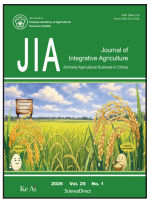
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RESEARCH ARTICLE

Cytogenetic characterization and molecular marker development of a novel wheat-*Thinopyrum ponticum* 5E (5D) disomic substitution line with resistance to powdery mildew and stripe rust

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Highlights

- CH97 is a stable wheat-*Thinopyrum ponticum* 5E (5D) disomic substitution line.
- CH97 showed high resistance to powdery mildew and stripe rust, with improved thousand-kernel weight and kernel length.
- Development success rate of 5E chromosome-specific molecular markers reached 33.3%.

Abstract

Thinopyrum ponticum (2n=10×=70), a wild relative of common wheat (*Triticum aestivum* L.), is considered an invaluable genetic resource for wheat improvement due to its abundance of genes conferring resistance to biotic and abiotic stresses. This study focused on the CH97 line, derived from the BC_1F_7 progeny of a cross between wheat cv. 7182 and *Th. ponticum*. Cytological evidence showed that CH97 has 42 chromosomes, forming 21 bivalents at meiotic metaphase I, with the bivalents subsequently separating and moving to opposite poles during meiotic anaphase I. Through a combination of fluorescence *in situ* hybridization (FISH), genomic *in situ* hybridization (GISH), multicolor GISH (mc-GISH), and liquid array analysis, it was determined that CH97 comprises 40 wheat chromosomes and two alien chromosomes from the E^e genome of *Th. ponticum*, featuring the absence of a pair of 5D chromosomes and variations in 1B, 6B, and 7B chromosomes. These findings confirm that CH97 is a stable wheat-*Th. ponticum* 5E (5D) alien disomic substitution line. Inoculation experiments revealed that CH97 exhibits high resistance to wheat powdery mildew and stripe rust throughout the growth period, in contrast to the highly susceptible common wheat parent 7182. Compared to 7182, CH97 displayed improvements in thousand-kernel weight and kernel length. Additionally, utilizing specific-locus amplified fragment sequencing (SLAF-seq) technology, chromosome 5E-specific molecular markers were developed and validated, achieving a 33.3% success rate, facilitating marker-assisted selection for disease resistance in wheat. Overall, the CH97 substitution line, with its resistance to diseases and improved agronomic traits, represents valuable new germplasm for wheat chromosome engineering and breeding.

Keywords: *Thinopyrum ponticum*, disomic substitution line, powdery mildew, stripe rust SLAF-seq, liquid array

1. Introduction

Wheat (*Triticum aestivum* L., 2n=42, AABBDD) is a crucial food crop anticipated to address global food shortages in the face of drastic climate changes and population growth (Lopes *et al.* 2015). Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *Tritici* (*Bgt*), and wheat stripe rust, caused by the strictly obligate parasitic fungus *Puccinia striiformis* f. sp. *Tritici* (*Pst*), significantly reduce wheat yields and economic returns worldwide (Everts and Leath 1992; Conner *et al.* 2003). While fungicides are commonly used, their extensive

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application poses risks to environmental sustainability and food security. The introgression of beneficial genes from wild relatives into wheat through distant hybridization and chromosome engineering presents a viable solution for enhancing wheat's genetic diversity, particularly in developing disease-resistant varieties (Blanco *et al.* 2008). The gene *Pm21*, which offers strong broad-spectrum resistance to powdery mildew, has been successfully transferred from *Dasypyrum villosum* into common wheat through the translocation line T6VS·6AL (Chen *et al.* 1995). Varieties carrying *Pm21* was more than 4 million hectares and continue to expand (Xing *et al.* 2018; Huang *et al.* 2023). Additionally, the 1RS·1BL translocation was one of the most widely used foreign chromosome segments, although its resistance gene *Yr9* has been overcome by newly evolved pathogens over time (Mago *et al.* 2005; Liu *et al.* 2017). Given these developments, there is an urgent need to consistently create broadly applicable germplasm to combat the critical challenge of losing disease resistance.

Thinopyrum ponticum, a part of the wheat tertiary gene pool, has contributed significantly to the development of numerous wheat-*Th. ponticum* chromosome engineering germplasms with resistance to powdery mildew and stripe rust (Linc *et al.* 2012). A complete set of derived addition lines was developed from Xiaoyan 7430, including addition line WTA55, which contains an alien group-6 chromosome from the E-genome and demonstrates high resistance to stripe rust during both seedling and grain-filling stages (Jia *et al.* 2022). The wheat-*Th. ponticum* substitution lines $1J^s$ (1B) SN19647 and $1J^s$ (1D) CH10A5 have shown resistance to powdery mildew and leaf rust (Wang *et al.* 2020; Li *et al.* 2021). A wheat-*Th. ponticum* TTh-1DS·1DL translocation line WTT80 possesses powdery mildew resistance and has a translocation breakpoint in the 21.5 Mb region of chromosome 1D, as revealed by wheat 660K SNP array analysis (Yang *et al.* 2022). The SN0224 translocation line, created by crossing *Th. ponticum* and Yannong 15, exhibited excellent resistance to powdery mildew. Inheritance analysis revealed that this trait is controlled by a single dominant gene, *PmSn0224*. Genetic mapping positioned *PmSn0224* on chromosome 2A and linked it to the molecular markers *Barc212*, *Xwmc522* and *Xbarc1138* (He *et al.* 2017). The introgression lines SN0293-2 and SN0293-7 are resistant to powdery mildew and carry the temporarily designated resistance gene *PmSN0293* (Li *et al.* 2022). Translocation lines (WTT139, WTT146 and WTT323), which were developed from the Blue 58 pollen of the wheat-*Th. ponticum* 4Ag (4D) disomic substitution line, showed resistance to powdery mildew. The *Pm* locus was mapped to 3.79–97.12 Mb on the short arm of chromosome 4Ag, as determined by *in situ* hybridization and specific-locus amplified fragment sequencing (SLAF-seq) analysis (Yang *et al.* 2023). Currently, *pm51* is the only officially designated *pm* gene from *Th. ponticum*, with genetic analysis indicating single dominant gene inheritance, and molecular markers locating the locus in the bin 2BL 0.89–1.00 region (Zhan *et al.* 2014). The stripe rust-resistant wheat introgression line CH7086, derived from *Th. ponticum*-based germplasm Xiaoyan 7430, features a single dominant resistance gene located within the bin 2AS-0.78–1.00 region, identified using eight molecular markers (Hou *et al.* 2016). These findings highlight the important role of *Th. ponticum* in increasing the genetic diversity of wheat, especially in improving resistance to powdery mildew and stripe rust.

Molecular marker technology plays a crucial role in identifying wheat heterologous germplasm and screening wheat alien chromosome lines, as well as in molecular marker-assisted breeding (MAS) (Liu and Wang 2005). The advent of next-generation high-throughput sequencing technologies, such as SLAF-seq has revolutionized *Thinopyrum*-specific molecular marker development. This advancement greatly increases the efficiency of developing molecular markers. For instance, in 2013, SLAF-seq technology facilitated the identification of 89 chromosome 7E-specific molecular markers for *Th. elongatum* (Chen *et al.* 2013). In 2018, the same technology led to the development of 621 blue-grain-related molecular markers from *Th. ponticum* chromosome 4Ag, achieving a high-efficiency rate of up to 65.9% (Liu *et al.* 2018). Despite these advancements, the development of specific molecular markers for *Th. ponticum* chromosome 5E based on SLAF-seq has not been reported.

In the present study, a novel wheat-*Th. ponticum* disomic substitution line (CH97) exhibiting high resistance to powdery mildew and stripe rust was developed. The objectives of the study were as follows: (1) to determine the chromosome count and analyse the genetic behaviour of chromosomes during meiosis using cytogenetic methods; (2) to ascertain the chromosome composition through *in situ* hybridization, liquid array analyses, and molecular markers; (3) to develop specific molecular markers using SLAF-seq (Specific-Locus Amplified Fragment Sequencing) technology for chromosome tracking and enhancing screening efficiency; and (4) to assess disease resistance and agronomic traits of the developed line.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study included hexaploid wheat lines 7182 (2n=6×=42, AABBDD), Shaanyou 225 (SY225), Huixianhong (HXH), Chinese Spring (CS), wheat relatives *Thionpyrum ponticum* (Podp.) Barkworth and D.R.Dewey (2n=10×=70, $E^eE^eE^bE^bE^{St}E^{St}$StStStSt), *Thionpyrum elongatum* (2n=2×=14, E^eE^e), and *Pseudoroegneria spicata* (2n=14, StSt). The novel wheat-*Th. ponticum* 5E (5D) disomic substitution line CH97 was selected from the BC_1F_7 pedigree of common wheat 7182/*Th. ponticum*//7182. The plant materials were preserved at the College of Agronomy, Northwest A&F University, China.

2.2. Cytogenetic identification

Seeds were soaked in distilled water for 24 h to initiate germination, after which the water was drained. The root tips were cut when they reached a length of 2–3 cm and were treated with nitrous oxide for 2 h. Following this treatment, the root tips were fixed in 90% acetic acid for 10 min and then preserved in 70% ethanol at –20°C, in accordance with

previously reported methods (Han *et al.* 2006). The root tips underwent enzymatic hydrolysis using a mixture of enzyme solutions, specifically pectinase (Y-23, Yakult Japan) and cellulase (R-10, Yakult Japan), following the established procedure (Feng *et al.* 2022). Young spikes were harvested during the optimal fertility periodand submerged in an ethanol-chloroform-acetic acid mixture (6:3:1, v/v) at 25°C for 1 wk. Anthers were then collected using forceps, tapped onto a microscope slide, and stained in a 1% acetocarmine solution (Wang *et al.* 2021). Observations and photographs of the chromosome numbers at the root tip and the chromosome pairing during meiosis I in pollen mother cells were made using an Olympus BX43 microscope (Tokyo, Japan).

2.3. *In situ* hybridization of CH97

For *in situ* hybridization, chromosomes were affixed to the microtiter slide by exposing them to ultraviolet cross-linking irradiation at 125,000 mJ cm^{-2} for 60 s using a Spectrolinker™ XL-1500 (Long Island, NY, USA). Total genomic DNA was extracted from seedling leaves using a modified CTAB procedure to achieve high-purity DNA (Allen *et al.* 2006). This gDNA of *Th. ponticum* was then labeled with Texas Red 5-dCTP for use as a probe DNA, following a previously described nick translation method (Fu *et al.* 2012). Sheared gDNA from CS at 120°C served as a blocker. Multicolor-genomic *in situ* hybridization (mc-GISH) was utilized to identify various exogenous genomes within *Th. ponticum*, with E^e gDNA from diploid *Th. elongatum* tagged with Texas Red-5-dCTP (Invitrogen, United States), and St gDNA from diploid *P. spicata* marked with Alexa Fluor 488-5-dUTP (Invitrogen). The mc-GISH procedure was based on a previously reported method (Kruppa *et al.* 2016). Probes oligo-pTa535 (Tamra-5´), oligo-pSc119.2 (6-FAM-5´) (Tang *et al.* 2014), and oligo-D (6-FAM-5´) (Tang *et al.* 2018), all synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China), were employed in a non-denaturing fluorescence *in situ* hybridization (ND-FISH) experiment to distinguish the chromosome karyotype between wheat and *Th. ponticum*. Chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI), and an Olympus BX-63 fluorescence microscope (Japan) equipped with a Photometrics DP80 camera was used for observing and capturing the hybridization signals.

2.4. Wheat-*Th. elongatum* liquid array analysis and molecular markers analysis

To clarify the exogenous chromosomes of CH97, a wheat-*Th. elongatum* liquid array (GenoBaits®WheatplusEE) was used to identify its homoeologous group. GenoBaits is based on the complementary binding of target probes to target sequences for targeted capture, elution, target amplification, library construction, and sequencing (Deng *et al.* 2023). The resulting sequencing data were quality controlled, mapped to the reference genome using BWA (Burrows-Wheeler Aligner) (Li *et al.* 2012), and finally plotted with R packets. Additionally, a total of 196 SSR and PLUG markers (Appendix A) were synthesized by AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China) to clarify the genome constitution of the wheat-*Th. ponticum* substitution line CH97 (Ishikawa *et al.* 2009). The PCR products were separated by 1% agarose gel electrophoresis or 8% non-denaturing polypropylene gel electrophoresis and subsequently silver stained following the previously described method (Yang *et al.* 2014).

2.5. Evaluation of resistance to powdery mildew and stripe rust in seedling and adult stages

The predominant isolate E09 of *Bgt* was utilized to evaluate powdery mildew resistance in seedlings within a greenhouse setting. The susceptible control, SY225, was inoculated with the *Bgt* race E09 once the first leaf had fully expanded, using a dusting method. Post-inoculation, the plants were maintained at a controlled environment of 22°C and 70% humidity for 15 d. Upon full infection of the SY225 leaves with powdery mildew spores, an assessment was made to determine the infection type (IT) on the plants. The IT is a measure of the extent of infection in wheat, with a scale ranging from 0 to 4; where 0 indicates immunity, and 0; (a semi-colon is used to denote near-immunity). Values from 1 to 4 represent increasing susceptibility levels, with 1 indicating high resistance, 2 indicating moderate resistance, 3 indicating moderate susceptibility, and 4 denoting full susceptibility.

During the adult phase, powdery mildew resistance evaluation was conducted in the field at the experimental station of the College of Agriculture, Northwest A&F University across two consecutive growing seasons (2021–2022). The susceptible control SY225 was also used to spread the inoculum in the field. Disease assessment for powdery mildew utilized the IT scale, which, in this context, ranged from 0–9. A score between 0 to 4 on this scale indicates resistance to the disease, while a score from 5 to 9 suggests susceptibility.

Resistance to stripe rust at the seedling stage for wheat lines 7182, CH97 and HXH was assessed over two consecutive years (2021–2022) within a cryogenic chamber at the College of Plant Protection, Northwest A&F University. The assessment commenced once the first leaves of the plants were fully expanded, utilizing mixed *Pst* races (CYR32 and CYR34) at a ratio of 1:30 with talcum powder for inoculation through shaking. Following inoculation, the seedlings were placed in a humid chamber at 10°C for a 24-h period in complete darkness. Subsequently, the plants were moved to a greenhouse, which maintained controlled temperatures of 15–18°C during the day and 11–14°C at night, with 16 h of light exposure daily. Disease resistance was evaluated tri-weekly until the 21-d mark post-inoculation, by which time the susceptible control HXH had fully manifested the disease.

An adult plant disease resistance identification nursery was established in the experimental field of Northwest A&F University. The trial materials were sown in rows of 100 cm length and 25 cm width, with 20 seeds per material. These rows were flanked by HXH, which served as both the induced row and susceptible control. A combination of *Pst* races CYR32, CYR33 and CYR34 was applied annually for resistance evaluation and grading. The resistance levels of 7182, CH97, and HXH at the adult stage were assessed,

recording the IT of plants on a scale of 0–4 for both seedling and adult-stage stripe rusts (Ma *et al.* 1995).

2.6. Characterization of agronomic traits

CH97 and its parent 7182 were cultivated in consecutive growing seasons in 2021 and 2022 at the experimental station of the Northwest A&F University. During each harvest season, 10 randomly selected plants were evaluated for their agronomic traits. The assessed traits included plant height, tiller number, peduncle length, spike length, kernel number per spike, florets number per spikelet, and spikelet number. The SC-G Seed Testing Instrument (Wseen Detection Technology Co., Ltd., Hangzhou, China) was employed to measure grain length, width, and thousand-kernel weight with five repetitions. Statistical analysis of the measured data was performed using GraphPad Prism8.0 software.

2.7. Development of specific markers based on SLAF-seq data

CH97 and *Th. ponticum* were sequenced using SLAF-seq technology at the BioMarker Technologies Company (Beijing, China) (Yang *et al.* 2022). For sequencing library construction, gDNA was digested with the restriction enzyme *Hae*III, and specific length fragments were selected. The libraries were sequenced on the Illumina HiSeq 2500 platform, generating numerous tag sequences. To optimize marker efficiency and specificity, candidate-specific SLAFs of CH97 were identified through alignment to the reference genome of CS (IWGSC_v2.1). Subsequently, reads without hits in the CS genome were retained and compared against the sequences of *Th. ponticum*. Reads showing 100% similarity to the *Th. ponticum* genome were retained as CH97 candidate-specific SLAFs and utilized for primer development. PCR primers were designed using Premier V5.0 software (PREMIER Biosoft, Palo Alto, CA, USA) based on the obtained SLAFs and synthesized by AuGCT (Beijing AuGCT Biotechnology Co. Ltd., Beijing, China). CH97, *Th. ponticum* and 7182 were used to validate the PCR amplification specificity and efficiency of developed markers.

3. Results

3.1. Cytological observation of CH97

The root tip cells (RTCs) and pollen mother cells (PMCs) of CH97 were observed for two consecutive years. Among the 154 identified RTCs, 149 (96.75%) had 42 chromosomes, 2 (1.30%) had 43 chromosomes, and 3 (1.95%) had 41 chromosomes. Among the 125 observed PMCs, 120 (96%) formed 21 pairs of bivalents, while univalents were found in five (4%) PMCs, with no occurrences of trivalents or quadrivalents in meiotic metaphase I (Appendix B). Taken together, the cytogenetic analysis indicated that CH97 possessed 42 chromosomes in the RTCs (Fig. 1-A) and 21 bivalents in the PMCs (Fig. 1-B). The chromosomes of CH97 were evenly distributed on both sides of the equatorial plate, forming a 21'+21' configuration, with no lagging chromosomes observed at meiotic anaphase I (Fig. 1-C). These results indicate that CH97 maintains high stability during meiosis.

3.2. *In situ* hybridization of CH97

Image analysis revealed that CH97 has two strong green signals, indicating the presence of *Th. ponticum* alien chromosomes. The remaining 40 chromosomes from wheat parent 7182 were stained blue with DAPI (Fig. 2-A). These results suggest that CH97 is a disomic substitution line comprising a pair of chromosomes from *Th. ponticum* and 40 chromosomes from common wheat. To confirm that the alien chromosomes are part of the E^e subgenome, mc-GISH identification was performed. The two chromosomes showed red signals from *Th. elongatum* but lacked green signals from *P. spicata* (Fig. 2-B).

The wheat chromosomes substituted by *Th. ponticum* in CH97 were validated through FISH and sequential FISH-GISH analyses in the same chromosome division phase. FISH analysis using probes oligo-pTa535 (red) and oligo-pSc119.2 (green) allowed differentiation of all 42 wheat chromosomes (Fig. 2-C). Subsequent GISH analysis of the FISH signals confirmed the absence of the pair of 5D chromosomes (Fig. 2-D), with red signals only detected *via* oligo-pTa535 hybridization on the alien chromosomes, and no signals from oligo-pSc119.2 hybridization. Twelve chromosomes from the D-genome were identified in CH97

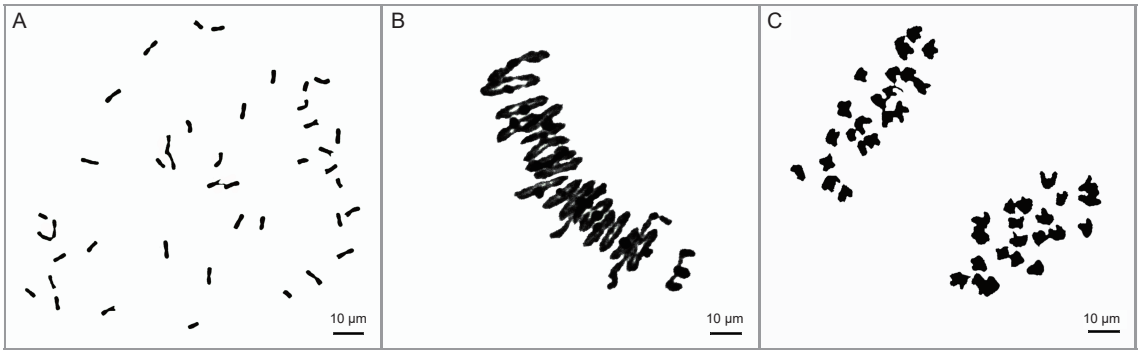


Fig. 1 Cytogenetic analysis of wheat-*Thinopyrum ponticum* disomic substitution line CH97. A, the number of chromosomes in root tip cells at mitotic metaphase, 2n=42. B, chromosomal behaviors of the pollen mother cell at meiotic metaphase I, 2n=21II. C, chromosomal behaviors of the pollen mother cell at meiotic anaphase I, 2n=21+21.

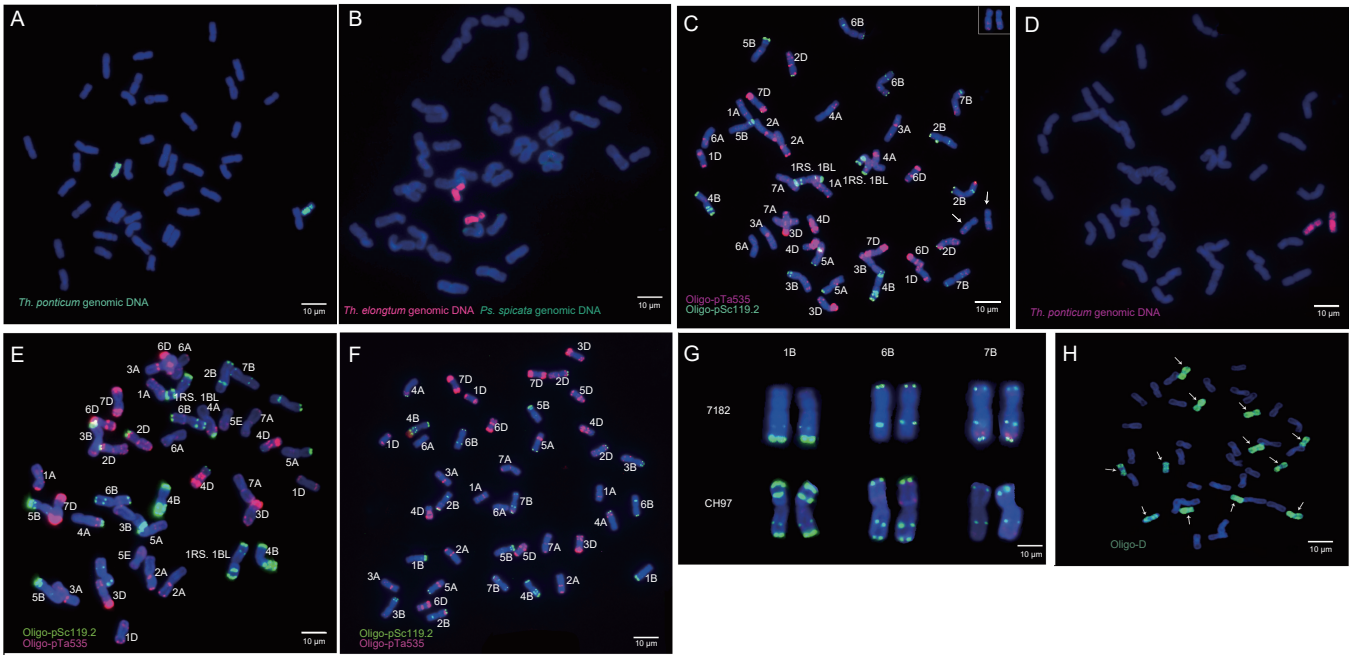


Fig. 2 *In situ* hybridization identification of wheat-*Thinopyrum ponticum* disomic substitution line CH97. Chromosomes were counterstained by 4,6-diamidino-2-phenylindole (DAPI) (blue). A, genomic *in situ* hybridization (GISH) showed wheat chromosomes (blue) and *Th. ponticum* chromosomes (green) by using *Th. ponticum* genomic DNA as a probe and Chinese Spring (CS) DNA as a blocker. B, multicolor (mc)-GISH, *Th. elongatum* (E^e) gDNA (red) and *Pseudoroegneria spicata* (St) gDNA (green) as probes; CS gDNA as a blocker. C, mc-non-denaturing fluorescence *in situ* hybridization (FISH) of CH97 with two probes oligo-pTa535 (red) and oligo-pSc119.2 (green) used simultaneously. D, sequential GISH was performed on the same cell following FISH analysis of CH97. The arrows note the pair of substituted 5E chromosomes. E, FISH karyotype analysis of CH97 using oligo-pTa535 and oligo-pSc119.2. F, FISH karyotype analysis of parent 7182 using oligo-pTa535 and oligo-pSc119.2. G, FISH signals comparison between 7182 and CH97 on chromosomes 1B, 6B and 7B. H, non-denaturing (ND)-FISH showed the detection of 12 D chromosomes using the oligo-D probe (green) from D genome.

through oligo-D probes, as opposed to the standard 14 common wheat chromosomes (Fig. 2-H). Further ND-FISH analysis confirmed the absence of two wheat chromosomes in CH97. The above results indicate that the two 5D chromosomes present in CH97 were replaced by two E^e chromosomes. Compared to the FISH karyotype of 7182 (Fig. 2-F), CH97 showed variations in chromosomes 1B, 6B and 7B (Fig. 2-E), as well as harboring a pair of rye 1RS·1BL translocation chromosomes (Fig. 2-G).

3.3. Identification of the alien chromosome homoeologous group in CH97

GenoBaits®WheatplusEE represents a cutting-edge liquid phase chip technology, incorporating 90,000 probes, comprising 10,000 from wheat and 80,000 from *Th. elongatum*. This innovative chip provides an intuitive method for presenting chromosome composition. Analysis of 7182 using this chip revealed an even distribution of signals across the 21 pairs of wheat chromosomes, with a notably low-abundance signal detected on the 1E-7E chromosomes of *Th. elongatum* (Fig. 3-A). In contrast, the CH97 exhibited significantly higher signal intensity on the *Th. elongatum* 5E chromosomes, the signals on the wheat 5D chromosomes appeared weak (Fig. 3-B). Intriguingly, the absence of the 1BS signal was attributed to the presence of a 1RS·1BL translocation line, aligning with findings from previous FISH findings.

The nine markers were mapped to the fifth homoeologous group in *Th. ponticum* and CH97, including five SSR markers (*Xwmc215*, *Xgwm159*, *Xgpw7346*, *Xgpw7309*, and *Xcfd12*) and four PLUG markers (TNAC1574-*Taq*I, TNAC1574-*Hae*III, TNAC1588-*Taq*I, and TNAC1588-*Hae*III). These markers amplified the same specific bands in both strains but not in the common wheat parent 7182 (Appendix C; Fig. 3-C). Molecular markers and liquid microarray analysis, combined with *in situ* hybridization results, provided sufficient evidence that CH97 was a wheat-*Th. ponticum* 5E (5D) alien disomic substitution line.

3.4. Evaluating disease resistance and agronomic performance for CH97

The response of CH97 to powdery mildew is detailed in Appendix D, highlighting the comparative resistance of wheat lines 7182, CH97 and SY225 against the *Bgt* isolate E09 of powdery mildew during the seedling stage in both 2021 and 2022. The experiments, conducted within a growth chamber, demonstrated that while SY225 was completely susceptible to the disease, CH97 exhibited near immunity, and 7182 showed high susceptibility (as shown in Fig. 4-A). Furthermore, the resistance to powdery mildew was assessed in the field over two years for adult plants under natural infection conditions. The evaluation was based on the most severe response noted in any of the years. The results were consistent with those observed at the seedling stage; *Th. ponticum* and

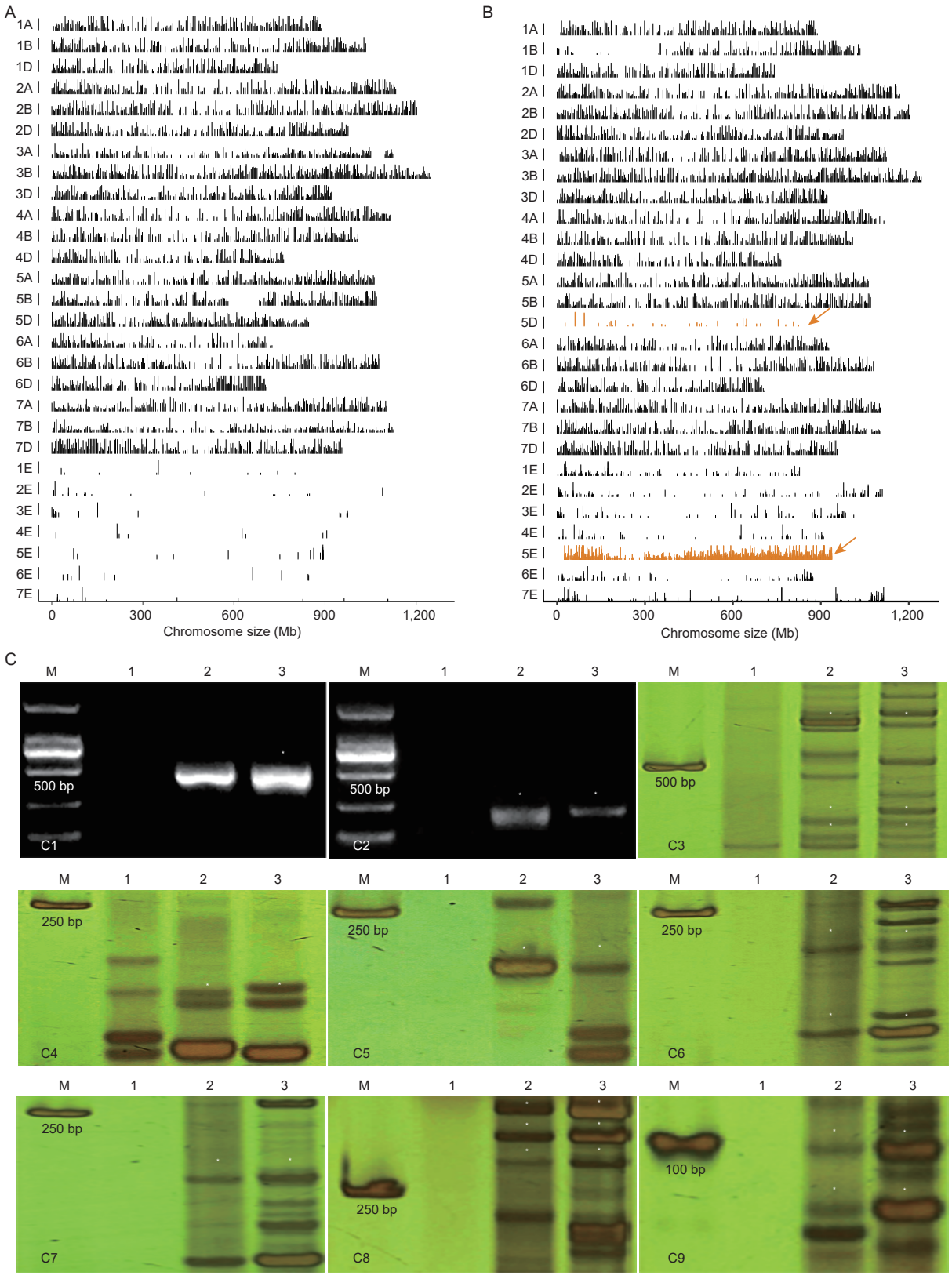


Fig. 3 Liquid array (GenoBaits®WheatplusEE) and molecular marker analysis of wheat-*Thinopyrum ponticum* disomic substitution line CH97. A, liquid array analysis of the common wheat parent 7182. Enriched signals were distributed on chromosomes 1A–7D. B, liquid array analysis of CH97 indicated a weak signal on chromosome 5D, but a significant enrichment on chromosome 5E, as shown by the orange arrows. The horizontal axis indicates the physical position in Mb, while the vertical axis indicates the distribution and abundance of sequencing depth per million along the chromosomes. C, simple sequence repeats (SSR) and PCR-based landmark unique gene (PLUG) molecular markers analysis. M, D2000; 1, 7182; 2, *Th. ponticum*; 3, CH97. C1, *Xwmc215*; C2, *Xgwm159*; C3, *Xgpw7346*; C4, *Xgpw7309*; C5, *Xcfd12*; C6, TNAC1574-*Taq*I; C7, TNAC1574-*Hae*III; C8, TNAC1588-*Taq*I; C9, TNAC1588-*Hae*III. * indicates specific bands of *Th. ponticum* and CH97.

CH97 displayed immunity, whereas 7182 remained highly susceptible to powdery mildew (Fig. 4-D).

CH97 exhibited resistance to the *Pst* CYR32 and CYR34, indicative of a strong defense mechanism against stripe rust (Appendix E). During the seedling stage, two consecutive surveys indicated that 7182 was susceptible to CYR32 and CYR34, while CH97 demonstrated high resistance to these races when HXH leaves were fully covered with spores (Fig. 4-B and C). Field tests were conducted in 2022 and 2023 to assess the responses of 7182, *Th. ponticum* and CH97 to mixed *Pst* races (CYR32, CYR33, and CYR34). The results indicated that both *Th. ponticum* and CH97 maintained healthy foliage without necrosis, unlike 7182, which displayed leaves with stripe necrosis (Fig. 4-E). This pattern underscores the role of the 5E chromosome from *Th. ponticum* in conferring resistance to both wheat powdery mildew and stripe rust.

Agronomic trait evaluations of CH97 and its wheat parent 7182 revealed that both lines share similar spike shape and awnedness, as well as comparable panicle traits including tiller number, spike length, peduncle length and kernels per spike (Fig. 5-A–I), with no significant differences ($P>0.05$). However, the thousand-kernel weight and kernel length of CH97 were significantly higher than those of 7182 ($P<0.01$). Meanwhile, CH97 exhibited a significantly greater plant height ($P<0.01$) (considered an unfavorable trait) and a significantly lower number of spikelets per spike ($P<0.05$) than 7182. These enhancements in CH97 suggest that while its germplasm is primarily derived from 7182, the observed improvements in thousand-kernel weight and kernel length are likely attributable to genes on the 5E chromosomes from *Th. ponticum*. The increased plant height and reduced number of spikelets per spike suggest that the introduction of elite alien genes may be accompanied by the transfer of undesirable genetic traits.

3.5. Specific molecular marker development of CH97

A total of 8,803,966 genomic sequences were obtained from CH97, with an average GC (guanine-cytosine) percentage and Q30 percentage of 46.95 and 93.98%, respectively. A total of 714,423 SLAF tags were developed following the enzyme digestion procedure, achieving an average sequencing depth of 10.23× for these tags. A total of 16,331 reads were identified as *Th. ponticum*-specific sequences using the Burrows-Wheeler Alignment (BWA) tool. These reads exhibited no similarity to the CS reference genome (IWGSC_v2.1) and complete similarity to the *Th. ponticum* genome (unpublished data). Of the 22 primer pairs randomly selected to develop *Th. ponticum* 5E chromosome-specific markers from the screened fragments, only nine showed identical bands between *Th. ponticum* and CH97. This resulted in a successful development rate of 33.3%, as indicated in Table 1 and Fig. 6. These markers can aid in recognizing foreign genetic components during the transfer of 5E chromosome fragments from *Th. ponticum* to wheat and expedite the molecular breeding process.

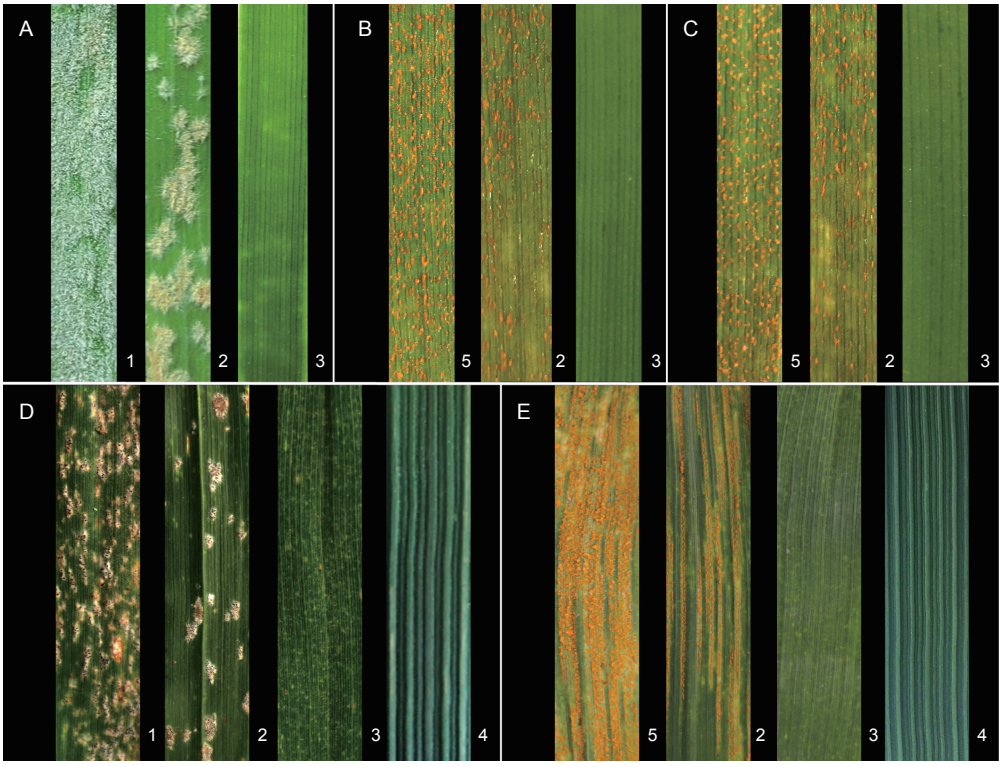


Fig. 4 Evaluation reactions of CH97 and its parents to powdery mildew and stripe rust. A, symptoms in response to *Bgt* race E09 in the seedling stage. B, symptoms to *Pst* race CYR32 in the seedling stage. C, symptoms to CYR34 in the seedling stage. D, response to powdery mildew after natural infection at adult stage. E, responses to inoculation with mixture of *Pst* races CYR32, CYR33 and CYR34 at adult stage. 1, SY225; 2, 7182; 3, CH97; 4, *Th. ponticum*; 5, HXH.

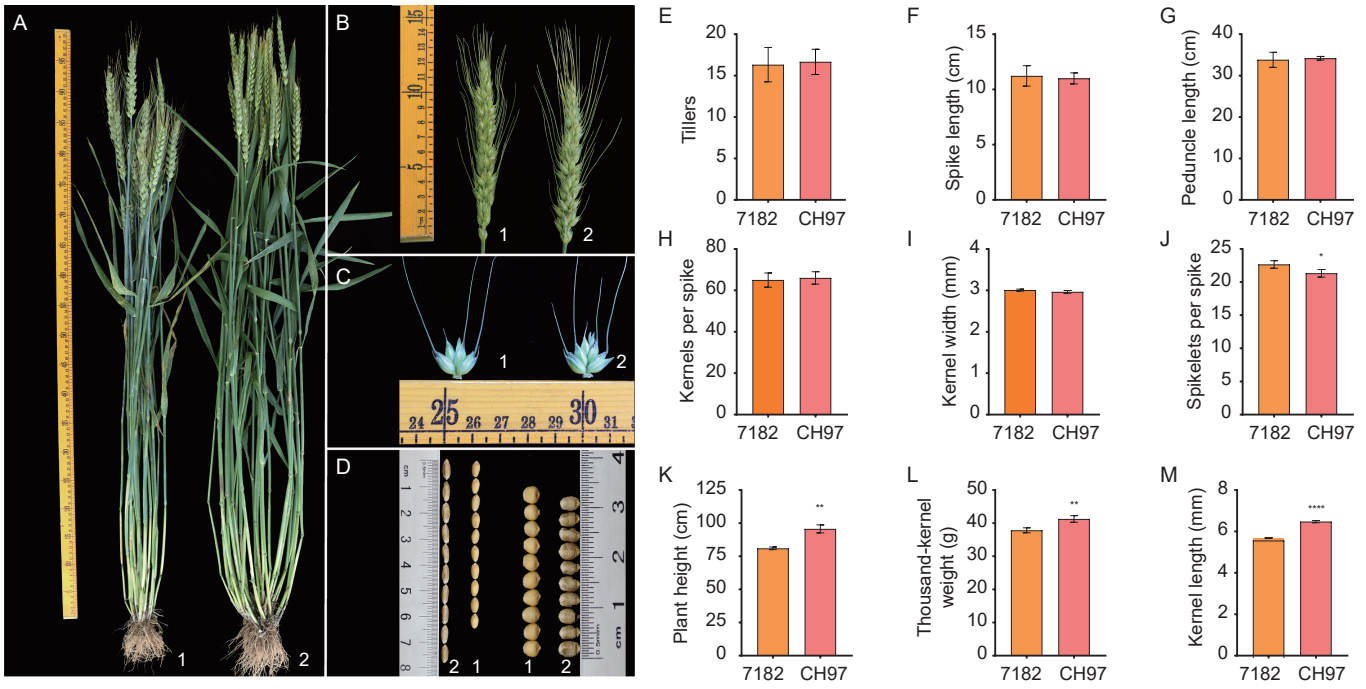


Fig. 5 Performance of agronomic traits. A, adult plants. B, spikes. C, spikelets. D, 10-seed length and width. 1, 7182; 2, CH97. E, tillers. F, spike length. G, peduncle length. H, kernels per spike. I, kernel width. J, spikelets per spike. K, plant height. L, thousand-kernel weight. M, kernel length.

4. Discussion

4.1. *Thinopyrum* is a significant source for mining novel wheat disease-resistance genes

The genetic diversity of resistance loci, lost during the domestication of wheat, can be effectively achieved through the development of chromosomally engineered materials. This method holds significant implications for augmenting the genetic diversity of common wheat, particularly for the cultivation of novel disease-resistant wheat varieties. Diploid *Th*. *elongatum* serves as the E^e genome donor of *Thinopyrum*, and it has been reported that the Ee genome possesses a multitude of advantageous genetic traits (Dvořák and Chen 1984; Li *et al.* 2018). Cytogenetic analysis and phenotyping have underscored the potential of chromosomal substitution lines in conferring disease resistance. For instance, the wheat-tetraploid *Th*. *elongatum* 1E (1D) substitution line K17-841-1 displayed high resistance to a mixture of *Pst* races (CYR-32, CYR-33, CYR-34, and V26/Gui22-14) at the adult stage, with an ITs of 0 (Li *et al.* 2019). K16-730-3 was identified as a wheat-*Th*. *scirpeum* 4E (4D) substitution line with resistance to powdery mildew and the mixture of *Pst* races (CYR 32, CYR 33, CYR 34, Sull-4, Sull-5, Sull-7 and G22-14) across two consecutive growing seasons (Gong *et al.* 2022). The wheat-tetraploid *Th*. *elongatum* 6E (6D) disomic substitution line (K17-1065-4), characterized by GISH and FISH, showed high resistance to a mixture comprising *Pst* races (CYR 32, CYR 33, CYR 34, Sull-4, Sull-5, Sull-7 and G22-14) at the adult stage (Gong *et al.* 2023). However, the wheat-*Th*. *ponticum* 5E (5D) disomic substitution line, notably CH97, represents a rare yet significant development in the field of wheat breeding, emphasizing its importance in enhancing disease resistance within wheat germplasm. The ability to accurately define the 5E FISH karyotype is essential for the efficient screening of wheat germplasm, facilitating the identification of resistance genes. CH97 has shown considerable resistance to wheat powdery mildew and stripe rust at both the seedling and adult stages, positioning it as a highly valuable resource for breeders aiming to improve disease resistance in wheat. In efforts to pinpoint the chromosome segments harboring disease-resistance

Table 1 Development of specific molecular markers for CH97 based on specific-locus amplifed fragment sequencing (SLAF-seq)

Marker	Temperature (°C)	Primer (5′→3′)
CH5-1	62	F: TGTGGTACGCTTAAGCCTCGC
		R: GCTGTAGGTCGGCACCTTCAA
CH5-9	59	F: TGAGTTCCAGACTGCTTTCCGTAC
		R: CCTGAAGATGGACTGTGCTTC
CH5-10	60	F: ATTCAATGCCCTCAGCTCGG
		R: GTGCCTCTGTTCCACATGGTAC
CH5-15	61	F: CCTCTATGCCTATGGCGTTCAG
		R: GCTTGTTGTGACTTGCTAGTCG
CH5-17	61	F: TCGACAGAAGCTTTGCTGAGTG
		R: GGTATCCACAAGTCGGAACCTG
CH5-18	60	F: TGATGTGGACTGTTTCAGTGGC
		R: CAATTTTGGTGTCCTGTCGAGC
CH5-20	59	F: AATACAGTATGCGTGGCATGAG
		R: CGTCTTCATCATGAGAACATGGC
CH5-24	58	F: CTTAGCATTAGATCGCACTATGC
		R: GACCACAGCCAGCATGTTTAC
CH5-27	59	F: TTCCACCAACAGTTGGCAGC
		R: CTGTATGGGACGCTAATGAACAC

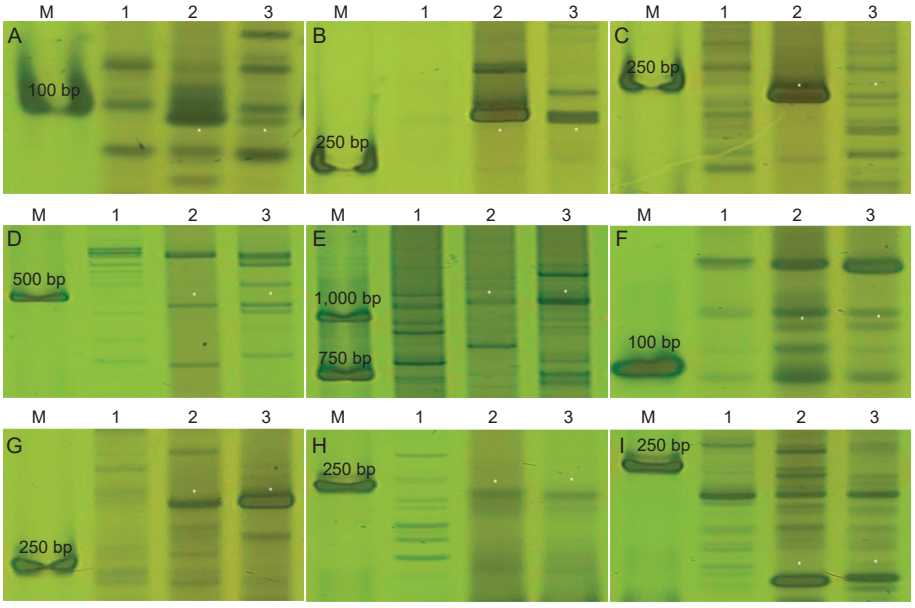


Fig. 6 Development of specific molecular markers for wheat-*Thinopyrum* ponticum disomic substitution line CH97. M, D2000; 1, 7182; 2, *Th. ponticum*; 3, CH97. A, CH5-1. B, CH5-9. C, CH5-10. D, CH5-15. E, CH5-17. F, CH5-18. G, CH5-20. H, CH5-24. I, CH5-27. * indicates specific bands of *Th. ponticum* and CH97.

genes, CH97 has undergone crossbreeding with the CS *ph1b* mutant (Qu *et al.* 1998; Gyawali *et al.* 2019). This strategic cross is anticipated to yield stable translocation lines that retain resistance to diseases, which will subsequently serve as the foundation for genetic populations aimed at mapping resistance genes for powdery mildew and stripe rust. These developments are pivotal in advancing the understanding and application of genetic resistance within wheat breeding programs.

Moreover, the 5E (5D) disomic substitution line CH97 exhibits superior disease resistance and enhanced agronomic performance compared to its common wheat parent, 7182. Notable improvements include a higher thousand-kernel weight and an elongation in kernel length. Given the positive correlation between yield and its individual components, the management of these yield components is crucial in wheat breeding. With its advantageous traits, CH97 offers a promising source for introducing excellent genes into wheat, aiding the development of varieties that meet the dual goals of disease resistance and improved yield.

4.2. FISH combined with GISH is a powerful tool for characterizing the origin and evolution of allopolyploids

Breeders aim to quickly and accurately identify foreign genetic material within wheat to enhance its traits, including disease resistance, yield and adaptability. Traditional morphological markers such as plant height, spike shape, grain shape, thousand-kernel weight, and disease resistance have been commonly employed for this purpose. However, relying solely on phenotypic characteristics for the identification of exogenous genetic material in wheat presents limitations. This is because wheat's physiological and morphological traits are influenced not just by genetic factors but also by environmental conditions, making phenotypic characteristics an unreliable sole indicator. To overcome these challenges, *in situ* hybridization techniques, such as GISH and ND-FISH, have become indispensable tools in the detection of heterochromatic lines in wheat. For example, D27, a wheat-Mexico Rye T1DL·1RS translocation line, demonstrated stripe rust and powdery mildew resistance which was attributed to a locus on chromosome 1RS using GISH (Li J C *et al.* 2023). Similarly, Li J J *et al.* (2023) developed three ND-FISH probes, HS-TZ3, HS-TZ4, and HS-CHTZ5, which were successfully employed to construct chromosome FISH karyotypes of seven homologous groups of *Psathyrostachys huashanica*. In the current study, we substantiated the introduction of a pair of chromosomes from *Th. ponticum* into CH97 through GISH, determining these chromosomes to belong to *Th. elongatum*. Further evidence from FISH highlighted the signaling morphology of these alien chromosomes. Additionally, structural variations in chromosomes 1B, 6B and 7B of CH97, when compared with its common wheat parent 7182, were unexpectedly discovered. These findings suggest that the incorporation of chromosome 5E might interfere with the wheat's homologous chromosome pairing genes, thereby enhancing recombination between wheat chromosomes and *Th. ponticum* homoeologous chromosomes. This alteration could stem from the selective spontaneous loss or addition of chromosomal segments during the process of polyploid evolution, which might serve to adapt to environmental changes, improve reproduction, and ensure coordinated genome functioning — a phenomenon often referred to as "genome shock" (Ha *et al.* 2009; Bashir *et al.* 2018; Fouche *et al.* 2018; Yu *et al.* 2019). Additionally, the FISH karyotype analysis suggests that CH97 may possess a pair of 1RS·1BL translocation chromosomes. This observation aligns with findings from a previous study on the $1J^s$ (1D) substitution

line (Wang *et al.* 2020), which may have resulted from low-probability cross-pollination events occurring in the field.

4.3. Liquid phase microarray served as efficient and economical genotypes for exploring biological species

While sequential FISH-GISH analysis can precisely identify chromosome lines, it is a time-consuming process. As an alternative, faster, more affordable high-throughput SNPs are becoming more widely used. Recently, liquid-phase microarrays, utilizing targeted capture sequencing technology have emerged as an approach for biochips (Guo *et al.* 2021). For instance, the liquid phase microarray has been employed to identify significant association signals with hair type in sheep (Guo *et al.* 2023), demonstrating its utility in genetic studies beyond crop sciences. In maize, the application of genotyping by target sequencing (GBTS) and in-solution capture has facilitated a comprehensive evaluation of germplasm for breeding purposes (Guo *et al.* 2019). Similarly, the chromosome composition of wheat-*L. mollis* 4Ns (4D) disomic substitution lines have been analyzed in a more visually intuitive manner using liquid phase microarrays (Du *et al.* 2022).

In the context of this study, the wheat-*Th. elongatum* liquid array (GenoBaits®WheatplusEE) can be used to visualize the chromosomal composition of CH97. The liquid array showed a distinct lack of signal on chromosome 5D in the wheat background, while significantly enriched signals of *Th. elongatum* were detected on chromosome 5E. The introduction of liquid-phase microarrays represents a shift in the methodology for identifying foreign chromosomes, offering a more efficient and less complex alternative to traditional cytological techniques. This advancement holds the potential to significantly accelerate the breeding process by facilitating the rapid identification and incorporation of beneficial genetic material from foreign species into wheat. Moreover, the five SSR markers and 4 PLUG markers distributed in the fifth homoeologous group of wheat successfully amplified target bands in *Th. ponticum* and CH97, but not in the common wheat parent 7182.

4.4. SLAF-seq markers allow rapid detection of alien chromosomes in a common wheat background

SLAF-seq, a simplified sequencing technique used for genome-specific DNA enzyme fragments, has been efficiently applied in the development of molecular markers for wheat relatives due to its high throughput, short sequencing cycle, and accuracy (Sun *et al.* 2013; Tong *et al.* 2022). For instance, SLAF-seq has facilitated the development of chromosome 4Ag-specific markers in *Th. ponticum* with an efficacy rate of up to 40% (Liu *et al.* 2018). Similarly, 89 specific molecular markers targeting the chromosome 7E of *Th. elongatum* was developed, achieving an efficiency of up to 65.9% (Chen *et al.* 2013). In *Aegilops geniculate*, specific markers for chromosome $3M^g$ were developed with a success rate of 47.66% (Wang *et al.* 2021). In the current study, a 33.3% developmental efficiency was achieved through PCR and electrophoretic assay in screening 9 markers from 22 primers for tracking the *Th. ponticum* 5E chromosome. These markers provide a convenient and reliable method for tracing the 5E chromosome from *Th. ponticum*, significantly aiding in the identification and incorporation of desirable genetic traits into wheat.

5. Conclusion

In this study, we successfully developed a novel wheat-*Th. ponticum* derivative line, CH97. Through a comprehensive evaluation involving cytological evidence, *in situ* hybridization, molecular markers, liquid array analysis and an assessment of agronomic traits, CH97 has been characterized as a disomic substitution line where chromosome 5E from *Th. ponticum* substitutes for wheat's chromosome 5D. This genetic modification has endowed CH97 with significant resistance to powdery mildew and stripe rust throughout its entire growth period. Compared to its common wheat parent, 7182, CH97 exhibited notable improvements, including higher thousand-kernel weight and longer kernel length. Additionally, leveraging the capabilities of SLAF-seq technology, specific molecular markers for the alien chromosome 5E were developed and validated with a success rate of 33.3%. These markers provide a practical tool for detecting 5E chromosome fragments within a common wheat background, facilitating the tracking and breeding of wheat lines with enhanced disease resistance and agronomic traits.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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