INVESTIGATION OF EPICHLOË FESTUCAE–STRONG CREEPING RED FESCUE MUTUALISTIC AND ANTAGONISTIC INTERACTION

by

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Plant Biology

Written under the direction of

Faith C. Belanger and Bruce B. Clarke

And approved by

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New Brunswick, New Jersey

OCTOBER, 2019

ABSTRACT OF THE DISSERTATION

Investigation of *Epichloë festucae*–Strong Creeping Red Fescue Mutualistic and Antagonistic Interaction

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Many cool-season grasses have symbiotic relationships with *Epichloë* (Ascomycota, Clavicipitaceae) fungal endophytes that reside in the intercellular spaces of the above-ground parts of host plants. The presence of the *Epichloë* endophytes is generally beneficial to host plants due to enhanced tolerance to biotic and abiotic stresses conferred by the endophytes. Many *Epichloë* spp. are asexual and those infections always remain asymptomatic. However, some *Epichloë* spp. have a sexual stage and produce macroscopic fruiting bodies (stromata) that envelop the developing inflorescences causing a syndrome termed "choke disease". To better understand this antagonistic association, a transcriptome analysis of fungal and plant genes was performed to compare stroma tissue and asymptomatic inflorescence tissue of *Epichloë festucae* infected strong creeping red fescue (*Festuca rubra* subsp. *rubra*). Hundreds of fungal genes and over 10% of the plant genes were differentially expressed between the two tissue types. The differentially expressed fungal genes in the stroma tissue indicated a change in carbohydrate and lipid metabolism. Plant stress related genes were up-regulated in the

stroma tissue suggesting the plant host was responding to the normally symbiotic fungal endophyte as a pathogen.

Genome and transcriptome analyses are often the first steps for gene discovery followed by gene function studies. CRISPR/Cas technology is a powerful molecular tool to genetically modify genes of interest for further functional characterization of those genes. Here CRISPR/Cas9 approach was utilized to knockout an *E. festucae* antifungal protein gene (*Efe-afpA*), whereas fungal transformation relying on homologous recombination was unsuccessful due to the lack of long and unique flanking regions of this gene. The mutants lacking the *Efe-afpA* gene were shown to have impaired growth in culture, and therefore, unlikely to form a symbiotic relationship by infecting and systematically colonizing its plant host, strong creeping red fescue. CRISPR/Cas9 approach is highly effective and precise compared to conventional homologous recombination approach. More importantly, the CRISPR/Cas9 approach is more versatile and is not restricted by the availability of long and unique homologous flanking regions of the target gene. CRISPR/Cas9 enables functional characterization of many more *Epichloë* genes to study the *Epichloë*–grass symbiosis.

ACKOWLEDGMENTS

I would like to thank my co-advisor Dr. Bruce Clarke for his continuous support and encouragement since my first day in graduate school. I am also grateful for the guidance and mentorship provided by my other co-advisor Dr. Faith Belanger. Her dedication and passion about research inspired me as a woman scientist and will be invaluable to me throughout my career. I am thankful for my committee Dr. James White and Dr. Scott Warnke who graciously contributed their time, expertise, and insight to assist in my research. I am also wish to thank Dr. William Meyer for his expertise in breeding endophyte-enhanced turfgrass and providing plant materials for my study.

I appreciate all the help from the faculty, staff and students in the Plant Biology Department during my graduate study. I thank Dr. Melinda Martin and Zipeng Tian for their help and I enjoyed working with them in the Belanger lab. I also wish to acknowledge Dr. Lisa Beirn and Limei Du as friends and colleagues and whom I learned a lot from and appreciate their encouragement and suggestions throughout graduate school. I am very lucky to be surrounded and inspired by these great women in science. I am especially grateful to the assistance provided by secretary staff, including Nalini Kaul, Anne Diglio, Barbara Fitzgerald, Barbara Connors, Karen Yudin, and Donna Jones.

I want to give special thanks to Dr. Guohong Cai and Dr. Bradley Hillman who were instrumental in getting me interested in molecular research and from whom I learned many techniques, as well as for the training I received from my M.S. advisor Dr. James Murphy. I would like to extend my thanks to people working in Dr. White's lab for their help as well as Dr. Dana Price and Ehud (Udi) Zelzion from Genome Cooperative at

the School of Environmental and Biological Science for their input and assistance with analyzing transcriptome data. I also thank the Rutgers Center for Turfgrass Science and United States Golf Association for their generous financial support of my research.

Finally, I would like to thank my family and friends for their love and support throughout my education. Most importantly, I am extremely grateful for my wonderful husband, Dr. Trent Tate. Achieving a doctoral degree would not have been possible without him and his unconditional love and support. Thank you for believing in me and supporting me above and beyond graduate school. I am truly blessed to have met him during graduate school and to have him in my life.

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CHAPTER 1. Literature Review

Introduction

Facing the challenge of climate change, reducing the use of fertilizers and pesticides is essential for more sustainable agriculture. Scientists have been seeking solutions for reducing inputs by studying microbial symbionts of crops and their host interactions to improve host fitness and resistance to biotic and abiotic stresses (Kauppinen et al., 2016). Fungal endophytes reside within host plants and form symbiotic relationships. Endophytic fungi are divided into two distinct categories – clavicipitaceous and non-clavicipitaceous (Rodriguez et al., 2009). Clavicipitaceous endophytes, symbionts of grasses, were first reported in *Lolium* in late 19th century Europe (Rodriguez et al., 2009). *Epichloë* endophytes are clavicipitaceous symbiotic fungi that grow intercellularly throughout the above-ground tissues of forage and turf grasses (Saikkonen et al., 1998). Utilizing other beneficial microbial symbionts (such as mycorrhizal fungi and rhizobacteria) in agriculture requires continuously applying those organisms to the production environment (Smith and Read, 2008; Saharan and Nehra, 2011), whereas *Epichloë* endophytes are systemic, maternally inherited, and persist in grass lines after being introduced. These endophytic fungi benefit their hosts with protection from vertebrate and invertebrate pests, resistance to fungal diseases, enhanced drought tolerance, and tolerance to poor soil conditions (Kuldau and Bacon, 2008).

Fungal Growth and Transmission

Hyphae of *Epichloë* endophytes branch sparsely in intercellular spaces of grass leaf blades and leaf sheaths, likely to acquire sugars and amino acids (Clay and Schardl,

2002). Unlike other fungi that grow exclusively by extension at the hyphal tip, *Epichloë* endophytes can grow by intercalary hyphal extension at the same rate of elongating grass leaves (Tan et al., 2001; Christensen et al., 2008b). Hyphal tip growth and branching occur when *Epichloë* endophytes grow between the undifferentiated plant cells, such as the shoot apical meristem and embryo (Scott et al., 2012). In addition to the intercellular hyphae growth, epiphyllous mycelial nets have been observed with some *Epichloë* species (White et al., 1996; Moy et al., 2000; Becker et al., 2016).

Research has shown that *in planta* branching is inhibited by the accumulation of reactive oxygen species (ROS) produced by the fungus (Tanaka et al., 2006). When the *noxA* gene encoding NADPH oxidase was disrupted, the endophyte increased in hyphal branching *in planta* and became pathogenic, as a result the host plants were stunted, senescent, and eventually died (Tanaka et al., 2006). Similarly, the *noxR* gene also regulates ROS production and therefore also controls hyphal branching (Takemoto et al., 2006). Other genes involved in ROS production were also seen to be critical to maintain the mutualistic relationship (Tanaka et al., 2008; Takemoto et al., 2011; Kayano et al., 2013; Kayano et al., 2018). Other gene function studies have shown that siderophore synthetase SidN (Johnson et al., 2013), transcription factor proA (Tanaka et al., 2013), kinases mkkA, mpkA and mobC (Becker et al., 2015; Green et al., 2016), GPI-anchored cell wall protein RhgA (Bassett et al., 2016), membrane associated proteins symB and symC (Green et al., 2017), calcineurin catalytic subunit cnaA (Mitic et al., 2018), velvet-domain protein VelA (Rahnama et al., 2018), heterochromatin 1 protein HepA (Chujo et al., 2019), and tetraspanin PlsA (Green et al., 2019) were required to maintain restrictive hyphal growth *in planta*. In another extreme case, *E. festucae* lacking the hyphal

anastomosis gene (*so*) killed the host plant within 2 months after the initial infection (Charlton et al., 2012). Hence, the regulation of symbiotic growth in planta is rather complex involving numerous genes from different pathways.

Epichloë endophytes are vertically transmitted via seeds, some are also horizontally transmitted by infecting neighboring uninfected plants (Schardl and Phillips, 1997). During the host plant's reproductive stage, hyphae grow into the developing inflorescence and seeds and infect the developing embryo (Zhang et al., 2017). This method of reproduction is known as the asexual life cycle of the endophyte. However, a recent study with red fescue (*Festuca rubra*) showed that vertical transmission can be unstable; 40% of the endophyte-infected maternal plants had some uninfected offspring seedlings (Saikkonen et al., 2010). Greater instability has been observed with manipulated inoculated grass–endophyte associations, likely due to genetic incompatibility (Saikkonen et al., 2010). Horizontal transmission occurs during the sexual life cycle; ascospores that land on the florets of a new host plant may germinate and colonize the ovary and developing seed (Chung and Schardl, 1997; Schardl et al., 1997; Schardl, 2001; Tadych et al., 2014), or alternatively the stem or leaf tissues (Brem and Leuchtmann, 1999). During the asexual stage, conidia and epiphyllous nets have also been shown to play a role in horizontal transmission (White et al., 1996; Meijer and Leuchtmann, 1999; Tadych and White, 2007; Tadych et al., 2012; Becker et al., 2016).

Choke Disease

Some endophyte strains are both mutualistic and antagonistic. When a plant host undergoes reproductive development, the sexual fungal species can proliferate and form a

stroma (choke) that envelops and arrests the development of an inflorescence (Sampson, 1933; Kirby, 1961). Schardl et al. (2009) and Tadych et al. (2014) have summarized the stroma-forming and non-stroma-forming *Epichloë* strains and their hosts. As a result, choke disease can significantly reduce seed yield (Kirby, 1961; Lam et al., 1995; Pfender and Alderman, 2006). Fungicides applied to orchardgrass exhibiting choke disease caused by *E. typhina* were reported to have little or no effect on the disease (Pfender and Alderman, 2003). Adequate nitrogen fertilization can reduce the level of choke symptoms (Sun et al., 1990), however, high nitrogen promotes vegetative growth and reduces seed yield (Funk and White, 1997). Selective breeding for endophyte-infected grasses not exhibiting choke symptoms has reduced the incidence of this disease, but choke disease can still be a problem (Meyer et al., 2013; Bushman et al., 2019).

Among the sexual endophytes, the degree of stromata formation can vary among grass and endophyte associations (White, 1988). In type 1 associations, such as that in orchardgrass, stromata are produced on almost all infected tillers so the sexual reproduction of the infected plants is completely suppressed, and endophytes are therefore only transmitted horizontally. In type 2 associations, such as that in *F. rubra*, only some of the flowering tillers from an infected plant bear stromata, while other tillers are healthy, allowing the endophyte to transmit vertically through infecting seeds. In type 3 associations, such as those with the hybrid *Epichloë* spp., stromata are never formed.

As the stroma develops, anthomyiid flies transfer mitotic spores of the opposite mating type to fertilize the stroma (White et al., 1993a). A group of fungal volatiles named chokol were discovered from stomata of *E. typhina* infecting timothy, *Phleum pretense* (Yoshihara et al., 1985; Tanimori et al., 1994). Chokol K plays an important role

to attract *Botanophila* flies to transfer gametes for fertilization (Schiestl et al., 2006), and also has antifungal activity (Steinebrunner et al., 2008a). Another volatile compound, methyl (Z)-3-methyldodec-2-enoate, was also reported to attract flies to the stromata (Steinebrunner et al., 2008b). The initial stromata are white; the mature stromata turn yellow or orange due to the formation of carotenes as the perithecia bearing ascospores formed in the stromata (White et al., 1993a). Dissemination of the meiotic ascospores produced on stromata to infect neighboring plants leads to horizontal transmission (Chung and Schardl, 1997).

Stroma-forming endophyte species generally produce low level of alkaloids compared to seed-transmitted (asexual) species (Leuchtmann et al., 2000; Tintjer and Rudgers, 2006). Genes involved in loline, ergot alkaloid, or indole-diterpene biosynthesis were not detected in *E. typhina*-infected orchardgrass (Bushman et al., 2019). Although, most loline-producing endophytes are asexual, the sexual endophyte, *E. festucae* and grass symbiota vary widely in alkaloid profiles (Bush et al., 1997). In the meadow fescue–*E. festucae* symbiota, anti-insect loline alkaloid genes were down-regulated in stromata, likely to minimize the toxic effect on flies that cross-fertilize the stromata (Zhang et al., 2010).

Stromata-forming strains generally grow faster than non-stroma forming (asymptomatic) strains *in vitro* (White et al., 1991a; White and Chambless, 1991; White et al., 1991b; White et al., 1993b). Early studies reported increased growth with stroma-forming strains on sugars that are abundant in the developing inflorescences, the authors therefore hypothesized that the ability to utilize the sugars and grow rapidly is critical for forming stromata (White et al., 1991b; White et al., 1993a). Nitrogen application was

reported to reduce choke disease (Sun et al., 1990). Also, applying gibberellic acid which promotes growth of the inflorescences has been shown to reduce the amount of stromata formation (Emecz and Jones, 1970). Together, research supported the hypothesis that rapid growth of the inflorescences is the key to outgrow the endophyte and therefore not becoming trapped in the massive fungal mycelia of the stromata. However, in type 2 associations, stromata are only formed on a fraction of the host plant inflorescences during the same developmental stage with similar sugar availability. This variation was explained as a way for the fungus to balance stroma formation and seed transmission, however, with an unknown mechanism (White et al., 1993a). Later, Lam et al. (1995) reported dramatically elevated invertase activity, which is likely to improve the sink strength for sucrose utilization by the fungus, leading to rapid proliferation. Although, the switching from restrictive, microscopic to proliferative, macroscopic mycelium occasionally was also observed on vegetative perennial ryegrass (*Lolium perenne* L.) leaves infected with *E. typhina*, ascospores in perithecia were unable to develop (Christensen et al., 2008a). The vegetative leaves are likely lacking in abundant nutrients to support the stromata long enough to develop perithecia, which suggests the importance of acquiring adequate sugars from the host plant's reproductive tissue.

Moreover, Bucheli and Leuchtmann (1996) reported isozyme data of *E. sylvatica* stroma and non-stroma-forming isolates from *Brachypodium sylvaticum*, and concluded there was genetic differentiation between the isolates. Meijer and Leuchtmann (1999) proposed that the fungal genome controls stromata formation and that the choked and non-choked tillers were infected with different strains of *E. sylvatica* in *B. sylvaticum* populations where stromata develop only on some inflorescences of an individual plant.

Data from Chewings and strong creeping red fescues cross-inoculated with *Epichloë* isolates, suggested incidence of choke disease may depend on both the host plant genotype and the *Epichloë* endophyte genotype (Johnson-Cicalese et al., 2000). White (1988) reported a plant-produced alkaloid inhibits the growth of endophyte in culture and therefore may have an effect on suppressing stromata formation. However, the interaction of host plants and endophytes on choke disease has not been studied intensively. Recently, research was conducted to investigate choke disease on meadow fescue via transcriptome analysis; half of the unigenes were differentially expressed, but further research is needed to understand their association with the development of choke disease (Dinkins et al., 2012). Some currently unknown signal or metabolite from the host that is unique to the flowering stage may be involved and the fungus must be able to respond to such a signal for stromata development to occur.

Fine Fescues

Recent studies to better understand the endophyte and grass interaction have been focused on the important forage grasses, such as tall fescue and perennial ryegrass. Fine fescues, a very diverse subgroup of *Festuca* species, are important turfgrasses but have not received as much research attention. Fine fescues were utilized for golf turf as early as the sixteenth century; they were broadly used on greens, fairways, roughs and other natural areas (Beard, 1973; Ruemmele et al., 2003). Fine fescues are adapted to cool-humid regions and acidic, infertile soils (Beard, 1973; Ruemmele et al., 1995; Ruemmele et al., 2003). The moderate shade tolerance, drought resistance, and low maintenance make fine fescues superior for erosion control, roadside turf, parks, school ground areas, and home lawns (Ruemmele et al., 2003). However, fine fescues are generally susceptible

to many common fungal diseases, such as red thread, dollar spot (Smith et al., 1989), *Drechslera* leaf spot (Ruemmele et al., 1995), and smut (Meyer and Funk, 1989; Ruemmele et al., 1995).

Fine fescues were categorized into two major groups known as *F. rubra* (red fescue) and *F. ovina* (sheep fescue) aggregates (Ruemmele et al., 2003). Strong creeping red fescue (*F. rubra* L. subsp. *rubra*) is valued for its strong, long creeping rhizomes for rapid spreading and recovery. Strong creeping red fescue is octoploid with 56 chromosomes (2n = 8x = 56) (Huff and Palazzo, 1998; Oliveira et al., 2008), however, no complete and annotated genome sequence has been reported to date.

Disease Resistance

Dollar spot, caused by *Clarireedia jacksonii* C. Salgado, L.A. Beirn, B.B. Clarke, & J.A. Crouch sp. nov. (formerly *Sclerotinia homoeocarpa* F.T. Bennett), is a major fungal disease affecting many turfgrasses and is the most economically important disease on golf courses (Walsh et al., 1999; Vargas, 2005). More money is spent to manage this disease than any other diseases on golf courses in the United States (Vargas, 2005). Although strong creeping red fescues are ideal for low-maintenance use as discussed above, they are generally very susceptible to dollar spot (Smith et al., 1989). Strong creeping red fescue was also reported to be more susceptible to red thread disease [*Laetisaria fuciformis* (McAlp.) Burdsall] than other fine fescues (Morris, 2002). Red thread is most severe under cool-humid, low-maintenance conditions where strong creeping red fescue is utilized, therefore, Bonos et al. (2005) considered this disease to be most important for this species of fine fescue.

Endophyte-mediated resistance to dollar spot and red thread has been observed with fine fescues in the field (Bonos et al., 2005; Clarke et al., 2006). In the forage grass *Bromus auleticus*, *Epichloë* sp. endophyte was reported to alleviate head smut (*Ustilago bullata*) potentially by competing for resources in the flowers (Iannone et al., 2017). In another field study, the endophyte *Neotyphodium occultans* protected *L. multiflorum* from a closely related pathogen *Claviceps purpurea*, however, this protective effect was not sustained under stress conditions (Pérez et al., 2013).

Epichloë endophytes produce bioprotective alkaloids that protect their hosts from insects, which is a well-studied phenomenon (Bush et al., 1997; Scott et al., 2009). However, the antibiosis mechanism against turfgrass fungal pathogens by the mutualistic endophytes is not clear. An *E. festucae* E437 isolate from soft fescue (*F. pulchella* Schrad.) was reported to inhibit the growth of *Drechslera erythrospila*, *D. siccans*, *D. dictyoides*, *Colletotrichum graminicola* and *Bipolaris sorokiniana*, but did not affect three important diseases in turf, *S. homoeocarpa* (renamed *C. jacksonii*, see above), *Rhizoctonia solani*, and *Magnaporthe grisea* (Niones and Takemoto, 2014). They also found that perennial ryegrass inoculated with *E. festucae* E437 or Fl1 (from hard fescue) alleviated the symptoms of *D. erythrospila* infection, however, this was only tested on detached leaves (Niones and Takemoto, 2014). In a subsequent study, they identified a transcription factor VibA to be involved in antibiosis in *E. festucae* E437. Transformants overexpressing *vibA* inhibited the pathogens *S. homoeocarpa* (renamed *C. jacksonii*, see above), *R. solani*, and *M. grisea in vitro* (Niones and Takemoto, 2015). The nonantifungal isolate Fl1 gained inhibitory activity by overexpressing *vibA*, and the transformants were observed to have more aerial hyphae but reduced radial growth

(Niones and Takemoto, 2015). However, the downstream gene (or genes) that is regulated by VibA is currently unknown.

In a transcriptome study of the *E. festucae*–strong creeping red fescue interaction, Ambrose and Belanger (2012) discovered that the second most abundant fungal transcripts encoded a small secreted protein similar to antifungal proteins from *Penicillium* and *Aspergillus* species. Later, the purified protein, *Efe*-AfpA, showed growth inhibition of dollar spot fungus in plate assays, likely due to permeabilizing cell membranes of this pathogen (Tian et al., 2017). *Efe*-AfpA has a shared gamma-core motif with the *A. niger* antifungal protein AnAFP; the conserved motif is critical for antifungal properties and was proposed to interact with fungal membranes (Paege et al., 2016). Interestingly, AnAFP was proposed to function intracellularly to induce autophagy during carbon-starvation and extracellularly to be secreted to inhibit the growth of fungal nutrient competitors (Paege et al., 2016).

Transcriptome Analysis to Study Endophyte and Grass Interaction

Research utilizing next-generation sequencing to study the endophyte–grass interaction has been growing. Whole genome sequences for many *Epichloë* spp. are available (Schardl et al., 2013), which has advanced the research in endophyte–grass symbiosis. Studies on mutualistic relationship of the endophyte and grass host often involve comparing E+ and E– RNA-Seq data from one or more tissues. The availability of the fungal genome sequences allows identification of fungal and plant transcripts, since most grass genomes have not been sequenced. The mRNA sequences can be first

mapped to the endophyte genome then the remaining sequences can be *de novo* assembled to generate a plant transcriptome.

Eaton et al. (2015) reported a transcriptome comparison of *E. festucae*–perennial ryegrass associations of wild-type and knockout lines of three of the symbiosis related endophyte genes; a component of the NADPH oxidase complex *noxA*, a stress-activated mitogen-activated protein kinase *sakA*, and a transcription factor *proA*. They identified a core set of 182 genes that were differentially expressed in all three knockout lines that were considered to be contributing to the antagonistic nature of the mutant lines. Dupont et al. (2015) and Dinkins et al. (2017) were focused on differentially expressed plant genes due to the presence and absence of the endophyte; Dinkins et al. (2019) reported the change of plant gene expression in response to endophyte and water deficit. Others discussed both plant and fungal gene expression (Ambrose and Belanger, 2012; Schmid et al., 2017; Nagabhyru et al., 2019).

Dupont et al. (2015) reported dramatic changes in the expression of over 38% of host genes when inoculating endophyte-free perennial ryegrass with an endophyte strain (*E. festucae* strain Fl1) originally isolated from hard fescue (*F. trachyphylla*). In contrast, Nagabhyru et al. (2019) reported almost no significant difference in plant gene expression profiles in transcriptome comparisons of endophyte-infected with endophyte-free tall fescue inflorescence tissues. Similarly, in comparisons of endophyte-infected and endophyte-free vegetative tissues of strong creeping red fescue, perennial ryegrass, and tall fescue, subtle to modest changes in plant gene expression have been reported (Ambrose and Belanger, 2012; Dinkins et al., 2017; Schmid et al., 2017; Dinkins et al., 2019).

Several reports provided evidence that the endophyte presence tended to reduce expression of plant genes likely involved in defense against fungi (Dupont et al., 2015; Dinkins et al., 2017; Dinkins et al., 2019), whereas Schmid et al. (2017) reported the presence of the endophyte induced host defense responses. Zhang et al. (2011) detected a pathogenesis-related class 10 protein from endophyte infected perennial ryegrass, but suggested the endophyte only elicited a very limited host defense response. It is likely that endophytes somehow suppress some plant host genes to avoid being attacked by plant defense responses but promote the expression of other host genes to enhance the host tolerance to other biotic stresses. Additional research is needed to understand the mutualistic relationship of *Epichloë* endophytes and their grass hosts.

Fungal Transformation

Fungal gene knock-outs and knock-ins are commonly generated to understand gene functions. Numerous studies mentioned before and more studies are undergoing to ultimately elucidate the interaction between grasses and their endophytes by utilizing fungal transformation and evaluating mutant phenotypes. Homologous recombination (HR) approach is broadly used in the *Epichloë* endophytes. Gene modification has become more efficient and precise due to the development of new technologies, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). The use of CRISPR for generating knockouts has been well established in plant and animal systems.

Homologous Recombination

Fungal transformation utilizing HR to swap a target gene with a selective marker gene is one of the most frequently used conventional methods. *Saccharomyces cerevisiae*

mainly uses the HR system to repair DNA double-strand breaks (DSB), therefore replacement of DNA fragments with as little as 20 bp homologous sequences on the both ends can be integrated at the target site (Wach et al., 1994; Takita et al., 1997). However, most fungi use non-homogenous end-joining (NHEJ) as the main process for repairing DSB, which results in ectopic integration of the marker gene (Rahnama et al., 2017). Therefore, for most fungi the efficacy of transformation that relies on HR is normally between 1% to 25% and requires long flanking regions (left and right flanks totaling >3 kb) (Scott et al., 2007). Many researchers have successfully generated knockouts for the *Epichloë* endophyte genes of their interests using HR transformation (Scott et al., 2007). A split marker system was shown to reduce ectopic integration and increase transformation efficacy in *E. festucae* to 33%−74%, which also required at least 1.5 kb for each flanking region (Rahnama et al., 2017). Alternatively, to increase homologous recombination, NHEJ deficiency mutants were generated by disrupting the NHEJ machinery-encoding genes such as *ku70* and *ku80* (Kück and Hoff, 2010). However, increased sensitivity to genotoxic stress, DNA damaging irradiation and chemicals has been observed with NHEJ deficiency mutants (Meyer, 2008; Kück and Hoff, 2010).

Clustered Regularly Interspaced Short Palindromic Repeats

Several scientists recently discovered a microbial repeat sequence named CRISPR, which is used by prokaryotes to record and target invading virus sequences as an adaptive immune system (Jansen et al., 2002; Mojica et al., 2005; Pourcel et al., 2005). CRISPR-associated (Cas) endonuclease, 20 nt CRISPR RNA (crRNA) and *trans*-activating CRISPR RNA (tracrRNA) duplex, which can be engineered as a single guide RNA (gRNA) were identified as the key components of the CRISPR/Cas system. The

most widely used Cas protein is Cas9. The crRNA directs the Cas9 nuclease to generate DSB at the site in the genome with the complementary sequence. When designing the gRNA sequence, the NGG protospacer-adjacent motif (PAM) is also required for the stable binding of Cas9 endonuclease to the target locus. When repairing the DSB via NHEJ, small insertions or deletions often occurred at the cutting site. CRISPR/Cas is a very powerful tool; with proper design of the protospacer sequence of the gRNA adjacent to a GG dinucleotide scientists can, in theory, edit any gene of interest (Jinek et al., 2012). This system has been utilized broadly as a new approach for genome modification.

Despite the broad use of CRISPR to edit genes in plants and animals, there is very limited research on non-model fungal organisms. In *Saccharomyces cerevisiae*, a transformation vector was constructed with a codon-optimized human version *cas9* gene and gRNA driven by the snoRNA SNR52 promoter; this vector was integrated into the genome and generated Cas9 and gRNA *in vivo* (DiCarlo et al., 2013). Zhang et al. (2016) demonstrated that codon-optimized *cas9* gene for human cells could be expressed in *A. fumigatus* driven by fungal promoters, while Nødvig et al. (2015) utilized a codon-optimized *cas9* for *A. niger* to edit other *Aspergillus* species. However, Liu et al. (2015) reported that the human version *cas9* gene did not function in *Trichoderma reesei*. Others have successfully edited fungal genomes by optimizing those two key components, codon-optimized *cas9* gene and gRNA driven by the endogenous U6 promoter from *U. maydis* (Schuster et al., 2016) and *A. oryzae* (Katayama et al., 2016). A polymerase III promoter, most often the U6 promoter, is used to generate abundant gRNAs. However, the U6 promoter has not been identified in many fungi because the fungal U6 gene often has multiple introns (Canzler et al., 2016). Common fungal RNA polymerase II

promoters, such as *trpC* (Arazoe et al., 2015) and *gpdA* (Nødvig et al., 2015), have been used in fungal systems; however, Arazoe et al. (2015) showed significantly less transformation efficiency with *trpC* than the endogenous U6 promoter. Alternately, Liu et al. (2015) introduced *in vitro* transcribed gRNAs into *T. reesei*.

While the CRISPR toolset is ideal for loss-of-function studies, gene editing utilizing the combination of CRISPR and HR is precise, efficient and versatile. Double strand breaks generated by Cas endonuclease boosted the efficient homology-directed repair using as little as 60 bp (Pohl et al., 2016) and 35 bp (referred to as microhomology-mediated end joining) (Zhang et al., 2016) recombination flanks.

The goals of my dissertation research are 1) use next-generation sequencing to study the changes at the gene expression level between asymptomatic inflorescence and stroma tissues in *E. festucae* infected strong creeping red fescue; and 2) to utilize the CRISPR/Cas9 technology to knock-out the *E. festucae* antifungal protein gene and to investigate its role in the endophyte-host grass interaction.

REFERENCES

Ambrose, K.V., and Belanger, F.C. 2012. SOLiD-SAGE of endophyte-infected red fescue reveals numerous effects on host transcriptome and an abundance of highly expressed fungal secreted proteins. PLoS ONE 7:e53214.

Arazoe, T., Miyoshi, K., Yamato, T., Ogawa, T., Ohsato, S., Arie, T., and Kuwata, S. 2015. Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. Biotechnology and Bioengineering 112:2543–2549.

Bassett, S.A., Johnson, R.D., Simpson, W.R., Laugraud, A., Jordan, T.W., and Bryan, G.T. 2016. Identification of a gene involved in the regulation of hyphal growth of *Epichloë festucae* during symbiosis. FEMS Microbiology Letters 363.

Beard, J.B. 1973. Turfgrass science and culture. Prentice-Hall, Englewood Cliffs, N.J.

Becker, M., Becker, Y., Green, K., and Scott, B. 2016. The endophytic symbiont *Epichloë festucae* establishes an epiphyllous net on the surface of *Lolium perenne* leaves by development of an expressorium, an appressorium-like leaf exit structure. The New Phytologist 211:240–254.

Becker, Y., Eaton, C.J., Brasell, E., May, K.J., Becker, M., Hassing, B., Cartwright, G.M., Reinhold, L., and Scott, B. 2015. 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*. Molecular Plant-Microbe Interactions 28:69–85.

Bonos, S.A., Wilson, M.M., Meyer, W.A., and Funk, C.R. 2005. Suppression of red thread in fine fescues through endophyte-mediated resistance. Applied Turfgrass Science 2.

Brem, D., and Leuchtmann, A. 1999. High prevalence of horizontal transmission of the fungal endophyte *Epichloë sylvatica*. Bull. Geobot. Inst. ETH 65:3–12.

Bucheli, E., and Leuchtmann, A. 1996. Evidence for genetic differentiation between choke-inducing and asymptomatic strains of the *Epichloë* grass endophyte from *Brachypodium sylvaticum*. Evolution 50:1879–1887.

Bush, L.P., Wilkinson, H.H., and Schardl, C.L. 1997. Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiology 114:1–7.

Bushman, B.S., Singh, D., Lamp, R., Young, C.A., Charlton, N.D., Robins, J.G., and Anderson, N. 2019. Variation among orchardgrass (*Dactylis glomerata*) germplasm for choke prevalence caused by *Epichloë typhina*. Plant Disease 103:324–330.

Canzler, S., Stadler, P.F., and Hertel, J. 2016. U6 snRNA intron insertion occurred multiple times during fungi evolution. RNA biology 13:119–127.

Charlton, N.D., Shoji, J.-Y., Ghimire, S.R., Nakashima, J., and Craven, K.D. 2012. Deletion of the fungal gene *soft* disrupts mutualistic symbiosis between the grass endophyte *Epichloë festucae* and the host plant. Eukaryotic Cell 11:1463–1471.

Christensen, M.J., Zhang, X., and Scott, B. 2008a. Regulation switching of *Epichloë typhina* within elongating perennial ryegrass leaves. Mycological Research 112:1056-1062.

Christensen, M.J., Bennett, R.J., Ansari, H.A., Koga, H., Johnson, R.D., Bryan, G.T., Simpson, W.R., Koolaard, J.P., Nickless, E.M., and Voisey, C.R. 2008b. *Epichloë* endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genetics and Biology 45:84–93.

Chujo, T., Lukito, Y., Eaton, C.J., Dupont, P.-Y., Johnson, L.J., Winter, D., Cox, M.P., and Scott, B. 2019. Complex epigenetic regulation of alkaloid biosynthesis and host interaction by heterochromatin protein I in a fungal endophyte-plant symbiosis. Fungal Genetics and Biology 125:71–83.

Chung, K.R., and Schardl, C.L. 1997. Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. Mycological Research 101:295–301.

Clarke, B.B., White, J.F., Jr., Hurley, R.H., Torres, M.S., Sun, S., and Huff, D.R. 2006. Endophyte-mediated suppression of dollar spot disease in fine fescues. Plant Disease 90:994–998.

Clay, K., and Schardl, C.L. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist 160:S99–S127.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., and Church, G.M. 2013. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Research 41:4336–4343.

Dinkins, R.D., Nagabhyru, P., Graham, M.A., Boykin, D., and Schardl, C.L. 2017. Transcriptome response of *Lolium arundinaceum* to its fungal endophyte *Epichloë coenophiala*. New Phytologist 213:324–337.

Dinkins, R.D., Nagabhyru, P., Young, C.A., West, C.P., and Schardl, C.L. 2019. Transcriptome analysis and differential expression in tall fescue harboring different endophyte strains in response to water deficit. The Plant Genome 12:180071.

Dinkins, R.D., Hesse, U., Arnaoudova, E.G., Nagabhyru, P., Moore, N., Liu, J., Jaromczyk, J., Zeng, Z., Dai, X., Zhao, P.X., Scheffler, B.E., Jaromczyk, J.W., and Schardl, C.L. (2012). Transcriptomes of *Lolium*/*Schedonorus*/*Festuca* species. In Epichloae, endophytes of cool season grasses: implications, utilization

and biology. Proceedings of the 7th International Symposium on Fungal Endophytes of Grasses, Lexington, Kentucky, USA, 28 June to 1 July 2010 (Samuel Roberts Noble Foundation), pp. 70–76.

Dupont, P.-Y., Eaton, C.J., Wargent, J.J., Fechtner, S., Solomon, P., Schmid, J., Day, R.C., Scott, B., and Cox, M.P. 2015. Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. New Phytologist 208:1227–1240.

Eaton, C.J., Dupont, P.-Y., Solomon, P., Clayton, W., Scott, B., and Cox, M.P. 2015. A core gene set describes the molecular basis of mutualism and antagonism in *Epichloë* spp. Molecular Plant-Microbe Interactions 28:218–231.

Emecz, T.I., and Jones, G.D. 1970. Effect of gibberellic acid on inflorescence production in cocksfoot plants infected with choke (*Epichloë typhina*). Transactions of the British Mycological Society 55:77–82, IN76.

Funk, C.R., and White, J.F., Jr. 1997. Use of natural and transformed endophytes for turf improvement. Pages 229–239 in: *Neotyphodium*/grass interactions, Springer.

Green, K.A., Becker, Y., Fitzsimons, H.L., and Scott, B. 2016. An *Epichloë festucae* homologue of MOB3, a component of the STRIPAK complex, is required for the establishment of a mutualistic symbiotic interaction with *Lolium perenne*. Molecular Plant Pathology 17:1480–1492.

Green, K.A., Eaton, C.J., Savoian, M.S., and Scott, B. 2019. A homologue of the fungal tetraspanin Pls1 is required for *Epichloë festucae* expressorium formation and establishment of a mutualistic interaction with *Lolium perenne*. Molecular Plant Pathology.

Green, K.A., Becker, Y., Tanaka, A., Takemoto, D., Fitzsimons, H.L., Seiler, S., Lalucque, H., Silar, P., and Scott, B. 2017. SymB and SymC, two membrane associated proteins, are required for *Epichloë festucae* hyphal cell–cell fusion and maintenance of a mutualistic interaction with *Lolium perenne*. Molecular Microbiology 103:657–677.

Huff, D.R., and Palazzo, A.J. 1998. Fine fescue species determination by laser flow cytometry. Crop Science 38:445–450.

Iannone, L.J., Vignale, M.V., Pinget, A.D., Re, A., Mc Cargo, P.D., and Novas, M.V. 2017. Seed-transmitted *Epichloë* sp. endophyte alleviates the negative effects of head smut of grasses (*Ustilago bullata*) on *Bromus auleticus*. Fungal Ecology 29:45–51.

Jansen, R., Embden, J.D.A.v., Gaastra, W., and Schouls, L.M. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology 43:1565–1575.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821.

Johnson, L.J., Koulman, A., Christensen, M., Lane, G.A., Fraser, K., Forester, N., Johnson, R.D., Bryan, G.T., and Rasmussen, S. 2013. An extracellular siderophore is required to maintain the mutualistic interaction of *Epichloë festucae* with *Lolium perenne*. PLOS Pathogens 9:e1003332.

Johnson-Cicalese, J., Secks, M.E., Lam, C.K., Meyer, W.A., Murphy, J.A., and Belanger, F.C. 2000. Cross species inoculation of Chewings and strong creeping red fescues with fungal endophytes. Crop Science 40:1485–1489.

Katayama, T., Tanaka, Y., Okabe, T., Nakamura, H., Fujii, W., Kitamoto, K., and Maruyama, J.-i. 2016. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnology Letters 38:637–642.

Kauppinen, M., Saikkonen, K., Helander, M., Pirttilä, A.M., and Wäli, P.R. 2016. *Epichloë* grass endophytes in sustainable agriculture. Nature Plants 2:15224.

Kayano, Y., Tanaka, A., and Takemoto, D. 2018. Two closely related Rho GTPases, Cdc42 and RacA, of the endophytic fungus *Epichloë festucae* have contrasting roles for ROS production and symbiotic infection synchronized with the host plant. PLOS Pathogens 14:e1006840.

Kayano, Y., Tanaka, A., Akano, F., Scott, B., and Takemoto, D. 2013. Differential roles of NADPH oxidases and associated regulators in polarized growth, conidiation and hyphal fusion in the symbiotic fungus *Epichloë festucae*. Fungal Genetics and Biology 56:87–97.

Kirby, E.J.M. 1961. Host-parasite relations in the choke disease of grasses. Transactions of the British Mycological Society 44:493–503.

Kück, U., and Hoff, B. 2010. New tools for the genetic manipulation of filamentous fungi. Applied Microbiology and Biotechnology 86:51–62.

Kuldau, G., and Bacon, C. 2008. Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. Biological Control 46:57–71.

Lam, C.K., Belanger, F.C., White, J.F., Jr., and Daie, J. 1995. Invertase activity in *Epichloë*/*Acremonium* fungal endophytes and its possible role in choke disease. Mycological Research 99:867–873.

Leuchtmann, A., Schmidt, D., and Bush, L.P. 2000. Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë*/*Neotyphodium* endophytes. Journal of Chemical Ecology 26:1025–1036.

Liu, R., Chen, L., Jiang, Y., Zhou, Z., and Zou, G. 2015. Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discovery 1:15007.

Meijer, G., and Leuchtmann, A. 1999. Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica*. New Phytologist 141:355–368.

Meyer, V. 2008. Genetic engineering of filamentous fungi — Progress, obstacles and future trends. Biotechnology Advances 26:177–185.

Meyer, W.A., and Funk, C.R. 1989. Progress and benefits to humanity from breeding cool-season grasses for turf. Pages 31–48 in: Contributions from Breeding Forage and Turf Grasses, D.A. Sleper, K.H. Asay, and J.F. Pedersen, eds. CSSA, Madison, WI.

Meyer, W.A., Torres, M.S., and White, J.F. 2013. Biology and applications of fungal endophytes in turfgrasses. Pages 713–731 in: Turfgrass: Biology, Use, and Management, J.C. Stier, B.P. Horgan, and S.A. Bonos, eds. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI.

Mitic, M., Berry, D., Brasell, E., Green, K., Young, C.A., Saikia, S., Rakonjac, J., and Scott, B. 2018. Disruption of calcineurin catalytic subunit (*cnaA*) in *Epichloë festucae* induces symbiotic defects and intrahyphal hyphae formation. Molecular Plant Pathology 19:1414–1426.

Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J., and Soria, E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. Journal of Molecular Evolution 60:174–182.

Morris, K.N. (2002). 1998 Fine-leafed fescue test. 2001 Data Prog. Rep. NTEP (USDA-ARS, Beltsville, MD.).

Moy, M., Belanger, F.C., Duncan, R., Freehoff, A., Leary, C., Meyer, W., Sullivan, R., and White, J.F., Jr. 2000. Identification of epiphyllous mycelial nets on leaves of grasses infected by clavicipitaceous endophytes. Symbiosis 28:291–302.

Nagabhyru, P., Dinkins, R.D., and Schardl, C.L. 2019. Transcriptomics of *Epichloë*-grass symbioses in host vegetative and reproductive stages. Molecular Plant-Microbe Interactions 32:194–207.

Niones, J.T., and Takemoto, D. 2014. An isolate of *Epichloë festucae*, an endophytic fungus of temperate grasses, has growth inhibitory activity against selected grass pathogens. Journal of General Plant Pathology 80:337–347.

Niones, J.T., and Takemoto, D. 2015. VibA, a homologue of a transcription factor for fungal heterokaryon incompatibility, is involved in antifungal compound

production in the plant-symbiotic fungus *Epichloë festucae*. Eukaryotic Cell 14:13–24.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E., and Mortensen, U.H. 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLOS ONE 10:e0133085.

Oliveira, J.A., Gutiérrez-Villarias, M.I., Fernández-Casado, M.A., Costal-Andrade, L., González-Arráez, E., Bughrara, S.S., and Afif, E. 2008. Agronomic, leaf anatomy, morphology, endophyte presence and ploidy characterization of accessions of *Festuca* group *rubra* collected in northern Spain. Spanish Journal of Agricultural Research 6: 586–598.

Paege, N., Jung, S., Schäpe, P., Müller-Hagen, D., Ouedraogo, J.-P., Heiderich, C., Jedamzick, J., Nitsche, B.M., van den Hondel, C.A., Ram, A.F., and Meyer, V. 2016. A transcriptome meta-analysis proposes novel biological roles for the antifungal protein AnAFP in *Aspergillus niger*. PLOS ONE 11:e0165755.

Pérez, L.I., Gundel, P.E., Ghersa, C.M., and Omacini, M. 2013. Family issues: fungal endophyte protects host grass from the closely related pathogen *Claviceps purpurea*. Fungal Ecology 6:379–386.

Pfender, W.F., and Alderman, S.C. 2003. Evaluation of postharvest burning and fungicides to reduce the polyetic rate of increase of choke disease in orchardgrass seed production. Plant Disease 87:375–379.

Pfender, W.F., and Alderman, S.C. 2006. Regional development of orchardgrass choke and estimation of seed yield loss. Plant Disease 90:240–244.

Pohl, C., Kiel, J.A.K.W., Driessen, A.J.M., Bovenberg, R.A.L., and Nygård, Y. 2016. CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*. ACS Synthetic Biology 5:754–764.

Pourcel, C., Salvignol, G., and Vergnaud, G. 2005. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151:653–663.

Rahnama, M., Johnson, R.D., Voisey, C.R., Simpson, W.R., and Fleetwood, D.J. 2018. The global regulatory protein VelA is required for symbiosis between the endophytic fungus *Epichloë festucae* and *Lolium perenne*. Molecular Plant-Microbe Interactions 31:591–604.

Rahnama, M., Forester, N., Ariyawansa, K.G.S.U., Voisey, C.R., Johnson, L.J., Johnson, R.D., and Fleetwood, D.J. 2017. Efficient targeted mutagenesis in *Epichloë festucae* using a split marker system. Journal of Microbiological Methods 134:62–65.

Rodriguez, R.J., White, J.F., Jr., Arnold, A.E., and Redman, R.S. 2009. Fungal endophytes: diversity and functional roles. New Phytologist 182:314–330.

Ruemmele, B.A., Brilman, L.A., and Huff, D.R. 1995. Fine fescue germplasm diversity and vulnerability. Crop Science 35:313–316.

Ruemmele, B.A., Wipff, J.K., Brilman, L., and Hignight, K.W. 2003. Fine-leaved *Festuca* species. Turfgrass biology, genetics, and breeding. John Wiley & Sons, Inc., Hoboken, NJ, USA:129–174.

Saharan, B., and Nehra, V. 2011. Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res 21:1–30.

Saikkonen, K., Wäli, P.R., and Helander, M. 2010. Genetic compatibility determines endophyte-grass combinations. PLOS ONE 5:e11395.

Saikkonen, K., Faeth, S.H., Helander, M., and Sullivan, T.J. 1998. Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Systematics 29:319–343.

Salgado-Salazar, C., Beirn, L.A., Ismaiel, A., Boehm, M.J., Carbone, I., Putman, A.I., Tredway, L.P., Clarke, B.B., and Crouch, J.A. 2018. *Clarireedia*: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. Fungal Biology 122:761–773.

Sampson, K. 1933. The systemic infection of grasses by *Epichloë typhina* (Pers.) Tul. Transactions of the British Mycological Society 18:30–47, IN31–IN33.

Schardl, C.L. 2001. *Epichloë festucae* and related mutualistic symbionts of grasses. Fungal Genetics and Biology 33:69–82.

Schardl, C.L., and Phillips, T.D. 1997. Protective grass endophytes: where are they from and where are they going? Plant Disease 81:430–438.

Schardl, C.L., Leuchtmann, A., Chung, K.-R., Penny, D., and Siegel, M.R. 1997. Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. Mol. Biol. Evol. 14:133–143

Schardl, C.L., Balestrini, R., Florea, S., Zhang, D., and Scott, B. 2009. *Epichloë* endophytes: Clavicipitaceous symbionts of grasses. Pages 275-306 in: Plant Relationships, H.B. Deising, ed. Springer-Verlag, Berlin, Heidelberg.

Schardl, C.L., Young, C.A., Hesse, U., Amyotte, S.G., Andreeva, K., Calie, P.J., Fleetwood, D.J., Haws, D.C., Moore, N., Oeser, B., Panaccione, D.G., Schweri, K.K., Voisey, C.R., Farman, M.L., Jaromczyk, J.W., Roe, B.A., O'Sullivan, D.M., Scott, B., Tudzynski, P., An, Z., Arnaoudova, E.G., Bullock, C.T., Charlton, N.D., Chen, L., Cox, M., Dinkins, R.D., Florea, S., Glenn, A.E., Gordon, A., Güldener, U., Harris, D.R., Hollin, W., Jaromczyk, J., Johnson, R.D., Khan, A.K., Leistner, E., Leuchtmann, A., Li, C., Liu, J., Liu, J., Liu, M., Mace, W., Machado, C., Nagabhyru, P., Pan, J., Schmid, J., Sugawara, K., Steiner, U., Takach, J.E., Tanaka, E., Webb, J.S., Wilson, E.V., Wiseman, J.L., Yoshida, R., and Zeng, Z.

2013. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitaceae reveals dynamics of alkaloid loci. PLOS Genetics 9:e1003323.

Schiestl, F.P., Steinebrunner, F., Schulz, C., von Reuß, S., Francke, W., Weymuth, C., and Leuchtmann, A. 2006. Evolution of 'pollinator'- attracting signals in fungi. Biology Letters 2:401–404.

Schmid, J., Day, R., Zhang, N., Dupont, P.-Y., Cox, M.P., Schardl, C.L., Minards, N., Truglio, M., Moore, N., Harris, D.R., and Zhou, Y. 2017. Host tissue environment directs activities of an *Epichloë* endophyte, while it induces systemic hormone and defense responses in its native perennial ryegrass host. Molecular Plant-Microbe Interactions 30:138–149.

Schuster, M., Schweizer, G., Reissmann, S., and Kahmann, R. 2016. Genome editing in *Ustilago maydis* using the CRISPR–Cas system. Fungal Genetics and Biology 89:3–9.

Scott, B., Becker, Y., Becker, M., and Cartwright, G. 2012. Morphogenesis, growth, and development of the grass symbiont *Epichlöe festucae*. Pages 243-264 in: Morphogenesis and Pathogenicity in Fungi, J. Pérez-Martín and A. Di Pietro, eds. Springer Berlin Heidelberg, Berlin, Heidelberg.

Scott, B., Takemoto, D., Tanaka, A., Young, C., Bryant, M., and May, K. (2007). Functional analysis of the *Epichloë festucae*–perennial ryegrass symbiosis. In 6th International symposium on fungal endophytes of grasses. New Zealand Grassland Association, pp. 433–441.

Scott, B., Wrenn, R.E., May, K.J., Takemoto, D., Young, C.A., Tanaka, A., Fleetwood, D.J., and Johnson, R.D. 2009. Regulation and functional analysis of bioprotective metabolite genes from the grass symbiont *Epichloë festucae*. Pages 199–213 in: Recent Developments in Management of Plant Diseases, U. Gisi, I. Chet, and M.L. Gullino, eds. Springer Netherlands, Dordrecht.

Smith, J.D., Jackson, N., and Woolhouse, A.R. 1989. Fungal diseases of amenity turf grasses. E. & FN Spon Ltd.

Smith, S.E., and Read, D.J. 2008. Mycorrhizal symbiosis. Academic press, New York.

Steinebrunner, F., Schiestl, F.P., and Leuchtmann, A. 2008a. Ecological role of volatiles produced by *Epichloë*: differences in antifungal toxicity. FEMS Microbiology Ecology 64:307–316.

Steinebrunner, F., Twele, R., Francke, W., Leuchtmann, A., and Schiestl, F.P. 2008b. Role of odour compounds in the attraction of gamete vectors in endophytic *Epichloë* fungi. New Phytologist 178:401–411.

Sun, S., Clarke, B.B., and Funk, C.R. (1990). Effect of fertilizer and fungicide applications on choke expression and endophyte transmission in Chewings fescue.

In Proceedings of the International Symposium on Acremonium/Grass Interactions (Louisiana Agricultural Experiment Station Baton Rouge), pp. 62–66.

Tadych, M., and White, J.F., Jr. 2007. Ecology of epiphyllous stages of endophytes and implications for horizontal dissemination. Spore 150:157–161.

Tadych, M., Bergen, M.S., and White, J.F., Jr. 2014. *Epichloë* spp. associated with grasses: new insights on life cycles, dissemination and evolution. Mycologia 106:181–201.

Tadych, M., Ambrose, K.V., Bergen, M.S., Belanger, F.C., and White, J.F., Jr. 2012. Taxonomic placement of *Epichloë poae* sp. nov. and horizontal dissemination to seedlings via conidia. Fungal Diversity 54:117–131.

Takemoto, D., Tanaka, A., and Scott, B. 2006. A $p67^{Phox}$-like regulator is recruited to control hyphal branching in a fungal–grass mutualistic symbiosis. The Plant Cell 18:2807–2821.

Takemoto, D., Kamakura, S., Saikia, S., Becker, Y., Wrenn, R., Tanaka, A., Sumimoto, H., and Scott, B. 2011. Polarity proteins Bem1 and Cdc24 are components of the filamentous fungal NADPH oxidase complex. Proceedings of the National Academy of Sciences 108:2861–2866.

Takita, Y., Takahara, M., Nogami, S., Anraku, Y., and Ohya, Y. 1997. Applications of the long and accurate polymerase chain reaction method in yeast molecular biology: direct sequencing of the amplified DNA and its introduction into yeast. Yeast 13:763–768.

Tan, Y.Y., Spiering, M.J., Scott, V., Lane, G.A., Christensen, M.J., and Schmid, J. 2001. *In planta* regulation of extension of an endophytic fungus and maintenance of high metabolic rates in its mycelium in the absence of apical extension. Applied and Environmental Microbiology 67:5377–5383.

Tanaka, A., Christensen, M.J., Takemoto, D., Park, P., and Scott, B. 2006. Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. The Plant Cell 18:1052–1066.

Tanaka, A., Takemoto, D., Hyon, G.-S., Park, P., and Scott, B. 2008. NoxA activation by the small GTPase RacA is required to maintain a mutualistic symbiotic association between *Epichloë festucae* and perennial ryegrass. Molecular Microbiology 68:1165–1178.

Tanaka, A., Cartwright, G.M., Saikia, S., Kayano, Y., Takemoto, D., Kato, M., Tsuge, T., and Scott, B. 2013. ProA, a transcriptional regulator of fungal fruiting body development, regulates leaf hyphal network development in the *Epichloë festucae–Lolium perenne* symbiosis. Molecular Microbiology 90:551–568.

Tanimori, S., Ueda, T., and Nakayama, M. 1994. Efficient chemical conversion of (±)-chokol G to (±)-chokols A, B, C, F, and K, chokolic acid B, and chokolal A. Bioscience, Biotechnology, and Biochemistry 58:1174–1176.

Tian, Z., Wang, R., Ambrose, K.V., Clarke, B.B., and Belanger, F.C. 2017. The *Epichloë festucae* antifungal protein has activity against the plant pathogen *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease. Scientific Reports 7:5643.

Tintjer, T., and Rudgers, J.A. 2006. Grass–herbivore interactions altered by strains of a native endophyte. New Phytologist 170:513–521.

Vargas, J.M., Jr. 2005. Management of turfgrass diseases. J. Wiley, Hoboken, N.J.

Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10:1793–1808.

Walsh, B., Ikeda, S.S., and Boland, G.J. 1999. Biology and management of dollar spot (*Sclerotinia homoeocarpa*); an important disease of turfgrass. HortScience 34:13–21.

White, J.F., Jr. 1988. Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. Mycologia 80:442–446.

White, J.F., Jr., Breen, J.P., and Morgan-Jones, G. 1991a. Substrate utilization in selected *Acremonium*, *Atkinsonella* and *Balansia* species. Mycologia 83:601–610.

White, J.F., Jr., Morgan-Jones, G., and Morrow, A.C. 1993a. Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. Agriculture, Ecosystems & Environment 44:13–37.

White, J.F., Jr., Martin, T.I., and Cabral, D. 1996. Endophyte-host associations in grasses. XXII. Conidia formation by *Acremonium* endophytes on the phylloplanes of *Agrostis hiemalis* and *Poa rigidifolia*. Mycologia 88:174–178.

White, J.F., Jr., Morrow, A.C., Morgan-Jones, G., and Chambless, D.A. 1991b. Endophyte–host associations in forage grasses. XIV. Primary stromata formation and seed transmission in *Epichloë typhina*: developmental and regulatory aspects. Mycologia 83:72–81.

White, J.F., Jr., Lewis, G., Sun, S., and Funk , C.R., Jr. 1993b. A study of distribution of *Acremonium typhinum* in populations of red fescue in southwest England and *in vitro* growth comparisons to isolates from North American collections. Sydowia 45:388–394.

White, J.F., Jr. , and Chambless, D.A. 1991. Endophyte-host associations in forage grasses. XV. Clustering of stromata-bearing individuals of *Agrostis hiemalis* infected by *Epichloë typhina*. American Journal of Botany 78:527–533.

Yoshihara, T., Togiya, S., Koshino, H., Sakamura, S., Shimanuki, T., Sato, T., and Tajimi, A. 1985. Three fungitoxic cyclopentanoid sesquiterpenes from stromata of *Epichloë typhina*. Tetrahedron Letters 26:5551–5554.

Zhang, C., Meng, X., Wei, X., and Lu, L. 2016. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. Fungal Genetics and Biology 86:47–57.

Zhang, D.-X., Nagabhyru, P., Blankenship, J.D., and Schardl, C.L. 2010. Are loline alkaloid levels regulated in grass endophytes by gene expression or substrate availability? Plant Signaling & Behavior 5:1419–1422.

Zhang, N., Zhang, S., Borchert, S., Richardson, K., and Schmid, J. 2011. High levels of a fungal superoxide dismutase and increased concentration of a PR-10 plant protein in associations between the endophytic fungus *Neotyphodium lolii* and ryegrass. Molecular Plant-Microbe Interactions 24:984-992.

Zhang, W., Card, S.D., Mace, W.J., Christensen, M.J., McGill, C.R., and Matthew, C. 2017. Defining the pathways of symbiotic *Epichloë* colonization in grass embryos with confocal microscopy. Mycologia 109:153–161.

Chapter 2. Transcriptome Analysis of Stroma and Asymptomatic Inflorescence Tissues Reveals Numerous Differentially Expressed *Epichloë festucae* and *Festuca rubra* subsp. *rubra* Genes

INTRODUCTION

Many cool-season grasses have symbiotic relationships with *Epichloë* (Ascomycota, Clavicipitaceae) fungal endophytes, which inhabit the intercellular spaces of the above-ground parts of the host plants (Schardl et al., 2009; Scott et al., 2018a). Plants infected with the asexual *Epichloë* spp. are symptomless throughout their lifecycle and the endophytes are dispersed to the next plant generation in seeds by infecting the ovary and developing embryo (Liu et al., 2017). In contrast, most of the haploid *Epichloë* spp. have a sexual cycle, which is strictly linked to the flowering of the host plant. At flowering, the normally asymptomatic intercellular fungal mycelium proliferates extensively and eventually forms an external stroma, the fungal fruiting structure, which envelops the developing inflorescence (Fig. 2.1).

The presence of the fungal endophytes is generally beneficial to the hosts due to the enhanced tolerance to biotic and abiotic stresses conferred by the endophytes (Kuldau and Bacon, 2008; Johnson et al., 2013b). Endophyte-infected forage and turf grasses are marketed for their insect deterrence and toxicity to pests or non-toxicity to livestock (Card et al., 2016). Despite the benefits of *Epichloë* endophytes to their host, some strains are also antagonistic. Because the development of the inflorescence is physically constrained by the fungus, this syndrome is referred to as “choke disease”.

Choke disease can be a serious problem on commercially important grasses since it reduces seed yields. The turfgrasses Chewings fescue (*Festuca rubra* subsp. *commutata*) and strong creeping red fescue (*F. rubra* subsp. *rubra*), and the forage orchardgrass (*Dactylis glomerata*) can experience choke disease. In Oregon losses due to choke disease in orchardgrass in 2004 were estimated to be 0.8 million dollars (Pfender and Alderman, 2006). The use of fungicides to control choke disease in orchardgrass caused by *E. typhina* has been ineffective (Pfender and Alderman, 2003). In fine fescues, years of selective breeding for endophyte-infected plants not exhibiting choke symptoms have successfully reduced the incidence of choke disease in new turfgrass cultivars, but choke disease can still be a problem (Meyer et al., 2013).

Schardl et al. (2009) and Tadych et al. (2014) and have summarized the stroma-forming and non-stroma-forming *Epichloë* strains and their hosts. White (1988) classified the *Epichloë*–grass host symbiosis into three types. In type 1 associations, such as that in orchardgrass, stromata are produced on most endophyte-infected inflorescences and therefore seed transmission of the endophyte is suppressed. In type 2 associations, such as that in *F. rubra*, stromata are produced on only some of the inflorescences, and the endophyte is transmitted through seed produced on non-choked inflorescences. In type 3 associations, such as those with the hybrid *Epichloë* spp., stromata are never formed.

What triggers the development of a fungal stroma in response to the host flowering is not known. The change from microscopic to macroscopic mycelium must involve an increase in fungal growth rate, which suggests an increase in nutrient supply to the fungus. Kirby (1961) proposed that the developmental stage and growth rate of the host's flowering apex were critical factors in stromata development. He reported that the

rapid growth of the fungus began after the floral apex developed to the double-ridge stage. If the growth rate of the floral apex was slow, then the fungus would be in contact with the relatively large volume of plant meristematic cells long enough to absorb adequate nutrients for enhanced fungal growth. Applying gibberellic acid which promotes growth of the inflorescences has been shown to reduce the amount of stromata formation (Emecz and Jones, 1970). These findings support the hypothesis that a faster growing floral apex could escape stroma development. However, there has not been a subsequent exploration of this hypothesis. A complementary hypothesis presented by White et al. (1991b) was that stroma-forming *Epichloë* isolates are better able to utilize host sugars for rapid growth. Lam et al. (1995) reported dramatically elevated fungal invertase activity in stroma tissue, which would likely improve the ability of the fungus to access sucrose produced by the plant, which would support rapid mycelial proliferation.

Bucheli and Leuchtmann (1996) reported isozyme data of *E. sylvatica* stroma and non-stroma-forming isolates from *Brachypodium sylvaticum*, and concluded there was genetic differentiation between the isolates. Additionally, studies suggested that stroma-forming strains generally grow faster than asymptomatic strains in vitro (White et al., 1991a; White and Chambless, 1991; White et al., 1991b; White et al., 1993c). Meijer and Leuchtmann (1999) proposed that the fungal genome controls stromata formation and that the choked and non-choked tillers were infected with different strains of *E. sylvatica* in *B. sylvaticum* populations where stromata develop only on some inflorescences of an individual plant. Data from Chewings and strong creeping red fescues cross-inoculated with *Epichloë* isolates, suggested incidence of choke disease may depend on both the host plant genotype and the *Epichloë* endophyte genotype (Johnson-Cicalese et al., 2000).

Both of these possibilities fit the hypothesis that some signal or metabolite from the host that is unique to the flowering stage may be involved and that the fungus must be able to respond to such a signal for stromata development to occur.

The type 2 association is particularly interesting, because stromata are only formed on a fraction of the host plant inflorescences during the same developmental stage with presumably similar sugar availability. This variation was explained as a way for the fungus to balance stroma formation and seed transmission, however, with an unknown mechanism (White et al., 1993b). As a next step in investigating the mechanism underlying the development of choke disease, we carried out a transcriptome study to determine the differential plant and fungal gene expression between asymptomatic inflorescence tissue and stroma tissue in *E. festucae* infected strong creeping red fescue. Our objectives were to determine which fungal genes may be underlying the increase in growth rate and how does the plant tissue respond to that change.

MATERIAL AND METHODS

Sample Preparation

Three replicates of healthy developing inflorescences and choked panicles were collected from strong creeping red fescue plant 6035-5 A10-484 from the Rutgers research farm in Adephia, NJ on 10 May 2016. Healthy developing inflorescences were collected prior to anthesis; choked panicles were sampled before the development of perithecia when the fungal stroma turns yellow to orange color. Samples were immediately frozen and stored at –80 °C before RNA extraction. Total RNA was extracted from both tissues using ZR Fungal/Bacterial RNA MiniPrep™ (Zymo Research, Orange, CA) and treated with DNase to remove DNA following the manufacture recommendations.

RNA Sequencing

RNA-Seq was performed by the Waksman Genomics Core Facility. In brief, RNA concentration and integrity was verified using BioAnalyzer 2100 with RNA 6000 Nano Labchips according to manufacturer's instructions (Agilent Technologies, Palo Alto, CA). Samples of RNA had 28S/18S ratios ranging from 1.8 to 2.0 and RIN (RNA Integrity Number) values of between 7.0 and 9.0. PolyA RNA was isolated from 5–10 ug total RNA with oligo(dT) beads using two rounds of oligo-dT purification. Fifty to one hundred ng mRNA was used for Illumina library preparation.

Directional cDNA libraries were prepared using the dUTP method with a NEB Ultra directional RNA Library Prep for Illumina kit (New England BioLabs Inc.,

Ipswich, MA). Each sample was ligated with different indexes and amplified with 12 PCR cycles. The quality and quantity of cDNA libraries were evaluated using Qubit 2.0 (Invitrogen, Life Technologies, Carlsbad, CA), BioAnalyzer 2100 with DNA 1000 kit and real-time PCR using the KAPA Library Quantification Kit (Kapa Biosystems Boston, MA). Adapter-ligated cDNA fragment libraries were pooled together and loaded into an Illumina NextSeq500 using NextSeq® 500/550 High Output Kit version 3 according to the manufacturer's protocol (Illumina, San Diego, CA).

Data Analysis

Raw sequence data were de-multiplexed and FastQC software (v0.11.4) was applied for quality control (QC) of raw sequences. Fastq output files were used for all downstream applications. Adaptor sequences and reads shorter than 36 bp and/or reads with a quality score lower than 15 were removed from the dataset using Cutadapt software (v1.3). Data were submitted to NCBI (BioProject PRJNA490735). The trimmed reads were then mapped to the mitochondrion and chloroplast sequences (GenBank accessions: NC_009950.1 and JX999996.1) of *Lolium perenne* – the closest species to *Festuca rubra rubra* for which there are mitochondrion and chloroplast sequences are publicly available. The mapped reads were discarded and all the unmapped reads were retained for further analysis.

The filtered reads were first mapped to the reference genome *E. festucae* E2368 from the *Epichloë festucae* Genome Project (Schardl et al., 2013b). The mapping was done with CLC Genomics Workbench v10.1.1 with the minimum reads length fraction and the read similarity fraction both set to 0.9; reads that mapped to more than 1 contig

were not counted. Differential expression was done using the RNA-Seq Analysis tool with across groups comparison (ANOVA-like). Genes with normalized reads averaged across replications less than 10 RPKM (reads per kilobase of exon model per million mapped reads) in both symptomatic and asymptomatic tissues were not used for differential expression analysis. Significance thresholds were set to a false discovery rate (FDR) adjusted *p*-value lower than 0.01 and greater than 4-fold (Log_2 fold change of 2) difference in expression level.

The remaining plant RNA-Seq reads were pooled for assembling the *Festuca rubra rubra* transcriptome using Trinity (Grabherr et al., 2011) and CLC Genomics Workbench. CD-HIT (Li and Godzik, 2006; Fu et al., 2012) with a threshold of 90% identity was used to reduce redundancy, multi-copy genes and gene families in the transcriptome assembly. The non-redundant assembly contigs were used as query for a homology search using BLASTx against the NCBI non-redundant protein database (NR) applying a 10^{-5} e-value cutoff. There were 78 BLASTx matches to non-Viridiplantae species, which were omitted from the assembly.

The next step was to identify differentially expressed plant genes. For that, we separately mapped each library to the transcriptome assembly and counted the number of reads mapped to each contig. Differentially expressed genes were analyzed using the R Bioconductor package DESeq2 (Love et al., 2014). A table with the unique read counts per contig per library was used as input to DESeq2, and genes with a FDR adjusted *p*-value lower than 0.01 were considered differentially expressed.

Gene ontology (GO) terms were assigned to plant and fungal transcripts in blast2GO (BioBam, Valencia, Spain). Plant GO Slim categorization was generated for plant transcripts and Fisher's Exact Test was performed in Blast2GO with FDR < 0.05. No over-represented GOs were identified with differentially expressed fungal transcripts.

RESULTS AND DISCUSSION

Over 96 million reads per replicate per sample were generated (Table 2.1). In the asymptomatic inflorescence tissue, the fungal reads were a small percentage, 1%–1.5%, of the total reads. Similarly low levels of fungal transcripts in asymptomatic plant tissues, 0.01%–3.41%, have been reported previously (Ambrose and Belanger, 2012; Dinkins et al., 2017; Schmid et al., 2017; Nagabhyru et al., 2019). In contrast, the fungal reads were a major percentage, 76%–83%, of the total reads in the stroma samples reflecting the increased fungal biomass.

There are 9,324 annotated genes in the *E. festucae* 2368 genome (Schardl et al., 2013b). Here, sequence reads were mapped to 9,142 of the annotated genes. It may be that the undetected 182 genes were not expressed or these genes may be missing from the *E. festucae* isolate in our samples. One hundred and eighteen genes were more highly expressed in the asymptomatic inflorescence tissue, and 150 genes were more highly expressed in the stroma tissue (Tables 2.2 and 2.3, respectively). The characteristics of the strong creeping red fescue transcriptome assembly are presented in Table 2.4. In contrast, 2,964 and 4,862 of 75, 264 plant contigs were up- and down-regulated in the stroma tissue, respectively.

Most Abundant Endophyte Gene Transcripts

Among the top 20 highly expressed *E. festucae* genes in the asymptomatic inflorescences, 17 genes were significantly down-regulated in the stroma tissue (Table 2.5). Intriguingly, our data suggested that the gene expression profile was dramatically altered when *E. festucae* switched from restricted, intercellular growth to proliferative

growth to arrest the host reproductive tissue. Three out of the four genes that were highly expressed in both tissues were not statistically different in expression at FDR $p<0.01$ (Tables 2.5 and 2.6). They encode a glucose repressible protein (EfM3.025530), a secreted serine/threonine rich protein (EfM3.028690), and an uncharacterized protein (EfM3.072910).

Five of the abundantly expressed genes in the asymptomatic inflorescences (Table 2.5) have been previously reported to be among the top 20 fungal genes in the *E. festucae*–strong creeping red fescue interaction in asymptomatic leaf sheath tissue (Ambrose and Belanger, 2012). *Epichloë* specific genes NC12 and NC25/*gigA* (Johnson et al., 2015) were abundantly expressed in the asymptomatic inflorescence tissue, which were also reported in leaf sheaths and blades of many *Epichloë* and grass associations (Johnson et al., 2003; Eaton et al., 2010; Ambrose and Belanger, 2012; Dinkins et al., 2017; Schmid et al., 2017). However, both genes were down-regulated in the stroma tissue. NC25 was found highly expressed in the *E. festucae* Fl1–perennial ryegrass (*Lolium perenne*) association, however, it was down-regulated in the mutant Fl1 **Δ***sakA* mutant-infected perennial ryegrass plants (Eaton et al., 2010). The **Δ***sakA* mutant was reported to have unrestricted growth *in planta*, therefore was antagonistic to the host plant and resulted in stunted plants (Eaton et al., 2010).

An antifungal protein gene (*Efe-afpA*, EfM3.063660), abundantly expressed in leaf sheaths (Ambrose and Belanger, 2012; Tian et al., 2017) and the asymptomatic inflorescence tissue, was down-regulated in the stroma tissue (Table 2.5). The purified protein, *Efe*-AfpA, has been shown to have activity against the dollar spot pathogen *in vitro* (Tian et al., 2017). In *Aspergillus niger*, a transcriptome study suggested that a

similar antifungal protein, AnAFP, might function during carbon-starvation to induce autophagy (Paege et al., 2016). This supported our findings that transcripts of *Efe-afpA* were more abundant in the asymptomatic association where nutrients are limited in the intracellular spaces. The other two genes both highly expressed in leaf sheaths and in the asymptomatic inflorescence tissue, EfM3.020500 and EfM3.067730, are predicted as candidate effector proteins (Hassing et al., 2019); however, their functions are currently unknown.

Surprisingly, EfM3.046900 encoding glyceraldehyde 3-phosphate dehydrogenase was down regulated in the stroma tissue (Table 2.5). Glyceraldehyde 3-phosphate dehydrogenase is a key enzyme in the glycolysis pathway to convert glucose to pyruvate. Translation elongation factor 1 alpha gene (*tefA*; EfM3.021690) was abundantly expressed in both tissues but down-regulated in the stromata (Tables 2.5 and 2.6), even though *tefA* was stably expressed across many other tissues. This suggested that using the *tefA* as the sole reference for expression studies could be problematic. Therefore, multiple reference genes should be used for RT-qPCR studies.

Only two of the 20 most highly expressed genes in the stroma tissue have been previously characterized in *Epichloë*, *rhgA* (EfM3.030930) (Bassett et al., 2016) and *esdC* (EfM3.019650) (Tanaka et al., 2013), neither of which was differentially expressed in the current study (Table 2.6). The functions of most of the other highly expressed genes in the stroma tissue are currently unknown. A highly expressed gene that may relate to development of stromata is aquaglyceroporin (EfM3.025350), which was more highly expressed in the stromata than the asymptomatic inflorescences (Table 2.6). The annotation of the gene as “aquaglyceroporin” is based on the detection of a conserved

domain through a BLAST search at NCBI. Fungal "aquaglyceroporins" often function as water channels (Nehls and Dietz, 2014), so functional characterization of the *E. festucae* gene would be required to determine the type of molecule transported. It may function in water transport. There are several instances of fungal aquaglyceroporins functioning in water transport and their genes being up-regulated due to the increased demand for water in rapidly growing fruiting bodies (Nehls and Dietz, 2014). Similarly, water would be essential for stromata development. White et al. (1993a) reported that transpiration was enhanced in stromal leaves compared to nonstromal leaves, and would function to draw water through the stromata.

A Previously Reported Five-gene Cluster

A five-gene cluster was proposed to function in the production of an as yet unidentified secondary metabolite that may regulate stromata development (Berry, 2016; Berry et al., 2018a). In the *E. typhina–D. glomerata* and *E. elymi–Elymus* sp. symbioses the five genes of the cluster were up-regulated in stroma tissue relative to asymptomatic inflorescence tissue (Berry, 2016). In the *E. festucae*–strong creeping red fescue interaction analyzed here, two of the five genes in the cluster, *fxbA* (EfM3.019620) and *mfsB* (EfM3.019610), were up-regulated in the stroma tissue relative to the asymptomatic inflorescence tissue (Table 2.7). This expression pattern is consistent with the model presented by Berry (2016) in which the activity of IrlA on an as yet unidentified compound results in a non-stroma producing product but the competing activity of FxbA on the precursor compound leads to a stroma-producing compound that is transported out of the cell by MfsB. Over expression of *fxbA* in the stroma tissue relative to the asymptomatic inflorescence tissue could increase the rate of conversion of the putative

precursor to the stroma-producing compound relative to the non-stroma producing compound.

E. festucae Carbohydrate-Active Enzymes (CAZymes)

CAZymes play major roles in breaking down, modifying, or synthesizing polysaccharides. Fungi often produce CAZymes to degrade plant cell walls, therefore, plant pathogenic fungi generally possess more CAZyme genes than saprophytic or symbiotic fungi (Zhao et al., 2014). Twenty-four *E. festucae* genes in the auxiliary activities (AA), carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyltransferases (GT), and carbohydrate-binging modules (CBM) classes were differentially expressed (Table 2.8). Another CAZyme class of enzymes, polysaccharide lyases, were not found in *E. festucae* (Eaton et al., 2015).

Differential expression of some of the CAZymes is likely relevant to the difference in fungal growth between the two tissues. Genes for two secreted proteins, beta-fructofuranosidase and alpha-L-arabinofuranosidase, that are likely involved in accessing host-produced carbohydrates were more highly expressed in the stromata compared to the asymptomatic inflorescences. Expression of beta-fructofuranosidase (EfM3.074710), a secreted invertase, was significantly higher in the stroma tissue. Secreted invertase hydrolyzes apoplastic sucrose, which would likely be abundant in the inflorescence tissue (Braun et al., 2014). This result is consistent with previous research that detected high invertase activity in stroma tissues of Chewings fescue and strong creeping red fescue (Lam et al., 1995). The invertase activity produced by *E. festucae* grown in culture was shown to be sucrose inducible rather than glucose repressible (Lam

et al., 1995). The expression of a cytoplasmic invertase (EfM3.072380) in this study did not differ between the two tissue types.

Alpha-L-arabinofuranosidase (EfM3.015180) catalyzes the hydrolysis of terminal alpha-L-arabinofuranosidic bonds in hemicelluloses such as arabinoxylan and L-arabinan, which are present in host cell walls, releasing L-arabinose (Scheller and Ulvskov, 2010; Seiboth and Metz, 2011). In culture, *E. festucae* isolates grew as well on arabinose as on sucrose (White et al., 1993b). The up-regulation of both beta-fructofuranosidase and alpha-L-arabinofuranosidase in the stroma tissue suggests an increase in sucrose and arabinose utilization, which agrees with the hypothesis that the ability to utilize sugars and grow rapidly is critical for stromata formation (White et al., 1991b; White et al., 1993b).

Plant cell walls are mainly comprised of celluloses, hemicelluloses, ligins, pectins, and proteins. In accordance with Eaton et al. (2015), our data suggested expression of genes responsible for cellulose degradation was not altered during symptomatic and asymptomatic associations. Cutinases are often produced in early infection stages by plant fungal pathogens to assist in penetrating the waxy cuticle of plant surfaces and play a potential role as important virulence factors (Davies et al., 2000; Skamnioti and Gurr, 2007). Fungal transcripts encoding cutinases were detected in our study, but were not differentially expressed. Unlike their importance in the infection process, cutinases were apparently not required when endophyte changes from endophytic growth to proliferation that emerges from the plants to envelop and arrest the development of an inflorescence.

In contrast to the up-regulation of alpha-L-arabinofuranosidase, genes for two other plant cell wall degrading enzymes were down-regulated in the stroma tissue, a pectin methylesterase gene (EfM3.008730) and an endo-1,4-beta-xylanase (EfM3.040190). Both genes are candidate host specialization genes reported by Schirrmann et al. (2018) (discussed in more detail below). The interactions of the fungal endophyte with the cell wall of host plant are apparently different in asymptomatic inflorescence and stroma tissues.

Expression of two genes involved in the synthesis of chitin and glucan, important fungal cell wall components, were up-regulated in the stroma tissue. A chitin synthase (EfM3.049120) and a beta-1,3-glucan synthase (EfM3.026320) were up-regulated in the stromata relative to the asymptomatic inflorescences (Table 2.8). Increased glucan and chitin synthesis would be expected during the proliferative growth of *E. festucae* stromata, which envelop the developing inflorescences.

Other CAZymes were down-regulated in the stroma tissue, being more highly expressed in the asymptomatic inflorescence tissue. A chitinase (EfM3.024310) (Li et al., 2004) and a beta-1,6-glucanase (EfM3.013890) (Moy et al., 2002; Bryant et al., 2007), both hydrolytic enzymes that act on fungal cell walls, were down-regulated in the stroma tissue. Another chitinase (EfM3.000810) did not change in expression in this study, whereas it was reported to be down-regulated in all three antagonistic mutants by Eaton et al. (2015). The down-regulation of these fungal cell wall hydrolytic enzymes combined with the up-regulation in the fungal cell wall synthesis enzymes is possibly a factor in the increased growth and biomass accumulation of fungal tissue in the stromata. However, a glycosylphosphatidylinositol (GPI)-anchored beta-1,3-endoglucanase (EfM3.044280)

was more highly expressed in the stroma tissue. A potential role of this protein is to degrade the plant cell wall (Hasper et al., 2002), but it may also function to modify its own wall as was reported for a similar enzyme in *A. nidulans* (Choi et al., 2005).

Another CAZyme protein that may be important in the interaction of *E. festucae* with the host grass was a secreted protein with two LysM domains (EfM3.029340) that was more highly expressed in the asymptomatic inflorescence tissue than in the stroma tissue. Transcripts for this LysM domain containing protein were also reported to be abundant in *E. festucae* infected strong creeping red fescue leaf sheath tissue (Ambrose and Belanger, 2012). In the asymptomatic association of *E. festucae* with the host grass, the chitin in intercellular hyphae does not stain with wheat germ agglutinin, indicating that it is somehow masked, presumably as a defense mechanism to avoid being degraded by plant chitinase (Dupont et al., 2015). *E. festucae* can penetrate the leaf cuticle and emerge from the interior of the plant, forming an epiphytic hyphal network (Becker et al., 2016). The epiphytic hyphae do stain, indicating the chitin masking agent is no longer associated with the hyphae. Here, transcripts for the LysM domain-containing protein were down-regulated in the stroma tissue, an external tissue like the epiphytic leaf surface hyphae. In other fungal species secreted LysM domain effector proteins have been shown to bind chitin and thereby suppress the chitin-triggered plant defense against fungal pathogens (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012). This suggests a potential role of this protein in maintaining the symbiotic interaction in internal asymptomatic tissue by preventing chitin-triggered plant defense responses. Together with the down-regulation of chitinase (EfM3.024310) discussed above, the

endophyte likely maintains its asymptomatic association with its host grass by somehow modifying/masking its cell wall chitin.

Fungal Cell Wall Glycoproteins

Glycosylphosphatidylinositol (GPI)-anchored proteins attached to the carbohydrate backbone of the fungal cell wall have been shown to be involved in virulence of plant pathogenic fungi (Rittenour and Harris, 2013; Zhu et al., 2017). In *E. festucae*, Eaton et al. (2015) listed 127 predicted GPI-anchored proteins. A GPI-anchored cell wall beta-1,3-endoglucanase gene *eglC* (EfM3.044280) increased in expression in the stroma tissue (Table 2.8). The potential role of this protein is to degrade plant cell wall (Hasper et al., 2002) or inhibit the growth of other fungi by degrading their cell walls (Champer et al., 2016). Scott et al. (2018b) reported deleting a GPI-anchored cell wall protein gene *gpiB* (EfM3.018200) resulted in no phenotype *in planta*, despite the high expression *in planta* compared to in culture and the differential regulation in symbiosis defective *E. festucae* mutants. However, this gene was not differentially expressed when *E. festucae* switched from restricted endophytic to proliferative growth during choke disease in our study. Another GPI-anchored cell wall protein RhgA required to maintain restrictive hyphal growth *in planta* (Bassett et al., 2016) also did not change in expression in our study.

In contrast, another GPI-anchored cell wall protein gene (EfM3.005300) was down-regulated in the stroma tissue (Table 2.2). This gene encoding a subtilisin-like protease *prtC* (Bryant et al., 2009)/At1 (Reddy et al., 1996) was reported also being down-regulated in the all three antagonistic mutants of *E. festucae* (Eaton et al., 2015).

Abundant transcripts and protein were detected *in planta*, therefore, proteinase Atl was speculated to be an important factor in the symbiotic interaction (Lindstrom et al., 1993; Reddy et al., 1996). In agreement with Eaton et al. (2015), we found that the down-regulation of EfM3.005300 was associated with the antagonistic interaction.

Differential Expression of *E. festucae* Genes Related to Transport

Genes involved in sugar, amino acid, and oligopeptide transport were among those more highly expressed in the stroma tissue (Table 2.9), suggesting a higher demand for nutrient uptake in the stromata. Major facilitator superfamily (MFS) transporters and ATP-binding cassette (ABC) transporters were also among the differentially expressed transport related genes, but the compounds transported for many of these genes are not yet known. Some of these transporter genes were up-regulated and some were down-regulated in the stroma tissue. The differential expression of transport related genes between stromata and asymptomatic inflorescences indicates differences in how the two tissue types are interacting with their environment.

Genes related to vesicle-mediated transport were differentially expressed. A transcription factor Hac1 (EfM3.047190) was down-regulated in the stroma tissue (Table 2.2). The endoplasmic reticulum (ER) is a critical cell organelle for synthesis, folding, and transport of membrane and secreted proteins. ER stress occurs when the folding capacity is exceeded, which triggers the unfolded protein response (UPR). In yeast, the bZIP transcription factor Hac1 functions to activate expression of ER stress response genes (Cox and Walter, 1996; Mori et al., 1996). The role of Hac1/HacA in regulating UPR was characterized in filamentous fungi *Aspergillus*, *Trichoderma*, and *Neurospora*

(Saloheimo et al., 2003; Mulder et al., 2004; Montenegro-Montero et al., 2015). Utilization of cellulose as carbon source by *Neurospora* was impaired in the absence of Hac1, suggesting Hac1 affected the secretion of the enzymes required to decompose cellulose (Montenegro-Montero et al., 2015). In spite of the down-regulation of *Hac1* in the stroma tissue, we did not detect changes in the expression of *Epichloë* genes encoding cellulose degrading enzymes, which was in agreement with a study on antagonistic mutants by Eaton et al. (2015). Interestingly, UPR was demonstrated to impact fungal virulence of plant pathogens (Joubert et al., 2011; Heimel et al., 2013). Loss of UPR regulator genes (including Hac1) can impair the virulence of fungal pathogens, likely by affecting the secretion of important proteins for colonizing plant host and acquiring nutrients. The secreted effector proteins produced by fungal pathogens have been identified as key factors in infecting plants (Presti et al., 2015). In symbiotic interactions, secreted effector proteins may play a role in communicating with the plant hosts and maintaining mutualism (Eaton et al., 2015; Scott et al., 2018b).

Soluble N-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) first characterized in yeast to mediate membrane fusion in the protein-trafficking of the secretory pathway (Jahn and Scheller, 2006). A putative vacuolar SNARE subunit Vam7 (EfM3.037580_2) was up-regulated in the stroma tissue. In yeast, the homotypic fusion and protein sorting (HOPS) complex binds Vam7 via its subunits vacuole protein sorting proteins Vps16 and Vps18 (Krämer and Ungermann, 2011). Two up-regulated genes EfM3.001040 and EfM3.070700 in the stromata encode proteins similar to yeast VPS16 and VPS18, respectively (Table 2.9). Both yeast proteins belong to class C Vps; mutants of this class were defective in vacuolar carboxypeptidase Y sorting and lacked vacuoles

(Raymond et al., 1992). The functions of VPS proteins in filamentous fungi have not been studied in depth. *A. nidulans digA* (VPS18) mutant exhibited dichotomous and subapical branches with clustered mitochondria, clustered nuclei, and a defect in polarization of the actin cytoskeleton (Geißenhöner et al., 2001). Up-regulating vacuole protein sorting proteins during stromata development may suggest that actin polarization was stimulated resulting in rapid fungal growth. Vacuole protein sorting-associated proteins mediates the localization of glycosyltransferases to Golgi (Schmitz et al., 2008). In addition to vacuolar protein sorting proteins, a gene (EfM3.059060) encoding Golgi transport complex subunit Cog4 was up-regulated in the stroma tissue.

A siderophore iron transporter gene (EfM3.017590_2), homologous to *mirB* of *A. nidulans* (Haas et al., 2003) and *MFS1* of *Histoplasma* (Hwang et al., 2008), was increased in expression in the stroma tissue (Table 2.9). Iron limitation induced the transcription of *MFS1* and a siderophore gene cluster (Hwang et al., 2008). In *Aspergillus*, *mirB* was shown to transport the siderophore triacetylfusarinine C (Haas et al., 2003). Johnson et al. (2013a) reported that a siderophore synthase gene (*sidN*, EfM3.029790) was essential to maintain the mutualistic interaction of *E. festucae* with perennial ryegrass. This extracellular siderophore was designated as epichloënin A (Johnson et al., 2013a). However, the expression of *sidN* was not altered in our study. The *E. festucae* genome possesses multiple siderophore synthase and transporter genes, so whether epichloënin A is transported by siderophore iron transporter encoded by EfM3.017590_2 is currently unknown. Our data and findings from Johnson et al. (2013a) suggested that iron homeostasis might be essential or iron could be a signal for maintaining symbiotic relationship between endophytes and their hosts. More research is needed to understand

the role of iron in the *Epichloë* endophyte and grass symbiotic interaction.

An up-regulated amino acid transporter gene (EfM3.073030), homologous to *Neurospora crassa arg*-13 (Liu and Dunlap, 1996), indicated increased activities in arginine biosynthesis during choke disease (Table 2.9). Additionally, putative sugar transporter genes (EfM3.028310 and EfM3.054490), an oligopeptide transporter gene (EfM3.076540), and an amino acid permease gene (EfM3.056950) were up-regulated in the stromata (Table 2.9). In general, genes involved in nutrient transport were up-regulated, suggesting a high demand for nutrients during choke disease.

Two genes (EfM3.038260 and EfM3.073880) that encode P-type ATPases were increased in expression in the stroma tissue (Table 2.9). Gene EfM3.038260 is likely to mediate magnesium influx to the cytosol. On the other hand, a permease gene (EfM3.047170) and a peptide transporter gene (EfM3.027570) were down-regulated in the stroma tissue. Unlike the general up-regulation of transporters for nutrients, more major facilitator superfamily transporters and ATP-binding cassette transporters were found to have decreased activity during choke disease. However, the functions of those genes are still unknown.

Differential Expression of *E. festucae* Lipid Metabolism Genes

There were several lipid metabolism genes that were up-regulated in the stroma tissue, suggesting there may be a change in lipid composition in the stroma tissue relative to the intercellular fungal tissue. The biosynthetic pathway for glucosylceramide, a sphingolipid, is known and all the genes have been functionally characterized in other fungal species (Cheng et al., 2001; Garton et al., 2003; Fornarotto et al., 2006; Li et al.,

2006; Fernandes et al., 2016). Four of the eight genes for the biosynthesis of glycosylceramide were significantly more highly expressed in the stroma tissue (Table 2.10). Glucosylceramide is a component of the plasma membranes and is also found in exosomes (Nimrichter et al., 2008). Glucosylceramide can alter the properties of the plasma membrane favoring hyphal growth (Del Poeta et al., 2014), which is clearly enhanced in the stroma tissue.

Other lipid metabolism genes overexpressed in the stroma tissue are phosphatidic acid phosphatase (EfM3.009180), fatty acyltransferase (EfM3.017140), long-chain fatty acid CoA ligase (EfM3.034500), phospholipid-translocating P-type ATPase (EfM3.040210), phosphatidylserine decarboxylase (EfM3.054610), linoleate (8R)-dioxygenase (EfM3.057040), aminophospholipid translocase (EfM3.073880), and hydroxyacyl-CoA dehydrogenase-like (EfM3.074020) (Table 2.3).

Differential Expression of *E. festucae* Secondary Metabolite Biosynthetic Genes

Alkaloid Biosynthetic Genes

The fungal production of antiherbivore alkaloids is a common feature of many of the *Epichloë*–host grass interactions. Because of the commercial importance of the alkaloids to forage grasses, their structures, biosynthesis, and genes have been the topics of intensive investigation (Schardl et al., 2013a). There are four main categories of alkaloids produced by *Epichloë* endophytes, the indole-diterpenes, lolines, ergot alkaloids, and peramine. There is considerable variation among *Epichloë* spp. and isolates within a species in the biosynthesis of particular alkaloids, generally due to variation in the presence or absence of particular genes among isolates of a species (Schardl et al., 2013a).

Alkaloids are not generally detected when the *Epichloë* endophytes are grown in culture, but rather are synthesized in the symbiosis with the host. Stroma-forming endophyte species generally produce lower levels of alkaloids compared to asexual species (Leuchtmann et al., 2000; Tintjer and Rudgers, 2006). Alkaloid gene expression can also vary depending on the plant tissue. Alkaloid genes of *E. coenophila*, a non-stroma-forming endophyte of tall fescue, were significantly down-regulated in the ovaries compared with pseudostems (Nagabhyru et al., 2019). Therefore, it was suspected that we would only detect low levels of alkaloid biosynthesis transcripts in the reproductive tissue of strong creeping red fescue infected with sexual *E. festucae* (Table 2.11).

Transcripts for the gene for peramine biosynthesis and for six out of the 11 genes involved in ergot alkaloid biosynthesis were significantly down-regulated in the stroma tissue as compared to the asymptomatic inflorescence tissue, although for some the RPKM values were low (Table 2.11). Some of these genes were not included in the differential expression table because their expression did not meet the filter threshold of 10 RPKM. Sequences for the genes involved in indole-diterpene and loline biosynthesis were not detected in either the asymptomatic inflorescences or the stromata raising the possibility that these genes are not present in this isolate of *E. festucae*. Others also reported that lolines were not detected in other natural red fescue–*E. festucae* associations (Siegel et al., 1990; Leuchtmann et al., 2000). Variation in alkaloid synthesis capability among *E. festucae* isolates has previously been reported (Schardl et al., 2012).

Peramine biosynthesis is carried out by a multifunctional non-ribosomal peptide synthetase, encoded by the *perA*-1 gene. A partially deleted *perA* gene, designated *perA*-2, is present in many *Epichloë* spp. isolates, including some *E. festucae* isolates infecting

strong creeping red fescue (Schardl et al., 2012; Schardl et al., 2013a). Peramine is not detected in grasses infected with an *Epichloë* isolate containing the *perA*-2 allele although gene expression may be detected, leading to the speculation that perhaps a different secondary compound is produced from this mutated allele (Schardl et al., 2013a). Berry et al. (2018b) reported that the perA-2 allele encodes a functional diketopiperazine synthetase. Here, *perA* transcripts were detected, but a 17-bp insert sequence common to the *perA*-2 allele (Berry et al., 2015) was present indicating that this *E. festucae* isolate carries the variant allele. The same mutation generating the *perA*-2 allele is found in many *Epichloë* isolates, suggesting it had a single origin (Berry et al., 2015).

In general, alkaloid genes were down-regulated in the *Festuca rubra–E. festucae* interaction when the fungal growth habit shifted from the asymptomatic growth within the plant tissue to the visible stromata. Our results supported other reports that within plants, stroma-bearing tillers experienced greater herbivory damage by arthropods (Tintjer and Rudgers, 2006) and insects (Brem and Leuchtmann, 2001) than nonstroma-bearing tillers. Alkaloid genes were down-regulated in a MAP kinase mutant of *E. festucae* which exhibits proliferative growth in the host plant (Eaton et al., 2010). The hypothesis is that secondary metabolites are produced by nongrowing hyphae (Scott et al., 2012). Alkaloids are not generally detected when the *Epichloë* endophytes are actively growing on nutrient rich medium (Tanaka et al., 2012). During choke disease, abundant nutrients from the plant host would be available to *E. festucae* mimicking the nutrient rich medium growth condition; therefore, alkaloid production would be minimized. Moreover, *E. festucae* relies on the anthomyiid flies to cross-fertilize stromata to complete the sexual life cycle, therefore, it is possible the endophyte minimizes the

production of alkaloids to avoid the anti-insect effect on the flies (Leuchtmann et al., 2000; Zhang et al., 2010).

Non-ribosomal Peptide Synthetase and Polyketide Synthetase Genes

Other secondary metabolite biosynthetic genes were also down-regulated in the stroma tissue. In addition to the *perA* alleles, there are other non-ribosomal peptide synthetase genes in the *Epichloë* spp. genomes. Non-ribosomal peptide synthetases (NRPS) are large multifunctional proteins involved in synthesis of a diverse range of bioactive compounds (Finking and Marahiel, 2004). Johnson et al. (2007) examined 20 *Epichloë* spp. and identified 12 NRPS genes, although not all were present in all isolates. Here, one of the previously identified fungal non-ribosomal peptide synthetases, NRPS7 (EfM3.081210), was overexpressed in the asymptomatic inflorescence tissue (Table 2.12). The *E. festucae* 2368 genome sequence (http://csbio-l.csr.uky.edu/ef2011/) was also searched for genes annotated as non-ribosomal peptide synthases and two additional genes were identified that were overexpressed in the asymptomatic inflorescence tissue. One non-ribosomal peptide synthetase appears to be from the two annotated genes EfM3.059650 and EfM3.059660. These two annotated genes are likely actually a single gene since they are adjacent to each other and both are most similar to different regions of the same *Lecanicillium* sp. (Cordycipitaceae) gene encoding the synthesis of verlamelin, an antifungal compound (Ishidoh et al., 2014).

In addition to the synthesis of secondary metabolites by non-ribosomal peptide synthetases, many bioactive compounds are produced by polyketide synthases (Keller et al., 2005). The *E. festucae* 2368 genome sequence (http://csbio-l.csr.uky.edu/ef2011/)

contains 17 genes that are annotated as polyketide synthases, the functions of which are not yet known. Most were not differentially expressed, but three were more highly expressed in the asymptomatic inflorescence tissue than in the stroma tissue (Table 2.12). One of the polyketide synthases (EfM3.081190) is part of a gene cluster with two of the non-ribosomal peptide synthetases (EfM3.081210 and EfM3.081180) that were also more highly expressed in the asymptomatic inflorescence tissue. None of the differentially expressed *E. festucae* non-ribosomal peptide synthetases or polyketide synthases have yet been characterized as to the compounds they produce.

Coordinately regulated gene clusters are often involved in biosynthesis of secondary compounds. In addition to differential expression of two genes from the five-gene cluster proposed by Berry (2016) to be involved in stroma formation (discussed above), genes from other gene clusters were also differentially expressed between the two tissue types. Transcripts from a cluster of four genes, EfM3.029270 to EfM3.029300, were more highly expressed in the stroma tissue than the asymptomatic inflorescence tissue (Table 2.3), with two of them, EfM3.029280 and EfM3.029300, being among the 20 most highly expressed fungal genes in the stroma tissue (Table 2.6). The gene EfM3.029270 encodes a subtilisin-like protease designated *Efe*-PrtL (Bryant et al., 2009). Another subtilisin-like protease gene EfM3.013900, *prtB* (Bryant et al., 2009), was also up-regulated in the stroma tissue.

The other three genes in the cluster encode as yet uncharacterized proteins. This gene cluster appears to be restricted to *E. festucae*, (accessions ADFL02000205.1, NRIB01000040.1, AFRX02000601.1, NDBD01002901.1), *E. mollis* (accession JFGW01000214.1), and some isolates of *E. bromicola* (accessions NRIC01000304.1,

JFHA01000306.1) since it is not found in other *Epichloë* spp. for which whole genome sequences are available. Although in this study some of the genes of the cluster are highly and differentially expressed in stroma tissue, it remains to be determined whether they are critical to stromata production since this gene cluster is not found in some *Epichloë* spp. that produce stromata, such as *E. typhina* subsp. *typhina* infecting *D. glomerata* (accession SRX2830678) and *E. typhina* subsp. *clarkii* infecting *Holcus lanatus* (accession SRX2830679).

Two genes from another gene cluster were more highly expressed in the stroma tissue relative to the asymptomatic inflorescence tissue. EfM3.014890 (dipeptidase) and EfM3.014910 (MFS transporter, Table 2.9) are members of a gene cluster similar to the epipolythiodiketopiperazine gene cluster in *Claviceps purpurea* (Dopstadt et al., 2016). The cluster consists of 11 genes (EfM3.014870 to EfM3.014970) and all were expressed in both tissue types with only the dipeptidase and the MFS transporter genes being differentially expressed. In the *C. purpurea* strain analyzed (Dopstadt et al., 2016) the cytochrome P450 enzyme of the cluster was dysfunctional and no epipolythiodiketopiperazine was produced, but new, previously unknown, metabolites were produced. Such compounds have not been reported in *Epichloë* spp., but the expression of genes from this cluster suggests they may be present.

All genes of an apparent five-gene cluster (EfM3. 057200–EfM3.057240) were down-regulated in the stroma tissue relative to the asymptomatic inflorescence tissue (Table 2.2). However, the specific functions of the encoded proteins are currently not known. It is possible the proteins may act individually and do not function as a cluster acting in the same pathway.

E. festucae Symbiotic-related Genes

Candidate Host Specialization Genes

Candidate host specialization genes have been identified and hypothesized to be involved in stromata formation (Schirrmann et al., 2018). Host specialization among *Epichloë* spp. is considered to involve secreted proteins (Schirrmann et al., 2018; Hassing et al., 2019). Comparative genome scans of *E. typhina* subsp. *typhina* and *E. typhina* subsp. *clarkii* identified five candidate host specialization secreted proteins based on high dN/dS ratios indicative of positive selection (Schirrmann et al., 2018). Three of these candidates encode enzymes that could directly interact with the host grass; a pectin methylesterase (EfM3.008730), a peroxidase (EfM3.007770), and an endo-1-4-beta-xylanase (EfM3.040190). One of the host specificity candidates is a small-secreted protein (EfM3.008740) also identified as a candidate effector protein (Hassing et al., 2019). One is an unannotated gene that has a cyanovirin-N domain. Schirrmann et al. (2018) hypothesized that the three enzymes may be involved in facilitating stroma formation. However, in the transcriptome comparison presented here, the pectinesterase and endo-1-4-beta-xylanase were differentially expressed, but were more highly expressed in the asymptomatic inflorescence tissue than in the stroma tissue (Table 2.8). The other host specialization candidate genes were not significantly differentially expressed.

Candidate Effector Genes

The interaction of fungal plant pathogens and symbionts with their hosts involves effector proteins, characterized as small-secreted proteins that can be important for

colonization or for evasion of host defenses (Plett and Martin, 2015; Uhse and Djamei, 2018). *E. festucae* expresses numerous small-secreted proteins that may function as effectors in its interaction with the host grass (Ambrose and Belanger, 2012). However, none of these small-secreted proteins have been functionally confirmed as effectors. Hassing et al. (2019) analyzed the *E. festucae* genome sequence for potential effectors and identified 141 candidate genes. Some of these candidate effector genes were differentially expressed between the stroma and the asymptomatic inflorescence tissues. Six were more highly expressed in the stroma tissue and 19 were more highly expressed in the asymptomatic inflorescence tissue (Tables 2.2 and 2.3). The change in expression between the asymptomatic inflorescence tissue and the stroma tissue of these candidate effectors suggests they may affect a change in interaction with the host that likely occurs in the switch from asymptomatic to macroscopic growth of the fungus.

Two other candidate effectors protein genes, EfM3.062880 and EfM3.062900 were down-regulated in the stroma tissue (Table 2.2). Transcripts of EfM3.062880 were reported to be the 3rd most abundant in a study by Ambrose and Belanger (2012), and this protein was detected in the apoplast of *E. festucae* infected strong creeping red fescue (Tian et al., 2017). This gene encodes a protein similar to *Neosartorya fischeri* anti-yeast protein NFAP2 recently characterized by Tóth et al. (2016) (Fig. 2.2). NFAP2 has been shown to have the potential to treat vulvovaginal candidiasis (Kovács et al., 2019). Another copy of this gene (EfM3.062900) closely located on the same contig_441 was also down-regulated. Other small secreted proteins are largely uncharacterized. Eaton et al. (2015) reported that the antagonistic mutants of *E. festucae* down-regulated genes

encoding small-secreted proteins and secondary metabolites. Those mutants exhibited proliferative growth *in planta* and increased fungal biomass.

Genes Related to Endophyte Life Cycle

Transcripts of two putative hydrophobins genes (EfM3.042980 and EfM3.079420) were highly abundant in the stroma tissue and were significantly more highly expressed compared to asymptomatic inflorescence (Table 2.3). Hydrophobins interact at the fungal cell surface creating a hydrophobic surface layer and are important in many morphogenetic processes such as development of infection structures, sporulation, and fruiting body formation (Kershaw and Talbot, 1998). The aerial conidia and hyphae of filamentous fungi are coated in hydrophobins to protect against desiccation and wetting, and aid in the dispersal of spores to attach to hydrophobic surfaces (Linder et al., 2005). The endophyte up-regulated genes encoding hydrophobins likely to prepare for developing perithecia to undergo its sexual cycle. During choke disease the *E. festucae* mycelia emerge from the plant tissue and are exposed in the air; hydrophobins function to make aerial hyphae hydrophobic, and protect mycelia from the outside environment.

An adhesive-like protein gene (EfM3.021490), *adsA* (Child, 2014), which encodes a homolog of *Mad1* from *Metarhizium anisopliae* (Wang and St Leger, 2007), increased in expression in the stroma tissue (Table 2.3). Interestingly, MAD1 is required for conidia to adhere to insect cuticle, orientate the cytoskeleton, and regulate genes involved in cell cycle in *M. anisopliae* (Wang and St Leger, 2007). Expressing *adsA* in yeast conferred adherence, however, deletion of *adsA* did not alter hyphae growth in culture or *in planta* (Child, 2014). Therefore, it suggests gene redundancy in the *E.*

festucae genome involved in the physical attachment of hyphae to the host tissues. Despite the lack of mutant phenotype of the *adsA* knockout in the asexual stage, the up-regulation of the adhesion transcripts in *E. festucae* during stromata development in the current study suggested that the protein might play a role in regulating cell differentiation and initiation of the endophyte sexual cycle similar to the function of MAD1 in *M. anisopliae*.

A transcriptional factor (EfM3.000040) containing a NDT80/ PhoG DNA binding domain was up-regulated in the stroma tissue (Table 2.3). NDT80 was identified as a meiosis-specific gene in yeast (Xu et al., 1995). A *Neurospora* gene encodes the vegetative incompatibility blocked protein 1 (VIB-1) in the same protein family (Xiang and Glass, 2002). The up-regulation of this gene prior to the *E. festucae* sexual life cycle is in accordance with its potential role in conidiation and meiosis. Another *Epichloë* gene *vibA* (EfM3.000150) that characterized as affecting an unknown antifungal compound production (Niones and Takemoto, 2015), did not change in expression in our study. Many ribosomal proteins and transcription factors were down-regulated in the antagonistic association; however, the downstream genes regulated by them are not known.

Previously Reported *E. festucae* Symbiotic-related Genes

Many genes that were previously functionally characterized in *Epichloë* as affecting the symbiotic relationship with the host were not differentially expressed in our study. However, a putative glutathione-S-transferase gene (EfM3.056530) was down-regulated during choke disease (Table 2.2). This gene is similar to GST II from

Schizosaccharomyces pombe which plays a role in response to oxidative stress and detoxification (Cho et al., 2002). Reactive oxygen species were reported to negatively regulate tip growth and therefore to maintain restricted *in planta* growth (Tanaka et al., 2006). In contrast to the synchronous intercalary growth in the symptomless association, unrestricted tip growth and branching have been reported in stromata (Christensen et al., 2008). Therefore, the down-regulation of the glutathione-S-transferase gene in our study could indicate low level of reactive oxygen species which corresponded to the unrestricted, massive fungal mycelia by tip growth during choke disease.

Eaton et al. (2015) reported a transcriptome comparison of *E. festucae–Lolium perenne* associations of wild-type and knockout lines of three of the symbiosis related genes; a component of the NADPH oxidase complex *noxA*, a stress-activated mitogen-activated protein kinase *sakA*, and a transcription factor *proA*. They identified a core gene set of 182 genes that were differentially expressed with the same direction in all three knockout lines that were considered to be contributing to the antagonistic nature of the mutant lines. Although development of stromata could also be considered as an antagonistic interaction, only 22 of the 182 core genes were differentially expressed between the stromata and the asymptomatic inflorescences. Of the differentially expressed genes only 13 of the 22 were in the same direction as reported for the three knockout lines (Table 2.13). Similar to Eaton et al. (2015), the NAD(+)-dependent glutamate dehydrogenase gene (EfM3.013550), homologous to *Saccharomyces cerevisiae* GDH2 (Miller and Magasanik, 1990), was up-regulated when *E. festucae* became antagonistic to its grass host (Table 2.13). The *S. cerevisiae* GDH2 was demonstrated to be induced by low nitrogen (Miller and Magasanik, 1991). Interestingly,

early field research indicated that adequate nitrogen fertilization can reduce choke disease (Sun et al., 1990). Despite sharing some features in common, the gene expression profiles of the antagonistic mutants (Eaton et al., 2015) and the stroma tissue reported here are substantially different.

Differentially Expressed Plant Genes

We identified dramatic changes in plant gene expression between asymptomatic and symptomatic inflorescences–7826 differentially expressed plant genes with greater than 4-fold change (log2 fold change > 2; adjusted $p < 0.01$) comprising over 10% of the total assembled plant transcriptome. A greater number of genes were down-regulated compared to up-regulated in the stroma tissue. The over-represented plant GO Slim annotation suggested that genes related to biotic and abiotic stresses were up-regulated, whereas genes related to reproductive development were down-regulated in the stroma tissue (Table 2.14).

Up-regulated Plant Genes in the Stroma Tissue

Forty-six genes in the GO category “response to biotic stimulus” were up-regulated in the stroma tissue (Table 2.15), suggesting that the host strong creeping red fescue plant was under biotic stress during stromata development. The differential fungal gene expression suggested the general down-regulation of genes encoding plant cell wall degrading enzymes. Although *E. festucae* was antagonistic to its grass host during choke disease, the fungal gene expression profile was unlike the way other plant pathogens access nutrients from the host. On the other hand, plant genes up-regulated in response to the *E. festucae* proliferation were related to biotic stimulus. Therefore, the plant was

indeed sensing the presence of the antagonistic endophyte and attempting to defend against it. In contrast, Nagabhyru et al. (2019) reported no significant difference in gene expression profiles with endophyte presence or absence in inflorescence tissues in an asymptomatic association.

Among the plant proteins overexpressed in the stroma tissue in the GO category "response to biotic stimulus" were several pathogenesis-related (PR) proteins; a class 1 chitinase (PR-3), PR-4, thaumatin-like proteins (PR-5), peroxidases (PR-9), PR-10, and lipid transfer proteins (PR-14) (Table 2.15). These proteins have all been associated with antifungal activity (Loon et al., 2006; Liu et al., 2010; Jain and Kumar, 2015; Ali et al., 2018). PR-10 was previously reported in *E. festucae* infected ryegrass vegetative tissues (Zhang et al. 2011). However, they suggested that the endophyte only elicited a weak host defense response during an asymptomatic association.

Transcripts of 12 non-specific lipid-transfer proteins were among the genes in the GO category of "response to biotic stimulus" that were over-expressed in the stroma tissue (Table 2.15). Non-specific lipid-transfer proteins are a family of small cysteine-rich proteins involved in various biological functions, including resistance to biotic and abiotic stresses (Kader, 1997; Carvalho and Gomes, 2007; Yeats and Rose, 2008). Five of the 12 over-expressed lipid transfer proteins were similar to the DIR1 (defective in induced resistance)-type lipid transfer proteins. The *A. thaliana* lipid-transfer protein DIR1 has been shown to be critical for the development of systemic acquired resistance (Maldonado et al., 2002). Based on its structure DIR1 was considered to be a specific category of lipid transfer protein (Lascombe et al., 2008). We detected eight putative

DIR1-like lipid transfer protein sequences in the strong creeping red fescue transcriptome, five of which were up-regulated in the stroma tissue (Table 2.15).

Other proteins in this GO category over-expressed in the stroma tissue were calcium-dependent protein kinases, lectin-domain containing receptor kinases, probable leucine-rich repeat (LRR) receptor-like serine/threonine-protein kinases, triacylglycerol lipases, 1-aminocyclopropane-1-carboxylate oxidase, and an ethylene-responsive transcription factor (Table 2.15).

The over-represented GO "response to water deprivation" and "response to abscisic acid" in the current study suggested that the plant was under abiotic stress during choke disease. Six up-regulated genes with "response to water deprivation" GO supported the finding of White et al. (1993a) that transpiration is enhanced in stromal leaves. Those six genes encode a putative late embryogenesis abundant protein, a putative ornithine aminotransferase, a dehydrin, a heat shock protein, an aquaporin, and a histidine kinase (Table 2.15). Moisture from plant transpiration is likely to play an important role in the development of the stromata (White et al., 1993a); they found that the epidermal cells of the stromal leaves were damaged leading to increased evaporation from the surface. Transpirational water loss through the damaged stromal leaf may result in water deprivation in the plant tissue within the stroma. The strong creeping red fescue plant may have been under water deprivation as a result of increased transpiration. Abscisic acid is known to regulate leaf stomatal closure to prevent water loss via transpiration. Three of the four genes (putative ornithine aminotransferase, dehydrin, and histidine kinase genes) in the category of "response to abscisic acid" also belong to the "response

to water deprivation" GO category. The fourth gene encodes a putative low-temperature induced protein.

Down-regulated Plant Genes in the Stroma Tissue

Plant genes related to reproductive development including the GO categories of "cell cycle", "flower development", and "cell differentiation" were down-regulated in the stromata (Table 2.14). This suggests that the normal reproductive process was interrupted in the stroma tissue and consequently seed development was suppressed. There are many reports of choke disease causing a significantly reduction in seed production (Kirby, 1961; Lam et al., 1995; Pfender and Alderman, 2006).

Endophyte and Host Interaction

An over-expressed fungal gene (EfM3.028290) in the stroma tissue encodes an eliciting plant response-like protein similar to Epl1 (Sm1) in *Hypocrea*/*Trichoderma* (Djonović et al., 2006; Seidl et al., 2006; Yu et al., 2018). This small secreted protein belongs to the cerato-platanin family. Cerato-platanin genes are found in many fungal genomes and appear to be unique to fungi (Baccelli, 2015). The name originates from the first described member of this family, which was isolated from the fungal pathogen *Ceratocystis fimbriata* f. sp. *platani* and was found to be phytotoxic (Pazzagli et al., 1999). Cerato-platanin gene expression from *C. platani* was higher during rapid hyphal growth and spore formation (Baccelli et al., 2012). Based on the similarity in protein structure of cerato-platinin and plant expansins, Baccelli (2015) proposed that the cerato-platanins may function in loosening of fungal and plant cell walls facilitating hyphal elongation during growth as well as aiding in the release of nutrients from plant cells.

Similar proteins have since been characterized from other fungi and have been reported to have numerous effects such as phytotoxicity, elicitation of plant defense responses, and virulence (Pazzagli et al., 1999; Gaderer et al., 2014). Many have reported the role of this group of protein is to trigger plant defense against various pathogens (Djonović et al., 2006; Djonović et al., 2007; Salas-Marina et al., 2015). Likely, this elicitor protein triggers production of reactive oxygen species in plants, and induces the expression of defense-related genes, including class I chitinase (Djonović et al., 2006). A *Sclerotinia sclerotiorum* produced cerato-platanin protein SsCP1 was reported to be important for virulence and it targets plant PR1 to trigger plant defense responses (Yang et al., 2018).

The *E. festucae* cerato-platanin-like protein has not been functionally characterized, but the reported features of similar proteins may be relevant to what is seen in choke disease, where the fungal growth rate is increased and the encased plant tissue is expressing more defensive proteins. Significantly fewer transcripts of EfM3.028290 were found in the asymptomatic inflorescences as compared to the stromata in the current study, suggesting that to maintain an asymptomatic association the endophyte has to avoid the induction of plant defense responses. The up-regulation of the eliciting plant response gene (cerato-platanin-like gene) in the stroma tissue also was in accordance with up-regulation plant genes involved in systemic acquired resistance. In our plant data, we also identified up-regulated class I chitinase and other PR genes during choke disease.

Additionally, a fungal chitinase (EfM3.024310) (Li et al., 2004) that hydrolyzes fungal cell walls and a LysM domain containing protein (EfM3.029340) were highly abundant in the asymptomatic tissue but were down-regulated in the stroma tissue. Both

proteins likely modify endophyte cell wall chitin to maintain the asymptomatic association with the host grass. The down-regulation of both genes and up-regulation of a chitin synthase gene (EfM3.049120) during choke disease likely triggered plant defense as plant chitinase and PR genes were up-regulated.

CONCLUSIONS

Many sexual *Epichloë* spp. can switch from their normally asymptomatic growth habit to development of macroscopic stromata that envelop the developing inflorescences, resulting in what is termed choke disease. For commercial grass species the reduction in seed yield can be significant. However, little is known about the underlying biology of choke disease. Here we have compared gene expression of both the fungal endophyte, *E. festucae*, and the host plant, strong creeping red fescue, between the asymptomatic inflorescences and the stromata. Hundreds of fungal genes were differentially expressed between the two tissue types, supporting the expectation of a change in fungal metabolism occurring in the change from asymptomatic to macroscopic growth.

Fungal genes overexpressed in the stroma tissue indicate a change in fungal carbohydrate and lipid metabolism relative to the asymptomatic state. Also over-expressed in the stroma tissue were genes encoding enzymes of fungal cell wall synthesis, which would support the generation of the enhanced mycelial mass of stromata. Several candidate effector genes (Hassing et al., 2019) were differentially expressed between the two tissue types. Since such genes may be involved in the interaction of *E. festucae* with its host, the identified differentially expressed candidate effector genes are good targets for future functional characterization, including the use of gene knockouts. Most of the alkaloid biosynthetic genes and genes encoding some previously identified secreted hydrolytic enzymes were down-regulated in the stroma tissue.

Over 7,000 plant genes were differentially expressed between the stroma tissue and the asymptomatic inflorescence tissue. In contrast to the numerous differentially expressed plant genes reported here, changes in plant gene expression during asymptomatic associations are limited. In transcriptome comparisons of endophyte-infected and endophyte-free tall fescue inflorescence tissues, Nagabhyru et al. (2019) reported almost no significant difference in plant gene expression profiles. Similarly, in comparisons of endophyte-infected and endophyte-free vegetative tissues of strong creeping red fescue, perennial ryegrass, and tall fescue, only modest changes in plant gene expression have been reported (Ambrose and Belanger, 2012; Dinkins et al., 2017; Schmid et al., 2017). Plant genes over-expressed in the stroma tissue included genes related to water and biotic stress. The over-expression of biotic stress genes indicates the plant was responding to the fungal endophyte as if it was a pathogen by inducing expression of defense related genes. The plant tissue was also apparently experiencing water stress, which may be attributable to an increased diversion of available water to the developing stroma tissue. Moreover, plant genes related to reproductive processes were down-regulated in the stroma tissue, likely due to the interference with seed development by the physically restrictive growth of the stroma.

This study provides a step towards ultimately understanding what triggers the initiation of stromata development in some *Epichloë* spp. What triggers the initial shift in fungal growth habit in stromata development is not yet known but presumably is related to a host metabolic change that occurs in the transition from vegetative tissue to inflorescence development. Another complementary question for future research is why

in Type 2 interactions (White, 1988) on the same plant experiencing the same environmental conditions do some inflorescences develop stromata and some do not.

REFERENCES

Ali, S., Ganai, B.A., Kamili, A.N., Bhat, A.A., Mir, Z.A., Bhat, J.A., Tyagi, A., Islam, S.T., Mushtaq, M., Yadav, P., Rawat, S., and Grover, A. 2018. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiological Research 212-213:29-37.

Ambrose, K.V., and Belanger, F.C. 2012. SOLiD-SAGE of endophyte-infected red fescue reveals numerous effects on host transcriptome and an abundance of highly expressed fungal secreted proteins. PLoS ONE 7:e53214.

Baccelli, I. 2015. Cerato-platanin family proteins: one function for multiple biological roles? Frontiers in Plant Science 5:769.

Baccelli, I., Comparini, C., Bettini, P.P., Martellini, F., Ruocco, M., Pazzagli, L., Bernardi, R., and Scala, A. 2012. The expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation in *Ceratocystis platani*. FEMS Microbiology Letters 327:155-163.

Bassett, S.A., Johnson, R.D., Simpson, W.R., Laugraud, A., Jordan, T.W., and Bryan, G.T. 2016. Identification of a gene involved in the regulation of hyphal growth of *Epichloë festucae* during symbiosis. FEMS Microbiology Letters 363.

Becker, M., Becker, Y., Green, K., and Scott, B. 2016. The endophytic symbiont *Epichloë festucae* establishes an epiphyllous net on the surface of *Lolium perenne* leaves by development of an expressorium, an appressorium-like leaf exit structure. The New Phytologist 211:240–254.

Berry, D. (2016). Characterisation of novel secondary metabolism genes in plant-endophytic *Epichloë* fungi (Massey University, Palmerston North, New Zealand).

Berry, D., Takach, J.E., Schardl, C.L., Charlton, N.D., Scott, B., and Young, C.A. 2015. Disparate independent genetic events disrupt the secondary metabolism gene *perA* in certain symbiotic *Epichloë* species. Applied and Environmental Microbiology 81:2797-2807.

Berry, D., Grage, K., Ram, A., Padmaja, N., Dijkwel, P., Young, C.A., Schardl, C.L., and Scott, B. (2018a). Regulation of stroma development in sexual *Epichloë* species. In 10th International Symposium on Fungal Endophytes of Grasses, pp. 46.

Berry, D., Mace, W.J., Bode, H.B., Grage, K., Young, C.A., Schardl, C.L., Popay, A.J., Dijkwel, P., and Scott, B. (2018b). More than just peramine: New pyrrolopyrazine alkaloids from the surprisingly diverse *PER* locus. In 10th International Symposium on Fungal Endophytes of Grasses, pp. 59.

Braun, D.M., Wang, L., and Ruan, Y.-L. 2014. Understanding and manipulating sucrose phloem loading, unloading, metabolism, and signalling to enhance crop yield and food security. Journal of Experimental Botany 65:1713-1735.

Brem, D., and Leuchtmann, A. 2001. *Epichloë* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126:522–530.

Bryant, M.K., May, K.J., Bryan, G.T., and Scott, B. 2007. Functional analysis of a β-1,6-glucanase gene from the grass endophytic fungus *Epichloë festucae*. Fungal Genetics and Biology 44:808–817.

Bryant, M.K., Schardl, C.L., Hesse, U., and Scott, B. 2009. Evolution of a subtilisin-like protease gene family in the grass endophytic fungus *Epichloë festucae*. BMC evolutionary biology 9:168.

Bucheli, E., and Leuchtmann, A. 1996. Evidence for genetic differentiation between choke-inducing and asymptomatic strains of the *Epichloë* grass endophyte from *Brachypodium sylvaticum*. Evolution 50:1879–1887.

Card, S., Johnson, L., Teasdale, S., and Caradus, J. 2016. Deciphering endophyte behaviour: the link between endophyte biology and efficacious biological control agents. FEMS Microbiology Ecology 92.

Carvalho, A.d.O., and Gomes, V.M. 2007. Role of plant lipid transfer proteins in plant cell physiology—A concise review. Peptides 28:1144–1153.

Champer, J., Ito, J.I., Clemons, K.V., Stevens, D.A., and Kalkum, M. 2016. Proteomic analysis of pathogenic fungi reveals highly expressed conserved cell wall proteins. Journal of fungi (Basel, Switzerland) 2:6.

Cheng, J., Park, T.S., Fischl, A.S., and Ye, X.S. 2001. Cell cycle progression and cell polarity require sphingolipid biosynthesis in *Aspergillus nidulans*. Mol Cell Biol 21:6198–6209.

Child, P. (2014). Adhesins–Do they play a role in the *Epichloë festucae* association with perennial rye grass? (Massey University, Palmerston North, New Zealand).

Cho, Y.-W., Park, E.-H., Fuchs, J.A., and Lim, C.-J. 2002. A second stress-inducible glutathione S-transferase gene from *Schizosaccharomyces pombe*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1574:399-402.

Choi, C.-J., Ju, H.-J., Park, B.-H., Qin, R., Jahng, K.-Y., Han, D.-M., and Chae, K.-S. 2005. Isolation and characterization of the *Aspergillus nidulans eglC* gene encoding a putative β-1,3-endoglucanase. Fungal Genetics and Biology 42:590-600.

Christensen, M.J., Zhang, X., and Scott, B. 2008. Regulation switching of *Epichloë typhina* within elongating perennial ryegrass leaves. Mycological Research 112:1056-1062.

Cox, J.S., and Walter, P. 1996. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell 87:391–404.

Davies, K.A., De Lorono, I., Foster, S.J., Li, D., Johnstone, K., and Ashby, A.M. 2000. Evidence for a role of cutinase in pathogenicity of *Pyrenopeziza brassicae* on brassicas. Physiological and Molecular Plant Pathology 57:63–75.

de Jonge, R., Peter van Esse, H., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H.A.J., and Thomma, B.P.H.J. 2010. Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329:953–955.

Del Poeta, M., Nimrichter, L., Rodrigues, M.L., and Luberto, C. 2014. Synthesis and biological properties of fungal glucosylceramide. PLoS pathogens 10:e1003832.

Dinkins, R.D., Nagabhyru, P., Graham, M.A., Boykin, D., and Schardl, C.L. 2017. Transcriptome response of *Lolium arundinaceum* to its fungal endophyte *Epichloë coenophiala*. New Phytologist 213:324–337.

Djonović, S., Pozo, M.J., Dangott, L.J., Howell, C.R., and Kenerley, C.M. 2006. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Molecular Plant-Microbe Interactions 19:838–853.

Djonović, S., Vargas, W.A., Kolomiets, M.V., Horndeski, M., Wiest, A., and Kenerley, C.M. 2007. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiology 145:875–889.

Dopstadt, J., Neubauer, L., Tudzynski, P., and Humpf, H.-U. 2016. The epipolythiodiketopiperazine gene cluster in *Claviceps purpurea*: dysfunctional cytochrome P450 enzyme prevents formation of the previously unknown clapurines. PLOS ONE 11:e0158945.

Dupont, P.-Y., Eaton, C.J., Wargent, J.J., Fechtner, S., Solomon, P., Schmid, J., Day, R.C., Scott, B., and Cox, M.P. 2015. Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. New Phytologist 208:1227–1240.

Eaton, C.J., Dupont, P.-Y., Solomon, P., Clayton, W., Scott, B., and Cox, M.P. 2015. A core gene set describes the molecular basis of mutualism and antagonism in *Epichloë* spp. Molecular Plant-Microbe Interactions 28:218–231.

Eaton, C.J., Cox, M.P., Ambrose, B., Becker, M., Hesse, U., Schardl, C.L., and Scott, B. 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. Plant Physiology 153:1780–1794.

Emecz, T.I., and Jones, G.D. 1970. Effect of gibberellic acid on inflorescence production in cocksfoot plants infected with choke (*Epichloë typhina*). Transactions of the British Mycological Society 55:77–82, IN76.

Fernandes, C.M., de Castro, P.A., Singh, A., Fonseca, F.L., Pereira, M.D., Vila, T.V.M., Atella, G.C., Rozental, S., Savoldi, M., Del Poeta, M., Goldman, G.H., and Kurtenbach, E. 2016. Functional characterization of the *Aspergillus nidulans* glucosylceramide pathway reveals that LCB Δ8-desaturation and C9-methylation are relevant to filamentous growth, lipid raft localization and *Psd1* defensin activity. Molecular Microbiology 102:488–505.

Finking, R., and Marahiel, M.A. 2004. Biosynthesis of nonribosomal peptides. Annual Review of Microbiology 58:453–488.

Fornarotto, M., Xiao, L., Hou, Y., Koch, K.A., Chang, E., O'Malley, R.M., Black, T.A., Cable, M.B., and Walker, S.S. 2006. Sphingolipid biosynthesis in pathogenic fungi: Identification and characterization of the 3-ketosphinganine reductase activity of *Candida albicans* and *Aspergillus fumigatus*. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1761:52–63.

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28:3150-3152.

Gaderer, R., Bonazza, K., and Seidl-Seiboth, V. 2014. Cerato-platanins: a fungal protein family with intriguing properties and application potential. Applied Microbiology and Biotechnology 98:4795-4803.

Garton, S., Michaelson, L.V., Beaudoin, F., Beale, M.H., and Napier, J.A. 2003. The dihydroceramide desaturase is not essential for cell viability in *Schizosaccharomyces pombe*. FEBS Letters 538:192–196.

Geißenhöner, A., Sievers, N., Brock, M., and Fischer, R. 2001. *Aspergillus nidulans* DigA, a potential homolog of *Saccharomyces cerevisiae* Pep3 (Vps18), is required for nuclear migration, mitochondrial morphology and polarized growth. Mol Genet Genomics 266:672–685.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29:644–652.

Haas, H., Schoeser, M., Lesuisse, E., Ernst, J.F., Parson, W., Abt, B., Winkelmann, G., and Oberegger, H. 2003. Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C. Biochemical Journal 371:505–513.

Hasper, A.A., Dekkers, E., van Mil, M., van de Vondervoort, P.J.I., and de Graaff, L.H. 2002. EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan. Applied and Environmental Microbiology 68:1556–1560.

Hassing, B., Winter, D., Becker, Y., Mesarich, C.H., Eaton, C.J., and Scott, B. 2019. Analysis of *Epichloë festucae* small secreted proteins in the interaction with *Lolium perenne*. PLoS ONE 14:e0209463.

Heimel, K., Freitag, J., Hampel, M., Ast, J., Bölker, M., and Kämper, J. 2013. Crosstalk between the unfolded protein response and pathways that regulate pathogenic development in *Ustilago maydis*. The Plant Cell 25:4262–4277.

Hwang, L.H., Mayfield, J.A., Rine, J., and Sil, A. 2008. *Histoplasma* requires *SID1*, a member of an iron-regulated siderophore gene cluster, for host colonization. PLOS Pathogens 4:e1000044.

Ishidoh, K.-i., Kinoshita, H., and Nihira, T. 2014. Identification of a gene cluster responsible for the biosynthesis of cyclic lipopeptide verlamelin. Applied Microbiology and Biotechnology 98:7501-7510.

Jahn, R., and Scheller, R.H. 2006. SNAREs — engines for membrane fusion. Nature Reviews Molecular Cell Biology 7:631–643.

Jain, S., and Kumar, A. 2015. The pathogenesis related class 10 proteins in plant defense against biotic and abiotic stresses. Adv Plants Agric Res 2:305–314.

Johnson, L.J., Johnson, R.D., Schardl, C.L., and Panaccione, D.G. 2003. Identification of differentially expressed genes in the mutualistic association of tall fescue with *Neotyphodium coenophialum*. Physiological and Molecular Plant Pathology 63:305-317.

Johnson, L.J., Koulman, A., Christensen, M., Lane, G.A., Fraser, K., Forester, N., Johnson, R.D., Bryan, G.T., and Rasmussen, S. 2013a. An extracellular siderophore is required to maintain the mutualistic interaction of *Epichloë festucae* with *Lolium perenne*. PLOS Pathogens 9:e1003332.

Johnson, L.J., de Bonth, A.C.M., Briggs, L.R., Caradus, J.R., Finch, S.C., Fleetwood, D.J., Fletcher, L.R., Hume, D.E., Johnson, R.D., Popay, A.J., Tapper, B.A., Simpson, W.R., Voisey, C.R., and Card, S.D. 2013b. The exploitation of epichloae endophytes for agricultural benefit. Fungal Diversity 60:171-188.

Johnson, R., Voisey, C., Johnson, L., Pratt, J., Fleetwood, D., Khan, A., and Bryan, G. 2007. Distribution of NRPS gene families within the *Neotyphodium*/*Epichloë* complex. Fungal Genetics and Biology 44:1180–1190.

Johnson, R.D., Lane, G.A., Koulman, A., Cao, M., Fraser, K., Fleetwood, D.J., Voisey, C.R., Dyer, J.M., Pratt, J., Christensen, M., Simpson, W.R., Bryan, G.T., and Johnson, L.J. 2015. A novel family of cyclic oligopeptides derived from

ribosomal peptide synthesis of an in planta-induced gene, *gigA*, in *Epichloë* endophytes of grasses. Fungal Genetics and Biology 85:14–24.

Johnson-Cicalese, J., Secks, M.E., Lam, C.K., Meyer, W.A., Murphy, J.A., and Belanger, F.C. 2000. Cross species inoculation of Chewings and strong creeping red fescues with fungal endophytes. Crop Science 40:1485–1489.

Joubert, A., Simoneau, P., Campion, C., Bataillé-Simoneau, N., Iacomi-Vasilescu, B., Poupard, P., François, J.M., Georgeault, S., Sellier, E., and Guillemette, T. 2011. Impact of the unfolded protein response on the pathogenicity of the necrotrophic fungus *Alternaria brassicicola*. Molecular Microbiology 79:1305–1324.

Kader, J.-C. 1997. Lipid-transfer proteins: a puzzling family of plant proteins. Trends in Plant Science 2:66–70.

Keller, N.P., Turner, G., and Bennett, J.W. 2005. Fungal secondary metabolism — from biochemistry to genomics. Nature Reviews Microbiology 3:937-947.

Kershaw, M.J., and Talbot, N.J. 1998. Hydrophobins and pepellents: proteins with fundamental roles in fungal morphogenesis. Fungal Genetics and Biology 23:18–33.

Kirby, E.J.M. 1961. Host-parasite relations in the choke disease of grasses. Transactions of the British Mycological Society 44:493–503.

Kovács, R., Holzknecht, J., Hargitai, Z., Papp, C., Farkas, A., Borics, A., Tóth, L., Váradi, G., Tóth, G.K., Kovács, I., Dubrac, S., Majoros, L., Marx, F., and Galgóczy, L. 2019. *In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of vulvovaginal candidiasis. Antimicrobial Agents and Chemotherapy 63:e01777-01718.

Krämer, L., and Ungermann, C. 2011. HOPS drives vacuole fusion by binding the vacuolar SNARE complex and the Vam7 PX domain via two distinct sites. Molecular Biology of the Cell 22:2601–2611.

Kuldau, G., and Bacon, C. 2008. Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. Biological Control 46:57–71.

Lam, C.K., Belanger, F.C., White, J.F., Jr., and Daie, J. 1995. Invertase activity in *Epichloë*/*Acremonium* fungal endophytes and its possible role in choke disease. Mycological Research 99:867–873.

Lascombe, M.-B., Bakan, B., Buhot, N., Marion, D., Blein, J.-P., Larue, V., Lamb, C., and Prangé, T. 2008. The structure of "defective in induced resistance" protein of *Arabidopsis thaliana*, DIR1, reveals a new type of lipid transfer protein. Protein Science 17:1522-1530.

Leuchtmann, A., Schmidt, D., and Bush, L.P. 2000. Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë*/*Neotyphodium* endophytes. Journal of Chemical Ecology 26:1025–1036.

Li, H.M., Sullivan, R., Moy, M., Kobayashi, D.Y., and Belanger, F.C. 2004. Expression of a novel chitinase by the fungal endophyte in *Poa ampla*. Mycologia 96:526–536.

Li, S., Du, L., Yuen, G., and Harris, S.D. 2006. Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. Molecular biology of the cell 17:1218–1227.

Li, W., and Godzik, A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22:1658-1659.

Linder, M.B., Szilvay, G.R., Nakari-Setälä, T., and Penttilä, M.E. 2005. Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiology Reviews 29:877–896.

Lindstrom, J.T., Sun, S., and Belanger, F.C. 1993. A novel fungal protease expressed in endophytic infection of *Poa* species. Plant Physiology 102:645–650.

Liu, J., Nagabhyru, P., and Schardl, C.L. 2017. *Epichloë festucae* endophytic growth in florets, seeds, and seedlings of perennial ryegrass (*Lolium perenne*). Mycologia 109:691-700.

Liu, J.-J., Sturrock, R., and Ekramoddoullah, A.K.M. 2010. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. Plant Cell Reports 29:419-436.

Liu, Q., and Dunlap, J.C. 1996. Isolation and analysis of the *arg-13* gene of *Neurospora crassa*. Genetics 143:1163–1174.

Loon, L.C.v., Rep, M., and Pieterse, C.M.J. 2006. Significance of inducible defense-related proteins in infected plants. Annual Review of Phytopathology 44:135–162.

Love, M.I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K. 2002. A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. Nature 419:399–403.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K.E., Thomma, B.P.H.J., and Rudd, J.J. 2011. Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals

novel functional properties and varying contributions to virulence on wheat. Plant physiology 156:756–769.

Meijer, G., and Leuchtmann, A. 1999. Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica*. New Phytologist 141:355–368.

Mentlak, T.A., Kombrink, A., Shinya, T., Ryder, L.S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B.P.H.J., and Talbot, N.J. 2012. Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. The Plant Cell 24:322–335.

Meyer, W.A., Torres, M.S., and White, J.F. 2013. Biology and applications of fungal endophytes in turfgrasses. Pages 713–731 in: Turfgrass: Biology, Use, and Management, J.C. Stier, B.P. Horgan, and S.A. Bonos, eds. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI.

Miller, S.M., and Magasanik, B. 1990. Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*. Journal of Bacteriology 172:4927–4935.

Miller, S.M., and Magasanik, B. 1991. Role of the complex upstream region of the *GDH2* gene in nitrogen regulation of the NAD-linked glutamate dehydrogenase in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 11:6229–6247.

Montenegro-Montero, A., Goity, A., and Larrondo, L.F. 2015. The bZIP transcription factor HAC-1 is involved in the unfolded protein response and is necessary for growth on cellulose in *Neurospora crassa*. PLOS ONE 10:e0131415.

Mori, K., Kawahara, T., Yoshida, H., Yanagi, H., and Yura, T. 1996. Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes to Cells 1:803–817.

Moy, M., Li, H.M., Sullivan, R., White, J.F., Jr., and Belanger, F.C. 2002. Endophytic fungal β-1,6-glucanase expression in the infected host grass. Plant Physiology 130:1298–1308.

Mulder, H.J., Saloheimo, M., Penttilä, M., and Madrid, S.M. 2004. The transcription factor HACA mediates the unfolded protein response in *Aspergillus niger*, and up-regulates its own transcription. Mol Genet Genomics 271:130–140.

Nagabhyru, P., Dinkins, R.D., and Schardl, C.L. 2019. Transcriptomics of *Epichloë*-grass symbioses in host vegetative and reproductive stages. Molecular Plant-Microbe Interactions 32:194–207.

Nehls, U., and Dietz, S. 2014. Fungal aquaporins: cellular functions and ecophysiological perspectives. Applied Microbiology and Biotechnology 98:8835–8851.

Nimrichter, L., Rodrigues, M.L., Barreto-Bergter, E., and Travassos, L.R. 2008. Sophisticated functions for a simple molecule: The role of glucosylceramides in fungal cells. Lipid Insights 2:61-73.

Niones, J.T., and Takemoto, D. 2015. VibA, a homologue of a transcription factor for fungal heterokaryon incompatibility, is involved in antifungal compound production in the plant-symbiotic fungus *Epichloë festucae*. Eukaryotic Cell 14:13–24.

Paege, N., Jung, S., Schäpe, P., Müller-Hagen, D., Ouedraogo, J.-P., Heiderich, C., Jedamzick, J., Nitsche, B.M., van den Hondel, C.A., Ram, A.F., and Meyer, V. 2016. A transcriptome meta-analysis proposes novel biological roles for the antifungal protein AnAFP in *Aspergillus niger*. PLOS ONE 11:e0165755.

Pazzagli, L., Cappugi, G., Manao, G., Camici, G., Santini, A., and Scala, A. 1999. Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*. Journal of Biological Chemistry 274:24959-24964.

Pfender, W.F., and Alderman, S.C. 2003. Evaluation of postharvest burning and fungicides to reduce the polyetic rate of increase of choke disease in orchardgrass seed production. Plant Disease 87:375–379.

Pfender, W.F., and Alderman, S.C. 2006. Regional development of orchardgrass choke and estimation of seed yield loss. Plant Disease 90:240–244.

Plett, J.M., and Martin, F. 2015. Reconsidering mutualistic plant–fungal interactions through the lens of effector biology. Current Opinion in Plant Biology 26:45-50.

Presti, L.L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., and Kahmann, R. 2015. Fungal effectors and plant susceptibility. Annual Review of Plant Biology 66:513–545.

Raymond, C.K., Howald-Stevenson, I., Vater, C.A., and Stevens, T.H. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. Molecular biology of the cell 3:1389–1402.

Reddy, P.V., Lam, C.K., and Belanger, F.C. 1996. Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. Plant Physiology 111:1209–1218.

Rittenour, W.R., and Harris, S.D. 2013. Glycosylphosphatidylinositol-anchored proteins in *Fusarium graminearum*: inventory, variability, and virulence. PLOS ONE 8:e81603.

Salas-Marina, M.A., Isordia-Jasso, M.I., Islas-Osuna, M.A., Delgado-Sánchez, P., Jiménez-Bremont, J.F., Rodríguez-Kessler, M., Rosales-Saavedra, M.T., Herrera-Estrella, A., and Casas-Flores, S. 2015. The Epl1 and Sm1 proteins from *Trichoderma atroviride* and *Trichoderma virens* differentially modulate systemic disease resistance against different life style pathogens in *Solanum lycopersicum*. Frontiers in Plant Science 6.

Saloheimo, M., Valkonen, M., and Penttilä, M. 2003. Activation mechanisms of the HAC1-mediated unfolded protein response in filamentous fungi. Molecular Microbiology 47:1149–1161.

Schardl, C.L., Balestrini, R., Florea, S., Zhang, D., and Scott, B. 2009. *Epichloë* endophytes: Clavicipitaceous symbionts of grasses. Pages 275-306 in: Plant Relationships, H.B. Deising, ed. Springer-Verlag, Berlin, Heidelberg.

Schardl, C.L., Young, C.A., Faulkner, J.R., Florea, S., and Pan, J. 2012. Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecology 5:331-344.

Schardl, C.L., Young, C.A., Pan, J., Florea, S., Takach, J.E., Panaccione, D.G., Farman, M.L., Webb, J.S., Jaromczyk, J., Charlton, N.D., Nagabhyru, P., Chen, L., Shi, C., and Leuchtmann, A. 2013a. Currencies of mutualisms: Sources of alkaloid genes in vertically transmitted epichloae. Toxins 5:1064-1088.

Schardl, C.L., Young, C.A., Hesse, U., Amyotte, S.G., Andreeva, K., Calie, P.J., Fleetwood, D.J., Haws, D.C., Moore, N., Oeser, B., Panaccione, D.G., Schweri, K.K., Voisey, C.R., Farman, M.L., Jaromczyk, J.W., Roe, B.A., O'Sullivan, D.M., Scott, B., Tudzynski, P., An, Z., Arnaoudova, E.G., Bullock, C.T., Charlton, N.D., Chen, L., Cox, M., Dinkins, R.D., Florea, S., Glenn, A.E., Gordon, A., Güldener, U., Harris, D.R., Hollin, W., Jaromczyk, J., Johnson, R.D., Khan, A.K., Leistner, E., Leuchtmann, A., Li, C., Liu, J., Liu, J., Liu, M., Mace, W., Machado, C., Nagabhyru, P., Pan, J., Schmid, J., Sugawara, K., Steiner, U., Takach, J.E., Tanaka, E., Webb, J.S., Wilson, E.V., Wiseman, J.L., Yoshida, R., and Zeng, Z. 2013b. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitaceae reveals dynamics of alkaloid loci. PLOS Genetics 9:e1003323.

Scheller, H.V., and Ulvskov, P. 2010. Hemicelluloses. Annual Review of Plant Biology 61:263-289.

Schirrmann, M.K., Zoller, S., Croll, D., Stukenbrock, E.H., Leuchtmann, A., and Fior, S. 2018. Genomewide signatures of selection in *Epichloë* reveal candidate genes for host specialization. Molecular Ecology 27:3070-3086.

Schmid, J., Day, R., Zhang, N., Dupont, P.-Y., Cox, M.P., Schardl, C.L., Minards, N., Truglio, M., Moore, N., Harris, D.R., and Zhou, Y. 2017. Host tissue environment directs activities of an *Epichloë* endophyte, while it induces systemic hormone and defense responses in its native perennial ryegrass host. Molecular Plant-Microbe Interactions 30:138–149.

Schmitz, K.R., Liu, J., Li, S., Setty, T.G., Wood, C.S., Burd, C.G., and Ferguson, K.M. 2008. Golgi localization of glycosyltransferases requires a Vps74p oligomer. Developmental cell 14:523–534.

Scott, B., Green, K., and Berry, D. 2018a. The fine balance between mutualism and antagonism in the *Epichloë festucae*–grass symbiotic interaction. Current Opinion in Plant Biology 44:32–38.

Scott, B., Becker, Y., Becker, M., and Cartwright, G. 2012. Morphogenesis, growth, and development of the grass symbiont *Epichlöe festucae*. Pages 243-264 in: Morphogenesis and Pathogenicity in Fungi, J. Pérez-Martín and A. Di Pietro, eds. Springer Berlin Heidelberg, Berlin, Heidelberg.

Scott, B., Hassing, B., Winter, D., Mesarich, C., Eaton, C., and Becker, Y. 2018b. Analysis of *Epichloë festucae* small secreted proteins in the interaction with *Lolium perenne*. bioRxiv:490425.

Seiboth, B., and Metz, B. 2011. Fungal arabinan and L-arabinose metabolism. Applied Microbiology and Biotechnology 89:1665–1673.

Seidl, V., Marchetti, M., Schandl, R., Allmaier, G., and Kubicek, C.P. 2006. Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. The FEBS Journal 273:4346–4359.

Siegel, M.R., Latch, G.C.M., Bush, L.P., Fannin, F.F., Rowan, D.D., Tapper, B.A., Bacon, C.W., and Johnson, M.C. 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. Journal of Chemical Ecology 16:3301–3315.

Skamnioti, P., and Gurr, S.J. 2007. *Magnaporthe grisea* Cutinase2 mediates appressorium differentiation and host penetration and is required for full virulence. The Plant Cell 19:2674–2689.

Sun, S., Clarke, B.B., and Funk, C.R. (1990). Effect of fertilizer and fungicide applications on choke expression and endophyte transmission in Chewings fescue. In Proceedings of the International Symposium on Acremonium/Grass Interactions (Louisiana Agricultural Experiment Station Baton Rouge), pp. 62–66.

Tadych, M., Bergen, M.S., and White, J.F., Jr. 2014. *Epichloë* spp. associated with grasses: new insights on life cycles, dissemination and evolution. Mycologia 106:181–201.

Tanaka, A., Takemoto, D., Chujo, T., and Scott, B. 2012. Fungal endophytes of grasses. Current Opinion in Plant Biology 15:462–468.

Tanaka, A., Christensen, M.J., Takemoto, D., Park, P., and Scott, B. 2006. Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. The Plant Cell 18:1052–1066.

Tanaka, A., Cartwright, G.M., Saikia, S., Kayano, Y., Takemoto, D., Kato, M., Tsuge, T., and Scott, B. 2013. ProA, a transcriptional regulator of fungal fruiting body development, regulates leaf hyphal network development in the *Epichloë festucae–Lolium perenne* symbiosis. Molecular Microbiology 90:551–568.

Tian, Z., Wang, R., Ambrose, K.V., Clarke, B.B., and Belanger, F.C. 2017. The *Epichloë festucae* antifungal protein has activity against the plant pathogen *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease. Scientific Reports 7:5643.

Tintjer, T., and Rudgers, J.A. 2006. Grass–herbivore interactions altered by strains of a native endophyte. New Phytologist 170:513–521.

Tóth, L., Kele, Z., Borics, A., Nagy, L.G., Váradi, G., Virágh, M., Takó, M., Vágvölgyi, C., and Galgóczy, L. 2016. NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. AMB Express 6:75–75.

Uhse, S., and Djamei, A. 2018. Effectors of plant-colonizing fungi and beyond. PLOS Pathogens 14:e1006992.

Wang, C., and St Leger, R.J. 2007. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryotic cell 6:808–816.

White, J.F., Jr. 1988. Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. Mycologia 80:442–446.

White, J.F., Jr., Breen, J.P., and Morgan-Jones, G. 1991a. Substrate utilization in selected *Acremonium*, *Atkinsonella* and *Balansia* species. Mycologia 83:601–610.

White, J.F., Jr., Glenn, A.E., and Chandler, K.F. 1993a. Endophyte-host associations in grasses. XVIII. Moisture relations and insect herbivory of the emergent stromal leaf of *Epichloë*. Mycologia 85:195–202.

White, J.F., Jr., Morgan-Jones, G., and Morrow, A.C. 1993b. Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. Agriculture, Ecosystems & Environment 44:13–37.

White, J.F., Jr., Morrow, A.C., Morgan-Jones, G., and Chambless, D.A. 1991b. Endophyte–host associations in forage grasses. XIV. Primary stromata formation and seed transmission in *Epichloë typhina*: developmental and regulatory aspects. Mycologia 83:72–81.

White, J.F., Jr., Lewis, G., Sun, S., and Funk , C.R., Jr. 1993c. A study of distribution of *Acremonium typhinum* in populations of red fescue in southwest England and *in vitro* growth comparisons to isolates from North American collections. Sydowia 45:388–394.

White, J.F., Jr. , and Chambless, D.A. 1991. Endophyte-host associations in forage grasses. XV. Clustering of stromata-bearing individuals of *Agrostis hiemalis* infected by *Epichloë typhina*. American Journal of Botany 78:527–533.

Xiang, Q., and Glass, N.L. 2002. Identification of *vib-1*, a locus involved in vegetative incompatibility mediated by *het-c* in *Neurospora crassa*. Genetics 162:89–101.

Xu, L., Ajimura, M., Padmore, R., Klein, C., and Kleckner, N. 1995. *NDT80*, a meiosis-specific gene required for exit from pachytene in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 15:6572–6581.

Yang, G., Tang, L., Gong, Y., Xie, J., Fu, Y., Jiang, D., Li, G., Collinge, D.B., Chen, W., and Cheng, J. 2018. A cerato-platanin protein SsCP1 targets plant PR1 and contributes to virulence of Sclerotinia sclerotiorum. New Phytologist 217:739–755.

Yeats, T.H., and Rose, J.K.C. 2008. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). Protein Science 17:191–198.

Yu, W., Mijiti, G., Huang, Y., Fan, H., Wang, Y., and Liu, Z. 2018. Functional analysis of eliciting plant response protein Epl1-Tas from *Trichoderma asperellum* ACCC30536. Scientific Reports 8:7974.

Zhang, D.-X., Nagabhyru, P., Blankenship, J.D., and Schardl, C.L. 2010. Are loline alkaloid levels regulated in grass endophytes by gene expression or substrate availability? Plant Signaling & Behavior 5:1419–1422.

Zhao, Z., Liu, H., Wang, C., and Xu, J.-R. 2014. Correction: Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. BMC Genomics 15:6.

Zhu, W., Wei, W., Wu, Y., Zhou, Y., Peng, F., Zhang, S., Chen, P., and Xu, X. 2017. BcCFEM1, a CFEM domain-containing protein with putative GPI-anchored site, is involved in pathogenicity, conidial production, and stress tolerance in *Botrytis cinerea*. Frontiers in Microbiology 8:1807.

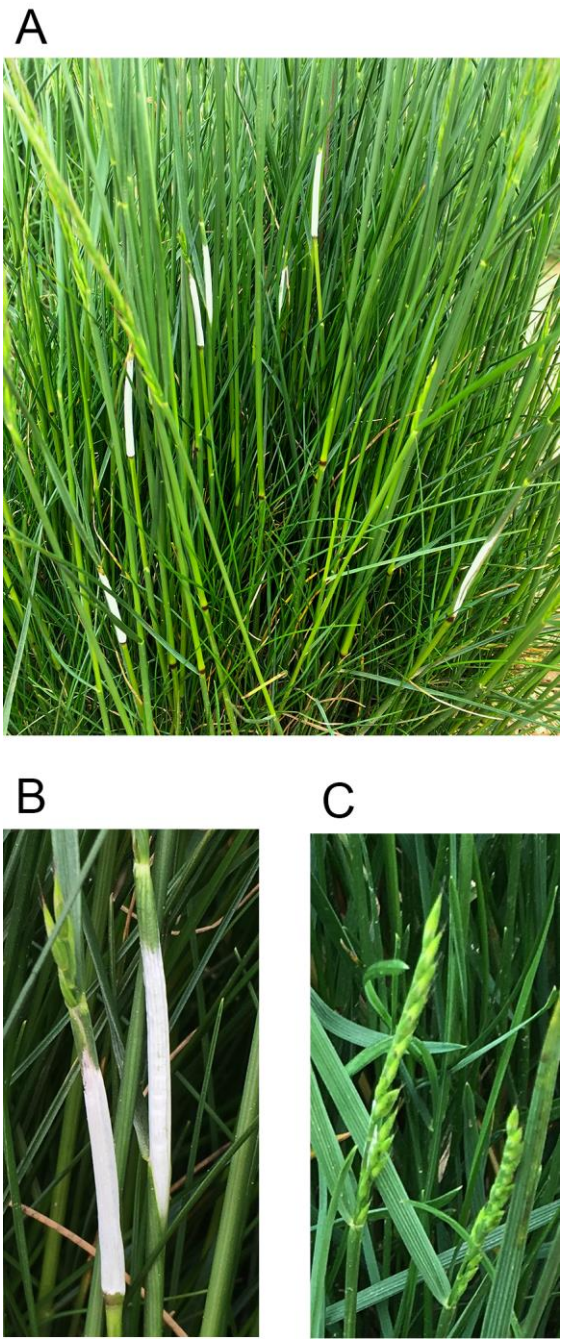

Figure 2.1. A, Choke disease in strong creeping red fescue. B, Stromata. C, Asymptomatic inflorescences.

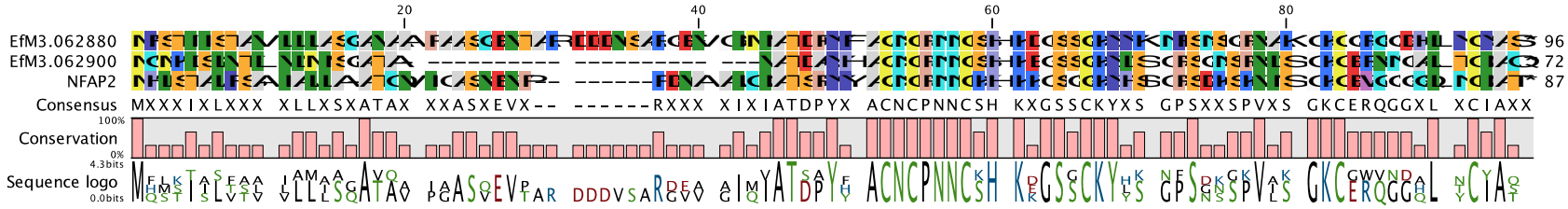

Figure 2.2. Protein sequence alignment of NFAP2 with EfM3.062880 and EfM3.062900.

Table 2.1. RNA-Seq mapping data.

	Total reads	**Fungal mapped reads, %**	**Plant mapped reads, %**
Choke stroma			
Replicate 1	124,013,190	82.93	9.09
Replicate 2	131,631,954	76.30	12.00
Replicate 3	188,024,471	76.04	14.23
Asymptomatic inflorescence			
Replicate 1	95,854,782	1.19	68.20
Replicate 2	102,612,960	1.04	68.08
Replicate 3	149,249,658	1.55	69.88

Table 2.2. *Epichloë festucae* differentially expressed genes significantly down-regulated in the stroma tissue relative to the asymptomatic inflorescence tissue at false discovery rate adjusted $p < 0.01$. Negative fold change (FC) value indicates expression in the stroma tissue was lower than in the asymptomatic inflorescence tissue.

Gene model	Protein annotation	Log_2 FC
EfM3.057200_1	2OG-Fe(II) oxygenase family	-6.2
EfM3.057240_1	2OG-Fe(II) oxygenase family Oxidoreductase; prolyl 4-hydroxylase	-9.1
EfM3.026500_1	40S ribosomal protein S17	-3.0
EfM3.066820_1	60S Ribosomal protein L17	-2.8
EfM3.020300_1	60S ribosomal protein L23	-2.1
EfM3.014790_1	ABC multidrug transporter	-6.8
EfM3.056220_1	ABC-type multidrug transport system	-5.7
EfM3.049610_1	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	-4.0
EfM3.073190_1	ADP-ribosylation factor	-2.2
EfM3.078640_1	Aldehyde dehydrogenase	-2.8
EfM3.040050_1	Arrestin domain-containing protein	-3.1
EfM3.059840_1	Basic leucine zipper transcription factor	-2.4
EfM3.020500_1	Candidate effector protein[a]	-12.0
EfM3.023030_1	Candidate effector protein	-4.0
EfM3.030230_1	Candidate effector protein	-15.0
EfM3.041770_1	Candidate effector protein	-7.9
EfM3.046430_1	Candidate effector protein	-6.2
EfM3.050840_1	Candidate effector protein	-8.4
EfM3.057230_1	Candidate effector protein	-6.7
EfM3.060210_1	Candidate effector protein	-7.4
EfM3.062880_1	Candidate effector protein	-5.6
EfM3.062900_1	Candidate effector protein	-4.5
EfM3.067730_1	Candidate effector protein	-3.7

EfM3.068730_1	Candidate effector protein	-7.8
EfM3.069580_1	Candidate effector protein	-2.9
EfM3.077900_1	Candidate effector protein	-4.8
EfM3.063660_1	Candidate effector protein, *Efe*-AfpA, Antifungal protein A (Tian et al., 2017)	-3.7
EfM3.006460_1	Candidate effector protein, syncollin-related protein	-17.1
EfM3.005070_1	Candidate effector protein; cupin superfamily protein	-4.5
EfM3.016770_1	Candidate effector protein; *Efe*-SspM	-11.1
EfM3.076450_1	Candidate effector protein; similar to Cladosporium extracellular protein 9-1	-5.2
EfM3.073810_1	Carbohydrate esterase family 3 protein; secreted	-4.2
EfM3.057830_1	Carboxyesterase type B, involved in lipid transport and metabolism	-3.8
EfM3.011910_1	Cytochrome P450	-5.4
EfM3.014800_1	Cytochrome P450	-6.5
EfM3.056990_1	Deoxyribonuclease nucA/nucB domain-containing protein	-4.0
EfM3.024310_1	*Efe*-ChiA, Chitinase; glycosyl hydrolase family 18 (Li et al., 2004)	-3.9
EfM3.049650_1	*Efe*-EasG, agroclavine dehydrogenase (Schardl et al., 2013b)	-14.0
EfM3.013890_1	*Efe*-GcnA, beta-1,6-glucanase (Moy et al., 2002; Bryant et al., 2007)	-5.4
EfM3.028480_1	*Efe*-GigA, cyclic peptide precursor (NC25) (Johnson et al., 2003)	-6.6
EfM3.018710_1	*Efe*-PerA, Peramine synthetase (Schardl et al., 2012)	-3.8
EfM3.005300_1	*Efe*-PrtC, subtilisin-like protease, secreted (Reddy et al., 1996; Bryant et al., 2009)	-2.7
EfM3.028570_1	*Efe*-PrtM, subtilisin-like serine protease, secreted (Bryant et al., 2009)	-5.5
EfM3.040190_1	Endo-1,4-beta-xylanase; secreted; glycoside hydrolase family 10 protein	-3.5
EfM3.057210_1	Eukaryotic cytochrome b561 domain-containing protein	-6.5
EfM3.064030_1	FAD-dependent pyridine nucleotide-disulfide oxidoreductase	-4.7
EfM3.001780_1	Fungal specific transcription factor	-5.6
EfM3.010300_1	Glutamate 5-kinase; step in proline biosynthesis	-3.3
EfM3.056530_1	Glutathione-S-transferase	-2.8
EfM3.046900_1	Glyceraldehyde-3-phosphate dehydrogenase	-2.9

EfM3.037010_1	Glycosyltransferase family 90 protein	-3.9
EfM3.041930_1	Glycosyltransferase family 90 protein	-2.3
EfM3.057470_1	GPI ethanolamine phosphate transferase	-9.0
EfM3.068720_1	GPR/FUN34 family protein; acetate transporter	-2.9
EfM3.054120_1	Intradiol dioxygenase	-3.1
EfM3.002010_1	LPS glycosyltransferase	-2.7
EfM3.029340_1	LysM domain protein, secreted (Ambrose and Belanger, 2012)	-5.1
EfM3.027570_1	Major facilitator superfamily, possible peptide transporter	-3.8
EfM3.082040_1	Major facilitator superfamily transporter	-2.4
EfM3.029870_1	Major facilitator superfamily transporter	-2.6
EfM3.017940_1	Major facilitator superfamily transporter	-5.5
EfM3.069880_1	Major facilitator superfamily transporter	-4.0
EfM3.066100_1	Methionine synthase; cobalamin independent	-4.2
EfM3.024560_1	Multicopper oxidase; laccase	-5.5
EfM3.001350_1	MYB DNA-binding domain-containing protein	-3.2
EfM3.003880_2	NADP-dependent alcohol dehydrogenase	-2.8
EfM3.059660_1	Non-ribosomal peptide synthetase	-6.4
EfM3.081210_1	Non-ribosomal peptide synthetase	-5.5
EfM3.059650_1	Non-ribosomal peptide synthetase[b]	-6.5
EfM3.081180_1	Nonribosomal peptide synthetase	-8.0
EfM3.008730_1	Pectinesterase; secreted; carbohydrate esterase family 8 protein	-3.1
EfM3.003560_1	Peptidoglycan binding domain	-2.5
EfM3.047170_1	Permease, cytosine/purine, uracil, thiamine, allantoin	-2.5
EfM3.081190_1	Polyketide synthase	-6.6
EfM3.081190_2	Polyketide synthase	-10.3
EfM3.043630_1	Polyubiquitin	-5.6
EfM3.048960_1	Related to integral membrane protein pth11	-5.0

EfM3.017060_1	Serine carboxypeptidase, secreted	-5.0
EfM3.047190_1	Transcriptional activator hac1	-2.0
EfM3.079050_1	Transglycosidase, crosslinks chitin to glucan	-2.7
EfM3.021690_1	Translation elongation factor 1-alpha	-4.4
EfM3.047160_1	UbiD family decarboxylase (ubiquinone biosynthesis)	-2.7
EfM3.005710_1	Ubiquitin-60S ribosomal protein L40 fusion	-5.7
EfM3.056300_1	Ubiquitin/ribosomal protein S27a fusion	-8.2
EfM3.007640_1	Uncharacterized protein	-3.1
EfM3.032325_1	Uncharacterized protein	-9.3
EfM3.035940_1	Uncharacterized protein	-3.6
EfM3.039600_1	Uncharacterized protein	-4.2
EfM3.053290_1	Uncharacterized protein	-5.1
EfM3.053300_1	Uncharacterized protein	-6.7
EfM3.057220_1	Uncharacterized protein	-6.6
EfM3.057590_1	Uncharacterized protein	-4.1
EfM3.057610_1	Uncharacterized protein	-2.3
EfM3.061540_1	Uncharacterized protein	-7.6
EfM3.071280_1	Uncharacterized protein	-4.4
EfM3.079040_1	Uncharacterized protein	-2.8
EfM3.066060.partial-1_1	Uncharacterized protein[c]	-6.2
EfM3.066060.partial-2_1	Uncharacterized protein[c]	-5.8
EfM3.071270_1	Uncharacterized protein, DUF 1237	-2.4
EfM3.028490_1	Uncharacterized protein, DUF 3328	-2.5
EfM3.051200_1	Uncharacterized protein, meiotically up-regulated domain	-7.5
EfM3.018170_1	Uncharacterized protein, NC12 (Johnson et al., 2003)	-8.1
EfM3.054640_1	Uncharacterized protein, secreted	-3.4
EfM3.057600_1	Uncharacterized protein, small	-3.5

EfM3.031770_1	Uncharacterized protein, small	-13.8
EfM3.047330_1	Uncharacterized protein, small	-4.8
EfM3.048860_1	Uncharacterized protein, small	-4.0
EfM3.051900_1	Uncharacterized protein, small	-3.9
EfM3.061530_1	Uncharacterized protein, small	-9.9
EfM3.069610_1	Uncharacterized protein, small	-3.5
EfM3.051210_1	Uncharacterized protein, small secreted	-6.7
EfM3.011570_1	Uncharacterized protein, small secreted	-3.1
EfM3.029680_1	Uncharacterized protein, small secreted	-4.1
EfM3.044610_1	Uncharacterized protein, small secreted	-5.1
EfM3.045520_1	Uncharacterized protein, small secreted	-9.9
EfM3.073520_1	Uncharacterized protein, small secreted	-4.0
EfM3.023050_1	Zinc finger transcription factor	-2.7
EfM3.033350_2	Zinc transcription factor	-3.7

[a] Protein annotation of "candidate effector protein" according to Hassing et al. (2019).
[c] Gene model EfM3.066060 split between two contigs.
[b] Possibly N-terminus of EfM3.059660_1.

Table 2.3. *Epichloë festucae* differentially expressed genes significantly more highly expressed in the stroma tissue than in the asymptomatic inflorescence tissue at false discovery rate adjusted $p < 0.01$. Positive fold change (FC) value indicates expression was higher in the stroma tissue than in the asymptomatic inflorescence tissue.

Gene model	Protein annotation	Log_2 FC
EfM3.057170_1	2-Epi-5-epi-valiolone synthase (converts SH7P to secondary products)	2.8
EfM3.000250_1	4-carboxymuconolactone decarboxylase (involved in benzoate degradation)	13.5
EfM3.054480_1	ABC type transporter	2.5
EfM3.053200_1	Aldehyde dehydrogenase	2.7
EfM3.029480_1	Alpha-N-acetylglucosaminidase	3.8
EfM3.010860_1	Amidinotransferase	4.1
EfM3.059800_1	Amidohydrolase family	13.2
EfM3.056950_1	Amino acid permease	13.6
EfM3.073880_1	Aminophospholipid translocase	3.9
EfM3.024210_1	Anthranilate/para-aminobenzoate synthase	3.5
EfM3.025350_1	Aquaglyceroporin	3.7
EfM3.002690_1	ATP synthase subunit C, DCCD-binding site	2.5
EfM3.079200_1	ATP-grasp fold protein	4.8
EfM3.052980_1	Autophagy-related protein 28	12.8
EfM3.026320_2	Beta-1,3-glucan synthase catalytic subunit	7.2
EfM3.074710_1	Beta-fructofuranosidase, secreted invertase	3.2
EfM3.012730_1	Bicupin, oxalate decarboxylase/oxidase	13.5
EfM3.053960_1	Calcium-dependent channel domain-containing protein	2.9
EfM3.007740_1	Candidate effector protein[a]	5.0
EfM3.028820_1	Candidate effector protein	5.2
EfM3.031680_1	Candidate effector protein	5.4
EfM3.075190_1	Candidate effector protein	4.9
EfM3.017130_1	Candidate effector protein; Phospholipase A2	3.9

EfM3.079420_1	Candidate effector protein; fungal hydrophobin domain-containing protein	6.4
EfM3.037330_1	Ceramide synthase	4.9
EfM3.028290_1	Cerato-platanin	3.4
EfM3.049120_1	Chitin synthase 1	2.8
EfM3.061150_1	Coenzyme A pyrophosphatase, a member of the Nudix hydrolase superfamily	4.8
EfM3.057920_2	Cysteine rich protein	12.8
EfM3.023600_1	Cytochrome P450	4.1
EfM3.071830_1	Cytochrome P450	2.3
EfM3.067500_2	DNA mismatch repair protein	14.1
EfM3.009880_1	DNA photolyase	3.8
EfM3.033770_1	DNase1	3.0
EfM3.021490_1	*Efe*-AdsA; Adhesin A; secreted; fungal specifc cysteine-rich domain (Child, 2014)	3.1
EfM3.019620_1	*Efe*-FxbA; FAD-binding oxidoreductase (Berry et al., 2016)	5.0
EfM3.019610_1	*Efe*-MfsB; membrane transporter protein (Berry et al., 2016)	4.5
EfM3.013900_1	*Efe*-PrtB; subtilisin-like protease, secreted (Bryant et al., 2009)	5.0
EfM3.029270_1	*Efe*-PrtL; subtilisin-like protease (Bryant et al., 2009)	4.8
EfM3.017140_1	Fatty acyltransferase	2.9
EfM3.059820_1	Ferritin-like superfamily of diiron-carboxylate proteins	3.2
EfM3.045830_1	Flavin-binding monooxygenase	3.9
EfM3.002510_3	Frag1/DRAM/Sfk1 family domain; calcofluor white hypersensitive protein	13.9
EfM3.039700_1	Fungal 1,3(4)-beta-D-glucanase	3.2
EfM3.015180_1	Fungal alpha-L-arabinofuranosidase, secreted	5.6
EfM3.001260_2	Fungal zinc cluster transcription factor	3.0
EfM3.057030_1	Fungal zinc cluster transcription factor	13.7
EfM3.080050_1	Glycoside hydrolase family 16	3.7
EfM3.037810_1	Glycosyl hydrolase 61 domain; may not have acitivity but enhance activity of other enzymes	3.6
EfM3.057650_1	Glycosyl hydrolase family 1	2.8

EfM3.080040_1	Glycosyl transferase, family 2	5.0
EfM3.059060_1	Golgi transport complex subunit COG4	13.5
EfM3.044280_1	GPI-anchored cell wall beta-1,3-endoglucanase EglC	2.3
EfM3.070970_2	Gtr1/RagA G domain containing protein	13.5
EfM3.072280_1	Haloacid dehalogenase-like hydrolase	3.0
EfM3.074020_1	Hydroxyacyl-CoA dehydrogenase-like; involved in beta oxidation of fatty acids	3.0
EfM3.031800_1	Lactonase family protein	2.9
EfM3.057040_1	Linoleate (8R)-dioxygenase	2.8
EfM3.034500_1	Long-chain fatty acid CoA ligase	2.9
EfM3.038260_1	Magnesium-translocating P-type ATPase	3.3
EfM3.014910_1	Major facilitator superfamily, multidrug resistance transporter	4.4
EfM3.017590_2	Major facilitator superfamily, possible iron siderophore transporter	12.9
EfM3.028310_1	Major facilitator superfamily, sugar transporter	4.1
EfM3.054490_2	Major facilitator superfamily, sugar transporter	4.6
EfM3.014890_1	Membrane dipeptidase	3.1
EfM3.073030_1	Mitochondrial amino acid transporter arg-13	2.5
EfM3.012750_1	N2227-like protein; methyltransferase domain	2.6
EfM3.081700_1	NAD(P)-dependent dehydrogenase; short chain alcohol dehydrogenase	13.0
EfM3.013550_1	NAD+ dependent glutamate dehydrogenase	2.5
EfM3.000040_1	NDT80 / PhoG like DNA-binding family protein	2.3
EfM3.057520_1	Nucleotidyltransferase/DNA polymerase involved in DNA repair	13.3
EfM3.079190_1	O-methyltransferase	2.8
EfM3.076540_1	Oligopeptide transporter OPT superfamily	3.6
EfM3.076150_1	Peptidase aspartic, secreted	3.6
EfM3.033210_1	Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A (cleaves glycopeptides)	3.2
EfM3.061580_1	Permease of the drug/metabolite transporter (DMT) superfamily	3.3
EfM3.010870_1	Phenylacetate-coenzyme A ligase PaaK, adenylate-forming domain	4.4

EfM3.009180_1	Phosphatidic acid phosphatase type 2 domain containing protein; lipid metabolism	2.6
EfM3.054610_1	Phosphatidylserine decarboxylase	3.5
EfM3.059410_1	Phosphoenolpyruvate phosphomutase; phosphonate synthesis	4.4
EfM3.040210_1	Phospholipid-translocating P-type ATPase, flippase	3.4
EfM3.018805_1	PLC-like phosphodiesterase	12.7
EfM3.058240_1	Pleckstrin homology domain; Domain commonly found in eukaryotic signalling proteins	4.3
EfM3.045990_2	Protein phosphatase	4.6
EfM3.070920_1	Putative serine esterase (DUF676)	3.9
EfM3.016080_1	PX domain protein; phosphoinositide binding domain	2.7
EfM3.040610_1	Regulator of G protein signaling superfamily	2.4
EfM3.067890_2	RNA binding protein of MSSP family	13.8
EfM3.033300_1	RNA polymerase II mediator complex component SRB4	12.6
EfM3.071430_1	RNA recognition motif containing protein	4.3
EfM3.013700_1	Serine palmitoyltransferase (sphingolipid biosynthesis)	2.6
EfM3.063400_2	Serine/threonine protein kinase	13.1
EfM3.016950_1	Sphingolipid C9-methyltransferase	4.2
EfM3.018540_1	Sphingolipid Δ8-desaturase	2.6
EfM3.032210_1	Src domain containing protein	3.6
EfM3.079780_1	Sugar O-acyltransferase	4.2
EfM3.074150_2	SWIB/MDM2 domain protein	14.5
EfM3.025130_1	Thioredoxin reductase	4.5
EfM3.042980_1	Trihydrophobin domain-containing protein	3.7
EfM3.001580_1	Uncharacterized protein	2.9
EfM3.004210_2	Uncharacterized protein	4.3
EfM3.010480_1	Uncharacterized protein	12.4
EfM3.014520_1	Uncharacterized protein	4.4
EfM3.026690_1	Uncharacterized protein	2.4

EfM3.029290_1	Uncharacterized protein	4.9
EfM3.029300_1	Uncharacterized protein	3.8
EfM3.030880_1	Uncharacterized protein	3.4
EfM3.032280_1	Uncharacterized protein	3.1
EfM3.032560_3	Uncharacterized protein	13.1
EfM3.037580_2	Uncharacterized protein	13.9
EfM3.045940_1	Uncharacterized protein	3.9
EfM3.047050_1	Uncharacterized protein	12.6
EfM3.047600_1	Uncharacterized protein	3.4
EfM3.048580_1	Uncharacterized protein	5.2
EfM3.051440_1	Uncharacterized protein	12.9
EfM3.052030_1	Uncharacterized protein	12.9
EfM3.053540_1	Uncharacterized protein	3.9
EfM3.063860_1	Uncharacterized protein	2.4
EfM3.064240_1	Uncharacterized protein	3.3
EfM3.064250_1	Uncharacterized protein	4.5
EfM3.066040_1	Uncharacterized protein	3.7
EfM3.068000_1	Uncharacterized protein	3.8
EfM3.068300_2	Uncharacterized protein	12.5
EfM3.070950_1	Uncharacterized protein	3.0
EfM3.073830_1	Uncharacterized protein	3.9
EfM3.075830_1	Uncharacterized protein	2.1
EfM3.078250_1	Uncharacterized protein	3.6
EfM3.082250_1	Uncharacterized protein	12.9
EfM3.037420_2	Uncharacterized protein	13.8
EfM3.049930_1	Uncharacterized protein	3.5
EfM3.004040_1	Uncharacterized protein, DUF 1765	3.2

EfM3.053070_2	Uncharacterized protein, DUF 1769	13.6
EfM3.023990_1	Uncharacterized protein, DUF 1996; secreted	2.9
EfM3.042820_1	Uncharacterized protein, DUF 2841	3.3
EfM3.071880_1	Uncharacterized protein, DUF 3405	3.6
EfM3.066800_1	Uncharacterized protein, DUF 3455	2.5
EfM3.002420_1	Uncharacterized protein, has DNA gyrase inhibitor domain	5.1
EfM3.003410_1	Uncharacterized protein, repetitive proline rich	5.0
EfM3.034060_1	Uncharacterized protein, retinal domain	5.3
EfM3.007650_1	Uncharacterized protein, small	2.2
EfM3.080780_1	Uncharacterized protein, small secreted	4.2
EfM3.059190_1	Uncharacterized protein, small secreted, serine rich	2.7
EfM3.045660_1	Uncharacterized protein, STLSQR repeats	3.5
EfM3.029280_1	Uncharacterized protein, vacuolating cytotoxin domain	5.1
EfM3.070700_1	Vacuolar protein sorting protein DigA	13.7
EfM3.001040_2	Vacuolar protein sorting vps16	14.6
EfM3.063630_1	WLM metalopeptidase domain containing protein	3.7
EfM3.061080_1	WSC domain; This domain may be involved in carbohydrate binding.	13.2
EfM3.003000_1	Zinc finger, C2H2-type/integrase, DNA-binding protein	4.8
EfM3.057810_1	Zinc-binding carboxypeptidase, secreted	3.0

[a] Protein annotation of “candidate effector protein” according to Hassing et al. (2019).

Table 2.4. Strong creeping red fescue assembly.

No. of contigs	75,264
Average length (bp)	516
Min/Max length (bp)	200/8,421
N50 (bp)	322
Total assembly length (bp)	38,860,733

Table 2.5. The 20 most abundant *Epichloë festucae* transcripts in asymptomatic strong creeping red fescue inflorescence tissue.

	Gene model and protein annotation		RPKM[a]	Log_2 FC[b]	FDR *p*
1	EfM3.056300	Ubiquitin – 40S ribosomal protein S27a fusion	68,394	-8.2	0
2	EfM3.043630	Polyubiquitin	16,619	-5.6	8.1e-13
3	EfM3.021690	Translation elongation factor EF-1 alpha	12,254	-4.4	4.4e-8
4	EfM3.005710	Ubiquitin – 60S ribosomal protein L40 fusion	11,137	-5.7	1.0e-11
5	EfM3.046430	Candidate effector protein[c,d]	10,899	-6.2	0
6	EfM3.020500	Candidate effector protein[c,d]	4,532	-12.0	0
7	EfM3.018170	Uncharacterized; NC12[e]	3,799	-8.0	0
8	EfM3.028480	GigA/NC25[f]; cyclic peptide precursor	3,421	-6.6	0
9	EfM3.025530	Glucose repressible protein, Grg1[d]	2,549	NS	NS
10	EfM3.029340	LysM domain containing protein	2,380	-5.1	4.3e-10
11	EfM3.072910	Uncharacterized small protein	1,672	NS	NS
12	EfM3.057230	Candidate effector protein[c]	1,398	-6.7	0
13	EfM3.067730	Candidate effector protein[c,d]	1,360	-3.6	4.8e-8
14	EfM3.024310	Chitinase[g]; glycosyl hydrolase family 18	1,106	-3.9	5.1e-7
15	EfM3.046900	Glyceraldehyde 3-phosphate dehydrogenase	1,098	-2.9	3.7e-4
16	EfM3.006460	Candidate effector protein[c]	1,004	-17.1	0
17	EfM3.026500	40S ribosomal protein S17	948	-3.0	3.3e-4
18	EfM3.028690	Secreted serine/threonine rich protein	931	NS	NS
19	EfM3.050840	Candidate effector protein[c]	828	-8.4	8.6e-10
20	EfM3.063660	Antifungal protein[d,h]; candidate effector protein[c]	815	-3.7	5.8e-10

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three asymptomatic inflorescence sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stromata with the asymptomatic inflorescences. Negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue. NS indicates there was no statistical difference in gene expression at the false discovery rate (FDR) adjusted *p* value < 0.01. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

[c] Hassing et al., 2019; [d] Ambrose and Belanger, 2012; [e] Johnson et al., 2003; [f] Johnson et al., 2015; [g] Li et al., 2004; [h] Tian et al., 2017.

Table 2.6. The 20 most abundant *Epichloë festucae* transcripts in strong creeping red fescue stroma tissue.

	Gene model and protein annotation		RPKM[a]	Log_2 FC[b]	FDR *p*
1	EfM3.025530	Glucose repressible, Grg1	34,478	NS	NS
2	EfM3.072910	Uncharacterized small protein	7,962	NS	NS
3	EfM3.019720	Clock-controlled protein-6; Mmc	6,484	NS	NS
4	EfM3.029300	Uncharacterized protein	5,815	3.8	7.0e-5
5	EfM3.007740	Candidate effector protein[c]	3,865	5.0	1.9e-4
6	EfM3.075190	Candidate effector protein[c]	2,779	4.9	4.5e-5
7	EfM3.059820	Ferritin/ribonucleotide reductase-like	2,428	3.2	0.002
8	EfM3.021690	Translation elongation factor EF-1 alpha	2,340	-4.4	4.4e-8
9	EfM3.025350	Aquaglyceroporin	2,241	3.7	5.2e-4
10	EfM3.059410	Pyruvate/phosphoenolpyruvate kinase	2,223	4.4	2.9e-4
11	EfM3.022140	Histone H3	2,155	NS	NS
12	EfM3.003250	Uncharacterized; coiled-coil domain	2,075	NS	NS
13	EfM3.043090	Hydrophobic surface binding protein	1,760	NS	NS
14	EfM3.031680	Candidate effector protein[c]	1,720	5.4	1.9e-5
15	EfM3.079420	Candidate effector protein[c]; Hydrophobin	1,539	6.4	1.9e-4
16	EfM3.030930	RhgA[d], secreted	1,520	NS	NS
17	EfM3.019650	EsdC[e], glycogen-binding domain	1,480	NS	NS
18	EfM3.064250	Uncharacterized	1,458	4.5	1.3e-5
19	EfM3.029280	Vacuolating cytotoxin domain protein	1,433	5.1	1.2e-4
20	EfM3.064240	Uncharacterized	1,399	3.3	1.3e-5

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three stroma tissue sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stromata with the asymptomatic inflorescences. Positive fold change value indicates the gene was more highly expressed in the stroma tissue and negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue. NS indicates there was no statistical difference in gene expression at the false discovery rate (FDR) adjusted *p* value < 0.01. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

[c] Hassing et al., 2019; [d] Bassett et al., 2016; [e] Tanaka et al., 2013.

Table 2.7. Expression of genes of the five-gene cluster proposed by Berry (2016) to be involved in regulation of stromata development.

Gene (gene model)	**RPKM[a]**	**Log_2 FC[b]**	**FDR *p* value**
pdtA (EfM3.019580)	45	NS	NS
afrA (EfM3.019590)	33	NS	NS
irlA (EfM3.019600)	630	NS	NS
mfsB (EfM3.019610)	226	4.5	0.0003
fxbA (EfM3.019620)	147	5.0	1.1e-05

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three stroma tissue sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stromata with asymptomatic inflorescences. Positive fold change value indicates the gene was more highly expressed in the stroma tissue. NS indicates there was no statistical difference in gene expression between the asymptomatic inflorescence and the stroma tissues at the false discovery rate (FDR) adjusted *p* value < 0.01. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

Table 2.8. Differential expression in *Epichloë festucae* CAZyme genes at false discovery rate adjusted $p < 0.01$.

CAZymes	Gene model	Predicted function	Log_2 FC[a]
AA11	EfM3.037810_1	Glycoside hydrolase	3.6
AA5	EfM3.061080_1	WSC domain	13.2
AA7	EfM3.019620_1	*Efe*-FxbA; FAD-binding oxidoreductase	5.0
CE16	EfM3.017140_1	Fatty acyltransferase	2.9
CE3	EfM3.073810_1	Carbohydrate esterase family 3; secreted	-4.2
CE8	EfM3.008730_1	Pectinesterase, secreted	-3.1
GH10	EfM3.040190_1	Endo-1,4-beta-xylanase, secreted	-3.5
GH125	EfM3.071270_1	DUF1237 family protein, secreted	-2.4
GH16	EfM3.039700_1	Fungal 1,3(4)-beta-D-glucanase	3.2
GH16	EfM3.080050_1	Glycoside hydrolase family 16	3.7
GH16	EfM3.079050_1	Transglycosidase; links chitin to glucan	-2.7
GH17	EfM3.044280_1	GPI-anchored cell wall beta-1,3-endoglucanase	2.3
GH18	EfM3.024310_1	*Efe*-ChiA, Chitinase	-3.9
GH32	EfM3.074710_1	Beta-fructofuranosidase	3.2
GH5/GH15	EfM3.013890_1	Beta-1,6-glucanase; *Efe*-GcnA	-5.4
GH54/CBM42	EfM3.015180_1	Alpha-L-arabinofuranosidase	5.6
GH89	EfM3.029480_1	Alpha-N-acetylglucosaminidase	3.8
GT2	EfM3.049120_1	Chitin synthase 1	2.8
GT2	EfM3.080040_1	Glycosyltransferase family 2	5.0
GT25	EfM3.002010_1	LPS glycosyltransferase	-2.7
GT48	EfM3.026320_2	Beta 1,3 glucan synthase	7.2
GT90	EfM3.037010_1	Glycosyltransferase	-3.9
GT90	EfM3.041930_1	Glycosyltransferase family 90	-2.3
CBM50	EfM3.029340_1	LysM domain-containing	-5.1

[a] Positive Log_2 fold change (FC) value indicates the gene was more highly expressed in the stroma tissue and negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue.

Table 2.9. Differentially expressed *Epichloë festucae* genes related to transport at false discovery rate adjusted $p < 0.01$.

Gene	Log_2 FC[a]
EfM3.017590_2 Major facilitator superfamily, possible iron siderophore transporter	12.9
EfM3.019610_1 *Efe*-MfsB; membrane transporter protein	4.5
EfM3.028310_1 Major facilitator superfamily, sugar transporter	4.1
EfM3.054480_1 ABC type transporter	2.5
EfM3.054490_2 Major facilitator superfamily, sugar transporter	4.6
EfM3.059060_1 Golgi transport complex subunit COG4	13.5
EfM3.073030_1 Mitochondrial amino acid transporter arg-13	2.5
EfM3.076540_1 Oligopeptide transporter OPT superfamily	3.6
EfM3.056950_1 Amino acid permease	13.6
EfM3.014910_1 Major facilitator superfamily, multidrug resistance transporter	4.4
EfM3.001040_2 Vacuolar protein sorting vps16	14.6
EfM3.025350_1 Aquaglyceroporin	3.7
EfM3.038260_1 Magnesium-translocating P-type ATPase	3.3
EfM3.070700_1 Vacuolar protein sorting protein DigA	12.7
EfM3.073880_1 Aminophospholipid translocase	3.9
EfM3.014790_1 ABC multidrug transporter	-6.8
EfM3.017940_1 Major facilitator superfamily transporter	-5.5
EfM3.027570_1 Major facilitator superfamily, possible peptide transporter	-3.8
EfM3.029870_1 Major facilitator superfamily transporter	-2.6
EfM3.047170_1 Permease, cytosine/purine, uracil, thiamine, allantoin	-2.5
EfM3.056220_1 ABC-type multidrug transport system	-5.7
EfM3.069880_1 Major facilitator superfamily transporter	-4.0
EfM3.082040_1 Major facilitator superfamily transporter	-2.4

[a] Positive Log_2 fold change (FC) value indicates the gene was more highly expressed in the stroma tissue and negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue.

Table 2.10. Expression of *Epichloë festucae* genes for glucosylceramide biosynthesis.

Gene (gene model)	**Mean RPKM[a]**	**Log_2 FC[b]**	**FDR *p***	**Reference (accession number)**
Serine palmitoyltransferase				
Subunit 1 (EfM3.026330)	73	NS	NS	Cheng et al., 2001 (AAK40364)
Subunit 2 (EfM3.013700)	604	2.6	0.0023	Cheng et al., 2001 (AAP47107)
3-Ketodihydrosphingosine reductase (EfM3.003080)	31	NS	NS	Fornarotto et al., 2006 (KMK61006)
Ceramide synthase (EfM3.037330)	134	4.9	0.0003	Li et al., 2006 (CBF77743)
Sphingolipid Δ4-desaturase (EfM3.079270)	1,000	NS	NS	Garton et al., 2003 (O59715)
Sphingolipid Δ8-desaturase (EfM3.018540)	708	2.6	0.0027	Fernandes et al., 2016 (CBF77189)
Sphingolipid C9-methyltransferase (EfM3.016950)	829	4.2	9E-05	Fernandes et al., 2016 (CBF81402)
Glucosylceramide synthase (EfM3.023750)	30	NS	NS	Fernandes et al., 2016 (CBF77985)

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three stroma sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stromata with the asymptomatic inflorescences. Positive fold change value indicates the gene was more highly expressed in the stroma tissue. NS indicates there was no statistical difference in gene expression between the asymptomatic inflorescence and the stroma tissues at the false discovery rate (FDR) adjusted *p* value < 0.01. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

Table 2.11. Expression of *Epichloë festucae* alkaloid biosynthetic genes.

Gene (gene model)	**RPKM[a]**	**Log_2 FC[b]**	**FDR *p* value**
Peramine biosynthesis			
PerA-2 (EfM3.018710)	24.7	-3.8	8.6e-10
Ergot alkaloid biosynthesis			
dmaW (EfM3.065770)	2.2	-7.1	0.0006
easF (EfM3.049640)	3.2	-6.5	0.001
easE (EfM3.049630)	9.9	-12.0	1.38e-5
easC (EfM3.065755)	1.5	NS	NS
easD (EfM3.065750)	<0.01	NS	NS
easA (EfM3.049660)	3.2	-7.9	0.0007
easG (EfM3.049650)	11.0	-14.0	1.44e-13
cloA (EfM3.065760)	5.0	NS	NS
lpsB (EfM3.049620)	9.7	-10.8	8.37e-12
lpsA (EfM3.063200)	1.3	NS	NS
easH (EfM3.049670)	17.6	NS	NS

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three asymptomatic inflorescence sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stroma with the asymptomatic inflorescences. Negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue. NS indicates there was no statistical difference in gene expression between the asymptomatic inflorescence and the stroma tissues at the false discovery rate (FDR) adjusted *p* value < 0.01. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

Table 2.12. Differential gene expression of some *Epichloë festucae* non-ribosomal peptide synthetases and polyketide synthases between the asymptomatic inflorescence and the stroma tissues at false discovery rate (FDR) adjusted $p < 0.01$.

Gene model	Mean RPKM[a]	Log_2 FC[b]	FDR *p* value
Non-ribosomal peptide synthetases			
EfM3.059650	13	-6.5	1.2e-7
EfM3.059660	12	-6.4	4.0e-10
EfM3.081180	174	-8.0	0
EfM3.081210	223	-5.5	0
EfM3.059650	13	-6.5	1.2e-7
Polyketide synthases			
EfM3.014820	6	-4.5	0.0017
EfM3.081190	108	-6.7	0

[a] RPKM value (reads per kilobase of exon model per million mapped reads) is the mean of three asymptomatic inflorescence sequence replicates.

[b] Fold change (FC) is the ratio of the largest mean RPKM values to the lowest mean RPKM values in the comparison of the stromata with the asymptomatic inflorescences. Negative fold change value indicates the gene was more highly expressed in the asymptomatic inflorescence tissue. The statistical model used by the analysis program corrects for differences in library size so the fold changes cannot be determined by simple algebraic calculations of the mean RPKM values of the two tissue types.

Table 2.13. Overlap of *Epichloë festucae* differentially expressed genes with the "core gene set" from Eaton et al. (2015).

	Tissue of higher expression	
Gene model and protein annotation	**Inflorescence or stroma[a]**	**Wild-type or mutant interaction[b]**
EfM3.001580 Uncharacterized protein	Stroma	Mutant
EfM3.005300 *Efe*-PrtC, subtilisin-like protease, secreted	Inflorescence	Wild-type
EfM3.013550 NAD+ dependent glutamate dehydrogenase	Stroma	Mutant
EfM3.016770 *Efe*-SspM, candidate effector protein	Inflorescence	Wild-type
EfM3.018170 Uncharacterized, NC12	Inflorescence	Wild-type
EfM3.027570 Major facilitator superfamily domain	Inflorescence	Mutant
EfM3.028490 Uncharacterized	Inflorescence	Wild-type
EfM3.040190 Endo-1,4-beta-xylanase, secreted	Inflorescence	Mutant
EfM3.041770 Candidate effector protein	Inflorescence	Mutant
EfM3.044610 Uncharacterized protein, small secreted	Inflorescence	Wild-type
EfM3.044630 Candidate effector protein	Inflorescence	Mutant
EfM3.045520 Uncharacterized, small secreted	Inflorescence	Wild-type
EfM3.047600 Uncharacterized protein	Stroma	Wild-type
EfM3.048860 Uncharacterized	Inflorescence	Mutant
EfM3.051900 Uncharacterized	Inflorescence	Mutant
EfM3.056220 ABC-type multidrug transport system	Inflorescence	Wild-type
EfM3.056300 Ubiquitin/ribosomal protein S27a fusion	Inflorescence	Wild-type
EfM3.057830 Cholinesterase	Inflorescence	Mutant
EfM3.064250 Uncharacterized protein	Stroma	Mutant
EfM3.069610 Uncharacterized protein	Inflorescence	Mutant
EfM3.073830 Uncharacterized protein	Stroma	Mutant
EfM3.074710 Secreted invertase	Stroma	Mutant

[a] Statistically different at false discovery rate adjusted $p < 0.01$ and Log_2 fold change > 2.

[b] Eaton, C.J., Dupont, P.-Y., Solomon, P., Clayton, W., Scott, B., and Cox, M.P. 2015. A core gene set describes the molecular basis of mutualism and antagonism in *Epichloë* spp. Molecular Plant-Microbe Interactions 28:218–231.

Table 2.14. Over-represented plant gene ontology (GO) biological process categories of differentially expressed (DE) plant transcripts in the choke stroma (CS) and the asymptomatic inflorescence (AI) tissues using Fisher's Exact Text at false discovery rate (FDR) < 0.05 in Blast2GO.

DE	Biological process GO name[a]	FDR	Nr DE	Nr reference[b]
CS > AI	Carbohydrate metabolic process	2.71E-06	130	1804
CS > AI	Response to water deprivation	0.001	6	7
CS > AI	Transport	0.001	238	4328
CS > AI	RNA phosphodiester bond hydrolysis, endonucleolytic	0.004	19	146
CS > AI	Cell-cell signaling	0.008	3	0
CS > AI	Response to biotic stimulus	0.013	46	603
CS > AI	Lignin catabolic process	0.023	3	1
CS > AI	Cofactor catabolic process	0.048	4	7
CS > AI	Response to abscisic acid	0.048	4	7
CS < AI	Cell cycle	5.07E-24	169	892
CS < AI	Flower development	4.58E-07	58	317
CS < AI	Anatomical structure morphogenesis	8.17E-07	70	442
CS < AI	Cellular component organization	1.00E-06	382	4048
CS < AI	Cell differentiation	2.70E-04	49	323
CS < AI	Lipid metabolic process	5.88E-04	145	1397
CS < AI	Carbohydrate metabolic process	0.005	169	1765
CS < AI	Negative regulation of translation	0.029	6	10

[a] Only the most specific GO categories are presented.

[b] Number (Nr) of contigs in the plant assembly with the GO category annotation minus the number up- or down-regulated.

Table 2.15. Up-regulated plant genes in the stroma tissue in the gene ontology (GO) categories of response to biotic stimulus and response to water deprivation (false discovery rate < 0.05).

Contig	Protein annotation	Log_2 FC[a]
Response to biotic stimulus; GO:0009607		
TRINITY_DN38410_c0_g1_i1	1-aminocyclopropane-1-carboxylate oxidase 1	3.4
_contig_31681	Bet v 1 allergen	3.4
_contig_10385	Calcium-dependent protein kinase 12	2.2
TRINITY_DN34300_c1_g1_i1	Calcium-dependent protein kinase 12	2.3
_contig_24170	Class I chitinase	5.9
_contig_3002	E3 ubiquitin-protein ligase RGLG4	2.5
_contig_33432	Ethylene-responsive transcription factor ABR1-like	3.4
TRINITY_DN8188_c0_g1_i1	Histidine kinase 3	2.2
_contig_10489	L-type lectin-domain containing receptor kinase IV.1	3.2
_contig_12768	L-type lectin-domain containing receptor kinase IV.1	2.8
_contig_17875	L-type lectin-domain containing receptor kinase IV.1	2.4
_contig_2947	L-type lectin-domain containing receptor kinase IV.1	3.6
_contig_6101	L-type lectin-domain containing receptor kinase IV.1	2.0
TRINITY_DN14979_c0_g1_i1	L-type lectin-domain containing receptor kinase IV.1	3.8
_contig_10466	L-type lectin-domain containing receptor kinase IV.1-like	3.4
TRINITY_DN10040_c0_g1_i1	L-type lectin-domain containing receptor kinase IX.1-like	2.6
_contig_1059	Non-specific lipid-transfer protein	5.6
_contig_1420	Non-specific lipid-transfer protein	6.0
_contig_628	Non-specific lipid-transfer protein	6.4
TRINITY_DN15788_c0_g1_i1	Non-specific lipid-transfer protein	6.5
TRINITY_DN30775_c0_g1_i1	Non-specific lipid-transfer protein	6.5
TRINITY_DN40235_c0_g1_i1	Non-specific lipid-transfer protein	4.0
TRINITY_DN56021_c0_g1_i1	Non-specific lipid-transfer protein 3	6.6
TRINITY_DN34047_c0_g1_i1	Pathogenesis-related protein 10	3.2
TRINITY_DN34047_c0_g1_i3	Pathogenesis-related protein 10	2.4
_contig_13205	Pathogenesis-related protein 4	2.6

_contig_16686	Peroxidase 21-like	2.7
_contig_19669	Peroxidase 21-like	2.5
_contig_16216	Probable LRR receptor-like serine/threonine-protein kinase At1g74360	3.7
_contig_2758	Probable LRR receptor-like serine/threonine-protein kinase At1g74360	3.3
TRINITY_DN8117_c0_g1_i1	Probable RNA-dependent RNA polymerase 1	2.1
_contig_22174	Probable serine/threonine-protein kinase PBL25	2.4
_contig_1704	Protein NRT1/ PTR FAMILY 4.3-like (MFS superfamily protein)	2.9
_contig_18851	Protein synthesis inhibitor II	4.7
TRINITY_DN51159_c0_g1_i1	Putative eukaryotic translation initiation factor 4 gamma	2.7
_contig_1969	Putative lipid-transfer protein DIR1	4.5
_contig_2249	Putative lipid-transfer protein DIR1	5.9
_contig_4625	Putative lipid-transfer protein DIR1	6.3
_contig_5137	Putative lipid-transfer protein DIR1	5.4
_contig_544	Putative lipid-transfer protein DIR1	4.6
TRINITY_DN26665_c0_g1_i1	Putative ornithine aminotransferase	2.1
_contig_18027	Thaumatin-like protein	2.8
TRINITY_DN34173_c0_g1_i2	Thaumatin-like protein	2.9
_contig_26838	Triacylglycerol lipase 2	4.7
_contig_27	Triacylglycerol lipase 2	6.9
TRINITY_DN31985_c0_g1_i3	WRKY transcription factor	2.2
Response to water deprivation; GO:0009414		
_contig_691	LEA protein	3.1
TRINITY_DN26665_c0_g1_i1	Putative ornithine aminotransferase	2.1
TRINITY_DN31632_c1_g1_i1	Dehydrin DHN3	3.3
TRINITY_DN43898_c0_g1_i8	Heat shock protein 90-5, chloroplastic	2.1
TRINITY_DN63715_c0_g1_i1	Aquaporin PIP1-5	3.5
TRINITY_DN8188_c0_g1_i1	Histidine kinase 3	2.2

[a] Positive Log_2 fold change (FC) value indicates the gene was more highly expressed in the stroma tissue.

Chapter 3. Application of CRISPR/Cas9 Technology to Study the *Epichloë festucae* Antifungal Protein Gene

INTRODUCTION

Strong creeping red fescue (*Festuca rubra* subsp. *rubra*) is a commercially important low-maintenance turfgrass and is often naturally infected with the fungal endophyte *Epichloë festucae*. Endophyte-mediated disease resistance to the fungal pathogen *Clarireedia jacksonii* C. Salgado, L.A. Beirn, B.B. Clarke, & J.A. Crouch sp. nov. (formerly *Sclerotinia homoeocarpa*), the causal agent of dollar spot disease, has been observed in the field (Clarke et al., 2006). In a previous transcriptome study of the *E. festucae*–strong creeping red fescue interaction, the second most abundant fungal transcripts were found to encode a protein similar to antifungal proteins from *Penicillium* and *Aspergillus* species (Ambrose and Belanger, 2012). The purified protein *Efe*-AfpA has been shown to inhibit the growth of *C. jacksonii* in plate assays (Tian et al., 2017).

The mechanism underlying the unique endophyte-mediated disease resistance in strong creeping red fescue is not yet established. The antifungal protein gene found in *E. festucae* infecting strong creeping red fescue is not present in most *Epichloë* genomes for which the whole genome sequence is available (Ambrose and Belanger, 2012; Tian et al., 2017). The antifungal activity, transcript abundance, and the limited existence of the gene among *Epichloë* spp. suggested the *E. festucae* antifungal protein may be a component of the unique endophyte-mediated disease resistance observed in strong creeping red fescue. If so, this protein may have the potential to be utilized as a biological control agent for fine fescues and other important turfgrasses.

A common approach to evaluate the role of a particular protein in a physiological process is to generate a gene knockout and evaluate the phenotype of the mutant. In *Epichloë* spp. knockouts of numerous genes have been reported using the approach of homologous recombination to replace the target gene with a selectable marker. However, *Efe-afpA* is the only gene in a genome sequence contig consisting of high A/T regions and flanked by repeated sequences (Tian et al., 2017), which makes the traditional transformation technique relying on homologous recombination rather challenging.

CRISPR/Cas9 technology has revolutionized gene modification and has become established broadly in gene function studies in animal, plant, and yeast systems. Whereas research using CRISPR/Cas9 in filamentous fungi is considerably less abundant but is increasing rapidly. CRISPR/Cas is a very powerful tool; with proper design of the protospacer sequence of the gRNA adjacent to a GG dinucleotide scientists could, in theory, edit any gene of interest (Jinek et al., 2012). With this method, a single guide RNA (gRNA) with a 20-bp target sequence directs Cas9 endonuclease to precisely generate double strand breaks. Repairing the breaks by nonhomologous end joining (NHEJ) is likely to introduce mistakes causing frame shifting, which knocks out the gene of interest.

This study was conducted to utilize CRISPR/Cas9 technology in a non-model system, the filamentous fungus *E. festucae*, to generate gene knockouts of the antifungal protein gene *Efe-afpA* efficiently. This study provides a first step towards ultimately understanding the biological role of *Efe-afpA*, and shreds light on gene modifications of many other *E. festucae* genes for which conventional fungal transformation approach was unsuccessful.

MATERIALS AND METHODS

Nucleic Acid Isolation

Genomic DNA of *E. festucae* isolates was extracted from cultures grown on 2.4% potato dextrose agar (PDA) or in potato dextrose broth (Difco Laboratories, Detroit, MI) using the method described by Moy et al. (2002) or using a DNeasy PowerPlant Pro Kit (Qiagen, Hilden, Germany).

Construction of Homologous Recombination Deletion Vector

Two methods of generating an antifungal protein gene knockout were used. The first attempt was the standard method of homologous recombination, which has been successful for many *Epichloë* genes (Scott et al., 2007). The transformation vector for this homologous recombination method was made by ligating the hygromycin resistance gene (*hph*) to the 598-bp left flank and the 634-bp right flank of the antifungal protein gene amplified from genomic DNA of the Rose City (RC) isolate (Tredway et al., 1999) of *E. festucae* (Fig. 3.1).

The 598-bp 5’ flank region was PCR amplified from the *E. festucae* genomic DNA using primers KpnI-EfAFP-f and ClaI-5'-r with the *Kpn*I and *Cla*I restriction enzyme sites introduced, respectively (Table 3.1). The 634-bp 3’ of *Efe-afpA* was PCR amplified using primers PstI-3'-f and SpeI-EfAFP-r with which the *Pst*I and *Spe*I restriction enzyme sites introduced, respectively (Table 3.1). The 1768-bp fragment of the hygromycin B resistance gene (*hph*) under the control of the *trpC* promoter from

pIGPAPA (Horwitz et al., 1999) was amplified using primers ClaI-hph-f and hph-r with the *ClaI* cite introduced by primer design (Table 3.1).

PCR amplification was carried out in a 100-µL reaction volume containing 0.8 µg of fungal genomic DNA or 88 pg of pIGPAPA, and each forward and reverse primer (Integrated DNA Technologies, Inc., Coralville, IA) at 500 nM and 50 µL Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Waltham, MA). PCR reactions were carried out in a GeneAmp 9700 thermo-cycler (Applied Biosystems, Foster City, CA) with one cycle at 98°C for 30 s; 30 cycles at 98°C for 10s, 60°C for 30 s and 72°C for 30 s; and one cycle at 72°C for 5 min. Each PCR product was sequenced (Genewiz, Inc., South Plainfield, NJ) after purification using 1.8X Agencourt AMPure XP (Beckman Coulter, Brea, CA).

The *Kpn*I/*Cla*I digested 5' flank of *Efe-afpA* was cloned into the *Kpn*I/*Cla*I site of pBlueScript SK(+) followed by sequentially ligating the *Cla*I/*Pst*I digested *hph* and the *Pst*I/*Spe*I digested fragment 3' flank of *Efe-afpA* (Fig. 3.1). The deletion construct was confirmed by sequencing to ensure that no mutations had been introduced during PCR amplification.

Construction of CRISPR/Cas9 Gene Knockout Vectors

The CRISPR/Cas9 gene knockout technique was used in the second approach. CRISPR/Cas9 endonuclease and a gRNA with 20-bp protospacer to target a genomic locus were identified as two key components of the CRISPR/Cas9 system. *Trichoderma* codon-optimized *cas9* gene from Liu et al. (2015) and *hph* as the selective marker gene were used in the CRISPR/Cas9 transformation vector. A unique 20-bp target sequence

upstream of NGG was designed (Fig. 3.2), and the gRNAs were generated *in vitro* using GeneArt™ Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacture's instructions with primers gF2 and gR2.

pD2P6 was modified from p-*hph*-P*tef1*-*cas9*-pksP-gRNA [received from Fuller et al. (2015)] to target 5′-GGCATTCTGATCACGTATGA-3' upstream of PAM sequence AGG in the first exon of *Efe-afpA*. Primers AFP-Del2-F and AFP-Del2-R were used to change the existing gRNA sequence of p-*hph*-P*tef1*-*cas9*-pksP-gRNA using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs Inc., Ipswich, MA). Since p-*hph*-P*tef1*-*cas9*-pksP-gRNA is a vey large plasmid (>13 kb), PrimeSTAR HS Premix (TaKaRa Bio Inc., Shiga, Japan) was used instead of Q5 Hot Start High-Fidelity 2X Master Mix in the Q5 Site-Directed Mutagenesis Kit. PCR was performed in 25 µL with 1.05 ng of p-*hph*-P*tef1*-*cas9*-pksP-gRNA, 0.3 µM of each forward and reverse primer (Integrated DNA Technologies, Inc., Coralville, IA), and 12.5 µL of PrimeSTAR HS Premix. Two-step PCR was performed by template denaturation at 98°C for 10 s followed by 6 min extension at 68°C for 30 cycles. The PCR product was used in the remaining steps following the Q5 Site-Directed Mutagenesis Kit manufacture protocol. pD2P6 was partially sequenced (Genewiz, Inc., South Plainfield, NJ).

pG4 plasmid (Fig. 3.3) was assembled in three fragments and used to transform into NEB 10-beta Electrocompetent *E. coli* cells (New England BioLabs Inc., Ipswich, MA). Fragments 1 and 2 were amplified from pD2P6 with primers sets BG2F/FullerGR and FullerGF2/PCseqR1, respectively. The human codon-optimized version of *cas9* gene was reported to not function in *Trichoderma reesei* (Liu et al., 2015). The *Trichoderma* codon-optimized *cas9* gene with a SV40 nuclear localization signal (*toCas9*) from

pDHt/sk-PC (Liu et al., 2015) was used in this study because *Trichoderma* and *Epichloë* are filamentous fungi in the same order *Hypocreales*. The Ppdc-*toCas9*-Tpdc fragment, *T. reesei* codon-optimized *cas9* gene and the *T. reesei* constitutive pyruvate decarboxylase promoter (Ppdc) and terminator (Tpdc), was amplified using primers GTCas9F and GTCas9R to also overlap fragment 1 and 2 (Table 3.1). The 100 µL reactions contained 2X Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 0.5 µM of each oligonucleotide, and either 125 pg of pD2P6 or 2 ng of pDHt/sk-PC as template. The PCR reaction conditions for fragment 1 and 2 were: an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of 10 s denaturation at 98 °C, 30 s annealing at 60 °C or 61 °C (for fragment 1 or 2, respectively), and 2 min extension at 72 °C, followed by a final extension at 72 °C for 5 min. Similarly, 30 cycles of 10 s denaturation at 98 °C, 30 s annealing at 65 °C, and 3.5 min extension at 72 °C, followed by an additional final 5 min extension at 72 °C was performed to amplify the Ppdc-*toCas9*-Tpdc. PCR products were then purified by using 0.7X Agencourt AMPure XP (Beckman Coulter, Brea, CA) and assembled with Gibson Assembly Master Mix (E2611S; New England BioLabs Inc., Ipswich, MA). In a 20 µL reaction, 0.11, 0.10, and 0.06 pmol of each fragment 1, 2 and Ppdc-*toCas9*-Tpdc, respectively, were mixed with 2X Gibson Assembly Master Mix incubated at 50 °C for 1 h. One µL of the assembly reaction was used to transform 25 µL NEB 10-beta Electrocompetent *E. coli* cells. The vector pG4 was partially sequenced.

Fungal Transformation and Screening Transformants

Both methods used polyethylene glycol-mediated protoplast transformation described by Turgeon et al. (2010). For the CRISPR-Cas9 approach, 10 µg of *in vitro*

transcribed gRNA and 10 µg of pG4 plasmid in a total of 25 µl was co-transformed to approximately 6×10^6 *E. festucae* RC protoplasts. Over 5 µg in 20 µl of uncut plasmid of the *Efe-afpA* deletion construct or PCR products amplified from the plasmid with primers KpnI-EfAFP-f and SpeI-EfAFP-r were used for transformation during the homologous recombination approach.

Fungal colonies were grown on PDA with hygromycin overlaid with cellophane, and genomic DNA of each colony was extracted. The *Efe-afpA* region of each hygromycin resistant colony was amplified by PCR with primers cafp-f and AFPr, and the thermocycler conditions were: one cycle at 98°C for 30 s; 30 cycles at 98°C for 10s, 60°C for 30 s and 72°C for 30 s; and one cycle at 72°C for 5 min. Each 5 µl PCR product was treated with 2 µL ExoSAP-IT (USB Corp., Cleveland, OH) incubated at 37°C for 15 min followed by heating at 80°C, and sequenced (Genewiz, Inc., South Plainfield, NJ).

Inoculating Strong Creeping Red Fescue and Screening for Endophyte Infection

A single spore isolate of each CRISPR-Cas9 mutant was generated and used to inoculate the mature tillers of strong creeping red fescue S1139 as described by Johnson-Cicalese et al. (2000). Additionally, endophyte free NT-24 Kent seeds were sterilized as described by Cicalese *et al.* (2000) and germinated in the dark on 3% (wt/vol) water agar. Ten-day-old seedlings were inoculated with 5 ul of spore suspension of *E. festucae* RC or mutants in sterile distilled water. Two to three weeks later, as mycelia started to grow around the hypocotyl, seedlings were wounded with a sterile 30 gauge needle (BD Ultra-Fine Insulin Syringes, Franklin Lakes, NJ) to insert a small piece of the mycelia at the hypocotyl. In addition, some seedlings were not wounded. Two weeks after wounding,

seedlings were transferred to Fafard Canadian Grow Mix 2 (Agawam, MA) in the greenhouse.

Inoculated plants were screened for endophyte infection by an immunoblot assay (Phytoscreen, Agrinostics, Ltd. Co., Watkinsville, GA) and microscopically. For microscopic examination, leaves were pre-treated by submerging in 95% ethanol for more than 4 h to remove chlorophyll. Leaf sheaths or pre-treated leaves were incubated in aniline blue–lactic acid stain (Bacon and White Jr, 1994) for 15 min at 50 °C and examined by microscopy for intercellular endophyte hyphae.

Phenotypic Comparison of Wild-type and Knockout Isolates

Single spore isolates of the wild-type and *Efe-afpA* knockouts were grown on PDA and 1.5% water agar in dark at room temperature. Experiments were arranged in a completely randomized design. Two diameter measurements were taken per plate and averaged for statistical analysis. Diameter measurements were subjected to analysis of variance using the General Linear Model procedure in the Statistical Analysis System software v. 9.4 (SAS Institute Inc., Cary, NC) and means were separated by Fisher's protected least significant difference at the 0.05 probability level.

RESULTS

CRISPR-Cas9 Was Effective at Knocking out *Efe-afpA*

The first attempt at generating a knockout of the antifungal protein gene was to use the well-established homologous recombination method, which has been successful for many *Epichloë* genes (Scott et al., 2007). However, screening over 250 hygromycin resistant transformants from the homologous recombination method reveled no correct gene replacement mutant. Although the HR approach is widely used in *Epichloë* species, this conventional fungal gene knockout method was unsuccessful in our study, likely because only short unique homologous regions were available for vector construction since this gene is flanked by repeated sequences. For most fungi the efficacy of transformation that relies on HR is normally between 1% to 25% and requires long flanking regions of more than 3 kb left and right flanks combined (Scott et al., 2007). A split marker system was shown to reduce ectopic integration and increase transformation efficacy in *E. festucae* to 33%−74%, which also requires relatively long flanking regions of at least 1.5 kb on each flanking region (Rahnama et al., 2017). We were unable to identify any **Δ***Efe-afpA* using the HR method because only a total of 1.2 kb left and right flanking regions was available for HR, apparently that is too short and resulted in transformation efficiency less that 1%.

However, we successfully applied the CRISPR-Cas9 technology to the non-model fungus *E. festucae*. The Cas9 endonuclease precisely generated a double-strand break at the target site of the antifungal protein gene, and a mutation was introduced when repairing the break. After screening 42 hygromycin resistant isolates we have identified

one, 1a-7t8s3, with a single base pair insertion precisely at the Cas9 target site (3 bp upstream of the PAM site), which immediately introduced an early stop codon (Fig. 3.4). Another mutant, 1c-3s5, had a large insertion of 1625 bp, which was confirmed to be a fragment from the transformation vector pG4 (Fig. 3.4). A random fragment of the CRISPR/Cas9 vector integrated at Cas9 cut site has been observed in other fungi (Fuller et al., 2015). The large insertion mutant 1c-3s5 also has a small (9 bp) deletion immediately upstream of the expected double-strand break (Fig. 3.4). Small deletions are common with CRISPR/Cas9 edited genes in other fungi (Nødvig et al., 2015; Katayama et al., 2016; Schuster et al., 2016). Here the CRISPR/Cas9 approach dramatically improved the transformation efficiency and was able to edit a gene that was not feasible using the conventional method due to short homologous flanking regions. In other systems, CRISPR/Cas9 technology was reported to have even higher efficiency, likely because when double strand breaks were generated, *E. festucae* was able to repair them correctly at a higher rate.

Possible Effect of *Efe-afpA* on the Ability of *E. festucae* to Infect Its Host

Single spore isolates of each independent mutant were generated and sequenced twice to ensure the purity of each mutant isolate. Attempts were made to reintroduce the knockout isolates into the host, strong creeping red fescue to evaluate the disease susceptibility of the host plant with the mutant endophyte to dollar spot disease. Inoculating mature S1139 strong creeping red fescue tillers with 1c-3s5 using methods reported by Johnson-Cicalese et al. (2000) was unsuccessful. Over 200 plants were examined by microscope and phytoscreen test were endophyte free (Table 3.2). Inoculating germinating strong creeping red fescue NT-24 Kent seedlings with wild-type

E. festucae RC was achieved at a relatively high success rate (5 out of 18). Wounding seemed to be an important factor in assisting the colonization of strong creeping red fescue by *E. festucae* RC, although, the comparison between inoculation with or without wounding has not been tested in replicated experiments. Previous research indicated that wounding facilitated the infection of orchardgrass by its endophyte *E. typhina* (Alderman, 2012), which supports our observation. However, inoculating NT-24 Kent seedlings with **Δ***Efe-afpA* mutants 1a-7t8s3 or 1c-3s5 was unsuccessful. We screened a total of 90 plants that were alive after inoculation using the same method that successfully inoculated with the wild-type (Table 3.2). It is possible that the **Δ***Efe-afpA* mutants could have a reduced ability to infect their host grass, but to test this hypothesis would require further investigation.

E. festucae Mutant Phenotype in Culture

We observed a significant reduction in fungal growth with **Δ***Efe-afpA* mutants as compared to the wild-type RC when measuring colony sizes in culture (Table 3.4). This reduction in fungal growth was more dramatic in the nutrient limited condition (1.5% water agar) than in the nutrient rich condition (PDA). Nutrients are often limited when the endophyte maintains the asymptomatic growth *in planta*. Therefore, the inability of the mutants to establish synchronized growth with the host seedlings may be related to impaired growth of the mutants under nutrient limited condition.

DISCUSSION

The lack of long homologous flanking regions required by the homologous recombination approach limited our research on functionally characterizing *Efe-afpA*. In filamentous fungi, homologous recombination efficiency is low due to the NHEJ system that promotes the ectopic insertion of the selective marker. Alternatively, to increase homologous recombination, NHEJ deficiency mutants were generated by disrupting the NHEJ machinery-encoding genes such as *ku70* and *ku80* (Kück and Hoff, 2010). However, increased sensitivity to genotoxic stress, DNA damaging irradiation and chemicals has been observed with NHEJ deficiency mutants (Meyer, 2008; Kück and Hoff, 2010). Additioanlly, large scale screening for correct gene knockouts involving DNA extraction, PCR, and gel electrophoresis can be intesive with homologous recombination approach.

The CRISPR/Cas9 approach dramatically improved transformation efficiency in the current study and was able to edit a gene that was not feasible using the conventional method due to lack of long enough unique flanking regions required for homologous recombination. The CRISPR/Cas9 gene editing approach that has been developed for eukaryotic systems requires expression of a functional Cas9 endonuclease gene and a gRNA, which targets the desired site of editing.

Prior to successfully editing *Efe-afpA* with the *Trichoderma* codon-optimized *cas9*, we first carried out the transformation with human codon-optimized *cas9* with no success after screening 20 hygromycin resistant colonies. Although, Zhang et al. (2016) reported that codon-optimized *cas9* gene for human cells could be expressed in *A.*

fumigatus driven by fungal promoters. Liu et al. (2015) reported that the human version of the *cas9* gene did not function in *T. reesei*. We speculate that the human version of *cas9* could only work for some species, or that it has low activity in *Epichloë* and *Trichoderma*.

In addition to codon-optimized *cas9* gene, another key component is gRNA driven by the endogenous U6 promoter. A polymerase III promoter, most often the U6 promoter is used to generate abundant gRNAs. However, the U6 promoter in many fungi has not been identified because the fungal U6 gene often has multiple introns (Canzler et al., 2016). Common fungal RNA polymerase II promoters such as *trpC* (Arazoe et al., 2015) and *gpdA* (Nødvig et al., 2015) have been used in the fungal systems; however, Arazoe et al. (2015) showed significantly less transformation efficiency with *trpC* than the endogenous U6 promoter. The U6 promoter of *Epichloë* species has not been identified.

As an alternative to *in vivo* expressed gRNA, we utilized *in vitro* transcribed gRNAs as reported by Liu et al. (2015) in *T. reesei*. We also transformed the wild-type with pG4 alone using the extract same method at the same time when we performed the co-transformation with pG4 and *in vitro* transcribed gRNAs. Although we did not identify any correct mutant when transforming with pG4 alone, we only screened 10 transformants before we identified **Δ***Efe-afpA* mutants 1a-7t8s3 and 1c-3s5 with co-transformation of pG4 and *in vitro* transcribed gRNAs. The pG4 vector has a yeast polymerase III promoter SNR52 driving gRNA expression (Fig. 3.3). Whether this yeast promoter functions in the *Epichloë* system or it generates less abundant gRNAs resulting in lower transformation efficiency in *Epichloë* compared to *in vitro* transcribed gRNAs

needs further investigation. We have not tested the transformation efficiency of co-transformed human codon-optimized version of *cas9* and *in vitro* transcribed gRNAs to determine whether human codon-optimized *cas9* functions in *Epichloë*.

This is the first reported use of CRISPR/Cas9 technology in *Epichloë* endophyte research. Many other genes can be edited with the same system by desgining unique gRNA targeting the gene of interest. Co-transformation of *Trichoderma* codon-optimized *cas9* gene and *in vitro* transcribed gRNAs, as developed by Liu et al. (2015), was successful in *E. festucae*. Generating *in vitro* transcribed gRNAs by changing the 20-bp protospacer of gRNA to the target any genomic locus allows for modification of many other genes without the time consuming vector construction steps for every gene. CRISPR/Cas9 techonology will empower us to generate gene knockouts to study functions of those genes that we were not able to with homologous recombination.

Two independent mutants of *Efe-afpA* were observed as being restrited in growth on water agar. During asympatomatic associations, *Epichloë* spp. are restricted to the intercellular spaces of the host, therefore, their ability to utilize the nutreint-poor fluids of the host is essential. Our hypothesis is that the mutants were defective in growing on nutrient limited media, and therefore were unlikely to systemically colonize the intercellular spaces of the host. We observed reduced ability of those mutants to infect the host plant as compared to wild-type *E. festucae* RC. However, whether they are unable to infect the host plant needs to be further investiagted.

In order to understand the biological role of *Efe-afpA* additional approaches are required. Whether *Efe-afpA* affects the ability to infect germinating seedlings can be

validated by rescuing the *Efe-afpA* knockout mutants generated in this study. Future studies should be conducted to investigate if the complementation restores the endophyte's ability to infect its host grass. Another approach to confirm the role of *Efe*-AfpA in disease resistance is to express its gene in another isolate *E. festucae* var. *lolii* that does not have an endogenous *Efe-afpA* gene. The association of *E. festucae* var. *lolii* with its host perennial ryegrass (*Lolium perenne*) did not exhibit disease resistance to dollar spot. Genetically modifying *E. festucae* var. *lolii* to introduce the *Efe-afpA* gene will allow us to test *in planta* if perennial ryegrass gains disease resistance, and therefore, to confirm the function of *Efe-afpA*. This type of research will shed light on manipulating fungal endophytes to gain disease resistance in economically important crops.

REFERENCE

Alderman, S.C. 2012. Survival, germination, and growth of *Epichloë typhina* and significance of leaf wounds and insects in infection orchardgrass. Plant Disease 97:323–328.

Ambrose, K.V., and Belanger, F.C. 2012. SOLiD-SAGE of endophyte-infected red fescue reveals numerous effects on host transcriptome and an abundance of highly expressed fungal secreted proteins. PLoS ONE 7:e53214.

Arazoe, T., Miyoshi, K., Yamato, T., Ogawa, T., Ohsato, S., Arie, T., and Kuwata, S. 2015. Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. Biotechnology and Bioengineering 112:2543–2549.

Bacon, C.W., and White Jr, J.F. 1994. Stains, media, and procedures for analyzing endophytes. Pages 47–56 in: Biotechnology of endophytic fungi of grasses, C.W. Bacon and J.F. White Jr, eds. CRC Press, Ann Arbor.

Canzler, S., Stadler, P.F., and Hertel, J. 2016. U6 snRNA intron insertion occurred multiple times during fungi evolution. RNA biology 13:119–127.

Clarke, B.B., White, J.F., Jr., Hurley, R.H., Torres, M.S., Sun, S., and Huff, D.R. 2006. Endophyte-mediated suppression of dollar spot disease in fine fescues. Plant Disease 90:994–998.

Fuller, K.K., Chen, S., Loros, J.J., and Dunlap, J.C. 2015. Development of the CRISPR/Cas9 system for targeted gene disruption in *Aspergillus fumigatus*. Eukaryotic Cell 14:1073–1080.

Horwitz, B.A., Sharon, A., Lu, S.-W., Ritter, V., Sandrock, T.M., Yoder, O.C., and Turgeon, B.G. 1999. A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. Fungal Genetics and Biology 26:19–32.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821.

Johnson-Cicalese, J., Secks, M.E., Lam, C.K., Meyer, W.A., Murphy, J.A., and Belanger, F.C. 2000. Cross species inoculation of Chewings and strong creeping red fescues with fungal endophytes. Crop Science 40:1485–1489.

Katayama, T., Tanaka, Y., Okabe, T., Nakamura, H., Fujii, W., Kitamoto, K., and Maruyama, J.-i. 2016. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnology Letters 38:637–642.

Kück, U., and Hoff, B. 2010. New tools for the genetic manipulation of filamentous fungi. Applied Microbiology and Biotechnology 86:51–62.

Liu, R., Chen, L., Jiang, Y., Zhou, Z., and Zou, G. 2015. Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discovery 1:15007.

Meyer, V. 2008. Genetic engineering of filamentous fungi — Progress, obstacles and future trends. Biotechnology Advances 26:177–185.

Moy, M., Li, H.M., Sullivan, R., White, J.F., Jr., and Belanger, F.C. 2002. Endophytic fungal β-1,6-glucanase expression in the infected host grass. Plant Physiology 130:1298–1308.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E., and Mortensen, U.H. 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLOS ONE 10:e0133085.

Rahnama, M., Forester, N., Ariyawansa, K.G.S.U., Voisey, C.R., Johnson, L.J., Johnson, R.D., and Fleetwood, D.J. 2017. Efficient targeted mutagenesis in *Epichloë festucae* using a split marker system. Journal of Microbiological Methods 134:62–65.

Schuster, M., Schweizer, G., Reissmann, S., and Kahmann, R. 2016. Genome editing in *Ustilago maydis* using the CRISPR–Cas system. Fungal Genetics and Biology 89:3–9.

Scott, B., Takemoto, D., Tanaka, A., Young, C., Bryant, M., and May, K. (2007). Functional analysis of the *Epichloë festucae*–perennial ryegrass symbiosis. In 6th International symposium on fungal endophytes of grasses. New Zealand Grassland Association, pp. 433–441.

Tian, Z., Wang, R., Ambrose, K.V., Clarke, B.B., and Belanger, F.C. 2017. The *Epichloë festucae* antifungal protein has activity against the plant pathogen *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease. Scientific Reports 7:5643.

Tredway, L.P., White, J.F., Gaut, B.S., Reddy, P.V., Richardson, M.D., and Clarke, B.B. 1999. Phylogenetic relationships within and between *Epichloë* and *Neotyphodium* endophytes as estimated by AFLP markers and rDNA sequences. Mycological Research 103:1593–1603.

Turgeon, B.G., Condon, B., Liu, J., and Zhang, N. 2010. Protoplast transformation of filamentous fungi. Pages 3–19 in: Molecular and Cell Biology Methods for Fungi, A. Sharon, ed. Humana Press, Totowa, NJ.

Zhang, C., Meng, X., Wei, X., and Lu, L. 2016. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. Fungal Genetics and Biology 86:47–57.

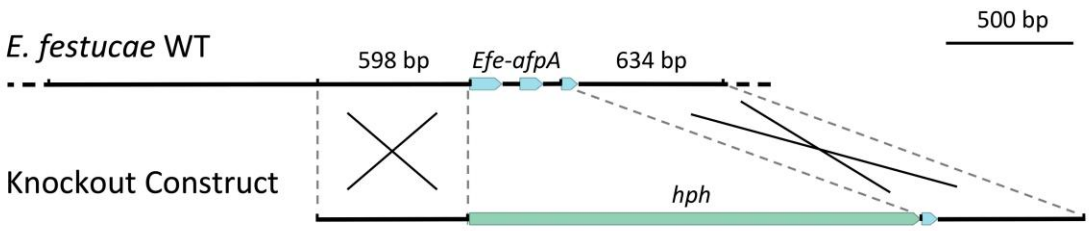

Figure 3.1. Schematic of gene replacement of *Efe-afpA* in the wild-type (WT) *Epichloë festucae* by homologous recombination.

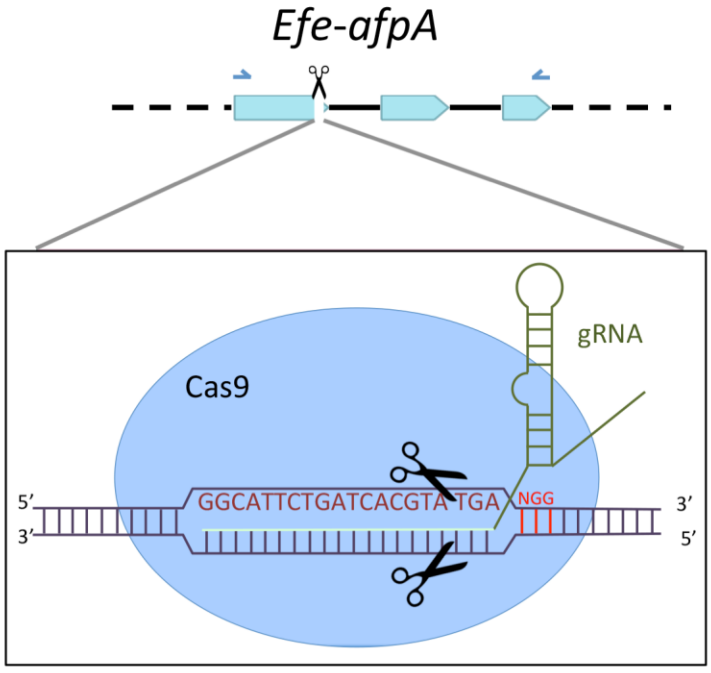

Figure 3.2. Illustration of RNA guided Cas9 nuclease specifically cleaves the target sequence in the first exon of the *Efe-afpA*. Blue arrows indicate PCR primers cafp-f and AFPr to screen and sequence mutants.

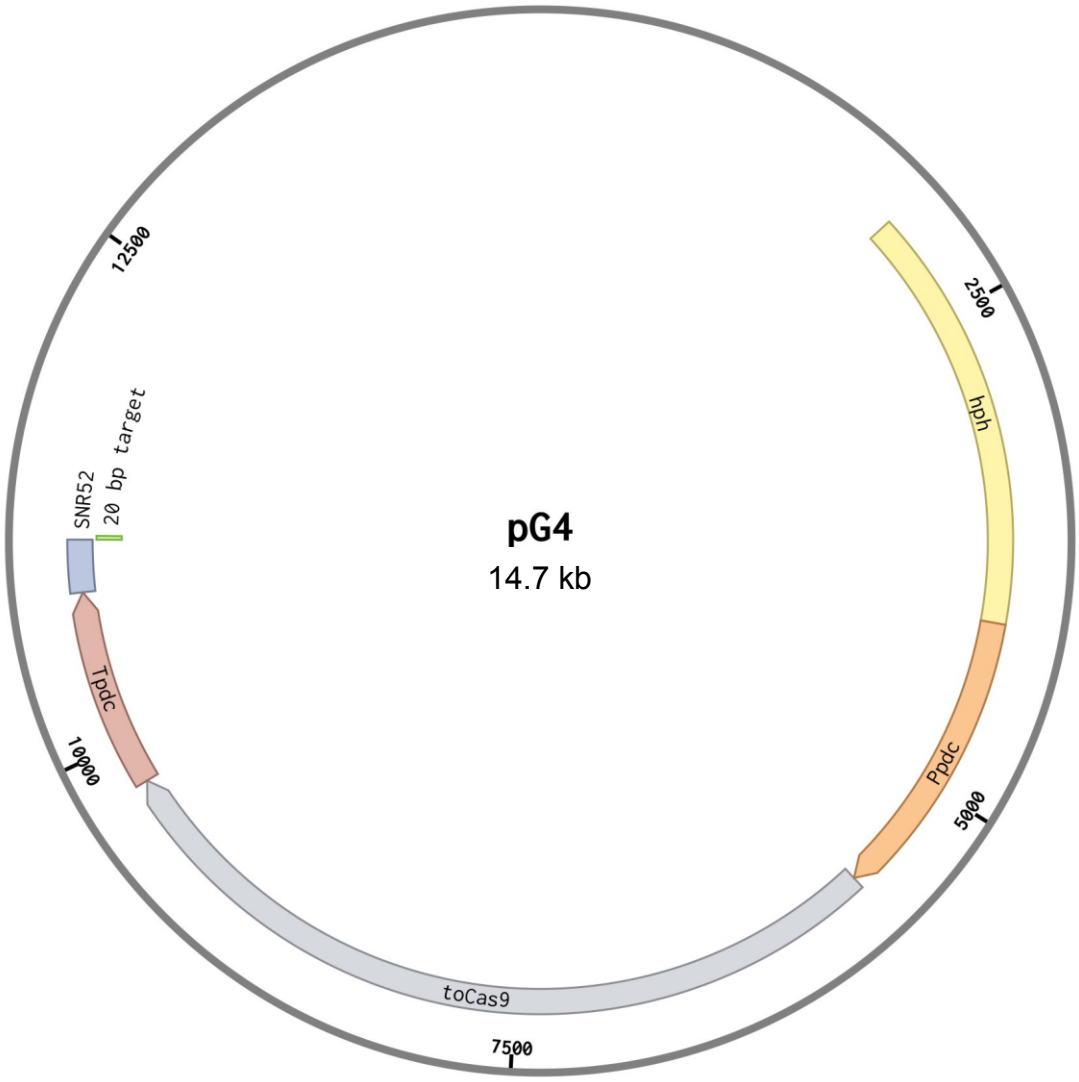

Figure 3.3 Plasmid map of pG4 with hygromycin B resistance gene (hph), *Trichoderma reesei* codon-optimized *cas9* gene (toCas9) with *T. reesei* constitutive pyruvate decarboxylase promoter (Ppdc) and terminator (Tpdc), guide RNA driven by SNR52 promoter, and 20 bp target sequence.

```
wild-type     ATGCAAATCACCGTGGTCGCGGTTTTCCTCCTCTCTGCAATGGGCGGAG
1a-7t8s3      ATGCAAATCACCGTGGTCGCGGTTTTCCTCCTCTCTGCAATGGGCGGAG
1c-3s5        ATGCAAATCACCGTGGTCGCGGTTTTCCTCCTCTCTGCAATGGGCGGAG

TAGCCACACCCATCAACTCCAGAATCAATCCTGTTGATGCCAGGGCGGAGACTGGCATT
TAGCCACACCCATCAACTCCAGAATCAATCCTGTTGATGCCAGGGCGGAGACTGGCATT
TAGCCACACCCATCAACTCCAGAATCAATCCTGTTGATGCCAGGGCGGAGACTGGCATT

CTGATCACGTA----------------------------------------------
CTGATCACGTAT---------------------------------------------
CT----------TACTTAGGGGAAATAAAGGTTCTTGGATGGGAAGATGAATATACTGA

------------------------------------------------------------
------------------------------------------------------------
AGATGGGAAAAGAAAGAGAAAAGAAAAGAGCAGCTGGTGGGGAGAGCAGGAAAATATGG

------------------------------------------------------------
------------------------------------------------------------
CAACAAATGTTGGACTGACGCAACGACCTTGTCAACCCCGCCGACACACCGGGCGGACA

------------------------------------------------------------
------------------------------------------------------------
GACGGGGCAAAGCTGCCTACCAGGGACTGAGGGACCTCAGCAGGTCGAGTGCAGAGCAC

------------------------------------------------------------
------------------------------------------------------------
CGGATGGGTCGACTGCCAGCTTGTGTTCCCGGTCTGCGCCGCTGGCCAGCTCCTGAGCG

------------------------------------------------------------
------------------------------------------------------------
GCCTTTCCGGTTTCATACACCGGGCAAAGCAGGAGAGGCACGATATTTGGACGCCCTAC

------------------------------------------------------------
------------------------------------------------------------
AGATGCCGGATGGGCCAATTAGGGAGCTTACGCGCCGGGTACTCGCTCTACCTACTTCG

------------------------------------------------------------
------------------------------------------------------------
GAGAAGGTACTATCTCGTGAATCTTTTACCAGATCGGAAGCAATTGGACTTCTGTACCT
```


AGGTTAATGGCATGCTATTTCGCCGACGGCTATACACCCCTGGCTTCACATTCTCCTTC

GCTTACTGCCGGTGATTCGATGAAGCTCCATATTCTCCGATGATGCAATAGATTCTTGG

TCAACGAGGGGCACACCAGCCTTTCCACTTCAGAAGCCGCAGGTGTCGAGCCGGGAGGA

GTTTTCGCAGTGGCTGTGCCGCGCGCACAACGATGTCAACCGGAAGCTGGGCAAGCCCG

AGTTTGACTGCTCGCGGGTGGACGAGCGGTGGAGGACGGGGTGGAAGGACGGTCGGTGC

GACTGATTGGCCATGAATTCCATCTTTCGAGGACGGACGAAGATACTGTACGATTAATG

AAAGGAGGGAGCATTCTTCGACTTGCGGCAATTGCATGCACATGTACGATTGGAAGCGC

GGGCGATGTATTCGCAATCATGTTTAGAAGGACGGCGTTTGGAAACGTTGGGATGCTGT

TGAAGCGTTGGAAACAGGGGCAATTAGAAACACCGAGCCAGACAGAGTCAATGGTACGA

GGTCAGCCAGTATCATGACCTGTGTGCGCATGGTGGCGAGAGATTCCGAGCCATGCCAC

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------------------------------------------------------------
------------------------------------------------------------
GGGAGACGAGCAATGAAAAAACTCTTCACTCACTTGTCGAGGCTCTCTCAACCTATCGA

------------------------------------------------------------
------------------------------------------------------------
CTTATCAAGTAGACGATGAAAGCCTTGCAACTGTGGTGATGTGGCTCATCAATGTGCGA

------------------------------------------------------------
------------------------------------------------------------
CGTCGTATCCATGTCTGAGGCCATTCGATATCGTGATGCGACTACCTAGTAAAGCCCGG

------------------------------------------------------------
------------------------------------------------------------
CCAGAGGGCAAACCGGGGCGACAGGGGCAGGCAATTGACCGGATGGCTGCATGTGCCGA

------------------------------------------------------------
------------------------------------------------------------
AGCAGCCCCGATGGAATCGAGATGTCTGTCGGATGGACCGCTGAGCGGCCTGGCAAGGT

------------------------------------------------------------
------------------------------------------------------------
GTCCCAGATACGAAGATGGAAGTGAAGTCAGAGGTGGTCGTTAATTGTCCGACGAGCGA

------------------------------------------------------------
------------------------------------------------------------
ATCGGCCGCTCCTTCGGATTGCCGGCTCTGCTGTATGTACCGTGCATGAAGCCACCCGG

------------------------------------------------------------
------------------------------------------------------------
GATCCATGTTACGATGGATAGGTTCCAACTCTCTAGTAGCTATAGTGGACCTGAGGCTA

------------------------------------------------------------
------------------------------------------------------------
TCTAGTATCACTGGAGGAGCAGCCGTCCACTATCGTCGAGCGCTGTAGAAGCAGCTGCA

------------------------------------------TGAAGGAGTGAGTTG
------------------------------------------TGAAGGAGTGAGTTG
TTAGCGGCTGCCCACCCGCGCAGAAATGGCCCCATTACATCACTTGAAGGAGTGAGTTG
```

ATTTTGTGTCACAATACCCGATTACTCTGAGTATTCAATAACCACGGAGTCCCCTCTAT
ATTTTGTGTCACAATACCCGATTACTCTGAGTATTCAATAACCACGGAGTCCCCTCTAT
ATTTTGTGTCACAATACCCGATTACTCTGAGTATTCAATAACCACGGAGTCCCCTCTAT

AAAGACATGTTCTAGAGCCAAAAACGAATGCAAGTACAAAAATCAGAACAACAAGGACA
AAAGACATGTTCTAGAGCCAAAAACGAATGCAAGTACAAAAATCAGAACAACAAGGACA
AAAGACATGTTCTAGAGCCAAAAACGAATGCAAGTACAAAAATCAGAACAACAAGGACA

CGTTCGTCAAGTGCCCGTCGTTCGCAAACAAGAAGGTAAAGGTTCTTTTTTGCCCCCTT
CGTTCGTCAAGTGCCCGTCGTTCGCAAACAAGAAGGTAAAGGTTCTTTTTTGCCCCCTT
CGTTCGTCAAGTGCCCGTCGTTCGCAAACAAGAAGGTAAAGGTTCTTTTTTGCCCCCTT

TTGAGGCTGCCTATTTCCAAGCTTACGTCTGATTAATCCACCGTTACAGTGCACCAAAG
TTGAGGCTGCCTATTTCCAAGCTTACGTCTGATTAATCCACCGTTACAGTGCACCAAAG
TTGAGGCTGCCTATTTCCAAGCTTACGTCTGATTAATCCACCGTTACAGTGCACCAAAG

ACAACGCCAAGTGTAGCTTTGACAGTTATTCTCGAGCTGTCACGTGTCATTAG
ACAACGCCAAGTGTAGCTTTGACAGTTATTCTCGAGCTGTCACGTGTCATTAG
ACAACGCCAAGTGTAGCTTTGACAGTTATTCTCGAGCTGTCACGTGTCATTAG

Figure 3.4. Sequence alignment of *Efe-afpA* region of *Epichloë festucae* wild-type, 1a-7t8s3, and 1c-3s5. 1a-7t8s3 has a single T (in red) insertion at the first exon of *Efe-afpA* resulted in frame shift and introduced early stop codon (boxed). A 1625-bp large insertion in 1c-3s5 highlighted in blue is a large fragment from the transformation vector pG4. Twenty base pair target for guide RNA sequence and PAM designed for CRISPR/Cas9 is highlighted in green and red in the wild-type, respectively. *Efe-afpA* introns are highlighted in yellow in the wild-type.

Table 3.1. Sequences of oligonucleotide primers used in this study.

Primer	**Sequence, 5' – 3'**
KpnI-EfAFP-f	NNNNNNGGTACCCTTTGCCTCTGTCTGTATCC[a]
ClaI-5'-r	NNNNNNATCGATATAGTGAATGATTTGGTTGCG[a]
PstI-3'-f	NNNNNNCTGCAGCACCAAAGACAACGCCAAGT[a]
SpeI-EfAFP-r	NNNNNNACTAGTATTCCTTCAACGCTATAGACG[a]
ClaI-hph-f	NNNNNNATCGATAATAGGGGTTCCGCGCACA[a]
hph-r	TCCGATGATAAGCTGTCAAAC
AFP-Del2-F	TCACGTATGAGTTTTAGAGCTAGAAATAGCAAG
AFP-Del2-R	TCAGAATGCCGATCATTTATCTTTCACTGCG
BG2F	GTATCACGAGGCCCTTTC
FullerGR	GAAGTGGAAAGGCTGGTG
FullerGF2	TCTTTGAAAAGATAATGTATGATTATGC
PCseqR1	TTCACCGTCATCACCGAAAC
GTCas9F	aggggcacaccagcctttccacttcAGAAGCCGCAGGTGTCGAG[b]
GTCas9R	taatcatacattatcttttcaaagaCGATGTCTTCCTCTTCGGC[b]
gF2	TAATACGACTCACTATAGGCATTCTGATCACGTA
gR2	TTCTAGCTCTAAAACTCATACGTGATCAGAATGC
cafp-f	CTATGCAAATCACCGTGGTC
AFPr	CTAATGACACGTGACAGCTC

[a] Where N=any. Restriction enzyme recognition cites are highlighted in red.
[b] Lower case letters indicate primer sequences to overlap pD2P6.

Table 3.2. Summary of manipulative inocluation of strong creeping red fescue (*Festuca rubra* L. subsp. *rubra*) with *Epichloë festucae* wild-type Rose City (RC) and mutants 1a-7t8s3 and 1c-3s5.

	Not infected	ME+[a]	Died	Total
E– NT-24 Kent[b]				
RC without wounding	46	0	7	53
RC with wounding	18	5	17	40
1a-7t8s3 with wounding	39	0	32	71
1c-3s5 with wounding	51	0	17	68
E– S1139[c]				
1c-3s5 with wounding	204	0	119	323

[a] Manipulatively endophyte-infected strong creeping red fescue plants.
[b] Sterile germinating seedlings.
[c] Mature tillers, same genotype.

Table 3.3. Colony diameter of *Epichloë festucae* wild-type Rose City (RC) and mutants 1a-7t8s3 and 1c-3s5 on potato dextrose agar (PDA) and 1.5% water agar (WA). Diameter means within a column followed by the same lower case letter are not significantly different based on Fisher's protected least significant difference at the 0.05 probability level.

	PDA		WA			
Colony	19 day		20 day		24 day	
	mm					
RC	37.0±2.2	a	26.4±8.5	a	37.9±8.5	a
1a-7t8s3	31.2±1.9	b	4.6±0.6	b	6.8±1.3	c
1c-3s5	31.8±1.5	b	12.0±1.8	b	16.0±0.8	b

Chapter 4. Summary

Many cool-season grasses have symbiotic relationships with *Epichloë* fungal endophytes that inhabit the intercellular spaces of the aerial parts of the host plants. The presence of the *Epichloë* endophytes is generally beneficial to the hosts due to enhanced tolerance to biotic and abiotic stresses conferred by the endophytes. Asexual *Epichloë* spp. are asymptomatic to the host plants. However, some *Epichloë* spp. have a sexual stage and produce macroscopic fruiting bodies, stromata, that envelop the developing inflorescences causing a syndrome termed "choke disease". However, the mechanism of this disease is unknown and chemical control is ineffective. We conducted a fungal and plant gene expression analysis of stroma tissue and asymptomatic inflorescence tissue of *Epichloë festucae* infected strong creeping red fescue (*Festuca rubra* subsp. *rubra*). Hundreds of fungal genes and over 10% of the plant genes were differentially expressed when comparing the two tissue types. The differentially expressed fungal genes in the stroma tissue indicated a change in carbohydrate and lipid metabolism as well as a change in the expression of numerous genes for candidate effector proteins. Moreover, plant stress related genes were up-regulated in the stroma tissue suggesting the plant host was responding to the normally symbiotic fungal endophyte as a pathogen.

Genome and transcriptome studies are often the first steps for gene discovery followed by gene function studies. CRISPR/Cas technology is a powerful molecular tool to genetically modify genes of interest for further functional characterization of those genes. CRISPR/Cas9 approach is highly effective compared to conventional fungal transformation relying on homologous recombination. More importantly, CRISPR/Cas9 approach is more versatile and is not restricted by the availability of long and unique

homologous flanking regions of the target gene. The second study utilized CRISPR/Cas9 techonology for the first time in *Epichloë* endophyte research. We succesfully generated knockouts of the *Efe-afpA* gene, whereas attempts made with the homologous recombination method were unsuccessful. Many other genes can be edited with the same system by desgining unique 20-bp protospacer of gRNA targting the gene of interest.