The wheat powdery mildew genome shows the unique evolution of an obligate biotroph

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Wheat powdery mildew, *Blumeria graminis forma specialis tritici*, is a devastating fungal pathogen with a poorly understood evolutionary history. Here we report the draft genome sequence of wheat powdery mildew, the resequencing of three additional isolates from different geographic regions and comparative analyses with the barley powdery mildew genome. Our comparative genomic analyses identified 602 candidate effector genes, with many showing evidence of positive selection. We characterize patterns of genetic diversity and suggest that mildew genomes are mosaics of ancient haplogroups that existed before wheat domestication. The patterns of diversity in modern isolates suggest that there was no pronounced loss of genetic diversity upon formation of the new host bread wheat 10,000 years ago. We conclude that the ready adaptation of *B. graminis* f.sp. *tritici* to the new host species was based on a diverse haplotype pool that provided great genetic potential for pathogen variation.

The onset of agriculture and the domestication of crops approximately 10,000 years ago resulted in drastic changes to plant pathogen environments. The genetically uniform agricultural ecosystems led either to rapid coevolution of the pathogen with its host during domestication (host tracking) or to the emergence of new pathogen species through host jump, host shift or hybridization1–3. For pathogen domestication (host tracking) or to the emergence of new pathogen species was accompanied by pronounced chromosomal changes and loss of genetic diversity1,7 (Supplementary Note). Powdery mildews are obligate biotrophic fungi that grow and reproduce only on living hosts. The accompanying disease occurs early in summer when haploid spores infect plants and asexually reproduce4. Sexual reproduction of isolates of opposite mating types in late summer results in the formation of overwintering chasmothecia (Supplementary Note). Cereal powdery mildew *B. graminis* has evolved into at least eight *formae specialis* that each specifically infect one host species5. It is assumed that the pathogen uses an arsenal of effector proteins to infect the host6–8. If such an effector is recognized by the plant, it renders the pathogen avirulent, and the effector gene becomes an avirulence gene9,10. This process allows selection for either effector changes or loss. It is postulated that the specific makeup of effectors determines the virulence spectrum of a particular mildew strain7,11–13 (Supplementary Note). Here we wanted to study genetic diversity between and within powdery mildew *formae specialis* and explore the impact of the introduction of the new host (bread wheat) on *B. graminis* f.sp. *tritici* evolution (Supplementary Note).

The reference genome sequence of *B. graminis* f.sp. *tritici* isolate 96224 consists of a backbone of 250 BAC contigs to which Roche 454 sequence scaffolds were anchored (Supplementary Table 1 and Supplementary Note). Having 250 large BAC contigs allowed the analysis of genome organization at the megabase level. In total, 82 Mb of the estimated 180-Mb genome could be assembled because many highly repetitive sequences were collapsed or removed from the assembly (Supplementary Note). We annotated 6,540 genes; however, over 90% of the genome was classified as transposable element (TE) sequences (Supplementary Note), making this the most repetitive fungal genome sequenced so far. Most of the gene space was covered, as 96% of the eukaryotic core genes were full length, and 98% were partially present (CEGMA evaluation; Supplementary Note). In comparison to non-obligate biotrophs, many gene families involved in primary and secondary metabolism were reduced or absent as in other obligate biotrophs7,14–16 (Supplementary Figs. 1–3 and Supplementary Note). Fewer than 50% of the genes had homologs in yeast. In the more closely related *Botrytis cinerea*, 72% of genes (4,731) had homologs. Almost 92% of the predicted

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Comparison of 5,258 bidirectionally most closely related B. graminis f.sp. tritici and B. graminis f.sp. hordei homologs. The x axis indicates the ratio of nonsynonymous to synonymous substitutions (dN/dS) for all gene pairs, and the y axis indicates the number of gene pairs in each class. The red series represents the 237 gene pairs of bidirectionally most closely related B. graminis f.sp. tritici and B. graminis f.sp. hordei homologs encoding CSEPs, and the blue series represents all 5,021 other gene pairs. For better visibility, the numbers for non-CSEP genes were divided by 10.

As in a previous study\textsuperscript{17}, we found gene order to be largely conserved between B. graminis f.sp. hordei and B. graminis f.sp. tritici, indicating that these two formae specialiae have very similar overall gene content and that there are a large number of genes that are specific to the Blumeria genus. Of these Blumeria-specific genes, 437 encoded candidate secreted effector proteins (CSEPs; Supplementary Table 2 and Supplementary Note).

On the basis of substitutions in synonymous sites of the 5,258 bidirectionally most closely related B. graminis f.sp. tritici and B. graminis f.sp. hordei homologs, we estimated that B. graminis f.sp. tritici and B. graminis f.sp. hordei diverged 6.3 ± 1.1 million years ago (Supplementary Note). This finding narrows down previous estimates, which ranged from 4.7 to 10 million years ago\textsuperscript{5,17}, and indicates that the two formae specialiae diverged several million years ago, after the divergence of their hosts 10–15 million years ago\textsuperscript{18,19}. As in a previous study\textsuperscript{17}, we found gene order to be largely conserved between B. graminis f.sp. tritici and B. graminis f.sp. hordei, whereas intergenic sequences were divergent owing to TE insertions and deletions (Supplementary Fig. 4 and Supplementary Note).

Of the 5,258 B. graminis f.sp. tritici and B. graminis f.sp. hordei gene pairs, 96.6% had a ratio of nonsynonymous-to-synonymous substitutions (dN/dS) of less than 0.5 (average of 0.24). In contrast, CSEPs showed much higher dN/dS ratios, with an average of 0.8, suggesting that they might be under divergifying selection (Fig. 1). Indeed, 55 of the 77 CSEP genes on which McDonald-Kreitman-like tests could be performed showed a positive direction of selection, which means that these genes are under selection pressure to evolve rapidly (Supplementary Note). This comparison of B. graminis f.sp. tritici and B. graminis f.sp. hordei genes allowed us to identify 165 novel genes that had no homologs in other fungi and lacked a sequence encoding a signal peptide but had a dN/dS ratio greater than 0.5. We propose that these genes may encode candidate effector proteins (CEPs; Supplementary Note) that are either non-secreted or secreted by non-conventional pathways\textsuperscript{20}. Taking CSEP and CEP genes together, B. graminis f.sp. tritici has 602 putative effector genes, comprising 9.2% of its total gene complement (Supplementary Note). Post-infection transcriptome analysis showed expression of 99% of all CSEP and CEP genes, further supporting their potential involvement in the host-pathogen interaction.

In addition to the reference genome of isolate 96224 (collected in 1996 in Switzerland), we sequenced isolate JIW2 (collected in 1980 in England), isolate 70 (collected in 1990 in Israel) and isolate 94202 (collected in 1994 in Switzerland) (Supplementary Note). Sequencing of these isolates allowed us to sample genetic diversity of the wheat powdery mildew gene pool in different geographic regions (from the UK and Israel) as well as within the same country (Switzerland).

The gene content of the four B. graminis f.sp. tritici isolates was almost completely identical. Besides expected differences in the mating type locus (Supplementary Fig. 5 and Supplementary Note), we identified 537 large deletions (>500 bp) in the 3 additional isolates. In 16 cases, these deletions led to presence-absence gene polymorphisms (Table 1). Notably, 13 of the 16 deleted genes were effector candidates. Considering that CEP and CSEP genes constitute only 9.2% of the gene content, they were highly over-represented in these presence-absence polymorphisms. CSEP analogs were described in fungal pathogens of humans and animals\textsuperscript{21,22}, but specific loss of such genes has, to our knowledge, not been reported. It is possible that loss of CEP and/or CSEP genes reflects selective pressure resulting from breeding for pathogen resistance, which, unlike in animals and humans, is a normal process in crop plants (Supplementary Note). The 16 affected genes were lost in deletions ranging from 0.6 to 44 kb in size. Highly diagnosable sequence motifs, such as perfect or near-perfect direct repeats immediately flanking the deletion breakpoints, indicate that gene loss is the result of double-strand break repair, similar to what was described in grasses such as rice and Brachypodium\textsuperscript{23,24} (Fig. 2a and Supplementary Note). One notable additional polymorphism was found in the Bgt-E-5692 gene, where a highly variable sequence fragment was probably introduced in a gene conversion event (Fig. 2b and Supplementary Fig. 6). Sampling of 6 additional isolates

### Table 1. Presence-absence polymorphisms of genes in the three B. graminis f.sp. tritici isolates JIW2, 94202 and 70 compared to reference isolate 96224

| Gene      | JIW2\(\bullet\) | 94202\(\bullet\) | 70\(\bullet\) | Deletion | Gene product                        |
|-----------|-----------------|-----------------|-------------|----------|-------------------------------------|
| Bgt-3306  | −               | −               | +           | >100 kb  | Mating type (Map1-2-I)              |
| Bgt-2805  | −               | −               | +           | >100 kb  | Mating type (SLA-1)                 |
| Bgt-E-5545| −               | +               | +           | 44 kb    | CSEP                                |
| Bgt-E-5597| −               | −               | −           | 25 kb    | CSEP                                |
| Bgt-E-5802| −               | −               | −           | 25 kb    | CSEP                                |
| Bgt-E-5845| −               | +               | +           | 13 kb    | CSEP                                |
| Bgt-E-5419| +               | −               | +           | 8 kb     | CSEP                                |
| Bgt-E-3419| +               | −               | +           | 6 kb     | CSEP                                |
| BgtAc-3046| +               | +               | −           | 5.3 bp   | CSEP                                |
| BgtAc-31249| +             | −               | +           | 15 kb    | CSEP                                |
| BgtAcSP-30824| +               | +               | −           | 4.5 kb   | CSEP                                |
| Bgt-E-40100| +              | +               | −           | 1.3 kb   | CSEP                                |
| BgtA-21525| −               | −               | −           | 0.6 kb   | CEP                                 |
| BgtA-20784| +               | −               | −           | 9.4 kb   | CEP                                 |
| Bgt-369   | −               | +               | +           | 13 kb    | Peptidyl-prolyl isomerase           |
| BgtAc-31336| +             | +               | −           | 0.8 kb   | \textit{ab initio}; no homolog     |
| BgtA-20381| +               | −               | +           | 2.3 kb   | \textit{ab initio}; no homolog     |

\(\bullet\)Presence or absence of a gene is indicated with plus and minus signs, respectively. The two genes Bgt-E-5802 and Bgt-E-5597 are paralogs that were deleted in the same event. The gene model originates from \textit{ab initio} gene prediction.
showed no correlation between the presence-absence of these genes and the geographic origin of the isolates (Supplementary Table 3 and Supplementary Note).

The three resequenced isolates differed at 113,967 to 161,117 SNPs from the 96224 reference sequence, with the Israeli isolate 70 being the most divergent. Small insertions and deletions of 1 to 4 bp were almost 100 times less frequent than SNPs (Supplementary Table 4 and Supplementary Note). Between 3.7 and 3.9% of the SNPs were found in the coding sequences of genes, and roughly 45% of these SNPs were nonsynonymous. For 57% of the genes, the predicted protein was identical. In 30% of all genes, we identified two protein variants, and 10% of genes had three different protein variants and 3% of genes four different protein variants (Supplementary Table 5). Candidate effector genes had more nonsynonymous substitutions than the average for all genes, indicating that they are under stronger diversifying selection, even within the same forma specialis (Supplementary Fig. 7 and Supplementary Note).

We observed that the SNP frequency in all isolates varied strongly in different regions of the genome compared to the reference sequence. For example, in isolate JIW2, approximately 25% of the genome consisted of large segments that were nearly identical to the 96224 reference genome (0.11 SNPs/kb). These regions were distinct from regions with an approximately 10 times higher SNP frequency (Fig. 2c, d, Supplementary Table 6 and Supplementary Note). This finding suggests that the isolates studied are mosaics of different haplogroups (chromosomal segments that are more closely related by descent than others). The average size of haplogroup segments ranged from 87.3 kb in isolate JIW2 to 150 kb in isolate 70. On the basis of the number of substitutions in the different haplogroup segments, we could distinguish two distinct groups representing more divergent haplogroups (H_{old}) and less divergent ones (H_{young}) (Fig. 3a). In approximately 40% of the genome, we could distinguish three different H_{old} haplogroups, whereas in about 25% of the genome four different H_{old} haplogroups were present. All four isolates shared the H_{young} haplogroup in only
2.2% of the genome. The \( H_{\text{old}} \) haplogroups diverged approximately 43,000 to 76,000 years ago from the 96224 reference. In contrast, \( H_{\text{young}} \) haplogroups diverged only approximately 2,100 to 8,600 years ago (for isolates JIW2 and 94202) and 5,600 to 11,700 years ago (for isolate 70) from the 96224 reference (Fig. 3a and Supplementary Table 7).

Notably, the divergence of the \( H_{\text{old}} \) haplogroups coincided with the last ice age (150,000–10,000 years ago), during which time it is assumed that wheat ancestors were restricted to the Fertile Crescent, which stretches from modern-day Israel to Iran\(^5\). We hypothesize that different \( B. graminis \) f.sp. tritici lineages (H1, H2 and H3 in the model in Fig. 3b) diverged by coevolving with different ancestral wheat populations in geographically separated areas and that the descendants of this diversification are represented in today’s \( H_{\text{old}} \) haplogroup segments (Fig. 3b). In contrast, the \( H_{\text{young}} \) haplogroups diverged within the time period after agriculture was introduced. We speculate that northbound agricultural migration approximately 10,000 years ago could have restricted genetic exchange between European and Israeli \( B. graminis \) f.sp. tritici lineages. This hypothesis would explain why the youngest haplogroup segments shared by these isolates were fewer and diverged 8,700 (±3,000) years ago, whereas the European isolates shared haplogroups that diverged more recently (Fig. 3b and Supplementary Table 7).

The large haplogroup segments indicate that the mildew isolates studied are descended from relatively few sexual recombination events and have since reproduced mainly clonally. \( B. graminis \) has very high sexual recombination rates (Supplementary Note). Thus, unrestricted mating of different \( B. graminis \) f.sp. tritici isolates would have completely homogenized SNP frequencies across the genomes and led to very low linkage disequilibrium (Supplementary Note). In contrast, our observations were consistent with clonal or near-clonal reproduction (for example, through inbreeding in small populations; Supplementary Note), which is key in pathogens, as it preserves successful combinations of genes and avoids the acquisition of undesirable avirulence genes\(^26\–28\). We conclude that the distinct haplogroup patterns in the \( B. graminis \) f.sp. tritici isolates reflect strong selection for clonal propagation and/or inbreeding (Supplementary Note). A similar mechanism was suggested recently for barley powdery mildew\(^29\).

The \( Blumeria \) genus shows unique evolutionary properties in that it has maintained high levels of adaptability and flexibility. The genomes of \( B. graminis \) f.sp. tritici isolates are composed of haplogroup segments that predate the formation of their hexaploid bread wheat host 10,000 years ago\(^30\). Thus, the shift from wild tetraploid to domesticated hexaploid wheat seemingly has not reduced genetic diversity in \( B. graminis \) f.sp. tritici (Supplementary Note), suggesting that the \( B. graminis \) f.sp. tritici gene pool provided all the necessary genetic diversity for adaption to a range of wheat species. This ability for adaptation is also demonstrated by its recent host range expansion.
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Phytophthora (Supplementary Note) and Mycosphaerella
genera, host changes were accompanied by the rapid formation of
new species and loss of genetic diversity. Indeed, the youngest
two Mycosphaerella species probably date back merely 10,000 and
500 years (Supplementary Note). Similarly, in Magnaporthe oryzae,
possibly as few as three genes determine host specificity and incom-
patibility (Supplementary Note). This scenario differs notably from
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maintain their ability to infect wild tetraploid wheat, even though
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ing. Thus, the formation of reproductive barriers as a consequence
of adaptation to new hosts might be detrimental to the lifestyle and
evolutionary success of mildew.

METHODS

Methods and any associated references are available in the online
version of the paper.

Accession codes. The genomes of the four powdery mildew isolates
96224, 94202, JIW2 and 70 have been deposited at the DNA Data Bank
of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL)
and GenBank under accessions ANZE01000000, ASJK01000000,
ASJL01000000 and ASJD01000000, respectively.

Note: Any Supplementary Information and Source Data files are available in the
online version of the paper.

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AUTHOR CONTRIBUTIONS

B.K., T.W. and K.K.S. designed the project. S.O., T.W., J.P.B., M.S., T.P. and
S.R. designed software and analyzed the genome sequence. R.B. performed
sequence assembly and pipeline development. F.P. and R.B.-D. conducted
the crossing experiments and sequencing. F.P., R.B.-D. and B.K. wrote the
manuscript. S.O., T.W. and K.K.S. commented on results and edited the manuscript. S.O., T.W. and B.K. wrote the manuscript and supplementary information and prepared the figures.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Roche 454 and Illumina genome sequencing. Genomic DNA from isolate 96224 was sequenced with Roche 454 Titanium technology at the Functional Genomics Center of the University of Zurich (Switzerland) to approximately 13× coverage using single-fragment (2.5 million reads, 900 Mb) and 3-kb insert (5 million reads, 1.653 Mb) paired-end libraries. Illumina sequencing was performed by GATC Biotech (Konstanz, Germany; isolates 96224 and JIW2) and The Genome Analysis Centre (Norwich, UK; isolates 94202 and 70). From each isolate, 5 µg of DNA was sequenced from paired-end libraries with insert sizes of 350–450 bp in length. Isolates 96224 and JIW2 were sequenced to approximately 24-fold coverage, and isolates 94202 and 70 were sequenced to approximately 50- to 70-fold coverage (Supplementary Fig. 8).

Production of the reference genome sequence of B. graminis f.sp. tritici isolate 96224. Quality-trimmed 454 reads were combined with 20,000 BAC end sequences33 and assembled using Roche's Newbler assembler (version 2.5; default parameters, minimum overlap identity of 99%, minimum overlap length of 50 bp). Reference genome sequences were generated by integrating the scaffolds from the 454 assembly into a BAC library fingerprint assembly, which consisted of 266 contigs (called FP contigs) with a total size of 180 Mb (ref. 33). BAC end sequences of BAGs present in the FP contigs were used as linker sequences between 454 scaffolds and FP contigs. Scaffolds were used as queries in BLAST searches against a database of all BAC end sequences. To avoid random anchoring of scaffolds to repetitive DNA in BAC end sequences, we used different stringency levels (from very stringent to less stringent) for the BLAST searches. Sequence space between anchored scaffolds was filled with strings of N bases (representing any nucleotide) of a length estimated on the basis of the FP contigs. The BAC end sequences of 16 short FP contigs were all repetitive and could therefore not be used to anchor any 454 scaffolds.

Illumina sequences from isolate 96224 were used to correct the reference sequence for 454-specific sequencing errors. About 47.9 million reads (2 runs on the same 350-bp insert paired-end library, read size of 96 bp, 4.3 and 4.6 Gb of sequence data) were quality trimmed and aligned to the reference using CLC Assembly Cell version 3.2 (CLC bio) using the program clc_ref_assemble_long with parameters -s 0.98 -l 0.95. Nucleotide differences that were present in all aligned Illumina reads and had minimal coverage of 2x were accepted as sequencing errors and were corrected in the reference sequence accordingly.

Gene annotation. Gene prediction in the B. graminis f.sp. tritici sequence was performed using two approaches. Genes conserved between B. graminis f.sp. tritici and B. graminis f.sp. hordei were identified by mapping the published B. graminis f.sp. hordei genes7 onto the B. graminis f.sp. tritici sequence using GMAP4. The recently sequenced B. graminis f.sp. hordei genome contains 5,854 annotated genes8. Before mapping, the B. graminis f.sp. hordei gene set was carefully searched for sequences with homology to TE or TR-related sequences (for example, EKA homologs)7 by running BLAST searches of all B. graminis f.sp. hordei genes against an updated version of our Blumeria repeat database33, which currently contains the predicted protein sequences of 74 TE families. On the basis of this analysis, 124 B. graminis f.sp. hordei genes were removed from the original B. graminis f.sp. hordei gene set. The remaining 5,730 B. graminis f.sp. hordei genes were mapped to the B. graminis f.sp. tritici genome using GMAP, which resulted in the annotation of 5,398 B. graminis f.sp. tritici gene models. Subsequently, the identified gene models and TEs were masked on the scaffolds of the 454 assembly. Augustus gene prediction software36 was run on the masked sequences after it was trained on 3,143 coding sequences of identified B. graminis f.sp. tritici genes. Ab initio gene models that had homology to TEs in our repeat library were discarded, and the remaining models were mapped to the draft genome. In a final step, the structure and location of all genes, including the ab initio models, were visualized on the draft genome using IGV (Integrative Genomics Viewer; see URLs) for manual curation.

To assign functions to gene models, we performed gene ontology (GO) analysis with Blast2Go software36 with the entire gene set using default settings. In addition, we performed a BLAST search of the protein sequences against the Pfam database and Botrytis cinerea genes (Broad Institute; see URLs). BLAST searches were performed with the BLASTALL program from NCBI (see URLs) on local Linux servers with local databases. For all analyses, BLAST hits with E values smaller than 1 × 10−10 were considered significant. We combined all the information available to provide detailed annotation in the definition line of each gene in the fasta file. These BLAST hit cutoffs were also used when gene families were determined (Supplementary Tables 8 and 9 and Supplementary Note).

CEGMA (Core Eukaryotic Genes Mapping Approach; see URLs)37 evaluation was run on the 454 scaffolds using CEGMA version v2.4.0.10312. CEGMA uses a reference set of conserved protein families that occur in a wide range of eukaryotes. The degree to which the gene set of a genome covers the CEGMA reference set is a measure of how completely the gene space of the genome is covered. Gene annotation was performed in the same way on the de novo assemblies of the three resequenced powdery mildew isolates (Supplementary Table 10).

Transcriptome sequencing. RNA was extracted from wheat leaves infected with B. graminis f.sp. tritici at 4, 8, 12, 24 and 48 h after infection. Equal amounts of RNA from each time point were mixed and sequenced with Illumina sequencing technology. About 1,109 million reads (50-bp read length) that represented fungal and wheat RNA from all 5 time points were pooled and mapped to the genome of isolate 96224. Mapping was performed with CLC Genomics Workbench version 6.0.1, thereby allowing only one mismatch per read and counting only reads that mapped to exons. A total of 7,442,144 reads (0.6%) could be mapped to the powdery mildew genome. This proportion was expected because most of the extracted RNA comes from wheat. For each gene, the number of reads per gene and the average coverage (total reads in base pairs divided by exon length in basepairs) was calculated to obtain a rough estimate of the overall expression level.

Evaluation of gene prediction using transcriptome data. The quality of annotation was evaluated with the exon discovery function of CLC used under default settings. We used a set of 4,000 genes to which at least 50 mRNA reads per kilobase of coding sequence were mapped. For each gene, we counted the number of reads that covered predicted exon-intron boundaries (thus contradicting the predicted exon-intron structure) and compared this number with the number of reads that mapped in exons or connected predicted exons. A low background number of reads that cover predicted exon-intron boundaries is expected owing to incorrectly or unprocessed mRNA. However, a high number of such reads indicates incorrect annotation (or alternative splicing). For more than 75% of the genes tested, fewer than 2% of transcriptome reads contradicted the predicted exon-intron structure, and, for 90% of genes, fewer than 6% of reads fell into this category. Furthermore, the 4,000 genes tested comprised 10,782 exons. Transcriptome data indicated the presence of only 45 additional exons. These data indicate high accuracy in gene prediction.

dN/dS analysis and tests for direction of selection. The aligned coding sequences of the bidirectionally most closely related homologs of B. graminis f.sp. tritici and B. graminis f.sp. hordei (Supplementary Note) were processed with the yn00 program of the PAML package38 (see URLs). yn00 implements the method of Yang and Nielsen39 for estimating synonymous and nonsynonymous substitution rates. For each of the gene pairs, the dS rate (synonymous substitutions per synonymous site) and dN rate (nonsynonymous substitutions per nonsynonymous site) was calculated. dN/dS ratios were assessed separately for the 5,021 non-CSEP and 237 CSEP gene pairs to test whether the group of CSEP genes showed characteristics of positive selection.

The dS values of all non-CSEP and CSEP genes were compared to test whether some of the bidirectionally most closely related homologs might represent deep paralogs. The distribution of dS values in the 5,021 non-CSEP alignments was used as a reference with which the dS values of CSEP genes were compared (Supplementary Fig. 9). We used the dN/dS ratio as a new criterion to identify previously unknown classes of candidate effector genes. We chose the cutoff of 0.5 for the dN/dS ratio for the following reasons. First, 96.6% of the non-CSEP genes had dN/dS values smaller than 0.5. Second, the dN/dS ratio distribution of CSEP genes had its peak at 0.5 (Fig. 1). Therefore, this value can be viewed as the expected dN/dS ratio of a given candidate effector. Third, the average dN/dS value of CSEP genes was 0.8, whereas the average dN/dS value of non-CSEP genes was 0.24; the average between the two was 0.52.
McDonald-Kreitman–like tests were employed to estimate the proportion of adaptive substitutions and the direction of selection in CSEP genes. We selected those bidirectional B. graminis f.sp. tritici and B. graminis f.sp. hordei CSEP homologs for which we had complete sequences for all four B. graminis f.sp. tritici isolates (Supplementary Tables 11 and 12).

**Divergence time estimate of B. graminis f.sp. tritici and B. graminis f.sp. hordei.** To estimate the divergence time of B. graminis f.sp. tritici and B. graminis f.sp. hordei, we used synonymous sites in the coding sequences of the bidirectionally most closely related homologs. We used only alignment positions corresponding to the third base of codons for Ala, Gly, Leu, Pro, Arg, Ser, Thr and Val. For Leu, Arg and Ser (which each have six possible codons), we used only the codons starting with CT, TC and CG, respectively. These are the codons in which the third base can be exchanged without causing an amino acid change. We concatenated all the synonymous sites into one alignment and applied the 1.3 × 10⁻⁸ substitutions per site per year to obtain a single estimate for the whole genome. The error estimate of 2.29 × 10⁻⁹ for the substitution rate was then applied to the calculated divergence time. The Kimura two-parameter criterion was applied to weight the transition-to-transversion ratio as previously described.

**Automated identification of B. graminis f.sp. tritici haplogroup segments.** Positions of all SNPs in the three resequenced isolates were mapped to the 96224 reference genome sequence and visualized with an in-house Perl script (Fig. 2). The genomes of the three resequenced isolates are mosaics of segments that are nearly identical to the 96224 reference sequence (referred to as H young) and regions that have roughly five- to tenfold higher SNP density (referred to as H old). The Kimura two-parameter criterion was applied to weight the transition-to-transversion ratio as previously described.

SNP distribution was surveyed in 20-kb sliding windows across the genome. Because the genome sequence contains large gaps caused by sequence scaffolds that were anchored on opposite ends of a BAC, sequence gaps larger than 2,000 bp were excised from the genome sequence and replaced by stretches of 200 N bases for the analysis. Using a 20-kb sliding window, gaps of 2,000 bp or less influenced SNP density by 10% at most. Because SNP densities of the different haplogroups differed roughly by a factor of ten, the different haplogroups could still be clearly distinguished. However, one has to be aware that large sequence gaps could sometimes contain additional haplogroup breakpoints that would be missed in this analysis.

This analysis was performed on the 128 largest FP contigs that contained at least 200 kb of sequence without gaps (10 times the size of the sliding window). The resulting SNP density distribution was an overlay of the densities of the SNP-rich and SNP-poor regions. For all three resequenced isolates, density in SNP-rich regions peaked at approximately 22 SNPs per 20 kb (1.1 SNPs/kb; SNP-rich and SNP-poor regions). For all three resequenced isolates, density in the different haplogroups could still be clearly distinguished. However, one has to be aware that large sequence gaps could sometimes contain additional haplogroup breakpoints that would be missed in this analysis.

The identification of the different haplogroup segments was automated as follows. SNP distribution was surveyed in 20-kb sliding windows across the genome. Because the genome sequence contains large gaps caused by sequence scaffolds that were anchored on opposite ends of a BAC, sequence gaps larger than 2,000 bp were excised from the genome sequence and replaced by stretches of 200 N bases for the analysis. Using a 20-kb sliding window, gaps of 2,000 bp or less influenced SNP density by 10% at most. Because SNP densities of the different haplogroups differed roughly by a factor of ten, the different haplogroups could still be clearly distinguished. However, one has to be aware that large sequence gaps could sometimes contain additional haplogroup breakpoints that would be missed in this analysis.

The resulting SNP density distribution was an overlay of the densities of the SNP-rich and SNP-poor regions. For all three resequenced isolates, density in SNP-rich regions peaked at approximately 22 SNPs per 20 kb (1.1 SNPs/kb; example in Supplementary Fig. 10). For simplicity, we divided the genome into segments with average SNP densities of 22 SNPs per 20 kb or higher (H young) and segments with a lower SNP density. To determine a suitable cutoff between SNP-rich and SNP-poor regions, we processed individually for each of the 250 FP contigs. For example, FP contig Bgt_crg_2 had a size of 898 kb of non-N bases. In isolate JW2, this contig contained six segments that corresponded to haplogroup H old. These 6 segments added up to 527 kb (59% of the FP contig), and they contained a total of 729 substitutions. From these numbers, two estimates for the divergence time of the H old haplogroup from the 96224 isolate were derived: one with a substitution rate of 1.071 × 10⁻⁹ and one with a substitution rate of 1.529 × 10⁻⁹. This was done to factor in the error in the substitution rate. In this case, two estimates are calculated of 43,800 and 62,600 years, respectively. The distribution of the individual divergence estimates for all FP contigs was used to calculate the overall standard deviation of the age estimate of the respective haplogroup. The variance was calculated as the square of the sum of all the segments assigned to haplogroups H old and H young were used for molecular dating. For dating, genes and the 1-kb regions up- and downstream of them were removed to avoid sequences that are under selection pressure. For the calculation of divergence times, we used the same synonymous substitution rate described above (1.3 × 10⁻⁸ ± 2.29 × 10⁻⁹ substitutions per site per year). To obtain an estimate for variance and standard deviation, haplogroup data were processed individually for each of the 250 FP contigs. For example, FP contig Bgt_crg_2 had a size of 898 kb of non-N bases. In isolate JW2, this contig contained six segments that corresponded to haplogroup H old. These 6 segments added up to 527 kb (59% of the FP contig), and they contained a total of 729 substitutions. From these numbers, two estimates for the divergence time of the H old haplogroup from the 96224 isolate were derived: one with a substitution rate of 1.071 × 10⁻⁹ and one with a substitution rate of 1.529 × 10⁻⁹. This was done to factor in the error in the substitution rate. In this case, two estimates are calculated of 43,800 and 62,600 years, respectively. The distribution of the individual divergence estimates for all FP contigs was used to calculate the overall standard deviation of the age estimate of the respective haplogroup. The variance was calculated as the square of the sum of all the differences from the average (Σ(Xᵢ–X̄)²)/N). The standard deviation was the square root of the variance.

**Molecular dating of B. graminis f.sp. tritici haplogroups.** The genomic segments assigned to haplogroups H old and H young were used for molecular dating. For dating, genes and the 1-kb regions up- and downstream of them were removed to avoid sequences that are under selection pressure. For the calculation of divergence times, we used the same synonymous substitution rate described above (1.3 × 10⁻⁸ ± 2.29 × 10⁻⁹ substitutions per site per year). To obtain an estimate for variance and standard deviation, haplogroup data were processed individually for each of the 250 FP contigs. For example, FP contig Bgt_crg_2 had a size of 898 kb of non-N bases. In isolate JW2, this contig contained six segments that corresponded to haplogroup H old. These 6 segments added up to 527 kb (59% of the FP contig), and they contained a total of 729 substitutions. From these numbers, two estimates for the divergence time of the H old haplogroup from the 96224 isolate were derived: one with a substitution rate of 1.071 × 10⁻⁹ and one with a substitution rate of 1.529 × 10⁻⁹. This was done to factor in the error in the substitution rate. In this case, two estimates are calculated of 43,800 and 62,600 years, respectively. The distribution of the individual divergence estimates for all FP contigs was used to calculate the overall standard deviation of the age estimate of the respective haplogroup. The variance was calculated as the square of the sum of all the differences from the average (Σ(Xᵢ–X̄)²)/N). The standard deviation was the square root of the variance.

All Perl programs used in this study are available upon request.

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