Role of a Mitogen-Activated Protein Kinase Cascade in Ion Flux-Mediated Turgor Regulation in Fungi

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Fungi normally maintain a high internal hydrostatic pressure (turgor) of about 500 kPa. In response to hyperosmotic shock, there are immediate electrical changes: a transient depolarization (1 to 2 min) followed by a sustained hyperpolarization (5 to 10 min) prior to turgor recovery (10 to 60 min). Using ion-selective vibrating probes, we established that the transient depolarization is due to Ca$^{2+}$ influx and the sustained hyperpolarization is due to H$^+$ efflux by activation of the plasma membrane H$^+$-ATPase. Protein synthesis is not required for H$^+$-ATPase activation. Net K$^+$ and Cl$^-$ uptake occurs at the same time as turgor recovery. The magnitude of the ion uptake is more than sufficient to account for the osmotic gradients required for turgor to return to its original level. Two osmotic mutants, os-1 and os-2, homologs of a two-component histidine kinase sensor and the yeast high osmotic glycerol mitogen-activated protein (MAP) kinase, respectively, have lower turgor than the wild type and do not exhibit the sustained hyperpolarization after hyperosmotic treatment. The os-1 mutant does not exhibit all of the wild-type turgor-adaptive ion fluxes (Cl$^-$ uptake increases, but net K$^+$ flux barely changes and net H$^+$ efflux declines) (os-2 was not examined). Both os mutants are able to regulate turgor but at a lower level than the wild type. Our results demonstrate that a MAP kinase cascade regulates ion transport, activation of the H$^+$-ATPase, and net K$^+$ and Cl$^-$ uptake during turgor regulation. Other pathways regulating turgor must also exist.

Osmotic shock is a perilous condition for any organism. The shock can be due to either hyperosmotic or hypoosmotic changes in the external environment. Unlike unwalled cells (mostly animal), which must remain isotonic to the external environment, organisms having walled cells can utilize a high internal hydrostatic pressure (turgor) created by trans-plasma membrane osmotic gradients to drive cell expansion during growth (15) and, therefore, may regulate turgor to maintain growth.

We are exploring turgor regulation in walled cells directly using pressure probe measurements of turgor. In two species we have examined, turgor is regulated within 60 min: in roots of the higher plant Arabidopsis thaliana (24) and in hyphae of the fungus Neurospora crassa (15). Prior to significant turgor recovery, both organisms exhibit similar electrical changes to hyperosmotic treatment: a hyperpolarization that is probably caused by activation of the plasma membrane proton pump that plays a role in turgor recovery (24).

Mitogen-activated protein (MAP) kinase cascades contribute to osmotic and/or turgor regulation. In yeast, hyperosmotic shock results in the synthesis and accumulation of glycerol, a response mediated by the HOG (high osmotic glycerol) pathway (reviewed by Mager and Siderius [16]). In N. crassa, the genes from a number of mutants sensitive to high osmolarity have been identified as MAP kinase cascade members. The OS-2 protein is homologous to the yeast HOG, a MAP kinase (31). The OS-4 and OS-5 proteins are homologous to the yeast SSK22 and PBS2 (MAP kinase kinase [MAPKK] and MAP kinase [MAPK] kinases, respectively) (5). In addition to MAP kinase cascade genes homologous to those of the yeast HOG pathway, the os-1 gene (18) encodes a histidine kinase homologous to the yeast SLN1, the osmosensing histidine kinase upstream of the yeast HOG pathway. In wild-type N. crassa, one adaptive response to hyperosmotic conditions is glycerol production (3). The os-1 mutant accumulates lower levels of glycerol (3), as do os-2, os-4, and os-5 mutant strains (4).

Besides osmolyte accumulation, other adaptive mechanisms may exist, such as ion accumulation (15). In N. crassa, the size of the hyphal trunk compartments are large enough to allow direct measurements of ion fluxes using noninvasive ion-selective electrodes (19) to assess the role of ion transport in regulating turgor, which is also directly measurable with a pressure probe (15). We examined turgor regulation, electrical responses, and ion fluxes in N. crassa. We compared the wild type to os-1, the putative osmosensor, to test whether the osmo-sensor mediates turgor regulation caused by changes in net ion fluxes. Turgor regulation and electrical changes were also examined in the os-2 mutant to determine whether the complete MAP kinase cascade mediates the electrical response to hyperosmotic shock. Our results indicate that a MAP kinase cascade regulates ion transport, activation of the plasma membrane H$^+$-ATPase, and net ion uptake during turgor recovery.

**MATERIALS AND METHODS**

**Strains.** Stock cultures of the wild type (strain 74-OR23-1A, FGSC no. 987), os-1 (allele B-135, FGSC no. 951), os-2 (allele UCLA80, FGSC no. 2238), and cut (allele LLM1, FGSC no. 2385) were obtained from the Fungal Genetics Stock.
phenylpyrrole fungicide fludioxonil (80% tested for growth from an agar plug of mycelium placed in the center of a 10-cm petri dish containing either VM, VM plus 4% (wt/vol) NaCl, or VM plus the dicarboximide fungicide vinclozolin (50 μg/ml; Supelco, Bellefonte, PA). Growth at 28°C was quantified every few hours by measuring colony diameter. Growth rates of os-1 were about 70% of those of the wild type in VM. Unlike the wild type, the os-1 mutant did not grow in 4% NaCl and was insensitive to vinclozolin, confirming the mutant phenotype (18). The os-2 UCLAB80 allele encodes a trp to stop codon mutation in the protein kinase domain and is expected to be nonfunctional (31). The os-2 mutant phenotype was tested for growth from an agar plug of mycelium placed in the center of a 10-cm petri dish containing either VM, VM plus 4% (wt/vol) NaCl, or VM plus the phenylpyrrole fungicide fumidoxidon (80 μM; Sigma-Aldrich, Oakville, Ontario). The wild type and os-2 had similar growth rates in VM. Unlike the wild type, the os-2 mutant was unable to grow in 4% NaCl and was insensitive to fumidoxidon, confirming the mutant phenotype (31).

Culture preparation for experiments. Cultures used for experiments were grown overnight from 3- by 5-mm agar plugs excised from the stock culture and with 3 ml of buffer solution (BS) containing (mM concentrations indicated in parentheses) KCl (10), CaCl2 (1), MgCl2 (1), sucrose (133), and Mes (10), with pH adjusted to 5.8 with KOH. The dish cover was transferred to the microscope, and the meniscus was brought to the edge of the dish cover to make sure that the bevel of the cover was not stuck to the glass, usually within 15 to 20 min. Then the colony was used for electrophysiological or turgor measurement experiments. The hyphae chosen for impalements were large-trunk hyphae (10- to 20-μm diameter), usually about 0.5 cm behind the growing edge. Hyperosmotic treatment was usually applied by adding 0.5 ml of BS plus 1,000 mM sucrose (about 1,425 mosmol kg−1) to the 3 ml of BS (about 195 mosmol kg−1). The final osmolality of BS after the addition of sucrose was 1,620 mosmol kg−1. The osmolality of BS after the addition of 100 mM cycloheximide (Sigma-Aldrich, Oakville, Ontario) from a 20 mM stock solution (dissolved in 95% [vol/vol] ethanol, then diluted to 17% ethanol with distilled H2O). The hyperosmotic treatment was given by adding 0.5 ml of BS plus 1,000 mM sucrose to the 3 ml BS as described above.

Ion flux measurements. Noninvasive ion-selective microelectrodes were used to measure the diffusive ion gradients at the surface of the hypha, from which the ion flux across the cell membrane can be calculated (19, 26). The instrumentation (the MIFE technique) was developed at the University of Tasmania (Hobart, Australia) (19, 23). As for electrical and pressure measurements, large-trunk hypae were selected. Care was taken to assure that there was unobstructed access to the hypha, with no other hyphae in the vicinity, to avoid interfering ion fluxes. The neighboring hyphae to be measured were monitored to assure that hyperhyphal growth had resumed, which it usually did within 15 to 20 min. Then the colony was used for electrophysiological or turgor measurement experiments. The hyphae chosen for impalements were large-trunk hyphae (10- to 20-μm diameter), usually about 0.5 cm behind the growing edge. Hyperosmotic treatment was usually applied by adding 0.5 ml of BS plus 1,000 mM sucrose (about 1,425 mosmol kg−1) to the 3 ml of BS (about 195 mosmol kg−1). The final osmolality of BS after the addition of sucrose was 1,620 mosmol kg−1. The osmolality of BS after the addition of 100 mM cycloheximide (Sigma-Aldrich, Oakville, Ontario) from a 20 mM stock solution (dissolved in 95% [vol/vol] ethanol, then diluted to 17% ethanol with distilled H2O). The hyperosmotic treatment was given by adding 0.5 ml of BS plus 1,000 mM sucrose to the 3 ml BS as described above.

Cycloheximide treatments. To test whether hyposmotically induced hyperosmolality was due to de novo synthesis of plasma membrane H+ -ATPase, hyphae were pretreated for 13.5 to 22.5 min by adding a final concentration of 100 μM cycloheximide (Sigma-Aldrich, Oakville, Ontario) from a 20 mM stock solution (dissolved in 95% [vol/vol] ethanol, then diluted to 17% ethanol with distilled H2O). The hyperosmotic treatment was given by adding 0.5 ml of BS plus 1,000 mM sucrose to the 3 ml BS as described above.

Concentrations in this section are given in mM or μM, and the molarity at 25°C and 1 atm is given in brackets. The pH for solutions was 5.8, unless otherwise stated.

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Equilibrate. In addition to the refractive wave observed as hyperosmotic solution entered the field of view, the decrease in pressure required to bring the oil meniscus to the micropipette tip confirmed that the hypha was being subjected to high external osmolarity.

If the tip of the pressure probe became irreversibly plugged during recording, it was withdrawn from the cell and a new probe was used to impale a new cell compartment. Every attempt was made to impale the same hypha, a few compartments away from the first impalement, if damage caused by withdrawal of the micropipette did not result in cytoplasm loss from adjacent compartments. Otherwise, a different hypha was used.

Electrophysiological measurements. As for pressure measurements, large-trunk hyphae, usually about 0.5 cm behind the growing edge, were selected for impalements. Double-barrel micropipettes (12, 13) were used to allow simultaneous current injection and potential monitoring for current-voltage measurements. Voltage clamping was performed using an operational amplifier configured as a current-voltage converter, controlled by a computer program via a data acquisition hardware unit (Labmaster DMA, Scientific Solutions, Inc., Solon, Ohio). A voltage range of −300 to 0 mV was clamped using a biphasic step protocol of alternating positive- and negative-going voltages to avoid membrane hysteresis. Clamping currents were not corrected for the cable properties of the hypha (7). We measured the cable length constant (20) along the hypha and across one septal pore to be about 200 μm. Cable-corrected currents should be added to obtain the measured current. However, the clamping protocol requires multiple impalements into adjacent hyphal compartments, very technically difficult when hyperosmotic treatments may cause small movements of the hypha, dislodging the micropipette(s).

After impalement and recording of a stable potential for about 4 min from the hypha in 3 ml of BS, 0.5 ml of BS plus 1,000 mM sucrose was added dropwise in a circle surrounding the objective. Observation of a refractive wave soon after addition confirmed the arrival of hyperosmotic solution. If the impaled hypha showed significant hysteresis, the micropipette was withdrawn and the hypha was impaled a second time.

After recording from two hyphal compartments, the BS was removed and the hypha was impaled a third time. The previous micropipette was withdrawn, and the BS was added again so that it was continuous with the BS at the previous impalement. Double-barrel micropipettes (12, 13) were used to allow simultaneous current injection and potential monitoring for current-voltage measurements.
BS osmolality was in the range of 140 to 170 mosmol kg\(^{-1}\), and the osmolality of the BS plus 1,000 mM sucrose was adjusted to 1,000 to 1,100 mosmol kg\(^{-1}\). The final osmolality was measured after experiments and was between 320 and 370 mosmol kg\(^{-1}\), so the net osmotic change was about 190 mosmol kg\(^{-1}\).

In initial experiments, there was a background K\(^+\) efflux (300 nmol m\(^{-2}\) s\(^{-1}\)) from the tape used to hold the cellophane securely in the dish. Initial net K\(^+\) fluxes were zero when the hyphae were immobilized with a Plexiglas frame. Some of the wild-type data had to be corrected for the background flux that occurred when tape was used.

Reverse transcription (RT)-PCR measurement of gene expression. Cellophane strips overlaid with the mycelium with BS and growth resumption, the three strains exhibited lower turgor, with a ranking of WT > os-2 > os-1. All differences were statistically significant.

FIG. 1. Initial turgor in the wild type (WT) and os-1 and os-2 mutants. Two-tailed \(t\) test comparisons between the three strains are shown. Data are jittered on the \(x\) axis for clarity. The mutant strains exhibited lower turgor, with a ranking of WT > os-2 > os-1. All differences were statistically significant.

The band sizes for the os-1, os-2, and tubulin bands calculated from molecular markers (0.41, 0.56, and 0.69 kbp, respectively) were very similar to the predicted sizes. Quantitation was performed by measuring integrated density of the bands using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/ij/).

Statistical analysis. Data are shown as means ± standard deviations (sample size) unless stated otherwise. Independent two-tailed \(t\) tests were performed in either SYSTAT (Systat, Inc.) or Excel (Microsoft).

FIG. 2. Turgor recovery in the wild type and os-1 and os-2 mutants. Lines show individual experiments; filled symbols show averages. The data for turgor recovery in the wild type (A) are redrawn from Lew et al. (15) with permission of the publisher and involved an osmolality increase of 250 mosmol kg\(^{-1}\). Hyperosmotic treatments for os-1 (B) and os-2 (C) were an increase of 155 mosmol kg\(^{-1}\); both osmotic mutants were capable of turgor recovery within a time frame similar to that for the wild type (60 to 90 min). Turgor recovery experiments when NaCl (BS plus 70 mM NaCl) was used as the osmoticum rather than sucrose are shown in panel D. Circles, wild type; triangles, os-1. Note the different time scale. Wild-type turgor recovery was faster after NaCl treatment.

RESULTS

Initial turgor is different in the os mutants. After flooding the mycelium with BS and growth resumption, the three strains had significantly different turgors. The wild type had the highest turgor (496 ± 71 kPa, \(n = 26\)), os-1 had the lowest turgor (302 ± 71 kPa, \(n = 26\)), and the os-2 turgor was intermediate (422 ± 64 kPa, \(n = 32\)) (Fig. 1). Since the os mutants are unable to grow at high osmolarities, we examined turgor regulation after hyperosmotic treatment.

The os mutants exhibit turgor recovery. Even though initial turgors were different in os-1 and os-2 compared to the wild type, both of the osmotic mutants did exhibit turgor recovery after hyperosmotic treatment with sucrose (Fig. 2B and C), similar to the wild type (Fig. 2A) (15). The recovery time was similar in all strains; turgor recovered to near initial level within 60 min. Since most phenotype analyses of osmotic mu-
tants have used NaCl to cause hyperosmotic shock, we performed preliminary experiments to show that turgor recovery also occurs after NaCl treatment in the wild type and os-1 (Fig. 2D). We used BS plus 70 mM NaCl (final concentration), which should have an osmolality increase of about 140 mosmol kg\(^{-1}\), similar in magnitude to the hyperosmotic treatment with sucrose. Wild-type turgor recovery was faster in NaCl compared to sucrose, consistent with uptake of the ions from the extracellular medium to counter the high external osmolarity. The time course of os-1 turgor recovery after NaCl treatment was similar to the time course after sucrose treatment.

Rather than taking up sucrose directly, *N. crassa* relies upon an extracellular invertase and diffusional hexose uptake through a neutral carrier or active uptake through a H\(^+\)/hexose symport (2). The mechanism underlying turgor recovery after sucrose addition could involve ion transport or osmotic production. If ionic influxes are required, it is expected they will affect the electrical properties of the hyphae, both potential and conductance.

**Wild-type hyperpolarization is absent in the os mutants.** The electrical differences between the wild type, os-1, and os-2 are shown in Fig. 3 and 4. In all three strains, hyperosmotic treatment caused a transient depolarization. In the wild type, the transient depolarization was followed by a prolonged hyperpolarization to a value about 40 mV more negative than the initial potential (Fig. 3A). In os-1 (B), the transient depolarization occurs, but the prolonged hyperpolarization does not. os-2 (C) exhibits a response intermediate between those of os-1 and the wild type. (Right panels) Current-voltage relations are shown for the WT (average of 16 experiments) and os-1 (9 experiments) and os-2 (9 experiments) mutants. The curves are the averages of current-voltage measurements (shown by vertical bars on the membrane potential trace) prior to the hyperosmotic treatment (initial), during the transient depolarized state, and during the final repolarized state, as marked. During the transient depolarization, the current-voltage relation shifts to depolarized potentials during the transient depolarization, but conductance remains the same. During repolarization, the WT conductance decreased at negative potentials and recovered completely at positive voltages. The recovery of the outward current at positive voltages, indicative of positive ion flux out of the cell, is consistent with activation of the plasma membrane proton pump. These changes during repolarization are not observed in os-1, which exhibited a lower conductance at positive voltages compared to the initial current-voltage relation. os-2 had consistently high conductances initially, during the transient depolarization, and during the repolarization.

**FIG. 3.** Electrical responses of the wild type (WT) (A) and os-1 (B) and os-2 (C) mutants to hyperosmotic treatment. (Left panels) The membrane potential, from impalement to removal of the micropipette from the hypha, is shown. Hyperosmotic treatment (an increase of 155 mosmol kg\(^{-1}\)) was applied as marked by the addition of 0.5 ml BS plus 1,000 mM sucrose to 3 ml BS. After hyperosmotic treatment, the plasma membrane potential undergoes a transient depolarization, followed by a prolonged hyperpolarization in the wild type (A). In os-1 (B), the transient depolarization occurs, but the prolonged hyperpolarization does not. os-2 (C) exhibits a response intermediate between those of os-1 and the wild type. (Right panels) Current-voltage relations are shown for the WT (average of 16 experiments) and os-1 (9 experiments) and os-2 (9 experiments) mutants. The curves are the averages of current-voltage measurements (shown by vertical bars on the membrane potential trace) prior to the hyperosmotic treatment (initial), during the transient depolarized state, and during the final repolarized state, as marked. During the transient depolarization, the current-voltage relation shifts to depolarized potentials during the transient depolarization, but conductance remains the same. During repolarization, the WT conductance decreased at negative potentials and recovered completely at positive voltages. The recovery of the outward current at positive voltages, indicative of positive ion flux out of the cell, is consistent with activation of the plasma membrane proton pump. These changes during repolarization are not observed in os-1, which exhibited a lower conductance at positive voltages compared to the initial current-voltage relation. os-2 had consistently high conductances initially, during the transient depolarization, and during the repolarization.
and os-2 mutants. Hyperosmotically induced conductance changes were similar between the wild type and os-1, with the exception of a large outward current in the wild type at positive potentials during hyperpolarization which was consistent with increased H⁺-ATPase activity (Fig. 3A, B). The os-2 conductances before and after hyperosmotic treatment were much larger than those of either the wild type or os-1 (Fig. 3C).

To ensure that the absence of hyperpolarization in the os-1 and os-2 mutants was due to a nonfunctional MAP kinase cascade rather than a pleiotropic consequence of osmotic sensitivity, we compared the electrical responses of the wild type with another osmotic mutant, cut (29). Like the os mutants, the cut mutant is unable to grow on VM plus 4% NaCl (data not shown) (29), but it is not a member of the MAP kinase cascade family (29). In an experimental run comparing the wild type and cut, the cut mutant exhibited electrical responses to hyperosmotic treatment that were very similar to those of the wild type (both the transient depolarization and the sustained hyperpolarization). The hyperpolarization observed in cut (−13 ± 28 mV, n = 11) was statistically the same as that in the wild type (−15 ± 35 mV, n = 11) (P = 0.926). Therefore, the hyperpolarization induced by hyperosmotic treatment appears to be mediated by the MAP kinase cascade, either by activating the H⁺-ATPase directly or by inducing expression of the H⁺-ATPase gene.

**Protein synthesis is not required for the wild-type hyperpolarization.** To determine whether the hyperpolarization caused by hyperosmotic treatment in the wild type was due to de novo
synthesis of the plasma membrane H⁺-ATPase, hyphae were preincubated with 100 μM cycloheximide for 13.5 to 22.2 min, followed by hyperosmotic treatment. Cycloheximide at 100 μM is reported to inhibit protein synthesis in N. crassa immediately (9). Tenfold-lower concentrations are reported to inhibit the induction of galactose transport under starvation conditions (21) and protein synthesis and conidial germination (8). Our treatment protocol is similar to that used to examine glucose activation of the H⁺-ATPase in *Fusarium oxysporum* (70 μM and 10 min preincubation) (1).

The initial potential in cycloheximide was \(-138 \pm 7\) mV \((n = 5)\), which is significantly lower than that in the untreated wild type \((-160 \pm 15\) mV, \(n = 18\) \((P = 0.007)\). After hyperosmotic treatment and transient depolarization \((-78 \pm 38\) mV, \(n = 5\)), 4/5 hyphae exhibited a sustained hyperpolarization similar to that of the wild type \((-171 \pm 15\) mV \([n = 4]\) compared to \(-188 \pm 24\) mV \([n = 18]\), respectively). Even including the depolarized outlier, the average change from the initial potential \((-21 \pm 27, n = 5\)) is not significantly different from the wild-type change \((-29 \pm 22, n = 18\) \((P = 0.596)\). Therefore, the sustained hyperpolarization in response to hyperosmotic stress in the wild type does not appear to require protein synthesis. H⁺-ATPase activation is the probable cause of the hyperpolarization, an explanation that can be confirmed with ion-selective probe measurements of H⁺ efflux. The ion-selective probe technique can also reveal the role of other fluxes in turgor recovery.

H⁺, K⁺, Cl⁻, and Ca²⁺ fluxes explain turgor recovery in the wild type but not in *os-1*. Net fluxes of ions prior to hyperosmotic treatment were compared to those after treatment. A modified BS solution with lower K⁺, Ca²⁺, Mg²⁺, and Cl⁻ was used to maximize the signal-to-noise ratio of the ion flux measurements. The *os-1* mutant was examined in detail, since it exhibits the lowest turgor and most significant electrical difference compared to the wild type (Fig. 5).

In unbuffered BS, net H⁺ flux was outward (net efflux of about 400 to 500 nmol m⁻² s⁻¹) in both the wild type and *os-1*, resulting in net acidification of the external medium over time. After hyperosmotic treatment, the wild-type net H⁺ efflux increased, while the *os-1* net H⁺ efflux declined (Fig. 5A). The onset of the increased H⁺ efflux in the wild type \((3\) to \(5\) min) is similar to the onset of the membrane potential hyperpolarization \((3\) to \(4\) min). Thus, H⁺-ATPase activation is the likely cause of both responses.

In the wild type, net K⁺ flux shifted to an uptake of about 400 nmol m⁻² s⁻¹ within 5 to 10 min after hyperosmotic treatment. This was at a time when turgor was beginning to recover and the membrane potential had hyperpolarized. In the *os-1* mutant, K⁺ flux barely changed from a net flux of zero (Fig. 5B).

Cl⁻ uptake \((400\) to 800 nmol m⁻² s⁻¹) occurred within 5 min of the hyperosmotic treatment. The net uptake was observed in both the wild type and the *os-1* mutant (Fig. 5C).

There was a transient uptake of Ca²⁺ in both the wild type and *os-1* that was observed immediately \((1\) min) after hyperosmotic treatment, tapering to zero net flux within 5 to 10 min (Fig. 5D). No Ca²⁺ transient was observed in control experiments when BS was added to the BS solution. The time course of the Ca²⁺ transient uptake (Fig. 5D) was similar to the transient depolarization (Fig. 3).

The net changes in K⁺ \((400\) nmol m⁻² s⁻¹) and Cl⁻ \((600\) nmol m⁻² s⁻¹) uptake are in the range appropriate for osmotic adjustment required to recover initial turgor. Typical dimensions of a hyphal compartment are 15-μm diameter and 100-μm length, so the surface area is \(5.1 \times 10^{-9}\) m⁻² and the volume is \(1.8 \times 10^{-11}\) liters. If the net uptake of K⁺ and Cl⁻ across the plasma membrane is about 1 μmol m⁻² s⁻¹, K⁺ and Cl⁻ accumulation in the cell will cause a concentration increase of 17.2 mM min⁻¹, or as much as 1,000 mM in the 60 min required for complete turgor recovery. Thus, ion uptake alone is more than sufficient to account for turgor recovery in the wild type but does not account for turgor recovery in *os-1*, which does not exhibit K⁺ influx and must rely upon an alternative pathway.

![Fig. 5. Ion fluxes in response to hyperosmotic treatment in the wild type (circles) and *os-1* mutant (triangles). Data are shown as means ± standard errors for 0.5-min intervals (before) and 1-min intervals (after hyperosmotic treatment) (sample size was 6 or 7). Negative values represent ion efflux from the trunk hyphal compartment; positive values represent ion uptake into the hyphal compartment. (A) H⁺ efflux is observed in both the wild type and *os-1*. After hyperosmotic treatment, H⁺ efflux increases in the wild type but declines in *os-1*. (B) K⁺ uptake occurs in the wild type after hyperosmotic treatment but not in *os-1*. (C) Cl⁻ uptake is induced in both the wild type and *os-1* after hyperosmotic treatment. (D) There is a transient Ca²⁺ uptake in both the wild type and *os-1* after hyperosmotic treatment, with a time course similar to the transient depolarization in the membrane potential (Fig. 3).](image-url)
Increased os-1 and os-2 gene expression is observed only in the os-1 and os-2 mutants. Induction of a MAP kinase cascade transduction pathway can result in increased expression of the MAP kinase cascade genes (28). In contrast with the expectation that members of the osmoresponse transduction pathway would be expressed upon osmotic treatment, gene expression of os-1 and os-2, as well as that of beta-tubulin, exhibited a modest increase in expression in the os-1 and os-2 mutants but not in the wild type (Fig. 6).

**DISCUSSION**

Walled cells such as fungi normally maintain a high internal hydrostatic pressure that can be used to drive cell expansion (15). Direct turgor measurements reveal a significant difference between the wild type and the os-1 and os-2 mutants that lack a complete MAP kinase cascade pathway. The wild-type turgor measurements, 496 ± 87 kPa (n = 26), are very similar to turgors measured by Lew et al. (15): 476 ± 124 kPa (n = 65).

In the two osmotic mutants, turgor is significantly lower, and os-1 turgor is significantly lower than the turgor of os-2 (Fig. 1). Even though the os-1 and os-2 mutants are unable to grow at high osmolality, they are still able to regulate turgor. Therefore, in the absence of a functional MAP kinase cascade, the turgor poise is lower and may be insufficient to maintain turgor when the mutants are subjected to high external osmolality. The results are consistent with a MAP kinase cascade regulating turgor, but other signal transduction systems must also contribute to turgor regulation. A similar conclusion was reached by Furukawa et al. (6) in an analysis of the Aspergillus nidulans HOG pathway. In N. crassa, in addition to glycerol production as in yeast (3, 5), the MAP kinase cascade regulates turgor (Fig. 2) by activating ion transport, based on our electrical (Fig. 3, 4) and ion flux measurements (Fig. 5).

As reported previously (15), the wild type (and the osmotic mutant cut, which is unrelated to the MAP kinase cascade) exhibits a transient depolarization followed by a sustained hyperpolarization. The transient depolarization was observed in both os mutants, but the hyperpolarization was not (Fig. 3). Unlike os-1, os-2 exhibited an intermediate return to the initial potential, an intermediate response similar to its intermediate turgor magnitude in BS. It is possible that there are multiple pathways after the os-1 step. That is, MAPKK and MAPK kinases may activate other targets besides MAP kinase (the OS-2 protein). This would explain the intermediate response of the os-2 mutant between those of the wild type and the os-1 mutant. On the basis of ion flux measurements, the transient depolarization observed in the wild type and the os mutants can be attributed to Ca\(^{2+}\) influx into the cell (Fig. 5). Stretch-activated Ca\(^{2+}\)-permeable channels have been identified in N. crassa (10), although one would expect them to be activated by a hyposmotic shock that would swell the hypha rather than a hyperosmotic shock that causes cell shrinkage unless they are mechanosensitive, responding to either tensile or compressive forces on the membrane. Other Ca\(^{2+}\) channels have not been characterized for N. crassa, although genomic analysis does identify a number of putative Ca\(^{2+}\) channels (30). N. crassa has homologs of the yeast Cch1p, Mid1p, and Yve1p Ca\(^{2+}\)-permeable channels (30) and two IP\(_3\)-activated Ca\(^{2+}\) channels (25). Thus, the cause of the hyperosmotically induced transient Ca\(^{2+}\) influx is not known, but it is probably due to a Ca\(^{2+}\) channel in the plasma membrane.

The hyperpolarization implicates the H\(^+\)-ATPase. This is corroborated directly by the hyperosmotically induced H\(^+\) efflux, which has a time course very similar to the hyperpolarization (Fig. 2, 5). Inhibiting protein synthesis does not affect the hyperpolarization, so the H\(^+\)-ATPase is activated directly, probably via phosphorylation. Elevated cytoplasmic Ca\(^{2+}\) is also reported to cause hyperpolarization in N. crassa by activating the H\(^+\)-ATPase (11), but Ca\(^{2+}\) influx cannot be implicated in the electrical response to hyperosmotic shock because the os-1 has a transient Ca\(^{2+}\) influx but does not hyperpolarize. Turgor recovery can be explained completely by the changes in net ion flux in the wild type: a MAP kinase cascade mediates turgor regulation by regulating ion fluxes, including the activity of the plasma membrane H\(^+\)-ATPase and K\(^+\) uptake. The fact that turgor recovery is also observed in the two os mutants, although they maintain a lower turgor than the wild type, is a clear indication that other signaling pathways are present, acting in concert to maintain turgor during cellular growth. This conclusion is corroborated by the fact that os-1 and os-2 gene expression is stimulated by osmotic treatment only in the os-1 and os-2 mutants, consistent with regulation by alternative osmoresponse pathway(s) (Fig. 6). The absence of osmotically induced expression of os-1 and os-4 was noted by Youssar et al. (29). Expression of cut, which encodes a member of the haloacid dehydrogenase family that may function as a phosphatase, is induced by hyperosmotic treatment (29). Alternative osmoresponse pathway(s) may involve Ca\(^{2+}\), given the presence of transient Ca\(^{2+}\) influx in the wild type and the os-1 mutant, but this possibility awaits further research.

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