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Ptk2 contributes to osmoadaptation in the filamentous fungus *Neurospora crassa* ☆

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ABSTRACT

Hyphal tip-growing organisms often rely upon an internal hydrostatic pressure (turgor) to drive localized expansion of the cell. Regulation of the turgor in response to osmotic shock is mediated primarily by an osmotic MAP kinase cascade which activates osmolyte synthesis and ion uptake to effect turgor recovery. We characterized a *Neurospora crassa* homolog (PTK2) of ser/thr kinase regulators of ion transport in yeast to determine its role in turgor regulation in a filamentous fungi. The *ptk2* mutant is osmosensitive, and has lower turgor poise than wildtype. The cause appears to be lower activity of the plasma membrane H^+-ATPase. Its role in osmoadaptation is unrelated to the activity of the osmotic MAP kinase cascade. Instead, it acts in an alternative pathway that, like the osmotic MAP kinase cascade, also involves ion transport mediated osmoadaptation.

1. Introduction

Fungal cell expansion often relies upon an internal hydrostatic pressure created by differences in the osmolarity of the cellular and extracellular media. This turgor causes wall expansion in localized regions of the cell; the result is tubular extension of the cell (tip growth). Fungal hyphae accumulate osmotically active solutes to maintain turgor during growth. In response to osmotic stress, they are able to actively adjust their osmolarity and thereby regulate turgor. The major pathway for turgor regulation is an osmotic MAP kinase cascade (Krantz et al., 2006). Downstream of osmosensors, a series of three kinases operate as 'amplifiers' to activate the expression of genes encoding various proteins that function in the synthesis of osmolytes – commonly glycerol – which may also act as protectants during cold (Panadero et al., 2006) and heat shock (Noguchi et al., 2007), as well as osmotic stress. In addition to biosynthesis of osmoprotectants, the filamentous fungi *Neurospora crassa* also actively accumulates ions from the extracellular media in response to hyperosmotic shock, mediated by the osmotic MAP kinase cascade (Lew et al., 2006). Either osmolyte synthesis or ion uptake is sufficient to maintain turgor in response to hyperosmotic shock (Lew and Levina, 2007).

Besides the osmotic MAP kinase cascade, other transduction mechanisms play a role in turgor regulation. Lew et al. (2006) showed that turgor regulation (albeit poor) is still observed when the osmotic MAP kinase cascade is inactivated in the *os-1* mutant (OS-1 encodes the osmosensor, Miller et al., 2000). The *os-1* mutant exhibits lower levels of glycerol synthesis (Ellis et al., 1991) and less activation of ion uptake (Lew et al., 2006), but phosphorylation of the OS-2 MAP kinase is still observed, suggesting that other activators of the cascade are present in wildtype (Noguchi et al., 2007). Even in the *os-2* mutant, turgor regulation is observed (Lew et al., 2006), as is glycerol synthesis (Fujimura et al., 2000). Thus, other pathways mediating osmoadaptation must be present.

Much of the research on osmotic stress responses in fungi has focused on *de novo* biosynthesis of osmolytes and regulation of gene expression. Given that ion transport also appears to play a key role in turgor regulation, we are exploring the possible roles of regulators of ion transport in *N. crassa*. In this paper, we explore the phenotype of a homolog of the yeast PTK2; ptk2p is known to regulate the activity of the yeast plasma membrane H^+-ATPase (Goossens et al., 2000). In the filamentous fungi *N. crassa*, the ptk2 homolog does affect osmosensitivity, and appears to function separately from the osmotic MAP kinase cascade.

2. Materials and methods

2.1. Strains and growth conditions

Stock cultures of wildtype *N. crassa* (strain 74-OR23-1A, FGSC No. 987), the *ptk2* mutant (NCU01940 knockout; Colot et al., 2006) (mating type a, FGSC No. 17932), a *cut* mutant (allele

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LLM1, mating type A, FGSC No. 2385), and the *os-1* mutant (allele B-135, mating type A, FGSC No. 951) were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) (McCluskey, 2003). Other knockout mutants (Colot et al., 2006) that were used in preliminary screens were FGSC No. 17954 (NCU06179), FGSC No. 14662 (NCU02806), and FGSC No. 17942 (NCU04335). The strains were maintained on Vogel's medium (plus 1.5% (w/v) sucrose and 2.0% (w/v) agar) (VM) (Vogel, 1956). To obtain a *cut ptk2* double mutant, the two strains (*cut*$^-$ mating type A and *ptk2*$^-$ mating type a) were crossed using standard procedures (Davis and de Serres, 1970). Progeny exhibiting the *cut* mutant phenotype (Kuwana, 1953) were selected and tested for the presence of the *ptk2* knockout. DNA was extracted from the progeny and screened by PCR using sequences that flanked the insertion site of the knockout cassette in *ptk2*$^-$ [left primer, 5′-GGGAGCAATGTGAAATGGAC-3′; right primer, 5′-AAGTTCGCTCCTCCTCTTCC-3′; predicted band sizes of 3.1 kb (knockout) or 1.7 kb (wildtype gene)] and, as an internal control, within the wildtype *ptk2* gene sequence [left primer, 5′-AAGACGTCGGTGATCGAAAT-3′; right primer, 5′-CTTTGTGCCCA GATCATCCT-3′; predicted band size 1.9 kb]. A double mutant (*cut*$^-$ *ptk2*$^-$) was identified by the presence of the *ptk2* knockout PCR product and absence of the control (wildtype gene) product.

2.2. Culture Preparation for experiments

Cultures were grown on strips (2.5 × 6 cm) of dialysis tubing that overlay VM in Petri dishes 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, gently placed inside the cover of a 30 mm Petri dish, and immobilized on the bottom with masking tape. The culture was flooded with 3 ml of buffer solution (BS) (in mM): KCl (10), $CaCl_2$ (1), $MgCl_2$ (1), sucrose (133), and MES (10), pH adjusted to 5.8 with KOH. Growth of hyphae at the colony edge normally resumed within 15 min in BS, indicating that there was no adverse effect on hyphal physiology.

For electrophysiology and turgor measurements, large trunk hyphae (10–20 μm diameter) *in situ* about 0.5 cm behind the colony edge were used.

2.3. Turgor measurements

Measurements of hyphal turgor have been described in detail elsewhere (Lew et al., 2004; Lew et al., 2006). The micropipettes were double pulled to obtain a large aperture tip, and filled with low viscosity silicone oil (polydimethylsiloxane, 1–1.5 centistoke, Dow Corning Corp., Midland MI). They were mounted in a custom-fabricated holder connected to a pressure transducer (XT-190-300G, Kulite Semiconductor Products) and, by thick-walled Teflon tubing, to a micrometer-driven piston. Upon impalement of the hypha, the hyphal turgor pushed the oil/cytoplasm interface into the micropipette. The pressure required to push the interface to the micropipette tip was taken as the normal hyphal turgor. To ensure that the micropipette tip did not become plugged, the pressure was adjusted every 1–2 min to ensure free movement of the oil/cytoplasm interface, and the pressure re-measured by bringing the interface back to the micropipette tip. Prior to hyperosmotic treatment, the turgor was measured about six times. Then, 0.5 ml of BS plus 1 M sucrose was added in a circle around the objective. Arrival of the hyperosmotic solution to the impaled hypha could be confirmed by the change in the refractive index, and occurred within about 1 min. The pressure was decreased to avoid movement of oil into the hypha as the turgor dropped (because of the high osmolarity of the extracellular solution). As turgor recovered, the pressure was adjusted periodically (usually every 2–5 min) to ensure the tip remained unplugged. In the event the tip did plug, a new micropipette was prepared and impaled into a nearby hyphal compartment to continue to monitor turgor recovery. During the experimental runs, measurements of the wildtype and *ptk2* mutant strains were normally interspersed.

2.4. Electrical measurements

After addition of 3 ml of BS to the dish, and confirmation that the hyphal tips had recommenced growth, double barrel micropipettes (Lew, 2006) were impaled into large hyphal trunks. To voltage-clamp the hyphae, a bipolar staircase of alternating positive and negative clamps, with intervening clamps at the resting potential was performed. The clamp duration was 50 ms; the clamped voltage and clamping current were measured during the last 5 ms of the clamp. If the clamped voltage did not reached the expected value, or the clamping current was outside the measurable range (±50 nA), the current–voltage measurements were discarded; potential measurements were, however, retained. Treatments were performed by adding 0.5 ml of BS plus 1 M sucrose (hyperosmotic), 0.5 ml of BS plus 0.5 M (low hyperosmotic treatment, which normally does not elicit a transient depolarization but does elicit the sustained hyperpolarization), 0.5 ml of BS plus 50 mM NaCN (cyanide treatment), or 0.3 ml of BS plus 0.8 mM fludioxonil (fludioxonil treatment).

2.5. Statistical analysis

Statistics are shown as mean ± SD (n = sample size) (SD was used as an estimator of population variance) unless stated otherwise. Independent two tail t-tests were performed in either SYSTAT (Systat, Inc.) or Excel (Microsoft). Least squares analysis of regression slopes (±standard error) were performed in SYSTAT.

3. Results

Our interest in the *ptk2* mutant arose from reports of its role in regulation of the yeast (*Saccharomyces cerevisiae*) H^+-ATPase, specifically activation of the H^+-ATPase by glucose (Goossens et al., 2000). The Ptk2 protein localizes to the plasma membrane and phosphorylates the C-terminus of the ATPase (Eraso et al., 2006), as part of a tandem two-site phosphorylation process in response to glucose (Lecchi et al., 2007). Ptk2p had been previously reported to regulate polyamine uptake (Nozaki et al., 1996), thus it was a candidate for regulation of transport during growth and development of a filamentous fungus. A BLAST search with the yeast ptk2 sequence of the *N. crassa* genome revealed a very similar gene, NCU01940 with 96 identities (25.5%) and 161 similar amino acids (45.1%) in an alignment of 357 amino acids. The gene is expressed in *N. crassa* (Tian et al., 2007). In reverse BLAST comparisons of the *S. cerevisiae* genome, the *N. crassa* Ptk2 protein sequence had strong similarities with a family of protein kinases known to regulate transport. These included SAT4 ([YCR008 W] alias HAL4, 32.0% identity) (which regulates activity of the potassium transporters trk1p and trk2p, Mulet et al., 1999), NPR1 ([YNL183C], 30.7% identity) (which regulates amino acid transporters, Vandenbol et al., 1987), HRK1 ([YOR267C], 28.9% identity) (which regulates activity of the H^+-ATPase, Goossens et al., 2000) as well as other serine/threonine kinase proteins. For example, PRR2 ([YDL214C], 32.0% identity), which is a regulator downstream of the mating MAP kinase cascade in yeast (Burchett et al., 2001).

We performed preliminary screens of the *N. crassa ptk2* mutant, as well as other candidate transport regulators (NCU06179 [stk5, a HAL4 homolog, FGSC17954], and NCU04335 [a HRK1 homolog, FGSC 17942]. Because of reports of the role of 14-3-3 proteins in activation of plant H^+-ATPases (Oecking et al., 1994), we also