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Molecular cytogenetic identification of a new wheat-*Thinopyrum* substitution line with stripe rust resistance

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Abstract A new wheat-*Thinopyrum* substitution line AS1677, developed from a cross between wheat line ML-13 and wheat-*Thinopyrum intermedium* ssp. *trichophorum* partial amphiploid TE-3, was characterized by fluorescence in situ hybridization (FISH), sequential Giemsa-C banding, genomic in situ hybridization (GISH), seed storage protein electrophoresis, molecular marker analysis and disease resistance screening. Sequential Giemsa-C banding and GISH using *Pseudoroegneria spicata* genomic DNA as probe indicated that a pair of St-chromosomes with strong terminal bands were introduced into AS1677. FISH using pTa71 as a probe gave strong hybridization signals at the nuclear organization region and in the distal region of the short arms of the St chromosome. Moreover, FISH using the repetitive sequence pAs1 revealed that a pair of wheat 1D chromosomes was absent in accession AS1677. Seed storage proteins separated by acid polyacrylamide gel electrophoresis (APAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed that AS1677 lacked the gliadin and glutenin bands encoded by *Gli-D1* and *Glu-D1*, further confirming the absence of chromosome 1D. The introduced St chromosome pair belonging to homoeologous group 1 was identified by newly produced genome specific markers. AS1677 is a new 1St (1D) substitution line. When inoculated with stripe rust and powdery mildew isolates, AS1677 expressed stripe rust resistance possibly derived from its *Thinopyrum* parent. AS1677 can be used as a donor source for introducing novel disease resistance genes to wheat in breeding programs aided by molecular and cytogenetic markers.

Keywords C-banding · In situ hybridization · Stripe rust resistance · *Thinopyrum intermedium* ssp. *trichophorum* · Alien substitution line

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Introduction

Stripe rust, caused by *Puccinia striiformis* Eriks. f. sp. *tritici*, is one of the most important foliar diseases of common wheat (*Triticum aestivum* L.) in the world. Yield reductions due to stripe rust can be as high as 75% in a severe epidemic on susceptible cultivars (Roelfs et al. 1992). Breeding resistant cultivars is the most economical method to control the disease. Many

stripe rust resistance genes have been identified and incorporated into high-yielding cultivars; however, many of the available resistance genes have been overcome by newly emerged races. The wild relatives of wheat provide a vast reservoir of disease resistance genes for wheat. Previous studies succeeded in transferring stripe rust resistance genes from *Secale*, *Aegilops* and *Dasypyrum* species to wheat (for references see McIntosh et al. 2008).

The wheatgrass, *Thinopyrum intermedium* (Host) Barkworth and Dewey (=*Agropyron intermedium* (Host) P. B.), a hexaploid species (2n = 6x = 42) with genomes E_1E_2St (Wang et al. 1994) or JJ^sSt (Chen et al. 1998), consists of two subspecies, namely intermediate wheatgrass, ssp. *intermedium*, and a close pubescent relative, ssp. *trichophorum* (Dewey 1984). *Th. intermedium* ssp. *intermedium* was hybridized extensively with wheat and proved to be a valuable source of genes for disease resistance (Larkin et al. 1995; Tang et al. 2000; Chen et al. 2003; Li and Wang 2009). We produced a wheat—*Th. intermedium* ssp. *trichophorum* partial amphiploid (TE-3) and found resistance to several foliar diseases including stripe rust (Yang et al. 2006).

In order to further introduce novel resistance from the *Th. intermedium* ssp. *trichophorum* to wheat, the production of wheat-*Thinopyrum* chromosome addition, substitution and translocation lines was an important step. Identifying wheat alien chromosomes and detecting genetic modifications in a wheat background are essential prior to their use in breeding programs (Gill 1987; Jiang et al. 1994; Schwarzacher 2003; Zhang et al. 2007).

Genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH) and C-banding are widely used and effective methods for identifying alien chromosomes and chromosome segments in wheat (Kato et al. 2005). Sequential C-banding and GISH are extremely useful in identifying wheat-alien introgressions (Jiang and Gill 1993). Multi-color FISH and an improved St-genome probe allowed the identification of wheat-*Thinopyrum* derivatives (Han et al. 2004; Chen 2005; Ji et al. 2009). Diverse molecular markers, including genome-specific and chromosome-specific markers, also enable reliable identification of alien chromosomes in wheat backgrounds (Zhang and Dvořák 1990; Li et al. 2007; Liu et al. 2009). In this study, we used both molecular and cytogenetic techniques to identify a new wheat-*Th. intermedium* ssp. *trichophorum* substitution line, and found that the *Thinopyrum* derived St-chromosomes contained new gene(s) for stripe rust resistance in wheat.

Materials and methods

Plant materials

Th. intermedium ssp. *trichophorum* accession PI440125 and *Pseudoroegneria spicata* (Pursh) A. Love (St genome, 2n = 2x = 14) accession PI 232131were obtained from the USA National Small Grains Collection at Aberdeen, Idaho. The wheat-*Th. intermedium* ssp. *trichophorum* partial amphiploid, TE-3 (Yang et al. 2006), and wheat lines SY95-71 and ML-13 are maintained at the Triticeae Research Institute, Sichuan Agricultural University, China.

Sequential C-banding and GISH

Seedling root tips were collected, pretreated in water at 0°C for 24 h and fixed in ethanol-acetic acid (3:1) for 1 week. Root-tip squashes were stained using the conventional Feulgen method for chromosome counting. C-banding was done according to Gill et al. (1991). For GISH analysis, total genomic DNA from *Ps. spicata* was labeled with digoxigenin-11-dUTP by nick translation following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Sheared genomic DNA of Chinese Spring wheat (CS, genomes ABD, 2n = 42) was used as blocking DNA. The hybridization mixture was prepared as described by Mukai et al. (1993). The GISH signal was detected with fluorescein-conjugated antidigoxigenin antibody (Roche Diagnostics, Indianapolis, IN), and the slide was mounted in propidium iodide dissolved in Vectrashield® antifade solution (Vector Laboratories, Burlingame, CA). Microphotographs of C-banded and GISH chromosomes were taken with an Olympus BX-51 microscope using a DP-70 CCD camera.

Fluorescence in situ hybridization

The probe pAs1, containing a 1-kb DNA fragment isolated from *Ae. tauschii* Coss. in the plasmid pUC8 (Rayburn and Gill 1986a), and pTa71, an 18S–5.8S–26S rDNA repetitive unit, isolated from *T. aestivum* (Gerlach and Bedbrook 1979), were generously

provided by Dr. B. Friebe, Wheat Genetic and Genomic Resources Centre, Department of Plant Pathology, Kansas State University, USA. The plasmid insert was labeled with digoxigenin-11-dUTP; hybridization and the detection were the same as in the GISH protocol.

Seed storage protein electrophoresis

Acid polyacrylamide gel electrophoresis (APAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to separate endosperm gliadin proteins and glutenin subunits, respectively. The procedures were described by Yang et al. (2001).

Disease resistance screening

Wheat line AS1677 and its parents were evaluated for adult-plant resistance to *P. striiformis* f. sp. *tritici* strains CRY30, CRY-31 and CRY-32, which were provided by the Plant Protection Institute, Sichuan Academy of Agricultural Sciences. During the 2008 and 2009 cropping seasons, adult plants were inoculated with these strains in the field at Qionglai city, Sichuan. Infection types (IT) were evaluated 2–3 weeks after inoculation when uredinia were fully developed. Stripe rust responses were recorded following Ma et al. (1995).

The seedling plants were evaluated for resistance to powdery mildew (*B. graminis* f. sp. *tritici*) using the procedure adopted by Ren et al. (2009).

Molecular marker analysis

DNA was extracted from fresh leaves of accession AS1677 and parental lines *Ps. spicata* and CS (Yang et al. 2006). PCR-based Landmark Unique Gene (PLUG) primers TNAC1021 (F: CTCATGCATGCG TTTGTTAAA, R: CCAGCTGAAACAAGCATCTTC) were made according to Ishikawa et al. (2009). Polymerase chain reaction (PCR) was performed in an Icycler thermalcycler (Bio-RAD Laboratories, Emeryville, CA) in reaction volumes of 25 µl, 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 primer. The cycling parameters were 94°C for 3 min for predenaturing; followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; and then a final extension at 72°C for 10 min. The amplified products were cloned and sequenced.

Results

Morphology and chromosome number of AS1677

The CS-*Th. intermedium* ssp. *trichophorum* partial amphiploid TE-3 was crossed to wheat line ML-13, then backcrossed and self-fertilized. Among BC_1F5 progenies, we selected line AS1677, which exhibited similar agronomic traits and high seed set similar to the ML-13 wheat parent.

Phenotypically, adult AS1677 plants were 90–105 cm in height, produced 7–10 spikes per plant, and a higher tilling ability than ML-13 (3–5 spikes). Plants of AS1677 had 20–23 spikelets per spike, closely resembling ML-13, but with longer spikes, a trait possibly inherited from *Th. intermedium* ssp. *trichophorum*. The pubescence on the heads and leaves of *Th. intermedium* ssp. *trichophorum* was not present in AS1677 plants.

Root-tip chromosome counts and meiotic observation of PMCs on 20 AS1677 plants showed that all had 42 somatic chromosomes and 21 bivalents at meiotic MI indicating line stability.

Sequential chromosome C-banding and in situ hybridization

C-banding was performed on AS1677 chromosomes at mitotic metaphase. As shown in Fig. 1a, 14 chromosomes were heavily banded and represented wheat chromosomes 1B–7B. Two of the remaining chromosomes had telomeric bands at the ends of the long arm and were presumably derived from *Thinopyrum*.

GISH, using total genomic DNA from *Ps. spicata* as a probe, on mitotic metaphase chromosomes of AS1677 is shown in Fig. 1b. The probe hybridized uniformly along the entire chromosome lengths, indicating that the two chromosomes were from *Th. intermedium* (Chen et al. 1998) and were St-genome chromosomes. However, neither ends the St chromosomes hybridized with the *Ps. spicata* probe.

A total of ten mitotic metaphase chromosomes in AS1677 contained hybridization signals from FISH with probe pTa71 (Fig. 1c). Two pairs of major

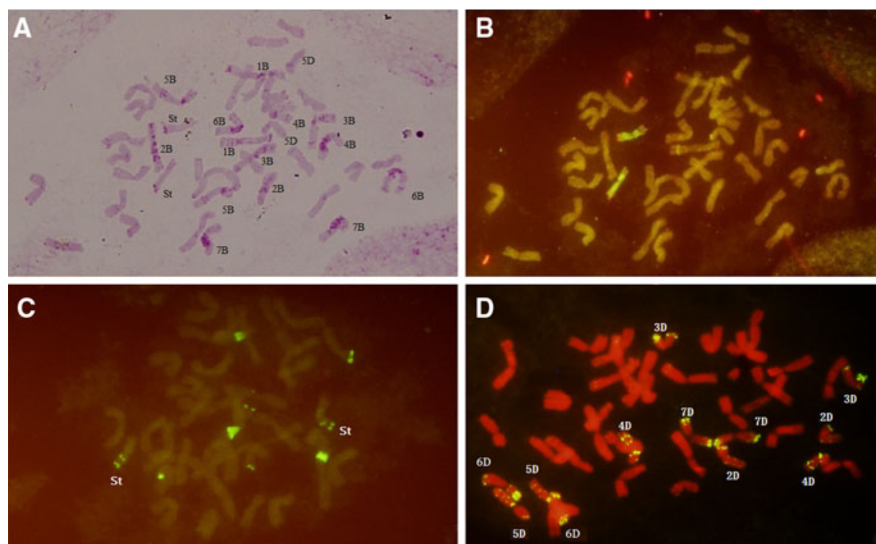

Fig. 1 Giemsa-C banding (**a**) and fluorescent in situ hybridization (**b**, **c**, and **d**) of AS1677. The probes used for in situ hybridization were *Ps. spicata* genomic DNA (**b**), pTa71 (**c**), and pAs1 (**d**)

hybridization signals occupied the secondary constriction of the 1B and 6B chromosomes, and two other minor hybridization signal pairs occurred at the ends of the 5D and 2A chromosome short arms. The hybridization of pTa71 to the St-genome chromosomes in AS1677 was located on both secondary constrictions and the end of the short arms.

We used the FISH of line AS1677 to determine which wheat chromosomes were substituted by *Th. intermedium* St-genome chromosomes. The presence of wheat D-genome chromosomes was demonstrated using probe pAs1. As shown in Fig. 1d, a total of 12 chromosomes clearly hybridized in AS1677. Compared with the standard pAs1 hybridization signals (Rayburn and Gill 1986b) we deduced that line AS1677 lacked a pair of 1D chromosomes.

Seed protein electrophoresis

APAGE of seed gliadin storage proteins revealed that CS, TE-3, ML-13 and AS1677 all produced distinctive bands in the ω, γ, β and α zones (Fig. 2a). Most of the AS1677 gliadin bands clearly originated from the parental ML-13 ω, γ and β zones whereas those in the α zone were likely from TE-3. Two particularly pronounced ω bands (arrows) in CS and TE-3, encoded by *Gli-D1* on short arm of chromosome 1D (Gianibelli et al. 2001), apparently were absent in AS1677.

The high-molecular-weight glutenin subunit (HMW-GS) composition of AS1677 and its parents TE-3 and ML-13 were analyzed by SDS-PAGE (Fig. 2b). The HMW-GS in TE-3 included *Glu-A1* null, *Glu-B1* subunits 7 + 8, and *Glu-D1* subunits 2 + 12. In ML-13, *Glu-A1* subunit 2*, *Glu-B1* subunits 17 + 18 and *Glu-D1* subunits 5 + 10 were present. Line AS1677 had the *Glu-A1* 2* subunit and

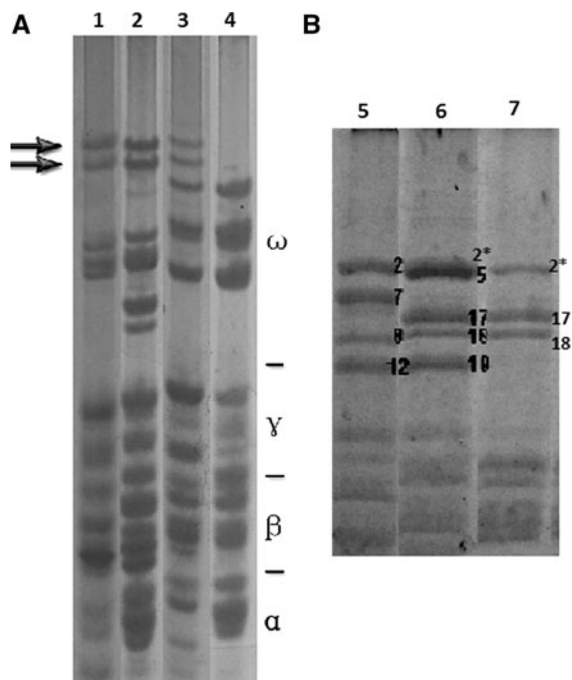

Fig. 2 Gliadin proteins separated by APAGE (**a**) and glutenins separated by SDS–PAGE (**b**) of CS (*lane 1*), TE-3 (*lane 2*, *5*), ML-13 (*lanes 3*, *6*) and AS1677 (*lanes 4*, *7*). Arrows indicate gliadin encoded by *Gli-D1*

Glu-B1 subunits 17 + 18, but there were no bands for the *Glu-D1* locus, which is on the long arm of chromosome 1D.

Molecular markers

The PLUG primers TNAC1021 amplified diagnostic fragments in wheat chromosomes 1A, 1B and 1D, respectively, in nulli-tetrasomic lines. The *Ps. spicata* and AS1677 lines produced a band on 1A and a strong band containing 1B and 1St (Fig. 3). The 1D-specific fragment was not amplified in AS1677, which also confirmed the lack of chromosome 1D in line AS1677. The sequences of the bands indicated that the A-, B- and D-genome specific bands were 1128, 1260 and 1430 bp, respectively. The St-genomic bands from *Ps. spicata* and AS1677 were 1,286 and 1,283 bp, respectively, and sequence alignment indicated that the St-derived bands had 99% homology to the St bands in AS1677 (Fig. 4). Similarly, another PLUG primer, TNAC1026, specific to homoeologous group 1 also generated the St band from AS1677 (data not shown). These results suggested that the St-chromosome in AS1677 belonged to homoeologous group 1.

Disease responses

Line AS1677 and the parental lines TE-3 and ML-13 were inoculated with *Blumeria graminis* f. sp. *tritici* isolates (seedling) and *P. striiformis* f. sp. *tritici* races CYR30, CYR31 and CYR32 (adult plant). TE-3 was immune to these isolates, whereas wheat parent ML-13 was highly susceptible. AS1677 was highly resistant to stripe rust (Table 1) but susceptible to powdery mildew. These results indicated that the stripe rust resistance in AS1677 was from the TE-3 parent, and was traced to *Th. intermedium* ssp. *trichophorum*.

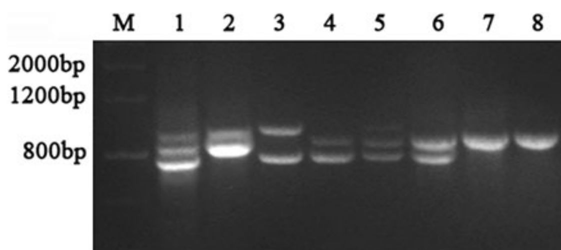

Fig. 3 PCR using PLUG primers TNAC1021. *M*: DNA ladder; *lane 1*: Chinese Spring; *lane 2*: nullisomic 1A-tetrasomic 1B (N1A-T1D); *lane 3*: N1B-T1D; *lane 4*: N1D-T1A; *lane 5*: ML13; *lane 6*: AS1677; *lane 7*: *Th. intermedium*; *lane 8*: *Ps. spicata*

Discussion

Chen (2005) listed various wheat-*Th. intermedium* partial amphiploid and derivatives including 25 wheat-*Th. intermedium* addition and substitution lines. The substituted or added *Thinopyrum* chromosomes were distributed on the St, J or J^s genomes and in different homoeologous groups. Wheat-*Thinopyrum* derivatives were screened for responses to leaf rust, stem rust, powdery mildew, barley yellow dwarf and wheat streak mosaic viruses (Friebe et al. 2009; He et al. 2009; Luo et al. 2009; Ohm et al. 2005; Zhang et al. 1999); however, few wheat-*Th. intermedium* germplasm lines were tested for resistance to stripe rust (Yang et al. 2006; Bao et al. 2009). Tang et al. (2000) tested derivatives from wheat-*Th. intermedium* partial amphiploid Zhong 5 and concluded that the St genome transferred to wheat did not condition stripe rust resistance. Other *Th. intermedium*-derived St chromosomes addition lines (Tang et al. 2006) and J chromosome addition lines (Shi et al. 2008) appeared to be resistant to stripe rust. Wheat-*Th. intermedium* introgression lines derived from different *Thinopyrum* parental lines will increase the potential of successful breeding for stripe rust resistance in wheat. In this study, we produced line AS1677 in which an St chromosome pair was derived from *Th. intermedium* ssp. *trichophorum*. AS1677 is a new 1St(1D) substitution line with high resistance to stripe rust, and contains a novel HMW-GS glutenin subunit combination of *Glu-A1* and *Glu-B1* while lacking *Glu-1D*. It could thus be interesting germplasm for both quality and disease resistance breeding.

GISH analysis using *Ps. strigosa* DNA as a probe readily distinguished three pairs of satellite chromosomes in *Th. intermedium*; viz. two J^S pairs and one pair of St chromosomes (Tang et al. 2000; Friebe et al. 1992a, b; Li et al. 2004). Based on studies of the SAT chromosomes in the addition lines derived from the wheat-*Th. intermedium* partial amphiploid TAF46, the addition line L5 contains a pair of *Th. intermedium* chromosomes with two minor satellites (SAT1) in the short arms and belongs to

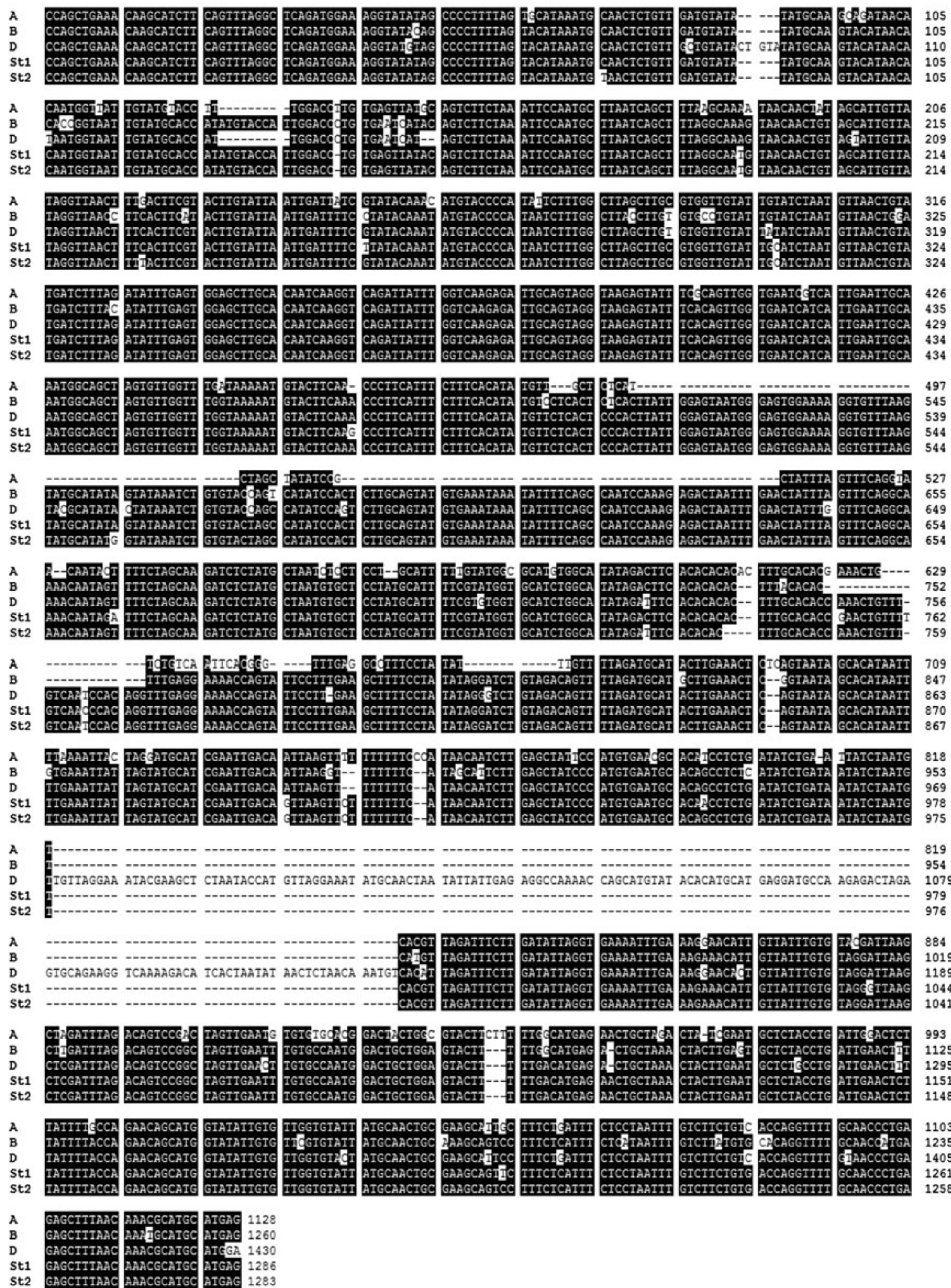

Fig. 4 ClustalW alignment of the nucleotide sequences from different genomes. **a**, **b** and **d**: sequences from the three genomes of *T. aestivum*; *St1* sequence from *Ps. spicata* 2; *St2* sequence from AS1677. Base similarities are indicated with *black boxes*; gaps introduced to optimize alignment are indicated with *dashes*

Table 1 Responses to *P. striiformis* f. sp. *tritici* races and genomic structures of AS1677 parents, and controls

Line	2n=	Alien chromosome type	Response to *P. s. tritici* race		
			CYR30	CYR31	CYR32
CS	42	–	S	S	S
Th. intermedium	42	14St + 14J + 14J^s	R	R	R
TE-3	56	8St + 4J + 2J^s + 2S/J	R	R	R
ML-13	42	–	S	S	S
AS1677	42	2St(1D)	R	R	R

R resistant, *S* susceptible

homoeologous group 5 (5J), whereas addition line L3 contains a pair of *Th. intermedium* chromosomes with two major satellites (SAT 1) in the short arms, and belongs to homoeologous group 1 (1J) (Forster et al. 1987; Friebe et al. 1992a). The shortest chromosome, SAT3, which is entirely labeled with the St genome probe, is homoeologous to group 1 (1St). RFLP analysis indicated that the added *Th. intermedium* in Z3 belonged to homoeologous group 1 (Larkin et al. 1995). Tang et al. (2000) showed that only one pair of St chromosomes had satellites in Zhong 5 and *Th. intermedium*, and stated that the St chromosomes with small satellites in *Th. intermedium* (SAT3), Zhong 5 and Z3 were identical. Our results indicated that both ends of the St chromosomes in AS1677 did not full hybridize in GISH with St genomic DNA, and similar results also were observed in the TE-3 amphiploid (Yang et al. 2006). It is likely that the parts without hybridization were the strongly C-banded heterochromatin regions (Fig. 1). Moreover, the satellites at the ends of the short arms of the St-chromosomes in AS1677 were generally suppressed in mitotic metaphase. Because the distribution of C-banding patterns and the pTa71 hybridization signals of St chromosomes in AS1677 differed from those in Z3 (Supplementary Fig. S1), we conclude that the St chromosome contained in AS1677 is not the same as that in Z3 and, therefore, is named 1St#2.

Molecular markers, especially markers from the conserved coding region, are useful for determining the homoeologous relationships of chromosomes from different grass species by comparative mapping (van Deynze et al. 1998; Heslop-Harrison 2000). PCR-based markers are more convenient in terms of manipulation and application. More recently, EST-PCR markers were used extensively as effective tools for genetic analysis. However, PCR products derived from orthologous genes are often simultaneously amplified with length polymorphisms. By using the similarities in gene structures between rice and wheat, Ishikawa et al. (2007) developed the PLUG system to design primers that amplified conserved EST regions including intron sequences, so that PCR products could be used as accurate anchor markers for genomic research when comparing rice and wheat homoeologous chromosome regions (Ishikawa et al. 2009). We used the PLUG marker TNAC1021 to amplify the A-, B-, D- and St-genome chromosomes. This marker easily distinguishes the different genomes by the different amplified lengths. Because the TNAC primer specific to the WD40-like domain containing protein on chromosome 5 of rice is presumably orthologous to wheat chromosome 1, we can assume that the marker can be used to trace linkage group 1 in Triticeae species. Based on sequence comparison, we determined that the introduced St chromosomes belong to homologous group 1 and note that PLUG markers can be an effective system when assigning genome homologies in Triticeae species.

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