A homologue of the fungal tetraspanin Pls1 is required for *Epichloë festucae* expressorium formation and establishment of a mutualistic interaction with *Lolium perenne*

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**SUMMARY**

*Epichloë festucae* is an endophytic fungus that forms a mutualistic symbiotic association with the grass host *Lolium perenne*. Endophytic hyphae exit the host by an appressorium-like structure known as an expressorium. In plant-pathogenic fungi, the tetraspanin Pls1 and the NADPH oxidase component Nox2 are required for appressorium development. Previously we showed that the homologue of Nox2, NoxB, is required for *E. festucae* expressorium development and establishment of a mutualistic symbiotic interaction with the grass host. Here we used a reverse genetics approach to functionally characterize the role of the *E. festucae* homologue of Pls1, PlsA. The morphology and growth of ΔplSA in axenic culture was comparable to wild-type. The tiller length of plants infected with ΔplSA was significantly reduced. Hyphae of ΔplSA had a proliferative pattern of growth within the leaves of *L. perenne* with increased colonization of the intercellular spaces and the vascular bundles. The ΔplSA mutant was also defective in expressorium development although the phenotype was not as severe as for ΔnoxB, highlighting potentially distinct roles for PlsA and NoxB in signaling through the NoxB complex. Hyphae of ΔplSA proliferate below the cuticle surface but still occasionally form an expressorium-like structure that enables the mutant hyphae to exit the leaf to grow on the surface. These expressoria still form a septin ring-like structure at the point of cuticle exit as found in the wild-type strain. These results establish that *E. festucae* PlsA has an important, but distinct, role to NoxB in expressorium development and plant symbiosis.

**Keywords:** tetraspanin, *Epichloë festucae*, expressorium, symbiosis.

**INTRODUCTION**

In mammalian cells, membrane-bound NADPH oxidase (Nox) flavoenzymes catalyse the reduction of dioxygen to superoxide anions using electrons provided by NADPH, which are then converted into further reactive oxygen species (ROS) (Lambeth, 2004; Sumimoto, 2008). Mammalian Nox complexes are generally composed of the integral membrane protein flavocytochrome b558, a heterodimer composed of the catalytic subunit gp91^phox^ and the adaptor protein p22^phox^, and the cytosolic regulatory components Rac1/2, p40^phox^, p47^phox^ and p67^phox^ which upon activation get recruited to the membrane to interact with gp91^phox^ and p22^phox^, enabling ROS production (Rastogi et al., 2017). Fungi generally contain two homologues of gp91^phox^ (Nox1/NoxA and Nox2/NoxB), a Rac1/2 homologue (RacA), a p67^phox^ homologue (NoxR), and two proteins which share similar functions to p40^phox^ and p47^phox^ (Cdc24 and BemA) (Aguirre et al., 2005; Lalucque and Silar, 2003; Takemoto et al., 2006, 2007). In comparison, *Botrytis cinerea* (Siegmund et al., 2015; Zhao et al., 2016). In many plant-pathogenic fungi, ROS production by the NoxA and NoxB complexes is required for successful host penetration and host colonization (Scott, 2015). To gain entry into a host plant, the fungal pathogen *Magnaporthe oryzae* forms a dome-shaped cell known as an appressorium that breaches the leaf cuticle by physical force through the formation of a narrow penetration peg at the base of the appressorium called the ‘appressorium pore’ (Wilson and Talbot, 2009). Similar appressorium-like structures have been observed in *Botrytis cinerea* (Siegmund et al., 2013) and *Colletotrichum lindemuthianum* (Veneault-Fourrey et al., 2005). In comparison, *Verticillium dahliae* hyphae differentiate into hyphopodia and penetrate host roots via penetration pegs (Zhao et al., 2016). Although the saprobic fungus *Podospora anserina* is not a plant pathogen, visualization of cellulose utilization has shown that *P. anserina* hyphae make specialized structures similar to appressoria that are able to penetrate and breach cellophane (Brun et al., 2009). In *Magnaporthe*, NOX1 and NOX2 are required for appressorium-mediated cuticle penetration (Egan et al., 2007). In *P. anserina*, Nox1 and NoxD mutants share similar defects in hyphal anastomosis, fruiting body formation.
and appressorium-like development on cellophane (Lacaze et al., 2015); results that are consistent with Nox1 (NoxA) and NoxD being components of the same Nox (Nox1) complex as supported by the demonstration that NoxA and NoxD from B. cinerea physically interact in vitro (Siegmund et al., 2015). In M. oryzae (Clergeot et al., 2001; Egan et al., 2007), B. cinerea (Gourgues et al., 2004) and C. lindemuthianum (Veneault-Fourrey et al., 2005), Pls1 and Nox2 are required for formation of appressoria penetration pegs, suggesting that Pls1 is the adaptor for the Nox2 complex. This hypothesis is further supported by the demonstration that in the saprobic fungus P. anserina (Brun et al., 2009) and the plant-pathogenic fungus V. dahliae (Zhao et al., 2016), Nox2 and Pls1 are required for appressorium-like cellophane penetration and the V. dahliae Pls1 and NoxB physically interact in vitro (Zhao et al., 2016). In M. oryzae, the F-actin cytoskeleton, septins Sep3, Sep4 and Sep5, and the exocyst components Sec3, Sec5, Sec6, Sec8, Sec15, Exo70 and Exo84 are recruited to the base of the appressorium in a ring-like structure (Dagdas et al., 2012; Gupta et al., 2015; Ryder et al., 2013). In M. oryzae, Nox2 is required for septin-mediated reorientation of the F-actin ring within the appressorium in the initial stages of host penetration, while Nox1 is required for maintaining the cortical F-actin network around the penetration pore for subsequent host colonization (Ryder et al., 2013). A similar F-actin, septin and exocyst component ring has been observed in V. dahliae hyphopodia penetration pegs (Zhao et al., 2016; Zhou et al., 2017).

In comparison to plant-pathogenic fungi, the fungal endophyte E. festucae forms mutualistic associations with temperate Festuca and Lollium grass hosts (Leuchtmann et al., 1994; Schardl, 1996). In vegetative plant tissues, hyphae grow within the intercellular space of host cells (Christensen et al., 1997) and systemically colonize the leaf sheath, leaf blade and inflorescences (May et al., 2008; Scott et al., 2012). Hyphae grow by tip growth within the live stem of the grass, where they enter into the developing leaf blade and sheath tissues, and become attached to the host cell wall by an adhesive matrix (Christensen and Voisey, 2007; Christensen et al., 2002). Intercalary growth allows attached hyphae to avoid mechanical shearing as the host cells expand around them (Christensen et al., 2008). Hyphal growth is tightly coordinated with host development. Once host cells stop elongating, hyphae cease growing, but remain metabolically active and maintain a mutualistic association within the host (Christensen and Voisey, 2007; Tan et al., 2001). E. festucae hyphae also grow epiphytically on the host plant (Christensen et al., 1997; Leuchtmann et al., 1994). Endophytic hyphae exit the host cuticle layer by an appressorium-like structure known as an expressorium (Becker et al., 2016) to form a restricted hyphal network on the leaf surface. These epiphytic hyphae may increase host resistance to fungal pathogens through ‘niche exclusion’ (Moy et al., 2000). To form expressoria E. festucae endophytic hyphae first colonize the tight spaces between the epidermal plant cells where they make contact with the host cuticle, thereby triggering formation of a swollen hyphal compartment, which differentiates and penetrates the undersurface of the leaf (Becker et al., 2016). The epiphytic hyphae which emerge on the leaf surface remain connected to the endophytic hyphal network but have a different cell wall structure; in endophytic hyphal chitin appears to be restricted to septa whereas epiphytic hyphae contain abundant chitin in the cell wall (Becker et al., 2016). This difference in cell wall structure may allow endophytic hyphae of E. festucae to avoid eliciting a host defence response when growing inside the plant (Becker et al., 2016). Similar to plant-pathogenic fungi that form appressoria, the NoxA and NoxB complexes are required for differentiation of expressoria (Becker et al., 2016) and maintenance of a mutualistic symbiotic interaction between E. festucae and its host (Takemoto et al., 2006, 2011; Tanaka et al., 2006, 2008). In comparison to mutualistic wild-type interactions, the ΔnoxA, ΔnoxB and ΔnoxR mutants all show antagonistic interactions with the host plant. Mutant hyphae proliferate within the intercellular spaces, colonize the vascular bundles of the leaves and grow as sub-cuticular hyphae beneath the host cuticle.

Given the novelty of the expressorium structure in E. festucae-host associations, the aim of this study was to determine whether the E. festucae homologue of Pls1, like NoxB (Becker et al., 2016), is required for expression formation and establishment of a mutualistic symbiotic association. Using a reverse genetics approach, we investigated this hypothesis and further determined whether the septin Sep3 is arranged in a ring-like structure in the expressorium, as observed in other fungi (Dagdas et al., 2012; Ryder et al., 2013; Zhao et al., 2016).

**RESULTS**

**E. festucae contains a Pls1 homologue**

To identify the E. festucae Pls1 homologue, a tBLASTn search of the wild-type Fl1 (E894) genome sequence (Schardl et al., 2013a) was carried out using M. oryzae Pls1 (MGG_12594) as the query sequence. This search identified the gene model EFm3.019170, subsequently named *plsA*, as the E. festucae homologue (Fig. S1A). *E. festucae* PlsA is predicted to be a 224 amino acid protein that shares 49% amino acid identity to *M. oryzae*, 60% identity to *F. graminearum*, 51% identity to *P. anserina*, and 50% identity to *N. crassa* and *S. macrospora* Pls1 homologues when aligned using ClustalW (Fig. S1C). Bioinformatic analysis using InterPro and TMHMM predicts that PlsA contains four trans-membrane domains and a tetraspanin EC2 domain (Fig. S1B,C). In *S. macrospora*, the transcription factor PRO1 binds to the consensus sequence GCGGCTTA...
within the promoter regions of nox1, pro41 (noxD), pls1 and nox2, suggesting PRO1 regulates Nox complex gene expression during fruiting body formation (Steffens et al., 2016). In E. festucae, the homologue of PRO1, ProA, binds to a motif identical to the S. macrospora PRO1 consensus sequence in the intergenic region of the divergently transcribed esdC and EF320, and in the promoter regions of symB and symC (Green et al., 2017; Tanaka et al., 2013). By aligning several Clavicipitaceae plsA promoter sequences, a putative ProA binding motif, which does not include the additional 3′ A of this consensus sequence, was identified 192 bp upstream of the E. festucae plsA ATG start site (Fig. S2). To determine whether ProA regulates plsA gene expression in planta, we examined the E. festucae RNAseq data sets generated from plants infected with wild-type and ΔproA mutants (Eaton et al., 2015). This analysis revealed that although the plsA promoter contains a putative ProA binding site, deletion of proA does not significantly alter plsA expression in planta (fold difference −1.08, corrected P < 0.05).

Deletion of plsA does not affect E. festucae culture morphology

To investigate the role of PlsA in regulating hyphal morphology and growth, expressorium formation, and the establishment of a mutualistic symbiotic interaction between E. festucae and the host Lolium perenne, the plsA gene was deleted in the wild-type strain background using a gene replacement approach (Fig. S3A). Protoplasts of wild-type were transformed with a 3781 bp PCR amplified fragment containing the plsA deletion cassette and transformants selected using hygromycin. PCR screening and Southern blot analysis identified ΔplsA#36 and ΔplsA#38 as single copy ‘clean’ deletion mutants (Fig. S3B). These strains were subsequently selected for all further experiments. In axenic culture, hyphae of E. festucae wild-type strain have a very distinct pattern of growth. Hyphal strands adhere to one another to form cables that extend outwards from the colony centre. Tip-to-side hyphal fusions occur frequently within these cables, and asexual conidiation and hyphal coiling are sparse (Becker et al., 2016; Kayano et al., 2013; Tanaka et al., 2013). Several E. festucae mutants, including ΔnoxA, ΔnoxR, ΔsymB, ΔsymC, ΔmobC, ΔproA, ΔmpkA and ΔmkkA, exhibit a loss or reduction of hyphal fusion and increased conidiation in culture (Becker et al., 2015, 2016; Green et al., 2016, 2017; Kayano et al., 2013; Tanaka et al., 2013). To determine whether deletion of plsA results in a similar culture phenotype to these mutants, cultures of wild-type and ΔplsA were grown on potato dextrose and water agar. No differences were observed in the overall radial growth, hyphal morphology, cell–cell fusion or coil formation between wild-type and ΔplsA cultures (Fig. 1A–C), as was previously reported for the ΔnoxB mutant (Kayano et al., 2013).

Deletion of plsA affects E. festucae growth on cellophane membranes

Several fungi have been documented as forming appressoria-like penetration pegs or needle-like structures in culture that allow hyphae to penetrate or degrade cellophane membranes (Brun et al., 2009; Gu et al., 2015; Gua et al., 2014; Kucheryav et al., 2008; Rispail and Pietro, 2009; Zhao et al., 2016). Furthermore,
several nox2 and pls1 mutants have been shown to exhibit cellophane penetration or degradation defects (Brun et al., 2009; Gu et al., 2015; Gua et al., 2014; Kucheryav et al., 2008; Rispaïl and Pietro, 2009; Zhao et al., 2016). To determine whether the E. festucae NoxA and NoxB complexes or the tetraspanin PlsA have similar or distinct roles in allowing E. festucae hyphae to penetrate or degrade cellophane, we examined wild-type, ΔnoxA, ΔnoxB, ΔnoxR and ΔplsA hyphal growth on cellophane membranes. In comparison to wild-type, ΔnoxA and ΔnoxR hyphae, which breached one to three layers of cellophane after five days of growth, both the ΔplsA and ΔnoxB hyphae were impaired in their ability to breach cellophane, with the latter completely defective (Fig. 1D).

### PlsA is required for expressorium development and mutualistic symbiotic interaction

We next examined whether plsA is required for expressorium development and establishment of a mutualistic symbiotic interaction with L. perenne by inoculating wild-type, ΔplsA and ΔnoxB cultures into ryegrass seedlings and analysing host morphology at eight weeks post-inoculation. Plants infected with the ΔplsA mutant had a reduced survival rate, a lower frequency of infection and mild host stunting (Fig. 2A–C; Table S1), whereas ΔnoxB associations caused severe host stunting, as previously reported (Becker et al., 2016). To determine the extent of host colonization and examine hyphal morphology in planta, host pseudostem tissue samples were harvested from wild-type and ΔplsA associations and examined by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). TEM analysis showed that ΔplsA hyphae colonize the host vascular bundles, whereas wild-type hyphae were never observed in this region (Fig. 3A). TEM and CLSM analysis showed that mutant hyphae were more abundant in the intercellular spaces between host cells compared to wild-type (Figs 3B, C and 4). Given expressorium formation requires the NoxA and NoxB complexes (Becker et al., 2016), we examined the development of these structures. Wild-type expressoria are characterized by formation of a swollen hyphal compartment that is delimited by a septum shortly after hyphal exit (Fig. 5). Hyphae then frequently branch and the cell wall is remodelled, as indicated by the abundant fluorescence of WGA-AF488 indicative of chitin throughout the cell walls. A range of expressorium phenotypes were observed in ΔplsA-infected L. perenne associations (Fig. 5), the most obvious being the presence of misshapen swellings with multiple hyphal exit points, or swellings, that were unable to penetrate the host cuticle layer, resulting in formation of subcuticular hyphae. Swellings that resembled normal wild-type-like expressoria were occasionally observed. Given NoxB plays a more crucial role than Pls1 in cellophane penetration (Fig. 1D), the ability of ΔplsA strains to form rare wild-type-like expressoria in planta (Fig. 4), a phenotype not observed for ΔnoxB-infected L. perenne associations (Becker et al., 2016), was not surprising. To confirm the observed...
phenotypes were due to deletion of the plsA gene a wild-type copy of the plsA gene was re-introduced into the two ΔplsA mutant backgrounds. Plants infected with these transformants had the same asymptomatic host interaction phenotypes as wild-type (Fig. S4). Collectively these results indicate that PlsA is required for proper expressorium differentiation and establishment of a mutualistic symbiotic interaction with *L. perenne*.

The septin Sep3 assembles in a ring-like structure in the expressorium

In *M. oryzae*, four septins, Sep3, Sep4, Sep5 and Sep6, have been characterized (Dagdas *et al.*, 2012). Sep3 is essential for host penetration while Sep4, Sep5 and Sep6 are required for full virulence progression. Furthermore Sep3, Sep4 and Sep5 form a localized septin ring at the appressorium pore. Given the
altered expressorium phenotypes observed for ΔnoxB (Becker et al., 2016) and ΔplsA strains, we examined how the E. festucae homologue of Sep3 is localized within the expressorium. Sep3 was chosen as it is essential for host penetration, rather than full virulence progression (Dagdas et al., 2012). To examine this phenotype, a Sep3-eGFP construct was transformed into wild-type protoplasts and the transformants examined in culture and in planta. Similar to M. oryzae (Dagdas et al., 2012), Sep3-eGFP in hyphae of E. festucae in axenic culture localized to a patchy network in hyphal tips and to septa (Fig. 6A). In planta, Sep3-eGFP localized to ring-like structures within wild-type expressoria at the points of host exit (Fig. 6B), results consistent with previous TEM analysis showing that septa occur at the base of the expressoria (Becker et al., 2016). In subcuticular ΔplsA hyphae, which reached but never breached the host cuticle, Sep3-eGFP was cytoplasmic and a polarized septin ring was never observed at the host cuticle (Fig. 6C). In comparison, a distinct Sep3-eGFP ring was observed in ΔplsA hyphae which reached and breached the host cuticle by means of rare wild-type-like expressoria swellings (Fig. 6B). Collectively these results show that a Sep3 ring occurs within wild-type-like E. festucae expressoria and that this ring is absent in subcuticular ΔplsA hyphae, which fail to polarize correctly and differentiate into expressoria when they reach the host cuticle.

DISCUSSION

In many plant–fungal interactions, ROS production from the catalytic activity of the NoxA and NoxB complexes is important for successful host penetration and colonization (Scott, 2015; Tuzynski et al., 2012). Here we have shown that the homologue of Pls1, PlsA, an adaptor protein for the NoxB complex (Zhao et al., 2016), is required for E. festucae expressorium differentiation and for establishment of a mutualistic symbiotic interaction with L. perenne. Imaging of Sep3-eGFP localization in planta showed that wild-type E. festucae expressoria contain a Sep3-eGFP ring that is absent in subcuticular hyphae of the ΔplsA mutant.
Fig. 5 Confocal depth series images of wild-type and mutant expressoria formation in *L. perenne* leaf sheaths. Wild-type (WT) like expressoria swellings are indicated by asterisks (*). Epiphyllous hyphae (Eh) and hyphae exiting the host cuticle layer from malformed expressoria (M-ex) and sub-cuticular hyphae (Sc) are indicated. Samples were stained with aniline blue (detects β-glucans, fluorescence shown in red pseudocolour) and WGA-AF488 (detects chitin, fluorescence shown in blue pseudocolour). Fluorescence of the host cuticle layer is shown in green pseudocolour. Bar = 20 μm.
Fig. 6  Sep3-eGFP localization in wild-type and ∆plsA cultures. (A) Wild-type hyphae growing on 1.5% water agar for seven days expressing Sep3-eGFP at hyphal tips and septa. Septa are marked with asterisks. Scale bar = 20 μm. (B) Incremental images showing Sep3-eGFP ring formation within in planta hyphae as they exit the host cuticle by means of an expressorium. Scale bar = 20 μm. (C) Sep3 eGFP localization in mutant subcuticular hyphae. Subcuticular hyphae do not contain a septin ring. Scale bar = 20 μm.
In filamentous fungi, ROS have an important role in the establishment and maintenance of hyphal polarity, co-ordinated hyphal growth, cell–cell communication and the development of sexual fruiting bodies (Herzog et al., 2015; Scott, 2015; Tudzynski et al., 2012). In many fungi, components of the NoxA complex are required for fruiting body formation and cell–cell fusion, whereas NoxB/Nox2 components are dispensable for these processes (Cano-Domínguez et al., 2008; Dirschnabe et al., 2014; Malagnac et al., 2004; Roca et al., 2012; Tong et al., 2014). In P. anserina, PaNox2 and PaPls1 are essential for hyphal re-orientation/polarization towards cellophane membranes, whereas PaNox1 is dispensable (Brun et al., 2009). Thus, in axenic culture there is a clear separation between Nox signalling, where NoxA/Nox1 contributes to cell–cell fusion and fruiting body formation, and Nox2 contributes to hyphal re-polarization on cellophane. Unlike many other fungi that form fruiting bodies in culture, the sexual cycle of E. festucae has only been studied in planta (Schardl, 1996). Previously, defects within either E. festucae NoxA or NoxB signalling in culture have only been distinguished using cell–cell fusion assays. Here we developed a second assay using cellophane membranes that further differentiates E. festucae NoxA and NoxB defects. In axenic culture, E. festucae ΔnoxA, ΔnoxR, ΔracA and ΔbemA hyphae are defective in cell–cell fusion, whereas ΔnoxB hyphae are indistinguishable from wild-type (Kayano et al., 2013). Here we have shown that similar to E. festucae NoxB (Kayano et al., 2013), PlsA is not required for NoxA-mediated hyphal fusion in culture. Furthermore, NoxB is essential, Pls1 is partially required, and NoxA and NoxR are dispensable for hyphae to breach cellophane. These results are consistent with Pls1 being an adaptor protein for NoxB/Nox2, which contributes to NoxB signalling (Scott, 2015; Tudzynski et al., 2012; Zhao et al., 2016). In P. anserina, PaNox2 and PaPls1 mutant hyphae do not re-orientation/re-polarize towards cellophane membranes, whereas PaNox1 hyphae reorient and establish contacts more prominently than wild-type and possess enhanced cellulolytic capacity, thereby allowing PaNox1 hyphae to efficiently degrade cellulose without penetration (Brun et al., 2009). PaNox1 and PaPls1 are also required to grow hyphae out of melanized structures, whether they are appressoria or ascospores (Lambou et al., 2008). E. festucae ΔnoxA hyphae appear to have a similar cellophane penetration phenotype, given they are able to breach cellophane and many genes involved in cell wall degradation are upregulated in the ΔnoxA mutant *in planta* compared to the wild-type strain (Eaton et al., 2015). Interestingly, E. festucae ΔnoxB and ΔplS4A phenotypes are not identical. NoxB is more important than Pls1 for hyphae to breach cellophane.

In *B. cinerea*, Nox2 is required for the differentiation of appressorium-like structures and host penetration, whereas Nox1 is required for virulent growth *in planta* (Segmüller et al., 2008). In *M. oryzae*, the F-actin cytoskeleton, septins Sep3, Sep4 and Sep5, and the exocyst components Sec3, Sec5, Sec6, Sec8, Sec15, Exo70 and Exo84 are recruited to the base of the appressorium in a ring-like structure. NoxR and Nox2 are required for Sep5-eGFP and Sec6-eGFP recruitment whereas Nox1 is dispensable, suggesting that septin-dependent host penetration requires the Nox2 rather than the Nox1 complex (Dagdas et al., 2012; Gupta et al., 2015; Ryder et al., 2013). Similar to *M. oryzae*, a Sep4-eGFP ring forms in *B. cinerea* appressoria (Feng et al., 2017), and an exocyst VdSec8-eGFP/VdExo70-eGFP and a NoxB-dependent Sep5-eGFP ring form in *V. dahliae* hyphopodia (Zhao et al., 2016, 2017). Furthermore, VdPls1 and VdNoxB localize to *V. dahliae* hyphopodia penetration points (Zhao et al., 2016). Collectively these studies indicate that in many fungi a septin ring forms within developing host penetration structures and there is a clear separation between Nox2 and Nox1 signalling *in planta*. Nox2 is required for the recruitment of septin and exocyst components during initial host penetration, whereas Nox1 is dispensable. In the initial stages of *E. festucae*–host colonization, endophytic hyphae grow by tip growth within the true stem, then switch to intercalary growth in the sheath and blade tissues to establish a restricted network of hyphae within the host (Eaton et al., 2012). Hyphal tip contact with the host cuticle triggers formation of a swollen hyphal compartment and differentiation of an appressorium and localized host penetration (Becker et al., 2016). Deletions of noxA, noxB, noxR, racA and bemA result in prolific hyphal growth, increased hyphal biomass and stunting of the host plant. Other phenotypes of these mutants *in planta* include the formation of convoluted hyphal structures with multiple hyphae packed in between host cells and increased colonization of the host vascular tissues (Becker et al., 2016; Takemoto et al., 2006, 2011; Tanaka et al., 2006, 2008). Furthermore, NoxA, NoxB and NoxR are essential for expression of the appressorium formation *in planta* (Becker et al., 2016). Here we have shown that in comparison to ΔnoxB, the ΔplS4A hyphae cause similar but less severe host interaction defects. Although ΔplS4A strains can occasionally form wild-type-like expression, hyphal tips predominantly fail to polarize or differentiate into expression and instead form a subcuticular hyphal network similar to that seen in ΔnoxA, ΔnoxB, ΔnoxR, ΔsymB, ΔsymC and ΔmobC associations (Becker et al., 2016; Green et al., 2016, 2017). Similar to plant-pathogenic fungi that contain a septin ring in appressoria (Dagdas et al., 2012; Feng et al., 2017; Gupta et al., 2015; Ryder et al., 2013; Zhao et al., 2016; Zhou et al., 2017), a Sep5-eGFP ring forms within *E. festucae* wild-type-like expression that is absent in subcuticular ΔplS4A hyphae. Collectively our results show that NoxB has a more important role than Pls1 in expression formation and in the establishment and maintenance of a mutualistic symbiotic interaction with the grass host. Furthermore, PlsA contributes to, but is not essential for, NoxB-mediated recruitment and/or maintenance of the septin ring. In contrast, *V. dahliae* (Zhao et al., 2016), *M. oryzae* (Clergeot et al., 2001; Egan et al.,...
components (Scott, 2015). Unlike E. festucae NoxA cytosolic scaffolding protein for tethering or recruiting other signalling components, Pls1/PlsA has been proposed to be a scaffolding protein for tethering or recruiting other signalling components (Scott, 2015). Unlike E. festucae NoxA cytosolic components, NoxB cytosolic components are largely unknown. While NoxB, Pls1, NoxR and Bem1 function together on the outside of the ER in B. cinerea, NoxB has been proposed to function alone within the ER, emphasising the dynamic nature of Nox complex assembly depending on the stage of development and the cellular localization (Marschall et al., 2016). In M. oryzae the Nox complex regulatory protein NoxR, the Nox1 adaptor protein NoxD and the Nox2 adaptor protein Pls1 are essential for septin ring formation in the appressorium, whereas Nox1 is dispensable (Galhano et al., 2017; Ryder et al., 2013). These findings suggest that Pls1 functions together with NoxD and Nox2 in regulating the formation of polarization structures (Galhano et al., 2017). Interestingly, E. festucae ΔplSΔ defects are less severe than ΔnoxB defects both on cellophane and in planta. Two hypotheses to explain these findings are (1) the catalytic component NoxB acts alone in certain differentiation processes as proposed for B. cinerea (Marschall et al., 2016) or (2) an alternative signalling mechanism or scaffold protein facilitates NoxB activation and hyphal polarisation in the absence of PlsA.

Although ROS play a major role in regulating E. festucae growth in planta, the NoxA and NoxB complexes are not the only signalling pathways required for controlling the establishment and maintenance of a mutualistic symbiotic association (Scott et al., 2018). A siderophore for maintaining iron homeostasis (Johnson et al., 2013), the transcription factor ProA (Green et al., 2017; Tanaka et al., 2013), the stress-activated and cell wall integrity mitogen-activated protein kinase (MAPK) signalling pathways (Becker et al., 2015; Eaton et al., 2010), the STRIPAK complex (Green et al., 2016), cAMP/PKA (Voisey et al., 2016) and calcineurin (Mitic et al., 2018) are all required for maintaining the symbiosis and cause similar host-association defects when disrupted. Given the similarities between mutant phenotypes, the molecular mechanisms which facilitate gene regulation and cross talk between the Nox complex components and the different signalling pathways in E. festucae–host associations are of considerable interest and still relatively poorly described. In S. macrospora the transcription factor PRO1 has been shown to bind to the promoter regions of nox1, pro41 (noxD), plS1 and nox2 during fruiting body development, suggesting PRO1 regulates the expression of Nox complex genes (Steffens et al., 2016). In E. festucae, however, although ProA is required for the symbiotic interaction (Tanaka et al., 2013) and the promoter region of plS contains a putative ProA binding site, plS is not differentially expressed in mutant ΔproA–host associations (Eaton et al., 2015), suggesting ProA does not regulate plS expression in vegetative tissue. In P. anserina, PaNox1 promotes nuclear translocation of the cell-wall integrity pathway MAPK PaMpk1 (Kicka et al., 2006) and in B. cinerea the IQGAP homologue BclqG1 interacts with the Nox complex, MAP kinase and calcium signalling proteins to regulate virulence and development (Marschall and Tudzynski, 2016). These results collectively suggest there is cross talk between Nox, MAPK and calcium signalling pathways. Interestingly, both the cell-wall integrity and stress-activated MAP kinase pathways (Becker et al., 2015; Eaton et al., 2010) and calcineurin (Mitic et al., 2018) signalling pathways are required for the E. festucae–L. perenne symbiotic interaction. Whether similar cross-talk between these pathways and the Nox complex occurs in E. festucae remains to be determined.

In summary, we have shown that the E. festucae tetraspanin PlsA contributes to expressorium differentiation and mutualistic colonization of the grass host L. perenne. Furthermore, analysis of Sep3-eGFP distribution indicates that a septin-based ring forms in expressorium that is absent in subcuticular ΔplSΔ hyphae. We have also developed a cellophane penetration assay for Epichloë that will help distinguish between NoxB and NoxA defects in culture. These findings provide new insights into how the proposed Nox2 complex adaptor protein PlsA affects E. festucae–host associations and how the septin Sep3 is organized in expressorium. Whether or not the Nox2 complex facilitates the recruitment of septins and/or exocyst components within E. festucae expressorium and what other signalling pathways are required for this differentiation process are key questions for the future.

**EXPERIMENTAL PROCEDURES**

**Growth conditions and endophyte inoculations**

*Saccharomyces cerevisiae* cultures were grown in yeast extract peptone dextrose (YPD) media or on YPD agar plates (Colot et al., 2006). *Escherichia coli* cultures were grown in Lysogeny Broth (LB) broth or on LB agar plates supplemented with 100 μg/mL ampicillin (Miller, 1972). *E. festucae* cultures were grown on 2.4% (w/v) potato dextrose (PD) agar plates or in PD broth (Moon et al., 1999, 2000). Endophyte-free seedlings of perennial ryegrass (*Lolium perenne* cv. Samson) were inoculated as previously described (Latch and Christensen, 1985). Plants were grown at 22°C with a photoperiod of 16 h of light (700 μmol/
m² per second) in an environmentally controlled growth room and tested for the presence of the endophyte by immunoblotting (Tanaka et al., 2005).

DNA isolation, PCR and sequencing

*S. cerevisiae* plasmid DNA was extracted as previously described (Colot et al., 2006). *E. coli* plasmid DNA was extracted using the High Pure Plasmid Isolation Kit (Roche, Indianapolis, IN, USA). Fungal DNA was extracted as previously described (Byrd et al., 1990). Cloning and deletion mutant screening PCR reactions were performed using Phusion® High Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and Taq DNA polymerase (Roche), respectively. Sequencing reactions were performed using the Big-DyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied BioSystems, Carlsbad, California, USA) and separated using an ABI3730 genetic analyser (Applied Bio Systems). Sequence data were assembled and analysed using MacVector sequence assembly software, version 12.0.5.

Construct preparation and transformations

Biological materials and primers can be found in Tables S2 and S3. The *plsA* replacement construct (pCE60) was prepared by yeast recombinational cloning (Gietz and Woods, 2002). EcoRI/Xhol restriction enzyme linearized pRS426 vector backbone was recombined with a 1616 bp *plsA* flank (primers pRS426-plsA-F/ plsA-hph-R, genomic *E. festucae* wild-type DNA), a 702 bp 3′ *plsA* flank (primers hph-plsA-F/ plsA-pRS426-R, genomic wild-type DNA) and a 1.4 kb *trpC-hph* cassette (primers hphF/R, plasmid pSF15.15 DNA). The *plsA* complementation construct (pKG34) was prepared by Gibson Assembly (Gibson et al., 2009). A 2.6-kb ampicillin resistance vector (primers pRS426- _F/pRS426- _R, plasmid pAN7-1 DNA) was recombined with a 3 kb fragment containing the native *plsA* gene (primers KG150/151, genomic wild-type DNA). The *E. festucae* homologue of Sep3 (EfM3.035910) was identified using BLASTN search of the *E. festucae* wild-type (E894) genome sequence using *M. oryzae* Sep3 (MGG_01521) as the query sequence. The Sep3-3xGA-eGFP construct (pKG36) was prepared by Gibson Assembly (Gibson et al., 2009) by recombining the *tefA* promoter, 3xGA-eGFP, TrpC terminator, ampicillin and hygromycin resistance cassettes (primers gfpF/ pteF_R, plasmid pPHN94 DNA) with the *sep3* gene sequence (primers KG158/155, genomic wild-type DNA). Recombined plasmids were transformed into electroporontent DH5α *E. coli*, transformants selected on ampicillin, and plasmids purified from *E. coli* and confirmed to be error-free by sequencing. *E. festucae* protoplasts were prepared (Young et al., 2005) and transformed with 2–3 μg of target DNA (Itoh et al., 1994) using hygromycin (150 μg/mL) or geneticin (200 μg/mL) selection. The *plsA* replacement fragment was amplified from pCE60 plasmid DNA using the primers pRS426-plsA-F and pLSA-pRS426-R, and transformed into *E. festucae* wild-type protoplasts using hygromycin selection. The *plsA* complementation construct pKG34 was co-transformed with pII99 into ΔplsA protoplasts using geneticin selection. The Sep3-eGFP construct pKG36 was transformed into wild-type protoplasts using hygromycin selection. Transformants were nuclear purified by three rounds of sub-culturing.

Southern analysis

*E. festucae* genomic digests, separated by agarose gel electrophoresis, were transferred to positively charged nylon membranes (Roche, Southern, 1975) and fixed by UV light cross-linking in a Cex-800 UV light cross-linker (Ultra-Lum, Claremont, California, USA) at 254 nm for 2 min. Digoxigenin-dUTP (DIG) labelling and hybridization of the *plsA* DNA probe and nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) visualization were performed as per the manufacturer’s instructions using the DIG High Prime DNA Labelling and Detection Starter Kit (Roche).

Microscopy

*E. festucae* cultures were grown on glass slides overlaid with 1.5% (w/v) water agar. Culture morphology and growth, and Sep3-eGFP localization in culture and in planta, were analysed using an Olympus IX83 inverted fluorescence microscope, using setups for both Differential interference contrast (DIC) and standard GFP filters. Infected pseudostem tissue for CLSM was stained and images captured as previously described (Becker et al., 2018) using a Leica SP5 DMM6000B confocal microscope (excitation wavelengths of 488 nm and 561 nm, emitted light was collected from 498–551, 593–625 and 661–796 nm, respectively, using either a 40 × or 63 × oil immersion objective, Numerical Aperture (NA) = 1.4) (Leica Microsystems, Wetzlar, Germany). Pseudostem sections for TEM analysis were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2 as previously described (Spiers and Hopcroft, 1993). Images were acquired using a Philips CM10 TEM and a SIS Morada digital camera.

Bioinformatics

The *E. festucae* wild-type (E894) genome is available at http://csbio-l.csr.uky.edu/ef894-2011/ (Schardl et al., 2013a). Psl1 and Sep3 protein sequences from several fungal species as listed in Fig. S1A were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). ClustalW pairwise protein sequence alignment (Thompson et al., 1994) was performed using MacVector 12.0.5 software. Domain analysis was performed using InterProScan (v. 5) (Quevillon et al., 2005; Zdobnov and Apweiler, 2001) and TMHMM (v. 2.0c) (Krogh et al., 2001; Sonnhammer et al., 1998).
ProA binding sites were identified manually using the *E. festucae* ProA binding domain described previously (Tanaka et al., 2013).

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 *Epichloë festucae* *plsA* gene structure, encoded protein domain structure and amino acid sequence alignment. (A) Gene structure of *E. festucae* *plsA* containing three exons of 402, 166 and 107 base pairs (bp), and two introns of 90 and 89 bp. (B) Protein domain structure of *E. festucae* PlsA. TMHMM software (v. 2.0c) (Krogh et al., 2001; Sonnhammer et al., 1998) predicts four trans membrane domains (TMD, blue). InterProScan (v. 5) (Quevillon et al., 2005; Zdobnov and Apweiler, 2001) predicts a tetraspanin domain between TMD3 and TMD4 (yellow). (C) Multiple amino acid (aa) sequence alignment (ClustalW) of PlsA homologues from *Ef, E. festucae* EFm3.019170; *Nc, Neurospora crassa* NCU07432 (XM_959526.2); Sm, Sordaria macrospora SMAC_05218 (XM_003346971.1); Pa, *Podospora anserina* Pa_1_19270 (XM_001907355.1); Mo, *Magnaportha oryzae* MGG_12594 (XM_003720928.1) and Fg, *Fusarium graminearum* FGSG_08695 (XM_011321741.1). TMDs and the EC2 domain are coloured as in B. Conserved cysteine residues (red) within the EC2 domain and predicted N-glycosylated residues (green) are indicated.

Fig. S2 Identification of ProA binding site in the *plsA* promoter of various *Epichloë* spp. Multiple sequence alignment of 1 kb *plsA* promoter regions from several *Epichloë* species including *E. ba- conii, E. bromicola, E. elymi, E. typhina, E. festucae* F1E and *E. amarillans*. The conserved predicted ProA binding site is shaded in red and ATG start site in green.
Fig. S3 plsA replacement strategy and Southern analysis of ΔplsA strains. (A) Physical maps of strain Fl1 wild type plsA and mutant ΔplsA loci and linear inserts of plsA replacement, pCE60 (top), and complementation, pKG34 (bottom) constructs. Regions of recombination are indicated by grey shading. Primer pairs used to amplify genomic 5′ and 3′ flanking regions and the hygromycin (hph) resistance cassette and primer pairs used for ΔplsA PCR screening are shown. EcoRI restriction enzyme sites used for Southern analysis are as shown. Bar 2 kb. (B) NBT BCIP stained Southern blot of EcoRI genomic DNA digests (1.5 μg) probed with (DIG) 11 dUTP labelled linear pCE60 PCR fragment (primers pRS426 plsA F plsA pRS426 R). Fragments of the expected size for wild type (6.6 kb) and clean (7.3 kb) integration are as shown.

Fig. S4 Complementation of ΔplsA defects in planta. (A) Host morphology in wild type, ΔplsA, ΔnoxB and ΔplsA plsA (C1 3) associations at seven weeks post planting. (B) Height of the tallest infected tiller (wild type, ΔplsA and ΔplsA/plsA associations n 14 18; ΔnoxB associations n 3). An asterisk indicates significant differences from wild type (P 0.05), as determined by one way ANOVA test.

Table S1 Survival and infection rates of wild type, mutant and complemented strains.
Table S2 Biological material.
Table S3 Primers used in this study.