Electrophysiological Response of the Scumbo (*sc* 5801 and *sc* R2386), *Smco8*, and *Smco9* mutants of *Neurospora crassa* to Hyperosmotic Treatment[1].

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OBJECTIVE

To determine whether the osmosensitive (and fungicide resistant) mutants of *Neurospora. crassa* undergo electrical changes similar to wild type when treated with hyperosmotic sucrose treatment, to test whether they are accessory proteins of the osmotic MAP kinase cascade.

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INTRODUCTION

Walled cells of plants, fungi and algae are normally sensitive to environmental conditions. One of these is modulation of the extracellular osmolarity. The cause of such changes can be as varied as drought, or even tidal flows causing changes in salinity. Fungi and plants respond to hyperosmotic challenges by modifying their intracellular osmolyte concentrations. In yeast, this has been studied extensively. Hyperosmotic treatment activates the high-osmolarity glycerol (HOG) mitogen activated protein (MAP) kinase pathway. This results in the accumulation of the osmolyte glycerol (reviewed by Mager and Siderius, 2002). Homologues of the MAP kinase pathway components have been found in *Neurospora crassa* (Zhang *et al*, 2002) and many other fungi (Krantz et al., 2006). In addition to accumulation of the osmolyte glycerol, at least in *N. crassa*, ion transport is induced within about 2 minutes (Lew et al., 2004), based on potential measurements, and net influx of ions commences within about 5 minutes, within the time frame of turgor recovery (15-20 minutes) (Lew et al., 2006). Similar changes are also observed in the higher plant *Arabidopsis thaliana* (Shabala and Lew, 2001). Thus ion transport plays a role in turgor regulation mediated by the osmotic MAP kinase cascade (Lew et al., 2006).

Mutations of the genes encoding the osmotic MAP kinase cascade in *N. crassa* result in osmosensitivity. But in addition, the mutants are reported to be resistant to fungicides (Grindle and Temple, 1983): both phenylpyrroles (e.g. fludioxonil) (Zhang et al., 2002) and dicarboximides (e.g. vinclozolin). The two fungicides are reported to activate glycerol accumulation in wildtype; substantial accumulations (2 to 3-fold higher than wildtype) are observed within 4 hours after fungicide treatment (Pillonel and Meyer, 1997). Fludioxonil causes activation of the H^+-ATPase, based upon a hyperpolarization observed within 2-4 minutes of treatment; a similar hyperpolarization is observed in response to hyperosmotic treatment in wildtype, presumably due to activation of the MAP kinase cascade. Thus the fungicide effects appear to be linked to ion transport activation mediated by the MAP kinase cascade.

Other osmosensitive mutants are reported to be resistant to fungicides, especially the dicarboximide vinclozolin (Grindle and Temple, 1983), including scumbo (*sc*), *smco8*, and *smco9*. These are not osmotic MAP kinase cascade mutants, but may play a role downstream of the cascade. The objective of my research was examine these mutants in detail and answer the following questions:

Do the *sc*, *smco8*, and *smco9* mutants lack the electrical changes observed in wildtype (electrical changes that are absent in mutants of the osmotic MAP kinase cascade)?

If the mutants lack the electrical changes, then the normal genes may play a role downstream of the MAP kinase cascade in osmoresponses.

Are the mutants osmosensitive?

If the mutants are osmosensitive, this would confirm a report by Grindle and Temple (1983) and corroborate that they may play a role as accessories to the osmotic MAP kinase cascade

And finally, does vinclozolin cause electrical and growth changes similar to fludioxonil (that is, hyperpolarization and growth inhibition)?

It's a very different molecule, but if it does cause similar electrical and growth changes, activation at some point in the same transduction pathway is likely.

MATERIALS AND METHODS

Strains. All strains were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) (McCluskey, 2003). The wildtype strain was 74-OR23-1VA (FGSC #2489). The scumbo strains were *sc* (allele 5801) (FGSC #49) and *sc* (allele R2386) (FGSC #1377). The smco (semi-colonial strains) were *smco8* (allele R2505)(FGSC #8247) and *smco9* (allele R2508) (FGSC #7365) (summarized in Table I). All strains were mating type A.

Table I. Strains of *Neurospora crassa* used in the present study.

Strain	Allele	FGSC Number
Wildtype	74-OR23-1VA	2489
scumbo (*sc*)	5801	49
scumbo (*sc*)	R2386	1377
smco8	R2505	8247
smco9	R2508	7365

Phenotype Characterization of the Mutants. The mutant strains were grown on Vogel's Minimal medium (VM)(with 1.5% w/v sucrose and 2% w/v agar) supplemented with either 4% NaCl or 50 µg/ml vinclozolin (at 28 º Celsius). The *smco8* strain was also grown on VM containing galactose instead of sucrose. The inoculum was a small agar block (about 3 mm square) of the strain from a culture previously grown on agar. The block was placed in the center of the dish. Colony diameters were measured over a period of time from 24 to 356 hours, depending upon the growth rate of the strain. Growth rates were calculated from the linear increase in colony diameter after inoculation.

Electrical Characterization of the Mutants. Details of the electrophysiology and fabrication of double barrel micropipettes are described in Lew (1996) and Lew (2006). Cultures of wildtype, *sc* (5801) and *smco*8 (R2505) were grown overnight on cellophane overlaying VM plus 1.5% sucrose and 2% agar at 28º Celsius. Strains of *smco9* (R2508) and *sc* (R2386) were grown for a week before the colonies were large enough for experimental manipulation. A strip of dialysis tubing (about 1 by 3 cm) and overlaying mycelium was cut with a razor blade, taped down in the cover of a 30 mm culture dish and flooded with 3 ml of BS (Buffer Solution: 10 mM KCl, 1 mM $CaCl_2$ and $MgCl_2$, 133 mM sucrose and 10 mM MES, pH adjusted to 5.8 with KOH). The colony edge was observed under the microscope to make sure hyphal tips were growing. Large trunk hyphae, behind the growing edge of the colony and aligned perpendicular to the direction of impalement, were selected for potential measurements. Double barrel micropipettes were used to allow simultaneous current injection and voltage measurement of wildtype, *sc* (5801) and *smco8* (R2505). Single barrel micropipettes were used for impaling *sc* (R2386) and *smco9* (R2508), since the hyphae were very delicate and easily damaged by impalement with the larger double barrel micropipettes.

After impalement, when the potential had stabilized at the resting potential, hyperosmotic treatment was applied by adding 1 ml of BS plus 1 M sucrose. The solution was added

dropwise in a circle around the objective. Before, during the hyperosmotic-induced transient potential change and after a final sustained potential had been reached, voltage clamping was performed to determine the membrane conductance.

In addition to hyperosmotic treatment with sucrose, the effects of vinclozolin (ED_{95}= 3.3 μg/ml) on electrophysiological properties was tested, but only with the wildtype strain. Vinclozolin is not very soluble in water, so methanol was used to dissolve the vinclozolin as a 100 X stock which was diluted into BS for a final concentration of 50 μg/ml. Perfusion at a rate of 6.4 ml/min was used, rather than stepwise addition of the inhibitor. BS plus 1% methanol was perfused as a control. Current-voltage relations were measured during perfusion with BS, and after switching to perfusion with either methanol or vinclozolin.

RESULTS

Characterization of the Mutant Phenotype. The phenotype of the mutants was compared to wildtype: both the responses of the mutants to hyperosmotic conditions and their sensitivity to the fungicide vinclozolin (Grindle and Temple, 1983). The scumbo mutant (*sc* 5801) exhibited wildtype-like colony morphology, but grew at a slower rate (Figure 1). It exhibited a slower growth rate under hyperosmotic conditions (VM + 4% NaCl), but the decline in growth (62% of the growth rate in VM) was similar to the decline in growth of wildtype (73%). Vinclozolin inhibited growth completely. Because hypersensitivity to osmotic treatment and resistance to vinclozolin resistance were not observed in this allele (5801), we selected another allele (R2386); the *sc* R2386 mutant was reported to be resistant to vinclozolin by Grindle and Temple (1982). The *sc* R2386 mutant was a very slow grower compared to wildtype, and exhibited a colonial to semi-colonial morphology. It was more sensitive to hyperosmotic treatment (38%) than wildtype and relatively insensitive to vinclozolin (Figure 1).

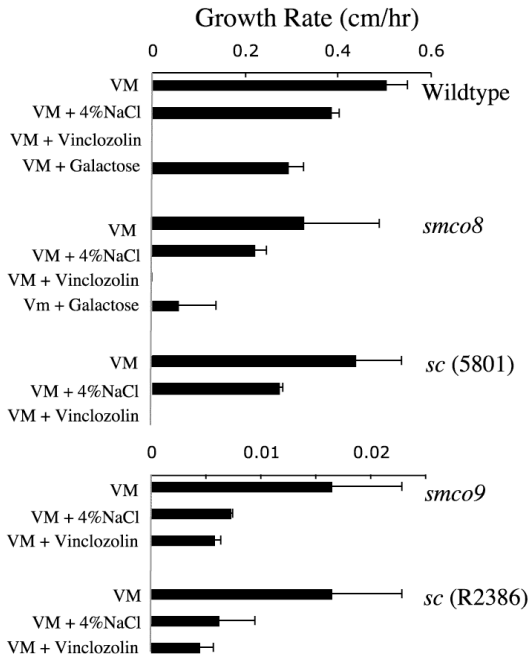

Figure 1: Phenotype Characterization of the Mutants. The growth rates of wildtype and osmotic mutants on Petri dishes under different growth conditions.

The other mutants examined were the semicolonial mutants *smco8* and *smco9*. The mutant *smco9* demonstrated semicolonial growth morphology. Semicolonial mutants are defined as mutants " which begin as small colony (from ascospore and upon transfer) and sooner or later produce flare of wild type-appearing hyphae (with or without conidia)" (Garnjobst and Tatum (1967). The *smco9* mutant was more sensitive to hyperosmotic treatment (44%) than the wild type and it was resistant to vinclozolin (Figure 1). The *smco8* mutant was less sensitive to osmotic treatment than *smco9*, and inhibited by vinclozolin.

Having established the sensitivity of the mutants to hyperosmotic treatment and resistance to vinclozolin, I next examined the electrical responses of the mutants to hyperosmotic treatment, to determine whether the osmotic MAP kinase cascade was functioning.

Electrical Characterization of the Mutants. The wildtype has a negative resting potential (the average potential of –153±21 mV). In response to hyperosmotic treatment, the potential generally undergoes transient depolarization to –85±44 mV (a potential change of +67±48 mV) and prolonged hyperpolarization to –178±29 mV (final potential change of –24±26 mV) (Figure 2). The mutant *sc* 5801 responded similarly to wildtype (Figure 2). The initial potential (–149±13mV) depolarized by +29±39 mV then hyperpolarized by –21±30mV (relative to the initial potential). The *sc* R2386 mutant exhibited a significantly different electrical response to the hyperosmotic shock compared to wildtype (Figure 2). It had more positive initial resting potential (–136±15 mV) with a transient depolarization by 65±40 mV, but the final potential change was positive (+64±59 mV); hyperpolarization was observed only rarely. The initial resting potential of the *smco8* mutant was –140±16 mV; it displayed a larger transient depolarization (+71±40mV) but a smaller hyperpolarization (–15±44 mV) compared to wildtype.

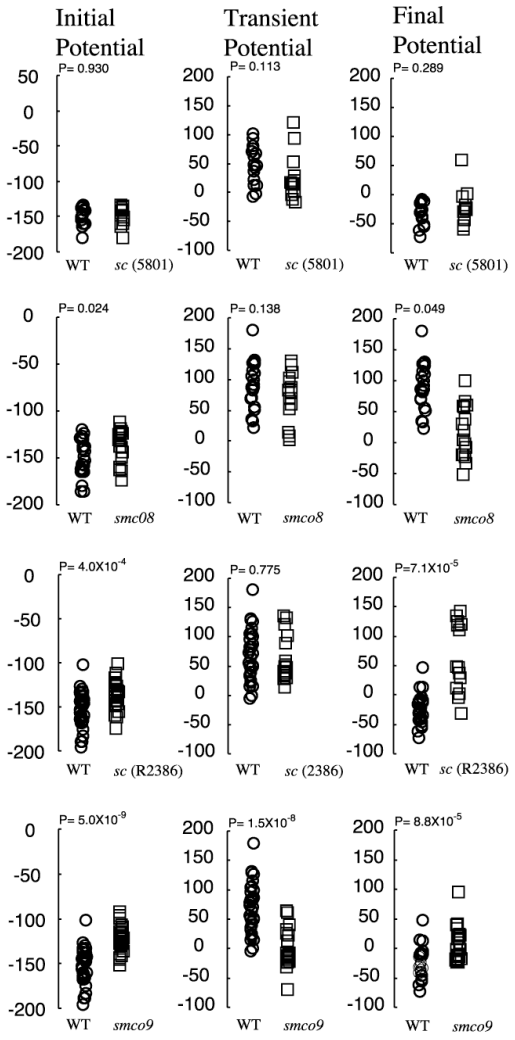

Figure 2: Electrical Comparison of the Mutants with the Wildtype. The resting potential and electrical changes (transient depolarization and final potential relative to the initial potential) of wildtype and the mutant strains (as marked) are shown.

The *smco9* mutant also had a slightly depolarized initial resting potential(–123±14mV), and a different response to hyperosmotic treatment. It hyperpolarized transiently by –2±30mV followed by a prolonged depolarization +15±30 mV (figure 2).

To compare the difference in ion channel conductances induced by hyperosmotic treatment, the current voltage relations for wildtype, *sc* 5801, *sc* R2386 and *smco8* were measured. The *smco9* hyphae were too small and fragile to be able to impale them with double barrel micropipettes (potential changes were measured with a single barrel micropipette), thus no current-voltage measurements were made of *smco9*. The slopes of the current-voltage relations were usually separated into three regions, depending on whether the slopes were visually distinct: with higher conductance at voltages negative to and positive to a lower conductance near the resting potential. Otherwise the

conductances were measured at voltage negative to and positive to the resting potential (Figure 3).

In comparison to wildtype, all three mutants had similar conductances prior to osmotic treatment. After hyperosmotic treatment, conductances tended to be higher in all four strains, and tended to decline similarly during the hyperpolarization phase.

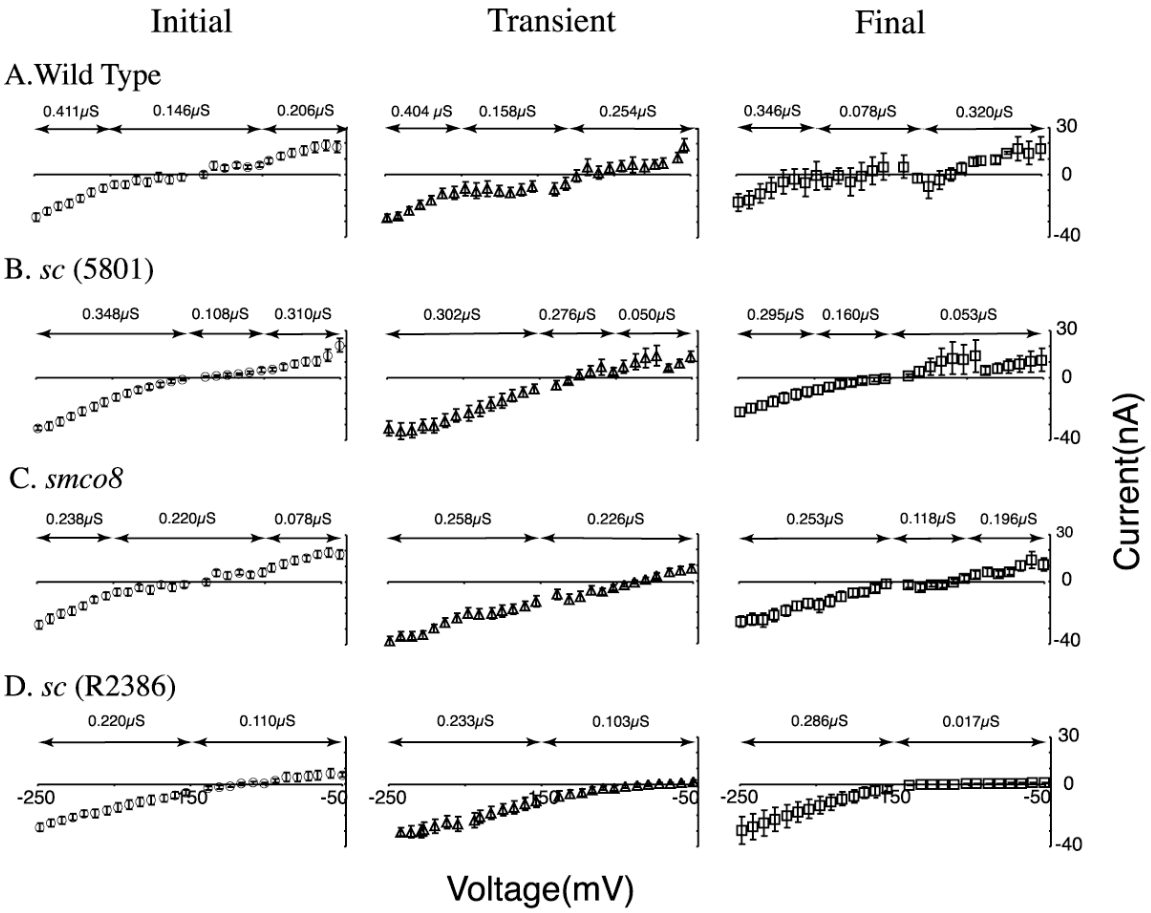

Figure 3: Current-voltage Relations Comparison of the Mutants with the Wildtype. Current-voltage relations are shown for the wildtype (A), *sc* 5801 (B), *smco8* (C) and *sc* R2386 (D) during the initial resting potential, and hyperosmotic-induced transient and final electrical responses. The values bracketed by arrows indicate the conductance across the membrane at the indicated voltage range.

Vinclozolin Induced Changes in Potential and Growth in Wildtype. Experiments with the fungicide fludioxonil had demonstrated that this phenylpyrrole compound induces hypepolarization of the membrane potential and stops the growth of the wildtype *N. crassa* (Lew and Levina, 2007). To see whether the dicarboximide fungicide vinclozolin also acts through the same pathway, it was tested for its effects on the membrane potential and hyphal growth.

Addition of the vinclozolin treatment to the wildtype cells induced hyperpolarization of the cells from –161±18mV to –213±7mV, a potential change of –49±14mV (Figure 4). Since methanol was used as solvent carrier for vinclozolin, a control experiment was done using methanol. Wildtype cells also hyperpolarized in response to methanol treatment from a resting potential of –162±9 mV to –177±12 mV(a change of –22±11 mV).

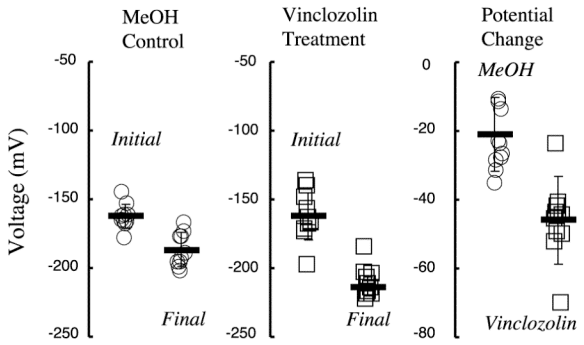

Figure 4: Electrical changes of the Wildtype Caused by Vinclozolin Treatment. Both Vinclozolin and the methanol (MeOH) control resulted in hypepolarization of the wildtype cells. The potential change caused by vinclozolin however, is significantly higher.

The methanol-induced hyperpolarization was significantly less than the vinclozolin-induced hyperpolarization (Figure 4). There were no significant conductance differences observed between the vinclozolin treatment and the methanol control (Figure 5). However, the conductances at the voltages close to the resting potential were much higher after either vinclozolin or control (methanol) treatment compared to the initial current-voltage relations.

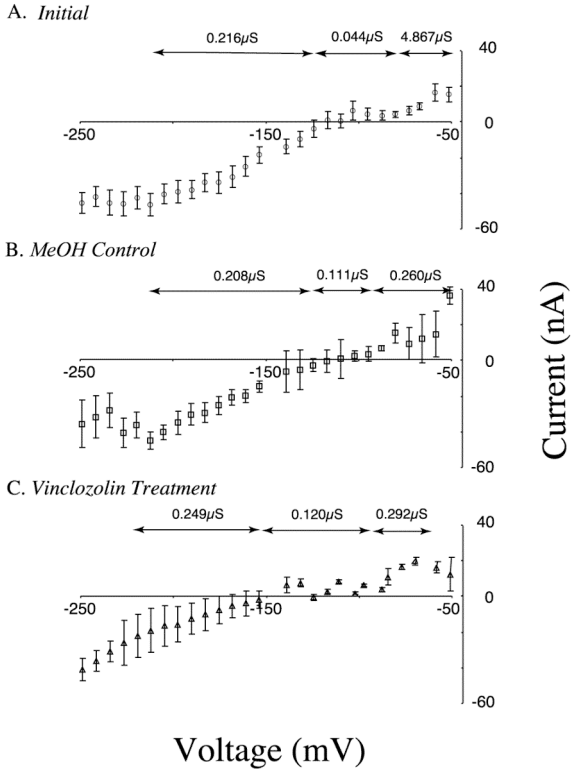

Figure 5: Current-Voltage Relations of Wildtype Induced by Vinclozolin or Control (methanol) Treatment. The conductances near the resting potential voltages are much higher after either vinclozolin or control (methanol). However, the differences in the conductance of the two treatment and the control at voltages negative, positive and close to resting potential were not statistically significant.

Treating the growing hyphae with vinclozolin solution reduced the growth rate to 33% of the normal growth after 12 minutes of applying the treatment (Figure 5). Methanol control also reduced the growth rate slightly to 65% of the initial growth rate.

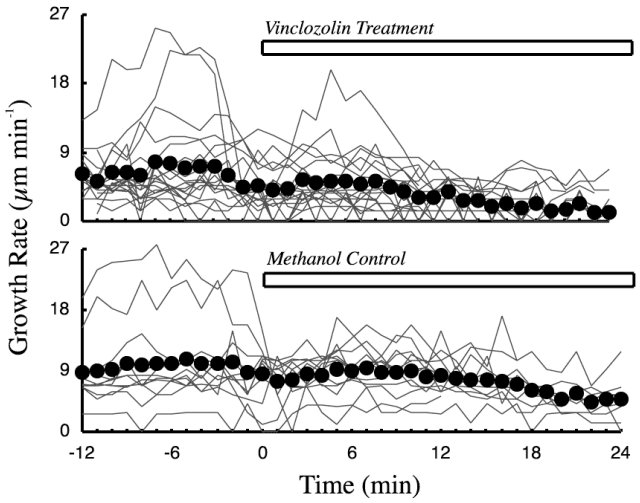

Figure 5: The Effects of the Vinclozolin on Wildtype Growth Rate. Vinclozolin treatment reduced the growth rate to 33% of its normal growth rate. The methanol control also decreased the growth rate to 65% of the normal growth rate.

DISCUSSION

The experiments were designed to examine whether a set morphological mutants reported to be sensitive to hyperosmotic conditions and resistant to fungicides contain mutations of genes that play a role in the *Neurospora crassa* osmotic MAP Kinase. They were selected based on a paper by Grindle and Temple (1982) reporting that these mutants are not only osmosensetive but also resistant to the dicarboximide fungicide vinclozolin.

I first confirmed the phenotypes of the mutants. The scumbo mutant *sc* 5801 was not a mutation of the allele of interest, therefore *sc* R2386 was used. Furthermore, the mutant *smco8* did not exhibit the phenotype reported by Grindle and Temple (1982). It grew with a wildtype-like colony morphology, was not resistant to vinclozolin and was less osmosensitive than *smco9*.

It is well known that the MAP kinase cascade regulates cells' recovery from the hyperosmotic challenge through intracellular accumulation of osmolytes. Activation of the MAP kinase cascade also stimulates ion transport across the cell membrane (Lew et al., 2006). This is observed as a transient depolarization followed by a sustained hyperpolarization in wildtype, concomitant with an increase in net ion uptake. The sustained hyperpolarization is not found in mutants of the osmotic MAP kinase cascade. Therefore, the changes in membrane potential changes in response to hyperosmotic shock were measured in mutants reported to be osmosensitive but not osmotic MAP kinase cascade members. The response of the scumbo mutant *sc* 5801 to hyperosmotic treatment was not significantly different from the wildtype, suggesting that the sc 5801 gene is not a component associated with the osmotic MAP kinase cascade. The other mutants, *sc* R2386, *smco8* and *smco9* did not follow the wildtype-like pattern of electrical potential changes in response to hyperosmotic treatment. Among these three mutants, *smco8* displayed potential changes that were closest to the wildtype electrical changes. *Smco8* and *sc* R2386 both exhibited wildtype-like depolarization following a hyperosmotic treatment. However, the depolarization is not always followed by a sustained hyperpolarization in both of these mutants. On average, *smco8* hyperpolarized by a small amount and *sc* R2386 remained depolarized. In contrast to *sc* R2386 and *smco8*, *smco9* did not depolarize in response to hyperosmotic treatment and instead hyperpolarized transiently followed by a sustained depolarization. These observations indicate that *sc* R2386 and *smco9* may play a role downstream of the osmotic MAP kinase cascade. This conclusion can not be made for *smco8* , since it did not exhibited electrical changes significantly different from the wildtype.

The effects of vinclozolin on membrane potential were examined to determine whether vinclozolin activates the osmotic MAP kinase cascade. The treatment of wildtype cells with vinclozolin induced cells to hyperpolarize. Methanol, the solvent used as a carrier for vinclozolin, also caused hyperpolarization of the wildtype cells. The methanol-induced hyperpolarization is however is significantly smaller than one caused by vinclozolin treatment. Therefore, vinclozolin alone hyperpolarizes the wild type cells similar to fludioxonil. Finally, examining the influence of vinclozolin on the growth of the wildtype cells, both vinclozolin treatment and methanol control reduced the growth

rate. Nevertheless, vinclozolin has inhibited growth significantly more compared to methanol. This decline in growth rate is not as pronounced as fludioxonil-induced growth inhibition, however. Therefore, vinclozolin may act through the same pathway as fludioxonil, the osmotic MAP kinase cascade, but fludioxonil may have additional effects directly on hyphal tip growth.

Overall, of the mutants examined the strongest case can be made that *sc* R2386 and *smco9* are accessories required for activation of ion transport downstream of the osmotic MAP kinase cascade. This conclusion is based on their lack of the final sustained hyperpolarization after hyperosmotic treatment and their resistance to vinclozolin. The fact that these mutants have reduced cell-wall peptides (Wrathall and Tatum, 1974) could also be another explanation for lack of hyperpolarizaion: That is, upstream osmo-sensing, associated with the plasma membrane and cell wall may be impaired. In contrast, the mutant *smco8* was not that different from wildtype, since hyperpolarization was often observed and was sensitive to vinclozolin; However, the phenotype of the *smco8* mutant did not match the phenotype reported by Grindle and Temple (1983) , the reason for this is unknown.

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