Cla4, but not Rac1, regulates the filamentous response of *Ustilago maydis* to low ammonium conditions

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Rho/Rac GTPases have been implicated in regulating cell polarity, cytoskeletal organization and cytokinesis; when activated, they modulate localization and activity of several downstream effectors, including p21-activated protein kinases (PAKs). The PAKs comprise a large family of serine/threonine protein kinases that regulate many cell processes including cell cycle, cytoskeletal organization and polar growth. As potential downstream effectors of the Rho/Rac GTPases, PAKs contribute to many of the GTPase specific roles.

*Ustilago maydis* is a basidiomycete fungus for which unicellular haploid cells reproduce by budding. Haploid cells of opposite mating backgrounds are able to fuse and form a diploid filamentous dikaryon. This dikaryon can subsequently infect maize (*Zea mays*). Cell fusion and pathogenic development are controlled by two separate loci, *a* and *b*. The *a* locus encodes a pheromone and pheromone receptor involved in cell recognition and cell fusion. Subsequent pathogenic development is dependent upon the *b* locus which encodes two homeodomain proteins, bE and bW. Differentiation into the infectious dikaryon is dependent upon the activity of this *b* heterodimer. However, *U. maydis* cells can also undergo similar filamentous differentiation in response to environmental conditions. Lipids, acidic pH and low ammonium conditions can trigger the filamentous response in a *b* locus-independent manner.

The signaling components responsible for such filamentous responses have been the subject of a number of investigations. In *U. maydis*, three small GTPases have been well-characterized: Rac1 establishes polar growth, while Cdc42 regulates cell separation, and Rho1 is required for cell polarity and cytokinesis. Cla4, a Ste20-like PAK kinase, has been identified as a downstream effector of Rac1, while downstream effectors of Cdc42 and Rho1 have yet to be identified, although Cla4 is...
Rac1 plays a role in polar growth and normal bud formation by its localization to the polar tip; Rac1 activity promotes localized cell wall formation in a Cla4 dependent manner.2 The Δcla4 mutant strains display a dramatic disruption in chitin deposition; however the strains display a dramatic disruption in polar growth.2 Thus it appears that Rac1, but is dispensable for bud formation and promote mother daughter cell separation, cell growth while Cdc42 is required to formation, and then promotes polarized apparatus assembly necessary for bud formation under conditions of low ammonium. In contrast the Ste20 homolog, Smu1, is known to have a role in the filamentous response to low ammonium conditions.9 Smu1 appears to regulate filament formation under low ammonium conditions in a positive fashion. In this study, we provide evidence that Cla4 also plays a role in the filamentous response to low ammonium conditions. In addition, Rac1 may not be the only upstream activator of Cla4. While neither Cla4 nor Rac1 are absolutely required for filament formation as a response to low ammonium, this ability is greatly reduced in rac1 deletion strains.

When grown in rich conditions, the Δcla4 mutant cells are morphologically distinct from the cells disrupted for the U. maydis PAK-like Ste20 homolog, Smu1. As seen previously in reference 8, Δcla4 cells were septated, containing several independent nuclei having failed to correctly separate (Fig. 1), unlike the Δsmu1 mutant strains which are affected only in cell length17 (Table 1). Moreover, the Δcla4 cells appear fatter than the wild type strains.8 In the current study, examination of the Δcla4 mutant strains identified a significant increase in cell length (Table 1). This increase in cell length stands in sharp contrast to the decrease in cell lengths observed in Δsmu1 mutant strains.17 These results are consistent with the observed roles of Cla4 in cytokinesis and polar growth in S. cerevisiae and C. albicans.10,18 In addition, the phenotypes observed in the Δcla4 mutants were similar to those exhibited in Cdk1, Cdk5 and Weel mutants, indicating that Cla4 may play a role in cell cycle similar to Cla4 from S. cerevisiae.19-21 The increased cell length of the Δcla4 mutant strains (Table 1) could indicate a delay in the G2-M transition in these U. maydis cells. Another possibility is that deletion of cla4 indirectly promotes cell elongation by failing to promote cell separation (via binding to Rac1), thus creating a cell cycle delay. Other pathways could then promote increased polar growth leading to a filamentous response. On low ammonium agar (SLAD), both the Δcla4 mutant and overexpression cla4mutant cells were filamentous, albeit less than the wild type (Fig. 2). Strikingly, in contrast to wild type, in liquid SLAD the Δcla4 mutant produced elongated filamentous cells (Fig. 3).

The role Rac1 plays in the filamentous response to low ammonium conditions is more complicated. Deletion of rac1 dramatically delayed filament formation, but did not eliminate the ability to form filaments.

### Table 1. Measures of cell length across all strains

| Strain          | n   | Length* (in μm) | Comparison         | p*  |
|-----------------|-----|-----------------|-------------------|-----|
| WT              | 213 | 19.14 ± 0.31    |                   |     |
| Δcla4           | 111 | 24.05 ± 0.83    | WT v. Δcla4       | >0.001 |
| Δsmu1           | 281 | 17.64 ± 0.18    | WT v. Δsmu1       | >0.05 |
| cla4mut         | 505 | 19.69 ± 0.20    | WT v. cla4mut     | N.S. |
| smu1mut         | 224 | 19.66 ± 0.30    | WT v. smu1mut     | N.S. |

*All mutants and wild type (WT) compared here were in the same genetic background, that of strain FB2.##. Cell length values are averages, ±S.E. Statistical analysis was performed using one way ANOVA with a Dunnett’s Multiple Comparison Test. N.S., Not Significant.
Cla4 to bind to the membrane and localize to polar growth sites as well as regulate cell elongation and hyphal growth. Subsequently Cla4 is involved in the hyper-phosphorylation of Swe1 promoting G_{1}-M transition. It is striking that the two *U. maydis* PAKs, Cla4 and Smu1, have opposite roles in the filamentous response pathway, where deletion of *cla4* alone or overexpressing *smu1* in a Δ*gsl7* background, leads to a hyper-filamentous response to low ammonium.

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Figure 3. Δclα4 strains produce filaments in liquid SLAD and display defects in cell wall localization. Δclα4 cells produce filaments in liquid SLAD, unlike WT or other mutant cells. The Δclα4 cells were highly branched displaying very few septa, with branch points occurring at cross wall septa (See arrows). The Δclα4 strains display massive chitin delocalization (WGA) but no defects in β-glucan localization (CFW). Neither clα4Δ nor either rac1 mutant type produced filaments; none of these latter mutants displayed defects in cell wall localization. Staining of U. maydis cells was obtained by treating 5 µL of cells with 1 µL of 10 µg/mL of Calcofluor white (CFW, 2 µg/mL final concentration; Fluorescent Brightener 28, Sigma, St. Louis, MO, specific for β-glucan and chitin of cell wall) or 100 µg/mL wheat germ agglutinin (WGA, 17 µg/mL final concentration; Tetramethylrhodamine conjugate, Invitrogen), specific for chitin of cell wall. Nucleic acid staining used 10 µM Syto 11 (5 nM green fluorescent nucleic acid stain). Scale bars, 10 µm.