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Dynamic nature of a wheat centromere with a functional gene

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Abstract Centromeric regions of higher eukaryotes are comprised mainly of tandem and non-tandem repeat sequences with variable copy number, spacing, order and orientation; are heterochromatic in nature, and are believed to be devoid of actively transcribing genes. Here, we report an actively transcribing wheat homolog of *HSP70* gene that maps in the functional wheat centromere, and copy number of which seems to change in response to centromeric breaks. The *HSP70* gene physically maps on the short arm of chromosomes *1A* and *1D* of Chinese Spring (CS) and *1R* of rye. Whereas, on chromosome *1B* in both 'CS' and Pavon background, the gene maps in the functional centromere as evident from its presence in both cytologically confirmed true ditelosomic lines Dt1BS and Dt1BL. Sequence comparison of 11 ESTs showed three sequence patterns suggesting that all three homoeologous copies of the gene are expressing. The cDNA-single stranded conformation polymorphism analysis confirmed expression of the 'CS' *1B* copy of the gene. Observed in two independently developed Dt1BL lines, the *1B* copy number of the gene showed three to fivefold increase in response to chromosomal breaks around the centromere. Putative gene duplications seem to involve large chromosomal segments as only one of the ten restriction enzymes used for DNA gel-blot analysis showed unique extra fragment band in the Dt1BL line. Further investigations are warranted to uncover the nature and mechanism of these duplications.

Keywords Centromere · Deletion mapping · N-banding · SSCP · Wheat

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Introduction

Centromeres are specialized regions of the eukaryotic chromosomes that are responsible for sister chromatid cohesion, kinetochore assembly and spindle fiber attachment to mediate proper chromosomal segregation during meiosis and mitosis. The size and complexity of centromeres may show tremendous variation across genera and even among chromosomes of the same organism (Birchler et al. 2009; Clarke 1998; Malik and Henikoff 2002). In spite of the sequence variation, centromeres show remarkable

conservation of function across eukaryotes. The evolutionary forces or the mechanisms responsible for this sequence variation are largely unknown.

Centromeres of higher eukaryotes are composed mainly of satellite repeats and centromeric retrotransposons (Ma et al. 2007). Significant variation in the arrangement and abundance of these repeats, however, are observed at every level. The size of the centromeric region in eukaryotes may range from 40 to 100 kb as in *Schizosaccharomyces pombe*, to 250–5,000 kb as found in human and other mammalian genomes (Clarke 1998). Plant centromeres are mainly composed of repetitive DNA arrays interspersed with centromere-specific retrotransposons (CRs) and are equally variable for their size. For example, the size of 180-base satellite repeats may range from 400 kb to 1.4 Mb among *Arabidopsis* centromere and size range for its rice counterpart (CentO satellite DNA) is 60 kb to 1.9 Mb (Ma et al. 2007). Furthermore, extensive variations in copy number of centromeric repeat sequences have also been reported among different ecotypes of *Arabidopsis* (Hall et al. 2003), inbred lines of maize (Kato et al. 2004) and among subspecies of rice (Cheng et al. 2002). As in other plants, wheat centromeres are also composed mainly of CRs that constitute major DNA component of functional centromeres (Liu et al. 2008).

Large segmental duplications, deletions, and rearrangements of centromeric DNA seem to be the common processes governing evolution of centromeres. For example, rice *Cen8* contains tandem duplication of three segments with sizes of 96, 90 and 26 kb (Ma and Bennetzen 2006). Similar segmental duplications were found for the CentO satellite arrays and CRs interspersed in the rice *Cen4* sequence (Ma and Jackson 2006). Comparison with the human orthologous region showed extensive duplication of a 250 kb segment on a relatively new centromere of macaque chromosome *4* (Ventura et al. 2007). Thus, rapid growth or shrinkage of centromeric regions seems to occur in relatively small evolutionary period, but the mechanism governing these changes (duplications and deletions) is not clear.

Most of the chromosomal regions in or around centromeres are believed to be devoid of functional genes although functional genes have been reported in the centromeric regions of *S. pombe*, *Drosophila melanogaster*, *A. thaliana* and *Oryza sativa* (Clarke 1998; Copenhaver et al. 1999; Nagaki et al. 2004; Wu et al. 2004). Seven genes were shown to be expressing from the kinetochore region of rice chromosome *8* (Nagaki et al. 2004). It is however unclear if the unusual deletion-duplication pattern seen in the centromeric regions is restricted to the repeat sequences or is common to all sequences present in the regions including genes.

With an average size of 11.2 μ/chromosome, wheat chromosomes are large and thus are ideal for cytological studies. At mitotic metaphase, functional centromeres show a distinct morphology that can easily be distinguished from the rest of the chromosomal parts (Friebe and Gill 1994). Clearly seen in telocentric chromosomes, wheat centromeres appear as distinct round structures that are remarkably different from the sub-telomeric regions present in acrocentric chromosomes (Gill et al. 1991). C-banding (staining constitutive heterochromatin) pattern and other evidence suggest that the wheat centromeric regions are highly heterochromatic, especially in the *B* genome. Physical mapping of more than 1,300 wheat genes using 35 ditelosomic (Dt) and 334 deletion lines showed that the centromeric regions are devoid of expressed genes (Erayman et al. 2004). With the exception of two that were present in the centromeric region of chromosome *1B*, no genic sequence was observed in the proximal 10% of the chromosomal region around the centromeres (Sandhu et al. 2001; Erayman et al. 2004). These two genes are *HSP70* homologs and are 89% similar at the DNA sequence level. The objectives of this study were to precisely localize these two genes on wheat chromosomes, study their expression, and understand the dynamics of centromeric regions in response to chromosomal breaks.

Materials and methods

Plant material

Various aneuploid stocks in wheat cultivar Chinese Spring (CS) and Pavon (P), and wheat–rye translocation lines were used for precise localization and expression analysis of the genes. The 'CS' aneuploids included group *1* nullisomic tetrasomic (NT) (lines missing a pair of homologous chromosomes, the deficiency of which is compensated by the double dose of either of the two homoeologous chromosomes) lines (N1AT1B^{CS}, N1BT1D^{CS}, and N1DT1A^{CS}), and six ditelosomic (Dt)

(lines missing a pair of chromosome arms) lines ($Dt1AS^{CS}$, $Dt1AL^{CS}$, $Dt1BS^{CS}$, $Dt1BL^{CS}$, $Dt1DS^{CS}$, and $Dt1DL^{CS}$; Sears 1954). Precise physical location of the genes was determined using 55 group *1* deletion lines (Sandhu et al. 2001). Ditelosomic 1BS ($Dt1BS^{P}$) and 1BL ($Ac1BL^{P}$) lines of cultivar 'P' were also used to confirm location of the genes in the *B* genome. The $Ac1BL^{P}$ line was actually acrocentric, containing a small chromosomal region of the short arm (Adam Lukaszewski, personal communication). The wheat–rye translocation lines for group *1* were $1AS^{P}$.1RL, $1RS.1AL^{P}$, $1BS^{P}$.1RL, $1RS.1BL^{P}$, $1DS^{P}$.1RL, $1RS.1DL^{P}$. The 'CS' aneuploid stocks were obtained from the Wheat Genetics Resource Center (Kansas State University, USA) and that for 'P' from Dr. A. Lukaszewski (University of California-Riverside, USA).

Gel-blot DNA analysis

DNA isolation and gel-blot analysis was performed as previously described (Gill et al. 1993; Sandhu et al. 2001).

N-banding analysis

The N-banding chromosome analysis procedure was performed as described by Endo and Gill (1984), with few modifications. Briefly, 1.5 cm long root tips were incubated in ice-cold water for 22 h and fixed in ethanol: acetic acid (3:1, v/v). After staining in acetocarmine for 2 h, the root-tips were squashed onto a microscope slide in 45% acetic acid. The cover-slips were removed from the slides frozen at −80°C, and the slides were immediately incubated in 45% acetic acid for 10 min at 60°C, followed by a 95% ethanol treatment for 10 min at room temperature. The slides were treated with 1 M NaH_2PO_4 for 2 min at 94°C, rinsed in water and air-dried overnight. The slides were then stained for 15–35 min in 66 mM of Na_2HPO_4 and 66 mM of KH_2PO_4 (1:1, v/v) containing 2% Giemsa stain (Richard-Allan scientific, MI, USA).

RNA and single-stranded conformation polymorphism analysis

All plants for RNA extractions were grown under uniform growth conditions of 16/8 h light/dark and 22°C/18°C day/night temperature in a growth chamber. For the gene expression analysis, RNA was isolated from tissue pooled from five different developmental stages: leaves at 5 and 20 days after germination, and spikes at flower initiation, pre- and post-anthesis stages. Total RNA was extracted using the guanidinium thiocyanate—cesium chloride density gradient method of Sambrook et al. (1989), with modifications as described by Sandhu et al. (2002). The first strand cDNA was prepared from 2 µg of DNaseI treated poly(A)$^{+}$ using the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Clonetech, CA, USA), following the manufacturer's recommendations.

PCR reactions were performed using 20 ng cDNA template in a 20 µl volume containing 5 µM each of dNTPs, 0.4 µl of Advantage® cDNA polymerase mix (Clonetech Lab Inc.), 20 µM of each primer, 2 µl of 10× cDNA PCR reaction buffer and 0.4 µl of [^{35}S]-ATP isotope. The PCR conditions were: one cycle of 1 min at 94°C, three cycles of 1 min at 92°C, 2.5 min at 60°C and 3 min at 72°C; 32 cycles of 45 s at 92°C, 1 min at 60°C and 45 s at 72°C, followed by 10 min at 72°C. The PCR product was mixed with an equal volume of loading buffer and about 5 µl of this mixture was loaded onto 0.4 mm thick denaturing 8% polyacrylamide/8 M urea gels (Sambrook et al. 1989). Gels were prepared and run in 0.5× TBE buffer at pH 8.3. Each sample was size separated on gels run under standard as well as single-stranded conformation polymorphism (SSCP) conditions. For a standard run, the gels were pre-run at a 33 mA constant current for 30 min followed by a 4 h run at 70 W constant power. For an SSCP run, the gels were pre-chilled at 4°C for at least 5–6 h before running it at 10 W for 12–13 h at 4°C. An X-ray film was placed on the dried gels and was exposed for 3–7 days.

Copy number analysis of PSR161 probe

ImageJ, a Java-based image processing software developed at the National Institutes of Health (http://rsbweb.nih.gov/ij) was used to measure the copy number of *PSR161* gene in $Dt1BL^{CS}$ in comparison to 'CS'.

Results

Physical mapping

Gel-blot DNA analysis of 'CS' group *1* nullisomic tetrasomic (NT) and ditelosomic (Dt) lines with

PSR161 gene probe is shown in Fig. 1. The probe *BCD1072* showed identical banding pattern as that of *PSR161* (data not shown). Sequence comparison revealed that these two probes shared 89% sequence similarity at nucleotide level. *BCD1072* is a barley cDNA probe and *PSR161* is a *Pst*I generated wheat genomic probe. Similar restriction fragment band pattern during gel-blot analysis and a high level (89%) of sequence similarity suggested that these probes represent orthologous sequences in wheat and barley. The probes detected three fragment bands, which by NT analysis were localized to each of the three homoeologs suggesting that the corresponding sequences are present on each of the chromosomes *1A*, *1B*, and *1D* (Fig. 1). On chromosomes *1A* and *1D* the sequence mapped on the short arms, as the corresponding fragment bands were present in Dt1AS^{CS} and Dt1DS^{CS} and missing in Dt1AL^{CS} and Dt1DL^{CS}. In order to precisely localize the *PSR161* gene sequence, physical mapping using 55 chromosome group *1* deletion lines was performed. Demarcated by deletion lines [1AS-1 fraction length (FL) 0.47 and 1AS-2 (FL 0.45)], the *A* genome band was present between FL 0.45 and FL 0.47 of chromosome *1AS* (data not shown). The *D* genome band was present in all of the short arm deletion lines. Since 1DS-1 (FL0.59) was the largest deletion of chromosome *1DS*, the probe sequence was localized to the region between the centromere and FL 0.59 (data not shown).

The *B* genome specific band, however, was present in both Dt1BS^{CS} and Dt1BL^{CS} suggesting that the *B* genome copy of the gene is present in the centromere. To rule out presence of any unexpected chromosomal abnormalities in the Dt lines, mitotic chromosomes of Dt1BS^{CS} and Dt1BL^{CS} lines were analyzed by Giemsa N-banding. As displayed by N-banding analysis, the centromeric end of both Dt1BS^{CS} and Dt1BL^{CS} lines were distinctly 'round' shaped and thus were true telosomics (Fig. 2). These results suggest that the *PSR161/BCD1072* probe sequence maps in the centromeric region of chromosome *1B*: the only common region between the two Dt lines. The *B* genome band was present in all 33 deletion lines involving chromosome *1B*. The largest deletion on the short arm was 1BS-1 with FL value of 0.35 and on the long arm was 1BL-11 with an FL value of 0.23. Thus, the gene mapped to the region between FL 0.35 of the short arm and FL 0.23 of the long arm supporting the Dt mapping results.

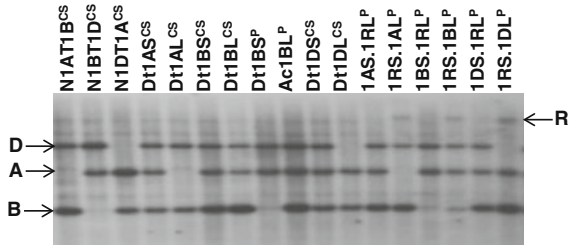

Fig. 1 Physical mapping of *PSR161* probe in Chinese Spring (*CS*), Pavon (*P*) and rye (*R*). An autoradiograph of genomic DNA gel-blot analysis involving CS group *1* nullisomic tetrasomic (*NT*) and ditleosomic (*Dt*) lines, Pavon Dt lines, and wheat (P)–rye translocation lines, digested with *Hind*III

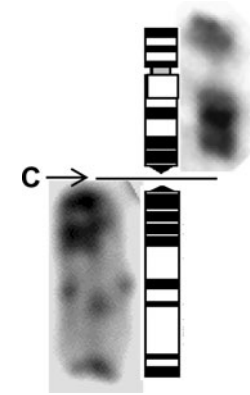

Fig. 2 The N-banded metaphase chromosome *1B* of ditelo lines Dt1BL^{CS} (*left*) and Dt1BS^{CS} (*right*). The N-banded ideogram of 'CS' 1BL (*middle*) is drawn to scale. 'C' marks the location of the centromere

Physical location of the *PSR161* probe sequence was studied in wheat cultivar Pavon (P) and in its translocation lines with rye. The probe mapped on 1RS as the rye (*R*) specific band was present in 1RS.1AL^{P}, 1RS.1BL^{P}, and 1RS.1DL^{P} but was absent in 1AS^{P}.1RL, 1BS^{P}.1RL, and 1DS^{P}.1RL (Fig. 1). The *A*- and the *D*-genome specific bands were missing in 1RS.1AL^{P} and 1RS.1DL^{P}, and present in 1AS^{P}.1RL and 1DS^{P}.1RL, mapping the *PSR161* probe on the short arms of *1A* and *1D* chromosomes of Pavon as well. The *B* specific band was absent in both wheat–rye translocation lines 1RS.1BL^{P} and 1BS^{P}.1RL (Fig. 1). Intensity of the *B* genome specific band in 'P' acrocentric line was similar to that of in normal 'CS' but was of very low intensity in Dt1BS^{P} (Fig. 3).

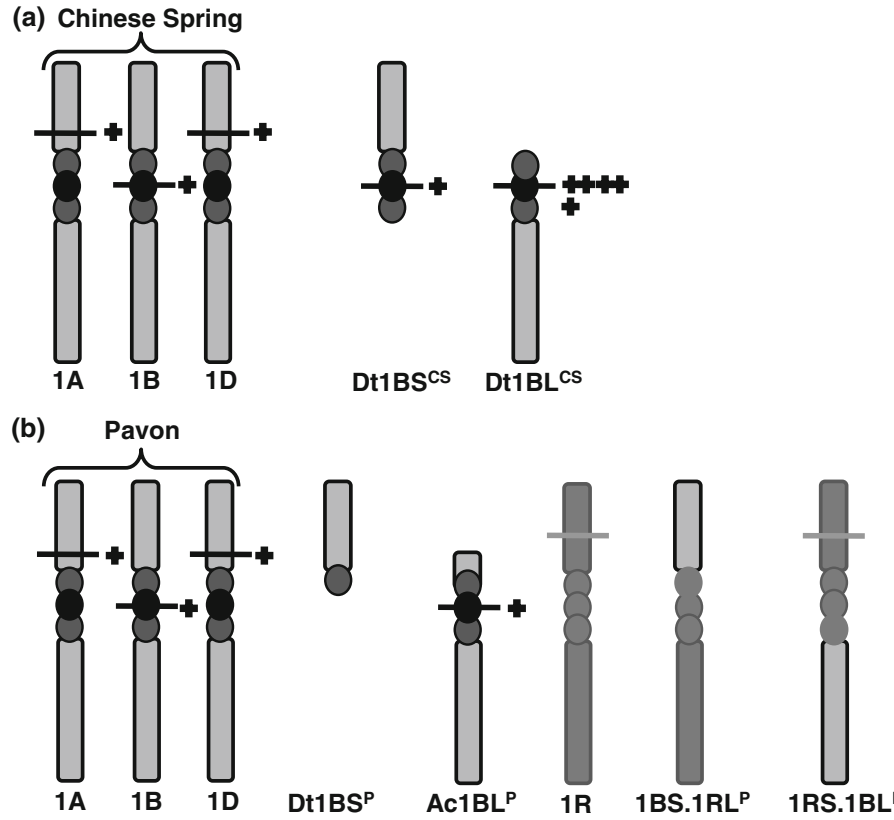

Fig. 3 Physical mapping of *PSR161* on wheat and rye chromosomes. *Black lines* represent the physical location of *PSR161* in 'CS' and Pavon, whereas, *light grey lines* represent the physical location of *PSR161* in rye. '+' represents number of *PSR161* copies on Dt line of 'CS' ($Dt1BL^{CS}$). Hypothetical partitioning of wheat centromeres is shown as *black circles* for core centromeres and *grey circles* for peri-centromeric regions, whereas *light grey circles* represent rye centromere

An interesting observation was that the intensity of the *B* genome specific gel-blot analysis band in $Dt1BL^{CS}$ was much higher than that in the normal 'CS' (Figs. 1, 4, 5). Band intensity comparison using *ImageJ* software showed the band intensity in $Dt1BL^{CS}$ to be significantly higher (Figs. 4, 5). Compared to the *D* genome bands, the *B* genome band intensity in $Dt1BL^{CS}$ was 2.8× higher (Fig. 4) and 5× higher in Fig. 5. The experiments in these two figures can be considered true replicates as they were performed on different plants and on independently isolated DNA.

Supernumerary chromosomes especially homologous to chromosome *1B* have been reported in wheat and its wild relatives. Supernumerary B chromosomes carrying 5S rDNA and other repeated DNA have been reported in *Triticum* species (Friebe et al. 1995). To rule out the possibility of a supernumerary chromosome explaining the unusual duplication of *PSR161* probe sequence, we carefully analyzed mitotically dividing root tip cells of $Dt1BL^{CS}$ and found no indication of any additional chromosome. Since copy number of supernumerary chromosomes

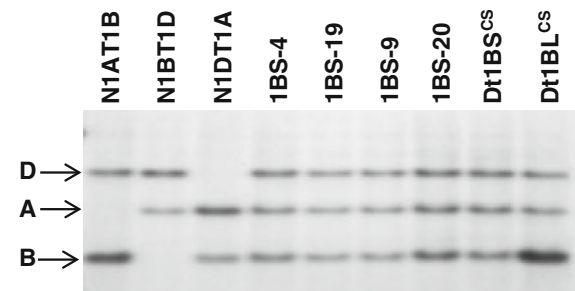

Fig. 4 An autoradiograph of DNA gel-blot analysis involving group *1* NT, Dt, and *1BS* deletion lines digested with *Hin*dIII and hybridized with *PSR161* probe. Note the increased *B*-band intensity in $Dt1BL^{CS}$ line. For the intensity comparison analysis, the *D* genome band in N1AT1B (*lane 1*) and $Dt1BL^{CS}$ (*lane 9*) were used as controls (see also results section "Physical mapping")

is known to change over generations, we evaluated 10 plants from each of the two-selfing generations of a $Dt1BL^{CS}$ plant by gel-blot analysis using *PSR161* probe. The *B* genome band intensity was very similar among these 20 plants further ruling out the presence of supernumerary chromosomes as an explanation for

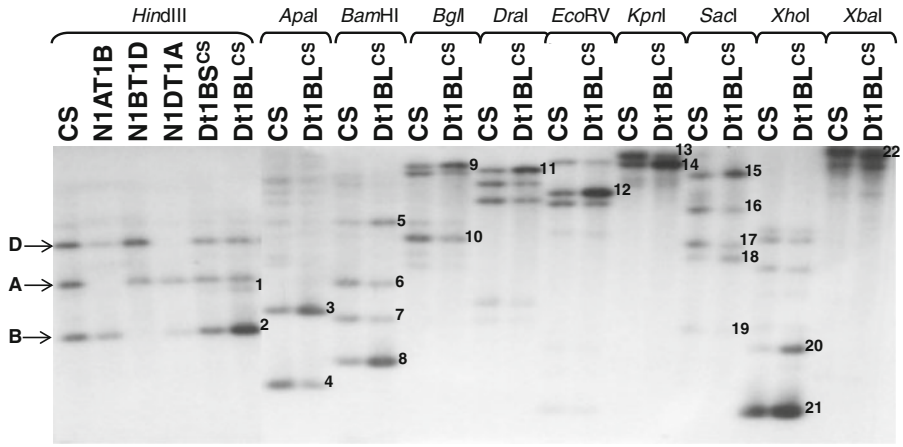

Fig. 5 DNA gel-blot analysis showing amplification of *PSR161* gene sequence. The genomic DNA in the *first six lines* was digested with *Hin*dIII enzyme and *nine* other restriction enzymes in the remaining lanes (*marked on the lanes*). The *band numbers* indicate the *PSR161* fragments showing differences between 'CS' and Dt1BL^{CS} lines. For the intensity comparison analysis, *A* and *D* genome bands in 'CS' (*lane 1*) and Dt1BL^{CS} (*lane 6*) lines were used as controls (see also results section "Physical mapping")

the duplication (data not shown). These results suggest that *PSR161* probe sequence is present in multiple copies in the Dt1BL^{CS} arm (Fig. 3a). In order to study the size of the putative duplicated region, 'CS' and Dt1BL^{CS} were analyzed by gel-blot analysis using ten restriction endonucleases. Except for *Kpn*I (Band #13, Fig. 5) we did not observe any extra band in Dt1BL^{CS} as compared to 'CS' indicating that the region involved in the duplication is much larger than the probe size and encompass restriction sites for almost all restriction enzymes (Fig. 5). Each enzyme detected one or more bands showing significantly higher intensity than its 'CS' counterparts. For example, band #s 9, 13, 14, 15, 18, 19, 21 and 22 showed 1.5 to 3× increase in intensity compared to its corresponding band in 'CS' line (Fig. 5). Whereas, band #s 3, 5, 8, 11 and 12 showed 3–5 times increase in intensity and band # 20 seemed to be amplified 16 times more than the corresponding band in 'CS' line. A unique band (#13) was detected that was present in 'CS' line but was absent in Dt1BL^{CS} line restriction digested with *Kpn*I. Some *PSR161*-specific fragment bands [# 6, 7, 10, 16, 17 and 19 (1.5–3 times), and #4 (3.5 times)] showed increased intensity in 'CS' compared to that in Dt1BL^{CS} (Fig. 5).

Expression of *PSR161* gene

To study homoeologue-specific gene expression of *PSR161*, specific primers were designed by comparing sequences of the corresponding 'CS' ESTs. The *PSR161* sequence is 1,569 nucleotides long (AF074969) with an open reading frame for 376 amino acids (Francki et al. 2002). This nucleotide sequence was used to perform 'MegaBLAST' with the 'CS' EST database in order to identify 'CS' ESTs showing a high level of sequence similarity over longer stretches of DNA (http://www.ncbi.nlm.nih.gov/BLAST/). With an *E*-value ranging from 0 to −133, 11 ESTs were identified. These included ESTs obtained from various tissues and from a variety of stages including crown of seedling, spikelet at early flowering stage, spikes at 5 and 10 days post anthesis, pistil at heading date, leaf, pre-anthesis spike, flag leaf from heat stressed flag leaf cDNA library and salt-stressed adult sheath. The ESTs along with the AF074969 sequence were assembled into contigs using the 'Contig Express' program of *Vector NTI* Suite. The aligned ESTs showed three distinct sequence patterns probably corresponding to the three homoeologs (Fig. 6a). Sequences from the variable regions were used to generate homoeologue specific

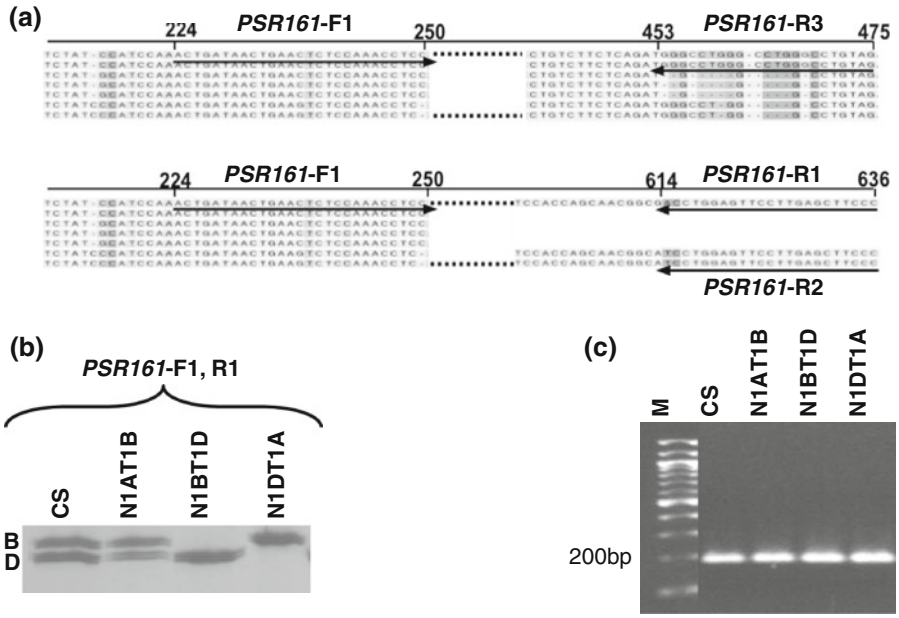

Fig. 6 Expression analysis of the *PSR161* gene sequence. **a** A portion of the contig showing three distinct sequence patterns among the aligned *PSR161* ESTs. The sequences used for designing primers are underlined by *arrows*. The nucleotide positions are shown on the *top* of the contigs. *Arrows* on the *left* of the contig indicate regions used to design the forward primer, *PSR161*-F1, and on the *right* indicate the regions used to design reverse primers *PSR161*-R1, -R2, and -R3. **b** cDNA-SSCP analysis on 'CS' and NT lines of group *1* using F_1/R_1 primer pair. **c** Expression analysis of *Actin* gene (control) performed on the same lines as used for **b**. 'M' is a size makers (100 bp ladder, Invitrogen)

primers for *PSR161*. For example, the reverse primers R1 and R2 had a two nucleotide difference at the 3′ end (Table 1; Fig. 6a). The R1 primer sequence was designed from an EST, BJ238735 that was identical to *PSR161* except for the two nucleotides. The R3 primer encompassed an eleven nucleotide deletion present in five of the 11 ESTs.

The expression analysis was performed on the PCR reactions using cDNA from tissue pooled from various stages of 'CS' and group *1* NT lines. Size separated on an 8% acrylamide gel, PCR products of the primer combinations *PSR161*-F1, *PSR161*-R1 and *PSR161*-F1, *PSR161*-R2 resolved into two bands mapping to chromosomes *1B* and *1D* as the corresponding bands were missing in N1BT1D and N1DT1A, respectively (Fig. 6b). These results clearly showed expression of the *B* genome copy of *PSR161*. PCR reactions with primer combination *PSR161*-F1 and *PSR161*-R3 failed to yield any product from any of the lines used for the analysis.

Table 1 List of DNA sequences of primers used to amplify *PSR161* and gene encoding *Actin*-like protein (Act) and, with their expected amplicon sizes

Forward primer	Sequence (5′–3′)	Reverse primer	Sequence (5′–3′)	Amplicon size (bp)
Act-F	TGTGCTTGATTCTGGTGATGGTGTG	Act-R	CGATTTCCCGCTCAGCAGTTGT	173
PSR161-F1	ACTGATAACTGAACTCTCCAAACCTCC	*PSR161*-R1	GGGAAGCTCAAGGAACTCCAGGAT	412
PSR161-F1	ACTGATAACTGAACTCTCCAAACCTCC	*PSR161*-R2	GGGAAGCTCAAGGAACTCCAGGTC	412
PSR161-F1	ACTGATAACTGAACTCTCCAAACCTCC	*PSR161*-R3	CTACAGGCCCAGGCCCAGGCCC	251

Underlined nucleotides indicate difference at the 3′ end between psr161-R1 and -R2

Discussion

Centromeric regions in the higher eukaryotes are known to be abundant in satellite repeats and centromeric retrotransposon sequences. These repeats make up bulk of the centromeric heterochromatin as seen in several eukaryotes including humans, mouse, *D. melanogaster*, *A. thaliana* and *O. sativa*. Centromeres usually lack any genes although some functional genes have been reported in the centromeric regions of *S. pombe*, *D. melanogaster*, *A. thaliana* and *O. sativa* (Clarke 1998; Copenhaver et al. 1999; Nagaki et al. 2004; Wu et al. 2004). These regions may correspond to an intermediate stage in the evolution of centromeres from genic regions to fully mature centromeres that are composed mainly of satellite DNA repeats and retrotransposon sequences (Nagaki et al. 2004). Here, we are reporting an actively transcribing gene in the functional centromere of a wheat chromosome. We have also shown that the single copy gene sequences present in the centromeric regions go through the unusual process of deletion and duplication similar to that of the satellite repeats of the region. Furthermore, we have made initial observations that some chromosomal break in the centromeric regions may trigger duplication of centromeric DNA including that of genes.

Sequence corresponding to *PSR161* probe mapped on the short arm of chromosome *1A* and *1D* but clearly mapped in the centromeric region of chromosome *1B* of cultivar 'CS'. Centromeric region is the only part common to the true telosomics of a chromosome. Careful evaluation of N-banded telocentric chromosomes in the two 'CS' Dt lines showed typical morphology of true telosomics. The centromeric ends of the 'CS' telocentric chromosomes were distinctly round (Fig. 2). These telosomic lines were isolated from the progeny of a plant monosomic for chromosome *1B* (Sears 1954). Crossway splits in the centromere is probably the main mechanism by which telosomics are generated from unpaired monosomic chromosomes (Steinitz-Sears 1966). Our data on physical mapping of *PSR161* both in wheat as well as in wheat–rye translocation lines clearly suggest that the breaks occur in the region around the centromere rather than in the middle of it. The exact mechanism by which an unpaired chromosome breaks to generate a stable telocentric chromosome is however unclear.

PSR161 sequence in cultivar Pavon mapped to the same physical location as that in 'CS'. The *1B* copy of *PSR161* was missing in Dt1BS^{P} and present in Ac1BL^{P} suggesting that it is either present in the centromere or on the long arm (Fig. 1). Lack of the *1B* specific band in both wheat–rye translocations 1BS^{P}.1RL and 1RS.1BL^{P} suggested that both of these lines carry rye centromere, further supporting the conclusion that the B genome copy both in 'CS' as well as in Pavon is present in the centromeric region (Figs. 1, 3b). This data also support the conclusion that most telomeres result from breaks around the centromeres rather than splitting.

Homoeologue-specific gene expression analysis is rather difficult in wheat as homoeologs are highly similar in gene sequences. Using in silico analysis combined with modified cDNA-SSCP analysis (Mutti 2007; Mutti and Gill 2008), we were able to show specific expression of the *B* genome copy of *PSR161*. It is however unclear if the gene expresses only in non-dividing cells where the DNA in the centromeric region is relatively relaxed or it expresses in the dividing cells as well.

The heat-shock proteins such as HSP70 are highly conserved chaperon proteins, specifically involved in proper protein folding (Gething and Sambrook 1992). Immunofluorescence microscopy showed degradation of microtubules when the yeast cells were subjected to a lethal heat shock at 46°C. But the microtubules remained stable after the lethal heat shock was preceded by a moderate heat shock of 37°C, suggesting that stress proteins induced by moderate heat shock were involved in maintaining microtubule stability (Holubarova et al. 2000). Abnormal or bent microtubules have been produced due to the absence of heat-shock proteins (Brown et al. 1996; Liang and MacRae 1997; Smertenko et al. 1997). The significance of an *HSP70* gene homolog expressing in the centromere of *1B* chromosome of wheat is unknown. Additional molecular analyses are required to understand if the gene is involved in the kinetochore assembly or if its location in the centromere has a regulatory function. It will be interesting to know if the gene expresses from both dividing and non-dividing cells and if proper chromosome segregation is one of its functions.

The DNA sequences associated with the centromeric region are known to be rapidly evolving (Henikoff et al. 2001; Jiang et al. 2003). Recent

detailed analyses of plant centromeres have shown the presence of high levels of duplications, inversions and deletions of the centromeric DNA sequences. More than 1/3 (212 of the ~750 kb) of the rice centromere *Cen8* is consisted of a triplicate segment (Ma and Bennetzen 2006). Similarly *Cen4* has several segmental duplications for CentO satellite arrays and centromeric retrotransposon sequences (Nagaki et al. 2004; Ma and Jackson 2006). Deletions or duplications are probably caused by unequal recombination that happens more frequently in the centromeric regions (Ma et al. 2007). The duplications were also suggested to be activated by deletions or rearrangements in the centromere or with the formation of neocentromeres. In the macaque genome, neocentromere formation was shown to be triggered by extensive intrachromosomal pericentromeric duplications (Ventura et al. 2007). Formation of neocentromeres with chromosomal breakage was also hypothesized in telosomic lines of barley *7H* chromosome in wheat background developed using wheat-Aegilops addition lines (Nasuda et al. 2005). In our analysis, multiple gene duplications were observed that appear to be triggered by certain chromosomal breakage. Duplications for *PSR161* sequence were observed in the Dt lines involving the long arm of chromosome *1B* but not for the short arm (Fig. 4).

Model for gene duplication in wheat centromere. We propose two different hypotheses to explain our results (Fig. 7). Wheat Dt lines were developed using wheat monosomic lines where unpaired centromeres may misdivide resulting into telocentric chromosomes (Sears 1954). Stability of the telocentric chromosomes depends upon the proportion of the centromeric region retained (Sears 1954; Steinitz-Sears 1966). As a result a very high percentage of telocentric chromosomes are lost during segregation. Often times the centromeric breaks trigger Breakage-Fusion-Bridge (BFB) cycle (Tsujimoto et al. 1997). The broken chromosomal ends without proper telomeres fuse for the sister chromatids to form dicentric isochromosomes (Tsujimoto et al. 1997). During chromosome segregation, mechanical break near the first break in isochromosome has been shown to result in a stable telocentric chromosome (Steinitz-Sears 1966). Depending upon the location of the break, this process may lead to deletion or duplication of the chromosomal regions.

Another possible explanation for our results is that a broken chromosome often loses part of its centromeric sequence thus results in the formation of a neocentromere. Neocentromere formation is implicated in triggering rearrangements including deletions and duplications via unequal crossover (Nasuda et al. 2005; Ventura et al. 2007). Unequal crossing over is known to be a common process for duplications or deletions of the centromeric regions (Smith 1976; Ma et al. 2007). It is quite possible that homologous Dt chromosomes undergo unequal crossing over resulting in deletions or duplications in the centromeric regions (Fig. 7). That may also explain multiple copy duplications observed in Dt1BL^{CS}. These duplication or deletion events are further

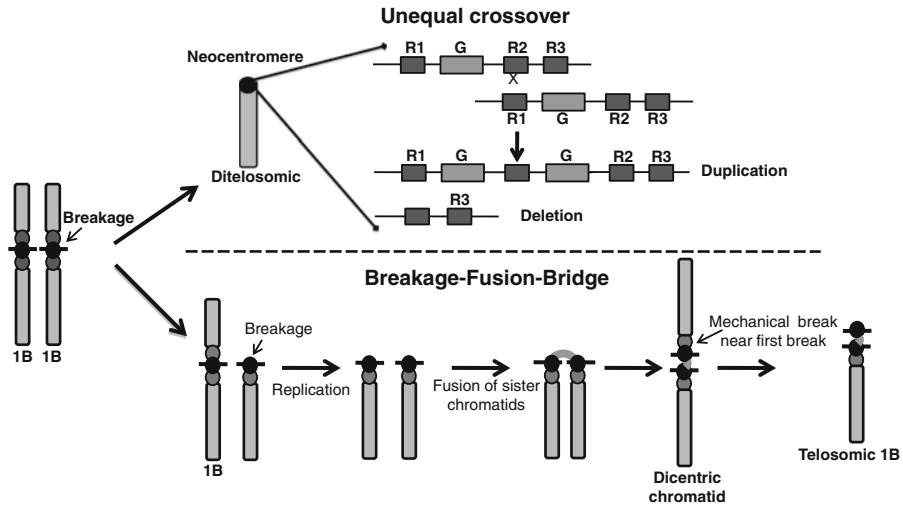

Fig. 7 Proposed models of gene duplication in wheat centromeres in response to chromosomal breakage. 'R' represents different DNA repeat sequences and 'G' denotes the functional gene mapping in the centromere

amplified possibly based on selection for the sequences or chromatin favoring successful segregation. It will be interesting to investigate if similar kinds of duplications are observed in the other Dt lines of wheat. Further studies are warranted to completely understand the mechanism involved in centromeric duplications in Dt lines.

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