Turgor regulation in the osmosensitive *cut* mutant of *Neurospora crassa*

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The internal hydrostatic pressure (turgor) of fungal cells is maintained at 400–500 kPa. The turgor is regulated by changes in ion flux and by production of the osmotically active metabolite glycerol. In *Neurospora crassa*, there are at least two genetically distinct pathways that function in adaptation to hyperosmotic shock. One involves a mitogen-activated protein (MAP) kinase cascade (kinases OS-4, OS-5 and OS-2 downstream of the osmosensing OS-1); the other is less understood, but involves the *cut* gene, which encodes a putative phosphatase. This study examined turgor regulation, electrical responses, ion fluxes and glycerol accumulation in the *cut* mutant.

Turgor recovery after hyperosmotic treatment was similar to that in the wild-type, for both time-course (~40 min) and magnitude. Prior to turgor recovery, the hyperosmotic shock caused a rapid transient depolarization of the membrane potential, followed by a sustained hyperpolarization that occurred concomitant with increased H⁺ efflux, indicating that the plasma membrane H⁺-ATPase was being activated. These changes also occurred in the wild-type. Net fluxes of Ca²⁺ and Cl⁻ during turgor recovery were similar to those in the wild-type, but K⁺ influx was attenuated in the *cut* mutant. The similar turgor recovery can be explained by the ion uptake, since glycerol did not accumulate in the *cut* mutant within the time frame of turgor recovery (but did accumulate in the wild-type). The results suggest that turgor regulation involves multi-faceted coordination of both ion flux and glycerol accumulation. Ion uptake is activated by a MAP kinase cascade, while CUT is required for glycerol accumulation.

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INTRODUCTION

Walled cells often maintain a high internal hydrostatic pressure (turgor) during growth. Turgor can be a driving force for cellular expansion; it is actively regulated in response to changes in the external osmolarity in some, but not all walled cells (Lew *et al.*, 2004). There are a number of mechanisms that may be used by a fungus to adjust its internal osmolarity, and therefore its turgor. One of these mechanisms is accumulation of osmotically active metabolites. Alcohol sugars (glycerol and erythritol) accumulate to high levels in *Aspergillus nidulans* grown in high salt (NaCl or KCl) concentrations (Beever & Laracy, 1986); glycerol levels begin to increase within an hour of hyperosmotic treatment. In *Neurospora crassa*, mannitol and glycerol accumulate within 22 h of treatment with high NaCl concentrations (Ellis *et al.*, 1991). Slightly lower accumulation is observed in the *os-1* mutant, which is defective in a histidine kinase that is homologous to the osmosensing SLN-1 histidine kinase upstream of the high osmolarity glycerol (HOG) pathway in yeast (Alex *et al.*, 1996; Schumacher *et al.*, 1997). The yeast HOG pathway is an MAP kinase cascade which is activated by high osmolarity

and causes the accumulation of glycerol. It is found in numerous fungal species, on the basis of comparative genomics (Krantz *et al.*, 2006). Kinetic models relate activation of the kinase members of the MAP kinase cascade to gene expression induced by Hog1p [the last of the three kinases in the MAP kinase cascade; the corresponding gene in *N. crassa* is *os-2* (Zhang *et al.*, 2002)]. Gene expression then results in the appearance of enzymes required for the biochemical changes that effect glycerol production (Klipp *et al.*, 2005). Glycerol production is not associated solely with hyperosmotic stress. The HOG pathway is also activated by low temperatures, since glycerol can confer freezing tolerance as a cryoprotectant (Panadero *et al.*, 2006). Glycerol synthesis is also activated at the level of gene expression in the homologous osmotic response MAP kinase cascade in *N. crassa* (Noguchi *et al.*, 2007).

An alternative to the production and accumulation of osmotically active metabolites is the accumulation of osmotically active ions from the external environment. This can be measured directly with an ion-selective self-referencing probe (Newman, 2001; Smith *et al.*, 1999). Net changes in ion influx that can explain turgor recovery after hyperosmotic treatment have been observed in higher plants (*Arabidopsis thaliana*) (Shabala & Lew, 2002) and *N. crassa* (Lew *et al.*, 2006).

Abbreviations: HOG, high osmolarity glycerol; MAP, mitogen-activated protein.

In addition to MAP kinase cascade-mediated turgor regulation [the sensor OS-1 (Miller *et al.*, 2002), MAPKKK OS-4 and MAPKK OS-5 (Fujimura *et al.*, 2003), and MAPK OS-2 (Zhang *et al.*, 2002)], there are other proteins that function in response to osmotic stress in *N. crassa* which are not homologues of MAP kinase cascade members. The osmosensitive mutant *cut* is one example. The *cut* mutant was originally isolated as a morphological mutant which exhibits a flat, as if it had been cut, conidiation zone when grown in slants (Kuwana, 1953). Temperature, pH and carbon source do not affect the mutant, but growth is affected by humidity (normal morphology is observed at 100 % relative humidity) and osmotic pressure (it is unable to grow at high osmolality) (Kuwana, 1953). The gene has been cloned. It is allelic to *ovc* (overaccumulator of carotenoids) and encodes a putative phosphatase (Youssar *et al.*, 2005). The expression of the *cut-1* gene increases after hyperosmotic and heat-shock treatment (Youssar & Avalos, 2006). Other osmosensitive mutants are known [e.g. *os-8*, *os-9*, *os-10* and *os-11* (Perkins *et al.*, 2001)] but the genes have not yet been identified. Scumbo (*sc*) and the semi-colonial mutants *smco-8* and *smco-9* are also osmosensitive and resistant to dicarboximide fungicides (Grindle & Temple, 1983). Mutations in the *os* genes encoding members of the osmo-responsive MAP kinase cascade in *N. crassa* are insensitive to dicarboximide (Grindle & Temple, 1982) and phenylpyrrole fungicides (Zhang *et al.*, 2002). The OS-1 family of histidine kinases apparently plays a central role in fungicide sensitivity, since its expression in yeast (normally insensitive to the fungicides) confers sensitivity to the dicarboximide, phenylpyrrole and aromatic hydrocarbon fungicides (Motoyama *et al.*, 2005).

In previous work, we have examined the role of ion transport in turgor regulation in wild-type and *os* mutants of *N. crassa*, and demonstrated turgor recovery within ~60 min directly with a pressure probe, as in Fig. 1 of the current study. Before the onset of turgor recovery, the membrane potential responds by depolarizing transiently, followed by a sustained hyperpolarization which is attributed to direct activation of the plasma membrane H^+ -ATPase (Lew *et al.*, 2006). Subsequently, there is net uptake of K^+ and Cl^- sufficient to explain turgor recovery. The electrical changes are not observed in the osmosensitive *os-1* and *os-2* mutants, leading to the conclusion that the HOG-like MAP kinase cascade functions as more than a gateway to changes in gene expression, and activates ion transport directly during turgor regulation.

In this paper, we report a similar functional characterization of the osmosensitive *cut* mutant, to identify its role as a mediator of turgor recovery, and present evidence that it may regulate glycerol accumulation during turgor recovery.

METHODS

Strains. Stock cultures of wild-type (strain 74-OR23-1A, FGSC No. 987), *os-2* (allele UCLA80, FGSC No. 2238) and *cut* (allele LLM1, FGSC no. 2385) were obtained from the Fungal Genetics

Fig. 1. Direct measurement of turgor in the *cut* mutant. Immediately after impalement, the hydrostatic pressure inside the hypha pushed the meniscus into the microprobe. The arrow shows the position of the oil/cytoplasm meniscus (0 s). Pressure was applied to push the meniscus to the microprobe tip (arrows, 24 and 39 s); the pressure required to bring the meniscus to the micropipette tip was equal to that of the hyphal turgor. The photomicrographs show that the hyphae continued mass flow after impalement (indicated by vacuole movement through the septal pore below the site of impalement). Bar, 20 μ m.

Stock Center (School of Biological Sciences, University of Missouri, Kansas City, MO, USA) (McCluskey, 2003). Stock cultures were maintained on Vogel's (plus 1.5 % w/v, sucrose and 2.0 % w/v, agar) medium (VM) (Vogel, 1956). We confirmed the phenotype of the *cut* mutant: an inability to grow on VM+4 % (w/v) NaCl (Perkins *et al.*, 2001).

Culture preparation for experiments. Cultures were grown at 28 °C (or room temperature, 21–24 °C) overnight by transferring conidia onto strips (2.5 × 6 cm) of dialysis tubing that overlaid the VM in Petri dishes. 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. The strip of dialysis tubing was placed inside the cover of a 30 mm Petri dish, and immobilized on the bottom with masking tape or double-sided sticky tape. The culture was flooded with 3 ml buffer solution (BS) containing KCl (10 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), sucrose (133 mM) and MES (10 mM), pH adjusted to 5.8 with KOH, or, for some of the ion-flux measurements, a modified BS containing $CaCl_2$ (0.05 mM), $MgCl_2$ (0.05 mM) and sucrose (150 mM), which was unbuffered. Growth of hyphae at the colony edge normally resumed within 15 min. In experiments comparing wild-type and mutants, we normally interspersed measurements of each of the strains.

Electrical measurements. For electrophysiology measurements, large-trunk hyphae (10–20 μ m diameter) ~0.5 cm behind the colony edge were impaled. After a stable membrane potential was obtained, the hyphae were treated in hyperosmotic conditions by adding 0.5 ml BS+1 M sucrose to 3 ml BS in the dish (a net increase of ~155 mosmol kg^{-1}). In other experiments, hyphae were treated with fludioxonil by adding 0.3 ml well-mixed 0.8 mM fludioxonil. The electrophysiological techniques have been described in detail previously (Lew, 1996). Briefly, double-barrel micropipettes (Lew, 2006) were used to voltage-clamp the hyphae, using a bipolar staircase of resting-potential clamps and alternating positive and negative voltages of 50 ms duration. Measurement of current density

in the hyphae is complicated by the cable properties of the hyphae. Voltage is attenuated $\sim 67\%$ at a distance of $\sim 200\ \mu\text{m}$ from the site of micropipette impalement (R. R. Lew and N. N. Levina, unpublished data). Correcting for current spread along the hyphae requires multiple impalements, which is technically challenging because vibration during treatment may dislodge the micropipettes. Thus, input currents are shown.

Turgor measurements. Impalements were made on large trunk hyphae (Fig. 1), similar to those for electrophysiological measurements. The technique has been described in detail previously (Lew *et al.*, 2004, 2006). Large-aperture micropipettes were fabricated using a double-pull technique. The micropipette was filled with low-viscosity silicone oil (polydimethylsiloxane, $1.5 \times 10^{-6}\ \text{m}^2\ \text{s}^{-1}$; 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 silicone oil per cell sap meniscus into the micropipette; the pressure required to bring the meniscus back to the tip was a measure of the turgor (Fig. 1). After turgor measurements for $\sim 6\ \text{min}$, $0.5\ \text{ml}\ \text{BS} + 1\ \text{M}$ sucrose was added to the dish containing $3\ \text{ml}\ \text{BS}$ for a hyperosmotic increase of $\sim 155\ \text{mosmol}\ \text{kg}^{-1}$. 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.

Ion-flux measurements. Large-trunk hyphae were selected for measurements of ion fluxes *in situ*. Construction of the ion-selective probe has been described in detail previously (Lew, 1999; Lew *et al.*, 2006). The following ion-selective cocktails were used (all from Sigma-Aldrich): H^+ , Fluka hydrogen ionophore II-cocktail A, catalogue no. 95297; Ca^{2+} , calcium ionophore I-cocktail A, catalogue no. 21048; K^+ , potassium ionophore I-cocktail B, catalogue no. 60398; and Cl^- , chloride ionophore I-cocktail A, catalogue no. 24902. The concentration of the selected ion was measured at two positions: as near to the hyphal wall as possible, and $20\ \mu\text{m}$ away, at a frequency of $0.3\ \text{Hz}$. To confirm the generation of steady-state diffusive gradients, and thus the ability to infer ion fluxes from the diffusive ion-concentration gradients, measurements were performed using K^+ and Cl^- point sources. The point sources were micropipettes pulled to a fine tip and filled with $3\ \text{M}\ \text{KCl}$, so that K^+ and Cl^- diffused from the tip into the solution. The gradients and corresponding ion fluxes were measured 10 to $50\text{--}100\ \mu\text{m}$ from the point source, and exhibited an exponential shape consistent with the generation of a diffusive gradient. The differences in ion concentrations 0 and $20\ \mu\text{m}$ from the hypha were converted to ion flux with the following equation, which accounts for the cylindrical geometry of the hyphae: $J\ (\text{nmol}\ \text{cm}^{-2}\ \text{s}^{-1}) = (D/r)[(c_2 - c_1)/\ln(r_2/r_1)]$, where D is the diffusion coefficient (H^+ , $9.31 \times 10^{-5}\ \text{cm}^2\ \text{s}^{-1}$; Ca^{2+} , $0.4 \times 10^{-5}\ \text{cm}^2\ \text{s}^{-1}$; K^+ , $1.96 \times 10^{-5}\ \text{cm}^2\ \text{s}^{-1}$; Cl^- , $2.03 \times 10^{-5}\ \text{cm}^2\ \text{s}^{-1}$); r is the hyphal radius; c_2 and c_1 the concentrations at the two excursion points; and r_2 and r_1 the distances from the hyphal centre to the two excursion points (Henriksen *et al.*, 1992). The ion-selective cocktail for Cl^- registered a higher than expected Cl^- concentration in the modified BS. However, the addition of Cl^- to the solution caused the predicted voltage change, indicating that there was interference, probably caused by an organic substance, which affected the voltage offset, but not the slope of voltage versus Cl^- concentration. To correct for this, the concentration was normalized to the expected concentration of $0.2\ \text{mM}\ \text{Cl}^-$ for flux calculations.

Immediately after addition of $0.5\ \text{ml}$ modified BS + $1\ \text{M}$ sucrose to the dish containing $3\ \text{ml}$ modified BS, the hyphae were observed to shrink and occasionally undergo incipient plasmolysis, followed by the

disappearance of incipient plasmolysis and reswelling. These observations corroborated the process of turgor recovery during measurements of ion fluxes.

Glycerol measurements. Colonies were grown overnight on a $7 \times 7\ \text{cm}$ strip of dialysis tubing overlaying VM. The dialysis tubing with the mycelial colony was transferred to a $100\ \text{mm}$ Petri dish, which was flooded with $15\ \text{ml}\ \text{BS}$. Either $2.5\ \text{ml}\ \text{BS} + 1\ \text{M}$ sucrose, or $2.5\ \text{ml}\ 1\ \text{M}\ \text{NaCl}$ was added to the dish to commence hyperosmotic treatments. The controls involved the addition of $2.5\ \text{ml}\ \text{BS}$. To harvest the mycelium, the strip of dialysis tubing with overlaying colony was transferred to a paper towel; the mycelium was scraped off the tubing, blotted, and placed in liquid N_2 in a mortar. After freezing, the mycelium was ground to a powder with a pestle, the powder was transferred to an Eppendorf tube, $0.5\ \text{ml}$ distilled water was added to the tube, and the tube was vortex-mixed, heated in a water bath at $80\text{--}90^\circ\text{C}$ for $20\ \text{min}$ and stored at -20°C . Mycelium yield was determined by measuring protein by the Bradford technique (Bradford, 1976). Glycerol was assayed using a commercially available kit (catalogue no. 10 148 270 035, Boehringer Mannheim/R-Biopharm). The assay comprised glycerokinase-mediated phosphorylation of glycerol, using ATP; ATP consumption was quantified by measuring NADH oxidation in the coupled reaction of pyruvate kinase ($\text{ADP} + \text{PEP} \leftrightarrow \text{ATP} + \text{pyruvate}$) and lactate dehydrogenase ($\text{pyruvate} + \text{NADH} + \text{H}^+ \leftrightarrow \text{lactate} + \text{NAD}^+$), where PEP stands for phosphoenolpyruvate.

Statistical analysis. Data are shown as means $\pm$ SD (sample size), unless stated otherwise. Independent two-tailed t tests were performed in either SYSTAT version 5.03 (Systat) or Excel (Microsoft).

RESULTS

Initial turgor in the *cut* mutant is the same as that in the wild-type

After flooding the mycelium with BS, and growth resumption, the two strains had similar turgor. Wild-type turgor was $427 \pm 63\ \text{kPa}$ ($n = 16$), and *cut* turgor was $436 \pm 83\ \text{kPa}$ ($n = 22$) (Fig. 2). The difference in turgor was not statistically significant (t test, $P = 0.684$). Thus, the turgor poise under non-stressed conditions was similar. Since osmosensitivity may be reflected in the response of the *cut* mutant to hyperosmotic stress, we examined the presence and time-course of turgor recovery.

The *cut* mutant exhibits turgor recovery

After hyperosmotic treatment with sucrose, the osmosensitive *cut* mutant exhibited turgor recovery (Fig. 3) similar to that in the wild-type (Lew *et al.*, 2004; Fig. 3). The recovery time was similar in both strains, with turgor recovering to near the initial level within $60\ \text{min}$. One mechanism of turgor recovery involves ion uptake, which may manifest itself in electrical changes.

The *cut* mutant exhibits electrical changes similar to those in the wild-type

One of the immediate responses of the wild-type to hyperosmotic treatment is transient depolarization of the membrane potential, followed by sustained hyperpolarization (Lew *et al.*, 2004). The sustained hyperpolarization is absent

Fig. 2. Initial turgor in the wild-type (WT) and the *cut* mutant. Data for the wild-type (circles) and the *cut* mutant (triangles) are jittered on the *x* axis for clarity. Means $\pm$ SD (solid symbols) are also shown. Two-tailed *t* test comparisons between the two strains indicated no significant difference between wild-type and *cut* ($P=0.684$).

in the *os-1* and *os-2* mutants of the osmotic response MAP kinase cascade (Lew *et al.*, 2006). Unlike the *os-1* and *os-2* mutants, the electrical response of the *cut* mutant was the same as that of the wild-type (Fig. 4A). The initial potentials for wild-type (-174 ± 23 mV, $n=11$) and *cut* (-169 ± 17 mV, $n=11$) depolarized to the same extent within 1–2 min after hyperosmotic treatment (-65 ± 23 and -54 ± 17 mV, respectively), followed by similar sustained

hyperpolarization (-189 ± 33 and -182 ± 24 mV, respectively) after ~ 3 –4 min. The electrical changes for the two strains were statistically the same, based on *t* tests ($0.367 < P < 0.926$, $n=11$). Current–voltage relations for the *cut* mutant revealed a higher conductance compared to that for the wild-type (Fig. 4B), but the responses to hyperosmotic treatment were similar. Thus, the *cut* mutant maintained the same array of electrical responses to hyperosmotic stress as did the wild-type, unlike the *os-1* and *os-2* mutants, for which sustained hyperpolarization is absent (Lew *et al.*, 2006).

Fludioxonil hyperpolarizes the potential in the wild-type and the *cut* mutant, but not the *os-2* mutant

Given that sustained hyperpolarization in the *cut* mutant and wild-type are absent in the *os-1* and *os-2* mutants of the osmotic MAP kinase cascade (Lew *et al.*, 2006), it appears that *cut* is not part of the cascade. To corroborate this, we examined the effect of the phenylpyrrole fludioxonil on the electrical properties of the wild-type and the *cut* and *os-2* mutants. Fludioxonil has been reported to activate the MAP kinase cascade, based upon the insensitivity of MAP kinase cascade mutants (including *os-2*) to fludioxonil, and the induction of glycerol synthesis and hyphal bursting in the wild-type by fludioxonil (Fujimura *et al.*, 2000). Similar to hyperosmotic treatment, fludioxonil caused hyperpolarization of the potential of both the wild-type and the *cut* mutant, but not the *os-2* mutant (consistent with *os-2* insensitivity to the fungicide fludioxonil) (Fig. 5A, B). Hyphal tip growth was inhibited within the same time frame as the hyperpolarization in the wild-type and *cut*, but not the *os-2* mutant (Fig. 5C).

The electrical responses of the wild-type and the *cut* mutant may be due to changes in ion fluxes, measurable with an ion-selective vibrating probe.

Fig. 3. Turgor recovery in the *cut* mutant. Lines show the results of seven individual experiments; filled triangles show running means for the compiled data ($n=8$). Hyperosmotic treatment was commenced at time 0. A similar time-course of turgor recovery has been previously reported for the wild-type (open circles) (Lew *et al.*, 2004).

Net ion-flux responses in the *cut* mutant

In response to hyperosmotic treatment, there was an initial H^+ influx into the hyphae, which corresponded to nearly plasmolytic conditions (incipient plasmolysis was often observed). Within 5 min, both the wild-type and the *cut* mutant shifted to H^+ efflux (corresponding to the disappearance of incipient plasmolysis) (Fig. 6). The time-course of H^+ efflux is similar to that of the sustained electrical hyperpolarization, suggesting that H^+ -ATPase activation is responsible for both (Lew *et al.*, 2006). Transient changes in Ca^{2+} (influx), K^+ (efflux) and Cl^- (a net shift to efflux) were also observed during the initial hyperosmotic shock (0–10 min). During the time interval associated with turgor recovery (10–40 min), Ca^{2+} efflux, and K^+ and Cl^- influx, occurred. Similar changes were observed in the wild-type and the *cut* mutant during turgor recovery, but the magnitudes varied. Compared to the wild-type, H^+ efflux was similar and Ca^{2+} efflux was lower in the *cut* mutant; there was a shift towards net K^+ influx

Fig. 4. Electrical responses of the wild-type and the *cut* mutant to hyperosmotic treatment. (A) Representative membrane potentials, from impalement to removal of the micropipette from the hypha (upper panel, wild-type; lower panel, *cut* mutant). Hyperosmotic treatment (an increase of 155 mosmol kg^{-1}) consisted of addition of 0.5 ml BS+1 M sucrose to 3 ml BS. After hyperosmotic treatment, the plasma membrane potential underwent transient depolarization, followed by prolonged hyperpolarization in both the wild-type and the *cut* mutant. (B) Mean current-voltage relations: initial (circles), during transient depolarization (triangles), and during sustained hyperpolarization (squares), for the wild-type (open symbols; six experiments) and the *cut* mutant (filled symbols; five experiments).

20–40 min after treatment, relative to the initial flux in *cut*, and Cl^- influx was similar to wild-type (Fig. 6). Other factors, such as glycerol production, may contribute to turgor recovery.

Glycerol does not accumulate in the *cut* mutant after hyperosmotic treatment

After pre-treatment for 15 min in BS, glycerol levels were measured 0, 20, 40 and 80 min after hyperosmotic treatment with either sucrose or NaCl, to compare glycerol accumulation during the same time interval as that for turgor recovery (Fig. 7). Even in the absence of hyperosmotic treatment (BS control), wild-type mycelia contained low levels of glycerol, while the *cut* mutant did not. Hyperosmotic treatment (either sucrose or NaCl) caused

Fig. 5. Effects of fludioxonil on the wild-type (WT) and the *cut* and *os-2* mutants. (A) Examples of the electrical responses to the addition of fludioxonil at $\sim 80 \mu\text{M}$. The responses of the three strains are summarized in (B) (wild-type, circles; *cut*, triangles; *os-2*, squares). Fludioxonil induced hyperpolarization in the wild-type and the *cut* mutant, but not the *os-2* mutant. For all three strains, initial potentials were similar (wild-type, $-139 \pm 10 \text{ mV}$; *cut*, $-150 \pm 7 \text{ mV}$; *os-2*, $-140 \pm 11 \text{ mV}$; $n=9$), but only the wild-type and the *cut* mutant were hyperpolarized after fludioxonil treatment (wild-type, $-18 \pm 10 \text{ mV}$; *cut*, $-20 \pm 6 \text{ mV}$; *os-2*, $-0.3 \pm 5 \text{ mV}$; $n=9$). (C) Fludioxonil treatment inhibited growth of the wild-type (upper panel, circles) and the *cut* mutant (middle panel, triangles), but not the *os-2* mutant (lower panel, squares), within the same time frame as the electrical hyperpolarization. Lines show growth rates of individual hyphae (measured every minute); filled symbols show the means at each time point [$n=13$ (*cut* and wild-type) or 10 (*os-2*)].

accumulation of glycerol in the wild-type, but not in the *cut* mutant. Glycerol accumulation by the wild-type varied within the three replicate experiments. After 80 min of hyperosmotic treatment, it ranged from 0.40 to 4.5 g glycerol (g protein) $^{-1}$, but it was always higher than that in the *cut* mutant [0.0–0.3 g glycerol (g protein) $^{-1}$], significantly so, after 80 min of treatment with either sucrose ($P=0.013$) or NaCl ($P=0.004$).

Fig. 6. Ion-flux responses to hyperosmotic treatment of the wild-type (circles) and *cut* mutant (triangles). Hyperosmotic treatment was given at time 0. Positive ion fluxes represent efflux, negative values represent influx. Data from four to six experiments are shown as running means $\pm$ SEM.

DISCUSSION

Walled cells often exist under internal hydrostatic pressure (turgor) as a consequence of the high osmolarity of the intracellular cytoplasm compared to the external osmolarity. Many walled organisms actively maintain their turgor, and when they do, there are different mechanisms that can be used. One mechanism is the accumulation of osmotically active metabolites [not only glycerol, but other alcohol sugars, amino acids and their derivatives, and other metabolites (Jennings, 1995; Bohnert & Jensen, 1996)]. This mechanism relies on modulation of biosynthetic pathways to divert carbon to osmolyte production. An alternative mechanism, ion uptake, depends upon the availability of ions in the extracellular milieu.

Fig. 7. Glycerol accumulation in the wild-type (circles) and the *cut* mutant (triangles). After pre-treatment in BS for 15 min, the mycelia were treated with hyperosmotic conditions for 0–80 min, or continued incubation with BS for 80 min (BS control). Both sucrose (empty symbols) and NaCl (filled symbols) treatment caused glycerol to accumulate in the wild-type, but not in the *cut* mutant (the differences were significant for either osmoticum by 80 min, $P < 0.015$). Individual measurements are shown, as well as linear regressions (calculated for combined sucrose and NaCl treatment). The time-course of glycerol accumulation in the wild-type was similar to that of turgor recovery.

Osmolyte accumulation has garnered much of the attention. In addition to a role in long-term acclimation of the organism to high external osmotic stress, osmolytes also confer osmoprotection to cytoplasmic constituents, and are even accumulated in response to other stresses, such as freezing (Panadero *et al.*, 2006). In contrast, ion uptake and accumulation cannot play a dual role of regulating both turgor and osmoprotection. Nevertheless, ion uptake does play a role in immediate responses to hyperosmotic conditions, and appears to be well-correlated with rapid turgor recovery in higher plants (Shabala & Lew, 2002) and fungi (Lew *et al.*, 2006).

In functional characterization of fast turgor regulation in *N. crassa*, it has become clear that the osmotic MAP kinase cascade not only regulates glycerol production, but also acts directly on ion transport, activating the H^+ -ATPase and mediating an increase in net ion influx to cause turgor recovery within 60 min of hyperosmotic stress (Lew *et al.*, 2006). In the present work, we extended our characterization of turgor regulation and ion transport to include the *cut* mutant, which is osmosensitive, but is defective in a pathway that is separate and distinct from the osmotic MAP kinase cascade.

In the absence of a functional *cut* gene, the initial turgor in BS was the same as that in the wild-type. After hyperosmotic treatment, both turgor recovery and electrical changes were very similar to those in the wild-type. The sustained hyperpolarization observed after hyperosmotic treatment can be attributed to activation of H^+ -ATPase, based upon the similar time-courses of hyperpolarization and increased H^+

efflux after hyperosmotic treatment. H^+ -ATPase activation is absent in the *os-1* and *os-2* mutants of the osmotic MAP kinase pathway, since they do not exhibit sustained hyperpolarization, and the *os-1* mutant (*os-2* has not been examined) does not exhibit H^+ efflux (Lew, 2006). The role of ion uptake in the wild-type and the *cut* mutant was corroborated by examining the effects of the fungicide fludioxonil.

Fludioxonil, reported to activate the osmotic MAP kinase cascade (Fujimura *et al.*, 2000), also causes sustained hyperpolarization of the potential in the wild-type and the *cut* mutant, but not in the *os-2* mutant, consistent with activation of H^+ -ATPase by the osmotic MAP kinase cascade. Fludioxonil inhibited hyphal elongation of the wild-type and the *cut* mutant (but not *os-2*) within ~5 min of treatment, similar to the time-course of the sustained hyperpolarization. This result suggests that there is a regulatory link between the osmotic MAP kinase cascade and tip growth. However, fludioxonil may have multiple targets. In the basidiomycete *Cryptococcus neoformans*, fludioxonil causes growth cessation before cell swelling, and mutations in the calcineurin pathway confer heightened sensitivity to fludioxonil (Kijima *et al.*, 2006). In addition, the *cut* mutant exhibits less sensitivity to fludioxonil than the wild-type or the allelic *ovc* mutant, which overproduces carotenoids in the light (Youssar *et al.*, 2005) (we did not examine the concentration-dependence of the effect of fludioxonil). Thus, fludioxonil may inhibit tip growth by a mechanism distinct from activation of the MAP kinase cascade, although a direct link between the MAP kinase cascade and tip growth seems more likely. If so, the inhibitor may offer insight into the linkages between turgor and tip growth.

After hyperosmotic stress, the *cut* mutant did not accumulate glycerol. Our results confirm a previous comprehensive characterization of glycerol accumulation in *os* and *cut* mutants, in which no glycerol accumulation was observed in the *cut* mutant (Fujimura *et al.*, 2000) when the osmotic stress was 4% NaCl, and accumulation was measured after 4 h. The alternative mechanism for turgor regulation that we examined is ion uptake. The *cut* mutant did take up ions from the extracellular medium, although the magnitude varied with respect to the wild-type. Of the two major osmotically active ions, Cl^- influx was similar to that in the wild-type, but only a shift towards net K^+ influx was observed in the *cut* mutant. Thus, we cannot ascribe turgor recovery in the *cut* mutant solely to ion uptake, although it will certainly contribute significantly.

Our results suggest a cause for the osmosensitivity of the *cut* mutant. The *os-1* and *os-2* mutants of the osmotic MAP kinase cascade maintain a lower turgor than the wild-type under non-stressed conditions (Lew *et al.*, 2006). It is easy to attribute their osmosensitivity to an inability to maintain a significantly high turgor poise by uptake of ions and accumulation of glycerol during osmotic stress. In the absence of glycerol accumulation in the *cut* mutant,

steady-state turgor poise is the same as that in the wild-type, as is turgor recovery, presumably due to ion uptake. However, long-term survival may not be possible in the absence of the osmoprotectant glycerol, hence the osmosensitivity of the *cut* mutant.

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