Comparative Analysis Highlights Variable Genome Content of Wheat Ruts and Divergence of the Mating Loci

Christina A. Cuomo,* 1 Guus Bakkeren,† 1 Hala Badr Khalil,† Vinay Panwar,‡ 2 David Joly,‡ 3 Rob Linning,† Sharadha Sakthikumar,* Xiao Song,† Xian Adiconis,* Lin Fan,† Jonathan M. Goldberg,* Joshua Z. Levin,* Sarah Young,* Qiandong Zeng,* Yehoshua Anikster,§ Myron Bruce,** Meinan Wang,†† Chuntao Yin,†† Brent McCallum,§ Les J. Szabo,†† Scot Hultberg,†† Xianming Chen,§§ and John P. Fellers** 1

*Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, 1 Agriculture and Agri-Food Canada, Summerland Research and Development Centre, British Columbia, V0H 1Z0, Canada, 2 Agriculture and Agri-Food Canada, Morden Research and Development Centre, Manitoba, R6M 1Y5, Canada, 3 The Institute for Cereal Crops Improvement, Tel Aviv University, Ramat Aviv 69978, Israel, 4 Department of Plant Pathology, Hard Winter Wheat Genetics Research Unit, United States Department of Agriculture-Agricultural Research Service, Pullman, Washington 99164, 5 Department of Plant Pathology, Washington State University and 6 Wheat Health, Genetics, and Quality Research Unit, United States Department of Agriculture-Agricultural Research Service, St. Paul, Minnesota 55108

ORCID IDs: 0000-0002-5778-960X (C.A.C.); 0000-0002-3065-6989 (G.B.); 0000-0003-3872-2909 (J.P.F.)

ABSTRACT Three members of the Puccinia genus, Puccinia triticina (Pt), P. striiformis f.sp. tritici (Pst), and P. graminis f.sp. tritici (Pgt), cause the most common and often most significant foliar diseases of wheat. While similar in biology and life cycle, each species is uniquely adapted and specialized. The genomes of Pt and Pst were sequenced and compared to that of Pgt to identify common and distinguishing gene content, to determine gene variation among wheat rust pathogens, other rust fungi, and basidiomycetes, and to identify genes of significance for infection. Pt had the largest genome of the three, estimated at 135 Mb with expansion due to mobile elements and repeats encompassing 50.9% of contig bases; in comparison, repeats occupy 31.5% for Pst and 36.5% for Pgt. We find all three genomes are highly heterozygous, with Pst (5.97 single nucleotide polymorphisms (SNPs)/kb) nearly twice the level detected in Pt (2.57 SNPs/kb) and that previously reported for Pgt. Of 1358 predicted effectors in Pt, 784 were found expressed across diverse life cycle stages including the sexual stage. Comparison to related fungi highlighted the expansion of gene families involved in transcriptional regulation and nucleotide binding, protein modification, and carbohydrate degradation enzymes. Two allelic homodomain pairs, HD1 and HD2, were identified in each dikaryotic Puccinia species along with three pheromone receptor (STE3) mating-type genes, two of which are likely representing allelic specificities. The HD proteins were active in a heterologous Ustilago maydis mating assay and host-induced gene silencing (HIGS) of the HD and STE3 alleles reduced wheat host infection.

KEYWORDS Puccinia genome comparisons effectors mating-type genes sexual stage

Rust fungi have the most complex life cycles among described fungi, with many stages having discrete morphologies and very distinguishable sexual and asexual propagation. For many rust fungi, these stages occur on two different, unrelated host plants requiring two different infection strategies (heteroecious). For many, such as the cereal rust fungi, the asexual stage can successfully propagate and lead to epidemics as long as the host is present. When this host becomes senescent, the fungus produces the more resilient teliospores allowing for the sexual cycle to occur (Mendgen 1984). In homoeocious rust fungi, like flax rust [Melampsora lini (Ehrenb.) Lév.], both stages evolved on the same host (autoecious). In heteroecious rust fungi, such as the cereal rust pathogens, major host jumps occurred through evolution of the asexual (uredinial) stage to infect a different host (Savile 1976). These complex interactions result in the production of up to five different rust spore types, requiring very discrete developmental programs, resulting in altered gene expression profiles (Huang et al. 2011; Xu et al. 2011; Upadhyaya et al. 2014).

The obligate biotrophic lifestyle of wheat rust pathogens hampers the ability to culture the fungus in vitro and thus limits biological studies.
Genetic studies by crossing individual strains is not trivial, but not impossible, due to the difficulty of breaking teliospore dormancy in order to infect the alternate hosts (Samborski and Dyck 1968; Rodriguez-Algaba et al. 2014). Most of what is known about wheat rust pathogen biology is based on extensive cytology (Bushnell and Roelfs 1984) and isolate interactions with host resistance genes. Many interactions between rust fungi and their cereal hosts have been shown to genetically conform to the gene-for-gene theory (Flor 1942; Loegering, and Powers 1962). The majority of wheat rust resistance genes (McIntosh et al. 1995) have been shown to be dominant or semidominant (Statter 1979, 1982, 2000), and current models imply an interaction between the resistance gene products and fungal effectors (Sperschneider et al. 2013; Petre et al. 2014).

Wheat leaf rust, caused by *Puccinia triticina* Eriks. (*Pt*), is the most commonly occurring and economically important cereal rust disease worldwide (Singh et al. 2002; Huerta-Espino et al. 2011). Leaf rust on wheat was first recognized as different from stem rust in 1718 (De Candolle 1815), included into a species complex (*P. rubigo-vera*), and taxonomic refinements resulted in the current classification based on differences in spore morphology and alternate host range (Eriksson 1899; Savile 1984; Anikster et al. 1997). *Pt* is an obligate biotroph that can complete its sexual cycle on either of two known alternate host species, *Thalictrum speciosissimum* Loefl. (meadow rue; Jackson and Mains 1921; Saari et al. 1968) or *Isopyrum* (Brizgalova 1935, 1937). The complete *Pt* cycle consists of five spore stages (Bolton et al. 2008). The urediniospore is the most common and is asexual and polycyclic. At maturity when leaves begin to senesce, the fungus will form black teliospores on the abaxial side of the leaf. Within the teliospores, karyogamy takes place and promycelia are formed when the teliospores germinate. Four haploid basidiospores, in which the mating types have been identified as *Pga* and *Pgs* (D’Sa 2004) and *Psf* (Chen et al. 2011), though single gene pairs are predicted in *Pst* (Statter 1979, 1982, 2000), and current models imply an interaction between the resistance gene products and fungal effectors (Sperschneider et al. 2013; Petre et al. 2014). Four haploid basidiospores, in which the mating types have segregated, are formed and infect the alternate host, forming the pycnidium. dikaryotization occurs through fusion between a receptive flexible hyphae and a pycnidiospore of a different mating type. After fusion, the dikaryotic state is reestablished and an aecium will form on the underside of the leaf from which aeciospores will be released and travel to the wheat host. After landing on wheat leaves, the aeciospore will germinate forming an appressorium over a stoma. The haustorial mother cell forms in the substomatal cavity and attaches to the host cell wall. The plant cell wall is breached between 24 and 48 hr, forming haustoria. The fungus will spread intercellularly and a urediniospore is formed at 7 d, from which urediniospores are produced to complete the life cycle. Wheat suffers from two other major rust diseases: stem rust, caused by *P. graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. (Pgt; Leonard and Szabo 2005), and stripe rust, caused by *P. striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*; Chen et al. 2014) with very similar biology and spore stages, except *Pgt* and *Pst* have *Berberis* spp. as an alternate host (Statter 1979, 1982, 2000; lin et al. 2010).

Rust fungi belong to the subphylum Pucciniomycotina that together with the Ustilaginomycotina, the true smut fungi, and the Agaricomycotina, which include mushroom-forming species, make up the phylum Basidiomycota. In this phylum, the sexual cycle typically requires cell-cell fusion governed by both pheromone (*mfa*) and pheromone receptors (*STE3*) genes, which then allows the formation of heterodimERIC transcription factors c0DEd for by two classes of homeodomain-containing protein genes, *HD1* and *HD2* (Raudaskoski and Køthe 2010; Køes et al. 2011). In the corn smut fungus *Ustilago maydis*, the mating-type locus contains both the pheromone receptor gene *pra* (the *STE3* equivalent) and a pheromone precursor gene *mfa* (Brefoet al. 2009).

In all basidiomycetes studied to date, heterodimeric HD-containing transcription factors have been implicated in the mating process. They are found in pairs of genes each encoding subunits of an HD1 and HD2-containing protein that are divergently transcribed. Originally found with their start sites within 1 kb in *Um*, many variations exist and, in mushrooms, multiple pairs are often found in arrays of linked diverged copies (Casselson and Køes 2007; Raudaskoski and Køthe 2010), though single gene pairs are predicted in *Pleurotus djamor* (James et al. 2004) and *Pholiot a nameko* (Yi et al. 2009). Supplemental Material, Figure S1 illustrates various mating-type genes and their organization in a few species of basidiomycete fungi. To date, the mating loci of the wheat rusts have not been carefully analyzed.

As for other obligate pathogens, genome sequencing of rust fungi has advanced the basic understanding of these organisms, which are otherwise recalcitrant to laboratory study. Initial molecular analyses and phylogenetic data indicated that, within each lineage of these three rust pathogens, adaptation to the wheat host had occurred independently (Zambino and Szabo 1993). Genome differences were identified in EST sequencing studies, where it was shown that 40% of *Pst* ESTs did not have a match to *Pgt* and *Pst* (Xu et al. 2011). Comparison of contigs from BAC and genome sequencing have shown synteny between the three genomes; however, there are regions of gene insertions, expansion by mobile elements, and inversions (Cantu et al. 2011; Fellers et al. 2013).

Prior to this work, the genomes of *Pgt* and the poplar leaf rust *M. larici-populina* (*Mlp*) were sequenced and compared. Out of 17,773 and 16,399 predicted genes, respectively, a core set of genes was identified representing the biotrophic nature of rusts (Duplessis et al. 2011). More recently, a second *de novo* genome assembly of *Pgt* was completed (Upadhyaya et al. 2014). Three genome sequencing projects have been described for *Pst* (Cantu et al. 2011, 2013; Zheng et al. 2013). The first two focused on identifying the effector complement, and the third study on identifying heterozygosity between two isolates of *Pst*. In each study, the total number of predicted genes varied across the projects (22,815 vs. 25,288, respectively). The genome of the flax rust *M. linii* (*Mli*) has also been sequenced (Nemri et al. 2014). Comparative analyses with other basidiomycete genomic resources have provided initial insights into the relatedness of subsets of genes (Xu et al. 2011; Nemri et al. 2014), but a comprehensive analysis among wheat rusts is missing.

Here, we have generated draft genome sequences of the wheat rust fungi *P. graminis* race 1 (*BBBD*) and *Pst* race PST-78, updated the gene set of *Pgt* race SCCL, and utilized these sets to define the shared and unique properties of these three related pathogens. To examine gene content evolution, we compared predicted proteins to those of other high-quality basidiomycete genomes. We examined the three wheat rust pathogens

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1 Corresponding authors: Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge MA 02142. E-mail: cuomo@broadinstitute.org; 4200 Hwv 97 Agriculture and Agri-Food Canada, Summerland Research and Development Centre, Summerland, BC, V0H 120, Canada. E-mail: guass.biakkeren@agr.gc.ca; and Department of Plant Pathology, Hard Winter Wheat Genetics Research Unit, USDA-ARS, 4004 Thirdmorton Hall, Manhattan, KS 66506. E-mail: john.fellers@ars.usda.gov
2 Present address: Plant Biotechnology Institute, National Research Council Canada, Saskatoon, Saskatchewan S7N OWN, Canada.
3 Present address: Department of Biology, Université de Moncton, New Brunswick E1A 3E9, Canada.
for conservation of gene families, including effector genes, and compared them to other sequenced rust fungi. We identified predicted secreted proteins including gene families found only in the wheat rust pathogens and found differences in expression levels between Pt life cycle stages, including sexual stages on the alternate host and wheat infection. In addition, we analyzed the mating-type gene complexes, revealed their evolutionary placement among basidiomycetes, tested the functionality of several Pt homeodomain proteins through heterologous expression in U. maydis (Um), and demonstrated a role for mating-type genes during wheat infection by HIGS.

**MATERIAL AND METHODS**

For detailed descriptions of isolates, sequencing strategies, genome assemblies and annotation, polymorphism analyses, DNA and RNA isolation procedures, RNA sequencing (RNA-Seq), and cloning methods, see File S1.

**Puccinia isolates and growth conditions**

Pt race 1, BBBD was selected as the race to be sequenced. This isolate was first collected in 1954 (Ordoñez and Kolmer 2009) and represents the earliest race characterized in North America. For Pt, isolate 2K41-Yr9 was selected (race PST-78). PST-78 was collected from the Great Plains in 2000 and is a representative of races that were first identified in the US in 2000 and then subsequently identified in other countries (Welling et al. 2003; Howmoller et al. 2008).

**DNA and RNA isolation**

Genomic DNA was isolated from Pt and Pt ureidiospores. RNA was isolated from three stages of Pt race 1: fresh mature, “dormant” ureidiospores that had been collected at 10 d postinoculation (DPI); ureidiospores that were germinated on water but harvested at 8 hr postgermination initiation; and from heavily infected wheat tissue at 6 DPI to represent the formation of ureidiospores, initiation of secondary infection, and most of the infection structures. RNA was also isolated and sequenced for two stages from the alternate host, for Pt, T. speciosissimum (meadow rue) and Pgt, Berberis spp. These stages represented pycnia with their pycniospores and a mixture of pycnia and aecia with aeciospores. RNA was isolated from two stages of Pt, infected wheat tissue at 8 DPI, isolated haustoria, and purified as described (Yin et al. 2009).

**Genome sequencing and assembly**

For Pt genome sequencing, various sizes of genomic DNA libraries and platforms were used. In short, libraries of 3 and 8 kb fragment inserts were sequenced using Roche 454 FLX chemistry and two large insert libraries were end-sequenced using Sanger technology: a 40 kb insert Fosmid library (30,731 clones) and 100 kb insert BAC library [15,000 clones (Fellers et al. 2013); Table S1]. An initial assembly of FLX and Sanger data was generated with Arachne (HybridAssembly) (Jaffe et al. 2003). The assembly was updated to incorporate the FLX+ data by first generating a new de novo assembly of all data using Newbler runAssembler, with parameters –het and –large, and merging the output with contigs uniquely present in the first assembly.

Three similar Pt genomic DNA insert libraries were sequenced using FLX chemistry with a Roche 454. In addition, paired-end Illumina reads were generated for three additional library sizes: fragment, 3–5 kb insert, and 40 kb Illumina-adapted Fosmids (Fosill library, Williams et al. 2012; Table S2). Three initial assemblies were generated using different algorithms: Life Technologies’ Newbler program, the CLC (Qiagen, Hilden, Germany) de novo assembler, and ALLPATHS-LG (Gnerre et al. 2011). To provide the most complete representation of the genome, contigs from the ALLPATHS-LG assembly were first selected, and then unique contigs from the CLC assembly were incorporated; see File S1 for details.

All assemblies were evaluated for regions that could correspond to both haplotypes that were independently assembled due to higher than typical divergence. One approach compared the ortholog representation (see below) across the three Puccinia genomes for two-copy paralogs in a single gene set, which could suggest either independent assembly of allelic copies in a single assembly or, alternatively, a gene duplication in that genome. As an independent approach, we aligned the repeat-masked sequence of each assembly to itself using nucmer (version 3.22, with parameter --maxmatch) (Kurtz et al. 2004); alignments were filtered to select alignments with 95% identity and 1 kb or greater length. Scaffolds with alignments covering 50% or greater of the repeat-masked contig length were considered as potentially representing the second haplotype and their total size was reported; alternatively, these could include segmental duplications present at different locations in the genome.

**Polymorphism analysis**

Heterozygous positions within the sequenced isolates of Pt and Pst were identified from Illumina data. Reads were aligned to each assembly using BWA (v0.5.9) (Li and Durbin 2010), and SNP positions were identified with GATK v2.1.9 UnifiedGenotyper, and then filtered by GATK VariantFiltration; see File S1 for details.

**RNA-Seq**

Strand-specific libraries were constructed with poly(A)-selected RNA samples using the dU5P second strand marking method (Parkhomchuk et al. 2009; Levin et al. 2010) for most samples. See File S1 for details of library construction and expression analysis.

**Genome annotation and protein set comparisons**

Gene sets were annotated by incorporating RNA-Seq data and predicted gene structures from multiple de novo predictions as previously described (Haas et al. 2011); see File S1 for a detailed description. The gene sets of the three Puccinia genomes were compared to each other and those of eight other fungi: M. lini, M. larici-populina, Microbotryum lycopersici, Mixia osmundae, Sporobolomyces roseus, Coprinopsis cinerea, Cryptococcus neoformans var. neoformans, and U. maydis. Orthologs were identified using OrthoMCL (Li et al. 2003) with expectation value 1e−5. The resulting orthologs were input to DAGchainer (Haas et al. 2004) to identify syntenic blocks, requiring a minimum of four genes in the same order and orientation in the compared genomes. Synteny plots were generated using a custom perl script, using the GDgraph library; code is available at https://github.com/gustavo11/zyntenia.

**Cloning, expression, and functional analysis of Pt mating-type genes**

The various Pt HD mating-type alleles were amplified by PCR from cDNA generated from total RNA isolated at 5 DPI from infected wheat cv. “Thatcher” leaves infected with Pt race 1 or from ureidiospores germinated over water. These alleles were subsequently cloned in a Ustilago-specific, integrative vector for heterologous expression from the strong constitutive Hsp70 promoter and terminator elements. Constructs were stably transformed into Um518 (Kronstad and Leong 1989) or strain FBI (Banuet 1991), and in U. hordei. To test the function of various mating-type genes during infection of wheat by Pt, HIGS experiments were performed. To create the RNAi silencing vectors, fragments of size 393, 430, 351, and 345 bp of the genes PtbW1, PtbE1,
PtSTE3.3, and PtSTE3.1, respectively, were amplified by PCR, cloned into the vector pENTR/D-TOPO (ThermoFisher, Waltham, MA), and subsequently recombined with the binary destination vector pPIPK007 (Himmelbach et al. 2007) using the LR GateWay recombination reaction to create the silencing vectors pRNAi-PtbW1, pRNAi-PtbE1, pRNAi-PtSTE3.3, and pRNAi-PtSTE3.1, respectively. Agroinfiltration assays, using Agrobacterium tumefaciens strain COR308, subsequent challenge by Pt, and fungal biomass measurements using quantitative PCR measurements were performed as described previously (Panwar et al. 2013). For details on these procedures, see File S1.

**Data availability**

Data access in NCBI: all genome assemblies and annotations are available with the following accesses ADAS00000000 (P. triticina), AJIL00000000 (P. striiformis f. sp tritici), and AAWC00000000 (P. graminis f. sp tritici). All sequence is linked to the following BioProjects: PRJNA36323 (P. triticina), PRJNA41729 (P. striiformis f. sp tritici), and PRJNA18535 (P. graminis f. sp. tritici). 

**RESULTS**

**Genome expansion in Pt associated with repetitive element proliferation**

High-quality genome assemblies of Pt and Pst were generated by combining data from multiple sequencing technologies. A range of insert size libraries for both genomes were sequenced using Roche 454, Illumina, and Sanger Technologies (Table S1 and Table S2). The assembled genome of Pt was the largest of the three wheat rust pathogens, totaling 135.3 Mb (Table 1); this assembly included 14,818 scaffolds of an N50 length of 544 kb. The assembly of Pst totaled 117.31 Mb and consisted of 9715 scaffolds with N50 length of 519 kb. The total contig size of 79.3 Mb is slightly larger than the total of contig assemblies generated for other strains at lower coverage levels (Cantu et al. 2011, 2013); both the scaffold and contig total are lower than those reported for the CY32 strain (Zheng et al. 2013). RNA-Seq was used to guide gene prediction for both Pt and Pst, and to improve the gene set of Pgt (Materials and Methods). Of the three rust pathogens, Pst had the highest number of genes predicted with 19,542, though fewer than the number reported for other Pst genomes (Cantu et al. 2011; Zheng et al. 2013), while Pt had the smallest total of the three with 14,880 genes. All three genomes have high coverage of a core eukaryotic gene (CEG) set (Parra et al. 2007; Table 1). The CEG coverage of this Pst gene set (97%) is notably higher than that of the PST-130 assembly (66%), the only other publicly available Pst gene set, due to a higher fraction of partial gene matches in PST-130 (Figure S2). In addition, comparison of a larger set of basidiomycete conserved orthologs supports the notion that few genes appear missing in the three wheat rust fungal genomes (Figure S3).

Together, these gene conservation metrics suggest that these assemblies contain highly complete gene sets. 

The assemblies of the three wheat rust fungi varied significantly in size, ranging from 89 Mb for Pgt to 135 Mb for Pt. While the Pst assembly totals 117 Mb, the genome may in fact be smaller than the assembly size, as the high percentage of gaps (32%) in scaffolds suggests that small contigs fall into some of the gap regions. In comparison, the Pt assembly consists of 21% and the Pgt assembly only 8% gap regions. While all assemblies are impacted by the high heterozygosity (see below), differences in repeat content and organization, as well as the use of different sequencing technologies, likely contribute to these differences. Each of the three wheat rust pathogen genomes was evaluated for content of repeated elements using both de novo predicted repeats and fungal elements from RepBase, which included 413 Puccinia sequences (File S1). The larger genome of Pt includes a higher fraction of repetitive elements, encompassing 50.9% of contig bases, whereas repeats covered only 31.5% of Pst and 36.5% of Pgt (Table 2). The expanded repeat content of Pst includes roughly twofold more of both class I retroelements and class II DNA elements. After excluding the identified repeats, the nonrepetitive portions of the genomes are very similar, totaling 53.4 Mb for Pt, 54.4 Mb for Pst, and 51.8 Mb for Pgt. 

Comparison of syntenic regions between Pt and Pst highlights that the genome expansion in Pt is due to disperse integration of repetitive elements. In total, 4319 orthologs are found between the two species in syntenic blocks of between 4 and 52 genes (Material and Methods). However, the size of the syntenic blocks in Pt are 30.1% larger overall than in Pgt; syntenic regions cover 46.7 Mb of Pt and 35.9 Mb of Pgt. In contrast, regions of Pst are 71.2% the size of syntenic regions of Pgt, suggesting a compaction of Pst; however, this analysis is impacted by the high percentage of gaps in the Pst assembly, reducing the resolution of blocks that can be detected. Within the expanded regions of Pt and Pst, there are larger blocks of repetitive sequence interleaved between the orthologs (Figure 1), highlighting that the genome expansion appears due to disperse integration of repetitive elements.

**High heterozygosity across all wheat rusts and evaluation of haploid assemblies**

The cereal rust pathogen species exist as dikaryotic (n + n) organisms for most of their life cycle, with a high level of heterozygosity between haplotype. For Pt, 269,370 heterozygous SNPs were identified across the genome based on Illumina read alignment (File S1). Across the genome, the average rate of heterozygosity was 2.53 SNPs/kb, though a higher rate was observed in intergenic regions (2.80 SNPs/kb) than in genic regions (1.69 SNPs/kb). In contrast, for Pst, 473,282 heterozygous SNPs were identified, for an average rate of 5.97 SNPs/kb; genic regions show a higher rate (7.49/kb) than intergenic regions (4.96/kb), a much higher rate than the 0.68 SNPs/kb previously reported for Pst with an
assembly of the CY32 strain (Zheng et al. 2013) but in the same range of 5.29 SNPs/kb averaged over another five Pst isolates (varying from 2.23 to 7.11 SNPs/kb; Cantu et al. 2013). The rate of heterozygosity for Pt is similar to that reported for Pgt (Duplessis et al. 2011), where higher rates in genic regions (2.28 SNPs/kb) were found compared to intergenic regions (1.72 SNPs/kb), although the sequencing technology and methods differ between these studies. Heterozygosity levels in both Pgt and Pt are more than double those reported for genic and intergenic regions of Mlp (0.84 and 0.87 SNPs/kb, respectively), supporting a high level of allelic diversity in these two wheat rust pathogens.

Regions of high heterozygosity could carry enough differences to prevent haploid assembly and could inflate the gene count, as alleles would appear as duplicated genes. Therefore, we examined the orthology assignment of genes found in all three wheat rust pathogens for conserved genes with two copies in only one species (Materials and Methods). Among the wheat rust fungi, Pt has only 230 species-specific two-copy paralogs (2:1:1; Pt:Pgt:Pst). Pt has an intermediate value of 361 species-specific paralogs while Pgt has 465 species-specific paralogs. This suggests that the new assemblies of Pt and Pst do not contain more duplicate conserved genes than the well-assembled Pgt genome. The presence of independently assembled haplotypes was also evaluated by identifying high identity and high coverage regions of self-alignment for each assembly (Materials and Methods); such regions cover 690 kb in Pt, 383 kb in Pgt, and 737 kb in Pst. The Pgt total is similar to the 326 kb estimated previously when sequence depth was also considered (Duplessis et al. 2011). While more stringently supported for Pgt, comparison of gene count conservation metrics between the assemblies suggests that independent assembly of both haplotypes is minimal in all three wheat rust pathogens, consistent with the use of assembly strategies that take heterozygosity into account.

### Core protein comparisons and orthology

To examine gene content variation between the three wheat rust pathogens and with other basidiomycetes, we compared the predicted proteins of Pt, Pgt, and Pst to those of related genomes. These included Mlp and Mli, the smuts Um and Mi. lycmidis-dioicae, the fern parasite M. osmundae, the unicellular plant phylloplane “red” yeast S. roseus, the human facultative pathogen C. neoformans, and the woodrotter Co. cinerea. By identifying orthologs across these genomes, we inferred the phylogenetic relationship of the species using single-copy orthologs; Pgt and Pt are most closely related, with Pst being an earlier diverged outgroup (Figure 2), consistent with previous findings from phylogenetic analysis of the ITS ribosomal DNA region (Zambino and Szabo 1993). While Pgt and Pt are most closely related based on phylogeny, some features may be more conserved or have evolved in parallel in Pgt and Pst, which share the same alternative host.

The wheat rust pathogens have very different gene content from other basidiomycetes, including a large fraction of species-specific genes. Less than half of the genes in each wheat rust pathogen, an average of 6867, were conserved among other basidiomycetes. All of the rust fungi (Pt, Pst, Pgt, Mlp, and Mli) contained an average of 6482 species-specific predicted genes. Among the other compared basidiomycetes, only Co. cinerea contained a similar number of species-specific genes. Among the wheat rust pathogens, Pt and Pst had similar numbers of species-specific genes (5443 and 4901, respectively), while the Pst number was higher at 8955. In addition, a large fraction of the genes were conserved across the wheat rust pathogens but not other fungi; an average of 3440 were conserved in at least two genomes and 2164 were found in all three.

To assess functional differences based on variation in genes between the three wheat rust pathogens and other fungi, we identified significant differences in the number of predicted protein domains. The three wheat rust pathogens were compared to Mli, Mlp, and to six other basidiomycetes, to determine what protein families exhibit significant gain or loss in rust fungi. The majority of these are protein families involved with nucleotide binding and modification, transcription factor regulation, and protein modification (Table 3). These include the NAM-associated transacting factor family, the most significantly enriched protein domain overall; this domain is specific to the rusts among basidiomycetes, with seven or 10 copies in Mlp and Mli and between 49 and 134 copies in the three wheat rusts. Three other Zn-finger transacting factors, and a fungal-specific transcription factor, families are also enriched. Four enriched protein families are associated with carbohydrate active enzymes; trehalose phosphatase, pectinesterases, glycoside hydrolase (GH) family 26 (GH26), which processes mannan and galactomannan, and the GH76 family of α-1,6 mannanases. Other families are involved in carbohydrate processing and transportation, cell metabolism, and metabolite transportation (Table 3). A notable depleted family, NmrA, belongs to a family of transcriptional repressors involved in controlling nitrogen metabolite repression in fungi (Table S3; Stammers et al. 2001). Other genes involved in nitrate metabolism are lost in Pt and Pst, as previously noted for Pgt (Duplessis et al. 2011). Overall, these domains highlight recent adaptation of gene regulation and host-interaction via gene duplication and diversification.

### Effector repertoire mining and conservation

Wheat rust pathogen candidate secreted effector proteins (CSEPs) are predicted to be expressed and secreted during host infection and are

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| Table 2 Repeat element content of the Pt, Pst, and Pgt genomes |
|-------------------|-----------------|-----------------|-----------------|
|                   | Pt              | Pst             | Pgt             |
| **Elements (#)**  | **Length (Mb)** | **Genome (%)**  | **Elements (#)** | **Length (Mb)** | **Genome (%)**  | **Elements (#)** | **Length (Mb)** | **Genome (%)**  |
| SINEs              | 254             | 0.04            | 0.04            | 21              | <0.00           | 0.00            | 17              | <0.00           | 0.00            |
| LINEs              | 1,380           | 0.51            | 0.48            | 344             | 0.26            | 0.33            | 419             | 0.32            | 0.40            |
| LTR elements       | 27,819          | 17.16           | 16.10           | 9,694           | 5.12            | 6.46            | 8,486           | 6.04            | 7.40            |
| DNA elements       | 46,961          | 12.55           | 11.78           | 19,863          | 5.38            | 6.78            | 20,526          | 5.61            | 6.88            |
| Unclassified       | 79,538          | 54.19           | 50.85           | 42,803          | 14.17           | 17.87           | 37,306          | 14.17           | 17.76           |
| Totals             | 155,952         | 5.29            | 5.08            | 72,725          | 24.94           | 31.45           | 66,754          | 29.73           | 36.47           |

Pt, P. triticina; Pst, P. striformis f.sp. tritici; Pgt, P. graminis f.sp. tritici; #, number; SINEs, short interspersed nuclear elements; LINEs, long interspersed nuclear elements; LTR, long terminal repeat.

*Percent of contig bases.
likely involved in host interactions. In this analysis, CSEPs predicted for Pt were compared to those previously identified in Pst and Pgt. From a starting set of 15,685 predicted proteins, including variant proteins encoded by alternate transcripts and novel genes detected by RNA-Seq data, a total of 1358 CSEP-encoding genes were predicted for Pt (Figure 5). Of these, a total of 914 Pt CSEPs grouped in 385 families or “tribes” previously assigned to Pst or Pgt CSEP tribes (Table S4; Cantu et al. 2013). From the remaining CSEPs, an additional 111 contained BLASTP sequence similarity (at $\leq e^{-20}$) to Pst and Pgt predicted proteins, of which 72 did not contain a predicted signal peptide at the expected initiation codon. The remaining 333 CSEPs were specific to Pt, of which 246 were unique without any paralogs in the Pt protein set. The remaining 87 CSEPs belonged to Pt-specific gene families having from two to eight members per tribe. This highlights that, while the vast majority of Pt CSEPs share sequence similarity with those in the other wheat rust pathogens, a subset is unique to each species. In addition, a disproportionate fraction of the wheat rust pathogen-specific genes mentioned above are predicted to code for secreted proteins. In Pt, a total of 17.0% of the wheat rust specific genes are predicted to encode secreted proteins compared to 9.6% of all predicted genes. This suggests that the genes specific to the wheat rust fungi include a high fraction of CSEP proteins.

Based on gene ontology (GO) term assignment and similarities to Pfam domains, the molecular functions of the Pt CSEPs appear highly diverse, though some frequent categories were observed. The largest subcategories include a total of 123 CSEPs that have hydrolase activity, 76 contain ion-binding activity, and 44 have oxidoreductase activity (Figure 3). Pfam domain comparisons also revealed some potential common and unique functions among rust fungi (Figure S5). Since protein targeting is dependent on intrinsic protein motifs such as a nuclear localization signal or a chloroplast transit peptide, we analyzed all predicted CSEPs in Pt to assess their signal peptides for their potential localization in the host to deduce possible functions. The distribution of their subcellular localization prediction in the plant indicates that 388 CSEPs are potentially targeted to the cytoplasm, 361 to the nucleus, and 292 to plastids. A total of 190 of these proteins are targeted to membranes, 16 to the apoplast, seven to the Golgi system, four to the vacuole, and one to the ER (Table S4).

**Expression profiles across life cycle stages and two hosts**

Functionally important gene expression was evaluated using RNA-Seq across diverse life cycle stages. RNA was sequenced from samples of dormant and germinating urediniospores, infected wheat leaf tissue at 6 DPI representing most of the infection structures of the uredinial spore genetics of the life cycle, and two stages collected from the alternate host, *T. speciosissimum* (meadow rue), at the pycnia sexual stage and a later stage mixture of both pycnia and aecia. Comparing normalized counts across conditions revealed that two urediniospore samples were most highly correlated, and that both were similar to the mixed sample of pycnia and aecia (Table S5). In contrast, the pycnia and infected wheat leaf samples appeared the most different from the others.

To closely evaluate how secreted proteins change in expression across these conditions, all Pt CSEP genes were assessed and 199 were identified with a minimum of fivefold change. From these, 138 Pt CSEP genes were highly induced in wheat-infected leaves (Figure 3) with 30 assigned to known proteins with hydrolase (10), peptidase (four), oxidoreductase (three), ion-binding (two), phosphatase (two), carbohydrate-binding (two), lipase (two), chitinase (one), protease (one), phospholipase (one), phosphogluconolactonase (one), and esterase (one) activities. Twenty-six CSEP genes were highly induced exclusively in germinating urediniospores. Among other stage-specific sets were 16 CSEP transcripts highly accumulated in dormant urediniospores compared to the germinated spore stage. When focusing on infection of the alternate host *Thalictrum*, 16 were highly expressed in pycnia and three during the later stage of the mixed pycnia and aecia sample (p + ae). One to 10 hydrolase-encoding CSEPs are highly induced in various datasets, except for the “mixed” p + ae stages. Among those, members of the GH superfamily were specifically highly expressed: GH18 in wheat-infected tissue, GH16 and GH17 in germinating urediniospores, GH26 in dormant urediniospores, and GH16 in pycnia.

The predominant gene classes expressed in each stage were also examined by testing for functional enrichment in differentially expressed genes. Roughly half of the genes are induced during wheat infection, compared to dormant spores, and encode predicted secreted proteins (Table S6). Other gene families that are enriched during wheat infection include the GH18 family, DNA binding proteins, peroxidases, and amino acid permeases (Table S7). In contrast, genes expressed in pycnia relative to dormant urediniospores are enriched for GMC oxidoreductase, LON proteases, potassium uptake, osmotic stress response, and chitin synthases (Table S8).

**Mating-type genes**

*Pheromone receptors and precursors*: Three homologs of the *Um* pheromone receptor gene *Pra* were found in each of the Pt, Pgt, and *Pst* dikaryotic genomes (Table S9). All corresponding genes had a
resulting in two nonsynonymous amino acid changes (Figure S7A). The same two SNPs were confirmed in 25 out of 29 other recently sequenced Pt genomes (data not shown). Race 1 RNA-Seq transcriptome analysis revealed two allelic transcript populations confirming the presence of two alleles, with both expressed roughly equally in various life cycle stages (Figure S7B). Complete digestion of a PCR product representing the 3’-end of the race 1 PtPRA3.1 gene with restriction enzyme Cac8I was able to distinguish one of the SNPs (Figure S7C). This analysis indicated the presence of two PtSTE3.1 alleles in the Pt race 1 genome. In contrast, no SNPs were found for the PtSTE3.2 and PtSTE3.3 genes, which may represent the two allelic specificities, one in each haplotype. Additional homologs, such as for Mfp, likely represent more recent, lineage-specific duplication and divergence events (gray box in Figure 4). Comparison among 16 resequenced Pgt genomes revealed 34 SNPs in STE3.1, no SNPs in STE3.2, and two SNPs in the STE3.3 gene, whereas these numbers are very similar among 15 resequenced Pgt isolate genomes: 38 SNPs in the STE3.1 gene, no SNPs in STE3.2, and one SNP in STE3.3.

Using annotated Pt EST or known mfa sequences (File S2), three contigs in Pt and Pgt and two in Pst were found to contain putative mfa genes (Table S10). A putative Ptmfa2 gene coding for a 33 amino acid protein with a characteristic C-terminal CAAX motif was identified on supercontig 2.517; while no gene model was initially predicted here, evidence of expression was detected in several life cycle stages (Figure S6). Extensive searches of genomic reads and RNA-Seq data could not identify other mfa genes. In all three Puccinia species, the predicted mfa2 and STE3.2 genes are divergently transcribed and are approximately 500-700 bp apart (605 bp in Pt; Figure S6), an organization reminiscent of Ustilagomycete a2 loci. The Pgt STE3.3 allele is 24 kb away from a potential Pgt mfa gene on supercontig 2.2 (Table S9 and Table S10). In Um and Sporisorium reilianum, the a2 loci each harbor two additional genes, lga2 and rga2, that are located in between the Pra2 and mfa2 genes. The LGA2 and RGA2 proteins localize to mitochondria and are implicated in mitochondrial fusion processes in that fungus (Bortfeld et al. 2004), but no obvious homologs could be identified in the Puccinia species.

Homeodomain-containing transcription factors: Two allelic homologs of both HD1- and HD2-containing protein genes were found in each of the three Puccinia species and were termed be-HD2 and bW-HD1 (Table S11). Gene models in the genome assemblies were found to be partial, and complete transcript sequences were constructed using de novo RNA-Seq assemblies (Figure S8). The predicted Puccinia HD2 proteins are ~374 amino acids in length whereas the HD1 proteins are ~620 amino acids in length. PtbE2-HD2 and PtbW2-HD1 are located close together and are divergently transcribed (Figure S9 and Table S11). However, in the fragmented genome assembly, PtbE1-HD2 and PtbW1-HD1 are each located on a small contig, so direct inferences of linkage could be made for this pair. Comparative analysis of aligned DNA and protein sequences for the two alleles of each PtbE and PtbW gene revealed the conserved HD-specific domains within an overall similar structure including five introns (Figure S6). The predicted proteins ranged in size from 379 to 395 amino acids, and had the characteristic seven transmembrane domains typical of these G protein-coupled membrane-inserted receptors (Bölker et al. 1992). A molecular phylogeny was calculated for these and the receptors from the poplar and pine rust pathogens, including known STE3/PRA proteins from several other basidiomycetes (Figure 4). This analysis revealed that the STE3 receptors from the Pucciniales formed a clade (blue and gray boxes) well-separated from the Agaricomycotina (no color), the Microbotryomycetes (yellow box). The rust clade encompassed two major groups, STE3.1 homologs (light blue box) and another branch with two subgroups, representing STE3.2 and STE3.3 (dark blue boxes). Since we did not have separate haplotype genome assemblies, we performed an in-depth analysis for Pt. Two allelic SNPs were found for PtSTE3.1 in Pt race 1, resulting in two nonsynonymous amino acid changes (Figure S7A).

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conserved C-terminal region, whereas the proteins were more diverged at the N-terminus, similar to the paradigm established in *Ustilago* species (Figure S8). A similar pattern of conservation was noted for the corrected *Ptg* and *Pst* alleles. A molecular phylogeny was generated to establish the relatedness among the HD-containing mating-type proteins in the three cereal rust fungi, compared to single homologs from the poplar and pine rust fungi (Figure 5). The allelic variants were closer to each other in each *Puccinia* species as they were among the species, since they are alleles and their sequences are evolving in a concerted fashion. Thus, among the rust fungi compared, the HD1- and HD2-containing transcription factors are each separated in different clades, as is seen when many basidiomycetes are compared, indicating an ancient system in which allelic specificities are maintained because of their functionality (Bakkeren *et al.* 2008; Kies *et al.* 2011).

In all three wheat rust pathogens, a large contig with a complete divergently-transcribed pair of *bE* and *bW* genes is found while the other sometimes partial alleles are found on small contigs. This analysis highlights the challenges faced when assembling very similar sequences such as the conserved C-terminal domains, likely belonging to two different haplotype genomes. Therefore, to investigate the physical arrangement of both *bE-bW* pairs in the *Pt* race 1 genome, primers to the conserved 3' ends of each gene (Table S12) were used in a PCR reaction, which yielded a single product of 3.9 kb from total gDNA isolated from germinating urediniospores. In dikaryotic urediniospores, both pairs are assumed to be present. Analysis of the sequences had revealed that nucleotide polymorphisms in restriction enzyme sites for *XmaI* and *SpeI* could be used to distinguish the allelic pairs. To verify whether or not the 3.9 kb PCR product contained both divergently-transcribed *bW* and *bE* gene pairs, it was digested with these enzymes for a prolonged period of time to yield fragments consistent with the presence of both allelic pairs, confirming the suspected organization (Figure 6).

### Pt HD genes can functionally interact in *U. maydis*  

We previously demonstrated the feasibility of using *Um* as a heterologous expression system for *Pt* genes (Hu *et al.* 2007). To examine the role of the candidate *Pt* mating-type genes, cDNA-derived *Pt* HD-containing transcripts were expressed in *Um*. Upon stable transformation of each of the *PtbE1*- or *PtbW1*-expressing constructs into either *Um* haploid strains *a1b1* or *a2b2*, the resulting transformants yielded cells that had changed morphology from growth by budding to a filamentous growth (Figure 7). When introduced into *Ustilago* cells, constructs expressing *b* mating-type genes of a different specificity or from different *Ustilago* species, transformants display these changed morphologies similar to regular mating interactions between cells of opposite mating types (Gillissen *et al.* 1992; Bakkeren and Kronstad

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**Table 3** Significantly enriched protein families found in wheat rusts when compared to eight other species of basidiomycetes

| Pfam Family | Enrichment Ratio* | Q Value Significanceb |
|-------------|------------------|-----------------------|
| Nucleotide-binding and modification | | |
| PF14303.1: NAM (No apical meristem-associated C-terminal domain) | 6.57 | 3.52E-100 |
| PF13873.1: Myb/SANT-like DNA-binding domain | 6.35 | 2.76E-04 |
| PF12776.2: Myb/SANT-like DNA-binding domain | 4.06 | 1.33E-28 |
| PF10443.4: RNA12 protein | 3.54 | 8.38E-03 |
| PF00891.13: O-methyltransferase | 3.52 | 6.43E-03 |
| PF13923.1: zinc finger, C3HC4 type (RING finger) | 3.11 | 3.45E-31 |
| PF13639.1: RING finger domain | 3.10 | 1.69E-38 |
| PF00997.20: zinc finger, C3HC4 type (RING finger) | 2.88 | 2.44E-23 |
| PF13920.1: zinc finger, C3HC4 type (RING finger) | 2.39 | 2.63E-03 |
| PF00270.24: DEAD/DEAH box helicase | 2.37 | 4.97E-19 |
| PF00271.26: Helicase conserved C-terminal domain | 2.09 | 3.04E-11 |
| Transcription factor regulation | | |
| PF10497.4: zinc finger-domain of monoamine-oxidase A repressor R1 | 4.63 | 1.71E-03 |
| PF00176.18: SNF2 family N-terminal domain | 2.56 | 5.77E-12 |
| Cell damage defense | | |
| PF00080.15: Copper/zinc superoxide dismutase | 4.43 | 2.03E-11 |
| Protein modification | | |
| PF12861.2: Anaphase-promoting complex subunit 11 RING-H2 finger | 3.94 | 3.57E-16 |
| PF11793.3: FANCL C-terminal domain | 3.75 | 2.26E-02 |
| PF02338.14: OTU-like cysteine protease | 3.24 | 6.72E-06 |
| PF12678.2: RING-H2 zinc finger | 2.34 | 3.50E-02 |
| Carbohydrate processing | | |
| PF03663.9: glyco hydro 76 (glycosyl hydrolase family 76) | 3.80 | 3.64E-03 |
| PF02156.10: glyco hydro 26 (glycosyl hydrolase family 26) | 3.50 | 1.22E-05 |
| PF02358.11: trehalose-phosphatase | 3.47 | 4.84E-02 |
| PF13813.1: membrane-bound O-acyl transferase family | 3.47 | 2.50E-03 |
| Cell cycle control | | |
| PF12678.2: RING-H2 zinc finger | 3.55 | 4.97E-19 |
| Other | | |
| PF03466.15: LysR substrate binding domain | 5.44 | 4.76E-02 |
| PF11937.3: protein of unknown function (DUF3455) | 3.67 | 4.79E-02 |
| PF01637.13: archaeal adenosine triphosphatase | 3.19 | 3.08E-03 |
| PF01735.13: lysophospholipase catalytic domain | 2.75 | 1.60E-03 |
| PF13813.1: membrane-bound O-acyl transferase family | 2.57 | 3.32E-03 |

* log2 weighted count wheat rusts/weighted count in nonwheat rust basidiomycetes.  
* Storey and Tibshirani (2003).
by the mating type (were paired on a mating-type plate assay. Transformants of opposite strains of opposite mating type but each deleted for both alleles [Uh553 (bW) and Uh530 (a2 b0); Bakkeren and Kronstad 1996] were each transformed with the above-tested single genes) allowing the respective Pt HD proteins to productively interact with the respective resident pair in reproductive heterodimer formation and initiation of filamentous growth. Whereas control pairings of Pt mating-type genes are functional during the sexual stage on the alternate host (certainly the pycniospore stage), a diversified role for them during infection could be envisaged. To examine such a role for the HD-containing alleles, PtSTE3.1 and PtSTE3.3, the Agrobacterium-mediated HIGS technique was used (Panwar et al. 2013). The silencing constructs containing the 3’ sequences representing PtbW1, PtbE1, and PtbE3.1 would each target both alleles because of their conserved nature (Figure S7, Figure S8, and File S1). Extensive searches by BLAST of the targeted Pt gene sequences to all available wheat and Pt genomic resources could not identify potential off-target sequences. Prior expression of silencing constructs in the wheat host targeted at these pathogen genes significantly reduced fungal development, as measured by biomass reduction and disease symptoms such as sporulation, upon infection with Pt urediniospores (Figure 8). The exception was the HIGS construct, which targeted the PtSTE3.1 alleles, resulting in no measurable reduction in Pt biomass, similar to the control wheat TaPDS silencing construct. This correlated with the very limited number of PtSTE3.1 transcripts in the Pt wheat-infected transcriptome (Figure S7 and Figure S10) and hence demonstrated the specificity of the system, as was previously extensively shown for several other pathogenicity genes (Panwar et al. 2013).

**DISCUSSION**

The genomes of Pt and Pst, sequenced here and compared to those from Pgt and other Basidiomycetes, are notable for their expanded size and high level of heterozygosity. While each genome was assembled using different sequencing technology, each of the gene sets appears to be of different sequencing technology, each of the gene sets appears to be of

**Figure 3** Highly expressed CSEPs vary across Pt developmental stages. The number of Pt CSEPs with high transcript levels in each of the five RNA-Seq datasets compared to other datasets, with their predicted GO annotations. The fold change of the transcript levels in each two datasets was calculated. Pt effectors revealing a ≥fivefold change in one dataset when compared to the remaining datasets were selected and their molecular functions (GO annotation) were assessed. (*) Pt transcript level from inoculated wheat at (6 DPI) was calculated from an average of two samples derived from independent wheat inoculations. CSEPs, candidate secreted effector proteins; DAI, days after inoculation; GO, gene ontology; Pt, Puccinia triticina; RNA-Seq, RNA sequencing.
Figure 4 Molecular phylogenetic relationship of STE3-like pheromone receptor protein sequences of basidiomycetes. The pheromone receptor sequences were C-terminally truncated to exclude the cytoplasmic tail and to optimize the alignment (as in Bakkeren et al. 2008; see File S2 for details), and were from Co. cinerea (CcinRCB1 and CcinRCB2), Cr. gattii (CgSTE3a and CgSTE3x), C. neoformans (CnSTE3a and CnSTE3x), L. scotii (LsSTE3.1 and LsSTE3.2), Malassezia globosa (MgPRA1), M. violaceum (MvPRA1 and MvPRA2), Pholiota nameko (PnamRCB1), Pt. djamor (PdjaSTE3), Puccinia protein IDs are given in Table S9 (N-terminal 287–294 amino acids), Rhodosporidium toruloides (RtSTE3.1 and RtSTE3.2), Schizoplyllum commune (SzcBBR1 and SzcBBR2), Spo. salmonicolor (SsSTE3.1 and SsSTE3.2), Sp. reilianum (SrPRA1, SrPRA2 and SrPRA3), U. hordet (UhPRA1 and UhPRA2), and U. maydis (UmPRA1 and UmPRA2). For the rust fungi: Cronartium quercuum f.sp. fusiforme (CqfSTE3.1, CqfSTE3.2, and CqfSTE3.3) and CqfSTE3.3 and CqfSTE3.3. STE2 of Saccharomyces cerevisiae (ScSTE2) served as outgroup. Blue boxes: Pucciniales; orange box: Ustilaginomycotina; and yellow box: Microbotryomycetes; Agaricomycotina, no color. STE names in red indicate tentative suggested groupings (see Discussion).
similar quality, with high representation of core genes. The genome of
Pt in particular has been expanded due to multiple classes of repetitive
elements; while this higher repeat content was found to be dispersed
across the genome assembly, repeat elements could impact the expres-
sion of nearby genes and could also contribute in this way to differences
between related strains of the same species. Notably, we
find that Pst
has the highest level of heterozygosity and that this measure is larger
than previously reported (Zheng
et al. 2013). While some of this dif-
ference could be attributed to the isolate sequenced, the much larger
size of the CY32 genome used in this previous study may result in an
underestimation of heterozygosity, such as in cases where both alleles
of a gene were assembled independently.

Prior to this work, gene content surveys focused on genes expressed
during infection and other life cycle stages. An extensive EST data set of
13,328 unique ESTs was created by sampling several stages in
Pt; however, functional annotation was generally low (Hu
et al. 2007; Xu
et al. 2011). During this genome project, ESTs and newly generated RNA
sequences were used to refine gene models and predict alternatively
spliced forms in each of the genomes. Notably, Pst contained the largest
set of predicted genes at 19,542, despite not having the largest genome.
This total is similar to what has been found in other Pt genome
projects. In the sequence of four other Pt races, the gene count varied from
18,149 to 21,030, which may have been impacted by differing
levels of heterozygosity (Cantu
et al. 2013). It is intriguing that in Pst
there are many more CSEPs than in Pt or Pgt; in one study, 2999 CSEPs
were predicted in five consolidated Pst genomes, compared to 1333
and 1173 in Pgt and Mlp, respectively (Cantu
et al. 2013). Virulence vari-
ability among Pst isolates is high and larger than for Pgt and Pt, likely
due to a CSEP gene expansion and diversification to elude host recog-
nition. In this regard, it may be significant that Pst can be found on
126 species of grasses among 20 genera (Line
2002; Cheng
et al. 2016).

Overall, the number of genes within the three rust fungal genomes is
higher than that in other plant pathogenic fungi. Smut fungi have fairly
low gene counts (6500–7000), but plant pathogenic fungi have as many
as 17,735 in Fusarium oxysporum (Ma
et al. 2010) and 16,448 in the
necrotroph Botrytis cinerea (Amselem
et al. 2011). Mli and Mlp have
gene numbers of 16,271 and 16,399, respectively (Duplessis
et al. 2011; Nemri
et al. 2014), indicating a similar number of genes to wheat rust pathogens. Higher gene numbers may support the multiple spore stages
and more complex life cycle in the rust fungi.

The large genome expansion in Pt due to repetitive elements was
suggested by an earlier study of selected genome regions (Fellers
et al. 2013). The genomes of other rust fungi are also enriched for repetitive
elements, though smaller in number and total DNA content. Pst and
Pgt have similar repeat element numbers, while Pt is more like Mli, for
which repeats occupy 87 Mbp or 46% of the genome (Nemri
et al. 2014). While in some fungi the process of repeat-induced point muta-
tion helps control the expansion of transposable elements, the activity
of repeat-induced point mutation in the rust fungi (Pgt and Mlp) ap-
pears much lower than in other fungi (Amselem
et al. 2015). Mobile
elements are now considered to be essential “genome modifiers” that
replicate and randomly reinsert to drive recombination, addition, and/
or deletion events, sometimes leading to protein neo-functionaliza-
tions. Regions of the genome enriched in repetitive elements have also
been shown to be a source of genetic diversity, particularly within
effector repertoires of pathogens for possible adaptation to their hosts
(Haas
et al. 2009; Raffaele and Kamoun
2012; Ali
et al. 2014).

Similar to two previously sequenced wheat rust pathogen genomes
(Duplessis
et al. 2011; Zheng
et al. 2013), 8% of the identified Pt
transcript repertoire encodes potential secreted effectors. The three _Puccinia_ species share a complement of secreted proteins, yet each has a group that is specific to its own species (Figure S5 and Table S4). Although all three are pathogens of wheat, their indigenous worldwide distribution and therefore evolutionary path, environmental (host) adaptation, and life histories are different, as are their symptom formation and alternate host selection; this will have likely translated into a varied complement of CSEPs. Comparison among available rust fungus inventories allowed us to identify a preliminary set of CSEPs specific to the wheat rusts. However, poor annotation of candidate effectors, currently a common challenge in plant pathology, makes deducing biological meaning from specific subsets difficult. Nevertheless, based on Pfam domain searches, specific wheat rust CSEPs were members of GH families (GH15, GH17, and GH88), trehalose-phosphatases, members of the DyP-type peroxidase family, glyoxal oxidase, and proteins with prokumamolisin, thaumatin, and alcohol dehydrogenase-like domains (Figure S5). Intriguingly, 140 of the unidentified proteins were predicted to target the cytoplasm of the host and could be candidates with a role in the interplay with the host immune system.

Gene expression during the key stages of the fungal life cycle is quite different. Many CSEPs were strongly expressed in plant host tissues in comparison to the (germinating) urediniospore stages, suggesting their particular role during infection. Although a large number of highly induced CSEPs could not be functionally annotated, a significant number fall into groups with hydrolase, peptidase, and oxidoreductase activities. In the uredinia, pycnial, and aecial spore stages, many of the genes are associated with sugar, amino acid, and membrane modification, or are amino acid transporters, nucleotide binding proteins, or transcription factors. However, prior to uredinia formation, the fungus induces the protein manufacturing machinery and the most highly expressed genes are associated with ribosomes.

A recent study of mating-type genes in a basal basidiomycete lineage, _Leucosporidium scotti_, strongly suggested a biallelic pheromone receptor recognition system to be ancestral in the basidiomycetes (Maia et al. 2015), separated into two ancient clades, tentatively called STE3.1 and STE3.2 in Figure 4 (in red). This is generally seen in genomes among the Ustilaginomycotina, Agaricomycotina, and the more recently identified Microbotryomycetes, though variations have become apparent. In the Ustilaginaceae, _Spacelotheca reilianum_ has three _pra_ (STE3) alleles, one possibly evolved through recombination (Schirawski et al. 2005), whereas in closely related _Uh_ and _Um_ only two are found. In the latter, a pseudo pheromone gene (_mfa_ in Figure S1) suggests one specificity might have been lost. A recent study among members of the Ustilaginaceae found three highly syntenic pheromone receptor alleles to be prevalent, which led Kellner et al. (2011) to propose a triallelic recognition system to be ancestral in this family. In the mushrooms, two clades of pheromone receptors are found but, in each, expansion by duplication and mutation is very common leading to several allelic series (Raudaskoski and Kothe 2010). Our analysis to date of members of the genus _Puccinia_ suggests that the biallelic recognition system is indeed ancestral in the basidiomycetes, represented by STE3.2 and STE3.3 (for consistency in this speculative scenario, we called them STE3.2-2 and STE3.2-3; dark blue boxes and red lettering in Figure 4). They each were found to be expressed during the sexual and the wheat infection stages, at approximately equal levels (Figure S10). The close proximity organization of the _PtSTE3.2_ and _PtMfa2_ genes is reminiscent of the P/R organization found in several basidiomycetes (Figure S6), whereas in _Pgt_ the STE3.3 allele is 24 kb away from a potential _Pgt_ _mfa_ gene on supercontig 2.2 (Table S9 and Table S10). In addition, almost no SNPs are identified for each of these two genes per species among a number of resequenced isolates, suggesting a biallelic recognition system. Further duplication and divergence of some of the allelic STE3 genes in certain species may have occurred, such as for _MlcSTE3.4_ (gray box), similar to mushrooms. The limited synteny, presence of homologous genes at variable spacing, and multiple TEs and repeats, are in agreement with accelerated evolutionary potential in _STE3_-containing regions (File S2). The well-separated clade containing the Pucciniales STE3.1 homologs (speculatively called STE3.2-1, light blue box and red lettering in Figure 4) could represent an ancient duplication and divergence event, with a possible neo-functionalization. This is supported by the finding of two alleles in each haplotype of _Pt_ race 1, the weak synteny that is apparent among the investigated three cereal rusts (Figure S12, Figure S13 and File S2), and the many SNPs found in this gene among resequenced isolate genomes for all three species.

Mating and compatibility have been very difficult to study in the (cereal) rusts because many are macrocyclic, completing their sexual
stage on a different (sometimes obscure or unknown) alternate host plant. Several studies have attempted to shed light on the mating-type system in rust fungi. Conclusions and speculations vary from rust fungi having a simple ± bipolar system in several *Puccinia* and *Uromyces* species (Anikster and Eliam 1999) to a more complicated tetrapolar system with multiple allelic specificities in *Mli* (Lawrence 1980) and the related oat crown rust pathogen, *P. coronata* (Narisawa et al. 1994). Our genome analysis demonstrates that the proposed simple ± bipolar system in the cereal rust fungi is more complex. The limited number of *a* locus *Pra* and *mfa* alleles in smuts indicates a small repertoire of haploid fusion capabilities in nature (though promiscuity has been observed; Bakkeren and Kronstad 1996); this contrasts with multiple (allelic) arrays often found in mushrooms. Similarly, single *be/bW* pairs are found in smuts with very few allelic variants identified in nature for the bipolar but many more (up to 33) for tetrapolar *Um*. The organization is often more complex in mushrooms where one to multiple HD1–HD2 pairs representing various alleles are found in arrays in many of their analyzed genomes, accounting for the myriad of sexually productive specificities recognized in nature (Fraser et al. 2007; Bakkeren et al. 2008; Kües et al. 2011; Nieuwenhuis et al. 2013; Kües 2015). Closer to the *Pucciniomycetes*, a bipolar system with limited number of alleles for the HD-pair and *pra/mfa* genes has been found in *Mi. violaceum* (Petit et al. 2012). A "pseudobipolar" system with loose linkage of the HD-pair and pheromone receptor genes, estimated to be 1.2 Mb apart, was described in *Sporidiobolus salmonicolor*, resulting in the discovery of multiple HD-pairs in nature (Coelho et al. 2010). The *Puccinia* species genome analyses described here did not indicate close linkage of the *STE3/mfa* and HD genes. The current assembly and preliminary mapping data in *Pt* indicate these loci to be at least 216 kb apart (File S2), though a loose linkage has not been ruled out. An inventory of HD alleles among a wide collection of isolates may answer some of these questions.

When introducing one particular *Pt be* or *bW* allele into a wild-type haploid *Um* strain, filamentous growth is triggered through the production of the respective HD protein, functional interaction with the *Ustilago* counterpart, and subsequent transcriptional activation of a subset of genes by the formed bispecies HD dimer, as shown for *Um* (Wahl et al. 2010). While we have shown such active interactions to occur between *b* alleles from different species within the smuts (Bakkeren and Kronstad 1993), such activity across quite diverged members of the basidiomycetes is astounding and suggests an ancient origin of these proteins. However, the experiment introducing *PbW1* and *PtbE2* alleles, each in a compatible *Uh* strain lacking *b* genes, did not trigger a switch to hyphal growth upon mating. Although fusion of mating hyphae was confirmed, this suggests that no productive interaction within the dikaryotic heterologous cell occurred. Given that we found only two allelic pairs of *be* and *bW* in these *Puccinia* species and the overwhelming evidence of the productive interaction between such heterodimers in many very diverse basidiomycetes studied to date, it is unlikely that the *PtbE* and *PbW* HD proteins would not interact. Failure to initiate filamentous growth in *Uh* then may indicate that the *Pt* HD proteins lack domains or the specificity necessary for *Ustilago*-specific downstream interactions, nuclear import, and/or for binding to *Ustilago* promoter elements that normally initiate the transcription of genes involved in the switch to filamentous growth (Scherer et al. 2006; Kahmann and Schirawski 2007); when *Pt-Uh* HD-heterodimers are formed, such functionality may be provided by the *Uh* component (Figure 7). Indeed, the predicted *PtbE* proteins are, at 374 amino acids, ~100 residues shorter than the *Ustilago* homologs. The compositions of the helices that constitute the HD are relatively well-conserved between the *Pt* and *Ustilago* b-proteins; however, their location within the protein is significantly different and may have evolved *Puccinia* lineage-specific adaptations.

The HIGS experiments demonstrated that some of the *Pt* mating-type genes were additionally functional in dikaryotic hyphae during wheat infection (Figure 8), as well as the assumed activity during the sexual stage on *Thalliclumssp*. The involvement of the *a* mating-type genes in pathogenicity of the dikaryotic cell type has been demonstrated in *Um* (Hartmann et al. 1996; Urban et al. 1996). Silencing of *PtSTE3.1* had less of an effect than of *PtSTE3.3* and was correlated with the observed expression levels during wheat infection (Figure S10). Differing expression levels for specific alleles at different life cycle stages could indicate functional diversification and possibly a loss in function in determining MAT-specificity, as seen in many mushrooms. The sequences in the HD silencing constructs were designed such that they would silence both alleles. This was clearly detrimental to the infection process, and therefore shows that they are important for pathogenicity.
They could play a role in the maintenance of the dikaryotic stage and/or induction or persistence of pathogenicity gene expression, such as demonstrated for Um where the bE/bW heterodimer was shown to be essential for initiating the induction of a set of genes involved in the pathogenic life style (Brachmann et al. 2001; Wahl et al. 2010). Wheat rust diseases are a major impediment to economic production of wheat in many areas in the world, and because of their rapid adaptation to newly introduced resistant cultivars and fungicides, they are a threat to envisaged increased yield for a growing population. Genome research on these elusive biotrophic pathogens has tremendously accelerated our understanding of their interaction with their host, and the presentation of a Pst and another Pst genome and the comparative analysis to other rust fungi in this study has highlighted similarities and differences that can now be exploited for targeted crop protection strategies. Conserved and essential effectors, expressed during infection, and their intended host targets, would be interesting components for further study; a search for natural or engineered resistance genes recognizing such effectors could be effective.

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LITERATURE CITED

Ali, S., J. D. Laurie, R. Linning, J. A. Cervantes-Chávez, D. Gaudet et al., 2014 An immunity-triggering effector from the Barley smut fungus *Ustilago hordei* resides in an Ustilaginaceae-specific cluster bearing signs of transposable element-assisted evolution. PLoS Pathog. 10: e1004223.

Amselem, J., C. A. Cuomo, J. A. L. van Kan, M. Viaud, E. P. Benito et al., 2011 Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet. 7(8): e1002230.

Amselem, J., M.-H. Lebrun, and H. Quesneville, 2015 Whole genome comparative analysis of transposable elements provides new insight into mechanisms of their inactivation in fungal genomes. BMC Genomics 16: 141.

Anikster, Y., and T. Elam, 1999 Pycnial nectar of rust fungi induces cap formation on pycniaeopores of opposite mating type. Mycologia 91: 858–870.

Anikster, Y., W. R. Bushnell, A. P. Roelfs, T. Elam, and J. Manisterski, 1997 *Puccinia recondita* causing leaf rust on cultivated wheats, wild wheats, and rye. Can. J. Bot. 75: 2082–2096.

Bakkeren, G., and J. W. Kronstad, 1993 Conservation of the b mating-type gene complex among bipolar and tetrapolar smut fungi. Plant Cell 5: 123–136.

Bakkeren, G., and J. W. Kronstad, 1996 The phenome cell signaling components of the *Ustilago* a mating-type loci determine intercompatibility between species. Genetics 143: 1601–1613.

Bakkeren, G., J. Kamper, and J. Schirawski, 2008 Sex in smut fungi: structure, function and evolution of mating-type complexes. Fungal Genet. Biol. 45: S15–S21.

Banuett, F., 1991 Identification of genes governing filamentous growth and tumor induction by the plant pathogen *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 88: 3922–3926.

Bölker, M., M. Urban, and R. Kahmann, 1992 The a mating type locus of *U. maydis* specifies cell signaling components. Cell 68: 441–450.

Bolton, M. D., J. A. Kolmer, and D. F. Garvin, 2008 Wheat leaf rust caused by *Puccinia triticina*. Mol. Plant Pathol. 9: 563–575.

Bortfeld, M., K. Auffarth, R. Kahmann, and C. W. Basse, 2004 *The Ustilago maydis* a2 mating-type locus genes lga2 and rga2 compromise pathogenicity in the absence of the mitochondrial p32 family protein Mrb1. Plant Cell 16: 2233–2248.

Brachmann, A., G. Weinzierl, J. Kämper, and R. Kahmann, 2001 Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. Mol. Microbiol. 42: 1047–1063.

Brefort, T., G. Doehlemann, A. Mendoza-Mendoza, S. Reissmann, A. Djeami et al., 2009 *Ustilago maydis* as a pathogen. Annu. Rev. Phytopathol. 47: 423–445.

Brizgalova, V. A., 1935 Brown rust of wheat under conditions of the Irkutsk-Nizhnyuyedinsk zone of the East Siberian District. Tr. Po. Zasch. Rast Vostoch Sob. 2: 99–174.

Brizgalova, V. A., 1937 On a new intermediate host of brown rust of wheat, *Puccinia triticina* Eriksk. Sb. Tr. Zasch. Rast Vostoch Sob. 5: 75–87.

Bushnell, W. R., and A. P. Roelfs, 1984 *The Cereal Rasts; Volume I. Origins, Specificity Structure, and Physiology*. Academic Press, Orlando.

Cantu, D., M. Govindarajulu, A. Kozik, M. Wang, X. Chen et al., 2011 Next generation sequencing provides rapid access to the genome of *Puccinia striiformis* f. sp. triticci, the causal agent of wheat stripe rust. PLoS One 6: e24230.

Cantu, D., V. Segovia, D. MacLean, R. Bayles, X. Chen et al., 2013 Genome analyses of the wheat yellow (stripe) rust pathogen *Puccinia striiformis* f. sp. *tritici* reveal polymorphic and haustorial expressed secreted proteins as candidate effectors. BMC Genomics 14: 270.

Casselton, L. A., and U. Kües, 2007 The origin of multiple mating types in the model mushrooms *Coprinopsis cinerea* and *Schizophyllum commune*, pp. 283–300 in Sex in Fungi, edited by Taylor, J. W., J. W. Kronstad, J. Heitman, and L. A. Casselton. American Society of Microbiology, Washington, DC.

Chen, W., C. Wellings, X. Chen, Z. Kang, and T. Liu, 2014 Wheat stripe (yellow) rust caused by *Puccinia striiformis* f. sp. *tritici*. Mol. Plant Pathol. 15: 433–446.

Cheng, P., X. M. Chen, and D. R. See, 2016 Grass hosts harbor more diverse isolates of *Puccinia striiformis* than cereal crops. Phytopathology 106: 362–371.

Coelho, M. A., J. P. Sampaio, and P. Gonçalves, 2010 A deviation from the bipolar-tetrapolar mating paradigm in an early diverged basidiomycete. PLoS Genet. 6: e1001052.

De Candolle, A. P., 1815 *Uredo rouille des céréales*, p. 83 in *Flora Française*, ou Descriptions Succinctes de Toutes les Plantes qui Croissent Naturelle- ment en France*, Disposées Selon une Novelle Méthode d’Analyse, et Pré- cédées par un Exposé des Principes Élémentaires de la Botanique, Vol. 6. Desray, Paris (in French).

Dufresne, S., C. A. Cuomo, Y.-C. Lin, A. Aerts, E. Tisserant et al., 2011 Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proc. Natl. Acad. Sci. USA 108: 9166–9171.

Eriksson, J., 1899 *Nouvelles Études sur la Rouille brune des Céréales*. Ann. Sci. Nat. Bot. 8: 241–288 (in French).

Fellers, J. P., B. M. Soltani, M. Bruce, R. Linning, C. A. Cuomo et al., 2013 Conserved loci of leaf and stem rust fungi of wheat share synteny interrupted by lineage-specific influx of repeat elements. BMC Genomics 14: 60.

Flor, H. H., 1942 Inheritance of pathogenicity in *Melampsora lini*. Phyto- pathology 32: 653–669.
Leonard, K. J., and L. J. Szabo, 2005 Stem rust of small grains and grasses caused by *Puccinia graminis*. Mol. Plant Pathol. 6: 99–111.

Levin, J. Z., M. Yassour, X. Adiconis, C. Nusbaum, D. A. Thompson et al., 2010 Comprehensive comparative analysis of strand-specific RNA sequencing methods. Nat. Methods 7: 709–715.

Li, H., and R. Durbin, 2010 Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26: 589–595.

Li, L., C. J. Stockert, and D. S. Roos, 2003 OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13: 2178–2189.

Line, R. F., 2002 Stripe rust of wheat and barley in North America: a retrospective historical review. Annu. Rev. Phytopathol. 40: 75–118.

Loegering, W. Q., and H. R. Powers, Jr., 1962 Inheritance of pathogenicity in a cross of physiological races 111 and 36 of *Puccinia graminis* f. sp. *triticit*. Phytopathology 52: 547–554.

Ma, L.-J., M. Rep, K. A. Borkovich, J. J. Coleman, M. Daboussi et al., 2010 *Pxaure* comparative genomics reveals lineage-specific chromosones related to pathogenicity. Nature 467: 367–373.

Maia, T. M., S. T. Lopes, J. M. G. C. Almeida, L. H. Rosa, J. P. Sampaio et al., 2015 Evolution of mating systems in basidiomycetes and the genetic architecture underlying mating-type determination in the yeast *Leucosporidium scottii*. Genetics 201: 75–89.

McIntosh, R. A., C. R. Wellings, and R. F. Park, 1995 *Wheat Ruts*. CSIRO, Melbourne.

Mendgen, K., 1984 Development and physiology of teliospores, pp. 375–398 in *The Cereal Ruts*, edited by Bushnell, W. R., and A. P. Roelfs. Academic Press, New York.

Narisawa, K., Y. Yamaoka, and K. Katsuya, 1994 Mating type of isolates derived from the spermogonial stage of *Puccinia coronata* var. *coronata*. Mycosenascience 35: 131–135.

Nemri, A., D. G. O. Saunders, C. Anderson, N. M. Upadhyaya, J. Win et al., 2014 The genome sequence and effector complement of the flax rust pathogen *Melampsora lini*. Front. Plant Sci. 5: 98.

Nieuwenhuis, B. P. S., S. Billiard, S. Vuilleumier, E. Petit, M. E. Hood et al., 2013 Evolution of uni- and bifactorial sexual compatibility systems in fungi. Heredity 111: 445–455.

Ordoñez, M. E., and J. A. Kolmer, 2009 Differentiation of molecular genotypes and virulence phenotypes of *Puccinia triticitica* from common wheat in *North America*. Phytopathology 99: 750–758.

Panwar, V., B. McCallum, and G. Bakkeren, 2013 Endogenous silencing of *Puccinia triticitica* pathogenicity genes through in planta-expressed sequences leads to the suppression of rust diseases on wheat. Plant J. 73: 521–532.

Parkhomchuk, D., T. Borodina, V. Amstislavskiy, M. Banaru, L. Hallen et al., 2009 Transcripome analysis by strand-specific sequencing of comple-mentary DNA. Nucleic Acids Res. 37: e123.

Parra, G., K. Bradmam, and I. Korff, 2007 CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. Bioinformatics 23: 1061–1067.

Petit, E., T. Giraud, D. M. de Vienne, M. A. Coelho, G. Aguilera et al., 2012 Linkage to the mating-type locus across the genus *Microbotryum*: insights into nonrecombining chromosomes. Evolution 66(6): 3519–3533.

Petre, B., D. L. Joly, and S. Duplessis, 2014 Effector proteins of rust fungi. Front. Plant Sci. 5: 416.

Raffaele, S., and S. Knoum, 2012 Genome evolution in filamentous plant pathogens: why bigger can be better. Nat. Rev. Microbiol. 10: 417–430.

Raudaskoski, M., and E. Kothe, 2010 Basidiomycete mating type genes and pheromone signaling. Eukaryot. Cell 9: 847–859.

Rodriguez-Algaba, J., S. Walter, C. K. Serensen, M. S. Hovmoller, and A. F. Justesen, 2014 Sexual structures and recombination of the wheat rust fungus *Puccinia striiformis* on *Berberis vulgaris*. Fungal Genet. Biol. 57: 77–85.

Saari, E. E., H. C. Young, and M. F. Kernkamp, 1968 Infection of North American Thalictrum sp. with *Puccinia recondita* f. sp. *triticitica*. Phytopa-thology 58: 939–943.

Samborski, D. J., and P. L. Dyck, 1968 Inheritance of virulence in wheat leaf rust on the standard differential wheat varieties. Can. J. Genet. Cytol. 10: 24–32.
Savile, D. B. O., 1976 Evolution of the rust fungi (uredinales) as reflected by their ecological problems, pp. 137–207 in Evolutionary Biology, edited by Hecht, M. K., W. C. Steere, and B. Wallace. Springer, Berlin.

Savile, D. B. O., 1984 Taxonomy of the cereal rust fungi, pp. 79–112 in The Cereal Rusts, edited by Bushnell, W. R., and A. P. Roelfs. Academic Press, Orlando, FL.

Scherer, M., K. Heimel, V. Starke, and J. Kämper, 2006 The Clp1 protein is required for clamp formation and pathogenic development of Ustilago maydis. Plant Cell 18: 2388–2401.

Schirawski, J., B. Heinze, M. Wagenknecht, and R. Kahmann, 2005 Mating type loci of Sporisorium reilianum: novel pattern with three a and multiple b specificities. Eukaryot. Cell 4: 1317–1327.

Singh, R. P., J. Huerta-Espino, and A. P. Roelfs, 2002 The wheat rusts, in Bread Wheat FAO Plant Production and Protection Series, Vol. 30, edited by Curtis, B. C., S. Rajaram, and H. Gómez Macpherson. FAO, Rome http://www.fao.org/docrep/006/y4011e/y4011e00.htm#Contents.

Sperschneider, J., D. M. Gardiner, J. M. Taylor, J. K. Hane, K. B. Singh et al., 2013 A comparative hidden Markov model analysis pipeline identifies proteins characteristic of cereal-infecting fungi. BMC Genomics 14: 807.

Stammers, D. K., J. Ren, K. Leslie, C. E. Nichols, H. K. Lamb et al., 2001 The structure of the negative transcriptional regulator NmrA reveals a structural superfamily which includes the short-chain dehydrogenase/reductases. EMBO J. 20: 6619–6626.

Statler, G. D., 1979 Inheritance of pathogenicity of culture 70–1, Race 1, of Puccinia recondita tritici. Phytopathology 69: 661.

Statler, G. D., 1982 Inheritance of virulence of Puccinia recondita Lsp. tritici on durum and spring wheat cultivars. Phytopathology 72: 210–213.

Statler, G. D., 2000 Inheritance of virulence of Puccinia triticina culture X47, the F1 of the cross 71–112 x 70–1. Can. J. Plant Pathol. 22: 276–279.

Storey, J. D., and R. Tibshirani, 2003 Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. USA 100: 9440–9445.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar, 2014 MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Upadhyaya, N. M., D. P. Garnica, H. Karaoglu, J. Sperschneider, A. Nemri et al., 2014 Comparative genomics of Australian isolates of the wheat stem rust pathogen Puccinia graminis f. sp. tritici reveals extensive polymorphism in candidate effector genes. Front. Plant Sci. 5: 759.

Urban, M., R. Kahmann, and M. Bölker, 1996 Identification of the pheromone response element in Ustilago maydis. Mol. Gen. Genet. MGG 251: 31–37.

Wahl, R., A. Zahiri, and J. Kämper, 2010 The Ustilago maydis b mating type locus controls hyphal proliferation and expression of secreted virulence factors in planta. Mol. Microbiol. 75: 208–220.

Wells, C. R., D. G. Wright, F. Keiper, and R. Loughman, 2003 First detection of wheat stripe rust in Western Australia: evidence for a foreign incursion. Australas. Plant Pathol. 32: 321–322.

Williams, L. J. S., D. G. Tabbaa, N. Li, A. M. Berlin, T. P. Shea et al., 2012 Paired-end sequencing of Fosmid libraries by Illumina. Genome Res. 22: 2241–2249.

Xu, J., R. Linning, J. Fellers, M. Dickinson, W. Zhu et al., 2011 Gene discovery in EST sequences from the wheat leaf rust fungus Puccinia triticina sexual spores, asexual spores and haustoria, compared to other rust and corn smut fungi. BMC Genomics 12: 161.

Yi, R., T. Tachikawa, M. Ishikawa, H. Mukaiyama, D. Bao et al., 2009 Genomic structure of the A mating-type locus in a bipolar basidiomycete, Pholiota nameko. Mycol. Res. 113: 240–248.

Yin, C., X. Chen, X. Wang, Q. Han, Z. Kang et al., 2009 Generation and analysis of expression sequence tags from haustoria of the wheat stripe rust fungus Puccinia striiformis f. sp. tritici. BMC Genomics 10: 626.

Zambino, P. J., and L. J. Szabo, 1993 Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. Mycologia 85: 401–414.

Zheng, W., L. Huang, J. Huang, X. Wang, X. Chen et al., 2013 High genome heterozygosity and endemic genetic recombination in the wheat stripe rust fungus. Nat. Commun. 4: 2673.

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