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ORIGINAL PAPER

Molecular cytogenetic analyses of hexaploid lines spontaneously appearing in octoploid Triticale

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Abstract Genome characterization of 14 hexaploid lines that spontaneously appeared in octoploid Triticales was carried out by sequential genomic in situ hybridization and fluorescence in situ hybridization, high molecular weight glutenin subunits and SSR marker analyses. All of the lines showed a chromosome constitution of complete A and B genomes, and a composite genome consisting of the chromosomes of D and R genomes. The composite genome of the 11 lines consisted of chromosomes 1R, 2D, 3R, 4R, 5R, 6R and 7R, that of the two lines were 1D, 2D, 3R, 4R, 5R, 6R and 7R, and that of one line was 1R, 2D, 3R, 4R, 5R, 6D and 7R. The incompatibility of the D and R genomes in common wheat genetic background, preferential retention of chromosome 2D and importance of these lines for the development of hexaploid Triticale are discussed in this report.

Introduction

Octoploid Triticale ($2n = 8x = 56$, AABBDDRR) was the first Triticale produced. As a man-made crop, octoploid Triticale was regarded as a new potential crop that could combine the resistance to environmental stress of rye and the quality of common wheat. However, later it was proven that the octoploid genome is very unstable. The extreme meiotic instability and resulting high aneuploid frequency reduced fertility well below acceptable levels (Lukaszewski and Gustafson 1987).

A large number of octoploid Triticale lines using many different Japanese or Korean common wheat cultivars and two rye lines (Petkus and Sanbongi-shu) as parents have been developed since the 1950s and 1960s of the last century in Tottori University, Japan. In addition, many hexaploid derivatives that spontaneously appeared in octoploid Triticales were obtained (Sasaki et al. 1985). Using acetocarmine–Giemsa staining and telosomes as markers, Nakata et al. (1984) identified three of these lines and demonstrated that the lines carried a composite genome consisting of the chromosomes of genomes D and R (1D, 2D, 3R, 4R, 5R, 6R, 7R, and 1R, 2D, 3R, 4R, 5R, 6R, 7R) together with the complete A and B genomes. Chromosome structural changes, such as a deficiency of the terminal heterochromatin in 3R and a translocation between wheat and rye chromosomes, were also reported. C-banding and telosomic analysis were efficient methods to identify chromosome constitution of Triticales at that time. However, biochemical markers such as chromosome mapped high molecular weight (HMW) glutenin subunits (GS) (Payne and Lawrence 1987), molecular markers such as SSR markers (Tams et al. 2004), genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) (Heslop-Harrison et al. 1992) are well used today as more accurate tools or techniques to analyze the chromosome constitution.

In this report we describe the genome constitution of 14 hexaploid derivatives of octoploid Triticales by

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sequential GISH and FISH using genomic DNA and different repetitive DNA probes in combination with the biochemical and molecular markers, and show general feature of the stable chromosome constitution of the hexaploid Triticale.

Materials and methods

Plant materials

We analyzed 14 hexaploid derivatives from the progenies of 13 primary octoploid Triticales developed by Sasaki et al. (1985; Table 1). These original lines were produced by the cross between Japanese or Korean common wheat cultivars (*Triticum aestivum*) and rye lines (*Secale cereale*). The series of wheat cultivars named 'Suwon' are Korean cultivars and the others are Japanese cultivars. All of these lines are kept in the Tottori Alien Chromosome Bank of Wheat supported by the National Bioresources Project—Wheat, Japan.

In situ hybridization

Seeds were germinated at room temperature. Root tips were collected at a length of 0.5–2 cm, pretreated in ice-water for 24 h, and fixed in 99% ethanol–glacial acid (3:1). Slides were prepared by squashing in 45% acetic acid.

Genomic DNA of rye and *Aegilops tauschii* were used as probes in GISH. Clones pAs1 (Rayburn and Gill 1986a, b), pSc74 (Bedbrook et al. 1980), pTa71 (45S rDNA of wheat; Gerlach and Bedbrook 1979) and a synthesized 30-base length $(AAG)_{10}$ repetitive oligomer were used as probes in FISH. Genomic DNA and cloned DNAs were labeled with fluorescein-12-dUTP or tetramethyl-rhodamine-5-dUTP using the random primer method (Gene Images™ Random Prime Labeling Module, Amersham Bioscience). Before labeling of the genomic and plasmid DNAs, the DNAs were fragmented by heat treatment at 120°C for 2 min. $(AAG)_{10}$ was labeled with tetramethyl-rhodamine-5-dUTP using the 3′-end method (Gene Images™ 3′-oligolabeling Module, Amersham Bioscience).

pSc74 carries the highly repeated 350–480-bp sequence element (Bedbrook et al. 1980). Usually the 350–480-bp sequence hybridizes to all rye chromosomes at the terminal and subterminal regions, in addition to the interstitial regions in some chromosomes (Cuadrado et al. 1995). The distribution patterns of microsatellite $(AAG)_5$ or GAA-satellites on the chromosomes of wheat, barley and related species in Triticeae are known to correspond to the N-band patterns (Cuadrado and Schwarzacher 1998; Pedersen et al. 1996). pAs1 is regarded to be a D-genome-specific clone. The pattern of FISH with this clone permits identification of the D-genome chromosomes, though there are other weak hybridization signals on some B- and A-genome chromosomes (Rayburn and Gill 1986a; Pedersen and Langridge 1997). The pattern of GAA-satellites together with pAs1 can identify the entire chromosome complement of bread wheat by two-color FISH (Pedersen and Langridge 1997).

The procedures for FISH and GISH were those reported by Kishii et al. (1999) with slight modifications. DNA denaturation was carried out in 0.15 M NaOH in 70% ethanol at room temperature for 10 min. The reprobing procedure was as follows: after photography of the first probing, cover slips were removed and

Table 1 Chromosome constitutions of hexaploid derivatives of octoploid Triticale

Line	Pedigree (wheat × rye)	No. of plants examined	No. of chromosomes in the genome				
			A	B	D	R	Total
S783-1	Suwon 92 × Sanbongi-shu	4	14	14	4 (2D, 6D)	10	42
S783-3	Suwon 92 × Sanbongi-shu	2	14	14	2 (2D)	12	42
S51-1	Suwon 85 × Sanbongi-shu	3	14	14	2 (2D)	12	42
S749-14	Honkei 124 × Petkus	2	14	14	4 (1D, 2D)	10	42
S78	Suwon 88 × Petkus	3	14	14	2 (2D)	12	42
S34	Kitakanto 41 × Petkus	2	14	14	2 (2D)	12	42
S88C	Hiraki-komugi × Petkus	4	14	14	2 (2D)	12	42
S7-1	Suwon 89 × Petkus	7	14	14	2 (2D)	12	42
S465a	Eshima-shinriki × Petkus	2	14	14	2 (2D)	12	42
S480	Norin 43 × Petkus	2	14	14	2 (2D)	12	42
S591	Shirodaruma × Sanbongi-shu	3	14	14	2 (2D)	12	42
S376	Sekitori 1 × Petkus	1	14	14	2 (2D)	12	42
S191	Hokuriku 30 × Petkus	3	14	14	4 (1D, 2D)	10	42
S710	Wase-nyubai × Petkus	1	14	14	2 (2D)	12	42

the slides were washed in 2 × SSC for 20 min at room temperature, and then briefly dried; and the hybridization mixture was directly applied to the slide with denaturation for 2 min or without denaturation. The other steps were the same as for the first hybridization. A 40-fold excess of genomic DNA of common wheat cv. Chinese Spring relative to the probe was included as blocking DNA in the GISH of rye. GISH of *A. tauschii* was directly carried out without adding blocking DNA of Chinese Spring. Images were acquired using a fluorescence microscope (Olympus BX61) with a CCD camera (Cool snapper, Photometrics), and were processed using Photoshop ver. 6.0 (Adobe).

SSR marker analysis

Seven SSR markers of chromosome 6D, *Xhbe387*, *Xhbg439*, *Xhbg269*, *Xhbg276* (kindly supplied by Dr. Torada, Hokkaido Greenbio Co., Japan) and *Xcfd76*, *Xbarc21*, *Xcfd60* (http://www.wheat.pw.usda.gov/GG2/index.shtml; Sourdille et al. 2004) are used in this study. *Xhbe387* was mapped on the short arm, while the others on the long arm. Polymerase chain reaction (PCR) was performed in 15 µl reaction volumes containing the following reagents: 25 ng of template DNA, 0.2 mM of each of the dNTP, *Taq* DNA polymerase buffer, 0.8 U of *Taq* DNA polymerase (Takara, Japan). The PCR program consisted of a 3-min initial denaturation step at 96°C, followed by 35 cycles with 30 s denaturation at 96°C, 30 s annealing at 60°C and 30 s extension at 72°C, and with a final extension step at 72°C for 5 min. The products were separated by electrophoresis with 8% polyacrylamide gels, and stained with ethidium bromide.

HMW glutenin analysis

High molecular weight GS compositions were determined by SDS-PAGE following the procedure described by Tanaka et al. (2003).

Results

First, karyotyping of rye cultivars Petkus and Sanbongi-shu was carried out by FISH using the pSc74 repetitive sequence and $(AAG)_{10}$ as probes. By combing the signal distributions of these repetitive sequences, we could completely discriminate the individual rye chromosomes (Fig. 1a). Remarkable hybridization sites of $(AAG)_{10}$ on chromosomes 2R, 3R and 6R are good markers to identify these chromosomes. Since rye is outcrossing species, polymorphism was observed in chromosomes 3R, 4R and 7R in Petkus, and 1R and 3R in Sanbongi-shu.

Next, GISH analyses were carried out for the 14 hexaploid derivatives from 13 octoploid Triticales. The results showed that 3 carried 10 rye chromosomes, and the others carried 12 rye chromosomes (Fig. 1b, e, i, l, for examples; Table 1). Further sequential GISH and FISH analyses showed that these derivatives could be grouped into three types with respect to their chromosome constitutions.

The majority of the lines (11 lines, 79% of the total) showed 12 rye chromosomes (Fig. 1b, e) and a pair of chromosome 2D (Fig. 1c, f). Further, reprobing with pSc74 revealed that chromosome 2R was not present (Fig. 1d). The patterns of $(AAG)_{10}$ and pAs1 signals indicated presence of complete A and B genomes in all of the lines except S51-1 (Fig. 1c). S51-1 showed a pair of unidentified A-genome chromosomes with a small pAs1 signal at the short arm end and a small $(AAG)_{10}$ signal on the centromere (Fig. 1f). Pedersen and Langridge (1997) reported that only the short arm of chromosome 1A carried the pAs1 signal within the A genome. However, typical chromosome 1A, as that in cultivar Chinese Spring, does not carry the $(AAG)_{10}$ signals on the centromere. Sequential FISH revealed that the chromosome carried 45S rDNA signal at the same portion of the pAs1 (Fig. 1g). In addition, GISH–FISH with *A. tauschii* DNA together with 45S rDNA denied possibility of existence of chromosome 5D that is known to carry 45 rDNA (Fig. 1h, Mukai et al. 1991). These results suggested that the chromosome in line S51-1 was chromosome 1A in variant form. In spite of different origin, these 11 hexaploid lines were found to carry a common composite D/R genome consisting of chromosome 1R, 2D, 3R, 4R, 5R, 6R and 7R, as well as complete A and B genomes.

Line S783-1 carried five pairs of rye chromosomes and two pairs of D-genome chromosomes in addition to the complete A and B genomes (Fig. 1i, j). One of the D-genome chromosomes was identified as 2D, and another chromosome seemed to be 3D or 6D because it carried strong pAs1 signals at both ends of the chromosomes. FISH with pSc74 and $(AAG)_{10}$ showed inclusion of chromosome 3R in this line (Fig. 1k). No other rye chromosomes with the $(AAG)_{10}$ signal were present in this line. This means absence of chromosome 2R and 6R. This result was confirmed by SSR markers mapped to the chromosome 6D (Fig. 2). All of the seven markers tested were present in Suwon 92 (wheat parent of S783-1) and S783-1 but absent in Sanbongi-shu (rye parent) and S783-3 (a sib line carrying chromosome 6R). This result indicated that S783-1 included chromosome 6D. Unexpectedly, two markers

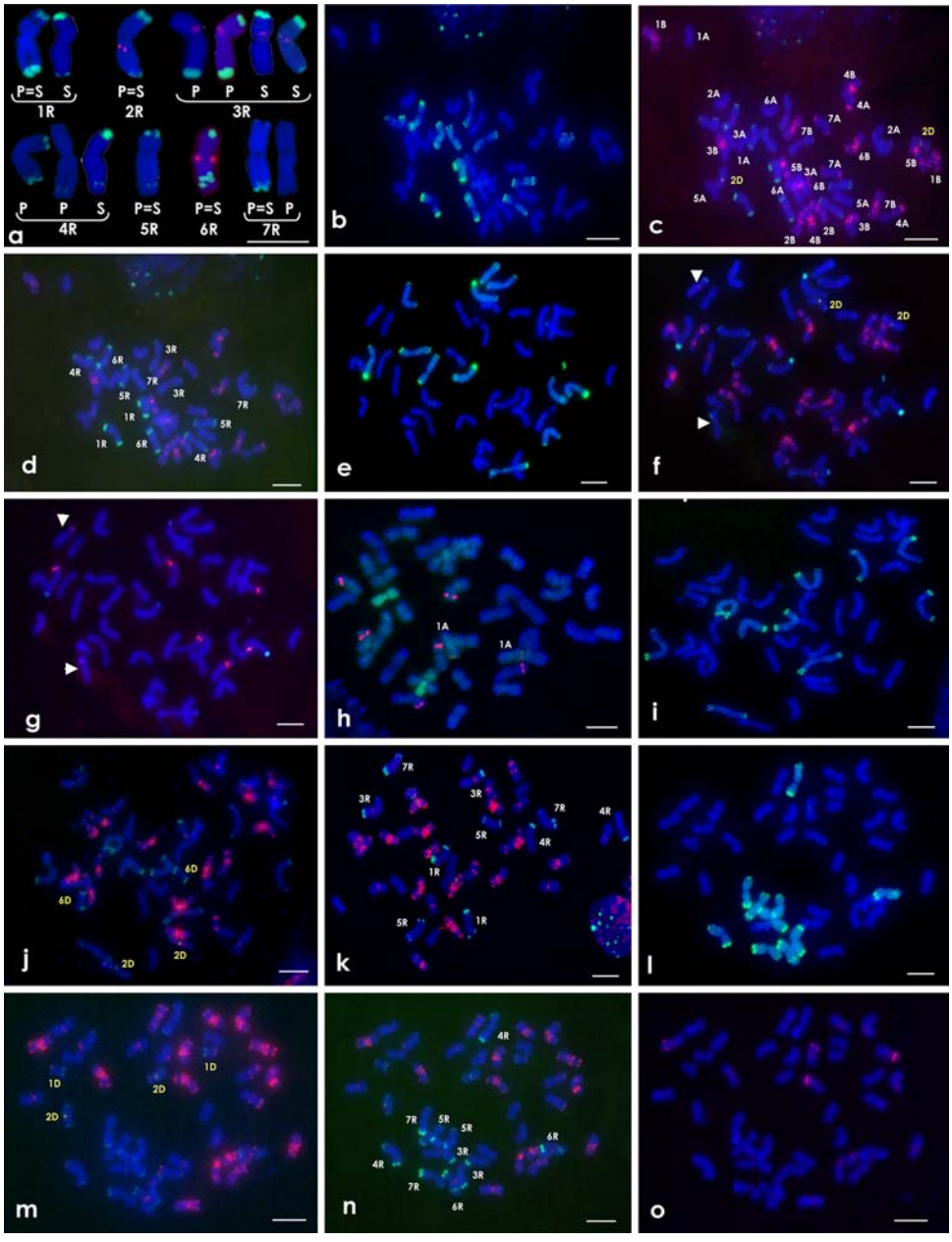

Fig. 1 FISH and GISH images of the chromosomes of rye and hexaploid derivates of octoploid Triticale. **a** Karyotype of rye cultivars 'Petkus' and 'Sanbongi-shu' probed with $(AAG)_{10}$ (*red*) and pSc74 (*green*). 'P' and 'S' indicate 'Petkus' and 'Sanbongi-shu', respectively. 'P = S' means no polymorphism between the cultivars. **b** GISH of S591 probed with genomic DNA of rye. **c** Sequential FISH of the same cell reprobed with $(AAG)_{10}$ (*red*) and pAs1 (*green*). A- and B-genome chromosomes are annotated together with chromosome 2D. **d** Sequential FISH of the same cell reprobed with pSc74 (*green*). Rye chromosomes are annotated. **e** GISH of S51-1 probed with rye DNA. **f** Sequential FISH of the same cell reprobed with $(AAG)_{10}$ (*red*) and pAs1 (*green*). *Arrowheads* indicate unidentified A-genome chromosome (see text). **g** Sequential FISH of the same cell reprobed with 45S rDNA. **h** FISH of S51-1 probed with genomic DNA of *A. tauschii* and 45S rDNA. Chromosome 1A is indicated. **i** GISH of S783-1 probed with rye DNA. **j** Sequential FISH of the same cell reprobed with $(AAG)_{10}$ (*red*) and pAs1 (*green*). Chromosomes 2D and 6D are indicated. **k** Sequential FISH of S783-1 reprobed with $(AAG)_{10}$ (*red*) and pSC74 (*green*). Rye chromosomes are annotated. **l** GISH of S191 probed with rye DNA. **m** Sequential FISH of the same cell reprobed with $(AAG)_{10}$ (*red*) and pAs1 (*green*). Chromosomes 2D and 1D are indicated. **n** Sequential FISH of the same cell reprobed with pSc74 (*green*). Rye chromosomes are annotated. **o** Sequential FISH of the same cell of reprobed with 45S rDNA. Scale bar = 10 μm

(*Xhbg276* and *Xcfd76*) showed different amplified fragments between Suwon 92 and S783-1. This may suggest sequence change due to the genome reorganization.

Lines S749-14 and S191 had ten rye chromosomes (Fig. 1l). Reprobing with $(AAG)_{10}$ and pAs1 showed that it included complete A and B genomes and two

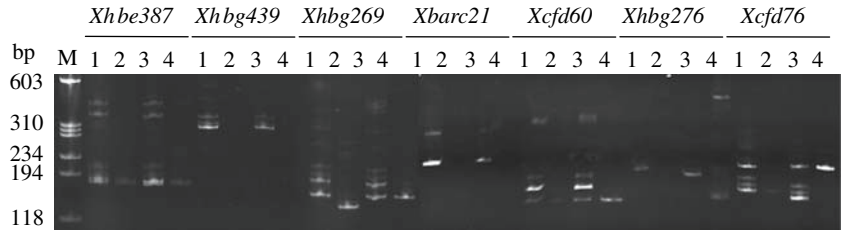

Fig. 2 Detection of chromosome 6D by SSR markers in hexaploid lines derived from octoploid Triticale: *M* molecular size marker (ϕX174/*Hae*III); *1* common wheat cultivar Suwon 92; *2* rye cultivar Sanbongi-shu; *3* hexaploid line S783-1; *4* hexaploid line S783-3. Both the hexaploid lines are the derivatives of a cross between Suwon 92 and Sanbongi-shu

pairs of D-genome chromosomes. One was identified as 2D, and the other was 1D, which showed a pAs1 signal on the subtelomeric regions of both arms and an $(AAG)_{10}$ signal on the subtelomere of the short arm (Fig. 1m). Reprobing with pSc74 revealed that rye chromosomes 1R and 2R were not included in this type (Fig. 1n). Further reprobing with 45S rDNA detected four large signals only on wheat chromosomes 1B and 6B (Fig. 1o), and no signals on the rye chromosomes. This confirmed lack of chromosome 1R.

The HMW-GS compositions of these 14 derivatives together with their common wheat and rye donor parents were analyzed by SDS-PAGE (Fig. 3). Lines S749-14 and S191 had 2+12 HMW-GS bands, which were encoded by the *Glu-D1a* gene located on the long arm of chromosome 1D of common wheat; while the other lines had bands encoded by the *Glu-R1* locus on 1RL of rye. These results were in agreement with the cytological analyses performed on these lines and described above. An exception to this was that in line S749-14, subunit 1 encoded by *Glu-A1a* gene was absent, but it was present in its donor common wheat Honkei 124. Since no apparent aberration of chromosome 1A of this line was observed in our study, this finding implied that the mutation or expression silencing of this locus might have occurred during the genomic reorganization.

Discussion

Octoploid Triticale is known to show meiotic instability and high aneuploid frequency (Lukaszewski and Gustafson 1987). Weimarck (1974) demonstrated by Giemsa banding that chromosomes from not only rye but also common wheat were eliminated. However, due to technical limitations, he could not discriminate whether different chromosomes of wheat were preferentially eliminated or not. In the present study, all of the hexaploid lines used were derived from octoploid Triticale lines due to spontaneous chromosome elimination. In the chromosome elimination process, the chance of chromosome loss of the A, B, D and R genomes would be equal because of the balanced genome constitution (AABBDDRR) of their original lines, if no selective advantages of these chromosomes existed. However, our results revealed that these lines retained the complete chromosomes of the A and B genomes, and most of the R-genome chromosomes, while most of the D-genome chromosomes were eliminated.

Tams et al. (2004) reported that when they determined genetic diversity in 128 European winter Triticale lines with SSR markers, 3 out of 39 D-genome-specific markers, on chromosomes 2D and 7D, amplified products in about 23.4% of total Triticale genotypes. Similarly, using SSR markers to characterize the genome

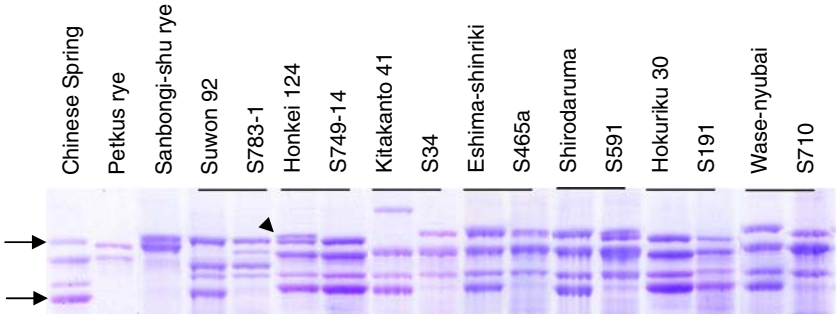

Fig. 3 HMW glutenin electrophoresis patterns of hexaploid Triticale representatives and their parents. *Arrows* indicate subunit 2 + 12 bands encoded by the *Glu-D1a* gene. The *arrowhead* indicates the subunit 1 band encoded by the *Glu-A1a* gene

constitution of seven hexaploid derivatives from octoploid Triticales in F_4–F_5 generation, Leonova et al. (2005) demonstrated that two lines exhibited SSR amplification specific to the 2DL and one to the 7D. Both researches revealed that hexaploid Triticale lines contained D-genome chromosomes in lower frequency, and the involved chromosomes are limited, i.e., 2D and 7D.

All these results suggest that the stability of the D genome is more strongly affected by the R genome in the octoploid Triticale, comparing to the A and B genomes of common wheat. Observation of the chromosome constitution of those original octoploid Triticale lines revealed very high chromosome variation of the R genome, as indicated by features such as telosomes, aneuploidy and translocation (unpublished data). This suggests that there might be also some genes in the D genome which can strongly promote the instability of the R genome in the common genetic background. Since 1D, 2D, 6D and 7D were found in this and the other studies, genes on chromosomes 3D, 4D and 5D may promote the instability of the R genome. In some way, the D genome and R genome are incompatible in the common wheat genetic background.

Meiotic instability, which always results in a high frequency of aneuploidy, is thought to be strongly affected by homoeologous chromosome pairing in allopolyploids. It is known that rye chromosomes can increase homologous chromosome pairing in hybrids between rye and wheat (Dvorak 1977; Gupta and Fedak 1986). It was reported that factors on 5R can affect homoeologous chromosome pairing: 5RS promotes homoeologous chromosome pairing, whereas 5RL has a weaker suppressor (Sears 1976). Cheng and Murata (2002) reported that 5RS was frequently lost in the advanced octoploid Triticale, and suggested that loss of 5RS would make the genome stable. Besides the *Ph1* (pairing homologous) gene on 5B, several loci that affect chromosome paring in wheat have been identified (Sears 1976). In the D genome, 3DS carries a promoter of pairing, while 3DL carries a suppressor (Driscoll 1972); 5D was suggested to carry a promoter on each arm (Feldman 1968), and the promoter on 5DL is not homoeoallelic to *Ph1* on 5BL (Mello-Sampayo 1972). Our study revealed that hexaploid derivatives, which nearly completely excluded the chromosomes of the D genome, contained the whole 5R chromosome, while octoploid Triticale containing the D genome appeared to show high instability of the R genome (unpublished data). If the instability of octoploid Triticale was affected by chromosome pairing controlled by the factors on chromosomes 3D, 5D and 5R, further investigations of the interaction between these factors should help to develop a more stable octoploid Triticale.

In the present study, 14 hexaploid lines derived from 13 cross-combinations including 13 common wheat and 2 rye cultivars. All of the lines showed 2D(2R) substitution, and additionally two showed 1D(1R) and one showed 6D(6R). The 6D(6R) substitution appeared in one of three combinations using Sanbongi-shu as the rye parent, while 1D(1R) occurred in two of the ten combinations using Petkus as the parent. Furthermore, chromosomes 2D and 7D were reported to be detected in some European hexaploid Triticales (Tams et al. 2004; Leonova et al. 2005). All these findings implied that the stability of some D-genome chromosomes in hexaploid Triticale may be affected by common wheat genetic background, rye genetic composition as well as their interaction.

All of the lines used here had lost chromosome 2R, which was substituted by chromosome 2D. Cheng and Murata (2002) also reported very frequent loss of chromosome 2R in the advanced lines of octoploid Triticale. They regarded it likely that chromosome 2R carries a gene affecting the threshing, and that artificial selection removed chromosome 2R from the advanced octoploid lines. Lukaszewski and Gustafson (1984, 1987) reported very frequent 2D(2R) substitution in secondary hexaploid Triticale produced by crosses between hexaploid Triticale and hexaploid wheat. They suggested that preferential 2D(2R) substitution was attributable to the selection pressure for a potent gene for day-length insensitivity located on wheat chromosome 2D. In the present lines no selections of agronomic traits were performed. It was reported that 2D(2R) substitution in hexaploid Triticale can prevent suppression of the activity of the rye-origin rDNA, which is usually inactivated in the AABBRR Triticales (Neves et al. 1997). These suggest that loss of chromosome 2R or substitution of 2D for 2R occurred because of selection due to genomic stability rather than the selection of agronomic traits or selection due to environmental stresses.

The D genome of wheat has a number of valuable genes for the improvement of quality, disease resistance, winter hardiness and other characteristics. It is believed that many characteristics of hexaploid Triticale could be improved by introducing D-genome chromosomes or their segments (Lukaszewski et al. 1987; Lukaszewski and Curtis 1994). Our study demonstrated stable substitution of 1D for 1R, 2D for 2R, 6D for 6R, in these hexaploid Triticale lines. It is known that chromosome 1D carries the *Glu-D1* locus, which plays a major role in the bread-making quality of bread wheat (Payne and Lawrence 1987). Two derivatives (S749-14 and S191) carrying chromosomes 1D substituted for 1R carried the expected HMW glutenin

(Fig. 3). Chromosome 6D carries the complex *Gli-2* locus controlling α and β gliadins, which are known to have beneficial effects on end-use quality characteristics (Payne et al. 1984). Chromosome 2D carries a gene for day-length insensitivity, *Ppd1* and an important dwarfing gene, *Rht8* (Worland et al. 1998). Thus, these lines might be potential materials for further hexaploid Triticale improvement.

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