Supplemental Material

Table S3 Biological Material

Biological material	Relevant characteristics	Reference
Epichloë festucae		
PN2278 (Fl1)	Wild-type	Young et al., 2005
PN2656	Fl1/Δ*sakA*::P*trpC-hph*; Hyg^R	Eaton et al., 2008
PN2658	Δ*sakA*/*sakA*; PN2656/pCE1; pII99; Hyg^R; Gen^R	Eaton et al., 2008
PN2327	Fl1/Δ*noxA*::P*trpC-hph*; Hyg^R	Tanaka et al., 2006
PN2497	Fl1/Δ*noxR*::P*trpC-hph*; Hyg^R	Takemoto et al., 2006

Protocol 1. Transcriptome Generation

Sample preparation. To compare plant and fungal gene expression patterns between wild-type and Δ*sakA* mutant associations, total RNA was isolated from wild-type *E. festucae* and Δ*sakA* mutant-infected perennial ryegrass plants and subjected to Illumina sequencing. Pseudostem tissue was harvested eight weeks after inoculation and snap frozen in liquid nitrogen. Approximately 1 g of infected pseudostem tissue was ground to a fine powder in liquid nitrogen. The tissue was then resuspended in 10 mL of TRIzol reagent (Invitrogen) and left to thaw. Cellular debris was pelleted by centrifugation at 12,000 g for 10 min at 4°C. The aqueous phase was then purified by chloroform extraction. Isopropanol was then added to the aqueous phase and the solution incubated at room temperature for 10 min to precipitate the RNA. The RNA was pelleted by centrifugation at 12,000 g for 10 min at 4°C. The pellet was washed once with 75% ethanol, air-dried and resuspended in DEPC-treated milliQ water. RNA samples were assessed for quality using a NanoPhotometer™ (Implen) and by SDS-gel electrophoresis. To confirm the samples contained fungal RNAs, RT-PCR analysis using primers designed to *E. festucae tubB* (T1.1 (5'-GAGAAAATGCGTGAGATTGT-3') and T1.2

(5'-TGGTCAACCAGCTCAGCACC-3'), *ltmJ* (lol214 (5'-GGTGACAAATCGATGCTTGG-3') and lol266 (5'-GACAGCGTCTTGAGGGAATC-3')) and *ltmC* (lol278 (5'-GAAACTGCCAATCGAGCATA-3') and lol279 (5'-TTCTTGCAATCATTTTGCAATTG-3')) was performed. RNA samples were ethanol precipitated and sent under absolute ethanol on dry ice to Cofactor Genomics (St Louis, MO).

Poly-A mRNA purification. Poly-A mRNA purification was performed as described in the manual for the Poly (A) Purist MAG kit (Ambion, Austin, TX). 50 µg of total RNA at 600 ng/µl was mixed with an equal volume of 2x binding solution. An equal mass of Oligo (dT) MagBeads were washed and hybridized with the RNA in 1x binding solution at 72°C for 5 min to denature secondary structures, and subsequently incubated at room temperature with gentle agitation for 1 h. After the hybridization, poly-A RNA attached beads were washed, and poly-A RNA eluted in 200 µl RNA Storage Solution at 72ºC. Eluted RNA was precipitated with ethanol, and the RNA pellet was re-suspended into 20 µl of RNA Storage Solution.

cDNA synthesis. 100 ng of purified poly-A RNA was fragmented by incubation with the fragmentation buffer included in Illumina's RNA-seq kit (llumina, San Diego, CA) for 5 min at 94ºC. Fragmented RNA was purified using ethanol precipitation. First strand cDNA synthesis was performed by priming fragmented RNA with random hexamers, and followed by reverse transcription using Superscript II (Invitrogen, Carlsbad, CA). Second strand synthesis was performed by incubating with second stranded buffer and dNTPs provided in the Illumina RNA-seq kit, and subsequently held on ice for 5 min. The reaction mix was then incubated with DNA Pol I and RNase H at 16ºC for 2.5 h (Invitrogen, Carlsbad, CA).

cDNA library construction. Double-stranded cDNA was purified using Qiagen Minelute columns (Qiagen, Valencia, CA), and eluted into 40 µl Qiagen EB buffer. Purified double stranded cDNA was treated with a cocktail of T4 DNA polymerase, Klenow large fragment and T4 polynucleotide kinase to create blunt-ended DNA. A single adenine base was added at the 3´ end by treating the DNA products with dATP and Klenow fragment (3´-to-5´ exonuclease action). This A-tailed DNA was ligated with paired end adaptors using T4-DNA quick ligase provided in Illumina's RNA-seq kit

(Illumina, San Diego, CA). The adaptor-ligated cDNA was size selected on a 4-12% acrylamide gel, and the amplified DNA library with ideal fragment size was obtained by in-gel PCR using the Phusion High-Fidelity system (New England Biolabs, Ipswich, MA).

Protocol 2 Transcriptome Analysis

Statistical analysis. Fold difference (ΔE), a measure of differential gene expression between the wild-type and mutant samples, was calculated using the following formula, where c_s is the smaller gene count and c_l is the larger gene count:

$$\Delta E = \frac{c_l - c_s}{c_s} \quad (1)$$

In cases where one sample produced a gene count of zero, fold difference was defined as the gene count of the other sample. A correction factor was applied to normalize gene counts based on the relative difference in total read counts observed for the wild-type and mutant lanes (*i.e.*, a technical correction that accommodates differences in read density between samples). For each gene i, the mutant read count (μ_i) was corrected by the difference in the total number of read counts observed for the wild-type and mutant samples across all genes (w_t and μ_t, respectively).

$$\mu_i' = \mu_i \frac{w_t}{\mu_t} \quad (2)$$

A ΔE of one means that the abundance of that mRNA transcript in the sample with the larger read count is, relative to the entire mRNA transcript pool, twice that of the sample with the smaller read count.

For reads that mapped to the *Epichloë festucae* sequence set, we observed 1,082,602 matches for the wild-type sample and 3,863,787 matches for the mutant sample, thus yielding a correction factor of 0.280. For reads assigned to the *Lolium perenne de novo* sequence set, we observed 13,962,929 matches for the wild-type sample and 15,959,338 matches for the mutant sample, thus yielding a correction factor of 0.875. Statistically significant differences in expression were determined from the gene counts.

We emphasize that the calculations for statistical significance and fold difference are independent. Statistical significance was determined with Fisher's Exact Test, which by default accounts for unequal sample sizes in the raw read counts. Fold difference was calculated primarily to provide a human-interpretable metric for differential gene expression.

Cluster analysis. The set of annotated gene models (EfM2) was mapped back to the *Epichloë festucae* genome sequence (November 2009 build, n = 844 scaffolds) using *gmap* (version 2007-09-28). Genes were ordered relative to their locations in the genome, and the expression of each was characterized as up-regulated, down-regulated or no change in the *ΔsakA* mutant relative to the wild-type (thresholds as defined above). Consecutive genes with identical patterns of expression (*i.e.*, runs of up- or down-regulated genes) were identified as possible gene clusters. To determine significance levels, we adopted a Monte Carlo simulation approach. Genes were randomly assigned to scaffolds (thus mimicking the fragmentary structure of the current genome sequence), cluster sizes were calculated, and the process repeated 10^5 times. To determine whether genes are more clustered in *Epichloë festucae* than would be expected by chance, we calculated the distribution of cluster sizes in the simulated dataset. Clusters falling outside the 0.025 and 0.975 quantiles of the simulated distribution (*i.e.*, the 95% confidence interval) were defined as outliers, and the likelihood of observing clusters of these sizes was calculated from additional simulated datasets ($n = 10^5$). Conversely, to determine whether *specific* clusters are statistically unlikely, we calculated the likelihood of observing each cluster size individually. Cluster sizes observed in fewer than 1-in-20 simulated datasets (one-tailed $p = 0.05$) were deemed significantly unlikely.

Fig. S1 Vasculature of plants infected with the *E. festucae* Δ*sakA* mutant

A. Light micrographs of perennial ryegrass blade tissue showing branching between the vasculature of plants infected with wild-type *E. festucae* (WT) and the Δ*sakA* mutant. Bar = 100 μm. **B**. Quantification of vasculature branching in plants infected with the WT and Δ*sakA* mutant strains. 1 cm sections of blade tissue were taken from multiple tillers on multiple plants and the number of branches within each section counted. n = 24 and 22

for the WT and Δ*sakA* strains respectively. Statistical significance in comparison to the WT strain was determined using Student's *t*-test ($t = 8.23$, df = 39.4 and $p = 4.3 \times 10^{-10}$).

Fig. S2 Fluorescence micrographs of FM4-64 stained *E. festucae* vacuoles

FM4-64 selectively stains the vacuolar membrane red and can be observed by fluorescence microscopy. No difference is seen between vacuoles at the hyphal tips in wild-type (WT) or the Δ*sakA* mutant. Vacuoles in regions distant from the tip are highly variable in size but no obvious difference is observed between WT and the Δ*sakA* mutant. Bar = 5 μm.

Fig. S3 Differentially expressed fungal and plant genes organised by catalytic activity ontology

Organisation of *E. festucae* (**A**) and *L. perenne* genes (**B**) differentially expressed between the wild-type and Δ*sakA* mutant-infected samples by catalytic activity GO category. Bars show the number of genes within each category that are up- or down-regulated in the Δ*sakA* mutant-infected sample relative to the WT-infected sample. Categories are: cyclase activity (GO:0009975), deaminase activity (GO:0019239), demethylase activity (GO:0032451), glycogen debranching enzyme (GDE) activity (GO:0004133), hydrolase activity (GO:0016787), isomerase activity (GO:0016853), ligase activity (GO:0016874), lipoic acid (LA) synthase activity (GO:0017140), lyase activity (GO:0016829), non-ribosomal peptide synthetase activity (NRPS; non-GO category), Mo-molybdopterin cofactor sulfurase activity (Mo sulfurase; GO:0008265), Mo-molybdopterin synthase activity (Mo synthase; GO:0030366), oxidoreductase activity (oxidoR; GO:0016491), polyketide synthase activity (PKS; GO:0016218), phytoene synthase activity (Psy; GO:0046905) and transferase activity (GO:0016740).

Fig. S4 Differential expression of fungal and plant oxidoreductase genes

Organisation of *E. festucae* (**A**) and *L. perenne* oxidoreductase genes (**B**) differentially expressed between the wild-type and Δ*sakA* mutant-infected samples by GO category. Bars show the number of genes within each category that are up- or down-regulated in

the Δ*sakA* mutant-infected sample relative to the WT-infected sample. Categories are oxidoreductase activity acting on: aldehyde or oxo groups of donors (GO:0016903), CH or CH_2 groups (GO:0016725), CH-CH group of donors (GO:0016627), CH-NH group of donors (GO:0016645), CH-NH_2 group of donors (GO:0016638), CH-OH group of donors (GO:0016614), diphenols and related substances as donors (GO:0016679), iron-sulfur proteins as donors (Fe-S; GO:0016730), oxidising metal ions (GO:0016722), NADH or NADPH (GO:0016651), other nitrogenous compounds as donors (N-groups; GO:0016661), paired donors with incorporation or reduction of molecular oxygen (GO:0016705), single donors with incorporation of molecular oxygen (GO:0016701), sulfur group of donors (S-groups; GO:0016667), superoxide radicals as acceptor (GO:0016721), dioxygenase activity (GO:0051213), fatty acid alpha-hydroxylase activity (FAH; GO:0080132), monooxygenase activity (MO; GO:0004497), phytanoyl-CoA dioxygenase activity (PHYD; GO:0016705) and unclassified oxidoreductase activity.

Fig. S5 Differential expression of fungal and plant transferase genes

Organisation of *E. festucae* (**A**) and *L. perenne* transferase genes (**B**) differentially expressed between the wild-type and Δ*sakA* mutant-infected samples by GO category. Bars show the number of genes within each category that are up- or down-regulated in the Δ*sakA* mutant-infected sample relative to the WT-infected sample. Categories are transferase activity transferring: one-carbon groups (GO:0016741), acyl groups (GO:0016746), aldehyde or ketonic groups (AH/ketonic; GO:0016744), alkyl or aryl (other than methyl) groups (GO:0016765), glycosyl groups (GO:0016757), nitrogenous groups (N-groups; GO:0016769), phosphorus-containing groups (P-groups; GO:0016772), and sulfur-containing groups (S-groups; GO:0016782).

Fig. S6 Putative gene clusters down-regulated in Δ*sakA* mutant association

A. Fold changes in expression of genes in a putative nitrogen metabolism cluster displaying significantly reduced expression in Δ*sakA* mutant-infected sample relative to the wild-type strain-infected sample. GTP, GTP-binding protein; Hyp, hypothetical protein; GOOX, gluco-oligosaccharide oxidase; MFS, MFS amine transporter; NmrA,

NmrA-like transcriptional regulator; NRPS, non-ribosomal peptide synthetase. Where the difference in expression between the two samples is non-significant (fold change <1 or p>0.05), the fold change is displayed as 0. **B**. Fold changes in expression of genes in a putative Nc25-associated cluster displaying significantly reduced expression in Δ*sakA* mutant-infected sample relative to the wild-type strain-infected sample. EF200-EF202, hypothetical proteins; Nc25, highly expressed novel endophyte gene; kexB, kexin-encoding gene.

Fig. S7 Putative gene clusters up-regulated in Δ*sakA* mutant association

A. Fold changes in expression of genes in a putative sugar metabolism cluster displaying significantly increased expression in Δ*sakA* mutant-infected sample relative to the wild-type strain-infected sample. TF, transcription factor; GL, glucosidase; MFS, major facilitator superfamily sugar transporter; DHO, dihydroorotase; Hyp, hypothetical protein; P450, cytochrome P450 monooxygenase. Where the difference in expression between the two samples is non-significant (fold change <1 or p>0.05), the fold change is displayed as 0. **B.** Fold changes in expression of genes in a putative cell integrity-associated cluster displaying significantly increased expression in Δ*sakA* mutant-infected sample relative to the wild-type strain-infected sample. RlmA, RlmA-like transcription factor; CAP, cell adhesion protein; FR, fumarate reductase; EfU, *E. festucae* unique gene; MFS, major facilitator superfamily monosaccharide transporter. **C**. Fold changes in expression of genes in a putative hydrolytic cluster displaying significantly increased expression in Δ*sakA* mutant-infected sample relative to the wild-type strain-infected sample. AP, aminopeptidase; EL, extracellular lipase; EfU, *E. festucae* unique gene; MP, membrane protein; Hyp, hypothetical protein.

Fig. S8 Differential expression of perennial ryegrass hydrolase genes

Organisation of *L. perenne* hydrolase genes differentially expressed between the wild-type and Δ*sakA* mutant-infected samples by GO category. Bars show the number of genes within each category that are up- or down-regulated in the Δ*sakA* mutant-infected sample relative to the WT-infected sample. Categories are: hydrolases acting on acid anhydrides

(GO:0016817), acid halide bonds (GO:0016824), carbon-nitrogen (but not peptide) bonds (C-N; GO:0016810), carbon-sulfur bonds (C-S; GO:0046508), ester bonds (GO:0016788), ether bonds (GO:0016801), glycosyl bonds (GO:0016798); peptidase activity (GO:0008233); deacetylase activity (GO:0019213) and unclassified hydrolase activity.

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