Histidine Kinase Two-Component Response Regulator Proteins Regulate Reproductive Development, Virulence, and Stress Responses of the Fungal Cereal Pathogens Cochliobolus heterostrophus and Gibberella zeae

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Histidine kinase (HK) phosphorelay signaling is a major mechanism by which fungi sense their environment. The maize pathogen Cochliobolus heterostrophus has 21 HK genes, 4 candidate response regulator (RR) genes (SSKI, SKN7, RIM15, REC1), and 1 gene (HPT1) encoding a histidine phosphotransfer domain protein. Because most HKs are expected to signal through RR, these were chosen for deletion. Except for pigment and slight growth alterations for rim15 mutants, no measurable altered phenotypes were detected in rim15 or rec1 mutants. Ssklp is required for virulence and affects fertility and proper timing of sexual development of heterothallic C. heterostrophus. Pseudothecia from crosses involving ssk1 mutants ooze masses of single ascospores, and tetrad cannot be found. Wild-type pseudohypha do not ooze. Ssklp represses asexual spore proliferation during the sexual phase, and lack of it dampens asexual spore proliferation during vegetative growth, compared to that of the wild type. ssk1 mutants are heavily pigmented. Mutants lacking Skn7p do not display any of the above phenotypes; however, both ssk1 and skn7 mutants are hypersensitive to oxidative and osmotic stresses and ssk1 skn7 mutants are more exaggerated in their spore-type balance phenotype and more sensitive to stress than single mutants. ssk1 mutant phenotypes largely overlap hog1 mutant phenotypes, and in both types of mutant, the Hog1 target gene, MST1, is not induced. ssk1 and hog1 mutants were examined in the homothallic cereal pathogen Gibberella zeae, and pathogenic and reproductive phases of development regulated by Ssk1 and Hog1 were found to mirror, but also vary from, those of C. heterostrophus.

Histidine kinase (HK) phosphorelay signaling is a major mechanism by which some organisms sense and adapt to their environment. HK signaling has been implicated in regulating diverse processes, including stress responses, chemotaxis, differentiation, secondary metabolite biosynthesis, and virulence in microbial pathogens of plants and animals (reviewed in references 15, 17, 34, 47, and 48). HKs have been characterized in microbial pathogens of plants and animals (reviewed in references 15, 17, 34, 47, and 48). The maize pathogen C. heterostrophus has 21 HK genes, 4 candidate response regulator (RR) genes (SSKI, SKN7, RIM15, REC1), and 1 gene (HPT1) encoding a histidine phosphotransfer domain protein. Because most HKs are expected to signal through RR, these were chosen for deletion. Except for pigment and slight growth alterations for rim15 mutants, no measurable altered phenotypes were detected in rim15 or rec1 mutants. Ssklp is required for virulence and affects fertility and proper timing of sexual development of heterothallic C. heterostrophus. Pseudothecia from crosses involving ssk1 mutants ooze masses of single ascospores, and tetrad cannot be found. Wild-type pseudohypha do not ooze. Ssklp represses asexual spore proliferation during the sexual phase, and lack of it dampens asexual spore proliferation during vegetative growth, compared to that of the wild type. ssk1 mutants are heavily pigmented. Mutants lacking Skn7p do not display any of the above phenotypes; however, both ssk1 and skn7 mutants are hypersensitive to oxidative and osmotic stresses and ssk1 skn7 mutants are more exaggerated in their spore-type balance phenotype and more sensitive to stress than single mutants. ssk1 mutant phenotypes largely overlap hog1 mutant phenotypes, and in both types of mutant, the Hog1 target gene, MST1, is not induced. ssk1 and hog1 mutants were examined in the homothallic cereal pathogen Gibberella zeae, and pathogenic and reproductive phases of development regulated by Ssk1 and Hog1 were found to mirror, but also vary from, those of C. heterostrophus.
of genes whose products protect against this type of stress. The Hog1 pathway in budding yeast is, foremost, an osmotic stress response pathway; induction of catalase expression by hydrogen peroxide, for example, is not affected by loss of Hog1 (39). The second S. cerevisiae RR, Skn7, is a transcriptional activator whose receiver domain is phosphorylated in response to hypertonic stress. Skn7, together with the redox-sensitive transcription factor Yap1, also induces genes for protection against oxidative stress. The oxidant response, in contrast to the osmotic response, does not depend on phosphorylation of the conserved receiver domain aspartate (19). In fission yeast, the picture is different. Mcs4 (Ssk1 ortholog) responds to oxidative (hydrogen peroxide) stress, activating the Sty1 (the S. pombe ortholog of Hog1) pathway. Prr1 (the S. pombe ortholog of Skn7) provides an independent pathway for the oxidative stress response (4, 9, 21). Strains carrying mutations of S. pombe Prr1 are sensitive to oxidative stress and also defective in sexual development (35). Comparison of S. cerevisiae and S. pombe RR roles highlights the mechanistic diversity between the two species.

For filamentous fungi, the situation is more complex, as their genomes encode a large number of HKs that are proposed to signal through a small number of RRs. Cochliobolus heterostrophus, for example, has 21 HK genes, four putative RR genes (SSK1, SKN7, RIM15, REC1), and one phosphorylation gene, HPT1 (12). Neurospora crassa rgg1 (the ScSSK1/C. heterostrophus SSK1 ortholog; see Table S1 in the supplemental material) mutants are sensitive to hyperosmotic stress, as might have been predicted from the role of Skn1 in the osmosensing pathways of S. cerevisiae. Rrg1, however, also controls cell integrity and development of protoperithecia (23). As in yeast, an HK initiates a multistep phosphorylation to phosphorylate Hog1. In N. crassa, the HK is Nik1/Os-1. Dichlorobimide and phenylpyrrole fungicides act by inappropriately activating the Hog1 stress response pathway, so that mutants in the relevant HK or its downstream effectors display fungicide resistance (22, 25, 50). Although the general principles are similar in different ascomycete species, the roles of orthologous proteins are not identical. In Aspergillus nidulans, nikA (the N. crassa Nik1/Os-1 ortholog; see Table S1 in the supplemental material) mutants are not sensitive to osmotic or oxidative stress, yet NikA does transmit fungicide signals. The Aspergillus RRs, SrrA (ortholog of Skn7) and SskA (ortholog of Ssk1), do function in stress response (44) as predicted, suggesting that a different HK might provide the input. An A. nidulans srrAΔ sskA double mutant is more sensitive to osmotic stress than the sskA single mutant. C. heterostrophus ssk1 and skn7 single mutants have been reported to be moderately sensitive to osmotic stress and moderately resistant to fungicidals, while double mutants are more sensitive to osmotic stress and more resistant to fungicidals (22). The two RRs apparently provide independent mechanisms to transmit signals from the HK Nik1/Dic1 (21, 22). When the stress response pathway was studied in filamentous fungi, it became clear that it is involved in sensing events that are not directly related to stress. Indeed, reactive oxygen species (ROS) (1) and osmolytes may have dual roles, acting as stressors and as signals for development. A. nidulans sskA or srrA mutants are defective in asexual sporulation and conidiospore viability (44). Rrg1 of N. crassa, as noted above, is required for protoperithecial development (23). For fungal pathogens, a further dimension to this picture is provided by host-generated ROS, which could act as both a stress and a signal. The Botrytis cinerea HK, encoded by BOS1 (NIK1/OS1 ortholog), is required for resistance to osmotic stress, confers fungicide sensitivity, and is also a virulence factor for infection of host plants, including bean, tomato, and apple fruit (45). The need for pathogens to contend with stress in order to survive in the host prompted studies of oxidant adaptation for the human pathogens Candida albicans and Aspergillus fumigatus. This work resulted in the identification of genes encoding antioxidant enzymes and upstream regulators of the stress pathways, including HK and RR genes (see Table S1 in the supplemental material). Oxidant resistance, which depends on these genes, is correlated with survival in host neutrophils (5, 14).

The necrotrophic plant pathogen C. heterostrophus has four predicted RRs and one HPT gene (12). A mutant lacking the single, central Hpt1/Ypd1 phosphorylase protein would, theoretically, lack all HK/RR signaling capability, as Hpt1 is predicted to have essential functions at the crossroads of all HK inputs. Indeed, deletion of A. nidulans ypd1 is lethal, although partial phenotypes can be studied in heterokaryons (44). Likewise, loss-of-function N. crassa mutants generated by repeat-induced point mutation of the single phosphorylase gene hpt-1 could be isolated only in a loss-of-function hog1Δos2 (MAPK) background (6). We therefore hypothesized that the most direct way to attain an overview of the involvement of two-component phosphorylases in C. heterostrophus reproductive and pathogenic development would be to construct deletion mutants corresponding to each of the four candidate RRs. Here, we report that two of the four candidate RRs (Ssk1, Skn7) play multiple developmental roles (in development, pigmentation, and virulence) and we confirm and extend previously reported stress response roles (21, 22). We compared our findings with heterothallic C. heterostrophus, a necrotrophic pathogen specific to maize, to those with Gibberella zeae, a more generalized necrotrophic cereal pathogen, with a different reproductive (homothallic) lifestyle. The common and different mutant phenotypes provide a view of which functions are general and which represent species-specific adaptations of the RR-controlled pathways.

MATERIALS AND METHODS

Culture conditions. All C. heterostrophus strains were started from 25% glycerol stocks stored at −80°C. All deletions were made in wild-type (WT) strain C4 (MAT1-2, ATCC 48331). Unless otherwise indicated, the fungus was grown on complete medium (CM) (26) or CM with xylose (CMX) (43) at 24°C under continuous light (Watt-Miser F34 WW/RS/WM, Warm White; General Electric). For liquid cultures, the fungus was grown in CM at 30°C with shaking at 175 rpm. CM without salts (CMNOS) was used for selection of transformatants on antibiotics.

Gene identification. Four candidate RR genes were identified previously (see Table S1 in the supplemental material; SSK1, AY456027.1/GI39656360; SKN7, AY456028.1/GI39656362; RIM15, AY456029.1/GI39656364; REC1, AY456026.1/GI39656358), as were the HK gene NIK1 (AY456025.1/GI39656352) and the MAPK gene HOG1 (AB208438.1/GI6868407). The SSK1 (AY456027.1) sequence was partial. To obtain the complete full-length sequence, the partial sequence was used to query the C. heterostrophus C. genome sequence (http://shake.gene.cbs.uchicago.edu; protein ID 128096). Five expressed sequence tags (ESTs) (FK685411, FK685410, FK674111, FK687523, and FK674110) in the vicinity of SSK1 were identified also. PCR primers (not shown) were designed in order to amplify the corresponding se-
quences from genomic and cDNA templates. Amino acid sequences of candidate orthologs of ChSSK1 in S. cerevisiae, in the available dothideomycete genomes (http://genome.jgi-psf.org/CocheC5_1/CocheC5_1.home.html), C. heterostrophus EST sequences, and cDNA sequences amplified from C. heterostrophus templates were aligned, and the putative coding sequence was deduced. The updated sequence has been deposited in GenBank (HM152026).

**Gene deletions.** All four candidate RR genes were deleted (Table 1 and see Table S1 in the supplemental material) using the split-marker approach (see Fig. S1 in the supplemental material) (11) and the transformation protocol described earlier (11, 41). Primers for deletion are listed in Table S2 in the supplemental material. Two types of deletion strain were made for SSK1, a partial deletion which began 201 bp upstream of the stop codon and ended 1,621 bp downstream of the stop codon and a full open reading frame (ORF) deletion that began 2 bp downstream of the start codon and ended 53 bp downstream of the stop codon. When examined for stress and virulence, both had the same phenotype. In addition, the HK gene NIKI (12) was deleted. A hog1 mutant (hog1-c5.1) constructed earlier (20) (Table 1) was crossed to WT strain C4. Single ascospore progeny with double gene deletions were used in this study (Table 1). ssk1 skn7Δ double mutants (Table 1) were constructed by crossing and assay of tetrads for those with double gene deletions. One of these progeny (1545-T1-2) was used as the background for constructing an ssk1Δ mutant strain using the split-marker procedure and the gene for bialaphos resistance (bar) as a selectable marker. All strains used or constructed in this study are listed in Table 1. C. heterostrophus gene deletions, using the split-marker protocol (11), were verified by PCR amplification using diagnostic primer pairs as described in Fig. S1B and Table S2 in the supplemental material.

**Complementation of C. heterostrophus skn7Δ and ssk1Δ mutants using the stress-sensitive ssk1Δ and ssk7Δ strains.** Complementation of skn7Δ mutant strain Chskn7-2 (skn7Δ hygBΔ, Table 1) was achieved by making use of the sensitivity to oxidative stress phenotype differential of the mutant versus the WT. A fragment containing the WT SKN7 gene and flanking DNA was amplified with Platinum Taq HIFI (Invitrogen) using primers ChSKN7U and ChSKN7D (see Table S2 in the supplemental material) and used directly to transform strain Chskn7-2. The transformed protoplasts were plated in minimal regeneration medium and overlaid with MM containing 3% (wt/vol) D-glucose and 0.4 mM hydroperoxide (r-butyl), to which the skn7 mutant is extremely sensitive, on the following day. Plates were held at 22°C under continuous light, and candidate colonies were picked at 6 days. Single spore isolates of the candidates were screened for restoration of the WT tolerance to r-butyl and loss of resistance to hygromycin B, followed by confirmation of correct integration of the WT SKN7 gene fragment by diagnostic PCR (see Fig. S1B in the supplemental material).

**Complementation of ssk1Δ deletion strain Chskp1-2 (skl1 hygBΔ, Table 1) was carried out essentially in the same way as described for the skn7 mutant strain, except that hyperosmotic stress was used as selection pressure. A PCR fragment containing the WT CHSSK1 gene and about 1 kb flanking sequences on both ends was used for transformation, and the transformed protoplasts were plated in molten regeneration medium. After 20 h of incubation at 30°C, the plates were overlayed with MM containing 5 M KCl. Transformation efficiencies were higher than those regenerated by our usual antibiotic resistance selection methods (candidates evident in 4 to 5 days), appearing at 9 to 14 days. Candidates were transferred to both CM and CMNOS-hygromycin B plates. Single conidia were isolated from the hygBΔ candidates and further tested for hygromycin B sensitivity and tolerance to hyperosmotic stress. Integration of the WT SKG1 gene was confirmed by diagnostic PCR in single conidiated strains (see Fig. S1B in the supplemental material). Chsk1-3-1 (a hygBΔ single conidial isolate of transformant Chsk1-C3) and Chsk1-C10-1 were used for further stress and virulence experiments after verifying the presence of the WT SKG1 gene at the native site by PCR using primers Chsk1-ORF4/Chsk1-ORF3R or Chsk1-ORF4/Chsk1-ORF2R.

**Pigmentation and growth response to light.** Strains were plated in triplicate on MM, and colony diameters were measured at 7 days. In addition, the WT and ssk1Δ, ssk1Δ[SSK1], skn7Δ, skn7Δ skn7Δ, and hog1Δ mutant strains were grown in liquid CM culture for 20 h at room temperature (12 h ambient light, 8 h ambient dark) with shaking at 175 rpm. Pigmentation was visually evaluated before and after the culture was spun down to determine whether pigment was associated with the mycelium or with a secreted product. Strains were also grown on CMX medium for 9 days at 23°C under three different light regimens (constant light, constant dark, and 16 h light:8 h dark).

**Stress sensitivity assays.** To evaluate sensitivity to hyperosmotic or oxidative stress, strains were plated on MM containing different stress agents. A cork borer (3-mm diameter) was used to cut plugs from the edges of 5-day-old cultures on CM plates and transferred to plates of MM with or without stress agents. Plates containing photoactive agents (i.e., hydrogen peroxide [H2O2], the hydroxyl radical generator r-butyl, or the superoxide generator potassium superoxide [KO2]) were incubated at 24°C in the dark as previously described (36). All others were incubated under continuous light. At least two independent strains were tested for each genotypic class. Three replicates were set up for each strain and each condition. Sensitivity of the strains to each stress was evaluated by comparing colony diameters of 5-day-old cultures. Experiments were repeated three times.

Oxidative stress conditions were generated by adding H2O2, r-butyl, or KO2 to MM (~ 48°C) after autoclaving. Fresh stock solutions of r-butyl and KO2 (100 mM and 1 M in water, respectively; Sigma) were prepared for each experiment. The final concentrations of the stress agents were adjusted as follows: 1.25, 2.5, 5, 10, and 16 mM H2O2; 0.1, 0.2, and 0.4 mM r-butyl; and 4, 8, 16, and 32 mM KO2. For hyperosmotic stress, 0.4, 0.8, or 1.6 M sorbitol or 0.5, 1, or 2 M KCl was added to MM before autoclaving.

**Expression of the Hog1 target marker gene MST1.** Expression of MST1 (Monosaccharide Transporter 1, accession no. ABY48860; JGI protein ID 110457) was examined in WT C4, ssk1Δ (Chskp1-2), skn7Δ (Chskn7-2), skn7Δ skk1Δ (1590-TI-6), ssk1Δ-complemented (Chsk1-C3-1), and hog1Δ mutant (1685-R-29) strains. Each strain was grown in liquid CM for 20 h at 30°C with shaking at 175 rpm, and the cultures were divided in two. One half was grown in CM, while the other half was grown in CM with 0.75 M KCl for an additional 1 h. The cultures were then filtered, and total RNA was isolated using the UltraClean Microbial RNA kit (MoBio) according to the manufacturer’s protocol. Approximately 15 μg of the total RNA (45 μg) of each sample was used for cDNA synthesis, and ~2 μg of the cDNA (40 μl) sample was used for each PCR. The primers used are listed in Table S2 in the supplemental material.

**Asexual reproduction.** To evaluate asexual spore production during vegetative growth, strains were grown on CMX plates (100 by 15 mm) for 12 days, each with three replicates. All asexual spores were collected from each plate by adding 20 ml of 0.05% Tween 20 solution and filtering through two layers of cheesecloth. The number of asexual spores in two 10-μl aliquots of thoroughly suspended spores was determined with a hemocytometer.

To evaluate asexual spore production during sexual development, 1-cm² pieces of corn leaf were excised from each cross plate, transferred to 1 ml of 0.05% Tween 20 solution, and vacuumed for 2 min to wash conidia off. Asexual spores were counted as described above, and the number of asexual spores/cm² of corn leaf was calculated.

Data were analyzed using analysis of variance (ANOVA) generated using the SAS program version 9.1 (SAS Institute Inc., Cary, NC). When a statistically significant difference (P value < 0.05) was observed, differences in least-square means were tested.

**Sexual reproduction.** Standard crossing protocols were followed (26, 27). Crosses between WT strains C4 (MATI-2), C2 (MATI-1 alb1), C5 (MATI-1), CB7 (MATI-1 alb1), and CB11 (MATI-2 alb1) of the appropriate opposite mating type (Table 1) were set up as a control for all experiments. Fertility was evaluated based on the average number of pseudothecia/cm² of senescent corn leaf and the average number of ascospores per pseudothecium. In certain cases, ascospores were spread on MM and counted as described above, and the number of asexual spores/cm² of corn leaf was calculated.

For evaluation of fertility of homoygous mutant crosses, progeny of the opposite mating type were collected from crosses of the mutant in question to the WT and then crossed to each other. Data were analyzed by ANOVA.

**Virulence.** Virulence of C. heterostrophus was evaluated as described previously (36). Eighteen- to 20-day-old corn plants (cultivar W84-A [normal cytoplasm]) were inoculated with 2 ml conidial suspension (~5 × 10⁶/ml). For each fungal strain, at least three replicates (i.e., inoculation of three independent plants) were set up, and experiments were repeated three times. The data were statistically analyzed by ANOVA.

**G. zeae protocols.** G. zeae WT strain GZ3639 (8) was used for gene deletion. Strain T394M1-1, derived from GZ3639, is a self-sterile, MATI-1 deletion-containing, Geneticin-resistant strain (26). Cultures were grown on potato dextrose agar (PDA) or Difco Laboratories, Detroit, MI). For vegetative growth, pigmentation, and tests of colony surface hydrophobicity, the strains were grown on either PDA or CM (29). Hydrophobicity of the skl1 mutant strain was examined for 7-day-old cultures of G. zeae on CM. Three or four drops of water were applied to the surface of a colony.
TABLE 1. *C. heterostrophus* and *G. zeae* strains used in this study

| Strain | Genotype | Comment(s) |
|--------|----------|------------|
| **C. heterostrophus** | | |
| C2 | MAT1-1 alb1 | WT, ATCC 48329 |
| C4 | MAT1-2 | WT, ATCC 48331 |
| C5 | MAT1-1 | WT, ATCC 48332 |
| CB7 | MAT1-1 alb1 | WT, B30-A3-R-20 |
| CB11 | MAT1-2 alb1 | WT, B30-A3-R-11 |
| Chskn7-1 | ssk1 hyg MAT1-2 | Strain C4 background, full-length deletion |
| Chskn7-6 | ssk1 hyg MAT1-2 | Strain C4 background, full-length deletion |
| Chskn7-7 | ssk1 hyg MAT1-2 | Strain C4 background, full-length deletion |
| Chskn7-8 | ssk1 hyg MAT1-2 | Strain C4 background, partial deletion |
| Chssk1p-2 | ssk1 hyg MAT1-2 | Strain C4 background, partial deletion |
| Chssk1p-3 | ssk1 hyg MAT1-2 | Strain C4 background, partial deletion |
| Chssk1p-5 | ssk1 hyg MAT1-2 | Strain C4 background, partial deletion |
| Chssk1p-7 | ssk1 hyg MAT1-2 | Strain C4 background, partial deletion |
| Chskn7-2 | ssk7 hyg MAT1-2 | Strain C4 background |
| Chskn7-3 | ssk7 hyg MAT1-2 | Strain C4 background |
| Chskn7-5 | ssk7 hyg MAT1-2 | Strain C4 background |
| Chrec1-2 | rec1 hyg MAT1-2 | Strain C4 background |
| Chrec1-5 | rec1 hyg MAT1-2 | Strain C4 background |
| Chrim1-5 | rim15 hyg MAT1-2 | Strain C4 background |
| Chrim1-2 | rim15 hyg MAT1-2 | Strain C4 background |
| Chniki-7 | nki1 hyg MAT1-2 | Strain C4 background |
| Chniki-8 | nki1 hyg MAT1-2 | Strain C4 background |
| 1533-T1-4 | skn7 hyg MAT1-2 alb1 | Progeny of Chskn7-2 × CB7 |
| 1533-T1-3 | skn7 hyg MAT1-2 | Progeny of Chskn7-2 × CB7 |
| 1533-T1-4 | skn7 hyg MAT1-2 | Progeny of Chskn7-2 × CB7 |
| 1533-T1-8 | skn7 hyg MAT1-2 | Progeny of Chskn7-2 × CB7 |
| 1534-T1-1 | skn7 hyg MAT1-1 | Progeny of Chskn7-3 × CB7 |
| 1534-T1-2 | skn7 hyg MAT1-1 | Progeny of Chskn7-3 × CB7 |
| 1534-T1-3 | skn7 hyg MAT1-1 | Progeny of Chskn7-3 × CB7 |
| 1534-T1-5 | skn7 hyg MAT1-2 | Progeny of Chskn7-3 × CB7 |
| 1542-T1-3 | rec1 hyg MAT1 | Progeny of Chrec1-2 × CB7, MAT is opposite of 1543-T1-1 |
| 1542-T1-7 | rec1 hyg MAT1 | Progeny of Chrec1-2 × CB7, MAT is opposite of 1543-T1-1 |
| 1543-T1-1 | rec1 hyg MAT1 | Chrec1-5 × CB7 |
| 1543-T1-3 | rec1 hyg MAT1 | Chrec1-5 × CB7, MAT is opposite of 1543-T1-1 |
| 1543-T1-4 | rec1 hyg MAT1 | Chrec1-5 × CB7, MAT is opposite of 1543-T1-1 |
| 1544-R1 | rim15 hyg MAT1-2 | Chrim1-5 × CB7 |
| 1544-R6 | rim15 hyg MAT1-1 | Chrim1-5 × CB7 |
| 1544-R8 | rim15 hyg MAT1-1 | Chrim1-5 × CB7 |
| 1545-T1-1 | skn7 hyg MAT1 | Progeny of 1533-T1-4 × Chskn7-2 |
| 1545-T1-2 | skn7 ssk1 hyg MAT1-1 | Progeny of 1533-T1-4 × Chskn7-1 |
| 1545-T1-3 | skn7 ssk1 hyg MAT1-1 | Progeny of 1533-T1-4 × Chskn7-1 |
| 1545-T1-4 | ssk1 hyg MAT1 | Progeny of 1533-T1-4 × Chskn7-1 |
| 1545-T1-5 | ssk1 hyg MAT1 | Progeny of 1533-T1-4 × Chskn7-1 |
| 1545-T1-6 | ssk1 hyg MAT1 | Progeny of 1533-T1-4 × Chskn7-1 |
| 1546-T1-3 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1543-T1-1 × Chskn7-1 |
| 1546-T1-8 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1543-T1-1 × Chskn7-1 |
| 1547-T1-3 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1543-T1-1 × Chskn7-1 |
| 1589-T1-4 | ssk1 hyg MAT1-1 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1589-T1-7 | ssk1 hyg MAT1-1 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1590-T1-2 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1590-T1-6 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1591-T1-1 | ssk1 hyg MAT1-2 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1591-T1-2 | ssk1 hyg MAT1-2 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1591-T1-8 | ssk7 ssk1 hyg MAT1-2 | Progeny of 1534-T1-1 × Chskn7-1 |
| 1545-T1-2niki-1 | ssk7 ssk1 nki1 bar hyg MAT1-1 | Strain 1545-T1-2 background |
| 1545-T1-2niki-3 | ssk7 ssk1 nki1 bar hyg MAT1-1 | Strain 1545-T1-2 background |
| ChSKN7-2 | skn7[SKN7] MAT1-2 | Strain Chskn7-2 complemented |
| ChSKN7-7 | skn7[SKN7] MAT1-2 | Strain Chskn7-2 complemented |
| Chssk1p-1 | ssk1[SSK1] MAT1-2 | Strain Chssk1p-2 complemented |
| Chssk1p-2 | ssk1[SSK1] MAT1-2 | Strain Chssk1p-2 complemented |
| 1685-R-23 | hog1 MAT1-2 | Progeny of hog1-C5.1(20) × C4 |
| 1685-R-29 | hog1 MAT1-1 | Progeny of hog1-C5.1(20) × C4 |

| **G. zeae** | | |
| GZ3639 | Lineage 7, self-fertile | 8 |
| GzSSK1 | ssk1 hyg | This study |
| GzHOG1 | hog1 hyg | This study |
| T92MAT-1 | mat1-1/MAT1-2 gen | Self-sterile, containing MAT1-1 deletion; 28 |

* Two types of SSK1 deletion were made as described in the text.
* Nomenclature for crosses, e.g., 1533-T1-1 indicates cross number 1533 between Chskn7-2 (parent 1) and CB7 (parent 2), T1-1 = tetrad number 1, ascospore progeny number 1.
The orthologs of SSK1 (FGSG_08943.3) and HOG1 (FGSG_09012.3) were identified in the genome database (http://www.broad.mit.edu/annotation /genome/fusarium_group). The GzHOG1 gene was identified previously (38; see Table S1 in the supplemental material). Gene deletions were carried out by a double-joint PCR procedure (49), with slight modification. The 5' and 3'-flanking regions of the GzSSK1 ORF were amplified by PCR with primer pairs GzSSK1-5' for/GzSSK1-5' rev-tail and GzSSK1-3' for-tail/GzSSK1-3' rev (see Table S2 in the supplemental material), respectively. For the 5' and 3' flanks of the GzHOG1 ORF, primer pairs GzHOG1-5' for/GzHOG1-5' rev-tail and GzHOG1-3' for-tail/FgHOG1-3' rev were used. The hygromycin B resistance cassette was amplified from pUCATPH (31) with primers hygB-for/hygB-rev (see Table S2 in the supplemental material). Three amplicons (5' flank, hygB, and 3' flank) were mixed in a 1:2:1 molar ratio and used as the template for the second-round PCR, followed by a nested PCR using specific primer pairs within those used in the first round of PCR (GzSSK1-5' nest/GzSSK1-3' nest for GzSSK1 and GzHOG1-5' nest/GzHOG1-3' nest for GzHOG1; see Table S2 in the supplemental material). For deletion of SSK1 a 2,426-bp SSK1 ORF region was replaced by hygB (see Fig. S1 in the supplemental material). For HOG1, the entire HOG1 ORF from the Gz3639 genome was removed as described for SSK1. Transformation was performed as previously described (28). Deletion was verified by using two sets of internal primers: GzSSK1-for/GzSSK1-rev and GzSSK1-flanking regions of the Gz

The expression of the RIM15, SSK1, or SKN7 affects pigmentation and vegetative growth of C. heterosporus. On MM plates, the rim15 mutant strain showed reduced pigmentation compared to that of the WT, while increased pigmentation was observed for the skn7 and ssk1 mutant strains (Fig. 1A). The pigmentation phenotype was more pronounced for the ssk1 mutant strain than the skn7 mutant strain, and the ssk1 skn7 double-mutant showed skn7 mutant-like pigmentation, suggesting that Skn7 is epistatic to Ssk1 with respect to this phenotype (Fig. 1A). On CMX, a medium on which C. heterosporus shows optimal asexual sporulation, under continuous light, no obvious differences in pigmentation were detected for any mutant strain compared to the WT in either spores or mycelium (see Fig. S2 in the supplemental material). On CMX in continuous darkness, ssk1 and skn7 mutants were darker than the WT, possibly because of reduced aerial hyphae, and grew more slowly, while nkl1 mutants had altered growth patterns (see Fig. S2 in the supplemental material). Under a 16-h light/8-h dark regimen, the ssk1 mutant strain grown on CMX was darker than the WT (Fig. 1B). In addition, under this dark/light cycling condition, all mutants displayed banding patterns associated with periods of condiation, suggesting that deletion of the RR genes does not affect the ability to sense light. The pigmentation phenotypes of the skn7, ssk1, and ssk1 skn7 mutants were further examined in liquid CM culture, which inhibits condiation. The ssk1 mutant strain showed markedly increased pigmentation compared to that of the WT, the skn7 mutant culture was slightly darker than the WT, and the ssk1 skn7 double mutant was intermediate in color between the ssk1 and skn7 mutants. WT pigmentation was restored in the ssk1(skn1) complemented strain (Fig. 1C). Pigment was associated with the mycelium and was not secreted into the medium under these conditions. The hog1 mutant strain showed a pigmentation phenotype identical to that of the ssk1 mutant strain (Fig. 1B and C), implying that Ssk1 regulates pigmentation through Hog1-dependent signaling. Apart from the pigmentation phenotype, a slight reduction in growth was observed for the rim15, ssk1, and hog1 mutant strains on CMX under dark/light cycling (Fig. 1B). Except for these defects in pigmentation and vegetative growth, no obvious phenotype was identified for the rim15 mutant strain. None of the screens described below identified a phenotype associated with rec1 mutant strains. Thus, the rest of this report focuses on altered phenotypes of the ssk1, skn7, and ssk1 skn7 mutant strains.

The ssk1 and ssk1 mutants strains are compromised in tolerance to oxidative and hyperosmotic stress. The skn7 mutant strains showed hypersensitivity to oxidative stress mediated by H2O2, t-butyl, and KO2 compared to the WT. Although the effect was less pronounced, deletion of SSK1 also compromised tolerance to H2O2 and t-butyl but not to KO2 (see Fig. S3 in the supplemental material). The ssk1 skn7 double-mutant strains showed further increased sensitivity to H2O2, t-butyl, and KO2, compared to that of each single-deletion strain (see Fig. S3 in the supplemental material), indicating that Ssk1 does contribute to tolerance to oxidative stress conferred by KO2.

Both the skn7 and ssk1 mutant strains showed increased sensitivity to hyperosmotic stress mediated by sorbitol or KCl (see Fig. S4 in the supplemental material). The ssk1 skn7 double-mutant strains were more sensitive to hyperosmotic stress mediated by KCl than either single-mutant strain. The hyperosmotic stress sensitivity of the ssk1 skn7 mutant strain was indistinguishable from that of a nkl1 HK mutant strain (data not shown). The hog1 mutant strains are also hypersensitive to hyperosmotic stress mediated by sorbitol or KCl (20). Previously we found that the hexose transporter gene MST1 is induced upon exposure to osmotic (sorbitol) or ionic (KCl) stress in a Hog1-dependent manner (20) and therefore examined MST1 expression in the ssk1, skn7, and ssk1 skn7 mutants under hyperosmotic stress to monitor activation of the Hog1 pathway. MST1 was strongly induced by exposure to 0.75 M KCl in the WT and skn7 mutant strains but not in the hog1, ssk1, or ssk1 skn7 mutant strain (Fig. 2). The ssk1(skn1) complemented mutant strain showed WT expression of MST1. This

RESULTS

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result shows that the Skn7 and Ssk1 pathways are distinct. Since both \( ssk1 \) and \( skn7 \) mutants are sensitive to hyperosmotic stress, taken together, the data imply that at least two pathways, one dependent on Skn7 and the other on Ssk1 and Hog1, regulate osmotic stress responses in \( C. \) heterostrophus as reported previously (22).

Loss of \( SSK1 \) reduces asexual reproduction during vegetative growth. When asexual sporulation was examined on CMX, the \( ssk1 \) mutant strains showed a >40% reduction compared to that of the WT, while no significant difference was observed between the \( skn7 \) mutant and WT strains (Fig. 3A). Asexual sporulation of the \( ssk1 skn7 \) mutant was like that of the WT.
suggesting antagonistic cross talk between the Ssk1 and Skn7 pathways. WT asexual sporulation of the hog1 mutant strain suggests that Ssk1 controls asexual sporulation in a Hog1-independent manner during vegetative growth of C. heterostrophus. Loss of SSK1 or HOG1 derepresses asexual sporulation during vegetative growth. During sexual development of WT C. heterostrophus, copious fruiting bodies are produced on cross plates (26) but asexual spore production is usually sparse. In contrast, increased numbers of asexual spores developed in crosses involving ssk1 mutants and this phenotype was even more pronounced in homozygous ssk1 × ssk1 crosses than in crosses between ssk1 mutant and WT strains (Fig. 3B). The WT phenotype was restored in the complemented ssk1[HOG1] mutant strain (not shown). This observation indicates that Ssk1 negatively regulates asexual sporulation during sexual development in C. heterostrophus. A role for Skn7 in repression of asexual sporulation was indicated by the observation that ssk1 skn7 double mutants showed a greater, but not statistically significant, increase in asexual sporulation over the ssk1 single mutant when crossed to the WT and in homozygous crosses (Fig. 3B). Homozygous ssk1 skn7 double-mutant and homozygous hog1 mutant crosses, however, produced massive numbers of asexual spores comparable to each other and statistically significantly different from the crosses between the WT and the single-deletion mutants, including the hog1 mutant. This suggests that Ssk1 and Skn7 synergistically control a Hog1-dependent pathway to repress asexual sporulation during sexual reproduction.

Loss of SSK1, HOG1, or SSK1 and SKN7 together affects sexual development. In general, when pigmented ssk1 mutant strains were crossed to albino WT testers of the opposite mating type, there were few or no pigmented pseudothecia (Fig. 2). Hog1-dependent C. heterostrophus MST1 gene expression in liquid culture with or without osmotic stress. WT C4, ssk1 mutant strain Chssk1p-2, skn7 mutant strain Chskn7-2, ssk1 skn7 mutant strain 1590-T1-6, complemented ssk1 mutant strain Chssk1-C3-1, and hog1 mutant strain 1685-R-29 were grown in liquid CM plus or minus 0.75 KCl. Note that WT C4, the skn7 mutant strain, and the complemented ssk1 mutant strain express MST1 under conditions of osmotic stress, while the ssk1 and hog1 mutant strains do not.

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FIG. 3. Asexual spore development during vegetative and sexual reproductive growth of C. heterostrophus mutants. (A) Asexual sporulation of ssk1, skn7, ssk1 skn7, and hog1 deletion-containing strains, WT strain C4, and the ssk1[HOG1] complemented control strain on CMX medium. Note that the ssk1 deletion-containing strains show reduced asexual sporulation during vegetative growth, while all of the other strains are like the WT, including the ssk1 skn7 double mutant and the hog1 mutant strain. Letters above bars indicate significant differences between strains (P < 0.05). (B) Asexual sporulation during sexual reproduction. The crosses examined are indicated in the inset legend. For WT × WT crosses, asexual spore production during the sexual reproductive phase is very low. Crosses involving skn7 single-deletion-containing strains are like the WT in this regard, whether to the WT or homozygous. In contrast, crosses of a ssk1 deletion-containing strain to a WT strain are increased in asexual sporule production and homozygous ssk1 crosses are even more so than WT crosses. Although asexual spore production for skn7 single-deletion-containing strain crosses is not statistically significantly different from that of WT crosses, a cross of ssk1 skn7 double-deletion mutant strains to the WT is statistically the same as WT × ssk1 or homozygous ssk1 crosses. Homozygous ssk1 skn7 crosses are even more derepressed in asexual sporule production, as are homozygous hog1 crosses, than when ssk1 or hog1 single-deletion-containing strains are involved in crosses. Letters above bars indicate significant differences between crosses (P < 0.05).
A and see Table S4 in the supplemental material), while the number of albino pseudothecia increased (Fig. 4B and see Table S4 in the supplemental material) compared to the number produced in crosses between pigmented and albino WT testers. As pseudothecium color is a marker for which strain acted as the female in the cross, these observations indicate that deletion of SSK1 influences, negatively and positively, the ability to act as the female or male mating partner, respectively. Homozygous ssk1 × ssk1 crosses showed a reduced total number of pseudothecia produced, compared to that of ssk1 mutant crosses with the WT or to homozygous WT crosses (Fig. 4C and see Table S4 in the supplemental material). Complemented ssk1[SSK1] mutant strains formed both pigmented and albino pseudothecia, comparable to WT crosses (Fig. 4). Crosses of the skin7 mutant strains to albino testers were like homozygous WT crosses in terms of segregation of pseudothecium color (not shown). However, reductions in pigmented pseudothecia and in total pseudothecia were observed when the pigmented ssk1 skin7 mutant strains were crossed to albino testers (Fig. 4 and see Table S4 in the supplemental material). In addition, the homozygous ssk1 skin7 crosses usually failed to make any pseudothecia (Fig. 4C and see Table S4 in the supplemental material), in contrast to the homozygous ssk1 crosses, where at least a few pseudothecia developed. These observations indicate that not only Ssk1 but also Snk7 plays a key role in the sexual development of C. heterostrophus and that both contribute to the ability to act as the female. Crosses involving hog1 mutants produced pseudothecia in color and numbers matching those of ssk1 mutant crosses (Fig. 4).

A remarkable phenotype was observed for the ssk1 mutant strains (Fig. 5). WT crosses are normally mature by 18 to 21 days after cross setup and typically do not eject ascospores through beaks (Fig. 5A). In contrast, at this time, pseudothecia from crosses involving the ssk1 mutants had oozed all of their contents through pseudothecial beaks as masses of single ascospores (Fig. 5B to F). No asci were found in the oozed contents, suggesting that sexual spores were released prematurely, either inside the pseudothecia or as they exited. WT pseudothecia did not ooze in this manner, even at 35 days after the cross had been set. The ssk1 skin7 mutant strain phenotype was identical to that of the ssk1 mutant strain with respect to oozing (data not shown). The pseudothecium development phenotypes of the hog1 mutant, including oozing of ascospores, were very similar to those of the ssk1 mutant, suggesting that Ssk1 and Hog1 are components of the same pathway controlling sexual reproduction.

We attempted to obtain a quantitative measurement of fertility in crosses involving ssk1 and ssk1 skin7 mutants by determining the numbers of ascis and ascospores per black and white pseudothecium from many replicate crosses; however, this was impossible because pseudothecia oozed their contents prematurely. Nevertheless, we can document that only a few, if any, ascis were found when both black and white pseudothecia from the crosses of the pigmented ssk1 mutant to albino WT strains were opened at 18 days. By this time point, pseudothecia from homozygous WT crosses normally contain 50 to 100 ascis/pseudothecium. No tetrads were found in pseudothecia from crosses involving the ssk1 or ssk1 skin7 mutant, consistent with the hypothesis that sexual spores were released from pseudothecia before completion of normal development in these
Loss of SSK1 or HOG1 reduces virulence of *C. heterostrophus* on maize. In tests of virulence on maize, skn7 mutants produced symptoms indistinguishable in number and severity from those produced by the WT. In contrast, maize leaves challenged by the ssk1 mutant strain developed lesions that were reduced in density and size and different in coloration from those formed on leaves infected by the WT (Fig. 6A). The complemented ssk1[SSK1] mutant strain showed WT virulence, confirming that the compromised virulence is a result of the deletion of SSK1. The disease symptoms of hog1-inoculated leaves were very similar to those developed following inoculation with the ssk1 mutant, except that the former tended to be more sharply defined (i.e., lesions often had brown perimeters; Fig. 6A). No significant difference (data not shown) in virulence in the host was found between the ssk1 and ssk1 skn7 mutant strains, further indicating that Skn7 does not contribute to the virulence of *C. heterostrophus* in maize.

Loss of SSK1 or HOG1 affects reproductive and pathogenic development of *G. zeae*. To extend our understanding of the roles of the Ssk1 and Hog1 homologs in fungal reproductive and pathogenic development, ssk1 and hog1 deletion-containing strains of homothallic *G. zeae* were constructed and the phenotypes of the mutants were compared to those of the corresponding *C. heterostrophus* mutants. ssk1 mutant strains were similar to their WT progenitor in mycelial growth rate on CM or carrot agar plates. However, on the same solid media, aerial mycelia of ssk1 mutant strains were more compact and the culture was more pigmented (see Fig. S5A in the supplemental material) than that of the WT. In addition, colony surfaces were highly hydrophilic, in contrast to the highly hydrophobic WT (see Fig. S5B in the supplemental material). Aerial mycelia of the WT retained water droplets for several days on the surface of the mycelium, whereas water droplets soaked rapidly (within 30 s) into the surface of ssk1 and hog1 mutant strains. hog1 mutant strains exhibited more dramatic changes in mycelial growth, producing fewer aerial mycelia and darker pigmentation (see Fig. S5B in the supplemental material), and grew more slowly than the ssk1 mutant strains on CM.

During vegetative growth on carrot agar, asexual sporulation was enhanced in the *G. zeae* ssk1 mutant strains compared to that of the WT (Fig. 7A). This observation is in marked con-
contrast to our finding with the *C. heterostrophus ssk1* mutant, which exhibited reduced asexual sporulation during vegetative growth, and suggests that the Ssk1 homologs play contrasting roles in the regulation of asexual reproduction in these two species. Although it was not as significant as that observed in the *ssk1* mutant strains, enhanced asexual sporulation compared to that of the WT was also shown by the *G. zeae hog1* mutant strains, in contrast to the *C. heterostrophus hog1* mutant strains, which were no different from the WT strains in terms of asexual development during vegetative growth (Fig. 7A). As for *C. heterostrophus*, asexual sporulation is generally repressed during WT *G. zeae* sexual development (Fig. 7A). Consistent with the observation with the *C. heterostrophus ssk1* mutant, however, *G. zeae ssk1* mutants continued to produce large numbers of asexual spores after the induction of sexual development (Fig. 7A). The asexual sporulation phenotype during the sexual development of *G. zeae hog1* mutant strains was very similar to that of the *ssk1* mutant, which indicates a slight difference from the *C. heterostrophus hog1* mutant, in which asexual sporulation was more enhanced than in the *ssk1* mutant during sexual development. The asexual spore production phenotypic differences are particularly notable, as most of the other phenotypes examined were similar for *ssk1* and *hog1* mutants of *C. heterostrophus* and *G. zeae*.

With respect to *G. zeae* sexual development, 7 days after perithecial induction on carrot agar, the number of perithecia produced by the *ssk1* mutant strains was less than 10% of the number from WT self-crosses (Fig. 7B). This phenotype mirrors the reduction in pseudothecium development in homozygous *C. heterostrophus ssk1* mutant crosses. However, the *ssk1* mutants of the two species showed the opposite phenotype in terms of ascospore release. Normally, perithecia of homothallic *G. zeae* eject their ascospores through ascospore beaks when mature. By 30 days after induction, only individual ascospores were found inside WT perithecia, indicating that the mature asci had already ejected their ascospores, as they normally do. In contrast, ascospore release was delayed in the *G. zeae ssk1* mutant and perithecia of *ssk1* self-crosses still contained intact asci at this time point. In addition, ~50% of the asci found in the *ssk1* perithecia contained no ascospores,
TABLE 2. Comparison of ssk1- and hog1-induced phenotypes in C. heterostrophus and G. zeae

| Phenotype                        | C. heterostrophus sskl | C. heterostrophus hog1 | G. zeae sskl | G. zeae hog1 |
|----------------------------------|------------------------|------------------------|--------------|--------------|
| Oxidative stress sensitivity     | Yes                    | Yes                    | Yes          | Yes          |
| Osmotic stress sensitivity       | Yes                    | Yes                    | Yes          | Yes          |
| Decreased conidiation in vitro   | Yes                    | No                     | Yes          | Yes          |
| Increased conidiation on cross   | Yes                    | Yes                    | Yes          | Yes          |
| plates                           |                        |                        |              |              |
| Early release of ascospores      | Yes                    | Yes                    | No           | No           |
| Delayed release of ascospores    | No                     | No                     | Yes          | Yes          |
| Decreased virulence              | Yes                    | Yes                    | Yes          | Yes          |
| Increased pigmentation           | Yes                    | Yes                    | Yes          | Yes          |
| Hydrophobic colony surface       | No                     | No                     | Yes          | Yes          |
| Lack MST1 expression on KCl      | Yes                    | Yes                    | ND          | ND          |

*ND, not determined.

while the rest contained normal tetrads. The G. zeae hog1 mutant strains failed to produce perithecia, indicating that sexual development is more severely affected in this mutant than in the ssk1 mutant and the C. heterostrophus ssk1 and hog1 mutants, which were similar to each other.

Finally, we examined the pathogenic development of G. zeae ssk1 and hog1 mutant strains (Fig. 6B). Wheat spikelets inoculated with the WT produced typical head blight symptoms that appeared at the inoculation point (third spikelet from the base of the head) and spread into neighboring spikelets (Fig. 6B). The ssk1 mutant strains were able to colonize inoculated spikelets but rarely spread to the adjacent ones, resulting in much-reduced disease severity compared to that produced by the WT. A similar reduction in virulence was observed in the hog1 mutant (Fig. 6B).

DISCUSSION

Functions of RRs. Here we report the association of RRs with asexual and sexual development, virulence, colony growth, pigmentation, and cell surface properties (in G. zeae) and confirm their central involvement in osmotic and oxidative stress responses. The data for both C. heterostrophus and G. zeae are consistent with the general paradigm in which Ssk1 (upstream of the Hog1 MAPK cascade) and Skn7 separately contribute to stress resistance in fungi. Apart from an alteration in pigmentation and slightly reduced growth under light cycling conditions, rim15 mutants were like the WT. rec1 mutants had no discernible altered phenotypes, leaving open the question of the function of the Rec1 (and perhaps Rim15) protein in C. heterostrophus and its categorization as an RR. We note that the RIM15 homolog in Magnaporthe oryzae is required for full virulence (33).

Evidence that C. heterostrophus Skn7 and Ssk1 belong to independent signaling pathways. The phenotypes associated with C. heterostrophus skn7 mutants differ from those of ssk1 mutants. In particular, individual loss of SKN7 has no significant effect on virulence (Fig. 6A), on sexual development (Fig. 4; see Table S4 in the supplemental material), on repression of asexual development in vitro (Fig. 3A), or on asexual development on cross plates (Fig. 3B and C). Both Skn7 and Ssk1, though, contribute to stress resistance, since loss of either compromises resistance to both oxidative and osmotic stresses (see Fig. S3 and S4 in the supplemental material). In the absence of epistasis, the phenotypes of the ssk1 skn7 double mutant would be expected to be augmented. The effects on stress resistance are consistent with this, as the double mutant is more sensitive to oxidative and osmotic stresses than is either single mutant (see Fig. S3 and S4 in the supplemental material). The clearest evidence of epistatic interaction is the in vitro production of conidia (Fig. 3A) and the pigmentation phenotype (Fig. 1; see Fig. S2 in the supplemental material), where loss of SKN7 fully or partially rescued defects associated with ssk1 single mutants.

Evidence that the MAPK Hog1 acts downstream of Ssk1. In C. heterostrophus, Hog1 phosphorylation in response to exposure to fungicides depends on Ssk1, providing direct biochemical evidence that Hog1 phosphorylation is downstream of Ssk1 (22). At first glance, this may seem paradoxical, because phosphorylated Ssk1 is considered to be an inhibitor of the Hog1 pathway. Our findings, however, are consistent with current signaling models (for example, see Fig. 2 in reference 14) in which loss of Ssk1 implies loss of the active signaling molecule nonphosphorylated Ssk1.

An exception is that the hog1 mutant phenocopies the ssk1 skn7 mutant with respect to derepression of asexual sporulation, rather than the ssk1 single mutant. This suggests a nonlinear pathway, some models for which are discussed in more detail below (see discussion of asexual development below). In G. zeae as well, there is indirect evidence that some Hog1-dependent responses are independent of the linear pathway from upstream HKs via Ssk1: activation of Hog1 by fungicide or sorbitol stress (and there is strong evidence that these signals act via Ssk1) did not affect secondary metabolite production (34). In the same study, loss of Hog1 increased secondary metabolite production (see discussion of pigmentation, below).

For G. zeae ssk1 and hog1 mutants, all of the phenotypes...
tested in this study overlapped (trended in the same direction; Table 2) but were not identical. For example, ssk1 mutants made fewer perithecia than the WT in self-crosses (Fig. 7) but hog1 mutants made no perithecia. Furthermore, ssk1 mutants produce more red pigment than the WT, but the phenotype of hog1 is much more pronounced (see Fig. S5A in the supplemental material). This is difficult to explain by a simple linear pathway. Our observations on the sensitivity of G. zeae mutants to osmotic stress support earlier findings of Ochial et al. (34). In C. heterostrophus, osmotic stress-induced expression of the hexose transporter gene MST1 is lost in both ssk1 and hog1 mutants (Fig. 2), supporting a linear pathway to gene expression. In summary, the majority of the phenotypes are consistent with a linear pathway signaling through Ssk1 and Hog1, with some exceptions where further study may uncover contributions of, or cross talk with, additional pathways (Fig. 8).

Stress responses. In C. heterostrophus, ssk1 and skn7 mutants are sensitive to osmotic and oxidative stresses, with the exception that the ssk1 mutant is similar to the WT in terms of resistance to oxidative stress mediated by KO2. Our findings regarding osmotic stress largely support those reported by Izumitsu et al. and He et al. (19, 21, 22). Presumably, the ability to withstand stress is key for survival and thus evolutionarily conserved. Indeed, comparison of the C. heterostrophus or G. zeae ssk1 mutant phenotypes with those of N. crassa and A. nidulans rss-1/sskA mutants emphasizes the conserved role of Ssk1. N. crassa Rrg1 is needed for osmotic stress response (23), while although the dominant role of A. nidulans SskA is the osmotic stress response, the downstream Hog1 homolog is not essential for this response (44). On the other hand, C. albicans Ssk1 is involved primarily in oxidant rather than osmotic stress sensing and in differentiation and cell wall properties (32). Skn7 provides a separate oxidant-responsive pathway. In C. neoformans, Ssk1 is essential for resistance to oxidants and is important, but not essential, for osmosensing. Skn7, in contrast to some other species, is not needed for resistance to oxidants but is specifically required to contend with high Na+ but not K+ levels (5). Thus, in all of these species, including C. heterostrophus and G. zeae, loss of SSK1, SKN7, or HOG1 orthologs results in sensitivity to osmotic and/or oxidative stress. The details vary, with either both or the RRs and the downstream MAPK Hog1 needed for full response to osmotic and/or oxidative stress. It seems that the “wiring” of the signaling pathways has evolved differently, such that the contribution of Ssk1 or Skn7 to oxidative or osmotic stress varies between species.

Asexual development. We found that during vegetative growth, C. heterostrophus and G. zeae ssk1 mutants differed with respect to asexual spore production. For C. heterostrophus, fewer conidia were produced by ssk1 mutants (<45%) than by the WT (Fig. 3A), while for G. zeae ssk1 mutants, the opposite was found (about four times more than the WT up to 8 days; Fig. 7A).

During C. heterostrophus WT mating, Ssk1 represses asexual sporulation (Fig. 3B), favoring fruiting body formation in response to appropriate signals. Loss of Ssk1 relieves this repression and promotes conidiation under conditions where sexual development is normally the priority. For WT G. zeae, asexual sporulation decreased upon entry into the sexual phase (starting on day 8; Fig. 7A). Although a reduction in asexual sporulation was observed also for the ssk1 and hog1 mutants, conidiation in the mutants was still much higher than in the WT throughout the sexual phase (Fig. 7A), indicating that the Ssk1/Hog1 pathway-dependent repression of asexual sporulation during sexual reproduction is operational also in G. zeae.

Dual loss of C. heterostrophus Ssk1 and Skn7 results in a more pronounced increase in asexual sporulation during sexual reproduction compared to the single loss of Ssk1, and this is mirrored by the loss of Hog1 (Fig. 3B). Despite the evidence that the two RRs, Ssk1 and Skn7, act independently in other respects, this observation suggests that the Hog1 pathway is under the control of Skn7, at least for this particular response (Fig. 8). How do Hog1 and the presumed transcription factor Skn7 act together? By analogy to S. cerevisiae Skn7, which interacts with other proteins to form transcription factor complexes (7, 37), C. heterostrophus Skn7 might collaborate with other proteins, some of which are perhaps regulated by Hog1. Loss of Hog1 would, in this model, prevent the action of Skn7 by removing its interacting partners, explaining why a hog1 mutant shows the same phenotype as an ssk1 skn7 mutant with respect to asexual sporulation during sexual reproduction.

Under the hypothesis that the Hog1 pathway is coregulated by Ssk1 and Skn7, Ssk1 is apparently the predominant determinant of Hog1 activity, as no significant alteration in asexual sporulation was observed in the skn7 single mutant.

In A. nidulans, the balance between sexual and asexual sporulation is set by light, acting through a VeA complex that includes the global regulator LaeA (10). To the extent that these regulators are conserved in the two plant pathogens studied here, it will be interesting to consider whether HK signaling is also involved. Indeed, fungal phytochromes are HKs (12). Current models show direct protein interaction of phytochrome with other members of the VeA complex (10). RRs downstream of phytochrome HKs remain to be identified.

Pigmentation. For the plant pathogens C. heterostrophus, G. zeae, and M. oryzae (33), as reported for the human pathogen C. neoformans (4), loss of either SSK1 or HOG1 results in strains that are much darker than the WT. Like C. heterostrophus, both ssk1 and skn7 mutants of M. oryzae (33) show increased pigmentation. Loss of C. heterostrophus SKN7 results in strains that are a little darker than the WT in liquid culture (Fig. 1C) while ssk1 skn7 double mutants are lighter than ssk1 mutants but darker than skn7 mutants in liquid culture. C. heterostrophus skn7 mutants are more like the WT on MM and CMX under various light conditions (Fig. 1B; see Fig. S2 in the supplemental material). Furthermore, M. oryzae rim15 mutants show decreased pigmentation, as do C. heterostrophus rim15 mutants (Fig. 1A). Note that this is one of the few phenotypes associated with rim15 that we identified. In contrast, skn7 mutants of C. neoformans produce more melanin (the 3,4-dihydroxynaphthalene type, synthesized by the laccase-dependent pathway [46]), than ssk1 mutants, although both produce more than the WT. The enhanced-melanin phenotype in both ssk1 and skn7 mutants was interpreted as evidence for a Hog1-independent pathway, since Skn7 does not signal through Hog1 in C. neoformans (4). Note that C. neoformans melanin is a well-known virulence factor, while 1,8-dihydroxynaphthalene (DHN) melanin is not considered a virulence factor for C. heterostrophus, as albino mutants display WT virulence in greenhouse experiments. hog1 mutants of G. zeae are much
more heavily pigmented (red) than ssk1 mutants (see Fig. S5A in the supplemental material). The phenotype of G. zeae hog1 is consistent with a previous report (34) which showed that loss of the upstream HK (FgOS1) decreased production of the red pigment (aurofusarin). G. zeae lacks genes for DHN melanin production.

Sexual reproduction. Ssk1 and Hog1 functions associated with development are perhaps even more diverged among fungal species than stress responses. For example, the dramatic abnormal ascospore release by C. heterostrophus ssk1 mutants is not found in G. zeae ssk1 mutants; G. zeae ssk1 mutants are delayed in normal ascospore release, the opposite of the C. heterostrophus phenotype. This difference may be related to different modes of WT ascospore release. The G. zeae L-type calcium channel protein Cch1 is necessary for spore discharge, perhaps conveying a Ca^{2+} message signaling for cytoskeletal rearrangements or other changes (18). C. heterostrophus ascospores are not released by rapid, forcible discharge. There is evidence from both Ascobolus (16) and G. zeae (40) that accumulation of osmolytes inside the ascus is responsible for the turgor pressure that leads to spore discharge. Perhaps a similar driving force is necessary for release of C. heterostrophus ssk1 ascospores as cirri (fungal spore masses exuded in a sticky matrix) (Fig. 5).

As a first test of this turgor hypothesis, we experimented with ascospore release from C. heterostrophus WT and ssk1 and hog1 mutant pseudothecia (26). Leaf sections with pseudothecia were taken 13 days after cross setup and floated on distilled water or a hyperosmotic solution (0.7 M NaCl, 50 mM CaCl_2, or STC [an osmoprotective buffer used in protoplast tillage] water or a hyperosmotic solution (0.7 M NaCl, 50 mM CaCl_2, or STC [an osmoprotective buffer used in protoplast tillage]) (Fig. 5).

Having established central roles for RRs in cereal pathogens, our next direction may be to study the functions of the individual HKs, which are mostly unknown. The results of this study define a number of pathways that can be used to report activation of the phosphorelays defined by Ssk1 and Skn7. It is too soon to speculate on why the Ssk1-mediated pathways studied here have opposite effects on sexual development in C. heterostrophus and G. zeae, but one avenue to follow would be to compare pseudothecial/perithecial development and the biophysics of ascospore release and to attempt to correlate these with gene expression (the genome sequences of both are available). The biophysical mechanism underlying the ascospore release in C. heterostrophus will be a fascinating topic to pursue. Likewise, further study of the reasons why ascospore release is affected in opposite ways by loss of Ssk1 in C. heterostrophus and G. zeae may uncover differences in the evolutionary forces that shaped the control of spore dispersal in the two species. Ascospore release is delayed in G. zeae ssk1 mutants, raising the possibility that the Ssk1-Hog1 pathway might convey a signal limiting turgor pressure in C. heterostrophus but raising it in G. zeae. In G. zeae, ascospores are the main vehicle for spread of disease, while C. heterostrophus disseminates in the field mainly by conidia.

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