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ORIGINAL PAPER

The introgression of chromosome 6P specifying for increased numbers of florets and kernels from *Agropyron cristatum* into wheat

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Abstract A wheat (*Triticum aestivum* L.) line 4844 with superior numbers of florets and grains per spike was derived from the cross between Fukohokomugi wheat and *Agropyron cristatum* (L.) Gaertn. In order to determine the genetic control of floret and kernel number per spike in this line, chromosome addition and substitution lines that were derived from line 4844 were characterized by means of in situ hybridization, microsatellite (SSR), and gliadin analyses. Genomic in situ hybridization analysis with biotinylated P genomic DNA of *A. cristatum* as a probe demonstrated that the increased number of florets and grains in a spike was associated with the introgression of an *A. cristatum* chromosome. Fluorescence in situ hybridization, using a repetitive sequence, pAs1, derived from *Aegilops squarrosa* L., indicated the replacement of chromosome 6D of wheat in the wheat-*A. cristatum* chromosome substitution lines. This was confirmed by microsatellite analyses with wheat SSR markers specific for chromosome 6D, suggesting that the *A. cristatum* chromosome was homoeologous to group 6 and was therefore designated as 6P. This conclvsion was further confirmed by amplification using EST-SSR markers and gliadin analysis. The increased number of florets and kernels within a spike of the wheat-*A. cristatum* hybrids thus was controlled by gene(s) located on *A. cristatum* chromosome 6P.

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Introduction

The wild relatives of wheat possess many agronomically important characteristics, e.g., wide adaptability, resistance to diseases, better quality and superior numbers of florets, which are desirable for the improvement of wheat (*Triticum aestivum* L., $2n = 6x = 42$; genomes AABBDD) (Dewey 1984; Chapman 1989; Dong et al. 1992). The transfer of useful genes from wild species is an effective method for development of cultivars. Species from genera *Secale*, *Elytrigia*, *Haynaldia*, and *Leymus* have been hybridized with common wheat to produce wheat genotypes with large spikes, more spikelets and florets (Li et al. 1985). Breeding for large spikes by increasing the number of kernels per spike is an option for improving yield potential of wheat (Millet 1983; Yen et al. 1993; Frederic and Bauer 2000).

Agropyron species, which are important wild relative species to wheat, occur at three ploidy levels (i.e., $2n = 14$, $2n = 28$, and $2n = 42$). Most species within the genus *Agropyron* have many useful traits, such as tolerance to abiotic stresses caused by drought, low temperature and salinity, and biotic stresses arising from various pathogens and pests (Dewey 1984; Dong et al. 1992). *A. cristatum* (L.) Gaertn. ($2n = 28$, genomes PPPP) is a typical representative species for genus *Agropyron* (Dewey 1984; Guo 1987). *Agropyron*

cristatum accessions that are native to northern China were shown to carry potentially valuable traits for wheat improvement in evaluations of collections within the tribe *Triticeae* (Li and Dong 1993).

In order to transfer desirable traits from *Agropyron* spp. into wheat, intergeneric hybrids of common wheat with diploid and tetraploid *A. cristatum* have been obtained (Chen et al. 1989, 1990; Limin and Fowler 1990; Jauhar 1992; Li et al. 1995). Tetraploid *Agropyron* species with the genomic designation PPPP, e.g., *A. desertorum* (Fisch) Schult. (Chen et al. 1990; Limin and Fowler 1990; Li and Dong 1991a), *A. michnoi* Roshev. (Chen et al. 1990; Li and Dong 1991b), and *A. fragile* (Roth.) Cand. (Ahmad and Comeau 1991), have been crossed with wheat. Progeny lines with single *A. cristatum* chromosomes incorporated into the wheat genome have been obtained either as chromosome additions or chromosome substitutions (Chen et al. 1994). The tetraploid *A. cristatum* accession Z559 (China National Gene Bank accession number), which originated from Xinjiang, China, possesses many desirable traits, such as high tiller and floret numbers and, resistance to wheat diseases, e.g., the wheat rusts, powdery mildew (*Blumeria graminis* (DC.) E. O. Speer. f. sp. *tritici*), and *Barley yellow dwarf virus* (Li et al. 1995). In the early 1990s, the Japanese wheat cultivar Fukohokomugi was used as maternal parent in cross with the *A. cristatum* accession Z559 to transfer the desirable traits of the wild species into wheat. A number of progeny lines have large spikes with multiple florets and grains (Li et al. 1998).

The purposes of this study were (1) to determine the genetic control of floret and grain numbers per spike of the wheat-*A. cristatum* progeny lines; and (2) to determine the homoeologus relationships of the *A. cristatum* chromosomes with those of wheat by genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH), microsatellite (also referred to as SSR), EST-SSRs, and gliadin analyses.

Materials and methods

Plant materials

In 1992, the wheat cultivar Fukohokomugi was crossed with *A. cristatum* accession Z559. A wheat-*A. cristatum* germplasm, designated 4844 ($2n = 42$–44), with large spikes, superior number of florets and kernels in a spike, was obtained (Li et al. 1998). Following three to four generations of selfing, accompanied by cytogenetic analysis, lines 4844–2, 4844–5, 4844–8, and 4844–12 with $2n = 42$ or 44 were derived from line 4844. Fifteen plants derived from line 4844 with different chromosome numbers of $2n = 42$, 43, or 44 were used to examine the association of the large spike traits with the alien chromosome. The parental wheat cultivar Fukohokomugi and *A. cristatum* accession Z559 were included in the present study in assessment of spike traits and cytogenetic analysis as controls. The Canadian spring wheat cultivar Marquis was used as the check in gliadin analysis. Chinese Spring wheat was used as a source of blocking DNA in GISH analysis.

Evaluation of spike traits

In 1998–1999 and 2004–2005 sowing seasons, a randomized complete block design with 3 replicates was used to evaluate the spike traits (i.e., the number of spikelets, florets, and kernels per spike) in Fukohokomugi, Z559, and the progeny lines 4844–2, 4844–5, 4844–8, and 4844–12 derived from the wheat-*A. cristatum* line 4844. After harvest, ten plants in each plot were examined for each trait and the means of different lines were compared using Duncan's multiple range procedure ($P < 0.01$) (Little and Hill 1978) generated by the General Linear Model procedure in the SAS package (version 6, SAS Institute Inc., Cary, NC, USA). The chromosome constitution of each line were confirmed by GISH analysis.

To determine the association of *A. cristatum* chromosomes with superior spike traits, 15 seeds were randomly chosen from line 4844-derived plants and examined for their chromosome constitution by GISH analysis, prior to assessing the number of spikelets, florets, and kernels per spike.

GISH and FISH analysis

Seeds of each line were germinated on moistened filter paper in petri dishes at 25°C in the dark in a growth chamber. Root tips 1–2 cm long were pretreated in ice water for 20–24 h, fixed in ethanol-acetic acid (3:1) fixative for two days, and stored in 70% (v/v) ethanol. Before squashing in 45% (v/v) acetic acid, root tips were stained with 1% w/v acetocarmine for 0.5–1 h. Slides were frozen in liquid nitrogen before cover slips were removed with a razor blade. The slides on which mitotic metaphase cells reside were stored at -20°C until use.

Genomic DNA was isolated from *A. cristatum* and Chinese Spring wheat separately using a modified CTAB method (Dellaport et al. 1983). Genomic DNA of *A. cristatum* was labeled with the DIG-Nick Translation Mix (Roche, Mannheim, Germany) to be used as a probe. The blocker DNA was prepared by sonicating

the Chinese Spring genomic DNA in a supersonic cleaner (model LED-50, Jiangshu, China) for 10 min. The hybridization mixture was denatured at 80°C for 10 min. Chromosome denaturation, hybridization, and hybridization signal detection were carried out as described by Han et al. (2004).

The probe pAs1 was labeled with fluorescent-16-dUTP (Roche, Mannheim, Germany). This probe includes a 1 kb insert from *Aegilops squarrosa* L. (syn. *Ae. tauschii* Coss.) repeated sequences (Rayburn and Gill 1986a) and enables identification of chromosome 1A and all the D-genome chromosomes of wheat (Pedersen and Langridge 1997). Slides of 4844–2 after GISH were washed in 2× SSC (0.3 M sodium chloride, 0.03 M trisodium citrate) at 37°C for 10 min, 4× SSC with 0.2% Tween-20 at room temperature for 10 min and then were used for FISH analysis. The hybridization mixture was denatured at 95°C for 10 min. The pAs1 probe hybridized with chromosome DNA at 37°C overnight. The detection of hybridization signals was carried out as described by Mukai et al. (1993).

Microsatellite analysis

Genomic DNA was isolated from the wheat-*A. cristatum* lines and the two parents as previously described (Dellaport et al. 1983). Two hundred and seventy eight microsatellite primer pairs (Röder et al. 1998; Pestsova et al. 2000a) and 21 EST-SSRs primer pairs (Chen et al. 2005) were used to characterize the genomic composition of the wheat-*A. cristatum* lines. These primers amplify clear and repeatable products from the wheat lines and distribute uniformly throughout all of the wheat chromosomes. Twenty microlitres of each reaction mixture contained 10 mM of Tris–HCl (pH 8.3), 50 mM of KCl, 3.0 mM of $MgCl_2$, 5.0 mM of dNTP each, 5.0 mM of primers each, 60 ng of genomic DNA and 1 unit of *Taq* polymerase. DNA amplification was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA), which was programmed for 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50–60°C, and 1 min at 72°C; and 10 min at 72°C for a final extension. The PCR products were separated on 6% polyacrylamide denaturing gels and were visualized following silver-staining.

Gliadin analysis

Seed proteins were extracted from the crushed endosperms of single kernels. Acid-polyacrylamide gel electrophoresis (A-PAGE) was conducted to fractionate gliadin proteins as described by Khan et al. (1985).

Results

Spike traits of the progeny lines derived from wheat-*Agropyron cristaum* line 4844

The progeny lines that originated from the wheat-*A. cristatum* line 4844, (i.e., 4844–2, 4844–8, and 4844–12) (Fig. 1), had significantly greater numbers of spikelets, florets and kernels per spike, compared with the wheat parent Fukohokomugi in both growing seasons in 1998–1999 and 2004–2005 (Table 1). Lines 4844–2, 4844–8, and 4844–12 had higher kernels and florets per spike than line 4844–5, which also was derived from line 4844 (Table 1).

GISH analysis of the wheat-*A. cristatum* progeny lines

Using *A. cristatum* genomic DNA as a probe and sheared Chinese Spring wheat DNA as a blocker, GISH detected two *A. cristatum* chromosomes in line

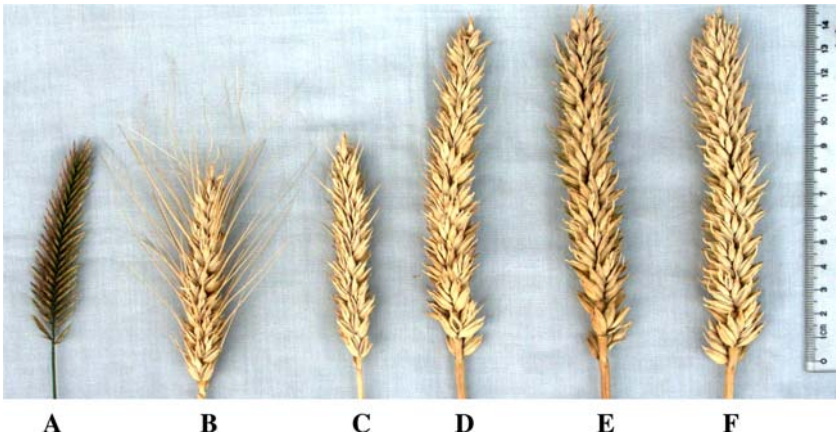

Fig. 1 Spike morphology of *Agropyron cristatum*: **a** *A. cristatum*, **b** Fukohokomugi, **c** 4844–5, **d** 4844–12, **e** 4844–2, **f** 4844–8

Table 1 Number of spikelets, florets, and kernels per spike of the wheat-*Agropyron cristatum* progeny lines

Lines	Chromosome composition	1998–1999			2004–2005		
		Spikelets per spike	Florets per spike	Kernels per spike	Spikelets per spike	Florets per spike	Kernels per spike
Z559	28 P[a]	46.0 A[b]	268.0 A	–	45 A	263 A	–
4844–2	40 W + 2 P	27.3 B	183.6 B	120.5 A	27.1 B	181.4 B	121.7 A
4844–8	40 W + 2 P	27.5 B	189.2 B	123.8 A	27.2 B	190.5 B	125.4 A
4844–12	42 W + 2 P	27.1 B	156.0 C	99.1 B	27.4 B	161.3 C	102.2 B
4844–5	42 W	21.0 C	112.5 D	50.5 C	21.9 C	109.2 D	49.2 C
Fukohokomugi	42 W	21.0 C	120.0 D	49.0 C	22.0 C	118.0 D	52.0 C

[a] P and W: *Agropyron cristatum* and wheat chromosomes, respectively, as determined by GISH using *A. cristatum* genomic DNA as a probe and Chinese Spring wheat genomic DNA as a blocker

[b] Means followed by the same letter were not significantly different at $P < 0.01$ based on Duncan's multiple range test

4844–12 ($2n = 44$) (Fig. 2a), indicating that this line is a wheat-*A. cristatum* chromosome addition line. Similarly, a pair of *A. cristatum* chromosomes were also observed in lines 4844–2 ($2n = 42$) and 4844–8 ($2n = 42$) (Fig. 2b), which replaced a pair of wheat chromosomes, indicating that these lines are wheat-*A. cristatum* chromosome substitution lines. GISH did not detect any *A. cristatum* chromatin in line 4844–5 ($2n = 42$).

Fifteen plants derived from line 4844, which were different in floret and kernel numbers per spike, were analyzed by GISH to confirm the association of *A. cristatum* chromosome with the increased numbers of florets and kernels per spike. Plants with more remarkably florets and kernels carried one or two *A. cristatum* chromosomes. Plants without *A. cristatum* chromosomes did not differ from wheat cultivar Fukohokomugi for numbers of florets and kernels

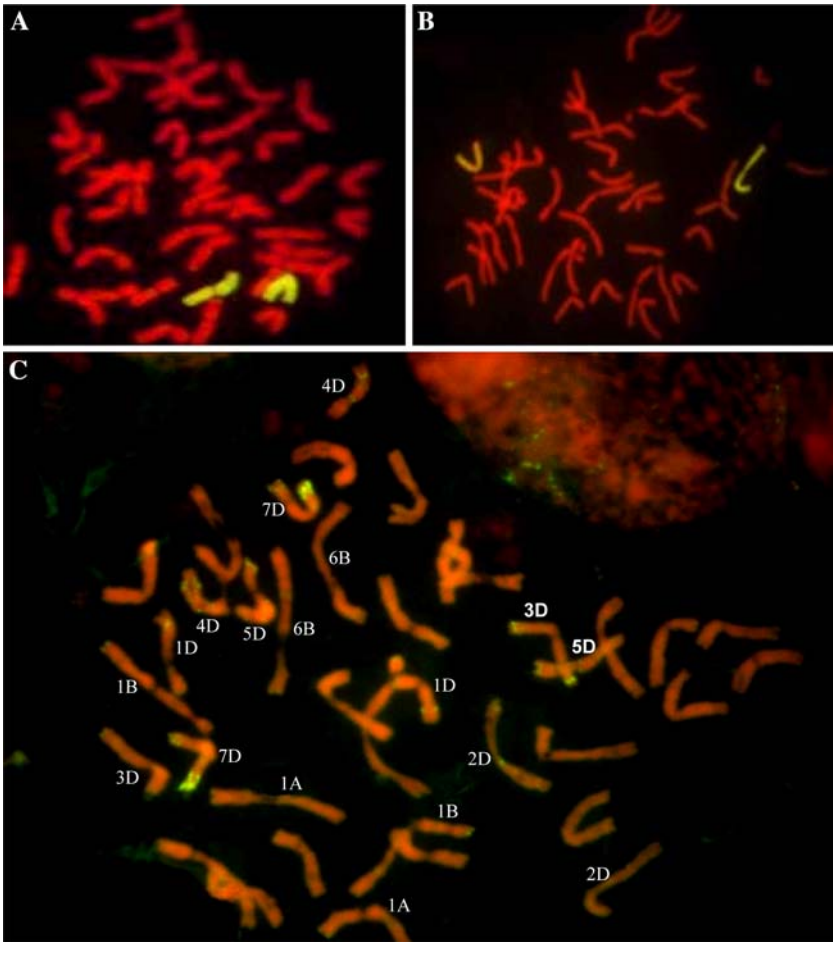

Fig. 2 Genomic in situ hybridization analysis with P genomic DNA from *Aropyron cristatum* (Z559) as a probe and ABD genomic DNA from Chinese Spring wheat as a blocker on mitotic metaphase of the disomic chromosome addition line 4844–12 (**a**) and the disomic chromosome substitution 4844–2 (**b**), a pair of *A. cristatum* chromosomes shows *yellow-greenish color*; and fluorescence in situ hybridization analysis with the repetitive sequence, pAs1, derived from *Aegilops suqrrosa*, as a probe on mitotic metaphase of 4844–2 (**c**), the hybridization signals of pAs1 on wheat chromosomes show a *yellow-greenish color*

Table 2 Agronomic trait of spike and cytogenic composition by GISH analysis of individual plants on the derivatives from 4844

Plant code	Spikelets per spike	Florets per spike	Kernels per spike	2*n*	Chromosome composition
Z559	48	268	—	28	28 P[a]
Fukohokomugi	21	120	49	42	42 W
1	27	164	102	44	42 W + 2 P
2	28	184	119	43	42 W + 1 P
3	28	193	121	42	40 W + 2 P
4	26	175	118	43	42 W + 1 P
5	28	165	105	44	42 W + 2 P
6	30	194	128	43	42 W + 1 P
7	20	109	48	42	42 W
8	27	189	117	42	40 W + 2 P
9	27	185	126	43	42 W + 1 P
10	22	116	53	42	42 W
11	27	187	122	43	42 W + 1 P
12	27	156	99	44	42 W + 2 P
13	28	180	118	43	42 W + 1 P
14	27	183	116	43	42 W + 1 P
15	27	181	117	43	42 W + 1 P

[a] P and W: *Agropyron cristatum* and wheat chromosomes, respectively, as determined by GISH using *A. cristatum* genomic DNA as a probe and Chinese Spring wheat genomic DNA as a blocker

(Table 2). These results demonstrated an association between the *A. cristatum* chromosome and the increased numbers of florets and kernels per spike in the wheat-*A. cristatum* hybrids.

FISH analysis

FISH analysis with the repetitive sequence derived from *Ae. squarrosa*, pAs1, as a probe showed that all the wheat D-genome chromosomes, except for chromosome 6D were present in line 4844–2, indicating that chromosome 6D of wheat was replaced by a pair of *A. cristatum* chromosomes (Fig. 2c).

Microsatellite analysis

Forty-five microsatellite primer pairs, which are polymorphic between Fukohokomugi and *A. cristatum* (Z559) and were located on different wheat chromosomes, were obtained through screening of the 278 microsatellite primer pairs. These SSR markers were used to analyze the wheat-*A. cristatum* lines 4844–12, 4844–2 and 4844–8 (Fig. 3a, b). The diagnostic bands of the SSR markers, such as *gdm*36 and *gdm*108 on chromosome 6D, were missing in lines 4844–2 and 4844–8, but all SSR markers on other chromosomes were present in these wheat-*A. cristatum* lines. This suggests that chromosome 6D of lines 4844–2 and 4844–8 had been substituted by a pair of *A. cristatum* chromosomes. The SSR markers on all wheat chromosomes amplified similar products in chromosome addition line 4844–12 (data not shown), indicating the presence of all the wheat chromosomes.

Twenty one pairs of EST-SSRs primers distributed on seven homologous groups were used to analyze the wheat-*A. cristatum* lines. The EST-SSR primers *SWES123* and *SWES2*, which have been mapped onto homoeologous group 6 of wheat, amplified alleles specific for *A. cristatum* in chromosome addition line 4844–12 and chromosome substitution lines 4844–2 and 4844–8, respectively (Fig. 3c, d), confirming that the *A. cristatum* chromosome that was introduced into Fukohokomugi was homoeologous to the group 6 of common wheat.

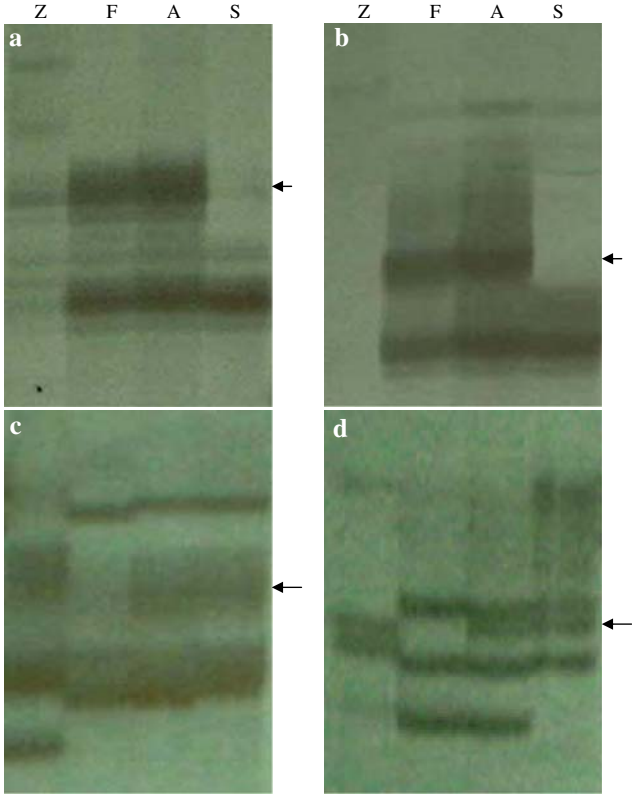

Fig. 3 PCR amplification profiles using SSR (a and b) and EST-SSR (c and d) primers. a, b, c, d: the PCR results of the primers *Xgdm*36, *Xgdm*108, *SWES*2, and *SWES*123. Z, Z559; F, Fukohokomugi; A, chromosome addition line 4844–12; S, chromosome substitution line 4844–2. The arrows indicate the band that are absent in the substitution line. Arrows indicate the diagnostic amplification products of wheat (a and b) or *Agropyron cristatum* (c and d)

Gliadin analysis

Gliadin patterns as revealed by A-PAGE showed that the chromosome addition line 4844–12 and chromosome substitution lines 4844–2 and 4844–8 had two bands that were specific for *A. cristatum* in α-gliadin section (Fig. 4). Common wheat gliadin coding genes are mainly located on wheat 1 and 6 of the homologous chromosome short arm. Since α- and β-gliadins are encoded by the short arm of group 6 of wheat, the presence of the bands of *A. cristatum* specific for α-gliadin further confirmed that the *A. cristatum* chromosome has homoeologous relationship with group 6 of wheat.

Discussion

The superior spike trait is useful in improving yield of wheat and can be achieved by the introgression of chromosomes from the wild species related to wheat. *A. cristatum* has many useful traits for wheat

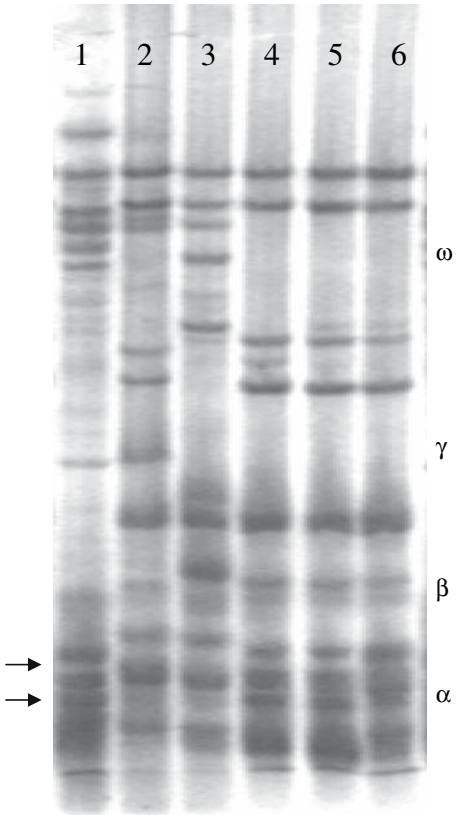

Fig. 4 The results of gliadin analysis. 1, Z559; 2, Fukohokomugi; 3, Marquis; 4, chromosome addition line 4844–12; 5, chromosome substitution line 4844–2; 6, chromosome substitution line 4844–8**.** Arrows indicate the two characteristic bands of *A. cristatum* in chromosome addition and substitution lines

improvement, so a project aimed at transferring *A. cristatum* traits into wheat was initiated by crossing Fukohokomugi wheat with *A. cristatum* accession Z559 in the 1990s, which resulted in a number of progeny lines (Li et al. 1998). Among them, line 4844 exhibited more florets and kernels within a spike in repeated field observations. In the present study, the progeny lines derived from the Fukohokomugi-*A. cristatum* line 4844 were identified as chromosome addition or substitution lines using *A. cristatum* genomic DNA as a probe in the presence of Chinese Spring wheat genomic DNA as a blocker. The association of the *A. cristatum* chromosome with more florets and kernels was confirmed via analyzing line 4844 progeny plants segregating for the *A. cristatum* chromosome. By means of FISH, SSR, EST-SSR, and gliadin analyses, the chromosome of *A. cristatum* in line 4844 proved to be homoeologous to group 6 of wheat. This *A. cristatum* chromosome, which is responsible for increased florets and kernels of wheat was thus designated as 6P. This is the first report on the transfer and genetic control of *A. cristatum* chromosome that enhances the floret and kernel numbers of wheat. Previously, Chen et al. (1994) reported monosomic chromosome addition lines that were derived from Chinese Spring wheat and *A. cristatum* accession that is different from Z559. However, these authors did not describe the agronomic performance of the wheat-*A. cristatum* lines, and they failed to produce any disomic chromosome addition lines from the wheat-*A. cristatum* cross. In the present study, wheat-*A. cristatum* chromosome addition and substitution lines were described, which had desirable agronomic traits that are useful for wheat improvement.

The relationships of chromosomes in the tribe Triticeae can be determined by homoeologous chromosome substitution between the substituted and substituting chromosome, based on the assumption that successful chromosome substitution will give rise to a healthy, viable and compensating organism. This strategy has been frequently used to determine homoeologous relationships between chromosomes of wheat and alien species, such as *Agropyron* spp. (Dvořak 1980; Driscoll 1983; Dou and Chen 2003) and rye (*S. cereale* L.) (Sears et al. 1968; Barber et al. 1968; Gupta 1971; Wang et al. 2004). The repetitive sequence, pAs1, which is derived from *Ae. squorrosa*, has previously been useful for the differentiation of wheat chromosomes by viewing different hybridization signal patterns (Rayburn and Gill 1986b; Mukai et al. 1993). This probe labelled chromosome 1A and all the D-genome chromosomes (Pedersen and Langridge 1997). Microsatellite markers can also be used to

distinguish chromosome substitution lines of wheat (Korzun et al. 1997; Pestsova et al. 2000b). In combination of FISH and SSR analyses, the wheat chromosome that was replaced by the alien chromosome in the wheat- *A. cristatum* lines proved to be chromosome 6D (Figs. 2c, 3a, b). Chromosome substitution lines 4844–2 and 4844–8 were produced by selfing the progenies of the wheat-*A. cristatum* line 4844. The *A. cristatum* chromosome compensated well for wheat chromosome 6D. Thus, the alien chromosome in the wheat-*A. cristatum* progeny lines is most likely homoeologous to the group 6 chromosomes of wheat. The results obtained by EST-SSRs and gliadin analysis further supported this conclusion (Figs. 3c, d, 4).

In this study, the presence of *A. cristatum* chromosome 6P increased the number of florets and kernels per spike in wheat. This indicates that gene(s) controlling high numbers of florets and kernels per spike of the wheat-*A. cristatum* progeny lines is located on chromosomes 6P. Zheng et al. (1993) concluded that chromosome 6D improved floret number per spike by increasing spikelet number within a spike. The results described in the present study demonstrated that the introgression of chromosome 6P not only increases the spikelet number in a spike, but also improves the kernel and floret number within a spikelet (Table 1, 2).

Yen et al. (1993) obtained a common wheat line, 10-A, with multispikelets through crossing rye with wheat. The progenies that are derived from crosses between wheat and *Thinopyrum* spp. often display super spikes with more florets (Li et al. 1985). Peng et al. (2004) described a common wheat mutation line producing three pistils within a floret. In the present study, line 4844 and its progeny lines derived from the wheat-*A. cristatum* cross were described, which exhibited significantly higher numbers of spikelets, florets and kernels per spike than the wheat parent. These wheat-*A. cristatum* progeny lines will be useful in wheat breeding programs for improving kernel numbers.

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