# *Оригинальные работы*

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# *THINOPYRUM* 7Ai-1-DERIVED SMALL CHROMATIN WITH BARLEY YELLOW DWARF VIRUS (BYDV) RESISTANCE GENE INTEGRATED INTO THE WHEAT GENOME WITH RETROTRANSPOSON

*Thinopyrum intermedium is a useful source of resistance genes for Barley Yellow Dwarf Virus (BYDV), one of the most damaging wheat diseases. In this study, wheat/Th. intermedium translocation lines with a BYDV resistance gene were developed using the Th. intermedium 7Ai-1 chromosome. Genomic in situ hybridization (GISH), using a Th. intermedium total genomic DNA probe, enabled detection of 7Ai-1-derived small chromatins containing a BYDV resistance gene, which were translocated onto the end of wheat chromosomes in the lines Y95011 and Y960843. Random amplified polymorphic DNA (RAPD) analyses using 120 random 10-mer primers were conducted to compare the BYDV-resistant translocation lines with susceptible lines. Two primers amplified the DNA fragments specific to the resistant line that would be useful as molecular markers to identify 7Ai-1-derived BYDV resistance chromatin in the wheat genome. Additionally, the isolated Th. intermedium-specific retrotransposon-like sequence pTi28 can be used to identify Th. intermedium chromatin transferred to the wheat genome.*

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**Introduction.** Barley Yellow Dwarf Virus (BYDV) results in yellowed, stunted plants and is one of the most serious pathogens of cultivated wheat globally (*Triticum aestivum*, 2*n* = 42, AABBDD) [1]. After 1966, large-scale outbreaks of BYDV occurred 5 times in areas under wheat cultivation in northwestern, northern, and northeastern China, during which damage to wheat production increased by 20–30 % [2]. Because wheat does not have a native BYDV resistance gene, it will be necessary to identify and introduce BYDV resistance into wheat from one of its relatives.

A wheat relative, *Thinopyrum intermedium* (2*n* = = 42, E1E1E2E2XX), is typically a useful gene source of BYDV resistance for improving common wheat cultivars. *Th. intermedium* has two kinds of BYDV resistance genes located on chromosomes 7Ai-1 [3, 4] and 2Ai-2 [5]. Several groups have developed various wheat–*Th. intermedium* chromosome addition lines, substitution lines, and translocation lines with BYDV resistance [3, 6–14]. Using cell culture to induce recombination between alien and wheat chromosomes, Banks et al. [8] developed a translocation line to introduce the BYDV resistance gene into Australian wheat cultivars by means of the *Th. intermedium* 7Ai-1 chromosome addition wheat line L1. However, in 5 out of 6 lines, part of both the long and short arms of the *Th. intermedium* 7Ai-1 chromosome carrying gene *Bdv2* was translocated to a wheat chromosome and, in one line, chromatin of 7Ai-1 origin comprised 44 % of the length of the entire long arm of chromosome 7D to which it had been translocated [15, 16]. In addition to the BYDV resistance gene, many other undesirable genes from wild species were introduced into the translocation line. On the other hand, in the United States, Ohm's group developed a resistant 7E/7D chromosome substitution line in which chromosome 7D of wheat was replaced by chromosome 7E of *Th. intermedium* carrying an additional gene, *Bdv3* [10, 17–20], and several resistant 7D-7E translocation lines [11]. It is only recently that translocations conferring BYDV resistance have become available in released wheat varieties [16, 21–23]. Therefore, to increase broad-spectrum resistance to various BYDV serotypes in wheat, the development of a line derived from 7Ai-1 carrying only the chromatin most closely flanking the BYDV resistance gene translocated to a wheat chromosome would be beneficial.

To create a BYDV-resistant wheat cultivar suitable for regions in which the barley yellow dwarf outbreaks historically occurred in China, a BYDV resistance gene located on *Th. intermedium* 7Ai-1 was introduced into a local wheat cultivar by several backcrosses, and a BYDV-resistant wheat line was developed whose agronomic characteristics such as yield were much improved. In order to use these lines effectively for breeding, it will be necessary to be able to detect introduced *Th. intermedium* chromatin carrying BYDV resistance and to clearly understand its structure. Furthermore, BYDV-bearing aphids (*Rhopalosiphum padi* L.) must be maintained for uniform inoculation of specimen plants for testing BYDV resistance. Therefore, development of a method for selection of BYDV resistance genes using molecular markers is critical.

The genomic *in situ* hybridization (GISH) method, in which total genomic DNA is hybridized as a probe to chromosome samples, is used for the detection of chromatin introduced into the wheat genome from related species such as *Th. intermedium* [4, 15], *Th. elongatum* [24], rye (*Secale cereale*) [25, 26], and barley (*Hordeum vulgare*) [25, 27]. Alternatively, random amplified polymorphic DNA (RAPD) markers linked to the rust resistance gene *Lr9* [28], *Lr24* [29], the wheat smut resistance gene [30], the powdery mildew resistance genes *Pm18* [31] or *Pm21* [32], and the Hessian fly resistance gene [33, 34] in common wheat have been developed.

In this study, both of the above techniques were applied. Chromatin carrying the BYDV resistance gene derived from *Th. intermedium* 7Ai-1 introduced into Chinese wheat cultivars was detected by GISH. Moreover, RAPD was developed to detect the translocated chromatin. In addition, to develop a DNA marker for *Th. intermedium* chromatin transferred to the wheat genome, we cloned a retrotransposon-like repetitive sequence that exists specifically in the *Th. intermedium* genome.

**Materials and methods**. *Plant materials.* In order to promote recombination between homoeologous chromosomes, the 7Ai-1 chromosome addition wheat line L1 (derived from Cauderon), carrying BYDV resistance was crossed with the wheat cultivar Chinese Spring (CS), carrying the inhibitor of homoeologous chromosome pairing (*Ph*), with the 5B nullisomic-5D tetrasomic (CS N5BT5D) line in which 5B carrying *Ph* was replaced with 5D. It was also backcrossed to the Chinese common wheat cultivars Shan 7859, Feng 8, FengKang 3, Zhong 8601, and Zhong 7902. Resistance to BYDV was tested by infecting the three-leaf stage of backcrossed plant materials with BYDV-infective aphids. Approximately 10 aphids infected with the BYDV-GAV serotype were deposited at the base of each seedling. After 2 weeks, the aphids were eliminated by insecticide. The infection type was recorded on the basis of the 0–9 scale of leaf discoloration or plant dwarfness (http://wheat.pw.usda.gov/ggpages/sxg/pheno.htm) 30 days after inoculation. The BYDV resistant wheat line PP9-1 was derived from the combination of Zhong 8601/4/Zhong 7902/3/2×CS *ph*/L1// CS N5BT5D. Furthermore, the BYDV-resistant wheat lines Y95011 ((PP9-1/Shan 7859×2) $F_4$) and Y960843 (Zhong 8601×4//Zhong 7902/5395-12) were developed using the BYDV-resistant wheat line 5395-12, which was derived from CS *ph*×2/L1//CS N5BT5D (subdivided from McIntosh) and PP9-1. The lines Y95011 and Y960843, as well as PP9-1, showed good resistance to the GAV serotype of BYDV and scored 0 on the infection type scale.

*GISH analysis.* The BYDV-resistant wheat lines Y95011 and Y960843 were used for GISH analysis. Chromosomal samples from somatic cells in metaphase were prepared by enzymatic maceration and air-drying by the method of Fukui [35, 36]. According to the method of Tomita et al. [37], genomic DNA of *Th. intermedium* was extracted and labeled with biotin-16-dUTP (Boehringer Mannheim) by the random primer method. Fifteen microliters of hybridization solution (50 % formamide, 2×SSC, 4.0–5.0 μg/ml probe DNA, 600 μg/ml unlabeled wheat cultivar CS genomic DNA, and 500 μg/ml salmon sperm DNA) was dropped onto each chromosome specimen and hybridized at 37 °C for 12 h. Samples were washed with 2×SSC at 40 °C for 10 min 3 times and with 4×SSC at 40 °C for 10 min. Detection of probe DNA fluorescence was performed according to the method of Fukui et al. [36]. In addition, the extent of the translocated chromatin was measured on each of 5 cells and evaluated using the Fraction Length (FL) of Friebe et al. [4]; specifically, translocation breakpoint

positions were calculated as a fraction of total chromosome arm length from the centromere. Therefore, we calculated the physical length of the translocated segments on the GISH photo by dividing by the whole arm length.

*RAPD analysis.* The BYDV-resistant lines Y95011, Y960843, parental cultivars Shan 7859, Zhong 8423, and the 7Ai-1 addition line L1, as well as its parental cultivar Vilmorin 27 were used for RAPD analysis. Ten progeny resistant individuals and ten disease-susceptible individuals from the $F_2$ hybrid of Y960843 and Shan 7859, were selected and their DNA was extracted and mixed in equal quantities as either bulked-resistant DNA or bulked disease-susceptible DNA. Forty-five cycles of PCR at 95 °C for 15 s, 37 °C for 45 s, and 72 °C for 1 min were performed in 25 μl reaction mixtures (2.0 μg/ml template DNA, 0.2 μM primer, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase (Takara) with a mixture of artificially synthesized 120 random 10-mer primers (Operon), namely, OPAB-01~20, OPE-01~20, OPF-01~20, OPH-01~20, OPI-01~20, and OPJ-01~20. PCR products were separated by gel electrophoresis on 1.5 % agarose.

*Cloning of a retrotransposon-like sequence of Thinopyrum intermedium.* In order to develop a DNA marker for *Th. intermedium* chromatin transferred to the wheat genome, we cloned repetitive sequences that exist specifically in the *Th. intermedium* genome. From an *Mbo*I library of genomic DNA from *Th. intermedium*, a repetitive clone, pTi28, which strongly hybridizes with *Th. intermedium*, was obtained by dot hybridization using genomic DNA from *Th. intermedium* and Chinese Spring (CS) wheat as probes. Recombinant plasmids were isolated and an aliquot of 1.5 μl was spotted on two sheets of nylon membranes. One membrane was probed with genomic DNA of *Th. intermedium* and the other was probed with CS DNA. The membranes were prehybridized at 65 °C for 3 h in hybridization solution containing 5×SSC, 2 % blocking reagent (Roche Diagnostics, Basel, Switzerland), 0.1 % N-lauroylsarcosine, and 0.02 % SDS. Genomic DNA probe (25 ng) labeled with digoxigenin-11-dUTP was added to the prehybridization solution and the mixture was allowed to hybridize at 65 °C for 14 h. Blots were washed twice in 2×SSC with 0.1 % SDS at room temperature for 5 min and twice in 0.1×SSC with 0.1 % SDS at 65 °C for 15 min and treated

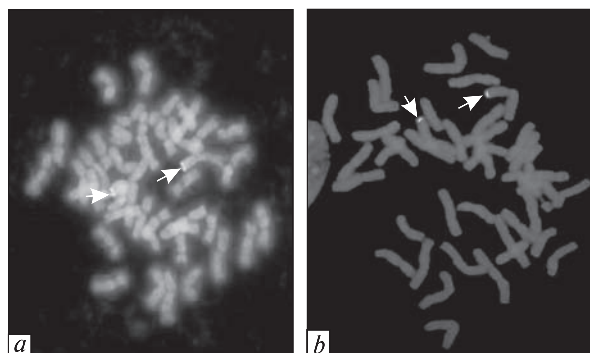



**Fig. 1.** *Thinopyrum* 7Ai-1-derived small chromatin with a Barley Yellow Dwarf Virus resistance gene was visualized in the 7Ai-1 translocated wheat line by genomic *in situ* hybridization: *a* – Y95011; *b* – Y960843. Arrows indicate *Thinopyrum* 7Ai-1 translocated segments

with CDP-Star (Roche Diagnostics, Basel, Switzerland) reagent before exposure to X-OMAT film (Eastman Kodak, NY). Then gel blot hybridization was conducted with the genomic DNA of *Th. intermedium* and CS using pTi28 as a probe. Genomic DNAs (10 μg) were digested to completion with the restriction enzyme *Eco*O109I or *Dra*I; subjected to electrophoresis on 1 % agarose gels; and then transferred and cross-linked to nylon membranes. The membranes were then probed with the repetitive sequence clone pTi28. Probe labeling, hybridization, stringency conditions and detection were conducted according to those of the dot blot analysis. Fluorescence *in situ* hybridization (FISH) analysis was comparable to the GISH analysis section. Furthermore, we determined the nucleotide sequence of pTi28, and conducted a homology search using the GenBank database.

**Results and discussion**. *GISH analysis.* The small amount of chromatin derived from *Th. intermedium* 7Ai-1 was detected by GISH on the end of the short arm of a pair of wheat chromosomes in Y95011 ($2n = 42$). Unlabeled genomic DNA of the common wheat cultivar CS was used as blocking DNA, and genomic DNA of *Th. intermedium* labeled with biotin-16-dUTP was as a probe (Fig. 1, *a*). The extent of the chromatin derived from 7Ai-1 introduced into this homozygous translocation Y95011 comprised 20 % of the full length of the short arm of the translocation chromosome. The distance between the translocation point and the kinetochore (FL) was 0.80. In contrast, a relatively small chromatin

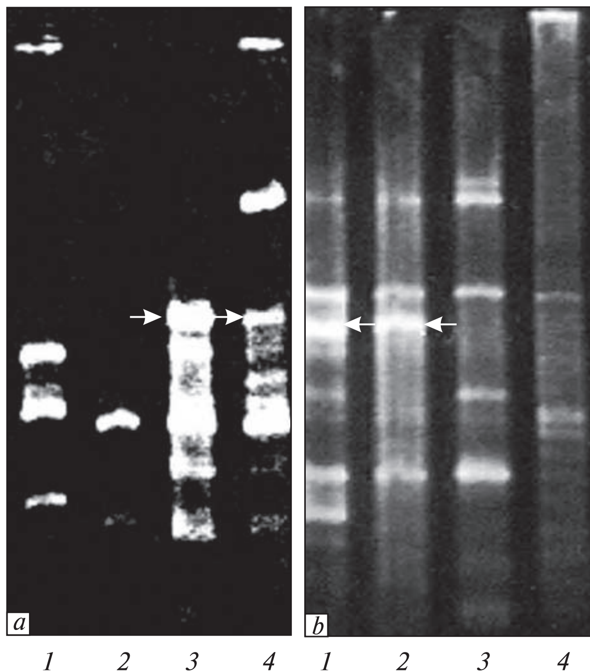



**Fig. 2.** *Thinopyrum* 7Ai-1 specific RAPD markers: *a* – Primer OPF-01, *1* – wheat variety Zhong 8423; *2* – wheat variety Vilmorin 27; *3* – 7Ai-1 translocated wheat line Y95011; *4* – 7Ai-1 addition wheat line L1; *b* – Primer OPAB-01, *1* – 7Ai-1 addition wheat line L1; *2* – Y960843 × Shan 7859 $F_2$ resistant bulk; *3* – Y960843 × Shan 7859 $F_2$ susceptible bulk; *4* – wheat variety Vilmorin 27. Arrows indicate *Thinopyrum* 7Ai-1-specific RAPD fragments

region derived from 7Ai-1 was translocated to the end of the short arm of a pair of wheat chromosomes and was detected in line Y960843 ($2n = 42$) (Fig. 1, *b*). The extent of the 7Ai-1 chromatin introduced into the homozygous translocation line Y960843 covered 17 % of the full length of the short arm of the translocation. The distance between the translocation point and the kinetochore (FL) was 0.83.

*RAPD analysis.* After RAPD analysis of 7Ai-1 translocation wheat lines for linkage to BYDV resistance or susceptibility with artificially synthesized 120 random 10-mer primers, polymorphism was detected in PCR products using two kinds of primers: OPF-01 (5′-ACGGATCCTG-3′) and OPAB-01 (5′-CCGTCGGTAG-3′).

Eight DNA fragments from the 7Ai-1 addition line L1 and six from the 7Ai-1 translocation line Y95011 were amplified using the OPF-01 primer. However, only two DNA fragments from the parental cultivar of the 7Ai-1 addition line L1, Vilmorin 27, were amplified (Fig. 2, *a*). Among these, a 2100 bp DNA fragment ($F\text{-}01_{2100}$) was amplified in the 7Ai-1 addition line L1, but parental Vilmorin 27 DNA was not amplified from the 7Ai-1 translocation line Y95011. Therefore, the DNA fragment $F\text{-}01_{2100}$ is likely derived from *Th. intermedium* 7Ai-1 chromatin carrying the BYDV resistance gene.

Six DNA fragments from the 7Ai-1 addition line L1, and five from the resistant bulk extracted from the $F_2$ progeny of the cross between Y960843 and Shan 7859, were amplified using the OPAB-01 primer. Three DNA fragments from the parental cultivar of the 7Ai-1 addition line L1, Vilmorin 27, and five from the disease-susceptible bulk of the $F_2$ from the cross between Y960843 and Shan 7859, were amplified (Fig. 2, *b*). Among these, a 1500 bp DNA fragment ($AB\text{-}01_{1500}$) amplified from the 7Ai-1 addition line L1 and from the resistant bulk was not amplified in the Vilmorin 27 parental sample or the disease-susceptible bulk. Therefore, $AB\text{-}01_{1500}$ is thought to have amplified from small region of chromatin carrying BYDV resistance derived from 7Ai-1. The specific DNA fragments $F\text{-}01_{2100}$ and $AB\text{-}01_{1500}$ detected in the chromatin derived from 7Ai-1 chromosome can be used as molecular markers for BYDV resistance derived from 7Ai-1 translocated to the common wheat genome. The diagnostic band of $AB\text{-}01_{1500}$ was present in the BYDV-resistant $F_2$ plants in Y960843×Shan 7859, but was absent in the susceptible $F_2$ plants (Fig. 3).

*Cloning of a retrotransposon-like sequence of Thinopyrum intermedium, and chromosome localization.* In order to develop a DNA marker for

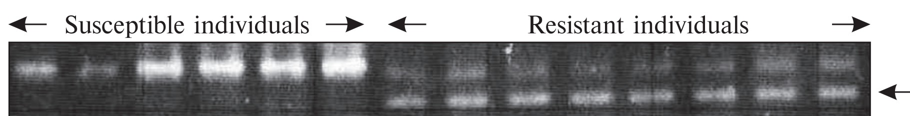



**Fig. 3.** PCR identification of BYDV-resistant individuals in Y960843 × Shan 7859 $F_2$ by *Thinopyrum* 7Ai-1-specific RAPD Primer OPAB-01. Arrows indicate *Thinopyrum* 7Ai-1-specific RAPD fragments

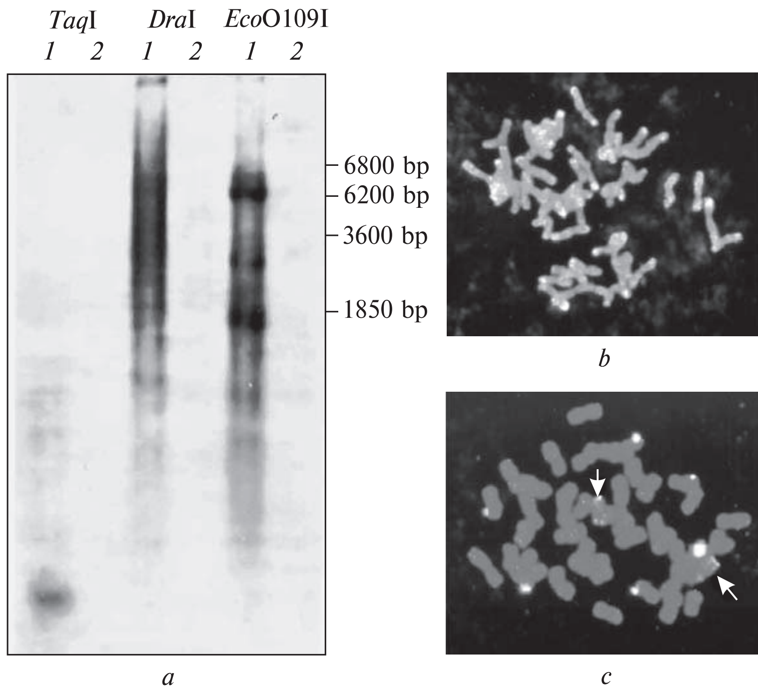
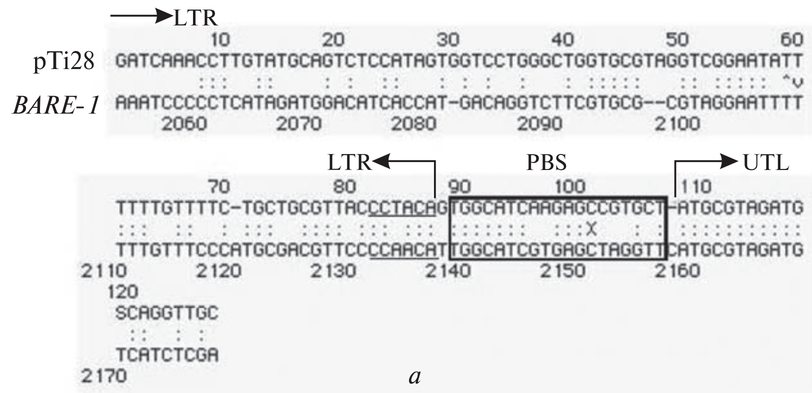



**Fig. 4.** The *Thinopyrum*-specific retrotransposon-like sequence pTi28: *a* – pTi28 showed *Thinopyrum*-specific hybridization on the genomic gel blot, *1* – *Th. intermedium; 2* – wheat; *b* – pTi28 was visualized on telomeric regions of *Thinopyrum* chromosomes; *c* – pTi28 was visualized on *Thinopyrum* segments translocated in the wheat genome. Arrows indicate *Thinopyrum* 7Ai-1 translocated chromosomes in Y960843



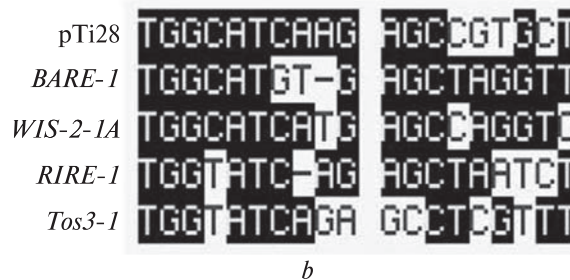




**Fig. 5.** Retrotransposon-like sequence pTi28 derived from *Th. intermedium*: *a* – pTi28 included LTR: 1–87 bp, PBS (primer binding site), 89–107 bp; UTL (untranslated leader sequence), 108–126 bp; *b* – alignment of PBS region of pTi28 and representative retrotransposons

*Th. intermedium* chromatin transferred to the wheat genome, we cloned repetitive sequences that exist specifically in the *Th. intermedium* genome. From an *Mbo*I library of genomic DNA from *Th. intermedium*, a repetitive clone, pTi28, which strongly hybridizes with *Th. intermedium*, was obtained by dot hybridization using genomic DNA from *Th. intermedium* and Chinese Spring

(CS) wheat as probes. Gel blot hybridization with the genomic DNA of *Th. intermedium* and CS using pTi28 as a probe showed that the retrotransposon pTi28 exists specifically in the *Th. intermedium* genome (Fig. 4, *a*). Furthermore, we determined the nucleotide sequence of a 126 bp-pTi28, and conducted a homology search using the GenBank database. Results of the database search showed that pTi28 contains part of the 5′ side of the LTR of a retrotransposon and PBS (Primer Binding Site), and that this region was 70.8 % homologous to Wis-2-1A of wheat (~1670–1783 bp region), 62.8 % homologous to BARE-1 of barley (~2050–2175 bp region) (Fig. 5, *a*, *b*) and 61.5 % homologous to RIRE-1 of rice (~1430–1558 bp region). The sequence pTi28 appeared to have been derived from this retrotransposon-like sequence.

When we performed fluorescence *in situ* hybridization (FISH) by labeling pTi28 with biotin-16-dUTP, hybridization signals at the telomeres and weak signals in intercalated portions were detected in all *Th. intermedium* chromosomes (Fig. 4, *b*). Furthermore, during FISH analysis of the translocated *Th. intermedium* wheat line Y960843, no signal was detected on the wheat chromosomes, but signals were certainly detected at the telomeric region of one pair of the translocated *Th. intermedium* chromosomes (Fig. 4, *c*). The signal of retrotransposon-like sequence pTi28 was considerably weaker than that of GISH (Fig. 1, *b*), because the copy number of the retrotransposon should be limited to a lower value than that of whole genomic DNA sequences. The results of the gel blot hybridization and FISH analyses indicate that the number of copies of pTi28 is quite small in wheat. As shown above, the retrotransposon-like sequence, pTi28, which is located on chromosomes of *Th. intermedium*, can be used to identify *Th. intermedium* chromatin transferred to the wheat genome.

Barley Yellow Dwarf Virus (BYDV) is one of the most serious pathogens in wheat worldwide. We developed a BYDV resistant wheat line, Y95011 and Y960843, by backcrossing common wheat and the wheat line L1 ($2n = 44$), to which the 7Ai-1 chromosome with a BYDV resistance gene from a wild relative of wheat, *Thinopyrum intermedium*, was translocated. The BYDV resistance gene of *Th. intermedium* is located on the long arm of the 7Ai-1 chromosome [3]. Banks et al. [8] introduced the chromatin derived from *Th. intermedium* 7Ai-1 carrying BYDV resistance into the wheat genome and developed six translocation lines with BYDV resistance. Among these, part of both the long and short arm of chromosome 7Ai-1 was introduced into five lines, and in one line, the chromatin derived from 7Ai-1 comprised 44 % of the full length of the long arm of chromosome 7D [15]. As mentioned above, the lines previously developed included most of the 7Ai-1 chromosome translocated to a wheat chromosome. From the two types of translocation lines found in this study, minute regions of chromatin derived from 7Ai-1 were detectable by GISH analysis. Thus, a translocation wheat line was developed for the first time which carried only the small amounts of chromatin regions flanking BYDV resistance derived from 7Ai-1. This was accomplished by inducing recombination of the 7Ai-1 chromosome with its homoeologous wheat chromosome, by crossing the CS *ph* mutant line and the CS N5BT5D line. These 7Ai-1 translocation lines will be useful as maternal lines for future breeding of BYDV-resistant wheat cultivars.

**Conclusion**. In this study, RAPD analysis was conducted on the BYDV-resistant and -susceptible lines using 120 different random primers enabling molecular markers specific to chromatin carrying BYDV resistance derived from 7Ai-1 to be characterized for the first time. Chromatin carrying the BYDV resistance gene derived from *Th. intermedium* 7Ai-1 can be detected by the DNA fragment F-$01_{2100}$ amplified using the primer OPF-01, or by the DNA fragment AB-$01_{1500}$ amplified by the primer OPAB-01. In addition, the retrotransposon-like sequence, pTi28, which is located on chromosomes of *Th. intermedium*, can be used to identify *Th. intermedium* chromatin which has been transferred to the wheat genome. As described above, in this study, a new type of BYDV-resistant wheat translocation line with minute amounts of chromatin derived from *Th. intermedium* 7Ai-1 was obtained, and molecular markers for the BYDV resistance gene were developed.

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THINOPYRUM 7AI-1-DERIVED SMALL CHROMATIN WITH BARLEY YELLOW DWARF VIRUS (BYDV) RESISTANCE GENE INTEGRATED INTO THE WHEAT GENOME WITH RETROTRANSPOSON

*Thinopyrum intermedium* является полезным источником генов устойчивости к вирусу желтой карликовости ячменя (BYDV), одного из наиболее серьезных заболеваний пшеницы. В настоящей работе транслокационные линии пшеница/*Th. intermedium* с геном устойчивости BYDV получены с использованием 7Ai-1 хромосомы *Th. intermedium*. Геномная гибридизация *in situ* (GISH) с использованием тотальной ДНК *Th. intermedium* в качестве зонда дала возможность показать наличие небольшого фрагмента хромосомы, происходящего от хромосомы 7Ai-1 и содержащего ген устойчивости BYDV, который транслоцировался в терминальний участок одной из пшеничных хромосом в каждой из линий Y95011 и Y960843. RAPD-анализ был проведен с использованием 120 случайных 10-нуклеотидных праймеров для сравнения BYDV-устойчивых транслокационных линий с восприимчивыми линиями. Два праймера амплифицировали фрагменты ДНК, специфичные для устойчивой линии, и они могут быть использованы как молекулярные маркеры для идентификации в геноме пшеницы хроматина, транслоцированного от 7Ai-1. Кроме того, выделенная *Th. intermedium*-специфичная ретротранспозон-подобная последовательность pTi28 может быть использована для идентификации хроматина *Th. intermedium*, перенесенного в геном пшеницы.

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