Characterization of progenies of *Triticum aestivum-Psathyrostachys juncea* derivatives by using genomic *in-situ* hybridization*

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Abstract Using genomic *in-situ* hybridization (GISH) technique, 7 translocation-addition lines, 6 translocation and translocation-addition lines, 2 ditelosomic addition lines and 1 translocation line were identified from *Triticum aestivum* L.-*Psathyrostachys juncea* (Fisch.) Nevski intergeneric hybrids, of which translocation-addition and translocation and translocation-addition lines were not found in other reports. No substitutions and disomic additions were detected in the hybrids and breakages occurred in all *P. juncea* chromosomes studied. Results have shown that the improved GISH technique is a rapid and economical method for use in this field.

Keywords: ***Triticum aestivum*, *Psathyrostachys juncea*, translocation-addition, translocation and translocation-addition, *in-situ* hybridization.**

Wheat relatives carry many valuable genes which are important sources for wheat improvement. In order to introduce alien genes into wheat, crosses need to be made between wheat and its relatives, followed by the selection of alien addition, substitution or translocation lines. The characterization of alien chromosomes is an essential step in this process and morphological, cytological and biochemical markers are often used. Genomic *in-situ* hybridization, as compared with the above-mentioned methods, is more accurate and easier to observe. *In-situ* hybridization was initially developed, independently, by Gall and Pardue (1969) and John *et al.* (1969). Using Digoxigenin-labelled total genomic DNA as probes, Heslop-Harrison *et al.* and Le *et al.* made successful *in-situ* hybridization in plants in the 1980s[1,2]. Reader *et al.* modified the original *in-situ* hybridization technique and established direct fluorescence-labelled *in-situ* hybridization procedure[3]. *In-situ* hybridization has been used to identify the chromosomal donors in hybrids *Hordeum vulgare-Hordeum chilense* and *H. vulgare-H. bulbosum* derivatives[4—7], and the chromosomes or segments of *Hordeum chilense*, *H. vulgare*, *L. multicaulis*, *Secale cereale* and diploid *Tinopyrum bessarabicum* in wheat background[8].

Psathyrostachys juncea, a species of the genus *Psathyrostachys* Nevski in *Triticeae* carrys valuable traits resistant to drought, cold, etc. Successful crosses were made, for the first time, between *P. juncea* and common wheat cultivar Chinese Spring by our laboratory in 1986[9]. Results from the characterization of these crosses using improved total genome *in-situ* hybridization procedure are reported in this paper.

1 Materials and methods

1.1 Materials

Twenty-five BC_2F_5 and BC_3F_4 accessions of *P. juncea*-wheat derivatives were used consisting

of 10 with $2n = 42$ and 15 with $2n = 44$ (95-125-3 being a doubled accession from anther culture).

1.2 Methods

1.2.1 Chromosome spreads preparation. Root tip cell chromosome spreads: Seeds were germinated at 25℃. Root tips were cut when root elongated to about 1 cm, pretreated in cold water at 0℃ for 24—36 h and fixed in Carnoy's fluid (alcohol: acetic acid = 3:1). Two days after fixation, the root tips were digested by 2% pectinase and 2% cellulase for 80 min at 37℃, and chromosome spreads were prepared in the presence of 45% acetic acid following removal of the root caps and epidermal cells.

Pollen-mother-cell (PMC) chromosome spreads: Anthers at meiosis metaphase were fixed in carnoy's fluid for 2 d, digested by 2% pectinase and 2% cellulase for 40—50 min and chromosome spreads were prepared in the presence of 45% acetic acid.

The slides were frozen by dry ice and the slips were removed. Then the slides with well scattered chromosomes separated from the cytoplasm were air-dried and stored at −20℃ for future use.

1.2.2 Labeling of probes. *P. juncea* total genomic DNAs were sonicated into 500-bp fragments with an ultrasonic instrument and labelled by nick translation with Digoxigenin-dUTP (Boehringer Mannheim) and Flurogreen (Amersham). Digoxigenin labelling followed the procedure of Leitch and Heslop-Harrison[10] and Flurogreen labelling followed the method described by Reader *et al.*[3], with some modifications, i. e. omitting all the steps for probe purification. Mixtures for labelling the probes included the following: 5 μL of 10 × NT buffer, 5 μL of unlabelled nucleotide mixture (0.5 mmol/L dATP, 0.5 mmol/L dCTP and 0.5 mmol/L dGTP in the ratio of 1:1:1), 3.5 μL of Digoxigenin-or Flurogreen-labelled nucleotide mixture (1 mmol/L Digoxigenin and 1 mmol/L dTTP in the ratio of 0.35:0.65; 1 mmol/L Flurogreen and 0.5 mmol/L dTTP in the ratio of 2.5:1), 1 μL of 100 mmol/L DTT and 1 μg of template DNA. Sterile water was poured into the mixtures to make up a total volume to 45 μL, 5 μL of DNA Polymerase I/DNase I was added and then the mixtures were gently stirred, briefly spanned, incubated in water bath at 15℃ for 3.5 h and stored at −20℃.

1.2.3 Hybridization. Rapid hybridization[3] was used for the probes labelled by Flurogreen. Chromosome spreads were digested by 5 μg/mL RNase for 30 min at 37℃, fixed with 4% paraformaldehyde for 10 min at room temperature and denatured with 70% formamide for 2 min at 70℃.

Hybridization mixture: For each slide the following was mixed: 20 μL of 25% DS, 4 μL of 20 × SSC, 1.25 μL of 10% SDS, 50 ng of labelled probe DNA, Chinese Spring DNA (10 times the probe concentration) used as block and sterile water added made a total of 45 μL. The mixtures were boiled for 5 min, chilled on ice for 3.5 min and centrifuged. Then they were pipetted onto the slides, covered with plastic slips and hybridized for 2 h at 65℃.

After incubation, the slides were washed, stained with DAPI (4, 6-diamidino-2-phenylindole, 0.125 μg/mL) for 2 min, washed again in 4 × SSC Tween 20 (0.2%) and counterstained

with 0.1 μg/mL PI (propidium iodide) for 1 min.

Hybridization using Digoxigenin labelled probes and examination procedures followed those of Leitch *et al.*[10]. Chromosome spreads were digested by 100 μg/mL RNase at 37℃ for 1 h, treated with 5 μg/mL pepsin at 37℃ for 10 min and fixed in 4% paraformaldehyde for 10 min at room temperature. For each slide, hybridization mixture was prepared by adding 20 μL 50% formamide, 4 μL 20 × SSC, 0.5 μL 10% SDS, 8 μL 50% DS, 1 μL of 1 μg/mL salmon sperm DNA, 0.5 μg of Chinese Spring DNA and 50 ng of probe DNA. Sterile water was added to the mixture to get a final volume of 40 μL. After denaturation at 80℃ for 5 min, the mixture was pipetted onto chromosome slide and put into a PCR device for denaturation for about 30 min together with the chromosomes. The PCR procedure used included: (i) 5 min at 65℃; (ii) 1 min at 50℃; (iii) 1.5 min at 45℃; (iv) 2 min at 40℃; (v) 5 min at 38℃ and overnight at 37℃. Hybridized slides were washed with 2 × SSC (30℃), 20% formamide (42℃), 0.1 × SSC (42℃) and 2 × SSC (42℃), and allowed to cool to 30℃.

1.2.4 Detection. After treating with 5% BSA (formulated using 4 × SSC Tween 20) for 15—20 min at 37℃. Antidigoxigenin-FITC solution diluted by 4 × SSC/Tween 20/BSA to 1 μg/mL its stock concentration was pipetted onto the slides which then were covered with plastic slips and put into a humid chamber at 37℃ for 1 h.

1.2.5 Counterstaining. Following staining with 4 μg/mL DAPI for 10 min, the slides were washed in 4 × SSC Tween 20 and counterstained using 0.1 μg/mL PI for 1—5 min.

1.2.6 Microscopic examination. Antifade (Vectashield, Vector Laboratories) was applied on the slides which were then viewed under a fluorescent photomicroscope and photographed with ASA 400 films.

2 Results

Under UV light, the DNA stained with DAPI emitted bright blue fluorescence (fig. 1(b), (g)) and chromatins or chromosomes in interphase, metaphase and anaphase nuclei could be clearly observed. Although it is not able to distinguish *P. juncea* and wheat chromosomes under this wave length, the division stages, chromosomal morphologies and numbers could be identified, allowing comparisons to be made with chromosomes detected under other wavelength. Under blue excitation, the *P. juncea* chromosomes hybridized to the probes labelled by Digoxigenin or Flurogreen emitted yellow or yellow-green fluorescence while those of wheat gave out green fluorescence (figs. 1(a), (c), (e), (f) and (h), 2(a)—(f)). If counterstained with PI, yellow or yellow-green fluorescence was produced due to interaction between green FITC and tangerine fluorescence; by this procedure, *P. juncea* chromosomes appeared to be yellow or yellow-green and wheat chromosomes orange (fig. 1(d)), allowing easy discrimination of wheat and *P. juncea* chromosomes in wheat-*P. juncea* derivatives. From the 25 accessions of wheat-*P. juncea* derivatives studied, 7 translocation addition lines, 6 translocation and translocation-addition lines, 2 ditelosomic addition lines and 1 translocation line were identified, and the other 9 accessions were not found to contain *P. juncea* chromosomes.

Fig. 1. (a) PMC at metaphase I for 95-125-3, showing 22 bivalents and 1 ring bivalent translocation; (b) root-tip cell at metaphase for 95-140-1, showing 44 chromosomes stained with DAPI; (c) the same cell as in 1(b), showing a pair of translocated chromosomes; (d) the recombination of 2 *P. juncea* chromosomal long arms with wheat chromosomes carrying satellites in the line 95-137-10, translocated wheat chromosomes being 1B or 6B (arrows); (e) PMC at meiosis metaphase I in the line 95-131-2 showing 22 bivalents and 2 translocated ring bivalents; (f) PMC at meiosis anaphase I in the line 95-131-2; (g) interphase cell stained with DAPI in the line 95-131-2; (h) the regionalized distribution of yellowish *P. juncea* segments in interphase cell (the same cell as 1(g)).

2.1 Translocation-addition lines

95-125-3 is a double haploid derived from anther culture of the hybrid F_1 between wheat-*P. juncea* BC_3F_2 and Laizhou 953, a wheat line easy to anther culture. *In-situ* hybridization of pollen mother cell showed that this material contained 22 bivalents consisting of 21 wheat chromosomes not hybridizing to the probes and 1 ring bivalent formed by a *P. juncea* chromosome which hybridized to the probes and appeared to be yellow, and a wheat chromosome which did not hybridize to the probes (fig. 1(a)). The result from *in-situ* hybridization of metaphase root-tip cells also revealed that the line had 42 wheat chromosomes and 2 wheat-*P. juncea* translocated chro-

mosomes, which is identical to the above findings. In the derivatives of wheat distant crosses, a line is called disomic alien addition if it carries 44 chromosomes which include a pair of intact alien chromosomes; or a line is termed translocation if it contains 42 chromosomes which include a pair of translocated chromosomes. 95-125-3 has $2n = 44$ chromosomes including a pair of wheat-*P. juncea* translocated chromosomes in the complex, and we call the line a translocation-addition line, which we did not find previously. Other translocation-addition lines identified include 95-130-9, 95-134-8, 95-139-6, 95-140-1, 95-141-8 and 95-143-9. Fig. 1(b) and (c) show the root-tip chromosomal spreads for 95-140-1 ($2n = 44$).

2.2 Translocation and translocation-addition lines

95-137-10, $2n = 44$. Meiosis observation on pollen mother cells showed 22 bivalents. *In-situ* hybridization of root-tip cells revealed that the line contained 4 wheat-*P. juncea* translocated chromosomes, 2 of wheat are formed by the long arms of *P. juncea* chromosomes with the short

Fig. 2. (a) PMC at metaphase I in the line 95-132-8 showing the translocation between 2 irregular bivalents with an arrow indicating the deficiency for a wheat arm; (b) PMC at anaphase in the line 95-132-8 showing the random movement to the 2 poles for the translocated chromosomes; (c) PMC at telophase Ⅱ in the line 95-132-8; (d) root-tip cell chromosomes in the line 95-144-5 showing the 2 yellow-green *P. juncea* chromosomal segments; (e) root-tip cell chromosomes (42) in the line 95-147-3, with translocation between 2 chromosomes; (f) bridge formed at mitosis telophase in the line 95-147-3.

arms of wheat chromosomes carrying satellites, indicating the translocated wheat chromosomes being 1B or 6B; the other 2 translocated chromosomes are composed of the short arms of *P. juncea* and the long arms of wheat chromosomes (fig. 1(d)). There were 2 pairs of translocated chromosomes in this line. If one of them is called translocation, the other is considered as addition by translocation. Therefore, we name the line of this type translocation and translocation-addition lines, which we did not find previously. 95-131-2 is similar to 95-137-10, and both are translocation and translocation-addition lines. *In-situ* hybridization results for pollen mother cells (metaphase to interphase) of the line 95-131-2 are given in figure 1(e)—(h).

95-132-8 is a translocation and translocation-addition line of different type. Meiosis observation on pollen mother cells showed that the line carried 22 bivalents which included 2 irregular bar bivalents composed of wheat-*P. juncea* translocation chromosomes (fig. 2(a)). The 2 irregular bivalents differed from each other, one without a wheat chromosome arm (indicated by arrow) and the other without a *P. juncea* chromosome arm. Obviously, such a line is not stable, for segregation will occur in its offsprings and may lead to the production of translocation and translocation-addition, dimonosomic addition, ditelosomic addition, etc. Fig. 2(b) shows the random movement to the 2 poles for the chromosomes with *P. juncea* arms at anaphase I. Fig. 2(c) shows the situation at telophase Ⅱ.

2.3 Telosomic addition line

Root-tip cell *in-situ* hybridization revealed that 95-144-5 carried 42 wheat chromosomes and 2 yellow segments from *P. juncea* chromosomes (fig. 2(d)), indicating that the line is a wheat-*P. juncea* ditelosomic addition.

2.4 Translocation line

95-147-3, $2n = 42$. Root-tip cell *in-situ* hybridization showed that the line contains two wheat-*P. juncea* translocation chromosomes (fig. 2(e)), and therefore represents a wheat-*P. juncea* translocation line. It can be seen from fig. 2(e) that the translocation occurred at the centromere, being a pericentric translocation. In addition, the chromosomes in the cell centre showed uncontinuous yellow color, indicating possible existence of small translocated segments. Fig. 2(f) shows the chromosomal bridge occurring at telophase in root-tip cell and small translocated chromatin segments may be present at both ends of the bridge. There were some smaller and scattered areas formed by yellowish *P. juncea* chromatin in addition to the two stronger yellowish areas in interphase cells.

3 Discussion

This study conducted *in-situ* hybridization with root-tip and pollen mother cells, respectively. The results showed that root-tip cell *in-situ* hybridization is able to clearly visualize alien chromosome numbers, translocation positions and segment sizes, while pollen mother cell *in-situ* hybridization can not only provide the above information, but also demonstrate the pairing patterns of alien chromosomes, thereby clarifying their homologous relations. At meiosis anaphase and telophase, the separation patterns of alien chromosomes can also be investigated on which projections to their segregating types in next generations can be based. It is evident that pollen mother

cell *in-situ* hybridization is capable of providing more information. Furthermore, the method for preparing chromosome spreads from pollen mother cell (PMC) is simpler and metaphase I PMCs with well-scattered chromosomes are easy to obtain.

The study used Digoxigenin and Flurogreen to label probes, and the results confirmed that both labels are equally effective and accurate in identifying alien chromosomes/chromatins. Direct Flurogreen labelling has the advantages of being simple (without the need for detection and rapid (1 d) while Digoxigenin labelling (and detection with Anti-Digoxigenin) leads to higher imaging sensitivity, but it requires more reagents and more complex procedure, and takes longer time (2 d). Therefore it is better to use the latter method for *in-situ* hybridization with oligo-copy and mono-copy probes and to use direct fluorochrome labelling for such probes as total DNA or multi-copy repeated sequences.

Previously used methods for labelling probes[1,3,10] have been improved in the present study by omitting all the steps for probe purification, which can prevent a substantial loss of probes from occurring in the purification process. Results from the experiment indicated that the utilization rates for non-purified probes was greatly increased as compared with purified probes. In the present study, each slide was treated with 50 ng probe DNA and the signal produced by non-purified probe was much stronger than that by purified probe and lasted longer time. The slides hybridized by non-purified probe emitted stronger signal after storage at $-20℃$ for 7 months than that by purified probe. Efforts will be made to reduce the dosage of probe in future experiments. As the reagents for labelling probe DNA used in *in-situ* hybridization are very expensive, the modification in this aspect will greatly reduce *in-situ* hybridization cost, which is of great significance for developing countries. Futhermore, the time required for labelling probe is reduced from 24 to 4 h by omitting all the steps for probe purification, saving both time and manpower.

Of the 25 lines studied, 16 lines were found to carry *P. juncea* chromosomes, including 7 translocation-addition, 6 translocation and translocation-addition, 2 ditelosomic addition and 1 translocation; no disomic additions were detected. The results meant that breakages had occurred in all *P. juncea* chromosomes with broken points located at centromeres, and the translocations thus formed were all pericentric ones. Pericentric translocation is also termed Robertsonian translocation. The generally recognized mechanism for this type of translocation is that, during meiosis, telocentric chromosomes are produced from univalents which sometimes misdivide and lead to split centromeres and recombination may occur between these alien telocentric chromosomes and their homoeologous telocentric chromosomes from wheat, resulting in translocation. It should be noted that the frequencies for pericentric translocations were high in this study, which might be due to the easily-broken nature of the *P. juncea* univalents during meiosis, or caused by the 2 culture cycles for the F_1 hybrid influorescence. In other words, the *P. juncea* univalents are "often" rather than "occasionally" broken during meiosis as compared with other wild relatives.

This study, using *in-situ* hybridization, examined only the chromosomal numbers, segment sizes and translocation positions of the *P. juncea* chromosomes in wheat-*P. juncea* derivatives. The homologous relations between wheat and *P. juncea* chromosomes will be studied by screening a set of RFLP probes.

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