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ARTICLE

Molecular cytogenetic identification of a wheat–rye 1R addition line with multiple spikelets and resistance to powdery mildew

Wujuan Yang, Changyou Wang, Chunhuan Chen, Yajuan Wang, Hong Zhang, Xinlun Liu, and Wanquan Ji

Abstract: Alien addition lines are important for transferring useful genes from alien species into common wheat. Rye is an important and valuable gene resource for improving wheat disease resistance, yield, and environment adaptation. A new wheat–rye addition line, N9436B, was developed from the progeny of the cross of common wheat (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD) cultivar Shaanmai 611 and rye (*Secale cereal* L., 2*n* = 2*x* = 14, RR) accession Austrian rye. We characterized this new line by cytology, genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH), molecular markers, and disease resistance screening. N9436B was stable in morphology and cytology, with a chromosome composition of 2*n* = 42 + 2t = 22II. GISH investigations showed that this line contained two rye chromosomes. GISH, FISH, and molecular maker identification suggested that the introduced R chromosome and the missing wheat chromosome arms were 1R chromosome and 2DL chromosome arm, respectively. N9436B exhibited 30–37 spikelets per spike and a high level of resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) isolate E09 at the seedling stage. N9436B was cytologically stable, had the trait of multiple spikelets, and was resistant to powdery mildew; this line should thus be useful in wheat improvement.

Key words: wheat–rye addition line, multiple spikelets, powdery mildew resistance, GISH and FISH, molecular makers.

Résumé : Les lignées d'addition exotiques constituent un moyen important pour transférer des gènes utiles d'espèces exotiques au blé. Le seigle constitue une ressource génétique importante et de grande valeur pour augmenter la résistance aux maladies, le rendement et l'adaptation environnementale chez le blé. Une nouvelle lignée d'addition blé–seigle, N9436B, a été développée à partir de la descendance du croisement entre le cultivar Shaanmai 611 du blé tendre (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD) et l'accession Austrian du seigle (*Secale cereale* L., 2*n* = 2*x* = 14, RR). Les auteurs ont caractérisé cette nouvelle lignée par le biais d'analyses de cytologie, d'hybridation génomique in situ (GISH), d'hybridation in situ en fluorescence (FISH), de marqueurs moléculaires et de la résistance à une maladie. La lignée N9436B s'est montrée stable tant en matière de morphologie que de cytologie, avec une formule chromosomique de 2*n* = 42 + 2t = 22II. Les analyses GISH ont montré que cette lignée possédait deux chromosomes du seigle. Les analyses GISH, FISH et les marqueurs moléculaires ont suggéré que le chromosome R introduit et le bras chromosomique manquant étaient respectivement le 1R et le 2DL. N9436B présente 30 à 37 épillets par épi et un niveau élevé de résistance à l'isolat E09 du blanc (*Blumeria graminis* f. sp. *tritici*, *Bgt*) au stade plantule. N9436B affichait une stabilité cytologique, de nombreux épillets et la résistance au blanc; cette lignée devrait ainsi s'avérer utile en amélioration génétique du blé. [Traduit par la Rédaction]

Mots-clés : lignée d'addition blé–seigle, épillets multiples, résistance au blanc, GISH et FISH, marqueurs moléculaires.

Introduction

Rye (*Secale cereale* L., 2*n* = 2*x* = 14, RR), a species closely related to wheat (*Triticum aestivum* L., 2*n* = 6*x* = 42, AABBDD), has been used extensively and successfully as a valuable and significant germplasm resource for wheat cultivar improvement in improving disease resistance, quality, yield, and environment adaptation (Friebe et al. 1996). Importing the exogenous gene of rye into wheat can lead to genetic variation, producing new materials that are of important value.

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The production of single spikes and yield has shown significant positive correlation on the basis of certain spikes number (Song et al. 1996), so the improvement of single spike productivity is the most important way to improve wheat yield, which has drawn wide-spread attention from international breeders (Smocek 1988; Li 1993; Ren 1995; Song et al. 1996). Multiple spikelets of rye, from 33 to 40, is a potential valuable source of genes for wheat yield improvement. Transferring rye's multiple spikelet characteristic to common wheat can cultivate wheat germplasms with multiple spikelets. To date, the multiple spikelets line 10-A, with 30–37 spikelets per spike, has been developed (Yen et al. (1993). 10-A was derived from the cross between an octaploid triticale and the common wheat cultivar Avrora, and the octaploid triticale was artificially synthesized from the common wheat cultivar Yaanai No.10 and *S. cereale* accession Qinling rye (Yen et al. 1993). Avrora was proven to be a 1B/1R translocation line (Zeller 1973), and 10-A was proven to carry the 1RS/1BL wheat–rye translocation chromosome (Wei et al. 1999). Therefore, rye is a potential reservoir for the improvement of wheat yield.

Powdery mildew of wheat caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) is one of the most damaging diseases. The pathogen can attack all above-ground wheat parts, including leaves, stems, and spikes, and is the most serious wheat disease effecting the production of wheat in China and other parts of the world. Powdery mildew can cause significant yield losses in most of the wheat production areas. Powdery mildew yield losses ranging from 17% to 34% have been reported (Johnson et al. 1979; Leath and Bowen 1989). Therefore, the deployment of resistant cultivars is the most reliable, economical, and environmentally safe approach to cope with this disease (Bennett 1984). To date, approximately 80 formally designated *Pm* genes have been identified at 49 loci in wheat and its wild relatives (*Pm1*–*Pm54*, *Pm18* = *Pm1c*, *Pm22* = *Pm1e*, *Pm23* = *Pm4c*, *Pm31* = *Pm21*, *Pm8* is allelic to *Pm17*), with the loci *Pm1*, *Pm2*, *Pm3*, *Pm4*, *Pm5*, and *Pm24* having 5, 3, 17, 4, 5, and 2 alleles, respectively (Hao et al. 2008; Hsam et al. 1998; McIntosh et al. 2013, 2014; Singrün et al. 2003; Xie et al. 2012; Ma et al. 2015; Hao et al. 2015; Xu et al. 2015). Among these genes or alleles, approximately 40 were derived from *T. aestivum*, whereas the others originated either from species closely related to common wheat, such as *T. monococcum*, *T. turgidum*, *T. timopheevii*, or from different genera, such as *Secale*, *Aegilops*, *Haynaldia*, and *Elytrigia* (Xiao et al. 2013). Rye offers a rich reservoir of genes for enhancing useful genetic variability in wheat breeding. The powdery mildew resistance genes derived from rye are *Pm7*, *Pm8*, *Pm17*, and *Pm20*, located on the 2RL, 1RS, 1RS, and 6RL chromosomes, respectively. Some of these resistant genes have already been successfully used in commercial wheat production. The extensive utilization of these resistant genes may make them susceptible to new pathogen races because of co-evolution of the host and pathogen, and thus the cultivars with these resistance genes may, over time, lose their resistance to pathogens. After widespread agricultural cultivation, the gene *Pm8* is now widely overcome by adapted mildew races (Lutz et al. 1992; Yang and Ren 1997). Therefore, it is important to identify and deploy new resistant gene sources in other rye genotypes. It has been reported that rye chromosomes 4R, 5R, and 6R carry powdery mildew resistant genes (Friebe et al. 1994; An et al. 2013; Fu et al. 2010, 2011, 2014*a*).

Distant hybridization can transfer the desirable traits from wild relatives into common wheat and promote the new alien germplasms with advantageous exogenous genes (Anamthawat-Jónsson 1995), including amphidiploids, addition, substitution, and translocation lines. Traditionally, addition line is not only a genetic material to research the origin and evolutionary of species, the relationship between genomes, and the interaction and expression of genes, but also an intermediate material playing a bridging role for developing substitution, translocation, and introgression lines in wheat breeding. To date, complete sets of wheat–rye addition lines have been produced including Holdfast–KingII, Kharkov–Dakold, Chinese Spring (CS)–Imperial, and CS–KingII (Xue et al. 1993). In addition, other wheat–rye addition lines have been reported (O'Mara 1940; Hu and Wang 1990; Liu and Xin 1993; Fu et al. 2011). Other rye genotypes should be used to create different wheat–rye addition lines for potential utilization in wheat improvement.

Winter rye cultivar Austrian rye (*S.cereale* L.) is a valuable resistant resource for wheat improvement owing to its superior and wide resistance to various isolates of powdery mildew pathogens prevalent in China. Common winter wheat cultivar Shaanmai 611 possesses the characteristics of high-yielding, dwarf in stature, and wide environmental adaptation. A new wheat–rye 1R chromosome addition line, N9436B, derived from the cross of Shaanmai 611 and Austrian rye has the characteristics of multiple spikelets and shows a high level of resistance to powdery mildew. The objectives of this study were to determine the genomic composition of N9436B using molecular cytogenetic methods, characterize its resistance to powdery mildew, and evaluate its agronomic performance.

Materials and methods

Plant materials

A wheat–rye addition line was produced by crossing winter wheat cultivar Shaanmai 611 with winter rye accession Austrian rye. Shaanmai 611 and Austrian rye were employed as controls in the agronomic trait assessment and in the DNA marker and electrophoretic analyses. Kavkaz, with the gene *Pm8*, Amigo, with the gene *Pm17*, both derived from rye chromosome 1RS, and wheat cultivar Shaanyou 225 were conserved in the College of Agronomy, Northwest A&F University, Yangling,

Shaanxi Province, China. These materials were used in this study as controls for testing resistance to powdery mildew. Total DNA extracted from rye cultivar Austrian rye was used as probe in genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) detection. Total DNA extracted from an 1R addition line of CS × Imperial was used as control to detect the Austrian rye chromosome in N9436B by polymerase chain reaction (PCR) analysis. All plant materials were maintained by strict selfing in the field of Northwest A&F University.

Production of wheat–rye chromosome addition line N9436B

Wheat cultivar Shaanmai 611 crossed with Austrian rye was performed in 1994. F_1 hybrids of this cross, which possessed the multiple spikelet property, were selected and maintained by strict selfing. Among the offspring, plants with the trait of multiple spikelet and resistance to powdery mildew were selected. Finally, N9436B, with multiple spikelets, resistance to powdery mildew, and a genetically stable genotype with chromosome composition of $2n = 42 + 2t = 22II$, was obtained.

Evaluation of agronomic performance

The wheat–rye derivative N9436B and its parents Shaanmai 611 and Austrian rye were planted in early October and harvested in the middle of June the following year. From seedling to maturity, growth conditions of N9436B and their parents were observed and recorded. Preharvest, 10 plants of each material were randomly selected, and the following traits were measured and recorded: plant type, plant height, spike length, spikelets per spike, kernels per spike, and resistance to powdery mildew. Postharvest, the characters of the kernel and thousand-kernel weight of each material were measured and recorded.

Identification of the cytology

The chromosome number of the root tip cell

When the roots were 1.5–2.0 cm in length, the root tips were removed and pretreated with ice-water at 0–4 °C for 24 h and fixed in Carnoy's fixative fluid (a 3:1 ethanol – acetic acid mixture) at 4 °C for at least 2 days. The root tips were stained with 1% (*w*/*v*) aceto-carmine solution overnight and then squashed in 45% (*v*/*v*) acetic acid. Finally, the chromosomes were counted and photographed using an Olympus BX-43 microscope (Japan) equipped with a PhotometricsSenSys CCD camera. In total, 30 or more cells were observed.

The configuration of pollen mother cell at metaphase I

Young spikes were sampled at appropriate stages, between 07:00 and 09:00, at a temperature of ~12–17 °C in early April 2014. The spikes were put into a 6:3:1 ethanol – chloroform – acetic acid mixture for at least 48 h. The anthers were then removed and squashed in 1% aceto-carmine solution. Finally, the cells at metaphase I, with a complete chromosome complement, were photographed using an Olympus BX-43microscope (Japan) equipped with a PhotometricsSenSys CCD camera. In total, 30 or more cells were observed.

Identification of resistance to powdery mildew

Assessment at seedling stage

Powdery mildew reactions at the seedling stage of N9436B were assessed via inoculation with *Bgt* isolate E09 (kindly provided by Xiayu Duan and Yilin Zhou, State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China). N9436B, its parents Shaanmai 611and Austrian rye, Shaanyou 225, Kavkaz, and Amigo were each potted with 20 seeds; Shaanyou 225 was used as the susceptible control. *Bgt* isolate E09 was maintained on Shaanyou 225 until the leaf was fully expanded by conidia. Plants were inoculated by dusting conidia from sporulating seedlings of Shaanyou 225 at the two to three leaf stage, and then transferred to a temperature-controlled greenhouse in the College of Agronomy, Northwest A&F University, Yangling, Shaanxi, China. After inoculated for approximately15 days, when the pustules were fully developed on Shaanyou 225, the plants were investigated and infection types (IT) were recorded; the procedure was repeated after 3 days. IT was recorded based on a 0–4 scale, of which 0 = immune, no visible symptoms and signs; 0; = almost immune, necrotic flecks without sporulation; 1 = high resistance, sparse aerial hypha and little sporulation, with diameter of colonies less than 1 mm; 2 = medium resistance, moderate aerial hypha and sporulation, with diameter of colonies less than 1 mm; 3 = medium susceptibility, thick aerial hypha and abundant sporulation, with diameter of colonies more than 1 mm; and 4 = high susceptibility, abundant sporulation with more than 80% of the leaf area covered with aerial hypha. Plants with an IT score of 0–2 were considered resistant, while those with an IT score of 3–4 were considered susceptible (Si et al. 1992).

Assessment at adult stage

Resistance to powdery mildew at the adult stage was tested on N9436B, its parents Shaanmai 611and Austrian rye, Kavkaz, and Amigo in the powdery mildew disease nursery at the College of Agronomy, Northwest A&F University, Yangling, Shaanxi, China, using a mixture of *Bgt* isolates prevalent in Guanzhong region of Shaanxi Province in China. Individual plants were spaced 10 cm apart within 1 m long rows, with row spacings of 25 cm. As the susceptible control, Shaanyou 225 was planted around the nursery. The tests with the mixture of the isolates were conducted using the procedures described by Duan et al. (1998). After wheat heading, and when the susceptible control Shaanyou 225 were fully infected, powdery mildew resistance was investigated and the level recorded for the test materials. Disease reaction was assessed on a 0–9 scale, of which 0 = whole plant

Table 1. Summary of SSR and EST-STS polymorphic markers applied to analysis introduced 1R chromosome of Austrian rye.

Marker	Type	Primer (5′–3′)	Location	Annealing temperature (°C)
TSM716	SSR	F: GTGCTCGTCCCACTTGATTC R: GCATGGAGAGGACGTTTGAC	1RS	60
NOR-1	STS-PCR	F: GCATGTAGCGACTAACTCATCG R: CCCAGTTTTCCATGTCGC	1RS	55
NOR-R1	STS-PCR	F: GACTGTAGCGACTAACTCATC R: CCCAGTTTTCCATGTCGC	1R	55

disease-free after heading; 1–2 = high resistance, plant disease extended to the top fourth leaf; 3–4 = medium resistance, the disease extended to the top third leaf; 5–6 = medium susceptibility, the disease extended to the second leaf; 7–8 = high susceptibility, flag leaf has disease; and 9 = complete susceptibility, the disease extends to the spike. The adult stage assessment was repeated in the following year's growing season using the same procedure.

DNA extraction

The genomic DNA of common wheat Shaanmai 611, Austrian rye, and wheat – rye addition line, N9436B, were isolated from seedling leaves using a modified CTAB method (Doyle and Doyle 1987), with one additional purification step using chloroform to obtain high-quality DNA, which were used for GISH, FISH, and molecular marker analysis.

Molecular marker screening and electrophoretic analysis

Polymerase chain reaction (PCR) assay was used to detect the alien chromosome in wheat–rye addition line N9436B. The materials including Shaanmai 611, Austrian rye, N9436B, and an 1R addition line of CS × Imperial.

To detect the alien chromosome in wheat–rye addition line N9436B, one SSR marker, *TSM716*, specific for rye chromosome arm 1RS, and two STS-PCR markers, *NOR-R1* and *NOR-1* (Koebner 1995), specific for rye chromosome 1R and rye chromosome arm 1RS, respectively (Table 1), were used. The primers were all synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. DNA amplification was conducted in a 10 μL reaction volume containing 6.54 μL of double-distilled water, 1.0 μL of 10× PCR buffer, 0.8 μL of dNTP mixture (Mg^{2+}) (2.5 mmol/L), 0.06 μL of *Taq* DNA polymerase (2.5 U/μL), 0.4 μL of each primer, and 0.8 μL of template DNA (100–150 ng/μL). The PCR was performed using a S1000TM Thermal Cycler (Bio-Rad, California, USA) with the following parameters: 1 cycle at 94 °C for 3 min; followed 35 cycles at 94 °C for 30 s, 50–60 °C (based on the primer information) for 30 s, and 72 °C for 45 s; with a final extension at 72 °C for 10 min before cooling to 4 °C. The PCR products were separated in 8% nondenaturing polyacrylamide gel and then silver-stained (Tixier and Sourdille 1997) and photographed.

GISH analysis

GISH analysis was conducted to detect the alien chromosome in N9436B. Seeds were germinated on moistened filter paper in petri dishes. Following seed germination, the petri dishes were placed into the refrigerator (4 °C) for 24 h, then into an incubator (23 °C) until roots developed to 1.5–2.0 cm. Roots were placed into a centrifuge tube filled with ice–water (0–4 °C) for 24 h, and then fixed in Carnoy's fixative fluid (a 3:1 ethanol – acetic acid mixture) at 4 °C for at least 2 days. The root tips were digested in 1% pectinase and 2% cellulase at 37 °C for 50–60 min (different materials were subjected to different digestion time), slides were then prepared using the drop technique (Han et al. 2004). Genomic DNA of Austrian rye was labeled with DIG-Nick-Translation Mix and used as a probe (Roche, Germany). The GISH procedure was performed as described by Liu et al. (2010) with minor modifications. Finally, the images captured for each color channel were viewed and photographed with a PhotometricsSenSys CCD camera (Olympus BX53F, Japan).

FISH and GISH analysis

Multicolor FISH and GISH analysis was conducted to detect the alien chromosome and the missing wheat chromosome arms in N9436B using Oligo-pTa535 (red) and Oligo-pSc119.2 (green) as probes on root tip metaphase chromosomes of Austrian rye, Shaanmai 611, and N9436B. Chromosome spreads of materials were prepared according to methods previously described by Han et al. (2004). Oligonucleotide probes, Oligo-pTa535 and Oligo-pSc119.2, were 5′ end-labelled with 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (Tamra), synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China), as described by Tang et al. (2014*b*). The genomic DNA of Austrian rye was labeled with Alexa Flour 488-5-dUTP (Invitrogen). Rye chromosome can be discriminated by Oligo-pSc119.2 signals. Chromosomes were counterstained with DAPI (blue). Finally, the images captured for each color channel were viewed and photographed with a PhotometricsSenSys CCD camera (Olympus BX53F, Japan).

Fig. 1. Chromosome characteristics of wheat–rye addition line N9436B at (*a*) mitotic metaphase and (*b*) meiotic metaphase I. (*a*) The arrows show the two telosomes and four satellite chromosomes ($2n = 42 + 2t$). (*b*) The arrow shows a bivalent from two telosomes ($2n = 22II$).

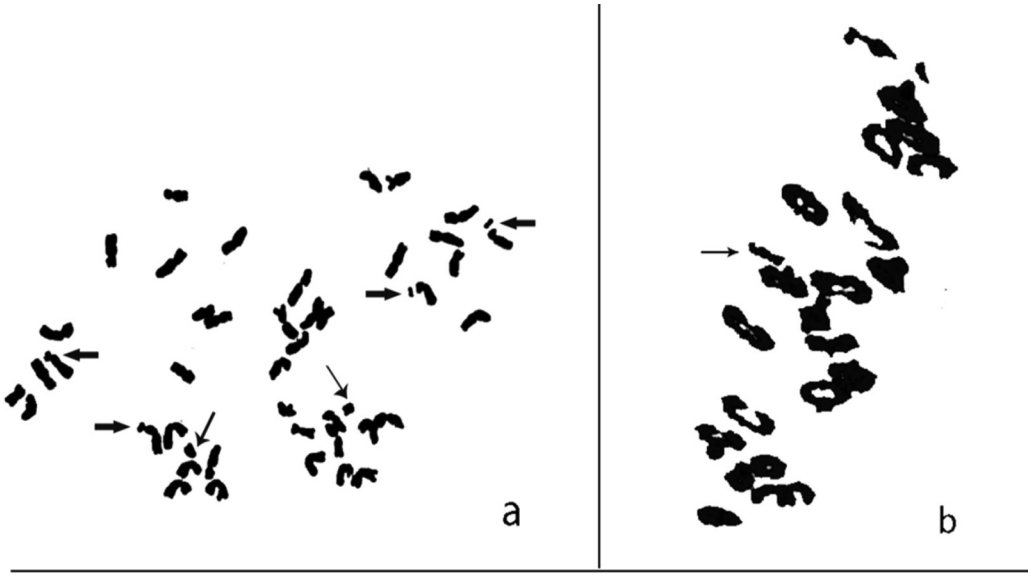
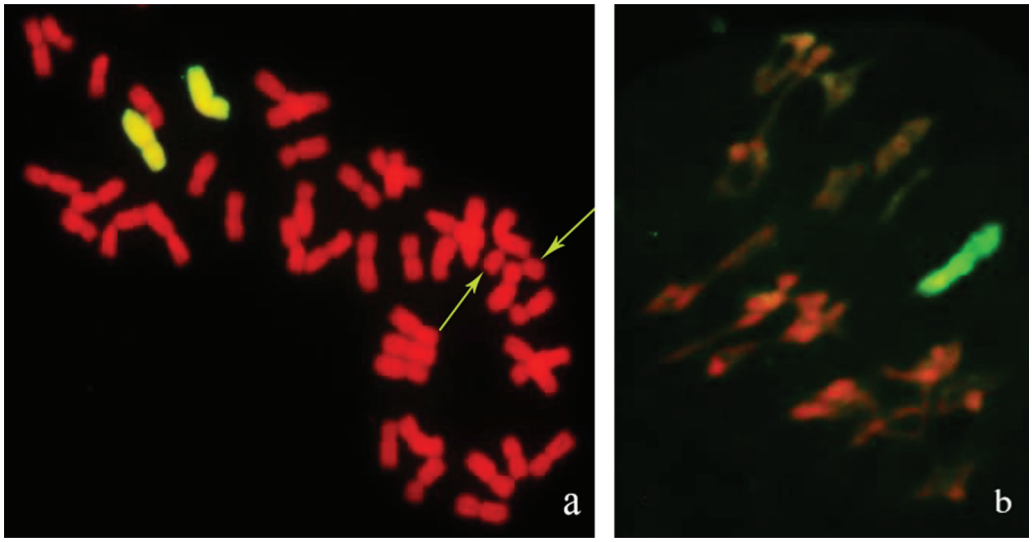


Fig. 2. Genomic in situ hybridization (GISH) results of wheat–rye addition line N9436B at (*a*) mitotic metaphase and (*b*) meiotic metaphase I using Austrian rye total genomic DNA labeled via nick translation with anti-digoxigenin-fluorescein Fab fragments (green) as a probe. (*a*) Mitotic metaphase GISH results of wheat–rye addition line N9436B showing two chromosomes with yellow–green hybridization signal. The arrows show the two telosomes ($2n = 42 + 2t = 40W + 2t_W + 2R$). (*b*) GISH results of wheat–rye addition line N9436B during meiotic metaphase I, showing a bivalent with yellow–green hybridization signal.



Results

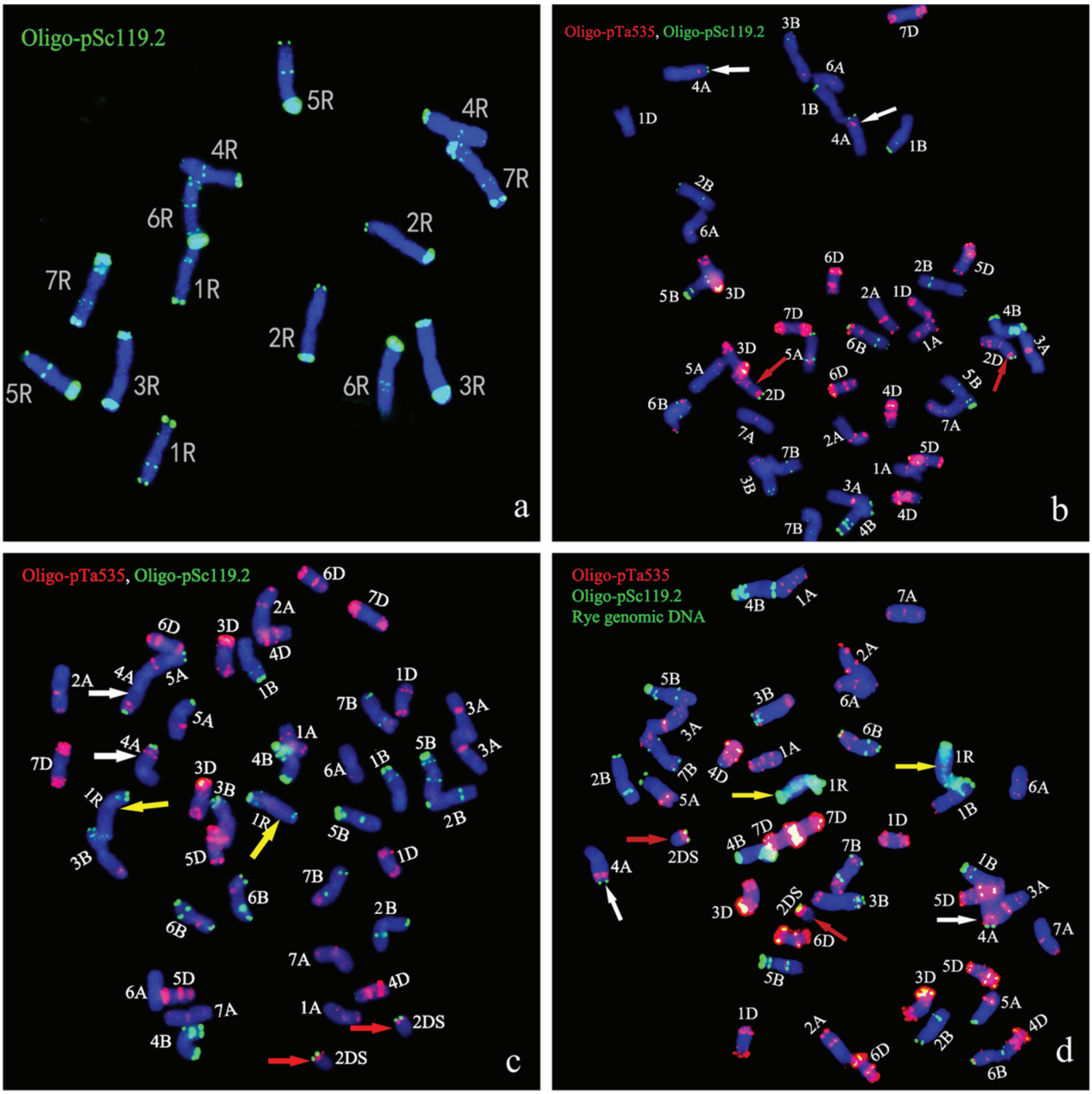
Cytological characterization of N9436B

The root tips and young spikes were sampled in the field at the appropriate time for the respective samplings. The results of the root tips and young spikes indicated that N9436B had a chromosome number of $2n = 42 + 2t$, with four satellite chromosomes at mitotic metaphase (Fig. 1*a*), and its configuration was $2n = 22II$ at metaphase I of pollen mother cell (Fig. 1*b*). Therefore, we confirmed that N9436B was cytogenetically stable.

GISH analysis of N9436B

GISH analysis was performed to determine the chromosome constitution of N9436B using genomic DNA of Austrian rye as a probe. The mitotic GISH of somatic cells showed that N9436B had two chromosomes with bright yellow–green hybridization signals (Fig. 2*a*), and the meiotic GISH of pollen mother cell metaphase I showed that N9436B possessed a bivalent with bright yellow–green hybridization signal (Fig. 2*b*). Therefore, N9436B contained two chromosomes from Austrian rye and 40 complete chromosomes and two telosomes of wheat,

Fig. 3. Fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH) analysis of Austrian rye, Shaanmai 611, and wheat–rye addition line N9436B. (*a*) FISH analysis using Oligo-pSc119.2 (green) as probe on root tip metaphase chromosomes of Austrian rye. (*b*) FISH analysis using Oligo-pTa535 (red) and Oligo-pSc119.2 (green) as probes on root tip metaphase chromosomes of Shaanmai 611. (*c*) FISH analysis using Oligo-pTa535 (red) and Oligo-pSc119.2 (green) as probes on root tip metaphase chromosomes of wheat–rye addition line N9436B. (*d*) FISH and GISH analyses using Oligo-pSc119.2 (green), Oligo-pTa535 (red), and rye genomic DNA (green) as probes on root tip metaphase chromosomes of wheat–rye addition line N9436B. Chromosomes were counterstained with DAPI (blue). The white arrows show two chromosome 4A in (*b*), (*c*), and (*d*); the red arrows show two chromosome 2D in (*b*) and two telesomes 2DS in (*c*) and (*d*); and the yellow arrows show two chromosome 1R in (*c*) and (*d*). (*a*) $2n = 14$, (*b*) $2n = 42$, (*c*) $2n = 42 + 2t = 40W + 2t_W + 2(1R)$, (*d*) $2n = 42 + 2t = 40W + 2t_W + 2(1R)$.



and the two alien chromosomes formed one paired bivalent during the meiotic stage. GISH analysis also showed other chromosomes displaying red signals counterstained with DAPI, indicating that these chromosomes originated from the wheat parent Shaanmai 611. Therefore, N9436B was proven to be a wheat–rye addition line. To further validate these results, 35 plants of N9436B were subjected to GISH analysis and 33 plants obtained

the same results, indicating that N9436B was a genetically stable wheat–rye addition line.

Multicolor FISH and GISH analysis of N9436B

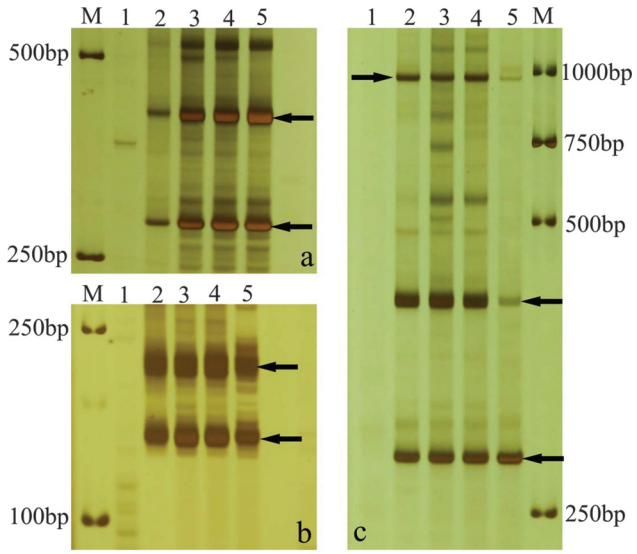
FISH analysis was conducted to detect the alien chromosome and the missing wheat chromosome arms in N9436B. From previous study, tandem repeat sequence pTa-535 (red) and pSc119.2 (green) can effectively identify wheat A-, B-, and D-genome chromosomes (Tang et al. 2014*b*), and pSc119.2 can discriminate R-genome chromosomes (McIntyre et al. 1990). FISH karyotypes of Austrian rye, Shaanmai 611, and N9436B were primarily established by employing Oligo-pSc119.2 (green), Oligo-pTa-535 (red) and Oligo-pSc119.2 (green), and Oligo-pTa-535 (red) and Oligo-pSc119.2 (green), respectively (Figs. 3*a*, 3*b*, 3*c*). Therefore, proving the two chromosomes from Austrian rye in N9436B is 1R chromosome. The chromosome constitution of N9436B was successfully karyotyped by combining Oligo-pTa-535, Oligo-pSc119.2, and rye's genomic DNA (green) as probes (Fig. 3*d*), which further demonstrated the karyotype of N9436B. Therefore, the chromosome constitutions of N9436B is $2n = 42 + 2t = 40W + 2t_W + 2(1R)$ (W, wheat chromosome; t_W, wheat telosome chromosome; 1R, rye 1R chromosome).

The karyotype of Austrian rye, Shaanmai 611, and N9436B has been previously established according to Tang et al. (2014*b*). Further proof that the rye chromosome in N9436B is 1R chromosome can be seen by comparing the karyotype of Austrian rye with that of N9436B, as seen by the lose of an Oligo-pSc119.2 green signal on 1RL (Figs. 3*a*, 3*c*, 3*d*). The missing wheat chromosome arms in N9436B were 2DL, as seen by comparing the karyotype of N9436B with that of Shaanmai 611 (Figs. 3*b*, 3*c*, 3*d*). The 4A chromosome in Shaanmai 611 and N9436B was also labelled because in whole chromosomes, chromosome 4A is similar to chromosome 2D, the short arms are the same. However, Oligo-pTa535 red signal was shown on chromosome arm 2DL but not on chromosome arm 4AL. N9436B has a complete 4A chromosome, so it was confirmed that the missing wheat chromosome arms in N9436B were 2DL.

More interestingly, some chromosomes' FISH signal patterns of N9436B were different from their parents' to some extent, especially the A- and B-genome's chromosomes, which indicate alterations of wheat chromosomes. Specifically, chromosome arm 5AL of N9436B displayed Oligo-pSc119.2 green signal loss and intercalary Oligo-pTa535 red signal gain. The 1BS and 1BL, 7BS and 7BL arms of N9436B contained intercalary terminal Oligo-pTa535 red signal. In addition, 3AL of N9436B presented greater Oligo-pTa535 red signals than Shaanmai 611.

Therefore, N9436B was demonstrated to be a wheat–rye addition line carrying A-, B-, and D-genome chromosomes (missing chromosome arms 2DL) of wheat and 1R chromosome of Austrian rye. The plentiful structural alterations of wheat chromosomes were observed in N9436B.

Fig. 4. Non-denaturing polyacrylamide gel electrophoretic analysis of the introduced R chromosome. M, DL2000; 1, Shaanmai 611; 2, Austrian rye; 3 and 4, wheat–rye addition line N9436B; 5, 1R addition line of Chinese Spring × Imperial. (*a*) *NOR-R1*, (*b*) *TSM716*, and (*c*) *NOR-1*. The arrows show the target bands.



Molecular marker screening and electrophoretic analyses

In this study, markers *TSM716*, *NOR-R1*, and *NOR-1* were used to detect the alien chromosome in wheat–rye addition line N9436B. DNA fragments ranging from 100 to 2000 bp, amplified from N9436B and Austria rye, indicate that N9436B contained the DNA region specific for chromosome 1R derived from Austria rye. The corresponding diagnostic fragments were also detected in an 1R addition line of CS × Imperial (Fig. 4). The results showed that the alien chromosome in N9436B was chromosome 1R, which were consistent with FISH detection results of N9436B.

Agronomic performance and reaction to powdery mildew of N9436B

After more than 10 generations of selfing, no segregation was observed in wheat–rye addition line N9436B, neither in morphology nor in cytology. The plant type of N9436B was similar to that of common wheat; it was compact but higher than its parent Shaanmai 611 (Table 2; Fig. 5*c*). The spikes of N9436B showed superior performance with respect to spike length, spikelets per spike, and kernels per spike (Table 2; Fig. 5*b*). The seeds of N9436B were red and similar in shape and size to its parent Shaanmai 611 (Fig. 5*a*). The average thousand-kernel weight of N9436B was 30.06 g, which was higher than Austrian rye but less than Shaanmai 611 (Table 2).

For testing the powdery mildew reaction at the seedling stage, N9436B, Austrian rye, Kavkaz, Amigo, and susceptible control Shaanyou 225 were inoculated with the *Bgt* isolate E09. Austrian rye and N9436B showed

Table 2. Agronomic traits of wheat–rye addition line N9436B and its parents Shaanmai 611 and Austrian rye.

Material	Plant height (cm)	Spike length (cm)	Spikelets/spike	Kernels/spike	Thousand-kernel weight (g)	Awnedness
Shaanmai 611	81±3	10.0±0.2	21±3	45±4	34.3±0.5	Long
Austrian rye	172±5	14.5±0.4	43±2	86±3	26.1±0.4	Long
N9436B	105±3	13.5±0.3	34±3	82±6	30.6±0.3	Short

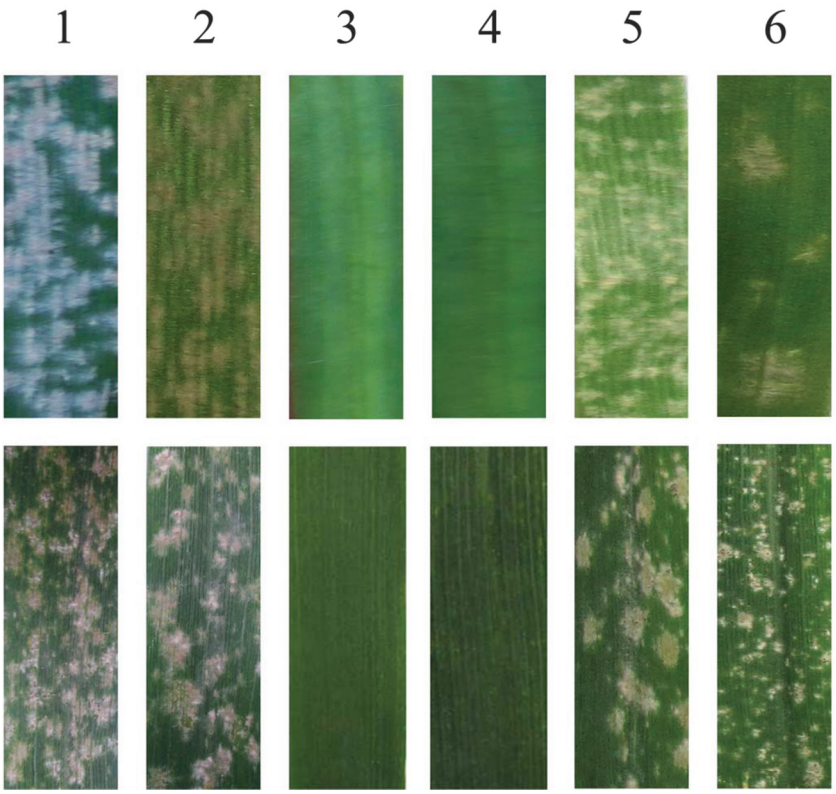
Fig. 5. Morphologic traits of wheat–rye addition line N9436B and its parents Shaanmai 611 and Austrian rye. (*a*) Kernels of Austrian rye (1), Shaanmai 611 (2), and wheat–rye addition line N9436B (3). (*b*) Spikes of Austrian rye (1), Shaanmai 611 (2), and wheat–rye addition line N9436B (3). (*c*) Plant of Austrian rye (1), Shaanmai 611 (2), and wheat–rye addition line N9436B (3).

immunity to E09 isolate with an IT score of 0. In contrast, the susceptible control Shaanyou 225 showed high susceptibility to E09 isolate with an IT score of 4, Shaanmai 611 showed medium susceptibility with an IT score of 3, and Kavkaz and Amigo showed complete susceptibility (Fig. 6). For testing the powdery mildew reaction at the adult stage, N9436B, Austrian rye, Shaanmai 611, Kavkaz, Amigo, and the susceptible control Shaanyou 225 were inoculated with a mixture of *Bgt* isolates prevalent in Guanzhong region of Shaanxi Province in China in two consecutive wheat growing seasons. Shaanmai 611 and the susceptible control Shaanyou 225 were covered by *Bgt* spores and showed high susceptibility with a disease reaction score of 7–8. Kavkaz, with the gene *Pm8*, and Amigo, with the gene *Pm17*, showed susceptibility with disease reaction scores of 8 and 6, respectively. Whereas Austrian rye and N9436B showed immunity to the mixture of *Bgt* isolates with a disease reaction of 0 (Fig. 6). Therefore, N9436B was immune to powdery mildew at the seedling and adult stages. The powdery mildew resistant gene(s) in N9436B should be from 1R chromosome of Austrian rye and the gene(s) could be a new gene in 1R for resistance or new alleles of *Pm8* and *Pm17*.

Discussion

There are three factors affecting the yield of wheat: spikes per acre, kernels per spike, and thousand-kernel weight. Therefore, the improvement of wheat spike traits is one way to increase yield. Rye, a species closely related to wheat, possesses the characteristic of multiple spikelets: about 30 generally, and up to 40. Transferring this characteristic to common wheat can cultivate wheat germplasm with multiple spikelets. To date, the multiple spikelets line 10-A, with 30–37spikelets per spike, has been developed (Yen et al. 1993), and it has been proven to carry a wheat–rye 1RS/1BL translocation chromosome (Wei et al. 1999). In the present study, N9436B, with 31–37 spikelets per spike, was proven to be a wheat–rye 1R addition line. Both 10-A and N9436B have the same rye chromosome 1R, so the gene(s) controlling the trait of

Fig. 6. Resistance of Shaanyou 225 (1), Shaanmai 611 (2), Austrian rye (3), wheat–rye addition line N9436B (4), Kavkaz (5), and Amigo (6) for powdery mildew at the seedling (above) and adult stages (below).



multiple spikelets may be related to rye chromosome 1R. However, other wheat–rye 1R addition, substitution, or translocation lines have not reported the characteristic of multiple spikelets (Xue et al. 1993). The gene loci located on wheat group 2 chromosomes has already been shown to be involved in the control of spikelets per spike and kernels per spike in wheat (Sears 1954; Klindworth et al. 1990; Peng et al. 1998; Dobrovolskaya et al. 2009; Li et al. 2012; Zhang et al. 2013). The gene on chromosome 2D has the strongest effect on the expression of the multiple spikelets character (Peng et al. 1998), and genes governing spike branching and supernumerary spikelets are located on chromosome arm 2DS (Dobrovolskaya et al. 2009). Sears (1954) found that hexaploid wheat nullisomic for chromosomes 2A or 2D might generate multiple spikelets trait of which the gene inhibiting this trait is located on chromosomes 2DS and 2AL. Chromosome 2D of common wheat has been shown to carry a strong inhibitor of multiple spikelets expression (Klindworth et al. 1990). Therefore, the missing 2DL arms may also have caused a significant spikelet number increase. These results suggest the possible influence of the genotype of rye and wheat or the missing 2DL arms on the appearance of multiple spikelets. However, N9436B has the shortcomings of late maturity, higher plant height, and low thousand-kernel weight (30.6 g), which need to be further improved. Therefore, the wheat–rye addition N9436B should be a useful bridge material to produce wheat–rye substitution and translocation lines with multiple spikelets.

As the tertiary gene pool for wheat, rye plays an important role in the genetic improvement of wheat, and as a cross-pollinated crop, rye offers significant and abundant genetic diversity within and between cultivars. The powdery mildew resistance genes derived from rye are *Pm7*, *Pm8*, *Pm17*, and *Pm20*. They are located on the 2RL, 1RS, 1RS, and 6RL chromosomes, respectively, and they have already been successfully used in commercial wheat production. *Pm8*, derived from rye cultivar Petkus (Hsam and Zeller 1997), and *Pm17*, derived from rye cultivar Insave (Heun et al. 1990), were proven to be allelic genes and are widely used in wheat breeding and improvement programs as translocation lines T1BL·1RS and T1AL·1RS, respectively (Rabinovich 1998). In China, approximately 38% of wheat cultivars contain the T1BL·1RS translocation (Zhou et al. 2004). However, because of the co-evolution of pathogen and host, new virulent pathogen isolates have rapidly emergence (McDonald and Linde 2002). The cultivars with 1RS translocation successively lost their resistance to powdery mildew; *Pm8* and *Pm17* are no longer resistant to powdery mildew (Zhuang and Li 1993; Zhuang 2003). In addition, many new wheat–rye germplasms and powdery mildew resistance genes from rye have been identified and reported. These *Pm* genes were located on 1R, 2R, 4R, and 6R chromosomes of rye, and their reaction patterns were different from the

four known *Pm* genes, *Pm7*, *Pm8*, *Pm17*, and *Pm20*, derived from rye (Li et al. 2004; Hysing et al. 2007; Tang et al. 2008; Ren et al. 2009; Fu et al. 2010, 2011, 2014*a*; Wang et al. 2009, 2010; Zhuang et al. 2011; An et al. 2006, 2013). In the present study, wheat cultivar Shaanmai 611 displayed high susceptibility to powdery mildew, and wheat–rye addition line N9436B that contained 1R chromosome of Austrian rye showed immunity to powdery mildew. Furthermore, Kavkaz and Amigo, which contain the genes *Pm8* and *Pm17* located on rye chromosome 1R, respectively, displayed susceptibility to powdery mildew. Therefore, the powdery mildew resistant gene in N9436B should be from 1R chromosome of Austrian rye and it could be a new gene in 1R for resistance or new alleles of *Pm8* and *Pm17*. Wheat–rye addition, substitutions, as well as translocations have been successfully used in wheat breeding and improvement programs.

Wide hybridization is one of the stresses that may cause reorganization of parental genomes (McClintock 1978). Wide hybridization between wheat and rye is an important tool in wheat breeding and for the development of more highly engineered introgression lines for wheat improvement programs. Wheat–rye derivatives include amphiploid, chromosome addition, substitution, and translocation lines. The introduction of rye chromatin into common wheat could result in changes of chromosome structure of common wheat (Ren 1991). Chromosome instability and genome rearrangements in wheat–rye disomic addition lines have been reported (Szakacs and Molnar-Lang 2010; Bento et al. 2010). A single 1R chromosome added to wheat might cause abnormal mitotic behaviour of both wheat and rye chromosomes and different genetic variations might occurr among the sibling 1R monosomic addition lines (Fu et al. 2014*b*). One 2D chromosome was broken and three 4A chromosomes were observed in one of the selfed progeny of a 7R monosomic addition line. The elimination of 1A and 4B chromosomes, the structural variation, and abnormal mitotic behaviour of 3D chromosome were detected in the selfed progeny of 6R monosomic addition line (Fu et al. 2013). The breakage and deletion of wheat chromosomes 7B, 3B, and 4D were observed in the selfed progenies of 5R monosomic addition line (Ge et al. 2014). The alterations of wheat chromosomes including 5A, 6A, 1B, 2B, 6B, 7B, 1D, 3D, and 7D were observed in the progeny of wheat–rye hybrids (Tang et al. 2014*a*). The results of preferential elimination of D-genome chromosomes, and alterations of wheat and rye chromosomes, were reported in the derivatives of synthetic hexaploid wheat and Qinling rye (Hao et al. 2013). Complete elimination of D-genome chomosomes, altered 5A, 5B, and 7A chromosomes, and restructured 2A chromosome were detected in two hexaploid triticales, N9116H and N9116M, derived from the cross of common wheat cultivar and Austrian rye (Li et al. 2015). In the present study, the deletion of 2DL chromosome arms and the alteration of chromosomes 3A, 5A, 1B, and 7B were detected in N9436B according to FISH karatypes of N9436B and its parent Shaanmai 611. These phenomena and results suggest that rye chromosome added to wheat might result in structural alterations of the wheat chromosome and that this variation randomly occurs.

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

Author contributions

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

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