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Response to Environmental Stresses, Cell-wall Integrity, and Virulence Are Orchestrated Through the Calcineurin Pathway in *Ustilago hordei*

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In eukaryotes, several biological processes are regulated through calcium signaling. Calcineurin is a calcium-calmodulin-regulated serine/threonine phosphatase consisting of catalytic subunit A and regulatory subunit B. Phosphatase activity resides in the catalytic subunit, which activates by dephosphorylation downstream components such as transcription factor Crz1. The importance of this pathway to respond to environmental stress has been explored in several fungal pathogens. The basidiomycete *Ustilago hordei* causes covered smut of barley. We addressed the role of the Ca^{2+}-calcineurin activated pathway by deleting *UhCna1* and *UhCnb1*. These genes were not essential in *U. hordei* but the corresponding mutants displayed a variety of phenotypes when applying environmental stress such as sensitivity to pH, temperature, H_2O_2, mono- and divalent cations; and to genotoxic, acid, or oxidative stresses. Cell-wall integrity was compromised and mutants displayed altered cell morphologies. Mating was delayed but not abolished, and combined sensitivities likely explained a severely reduced virulence toward barley plants. Expression analyses revealed that response to salt stress involved the induction of membrane ATPase genes *UhEna1* and *UhEna2*, which were regulated through the calcineurin pathway. Upregulation of *UhFKS1*, a 1,3-β-D-glucan synthase gene, correlated with the increased amount of 1,3-β-D-glucan in the calcineurin mutants grown under salt stress.

Calcium is an important ubiquitous second messenger that functions in signaling pathways in eukaryotes. In multicellular organisms, signaling through calcium regulates physiological processes as diverse as muscle contraction, motility, programmed cell death, cell division, differentiation, and chromatin remodeling (Carafoli 2005). In the fungal kingdom, this pathway is involved in morphogenesis, circadian rhythm, cell cycle progression, stress response, and virulence (Cyert 2003; Kraus and Heitman 2003; Miyakawa and Mizunuma 2007; Stie and Fox 2008). Upon increase of intracellular levels of calcium, signaling pathways are activated through the action of two calcium-binding proteins: calmodulin and calcineurin. Calcineurin is a highly conserved serine/threonine phosphatase also knows as phosphatase 2B. The inactive protein is a heterodimer formed by one catalytic subunit A (Cna) and one regulatory subunit B (Cnb). The enzyme is activated after the interaction of the catalytic subunit with calmodulin. This association produces conformational changes in the catalytic subunit leading to release of its active site from the autoinhibitory regulatory domain (Kissinger et al. 1995). Specific and strong inhibitors of calcineurin phosphatase are the immunosuppressive drugs cyclosporine A (CsA) and FK506 (tracolimus), which bind irreversibly to the immunophilins cyclophilin or FKB12, respectively, resulting in a ternary complex between calcineurin with CsA-cyclophilin or FK506-FKB12 (Matsuda and Koyasu 2000). These complexes inhibit the phosphatase function, thereby preventing the activation of one of the key downstream effectors of this pathway, the transcription factor Crz1 (Cyert 2001; Fox et al. 2001; Karababa et al. 2006). Calcineurin-dependent dephosphorylation of Crz1 causes its nuclear import and, through its C2H2 zinc finger domain, Crz1 binds to the calcineurin-dependent response element (CDRE) present in calcineurin-responsive genes, thereby activating transcription (Karababa et al. 2006; Matheos et al. 1997; Stathopoulos and Cyert 1997).

In the yeast *Saccharomyces cerevisiae*, mutants defective in this pathway are not able to grow at alkaline pH, nor in the presence of high concentrations of Na^+, Li^+, or Mn^{2+} ions. However, under standard growing conditions, the function of this pathway is dispensable (Matheos et al. 1997; Mendoza et al. 1996). Similarly, in the human pathogens *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, the calcineurin pathway regulates not only growth at alkaline pH or in the presence of some cations but also is involved in virulence (Bader et al. 2003; Cramer et al. 2008; Fox et al. 2001; Karababa et al. 2006; Odom et al. 1997; Steinbach et al. 2006). Similar effects on virulence and pathogenicity were described in the plant pathogens *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Magnaporthe grisea* for mutants defective in this signaling pathway (Harel et al. 2006; Schumacher et al. 2008; Zhang et al. 2009). Recently, the relationship of calcineurin and virulence was addressed in *Ustilago maydis*, where mutants in the catalytic subunit (*ucn1*) were severely reduced in virulence toward maize plants (Egan et al. 2009). Another physiological process regulated through this pathway, at least in *A. parasiticus*, is the synthesis of secondary metabolites (Chang 2008).

We are interested in the possible roles the calcineurin phosphatase catalytic (*Cna1*) and regulatory (*Cnb1*) subunits have by interrupting calcium signaling through deletion of the respec-

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tive genes and investigating resulting pleiotropic effects in the plant-pathogenic fungus *U. hordei*. *Ustilago* spp. have become the paradigm for basidiomycete plant pathogens, with *U. maydis* leading the way. However, in comparison, the close relative *U. hordei* has some additional unique properties such as single genetic elements which can elicit specific host resistance (so-called "avirulence genes") (Linning et al. 2004) and an RNAi machinery (which *U. maydis* lacks) (Laurie et al. 2008). The dimorphic fungus *U. hordei* is a pathogen to small grain cereals such as barley and oat, generally found in nature as black pigmented masses of teliospores on infected ears of the host (Fisher and Holton 1957). The fungus has a worldwide distribution, causing considerable losses due to decreased yield and to contamination of healthy seed with teliospores. In nature, barley seed come into contact with wind-dispersed teliospores or teliospores from infested neighboring seed. Teliospores overwinter under the seed hull and then germinate with the seed in the spring to form a promycelium. Under favorable conditions, two basidiospores of opposite mating type fuse and form a dikaryotic mycelium, which needs the host for survival and to complete its life cycle (Hu et al. 2002). After penetrating the plant cuticle through an appressorium-like structure, the fungus enters its biotrophic phase, eluding and suppressing host defenses. Hyphae colonize intercellular spaces and transverse cell layers to reach the shoot meristem, where it establishes itself quiescently until differentiation of this meristem to floral tissue takes place. In the spikelets of the inflorescence, the fungus proliferates and, upon emergence, barley kernels have been replaced with masses of black sooty teliospores (Hu et al. 2002).

In this study, we present the analysis of *U. hordei Cna1* and *Cnb1* deletion mutants. Aspects of the effects of environmental stresses, including the host environment of similar mutations in a variety of fungi, have been reported individually in different publications. We carried out a comprehensive study in this pathosystem and revealed that the *U. hordei* mutants were sensitive to agents that impose stress in the endoplasmic reticulum (ER) and showed severe defects in cell-wall construction; intact cell walls or the capability to modify them upon host infection are important for fungal virulence (Arbelet et al. 2010; Joubert et al. 2010; Klippel et al. 2010; Treitschke et al. 2010). We also identified novel response genes. Through the calcineurin pathway, *U. hordei* orchestrates proper responses to several types of environmental stress such as variation in the pH, salinity, and presence of heavy metals in the culture media, as well as temperature, oxidative, acid, nitrosative, and genotoxic stresses. As a result, Δ*cna1* and Δ*cnb1* deletion mutants are also severely affected in virulence toward barley plants.

RESULTS

Identification and cloning of *U. hordei* calcineurin genes.

U. hordei genes encoding the calcineurin catalytic (*UhCna1*) and regulatory (*UhCnb1*) subunits were identified by in silico searches of the *U. hordei* genome database at the Munich Information Center for Protein Sequences MIPS (R. Kahmann, J. Schirawski, and G. Bakkeren, *unpublished*). Using the blastx algorithm (Altschul et al. 1997), this database was searched with previously reported fungal calcineurin proteins as queries. To obtain *UhCna1*, we used Cna1 protein sequences from *U. maydis* (AAP48999), *C. neoformans* (XP_567518), *A. fumigatus* (XP_753703), *Candida albicans* (XM_713902), *B. cinerea* (XP_001558972), *S. sclerotiorum* (XP_001597594), and *M. grisea* (XP_367545); and, to retrieve *UhCnb1*, we used Cnb1-homologous sequences from *Cryptococcus neoformans* (XP_775641), *U. maydis* (EAK82139), *B. cinerea* (ABN54442), *Saccharomyces cerevisiae* (NP_012731), and *Sclerotinia sclerotiorum* (XP_001598128). Putative homologous genes UH_01405 (catalytic subunit) and UH_01914 (regulatory subunit; Supplementary Table S1) were selected according to the highest score obtained. Both genes appeared intron-less and encoded the predicted proteins UhCna1 (629 amino acids) and UhCnb1 (176 amino acids). The closest homologs were found in other basidiomycetes: for *UhCna1*, in *U. maydis, Malazzesia globosa, C. neoformans*, and *Coprinopsis cinerea*, with 95, 76, 74, and 71% amino acid identity, respectively; and, for *UhCnb1*, in *U. maydis, Postia placenta*, and *Cryptococcus neoformans*, sharing 97, 80, and 78% amino acid identity, respectively. UhCnb1 appeared to have two calcium-binding motifs (EFh), and the well-conserved phosphatase catalytic domain (PP2Ac), a signature in proteins belonging to the serine-threonine phosphatase family, was present at the amino terminus of UhCna1. Genes with estimated promoter and terminator elements on either side of the presumed start and stop codons, respectively, were amplified by polymerase chain reaction (PCR) and cloned into either episomal or integrative plasmids for genetic complementation of generated mutants.

Deletion of *UhCna1* and *UhCnb1* genes.

U. hordei protoplasts from wild-type strains Uh364 (*MAT-1*) or Uh365 (*MAT-2*) (Table 1) were each transformed with plasmids 1,178 or 1,176 to delete the *UhCna1* and *UhCnb1* genes, respectively, by marker-exchange (discussed below). Proper gene deletion was confirmed by DNA blot (Supplementary Fig. S1) and the following set of mutants was selected to conduct further experiments: Uh1011 and Uh1013 (Δ*cna1*, *MAT-1*), Uh1015 and Uh1016 (Δ*cna1, MAT-2*), Uh1123 and Uh1176 (Δ*cnb1*, *MAT-1*), and Uh976 and Uh978 (Δ*cnb1*, *MAT-2*), collectively referred to as calcineurin mutants. The recovery of deletion mutants of either gene in haploid strains indicated that these genes are not essential in *U. hordei* when grown under normal culture conditions.

Phenotypic analysis of calcineurin mutants.

Morphological changes caused by impaired function of calcineurin. In standard growing media such as complete medium (CM), yeast extract-peptone-sucrose (YEPS) or potato dextrose broth (PDB), calcineurin mutants showed altered cell morphology. Cells looked hyperbranched and a clear tendency to form small microscopic aggregates was observed, in contrast to the parental strains, Uh364 or Uh365, which, under similar growing conditions, grew normally as a uniform population of yeast-like cells (Fig. 1). Similar cell morphological changes were observed in *ucn1* (calcineurin catalytic subunit) mutants of *U. maydis*, which also resulted in a wrinkled colony morphology (Egan et al. 2009), similar to that found for *Candida albicans cna1* homozygous mutants (Sanglard et al. 2003). In contrast, the colony morphology of the *U. hordei* calcineurin mutants was not altered, and creamy-looking colonies of similar shape and size were observed for both wild-type and mutant strains (data not shown). Cell morphology was also reported to be affected in *A. fumigatus* Δ*cnaA* strains (Steinbach et al. 2006).

Calcineurin pathway is involved in cell-wall integrity. Wild-type, Δ*cna1* and Δ*cnb1* strains were grown on CM supplemented with compounds known to reveal cell-wall defects. Mutants were not viable when either 0.002% sodium dodecyl sulfate (SDS), Congo red (CR) at 8.5 μg ml^{-1}, Calcofluor white (CFW) at 20 μg ml^{-1}, or 0.09 mM caffeine were present in the culture media (Fig. 2), although caffeine seemed to be the least harmful agent. Similar responses to all compounds were observed for both mutants. A cumulative effect was observed with SDS plus CR, because mutants were not able to grow when lower concentrations of both compounds were added together in the medium (0.0003% SDS and CR at 4 μg ml^{-1}; data not shown). The effect of high osmotic medium when supplemented with 1.2 M sorbitol or mannitol was tested but found to not affect growth because both wild-type and mutant colonies grew at the same extent (data not shown). The effect of the above compounds was also tested in the presence of 1 M sorbitol but no improvement in growth was observed for the calcineurin mutants (data not shown). Overall, these experiments suggested that cell-wall defects might be present. This was further substantiated by the reduced number of CFU recovered after partial cell-wall digestion with lysing enzymes from *Trichoderma harzianum* and subsequent incubation on CM without osmotic support such as 1 M sorbitol normally used for protoplast regeneration. For example, in a comparative test, $1.41 \pm 0.19 \times 10^6$ CFU were recovered from the Uh365 wild-type strain, whereas only $1.4 \pm 0.35 \times 10^4$ and $2 \pm 0.28 \times 10^4$ CFU were obtained from Δ*cna1* and Δ*cnb1* mutants, respectively. Data represent an average of three experiments with triplicate samples each.

Finally, sensitivity to Fludioxonil was assayed. Addition of this fungicide to the medium results in cell lysis due to an increase of internal glycerol through the activation of the Hog1 pathway (Kojima et al. 2006). The effect of this compound

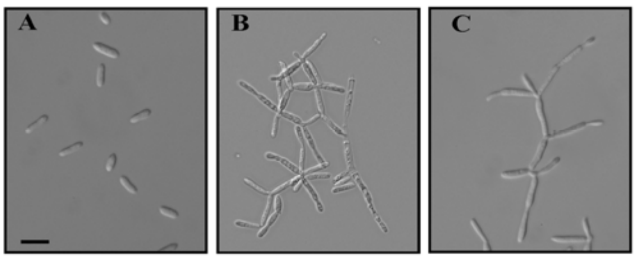

Fig. 1. In vitro cell morphology is altered due to deletion of *UhCna1* or *UhCnb1* genes. The indicated strains were grown in liquid complete medium at 22°C in constant shaking. Samples were withdrawn after 36 h and cells were observed with a Zeiss Axiophot microscope using DIC optics. Scale bar is 5 μm. **A,** Uh365 wild-type strain; **B,** Δ*cna1* (Uh1015); and **C,** Δ*cnb1* (Uh978).

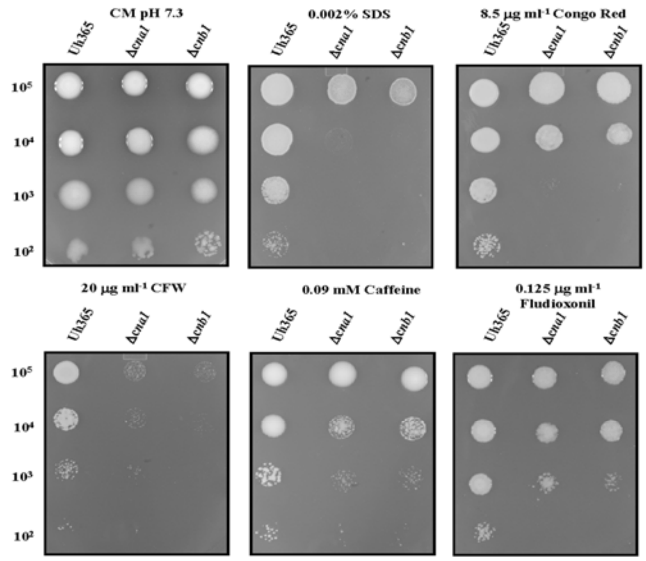

Fig. 2. Cell-wall-perturbing agents are harmful to Δ*cna1* and Δ*cnb1* mutants. Strains were grown in liquid complete medium (CM) at 22°C for 36 h. Optical density at 600 nm of the cultures was adjusted to 0.8; 10 μl of 10-fold serial dilutions were spotted on CM adjusted to pH 7.3 as control condition. Similar media were amended with the compounds indicated. Plates were incubated at 22°C for 5 days, after which they were photographed. Uh365, wild-type strain; Δ*cna1* (Uh1015); Δ*cnb1* (Uh978).

Table 1. Strains used in this work[a]

Strain ID	Relevant genotype	Source
Uh364	*MAT-1*	Wild type; Lining et al. 2004
Uh365	*MAT-2*	Wild type; Lining et al. 2004
Deletion strains		
Uh1011	*MAT-1*, Δ*cna1* cbx^r	This work
Uh1013	*MAT-1*, Δ*cna1* cbx^r	This work
Uh1015	*MAT-2*, Δ*cna1* cbx^r	This work
Uh1016	*MAT-2*, Δ*cna1* cbx^r	This work
Uh1123	*MAT-1*, Δ*cnb1* cbx^r	This work
Uh1176	*MAT-1*, Δ*cnb1* cbx^r	This work
Uh976	*MAT-2*, Δ*cnb1* cbx^r	This work
Uh978	*MAT-2*, Δ*cnb1* cbx^r	This work
Complemented strains (Δ*cna1*; cbx^r)		
Uh1094	Uh1015 plus 1223^{ep} *UhCna1*, hyg^r	This work
Uh1095	Uh1015 plus 1223^{ep} *UhCna1*, hyg^r	This work
Uh1096	Uh1015 plus 1220^{ep} *Ucn1*, hyg^r	This work
Uh1097	Uh1015 plus 1220^{ep} *Ucn1,* hyg^r	This work
Uh1099	Uh1011 plus 1223^{ep} *UhCna1*, hyg^r	This work
Uh1100	Uh1011 plus 1223^{ep} *UhCna1*, hyg^r	This work
Uh1102	Uh1011 plus 1220^{ep} *Ucn1*, hyg^r	This work
Uh1103	Uh1011 plus 1220^{ep} *Ucn1*, hyg^r	This work
Uh1216	Uh1015 plus 1223^{int} *UhCna1*, hyg^r	This work
Uh1217	Uh1015 plus 1223^{int} *UhCna1*, hyg^r	This work
Uh1219	Uh1011 plus 1223^{int} *UhCna1*, hyg^r	This work
Uh1220	Uh1011 plus 1223^{int} *UhCna1*, hyg^r	This work
Complemented strains (Δ*cnb1*; cbx^r)		
Uh1080	Uh978 plus 1219^{ep} *UmCnb1*, hyg^r	This work
Uh1081	Uh978 plus 1219^{ep} *UmCnb1*, hyg^r	This work
Uh1105	Uh978 plus 1222^{int} *UhCnb1*, zeo^r	This work
Uh1106	Uh978 plus 1222^{int} *UhCnb1*, zeo^r	This work
Uh1262	Uh1123 plus 1222^{int} *UhCnb1*, zeo^r	This work
Uh1263	Uh1123 plus 1222^{int} *UhCnb1*, zeo^r	This work

[a] All mutants were generated in the Uh364 or Uh365 genetic background as indicated. Superscripts: r = resistant to the indicated antibiotic, ep = episomal complementing plasmid, and int = integrative complementing plasmid.

will be exacerbated in strains with fragile cell walls. The wild-type strains, Uh364 or Uh365, were sensitive to fludioxonil at concentrations as low as 2.5 µg ml^{-1}. However, the calcineurin mutants Δ*cna1* or Δ*cnb1* were more sensitive to this fungicide, with growth inhibited by the addition of just 0.125 µg ml^{-1} (Fig. 2). This result is in agreement with the sensitivity observed in a *Cryptococcus neoformans* Δ*cna1* mutant (Fan et al. 2007). Fludioxonil is prepared in dimethyl sulfoxide (DMSO) but no detrimental effect was observed by the addition of DMSO alone (data not shown).

Synthesis of 1,3-β-D-glucan is increased in calcineurin mutants under salt stress. Proper cell-wall construction is directly related to the synthesis and assembly of 1,3-β-*D*-glucan (Lesage and Bussey 2006). This fact prompted us to determine whether the compromised cell-wall integrity exhibited by Δ*cna1* and Δ*cnb1* mutants could be related to the total amount of this polysaccharide. Both wild-type cells and mutants revealed similar amounts of 1,3-β-D-glucan when grown under standard conditions (Uh365: 0.24 ± 0.027 µg mg wt^{-1}; Δ*cna1*: 0.26 ± 0.006 µg mg wt^{-1}; Δ*cnb1*: 0.26 ± 0.027 µg mg wt^{-1}). This result contrasts with the reduction of 1,3-β-D-glucan content observed in wild-type *S. sclerotiorum* cells grown in PDB media amended with CsA, which mimics calcineurin mutants by strongly inhibiting calcineurin phosphatase (Harel et al. 2006), or in *A. fumigatus* Δ*cnaA* cells grown in RPMI medium (Cramer et al. 2008). On the other hand, when a stress was imposed to the cells (CM, pH 7.3, amended with 500 mM NaCl for 30 min), the amount of 1,3-β-D-glucan increased 1.6-fold in the wild-type Uh365 strain but more than fivefold in the calcineurin mutants (Δ*cna1*: 1.3 ± 0.09 µg mg wt^{-1} and Δ*cnb1*: 1.5 ± 0.08 µg mg wt^{-1}). This effect was exacerbated when cells were subjected to a less severe saline stress but for longer (CM, pH 7.3, amended with 250 mM NaCl for 4 h), resulting in 10- to 12-fold higher 1,3-β-D-glucan content in the calcineurin mutants (Δ*cna1*: 2.6 ± 0.07 µg mg wt^{-1} and Δ*cnb1*: 2.7 ± 0.09 µg mg wt^{-1}) compared with wild-type strain Uh365 (0.22 ± 0.16 µg mg wt^{-1}).

The finding that the 1,3-β-D-glucan content in the calcineurin mutants was more then 10-times higher when grown under moderate saline stress (CM, pH7.3, and 250 mM NaCl for 4 h), prompted us to determine whether this had any bearing on their cell wall or would change their sensitivity to the effects of *T. harzianum* lysing enzymes. However, Δ*cna1* and Δ*cnb1* mutant cells grown under these moderate saline stress conditions were still more sensitive than wild-type Uh365 cells to cell-wall digestion treatment, according to the number of CFU recovered (data not shown). Even though higher amounts of glucan are present in the calcineurin mutants, its distribution or incorporation in the cell wall might be compromised or an impaired calcineurin pathway might affect the proportion or distribution of other important cell-wall components such as chitin or chitosan, thereby maintaining their increased sensitivity to the lysing enzymes. All specificities of the various cell-wall-degrading activities in the complex lysing enzyme cocktail are not known.

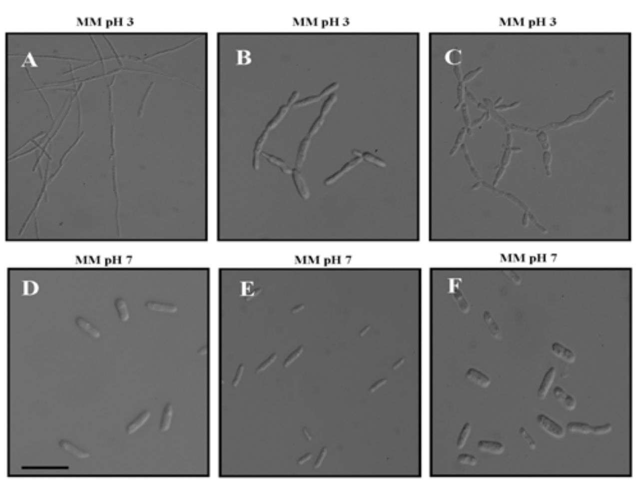

Fig. 3. Effect of *UhCna1* and *UhCnb1* deletion on cell-morphological transition under acidic conditions. The indicated strains were grown in liquid complete medium at 22°C for 36 h. Cells were inoculated in liquid minimal medium with pH as indicated and samples were observed after 32 h with a Zeiss Axiophot microscope using DIC optics. **A** and **D,** Uh365 wild-type strain; **B** and **E,** Δ*cna1* (Uh1015); and **C** and **F,** Δ*cnb1* (Uh978). Scale bar: 10 µm.

Sensitivity to pH and peptone. U. hordei wild-type strains displayed hyphal growth when cultured under acidic conditions. After transfer of *U. hordei* Uh364 or Uh365 wild-type cells from CM to liquid minimal medium (MM) at pH 3, elongated hyphal cells were observed. In contrast, a uniform population of yeast-like cells is maintained in MM at neutral pH (Fig. 3, compare panels A and D). Interestingly, the change in cell morphology of the calcineurin mutants cultured under the same conditions was different: cells were still elongated in acidic medium but shorter and thicker hyphae with some visible constrictions were observed (Fig 3, compare panels B and C to A). Additionally, Δ*cnb1* cells seemed to produce branched hyphae (Fig. 3, panel C). On the other hand, at neutral pH (MM, pH 7), the mutants grew as budding yeast, as was seen for the wild-type cells, although Δ*cna1* cells were slightly more elongated than the wild-type or Δ*cnb1* cells.

We have observed that *U. hordei* strains are sensitive to small changes in the pH of the culture media. Essentially, values from neutral to a mildly alkaline pH resulted in an increasing detrimental effect on growth. Uh364 or Uh365 wild-type strains were not able to grow on CM adjusted to pH higher than 8.3 (data not shown). The calcineurin mutants (Δ*cna1* or Δ*cnb1*) were affected much more strongly by pH because they grew poorly on CM adjusted to pH 7.6, whereas no growth was observed when the pH was raised to 7.8 (Supplementary Fig. S2).

For an unknown reason, Δ*cna1* or Δ*cnb1* mutants were not able to properly grow when peptone was present in media (such as in YEPS). This effect was also reported for the *U. maydis* Δ*ucn1* mutant (Egan et al. 2009). In contrast, *U. hordei* mutants (Δ*cna1* or Δ*cnb1*) grew at the same rate as the wild-type strain in CM, which does not contain peptone (data not shown).

Δcna1 and Δcnb1 mutants are not resistant to a calcineurin inhibitor. In the human pathogens *C. neoformans* or *A. fumigatus*, mutation of the CsA target site or deletion of the calcineurin A gene renders these mutants resistant to inhibitors of this pathway such as CsA or FK506 (Cruz et al. 2001; da Silva Ferreira et al. 2007). We tested how the *U. hordei* calcineurin mutants responded to the presence of CsA in the culture media. Wild-type strains Uh364 or Uh365 were sensitive to this compound, because a growth inhibitory halo was formed after 4 days of incubation (2.0 ± 0.1 cm; n = 3). In contrast to the findings for the human fungal pathogens, the *U. hordei* Δ*cna1* and Δ*cnb1* mutants were sensitive to this compound as well, because growth inhibitory halos of similar sizes were formed by Δ*cna1* (2.2 ± 0.2 cm; n = 3) or by Δ*cnb1* (2.0 ± 0.2 cm; n = 3) cells. No significant inhibition was observed when the CsA solvent ethanol was tested alone (data not shown). This suggests that CsA has additional targets in *U. hordei* cells whose inhibition affects growth.

Sensitivity to monovalent and divalent cations. The presence in the media of mono or divalent cations was toxic to calcineurin mutants. A dramatic inhibition in their growth was observed on CM (pH 7.3) plates supplemented with the following cations separately at different concentrations: 30 mM CsCl, 40 mM LiCl, 600 mM NaCl, 30 mM $MnCl_2$, 20 mM $MgCl_2$, or 600 mM $CaCl_2$. Regarding the monovalent cations, the most dramatic effect was observed with Cs^+, because both mutants did not grow at all in its presence. Both mutants behaved similarly in the presence of Li^+ but, curiously, Δ*cna1* was more sensitive to the effect of Na^+ than Δ*cnb1* (Fig. 4; Supplementary Fig. S3). With respect to divalent cations, calcineurin mutants showed similar responses to Mn^{2+} and Mg^{2+} but seemed more sensitive to Ca^{2+}; for both wild-type strain and mutants, growth was delayed in the presence of this cation given that plates were incubated for 9 days (Fig. 4).

The result that high levels of calcium inhibited the growth of calcineurin mutants suggested that they might not be able to respond as efficiently to Ca-ion fluctuations in the environment. Therefore, we also evaluated the response of these strains to depletion of calcium ions by adding the calcium chelating agent EGTA to the growing media. Low concentrations of this compound (1 mM) were sufficient to completely inhibit the growth of the Δ*cnb1* mutant and a drastic reduction in growth was observed for the Δ*cna1* mutant, whereas EGTA addition had almost no effect on a wild-type strain (Fig. 4). These data suggest that the calcineurin pathway is very important in sensing or responding to fluctuations in environmental calcium levels, given that either high concentration or calcium depletion in the medium drastically compromised viability. To corroborate this further, we tested the response of the calcineurin mutants to the effect of the calcium ionophore, A23187, which increases intracellular Ca^{2+} levels. In *Claviceps purpurea*, a calcium channel 1 (*Mid1*) deletion mutant was unable to grow in the presence of this compound whereas the wild-type strain was unaffected (Bormann and Tudzynski 2009). However, in *U. hordei*, we found that both the wild-type strain and the calcineurin mutants were equally sensitive to the presence of A23187, because a similar growth inhibition was observed (data not shown). The inhibitory growth effect of the ionophore and presumed increase in internal Ca^{2+} levels could mimic the overall slower growth we observed in the medium with high Ca^{2+} levels (Fig. 4).

Enhanced sensitivity of calcineurin mutants to chemical ER stress. Intracellular calcium levels are maintained by the action of Ca^{2+} pumps located in membranes of vacuoles, Golgi apparatus, and ER. The ER is the main dynamic calcium storage compartment, where a physiological threshold of calcium is required for the proper folding and secretion of proteins (Meldolesi and Pozzan 1998). Bonilla and associates (2002) described the calcium cell survival (CCS) pathway in *Saccharomyces cerevisiae* as a protective mechanism whose activation is triggered in cells undergoing ER stress. Calcium influx is conducted through the Cch1p-Mid1p channel, which subsequently activates the calcineurin pathway, thereby improving cell survival (Bonilla and Cunningham 2003; Bonilla et al. 2002). Therefore, we measured the behavior of the calcineurin mutants upon treatment with chemical agents that impose ER stress. We assayed the effect of tunicamycin (Tm), which blocks the synthesis of N-linked glycoproteins in the ER (Kukuruzinska and Lennon 1995), and dithiothreitol (DTT), which disrupts formation of disulfide bonds and leads to retention of proteins in the ER (Jamsa et al. 1994). *U. hordei* calcineurin mutants were unable to manage the ER stress imposed by these chemicals, because we observed that both, Δ*cna1* and Δ*cnb1* mutants barely grew in the presence of Tm (0.1 μM) and were severely impaired by the effect of 50 μM DTT, whereas no significant growth reduction was seen for the wild-type strains in the presence of these agents (Fig. 4). Interestingly, the Δ*cna1* mutant was more resistant to the effect of DTT than the Δ*cnb1* mutant (Fig. 4). This data is in agreement with the phenomenon observed in *S. cerevisiae*, where cell death under ER stress is prevented via signaling through the calcineurin pathway (Bonilla and Cunningham 2003; Bonilla et al. 2002).

Deleterious effect of physical and chemical challenges. Both Δ*cna1* and Δ*cnb1* calcineurin mutants were not able to tolerate a constant incubation temperature of 28°C (6°C above optimal of 22°C) (Fig. 5). They were not able to recover after a heat shock treatment at 39°C for 40 min was applied; in contrast, wild-type strains were not affected by such treatment (Fig. 5). The calcineurin mutants Δ*cna1* or Δ*cnb1* seemed equally sensitive to the harmful effect of UV light (Fig. 5), suggesting inability of mutants to properly respond to genotoxic agents such as UV-generated oxygen radicals (and subsequent DNA damage). In the same way, these mutants were sensitive to ZeocinTM, another genotoxic agent. A low concentration of this compound (10 ng ml^{-1}) was enough to inhibit the growth of Δ*cna1* and Δ*cnb1* cells, in contrast to the wild-type strain, whose growth was not inhibited by this low concentration of ZeocinTM (data not shown). Exposure of the Δ*cna1* and Δ*cnb1* mutants to oxidative or acid stresses imposed by H_2O_2 (17.4 mM for 90 min) or acetic acid (161 mM for 25 min), respectively, resulted in a great reduction in their growth, especially when treated with acetic acid (Fig. 5). Interestingly, the Δ*cna1* mutants were more sensitive to oxidative stress but more resistant to acid stress than the Δ*cnb1* mutants. Because the calcineurin mutants were considerably damaged by oxidative stress generated by H_2O_2, we also tested their response to singlet oxygen ($^1O_2$), another very harmful reactive oxygen species which can be produced by the addition to the medium of the dye Rose Bengal (RB) (Brombacher et al. 2006). Both Δ*cna1* and Δ*cnb1* mutants were severely affected by this compound, given that they barely grew on CM (pH 7.3) plates amended with 100 μM RB (Fig. 5). Calcineurin mutants were also incapable of overcoming the nitrosative stress damage produced by the addition of $NaNO_2$ (20 mM), whereas this detrimental effect was not observed in wild-type strains (data not shown).

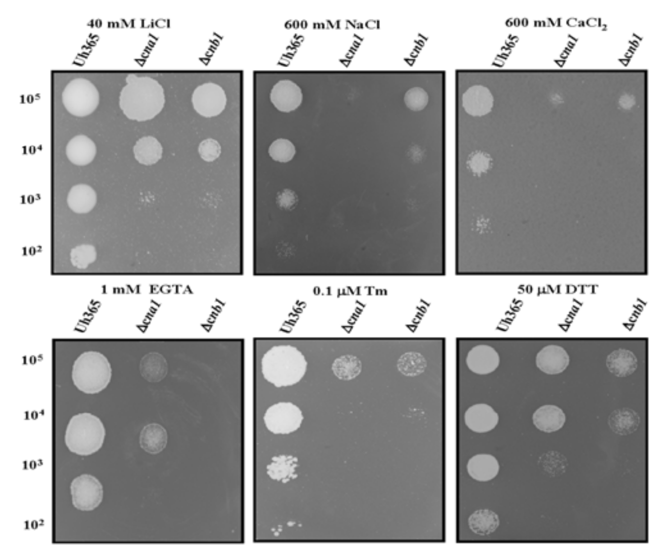

Fig. 4. Calcineurin is important for protection against cations and chemical endoplasmic reticulum stress. Strains were grown in liquid complete medium (CM) at 22°C for 36 h. Optical density at 600 nm of the cultures was adjusted to 0.8 and aliquots of 10 μl were spotted onto CM (pH 7.3) amended with the amount of the compound indicated in each panel. Plates were incubated at 22°C and photographed after 4 days, except plates with NaCl or $CaCl_2$, which were incubated for 9 days, respectively. Uh365, wild-type strain; Δ*cna1* (Uh1015); Δ*cnb1* (Uh978).

Effect of heavy metals. U. hordei calcineurin mutants were not able to tolerate very well the presence of heavy metals in the culture media. Compared with wild-type cells, we observed severe growth reduction at the same degree in both Δ*cna1* and Δ*cnb1* mutants in the presence of 10 mM Zn^{2+}, 1 mM Cu^{2+}, 15 mM Fe^{3+}, 1 mM Cr^{6+}, 0.5 mM Co^{2+} or, to a lesser extent, 1 mM Ni^{+}, respectively (Supplementary Fig. S4; data not shown). Curiously, Δ*cnb1* was more sensitive than Δ*cna1* to 1 mM Cu^{2+} but was more resistant to the effect of 1 mM CrO_3.

Mating is not impaired but delayed in calcineurin mutants. Before infecting a host plant, all smut species require successful mating between compatible haploid cells to form the dikaryotic infection hyphae (Bakkeren et al. 2006). We investigated whether the ability to mate was affected in the *U. hordei* calcineurin mutants. On solid MM pH 7 medium, a compatible wild-type cross of strains Uh364 (*MAT-1*) × Uh365 (*MAT-2*) produced a positive mating reaction after 24 h, typified by short, meandering mating hyphae and long, septated infection hyphae after fusion (Fig. 6A and C). Similar structures were observed in a compatible Δ*cna1* cross, (Uh1011 × Uh1015) but only after 48 h incubation (Fig. 6B and D); comparable results were obtained for a compatible Δ*cnb1* cross (Uh1123 × Uh978) (data not shown). Under our assay conditions, similar fractions of cells produced the mating interactions in both wild-type and mutant cells. Our findings show that the *U. hordei* calcineurin mutants are still able to sense and respond to pheromone but produce a delayed morphological response, possibly due to a less effective cell-wall remodeling. For *U. maydis ucn1* (calcineurin catalytic subunit) mutants, a drastic reduction in mating was reported (Egan et al. 2009); however, this was assayed as a "fuzzy" colony phenotype on conventional charcoal-containing CM plates, which represents a less sensitive assay than microscopic observation of cell interactions.

Virulence is impaired in calcineurin mutants. The calcineurin pathway has been shown to be involved in virulence in both plant and human fungal pathogens (Bader et al. 2003; Cramer et al. 2008; Choi et al. 2009; Fox et al. 2001). Mating did not seem to be (grossly) impaired; therefore, we investigated whether *U. hordei* calcineurin mutants were affected in their virulence toward barley plants. Barley seed were inoculated with mixtures of sexually compatible crosses *MAT-1* × *MAT-2* (mutant × mutant or wild-type × mutant; two sets of mutants were tested) and plants were scored for disease symptoms upon heading. Indeed, disabling the calcineurin pathway affected virulence, given that drastically reduced numbers of infected plants with smutted heads were obtained when inoculated with compatible mixtures in which both mating partners had the mutant genotype Δ*cna1* or Δ*cnb1* (Fig. 7, representative data). In contrast, over 60% of diseased plants were obtained from seed inoculated with the wild-type cross Uh364 × Uh365 (Fig. 7). Importantly, when seed were inoculated with a cross of Δ*cna1* (*MAT-1*) × Δ*cnb1* (*MAT-2*), an intermediate level of disease was obtained in which 44% of inoculated plants developed smutted heads; this result indicates the recessive nature of each mutation, which can be compensated for by a single wild-type allele in one of each partner in the mated dikaryon (Fig. 7). It is unclear why, in those crosses, full virulence is not restored; there could be a gene-dosage effect because each wild-type allele resides in a separate nucleus in the dikaryotic infection hyphae, or effects on early mating interactions between single mutant haploid partners, as described above, might delay infection. Virulence was regained to a comparable wild-type level when deletion mutants were complemented with the respective *U. hordei* wild-type *UhCna1*

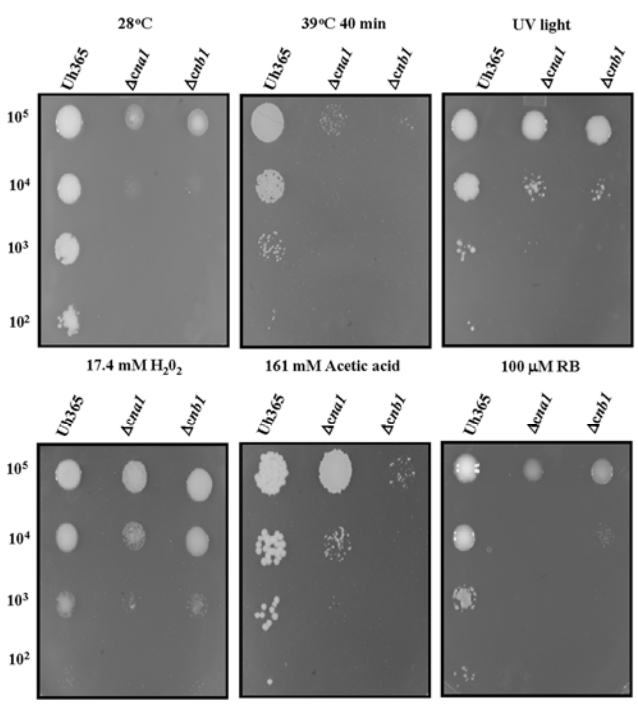

Fig. 5. Deletion of calcineurin-encoding genes render mutants susceptible to several stresses. The indicated strains were grown in liquid complete medium (CM) at 22°C for 36 h. Optical density at 600 nm (OD_{600}) of the cultures was adjusted to 0.8. Then, 10-µl aliquots were spotted on CM (pH 7.3). For suboptimal incubation temperature, one plate was incubated at 28°C. For heat shock, 1 ml of cells (OD_{600} of 0.8) was heated at 39°C for 40 min, diluted, and spotted as above onto CM (pH 7.3). For UV light treatment, cells were spotted as above onto CM (pH 7.3) and plates allowed to dry, whereafter UV light was applied. For oxidative or acid stresses, 1 ml of cells (OD_{600} of 0.8) was treated with H_2O_2 (17.4 mM) for 25 min or with acetic acid (161 mM) for 90 min with shaking at 22°C. Next, cells were diluted as above and 10 µl-aliquots were spotted on CM (pH 7.3). For the Rose Bengal treatment, cells were diluted and spotted as above on plates amended with the compound. Plates were photographed after 4 days of incubation at 22°C, except for the suboptimal temperature experiment which was incubated at 28°C during the same time. Uh365, wild-type strain; Δ*cna1* (Uh1015); Δ*cnb1* (Uh978).

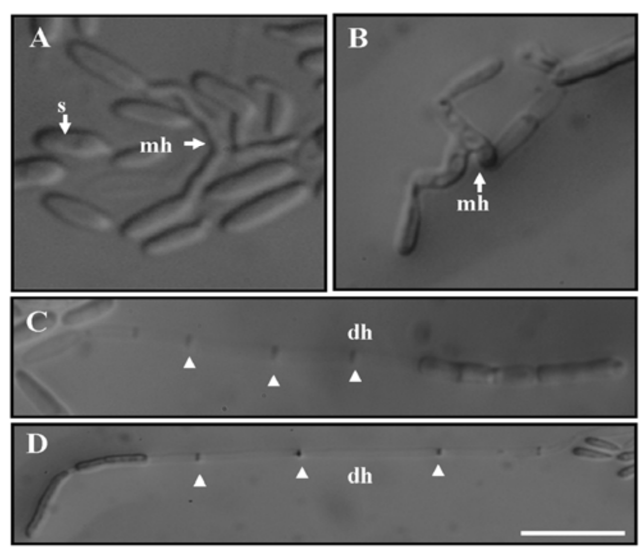

Fig. 6. Mating is delayed but not abolished in calcineurin mutants. Strains were grown in liquid complete medium for 24 h, after which cells of compatible mating type were diluted, mixed, and spread on a thin layer of minimal medium (pH 7) agar applied on a microscope slide. DIC images were taken after microscope slides were incubated at 22°C for **A** and **C,** 24 h, Uh364 × Uh365 (wild-type cross); or **B** and **D,** after 48 h, Uh1011 × Uh1015 (Δ*cna1* cross). Abbreviations: s, sporidia; mh, mating hyphae; dh, dikaryotic hyphae. **C** and **D,** Septa are indicated by arrow heads. Scale bar: 10 µm.

(Uh1216 × Uh1219, Uh1217 × Uh1229) or *UhCnb1* (Uh1105 × Uh1262; Uh1106 × Uh1263) genes, randomly integrated into the genome (Fig. 7, representative data).

Effect on genes possibly regulated by the calcineurin pathway.

The expression of genes involved in ion homeostasis is partly regulated through the calcineurin pathway in U. hordei. In view of the increased sensitivity of calcineurin mutants to mono- or divalent cations, we investigated whether the calcineurin pathway regulates transcription of genes involved in maintenance of cell-ion homeostasis. Salt tolerance in yeast is orchestrated through the action of plasma membrane ATPases (Ena system), which are involved in Na^+ and Li^+ efflux (Ariño et al. 2010). Using the Ena1p, Ena2p, and Ena5p protein sequences from *S. cerevisiae* and the corresponding Ena1 proteins from *U. maydis* and *Cryptococcus neoformans*, several genes with similar annotations were retrieved from the *U. hordei* database. Two genes, UH_02598 and UH_00318, which had the highest similarity score and were annotated as *ENA2* plasma membrane ATPase and Ca^{2+}-transporting ATPase, respectively, were named *UhEna1* and *UhEna2*, respectively, following the nomenclature in *U. maydis* (Benito et al. 2009). We investigated their expression patterns by quantitative reverse-transcription (qRT)-PCR in the wild-type strain Uh365 and Δ*cna1* cells (Uh1015), grown in CM (pH 7.3) medium with the addition of either 80 mM LiCl or 500 mM NaCl. Both salt treatments induced the expression of both genes in wild-type cells, whereas their expression was reduced in Δ*cna1* cells (Fig. 8 A and B, representative data). The residual expression seen indicates the involvement of other signaling pathways as is observed in *ENA1* from *S. cerevisiae* (Platara et al. 2006). It appears that both *UhEna1* and *UhEna2* are induced upon salt stress in a calcineurin-dependent manner.

Regulation of cell-wall biosynthetic genes by the calcineurin pathway. Whatever the cause, the deletion of *UhCna1* or *UhCnb1* results in increased 1,3-β-D-glucan synthesis (discussed above). Therefore, we wanted to investigate whether the level of this polysaccharide quantified in calcineurin mutants could be correlated with the expression of genes involved in its synthesis. In *S. cerevisiae*, these processes are carried out by *GAS1* (1,3-β-glucanosyltransferase) and *FKS1* and *FKS2* (1,3-β-D-glucan synthase) (Lesage and Bussey 2006). Several fungal 1,3-β-glucanosyltransferase homologs to *GAS1* were found at the National Center for Biotechnology Information (NCBI) and were used as queries against the *U. hordei* database. A single gene, UH_02432, was identified as the putative *UhGas1* homolog. According to our qRT-PCR data, *UhGas1* was upregulated in wild-type Uh365 cells grown in CM (pH 7.3) amended with 500 mM NaCl for 30 min as compared with the control condition (Fig. 9A). However, the same change in expression level was seen for the Δ*cna1* mutant when presented with the same saline stress, suggesting no regulation of *UhGas1* by the calcineurin pathway (Fig. 9A). On the other hand, UH_02430 was the only putative gene homolog found in the genome of *U. hordei* encoding the 1,3-β-D-glucan synthase (*UhFks1*). Similarly, one copy has been identified in the close relative to *U. hordei*, *U. maydis* (Ruiz-Herrera et al. 2008), whereas *S. cerevisiae* has two genes, *FKS1* and *FKS2*, with essential, overlapping function (Mazur et al. 1995). When the same saline stress was applied to wild-type Uh365 cells, the expression of *UhFks1* was not significantly altered (Fig. 9B). However, in Δ*cna1* calcineurin mutant cells, an induction of transcription was seen (Fig. 9B). In *C. neoformans*, impairment of the calcineurin pathway through deletion of *CNB1* or by FK506 addition highly induces the expression of *FKS1* under standard growing conditions (Kraus et al. 2003). In contrast, in *B. cinerea* which has only one *FKS1* homolog, no significant difference in the expression of this gene was observed by deletion of the transcription factor *BcCRZ1* (Schumacher et al. 2008). In *Magnaporthe oryzae*, *FKS1* expression was reduced by deletion of *MoCRZ1* in cells grown in $CaCl_2$ (Choi et al. 2009) as well as in *Candida albicans* Δ*cna1* homozygous cells grown under several culture conditions (Sanglard et al. 2003). On the other hand, in *S. cerevisiae*, *FKS2* expression is positively regulated by the calcineurin pathway (Zhao et al. 1998). Our data and data from the literature suggest that the calcineurin pathway is involved in the regulation of *UhFKS1*, and its upregulation correlates with the observed increase in the amount of 1,3-β-D-glucan quantified in the calcineurin mutants grown under salt stress imposed by NaCl.

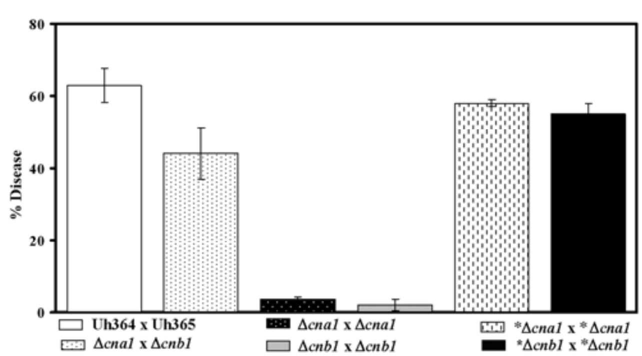

Fig. 7. Effect of calcineurin deletion on virulence. Barley seeds, cv. Odessa, were inoculated with a mixture of sexually compatible strains affected or not in the calcineurin-encoding genes. Disease symptoms were scored 2 months after inoculation. Results are the average of three independent experiments, conducted in duplo. Uh364 and Uh365, wild-type strains; Δ*cna1*, Uh1011 or Uh1015; Δ*cnb1* Uh1123 or Uh978; *Δ*cna1* or *Δ*cnb1* mutant strains complemented with wild-type *UhCna1* (Uh1220, Uh1216) or *UhCnb1* (Uh1262, Uh1105) genes.

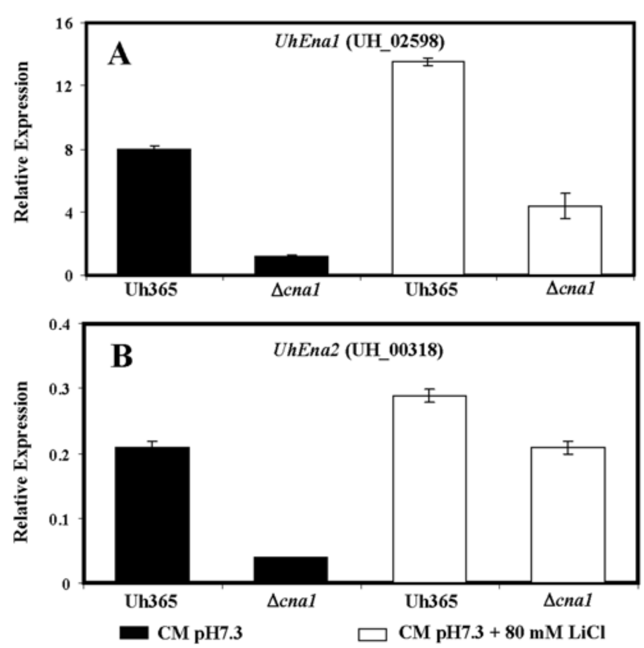

Fig. 8. Relative expression of *UhEna1* and *UhEna2* genes. Strains Uh365 (wild type) and Δ*cna1* (Uh1015) were grown in liquid complete medium (CM) at 22°C for 24 h. Cells were collected and inoculated in liquid CM (pH 7.3) without or with 80 mM LiCl. Cells were recovered after 1 h and RNA was purified. Expression levels of *UhEna1* and *UhEna2* were analyzed by quantitative reverse-transcription polymerase chain reaction and normalized to the *UhActin* and *UhelF2B* reference genes. Error bars represent standard error of mean expression values.

Rescue of mutant phenotypes.

Genetic complementation of calcineurin mutants. Strains Uh1011 and Uh1015 (Δ*cna1 MAT-1* or *MAT-2*) were each transformed with episomal plasmids Uh1223 or Uh1220 harboring the calcineurin catalytic subunit gene from *U. hordei* (e.g., Uh1094, Uh1095, Uh1099, and Uh1100) or its homolog from *U. maydis* (e.g., Uh1096, Uh1097, Uh1102, and Uh1103), respectively. Similarly, strains Uh1123 and Uh978 (Δ*cnb1 MAT-1* or *MAT-2*) were transformed with integrative plasmid 1222 or episomal plasmid 1219 bearing the wild-type regulatory subunit gene from *U. hordei* (Uh1105, Uh1106, Uh1262, and Uh1263) or *U. maydis* (Uh1080 and Uh1081), respectively (Materials And Methods). Double resistant transformants (carboxin and hygromycin B or carboxin and Zeocin, depending on the complementing plasmid used) were selected on double CM plates supplemented with 1 M sorbitol (DCM-S). Details on the genotypes of the set of complemented strains selected for further experiments are shown in Table 1.

All complemented deletion strains, whether harboring the *U. hordei* or the *U. maydis* complementing homolog, were undistinguishable from the wild-type *U. hordei* parental strains in colony or cellular morphology when grown on CM, YEPS, or PDB media (data not shown). The ability to grow as hyphae was recovered as well (data not shown). Complemented *U. hordei* deletion strains were able to grow in CM adjusted to pH 7.8, as well as in media supplemented with 2% peptone. On CM (pH 7.3) supplemented with cell-wall-disturbing agents (0.002% SDS, CR at 8.5 µg ml^{-1}, 0.5 mM caffeine, CFW at 1 µg ml^{-1}, or fludioxonil at 0.125 µg ml^{-1}), all complemented strains were able to overcome the defects caused by the deletions (Supplementary Fig. S5; data not shown). The number of CFU recovered on media without osmotic support after cell-wall digestion was also similar to that obtained from wild-type strains (data not shown). They were also able to grow on CM (pH 7.3) supplemented with monovalent cations (30 mM CsCl, 30 mM LiCl, or 600 mM NaCl) or divalent cations (30 mM $MgCl_2$, 20 mM $MnCl_2$, or 400 mM $CaCl_2$ (data not shown). Complemented mutants survived equally well compared with wild-type cells after treatment with oxidative agents, UV light, or under acid conditions, as well as after enduring heat shock treatment (39°C for 40 min) or continuous growth at 28°C (data not shown). When challenged by chemically induced ER stress conditions (0.1 µM Tm, 1 mM EGTA, or 50 µM DTT), all complemented strains revealed a wild-type phenotype (Supplementary Fig. S6). Similarly, the presence of heavy metals in CM (pH 7.3) (7 mM $ZnCl_2$, 15 mM $FeCl_3$, 1 mM $NiNO_3$, 0.1 mM CrO_3, or 1 mM $CuCl_2$) also did not cause growth inhibition in this set of complemented strains.

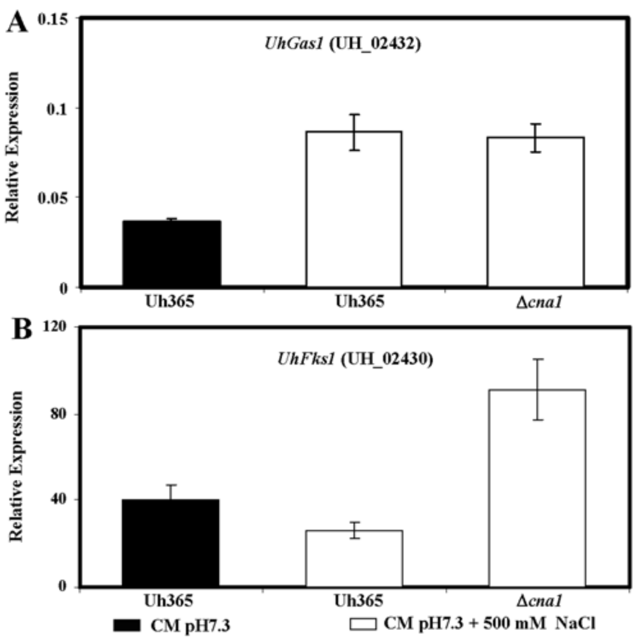

Fig. 9. Relative expression of *UhFks1* and *UhGas1* genes. Strains Uh365 (wild type) and Δ*cna1* (Uh1015) were grown in liquid complete medium (CM) at 22°C for 24 h. Cells were collected and inoculated in liquid CM (pH 7.3) without or with 500 mM NaCl. Cells were recovered after 30 min and RNA was purified. Relative expression levels of *UhFks1* and *UhGas1* were analyzed by quantitative reverse-transcription polymerase chain reaction and normalized to the *UhActin* and *UhelF2B* reference genes. Error bars represent standard error of mean expression values.

DISCUSSION

Recently, calcium signaling via the calcineurin pathway has been shown to play a role in fungal virulence toward human or plant hosts (Choi et al. 2009; Egan et al. 2009; Fox et al. 2001; Karababa et al. 2006; Schumacher et al. 2008). In the present study, through deletion of calcineurin-encoding genes *UhCna1* and *UhCnb1* in *U. hordei*, we analyzed the roles played by this pathway in this basidiomycete. We found that calcineurin mutants showed pleiotropic phenotypes being affected in several biological aspects involving environmental stresses and impacting on virulence, which was dramatically reduced. All phenotypes displayed by the *U. hordei* calcineurin mutants were alleviated to the same degree by the expression of *Cna1* and *Cnb1* genes from *U. hordei* as well as the homologs from *U. maydis*. This not only confirmed that the phenotypes of the calcineurin mutants were caused by the impairment of *UhCna1* and *UhCnb1* but also showed the conservation of gene functions in both species.

In yeast, CsA binds peptidyl-prolyl isomerase cyclophilin A, and this complex then targets calcineurin, resulting in cell-growth inhibition (Cardenas et al. 1995). In general, abolishment of CsA binding by calcineurin/cyclophilin renders fungal cells resistant to the toxic effect of this calcineurin inhibitor (Cruz et al. 2000; da Silva Ferreira et al. 2007). However, our findings revealed a different scenario in *U. hordei*, considering the fact that deletion of calcineurin-encoding genes did not improve the tolerance to CsA's toxicity and calcineurin mutants were as sensitive as the wild-type strain. It is possible that the CsA-cyclophilin complex targets other proteins affecting downstream processes, resulting in cell growth arrest. Mutants lacking *BcCRZ1*, a target of calcineurin in *B. cinerea*, are still able to respond to calcineurin inhibitors (CsA or FK506), resulting in growth reduction (Schumacher et al. 2008). To our knowledge, this is the first report in basidiomycetes (second in fungi) showing sensitivity of calcineurin deletion mutants to this compound, opening avenues to study CsA toxicity mechanisms which could be different among fungi.

The fungal cell wall is a well-organized structure subjected to an accurate remodeling process in response to stressful environmental conditions, and also during cell growth and development (Lesage and Bussey 2006). It has been documented that glucan and chitin are the most important polysaccharides used for its construction (Klis et al. 2006). Our study revealed that *U. hordei* calcineurin mutants were sensitive to several indicator compounds of compromised cell-wall integrity. *U. hordei* calcineurin mutants stressed by nonphysiological levels of NaCl produced increased amounts of 1,3-β-D-glucan due to higher expression of the of the unique 1,3-β-D-glucan synthase gene present in *U. hordei* (*UhFks1*), suggesting a negative transcriptional regulatory mechanism by the calcineurin pathway. These data contrast with the regulation of the *FKS1* homolog in *C. albicans*. In this fungus, a reduction of *FKS1* expression was scored in a calcineurin-dependent manner under several conditions tested (Sanglard et al. 2003). Similarly, a downregulation of the corresponding homologs in the ascomycetes *A. fumigatus* (*fksA*) and *M. oryzae* (*FKS1*) was detected in cells growing in the presence of calcium, and calcineurin signaling was impaired by either genetic or chemical means (Cramer et al. 2008; Choi et al. 2009). It seems that different regulatory mechanisms for *FKS1* evolved in basidiomycetes, given that high expression of the corresponding homolog was also reported in a Δ*cnb1* mutant of *Cryptococcus neoformans* grown under standard conditions (Kraus et al. 2003). In *S. cerevisiae*, the synthesis of glucan and chitin is induced during cell stress and is mediated by the PKC and calcineurin pathways (Zhao et al. 1998). Similarly, in *C. neoformans*, mutants in the *PKC1* gene revealed an abnormal distribution of chitin and chitosan in addition to several phenotypes similar to our *U. hordei* calcineurin mutants (Gerik et al. 2008). Interestingly, a communication between PKC and the calcineurin pathway has been demonstrated in this human pathogen (Kraus et al. 2003). Although we only measured glucan content in the *U. hordei* calcineurin mutants, it is possible that differences in the chitin content or its distribution occurred as well, adding to the observed cell-wall defects to a certain extent. In general, chitin synthesis is a tightly regulated process. For example, in *Candida albicans*, such exquisite regulation proceeds through the coordinated function of Ca^{2+}/calcineurin, PKC, and HOG pathways (Munro et al. 2007); and, in *A. fumigatus*, recent evidence was presented for the regulation of chitin synthase genes orchestrated through the calcineurin pathway (Fortwendel et al. 2010).

Impairment of calcineurin signaling in fungi results in pleiotropic phenotypes, several of which might contribute to a reduction in virulence toward hosts. In the plant pathogen *B. cinerea*, deletion of *BcCRZ1*, one of the targets of calcineurin, shows that it is required for full virulence (Schumacher et al. 2008). In this regard, two genes potentially coding for CRZ1 and, possibly, targets for calcineurin were identified in the *U. hordei* genome (data not shown), represented by *U. maydis* homologs um12004 and um10181. Several phenotypic traits are shared between *U. hordei* Δ*cna1* and Δ*cnb1* and *B. cinerea* Δ*Bccrz1* mutants, one of them being cell-wall defects. *Bccrz1* cells were unable to penetrate plant cells but reinforcement of the fungal cell wall by addition of Mg^{2+} restored this capability (Schumacher et al. 2008). The addition of Mg^{2+} did not improve the growth of *U. hordei* calcineurin mutants under any of the applied stresses tested (data not shown). The importance of cell-wall integrity during the infection process was recently demonstrated in *U. maydis*, where deletion of the chitin synthase V gene resulted in changes in cell-wall composition and mutants showed reduced infection in corn plants (Treitschke et al. 2010). Accumulation of certain cell-wall components or remodeling, including masking of pathogen-associated molecular pattern epitopes during infection, may help the pathogen to bypass the plant surveillance mechanism. *M. grisea* undergoes dynamic changes in cell-wall composition during the infection process and, indeed, preferential accumulation of α-1,3-glucan protects the cell against the degradative effect of plant chitinase (Fujikawa et al. 2009). In light of revealed communication between the PKC1 mitogen-activated protein kinase (MAPK) signaling cascade and the calcineurin pathway in *Cryptococcus neoformans* (Kraus et al. 2003), it is possible that the *U. hordei* calcineurin mutants are more susceptible to cell-wall digestion by host digestive enzymes caused by a lack of interaction with the cell-wall integrity MAPK cascade. Indeed, *U. hordei* calcineurin mutants were more sensitive to cell-wall-degrading enzymes in vitro, and it is worth mentioning in this context that *U. hordei* calcineurin mutants were also sensitive to nitrosative stress, similar to *C. neoformans* Δ*pkc1* cells, which were also damaged by SDS, caffeine, CFW, or CR (Gerik et al. 2008). Another pleiotropic phenotype observed in the *U. hordei* calcineurin mutants, an increased sensitivity compared with wild-type cells toward reactive oxygen species (ROS) (produced in the presence of RB or by addition of H_2O_2), could also contribute to reduced virulence. Recognition of pathogens by host plants is often followed by ROS production (Nurnberger et al. 2004). We hypothesized that these mutants are not able to properly activate mechanism involved in ROS detoxification, normally allowing them to successfully infect their host. It has recently been shown that deletion mutants of *U. maydis yap1*, a transcription factor that protects cells against ROS damage, were sensitive to H_2O_2 and, as a consequence, were reduced in virulence owing to the cells being killed by the ROS produced by the host (Molina and Kahmann 2007).

Mating in *Ustilago* spp. is the prelude for several cellular processes that result in the establishment of disease, and we analyzed whether mating was affected in *U. hordei* calcineurin mutants. In a cross of *MAT-1* Δ*cna1* × *MAT-2* Δ*cna1* or *MAT-1* Δ*cnb1* × *MAT-2* Δ*cnb1*, we observed that the response to mating pheromone was delayed but not abrogated in these haploid mutant cells. In the wild-type cross, cell conjugation occurred within 24 h whereas the same structures were observed, on average, 24 h later in the mutant cross. Because the disease rating achieved with Δ*cna1* × Δ*cnb1* crosses was similar to wild-type infection, this revealed not only that each deletion was recessive but also that early steps leading to conjugation were occurring and that the wild-type gene harbored in the other mating partner complemented the deletion once mated (i.e., the dikaryotic cell). Mutants in the *U. maydis* calcineurin catalytic subunit gene, *Ucn1*, did not seem to mate in a less-sensitive plate mating assay (Egan et al. 2009). Similarly, *C. neoformans* mutants affected in *CNA1* or *CNB1* also revealed impaired mating (Cruz et al. 2001). Our experiments suggest that the reduction in virulence scored for the calcineurin mutants might be caused by post-mating events. This assumption is reinforced by data obtained in *U. maydis*, where this possibility was addressed by deletion of the *Ucn1* gene in the solopathogic strain SG200, in which the mating step is bypassed but a reduction in virulence similar to that observed for a cross with haploid mutants was obtained (Egan et al. 2009).

The importance of the Ena system in salt detoxification has been thoroughly addressed in *S. cerevisiae* (Ariño et al. 2010; Platara et al. 2006; Ruiz and Ariño 2007): exposure to high NaCl concentration in yeast leads to an intracellular calcium burst and a subsequent activating of the calcineurin pathway (Matsumoto et al. 2002). Given the sensitivity of the *U. hordei* calcineurin mutants to mono- or divalent cations, we analyzed the expression of the corresponding *UhEna1* and *UhEna2* genes. According to our data, under salt stress, expression of both genes is positively regulated at the transcriptional level through the calcineurin pathway (Fig. 8), showing the importance of this system in ion detoxification in *U. hordei* as well. Benito and associates (2009) showed that expression of the corresponding *U. maydis* homologs, *UmEna1* and *UmEna2*, was induced by NaCl (and high pH) in a fashion similar to *U. hordei*. In *S. cerevisiae*, expression of *ENA1* is regulated not only by NaCl but also by high pH (Serrano et al. 2002), and the homologous gene in *Fusarium oxysporum* is induced only when the two conditions are present together (high pH and NaCl) (Caracuel et al. 2003). This indicates a complex regulation of this gene given that, in *S. cerevisiae*, *ENA1* transcriptional regulation proceeds through the joint participation of several pathways, including calcium/calcineurin (Platara et al. 2006; Ruiz and Ariño 2007; Serrano et al. 2002). *U. hordei* Δ*cna1* and Δ*cnb1* cells were highly sensitive to changes in the pH of the growth media (neutral to mildly alkaline), in contrast to *M. oryzae* cells deleted for the *MCNA* gene (Choi et al. 2009) but similar to *Candida albicans* Δ*cna1* mutants (Bader et al. 2003) or *Cryptococcus neoformans* cells treated with calcineurin inhibitors (Odom et al. 1997). In fungi, adaptation to changes in environmental pH is mediated by the Rim101/PacC pathway (Peñalva et al. 2008), and impairment of this pathway in *U. maydis* renders mutants sensitive to alkaline pH and reveals pleiotropic defects that resemble the *U. hordei* calcineurin mutant phenotypes (Cervantes-Chávez et al. 2010).

In *U. hordei*, the calcineurin pathway plays a role in cell-wall construction and the adaptation to changes in the environment such as pH, salinity, and many other stresses, including adjustment to the host environment upon infection. Our work and the mentioned related studies indicate that likely more than one pathway is required to orchestrate proper cell responses, and the paucity in the understanding of the interplay between the various signaling cascades deserves further examination. Studies should focus on establishing the link between calcineurin and other signaling cascades and, with regard to fungal pathogens, further elucidate the role of the calcineurin pathway in infection and disease progression.

MATERIALS AND METHODS

Strain growth conditions.

U. hordei haploid strains (Table 1) were preserved at –80°C in liquid CM (Holliday 1961) supplemented with 9% DMSO and were recovered on solid CM, or YEPS (1% yeast extract, 2% peptone, 2% sucrose), incubated at 22°C for 3 days. *U. hordei* genetic transformation was achieved by making protoplasts according to (Barrett et al. 1993) using *T. harzianum* lysing enzymes (Sigma-Aldrich, St. Louis). Transformants were selected on hypertonic DCM-S plus carboxin (2 µg ml^{-1}; Sigma-Aldrich), Zeocin (40 µg ml^{-1}; Invitrogen, Valencia, CA, U.S.A.), or hygromycin B (120 µg ml^{-1}; Calbiochem, La Jolla CA, U.S.A.) where appropriate. CsA, CFW, Tm, SDS, EGTA, A23187, DTT, laminarin, and fludioxonil were obtained from Sigma-Aldrich; acetic acid, H_2O_2, RB, sodium nitrite ($NaNO_2$), and caffeine were obtained from Fluka. *Escherichia coli* DH5α and DH10BR strains were used for routine plasmid propagation, cloning, and subcloning steps; transformation was performed by standard procedures (Sambrook and Russell 1999).

Nucleic acid manipulation.

Purification of genomic DNA for PCR was carried out according to Hoffman and Winston (1987). For DNA blots, genomic DNA was purified using the Qiagen plant genomic DNA extraction kit (Qiagen, Mississauga, Ontario, Canada). PCR was conducted using Taq polymerase or, when required, high fidelity Taq *Pfx* DNA polymerase (Invitrogen). Purification of PCR products for labeling or cloning reactions was carried out using the PCR purification Kit from Qiagen. Vector dephosphorylation, ligation, and DNA digestion were done according to suppliers' instructions (Invitrogen). Sequences of primers used are given in Table 2. Sequencing reactions were performed using the Big Dye terminator mix from Applied Biosystems and an ABI310 Genetic Analyzer (Foster City, CA, U.S.A.). For DNA blot hybridization, 10 µg of genomic DNA was digested with selected restriction enzymes and run out in 1.1% agarose in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) gels. Blotting to Nylon membranes (Amersham Biosciences, Buckinghamshire, U.K.) and hybridization were carried out following standard procedures (Sambrook and Russell 1999). DNA probes to identify *U. hordei* Δ*cna1* and Δ*cnb1* mutants, corresponding to their 3′ flank sequence, were synthesized by PCR as follows: for Δ*cna1*, primers 1321 and 1322 with plasmid 1178 as a template (discussed below); for Δ*cnb1*, primers 1317 and 1318 with plasmid 1176 as a template (discussed below). Probes were labeled with [α-^{32}P] dCTP using the random primer labeling system kit (Amersham Biosciences) according to manufacturer's recommendations.

qRT-PCR analysis.

U. hordei strains of interest were grown as indicated in each experiment. Total RNA (1 µg) was purified according to Jones and associates (1985) and treated with amplification-grade DNAseI (Invitrogen). First-strand cDNA was synthesized using the Dynamo SYBR green 2-step qRT-PCR kit from FINNZYMES following their recommendations. Samples were run on a Mx3000P qPCR instrument (Stratagene, La Jolla, CA, U.S.A.) and the PCR-amplification program involved 15 min at 95°C; followed by 40 cycles of 30 s at 94°C, 30 s at 63°C,

Table 2. Primer list[a]

Number	Sequence
1078 R	ATCGCGGCCTCGACGTTTCC
1079 F	GACAGCCTATTGTGGCAGCC
1315 R	AAAAT<u>AGGGATAACAGGGTAAT</u>GGTTCTTCCTTGCGATGAAA
1316 F	**GGGGACAAGTTTGTACAAAAAAGCAGGCTA**TCATCTTTGCCGAGTATTGGG
1317 F	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**GATTACCAGTTTTGTCCTGC
1318 R	AAAA<u>ATTACCCTGTTATCCCTA</u>GTGTTGTGGTGAGTGTGAGC
1321 F	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TGAGCGGACAGTATACGGCA
1322 R	<u>AAAAATTACCCTGTTATCCCTA</u>GAGCAGGAAATGCAAGGAAA
1344 F	<u>AAAATAGGGATAACAGGGTAAT</u>CGTGCAGTGTCAGTCCAACT
1345 R	**GGGGACAAGTTTGTACAAAAAAGCAGGCTA**TGGCAAGGTGCAGAAACTCTC
1368 F	CTGGGGGTGGCACGACTGGAT
1369 R	GTCCATCGCTTCTTCCTCCAT
1412 F	ggat<u>**ggatccgcggccg**</u>cCGAAGCACATCAACGAACACA
1413 R	caaa<u>**ggatccgcggccgc**</u>CTGCTGGCGGCTCTGGTGGTAG
1416 F	cgaa<u>**ggatccgcggccgc**</u>GCCGACGTCTGCTGGATGTGC
1417 R	cttc<u>**ggatccgcggccgc**</u>CCAGCTCTCGGCATACCTTTCT
1420 R	ggta<u>**gcggccgc**</u>GTTGGACGACTGGCGAAAGA
1421 F	ggat<u>**gcggccgc**</u>AACACTCAACCCAGCCCACAC
1422 R	cccc<u>**tagatct**</u>CCTCGACAAGCGCGGACGAGACT
1423 F	cccc<u>**tagatct**</u>TCGCCAACGCCAAAGCATTCT

[a] F = forward and R = reverse. I-*Sce*I recognition sequence is underlined on primers 1315 and 1344 (forward orientation) and primers 1318 and 1322 (reverse orientation). Bold text represents the *attB1* sequence on primers 1316 and 1345 and the *attB2* sequence on 1317 and 1321. Lower and bold text on primers 1412, 1413, 1416, and 1417 represent restriction recognition sequence for *Bam*HI and *Not*I enzymes, *Not*I on primers 1420 and 1421, or *Bgl*II on primers 1422 and 1423.

and 30 s at 72°C; followed by acquisition of a dissociation curve to test for product purity. Standard error presented was obtained on the basis of duplicate RT-PCR assays with two biological replicates analyzed. The level of gene expression was normalized to the *U. hordei* reference genes actin (UH_08813; primers 1604 and 1605) and the translation initiation factor *UhelF2B*, epsilon subunit (UH_07772, primers 1595 and 1596). Gene sequences were identified in the *U. hordei* genome database at the MIPS (collaborative project of R. Kahmann, J. Schirawski, and G. Bakkeren, *unpublished*). Other *U. hordei* genes for which expression levels were determined were a plasma membrane P-type ATPase, *UhEna1* (UH_02598, primers 1608 and 1069); *UhEna2*, related to a calcium-transporting ATPase (UH_00318, primers 1610 and 1611); *UhFks1*, 1,3-β-D-glucan synthase (UH_02430, primers 1589 and 1627); and *UhGas1*, glucanosyl transferase (UH_02432, primers 1628 and 1629). Primers sequences are shown in Supplementary Table S2 online.

Plasmid constructs.

To delete the *U. hordei Cna1* (*UhCna1*, UH_01405) and *Cnb1* (*UhCnb1*, UH_01914) genes, plasmids 1178 and 1176 were constructed, respectively, according to the DelsGate technology (García-Pedrajas et al. 2010). Briefly, 5′ and 3′ sequences flanking the *UhCna1* gene were amplified with primer pairs 1344–1345 and 1321–1322, respectively (Table 2). For *UhCnb1*, 5′ and 3′ flanks were generated with primer pairs 1315–1316 and 1317–1318, respectively (Table 2). Genomic DNA from *U. hordei* strain Uh365 (Table 1) was used as a template in PCR reactions: the 5′ and 3′ flanks (PCR-amplified) were subsequently recombined into pDnorCbx vector (NCBI accession number EU360889) using BP Clonase enzyme (Invitrogen). Then, plasmids were linearized at the unique restriction site I-*Sce*I using the corresponding enzyme from New England Biolabs (Beverly, MA, U.S.A.), and the DNA was precipitated and used for transformation of *U. hordei* as indicated above. Putative transformants resistant to carboxin were recovered on DCM-S and were repurified on CM plus carboxin to confirm their resistance. Putative mutants were identified by PCR-based screening using primer pairs for Δ*cna1* (1369 and 1078) or Δ*cnb1* (1368 and 1079). In both cases, the expected PCR products of approximately 2 kb were successfully amplified (data not shown). Deletions were confirmed by DNA blot analysis.

Plasmids to complement U. hordei *Δcna1 mutants.* Plasmid 1220, an episomal plasmid derivative from pHyg101 (Mayorga and Gold 1998), confers resistance to hygromycin B and was constructed to harbor the calcineurin catalytic subunit gene, *Ucn1*, from *U. maydis* (um00936; MIPS). The complementing gene fragment, including a 764-bp 5′ upstream promoter region, the 1,884-bp open reading frame (ORF), and 757-bp 3′ downstream terminator sequence, was amplified by PCR with primers 1412 and 1413, which each had a *Bam*HI and *Not*I restriction enzyme site at their 5′ termini (Table 2). The PCR product was digested with *Not*I and cloned into the unique *Not*I site of pHyg101.

Plasmid 1223, an episomal plasmid derivative from pHyg101, was constructed similarly to bear the *UhCna1* gene (UH_01405). The full-length gene (806-bp 5′ flank, 1,890-bp ORF, 970-bp 3′ flank) was amplified by PCR with primers 1420 and 1421 which each had a *Not*I restriction enzyme site at their 5′ termini, to allow cloning into the ditto restriction site of pHyg101. With the aim to obtain stable transformants to carry out the pathogenicity test, this plasmid was converted to an integrative one by digestion with *Ssp*I to eliminate the autonomous replication sequence element.

Plasmids to complement U. hordei *Δcnb1 mutants.* Plasmid 1219 is an episomal plasmid harboring the *U. maydis Cnb1* gene (um10226, MIPS; 946-bp 5′ flank, 528-bp ORF, 865-bp 3′ flank), amplified with primers 1416 and 1417, which each have a *Bam*HI and *Not*I restriction enzyme site at their 5′ termini. The PCR product was digested with *Bam*HI and cloned into the ditto site of plasmid pHyg101.

Plasmid 1222 is an integrative plasmid conferring resistance to the antibiotic phleomycin (bleomycin, zeomycin, or Zeocin) and bears the *UhCnb1* gene (UH_01914; 1,358-bp 5′ flank, 528-bp ORF, 784-bp 3′ flank), amplified with primers 1422 and 1423, which each had a *Bgl*II restriction enzyme site at their 5′ termini. After amplification, the PCR product was digested with *Bgl*II and cloned in the unique *Bgl*II site of plasmid pUBleX1Int, between the *U. maydis* HSP70 promoter and terminator elements (Hu et al. 2007). All constructs generated during this study were verified by sequencing.

Cell morphology and microscopy.

Wild-type Uh364 or Uh365 and mutant strains Uh1011 and Uh1015 (Δ*cna1*) or Uh1123 and Uh978 (Δ*cnb1*) were grown in CM plus amendments and incubated under standard growing conditions for 36 h. The optical density at 600 nm (OD_{600}) of the cultures was measured in a Heλiosβ spectrophotometer (Thermo Spectronic) and was adjusted to 1.2. Next, 5 ml of MM (Holliday 1961), adjusted to pH 3 or 7 according to Ruiz-Herrera and associates (1995), was inoculated with 100 µl of the preculture. Tubes were incubated under standard conditions and cell morphology was scored after 32 h. Photomicrographs of cells were taken using a Zeiss Axiophot microscope, using DIC optics and ×40 magnification. Images were captured using a Nikon D700 digital camera and processed with Photoshop software (Adobe, San Jose, CA, U.S.A.).

Mating test.

Strains of interest were grown in CM plus amendments and incubated under standard growing conditions for 24 h. The OD_{600} of the cultures was adjusted to 0.8 and cells of compatible mating type were mixed in a 1:1 proportion. Next, a microscope slide was covered with a thin layer of 1% agar in MM, pH 7, without glucose, and 10 µl of various dilutions of the cell mixture were spread on the solidified agar. The slides were incubated at 22°C in a petri dish with wet paper towels to keep the humidity. Mating reactions were scored after 24 and 48 h incubation using DIC optics as mentioned above. Experiments were conducted twice obtaining similar results.

Pathogenicity assay.

Strains were grown in CM plus amendments as above. The OD_{600} of the cultures was adjusted to 0.5 and mating-type compatible strains (*MAT-1* × *MAT-2*) (Table 1) were mixed in a 1:1 proportion in CM and incubated for 12 h at 22°C with slow shaking (75 rpm). Then, cells were collected by centrifugation and resuspended in 10 ml of sterile distilled water. Seed of universal susceptible barley cv. Odessa, previously surface sterilized with 1% bleach solution, were submerged into the cell suspension and a vacuum of 20 lbs was applied for 20 min. Subsequently, the seed were drained and dried at RT for 24 h, whereafter they were sown in general potting mix (Pro-MixBX). Plants were grown under controlled conditions, 24 h of continuous light at 25°C, and were scored at heading (after approximately 2 months) for smut symptoms. Data shown are the average of three independent experiments, each conducted in duplo.

Inhibition of calcineurin pathway and calcium ionophore (A23187) treatment.

Strains were grown in liquid CM plus amendments for 1.5 days at 22°C under constant shaking. The OD_{600} of the cultures was adjusted to 0.8 and 120 µl of each strain was spread with a wet cotton swab on solid CM (pH 7.3) plates (adjusted with 100 mM Tris-HCl) for CsA treatment or in CM (pH 7.3) amended with 300 mM $CaCl_2$ for A23187 treatment. A filter paper disc (0.6 cm) with CsA (100 or 200 µg dissolved in ethanol) or with A23187 (4.5 mM) dissolved in DMSO was placed in the center of the corresponding petri dish. Plates were incubated at 22°C for 4 days, after which the size of the growth-inhibitory halo was measured. As a control, a disc with ethanol or DMSO was included and, for the A23187 treatment, no detrimental effect was observed in the calcineurin mutants by the presence of $CaCl_2$. These experiments were repeated three times.

Stress assays.

Strains were grown and diluted as above. Then, 10-fold serial dilutions were prepared and 10 µl of each dilution was spotted on CM (pH 7.3) plates amended with the compounds as indicated for each experiment; compounds were added after agar was cooled to 55°C. For UV light treatment, cells were spotted as above and dried, after which UV light (42 J cm^{-2}) was applied using a UVP HL-2000 Hybrilinker-Hybridization oven (UVP). To measure the response to acid, oxidative, or heat shock stresses, 1 ml of culture (OD_{600} of 0.8) was treated with (17.4 mM) H_2O_2 for 25 min, (161 mM) acetic acid for 90 min, or incubated at 39°C for 40 min with shaking. Next, cells were diluted and spotted as above on CM plates, pH 7.3. Plates were incubated at 22°C, or at 28°C for the suboptimal incubation temperature test, after which time they were photographed using a Nikon D700 digital camera. The cell-wall fragility analysis of *U. hordei* mutants was conducted as follows. Strains of interest were grown in CM. Then, cells were recovered by centrifugation and the number of cells was adjusted to 1×10^7 cells ml^{-1} in SCS buffer (20 mM sodium citrate, pH 5.8, and 1 M sorbitol). *T. harzianum* (Sigma-Aldrich) lysing enzymes (1.5 mg) was added, and the mixture was incubated with constant shaking at 22°C for 60 min. Subsequently, cells were serially diluted in water and 100-µl aliquots of a 10^{-3} dilution were spread on freshly prepared CM (pH 7.3) plates without osmotic support. Plates were incubated at 22°C for 4 to 6 days and the number of CFU counted. Data presented are the result of three independent assays.

Quantification of 1,3-β-D-glucan content.

1,3-β-D-glucan content was determined using aniline blue (AB) as reported by Shedletzky and associates (1997). Briefly, strains of interest were grown in CM under standard conditions for 24 h. Next, 50 ml of CM was inoculated with 1 ml of preculture and incubated for 18 h. Cells were recovered by centrifugation, cultured in CM (pH 7.3) amended with 500 or 250 mM NaCl, and incubated for 30 min or for 4 h, respectively, as above. Control cells were incubated as above but no salt was added. Subsequently, cells were ground in liquid nitrogen and 50 mg was mixed with 250 µl of a 1 N NaOH solution. The resulting mixture was incubated at 80°C for 40 min in a water bath. Three aliquots of 50 µl each were withdrawn and mixed with 200 µl of AB solution (0.03% AB, 0.18 N HCl, 0.98 M glycine-NaOH, pH 9.5) and incubated for 30 min at 52°C, followed by a 30-min incubation at 22°C. Samples were placed in a 96-well fluorescence plate (Microlon; Greiner Bio-One, Solingen-Wald, Germany), whereafter the 1,3-β-D-glucan content was quantified using a spectrofluorometer microtiter-plate reader (SpectraMAX GeminiEM; Molecular Devices, Menlo Park, CA, U.S.A.). Excitation and emission wavelengths were 400 and 460 nm, respectively. Concentration of 1,3-β-D-glucan was expressed as micrograms of glucan per milligrams of fresh weight and was calculated on the basis of a standard curve prepared with laminarin. The results shown are the average of three independent experiments.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

MIPS Fungal Genomics Group website:
www.helmholtz-muenchen.de/en/mips/projects/fungi/index.html