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LETTER TO THE EDITOR

Wheat stripe rust resistance gene *Yr9*, derived from rye, is a *CC-NBS-LRR* gene in a highly conserved *NLR* cluster

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Dear Editor,

Crop pathogens cause substantial economic losses and reduce food security globally. Harnessing disease resistance genes and breeding resistant crops are the most effective ways to reduce losses caused by pathogens. Domestication and cultivar breeding of wheat and other crops have significantly reduced their genetic diversity. The reduced diversity has resulted in a shortage of disease resistance genes, which is a severe obstacle for wheat breeding. Wheat relatives are valuable sources of genetic diversity that can improve wheat's disease resistance. In wheat breeding history, the replacement of the short arm of chromosome 1B from wheat with the 1RS from rye (*Secale cereale* L.) has been one of the most impactful incorporations of alien genetic diversity. The 1BL/1RS translocation enhances wheat resistance to biotic and abiotic stresses, and provides resistance to stripe rust (*Yr9*), leaf rust (*Lr26*), and stem rust (*Sr31*). Previous studies revealed that *Yr9*, *Lr26*, and *Sr31* are separate but closely-linked genes that co-segregate with *Mla-LRR* markers (Mago et al., 2005). However, attempts to clone these genes have been unsuccessful. Despite significant global efforts to clone resistance genes in wheat, only around ten genes for resistance to *Pst* have been successfully cloned to date (Tong et al., 2024). Although *Yr9*, *Lr26*, and *Sr31* have been defeated by newly emergent virulent races, revealing the resistance locus on 1RS will facilitate mining of untapped genetic diversity and provide new insights into the evolution of plant resistance genes.

In this study, we cloned *Yr9* from 'Lumai 15' (LM15), a predominant Chinese wheat cultivar released in the 1990s. LM15 is a 1BL/1RS derivative (Datasets S1 and S2) that yields well and is disease resistant. Because of the 1RS translocation, when LM15 was challenged with *Pst* races CYR23 and CYR34, seedling leaves were immune (infection type IT=0) (Figure 1A). Another 1BL/1RS cultivar 'Aikang 58' (AK58) shows slight chlorotic and necrotic flecks with a resistant infection type (IT=1) (Figure 1A). To identify the *Pst*-resistance genes in LM15, we propagated an ethyl-methanesulfonate (EMS) mutagenized population and used it to screen for *Pst*-susceptible mutants. Among the 2,800 M_3 generation lines, individual plants from four lines (M2333, M2365, M3289, and M3530), were identified as highly susceptible (ITs=7–9) (Figure 1B). In the M_5 generation, the most susceptible plant was selected from each line, and then used for sequencing trait-associated mutations (STAM) analysis (Ni et al., 2023).

In STAM analysis, the Iso-seq from wild-type (WT) LM15 produced 55,716 non-redundant isoforms that were used as a reference. RNA-Seq reads from the four mutants were used to call SNPs between the WT and *Pst*-susceptible mutants. A total of 2,083 transcripts that have SNP (s) in at least one mutant line were identified. Only one transcript (#1460) (GenBank PQ412819) was mutated in all four lines; M2365 has two missense mutations, M3530 has one nonsense mutation, and M2333 and M3289 each have a missense mutation (Figure 1C). We named transcript #1460 as *YrChr1B*; its coding region is 2,889 bp and encodes an iconic, intracellular CC-NBS-LRR disease resistance gene (Figure 1C). In addition to using full-length isoforms as the reference for STAM analysis, we also performed STAM analysis using the HiFi assembly of LM15 (9,131 contigs with an N50 of 6.1 Mb) as the reference genome. Both methods identified *YrChr1B* as the sole candidate for *Yr9*.

To determine if *YrChr1B* confers *Pst-resistance*, we constructed PC1332, a natural expression vector with a 9.3 kb genomic sequence. After transforming the *Pst*-susceptible cultivar 'Fielder', we showed that *YrChr1B* significantly enhanced *Pst*-resistance (Figure 1D); however, it was ineffective against the *Yr9*-virulent race CYR32 (Dataset S3). The over-expression of *YrChr1B* (PC1331) also confers *Pst*-resistance (Figure 1E). *YrChr1B* function also was confirmed with CRISPR/Cas9 gene-edited plants (PC1315 transgenics). Using a 1BL/1RS cultivar 'CB037-*Pst*R' (CBR) (Dataset S1) as WT, *YrChr1B* knock-out plants were highly susceptible, whereas WT CBR was resistant (Figure 1F; Dataset S4). Thus,

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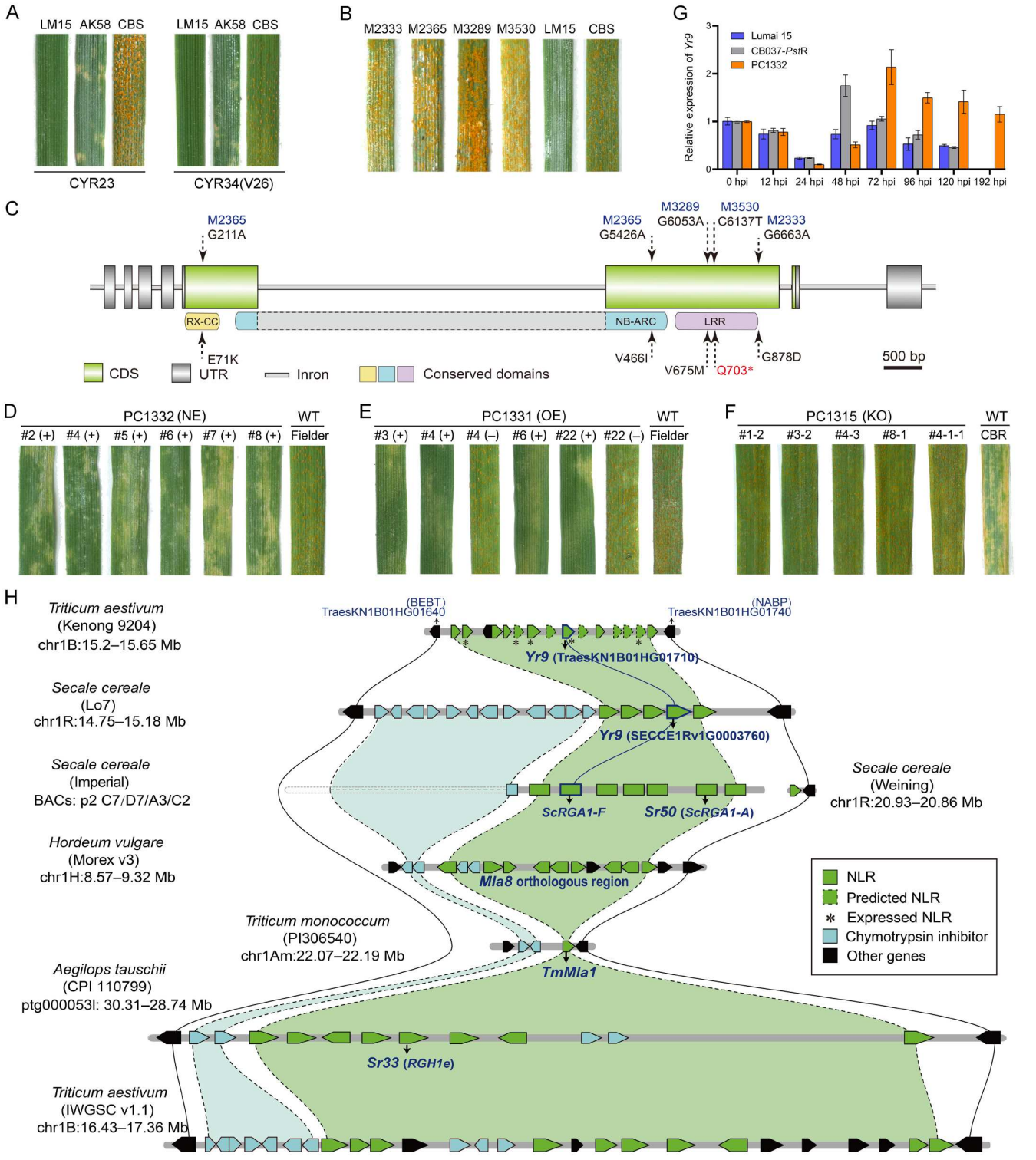

Figure 1. The cloning, functional validation, and micro-collinearity of *Pst*-resistance gene *Yr9*. A, Phenotypic responses of 1BL/1RS cultivars Lumai 15 (LM15) and Aikang 58 (AK58) to *Pst* races CYR23 and CYR34 (V26), with CB037-*Pst*S (CBS) as the susceptible control. B, Four *Pst*-susceptible mutants of LM15. C, The *YrChr1B* (*Yr9*) gene and conserved domains. Mutations and amino acid changes are labeled. D, Responses of six independent T_1 transgenic lines transformed with natural-expression (NE) vector PC1332, with Fielder as the susceptible control. E, Responses of T_1 transgenic lines derived from the over-expression (OE) vector PC1331. F, CRISPR/Cas9 editing of *Yr9* in 1BL/1RS cultivar CB037-*Pst*R (CBR). WT CBR is resistant to CYR34, and *Yr9*-edited plants are susceptible. Small insertions or deletions occurred at the sgRNA target of T_2 (#4-1-1) and T_1 (four other) plants. '(+)' and '(−)' in D and/or E represent positive and negative siblings, respectively. G, Relative expression levels of *Yr9* in LM15, CBR, and PC1332 transgenics, respectively, at various time points following inoculation with *Pst* race CYR34 (V26). The expression level of *Yr9* at 0 hpi (pre-inoculation) serves as the baseline (normalized to 1). Error bars represent the mean±standard error (SE) calculated from three biological replicates. H, The conserved *NLR* gene cluster harboring *Yr9*. Using the Triticeae-Gene Tribe tool, we modified the output to show the collinearity between different species. The physical maps of *Sr50* and *Sr33* were added manually. In KN9204, six additional *NLR* genes were annotated manually and highlighted with dashed boxes; *NLR*s with confirmed expression are indicated by an asterisk.

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YrChr1B confers *Pst*-resistance in 1BL/1RS cultivars LM15 and CBR. Given that only one *Pst*-resistance gene (*Yr9*) was mapped to 1RS (Mago et al., 2005), *YrChr1B* was deduced to be *Yr9*. *Yr9* expression exhibits a marked decrease, reaching its lowest level 24 h post-*Pst* inoculation in LM15, CBR, and PC1332 transgenics (Figure 1G).

The four 1BL/1RS cultivars (LM15, CBR, AK58, and Kenong 9204), which are all *Pst*-resistant, have an identical *Yr9* sequence. In Blastn searches against assembled wheat genomes, *Yr9* is 100% identical to TraesKN1B01HG01710.1 (located at 15.47 Mb) in 'Kenong 9204' (KN9204) (Shi et al., 2022). *Yr9* is 99% similar to SECCE1Rv1G0003760.1 (located at 14.97 Mb) in rye 'Lo7' (Rabanus-Wallace et al., 2021).

In KN9204, *Yr9* is a member of a *NLR* gene cluster, flanked by TraesKN1B01-HG01640 (benzyl alcohol O-benzoyltransferase-like, BEBT) and TraesKN1B–01HG01740 (nucleic acid-binding protein, NABP) (Figure 1H). In this 430 kb region, eight *NLRs* are annotated in a whole genome sequencing project (Shi et al., 2022). By sequence alignment and structure prediction, we annotated six more *NLRs* in this cluster (Dataset S5). Based on our Iso-Seq transcripts, five of 14 *NLRs* were transcribed (Figure 1H). This *NLR* gene cluster was conserved among *Triticeae* species, alongside the conserved *NABP* gene (Figure 1H). Interestingly, this region is orthologous to the barley *Mla* cluster that confers powdery mildew resistance (Wei et al., 2002). *TmMla1* provides resistance to powdery mildew in both wheat and barley (Jordan et al., 2011), and *Mla8* confers resistance to both barley powdery mildew and wheat stripe rust (Bettgenhaeuser et al., 2021). Stem rust resistance gene *Sr50* from *S. cereale* and *Sr33* from *Ae. tauschii* are also in this orthologous region (Cavalet-Giorsa et al., 2024; Mago et al., 2015). Therefore, diverse genes resistant to fungal diseases have evolved in the *Yr9* ortholog region (Dataset S6). In addition, chymotrypsin inhibitor 2 (*CI2*), which is another type of gene that is correlated with plants' resistance to wounding and pests, was also enriched around the *Yr9* locus (Figure 1H). We further investigated the distribution of *Yr9* in rye germplasm. Using two primer pairs, *Yr9* was amplified from 6 out of 19 lines, all of which exhibited *Pst* resistance (Dataset S7). Additionally, we analyzed public re-sequencing data of 122 worldwide accessions of wild, weedy, and cultivated rye. Based on SNP analysis of the coding region and the presence of a specific transposon insertion (3.1 kb) in the *Yr9* intron, approximately 52% of the rye germplasm carried *Yr9* homologues (Datasets S8). Notably, none of the nine *Secale sylvestre* accessions contained *Yr9*.

In conclusion, we identified a single candidate gene and validated it as *Yr9* through genetic complementation and gene editing. The *Yr9* gene encodes a coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR or NLR) protein and is part of a 14-member *NLR* gene cluster. This cluster is conserved among *Triticeae* species and is an ortholog of barley *Mla*, which evolved diverse genes resistant to fungal diseases. Exploration of the genetic diversity of this region in more species and cultivars will help identify more functional resistance genes and provide insights into the evolution of *NLR* clusters.

Compliance and ethics

The authors declare that they have no conflict of interest.

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Supporting information

The supporting information is available online at https://doi.org/10.1007/s11427-024-2932-5. The raw sequencing data reported in this study have been deposited to the NCBI Sequence Read Archive (SRA) under accession number PRJNA1166628. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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