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Turgor and net ion flux responses to activation of the osmotic MAP kinase cascade by fludioxonil in the filamentous fungus *Neurospora crassa*

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ABSTRACT

The internal hydrostatic pressure (turgor) of the filamentous fungus *Neurospora crassa* is regulated at about 400–500 kiloPascals, primarily by an osmotic MAP kinase cascade which activates ion uptake from the extracellular medium and glycerol synthesis. In the absence of hyperosmotic stress, the phenylpyrrole fungicide fludioxonil activates the osmotic MAP kinase cascade, resulting in cell death. Turgor, the electrical potential and net ion fluxes were measured after treatment with fludioxonil. In wildtype, fludioxonil causes a hyperpolarization of the plasma membrane and net H^+ efflux from the cell, consistent with activation of the H^+-ATPase. At the same time, net K^+ uptake occurs, and turgor increases (about 2-fold above normal levels). None of these changes are observed in the *os–2* mutant (which lacks a functional MAP kinase, the last of the three kinases in the osmotic MAP kinase cascade). Tip growth ceases as hyperpolarization, net ion flux changes, and turgor increases begin. The inappropriate turgor increase is the probable cause of eventual lysis and death. The results corroborate a multi-pathway response to hyperosmotic stress that includes activation of plasma membrane transport. The relation to cell expansion (tip growth) is not direct. Increases in turgor due to ion transport might be expected to increase growth rate, but this does not occur. Instead, there must be a complex regulatory interplay between the growth and the turgor driving force, possibly mediated by regulation of cell wall extensibility.

1. Introduction

Fungi often maintain a high internal hydrostatic pressure (turgor) during cellular expansion (Lew et al., 2004), a trait also common in cells of higher plants and algae (Zimmermann, 1978; Tomos and Leigh, 1999). In *Neurospora crassa*, hyperosmotic stress causes a rapid turgor decrease, but turgor recovers within an hour (Lew and Nasserifar, 2009). Turgor recovery is caused by two different mechanisms: *de novo* synthesis of osmolytes (Ellis et al., 1991) and ion accumulation from the extracellular medium (Lew et al., 2006). Both responses are activated by an osmotic MAP kinase cascade (O'Rourke et al., 2002; Krantz et al., 2006). A histidine kinase sensor, OS–1 (Alex et al., 1996; Miller et al., 2002), activates the kinase cascade comprised of MAPKKK OS–4 and MAPKK OS–5 (Fujimura et al., 2003), and MAPK OS–2 (Zhang et al., 2002). Upon activation of OS–2, one response is the expression of a number of genes, including genes for glycerol synthesis (Noguchi et al., 2007) as well as sugar and amino acid metabolism (Irmler et al., 2006); at least one of the transcription factors activated by OS–2 has been identified, ATF–1 (Yamashita et al., 2008). Independent of gene expression, the plasma membrane H^+-ATPase and ion uptake are rapidly (within 10–15 min) activated by hyperosmotic treatment via the osmotic MAP kinase cascade (Lew et al., 2006) and can regulate turgor in the absence of glycerol synthesis (in the osmosensitive *cut* mutant) (Lew and Levina, 2007).

A useful tool in analysis of the osmotic response is the phenylpyrrole fungicide fludioxonil (Pillonel and Meyer, 1997). It activates the osmotic MAP kinase cascade directly (Zhang et al., 2002; Irmler et al., 2006). Mutants in the sensor and kinase cascade (*os–1*, *os–4*, *os–5*, *os–2*) are insensitive to the fungicide (Fujimura et al., 2000; Ochiai et al., 2001; Zhang et al., 2002). Fludioxonil activation of the osmotic MAP kinase cascade occurs at the OS–1 osmosensor; *os–1* mutant alleles fall into two classes of resistance to fludioxonil (high and low) that are related to the site of the mutation in the OS–1 gene (Ochiai et al., 2001). When an *os–1* homolog (*HIK1* from the rice blast fungus *Pyricularia oryzae*) is expressed in *Saccharomyces cerevisiae* (which lacks a homolog of *os–1* and is normally insensitive to the fludioxonil), it confers sensitivity to fludioxonil (Motoyama et al., 2005). Another yeast (*Candida albicans*) contains a homolog of the *os–1* gene and is sensitive to fludioxonil (Ochiai et al., 2001, 2002). Thus, the phenylpyrrole fungicide is specific to the osmotic MAP kinase cascade, and offers an alternative way to activate the osmotic MAP kinase cascade and explore its mechanism in detail, without perturbing the cells with the stress of high osmolarity, a stress that may activate multiple osmoresponsive pathways. We have previously shown that fludioxonil

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causes hyperpolarization of the plasma membrane potential in wildtype, a response that is absent in the *os–2* mutant (Lew and Levina, 2007). In this paper, the effect of fludioxonil on turgor, potential, and ion fluxes is examined. Fludioxonil activation of the osmotic MAP kinase cascade activates ion transport and elevates turgor well above normal levels. The results corroborate previous work on ion transport regulation by the kinase cascade, and demonstrates directly the turgor increases that are the likely cause of fungal death.

2. Materials and methods

2.1. Strains

Stock cultures of wildtype (FGSC No. 2489) and *os–2* (allele UCLA80, FGSC No. 2238) were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA)(McCluskey, 2003).

Stock cultures of the strains were maintained on Vogel's (plus 1.5% (w/v) sucrose and 2.0% (w/v) agar) medium (VM) (Vogel, 1956).

2.2. Culture preparation for experiments

Cultures were prepared by inoculating conidia onto strips (2.5 × 6 cm) of dialysis tubing that overlay the VM in Petri dishes and incubated at 28 °C overnight. The dialysis tubing was cut with a razor blade to a size of about 1 × 3 cm, which included the growing edge of the colony, placed inside the cover of a 30 mm Petri dish, and immobilized on the bottom with narrow strips of masking tape. The culture was flooded with 3 ml of a modified buffer solution (BS) (in mM): $CaCl_2$ (0.05), $MgCl_2$ (0.05), and sucrose (150), unbuffered. The use of unbuffered media with low salt concentrations was to avoid 'hidden' H^+ fluxes due to H^+ binding to buffer (Arif et al., 1995; Messerli et al., 2006) and avoid high background concentrations of K^+, to maximize the signal to noise ratio of K^+ flux measurements. Since the fungal colonies were grown on nutrient-replete Vogel's medium, there was carry-over of K^+. Background [K^+] in the submersed fungal colonies (measured with the ion-selective probe) was about 230 ± 106 μM (n = 24). The pH remained fairly constant (5.81 ± 0.37, n = 27) over the course of the experiments. Growth of hyphae at the colony edge resumed within 15 min after flooding with the modified BS.

2.3. Electrical measurements

Large trunk hyphae (10–20 μm diameter) about 0.5 cm behind the colony edge were selected for potential and concomitant net ion flux measurements. The hypha was first impaled with a micropipette, then the ion-selective probe was positioned nearby for measurements of the ion flux. Em values were measured when the Em had stabilized immediately before fludioxonil addition, and at a point where the Em value was stable (that is, unchanging) after treatment. For complete time course experiments, Em values were digitized at 30 s intervals for presentation.

2.4. Ion flux measurements

Measurements with the ion-selective probe have been described in detail previously (Lew, 1999; Lew et al., 2006; Lew and Levina, 2007). The following ion-selective cocktails were used (Sigma–Aldrich): H^+ (hydrogen ionophore II-cocktail A, Catalogue No. 95297); K^+ (a modified version of potassium ionophore I-cocktail A, Catalogue No. 60398 in which 25% w/w 2-nitrophenyl octyl ether was replaced with 25% w/w 1,2-dimethyl-3-nitrobenzene [Messerli et al., 2006]). The ion-selective probes were calibrated at pH 7, 6, 5 and 4 (H^+) using pH standards (Fisher) or 0.01, 0.1, 1.0, 10.0 mM KCl (K^+). The concentration of the selected ion was measured at two positions: as near to the hyphal wall as possible, and 20 μm away, at a frequency of about 0.3 Hz. The differences in ion concentrations at the hypha and 20 μm from the hypha were converted to net ion flux (J, nmol m^{-1} s^{-1}) with the following equation, which accounts for the cylindrical geometry of the hyphae:

$$J = \frac{D}{r}\frac{(c_2 - c_1)}{\ln\left(\frac{r_2}{r_1}\right)}$$

where D is the diffusion coefficient (H^+, 9.31×10^{-9} m^2 s^{-1}; K^+, 1.96×10^{-9} m^2 s^{-1}); r is the hyphal radius; c_1 and c_2 are the concentrations at the two excursion points; and r_1 and r_2 are the distances from the hyphal centre to the two excursion points (Henriksen et al., 1992). Background measurements were performed 100 μm above the hypha twice during the experiments (during the pre-treatment measurement and at the end of the experiment); these were used to correct any offset in the measured net ion flux.

Net influxes are shown as negative fluxes; net effluxes are shown as positive fluxes.

After about 15 min of recording the electrical potential and the net ion flux, the hyphae were treated with fludioxonil, added as an 0.5 ml aliquot of 0.8 mM fludioxonil in modified BS (final concentration 115 μM), and measurements continued for about 14 min. At this concentration, fludioxonil is expected to have maximal inhibitory effect. Pillonel and Meyer (1997) reported half-maximal effects at 0.06 μM; complete growth inhibition is observed at 100 μM (Ochiai et al., 2001). For compiled data, the ion fluxes were obtained by averaging all of the measurements before and after fludioxonil treatment.

2.5. Turgor measurements

Impalements were made on large trunk hyphae of cultures prepared the same way as for electrophysiological and ion flux measurements. The technique has been described in detail previously (Lew et al., 2004, 2006; Lew and Levina, 2007). Large-aperture micropipettes were fabricated using a double-pull technique. The micropipette was filled with low-viscosity silicone oil (polydimethylsiloxane, 2.5 centistokes; Dow Corning), and mounted in a holder attached to a micrometer-driven piston by thick-wall Teflon tubing. Pressure was measured with a transducer (XT-190–300G; Kulite Semiconductor Products) mounted on the holder. After impalement, the internal hydrostatic pressure pushed the meniscus between the silicone oil and the cell sap into the micropipette; the pressure required to bring the meniscus back to the tip was a measure of the turgor. After turgor measurements for ~6 min, 0.5 ml of 0.8 mM fludioxonil in modified BS was added to the dish and turgor measurements continued for about 40 min. If the micropipette tip became plugged during the turgor measurements, every attempt was made to re-impale the same hypha, or another one nearby, with a fresh micropipette. Initial cell turgor measurements include multiple attempts to obtain long-term measurements from single dishes. For turgor recovery experiments, five independent experiments were performed for each strain.

2.6. Statistical analysis

Data are shown as mean ± SD (sample size) unless stated otherwise. Independent two-tail t-tests and non-linear regressions were