High-quality genome assembly of the soybean fungal pathogen Cercospora kikuchii

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Abstract

Plant diseases caused by the Cercospora genus of ascomycete fungi are a major concern for commercial agricultural practices. Several Cercospora species can affect soybeans, such as Cercospora kikuchii which causes soybean leaf blight. Speciation in Cercospora on soybean has not been adequately studied. Some cryptic groups of Cercospora also cause diseases on soybean. Moreover, it has been known C. kikuchii population genetic structure is different between countries. Consequently, further genomic information could help to elucidate the covert differentiation of Cercospora diseases in soybean. Here, we report for the first time, a chromosome-level genome assembly for C. kikuchii. The genome assembly of 9 contigs was 34.44 Mb and the N50 was 4.19 Mb. Based on ab initio gene prediction, several candidates for pathogenicity-related genes, including 242 genes for putative effectors, 55 secondary metabolite gene clusters, and 399 carbohydrate-active enzyme genes were identified. The genome sequence and the features described in this study provide a solid foundation for comparative and evolutionary genomic analysis for Cercospora species that cause soybean diseases worldwide.

Keywords: Cercospora kikuchii; soybean; Cercospora leaf blight; purple seed stain

Introduction

Cercospora is one of the major groups of plant pathogenic fungi and Cercospora spp. can cause necrotic leaf spots on many different species of plants (Groenewald et al. 2013). Cercospora kikuchii (Tak. Matsumoto & Tomoy.) M. W. Gardner is a soybean pathogen, identified in eastern Asia in the early 20th century (Suzuki 1921; Matsumoto and Tomoyasu 1925; Walters 1980). It causes two types of pathogenic symptoms in soybean: purple seed stain (PSS) on seed pods and seeds, and Cercospora leaf blight (CLB) on leaves and petioles. Both symptoms present a typical dark-purple-colored lesion. This is caused by cercosporin, a pigment produced by the pathogen. The pigment induces cell death of the host plant in conditions with light (Kuyama and Tamura 1957; Yamazaki et al. 1975; Daub and Hangarter 1983).

Symptoms caused by C. kikuchii are frequently observed in soybean fields. Currently, CLB is one of the biggest problems for soybean production in many areas of South America, such as Argentina (Wrather et al. 2010). It has been suggested that the C. kikuchii populations in South America and Japan are different, based on phylogenetic analysis (Imazaki et al. 2006). It has also been reported that other Cercospora species such as C. cf. flagellalis, C. cf. sigesbeckiae (Albu et al. 2016), and C. cf. nicotianae (Sautua et al. 2020) can also cause soybean leaf blight. Moreover, some cryptic species of Cercospora infect soybean (Soares et al. 2015). Phylogenetic studies of Cercospora species, including CLB pathogens, have been reported (Groenewald et al. 2013; Soares et al. 2015). Such investigations will significantly benefit from additional high-quality genomic resources.

The genomes of the Cercospora spp. such as C. zeae-maydis (causal agent of gray leaf spot on corn, Haridas et al. 2020) and C. beticola (causal agent of leaf spot on sugar beet and other plant species, Vaghefi et al. 2017) have been analyzed and deposited in public databases. Additionally, genome sequences of the species infecting soybean have been published, such as Cercospora sojina (frogeye leaf spot, Luo et al. 2018; Gu et al. 2020) and Cercospora cf. sigesbeckiae (leaf blight, Albu et al. 2017). For C. kikuchii, two genomes have recently been deposited. These genomes were obtained from isolates originating from the United States (isolate A3, NCBI GenBank accession: GCA_005356855.1, contig N50: 364,948 bp) and Argentina (isolate ARG_18_001, NCBI GenBank accession: GCA_009193115.1, Sautua et al. 2019, contig N50: 675,846 bp), but have low contiguity.

The aim of this study was to obtain a reference genome sequence for C. kikuchii for further comparative genomic studies of the Cercospora species. The Japanese C. kikuchii isolate MAFF 305040 was sequenced. The isolate was subjected to species-wide phylogenetic study of the genus Cercospora in the previous study (Groenewald et al. 2013). Sufficient depth of sequencing coverage by long-read sequences generated a chromosome-level assembly. This high-quality genome sequence data provide fundamental genomic information and deepen our understanding of the pathogen.
Materials and methods

Fungal strain and culture conditions

*Cercospora kikuchii* MAFF 305040 was provided by the National Agriculture and Food Research Organization (NARO) Genebank (Tsukuba, Ibaraki, Japan). The isolate was maintained and cultured by following the method (Kashiwa et al. 2021).

DNA extraction from the fungus

Mycelia were collected from 3- to 4-day-old liquid cultures and dehydrated on paper towels. Collected mycelia (approximately 1 g) was ground in liquid nitrogen and extracted with 10 mL of CTAB lysis buffer (100 mM Tris-HCl, 2% CTAB, 1.4 M NaCl, 10 mM EDTA) at 65°C for 1 h. DNA was purified using a double extraction by mixing for 15 min with an equal volume of chloroform: isoamylalcohol mixture (24:1) and then centrifuged at 8,370 × g for 10 min. The DNA was precipitated with ethanol and then centrifuged at 20,400 × g for 5 min at 4°C. The pellet was washed with 70% ethanol and dried. The DNA was resuspended in 10 mM Tris-HCl solution and stored at −30°C prior to genome sequencing.

Genome sequencing and data manipulation

The genome DNA library was prepared and subjected to sequencing by PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA). RNA removal from the DNA solution, DNA library preparation, and sequencing was performed by Macrogen Japan (Tokyo, Japan). Statistics for the sequence data was calculated by SeqKit 0.12.1 (Shen et al. 2016). The obtained sequence data were assembled by Canu 1.9 (Koren et al. 2017). Default values for Canu were kept for the assembly step. The estimated genome size was set to 34 Mb. Assembled sequences were polished by Arrow 2.3.3 (Pacific Biosciences). The polished assembly was checked by Tapestry 1.0.0 (Davey et al. 2021) with default values. The telomeric repeat TTAGGG (Cohn et al. 2016) was used for the detection of telomeric repeats. The software Tapestry is also used for the evaluation of read depth and GC content of each contigs.

The Dfam TE Tools 1.4 for Docker was used for soft masking the genome based on *de novo* repeat finding by RepeatModeler (Flynn et al. 2020). The container incorporating the programs obtained from http://www.repeatmasker.org/ (RepeatModeler, RepeatMasker, coseg, RMBlast, RepeatScout, and RECON) and other tools such as HMMER (http://hmmer.org/), CD-HIT (Fu et al. 2005) was for the detection of transposable elements. The obtained sequence data were assem-

Results and discussion

Assembly and completeness of the genome sequence

Genome sequencing generated more than 3.5 Gb from 3 SMRT cells of the PacBio RS II. Read length N50 was approximately 14 kb. The polished genome assembly for MAFF 305040 consisted of 15 contigs comprising approximately 34.55 Mb. GC content of the polished assembly was 53.0%. Based on the size of contigs, we obtained nine large contigs (2.36–5.86 Mb) and six small contigs (1.2–54.3 kb). To filter contigs, we used two criteria based on Tapestry results: contig GC content ranges 53 ± 10%; and median of read depth for contig is above 20. After filtering, six small contigs were excluded. Telomeric repeats were detected on both ends for eight contigs out of the nine selected contigs. This suggested that the eight contigs were close-to-complete chromosomes. The nine contigs comprising approximately 34.44 Mb were subjected to downstream analysis (Table 1). GC content of the nine contigs was 53.0% (Table 1).

As a result of repeat masking, approximately 1.45 Mb (4.21% of total sequence) was masked. Among the masked regions, 119 LINEs, 426 LTR elements, and 92 DNA transposons were detected.

Recently, genome sequences of soybean pathogens were deposited in public databases, and some of their assemblies produced chromosome-level contigs. Compared to *C. sojina* (12 chromosomes comprising approximately 40.12 Mb, NCBI GenBank accession: GCA_004299825.1, Gu et al. 2020), the genome assembly generated by the pipeline of this study consisted of nine total contigs and showed a similar range in size as compared to the genome of *C. sojina* Race 15 (Gu et al. 2020, NCBI GenBank accession: GCA_004299825.1, Gu et al. 2020).

| Features                        | Value             |
|--------------------------------|-------------------|
| Isolate name                   | MAFF 305040       |
| Culture collection             | NARO Genebank, Japan |
| Origin of isolate             | Kagoshima, Japan  |
| Assembly size (bp)             | 34,440,063        |
| Number of contigs              | 9                 |
| Maximum contig length (bp)     | 5,855,908         |
| N50 of contigs                 | 4,190,438         |
| GC content (%)                 | 53.0              |
| BUSCO completeness (%)         | 99.4              |
| Predicted genes                | 13,001            |
| Genome accession               | BOLY00000000      |

Table 1 Summary of the genome assembly

Notes:

- Data obtained from NARO Genebank (https://www.gene.affrc.go.jp, accessed on August 31, 2020).
- DDBJ/EMBL/GenBank accession number for the assembled contigs.
of C. kikuchii MAFF 305040 was found to be somewhat smaller, while that of C. cf. sigesbeckiae (approximately 34.94 Mb, NCBI GenBank accession: GCA_002217505.1, Albu et al. 2017) was of a similar size. However, consideration should be given to the fragmentation of the C. cf. sigesbeckiae genome (1,945 contigs) for size comparison.

A total of 13,001 genes were predicted by Augustus that was trained by the BRAKER2 pipeline. The pipeline used a fungal gene ortholog database, and Augustus was trained by the proteins from any evolutionary distance for gene prediction. Based on the predicted coding protein sequences, the completeness of the genome assembly was estimated to be 99.4% by BUSCO (Table 1). In the 3,786 groups, there were only 21 missing and 4 fragmented BUSCOs for MAFF 305040.

Pathogenicity-related genomic features and comparisons to C. sojina, one of the soybean pathogen of genus Cercospora

To obtain information about the pathogenicity-related genes in the genome, several programs were used for functional annotation. Effectors are an important feature of plant pathogenic fungi, as they suppress host immunity. Effector proteins possess several characteristics, such as being small, secreted, and they have a high proportion of cysteine residues (Siperschneider et al. 2018). The DeepSig program estimated that a total of 1,393 proteins harbored an N-terminus signal peptide sequence for secretion. From the predicted secretome proteins, the EffectorP 2.0 program identified 245 proteins as effector candidates. The number of the genes for effector candidates was 242 (Table 2) after removing duplicated predictions for two transcripts from one gene.

In some plant pathogenic fungi, specific genomic compartments can function as toolboxes for effector genes. For example, in Fusarium oxysporum, effector genes such as SIX (Secreted In Xylem) are enriched in small dispensable chromosomes (approximately 2 Mb). Transmission of the chromosomes between the isolates enables the acquisition of pathogenicity to nonpathogens (Ma et al. 2010). For the genome of C. kikuchii MAFF 305040, the effector candidate genes were distributed among the nine contigs (Figure 1B). However, the frequency of the genes for effector candidates was different among the contigs. For the largest contig_00001, the frequency of the genes for effector candidates in 100 kb of nucleotide sequences was 0.60 (35 genes in 5,855,908 bp). Meanwhile, the frequency was more than double (1.23, 29 genes in 2,357,730 bp) for contig_00009. It is assumed that the benefit of effector-enriched chromosomes is a facile adaptation to environmental changes (Croll and McDonald 2012). Namely, conditionally dispensable small chromosomes fulfill the role of the toolbox for pathogenicity-related factors and accelerate changes in the pathogenicity by altering the content and/or expression profile of the effector genes on the chromosomes (Seidl et al. 2016). It is uncertain whether such genomic regions or chromosomes are in the C. kikuchii genome. Further studies of the gene composition of each chromosome and the virulence functions of the predicted effector candidates are necessary to define the properties of chromosomes.

SM gene clusters were also predicted in the nine contigs (Figure 1C, Table 2). A total of 55 gene clusters were predicted from the genome (Table 2). Numbers of each metabolite types from the cluster are also listed in Table 2. For instance, 15 polyketides, 22 nonribosomal peptides, and 6 terpenes were predicted. A cluster harboring multiple core biosynthetic genes was grouped into “others” in Table 2.

Carbohydrate-active enzymes (CAZymes) are also important for fungi, for successful infections of their plant hosts. CAZymes are used to degrade plant polysaccharides to obtain nutrients and to enhance the infection in the host (Zhao et al. 2014). A total of 399 genes encoding CAZymes were predicted by dbCAN and classified into six categories (Figures 1D and 2, Table 2). Some of the proteins were matched multiple categories. Among these, 220 genes were categorized as glycoside hydrolase (GH), 86 as glycosyltransferase (GT), 10 as carbohydrate-binding module (CBM), 66 as auxiliary activity (AA), 19 as carbohydrate esterase (CE), and 7 as polysaccharide lyase (PL).

The genomic composition of CAZymes is key to understanding the infection strategy of plant pathogenic fungi (Zhao et al. 2014; Hane et al. 2020). As described, different species of the genus Cercospora can utilize soybeans as their hosts. To understand their difference, their CAZyme profiles were compared. The CAZymes in the C. sojina Race 15 genome were also checked for this purpose. A total of 13,253 genes were predicted from 12 chromosomes of Race 15 by BRAKER2-trained Augustus. Then, the dBCAN meta server selected 359 genes as having CAZyme encoding sequences. This number is higher than in previous C. sojina genome analyses (number of CAZyme genes was 340, Gu et al. 2020). Interestingly, the number of genes encoding CAZymes in the C. kikuchii genome was greater than in C. sojina (Figure 2). As described, C. kikuchii has a smaller genome (34.44 Mb) than C. sojina (40.12 Mb, Gu et al. 2020). For instance, the number of genes coding for the GH family enzyme was different between the two genomes. The genome of C. kikuchii has some additional genes coding GH (Figure 2 and Table 3). It is known that some enzymes belonging to the GH family are critical for the infection of the host plant. For example, PsGh7a (GH7) contributes for virulence in soybean oomycete pathogen Phytophthora sojae (Tan et al. 2020). Based on the previous report (Zhao et al. 2014), 61 genes from C. kikuchii MAFF 305040 were grouped into plant cell wall (PCW in Table 3) as substrate of the encoding enzyme. For C. sojina Race 15, 53 genes were grouped into PCW (Table 3). Functional analysis of their pathogenicity-related factors, such as CAZymes, is needed for further study.

Table 2 Candidates for pathogenicity-related genes and gene clusters

| Features | Number |
|----------|--------|
| Effectors candidates | 242 |
| SM gene clusters | 55 |
| Polyketide | 15 |
| Nonribosomal peptide | 22 |
| Terpene | 6 |
| Beta-lactone | 1 |
| Siderophore | 2 |
| Fungal-RiPP | 1 |
| Others | 8 |
| CAZymes | 399 |
| GH | 220 |
| GT | 86 |
| CBM | 10 |
| AA | 66 |
| CE | 19 |
| PL | 7 |

* Genes encoding effector candidates predicted by EffectorP
* Numbers of SM gene clusters for each metabolite type predicted by antiSMASH. Fungal-RiPP, fungal ribosomally synthesized and post-translationally modified peptide
* Genes encoding carbohydrate-activate enzymes (CAZymes) predicted by dbCAN. Some genes were annotated with more than one category. GH, glycoside hydrolase; GT, glycosyltransferase; CBM, carbohydrate-binding module; AA, auxiliary activity; CE, carbohydrate esterase; and, PL, polysaccharide lyase

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**References**

- Albu et al. 2017
- Croll and McDonald 2012
- Seidl et al. 2016
- Ma et al. 2010
- Zhao et al. 2014
- Hane et al. 2020
- Gu et al. 2020
- Tan et al. 2020
As described, several *Cercospora* species, including cryptic groups, are known to cause diseases in soybeans. The ability to elucidate the relationships between these pathogens provides a strong incentive to define their diversity and classification. This is the first chromosome-level assembly for *C. kikuchii*, causal agent of CLB and PSS. Our highly contiguous genome assembly of *C. kikuchii* and the results obtained in this study provide a solid foundation for genome analysis of the genus *Cercospora*.

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**Data availability**

The data described herein have been deposited at DDBJ/EMBL/GenBank under the BioProject accession PRJDB10872. The BioSample accession number for MAFF 305040 is SAMD00262043. The sequencing reads have been deposited under the accession number DRX296098. The genome assembly has been deposited under accession number BOLY0000000.
### Table 3 Number of genes related to GH families identified from C. kikuchii MAFF 305040 and C. sojina Race 15

| Family | MAFF 305040 | Race 15 | Substrate*<sup>a</sup> |
|--------|-------------|---------|------------------------|
| GH64   | 4           | 4       | CW (β-1,3-glucan)      |
| GH1    | 3           | 3       | CW (β-glycans)         |
| GH2    | 6           | 6       | CW (β-glycans)         |
| GH3*   | 17          | 16      | CW (β-glycans)         |
| GH5*   | 14          | 13      | CW (β-glycans)         |
| GH32   | 3           | 3       | ESR (sucrose/insulin)  |
| GH37   | 2           | 2       | ESR (trehalose)        |
| GH65   | 1           | 1       | ESR (trehalose)        |
| GH15*  | 2           | 1       | ESR (α-glucans)        |
| GH30   | 2           | 2       | FCW                    |
| GH45   | 1           | 1       | FCW                    |
| GH16*  | 5           | 5       | FCW (chitin)           |
| GH20   | 1           | 1       | FCW (chitin)           |
| GH76   | 9           | 9       | FCW (chitin)           |
| GH17   | 4           | 5       | FCW (β-1,3-glucan)     |
| GH52   | 5           | 5       | FCW (β-1,3-glucan)     |
| GH71   | 2           | 2       | FCW (β-1,3-glucan)     |
| GH72   | 6           | 6       | FCW (β-1,3-glucan)     |
| GH81   | 1           | 1       | FCW (β-1,3-glucan)     |
| GH16*  | 11          | 9       | FCW (β-glycans)        |
| GH13   | 15          | 15      | FCW + ESR (α-glucans)  |
| GH7    | 1           | 1       | PCW (cellulose)        |
| GH12   | 1           | 1       | PCW (cellulose)        |
| GH10*  | 4           | 3       | PCW (hemicellulose)    |
| GH11   | 3           | 3       | PCW (hemicellulose)    |
| GH27   | 2           | 3       | PCW (hemicellulose)    |
| GH29*  | 3           | 2       | PCW (hemicellulose)    |
| GH35*  | 2           | 2       | PCW (hemicellulose)    |
| GH36*  | 2           | 2       | PCW (hemicellulose)    |
| GH39   | 0           | 1       | PCW (hemicellulose)    |
| GH51   | 1           | 1       | PCW (hemicellulose)    |
| GH54   | 1           | 1       | PCW (hemicellulose)    |
| GH62   | 1           | 1       | PCW (hemicellulose)    |
| GH67   | 1           | 1       | PCW (hemicellulose)    |
| GH93*  | 1           | 0       | PCW (hemicellulose)    |
| GH115  | 1           | 2       | PCW (hemicellulose)    |
| GH43   | 12          | 10      | PCW (pectin + hemicellulose) |
| GH28   | 5           | 5       | PCW (pectin)           |
| GH78*  | 2           | 2       | PCW (pectin)           |
| GH88   | 2           | 1       | PCW (pectin)           |
| GH105  | 3           | 3       | PCW (pectin)           |
| GH38   | 1           | 1       | PG (N-/O-glycans)      |
| GH47   | 9           | 9       | PG (N-/O-glycans)      |
| GH63   | 1           | 1       | PG (N-glycans)         |
| GH125  | 3           | 3       | PG (N-glycans)         |
| GH31   | 9           | 8       | PG + ESR + PCW (hemicellulose) |
| GH33   | 1           | 1       | NA                     |
| GH42   | 1           | 1       | NA                     |
| GH79   | 3           | 4       | NA                     |
| GH92   | 7           | 7       | NA                     |
| GH95*  | 2           | 0       | NA                     |
| GH97*  | 1           | 0       | NA                     |
| GH106  | 1           | 1       | NA                     |
| GH114  | 1           | 1       | NA                     |
| GH127  | 1           | 1       | NA                     |
| GH128* | 2           | 1       | NA                     |
| GH130  | 1           | 1       | NA                     |
| GH131  | 1           | 1       | NA                     |
| GH132  | 1           | 1       | NA                     |
| GH135  | 1           | 2       | NA                     |
| GH139* | 1           | 0       | NA                     |
| GH141* | 1           | 0       | NA                     |
| GH142  | 1           | 1       | NA                     |
| GH152  | 1           | 1       | NA                     |
| GH154* | 2           | 1       | NA                     |

* Asterisk indicates that the number of genes identified from C. kikuchii MAFF 305040 was greater than the number identified from C. sojina Race 15.

**Information on the substrate was obtained from Zhao et al. (2014). CW, cell wall; ESR, energy storage and recovery; FCW, fungal cell wall; PCW, plant cell wall; PG, protein glycosylation; NA, not assigned.**

### Author contributions
T.K. conceived, designed, and performed the experiments. T.K. and T.S. analyzed the genome sequence. T.K. wrote the manuscript in consultation with T.S. All authors have approved the manuscript.

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### Conflicts of interest
None declared.

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