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Molecular Cytogenetic Identification of a Wheat-*Thinopyrum ponticum* Substitution Line with Stripe Rust Resistance

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Thinopyrum ponticum ($2n = 10x = 70$) has donated rust resistance genes to protect wheat from this fungal disease. In the present study, the line ES-7, derived from the progeny of the crosses between common wheat cultivar Abbondanza and *Triticum aestivum–Th. ponticum* partial amphiploid line Xiaoyan784, was characterized by cytological, fluorescence *in situ* hybridization (FISH), genomic *in situ* hybridization (GISH) and EST-STS marker techniques. Cytological observations revealed that the configuration of ES-7 was $2n = 42 = 21$ II. GISH and FISH results showed that ES-7 had two St chromosomes and lacked 5A chromosomes compared to common wheat. The 4A chromosome of ES-7 had small alterations from common wheat. Two EST-SSR markers *BE482522* and *BG262826*, specific to *Th. ponticum* and tetraploid *Pseudoroegneria spicata* ($2n = 4x = 28$), locate on the homoeologous group 5 chromosomes of wheat, could amplify polymorphic bands in ES-7. It was suggested that the introduced St chromosomes belonged to homoeologous group 5, that is, ES-7 was a 5St (5A) disomic substitution line. Furthermore, ES-7 showed highly resistance to mixed stripe rust races of CYR32 and CYR33 in adult stages, which was possibly inherited from *Th. ponticum*. Thus, ES-7 can be used for wheat stripe rust resistance breeding program.

Keywords: wheat, *Thinopyrum ponticum*, disomic substitution line, stripe rust resistance, *in situ* hybridization, EST-STS markers

Introduction

The stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), generally impairs annual yield. Although stripe rusts generally produce fewer losses than stem rust and leaf rust (Huerta-Espino et al. 2011), it is widespread due to high variability of physiological race. Even though nearly 70 stripe rust resistance (Yr) genes have been cataloged and mapped to specific chromosomes, and plenty of addition lines, substitution lines, translocation lines and introgression lines have been developed between wheat and its relatives, such as *Secale* and *Aegilops* (Mcintosh 2003), many of the resistance genes may have been overwhelmed by newly emerged stripe rust races. Hence, the continuous production of resistance resources and characterization of novel resistance genes are essential to wheat breeding.

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Thinopyrum ponticum (Podp.) Z.-W. Liu & R.-C. (syn. *Agropyron elongatum* (Host) Beauv., *Lophopyrum ponticum* (Podp.) A. Löve, and *Elytrigia elongata* (Host) Nevski) has been widely used in wheat breeding (Li and Wang 2009). Massive disease-resistant genes from *Th. ponticum* have been transferred into wheat, such as soil-borne diseases common root rot (Li et al. 2004), wheat streak mosaic virus (WSMV), wheat curl mite (WCM) (Chen et al. 2002) and Cephalosporium stripe (Cai et al. 1998) and several multi-resistant wheat-*Th. ponticum* partial amphiploids were developed, such as Xiaoyan693, Xiaoyan7631, Xiaoyan7430, Xiaoyan68 and Xiaoyan784 (Li et al. 1985). Xiaoyan784 contains 10 A-chromosomes, 14 B-chromosomes, 12 D-chromosomes, 4 A-D translocation chromosomes and 2 A-B translocation chromosomes according to Zheng et al. (2014).

Since multivalent formation was reported in *Th. ponticum* chromosomes (Muramatsu 1990) and wheat-*Th. ponticum* hybrids during meiosis, the genomic composition of *Th. ponticum* has not been clarified yet. Chen et al. (1998) suggested that the genomic composition of *Th. ponticum* was $JJJ^sJ^sJ^s$, in which the J genome of *Th. ponticum* was closely related to the J genome of the diploid *Th. bessarabicum* and the J^s genome was a modified J genome of unknown origin characterized by the presence of an St (*P. spicata*) genome-specific hybridization signal near the centromere. However, Zhang et al. (1996) presumed that *Th. ponticum* consisted of two basic genomes St and E, shown by the formula $StStE^eE^bE^x$, with E^e from *Th. elongatum*, E^b from *Th. bessarabicum* and St from *Pseudoroegneria* species, respectively.

Because of the efficiency and high accuracy, genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) are employed continuously to detect the alien chromosomes or introgression segments transferred into the wheat backgrounds. Diverse functional molecular markers, especially genome-specific markers, were applied to check the targeted alien chromosome, chromosome segments or genes introduced to common wheat (Wang et al. 2010). Therefore, it is convenient to characterize the constitution of the progeny from wheat and its wild relatives by combining those techniques.

In the present study, a) a novel wheat-*Th. ponticum* 5St (5A) disomic substitution line ES-7 was developed, which was derived from common wheat cultivar Abbondanza and wheat-*Th. ponticum* partial amphiploid line Xiaoyan784. It was revealed that the introduction of new stripe rust resistance gene(s) from *Th. ponticum* 5St chromosomes into wheat, which can be used as the bridge material to incorporate the gene into the wheat genome through chromosome translocation. b) the chromosome constitution of this substitution line was characterized, based on mitotic and meiotic cytogenetics and FISH and GISH. c) 5St specific EST-SSR markers were developed and characterized.

Materials and Methods

Materials

Materials included *Th. ponticum* ($2n = 10x = 70$), *Th. elongatum* ($2n = 2x = 14$), *Th. bessarabicum* ($2n = 2x = 14$), tetraploid *P. spicata* ($2n = 4x = 28$), *Triticum aestivum* cultivar Chinese Spring (CS), Abbondanza, wheat-*Th. ponticum* partial amphiploid line

Xiaoyan784, and the substitution line ES-7. All plant materials were provided by the College of Agronomy, Northwest A & F University. Huixianhong (HXH) was employed as the susceptible controls in the stripe rust resistance tests at the adult stage in the field.

Cytological identification

Root tips and young spikes were sampled in the field at appropriate stage, and transferred into the Carnoy's fixative fluid (3:1 ethanol-acetic acid mixture and 6:3:1 ethanol-chloroform-acetic acid mixture, respectively) for at least 48 h. The root tips were tinted by 1% (w/v) aceto-carmine solution for 1–2 h and squashed in 45% (v/v) acetic acid. The appropriate anthers contain metaphase I cells were squashed in 1% aceto-carmine solution (Li et al. 2014). The cell with complete chromosomes complements were photographed by an Olympus BX-43 microscope (Japan) equipped Photometrics SenSys CCD camera.

GISH and FISH

Seeds were germinated in the dark at 23 °C until roots reached 1–2 cm. The root tips were removed and pretreated in nitrous oxide with 0.8–1.0 MPa for 2 h, after that it were placed in ice, submerging in acetic acid, for 10 min, and then stored in 70% ethanol at –20 °C for later use. The treated root tips were digested in 2% cellulase mingled 1% pectinase at 37 °C for 52–58 min (different digestion time in accordance with difference between materials); the slides were then prepared using the drop technique as Han et al. (2004).

The total genomic DNA of common wheat Chinese Spring (CS) and *Th. ponticum*, *Th. elongatum, Th. bessarabicum,* tetraploid *P. spicata*, which were used for GISH blocks and probes, respectively, were isolated from seedling leaves using a modified CTAB method (Doyle 1987) and purified by using chloroform. The total genomic DNA of *Th. ponticum, Th. elongatum, Th. elongatum, Th. bessarabicum,* tetraploid *P. spicata* as probe was labeled with a Dig-Nick Translation Mix (Roche, Germany). The sheared (121 °C, 5 min) genomic DNA of CS was used as blocking DNA and the ratio of blocking DNA and probe is 330:1. The GISH procedure was performed as described in Yang et al. (2014) with minor modifications. Oligonucleotide probes Oligo-pSc119.2 and Oligo-pTa535, with 5' end-labelled by 6-carboxyfluorescein (6-FAM) or 6-carboxy tetramethylrhodamine (Tamra) were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China), could be used to identify the whole set of wheat chromosomes by FISH analysis, the steps of labeling probe and *in situ* hybridization were according to Tang et al. (2014). Fluorescent signals were viewed and photographed by an Olympus BX53 (Japan) equipped Photometrics SenSys CCD camera DP 80.

EST analysis

EST markers (http://wheat.pw.usda.gov/SNP/new/pcr_primers.shtml) of wheat chromosomes were all synthesized in AuGCT DNA-SYN Biotechnology Co., Ltd (Beijing, China). These markers were used to further detect the introduced alien chromosomes in the

wheat-*Th. ponticum* disomic substitution line ES-7. The volumes of polymerase chain reaction (PCR) assays were 10 µl, containing 0.1 µl of *Taq* DNA polymerase (2.5 U/µl) (TAKARA, Japan), 0.5 µl of each primer, 0.8 µl of dNTP mixture (Mg^{2+}) (2.5 mmol/L) (TAKARA, Japan), 1.0 µl of DNA template (40–100 ng/µl), 1.0 µl of 10 ×*Taq* buffer (TAKARA, Japan) and 6.1 µl ddH_2O. PCR was performed in an S1000TM Thermal Cycler (Bio-Rad, California, USA) using the following program: initial denaturation at 94 °C, 3 min, followed by 35 cycles at 94 °C, 30 s, at 50–60 °C (based on the primer information from the GrainGenes database), 45 s, at 72 °C, 50 s and a final extension step at 72 °C, 10 min before cooling to 4 °C. The PCR products of EST-STS markers were separated in 8% non-denaturing polyacrylamide gel and visualized with silver staining.

Disease resistance and agronomic trait evaluation

A mixture of *Puccinia striiformis* f. sp. *tritici* (*Pst*) races CYR32, CYR33 was used to evaluate resistance to stripe rust at the adult stage. Common wheat cv. Abbondanza, Xiaoyan784, the substitution line ES-7 and the susceptible control HXH was separately tested in the field at the College of Agronomy, Northwest A & F University.

When HXH were fully infected after the artificial inoculation, the reactions to the mixed *Pst* races were ranked according to a previously published method, the infection types (IT) scores of wheat stripe rust at adult stage was assessed on a scale from 0–4, as follows: 0, 0; and 1 were considered to be resistant, 2 was recorded to be moderately resistant, 3 and 4 was assessed to be moderately susceptible and susceptible, respectively (Ma et al. 1995).

Morphological traits of line ES-7 and its parents, common wheat cv. Abbondanza, Xiaoyan784, i.e. plant height, spike length, spikelet numbers and grains per spike were all sampled randomly and investigated.

Results

Morphology and cytological characterization

ES-7 was derived from the progeny of wheat cv. Abbondanza and wheat-*Th. ponticum* partial amphiploid Xiaoyan784. Mitotic and meiotic configurations of line ES-7 showed that there were 42 chromosomes in root tip cells (RTCs) and 21 II in pollen mother cells (PMCs) (Fig. S1*). The chromosome pairing behavior in PMCs was assayed during metaphase I, while no chromosomes was lagged at anaphase I. Therefore, line ES-7 revealed a highly cytological stability.

The plant height, spike length, spikelet numbers and kernels per spike of Xiaoyan784, Abbondanza and ES-7 were shown (Table S1). The plant height of ES-7 was definitely shorter than both parents, and the per spike grains of ES-7 was less. The spikelet numbers of ES-7 were closely resembled to Abbondanza and Xiaoyan784. The spike length of

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

ES-7 and Abbondanza showed high similarity, which was significantly shorter than Xiaoyan784.

Stripe rust resistance evaluation

The reactions of adult plants to the mixed *Pst* races (CYR32 and CYR33) were tested in the field. Wheat parent cv. Abbondanza and control variety HXH were susceptible, while Xiaoyan784 was almost immune to these two races, meanwhile ES-7 also performed highly resistance to stripe rust at the adult stage (Table S2, Fig. S2). The results indicated that the stripe rust resistance of ES-7 was inherited from Xiaoyan784, which traced to *Th. ponticum*.

GISH and FISH analysis

The total genomic DNA of *Th. ponticum* was used as probes and that of wheat CS were used as block, Mitotic GISH were conducted to identify the introduced chromosomes from *Th. ponticum* into ES-7. Two green signals were clearly, which means that these two chromosomes in ES-7 were from *Th. ponticum* (Fig. 1A). And then, the DNA of *Th. elongatum, Th. bessarabicum* and *P. spicata* were used as probes, respectively, while the green signals only appear in the condition using the DNA of *P. spicata* (the results that using the DNA of *Th. elongatum, Th. bessarabicum* are not shown). These results of somatic cells revealed that ES-7 had two alien chromosomes, with clear hybridization signals, from *P. spicata* and namely from St genome (Fig. 1B).

To further determine which wheat chromosomes were replaced by *Th. ponticum* in ES-7, FISH analysis was performed. Oligo-pTa535 and Oligo-pSc119.2 were used to hybridize with ES-7, Abbondanza and Xiaoyan784 mitotic chromosomes by multi-color FISH (Fig. 2, Fig. S3). According to the standard FISH karyotype of CS made by Tang et al. (2014), common wheat chromosomes 5A were missing in line ES-7.

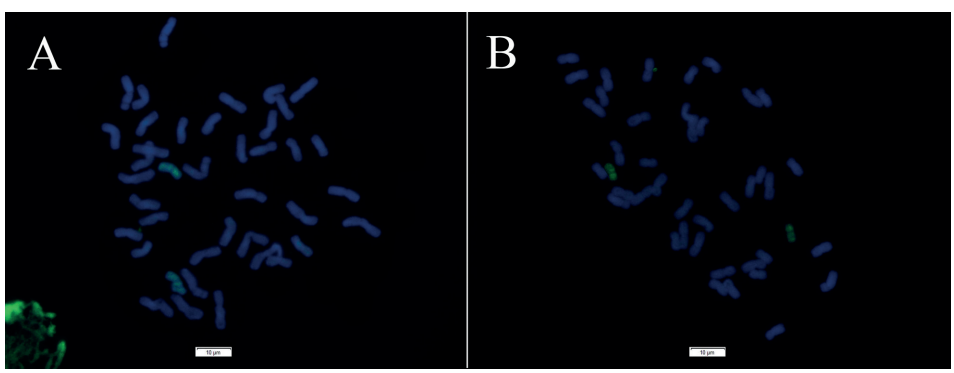

Figure 1. Genomic *in situ* hybridization (GISH) analysis of ES-7 using *Th. ponticum* genomic DNA (A: green) and *Pseudoroegneria* genomic DNA (B: green) as probe, respectively, and CS genomic DNA as block in the ratio of 330:1. Chromosomes were counterstained with DAPI (blue)

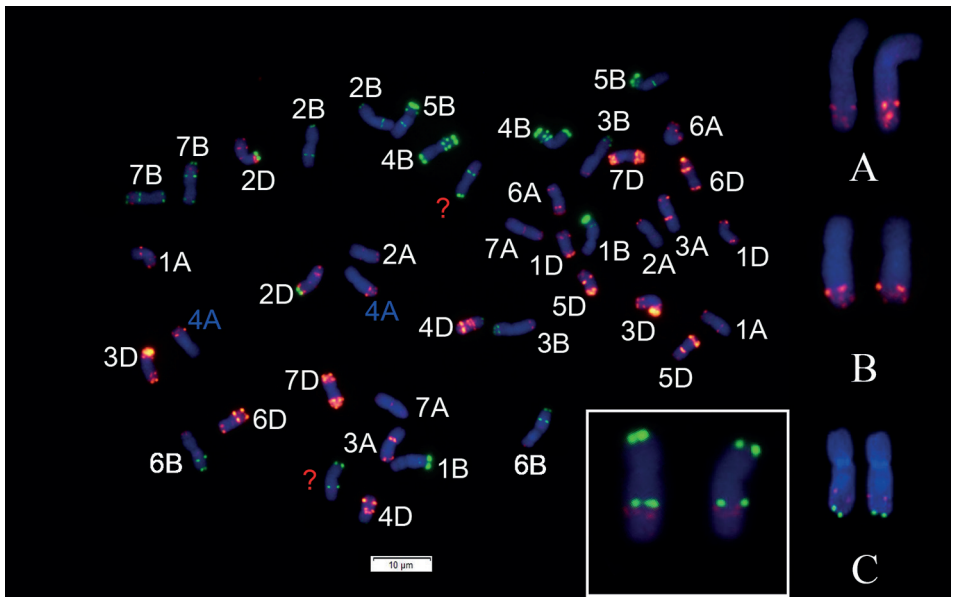

Figure 2. Result of FISH using Oligo-pTa535 (red), Oligo-pSc119.2 (green) and chromosomes were counterstained with DAPI (blue) of ES-7, two interrogations in red show the alien chromosomes (which, are supposed to be 5 St, has been cut out and magnified in the bottom right with white square) in ES-7; 4A in Abbondanza (A), 4A in ES-7 (B) and 4A in CS made by Tang et al. (2014) (C) has been cut out and magnified in the right side. Compared to 4A of CS, two green signals in long arm disappeared in Abbondanza and ES-7

Furthermore, two chromosomes, share a similar FISH karyotype and this karyotype was distinct from common wheat chromosomes, were tracked (Fig. 2), which were supposed to be a pair of alien chromosomes from Xiaoyan784, namely, from *Th. ponticum* and *P. spicata.* Moreover, there were some small alterations between the FISH karyotypes of ES-7, Xiaoyan784 and Abbondanza. ES-7 and Abbondanza lack two green signals (Oligo-pSc119.2) in the long arm of the 4A chromosome, in comparison with published FISH karyotype, and this difference indicated that the 4A in ES-7 was derived from Abbondanza. This occurrence may result from the quite complex genotypic milieu of Xiaoyan784 and Abbondanza.

Molecular marker analysis

Expressed sequence tag (EST) markers were used to identify which chromosome of *Th. ponticum* replaced 5A in ES-7. After testing 66 EST molecular markers, two markers (*BE482522* and *BG262826*) (Table S3), which were located on the fifth homoeologous groups of wheat chromosomes, amplified the specific bands of *Th. ponticum* and *P. spicata* in ES-7 and Xiaoyan784 (Fig. 3). The result showed that the substitution line possessed a wheat background while it also amplified unique bands from *Th. ponticum* and *P. spicata,* which was consistent with the analysis of cytogenetic and GISH. Thus, the

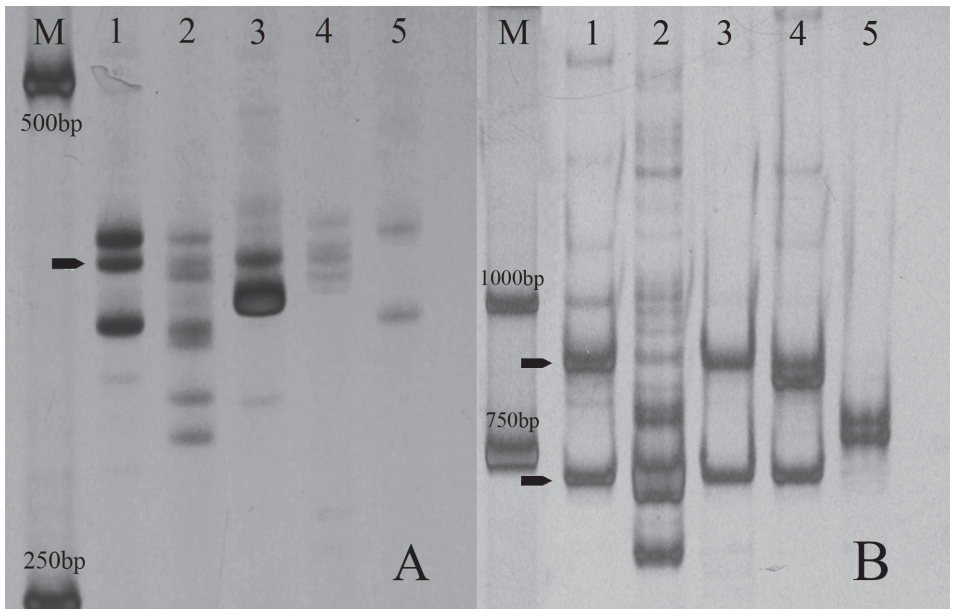

Figure 3. Eight percent non-denaturing polyacrylamide gel electrophoretic analysis of ES-7 A is primer *BG262826* and B is primer *BE482522*. M: DL2000 (Takara) 1: ES-7, 2: *Th. ponticum,* 3: *P. spicata,* 4: Xiaoyan 784, 5: Abbondanza. The arrows show the polymorphic bands

alien chromosomes may source from fifth homoeologous groups substituting 5A chromosomes of common wheat and two markers (*BE482522* and *BG262826*) can be specific to detect DNA from *Th. ponticum* and *P. spicata*.

Discussion

In summary, a new wheat-*Th. ponticum* disomic substitution line ES-7 was spontaneously produced from the progeny of a cross between Xiaoyan784 and Abbondanza. This alien disomic substitution line ES-7 was verified by GISH, FISH and EST molecular markers analysis, and the alien chromosomes were most likely to be 5St chromosome. Meanwhile, FISH analysis demonstrated the pair of common wheat chromosomes 5A were absent in ES-7. Therefore, ES-7 was a new 5St (5A) substitution line. Since the self-fertility of ES-7 was as high as 91.0%, we considered that all of the chromosomes in ES-7 accurately paired and separated. Furthermore, wheat-*Th. ponticum* disomic substitution line ES-7 obtained a significant resistance to stripe rust at adult stage, which could contribute to a higher yield cultivar without this kind of resistance. These traits overall revealed that this new wheat-*Th. ponticum* disomic substitution line ES-7 can be a better bridge material in wheat breeding than Xiaoyan784 or other wheat-*Th. ponticum* partial amphiploids due to relatively similar chromosomal composition with common wheat, especially in culturing new variety with strong stripe rust resistance.

Th. ponticum was considered as a superior source of resistance to wheat rust (Yin et al. 2006; Li and Wang 2009) and some rust resistance genes have been reported, such as *Lr19* (Sarma and Knott 1966), *Sr24* (Jiang et al. 1994), *Sr25* (Friebe et al. 1994), *Sr26* (Friebe et al. 1994) and so on. It was reported that 6 J^s of *Th. ponticum* contained relevant genes (Hu et al. 2011). As it was reported that Xiaoyan784 was highly resistant to all races of stem-rust (Zheng et al. 2014), it is worth noticing that *Th. ponticum* chromosomes contained novel stripe rust resistance genes and it can be expressed in the wheat back grounds. ES-7 also presented high stripe rust resistance. Accordingly, we supposed that the 5St also had gene(s) that can help wheat from being destroyed by stripe rust. However, there was no systematical study in *Th. ponticum*. Thus, novel gene(s) relating to stripe rust may be found in *Th. ponticum.*

Repetitive sequences, pSc119.2 and pTa-535 were usually used as probes in FISH analysis to distinguish wheat A-, B-, and D-genome chromosomes (Pedersen and Langridge 1997; Komuro et al. 2013; Tang et al. 2014). Thus, these two probes proved the absence of 5A clearly in this study, which provided facility in the research. However, these probes displayed some small alterations, such as 4A in ES-7, Xiaoyan784 and Abbondanza, in karyotypes between different verities, and this phenomenon may due to two reasons: 1) complex genotypic milieu of parents resulted in some differences in repetitive sequences where the probes hybridizing, so that some signals showed absence or appearance. 2) Introgression that is happening in hybridizing between the wheat and its relative. GISH could detect some chromosome segments coming from non-wheat chromosome. Thus, we did suppose that the main reason that the small alterations existing between the karyotype of Xiaoyan784, Abbondanza, ES-7 and CS was complexity of genotypic milieu.

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Author contributions

W. J. and C. W. designed the experiments; Q. M., C. W., C. C. and Y. W. performed the experiments; Q. M., C. W., H. Z. and X. L. analyzed the data; Q. M., C. W. and W. J. wrote the paper.

Disclosure of potential conflict of interest

The authors declare no conflict of interest.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Agronomic traits of the alien substitution line ES-7 and its parents

Electronic Supplementary *Table S2*. Evaluation of the disease resistance to stripe rust of *Th. ponticum*, Abbondanza, Xiaoyan784 and ES-7

Electronic Supplementary *Table S3*. Two specific markers on the fifth homoeologous groups

Electronic Supplementary *Figure S1*. Mitotic (A $2n = 42$), meiotic I (B $2n = 21$ II) and anaphase I (C $2n = 21 + 21$) chromosome characteristics of ES-7

Electronic Supplementary *Figure S2*. Stripe rust tests of Abbondanza (A), Xiaoyan784 (B) and ES-7 (C) and HXH (D) at adult stages

Electronic Supplementary *Figure S3*. Result of FISH using Oligo-pTa535 (red), Oligo-pSc119.2 (green) and chromosomes were counterstained with DAPI (blue) of A: Xiaoyan784 the lower cases from a to g in red indicate these chromosomes are different from the standard karyotype, and the 1B in blue show the chromosomes 1B which have small alterations from the common one; B: Abbondanza the 4A in blue show the chromosomes 4A which have small alterations from standard karyotype