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The L-Type Calcium Ion Channel Cch1 Affects Ascospore Discharge and Mycelial Growth in the Filamentous Fungus Gibberella zeae (Anamorph Fusarium graminearum) *2†

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Cch1, a putative voltage-gated calcium ion channel, was investigated for its role in ascus development in Gibberella zeae. Gene replacement mutants of CCH1 were generated and found to have asci which did not forcibly discharge spores, although morphologically ascus and ascospore development in the majority of asci appeared normal. Additionally, mycelial growth was significantly slower, and sexual development was slightly delayed in the mutant; mutant mycelia showed a distinctive fluffy morphology, and no cirrhi were produced. Wheat infected with Δcch1 mutants developed symptoms comparable to wheat infected with the wild type; however, the mutants showed a reduced ability to protect the infected stalk from colonization by saprobic fungi. Transcriptional analysis of gene expression in mutants using the Affymetrix Fusarium microarray showed 2,449 genes with significant, twofold or greater, changes in transcript abundance across a developmental series. This work extends the role of CCH1 to forcible spore discharge in G. zeae and suggests that this channel has subtle effects on growth and development.

Forcible discharge of ascospores from asci is a mechanism common to the majority of fungi in the phylum Ascomycota. The mechanism is vital for initiating the disease cycle in many plant-pathogenic species, including Gibberella zeae (sexual stage Fusarium graminearum), the causal agent of fusarium head blight of wheat. Analysis of the epiplasmic fluid surrounding the spores within the ascus indicates that sugars (Ascobolus immersus) (14) or ions (G. zeae) (56) accumulate and act as osmoles for generation of the turgor pressure necessary to fire the spores. In the case of G. zeae, the pressure drives an impressive acceleration—$870,000 \times g$—the highest yet recorded in a biological system (56). While the physiological basis of the mechanism of discharge is beginning to be elucidated, the genetic basis remains largely unexplored.

Shifts in calcium levels across both internal and plasma membranes regulate many cellular processes (2). Calcium fluxes are generated by internal release or by influx through calcium channels. Although calcium signaling is not well understood in fungi, the components are conserved in fungal genomes (59), and several parts of the signaling machinery have been studied, including calcineurin (43, 10, 4, 48, 13), calmodulin (37), $\mathrm{Ca}^{2+}$/calmodulin-dependent kinases (11, 12, 35, 31, 58, 52), and phospholipase C (19).

CCH1 encodes a calcium ion channel localized in yeast to the plasma membrane (39). Cch1 has been described as a voltage-gated $\mathrm{Ca}^{2+}$ channel due to its highly conserved transmembrane voltage-sensing S4 domains related to the mammalian L-type voltage-gated calcium channel (15, 39). However, a recent comparison of the conserved charged residues (arginine and lysine) in the S4 region indicates that the full complement of residues is not present in the fungal proteins, and thus fungal Cch1 may be less sensitive to voltage than its mammalian orthologs (38). In Saccharomyces cerevisiae, Cch1 has been proposed to interact with the stretch-activated channel Mid1 (32). Both are implicated in a phenotype described as “mating pheromone-induced death”: on exposure to α-factor, deletion mutants of either gene in a MATa background die shortly after forming shmoos (26, 15). Based on phenotype and double-mutant studies, Locke and colleagues (39) suggest that Cch1 and Mid1 may function together in yeast, and coimmunoprecipitation studies have supported their association in vivo. However, Kanazaki and colleagues (32) have shown that Mid1 is capable of forming an independent, functional channel in Chinese hamster ovary cells (32), and Liu et al. (38) present evidence that Mid1 and Cch1 have independent roles under certain culture conditions.

G. zeae is a major crop pathogen, infecting wheat, barley, maize, and other cereal crops worldwide and causing extensive losses. In addition to reduced yield, the infected crop can be rendered unusable by the production of mycotoxins (9, 56), notably zearalenone (estrogenic) and trichothecenes (antifeedant and emetic). While the fungus possess an asexual stage (F. graminearum) capable of producing abundant conidia, the primary inoculum of the disease is believed to be ascospores (49), which are forcibly discharged from perithecia forming on crop debris. The process of sexual development both in culture and in planta has been well described (54, 23). Recently, a detailed transcriptional analysis during the developmental stages of the peritheciun was completed (24) on the Fusarium GeneChip (22).

As forcible ascospore discharge can be considered a possible target for control measures, we have been focusing on the physiological and genetic aspects of this phenomenon. In G. zeae, pharmacological evidence suggests that calcium plays a
Fungal strains and growth conditions. The strain of G. zeae used for this study was a Michigan field isolate, PH-1 (FGSC 9075; NRRL 31084), and mutants derived from this wild-type strain. The genome of PH-1 has been sequenced (http://www.broad.mit.edu/annotation/fusarium/graminearum/Home.html), and considerable annotation has been completed (http://mips.gsf.de/genre/proj/fusarium/). All strains were maintained as mycelia and conidia (10^6 conidia ml^-1) in 30% glycerol at −80°C and on sterile soil at 4°C. Conidia were generated for stocks and for quantification in carboxymethylcellulose (Sigma Chemical Co., St. Louis, MO) following standard techniques (17). The ortholog of CCH1 was homothallic, and sexual development was induced by the tetrad-mating type (18). CCH1 was homologous to Hygromycin B phosphotransferase gene (hph) probe, and once with the CCH1 internal probe (amplified by primers 7 and 8).

Characterization of mutant phenotypes. Mutants were selected by their ability to grow on 500 µg ml^-1 G418. Transformants were probed twice with [32P]CTP-labeled probes, once with the 1G418. Transformants were selected by their ability to grow on 500 µg ml^-1 G418. Transformants were selected by their ability to grow on 500 µg ml^-1 G418. Transformants were selected by their ability to grow on 500 µg ml^-1 G418.

Construction of Δcch1 mutants. The ortholog of S. cerevisiae CCH1 was identified in the G. zeae genome on the basis of BLAST sequence homology as NCBI accession number AAL02613. To construct the CCH1 gene deletion construct, primers 11 and 12 were used as probes in Southern analysis, and pair 1 and 2 were reacted with [32P]CTP-labeled probes, once with the CCH1 internal probe, and once with the hph probe. The residual probe was removed by imidazole and collected by centrifugation, collected by centrifugation, and stored as above. Several mutants were confirmed by PCR and Southern analysis and used for further characterization. Transformants Δcch1-T11 were representative of the mutant phenotype and genotype and were used for further study, along with ectopic transformant Δcch1-T11.

When the expected amplicon was <0.2000 bp, PCRs used Invitrogen Tag (Invitrogen, Carlsbad, CA), and the reaction protocol followed Tank and Sang (53). For amplicons of ≥0.2000 bp (primer pair 1 and 5; assembling the deletion construct and amplifying the complementation template), Expand Long-Term-plate Polymerase was used, following manufacturer’s instructions (Roche Applied Science, Indianapolis, IN).

For Southern analysis, genomic DNA was cut to completion with BamHI, which was predicted to cut CCH1 twice, yielding one fragment of 3,507 bp that would hybridize to the CCH1 internal probe (amplified by primers 7 and 8). BamHI was not predicted to restrict the gene deletion construct carrying the hph gene. Restriction fragments were resolved by electrophoresis in 0.8% agarose in 1× Tris-acetate-EDTA (47) and transferred onto a Nytran Supercharge nylon membrane (Schleicher and Schuell Bioscience, Keene, NH). The membranes were probed twice with [32P]CTP-labeled probes, once with the CCH1 internal probe, and once with the hph probe. The residual probe was removed by imidazole and collected by centrifugation, collected by centrifugation, and stored as above. Several mutants were confirmed by PCR and Southern analysis and used for further characterization. Transformants Δcch1-T11 were representative of the mutant phenotype and genotype and were used for further study, along with ectopic transformant Δcch1-T11.

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Microarray analysis. The mycelium harvested at the time of induction, i.e., when the mycelium reached the edge of the petri dish, was considered the 0 h vegetative mycelium (4 days for the wild type and 5 days for the mutant). Surface mycelia or developing perithecium were similarly harvested from the induced cultures, at the stage in which ascus initials were present in immature perithecia (96 h) and at the time of ascus and ascospore maturity (multiseptate ascospores present in well-developed asci; 144 h). Three replicates were harvested for each developmental stage. Collection and analysis of developmental stages from the wild type have been previously described (24). All harvested samples were lyophilized and frozen at −80°C until RNA extraction.

RNA was extracted from lyophilized samples using the Trizol reagent (Invitrogen). A CTAB-chloroform step was incorporated into all RNA preparations due to high levels of polysaccharides in the 96-h and 144-h stages, as described in Hallen et al. (24). The samples were purified using an RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Purified RNA was processed using the Affymetrix One-Cycle Target Labeling procedure, following the Affymetrix manual (1) and hybridized to the Fusarium sp. GCS3000 scanner (Affymetrix) and the cell intensity (CEL) files were obtained from GCO 2.1 software (Affymetrix). CEL files are available at PLEXdb (http://www.plexdb.org/), accession numbers FG5 (wild type) and FG6 (mutant). CEL files were normalized in the Bioconductor package of R, version 2.3.0 (20, 45) using RMA, an expression measure that accounts for background correction, quantile normalization, and variation between arrays (27, 28).

Comparisons between Δcch1 and the wild type for each developmental stage were conducted using the Limma package in Bioconductor (51). The list of differentially expressed genes was ranked based on the moderated t statistic introduced by Smyth (50). Statistical significance was empirically determined for each comparison by selecting the cutoff P value lower than the smallest P value found in any of the Affymetrix control probe sets, as recommended by Smyth (51). Genes for which significant differential transcript abundance was detected were functionally characterized using FunCat (46).

RESULTS

The putative G. zeae homolog of the yeast CCH1 gene was replaced with the coding sequence of the hph gene imparting hygromycin resistance. Sixteen hygromycin-resistant colonies were obtained from a single transformation experiment with the CCH1 deletion construct. Of the 16, six showed abnormal vegetative growth on carrot agar. All 16 were analyzed by PCR, and 5 of the abnormal colonies, designated Δcch1-T10, -T12, -T13, -T14, and -T15, were shown to be consistent with hph insertion, replacing CCH1. Of the transformants demonstrating wild-type growth (ectopic insertions of the hph gene), one isolate, designated Δcch1-T11, was used in further studies as a control. The status of transformants Δcch1-T10 through Δcch1-T14 was confirmed by Southern analysis with an internal fragment of CCH1 and with hph (Fig. 1).

Mutants of G. zeae lacking a functional CCH1 displayed abnormal, delayed vegetative growth on carrot agar. Colony morphology was distinct, with a dense, fluffy growth not observed in the wild type (Fig. 2). Radial colony growth of the wild type was 17.8 mm 24 h−1 until the edge of the petri plate was reached, compared with 10.3 for Δcch1-T12 and 10.6 for Δcch1-T14 (standard deviations of 1.4, 0.5, and 0.3, respectively). The ectopic transformant Δcch1-T11 exhibited radial colony growth of 21.2 mm 24 h−1 (standard deviation, 2.6), and the complemented strain Δcch1comp6 grew 20.7 mm 24 h−1 (standard deviation, 0.7).

Following induction of sexual development, the Δcch1 mu-
tants continued to exhibit slower development, with a delay of approximately 24 h compared with the wild type to full maturity. While there were obvious differences in gross colony morphology, these were not quantifiable at the microscopic level when we examined hyphal tip growth. Normal morphological development of the perithecia was present in the mutants. Asci viewed under the microscope were morphologically normal, and ascospores were viable (86 out of 97 [88%] ascospores germinated, compared with 130 out of 140 [92%] for the wild type; results not significantly different, with the chi-square test). Spore discharge was not detected either at the time of mutant peritheium maturity (168 h postinduction; multiseptate ascospores present in peritheium) or 24 h after attaining maturity (192 h postinduction) (Fig. 3). Examination of lids of petri dishes containing mutant cultures up to 3 weeks after maturation failed to reveal any released ascospores, whereas the lids of wild-type cultures exhibited copious amounts of spores that had been discharged from the peritheium. Notably, 38% of the Δcchl mutant asci contained at least one abnormal spore (Fig. 4). Abnormal ascospores have also been occasionally observed in the wild type. Abnormal spores were much reduced in size and were not included in the germination assays.

Wild-type perithecia will commonly exude spores which have not been discharged as the colonies age and dry. These exuded spore masses, called cirrhi, are prominent within several days of maturation in the wild type (Fig. 4A). No cirri were observed at any time as the mutant cultures aged (Fig. 4B). The presence of a single cluster of conidia was frequently observed on the surface of the perithecia (Fig. 4B). Strikingly, older cultures of the wild type normally become covered with conidial clusters (reduced sporodochia), but the Δcchl mutants never accumulated more than these single clusters. There was no difference in conidia production between the wild type and the mutants in standard conidia-inducing medium made with carboxymethylcellulose.

A complementation construct was prepared by subcloning a 7.6-kb fragment surrounding and including the CCH1 coding region into pYN06 and using that construct to complement the Δcchl-T14 mutant. Two G418-resistant colonies, Δcchlcomp5 and Δcchlcomp6, were obtained from one transformation experiment, and both harbored the wild-type CCH1. The wild-type vegetative phenotype and active spore discharge were restored with the complementation (Fig. 2 and 3), which was confirmed initially by PCR analysis (results not shown) and subsequently by Southern analysis (Fig. 1). To test whether additional calcium could complement the Δcchl mutation, as had been shown in S. cerevisiae (15), transformants were supplemented with calcium (CaCl2) during several different cultural and developmental stages. Carrot agar was amended to a final concentration of 0.77, 7.69, 38.46, or 76.92 mM CaCl2 at each of three time points: at the time of initial inoculation, concurrent with induction of sexual development, and at the initiation of the ascospore discharge assay. To ensure that higher than average calcium levels did not exert a negative effect, wild-type PH-1 was also supplemented. No phenotypic differences in either wild-type or mutant cultures were noted at the two lowest concentrations of CaCl2. At the higher levels, the wild-type vegetative phenotype was partially restored in the mutants (Fig. 5). In addition, spore discharge was restored in the mutants at the two highest levels of CaCl2 (shown for 38.46 mM CaCl2 in Fig. 3), although at the highest level (76.92 mM) spore discharge began to diminish in the wild type, probably due to a reduction in the turgor in the ascus (57). To test, by contrast, the phenotype of the wild type and mutants under calcium stress, the calcium chelator BAPTA was added to solid Bilay’s medium, which was then center inoculated with conidia of the wild type and Δcchl mutants. Growth was poor for all cultures; however, wild-type PH-1 was capable of extending hyphae beyond the point of inoculation, while the Δcchl cultures never grew beyond the

FIG. 2. Vegetative growth on carrot agar 96 h after inoculation. (A) Wild type. (B) Ectopic transformant Δcchl-T11. (C and D) Mutants Δcchl-T12 and Δcchl-T14. Note the dense, fluffy growth. (E) Δcchlcomp6. Complementation of the Δcchl deletion mutant restores the wild-type phenotype.
point of inoculation (Fig. 6), although some aerial hyphal
growth was visible.

Examination of wheat infected with \(cch1\) and with the
wild type revealed no differences in symptom severity, with
all inoculated plants (10 plants each for the wild type and
the \(cch1\) mutants) exhibiting well-developed symptoms
(senescence of heads and stems extending below the top
node) by 14 days postinoculation. Mycelia were readily ob-
served within the pith of the stems when the plants were
harvested at 28 days postinoculation. Perithecia were
formed in 5 days when dry wheat stem sections were incu-
bated in moist vermiculite under light. Perithecia were pro-
duced abundantly by both the wild type and the mutants in
approximately equal numbers (average, 114 perithecia per 7
cm stem fragment). Stems colonized by the \(cch1\) mutants
and by the wild type revealed both obvious colonization and
conidiation by both \(G. \text{zeae}\) and other nonpathogenic fungi
from the greenhouse (largely \(Penicillium\) and \(Aspergillus\)
spp.). In both wild-type- and mutant-infected stems, \(G. \text{zeae}\)
conidia were in the minority compared with those of the
other fungi; however, the number of \(G. \text{zeae}\) conidia was
smaller, and the number of other conidia was greater in the
stems colonized by the mutants. Thus, there were 8.75 \(10^3\)
\(G. \text{zeae}\) conidia per stem in the \(cch1\) mutants, compared
with 5.04 \(10^5\) conidia from other organisms. In the wild-
type-infected stems, there were 1.32 \(10^4\) \(G. \text{zeae}\) conidia
per stem and 2.08 \(10^5\) conidia from other organisms
(results were averaged over seven stems). The ratios were
1/59 (\(G. \text{zeae}\) conidia/other conidia) in wheat stems infected
by a \(cch1\) mutant and 1/16 in stems infected by the wild type.
These values differed significantly at a $P$ of $<0.001$ according to the chi-square distribution.

As calcium fluxes are known to affect gene transcription, gene expression was compared between the wild type and Δcch1-T14. RNA was extracted at three time points, representing vegetative growth, immature perithecia (containing paraphyses, croziers, and immature asci), and mature perithecium formation at 0, 96 h, and 144 h, respectively (24). In total, 2,449 probe sets showed a statistically significant change in abundance of twofold or greater between mutant and the wild type for all three time points in Δcch1 mutants compared with the wild type, while FunCats 30 and 40 (cellular protein fate, respectively) also contained significant proportions of genes with differentially expressed transcripts. There is comparatively little overlap between time points, with only 111 transcripts showing differential accumulation versus the wild type at more than one time point.

Table 2 shows those significantly different from the wild type at both 0 and 96 h. CCH1 (probe set fgd69-40_at) shows a decrease in transcript abundance of 6.18- to 8.62-fold in the Δcch1 mutants compared with the wild type, indicating that hybridization on the GeneChip to the CCH1 probe set is essentially absent in the mutants but present in the wild type, as expected for gene replacement mutants.

A functional categorization of genes showing a statistically significant decrease in transcript accumulation of twofold or greater in the Δcch1 mutant compared to wild type is shown in Table 3 (for the list of individual genes and complete breakdown into FunCat categories see Table S2 in the supplemental material). Of particular interest are those categories for which there is an enrichment of genes showing a decrease in transcript abundance in the mutant compared to the genome as a whole. This enrichment is significant at $P$ values of less than 0.05 (29). This analysis shows a significant enrichment of genes involved in metabolism (FunCat category 01) in the pool of down-regulated Δcch1 genes. FunCats 02 and 14 (energy and protein fate, respectively) also contained significant proportions of genes with lowered transcript levels at 0 and 96 h compared to the wild type, while FunCats 30 and 40 (cellular communication/signal transduction mechanism and cell fate, respectively) contained significant proportions of genes with lowered abundance at 96 and 144 h for the same comparison. Genes involved in cell cycle and DNA processing (FunCat 10) were significantly reduced in transcript levels at all time points in the mutant.

**DISCUSSION**

Arguably, the most unique cell in the life cycle of the ascomycete fungus is the ascus. Asci elongate in response to increased turgor pressure and eventually rupture at the tip to fire spores into the air (54). In the perithecium-forming fungi, the ascus fire singly in succession—approximately 45 s apart in *G. zeae* under optimal conditions (56)—which suggests the presence of a regulatory mechanism that coordinates discharge. One possibility for a regulatory mechanism is a calcium signaling cascade. In this study, we have identified one component of the calcium signaling machinery, CCH1, which affects discharge in *G. zeae* when genetically disrupted.

Cch1 has been implicated as part of the high-affinity calcium-acquiring machinery (42). The role of Cch1 in *G. zeae* in facilitating calcium influx at low concentrations was aptly demonstrated by the lack of growth of the mutants in the presence of the calcium chelator BAPTA in contrast to the wild type and ectopic Δcch1 mutant, which both exhibited growth (Fig. 6). These results support the role of Cch1 in obtaining calcium in low-calcium environments, as has been suggested in studies of *C. neoformans* (38) and yeast (39, 42). However, the fact that discharge was restored at high concentrations of Ca$^{2+}$ indicates that another channel is present—possibly a distinct low-affinity Ca$^{2+}$ influx system, as postulated by Muller and colleagues (42)—and able to substitute for Cch1.

In an effort to simulate peritheci um formation in crop residue in the field, we have established a protocol to induce development of perithecia on colonized wheat stems, a process which also allows conidiation. An unexpected outcome of this experiment was the observation that the Δcch1 mutant allowed more conidiation from contaminating fungi than did the wild type. This suggests that, while the Δcch1 mutant is competent in infecting and colonizing wheat, it may not be as capable as the wild type in defending its resources against competitors in the crop residue. One possible explanation may be the reduced expression of the polyketide synthase gene responsible for aurofusarin production (fg12040) (17, 34, 41). fg12040 showed a 16-fold reduction in transcript abundance in the Δcch1 mutant compared to the wild type during vegetative growth; this is of interest as rubrofusarin, a precursor to aurofusarin and also...
TABLE 2. Transcripts showing statistically significant twofold or greater differential accumulation between mutant and wild type at multiple time points

| Type of change in transcript abundance and transcript identifier | Transcript abundance in the mutant relative to wild type at the indicated time point<sup>a</sup> | Annotation |
|---------------------------------------------------------------|---------------------------------|------------|
| Twofold or greater increase between wild type and mutant at all time points | fgd246-110_at | Hypothetical protein |
| fgd124-460_at | 3.049206 2.09E-08 3.103217 0.000159 | Related to PKC2-related protein |
| fgd197-46_at | 3.049206 2.09E-08 3.103217 0.000159 | Related to PKC2-related protein |
| fgd35-590_at | 3.030282 0.001579 | Related to PRO3 protein |
| fgd422-230_at | 3.019806 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd458-640_at | 3.019806 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd458-640_at | 3.019806 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |
| fgd110-110_at | 3.009608 0.000159 | Related to ATP-binding cassette transporter protein |

<sup>a</sup> Significant changes in abundance in the mutant are indicated by an asterisk.

<sup>b</sup> Log 2 relative change. Negative numbers and positive numbers represent a decrease and increase, respectively, in mutant transcript abundance compared with wild type. P Values are identified by time point (e.g., P<sub>0h</sub> value for change in transcript abundance at 0 h). The cutoffs for statistical significance vary between treatments.
produced by the fg12040 protein, is known to possess antimicrobial properties (16, 21). The antimicrobial properties of aurofusarin have not been determined. More rigorous testing of this hypothesis using a strain deficient in aurofusarin production is under way. Although the growth of the mutant is slower in culture, the progress of disease symptoms is identical to that of the wild type, suggesting that its development and differentiation may reflect formation of discrete cell types (perithecium initials, ascogenous hyphae, paraphyses, and ascospores), and therefore our method of sampling based on developmental stages should ensure an equitable comparison. The decrease in these classes of genes may explain the delay in mycelial growth and development. It is surprising, with the large number of genes whose expression is affected, that the in vitro effects of the mutation are so subtle. The mutant might include differences in mycelial age. However, developmental stages reflect formation of discrete cell types (perithecium initials, ascogenous hyphae, paraphyses, and ascospores), and therefore our method of sampling based on developmental stage should ensure an equitable comparison.

For the study of sexual development and differentiation, 96 h is arguably the most interesting of the time points we examined: the perithecium has reached its full size and pigmentation, and asci are forming. Ascus structure will be completed over the next 24 h, and the spores will mature over the next 48 h (55, 44). A decline in meiosis-related transcript abundance was observed in each of the time points in the mutant; however, meiosis takes place during ascus development from about 96 to 120 h (24). It is not surprising, therefore, that 96 h

### Table 3. Functional categories of genes showing a statistically significant decrease in transcript accumulation of twofold or greater in the ∆cch1 mutant compared to wild type

| FunCat code | Category name (description) | % of genome | 0 h | 96 h | 144 h |
|-------------|-----------------------------|-------------|-----|------|------|
| 01          | Metabolism                  | 13.7        | 95 (24.2) | 9.43E-09 | 172 (20.7) | 6.54E-09 | 37 (9.86) | 1 |
| 02          | Energy                      | 2.96        | 22 (5.62) | 0.003189 | 44 (5.3) | 0.000129 | 4 (1.06) | 1 |
| 10          | Cell cycle and DNA processing | 32 (8.18) | 0.000491 | 79 (9.52) | 3.05E-11 | 32 (8.53) | 0.000235 |
| 11          | Transcription               | 4.77        | 20 (5.11) | 0.408944 | 55 (6.63) | 0.008169 | 31 (8.26) | 0.002126 |
| 12          | Protein synthesis           | 2.84        | 4 (1.02) | 1 | 26 (3.13) | 0.32809 | 9 (2.4) | 1 |
| 14          | Protein fate (folding, modification, destination) | 6.44 | 38 (9.71) | 0.007318 | 124 (14.9) | 1.79E-19 | 31 (8.26) | 0.091332 |
| 16          | Protein with binding function or cofactor requirement (structural or catalytic) | 7.36 | 39 (9.97) | 0.032521 | 101 (12.1) | 2.53E-07 | 25 (6.66) | 1 |
| 18          | Regulation of metabolism and protein function | 1.14 | 10 (2.55) | 0.014227 | 27 (3.25) | 6.93E-07 | 10 (2.66) | 0.010869 |
| 20          | Cellular transport, transport facilities and transport routes | 7.5 | 40 (10.2) | 0.027795 | 107 (12.9) | 1.35E-08 | 27 (7.2) | 1 |
| 30          | Cellular communication/signal transduction mechanism | 1.86 | 9 (2.3) | 0.307383 | 29 (3.49) | 0.000818 | 17 (4.53) | 0.000686 |
| 32          | Cell rescue, defense and virulence | 4.73 | 23 (5.88) | 0.16665 | 61 (7.35) | 0.00378 | 16 (4.26) | 1 |
| 34          | Interaction with the environment | 3.29 | 15 (3.83) | 0.308379 | 42 (5.06) | 0.003558 | 19 (5.06) | 0.042005 |
| 36          | Systemic interaction with the environment | 0.18 | 3 (0.76) | 0.035313 | 4 (0.48) | 0.065461 | 1 (0.26) | 0.508159 |
| 40          | Cell fate                   | 1.49        | 9 (2.3) | 0.131456 | 33 (3.98) | 2.15E-07 | 14 (3.73) | 0.001518 |
| 41          | Development (systemic)      | 0.3         | 2 (0.51) | 0.34069 | 5 (0.6) | 0.110039 | 1 (0.26) | 1 |
| 42          | Biogenesis of cellular components | 4.34 | 20 (5.11) | 0.257292 | 74 (8.92) | 2.12E-09 | 18 (4.8) | 0.366487 |
| 43          | Cell type differentiation    | 2.5         | 16 (4.09) | 0.037512 | 47 (5.66) | 1.15E-07 | 15 (4) | 0.050677 |
| 99          | Unclassified proteins        | 69.3        | 215 (54.9) | 1 | 413 (49.8) | 1 | 259 (69) | 0.568122 |

* As some genes can be classified in more than one FunCat category, percentages sum to >100.
showed the most meiosis genes down-regulated (12; also see Table S2 in the supplemental material). Also at 96 h, more genes are differentially regulated in the Δcch1 mutant than in the wild type, and this difference is greater than that at 0 h and 144 h (Table 3; also see Table S2 in the supplemental material).

Some of these changes in gene expression may be reflected in the increased number of spores with aberrant morphologies in the asci of the mutant.

Understanding the mechanism by which Cch1 affects discharge will reveal important aspects of ascus function. In mammalian cells, the L-type ion channels are triggered to open by membrane depolarization. Previous work has indicated that the fungal channels are less sensitive to voltage fluctuations and may have a different gating mechanism (38). Interestingly, we previously reported the effect of the L-type ion channel inhibitor, verapamil, resulting in nearly 50% reduction of the Δcch1 mutation, and the target of verapamil is, therefore, probably Cch1. Because verapamil is effective on perithecia whose asci are mature and whose nuclei are already enclosed in the spores, it seems likely that the effect of Cch1 on discharging asci is directly on the cell components to effect spore propulsion rather than by influencing transcription. This hypothesis is further supported by the calcium addition experiment reported here, in which the addition of exogenous calcium elicits discharge, indicating that the structural and physiological components of the discharge apparatus are intact. Calcium signaling is known to regulate cytoskeletal rearrangements (for example, Mace et al. [40]) and other physiological functions (3) in mammals. There are no gross cytoskeletal defects in the mutant, or the shape of the asci would be affected. However, more subtle defects in cytoskeleton function may be preventing ascus function. We will explore mechanisms both upstream and downstream of Cch1 in future experiments.

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REFERENCES

1. Affymetrix. 2004. GeneChip expression analysis. Affymetrix, Santa Clara, CA.
2. Berridge, M. J., P. Lipp, and M. D. Bootman. 2000. The versatility and universality of calcium signaling. Nat. Rev. Mol. Cell Biol. 1:11–21.
3. Berridge, M. J., M. D. Bootman, and H. L. Roderick. 2003. Calcium signaling: dynamics, homeostasis and remodeling. Nat. Rev. Mol. Cell Biol. 4:517–529.
4. Blankschien, J. R., F. L. Wormley, M. K. Boyce, W. A. Schell, S. G. Filler, J. R. Perfect, and J. Heitman. 2003. Calcineurin is essential for Candida albicans survival in serum and virulence. Eukaryot. Cell 2:422–430.
5. Booth, C. 1971. The genus Fusarium. Commonwealth Mycological Institute, Kew, Surrey, England.
6. Bowden, R. L. and J. L. Leslie. 1999. Sexual recombination in Gibberella zeae. Phytopathology 89:182–188.
7. Cappellini, R. A., and J. L. Peterson. 1965. Macronoid formation in submerged cultures by a non-spurulating strain of Gibberella zeae. Mycologia 57:962–966.
8. Carroll, A. M, J. A. Swiegard, and B. Valent. 1994. Improved vectors for selecting resistance to hygromycin. Fungal Genet. Newslett. 41:22.
9. Council for Agricultural Science and Technology. 2003. Mycotoxins: risks in plant, animal, and human systems. Task force report no. 139. Council for Agricultural Science and Technology, Ames, IA.
10. Cyert, M. S. 2001. Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. Annu. Rev. Genet. 35:647–672.
11. Dayton, J. S., and A. R. Means. 1996. Ca2+/calmodulin-dependent kinase is essential for both growth and nuclear division in Aspergillus nidulans. Mol. Biol. Cell 7:1511–1519.
12. Deroome, J. S., M. Sunjic, N. N. Nanthakumar, and A. R. Means. 1997. Expression of a constitutively active Ca2+/calmodulin-dependent kinase in Aspergillus nidulans spores promises germination and entry into the cell cycle. J. Biol. Chem. 272:3223–3230.
13. Deng, L., R. Sugimura, M. Takenchi, M. Suzuki, H. Ebina, T. Takamai, A. Koike, S. Iba, and T. Kuno. 2006. Real-time monitoring of calcineurin activity in living cells: evidence for two distinct Ca2+/dependent pathways in fission yeast. Mol. Biol. Cell 17:4790–4800.
14. Fischer, M., J. Cox, D. J. Davis, A. Wagner, R. Taylor, A. J. Huerta, and N. P. Money. 2004. New information on the mechanism of forcellin discharge from Ascbobula immersa. Fus. Genet. 41:698–707.
15. Fischer, M., N. Schnell, J. Chattaway, P. Davies, G. Dixon, and D. Sanders. 1997. The Saccharomyces cerevisiae CCH1 gene is involved in calcium influx and calcium binding. FEBS Lett. 419:259–262.
16. Frandsen, R. J. N., N. J. Nielsen, N. Maaløn, J. C. Sorensen, S. Olsson, J. Nielsen, and H. Giese. 2006. The biosynthetic pathway for aurofusarin in Fusarium graminearum reveals a close link between the naphthoquinones and naphthopyrones. Mol. Microbiol. 61:4169–4180.
17. Gaffoor, I., D. W. Brown, R. Plattner, R. H. Proctor, W. Qi, and F. Trail. 2005. Functional analysis of the polyketide synthase genes in the filamentous fungus Gibberella zeae (anamorph Fusarium graminearum). Eukaryot. Cell 4:1926–1933.
18. Gaffoor, I., and F. Trail. 2006. Characterization of two polyketide synthase genes involved in zearalanone biosynthesis in Gibberella zeae. Appl. Environ. Microbiol. 72:1793–1799.
19. Gavric, O., D. Becker de los Santos, and A. Griffiths. 2007. Mutation and divergence of the phospholipase C gene in Neurospora crassa. Fungal Genet. Biol. 44:224–229.
20. Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gentle, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. H. Yang, and J. Zhang. 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5:R8.
21. Graham, J. G., H. J. Zhang, S. I. Pendland, B. D. Santarsiero, A. D. Mecear, F. Cabieses, and N. R. Farnsworth. 2004. Antimycobacterial naphthopyrones from Senna obtusa. J. Nat. Prod. 67:225–227.
22. Guldener, U., K.-Y. Seong, J. Boddhu, S. Cho, F. Trail, J.-R. Xu, G. Adam, H.-W. Mewes, G. J. Muehlbauer, and H. C. Kistler. 2006. Development of a Fusarium graminearum Affymetrix GeneChip for profiling fungal gene expression in vitro and in planta. Fungal Genet. Biol. 43:316–345.
23. Guenther, J. C., and F. Trail. 2005. The development and differentiation of Gibberella zeae (anamorph Fusarium graminearum) during colonization of wheat. Mycologia 97:229–237.
24. Hallen, H. E., M. Huebner, S.-H. Shiu, U. Guldener, and F. Trail. 2007. Gene expression shifts during perithecium development in Gibberella zeae (anamorph Fusarium graminearum), with particular emphasis on ion transport proteins. Fungal Genet. Biol. 44:1146–1156.
25. Hou, Z., C. Yuo, Y. Peng, T. Katan, H. C. Kistler, and J.-R. Xu. 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.
26. Iida, H., H. Nakamura, T. Ono, M. S. Okumura, and Y. Anraku. 1994. MD1, a novel Saccharomyces cerevisiae gene encoding a plasma membrane protein, is required for Ca2+/ influx and mating. Mol. Cell. Biol. 14:8259–8271.
27. Irizarry, R. A., B. M. Bolstad, S. F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31:e15.
28. Irizarry, R. A., B. Hobbs, F. Collin, F. Beaver-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Bioinformatics 20:249–254.
29. Jansen, R., and M. Gerstein. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Bioinformatics 20:249–254.
30. Jin, Y., and X. Zhang. 1998. Mass production of ascospores of Gibberella zeae in solid-state fermentation. Phytopathology 88:544. (Abstract).
31. Joseph, J. D., and A. R. Means. 2000. Identification and characterization of two Ca2+/CaM-dependent protein kinases required for normal nuclear division in Aspergillus nidulans. J. Biol. Chem. 275:38230–38238.
32. Kanzaki, M., M. Nagasawa, I. Kojima, C. Sato, K. Naruse, M. Sokabe, and H. Ida. 1999. Molecular identification of a eukaryotic, stretch-activated, non-selective cation channel. Science 285:882–886.

33. Kerenyi, Z., K. Zelter, L. Hornok, and J. F. Leslie. 1999. Molecular standardization of mating type terminology in the Gibberella fujikuroi species complex. Appl. Microbiol. Biotechnol. 65:4071–4076.

34. Kim, J.-E., K.-H. Han, J. Jin, H. Kim, J.-C. Kim, S.-H. Yun, and Y.-W. Lee. 2005. Putative polyketide synthase and laccase genes for biosynthesis of aurofusarin in Gibberella zeae. Appl. Environ. Microbiol. 71:i701–1788.

35. Kim, Y. K., D. X. Li, and P. E. Kolattukudy. 1998. Induction of Ca2+-calmodulin signaling by hard-surface contact primes Colletotrichum gloeosporioides conidia to germinate and form appressoria. J. Bacteriol. 180:5144–5150.

36. Klittich, C. J. R., and J. F. Leslie. 1988. Nitrate reduction mutants of Fusarium moniliforme (Gibberella fujikuroi). Genetics 118:417–423.

37. Kraus, P. R., C. B. Nichols, and J. Heitman. 2005. Calcium- and calcineurin-independent roles for calmodulin in Cryptococcus neoformans morphogenesis and high-temperature growth. Eukaryot. Cell 4:1079–1087.

38. Liu, M., P. Du, G. Heinrich, G. M. Cox, and A. Gelli. 2006. Cch1 mediates calcium entry in Cryptococcus neoformans and is essential in low-calcium environments. Eukaryot. Cell 5:1786–1796.

39. Locke, E. G., M. Bonilla, L. Liang, Y. Takita, and K. W. Cunningham. 2000. A homolog of voltage-gated Ca2+ channels stimulated by depletion of secretory Ca2+ in yeast. Mol. Cell. Biol. 20:6868–6894.

40. Mace, O. J., E. L. Morgan, J. A. Affleck, N. Lister, and G. L. Kellett. 2007. Calcium absorption by CaV1.3 induces terminal web myosin II phosphorylation and apical GLUT2 insertion in rat intestine. J. Physiol. 586:605–616.

41. Malz, S., M. N. Grell, C. Thrane, F. J. Maier, A. Felk, K. S. Albertsen, S. Salomon, L. Bohn, W. Schafer, and H. Giese. 2006. Cch1 mediates calcium entry in Cryptococcus neoformans and is essential in low-calcium environments. Eukaryot. Cell 5:1786–1796.

42. Muller, E. M., E. G. Locke, and K. W. Cunningham. 2001. Differential regulation of two Ca2+ influx systems by pheromone signaling in Saccharomyces cerevisiae. Genetics 159:1527–1538.

43. Odom, A., S. Muir, E. Lim, D. L. Toftaletter, J. Perfect, and J. Heitman. 1997. Calcineurin is required for virulence of Cryptococcus neoformans. EMBO J. 16:2576–2580.

44. Qi, W., C. Kwon, and F. Trail. 2006. Microarray analysis of transcript accumulation during perithecium development in the filamentous fungus Gibberella zeae (anamorph Fusarium graminearum). Mol. Genet. Genomics 276:87–100.

45. R Development Core Team. 2006. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.