Molecular Cytogenetics of Chromosome 2St as Well as Chromosome 3St Derived from *Thinopyrum Intermedium* and *Thinopyrum Ponticum*

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Abstract

Owing to the excellent resistance to abiotic and biotic stress, *Thionpyrum intermedium* (2*n* = 6*x* = 42, JJJ^{s}J^{s}StSt) and *Thinopyrum ponticum* (2*n* = 10*x* = 70) are both widely utilized in wheat germplasm innovation programs. Disomic substitution lines (DSLs) carrying one pair of alien chromosomes are valuable bridge materials for novel genes transmission. In this study, six wheat-*Thinopyrum* DSLs were derived from crosses between Abbondanza nullisomic lines (2*n* = 40) and two octoploid *Trititrigia* lines (2*n* = 8*x* = 56), characterized by a sequential fluorescence *in situ* hybridization (FISH)-genome *in situ* hybridization (GISH), a multicolor GISH (mc-GISH), and an analysis of wheat 15K SNP array combined with molecular marker selection. ES-9 and ES-10 were two wheat- *Th. ponticum* disomic substitution lines, DS2St (2A) and DS3St (3D). While ES-23, ES-24, ES-25, and ES-26 were four wheat- *Th. intermedium* disomic substitution lines, DS2St (2A), DS3St (3D), DS2St (2B), DS2St (2D). The FISH karyotypes of *Th. ponticum* 2St/3St chromosomes were well coincident with the ones of *Th. intermedium*. The chromosome configurations of F_1 hybrids derived from crosses between ES-23 and ES-9, as well as ES-24 and ES-10 were mostly formed 21Ⅱ. Four St-chromosome-specific markers were developed by specific-locus amplified fragment sequencing (SLAF-seq). Additionally, the substitution lines containing chromosome 2St conferred higher thousand-kernel weight and stripe rust resistance at adult stages, while the substitution lines containing chromosome 3St were highly resistant to stripe rust at all stages. Therefore, these six substitution lines could serve as useful bridging parents for wheat genetic improvement.

Introduction

Intermediate wheatgrass (*Thinopyrum intermedium* Barkworth & D.R. Dewey, JJJ^{s}J^{s}StSt, 2*n* = 6*x* = 42) and tall wheatgrass (*Thinopyrum ponticum* (Podp.) Barkworth & D. R. Dewey, 2*n* = 10*x* = 70) are important allopolyploids of *Thinoprum* species. Because of the desirable tolerance to biotic and abiotic stresses, both of them have been widely used in wheat chromosome engineering breeding for decades (Chen et al. 2003; Li et al. 2008). According to previous studies, neither the chromosomal composition of *Th. intermedium* nor *Th. ponticum* has been fully characterized. In terms of *Th. intermedium*, the chromosomal composition is generally regarded as JJJ^{s}J^{s}StSt (Chen et al. 1998) or J^{r}J^{r}J^{vs}J^{vs}StSt (Wang et al. 2015). The subgenome J or J^r is highly homologous with genome J (*Th. bessarabicum*, J^b, E^b)/E (*Th. elongatum*, J^e, E^e) (Liu and Wang 1993), and the main controversy has been whether the genome V originating from *Dasypyrum villosum* (2*n* = 2*x* = 14, VV) was involved in the recombinant subgenome J^s or not (Wang and Lu 2014; Deng et al. 2013). Additionally, it was convinced that *Th. intermedium* contained a set of St chromosomes which probably derived from diploid *Pseudoroegneria spicata* (2*n* = 2*x* = 14, StSt) (Mahelka et al. 2011; Cseh et al. 2019). However, it has been still unclear that whether *Th. ponticum* contains St chromosomes and if the St genome as well as the J/E genome were affected by recombination during the allopolyploidization process (Kruppa and Molnár-Láng 2016; He et al. 2017).

Although there are aspects of *Th. intermedium* genome and *Th. ponticum* genome remain undiscovered, numerous partial amphiploid lines have been successfully developed during the past decades (Fedak et al. 2000; Han et al. 2004; Zheng et al. 2014; Kruppa et al. 2016). Octoploid *Trititrigia* with advantageous traits served as significant cytogenetic resources to develop alien introgression lines and could be further applied to wheat breeding programs (Li et al. 2016b; Li et al. 2019a; Li et al. 2019b; Zheng et al. 2020). Furthermore, octoploid *Trititrigia* lines mostly carry a synthetic genome inherited from *Th. intermedium* or *Th. ponticum*. According to the defined genome composition of partial amphiploids by molecular cytogenetic mothed, to some degree, it is possible to understand the chromosomal compositions of *Thinopyrum* allopolyploids. TAF46 is an important wheat-*Th. intermedium* partial amphiploid with a common wheat Vilmorin 27 background, and the six disomic addition lines L1, L2, L3, L4, L5 with L7 were developed from TAF46 (Figueiras et al. 1986). Subsequently, molecular cytogenetic identification of TAF46 as well as the derived six addition lines revealed that the genome composition of TAF46 is 14A + 14B + 14D + 2(1J) + 2(2St) + 2(3J) + 2(4St) + 2(5J) + 2(6St) + 2(7J) (Friebe et al. 1992; Chen et al. 1999; Forster et al. 2009). It is suggested that chromosomes of St genome contained in *Th. intermedium* could be stably inherited. It is feasible to introduce the St chromosomes into common wheat background for wheat genetic improvement.

Stripe rust (*Puccinia striiformis f.sp. tritici*, *Pst*) is a recurrent damaging disease causes serious yields decrease of wheat annually (Chen 2005). Development and transfer of novel resistant genes contained in wheat related wild species is one of the most efficient and environment-friendly solutions. According to previous studies, St chromosomes originating from *Th. intermedium* carry several new stripe rust resistance genes, which are potentially optimal genetic resources for wheat breeding. Except the named wheat- *Th. intermedium* disomic addition lines, L4 (DA4St) and L7 (DA6St), a DS1St (1D) with stripe rust resistance was produced (Hu et al. 2010). Additionally, a DA3St (Nie et al. 2019) and a DA7St (Song et al. 2013) were characterized, both carrying stripe rust resistant gene(s). Now FISH karyotypes of *Th. intermedium* have yet to be constructed, which limits the improvement of germplasm materials identification efficiency. Based on molecular cytogenetic identification of wheat- *Th. intermedium* DALs or DSLs, the St chromosome FISH karyotypes are able to be established. However, at present no wheat- *Th. intermedium* 2St disomic substitution lines or 2St chromosome FISH karyotypes have been reported.

In the present study, four wheat- *Th. intermedium* disomic substitution lines, ES-22, ES-23, ES-25 and ES-26, were generated from crosses between Abbondanza nullisomic lines ($2n = 40$) and Zhong4 (a wheat-*Th. intermedium* partial amphiploid with stripe rust, $2n = 8x = 56$) with consecutive self-crosses for several years. While two wheat- *Th. ponticum* disomic substitution lines, ES-9 and ES-10, were derived from Xiaoyan784 (a wheat- *Th. ponticum* partial amphiploid with stripe rust, $2n = 8x = 56$) by the same procedure. Molecular cytogenetic analysis was to determine and compare the genome composition of the six alien lines, and two 2St-chromosome-specific markers and two 3St-chromosome-specific markers were developed by SLAF-sEq. Disease evaluation results indicated that the alien lines containing *Thinopyrum* chromosome 2St (ES-9, ES-23, ES-25 and ES-26) conferred high level stripe rust resistance at adult stages, while alien lines containing *Thinopyrum* chromosome 3St (ES-10 and ES-24) are highly

resistant to stripe rust at all stages. In addition, potential value of the morphological characteristics for wheat breeding was evaluated.

Materials And Methods

Plant materials

The plant materials include *Thinopyrum intermedium* (2*n* = 6*x* = 42, JJJ^{s}J^{s}StSt), *Thinopyrum ponticum* (2*n* = 10*x* = 70), diploid *Pseudoroegneria spicata* (2*n* = 2*x* = 14, StSt), tetraploid *Pseudoroegneria spicata* (2*n* = 4*x* = 28, StStStSt), *Thinopyrum bessarabicum* (2*n* = 2*x* = 14, JJ), *Thinopyrum elongatum* (2*n* = 2*x* = 14, EE), wheat cv. Chinese Spring (CS), the Abbondanza lines, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, Zhong4 and Xiaoyan784, as well as two wheat- *Th. intermedium* disomic addition lines, L4 (DA4St) and L7 (DA6St). Twenty-six F_1 hybrids obtained from hybridizations between two pairs of cross combinations, ES-9 and ES-23 (15 plants) as well as ES-10 and ES-24 (11 plants). The BC_1F_2 population comprising 60 individuals were derived from crosses between ES-24 and the wheat landrace Huixianhong (HXH). Five wheat- *Th. intermedium* disomic addition lines were developed via hybridization between Abbondanza nullisomic lines and Zhong4, including DA1St, DA2St, DA3St, DA5St and DA7St (unpublished data). All the above-mentioned plant materials were preserved at the College of Agronomy, Northwest A&F University, China. HXH was served as a susceptible control in the stripe rust resistance evaluation. The *Pst* races CYR32 were used for seedling stage of stripe rust resistance evaluation as well as the CYR31 and CYR32 mixture were used for adult stage evaluation. All the *Pst* races were provided by the College of Plant Protection, Northwest A&F University, China.

In situ hybridization

Chromosome spreads by drop method (Han et al. 2004) were used for *in situ* hybridization analyses. The protocols of genomic DNA extracting and sequential FISH–GISH as well as mc-GISH were conducted by Wang et al. (Wang et al. 2019). According to the nick translation method, total genomic DNA of *Th. bessarabicum*, *Th. intermedium*, as well as *Th. ponticum* was labeled with fluorescein-12-dUTP, while St genomic DNA from diploid and tetraploid *P. spicata* was labeled with Texas Red-5-Dutp, respectively, used as GISH and mc-GISH probes. And the sheared DNA of CS was as a blocking DNA. The Oligonucleotide probes combination of Oligo-pTa535 (red) and Oligo-pSc119.2 (green) were used for FISH analyses. Hybridization signals were observed and acquired under an Olympus BX53 fluorescence microscope.

Wheat 15K SNP array analysis

Wheat 15K SNP genotyping arrays were used to genotype the 9 samples, including Abbondanza, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, *Th. ponticum* and *Th. intermedium*, by using Illumina SNP genotyping technology (China Golden Marker Biotechnology Company). There were 13199 SNP loci contained in the wheat 15K array and distributed on all 21 wheat chromosomes. The calculation of the percentage of the

same genotype between two materials in each chromosome was carried out as the total number of markers divided by the loci number of the same genotypes. The software Origin (OriginLab, USA) was used for data analysis and graphing.

PLUG markers analysis

The polymerase chain reaction (PCR)–based landmark unique gene (PLUG) markers (http://wheat.pw.usda.gov/SNP/new/pcr_primers.shtml) were selected for 21 wheat chromosomes among homoeologous groups 1 to 7 and then synthesized by AuGCT DNA-SYN Biotechnology Co. (Beijing, China). PCR assays and electrophoresis procedures were conducted as described (Zhu et al. 2017).

Stripe rust resistance and agronomic traits evaluation

The stripe rust resistance evaluation was conducted in the field at the adult stage, while seedling stage test was conducted in the greenhouse. A mixture *Pst* races of CYR31 and CYR32 was used to evaluated the adult plant resistance of Abbondanza, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, Xiaoyan 784 and Zhong 4, with HXH severed as susceptible control. For further genetic analyses of the resistance, *Pst* races CYR32 was used to inoculate the above-mentioned materials at the seedling stage as well as the BC_1F_2 population individuals of ES-24 and HXH. The infection type (IT) was scored with a scale of 0-4 (Ma et al. 1995).

To assess the morphological traits, ten plants of each materials (Abbondanza, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, Xiaoyan 784 and Zhong 4,) at physiology maturity stage were randomly selected during the 2019-2020 growing season. There were totally six agronomic traits recorded in the field which involved in plant height, spike length, number of spikelets per plant, number of tillers, number of spikelets per spike, awnedness, and thousand kernel weight. The significant differences of each agronomic trait were analyzed by Duncan's multiple range test ($P < 0.05$).

Meiotic chromosome pairing analysis of the F_1 hybrids

Young spikes of F_1 hybrids derived from the two crosses combinations (ES-9×ES-23 as well as ES-10×ES-24) at propriate stage were extracted at the suitable temperature under field conditions, and immediately treated with Carnoy's fixative fluid II (6:3:1 ethanol-chloroform-glacial acetic acid solution). Before cytological observation of pollen mother cells, anthers were extracted and stained with 1% acetocarmine. The chromosome configurations in the miosis period were observed, recorded and photographed.

Genomic polymorphism analysis by pairwise comparisons

On the basis of SLAF-seq (Sun et al. 2013), genomic DNA of Abbondanza, ES-9, ES-10, ES-23, ES-24, *Th. intermedium* and *Th. ponticum* was sequenced, carried out by Biomarker Technologies Co. (Beijing, China). The restriction endonuclease, *Hae* III was selected to digest the genomic DNA. According to the sequence similarity, the filtered SLAF pair-end reads (150 bp per read) were clustered. By using BLAST software, sequences with over 90% identity were divided into one SLAF locus. Genomic polymorphism analyses were conducted by intercomparisons between ES-9 and ES-23, as well as ES-10 and ES-24. Firstly, all the SLAFs from ES-9, ES-10, ES-23 and ES-24 were blasted with wheat genome, removing the sequences with high wheat homology (over 80%). Secondly, the remaining SLAFs of ES-9 and ES-10 were further blasted with the sequences of *Th. ponticum*, while the SLAFs of ES-23 and ES-24 were blasted with *Th. intermedium*. Then the SLAFs with high identity (over 90%) of each material were remained, which were served as specific sequences of *Th. ponticum* attributing to ES-9 and ES-10, as well as the specific sequences of *Th. intermedium* attributing to ES-23 and ES-24. Finally, pairwise comparisons were conducted and the respective specific SLAFs with high identity (over 90%) were acquired.

Development and validation of the St-chromosome-specific markers

Based on the respective specific SLAFs obtained from the intercomparisons, PCR primers were designed for the amplification of the two groups of materials (ES-9 and ES-23 as well as ES-10 and ES-24). All the primers were designed by using the online tool (Primer3 Plus, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by AuGCT DNA-SYN Biotechnology Co. (Beijing, China). The amplified products were examined by using 2% agarose gel electrophoresis. The markers amplificated specific sequences in *Th. ponticum*, tetraploid *P. spicata*, *Th. intermedium*, diploid *P. spicata*, DA2St, ES-9 and ES-23, but not in CS, Abbondanza, *Th. bessarabicum*, *Th. elongatum*, the 1St and 3-7St addition lines, were served as 2St-chromosomes-specific molecular markers. While the markers presented in ES-10, ES-24, whereas absent in the 1-2St and 4-7St addition lines, were served as 3St-chromosomes-specific molecular markers. Subsequently, the 3St-chromosomes-specific markers were utilized in BC_1F_2 individuals of ES-24 and HXH for specificity validation.

The PCR amplifications were performed in a reaction of 20μl, containing 1.6μl of template DNA (100ng/μl), 1.6μl dNTP mixture (2.5 mM each), 2μl of 10× PCR buffer (Mg^{2+} plus), 1.4μl of each primer (10 μM), 0.1μl *rTaq* DNA polymerase (2.5 U/μL, Takara) and 13.3μL double-distilled water. The PCR protocol was as follows: 94 °C for 4min, 32 cycles of 94 °C for 30s, 54–60 °C for 35s, 72 °C for 30s, and 72 °C for 30s, 72 °C for 10 min.

Results

In situ hybridization of the six substitution lines

By GISH analysis of somatic cells, alien chromosomes derived from *Th. ponticum* or *Th. intermedium* were able to be traced. It was showed that all the six lines, ES-9, ES-10, ES-23, ES-24, ES-25 and ES-26

contained 42 chromosomes (Fig. 1). ES-9 and ES-10 both carried two *Th. ponticum* chromosomes with a bright-green hybridization signal by using *Th. ponticum* genome DNA as a probe (Fig. 1, b1 and b2). Whereas ES-23 (Fig. 1, b3), ES-24 (Fig. 1, b4), ES-25 (Fig. 1, b5) and ES-26 (Fig. 1, b6), each of them carried two *Th. intermedium* chromosomes with a bright-green hybridization signal, by using the GISH probe of *Th. intermedium*. Therefore, ES-9 and ES-10 were wheat- *Th. ponticum* disomic substitution lines, and ES-23, ES-24, ES-25, as well as ES-26 were wheat- *Th. intermedium* disomic substitution lines.

Two Oligonucleotide probes of pTa535 and pSc119.2 were combined for a sequential FISH–GISH to simultaneously examine the elimination of wheat chromosomes in the six substitution lines. Pairwise comparisons for the FISH results between substitution lines and the corresponding parent lines, Abbondanza, Zhong4 and Xiaoyan784, were conducted. It was revealed that chromosome 2A was eliminated in ES-9 and substituted by one pair of *Th. ponticum* chromosomes with three specific signal bands, including the terminal pTa535 hybridization sites detected on short arms and long arms as well as an interstitial pTa535 signal on the long arms, which was different from the FISH patterns of other wheat chromosomes (Fig. 1, a1). ES-10 lost chromosome 3D and contained one pair of *Th. ponticum* chromosomes carrying terminal pSc119.2 hybridization sites on short arms with terminal pTa535 hybridization segments on the long arms and short arms (Fig. 1, a2). Wheat chromosome 2A, chromosome 2B, and wheat chromosome 2D were eliminated in ES-23 (Fig. 1, a3), ES-25 (Fig. 1, a5) and ES-26 (Fig. 1, a6), respectively, and replaced by the same pair of *Th. intermedium* chromosomes with the identical FISH patterns of the alien chromosomes presenting in ES-9. Moreover, the telomeric region of chromosome 5B carrying a bright-green fluorescence signal was eliminated in ES-25 compared with other related materials. In terms of ES-24, chromosome 3D was substituted by a pair of *Th. intermedium* chromosomes with the FISH patterns almost consistent with the alien chromosomes detected in ES-10 (Fig. 1, a4).

In addition, according to the mc-GISH results, each of the six derived lines contained two alien chromosomes carrying a bright-red fluorescence signal originating from *P. spicata* (St) genome DNA (Fig. 1, c1-c6). It was suggested that ES-9 and ES-10 carried two different pairs of St chromosomes derived from *Th. ponticum*. While ES-23, ES-25 and ES-26 contained the same pair of St chromosomes from *Th. intermedium* which was distinguished from the pair of St chromosomes in ES-24.

Wheat 15K SNP array analysis of the six substitution lines

The chromosomal composition of the six substitution lines were determined based on genotype data by using a wheat 15K SNP array (Table S1-6). Generally, the common SNP sequences detected between the substitution lines and the same wheat parent line Abbondanza were much higher than between the substitution lines and *Th. ponticum* or *Th. intermedium*. However, obvious point of intersection was found in each of the substitution lines (Fig. 2 a-f). As shown in ES-9 (Fig. 2a), an intersection point was distinctly observed in chromosome 2A, where ES-9 had the most of the same SNP marker loci as *Th. ponticum* but few SNP marker loci as Abbondanza. According to the same genotype SNP loci number in

chromosome 2A, ES-9 contained more of the same genotype SNP loci as *Th. ponticum* rather than Abbondanza. It suggested that chromosome 2A in ES-9 were replaced by the pair of *Th. ponticum* chromosome, which was consistent with the FISH result. In ES-10 (Fig. 2b), the intersection point was detected in chromosome 3D where ES-10 had the most of the same SNP marker loci as *Th. ponticum* but few SNP marker loci compared with Abbondanza, which was consistent with the FISH result, suggesting that chromosome 3D of ES-10 were substituted by the pair of *Th. ponticum* chromosomes. While in ES-24, the intersection point was also detected in chromosome 3D, but the most of the same SNP marker loci was obtained from the comparison between *Th. intermedium* and ES-24, which meant that chromosome 3D of ES-24 was replaced by the pair of *Th. intermedium* chromosomes (Fig. 2d). It was consistent with the FISH analysis of ES-24. In terms of ES-23, ES-25 and ES-26, the intersection point of each material was undoubtedly identified in chromosome 2A (Fig. 2c), chromosome 2B (Fig. 2e), as well as chromosome 2D (Fig. 2f). Combined with the FISH results, it was revealed that chromosome 2A in ES-23, chromosome 2B in ES-25, as well as chromosome 2D in ES-26 were substituted by the same pair of *Th. intermedium* chromosomes.

PLUG marker analysis of the six substitution lines

The 135 PLUG markers were screened to further validated the homoeologous groups for the alien chromosomes. There were four PLUG markers (*TNAC1142-Hae*III, *TNAC1142-Taq*I, *TNAC1132-Taq*I, *TNAC1140-Taq*I) mapped to the second homoeologous group in ES-9, ES-23, ES-25 and ES-26 (Table S7, Fig. 3a-d). While three pairs of primers (*TNAC1326-Hae*III, *TNAC1326-Taq*I, *TNAC1359-Taq*I) were distributed in the third homoeologous group in ES-10 and ES-24 (Table S7, Fig. 3e-g). Combined with the mc-GISH results of each substitution lines, it was showed that 2St-chromosome-specific bands could be amplified in ES-9, ES-23, ES-24, ES-25, ES-26, *Th. intermedium* and *Th. ponticum*, in addition, 3St-chromosome-specific bands were identified in ES-10, ES-24, as well as, *Th. intermedium* and *Th. ponticum*, whereas the above polymorphic bands could not be amplified in Abbondanza.

The FISH karyotypes of *Th. intermedium* chromosomes 2St/3St, as well as, *Th. ponticum* chromosomes 2St/3St were characterized by *in situ* hybridization combined with wheat 15K SNP array analyses and a further PLIG marker screening. The genome composition of ES-25 (Fig. 4d) was 14A + 12B + 14D + 2(2St), while that of ES-26 (Fig. 4f) was 14A + 14B + 12D + 2(2St). Remarkably, chromosome 2St contained in ES-23 (Fig. 4b) were derived from *Th. intermedium* whereas the chromosome 2St of ES-9 (Fig. 4a) were derived from *Th. ponticum*, but they were for the same genome composition of 12A + 14B+ 14D+ 2(2St). In addition, chromosome 3St of ES-24 (Fig. 4e) and ES-10 (Fig. 4c) derived from *Th. intermedium* and *Th. ponticum*, respectively, were for the same genome composition of 14A+ 14B+ 12D+ 2(3St).

Evaluation of resistance to stripe rust and agricultural performance of the six substitution lines

The agronomic traits of the six substitution lines as well as their parents Abbondanza and Xiaoyan784 (Table 1, Fig 5) or Zhong4 (Table 2, Fig 5) were compared. On average, the tiller number of ES-9 was higher and the spikes exhibited longer than those of Abbondanza. In terms of the other substitution lines derived from Zhong4, both ES-23 and ES-26 showed much more tillers, and the spikelets per spike number of ES-26 was higher than that of Abbondanza as well as Zhong4. Surprisingly, the average thousand kernel weight of the alien lines containing chromosome 2St (ES-9, ES-23, ES-25 and ES-26) were more than 43g. It was indicated that the chromosome 2St whether originating from *Th. ponticum* or *Th. intermedium* increased thousand-kernel weight.

At the adult stage, stripe rust reaction test of the six substitution lines was conducted by comparisons with the susceptible control (HXH). Sequentially, the IT score of the six substitution lines, Abbondanza, Xiaoyan784, Zhong4, as well as *Th. ponticum* and *Th. intermedium* were recorded under field conditions. The IT score of the above-mentioned materials were as follows: *Th. ponticum*, IT = 0, *Th. intermedium*, IT = 0, Xiaoyan784, IT = 0, Zhong4, IT = 0, ES-9, IT = 1, ES-10, IT = 0, ES-23, IT = 1, ES-24, IT = 0, ES-25, IT = 1, ES-26, IT = 1, Abbondanza, IT = 3, HXH, IT = 4 (Fig 5c). Furthermore, the seedling stage stripe rust infection was conducted in the greenhouse, and the IT scores were recorded at 24 days post-inoculation (Fig 5d). With an IT score of 0, Zhong4 and Xiaoyan784 were immune to the disease. Additionally, ES-10 and ES-24 were nearly immune (IT score of 1). In contrast, Abbondanza, ES-9, ES-23, ES-25 and ES-26 were susceptible (IT score of 3). The results suggested that ES-9, ES-23, ES-25 and ES-26 carried chromosome 2St of *Th. ponticum* or *Th. intermedium* showed highly resistant to stripe rust at the adult stage. While ES-10 and ES-24 contained chromosome 3St of *Th. ponticum* or *Th. intermedium* were highly resistant at all stages.

Meiotic chromosome pairing analysis of F_1 hybrids

Based on molecular cytogenetic identification of the six substitution lines, crosses were made between the alien lines with the same genome compositions, respectively. There were 15 F_1 plants obtained from the cross between ES-9 and ES-23, and 11 F_1 plants obtained from the cross between ES-10 and ES-24. Meiotic chromosome pairing analysis of the F_1 hybrids was conducted to further validate the related genome constitution (Table 3). More than half of the pollen mother cells (PMCs) of ES-9×ES-23 and ES-10×ES-24 had 21 bivalents at metaphase I, and there was no trivalents or quadrivalents, as well as lagging chromosomes observed at meiosis anaphase I. It was indicated that chromosome 2St originating from *Th. ponticum* and *Th. intermedium* exhibited the close homologous relationship between each other, so did the *Thinopyrum* chromosome 3St.

Pairwise comparisons of genomic polymorphism analyses and St-chromosomes-specific molecular markers development

After high-throughput sequencing, SLAF library was constructed with the sequencing details (Supplementary table 8). A total of 1,055,234 (ES-9), 938,861 (ES-10), 524,288 (ES-23), 1,026,271 (ES-24), 974,634 (Abbobdanza), 572,791 (*Th. intermedium*), and 513,056 (*Th. ponticum*) SLAFs were obtained. By bioinformatics analysis, 3203 (ES-9), 4455 (ES-23), 2775 (ES-10), and 3148 (ES-24) specific sequences were selected for further sequence alignments. There were 78 out of 263 sequences from ES-24 with homology more than 90% of ES-10 (78/153). In addition, 114 out of 221 sequences from ES-23 were more than 90% homologous with ES-9 (114/177). To some degree, these results revealed the possible genomic similarity between chromosome 2St/3St of *Th. intermedium* and *Th. ponticum*.

According to the above sequence alignment results, 110 fragments from ES-23 were selected, which were regarded as 2St chromosome-specific fragments and then 73 of 3St chromosome-specific fragments from ES-24 were also selected. Subsequently, 183 pairs of primers were designed to amplify fragments from CS, Abbondanza, Zhong4, Xiaoyan784, ES-9, ES-23, ES-10, ES-24. In addition, specificity of the primers was further confirmed by analysis of *Th. ponticum*, *Th. intermedium*, tetraploid *P. spicata*, diploid *P. spicata*, *Th. bessarabicum*, *Th. elongatum*, and the wheat- *Th. intermedium* 1-7St addition line. A total of two 2St-chromosome-specific molecular markers, PTH-005 and PTH-013, and two 3St-chromosome-specific molecular markers, PTH-113 and PTH-135, were developed (Fig 6, Table 4).

Utility of the 3St-chromosome-specific markers in BC_1F_2 population

In order to validate that the stripe rust resistance gene(s) were carried by chromosome 3St, 60 BC_1F_2 individuals of ES-24 and HXH were further used for a genetic analysis. The evaluation of stripe rust resistance revealed that Zhong4, ES-24, and the 33 F_2 individuals were highly resistant to *Pst* race CYR32 at the seedling stage (Fig 7a). Subsequently, 10 resistant F_2 individuals as well as 10 susceptible ones were randomly selected for FISH analysis. Compared with the FISH karyotype of ES-24, chromosome 3St were actually detected in the resistant individuals (Fig 7b) and susceptible ones had undetectable FISH pattern of chromosome 3St (Fig 7b). It was indicated that the novel stripe rust resistant gene(s) originated from the chromosome 3St of *Th. intermedium*.

Furthermore, the specificity of newly developed 3St-chromosome-specific molecular markers was confirmed by PCR analyses of the 60 BC_1F_2 individuals of ES-24 and HXH (Fig 8). Combined with the result of seedling stage stripe rust resistance evaluation, it was revealed that Xiaoyan784, Zhong4, ES-9, ES-24, and the 33 BC_1F_2 plants conferring strong resistance to *Pst* race CYR32 carried 3St chromosome-specific markers. Oppositely, the other 26 BC_1F_2 plants without specific amplification as well as the parental line Abbondanza, and susceptible control HXH were seriously susceptible to *Pst* race CYR32. It was indicated that the newly developed St-chromosome-specific molecular markers could be used to trace the chromosome 3St in a common wheat background.

Discussion

On the basis of distant hybridization, chromosome manipulation has been widely utilized for wheat improvement programs, especially for breeding novel disease-resistant wheat lines. During the past few decades, numerous disease-resistant genes contained in wild related species have been successfully transferred to common wheat background by developing introgression lines (Zhan et al. 2014; Ma et al. 2016; Ceoloni et al. 2017; Yang et al. 2021). Disomic substitution lines contained one pair of defined alien chromosomes with desirable resistant genes are vital bridge materials for small segments of introgression (Guo et al. 2015; Mago et al. 2019), which are valuable germplasm resources for wheat disease-resistant breeding. In the current study, six stable wheat-*Thinopyrum* derived lines with remarkable stripe rust resistance were obtained from wide crosses between Abbondanza nullisomic lines and two different octoploid *Trititrigia* lines, which can be served as novel resistant germplasms for wheat breeding.

As one of the most commonly used technique, FISH analysis is generally used with GISH to discriminate genomic composition and construct the karyotype (Wang et al. 2019; Wang et al. 2020b). In this study, wheat- *Th. intermedium* disomic substitution lines DS2St (2A), DS2St (2B) and DS2St (2D), as well as wheat- *Th. ponticum* 2St(2A) disomic substitution line were developed by nullisomic backcross method. After characterized by sequential FISH–GISH and mc-GISH analysis, specific karyotype patterns of chromosome 2St were elucidated, which is significant for rapidly identifying the pair of alien chromosomes in germplasm materials. Furthermore, genomic changes in specific regions frequently happened following the process of distant hybridization (Liu et al. 2009; Li et al. 2015), which could be accurately detected by FISH. Compared with the parental lines, Abbondanza and Zhong4, telomere with subtelomeric region of chromosome 5BS carrying a blight pSc119.2 hybridization signal was eliminated in ES-25, which resulted a similar FISH pattern to chromosome 2B of common wheat. For chromosome 2B is almost metacentric whereas chromosome 5B is absolutely submetacentric, it was clear that chromosome 2B in ES-25 were replaced by chromosome 2St of *Th. intermedium* (Fig. 4h). Subtelomeres of *Triticeae* species were regarded as dynamic and relatively high frequent variable genome organization with constant homogenization between different chromosome ends (Zhang et al. 2004). Additionally, subtelomere regions of *Schizosaccharomyces pombe* showed high sequence variation, but no severe effects on the RNA expression (Oizumi et al. 2021). In terms of the deletion of subtelomeric region of chromosome 5BS in ES-25, it is difficult to access the possible function(s) of the regions for the variable nature. The segment elimination may have been resulted from chromosomal rearrangement via the process of chromosome 2St introduction. There were no severe effects detected on viability of ES-25, which suggested that the subtelomeric region eliminations of chromosome 5BS presumably contributed to genome diversity.

Based on molecular cytogenetic identification results of the six substitution lines, ES-23 and ES-9 contained the same genome composition of 12A + 14B + 14D + 2(2St), and ES-24 as well as ES-10 were for the same genome composition of 14A + 14B + 12D + 2(3St). It is surprised that FISH patterns of *Th. intermedium* 2St/3St chromosomes and *Th. ponticum* are consistent with each other. What else, the agricultural performance evaluation suggested that chromosome 2St derived from *Th. intermedium* and *Th. ponticum* both conferred higher thousand-kernel weight, more tillers and stripe rust resistance at adult

stages. And chromosome 3St of *Th. ponticum* and *Th. intermedium* were both highly resistant to stripe rust at all stages. Because of the same genome compositions, consistent FISH patterns and the similar specific agricultural performances, comparisons between ES-23 and ES-9, as well as ES-24 and ES-10, were further conducted. As one of the most traditional methods, meiotic chromosome pairing analysis of species hybrids has been used to study *Triticeae* species genome constitution for several decades (Lu and Vonbothmer 1993; Yang et al. 2015). In terms of the two groups of germplasm materials with the same genome compositions, more than half of the pollen mother cells of the F_1 hybrids perfectly formed 21 bivalents at metaphase I without any trivalents or quadrivalents, which revealed the close homologous relationship between *Th. ponticum* chromosome 2St/3St and *Th. intermedium* chromosome 2St/3St, respectively. Furthermore, genomic polymorphism intercomparisons between ES-23 and ES-9, as well as ES-24 and ES-10, were analyzed by SLAF-sEq. According to the sequence alignment results, 114 specific sequences of 221 (ES-23) and 177 (ES-9), as well as 78 specific sequences of 263 (ES-24) and 153 (ES-10) were with homology more than 90%. Overall, it was suggested the possible genomic similarity between chromosome 2St/3St of *Th. intermedium* and *Th. ponticum*.

The genomic composition of *Th. ponticum* and *Th. intermedium* has been an interesting subject for a considerable time (Wang 1992; Tiryaki et al. 2021). During the past several decades, it was convinced that the set of St chromosomes contained in *Th. intermedium* were probably derived from *P. spicata*, whereas it has been still undefined that whether the St genome is one of the sets of chromosomes of *Th. ponticum* or not (Kruppa and Molnár-Láng 2016). In this study, *Th. ponticum* chromosome 2St/3St and *Th. intermedium* chromosome 2St/3St were simultaneously identified in the six alien substitution lines derived from two different octoploid *Trititrigia* lines, Xiaoyan784 (a wheat- *Th. ponticum* partial amphiploid) and Zhong4 (a wheat- *Th. intermedium* partial amphiploid). In terms of the FISH patterns of chromosome 2St/3St, no obvious variations were detected in Xiaoyan784, Zhong4 and the six substitution lines (Fig. 4h). It was implied that St chromosomes were not only included in *Th. ponticum*, but also could be stably inherited. Furthermore, combined with the previous results of the close homologous relationship between *Th. ponticum* chromosome 2St/3St and that of *Th. intermedium*, it is convinced that *P. spicata* representing the complete set of St chromosomes played an important role during the speciation of *Th. ponticum*, but the effects of the recombination events happened between diverse genomes through the allopolyploidization process need further analyses.

Although FISH–GISH analysis has been widely utilized to precisely characterized wheat- *Th. intermedium* lines for several decades, it is time-consuming. Specific molecular markers are able to rapidly trace the alien chromosome or even small segment introgression with the advantage traits for wheat improvement breeding programs. However, for the complete *Th. intermedium* genome has not been sequenced, there are only a few chromosome-specific markers enabled to be used (Zhang et al. 2001; Hu et al. 2012b; Li et al. 2016a). With the development of sequencing technology, 635 unique *Th. intermedium* SNP markers have been successfully developed, including 135 St-chromosome-specific markers, with 15 of 2St-chromosome-specific markers and 10 of 3St-chromoosme-specific markers (Cseh et al. 2019). Due to the much more complex genomic composition of *Th. ponticum*, molecular marker development work was

mainly focused on genome E (Hu et al. 2012a; Baker et al. 2020), especially in the following of the published complete genome of *Th. elongatum* (Wang et al. 2020a). In the present study, four wheat- *Th. intermedium* disomic substitution lines were clearly characterized, of which, ES-23(DS2St (2A)) and ES-24(DS3St (3D)) were further sequenced by SLAF-seq for St-chromosome-specific marker development. And two 2St-chromosome-specific molecular markers, PTH-005 and PTH-013, as well as two 3St-chromosome-specific molecular markers, PTH-113 and PTH-135 were obtained. The FISH analysis results showed chromosome 3St were merely detected in the resistant individuals of the BC_1F_2 population of ES-24 and HXH (Fig. 7), which meant the stripe rust resistance gene(s) was derived from chromosome 3St of *Th. intermedium*. The utility of PTH-113 and PTH-135 amplification in the BC_1F_2 individuals indicated that the St-chromosome-specific molecular markers enabled to serve as useful tools for tracing the St chromosomes of *Th. intermedium* in common wheat background. In addition, according to the close genetic relationship between *Th. ponticum* chromosome 2St/3St and that of *Th. intermedium* analyzed in this study, the four St-chromosome-specific markers could be simultaneously amplified in *Th. ponticum*, tetraploid *P. spicata*, *Th. intermedium* and diploid *P. spicata*, as well as the corresponding substitution lines, ES-9, ES-23, ES-10 and ES-24 (Fig. 4h). It was speculated that the four St-chromosome-specific markers could also be utilized for tracing the St genome chromosomes of *Th. ponticum*, which need to be validated in future genetic analyses.

Conclusions

Four wheat- *Thinopyrum intermedium* and two wheat- *Thinopyrun ponticum* alien disomic substitution lines were characterized and compared by molecular cytogenetic analysis. ES-9, ES-23, ES-25 and ES-26 containing chromosome 2St conferred stripe rust resistance at adult stages and higher thousand-kernel weight, and ES-10 as well as ES-24 containing chromosome 3St conferred stripe rust resistance at all stages. What else, four St-chromosome-specific molecular markers were developed.

Declarations

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Author contribution

WQJ and CYW designed the project, SWW performed the experiments and drafted the manuscript, JXZ provided help in analysis of wheat 15K SNP array, XBF and PCD provided help in analyzing the morphological characters, YJW and CHC provided help in preparing materials, BTW provided the *Pst* races.

Conflict of interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Ethical standards

The authors declare that the experiments comply with the current laws of the country in which they were performed.

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Tables

Table 1 Agronomic traits of the alien substitution lines ES-9, ES-10, as well as their parents (Abbondanza, Xiaoyan784)

Materials	Plant height (cm)	Tillers	Spike length (cm)	Spikelets/ spike	Florets/ spikelet	Thousand Kenel Weight (g)	Awnedness
Xiaoyan784	112±6a	11±3b	21±1.5a	25±2a	6±1a	31±0.5c	awnless
ES-9	105±5a	25±4a	13.9±1b	24±1ab	4±1b	43±1a	awnless
ES-10	90±5b	18±4ab	14±1.5b	20±1c	5±1ab	36±1b	awnless
Abbondanza	107±6a	14±3b	14.4±1b	22±1b	4±1b	42±2a	awnless

Note: Different letters a, b and c indicate significant differences between ES-9, ES-10 and its wheat parent ($P < 0.05$)

Table 2 Agronomic traits of the substitution lines ES-23, ES-24, ES-25, ES-26, as well as their parents (Abbondanza and Zhong4)

Materials	Plant height (cm)	Tillers	Spike length (cm)	Spikelets/ spike	Florets/ spikelet	Thousand Kenel Weight (g)	Awnedness
Zhong4	127±4a	12±3b	16±1.5a	23±2ab	5±1a	31±0.5c	Long awn
ES-23	114±5b	23±4a	17±1.5a	21±2bc	4.4±1ab	43±1a	awnless
ES-24	87±6c	17±4b	11.6±1c	21±1c	4.4±1ab	37±2b	Short awn
ES-25	93±5c	15±4b	13±1.5b	23±1b	3.7±1b	45±1.5a	Short awn
ES-26	113±5b	27±4a	14±1.5b	25±1a	4.4±1ab	43±1.5a	Short awn
Abbondanza	107±6b	14±3b	14.4±1b	22±1b	4±1b	42±2ab	awnless

Note: Different letters a, b and c indicate significant differences between ES-23, ES-24, ES-25, ES-26 and its wheat parent ($P < 0.05$)

Table 3 Chromosome pairing in the meiotic and meiotic phases for the hybrid F_1 individuals

Material	No. of cells	Chromosome configuration					
		Univalent	Bivalent			Trivalent	Quadrivalent
			Rod	Ring	Total		
ES-9×ES-23	144	0.47(0-2)	2.69(1-4)	17.93(17-21)	20.62(20-21)	0	0
ES-10×ES-24	135	0.39(0-2)	2.61(1-4)	18.19(17-21)	20.8(20-21)	0	0

Table 4 Specific amplification markers of chromosome 2St and chromosome 3St.

Specific primers	Primers (5'-3')	Amplified chromosomes	Annealing temperatures
PTH-005	F: TCCTCAACTGGAAACAAAGGA	2St	56
	R: TTGGGAGTGAGTGTAGTTCAC		
PTH-013	F: AGCCCTCCGGAAAGAATGAA	2St	62
	R: CCGCTCAAACAATCGCTACC		
PTH-113	F: AACAGGGTCAACGGGTTTGA	3St	60
	R: TTGGTGCAGAAACAATGCGG		
PTH-135	F: TGCCTCTAACACATGCATGT	3St	60
	R: TCCAGTAGGTCTTGGCTCCA		

Supplementary

Supplementary table 4 to 8 are not available

Figures

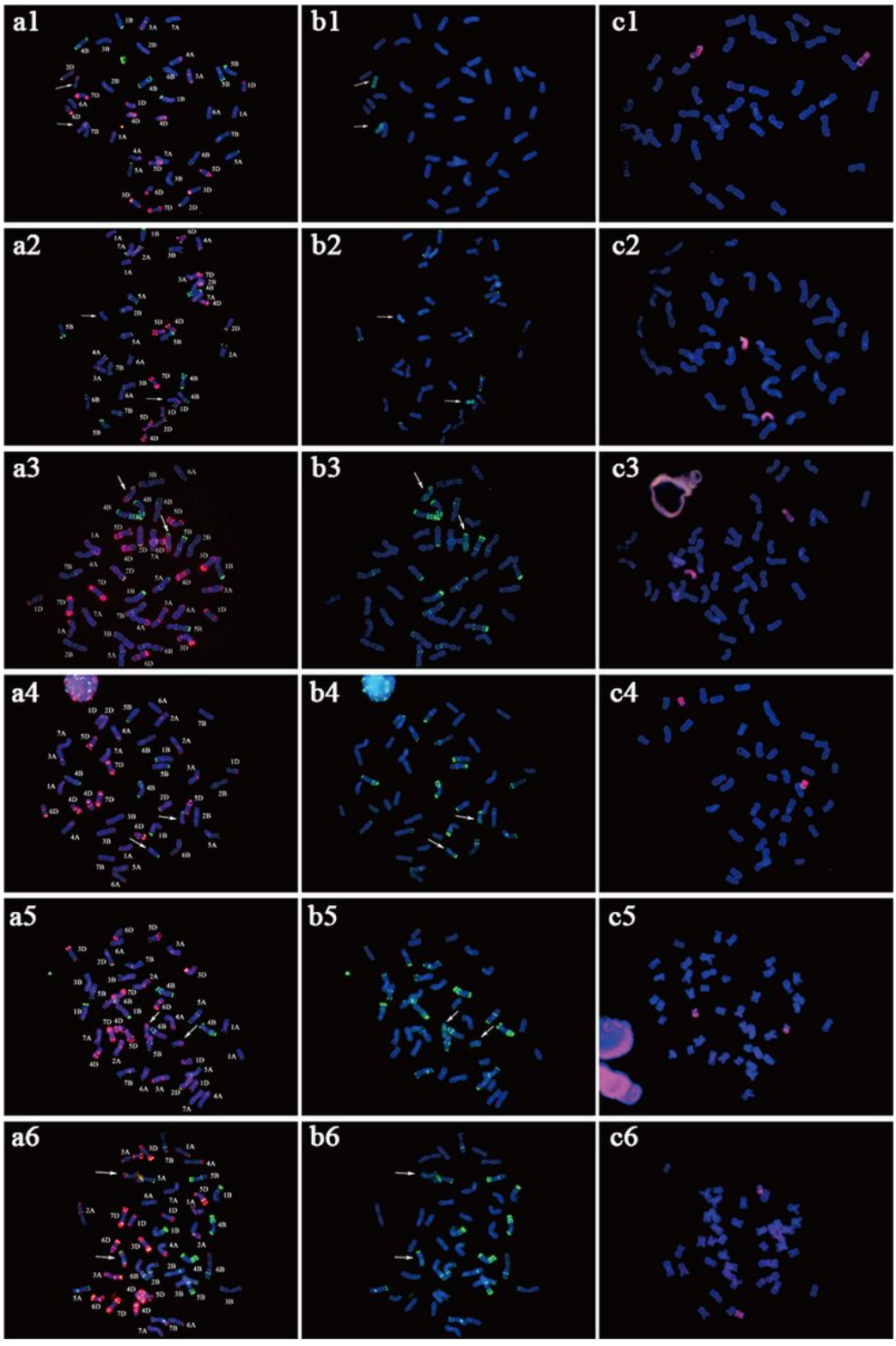

Figure 1

In situ hybridization patterns of the six alien substitution lines. a FISH patterns of ES-9 (a1), ES-10 (a2), ES-23 (a3), ES-24 (a4), ES-25 (a5) and ES-26 (a6): Oligo-pSc119.2 (green) and Oligo-pTa535 (red) as probes. b GISH patterns of ES-9 (b1) and ES-10 (b2): Thinopyrum ponticum genomic DNA (green) as probe and CS genomic DNA as a blocker; GISH patterns of ES-23 (b3), ES-24 (b4), ES-25 (b5) and ES-26 (b6): Thinopyrum intermedium genomic DNA (green) as probe and CS genomic DNA as a blocker. c Mc-

GISH patterns of ES-9 (c1), ES-10 (c2): Thinopyrum bessarabicum (J) genomic DNA (green) and tetraploid Pseudoroegneria spicata (St) genomic DNA (red) as probes, CS genomic DNA as a blocker; ES-23 (c3), ES-24 (c4), ES-25 (c5) and ES-26 (c6): Th. bessarabicum (J) genomic DNA (green) and diploid P. spicata (St) genomic DNA (red) as probes, CS genomic DNA as a blocker. The arrows indicate the alien chromosomes of the six substitution lines.

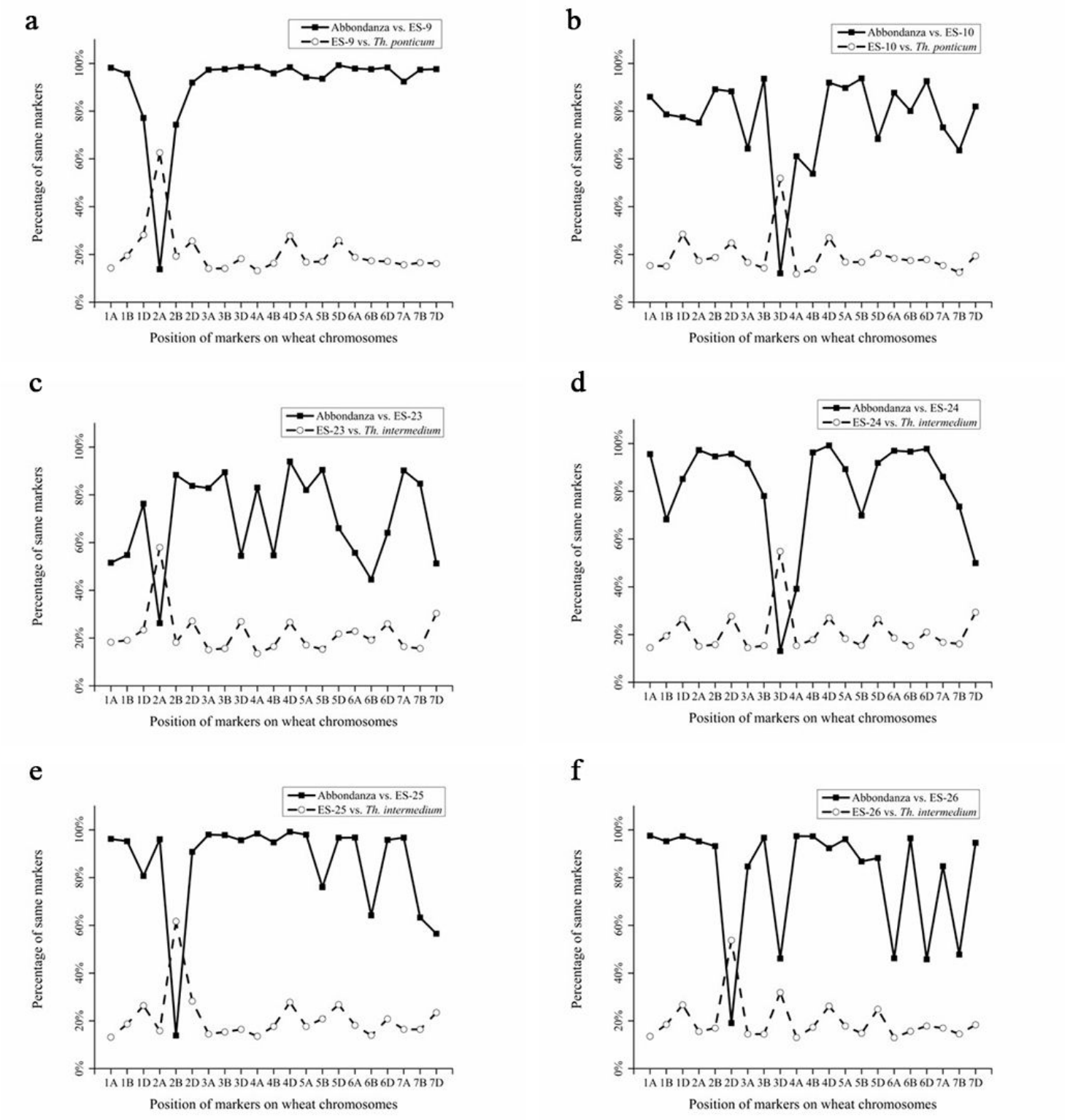

Figure 2

Wheat 15K SNP array analysis of the six alien substitution lines. a Wheat 15K SNP array analysis of ES-9. Obvious crossing point were detected in terms of the position of chromosome 2A. b Wheat 15K SNP array analysis of ES-10. Obvious crossing point were detected in terms of the position of chromosome 3D. c Wheat 15K SNP array analysis of ES-23. Obvious crossing point were detected in terms of the position of chromosome 2A. d Wheat 15K SNP array analysis of ES-24. Obvious crossing point were detected in terms of the position of chromosome 3D. e Wheat 15K SNP array analysis of ES-25. Obvious crossing point were detected in terms of the position of chromosome 2B. f Wheat 15K SNP array analysis of ES-26. Obvious crossing point were detected in terms of the position of chromosome 2D.

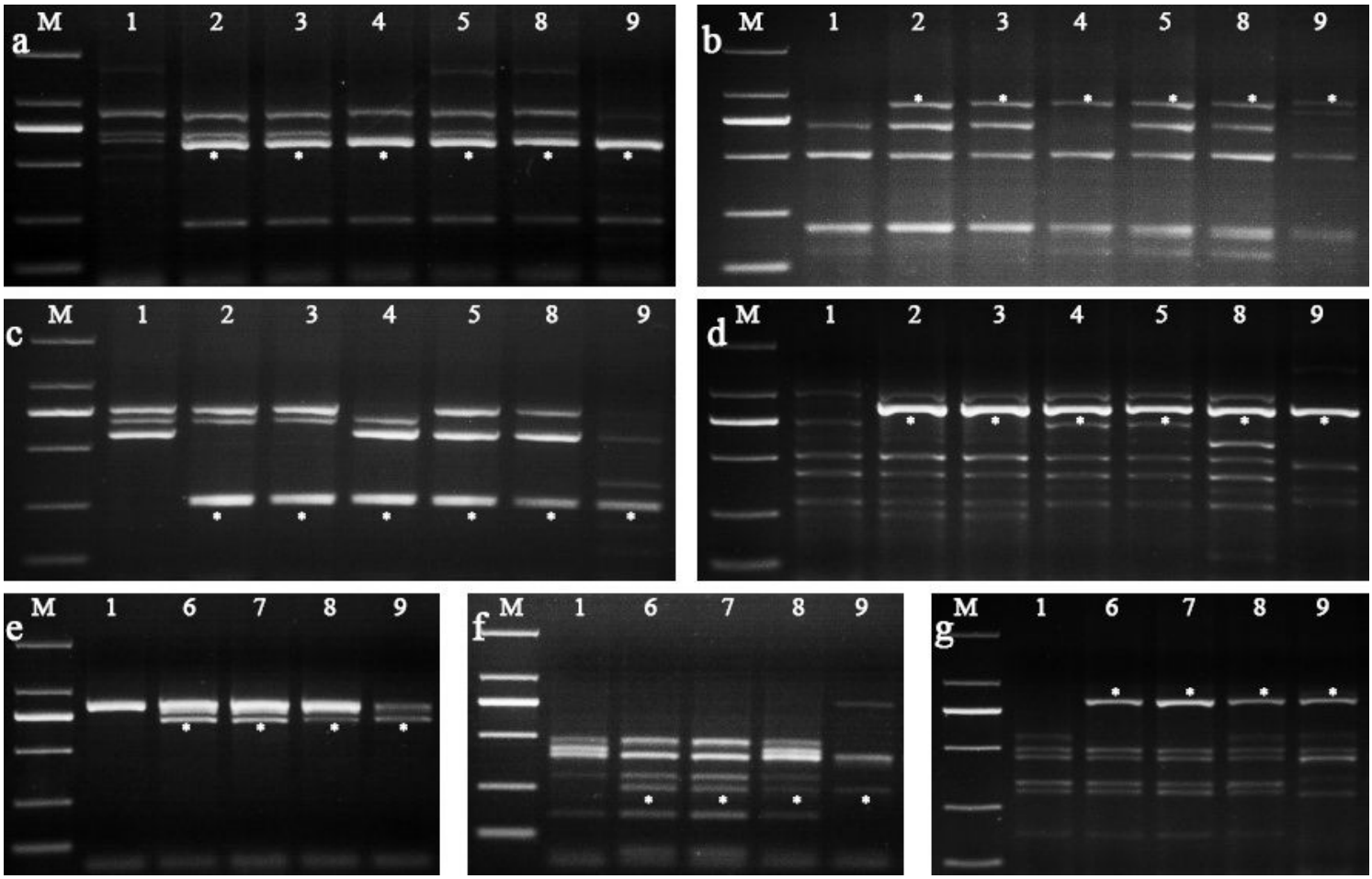

Figure 3

PLUG markers analysis of Abbondanza, the six alien substitution lines, Thinopyrum intermedium and Thinopyrum ponticum. a TNAC1142-Hae□; b TNAC1142-Taq□; c TNAC1132-Taq□; d TNAC1140-Taq□; e TNAC1326-Hae□; f TNAC1326-Taq□; g TNAC1359-Taq□. Lane M: DL2000; lane 1: Abbondanza; lane 2: ES-9; lane 3: ES-23; lane 4: ES-25; lane 5: ES-26; lane 6: ES-10; lane 7: ES-24; lane 8: Th. intermedium; lane 9: Th. ponticum. The * indicates specific band of Th. ponticum and Th. intermedium.

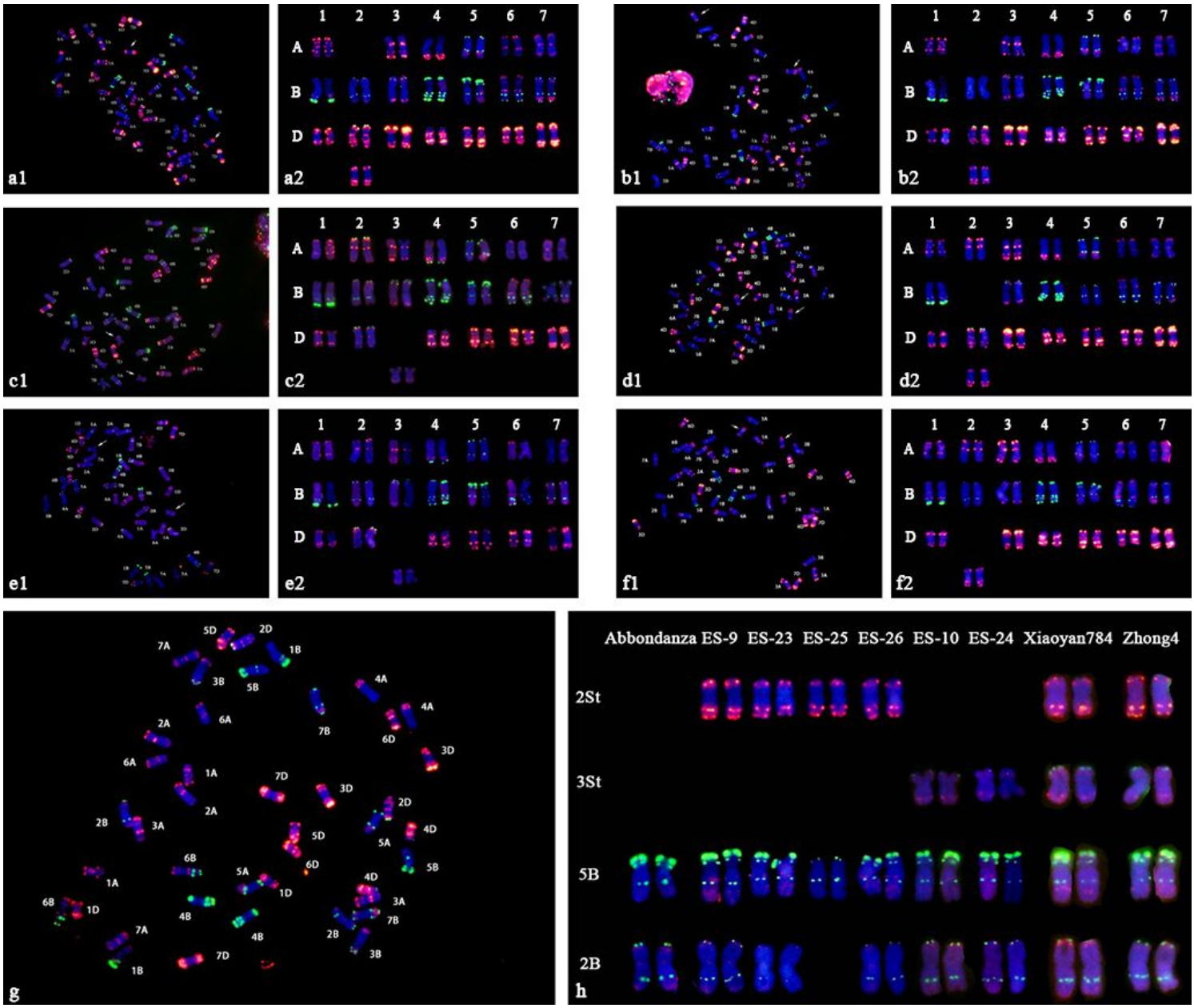

Figure 4

Karyotypes of the six alien substitution lines with the genomic composition variations. a Karyotype analysis of ES-9. Wheat chromosome 2A were replaced by Thinopyrum ponticum chromosome 2St. b Karyotype analysis of ES-23. Wheat chromosomes 2A were replaced by Thinopyrum intermedium chromosome 2St. c Karyotype analysis of ES-10. Wheat chromosome 3D were replaced by Th. ponticum chromosome 3St. d Karyotype analysis of ES-25. Wheat chromosome 2B were replaced by Th. intermedium chromosome 2St. e Karyotype analysis of ES-24. Wheat chromosome 3D were replaced by Th. intermedium chromosome 3St. f Karyotype analysis of ES-26. Wheat chromosome 2D were replaced by Th. intermedium chromosome 2St. g FISH analysis of Abbondanza. h FISH pattern comparisons of chromosome 2St, chromosome 3St, chromosome 5B and chromosome 2B between the six alien substitution lines and their parent lines Abbondanza, Xiaoyan784, and Zhong4. The telomeric region of

chromosome 5BS carrying a bright-green fluorescence signal was eliminated in ES-25. Chromosome 2B are metacentric in all the above-mentioned materials except ES-25.

Figure 5

Evaluation of agronomic traits and stripe rust resistance. a Adult plants; b seeds; c symptoms in response to inoculation with the mixture of Pst races at the adult stage; d seedling stage reactions to Pst races

CYR32. (1) Huixianhong; (2) Abbondanza; (3) ES-9; (4) ES-23; (5) ES-25; (6) ES-26; (7) ES-10; (8) ES-24; (9) Xiaoyan784; (10) Zhong4; (11) Thinopyrum ponticum; (12) Thinopyrum intermedium.

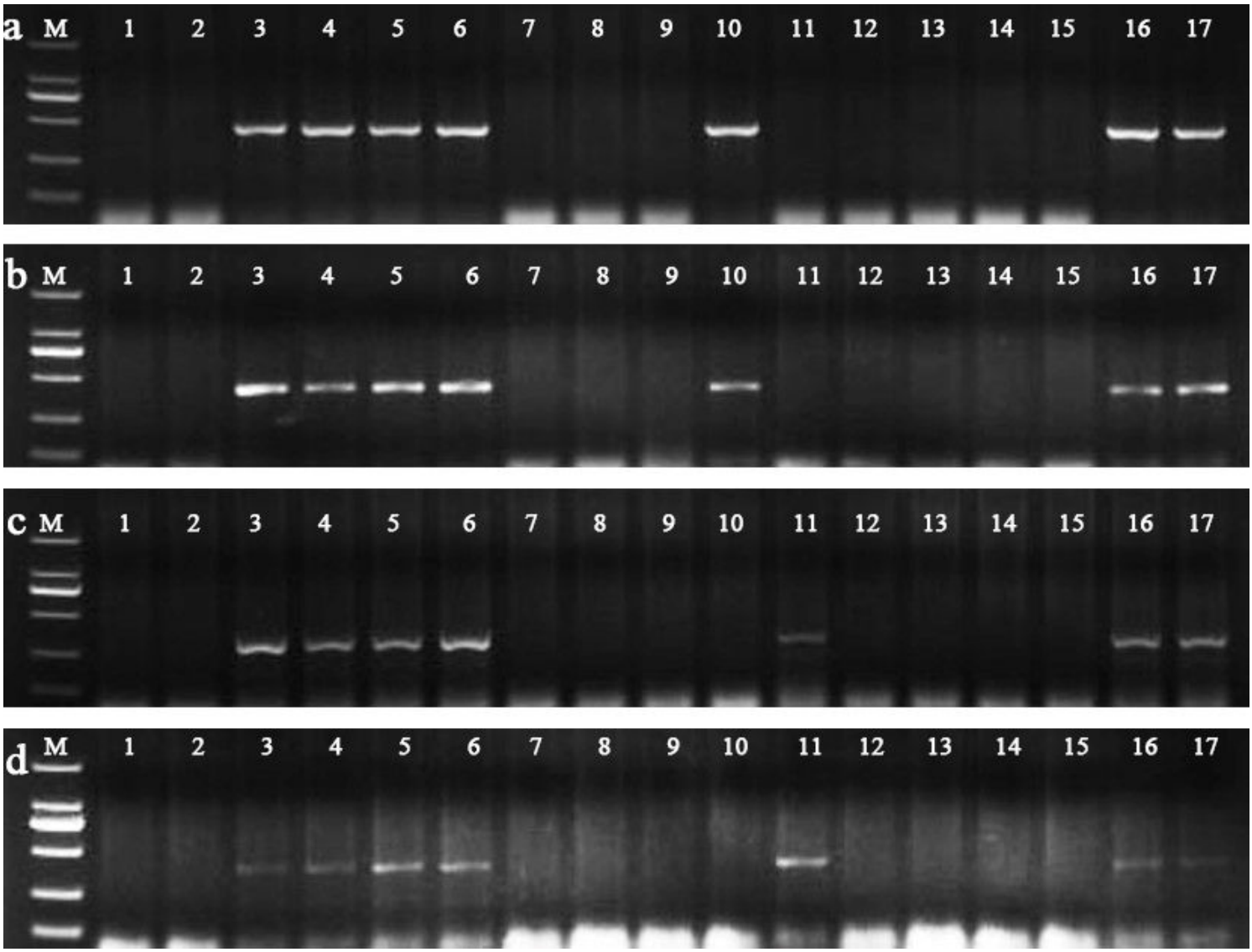

Figure 6

Specific amplification markers of chromosome 2St (a and b) and chromosome 3St (c and d). a PTH-005; b PTH-013; c PTH-113; d PTH-135. Lane M: DL2000; lane 1: Chinese Spring; lane 2: Abbondanza; lane 3: Thinopyrum ponticum; lane 4: Thinopyrum intermedium; lane 5: tetraploid Pseudoroegneria spicata; lane 6: diploid Pseudoroegneria spicata; lane 7: Thinopyrum bessarabicum; lane 8: Thinopyrum elongatum; lane 9-15: wheat- Th. intermedium disomic addition lines (DALs), DA1St, DA2St; DA3St; DA4St; DA5St; DA6St; DA7St; lane 16: ES-9 (a and b), ES-10 (c and d); lane 17: ES-23 (a and b), ES-24 (c and d).

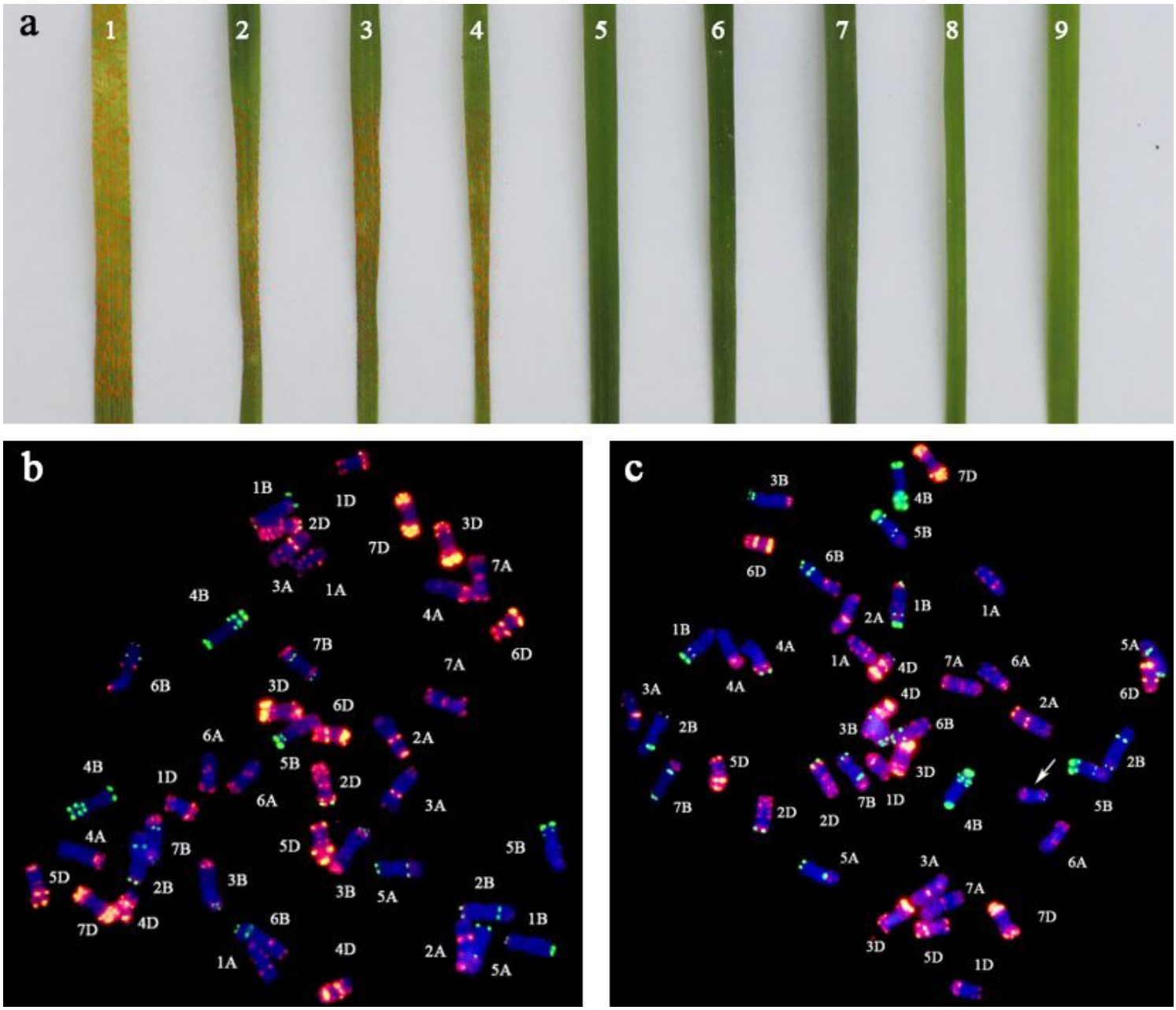

Figure 7

Stripe rust resistance evaluation and FISH analysis in BC1F2 individuals of ES-24 and Huixianhong. a Reactions to inoculation with the Pst race CYR32 of the BC1F2 individuals at the seedling stage; b FISH patterns of susceptible BC1F2 individuals; c FISH patterns of resistant BC1F2 individuals. (1) Huixianhong; (2)-(4) susceptible BC1F2 individuals; (5)-(7) resistant BC1F2 individuals; (8) Zhong4; (9) ES-24. The arrow indicates the chromosome 3St of Th. intermedium.

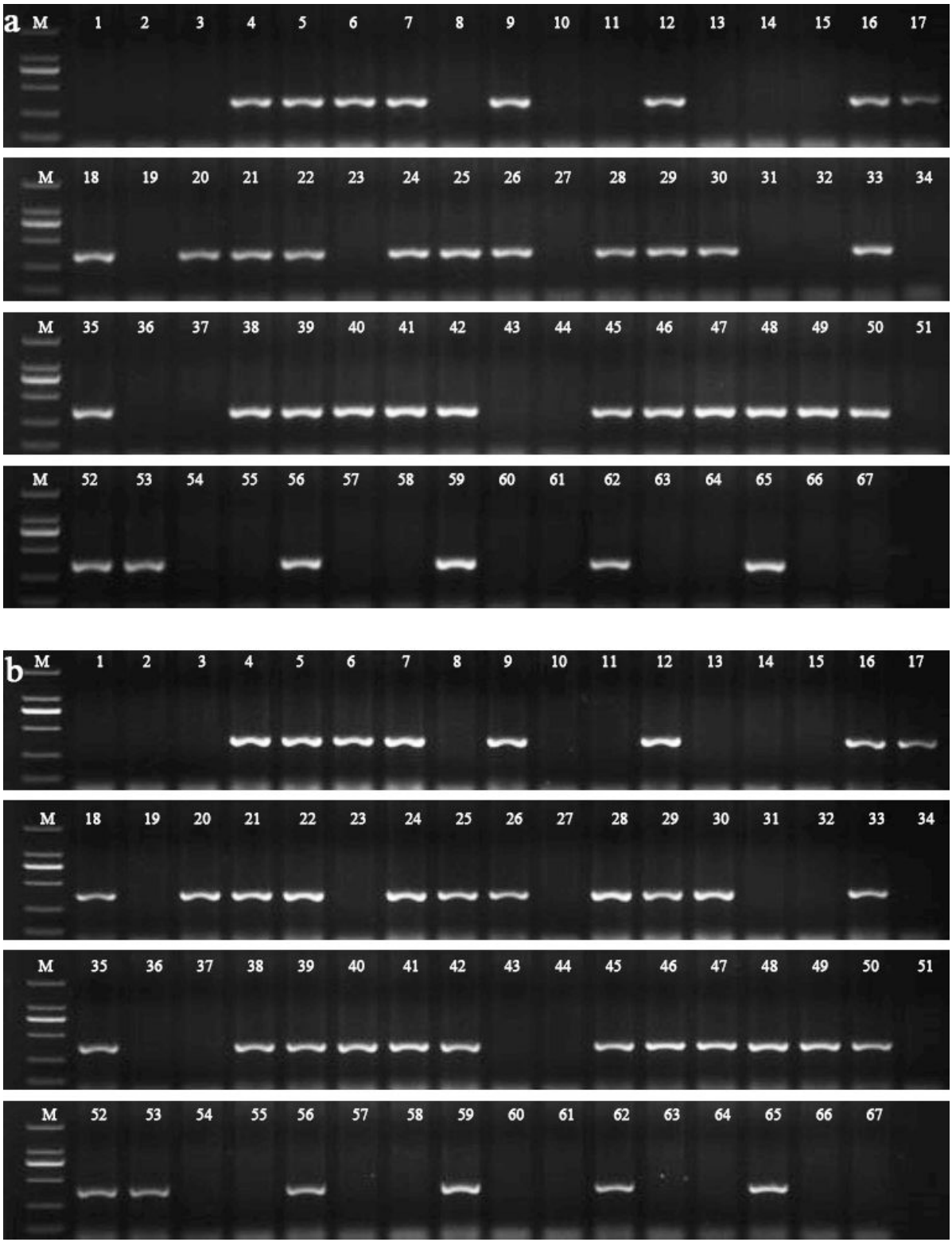

Figure 8

Utility of newly developed 3St-chromosome-specific markers in 60 BC1F2 individuals of ES-24 and Huixianhong (HXH). a PTH-113; b PTH-135. Lane M: DL2000; lane 1: Chinese Spring; lane 2: Abbondanza; lane 3: HXH; lane 4: Xiaoyan784 (wheat- Th. ponticum partial amphiploid with stripe rust); lane 5: Zhong4 (wheat- Th. intermedium partial amphiploid with stripe rust); lane 6: ES-10; lane 7:ES-24; lane 8-67: 60 BC1F2 individuals.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Thesupplementarytable1.docx
- Thesupplementarytable2.docx
- Thesupplementarytable3.docx