An ancestral NB-LRR with duplicated 3’UTRs confers stripe rust resistance in wheat and barley

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Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is a global threat to wheat production. *Aegilops tauschii*, one of the wheat progenitors, carries the YrAS2388 locus for resistance to *Pst* on chromosome 4DS. We reveal that YrAS2388 encodes a typical nucleotide oligomerization domain-like receptor (NLR). The *Pst*-resistant allele YrAS2388R has duplicated 3’ untranslated regions and is characterized by alternative splicing in the nucleotide-binding domain. Mutation of the YrAS2388R allele disrupts its resistance to *Pst* in synthetic hexaploid wheat; transgenic plants with YrAS2388R show resistance to eleven *Pst* races in common wheat and one race of *P. striiformis* f. sp. *hordei* in barley. The YrAS2388R allele occurs only in *Ae. tauschii* and the *Ae. tauschii*-derived synthetic wheat; it is absent in 100% (*n* = 461) of common wheat lines tested. The cloning of YrAS2388R will facilitate breeding for stripe rust resistance in wheat and other Triticeae species.
Wheat (Triticum spp.) is the largest acreage crop in the world. With an approximate 220 million hectares and 760 million tons in 2018, wheat was ranked second in global production after maize. As a staple food crop, wheat provides about 20% of global calories for human consumption. Because the world population is projected to increase by nearly two billion people within the next three decades, the increasing human population worldwide will place an even greater demand for wheat production globally.

Wheat stripe rust (or yellow rust; abbreviated as Yr), caused by Puccinia striiformis f. sp. tritici (Pst), is a serious fungal disease that poses a huge threat to wheat production in regions with cool and moist weather conditions, including major wheat-producing countries, such as Australia, Canada, China, France, India, the United States, and many others. Planting wheat cultivars with adequate levels of resistance is the most practical and sustainable method to control stripe rust. Host resistance of wheat against Pst is normally classified as either all-stage resistance (ASR) or adult-plant resistance (APR). Whereas ASR is effective starting at the seedling stage through the late stages of plant growth, APR is main effective at the late stages of plant growth. In wheat, ASR confers high levels of resistance to specific Pst races, but the underlying genes, such as Yr9 and Yr17, are often circumvented by the emergence of new virulent races. In contrast, APR typically provides a partial level of resistance, but is more durable and is effective against all or a wider spectrum of Pst races than ASR. High-temperature adult-plant (HTAP) resistance is a major type of APR; HTAP typically provides durable and non-race-specific resistance to Pst. Incorporating multiple ASR and HTAP genes appears to be an excellent strategy for maintaining sustainable resistance to wheat stripe rust.

Over 80 wheat stripe rust resistance (R) genes (Yr1–Yr81) have been permanently named. Of the seven genes cloned so far, Yr5, Yr7 and YrSP, a gene cluster, encodes nucleotide-binding (NB) and leucine-rich repeat (LRR) proteins; Yr15 has two kinase-like domains; Yr36 has a kinase domain and a lipid binding domain; Lr34/Yr18 encodes a putative ABC transporter; and Lr67/Yr46 encodes a predicted hexose transporter. While the Yr5/Yr7/YrSP cluster and Yr18 stripe rust, widely used in wheat cultivars, however, Yr18 alone does not confer adequate resistance under high disease pressures. Yr7 and YrSP confer high levels of resistance, but Pst races virulent to Yr7 are common globally and those virulent to YrSP occur in some countries. Yr5 and Yr15 confer high levels of resistance to a wide range of Pst races, but the increasing adoption of them in wheat cultivars may cause the emergence of virulent races. Characterization of additional R genes is essential in order to assemble effective resistance to constantly changing populations of Pst.

Aegilops tauschii Coss. (2n = 2x = 14, DD) is the D genome progenitor of common wheat. The diverse Ae. tauschii D genome offers a valuable gene pool for stripe rust resistance. To date, several stripe rust resistance genes have been mapped in Ae. tauschii, including YrAS2388 and Yr28 on 4DS, and YrY201 on 7DL. Synthetic hexaploid wheat (SHW) lines, which contain a diversity of Ae. tauschii accessions, are potential breeding stocks. However, many biotic and abiotic resistance genes are suppressed in the hexaploid background. To prevent a linkage drag of undesirable traits and resistance suppression, it is best to identify R genes and use them precisely in gene pyramids.

In this study, we cloned the stripe rust resistance gene YrAS2388 from Ae. tauschii. Additionally, we have demonstrated that this gene can express effectively in hexaploid wheat and barley. Deployment of YrAS2388R in wheat cultivars together with other effective genes should sustainably protect wheat production from the devastating disease stripe rust.

Results

YrAS2388R confers resistance to wheat stripe rust. Aegilops tauschii Clae9, PI511383 and PI511384 (all from the subspecies (subsp.) strangulata) possess YrAS2388R. At the two-leaf seedling (juvenile) stage, Clae9, PI511383, and/or PI511384 were resistant with infection types (IT) between 1 and 5 to nine Pst races (PSTv-3, PSTv-4, PSTv-11, PSTv-18, PSTv-37, PSTv-41, PSTv-51, PSTv-52, and PSTv-172), under low temperature (LT) and/or high temperature (HT) regimes (Table 1). These races are virulent on a wide range of wheat germplasm (Supplementary Data 1). Clae9, PI511383 and PI511384 have shown Pst resistance (IT scores = 1–3; Fig. 1a) under natural infections in the Sichuan basin in China since 1995. In contrast, Ae. tauschii AS87, PI486274 and PI560536 (all subsp. tauschii accessions) do not

Table 1 Seed responses of selected lines to Puccinia striiformis f. sp. tritici

| Materials | Genomes | YrAS2388Rb | Infection typesc | TRd |
|-----------|---------|------------|------------------|-----|
| Clae9     | DD      | +          | PSTv-3 PSTv-11 PSTv-41 PSTv-172 | HT/LT1 |
| PI511383  | DD      | +          | 2 2 8 9         | LT1  |
| AvS5Yr28NIL | ABBDD  | +          | 1 1 8 9         | LT1  |
| AvS5     | ABBDD  | +          | 2 2 2 2         | LT1  |
| PI511383  | DD      | +          | 2 2 2 2         | LT1  |
| AvS5Yr28NIL | ABBDD  | +          | 2 2 2 2         | LT1  |
| AvS5     | ABBDD  | +          | 9 9 9 9         | LT1  |

*PI511384 = AS2388; SW3 = Langdon/Clae9; SW58 = Langdon/AvS18; AvS = Avocet Susceptible; AvS5Yr28NIL = Avocet + Y28
+ plus sign = positive for the YAS2388R (or Y28) locus; a minus sign = negative for the YAS2388R (or Y28) locus.
*Responses from 0 (immune) to 9 (massive sporulation) are according to McNeal’s scale. Unexpected Pst responses are highlighted by an italicized font.
*TR, temperature regimes: HT = high diurnal temperature cycle of 12°C/30°C; LT = low diurnal temperature cycle of 4°C/20°C.
*All seedling test with field spores (from Parker Farm) under a low temperature regime in 2018. Field spores likely included PSTv-37 and/or PSTv-52.
have the YrAS2388R gene\textsuperscript{22}, and were always susceptible under either natural infections or controlled inoculation (IT scores = 7–9; Fig. 1a, Table 1).

Previously, we hypothesized that YrAS2388 and Yr28 are the same gene\textsuperscript{22}. AvSYr28NIL and AvS are near-isogenic lines (NILs) for the Yr28 gene. During a Pst test with PSTv-3, PSTv-11, PSTv-41, and PSTv-172, AvS was susceptible to all races under both LT and HT, but AvSYr28NIL was highly resistant to PSTv-3, PSTv-11, and PSTv-172 (Table 1). We additionally tested two synthetic hexaploid wheat (SHW) lines, SW3 and SW58, that were derived from the durum wheat Langdon but have different D-genome donors: the Pst-resistant Clae9 and the Pst-susceptible AL8/78, respectively. Despite a dominant YrAS2388R gene in Clae9, SW3 was highly susceptible to PSTv-4, PSTv-18, PSTv-37 and PSTv-52 (Table 1), which was comparable to SW58 under LT. Thus, YrAS2388R can be suppressed when it is introgressed into certain hexaploid wheat genotypes.

YrAS2388R was delimited to a 50-kb region in PI511383. Clae9, PI511383, PI511384 and eleven other accessions were
found to have the YrAS2388R gene or allelic genes on 4D. We previously developed three F2 populations: popA (P1511383/P1486274), popB (Clas9/P1560536) and popC (P1511384/AS87). The YrAS2388R-based Pst responses are inherited as a Mendelian trait in all three F2 populations. Here, among 1910 F2 plants of popC-P2 (P1511384/AS87), 1,432 were resistant and 478 were susceptible in Wenjiang, Sichuan, China, which fits single dominant gene inheritance (Chi-Square goodness of fit test, $\chi^2_{3,1} = 0.001, P = 0.98$).

Using the wheat 10k iSelect array, we genotyped Clas9, P1486274, P1511383, P1560536, and 17 Pst-susceptible F2 plants (10 from popA and 7 from popB) for bulked segregant analysis (BSA) of the Pst-susceptible allele of YrAS2388 (YrAS2388S). Among 3276 applicable single nucleotide polymorphisms (SNPs), we selected 20 SNPs that were mostly associated with a Pst-susceptible phenotype; eight of them, including AT4D3406, AT4D3410, AT4D3411, AT4D3412, AT4D3413, AT4D3417, AT4D3418, and AT4D3419 (Supplementary Table 1), were in the 4D35 distal region. Based on specific genotypes per marker per plant, YrAS2388 (Supplementary Fig. 1a) was mapped distal to the AT4D3406 region (Supplementary Table 1).

The AT4D3406 region (Supplementary Fig. 1a) was initially targeted to map the YrAS2388 gene in popA and popC. Using the F2 and F3 data, we mapped YrAS2388 to the same region in popA-1 and popC-1 (Supplementary Fig. 1b, c). In popC-1, YrAS2388 is between Xsdauw2b (= AT4D3403) and Xsdauw3a (= AT4D3405) (Supplementary Table 2), an approximate 2.4-cM interval (Supplementary Fig. 1c). To assure that we defined the correct region, we targeted a large interval, Xsdauw2a (= AT4D3403)-Xsdauw3a (= AT4D3410), for screening recombinants in popA-2. Additional markers were designed from the linkage map and genome sequence of Ae. tauschii. First, we retrieved the AT4D3403, AT4D3404 and AT4D3405 corresponding genomic sequence, prioritized the low-copy number regions, created nine PCR markers (Xsdauw86 to Xsdauw91, Xsdauw93, Xsdauw95 and Xsdauw97) among six parental lines, and placed YrAS2388 between Xsdauw91 and Xsdauw97 (Supplementary Fig. 1d). Second, we constructed a fosmid genomic library from the Pst-resistant genotype P1511383. Xsdauw92, Xsdauw94 and Xsdauw96 were then developed using the fosmid clones of the YrAS2388 region. After analyzing 4205 popA F2 plants, which were from 11 F2 plants heterozygous in the Xsdauw2a-Xsdauw36a region, we precisely mapped YrAS2388 between Xsdauw92 and Xsdauw96, about a 0.13-cM interval, and added to the YrAS2388 interval with three completely linked markers (Xsdauw93-Xsdauw95) (Fig. 1b, Supplementary Fig. 1d, Supplementary Table 2).

The fosmid genomic library of P1511383 has approximately one million clones and represents an eight-fold coverage of the Ae. tauschii genome (= 4.3 Gb). Twenty fosmid clones were identified in the YrAS2388 region. In the physical map (Fig. 1c, Supplementary Fig. 2), Xsdauw91 and Xsdauw96, which delimited the YrAS2388 gene, were anchored to the two overlapping fosmids, F2-1 and Fe-19. After sequencing F2-1 and Fe-19, we primarily analyzed the Xsdauw91-Xsdauw96 region (ca. 50 kb). RNA sequencing (RNA-seq) in P1511383 revealed three active gene candidates for the YrAS2388 region. In the parental lines, and placed YrAS2388 between Xsdauw91 and Xsdauw97 (Supplementary Fig. 1d), a coiled-coil, a NB domain and a LRR domain with a classical four-helix bundle (4HB). Xsdauw91, Xsdauw93 and Xsdauw95 have only the STK domain. Xsdauw92, Xsdauw94 and Xsdauw96 have a classic four-helix bundle (4HB) that was previously classified as a coiled-coil, a NB domain and a LRR domain with a classic four-helix bundle (4HB). Xsdauw91, Xsdauw93 and Xsdauw95 have only the STK domain. Xsdauw92, Xsdauw94 and Xsdauw96 have a classic four-helix bundle (4HB) that was previously classified as a coiled-coil, a NB domain and a LRR domain with a classic four-helix bundle (4HB). Xsdauw91, Xsdauw93 and Xsdauw95 have only the STK domain. Xsdauw92, Xsdauw94 and Xsdauw96 have a classic four-helix bundle (4HB) that was previously classified as a coiled-coil, a NB domain and a LRR domain with a classic four-helix bundle (4HB).

Haplotype markers indicated that NLR4DS-1 is YrAS2388. To help identify the correct gene, we genotyped 159 Ae. tauschii...
accessions using five markers for NLR4DS-1 (HTM3a to HTM3e, or collectively called HTM3S), one for RLK4DS-1 (HTM1a) and one for RLK4DS-2 (HTM2a) (Supplementary Tables 2 and 3, Supplementary Data 3). The R-type allele (e.g. “A” in PI511383) of NLR4DS-1 was completely associated with Pst resistance in resistant haplotypes R1 to R3 (Supplementary Data 3). All non-A scores of the NLR4DS-1 markers were associated with Pst susceptibility. The coding region (ATG to 3′UTR2; Fig. 1d) of NLR4DS-1 is identical amongst eight Pst-resistant Ae. tauschii accessions, including AS2386, AS2387, AS2399, AS2402, CIae9, PI349037, PI511383, and PI511384. In contrast, in RLK4DS-1 and RLK4DS-2, the R-type allele (e.g. “A” in PI511383) was present in the Pst-susceptible genotypes (S1-S3 and S5), indicating that both genes do not confer Pst resistance. Similarly, the absence of RLK4DS-1 and/or RLK4DS-2 in the R2 and R3 haplotypes suggested that neither gene is essential for Pst resistance. Thus, NLR4DS-1 is the only candidate for YrAS2388R.

Pst-susceptible SHW mutants have more mutations in NLR4DS-1.

Synthetic hexaploid wheat (SHW) SW332 and Syn-SAU-933 acquire the YrAS2388R gene from their D-genome donor; both SW3 and Syn-SAU-93 displayed moderate Pst resistance (IT scores = 3–5; Fig. 2a) in Sichuan, China. Using ethyl methanesulfonate (EMS), we generated 1132 M2 families of SW3 and 613 M2 families of Syn-SAU-93. Under field conditions, we identified 103 Pst-susceptible plants (IT scores = 7–9; Fig. 2a, Supplementary Data 4). For the NLR4DS-1, RLK4DS-1, and RLK4DS-2 genes, 51 Pst-susceptible mutants (49.5%) had a deletion in the NLR4DS-1 gene, of which 50 deletion events extended into RLK4DS-2 but only 11 deletion events extended further to RLK4DS-1 (Supplementary Data 4). However, no deletion only occurred in RLK4DS-1 and/or RLK4DS-2. Among the remaining 52 non-deletion mutants, 18 Pst-susceptible mutants had at least one base change in the NLR4DS-1 gene, and 16 of those mutations (89%) either caused an amino acid change or formed a premature stop codon (Supplementary Data 4).

Fig. 2 The YrAS2388 locus confers stripe rust resistance in wheat and barley. a Syn-SAU-93 and SW3 are two synthetic hexaploid wheat (SHW) lines that express the YrAS2388R gene. WT is the resistant wild-type control with necrotic lesions. L68 (G117D), L91 (V267I), F7 (S394N) and F43 (V482I) are susceptible mutants with sporulating Pst. Plant responses (MR = moderate resistance; R = resistant; S = susceptible) to Pst are indicated in parentheses. b The susceptible hexaploid wheat CB037 was transformed with the intact PCI1104 (= F2-1). Transgenic T3 wheat (all from the No. 5 and 10 T2 subfamilies) was challenged with PSTv-239 at the adult plant stage. Under each picture, PCR results as positive (plus signs) or negative (minus signs) for DNA amplification (upper) and RNA expression (lower) of the three target genes: RLK4DS-1 (left), RLK4DS-2 (middle) and NLR4DS-1 (right). RT-PCR is illustrated in Supplementary Fig. 4. c The susceptible barley Golden Promise (GDP) was transformed with the intact PC1104 (= F2-1). Transgenic T1 barley seedlings were inoculated with race PSH-72 of Puccinia striiformis f. sp. hordei (Psh). Scale bar = 1 cm. Source data are provided as a Source Data file.
Seven amino acid variations were identified in the NLR4DS-1 gene, including Gly117Asp, Val267Ile, Ser394Asn, Leu421Phe, Glu426Lys, Gly430Ser, Gly431Ser, Gly432Pro, Ser433Cys, Lys435Asn, and Gly436Asp. Only transgenic plants that expressed NLR4DS-1 were resistant to Pst (Table 2, Supplementary Fig. 4). Therefore, NLR4DS-1 represents a strong candidate for YrAS2388 (Table 2).

The Pst-resistant NLR4DS-1 has duplicated 3′UTRs (Fig. 1d) in all Pst-resistant parents (CIae9, PI511383 and PI511384) and each 3′UTR is associated with multiple transcript variants: TV1 and TV2 with 3′UTR1, TV3 and TV4a (and 4b) with 3′UTR2 (Fig. 1e). We overexpressed the Pst-resistant NLR4DS-1 cDNA under the maize Ubi promoter (Supplementary Table 4). All 36 transgenic wheat and barley lines that expressed TV1 (or TV2) did not confer resistance to stripe rust (Table 2), suggesting that one cDNA isoform was insufficient to confer stripe rust resistance. For stripe rust resistance, the NLR4DS-1 gene may require the activity of multiple cDNA isoforms and/or regulatory elements in the genomic sequence.

**Innate and external factors regulate NLR4DS-1 expression.** In the Pst-resistant NLR4DS-1, the most abundant isoforms are TV1 (for a 1068-aa protein with complete 4HB, NB, and LRR domains) and TV4 (for a 471-aa protein with a complete 4HB and a partial NB domain) (Fig. 1e, Supplementary Fig. 7a). The less abundant isoform TV2 might result from either a partial exon skipping from TV1 or the retention of an 833-bp cryptic intron in exon 5, which disrupts the NB and LRR domains. TV3 is also a less abundant isoform and is structurally similar to TV4, but retains the first 244 bp in the 833-bp cryptic intron, which only disrupts the LRR domain. In contrast, the Pst-susceptible NLR4DS-1 either remained completely silent in PI486274 and PI560536 or produced only the TV1-type transcript in AL8/78 and AS87 (Supplementary Fig. 3b).

In Pst-resistant PI511383, TV1 to TV4 cDNAs were all expressed in the seedling and adult leaves (Supplementary Fig. 3c). When exposed to alternating low (10°C) and high (25°C) temperatures, the high temperature upregulated TV2 and downregulated TV4 (Supplementary Fig. 3d), which is correlated with increased Pst-resistance at elevated temperatures. In response to Pst race PSTv-306, the TV1 cDNA levels in the Pst-infected plants were comparable to those in the mock-inoculated control plants (Supplementary Fig. 3e). In contrast, Pst infections upregulated TV2 at 2, 5, and 10 days post inoculation (dpi) but not at 3 dpi, downregulated TV3 at 3, 7, and 14 dpi, and downregulated TV4 at 3 dpi (Supplementary Fig. 3e). Thus, both temperature and Pst infection regulate the transcription of NLR4DS-1. However, a change in the relative levels of either the individual four transcripts and/or the proteins or protein complexes may affect the induction of stripe rust resistance. Among the Pst-susceptible mutations of NLR4DS-1, Ser394Asn and Gln557Stop(∗) only affect TV1 and Thr456Ile only affects TV4, which indicates that both

### Table 2 Transgene expression and plant responses to *Puccinia striiformis*

| Groups | Constructs (treatment) | Events | RLK4DS-1 | RLK4DS-2 | NLR4DS-1d | Responses to Pst (or Psh) |
|--------|------------------------|--------|----------|----------|-----------|--------------------------|
| G1     | PC1104 (I)             | 1+3    | +        | +        | +         | Resistant                |
| G2     | PC1104 (XI)            | 1      | +        | −        | −         | Susceptible              |
| G3     | PC1104 (I)             | 1      | −        | +        | +         | Resistant                |
| G4     | PC1104 (XI, X1I)       | 2      | +        | −        | −         | Susceptible              |
| G5     | PC1104 (B1, XI, X1I)   | 5      | −        | +        | −         | Susceptible              |
| G6     | PC1104 (B1, XI, X1)    | 7+2    | −        | −        | −         | Susceptible              |
| G7     | PC101 (Ubi::NLR4DS-1 TV1) | 9+4    | −        | −        | +         | Susceptible              |
| G8     | PC1012 (Ubi::NLR4DS-1 TV2) | 10+13  | −        | −        | +         | Susceptible              |

*Groups G1-G6 are based on genomic DNA, and Groups G7-G8 are based on cDNA.

### Notes:
- PC1104 was either intact (I) or linearized with BsrGI (B), NotI (N1), XbaI (X1I) and XbaI plus KpnI (XK1). Intact or linearized plasmid per enzyme was introduced into recipient genotypes separately.
- Per cell, the first number indicates the number of wheat transfectants; when there are two numbers, the second number indicates the number of barley transfectants.
- A plus sign (+) indicates full-length gene expression by PCR; a minus sign (−) indicates no gene expression.
- RT-PCR is illustrated in Supplementary Fig. 4.
- PSTv-40 and PSH-72 were used to test the transgenic wheat and barley, respectively.
- The NLR4DS-1 cDNAs were under the maize Ubi promoter; no digestion was applied to them.
TV1 and TV4 are essential for stripe rust resistance (Supplementary Data 4). Collectively, we hypothesize that TV1 plays a major role in the induction of stripe rust resistance, TV2 acts as a positive co-factor, and TV4 (or possibly TV3) act either as negative regulators when its expression is high or as positive regulators when its expression is low (Supplementary Fig. 8).

Using a yeast two-hybrid system, we tested the interaction among the native (TV1, TV2, and TV4) and mutant (TV1G117D, TV2G117D, and TV2V267I; Supplementary Data 4, Supplementary Fig. 7a) isoforms of the Pst-resistant NLR4DS-1. The NLR4DS-1 isoforms, both native and mutant forms (NM forms) had no autoactivity. A strong interaction occurred amongst the TV2 proteins (NM forms; Supplementary Fig. 7b). We observed a weak interaction between TV2 mutants and TV1 (NM forms), and between TV2 proteins (NM forms) and TV4. Apparently, TV2 can mediate protein interactions amongst multiple isoforms of NLR4DS-1.

The Pst-resistant NLR4DS-1 occurs only in Aegilops tauschii. The D genome of common wheat was derived from Ae. tauschii subsp. straunferata or tauschiia. The resistance allele of NLR4DS-1 is present in 100% (n = 37) and 19% (n = 122) of the accessions of subsp. straunferata and tauschiia tested, respectively (Supplementary Data 3). Similarly, the resistance allele of NLR4DS-1 is present in 30% (n = 23) of the Ae. tauschiia accessions used as a parent in developing SHW lines (Supplementary Data 5). Surprisingly, the resistance allele is absent in all (n = 461) of the common wheat lines tested (Supplementary Table 5, Supplementary Data 6). The NLR4DS-1 allele in Chinese Spring (CS) is nearly identical to the Pst-susceptible alleles from the subsp. tauschiia accessions PI486274 and PI560636 (Supplementary Data 2). In addition, the resistant NLR4DS-1 allele is also absent in all the tested T. monococcum subsp. aegilopaides (n = 24), T. monococcum subsp. monococcum (n = 24), T. turgidum subsp. dicoccoides (n = 140), Ae. comosa (n = 17), Ae. comosa var. subventrica (n = 6), Ae. longissima (n = 8), Ae. sharonensis (n = 28), Dasypyrum villosum (n = 10), and Hordeum vulgare subsp. spontaneum (n = 5) (Supplementary Table 5, Supplementary Data 6).

The Pst-resistant NLR4DS-1 may arise from paralogous genes. All Pst-resistant NLR4DS-1 genes contain two duplicated regions. The first region includes the 3′ end of exon 5, exons 6 and 7, and intron 7a; and the second region includes the pseudo-exon 5′, exons 6′ and 7′, and intron 7a′ (Fig. 1d, e, Supplementary Fig. 9a). This duplication is not present either in Pst-susceptible NLR4DS-1 alleles or in any NLR4DS-1-like genes. To examine the origin of the duplicated regions, we built separate phylogenetic trees for each of six selected fragments (exons 5, 6, 7, and 8; and introns 7a and 7b) of 7 to 15 NLR4DS-1 homologues in Triticeae (Supplementary Fig. 9b). The trees indicate that exons (5–8) and introns (7a and 7b) of the Pst-resistant NLR4DS-1 are more related to those of the Pst-susceptible NLR4DS-1 in CS (CS-4D:1821950..1825589); all the duplicated fragments (exons 5′ to 7′ and intron 7a′) are in separate clades. In addition, the duplicated 3′UTR1 and 3′UTR2 DNA of NLR4DS-1 in PI511383 are only 87% identical in the conserved 373 bp (GenBank MK736661: 3735..4107 versus 6409..6781, counted forward from the start codon ATG). Thus, the Pst-resistant NLR4DS-1 likely arose after a shuffling event between two paralogous genes. Specifically, the 3′UTR2 contains part of a 2668-bp insertion (within a 6-bp target site duplication = TACTGG) that occurred in intron 7 of the ancestral 3′UTR1 region. A similar 3′UTR duplication in the Pst-resistant NLR4DS-1 gene is present in the synthetic wheat W7984. In the 2668-bp insertion, a 496-bp region (pseudo-exon exon 5′) is 90% identical to the ancestral exon 5. The insertion also has two miniature inverted-repeat transposable elements, which are frequently adjacent to transcriptionally active genes. Likely, the 2668-bp fragment was derived from another, currently unidentified, NLR4DS-1 homologue in Ae. tauschii.

In Triticeae, there are multiple NLR4DS-1-like genes; three copies were identified in the YrAS2388 region (Supplementary Fig. 2). In common wheat CS, there are at least five transcriptionally active homologues of the NLR4DS-1 gene (Supplementary Fig. 10). None of the NLR4DS-1-like homologues in CS has duplicated 3′ UTRS. The Pst-susceptible NLR4DS-1 homologues in CS share only 86%-94% identity with the Pst-resistant NLR4DS-1 in PI511383 at the cDNA level.

NLR4DS-1 offers a toolbox for solving stripe rust problems. We compared the stripe rust resistance in 81 SHW lines and their original parents, including 30 SHW lines with the YrAS2388R gene (Supplementary Data 5). YrAS2388R confers a strong Pst resistance (IT scores = 1–3) in Ae. tauschii. However, 27% of SHW wheat had significantly less resistance than the parental lines (T. turgidum and/or Ae. tauschii). In this study, SW3 has the Pst-resistant NLR4DS-1 allele and shows the characteristic expression of alternatively spliced transcripts. However, SW3 was susceptible (IT scores = 7–9) to Pst in Moscow, ID, USA (Table 1), presumably because of a suppressor in its genetic background. Nonetheless, Ae. tauschii accessions with a strong Pst resistance frequently conferred moderate to high Pst resistance in a derived SHW wheat (Supplementary Data 5), indicating that Ae. tauschii is valuable for breeding resistant NLR4DS-1. For example, the SHW wheat Syn-SAU-S9 is based on Langdon/AS313//AS2399, in which the Ae. tauschii AS2399 is positive for the YrAS2388R gene. Although Syn-SAU-S9 displayed only moderate resistance to Pst (IT scores = 4–5), we used Syn-SAU-S9 to transfer the YrAS2388R gene into common wheat. Three co-segregating markers were used for marker-assisted selection of YrAS2388R (Supplementary Fig. 11, Supplementary Table 2). In 2015, we developed an elite line Shumai 1675, which is an F6 line of Syn-SAU-S9/Chuan 07001/Shumai 969. Shumai 1675 is highly resistant to Pst in Sichuan, China. In 2017, Shumai 1675 outcompeted the check variety Miaimi 367 with an 11% increase in yield in the regional variety trials of the Sichuan province, China (Supplementary Table 6).

Discussion

YrAS2388R provides robust resistance in a wide spatial and temporal range, including China (current study), Canada, Norway, the United Kingdom and the United States (TA2450 = C1ae9, TA2452 = PI511384; current study). However, YrAS2388R has had limited use probably for two reasons: it is absent in common wheat; and it can be suppressed in hexaploid wheat. In the present study, YrAS2388R, when separated from potential linkage drag, conferred strong stripe rust resistance in transgenic wheat and barley, indicating that YrAS2388R offers a practical solution for stripe rust resistance in Triticeae. The YrAS2388R gene-based markers (e.g. Xsdaau95, Supplementary Fig. 11) can be used for marker-assisted selection.

YrAS2388R is another example of a gene that was either not transferred or lost during domestication. Nevertheless, genes from both progenitors and distantly-related species of wheat can be used to enhance contemporary common wheat. Of the 81 permanently named Yr resistance genes, 21 were transferred from either related species or wild relatives of wheat, such as Yr5 from Triticum spelta, Yr15 and Yr36 from T. dicoccoides and Yr28 from Ae. tauschii. However, alien genes can be accompanied by linkage drag. For example, linked genes to Yr8 from Ae. comosa...
are associated with tall height and delayed maturity.42 The Yr9 gene from the IBL/1RS translocation improves grain yield but causes inferior quality,43 which limits its use in wheat especially in the U.S. Pacific Northwest44. YrAS2388R could be transferred into wheat through a cisgenic approach. Thus, cisgenic YrAS2388RR can provide an advantage to consumers in comparison to traditional breeding. YrAS2388RR (or Pst-resistant NLR_{ADS-1}) is associated with duplicated 3’UTRs, which is an apparently rare phenomenon. The ancestral 3’UTR of NLR_{ADS-1} adjoined the 3’-end of an unknown NLR_{ADS-1} paralog, resulting in duplicated 3’UTRs in Pst-resistant NLR_{ADS-1}. The 3’UTR is an important component of eukaryotic genes.52 More than half of human genes use alternative polyadenylation to generate mRNAs that differ in the 3’UTR length but encode the same protein.53 In contrast, there are few reports of genes with two separate 3’UTRs that cause a difference in the protein product. In wheat, the stripe rust resistance gene WKS1I generated six transcript variants, of which WKS1I differs from the others in the 3’UTR.14 Pst-resistant NLR_{ADS-1} also shows alternative splicing (AS) in the NB-LRR region of the gene. AS is prevalent in eukaryotes,54 95% of multi-exon genes in human and 44% of multi-exon genes in Arabidopsis display AS. In Arabidopsis, the bacterial-resistance gene RPM1 produces alternative transcripts in response to infection by pathogen Pseudomonas syringae pv. tomato.50 Both environmental and developmental stimuli precisely regulate the abundance of functional mRNA isoforms.51 Here, in keeping with resistance, expression of the NLR_{ADS-1} isoforms also depends on pathogen infection and the temperature. Thus, abundance of NLR_{ADS-1} isoforms appears to be a mechanism that wheat can use to robustly resist stripe rust pathogen invasion.

The NLR_{ADS-1} protein is a member of the CC-NB-LRR (CNL) proteins. The coiled-coil domain of the potato virus X resistance protein (Rx) actually forms a four-helix bundle (4HB).32 The N-terminal domain of NLR_{ADS-1} is predicted to fold into four helices, and it is also classified as an Rx-CC-like in the NCBI CDD (E = 9 × 10^-9) and Rx_N in the Pfam database (E = 6 × 10^-16). Although CNL genes are often race-specific and not durable,53 some CNL genes such as the rice blast resistance gene Pigm R34 have been durable. Here, we showed that YrAS2388RR confers resistance to a broad array of Pst races and has been effective to all natural infections of Pst in China since 1995. As a typical NLR gene, we hypothesize that the NLR_{ADS-1} proteins change their state via a competition model (Supplementary Fig. 8). The full-length TV1 protein plays a central role in signal transduction, but it requires other variant proteins (TV2 and TV4) for a proper conformation, which together form an active TV1 complex for defense signaling.

Here, YrAS2388RR was fully expressed without suppression in transgenic hexaploid wheat and in barley. In addition, we have produced Shumai 1675, which has YrAS2388RR and is strongly resistant to Pst, suggesting that either YrAS2388R is not suppressed in Shumai 1675 or that YrAS2388R worked positively with other Yr genes to confer resistance to Pst. However, in the current study, the resistance levels of parental lines (T. turgidum and/or Ae. tauschii) were suppressed in nearly 27% of the SHW wheat lines. Yr28, which is probably the same gene as YrAS2388R,24 was effective in seedlings and adult plants of SHW Altar 84/Ae. tauschii accession W-2194. Here, we observed that YrAS2388RR in SHW SW3 was suppressed, i.e., it was fully susceptible to natural Pst races at adult-plant stages in Moscow (ID, USA), probably because the suppressor responds more to the cooler night temperatures in this area. When YrAS2388RR is suppressed in a specific hexaploid wheat such as SW3, Pst-resistance levels might be increased by disrupting the unknown suppressor, as was previously done by inactivating a suppressor of stem rust resistance.55

In the case of wheat powdery mildew, pyramiding of closely related NLR genes can cause dominant-negative interactions and that lead to R gene suppression.56 For example, the Pm8 resistance gene from rye was suppressed in wheat by a susceptible allele of the wheat ortholog Pm3.57 In the present study, the Pst-resistant NLR_{ADS-1} in PI511383 shares 86–94% identity with cDNA from the transcriptionally active homologues in common wheat (Supplementary Fig. 10). Thus, YrAS2388RR suppression might conceivably be caused by close homologues of NLR_{ADS-1} that are present in Triticeae. To test this hypothesis, in the future, one could mutate a SW3 line, screen for truncation mutations in the NLR_{ADS-1} homologues, and test whether the homologues' mutations have any effect on stripe rust resistance. Regardless, because the transgene NLR_{ADS-1} induces effective Pst resistance in hexaploid wheat, we predict that sustainable Pst resistance can be achieved with either a cisgenic strategy with Pst-resistant NLR_{ADS-1} or a conventional strategy that combines both the incorporation of a Pst-resistant NLR_{ADS-1} and either avoidance or inactivation of the apparently linked latent suppressor(s) from Ae. tauschii.

Methods

Plant materials. This study was performed on Aeolops tauschii, Hordeum vulgare, Triticum aestivum and synthetic hexaploid wheat (SHW) (Supplementary Table 7). Sources of accessions used for haplotype analysis are indicated in Supplementary Data 3 and 6. To map YrAS2388RR, we used six Ae. tauschii accessions (Supplementary Table 7), in which the Pst-resistant parents, Clea9, PI511383 and PI511384 (AS8723), all have YrAS2388RR.

We developed three F2 populations (popA: PI511383/PI466272; popB: Clea9: PI560536; and popC: PI511384/AS8723). These populations were used for preliminary and fine mapping, and popC was also used to confirm the single Mendelian inheritance of YrAS2388RR. In popA, we selected 11 F2 plants that were heterozygous in the YrAS2388RR region (Xsdauw2-Xsdauw36), and allowed them to self-pollinate to produce F3 seeds. After screening 4,205 F3 plants, we identified 467 plants with crossovers in the Xsdauw2-Xsdauw36 interval, and used them to generate a high-density map.

Stripe rust inoculum and infection assays. Wheat stripe rust tests were conducted in four institutions: Shandong Agricultural University (SDAU), Tai’an, China; Sichuan Agricultural University (SCAU), Chengdu, China; Washington University (WSU), Pullman University of Idaho (UI), Moscow, USA. Avocet Susceptible (AvS), Huixianhong, Mingxian, 169, and/or SY95-71 were used as susceptible checks and also planted surrounding the plots to increase and spreaduredinisporoes for adequate and uniform rust levels for reliable screening. For winter-growth genotypes tested in greenhouses or growth chambers, seeds were vernalized in wet germination paper (Anchor Paper Co., Saint Paul, MN, USA) at 4 °C in darkness for 45 d; vernalized shoots were transplanted into soil in the greenhouse and maintained at 25 °C during the day and 15 °C at night with 16 h photoperiod.

Infection types (IT) were recorded using a 0–9 scale39 and the following categories: resistant (R, IT scores = 0–3), moderate reactions (M, IT scores = 4–6) that include moderate resistance (MR, IT scores = 4–5) and moderate susceptibility (MS, IT score = 6), and susceptible (S, IT scores = 7–9). IT scores were recorded 15–18 days post inoculation (dpi) when the uredinial pustules were clearly visible on susceptible plants. Responses of SHW and their parental lines Pst were shown in Supplementary Data 5.

At SDAU, uredinisporoes were obtained from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. Due to changes in race frequency and spore availability, different Pst races were used in different years (mixed spores of Chinese Pst races CYR29, CYR31, CYR52, CYR33, Su11 and/or Su14 during 2010 to 2012; CYR29 and CYR12 in 2013; and CYR29, CYR31, CYR52 and CYR33 in 2014–2016). Collectively, these races represent the predominant Pst races in China in different periods since the 1990’s. Field trials were performed to assess the responses to Pst in the parental lines, F1, F2 and advanced progenies of popA and popB. At the seedling stage, an aqueous spore suspension was manually injected with a 2.5 ml syringe into leaf bundles and repeated after 10 days. For preliminary mapping, F1 plants of popA and popB were evaluated in 2011, and the corresponding F2 progeny were then tested in 2012. Critical recombinants of popA were evaluated in 2013–2016 (F2 to F4 generations, one generation per year), and the F4-F5 generations were additionally tested in SCAU in 2014–2016. For SCAU, we primarily conducted the Pst test in Dunhuang and Weinjiang, two experimental stations of the Triticaceae Research Institute at SCAU. Uredinisporoes were obtained from the Research Institute of Plant Protection,
Gansu Academy of Agricultural Sciences, Lanzhou, China. Using the mixture of Chinese *Pst* races CYR30, CYR31, CYR32, SY11-4, SY11-14, and HY46-8, we evaluated the *Ae. tauschii* germplasm in Dujianyuan for three growing seasons (2006–2009). In 2008–2009, we also tested synthetic wheat and their polyploid parents in Dujianyuan (Supplementary Data 5). A mixture of CYR30, CYR31, CYR32, SY11-4 and HY46-8, we tested synthetic wheat and their parent lines in Wenjiang in 2011–2012 (Supplementary Data 5), and then retested five synthetic wheat and their parent lines in Wenjiang in 2016–2017 using a mixture of CYR32, CYR33, CYR34 (= Gu22-9), Gu22-14, and SY11-1 (Supplementary Data 5). To identify *Pst*-susceptible mutants, we screened the Syn-SAU-93 population in 2016–2018 using udnosporic acid of similar races as 2016 supplemented with 12.5 μg chloramphenicol ml −1 (LB-C), and cultured on a 250 rpm shaker at 37 °C for 4 h. The culture was diluted in a 10-fold series (10−1 to 10−5) using liquid LB, and the serial dilutions (300 μl per level) were plated onto the LB-C agar. An ideal dilution yielded 4,000–5,000 clones per 15-cm-diameter plate, from which colonies were collected using the 384-pin replicator with four repeated contacts to collect more representative colonies. After the replicator was used to inoculate a 384-well plate with 50-μl liquid LB-C, the plate was incubated at 37 °C overnight. Each well was screened by PCR. For positive wells, 20-μl culture was enriched in 2-ml liquid LB-C, and grown in a 250 rpm shaker at 37 °C for 2 h. The end culture was diluted 10-fold (from 10−1 to 10−5) using liquid LB-C, and used for transformation. There were plated onto LB-C agar. An ideal dilution yielded 50–200 clones per 9-cm-diameter plate, from which a positive clone would be revealed among 24 clones.

**Mutagenesis and mutation screening.** Synthetic hexaploid wheat SW3 and Syn-SAU-93 were treated with 0.8% EMS (78 mM in water; Sigma-Aldrich Co., St. Louis, MO, USA). Briefly, lots of 400 seeds (M0) were soaked in 100 mM EMS solution, treated on a shaker at 150 rpm, at 25 °C for 10 h, and washed with running water at room temperatures for 4 h. M1 plants of SW3 were grown in a greenhouse in Taian, China. M1 plants of Syn-SAU-93 were grown in the field in Chongzhou, China. To simplify the fieldwork, mutant seeds of SW3 and Syn-SAU-93 were bulk plated at M0 to M4 generations in Chongzhou and Wenjiang. Screening for resistance and screening for female fertility using *Pst* races CYR30, CYR31, CYR32, SY11-4, and HY46-8 in 2016–2018.

**PCR screening.** We screened on each of 622 super colon plants, with 2-μl 2× PCR master mix as a template. We screened for markers Xsau93, Xd605 and O13 (PCR primers P160/P161 (Supplementary Tables 2 and 8). PCR amplification was performed as follows: 95 °C for 5 min, 32 cycles with 95 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s, and a final extension at 72 °C for 10 min. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. For positive super colon plants, 25-μl gDNA was inoculated into 5-ml liquid LB supplemented with 12.5 μg chloramphenicol ml −1 (LB-C), and cultured on a 250 rpm shaker at 37 °C for 4 h. The culture was diluted in a 10-fold series (10−1 to 10−5) using liquid LB, and the serial dilutions (300 μl per level) were plated onto the LB-C agar. An ideal dilution yielded 4,000–5,000 clones per 15-cm-diameter plate, from which colonies were collected using the 384-pin replicator with four repeated contacts to collect more representative colonies. After the replicator was used to inoculate a 384-well plate with 50-μl liquid LB-C, the plate was incubated at 37 °C overnight. Each well was screened by PCR. For positive wells, 20-μl culture was enriched in 2-ml liquid LB-C, and grown in a 250 rpm shaker at 37 °C for 2 h. The end culture was diluted 10-fold (from 10−1 to 10−5) using liquid LB-C, and used for transformation. There were plated onto LB-C agar. An ideal dilution yielded 50–200 clones per 9-cm-diameter plate, from which a positive clone would be revealed among 24 clones.
transgenic plants, respectively. Using the bombardment protocol for wheat\footnote{10}, we also transferred the intact fosmid PC1104 into barley Golden Promise, however the tissue culture and regeneration procedures were specific for barley\footnote{16}. We bombarded 2,200 immature embryos of Golden Promise and generated 300 putative transgenic plants.

We also overexpressed the NLR\textsubscript{DS-1} DNA under the maize Ubi promoter in wheat and barley. For the NLR\textsubscript{DS-1} \textit{TV1} cDNA (in PC1101), we bombarded 2,200 wheat immature embryos (Bobwhite or CB037), obtained 40 putative T\textsubscript{0} plants, and tested nine NLR\textsubscript{DS-1} \textit{TV1} expressing \text{T1} families against PSH-72. For NLR\textsubscript{DS-1} \textit{TV2} cDNA (PC1102), we bombarded 2,500 wheat immature embryos (Bobwhite), obtained 54 putative T\textsubscript{0} plants, and tested ten NLR\textsubscript{DS-1} \textit{TV2} expressing \text{T1} families for their response to PSTV-40. Using a standard \textit{Agrobacterium}-mediated transformation\footnote{10}, we then infected 800 barley immature embryos (Golden Promise), obtained 28 putative transgenic T\textsubscript{0} plants and tested 13 NLR\textsubscript{DS-1} \textit{TV2} expressing \text{T1} families against PSH-72.

Transgene integration was confirmed by a positive amplification of \textit{BAR} with primers P184/P185, \textit{RLK\textsubscript{DS-1}}, with primers P208/P209, \textit{RLK\textsubscript{DS-2}}, with primers P203/P204 and NLR\textsubscript{DS-1}, with primers P213/P214 (or in the overexpression experiment with primers P166/P160). Transcription was assessed by RT-PCR with primers P208/P209 for \textit{RLK\textsubscript{DS-1}}, Primers P187/P188 for \textit{RLK\textsubscript{DS-2}}, and primers P189/P190 for NLR\textsubscript{DS-1}. \textit{ACTIN} primers P191/P192 were used as an internal control for both wheat and barley. PCR primers are described in Supplementary Table 8.

**Haplotype analysis.** Haplotype analysis was performed to understand the association of haplotypes and responses to \textit{Pst} and the evolution of the \textit{YaRS2388} region. Haplotype markers (HTM) were specifically designed for \textit{RLK\textsubscript{DS-1}}, \textit{RLK\textsubscript{DS-2}}, and \textit{NLR\textsubscript{DS-1}} (Supplementary Table 5, Supplementary Data 3) and counted from “A” in the start codon (ATG) in the genomic allele (GenBank accession number MK288012); for each marker, two periods separate the starting and ending nucleotides, and a minus sign indicates a backward count from “A” and a plus sign indicates a forward count from “A”. First, 159 \textit{Ae. tauschii} accessions were genotyped in Sichuan, China using seven markers: HTM1a (= \textit{RLK\textsubscript{DS-1}}), HTM2a (= \textit{RLK\textsubscript{DS-2}}) and HTM3a (= \textit{NLR\textsubscript{DS-1}}) (Supplementary Table 3, Supplementary Data 3). Second, 874 Triticale lines were genotyped in Shandong, China using four markers: HTM1b (= \textit{RLK\textsubscript{DS-1}}), HTM2b (= \textit{RLK\textsubscript{DS-2}}) and HTM3b (= \textit{NLR\textsubscript{DS-1}}). PCR primers are described in Supplementary Table 2. Markers used to genotype the Triticale collection in Shandong were different from those used for genotyping the \textit{Ae. tauschii} collection in Sichuan. Genotypes per gene per accession were not necessarily identical between the two tested collections. Thus, grouping of haplotypes should be considered separately for these two collections.

**Gene expression analysis.** RT-PCR was used to detect the expression of \textit{RLK\textsubscript{DS-1}}, \textit{RLK\textsubscript{DS-2}}, \textit{NLR\textsubscript{DS-1}}, and \textit{ACTIN} (internal control). Plants were maintained at 25 °C during the day and 15 °C at night with a 16 h photoperiod. Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY, USA). First strand cDNA was synthesized using the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RT-PCR was conducted on the \textit{2}\textsuperscript{nd}-leaf of the juvenile (two-leaf stage) plants. Primers used were P193/P194 for \textit{RLK\textsubscript{DS-1}}, P195/P196 for \textit{RLK\textsubscript{DS-2}}, P197/P198 for \textit{NLR\textsubscript{DS-1}}, and P199/P200 for \textit{ACTIN}. Fosmid clones were extracted using QIAGEN Large Con-}
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Author contributions
D.F. conceived the project; D.L., J.Wu, J.Wu, L.E., M.C.L., X.C. and Y.Z. contributed ideas and resources, B.L., C.K., C.Z., F.C., F.N., G.G., H.Z., J.Q., L.H., L.Z., M.H., M.Li, M.Liu, M.W. and Q.H. performed the experiments; C.Z., D.F. and L.H. analyzed the data; C.Z., D.F. and L.E. wrote the paper; and all authors discussed the results and the paper.

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