REVIEW OPEN ACCESS

Genomics Research on the Road of Studying Biology and Virulence of Cereal Rust Fungi

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ABSTRACT

Rust fungi are highly destructive pathogens that pose a significant threat to crop production worldwide, especially cereals. Obligate biotrophy and, in many cases, complex life cycles make rust fungi particularly challenging to study. However, recent rapid advances in sequencing technologies and genomic analysis tools have revolutionised rust fungal research. It is anticipated that the increasing availability and ongoing substantial improvements in genome assemblies will propel the field of rust biology into the post-genomic era, instigating a cascade of research endeavours encompassing multi-omics and gene discoveries. This is especially the case for many cereal rust pathogens, for which continental-scale studies of virulence have been conducted over many years and historical collections of viable isolates have been sequenced and assembled. Genomic analysis plays a crucial role in uncovering the underlying causes of the high variability of virulence and the complexity of population dynamics in rust fungi. Here, we provide an overview of progress in rust genomics, discuss the strategies employed in genomic analysis, and elucidate the strides that will drive cereal rust biology into the post-genomic era.

1 | Introduction

Rust fungi, belonging to the phylum *Basidiomycota*, are a diverse and destructive group of plant pathogens that have impacted human civilizations significantly. As largely obligate biotrophs, rust fungi are not easily cultured on artificial media and rely on living plant hosts for their growth and reproduction (Lorrain et al. 2019; Voegele and Mendgen 2011). Rust pathogens produce large quantities of orange, yellow or brown spores on leaves, leaf sheaths, stems and/or fruits. In severe cases, a dense accumulation of spores results in rapid plant death due to excessive water loss (Guest and Brown 1997). Rust disease poses a serious threat to important cereal crops such as wheat, barley, oats, rye, triticale and maize, thereby affecting global food security (Zhao

et al. 2016; Chaves et al. 2008). The historical impact of rust fungi dates back to ancient times. The first knowledge of famine caused by rust can be traced back to Joseph in Egypt in 1800 BC (Carefoot and Sprott 1967). In 1767, the Italians Fontana and Tozzetti independently made the first unequivocal and detailed reports on wheat stem rust (Fontana 1932; Tozzetti 1952). Additional records of grain rust can be found in ancient texts such as the Bible and the Greek and Roman documents (McIntosh et al. 1995). The devastating Ug99, a lineage of the wheat stem rust pathogen, *Puccinia graminis* f. sp. *tritici*, was first detected in Uganda in 1998 and has since caused epidemics in Africa and the Middle East (Singh et al. 2011; Li, Upadhyaya, et al. 2019). Very recently, it was detected in Nepal (Patpour et al. 2024), which may have a huge impact on wheat production in South Asia.

Within species of rust fungi, it is common to find morphologically indistinguishable variants that are adapted to parasitism on different host species. Jakob Eriksson, a Swedish plant pathologist, was the first to coin the term forma specialis (abbreviated f. sp.) to designate these variants (Eriksson 1894). Cereal rust species have been classified into different formae speciales based on host specificity and morphological characteristics. For instance, *P. graminis* f. sp. *tritici* (Pgt) and *P. striiformis* f. sp. *tritici* (Pst) cause stem rust and stripe rust on wheat, respectively; *P. graminis* f. sp. *tritici* (Pgt), *P. hordei* (Ph), *P. coronata* f. sp. *hordei* (Pch), and *P. striiformis* f. sp. *hordei* (Psh) cause stem rust, leaf rust, crown rust, and stripe rust on barley, respectively; *P. graminis* f. sp. *avenae* (Pga) and *P. coronata* f. sp. *avenae* (Pca) cause stem rust and crown rust on oat, respectively; *P. graminis* f. sp. *secalis* (Pgs) and *P. recondita* f. sp. *secalis* (Prs) cause stem rust and leaf rust on rye, respectively; and *P. sorghi* f. sp. *zeae* (Psz) and *P. polysora* f. sp. *zeae* (Ppz) cause common and southern corn rust on maize, respectively.

Despite the economic importance of cereal rusts, difficulties in artificial culture due to obligate biotrophy and complex and polymorphic life cycles have impeded progress in rust research. The rust biology research community recognised the necessity to obtain the first rust genome sequences approximately 15 years ago. The first sequenced rust genomes, *Melampsora laricipopulina* (101 Mb) and Pgt (89 Mb), marked a breakthrough in rust genomics (Duplessis et al. 2011). Since then, advances in sequencing technologies have facilitated the assembly of numerous cereal rust genomes, from fragmented-hybrid assemblies to more recent nearly gap-free and phased genome assemblies (Table 1). These efforts have revealed fundamental genomic characteristics of rust fungi, including their large genome sizes, high repeat content, gene family expansions, and extensive structural variations.

In this review, we describe the progress in cereal rust genome research over the past decade, with a focus on the latest genomics-accelerated discoveries related to genome composition, pathogen virulence and population dynamics. We discuss the most recent strategies and pipelines developed for the study of rust genomics. Furthermore, we identify and discuss emerging research trends in cereal rust biology facilitated by genomics research.

2 | Cereal Rust Genomics

2.1 | Rust Pathogen Genomes

Because of the complex life cycles and diverse host ranges of cereal rusts, almost all studies of host infection by rust fungi after sequencing purified fungal materials have used urediniospores, which are dikaryotic and remarkably heterozygous (Voegele et al. 2009). Genome sequencing studies have revealed that rust fungi have large genomes compared to other plant-pathogenic fungi. Flow cytometry analysis of 30 rust species revealed that genome sizes varied over 10-fold, from 70 to 893 Mb, with an average genome size of 380.2 Mb (Tavares et al. 2014). The largest rust genome reported is that of *Uromyces bidentis*, estimated to be more than 2.4 Gb (Ramos et al. 2015). For cereal rust fungi, there are significant variations in genome sizes among families, genera, species, and even isolates, with a haplotype-phased genome assembly size that could range from around 150 Mb to 1.71 Gb (Table 1).

The large genome size of rust fungi can be attributed to their heteroecious and macrocyclic nature, as well as the invasion of transposable elements (TEs) (Aime et al. 2017). The long-term accumulation of LTR retrotransposons (LTR-RTs) has greatly contributed to genome expansion in rust fungi (Li, Qiao, et al. 2023). According to published reports, the TE content in cereal rust pathogen genomes is very high, accounting for 17.8%–85% of the whole genome assemblies (Aime et al. 2017; Li, Qiao, et al. 2023; Krishnan et al. 2018; Raffaele and Kamoun 2012; Liang et al. 2023). The results comparing the genomes of Pt, Pst and Pgt showed that Pt had the largest genome of the three, estimated at 135 Mb, with expansion due to TEs and repeats encompassing 50.9% of contig bases; in comparison, repeats occupied 31.5% for Pst and 36.5% for Pgt (Cuomo et al. 2017). Moreover, the identification and classification of TEs showed that more than half of the genome of strain Pst-104E was repeat elements and the composition of the TE superfamily was very similar, regardless of primary contigs or haplotypes (Schwessinger et al. 2018). In addition, a comprehensive whole-genome comparative genomic analysis was conducted on two strains: Pst-104E, originating from a strictly clonal population (PstS0 lineage), and Pst-DK0911, derived from sexual reproduction (PstS7 lineage). The comparison showed that the difference in genome size between Pst-DK0911 and Pst-104E was attributable to variations in the content of TEs (Schwessinger et al. 2020). Notably, the gigascale rust Ppz genome contains 85% repeats, which is among the highest repeat content reported to date for rust fungi. The large genome size is largely attributed to a significant historical expansion of TEs (Liang et al. 2023).

Rust genomes also exhibit a higher average range of predicted genes compared to other basidiomycetes, and significant intraspecific differences were observed (Cuomo et al. 2017; Kiran et al. 2016). High-quality assembled genomes have shown clearer patterns of gene content and the genomic landscape of cereal rust fungi. Comparative genomic analyses of rust fungi have shown that gene families underwent a large expansion and the number of genes increased during the evolutionary process (Raffaele and Kamoun 2012; Aime et al. 2017). Compared with the wheat rust pathogens, Ppz underwent a massive genome expansion during the evolutionary process (Liang et al. 2023). Moreover, it was shown that the Ppz isolate Ppz-GD1913 has more predicted genes than its two closely related cereal rust pathogens Pca and Pst. In addition, the annotation of expanded gene families in rust fungi showed that most proteins had unknown functions that were speculated to include virulence effector genes, different types of transporters, transcription factors, and functions associated with nucleic acid interactions and modifications (Aime et al. 2017). These special gene expansions may reveal the successful specialisation of rust pathogens.

2.2 | Genomic Approaches to Effector Gene Identification

The emergence of new virulent strains of rust fungi poses a significant threat to global cereal production. Rust pathogens and their host plants often interact through a gene-for-gene

TABLE 1 | Cereal rust pathogens genomes available.

Species	Strain	Total assembly size (Mb)[a]	GC (%)	Coding genes	Strategy	Phasing condition	Reference
Pgt	CDL 75-36-700-3	88.6	42.35	15,800	Sanger	Unphased	Duplessis et al. 2011
Pgt	21-0	92	—	22,391	Illumina	Unphased	Upadhyaya et al. 2015
Pgt	59KS19	93.3	—	18,166	PacBio CLR + Illumina	Unphased	Rutter et al. 2017
Pgt	99KS76A-1	107.31	—	18,777	PacBio CLR + Illumina	Unphased	Rutter et al. 2017
Pgt	Pgt 21-0	176.9	—	—	PacBio CLR + Illumina + Hi-C	Phased	Li, Upadhyaya, et al. 2019
Pgt	Ug99	176.2	—	—	PacBio CLR + Illumina + Hi-C	Phased	Li, Upadhyaya, et al. 2019
Pgt	UK-01	164.3	43.45	—	Nanopore + Illumina	Unphased	Kangara et al. 2020
Pgt	99KS76A-1	181	—	22,974	PacBio CLR + Illumina + Hi-C	Phased	Guo et al. 2022
Pt	77	100.6	46.64	27,678	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-1	95.12	46.71	32,824	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-2	97.25	46.71	32,769	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-3	97.33	46.71	32,894	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-4	97.22	46.71	32,745	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-5	97.25	46.71	32,851	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-6	97.22	46.71	32,822	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-7	97.26	46.71	32,757	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-8	96.41	46.71	32,366	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-9	96.14	46.71	32,211	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-10	93.66	46.71	32,180	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-11	93.54	46.71	32,203	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-a	97.02	46.69	32,772	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	77-A1	97.25	46.71	32,747	454 sequencing + Sanger	Unphased	Kiran et al. 2016
Pt	106	106.5	46.62	26,384	454 sequencing	Unphased	Kiran et al. 2016
Pt	BBBD	135.34	46.72	14,880	Illumina	Unphased	Cuomo et al. 2017
Pt	Pt104	268.5	46.7	29,043	PacBio CLR + Illumina	Unphased	Wu et al. 2020
Pt	Pt64	295.3	46.64	59,692	PacBio CLR + Illumina + Hi-C	Phased	Wu et al. 2021

(Continues)

TABLE 1 | (Continued)

Species	Strain	Total assembly size (Mb)[a]	GC (%)	Coding genes	Strategy	Phasing condition	Reference
Pt	Pt76	260.3	46.3	—	PacBio CCS (or HiFi)+Nanopore + Illumina + Hi-C	Phased	Duan et al. 2022
Pt	Pt15	243.93	46.63	25,429	PacBio CCS (or HiFi)+Illumina + Hi-C	Phased	Li, Qiao, et al. 2023
Pt	19NSW04	245.9	46.6	36,077	Pacbio CCS (or HiFi)+Hi-C	Phased	Sperschneider et al. 2023
Pt	20QLD87	245.1	46.6	37,756	Pacbio CCS (or HiFi)+Hi-C	Phased	Sperschneider et al. 2023
Ph	Ph560	206.91	41.65	25,543	PacBio CLR+Illumina	Unphased	Chen et al. 2019
Pst	PST-130	64.8	44.1	18,149	Illumina	Unphased	Cantu et al. 2011
Pst	CYR32	130.7	44.77	25,288	Illumina + Sanger (fosmid-to-fosmid)	Unphased	Zheng et al. 2013
Pst	P5T-21	73	43.9	20,653	Illumina	Unphased	Cantu et al. 2013
Pst	P5T-43	71	43.3	21,036	Illumina	Unphased	Cantu et al. 2013
Pst	P5T-87/7	53	44.5	20,688	Illumina	Unphased	Cantu et al. 2013
Pst	P5T-08/21	56	44.5	20,875	Illumina	Unphased	Cantu et al. 2013
Pst	PST-78	117.31	44.43	19,542	454 sequencing + Illumina	Unphased	Cuomo et al. 2017
Pst	31 (67S 64)	66.26	44.44	18,362	Illumina	Unphased	Kiran et al. 2016
Pst	K (47S 102)	69.77	44.41	18,880	Illumina	Unphased	Kiran et al. 2016
Pst	46S 119	70.24	44.41	19,795	Illumina	Unphased	Kiran et al. 2016
Pst	93–210	84.62	44.39	16,513	PacBio CLR+Illumina	Unphased	Xia et al. 2018
Pst	104E	156	44.4	30,249	PacBio CLR+Illumina	Phased	Schwessinger et al. 2018
Pst	11–281	144.84	44.37	29,014	PacBio CLR+Illumina	Phased	Li, Wang, et al. 2019
Pst	DK0911	126.07	—	25,873	PacBio CLR+Illumina	Phased	Schwessinger et al. 2020
Pst	PST-130	155.1	44.4	32,054	PacBio CLR+Illumina	Phased	Vasquez-Gross et al. 2020
Pst	CYR34	82.67h	44.37	17,095	PacBio CLR+Illumina + Hi-C	Unphased	Xia, Huang, et al. 2022
Pst	93–210	83.95h	44.39	17,946	PacBio CLR+Illumina + Hi-C	Unphased	Xia, Huang, et al. 2022
Pst	110S119	58.62	44.5	13,744	Illumina	Unphased	Yadav et al. 2022
Pst	46S119	58.33	44.4	15,163	Illumina	Unphased	Yadav et al. 2022
Pst	78S84	55.78	44.4	13,758	Illumina	Unphased	Yadav et al. 2022

(Continues)

TABLE 1 | (Continued)

Species	Strain	Total assembly size (Mb)[a]	GC (%)	Coding genes	Strategy	Phasing condition	Reference
Pst	134 E16 A+ 17+ 33+	167.67	—	34,075	Nanopore + Illumina + Hi-C	Phased	Schwessinger et al. 2022
Pst	AZ2	151.5	44.4	30,096	Pacbio CCS (or HiFi) + Hi-C	Phased	Wang, Xu, et al. 2024
Psh	93TX-2	77.36	44.4	15,976	PacBio CLR + Illumina	Unphased	Xia et al. 2018
Pca	12SD80	150.5	44.7	26,796	PacBio CLR + Illumina	Phased	Miller et al. 2018
Pca	12NC29	166.2	44.7	28,801	PacBio CLR + Illumina	Phased	Miller et al. 2018
Pca	203	208.1	44.64	36,493	PacBio CLR + Illumina + Hi-C	Phased	Henningsen et al. 2022
Psz	RO10H11247	99.62	—	21,087	Illumina	Unphased	Rochi et al. 2018
Ppz	GD1913	1710	40	47,512	PacBio CCS (or HiFi) + Illumina + Hi-C	Phased	Liang et al. 2023

Abbreviations: Hi-C, chromosome conformation capture sequencing; PacBio CCS (or HiFi), circular consensus sequencing (or High Fidelity sequencing); PacBio CLR, continuous long read (CLR) sequencing; Pca, *Puccinia coronata* f. sp. *avenae*; Pgt, *P. graminis* f. sp. *tritici*; Ph, *P. hordei*; Ppz, *P. polysora* f. sp. *zeae*; Psh, *P. striiformis* f. sp. *hordei*; Pst, *P. striiformis* f. sp. *tritici*; Psz, *P. sorghi*; Pt, *P. triticina*.

[a]A haploid assembly size with 'h'.

system (Flor 1959), where pathogen avirulence (Avr) proteins (effectors) are recognised by corresponding host resistance (R) proteins, triggering defence responses (Sharpee and Dean 2016; Flor 1971; Ramirez-Prado et al. 2018; De Wit et al. 2009). The four models describing these R–Avr interactions in host–pathogen systems contributing to plant immune responses include the elicitor–receptor model, the guard model, the decoy model and the integrated decoy model (Petit-Houdenot and Fudal 2017; Catanzariti et al. 2010; Steinbrenner et al. 2015; Dangl and Jones 2001; van der Hoorn and Kamoun 2008; Cesari et al. 2014; Le Roux et al. 2015). Mutations in Avr products can disrupt recognition by R proteins across all four models, leading to pathogen evasion and resistance breakdown, which can be adequately evaluated by genome-driven surveillance approaches.

Genomic analyses have been instrumental in identifying *Avr* genes and understanding R–Avr interactions, which are crucial for *R* gene deployment in crop breeding (Prasad et al. 2019). While many rust *R* genes have been identified in cereals, only a few *Avr* genes were characterised. Notable examples include AvrRpg1, AvrSr50, AvrSr35, AvrSr27, AvrSr13 and AvrSr22 in Pgt, shown to be recognised by their paired NLR immune receptors (Nirmala et al. 2011; Chen et al. 2017; Salcedo et al. 2017; Upadhyaya et al. 2021; Arndell et al. 2024). Genomic accelerated approaches, such as whole-genome sequencing, transcriptome and comparative genomics, have facilitated the identification of these *Avr* genes. *AvrSr50* and *AvrSr27* were identified from spontaneous mutations, *AvrSr35* from chemically induced mutagenesis, and *AvrSr13* and *AvrSr22* through large-scale effector library screening (Chen et al. 2017; Salcedo et al. 2017; Upadhyaya et al. 2021). Their subcellular localisation and expression patterns suggest distinct roles in the establishment of host infection prior to or during haustoria formation (Nirmala et al. 2011; Upadhyaya et al. 2021; Salcedo et al. 2017; Lubega et al. 2024). Different patterns of virulence evolution were also revealed by the cloning of two Ppz *R* genes (*RppC* and *RppK*) and the cognate *Avr* genes (*AvrRppC* and *AvrRppK*, supported by recent functional genomics studies (Chen et al. 2022; Deng et al. 2022). Allelic variation in *AvrRppC* directly determines the effectiveness of *RppC*-mediated resistance to Ppz (Deng et al. 2022). In contrast, *AvrRppK* cloning showed that it is conserved in all tested isolates of Ppz, elucidating the molecular mechanism by which the *RppK* gene confers broad-spectrum resistance to Ppz and a putative role of *AvrRppK* in the suppression pattern-triggered immunity (Chen et al. 2022).

Plant fungal pathogen *Avr* genes evolve rapidly under selection pressure, allowing pathogens to evade host R protein recognition and cause disease outbreaks. The current discovery of cereal rust *Avr* genes has been exclusively based on large-scale screening of candidate secreted effector proteins (CSEPs), which are believed to exhibit taxonomic specificity and be enriched in haustoria crucial for infection (Duplessis et al. 2013; Petre et al. 2014). Despite often lacking conserved motifs, CSEPs share common characteristics such as secretion signals, small size, and cysteine enrichment, aiding their identification (Duplessis et al. 2011; Sperschneider et al. 2016). These criteria, combined with genomics and transcriptomics, have been applied to effector mining pipelines, revealing

TABLE 2 | Catalogue of currently identified/predicted candidate secreted effector proteins (CSEPs) in cereal rust pathogens.

Species	Isolate/race	Effector	Function	Reference
Pgt	Race MCCF	PGTG_10537.2, PGTG_16791	Triggering RPG1-mediated host hypersensitive response (HR) and resistance	Nirmala et al. 2011
Pgt	21-0	PGTAUSPE10-1	Triggering host cell death	Upadhyaya et al. 2015
Pgt	Pgt279 and Pgt632	AvrSr50	Race-specific Avr recognised by paired R	Chen et al. 2017; Ortiz et al. 2022
Pgt	99KS76A-1	AvrSr35	Race specific Avr recognised by paired R	Salcedo et al. 2017; Bolus et al. 2020; Förderer et al. 2022; Zhao et al. 2022
Pgt	Pgt7A	PgtSR1	Suppressing RNA silencing in host plants and impeding plant defences	Yin et al. 2019
Pgt	21-0	AvrSr27	Race-specific Avr recognised by paired R; zinc-bound effector	Upadhyaya et al. 2021; Outram et al. 2024
Pgt	21-0	AvrSr13, AvrSr22	Race-specific Avrs recognised by paired R	Arndell et al. 2024
Pst	98AG31	PST02549	Suppressing host HR and basal immunity	Petre et al. 2016
Pst	GB75/30c	PEC6	Suppressing pattern-triggered immunity (PTI) and targeting host adenosine kinases	Liu et al. 2016
Pst	PST-130	PNPi	Suppressing host basal immunity targeting NPR1	Wang et al. 2016
Pst	PST-100	Shr7	Suppressing host production of reactive oxygen species (ROS)	Ramachandran et al. 2017
Pst	PST-78	PstSCR1	Host cell death elicitor	Dagvadorj et al. 2017
Pst	CYR32	Pst-milR1	Suppressing host immunity	Wang et al. 2017
Pst	CYR32	PST_8713	Host PTI suppression	Zhao et al. 2018
Pst	CYR32	PstGSRE1	Suppressing host programmed cell death	Qi et al. 2019
Pst	CYR32	PST_12806	Suppressing host HR and basal immunity	Xu et al. 2019
Pst	PST-78	PstCTE1	Chloroplast translocation in planta	Andac et al. 2020

(Continues)

TABLE 2 | (Continued)

Species	Isolate/race	Effector	Function	Reference
Pst	CYR32	PNPi	Suppressing host multiple defence responses	Bi et al. 2020
Pst	CYR32	PST_18363	Suppressing host ROS accumulation	Yang et al. 2020
Pst	CYR31	Pst13661	Suppressing host BCL2 associated X protein (BAX)-induced cell death	Xu et al. 2020
Pst	CYR32	PSEC2, PSEC17	Host PTI suppression	Su et al. 2021
Pst	CYR32	Pst_4, Pst_5	Interfering with host chloroplast-mediated defence	Wang et al. 2021
Pst	PstTR	PSTG_10917	Suppressing host cell death	Ozketen et al. 2020
Pst	CYR31	Pst27791	Interfering with host immunity	Wan et al. 2022
Pst	CYR31	PstCFEM1	Suppressing host ROS accumulation	Bai et al. 2022
Pst	CYR32	PstCEP1 (PSTG_13342)	Suppressing host PTI and effector-triggered immunity (ETI)	Tao et al. 2020
Pst	CYR31	Pst_A23	Regulating host pre-mRNA splicing and interfering with host immunity	Tang et al. 2022
Pst	CYR31	PsSpg1	Suppressing host ETI	Wang, Tang, et al. 2022
Pst	Pst-104E; Yr9 vir. (46S 119)	Pst_13960	Suppressing BAX-induced host cell death	Jaswal, Dubey, et al. 2021
Pst	Yr9 vir. (46S 119)	Pstr-11,677, Pstr_09735	Suppressing host cell death	Jaswal, Rajarammohan, et al. 2021
Pst	CYR32	PSTha5a23	Host PTI suppression and related to pathogenicity	Cheng et al. 2017
Pst	CYR32	PstGSRE4	Reducing host H_2O_2 accumulation and HR	Liu et al. 2022
Pst	CYR32	PSTG_01766	Suppressing high-temperature seedling resistance	Hu et al. 2022
Pt	Pt104	AvrLr26, AvrLr2a, AvrLr3ka	In silico prediction	Wu et al. 2020
Pt	20 Pt isolates; Pt races (10, 26, 122, 135, 162, 104, and 68)	AvrLr20	In silico prediction	Wu et al. 2017
Pt	PHNT (isolate 07-10-426-1)	Pt1234	Modulating host immunity by targeting host transcription factor	Geng et al. 2024

(Continues)

TABLE 2 | (Continued)

Species	Isolate/race	Effector	Function	Reference
Pt	PHNT (isolate 07-10-426-1)	AvrLr15	Inducing Lr15-dependent immune responses	Cui et al. 2024
Pt	PHNT (isolate 07-10-426-1)	AvrLr21	Directly interacting with Lr21	Shen et al. 2024
Pca	60 Pca isolates collected from across the United States in 1990 and 2015	PCANC_11327 (AvrPc14), PCANC_10462 (AvrPc50), PCANC_18844 (AvrPc51), PCANC_25211 (AvrPcStainless)	In silico prediction	Miller et al. 2020
Ppz	Multiple isolates of collection	AvrRppK	Recognised by RppK; conserved in multiple isolates; PTI suppression	Chen et al. 2022
Ppz	PP.CN1.0, PP.CN2.0, and PP.CN3.0	AvrRppC	Race-specific Avr recognised by paired R	Deng et al. 2022

Abbreviations: Pca, *Puccinia coronata* f. sp. *avenae*; Pgt, *P. graminis* f. sp. *tritici*; Ppz, *P. polysora* f. sp. *zeae*; Pst, *P. striiformis* f. sp. *tritici*; Pt, *P. triticina*.

catalogues of extremely abundant rust CSEPs including Avrs (Table 2). The development of tools for predicting effectors and the increasing number of CSEPs have led to the emergence of a new research area known as effectoromics (Du et al. 2014; Sonah et al. 2016).

Historically, the gene-for-gene relationship between rust pathogen *Avr* genes and host *R* genes was established through genetic studies (Samborski and Dyck 1968; Dyck and Samborski 1982). Early methods for effector identification included map-based cloning, fungal secretome analysis and expressed sequence tag (EST) screening (van Kan et al. 1991; Torto et al. 2003; Guyon et al. 2014). Comparative genomics of Pgt wild-type and mutants pioneered the identification of the first cereal rust Avrs (Chen et al. 2017; Salcedo et al. 2017). The advent of computational tools (illustrated in Figure S1) has further refined CSEP prediction, leveraging machine-learning algorithms such as FunEffector-Pred (Wang et al. 2020) and Effector-GAN (Wang, Luo, and Zou 2022). More recently, whole-genome mining and functional genomics-based validation using wheat and maize protoplast systems have enhanced *Avr–R* gene pair screening (Chen et al. 2022; Deng et al. 2022; Arndell et al. 2024; Wilson et al. 2024). It is expected that advances in these genomic approaches will continue to accelerate effector gene discovery and their application in crop protection strategies.

2.3 | Genomic Variation and Pathogen Virulence

Diverse virulence is a typical feature of cereal rusts due to the high abundance of variations within the pathogen genomes arising at each stage of the macrocyclic, heteroecious life cycles (Figure 1). Research has shown that variation in virulence in rust pathogens leads to the loss of disease resistance in cereal varieties. As the saying goes, "Rust never sleeps", emphasising the ongoing efforts required to combat rust diseases (Prasad et al. 2020). To effectively control rust diseases in cereals, it is essential to gain a better understanding of the mechanisms underlying virulence variation in rust fungi, particularly those associated with the emergence of strains with new virulence profiles.

Different mechanisms in the sexual and asexual stages on different hosts may contribute to the generation of new cereal rust races, significantly influencing the evolutionary potential of rust pathogens (Figueroa et al. 2020). Cereal rusts can produce new virulence combinations on alternate host plants during sexual reproduction (Figure 1). For example, the role of sexual recombination in generating genetic variation in Pgt, facilitated by its sexual cycle on barberry, has been well established, creating new varieties through genetic recombination (Villegas et al. 2022; Olivera et al. 2019; Berlin et al. 2013; Jin 2011; Roelfs 1982). A study demonstrated that the large-scale eradication of *Berberis* in the United States between 1918 and 1974 significantly reduced the genetic variability of Pgt isolates (Roelfs 1982; Peterson et al. 2005). However, the sexual reproduction process of wheat stripe rust fungus was not discovered until 2010 in barberry (Jin et al. 2010). Since then, many studies have shown that Pst that infects barberry can produce a highly diverse progeny population through self-crossing or hybridisation,
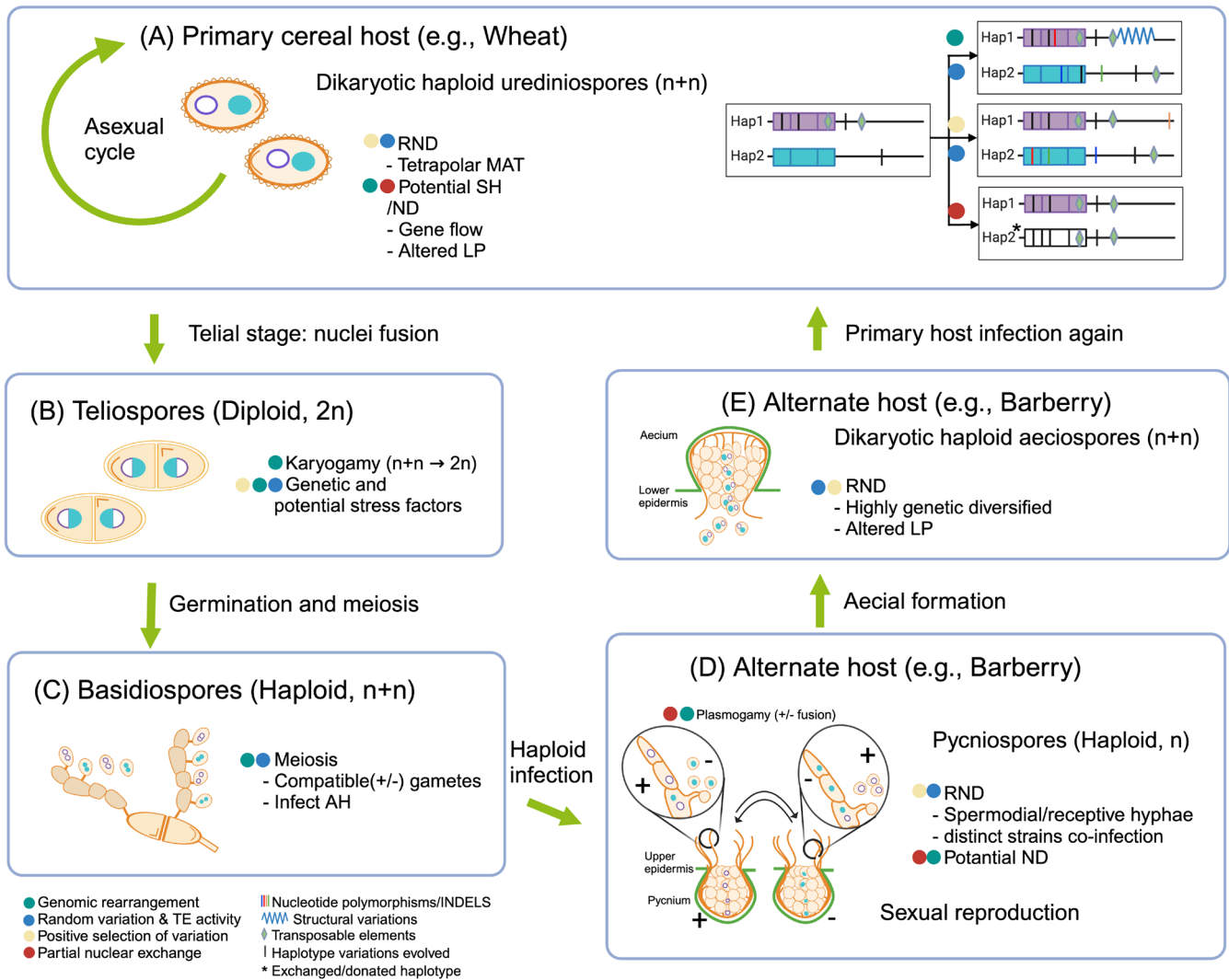
FIGURE 1 | Schematic representation of genomic variations arising at each stage of the macrocyclic, heteroecious life cycle of cereal rusts. Overall high mutation rate in cereal rusts is along with spontaneous mutations, movements of high abundance of transposable elements (TEs), and genome rearrangements in all steps (A–E) involving fungal nuclear divisions, genetic processes such as karyogamy and meiosis, and the drive by host and environmental pressures (stress factors). Selection (e.g., by host resistance) shapes which variants persist, with avirulent genes frequently under positive selection for instance. Noted that the colours of nucleus do not necessarily indicate the same strains and actual genotypes. Dikaryotic $(n+n)$ urediniospores on the "primary" cereal host (e.g., wheat) (A) can undergo continuous asexual cycle, repeated nuclear divisions (RND), and there are potential for somatic hybridisation (SH; nuclear exchange and/or followed mitotic and genomic recombination between dikaryotic hyphae) with the presence of host co-infection by mycelium of distinct strains. The two nuclei carry linked or unlinked mating-type (MAT) loci forming a tetrapolar system, which further governs genomic organisation and segregation patterns through the whole macrocycle. Compatibility depends on the identity of alleles at both MAT loci. Predominant genome studies of cereal rust pathogens (reviewed here in the main text) so far on haplotype genomes (A, right panel) from this stage have depicted extensive haplotype variations; local population (LP) adapted gene flows, sequence polymorphisms/small insertion/deletions (indels), genomic rearrangements and structural variations including insertion, inversion, translocation and copy number variation; partial loss of heterozygosity (probably due to propagations of unstable chromosomal regions under stress within host cells); as well as large genomic recombination such as nucleus donation (ND) or exchange. (B) Under factors such as race type, temperature, and host variety, teliospore stage may occur on sites of infection, where the two haploid nuclei fuse via karyogamy. The emerged diploid phase provides gateway through sexual production cycle. (C) Germination and meiosis occur within the teliospore, yielding haploid basidiospores (n); each carries one mating locus allele set (from the tetrapolar system) generating genetically unique propagules and are infectious on alternate host (AH; e.g., *Berberis* spp.), initiating the haploid phase. (D) In the alternate host, pycniospores (haploid, n) form in specialised structures (pycnia or spermogonia) enabling the fusion (plasmogamy) of compatible haploid cells. This re-establishes the dikaryotic condition and leads to the production of aeciospores $(n+n)$ (E), which can reinfect the primary hosts. Icons indicate key genome variation mechanisms: Genomic rearrangement and partial nuclear exchange (possible via asexual routes and bi-mon during sexual routes where dikaryon hyphae fusion adjacent to haploid spermogonium); random variation and TE activity generating mutations and structural changes; positive selection of variants (e.g., during host co-evolving of virulence and under temperature stresses; SNPs/multiple nucleotide polymorphisms [MNPs]/indels), and structural variations adding new layers of allelic variations and haplotype evolution in the local pathogen populations and disperse distantly.

indicating sexual reproduction plays an important role in the process of virulence variation of the wheat stripe rust pathogen (Tian et al. 2016; Wang et al. 2018; Xia et al. 2020). Tian et al. (2016) used 24 wheat stripe rust-resistant near-isogenic lines to measure the virulence of Pst race V26 (Pinglan 17–7) and its sexual progeny. Through virulence testing of the progeny, they found avirulence to *Yr6*, *Yr27*, *Yr28*, *Yr43* and *YrSp* was controlled by a single locus, and avirulence to *Yr4*, *Yr32* and *Yr44* was controlled by two loci. Similarly, a sexual population of 115 isolates was generated by selfing the dominant Pakistani race (574232) of Pst on the Himalayan barberry (*Berberis pseudumbellata*), and they had varying virulence (Mehmood et al. 2020). Due to the prevalence of the alternate host buckthorn and the production of sexual spores with new virulence combinations, Pca has been considered the most variable cereal rust pathogen in North America (Leonard 2003).

However, in the absence of sexual reproduction, the emergence of new virulent races is thought to occur primarily via random mutation and asexual or somatic hybridisation (Park and Wellings 2012) (Figure 1). Genetic variation is often caused by the slow, gradual accumulation of mutational changes, resulting in gradual changes in allele frequency. Mutations in the *Avr* gene can allow the rust fungi to escape recognition by the host and initiate infection. Long-term pathogenicity investigations of cereal rust pathogens have provided many examples of virulence acquisition via mutation (Park and Wellings 2012). The copy number variations (CNVs) of eight Australian isolates of Pst representing four clonal lineages (PstS0, PstS1, PstS10 and PstS13) showed that gains on haplotigs of isolates within the PstS0 and PstS1 lineages were more than losses; in PstS10 lineage isolates, there were excessive copy losses and balanced gains in interhaplotype regions; and in PstS13 lineage isolates, losses were more than gains on haplotigs (Ding et al. 2021). Guo et al. (2022) analysed single-nucleotide polymorphisms (SNPs) and structural variation (SV) between the four haplotypes of 99KS76A-1 and Pgt21-0 and found that there were many effector coding genes with SVs, which are under stronger purifying selection in the evolution of virulence. The induced mutations mainly come from the influence of chemicals, radiation and viruses. By treating urediniospores of Pst race PSTv-18 with ethyl methanesulfonate (EMS), 33 mutants were isolated and identified as 24 races by virulence tests, of which 19 were new. More importantly, except for Pst isolates M11-Yr8 and M11-Yr31, other mutant strains had different virulence patterns from the wild-type isolate, demonstrating that EMS-induced mutations can change avirulence genes to virulence genes (Li, Wang, et al. 2019). Similarly, Li et al. (2020) identified SNPs and insertions and deletions (indels) from Pst mutants induced by EMS. They identified a series of Pst candidate effectors that may be responsible for the virulence acquisition of many wheat resistance genes.

Somatic hybridisation (SH) between formae speciales or different strains of the same forma specialis of cereal rusts has been associated with the origin of novel virulence combinations (Burdon et al. 1981). Early studies using limited molecular markers, such as shared isozyme or random amplified polymorphic DNA (RAPD) markers, have shown that somatic hybridisations occurred in both Pgt and Pt populations (Burdon et al. 1981; Park et al. 1999). Based on isozyme differences between Pgs and Pgt, it was shown that new strains of *P. graminis* arose via

SH on the Australian native grass *Agropyron scabrum* (Burdon et al. 1981). The Australian Pt pathotype 64-(6),(7),(10),11 was shown to have evolved from SH between isolates of pathotypes 104–2,3,(6),(7),11 and 53–1,(6),(7),10,11 (Park et al. 1999). Characterisation of genetic relationships of rust pathogens in populations was further implemented to determine the origin of virulent strains via hybridisation. The evolving virulence of the Ug99 lineage of Pgt poses a serious threat to wheat production due to its high mutation rate and was proposed to emerge via SH (Singh et al. 2015). The Ug99 lineage shares a single nucleus-specific haplotype with the more ancient South African lineage (Li, Qiao, et al. 2023). Guo et al. (2022) provided substantial evidence that admixture played a crucial role in the origin and evolution of Pgt races, where the Ug99 race haplotypes conformed to a two-way admixture model and had an equal probability of duplication from ancestral clades. Genotype comparative analysis between Ug99 and its two ancestral clades showed over 95% of homozygous SNP sites in the ancestors were heterozygous in Ug99, suggesting a hybrid origin of Ug99 and haplotype contribution from ancestral clade isolates. Studies focusing on the molecular genotypes and virulence phenotypes of Pt in different geographical regions suggested that the main mode of reproduction of Pt populations was asexual reproduction, associated with high levels of heterozygosity and linkage disequilibrium and limited diversity (Ordoñez and Kolmer 2009; Ordoñez et al. 2010; Kolmer et al. 2013; Kolmer 2019). More recently, comparative analysis of haplotype genomes and phylogenetic trees based on haplotype-specific SNPs using Australian Pt isolates revealed near-identical haplotype sequence features between putative parental and hybridised isolates (Sperschneider et al. 2023). It was speculated that repeated somatic exchange events shuffled haploid nuclei among long-lived clonal lineages, resulting in a global population of Pt representing different combinations of limited haploid genomes.

In addition, a notable feature of the rust genome is the relative abundance of multigene families and genomic plasticity driven by repeated invasions, which may contribute to the diversification of virulence genes (Aime et al. 2017). Near-complete genomes and the illustration of complex genome contents aid in unravelling the dynamics of virulence variation in cereal rust pathogens and their virulence evolution. Understanding how virulence evolves and monitoring the variability of fungal virulence is a crucial part of rust disease management, benefits the development of crop cultivars with genetic resistance, and enables the predictions of future virulence changes.

2.4 | Genome Evolution

The rapid evolution and ability of rust fungi to overcome the resistance of cereal varieties pose significant challenges for breeders. The ability to sequence hundreds of rust pathogen genomes is now providing resources that can be used to unravel the processes occurring at the genome level that drive this evolution. Fungi belonging to the Basidiomycota phylum have developed distinctive mating systems regulating nuclear compatibility, preferences, and life cycle progression (Coelho et al. 2017). Genomic comparative studies have revealed that rust fungi possess two unlinked mating-type loci (MAT), that is, the homeodomain (HD) locus and the pheromone receptor (PR)

locus, and these loci are heterozygous in representative dikaryotic genome assemblies, supporting the conclusion that cereal rust fungi operate under a tetrapolar mating compatibility system (Cuomo et al. 2017; Wu et al. 2021; Henningsen et al. 2024; Luo et al. 2024). Allelic differences at the MAT loci contribute to speciation, with the HD genes displaying significant allelic diversity, particularly in Pca (Luo et al. 2024). Moreover, the PR locus shows distinct features across cereal rust fungi, including notable macro- and microsynteny disruptions, the accumulation of repetitive sequences, and elevated rates of synonymous substitutions. These findings highlight the high plasticity of MAT loci, driven by strong selection pressures, which play a pivotal role in the evolution of rust fungi.

A noteworthy discovery was the considerable variation in genome size and TE content among closely related species. Genomes with high mutation or recombination rates have the potential to evolve rapidly by increasing the production of new mutations or recombinants. The difference in the TE content is one of the reasons for the change in genome structure. A study has shown that plastic genome compartments in rust pathogens are enriched in repetitive sequences, accelerating pathogen adaptive evolution (Faino et al. 2016). Schwessinger et al. (2020) examined the variation and presence–absence polymorphisms between the genomes of Pst isolates Pst-104E (PstS0 lineage) and Pst-DK0911 (PstS7 lineage) and found that the TEs were significantly enriched in unique regions of the two isolates. This suggested that sequence variation between lineages was caused by TE content. Moreover, the presence of a great number of genetic variants, including SNPs, indels, gene presence and absence polymorphisms (PAPs), and segmental duplications (SDs), could be part of the reasons for the rapid evolution of rust fungi. Studies have shown that there are significant differences in heterozygosity at the SNP level between different Pst isolates; for example, Pst 93–210 with 6.4 SNPs/kb, Pst-21 with 5.0 SNPs/kb, Pst-43 with 5.3 SNPs/kb, Pst-130 with 5.4 SNPs/kb, Pst-87/7 with 6.6 SNPs/kb, Pst-08/21 with 7.6 SNPs/kb, Pst-78 with 6.0 SNPs/kb, Pst-104E with 7.1 SNPs/kb, and Pst-DK0911 with 1.6 SNPs/kb (Cantu et al. 2011, 2013; Zheng et al. 2013; Hubbard et al. 2015; Cuomo et al. 2017; Bueno-Sancho et al. 2017; Schwessinger et al. 2018, 2020; Xia et al. 2018). In addition, the identification of SNPs in candidate effector protein genes among different rust pathogen races may reflect the rapid adaptation to specific host plants (Cantu et al. 2013).

In natural ecosystems, adversarial interactions between plants and their pathogens drive coevolutionary dynamics, in which plants evolve mechanisms to recognise pathogens, whereas pathogens evolve strategies to evade plant defence systems. These coevolutionary processes shape genetic variation at the genomic and population levels of plants and pathogens (Möller and Stukenbrock 2017). In recent years, comparative phylogenetic studies of host plants and rust fungi have confirmed the coevolutionary relationship between the two and supported the relative importance of the aecial host in the evolution of *Pucciniales* (Aime et al. 2017, 2018). In cereal rust fungal genomes, virulence-related genes such as those that code for secreted proteins are subjected to high evolutionary selection pressure imposed by host plants, leading to various types of variations including gene loss. For example, the virulence of *P. graminis* to *Sr27* has been shown to arise through various mechanisms,

including deletion mutations, copy number variation, and expression level polymorphisms at the corresponding *AvrSr27* locus (Upadhyaya et al. 2021). Gene losses have been reported in many filamentous plant pathogens as a response to changes in the host and environmental conditions (Pendleton et al. 2014; Albalat and Cañestro 2016; Duplessis et al. 2011). Reductions of genes, such as active enzymes encoding carbohydrates, secondary metabolites, and enzymes in nitrogen and sulphur assimilation pathways, allow biotrophs to avoid recognition by plant defence systems and adapt to plant hosts (Duplessis et al. 2011; Albalat and Cañestro 2016; Ding et al. 2021; Spanu 2012). Xia et al. (2018) used comparative genomics to analyse the genomes of Pst and Psh and showed that both experienced extensive gene loss events after separation from their most recent common ancestor to adapt to their respective hosts.

3 | Strategies for Genomic Analysis of Cereal Rust Fungi

3.1 | Genome Sequencing and Assembly

Advances in sequencing technologies have made it possible to generate whole-genome sequences and comprehensive assemblies for rust isolates. Next-generation sequencing (NGS) technologies have significantly reduced the cost of sequencing genomes and enabled whole genome sequencing for different rust species. The first hybrid draft genome assembly of Pst was successfully completed based on short-read sequences generated by the Illumina sequencing platform (Cantu et al. 2011). Since then, genomes of several cereal rust species and other important rust fungi have been sequenced and published. However, due to high heterozygosity and repeat content of the dikaryon of rust pathogen genomes, short-read genome assemblies are highly fragmented and lack haplotype-specific information that does not fully capture sequences from haploid nuclei.

The application of third-generation sequencing technologies, including Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), has significantly reduced drawbacks associated with short-read sequences. Long-read sequencing (LRS) platforms can generate ultra-long reads ($>10\,kb$ on average) with high accuracy, thus resolving complex genomic regions for more complete genome assemblies and enabling pseudo-phasing of haplotypes in dikaryotic rust genomes (Schwessinger et al. 2018; Wu et al. 2020; Henningsen et al. 2022) (Table 1). Notably, the sequencing and assembly of cereal rust genomes was made possible through extensive long-read sequencing, particularly using PacBio high-fidelity (HiFi) sequencing technology (Liang et al. 2023; Li, Qiao, et al. 2023). Moreover, haplotype phasing within a heterozygous diploid genome is of great importance for rust fungal genomic analysis. Advances in sequencing technologies and haplotype resolution software have contributed to higher continuity and haplotype-phased genomes of several *Puccinia* species. In addition, chromatin contact data such as chromosome conformation capture (Hi-C) have greatly enhanced the assembly of haplotype-phased contigs to the chromosomal or scaffold scale without genetic and physical maps (Kronenberg et al. 2021). Given the complexity of the rust fungal genome, it took about a decade to achieve haplotype-phased genome telomere-to-telomere assembly of all chromosomes (Petre

and Duplessis 2022). Combining LRS and Hi-C sequencing technologies, researchers have successfully assembled and resolved 18 chromosome haplotypes from several different cereal rust pathogens (Table 1).

To date, numerous assembly programs and analysis tools have been developed for long-read sequencing, including Canu (Koren et al. 2017), FALCON (Chin et al. 2016), SOAPdenovo (Li et al. 2010), and wtdbg2 (Ruan and Li 2020). Early rust genome assemblies collapsed homologous haplotypes into a consensus representation, failing to fully capture sequences from the two haploid nuclei. To address this issue, de novo assemblers have been developed that take advantage of high-quality long-read sequences to represent the haplotype information. These tools include FALCON-Unzip (Chin et al. 2016), HaploMerger2 (Huang et al. 2017), Purge_Haplotigs (Roach et al. 2018), TrioCanu (Koren et al. 2018), NuclearPhaser (Duan et al. 2022), and FALCON-Phase (Kronenberg et al. 2021). In addition, assemblers, such as HiCanu (Nurk et al. 2020) and Hifiasm (Cheng et al. 2021), can exploit long and highly accurate HiFi data to faithfully represent the haplotype information in a phased assembly graph. With the advancement of sequencing technologies and the widespread application of haplotype resolution software, assemblies of cereal rust pathogens have been improved significantly, revealing the genome structure of rust fungi with two haploid nuclei accurately.

3.2 | Comparative Genomic Analysis

In the early stages of the comparative analysis of rust genomes, fragmented rust genome assemblies meant that little synteny could be resolved between the different rust fungi (Duplessis et al. 2011, 2014). However, in recent years, with the increasing number of high-quality genomes, comparative genomic analysis has been used widely to study polymorphisms in cereal rust pathogens (Xia, Qiu, et al. 2022). Due to the dikaryotic and heterozygous nature of rust spores, substantial genetic variation occurs and is especially distributed between the two haplotypes (Figure 1). Chromosome-level and haplotype-phased assemblies for several cereal rust species have revealed an unexpectedly high level of diversity between haplotypes in a single rust isolate, which has been shown to be associated with adaptive evolution (Li, Qiao, et al. 2023). In the case of Pgt race 21–0, the overall sequence identity between the two haploid nuclei is only 87% (Li, Upadhyaya, et al. 2019). The interhaplotype variation of Pca isolates 12SD80 and 12NC29 was mainly located in the intergenic regions, with 3.66 and 4.88 variants/kb, respectively (Miller et al. 2018). The interhaplotype diversity of Pst104E is over 6.4% with high levels of nonallelic genes (~25%) and single-haplotype genes (~5%) (Schwessinger et al. 2018). The large-scale structural variants of interhaplotype were found in Pst-104E and Pst-DK0911, at 5.10 Mb (~6.39%) and 2.00 Mb (~2.66%), respectively (Schwessinger et al. 2020). Similarly, significant interhaplotype structural variation was also observed in Pt64 at 10% of the total genome (Wu et al. 2021); Pt15 at 12% in haplotype A and 10.5% in haplotype B (Li, Qiao, et al. 2023); and Ppz-GD1913 at 3.0% of the genome size (Liang et al. 2023).

Comparative genomics of cereal rusts focusing on the conservation of homologous genes between two or more genomes of different species has provided valuable insights into their evolutionary processes (Figure 2). Based on single copy orthologues, Cuomo et al. (2017) constructed the phylogenetic relationship of different rust species and found weak synteny among the three rust pathogens of wheat, namely, Pt, Pst and Pgt. There is significant genome variability among these three wheat rust pathogens, especially in the mating sites, which reflect their genetic diversity and evolutionary divergence. The comparison of virulence profiles of different strains is helpful in revealing the molecular basis of virulence and the pathogenic mechanism within and between species. For example, compared with previous Pca isolates 12SD80 and 12NC29, the virulence profile analysis showed that Pca203 has unique virulence and Avr effector profiles (Henningsen et al. 2022). In addition, comparative genomics combined with association analysis has also been used to identify candidate *Avr* genes in pathogenic strains with similar genetic backgrounds (Xia et al. 2020; Wu et al. 2017; Chen et al. 2019).

Comparative genomics is one of the most potent tools in genomic research, facilitating predictions of gene function and understanding the evolutionary relationships between species based on differences in genome structure (Figure 2). Numerous strategies are commonly employed for comparative genomic research (Sivashankari and Shanmughavel 2007). Genome-wide association study (GWAS) is a critical tool in comparative genomics research. It can identify existing sequence variations, such as SNPs and CNVs, through whole-genome sequencing at the population level, and then identify the genetic loci associated with the traits (Uffelmann et al. 2021). In the context of rust pathogen genomics, GWAS would be effective in predicting genetic variation in virulence among different races. Furthermore, integrating phylogenetic analysis with comparative genomics can provide insight into evolutionary history and relationships among diverse species. Core orthologous genes, characterised by high conservation, can be used to reconstruct phylogenetic relationships, which can reveal important evolutionary patterns such as differences in morphology, virulence, adaptations and speciation (Aime et al. 2017). Additionally, synteny analysis involves comparing the order and arrangement of genes and genomic regions between species to uncover conserved segments and variation structures (Liu et al. 2018). Stronger synteny indicates conserved family and genetic structure among isolates, showing a close evolutionary relationship.

3.3 | Pan-Genomic Analysis

The concept of the pan-genome, first proposed in 2005, refers to all the genes in a species, including the core and dispensable genomes (Tettelin et al. 2005). The core genome comprises genes shared by all individuals of the species and is mainly responsible for essential functions. In contrast, the dispensable genome comprises accessory genes (present in two or more strains) and unique genes (specific to a particular strain), which are related to environmental adaptations (Vernikos et al. 2015). With the continuous development of sequencing technology, an increasing number of high-quality genome assemblies have emerged, providing the opportunity to construct pan-genomes. Pan-genome strategies have been widely employed in microorganisms, animals and plants to identify more genes responsible for important
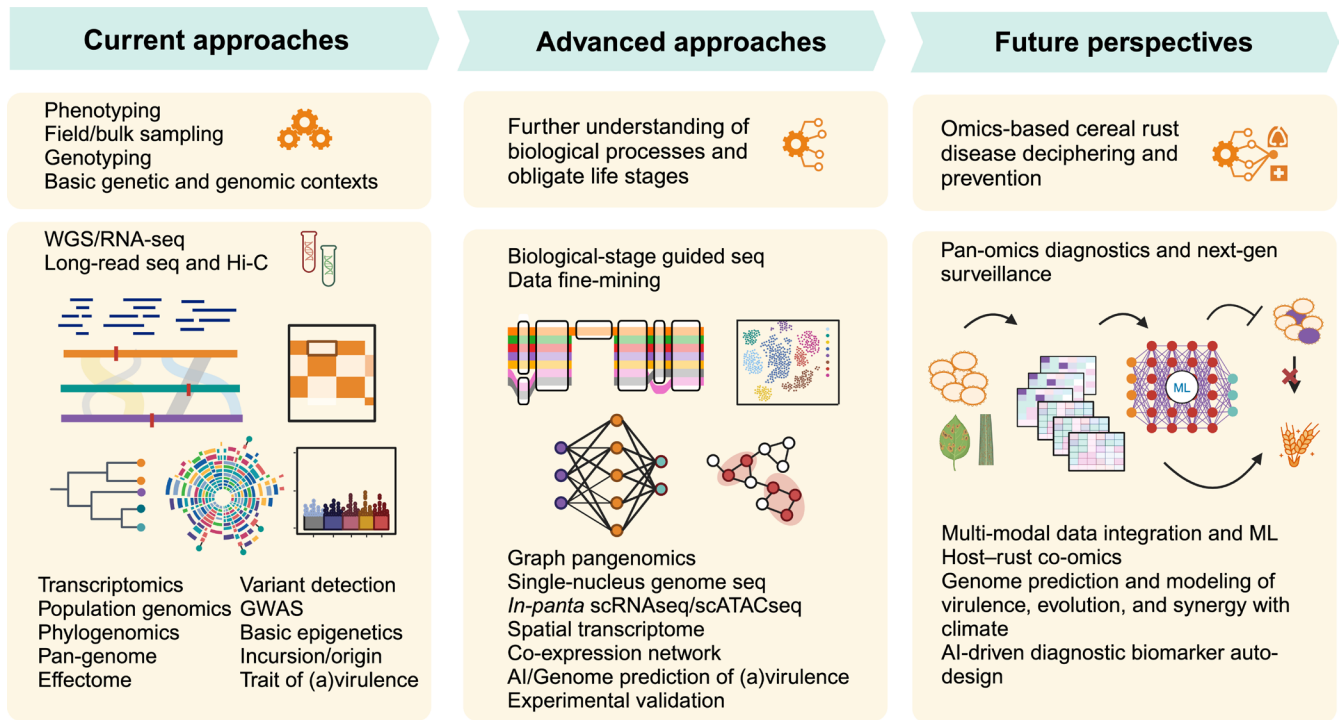
FIGURE 2 | A schematic overview of cereal rust genomic research strategies, advanced approaches, and future perspectives. The current research stage is around basic genetic and genomic contexts (left) centred in accurate phenotyping, field/bulk sampling and basic genotyping. Long-read seq combined with Hi-C, as well as whole-genome seq (WGS) and RNA-seq have enabled chromosome-scale phased assemblies, with population genomics, genome-wide association studies (GWAS) and early epigenetic profiling (e.g., DNA methylation), and support variant detection and trait analyses for (a)virulence. The advanced approaches column (centre) highlights emerging needs for deeper biological insights through life stage (e.g., both sexual and asexual)-guided sequencing and sophisticated computational pipelines for fine-mining of complexed datasets. Examples include graph-based pangenomics for capturing complex structural variations across multiple isolates/races/species in a single, dynamic representation; single-nucleus genome sequencing for phasing-free genome assemblies; spatial transcriptomics and co-expression networks integrating epigenetics (scATAC-seq) and metabolomics to map and correlate pathogen key gene activities and pathways within infected tissues; leveraging artificial intelligence (AI) for genome prediction of (a)virulence. The demands for real-time genome prediction of virulence shifts, AI-driven biomarker discovery, and rapid strategies for cereal rust disease prevention make it more clear for a few future perspectives (right panel). It is anticipated that, in the coming years, machine-learning (ML) accelerated genomic and pan-omic data will help dissect cereal rust biology along with host genetics components in the changing climate, providing a multilayered perspective on rust–host–environment interactions and, ultimately, precision breeding or tailored fungicidal treatments. Figure created in BioRender.

phenotypic variation. However, pan-genome research in rust fungi is still at the early stage. In 2015, Upadhyaya et al. (2015) constructed a pan-genome based on genome sequence information from five Australian Pgt isolates, which built on and extended the previous reference assembly of the North American Pgt isolate CDL 75-36-700-3 (p7a). More recently, a pan-genome of Pst was constructed by combining the genome of the Pst isolate FH11 with 7483 non-redundant sequences (NRS) identified across nine additional Pst genomes that were absent in FH11 (Wang, Peng, et al. 2024). A total of 23,526 protein-coding genes were predicted in the Pst pan-genome, including 17,062 in the FH11 genome and 6464 in the NRS.

Rust pathogen adaptation is driven by extensive genetic variation in populations, and different strains may not share the same set of genes (Badet and Croll 2020). Presence/absence variants (PAVs) represent an important aspect of genomic diversity within eukaryotic species, providing significant insights into the evolutionary history of a species (Hu et al. 2020). Using the pan-genome, researchers can explore PAVs in each isolate by mapping reads and examining gene coverage. The

non-redundant secreted protein clusters (SPCs) PAV patterns of a global collection of 42 Pst isolates revealed variations in the number of SPCs per isolate, with 282 SPCs absent in at least one isolate. Moreover, dispensable secretome SPCs exhibited significantly more PAV compared to the core secretome SPCs, highlighting the extensive polymorphism within the Pst genome (Wang, Peng, et al. 2024). In the pan-genome of rust fungi, core genes are typically conserved through evolutionary selection. Such genes could be targeted to design fungicides. In contrast, dispensable genes are highly diverse within the species and are often associated with host specificity, which could be used to analyse the genetic diversity of rust fungi. For instance, the *Avr* genes of rust pathogens are usually a part of dispensable genomes (Syme et al. 2018). Moreover, the pan-genome, being a much broader gene repertoire than a single reference genome, can provide more reliable genetic annotations than a reference genome.

Pan-genomic analysis holds great promise as an effective strategy for studying rust pathogens. However, constructing an optimal pan-genome for a given species, especially rust

pathogens, requires several considerations. Firstly, it is important to include an adequate number of representative isolates that accurately reflect the characteristics of the species. Secondly, ensuring the integrity of the entire genome assembly is crucial because pan-genome analysis follows a homologue-based approach. Additionally, homologous protein similarity threshold settings may influence the size of the core genome and pan-genome, as well as their relative sizes (Hu et al. 2020). Furthermore, dikaryotic rust pathogens pose a unique challenge for pan-genome construction because they have two genetically distinct nuclei (Badet and Croll 2020). Therefore, tracing dikaryon variation and capturing full genome content are necessary to construct the high-quality pan-genome of cereal rust fungi.

3.4 | Epigenomic Analysis

Epigenetic regulation between pathogens and hosts is complex. Common epigenetic regulatory mechanisms include DNA methylation, histone modifications, chromatin remodelling and RNA interference (RNAi) (Gibney and Nolan 2010). Epigenetic regulation simply changes the pattern of gene expression without altering the underlying nucleotide sequence (Kumar et al. 2017). To maximise survival and spread, rust pathogens induce various physiological, morphological and behavioural changes in their host plants. These changes can involve insertions/deletions, extensive chromosomal duplications, and unequal crossing over occurring in *R* genes, among other mechanisms (Shilpa et al. 2022). Pathogens can regulate their development during infection through a series of epigenetic changes, such as DNA methylation and histone modification, to enhance virulence and evade host recognition (Jeon et al. 2015; Gómez-Díaz et al. 2012). When a pathogen infects a host, the corresponding *Avr* gene needs to be altered in some way so that the *R* gene no longer confers effector-triggered immunity (ETI), allowing the pathogen to grow, multiply, and infect. Different virulence increases in pathogens may be affected by genetic variation in the *Avr* gene (Sacristán and García-Arenal 2008). However, some studies have shown that epigenetic modification to *trans*-acting elements can regulate transcription or translation of *Avr* genes, thereby increasing virulence (Shilpa et al. 2022). RNAi, as an epigenetic regulatory mechanism, can degrade and inhibit translation as well as modify chromatin and target gene expression. Using an epigenetic approach, Mueth et al. (2015) demonstrated the presence of a fungal endogenous small RNA repository during wheat and stripe rust interaction. In wheat, transgenic plants expressing RNAi constructs targeting Pt pathogenicity genes successfully disrupted the transcription of these genes during infection, thereby significantly suppressing the pathogen virulence (Panwar et al. 2018). Similar to the repeat-associated siRNA pathway of plants, fungal pathogens can use small RNAs (sRNAs) to direct DNA methylation of TEs. DNA methylation serves as a crucial regulatory mechanism in many eukaryotic organisms, silencing transcriptional targets by methylating cytosine residues in complementary DNA molecules (Zhang et al. 2018). In Pgt, the sRNA and gene silencing were differentially expressed over time during infection. A comparative analysis of sRNA-mapped versus sRNA-unmapped TE regions at the late stage of infection revealed a strong association between Pgt sRNAs and TE methylation (Sperschneider et al. 2021). Epigenetic regulation can provide valuable information to explain the molecular basis of the host–pathogen interaction, thus unravelling the complex pathogenic pathways involved.

3.5 | Multi-Omics Analysis

To develop effective strategies for controlling rust pathogens, it is crucial to gain a detailed understanding of the infectious process and pathogenic mechanisms at the molecular level. In this context, multi-omics encompassing reference-guided transcriptomics, proteomics, metabolomics, epigenomics and metagenomes has become a powerful tool for the study of plant–pathogen interactions and molecular mechanisms (Shen et al. 2022). Integration of multi-omics can study the relationship across variations at different omics levels, which is conducive to revealing the changes in the evolution of rust pathogen populations at different levels and analysing the molecular mechanism of the formation and regulation of complex phenotypic traits (Figure 2). The integration of transcriptomic, proteomic and metabolomic analyses can help identify key biological changes in fungi during infection. For example, early work by Song et al. (2011) integrated proteomics with the early reference genome of Pgt to explore haustorial-enriched proteins. Moreover, multi-omics integrating Hi-C, genomic and transcriptomic data was also used to analyse the gene expression/structural characteristics in cereal rust pathogen genomes. Xia, Huang, et al. (2022) generated chromosome-level genomes of two Pst isolates (CYR34 and 93-210) and combined genomics, transcriptomics and epigenomics to study their three-dimensional (3D) genomic architectural characteristics. They found that the changes in 3D genome structure were not associated with the changes in gene expression at the urediniospore and germ-tube stages of Pst.

Efficient data integration is essential for reliable multi-omics studies. One of the main challenges of multi-omics is integrating data from different omics due to differences in data structure, resolution and complexity (Subramanian et al. 2020; Argelaguet et al. 2021). Because of the current study stage of cereal rusts only being on the basic contexts of genetics, genomics and biology, relatively few studies have been conducted integrating comprehensive multi-omics for cereal rust pathogens (Figure 2). As the emergence of advanced omics data types, sophisticated data integration strategies need to be designed. Here, we briefly summarise strategies that may be adopted for cereal rust pathogen genomics-based multi-omics data integration. Depending on the choice of common features (or "anchors") of the dataset, there are three types of data integration strategies; that is, horizontal integration, vertical integration and diagonal integration (Zheng et al. 2024). Horizontal integration is used for diverse datasets of a single omics type, such as two RNA-seq datasets, and genes are acting as anchors. Vertical integration is used for datasets collected from the same cells and combines genomic, transcriptomic and metabolomic data for analysis, with the cell acting as the anchor. Diagonal integration is used for different omics from different samples or cells. With the advent of the availability of multi-omics data generated from different species, computational

methods for integrating multi-omics data (including data mining, machine learning, deep learning, statistical methods and meta-heuristic techniques) have been developed (Kaur et al. 2021; Mansoor et al. 2024). Integrative tools for multi-omics data analysis can be categorised as data-driven analysis and knowledge-based analysis (Kaur et al. 2021). Data-driven analysis is based on verified information or public resources, such as MetaboAnalyst (Sun et al. 2014), MetaMapR (Grapov et al. 2015), integrated tool for heterogeneous multi-omics data analysis (iODA) (Yu et al. 2020) and PaintOmics 3 (Hernández-de-Diego et al. 2018). Data-driven integration is based on different metrics and can reveal pairwise associations through network visualisation. The available tools include SAMNetWeb (Gosline et al. 2015), Metscape (Gao et al. 2010), get Residue Interaction eNergies and Networks (gRINN) (Serçinoğlu and Ozbek 2018), MONGKIE (Jang et al. 2016), MixOmics (Rohart et al. 2017), and Multiomics Factor Analysis (MOFA) (Argelaguet et al. 2018). Although there are still limitations in the current multi-omics analysis and data integration, multi-omics technology has a wide range of potential applications in the study of cereal rust fungi that should enhance our understanding of how they and other fungal pathogens interact with their plant hosts.

3.6 | Population Genomics

Whole-genome sequencing and comparative genomics have laid a robust foundation for advancing population genomics research. Population genomics facilitates the investigation of population structure, evolutionary dynamics, speciation and adaptive mechanisms at the whole-genome level, enabling the tracing of the transmission pathways and biology of cereal rust pathogens through comprehensive analyses (Guo et al. 2022; Li, Dai, et al. 2023; Ding et al. 2021). For instance, pathogenomic pipelines have been developed in recent years to analyse population structures of Pst by integrating genomic and transcriptomic data, which could combine in-field rust infected samples of wheat (Hubbard et al. 2015; Radhakrishnan et al. 2019). This approach enables fast tracking of pathogen populations based on selected variant gene markers, allowing for the identification of specific lineages. Moreover, population genomics analyses have revealed the global spread of rust pathogens, including the transcontinental dispersal routes of Pst (Ali et al. 2014) and the worldwide population dynamics of Pt (Sperschneider et al. 2023). Additionally, studies have identified sexual recombination in the Pst populations, highlighting its role in generating genetic diversity and facilitating pathogen adaptation (Ali et al. 2010; Holden et al. 2023). Further developed population genomic pipelines have revealed that shorter gene ranges in Pst may represent both shared and unique genetic changes within populations, some of which may additionally account for the local adaptation of these isolates to triticale (Ding et al. 2021). With the emergence of advanced sequencing techniques and graph-based pan-genome references (Figure 2), high-resolution population genomics may be achieved for local rust populations (Figure 1) to allow more accurate pathogen surveillance programmes and a deeper understanding of the evolutionary forces driving rust pathogen diversity. Such integration provides a framework for updating disease surveillance systems

systematically and rapidly, ultimately supporting more effective disease management strategies.

3.7 | Advanced Genomics

Single-nucleus sequencing and single-cell genomics/spatial transcriptomics technologies have emerged as invaluable tools in diverse fungal genomics research (Xu et al. 2024; Serrano, Tedeschi, et al. 2024; Serrano, Bezrutczyk, et al. 2024; Wang, Xu, et al. 2024; Ahrendt et al. 2018; Lin et al. 2014). Integration of these sophisticated technologies is necessary to propel the field of rust genomics forward. Single-nucleus sequencing, despite the current coverage issue, represents a powerful tool for dealing with complex or heterogeneous genomes, enabling the resolution of genomic variations at the single-nucleus level (Lin et al. 2014). It thus may provide insights into the genetic heterogeneity within cereal rust pathogens and was recently applied in assisting haplotype phasing of Pst genome assemblies and achieving gap-free genome assemblies (Wang, Xu, et al. 2024). Single-cell omics can analyse genetic material from individual cells that are isolated from natural samples and subjected to whole-genome amplification (WGA) (Roy et al. 2014), which is a promising approach to study adaptation in cereal rust pathogens. This approach could illuminate the dynamics of infection and provide insights into interactions with host plants at the cellular level.

3.8 | Functional Genomics and Manipulation of Cereal Rust Genes

Gene functional verification often explores changes in phenotype and function by manipulating the expression of target genes. Advances in genomic and transcriptomic sequencing, along with improved reference assemblies, have facilitated comparative analyses of representative rust pathogens to identify putative candidate genes of interest contributing to pathogen development or virulence that will need to be functionally validated. Functional genomics incorporated hetero-expression systems (e.g., in planta and in-yeast expression) have been broadly used for validating the function of cereal rust genes, particularly Avr genes, as aforementioned. Regarding gene disruption, methods including RNAi and gene editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems have been successfully applied to study gene functions in various fungal species (Touzdjian Pinheiro Kohlrausch Távora et al. 2022; Bahrini et al. 2011; Schwessinger 2016; Li et al. 2024). However, disrupting specific target genes in the cereal rust pathogens is extremely challenging due to the highly complex macrocyclic-staged and obligate biotrophic feature (Figure 1) causing the lack of an efficient stable genetic manipulation tool. Until now, transient expression in hetero-systems as well as RNAi-based approaches (i.e., exogenous delivery of small RNAs, such as host-induced gene silencing [HIGS], virus-induced gene silencing [VIGS] and spray-induced gene silencing [SIGS]) have been predominant approaches used for silencing candidate genes, which are well documented in previous reviews and reports (Panwar et al. 2018; Bakkeren and Szabo 2020; Degnan et al. 2023). The CRISPR/Cas system

can not only knock out genes but also perform precise point mutations or insertions (Sander and Joung 2014; Muñoz et al. 2019; Vercauteren et al. 2024), making it a powerful tool for functional validation of candidate genes and variations of rust pathogens. It can be expected that in the near future, the CRISPR/Cas system may be available with externally assisted delivery methods such as particle bombardment or nanoparticle-based approaches to assist large-scale functional genomics in cereal rust.

4 | Conclusion and Prospects

Advanced genomic and computational approaches will play a pivotal role in unravelling the multilayered biology of complex cereal rust pathogens (Figure 1). Moving beyond basic reference and pan-genomes and bulk assays, life stage-guided sequencing across sexual and asexual phases, supported by sophisticated computational pipelines, is poised to enhance functional and epidemiological studies significantly (Figure 2). For instance, the recently developed graph-based pan-genomics approach (Li et al. 2022) may allow for the obtaining of extensive structural variations among diverse isolates or races that have never been easily captured before, while single-nucleus genome sequencing may promote phasing-free and high-resolution assemblies of each haploid nucleus. In parallel, integrated multi-omics, including spatial transcriptomics, epigenetic profiling (e.g., scATAC-seq), and metabolomics will offer deeper insights into pathogen gene activities within host tissues, allowing researchers to pinpoint essential virulence and key biological pathways. Moreover, artificial intelligence (AI) and machine-learning (ML) methodologies may become essential for real-time virulence prediction, AI-driven biomarker discovery, and rapid surveillance of emerging cereal rust threats.

With global warming and increasing environmental challenges in modern agriculture, analyses of the rust–host–environment interplay, integrating rust genomic data, host genetic components, and climate factors to refine disease management strategies are becoming more critical (Figure 2). Multilayered integration of rust omics data will be needed more than ever to support precision breeding (e.g., stacking robust durable resistance alleles) and targeted fungicidal interventions. It can be foreseen that an ultimate goal is to establish a predictive framework for cereal rust genomic and omics research, one that brings together diverse data streams and computational innovations to protect global cereal production against rust diseases more effectively.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.