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Mediation of fludioxonil fungicide activity and resistance through *Cochliobolus heterostrophus* G-protein and MAPK signaling pathways

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Summary. The heterotrimeric G proteins and mitogen-activated protein kinases (MAPKs) conserved signaling pathways are involved in the development, reproduction and pathogenicity in filamentous fungi. The two-component histidine kinase, known also as the *HOG* MAPK pathway, regulates a similar complex set of responses and is known to mediate the phenylpyrrole fludioxonil fungicide response in fungi. We used *Cochliobolus heterostrophus* mutant strains deficient in G-protein α (*cga1*) and/or β (*cgb1*) subunits or MAPK (*chk1*, *mps1* and *hog1*) to uncover their role in the mediation of this fungicide activity and resistance. The results revealed complex interactions between the G-protein subunits and the MAPK in response to osmotic/ionic and fludioxonil stresses. Under normal conditions, the Hog1 pathway restricts glycerol accumulation since its disruption led to hyperosmosensitivity and very high cellular glycerol accumulation. Moreover, our results suggest that *cgb1*, *chk1* and *mps1* are also weak repressors of this response since mutation in these genes caused relatively high elevation in glycerol levels in the cells. Supporting this is the finding that these three strains have resistance to KCl stress. In contrast, the *cga1* strain has only moderate levels of cellular glycerol (higher than those of the WT, but lower than those of the other mutants) that are little affected by KCl stress. Indeed, these mutants are highly sensitive to KCl stress. This suggests that Cga1 is a moderate repressor of cellular glycerol under normal conditions and an enhancer of glycerol accumulation under osmotic/ionic stress conditions. This study provides insight into the roles of G-protein in mediating the anti-fungal fludioxonil response. A model is proposed for the interactions between the G-protein and MAPK signaling pathways.

Keywords: *Bipolaris maydis*; *Cochliobolus*; fludioxonil fungicide; G-protein; MAPK; osmotic sensitivity

INTRODUCTION

The appearance of fungicide resistance is a key factor in the limitation of the efficacy and lifetime of disease control strategies. However, the growing basic understanding at the molecular level of signaling mechanisms for sensing the environment can now be used to develop new strategies in controlling plant diseases (Steffens et al. 1996). Such biotechnology tools are especially important for protecting crops against pathogens and preventing epidemics. *Cochliobolus heterostrophus* (anamorph; *Bipolaris maydis*), the agent of Southern Corn Leaf Blight, is an excellent model for this purpose (Gafur et al. 1998; Kojima et al. 2006; Yoshimi et al. 2003).

Signal transduction pathways in filamentous fungi. Phytopathogenic fungi depend on signaling cascades in order to recognize their plant host. These intermediate pathways include signaling such as G-protein-coding genes, mitogen-activated protein (MAP) kinase genes and cyclic AMP-dependent protein kinase genes (Lengeler et al. 2000). They function coordinately to regulate mating, development and virulence. Mitogen-activated protein kinase (MAPK) modules are known targets of Gβγ dimers in yeast and mammalian cells (Crespo et al. 1994; Gudermann 2001; Leeuw et al. 1998), and most likely in filamentous fungi as well based on the interpretation of common phenotypes (Lee et al. 2003; Nishimura et al. 2003).

In *C. heterostrophus*, G-protein Gα (*cga1*) and Gβ (*cgb1*) subunits as well as MAPK (*chk1*) participate in several developmental pathways, as summarized in Degani (2013, 2014). Disruption of these components led to severe developmental, reproduction and virulence defects. Loss of the Gβ subunit *cgb1* or the MAPK *chk1, mps1* and *hog1* also led to severe loss of virulence (Ganem et al. 2004; Igbaria et al. 2008; Lev et al. 1999). In contrast, mutants deleted for *cga1* are able to cause wild-type symptoms in maize (Horwitz et al. 1999), but

cga1 is required for full virulence depending on host physiology (Degani 2014; Degani et al. 2004).

The regulation of MAPK in some eukaryotes is often controlled by two-component signaling systems (Bourret et al. 1991; Parkinson and Kofoid 1992; Alex and Simon 1994; Stock et al. 2000). A typical two-component system consists of a histidine kinase sensor that is regulated by environmental stimuli and a regulatory protein referred to as a response regulator (for review, see Smith et al. 2010). In fungi and other eukaryotes, these two proteins are typically fused (Chang et al. 1993; Ota and Varshavsky 1993; Alex et al. 1996), and the hybrid histidine kinases often regulate a MAPK pathway that ultimately regulates gene expression (Kieber et al. 1993; Maeda et al. 1994; Fassler et al. 1997). Recent lines of evidence in *C. heterostrophus*, *Candida albicans* and *Neurospora crassa* indicate that some filamentous fungi use several histidine kinases to detect environmental changes and transmit signals via several signaling pathways (Calera et al. 1998; Liu et al. 2007; Yoshimi et al. 2004).

Fungicide activity through interference with fungal signaling pathways. Three groups of fungicides are effective against filamentous fungi: phenylpyrrole (fludioxonil and fenpiclonil are fungicides derived from the antibiotic pyrrolnitrin (Gehmann et al. 1990)), dicarboximides (e.g., iprodione, procymidone, vinclozolin) and aromatic hydrocarbons (chloroneb, dicloran, quintozene). Some of these fungicides affect their target fungi by interfering with cellular signaling in fungi. Quinoxyfen [5,7-Dichlor-4-(p-fluorphenoxy)chinolin, CAS no. 124495-18-7] is a relatively novel protectant fungicide that controls powdery mildew diseases. It has been shown (Wheeler et al. 2003) that quinoxyfen alters the accumulation of transcripts of protein kinase C and the catalytic subunit of cAMP-dependent protein kinase (PKA) genes. Another anti-fungal agent, Fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl) pyrrole-3-

carbonitrile, CAS no.131341-86-1], is a phenylpyrrole antifungal drug derived from the antibiotic pyrrolnitrin (Gehmann et al. 1990); it has a broad antifungal spectrum and is now used to control a variety of important plant-pathogenic fungi. It is a unique antifungal drug that targets signal transduction.

It was previously demonstrated that antifungal fludioxonil interferes with osmosensing regulation controlled by the two-component histidine kinases. In yeast, the Sln1 protein (a transmembrane histidine kinase receptor) acts as an osmosensor through the negative regulation of the high osmolarity glycerol (HOG) MAP kinase signaling pathway (Hohmann 2009). In response to osmotic stress, Sln1 controls the induction of a range of osmotic responses in *S. cerevisiae*, including increased glycerol synthesis. In *N. crassa*, Os-2, Os-4 and Os-5 mediate signaling downstream from Os-1 (the Sln1 homolog), and are required for sensitivity to osmotic stress; however, unlike in yeast, they are also activated by fungicides (phenylpyrrole, dicarboximides and aromatic hydrocarbons) (Fujimura et al. 2003). Thus, it seems that under "normal" growth conditions, pathway activity is modulated by the osmosensor (Sln1 or Os1) to maintain a low level of glycerol through negative regulation of glycerol synthesis enzymes (Gustin et al. 1998). Addition of an osmoticum triggers a response in the osmosensor, which stimulates the dephosphorylation of the histidine kinase (*sln1*) and activation of the cascade leading to (HOG) MAP kinase signaling pathway activation and to glycerol synthesis, and thus an increase in the cellular osmotic potential of the cell to counteract the increased external osmotic pressure (Motoyama et al. 2005).

In *C. heterostrophus*, *Dic1*, a gene for two-component histidine kinase, was cloned and characterized (Yoshimi et al. 2004). *Dic1* mutants were resistant to dicarboximide and phenylpyrrole fungicides, and sensitive to osmotic stress. Another study (Izumitsu et al. 2007) showed that the disruption of the response regulator genes Ch*Ssk1* and Ch*Skn7* in *C. heterostrophus* led to little sensitivity to high-osmolarity stress and moderate resistance to the

iprodione/fludioxonil fungicides. The phosphorylation of Hog1-type MAPK BmHog1p induced by high-osmolarity stress and fungicide treatments was regulated only by ChSsk1p, indicating that ChSkn7p plays a role in high-osmolarity adaptation and fungicide sensitivity, which are independent of BmHog1p activation.

Despite these achievements in understanding the role of the MAPK cascade and in mediating fungicide action and resistance, the role of other signaling pathways remains obscure. It has been reported in *N. crassa* that activity of a cAMP and calcium-independent protein kinase was inhibited by phenylpyrroles (Pillonel and Meyer 1997). Ramesh et al. (2001) further reported that the cAMP signal transduction pathway is involved in the action of dicarboximide and aromatic hydrocarbon fungicides in the basidiomycete fungus *Ustilago maydis* (Ramesh et al. 2001). Although the *ADR1* gene, which encodes the major catalytic subunit of *U. maydis* cAMP-dependent protein kinase (PKA) (Durrenberger et al. 1998), was assumed to be responsible for resistance against vinclozolin (a dicarboximide fungicide) (Orth et al. 1995), PKA is not a direct target of inhibition by vinclozolin (Ramesh et al. 2001). The mechanism by which a protein kinase might confer resistance to vinclozolin was further revealed when Ramesh et al. (2001) showed that mutants with disruptions in the *ubc1* gene, which encodes the regulatory subunit of PKA, were resistant to both vinclozolin and chloroneb (Ramesh et al. 2001). They also found that mutants defective in *ubc1* display osmotic sensitivity, a property often associated with vinclozolin and chloroneb resistance in other fungi (Ramesh et al. 2001). Interestingly, the *rpk1* encoding a PKA regulatory subunit in *C. lagenarium* deletion strain showed significantly increased sensitivity to the fludioxonil fungicide (Kojima et al. 2004). These observations raise the question as to whether the dose G-protein signal cascade is involved in MAPK fludioxonil signal mediation.

This study focused on the response of *C. heterostrophus* wild-type (WT) and G-protein and MAPK signaling mutant strains to the fludioxonil fungicide. A detailed map of

the fungicide sensitivity of each mutant strain is important for identifying new roles of these components in mediating fungicidal response. Furthermore, crossing this new database with the osmotic stress response phenotype of each strain may point to common signaling pathways and target genes.

MATERIALS AND METHODS

In this study we used *C. heterostrophus* wild-type strains (C4 and C5) and G-protein and MAPK signaling deficiency strains to conduct a detailed study of their role of in mediating the response to hyperosmotic/ionic environments and the action of fludioxonil.

Strains. The strains used in this study are listed in Table 1.

Normal and osmotic stress culture conditions. Fungal strains were grown on solid standard complete medium (CM) (Turgeon et al. 1987) at room temperature (26-27°C) under continuous light from cool white fluorescent tubes (Philips, Eindhoven, The Netherlands). Media for the stress-response experiments were prepared by adding KCl (to 750 mM) or sorbitol (to 1.5 M) to CM prior to autoclaving. Mycelial disks (6 mm in diameter) were cut with a sterilized pipette from the margin of 6-7-day-old colonies, and each disk was placed upside down on a series of CM plates containing the osmotic stress-causing agent (KCl or sorbitol). Fungal growth rate was determined after six days of incubation under the above-mentioned conditions. Each assay was performed in five replications and in two independent experiments. Statistical analysis, conducted using Student's *t* test, verified whether differences were statistically significant ($P \leq 0.05$).

For liquid growth media cultures, six CM culture agar disks, 6 mm in diameter, were cut from the margins of 4-6-day-old colonies of each strain and transferred to a 50 ml

polycarbonate screw-capped test tube containing 20 ml of liquid CM (complete medium without agarose). The cultures were incubated diagonally for three days in a rotary shaker at 230 rpm and at a temperature of 30°C under continuous light from cool white fluorescent tubes. Mycelia were collected by centrifugation (10 min, 8,000 rpm) and briefly homogenized (20 s, Polytron, Brinkmann Instruments, Westbury, NY).

Fludioxonil stress sensitivity. The sensitivity of the WT and signaling mutants (Table 1) to fludioxonil was evaluated at the minimum mycelial growth inhibitory concentration (MIC). The MIC was determined using the plate dilution method (Yoshimi et al. 2003) using CM plates containing the fludioxonil fungicide (0.01-100 μg/ml, active ingredient) obtained from two manufacturers: Scholar (commercial name), 230 g/l, purchased from C.T.S. Ltd., Israel (manufacturer, Syngenta USA), and Celest (commercial name), 100 g/l, purchased from Milchan Bros. Ltd., Israel (manufacturer, Syngenta USA). These two formulas are used widely to protect crops against plant pathogenic fungi. Each analysis included an untreated control that contained the solvent used to dissolve the inhibitor. The effectiveness of the fungicide was determined six days after incubation under the above-mentioned conditions by plotting the percentage decrease in colony diameter against the log concentration of the chemicals. The experiment included two replications and was repeated twice. The minimum inhibitory concentration (MIC) of both fludioxonil drugs was defined as the lowest drug concentration in the medium producing a 100% decrease in growth rate compared with the control group. In a subsequent experiment, CM media plates containing 5 μg/ml fludioxonil (MIC active ingredient) from the two commercial sources (described above) were used to evaluate the WT and signal deficiency strains growth rate in the presence of the fungicide. Cultures were incubated under the same light and temperature conditions (described above) for 14 days. Each assay was performed in five replications in two independent experiments.

Statistical analysis, conducted using Student's *t* test, verified whether differences were statistically significant ($P \leq 0.05$).

Disk diffusion halo assay for spore germination and the initial hypha extension. This assay aimed at evaluating Cga1 pathway involvement in fludioxonil sensitivity. The recovery, germination and initial hypha extension of a massive amount of spores were examined under the suppressive effect of the antifungal drugs. This assay did not include the other mutant strains since they did not sporulate. To induce sporulation, cultures in a humid atmosphere on the CM surface were grown at 22-26°C under continuous light from cool white fluorescent tubes for 8-14 days. Spores were harvested by washing and scraping them off the agar surface with sterile deionized water. The spore suspension was adjusted to 78±22 spores per µl and used immediately. The spores' suspension was sown on the surface of 15 cm (in diameter) CM growth media plates. Immediately afterwards, Whatman filter paper disks (5 mm diameter) soaked with fludioxonil concentrations (active ingredient) of 0, 0.5, 0.05, $5x10^{-3}$ (MIC), $5x10^{-4}$ and $5x10^{-5}$ mg/ml were placed over the spores layer. Cultures were then incubated under the above conditions. The influence of fludioxonil fungicide on germinating conidia was determined after four days by qualitatively evaluating the halo area around the fludioxonil-soaked disk paper. The experiment was repeated twice.

Effect of fungicides on intracellular glycerol accumulation. It was previously reported that treatment with fludioxonil fungicides stimulates glycerol accumulation (a response mechanism for osmotic stresses) in many fungal strains (see Introduction). To measure the glycerol content of the cells, the strains of *C. heterostrophus* were cultured in liquid CM medium at 30°C for three days, as described above. One mg wet weight mycelia of each strain was then transferred into a new 15 ml polycarbonate screw-capped test tube containing fresh

CM medium with Scholar fludioxonil fungicide (at the MIC determined in the plate's sensitivity assay to 5 μg/ml) or KCl (750 mM). The tubes were incubated under chronic (16 h) stress conditions in a rotary shaker at 230 rpm and at a temperature of 30°C under continuous light from cool white fluorescent tubes. After incubation, the cells were collected by centrifugation (8,000 rpm) and washed twice with double-distilled water (addition of 10 ml double-distilled water, centrifugation and removing the supernatant); after adding 4 ml double-distilled water, they were extracted by extensive sonication followed by heating to 100°C for 5 min. Following additional centrifugation at 15,000 rpm (to remove cell debris), the tube was cooled to room temperature and the pH of each solution was adjusted to pH=8 with sodium hydroxide solution. The glycerol concentration in the supernatant was measured using the UV-glycerol assay kit (EnzytecTM fluid Glycerol kit N5360, Scil diagnostis, GmbH, Darmstadt, Germany) previously described (Fujimura et al. 2000b; Pillonel and Meyer 1997) in 340 nm (double-distilled water used as blank solution). The same samples were dried for 24 h at 85°C to measure their dry weight. Each treatment was conducted in duplicate. The experiment was repeated twice.

RESULTS

Osmotic and ionic stress sensitivity. During growth on maize leaves, *C. heterostrophus* is likely to be subject to various kinds of stress. We conducted a well-controlled comparison of all of our available G-protein and MAPK signaling mutant strains (Table 1) in order to identify specific and common roles in mediating osmotic and ionic environments. Since the *chk1*, *mps1*, *cgb1* and *cga1 cgb1* mutants do not sporulate, or produce only a few or non-viable spores, it was not possible to test the effect of osmotic and ionic shock on germinated spores. Instead, mycelium was grown on complete medium (CM) amended with 1.5 M sorbitol (hyperosmotic stress) or 0.75 M KCl (hyperosmotic and salt stress).

MAPK deficiency strains resistance to osmotic and ionic stress. Mps1 and Hog1 are closely related to MAPKs needed for cell integrity and stress resistance, respectively, in yeast and other fungi. To investigate this overlap of phenotypes and in order to compare all three MAPKs, a *chk1* strain was included in the following experiments. The results are shown in Figure 1A. Growth of all strains was inhibited by 0.75 M KCl ($p<0.005$), but *mps1* and *chk1* were more resistant to this combined ionic and osmotic stress than the WT ($p<0.005$). The *hog1* strain was severely affected by this stress ($p<0.0005$) and practically did not grow at all. This tendency was maintained on 1.5 M sorbitol (Fig. 1A). All strains showed decreased growth in comparison to normal growth conditions (CM), but this decrease was less pronounced than in the KCl medium for the WT and *mps1* strains.

G-protein subunits deficiency strains resistance to osmotic and ionic stress. Hypertonic stress (CM with KCl or sorbitol) caused growth reduction in all strains (Fig. 1B, $p<0.0005$). Surprisingly, *cgb1* exhibited less pronounced reduction in radial growth in KCl medium (in a similar decrease in growth to the *mps1* in Fig. 1A) while the WT and especially the *cga1* and double mutant *cga1 cgb1* strains were severely affected (Fig. 1B). Sorbitol containing medium caused a severe reduction in *cga1* strains radial growth ($p< 0.0001$), but all other strains (mainly WT) showed only moderate sensitivity to this stress in comparison to KCl (Fig. 1B). The *cga1* strains had a growth rate in both media very similar to *hog1* (Fig. 1B).

Fludioxonil stress sensitivity. To investigate whether *C. heterostrophus* is sensitive to fludioxonil, fungal growth was tested on CM agar containing the drug. Fludioxonil severely inhibited growth of the C4 wild-type (WT) strain in a dose-dependent manner (Fig. 2). The minimum inhibitory concentration at which growth of *C. heterostrophus* was completely inhibited (MIC) was > 5 μg/ml for both fludioxonil fungicides tested: Celect and Scholar. To

elucidate the role of G-protein and MAPK pathways in fludioxonil sensitivity, we tested the sensitivity of various signaling mutants that had been constructed before (Table 1). All strains, especially the two WT strains, were severely affected by the anti-fungal compound ($p<0.05$), as expected (according to Fig. 2), although the WT C5 strain (which is also the more abundant strain (Carson 1998; Leonard 1977) was less sensitive than the WT C4 strain (Fig. 3). All mutants exhibited significant ($p<0.05$) resistance to 5 μg/ml fludioxonil (in comparison to the WT strain from which they were created), indicating that both G-protein and MAPK pathways are involved in fludioxonil sensitivity of C. *heterostrophus* (Fig. 3). Interestingly, the double *cga1 cgb1* mutant showed the highest resistance to this antibiotic, implying a synergistic effect caused by the disruption of the two signaling components.

The ability of *cga1* strains to sporulate enabled us to evaluate the effect of fludioxonil fungicide on spore germination and initial hypha extension. A disk diffusion halo assay for spore germination was applied in order to identify spontaneous germination of numerous amounts of spore under this fungicidal stress. As shown in Figure 4, the *C5cga1* strain had slightly increased fludioxonil susceptibility in the Scholar treatment compared to the WT C5. This tendency was more noticeable in the Celest treatment (which is in agreement with the more pronounced effect of the Celest fungicide, as shown in Figs. 2 and 3). The WT C4 and *C4cga1* strains showed similar tendencies but with lower resistance to fludioxonil than the C5 strains (data not shown).

Cellular glycerol accumulation in response to KCl stress or fludioxonil. Since glycerol accumulation is required for a response to high osmolarity and is also closely related to the action of three groups of fungicides (phenylpyrroles, dicarboximides and aromatic hydrocarbons) (Fujimura et al. 2000b), we measured and compared the accumulation of glycerol between the wild-type strain and G-protein and MAPK mutant strains under high

osmolarity conditions and in the presence of Scholar fludioxonil (Fig. 5). The WT and mutant strains selected for this assay were all C4 in background (*MAT1-2*; *Tox1*$^{+}$) since these strains showed higher sensitivity or resistance responses than the C5 in background strains (*MAT1-1*; *Tox1*$^{-}$, Fig. 3). Initial glycerol levels in the WT C4 strain were 0.17 μg/ml. As expected, the WT C4 strain accumulated glycerol as compatible solutes in response to 750 mM KCl (24-fold increase) or 5 μg/ml fludioxonil fungicide (54-fold increase) chronic (16 h) stress conditions (reaching 4 and 9 μg/ml, respectively) (Fig. 5). All signaling mutant strains had basal high levels of cellular glycerol (measured on CM growth media), ranging from a 17-fold increase in the *C4cga1* strain (in comparison to the WT) to a 71-fold increase in the *hog1* (3 and 12 μg/ml, respectively) (Fig. 5). For comparison, both MAPK mutants, *chk1* and *mps1*, had initial glycerol levels (8.5 μg/ml, 51-fold higher than the WT on CM media) equivalent to the highest levels reached by the WT strain under fludioxonil stress. Thus, similar but less pronounced (approximately a two-fold increase under fludioxonil stress) tendencies were measured for the *C4cga1* and *cgb1*, and only a minor elevation (13-fold increase) in glycerol accumulation was measured for the *chk1* and *mps1* strains. Remarkably, *hog1* had high and almost constant levels of glycerol under all conditions tested. Chronic treatment by fludioxonil caused an evident reduction in glycerol level in the *hog1* strain (from 12 μg/ml to 7 μg/ml) (Fig. 5). This difference in glycerol accumulation may reflect the strains-specific level of resistance to the fungicide, as demonstrated for *N. crassa* (Fujimura et al. 2000a). Indeed, in the Scholar plate's sensitivity assay (Fig. 3), this strain had a weaker ability to grow compared to the other C4 in background signaling mutants.

Shared and distinct roles conferred by mutations in G-protein subunits and MAPK genes. The above results revealed complex interactions between G-protein subunits and the MAPK in response to osmotic/ionic and fludioxonil stresses (summarized in a model

presented in Fig. 6 based on findings presented in this study and from previous studies of *C. heterostrophus* (Degani 2013; Eliahu et al. 2007; Igbaria et al. 2008; Izumitsu et al. 2007; Kojima et al. 2004; Oide et al. 2010; Yoshimi et al. 2003)). Under normal conditions, the Hog1 pathway restricts glycerol accumulation since its disruption led to hyperosmosensitivity and very high cellular glycerol accumulation (Fig. 5). Moreover, our results suggest that *cgb1*, *chk1* and *mps1* are also weak repressors of this response (Fig. 6) since mutation in these genes caused a relatively high elevation in glycerol levels in the cells (Fig. 5). Supporting this is the finding that these three strains have resistance to KCl stress (i.e., they grew significantly faster than the WT under high osmolality conditions) (Fig. 1). In contrast, the *cga1* strain has only moderate levels of cellular glycerol (higher than those of the WT but lower than those of the other mutants), which are not affected by KCl stress (Fig. 5). Indeed, these mutants are highly sensitive to KCl stress (Fig. 1). This suggests that *cga1* is a moderate repressor of cellular glycerol under normal conditions and an enhancer of glycerol accumulation under osmotic/ionic stress conditions (Fig. 6). Other aspects that are included in this model and based on previous studies of *C. heterostrophus* will be discussed in detail below.

DISCUSSION

In this study, we examined the involvement of G-protein and MAPK signaling hierarchies in the phenylpyrrole drug fludioxonil's antifungal activity against the fungal pathogen *C. heterostrophus*. A growing number of reports indicate that resistance to dicarboximides and phenylpyrrole fungicides is often the result of mutations in histidine kinases regulating osmotic signal transmission (Cui et al. 2002; Dry et al. 2004; Ochiai et al. 2001; Oshima et al. 2002; Yoshimi et al. 2004). Yet, some different signaling pathways may also be involved. Studies on the role of G-protein signal transduction in the response to environmental stresses were reported for *Cryphonectria parasitica* (Segers and Nuss 2003),

N. crassa (Ivey et al. 1996; Yang and Borkovich 1999; Yang et al. 2002), *C. heterostrophus* (Degani 2013) and other fungi. Lines of evidence implying the involvement of the G-protein and MAPK signaling in osmoregulation are, like other growth phenotypes, species-specific. In this study, hypertonic stress (CM with KCl or sorbitol) caused growth reduction in all *C. heterostrophus* strains (Fig. 1). The more pronounced decreasing effect in the KCl medium for the WT and *mps1* strains suggests that the difference between the two media (CM with KCl or sorbitol) is caused by the ionic stress present only in the KCl treatment. Mutation in C. *heterostrophus cgb1* led to KCl high salt/osmotic stress resistance (Fig. 1B) that was not observed in *N. crassa* or *C. parasitica* (Ivey et al. 1996; Segers and Nuss 2003). MAPK deficiency mutants *chk1* and especially *mps1* were also relatively resistant to KCl stress, while the WT and moreover *cga1* strains were severely affected (Fig. 1A). Furthermore, unlike *N. crassa* and *C. parasitica* in which no different tendency between KCl and sorbitol treatment was observed (Ivey et al. 1996; Segers and Nuss 2003), the wild-type, *mps1* and *cga1 cgb1* double mutant strains showed only moderate sensitivity to sorbitol in comparison to KCl (Fig. 1), suggesting that ionic stress and not osmotic stress is the cause of the significant growth reduction of these strains in the KCl treatment.

Further investigation of the roles of these hierarchies in mediating fludioxonil response suggests an intricate relationship between the individual signaling components. Although the *C. heterostrophus* WT strains obtained after six backcrosses are nearly isogenic (Table 1), some variations occur in their phenotypic behavior measurement results that are not significant. These variations can most likely be attributed to experimental (measurement) variations (limits in accuracy) and differences in morphology, behavior and probably other aspects that exist between individual colonies of the same species (phenotypic variability). It is also possible that since Race O (WT C5) is considered to be the most common race (Carson 1998), it acquired some resistance to fludioxonil. This explanation is yet to be established.

The differences between disk diffusion halo assay for spore germination/initial hypha extension (Fig. 4) and the fungal growth experiment (Fig. 3) in which WT C5 resistance exceeded the *C5cga1* mutant strains implied that the antifungal compound fludioxonil in this strain mainly affects the hypha elongation stage with a lesser influence on spore germination.

The signaling pathways mediating fludioxonil and KCl effects on *C. heterostrophus* are summarized in the proposed model (Fig. 6). This model was built based on the findings presented in this study and in previous studies of *C. heterostrophus* (Degani 2013; Eliahu et al. 2007; Igbaria et al. 2008; Izumitsu et al. 2007; Kojima et al. 2004; Oide et al. 2010; Yoshimi et al. 2003). It appears that in *C. heterostrophus*, fludioxonil treatment activates the Hog1 by phosphorylation (Igbaria et al. 2008; Kojima et al. 2004) and the Ch*Ssk1*/Ch*Skn7* response regulators (Izumitsu et al. 2007), and causes a rapid excessive accumulation of intracellular glycerol, which may lead to cell swelling, cytokinesis defects and cell growth inhibition (Kojima et al. 2006). Supporting this conclusion is the osmosensitivity of the Hog1 disrupted mutants (Fig. 1), their constant high cellular glycerol levels (Fig. 5, and the induction of glycerol-3-phosphate dehydrogenase (Igbaria et al. 2008)) and their resistance to fludioxonil (Fig. 3) (Yoshimi et al. 2004). The results of this work revealed that the sensitivity to fludioxonil is not only positively controlled by the Hog1 pathway, but also negatively controlled by *chk1*, *mps1* (MAPK pathways), *cga1* and *cgb1* (G-protein pathways) (Fig. 6), which were previously shown to be involved in maintaining cell wall integrity and appressoria formation (Degani 2013; Igbaria et al. 2008). Similar phenotypic traits (resistance to phenylpyrroles and high osmolality sensitivity) were also found in other filamentous fungi, such as *N. crassa* (Fujimura et al. 2000b; Miller et al. 2002), *B. cinerea* (Cui et al. 2002), *Alternaria alternate* (Dry et al. 2004) and *Alternaria brassicicola* (Avenot et al. 2005).

Previous observations have shown a possible common mechanism for hyper osmotic cell integrity and melanin biosynthesis associated genes. Colonies of *mps1*, *chk1* and the

double *cga1 cgb1* deletion mutants have a white and autolytic appearance, which was partially rescued by a hyperosmotic environment (Degani 2013; Eliahu et al. 2007). Furthermore, genes involved in melanin biosynthesis were significantly induced in *chk1* and *mps1* mutants under hyperosmotic conditions compared to invariably high expression in the wild-type (Eliahu et al. 2007). Cell integrity may be related to glycerol accumulation as suggested by Kojima (2006) (Kojima et al. 2006) and supported by integrating the results presented here (Figs. 1, 5) with our previous work (Degani 2013). This glycerol accumulation may also disrupt the appressoria formation (de Jong et al. 1997b; Dixon et al. 1999), as will be discussed in detail below, resulting in the inability to infect the host plant (Fig. 6).

Indeed, in many cases, disturbance of the two-component histidine kinase signal transduction pathway by the three groups of filamentous fungi-specific fungicides leads to growth defects that are associated with abnormal glycerol synthesis (Fujimura et al. 2000a; Ochiai et al. 2001; Pillonel and Meyer 1997; Zhang et al. 2002). For example, in the presence of fludioxonil, the wild-type fungus *C. lagenarium* was not able to infect the host plant because of a failure of appressorium-mediated penetration, whereas *osc1* mutants (lack of the gene encodes a MAPK related to yeast Hog1) successfully infected plants (Kojima et al. 2004). Appressoria generate enormous turgor for penetrating the host tissue (Bechinger et al. 1999). *M. grisea* accumulates glycerol in appressoria and this glycerol accumulation is suggested to contribute to turgor generation (de Jong et al. 1997a). Activation of *M. grisea* Osm1, which is related to yeast Hog1 MAPK, induces the accumulation of arabitol but not glycerol under high osmotic stress (Dixon et al. 1999). Consistent with this, appressoria formed by *M. grisea osm1* mutants are functional, just like those formed by the *C. lagenarium osc1* mutants. It is possible that fungicide activation of Osc1 induces improper accumulation of polyols, such as arabitol, and therefore interferes with appressorium functionality.

To date, there are no reports on the role of G-protein cascade in the transmission of fungicide response, but the relationship of the G-protein and MAPK signaling hierarchies is gradually being revealed. A previous study of *S. cerevisiae* indicates that PKA promotes the exclusion of stress transcription factors Msn2p and Msn4p from the nucleus to negatively regulate the stress response (Gorner et al. 1998). The activity of these transcription factors is also influenced by osmotic stress in *S. cerevisiae*, suggesting a complex interplay between cAMP signaling and the Hog1-dependent pathway for responding to hyperosmolarity (Schuller et al. 1994). It has been reported recently that in the rice blast fungus *M. grisea*, an activated allele of the MAPKK belonging to the PMK1 (ortholog of *C. heterostrophus* Chk1) pathway decreases steady-state cAMP levels (Zhao et al. 2005). This finding supports the possibility of crosstalk between the G-protein and MAPK signaling cascades. The osmosensing mediation role of G-protein subunits and evidence of the active role for cAMP may hint to the possibility that G-protein is another mediator of fungicide response.

The findings presented in this study support the indirect evidence presented above and indicate a role for G-protein subunits in the mediation of fungicide responses. The crucial role of G-protein signaling hierarchies in the development of virulence and reproduction may explain why to date no field-resistant strains have been found with impaired G-protein associated elements. Thus, understanding the basis of resistance and the relevance of parallel signaling pathways such as PKA and the HOG-related signaling mechanism is significant for the practical control of plant diseases and the proper use of fungicides. The establishment of the G-protein-MAPK role as a possible mediator of fungicide responses may later help us improve existing anti-fungal agents and explore new possibilities of protecting crops against pathogens. New strategies may include enhancing the fungicidal effect by manipulating the signal transduction pathways and developing new antibiotics that act directly on the signaling mechanism.

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Table 1. Strains used in this study.

Strain	**Genotype**	**Reference or source**	**comments**
WT C4 (Race T)	*MAT1-2*; *Tox1*$^{+}$ ATCC 48331	(Leach et al. 1982)	Wild type strains, obtained after six backcrosses and are nearly isogenic.
WT C5 (Race O)	*MAT1-1*; *Tox1*$^{-}$ ATCC 42332	(Leach et al. 1982)	
C5cga1	*MAT1-1*; *Tox1*$^{-}$ ATCC 42332	(Horwitz et al. 1999)	G-protein α1 subunit disrupted mutant (WT C5 in background), created by insertion of the hygromycin cassette into the coding region, combined with an 18-bp deletion.
C4cga1	*MAT1-2*; *Tox1*$^{+}$ ATCC 48331 GFP	(Horwitz et al. 1999)	G-protein α1 subunit disrupted mutant (WT C4 in background), created by complete deletion of the coding region and insertion of hygromycin cassette.
cgb1	*MAT1-1*; *Tox1*$^{-}$ ATCC 42332	(Ganem et al. 2004)	G-protein β subunit disrupted mutant (WT C5 in background), created by insertion of the hygromycin cassette into the coding region, combined with a 473 bp deletion.
cga1 cgb1	*MAT1-1*; *Tox1*$^{-}$ ATCC 42332	(Degani 2013)	G-protein α and β subunits double mutant (WT C5 in background), created by insertion of the Bar cassette into the coding region, combined with a 473 bp deletion.
chk1	*MAT1-2*; *Tox1*$^{+}$ ATCC 48331	(Lev and Horwitz 2003)	MAPK disrupted mutant (WT C4 in background), created by replacement of the coding region with the hygromycin resistance cassette.
hog1	*MAT1-2*; *Tox1*$^{+}$ ATCC 48331	(Igbaria et al. 2008)	MAPK disrupted mutant (WT C4 in background), created by replacement of the coding region with the hygromycin resistance cassette.
mps1	*MAT1-2*; *Tox1*$^{+}$ ATCC 48331	(Igbaria et al. 2008)	MAPK disrupted mutant (WT C4 in background), created by replacement of the coding region with the hygromycin resistance cassette.

FIGURES

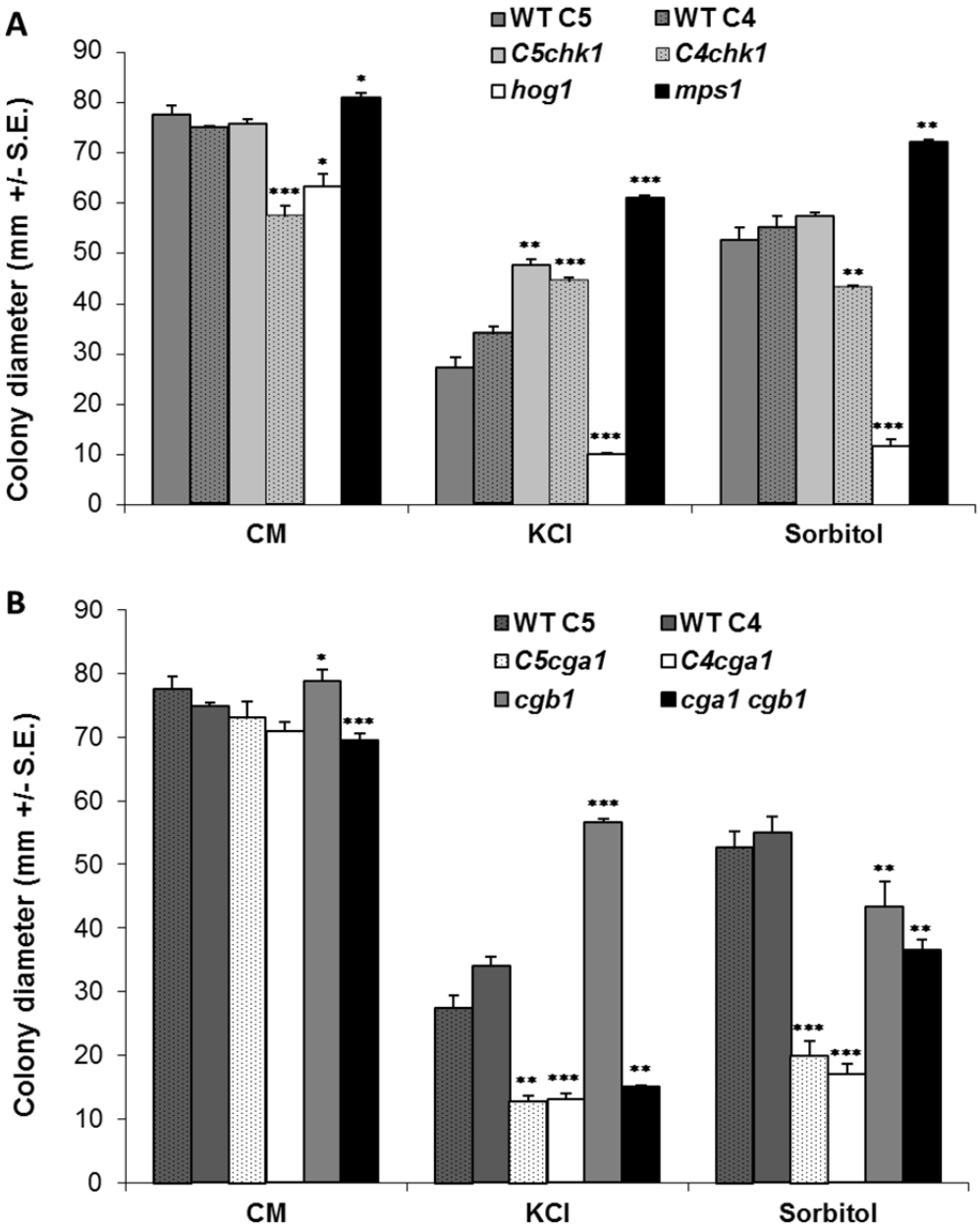

Fig. 1 *Cochliobolus heterostrophus* colonies' radial growth under conditions of osmotic and ionic stress. Wild type (WT C4, MAT1-2; Tox1+ and WT C5, MAT1-1; Tox1-) and mitogen-activated protein kinase (MAPK, A) or G-protein (B) deficiency strains. Mutant strains: chk1, hog1, mps1 (ΔMAPK), cga1 (ΔGα1), cgb1 (ΔGβ1) and the double mutant cga1 cgb1 (Table 1). All strains were grown for 6 days at 26–27°C under continuous white fluorescent light. CM, complete medium. KCl and sorbitol (final concentration of 0.75 and 1.5 M, respectively) were incorporated into complete medium to evaluate ionic and osmotic stress. Values represent the average of five replicates. Error bars represent standard error. Asterisks

represent significant (*P=0.05, **P=0.005, ***P<5*10-4) differences from the WT strain from which they were created under the same environmental conditions.

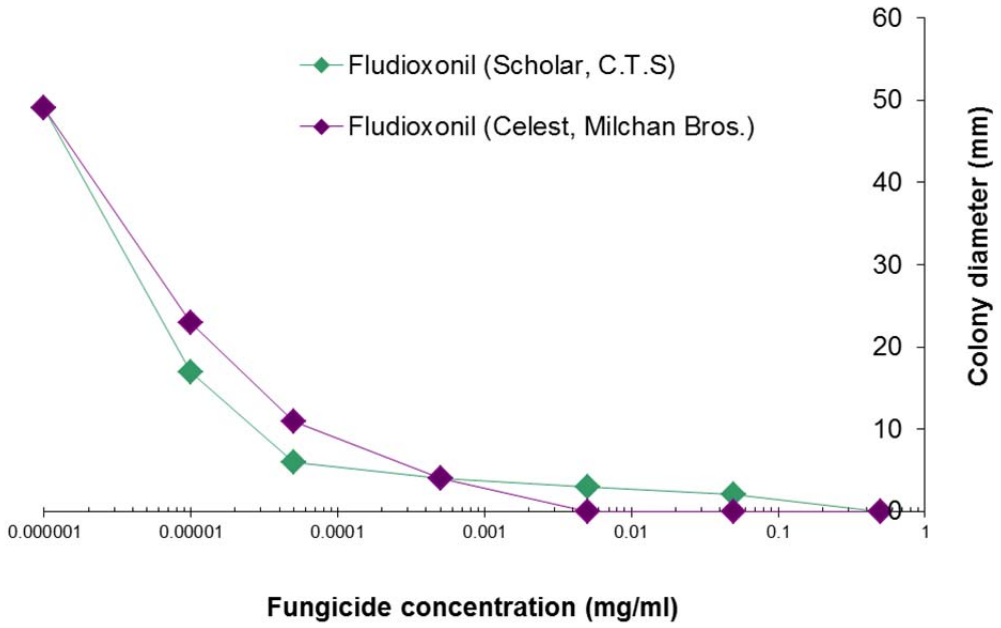

Fig. 2 Fludioxonil stress sensitivity. The sensitivity of the WT C5 to fludioxonil was evaluated by a series of complete medium (CM) plates containing the fungicide (1*10-6–0.5 mg ml-1 active ingredient) from two manufacturers: Scholar (commercial name), 230 g l-1, purchased from C.T.S. Ltd., Israel (manufacturer, Syngenta USA), and Celest (commercial name), 100 g l-1, purchased from Milchan Bros. Ltd., Israel (manufacturer, Syngenta USA). Each plate was inoculated by deposition of mycelial disk (6 mm in diameter) cut with a sterilized pipette from the margin of 6–7-day-old colonies. The cultures were incubated under the same conditions as in Figure 1 for 6 days. Each treatment was conducted in duplicate.

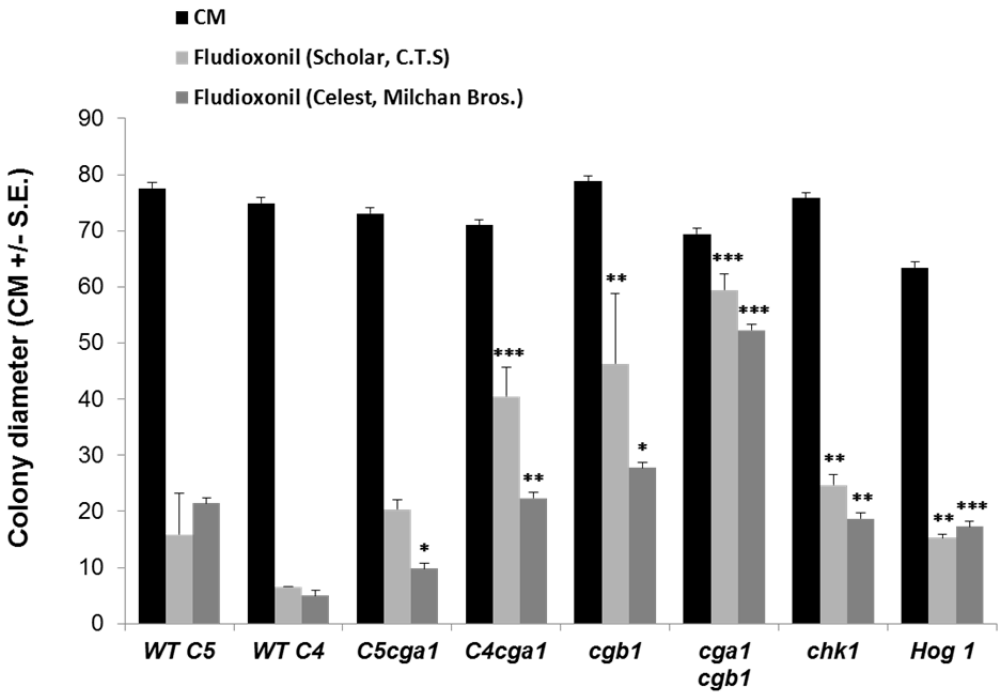

Fig. 3 G-protein and MAPK involvement in resistance to fludioxonil. Complete medium (CM) media plates containing 5 µg ml-1 fludioxonil from two commercial sources (described in Fig. 2) were used to evaluate the wild type (WT) and signal deficiency strains' growth rate. The cultures were incubated under the same conditions as in Figure 1 for 14 days. Abbreviations as in Figure 1. Values represent the average of five replicates. Bars indicate standard error. Asterisks represent significant (*P=0.05, **P=0.005, ***P<5*10-4) differences from the WT strain from which they were created in the same fungicide treatment. All strains, especially the WT strains, which grew on CM medium with fludioxonil, grew significantly (P<0.05) more slowly than in the control (CM without fungicide).

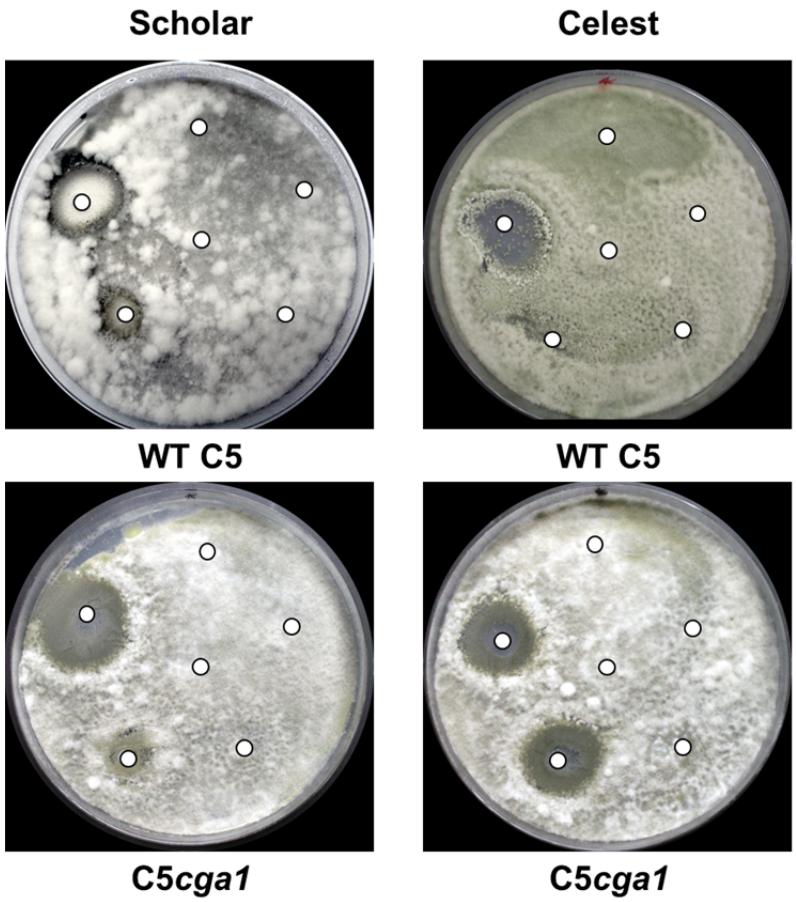

Fig. 4 Spore germination assay to evaluate cga1 pathway involvement in fludioxonil sensitivity. *Cochliobolus heterostrophus* strains, wild type (WT) C5 and C5cga1, were grown at 22–26°C for 8–14 days in complete medium (CM) under continuous light from cool white fluorescent tubes. Spore suspension at a concentration of 78±22 spores per µl was sown on the surface of 15-cm diameter CM growth media plates. Immediately afterwards, Whatman filter paper disks (5 mm diameter) soaked with the fludioxonil concentrations (active ingredient) of 0 (at the center of the plate) and 5x10-5, 5x10-4, 5x10-3, 0.05, 0.5 mg ml-1 (clockwise, starting from the upper pole of the plate), were placed over the spores layer. Cultures were then incubated for 4 days under the above conditions. The influence of fludioxonil fungicide on germinating conidia was determined after 4 days by qualitatively evaluating the inhibition of fungal growth in regions surrounding the disc paper producing a halo area. The experiment was repeated twice.

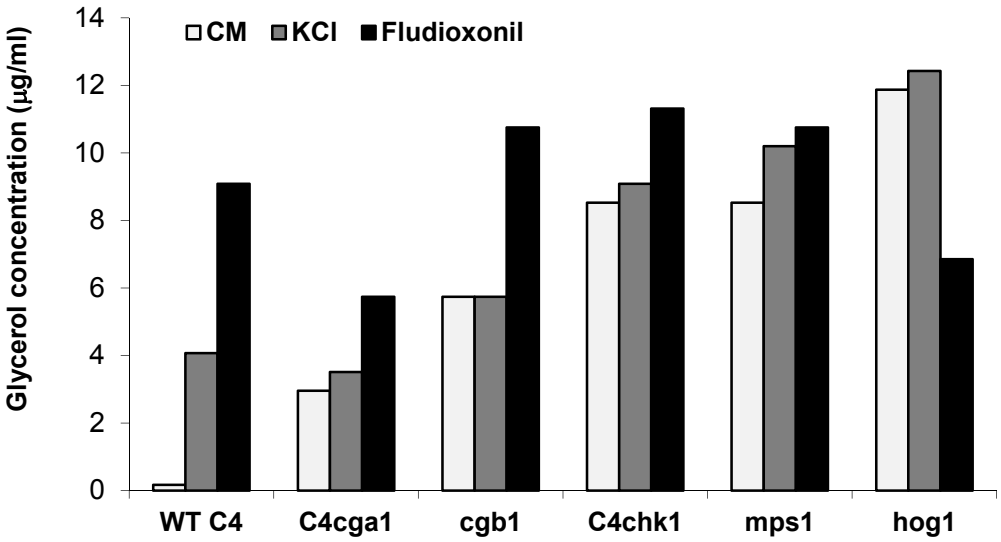

Fig. 5 Glycerol accumulation in G-protein and MAPK signaling mutant strains under fludioxonil and KCl treatments. To measure glycerol content, wild type (WT) C4 and signaling deficiency strains were grown on complete medium (CM) medium with 5 µg fludioxonil (Scholar, C.T.S., Israel) ml−1 or 750 mM KCl for 16 h at a temperature of 30°C under continuous light from cool white fluorescent tubes. Glycerol content in cell extracts was measured by a UV-glycerol assay procedure, as described in Materials and Methods, and normalized to dry cell weight. Each treatment was conducted in duplicate and two individual experiments were performed.

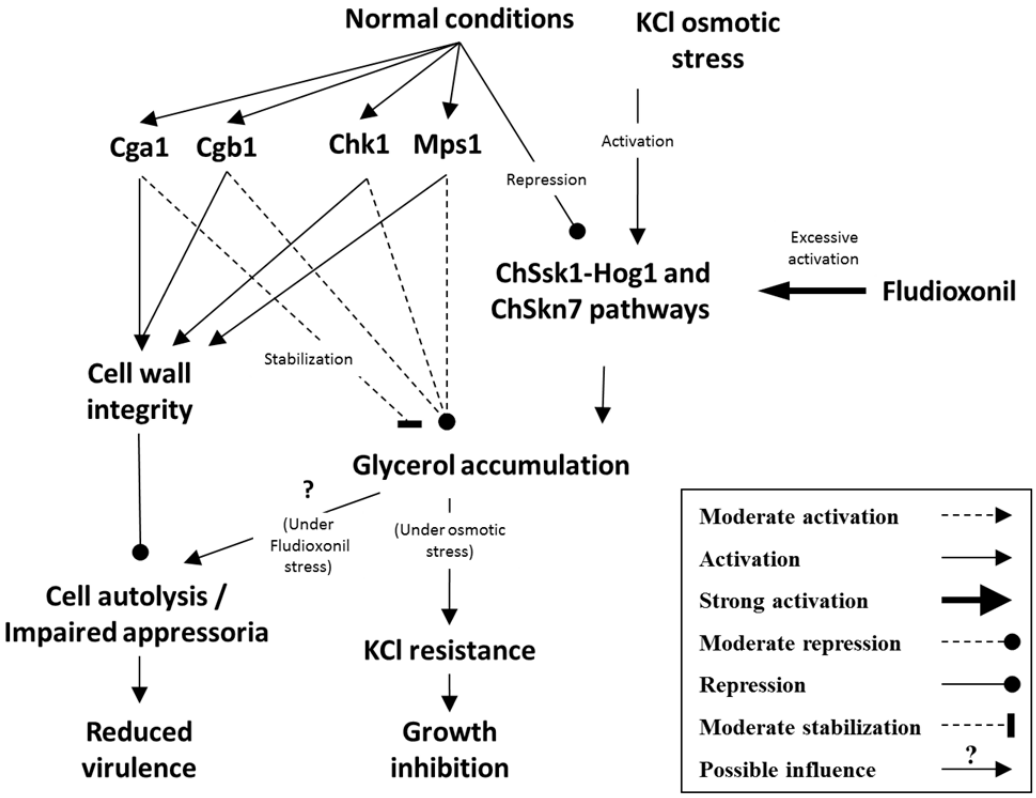

Fig. 6 Schematic diagram of pathways mediating fludioxonil and KCl effects on Cochliobolus heterostrophus. This model was built based on the findings presented in this study and in previous studies of C. heterostrophus. Both fludioxonil and KCl treatments activate the HOG pathway by rapid dephosphorylation of the hog1 MAPK in the C. heterostrophus strains in which hog1 is phosphorylated under normal conditions. Independently, both stresses can activate the ChSkn7 response regulator. The result of the activation of these pathways contributes to intracellular glycerol accumulation, causing KCl resistance and growth inhibition. Alternatively, glycerol accumulation may affect cell integrity, interfere in appressoria formation and disrupt normal virulence. The cell surface integrity is maintained under normal conditions by the G-protein and MAPK pathways.