The fusion of vegetative cells from a single individual, or self-anastomosis, is one of the basic characteristics of fungal mycelia in the majority of filamentous fungi. Hyphal anastomosis establishes the interconnection of individual hyphal strands into an integrated network, presumably allowing for distribution and sharing of water, nutrients, and signals throughout a mycelium (1, 18). As such, self-anastomosis may promote efficient fungal colonization in natural environments where resources are heterogeneously distributed.

Our understanding of the molecular mechanisms underlying hyphal anastomosis has significantly improved in the past decade, largely through the characterization of mutant strains of the model filamentous fungus Neurospora crassa that lack the ability to anastomose (4, 12–14, 17, 21, 22, 28, 31, 34, 38, 44; see references 15, 30, and 32 for reviews). Of the genes documented thus far as contributors to anastomosis, perhaps the best characterized is the soft (so) gene of N. crassa, which encodes a protein that is largely undefined but contains a WW domain that typically facilitates protein-protein interactions. The so gene has been shown to have an essential role in hyphal anastomosis, presumably by regulating the release of an undefined chemoattractant under the control of the mitogen-activated protein kinase (MAPK) MAK-2 (13, 14, 32).

In contrast to these recent advances in our understanding of the molecular basis for hyphal anastomosis, our knowledge of its physiological role has lagged far behind. This may be attributed to the fact that studies on hyphal anastomosis have been performed mainly using model filamentous fungi under laboratory conditions. One would imagine that the importance of hyphal anastomosis (e.g., in the transport of water, nutrients, and signals across a mycelium) would be less pronounced when a fungus is growing on an artificial medium where nutrients and water are homogeneously distributed. Thus, empirical studies conducted under artificial conditions may significantly underestimate the physiological importance of hyphal anastomosis. This issue is probably magnified for fungi that live in close association with other organisms, such as pathogenic and endophytic fungi, whose natural habitats have exceedingly little in common with artificial growth conditions.

To date, two studies have addressed the roles of hyphal anastomosis in fungal pathogen-host plant interactions. Craven et al. demonstrated that a strain with a deletion of the so ortholog in a pathogen of crucifers, Alternaria brassicicola, lacks hyphal anastomosis and virulence (7). In contrast, Prados Rosales and Di Pietro showed that a hyphal anastomosis mutant of Fusarium oxysporum lacking the so ortholog loses the ability to efficiently colonize tomato roots, but its virulence is only slightly decreased (29). These findings suggest that the extent to which anastomosis contributes to pathogenicity might depend on the individual infection strategies of different phytopathogenic species (30).

Epichloë endophytes are fungal symbionts of cool-season grasses that generally grow as sparse unbranched hyphae in the intercellular spaces of host tissues (5). They are known to confer protection to the host plant against a wide range of biotic and abiotic stresses (2, 3, 6, 23, 43). Thus, fungal phytopathogens and endophytes represent two poles of fungus-plant interactions with similar habitats but opposite outcomes, both feeding on plants but one killing the host, whereas the other forms a mutual alliance with the host plant. Therefore, fungal endophytes provide an excellent opportunity to study further the physiological importance of hyphal anastomosis in the natural environment.

In this study, we deleted the so ortholog of the endophytic symbiont Epichloë festucae and inoculated the E. festucae so dele-
TABLE 1 Primers used in this study

| Primer         | Sequence (5' to 3')               | Function                          |
|---------------|-----------------------------------|-----------------------------------|
| so1_5'flank_F | CGCAAGCTTTAGTTCTCAGTGGTTGATCTCT   | Cloning of the so1 5' flanking region |
| so1_5'flank_R | CGCCCTTACGGTCTCTGACGTGCTTCTCT     | Cloning of the so1 5' flanking region |
| 3'flank_R     | CGAGAATTTGGCAGACAGCAGACAAAGC      | Cloning of the so1 3' flanking region |
| 3'flank_F     | CGGCGTACGGTCTCTGACGTGCTTCTCT     | Cloning of the so1 3' flanking region |
| 5'comp-F      | CGCGATTTGACGCACATCTTACCAATGG    | Cloning of the so1 gene            |
| 3'comp-R      | CGGGAATTTGGCAGACAGCAGACAAAGC      | Cloning of the so1 gene            |
| Ef_so1_2F     | CGCGTATGGACGACAGCAGACAAAGC       | PCR of the deletion cassette        |
| pUChph4       | ACAGGAGAAGCTCGCAGGGAAT           | PCR of the deletion cassette        |
| so1_ORF_F     | ATGAGCAAGAAGCTCGCAGGGAAT          | PCR of the so1 gene                |
| so1-ORF-415-396R | GTTCTTCTGACCCCTGTCTTCAAG       | PCR of the so1 gene                |
| 5'flank_probeF | TACGGACCTACACCCCCACTGT          | Probe for Southern analysis        |
| 5'flank_probeR | GTTCTTCTGACCCCTGTCTTCAAG         | Probe for Southern analysis        |
| ENDO.EF1.RTF  | CGCAGATGCGCCTTCGAGAAT           | qPCR of E. festucae tefA            |
| ENDO.EF1.RTR  | CGCAGATGCGCCTTCGAGAAT           | qPCR of E. festucae tefA            |
| So1-RT-1F     | CTACCCTCATCTCACCAG              | RT-PCR of the deletion cassette     |
| So1-RT-1R     | ATCCGAGAATCTTCTCACCAG           | RT-PCR of the deletion cassette     |
| TGap_SuF      | CTAAGGCGATTTTTCGGTTAT           | qPCR of tall fescue gDNA           |
| TGap_SuR      | TCTAGGCGAATCCCTCCCAGCTT         | qPCR of tall fescue gDNA           |

a B. H. R. R. Amarasinghe and C. A. Young, unpublished data.
b Quantitative PCR.
c Reverse transcription-PCR.

Fungal strain and culture conditions. Epichloë festucae strain E2368 was obtained from C. Schardl (University of Kentucky, Lexington, KY). The cultures were stored on potato dextrose agar (PDA) slants under mineral oil at 4°C. Active cultures were maintained on PDA at 23°C. For confocal microscopy, fungal isolates were grown on 100-times-diluted potato dextrose broth (PDB) with 2% agar.

Preparation of the E. festucae so deletion and complementation plasmids. Genomic DNA (gDNA) was extracted from E. festucae strain E2368 cultures grown in PDB for 5 to 7 days at 23°C while shaking at 180 rpm. The primers incorporating enzyme recognition sites were designed to amplify a 1.8-kb fragment of the 5' flanking region (primer set so1_5'flank_F [forward] and so1_5'flank_R [reverse]) (Table 1) and a 2.1-kb fragment of the 3' flanking region (primer set so1_3'flank_F and so1_3'flank_R) of the E. festucae so open reading frame (Fig. 1A). PCR was performed in a total volume of 50 μL containing 200 ng of gDNA, 1 U Platinum PfX DNA polymerase (Invitrogen, Carlsbad, CA), 1× PfX amplification buffer, 1 mM MgSO4, 0.3 mM each deoxynucleoside triphosphate (dNTP), and 0.3 μM each primer. The purified PCR products were ligated into plasmid pPN1688 (46) following digestion with the appropriate enzymes to flank the hygromycin resistance (hph) gene (Fig. 1B) and were introduced into One Shot Top10 competent cells (Invitrogen). The resulting plasmid was named pEsfo-DC. In order to reintroduce the E. festucae so gene into the Δso deletion strains, the primers 5'comp-F and 3'comp-R (Table 1) were designed with the appropriate enzyme restriction sites to amplify the 5.2-kb E. festucae so gene fragment, including its 1-kb upstream and 0.3-kb downstream regulatory regions. The PCR product was ligated into the plasmid pSF16.9, derived from pL199 (26) containing a geneticin resistance gene, and the resulting plasmid was named pEsfo-Comp. The PCR cycling parameters were an initial denaturation step for 2 min at 94°C, 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min (per kb), followed by a final synthesis step at 68°C for 7 min. All plasmids were checked by DNA sequencing.

Transformation of E. festucae strain E2368. The hph gene with the flanking regions of the E. festucae so gene were amplified from the replacement plasmid pEsfo-DC and purified. The protoplasts obtained from E. festucae strain E2368 were transformed with 5 μg of this linear PCR product, using the method described previously (45). The transformants were selected on regeneration medium (RG) (PDA with 0.8 M sucrose [pH 6.5]) containing 150 μg/ml of hygromycin. The putative transformants for E. festucae so gene replacement were confirmed by amplifying the hph gene using a forward primer in the 5' flanking region (Ef_so1_2F) and a reverse primer in the hph gene (pUChph4) and by amplifying the E. festucae so gene using internal so1 gene primers (so1_ORF_F and so1-ORF-415-396R) (Table 1). Secondary confirmation by Southern hybridization was performed by digesting gDNA with SacI (Fig. 1), Sall, or PstI (not shown) restriction enzymes. Hybridization and detection were performed using the AlkPhos Direct labeling and detection system in conjuction with the CDP-Star detection kit (GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions. For E. festucae so gene complementation, protoplasts of the confirmed deletion mutants were generated and transformed with 5 μg of plasmid DNA (pEsfo-Comp) containing the complete E. festucae so gene, including its regulatory regions. The complement transformants were selected on RG medium with 150 μg/ml hygromycin and 200 μg/ml geneticin. The putative complements were confirmed by amplifying the so1 gene.

Plant inoculations. Tall fescue (Lolium arundinaceum) seeds (ecotype PDF) were treated to eliminate the potential presence of endophytes by placing the seeds on a ceramic plate in a desiccator with 75% glycerol, applying a vacuum, and placing the desiccator in an oven at 48°C for 21 days. The seeds were surface sterilized and germinated at 24°C in the dark on 3% (wt/vol) water agar. A fungal mycelium from the edge for an actively growing culture was placed in a small incision in the region between the mesocotyl and coleoptile tissues of 1-week-old seedlings. Additional seedlings were mock inoculated with agar only as a control. The inoculated seedlings were incubated at 24°C in the dark for 5 days followed by light for 5 to 7 days as described previously (20). The seedlings were then transplanted into MetroMix 300 soil (Scotts-Sierra Horticultural Products Co., Marysville, OH) in 72-cell plastic trays and maintained in a growth chamber before being transferred to 4-in. plastic pots and maintained in a growth chamber (16-h day length with a temperature of 19°C).

Microscopy. We used the inverted-agar block method (19) with modifications for cell wall staining with calcofluor white (CFW) (Sigma-Aldrich, St. Louis, MO). A growing edge of a fungal mycelium was ground...
FIG 1 Graphical representation of the *E. festucae* so gene locus from the wild-type (A) and the *E. festucae* Δso mutant (Efso) (B) strains. In the *E. festucae* Δso strain, the wild-type so gene (3.8 kb) is replaced by the 1.5-kb hph gene. An approximately 0.5-kb 5' flanking sequence was used as a probe for Southern analysis. The positions of the SacI restriction sites are given. The wild-type so locus yields a 1.4-kb signal by Southern analysis after SacI digestion, whereas the mutant so locus replaced by the hph gene yields a 3.8-kb band. (C) Southern analysis of SacI-digested gDNA from the *E. festucae* wild-type E2368 strain (WT) and potential *E. festucae* Δso transformants. Among those that have a 3.8-kb band but lack a 1.4-kb wild-type signal, the strains Δso-75, Δso-238, and Δso-268 were selected for further analyses. Note that only the strains that were used for subsequent experiments are labeled.

Real-time quantitative PCR for fungal biomass analysis. To determine the biomass of the endophyte at 5 weeks in planta by quantitative PCR, gDNA was isolated from strain E2368 and endophyte-infected and endophyte-free tall fescue and quantified using a DyNA Quant 200 fluorometer (Hoefer, San Francisco, CA). A standard curve of tall fescue gDNA was generated by quantitative PCR of gDNA samples from endophyte-free tall fescue diluted to a concentration range of 0.01 to 5 ng/μl. A standard curve was also established for endophyte gDNA by quantitative PCR of fungal gDNA at concentrations of 0.0001875 to 0.5 ng/μl in a 5 ng/μl solution of tall fescue gDNA as described previously (46). The amounts of fungal and plant gDNA in endophyte-infected plants were then determined by quantitative PCR by using the generated standard curves as references. Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 10 μl containing 5 μl of SYBR green, 4 μl (20 ng except for generating standard curves) of sample DNA, and each primer at 1 μM. The primer pairs ENDO.EF1.RTF and ENDO.EF1.RTR and Tfgap_1F and Tfgap_1R (Table 1) were used to amplify endophyte and tall fescue gDNA, respectively. For each endophyte genotype, 12 fungus-inoculated plants were harvested and analyzed by quantitative PCR in triplicate for each primer combination. The endophyte biomass was calculated as a percentage of the endophyte DNA relative to tall fescue DNA using the formula \((E/F) \times 100\), where \(E\) and \(F\) are the DNA quantities of endophyte and tall fescue in the sample, as described previously (46).

RESULTS

Generation of the *E. festucae* so deletion and complement strains. To investigate the role of hyphal anastomosis in fungal endophyte-plant symbiosis, the so gene homolog (EfM2.004880) was identified in the *E. festucae* genome database (http://www.endophyte.uky.edu/) by sequence homology to the so gene in *N. crassa*. The predicted *E. festucae* So protein showed 76% identity with *F. oxysporum* Fso1 (29), 63% identity with *N. crassa* So (14),...
62% identity with Sordaria macrospora Pro40 (10), 52% identity with Aspergillus oryzae AoSo (24), and 51% identity with A. bras-

sicicola Aso1 (7) (see Fig. S1 in the supplemental material).

The E. festucae so gene deletion mutant was generated by replacing the so gene with the hygromycin resistance (hph) gene via homologous recombination. Over 400 putative transformants were checked by PCR, and following Southern hybridization analysis, three E. festucae so gene deletion strains (Δso-75, -238, and -268) were selected for further analyses (Fig. 1C). The Δso-238 mutant strain possessed a small amount of the E. festucae so gene that was still detectable by PCR after successive subculturing on the selective medium; this suggests that it is a heterokaryon and a knockdown mutant rather than a knockout mutant. These three mutant strains were further transformed by a so complement plasmid containing the full-length E. festucae so gene with its regulatory regions. Potential complement transformants were screened by PCR, and at least four complement strains from each E. festucae Δso mutant were further checked for their phenotypes.

 Colonies of the E. festucae Δso mutants. E. festucae Δso mutants and their complement strains were grown on PDA to investigate their colony morphologies (Fig. 2). The three E. festucae Δso mutants showed similar phenotypes, although Δso-75 tended to form more hyphal bundle-like structures than did the other strains (data not shown). Deletion of the E. festucae so gene did not affect the growth rate, but it resulted in a slight decrease in aerial hypha formation, as can be seen by the smaller white cottony mycelial regions (Fig. 2B and D). This defect was restored in the complement strains (Fig. 2C and E). Our results are consistent with earlier observations in N. crassa, where the lack of the so gene led to a decrease in aerial hyphae without dramatic effects on the colony growth rate (14) and suggests that E. festucae so is a functional ortholog of the So protein. To quantify the amount of conidia, ground mycelia were inoculated on 103-diluted PDB with 2% agar, and conidia were harvested in distilled water after 2 weeks for quantification with a hemocytometer. Whereas the wild-type and Δso-75 strains formed slightly more conidia than other strains, we did not note a difference in the numbers of conidia between the Δso mutant strains and their complement strains (wild type, 8.8 ± 5.5 [average ± standard deviation (SD)]; Δso-75, 5.3 ± 4.4; Δso-238, 2.5 ± 1.5; its complement strain 238C1, 1.8 ± 1.0; Δso-268, 2.0 ± 1.1; its complement strain 268C53, 2.0 ± 1.3; all × 105, per 9-cm petri dish, n = 3).

 Hyphal anastomosis defects of E. festucae Δso in culture. To investigate further whether the E. festucae so gene is functionally similar to its orthologs in other fungi, we analyzed the presence of hyphal anastomosis in the E. festucae Δso mutants. Cell wall staining using calcofluor white revealed hyphal anastomosis connecting adjacent hyphae in the wild-type E2368 strain (Fig. 3A and B). Hyphal anastomosis was more frequently observed at the subepithelial regions than in the peripheral regions, where hyphae are sparser (Fig. 3C and D; see also Fig. S3A to D in the supplemental material), indicating that the E. festucae so gene plays an essential role in hyphal anastomosis, as has been described in other fungi (19). Hyphal anastomosis has also been observed during endophytic growth in host plants (37). In contrast, hyphal anastomosis was absent in the three E. festucae Δso mutants (Fig. 3C and D; see also Fig. S3A to D in the supplemental material), confirming that the hyphal anastomosis defect of the mutants is due to the lack of the E. festucae so gene. We did not note any difference in hyphal morphology between the wild-type/complement strains and the Δso mutant strains, and all the strains grew more or less as straight hyphae, occasionally forming a bundle of hyphae with rare branches (see Fig. S2 in the supplemental material), as wild-type E. festucae normally does (37).

Deletion of E. festucae so disrupts symbiotic association in tall fescue. To investigate the role of so in E. festucae-plant interaction, tall fescue seedlings were artificially infected with an agar block without the fungus (wound control), the wild-type E2368 strain, E. festucae Δso mutants, or the complement strains. The fungal infection rates were between 90 and 100% for all fungal isolates (see Table S1 in the supplemental material). In stark contrast with the asymptomatic infections of host plants infected with either wild-type or complement strains of endophytes, tall fescue infected with E. festucae Δso became severely stunted. One month after inoculation, when plants infected with either the wild type or complements had multiple tillers, tall fescue infected with the E. festucae Δso mutants had only one or two tillers (Fig. 4A; see also Fig. S4 and S5A in the supplemental material). These tillers did not grow further and died soon afterward, in contrast to the plants infected with the wild-type or complement fungus, which grew into bigger healthy plants similar to the wound control plants (Fig.
4B; see also Fig. S5B and S6 and Table S1 in the supplemental material). This indicates an essential role of the \( E. \) \( \text{festucae so} \) gene in the establishment and/or maintenance of the symbiotic interaction between \( E. \) \( \text{festucae} \) and tall fescue.

\( E. \) \( \text{festucae so} \) strains do not show increased in planta biomass. Although our knowledge of the molecular basis for fungal endophyte-plant interactions is still very limited, it has been proposed that the key to the establishment of mutualistic and symp-
tomless symbiosis is the restricted intercellular growth of endophytes, which is synchronized to host plant growth (5, 8, 37). In support of this view, all E. festucae mutant isolates studied so far that stunt host plants showed increased endophytic biomass due to presumably unrestricted growth in planta (39–42). To test whether the stunting of host plants infected with the E. festucae Δso mutant strains is similarly due to the unrestricted endophytic growth of the fungus, we collected plant samples infected with the endophyte 5 weeks after inoculation and performed quantitative PCR to measure the amounts of plant and endophyte gDNA. E. festucae has uninculturated hyphal compartments (37), and since we did not observe any significant differences in the sizes of the hyphal compartments between the wild-type and Δso mutant strains (e.g., see Fig. 3; see also Fig. S2 and S3 in the supplemental material), we reasoned that the amount of fungal gDNA relative to plant gDNA can be used as an indirect measurement of endophytic fungal biomass. gDNA from wild-type E. festucae (E2368 strain) and endophyte-free plants with known concentrations was used for quantitative PCR to generate standard curves for the quantification of fungal and plant gDNA, respectively. The amounts of fungal and plant gDNA in endophyte-infected plants were then determined by quantitative PCR by using the generated standard curves as references. Fungal gDNA in wild-type-infected plants constituted 0.75% ± 0.31% (average ± SD) (n = 12) of plant gDNA. This was within the range of the wild-type endophytic biomass (0.3 to 1.9%) in perennial ryegrass reported in previous studies (27, 46). In E. festucae Δso-infected plants, fungal gDNA constituted 1.11% ± 0.43% (Δso-238 mutant) or 1.34% ± 1.34% (Δso-268 mutant) of plant gDNA (average ± SD) (n = 12 for each strain). Fungal gDNA in plants infected with the complement strains was 1.28% ± 0.42% (238C1 strain) and 1.04% ± 0.49% (268C53 strain) (average ± SD) (n = 12 for each strain). Thus, there was no statistically significant difference in the proportions of fungal gDNA between the plants infected with the E. festucae Δso mutants and their complements. This is in stark contrast to all E. festucae mutant strains reported so far that induce stunting of host plants, where fungal biomass is increased up to 14-fold higher than that of the wild type (41); therefore, E. festucae Δso strains represent the first documented mutants that restrain host plant growth without overcolonization. This suggests that restricted endophytic growth of fungal endophytes is not the only key to the establishment of mutualistic fungus-plant symbioses.

E. festucae Δso mutants induce distortion and disorganization of tall fescue plant cells. To further investigate the mechanism by which E. festucae Δso mutant strains cause death of the host plant, tall fescue plants infected with the wild-type strain, E. festucae Δso mutants, or the so complements were chemically fixed with glutaraldehyde 1 month after fungal infection. Serial cross sections of the plant samples were taken from a pseudostem approximately 10 mm above ground and stained with toluidine blue O dye to visualize the positional relationship between cell walls and the distribution of the fungal hyphae. Consistent with the quantitative PCR results, we did not see a significant difference in the numbers of endophytic fungal hyphae observed in cross sections between plants infected with the different endophyte strains (e.g., see Fig. 5). Tall fescue cells from plants infected with either the wild type or E. festucae so complements had a hierarchical organization in an orderly manner (Fig. 5A and C). In contrast, the cells from plants infected by the E. festucae Δso mutants were generally smaller and distorted, especially in leaf sheaths, such that their epidermal layers were not clearly recognizable (Fig. 5B). It appeared that the defective plant cells were distributed throughout the tissue rather than restricted to the areas adjacent to the fungal hyphae. This might further suggest that the deletion of E. festucae so leads to the loss of the delicate symbiotic balance between the host plant and the endophyte and potentially results in the induction of some form of global stress affecting large portions of the plant.

**DISCUSSION**

Our current knowledge about the physiological roles of hyphal anastomosis is still very limited, partly due to the lack of studies investigating the effects of hyphal anastomosis in the natural habitats of filamentous fungi. This study demonstrated that the so gene plays an essential role in hyphal anastomosis in a grass endophytic fungus, E. festucae, and that its deletion results in disruption of the symbiotic relationship and early death of the host plant. Plants infected with the E. festucae Δso mutant strains exhibited severe stunting and collapsed within 5 to 7 weeks following fungal inoculation.

The so gene and its orthologs have been widely used as model genes to study hyphal anastomosis in numerous filamentous fungi (7, 13, 14, 29, 33). Even though its exact molecular function still remains unclear, the So protein has been proposed to act downstream of the MAPK MAK-2 and to function in chemoattractant release during hyphal homing just prior to anastomosis (13, 32). However, So orthologs are large proteins with an estimated molecular mass of 140 kDa (10) and may have multiple molecular functions. Indeed, the So orthologs have also been implicated in the plugging of septal pores in aging hyphae or after hyphal injury (10, 16, 24). It is currently unknown whether this function of So orthologs is associated with their role in hyphal anastomosis. For this reason, we cannot eliminate the possibility that the essential function of the E. festucae so gene in maintaining mutualistic symbiosis may be independent of its role in hyphal anastomosis. Nevertheless, it should be noted that E. festucae strains lacking any one of the components of the NADPH oxidase complex (40–42), whose orthologs are essential for hyphal anastomosis (17, 31, 35), also showed a defect in the establishment/maintenance of symbiosis similar to that in our E. festucae Δso mutant strains. Thus, it seems likely that the lack of hyphal anastomosis is at least partly responsible for disrupting mutualism, leading to the endophyte-mediated death of the host plant. However, we also noted some phenotypic differences between our E. festucae Δso mutant strains and the mutant strains of the NADPH oxidase components, including the absence of an increase in in planta fungal biomass and a more severe stunting of the host plant, which resulted in earlier death of the plant caused by the E. festucae Δso mutants. This difference may be simply due to the combination of different E. festucae isolates with different host plants. Alternatively, this may suggest that an additional role of So, which is independent of hyphal anastomosis, is also required for grass-endophyte symbiosis. Collectively, mutations in hyphal anastomosis-related proteins in E. festucae, including the NADPH oxidase components and our E. festucae So protein, have thus far always resulted in the disruption of endophyte-plant symbiosis, leading to the eventual demise of the host. It is becoming increasingly obvious that the maintenance of these symbioses requires signaling between the host and the fungus (9), and consequently, the disruption of anastomosis may impair the signaling ability within the endophyte or...
potentially between the two partners. An alternative explanation that warrants consideration is that hyphal anastomosis and fungus-plant interaction are two independent phenomena that are regulated by partially overlapping pathways involving both the So protein and the NADPH oxidase complex. An analogous example is the MAPK cascade, involving Fus3 orthologs, that regulates three cellular processes: hyphal anastomosis, sexual development, and pathogenicity (7, 28, 29, 32).

Our finding that the deletion of the *E. festucae* so gene leads to death of the host plant provides an interesting contrast to two
previous studies that analyzed the role of so orthologs in phyto-
pathogen-plant interactions. In the crucifer pathogen A. brassi-
cola, deletion of the so ortholog led to a complete loss of its patho-
genicity (7). In F. oxysporum, the deletion of the so ortholog
resulted in inefficient fungal colonization on host roots; however,
it showed a wild-type level of virulence (29). Thus, three studies
performed so far to investigate a role of so orthologs in fungus-
plant interactions, including this study, yielded three apparently
conflicting results: a loss of pathogenicity in A. brassicicola, unaf-
fected virulence in F. oxysporum, and a gain of pathogenicity in the
E. festucae endophyte. These findings may suggest that whether
hyphal anastomosis contributes to pathogenicity depends on the
individual strategies of the fungi colonizing the host plants, as
discussed previously (30). Alternatively, these results may repre-
sent specific roles of hyphal anastomosis at different stages of fun-
gus-plant interactions. Apparently, the A. brassicicola Δso mutant
strain was unable to spread from the site of inoculation on cabbage
leaves (7). The F. oxysporum Δso mutant strain was also defective
in efficiently colonizing tomato roots (29). Thus, these results may
indicate an important role of hyphal anastomosis in facilitating
the efficient colonization of host plants. Since we artificially inoc-
ulated E. festucae mycelium into tall fescue seedlings, our data may
not relate to a role of the E. festucae so gene during colony estab-
ishment in planta but instead may represent an apparent pathog-
genicity of the E. festucae Δso strain at a later stage. In this regard,
it is noteworthy that even though its colonization efficiency on the
host plant was significantly reduced, the F. oxysporum Δso mutant
strain showed virulence comparable to that of the wild type, which
may be associated with higher virulence of its individual hyphae to
compensate for the reduced initial infection.

Collectively, our results demonstrate an essential role of the
anastomosis gene E. festucae so in the establishment/maintenance
of a symptomless symbiotic relationship between the fungal en-
dophyte E. festucae and the host plant tall fescue. In addition to the
apparent importance in mutasic symbiosis, hyphal anastomo-
sis in epichloa endophytes is also significant in the context of their
evolution and potential agricultural applications. Asexual deriv-
aves of epichloa endophytes, commonly referred to as Neothypto-
dium, include many interspecies hybrids which have presumably
arisen from vegetative hyphal anastomosis between different
epichloa isolates (36). The formation of new hybrids is postulated
to promote the diversification of various phenotypic traits of
epichloa endophytes, such as the reorganization and pyramiding of
fungal alkaloid genes, which are crucial for protecting the host
plant from grazing herbivores (3, 6). Thus, molecular and struc-
tural insights into hyphal anastomosis obtained from the current
study will form an important basis for engineering agriculturally
useful isolates of the endophyte that protect plants from a wide
range of biotic and abiotic stresses, as well as furthering our un-
derstanding of the molecular underpinnings of fungus-plant in-
teractions.

ACKNOWLEDGMENTS
We thank The Samuel Roberts Noble Foundation (internal grant) and the
NSF (equipment grant DBI 0400580) for financial support.

We thank Chris Schardl, University of Kentucky, for the strain used in
this research, and Simon Foster and Barry Scott, Massey University,
for providing the plasmid pSFr16.9, containing the geneticin resistance
gene. We also acknowledge Carolyn Young, The Samuel Roberts Noble
Foundation, for her contributions to this research and critical review
of the manuscript. We also thank Jeremy Bell and Broderick Sterns for
technical assistance.

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