ONLINE RESOURCES

Genomic restructuring in F_1 *Hordeum chilense* × durum wheat hybrids and corresponding hexaploid tritordeum lines revealed by DNA fingerprinting analyses

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Introduction

Allopolyploidization induces irreversible changes and some of which can be detected by molecular markers. Such alterations may occur at the hybrid stage and/or after polyploidization. In this study, we aimed to determine the exact stage at which they occur during the synthesis of hexaploid tritordeum. We characterized two F_1 hybrids, genomic constitution $H^{ch}AB$ ($2n = 3x = 21$) derived from crosses between *Hordeum chilense* (lines H1 and H75; $H^{ch}H^{ch}$; $2n = 2x = 14$) and durum wheat (line T846; AABB; $2n = 4x = 28$), their corresponding tritordeum lines ($H^{ch}H^{ch}AABB$; $2n = 6x = 42$) produced through chromosome doubling of the sterile F_1 hybrids, and the parental species using four molecular-marker systems. The interretrotransposon amplified polymorphism, retrotransposon-microsatellite amplified polymorphism, intersimple sequence repeat and interprimer-binding site markers were suitable for DNA fingerprinting, and also detected molecular rearrangements in both F_1 hybrids and/or respective tritordeums relative to their parents. The number of bands of wheat-origin inherited by the descendants of both crosses were almost double than those of *H. chilense* origin. The rearrangements consisted of two novel bands detected in the F_1 hybrid H75 × T846 and/or its tritordeum, and lost parental bands (only amplified in one or both parents, not transmitted to the descendants). The number of lost parental bands of *H. chilense* origin and wheat origin were almost equal. The novel and lost parental bands were mostly detected in F_1 hybrids revealing that the genomic rearrangements took place at the hybrid stage.

The key role of polyploidy in the evolution of plants has been long recognized by biologists (see Matsuoka *et al.* 2014 for a review). Allopolyploidy consists of two major events starting with the hybridization of two or more divergent genomes into a single nucleus, followed by polyploidization (Stebbins 1971). The nucleus of a newly formed allopolyploid responds to allopolyploidization with several changes at the chromosomal levels (Leitch and Bennett 1997) and DNA sequence levels (Song *et al.* 1995; Wendel *et al.* 1995). In addition, alterations of gene expression and regulation (Scheid *et al.* 1996; Comai *et al.* 2000); transposon activity (Matzke and Matzke 1998); amplification, segregation and elimination of highly repetitive (Salina *et al.* 2000); and low-copy sequences (Feldman *et al.* 1997; Liu *et al.* 1998) were also reported.

Over the years, the occurrence of rearrangements was ascribed to various allopolyploid species, such as wheat (*Aegilops–Triticum*) group (Ozkan *et al.* 2001, 2003; Shaked *et al.* 2001), triticale and wheat–rye addition lines (Bento *et al.* 2008, 2010), and newly formed allotetraploids

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Cucumis × *hytivus* Chen & Kirkbride (Chen *et al.* 2007; Jiang *et al.* 2011) among others.

Tritordeum (× *Tritordeum* Ascherson et Graebner) is the synthetic allopolyploid produced by crossing wild barley (*Hordeum chilense* Roem. et Schult.) and cultivated durum wheat, followed by the chromosome doubling of the sterile F_1 interspecific hybrids with colchicine (Martín and Sánchez-Monge Laguna 1982). This synthetic allopolyploid has been considered as a potential new crop, since it shows good agronomic performance, chromosomal stability, fertility among other interesting traits (Alvarez *et al.* 1992; Martín *et al.* 1999; Atienza *et al.* 2007; Mellado-Ortega and Hornero-Méndez 2012; Navas-Lopez *et al.* 2014). The tritordeum accessions have been characterized by cytogenetic tools and with a small set of DNA markers (Castilho *et al.* 2013). However, the recent development of diversity array technology (DArT) in *H. chilense* will offer new possibilities for screening a large number of tritordeum accessions (Castilho *et al.* 2013).

The screening of tritordeum accessions is needed due to the possible occurrence of rearrangements induced by allopolyploidization. The level and type of DNA sequences involved in genomic restructuring induced by allopolyploidization remains to be determined. However, the use of interretrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP) and intersimple sequence repeat (ISSR) markers have provided valuable information about genetic rearrangements in triticale (Bento *et al.* 2008), wheat–rye addition lines (Bento *et al.* 2010) and hexaploid tritordeum (HT) (Cabo *et al.* 2014b). In our previous work, developed in newly formed HT (Cabo *et al.* 2014b, c), it was not possible to determine if the rearrangements occurred early at the hybrid stage or after the genomes duplication, since no interspecific F_1 hybrids were included.

The main limitation of most of the retrotransposon (RTN)-based markers is the previous need of sequence knowledge for specific primer design (Wegsheider *et al.* 2009). Since the reverse transcription of the RNA intermediate of long terminal repeat (LTR)-RTNs starts at the 5′ end of the internal domain, referred to as the primer-binding site (PBS) (Havecker *et al.* 2004), the use of a single primer based on conserved PBS sequences may allow the production of interprimer-binding site (iPBS) markers (Kalendar *et al.* 2010). The primer anneals to the PBS of two proximal LTR–RTNs with head-to-head orientation, amplifying the two complete LTRs and the genomic DNA region between them (Kalendar *et al.* 2010). The iPBS markers are useful for the detection of polymorphism among individuals and isolation of RTNs (Kalendar *et al.* 2010; Poczai *et al.* 2013).

With the present work, we aim to perform the DNA fingerprinting of two F_1 *H. chilense* × durum wheat hybrids ($H^{ch}AB$; $2n = 3x = 21$), their corresponding tritordeum lines ($H^{ch}H^{ch}AABB$; $2n = 6x = 42$) and parental species using IRAP, REMAP, ISSR and iPBS markers, and to screen the potential molecular rearrangements during the synthesis of HT.

Materials and methods

Plant material

The exact parental lines of the F_1 interspecific hybrids and corresponding tritordeum lines (synthetic allopolyploids) were highly inbred and resulted from several years of self-fertilization. Thus, only one plant per parental line was used. Due to the reduced rates of successful hybridization and survival of the resultant F_1 hybrid plants (even under greenhouse conditions), only one F_1 hybrid plant was produced per interspecific cross and used in this study. The two tritordeum (HT) lines studied here constitute the first generation after polyploidization (S1 individuals), and produced viable seeds. A single HT plant per cross was also analysed. The plant material was obtained from Antonio Martín in Córdoba (Spain) at the IAS-CSIC. The plant material used in this work is presented in table 1.

Young leaves of 6–8 week old plants of the parental species, F_1 hybrids and HT lines were collected, immediately frozen in liquid nitrogen, and maintained at −80°C till the extraction of genomic DNA.

Table 1. Plant materials.

Plant material	Designation	Genomic constitution
H. chilense line H1	Female parent of cross A	$2n = 2x = 14$ ($H^{ch}H^{ch}$)
H. chilense line H75	Female parent of cross B	$2n = 2x = 14$ ($H^{ch}H^{ch}$)
T. turgidum line T846	Male parent of crosses A and B	$2n = 4x = 28$ (AABB)
F_1 hybrid H1 × T846	F_1 hybrid A	$2n = 3x = 21$ ($H^{ch}AB$)
F_1 hybrid H75 × T846	F_1 hybrid B	$2n = 3x = 21$ ($H^{ch}AB$)
Tritordeum H1 × T846	HT line A (synthetic allopolyploid)	$2n = 6x = 42$ ($H^{ch}H^{ch}AABB$)
Tritordeum H75 × T846	HT line B (synthetic allopolyploid)	$2n = 6x = 42$ ($H^{ch}H^{ch}AABB$)

Table 2. Primers for IRAP, REMAP, ISSR and iPBS markers amplification.

Primer	RTN source and orientation	Origin	GenBank accession	Sequence 5′ → 3′	Reference
LTR					
3′LTR	*BARE-1*→	*H. vulgare*	Z17327	TGTTTCCCATGCGACGTTCCCCAACA	Teo *et al.* (2005)
5′LTR2	*BARE-1*←	*H. vulgare*	Z17327	ATCATTGCCTCTAGGGCATAATTC	Teo *et al.* (2005)
LTR 6149	*BARE-1*→	*H. vulgare*	Z17327	CTCGCTCGCCCACTACATCAACCGCGTTTATT	Kalendar *et al.* (1999)
LTR 6150	*BARE-1*←	*H. vulgare*	Z17327	CTGGTTCGGCCCATGTCTATGTATCCACACATGGTA	Kalendar *et al.* (1999)
LTR 7286	*BARE-1*←	*H. vulgare*	Z17327	GGAATTCATAGCATGGATAATAAACGATTATC	Kalendar *et al.* (1999)
Nikita	*Nikita* →	*T. turgidum*	AY078073 AY078074 AY078075	CGCATTTGTTCAAGCCTAAACC	Teo *et al.* (2005)
Sukkula	*Sukkula* →	*H. vulgare*	AY034376	GATAGGGTCGCATCTTGGGCGTGAC	Teo *et al.* (2005)
Stowaway	*Stowaway* →	*H. vulgare*		CTTATATTTAGGAACGGAGGGAGT	Bento *et al.* (2008)
SSR					
8081				$(GA)_9C$	Kalendar *et al.* (1999)
8082				$(CT)_9G$	Kalendar *et al.* (1999)
8564				$(CAC)_7T$	Kalendar *et al.* (1999)
PBS					
F0100	F0100 →	Universal RTN-based sequence		TAGGTCGGAACAGGCTCTGATACCA	Kalendar *et al.* (2008) Wegscheider *et al.* (2009)

Table 3. Combinations of primers tested for the amplification of IRAP, REMAP, ISSR and iPBS markers.

Marker	Combination of primers
IRAP	*Sukkula*
	Nikita + *Sukkula*
	5′LTR2-*BARE-1* + *Sukkula*
	LTR 6149 + *Sukkula*
	LTR 6150 + *Sukkula*
	3′LTR-*BARE-1*
	Stowaway
REMAP	*Sukkula* + 3′LTR-*BARE-1*
	Sukkula + *Stowaway*
	Sukkula + 8081
	Sukkula + 8082
	Sukkula + 8564
	Nikita + 8081
	Nikita + 8082
	LTR 7286 + 8081
	LTR 7286 + 8082
	LTR 7286 + 8564
	3′LTR-*BARE-1* + 8081
	3′LTR-*BARE-1* + 8654
	F0100 + 8081
	Stowaway + 8081
ISSR	SSR 8081 [$(GA)_9C$]
	SSR 8082 [$(CT)_9G$]
	SSR 8564 [$(CAC)_7T$]
iPBS	F0100

Fluorescence in situ hybridization (FISH) experiments

Prior to the molecular analyses, the success of the interspecific crosses was confirmed by the identification of the parental genomes using the FISH technique. Thus, the fixed root-tips from germinated seeds of hexaploid tritordeum (HT lines A and B) were used for the preparation of mitotic chromosome spreads according to Lima-Brito *et al.* (1997). Then, the chromosome spreads were hybridized with genomic DNA of *H. chilense* labelled with biotin-16-dUTP (Roche Applied Science, Mannheim, Germany) and 45S rDNA sequence, pTa71 (Gerlach and Bedbrook 1979) labelled with digoxigenin-11-dUTP (Roche Applied Science). Both probes were labelled by nick translation. The FISH experiments were developed according to Schwarzacher and Heslop-Harrison (2000).

Isolation of genomic DNA and amplification of molecular markers

The genomic DNA was extracted from the frozen leaves using CTAB method of Doyle and Doyle (1987). The DNA samples were quantified in the spectrophotometer Nanodrop^{TM} ND-1000 (Thermo Scientific) and their integrity was evaluated after electrophoresis on 0.8% agarose gels stained with ethidium bromide. The DNA samples were diluted to working solutions with a

concentration of 40 ng/μL for further amplification of molecular markers.

Different primers (table 2) were tested in this study for the amplification of IRAP, REMAP, ISSR and iPBS markers using a total of 25 combinations (table 3) which were previously successful in some Triticeae species, including those studied here.

The amplification and visualization of IRAP, REMAP and ISSR markers were performed according to the conditions described by Cabo *et al.* (2014b). The same conditions and procedures were followed for the production of iPBS markers with the single primer F0100.

To assess if the rearranged bands detected in the F_1 hybrids and corresponding tritordeums are an effective result of polyploidization and to discard the hypothesis of being generated by competition between primer hybridization sites of one parent or the other, control PCR experiments containing the mixture of the DNAs of the parental lines were performed.

Each band was considered as an IRAP, REMAP, ISSR or iPBS marker. Although there is the potential for the occurrence of homoplasy among different genera for the four dominant marker systems, we assumed that similar-sized bands among the studied individuals produced with the same combination of primers, would correspond to the same locus.

Each PCR reaction was repeated at least twice and only reproducible bands were considered for the presence (1) / absence (0) analysis.

Since the amplification conditions were the same for the four-marker systems in each REMAP matrix, bands with similar molecular weight to ISSRs and/or IRAPs produced with the same simple sequence repeat or LTR primers, respectively, were discarded to ensure the analysis of effective REMAPs as suggested by Kalendar *et al.* (1999).

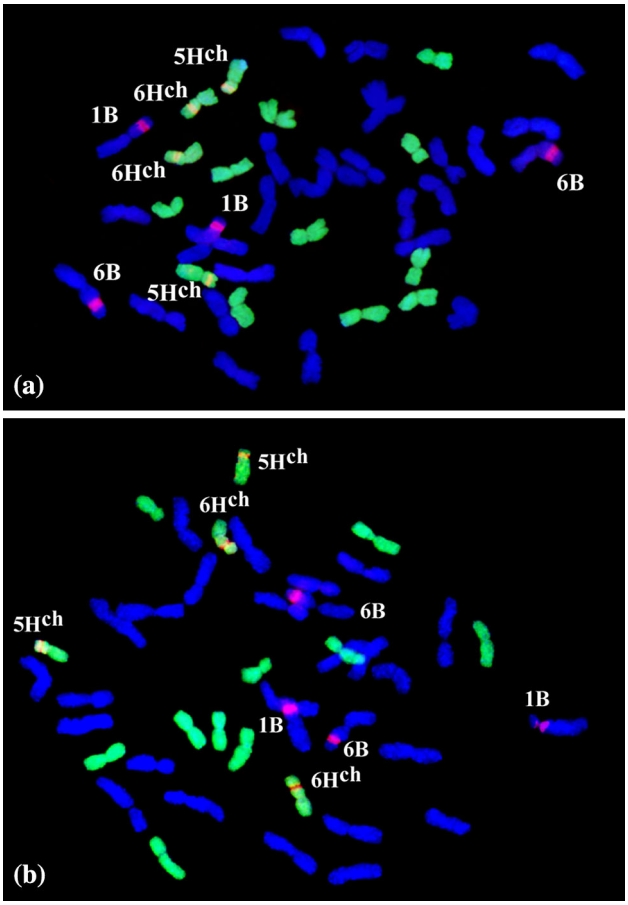

Figure 1. Root-tip metaphases of tritordeum ($H^{ch}H^{ch}AABB$; $2n = 42$), (a) HT line A and (b) HT line B, after FISH experiments probed with genomic DNA of *H. chilense* and pTa71. The 28 wheat-origin chromosomes were counter-stained with DAPI (blue). Genomic DNA of *H. chilense* probe was labelled uniformly the 14 *H. chilense*-origin chromosomes (green). All nucleolar chromosomes of wheat origin (pairs 1B and 6B) and *H. chilense* origin (pairs $5H^{ch}$ and $6H^{ch}$) were identified by the rDNA probe pTa71 (red).

Results

Figure 1 shows root-tip metaphase cells of tritordeum (HT lines A and B) with chromosome constitution $H^{ch}H^{ch}AA$ BB ($2n = 6x = 42$). The 14 chromosomes originating from *H. chilense* were strongly hybridized with the *H. chilense* genomic DNA probe (green). The 28 wheat-origin chromosomes were also observed (blue). The pTa71 probe identified eight major 18S-25S rDNA sites (red) per metaphase cell of tritordeum, four being of wheat origin (chromosome pairs 1B and 6B) and four of *H. chilense*-origin (chromosome pairs $5H^{ch}$ and $6H^{ch}$) (figure 1).

Among 25 combinations of primers tested, 14 were selected based on the successful amplification and/or production of clear bands (table 4).

The LTR primers *Sukkula* and 3′LTR-*BARE1* produced IRAPs when used alone (table 4). Also, the single PBS primer F0100 generated iPBS markers (table 4).

The highest total percentages of REMAP and IRAP polymorphism were observed among the parental lines and descendants of cross B (table 4). The highest percentage values of ISSR and iPBS polymorphism were detected in cross A (table 4).

The different cases of polymorphic bands, such as those shared among each parent, the F_1 hybrid and/or respective tritordeum (HT) as well as the rearranged bands (novel and lost parental bands) are indicated in table 5. Considering the two crosses and all marker systems, the fingerprinting analyses revealed that the bands of wheat origin inherited by the descendants (F_1 hybrids and HTs) of each cross (61) were almost the double of those of *H. chilense*-origin (32). Regarding the IRAP markers of cross A, the number of bands inherited by both the F_1 hybrid H1 × T846 and its respective HT with origin in the female parent (19) was slightly higher than that of wheat origin (17) (table 5). Most

Table 4. REMAP, IRAP, ISSR and iPBS data achieved per combination of primers in each cross.

Marker	Cross	Primer	T	M	P	%P
REMAP	A (H1 × T846)	*Sukkula*+8081	6	1	5	83.33
		Sukkula+8082	4	0	4	100
		Sukkula+8564	4	2	2	50.0
		3′LTR+8081	5	1	4	80.00
		3′LTR+8564	3	2	1	33.33
		Nikita+8081	6	3	3	50.00
		Total	28	9	19	67.86
	B (H75×T846)	*Sukkula*+8081	3	0	3	100
		Sukkula+8082	7	0	7	100
		Sukkula+8564	10	0	10	100
		3′ LTR+8081	6	2	4	66.67
		3′ LTR+8564	6	0	6	100
		Nikita+8081	10	6	4	40.00
		Total	42	8	34	80.95
IRAP	A (H1 × T846)	*Sukkula*	21	9	12	57.14
		3′LTR-*BARE1*	10	9	1	10.00
		Sukkula+*Nikita*	6	1	5	83.33
		Sukkula+3′LTR	8	5	3	37.50
		Total	45	24	21	46.67
	B (H75 × T846)	*Sukkula*	13	1	12	92.30
		3′LTR-*BARE1*	7	5	2	28.57
		Sukkula+*Nikita*	10	1	9	90.00
		Sukkula+3′LTR	6	2	4	66.67
		Total	36	9	27	75.00
ISSR	A (H1 × T846)	8081	8	2	6	75.00
		8082	10	3	7	70.00
		8564	6	4	2	33.33
		Total	26	9	17	65.38
	B (H75 × T846)	8081	7	1	6	85.71
		8082	6	4	2	33.33
		8564	9	6	3	33.33
		Total	22	11	11	50.00
iPBS	A (H1 × T846)	F0100	6	3	3	50.00
	B (H75×T846)	F0100	12	9	3	25.00

T, total number of amplified bands; M, number of monomorphic bands; P, number of polymorphic bands; %P, percentage of polymorphism (calculated by P/T × 100).

of the markers were inherited by both the F_1 hybrid and its corresponding HT, but six *H. chilense*-origin markers and five wheat-origin bands were only transmitted to the F_1 hybrids being absent in tritordeum (table 5).

Considering the lost parental bands (transmitted neither to the F_1 hybrid nor to the amphiploid tritordeum), the number of bands with *H. chilense* origin (16) was similar to those of wheat origin (13) (table 5). Such feature was verified with the four-marker systems (table 5). Two novel bands (absent in the parental species) were detected (table 5; figure 2). One novel REMAP band (~900 bp) was amplified in the F_1 hybrid B and respective tritordeum, and one novel IRAP (~400 bp) was exclusively amplified in the F_1 hybrid B (table 5; figures 2 and 3).

Discussion

FISH technique confirmed the success of interspecific crosses and genome duplication, since it was possible to differentiate the parental genomes and to localize physically the rDNA loci on mitotic chromosome spreads of both tritordeum lines (HT lines A and B). The same results were previously obtained in other lines of hexaploid tritordeum by Lima-Brito *et al.* (1998) and Cabo *et al.* (2014b, c).

An intraspecific analysis of the IRAP molecular patterns achieved among three individual plants of each parental line, *H. chilense* (lines H1 and H75) and durum wheat line T846 was performed using the LTR primer *Sukkula* (figure 4). All plants of the same parental line presented identical IRAP patterns confirming that they were highly inbred, and justifying the use of a single plant per parental line in the following molecular analyses. Further, the IRAP, REMAP and ISSR profiles obtained for the mixture of the parental genomes, and their comparison with those produced individually in wheat, *H. chilense*, F_1 hybrid and corresponding tritordeum, evidenced that the rearranged bands (novel and/or lost parental bands) resulted from hybridization

Table 5. Polymorphic REMAP, IRAP, ISSR and iPBS markers among each parent (H^{ch}, line H1 or H75; wheat, line T846), the F_1 hybrid (F_1) and/or respective tritordeum (HT) and rearranged bands (novel and lost parental bands) detected per cross and combination of primers.

			Number of polymorphic bands										
			Common to H^{ch}			Common to wheat			Rearranged bands: Novel bands		Rearranged bands: Lost parental bands		
Marker	Cross	Primers	F_1	HT	F_1 and HT	F_1	HT	F_1 and HT	F_1	F_1 and HT	H^{ch}	Wheat	H^{ch} and wheat
REMAP	A (H1 × T846)	*Sukkula*+8081	0	0	2	0	0	3	0	0	0	0	0
		Sukkula+8082	0	0	1	0	0	2	0	0	1	0	0
		Sukkula+8564	0	0	0	0	0	1	0	0	0	1	0
		3′LTR+8081	1	0	1	0	0	1	0	0	0	1	0
		3′LTR+8564	0	0	0	0	0	0	0	0	0	1	0
		Nikita+8081	0	0	1	0	0	1	0	0	0	1	0
		Total	1	0	5	0	0	8	0	0	1	4	0
	B (H75 × T846)	*Sukkula*+8081	1	0	0	0	0	0	0	1 (900 bp)	1	0	0
		Sukkula+8082	1	0	0	1	0	3	0	0	1	1	0
		Sukkula+8564	0	0	2	0	0	2	0	0	3	2	1
		3′LTR+8081	0	0	0	0	0	1	0	0	1	1	1
		3′LTR+8564	0	0	1	0	0	2	0	0	1	2	0
		Nikita+8081	0	0	0	0	0	2	0	0	1	1	0
		Total	2	0	3	1	0	10	0	1	8	7	2
IRAP	A (H1 × T846)	*Sukkula*	0	0	9	0	0	3	0	0	0	0	0
		3′LTR-*BARE1*	0	0	1	0	0	0	0	0	0	0	0
		Sukkula+*Nikita*	0	0	1	0	0	3	0	0	1	0	0
		Sukkula+3′LTR	0	0	0	0	0	1	0	0	2	0	0
		Total	0	0	11	0	0	7	0	0	3	0	0
	B (H75 × T846)	*Sukkula*	2	0	1	4	0	3	1 (400 bp)	0	0	1	0
		3′LTR-*BARE1*	0	0	0	0	0	2	0	0	0	0	0
		Sukkula+*Nikita*	1	0	5	0	0	3	0	0	0	0	0
		Sukkula+3′LTR	0	0	2	0	0	2	0	0	0	0	0
		Total	3	0	8	4	0	10	1	0	0	1	0
ISSR	A (H1 × T846)	8081	0	0	2	0	0	4	0	0	2	0	0
		8082	0	0	1	0	1	5	0	0	1	0	0
		8564	0	0	0	0	0	2	0	0	0	0	0
		Total	0	0	3	0	1	11	0	0	3	0	0
	B (H75 × T846)	8081	0	0	2	0	0	3	0	0	1	0	0
		8082	0	0	0	0	0	4	0	0	0	0	0
		8564	0	0	0	0	0	3	0	0	0	0	0
		Total	0	0	2	0	0	10	0	0	1	0	0
iPBS	A (H1 × T846)	F0100	0	0	0	0	0	3	0	0	0	0	0
	B (H75 × T846)	F0100	0	0	0	0	0	2	0	0	0	1	0

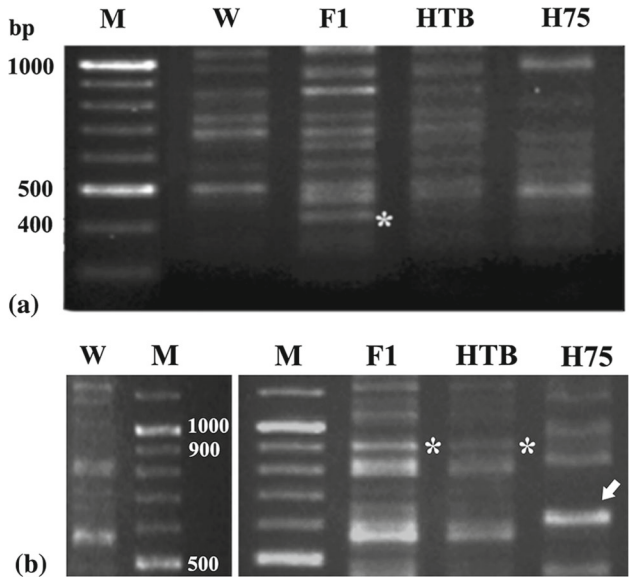

Figure 2. Detailed sections of (a) IRAP and (b) REMAP profiles produced by the primer *Sukkula* and primers *Sukkula* + 8081, respectively, in the F_1 hybrid B, respective tritordeum HT line B (HTB) and the parents, *H. chilense* (H75) and durum wheat line T846 (W). One novel IRAP with ~400 bp (*) was uniquely detected in the F_1 hybrid B. One novel REMAP with ~900 bp (*) was amplified in both the F_1 hybrid B and corresponding tritordeum (HTB). The arrow indicates a specific band of line H75 that was not transmitted to the descendants of the cross B (lost parental band). M, GeneRuler DNA Ladder Mix (Thermo Scientific).

and/or polyploidization (figure 3). Thus, any detectable polymorphism in the F_1 hybrids or tritordeums was related to genomic rearrangements induced by hybridization and/or polyploidization.

Several investigations have been focussed on genomic changes induced by allopolyploidization, but the involved mechanisms can remain elusive when the exact parental species of the allopolyploids are unknown (Wendel 2000). Here and in our previous studies (Cabo *et al.* 2014b, c), the exact parental species of the newly formed hexaploid tritordeums or F_1 hybrids were used for comparison.

The present study revealed: (i) high percentage values of REMAP and IRAP polymorphisms among the F_1 hybrids, corresponding tritordeums and their parental species; (ii) a higher number of markers with wheat origin inherited by the F_1 hybrids and tritordeums; (iii) the number of lost parental bands (not transmitted to the descendants of each cross) with *H. chilense* origin was similar to that of wheat origin; and (iv) the amplification of two novel bands (tables 4 and 5). The IRAP data obtained in cross A (table 5) constitute an exception to the case (ii) because of the higher number of IRAPs inherited by both the F_1 hybrid A, and HT line A had origin in the female parent, *H. chilense*. Such molecular results could be explained by the use of LTR primers that were designed for the RTN family *BARE-1* of *H. vulgare* L. (Kalendar *et al.* 1999) and by the preferential elimination of SSR sequences of *H. chilense* origin for the homogenization of the divergent parental genomes. In other tritordeum lines, the number of IRAPs of *H. chilense* origin was almost double than the wheat origin (Cabo *et al.* 2014b) and these molecular results were supported by cytogenetic analyses that showed the inheritance of SSR-rich regions with wheatorigin by tritordeum (Cabo *et al.* 2014a).

The loss of parental bands as well as the appearance of novel bands in the F_1 hybrids and/or corresponding tritordeum lines can be assumed as evidence of genomic restructuring (table 5).

As far as we know, this study constitutes for the first time the use of iPBS markers for detecting genomic restructuring in F_1 interspecific hybrids and/or allopolyploids. Such markers enabled the detection of one lost parental band (table 5), and are not only useful for the study of interspecific hybrids and allopolyploids of Triticeae tribe, but also in other plant species regarding the use of a conserved PBS sequence as primer. According to Kalendar *et al.* (2010), the iPBS markers are applicable to any organism with RTNs containing PBS sites complementary to tRNA.

In this study, the loss of parental bands in both the F_1 hybrids and tritordeum lines was more frequent than the appearance of novel bands (table 5). Only one novel IRAP band in the F_1 hybrid B (F_1 H75 × T846 hybrid), and one novel REMAP band in both F_1 hybrid B and tritordeum line B were detected (table 5). The novel REMAP band was detected using primers *Sukkula* + 8081, which demonstrated that some rearrangements occurred during or after hybridization in the F_1 hybrid B, being maintained in the HT line B. On the other hand, the novel IRAP band was not amplified in tritordeum, but being exclusively amplified in the F_1 hybrid B (table 5; figures 2 and 3).

The number of rDNA loci observed in hexaploid tritordeum (eight) detected by the probe pTa71 constitutes an additive pattern since it results from the inheritance of four rDNA loci of wheat origin and four rDNA loci of *H. chilense* origin (see figure 1). The detection of lost parental bands and novel bands in the F_1 hybrids and/or corresponding amphiploids (table 5) constitute exceptions to the expected additive pattern of the parental REMAP, IRAP, ISSR (figure 3) and iPBS markers.

The occurrence of genomic restructuring was previously detected in other newly formed tritordeum lines not only by using REMAP, IRAP and ISSR (Cabo *et al.* 2014b), but also the start codon targeted (SCoT) markers (Cabo *et al.* 2014c). Novel and lost parental bands were also revealed by these markers in other allopolyploids of the Triticeae tribe (Bento *et al.* 2008, 2010). The rearranged bands detected until now in both triticale and tritordeum have been considered as being induced by polyploidization (Bento *et al.* 2008, 2010; Cabo *et al.* 2014b, c). Here, due to the inclusion of the F_1 hybrids, we were able to

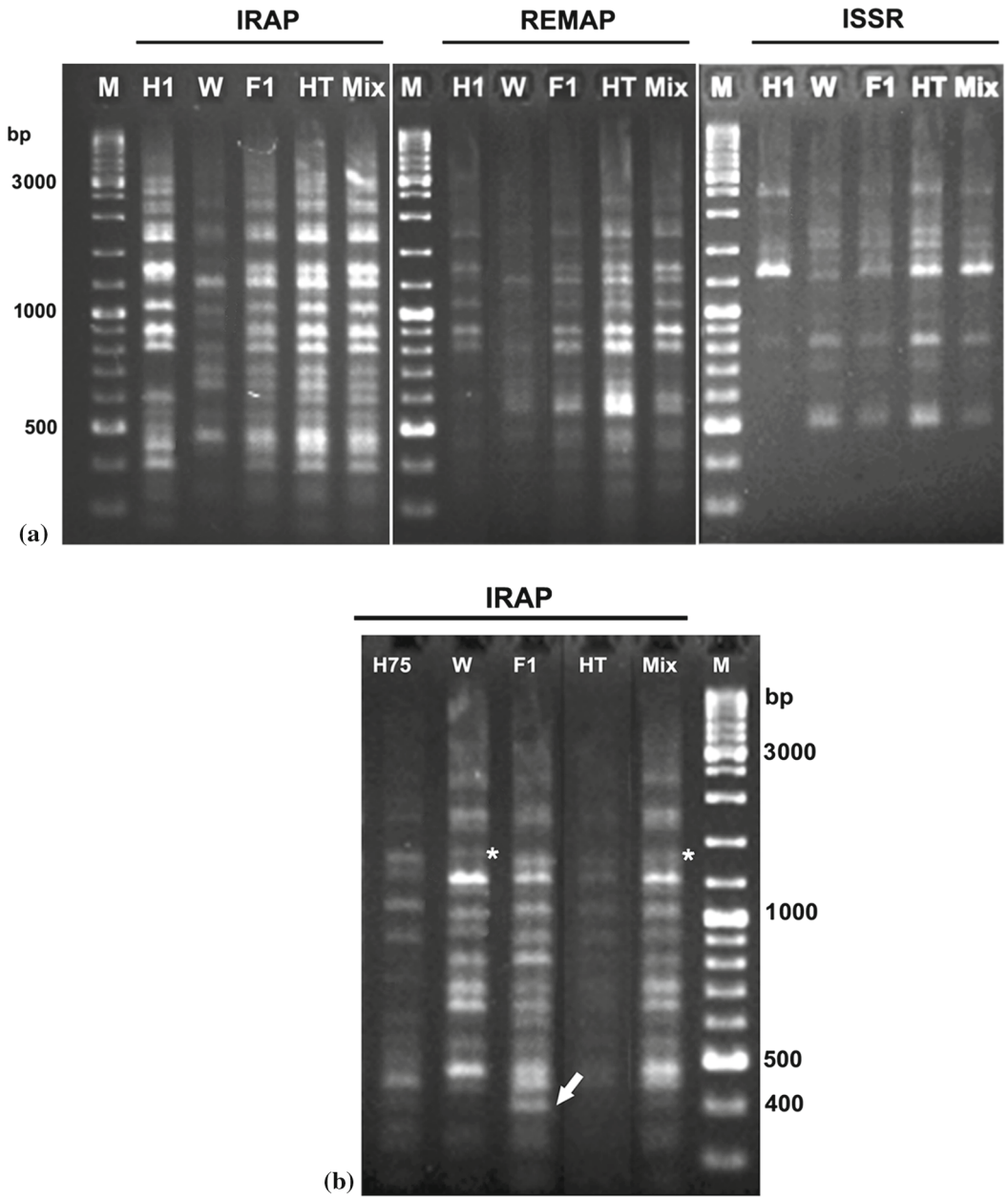

Figure 3. (a) IRAP, REMAP and ISSR patterns produced with the primers *Sukkula*, *Sukkula* + 8081 and SSR 8081, respectively, in the parental lines, F_1 hybrid and tritordeum of cross A. The control (Mix) is composed by the mixture of DNAs of both parents *H. chilense* (line H1) and wheat line T846. (b) IRAP patterns produced with primer *Sukkula* in the parental lines, F_1 hybrid and tritordeum of cross B. The control (Mix) is composed by the mixture of DNAs of both parents *H. chilense* (line H75) and wheat line T846. Rearranged bands, such as one lost parental band (*) and one novel IRAP band with 400 bp (arrow) are indicated.

verify the early occurrence of genomic restructuring at the hybrid stage, suggesting that the observed molecular rearrangements were induced at the hybridization step. Additionally, such rearrangements seem to involve RTN and/or SSR regions, once they were detected with IRAP, REMAP, ISSR and iPBS markers. The early occurrence of genomic rearrangements (in the F_1 generation) was previously reported in plant species of the genera *Brassica* (Song *et al.* 1995), *Cucumis* (Chen *et al.* 2007; Jiang *et al.* 2011), *Nicotiana* (Petit *et al.* 2010) and *Arabidopsis* (Bento *et al.* 2013).

The different results obtained when compared to our previous studies can be explained by the use of distinct lines of tritordeum, durum wheat and *H. chilense*, and by the high genetic variability among lines of *H. chilense* (Prieto *et al.* 2004; Marín *et al.* 2008). These assumptions could also explain why some combinations of primers that were previously successful did not amplify IRAP

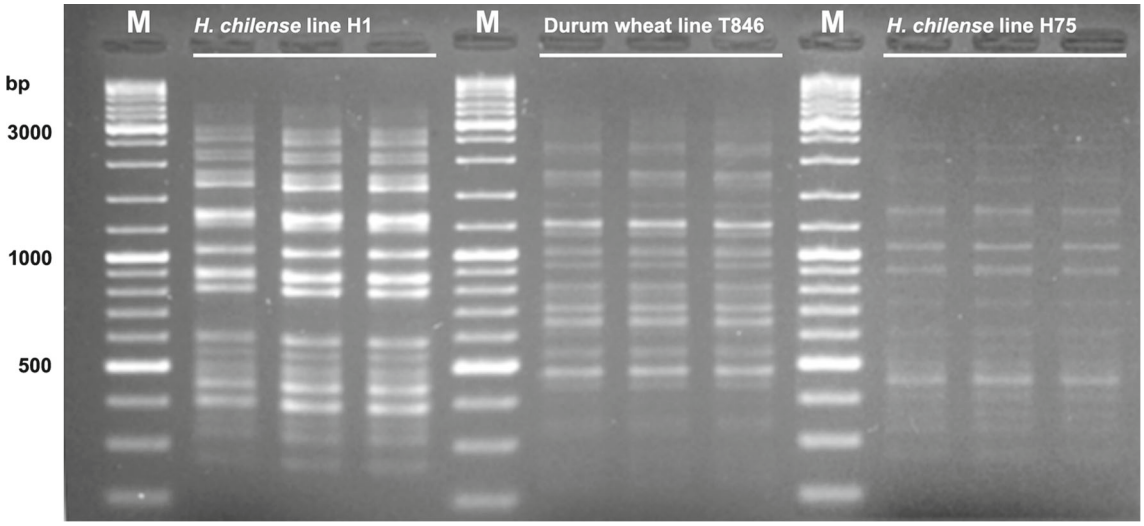

Figure 4. IRAP molecular patterns produced with the LTR primer *Sukkula* in three individual plants of each parental line: *H. chilense* line H1, durum wheat line T846 and *H. chilense* line H75.

and REMAP markers in the present work. Nonetheless, two interesting features have been reiterated through our studies of newly formed tritordeum, namely the nonrandom elimination of sequences of *H. chilense* origin, and the preferential inheritance of markers with wheat origin, independently of the tritordeum line studied. A parallelism could be made with studies developed in triticale and wheat–rye addition lines (Bento *et al.* 2008, 2010), where the nonrandom elimination of bands of rye origin genome was reported. These authors studied genomic rearrangements in the seven wheat–rye addition lines and triticale, and explained that the degree of genomic reshuffling increases with the reduction of alien (rye) chromatin introgressed into the wheat background (Bento *et al.* 2008, 2010). The rearranged RTN and SSR sequences seem to be preferentially allocated in major heterochromatic domains, and affect mainly the genome with more reduced nuclear DNA content, as the rye genome does in triticale (Bento *et al.* 2008). Similarly, the genome with reduced nuclear DNA content in hexaploid tritordeum is *H. chilense*, justifying the preferential elimination of its markers in both the F_1 hybrids and corresponding tritordeum lines.

Overall, our molecular data demonstrated that the rapid and nonrandom elimination of repetitive DNA sequences probably occurs early at the F_1 hybrid stage. The nonrandom elimination of sequences might constitute a way to homogenize the divergent genomes of new allopolyploids, improving their diploid-like behaviour, ensuring their fertility and success as a new species in nature (Feldman *et al.* 1997; Comai 2000). Other authors proposed that in the first generation of a new allopolyploid, the elimination events are more frequent than the genetic gain (Song *et al.* 1995; Ozkan *et al.* 2001; Shaked *et al.* 2001; Kashkush *et al.* 2002; Chen *et al.* 2007; Bento *et al.* 2008, 2010). Although, the present results agreed with this assumption, two novel bands were detected once; but this should not be considered as a rule. Different studies have demonstrated that genomic restructuring mostly involves sequences associated with RTN-rich and SSR-rich regions. These genomic regions do not obey a predictive mechanism of elimination or genetic gain throughout allopolyploidization. In fact, novel SSR regions may arise in response to allopolyploidization (Cabo *et al.* 2014a). We could not discard the hypothesis that the amount of genetic gain could be undetectable with the approaches used, and it is probably easier to detect sequences elimination. In addition, the procedures of homogenization are highly dependent on the level of divergence between or among the genomes merging in the nascent allopolyploid, and on the genotype of the parental species. Further studies on this and other synthetic allopolyploids should be performed through successive generations after polyploidization, based on these or other approaches before establishing a trend.

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