The genome sequence of *Bipolaris cookei* reveals mechanisms of pathogenesis underlying target leaf spot of sorghum

Alex Z. Zaccaron & Burton H. Bluhm

*Bipolaris cookei* (=*Bipolaris sorghicola*) causes target leaf spot, one of the most prevalent foliar diseases of sorghum. Little is known about the molecular basis of pathogenesis in *B. cookei*, in large part due to a paucity of resources for molecular genetics, such as a reference genome. Here, a draft genome sequence of *B. cookei* was obtained and analyzed. A hybrid assembly strategy utilizing Illumina and Pacific Biosciences sequencing technologies produced a draft nuclear genome of 36.1 Mb, organized into 321 scaffolds with L50 of 31 and N50 of 378 kb, from which 11,189 genes were predicted. Additionally, a finished mitochondrial genome sequence of 135,790 bp was obtained, which contained 75 predicted genes. Comparative genomics revealed that *B. cookei* possessed substantially fewer carbohydrate-active enzymes and secreted proteins than closely related *Bipolaris* species. Novel genes involved in secondary metabolism, including genes implicated in ophiobolin biosynthesis, were identified. Among 37 *B. cookei* genes induced during sorghum infection, one encodes a putative effector with a limited taxonomic distribution among plant pathogenic fungi. The draft genome sequence of *B. cookei* provided novel insights into target leaf spot of sorghum and is an important resource for future investigation.

Sorghum (*Sorghum bicolor* (L.)) was domesticated in northeast Africa 4–5000 years ago, although anthropological records indicate it has been consumed by humans as early as 8000 BC\(^1,2\). In 2016, the U.S. was the world's leading producer of sorghum, followed by Nigeria, Sudan, Mexico, India, and China (https://www.worldsorghumproduction.com/previous-year.asp). In the U.S., sorghum is mostly grown as a grain crop, although it is also produced for silage and syrup\(^3,4\), and has recently garnered interest as a feedstock for cellulosic ethanol production\(^5\) and in culinary applications due to its gluten-free properties\(^6\). Sorghum is naturally adapted to harsh growing environments, particularly locations where heat, drought, and marginal soils limit the profitability of crops such as corn or wheat\(^7\).

Sorghum production, particularly in warm, humid growing areas, is hindered by a wide variety of fungal foliar diseases\(^8\). Noteworthy in the southeastern U.S. is target leaf spot, caused by *Bipolaris cookei* (=*B. sorghicola* = *Drechslera sorghicola* = *Helminthosporium sorghicola*\(^9\)). For many decades after its initial description\(^10\), target leaf spot was considered to be a disease of minor concern in the U.S.\(^11\) and other regions of the world\(^12,13\). However, within two decades after its first reported observation on grain sorghum in Mississippi in 1986\(^14\), target leaf spot has become widespread in the lower Mississippi river valley. Foliar lesions are distinctly rectangular (Fig. 1A and B), and leaf spots often contain scalariform bands of pigmentation that inspired the disease's name. The pathogen is generally restrained by the major veins of leaves, but lesions can coalesce under heavy disease pressure, resulting in irregular patches of chlorosis and/or premature leaf death\(^10\). Most species of *Bipolaris* with defined pathogenicity lifestyles are categorized as necrotrophic pathogens\(^15\), although hemibiotrophic species have been noted\(^16\). In *B. cookei*, the growth habit underlying pathogenesis on sorghum has not yet been determined. However, the rapid onset of necrosis after infection, unimpeded intracellular growth during host colonization, and an apparent lack of specialized infectious structures associated with biotrophy (such as infectious hyphae or haustoria) collectively suggest a necrotrophic lifestyle\(^12\).

The molecular basis of target leaf spot is poorly understood. Quantitative trait loci underlying resistance to target leaf spot have been explored to some extent\(^17,18\). A single recessive resistance gene (*ds1*) was positionally...
cloned and postulated to encode a leucine-rich receptor kinase family protein. Comparative transcriptomics during infection of resistant and susceptible hybrids with B. cookei identified both plant and fungal genes 21,22. However, only 160 transcripts were attributed to B. cookei, likely due to the low amount of fungal biomass and difficulty mapping transcripts due to the lack of a reference genome sequence for the pathogen.

To address the lack of genomic resources of B. cookei and provide new insights into the mechanistic basis of target leaf spot, a draft genome assembly of B. cookei was assembled and analyzed. Broad categories of genes involved in pathogenesis, such as carbohydrate-active enzymes and genes involved in secondary metabolism, were identified and assessed through comparative genomics. Additionally, novel genes were identified that could play important roles in pathogenicity.

Results

Genome sequencing, assembly, and annotation. The B. cookei genome was sequenced with Illumina and Pacific Biosciences (PacBio) sequencing technologies. Illumina sequencing produced 176,463,088 reads (2 × 100 bp paired-end sequences), and PacBio sequencing produced 251,565 reads with lengths of 35 to 21,246 bp (average length = 2,427 bp). A hybrid assembly pipeline (Supplementary Fig. S1) incorporated both types of reads to produce a draft genome assembly of 36,171,030 bp organized into 321 scaffolds (average length = 112 kb) (Table 1).

Approximately 14% of the B. cookei draft genome sequence was comprised of transposable elements (TEs). Of these, DNA transposons (class 2 TEs) corresponded to 9% of the B. cookei genome, and retrotransposons (class 1 TEs), comprised 3% (Supplementary Fig. S2). The majority of the classified DNA transposons contained a DDE superfamily transposase motif, which comprised approximately 3 Mb of the draft assembly.

To assist gene prediction, RNA-seq was performed from in vitro cultures of B. cookei. RNA was sequenced with Ion Torrent technology, which generated 6,273,768 reads (average length = 247 bp). After RNA-seq reads were mapped to the B. cookei genome assembly, 17,998 transcripts were reconstructed, corresponding to 11,451 distinct loci. With reconstructed transcripts and proteins from closely related species as evidence, 11,189 distinct protein-encoding genes were predicted (average length = 1,707 bp) (Table 2). Assessment of predicted genes with BUSCO software showed 95.7% gene completeness, with 0.2% duplication, and 2.9% fragmentation. Functional characterization of the predicted B. cookei proteins revealed that 10,801 proteins (96%) had at least one homologous sequence in the NCBI nr database (e-value < 1e-5), 8,422 proteins (75%) had a conserved domain matching the InterPro database, and 9,034 proteins (80%) had a GO term attributed by Blast2GO.

Homology-based analyses with mating-type loci from related Bipolaris species indicated that B. cookei strain LSLP18.3 contained a MAT-1 gene (Bc_02106). The predicted protein encoded by Bc_02106 shared 87% amino...
acid identity with MAT-1 from *B. maydis*, and was located within a genomic region conserved between *B. maydis* and *B. cookei* (Supplementary Fig. S3).

In addition to the nuclear genome, the *B. cookei* mitochondrial genome was assembled as a circular sequence of 135,790 bp with a GC content of 30% (Fig. 2). A total of 75 mitochondrial genes were predicted, including 12 of 14 highly conserved genes among fungal mitochondria: four subunits of the respiratory chain complexes (*cox1*, *cox2*, *cox3* and *cob*), seven NADH dehydrogenase subunits (*nad1–6*), one ATP synthase (*atp6*) in yellow, rDNAs (*rns* and *rnl*) in red, tRNAs in black and ribosomal protein S3 (*rps3*) in blue. ORFs encoding homing endonucleases are drawn in purple, and introns are represented as white regions. The innermost circle represents the GC content, calculated with a sliding window of 1 kb and step size of 100 bp. Regions with the GC content below and above the average (30%), are in blue and orange, respectively. A grey circle designates the 50% GC content.

### Table 2. Gene prediction statistics of *B. cookei* and other *Bipolaris* species.

|          | *B. cookei* | *B. maydis* C5 | *B. sorokiniana* | *B. zeicola* | *B. oryzae* | *B. victoriae* |
|----------|-------------|----------------|------------------|-------------|-------------|---------------|
| Genes    | 11,189      | 13,336         | 12,250           | 12,857      | 12,007      | 12,894        |
| Average length of: |          |                |                  |             |             |               |
| Genes (bp) | 1,707      | 1,580          | 1,691            | 1,418       | 1,479       | 1,432         |
| ORFs (bp)  | 1,580      | 1,439          | 1,474            | 1,418       | 1,479       | 1,432         |
| Proteins (aa) | 465        | 427            | 443              | 427         | 445         | 431           |
| Introns (bp) | 99         | 97             | 91               | 87          | 88          | 88            |
| Exons (bp) | 493        | 535            | 587              | 505         | 416         | 411           |
| Gene completeness | 95.7%      | 97.6%          | 98.2%            | 97.0%       | 97.1%       | 96.9%         |
| Duplicated genes | 0.2%       | 2.4%           | 0.2%             | 0.2%        | 0.2%        | 0.2%          |
| Fragmented genes | 2.9%       | 1.5%           | 0.8%             | 1.0%        | 0.9%        | 0.8%          |
| Missing genes | 1.4%       | 0.9%           | 1.0%             | 2.0%        | 2.0%        | 2.3%          |

![Figure 2. Circular representation of the *B. cookei* mitochondrial genome.](image)

Bipolaris cookei Mitochondrial genome 135790 bp

In addition to the nuclear genome, the *B. cookei* mitochondrial genome was assembled as a circular sequence of 135,790 bp with a GC content of 30% (Fig. 2). A total of 75 mitochondrial genes were predicted, including 12 of 14 highly conserved genes among fungal mitochondria: four subunits of the respiratory chain complexes (*cox1*, *cox2*, *cox3* and *cob*), seven NADH dehydrogenase subunits (*nad1–6*), one ATP synthase (*atp6*) in yellow, rDNAs (*rns* and *rnl*) in red, tRNAs in black and ribosomal protein S3 (*rps3*) in blue. ORFs encoding homing endonucleases are drawn in purple, and introns are represented as white regions. The innermost circle represents the GC content, calculated with a sliding window of 1 kb and step size of 100 bp. Regions with the GC content below and above the average (30%), are in blue and orange, respectively. A grey circle designates the 50% GC content.
resistance to QoI fungicides, is not present in *B. cookei*

Family GH43 includes enzymes such as xylanases, genes (Fig. 3). Family GH16 is comprised of numerous endoglucanases, which catalyze hemicellulose degradation from family GH13, whereas members of the Pleosporales had 6.9. Interestingly, hierarchical clustering grouped *Setosphaeria turcica* most closely with *B. cookei*

*ygenases (LPMOs) that help cellulases and hemicellulases break down cellulose and hemicellulose*32. CAZymes includes gluco- and chitooligosaccharide oxidases, family AA9 is represented by lytic polysaccharide monooxygenases (LPMOs) that help cellulases and hemicellulases break down cellulose and hemicellulose32. CAZymes from family AA9 often act in conjunction with cellobiose dehydrogenases (CDHs), found in family AA3, to accelerate oxidative degradation of cellulosic materials, including foliar tissues of plants32–34.

GIY-YIG family) located within intronic regions. The *B. cookei* mitochondrial genome assembly contained 36 introns (average length = 2,284 bp), which comprised a total of 82,220 bp. Comparative genomic analyses with the mitochondrial gene cob from *Cercospora beticola*25 indicated that the mutation G143A, which is associated with resistance to QoI fungicides, is not present in *B. cookei* strain LSLP18.3 (Supplementary Fig. S4).

**Carbohydrate-active enzymes (CAZymes).** A total of 589 genes encoding CAZymes were identified in the *B. cookei* genome (Table 3; Supplementary Table S1). The three most populated CAZyme families were CE10 with 38 genes, AA7 with 32 genes, and CE1 with 26 genes. Both CE10 and CE1 families include a great variety of esterases, such as acetyl xylan esterases (EC: 3.1.1.72), carboxylesterases (EC: 3.1.1.1), and acetylcholinesterases (EC: 3.1.1.7)26, whereas family AA7 includes gluco- and chitooligosaccharide oxidases (EC: 1.1.3.-)27.

CAZymes categorized as glycoside hydrolases (GHs) receive particular attention for their ability to hydrolyse glycosidic bonds between carbohydrates or between a carbohydrate and a non-carbohydrate moiety26. In *B. cookei*, the most populated GH families were GH16 with 16 genes, GH43 with 14 genes, and 13 genes (Fig. 3). Family GH16 is comprised of numerous endoglucanases, which catalyze hemicellulose degradation26. Family GH43 includes enzymes such as xylanases, α-L-arabinofuranosidases, and β-D-galactosidases, that debranch and degrade hemicellulose and pectin26. Most *B. cookei* CAZymes from family GH3 were functionally annotated as β-glucosidases (EC: 3.2.1.21). Beta-glucosidases play an important role in the degradation of cellulose by converting cellobiose into glucose26.

Many members of the AA class of CAZymes are carbohydrate oxidases that assist other enzymes from GH, PL and CE classes to gain access to carbohydrates present in plant cell walls31. Two-thirds of the AA CAZymes in the *B. cookei* genome belonged to families AA7 (32 genes), AA9 (23 genes), and AA11 (11 genes). While family AA7 includes gluco- and chitooligosaccharide oxidases, family AA9 is represented by lytic polysaccharide monooxygenases (LPMOs) that help cellulases and hemicellulases break down cellulose and hemicellulose26. CAZymes from family AA9 often act in conjunction with cellobiose dehydrogenases (CDHs), found in family AA3, to accelerate oxidative degradation of cellulosic materials, including foliar tissues of plants32–34.

*B. cookei* and 16 other plant pathogenic members of the Dothideomycetes were clustered based on the number of CAZymes belonging to families likely involved in plant cell wall degradation. In concordance with previous studies, species within the Capnodiales and Pleosporales were organized in two distinct branches that resemble their phylogenetic placement35–37 (Fig. 3). The number of enzymes from families AA9 (LPMOs), GH64 (3-1,3-glucanases), CE5 (cutinases), PL1, and PL3 (pectin lyases) were notably different between Capnodiales and Pleosporales members. The number of GH13 enzymes, which include α-amylases and related enzymes involved in starch degradation38, was also markedly different. On average, members of the Capnodiales had 12.6 proteins from family GH13, whereas members of the Pleosporales had 6.9. Interestingly, hierarchical clustering grouped all analyzed *Bipolaris* spp. together in a distinct branch with the exclusion of *B. cookei*. *B. cookei* instead grouped most closely with *Setosphaeria turcica* in a different branch that also included *Pyrenophora tritici-repentis, P. teres f. teres*, and *Stagonospora nodorum* (Fig. 3).

**Secondary metabolism genes.** Plant pathogenic fungi possess a diverse array of genes involved in the biosynthesis of secondary metabolites (SMs). SMs are often produced by genes organized into clusters that contain one or more genes referred to as backbone genes, which are primarily classified as polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), hybrid PKS-NRPSs, dimethylallyl tryptophan synthases (DMATs), or terpene synthases (TSs)39. *B. cookei* had 47 backbone genes involved in secondary metabolism, categorized as 20 PKSs, 17 NRPSs, 3 DMATs, and 7 TSs, which is consistent with other *Bipolaris* species analyzed so far (Supplementary Table S2). One of the PKSs was a type III PKS, and the remaining 19 were type I PKSs, which were further classified into 10 highly reducing (HR-PKS), 2 partially reducing (NR-PKS), and 7 non-reducing (NR-PKS) (Supplementary Table S3). Orthologs of one NR-PKS from *B. cookei* (Bc_10041) could not be identified in the genomes of other *Bipolaris* species via homology searches (Supplementary Table S4), and the most similar protein identified among all fungi corresponded to a hypothetical gene from another sorghum pathogen, *Colletotrichum sublineolum* (55% amino acid identity). Gene Bc_10041 was located in a genomic region enriched with repetitive DNA, in close proximity to two other backbone genes encoding NRPSs, Bc_10039 and Bc_10040 (Fig. 4A). Interestingly, putative orthologs of Bc_10039 were not detected in the genomes of other *Bipolaris* species, and Bc_10040 was orthologous to NPS9 (78% identity), a NRPS-encoding gene from *B. maydis* that was previously thought to be unique to this species15 (Supplementary Table S4).

| GH | GT | PL | CE | CBM | AA | Total |
|----|----|----|----|-----|----|-------|
| B. cookei | 238 | 95 | 16 | 105 | 52 | 115 | 589 |
| B. maydis | 279 | 102 | 15 | 135 | 66 | 145 | 705 |
| B. zeicola | 264 | 95 | 15 | 129 | 63 | 130 | 656 |
| B. oryzae | 259 | 98 | 15 | 124 | 58 | 131 | 648 |
| B. sorokiniana | 260 | 99 | 15 | 125 | 66 | 125 | 651 |
| B. victoriae | 267 | 95 | 15 | 131 | 67 | 130 | 664 |

Table 3. Number of genes encoding carbohydrate-active enzymes across different *Bipolaris* species. GH: glycoside hydrolases; GT: glycosyltransferases; PL: polysaccharide lyases; CE: carbohydrate esterases; CBM: carbohydrate-biding modules; and AA: auxiliary activities.
Phytotoxic sesquiterpenoids of the ophiobolin family have reportedly been produced by *B. cookei* and other *Bipolaris* species.\(^40-42^\). Recently, the gene cluster responsible for ophiobolin F biosynthesis was characterized in *Aspergillus clavatus*.\(^43^\) Based on this report, a similar gene cluster was identified in *B. cookei* that contained homologs of the terpene synthase *oblA* (*Bc_07938, 62% identity*), the cytochrome P450 *oblB* (*Bc_07937, 68% identity*), the FAD dependent oxidoreductase *oblC* (*Bc_07941, 52% identity*), and the ABC transporter *oblD* (*Bc_07943, 76% identity*) (Fig. 4B). Two additional ORFs present in the *A. clavatus* ophiobolin cluster were also conserved in *B. cookei*: a homolog of Aspc_1602 (*Bc_07939, 38% identity*; a hypothetical protein with...
no conserved domains), and a homolog of Aspcl_1604 (Bc_07940, 43% identity; a putative bZIP transcription factor). The additional ORF in the B. cookei ophiobolin cluster that is not present in the A. clavatus cluster (Bc_07942) encoded a hypothetical protein containing a NAD(P)-binding domain.

The PKS-encoding gene Bc_07901 of B. cookei was highly homologous to PKS18 of B. maydis (98% amino acid identity), which is involved in melanin biosynthesis. The draft genome sequence of B. cookei contained additional genes involved in melanin biosynthesis, including the 1,3,8-trihydroxynaphthalene reductase BRN1 (Bc_07806, 92% identity), the transcription factor CMR1 (Bc_0788, 97% identity), the 1,3,6,8-tetrahydroxynaphthalene reductase BRN2 (Bc_07555, 99% identity), and the scytalone dehydratase SCD1 (Bc_10431, 98% identity) (Supplementary Fig. S5).

**Secretome and candidate effectors.** A total of 1035 genes were identified in the B. cookei nuclear genome that encoded proteins containing a signal peptide for secretion. Among these genes, 170 were predicted to encode proteins containing transmembrane domains or a glycosylphosphatidylinositol (GPI) anchor (Supplementary Table S5), and thus were predicted to be cell surface proteins. The remaining 865 proteins were determined to comprise the B. cookei secretome. Nearly half (42%) of the predicted secretome consisted of proteins implicated in aspects of primary metabolism. Specifically, the putative secretome contained 253 CAZymes, with AA families being the most abundant: 22 proteins from family AA9, 18 proteins from family AA7, and 15 proteins from family AA3. Additionally, 74 proteases, 43 peroxidases, and 107 lipases were identified in the secretome. Nearly half (42%) of the predicted secretome consisted of proteins implicated in aspects of primary metabolism. Specifically, the putative secretome contained 253 CAZymes, with AA families being the most abundant: 22 proteins from family AA9, 18 proteins from family AA7, and 15 proteins from family AA3. Additionally, 74 proteases, 43 peroxidases, and 107 lipases were identified in the secretome.

A set of 233 proteins were classified as candidate effectors, or small secreted proteins (SSPs). Among these, 133 could not be assigned a putative function based on homology, and 149 had no conserved domains. One of the B. cookei SSPs (Bc_04981) was homologous to EcP6 from the tomato pathogen Cladosporium fulvum. EcP6 is an effector that contains lysin motif (LysM) domains, and sequesters chitin oligosaccharides released from the fungal cell wall to avoid host chitin-triggered immune responses. The architecture of the three LysM domains found in EcP6 was conserved in the B. cookei homolog Bc_04981 (Supplementary Fig. S6).

**Expression-based analyses to identify candidate genes involved in pathogenesis.** Fungal genes preferentially expressed during host infection are potentially involved in pathogenicity. To qualitatively identify B. cookei genes highly or exclusively expressed in planta, an RNA-seq data set was obtained from B. cookei grown on various defined culture media in vitro, representing the basal transcriptome, which was then compared to in planta expression data from sorghum leaves infected by B. cookei (accessions: DRR060371 and DRR06373). Of the 66 million reads obtained by Yazawa and co-authors, 537,582 (0.8%) mapped to the B. cookei draft genome assembly. Fungal RNA-seq reads identified in planta accounted for 54,295,782 bp, as compared to 1,422,960,112 bp (5,531,920 reads) obtained in vitro. A total of 37 genes were considered to be induced in planta, 16 of which had no evidence of expression in vitro (Table 4). Several of the identified genes induced in planta, including putative oxidoreductase activity enzymes, major facilitator superfAMILY transporters, and SM backbone enzymes, were associated with fungal secondary metabolite biosynthesis or detoxification of compounds. Additionally, four genes encoding candidate effectors were also considered to be induced in planta.

Interestingly, some of the genes induced in planta were clustered in two regions of the B. cookei genome (Fig. 5A and B). Both regions were rich in repetitive DNA, and contained signs of repeat-induced point (RIP) mutations. One region (Fig. 5B) contained four genes exclusively expressed in planta. These four genes corresponded to a CAZyme from family AA7, annotated as 6-hydroxy-d-nicotine oxidase (Bc_11188), a putative oxidoreductase activity enzyme (Bc_10413), an oxidoreductase activity enzyme (Bc_10413), and a hypothetical protein (Bc_10413). The other region (Fig. 5A) contained four genes that are part of the cluster are labelled oblA-oblD, and other genes are labelled with the JGI protein ID. oblA encodes a terpene synthase, oblB encodes a cytochrome P450, oblC encodes a flavin-dependent oxidase, and oblD encodes a major facilitator transport protein. Genes Bc_07939, Bc_07940, and Bc_07942 encode hypothetical proteins. Bc_07939 has a bZIP domain, and Bc_07942 has a NAD(P)-binding domain. Reproductive DNA is represented as small rectangles.
cytochrome P450 (Bc_10329), a putative dehydrogenase reductase (Bc_10330), and a protein containing a nuclear transport factor 2 superfamily domain, annotated as SnoaL-like polyketide cyclase family 2 (Bc_10331). Homology searches revealed that these four genes were not conserved in other *Bipolaris* species or members of the Pleosporaceae family (Supplementary Table S4). Additionally, the closest homolog of Bc_10331 was a hypothetical protein from the sorghum pathogen *C. sublineolum* (67% amino acid identity).

Additional genes with exclusive in planta expression evidence included a serine protease (Bc_04816), an additional AA7 CAZyme (Bc_06321), and two candidate effectors (Bc_06468 and Bc_04701) (Table 4). Bc_06468 was located within a genomic region rich in repetitive DNA that showed signs of RIP mutation (Fig. 5C), and was classified as a putative effector due to its small size (98 amino acids), predicted signal peptide for secretion, and lack of conserved domains. Interestingly, homologs of Bc_06468 were found only in *B. maydis*, *B. zeicola*, *B. victoriae*, *B. sorokiniana*, *B. oryzae*, *S. turcica*, *P. tritici-repentis*, *Fusarium oxysporum* and *F. oxysporum f. sp. cubense* (Supplementary Table S4).

The PKS-encoding gene exclusively expressed in planta (Bc_04579) was homologous to alt5 (82% identity), a PKS gene from *Alternaria solani* postulated to be involved in alternapyrone biosynthesis47. Homologs of other genes also predicted to be involved in alternapyrone biosynthesis were clustered with Bc_04579, including Bc_11189 (82% identity with the cytochrome P450 alt2), Bc_11189 (87% identity with the cytochrome P450

### Table 4. *B. cookei* genes considered induced during infection. The number of *in planta* and *in vitro* RNA-seq reads mapped to each gene ORF is shown. *In planta* RNA-seq reads were sequenced from sorghum leaves infected by *B. cookei*21, and *in vitro* RNA-seq reads were sequenced from *B. cookei* grown on various culture media. Genes with evidence of expression only *in planta* (at least four reads), or with a ratio of reads *in planta*/reads *in vitro* of at least four, were considered induced during infection.

| Gene ID | ORF size (bp) | Reads in planta | Reads in vitro | Secreted | Blast2GO functional description |
|---------|---------------|-----------------|---------------|----------|---------------------------------|
| Bc_10331 | 456          | 421             | 0             | NO       | Snoal-like polyketide cyclase family 2 |
| Bc_11188 | 1455         | 259             | 0             | NO       | 6-hydroxy-d-nicotine oxidase     |
| Bc_10330 | 1192         | 226             | 0             | NO       | Dehydrogenase reductase SDR family member 7B |
| Bc_10329 | 1618         | 176             | 0             | NO       | Cytochrome P450                  |
| Bc_04819 | 1894         | 78              | 0             | NO       | X-Pro dipeptidyl-peptidase (S15 family) |
| Bc_06468 | 415          | 70              | 0             | YES      | Hypothetical protein             |
| Bc_06321 | 1724         | 43              | 0             | NO       | Glucooligosaccharide oxidase     |
| Bc_04208 | 915          | 12              | 0             | NO       | Hypothetical protein             |
| Bc_04302 | 309          | 11              | 0             | NO       | Hypothetical protein             |
| Bc_09129 | 537          | 8               | 0             | NO       | djl family                      |
| Bc_09118 | 483          | 8               | 0             | NO       | Mitochondrial carrier           |
| Bc_09119 | 1290         | 8               | 0             | NO       | Mitochondrial carrier           |
| Bc_04701 | 512          | 7               | 0             | YES      | Hypothetical protein             |
| Bc_03870 | 1924         | 6               | 0             | YES      | Glutamyl-tRNA(Gln) amidotransferase subunit A |
| Bc_00034 | 1853         | 5               | 0             | NO       | Cobalamin synthesis             |
| Bc_11121 | 1898         | 4               | 0             | NO       | High affinity methionine permease |
| Bc_01083 | 1468         | 9               | 2             | No       | Major facilitator superfamily transporter |
| Bc_01920 | 2277         | 363             | 36            | No       | Catalase A                      |
| Bc_03407 | 1789         | 14              | 1             | No       | Dibenzothiophene desulfurization enzyme A |
| Bc_04579 | 7882         | 6               | 1             | No       | Polyketide synthase             |
| Bc_04816 | 1671         | 67              | 3             | No       | Aldehyde desulfurization enzyme A |
| Bc_04818 | 1057         | 79              | 2             | No       | Aromatic ring-opening dioxygenase family |
| Bc_04820 | 1176         | 110             | 9             | No       | Galactonate dehydratase         |
| Bc_05113 | 497          | 147             | 27            | No       | Cupin domain containing         |
| Bc_06848 | 651          | 207             | 28            | Yes      | Hypothetical protein             |
| Bc_06913 | 2635         | 24206           | 4976          | No       | Heat shock 70 kDa               |
| Bc_07074 | 345          | 11              | 2             | Yes      | Hypothetical protein             |
| Bc_08665 | 984          | 12              | 1             | No       | Acetate transporter             |
| Bc_09116 | 231          | 8               | 2             | No       | Hypothetical protein             |
| Bc_09501 | 1800         | 14              | 3             | No       | Major facilitator superfamily transporter |
| Bc_09836 | 3398         | 92              | 6             | No       | Salicylate hydroxylase          |
| Bc_10839 | 3324         | 4               | 1             | No       | Non-ribosomal peptide synthetase |
| Bc_10847 | 1047         | 37              | 1             | No       | Cinnamyl-alcohol dehydrogenase   |
| Bc_10957 | 930          | 47              | 1             | Yes      | Dimeric alpha-beta barrel       |
| Bc_11123 | 1532         | 33              | 8             | No       | Pusatin demethylase             |
| Bc_11127 | 1135         | 30              | 1             | No       | Gentisate 1,2-dioxygenase       |
| Bc_11189 | 1846         | 7               | 1             | No       | Cytochrome P450                  |

The PKS-encoding gene exclusively expressed in planta (Bc_04579) was homologous to alt5 (82% identity), a PKS gene from *Alternaria solani* postulated to be involved in alternapyrone biosynthesis47. Homologs of other genes also predicted to be involved in alternapyrone biosynthesis were clustered with Bc_04579, including Bc_11190 (82% identity with the cytochrome P450 alt2), Bc_11189 (87% identity with the cytochrome P450
alt3), and Bc_04580 (76% identity with the FAD-dependent oxygenase/oxidase alt4) (Supplementary Fig. S7). Interestingly, these four genes were located on a 30 kb region of the B. cookei genome with little or no evidence of expression in vitro (Supplementary Fig. S8). This genomic region also contained four other genes predicted to encode an NAD-dependent epimerase (Bc_04582), a major facilitator superfamily transporter (Bc_04583), an acetylcholinesterase (Bc_04584), and a hypothetical protein (Bc_04585).

Enrichment of repetitive DNA in proximity to pathogenicity-related genes. In some plant pathogenic fungi, genes important for pathogenicity are in close proximity to repetitive genomic regions. Genes co-localized with repetitive elements are postulated to provide pathogens an evolutionary advantage due to allelic diversification induced by RIP activity. Consistent with other plant pathogenic fungi, candidate effectors and backbone genes for secondary metabolite production in B. cookei were localized in closer proximity to repetitive elements than the overall predicted genes and genes highly conserved among members of the Ascomycota (Fig. 6). More specifically, nearly all B. cookei backbone genes (40 out of 47; 85%) and most candidate effectors (153 out of 233; 65%) had repetitive DNA within 5 kb up- or downstream of their ORFs, while about half of all predicted genes (5,478 out of 11,189; 49%) had an analogous arrangement. We also observed similar enrichment of repeats near SM backbone genes and SSPs in the close relatives B. maydis C5 and B. sorokiniana, albeit not as pronounced as in B. cookei (Fig. 6). Interestingly, only 26% and 24% of the B. maydis C5 and B. sorokiniana predicted genes, respectively, had a repeat within a neighbor region of 5 kb, which is much less than the ratio in B. cookei (49%). These results indicate that B. cookei has a substantial enrichment of repetitive elements near genes potentially important for pathogenicity, and that repeats are more dispersed in the B. cookei genome as compared to closely related species.

Discussion
The draft genome sequence of B. cookei strain LSLP18.3 expanded comparative genomics analyses among members of the order Pleosporales within the Dothidiomycetes class of fungi. To date, the genomes of at least 60 members of the Pleosporales have been sequenced. Although the genome size of B. cookei is very similar to other members of the Pleosporales, the set of predicted genes in B. cookei is notably smaller than many close relatives (Supplementary Fig. S9; Supplementary Table S6). Particularly in the context of other Bipolaris species, B. cookei had contracted sets of CAZymes, secreted proteins, and candidate effectors. Conversely, B. cookei had...
Figure 6. Proximity of classes of genes to repetitive elements. The charts show the fraction of genes from *B. cookei*, *B. maydis* C5, and *B. sorokiniana* that have a repetitive element within an adjacent region up- or downstream of their ORFs of size shown on the horizontal axis. SM backbones: secondary metabolite backbone genes (PKSs, NRPSs, TPs, hybrid PKS-NRPSs, DMATs, and TPs); SSPs: small secreted proteins (candidate effectors); CAZymes: carbohydrate-active enzymes; USCOs: universal single-copy orthologs among members of the Acomycota.

A substantially larger mitochondrial genome than most other species of Dothideomycetes for which a complete mitochondrial genome is available. The proliferation of introns and ORFs encoding putative homing endonucleases were primarily responsible for the expanded size. Fungal mitochondria usually contain a set of 14 conserved protein-encoding genes required for electron transfer and oxidative phosphorylation. However, exceptions have been previously noted. More precisely, the absence of the ATP synthase genes *atp8* and *atp9* was also observed in the *B. cookei* close relatives *Stagonospora nodorum* and *Shirata bambusicolor*, the only Pleosporales members with mitochondrial genomes published to date. This result supports the hypothesis that the absence of *atp8* and *atp9* is a common genomic feature among members of the Pleosporales. Interestingly, *B. cookei* strain LSLP18.3 does not contain a G143A mutation within the mitochondrial *cytochrome b* gene, which is associated with resistance to QoI fungicides. At the time strain LSLP18.3 was isolated (2009), fungicides were not commonly applied to sorghum grown in Arkansas, although applications of foliar fungicides on grain sorghum in Arkansas have become increasingly common in recent years. Resistance to QoI fungicides recently emerged in related Dothideomycetes pathogens of soybean (e.g., *Cercospora sojina* and *Cercospora c. flagellaris*) and spread rapidly throughout the southeastern U.S. The finished mitochondrial genome of *B. cookei* will help facilitate future population-level studies to explore the potential emergence of fungicide resistance in this pathosystem.

Pathogens must adapt rapidly to changing environments in order to win ongoing evolutionary arms races with their hosts. The genome sequence of *B. cookei* provides evidence for diverse mechanisms promoting genetic variability and genome evolution. Regarding sexual reproduction, *B. cookei* strain LSLP18.3 possesses an ortholog of *MAT-1* that lacks obvious evidence of inactivation associated with RIP mutations, transposon insertion, or other disruptive mutations. The presence of a presumably functional *MAT-1* mating-type locus indicates the potential for heterothallic sexual reproduction in *B. cookei*, which would be consistent with other closely related *Bipolaris* species. Sexual recombination is a central driver of adaptation among many plant pathogenic fungi by creating new combinations of genes and alleles involved in pathogenesis. Byproducts of sexual recombination can also modify genome architecture by inducing genome modifications and rearrangements, such as duplications, inversions, and deletions. In plant pathogenic fungi, the ability to induce changes in gene content and genomic architecture is a key component of rapid adaptive evolution. Mobile genetic elements such as transposons are associated with genomic rearrangements and the creation of novel genes and alleles in many plant pathogenic fungi, and specific selective advantages have been documented to result from such genomic modifications. Similar to the genomes of many other plant pathogenic fungi, the genome of *B. cookei* contains a large number of taxonomically diverse transposable elements. The substantial enrichment of SM backbone genes and SSPs near repetitive elements, compared to a negative association of housekeeping genes and other predicted elements of the core genome, is consistent with the presence of ‘repetitive islands’, in which pathogenicity-associated genes are enriched and isolated from components of the core genome. The dynamic nature of such repetitive islands is associated, in part, through genome defense mechanisms such as RIP mutations. Although RIP presumably evolved as a mechanism for fungi to inactivate duplicated sequences in their genomes, the sequences of single-copy genes in close proximity to repetitive elements can be altered via RIP slippage, thus leading to the generation of novel alleles. Considering the widespread distribution of repetitive elements in the *B. cookei* genome, and their notable proximity to secondary metabolite genes and candidate effectors, repetitive elements are a plausible source of genomic variability and evolution in *B. cookei*. Yet another mechanism of fungal genome diversification is horizontal gene transfer (HGT), in which genetic material is exchanged between reproductively isolated organisms. Circumstantial evidence for horizontal gene transfer in *B. cookei* is provided by the NR-PKS gene *Bc_10041* and the hypothetical gene *Bc_10331*. Homologs of *Bc_10041* and *Bc_10331* were present in *Colletotrichum sublineolum*, yet absent from other sequenced *Bipolaris* species, which is inconsistent with a pattern of vertical inheritance from a common ancestor. Because *B. cookei* and *C. sublineolum* are both pathogens of...
the same host, share overlapping geographical distributions, and have been noted to co-infect individual sorghum leaves, populations of both pathogens have presumably been in more than adequate physical proximity for HGT events to have occurred.

At the current time, whether *B. cookei* utilizes a hemibiotrophic or necrotrophic infection strategy is not fully resolved. Most plant pathogenic members of *Bipolaris* are generally considered to be necrotrophs, although a few hemibiotrophic species are postulated to exist within the genus. When considered in the context of the limited histopathological analyses of *B. cookei* infection, the genome sequencing and analysis presented in this study lends support to the hypothesis that *B. cookei* is a necrotrophic pathogen. In particular, available information about gene content, such as the reduced set of candidate effectors, and gene expression are mostly consistent with a necrotrophic growth habit. The *in planta* expression set examined in this study was derived from an early stage of the infection process (24 hr after inoculation), at which time hemibiotrophs are typically initiating latent (asymptomatic) infections. As such, hemibiotrophic pathogens are thought to rely more heavily on suites of effectors that manipulate host defense reactions at early stages of infection, rather than phytotoxic secondary metabolites. However, genes for secondary metabolite production or detoxification in some hemibiotrophic fungi have been previously reported upregulated at early stages of infection. Furthermore, it is important to note that the distinction between a necrotroph and a hemibiotroph is currently predicated on the existence of a consistent, definable latent phase before the visible onset of necrosis, rather than a specific set of genetic or biochemical criteria. Thus, if the period between inoculation and symptom expression is compressed, as has been reported for *B. cookei*, the potential existence of an unusually short-lived hemibiotrophic phase cannot fully be discounted. Future histopathological experiments utilizing cell biology approaches, in conjunction with extensive transcriptional profiling, will be required to more conclusively define the infection strategy utilized by *B. cookei*.

The known host range of *B. cookei* is limited to certain members of the grass family (Poaceae), specifically species within the genera *Sorghum* and *Zea*. The current study provides a degree of insight into potential mechanisms underlying host specificity. In other fungal pathosystems, host specific toxins (HSTs) are important components of host-specific necrotrophy. The *B. cookei* LSLP18.3 draft genome contains a substantial number of secondary metabolism genes and clusters, some of which have not yet been described in other fungal genome sequencing projects and thus are potentially unique to *B. cookei*. Interestingly, the SM backbone gene Bc_10041 is taxonomically restricted to *B. cookei* among *Bipolaris* genomes sequenced to date, yet an ortholog is present in the genome of the sorghum pathogen *C. sublineolatum*. An intriguing possibility is that one or more metabolites requiring Bc_10041 for their biosynthesis could conceivably function as HSTs on sorghum. The gene expression profiling data analyzed in this study also suggest the potential existence of HSTs in this pathosystem. Among the 37 genes highly expressed *in planta* as opposed to *in vitro*, the majority encoded genes implicated in secondary metabolism, as well as some that appear to be involved in modification or detoxification of host phytoalexins. At this time, it is not clear whether *B. cookei* might utilize a suite of SM-derived HSTs, or if it may instead utilize a limited number of HSTs in conjunction with an arsenal of broad-spectrum necrosis-inducing toxins. Additionally, given that HSTs can be proteins rather than SMs, such as ToxA, the potential contribution of diverse HSTs throughout various stages of the infection process is worthy of further investigation. When considering the potential roles of HSTs in host-specific necrotrophy, much less clear is the potential role of effectors, e.g., pathogen-derived proteins that modulate host defense. It is conceivable that necrotrophs could utilize effectors to manipulate host defense responses in such a way to accelerate cell death, which would be advantageous for pathogen growth. Gene Bc_06468 is particularly interesting as a candidate gene involved in necrotrophic pathogenesis. Although it may have a relatively conventional function such as suppressing host defense responses, it could potentially have a biochemical function related to the induction or maintenance of necrotrophy. However, its function is difficult to infer based on its structure, which contains no conserved domains, or its taxonomic distribution; putative orthologs were found only in related *Bipolaris* spp., *S. turcica*, *P. triticis-repentis*, and species of the *F. oxysporum* complex. Thus, functional characterization of Bc_06468 and other genes underlying pathogenesis will be required to clarify the molecular basis of host-specific necrotrophy in *B. cookei*.

In summary, this work presented a comprehensive analysis of genomic features in *B. cookei* and expression analysis of *B. cookei* genes induced at early stages of infection. The results provided important and novel insights into the molecular basis of target leaf spot on sorghum, and highlighted several candidate pathogenicity-related genes that were taxonomically restricted among *Bipolaris* species. Lastly, the draft genome assembly of *B. cookei* will serve as a valuable genomic resource for functional genomics studies and population genetics in this organism.

**Materials and Methods**

**Fungal strain and culture conditions.** Wild-type strain LSLP18 of *B. cookei* was isolated from a diseased sorghum plant collected at the University of Arkansas Lon Mann Cotton Research Station in Lee County, Arkansas, during the 2009 growing season. The isolate was selected for genome sequencing due to a high level of virulence and stable growth in routine laboratory culture. To confirm Koch’s postulates, the sorghum line BTx623 was grown in a growth chamber maintained at 25 °C under a 12 h photoperiod. A conidial suspension (5 × 10⁶ conidia ml⁻¹) in 0.01% Tween-20 was atomized onto the adaxial surface of sorghum leaves three weeks after planting until just before runoff. Inoculated plants were placed in transparent plastic bags and incubated in a growth chamber at 25 °C and near 100% relative humidity under a 12 h photoperiod. After 48 h, plants were removed from the bags and maintained in a growth chamber at 25 °C under a 12 h photoperiod until symptoms developed. Working cultures were maintained on V8 agar medium. The fungus was stored as colonized agar in 30% (v/v) glycerol at −80 °C. For DNA isolation, a single-spore isolate (LSLP18.3) was derived from LSLP18 and grown in yeast extract peptone dextrose liquid medium for four days at 25 °C with shaking at 150 RPM.

For RNA sequencing, cultures of *B. cookei* were grown on eight different conditions on solidified media plates overlaid with cellophane (V8 juice agar at constant darkness and constant light; 0.2 × strength potato dextrose agar)
DNA and RNA extraction and sequencing. For DNA isolation, strain LSLP18.3 was grown as described above. Tissue was collected by filtration, frozen in liquid nitrogen, and ground in a pre-chilled mortar and pestle containing sterile glass beads (0.5 mm) (Research Products International, Mt. Prospect, IL, USA). Genomic DNA was isolated with a modified cetyltrimethylammonium bromide (CTAB) method71. DNA was further purified with a Qiagen Genomic-tip 500/G column (Qiagen, Germantown, MD, USA) following the manufacturer's recommendations. DNA quality and quantity was determined by agarose gel electrophoresis and a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genome sequencing was performed with a hybrid sequencing approach. Library preparation (fragment size = 500 bp) and sequencing (one lane, paired-end, 100 bp read length) was performed by BGI Americas (Cambridge, MA, USA) with an Illumina HiSeq 2000 Sequencing System (Illumina Inc., San Diego, CA, USA). Additionally, library preparation (library size = 3–10 kb) and sequencing (two SMRT cells) was performed by the Yale Center for Genome Analysis (Orange, CT, USA) with a PacBio RS II Sequencing System (Pacific Biosciences, Menlo Park, CA, USA).

For RNA isolation, strain LSLP18.3 was grown as described above. Tissue was collected, frozen in liquid nitrogen, and ground in a pre-chilled mortar and pestle containing sterile glass beads (0.5 mm) (Research Products International). Total RNA was isolated with a Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's recommendations. DNA quality and quantity was determined by agarose gel electrophoresis and a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Total RNA from each condition was mixed in equal amounts. Pooled total RNA (10 μg) was subjected to mRNA enrichment with a MagIE7 mRNA Enrichment Kit (Thermo Fisher Scientific). Ribosomal RNA-free mRNA was fragmented with RNase III (New England Biolabs, Ipswich, MA, USA) at 37 °C for 5 minutes. First strand cDNA was synthesized from the fragmented mRNA with random hexamers (Integrated DNA Technologies, Inc., Coralville, IA, USA) and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), and second strand synthesis was performed with a NEBNext mRNA Second Strand Synthesis Module (New England Biolabs). End repair was performed on the second strand DNA with a NEBNext End Repair Module (New England Biolabs). Following end repair, sequencing adapters were ligated to the double stranded cDNA with a NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs). Size selection for 480 bp was performed with an Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). A final PCR enrichment step was performed following the recommendations provided with the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent to amplify fragments containing ligated adapters at both ends. The size distribution and quality of the library was determined with an Agilent Tapestation 2200 D1K (Agilent Technologies, Santa Clara, CA, USA). The library was sequenced on an Ion Torrent Personal Genome Machine System with an Ion 318 Chip Kit v2 (Thermo Fisher Scientific).

Genome assembly and repetitive DNA identification. The draft genome of B. cookei was assembled based on a custom hybrid strategy (Supplementary Fig. S1). The Illumina reads were corrected with BayesHammer within SPAdes v3.1 assembler72. Corrected Illumina reads were used to correct the PacBio reads with LoRDEC v0.27. Illumina and PacBio reads were analyzed with SPAdes v3.1, which produced a raw draft assembly. Contigs from the draft assembly were merged into scaffolds with successive iterations of SSPACE-Standard v3.075 and AHA from smrtanalysis suite 2.3 (http://www.pacb.com/support/software-downloads/). Genomic regions containing gaps were filled with GapFiller v1.176 and PBJelly v14.7.1477.

A homology search with genes from the Stagonospora nodorum mitochondrial genome50 identified one assembled scaffold (scaffold75) corresponding to the B. cookei mitochondrial genome. Illumina and PacBio reads were mapped to the B. cookei assembly with Bowtie v2.2.978 and BLASR v1.3.179, respectively, and the reads that mapped to scaffold75 were given to SPAdes to produce a new assembly, which was improved with SSPACE-Standard v3.0. This produced a single contig with overlapping ends.

Repetitive DNA sequences in the B. cookei genome were identified with RepeatScout v1.0.580 using an l-mer value of 13, and filtered with the accessory script filter-stage-1.prl. Repetitive elements that appeared less than 10 times in the genome were filtered out with the accessory script filter-stage-2.prl and RepeatMasker open-4.0.5 (http://www.repeatmasker.org). Annotation of transposable elements was performed with TransposonPSI (http://transposonspsi.sourceforge.net). The Repeat-Induced Point (RIP) mutation index (TpA/ApT) was calculated with the dinucleotide Frequency function within the R package Biostrings v2.44.281 based on a sliding window approach with window size of 300 bp and step size of 50 bp. The distance of genes to repetitive elements was calculated with the function closest within BEDtools v2.2682.

Gene prediction and functional annotation. For gene prediction, RNA sequencing reads were first mapped to the draft genome with GSNAP v2014-10-0983 with the splicing option (-N) enabled. Then, mapped RNA-seq reads were analyzed with Cufflinks v2.2.184 to reconstruct transcripts with default parameters. Gene models were predicted with Maker pipeline v2.32.685 with the reconstructed transcripts from B. cookei as EST evidence, and protein sequences from B. maydis C5 and B. zeicola (http://genome.jgi.doe.gov/programs/fungi/index.jsf) as protein homology evidence. Gene models were selected with the accessory script Maker2zff within the Maker software package with default parameters to train the ab initio predictors SNAP v2006-07-2886 and Augustus v3.0.287. A new set of gene models was predicted with Maker adjusted to use SNAP and Augustus simultaneously with the B. cookei reconstructed transcripts, and the protein sequences from B. maydis C5 and B. zeicola. Gene completeness was assessed with BUSCO software v286 using protein assessment mode (-m protein) and the Ascomycota lineage (-l).
To functionally characterize genes, predicted protein sequences were queried against the NCBI nr database (update 03/2016) with BLAST v2.2.31+ and an e-value < 1e-5. BLAST results were analyzed with Blast2GO v3.0.06 to attribute functional descriptions and GO terms for each protein. Mitochondrial genes were identified and annotated with MFannot (http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl) and MITOS2. Uncharacterized ORFs in the mitochondrial genome were annotated with Blast2GO as described previously.

The mating type of *B. cookei* was determined with queries the homology searching using as queries the mating genes MAT-1 (GenBank accession: CA4A8464) and MAT-2 (GenBank accession: CA4A8465) from *B. maydis*.

Carbohydrate-active enzymes were identified with a local installation of dbCAN v5.093 and HMM v3.1b1 (http://hmm.org). Polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and dimethylallyl transferases (DMTs) were identified with SMURF39, and terpene synthases (TSSs) were identified with antiSMASH v3.0.584. PKSs were queried against the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) for further categorization. PKSs containing a starter unit:ACP transacylase (SAT) domain were classified as non-reducing PKSs, those containing an enoyl reductase (ER) domain were classified as highly reducing PKSs, and those that lacked both SAT and ER domains were classified as partially-reducing PKSs.

To identify putative secreted proteins, all predicted proteins were evaluated for the presence of signal peptides with SignalP v4.195 and TargetP v1.196. Proteins containing a signal peptide as well as two or more transmembrane domains, as determined with TMHMM v2.097, or a glycosylphosphatidylinositol (GPI) anchor, as determined with PredGPI98, were categorized as plasma membrane-associated rather than secreted. The remaining proteins (comprising the putative secretome) were further categorized into secreted proteases, lipases and peroxidases by performing BLAST searches against the MEROPS database v1199, Fungal Peroxidase Database100, and Lipase Engineering Database101, respectively, with a minimum e-value of 1e-5. Secreted proteins classified as effectors according to EffectoR v1.0102 or containing less than 300 amino acids with at least 2% of predicted residues corresponding to cysteine, calculated with EMBOSS package v6.6103, were considered to be candidate effectors.

**Gene expression and comparative genomic analyses.** RNA-seq reads from sorghum leaves infected with *B. cookei* 12h and 24h post-inoculation11 were combined and mapped to the *B. cookei* genome assembly with GSNAP v2014-10-09, and then compared with RNA-seq data obtained from *B. cookei* grown on various defined culture media in vitro. Reads mapped to each predicted ORF were counted with the function coverage within BEDtools v2.26. Genes with more than three in planta RNA-seq reads and zero in vitro RNA-seq reads were considered exclusively expressed in planta. In addition, genes with a ratio of at least 4:1 regarding in planta and in vitro RNA-seq reads, respectively, were also considered induced during sorghum infection.

Comparative genomic analyses with other *Bipolaris* species were performed with the genomes of *B. maydis* (=Cochliobolus heterostrophus) C5, *B. sorokiniana* (=*C. sativus*) ND90Pr, *B. zicola* (=*C. carbonum*) 26-R-13, *B. oryzae* (=*C. miyabeanus*) ATCC 44560, and *B. victoriae* (=*C. victoriae*) FI3104,35, obtained from the JGI website (http://genome.jgi.doe.gov/programs/fungi/index.jsf).

**Data availability.** This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NR5V0000000. The version described in this paper is version NR5V0100000. The complete mitochondrial genome has been deposited at GenBank under accession MF784482. RNA-seq data obtained from culture media conditions have been deposited at NCBI sequence read archive (SRA) under accession SRR5957114.

**References.**

1. Smith, C. W. & Frederiksen, R. A. Sorghum: origins, history, technology, and production. (Wiley, 2000).
2. Dillon, S. L. et al. Domestication to crop improvement: Genetic resources for Sorghum and Saccharum (Andropogonaeae). *Annals of Botany* 100, 975–989 (2007).
3. Undersander, D., Smith, L., Kaminski, A., Kelling, K. & Doll, J. Sorghum-forage. *Alternative field crops manual. Wisconsin and Minnesota Cooperative Extension, Univ. of Wisconsin, Madison, and Univ. of Minnesota, St. Paul* (2004).
4. Undersander, D. & al Sorghum for syrup. *Alternative Field Crops Manual. University of Minnesota Extension Service, Center of Alternative Plant and Animal Products* (1990).
5. Calviño, M. & Messing, J. Sweet sorghum as a model system for bioenergy crops. *Current Opinion in Biotechnology* 23, 323–329 (2012).
6. Pontieri, P. et al. Sorghum, a healthy and gluten-free food for celiac patients as demonstrated by genome, biochemical, and immunological analyses. *J. Agric. Food Chem.* 61, 2565–2571 (2013).
7. Espinoza, L. & Kelley, J. *Grain sorghum production handbook*. (Cooperative Extension Service University of Arkansas, 2004).
8. Frederiksen, R. A. *Compendium of Sorghum Diseases*. (American Phytopathological Society, 2000).
9. Manamgoda, D. S. et al. The genus Bipolaris. *Stud. Mycol.* 79, 221–288 (2014).
10. Lefebvre, C. L. & Sherwin, H. S. An Undescribed Species of Helminthosporium on Sudan Grass and Sorghum. *Mycologia* 40, 708–716 (1948).
11. Odvody, G. N. & Dunkle, L. D. Occurrence of Helminthosporium sorghicola and other minor pathogens of sorghum in Nebraska. *Plant Dis. Report.* 59, 120–122 (1975).
12. Elazegui, F. & Exconde, O. Host-parasite relationship in Helminthosporium leaf spot of sorghum. *Philipl. Agric.* 57, 210–218 (1973).
13. Borges, O. Pathogenicity of Drechslera sorghicola isolates on sorghum in Venezuela. *Plant Dis. 67*, 996–997 (1983).
14. Zummo, N. & Gourley, L. Occurrence of Target Leaf Spot (Bipolaris sorghicola) on Sorghum in Mississippi. *Plant Dis.* 71, 1045 (1987).
15. Condon, B. J. et al. Comparative Genome Structure, Secondary Metabolite, and Effector Coding Capacity across Cochliobolus Pathogens. *PLoS Genet.* 9, e1003233 (2013).
16. Kumar, J. et al. Bipolaris sorokiniana, a cereal pathogen of global concern: cytological and molecular approaches towards better control. *Mol. Plant Pathol.* 3, 185–195 (2002).
17. Mohan, S. M. et al. Co-localization of quantitative trait loci for foliar disease resistance in sorghum. *Plant Breed.* 128, 532–535 (2009).
18. Murali Mohan, S. et al. Identification of quantitative trait loci associated with resistance to foliar diseases in sorghum [Sorghum bicolor (L.) Moench]. *Euphytica* **176**, 199–211 (2010).

19. Kawahigashi, H. et al. Positional cloning of ds1, the target leaf spot resistance gene against Bipolaris sorghicola in sorghum. *Theor. Appl. Genet.* **123**, 131–142 (2011).

20. Kawahigashi, H. et al. Classification of Genotypes of the Target Leaf Spot-Resistant Gene (ds1) in a Sorghum Collection. *Crop Sci.* **51**, 2095–2103 (2011).

21. Yagawa, T., Kawahigashi, H., Matsumoto, T. & Mizuno, H. Simultaneous Transcriptome Analysis of Sorghum and Bipolaris sorghicola by Using RNA-seq in Combination with De Novo Transcriptome Assembly. *PloS One* **8**, e62460 (2013).

22. Mizuno, H. et al. Global transcriptome analysis reveals distinct expression among duplicated genes during sorghum-Bipolaris sorghicola interaction. *BMC Plant Biol.* **12**, 121 (2012).

23. Doak, T. G., Doerder, F. P., Jahn, C. L. & Herrick, G. A proposed superfamily of transposable genes: transposon-like elements in ciliated protozoa and a common 'DDE' motif. *Proc. Natl. Acad. Sci. USA* **91**, 942–946 (1994).

24. Aguileta, G. et al. High Variability of Mitochondrial Gene Order among Fungi. *Genome Biol. Evol.* **6**, 451–464 (2014).

25. Bolton, M. D., Rivera, V. & Secor, G. Identification of the G143A mutation associated with QoI resistance in Cercospora beticola field isolates from Michigan, United States. *Pest Manag. Sci.* **69**, 35–39 (2013).

26. Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M. & Henrissat, B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **42**, (2014).

27. Kuan, C. S. et al. Dissecting the fungal biology of Bipolaris papendorfii from phylogenetic to comparative genomic analysis. *DNA Res.* **22**, 219–232 (2015).

28. Scheller, H. V. & Ulvskov, P. Hemicelluloses. *Annu. Rev. Plant Biol.* **61**, 263–289 (2010).

29. Mewis, K., Lenfant, N., Lombard, V. & Henrissat, B. Dividing the large glycoside hydrolase family 43 into subfamilies: A motivation for detailed enzyme characterization. *Appl. Environ. Microbiol.* **82**, 1866–1862 (2016).

30. Sorensen, A., Lübeck, M., Lübeck, P. & Åhring, B. Fungal Beta-Glucosidases: A Bottleneck in Industrial Use of Lignocellulosic Materials. *Biomolecules* **3**, 612–631 (2013).

31. Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M. & Henrissat, B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Biofuels* **6**, 41 (2013).

32. Agger, J. W. et al. Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. *Proc. Natl. Acad. Sci. USA* **111**, 6287–6292 (2014).

33. Beeson, W. T., Phillips, C. M., Cate, J. H. & Marletta, M. A. Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. *J. Am. Chem. Soc.* **134**, 890–892 (2012).

34. Phillips, C. M., Beeson, W. T., Cate, J. H. & Marletta, M. A. Cellulose Dehydrogenase and a Copper-Dependent Polysaccharide Monooxygenase: Potential Cellulose Degradation by Neurospora. *ACS Chem. Biol.* **6**, 1399–1402 (2011).

35. Ohm, R. A. et al. Diverse Lifestyles and Strategies of Plant Pathogenesis Encoded in the Genomes of Eighteen Dothideomycetes Fungi. *PloS Pathog.* **8**, (2012).

36. Peter, M. et al. Ectomycorrhizal ecology is imprinted in the genome of the dominant symbiotic fungus *Cenococcum geophilum*. *Nat. Commun.* **7**, 12662 (2016).

37. McGrann, G. D. & other et al. The genome of the emerging barley pathogen Ramularia collo-cygni. *BMC Genomics* **17**, 584 (2016).

38. Strobl, M. R., Danchin, E. G. J., Rancurel, C., Coutinho, P. M. & Henrissat, B. Dividing the large glycoside hydrolase family 43 into subfamilies: Towards improved functional annotations of α-amylase-related proteins. *Protein Eng. Des. Sel.* **19**, 555–562 (2006).

39. Khali, N. et al. SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet. Biol.* **47**, 736–741 (2010).

40. Sugawara, E. et al. Phytootoxins from the Pathogenic Fungi *Drechslera maydis* and *Drechslera sorghicola*. *Proc. Natl. Acad. Sci. USA* **84**, 3081–3085 (1987).

41. Penás-Rodríguez, L. & Chilton, W. 3-Anhydroribofuranosin A and 3-anhydro-6-epi-ribofuranosin A, phytoalexin metabolites of the Johnson grass pathogen Bipolaris sorghicola. *J. Nat. Prod.* **52**, 1170–1172 (1989).

42. Au, T. K., Chick, W. S. H. & Leung, P. C. The biology of ophiobolins. *Life Sci.* **67**, 733–742 (2000).

43. Narita, K. et al. Multiple Oxidative Modifications in the Ophiobolin Biosynthesis: P450 Oxidations Found in Genome Mining. *Org. Lett.* **18**, 1980–1983 (2016).

44. Eliash, N., Igbaria, A., Rose, M. S., Horwitz, B. A. & Lev, S. Melanin biosynthesis in the maize pathogen Cochliobolus heterosporus depends on two mitogen-activated protein kinases, Chk1 and Mps1, and the transcription factor Cmr1. *Eukaryot. Cell* **6**, 421–429 (2007).

45. Guyon, K., Balagué, C., Roby, D. & Raffaele, S. Secretome analysis reveals effector candidates associated with broad host range necrotrophy in the fungal plant pathogen *Sclerotinia sclerotiorum*. *BMC Genomics* **15**, 336 (2014).

46. Yoshida, Y. et al. Chloroplastic Divide by Contraction of a Bundle of Nanofilaments Consisting of Polyglycan. *Science* **(80-)**. **329**, 949–953 (2010).

47. Fuji, I., Yoshida, N., Shimomaki, S., Oikawa, H. & Ebizuka, Y. An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. *Chem. Biol.* **19**, 1579–1584 (2012).

48. Zhang, G. R. et al. Multiple Oxidative Modifications in the Ophiobolin Biosynthesis: P450 Oxidations Found in Genome Mining. *Org. Lett.* **18**, 736–741 (2010).

49. Zhang, G. R., Newman, M. A. & Bradley, C. A. First Report of the Soybean Fungus *Fusarium avenaceum* Resistant to Quinone Outside Inhibitor Fungi in North America. *Plant Dis.* **96**, 767–767 (2012).

50. Nelson, R. Heterothallism in *Helminthosporium maydis*. *Phytopathology* **47**, 191–192 (1957).

51. Nelson, R. Cochliobolus carbonum, the perfect stage of *Helminthosporium carthorum*. *Phytopathology* **49**, 12 (1959).

52. Nelson, R. *Cochliobolus victoriae*, the perfect stage of *Rhizoctonia solani*. *Phytopathology* **50**, 10 (1960).

53. Burdon, J. J. & Silk, J. Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology* **87**, 664–669 (1997).

54. Kistler, H. C. & Miao, V. P. W. New modes of genetic change in filamentous fungi. *Annu. Rev. Phytopathol.* **30**, 131–152 (1992).

55. Stuntenbrock, E. H. & Croll, D. The evolving fungal genome. *Fungal Biology Reviews* **28**, 1–12 (2014).

56. Raffaele, S. & Kamoun, S. Genome evolution in filamentous plant pathogens: why bigger can be better. *Nat. Rev. Microbiol.* **10**, 417–430 (2012).

57. Selker, E. U. Premiceotic instability of repeated sequences in Neurospora crassa. *Annu. Rev. Genet.* **24**, 579–613 (1990).

58. van de Wouw, A. P. et al. Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PloS Pathog.* **6**, (2010).

59. Soanes, D. & Richards, T. A. Horizontal gene transfer in eukaryotic plant pathogens. *Annu. Rev. Phytopathol.* **52**, 583–614 (2014).
65. O’Connell, J. R. et al. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. *Nat. Genet.* **44**, 1060–1065 (2012).

66. Palma-Guerrero, J. et al. Comparative transcriptomic analyses of *Zyromospora triitici* strains show complex lifestyle transitions and intraspecific variability in transcription profiles. *Mol. Plant Pathol.* **17**, 845–859 (2016).

67. Horbach, R., Navarro-Quesada, A. R., Knooge, W. & Deising, H. B. When and how to kill a plant cell: Infection strategies of plant pathogenic fungi. *Journal of Plant Physiology* **168**, 51–62 (2011).

68. Wolpert, T. J., Dunkle, L. D. & Ciufletta, L. M. Host-selective toxins and avirulence determinants: what’s in a name? *Annu. Rev. Phytopathol.* **40**, 251–285 (2002).

69. Walton, J. D. Host-selective toxins: agents of compatibility. *Plant Cell* **8**, 1723–1733 (1996).

70. Ballance, G. M., Lamari, L. & Bernier, C. C. Purification and characterization of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. *Physiol. Mol. Plant Pathol.* **35**, 203–213 (1989).

71. Leslie, J. F. & Summerville, B. A. The Fusarium laboratory manual. (Blackwell, 2006).

72. Flaherty, I. E., Pirttula, A. M., Bluhm, B. H. & Woloshuk, C. P. PAC1, a pH-regulatory gene from *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **69**, 5222–5227 (2003).

73. Bankevich, A. et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **19**, 453–477 (2012).

74. Salmela, L. & Rivals, E. LoRD: efficient and accurate long read error correction. *Bioinformatics* **30**, 3506–3514 (2014).

75. Boetzer, M., Henkel, C. V., Jansen, H. J., Butler, D. & Pirovano, W. Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* **27**, 578–579 (2011).

76. Boetzer, M. et al. Toward almost closed genomes with GapFiller. *Genome Biol.* **13**, R56 (2012).

77. English, A. C. et al. Mind the Gap: Upgrading Genomes with Pacific Biosciences RS Long-Read Sequencing Technology. *PLoS One* **7**, e47768 (2012).

78. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Bioinformatics* **21**, 35–40 (2015).

79. Chaisson, M. J. & Tesler, G. Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. *BMC Bioinformatics* **13**, 258 (2012).

80. Price, A. L., Jones, N. C. & Pevzner, P. A. De novo identification of repeat families in large genomes. *Bioinformatics* **21**, 351–358 (2005).

81. Pagès, H., Gentleman, R., Abouyoun, P. & DebRoy, S. Biostrings: String objects representing biological sequences, and matching algorithms. *R Pacakge, version 2.4.2* (2017).

82. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841–842 (2010).

83. Wu, T. D. & Watanabe, C. K. GMAP: A genomic mapping and alignment program for mRNA and EST sequences. *Bioinformatics* **21**, 1859–1875 (2005).

84. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–5 (2010).

85. Cantarel, B. L. et al. MAKER: An easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Res.* **18**, 188–196 (2008).

86. Korf, I. Gene finding in novel genomes. *BMC Bioinformatics* **5**, 59 (2004).

87. Stanke, M. & Waack, S. Gene prediction with a hidden Markov model and a new intron submodel. *BMC Bioinformatics* **19**, ii215–ii225 (2018).

88. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol Biol.* **238**, 403–410 (1994).

89. Novák, P. et al. AntiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* **40**, W445–W451 (2012).

90. Weber, T. et al. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* **43**, W237–W243 (2015).

91. Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786 (2011).

92. Emanuelsson, H., Nielsen, S. B. & von, G. H. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005–1016 (2000).

93. Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567–580 (2001).

94. Pellegrini, A., Martelli, P. & Casadio, R. PredGPI: a GPI-anchor predictor. *BMC Bioinformatics* **9**, 392 (2008).

95. Rawlings, N. D., Waller, M., Barrett, A. J. & Bateman, A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* **42**, D503–D509 (2014).

96. Choi, J. et al. PacBio: fungal peroxidase database for comparative genomics. *BMC Microbiol.* **14**, 117 (2014).

97. Eisen, J. A. & Plesy, J. The Lipase Engineering Database: A navigation and analysis tool for protein families. *Nucleic Acids Res.* **31**, 319–321 (2003).

98. Schlotterer, C. et al. EffectorP: Predicting fungal effector proteins from secretomes using machine learning. *New Phyto* **210**, 743–761 (2016).

99. Rice, P., Longden, I. & Bleasby, A. EMBOS: The European Molecular Biology Open Software Suite. *Trends Genet.* **16**, 276–277 (2000).

100. Margolin, B. S. et al. A methylated neurospora SS RNA pseudogene contains a transposable element inactivated by repeat-induced point mutation. *Genetics* **149**, 1787–1797 (1998).

**Acknowledgements**

We thank John B. Ridenour for preparing DNA for sequencing, and Sandeep Sharma for preparing and sequencing RNA. We also thank Braham D. S. Dhillon and Marcio L. Zaccaron for helpful suggestions. We are grateful to BGI Americas (Cambridge, MA, USA) as the Illumina sequencing provider, and Yale Center for Genome Analysis (Orange, CT, USA) as the PacBio sequencing provider. This material is based upon work that is supported by the Arkansas Corn and Grain Sorghum Promotion Board. Additionally, this work was supported in part by the USDA National Institute of Food and Agriculture, Hatch project 1008895.
Author Contributions
A.Z.Z. performed data analyses, interpreted data, and wrote the manuscript. B.H.B. conceptualized the experiments, interpreted data, and wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-17476-x.

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017