Delta cpku80$ are shown. Representative photographs from one of three experiments are shown.

variation was not confirmed in liquid culture containing tannic acids (not shown), linking a possible phenol oxidase up-regulation only to growth on solid medium. No specific growth inhibition caused by tannic acid was observed, and no differences were detected between the wild-type laccase phenotype and the other two MAP2K-null mutants (data not shown).

In order to evaluate a putative involvement of the three MAP2Ks in temperature sensitivity, the diameter of each fungal colony was measured after 5 days of growth on PDAMB plates at 8, 12, 30 and 37 °C. The absence of growth was noted at the two extreme temperatures tested (8 and 37 °C) for all isolates, including the control, whereas significant differences were observed at 12 °C. More specifically, although all the isolates grew more slowly than normal, the $\Delta cpkk1$ mutant failed to grow at all (Fig. S5, see Supporting Information). At 30 °C, all strains were equally delayed in growth (data not shown).

Experiments to evaluate the responses to oxidative and detergent/surfactant stresses were also carried out. The MAP2K-null mutants and the wild-type isolate were grown on PDAMB supplemented with 0.125 mM menadione, 100 mM hydrogen peroxide (H_2O_2) or 0.005% sodium dodecylsulphate (SDS). Sensitivity to menadione was observed in the $\Delta cpkk1$ and $\Delta cpkk2$ strains, whereas absence of growth was detected for $\Delta cpkk1$ in the presence of SDS, which also partially influenced the growth of the other strains. Hydrogen peroxide proved to be extremely toxic for the $cpkk1$ -null mutant, as no growth was recorded; under the

same conditions, growth of the $\Delta cpkk2$ strain was also strongly reduced (Table 1, Figs S6–S8, see Supporting Information). Sensitivity to growth on solid media containing calcofluor white (CFW) and Congo red was evaluated: the results (Fig. S9, see Supporting Information) showed a statistically significant specific inhibition of $\Delta cpkk1$ in both media, whereas specific inhibition of $\Delta cpkk2$ was only present in CFW-containing medium.

Four different antifungal compounds commonly used for selection in *C. parasitica* were also tested at different concentrations in PDAMB medium: no difference among the various mutants and wild-type was observed for benomyl and hygromycin B at the high inhibitory concentration tested (data not shown). Surprisingly, $\Delta cpkk2$ strains exhibited specific higher sensitivity to G418 (Fig. S10, see Supporting Information): the minimal concentration to completely inhibit growth of the wild-type and $\Delta cpkk1$ and $\Delta cpkk3$ strains was 50 μ g/mL, whereas a concentration of 3.75 μ g/mL was sufficient to completely inhibit growth of $\Delta cpkk2$ strains; on the contrary, $\Delta cpkk3$ strains were fully resistant to inhibitory working concentrations of fludioxonil (Fig. S11, see Supporting Information).

Cell wall digestion assay

Resistance to cell wall-degrading enzymes was tested on each of the *C. parasitica* MAP2K-null mutants and the wild-type isolate. Starting with the same amounts of young mycelia grown in liquid medium for 16 h in the dark, the number of spheroplasts produced after 2 h of digestion was measured. Only in the case of the $\Delta cpkk1$ strain was the number of spheroplasts significantly higher than for the other strains, indicating a strong sensitivity to digestion and a low level of cell wall resistance (Fig. 4A).

Confocal microscopic observations of CFW-stained hyphae and hyphal fusion during colony growth

After CFW staining, mycelia of the MAP2K-null mutants were microscopically inspected and compared with those of $\Delta cpku80$. The $\Delta cpkk1$ strain showed thinner hyphae than the control (Fig. S12A, see Supporting Information), and the frequency of septa per length unit of mycelium was lower (Fig. 4B,C). The observed fluorescence was also lower in the $\Delta cpkk1$ strain (Fig. S12B). No differences were noted in the other MAP2K mutants (data not shown).

We also observed hyphal fusion events in the subperipheral region of a growing colony: fusions among different hyphae were commonly found within $\Delta cpkk1$, $\Delta cpkk3$ and $\Delta cpku80$ strains, but we failed to detect any fusion in $\Delta cpkk2$ strains (Fig. S13, see Supporting Information).

Mating experiments

MAP2K-null mutants (derived from $\Delta cpku80$, a mating type Mat-2 strain) were compared with the wild-type strain ($\Delta cpku80$) with

Table 1 Comparison of mitogen-activated protein kinase kinase (MAP2K)-null mutants, complemented strains and wild-type isolate ($\Delta cpku80$). Reported values are means $\pm$ standard deviation. Data followed by the same letter are not significantly different according to Tukey's test ($P < 0.05$). Colony diameter was taken after 7 days of growth.

Characteristic	$\Delta cpkk1$	$\Delta cpkk2$	$\Delta cpkk3$	$\Delta cpku80$	$\Delta cpkk1$ -compl	$\Delta cpkk2$ -compl
Growth on PDAMB (cm)	2.69 $\pm$ 0.23a	1.95 $\pm$ 0.30b	7.23 $\pm$ 0.41c	7.42 $\pm$ 0.60c	7.23 $\pm$ 0.35c	7.59 $\pm$ 0.31c
Biomass dry weight (g) from liquid culture	0.07 $\pm$ 0.01a	0.27 $\pm$ 0.03b	0.28 $\pm$ 0.01b	0.28 $\pm$ 0.02b	0.29 $\pm$ 0.04b	0.27 $\pm$ 0.01b
Growth on PDAMB + 2 M sorbitol (cm)	1.64 $\pm$ 0.14a	1.21 $\pm$ 0.18b	No	5.95 $\pm$ 0.30c	5.77 $\pm$ 0.12c	5.86 $\pm$ 0.16c
Growth on PDAMB + 4% NaCl (cm)	0.75 $\pm$ 0.41a	1.05 $\pm$ 0.11b	No	2.00 $\pm$ 0.10c	1.88 $\pm$ 0.14c	1.97 $\pm$ 0.11c
Growth on PDAMB + 0.125 mM menadione (cm)	0.76 $\pm$ 0.09a	0.89 $\pm$ 0.13a	2.33 $\pm$ 0.15b	2.41 $\pm$ 0.10b	2.18 $\pm$ 0.17b	2.27 $\pm$ 0.20b
Growth on PDAMB + 0.005% SDS (cm)	No	0.68 $\pm$ 0.10a	2.86 $\pm$ 0.33b	4.68 $\pm$ 0.34c	4.24 $\pm$ 0.54c	4.61 $\pm$ 0.27c
Growth on PDAMB + 100 mM H ₂ O ₂ (cm)	No	0.86 $\pm$ 0.12a	5.19 $\pm$ 0.32b	5.02 $\pm$ 0.23b	4.95 $\pm$ 0.31b	5.05 $\pm$ 0.27b
Asexual sporulation (conidiospores $\times 10^7$ /mL)	No	No	2.91 $\pm$ 0.15a	2.72 $\pm$ 0.25a	2.34 $\pm$ 0.18a	2.47 $\pm$ 0.11a
Stromal pustule production	No	No	Yes	Yes	Yes	Yes
Canker area on chestnut at 21 days post-inoculation (cm ²)	0.27 $\pm$ 0.14a	0.21 $\pm$ 0.06a	2.91 $\pm$ 0.69b	3.12 $\pm$ 0.58b	2.98 $\pm$ 0.58b	2.96 $\pm$ 0.54b

PDAMB, potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L); SDS, sodium dodecylsulphate.

Fig. 3 Polyphenol oxidase activity on Bavendamm's medium at 5 days post-inoculation. The level of brown coloration is correlated with the phenol oxidase activity of the strain degrading tannic acid contained in the medium. A very strong dark-brown halo was observed around the $\Delta cpkk2$ colony, whereas, in these specific experimental conditions, the colour intensity of the halo was much lower for the wild-type strain $\Delta cpku80$.

regard to the production of conidia (also serving as spermatia in mating experiments) and the ability to serve as the female parent in sexual crosses with the opposite mating type strain EP67 (Mat-1). Asexual sporulation was evaluated after 1 month of growth on PDAMB (Table 1). Conidiation was completely absent for the $\Delta cpkk1$ and $\Delta cpkk2$ strains; thus, spermatization of strain EP67 was not possible. When inoculated on agar substrate adjacent to chestnut twigs as female parent, strains $\Delta cpkk1$ and $\Delta cpkk2$ were unable to colonize the bark tissue and erupt to produce stroma and stromal pustules; no mating products were observed after the addition of a conidial suspension from strain EP67 (Fig. 5). Taken together, these findings show that the developmental impairments in both the $cpkk1$ and $cpkk2$ knockout strains are such that it was impossible to evaluate their competence for mating, either as female or male. On the contrary, mature perithecia with protruding ostiolar necks were present in abundance after 3 months in the control crosses EP67 (♀ or ♂) $\times$ $\Delta cpku80$ (♂ or ♀) and for $\Delta cpkk3$ (♂ or ♀) (Fig. 5).

Deletion of *cpkk1* and *cpkk2* reduces *C. parasitica* virulence

Pathogenicity assays were performed to evaluate whether disruption of the MAP2K genes affected virulence. Wild-type and MAP2K *C. parasitica* mutants were inoculated onto dormant European chestnut stems. At 21 days post-inoculation, the canker diameter area was measured: deletion of *cpkk1* or *cpkk2* resulted in a dramatic reduction in virulence, as the mutants were unable to form any cankers even 8 weeks after inoculation. The pathogenic behaviour was not affected in the *cpkk3* knockout strains, suggesting that Cpkk3 is dispensable for virulence (Table 1 and Fig. 6).

Complementation experiments

Considering the intriguing phenotypic changes in the *cpkk1*- and *cpkk2*-null mutants of *C. parasitica*, complementation experiments were carried out. Vectors expressing each specific MAP2K wild-type allele, and conferring resistance to G418, were assembled and used to transform the respective mutants. G418-resistant transformants were then analysed for their morphology, growth on different media, asexual sporulation and virulence. PCR analyses confirmed that the complemented transformants contained an additional wild-type allele of the specific MAP2K gene (Fig. S14, see Supporting Information). All altered functions were restored and the complemented strains were closely similar to the wild-type $\Delta cpku80$, confirming unequivocally that the differential phenotypic characters of the null mutants were caused by their gene disruptions (Table 1 and Fig. 6).

DISCUSSION

Three conserved MAPK pathways involved in the transduction of a variety of extracellular signals and the regulation of different developmental processes have been described in fungal patho-

Fig. 4 Characterization of the *cpk1*-null mutant strain. (A) Resistance to cell wall enzymatic digestion. The $\Delta cpk1$ strain showed an extreme sensitivity to enzymatic digestion, resulting in a larger number of spheroplasts per millilitre, when compared with the $\Delta cpku80$ strain ($1.7 \times 10^7 \pm 0.56 \times 10^7$ versus $0.24 \times 10^7 \pm 0.07 \times 10^7$). (B) Confocal micrographs of $\Delta cpk1$ and $\Delta cpku80$ mycelia after calcofluor white staining. Hyphae of the *cpk1*-null mutant are thinner and less septated than those of the wild-type. (C) Graphical representation of the number of septa per micrometre observed in the $\Delta cpk1$ and $\Delta cpku80$ strains (0.29 ± 0.04 versus 0.54 ± 0.12). Data were analysed with one-way analysis of variance (ANOVA), Tukey's test, $P < 0.05$.

gens (Hamel *et al.*, 2012; Rispaill *et al.*, 2009; Zhao *et al.*, 2007). The most studied and best characterized is the Fus3/Kss1 pathway, also known as the pheromone response pathway, because of its activation on binding of pheromone to its membrane receptor. This MAPK cascade regulates various phenomena of fungal biology, ranging from conidiation, gamete maturation, haploid fruiting (morphological switch from a budding yeast to hyphal growth),

hyphal differentiation and, in a number of cases, virulence (Müller *et al.*, 2003; Tschärke *et al.*, 2003; Zhao *et al.*, 2007).

The Pkc1-Slt2 signalling pathway is a phosphorylation cascade regulating cell integrity and promoting cell wall biosynthesis. Impairment of this cascade results in perturbed growth, defects in cell wall integrity and the accumulation of secretory vesicles (Irie *et al.*, 1993; Loewith *et al.*, 2000; Mazzoni *et al.*, 1993). In yeast,

Fig. 5 Stromal pustule formation on chestnut twigs. The $\Delta cpk1$ and $\Delta cpk2$ strains are unable to erupt from bark to produce stromal pustules. Photographs were taken 3 months after inoculation with each respective strain.

Fig. 6 Virulence assay on chestnut stems inferred from the area of the cankers induced by each tested strain. Photographs were taken at 21 days post-inoculation.

this MAPK pathway is also involved in responses to low osmolarity, high temperature, alkaline pH and nutrient starvation (Heinisch, 2005). Considering that the ability to maintain cell wall integrity and resistance is critical for the establishment of disease in a host, the Pkc1-Slt2 MAPK cascade has also been found to play an important role in virulence, as demonstrated for several plant-pathogenic fungi, including *Claviceps purpurea*, *Fusarium graminearum*, *Botrytis cinerea* and *Colletotrichum lagenarium* (Hou *et al.*, 2002; Kojima *et al.*, 2002; Mey *et al.*, 2002; Rui and Hahn, 2007).

The third MAPK cascade, known as the high-osmolarity glycerol (Hog) response pathway, is primarily required for growth under hyperosmotic conditions, but recent studies in yeast have also related it to resistance to non-osmotic stresses, such as arsenite, low temperature and acid pH (Mollapour and Piper, 2006; Panadero *et al.*, 2006; Thorsen *et al.*, 2006). Moreover, it is involved in fungicide resistance in *Magnaporthe oryzae* and *Neurospora crassa* (Motoyama *et al.*, 2008; Zhang *et al.*, 2002),

and in the virulence of several filamentous fungi (Hamel *et al.*, 2012).

Several MAPKs and their downstream effectors have been characterized already in *C. parasitica*. In the pheromone response pathway, Fus3 and Ste11 homologues (encoded by *cpmk2* and *cpst11*), the kinases activated by and activating MAP2K Cpkk2, respectively, have been analysed and addressed for possible role(s) in fungal development, pathogenesis and hypovirus infection (Choi *et al.*, 2005; Park *et al.*, 2012). Transcription factors, such as Ste12 and Pro1, activated by Cpmk2, and downstream target genes, such as *Mf2/1* and *Mf2/2*, have also been studied to better define the molecular mechanisms triggered by or regulating the pheromone response (Deng *et al.*, 2006; Sun *et al.*, 2009; Turina *et al.*, 2003; Zhang *et al.*, 1993).

Concerning the high-osmolarity growth cascade, only Cpmk1, the Hog1 homologue activated by MAP2K Cpkk3, has been analysed (Park *et al.*, 2004). In addition to osmosensitivity, disruption of the *cpmk1* gene resulted in several pleiotropic hypovirulence-

associated changes, such as reduced pigmentation, conidiation, laccase production and virulence expression. Moreover, this MAPK seems to be regulated by hypovirus infection after hypertonic stress (Park *et al.*, 2004).

In contrast, no elements of the cell integrity pathway have been investigated so far in *C. parasitica*, except Cpkk1, whose function was recently revealed in our laboratory using silencing techniques (Rostagno *et al.*, 2010). Experiments demonstrated that Cpkk1 plays an important role in conidiation, growth on minimal medium and virulence. A previous study has shown that hypovirus infection does not regulate mRNA accumulation of the three MAP2Ks (Rostagno *et al.*, 2009), but is able to induce hyperphosphorylation of Cpkk1, observed during active mycelial growth and juvenile status of the fungus (Turina *et al.*, 2006). Apart from these studies, knowledge of this MAPK pathway in *C. parasitica*, especially during pathogen–plant interactions, is still limited.

In the present study, we have characterized the three MAP2K genes, *cpkk1*, *cpkk2* and *cpkk3*, identified in the *C. parasitica* genome, which appear to be functional homologues of the corresponding MAPK genes in yeast and filamentous fungi. Our functional analysis generally confirmed the assignment of each MAP2K to the expected signalling cascade described above—specifically, the cell integrity pathway for Cpkk1, the high-osmolarity growth cascade for Cpkk3 and the pheromone response pathway for Cpkk2—although we could not demonstrate a direct role in mating for Cpkk2 because of major impairments in developmental processes not strictly related to mating, but necessary for mating to occur.

Cpkk1 and Cpkk2 are necessary for growth, sporulation, pathogenesis and developmental steps important for mating

Phenotypic analysis of the $\Delta cpkk1$ and $\Delta cpkk2$ strains revealed common vegetative growth defects.

The importance of MAPK cascades in fungal growth is universally recognized; therefore, it is evident that, in the absence of a correct signalling system, severe growth defects may result because of the fact that environmental conditions or internal stimuli are not well perceived and/or transduced (Lew, 2011). Nevertheless, in different fungi, the roles of MAPK homologues in vegetative growth may differ greatly, and the same MAPK deletion can give rise to diverse morphofunctional behaviour. For instance, in the basidiomycete *Ustilago maydis*, contrary to the situation in *C. parasitica*, no influence on cell morphology or growth was observed as a result of deletion of *mkk1* (Carbó and Pérez-Martín, 2010). In *M. oryzae*, mutants deleted for *Mmk2* were defective in cell wall integrity, conidiation and virulence, but not in growth rate (Mehrabi *et al.*, 2009). The *PaMkk1* mutant of the model saprophytic filamentous fungus *Podospora anserina* showed no pigmentation, differentiation of aerial hyphae or fruiting body development, but growth was

not affected (Kicka *et al.*, 2006). In addition, no influence on growth was observed in the *Mkk2* mutant of the human pathogen *Cryptococcus neoformans* (Gerik *et al.*, 2005).

Concerning the pheromone pathway, the Fuz7 (a Cpkk2 homologue) gene of *U. maydis*, similar to the situation in *C. parasitica*, has been shown to be fundamental for growth and spore formation (Kahmann and Kamper, 2004). By contrast, Ste7 homologues are dispensable for vegetative growth in *M. oryzae* and *F. graminearum* (Ramamoorthy *et al.*, 2007; Zhao *et al.*, 2005).

The impossibility of performing complete mating experiments because of the absence of stromal pustule formation on chestnut wood and a lack of sporulation suggests that both Cpkk1 and Cpkk2 are required for essential developmental prerequisites to mating, which are not mating dependent. Thus, we cannot provide direct evidence for the likely role of Cpkk2 in mating.

Considering that *C. parasitica* is a phytopathogenic fungus, effects on virulence were of major interest. Neither the *cpkk1*- nor *cpkk2*-null mutant was able to produce canker areas after artificial inoculation on chestnut stems. Among other possibilities, we envision three different scenarios to explain such behaviour: (i) the two serine/threonine kinases may influence directly the expression of genes involved in pathogenesis (thus, the absence of virulence would be a result of a direct impairment in the production of pathogenic effectors); (ii) the deletion of the MAP2K genes results in such strong destabilizing events for metabolism that even virulence is affected secondarily (therefore the fungus is avirulent, just as it was unable to mate, in response to the many defects that perturb its survival); (iii) Cpkk1 and Cpkk2 are involved, independently, in the recognition of the host plant or, in other words, in the transduction of a signal activated after the interaction with the plant; therefore, in the absence of either, the plant would not be perceived and the activation of the virulence machinery would not occur.

As reported above for filamentous growth, virulence may also be influenced in different ways by orthologous MAPKs in different fungal pathogens. Similar effects to those observed in *C. parasitica* were reported in *U. maydis*, in which the Fuz7-null mutant is non-pathogenic (Kahmann and Kamper, 2004). Ste7 orthologues also play a fundamental role in the virulence of *M. oryzae* and *F. graminearum*, (Ramamoorthy *et al.*, 2007; Zhao *et al.*, 2005).

Considering the only MAPK pathway completely characterized in *C. parasitica*—the mating response pathway— $\Delta cpmk2$, $\Delta cpkk2$ and $\Delta cpste11$ share some commonalities in their phenotype, but have three different vegetative and pathogenic behaviours. The $\Delta cpste11$ isolate showed no significant morphological changes, except for enhanced sporulation, and is still pathogenic, even if less than the wild-type (Park *et al.*, 2012). On the contrary, the $\Delta cpmk2$ strain is unable to sporulate, like the $\Delta cpkk2$ mutant, and displays reduced growth on both solid and liquid media, but is partially virulent on chestnut (Choi *et al.*, 2005). As the $\Delta cpkk2$ strain is totally apathogenic, it is likely that Cpkk2 has additional

downstream targets in addition to Cpmk2 that contribute to virulence. The knockout of one of the transcription factors (CpST12) controlled by the pheromone response cascade did not lead to impaired growth on PDA medium, but produced abundant conidia (both features strongly impaired in our $\Delta cpkk2$ mutant); however, like the latter, there was impairment of canker production on chestnut stems, but the production of stromal pustules on autoclaved chestnut twigs was normal (Deng *et al.*, 2006).

Cpkk1 is essential for correct hyphal development and cell wall structure

The $\Delta cpkk1$ strain displayed no virulence and lacked aerial mycelium; such features may be a direct consequence of the morphological problems detected through evaluation of the mycelia by confocal microscopy. More specifically, thinner hyphae, reduced frequency of septa per length unit of mycelium, thinner cell walls and hypersensitivity to enzymatic digestion were all recorded in comparison with the control $\Delta cpku80$. Moreover, reduced fluorescence after CFW staining was observed. Considering that CFW is a strong non-specific fluorochrome that binds to cellulose and chitin, a fainter signal may imply a lower or altered biosynthesis of cell wall components. Growth itself in CFW- and Congo red-containing media was impaired. These data confirmed that Cpkk1, as part of the cell integrity pathway, plays a fundamental role in the regulation of cell wall properties, such as mechanical resistance, composition, structure and susceptibility to chemical compounds, such as surfactant agents or oxidants. The observed hypersensitivity to SDS, which disrupts plasma membranes and lyses cells with cell wall defects, and to menadione or H_2O_2 , which have been used extensively to study cell integrity phenotypes in fungi (Carbó and Pérez-Martin, 2010; Nikolaou *et al.*, 2009; Rui and Hahn, 2007), may be a consequence of the abnormal cell wall composition and thickness. Mutants in the cell integrity MAPK cascade in *M. oryzae*, *Claviceps purpurea*, *Colletotrichum lagenarium*, *B. cinerea* and *F. graminearum* all show similar effects to those observed in the *C. parasitica* $\Delta cpkk1$ strain (Hou *et al.*, 2002; Kojima *et al.*, 2002; Mey *et al.*, 2002; Rui and Hahn, 2007; Xu *et al.*, 1998), but septal frequency is correlated to a functional Cpkk1 for the first time.

Cpkk1 is required for growth at low temperature

Interestingly, after a temperature growth assay, growth of the $cpkk1$ -null mutant was completely abolished at 12 °C, in comparison with other strains in which growth was only partially reduced. This result suggests that the cell integrity MAPK pathway may transduce signals derived from the perception of low temperature. Currently, we do not know exactly how the stimulus is perceived, but it is likely that low-temperature sensing receptors may be involved in the activation of the cascade. A similar outcome has been reported for the Hog response pathway in yeast (Panadero

et al., 2006), whereas the Pkc1-Slt2 pathway seems to play a role in the response to high temperature (Heinisch, 2005). These differences clearly illustrate that, although the components of the MAPK cascade are homologous in different organisms, the biological functions controlled by them may be quite dissimilar.

Hard surface perception, medium composition sensing and hyphal fusions are regulated by Cpkk2

Loss of function of Cpkk2 was responsible for the reduced growth of the $\Delta cpkk2$ mutant on solid substrates. Nevertheless, the vegetative growth rate in liquid medium was not affected. This finding suggests that Cpkk2 (but not Cpkk1) may be involved in the sensing of hard surfaces. A similar phenotype has already been reported for the $\Delta cpmk2$ mutant (Choi *et al.*, 2005). Ste-7 homologues are also involved in surface sensing in *M. oryzae*, *F. oxysporum* and *U. maydis* (Lanver *et al.*, 2010; Liu *et al.*, 2011; Pérez-Nadales and Di Pietro, 2011). How the pheromone response MAPK cascade is activated by the hard surface stimulus is still unknown. It has been proposed that the transmembrane protein Msb2 may play a 'sensor' role in surface perception upstream of the MAPK pathway.

Cpkk2 seems to be involved in some way in the growth response to substrate composition. On malt agar plates, growth of the $cpkk2$ -null mutant was strongly affected in comparison with growth on PDAMB and with the effects produced in the other two knockout strains. This finding indicates that nutrients are perceived in a different manner by $\Delta cpkk2$ and the other two MAP2K mutants. Among the possible explanations, we can envision either a direct toxic effect of the maltose medium or a lack of ability to use maltose as a carbon source specific to the $\Delta cpkk2$ strain. To our knowledge, this is the first evidence of direct involvement of a pheromone response cascade element in nutrient substrate sensing in filamentous fungi.

A further characterization of the $\Delta cpkk2$ mutants revealed increased sensitivity towards the antibiotic G418 sulphate (Geneticin) when compared with the wild-type and other MAP2K knockout strains. Such a phenotype may be related to decreased detoxification in the mutant or a higher specific sensitivity during protein translation in the specific steps affected by G418. Regulation of translation was shown to be dependent on a MAPK cascade orthologous to Cpkk2 in mammals: MNK1—a MAPK-activated protein kinase—phosphorylates directly the protein eIF4E (Cargnello and Roux, 2011), but the biological significance of such a phosphorylation event is still debated; the MNK1 catalytic domain belongs to the CAMK family of kinases and orthologous sequences are present in the *C. parasitica* genome, but, so far, they remain uncharacterized.

An additional feature of $cpkk2$ -null mutants has been noted by microscopic analysis: hyphae are not able to carry out vegetative self-fusion. This result has been reported previously in filamentous

fungi, in which elements of the pheromone response MAPK cascade or homologues have been deleted (Pandey *et al.*, 2004; Prados Rosales and Di Pietro, 2008). Vegetative hyphal fusion is a ubiquitous phenomenon during the growth of filamentous fungi: a model system for studying its genetic regulation is *N. crassa* (Fleissner *et al.*, 2009); the biological role of self-fusion seems to be related to general homeostasis, translocation of nutrients and water, developmental processes and non-meiotic gene transfer within the fungal colony (Read *et al.*, 2009). The pheromone signaling pathway has been suggested to play a role in the transduction of all of these intrahyphal communication events (Fleissner *et al.*, 2009).

Tannin degradation may be controlled by Cpkk2 on solid media

Cpkk2 may be involved in the control of the phenol oxidase activity of *C. parasitica*. Despite the slight growth inhibition on malt agar plates containing tannic acid, a strong dark brown colour was detected around colonies of the *cpkk2*-null mutant. Browning of the medium is the result of the breakdown of tannic acid by the phenol oxidase activities of extracellular fungal enzymes. Tannic acid is a toxic compound that may resemble either a breakdown product of lignin or an antimicrobial agent secreted by the host plant. As part of their defence barrier, tannic acid is abundant in plants; thus, for phytopathogenic fungi, extracellular phenol oxidase activity most probably represents a response to eliminate this compound. The strong brown halo observed around $\Delta cpkk2$ colonies may be caused by an alteration in laccase pattern expression: possibly, the absence of the pheromone response MAP2K leads to higher phenol oxidase activity.

Roles of Cpkk3 in *C. parasitica*

Cpkk3 is the putative homologue of *S. cerevisiae* Pbs2. In the present study, we observed phenotypic growth defects of the *cpkk3*-null mutant only under hyperosmotic conditions, confirming that it belongs functionally to the high-osmolarity growth response MAPK cascade. Furthermore, as *cpkk3*-null mutants showed high resistance to the phenylpyrrole compound fludioxonil, it is probable that, as observed in *N. crassa* and *M. oryzae* (Motoyama *et al.*, 2008; Noguchi *et al.*, 2007), Cpkk3 may be positioned downstream of a histidine kinase involved in sensitivity to fludioxonil.

Unlike the observations in Cpkk1 and Cpkk2, Cpkk3 does not affect vegetative growth and is dispensable for conidiation, mating, resistance to oxidative stress or heat shock, eruption from bark, dead wood colonization and virulence. The deletion of *cpmk1*, which encodes the Hog1 homologue, putatively acting downstream of Cpkk3, produced different alterations from those observed in the *cpkk3*-null mutant, such as reduced pigmentation, conidiation, laccase production and virulence (Park *et al.*, 2004).

These data suggest that Cpmk1 can either control several functions or take part in different pathways (resulting in the activation of different sets of genes) which are not influenced by the upstream MAP2K Cpkk3; alternatively, in the absence of Cpkk3, other kinases can partially complement its function. Therefore, we might speculate that Cpmk1 can be activated by various signals through different kinases and, in the absence of Cpkk3, only the 'transduction pathway' responding to high osmolarity is affected.

The opposite situation from *C. parasitica* was reported in *B. cinerea* and *Colletotrichum lagenarium*, in which the Pbs2 homologue is very important, not only for the osmolarity response, but also in pathogenesis, as these mutants failed to cause disease in host plants (Kojima *et al.*, 2002; Yan *et al.*, 2010), whereas Hog1 deletion strains were fully virulent (Kojima *et al.*, 2006; Segmuller *et al.*, 2007). In these cases, the MAP2K Pbs2 can control functions in which the downstream MAPK is not involved. This implies that, in such fungi, additional stimuli (for example, the presence of the host plant which induces the pathogenic response) can activate Pbs2.

A further case was reported for the human pathogens *Candida albicans* and *Cryptococcus neoformans*, in which both the Hog1 and Pbs2 homologues are necessary for virulence (Alonso-Monge *et al.*, 2006; Kojima *et al.*, 2006).

All of these studies suggest that osmolarity-responsive MAPKs display different involvements and levels of importance in different fungal species.

The results presented here highlight portions of the complex cellular networks occurring in *C. parasitica* in response to environmental stimuli; additional studies specifically aimed at elucidating the activation of each MAPK module or the involvement of MAPK proteins in hypovirus interaction are still required to better characterize the complex interplay at the base of virulence and virus-induced hypovirulence phenomena.

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Cryphonectria parasitica strains used in the present study were maintained on PDAMB and kept at 6 °C. For long storage, strains were conserved under 15% glycerol at -80 °C. Evaluation of vegetative growth was performed in Petri plates on PDAMB at 25 °C under a constant level of light (Kim *et al.*, 1995). Growth inhibition and resistance to stresses were tested on PDAMB supplemented with SDS, sorbitol, NaCl, H₂O₂, menadione, G418 sulphate or fludioxonil (see Figs S3, S4, S6, S7, S8, S10 and S11 for the concentrations used). Radial growth was assessed by measuring the diameters of colonies after 7 days. Liquid mycelial cultures were grown in 500 mL of EP complete medium for 3 days with shaking at 200 rpm (Puhalla and Anagnostakis, 1971). The primary inoculum was prepared as described previously (Kim *et al.*, 1995). To determine biomass,

100 mL of liquid cultures were filtered through Miracloth (Calbiochem-Merk4biosciences, Darmstadt, Germany), washed with sterile water and lyophilized for 24 h to measure the dry weight. Each experiment was repeated three times, and three biological replicates were included in each experiment. All statistical analyses were performed using one-way analysis of variance (ANOVA) with Systat software (Cranes Software International Ltd, Bangalore, Karnataka, India). The significance of the differences was assessed with Tukey's test ($P < 0.05$).

Microscopy

Confocal observations of hyphae were made on mycelia grown in liquid medium, washed once with 0.6 M MgSO_4 and stained with CFW. Photographs were taken with a Leica TCS-SP2 microscope (Leica Microsystems, Wetzlar, Germany). Calcofluor fluorescence was excited with the 405-nm line of a mercury lamp and recorded at 420–440 nm. For light microscopy, mycelium was grown on PDAMB for 2 days and observed using a bright field.

Construction of knockout strains

A hygromycin B resistance cassette (*hph*) was used to replace the *cpkk1*, *cpkk2* and *cpkk3* genes in *C. parasitica*. The *hph* cassette was obtained from the plasmid pCB1004 (Carroll *et al.*, 1994) after digestion with *HpaI*, and introduced into a fragment of the coding sequence of each MAP2K amplified by PCR. Primers were designed on the basis of known MAP2K sequences (Rostagno *et al.*, 2010; Turina *et al.*, 2006) (Table S1, see Supporting Information). Briefly, MAP2K amplified fragments were cloned into the TOPO TA Vector (Invitrogen Life Technology, Carlsbad, CA, USA), digested with a specific single-site-cutting enzyme (*NruI* for *cpkk1*, *SnaBI* for *cpkk2* and *BsaBI* for *cpkk3*) and fused with the 1.4-kb *hph* cassette. The resulting plasmids were linearized in a unique site flanking the MAP2K gene fragment prior to transformation (*BamHI* was employed for p Δ Cpkk1, *SmaI* for p Δ Cpkk2 and *EcoRV* for p Δ Cpkk3), and used to transform the Δ *cpku80* strain (Lan *et al.*, 2008). Spheroplast preparation and transformation were performed as described previously (Churchill *et al.*, 1990). Transformants were selected from agar plates supplemented with 150 mg/L hygromycin B (Duchefa, Haarlem, The Netherlands). Initial screens were carried out by PCR with specific *hph* inner primers and MAP2K primers designed outside the region used for the knockout vector. After obtaining nuclear homogeneity and stability through single-spore isolation, Southern blot analysis was carried out as described previously (Turina *et al.*, 2003).

Complementation of *cpkk1*- and *cpkk2*-null mutant strains

As the *C. parasitica* genome has been released and can be accessed at <http://genome.jgi-psf.org/Crypa2/Crypa2.home.html>, DNA copies of the full-length *cpkk1* and *cpkk2* genes (the entire protein coding sequence plus approximately 800 and 300 bp upstream and downstream of the mRNA of the gene) were amplified by PCR using the primers cpkk1-comp1F/cpkk1-comp1R and cpkk2-comp1F/cpkk2-comp1R (Table S1). Fragments were cloned into pGEMT vector (Promega, Madison, WI, USA) to obtain pCpkk1 and pCpkk2. A Geneticin (G418) resistance cassette of 1.2 kb, consisting of the *Escherichia coli* neomycin phosphotransferase gene (*NptII*), under the

control of the *Aspergillus nidulans* tryptophan synthase gene promoter (TrpC), was inserted into each vector (in the *EcoRV* site in pCpkk1 and the *EcoRI* site in pCpkk2). Briefly, the cassette was constructed as follows: *NptII* and TrpC were amplified from the plasmids TOPO TA Vector and pCB1004, respectively, using the primers Npt1IF/Npt1IR and TrpCF/TrpCR (Table S1). Subsequently, the TrpC promoter was cloned in the appropriate orientation at the 5' end of the *NptII* gene in a *SmaI* site of a pBSK plasmid (Stratagene, La Jolla, CA, USA). The pCpkk1 and pCpkk2 plasmids were then used to transform Δ *cpkk1* and Δ *cpkk2* mutant spheroplasts after linearization with *PstI* (pCpkk1) or *EcoRV* (pCpkk2). The selected transformants were subjected to single-spore purification using G418 (20 $\mu\text{g/mL}$) as marker and further verified by PCR analyses to confirm both the presence of the respective wild-type allele and the original deleted allele, in order to exclude external contamination.

DNA manipulations

Genomic DNA from *C. parasitica* strains was extracted using the method described by Turina *et al.* (2003). Southern blot analyses were performed according to standard protocols (Sambrook *et al.*, 1989). One microgram of DNA was digested with appropriate restriction enzymes (*SacI* for *cpkk1*, *BamHI* for *cpkk2* and *SmaI* in case of *cpkk3*), blotted onto a Hybond N+ nylon membrane (GE Healthcare, Uppsala, Sweden) after separation and hybridized with radioactively labelled specific probes synthesized as described previously (Rastgou *et al.*, 2009) using the T7 RNA polymerase.

Protein analysis and laccase activity assay

Total proteins of the selected strains were extracted in buffer A after 24 h as described by Turina *et al.* (2006). Western blot analyses were carried out as detailed previously using polyclonal anti-*cpkk1* and anti-*cpkk2* primary antibodies (Turina *et al.*, 2006).

Laccase activity was gauged by growing fungal strains on Bavendamm's medium (0.05% tannic acid, 1.5% malt extract, 2.0% agar) and assessing the resultant darkening of the medium after 5 days (Rigling *et al.*, 1989). Furthermore, the same test was carried out in liquid medium containing 1.5% malt extract or EP minimal medium (Puhalla and Anagnostakis, 1971) containing 0.05% tannic acid.

Spheroplast cell wall resistance evaluation

To test the susceptibility of the Δ *cpkk1*, Δ *cpkk2* and Δ *cpkk3* strains to cell wall-degrading enzymes, fungal mycelium was cultured in 200 mL of EP complete liquid medium in the total absence of light at 120 rpm for 23 h. One milligram of harvested mycelium was washed once with 0.6 M MgSO_4 , transferred into a flask with 30 mL of osmotically stabilized protoplasting solution containing β -glucuronidase and lytic enzyme from *Trichoderma harzianum*, and incubated at 26 °C under slow shaking (Churchill *et al.*, 1990). After 2 h, the concentration of spheroplasts was determined with a haemocytometer. The Δ *cpku80* strain was used as a control.

Analysis of septa

In order to evaluate the frequency of septa per micrometre of mycelium, five photographs taken by confocal microscopy from different positions in

the fungal colonies were analysed. Three hyphae of different lengths for each photograph were considered for the septal count. Frequency data were obtained by dividing the number of septa by the hyphal length. For each MAP2K mutant and the *Δcpku80* strain, three different colonies from different experimental plates were subjected to analysis.

Male and female fertility analysis

To test the sexual fertility of MAP2K-null mutants, mating experiments were carried out. Mating was performed on autoclaved twigs of chestnut tree embedded in 2% water agar as described previously (Anagnostakis, 1979). Mutant and complemented (mating type Mat-2) strains were tested as both male and female in crosses with strain EP67 (mating type Mat-1). Reciprocal *Δcpku80* × EP67 crosses served as controls. After spermatization with conidia from the male parent, plates were incubated at 25 °C until perithecia could be observed in the stroma.

Conidiation was evaluated after 1 month of growth on PDAMB plates under standard conditions at 25 °C. Conidia from each plate were harvested with 10 mL of sterile water and their concentrations were determined using a haemocytometer.

Virulence assay

Virulence assays were performed on stems of European chestnut tree (*Castanea sativa*) with four replicates per fungal strain as described previously (Rostagno *et al.*, 2010). Inoculated stems were kept at 24 °C under normal light covered with plastic bags to maintain moisture. Three independent experiments were carried out.

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REFERENCES

- Alonso-Monge, R., Roman, E., Nombela, C. and Pla, J. (2006) The MAP kinase signal transduction network in *Candida albicans*. *Microbiology*, **152**, 905–912.
- Anagnostakis, S.L. (1979) Sexual reproduction of *Endothia parasitica* in the laboratory. *Mycologia*, **71**, 213–215.
- Bhalla, U.S., Ram, P.T. and Iyengar, R. (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science*, **297**, 1018–1023.
- Carbó, N. and Pérez-Martín, J. (2010) Activation of the cell wall integrity pathway promotes escape from G2 in the fungus *Ustilago maydis*. *PLoS Genet.* **6**, e1001009.
- Cargnello, M. and Roux, P.P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75**, 50–83.
- Carroll, A., Sweigard, J. and Valent, B. (1994) Improved vectors for selecting resistance to hygromycin. *Fungal Genet. Newsl.* **41**, 22.
- Choi, E.S., Chung, H.J., Kim, M.J., Park, S.M., Cha, B.J., Yang, M.S. and Kim, D.H. (2005) Characterization of the ERK homologue Cpmk2 from the chestnut blight fungus *Cryphonectria parasitica*. *Microbiology*, **151**, 1349–1358.
- Churchill, A.C.L., Ciuffetti, L.M., Hansen, D.R., Van Etten, H.D. and Van Alfen, N.K. (1990) Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. *Curr. Genet.* **17**, 25–31.
- Dawe, A.I. and Nuss, D.L. (2013) Hypovirus molecular biology: from Koch's postulates to host self-recognition genes that restrict virus transmission. *Adv. Virus Res.* **86**, 109–147.
- Deng, F., Allen, T.D. and Nuss, D.L. (2006) Ste12 transcription factor homologue CpsT12 is down-regulated by hypovirus infection and required for virulence and female fertility of the chestnut blight fungus *Cryphonectria parasitica*. *Eukaryot. Cell*, **6**, 235–244.
- Fleissner, A., Leeder, A.C., Roca, M.G., Read, N.D. and Glass, N.L. (2009) Oscillatory recruitment of signaling proteins to cell tips promotes coordinated behavior during cell fusion. *Proc. Natl. Acad. Sci. USA*, **106**, 19 393–19 398.
- Gerik, K.J., Donlin, M.J., Soto, C.E., Banks, A.M., Banks, I.R., Maligie, M.A., Selitrennikoff, C.P. and Lodge, J.K. (2005) Cell wall integrity is dependent on the PKC1 signal transduction pathway in *Cryptococcus neoformans*. *Mol. Microbiol.* **58**, 393–408.
- Hamel, L.P., Nicole, M.C., Duplessis, S. and Ellis, B.E. (2012) Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct messages from conserved messengers. *Plant Cell*, **24**, 1327–1351.
- Heinisch, J.J. (2005) Baker's yeast as a tool for the development of antifungal kinase inhibitors targeting protein kinase C and the cell integrity pathway. *Biochim. Biophys. Acta*, **1754**, 171–182.
- Hou, Z., Xue, C., Peng, Y., Katan, T., Kistler, H.C. and Xu, J.R. (2002) A mitogen-activated protein kinase gene (*MGV1*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol. Plant-Microbe Interact.* **15**, 1119–1127.
- Imajo, M., Tsuchiya, Y. and Nishida, E. (2006) Regulatory mechanisms and functions of MAP kinase signaling pathways. *IUBMB Life*, **58**, 312–317.
- Irie, K., Takase, M., Lee, K.S., Levin, D.E., Araki, H., Matsumoto, K. and Oshima, Y. (1993) MKK1 and MKK2, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. *Mol. Cell. Biol.* **13**, 3076–3083.
- Kahmann, R. and Kamper, J. (2004) *Ustilago maydis*: how its biology relates to pathogenic development. *New Phytol.* **164**, 31–42.
- Kicka, S., Bonnet, C., Sobering, A.K., Ganesan, L.P. and Silar, P. (2006) A mitotically inheritable unit containing a MAP kinase module. *Proc. Natl. Acad. Sci. USA*, **103**, 13 445–13 450.
- Kim, D.H., Rigling, D., Zhang, L. and Van Alfen, N.K. (1995) A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of Lac1. *Mol. Plant-Microbe Interact.* **8**, 259–266.
- Kojima, K., Kikuchi, T., Takano, Y., Oshiro, E. and Okuno, T. (2002) The mitogen-activated protein kinase gene *MAF1* is essential for the early differentiation phase of appressorium formation in *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* **15**, 1268–1276.
- Kojima, K., Bahn, Y.S. and Heitman, J. (2006) Calcineurin, Mpk1 and Hog1 MAPK pathways independently control fludioxonil antifungal sensitivity in *Cryptococcus neoformans*. *Microbiology*, **152**, 591–604.
- Lan, X., Yao, Z., Zhou, Y., Shang, J., Lin, H., Nuss, D.L. and Chen, B. (2008) Deletion of the *cpku80* gene in the chestnut blight fungus, *Cryphonectria parasitica*, enhances gene disruption efficiency. *Curr. Genet.* **53**, 59–66.
- Lanver, D., Mendoza-Mendoza, A., Brachmann, A. and Kahmann, R. (2010) Sho1 and Msb2-related proteins regulate appressorium development in the smut fungus *Ustilago maydis*. *Plant Cell*, **22**, 2085–2101.
- Lew, R.R. (2011) How does a hypha grow? The biophysics of pressurized growth in fungi. *Nat. Rev. Microbiol.* **9**, 509–518.
- Liu, W., Zhou, X., Li, G., Li, L., Kong, L., Wang, C., Zhang, H. and Xu, J.R. (2011) Multiple plant surface signals are sensed by different mechanisms in the rice blast fungus for appressorium formation. *PLoS Pathog.* **7**, e1001261.
- Loewith, R., Hubberstey, A. and Young, D. (2000) Skh1, the MEK component of the mkh1 signaling pathway in *Schizosaccharomyces pombe*. *J. Cell Sci.* **113**, 153–160.
- Mazzoni, C., Zarzov, P., Rambourg, A. and Mann, C. (1993) The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. *J. Cell Biol.* **123**, 1821–1833.
- Mehrabi, R., Zhao, X., Kim, Y. and Xu, J.R. (2009) The cAMP signaling and MAP kinase pathways in plant pathogenic fungi. In: *The Mycota*, Vol. 5. Part 2 (Esser, K., ed.), pp. 157–172. Berlin, Heidelberg: Springer-Verlag.
- Mey, G., Held, K., Scheffer, J., Tenberge, K.B. and Tudzynski, P. (2002) CPMK2, an SLT2-homologous mitogen-activated protein (MAP) kinase, is essential for pathogenesis of *Claviceps purpurea* on rye: evidence for a second conserved pathogenesis-related MAP kinase cascade in phytopathogenic fungi. *Mol. Microbiol.* **46**, 305–318.
- Milgroom, M.G. and Cortesi, P. (2004) Biological control of chestnut blight with hypovirulence: a critical analysis. *Annu. Rev. Phytopathol.* **42**, 311–338.
- Mollapour, M. and Piper, P.W. (2006) Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **6**, 1274–1280.

- Motoyama, T., Ochiai, N., Morita, M., Iida, Y., Usami, R. and Kudo, T. (2008) Involvement of putative response regulator genes of the rice blast fungus *Magnaporthe oryzae* in osmotic stress response, fungicide action, and pathogenicity. *Curr. Genet.* **54**, 185–195.
- Müller, P., Weinzierl, G., Brachmann, A., Feldbrügge, M. and Kahmann, R. (2003) Mating and pathogenic development of the smut fungus *Ustilago maydis* are regulated by one mitogen-activated protein kinase cascade. *Eukaryot. Cell*, **2**, 1187–1199.
- Nikolaou, E., Agrafioti, I., Stumpf, M., Quinn, J., Stansfield, I. and Brown, A.J.P. (2009) Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol. Biol.* **9**, 44. doi:10.1186/1471-2148-9-44.
- Noguchi, R., Banno, S., Ichikawa, R., Fukumori, F., Ichishi, A., Kimura, M., Yamaguchi, I. and Fujimura, M. (2007) Identification of OS-2 MAP kinase-dependent genes induced in response to osmotic stress, antifungal agent fludioxonil, and heat shock in *Neurospora crassa*. *Fungal Genet. Biol.* **44**, 208–218.
- Nuss, D.L. (2005) Hypovirulence: mycoviruses at the fungal–plant interface. *Nat. Rev. Microbiol.* **3**, 632–642.
- Nuss, D.L. (2010) Mycoviruses. In: *Cellular and Molecular Biology of Filamentous Fungi* (Borkovich, K.A. and Ebbel, D.J., eds), pp. 145–152. Washington, DC: ASM Press.
- Panadero, J., Pallotti, C., Rodriguez-Vargas, S., Rande-Gil, F. and Prieto, J.A. (2006) A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281**, 4638–4645.
- Pandey, A., Roca, M.G., Read, N.D. and Glass, N.L. (2004) Role of a mitogen-activated protein kinase pathway during conidial germination and hyphal fusion in *Neurospora crassa*. *Eukaryot. Cell*, **3**, 348–358.
- Park, J.A., Kim, J.M., Park, S.M. and Kim, D.H. (2012) Characterization of CpSte11, a MAPKKK gene of *Cryphonectria parasitica*, and initial evidence of its involvement in the pheromone response pathway. *Mol. Plant Pathol.* **13**, 240–250.
- Park, S.M., Choi, E.S., Kim, M.J., Cha, B.J., Yang, M.S. and Kim, D.H. (2004) Characterization of HOG1 homologue, CpmK1, from *Cryphonectria parasitica* and evidence for hypovirus-mediated perturbation of its phosphorylation in response to hypertonic stress. *Mol. Microbiol.* **51**, 1267–1277.
- Pérez-Nadales, E. and Di Pietro, A. (2011) The membrane mucin Msb2 regulates invasive growth and plant infection in *Fusarium oxysporum*. *Plant Cell*, **23**, 1171–1185.
- Prados Rosales, R.C. and Di Pietro, A. (2008) Vegetative hyphal fusion is not essential for plant infection by *Fusarium oxysporum*. *Eukaryot. Cell*, **7**, 162–171.
- Puhalla, J.E. and Anagnostakis, S.L. (1971) Genetic and nutritional requirements of *Endothia parasitica*. *Phytopathology*, **61**, 169–173.
- Ramamoorthy, V., Zhao, X., Snyder, A.K., Xu, J.R. and Shah, D.M. (2007) Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defenses in *Fusarium graminearum*. *Cell. Microbiol.* **9**, 1491–1506.
- Rastgou, M., Koohi Habibi, M., Izadpanah, K., Luisoni, E., Masenga, V., Milne, R.G., Wolf, Y.I., Koonin, E.V. and Turina, M. (2009) Molecular characterization of the plant virus genus *Ourmiavirus* and evidence of inter-kingdom reassortment of viral genome segments as its possible route of origin. *J. Gen. Virol.* **90**, 2525–2535.
- Read, N.D., Lichius, A., Shoji, J.Y. and Goryachev, A.B. (2009) Self-signalling and self-fusion in filamentous fungi. *Curr. Opin. Microbiol.* **12**, 608–615.
- Rigling, D., Heiniger, U. and Hohl, H.R. (1989) Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria (Endothia) parasitica*. *Phytopathology*, **79**, 219–223.
- Rispail, N., Soanes, D.M., Ant, C., Czajkowski, R., Grünler, A., Huguet, R., Perez-Nadales, E., Poli, A., Sartorel, E., Valiente, V., Yang, M., Beffa, R., Brakhage, A.A., Gow, N.A., Kahmann, R., Lebrun, M.H., Lenasi, H., Perez-Martin, J., Talbot, N.J., Wendland, J. and Di Pietro, A. (2009) Comparative genomics of MAP kinase and calcium-calmodulin signalling components in plant and human pathogenic fungi. *Fungal Genet. Biol.* **46**, 287–298.
- Rostagno, L., Crivelli, G. and Turina, M. (2009) Study of mRNA expression by real time PCR of Cpkk1, Cpkk2 and Cpkk3, three MEKs of *Cryphonectria parasitica*, in virus-free and virus-infected isogenic isolates. *J. Phytopathol.* **158**, 409–416.
- Rostagno, L., Prodi, A. and Turina, M. (2010) Cpkk1, MAPKK of *Cryphonectria parasitica*, is necessary for virulence on chestnut. *Phytopathology*, **100**, 1100–1110.
- Rui, O. and Hahn, M. (2007) The Slit2-type MAP kinase Bmp3 of *Botrytis cinerea* is required for normal saprotrophic growth, conidiation, plant surface sensing and host tissue colonization. *Mol. Plant Pathol.* **8**, 173–184.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Segmüller, N., Ellendorf, U., Tudzynski, B. and Tudzynski, P. (2007) BcSak1, a stress-activated mitogen-activated protein kinase, is involved in vegetative differentiation and pathogenicity in *Botrytis cinerea*. *Eukaryot. Cell*, **6**, 211–221.
- Sun, Q., Choi, G.H. and Nuss, D.L. (2009) Hypovirus-responsive transcription factor gene pro1 of the chestnut blight fungus *Cryphonectria parasitica* is required for female fertility, asexual spore development, and stable maintenance of hypovirus infection. *Eukaryot. Cell*, **8**, 262–270.
- Thorsen, M., Di, Y.J., Tangemo, C., Morillas, M., Ahmadpour, D., Van der Does, C., Wagner, A., Johansson, E., Boman, J., Posas, F., Wysocki, R. and Tamás, M.J. (2006) The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. *Mol. Biol. Cell* **17**, 4400–4410.
- Tscharke, R.L., Lazera, M., Chang, Y.C., Wickes, B.L. and Kwon-Chung, K.J. (2003) Haploid fruiting in *Cryptococcus neoformans* is not mating type alpha-specific. *Fungal Genet. Biol.* **39**, 230–237.
- Turina, M. and Rostagno, L. (2007) Virus-induced hypovirulence in *Cryphonectria parasitica*: still an unresolved conundrum. *J. Plant Pathol.* **89**, 165–178.
- Turina, M., Prodi, A. and Van Alfen, N.K. (2003) Role of the Mf1-1 pheromone precursor gene of the filamentous ascomycete *Cryphonectria parasitica*. *Fungal Genet. Biol.* **40**, 242–251.
- Turina, M., Zhao, L. and Van Alfen, N.K. (2006) Effect of *Cryphonectria* hypovirus 1 (CHV1) infection on Cpkk1, a mitogen-activated protein kinase of the filamentous fungus *Cryphonectria parasitica*. *Fungal Genet. Biol.* **43**, 764–774.
- Xu, J.R., Staiger, C.J. and Hamer, J.E. (1998) Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proc. Natl. Acad. Sci. USA*, **95**, 12 713–12 718.
- Yan, L., Yang, Q., Sundin, G.W., Li, H. and Ma, Z. (2010) The mitogen-activated protein kinase B055 is involved in regulating vegetative differentiation and virulence in *Botrytis cinerea*. *Fungal Genet. Biol.* **47**, 753–760.
- Zhang, L., Churchill, A.C., Kazmierczak, P., Kim, D.H. and Van Alfen, N.K. (1993) Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. *Mol. Cell. Biol.* **13**, 7782–7792.
- Zhang, Y., Lamm, R., Pillonel, C., Lam, S. and Xu, J.R. (2002) Osmoregulation and fungicide resistance: the *Neurospora crassa* os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. *Appl. Environ. Microbiol.* **68**, 532–538.
- Zhao, X., Kim, Y., Park, G. and Xu, J.R. (2005) A mitogen-activated protein kinase cascade regulating infection-related morphogenesis in *Magnaporthe grisea*. *Plant Cell*, **17**, 1317–1329.
- Zhao, X., Mehrabi, R. and Xu, J.R. (2007) Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot. Cell*, **6**, 1701–1714.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Restriction maps and Southern blot analyses of mitogen-activated protein kinase kinase (MAP2K)-null mutants and $\Delta cpk80$ strain (used as wild-type). (A) Gene *cpkk1*. (B) Gene *cpkk2*. (C) Gene *cpkk3*. Gene disruption was generated for each MAP2K through the insertion of a 1.4-kb hygromycin cassette (Hyg) in the coding sequence. Total fungal DNA from the $\Delta cpkk1$, $\Delta cpkk2$ and $\Delta cpkk3$ mutants was digested with *SacI*, *BamHI* and *SmaI*, respectively. The digested fragments were separated on a 1% agarose gel by electrophoresis, and hybridized with 32 P-labelled specific probes indicated in the restriction maps: each probe detected a digested fragment that differed in size compared with the wild-type, indicating that transformants underwent the desired replacement at the MAP2K site through double homologous recombination.

Fig. S2 Comparison of mitogen-activated protein kinase kinase (MAP2K)-null mutant colony morphology after 7 days of growth on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] (left) versus malt agar plates (right). (A) Strain $\Delta cpkk1$. (B) Strain $\Delta cpkk2$. (C) Strain $\Delta cpkk3$. (D)

Strain *Δcpku80*. Medium composition significantly affected the growth of the *cpkk2*-null mutant.

Fig. S3 Colony morphology of mitogen-activated protein kinase kinase (MAP2K)-null mutants grown for 7 days on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with 2 M sorbitol: no growth was observed in the *cpkk3*-null mutant and a strong reduction in growth was reported for the other strains.

Fig. S4 Growth of mitogen-activated protein kinase kinase (MAP2K)-null mutants after 7 days on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with different concentrations of NaCl. The *cpkk3*-null mutant displayed the strongest impairment in growth with increasing NaCl concentration in the medium.

Fig. S5 Growth experiment at different temperatures. (A) One-way analysis of variance (ANOVA) of mitogen-activated protein kinase kinase (MAP2K)-null mutant growth compared with the wild-type strain at 12 °C (Tukey's test, $P < 0.05$). Fungal strains were grown on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] for 5 days. The *Δcpkk1* strain could not grow at 12 °C. Results from three different experiments are shown: *Δcpkk1*, 0 cm; *Δcpkk2*, 0.60 ± 0 cm; *Δcpkk3*, 1.87 ± 0.17 cm; *Δcpku80*, 1.95 ± 0.12 cm. (B) Growth at 25 °C (Tukey's test, $P < 0.05$). Fungal strains were grown on PDAMB for 5 days. Results from three different experiments are shown: *Δcpkk1*, 2.08 ± 0.16 cm; *Δcpkk2*, 1.38 ± 0.13 cm; *Δcpkk3*, 6.32 ± 0.22 cm; *Δcpku80*, 6.80 ± 0.41 cm.

Fig. S6 Colony morphology of wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants grown for 7 days on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with 0.125 mM menadione. *cpkk1*- and *cpkk2*-null mutants displayed a strong sensitivity to the oxidative stress induced by menadione.

Fig. S7 Colony morphology of wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants grown for 7 days on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with 0.005% sodium dodecylsulphate (SDS). Reduction of growth was reported for all strains, except the *Δcpkk1* isolate, in which the absence of growth was observed.

Fig. S8 Colony morphology of wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants grown for 7 days on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with 100 mM H₂O₂. All strains, including the wild-type, were affected, but only the *cpkk1*-null mutant displayed absence of growth under the same conditions.

Fig. S9 Wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants growth in PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] containing Congo red (1 mg/mL) and calcofluor

white (0.5 mg/mL). The y axis represents the average of the difference between the diameter on PDAMB and the diameter on Congo red- and calcofluor white-containing media. Error bars represent the standard deviation. Red type: *t*-test values for two-sample comparisons (each knockout strain compared with the wild-type). Asterisks represent a statistically significant difference when compared with the wild-type strain.

Fig. S10 Wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants growth in PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] containing different concentrations of G418 (Geneticin) antibiotic. Four representative concentrations are shown to illustrate the sensitivity of *Δcpkk2* to the presence of Geneticin in the medium.

Fig. S11 Wild-type (*Δcpku80*) and mitogen-activated protein kinase kinase (MAP2K)-null mutants growth on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] supplemented with fludioxonil. Concentrations of 100, 50, 25, 12.5 and 6.25 mg/L were tested: at 100 mg/L, only the *Δcpkk3* strain displayed normal growth, whereas, at the same concentration, the growth of all the other strains was fully inhibited.

Fig. S12 *cpkk1*-null mutants show lower diameter hyphae and lower fluorescence on calcofluor staining of cell walls compared with *Δcpku80*. Quantification of the cell diameter and calcofluor fluorescence intensity was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In both cases, the numbers are relative to measurements taken for strain *Δcpku80*, arbitrarily given the value of 100. For each strain, the diameters of 100 hyphae were measured on five different slides.

Fig. S13 Representative photographs of *Δcpkk2* and *Δcpku80* hyphal fusions. Fungal strains were inoculated on PDAMB [potato dextrose agar (PDA) containing L-methionine (100 mg/L) and biotin (1 mg/L)] and observed after 2 days by light microscopy. Several fusion events were observed in *Δcpku80* mycelium (white arrowheads), but no hyphal fusion was detected in *Δcpkk2* colonies. Lack of hyphal fusion was evident at the hyphal contact points (asterisks). Bars, 20 μm.

Fig. S14 Agar gel electrophoresis of polymerase chain reaction (PCR) products obtained using DNA templates from knockout and complemented strains. Oligonucleotides were designed immediately upstream and downstream of the hygromycin resistance gene insertion point in each of the two genes. Oligonucleotides were: for *Δcpkk1*, Cpkk1-screenF (5'-GCCGAGGGTGTCTGCAGGG-3') and Cpkk1-screenRev (5'-GCACAACAGGATATTCGAGG-3'); for *Δcpkk2*, Cpkk2-koreaIF (5'-GGAGGATATGATTGAGAAGTGTCTTT-3') and Cpkk2-koreaIRev (5'-ACGCTGGCTGTATCTGGTG-3'). The lower band in both gels represents the c. 100-bp fragment amplified from the locus without insertion, whereas the higher band running with the 1.5-kbp fragment represents the locus with the hygromycin resistance gene insertion.

Table S1 Oligonucleotide primers used in this study.