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Characterization of Chromosomal Rearrangement in New Wheat—*Thinopyrum intermedium* Addition Lines Carrying *Thinopyrum*—Specific Grain Hardness Genes

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Abstract: The wild species, *Thinopyrum intermedium*. (Genome StStJ^{S}J^{S}JJ), serves as a valuable germplasm resource providing novel genes for wheat improvement. In the current study, non-denaturing fluorescence in situ hybridization (ND-FISH) with multiple probes and comparative molecular markers were applied to characterize two wheat-*Th. intermedium* chromosome additions. Sequential ND-FISH with new labeled *Th. intermedium* specific oligo-probes were used to precisely determine the chromosomal constitution of *Th. intermedium*, wheat—*Th. intermedium* partial amphiploids and addition lines Hy36 and Hy37. The ND-FISH results showed that the added J^S-St translocated chromosomes in Hy36 had minor Oligo-5S ribosomal DNA (rDNA) signals at the short arm, while a pair of J-St chromosomes in Hy37 had major Oligo-pTa71 and minor Oligo-5S rDNA signals. The 90K SNP array and PCR-based molecular markers that mapped on wheat linkage group 5 and 3 facilitated the identification of *Thinopyrum* chromosome introgressions in the addition lines, and confirmed that added chromosomes in Hy36 and Hy37 were 5J^{S}S.3StS and 5JS.3StS, respectively. Complete coding sequences at the paralogous puroindoline-a (*Pina*) loci from *Th. intermedium* were cloned and localized on the short arm of chromosome 5J^S of Hy36. Line Hy36 showed a reduction in the hardness index, which suggested that *Th. intermedium*-specific *Pina* gene sequences may be associated with the softness trait in wheat background. The molecular cytogenetic identification of novel wheat—*Th. intermedium* derivatives indicated that the frequent chromosome rearrangement occurred in the progenies of wheat-*Thinopyrum* hybridization. The new wheat-*Thinopyrum* derived lines may increase the genetic diversity for wheat breeding.

Keywords: fluorescence in situ hybridization; grain hardness; *Thinopyrum intermedium*; wheat-alien introgression

1. Introduction

Thinopyrum intermedium (Host) Barkworth & D.R. Dewey, a hexaploid perennial species, is widely distributed in Europe, western Asia, and northern Africa [1]. The great geographical and climatic diversity of the native distribution has resulted in high variability and accumulated genetic rearrangement among *Th. intermedium* subspecies [2]. The relative ease of crossability between wheat and *Th. intermedium* has led to studies involving the transfer of valuable genetic traits from *Th. intermedium to* wheat [2,3]. Different types of wheat—*Th. intermedium* partial amphiploids have

been developed [4–8], and a number of wheat-*Th. intermedium* chromosome addition, substitution, and translocation lines were produced for the localization of novel gene(s) for disease resistances and agronomic traits in diversified wheat background [9–12]. The genome in situ hybridization (GISH) revealed that *Th. intermedium* consists of three genomes named J, J^S, and St, among which the St genome shows a high degree of similarity to that of *Pseudoroegneria strigosa* [2,4], while the J and J^S genomes appear to be related to the modified *Th. elongatum/ Th. bessarabicum/ Dasypyrum villosum* genomes [13–16]. So far, the complete set of *Th. intermedium* chromosomes involving seven linkage groups and three individual genomes still have not been transferred to a wheat background [12]. Thus continuous production and precise characterization of new wheat-*Th. intermedium* addition lines will be important for wheat breeding programs, including grain quality enhancement by chromosome engineering.

Grain endosperm texture responsible for hardness or softness is an important characteristic for wheat quality improvement, since the hard-textured grains require more grinding energy than soft-textured grains to reduce endosperm into flour [17]. The flours from hard wheat are preferred for yeast-leavened bread baking, while flours from soft wheat are preferred for manufacturing cookies and cakes [18]. Grain hardness is inherited and controlled primarily by the Hardness locus (*Ha*), and the *Puroindoline* genes in *Ha* locus were located in the extreme distal end of chromosome 5D in common wheat [17,18]. The *Puroindoline* homologous alleles and mutations from barley [19] and wheat related species including *Secale cereale* [20], *Dasypyrum villosum* [21] and *S. africanum* [22] have been introduced into common wheat for increasing the genetic diversity of the wheat hardness locus. The identification of novel *Puroindoline* alleles from *Thinopyrum* species will be useful in improving the wheat quality for end-use products and better understanding *Puroindoline* gene function.

The objectives of the present study were to characterize new wheat-*Th. intermedium* chromosome addition lines by fluorescence in situ hybridization (FISH) and molecular markers, and to target the *Puroindoline* gene sequences on specific *Th. intermedium* chromosomes. The chromosomal rearrangement of *Th. intermedium* derived lines and new *Th. intermedium* specific markers are potentially useful in wheat quality improvement.

2. Materials and Methods

2.1. Plant Materials

Th. intermedium accession PI440043 (StJ^SJ genomes, 2n = 6x = 42) was obtained from the National Small Grains Collection at Aberdeen, Idaho, USA. Wheat-*Th. intermedium* addition lines Z series, the octoploid wheat and *Th. intermedium* partial amphiploid Zhong 5 and Zhong 2 [23] were provided by Dr. Zhijian Chang of Shanxi Academy of Agricultural Sciences, China. The wheat—*Th. intermedium* addition lines L series originated from TAF46 were from National Plant Germplasm System (NPGS), United States Department of Agriculture (USDA). Two wheat Chinese Spring (CS)—*Th. intermedium* addition named Hy36 and Hy37 were selected from a BC_1F_6 generation of the wheat CS crosses to Zhang 5 and Zhong 2, respectively.

2.2. Fluorescence In Situ Hybridization (FISH)

Seedling root tips of Hy36 and Hy37 and their parents were collected and treated with nitrous oxide followed by enzyme digestion, using the procedure of Han et al. [24]. The synthesized oligo-nucleotide probes Oligo-pSc119.2, Oligo-pTa535, Oligo-pTa71 and Oligo-$(GAA)_7$ were used for identifying the wheat chromosomes according to the description of Tang et al. [25]. Probe Oligo-pSt122 is specific for terminal regions of *Th. intermedium* chromosomes [26]. Oligo-probe Oligo-5S ribosomal DNA (rDNA) (TCAGAACTCC GAAGTTAAGC GTGCTTGGGC GAGAGTAGTAC) for specifically hybridized 5S rDNA region was based on the sequence of pTa794 probe [27]. A new oligo probe named Oligo-pDb12H (TCAGAATTTT TAGG ATAGCA GAAGTATTCG AAATACCCAG ATTGCTACAG) was labeled for replacing the long terminal repeat (LTR) probe pDb12H [28] to clearly

distinguish the J^S chromosomes in *Th. intermedium* [14]. The *Thinopyrum* specific probe Oligo-B11 (TCCGCTCACC TTGATGACAA CATCAGGTGG AATTCCGTTC GAGGG) was developed from a *Thinopyrum*-specific LTR sequence B11 [29]. Oligonucleotide probes were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The synthetic oligonucleotides were either 5′ end-labelled with 6-carboxyfluorescein (6-FAM) for green or 6-carboxytetramethyl-rhodamine (Tamra) for red signals. The protocol of non-denaturing FISH (ND-FISH) using the synthesized probes was described by Fu et al. [30]. Photomicrographs of FISH signals for chromosomes were taken with an Olympus BX-51 microscope equipped with a DP-70 CCD camera.

2.3. 90K SNP Arrays Analysis

Plants from lines CS, Hy36 and Hy37 were grown in a greenhouse at 23 °C under 12-h photoperiod. At the two-leaf stage, the leaves were used for the isolation of genomic DNA by CTAB methods [30]. The DNA of plants was subjected to genome-wide scanning using an Illumina wheat 90K iSelect SNP array (containing 81,587 SNPs) using a combination of GenomeStudio v2011.1, as described [31]. All of the genotyping experiments were performed by CapitalBio Corporation (Beijing, China). SNP genotype calling and clustering were performed as described by Yuan et al. [32].

2.4. Molecular Marker Analysis

DNA was extracted from the young leaves of CS, Hy36, Hy37, *Th. intermedium*, and Zhong 5, using a sodium dodecyl sulfate (SDS) protocol [14]. PCR-based Landmark Unique Gene (PLUG) primers [33] and CINAU (Cytogenetics Institute, Nanjing Agricultural University, Nanjing, China) markers [34] were designed from rice genomic DNA sequences specific for the wheat A, B, and D syntenic regions. Polymerase chain reaction (PCR) was performed in an Icycler Thermal Cycler (Bio-Rad Laboratories, Emeryville, CA, USA) in a 25 μ: reaction, containing 10 mmol Tris-HCl (pH 8.3), 2.5 mmol $MgCl_2$, 200 μmol of each dNTP, 100 ng template DNA, 0.2 U Taq polymerase (Takara, Japan), and 400 nmol of each primer. The cycling parameters were 94 °C for 3 min for denaturation, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified products were restriction enzyme-digested and electrophoresis was conducted as described by Li et al. [26]. The markers for chromosomal physical location were searched using the wheat genome database The International Wheat Genome Sequencing Consortium hole genome assembly (IWGSC) WGA v0.4 from https://urgi.versailles.inra.fr/blast/, and confirmed by PCR.

2.5. Cloning and Sequencing of the Pina Gene

Primers for *Pina* (Pina-F and Pina-R) and PCR protocol followed the reports of Li et al. [21]. The target genes identified by PCR were excised from 1.0% agarose gels, and purified using a gel extraction kit (Qiagen, Valencia, CA, USA). The purified products were ligated into the pT7 Blue R-Vector (Novagen, Madision, WI, USA) using T4 DNA ligase, and then introduced into *Escherichia coli* DH5α using heat shock transformation. Nucleotide sequencing was performed on a polyacrylamide gel with the ABI prism 377 sequencer (Perkin Elmer, Waltham, MA, USA) as an automated fluorescent sequencing system. The nucleotide sequences based on two identically independent sequences of each clone were assembled, and the sequence alignment among different alleles and neighbor-joining (NJ) phylogenetic tree construction was carried out using BIOEDIT software v7.0.5 (http://www.mbio.ncsu.edu/Bioedit) [35].

2.6. Agronomic Traits Survey and Grain Hardness Tests

Field trials were performed by each plot contained three 2 m rows spaced 20 cm apart with three replicates at the Xindu Experimental Station, Chengdu, China during the 2016–2018 wheat growing seasons. The protein, wet gluten content, test weight, water absorption, and Zeleny sedimentation value of whole grains were determined using the near-infrared spectroscopy DA7250 (Perten, Sweden), according to the approved methods at Northwest A & F University, China. The hardness of grains

was determined by a Perten Single Kernel Characterization System (SKCS), and the classification of hardness types referred to the report of Li et al. [21].

3. Results

3.1. FISH of Th. Intermedium by Multiple Oligo Probes

Sequential FISH analyses of metaphase cell of *Th. intermedium* accession PI440043 were done by using multiple probes including Oligo-B11, Oligo-pSc119.2, Oligo-pTa535, Oligo-pTa71 [25], Oligo-pSt122 [12], Oligo-3A1 [36], Oligo-pDb12H, and Oligo-5S rDNA. The new Oligo-pDb12H was used to distinguish the J^S genome, instead of the conventional LTR probe pDb12H [14,28]. As shown in Figure 1a, the Oligo-pDb12H was able to clearly classify the 14 J^S chromosomes of *Th. intermedium*, while the full abundant signals by Oligo-B11 were St-chromosomes, and the mostly telomeric and sub-telomeric signals by Oligo-B11 were J-chromosomes. Thus, the probes Oligo-B11 and Oligo-pDb12H can be used to distinguish the *Th. intermedium* chromosomes into three groups, each with 14 chromosomes, by using ND-FISH (Figure 1a). The sequential ND-FISH demonstrated that a total of 32 *Th. intermedium* chromosomes had clear signals of the probes Oligo-pSc119.2 and Oligo-pTa535 (Figure 1b), and probes Oligo-5SrDNA, Oligo-3A1 (Figure 1c), Oligo-pTa71, and Oligo-pSt122 signals (Figure 1d) were observed in 12, four, six, and 24 *Th. intermedium* chromosomes, respectively. The ND-FISH patterns from the above probes were used to construct the *Th. intermedium* karyotypes (Figure 1e). The probes Oligo-pTa71 and Oligo-5SrDNA representing 18S-5.8-45S rDNA and 5S rDNA were mostly localized in the chromosomes of groups 1 and 5. Based on the different arm ratios between the two groups of chromosomes, they were presumably designated as 1J, 5J, $5J^S$, and 1St (Figure 1).

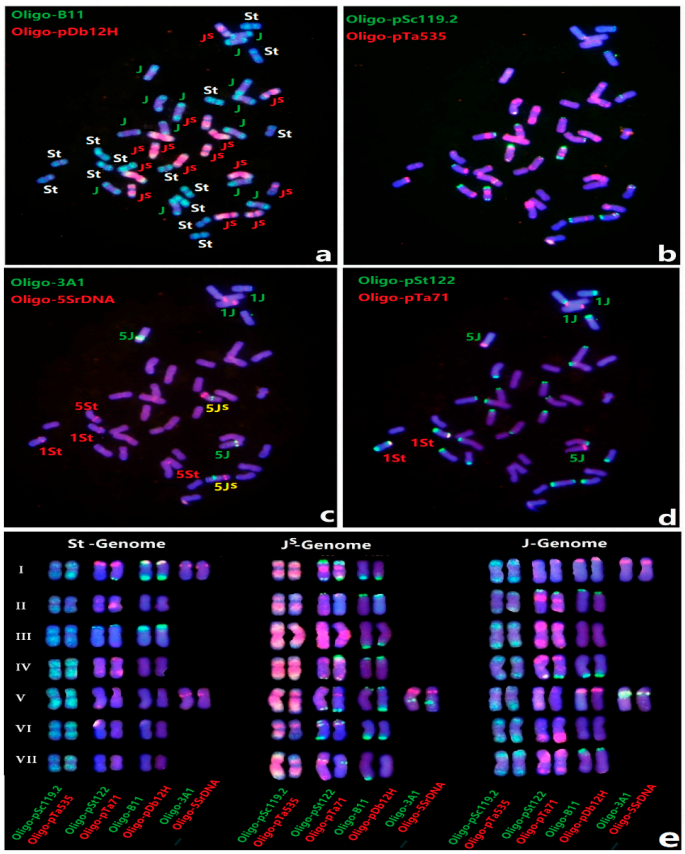

Figure 1. Sequential ND-FISH of *Thinopyrum intermedium* (accession PI440043), each carrying 14 J, Js, and St chromosomes, using Oligo-B11 (green) + Oligo-pDb12H (red) (**a**), Oligo-pSc119.2 (green) + Oligo-pTa535 (red) (**b**), Oligo-3A1(green) + Oligo-5S rDNA (red) (green) (**c**), and Oligo-pTa71 (red) + Oligo-pSt122 (**d**) as probes, respectively.

3.2. An Updated FISH Karyotype of Zhong 5 and Zhong 2

Cytological analysis of wheat- *Th. intermedium* partial amphiploid Zhong 5 with 2n = 56 were identified by sequential ND-FISH using probes Oligo-B11, Oligo-pDb12H, Oligo-pSc119.2, Oligo-pTa535, Oligo-pTa71, Oligo-3A1, and Oligo-$(GAA)_7$ (Figure 2). The 14 chromosomes showed hybridization signals of probe Oligo-B11, and eight of 14 chromosomes had the signals of Oligo-pDb12H including two chromosomes with the signal of the entire arms, and six chromosomes with the signal only on one arm (Figure 2a). It indicated that a total of 14 *Th. intermedium* chromosomes, including six St, two J^S and six St-J^S translocations, were observed in Zhong 5. Based on the comparison of current FISH patterns of Zhong 5 to CS by the probes Oligo-pSc119.2 and Oligo-pTa535 [25], we found that 39 of the 42 wheat chromosomes from Zhong 5 had normal FISH patterns, except that a strong Oligo-pSc119.2 signal appeared on the end of the chromosome 1AL, and a clear Oligo-pSc119.2 signal on chromosome 2AS, as well as a distinct Oligo-pTa535 signal added on chromosome 3AS (Figure 2b). The probe Oligo-pTa71 hybridized to the nucleolus regions of both wheat and *Thinopyrum* chromosomes. The chromosomes 1B and 6B and two *Th. intermedium* chromosomes of Zhong 5 displayed hybridization signals by Oligo-pTa71 (Figure 2c). The FISH were conducted in the Zhong 5 derived addition lines Z1 to Z6, by using Oligo-pSc119.2, Oligo-pTa535 and Oligo-pSt122. The chromosomes 1St in Z3, 7J^S in Z4 and Z6, 2St.J^S translocation in Z5 were defined, respectively (Figure 2e). The remaining three pairs of *Thinopyrum* chromosomes in Zhong 5 were designated as E, C, and A with unidentified linkage group (Figure 2e). Similarly, the sequential ND-FISH with above eight Oligo probes were applied to identify the chromosome constitution of *Th. intermedium* chromosomes in Zhong 2, with 54 chromosomes (Figure S1). The karyotype of FISH patterns of six pairs of *Th. intermedium* chromosomes of Zhong 2 were shown in Figure 2f. A pair of J^S chromosomes with the terminal region on the short arm contained strong signals from Oligo-pSc119.2 and Oligo-pSt122, which reassembled the 4J^S chromosome [12]. An undetermined J^S chromosome was designated as F, and the remaining four pairs of chromosomes were consistent to those from Zhong 5. Therefore, Zhong 2 contained six J^S, two St, and four St-J^S chromosomes of *Th. intermedium* in the wheat background (Figure 2f).

3.3. ND-FISH of Lines Hy36 and Hy37

The mitotic metaphase cells of wheat-*Th. intermedium* derived lines Hy36 and Hy37 were identified by using ND-FISH with the probes Oligo-B11 and Oligo-pDb12H. Both Hy36 and Hy37 contained 44 chromosomes including a pair of *Th. intermedium* chromosomes, and only the short arm of the alien chromosome in Hy36 has Oligo-pDb12H hybridization signals (Figure 3a,e). Compared to the length (8.0 μm) of wheat chromosome 3B, the averaged relative length of *Thinopyrum* chromosomes in Hy36 and Hy37 were 5.0 μm and 4.6 μm, respectively. The short arm of the *Th. intermedium* chromosomes in Hy37 was a short satellite with clear nucleolus regions. FISH using probes Oligo-pSc119.2 and Oligo-pTa535 showed that the added *Th. intermedium* chromosomes in both Hy36 and Hy37 have weak Oligo-pSc119.2 signals on the long arm (Figure 3b,f). The FISH signals from Oligo-5SrDNA were observed in the interstitial regions of the short arms of *Th. intermedium* chromosomes in both Hy36 and Hy37 (Figure 3c,g). The alien chromosome in Hy37 has strong Oligo-3A1 hybridization signals on the short arm closed to centromere (Figure 3g). The sequential ND-FISH with Oligo-$(GAA)_7$ revealed that the added *Th. intermedium* chromosomes of Hy36 had strong hybridization signals on the pericentromeric region on the short arm (Figure 3d). Oligo-pTa71 had the FISH signals on the alien chromosome of Hy37 (Figure 3h). The ND-FISH patterns of the added *Th. intermedium* chromosomes in Hy36 and Hy37 were indicated in Figure 3i,j, respectively. It is likely that the short arm of chromosome C contributed to the long arm of alien chromosome in both Hy36 and Hy37, while the short arm of alien chromosome in Hy37 were originated from the short arm of chromosome E in Zhong 5, and the short arm of alien chromosome in Hy36 originated from short arm of chromosome F in Zhong 2. Therefore, the sequential ND-FISH with multiple probes can be used to trace the chromosome transmission or variation during the processes of hybridization and selection.

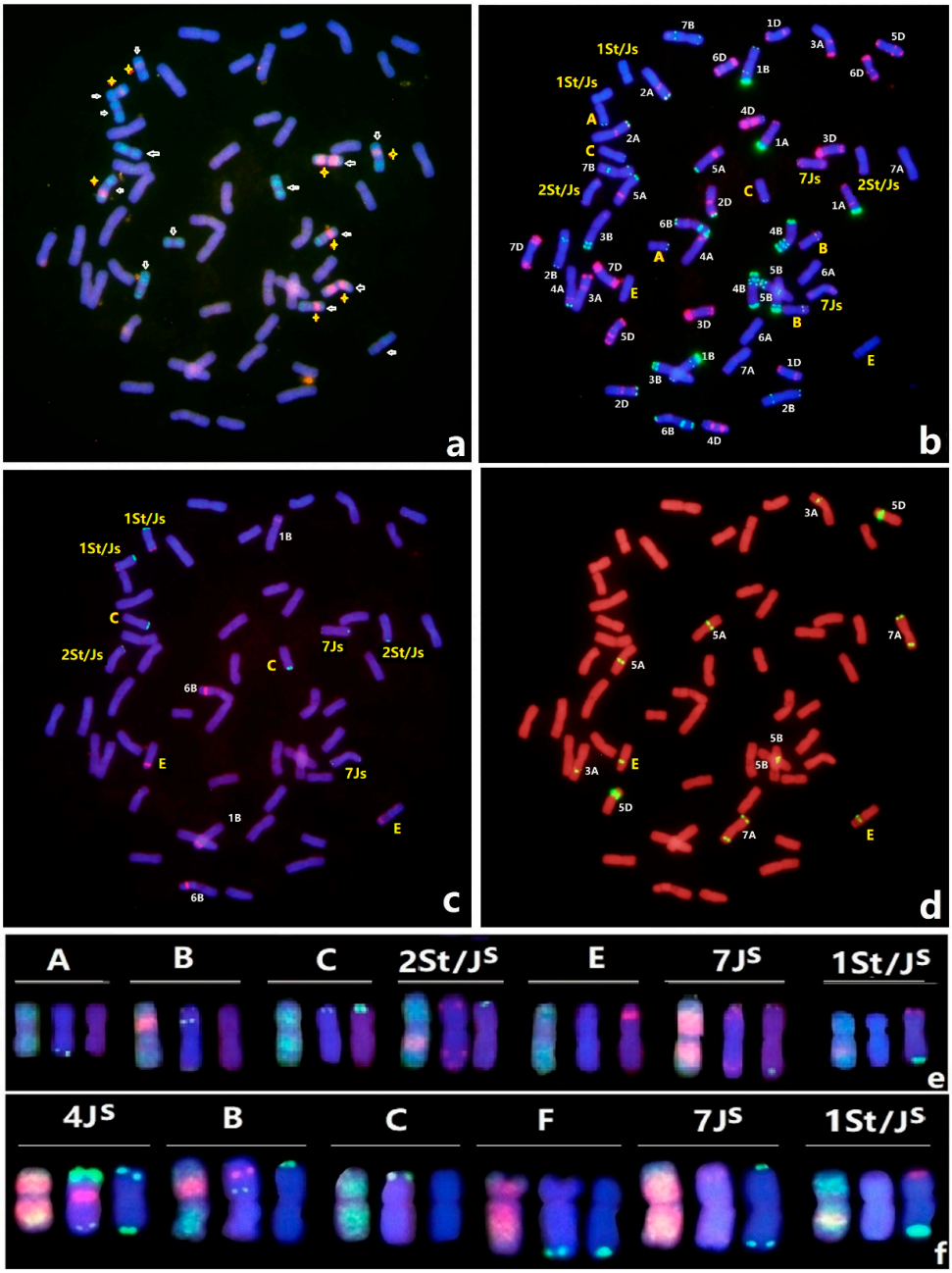

Figure 2. Sequential ND-FISH of Zhong 5 and Zhong 2 by multiple probes. The probes Oligo-B11 (green) + Oligo-pDb12H (red) (**a**), Oligo-pSc119.2 (green) + Oligo-pTa535 (red) (**b**), Oligo-pTa71 (red) + Oligo-pSt122 (green) (**c**), and Oligo-3A1 (green) (**d**) were used. The karyotype of non-wheat chromosomes in Zhong 5 (**e**) and Zhong 2 (**f**) as shown by ND-FISH, with probes of Oligo-B11 (green) + Oligo-pDb12H (red) for left chromosomes, Oligo-pSc119.2 (green) and Oligo-pTa535 (red) for middle chromosomes, Oligo-$(GAA)_7$ (red) + Oligo-pSt122 (green) and pTa71 (red) + Oligo-pSt122 (green) for right chromosomes, respectively.

3.4. Molecular Markers Validation of Linkage Groups

The 90K iSelect SNP array was performed to detect the polymorphisms among CS, Hy36, and Hy37. A total of 7470 SNP markers showed the polymorphism among CS, Hy36, and Hy37. Most of markers (90.89%) from Hy36 and Hy37 were identical to those from CS, implying the two lines were closely related to CS. The 32.4% categorized heterozygous SNPs from both Hy36 and Hy37 were

distributed on the chromosomes 3D, 5A, 5B, and 5D. These highly heterozygous SNPs may be resulted from the homologous groups 3 and 5 of *Thinopyrum* chromosomes added in Hy36 and Hy37.

We analyzed the amplification patterns of the DNAs from Zhong5, Zhong 2, Hy36, Hy37, and CS, respectively, by using 350 PLUG markers [33] which were located on seven wheat homoeologous groups and 890 CINAU markers [34] derived from all *D. villosum* chromosomes (Figure 4). The 51 markers from the short arm of group 5, and the 44 markers from the short arm of group 3 generated *Th. intermedium*- specific bands in Hy36 and Hy37, respectively. As shown in Figure 4a, the *Th. intermedium*- specific bands in Hy36 were identical to Zhong 5, and those in Hy37 were identical to Zhong 2. Among 51 markers located on short arm group 5, a total of 34 and 22 markers produced *Th. intermedium*-specific polymorphic bands in Hy36 and Hy37, respectively (Figure 4b). The results showed that these two group 5 *Th. intermedium* chromosomes were genetically different between Hy36 and Hy37. A total of 30 and 28 of 44 markers located on short arm of group 3 produced *Th. intermedium*-specific polymorphic bands in Hy36 and Hy37, respectively (Figure 4b). The FISH results and molecular marker surveys demonstrated that the *Th. intermedium* chromosomes in Hy36 and Hy37 were 5J^{S}S.3StS and 5JS.3StS, respectively.

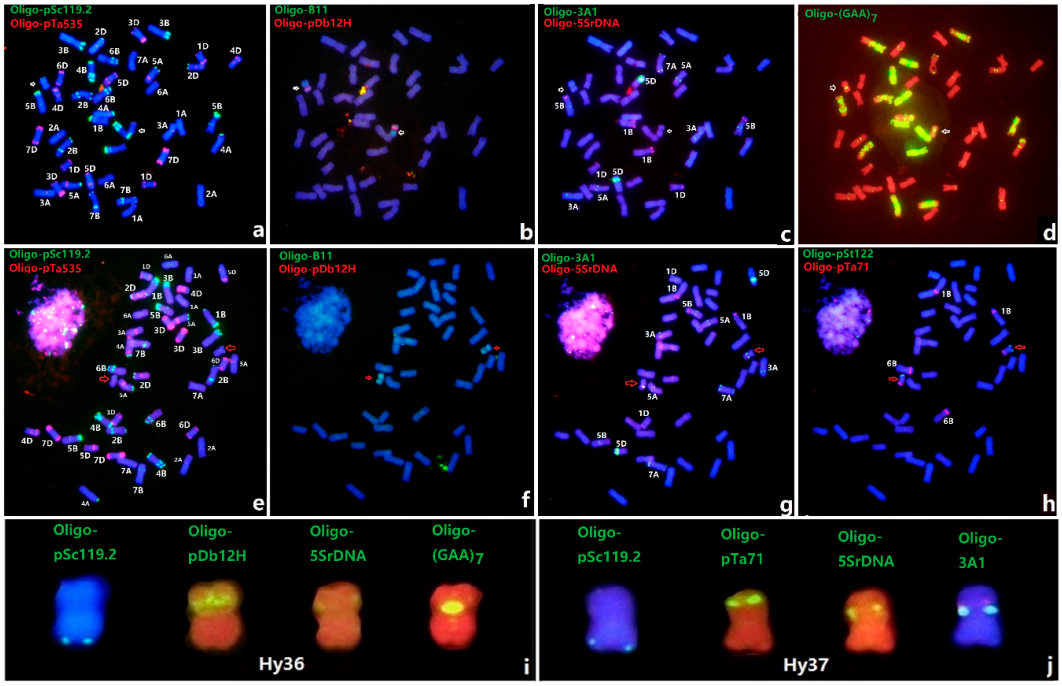

Figure 3. Sequential FISH of Hy36 and Hy37. The probes Oligo-pSc119.2 (green) + Oligo-pTa535 (red) (**a**,**e**), Oligo-B11 (green) + Oligo-pDb12H (red) (**b**,**f**) Oligo-5S rDNA (red) + Oligo-3A1 (green) (**c**,**g**), Oligo-(GAA)$_7$ (**d**), and Oligo-pSt122 (green) + Oligo-pTa71(red) (**h**) were used. The ND-FISH karyotype of *Th. intermedium* chromosomes in Hy36 (**i**) and Hy37 (**j**) were indicated. Arrows showed the *Th. intermedium* chromosomes.

3.5. Molecular Cloning of Th. Intermedium—Specific Pina-like Genes

The primer pair Pina-F/Pina-R for targeting *puroindoline* alleles were used to amplify the DNA of *Th. intermedium*, Hy36, Hy37 and *D. villosum*. The amplicon of about 500 bp for *Pina* gene was cloned and sequenced. A total of 20 clones from *Th. intermedium*, 25 each from Hy36 and Hy37, and ten from *D. villosum* were sequenced. Two clones from Hy36 (Genbank numbers: MH745807 and MH745808) were different from the reported wheat *Pina* sequences. All clones from Hy37 were identical to the registered wheat *Pina* gene sequences. Four redundant *Th. intermedium* and three *D. villosum* sequences were also obtained, respectively. The conserved tryptophan-rich region (WRWWKWWK) of the predicted PINA protein sequences was located between cysteine residues 3 and 4, and the substitution

from glutamine to arginine at position 69 (K65R) was identified from *Th. intermedium* PINA for the first time (Figure S2). The *Pina* sequences from wheat, *Secale* species, and the present *Th. intermedium* and *D. villosum* were used to construct a phylogenetic tree. As shown in Figure 5, the *Pina* gene sequences from *Secale* were first separated, and *Pina* genes of wheat were categorized as the second subgroup. The *Pina* sequences from *D. villosum*, *Th. intermedium*, and the Hy36 were clustered as the third subgroup. The results supported that the genome of *D. villosum* was genetically close to *Th. intermedium* genomes.

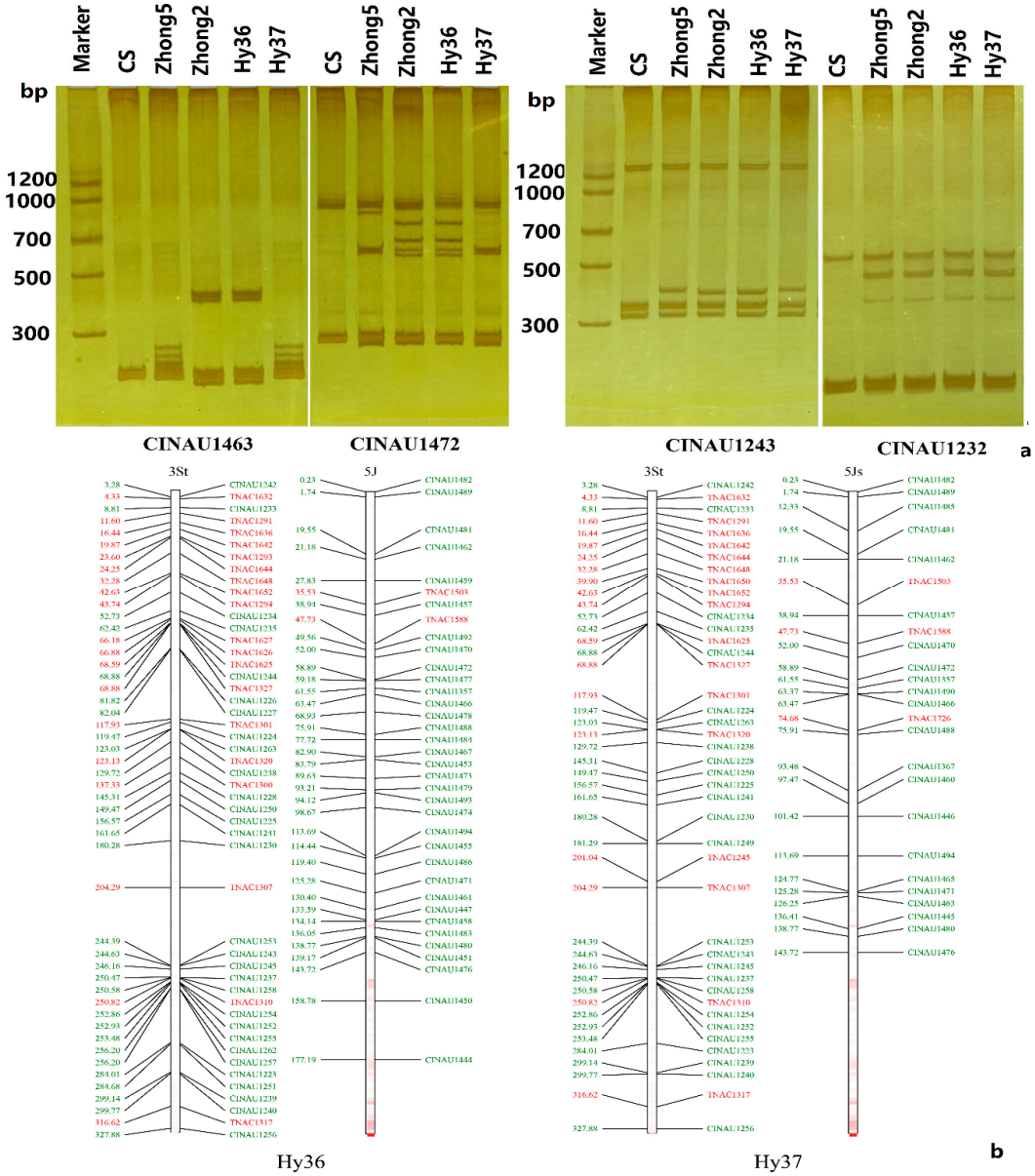

Figure 4. PCR profiling (**a**) and physical localization (**b**) of *Th. intermedium*-polymorphic markers in wheat chromosomes 5B and 3B by a BLAST search the reference genomic sequences version 1.0 at https://urgi.versailles.inra.fr/blast/. The red colors in the chromosomes (**b**) indicate the location of centromeric specific repeats.

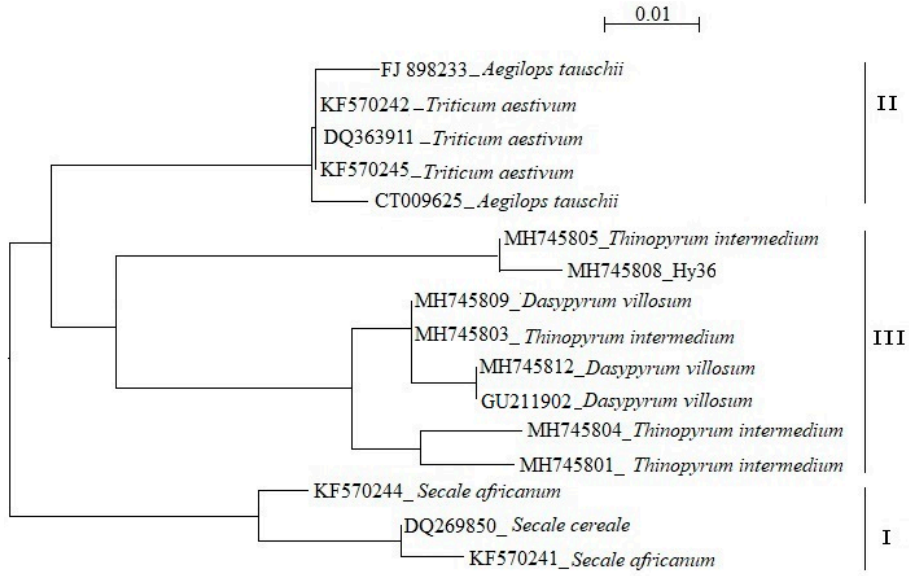

Figure 5. A phylogenetic tree generated from the nucleotide sequence alignment of the *Pina* genes by neighbor joining method by 1000 bootstrap replicates. Scale bar 0.01 = 1% difference among nucleotide sequences.

3.6. Agronomic Traits and Grain Quality Observation

The plants Hy36, Hy37, CS, and Zhong 5 were grown under field conditions, and their agronomic traits and grain quality were measured (Table 1, Figure S3). The Hy36 line showed a higher number of spikelet per spike, longer grain length, but a shorter spike length compared to those of Hy37. The plant height and the tiller per plant of Hy36 and Hy37 were significantly reduced compared to CS. The kernel protein contents, wet gluten content, and Zeleny value of Hy36 and Hy37 were significantly increased, in comparison with those in wheat parent CS, showing the end-product quality improved. Hy36 became soft, according to the SKCS hardness index, comparing to Hy37 and the recurrent parent CS, to an extent of 17.4%. The results imply that the addition of *Th. intermedium* chromosome $5J^S$ in Hy36 may reduce the hardness. The increase of protein and gluten contents in Hy36 and Hy37 proved that the introgression of *Th. intermedium* group 5 and 3 chromatin may be associated with grain end-use quality characteristics.

Table 1. Agronomic and grain quality traits of wheat- *Th. intermedium* lines and the wheat parent CS.

Agronomic and Quality Traits	CS	Hy36	Hy37
Plant height (cm)	142 ± 2.65	118.91 ± 6.34 **	111.08 ± 8.99 **
Tillers per plant	28 ± 1.00	20.09 ± 7.44 *	10.75 ± 7.11 **
Spike length (cm)	9.93 ± 0.12	10.54 ± 1.36	11.72 ± 0.96 *
Spikelet number	26.33 ± 0.58	23.10 ± 1.55	21.83 ± 1.47
Grain length (cm)	5.93 ± 0.07	6.61 ± 0.11 *	5.92 ± 0.07
Grain width	2.7 ± 0.04	2.92 ± 0.12	3.00 ± 0.06
Thousand-kernel weight (g)	25.30 ± 0.68	25.97 ± 0.47	24.87 ± 0.39
Protein content (%)	11.86 ± 0.28	14.80 ± 0.42 *	14.51 ± 0.36 *
Test weight (g/L)	732.10 ± 12.00	706.20 ± 6.08 *	741.00 ± 10.50
Wet gluten content (%)	29.58 ± 0.85	36.41 ± 0.70 *	36.25 ± 1.22 *
Stable time (min)	2.5 ± 0.28	4.08 ± 0.30 *	5.60 ± 0.56 *
Grain hardness	55.0 ± 3.28	47.8 ± 2.16 *	56.0 ± 3.11
Zeleny sedimentation value (mL)	36.8 ± 4.08	50.98 ± 2.08 **	44.47 ± 2.77 *
Milling yield (%)	62.9 ± 3.25	50.92 ± 2.20 *	59.10 ± 4.80

*, ** showed significant difference at $p < 0.05$, and 0.01 to their wheat parents, respectively.

4. Discussion

The ND-FISH based chromosome painting technique with the labelled oligonucleotide (oligo) is widely applied, due to obvious advantages over conventional FISH procedures, such as easy probes synthesis, flexible design, relatively low cost, and high-throughput [25,30,36–39]. This technique has been successfully used for distinguishing different genomes, such as Oligo-1162 for *Secale cereale* [30] and Oligo-B and Oligo-D for wheat B- and D-chromosomes [39], respectively. GISH by using *D. villosum* genomic DNA as a probe [13,15], and FISH using *Dasypyrum* specific LTR pDb12H probe can distinguish the J^S chromosomes of *Th. intermedium* genome [12,14,40]. In the present study, the hybridization patterns of ND-FISH using probe Oligo-pDb12H can substitute the conventional FISH with the pDb12H probe. All 14 J^S-chromosomes were clearly visualized by ND-FISH with Oligo-pDb12H in *Th. intermedium* (Figure 1). Another new LTR probe B11 [29] was also converted to the Oligo-probe, which was helpful for detecting wheatgrass fragments in common wheat by using ND-FISH. Our results on the constitution of alien chromosomes in wheat-*Th. intermedium* partial amphiploid Zhong 5 (Figure 2) are identical to the St-genome DNA-based GISH and FISH [5,36,41]. Taking advantage of a combination of Oligo-pDb12H and Oligo-B11 for multicolor ND-FISH, we can effectively distinguish St, J^S, and J-chromosomes of *Th. intermedium*, and precisely trace the specific *Th. intermedium* chromosome segments in large numbers of wheat-*Th. intermedium* hybridization progenies.

By using GISH with St genomic DNA as a probe, Tang et al. [42] observed that Zhong 5 has three pairs of St chromosomes, two pairs of J^S chromosomes, and two pairs of St-J^S reciprocal translocation chromosomes. Chen et al. [41] reported that Zhong 5 consists of four St and two J^S chromosomes, plus two J^S-W translocations, and six St and J^S or J translocated chromosomes. Han et al. [43] demonstrated that Zhong 5 contains 42 wheat chromosomes plus 14 *Th. intermedium* chromosomes by multi-color GISH. In the present study, we observed that the composition of *Th. intermedium* chromosomes in Zhong 5 is six St-chromosomes, two J^S-chromosomes, and six St/J^S translocated chromosomes. Recently, Lang et al. [36] found that Zhong 5 contains two pairs of J^S-W translocations, which were confirmed as T3DS-3AS.3AL-7J^SS and T3AL-7J^SS.7J^SL translocations. Consequently, Zhong 5 may have different kinds of karyotypes. Among them, one has normal 7J^S, and the other has translocation between 7J^S and 3A. The above 7J^S -wheat chromosomes in Zhong 5 were transmitted to wheat—*Th. intermedium* addition line Z4 [36]. The relatively high variation of *Th. intermedium* chromosome constitution in Zhong 2 was also observed [41,43]. The karyotypic variation found in the wheat-*Thinopyrum* partial amphiploids is due to the high frequency of paired wheat and *Th. intermedium* chromosomal rearrangement during the period of maintenance by successive self-selection [5–7,44–46].

The localization of rDNA in the wheat and Triticeae species was observed by cytological methods [47]. The probes pTa794, a 420 bp sequence of wheat encoding the 5S rRNA gene [27], and pTa71, a 9 kb sequence that encodes 18S-5.8S-26S rRNA genes [48] are commonly used for FISH analyses. The 45S rDNA sites preferentially localize on the chromosomes of homoeologous groups 1, 5, and 6 [49], while the 5S rDNA sites appear on the short arms of groups 1 and 5 chromosomes [50]. Li et al. [51] reported that six or eight major pTa71 signals of rDNA loci are detected in 42 chromosomes of three *Th. intermedium* accessions, with two or three pairs located in terminal parts, one pair of loci located interstitially. Mahelka et al. [15] used FISH with pTa794 probe to reveal that three or four pairs of major loci were detected in all accessions of *Th. intermedium*. In the present study, we used the Oligo-5SrDNA representing pTa794 probe for ND-FISH, and revealed that the signals of Oligo-5S rDNA were presented on the ends of two pairs of St-chromosomes, one pair of J^S- chromosome and two pairs of J-chromosomes in *Th. intermedium* accession (Figure 1). We suggested that a pair of major 5SrDNA loci in J^S chromosomes were probably lost during the evolution of the hexaploid species of *Th. intermedium*. Moreover, the FISH signals of Oligo-3A1 were located on 5AL, 5BL and 5DS [36]. In the present study, we found the hybridization signals of Oligo-3A1 on 5JS and 5J^SL (Figure 1). The varied distribution patterns of FISH hybridization signals of Oligo-pTa71, Oligo-5SrDNA and Oligo-3A1 sites among different wheat and *Th. intermedium* chromosomes' arms (Figures 1 and 2) indicate the

complexity of chromosome rearrangement during the evolutionary process of both allopolyploid wheat and *Th. intermedium* species.

Chromosome rearrangement is commonly observed in wheat- *Th. intermedium* derivatives. Different St–J^S or J-St genome chromosomes as either Robertsonian, intercalary, or terminal interchanges are observed in several wheat—*Th. intermedium* partial amphiploids and addition lines [2]. The wheat-*Th. intermedium* addition lines Yi 4212, HG295, Z1-Z6 originated from Zhong 5 have extensively rearranged *Th. interemedium* chromosomes [10,36,41]. We previously observed non-Roberstanian St–J^S translocations among homologous group 4 chromosomes in wheat—*Th. intermedium* substitution lines X479 and X482, by GISH–FISH and molecular marker analysis [52]. The present study also showed that the lines Hy36 and Hy37 contained the translocation between chromosomes $5J^S$, 5J with 3St, respectively. It is likely that St-genome is closely related to J or J^S genomes than between J and J^S, which is resulted in the high frequency occurrences of St–J and St–J^S chromosome translocation [2,51].

Kernel texture controlled by the hardness (Ha) locus including *Puroindoline* genes in wheat has long been recognized as a highly heritable trait [53]. The hardness loci, including *Pina* and *Pinb* genes are reported in species of genera such as *Triticum*, *Aegilops*, *Hordeum*, *Secale*, and *Avena* [17,54]. These genes were not detected in species of other Poaceae subfamilies, such as rice, maize, or sorghum [53]. The sequence variation of the *Pina* locus constitutes an excellent model for studying the mechanisms of evolution in the tribe Triticeae [54,55]. In the present study, the phylogenetic analysis indicates that the distinct evolutionary patterns of *Pina* genes occurred among the different Triticeae genomes (Figure 5). The *Pina* gene sequences from *Th. intermedium* showed great variation, possibly due to hexaploid genome or the cross pollination which resulted in heterozygosity of genes in the *Th. intermedium* accession. The *Puroindoline* genes were localized on the group 5 chromosomes of all Triticeae diploids. However, the *Puroindoline* genes have been deleted from chromosomes 5A and 5B, and only a gene was found on the short arm of chromosome 5D of common wheat [54]. In the present study, we found that the chromosome $5J^S$ in Hy36 contained the *Th. intermedium* specific *Pina* gene sequences, however, chromosome 5J in Hy37 had no *Pina* genes. The evolutionary dynamics of *Pina* genes along with the chromosome rearrangements in polyploid Triticeae species including *Th. intermedium* genomes needs to be further investigated. Moreover, the *hordoindolines* on chromosome 5HS of *Hordeum chilense* plays a role in reducing grain hardness in a wheat genetic background [19]. The *Dasypyrum villosum* derived *Pina* genes are associated with soft grain texture [22]. Our previous studies indicated that the introduction of *Pina* gene from *S. africanum* chromosome $5R^{afr}S$ to wheat also clearly reduced the grain hardness [21]. Gazza et al [56] also reported that wheat—*Th. intermedium* introgression lines inherited PINA proteins from *Th. intermedium*, and produced medium-hard kernels. Therefore, it is reasonable to find that Hy36 with $5J^S$S.3StS chromosome displayed soft grain texture, with an approximately 17% reduction in the SKCS hardness index compared with that of its parent CS and Hy37 with 5JS.3StS chromosome. Furthermore, the *Puroindoline* genes have been proven to have a broad range of antibacterial and antifungal activities, the effects of *Pina* genes on wheat rust are also recognized [57]. Further studies will be conducted to develop different types of wheat $5J^S$ translocation lines by chromosome manipulation, and to reveal the expression between *Th. intermedium* derived genes and wheat native genes for better quality and elite plant defense characteristics. As shown in Table 1, Hy36 and Hy37 showed shorter plant height and tillers per plant for tight plant architectures, which may overcome the defects of the wheat line Chinese Spring, for direct yield improvement [58]. Moreover, Hy36 and Hy37 displayed higher grain wet gluten content, stable time, and sedimentation value, which are in favor of dough processing quality. The deviation of morphology and grain quality between Hy36 and Hy37 were mostly due to the different chromosome arms of 5Js and 5J, and their interaction with wheat background. It is convincingly shown that the wheat—*Th. intermedium* hybrids are useful for future wheat breeding for yields, end-use quality, market classification, and economic benefits [3,11,12].

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/1/18/s1, Figure S1: ND-FISH of Zhong 2 by multiple Oligo probes. Figure S2: Alignment of PINA genes sequences among wheat, *Thinopytum intermedium* and *Dasypyrum villosum*; Figure S3: The spikes (a) and grains (b) of the lines CS, Zhong 5, Hy36 and Hy37.

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References

1. Dewey, D.R. The genomic system of classification as a guide to intergeneric hybridization in the perennial Triticeae. In *Gene Manipulation in Plant Improvement*; Gustafson, J.P., Ed.; Plenum: New York, NY, USA, 1984; pp. 209–279. [CrossRef]
2. Chen, Q. Detection of alien chromatin introgression from *Thinopyrum* into wheat using S genomic DNA as a probe—A landmark approach for *Thinopyrum* genome research. *Cytogenet. Genome Res.* **2005**, *109*, 350–359. [CrossRef] [PubMed]
3. Li, H.; Wang, X. *Thinopyrum ponticum* and the promising source of resistance to fungal and viral diseases of wheat. *J. Genet. Genom.* **2009**, *36*, 557–565. [CrossRef]
4. Cauderon, Y.; Saigne, B.; Dauge, M. The resistance to wheat rusts of *Agropyron intermedium* and its use in wheat improvement. In Proceedings of the fourth International Wheat Genetics Symposium, Columbia, MI, USA, 6–11 August 1973; Sears, E.R., Sears, L.M.S., Eds.; University of Missouri: Columbia, MI, USA, 1973; pp. 401–407.
5. Chen, Q.; Conner, R.L.; Laroche, A.; Ji, W.; Armstrong, K.C.; Fedak, G. Genomic *in situ* hybridization analysis of *Thinopyrum* chromatin in a wheat—*Th. intermedium* partial amphiploid and six derived chromosome addition lines. *Genome* **1999**, *42*, 1217–1223. [CrossRef] [PubMed]
6. Yang, Z.J.; Li, G.R.; Chang, Z.J.; Zhou, J.P.; Ren, Z.L. Characterization of a partial amphiploid between *Triticum aestivum* cv. Chinese Spring and *Thinopyrum intermedium* ssp. *trichophorum*. *Euphytica* **2006**, *149*, 11–17. [CrossRef]
7. Bao, Y.G.; Wu, X.; Zhang, C.; Li, X.F.; He, F.; Qi, X.L.; Wang, H.G. Chromosomal constitutions and reactions to powdery mildew and stripe rust of four novel wheat—*Thinopyrum intermedium* partial amphiploids. *J. Genet. Genom.* **2014**, *12*, 663–666. [CrossRef] [PubMed]
8. Cui, Y.; Zhang, Y.; Qi, J.; Wang, H.; Wang, R.C.C.; Bao, Y.; Li, X. Identification of chromosomes in *Thinopyrum intermedium* and wheat *Th. intermedium* amphiploids based on multiplex oligonucleotide probes. *Genome* **2018**, *61*, 515–521. [CrossRef] [PubMed]
9. Larkin, P.J.; Banks, P.M.; Lagudah, E.S.; Apple, R.; Chen, X.; Xin, Z.Y.; Ohm, H.W.; McIntosh, R.A. Disomic *Thinopyrum intermedium* addition lines in wheat with barley yellow dwarf virus resistance and with rust resistances. *Genome* **1995**, *38*, 385–394. [CrossRef] [PubMed]
10. Han, F.; Liu, B.; Fedak, G.; Liu, Z. Genomic constitution and variation in five partial amphiploids of wheat-*Thinopyrum intermedium* as revealed by GISH, multicolor GISH and seed storage protein analysis. *Theor. Appl. Genet.* **2004**, *109*, 1070–1076. [CrossRef]
11. Wang, Y.; Wang, H. Characterization of three novel wheat-*Thinopyrum intermedium* addition lines with novel storage protein subunits and resistance to both powdery mildew and stripe rust. *J. Genet. Genom.* **2016**, *43*, 45–48. [CrossRef]
12. Li, J.; Lang, T.; Li, B.; Yu, Z.; Wang, H.; Li, G.; Yang, E.; Yang, Z. Introduction of *Thinopyrum intermedium* ssp. *trichophorum* chromosomes to wheat by trigeneric hybridization involving *Triticum*, *Secale* and *Thinopyrum* genera. *Planta* **2017**, *245*, 1121–1135. [CrossRef]
13. Kishii, M.; Wang, R.R.C.; Tsujimoto, H. GISH analysis revealed new aspect of genomic constitution of *Thinopyrum intermedium*. *Czech. J. Genet. Plan.* **2005**, *41*, 91–95. [CrossRef]

14. Liu, C.; Yang, Z.J.; Jia, J.Q.; Li, G.R.; Zhou, J.P.; Ren, Z.L. Genomic distribution of a long terminal repeat (LTR) Sabrina-like retrotransposon in Triticeae species. *Cereal. Res. Commun.* **2009**, *37*, 363–372. [CrossRef]
15. Mahelka, V.; Kopecky, D.; Baum, B.R. Contrasting patterns of evolution of 45S and 5S rDNA families uncover new aspects in the genome constitution of the agronomically important grass *Thinopyrum intermedium* (Triticeae). *Mol. Biol. Evol.* **2013**, *30*, 2065–2086. [CrossRef] [PubMed]
16. Mahelka, V.; Kopecky, D.; Pastova, L. On the genome constitution and evolution of intermediate wheatgrass (*Thinopyrum intermedium*, Poaceae, Triticeae). *BMC Evol. Biol.* **2011**, *11*, 127. [CrossRef] [PubMed]
17. Morris, C.F. Puroindolines: The molecular genetic basis of wheat grain hardness. *Plant Mol. Biol.* **2002**, *48*, 633–647. [CrossRef] [PubMed]
18. Turner, M.; Mukai, Y.; Leroy, P.; Charef, B.; Appels, R.; Rahman, S. The Ha locus of wheat: Identification of a polymorphic region for tracing grain hardness in crosses. *Genome* **1999**, *42*, 1242–1250. [CrossRef] [PubMed]
19. Yanaka, M.; Takata, K.; Terasawa, Y.; Ikeda, T.M. Chromosome 5H of *Hordeum* species involved in reduction in grain hardness in wheat genetic background. *Theor. Appl. Genet.* **2011**, *123*, 1013–1018. [CrossRef]
20. Escobar, J.S.; Scornavacca, C.; Cenci, A.; Guilhaumon, C.; Santoni, S.; Douzery, E.J.; Ranwez, V.; Glémin, S.; David, J. Multigenic phylogeny and analysis of tree incongruences in Triticeae (Poaceae). *BMC Evol. Biol.* **2011**, *11*, 181. [CrossRef]
21. Li, G.; Gao, D.; La, S.; Wang, H.; Li, J.; He, W.; Yang, E.; Yang, Z. Characterization of wheat-*Secale africanum* chromosome 5Ra derivatives carrying *Secale* specific genes for grain hardness. *Planta* **2016**, *243*, 1203–1212. [CrossRef]
22. Zhang, R.Q.; Cao, Y.P.; Wang, X.E.; Feng, Y.G.; Chen, P.D. Development and characterization of a *Triticum aestivum – H. villosa* T5VS·5DL translocation line with soft grain texture. *J. Cereal. Sci.* **2010**, *51*, 220–225. [CrossRef]
23. Sun, S.C. The approach and methods of breeding new varieties and new species from Agrotriticum hybrids. *Acta Agron. Sin.* **1981**, *7*, 51–58. (In Chinese)
24. Han, F.P.; Lamb, J.C.; Birchler, J.A. High frequency of centromere inactivation resulting in stable dicentric chromosomes of maize. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3238–3243. [CrossRef] [PubMed]
25. Tang, Z.; Yang, Z.; Fu, S. Oligonucleotides replacing the roles of repetitive sequences pAs1.; pSc119.2.; pTa-535.; pTa71.; CCS1.; and pAWRC.1 for FISH analysis. *J. Appl. Genet.* **2014**, *55*, 313–318. [CrossRef] [PubMed]
26. Li, G.; Wang, H.; Lang, T.; Li, J.; La, S.; Yang, E.; Yang, Z. New molecular markers and cytogenetic probes enable chromosome identification of wheat-*Thinopyrum intermedium* introgression lines for improving grain quality. *Planta* **2016**, *244*, 865–876. [CrossRef] [PubMed]
27. Gerlach, W.L.; Dyer, T.A. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. *Nucleic Acids Res.* **1980**, *8*, 4851–4865. [CrossRef]
28. Yang, Z.J.; Liu, C.; Feng, J.; Li, G.R.; Zhou, J.P.; Deng, K.J.; Ren, Z.L. Studies on genomic relationship and specific marker of *Dasypyrum breviaristatum* in Triticeae. *Hereditas* **2006**, *143*, 47–54. [CrossRef] [PubMed]
29. Yao, H.; Tang, C.G.; Zhao, J.; Zheng, Q.; Li, B.; Hao, C.Y.; Li, Z.S.; Zhang, X.Y. Isolation of *Thinopyrum ponticum* genome specific repetitive sequences and their application for effective detection of alien segments in wheat. *Sci. Agric. Sin.* **2016**, *49*, 3683–3693. [CrossRef]
30. Fu, S.; Chen, L.; Wang, Y.; Li, M.; Yang, Z.; Qiu, L.; Yan, B.; Ren, Z.; Tang, Z. Oligonucleotide probes for ND-FISH analysis to identify rye and wheat chromosomes. *Sci. Rep.* **2015**, *5*, 10552. [CrossRef]
31. Wang, S.; Wong, D.; Forrest, K.; Allen, A.; Chao, S.; Huang, B.E.; Maccaferri, M.; Salvi, S.; Milner, S.G.; Cattivelli, L.; et al. Characterization of polyploid wheat genomic diversity using the high-density 90.;000 SNP array. *Plant Biotechnol. J.* **2014**, *12*, 787–796. [CrossRef]
32. Yuan, F.; Zeng, Q.; Wu, J.; Wang, Q.; Yang, Z.; Liang, B.; Kang, Z.; Chen, X.X.; Han, D. QTL mapping and validation of adult plant resistance to stripe rust in Chinese wheat landrace Humai 15. *Front. Plant Sci.* **2018**, *9*, 968. [CrossRef]
33. Ishikawa, G.; Nakamura, T.; Ashida, T.; Saito, M.; Nasuda, S.; Endo, T.; Wu, J. Localization of anchor loci representing five hundred annotated rice genes to wheat chromosomes using PLUG markers. *Theor. Appl. Genet.* **2009**, *118*, 499–514. [CrossRef] [PubMed]
34. Wang, H.; Dai, K.; Xiao, J.; Yuan, C.; Zhao, R.; Doležel, J.; Wu, Y.; Cao, A.; Chen, P.; Zhang, S.; Wang, X. Development of intron targeting (IT) markers specific for chromosome arm 4VS of *Haynaldia villosa* by chromosome sorting and next-generation sequencing. *BMC Genom.* **2017**, *18*, 167. [CrossRef]

35. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **1999**, *41*, 95–98.
36. Lang, T.; La, S.; Li, B.; Yu, Z.; Chen, Q.; Li, J.; Yang, E.; Li, G.; Yang, Z. Precise identification of wheat-*Thinopyrum intermedium* translocation chromosomes carrying resistance to wheat stripe rust in line Z4 and its derived progenies. *Genome* **2018**, *61*, 177–185. [CrossRef] [PubMed]
37. Danilova, T.V.; Friebe, B.; Gill, B.S. Single–copy gene fluorescence *in situ* hybridization and genome analysis: Acc–2 loci mark evolutionary chromosomal rearrangements in wheat. *Chromosoma* **2012**, *121*, 597–611. [CrossRef]
38. Lang, T.; Li, G.; Wang, H.; Yu, Z.; Chen, Q.; Yang, E.; Fu, S.; Tang, Z.; Yang, Z. Physical location of tandem repeats in the wheat genome and application for chromosome identification. *Planta* **2018**. [CrossRef] [PubMed]
39. Tang, S.; Tang, Z.; Qiu, L.; Yang, Z.; Li, G.; Lang, T.; Zhu, W.; Zhang, J.; Fu, S. Developing new Oligo probes to distinguish specific chromosomal segments and the A.; B.; D genomes of wheat (*Triticum aestivum* L.) using ND-FISH. *Front. Plant Sci.* **2018**, *9*, 1104. [CrossRef]
40. Deng, C.; Bai, L.; Fu, S.; Yin, W.; Zhang, Y.; Chen, Y.; Wang, R.R.; Zhang, X.; Han, F.; Hu, Z. Microdissection and chromosome painting of the alien chromosome in an addition line of wheat—*Thinopyrum intermedium*. *PLoS ONE* **2013**, *8*, e72564. [CrossRef]
41. Chen, Q.; Conner, R.L.; Li, H.J.; Sun, S.C.; Ahmad, F.; Laroche, A.; Graf, R.J. Molecular cytogenetic discrimination and reaction to wheat streak mosaic virus and the wheat curl mite in Zhong series of wheat—*Thinopyrum intermedium* partial amphiploids. *Genome* **2003**, *46*, 135–145. [CrossRef]
42. Tang, S.; Li, Z.; Jia, X.; Larkin, P.J. Genomic *in situ* hybridization (GISH) analyses of *Thinopyrum intermedium*, its partial amphiploid Zhong 5, and disease-resistant derivatives in wheat. *Theor. Appl. Genet.* **2000**, *100*, 344–352. [CrossRef]
43. Han, F.P.; Fedak, G.; Benabdelmouna, A.; Armstrong, K.; Ouellet, T. Characterization of six wheat × *Thinopyrum intermedium* derivatives by GISH.; RFLP.; and multicolor GISH. *Genome* **2003**, *46*, 490–495. [CrossRef] [PubMed]
44. Banks, P.M.; Xu, S.J.; Wang, R.R.-C.; Larkin, P.J. Varying chromosome composition of 56-chromosome wheat × *Thinopyrum intermedium* partial amphiploids. *Genome* **1993**, *36*, 207–215. [CrossRef] [PubMed]
45. Fedak, G.; Chen, Q.; Conner, R.L.; Laroche, A.; Petroski, R.; Armstrong, K.W. Characterization of wheat-*Thinopyrum* partial amphiploids by meiotic analysis and genomic in situ hybridization. *Genome* **2000**, *43*, 712–719. [CrossRef] [PubMed]
46. Georgieva, M.; Sepsi, A.; Tyankova, N.; Molnár-Láng, M. Molecular cytogenetic characterization of two high protein wheat-*Thinopyrum intermedium* partial amphiploids. *J. Appl. Genet.* **2011**, *52*, 269–277. [CrossRef] [PubMed]
47. Appels, R.; Gerlach, W.L.; Dennis, E.S.; Swift, H.; Peacock, W.J. Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. *Chromosoma* **1980**, *78*, 293–311. [CrossRef]
48. Gerlach, W.L.; Bedbrook, J.R. Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res.* **1979**, *7*, 1869–1885. [CrossRef]
49. Badaeva, E.D.; Friebe, B.; Gill, B.S. Genome differentiation in *Aegilops*. 2. Physical mapping of 5S and 18S–26S ribosomal RNA gene families in diploid species. *Genome* **1996**, *39*, 1150–1158. [CrossRef]
50. Mukai, Y.; Endo, T.R.; Gill, B.S. Physical mapping of the 5S rRNA multigene family in common wheat. *J. Hered.* **1990**, *81*, 290–295. [CrossRef]
51. Li, D.Y.; Ru, Y.Y.; Zhang, X.Y. Chromosomal Distribution of the 18S-5.8S–26S rDNA Loci and Heterogeneity of Nuclear ITS Regions in *Thinopyrum intermedium* (Poaceae, Triticeae). *Acta Bot. Sin.* **2004**, *46*, 1234–1241.
52. Li, G.; Lang, T.; Dai, G.; Li, D.; Li, C.; Song, X.; Yang, Z. Precise identification of two wheat-*Thinopyrum intermedium* substitutions reveals the compensation and rearrangement between wheat and *Thinopyrum* chromosomes. *Mol. Breed.* **2015**, *35*, 1. [CrossRef]
53. Gautier, M.F.; Cosson, P.; Guirao, A.; Alary, R.; Joudrier, P. Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species. *Plant Sci.* **2000**, *153*, 81–91. [CrossRef]
54. Massa, A.N.; Morris, C.F. Molecular evolution of the Puroindoline-a, Puroindoline-b, and grain softness Protein-1 genes in the tribe Triticeae. *J. Mol. Evol.* **2006**, *63*, 526–536. [CrossRef] [PubMed]

55. Turnbull, K.M.; Turner, M.; Mukai, Y.; Yamamoto, M.; Morell, M.K.; Appels, R.; Rahman, S. The organization of genes tightly linked to the Ha locus in *Aegilops tauschii*, the D-genome donor to wheat. *Genome* **2003**, *46*, 330–338. [CrossRef] [PubMed]
56. Gazza, L.; Galassi, E.; Ciccoritti, R.; Cacciatori, P.; Pogna, N.E. Qualitative traits of perennial wheat lines derived from different *Thinopyrum* species. *Genet. Resour. Crop Evol.* **2016**, *63*, 209–219. [CrossRef]
57. Alfred, R.L.; Palombo, E.A.; Panozzo, J.F.; Bariana, H.; Bhave, M. Stability of puroindoline peptides and effects on wheat rust. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1409–1419. [CrossRef] [PubMed]
58. Liu, D.; Zhang, L.; Hao, M.; Ni, S.; Yuan, Z.; Dai, S.; Huang, L.; Wu, B.; Yan, Z.; Lan, X.; et al. Wheat breeding in the hometown of Chinese Spring. *Crop J.* **2018**, *6*, 82–90. [CrossRef]