The Response Regulator RRG-1 Functions Upstream of a Mitogen-activated Protein Kinase Pathway Impacting Asexual Development, Female Fertility, Osmotic Stress, and Fungicide Resistance in *Neurospora crassa*[□]

Carol A. Jones,^{*†‡} Suzanne E. Greer-Phillips,^{*‡§} and Katherine A. Borkovich^{*†}

^{*}Department of Plant Pathology and Microbiology and [†]Program in Biochemistry and Molecular Biology, University of California, Riverside, Riverside, CA 92521

Submitted March 23, 2006; Revised March 6, 2007; Accepted March 16, 2007

Monitoring Editor: Ralph Isberg

Two-component systems, consisting of proteins with histidine kinase and/or response regulator domains, regulate environmental responses in bacteria, Archaea, fungi, slime molds, and plants. Here, we characterize RRG-1, a response regulator protein from the filamentous fungus *Neurospora crassa*. The cell lysis phenotype of $\Delta rrg-1$ mutants is reminiscent of osmotic-sensitive (*os*) mutants, including *nik-1/os-1* (a histidine kinase) and strains defective in components of a mitogen-activated protein kinase (MAPK) pathway: *os-4* (MAPK kinase kinase), *os-5* (MAPK kinase), and *os-2* (MAPK). Similar to *os* mutants, $\Delta rrg-1$ strains are sensitive to hyperosmotic conditions, and they are resistant to the fungicides fludioxonil and iprodione. Like *os-5*, *os-4*, and *os-2* mutants, but in contrast to *nik-1/os-1* strains, $\Delta rrg-1$ mutants do not produce female reproductive structures (protoperithecia) when nitrogen starved. OS-2-phosphate levels are elevated in wild-type cells exposed to NaCl or fludioxonil, but they are nearly undetectable in $\Delta rrg-1$ strains. OS-2-phosphate levels are also low in $\Delta rrg-1$, *os-2*, and *os-4* mutants under nitrogen starvation. Analysis of the *rrg-1*^{D921N} allele, mutated in the predicted phosphorylation site, provides support for phosphorylation-dependent and -independent functions for RRG-1. The data indicate that RRG-1 controls vegetative cell integrity, hyperosmotic sensitivity, fungicide resistance, and protoperithecial development through regulation of the OS-4/OS-5/OS-2 MAPK pathway.

INTRODUCTION

Two-component regulatory systems are signal transduction pathways found in bacteria, Archaea, slime molds, fungi, and plants (for reviews, see Wolanin *et al.*, 2002). Two-component systems have been implicated in responses to light, osmolarity, cellular redox status, nutrient and oxygen levels, virulence, and other factors (for review, see Wolanin *et al.*, 2002; Borkovich *et al.*, 2004). A basic two-component system consists of a histidine kinase and a response regulator (for review, see West and Stock, 2001; Wolanin *et al.*, 2002). Histidine kinases autophosphorylate on a conserved histidine residue in the histidine kinase domain. The phosphoryl group is then transferred to an aspartate residue in the receiver domain on the response regulator. Complex two-component signaling pathways (also termed phosphorelays) contain hybrid histidine kinases that have both a histidine kinase domain and a response regulator receiver

domain in the same protein, thus facilitating an intramolecular phosphate transfer reaction. The phosphate group on the aspartate residue is transferred to a histidine phosphotransfer (HPT) protein. Subsequently, the histidine phosphotransfer protein phosphorylates the receiver domain on a response regulator. Phosphorelays are the major two-component pathways in eukaryotic systems; however, two-component systems are not present in animals, making them ideal candidate targets for antimicrobial drugs (for review, see Alex *et al.*, 1998; Wolanin *et al.*, 2002; Borkovich *et al.*, 2004).

Many response regulators contain a transcriptional regulatory domain that is controlled by the phosphorylation status of the aspartate residue on the receiver domain, leading to changes in gene expression. The phosphorylation status of the response regulator can also determine its ability to interact with other regulatory proteins, such as mitogen-activated protein kinase (MAPK) cascades in eukaryotes (Maeda *et al.*, 1994; Buck *et al.*, 2001), and with proteins that control flagellar motor rotation in bacteria (Baker *et al.*, 2006). Two-component systems have been well studied in the yeast *Saccharomyces cerevisiae*. The *S. cerevisiae* genome contains one histidine kinase (Sln1p), one HPT protein (Ypd1p), and two response regulators (Ssk1p and Skn7p). The Sln1p/Ypd1p/Ssk1p system regulates growth under hyperosmotic conditions through modulation of the Ssk2p (Ssk22p)/Pbs2p/Hog1p MAPK pathway (for review, see Saito and Tatebayashi, 2004). The Hog1p MAPK can also be regulated by the transmembrane protein Sho1p in response to hyperosmotic stress in a pathway that includes the Ste11p MAPK kinase kinase (MAPKKK) and the MAPK kinase (MAPKK) Pbs2p (Posas and Saito, 1997). The Ssk1p response

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-03-0226>) on March 28, 2007.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

[‡] These authors contributed equally to this work.

[§] Present address: Biology Department, Southwestern Adventist University, Keene, TX 76059.

Address correspondence to: Katherine A. Borkovich (katherine.borkovich@ucr.edu).

regulator has a structure that includes the phospho-accepting receiver domain at the carboxy terminus. In addition to a carboxy-terminal receiver domain, the response regulator Skn7p also contains an amino-terminal heat-shock factor-like helix-turn-helix DNA binding domain. Various functions of Skn7p differ in their requirement for the conserved phospho-accepting aspartate residue Asp-427. For example, cell wall assembly and regulation of the cell cycle requires Asp-427 (Brown *et al.*, 1994; Morgan *et al.*, 1995), whereas the requirement for Skn7p during resistance to oxidative stress does not depend on Asp-427 (Morgan *et al.*, 1997).

NIK-1 and NIK-2 from *Neurospora crassa* were the first histidine kinases to be identified in filamentous fungi (Borkovich *et al.*, 2004). $\Delta nik-1$ strains are phenotypically similar to osmotic-sensitive (*os*) mutants, with swollen hyphae and asexual spores that lyse easily and are sensitive to hyperosmotic conditions. Subsequent work demonstrated that *nik-1* is allelic with *os-1* (Schumacher *et al.*, 1997) and that three additional *os* genes (*os-4* MAPKKK, *os-5* MAPKK, and *os-2* MAPK) comprise an MAPK pathway in *Neurospora* whose members are similar to those of the *S. cerevisiae* Hog1p system (Zhang *et al.*, 2002; Fujimura *et al.*, 2003). Deletion of *os* genes also leads to increased resistance to phenylpyrrole and dicarboximide fungicides (Fujimura *et al.*, 2000a,b; Ochiai *et al.*, 2001; Zhang *et al.*, 2002). Of interest, wild-type *S. cerevisiae* strains are resistant to these fungicides (Zhang *et al.*, 2002; Motoyama *et al.*, 2005), but they are rendered sensitive by introduction of an OS-1/NIK-1-related histidine kinase gene from the filamentous fungus *Magnaporthe grisea* (Motoyama *et al.*, 2005). In the yeast *Candida albicans*, deletion of the *os-1/NIK-1* homologue (*cos-1*) leads to defects in hyphal growth and reduced virulence (Alex *et al.*, 1998; Selitrennikoff *et al.*, 2001).

In contrast to results with *nik-1*, $\Delta nik-2$ mutants have no obvious phenotypes (Alex *et al.*, 1996; Borkovich *et al.*, 2004). However, analysis of the recently completed *Neurospora* genome sequence suggests that the lack of an identified function for NIK-2 may result from gene redundancy, because *Neurospora* possesses 11 genes encoding hybrid histidine

kinases (Galagan *et al.*, 2003; Borkovich *et al.*, 2004). The *Neurospora* genome also predicts one HPT protein (HPT-1) and two response regulators, RRG-1 and RRG-2. RRG-1 and RRG-2 are most similar to the class of response regulators represented by *S. cerevisiae* Ssk1p and Skn7p, respectively. The expansion of histidine kinases in *Neurospora* relative to *S. cerevisiae*, coupled with the same number of HPTs and response regulators, suggests either a signaling bottleneck downstream of the histidine kinases or differential regulation of the HPT and response regulators due to time or tissue-dependent expression of the histidine kinases or downstream effectors (Galagan *et al.*, 2003; Borkovich *et al.*, 2004). It is also possible that histidine kinases regulate cellular functions independently of the HPT and response regulators in *Neurospora* (Galagan *et al.*, 2003; Borkovich *et al.*, 2004).

In this study, we characterize the cellular role of the response regulator RRG-1 in *Neurospora*. We determine the gene structure and pattern of expression of *rrg-1* during the life cycle. We create gene replacement mutants as well as strains carrying a mutation at the presumed site of phosphorylation. We observe all strains for phenotypes during growth and development as well as for sensitivity to hyperosmotic conditions and fungicides. We also monitor the phosphorylation status of the OS-2 MAPK protein, and we identify downstream effects on gene expression. Our results demonstrate functions for RRG-1 in cell integrity, osmotic stress responses, fungicide sensitivity, and female fertility. We also present evidence that RRG-1 regulates the OS-2 MAPK pathway in *Neurospora*.

MATERIALS AND METHODS

Wild-Type *Neurospora* Strains and Culture Conditions

Neurospora strains used in this study are listed in Table 1. For vegetative growth, strains were cultured on Vogel's minimal medium (VM; Vogel, 1964), whereas synthetic crossing medium (SCM) was used to induce the sexual cycle (Westergaard and Mitchell, 1947). For hyperosmotic conditions, VM solid medium was supplemented with 0.75 M NaCl, 0.75 M KCl, or 1.5 M

Table 1. *Neurospora* strains

Strain	Relevant genotype	Comment(s)	Source or reference
74A-OR23-1A (74A)	Wild type, <i>mat A</i>	FGSC 987	FGSC
74a-OR8-1a (74a)	Wild type, <i>mat a</i>	FGSC 988	FGSC
6.2	$\Delta rrg-1::hph^+$, <i>mat a</i>	$\Delta rrg-1$ homokaryon	This study
11.4	$\Delta rrg-1::hph^+$, <i>mat A</i>	$\Delta rrg-1$ homokaryon	This study
15.20	$\Delta rrg-1::hph^+$, <i>mat A</i>	$\Delta rrg-1$ homokaryon	This study
32.15	$\Delta rrg-1::hph^+$, <i>mat a</i>	$\Delta rrg-1$ homokaryon	This study
10.10	$\Delta rrg-1::hph^+$, <i>mat a</i>	$\Delta rrg-1$ homokaryon	This study
96.1	$\Delta rrg-1::hph^+$, <i>rrg-1^+::bar^+</i> , <i>mat A</i>	Complemented $\Delta rrg-1$ mutant	This study
96.9	$\Delta rrg-1::hph^+$, <i>rrg-1^+::bar^+</i> , <i>mat A</i>	Complemented $\Delta rrg-1$ mutant	This study
Y256M209; FGSC 3625	<i>os-1</i> , <i>mat a</i>	HK mutant	FGSC
NM2010; FGSC 2430	<i>os-4</i> , <i>mat a</i>	MAPKKK mutant	FGSC
NM2160; FGSC 1638	<i>os-5</i> , <i>mat A</i>	MAPKK mutant	FGSC
UCLA80; FGSC 2239	<i>os-2</i> , <i>mat a</i>	MAPK mutant	FGSC
PB-1	$\Delta mak-2::hph^+$, <i>mat a</i>	MAPK mutant	Daniel Ebbole (Texas A&M University); Li <i>et al.</i> (2005)
1.12	$\Delta rrg-1::hph^+$, <i>his3 mat a</i>	$\Delta rrg-1$ <i>his-3</i> homokaryon	This study
7.1D921N	$\Delta rrg-1::hph^+$, <i>his3^+::rrg-1^{D921N}::FLAG</i> , <i>mat a</i>	<i>rrg-1^{D921N}</i> allele	This study
7.3D921N	$\Delta rrg-1::hph^+$, <i>his3^+::rrg-1^{D921N}::FLAG</i> , <i>mat a</i>	<i>rrg-1^{D921N}</i> allele	This study
11.4D921N	$\Delta rrg-1::hph^+$, <i>his3^+::rrg-1^{D921N}::FLAG</i> , <i>mat a</i>	<i>rrg-1^{D921N}</i> allele	This study
12.1	$\Delta rrg-1::hph^+$, <i>his3^+::rrg-1::FLAG</i> , <i>mat a</i>	Complemented $\Delta rrg-1$ mutant	This study
12.2	$\Delta rrg-1::hph^+$, <i>his3^+::rrg-1::FLAG</i> , <i>mat a</i>	Complemented $\Delta rrg-1$ mutant	This study

FGSC, Fungal Genetics Stock Center (Kansas City, MO).

Table 2. Primers used in this study

Gene	Primer	Sequence (5'-3')	Template
<i>rrg-1</i>	Intron left	ATCTTGACGTGATGCCCGAGGAAG	RNA and genomic DNA
<i>rrg-1</i>	Intron right	CCGTCAAAGTCGATAAGGGCTTGC	RNA and genomic DNA
<i>rrg-1</i>	RG1vL	ATCCGTCTTAAGAAACCATTATTATCATGACAGCGGCCGCCCATGTAGC GGGACGTTGTCGTCTG	Genomic DNA
<i>rrg-1</i>	RG1vR	AAGCGTGGTGCACCTCTCAGTACAATCTGCTCTGCGGCCGCCGGGCGA GCCAGGGGCTTATACTAT	Genomic DNA
<i>rrg-1</i>	RG1cL	CGGCCGCGGATCTGCCGGTCTCCCTATAGTGAGTCGTATCCGTGCA GACGCGTGTGTGAAAGCG	Genomic DNA
<i>rrg-1</i>	RG1cR	GCTGGCGGCCGCGTTCTATAGTGTACCTAAATCGTATGTGTTCCG GCGCTGACTCTGACTGGCGT	Genomic DNA
<i>rrg-1</i>	RG1-1	GTAACGCCAGGGTTTTCCAGTCACGACGGATATCGTGCGCAGGGTT CGTCTG	pCJ2
<i>rrg-1</i>	RG1-2	GCGGATAACAATTTACACAGGAAACAGCCTCGAGGTCGACGGTA TCGTTG	pCJ2
<i>rrg-1</i>	RG1-3	GGCGACTACAAGGACGACGACGACAAGTGAGGTGTCGGCGCTGACTC TGACTG	pCJ2
<i>rrg-1</i>	RG1-4	TCACTTGTCTGTCGTCGTCCTTGTAGTCGCCCTTATCGTCGCTCGAAGTC TGACTC	pCJ2
<i>rrg-1</i>	RG1-5 ^a	CTTTCACCTGGTGTGATGAACATTGAGCTGCCCATCATG	pCJ2
<i>rrg-1</i>	RG1-6 ^a	CATGATGGGCAGCTGAATGTTTCATCAACACCAAGTGAAG	pCJ2
<i>rrg-1</i>	RG1-7	CCTTGCGCTTTTACACACGCGCTCTG	pCJ2
<i>rrg-1</i>	RG1-8	GAGCGTTCGAATGAGATCGTCTGG	pCJ2
<i>hph</i>	<i>hph</i> cassette 1 (5')	ACATACGATTTAGGTGACAC	Plasmid pAG32
<i>hph</i>	<i>hph</i> cassette 2 (3')	ATACGACTCACTATAGGGAG	Plasmid pAG32
<i>poi-2</i>	<i>poi-2</i> forward ^b	TATGTGATGCCGCAGGTGAG	Cosmid pMOcosX G15 A5
<i>poi-2</i>	<i>poi-2</i> reverse ^b	GGGGTTGTTGCTTCGTTC	Cosmid pMOcosX G15 A5

^a Codon 921 (with D/N mutation) is underlined.

^b Sequences from Kim and Nelson (2005).

sorbitol, whereas VM liquid medium was supplemented with 0.1 or 0.8 M NaCl. Sorbose-containing medium (FIGS or FGS) was used to facilitate colony formation on plates (Davis and deSerres, 1970). When required, hygromycin B (Calbiochem, EMD Biosciences, San Diego, CA) was added to media at a concentration of 200 µg/ml. Plasmids were maintained in *Escherichia coli* strain DH5α (Hanahan, 1983). Fludioxonil and iprodione (gifts from Drs. Allison Tally [Syngenta Crop Protection, Greensboro, NC] and Frank Wong [University of California, Riverside, CA]) were used at final concentrations of 10 or 100 µg/ml (see figure legends), from stock solutions prepared at 100 mg/ml in 100% dimethyl sulfoxide.

Macroconidia, various plate tissues, and submerged sexual and vegetative cultures were used for RNA and protein isolation. Plate cultures were grown on solid medium (VM or SCM) overlaid with cellophane (Bio-Rad, Hercules, CA). VM plates were grown in the dark at 30°C for 3 d, whereas SCM plates were grown in constant light at 25°C for 6 d. Submerged vegetative cultures were obtained by inoculation of liquid VM with 5–8-d-old macroconidia to a final concentration of 1×10^6 macroconidia/ml followed by culturing at 30°C for 16 h with shaking at 200 rpm, whereas 3-d-old liquid SCM cultures were grown with constant light at room temperature at 60 rpm. Total RNA was extracted as described previously (Sachs and Yanofsky, 1991) or with the TRIzol reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA) or by using the PureScript kit according to the manufacturer's directions for plant tissue (Gentra Systems, Minneapolis, MN).

rrg-1 Gene Structure, Intron Verification, and Expression Analysis

The *rrg-1* open reading frame (ORF; NCU01895.1) was originally identified during homology searches of the *N. crassa* genome sequence (<http://www.broad.mit.edu/annotation/fungi/neurospora/>; Galagan *et al.*, 2003) by using BLAST (Altschul *et al.*, 1997) with response regulators from *S. cerevisiae* and *Schizosaccharomyces pombe* as queries. The predicted gene structure includes one 85-base pair intron near the 3' end of the ORF. The primer pair intron left and intron right (Table 2) was used with RNA isolated from SCM plate cultures in reverse transcriptase-polymerase chain reaction (RT-PCR) reactions (as described in Krystofova and Borkovich, 2005), to verify the existence and size of the intron. Amplification reactions containing cosmid pMOcosX G18F7 (Orbach *et al.*, 1990; includes the entire *rrg-1* region) were used to generate a genomic control fragment. Products were detected by subjecting samples to agarose gel electrophoresis, blotting and then Southern analysis. The same primers used in RT-PCR reactions were used to produce probe

templates during PCR reactions with the pMOcosX G18F7 cosmid. All probe templates were labeled using the Prime-a-Gene labeling kit method according to the manufacturer's protocol (Promega, Madison, WI). The RT-PCR products were subsequently cloned into pGEMT (Promega) and sequenced.

To assess expression levels of *rrg-1* during the life cycle, quantitative RT-PCR (16 cycles) was performed using 1 µg of total RNA isolated from macroconidia, 16-h submerged cultures, and VM or SCM plate tissues of wild-type strain 74A (Table 1; Krystofova and Borkovich, 2005). This method was also used to check the $\Delta rrg-1$ and *rrg-1*^{D921N} mutants and $\Delta rrg-1$, *rrg-1*⁺ complemented strains (Table 1) for the presence of the *rrg-1* transcript by using 6-d-old SCM plates or 3-d-old liquid SCM cultures. Reaction samples were processed using the procedure described for intron verification above.

For Northern analysis of *poi-2* (NCU05768.2), samples containing 25 µg of total RNA were analyzed as described previously (Krystofova and Borkovich, 2005). The *poi-2* probe DNA was generated by PCR by using a cosmid template (Table 2) and then labeled as described above.

Construction of $\Delta rrg-1$, *rrg-1*^{D921N}, and Rescued Strains

The *rrg-1* gene replacement construct was generated using a modification of a yeast recombinational cloning procedure (Colot *et al.*, 2006; Raymond *et al.*, 1999). Fragments corresponding to the 2-kb regions just 5' and 3' to the *rrg-1* ORF (5' and 3' flanks) were amplified by PCR by using genomic DNA from wild-type strain 74A as a template. Primers contained 45 bases of overlap to the SrfI gapped pCRG26 vector at the 5' end and 45 bases of overlap to the cassette at the 3' end, in addition to 20 bases of gene specific sequence (Table 2). RG1vL was paired with RG1cL, and RG1cR was paired with RG1vR to generate the 5' flank and 3' flank, respectively. Cycling conditions were as follows: 94°C for 5 min; 98°C for 20 s; 60°C for 30 s and 68°C for 2 min (35 cycles total), with a final incubation at 72°C for 10 min.

The antibiotic resistance cassette was generated by PCR of vector pAG32 by using the cassette 1 and cassette 2 primers (Table 2). The cassette contains the *E. coli hph* gene (conferring hygromycin B resistance) with the *Ashbya gossypii* TEF (translation elongation factor) promoter and terminator that are expressed in *S. cerevisiae* and *N. crassa* (Pitt, Colot, and Dunlap, personal communication). The cassette, 5' flank, 3' flank, and SrfI gapped pCRG26 were transformed into *S. cerevisiae* strain CRY1-2 for recombination via the overlapping 45mers into a circular plasmid. Colonies harboring correctly recombined constructs were selected by plating cells on YPAD containing 200 µg/ml hygromycin B. DNA was isolated from the population of yeast transformant colonies, and plasmids were recovered by transformation into *E. coli*

and plating on ampicillin-containing medium. Plasmids were analyzed for the correct recombination event by restriction digestion and PCR. The resulting $\Delta rrg-1$ gene replacement construct was named pSP1.

The *rrg-1* gene is located on linkage group 1 near the mating-type locus. To obtain the $\Delta rrg-1$ mutation in both mating types, the gene replacement construct was transformed into wild-type strains 74A and 74a and histidine auxotrophic strain *his3a* (Krystofova and Borkovich, 2005). Plasmid pSP1 was linearized by XhoI digestion, and 1 μ g was used to electroporate 10-d-old macroconidia as described previously (Vann, 1995; Ivey *et al.*, 1996). Transformants were suspended in regeneration agar (Case *et al.*, 1979) and then plated on FIGS medium containing 150 or 200 μ g/ml hygromycin B.

Genomic DNA was extracted from transformants using the PureGene kit according to the manufacturer's protocol (Gentra Systems). Homologous recombination events were identified by Southern analysis (Ivey *et al.*, 1996) of genomic DNA from the transformants after digestion with HindIII (Supplemental Figure 1B; data not shown). A 4-kb fragment containing the *hph* cassette and 3' flank from the $\Delta rrg-1$ cassette (Supplemental Figure 1A) was used as the probe. Heterokaryotic strains containing the gene replacement event without ectopic integrations were crossed to the opposite mating type and homokaryotic progeny were selected on FIGS medium containing 150 μ g/ml hygromycin B. The purity of strains was verified by Southern analysis as described above (Supplemental Figure 1, A and B). $\Delta rrg-1$ strains 11.4 (*mat A*) and 6.2 (*mat a*) were chosen for further analysis.

Two different approaches were used to complement the $\Delta rrg-1$ mutation in trans. In the first approach, a construct containing the *rrg-1* gene and the *bar* dominant-selectable marker (confers resistance to Ignite; Pall, 1993) was integrated at a random ectopic site in the genome of a $\Delta rrg-1$ strain. The construct (pCJ2) was generated as follows. First, pTJK1 was made by inserting the XbaI-SpeI fragment containing the *bar* cassette from plasmid pBAR-GEM7-2 (Pall and Brunelli, 1993) into pBluescript II KS+ (Stratagene, La Jolla, CA). The pMOcosX G18F7 cosmid was digested with XmnI and AclI, which cuts 2.05 kb upstream and 0.87 kb downstream of the *rrg-1* ORF, respectively, resulting in a 5.96-kb fragment. This fragment was subcloned into pTJK1 digested with ClaI and EcoRV (ClaI generates the same overhang as AclI, whereas EcoRV and XmnI create blunt ends) to yield pCJ2, the *rrg-1*⁺ *bar*⁺ rescue construct. Aliquots containing 300–600 ng of XmnI-linearized pCJ2 plasmid were transformed into 8-d-old conidia from strain $\Delta rrg-1$ (11.4) via electroporation as described previously, by using water instead of 1 M sorbitol to suspend the conidia. Transformants were selected on FIGS medium supplemented with 400 μ g/ml Ignite, and then they were screened for integration of the rescue construct by growth on VM plates containing 1 M sorbitol and by Southern analysis (using an XhoI-NotI digest of genomic DNA and with the 2-kb 5' flanking region of *rrg-1* as the probe). Rescue strains produce a hybridizing fragment of 7.5 kb, whereas the $\Delta rrg-1$ gene replacement mutant produces a 4.8-kb fragment. Homokaryotic $\Delta rrg-1$ *rrg-1*⁺-complemented strains were obtained after cultivation of transformants with the desired integration event on microconidiation medium (Ebbole and Sachs, 1990) supplemented with 400 μ g/ml Ignite.

The second approach for complementation involved targeting *rrg-1*⁺ to the *his-3* locus of a $\Delta rrg-1$ *his-3* strain. Because this rescue strain served as a control for the *his-3*-targeted *rrg-1*^{D921N} allele, construction of both strains will be discussed together. First, the pMOcosX G18F7 cosmid was digested with NotI (made blunt with Klenow) and then SacI, releasing an 8.5-kb *rrg-1* genomic fragment. This fragment was ligated into pTJK1 at StuI/SacI forming pCJ1. Separately, C-terminal *rrg-1* gene fragments containing a FLAG tag and the *RRG-1*^{D921N} point mutation were generated via PCR by using primers containing one copy of the FLAG sequence in place of the *rrg-1* stop codon (for both constructs) and AAC in place of GAT at codon 921 (for the D921N mutation). All fragments were amplified using pCJ2 template and assembled using yeast recombinational cloning with yeast plasmid pRS426 as described previously (Raymond *et al.*, 1999; Colot *et al.*, 2006). Fragments A and B and D were used for the *RRG-1*^{D921N} point mutation, whereas fragments C and D were used for the wild-type allele. Fragment A was generated using the RG1-1 and RG1-6 primers (Table 2), creating a 1.6-kb fragment extending from the EcoRV site in the *rrg-1* ORF to amino acid 921. Fragment A also contained sequence homologous to pRS426 at the 5' end. Fragment B was amplified using RG1-5 and RG1-4 primers, generating a 0.6-kb fragment spanning from amino acid 921 to the position of the stop codon (a FLAG tag has been inserted upstream of a new stop codon). For the wild-type allele, fragment C was amplified using the RG1-1 and RG1-4 primers; this generates a 2.2-kb fragment extending from the EcoRV site in *rrg-1* (with a pRS426 adaptor) to the end of the *rrg-1* ORF (stop codon replaced with the FLAG sequence). Fragment D was generated using primers RG1-3 and RG1-2; the resulting 0.9-kb fragment extends from the FLAG tag to the XhoI site in pCJ2 (also includes sequence homologous to pRS426 vector). pCJ3 (D921N mutation with FLAG tag) and pCJ4 (wild-type allele, with FLAG tag) were then generated by yeast recombinational cloning of the appropriate fragments. pCJ2 was digested with XhoI/EcoRV, and the 7.2-kb fragment (5' end of *rrg-1* ORF and promoter region) was ligated with the 3.1-kb fragment from the digestion of pCJ4 or pCJ3 with EcoRV/XhoI to generate pCJ5 and pCJ6, respectively. Finally, SmaI/SpeI-digested pBM61 (Margolin *et al.*, 1997), the 2.7-kb XmnI/BamHI fragment from pCJ1, and the 3.3-kb BamHI/SpeI frag-

ment from either pCJ5 or pCJ6 were ligated to generate the *his-3*-targeted *rrg-1*⁺ (pCJ7) or *rrg-1*^{D921N} (pCJ8) vectors.

Eight-day-old conidia from the histidine auxotrophic strain $\Delta rrg-1$ *his-3* *mat a* (1.12) were transformed with 300- to 600-ng aliquots of BssHII-linearized pCJ7 or pCJ8. Transformation was performed as described above for the *bar*⁺ rescue strains. Histidine prototrophs were selected on FGS medium and screened for successful *his-3* targeting by Southern analysis by using a HindIII digest of genomic DNA and a 1.5-kb probe corresponding to the 5' end of the *rrg-1* ORF (amplified using primers RG1-7 and RG1-8; Table 2; see "probe *his-3* targeting" in Supplemental Figure 1A). Strains with *rrg-1* targeted to the *his-3* locus produce a hybridizing fragment of 8.7 kb, whereas the $\Delta rrg-1$ *his-3* recipient strain does not. Further Southern analysis by using alternative probes and enzymes confirmed the original results. Homokaryotic $\Delta rrg-1$ *rrg-1*⁺:*his-3*⁺ and $\Delta rrg-1$ *rrg-1*^{D921N}:*his-3*⁺ strains were obtained after cultivation of transformants with the desired integration event on microconidiation medium (Ebbole and Sachs, 1990) and plating on FGS medium.

Phenotypic Testing and Western Analysis

Assessment of colony growth on VM plates, macroconidiation in slants, and protoperithecial and perithecial development during the sexual cycle were as described previously (Krystofova and Borkovich, 2005). Growth under hyperosmotic conditions was assayed as described previously (Ivey *et al.*, 1996), with details in the figure legend. Photomicroscopy was performed using a SZX9 stereomicroscope or a BX41 compound microscope with a C-4040 digital camera (Olympus, Lake Success, NY).

For Western analysis, whole cell extracts were isolated from the tissues indicated in the figure legends by using a modified version of a published protocol (Borkovich *et al.*, 1989). *Neurospora* tissues, ground in liquid nitrogen, were added to 1 ml of chilled 3 mM phenylmethylsulfonyl fluoride in 95% ethanol with 0.2 g of 0.5-mm glass beads in a 2-ml screw-top centrifuge tube. Samples were vortexed 3 times for 60 s with 60-s rests on ice in between. Extracts were chilled at –20°C for at least 16 h. Samples were then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was removed, and precipitated proteins were dried on the beads for 30 min in a vacuum concentrator (Eppendorf, Westbury, NY). Samples were reconstituted in 250 μ l of 1% SDS, heated at 85°C for 5 min, and centrifuged at 14,000 rpm for 5 min at room temperature. The protein supernatant was removed and saved. This step was repeated once, and the supernatants were combined. The combined supernatants were subjected to a final heating, centrifugation, and supernatant recovery as described above to remove residual cellular debris. Protein concentration was determined using the BCA protein assay (Pierce Chemical, Rockford, IL), and a volume containing 50 μ g of protein was subjected to SDS-polyacrylamide gel electrophoresis as described previously (Krystofova and Borkovich, 2005).

The POI-2 antibody (Kim and Nelson, 2005) was used at a dilution of 1:1000. Commercially available antibodies against mammalian or *S. cerevisiae* MAPKs were used to detect phospho-OS-2 (anti-p38 9211; 1:1000 dilution; Cell Signaling Technology, Beverly, MA; Kawasaki *et al.*, 2002), and OS-2 (anti-Hog1p sc-9079; 1:600 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Incubation with secondary antibody and chemiluminescent detection were performed as described previously (Krystofova and Borkovich, 2005).

RESULTS

rrg-1 Structure and Expression and Construction of a Gene Replacement Mutant

The *rrg-1* gene was identified during BLAST searches of the Broad Institute *Neurospora* genome sequence (Galagan *et al.*, 2003; Borkovich *et al.*, 2004). The autocalled gene has one predicted intron in the carboxy terminus. Because there are no cDNA clones available for *rrg-1*, the existence of this intron was verified with RT-PCR. Primers were designed that would produce 220- and 305-base pair products from mRNA and genomic DNA, respectively, if an intron was present. A product of 220 base pairs was obtained using mRNA as a template in RT-PCR reactions (Figure 1A, left), and sequencing of this product verified the predicted position and size of the intron (data not shown).

The CDART protein domain prediction program (Geer *et al.*, 2002) predicts a carboxy-terminal response regulator receiver domain for RRG-1. RRG-1 belongs to the Ssk1p class of response regulators, implicated in resistance to hyperosmotic stress in other fungi. Under normal conditions, *S. cerevisiae* Ssk1p is phosphorylated at Asp-554, blocking activation of the Hog1 MAPK cascade (Maeda *et al.*, 1994; Posas

Figure 1. Expression and gene structure of *Neurospora rrg-1*. (A) *rrg-1* expression and intron verification. Left, RT-PCR was used to determine the level of *rrg-1* mRNA in tissues isolated from wild-type strain 74A. Tissues were collected from 8-d-old macroconidia (Conidia), 16-h submerged cultures (Liq), 3-d VM plates (VM), and 6-d SCM plates (SCM). Genomic DNA was amplified as a size control for intron-containing sequence. Middle, RT-PCR was used to quantitate *rrg-1* message levels in RNA isolated from 6-d-old SCM plates. Strains were WT (wild type; 74A), $\Delta rrg-1$ (strain 11.4), and *rrg-1*⁺::*bar*⁺ rescue (strain 96.1). Right, RT-PCR was used to quantitate *rrg-1* message levels in RNA isolated from 3-d SCM liquid cultures. Strains were $\Delta rrg-1$ (strain 11.4), *rrg-1*^{D921N}::*his-3*⁺ (11.4D921N) and *rrg-1*⁺::*his-3*⁺ rescue (12.1). (B) Schematic showing conserved regions in the RRG-1 protein sequence. The response regulator domain (RR) is shown in black and contains the Asp-921 residue, the predicted site of phosphorylation. The gray bar indicates a region conserved in other filamentous fungi (FFS; filamentous fungi similar region). White regions share more limited homology with related response regulators from filamentous fungi and yeasts.

et al., 1996). Asp-921 in RRG-1 aligns with Asp-554 in Ssk1p, suggesting that Asp-921 is phosphorylated in RRG-1. The predicted 1114-amino acid sequence of RRG-1 is also similar to several predicted proteins in the genomes of other ascomycete filamentous fungi (Figure 1B and Supplemental Figure 2). Sequence conservation between these putative fungal response regulators is highest in and around the receiver domain (amino acids 869–1027 in RRG-1), and all contain the conserved aspartyl residue (indicated by an asterisk in Supplemental Figure 2). Areas of high similarity also exist 5' of the receiver domain; the first 17 amino acids of *Neurospora* RRG-1 are 82% similar to predicted response regulator proteins from other filamentous fungi. A region of 95% similarity spans from amino acids 485–1077, with highest conservation occurring between residues 527–642 and 688–743 (both regions are outside the response regulator domain).

The *rrg-1* mRNA is of relatively low abundance and cannot be detected using Northern analysis (data not shown). Therefore, quantitative RT-PCR was used to determine relative expression levels of *rrg-1* during the *Neurospora* life cycle (Figure 1A, left). We tested tissues representing vegetative growth, asexual and sexual development (see below). The *rrg-1* message was present in all tissues analyzed, but was most abundant in sexually differentiated (SCM) plate cultures.

The *rrg-1* gene was mutated in *Neurospora* by using a gene replacement procedure. The knockout construct was made using a yeast recombinational cloning method (see *Materials and Methods*), with the selectable marker consisting of the *E. coli hph* gene under the control of a fungal promoter (Staben *et al.*, 1989; see diagram in Supplemental Figure 1A). Southern analysis was used to verify the final homokaryotic $\Delta rrg-1$ gene replacement mutants (Supplemental Figure 1B). To ensure that the observed phenotypes resulted from deletion of the *rrg-1* gene, the $\Delta rrg-1$ mutant was complemented via two different methods in trans with wild-type *rrg-1*⁺, by using the *bar* gene (Pall, 1993) or *his-3*⁺ as selectable markers (Margolin *et al.*, 1997). Results from RT-PCR analysis of RNA showed that the *rrg-1* transcript was absent from $\Delta rrg-1$ gene replacement mutants (Figure 1A, middle and right). Rescued $\Delta rrg-1$::*rrg-1*⁺ strains were similar to wild type with respect to all traits tested, with the exception of fungicide sensitivity in the *rrg-1*⁺::*bar*⁺ strain (data not shown; see below). Complemented *rrg-1*⁺::*bar*⁺ and *rrg-1*⁺::*his-3*⁺ targeted strains possessed reduced and comparable levels of *rrg-1* transcript compared with wild type, respectively (Figure 1A, middle and right; data not shown). The lower level of expression in the *rrg-1*⁺::*bar*⁺ strain may

explain the complementation of most, but not all phenotypes in these strains.

$\Delta rrg-1$ Strains Resemble *os* Mutants during Vegetative Growth

$\Delta rrg-1$ mutants were first analyzed for defects during vegetative growth. *Neurospora* grows vegetatively by apical extension of basal hyphae (for review, see Springer, 1993; Borkovich *et al.*, 2004). Hyphae fuse and branch to form a multicellular web-like structure, the mycelium. A variety of environmental stimuli, including desiccation, heat, and carbon or nitrogen starvation, can induce *Neurospora* to commit to an asexual sporulation pathway, macroconidiation. The timing of macroconidiation is also controlled by a circadian rhythm. The macroconidiation pathway begins with differentiation of aerial hyphae from the mycelium. Vegetative spore-forming structures, conidiophores, develop from the tips of aerial hyphae. Constrictions in the aerial hyphae then form, yielding a chain of multinucleated spores, the macroconidia. Macroconidia are hydrophobic, and they are easily released by mechanical perturbation. Because of the requirement for desiccation, submerged liquid cultures of wild-type *Neurospora* do not develop aerial hyphae or macroconidia.

$\Delta rrg-1$ mutants have a hyphal apical extension rate similar to wild-type (data not shown). However, $\Delta rrg-1$ strains exhibit premature aerial hyphae development and macroconidiation on solid medium (data not shown). Under light exposure, *Neurospora* develops a characteristic orange color, which results from the production of carotenoid pigments. Macroconidia from $\Delta rrg-1$ strains eventually form a deep orange or red pigment due to lysis and leakage of cytoplasmic contents, including carotenoids (Figure 2A). This phenotype is enhanced when spores are collected in 1 M sorbitol (data not shown), greatly reducing the viability of $\Delta rrg-1$ macroconidia during preparation for electroporation. This trait made $\Delta rrg-1$ macroconidia poor candidates for complementation in trans with wild-type *rrg-1* (see *Materials and Methods*; data not shown).

The phenotypes of $\Delta rrg-1$ strains on vegetative minimal medium are reminiscent of previously characterized *Neurospora os* mutants. As a group, the *os* mutants develop macroconidia that acquire a deep orange pigment and tend to lyse (Figure 2A; Perkins *et al.*, 2001; Zhang *et al.*, 2002). The *os-1/nik-1*, *os-4*, *os-5*, and *os-2* genes encode a hybrid histidine kinase, MAPKKK, MAPKK, and MAPK, respectively. OS-4, OS-5, and OS-2 are similar to *S. cerevisiae* Ssk2p, Pbs2p, and Hog1p, which are required for the response to hyperosmotic stress in budding yeast (Maeda *et al.*, 1994). OS-4, OS-5, and

Figure 2. Growth of $\Delta rrg-1$ and *os* mutants on normal, hyperosmotic, and fungicide-containing medium. (A) Phenotypes during vegetative growth. Wild-type (74A), $\Delta rrg-1$ (11.4), *os-4* (FGSC 2430; MAPKKK), *os-5* (FGSC 1638; MAPKK), and *os-2* (FGSC 2239; MAPK) strains were cultured in VM agar medium flasks for 3 d in the dark at 30°C followed by 5 d in the light at 25°C. The presence of ruptured, deep-pigmented macroconidia in the $\Delta rrg-1$ and *os* mutants is evidenced by the dark red-orange pigmentation along the top of the growth margin in the flasks. (B) Sensitivity to hyperosmotic conditions. Strains indicated in A were inoculated in the center of VM plates with no additions (–) or VM plates supplemented with 0.75 M NaCl (NaCl), 0.75 M KCl (KCl), or 1.5 M sorbitol (Sorbitol). The plates were incubated at 30°C in the dark for 48 h. (C) Fungicide resistance. Strains indicated in A were inoculated in the center of VM plates with no additions (–) or VM plates supplemented with 10 μ g/ml fludioxonil or iprodione. Plates were incubated at 30°C in the dark for 22 h.

OS-2 also have homologues in *S. pombe*, making up a related MAPK pathway that controls resistance to oxidative stress (Degols *et al.*, 1996). *OS-1* does not have a close homologue in *S. cerevisiae* or *S. pombe*. In *S. cerevisiae*, the Sln1p hybrid histidine kinase operates upstream of the Hog1p MAPK cascade. The Mak2 and Mak3 hybrid histidine kinases phosphorylate response regulator Msc4 (similar to RRG-1) to control the oxidative stress response in *S. pombe* (Buck *et al.*, 2001).

$\Delta rrg-1$ Mutants Are Sensitive to Hyperosmotic Stress and Resistant to Phenylpyrrole and Dicarboximide Fungicides

It was previously shown that *os* mutants are sensitive to hyperosmotic stress (Selitrennikoff *et al.*, 1981; Perkins *et al.*, 1982). The similar growth phenotypes of $\Delta rrg-1$ and *os* strains prompted comparison of the $\Delta rrg-1$ mutant to wild-type, *os-5*, *os-4*, and *os-2* strains during growth on medium containing 0.75 M NaCl, 0.75 M KCl, or 1.5 M sorbitol (Figure 2B). Consistent with the previous studies, *os-5*, *os-4*, and *os-2* mutants are more sensitive to all three hyperosmotic media than wild type. Similarly, growth of the $\Delta rrg-1$ mutant was severely restricted

under osmotic stress, with the largest reduction on sorbitol-containing medium (Figure 2B).

There are conflicting reports regarding the sensitivity of *os* mutants to phenylpyrrole and dicarboximide fungicides (Grindle and Temple; 1982; Fujimura *et al.*, 2000a,b). For example, one analysis reported that *os-1/nik-1*, *os-2*, *os-4*, and *os-5* mutants are resistant to the phenylpyrrole, fludioxonil, and the dicarboximide iprodione (Grindle and Temple, 1982; Fujimura *et al.*, 2000a,b), whereas a subsequent study showed that *os-1/nik-1* and *os-4* mutants were sensitive to fludioxonil (Zhang *et al.*, 2002). These inconsistencies may be due to differences in the mutant alleles used for each analysis. Because $\Delta rrg-1$ strains and *os* pathway mutants exhibit osmotic sensitivity, and in light of the discrepancies in the literature, we analyzed the growth of wild-type, $\Delta rrg-1$, *os-5*, *os-4*, and *os-2* mutants on solid medium containing iprodione or fludioxonil. The results show that $\Delta rrg-1$, *os-5*, *os-4*, and *os-2* strains are indeed resistant to fludioxonil and iprodione (Figure 2C), suggesting that RRG-1 and proteins of the OS signaling cascade control fungicide sensitivity in *Neurospora*.

Figure 3. Phenotypes of $\Delta rrg-1$, *os*, and rescued $\Delta rrg-1$ *rrg-1*⁺ strains during the sexual cycle. (A) Wild-type (74A), $\Delta rrg-1$ (11.4A), *os-4* (FGSC 2430), *os-5* (FGSC 1638), and *os-2* (FGSC 2339) strains were inoculated onto SCM plates and incubated in constant light at 25°C for 6 d. At that time, half of each plate was inoculated with macroconidia (males) of opposite mating type, and incubation continued for an additional 3 d. Both sides of the plates were then photographed. Examples of protoperithecia (unfertilized) and perithecia (fertilized) are indicated by the white arrows. (B) Wild-type (74A), $\Delta rrg-1$ (11.4), and *rrg-1*⁺:*bar*⁺ rescue (96.9) strains were analyzed as described in A. (C) Wild-type (74A), $\Delta rrg-1$ (11.4), *os-1* (FGSC 3625), and *os-2* (FGSC 2339) strains were analyzed as in described in A.

rrg-1 and Components of the *os-2* MAPK Pathway Are Essential for Protoperithelial Development and Female Fertility

We next analyzed $\Delta rrg-1$ strains for defects in sexual fertility. Nitrogen starvation induces differentiation of female reproductive structures, protoperithecia, in *Neurospora* (for review, see Raju, 1992). Protoperithecia extend specialized hyphae called trichogynes that are positively chemotropic, growing toward male cells (any vegetative cell type; typically, macroconidia) of opposite mating type (Bistis, 1981). Successful attraction requires that the male cell produces pheromone and that the female contains the appropriate pheromone receptor (Bistis, 1983; Bobrowicz *et al.*, 2002; Kim *et al.*, 2002; Kim and Borkovich, 2004). Once physical contact is established, the trichogyne coils around and fuses with the male cell (Bistis, 1981; Kim and Borkovich, 2004). It is thought that the nucleus from the male then traverses the trichogyne tube and that fertilization takes place at the base of the protoperithecium where the female gametes are located. The diploid phase of the life cycle is transient, and nuclear fusion is followed immediately by meiosis and the onset of sexual spore (ascospore) development. Early during the process of fertilization and meiosis, the protoperithecium enlarges and becomes the perithecium. Once ascospore maturation is complete, the spores are ejected through a hole in the tip of the perithecium (ostiole). Under appropriate conditions (high temperature and available nutrients), ascospores can germinate to form a new mycelium.

$\Delta rrg-1$ mutants of either mating type are fertile as males when crossed with wild-type females of opposite mating type (data not shown). $\Delta rrg-1$ strains exhibit reduced mass accumulation on nitrogen-limited SCM compared with wild-type (data not shown). More importantly, protoperithecia cannot be observed in $\Delta rrg-1$ strains cultivated on

SCM, and application of wild-type macroconidia does not result in production of perithecia or ascospores (Figure 3, A–C; data not shown). This defect is rescued by introduction of the wild-type *rrg-1* gene in trans (Figure 3B). Thus, loss of *rrg-1* leads to an early and complete block in female fertility in *Neurospora*. It has been reported that *os-4* mutants are female sterile, but the exact defect was not specified (Fujimura *et al.*, 2003). Therefore, we analyzed the *os* mutant strains for protoperithelial and perithelial development. Protoperithecia could not be detected in the *os-4*, *os-5*, or *os-2* mutants, and perithecia were not observed after fertilization with wild-type macroconidia during our study (Figure 3A). This experiment was repeated several times, and an occasional rare perithecium could be observed in cultures of *os-2* and *os-5* mutants, but never in *os-4* strains (data not shown). These results suggest that the OS-2 MAPK pathway is required for protoperithelial development and female fertility in *Neurospora*. This is in contrast with previous observations with *nik-1/os-1* mutants, demonstrating that this histidine kinase is not required for protoperithelial or perithelial development (Alex *et al.*, 1996). We analyzed a *nik-1/os-1* mutant (allele Y256M209) during our study and observed that it was able to produce protoperithecia and perithecia, but at reduced numbers compared with wild type (Figure 3C). Further analysis showed that the *os-1* mutant did not produce viable ascospores 10 d postfertilization (data not shown). The Y256M209 *os-1* mutant allele was generated using UV light and contains a frameshift mutation at amino acid 294 (Ochiai *et al.*, 2001). The difference in phenotypes observed for $\Delta rrg-1$ and *os-2* versus *os-1* mutants supports the hypothesis that the OS-2 MAPK pathway is regulated by multiple histidine kinases under different environmental conditions (e.g., osmotic stress vs. nitrogen starvation) in *Neurospora*.

Figure 4. Analysis of OS-2 protein levels and phosphorylation. (A) Hyperosmotic conditions. Wild-type (74A), $\Delta rrg-1$ (11.4), and *os-2* (FGSC 2239) strains were grown for 16 h in submerged VM cultures. The cultures were brought to 0, 0.1, or 0.8 M NaCl and incubated with shaking for the last 10 min of growth. The cells were collected, and whole cell extracts were prepared and then subjected to Western analysis (see *Materials and Methods*). Top, Western blot reacted with anti-p38 that recognizes only the phosphorylated form of OS-2. Bottom, Western blot reacted with anti-Hog1p that recognizes all forms of the OS-2 protein. (B) Fungicide exposure. Sixteen-hour submerged VM cultures of the strains indicated in A were treated with 0, 10, or 100 $\mu\text{g/ml}$ fludioxonil for the last 10 min of incubation. Whole cell extracts were prepared and subjected to Western analysis with the antibodies described in A. (C) Nitrogen starvation. Wild-type (74A), $\Delta rrg-1$ (11.4), *os-2* (FGSC 2239), *os-4* (FGSC 2430), and $\Delta mak-2$ (PB-1) mutants were inoculated onto SCM plates and incubated in constant light at 25°C for 6 d, at which time protoperithecia were present in wild-type cultures. Whole cell extracts were prepared and subjected to Western analysis with the antibodies described in A.

Loss of *rrg-1* Affects Phosphorylation of the OS-2 MAPK

The shared sensitivity of $\Delta rrg-1$ and *os-2* strains to hyperosmotic conditions and their resistance to fludioxonil and iprodione prompted us to investigate whether 1) OS-2 phosphorylation varied after exposure to high salt or fungicide and 2) the possibility that $\Delta rrg-1$ mutants would have lower levels of phosphorylated OS-2 than wild-type cells. For these studies, we took advantage of commercially available peptide antibodies raised against the mammalian MAPK p38, and *S. cerevisiae* Hog1p, which are homologous to *Neurospora* OS-2. Anti-Hog1p recognizes both nonphosphorylated and phosphorylated forms of the protein, whereas anti-phospho p38 is specific for the phosphorylated MAPK. The phospho-p38 antibody was previously shown to cross-react with the homologous MAPK protein, SakA, from *Aspergillus nidulans* (Kawasaki *et al.*, 2002), whereas both antibodies were shown to recognize the homologous MAPK protein OSC-1 from *Colletotrichum lagenarium* (Kojima *et al.*, 2004). We tested the antibodies for binding to OS-2 via Western analysis of extracts from wild-type, $\Delta rrg-1$, and *os-2* strains (Figure 4, A–C). The two antisera cross-react with a protein of the expected size of *Neurospora* OS-2 in wild-type extracts (41.3 kDa; Figure 4, A–C). The cross-reactive species is absent from the *os-2* mutant, supporting the specificity of the antibodies for OS-2.

We next used the two antisera to analyze levels of OS-2 protein and the extent of phosphorylation in wild-type and

$\Delta rrg-1$ strains during short-term exposure to different concentrations of NaCl (Figure 4A). We found levels of OS-2 protein are reduced two-fold in $\Delta rrg-1$ mutants compared with wild-type (Figure 4A, bottom). Furthermore, the amount of OS-2-phosphate is much lower in $\Delta rrg-1$ strains than in wild-type after a 10-min treatment with 0.8 M NaCl, and no reactive species were evident in $\Delta rrg-1$ mutants in the presence of 0 or 0.1 M NaCl (Figure 4A, top).

Similar results were obtained when we tested the effects of growth to different concentrations of the fungicide fludioxonil (Figure 4B) in wild-type and $\Delta rrg-1$ strains. Again, levels of OS-2 protein are reduced by ~50% in $\Delta rrg-1$ mutants (Figure 4B, bottom). In wild-type cells, the amount of phosphorylated OS-2 is maximal at 10 $\mu\text{g/ml}$ fludioxonil, whereas OS-2-phosphate cannot be detected in $\Delta rrg-1$ mutants in the presence or absence of fludioxonil, even at 100 $\mu\text{g/ml}$ (Figure 4B, top). During the preparation of this manuscript, another group reported that treatment with fludioxonil or iprodione leads to increased levels of phosphorylated OS-2 in wild-type *Neurospora* cells (Yoshimi *et al.*, 2005). Taken together, these results suggest that RRG-1 is required for optimal phosphorylation of OS-2, particularly after fungicide exposure. The small amount of residual phosphorylated OS-2 in $\Delta rrg-1$ mutants treated with 0.8 M NaCl indicates that the RRG-2 response regulator or other proteins can activate phosphorylation of OS-2 to a limited extent in the absence of RRG-1 *Neurospora*.

The results mentioned above provide evidence that the OS-2 MAPK cascade is required for differentiation of female reproductive structures. Previous studies have demonstrated that mutation of *nrc-1* (MAPKKK) or *mak-2* (MAPK) abolishes formation of protoperithecia in *Neurospora* (Kothe and Free, 1998; Pandey *et al.*, 2004; Li *et al.*, 2005). MAK-2 is homologous to *S. cerevisiae* Fus3p/Kss1p (Pandey *et al.*, 2004; Li *et al.*, 2005). Other work has shown that phosphorylation of MAK-2 is dependent upon NRC-1 in vegetative cultures of *Neurospora* (Pandey *et al.*, 2004). To explore the possible involvement of RRG-1 in regulation of OS-2 phosphorylation during sexual development and to investigate potential connections between OS-2 and MAK-2, we next analyzed levels of OS-2 protein and the extent of phosphorylation in extracts from wild-type, $\Delta rrg-1$, *os-4*, $\Delta mak-2$, and *os-2* strains cultured under conditions that favor formation of protoperithecia (SCM plates; Figure 4C). We found that OS-2 protein levels were similar in all strains tested, except for *os-2*, where it is absent (Figure 4C, bottom). OS-2-phosphate was detected in wild-type and $\Delta mak-2$ strains but not in $\Delta rrg-1$, *os-4*, or *os-2* mutants (Figure 4C, top), further supporting the hypothesis that RRG-1 regulates the OS-2 MAPK pathway.

$\Delta rrg-1$ Mutants Exhibit Reduced Expression of *poi-2*, a Gene Required for Protoperithelial Development

The *poi-2* gene encodes a predicted secreted protein containing 16 tandem repeats consisting of 13–14 amino acids each. These tandemly repeated sequences are separated by apparent Kex2 protease sites (Kim and Nelson, 2005). POI-2 is required for normal growth and asexual and sexual development in *Neurospora* (Kim and Nelson, 2005). *poi-2* mutants have reduced vegetative growth, form white conidia and few aerial hyphae, and they are prone to reversion (Kim and Nelson 2005; data not shown). *poi-2* repeat-induced point mutants with truncated ORFs and complete gene replacement mutants do not produce protoperithecia and are thus female sterile (Kim and Nelson, 2005; Kim and Borkovich, unpublished data). Levels of *poi-2* mRNA are also decreased in $\Delta mak-2$ strains (Li *et al.*, 2005).

Figure 5. Measurements of *poi-2* mRNA and POI-2 protein levels. (A) *poi-2* mRNA amount. RNA was isolated from SCM and VM plate cultures of wild-type (74A and 74a) and $\Delta rrg-1$ strains (11.4 and 6.2) and used to prepare Northern blots. Northern blots were probed using a DNA fragment containing the *poi-2* ORF (top). Blots were photographed before hybridization to reveal the amount of rRNA subunits (loading control; bottom). (B) POI-2 protein levels during vegetative growth and development. Whole cell extracts were isolated from VM plate cultures of wild-type (74A), $\Delta rrg-1$ (11.4), *os-2* (FGSC 2339), and *os-1* (FGSC 3625) strains. Western blots were probed using an antiserum that recognizes POI-2 (top). A duplicate Coomassie-stained gel is shown as a loading control (bottom); numbers to left indicate protein size markers in kilodaltons. (C) POI-2 protein amounts under nitrogen starvation. Whole cell extracts were isolated from SCM plate cultures of the strains used in B. Western blots were probed using the POI-2 antiserum (top). A duplicate Coomassie-stained gel is shown as a loading control (bottom); numbers to left indicate protein size markers in kilodaltons.

Based on the shared inability of $\Delta mak-2$ and *poi-2* mutants to differentiate protoperithecia and the observed dependence of *poi-2* expression on *mak-2*, we investigated expression of *poi-2* in $\Delta rrg-1$ strains. We began our analysis by measuring *poi-2* mRNA levels in VM and SCM plate cultures of $\Delta rrg-1$ and wild-type strains. *poi-2* transcript levels were reduced in $\Delta rrg-1$ compared with wild type in SCM cultures and *poi-2* transcript could not be detected in VM cultures of the $\Delta rrg-1$ mutant (Figure 5A). These results suggest that RRG-1 is required for normal expression of *poi-2* in *Neurospora*.

We next analyzed levels of POI-2 protein in VM and SCM cultures of wild-type, $\Delta rrg-1$, *os-2*, and *os-1* strains by using a specific antiserum (Kim and Nelson, 2005). Consistent with previous results, POI-2 can be detected in wild type under both growth conditions, with highest levels in sexually differentiated cultures (Figures 5, B and C; data not shown; Kim and Nelson, 2005). POI-2 expression was greatly reduced in the $\Delta rrg-1$ mutant cultured on VM or SCM (Figure 5, B and C), similar to results with the *nrc-1* and $\Delta mak-2$ mutants (data not shown). POI-2 levels were similar in *os-2* mutants compared with wild type when cultured on VM (Figure 5B), but they were greatly reduced in SCM cultures (Figure 5C). In contrast, POI-2 could not be detected in VM cultures of

os-1 mutants, but it was present at slightly reduced levels relative to wild-type strains in SCM plates (Figure 5, B and C). The residual level of POI-2 observed in *os-1* mutants on SCM could be explained by functional redundancy to give the following model: NIK-1/OS-1 and other histidine kinases regulate expression of POI-2 (and protoperithecial development) through RRG-1 and the OS-2 MAPK pathway under nitrogen starvation conditions (SCM medium).

The wild-type amount of POI-2 observed in *os-2* mutants in VM cultures suggests that the OS-2 MAPK pathway does not regulate POI-2 levels under conditions of ample nitrogen. However, the drastic reduction in POI-2 amount in VM cultures of *os-1* and $\Delta rrg-1$ mutants suggests that the NIK-1/OS-1 hybrid histidine kinase and RRG-1 response regulator may regulate an alternative downstream pathway under conditions of nitrogen excess.

Analysis of the rrg-1^{D921N} Allele, Containing a Mutation in the Predicted Phosphorylation Site

To determine the importance of the phosphorylation status of RRG-1^{D921}, an *rrg-1^{D921N}* strain was generated. Asp-921 is highly conserved among fungal response regulators, corresponding to Asp-554 in *S. cerevisiae* Ssk1p and Asp-427 in *S. pombe* Mcs4. *S. pombe* Mcs4 is required for activation of the Sty1 MAPK cascade, because deletion of *mcs4* prevents phosphorylation of Sty1 under all conditions (Shieh *et al.*, 1997). Although the Mcs4^{D421N} protein does not support phosphorylation of Sty1 in response to hydrogen peroxide stress, phosphorylation is observed during salt or heat stress (Buck *et al.*, 2001), suggesting that some functions of Mcs4 are independent of phosphorylation.

The *Neurospora rrg-1^{D921N}* mutant and corresponding *rrg-1⁺::his-3⁺* control strain possessed comparable levels of *rrg-1* message (Figure 1A, right). Phenotypic analysis revealed that the *rrg-1^{D921N}* strain differs from wild-type and the $\Delta rrg-1$ gene replacement mutant. In VM flask cultures, the *rrg-1^{D921N}* strain is slower to conidiate (faint orange color) and has shorter aerial hyphae than wild type or $\Delta rrg-1$ (Figure 6A). The *rrg-1^{D921N}* mutant exhibits greater growth than $\Delta rrg-1$ strains under hyperosmotic conditions (Figure 6B), and it is sensitive to fungicide (Figure 6C). However, the *rrg-1^{D921N}* mutant grows considerably slower than wild-type or $\Delta rrg-1$ strains on normal medium (see fungicide – column in Figure 6C).

The observation of normal responses to salt and fungicide in the *rrg-1^{D921N}* strains prompted us to ask whether the RRG-1^{D921N} protein can support phosphorylation of the OS-2 MAPK under these conditions. The *rrg-1^{D921N}* strain has levels of OS-2 protein similar to those of wild-type and the rescue strain (Figure 6, E and F, bottom). Importantly, the *rrg-1^{D921N}* strain produced phospho-OS-2 when treated with NaCl or fungicide (Figure 6, E and F, top). There is an increase in phosphorylated-OS-2 in *rrg-1^{D921N}* strains with the higher concentration of fungicide; however, the increase is not as great as that observed in wild type or the rescue strain (Figure 6F, top). The production of phosphorylated-OS-2 with fungicide or NaCl treatment in the *rrg-1^{D921N}* strain correlates with the phenotype observed on fungicide and salt plates.

We also observed that the *rrg-1^{D921N}* mutant exhibits delays during sexual development. Protoperithecia are fewer but larger than those observed in wild type, and they take longer to develop (Figure 6D). After fertilization, perithecia from the *rrg-1^{D921N}* strains often were deformed with a “hairy” appearance and were elongated either side to side or top to bottom. Crushing of perithecia showed that ascospores were produced 7 d after fertilization in wild-type strains, whereas

Figure 6. Phenotypes and OS-2 phosphate levels in the *rrg-1^{D921N}* mutant. (A) Phenotypes during vegetative growth. Wild-type (74A), $\Delta rrg-1$ (11.4), *rrg-1^{D921N}::his-3⁺* (11.4D921N), and *rrg-1⁺::his-3⁺* rescue (12.2) strains were cultured in VM agar flasks for 3 d in the dark at 30°C followed by 5 d in the light at 25°C. (B) Sensitivity to hyperosmotic conditions. Wild-type (74A), $\Delta rrg-1$ (11.4), *rrg-1^{D921N}::his-3⁺* (7.3D921N), and *rrg-1⁺::his-3⁺* (12.2) strains were inoculated in the center of VM plates with no additions (–) or supplemented with 0.75 M NaCl (NaCl). The plates were incubated at 30°C in the dark for 50 h. (C) Fungicide resistance. Wild-type (74A), $\Delta rrg-1$ (11.4), *rrg-1^{D921N}::his-3⁺* (7.1D921N), and *rrg-1⁺::his-3⁺* (12.1) strains were inoculated in the center of VM plates with no additions (–) or supplemented with 10 μ g/ml fludioxonil. Plates were incubated at 30°C in the dark for 28 h. (D) Strains indicated in C were inoculated onto SCM plates and incubated in constant light at 25°C for 6 d. At this time, half of each plate was inoculated with wild-type macroconidia (males) of opposite mating type, and incubation continued for an additional 3 d. Both sides of the plates were then photographed. Examples of protoperithecia and perithecia are indicated by the white arrows. (E) Analysis of OS-2 protein levels and phosphorylation in response to hyperosmotic conditions. Wild-type (74A), *rrg-1^{D921N}::his-3⁺* (7.3D921N), *rrg-1⁺::his-3⁺* (12.2), and *os-2* (FGSC 2239) strains were grown for 16 h in submerged VM cultures. The cultures were brought to 0, 0.1, or 0.8 M NaCl and incubated with shaking for the last 10 min of growth. The cells were collected, whole cell extracts were prepared and then subjected to Western analysis (see *Materials and Methods*). Top, Western blot using anti-p38 that recognizes only phosphorylated OS-2. Bottom, Western blot using anti-Hog1p that reacts with all forms of the OS-2 protein. (F) Analysis of OS-2 protein levels and phosphorylation during fungicide exposure. Sixteen-hour submerged VM cultures of the strains indicated in E were treated with 0, 10, or 100 μ g/ml fludioxonil for the last 10 min of incubation. Whole cell extracts were prepared and subjected to Western analysis with the antibodies described in E.

the *rrg-1^{D921N}* mutant did not form ascospores until 15 d postfertilization. Similarly, the wild-type strain shot ascospores 14 d postfertilization, whereas the *rrg-1^{D921N}* mutants did not shoot ascospores until just after 30 d (data not shown). The observed delays in sexual development suggest that *rrg-1^{D921N}* may be involved in nitrogen sensing. Other pathways, absent in the $\Delta rrg-1$ gene replacement mutant, must activate down-

stream effectors allowing protoperithecial development in the *rrg-1^{D921N}* mutant. Analysis of OS-2 phosphorylation showed that the *rrg-1^{D921N}* mutant supports production of phospho-OS-2 in 3-d liquid and 6-d solid SCM cultures (data not shown), suggesting that phosphorylation of RRG-1 is not absolutely essential for activation of the downstream MAPK cascade during sexual development.

DISCUSSION

RRG-1 is required for conidial integrity, resistance to hyperosmotic conditions, sensitivity to phenylpyrrole and dicarboximide fungicides, and protoperithelial development in *Neurospora*. The wide range of functions described for RRG-1 is novel among fungal response regulators, but it is consistent with the expansion of histidine kinases in filamentous fungi along with conservation of the same number of response regulators as in yeast. It seems that the increased number of developmental pathways and cell types (28 cell types; Bistis *et al.*, 2003) in *Neurospora* versus yeast is also reflected in the greater number of phenotypes observed upon mutation of a single response regulator gene.

RRG-1 and Osmotolerance

The ability of fungi to respond to osmotic stress in their environment is pivotal to their survival. In *Neurospora*, the OS pathway is essential for osmotic stress responses. We found that *Δrrg-1* mutants share many phenotypes with *os* mutants. For example, macroconidia from *Δrrg-1* mutants develop a deep orange pigment and lyse easily and *Δrrg-1* strains grow poorly in high concentrations of NaCl, KCl, and sorbitol. We determined that *Δrrg-1* mutants were resistant to certain fungicides, a characteristic also associated with *os* mutants (Fujimura *et al.*, 2000a,b; Ochiai *et al.*, 2001; Zhang *et al.*, 2002). We uncovered evidence suggesting that RRG-1 is upstream of the OS-4/OS-5/OS-2 MAPK pathway, in that OS-2-phosphate levels were greatly reduced in *Δrrg-1* mutants relative to wild type. Previous results support OS-1/NIK-1 acting upstream of the OS-2 MAPK cascade with regard to hyperosmotic stress and fungicide resistance (Fujimura *et al.*, 2003). Because there is only one HPT protein, we expect that NIK-1/OS-1 and perhaps all of the 11 histidine kinases operate upstream of HPT-1 to regulate the response regulators RRG-1 and RRG-2 in *Neurospora* (Figure 7).

Similar to the yeasts *S. cerevisiae* and *S. pombe*, *Neurospora* possesses an osmosensing MAPK module that is regulated by an upstream two-component signaling pathway. Osmotolerance in *S. cerevisiae* is mediated through activation of the Hog1p MAPK, resulting in increased expression of the enzymes glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase (for review, see Gustin *et al.*, 1998). This leads to cytosolic glycerol accumulation, restoring the osmotic gradient between the cell and the environment. A striking difference between *Neurospora* and the two yeast is that both the *Δsln1* (hybrid histidine kinase) and *ssk1*^{D554N} (response regulator) alleles are lethal in *S. cerevisiae*, due to constitutive phosphorylation of the Hog1p MAPK and subsequent build up of glycerol, causing cell lysis (Maeda *et al.*, 1994). Conversely, the *S. pombe* Msc4 response regulator is not required for the response to osmotic stress (Shieh *et al.*, 1997). Deletion of *mcs4* prevents the phosphorylation of the Sty1 MAPK under all conditions (Shieh *et al.*, 1997). However, the *mcs4*^{D412N} allele supports phosphorylation of Sty1 in response to salt or heat stress but not in response to hydrogen peroxide (Buck *et al.*, 2001). This suggests that the conserved aspartate residue is required for certain functions (i.e., the oxidative stress response) but not others (i.e., hyperosmotic stress, heat, etc.) in *S. pombe*. A similar situation may exist for *Neurospora* RRG-1 with respect to hyperosmotic stress and fungicide resistance versus female fertility. Furthermore, the observation that the *rrg-1*^{D921N} and *Δrrg-1* alleles are not equivalent supports the hypothesis that this mutant allele possesses at least partial activity during signaling.

OS-2-phosphate levels are greatly reduced, but still detectable, in *Δrrg-1* mutants under hyperosmotic conditions (Fig-

Figure 7. Model for the action of RRG-1 in *Neurospora*. A possible mechanism that incorporates our experimental results is presented. The response regulator RRG-1 operates downstream of the hybrid histidine kinase NIK-1/OS-1 (and perhaps others) and the histidine phosphotransfer protein HPT-1. It is also possible that RRG-1 is controlled by as yet unknown signaling proteins. The phenotypes of strains carrying the predicted nonphosphorylatable allele D921N suggest that RRG-1 activity can be regulated independently of phosphorylation in some instances. RRG-1 regulates the OS-4(MAPKKK)/OS-5(MAPKK)/OS-2(MAPK) cascade to control osmotic and fungicide sensitivity and protoperithelial development. Production of POI-2, a protein required for protoperithelial development, is regulated by RRG-1 and the OS-2 MAPK pathway. The observation that *nik-1/os-1* mutants are able to produce protoperithelia and the POI-2 protein under nitrogen starvation suggests that other histidine kinases may contribute to regulation of RRG-1 and the OS-2 MAPK pathway during protoperithelial development. Other inputs may influence female fertility through the OS-2 MAPK cascade and the MAK-2 MAPK pathway has been previously shown to control protoperithelial development and POI-2 levels.

ure 4A). This suggests that some other pathway can activate OS-2 phosphorylation in the absence of the NIK-1/HPT-1/RRG-1 system. *Neurospora* contains a Sho1p homologue (NCU08067.3) that is predicted to have four transmembrane helices and a Src homology 3 domain (<http://www.broad.mit.edu/annotation/fungi/neurospora/>). It is possible that the *Neurospora* Sho1p homologue and/or another pathway using the response regulator RRG-2 mediates the residual osmotic response via the OS-2 MAPK cascade.

Homologues of RRG-1 and the OS-2 MAPK also exist in *A. nidulans* and the human pathogens *C. albicans* and *Cryptococcus neoformans*. *A. nidulans* contains a Hog1p/OS-2 family MAPK, SakA. *sakA* mutants are not sensitive to hyperosmotic conditions, but they undergo premature sexual development and produce asexual spores that are sensitive to oxidative and heat stress (Kawasaki *et al.*, 2002). The lack of an osmotic phenotype is of interest, because phosphorylation of SakA is induced by exposure to both 0.8 M KCl and 10 mM hydrogen peroxide. In *C. albicans*, deletion of *ssk1* (a RRG-1 homologue) leads to avirulence in a mouse model of hematogenously disseminated candidiasis (Du *et al.*, 2005)

and sensitivity to oxidative stress but only partial sensitivity to hyperosmotic stress (Chauhan *et al.*, 2003). Loss of *skn7*, another *C. albicans* response regulator, leads to sensitivity to hydrogen peroxide but not to hyperosmotic conditions (Singh *et al.*, 2004). *C. neoformans* *ssk1Δ* deletion mutants also exhibit sensitivity to UV irradiation and oxidative and osmotic stress (Bahn *et al.*, 2006). In contrast to *C. albicans*, mutation of the *C. neoformans* *skn7* gene leads to reduced virulence in a mouse inhalation model as well as sensitivity to oxidative stress (Wormley *et al.*, 2005; Bahn *et al.*, 2006). Thus, although a response regulator gene is necessary for pathogenesis in *C. albicans* and *C. neoformans*, there is currently no clear pattern for predicting whether the *ssk1* or *skn7* homologue will fulfill this requirement.

The *os-1*, *os-2*, *os-4*, and *os-5* mutants exhibit reduced glycerol accumulation under hyperosmotic stress conditions (Ellis *et al.*, 1991; Fujimura *et al.*, 2000b), which may explain why these mutants are unable to grow on hyperosmotic media. New evidence also suggests that, in addition to osmolyte regulation/accumulation, modulation of turgor pressure plays a role in this hyperosmotic adaptation. It has been shown that *os-1* and *os-2* mutants have lower turgor than wild-type *Neurospora* strains (Lew *et al.*, 2006), suggesting that ion regulation/accumulation also contributes to the phenotype seen in *os* mutants.

We have demonstrated that hyperosmotic sensitivity and fungicide-resistant phenotypes place RRG-1 upstream of the OS-2 MAPK cascade. However, when treated with high concentrations of salts, *Δrrg-1* mutants exhibit measurable, but severely reduced, levels of phosphorylated OS-2. In contrast, the *rrg-1^{D921N}* mutant produced slightly lower but comparable levels of phosphorylated OS-2 in response to treatment with fungicide or NaCl compared with wild type. This suggests that an alternate pathway, possibly involving an alternative site of RRG-1 phosphorylation and/or the other response regulator RRG-2, may be capable of activating OS-2 under hyperosmotic stress conditions.

RRG-1 and Sexual Development

We found that *rrg-1* mutants were unable to produce female reproductive structures during growth under nitrogen starvation and therefore were female sterile. In contrast, the *rrg-1^{D921N}* mutant is not female sterile, but instead exhibits delayed female fertility and ascospore development. This is the first such report in two-component signaling pathways of filamentous fungi. The conidial lysis phenotype of *Δrrg-1* strains coupled with the inability to produce protoperithecia may indicate that protoperithecial development is very closely tied to cell integrity. To further investigate this phenomenon, we measured levels of a candidate regulated gene, *poi-2*, in wild type versus *Δrrg-1* strains. We found that *poi-2* mRNA and protein levels were greatly reduced or absent in *rrg-1* mutants. It is known that *poi-2* expression is also controlled by the MAPK MAK-2 (Li *et al.*, 2005), which is dependent on the MAPKKK NRC-1 for phosphorylation (Pandey *et al.*, 2004). We found that *mak-2* and *rrg-1* mutants had greatly reduced levels of POI-2 under all conditions and that *os-2* mutants had lower amounts of POI-2 on low-nitrogen medium. This shows that RRG-1 regulates *poi-2*. Other genes affecting *Neurospora* sexual development, such as the peptide pheromones *mfa-1* and *cgg-4* (Bobrowicz *et al.*, 2002; Kim and Borkovich, 2006), were expressed at normal levels in *Δrrg-1* mutants (data not shown). Because *nik-1/os-1* mutants differentiate protoperithecia, whereas strains lacking *rrg-1* and the downstream *os* mutants do not, additional histidine kinase(s) likely regulates RRG-1 to direct protoperithecial development in *Neurospora*.

Interestingly, when *Δrrg-1* is deleted from the genome, the ability to induce protoperithecia in a nitrogen-limited environment is abolished; however, the *rrg-1^{D921N}* mutant produces protoperithecia, which can undergo fertilization and eventually shoot viable ascospores. The slowing of maturation of female reproductive structures in the *rrg-1^{D921N}* mutant suggests that phosphorylation of the conserved aspartate accelerates sexual development but is not essential for this process. The functions of RRG-1 during sexual development in *Neurospora* contrast with the known roles of two-component systems in the ascomycete yeast *S. pombe* and the basidiomycete yeast *C. neoformans*. *S. pombe* possesses three hybrid histidine kinases (Mak1-3p), one HPT protein and two response regulators (Mcs4 and Prr1). A triple *mak1 mak2 mak3* mutant mates inappropriately in high nitrogen and enters the cell cycle precociously, producing cells that are smaller in size than wild type (Nakamichi *et al.*, 2002). Of the two *S. pombe* response regulators, Mcs4 shares homology with *Neurospora* RRG-1, whereas Prr1 is most similar to RRG-2. Loss of either *mcs4* or *prr1* results in sexual sterility in *S. pombe*. Interestingly, deletion of the *ssk1* or *skn7* response regulator in *C. neoformans* results in enhanced mating ability during sexual reproduction (Bahn *et al.*, 2006).

The relatively slow sexual development and conidiation observed in *rrg-1^{D921N}* mutants relative to wild type suggests that the conserved aspartate residue, although not essential, is important for timely completion of these processes in *Neurospora*. RRG-1 may contain other unknown site(s) of phosphorylation that, if mutated, would eliminate the ability of RRG-1 to activate the OS-2 MAPK. Alternatively, RRG-1 may participate in a protein-protein complex with the presumed upstream protein HPT-1 and/or downstream MAPKKK OS-4; this interaction may be stronger when RRG-1 can be phosphorylated, leading to a higher rate of downstream MAPK activation.

We have shown that RRG-1 acts upstream of the OS-2 MAPK pathway with regards to hyperosmotic stress resistance, fungicide sensitivity, conidial integrity, and female fertility. Future investigations will reveal networking interactions that link upstream histidine kinases with the downstream response regulators and MAPK cascades or other effector pathways in *Neurospora*. Such studies will provide insight into the roles that two-component regulatory systems play during growth, development, and pathogenesis in filamentous fungi.

ACKNOWLEDGMENTS

We are indebted to Drs. Christopher Pitt and Hildur Colot for construction and testing of the *Ashbya* TEF promoter *hph* cassette. We thank Timothy Kirkpatrick for construction of plasmid pTJK1. We acknowledge the "Capture" protocols for yeast recombinational cloning from Dr. Christopher Raymond at the University of Washington. We thank Dr. Svetlana Krystofova for helpful discussions. We thank Dr. Lisa Alex for annotation of the *Neurospora* two-component regulatory system genes and for many helpful discussions. We are grateful to Drs. Frank Wong (University of California, Riverside) and Allison Tally (Syngenta) for providing us with the fungicides iprodione and fludioxonil.

REFERENCES

- Alex, L. A., Borkovich, K. A., and Simon, M. I. (1996). Hyphal development in *Neurospora crassa*: involvement of a two-component histidine kinase. *Proc. Natl. Acad. Sci. USA* 93, 3416–3421.
- Alex, L. A., Korch, C., Selitrennikoff, C. P., and Simon, M. I. (1998). COS1, a two-component histidine kinase that is involved in hyphal development in the opportunistic pathogen *Candida albicans*. *Proc. Natl. Acad. Sci. USA* 95, 7069–7073.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.

- Bahn, Y., Kojima, K., Cox, G., and Heirman, J. (2006). A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of *Cryptococcus neoformans*. *Mol. Biol. Cell* 17, 3122–3135.
- Baker, M. D., Wolanin, P. M., and Stock, J. B. (2006). Signal transduction in bacterial chemotaxis. *Bioessays* 28, 9–22.
- Bistis, G. N. (1981). Chemotropic interactions between trichogynes and conidia of opposite mating-type in *Neurospora crassa*. *Mycologia* 73, 959–975.
- Bistis, G. N. (1983). Evidence for diffusible, mating-type-specific trichogyne attractants in *Neurospora crassa*. *Exp. Mycol.* 7, 292–295.
- Bistis, G. N., Perkins, D. D., and Read, N. D. (2003). Cell types of *Neurospora crassa*. *Fungal Gen. Newsl.* 50, 17–19.
- Bobrowicz, P., Pawlak, R., Correa, A., Bell-Pedersen, D., and Ebbola, D. J. (2002). The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. *Mol. Microbiol.* 45, 795–804.
- Borkovich, K. A. et al. (2004). Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. *Microbiol. Mol. Biol. Rev.* 68, 1–108.
- Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulien, J., and Lindquist, S. (1989). Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell Biol.* 9, 3919–3930.
- Brown, J. L., Bussey, H., and Stewart, R. C. (1994). Yeast Skn7p functions in a eukaryotic two-component regulatory pathway. *EMBO J.* 13, 5186–5194.
- Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B. A., and Millar, J. B. (2001). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol. Biol. Cell* 12, 407–419.
- Case, M. E., Schweizer, M., Kushner, S. R., and Giles, N. H. (1979). Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. *Proc. Natl. Acad. Sci. USA* 76, 5259–5263.
- Chauhan, N., Inglis, D., Roman, E., Pla, J., Li, D., Calera, J. A., and Calderone, R. (2003). *Candida albicans* response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress. *Eukaryot. Cell* 2, 1018–1024.
- Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., and Dunlap, J. C. (2006). A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. USA* 103, 10352–10357.
- Davis, R. H., and deSerres, F. J. (1970). Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* 71A, 79–143.
- Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the *Spc1* stress-activated protein kinase in *Schizosaccharomyces pombe*. *Mol. Cell Biol.* 16, 2870–2877.
- Du, C., Calderone, R., Richert, J., and Li, D. (2005). Deletion of the *SSK1* response regulator gene in *Candida albicans* contributes to enhanced killing by human polymorphonuclear neutrophils. *Infect. Immun.* 73, 865–871.
- Ebbola, D., and Sachs, M. S. (1990). A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. *Fungal Genet. Newsl.* 37, 17–18.
- Ellis, S. W., Grindle, M., and Lewis, D. H. (1991). Effect of osmotic stress on yield and polyol content of dicarboximide-sensitive and -resistant strains of *Neurospora crassa*. *Mycol. Res.* 95, 457–464.
- Fujimura, M., Ochiai, N., Ichiishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2000a). Fungicide resistance and osmotic stress sensitivity in *os* mutants of *Neurospora crassa*. *Pestic. Biochem. Physiol.* 67, 125–133.
- Fujimura, M., Ochiai, N., Ichiishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2000b). Sensitivity to phenylpyrrole fungicides and abnormal glycerol accumulation in *os* and *cut* mutant strains of *Neurospora crassa*. *J. Pestic. Sci.* 25, 31–36.
- Fujimura, M., Ochiai, N., Oshima, M., and Motoyama, T. (2003). Putative Homologs of SSK22 MAPKK Kinase and PBS2 MAPK Kinase of *Saccharomyces cerevisiae* Encoded by *os-4* and *os-5* Genes for Osmotic Sensitivity and Fungicide Resistance in *Neurospora crassa*. *Biosci. Biotechnol. Biochem.* 67, 186–191.
- Galagan, J. E. et al. (2003). The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* 422, 859–868.
- Geer, L. Y., Domrachev, M., Lipman, D. J., and Bryant, S. H. (2002). CDART: protein homology by domain architecture. *Genome Res.* 12, 1619–1923.
- Grindle, M., and Temple, W. (1982). Fungicide-resistance of *os* mutants of *Neurospora crassa*. *Neurospora Newsl.* 29, 16–17.
- Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 62, 1264–1300.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580.
- Ivey, F. D., Hodge, P. N., Turner, G. E., and Borkovich, K. A. (1996). The *Gai* homologue *gna-1* controls multiple differentiation pathways in *Neurospora crassa*. *Mol. Biol. Cell* 7, 1283–1297.
- Kawasaki, L., Sanchez, O., Shiozaki, K., and Aguirre, J. (2002). SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. *Mol. Microbiol.* 45, 1153–1163.
- Kim, H., and Borkovich, K. A. (2004). A pheromone receptor gene, *pre-1*, is essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa*. *Mol. Microbiol.* 52, 1781–1798.
- Kim, H., and Borkovich, K. A. (2006). Pheromones are essential for male fertility and sufficient to direct chemotropic polarized growth of trichogynes during mating in *Neurospora crassa*. *Eukaryot. Cell* 5, 544–554.
- Kim, H., Metzberg, R. L., and Nelson, M. A. (2002). Multiple functions of *mfa-1*, a putative pheromone precursor gene of *Neurospora crassa*. *Eukaryot. Cell* 1, 987–999.
- Kim, H., and Nelson, M. A. (2005). Molecular and functional analyses of *poi-2*, a novel gene highly expressed in sexual and perithecial tissues of *Neurospora crassa*. *Eukaryot. Cell* 4, 900–910.
- Kojima, K., Takano, Y., Yoshimi, A., Tanaka, C., Kikuchi, T., and Okuno, T. (2004). Fungicide activity through activation of a fungal signaling pathway. *Mol. Microbiol.* 53, 1785–1796.
- Kothe, G. O., and Free, S. J. (1998). The isolation and characterization of *nrc-1* and *nrc-2*, two genes encoding protein kinases that control growth and development in *Neurospora crassa*. *Genetics* 149, 117–130.
- Krystofova, S., and Borkovich, K. A. (2005). The heterotrimeric G-protein subunits GNG-1 and GNB-1 form a G $\beta\gamma$ dimer required for normal female fertility, asexual development, and galactose protein levels in *Neurospora crassa*. *Eukaryot. Cell* 4, 365–378.
- Lew, R. R., Levina, N. N., Shabala, L., Anderca, M. I., and Shabala, S. N. (2006). Role of a mitogen-activated protein kinase cascade in ion flux-mediated turgor regulation in fungi. *Eukaryot. Cell* 5, 480–487.
- Li, D., Bobrowicz, P., Wilkinson, H. H., and Ebbola, D. J. (2005). A mitogen-activated protein kinase pathway essential for mating and contributing to vegetative growth in *Neurospora crassa*. *Genetics* 170, 1091–1104.
- Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* 369, 242–245.
- Margolin, B. S., Freitag, M., and Selker, E. (1997). Improved plasmid for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. *Fungal Gen. Newsl.* 44, 34–36.
- Morgan, B. A., Banks, G. R., Toone, W. M., Raitt, D., Kuge, S., and Johnston, L. H. (1997). The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. *EMBO J.* 16, 1035–1044.
- Morgan, B. A., Bouquin, N., Merrill, G. F., and Johnston, L. H. (1995). A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in budding yeast has homology to bacterial signal transduction proteins. *EMBO J.* 14, 5679–5689.
- Motoyama, T., Ohira, T., Kadokura, K., Ichiishi, A., Fujimura, M., Yamaguchi, I., and Kudo, T. (2005). An *Os-1* family histidine kinase from a filamentous fungus confers fungicide-sensitivity to yeast. *Curr. Genet.* 47, 298–306.
- Nakamichi, N., Yamada, H., Aoyama, K., Ohmiya, R., Aiba, H., and Mizuno, T. (2002). His-to-Asp phosphorelay circuitry for regulation of sexual development in *Schizosaccharomyces pombe*. *Biosci. Biotechnol. Biochem.* 66, 2663–2672.
- Ochiai, N., Fujimura, M., Motoyama, T., Ichiishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2001). Characterization of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the *os-1* mutants of *Neurospora crassa*. *Pest. Manag. Sci.* 57, 437–442.
- Orbach, M. J., Sachs, M. S., and Yanofsky, C. (1990). The *Neurospora crassa arg-2* locus. Structure and expression of the gene encoding the small subunit of arginine-specific carbamoyl phosphate synthetase. *J. Biol. Chem.* 265, 10981–10987.
- Pall, M. L. (1993). The use of Ignite (Basta;glufosinate;phosphinothricin) to select transformants of *bar*-containing plasmids in *Neurospora crassa*. *Fungal Gen. Newsl.* 40, 58.

- Pall, M. L., and Brunelli, J. P. (1993). A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. *Fungal Gen. Newsl.* 40, 59–62.
- Pandey, A., Roca, M. G., Read, N. D., and Glass, N. L. (2004). Role of a mitogen-activated protein kinase pathway during conidial germination and hyphal fusion in *Neurospora crassa*. *Eukaryot. Cell* 3, 348–358.
- Perkins, D. D., Radford, A., Newmeyer, D., and Bjorkman, M. (1982). Chromosomal loci of *Neurospora crassa*. *Microbiol. Rev.* 46, 426–570.
- Perkins, D. D., Radford, A., and Sachs, M. S. (2001). *The Neurospora Compendium: Chromosomal Loci*, San Diego, CA: Academic Press.
- Posas, F., and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* 276, 1702–1705.
- Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E. A., Thai, T. C., and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 “two-component” osmosensor. *Cell* 86, 865–875.
- Raju, N. B. (1992). Genetic control of the sexual cycle in *Neurospora*. *Mycol. Res.* 96, 241–262.
- Raymond, C. K., Pownder, T. A., and Sexson, S. L. (1999). General method for plasmid construction using homologous recombination. *Biotechniques* 26, 134–138, 140–141.
- Sachs, M. S., and Yanofsky, C. (1991). Developmental expression of genes involved in conidiation and amino acid biosynthesis in *Neurospora crassa*. *Dev. Biol.* 148, 117–128.
- Saito, H., and Tatebayashi, K. (2004). Regulation of the osmoregulatory HOG MAPK cascade in yeast. *J. Biochem.* 136, 267–272.
- Schumacher, M. M., Enderlin, C. S., and Selitrennikoff, C. P. (1997). The *osmotic-1* locus of *Neurospora crassa* encodes a putative histidine kinase similar to osmosensors of bacteria and yeast. *Curr. Microbiol.* 34, 340–347.
- Selitrennikoff, C., Lilley, B., and Zucker, R. (1981). Formation and regeneration of protoplasts derived from a temperature-sensitive osmotic strain of *Neurospora crassa*. *Exp. Mycol.* 5, 155–161.
- Selitrennikoff, C. P., Alex, L., Miller, T. K., Clemons, K. V., Simon, M. I., and Stevens, D. A. (2001). COS-1, a putative two-component histidine kinase of *Candida albicans*, is an *in vivo* virulence factor. *Med. Mycol.* 39, 69–74.
- Shieh, J. C., Wilkinson, M. G., Buck, V., Morgan, B. A., Makino, K., and Millar, J. B. (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. *Genes Dev.* 11, 1008–1022.
- Singh, P., Chauhan, N., Ghosh, A., Dixon, F., and Calderone, R. (2004). SKN7 of *Candida albicans*: mutant construction and phenotype analysis. *Infect. Immun.* 72, 2390–2394.
- Springer, M. L. (1993). Genetic control of fungal differentiation: the three sporulation pathways of *Neurospora crassa*. *Bioessays* 15, 365–374.
- Staben, C., Jensen, B., Singer, M., Pollock, J., Schechtman, M., Kinsey, J., and Selker, E. (1989). Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. *Fungal Genet. Newsl.* 36, 79–81.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Vann, D. C. (1995). Electroporation-based transformation of freshly harvested conidia of *Neurospora crassa*. *Fungal Gen. Newsl.* 42A, 53.
- Vogel, H. J. (1964). Distribution of lysine pathways among fungi: evolutionary implications. *Am. Nat.* 98, 435–446.
- West, A. H., and Stock, A. M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem. Sci.* 26, 369–376.
- Westergaard, M., and Mitchell, H. K. (1947). *Neurospora V*. A synthetic medium favoring sexual reproduction. *Am. J. Bot.* 34, 573–577.
- Wolanin, P. M., Thomason, P. A., and Stock, J. B. (2002). Histidine protein kinases: key signal transducers outside the animal kingdom. *Genome Biol.* 3, REVIEWS3013.
- Wormley, F. L., Jr., Heinrich, G., Miller, J. L., Perfect, J. R., and Cox, G. M. (2005). Identification and characterization of an SKN7 homologue in *Cryptococcus neoformans*. *Infect. Immun.* 73, 5022–5030.
- Yoshimi, A., Kojima, K., Takano, Y., and Tanaka, C. (2005). Group III histidine kinase is a positive regulator of Hog1-type mitogen-activated protein kinase in filamentous fungi. *Eukaryot. Cell* 4, 1820–1828.
- Zhang, Y., Lamm, R., Pillonel, C., Lam, S., and Xu, J.-R. (2002). Osmoregulation and fungicide resistance: the *Neurospora crassa os-2* gene encodes a HOG1 mitogen-activated protein kinase homologue. *Appl. Environ. Microbiol.* 68, 532–538.