Molecular cytogenetics and St-chromosome-specific molecular markers development of novel stripe rust resistant wheat– *Thinopyrum intermedium* as well as wheat– *Thinopyrum ponticum* substitution lines

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Abstract

Background

Owing to the excellent resistance to abiotic and biotic stress, *Thinopyrum intermedium* ($2n = 6x = 42$, JJJ^sJ^sStSt) and *Thinopyrum ponticum* ($2n = 10x = 70$) 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, FISH karyotype construction and specific molecular marker development.

Results

Six wheat–*Thinopyrum* DSLs derived from crosses between Abbondanza nullisomic lines ($2n = 40$) and two octoploid *Trititrigia* lines ($2n = 8x = 56$), were 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 (DS2St (2A)) and ES-10 (DS3St (3D)) are wheat–*Th. ponticum* DSLs, while ES-23 (DS2St (2A)), ES-24 (DS3St (3D)), ES-25 (DS2St (2B)), and ES-26 (DS2St (2D)) are wheat–*Th. intermedium* DSLs. ES-9, ES-23, ES-25 and ES-26 conferred higher thousand-kernel weight and stripe rust resistance at adult stages, while ES-10 and ES-24 performed highly resistant to stripe rust at all stages. Furthermore, cytological analysis showed that the alien chromosomes (2St/3St) belonging to the same homoeologous group derived from different donors carried the same FISH karyotype and could normally form a bivalent. Based on specific-locus amplified fragment sequencing (SLAF-seq), two 2St-chromosome-specific markers (PTH-005 and PTH-013) and two 3St-chromosome-specific markers (PTH-113 and PTH-135) were developed.

Conclusions

The six wheat–*Thinopyrum* disomic substitution lines conferring stripe rust resistance will be used as bridging parents for valuable resistant genes transmission. And the utility of PTH-113 and PTH-135 in a BC1F2 population showed the newly developed markers could be useful tools for efficient identification of St chromosomes in a common wheat background.

Background

Intermediate wheatgrass (*Thinopyrum intermedium* Barkworth & D.R. Dewey, JJJ^sJ^sStSt, $2n = 6x = 42$) and tall wheatgrass (*Thinopyrum ponticum* (Podp.) Barkworth & D. R. Dewey, $2n = 10x = 70$) are important allopolyploids of *Thinopyrum* 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 [1, 2]. According to previous studies, the chromosomal composition of *Th. intermedium* and *Th. ponticum* have not been fully characterized. In terms of *Th. intermedium*, the chromosomal composition is generally regarded as JJJ^sJ^sStSt [3] or J^rJ^rJ^{vs}J^{vs}StSt [4]. The subgenome J or J^r is highly homologous with genome J (*Th. bessabicum*, J^b, E^b)/E (*Th. elongatum*, J^e, E^e) [5], and the main controversy has been whether the genome V originating from *Dasypyrum villosum* ($2n = 2x = 14$, VV) was involved in the recombinant subgenome J^s or not [6, 7]. Additionally, it was convinced that *Th. intermedium* contained a set of St chromosomes which probably derived from diploid *Pseudoroegneria spicata* ($2n = 2x = 14$, StSt) [8–10]. 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 [11, 12].

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 [13–18]. Octoploid *Trititrigia* with advantageous traits served as significant cytogenetic resources to develop alien introgression lines and be further applied to wheat breeding programs [19–23]. Generally, octoploid *Trititrigia* lines carry a synthetic genome inherited from *Th. intermedium* or *Th. ponticum*. Therefore, according to the defined genome compositions of partial amphiploids by molecular cytogenetic method, 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 [24]. Subsequently, molecular cytogenetic identification of TAF46 as well as the derived six addition lines revealed that the genome composition of TAF46 was 14A + 14B + 14D + 2(1J) + 2(2St) + 2(3J) + 2(4St) + 2(5J) + 2(6St) + 2(7J) [25–27], which suggested that chromosomes of St genome contained in *Th. intermedium* could be stably inherited, and 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 [28, 29]. 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 (DALs), L4 (DA4St) and L7 (DA6St), a DS1St (1D) with stripe rust resistance was produced [30]. Additionally, a DA3St [31] and a DA7St (Song et al. 2013) were

characterized, both carrying stripe rust resistant gene(s). However, at present no wheat– *Thinopyrum intermedium* 2St disomic substitution lines with stripe rust resistance 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, $2n = 8x = 56$). Molecular cytogenetic analysis was to determine and compare the genome composition of the six alien lines. In addition, stripe rust resistance and potential value of the morphological characteristics for wheat breeding was evaluated. Finally, St-chromosome-specific markers were developed and validated by specific-locus amplified fragment sequencing (SLAF-seq), which could be useful tools for efficient identification of St chromosomes in a common wheat background.

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 alien 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. Comparisons of 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 both on the long arms and short arms (Fig. 1, a2). Wheat chromosome 2A, chromosome 2B, as well as 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 detected 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).

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 shown 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 alien chromosomes of ES-24.

Wheat 15K SNP array analysis of the six substitution lines

The chromosomal composition of the six substitution lines were further determined based on genotype data by using a wheat 15K SNP array (Table S1). Generally, compared with *Th. ponticum* or *Th. intermedium*, the number of common SNP sequences detected between the substitution lines and Abbondanza was much higher. 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. It suggested that chromosome 2A in ES-9 were replaced by the alien chromosomes of *Th. ponticum*, 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 same SNP marker loci as *Th. ponticum* but few SNP marker loci compared with Abbondanza, which suggested that chromosome 3D of ES-10 were substituted by the pair of *Th. ponticum* chromosomes.

In terms of the wheat– *Th. intermedium* alien lines, the intersection point was detected in chromosome 3D of ES-24, and the most same SNP marker loci was obtained from the comparison between *Th. intermedium* and ES-24. It was meant that chromosome 3D of ES-24 was replaced by the pair of *Th. intermedium* chromosomes (Fig. 2d). The intersection point of ES-23, ES-25 and ES-26 was undoubtedly identified in chromosome 2A (Fig. 2c), chromosome 2B (Fig. 2e), as well as chromosome 2D (Fig. 2f), respectively. 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 validated the homoeologous groups for the alien chromosomes. There were four PLUG markers (*TNAC1142-HaeIII*, *TNAC1142-TaqI*, *TNAC1132-TaqI*, *TNAC1140-TaqI*) mapped to the second homoeologous group in ES-9, ES-23, ES-25 and ES-26 (Table S2; Fig. 3a-d). While three pairs of primers (*TNAC1326-HaeIII*, *TNAC1326-TaqI*, *TNAC1359-TaqI*) 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*. While 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.

Based on chromosomal composition analysis, the genome composition of ES-25 (Fig. 4d) was 14A + 12B + 14D + 2(2St), and ES-26 (Fig. 4f) was 14A + 14B + 12D + 2(2St). ES-23 and ES-9 were for the same genome composition of 12A + 14B+ 14D+ 2(2St), while ES-24 and ES-10 were for the same genome composition of 14A+ 14B+ 12D+ 2(3St). Remarkably, the alien chromosomes contained in ES-23 (Fig. 4b) and ES-24 (Fig. 4e) were derived from *Th. intermedium*, while the alien chromosomes carried by ES-9 (Fig. 4a) and ES-10 (Fig. 4c) were derived from *Th. ponticum*. Although the alien chromosomes belonging to the same homoeologous groups were derived from two different donors, identical FISH karyotypes of the alien chromosomes were respectively detected between ES-23 and ES-9 (2St), as well as ES-24 and ES-10 (3St).

Evaluation of agricultural performance and resistance to stripe rust 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 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 Abbondanza and 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.

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)

At the adult stage, stripe rust reaction test of the six substitution lines was carried out 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 chromosome 2St originating from *Th. ponticum* and *Th. intermedium* both conferred resistance to stripe rust at the adult stage, while chromosome 3St of *Th. ponticum* and *Th. intermedium* both carried remarkable resistance at whole stage.

Meiotic chromosome pairing analysis of F₁ hybrids

Crosses were made between the alien lines with the same genome compositions. There were 15 F₁ plants obtained from the cross between ES-9 and ES-23, and 11 F₁ plants obtained from the cross between ES-10 and ES-24. Meiotic chromosome pairing analysis of the F₁ hybrids was conducted to further validate the related genome constitution (Table 3). Mostly, the alien chromosomes derived from *Th. intermedium* and *Th. ponticum* but belonging to the same homoeologous group (2/3) could form a bivalent at metaphase I, and no trivalent or quadrivalent was observed at meiosis anaphase I. It was further revealed the close homoeologous relationship between the alien chromosomes derived from the two different donors.

Table 3 Chromosome pairing in the meiotic and meiotic phases for the hybrid F₁ 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

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 3). 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 the alien chromosomes of *Th. intermedium* and *Th. ponticum*.

According to the above sequence alignment results, 110 SLAFs from ES-23 regarded as 2St chromosome-specific fragments and 73 SLAFs from ES-24 regarded as 3St chromosome-specific fragments were 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. Totally, 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).

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

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

Utility of the 3St-chromosome-specific markers in BC₁F₂ population

In order to validate that the stripe rust resistance gene(s) at whole stage were carried by chromosome 3St, 60 BC₁F₂ individuals of ES-24 and HXH were used for a genetic analysis. The evaluation of stripe rust resistance at the seedling stage revealed that Zhong4, ES-24, and the 33 F₂ individuals were highly resistant to *Pst* race CYR32 (Fig 7a). Subsequently, 10 resistant F₂ individuals as well as 10 susceptible ones were randomly selected for FISH analysis. Compared with the FISH karyotype of ES-24, chromosome 3St were detected in all the resistant individuals (Fig 7c) and susceptible ones had undetectable FISH pattern of chromosome 3St (Fig 7b). It was shown that the novel stripe rust resistant gene(s) originated from the alien chromosome 3St of *Th. intermedium*.

Furthermore, the specificity of PTH-113 and PTH-135 was confirmed by PCR analyses of the 60 BC₁F₂ individuals (Fig 8). Combined with the result of seedling stage stripe rust resistance evaluation, Xiaoyan784, Zhong4, ES-9, ES-24, and the 33 BC₁F₂ plants conferring strong resistance to *Pst* race CYR32 also carried 3St chromosome-specific markers. Oppositely, the other 26 BC₁F₂ plants, the parental line Abbondanza and susceptible control HXH, without specific amplification, were seriously susceptible to *Pst* race CYR32. Hence, the new developed chromosome-specific molecular markers could be used to rapidly trace the alien 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 carried by wild related species have been successfully transferred to common wheat background by developing introgression lines [32-37]. Disomic substitution lines contained one pair of alien chromosomes with desirable resistant genes are vital bridge materials for small segments of introgression [38-40], which are valuable germplasm resources for wheat disease-resistant breeding. In the current study, six stable wheat–*Thinopyrum* derived lines were developed by nullisomic backcross method. Molecular cytogenetic analysis confirmed that ES-23 (DS2St (2A)), ES-24 (DS3St (3D)), ES-25 (DS2St (2B)) and ES-26 (DS2St (2D)) are wheat–*Th. intermedium* disomic substitution lines, while ES-9 (DS2St (2A)) and ES-10 (DS3St (3D)) are wheat–*Th. ponticum* disomic substitution lines. It was shown that the four alien lines, ES-9, ES-23, ES-25 and ES-26 carrying chromosome 2St conferred higher thousand-kernel weight and stripe rust resistance at adult stages, while ES-10 and ES-24 both containing chromosome 3St performed highly resistant to stripe rust at all stages. Therefore, all of the six substitution lines may 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 characterize the alien chromosomes [41, 42] and to detect genomic changes in specific regions [43-45]. In this study, after characterized by sequential FISH–GISH and mc-GISH analysis, specific karyotype patterns of chromosome 2St and chromosome 3St derived from *Th. intermedium* and *Th. ponticum* were elucidated, which is significant for rapidly identifying the alien chromosomes in germplasm materials. Furthermore, chromosomal structure variation happened in ES-25 following the process of distant hybridization was 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 were replaced by chromosome 2St of *Th. intermedium* (Fig 4h). Due to the dynamic and high frequent variable nature of subtelomeres of *Triticeae* species [46, 47], it is difficult to access the possible function(s) of the deleted regions of chromosome 5BS. Because of no severe effects on viability of ES-25, the subtelomeric region elimination presumably contributed to genome diversity [48].

The genomic composition of *Th. ponticum* and *Th. intermedium* has been an interesting subject for a considerable time [49, 50]. During the past several decades, it was convinced that the set of St chromosomes contained in *Th. intermedium* were probably derived from *P. spicata* [51], whereas it has been still undefined that whether the St genome is one of the sets of chromosomes of *Th. ponticum* [11, 52]. According to molecular cytogenetic identification results, ES-23 and ES-9 (group 1) contained the same genome composition of 12A + 14B + 14D + 2(2St), while ES-24 and ES-10 (group 2) were for the same genome composition of 14A + 14B + 12D + 2(3St). Although the alien chromosomes derived from the two different donors, *Th. intermedium* and *Th. ponticum*, identical alien chromosome FISH patterns and similar specific agricultural performances were identified in each group of the plant materials. It was implied that St chromosomes were included in *Th. ponticum*, and could be stably inherited with remarkable genes. Furthermore, combined with the close homoeologous relationships between the alien chromosomes analyzed by meiotic chromosome pairing and genomic polymorphism, it was 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. All up, based on the specific SLAFs obtained in this study, it was feasible for us to develop transferable St-chromosome-specific molecular markers from *Th. intermedium* to *Th. ponticum*.

Although FISH–GISH analysis has been widely utilized to precisely characterized wheat–*Th. intermedium* lines for several decades, it is severely 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 [53-55]. With the development of sequencing technology, the first consensus genetic map of *Th. intermedium* was developed by genotyping-by-sequencing [56]. Subsequently, 635 [9] and 745 [57] unique *Th. intermedium* SNP

markers have been successfully developed, totally including 225 St-chromosome-specific markers, with 27 of 2St-chromosome-specific markers and 25 of 3St-chromosome-specific markers. Due to the much more complex genomic composition of *Th. ponticum*, the molecular markers development work was mainly focused on genome E [52, 58, 59], especially in the following of the published complete genome of *Th. elongatum* [60]. In the present study, the wheat–*Th. intermedium* disomic substitution lines, ES-23(DS2St (2A)) and ES-24(DS3St (3D)) were sequenced by SLAF-seq for further St-chromosome-specific marker development. 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. FISH analysis of the BC₁F₂ population of ES-24 and HXH combined with stripe rust resistance test (Fig 7) was to confirm that 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 BC₁F₂ individuals indicated that the St-chromosome-specific molecular markers enabled to serve as useful tools for tracing chromosome 3St of *Th. intermedium* in common wheat background. In addition, according to the close genetic relationship between the alien chromosomes of *Th. ponticum* and *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. It suggested that the four St-chromosome-specific markers could also be utilized for rapidly detecting the St genome chromosomes of *Th. ponticum*. And the remarkable stripe rust resistance of ES-24 and ES-10 probably originated from the same gene(s), which need to be validated in future genetic analyses.

Conclusions

Four wheat–*Th. intermedium* and two wheat–*Th. ponticum* disomic substitution lines conferring stripe rust resistance were characterized and compared by molecular cytogenetic analysis, which will be used as bridging parents for valuable resistant genes transmission. Furthermore, according to the related homoeologous relationships, two 2St-chromosome-specific and two 3St-chromosome-specific molecular markers were developed by SLAF-seq for rapidly detecting the alien chromosomes of *Th. intermedium* and *Th. ponticum* in a common wheat background.

Materials And Methods

Plant materials

The plant materials include *Thinopyrum intermedium* ($2n = 6x = 42$, JJJ^sJ^sStSt), *Thinopyrum ponticum* ($2n = 10x = 70$), diploid *Pseudoroegneria spicata* ($2n = 2x = 14$, StSt), tetraploid *Pseudoroegneria spicata* ($2n = 4x = 28$, StStStSt), *Thinopyrum bessarabicum* ($2n = 2x = 14$, JJ), *Thinopyrum elongatum* ($2n = 2x = 14$, 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 F₁ hybrids obtained from two cross combinations, ES-9 × ES-23 (15 plants), and ES-10 × ES-24 (11 plants). The BC₁F₂ population comprising 60 individuals were derived from ES-24 and the wheat landrace Huixianhong (HXH). Five wheat–*Th. intermedium* DALs 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 [14] were used for *in situ* hybridization analyses. The protocols of genomic DNA extracting, sequential FISH–GISH and mc-GISH were conducted by Wang et al. [41]. 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. Percentage of the same genotypes in each chromosome between two materials was obtained by calculating the rate of the same genotype loci number in total number of markers. 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 [61].

Agronomic traits and stripe rust resistance 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 evaluate the adult plant resistance of Abbondanza, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, Xiaoyan784 and Zhong4, 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 BC₁F₂ population individuals of ES-24 and HXH. The infection type (IT) was scored with a scale of 0-4 [62].

To assess the morphological traits, ten plants of each material (Abbondanza, ES-9, ES-10, ES-23, ES-24, ES-25, ES-26, Xiaoyan784 and Zhong4,) 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 F₁ hybrids

Young spikes of F₁ hybrids derived from the two crosses combinations (ES-9×ES-23 and ES-10×ES-24) at appropriate 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 meiosis period were observed, recorded and photographed.

Genomic polymorphism analysis by pairwise comparisons

On the basis of SLAF-seq [63], 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 in two groups, ES-9 and ES-23 (group 1), as well as ES-10 and ES-24 (group 2). Firstly, all the SLAFs from the two groups were blasted with wheat genome, removing the sequences with high wheat homology (over 80%). Secondly, the remaining SLAFs were further blasted with the sequences of *Th. ponticum* or *Th. intermedium*. Then the SLAFs with high identity (over 90%) were remained, 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, intercomparisons within groups were conducted and the specific SLAFs with high identity (over 90%) were acquired.

Development and validation of the St-chromosome-specific markers

Based on the obtained specific SLAFs, PCR primers were designed for the amplification of the two groups of materials. 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 amplified specific sequences in tetraploid *P. spicata*, diploid *P. spicata*, *Th. ponticum*, *Th. intermedium*, 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 BC₁F₂ individuals of ES-24 and HXH for a further genetic analysis.

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 (Mg²⁺ 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.

Declarations

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Conflicts of interest

It should be understood that none of the authors have any financial or scientific conflicts of interest with regard to the research described in this manuscript.

Ethics approval

We hereby confirm that this manuscript is our original work and has not been published nor has it been submitted simultaneously elsewhere.

Consent to participate and consent for publication

We further confirm that all authors have checked the manuscript and have agreed to the submission.

Availability of data and material

The datasets used or materials during the current study are available from the corresponding author on reasonable request.

Code availability

The codes during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WJ and CW designed the project, SW performed the experiments and drafted the manuscript, JZ and PD provided help in analysis of wheat 15K SNP array, XF, HZ and XL provided help in analyzing the morphological characters, YW, TL and CC provided help in preparing materials, BW provided the *Pst* races.

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Supplementary Tables

Supplementary Table 1-1 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-9	No. of valid markers in <i>Th. ponticum</i>	No. of same markers (Abbondanza vs. ES-9)	Percentage of same markers (Abbondanza vs. ES-9)	No. of same markers (ES-9 vs. <i>Th. ponticum</i>)	Percentage of same markers (ES-9 vs. <i>Th. ponticum</i>)
1A	607	601	605	259	596	98.19%	87	14.33%
1B	670	661	659	303	641	95.67%	131	19.55%
1D	337	333	334	185	260	77.15%	95	28.19%
2A	907	893	284	327	125	13.78%	568	62.62%
2B	736	728	716	377	547	74.32%	142	19.29%
2D	597	579	576	328	549	91.96%	153	25.63%
3A	594	585	579	264	578	97.31%	84	14.14%
3B	997	986	985	391	973	97.59%	141	14.14%
3D	505	498	503	256	497	98.42%	92	18.22%
4A	766	762	759	285	754	98.43%	101	13.19%
4B	589	584	580	230	564	95.76%	96	16.30%
4D	248	246	246	155	244	98.39%	69	27.82%
5A	690	687	683	300	650	94.20%	116	16.81%
5B	763	755	756	355	714	93.58%	130	17.04%
5D	518	514	514	263	514	99.23%	134	25.87%
6A	463	456	456	201	453	97.84%	87	18.79%
6B	768	754	753	336	749	97.53%	133	17.32%
6D	404	400	400	179	397	98.27%	69	17.08%
7A	735	729	728	329	679	92.38%	115	15.65%
7B	640	635	634	265	623	97.34%	106	16.56%
7D	665	659	659	328	649	97.59%	108	16.24%
Total	13199	13045	12409	5916	11756	89.07%	2757	20.89%

Supplementary Table 1-2 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-10	No. of valid markers in <i>Th. ponticum</i>	No. of same markers (Abbondanza vs. ES-10)	Percentage of same markers (Abbondanza vs. ES-10)	No. of same markers (ES-10 vs. <i>Th. ponticum</i>)	Percentage of same markers (ES-10 vs. <i>Th. ponticum</i>)	
1A	607	601	603	259	522	86.00%	93	15.32%	
1B	670	661	665	303	527	78.66%	101	15.07%	
1D	337	333	334	185	261	77.45%	96	28.49%	
2A	907	893	849	327	682	75.19%	158	17.42%	
2B	736	728	728	377	656	89.13%	138	18.75%	
2D	597	579	577	328	527	88.27%	148	24.79%	
3A	594	585	582	264	382	64.31%	99	16.67%	
3B	997	986	981	391	933	93.58%	143	14.34%	
3D	505	498	159	256	62	12.28%	262	51.88%	
4A	766	762	759	285	468	61.10%	91	11.88%	
4B	589	584	584	230	317	53.82%	81	13.75%	
4D	248	246	245	155	228	91.94%	67	27.02%	
5A	690	687	685	300	619	89.71%	116	16.81%	
5B	763	755	753	355	715	93.71%	128	16.78%	
5D	518	514	517	263	354	68.34%	106	20.46%	
6A	463	456	457	201	406	87.69%	85	18.36%	
6B	768	754	754	336	615	80.08%	134	17.45%	
6D	404	400	402	179	374	92.57%	72	17.82%	
7A	735	729	728	329	538	73.20%	113	15.37%	
7B	640	635	635	265	407	63.59%	80	12.50%	
7D	665	659	660	328	545	81.95%	129	19.40%	
Total	13199	13045	12657	5916	10137	76.80%	2440	18.49%	

Supplementary Table 1-3 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-23	No. of valid markers in <i>Th. intermedium</i>	No. of same markers (Abbondanza vs. ES-23)	Percentage of same markers (Abbondanza vs. ES-23)	No. of same markers (ES-23 vs. <i>Th. intermedium</i>)	Percentage of same markers (ES-23 vs. <i>Th. intermedium</i>)
1A	607	601	570	235	313	51.57%	111	18.29%
1B	670	661	622	294	367	54.78%	128	19.10%
1D	337	333	321	174	257	76.26%	79	23.44%
2A	907	893	421	299	238	26.24%	526	57.99%
2B	736	728	712	343	650	88.32%	134	18.21%
2D	597	579	569	339	500	83.75%	162	27.14%
3A	594	585	582	246	492	82.83%	90	15.15%
3B	997	986	970	379	892	89.47%	156	15.65%
3D	505	498	484	267	275	54.46%	136	26.93%
4A	766	762	743	266	636	83.03%	104	13.58%
4B	589	584	550	219	322	54.67%	97	16.47%
4D	248	246	247	154	233	93.95%	66	26.61%
5A	690	687	659	298	566	82.03%	118	17.10%
5B	763	755	741	335	690	90.43%	117	15.33%
5D	518	514	487	276	342	66.02%	113	21.81%
6A	463	456	412	202	258	55.72%	106	22.89%
6B	768	754	714	311	342	44.53%	147	19.14%
6D	404	400	384	192	259	64.11%	105	25.99%
7A	735	729	725	313	663	90.20%	121	16.46%
7B	640	635	619	261	542	84.69%	100	15.63%
7D	665	659	619	336	341	51.28%	202	30.38%
Total	13199	13045	12151	5739	9169	69.47%	2918	22.11%

Supplementary Table 1-4 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-24	No. of valid markers in <i>Th. intermedium</i>	No. of same markers (Abbondanza vs. ES-24)	Percentage of same markers (Abbondanza vs. ES-24)	No. of same markers (ES-24 vs. <i>Th. intermedium</i>)	Percentage of same markers (ES-24 vs. <i>Th. intermedium</i>)
1A	607	601	603	235	580	95.55%	88	14.50%
1B	670	661	663	294	457	68.21%	130	19.40%
1D	337	333	331	174	287	85.16%	89	26.41%
2A	907	893	890	299	882	97.24%	137	15.10%
2B	736	728	732	343	696	94.57%	116	15.76%
2D	597	579	588	339	571	95.64%	165	27.64%
3A	594	585	584	246	544	91.58%	86	14.48%
3B	997	986	975	379	778	78.03%	153	15.35%
3D	505	498	174	267	66	13.07%	277	54.85%
4A	766	762	748	266	300	39.16%	118	15.40%
4B	589	584	582	219	567	96.26%	105	17.83%
4D	248	246	246	154	246	99.19%	67	27.02%
5A	690	687	680	298	616	89.28%	126	18.26%
5B	763	755	754	335	533	69.86%	118	15.47%
5D	518	514	513	276	476	91.89%	137	26.45%
6A	463	456	456	202	449	96.98%	86	18.57%
6B	768	754	753	311	742	96.61%	118	15.36%
6D	404	400	400	192	395	97.77%	85	21.04%
7A	735	729	726	313	633	86.12%	123	16.73%
7B	640	635	630	261	471	73.59%	103	16.09%
7D	665	659	665	336	332	49.92%	195	29.32%
Total	13199	13045	12693	5739	10621	80.47%	2622	19.87%

Supplementary Table 1-5 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-25	No. of valid markers in <i>Th. intermedium</i>	No. of same markers (Abbondanza vs. ES-25)	Percentage of same markers (Abbondanza vs. ES-25)	No. of same markers (ES-25 vs. <i>Th. intermedium</i>)	Percentage of same markers (ES-25 vs. <i>Th. intermedium</i>)
1A	607	601	605	235	584	96.21%	80	13.18%
1B	670	661	662	294	638	95.22%	125	18.66%
1D	337	333	332	174	272	80.71%	89	26.41%
2A	907	893	888	299	871	96.03%	144	15.88%
2B	736	728	251	343	102	13.86%	454	61.68%
2D	597	579	578	339	542	90.79%	169	28.31%
3A	594	585	589	246	582	97.98%	86	14.48%
3B	997	986	984	379	975	97.79%	153	15.35%
3D	505	498	502	267	483	95.64%	83	16.44%
4A	766	762	764	266	754	98.43%	104	13.58%
4B	589	584	583	219	558	94.74%	104	17.66%
4D	248	246	246	154	246	99.19%	69	27.82%
5A	690	687	683	298	676	97.97%	122	17.68%
5B	763	755	671	335	580	76.02%	159	20.84%
5D	518	514	512	276	501	96.72%	139	26.83%
6A	463	456	459	202	448	96.76%	84	18.14%
6B	768	754	762	311	493	64.19%	107	13.93%
6D	404	400	402	192	387	95.79%	84	20.79%
7A	735	729	732	313	711	96.73%	121	16.46%
7B	640	635	622	261	405	63.28%	105	16.41%
7D	665	659	658	336	376	56.54%	156	23.46%
Total	13199	13045	12485	5739	11184	84.73%	2737	20.74%

Supplementary Table 1-6 Genotype data of wheat 15K SNP arrays

Chromosome	No. of markers	No. of valid markers in Abbondanza	No. of valid markers in ES-26	No. of valid markers in <i>Th. intermedium</i>	No. of same markers (Abbondanza vs. ES-26)	Percentage of same markers (Abbondanza vs. ES-26)	No. of same markers (ES-26 vs. <i>Th. intermedium</i>)	Percentage of same markers (ES-26 vs. <i>Th. intermedium</i>)
1A	607	601	604	235	592	97.53%	82	13.51%
1B	670	661	658	294	638	95.22%	124	18.51%
1D	337	333	332	174	328	97.33%	90	26.71%
2A	907	893	886	299	863	95.15%	141	15.55%
2B	736	728	717	343	686	93.21%	125	16.98%
2D	597	579	236	339	114	19.10%	321	53.77%
3A	594	585	572	246	503	84.68%	86	14.48%
3B	997	986	987	379	964	96.69%	144	14.44%
3D	505	498	500	267	233	46.14%	161	31.88%
4A	766	762	763	266	746	97.39%	100	13.05%
4B	589	584	587	219	573	97.28%	102	17.32%
4D	248	246	246	154	229	92.34%	65	26.21%
5A	690	687	686	298	663	96.09%	123	17.83%
5B	763	755	755	335	662	86.76%	113	14.81%
5D	518	514	506	276	457	88.22%	129	24.90%
6A	463	456	460	202	214	46.22%	60	12.96%
6B	768	754	754	311	741	96.48%	120	15.63%
6D	404	400	400	192	185	45.79%	72	17.82%
7A	735	729	729	313	623	84.76%	125	17.01%
7B	640	635	634	261	306	47.81%	93	14.53%
7D	665	659	655	336	629	94.59%	122	18.35%
Total	13199	13045	12667	5739	10949	82.95%	2498	18.93%

Supplementary table 2 PLUG polymorphic markers mapped on homoeologous group 2 and 3 used to linkage analysis of *Thinopyrum ponticum* and *Thinopyrum intermedium* chromosome

Markers	Type	Primers (5'-3')	Location	Gel type/Restriction enzyme	Tm (°C)/ time of enzyme digestion (h)
<i>TNAC1142</i>	PLUG	F: GCCTACGAGTACATGGTCGAG R: CAGCATCCATAACCAGGATGT	2AL 2BL 2DL	1.5% agarose gel/ <i>TaqI</i> / <i>HaeI</i>	60/ 2 or 3
<i>TNAC1132</i>	PLUG	F: TATTGGTAGCCTTGTCGCTCT R: TATGCTGCATGTGCTATCGAC	2AL 2BL 2DL	1.5% agarose gel/ <i>TaqI</i>	60/ 2
<i>TNAC1140</i>	PLUG	F: TCCCAGAAATTACAAGGCTCA R: AGGAACCCTATGCATTGGAAA	2AL 2BL 2DL	1.5% agarose gel/ <i>TaqI</i>	60/ 2
<i>TNAC1326</i>	PLUG	F: ACAGATCGAGATGTTTATTGAAA R: GATCAAAGAGATGCGCTGAAG	3AS 3BS 3DS	1.5% agarose gel/ <i>TaqI</i> / <i>HaeI</i>	60/ 2 or 3
<i>TNAC1359</i>	PLUG	F: GTAAATAGCGCCATCTGCGTA R: CTCTGGATGCAGTTGGAATGT	3AL 3BL 3DL	1.5% agarose gel/ <i>TaqI</i>	60/ 2

Supplementary table 3 Quality of SLAF data

Genotype	Clean base (bp)	Clean reads	Q20(%)	Q30(%)	GC(%)
ES-9	1,277,894,774	6,401,833	97.16	91.82	47.77
ES-10	1,054,658,932	5,284,631	96.36	90.29	47.83
ES-23	1,906,494,968	9,555,684	97.06	91.55	47.76
ES-24	1,228,787,742	6,155,836	97.08	91.59	47.72
Abbondanza	1,297,710,674	6,495,947	96.31	90.13	47.65
<i>Thinopyrum ponticum</i>	1,520,267,864	7,613,789	96.84	90.98	47.45
<i>Thinopyrum intermedium</i>	2,038,483,374	10,205,879	97.10	91.50	46.77

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-HaeIII; b TNAC1142-TaqI; c TNAC1132-TaqI; d TNAC1140-TaqI; e TNAC1326-HaeIII; f TNAC1326-TaqI; g TNAC1359-TaqI. 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.