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Identification of a Novel Wheat-*Thinopyrum ponticum* Addition Line Revealed with Cytology, SSR, EST-SSR, EST-STS and PLUG Markers

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Thinopyrum ponticum is particularly a valuable source of genes for wheat improvement. A novel wheat-*Th. ponticum* addition line, 1–27, was identified using cytology, SSR, EST-SSR, EST-STS and PCR-based landmark unique gene (PLUG) markers in this study. Cytological studies showed that 1–27 contained 44 chromosomes and formed 22 bivalents at meiotic metaphase I. Genomic in situ hybridization (GISH) analysis indicated that two chromosomes from *Th. ponticum* had been introduced into 1–27 and that these two chromosomes could form a bivalent in wheat background. Such results demonstrated that 1–27 was a disomic addition line with 42 wheat chromosomes and a pair of *Th. ponticum* chromosomes. One SSR marker (BARC235), one EST-STS marker (MAG3284) and 8 PLUG markers (TNAC1210, TNAC1787, TNAC1803, TNAC1805, TNAC1806, TNAC1821, TNAC1867 and TNAC1957), which were all from wheat chromosome group 7, produced the specific band in *Th. ponticum* and 1–27, indicating that the introduced *Th. ponticum* chromosomes belonging to the group 7 of wheat. Sequence analysis on specific bands from *Th. ponticum* and 1–27 amplified using the PLUG marker TNAC1867 further confirmed this result. The 1–27 addition line was also observed to be high resistant to powdery mildew though it is not clear if the resistance of 1–27 inherited from *Th. ponticum*. This study provided some useful information for effective exploitation of the source of genetic variability in wheat breeding.

Keywords: alien addition line, *Th. ponticum*, wheat, cytology, molecular marker

Introduction

Wild relatives of bread wheat (*Triticum aestivum* L.) constitute a valuable reservoir of genes. The transfer of alien genetic material from related species into wheat through chromosome engineering is an important approach for enriching genetic diversity available to wheat (Sepsi et al. 2008; Luan et al. 2010). *Thinopyrum ponticum* (Popd.) Barkworth & D. R. Dewey [syn *Agropyron elongatum* (Host) Beauvoir ssp. *ruthenicum* Beldie]

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(2n = 10x = 70) is particularly a valuable source of genes carrying potentially favorable traits such as high-seed protein content, large biomass, and tolerance/resistance against a number of both biotic and abiotic stresses. Therefore, it was hybridized extensively with wheat to develop valuable germplasm lines (Cui et al. 2009).

The first step to transfer desirable traits from *Th. ponticum* to wheat is to develop wheat *Th. ponticum* introgression lines. So far, a large number of wheat *Th. ponticum* derivatives have been produced from wheat-*Th. ponticum* hybrids, such as octoploid amphiploids, hexaploid amphiploids, alien addition, substitution and translocation lines (Friebe et al. 1996; Fedak et al. 2001; Sepsi et al. 2008). Furthermore, a few useful genes have been transferred from *Th. ponticum* into commercially grown wheat cultivars, which have led to the production of a series of wheat cultivars. For example, a famous wheat cultivar, Xiaoyan 6, was derived from a wheat-*Thinopyrum ponticum* cross in China. It has many valuable traits such as high-yielding, good bread-making quality with high molecular weight glutenin subunits (HMW-GS) 1Bx14 and 1By15, high protein content, stress tolerance and wide adaptation (An et al. 2013). Further development of wheat-*Th. ponticum* introgression lines diverse parental germplasm sources may possess valuable genes or alleles, which could increase the potential of successful breeding in wheat (Hu et al. 2011; Lei et al. 2011).

In wheat breeding programs, chromosome identification and alien chromatin detection in wheat background are essential prior to their use. With the development of molecular biology, the identification of alien chromosome has greatly developed. Genomic in situ hybridization (GISH) is widely used for determining the origin of genome, chromosome and chromosome segment in hybrid (Schwarzacher et al. 1989), however it can not determine which chromosome(s) that have been added or transferred (Du et al. 2013a). Polymerase chain reaction (PCR) markers are more convenient to achieve much higher throughput analysis in terms of manipulation and application. PCR-based markers have become a main tool for genetic analysis in wheat (Ishikawa et al. 2007; Li et al. 2013). In recent years, diverse PCR-based markers, such as simple sequence repeat (SSR) and sequence-tagged site (STS), have been used extensively for determining the homoeologous relationships of chromosomes from different grass species by comparative mapping (Heslop-Harrison 2000).

In our research team, a F_1 plant was obtained firstly from a cross between *Thinopyrum ponticum* introduced from Australia and bread wheat Lankaoaizaoba, and it was further crossed to wheat line Cp02-3-5-5. Subsequently, the resulting progeny was backcrossed two times using another excellent wheat line YN001 successively. During these crosses, resistant derivatives to powdery mildew were persisted continually in each generation. Finally, we selected line 1–27 based on its powdery mildew resistance under natural conditions and identical agronomical traits. In this study, we identify the addition line using cytology, SSR, EST-SSR, EST-STS and wheat PLUG markers reported by Ishikawa et al. (2009). This will lead to effective exploitation of the source of genetic variability for the genetic improvement of wheat crops.

Materials and Methods

Plant materials

The materials used in this study included *Thinopyrum ponticum*, bread wheats (Chinese Spring, Lankaoaizaoba, Cp02-3-5-5 and YN001) and a wheat-*Th. ponticum* derivate line 1–27. Chinese Spring genomic DNA was used as a blocker in the GISH analysis.

Mitosis and meiosis analysis

Seeds were germinated on moistened filter paper in petri dishes maintained in a growth chamber at 25 °C in the dark. Root tips were collected and placed immediately in ice water for 20–24 h, fixed in ethanol-acetic acid (3:1) fixative for two days and stored in 70% (v/v) ethanol. Root tips were squashed in 45% (v/v) acetic acid, and then used for cytological observation. Young spikes were excised at the appropriate stage and fixed in ethanol-acetic acid-chloroform (6:1:3) fixative for 12 h and stored in 70% (v/v) ethanol. The anthers were squashed in 45% (v/v) acetic acid to examine the meiotic chromosomes.

GISH analysis

For GISH analysis, slides were frozen in liquid nitrogen and cover slips were removed using a razor blade. The slides on which mitotic metaphase cells reside were stored at –20 °C until use. Genomic DNA of *Th. ponticum* and Chinese Spring wheat was extracted separately using the method by Sharp et al. (1988). Genomic DNA of *Th. ponticum* was labeled with the DIG-Nick Translation Mix (Roche, Mannheim, Germany) and used as a probe. The genomic DNA from Chinese Spring wheat was sheared for 5 min by ultrasonication and used as a blocker. The hybridization mixture was added onto the slides and denatured at 80 °C for 6 min. Chromosome denaturation, hybridization, and hybridization signal detection were carried out as described by Han et al. (2004).

Molecular marker analysis

Genomic DNA was extracted from the wheat-*Th. ponticum* derivate line 1–27 and its parents following the method by Sharp et al. (1988). Total 1210 primer pairs were used to characterize the genomic composition of the wheat-*Th. ponticum* addition line. Of these markers, 600 SSR (GWM, GDM, WMC, BARC, CFA and CFD), 391 EST-SSR (CFE, SWES and CWEM) and 35 EST-STS (MAG) that were from seven homoeologous groups of wheat, were derived from a previous report (Chen et al. 2005) or http://wheat.pw.usda.gov/GG2/index.shtml. In addition, 149 PCR-based landmark unique gene (PLUG) primers reported by Ishikawa et al. (2009) were also used in this analysis.

PCR reaction were performed as described by Li et al. (2012) using a PTC-200 thermocycler (MJ Research, Watertown, MA). The cycling parameters were, 94 °C for 4 min to pre-denature; followed by 36 cycles of 94 °C for 1 min, 55~60 °C for 1 min, and 72 °C

for 1 min (SSR and EST-SSR amplification) or 2 min (EST-STS and PLUG amplification); and then a final extension at 72 °C for 8 min. PCR products were separated on 8% polyacrylamide non-denatured gels and were visualized following silver-staining. The PCR products from the PLUG marker TNAC1867 were separated by 1% agarose gel, and the amplified *Th. ponticum* specific bands were extracted from gel. The cloning and sequencing of PCR product was conducted as described by Tang et al. (2008).

Powdery mildew assessment

Adult plant resistance to powdery mildew (*Erysiphe graminis* DC. f. sp. tritici Em. Marchal) of 1–27, together with its parents, was assessed under natural conditions in field and greenhouses in 2012 and 2013. Chinese Spring was used as the susceptible control.

Results

Cytology and resistance to powdery mildew of 1–27

Root-tip chromosome counts indicated that 1–27 had a chromosome number of 2n = 44 (Fig. 1a). We also examined the chromosome pairing behavior of 1–27 in the PMCs during metaphase I. Twenty-two bivalents were mostly found in the PMCs (Fig. 1b). These results indicated that 1–27 is a cytogenetically stable wheat-*Th. ponticum* disomic addition line. Adult plant resistance to powdery mildew indicated that the wheat parent Lankaoaizaoba and Cp02-3-5-5 were high susceptible when the susceptible control Chinese spring were full of spores. In contrast, *Th. ponticum*, the wheat parent YN001 and 1–27 was immune or high resistant. However, it is not clear if the resistance of 1–27 inherited from *Th. ponticum* or the wheat parent YN001.

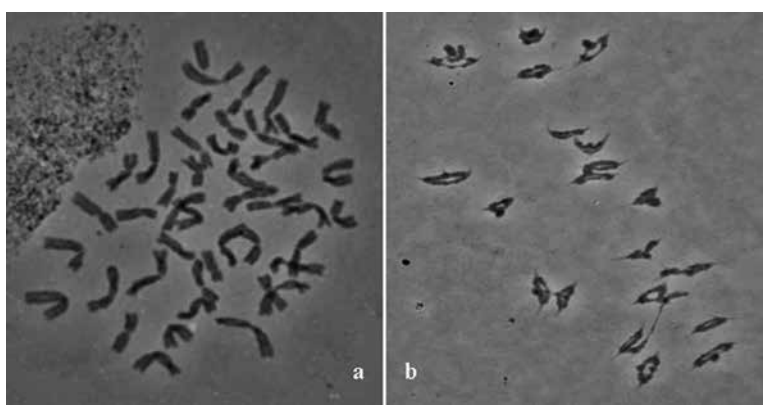

Figure 1. Mitotic (a) and meiotic (b) patterns of the wheat-*Th. ponticum* addition line 1–27. a) Somatic chromosomes in root tips, 2n = 44; b) Pollen mother cells at meiotic metaphase I, 2n = 22 II

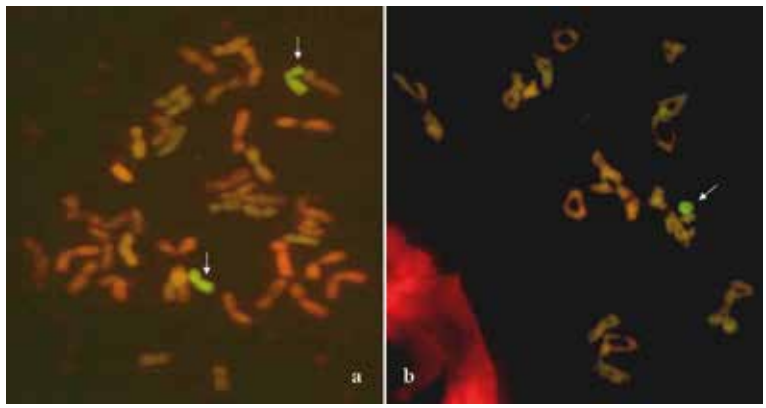

Figure 2. Mitotic (a) and meiotic (b) GISH analysis of 1–27. a) Somatic chromosomes after GISH with genomic DNA of *Th. ponticum* labeled as a probe. There are 44 chromosomes with 2 *Th. ponticum* chromosomes visualised in green. b) Pollen mother cells at meiotic metaphase I after GISH showing a bivalent with green hybridized signals. Arrows indicate the *Th. ponticum* chromosomes

GISH analysis

GISH was conducted to analyze chromosome configuration and composition of 1–27 during the mitotic and meiotic metaphases. When using digoxigenin labeled *Th. ponticum* genomic DNA as a probe and Chinese Spring DNA as a blocker, a pair of alien chromosomes in the root tip cell of 1–27 showed strong yellowish-green hybridization signals (Fig. 2a). Meiotic GISH of PMCs at metaphase I indicated that 1–27 contained a bivalent with a hybridization signal (Fig. 2b). These results indicated that two chromosomes from *Th. ponticum* had been introduced into 1–27 and that these two chromosomes could form a bivalent in wheat background. Such results demonstrated that 1–27 was a disomic addition line with 42 chromosomes of wheat and a pair of chromosomes of *Th. ponticum*.

Molecular marker analysis

Molecular markers were used to determine the homoeologous relationships between the added *Th. ponticum* chromosomes and wheat. First, 1210 primer pairs, including 600 SSR, 391 EST-SSR, 35 EST-STS and 149 PLUG markers, which were all from seven homoeologous groups of wheat, were screened in the parents. We found that 466 pairs, i.e., a ratio of 38.5%, amplified specific bands for *Th. Ponticum*, including 201 SSR, 149 EST-SSR, 22 EST-STS and 94 PLUG pairs with the frequencies of 33.5%, 38.1%, 62.6% and 63.1%, respectively. These selected primers were used to amplify DNA samples from the disomic addition line 1–27 and its parents. Only one SSR marker, BARC235 which were mapped on chromosome 7D, produced the specific band in *Th. ponticum* and 1–27 (Fig. 3). All EST-SSR markers did not amplify fragments specific for *Th. ponticum* in 1–27. In addition, we found that one EST-STS marker, i.e., MAG3284 which was located

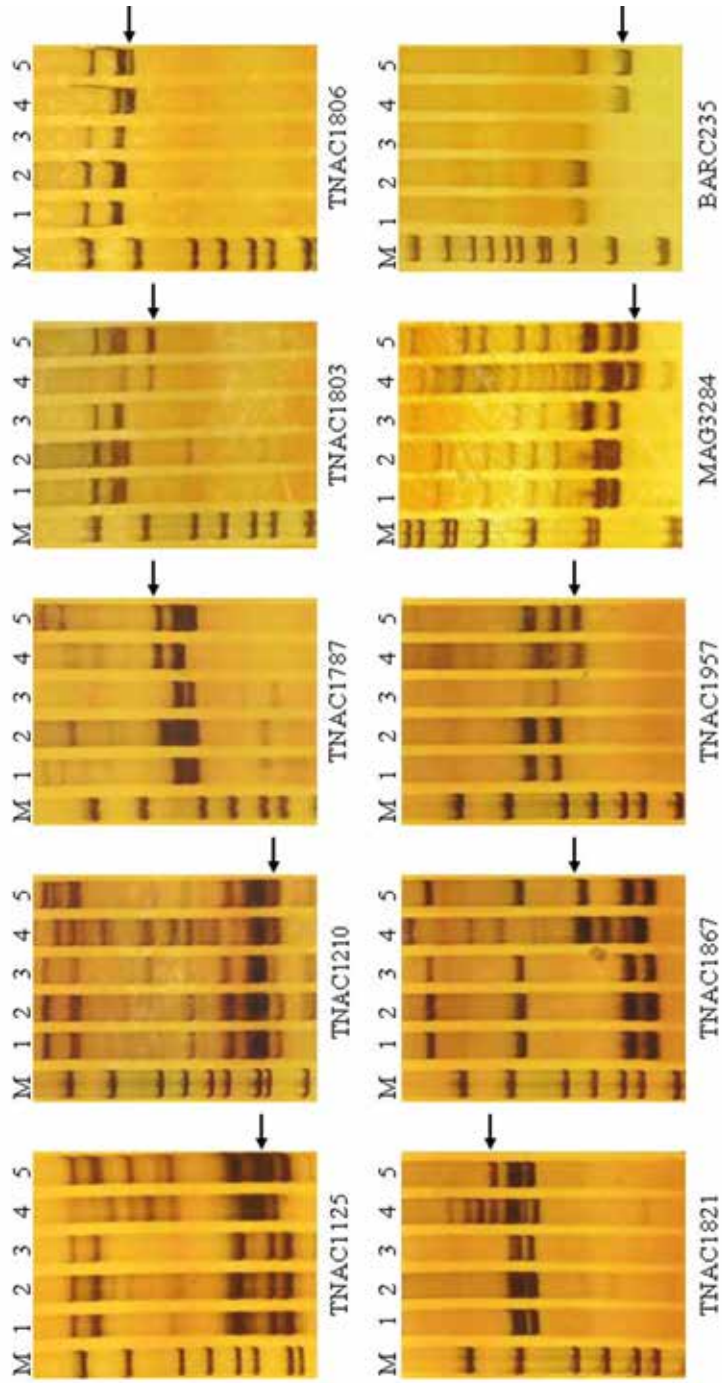

Figure 3. Identification of *Th. ponticum* chromosomes using SSR, EST-STS and PLUG markers. M marker; lane 1 Lankaoaizaoba; lane 2 Cp02-3-5-5; lane 3 YN001; lane 4 *Th. ponticum*; lane 5 addition line 1–27. Arrows indicate the diagnostic amplification products of *Th. ponticum*

on chromosome 7A, and 8 PLUG markers, i.e., TNAC1210, TNAC1787, TNAC1803, TNAC1805, TNAC1806, TNAC1821, TNAC1867 and TNAC1957 that mapped onto 7A, 7B and 7D, amplified diagnostic fragments of *Th. ponticum* in the disomic addition line 1–27. These results suggested that the two *Th. ponticum* chromosomes in addition line 1–27 were homoeologous to the group 7 of wheat. These detected markers, one SSR, one EST-STS and eight PLUG markers, could be used as unique markers to trace the *Th. ponticum* chromosome.

In addition, the specific bands from *Th. ponticum* and the addition line 1–27 amplified using the PLUG marker, TNAC1867, were sequenced to gain further insights into their homoeologous relationships. Both specific bands length were 720 bp with the same sequence (Fig. 4). A 137 bp fragment of the sequence displayed 89% homology to wheat EST BE404240 mapped on 7AL, 7BL and 7DL by Blast on http://wheat.pw.usda.gov/GG2/. Such result further indicated that these specific bands in *Th. ponticum* and addition line 1–27 also belonged to homologous group 7.

Figure 4. Alignment of the nucleotide sequences from *Th. ponticum* specific band with sequence of wheat EST (BE404240). Base similarities are indicated with dots in black boxes, and gaps introduced to optimize alignment are indicated with dashes

Discussion

Relatives of wheat have been widely used as valuable sources for introgression of useful traits in wheat improvement. As *Th. ponticum* is easily crossed with common wheat, many agronomically important genes, such as various disease-resistant genes (Nocente et al. 2007; Niu et al. 2014), as well as the genes controlling salt-tolerance (Chen et al. 2004) and dwarf stalk (Chen et al. 2012), have been transferred into wheat. Until now, in order

to introduce novel genes from the *Th. ponticum* to wheat, there are many studies in developing wheat-*Th. ponticum* introgression lines. In this study, a new wheat-*Th*. *ponticum* addition line was obtained and the germplasm of 1–27 may be useful in genetic studies and wheat breeding.

In wheat-related species such as *Th. ponticum*, where there is a lack of molecular biology information, the transfer of wheat molecular markers to the species is a feasible method for genetic analysis (Dobrovolskaya et al. 2009). Over the last two decades, SSR markers have been used extensively as a effective tool for the genetic analysis of alien chromosomes in various species, such as *Thinopyrum ponticum* (Hu et al. 2011), *Thinopyrum intermedium* (Lin et al. 2007), *Triticum timopheevii* (Uhrin et al. 2012), *Leymus mollis* (Zhao et al. 2013) and *Secale cereale* (Tang et al. 2008).

More recently, with the development of functional molecular markers, SSRs and STS markers based on EST sequences are continually increasing. EST-SSR markers originated from transcribed regions of genomes, whereas STS markers are derived from the transcribed spacers of genes. Compared with unexpressed sequence markers (e.g. SSR), these markers are highly conserved and particularly effective and accurate for determining the homologous relationships of alien chromosomes in wheat background (Dobrovolskaya et al. 2009). Du et al. (2013a and b) successfully identified alien chromosomes in two wheat-*P. huashanica* addition lines, 59-11 and 3-8-10-2, which were designated as 6Ns and 5NS based on EST-SSR and EST-STS marker analysis, respectively. Thirty-three EST-SSR markers were used to identify the sub-arm of prolamin loci on the $1H^{ch}$ chromosome in a wheat background (Cherif-Mouaki et al. 2011). In our study, of these EST-SSR and EST-STS markers tested, only one EST-STS marker, MAG3284 mapped on chromosome 7A in wheat, produced the specific bands in *Th. ponticum* and disomic addition line 1–27. Likewise, the marker was found to amplify specific bands of the homologous group 7 in a wheat-*Th. ponticum* partial amphiploid 7430 (Hu et al. 2012).

The PCR-based landmark unique gene (PLUG) marker system was developed by Ishikawa et al. (2007) and becomes an effective tool for large-scale marker mining in wheat. PLUG markers that amplify wheat genic sequences including introns are based on the sequence conservation of orthologous genes between rice and wheat, and therefore, their transferability between *Triticeae* species should be high (Ishikawa et al. 2009; Li et al. 2013). In this study, of 149 PLUG markers tested, 121 (81.2%) could amplify PCR products in *Th. ponticum*, and 94 (63.1%) produced the allele-specific bands of *Th. ponticum* compared to wheat parents. Finally, we found that eight markers, i.e., a ratio of 5.4%, could successfully amplified diagnostic fragments of *Th. ponticum* in the addition line 1–27. Likewise, Li et al. (2013) found that 131 PLUG markers successfully amplified PCR products with the template of the rye genomic DNA, and 110 (76.4%) markers showed rye-specific PCR amplification. Lei et al. (2011) also revealed a new wheat-*Secale africanum* $2R^{a}$(2D) substitution line using five PLUG markers. On the other hand, these specific markers found in this study should be useful to monitor the *Th. ponticum* chromosome during wheat breeding.

Ascertaining homoeologous relationship between alien chromosomes and wheat chromosomes is important. In the present study, we used SSR, EST-SSR, EST-STS and PLUG

markers to assign the linkage group of introduced *Th*. *ponticum* chromosomes in 1–27. The PLUG marker TNAC1867 mapped on chromosome 7A, 7B and 7D was from an EST of wheat. Since we sequenced the specific bands from *Th*. *ponticum* and 1–27, and the result of BLAST also supported that it belongs to the group 7. Similarly, Hu et al. (2011) sequenced the amplification of the EST-STS marker MAG2114 on specific bands from wheat-*Thinopyrum ponticum* substitution line X005, and sequence analysis confirmed that the introduced *Th. ponticum* chromosomes belonged to homologous group 6. Such result indicated that PLUG markers can be used as accurate anchor markers for genome research.

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