Involvement of a putative response regulator FgRrg-1 in osmotic stress response, fungicide resistance and virulence in *Fusarium graminearum*

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SUMMARY

Response regulator (RR) proteins are core elements of the high-osmolarity glycerol (HOG) pathway, which plays an important role in the adaptation of fungi to a variety of environmental stresses. In this study, we constructed deletion mutants of two putative RR genes, *FgRRG-1* and *FgRRG-2*, which are orthologues of *Neurospora crassa* *RRG-1* and *RRG-2*, respectively. The *FgRRG-1* deletion mutant (Δ FgRrg1-6) showed increased sensitivity to osmotic stress mediated by NaCl, KCl, sorbitol or glucose, and to metal cations Li^+ , Ca^{2+} and Mg^{2+} . The mutant, however, was more resistant than the parent isolate to dicarboximide and phenylpyrrole fungicides. Inoculation tests showed that the mutant exhibited decreased virulence on wheat heads. Quantitative real-time polymerase chain reaction assays indicated that the expression of *FgOS-2*, the putative downstream gene of *FgRRG-1*, was decreased significantly in Δ FgRrg1-6. All of the defects were restored by genetic complementation of Δ FgRrg1-6 with the wild-type *FgRRG-1* gene. Different from the *FgRRG-1* deletion mutant, *FgRRG-2* deletion mutants were morphologically indistinguishable from the wild-type progenitor in virulence and in sensitivity to the dicarboximide fungicide iprodione and osmotic stresses. These results indicate that the RR FgRrg-1 of *F. graminearum* is involved in the osmotic stress response, pathogenicity and sensitivity to dicarboximide and phenylpyrrole fungicides and metal cations.

INTRODUCTION

The high-osmolarity glycerol (HOG) pathway regulates cellular responses to a variety of environmental stresses in eukaryotes (Stock *et al.*, 2000). To date, the HOG pathway in *Saccharomyces cerevisiae* has been well characterized, and consists of a sensor histidine kinase Sln1, a phosphotransfer (HPT) protein Ypd1, two response regulators (RR) Ssk1 and Skn7, and the downstream mitogen-activated protein kinase (MAPK) cascades (Hohmann,

2002). Under nonosmotic stress conditions, Sln1 is active and phosphoryl groups are shuttled through Ypd1 to Ssk1, therefore maintaining the RR protein in a phosphorylated state (Posas *et al.*, 1996). Under hyperosmotic stress conditions, Sln1 is inactive and the accumulation of unphosphorylated Ssk1 activates downstream MAPK cascades (Ssk2/Ssk22-Pbs2-Hog1) by sequential phosphorylation (Krantz *et al.*, 2006; Posas *et al.*, 1996). Phosphorylation and dephosphorylation of Ssk1 therefore function as an on/off switch in controlling the activity of downstream MAPK cascades (Horie *et al.*, 2008; Posas and Saito, 1998).

Similar to observations in *S. cerevisiae*, the HOG pathway plays an important role in the process of osmotic adaptation in many filamentous fungi, including *Neurospora crassa*, *Aspergillus nidulans* and *Fusarium graminearum* (Furukawa *et al.*, 2005; Jones *et al.*, 2007; Ochiai *et al.*, 2007; Zhang *et al.*, 2002). In the model filamentous fungus *N. crassa*, components of the HOG pathway include an osmosensor histidine kinase Os-1, a histidine phosphotransfer protein Hpt-1, two RRs Rrg-1 and Rrg-2, and downstream MAPK cascades Os-4, Os-5 and Os-2, which are homologous to *S. cerevisiae* Ssk2/Ssk22, Pbs2 and Hog1, respectively (Banno *et al.*, 2007). In contrast with the unique histidine kinase Sln1 in *S. cerevisiae*, 11 histidine kinases (including Os-1) have been identified in *N. crassa* (Catlett *et al.*, 2003). In addition to the response to osmotic stress, the HOG pathway is involved in regulating fungicide resistance, morphogenesis and virulence in filamentous fungi (Rispaill *et al.*, 2009).

Fusarium head blight (FHB), caused primarily by *F. graminearum*, is a devastating disease of cereal crops worldwide (Starkey *et al.*, 2007). Although yield loss caused by the disease is a major concern, mycotoxins produced in infected grains pose a serious threat to human and animal health. One of the recent concerns is whether *F. graminearum* can adapt to environmental stresses, particularly fungicide applications, and result in a population shift that may lead to more severe diseases and mycotoxin contamination.

A genome-wide search for the HOG pathway-related genes in *F. graminearum* revealed that the fungus possesses many putative HOG elements, including one osmosensor histidine kinase FgOs-1 and 15 other histidine kinases, one histidine

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phosphotransfer protein FgHpt-1, two RRs FgRrg-1 and FgRrg-2, and the downstream MAPK cascades (FgOs-4, FgOs-5 and FgOs-2). To date, however, information on the functions of the HOG pathway in *F. graminearum* is very limited, and the only study was conducted by Ochiai *et al.* (2007). They found that null mutation of *FgOs-1*, *FgOs-2*, *FgOs-4* or *FgOs-5* led to increased resistance to iprodione and fludioxonil. As expected, all mutants exhibited increased sensitivity to osmotic stress. Interestingly, the *FgOs-1* deletion mutant produced a reduced level of aurofusarin, and exhibited no change in its ability to produce trichothecenes. In contrast, all other null mutants of *FgOs-2*, *FgOs-4* and *FgOs-5* showed markedly enhanced pigmentation and failed to produce trichothecenes in aerial hyphae.

Several previous reports have described that RR proteins in different filamentous fungi show distinct functions. In *Cochliobolus heterostrophus*, both RR proteins ChSsk1 and ChSkn7 contribute to osmoadaptation and sensitivity to the phenylpyrrole fungicide fludioxonil (Izumitsu *et al.*, 2007). In *Candida lusitanae*, Ssk1 is involved in osmotolerance and pseudohyphal development, whereas Skn7 appears to be crucial for oxidative stress adaptation (Ruprich-Robert *et al.*, 2008). In *N. crassa*, the RR proteins perform distinct functions. RRG-1 controls vegetative cell integrity, hyperosmotic sensitivity, fungicide resistance and protoperithecial development (Jones *et al.*, 2007), whereas RRG-2 participates only in the oxidative stress response (Banno *et al.*, 2007). In the rice blast fungus *Magnaporthe oryzae*, in addition to adaptation to osmotic stress and fludioxonil fungicide, RR proteins are involved in pathogenicity (Motoyama *et al.*, 2008). To date, the functions of RR proteins in *F. graminearum* remain unidentified.

The key aim of this study was to characterize the functional role of two RR proteins in *F. graminearum*. Previous reports and our preliminary *in silico* analysis led us to hypothesize that the RR proteins of the HOG pathway in *F. graminearum* are involved in the stress response. We were particularly interested in determining how RR proteins mediate fungicide resistance. Here, we tested this hypothesis by generating null mutants of *FgRRG-1* and *FgRRG-2*, which encode putative RR proteins, by a gene replacement strategy, and applying a variety of stress factors on the mutants.

RESULTS

Sequence analysis of *FgRRG-1* and *FgRRG-2*

We searched the *F. graminearum* genome with amino acid sequences of *N. crassa* RRs Rrg-1 and Rrg-2 (Jones *et al.*, 2007) as queries using BLASTP, and subsequently identified nucleotide sequences of *FgRRG-1* and *FgRRG-2*. *FgRRG-1*, which is 2497 bp in length, is predicted to have two introns, and encodes a 784-amino-acid protein. *FgRRG-2* is 2083 bp in length with six

introns, and encodes a 587-amino-acid protein. The locations of the introns in the *FgRRG-1* and *FgRRG-2* genes were verified by sequencing respective 2355-bp and 1764-bp cDNAs. We confirmed that both genes were transcribed in *F. graminearum* mycelium by reverse transcription-polymerase chain reaction (RT-PCR) (data not shown).

Phylogenetic analysis with a neighbour-joining method using Mega 4.1 software (Kumar *et al.*, 2008) showed that *F. graminearum* FgRrg-1 and FgRrg-2 were homologous to RR proteins in other fungi (Fig. S1, see Supporting Information). The predicted amino acid sequences of FgRrg-1 and FgRrg-2 shared 51% and 47% identity with Rrg-1 (GenBank accession number NCU01895.4) and Rrg-2 (NCU02413.4) of *N. crassa*, respectively. The CDART (conserved domain architecture retrieval tool) program (Geer *et al.*, 2002) predicted a conserved RR receiver domain in both FgRrg-1 and FgRrg-2 (Fig. S2, see Supporting Information).

Disruption of *FgRRG-1* and *FgRRG-2* genes in *F. graminearum*

To investigate the function of the two RRs FgRrg-1 and FgRrg-2, we generated gene deletion mutants using a homology recombination strategy (Fig. 1A). For *FgRRG-1*, eight deletion mutants were identified from 12 hygromycin-resistant transformants by PCR analysis with the primer pair R15 + R16 (Table 1). The primer pair amplified 1752- and 1102-bp fragments from *FgRRG-1* deletion mutants and the wild-type progenitor PH-1, respectively. Both amplicons were detected in ectopic transformants. When probed with a 1008-bp upstream DNA fragment of *FgRRG-1*, the deletion mutant Δ FgRrg1-6 had an anticipated 2.6-kb band, but lacked a 4.3-kb band which was present in PH-1 (Fig. 1B). This Southern hybridization pattern confirmed that the transformant Δ FgRrg1-6 is a null mutant resulting from a single homologous recombination event at the *FgRRG-1* locus. The complemented strain Δ FgRrg1-6C harbours a single copy of wild-type *FgRRG-1* inserted into the genome of the disruption mutant Δ FgRrg1-6 (Fig. 1B).

For *FgRRG-2*, six deletion mutants were identified from 11 hygromycin-resistant transformants by PCR analysis (data not shown). Southern analysis of two transformants (Δ FgRrg2-2 and Δ FgRrg2-10) further confirmed that *FgRRG-2* was disrupted in these transformants (data not shown).

Morphology of *FgRRG-1* and *FgRRG-2* deletion mutants

The Δ FgRrg1-6 strain was not significantly different from the wild-type progenitor PH-1 in mycelial growth rate on both potato dextrose agar (PDA) and minimal medium (MM) plates, but showed slightly enhanced pigmentation on PDA (data not

Fig. 1 (A) Schematic representation of the *FgRRG-1* disruption strategy. *FgRRG-1* and the hygromycin resistance cassette (*HPH*) are denoted by large black and grey arrows, respectively. The annealing sites of primers R11, R12, R13, R14, R15 and R16 are indicated by small black arrows (see Table 1 for the primer sequences). (B) A 1008-bp upstream fragment of *FgRRG-1* was used as a probe in Southern blot hybridization analysis. Genomic DNA preparations of the wild-type PH-1, the *FgRRG-1* deletion mutant Δ FgRrg1-6 and the complemented strain Δ FgRrg1-6C were digested with *Nco*I.

Table 1 Oligonucleotide primers used in this study.

Primer code	Primer	Sequence (5'–3')*	Relevant characteristics
1	Frg1-F1	ATGGAGATGGAGATCGTTCAT	A pair of polymerase chain reaction (PCR) primers for amplification of the full cDNA sequence of the <i>FgRRG-1</i> gene
2	Frg1-R1	TCAGCCAGGCTTGGTCAT	
3	R11	ATctcgagTAGTTCTAGCGACCAGCACCA	A pair of PCR primers for amplification of the 661-bp <i>FgRRG-1</i> upstream fragment for construction of the gene deletion vector
4	R12	ATgtcgacTATCATATCGCGGACGTCGT	
5	R13	ATAagcttGGCTTTCTGTTAAGCGCCTTAA	A pair of PCR primers for amplification of the 610-bp <i>FgRRG-1</i> downstream fragment for construction of the gene deletion vector
6	R14	ATctcgagTGGTCATGGAACTCGATTCT	
7	R15	ACAACCTCCTACATACGCGCC	A pair of PCR primers for identification of <i>FgRRG-1</i> deletion mutants
8	R16	GGGAGCTGAATATCCATCAA	
9	R1-com-F	ATggtaccTGGGCGCAAAGTCAGGTT	A pair of PCR primers to amplify the full <i>FgRRG-1</i> including 1704-bp upstream and 472-bp downstream fragments
10	R1-com-R	ATccgggTCATGCGAGAGATAGCGACCAA	
11	R1-probe-F	ATGGAGATGGAGATCGTTCAT	PCR primers to amplify the 1008-bp <i>FgRRG-1</i> fragment used as the probe for Southern blot analysis
12	R1-probe-R	CGTCGTCAACAAGGTCATCTT	
13	Frg2-F1	ATGTCTGCCGGAGATGGAA	PCR primers for amplification of full cDNA sequence of the <i>FgRRG-2</i> gene
14	Frg2-R1	CATAGGTACGCTGTCGCTTTT	
15	R21	ATctcgagAAAGATGGCGACACCTTTGT	PCR primers to amplify the 644-bp <i>FgRRG-2</i> upstream fragment for construction of the gene deletion vector
16	R22	ATgtcgacAATCATCGCTGCCATCTAGAG	
17	R23	ATAagcttTAAGCCATTACCAAGAGCG	PCR primers to amplify the 631-bp <i>FgRRG-2</i> downstream fragment for construction of the deletion vector
18	R24	ATggatcTTAAAGGGTCCGATTCCAA	
19	R25	AAACCGTCTCCTTGTAAACGA	PCR primers for identification of <i>FgRRG-2</i> deletion transformants
20	R26	TGGGAGGGTTCATGTAACCTG	
21	neo-F	ATctcgagGGAGGTCAACATCAATGCT	PCR primers for amplification of neomycin resistance gene
22	neo-R	ATggtaccTCAGAAGAACTCGTCAAGAA	
23	FgOs2-F	TTGTCAAGTCGCTACCCAAG	PCR primers for amplification of the partial <i>FgOS-2</i> gene in quantitative real-time PCR assays
24	FgOs2-R	ATGTATCAACAGGCAGATCGG	
25	Tri5-F	TCACCCAGGAAACCTACACT	PCR primers for amplification of the partial <i>TRI5</i> gene in quantitative real-time PCR assays
26	Tri5-R	ACGTTTGCCAGTTGTCAA	
27	Tri12-F	TTCCACAGTCATCTTTCCCA	PCR primers for amplification of the partial <i>TRI12</i> gene in quantitative real-time PCR assays
28	Tri12-R	TCAAGTACGTCCTTATCCGCT	
29	actin-F	ATCCACGTCAACACTTTCAA	PCR primers for amplification of the reference actin gene in quantitative real-time PCR assays
30	actin-R	TGCTTGGAGATCCACATTG	

*The respective restriction enzyme sites included in the primers are listed in lowercase letters in the sequences.

Fig. 2 Comparisons of conidiation in the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C after 4 days of incubation in mung bean liquid medium. Bars with the same letter indicate no significant statistical difference ($P < 0.05$).

shown). The mutant showed a significant decrease in conidiation in mung bean liquid (MBL) medium (Fig. 2), although the conidia of the mutant were morphologically indistinguishable from those of the wild-type progenitor.

The *FgRRG-2* deletion mutant, Δ FgRrg2-2, exhibited enhanced pigmentation on PDA. The mutant also showed increased sensitivity to oxidative stress generated by H_2O_2 , but was indistinguishable from PH-1 with regard to the growth rate under other various stress conditions (Fig. S3, see Supporting Information), sporulation and virulence (data not shown).

Resistance of the *FgRRG-1* deletion mutant to phenylpyrrole and dicarboximide fungicides, and to osmotic and metal cation stresses

As phenylpyrrole and dicarboximide fungicides have been shown to activate the HOG pathway in several fungal pathogens (Kojima *et al.*, 2004), we tested the sensitivity of the *FgRRG-1* deletion mutant to the phenylpyrrole fungicide fludioxonil and the dicarboximide fungicide iprodione. As shown in Fig. 3, Δ FgRrg1-6 was highly resistant to both fungicides and was able to grow very well on PDA amended with 0.5 μ g/mL fludioxonil or 10 μ g/mL iprodione, whereas the complemented strain Δ FgRrg1-6C and wild-type PH-1 were sensitive to these two fungicides. In addition, we observed that PH-1 was unable to grow on PDA containing 0.5 μ g/mL fludioxonil, but was still able to grow on 10 μ g/mL iprodione. This indicates that fludioxonil is more effective than iprodione in suppressing wild-type *F. graminearum*. When the fungal strains were tested for their sensitivity to other fungicides, PH-1, Δ FgRrg1-6 and Δ FgRrg1-6C exhibited similar sensitivity to the benzimidazole fungicide carbendazim and the sterol demethylation inhibitors tebuconazole and triadimefon (Fig. 3). The results indicate that Δ FgRrg1-6 is resistant only to phenylpyrrole and dicarboximide fungicides, but not to other groups of fungicide.

To investigate the role of FgRrg-1 in osmoadaptation, Δ FgRrg1-6 and PH-1 were incubated on PDA amended with 1.2 M NaCl, 1.2 M KCl, 1 M sorbitol or 1 M glucose to generate osmotic stress. As shown in Fig. 4, Δ FgRrg1-6 was far more sensitive than PH-1 and Δ FgRrg1-6C to osmotic stress. Sensitivity to oxidative stress tests showed that, similar to Δ FgRrg2-2, Δ FgRrg1-6 exhibited slightly increased sensitivity to oxidative stress generated by 15 mM H_2O_2 in PDA medium (Fig. 4).

Previous studies have shown that the HOG pathway is involved in the regulation of the sensitivity of the Gram-negative bacterium *Pseudomonas aeruginosa* to several metal cations, including copper, zinc and cadmium (Caille *et al.*, 2007; Perron *et al.*, 2004), but a similar phenomenon has not been reported in fungi to date. In this study, when we tested the sensitivity of Δ FgRrg1-6 to metal cations, we were surprised to find that the mutant was much more sensitive than PH-1 and Δ FgRrg1-6C to LiCl, CaCl₂ and MgCl₂, but not to MnCl₂ and ZnCl₂ (Fig. 5).

A previous study has shown that the expression of *Os-2*, a putative downstream gene of *RRG-1*, is up-regulated by fungicide and osmotic stresses in *Botrytis cinerea* (Segmüller *et al.*, 2007). Therefore, to verify whether FgRrg-1 regulates the sensitivity of *F. graminearum* to osmotic stress and fungicide resistance through an *Os-2* orthologue, we evaluated the expression of *FgOS-2* in Δ FgRrg1-6 and PH-1 under various stress conditions. As shown in Fig. 6, in all chemical treatments except 0.2 M LiCl, the expression of *FgOS-2* in PH-1 was significantly higher than that in Δ FgRrg1-6, suggesting that FgRrg-1 plays an important role in the appropriate transcriptional activation of *FgOS-2* in *F. graminearum*.

Requirement for FgRrg-1 in full virulence of *F. graminearum*

The virulence of Δ FgRrg1-6 was evaluated by point inoculation of conidial suspension on wheat heads. Twenty days after inoculation with Δ FgRrg1-6, scab symptoms were observed only in the point-inoculated spikelets or adjacent one to two spikelets (Fig. 7). Similarly, when point inoculated with mycelia of Δ FgRrg1-6, scab symptoms were observed in the inoculated or adjacent one to two spikelets (data not shown). Under the same conditions, however, scab symptoms developed in more than 90% of spikelets when wheat heads were point inoculated with the wild-type PH-1 or the complemented strain Δ FgRrg1-6C.

Involvement of FgRrg-1 in the regulation of deoxynivalenol (DON) biosynthesis and expression of the *TRI5* and *TRI12* genes

Several studies have shown that DON is an important virulence factor in *F. graminearum* (Desjardins *et al.*, 1996; Proctor *et al.*, 1995; Seong *et al.*, 2009). Because *FgRRG-1* gene deletion

Fig. 3 Sensitivity of the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C to different fungicides. Comparison was made on potato dextrose agar (PDA) plates amended with each fungicide at the concentrations indicated in the figure.

Fig. 4 Sensitivity of the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C to osmotic stresses generated by sorbitol, glucose, NaCl and KCl. Comparison was made on potato dextrose agar (PDA) plates amended with each osmotic stress agent at the concentration indicated in the figure.

mutants showed reduced virulence, we compared the amount of DON produced by PH-1 and Δ FgRrg1-6. As shown in Fig. 8A, the amount of DON produced by Δ FgRrg1-6 was about six- to seven-fold less than that of the wild-type progenitor or the complemented strain.

To further confirm the finding that FgRrg-1 affects DON biosynthesis, we assayed the expression of the trichodiene synthase gene *TRI5* by quantitative real-time PCR. RNA

samples were isolated from mycelia grown in GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) for 2 days. The expression level of *TRI5* in the mutant Δ FgRrg1-6 decreased by approximately seven-fold in comparison with that of PH-1 or Δ FgRrg1-6C (Fig. 8B). Similar to *TRI5*, in Δ FgRrg1-6, the expression of *TRI12*, which encodes a trichothecene efflux pump, was significantly lower than that in PH-1 (Fig. 8B).

Fig. 5 Sensitivity of the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C to different metal cations. Comparison was made on minimal medium (MM) plates amended with each metal cation at the concentration indicated in the figure.

Fig. 6 Comparison of *FgOS-2* expression in the wild-type strain PH-1 with that in the *FgRRG-1* deletion mutant Δ FgRrg1-6 under various stress conditions. The relative expression of *FgOS-2* in PH-1 or Δ FgRrg1-6 under fungicide, osmotic or metal cation stress conditions is the relative amount of *FgOS-2* mRNA in PH-1 without treatment. Bars denote standard errors from three repeated experiments.

DISCUSSION

The ability of fungi to adapt to stress is pivotal to their survival in the environment, and this adaptation ability is one of the key factors leading to population shifts or mutations that can give rise to more aggressive crop pathogens in an agricultural setting. The aim of our investigation was to functionally characterize the role of *F. graminearum* RR proteins in the stress response and pathogenicity. In this study, we learned that *FgRRG-1* deletion mutants share many phenotypes with *FgOS* mutants (Ochiai *et al.*, 2007). For example, both *FgRRG-1* and *FgOS* deletion mutants showed increased sensitivity to osmotic stress generated by NaCl, KCl and sorbitol. *FgRRG-1* mutants were highly resistant to phenylpyrrole and dicarboximide fungicides, a char-

acteristic also associated with *FgOS* mutants. Our results, combined with a previous report by Ochiai *et al.* (2007), suggest that the HOG pathway plays an essential role in osmoadaptation in *F. graminearum*.

Many fungi have more than one RR protein in the HOG pathway. In *N. crassa*, Rrg-1 controls hyperosmotic sensitivity and fungicide resistance (Jones *et al.*, 2007). Unlike Rrg-1, Rrg-2 is not involved in osmotic stress and fungicide resistance, but participates in the oxidative stress response (Banno *et al.*, 2007). Similar to RR proteins in *N. crassa*, *Candida lusitanae* Ssk1 is involved in osmotolerance and pseudohyphal development, whereas Skn7 serves a crucial role in oxidative stress adaptation (Ruprich-Robert *et al.*, 2008). A different scenario can be found in *M. oryzae*, where there are three RR proteins (MoSsk1,

Fig. 7 Virulence of the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C on wheat heads. Wheat heads were point inoculated with a conidial suspension of each strain, and infected wheat heads were examined 20 days after inoculation.

Fig. 8 Deoxynivalenol (DON) production and expression of *TRI5* and *TRI12*. (A) Amount of DON per gram of wheat kernels (μg/g) infected with the wild-type strain PH-1, *FgRRG-1* deletion mutant Δ FgRrg1-6 and complemented strain Δ FgRrg1-6C. (B) Expression levels of *TRI5* and *TRI12* were measured by quantitative real-time polymerase chain reaction (PCR). The relative expression of *TRI5* and *TRI12* in Δ FgRrg1-6 is the relative amount of *TRI5* and *TRI12* mRNA in PH-1, respectively. Bars denote standard errors from three repeated experiments.

MoSkn7 and MoRim15), but none of the three RR gene disruption mutants exhibit increased sensitivity to oxidative stresses (Motoyama *et al.*, 2008). Our study showed that both *FgRRG-1* and *FgRRG-2* *F. graminearum* mutants are more sensitive to oxidative stresses. A previous study showed that *FgOS-4*, *FgOS-5*

and *FgOS-2* mutants, but not the *FgOS-1* mutant, exhibited increased sensitivity to H₂O₂ and *tert*-butylhydroperoxide (Ochiai *et al.*, 2007). These results suggest that other histidine kinases, not FgOs-1, may mediate the oxidative response via downstream RR proteins and MAPKs in *F. graminearum* (Fig. 9).

FgRRG-1 mutants were more sensitive to LiCl, MgCl₂ and CaCl₂. To our knowledge, this is the first report of an RR protein being involved in the mediation of sensitivity to metal cations in a filamentous fungus. In *P. aeruginosa*, the expression of *czcR* (encoding an RR protein) and *czcS* sensor (encoding a sensor kinase) was strongly enhanced by zinc treatment, leading to the transcriptional activation of *czcCBA*, an RND-type efflux pump for metal cations (Caille *et al.*, 2007; Hassan *et al.*, 1999). In *S. cerevisiae*, tolerance to high salt is strongly related to the activity of an Na⁺-ATPase, Ena1, which pumps out toxic sodium and lithium cations from cells (Garcia-deblas *et al.*, 1993; Haro *et al.*, 1991). Under osmotic stress, activated Hog1 prevents the binding of the transcriptional inhibitor Sko1 to the cyclic AMP response element present in the *ENA1* promoter, which results in enhanced expression of the *ENA1* gene (Proft *et al.*, 2001). Currently, we know little about the relationships between osmotic stress and metal tolerance in *F. graminearum*, and whether the HOG pathway is associated with this mechanism. Further investigation is needed to determine how the HOG pathway components regulate *F. graminearum* adaptation to metal cations.

In this study, we found that *FgRRG-1* deletion mutants exhibited reduced virulence on wheat heads. In addition, we found that the *FgOS-2* mutant was compromised in its ability to infect wheat heads (J. Jiang and Z. Ma, unpublished data). These results indicate that the HOG pathway is involved in the pathogenicity of *F. graminearum*. Similar to *F. graminearum*, *Mycosphaerella graminicola* *HOG1* (homologous of *FgOS-2*) deletion mutants failed to infect wheat leaves (Mehrabi *et al.*, 2006). However, in contrast, *HOG1* deletion mutants of the rice pathogens *Magnaporthe grisea* and *Bipolaris oryzae* were fully pathogenic (Dixon *et al.*, 1999; Moriwaki *et al.*, 2006), which suggests that

Fig. 9 Proposed functions of the high-osmolarity glycerol (HOG) pathway in *Fusarium graminearum*. FgRrg-2 is only involved in adaptation to oxidative stress, whereas FgRrg-1 regulates the sensitivity of *F. graminearum* to fungicide, metal cation, oxidative and osmotic stresses. FgRrg-1 is also associated with the regulation of deoxynivalenol (DON) production and virulence in *F. graminearum*. In addition, the HOG pathway may influence aurofusarin production via unidentified inputs.

the role of the HOG pathway in regulating the virulence of phytopathogenic fungi varies significantly.

DON is an end product of the trichothecene biosynthetic pathway in *F. graminearum*, and the toxin has been identified as an important virulence factor (Desjardins *et al.*, 1996; Proctor *et al.*, 1995; Seong *et al.*, 2009). When the role of DON in pathogenesis was further investigated, Bai *et al.* (2002) found that DON production played a significant role in the spread of FHB within a spike, but was not necessary for initial infection by the fungus. In this study, we found that Δ FgRrg1-6 produced a significantly reduced level of DON. This result is consistent with the observation that Δ FgRrg1-6 causes scab symptoms only in inoculated or nearby spikelets. In addition, the decrease in DON production in *FgRRG-1* mutants further supports the previous finding that the HOG pathway is involved in the regulation of

trichothecene production in *F. graminearum* (Ochiai *et al.*, 2007).

Accumulating data have shown that the HOG pathway plays an important role in virulence, sexual and asexual development, and adaptation to various environmental stresses, which prompted our further investigation of the HOG pathway in *F. graminearum*. Comparative analyses have shown that *F. graminearum* shares many conserved components of the HOG pathway (Fig. 9). As the functional role of the HOG pathway varies significantly among different fungal species, it will be necessary to perform further molecular characterization of other components of the pathway, including FgHpt-1 and other histidine kinases, in *F. graminearum*. Detailed information on the HOG pathway in pathogenic fungi will be helpful in developing new antifungal compounds targeting this pathway, as it is absent in mammalian cells (Catlett *et al.*, 2003).

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Fusarium graminearum wild-type strain PH-1 and the transformants generated in this study were grown on PDA (200 g potato, 20 g dextrose, 20 g agar and 1 L water) or MM (10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 4 mM $(NH_4)_2SO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 9 μ M $FeSO_4$, 10 mM glucose and 1 L water, pH 6.9) for mycelial growth tests, and in MBL (40 g mung beans boiled in 1 L water for 20 min and filtered through cheesecloth) medium (Bai and Shaner, 1996) for sporulation analysis.

Sequence analysis of *FgRRG-1* and *FgRRG-2* genes

FgRrg-1 (*F. graminearum* genome accession number FGSG_08948.3) and FgRrg-2 (FGSG_06359.3) were originally identified through homology searches of the *F. graminearum* genome sequence (available at http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) using the BLASTP algorithm with the RRs RRG-1 and RRG-2 from *N. crassa* (Jones *et al.*, 2007) as queries. To verify the existence and size of the introns in *FgRRG-1* and *FgRRG-2*, RNA was extracted from mycelia of the wild-type PH-1 using TaKaRa RNAiso Reagent (TaKaRa Biotech. Co., Dalian, China) and employed for RT with a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, ON, Canada) according to the manufacturer's instructions. RT-PCR of *FgRRG-1* and *FgRRG-2* cDNAs was performed using the primer pairs Frg1-F1 + Frg1-R1 and Frg2-F1 + Frg2-R1 (Table 1), respectively. PCR amplifications were performed using the following parameters: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 53 °C for 40 s, extension at 72 °C for 2 min and a final extension at 72 °C for

10 min. The resulting PCR products were purified, cloned and sequenced.

Construction of replacement vectors for deletion of *FgRRG-1* and *FgRRG-2*

The *FgRRG-1* gene disruption vector pCA-FgRrg1-Del was constructed by inserting two flanking sequences of the *FgRRG-1* gene into two sides of the *hph* (hygromycin resistance) gene in the pBS-HPH1 vector (Liu *et al.*, 2007). The upstream flanking sequence fragment of *FgRRG-1* was amplified from PH-1 genomic DNA using the primers R11 + R12 (Table 1). The 661-bp fragment was inserted into the *XhoI*–*SalI* site of the pBS-HPH1 vector to generate a plasmid pBS-FgRrg1-up. Subsequently, a 610-bp downstream flanking sequence fragment of *FgRRG-1* was amplified from PH-1 genomic DNA using the primers R13 + R14 (Table 1) and was inserted into the *HindIII*–*PstI* site of the pBS-FgRrg1-up vector to generate a plasmid pBS-FgRrg1-UD. Finally, the 2775-bp fragment containing the FgRrg1-upstream-*hph*-FgRrg1-downstream cassette (Fig. 1A) was obtained by digestion of plasmid pBS-FgRrg1-UD with *XhoI* and *PstI*, and ligated into the *XhoI*–*PstI* site in pCAMBIA 1300 (CAMBIA, Canberra, Australia). The resulting *FgRRG-1* deletion vector pCA-FgRrg1-Del was transformed into *Agrobacterium tumefaciens* strain C₅₈C₁ by electroporation. Using the same strategy, a pCA-FgRrg2-Del vector was constructed for disruption of the *FgRRG-2* gene.

Transformation of *F. graminearum*

Agrobacterium tumefaciens-mediated fungal transformation was performed as described previously (Mullins *et al.*, 2001). Briefly, *A. tumefaciens* strain C₅₈C₁, containing an appropriate binary vector, was grown at 28 °C for 2 days in MM supplemented with kanamycin (100 µg/mL). *Agrobacterium tumefaciens* cells were diluted to an optical density at 600 nm (OD₆₀₀) of 0.15 in induction medium (IM) containing 200 µM acetosyringone (AS). The cells were grown for an additional 6 h before mixing with an equal volume of a fresh *F. graminearum* conidial suspension (1 × 10⁶ conidia/mL). A 200-µL sample of the mixture was sprayed onto a nylon membrane (3 × 3 cm²) (Millipore Co., Bedford, MA, USA) and plated on IM amended with 200 µM AS. After incubation at 20 °C for 2 days in the dark, the membrane was cut into small pieces (3 × 0.1 cm²) and transferred upside down on PDA plates supplemented with hygromycin B (100 µg/mL), as a selection agent for transformants, and cefotaxime (200 µM), to kill the *A. tumefaciens* cells. After 5–7 days, hygromycin-resistant colonies appeared, and individual transformants were transferred onto fresh PDA plates amended with hygromycin B at 100 µg/mL. For conidiation, 10 mycelial plugs (5 mm in diameter) of each transformant, taken from the periphery of a 3-day-old colony, were inoculated into a 50-mL flask

containing 10 mL of MBL medium. The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm). To create monoconidial transformants, a single germinated conidium from each transformant was transferred with a sterile needle to PDA containing hygromycin B (100 µg/mL) in a small Petri dish (60 × 15 mm²) for use in subsequent experiments.

Complementation of the *FgRRG-1* gene deletion mutant

The *FgRRG-1* deletion mutant (ΔFgRrg1-6) was complemented with the full-length *FgRRG-1* gene to confirm that the phenotype changes in the *FgRRG-1* deletion mutant were a result of disruption of the gene. The *FgRRG-1* complement plasmid pCA-FgRrg1-C was constructed using the backbone of pCAMBIA1300. First, a *XhoI*–*KpnI* *neo* cassette containing a *trpC* promoter was amplified from plasmid pBS-RP-Red-A8-NEO (Dong *et al.*, 2009) with primers neo-F + neo-R (Table 1), and cloned into the *XhoI*–*KpnI* site of pCAMBIA1300 to create plasmid pCA-*neo*. Then, a full-length *FgRRG-1* gene including a 1704-bp promoter region and a 472-bp terminator region was amplified from genomic DNA of wild-type PH-1 using the primers R1-com-F + R1-com-R (Table 1), and subsequently cloned into the *KpnI* and *SmaI* sites of pCA-*neo* to generate the complement plasmid pCA-FgRrg1-C. Before plasmid pCA-FgRrg1-C was transformed into *A. tumefaciens* strain C₅₈C₁, *FgRRG-1* in this plasmid was sequenced to ensure flawlessness of the sequence. Transformation of ΔFgRrg1-6 with the full-length *FgRRG-1* gene was conducted as described above, except that neomycin was used as a selection agent.

Mycelial growth and conidiation assays

Growth tests under different conditions were performed on PDA or MM plates supplemented with the following products: D-sorbitol (Shangon Co., Shanghai, China), glucose, NaCl, KCl, LiCl, CaCl₂, MgCl₂, MnCl₂, ZnCl₂, H₂O₂, iprodione (96.5% a.i., Heyi Agricultural Chemical Co., Ltd., Zhejiang, China), fludioxonil, carbendazim, triadimefon and tebuconazole, at the concentrations indicated in the figures. The fungicides were kindly provided by the Institute of Zhejiang Chemical Industry or the Institute for the Control of Agrochemicals, Ministry of Agriculture (ICAMA), Beijing, China. Each plate was inoculated with a 5-mm mycelial plug taken from the edge of a 3-day-old colony. Plates were incubated at 25 °C for 3 days in the dark, and then the colony diameter in each plate was measured and the original mycelial plug diameter (5 mm) was subtracted from each measurement. The percentage of mycelial radial growth inhibition (RGI) was calculated using the formula $RGI = [(C - N)/(C - 5)] \times 100$, where *C* is the colony diameter of the control and *N* is the colony diameter of the treatment. Each experiment was repeated three times.

For conidiation assays, 10 mycelial plugs (5 mm in diameter) of each strain, taken from the periphery of a 3-day-old colony, were inoculated in a 50-mL flask containing 10 mL of MBL medium. The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm). For each strain, the number of conidia in the broth was determined using a haemocytometer. The experiment was repeated three times.

Determination of *FgOS-2* expression

To extract total RNA, mycelia of PH-1 and $\Delta FgRrg1-6$ were inoculated into potato dextrose broth and cultured for 2 days at 25 °C in the dark. Before mycelia were harvested for RNA extraction, the culture was treated for 3 h with iprodione, fludioxonil, LiCl, CaCl₂, MgCl₂, NaCl or sorbitol at the concentrations indicated in Fig. 5. Total RNA was extracted from mycelia of each sample using the TaKaRa RNAiso Reagent, and 10 µg of each RNA sample was used for RT with a RevertAid H Minus First Strand cDNA Synthesis Kit employing the oligo(dT)₁₈ primer. Expression of *FgOS-2* was determined by quantitative real-time PCR with the primer pair *FgOs2-F* + *FgOs2-R*. RT-PCR amplifications were performed in a DNA Engine Opticons 4 System (MJ Research, Massachusetts, USA) using SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20-µL volume containing 10 µL of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1 µL of RT product and 0.5 µL of each primer (Table 1). There were two replicates for each sample. The quantitative real-time PCR amplifications were performed with the following parameters: an initial preheat at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 56 °C for 20 s, 72 °C for 20 s and 75 °C for 3 s in order to quantify the fluorescence at a temperature above the denaturation of primer dimers. Once the amplifications were completed, melting curves were obtained to identify the PCR products. For each sample, PCR amplifications with the primer pair *actin-F* + *actin-R* (Table 1) for the quantification of expression of the *actin* gene were performed as a reference. The experiment was repeated three times. The expression of *FgOS-2* in the parent isolate and $\Delta FgRrg1-6$ under fungicide, osmotic and cation stress conditions, relative to that in the parent isolate PH-1 without treatment, was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Pathogenicity assays on flowering wheat heads

After incubation in MBL medium for 4 days, conidia of each strain were collected by filtration through three layers of gauze and subsequently resuspended in sterile distilled water to a concentration of 1×10^5 conidia/mL. A 10-µL aliquot of conidial suspension was injected into a floret in the central section spikelet of single flowering wheat heads of susceptible cultivar Zimai12. There were 10 replicates for each strain. After inoculation, the plants were kept at 22 ± 2 °C and 100% humidity for

2 days, and then maintained in a glasshouse. Twenty days after inoculation, the infected spikelets in each inoculated wheat head were recorded. The experiment was repeated four times. Using a similar method, mycelia of each strain were also used for inoculation tests.

Analysis of DON production and expression level of *TRI5* and *TRI12*

A 30-g aliquot of healthy wheat kernels was sterilized and inoculated with 1 mL of spore suspension (10^6 spores/mL) of the wild-type strain PH-1, the complemented strain $\Delta FgRrg1-6C$ or $\Delta FgRrg1-6$. After incubation at 25 °C for 20 days, DON was extracted using a previously described protocol (Mirocha *et al.*, 1998). The extracts were purified with PuriTox^{SR} DON column TC-T200 (Trilogy Analytical Laboratory, Missouri, USA), and the amount of DON in each sample was determined using a Waters 1525 (Massachusetts, USA) high-performance liquid chromatography (HPLC) system. The experiment was repeated three times, and data were analysed using analysis of variance (SAS version 8.0; SAS Institute, Cary, NC, USA).

To determine the expression levels of *TRI5* and *TRI12*, mycelia of the parental strain PH-1 and $\Delta FgRrg1-6$ were inoculated into GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) and cultured for 2 days at 25 °C in the dark. Total RNA was extracted from the mycelia of each sample using TaKaRa RNAiso Reagent, and 10 µg of each RNA sample was used for RT with a RevertAid H Minus First Strand cDNA Synthesis Kit employing the oligo(dT)₁₈ primer. The expression of *TRI5* and *TRI12* was determined using a quantitative real-time PCR method as described above. The experiment was repeated three times.

Standard molecular methods

Fungal genomic DNA was isolated as described by McDonald and Martinez (1990). Plasmid DNA was isolated using a Plasmid Miniprep Purification Kit (BioDev Co., Beijing, China). Southern analysis of *FgRRG-1* in *F. graminearum* was performed using the 1008-bp *FgRRG-1* upstream fragment as a probe, which was amplified by the primer pair R1-probe-F + R1-probe-R (Table 1). The probe was labelled with digoxigenin (DIG) using a High Prime DNA Labelling and Detection Starter Kit II according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). DNA extracted from *F. graminearum* was digested with *NcoI* and used for Southern hybridization analysis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phylogenetic analysis of amino acid sequences of the putative response regulators from *Aspergillus nidulans* (*A. nid*

SskA and *A. nid* *SrrA*), *Fusarium graminearum* (*F. gra* *Rrg1* and *F. gra* *Rrg2*), *Neurospora crassa* (*N. cra* *Rrg1* and *N. cra* *Rrg2*) and *Saccharomyces cerevisiae* (*S. cer* *Ssk1* and *S. cer* *Skn7*). The tree shown was obtained by a neighbour-joining method using Mega 4.1 software.

Fig. S2 Alignments of (A) *Rrg-1* and (B) *Rrg-2* from *F. graminearum* (*F. gra*) with the related response regulators from other fungi, including *Aspergillus nidulans* (*A. nid*), *Neurospora crassa* (*N. cra*) and *Saccharomyces cerevisiae* (*S. cer*). Boxshade program was used to highlight identical (black shading) or similar (grey shading) amino acids. The receiver domain is underlined.

Fig. S3 Sensitivity of the wild-type strain PH-1 and *FgRRG-2* deletion mutant Δ FgRrg2-2 to different stresses. Comparison was made on PDA or MM plates amended with each compound at the concentration described in the figure.

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