Theor Appl Genet (2010) 121:1093–1101
DOI 10.1007/s00122-010-1374-x

ORIGINAL PAPER

Molecular and cytological characterization of an extra acrocentric chromosome that restores male fertility of wheat in the msH1 CMS system

A. C. Martín · S. G. Atienza · M. C. Ramírez · F. Barro · A. Martín

Received: 4 April 2010 / Accepted: 12 May 2010 / Published online: 12 June 2010

Abstract A new CMS system designated as 'msH1' has been reported in bread wheat using the cytoplasm of *H. chilense*. While testing this system in different wheat backgrounds, a highly fertile line with chromosome number 42 plus an extra acrocentric chromosome was obtained. The extra chromosome did not pair with any wheat chromosome at meiosis, and progeny from this line which lack the acrocentric chromosome showed pollen abortion and male sterility. In order to establish the origin of this chromosome, FISH using *H. chilense* genomic DNA as probe was used and showed that it had originated from *H. chilense* chromosome(s). The novel chromosome did not possess sequences similar to wheat rDNA; however, the probe pSc119.2 from *S. cereale* containing the 120 bp family was found to occur at the end of its long arm. Data obtained from FISH and EST molecular markers confirm that the long arm of the acrocentric chromosome is indeed, the short arm of chromosome $1H^{ch}$ from *H. chilense*. We suggest that the novel chromosome originated from a deletion of the distal part of the long arm of chromosome $1H^{ch}$. Neither the $1H^{ch}S$ short arm, nor the whole chromosome $1H^{ch}$ restores pollen fertility of the alloplasmic wheat. Therefore, the restorer gene on the acrocentric chromosome must be located on the retained segment from the hypothetical $1H^{ch}L$, while some pollen fertility inhibitor could be present on the deleted $1H^{ch}L$ distal segment. Disomic addition of the acrocentric chromosome was obtained and this line resulted fully stable and fertile.

Introduction

Developing commercially viable hybrid wheat remains a goal in wheat breeding, as an additional approach to break the yield ceiling. The autogamous nature of wheat makes hybrid seed production a challenging task, but several hybrid seed production systems have been developed based either on chemical hybridizing agents (CHAs) or the use of genetic systems. Utilization of a CHA is expensive, polluting and highly influenced by environmental conditions, and up to date, no CHA is in commercial use. Since Wilson and Ross (1962) described a nuclear-restoration system with *Triticum timopheevii* as the source of cytoplasmic male sterility (CMS); many cytoplasms of the genera *Triticum*, *Secale*, *Haynaldia* and *Aegilops* have been recognized as the basis of a CMS system for wheat. The *T. timopheevii* system has gained general use because of deleterious the effects of other cytoplasms on various traits and/or because no advantage existed over the *T. timopheevii* system (Pickett 1993). However, practical application of this system has been disappointing, mainly due to the complexity of the fertility restoration procedure.

Hordeum is a genus that has been poorly explored as a source of CMS for wheat. Recently, a new CMS source, designated 'msH1', has been reported in bread wheat (*Triticum aestivum* L.) using the cytoplasm of *Hordeum chilense* Roem. et Schult (Martín et al. 2008a; Martín et al. 2009).

H. chilense is a diploid wild barley included in the section Anisolepis Nevski, native to Chile and Argentina. This species shows some traits potentially useful for wheat

Communicated by B. Friebe.

A. C. Martín (✉) · S. G. Atienza · M. C. Ramírez · F. Barro · A. Martín
Departamento de Mejora Genética Vegetal, I.A.S-C.S.I.C, Apdo. 4084, 14080 Córdoba, Spain
e-mail: a62maraa@uco.es

breeding, including drought and salt tolerance, resistance to several pests and diseases (Martín et al. 1996) and high seed carotenoid content (Atienza et al. 2004; Atienza et al. 2007). In addition, a major interest in this species relates to its high crossability with other members of the *Triticeae* tribe: *Aegilops*, *Agropyrum*, *Dasypirum*, *Secale*, *Triticum*, and ×*Triticosecale* (Bothmer et al. 1986; Martín et al. 1998). The compatibility of the *H. chilense* genome with that of *Triticum* spp. is high, and fertile amphiploids named tritordeums (×*Tritordeum* Ascherson et Graebner) were obtained after chromosome doubling of the hybrid between *H. chilense* and *T. aestivum* (Martín et al. 1999). When *Tritordeum* is backcrossed repeatedly to wheat, alloplasmic wheat is obtained possessing the wheat nuclear genome in barley cytoplasm. This alloplasmic wheat is either self-fertile or male sterile depending on the *H. chilense* accession used to synthesize the tritordeum (Martín et al. 2008a). It was observed that when using the *H. chilense* accession H1 and *T. aestivum* cv. Chinese Spring as female and male parents, respectively, the corresponding alloplasmic wheat was male sterile. The new 'msH1' cytoplasmic male sterility (CMS) source in bread wheat (*T. aestivum*) is stable under different environmental conditions and it does not exhibit developmental or floral abnormalities as alloplasmic wheat with *H. vulgare* cytoplasm does, showing only slightly reduced height and some delay in heading (Martín et al. 2008a). Neither grain shrivelling nor germination disorders, common in other alloplasmic wheats, have been observed. When the alloplasmic ditelosomic addition line of $6H^{ch}S$ was obtained, it resulted to be fully fertile. Therefore, fertility restoration of the CMS phenotype caused by the *H. chilense* cytoplasm was associated with the addition of the short arm of chromosome $6H^{ch}$ of *H. chilense* accession H1 (Martín et al. 2008a) and therefore, one or more fertility restoration genes appear to be located on this chromosome arm.

With the aim of obtaining the alloplasmic *T. aestivum* cv. T26 in *H. chilense* accession H1 cytoplasm, the amphiploid ×*Tritordeum* was obtained after chromosome doubling of the hybrid between wheat and barley. After repeated backcrosses to wheat, a highly fertile line with chromosome number 42 plus an extra acrocentric chromosome of morphology distinct to both wheat and barley was isolated. In this work, the molecular and cytological characterization of this restored line is reported.

Materials and methods

Plant material

The genetic stocks used in this study are shown in Table 1. Lines T21A1H_1S, T21A1H_1-1H_1S, T551 and T552 were kindly provided by Steve Reader, JIC, Norwich, UK. Lines T526 and T528 were obtained in this work.

Development of alloplasmic lines

Tritordeum HT26 ($2n = 8x = 56$, $H^{ch}H^{ch}$AABBDD) was obtained after chromosome doubling of hybrids between *H. chilense* accession H1 ($2n = 2x = 14$, $H^{ch}H^{ch}$) and *T. aestivum* cv. T26 ($2n = 6x = 42$, AABBDD), as described by Martín and Chapman (1977). The amphiploid obtained, HT26, was recurrently back-crossed to the wheat parental T26, resulting in the definitive alloplasmic line named T236 (AABBDD). This line carrying *H. chilense* cytoplasm was fully male sterile.

Cytological observations

For somatic chromosome counting, root tips of 1-cm length were collected from germinating seeds and pre-treated for 4 h in an aqueous colchicine solution (0.05%) at 25°C. They were fixed in a freshly prepared 3 absolute ethanol:1 glacial acetic acid (*v*/*v*) mixture and stained by the conventional Feulgen technique.

For meiotic chromosomes observation, anthers were collected and stained directly with 0.1% acetocarmine.

Microsporogenesis was studied at different developmental stages. Spikelets were collected and fixed in 3 absolute alcohol:1 glacial acetic acid (*v*/*v*) mixture. The material was transferred to fresh fixative after 1–2 h and stored at 4°C. Anthers were stained with 0.1% acetocarmine.

Molecular analysis

DNA was extracted from 5 to 6-week-old seedlings from every genotype according to the procedure of Doyle and Doyle (1990) with some minor modifications. The consensus chloroplast simple sequence repeat ccSSR-4 developed by Chung and Staub (2003) was used to verify the presence of the *H. chilense* cytoplasm in the alloplasmic lines. The polymerase chain reaction (PCR) was carried out in 25 ml of reaction mixture (as described by Chung and Staub 2003).

The expressed sequence tag (EST) markers described in Table 2 were used to identify the origin of the extra acrocentric chromosome. PCR was carried out as described by Nasuda et al. (2005).

The B3-hordein gene-specific primers HoB*9 and HoB*10 (Pistón et al. 2005) that amplify specifically in *H. chilense* chromosome $1H^{ch}$, were used to confirm the identity of the acrocentric chromosome. PCR was carried out as described by Pistón et al. (2005). All amplification products were resolved by agarose gel electrophoresis and visualized with ethidium bromide.

Table 1 Description of the genetic material used in this study

Line 1	Standard abbreviation 2	Germplasm 2	Chromosome number 2	Chromosome configuration 2	Male fertility
H1	H1	*H. chilense* Roem. et Schultz. accession H1	14	7″	Fertile
Hv	Hv	*H. vulgare* cv. Betzes	14	7″	Fertile
R	I	*Secale cereale* cv. Imperial	14	7″	Fertile
T21	CS	*T. aestivum* cv. Chinese Spring	42	21″	Fertile
T26	T26	*T. aestivum* cv. T26	42	20″ + 1″ T1RS·1BL	Fertile
T236	(H1)T26	*T. aestivum* cv. T26 in H1 cytoplasm	42	20″ + 1″ T1RS·1BL	Male sterile
T526	(H1)T26-H^{ch} MAH^{ch}ac	*T. aestivum* cv. Chinese Spring–*H. chilense* monosomic addition acrocentric chromosome in H1 cytoplasm	42 + ac	20″ + 1″ T1RS·1BL +1′ H^{ch}ac	Fertile
T528	(H1)T26-H^{ch} DAH^{ch}ac	*T. aestivum* cv. Chinese Spring–*H. chilense* disomic addition acrocentric chromosome in H1 cytoplasm	42 + ac″	20″ + 1″ T1RS·1BL + 1″ H^{ch} ac	Fertile
T21A1H1-1H1S	CS-H^{ch} $MA1H^{ch}MtA1H^{ch}S$	*T. aestivum* cv. Chinese Spring–*H. chilense* monosomic addition $1H^{ch}$ monotelosomic addition $1H^{ch}S$	43 + t′	21″ + 1′ $1H^{ch}$ + t′ $1H^{ch}S$	Fertile
T21A1H1S	CS-H^{ch} $DtA1H^{ch}S$	*T. aestivum* cv. Chinese Spring–*H. chilense* ditelosomic addition $1H^{ch}S$	42 + t″	21″ + t″ $1H^{ch}S$	Fertile
T551	CS-H^{ch} $DS1H^{ch}$(1A)	*T. aestivum* cv. Chinese Spring–*H. chilense* disomic substitution $1H^{ch}$(1A)	42	20″ + 0″ 1A + 1″ $1H^{ch}$	Fertile
T552	CS-H^{ch} $DT1H^{ch}S$·1BL	*T. aestivum* cv. Chinese Spring–H. chilense double translocation $T1H^{ch}S$·1BL	42	20″ + 1″ $T1H^{ch}S$·1BL	Fertile

The extra acrocentric chromosome is abbreviated as H^{ch}ac

[1] Abbreviation used in this work

[2] Nomenclature suggested by Raupp et al. (1995) for the genetic stocks of wheat and its relatives

Table 2 Expressed sequence tag (EST) markers used to identify the origin of the extra acrocentric chromosome and assignment to chromosomes arms

EST marker (Nasuda et al. 2005)	Primer (L)	Primer (R)	Chromosomal location
k00183	TGTGTTTTATTCAGCTGGCG	TGCTCTTTCCTCACCAACCT	$1H^{ch}S$
k04031	CCGGT AT T GACAAGCAT CAA	AT T CAT GGCACT CCGT CTTC	$1H^{ch}L$
k01360	AT GGGCCAAT AT CAAT T CCA	TGCTGCGTTCAGCTTTAAGA	$2H^{ch}S$
k02539	AAT GT GCCT CCACAGGGT AG	GAT ACT GAGT GGAAAGCGGC	$4H^{ch}S$
k04725	AAACGGGAAAT GTTTT GCAG	TCAAGAAGGCCATCGAGAAG	$4H^{ch}L$
k05021	T GCCCCCAAACT TTATATGC	GAGGGT CT T CCT GT T GT GGA	$4H^{ch}L$
k03014	GCT GCAT GCT GGACT CAAT A	GCACTCATTGAACAGCAGGA	$6H^{ch}L$
k01294	T GAT CTTT GGCAGAGCACAC	GGTTGGTGGGGAGTTGTATG	$6H^{ch}L$
k04783	AGAACCGAGAT GAGGAAT GT G	AGT CT CT T CGCGGAAT CAAG	$7H^{ch}S$
k03325	GGT T CAGGGACGAAGGT ACA	CTACGCCTAGCGGAGATGAG	$7H^{ch}S$

Fluorescence in situ hybridisation (FISH)

Root tips were fixed as described in "Cytological observations". Preparations were made as described by Prieto et al. (2001).

Total *H. chilense* genomic DNA was labelled by nick translation with biotin-11-dUTP (Roche Corporation, Basel, Switzerland) and total *S. cereale* genomic DNA was labelled with digoxigenin-dUTP. Both probes were mixed in the hybridization solution to a final concentration of 5 ng/ml.

After examination of nuclei hybridized by GISH (genomic in situ hybridization), some preparations were examined by using two repetitive probes: probe pTa71, containing 1 unit of 18S-5.8S-26S rDNA (8.9 kb) from *T. aestivum* (Gerlach and Bedbrook 1979) was labelled by nick translation with digoxigenin-dUTP (Roche Corporation, Basel, Switzerland) and probe pSc119.2 from *S. cereale* containing the 120-bp family isolated by McIntryre et al. (1990) was labelled with biotin-11-dUTP.

The in situ hybridization protocol was that of Cabrera et al. (2002). Digoxigenin- and biotin-labelled probes were detected with antidigoxigenin-FITC (Roche Corporate) and streptavidin-Cy3 conjugates (Sigma, St Louis, MO, USA), respectively. Chromosomes were counterstained with DAPI (40,6-diamidino-2-phenylindole) 339 and mounted in Vectashield (Vector Laboratories Inc.). Slides were examined by using a Zeiss LSM 5 Pa confocal laser scanning microscope with LSM 5 Pa software version 3.0 (Zeiss, Jena, Germany).

Results

Development of the alloplasmic line and identification of the acrocentric chromosome

Tritordeum HT26 ($2n = 8x = 56$, $H^{ch}H^{ch}AABBDD$) was obtained after chromosome doubling of the hybrid between *H. chilense* accession H1 and *Triticum aestivum* cv. T26, as described by Martín et al. (1999). The amphiploid obtained, HT26, was recurrently back-crossed to the wheat parental T26. A line with chromosome number 45 resulted to be self-fertile. Progeny of this plant presented a chromosome number ranging from sterile 42 to fertile 45 chromosomes, with some lines containing telosomic chromosomes. Male sterile plants with chromosome number 42 were pollinated by fertile sister plants, and among their progeny, plants with chromosome number close to 42 together with self-fertility were selected.

A fertile plant with chromosome number 42 plus a small acrocentric chromosome was identified (Fig. 1a). Selfing of this plant gave rise to two kinds of plants: plants with chromosome number 42 that resulted to be male sterile, and plants with chromosome number 42 plus the extra acrocentric chromosome that resulted again to be male fertile. The first group of plants, i.e. T26 in *H. chilense* cytoplasm, was named T236; and male fertile plants possessing the acrocentric chromosome were named T526. As can be observed in Fig. 1b, the extra chromosome did not pair with any wheat chromosome at meiosis.

It was important to check that fertility restoration was the consequence of the extra acrocentric chromosome addition and not the result of paternal transmission of cytoplasm and loss of alloplasmy as described previously (Aksyonova et al. 2005; Badaeva et al. 2006; Martín et al. 2008b). In the progeny of T526 appeared plants with and without the acrocentric chromosome, the latter group being completely sterile and therefore confirming the alloplasmic nature of T526. However, in order to prove that there was no transmission of paternal cytoplasm in the restored line T526, the chloroplast simple sequence repeat ccSSR-4 (Chung and Staub 2003) was used. This chloroplast marker is polymorphic with amplification products of 200 and 225 bp, respectively, in wheat and H1 cytoplasms (Martín et al. 2008b). DNA from line T526

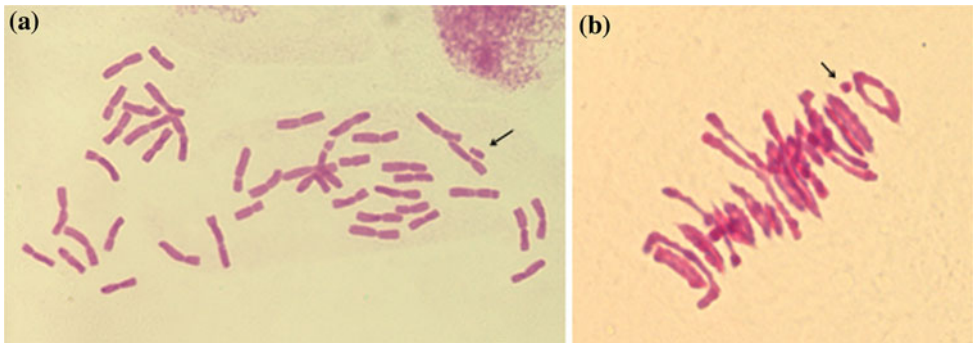

Fig. 1 Chromosomes observation in restored line T526: (H1)T26 + acrocentric chromosome. **a** Root-tip metaphase cell stained by the conventional Feulgen technique. **b** Meiotic chromosomes stained directly with 0.1% acetocarmine. The extra acrocentric chromosome is indicated by an *arrow*

amplified a 225-bp band, confirming that it was an alloplasmic line in H1 cytoplasm. After more than ten generations of crosses with wheat, the reversion of the *H. chilense* cytoplasm has never been observed.

Cytogenetic characterization by FISH

In order to identify the origin of the acrocentric chromosome, the fertile line T526 was analyzed by genomic in situ hybridization (GISH) using *H. chilense* H1, and *S. cereale* genomic DNA as probes, labelled with biotin-11-dUTP and digoxigenin-dUTP, respectively. Our first hypothesis considered the possibility of a barley and rye origin for the novel acrocentric chromosome, since some rearrangement involving rye chromatin (present in the 1RS/1BS translocation) could have taken place. As it is observed in Fig. 2a, the entire length of the extra acrocentric chromosome displayed an intense red color, indicating that it is only of barley origin. It was also possible that some introgression of *H. chilense* was present in this material but, as can be seen in Fig. 2a, no introgression of barley or rearrangements of rye sequences were observed.

Once the barley origin of this acrocentric chromosome was established, fluorescence in situ hybridization (FISH) was carried out using pTa71 and pSc119.2 probes to elucidate which *H. chilense* chromosome was implicated. Probe pTa71 contains 1 unit of 18S-5.8S-26S rDNA, located in the nucleolar organizer regions (NORs) of chromosomes. Probe pSc119.2 from *S. cereale* marks a series of very useful diagnostic signals at the telomeres of chromosome arms $1H^{ch}S$, $2H^{ch}S$, $4H^{ch}S$, $4H^{ch}L$, $5H^{ch}S$ and at a subtelomeric position of $6H^{ch}L$ and $7H^{ch}S$ chromosome arms (de Bustos et al. 1996; Marín et al. 2008). The NORs in *Hordeum* are localized in chromosome segments 5HS and 6HS. As can be observed in Fig. 2b, no pTa71 signal was detected on the acrocentric chromosome, but was confined to wheat chromosomes, and thus, chromosomes $5H^{ch}S$ and $6H^{ch}S$ are ruled out as components of the acrocentric chromosome. On the other hand, a pSc119.2 signal appeared in the long arm of the acrocentric chromosome, which indicates that chromosomes $1H^{ch}S$, $2H^{ch}S$ $4H^{ch}S$, $4H^{ch}L$, $6H^{ch}L$ and $7H^{ch}S$ are the possible candidates for the origin of the acrocentric chromosome.

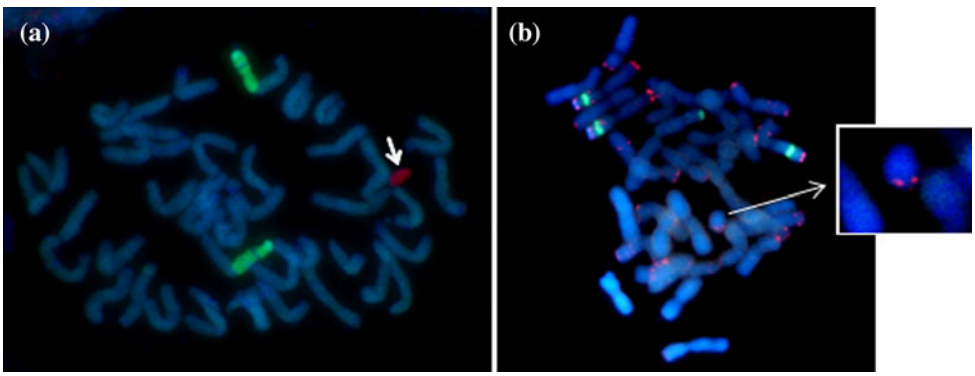

Fig. 2 In situ hybridization to root-tip metaphase cells from restored line T526. The acrocentric chromosome is indicated by an *arrow*. **a** GISH using *H. chilense* genomic DNA and *S. cereale* genomic DNA probes detected with streptavidin-Cy3 (*red*) and antidigoxigenin-FITC (*green*), respectively. *Blue* DAPI staining shows the wheat chromosomal DNA. The acrocentric chromosome shows an intense *red* color, indicating its purely barley origin. **b** Double FISH signals using the pTa71 probe detected with antidigoxigenin-FITC (*green*) and pSc119.2 probe detected with streptavidin-Cy3 (*red*). The pTa71 probe only hybridizes to satellited wheat chromosomes; however, the pSc119.2 probe displays a telomeric signal at the long arm of the extra acrocentric chromosome

Molecular characterization

After GISH and FISH analyses, chromosome specific EST markers (Table 2) addressed to different *H. chilense* chromosome arms were used to identify the acrocentric chromosome. These markers either produce different amplification products in *H. chilense* and wheat or exclusively amplify the *H. chilense* genome.

PCR results on T526 showed that marker k00183 was the only one that produced an amplification fragment corresponding to *H. chilense*. This marker amplifies with PCR in barley (*H. vulgare* cv. Betzes), and it is assigned to the short arm of chromosome 1. As can be observed in Fig. 3a, it also amplifies in chromosome $1H^{ch}$ in *H. chilense* and do not amplify in bread wheat. In order to further confirm, that the $1H^{ch}S$ chromosome arm was part of the extra acrocentric chromosome, the B3-hordein gene-specific primers HoB*9 and HoB*10 (Pistón et al. 2005), that amplify specifically in *H. chilense* chromosome $1H^{ch}$ (but not in *H. vulgare*), were used. As can be observed in Fig. 3b, the presence of $1H^{ch}S$ was definitely established.

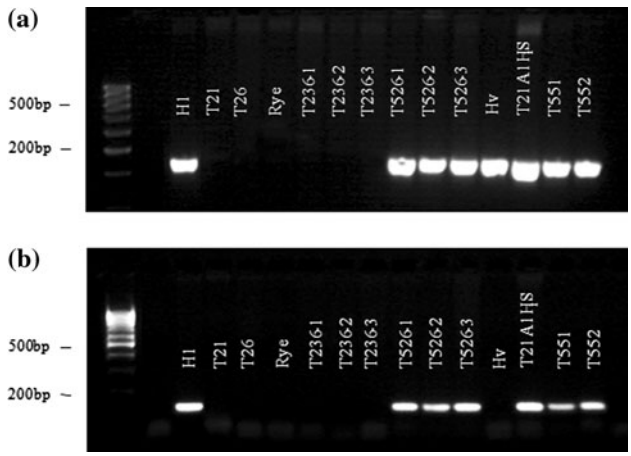

Fig. 3 PCR amplification products using **a** EST marker k00183 that amplifies specifically the $1H^{ch}S$ chromosome in *H. chilense* and *H. vulgare* and does not produce an amplification product in wheat and rye. **b** B3-hordein genes-specific primers HoB*9 and HoB*10 that amplifies specifically in the *H. chilense* $1H^{ch}S$ chromosome. The identity of $1H^{ch}S$ as the long arm of the extra acrocentric chromosome is therefore established. H1, *H. chilense* accession H1; T21, *T. aestivum* cv. Chinese Spring; T26, *T. aestivum* carrying the translocation T1RS·1BL; T236, alloplasmic T26 in H1 cytoplasm; T526, restored T236 + acrocentric chromosome; Hv, *H. vulgare* cv. Betzes; $T21A1H_1S$, T21 ditelosomic addition $1H^{ch}S$; T551, T21 substitution $1H^{ch}$ (1A); T552, T21 double translocation $T1H^{ch}S{\cdot}1BL$

Crosses carried out

In order to investigate whether the restorer gene is present in the long arm of the acrocentric chromosome (i.e. $1H^{ch}S$) or in the short arm, line T526 was used as female parent and pollinated with the heteromorphic $1H^{ch}$-$1H^{ch}S$ addition ($T21A1H_1$-$1H_1S$) in Chinese Spring. The genomic combinations listed in Table 3 were obtained. None of the combinations except the one with chromosome number 42 + $1H^{ch}S$ + $H^{ch}ac$, was fertile. These results indicate that neither the $1H^{ch}S$ nor the whole chromosome $1H^{ch}$ restore pollen fertility and therefore, either the entire extra acrocentric chromosome or only its short arm, is needed to restore male fertility of wheat in *H. chilense* cytoplasm.

Obtention of the disomic addition of the acrocentric chromosome

T526 line was male fertile, but in order to use it as a restorer line, it is necessary to develop this acrocentric chromosome addition in disomic condition. Therefore, self-progeny of T526 was analyzed cytologically by somatic chromosome counting and plants with 42 chromosomes plus 2 acrocentric chromosomes were identified. This line was fully fertile and named T528. When selfing, all individual in the progeny keep the 2 acrocentric chromosomes perfectly stable. T528 was also characterized using genomic in situ hybridization with *H. chilense* H1 and *S. cereale* genomic DNA as probes (Fig. 4).

Morphology of alloplasmic lines and cytological analysis of pollen development

CMS of the alloplasmic line T236, as well as the fertility of the restored lines T526 and T528 were stable under different environmental conditions: growth chamber, greenhouse and open field. Mature anthers of male sterile T236 were

Table 3 Fertility of different genomic combinations of alloplasmic wheat in *H. chilense* cytoplasm, involving chromosome $1H^{ch}$, $1H^{ch}S$ and the acrocentric chromosome

Origin	Chromosome number	Chromosome configuration 1	Fertility
T526xT21A1H1-1H1S	42 + t′	20″ + 1′ 1B + 1′ T1RS-1BL + t′ $1H^{ch}S$	Sterile
	43	20″ + 1′ 1B + 1′ T1RS-1BL + 1′ $1H^{ch}$	Sterile
	42 + t′ + 1′ac	20″ + 1′ 1B + 1′ T1RS-1BL + t′ $1H^{ch}S$ + 1′ $H^{ch}ac$	Fertile
	43 + 1′ac	20″ + 1′ 1B + 1′ T1RS-1BL + 1′ 1ffh + 1′ H = hac	Fertile

Nomenclature suggested by Raupp et al. (1995) for the genetic stocks of wheat and its relatives

$H^{ch}ac$ acrocentric chromosome, *T526* restored T236 + $H^{ch}ac$, *$T21A1H_1$-$1H_1S$* T21 heteromorphic $1H^{ch}$-$1H^{ch}S$ addition

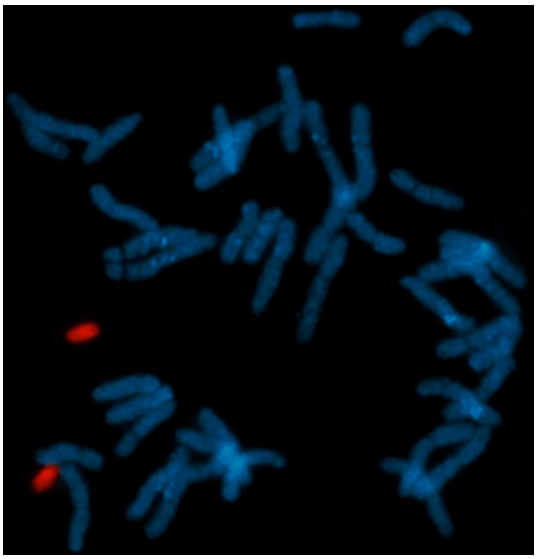

Fig. 4 Genomic in situ hybridization to root-tip metaphase cells from T528 line using *H. chilense* H1 genomic DNA probe detected with streptavidin-Cy3 (*red*). Acrocentric chromosomes H^{ch}ac show *red color* indicating its barley origin

dry, pale and devoid of viable pollen, but apart from that, the plants did not exhibit other developmental or floral abnormalities. The restored lines T526 and T528 with one and two acrocentric chromosomes, respectively, did not present any abnormality and neither shrivelling nor decrease of germination was observed in the seeds of these lines. As alloplasmic T21 in *H. chilense* cytoplasm, T236, T526 and T528 were 3 or 4 days later to anthesis when grown in greenhouse and growth chamber under long day conditions and this difference is about 6 or 7 days when grown in open field conditions. Alloplasmic lines also showed lower height; actually, decrease in height was directly correlated with the amount of *H. chilense* DNA present. Therefore, T236 presented the highest, T528 the lowest height and the T526 line being in between them. Fertility of restored line in T526 line was stable but lower than euplasmic wheat, however, no significant difference was found between euplasmic T26 and alloplasmic T528.

Cytological studies comparing alloplasmic (T236), euplasmic (T26) and restored lines (T526 and T528) at successive stages of pollen development revealed that formation of microspores followed a normal course in every line until the tetrad stage. However, soon after the microspores were released from the tetrad and before mitosis I, degeneration of pollen occurred in line T236. At this point, most pollen cells of T236 line appeared shrunken and heterogeneously stained, as happens with alloplasmic T21 in *H. chilense* cytoplasm (Martín et al. 2008a). Pollen development was completely asynchronous from this stage, and it was possible to observe cells at different sizes, shapes, and stages, although none of the pollen cells reached the mature tricellular pollen stage. Pollen development in the restored line T528 was completely normal, with no difference when compared with the euplasmic T26 wheat. However, pollen development in the restored line T526 was different from both T528 and T236. Thus, some of the microspores degenerate after being released from the tetrad at different development stages, while some others managed to reach the mature tricellular pollen and seed set is accomplished (Fig. 5).

Discussion

While trying to obtain alloplasmic wheat in *H. chilense* cytoplasm, an extra acrocentric chromosome capable of restoring male fertility of the alloplasmic wheat was observed. The line possessing this acrocentric chromosome was named T526. The extra chromosome does not pair with any wheat chromosome at meiosis, and progeny from the T526 line which lack the acrocentric chromosome showed pollen abortion and male sterility. These facts suggest that this chromosome derived from *H. chilense* chromosome/s has gene/s essential for maintaining male fertility in *H. chilense* cytoplasm. Disomic addition of the acrocentric chromosome (named T528) was obtained and this resulted in a fully stable and fertile seed set which was not different when compared with that of euplasmic T26.

In contrast to the fertility restorer gene(s) (*Rf*) present on chromosome $6H^{ch}S$ which is ineffective in hemicygosis, the *Rf* gene(s) on the acrocentric chromosome restores pollen fertility with a single dose. In consequence, we believe that it offers a real potential for the development of viable technology for hybrid wheat production. Seed set of T526 was nevertheless lower than euplasmic wheat, what can be due not only to the alloplasmic state, but also due to aneuploidy, that frequently causes a decrease in fertility rates. However, it may be possible to obtain a workable restorer line for the 'msH1' CMS system by introgressing the segment carrying the *Rf* gene into a group homeologous 1 wheat chromosome.

Data obtained using fluorescent in situ hybridization (FISH) and molecular markers support the hypothesis that part of this acrocentric chromosome was derived from chromosome $1H^{ch}$ from *H. chilense*, most likely, after a deletion of the distal part of the long arm of chromosome $1H^{ch}$. Therefore, the extra chromosome would be the $1H^{ch}S{\cdot}del1H^{ch}L$ chromosome.

Crosses carried out between T526 and the heteromorphic $1H^{ch}$-$1H^{ch}S$ addition line ($T21A1H_1$-$1H_1S$) and ditelosomic addition $1H^{ch}S$ ($T21A1H_1S$) in Chinese Spring, indicated that neither the arm $1H^{ch}S$ nor the chromosome $1H^{ch}$, restore male fertility in alloplasmic T236, which suggest that the putative fertility restorer gene (*Rf*) on the acrocentric chromosome must be located on the segment retained

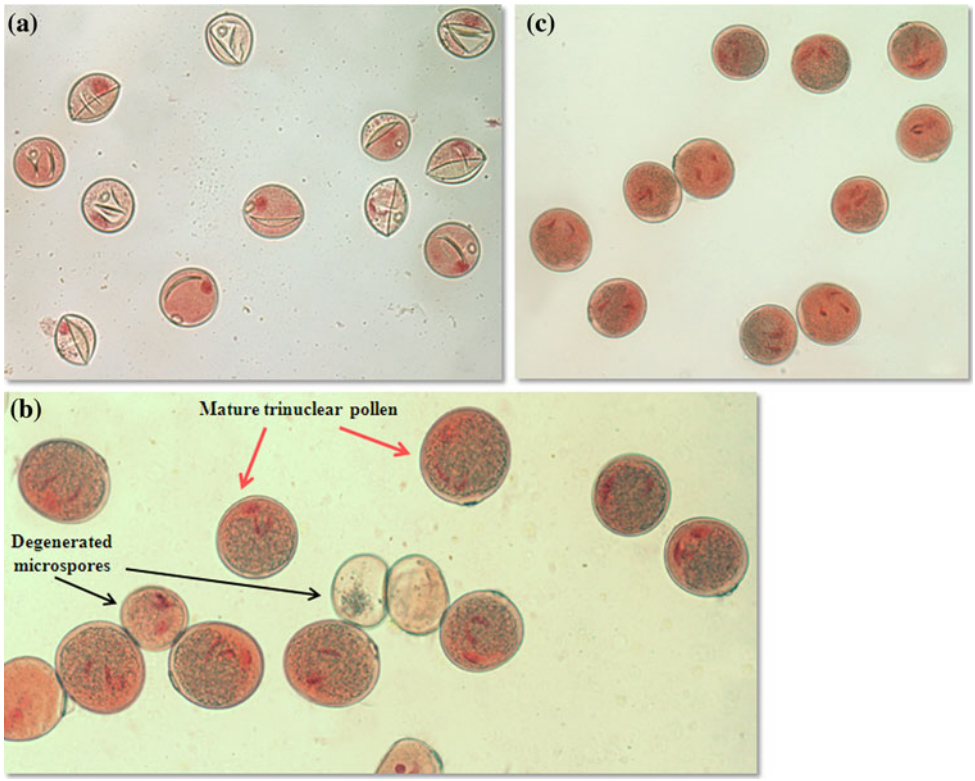

Fig. 5 Pollen development at anthesis in alloplasmic male sterile line T236 (**a**), and restored lines T526 (**b**) and T528 (**c**). **a** Example of the different degeneration stages at the end of microgametogenesis in the male sterile line T236. **b** Normal and degenerated microspores in restored line T526 (T236 + 1′ H^{ch}ac). **c** Normal trinuclear pollen grains at anthesis in restored line T528 (T236 + 1″ H^{ch}ac)

from $1H^{ch}L$. However, the presence of this *Rf* gene does not explain why the addition of the whole chromosome $1H^{ch}$ does not restore male fertility either. To explain this observation, we suggest the existence of a pollen fertility inhibitor gene F_i on the deleted distal $1H^{ch}L$ segment. The presence of gene/s on homeologous group 1 long arm chromosomes, negatively affecting the fertility of hybrids has been proposed previously by others authors. For example, addition lines of barley (*H. vulgare*) chromosome $1H^{v}$ in wheat could not be produced, because a gene present on the long arm of this barley chromosome causes extreme cytological abnormalities at meiosis, and results in the complete sterility of the plants. Because of that reason, fertile disomic $1H^{v}$ and ditelosomic $1H^{v}L$ addition lines are not available (Islam et al. 1981; Islam and Shepherd 1990, 2000; Taketa and Takeda 1997; Taketa et al. 2001). A sterility gene named *Shw*, located in the interstitial 25% region of the $1H^{v}L$ arm (Taketa et al. 2002) has been proposed. Different sources of barley $1H^{v}$ chromosomes have been tested causing all of them sterility when introduced to different wheat backgrounds, which suggests the generality of this phenomenon in wheat–barley hybrids (Islam and Shepherd 1990; Taketa and Takeda 1997). Our results suggest that *H. chilense* $1H^{ch}$ chromosome have the same activity as barley $1H^{v}$ chromosomes in causing cytological abnormalities at meiosis in wheat. The $1H^{ch}$ *H. chilense* addition line in wheat is only available as a heteromorphic $1H^{ch}$-$1H^{ch}S$ pair because two doses of $1H^{ch}L$ seems to be non-viable. Therefore, the pollen fertility inhibitor gene proposed in the present work, may be similar or even the same gene as the *Shw* gene in $1H^{v}L$.

The appearance of extra chromosomes has been described in previous studies on alloplasmic crops. In some lines of common and durum wheat containing alien cytoplasms such as *Aegilops*, *Secale*, and *Agropyron*, specific chromosomes are retained in addition to a normal set of wheat chromosomes even after a large number of backcrosses with the nuclear donor (Tsunewaki 1980; Murata et al. 1992). Furthermore, common wheats with the cytoplasm of *Agropyron trichophorum* need a specific submetacentric or telocentric chromosome to restore endosperm development and seed viability (Tsujimoto et al. 1987).

In conclusion, for the data obtained in the present work, we suggest that the extra acrocentric chromosome described here could be the $1H^{ch}S{\cdot}del1H^{ch}L$. We proved by FISH and molecular markers that the long arm of the acrocentric chromosome was the $1H^{ch}S$ arm. However, even if we tested many molecular markers aimed at confirming the short arm of the acrocentric chromosome ($del1H^{ch}L$) as part of the $1H^{ch}L$ arm, no positive results were obtained. Nevertheless, this could be due to the small size of the short arm of the acrocentric chromosome and its proximity to the centromere, since low marker density and suppressed recombination around the centromeric regions of barley chromosome $1H^{v}$ has been reported previously (Künzel et al. 2000; Taketa et al. 2002). In addition, it can not be

excluded that the extra acrocentric chromosome might have been produced by a more complicated process than that proposed above, and further studies will be necessary to unravel its identity.

Acknowledgments This work was supported by MICINN (Ministerio de Ciencia e Innovación) project AGL2007-65685-C02-01 and AGL2009-11359 of the Spanish Government.

References

Aksyonova E, Sinyavskaya M, Danilenko N, Pershina L, Nakamura C, Davydenko O (2005) Heteroplasmy and paternally oriented shift of the organellar DNA composition in barley–wheat hybrids during backcrosses with wheat parents. Genome 48:761–769

Atienza SG, Ramírez MC, Hernández P, Martín A (2004) Chromosomal location of genes for carotenoid pigments in *Hordeum chilense*. Plant Breed 123:303–304

Atienza SG, Ballesteros J, Martín A, Hornero-Mández D (2007) Genetic variability of carotenoid concentration and degree of esterification among Tritordeum (*Tritordeum* Ascherson et Graebner) and durum wheat accessions. J Agric Food Chem 55:4244–4251

Badaeva ED, Pershina LA, Bil'danova LL (2006) Cytogenetic analysis of alloplasmic recombinant lines (*H. vulgare*)-*T. aestivum* unstable in fertility and viability. Rus J Genet 42:140–149

Bothmer Rv, Giles BE, Jacobsen N (1986) Crosses and genome relationship in the *Hordeum patagonicum* group. Genetica 71:75–80

Cabrera A, Martín A, Barro F (2002) In situ comparative mapping (ISCM) of Glu-1 loci in *Triticum* and *Hordeum*. Chromosome Res 10:49–54

Chung SM, Staub JE (2003) The development and evaluation of consensus chloroplast primer pairs that possess highly variable sequence regions in a diverse array of plant taxa. Theor Appl Genet 107:757–767

de Bustos A, Cuadrado A, Soler C, Jouve N (1996) Physical mapping of repetitive DNA sequences and 5 s and 18 s–26 s rDNA in five wild species of the genus Hordeum. Chromosome Res 4:491–499

Doyle JJ, Doyle JH (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Islam AKMR, Shepherd KW (1990) Incorporation of barley chromosomes into wheat. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 13. Springer, Berlin, pp 128–151

Islam AKMR, Shepherd KW (2000) Isolation of a fertile wheat–barley addition line carrying the entire barley chromosome 1H. Euphytica 111:145–149

Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat–barley chromosome addition lines. Heredity 46:161–174

Künzel G, Korzum L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Marín S, Martín A, Barro F (2008) Comparative FISH mapping of two highly repetitive DNA sequences in *Hordeum chilense* (Roem et Schult.). Genome 51:580–588

Martín A, Chapman V (1977) A hybrid between *Hordeum chilense* and *Triticum aestivum*. Cereal Res Commun 4:365–368

Martín A, Martínez C, Rubiales D, Ballesteros J (1996) Tritordeum: triticale′s new brother cereal. In: Güedes-Pinto H, Darvey N, Carnide VP (eds) Triticale: today and tomorrow. Kluwer, Dordrecht, pp 57–72

Martín A, Martín LM, Cabrera A, Ramírez MC, Giménez MJ, Rubiales D, Hernández P, Ballesteros J (1998) The potential of *Hordeum chilense* in breeding Triticeae species. In: Jaradat AA (ed) Triticeae III. Science Publishers, Enfield, pp 377–386

Martín A, Álvarez JB, Martín LM, Barro F, Ballesteros J (1999) The development of tritordeum: a novel cereal for food processing. J Cereal Sci 30:85–95

Martín AC, Atienza S, Ramírez MC, Barro F, Martín A (2008a) Male fertility restoration of wheat in *Hordeum chilense* cytoplasm is associated with $6H^{ch}S$ chromosome addition. Aust J Agric Res 59:206–213

Martín AC, Atienza SG, Barro F (2008b) Use of ccSSR markers for the determination of the purity of alloplasmic wheat in different *Hordeum* cytoplasms. Plant Breed 127:470–475

Martín AC, Ramírez MC, Atienza SG, Barro F, Martín A (2009) Chromosome engineering in wheat to restore male fertility in the msH1 CMS system. Mol Breed 24:397–408

McIntryre C, Pereira S, Moran L, Appels R (1990) New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. Genome 33:635–640

Murata M, Nakata N, Yasumuro Y (1992) Origin and molecular structure of a midget chromosome in a common wheat carrying rye cytoplasm. Chromosoma 102:27–31

Nasuda S, Kikkawa Y, Ashida T, Rafiqul AKM, Sato K, Endo TR (2005) Chromosomal assignment and deletion mapping of barley EST markers. Genes Genet Syst 80:357–366

Pickett AA (1993) Hybrid wheat: results and problems. Adv Plant Breed Supplement 15:1–259

Pistón F, Martín A, Dorado G, Barro F (2005) Cloning and molecular characterization of B-hordeins from *Hordeum chilense* (Roem. et Schult.). Theor Appl Genet 111:551–560

Prieto P, Ramírez MC, Ballesteros J, Cabrera A (2001) Identification of intergenomic translocations involving wheat, *Hordeum vulgare* and *Hordeum chilense* chromosomes by FISH. Hereditas 135:171–174

Raupp WJ, Friebe B, Gill BS (1995) Suggested guidelines for the nomenclature and abbreviation of the genetic stock of wheat and its relatives. Wheat Inf Serv 81:51–55

Taketa S, Takeda K (1997) Expression of dominant marker genes of barley in wheat–barley hybrids. Genes Genet Syst 72:101–106

Taketa S, Ando H, Takeda K, Bothmer Rv (2001) Physical locations of 5S and 18S–25S rDNA in Asian and American diploid Hordeum species with the I genome. Heredity 86:522–530

Taketa S, Choda M, Ohashi R, Ichii R, Takeda K (2002) Molecular and physical mapping of a barley gene on chromosome arm 1HL that causes sterility in hybrids with wheat. Genome 45:617–625

Tsujimoto H, Panayotov I, Tsunewaki K (1987) Behaviour of an extra chromosome carried by alloplasmic common wheat lines having *Agropyron trichophorum* cytoplasm. Jpn J Genet 62:291–299

Tsunewaki K (1980) Basic studies on hybrid wheat breeding utilizing the timopheevi cytoplasm and Rf3 gene—summary of the results. Seiken Ziho 29:40–56

Wilson JA, Ross VM (1962) Male sterility interaction of *Triticum aestivum* nucleous and *Triticum timopheevii* cytoplasm. Wheat Inf Serv 14:29–30