The Fungal Cell-Wall Integrity MAPK Cascade Is Crucial for Hyphal Network Formation and Maintenance of Restrictive Growth of *Epichloë festucae* in Symbiosis With *Lolium perenne*

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Epichloë festucae is a mutualistic symbiont that systemically colonizes the intercellular spaces of *Lolium perenne* leaves to form a highly structured and interconnected hyphal network. In an *Agrobacterium tumefaciens* T-DNA forward genetic screen, we identified a mutant TM1066 that had a severe host interaction phenotype, causing stunting and premature senescence of the host. Molecular analysis revealed that the mutation responsible for this phenotype was in the cell-wall integrity (CWI) mitogen-activated protein kinase kinase (MAPKK), *mkkA*. Mutants generated by targeted deletion of the *mkkA* or the downstream *mpkA* kinase recapitulated the phenotypes observed for TM1066. Both mutants were defective in hyphal cell–cell fusion, formed intrahyphal hyphae, had enhanced conidiation, and showed microcyclic conidiation. Transmission electron microscopy and confocal microscopy analysis of leaf tissue showed that mutant hyphae were more abundant than the wild type in the intercellular spaces and colonized the vascular bundles. Hyphal branches failed to fuse but, instead, grew past one another to form bundles of convoluted hyphae. Mutant hyphae showed increased fluorescence with AF488-WGA, indicative of increased accessibility of chitin, a hypothesis supported by changes in the cell-wall ultrastructure. These results show that the CWI MAPK pathway is a key signaling pathway for controlling the mutualistic symbiotic interaction between *E. festucae* and *L. perenne*.

The endophytic fungus *Epichloë festucae* is a mutualistic symbiont of *Festuca* and *Lolium* cool-season grasses (Christensen et al. 2002; Leuchtmann et al. 1994; Schardl 2001; Scott et al. 2012). This biotrophic fungus systemically colonizes the intercellular spaces of both vegetative and reproductive aerial tissues of the plant to form a highly structured and interconnected symbiotic hyphal network (Takemoto et al. 2006; Tanaka et al. 2006). Hyphae grow within the true stem of the grass by hyphal tip growth, from where they ramify into

leaf primordia and axillary buds that give rise to new leaves and new shoots, respectively. Within each leaf primordium, there are two zones of cell division, one that gives rise to the blade and the other to the sheath. Intercalary division of plant cells in both zones combined with leaf expansion results in stretching of hyphae. Remarkably, hyphae within these leaf expansion zones grow by intercalary extension; a pattern of growth that allows synchronization of hyphal growth with that of the rapidly expanding leaf (Christensen et al. 2008). Given that hyphae are firmly attached to the cell walls, this form of growth prevents mechanical shear of the hyphae as the leaves expand. Once leaf expansion ceases, growth of the hyphae also ceases, suggesting that signaling from the host controls fungal growth (Tan et al. 2001).

Establishment and maintenance of this symbiotic network of *E. festucae* within its host is dependent on a number of important fungal signaling pathways, including reactive oxygen species signaling from the NADPH oxidase (Nox) complex and signaling through the stress-activated mitogen-activated protein kinase (MAPK) pathway (Eaton et al. 2010; Tanaka et al. 2006). Disruption of *noxA* and fungal genes that encode additional components of this complex, including *noxR*, *racA*, and *bemA*, leads to a breakdown in the symbiosis; mutant hyphae switch from a pattern of restrictive to proliferative growth resulting in premature senescence and, frequently, death of the host (Takemoto et al. 2006, 2011; Tanaka et al. 2006, 2008). The demonstration that *Neurospora crassa* homologues of *noxA* (*nox-1*) and *noxR* (*nor-1*) were required for fusion of conidial anastomosis tubes (CATs) (Read et al. 2012) led us to propose that a lack of hyphal cell fusion in planta was responsible for disruption of the *E. festucae* symbiotic hyphal network in leaves of *Lolium perenne*. In support of this hypothesis, we showed that *noxA*, *noxR*, *racA*, and, to a lesser extent, *bemA* mutants were defective in cell–cell fusion when grown on water agar in axenic culture (Kayano et al. 2013). In addition, the *noxA* mutant was found to hyperconidiate, suggesting that the NoxA complex represses conidiation in *E. festucae*.

Disruption of *proA*, encoding a C6 zinc finger transcription factor, also led to a breakdown in the mutualistic symbiotic interaction between *E. festucae* and *L. perenne* (Tanaka et al. 2013). Homologues of this gene in *Sordaria macrospora*, *N. crassa*, and *Aspergillus nidulans* are required for sexual fruiting body development (Colot et al. 2006; Masloff et al. 1999; Vienken and Fischer 2006) and are defective in cell–cell fusion (Fu et al. 2011; Masloff et al. 2002). Like $\Delta noxA$, the *E. festucae*

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proA mutant was also defective in hyphal cell fusion in axenic culture and had a hyperconidiation phenotype (Tanaka et al. 2013). Deletion of the *E. festucae soft* (*so*) gene, encoding a protein of unknown function required for chemotropic signaling during CAT fusion in *N. crassa* (Fleissner et al. 2009), also led to a hyphal cell fusion defect in culture and a severe symbiotic interaction phenotype (Charlton et al. 2012).

In the course of an *Agrobacterium tumefaciens* T-DNA mutagenesis screen for mutations that regulate the expression of indole-diterpene alkaloid biosynthetic genes (Young et al. 2005; Young et al. 2006), we isolated TM1066, a mutant that induced a host interaction phenotype very similar to that observed for the $\Delta noxA$, $\Delta noxR$, $\Delta racA$, $\Delta proA$, and Δso mutants. Here, we report that the gene responsible for this symbiosis phenotype, *mkkA*, encodes the cell-wall integrity (CWI) MAPKK.

RESULTS

T-DNA induced mutant TM1066 is defective in cell fusion and symbiosis.

To identify *E. festucae* genes that regulate the in-planta-specific expression of lolitrem (*lrm*) biosynthetic genes, a forward genetics screen was carried out using *A. tumefaciens* T-DNA-mediated mutagenesis (Tanaka et al. 2007) of a *PltmM-gusA* “knock-in”, strain KM2.49 (Supplementary Fig. S1A), to identify mutants that were either β -glucuronidase (GUS) positive in culture or GUS negative in planta. From approximately 1,400 T-DNA mutants screened, no mutants were obtained that were GUS negative in *L. perenne* seedlings. However, strain TM1066 induced severe stunting of the *L. perenne* host (Fig. 1A), a phenotype similar to that previously observed for plants infected with mutants in the Nox complex, including $\Delta noxA$ (Tanaka et al. 2006), $\Delta noxR$ (Takemoto et al. 2006), and $\Delta racA$ (Tanaka et al. 2008); a stress-activated MAPK mutant $\Delta saka$ (Eaton et al. 2010); and the Zn(II)₂Cys₆ transcription factor mutant $\Delta proA$ (Tanaka et al. 2013). Plants infected with TM1066 showed varying degrees of stunting, drastically reduced infection rates (6 to 18%) compared with wild-type- or KM2.49-infected plants (75 to 85%), and decreased seedling survival rates (33%). Transmission electron microscopy (TEM) analysis of hyphae in the intercellular space of leaf sheath cross sections showed that mutant hyphae were more abundant than wild-type hyphae, readily colonized the plant vascular bundles, and formed intrahyphal hyphae (Fig. 1B). In wild-type associations, hyphae are generally excluded from vascular bundles, intercellular spaces are mostly colonized by one or two hyphae, and intrahyphal hypha are not formed (Christensen et al. 2008; Scott et al. 2012; Tanaka et al. 2006).

In axenic culture, the radial growth of TM1066 was slightly reduced compared with KM2.49 and the wild type (Fig. 1C). Both KM2.49 and TM1066 have an altered colony morphology compared with the wild-type strain, characterized by an increase in surface hydrophobicity and reduced formation of aerial hyphae. The fact that KM2.49 also has this phenotype suggests that this morphological change arose from generation of the *PltmM-gusA* knock-in. Light microscopy analysis showed that TM1066 is defective in cell–cell fusion and forms intrahyphal hyphae in culture (Fig. 1D), a phenotype indicative of a septum defect (Liu et al. 2008). A closer examination of the cell–cell fusion process occurring in the wild type revealed that *E. festucae* undergoes apical tip fusion (anastomosis) with an adjacent subapical cell (Fig. 2A). As the tip of the growing hypha extends, further cell–cell fusions occur with subapical compartments of the adjacent hypha to build a ladder-like structure connecting the two hyphae (Fig. 2C). Imaging of an F11 strain expressing mRFP1, growing alongside an F11 strain

expressing enhanced green fluorescent protein (eGFP), demonstrated that, following cell–cell fusion, there is cytoplasmic migration and mixing between the two fused hyphae (Fig. 2B). The monomeric red fluorescent protein (mRFP1)–expressing hypha (top) appears to have undergone fusion with the subapical cell of an eGFP (green)–expressing hypha (bottom), followed by further growth of the hyphal tip and mixing of the cytoplasmic contents of the two hyphae. The merge of the mRFP1 and eGFP channels shows the progression of the cytoplasmic mixing.

Conidiation of *E. festucae* wild-type F11 on potato dextrose agar (PDA) is sparse but, when grown under nutrient-limiting conditions such as on water agar, a few conidia are produced predominantly from conidiophores that arise as lateral branches from coil-like hyphal structures (Kayano et al. 2013; Scott et al. 2012). TM1066 showed an increase in conidiation compared with the wild type and KM2.49 when grown on either water agar (Fig. 1D) or PDA (Fig. 3).

Molecular analysis of the T-DNA integration event in TM1066 and complementation.

Southern analysis of genomic digests of TM1066 probed with [³²P]-labeled pBSYT7 revealed the presence of a single-copy T-DNA insertion. The sequence flanking the left border of the T-DNA was isolated by thermal asymmetrical interlaced polymerase chain reaction (TAIL-PCR). Subsequent PCR, sequence, and Southern blot analyses revealed that TM1066 contained a 2,221-bp deletion of the T-DNA, including the right border, and a 1,081-bp deletion of the *E. festucae* genome that included 3' coding sequences of *pusA* (EfM3.016600), encoding a pseudouridine synthase, and *mkkA*, encoding a CWI MAPKK.

The *E. festucae pusA* coding sequence is predicted to encode a polypeptide of 755 amino acids that shares 56% sequence identity with a *Fusarium graminearum* hypothetical protein, 31% with *N. crassa* pseudouridine synthase TruD/Pus7, and 31% with *Magnaporthe oryzae* tRNA pseudouridine synthase D (Supplementary Fig. S2). Pseudouridine synthases catalyze the isomerization of uridine to pseudouridine in a variety of RNA molecules, and may function as RNA chaperones (Ramamurthy et al. 1999). *E. festucae* PusA shows highest homology to the TruD family of pseudouridine synthases that modify uracil-13 in tRNA (Chan and Huang 2009).

The *E. festucae mkkA* coding sequence is predicted to encode a polypeptide of 532 amino acids that shares 82% sequence identity with *F. graminearum* Fgmkk2 (Fgmkk1), 68% with *N. crassa* MEK-1, and 76% with *M. oryzae* Mkk1 (Supplementary Fig. S3). The MkkA/Mek1 protein is a central component of the CWI MAPK signal transduction pathway, where it acts as the kinase kinase phosphorylating the MpkA MAPK. The MkkA protein kinase domain is located between amino acids 235 and 505. *Saccharomyces cerevisiae* has two functionally redundant MEK (Mkk1/2) but filamentous fungi have just one copy (Irie et al. 1993). A tBLASTn analysis of the *E. festucae* genome using *N. crassa* Mak1 as the query sequence identified a putative homologue of the CWI MAPK downstream of MkkA, which we have called MpkA (EfM3.074990). The *E. festucae mpkA* coding sequence is predicted to encode a polypeptide of 418 amino acids that shares 92% sequence identity with *F. graminearum* Mgv1, 86% with *N. crassa* Mak1, and 88% with *M. oryzae* Mps1 (Supplementary Fig. S4).

To determine which of the two genes deleted in TM1066 was responsible for the culture and symbiosis phenotypes, the *mkkA* and *pusA* genes, under the control of their native promoters and terminators, were separately introduced into the genome of TM1066. Reintroduction of pYR31, containing

mkkA, rescued the culture fusion and plant symbiotic interaction defects but not pYR29, containing *puaA* (Fig. 4).

The CWI MAPK pathway is essential for vegetative hyphal fusion and regulates conidiation.

Because TM1066 was not derived directly from the wild type and KM2.49 had a different culture morphology from the wild

type, we made a targeted deletion of *mkkA* and of the downstream MAPK *mpkA* to confirm that a functional CWI MAPK pathway is essential for hyphal fusion, regulation of conidiation, and symbiosis. Linear fragments of pCE63 and pCE64 containing replacement cassettes for *mkkA* and *mpkA*, respectively, were generated by PCR and transformed into protoplasts of F11. PCR screening of 72 arbitrarily chosen hygromycin-

Fig. 1. TM1066 mutant phenotype. **A**, Phenotype of perennial ryegrass plants infected with *Epichloë festucae* wild-type (WT) strain F11 and T-DNA mutant TM1066. Photograph was taken 9 weeks postinoculation. **B**, Transmission electron micrographs of cross-sections of *E. festucae* WT and mutant TM1066 growing in *Lolium perenne*. Hyphae colonizing a plant vascular bundle are circled in box 6. Pseudostem cross sections were made 9 weeks postinoculation. Symbols: * = inner hypha, ** = outer hypha, h = hypha, pc = plant cell, pcw = plant cell wall, ics = intercellular space. Bars = 1 µm in boxes 1 to 5 and 10 µm in box 6. **C**, Culture phenotype of *E. festucae* WT strain F11, *PltmM-gusA* knock-in strain KM2.49, and T-DNA mutant TM1066 grown on potato dextrose agar for 6 days. **D**, Confocal laser-scanning and differential interference contrast microscopy images of hyphal morphology of *E. festucae* hyphae grown on water agar (1.5%) for 6 days. Cultures were stained with Calcofluor white (CFW). Hyphal anastomoses in WT and KM2.49 are circled. Symbols: c = conidium, cp = conidiophore, * = inner hypha, ** = outer hypha. Bars = 20 µm.

resistant transformants from each transformation with primer sets *mkk5/6* and *mpk5/6* identified 10 and 13 putative replacements, respectively. Additional PCR screening with primer sets that amplify the left and right flanks of the insertion (*mkkA*: *mkk3/TC45* and *mkk4/TC44*; *mpkA*: *mpk3/TC45* and *mpk4/*

TC44) identified five (numbers 2-4, 3-9, 4-2, 4-7, and 8-3) putative replacements of *mkkA* and seven (numbers 7-4, 8-3, 8-5, 10-8, 12-6 13-6, and 13-7) of *mpkA* (Supplementary Fig. S5). Southern blot analysis of genomic digests of these transformants confirmed that all were true replacements; four of the

Fig. 2. Confocal laser-scanning and differential interference contrast (DIC) microscopy images of hyphal fusion in *Epichloë festucae* wild-type cultures grown on water agar for 6 days. Cultures were stained with Calcofluor white (CFW) in A and C. **A**, Hyphal tip fusion with adjacent subapical cell compartment. The CFW image is a 2.7-nm Z stack, whereas the DIC image is a single plane. **B**, Cytoplasmic mixing after cell fusion between WT strains containing mRFP1 (PN2832) and eGFP (PN2833). The tip of the mRFP1 strain has fused with a subapical eGFP expressing hyphal cell compartment, followed by cytoplasmic mixing on further hyphal growth. **C**, Formation of a ladder of hyphal cell fusions following a series of hyphal tip fusions of a growing hypha with the subapical cells of an adjacent hypha (1,2,3) to build an interconnected network. The frequency of fusions is variable. Bar = 10 μ m.

Fig. 3. *Epichloë festucae* cell-wall integrity MAPK pathway suppresses conidiation under axenic conditions. **A**, Conidia were isolated from 16 colonies grown on potato dextrose (PD) plates for 6 days, with 50 μ l (top) and 100 μ l (bottom) of wild-type (WT), TM1066, Δ mkkA, and Δ mpkA suspensions spread on PD plates. Colony formation from single spores was photographed and counted after 6 days of growth. **B**, Mean of spore germination from three independent experiments. Although total colony numbers varied in each independent experiment, conidiation was increased in TM1066 and Δ mkkA compared with the WT and significantly increased in Δ mpkA. Bars represent mean $\pm$ standard error of the mean ($n = 3$). Letters show significant differences where $P < 0.05$, as determined by one-way analysis of variance with Tukey post hoc test.

five *mkkA* strains had ectopic integrations and the fifth, $\Delta mkkA4-2$ (henceforth $\Delta mkkA$; PN2892), a single-site multi-copy integration; five of the seven *mpkA* strains had ectopic integrations but two, $\Delta mpkA10-8$ (henceforth $\Delta mpkA$, PN2988) and $\Delta mpkA13-7$, were clean single-copy replacements.

To confirm that MpkA acts downstream of MkkA, its phosphorylation was measured in the wild-type, TM1066, and $\Delta mkkA$ strains (Fig. 5). Two bands were detected in the wild type, corresponding to phosphorylated MpkA at 47 kDa and phosphorylated MpkB (the Fus3 homolog) at 41 kDa (Fig. 5A). The absence of the upper band in TM1066 and $\Delta mkkA$ indicates that MkkA phosphorylates MpkA. We then checked whether the absence of MpkA phosphorylation in the $\Delta mkkA$ and $\Delta mpkA$ mutant backgrounds would affect nuclear localization of MpkA in arthroconidia. These asexual spore-like structures readily form when *E. festucae*-regenerating protoplasts grow through a regeneration media overlay. Under these conditions of stress, MpkA-eGFP localizes to the nucleus (Cartwright et al. 2014). MpkA-eGFP localized to the nucleus

in both the $\Delta mkkA$ and $\Delta mpkA$ mutant backgrounds, demonstrating that phosphorylation of MpkA by MkkA is not necessary for nuclear localization (Fig. 5B).

Several developmental phenotypes have been observed for CWI MAPK mutants in other fungi, including reduced colony radial growth (Maddi et al. 2012; Maerz et al. 2008; Park et al. 2008; Valiante et al. 2009; Xu et al. 1998), defects in conidiation and spore germination (Igbaria et al. 2008; Jeon et al. 2008; Kojima et al. 2002; Mey et al. 2002; Xu et al. 1998), an absence of cell-cell fusion (Lichius et al. 2012; Maddi et al. 2012; Maerz et al. 2008), and impaired sexual fruiting body development (Hou et al. 2002; Kicka and Silar 2004; Lichius et al. 2012; Maddi et al. 2012; Maerz et al. 2008; Park et al. 2008; Xu et al. 1998). Radial growth of *E. festucae* $\Delta mkkA$ and $\Delta mpkA$ mutants on PDA and water agar was normal and the colony morphology of both mutants was identical (Fig. 6A) and indistinguishable from that of the wild-type strain (Fig. 1C). Light microscopy analysis showed that both $\Delta mkkA$ and $\Delta mpkA$ hyphae grew as attached bundles with branching restricted to

Fig. 4. Complementation with *mkkA* but not *pusA* rescues the cell fusion and plant symbiosis defects of TM1066. **A**, Colony morphology of *Epichloë festucae* mutants TM1066/*pusA* (PN2875) and TM1066/*mkkA* (PN2989) grown on potato dextrose agar for 5 days. **B**, Confocal laser-scanning and differential interference contrast microscopy images of hyphal anastomosis in TM1066/*mkkA* but not TM1066/*pusA* cultures grown on water agar coated slides and stained with Calcofluor white (CFW). Bar = 20 μ m. **C**, Number of hyphal fusions per septa beginning from the first contact point. Cultures of *E. festucae* wild-type (WT) *PltM-gusA* knock-in strain KM2.49, T-DNA mutant TM1066, and strain TM1066 containing *pusA* (TM1066/*pusA*) or *mkkA* (TM1066/*mkkA*) were grown on water-agar-coated slides, stained with CFW, and 10 fusion ladders each from four colonies were counted under the fluorescence microscope. Bars represent mean $\pm$ standard error of the mean ($n = 40$). Letters show significant differences determined by one-way analysis of variance ($P < 0.05$). **D**, Phenotype of perennial ryegrass plants infected with *E. festucae* WT, KM2.49, TM1066/*pusA*, and TM1066/*mkkA*. Photograph was taken 10 weeks postinoculation.

the subapical region, growth phenotypes similar to that of the wild type. As observed for TM1066, both mutants were defective in hyphal cell-cell fusion and formed intrahyphal hyphae, although the latter phenotype was observed more frequently in older cultures (Fig. 6B). To confirm the defect in cell-cell fusion, wild-type and mutant strains were transformed with constructs containing either eGFP or mRFP1 and analyzed for cell fusion events between the different strains after co-cultivation on water-agar-coated slides (Fig. 7). Whereas cytoplasmic mixing was observed between F11-eGFP and F11-mRFP1, no cytoplasmic mixing was observed between $\Delta mkkA$ -eGFP $\times$ $\Delta mkkA$ -mRFP1 and $\Delta mpkA$ -eGFP $\times$ $\Delta mpkA$ -mRFP1 or in mutant strains co-cultivated with the wild type.

Conidiation was enhanced in both $\Delta mkkA$ and $\Delta mpkA$, with the frequency in the former similar to that seen in TM1066, whereas the frequency of conidiation in the latter was five times greater than that of $\Delta mkkA$ and TM1066 (Fig. 3). The frequency of conidia germination appeared to be nutrient independent because similar numbers of colonies arose from spore suspensions spread on PDA as on water agar. Likewise, germ

tube morphology was the same under both growth conditions, with most conidia forming unipolar germ tubes upon germination (Fig. 6C). Occasionally, we observed concerted bipolar germ tube emergence in the mutants. Although this phenotype has not yet been observed in the wild-type strain, this may be due to the very low rate of sporulation that occurs in F11, making it more difficult to find them. Conidiophores in F11 frequently arise from hyphae that are organized as coil-like structures; laterals from these coils give rise to conidiophores from which holoblastic conidiation occurs. Close microscopic examination of the coil-like structures in the $\Delta mpkA$ and $\Delta mkkA$ mutants (Fig. 6C) revealed microcyclic conidiation in both mutants, a process previously reported for *E. typhina* ascospore germination (Bacon and Hinton 1991), where conidiophores arise directly from germinating spores to form more spores. Microcyclic conidiation was more frequent in $\Delta mpkA$ than in $\Delta mkkA$ and may account for the greater rate of conidiation observed in this mutant (Fig. 3).

The CWI MAPK pathway is dispensable for growth under stress conditions.

Deletion of the CWI MAPKK or MAPK in yeast and filamentous fungi generally results in an increased sensitivity to cell-wall stress agents and cell-wall-degrading enzymes (Hou et al. 2002; Kojima et al. 2002; Mey et al. 2002; Valiante et al. 2009). Therefore, we tested the effect of a range of stress agents on the vegetative growth of *E. festucae* TM1066 compared with KM2.49 and the $\Delta mkkA$ and $\Delta mpkA$ mutants compared with the wild-type strain F11 (Supplementary Fig. S6). Surprisingly, *E. festucae mkkA* and *mpkA* mutants grew as well as the wild type on media containing the chitin synthase inhibitor nikkomycin Z and the cell-wall stress agents Calcofluor white and Congo red. Treatment with sodium dodecyl sulfate (SDS), a chemical known to readily lyse cells with membrane defects, also had no effect on the growth rate of mutants over and above that seen with the wild type. Addition of caffeine, an alkaloid known to trigger activation of the CWI pathway in yeast, did not affect growth of mutants. In yeast, the CWI MAPK pathway is essential for response to high temperature but the growth rate of *E. festucae* $\Delta mkkA$ and $\Delta mpkA$ mutants at 30°C was the same as the wild type. We also tested sensitivity to osmotic and salt stress using 0.7 mM sorbitol and 0.3 mM NaCl but neither of these treatments affected the growth of mutants compared with the wild type. Treatment with the oxidative stress agents H₂O₂, diamide, and menadione revealed no differences between the growth of mutants and the wild type. These results suggest that *E. festucae* has a highly redundant system for protection against cell wall and other stresses.

The *E. festucae* CWI MAPK pathway is crucial for plant symbiosis.

Both $\Delta mkkA$ and $\Delta mpkA$ had an *L. perenne* symbiotic interaction phenotype very similar to TM1066; only a small number of plants were infected (4 to 15%) and these were severely stunted and underwent premature senescence (Fig. 6D). Hyphal growth within the pseudostem of these leaves was examined by fluorescence confocal microscopy (Fig. 8) and TEM (Fig. 9). In contrast to the restrictive growth of the wild-type strain, hyphae of the $\Delta mkkA$ and $\Delta mpkA$ mutants were much more prolific within the leaf tissue, had an irregular pattern of growth, and frequently formed highly convoluted structures (Fig. 8A4 and A6). Whereas just the septa of the wild-type hyphae stained with AF488-WGA, in the mutant hyphae, large patches of AF488 fluorescence (imaged in blue) were visible, suggesting a change in structure or composition of the cell wall resulting in greater accessibility of the cell-wall chitin to

Fig. 5. Phosphorylation and localization of MpkA. **A**, Western blot analysis of the wild type (WT) and TM1066, $\Delta mkkA$, and $\Delta mpkA$ mutants. Phosphorylated MpkA (47 kDa) and MpkB (41 kDa) were detected using anti-phospho p42/p44 MAPK antibodies. The membrane was reprobed with anti- α tubulin as loading control. **B**, Localization of MpkA-eGFP in regenerating hyphae after protoplasting. $\Delta mkkA$ and $\Delta mpkA$ cultures expressing MpkA-eGFP and stained with 4',6-diamidino-2-phenylindole were examined using confocal laser-scanning and differential interference contrast microscopy during growth through the antibiotic overlay layer. Bar = 10 μ m.

the fluorophore-labeled lectin (Fig. 8B1 and B3). The increased fluorescence of the host cells, particularly in tissues infected with $\Delta mpkA$, may be indicative of a defense response which could be a consequence of the change in cell-wall structure or composition of the mutant hyphae or to the degradation of host chloroplasts (Fig. 8A5 and B4). Mutant hyphae don't appear to fuse but, instead, grow alongside one another to form bundles of hyphae in the intercellular spaces as well as repeatedly fold around one another to form highly convoluted structures (Fig. 8A4, A5, B2, and B4). These structures were more frequently found adjacent to vascular bundles, where there is a greater proliferation of the mutant hyphae compared with wild-type hyphae.

As observed for TM1066, the $\Delta mkkA$ and $\Delta mpkA$ mutants extensively colonized the vascular bundles of the host tissue (Fig. 9A) and formed intrahyphal hyphae (Fig. 9C). Hyphae

within the vascular bundles were more cytoplasmically dense than those growing outside these tissues (Fig. 9A and B). The number of hyphae in the intercellular space was much greater in the mutants than the wild type (Supplementary Fig. S7). A close examination of the cell walls of the mutant revealed the absence of the darkly staining outer layer in some hypha that was present in the wild type (Fig. 9D), a change consistent with the increased fluorescence found with AF488-WGA staining and increased frequency of ruptured cells of the mutant hyphae (Fig. 8B1, B3, and A4).

To confirm that the $mkkA$ and $mpkA$ deletions were responsible for the altered symbiotic interaction phenotype and cell fusion defect, plasmids pYR33 and pYR32, containing the full-length $mkkA$ and $mpkA$ genes under the control of their native promoters and terminators, were transformed into protoplasts of $\Delta mkkA$ and $\Delta mpkA$, respectively. These transformants estab-

Fig. 6. Strain $\Delta mkkA$ and $\Delta mpkA$ culture and in planta phenotypes. **A**, Culture phenotype of *Epichloë festucae* cell-wall integrity MAPK mutants $\Delta mkkA$ and $\Delta mpkA$ grown on potato dextrose agar for 6 days. **B**, Confocal laser-scanning and differential interference contrast (DIC) microscopy images of hyphal morphology of *E. festucae* grown on water agar (1.5%) for 6 days. Cultures were stained with Calcofluor white (CFW) as indicated; $\Delta mkkA$ and $\Delta mpkA$ are fusion negative and form intrahyphal hyphae. Symbols: * = inner hypha and ** = outer hypha. Bar = 10 μ m. **C**, DIC microscopy images of $\Delta mkkA$ and $\Delta mpkA$ hyperconidiation and microcyclic conidiation. Symbols: c = conidium, cp = conidiophore, mcc = microcyclic conidium, mccp = microcyclic conidiophore, ht = hyphal tip. Bar = 10 μ m. **D**, Phenotype of perennial ryegrass plants infected with *E. festucae* wild-type and $\Delta mkkA$ and $\Delta mpkA$ mutants. Photograph was taken 10 weeks postinoculation.

lished a symbiotic interaction with perennial ryegrass like the wild type (Fig. 10A) and regained the ability to fuse in culture (Fig. 10B and C).

DISCUSSION

The CWI pathway is one of three conserved MAPK pathways found in filamentous fungi for transducing signals, perceived by upstream membrane localized environmental sensors, to downstream effectors (Levin 2005; Risipail et al. 2009). This particular signaling cascade is responsible for mediating changes to the cell wall during certain stages in the cell cycle and in response to various forms of stress. In filamentous fungi, this pathway is important for a number of cellular developmental processes, including hyphal fusion (Lichius et al. 2012; Maddi et al. 2012; Maerz et al. 2008), conidiation (Igbaria et al. 2008; Jeon et al. 2008; Kojima et al. 2002; Mey et al. 2002; Xu et al. 1998), appressorium development (Igbaria et al. 2008; Jeon et al. 2008; Xu et al. 1998), plant infection (Hou et al. 2002; Igbaria et al. 2008; Jeon et al. 2008; Kojima et al. 2002; Mey et al. 2002; Xu et al. 1998), and female fertility and fruiting body development (Hou et al. 2002; Kicka and Silar 2004; Lichius et al. 2012; Maddi et al. 2012; Maerz et al. 2008; Park et al. 2008; Xu et al. 1998). Here, we demonstrate

for the first time a key role for this signaling pathway in establishing and maintaining a mutualistic symbiotic interaction between *E. festucae* and its host, *L. perenne*. The inability of *E. festucae* CWI mutants to undergo hyphal fusion appears to be a crucial developmental defect that leads to breakdown of this fungus–plant symbiotic interaction.

Homologues of nearly all components of the *S. cerevisiae* CWI pathway have been identified in the genomes of *E. festucae* and other filamentous fungi, including the three-tiered kinase module comprising a MAPKKK, a MAPKK, and the MAPK (Eaton et al. 2011; Risipail et al. 2009). Complementation analysis demonstrated that deletion of the *E. festucae* *mkkA* (*MKK1*), rather than *pusA* (Chan and Huang 2009) was responsible for the culture and symbiosis phenotypes observed for the *A. tumefaciens* T-DNA-induced deletion mutant, TM1066. Gene deletions or genome rearrangements appear to be a common outcome of T-DNA-induced mutagenesis in filamentous fungi (Choi et al. 2007; Jeon et al. 2007). A targeted deletion of *mkkA* recapitulated the phenotypes observed for TM1066, as did deletion of the downstream *mpkA*, providing strong support that MkkA and MpkA are components of the same MAPK pathway. This was confirmed by the lack of MpkA phosphorylation in the $\Delta mkkA$ background.

Fig. 7. Strain $\Delta mkkA$ and $\Delta mpkA$ cell-cell fusion culture phenotypes. *Epichloë festucae* wild-type (WT) and cell-wall integrity mutants $\Delta mkkA$ and $\Delta mpkA$, expressing either eGFP (WT: PN2833; $\Delta mkkA$: PN2996 $\Delta mpkA$: PN2998) or mRFP1 (WT: PN2832; $\Delta mkkA$: PN2997 $\Delta mpkA$: PN2999), were co-cultured on water agar for 5 days either with themselves or in combination with the WT and then examined for cell–cell fusion by confocal microscopy. Hyphal anastomoses (circled) were detected as appearance of both fluorescent proteins in the same hyphal cell in the WT–WT combination. No anastomoses were observed in the mutant–mutant and mutant–WT combinations. Bar = 10 μ m.

Both *mkkA* and *mpkA* mutants are defective in cell–cell fusion when grown on water agar, a culture phenotype observed for four other *E. festucae* mutants that have a severe plant interaction phenotype, including $\Delta noxA$ and $\Delta noxR$, genes that en-

code components of the Nox complex (Kayano et al. 2013); $\Delta proA$, a gene encoding a $Zn(II)_2Cys_6$ transcription factor (Tanaka et al. 2013); and Δso , a gene of unknown function (Charlton et al. 2012). Hyphal fusion is an important mecha-

Fig. 8. Strain $\Delta mkkA$ and $\Delta mpkA$ in planta cellular phenotypes. Confocal depth series images of aniline blue- or Alexafluor 488 WGA-stained *Lolium perenne* leaf sheath tissue infected with the wild type (WT), $\Delta mkkA$, and $\Delta mpkA$. Hyphae stained with aniline blue appear as red and chitin in septa and cell wall stained with Alexafluor 488-WGA as blue. Chloroplasts of plant cells appear as light orange. Leaf images were captured 7 weeks after inoculation. Bar = 20 μm .

Fig. 9. Strain $\Delta mkkA$ and $\Delta mpkA$ in planta cellular phenotypes compared with the wild type (WT). Transmission electron micrographs of cross-sections of *Lolium perenne* pseudostems infected with the WT endophyte and $\Delta mkkA$ and $\Delta mpkA$ deletion mutants. **A**, Hyphae in vascular bundles of mutant-infected plants. **B**, Hyphal colonization of plant intercellular spaces. **C**, Intrahyphal hyphae formed by $\Delta mkkA$ and $\Delta mpkA$ strains. **D**, Close up of cell walls of WT and $\Delta mkkA$ and $\Delta mpkA$ strains. Symbols: * = inner hypha, ** = outer hyphae, h = hypha, pc = plant cell. Bar = 2 μm (A and B) or 0.2 μm (C and D).

nism for establishment of a colony hyphal network, enabling sharing of resources and interhyphal communication (Fricker et al. 2007; Leeder et al. 2013; Read et al. 2009; Simonin et al. 2010). In *N. crassa*, there are two developmental stages where fusion is important. When conidia germinate, they initially put out small germ tubes called CATs, which home toward one another, then fuse (Roca et al. 2005a and b; Read et al. 2009, 2010). As the colony size increases, lateral hyphal branches also home and fuse, allowing the rapid development of an interconnected hyphal network (Hickey et al. 2002; Read et al. 2009, 2010). Many genes defective in hyphal anastomosis (*ham*) have been identified, principally by screening for a defect in heterokaryon formation (Aldabbous et al. 2010; Fu et al. 2011). Formation of CATs in *N. crassa* involves oscillation of key signaling network proteins at the tips of hyphae about to fuse, the paradigm for this being the oscillation of SO, a protein of unknown function, with MAK-2, the MAPK of the pheromone response pathway (Fleissner et al. 2009).

In contrast to *N. crassa*, development of conidia in axenic cultures of *E. festucae* is rather sparse, making it technically

difficult to study colony establishment from germinating conidia. Instead, we use small blocks of vegetative hyphae as the initial inoculum. A distinct feature of *E. festucae* hyphal growth under these nonsymbiotic conditions is the adherence of individual hyphae to one another to form bundles of hyphae, interconnected by branches, that radiate from the center of the colony rather than the highly branched single hyphae network observed for *N. crassa* and other filamentous fungi (Scott et al. 2012). A distinctive feature of this pattern of growth is the formation of lateral hyphal fusions, observed as small pegs between adjacent hyphae, rather than the more typical tip-to-tip fusions from lateral branches. However, the location of these hyphal connections on the lateral edges of the cells belies the origin of the underlying hyphal fusion mechanism. Microscopy of growing hyphae revealed that hyphal anastomosis in *E. festucae* occurs by fusion of the tip of a growing hyphal cell with the lateral edge of a subapical adjacent hypha cell (so-called tip-to-side fusion) (Hickey et al. 2002), followed by repolarization of the tip cell and ongoing tip growth: a fusion process very similar to that observed in *F. oxysporum* (Ruiz-

Fig. 10. In planta complementation and fusion counts of $\Delta mkkA$ and $\Delta mpkA$ complementation strains compared with the wild type (WT). **A**, Phenotype of perennial ryegrass plants infected with *Epichloë festucae* WT and complemented deletion mutants of $\Delta mkkA$ ($\Delta mkkA/mkkA$) and $\Delta mpkA$ ($\Delta mpkA/mpkA$). Photographs were taken 10 weeks postinoculation. **B**, Confocal laser-scanning and differential interference contrast microscopy images of hyphal anastomosis in complemented strains of cultures grown on water-agar-coated slides stained with Calcofluor white (CFW). **C**, Number of hyphal anastomosis of *E. festucae* WT and complemented strains. Cultures grown on water-agar-coated slides were stained with CFW and 10 fusion ladders each from four colonies were counted under the fluorescence microscope. Bars represent mean $\pm$ standard error of the mean ($n = 40$). Letters show significant differences determined by one-way analysis of variance ($P < 0.05$).

Roldán et al. 2010). A series of sequential fusions by the tip of actively growing hypha with a subapical cell of an adjacent hypha gives rise to a ladder-like hyphal network.

Another striking phenotype of the *mkkA* and *mpkA* mutants was the formation of intrahyphal hyphae. These structures have been observed in a number of naturally occurring fungi within the ascomycetes and are thought to form under conditions of hyphal stress or in response to cell damage (Buller 1933; Calonge 1968; Chan and Stephen 1967). A very detailed TEM analysis of intrahyphal hyphae in *Sclerotinia fructigena* provides important insights as to how these structures arise from parental hyphae at the ultrastructural level (Calonge 1968). A secondary cell wall is formed in the parent cell that is closely attached to the outer wall. From here, the new hypha pushes through the plugged septal pore into the adjacent hypha. The internal hypha is able to form septa and eventually exits the enveloping hypha. Interestingly, intrahyphal hyphae have also been observed in other mutants that perturb CWI, including *Aspergillus nidulans csmA*, encoding a class V chitin synthase (Horiuchi et al. 1999), and *N. crassa gpi-3* and *gpi-1*, encoding the glycoposphatidylinositol (GPI) anchor biosynthetic pathway enzymes, GPI *N*-acetylglucosamine transferase and GPI transaminase (Bowman et al. 2006). The increased sensitivity of these mutants to cell-wall stress agents such as Congo red and Calcofluor white is indicative of defects in CWI. Although the *E. festucae mkkA* and *mpkA* mutants were not defective in aerial growth, were not inhibited by the chitin synthase inhibitor nikkomycin Z, and were tolerant to a wide range of cell-wall stress agents, including Calcofluor white, Congo red, SDS, and caffeine, the propensity to form intrahyphal hyphae is indicative of an underlying defect in CWI.

The mechanisms underlying the formation of intrahyphal hyphae are not yet understood but studies on Woronin body biogenesis and cellular dynamics of these organelles are providing some important insights. Woronin bodies are important organelles that seal the septal pore in response to wounding and are found exclusively within the filamentous ascomycetes (Pezizomycotina). A key component of this organelle is the Woronin body sorting complex (WSC), which is required for both biogenesis and partitioning of the organelle within the cell (Liu et al. 2008). Deletion of *wsc* in *N. crassa* increases the formation of hexagonal rods of the HEX structural protein (HEX assemblies) at hyphal tips but depletes assemblies in subapical compartments. This mutation also leads to formation of intrahyphal hyphae, a response triggered by septal pore sealing in damaged hyphae. Both *hex* and *pex14* mutants fully suppress these *wsc* growth phenotypes. Intrahyphal hyphae are also observed in a mutant of the septal pore-associated (SPA) protein, SPA9 (Lai et al. 2012). This protein is one of a set of nonhomologous proteins that localize as rings at the septal pore. Upon cellular wounding, a Woronin body is translocated to the closest septal pore, where it associates with the SPA proteins to form an occlusion complex. Inactivation of SPA9 appears to trigger aberrant activation of Woronin bodies, leading to a reduction in radial growth and formation of intrahyphal hyphae.

While hyphal anastomosis on water agar is blocked in the *mkkA* and *mpkA* mutants, conidiation is derepressed, a phenotype also observed for $\Delta noxA$, $\Delta noxA/B$, and $\Delta proA$ (Kayano et al. 2013; Tanaka et al. 2013). These common culture phenotypes suggest that the NoxA Nox complex, ProA, and the CWI MAPK pathway are all components of a cellular regulatory network that controls hyphal fusion and conidiation. In contrast, disruption of components of the CWI MAPK pathway in most other filamentous fungi results in either reduction or complete abolition of conidiation (Igbaria et al. 2008; Jeon et

al. 2008; Kojima et al. 2002; Mey et al. 2002; Xu et al. 1998); an exception being *N. crassa* CWI mutants, which show an increase in arthroconidiation (Maerz et al. 2008; Park et al. 2008). Light and scanning electron microscopy examination of asexual development in *E. festucae* suggests that conidiophores arise from individual hyphae and hyphae organized as coiled structures (Scott et al. 2012). In this study, these coiled structures were more frequently observed in the *mkkA* and *mpkA* mutants than in the wild-type strain. A close examination of the conidiophores arising from these structures revealed that many of the conidia released immediately germinate to give rise to a new conidiophore rather than vegetative hyphae, a process known as microcycle conidiation (Anderson and Smith 1971). The greater frequency of microcycle conidiation in the *mpkA* mutant probably accounts for the greater number of conidia observed for this mutant compared with the *mkkA* mutant. There are numerous reports of microcycle conidiation by fungi grown under laboratory conditions but the role of this process in nature is not clear (Hanlin 1994; Smith et al. 1981). Bacon and Hinton (1991) proposed that microcycle conidiation might be a mechanism for increasing spore numbers, thereby enhancing plant infection. Floral infection by conidia appears to be the main route for horizontal transfer of *Epichloë* and *Claviceps* species in nature (Chung and Schardl 1997; Pazantova et al. 1978). In the case of *E. typhina*, ascospores released by perithecia from stromata on the leaves of the grass host germinate to produce conidia rather than vegetative hyphae and these conidia, in turn, undergo microcycle conidiation to generate large numbers of conidia which infect the ovules of florets (Bacon and Hinton 1991). Once the hyphae colonize the vegetative tissues of the host, conidiation is suppressed. Our work here suggests that the CWI MAPK pathway is an important signaling system for controlling conidiophore development and microcycle conidiation in *E. festucae*. Based on our observation that the *mpkA* mutant produces significantly more conidia than the *mkkA* mutant, and the fact that MpkA phosphorylation but not localization to the nuclei of arthroconidia is MkkA dependent, we propose that nuclear localization of MpkA is more important for repression of conidiation than its phosphorylation status. In contrast, conidiation in *F. graminearum* seems to be regulated by Mkk1 (=MkkA/MEK1) independent of the downstream-acting MpkA homolog Mgv1 (Yun et al. 2013).

The severe host interaction phenotype observed for the *mkkA* and *mpkA* mutants was very similar to that previously reported for the *E. festucae* $\Delta noxA$, $\Delta noxR$, $\Delta racA$, $\Delta proA$, and Δso mutants (Charlton et al. 2012; Takemoto et al. 2006; Tanaka et al. 2006, 2008, 2013); seedlings either died or formed severely stunted plants that underwent premature senescence. This host phenotype may be the result of an upregulation of host defense genes, including the pathogenesis-response (PR) genes, as previously observed for plants infected with *E. festucae* stress-activated MAPK (*sakA*) and Nox (*noxA*) mutants (Eaton et al. 2010; Tanaka et al. 2006). Instead of the restrictive pattern of hyphal growth observed for plants infected with the wild-type strain, the *mkkA* and *mpkA* mutants had a proliferative pattern of growth. There was an increase in the number of hyphae in the intercellular spaces of the leaves and colonization of the large vascular bundles, phenotypes not seen in wild-type associations. The apparent increase in host “virulence” observed for the *E. festucae* CWI MAPK mutants and related symbiosis mutants is in contrast to a reduction in virulence observed for the same mutations in a number of phytopathogenic fungi (Hou et al. 2002; Igbaria et al. 2008; Jeon et al. 2008; Kojima et al. 2002; Mey et al. 2002; Xu et al. 1998). In some cases, the inability of these fungi to infect the host was due to a defect in appressorium peg formation, thereby preventing breach

of the host cuticle (Igbaria et al. 2008; Jeon et al. 2008; Xu et al. 1998). But even when this step was bypassed by direct injection of hyphae into the plant, colonization of the host was still blocked (Jeon et al. 2008), a result that suggests that the CWI MAPK pathway is required for specific pathogen differentiation steps and invasive growth of the host. The pattern of proliferative growth observed for the *mkkA* and *mpkA* mutants appears to be a consequence of the inability of the lateral branches of hyphae, that grow between the junctions of the columns of plant cells that separate individual hyphae, to undergo cell–cell fusion. Instead of forming hyphal bridges through columns of plant cells that separate longitudinal hyphae, the lateral branches appear to grow past and around one another to form complex bundles of unfused hyphae. The failure of these mutants to form an interconnected symbiotic hyphal network within the leaf could lead to a breakdown in intrahyphal communication and nutrient transfer, possibly triggering a nutrient starvation response.

In addition to the fusion defect, TEM revealed major structural changes to the fungal cell wall; the outer cell-wall layer of some hyphae appears to be absent in both the *mkkA* and *mpkA* mutants. Consistent with this ultrastructural change was the unmasking of cell-wall chitin as detected by the increase in hyphal cell-wall fluorescence following staining of the leaves with Alexafluor 488 WGA. In leaves infected with the wild type, only the septa of the hyphae fluoresce; whereas, in leaves infected with the *mkkA* and *mpkA* mutants, large sections of hyphae fluoresce. This unmasking of *E. festucae* cell-wall chitin is likely to cause a pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) response in the host, which may account for the dramatic whole-plant and cellular responses observed in plants infected with *mkkA* and *mpkA* mutants. In the *M. oryzae*–rice pathogenic interaction, fungal cell-wall PAMPs are masked by α -1,3 glucans, thereby enhancing the virulence of the pathogen (Fujikawa et al. 2009, 2012). However, the absence of a gene encoding α -1,3-glucan synthase in the *E. festucae* genome does not support a role for α -1,3-glucans in chitin masking. In the *Cladosporium fulvum*–tomato pathogenic interaction, the fungal small-secreted effector proteins Avr4 and Ecp6 have been shown to bind chitin, thereby enhancing the virulence of the pathogen (de Jonge et al. 2010; van den Burg et al. 2006; van Esse et al. 2007). Avr4 is proposed to bind to chitin in the fungal cell wall, thereby preventing hydrolysis of the cell wall by plant chitinases and release of chitin fragments that could trigger a host immune response (van den Burg et al. 2006; van Esse et al. 2007). Ecp6 is proposed to sequester chitin oligosaccharides released from the cell wall of invasive hyphae, thereby preventing PTI (de Jonge et al. 2010). In an RNAseq comparison of genes differentially expressed between the *E. festucae* wild type and Δ *sakA*, a number of genes encoding small-secreted proteins, including a chitinase, were identified; however, whether any of these fulfil similar roles to Avr4 and Ecp6 in the *E. festucae*–*L. perenne* symbiosis remains to be determined (Eaton et al. 2010).

In conclusion, we have demonstrated that the CWI MAPK pathway is a key signaling pathway for controlling the mutualistic symbiotic interaction between *E. festucae* and *L. perenne*. The similarities in both culture and plant interaction phenotypes of the *mkkA* and *mpkA* mutants with the *noxA*, *noxR*, *proA*, and *so* mutants would suggest that these genes are components of a conserved regulatory network controlling fungus–plant symbiosis. In other fungi, this same regulatory network is used for establishment of vegetative hyphal networks, sexual fruiting body development, and host colonization, underscoring its general importance for regulating multicellular developmental processes in fungi. Identifying the other components of this regula-

tory network and the connections between them will be of considerable future interest.

MATERIALS AND METHODS

Strains and growth conditions.

Escherichia coli cultures were grown overnight in Luria-Bertani (LB) broth or on LB agar, as previously described (Miller 1972).

Cultures of *Epichloë festucae* were grown on 2.4% PDA and 1.5% water agar under conditions previously described (Moon et al. 1999, 2000). To test for stress sensitivity, cultures were grown on PDA supplemented with 0.01% SDS, 3 mM caffeine, nikkomycin at 50 μ g/ml, 0.7 mM sorbitol, 0.3 mM NaCl, 7 mM H₂O₂, 1 mM diamide, and 40 mM menadione. Sensitivity to Calcofluor white (Fluorescent Brighter 28; Sigma) at 10 and 25 μ g/ml and 50 mM Congo red was tested on buffered PDA (50 mM HEPES, pH 6.5) to ensure that the test compounds remained soluble (Ram and Klis 2006). For temperature stress, strains were grown on PDA at 30°C. A description of all biological material is provided in Supplementary Table S1.

Plant growth and endophyte inoculation conditions.

Endophyte-free seedlings of perennial ryegrass (*L. perenne* ‘Samson’) were inoculated with *E. festucae* by the method of Latch and Christensen (1985). Plants were grown in root trainers in an environmentally controlled growth room at 22°C with a photoperiod of 16 h of light (approximately 100 μ E/m²/s).

DNA isolation, PCR, TAIL-PCR, and sequencing.

Fungal genomic DNA was extracted from freeze-dried mycelium by the method of Byrd and associates (1990). Isolation of plasmid DNA was performed using a High Pure plasmid isolation kit (Roche) according to the manufacturer’s instructions. Standard PCR and TAIL-PCR amplification was performed with *Taq* DNA polymerase (Roche). When proofreading activity was required, PCR was performed with the Expand High-Fidelity PCR system (Roche) or Phusion High-Fidelity DNA polymerase (NEB). The reaction mixture for *Taq* DNA polymerase or the Expand High-Fidelity PCR system (50 μ l) contained 1 $\times$ *Taq* PCR Reaction buffer or 1 $\times$ Expand High-Fidelity Buffer, 0.2 μ M forward and reverse primers, 200 μ M dNTPs, 1 ng of template DNA, and 1 U of *Taq* polymerase or 1.75 U of Expand High-Fidelity Enzyme Mix (Roche). The cycle conditions used were 1 cycle at 94°C for 2 min; 36 cycles at 94°C for 15 s, 50 to 65°C for 30 s, and 68 to 72°C for 1 min/kb; followed by a final extension cycle of 7 min at 68 to 72°C. Reaction mixtures were stored at 4°C until analyzed. The reaction mixture for Phusion High-Fidelity DNA polymerase (50 μ l) contained 1 $\times$ Phusion HF buffer, 0.5 μ M forward and reverse primers, 200 μ M dNTPs, 1 ng of template DNA, and 1 U of Phusion High-Fidelity DNA polymerase. The cycle conditions used were 1 cycle at 98°C for 30 s; 10 cycles at 98°C for 10 s, 50 to 65°C for 30 s, and 72°C for 30 s/kb; 20 cycles at 98°C for 10 s and 72°C for 30 s/kb; followed by a final extension cycle of 5 min at 72°C. TAIL-PCR was performed as previously described (Liu and Whittier 1995; Terauchi and Kahl 2000), with minor changes described by Giesbert and associates (2012). Lists of the primer sequences used for TAIL and standard PCR can be found in Supplementary Tables S2 and S3.

DNA fragments were sequenced using the dideoxynucleotide chain termination method with the Big-Dye Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied BioSystems) and separated using an ABI3730 genetic analyzer (Applied BioSystems). Sequence data were assembled and analyzed using the MacVector sequence assembly software, version 12.0.5.

DNA hybridization.

E. festucae genomic digests separated by agarose gel electrophoresis were transferred to positively charged nylon membranes (Roche) and fixed by UV light cross-linking in a Cex-800 UV light cross-linker (Ultra-Lum) at 254 nm for 2 min. DNA was labeled by random priming with Klenow DNA polymerase and [α - 32 P]-dCTP (3,000 Ci/mmol; Amersham Biosciences), using a High Prime DNA labeling kit (Roche), or by the incorporation of digoxigenin (DIG)-11-dUTP into DNA by PCR, using a PCR DIG probe synthesis kit (Roche). Hybridizations were performed according to the manufacturer's instructions and visualized by either autoradiography (32 P-labeled probes) or by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate color detection (DIG-labeled probes).

Preparation of expression and deletion constructs.

The *PltmM-gusA* knock-in vector pKM6 (PN1921) was generated by cloning a 2.6-kb *Xma*I fragment containing *uidA-TtrpC* from *phFunGus* into pCY39 (*ltmM* KO construct, PN1856) (Young et al. 2005).

The *mpkA* and *mkkA* replacement constructs were generated using yeast recombinational cloning (Colot et al. 2006). Phusion High-Fidelity DNA polymerase (NEB) was used to amplify the fragments. For generation of the *mpkA* replacement construct, pCE64 (PN4155), a 968-bp PCR fragment 5' of *mpkA*, and a 1,315-bp PCR fragment 3' of *mpkA* were amplified. For generation of the *mkkA* replacement construct, pCE63 (PN4154), a 1,515-bp PCR fragment 5' of *mkkA*, and a 1,432-bp PCR fragment 3' of *mkkA* were amplified. To facilitate yeast recombinational cloning, primers for amplification of the *mpkA* and *mkkA* 5' flanking fragments contained overlap to the yeast vector pRS426 (YB165 and YB169) and to the *hph* hygromycin resistance cassette (YB166 and YB170), and primers for amplification of the 3' flanking fragment contained overlap to the *hph* resistance cassette (YB167 and YB171) and to pRS426 (YB168 and YB172). The *PtrpC-hph* cassette was amplified with primers YB173 and YB174. Yeast cells were transformed with *Eco*RI/*Xho*I-linearized pRS426 together with PCR-amplified fragments of the *PtrpC-hph* cassette and *mpkA* or *mkkA* 5' and 3' flanking regions, as previously described (Gietz and Woods 2002). Transformants were selected on media lacking uracil, and plasmid DNA were isolated and transformed into *E. coli*.

The *pusA* complementation construct pYR29 was prepared by cloning the full-length *pusA* gene containing 905-bp 5' and 595-bp 3' PCR amplified from *E. festucae* Fl1 genomic DNA with primers YB124 and YB125 into pCR4-TOPO (Invitrogen).

The *mkkA* complementation constructs pYR31 and pYR33 were prepared by cloning the full-length *mkkA* gene containing 998-bp 5' and 1,682-bp 3' of *mkkA* amplified from *E. festucae* Fl1 genomic DNA with primers YB118 and YB119 into pAN8-1 (containing a *ble* cassette) and pII99 (containing the *npII* cassette), respectively.

The *mpkA* complementation construct pYR32 was prepared by cloning the full-length *mpkA* gene containing 1-kb 5' and 446-bp 3' of *mpkA* amplified from *E. festucae* Fl1 genomic DNA with primers 137 and 138 into pSF17.1, a pII99-based vector containing the *npII* cassette (Tanaka et al. 2008).

E. festucae transformation and screening for mutants.

For *A. tumefaciens* T-DNA-mediated mutagenesis, *A. tumefaciens* EH105 (PN1828) was used to transform *E. festucae* strain KM2.49 (PN2599), as previously described (Tanaka et al. 2007, 2013).

E. festucae protoplasts were prepared as previously described (Young et al. 2005). Protoplasts were transformed with 3 to 5 μ g

of linear PCR-amplified, restriction enzyme digested, or circular plasmid DNA using the method previously described (Itoh et al. 1994). Transformants were selected on regeneration medium (PD with 0.8 M sucrose) containing either hygromycin (150 μ g/ml), geneticin (200 μ g/ml), zeocin (250 μ g/ml), or nourseothricin (250 μ g/ml) and nuclear purified by three rounds of subculturing on PD medium containing the same antibiotic selection.

To generate the *PltmM-gusA* knock-in, Fl1 protoplasts were transformed with a linear *Fsp*I/*Sap*I pKM6 fragment and transformants were selected on medium containing hygromycin at 150 μ g/ml. Transformants were screened with primers lol134 and lol135 to distinguish the wild type (1.7 kb) from replacement sequences (4.2 kb).

For *pusA* complementation of the T-DNA mutant TM1066, protoplasts were co-transformed with pYR29 and pNR1 and transformants selected on medium containing nourseothricin (250 μ g/ml). Reintroduction of the *pusA* gene was confirmed by PCR using primers YB163 and YB164.

For *mkkA* complementation of the T-DNA mutant TM1066, protoplasts were co-transformed with pYR31 and pAN8-1 and transformants were selected on medium containing zeocin (250 μ g/ml). Reintroduction of the *mkkA* gene was confirmed by PCR using primers YB148 and YB149.

Putative *mkkA* replacement mutants were screened by PCR using primers that flank the *hph* cassette (YB175 and YB176; 2.5 kb for wild-type sequence and 1.5 kb for replacement sequence) and the 5' (YB181 and TC45; 2.2 kb) and 3' (YB182 and TC44; 2 kb) flanking regions of the replacement. Deletion of the *mkkA* region was confirmed by PCR using primer YB185 and YB186 (457 bp in the wild type).

Putative *mpkA* replacement mutants were screened by PCR using primers that flank the *hph* cassette (YB177 and YB178; 1.3 kb for the wild type and 1.5 kb for replacement sequence) and the 5' (YB179 and TC45; 1.6 kb) and 3' (YB180 and TC44; 2 kb) flanking regions of the replacement. Deletion of the *mpkA* region was confirmed by PCR using primer YB183 and YB184 (329 bp in the wild type).

For complementation of knock-out strains, protoplasts of Δ *mkkA*4-2 and Δ *mpkA*10.8 were transformed with pYR33 and pYR32, respectively, and transformants selected on medium containing geneticin (200 μ g/ml). Reintroduction of the *mkkA* or *mpkA* gene was confirmed by PCR using primers YB148 and YB149 (*mkkA*) and YB183 and YB184 (*mpkA*), respectively.

mRFP and eGFP transformants of the *E. festucae* wild type, Δ *mkkA*, and Δ *mpkA* were generated by transforming protoplasts of each of these strains with pCA56 (pToxA-mRFP) and pCT74 (pToxA-eGFP), respectively (Andrie et al. 2005).

Western blot analysis.

E. festucae cultures were grown for 5 days in 50 ml of PD at 22°C and 180 rpm. After harvesting, the mycelium was washed three times with sterile Milli-Q water, frozen in liquid nitrogen, and freeze dried overnight. The mycelium was ground to a fine powder under liquid nitrogen. For protein extraction, 50 mg of the mycelium powder was homogenized in 1 ml of lysis buffer (50 mM Tris-HCl [pH 8], 100 mM NaCl, 10 mM EDTA, IGEPAL CA-630 [Sigma-Aldrich] at 1 μ l/ml, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and phosphatase inhibitor [Sigma-P5726] at 10 μ l/ml) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was retained and centrifuged a second time as described above. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad catalog number 500-0006) following the manufacturer's instructions. Samples of protein (50 μ g each) were separated on a 10% (wt/vol) SDS polyacrylamide gel and transferred to polyvinylidene difluoride mem-

branes (Roche). The phosphorylation of MpkA and MpkB was examined using anti-phospho-p44/42 MAPK (Erk1/2) antibody (number 9102; Cell Signalling Technology). To check the amount of protein loaded for each sample, the membrane was reprobed with 12G10 anti- α -tubulin (DSHB). Primary antibodies were detected using HRP-conjugated secondary antibodies and ECL Prime Western Blotting Detection Reagent (GE Healthcare Amersham).

Microscopy.

Cultures to be analyzed by microscopy were inoculated at the edge of a thin layer of PD agarose (1.5%) or water agarose (1.5%) layered on top of a glass microscopy slide embedded in a layer of PDA (1.5%) or water agar (1.5%) and grown for 5 days. Images of Calcofluor white (Fluorescent Brighter 28; Sigma)-stained hyphae and eGFP and mRFP1 fluorescence in *E. festucae* hyphae were captured by confocal laser-scanning microscopy using a Leica SP5 DM6000B confocal microscope (488 nm argon laser, $\times 40$ oil immersion objective, numerical aperture = 1.3) (Leica Microsystems) or an Olympus IX71 inverted fluorescence microscope. For statistical analyses of hyphal fusion, 10 fusion sites were analyzed starting from the tip of a hypha and counting fusions along the two partner hyphae as well as the number of septa of each hypha. Fusion frequency was calculated as the number of fusions per septum from four independent colonies. For analyses of conidiation, four PDA plates with four colonies of each strain were grown at 22°C for 6 days. Spores were harvested by scrubbing the colonies with 3 ml of sterile water. The suspension was filtered through glass wool filtered tips and 100, 200, and 300 μ l was spread on PDA (1.5%) or water agar (1.5%) plates. Spore germination and colony formation were monitored after 4 to 5 days. MpkA-gfp localization and 4',6-diamidino-2-phenylindole staining of arthroconidia was performed as previously described (Cartwright et al. 2014).

Growth and morphology of *E. festucae* hyphae in planta was determined by staining leaves with aniline blue diammonium salt (Sigma) and AlexaFluor-488 WGA (Molecular Probes) as follows. Tillers of perennial ryegrass infected with *E. festucae* were soaked in 95% (vol/vol) ethanol overnight at 4°C, then treated with 10% potassium hydroxide for 3 h. The tissue was washed three times with phosphate-buffered saline (PBS) (pH 7.4) and incubated in staining solution (0.02% aniline blue, AF488-WGA at 10 ng/ml, and 0.02% Tween 20 in PBS [pH 7.4] for 5 min, followed by 30 min of vacuum infiltration). Images of *E. festucae* hyphae and septa in planta were captured by confocal laser-scanning microscopy using a Leica SP5 DM6000B confocal microscope (488 nm argon and 561 nm DPSS laser, $\times 40$ oil immersion objective, NA = 1.3) (Leica Microsystems).

For TEM, pseudostem sections were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h, as described by Spiers and Hopcroft (1993). A Philips CM10 TEM was used to examine the fixed samples and the images were acquired using an SIS Morada digital camera.

Bioinformatic analysis and accession numbers.

E. festucae genes were identified by tBLASTn analysis of the *E. festucae* F11 (E894) genome with protein sequences obtained from either the National Center for Biotechnology Information GenBank database or the Broad Institute. The *E. festucae* genome sequence data (Schardl et al. 2013) is available from the University of Kentucky. Identity and similarity scores were calculated after ClustalW pairwise alignments of sequences, using MacVector version 12.0.5. Multiple sequence alignments were edited using Jalview. For protein functional analysis, InterProScan 4 EMBL/EBI was used.

The *pusA*, *mkkA*, and *mpkA* gene sequences of *E. festucae* strain F11 (894) are available in the GenBank database under accession numbers KJ605834 (*pusA*), KJ605833 (*mkkA*), and KJ605832 (*mpkA*), respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute: www.broad.mit.edu
E. festucae genome sequence data: csbio-l.csr.uky.edu/ef894-2011
 GenBank: www.ncbi.nlm.nih.gov/genbank/index.html
 InterProScan 4 EMBL/EBI: www.ebi.ac.uk/Tools/pfa/iprscan
 Jalview: www.jalview.org
 National Center for Biotechnology Information: www.ncbi.nlm.nih.gov