

Cytogenetic identification and molecular marker development of a novel Wheat - *Psathyrostachys huashanica* 2NsS (2D) fragment disomic substitution line with resistance to stripe rust

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Abstract

Background

Psathyrostachys huashanica Keng ($2n = 2x = 14$, NsNs) is a rare and unique species of grass that is native to China. It is a significant genetic resource for wheat breeding because of the multiple beneficial features that can be used in wheat improvement programs.

Results

The study isolated a novel wheat - *P. huashanica* derived line 18TR61 from the progeny of common wheat 7182 and *P. huashanica*. Cytological studies revealed that 18TR61 has chromosome karyotype of $2n = 42 = 21II$; Genomic *in situ* hybridization (GISH) and non-denaturing fluorescence *in situ* hybridization (ND-FISH) experiments confirmed that two 2D chromosomes in line 18TR61 were replaced by two incomplete Ns chromosomes. Meanwhile, it has been observed that 18TR61 exhibits structural variations in chromosomes 2B, 7B, and 5A when compared to its parents. Molecular markers and GenoBaits®WheatplusPh microarray analysis indicated that the alien chromosomes originated from *P. huashanica* 2Ns. Based on karyotype observations, it was incomplete in length, and contains all of the short arm, centromere, and fragment of the long arm. Assessment of agronomic traits showed that 18TR61 outperformed its parents in terms of stripe rust resistance in the adult plant stage, as well as spike length and grain size.

Conclusions

As a result, 18TR61 was identified as a wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution. Furthermore, the established 15 specific markers were beneficial for the quick identification of heterologous material containing the 2Ns chromosome in the wheat background. This line offered a possible bridge for wheat genetic improvement on disease resistance to stripe rust and yield.

Background

Wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD) is an important ration crop and the world's largest cultivated crop. In addition to being a major source of starch and energy, wheat provides a wide range of healthful and beneficial components such as proteins, vitamins, dietary fibers, and phytochemicals [1]. Population increase, environmental degradation, and climate warming all contribute to common wheat's reduced susceptibility to both biological and abiotic stressors, resulting in a decline in production [2]. The successful fusing of the common wheat with its wild cousins, also known as distant hybridization, offered an efficient means to incorporate beneficial genes from the wild relatives into the genetic material of common wheat [3, 4].

Psathyrostachys huashanica Keng ($2n = 2x = 14$, NsNs) is a rare plant found on Huashan mountain in Qinling, Shaanxi, China [5]. It is characterized by early maturity, drought tolerance, salinity tolerance, resistance to stripe rust, *Fusarium* head blight, powdery mildew, downy mildew, and yellow dwarf disease, in addition to many yield-related beneficial genes contained in its genetic material [6]. Since 1990, many of its excellent traits have been transferred to common wheat by wide hybridization [7, 8] and created some derived lines with *P. huashanica* chromosome fragments: Such as addition lines 20JH1155 [9], substitution lines H139 [10], translocation lines TR77 [11], etc., these derived offspring outperform their parents in biological and abiotic stressors, as well as agronomic qualities. So far, some important genes controlling excellent traits in derived lines have also been explored, such as the identification of stripe rust resistance genes *YrHu* [12] and *YrH9015* [13]; The resistance to take-all gene of wheat - *P. huashanica* introgression line H148 was located in the 68.8–70.1 Mb interval of chromosome 2As [14]; 7Ns of wheat - *P. huashanica* disomic addition line 2-1-6 showed resistance to leaf rust [15]. The development of these materials demonstrates that *P. huashanica* is an important germplasm resource for wheat genetic improvement.

Wheat Stripe Rust is a disease of wheat caused by the fungus Stripe Stalk Rust (*Puccinia striiformis* f. sp. *Tritici*) that may reduce wheat output. The present typical technique of managing stripe rust is pharmacological control, which has a significant impact on the environment, thus developing resistant types is a more cost-effective and ecologically benign approach. [16]. The genetic resistance to stripe rust in wheat is classified as all-stage resistance (ASR) and adult-plant resistance (APR) [17]. *P. huashanica* has good stripe rust resistance, and according to the current study, the materials of its 2Ns, 3Ns, 4Ns, and 5Ns derived lines [18] are all highly resistant to stripe rust during the all-stage period or adult-plant period, indicating that the *P. huashanica* genome contains many stripe rust resistance genes, and all of these genes play certain roles directly or indirectly in the derived lines. However, environmental changes have mutated *Pst* minor, resulting in the failure of certain stripe rust resistance genes [19, 20]. Therefore, the exploration of new germplasm resources and the development of superior genes are key to genetic improvement of wheat.

The insertion of foreign chromosomes or fragments into the wheat background is merely the first stage in using germplasm resources; the objective is to develop Wheat - *P. huashanica* derived materials with exceptional disease resistance or other desirable characteristics. The wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61 was identified by cytology, genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH), and molecular marker (EST, PLUG) analysis, and its disease resistance and agronomic properties were assessed. The findings indicated that it had strong resistance to stripe rust and good agronomic features; the 2Ns-specific molecular markers created concurrently would speed up wheat breeding.

Results

Observations of root tip cells

The 18TR61 metaphase root tip cells (RTC) were screened using an Olympus BX-43 microscope. Mitotic observations of grains obtained in 2022 and 2023 revealed that 103 (93.65%) of 110 RTCs had 42 chromosomes, suggesting that the chromosomal number of 18TR61 was $2n = 42$ (Fig. 1a).

GISH and sequential FISH-GISH analysis

The chromosomal composition of 18TR61 was determined using genomic in situ hybridization (GISH) using the whole genome of *P. huashanica* as a probe. The results showed that 18TR61 contained 40 wheat chromosomes plus two alien chromosomes with green hybridization signals, which originated from *P. huashanica* (Fig. 1b). the two alien chromosomes are shorter in length than common chromosome. To determine whether the short chromosome contains a centromere or not, the centromere-specific probe HS-CHTZ5 (green) were used in FISH examination to confirm the chromosome structure. The result revealed that the chromosome had a mitotic spindle (Fig. 1c) which suggests that the alien short chromosomes could associate and pair properly during division, but it was incomplete compared to the standard 2Ns karyotype in *P. huashanica*.

To further characterize constitution of 18TR61, sequential FISH-GISH with two oligonucleotide probes oligo-pSc119.2 (green) and oligo-pTa535 were employed. The fluorescence signals indicated that the RTC of 18TR61 did not exhibit the expected karyotypes for the 2D chromosome, leading to the conclusion that it lacked 2D chromosome. Among the remaining 40 wheat chromosomes, all were accurately identified except for chromosomes 2B, 7B and 5A, which displayed different chromosome karyotypes compared to their parental counterparts. Two incomplete chromosomes had large clusters of red fluorescent signals at one end of the chromosome arms, and a combination of GISH and sequential FISH-GISH analyses indicated that the 2D chromosomes were replaced by a fragment 2Ns chromosomes of *P. huashanica* in 18TR61 (Fig. 1d, e).

Structural variation was analyzed using the FISH karyotype of wheat chromosome 7182 constructed with oligo-pSc119.2 (green) and oligo-pTa535 (red) and compared to 18TR61 (Fig. 1f). It was discovered that the end of chromosome 2BL of 18TR61 had a green signal, whereas the end of chromosome 2BL of 7182 did not; additionally, 18TR61 had a green signal in the middle of the short arm of chromosome 7B, whereas 7182 had a green signal in the middle of the long arm of chromosome 7B; and, most notably, the chromosome 5A of 18TR61 was obviously different from that of 7182, which was more similar to the combination of 7182 and Trs-372. This suggests that heterologous substitutions of wheat chromosomes may be accompanied by structural variation in other chromosomes during the formation process.

Figure 1. Cytological observation and in situ hybridization identification for chromosomes of root tip cell at mitotic metaphase of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. **(a)** Chromosomes of root tip cell at mitotic metaphase, $2n = 42$. **(b)** GISH analysis of 18TR61 using *P. huashanica* genomic DNA as probe (green). **(c)** FISH karyotype using HS-CHTZ5 (green) of 18TR61. Incomplete two chromosomes contain centromere. **(d)** mc-FISH analysis of 18TR61 using probes Oligo-pSc119.2 (green) and Oligo-pTa535 (red). **(e)** GISH analysis of 18TR61 using *P. huashanica* genomic

DNA as probe (green). (f) FISH signal comparison among 7182, Trs-372, and 18TR61 in 2B, 7B and 5A chromosomes. Chromosomes were counterstained with DAPI(blue). Scale bar = 10 µm.

Homoeologous group identification

A liquid microarray GenoBaits®WheatplusPh, included a *P. huashanica* 80K probe mix and hybridization capture reagents, were employed in this work to visualize the homoeologous group of alien chromosome by utilizing read depth data. For 7182, the detection signal was uniformly distributed over the 21 wheat chromosome pairs, but the signal strength was lower for *P. huashanica* chromosomes (1Ns-7Ns). The results showed that the detection signal of 18TR61 rarely appeared on wheat chromosome 2D, whereas it was significantly enriched between 0-400 Mb of *P. huashanica* chromosome 2Ns (Fig. 2a), implying that the chromosome 2Ns of *P. huashanica*, which was replaced by 18TR61, consisted of all the short arms plus some of the long arms (Fig. 2b). Combined with the result of FISH analysis, it was confirmed that 18TR61 was a wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line.

Figure 2. Wheat - *P. huashanica* liquid array GenoBaits®WheatplusPh analysis. (a, b) The sequences of 18TR61 are rare distribution in 2D, but intensively distributed in the 0-400 Mb of 2Ns.

Molecular markers analysis

The homology of alien chromosomes was verified using PLUG and EST-STS markers spread across seven wheat homologous taxa. Three PLUG markers (TNAC1204-*TaqI*/*HaeIII*, TNAC1137-*TaqI*/*HaeIII*, and TNAC1200-*TaqI*/*HaeIII*) (Fig. 3a-f) and three EST markers (CD453463-2AS/2DS, BQ160588-2AS/2BS/2DS, BQ162235-2AL/2BL/2DL) (Fig. 3g-i) amplified the expected bands in *P. huashanica* and 18TR61, but not in the two parents 7182 and Trs-372 (Table 1, Fig. 3). Thus, it was determined that the alien chromosome of 18TR61 belonged to the second homologous group. Validated polymorphic markers may be used to trace the chromosomes of *P. huashanica* 2Ns in a wheat background.

Figure 3. EST-STS and PLUG markers analysis of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. The black arrows indicate the *P. huashanica* specific band. (M) DNA marker DL2000. (1) 7182. (2) Trs-372. (3) *P. huashanica*. (4) 18TR61. (a) TANC1137-*TaqI*. (b) TANC1137-*HaeIII*. (c) TANC1200-*TaqI*. (d) TANC1200-*HaeIII*. (e) TANC1204-*TaqI*. (f) TANC1204-*HaeIII*. (g) CD453463. (h) BQ160588. (i) BQ162235.

Development of Ph#2Ns chromosomes specific molecular markers

Using FastQC software, we evaluated sequencing data from GenoBaits®WheatplusPh liquid arrays and acquired 9350944 reads from 18TR61's QC with an average GC percentage of 49.0% and Q30 percentage of 95.8%. The analysis using BWA and Local BLAST + tools revealed 0% similarity to the CS reference genome (IWGSC-RefSeqv1.1) and higher similarity to the *P. huashanica* simplified genome (unpublished data), which was considered *P. huashanica* specific. The results yielded a total of 77,886

sequences, which were then filtered based on scores greater than 250, yielding 259 specific fragments (Table S1).

Primer design for these specific fragments was carried out using Primer Premier 5.0 software in accordance with the primer design principle, and a total of 30 pairs of primers were successfully designed, which were then used for identification in materials 7182, Trs-372, *S. cereale*, *Th. ponticum*, *P. huashanica*, *L. mollis*, and 18TR61. Among these, 15 primers amplified particular target bands solely between *P. huashanica*, *L. mollis* and 18TR61 with a primer design effectiveness of 37.5% (Fig. 4, Table 2). These distinct markers may be used to quickly identify the 2Ns chromosome in a wheat background, considerably increasing the detection rate of exogenous *P. huashanica*.

Figure 4. Molecular marker development and PCR amplification of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. The black arrows indicate the *P. huashanica* and *L. mollis* specific bands. (M) DNA marker DL2000. (1) 7182. (2) Trs-372. (3) *S. cereale*. (4) *Th. ponticum*. (5) *P. huashanica*. (6) *L. mollis*. (7) 18TR61.

Evaluation of agronomic traits and rust disease resistance

Agronomic characteristics were evaluated for 18TR61, parents 7182 and Trs-372 (Table 3, Fig. 5b-f). The plant height of 18TR61 was shorter than that of the parents, while the quantity of tillers, awn, spikelets per spike, and kernels per spike were all similar to the parents. In addition, the line 18TR61 have considerably longer spike length than parents, greater thousand kernel weight, kernel length and breadth than parents.

The reactions of mature plants to mixed rust races were studied in the field. The wheat parents 7182 and HXH showed signs of infection, whereas *P. huashanica* and 18TR61 were resistant to the mixed *Pst* races (Fig. 5a). There were the following immunity types (ITs): line 7182, IT = 4; *P. huashanica*, IT = 0; 18TR61, IT = 1; HXH, IT = 4; it may be revealed that the materials contained alien chromosomes were showing the resistance to stripe rust. In conclusion, 18TR61 exhibits outstanding features such as stripe rust resistance, increased thousand kernel weight. This makes it as potentially germplasm for wheat high-yielding and disease-resistant improvement.

Figure 5. Agronomic traits of line 18TR61 and its wheat parents. (1) 7182. (2) Trs-372. (3) *P. huashanica*. (4) 18TR61. (5) Huixianhong. (a) Identification of stripe rust resistance in 18TR61 and its parents at the adult stage. (b) Adult plant. (c) Spikes. (d) Spikelets. (e-f) 10-seeds length and width.

Discussion

Long-term intervarietal hybridization of wheat throughout contemporary breeding has diminished its genetic diversity, resulting in the loss of several biotic and abiotic stress resistance genes. In contrast, the purpose of distant hybridization in wheat is to cross common wheat with its related species and introduce foreign genetic material into common wheat to develop new wheat germplasm with better features [21]. *P. huashanica* is a tertiary gene pool of wheat that has been explored later than other

cousins, and some advances have been achieved in its exceptional disease resistance and agronomic traits, such as 1Ns disomic addition lines that can increase micronutrient storage in seeds and have leaf rust resistance [22, 23]; 1Ns disomic addition line H5-5-4-2 has strong powdery mildew resistance at both seedling and mature plant stages [24]; 3Ns disomic addition 20JH1155 has long glumes and long grains [9]; 4Ns disomic addition that increase the number of tillers and provide resistance to stripe rust [25]; 6Ns disomic addition lines are more precocious than the parent lines [26]; 7Ns disomic addition lines with earlier maturity and better leaf rust resistance than the parent lines [15, 27]; 2Ns (2D) disomic substitution lines to control total blight in wheat [10]; 3Ns(3D) disomic substitution line DH109 with powdery mildew resistance at seedling stage [28]; 5Ns (5D) disomic substitution line DH66 has stripe rust resistance at the adult plant stage [29]; Liu et al. [30] identified wheat - *P. huashanica* T3DS·3DL-4NsL and T3DL-4NsS translocation lines with adult stage powdery mildew resistance; Li et al. [11] identified the wheat - *P. huashanica* dual translocation line T3DS-5NsL·5NsS and T5DL-3DS·3DL with powdery mildew resistance at both seedling and adult plant stages. In the study, the wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61 identified differs from the above in that the *P. huashanica* chromosome imported into this material is incomplete. Compared by the previous authors [10], the line was shown to be stable with dwarf phenotype, long spike length, large grain size and resistance to stripe rust, which can be used to improve wheat yield and disease resistance improvement.

Non-denaturing fluorescence in situ hybridization (FISH) technology widely used for the identification of exogenous chromosomes in wheat germplasm and the detection of their structural variation[31]. The introduction of exogenous chromosomes into the wheat background can often cause structural variations in other chromosomes, such as Li et al. identified the wheat - *P. huashanica* T3DS-5NsL·5NsS and T5DL-3DS·3DL dual translocation line TR77, the *P. huashanica* 5Ns were introduced into wheat and translocated with the 5D chromosome, causing a structural mutation on the 3D chromosome. Wheat - *P. huashanica* 3Ns additional line 20JH1155 exhibits structural variation on chromosome 5A,7B[9]. This research used GISH and sequential FISH-GISH studies to show the introduction of incomplete 2Ns chromosomes into the wheat background, which replaced 2D chromosomes from common wheat. Chromosome 5A of 18TR61 differs greatly from that of wheat 7182 and Trs-372. It is thought that a replacement between 2Ns and 2D occurred during the parental crossings, as well as maybe a recombination between chromosome 5A in 7182 and Trs-372. However, whether these chromosomal structural variations in 18TR61 are caused by 2Ns substitution or by wheat's own genes needs to be further investigated.

Currently, 15K, 35K, 55K, and 660K SNP arrays have been developed for wide application in identifying exogenous chromosomes in wheat [32]. The liquid array GenoBaits®WheatplusPh allows the tracing of Ns chromosomes in wheat - *P. huashanica* derived lines. Compared to the SNP arrays for wheat, the liquid array preserve wheat specific fragment intervals while additionally include *P. huashanica* specific fragment intervals. This modification allows for a more intuitive characterization of the chromosome composition of wheat - *P. huashanica* derived lines. In the study, it was verified that the 2Ns chromosome substituted by 18TR61 was incomplete, as shown by the liquid array results. The retained

length of the chromosome was found to be 400 Mb based on the depth of the covered region. Due to the lack of information on the physical mapping of the sequenced *P. huashanica* genome and location of the centromere site, it has not yet been possible to determine the length of the long arm of the 2Ns chromosome in the substitution line.

Molecular marker analysis is a standard tool for detecting and tracking exogenous genetic material in a similar wheat background [4], its kinds include simple sequence repeats (SSRs), expressed sequence tags (ESTs), and PCR-based landmark unique genes (PLUGs) [33, 34]. Currently, high-throughput sequencing technology is extensively employed because of its great efficiency and cheap cost. Specific markers produced based on SNPs (single nucleotide polymorphisms) such as KASP, SLAF- sequence, and GBS [35, 36]. Zhang et al. [37] sequenced the transcriptome of *P. huashanica*, which not only verified the Ns genome-specific SNPs in the common wheat background using KASP genotyping, but also revealed genetic links between *Psathyrostachys*, *Leymus*, and other related species. In this study, we discovered that EST-STS and PLUG markers on the second homologous group amplified Ns genome-specific bands of 18TR61 and *P. huashanica*. We then designed 30 pairs of specific primers to observe their amplification in materials 7182, Trs-372, *S. cereale*, *Th. ponticum*, *P. huashanica*, *L. mollis*, and 18TR61. Four pairs of specific primers amplified target bands only in *P. huashanica*, 18TR61, but not in *L. mollis* (Fig. 5, Table 3), despite the reference that Sha et al. [38] confirmed that the Ns subgenomic donor in *L. mollis* originated from *P. huashanica*. The results indicate that *L. mollis* differs from *P. huashanica*, presumably as a result of distributed multi-base differences.

Because resistance genes in cultivated wheat are limited and new pathogenic species continue to evolve, it is necessary to constantly search for new sources of resistance [11]. So far, remote hybridization procedures have been employed to generate a spectrum of germplasm resistant to stripe rust. For example, wheat - *L. mollis* 2Ns (2D) disomic substitution line, which were created by crossing wheat with *L. mollis*, exhibit resistance to stripe rust [39]; wheat-tetraploid *T. elongatum* 6E (6D) disomic substitution line, created by crossing wheat with tetraploid *T. elongatum*, exhibits high resistance to stripe rust during the adult plant stage [40]; wheat-Mexico Rye T1DL·1RS translocation line, created by crossing wheat with rye, exhibits resistance to stripe rust and powdery mildew [18]; wheat-*T. intermedium* translocation lines created by crossing wheat with *T. intermedium* with stripe rust resistance [41]. The research discovered that 18TR61 was highly resistant to stripe rust during the adult plant stage. Its parents were sensitive to stripe rust, suggesting that the resistance genes in 18TR61 may have come from the 2Ns. Furthermore, 18TR61 exhibited a lengthy spike length, huge kernel size, and increased thousand kernel weight. These data show that 18TR61 might be a useful intermediate material for wheat development and breeding.

Conclusion

The study investigates line 18TR61, which was identified as the wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line using molecular and cytogenetic methods. At the adult plant stage, this line demonstrated significant resistance to stripe rust as well as good characteristics such as long

spikes and large grains. The 15 markers identified can identify and track the 2Ns chromosome of *P. huashanica* in a wheat background. 4 of these markers also indicate that the Ns genomes of *L. mollis* and *P. huashanica* vary. This research enriches the knowledge base of *P. huashanica* and broadens the genetic resources for wheat breeding, it also provides further research opportunities on seed grain traits and stripe rust resistance.

Materials and Methods

Development of Plant Materials

The materials used in this study include common wheat line 7182 (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD), *Psathyrostachys huashanica* Keng ($2n = 2x = 14$, NsNs), *Triticum durum* line Trs-372 ($2n = 4x = 28$, AABB), and wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. 18TR61 was the progeny of wheat - *P. huashanica* heptaploid line H8911 $\times$ line Trs-372. H8911 ($2n = 49$, AABBDDNs) was generated from the cross between common wheat line 7182 and *P. huashanica*. Huixianhong (HXH) were vulnerable controls during disease resistance testing. All of the materials listed above were deposited at College of Agronomy, Northwest A&F University in Yangling city, Shaanxi, China.

Cytological Observation

To obtain suitable root tip mitotic cells, the seeds were germinated on moist filter paper to obtain root tips at a suitable growth period. Suitable root tips are cut out and placed in centrifuge tubes, after nitrous oxide treatment, the root tips were fixed in 90% acetic acid for 10 min, ddH₂O washes 3 times, which subsequently were stored in 70% ethanol at -20 °C for later use. Enzymatic hydrolysis with cellulase (R-10, Yakult Japan, Tokyo, Japan) and pectinase (Y-23, Yakult Japan, Tokyo, Japan) was performed at 37°C for 55 minutes, followed by The enzyme-treated root tips were then made into a suspension [42]. The number and configuration of Root tip chromosomes were observed and photographed with Olympus BX-43 microscope (Olympus Optics, Tokyo, Japan).

GISH, FISH and Sequential FISH–GISH

Mark the clear cleavage phase on the RTC slides and apply a UV radiation instrument (SpectrolinkerTM XL – 1500, Long Island, NY, USA) at a UV intensity of 1250 KJ/cm² for 60 seconds. Fluorescein-12-dUTP was used to mark the whole genomic DNA of *P. huashanica* as a probe for GISH. FISH analysis and sequential FISH-GISH analysis were performed utilizing oligonucleotide probes Oligo-pSc119.2 (6-FAM-5'CCGTTTTGTG GACTATTACT CACCGCTTTG GGGTCCCATA GCTAT), green) and Oligo-pTa535 (Tamra-5'AAAACTTGA CGCACGTCAC GTACAAATTG GACAACTCT TTCGGAGTAT CAGGGTTC, red) [43]. The GISH hybridization solution comprised 0.3 µL of probe DNA and 8.7 µL of GISH buffer (2× SSC/1× TE), while the FISH hybridization solution included 0.3 µL of Oligo-pSc119.2, 0.3 µL of Oligo-pTa535, and 8.4 µL of FISH buffer (2× SSC/1× TE). After adding the hybridization droplets to slides containing RTC, the slides for GISH analysis require denaturation at 100°C for 4:30 minutes, whereas FISH analysis does not necessitate denaturation and is subsequently renatured in an oven at 42°C for 16 hours, after which

DAPI was used to counterstain the chromosomes [39]. Eventually, fluorescent signals were detected and photographed using an Olympus BX-53 microscope.

Molecular Markers Analysis

Seventy-two Expressed Sequence Tag-Sequence-Tagged Site (EST-STS) and 131 PCR-Based Landmark Unique Genes (PLUG) were used to detect homoeologous groupings of foreign chromosomes. AuGCT DNA-SYN Biotechnology Co., Ltd. in Beijing, China, developed expressed sequence tag-sequence-tagged site (EST-STS) markers (available at https://wheat.pw.usda.gov/SNP/new/pcr_primers.shtml) and PCR-Based Landmark Unique Gene (PLUG) markers [44, 45]. Polymerase chain reaction (PCR) analysis was performed on all of these molecular markers between 7182, Trs-372, *P. huashanica*, and 18TR61. PCR amplification [27] and product separation [46] followed previously described protocols. 1.2% agarose gel electrophoresis was used to separate the PCR amplification products, which were provided by Tiangen Biochemical Technology (Beijing) Co., LTD.

Chip analysis by Wheat - *P. huashanica* Liquid Array

Genotyping by Target Sequencing (GBTS) is based on target capture using complementary combinations of probe and target sequences [47, 48], and the GBTS method is combined with solution capture technology to create liquid-phase microarrays. The liquid-phase microarray (GenoBaits®WheatplusPh) for this study consisted of individually packaged *P. huashanica* 80K probe mix and hybridization capture reagents, where the *P. huashanica* 80K probe mix contained 49K oligonucleotide probe sites from the entire genome of *P. huashanica*, for a total of 80K liquid phase-targeted capture probes. The liquid microarray allows for quick and precise assessment of *P. huashanica* chromosome and fragment composition in a wheat genetic background. Molbreeding Biotechnology (Shijiazhuang) Co., LTD. did the chip detection. FastQC was used for data quality control of the sequencing data, followed by comparing the BWA to the reference genome [49]. The probe's sequencing depth was computed using bamdst v. 1.0.6 (<https://github.com/shiquan/bamdst>), and the plots were analyzed using R (versions 4.3) software tools (ChIPseeker, clusterProfiler, RIdeogram, ggplot2).

Molecular marker development

The young leaves of the 18TR61 line were harvested, and DNA samples that satisfied the qualifying requirements underwent restriction enzyme *Hae*III digestion. The library was then sequenced after quality detection. The data filtering stages included: (1) removing reads including adapter sequences, (2) eliminating reads with N content over 10%, and (3) excluding more than 50% of reads with a base mass fraction less than 10%. Sequence quality was assessed after screening sequence readings [50]. The sequencing data were processed using Burrows-Wheeler-Alignment V0.1.17 (BWA) [51], and Basic Local Alignment Search Tool V2.10.1 (Index of /blast/executables/blast+/ LATEST (nih.gov)). Sequences having 0% similarity to CS (IWGSC-RefSeq-v1.1) but greater similarity to *P. huashanica* (unpublished data) were chosen for molecular marker development. Primers for these sequences were created using Primer Premier 5.0 (Premier Biosoft, CaPalo Alto, CA, USA). Following PCR amplification, the particular target bands were identified on a 1.2% agarose gel.

Agronomic traits and stripe rust characterization

The parental materials 7182, Trs-372 and 18TR61 were evaluated for agronomic traits including plant height, tiller number, spike length, spikelets per spike, kernels per spike, thousand kernel weight and awnedness at physiological maturity stage. The pathogenic responses of 7182, Trs-372, 18TR61, and HXH to stripe rust during the adult stage were investigated. Artificial inoculation was performed using a combination of tiny strains of stripe rust bacteria CYR32, CYR32 and CYR33. When the stripe rust susceptible control variety HXH was completely infected, the kind of disease development was evaluated using the prior standard [52]. Among these parameters, the type of infection (IT) in the adult stage of wheat stripe rust was classified as 0–4: 0, immune; 0,, practically immune; 1, extremely resistant; 2, moderately resistant; and 3 and 4, moderately sensitive and very susceptible, respectively.

Declarations

Ethics approval and consent to participate

There is no research requiring ethical approval and consent to participate in this manuscript. All related plant materials are available and comply Wild Plants Protection Regulation of China.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or materials and/or codes during the current study are available from the corresponding author on reasonable request. The data reported in this paper have been deposited in the GenBase in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation, under accession number PRJCA026478 that is publicly accessible at <https://ngdc.cnbc.ac.cn/genbase>.

Competing interests

The authors declare that they have no conflict of interests.

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Author Contributions

W.J. and J.Z. designed the research. Y.W. performed the research and wrote the article. Y.W. and P.D. analyzed the data. Y.W. and D.G. contributed to cytological analysis and molecular markers analysis. J.Z. and C.W. contributed to the development of the material. X.L. and C.C. contributed to resistance and agronomic trait evaluation. J.Z. and T.L. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Tables

Tables 1-3 is available in the Supplementary Files section.

Figures

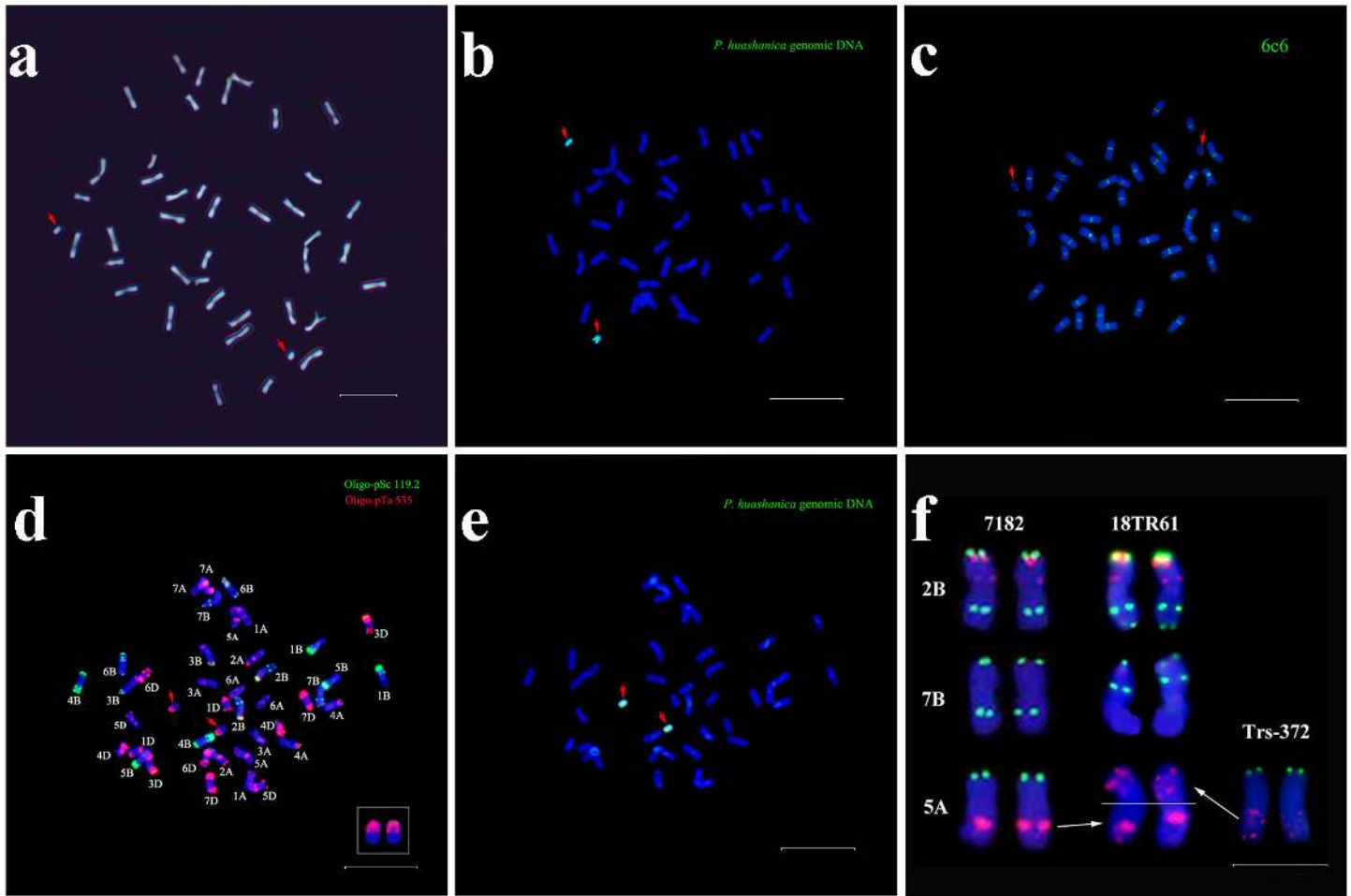


Figure 1

Cytological observation and in situ hybridization identification for chromosomes of root tip cell at mitotic metaphase of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. **(a)** Chromosomes of root tip cell at mitotic metaphase, $2n = 42$. **(b)** GISH analysis of 18TR61 using *P. huashanica* genomic DNA as probe (green). **(c)** FISH karyotype using HS-CHTZ5 (green) of 18TR61. Incomplete two chromosomes contain centromere. **(d)** mc-FISH analysis of 18TR61 using probes Oligo-pSc119.2 (green) and Oligo-pTa535 (red). **(e)** GISH analysis of 18TR61 using *P. huashanica* genomic DNA as probe (green). **(f)** FISH signal comparison among 7182, Trs-372, and 18TR61 in 2B, 7B and 5A chromosomes. Chromosomes were counterstained with DAPI(blue). Scale bar = 10 μ m.

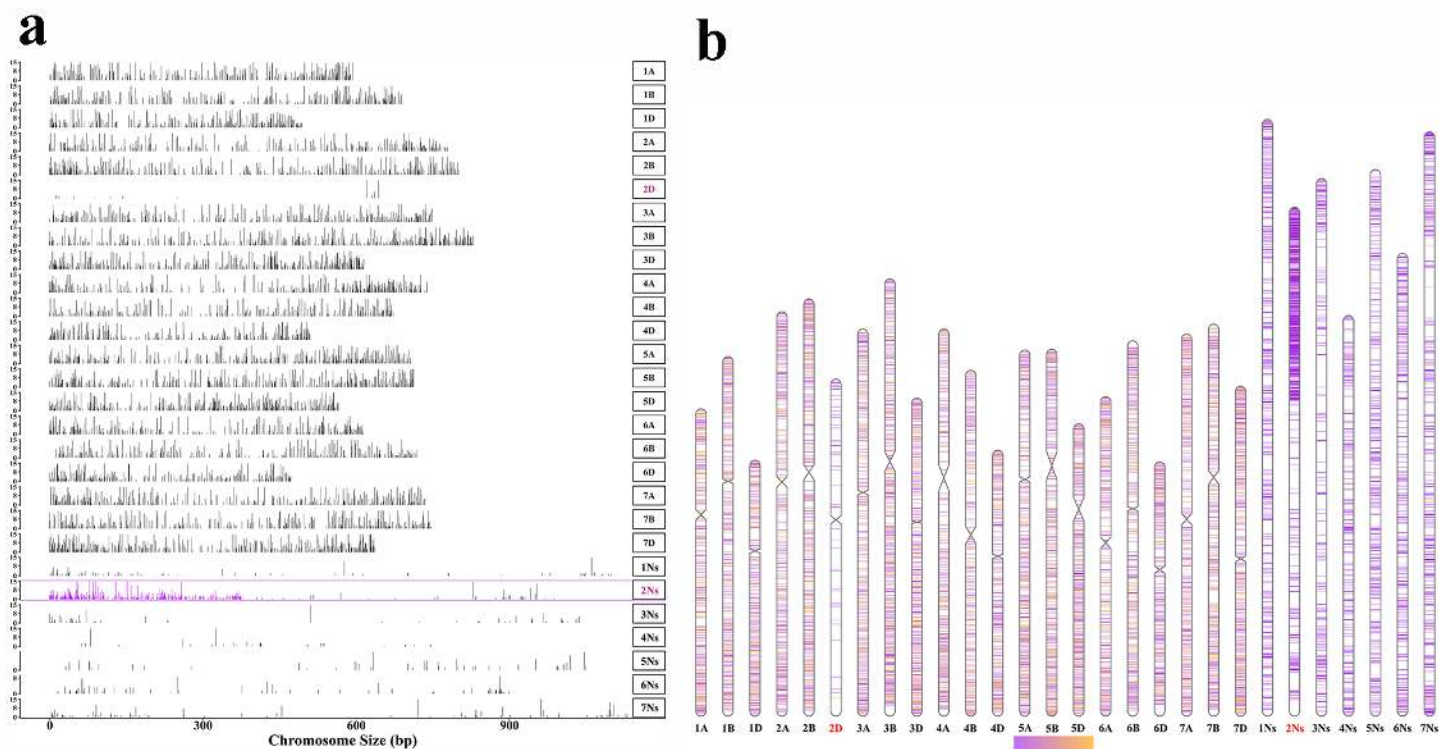


Figure 2

Wheat - *P. huashanica* liquid array GenoBaits®WheatplusPh analysis. **(a, b)** The sequences of 18TR61 are rare distribution in 2D, but intensively distributed in the 0-400 Mb of 2Ns.

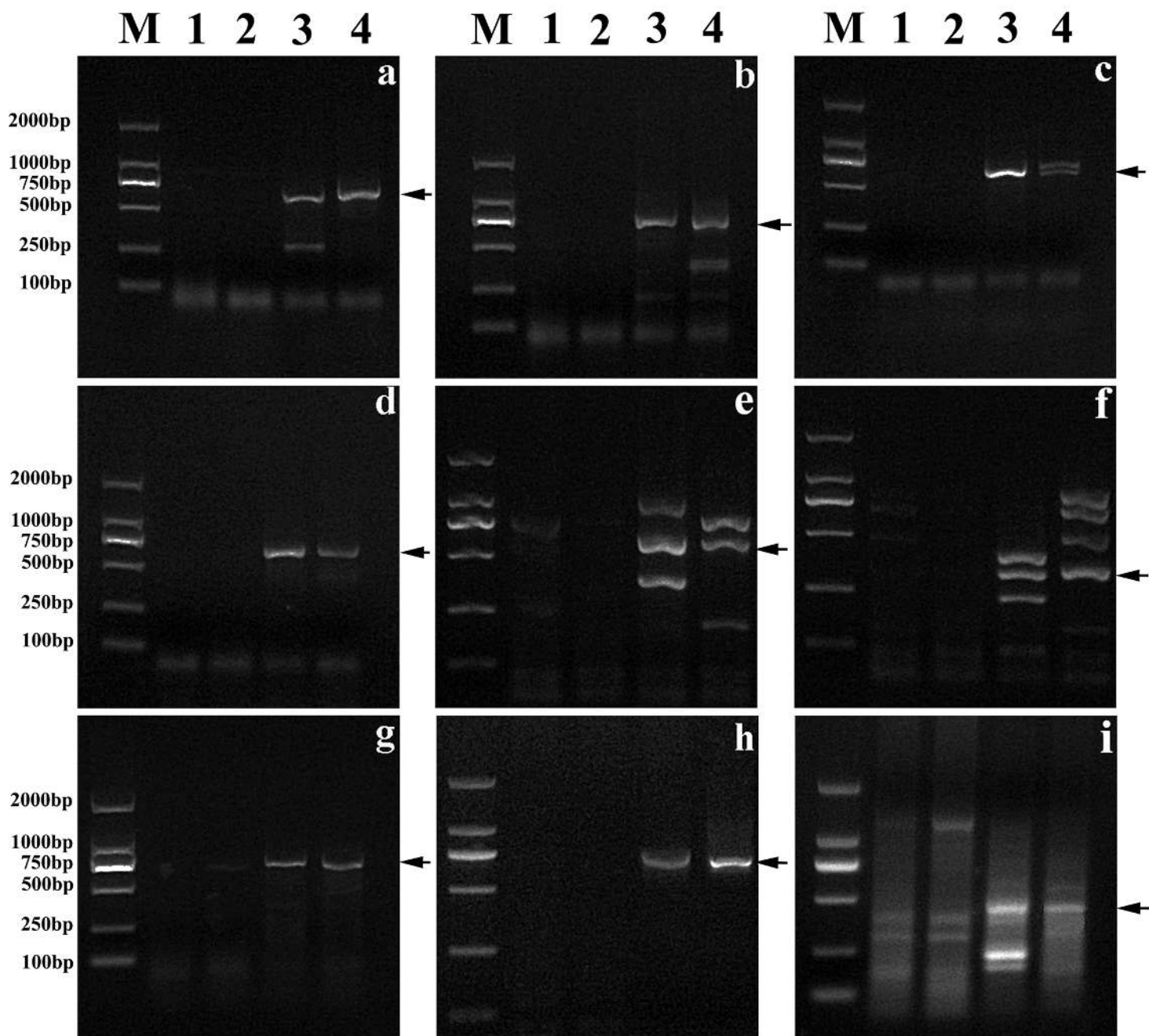


Figure 3

EST-STS and PLUG markers analysis of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. The black arrows indicate the *P. huashanica*-specific band. (M) DNA marker DL2000. (1) 7182. (2) Trs-372. (3) *P. huashanica*. (4) 18TR61. (a) TANC1137-*TaqI*. (b) TANC1137-*HaeIII*. (c) TANC1200-*TaqI*. (d) TANC1200-*HaeIII*. (e) TANC1204-*TaqI*. (f) TANC1204-*HaeIII*. (g) CD453463. (h) BQ160588. (i) BQ162235.

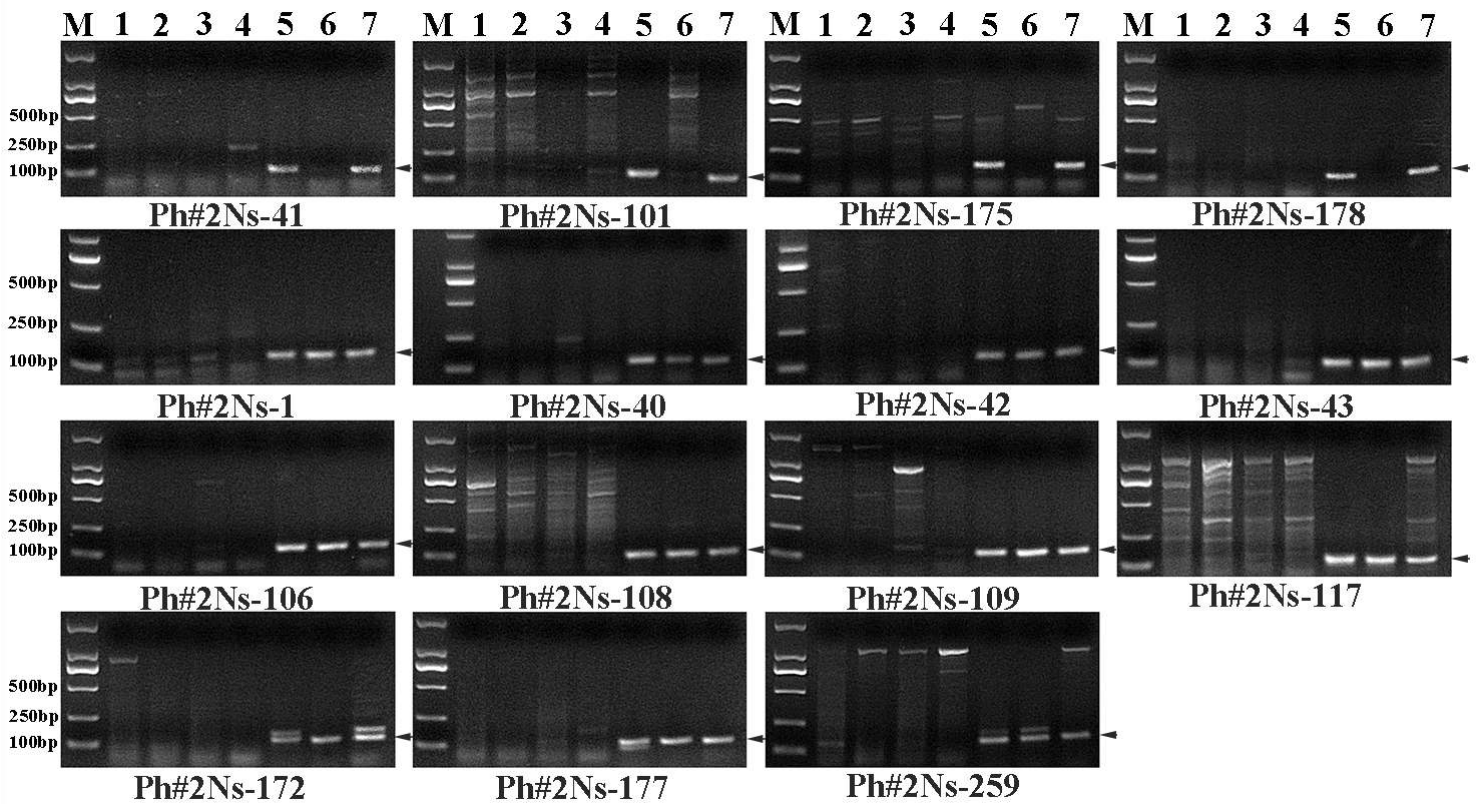


Figure 4

Molecular marker development and PCR amplification of wheat - *P. huashanica* 2NsS (2D) fragment disomic substitution line 18TR61. The black arrows indicate the *P. huashanica* and *L. mollis* specific bands. (M) DNA marker DL2000. (1) 7182. (2) Trs-372. (3) *S. cereale*. (4) *Th. ponticum*. (5) *P. huashanica*. (6) *L. mollis*. (7) 18TR61.

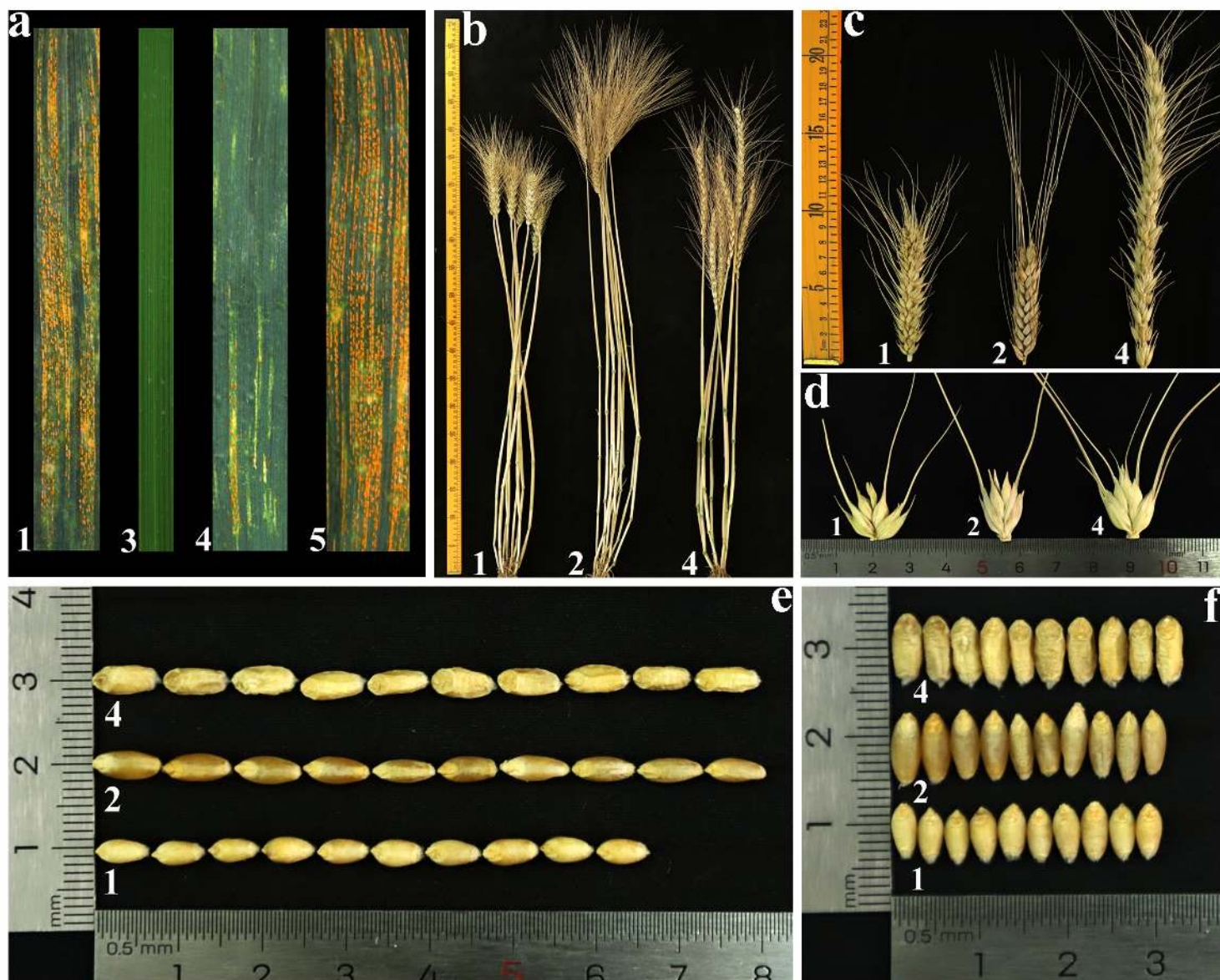


Figure 5

Agronomic traits of line 18TR61 and its wheat parents. (1) 7182. (2) Trs-372. (3) *P. huashanica*. (4) 18TR61. (5) Huixianhong. (a) Identification of stripe rust resistance in 18TR61 and its parents at the adult stage. (b) Adult plant. (c) Spikes. (d) Spikelets. (e-f) 10-seeds length and width.

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