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Identification, mapping and evolutionary course of wheat *lipoxygenase-1* genes located on the A genome

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ABSTRACT

Durum wheat (*Triticum turgidum*) is the cereal of preference for semolina and pasta production. Bright yellow color, which is desirable for pasta making, depends on the amount of carotenoid pigments present in the grain. Lipoxygenases (LPXs) account for most of the carotenoid degradation activity. Although B genome *Lpx* genes have been extensively described, little information about the A genome has been reported. Here, we demonstrate that the *Lpx-A1* locus is represented by a single gene in the diploid *Triticum urartu*, the tetraploid *T. turgidum* and the hexaploid *Triticum aestivum* wheats in contrast to the multiple copies reported in the B genome. The *Lpx-A1_like* pseudogene previously identified in *T. turgidum* genome A was also identified in the *T. aestivum* cv Chinese Spring wheat, whereas *T. urartu* possesses a complete copy, suggesting that pseudogenization occurred after the formation of the tetraploid and then passes to the hexaploid wheat. The nucleotide sequence of *T. urartu Lpx* is more closely related to genome B *Lpx-1* than to *Lpx* genes of genome A from *T. turgidum* and *T. aestivum*, probably due to the deletions and insertions that occur. Thus, the present paper extends our knowledge of lipoxygenase gene organization and evolution in the wheat A genome.

1. Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum*, genomes AABB) is the preferred grain for semolina and pasta production. The key quality factors for pasta production include high grain protein content, strong gluten and bright yellow color. The latter parameter is mainly determined by the concentration of carotenoid pigments in the grain. Carotenoid pigments are not only important to satisfy consumers' preferences but also to improve the nutritional value of pasta since they act as antioxidant compounds, reducing oxidative damage to biological membranes by scavenging peroxide radicals (reviewed in Troccoli et al., 2000). However, a high initial carotenoid level in semolina does not guarantee a high color score in the final product, since these pigments can be lost during milling or degraded by enzymatic activity during pasta processing (Borrelli et al., 1999). The main enzymes involved in the oxidative degradation of carotenoid pigments are the lipoxygenases (LPXs) (reviewed in Troccoli et al., 2000); thus, wheat genotypes with low LPX activity are preferred (Carrera et al., 2007; Zhang et al., 2008; Verlotta et al., 2010). The bleaching of dough and pasta products results from coupled oxidation of pigments due to free radical species generated by fatty acid oxidation (reviewed in Troccoli et al., 2000).

LPX1 isozymes were first identified by Hart and Langston (1977) at locus *Lpx-1* of homeologous chromosome group 4 (*Lpx-A1*, *Lpx-B1* and *Lpx-D1*, where A, B and D refer to the wheat genome and 1 to the isoform), using Chinese Spring nulli-tetrasomic lines (AABBDD). *Lpx* loci were further mapped to the same homeologous chromosomal group using a RIL population, but no information about isoforms was provided (Li et al., 1999). The number and location of *Lpx-1* genes in durum wheat (*Triticum turgidum* L. ssp. *durum)* is not completely known. The *Lpx-1* locus was mapped to chromosome 4B (Nachit et al., 2001; Hessler et al., 2002; Zhang et al., 2008; Carrera et al., 2007). Three copies of the gene, *Lpx-B1.1,* Lpx-*B1.2 and Lpx-B1.3,* were reported (Carrera et al., 2007; Verlotta et al., 2010), and deletion of *Lpx-B1.1* was found to be associated with reduction in LPX activity (Carrera et al., 2007). Furthermore, three alleles were identified for *Lpx-B1.1*, *Lpx-B1.1a*, *Lpx-B1.1b* and *Lpx-B1.1c*, (Verlotta et al., 2010). By contrast, less information is available about genome A *Lpx* genes. A partial copy of *Lpx*-1 on chromosome 4A

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(*Lpx-A1_like*) was identified using a tetraploid durum wheat BAC library constructed from the var. Langdon (Cenci et al., 2003) with barley (*Hordeum vulgare*)-based probes (Garbus et al., 2009). Another lipoxygenase locus, *Lpx-A3* associated with better semolina and pasta color (Carrera et al., 2007), was mapped on chromosome 4A (Carrera et al., 2007; Zhang et al., 2008) and co-localizes with *Lpx-A1_like* within a 42 kbp region (Garbus et al., 2009). The linkage among *Lpx* loci in durum wheat cannot be established since *Lpx-1* and *Lpx-3* are not mapped to the same chromosome in any of the currently available wheat maps. In barley, they were mapped 1 cM apart on the short arm of chromosome 4H (van Mechelen et al., 1999). Knowledge of the physical location of these *Lpx* genes is important to understand the evolution of the family but also has practical implications for breeding since closely linked genes are difficult to separate by recombination. Each *Lpx* gene has a distinct role in wheat development, but they likely all contribute to the total LPX activity in seeds (Feng et al., 2012).

Here, we examined lipoxygenase gene organization and evolution in the A genome from the diploid *Triticum urartu,* the tetraploid *T. turgidum* and the hexaploid *Triticum aestivum*, finding that *Lpx-A1_like* pseudogenization occurred at the tetraploid level and was fixed in modern hexaploid wheat. Additionally, *Lpx-A1_like* and *Lpx-3* were mapped together on chromosome 4A, 3.57 cM apart.

2. Experimental

2.1. Plant materials

The tetraploid wheat (*Triticum turgidum* L. ssp. *durum*) used in this study included the variety Kofa, the breeding line UC1113 and a single-seed-descent mapping population consisting of 93 F_9 recombinant inbred lines (RILs) derived from a cross between Kofa and UC1113. Kofa is a desert durum variety with excellent pasta quality and optimal semolina and pasta color developed by the company West-Bred. UC1113 is a breeding line from the University of California, Davis (UCD) with excellent agronomic performance but intermediate pasta color (Zhang et al., 2008). Other analyses were performed with the diploid *T. urartu*. Plants were grown in the greenhouse at 25 °C under a 16 h/8 h (light/dark) cycle.

2.2. Molecular methods

Nuclear DNA was extracted from leaves using a CTAB protocol as in Garbus et al. (2009), quantified in a spectrophotometer (Merck), and diluted as appropriate for PCR. The primers LpxLike1R (GTACGGGTAATCCGACACCA), LpxLike2L (TCCGAGTTCCTGCTCAAGAC) and LpxLike2R (CATGCACGTTCCAATCGTAT), previously designed to amplify *Lpx* sequences from genome A (Garbus et al., 2009), were used in different combinations. Primers LOXA F (CTGATCGACGTCAACAAC) and LOXA R (CAGGTACTCGCTCACGTA), together called LOXAF/R, specifically amplify *Lpx-1* over the *Lpx-2* and *Lpx-3* (Carrera et al., 2007). Primers LOXANI1 (TGCTGGAGCAGATCTACGTG) and LOXANI2 (TCGTGGCGTAGATGAAGTTG), together called LOXANI1/2, were designed based on the barley *Lox A* cDNA sequence to amplify a fragment of the *Lpx-B1* locus that includes the last two-thirds of exon 3 through the first third of exon 5, located upstream of the fragment amplified by LOXAF/R.

Amplification reactions were performed in a BIORAD thermal cycler in a 25 μL reaction mixture. Each reaction consisted of 200 mM dNTPs, 1.5 mM $MgCl_2$, 100 nM each primer, 1 U Taq polymerase (Promega) and 50 ng DNA. PCR amplification conditions for *Lpx-A1_like* were: 3 min at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at 56 °C, and 1 min at 72 °C followed by a final extension step of 10 min at 72 °C. For the primer pairs LOXAF/R and LOXANI1/2, PCR amplification conditions were as follows: 3 min at 94 °C; five touchdown cycles (1 °C each step) of 45 s at 94 °C, 45 s at 60–55 °C and 1 min at 72 °C; and after that, 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C followed by a final extension step of 10 min at 72 °C. For sequencing purposes, PCR products were cloned using the pGEM®-T Easy Vector System (Promega) according to the manufacturer's protocols. Competent *E. coli* cells (strain DH5α) were transformed with the recombinant vector and plated onto LB-agar ampicillin XGal − IPTG plates. White colonies were picked and plasmids plus insert were amplified and purified. The presence of the PCR fragment was checked based on restriction profile with the enzyme EcoRI. Three clones per PCR reaction were sequenced in SIGYSA (INTA Castelar, Argentina). The interface VecScreen was used in order to eliminate vector contamination from the obtained sequences (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Consensus sequences were constructed from the three replicates using the software BioEdit 7.0 Sequence Alignment Editor (Hall, 1999). Homology searches were performed using BLAST (http://www. ncbi.nlm.nih.gov/).

2.3. Genetic mapping

Genetic distances between *Lpx* loci and flanking microsatellite markers were estimated with the program Map-Maker (Lander et al., 1987) using the Kosambi function (Kosambi, 1944). Mapping was performed using the linkage map of durum wheat reported by Zhang et al. (2008). The total length of the map was 2140 cM (giving a mean chromosome length of 153 cM), based on 269 markers including molecular (230 SSR, 23 SNP, 10 RFLP and 3 STS), morphological and protein markers.

2.4. Phylogenetic tree construction

A phylogenetic tree was constructed from *Lpx* nucleotide sequences of diploid *T. urartu*, tetraploid *T. turgidum* and hexaploid *T. aestivum* either identified in this work or obtained from public databases (http://blast.ncbi.nlm.nih.gov). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Aligned sequences were used to generate trees using the Neighbor-Joining method (Saitou and Nei, 1987) and were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site. All positions containing gaps or missing data were eliminated from the dataset (Complete deletion option).

3. Results

3.1. Identification and mapping of Lpx-A1 sequences in durum wheat genotypes

The reported durum wheat genome B *Lpx1.1b* gene (genomic sequence, HM126468; cDNA, HM126473; Verlotta et al., 2010) is composed of seven exons (E1-E7) and six introns (I1–I6). Comparison of the *Lpx-A1_like* pseudogene (FJ518909; Garbus et al., 2009) to these sequences showed that it contains the second half of E2 through the first quarter of I2, followed by a 175 bp region with no similarity to *Lpx* sequences and a 393 bp region including the entire E6 and ~16% of E7 (Fig. 1a). This last segment lacks I6, which is present in orthologous regions of the *Lpx-B1* genes (HM126468, HM126473, DQ474240 and DQ474241). *Lpx1.1b* and *Lpx-A1_like* display 89% identity at the nucleotide level. The region comprising bases 431 to 504 shares 83% identity with a portion of a TIR Mariner DNA transposon of the DTT Icarus Stowaway MITE family. We found no evidence for expression of the *Lpx-A1_like*

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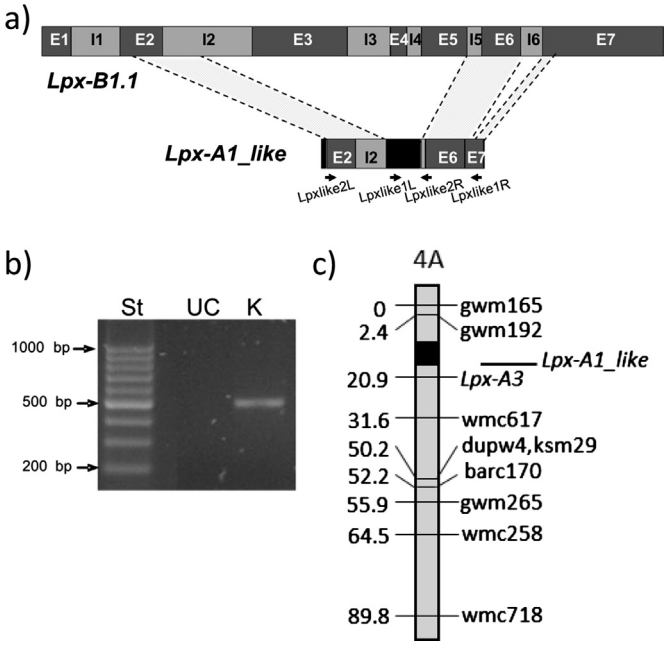

Fig. 1. *Lpx-A1_like* pseudogene analysis and mapping. a) Schematic diagram of the genomic loci of *Lpx-B1.1* (HM126468) and *Lpx-A1_like* (FJ518909) illustrating that *Lpx-A1_like* corresponds to a *Lpx* pseudogene, with several deleted regions and a TE insertion. Arrows indicate positions and orientations of the *Lpx-A1_like*-based primers. Shaded bars indicate the conserved regions between both sequences; b) Polymorphic amplification by the primer pair Lpxlike1R/1L. St: molecular weight marker; UC: UC1113 breeding line, K: Kofa variety; c) Mapping of the amplified fragment to the short arm of chromosome 4A.

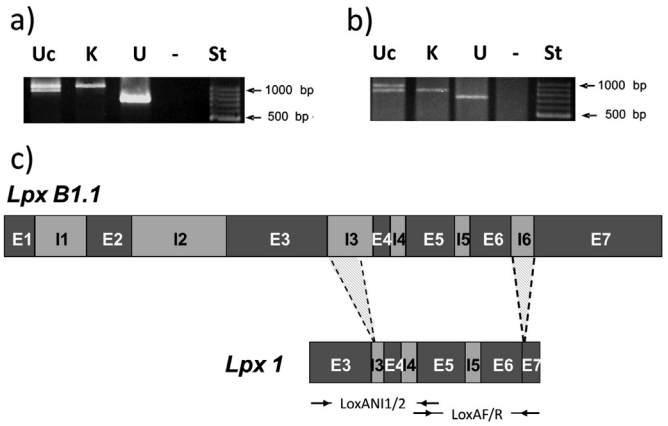

Fig. 2. *T. urartu Lpx-1* locus. Amplification of *the Lpx-1* locus from the tetraploid wheat line UC1113 (UC) and the variety Kofa (K) and the diplod wheat *T. urartu* (U) by the primer pairs a) LoxANI1/2 and b) LoxAF/R. c) Schematic view comparing the intron/exon structure of the *T. turgidum Lpx-B1.1* gene and the amplified region of *T. urartu Lpx-1* locus. The locations of the primers LoxANI1/2 and LoxAF/R are shown in the scheme. St.: molecular weight marker "-": negative control for the reactions.

pseudogene in searches of the EST and SRA triticum databases using the predicted mRNA sequence generated *in silico* by intron elimination. Additionally, no protein sequences with high identity to the predicted *Lpx-A1_like* pseudogene protein were identified.

Lpx-A1_like-based primers (Garbus et al., 2009) were used for PCR amplification from genomic DNA of the Kofa variety and the UC1113 line to identify polymorphisms that could be used to delineate the precise location of the sequences in the current durum wheat map. The primer pair LpxLike2L/LpxLike2R amplified from each genotype a fragment of ~500 bp with identical nucleotide sequence. In the Kofa variety, amplification with LpxLike2L/LpxLike1R and LpxLike1L/LpxLike1R primer pairs gave rise to 805 bp and 500 bp fragments, respectively, identical to *Lpx-A1_like* (FJ518909). Both primer pairs failed to amplify any product from UC1113. Thus, the primer pair LpxLike1L/LpxLike1R was used for mapping purposes, taking advantage of this absence/presence polymorphism (Fig. 1b). The *Lpx-A1_like* pseudogene was then amplified from 93 RILs of a Kofa x UC1113 mapping population (Zhang et al., 2008). Based on the presence/absence polymorphism between Kofa and UC1113, the *Lpx-A1_like* pseudogene could be placed on the current durum wheat map at the long arm of chromosome 4A (Fig. 1c), 15.07 cM distal to the microsatellite gwm192b (Zhang et al., 2008) and 3.57 cM proximal to the *Lpx-A3* locus (Carrera et al., 2007).

3.2. Identification of Lpx-1 sequences in diploid wheat T. urartu (AA)

We next searched for the presence of *Lpx* genes in the diploid *T. urartu*, the genome A donor (Dvorak et al., 1993), in order to make inferences with regard to their evolution. In *T. urartu*, PCR performed with the primer pair LOXA1F/R amplified a 753 bp fragment (Fig. 2a) that showed higher identity to wheat *Lpx-1* genes (95%) than to *Lpx-2* (88%) and *Lpx-3* (85%), thus confirming that it belongs to the *Lpx-1* locus (Table 1). The *Lpx-A1_like*-specific primers did not give rise to any amplification products, suggesting that those primer sites are absent in the *T. urartu* genome or, alternatively, that they are located more distant from each other than in the reported tetraploid sequence, and thus amplification could not occur under the utilized PCR conditions.

To examine the *T. urartu Lpx-1* locus further, a set of primers (LOXANIF/R) was designed based on barley *Lox-A* cDNA to amplify a region located 5′ of the fragment generated with LOXA1F/R. The amplification was first performed in the variety Kofa and the breeding line UC1113, aiming to characterize amplification carried out by these primers in tetraploid wheat and thus extend the *Lpx-B1* reported sequences for these two genotypes. As reported for the primer pair LOXAF/R (Carrera et al., 2007; Garbus et al., 2009), two fragments were obtained in the UC1113 line, whereas only one was detected in Kofa (Fig. 2b). The upper amplification band (1114 bp) was identical in the two lines and was also identical to the *LpxB1.2* gene (HM126467). It overlapped with the 5′ region of the *LpxB1.2* locus previously reported for Kofa and UC1113 (DQ474241); these regions combined would correspond to a 2253 bp sequence (KC679782). The lower band (1001 bp), detected only in UC1113, showed 99% identity to the *Lpx-B1.1b* and *Lpx-B1.1a* loci (HM126468, HM126466). This sequence was also annotated (KC679304).

An amplification product of 801 bp was obtained when these primers were used on *T. urartu* genomic DNA (Fig. 2b). Analysis of the sequence confirmed that the product was amplified from a *Lpx-1* locus since it showed higher identity to wheat *Lpx-1* genes (90–93%) than to *Lpx-2* (82%) or the portions of *Lpx-3* that have been reported (Table 1). A 1507 bp sequence corresponding to *T. urartu Lpx-1* was generated taking advantage of the overlapping sequences between both amplification products (KC679299).

We further used a complementary strategy to demonstrate that *T. urartu* possesses a non-deleted copy of *Lpx-1*. BLAST searches of the whole genome shotgun sequence libraries from *T. urartu* (Ling et al., 2013) using *T. urartu Lpx-1* as probe identified a contig (AOT I011454267, 2217 bp) that covers the complete *T. urartu Lpx-1* sequence (99% identity) and possesses an additional 645 bp that corresponds to the 3′ region of exon 7. Then, the *T. turgidum Lpx-B1.1b* gene (HM126468) was used as a probe in order to find the 5′ region, identifying a contig (AOTI010409062, 8688 bp) that aligned with an average identity of 92% with the 5′ region of *Lpx-B1.1b*. This contig

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Table 1
Comparison of *Lpx* sequences identified in the diploid wheat *T. urartu* to previously reported *Lpx* genes. The sequences obtained with LoxAF/R and LOX ANIF/R primers pairs were compared to the reported *Lpx-1*, *Lpx-2* and *Lpx-3* genes. Four *Lpx-1* sequences were included in the analysis.

		Lpx-1				*Lpx-2*	*Lpx-3*
		Lpx-B1.1b (HM126468)	*Lpx-B1.2* (HM126467)	*Lpx-B1.3* (HM126469)	*Lpx-A1_like* (FJ518909)	GU167921	DQ474243[a]
T. urartu (LoxAF/R)	Identity (%)	95	90	93	92	88	85
	Coverage (%)	99	99	99	57	17	35
T. urartu (LOX ANIF/R)	Identity (%)	92	93	93	–	82	–
	Coverage (%)	99	95	95	–	48	–

[a] Only a partial *Lpx-3* gene was found in the databases, and it does not include the region amplified by the primer pair LOX ANIF/R.

included exon 1 through the 5′region of exon 3. Thus, the two contigs together provide the complete *T. urartu Lpx-A1* gene sequence. The lower identity in the latter contig is consistent with the fact that the *Lpx-B1.1b* and *T. urartu Lpx-1* belong to different wheat genomes. No contig attributable to the *Lpx-A1_like* pseudogene was identified, but only partial sequences were shared with Lpx-1. Taking into account that the genome coverage of the library is 90x (Ling et al., 2013), it can be concluded that the *T. urartu* genome possesses a single non-deleted copy of *Lpx-1*. Additionally, analysis of exon-intron gene structure showed that *T. urartu Lpx-1* lacks intron 6 (Fig. 2c), as observed for *Lpx-A1_like* and a portion of intron 3.

3.3. Identification of Lpx-1 sequences in hexaploid wheat

The International Wheat Genome Sequencing Consortium (IWGSC) has adopted a chromosome-based approach that relies on the construction of physical maps of *T. aestivum* cv. Chinese Spring from isolated chromosomes and chromosome arms (Doležel et al., 2007). In wheat, chromosome arms represent 1.3–3.4% of the whole genome and thus chromosome genomics offers a great reduction in sample complexity (Bartos et al., 2012). The database of separate chromosome arms was searched using *T. turgidum LpxB1.1b* (HM126468) and Lpx-*1A_like* (FJ518909) and *T. urartu Lpx-1* (KC679299, identified in the present paper) genomic sequences as probes. For each identified sequence, we calculated the identity to the probe, i.e., the number of aligned nucleotides that were the same in both sequences, and the coverage of the alignment, expressed as the quotient of the number of aligned base pairs and the total length of the probe.

Four genomic sequences were identified in the long arm of chromosome 4A (Table 2, Supplementary Table 1). Sequence #7172128 contains the *Lpx-A1* gene, as judged based on the identity

Table 2
Analysis of hexaploid wheat *Lpx* genes. A repository containing sequences of individual wheat chromosome long and short arms (4AL, 4AS, 4BL, 4BS, 4DL and 4DS) was searched using *T. turgidum Lpx-A1_like* (FJ518909), *T. urartu Lpx-A1* (KC679299), and *T. turgidum Lpx-B1.1* (HM126468). The identification number and sequence lengths are shown in the 2nd and 3rd columns. For each tested probe, the percentage of identity and coverages are indicated. Additionally, schematic illustrations of the coverages are shown, being the upper and the lower bars representatives of the query and the subject, respectively. The symbol "–" denotes that no alignments were obtained between the indicated sequences. Additional information, including the range and length of alignments, are provided in Supplementary Table 1.

	Database sequence		***Lpx-A1_like*** (915 bp)		***Lpx-1*** (1503 bp)		***Lpx-B1.1b*** (4225 bp)	
	Identification	**Length**	**Identity (%)**	**Coverage (%)**	**Identity (%)**	**Coverage (%)**	**Identity (%)**	**Coverage (%)**
4AL	#7172128	17597	100	97	99	26	95	28
	#2062251	9660	-	-	85	18	85	6
	#7172135	12092	-	-	85	25	83	14
	#7173069	3979	-	-	-	-	86	5
4AS	-	-	-	-	-	-	-	-
4BL	#6852878	1529	87	40	-	-	90	4
4BS	#4959255	6281	96	43	94	96	99	92
	#4963368	5795	96	44	94	91	96	75
	#4959256	10907	-	-	-	-	96	18
	#4868582	5202	-	-	-	-	99	9
	#4957383	14297	-	-	84	18	84	7
4DL	-	-	-	-	-	-	-	-
4DS	#2311633	23672	95	70	94	96	94	79
	#2306653	10116	-	-	85	18	85	7

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(100%) and coverage (99%) showed with *Lpx-A1_like* (FJ518909). The coverage of *T. urartu Lpx-1* was lower and the alignment was fragmented (see Supplementary Table 1), suggesting that the *Lpx-A1* locus in hexaploid wheat possesses deletions similar to those described in tetraploid wheat. The high identity (95%) shared with *Lpx-B1.1b* (HM126468) was expected considering that they are homeologous loci. The low identities of the chromosome 4AL sequences #2062251, #7172135 and #7173069 to the probes tested and the very low coverage, suggest that they correspond to other *Lpx*s, probably *Lpx-A3,* which has been mapped to this chromosome arm (Carrera et al., 2007). To test this hypothesis, we searched the database using *Lpx-A3* sequences, finding that sequence #2062251 displayed ~100% identity to *T. turgidum Lpx-3* (DQ474242 and DQ474244) and 99% to *T. aestivum Lpx-3* mRNA (HQ913602). Sequence #7172135 showed a high level of identity (91%) to *Lpx-2* (GU167921.1), suggesting the existence of another locus for *Lpx* in this chromosome arm, different from *Lpx-1* and *Lpx-3*. The aligned region between #7173069 and HM126468 was not long enough to assign this sequence to a specific *Lpx*. Thus, we can conclude that one of the four sequences identified in chromosome 4AL corresponds to *Lpx-A1* and that the *T. aestivum* genome A has a partial deleted copy in accordance with the results obtained in *T. turgidum.*

Five sequences were identified when the chromosome arm 4BS was searched with the *Lpx-1* probes (Table 2, Supplementary Table 1). Their identities to HM126468 suggest that #4959255 and #4963368 are two different copies of the *Lpx-B1* locus. Sequences #4868582 and #4959256 aligned with HM126468 in regions that are absent in #4959255 and #4963368 thus correspond to the same loci (Supplementary Table 1). Sequences #4957383 showed ~98% identity to *Lpx-3.* Therefore, this strategy allowed the identification of three *Lpx* sequences on chromosome arm 4BS, two of which were *Lpx-1* genes and the other *Lpx-3*. In accord with these findings, two *Lpx-B1* genes of (*Lpx-B1.1* and *Lpx-B1.2*) were previously reported (Carrera et al., 2007), with three alleles detected in specific varieties (Verlotta et al., 2010), and *Lpx-B3* is located on this chromosome arm (Carrera et al., 2007; Zhang et al., 2008).

Two sequences were identified in chromosome 4DS, one with high identity to the *Lpx-1* locus (#2311633) and the other representing a *Lpx-3* locus (#2306653). No sequences with significant identity to *Lpx* genes were found in chromosomes 4AS or 4DL.

Additionally, there were identified and annotated, four *Lpx* sequences in *T. aestivum* genome including a *Lpx* pseudogen in the A genome (*Lpx-A1*; KC679303), the *LpxB1.1* (KC679300) and *LpxB1.2* (KC679301) genes in the B genome and *Lpx-D1* (KC679302) in the D genome. Finally, *T. aestivum* whole genome shotgun sequence libraries from NCBI were searched with *Lpx-1* sequences. Whereas a copy of *Lpx-A1_like* could be identified, no success was obtained searching either with the complete gene from the *T. turgidum* genome B or with *T. urartu Lpx-1*, providing complementary support for our data.

3.4. Phylogenetic analysis of Lpx-1 sequences

The information about *Lpx-1* sequences provided by the present investigation is summarized in Table 3 together with the previously reported one. Such sequences were used to perform a phylogenetic analysis of the genome A, B and D *Lpx* sequences (Fig. 3). The *Lpx-A1* from tetraploid and hexaploid wheat clustered separately from all the other sequences, including the diploid wheat one, suggesting that the insertion and deletions caused the divergence. Moreover, *T. urartu Lpx-A1* is closely related to *Lpx*s from genomes B and D. The linked clustering of sequences according to species and not to *Lpxs* coding isoforms observed for *T. turgidum* and *T. aestivum,* must be related to the species origin.

Table 3
Summary of the *lipoxygenase-1* (*Lpx-1*) genes from diploid, tetraploid and hexaploid wheat.

Species	Ploidy	*Lpx*-1 Gene	Chromosome	Reference
T. urartu	*2x*	*Lpx-A1*	4	This work
T. turgidum	*4x*	*Lpx-A1_like*	4A	Garbus et al., 2009
T. turgidum	*4x*	*Lpx-B1.1*	4B	Carrera et al., 2007, Verlotta et al., 2010
T. turgidum	*4x*	*Lpx-B1.2*	4B	Carrera et al., 2007, Verlotta et al., 2010
T. turgidum	*4x*	*Lpx-B1.3*	4B	Verlotta et al., 2010
T. aestivum	*6x*	*Lpx-A1_like*	4A	This work
T. aestivum	*6x*	*Lpx-B1.1*[a]	4B	This work
T. aestivum	*6x*	*Lpx-B1.2*	4B	This work
T. aestivum	*6x*	*Lpx-D1*	4D	This work

According to the accepted nomenclature, the letters A and B in the names refer to the wheat genomes A and B, respectively. The number 1 indicates the *Lpx* isoform (1 and 3 were reported to be in chromosome homeologous group 4), whereas the numbers after the periods represent the different copies of the genes in the locus.
[a] Three alleles have been reported: *Lpx-B1.1a, Lpx-B1.1b, Lpx-B1.1c.*

4. Discussion

The genetic localization of *Lpx* genes and their role in pasta and semolina color have been the subject of several studies. In the B genome, the *Lpx* locus has been extensively characterized (Hart and Langston, 1977; Li et al., 1999; Carrera et al., 2007; Garbus et al., 2009; Verlotta et al., 2010). However, little information about *Lpx* genes in the A genome has been reported (Garbus et al., 2009; Feng et al., 2012). A *Lpx-1* pseudogen, *Lpx-A1_like,* was described, physically located together with *Lpx-A3* within a region of less than 42 kbp (Garbus et al., 2009). Compared to *Lpx-B1.1*, one of the *Lpx-1* copies from the genome B, *Lpx-A1_like* has a ~2000 bp deletion in the region comprised from the last three quarters of I2 to E5, inclusively. Additionally, a ~180 bp insertion carrying portion of a TIR Mariner DNA transposon of the DTT Icarus Stowaway MITE family was detected. The detection of polymorphic regions in this pseudogene between Kofa and UC1113 allowed us to map it to the long arm of chromosome 4A, 3.57 cM proximal to *Lpx-A3,* near the centromeric region. To our knowledge, this is the first time that *Lpx-1* and *Lpx-3* could be mapped in the same chromosome. The loci are excessively close to each other to make recombination likely. The fact that the *Lpx-1* loci reside in the short arms of chromosomes 4B and 4D (Carrera et al., 2007; Hessler et al., 2002, Table 2) but in the long arm of chromosome 4A (Fig. 1c, Table 2), suggests that the locus was affected by the inversions that rearranged chromosome 4A (Devos et al., 1995; Mickelson-Young et al., 1995).

Aiming to understand the evolutionary course of this locus, *Lpx-1* sequences of ancient diploid and modern polyploid wheats were searched and compared. Wheat species form a polyploid series

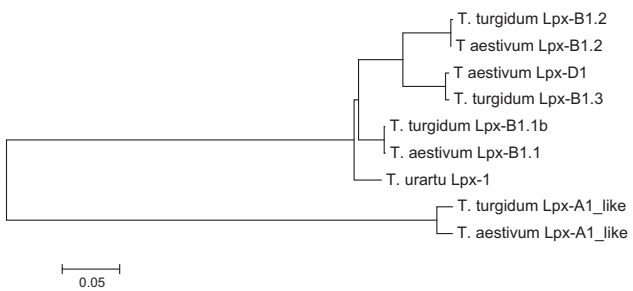

Fig. 3. Molecular phylogenetic analysis. Evolutionary relationships of 9 Lpx-1 sequences are shown. The evolutionary history was inferred using the Maximum Likelihood method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.15248002 is shown.

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with three ploidy levels, including tetraploids and hexaploids originating from hybridization events among the wild diploid species. Thus, the hybridization of wild diploid einkorn wheat, *T. urartu* (AA genome), with a close relative of the goat grass *Aegilops speltoides* (SS genome, closely related to the B genome) originated the ancestor of the cultivated durum wheat *T. turgidum* (AABB) (Sarkar and Stebbins, 1956; Dvorak et al., 1993), which, in via hybridization with diploid *A. tauschii* originated the hexaploid *T. aestivum* (AABBDD) (Kihara, 1944; McFadden and Sears, 1946). Thus, sequence comparison among *Lpx-1s* from diplod *T. urartu*, tetraploid *T. turgidum* and hexaploid *T. aestivum* revealed the existence of *Lpx-A1* homologs with the complete ORF at the chromosome 4A locus of diploid *T. urartu* whereas *Lpx* pseudogenes were identified in A genomes from the other two species, suggesting that pseudogenization has taken place after the formation of polyploid wheat. Pseudogenes have been proposed to arise from insertions of imperfect sequences either through double-strand break repair or TE capture (Wicker et al., 2011). Recently, Bartos et al. (2012) performed detailed comparisons of four gene–pseudogene pairs, identifying TE insertions in exons, frame-shift mutations caused by a single nucleotide insertion and partial deletions of coding sequence as the causes of pseudogene formation. The presence of a partial TE sequence in the *Lpx-A1_like* pseudogene suggests that TE insertion could have been involved in its pseudogenization.

Most of the deletions detected in wheat originated at the polyploid level (Dvorak et al., 2004). The deletions that were fixed in *T. turgidum* passed through the bottleneck that accompanied the speciation of hexaploid *T. aestivum* (Dvorak et al., 2004), as observed for the deletion in the *Lpx-A1* locus. Deletions of one of the three homoeologous copies of a gene in hexaploid wheat occur with high frequency and are not eliminated from the population because of the buffering effect of polyploidy (Dubcovsky and Dvorak, 2007).

Although intron/exon structure in general is highly conserved among species, introns 3 to 5 of *Lpx* genes have been lost in the durum wheat B genome with respect to the gene structure observed in rice, and the maize and sorghum orthologues (AF465643 and CM000760, respectively) lack introns 6 and 8 (Verlotta et al., 2010). Several intron losses have occurred in the course of the evolution of plant genomes, probably as a consequence of homologous recombination between cDNA and the corresponding genomic intron-containing locus (Hu and Leung, 2006). Comparisons with the orthologous barley (L35931) and rice (XM_469409) *Lox-1* cDNAs revealed that intron 6 is absent in both *T. urartu Lpx-1* and *T. turgidum* genome A *Lpx-A1_like*, whereas it is present in *T. turgidum LpxB1.1* (Figs. 1 and 2, Verlotta et al., 2010). Our results support the suggestion that independent intron loss events occurred in *Lpx-1* genes in grasses after the divergence of the different subfamilies from their common ancestor (Verlotta et al., 2010). It was previously proposed that the duplication that led to the *Lpx1* and *Lpx3* genes occurred before the wheat-rice-sorghum divergence and then, independent *Lpx1* duplications occurred in the different lineages (Garbus et al., 2009). However, in light of these new results, it seems that the evolution of these loci is characterized by duplication and deletion events.

5. Conclusions

This work sheds light on the evolution of the *Lpx* genes of genome A. *Lpx-A3* and *Lpx-A1_like* were mapped together to the proximal region of the long arm of chromosome 4A at a distance of 3.57 cM. The identification of a *Lpx-A1_like* sequence in chromosome 4AL from *T. aestivum* cv. Chinese Spring together with the identification of an intact copy of the gene in *T. urartu* strongly suggests that the deletion occurred close to tetraploid formation and then passed to the hexaploid wheat. Thus, contrary to the *Lpx-B1* gene family described in the durum wheat germplasm (Verlotta et al., 2010), a single copy of *Lpx* characterized by several intron/exon deletions and a TE insertion was identified in genome A of the modern wheat species assessed in the present study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2013.05.012.

References

Bartos, J., Vlček, C., Choulet, F., Džunková, M., Cviková, K., Šafář, J., Šimková, H., Pačes, J., Strnad, H., Sourdille, P., Bergès, H., Cattonaro, F., Feuillet, C., Doležel, J., 2012. Intraspecific sequence comparisons reveal similar rates of non-collinear gene insertion in the B and D genomes of bread wheat. BMC Plant Biology 12, 155.

Borrelli, G.M., Troccoli, A., Di Fonzo, N., Fares, C., 1999. Durum wheat lipoxygenase activity and other quality parameters that affect pasta color. Cereal Chemistry 76, 335–340.

Carrera, A., Echenique, V., Zhang, W., Helguera, M., Manthey, F., Schrager, A., Picca, A., Cervigni, G., Dubcovsky, J., 2007. A deletion at the Lpx-B1 locus is associated with low lipoxygenase activity and improved pasta color in durum wheat *Triticum turgidum* ssp. *durum*. Journal of Cereal Science 45, 67–77.

Cenci, A., Chantret, N., Kong, X., Gu, Y., Anderson, O.D., Fahima, T., Distelfeld, A., Dubcovsky, J., 2003. Construction and characterization of a half million clone BAC library of durum wheat, Triticum turgidum ssp. durum. Theoretical and Applied Genetics 107, 931–939.

Devos, K.M., Dubcovsky, J., Dvorak, J., Chinoy, C.N., Gale, M.D., 1995. Structural evolution of wheat chromosomes 4A, 5A, and 7B and its impact on recombination. Theoretical and Applied Genetics 91, 282–288.

Doležel, J., Kubaláková, M., Paux, E., Bartoš, J., Feuillet, C., 2007. Chromosome-based genomics in the cereals. Chromosome Research 15, 51–66.

Dubcovsky, J., Dvorak, J., 2007. Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316, 1862–1866.

Dvorak, J., di Terlizzi, P., Zhang, H.B., Resta, P., 1993. The evolution of polyploid wheats: identification of the A genome donor species. Genome 36, 21–31.

Dvorak, J., Yang, Z.-L.., You, F.M., Luo, M.C., 2004. Deletion polymorphism in wheat chromosome regions with contrasting recombination rates. Genetics 168, 1665–1675.

Feng, B., Dong, Z., Xu, Z., Wang, D., Wang, T., 2012. Molecular characterization of a novel type of lipoxygenase LOX. gene from common wheat Triticum aestivum L. Molecular Breeding 30, 113–124.

Garbus, I., Carrera, A., Dubcovsky, J., Echenique, V., 2009. Physical mapping of durum wheat lipoxygenase. Journal of Cereal Science 50, 67–73.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Hart, G.E., Langston, P.J., 1977. Chromosome location and evolution of isozyme structural genes in hexaploid wheat. Heredity 39, 263–277.

Hessler, G., Thomson, M.J., Bensher, D., Nachit, M.M., Sorrells, M.E., 2002. Association of a lipoxygenase locus, Lpx-B1, with variation in lipoxygenase activity in durum wheat seeds. Crop Science 42, 1695–1700.

Hu, K.J., Leung, P.C., 2006. Complete, precise, and innocuous loss of multiple introns in the currently intronless, active cathepsin-L-like genes, and inference from this event. Molecular Phylogenetics and Evolution 38, 685–696.

Kihara, H., 1944. Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. (Japanese). Agriculture and Horticulture (Tokyo) 19, 13–14.

Kosambi, D.D., 1944. The estimation of map distances from recombination values. Ann Eugen 12, 172–175.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S..E., Newburg, L., 1987. MAPMAKER: an interactive computer package for maps of experimental and natural populations. Genomics 1, 174–181.

Li, W.L., Faris, J.D., Chittoor, J.M., Leach, J.E., Hulbert, S.H., Liu, D.J., Chen, P.D., Gill, B.S., 1999. Genomic mapping of defense response genes in wheat. Theoretical and Applied Genetics 98, 226–233.

Ling, H.Q., Zhao, S., Liu, D., Wang, J., Sun, H., Zhang, C., Fan, H., Li, D., Dong, L., Tao, Y., Gao, C., Wu, H., Li, Y., Cui, Y., Guo, X., Zheng, S., Wang, B., Yu, K., Liang, Q., Yang, W., Lou, X., Chen, J., Feng, M., Jian, J., Zhang, X., Luo, G., Jiang, Y., Liu, J., Wang, Z., Sha, Y.,

Zhang, B., Wu, H., Tang, D., Shen, Q., Xue, P., Zou, S., Wang, X., Liu, X., Wang, F., Yang, Y., An, X., Dong, Z., Zhang, K., Zhang, X., Luo, M.C., Dvorak, J., Tong, Y., Wang, J., Yang, H., Li, Z., Wang, D., Zhang, A., Wang, J., 2013. Draft genome of the wheat A-genome progenitor *Triticum urartu*. Nature 496, 87–90.

McFadden, E.S., Sears, E.R., 1946. The origin of Triticum spelta and its free-threshing hexaploid relatives. Journal of Heredity 37 (81–9), 107–116.

Mickelson-Young, L., Endo, T.R., Gill, B.S., 1995. A cytogenetic ladder-map of the wheat homoeologous group-4 chromosomes. Theoretical and Applied Genetics 90, 1007–1011.

Nachit, M.M., Elouafi, I., Pagnotta, M.A., Salen, A.E., Iacono, E., Labhilili, M., Asbati, A., Azrak, M., Hazzam, H., Bensher, D., Khairallah, M., Ribaut, J.M., Tanzarella, O.A., Porceddu, E., Sorrells, M.E., 2001. Molecular linkage map for an intraespecific recombinant inbred population of durum wheat *Triticum turgidum* L. var. *durum*. Theoretical and Applied Genetics 102, 177–186.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.

Sarkar, P., Stebbins, G.L., 1956. Morphological evidence concerning the origin of the B genome in wheat. American Journal of Botany 43, 297–304.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences USA 101, 11030–11035.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis MEGA. Software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Troccoli, A., Borrelli, G.M., De Vita, P., Fares, C., Di Fonzo, N., 2000. Durum wheat quality: a multidisciplinary concept. Journal of Cereal Science 32, 99–113.

van Mechelen, J.R., Schuurink, R.C., Smits, M., Graner, A., Douma, A.C., Sedee, N.J.A., Schmitt, N.F., Valk, B.E., 1999. Molecular characterization of two lipoxygenases from barley. Plant Molecular Biology 39, 1283–1298.

Verlotta, A., De Simone, V., Mastrangelo, A.M., Cattivelli, L., Papa, R., Trono, D., 2010. Insight into durum wheat Lpx-B1: a small gene family coding for the lipoxygenase responsible for carotenoid bleaching in mature grains. BMC Plant Biology 10, 263.

Wicker, T., Mayer, K.F.X., Gundlach, H., Martis, M., Steuernagel, B., Scholz, U., Šimková, H., Kubaláková, M., Choulet, F., Taudien, S., Platzer, M., Feuillet, C., Fahima, T., Budak, H., Doležel, J., Keller, B., Stein, N., 2011. Frequent gene movement and pseudogene evolution is common to the large and complex genomes of wheat, barley, and their relatives. The Plant Cell 23, 1706–1718.

Zhang, W., Chao, S., Manthey, F., Chicaiza, O., Brevis, J.C., Echenique, V., Dubcovsky, J., 2008. QTL analysis of pasta quality using a composite microsatellite - SNP map of durum wheat. Theoretical and Applied Genetics 117, 1361–1377.

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