Theor Appl Genet (2013) 126:2381–2390
DOI 10.1007/s00122-013-2142-5

ORIGINAL PAPER

PCR and sequence analysis of barley chromosome 2H subjected to the gametocidal action of chromosome 2C

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Received: 18 February 2013 / Accepted: 3 June 2013 / Published online: 14 June 2013

Abstract Gametocidal (Gc) chromosomes induce various types of chromosomal mutations during gametogenesis in the chromosomes of common wheat and alien chromosomes added to common wheat. However, it is not yet known whether the Gc chromosome causes aberrations at the nucleotide level because mutations caused by Gc chromosomes have been studied only by cytological screening. In order to know whether the Gc chromosome induces point mutations, we conducted PCR analysis and sequencing with the progeny of a common wheat line that is disomic for barley chromosome 2H and monosomic for Gc chromosome 2C. We analyzed 18 2H-specific EST sequences using 81 progeny plants carrying a cytologically normal-appearing 2H chromosome and found no nucleotide changes in the analyzed 1,419 sequences (in total 647,075 bp). During this analysis, we found six plants for which some ESTs could not be PCR amplified, suggesting the presence of chromosomal mutations in these plants. The cytological and PCR analyses of the progeny of the six plants confirmed the occurrence of chromosomal mutations in the parental plants. These results suggested that the Gc chromosome mostly induced chromosomal aberrations, not nucleotide changes, and that the Gc-induced chromosomal mutations in the six plants occurred after fertilization.

Communicated by B. Friebe.

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Abbreviations

Gc	Gametocidal
PCR	Polymerase chain reaction
EST	Expressed sequence tag
FISH	Fluorescence in situ hybridization
GISH	Genomic in situ hybridization

Introduction

Mutations, heritable changes in genetic material, occur spontaneously or can be induced by mutagens in all plant and animal species. After Stadler's (1928) first experiment on barley, physical and chemical mutagens have been successfully used in plant breeding programs to generate artificial genetic variations leading to new varieties with improved traits (Maluszynksi 2001). Each mutagen has a specific behavior and can be used according to need for the plant improvement. Physical mutagens, such as non-ionizing (e.g. UV rays) and ionizing (e.g. X-rays, alpha rays, gamma rays, and fast and slow neutrons) radiations, induce breakage of DNA strands, resulting in large scale changes in chromosomal structure. By contrast, chemical mutagens like ethyl methane sulphonate (EMS), sodium azide, *N*-methyl-*N*-nitrosourea and hydroxylamine all of which are widely used to induce mutations in plants, affect single nucleotide pairs and cause nucleotide changes like base substitutions and frame shifts. Genetic variations caused by chemical mutagens can be screened by reverse genetic approaches, one of which is TILLING (Targeting induced local lesions in genomes). For this technique, mutant populations have been developed in many cereal crops including rice (Suzuki et al. 2008), maize (Till et al. 2004), sorghum (Xin et al. 2008), barley (Talamè et al. 2008), and

tetraploid and hexaploid wheat (Slade et al. 2005; Uauy et al. 2009). Besides physical and chemical mutagens, specific DNA sequences known as mobile genetic elements or transposable elements (transposons and retrotransposons) are responsible for creating genetic variability due to their ability to cause mutations through excision, insertion, translocation, and irregular recombination (Hirochika et al. 1996; Cowan et al. 2005; Kumar and Bennetzen 1999; Havecker et al. 2004).

In addition, certain chromosomes or genes in the members of Triticeae (a tribe in the grass family including wheat) are known to induce structural changes in chromosomes. Many alien-chromosomes were introduced into common wheat (or hexaploid wheat) from its wild relatives in the genus *Aegilops* by backcrossing common wheat-*Aegilops* hybrids to common wheat. In the course of backcrossing, some of the alien chromosomes had effects like mutagenic agents in the host, namely common wheat (Endo 1990). The chromosomes or genes were named Gametocidal (Gc) chromosomes or genes, and they cause chromosomal mutations in a unique manner during gametogenesis, i.e. only in the gametes that lack the Gc chromosomes (Endo 1990, 2007). The Gc chromosome induces breakage not only in wheat chromatin but also in alien chromatin, such as the chromosomes of barley and rye introduced into the wheat genetic background as alien additions (Endo 2007).

Among the Gc chromosomes, chromosomes 2C and $3C^{SAT}$ derived from *Ae. cylindrica* ($2n = 4x = 28$, DDCC) and *Ae. triuncialis* ($2n = 4x = 28$, UUCC), respectively, have been widely used to induce chromosomal breakage in wheat and alien chromosome addition lines of wheat (Endo and Gill 1996; Shi and Endo 1997, 1999, 2000; Friebe et al. 2000; Endo 2007). Structural aberrations of barley chromosomes can be identified cytologically by in situ hybridization using a barley genomic DNA probe and the barley-specific, subtelomeric repetitive sequence probe HvT01. Thus, various structural changes were singled out to develop common wheat lines carrying single barley chromatin segments, the so-called dissection lines, for individual barley chromosomes. The dissection lines have been used for cytological mapping of barley chromosomes using molecular markers, namely ESTs (Nasuda et al. 2005; Ashida et al. 2007; Sakai et al. 2009; Sakata et al. 2010; Joshi et al. 2011); however, in situ hybridization cannot reveal sub-microscopic chromosomal aberrations, much less point mutations if they occur at all. It is important to know whether the Gc chromosome induces point mutations in terms of not only clarifying the molecular mechanism of Gc action but also exploiting the Gc system for mutation breeding of wheat.

We planned this study to demonstrate whether the Gc chromosome induces changes in nucleotide sequences. We used the progeny of an alien addition line of 'Chinese Spring' wheat that is disomic for barley chromosome 2H and monosomic for a gametocidal chromosome 2C. We cytologically screened the progeny for plants carrying a 2H chromosome of normal appearance, with which we PCR-amplified 2H-specific EST markers and sequenced them. We did not use plants carrying 2H chromosomal segments to avoid being confused in the sequence analysis.

Materials and methods

Plant materials

We used two alien addition lines of common wheat (*Triticum aestivum* L., $2n = 6x = 42$, genome formula AABBDD) cultivar Chinese Spring (CS). One line is a disomic addition for barley (cv. Betzes) chromosome 2H produced by Islam et al. (1981), and the other line is a disomic addition for gametocidal (Gc) chromosome 2C produced by Endo (1988). The 2C chromosome causes chromosomal structural changes in gametes in which the 2C chromosome is not present (Endo 1988). In addition to the two addition lines, euploid CS was used as the control. We obtained these lines from the National BioResource Project-Wheat (NBRP-Wheat) (http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp), where the lines are stored with accession numbers LPGKU2269 (euploid CS), LPGKU2088 (disomic addition of 2H in CS), and LPGKU2153 (disomic addition of 2C in CS). We made a cross between the 2H and 2C addition lines to produce an F_1 hybrid ($2n = 44$, $21'' + 1'$ 2H $+ 1'$ 2C) and then backcrossed the F_1 to the 2H addition line to obtain BC_1 plants ($2n = 45$, $21'' + 1''$ 2H $+ 1'$ 2C). We hand-pollinated the BC_1 progeny with euploid CS pollen to obtain the BC_2 progeny. We found three types of chromosome constitutions in the progeny: normal 2H plus 2C, non-aberrant 2H plus no 2C, and aberrant 2H plus no 2C. For our study, we selected 81 plants with non-aberrant 2H plus no 2C to examine Gc-induced point mutations, if they occur at all, along the entire 2H chromosome. We checked for the presence of the 2C chromosome by the spike morphology of the plants; the presence of 2C was indicated by a tip-awned spike that was tougher and more slender (Fig. 1). We surveyed the 2H chromosome by fluorescence in situ hybridization (FISH) using the HvT01 probe that is specific to subtelomeric regions of all barley chromosomes (Belostotsky and Ananiev 1990) and by genomic in situ hybridization (GISH) using a total barley genomic DNA probe (Fig. 2). The procedures for chromosome preparation and simultaneous FISH/GISH were conducted as described by Sakai et al. (2009).

Fig. 1 Two types of spikes appeared in the backcrossed progeny of the monosomic 2C addition line of 'Chinese Spring' wheat. Plants with tip-awned spikes **a** were assumed to have chromosome 2C and those with awnless spikes **b** were assumed to have no 2C chromosome

Selection of EST markers

Among the EST markers that had previously been mapped on the 2H chromosome (Joshi et al. 2011), we selected 18 representative markers that showed clear single PCR products of ca. 500 bp and were distributed rather evenly over the length of the 2H chromosome for sequence analysis (EST clone sequences were according to Sato et al. 2009, Stein et al. unpublished, and Biemelt et al. unpublished), and an additional 40 2H-specific EST markers (Table 1) to characterize aberrant 2H chromosomes that were found during the PCR analysis in this study. The fraction length (FL) reported in Table 1 indicates the marker position on the chromosome. The FL values, cited from Joshi et al. (2011), represent the breakpoints of the aberrant 2H chromosomes flanking the EST markers.

PCR analysis

We conducted PCR using template DNA extracted from leaves by the DNeasy Plant Mini Kit (QIAGEN), and the 58 primer sets for the EST markers (Table 1). We added 2 µl of DNA solution (ca. 15 ng/µl) as a template to a PCR mixture consisting of 10 µl of 5× PCR buffer, 1.0 µl of dNTP (10 mM each), 3.75 µl of $MgCl_2$ (25 mM), 2.5 µl of primers (10 pmol/µl), 0.25 µl of KAPATaq Extra DNA Polymerase (5 U/µl KAPABIOSYSTEMS, USA), and 30.5 µl of dH_2O. We conducted thermal cycling with an iCycler (BioRad, USA) using the following conditions: 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 65 °C for 30 s (with the temperature subsequently decreased 1 °C per cycle), and 72 °C for 1 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and 72 °C for 7 min. PCR products are fractionated and visualized by agarose gel electrophoresis (1.5 % Agarose S (Nippon Gene, Japan), 200 V, 40 min) and ethidium bromide staining.

Sequencing

We purified the checked PCR products of the 18 representative ESTs by PEG precipitation and sequenced the purified PCR products in both orientations at the Dragon Genomics Center, Takara-Bio Inc., Japan. Direct sequencing was performed using Big Dye Terminator v3.1

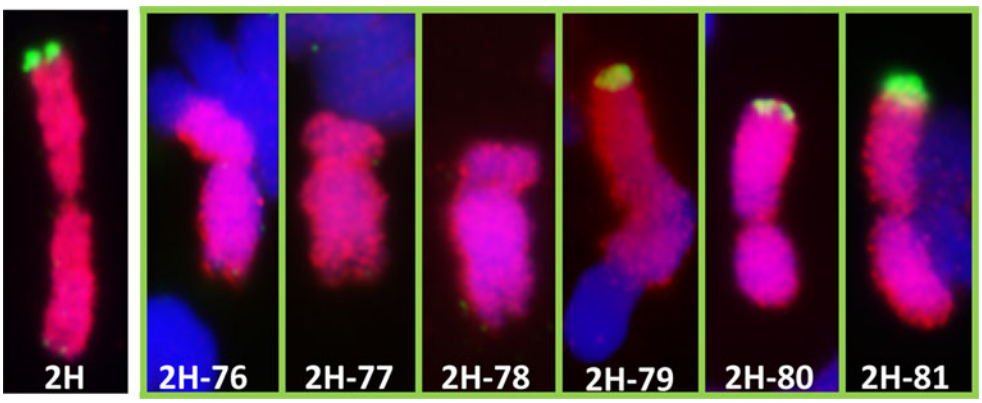

Fig. 2 GISH (*red*) and FISH (HvT01, *green*) photographs of normal chromosome 2H (*leftmost*) and six aberrant 2H chromosomes. *Bar* = 10 µm

Table 1 Primer sequences of the 18 EST markers used for PCR and sequence analyses and the additional 40 EST markers used to characterize the breakpoints of aberrant 2H chromosomes obtained in this study

EST ID	Forward[a]	Reverse[a]	FL[b]	Arm location
PCR and sequence analyses				
k01312	AAAGTTTGTATTGCCCGCTG	AGCCTGTCAAACTTCCCTGA	0.85–1.00	short arm
k04629	ATGCTAAGCAGAGAGCCGAG	CTGTACGGGAACCTCGACAT	0.85–1.00	short arm
k03538	AGGAACCACAAAGGCTCAGA	CAACATCGTTGTGGATGGAG	0.66–0.77	short arm
k00852	TGGCTCAATTCACGTTTCTG	CGCCTCAAACACGATCTACA	0.60–0.63	short arm
k00186	GTTCGATCAGACATTCCGGT	TTTTCTCTAAACCCCCTCGC	0.59–0.60	short arm
k00776	CCAAACAAAACAGCACATGG	GAGGGAACATCCTACAGCCA	0.52–0.58	short arm
k00091	CAACATGCACGAGCAAAACT	ACATTTGTTCAAGGCGTTCC	0.30–0.40	short arm
k02121	TCCTTGCAGGACTCGAAGTT	CTACAACTGGCCTGATGGGT	0.25–0.26	short arm
k03501	CGTGGCAACACACAGCTATT	AGAATGCCACACCAAAGACC	0.00–0.11	long arm
k03201	TACAGCGCTGAAAAATGCAC	CAGAACAAGCGAGCACAGAG	0.11–0.17	long arm
k05033	GCGAAAGCCAAAACTTGAAC	TCACAGATGTCTCAGCAGGG	0.17–0.30	long arm
k00679	CAAATTGGCATCCTTGTCCT	GCAGTAGAGCGAGCGAAGAC	0.45–0.47	long arm
k02551	TCAGCAAGCAAACATTCAGG	GGTTGGTCGCTGTTGGTACT	0.47–0.57	long arm
k01321	CACCATGTTACCACTGTCGC	TACGAGGTTTGTTCTGCACG	0.57–0.60	long arm
k01975	GGATCCCTGCATCGACTTTA	TCCAGATTTAAGGCCACCAC	0.60–0.64	long arm
k04771	TATACCAGCGCTGCACTTTG	ACCCAAACGCAAACAGACTC	0.60–0.64	long arm
k00932	GATGCAACGAACGAGCACTA	AAGACGAGGACACGGAGAGA	0.64–1.00	long arm
k00730	TTGATCTCACGATCTCACGG	GAGATCGACGAACTTGGAGC	0.64–1.00	long arm
Characterization of breakpoints				
k04935	TCCAAAGTTGGACCGTTCTC	ACATGAGCAGCATTAGCACG	0.85–1.00	short arm
k00074	ACCTTGGTGGCTCGTATTTG	GGTGTTTACGGAGGAGTCCA	0.85–1.00	short arm
k04102	TCTTTGCCTGGAAGAAGGAA	ACTCCCCACAATCAAGCAAG	0.85–1.00	short arm
k04782	CCGGGTCGTAGTCTTGTTGT	GAACGGAACGAGCTCAACAT	0.85–1.00	short arm
k04909	TCGAAGCGACAAACTTCAAA	GACCCAGAGAAATCCGATGA	0.85–1.00	short arm
k00434	CCACGAAAATGCATGAAACA	AAGTTCATCGCGAGTCCTGT	0.85–1.00	short arm
k01216	GAGGGAATCGACCAATTCAA	GGCGTACCAAACGACAGTCT	0.85–1.00	short arm
k01603	TCCAAATTGCAGCACTTGTC	GGCGTACCACCAGTACATCC	0.85–1.00	short arm
k00144	CCTTCCCTTCACACATTCGT	CTGCTCAAGCTCGTCAGTTG	0.85–1.00	short arm
k00265	CTACCAAATCTTGGCCCTCC	CGCACAAACACAGACACACA	0.58–0.59	short arm
k00376	CCGTCGATTGACCATTTCTT	GTCCGTCTTCAGGGAGACAA	0.52–0.58	short arm
k00168	CGGAGCTTCTGGTTGATTGT	AACTGCCAGTCCTTCCAATG	0.52–0.58	short arm
k01932	ATGGACAACTACAAAGCGGG	TAGGTTCCACATGGACGACA	0.52–0.58	short arm
k03300	AACATTCGGTAGATGGCAGC	GTGGCAAGTATGGCCTTGTT	0.52–0.58	short arm
k03744	CTCGCTAGCTCAGTTGAGGG	CAGGGTCGTTCCCAGTGTAT	0.52–0.58	short arm
k04759	GGTTAAATCCTCCATGCCAA	CTACGTGGAGAGGATCCAGC	0.52–0.58	short arm
k04039	GGCCCAAAACGAGTCTACAA	GACGCTACTACGTCGCTCCT	0.52–0.58	short arm
k00838	TAGCTGCTTCCGTTCTTCGT	CATCATGCCTAAGCCAGACA	0.26–0.40	short arm
k03231	GCTAGACACAACCGTCCCAT	GGGTTGCAGTTGACAAGGAT	0.47–0.57	long arm
k03187	AGGATCAACTGCGACCTGAC	GCATCCTCTTGCTGTTGTGA	0.57–0.60	long arm
k00246	ACAGCTCTCGCCTTTTCTTG	CCGGGGGTGCTATAGTTCTT	0.57–0.60	long arm
k05173	GCCAAGCTCATATAGGGCAG	TATCTGTGATGCCACATCCG	0.60–0.64	long arm
k02245	ACTCCTTGAACACCAATCCG	TAAGTTGGTTTGGGGCACTC	0.60–0.64	long arm
k00289	CAGCTTCCTCTTGTTTTGCC	ACGGTGTCCTTGCTGGTTAC	0.60–0.64	long arm
k04784	TATGTTTCGGCACCGTACAA	CCCATAGTCAAAGCCAGGAA	0.60–0.64	long arm
k04929	TGCTTCAGTACCCTGCTCCT	GGACAATACTGAGCCTGGGA	0.60–0.64	long arm
k04939	CCCACCCTTACCACTAGGCT	GACCGTGGAGTAGAGAAGCG	0.60–0.64	long arm

Table 1 continued

EST ID	Forward[a]	Reverse[a]	FL[b]	Arm location
k04446	ACCAAGCATGTACCCCAAAG	TCACTGAAGGCATAACTGCG	0.64–1.00	long arm
k03899	TCCAACACCATCCACTACGA	ATGACCCGGTCGATACAAGA	0.64–1.00	long arm
k03370	AAAGGGAAAAGGCGACTCAT	ATTCTTAGTGCGGCAATGCT	0.64–1.00	long arm
k01224	CATGTCTGGTGTCTGGCTTG	GTGCAAGAGGTCAAGGCTTC	0.64–1.00	long arm
k01423	TATCCCTTGCTCTTGCTCGT	GGAGCTTTTGTCGTCCTCTG	0.64–1.00	long arm
k01467	ACATAAGTGGGCTCAATGCC	CTCGCTTGATGAAGTGTCCA	0.64–1.00	long arm
k01418	TAGCTCCGGCTGTTCTTGAT	ACCAAGCCATGGCCATATAA	0.64–1.00	long arm
k01408	TCTAGCGGACAGCTAAACAGC	CCATCCTCATCACCCTCACT	0.64–1.00	long arm
k03044	GATGGGAGCACACCAGCTAT	TGCTATTCGCAGTGAGGATG	0.64–1.00	long arm
k00323	ATTCCAAGCACAACACACCA	CGGTGAAATGGTGCCTAACT	0.64–1.00	long arm
k03085	TCAAACTGCATGACAGCCTC	CTACGAGAAGCTGACCCTCG	0.64–1.00	long arm
k00579	ATCCTCGGCCATTCTACCTC	CGTCATCTTCCTCAAGCACA	0.64–1.00	long arm
k03626	CAAACAAATTCGGCAGGTG	TCAGTTGAGAAAGAAGCGCA	0.64–1.00	long arm

[a] Primer sequences are given in the 5′ to 3′ orientation

[b] Fraction lengths flanking the EST markers, cited from Joshi et al. (2011)

Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed with ABI 3130xl Genetic Analyzer (Applied Biosystems, USA). We analyzed the sequences with MEGA5 (Tamura et al. 2011) and aligned the sequences with CLUSTALW (Thompson et al. 1994).

Results

PCR analysis

Out of the 81 plants, 75 plants showed clear PCR amplification for all 18 markers as expected, but only six plants showed irregular patterns of PCR amplification (Table 2). Plants 2H-77 and 2H-78 lacked five markers within FL0.59–1.00 and six markers within FL0.52–1.00 on the short arm, respectively. Plant 2H-79 lacked five markers within FL0.57–1.00 on the long arm. Plant 2H-80 showed no PCR amplification for four markers within FL0.60–1.00 on the long arm. Plant 2H-76 lacked one marker close to the HvT01 sequences on the short arm, and plant 2H-81 lacked two markers close to the HvT01 sequences on the long arm. The above PCR amplification patterns suggested that two plants (2H-76 and 2H-81) had aberrant 2H chromosomes with minor deletions in the distal regions and that the other four plants (2H-77, 2H-78, 2H-79 and 2H-80) had aberrant 2H chromosomes with extensive deletions. This PCR result obviously contradicted the FISH/GISH result that had showed the presence of a normal-appearing 2H chromosome in root tip meristems of the six plants, and therefore implied that the breakage of the 2H chromosomes occurred during very early stages of differentiation after fertilization.

Table 2 PCR analysis of the three control lines (CS, 2C, and 2H) and 81 Gc-subjected plants (+ and − represent amplification and non-amplification of PCR products, respectively)

EST ID	Lines									
	Controls			Gc-subjected plants						
	CS	2C	2H	1–75[a]	76	77	78	79	80	81
k01312	−	−	+	+	−	−	−	+	+	+
k04629	−	−	+	+	+	−	−	+	+	+
k03538	−	−	+	+	+	−	−	+	+	+
k00852	−	−	+	+	+	−	−	+	+	+
k00186	−	−	+	+	+	−	−	+	+	+
k00776	−	−	+	+	+	+	−	+	+	+
k00091	−	−	+	+	+	+	+	+	+	+
k02121	−	−	+	+	+	+	+	+	+	+
k03501	−	−	+	+	+	+	+	+	+	+
k03201	−	−	+	+	+	+	+	+	+	+
k05033	−	−	+	+	+	+	+	+	+	+
k00679	−	−	+	+	+	+	+	+	+	+
k02551	−	−	+	+	+	+	+	+	+	+
k01321	−	−	+	+	+	+	+	−	+	+
k01975	−	−	+	+	+	+	+	−	−	+
k04771	−	−	+	+	+	+	+	−	−	+
k00932	−	−	+	+	+	+	+	−	−	−
k00730	−	−	+	+	+	+	+	−	−	−

[a] All EST markers were amplified in plants 1–75

Analysis of the progeny of the six plants with irregular PCR amplification

We cytologically analyzed the progeny of the six plants with irregular PCR amplification and found no normal

appearing of 2H chromosome that we had observed in the root tip cells of the parental plants; instead, we found aberrant 2H chromosomes (Fig. 2). We measured the FL values of the aberrant 2H chromosomes (an average of five photomicrographs for each), according to the procedure employed by Endo and Gill (1996). Chromosomes 2H-77 and 2H-78 had terminal deletions with breakpoints at FL0.53 and FL0.40 in the short arm, respectively, and chromosomes 2H-80 and 2H-81 had terminal deletions with breakpoints at FL0.58 and FL0.75 in the long arm, respectively. Chromosomes 2H-76 and 2H-79 were translocation chromosomes between 2H and wheat chromosomes with translocation points at FL0.89 in the short arm and at FL0.49 in the long arm, respectively. We further examined the progeny from a second spike for each of the six plants and found the same aberrant 2H chromosomes. These findings indicated that these 2H aberrations had occurred after fertilization, most likely after differentiation into root and shoot meristems.

The FL lengths of the aberrant 2H chromosomes in the progeny plants were consistent with the PCR result of the parental plants, i.e. the FL values were within the ranges estimated from the chromosomal positions of the 18 EST markers (Table 1). We further conducted PCR analysis of the 18 markers in the progeny of the six parental plants and obtained the same PCR results as found for the parental plants.

Plant 2H-76 lacked one representative marker (k01312), and further PCR analysis using nine additional markers in the vicinity of the breakpoint of chromosome 2H-76 in the short arm (FL0.85–1.00) divided the 11 markers, including the two representative ones (k01312 and k04629), into two groups, nine distal and two proximal to the breakpoint (FL0.89) (Fig. 3). Although the breakpoints of the other aberrant 2H chromosomes did not divide the markers, they narrowed the bin areas where the markers were located. For example, plant 2H-81 lacked two representative markers (k00932 and k00730), and also 13 additional markers located in the most distal bin (FL0.64–1.00) in the long arm (Joshi et al. 2011). This result, therefore, indicated that all 15 markers lay in a narrower bin distal to the breakpoint of chromosome 2H-81 (FL0.75) (Fig. 3). Figure 4 shows the latest version of the cytological map of chromosome 2H constructed after incorporating the data obtained in this study and those from Joshi et al. (2011).

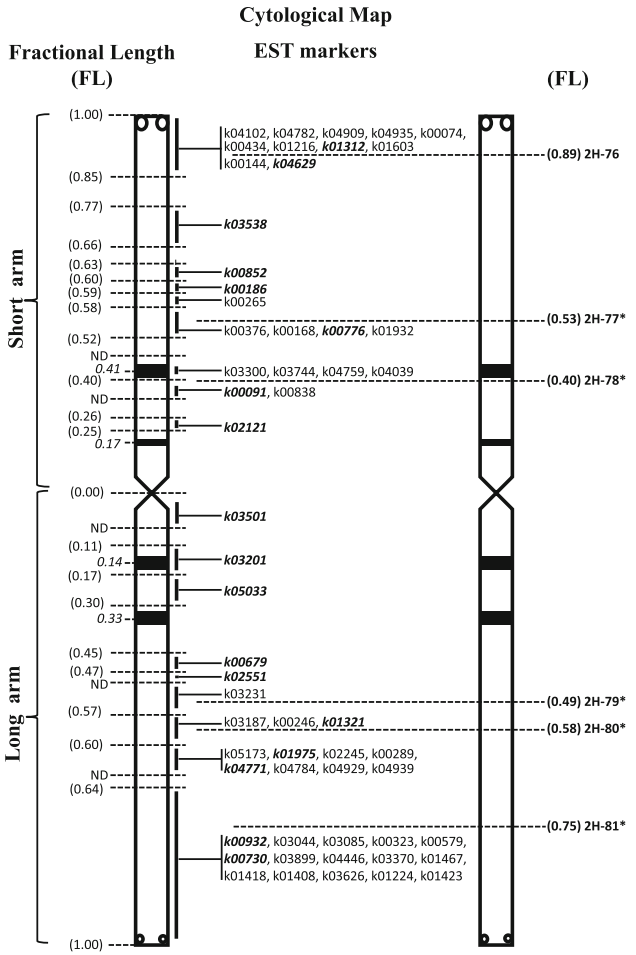

Fig. 3 Addition of six breakpoints to the cytological map of chromosome 2H constructed by Joshi et al. (2011). FL in *parentheses*: representative breakpoints flanking the 18 representative markers, FL in *italics*: position of C bands, *ND* FL non determined, EST markers (*boldface italics*): 18 representative markers used for PCR and sequence analyses, EST markers (*regular face*): additional 40 markers used to characterize the aberrant 2H chromosome

Sequence analysis

The amplified PCR products of the 18 representative ESTs ranged in length from 370 to 650 bp, accounting for ca. 9 kb (Table 3) altogether. We used the sequence data for BLASTN searches of the barley expressed sequence tags (ESTs) at the NCBI (National Center for Biological Information) database and found that all the sequences were in clones of *Hordeum vulgare* (Table 3). Chromosome 2H has been estimated to be 790 Mbp long (Suchánková et al. 2006) out of 5.1 Gbp of barley genome (Dolezel et al. 1998). We analyzed a total of 1,419 sequences of the 81 plants and the control 2H addition line to check for the occurrence of point mutations in the amplified regions. We examined the forward and reverse reads of each PCR product separately, and rechecked any ambiguous nucleotide changes found in one read in the other complementary reads. In this manner, we found no confirmed nucleotide changes, i.e. the same nucleotide change in the forward and reverse reads, in any of the sequences (Table 3).

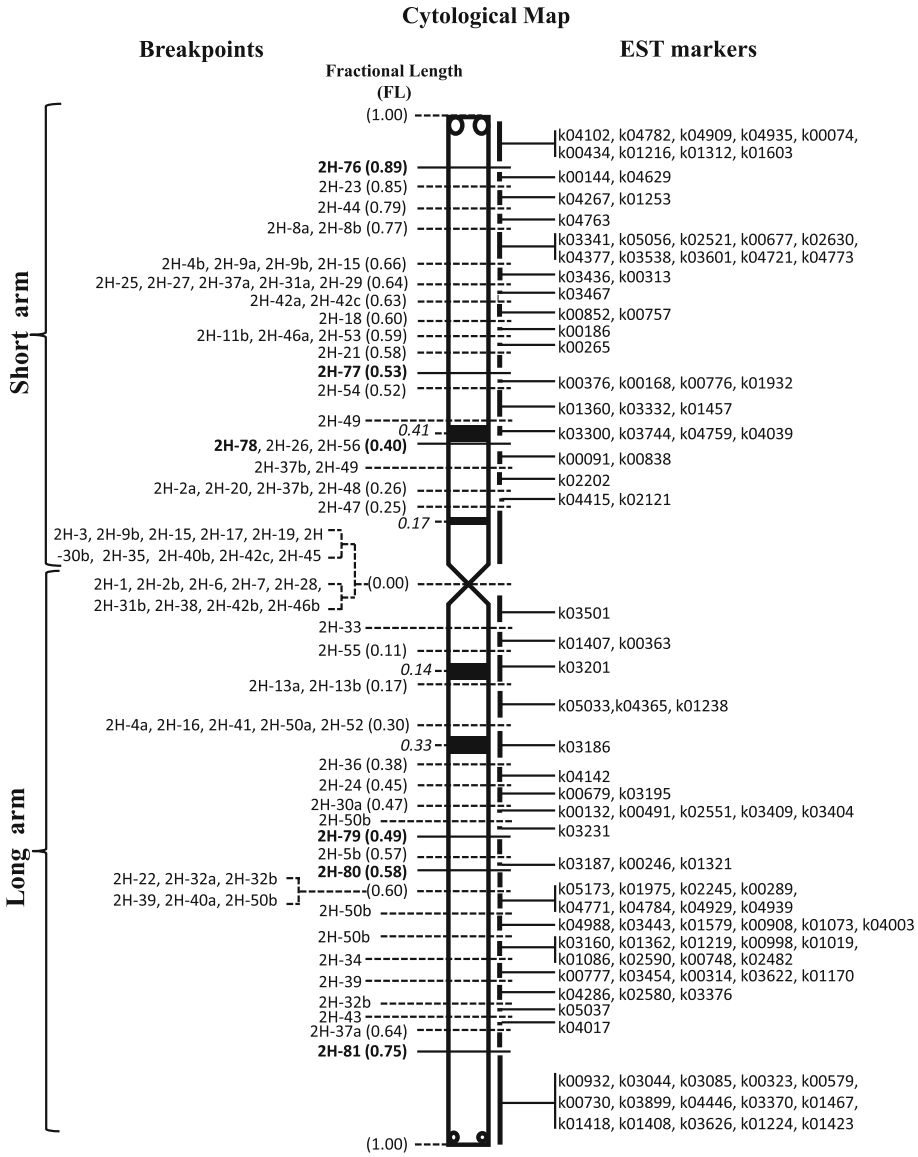

Fig. 4 Revision of the cytological map of chromosome 2H constructed by Joshi et al. (2011). FL in *parentheses*: breakpoints of 72 aberrant 2H chromosomes including six obtained in this study (*boldface*), FL in *italics*: position of C bands

Discussion

When present in the monosomic condition, chromosome 2C causes various kinds of chromosomal aberrations in wheat and also in wheat–barley addition lines during the gametogenesis of meiocytes (Endo and Gill 1996; Shi and Endo 1999; Sakai et al. 2009; Sakata et al. 2010; Joshi et al. 2011). Several previous studies suggested that the Gc system induces chromosomal aberrations at different developmental stages in embryos, endosperms as well as in meiocytes. For example, Nasuda et al. (1998) observed that three different Gc chromosomes (T2B-2S of *Ae. speltoides*, T4B-4S^{sh} of *Ae. sharonensis* and 2C^{c}L of *Ae. cylindrica*, the same as the 2C chromosome used in the study) induce chromosomal breaks in the first postmeiotic mitosis of pollen grains. Tsujimoto et al. (2001) reported that the 2C chromosome induces breakage of chromosomes after the formation of megaspores and found that different chromosomal breakages occurred in embryos and endosperms. Similarly, King and Laurie (1993) observed chromosomal aberrations during early embryo and endosperm development in reciprocal crosses between 'Chinese Spring' monosomic 4B and an alien substitution line in which chromosome 4B is replaced by a Gc chromosome 4S^{l} of *Ae. sharonensis*. Tsujimoto and Noda (1990) also suggested that the Gc chromosome T2B-2S$^{sp.au}$ of *Ae. speltoides* induces mutations after fertilization and ceases before differentiation of the shoot primordia.

If Gc-induced chromosomal breakage occurs exclusively during gametogenesis and stabilizes at the time of zygote formation, identical results should be obtained from cytological observations of root tip cells and PCR analysis of leaf tissues from the same plant; however, this was not always the case in this study. The contradictory results of

Table 3 Sequencing results with the 81 Gc-subjected plants and the 18 EST primer pairs

EST ID	PCR product (bp)	No. of plants examined[a]	No. of nucleotides aligned (bp)[b]	Total no. of nucleotides analyzed (bp)	Query for BLAST cDNA clone	Nucleotide (nt) positions compared with respect to the sequences indicated by the GenBank Accession numbers[c] (nt)	(Accession number)
k01312	650	74	623	46,102	baal39d15	216–604	BJ476997
k04629	450	77	418	32,186	bah62i11	179–604	BJ486709
k03538	465	79	422	33,338	GCN004P09	286–647	DN156539
k00852	590	79	556	43,924	HDP37P03	1–528	EX599848
k00186	415	79	374	29,546	baak16f14	329–623	BJ455326
k00776	650	80	635	50,800	baak46f04	162–532	BJ461678
k00091	610	80	584	46,720	bah15p15	111–418	AV943139
k02121	455	80	425	34,000	baak38c05	99–533	BJ459753
k03501	400	81	372	30,132	bags39p16	122–519	BJ468721
k03201	425	81	394	31,914	bags39m04	134–546	BJ468666
k05033	440	80	408	32,640	basd26h07	198–614	AV931854
k00679	450	80	414	33,120	baak41m17	52–358	BJ460676
k02551	370	81	335	27,135	bags22j12	156–525	AV918884
k01321	460	80	433	34,640	baal36n07	170–607	BJ476747
k01975	600	78	565	44,070	bags10e15	245–493	AV833891
k04771	550	74	485	35,890	basd13f02	344–658	AV927933
k00932	450	78	410	31,980	baal25p24	61–466	BJ475503
k00730	400	78	371	28,938	baak43c03	37–419	BJ461002
Total	8,830	1,419	8,224	647,075			

[a] The number of plants varied due to the exclusion of plants with unclear PCR or sequence results

[b] The sequences were aligned by CLUSTALW

[c] Data were cited from Sato et al. (2009) except for EX5999848 (Stein et al. unpublished cDNA library) and DN156539 (Biemelt et al. unpublished cDNA library)

cytological screening and PCR analysis in this study provided other positive evidence that the Gc chromosome 2C caused postzygotic chromosomal aberrations. The 2C chromosome might have affected DNA replication in postzygotic cell divisions to cause aberrations only in either of the newly replicated DNA strands or sister chromatids, and then the intact and aberrant chromatids might have moved into separate daughter cells that eventually developed into root and shoot meristems. If this was the case, the contradictions between FISH/GISH observations and PCR analysis in this study would be explicable because we used root tip cells for in situ hybridization and aerial parts to extract DNA for PCR amplification. Thus, whatever the mechanism of the Gc action of chromosome 2C, FISH/GISH screening using root tip cells could not detect a significant percentage of aberrations induced by the Gc system. Therefore, it is advisable to employ PCR, in addition to FISH/GISH, in screening for Gc-induced structural changes of alien chromosomes in common wheat, as long as some alien chromosome-specific terminal PCR markers, preferably on both chromosome arms, are available. The detection rate (7.4 %) of aberrant 2H chromosomes by PCR in this study was much higher than by FISH/GISH screening (4.4 %) conducted by Joshi et al. (2011).

Thuillet et al. (2002) directly estimated the mutation rate for microsatellite loci in *Triticum turgidum* ssp. *durum* (Durum wheat) to be 2.4×10^{-4} per allele per generation. By contrast, the frequency of induced mutations varies according to the type of mutagen and their intensity or concentration applied to plants. Ethylmethane sulphonate (EMS) induces mutations at a rate of at least one mutation per 24 and 40-kb screened in the hexaploid and tetraploid wheat TILLING populations, respectively (Slade et al. 2005). Using a similar EMS concentration, Uauy et al. (2009) reported one mutation per 49.4- and 68-kb in the hexaploid and tetraploid wheat populations, respectively. The frequency of chromosomal mutations observed in this study, 6 out of 81 plants (7.4 %), seems to be very high. By contrast, no point mutations were observed in the analysis of a ca. 650-kb sequence (ca. 8-kb region in total of the 18 loci excluding primer annealing sites of the aligned sequences for 81 Gc-subjected plants) (Table 3). We cannot rule out the possibility of point mutations in non-coding sequences that have not examined in the present study. Alternatively, the point mutations induced by the Gc

chromosome 2C are so less-frequent as to be detected by the survey of the 640 kb coding sequence. It is worth mentioning that the large volume bin-mapping efforts in wheat and relatives using deletion lines induced by the Gc system with various types of DNA markers have not detected point mutations (reviewed in Endo 2007). To have a genome-wide view of point mutations induced by the Gc system, broader re-sequencing effort, which is not applicable in hexaploid wheat yet, would be required. Genome scanning by recently developed genotyping-by-sequencing approach (Poland et al. 2012) would be adopted in future experiments.

Since Gc-induced chromosomal breakage is stabilized in subsequent generations, the translocation lines carrying alien segments without point mutations would be suitable for wheat improvement programs. Although most of these translocations are between non-homoeologous segments, selection of rare compensating translocations could also be achieved by screening a large number of Gc-induced progeny plants. In this regard, employing both screening methods, namely cytological (FISH/GISH) and PCR-based screening, will ensure the identification of almost all kinds of chromosomal aberrations caused by the action of the Gc chromosome.

Acknowledgments The aberrant 2H lines developed in this study will be deposited with the National BioResource Project-Wheat. This work was partially supported by the National BioResource Project, MEXT Japan. Contribution number 609 from the Laboratory of Plant Genetics, Graduate School of Agriculture, Kyoto University.

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