Elsevier Editorial System(tm) for Journal of Cereal Science
Manuscript Draft

Manuscript Number: JCS08-310R1

Title: Physical mapping of durum wheat lipoxygenase genes

Article Type: Standard Research Paper

Keywords: BAC library
durum wheat
lipoxygenase genes
fingerprinting

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Abstract: A bright yellow color is an important quality criterion for pasta making. Yellow color depends on the amount of carotenoid pigments in grain, which is the result of the balance between pigment synthesis and degradation by lipoxygenases (LPX). The organization of genes coding for lipoxygenases in the tetraploid wheat genome is not completely understood. Here, we report the screening of a durum wheat BAC library with barley probes to characterize the physical distribution of Lpx genes. PCR characterization and BAC fingerprinting of the positive clones suggests that Lpx-B1.1 and Lpx-B3 are less than 103-kb apart, whereas Lpx-B1.2 is further apart from them. In the A genome a partially deleted copy of Lpx-1 (Lpx-A1_like) was found, colocalizing within a 42 kbp region with Lpx-A3, confirming that in both genomes these two genes are close to each other. The knowledge of the physical location of these two genes is important to understand the evolution of this family but also has practical implications since closely linked genes are difficult to separate

by recombination. This may limit the number of Lpx allele combinations that can be obtained and affect the selection of optimal Lpx allele combinations for pasta quality improvement.

*** Revision Notes**

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Dear Dr Domenico Lafiandra:

We appreciate very much the comments about our manuscript entitled “Physical mapping of durum wheat lipoxygenase genes”.

The present version of the manuscript was updated following the reviewer´s recommendations.

We also followed the Checklist required for revision of the manuscript.

Yours sincerely,

Dr. Viviana Echenique

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Response to reviewers:

Reviewer #1: no suggestions about the manuscript were made by Reviewer #1.

Reviewer #2: we copied each comment for in improving the manuscript performed by the reviewer #2 (*in italics*), followed by its response.

1) *Lanes 186-188: Authors say that no polymorphism could be deduced….. This sentence is superfluous because polymorphisms based on small indel can not be detected on a 1000bp long DNA fragment on 1% agarose gel.*

We agree with the reviewer in his appreciation about detecting indels from 1% agarose gel. The sentence only intended to emphasize the concordance between the number and the size of the bands obtained from each genotype with these primers pairs in contrast to the profile obtained with the primers LoxAF/R. Moreover, a detailed analysis of the sequences obtained was performed in the subsequent sections of the paper.
However, the sentence was changed in order to clarify the misinterpretation.

The sentence (line 186):

“Here, no polymorphism could be deduced from the agarose gel electrophoresis profile among Langdon, UC1113 and Kofa”.

Was replaced by:

“Here, no differences could be observed from the agarose gel electrophoresis profile among Langdon, UC1113 and Kofa”.

2) *Lane 191: the sentence refers to Fig. 1C, not to Fig. 2.*

The manuscript was modified according to this suggestion

The sentence (line 193 in the revised version):

“….whereas the presence of both bands was no detected in any BAC (Fig 2), …”

Was replaced by:

“….whereas the presence of both bands was no detected in any BAC (Fig. 1c),…”

3) *Lanes 237-240: are there polymorphisms among Langdon, Kofa and UC1113 for Lpx-A1_like at sequence level? If the sequence refers to a pseudogene, a high level of mutation is expected. If present, some polymorphisms between Kofa and UC1113 could be used to genetically map Lpx-A1_like and link physical to genetic map. At this purpose, did the Authors try to amplify some anchor markers on selected BAC clones?*

No polymorphisms between the *Lpx-A1_like* sequence amplified from Kofa and Langdon were detected, whereas the amplification product obtained from UC1113 showed some differences.

Our current work is directed to define polymorphisms for the pseudogene that will make possible the precise location of the region in the current wheat map, making use of a RIL population obtained from Kofa and UC1113. At the same time, we are inquiring about the evolutionary origin of the pseudogene, studying a wide durum wheat collection and analyzing the *Lpx* genes in the genome A donor specie, *T. urartu*. This will be the subject of another future publication.

Physical mapping of durum wheat lipoxygenase genes

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Abbreviations:

BAC: bacterial artificial chromosome; BLAST: Basic Local Alignment Search Tool; CTAB: Cetyl Trimethyl Ammonium Bromide; EST: expressed tagged sequence; LOX: lipoxygenase; LPX: wheat lipoxygenases; MITE: miniature inverted transposable element; PCR: polymerase chain reaction; QTL: quantitative trait loci; SNP: single nucleotide polymorphism.

1. Abstract

A bright yellow color is an important quality criterion for pasta making. Yellow color depends on the amount of carotenoid pigments in grain, which is the result of the balance between pigment synthesis and degradation by lipoxygenases (LPX). The organization of genes coding for lipoxygenases in the tetraploid wheat genome is not completely understood. Here, we report the screening of a durum wheat BAC library with barley probes to characterize the physical distribution of *Lpx* genes. PCR characterization and BAC fingerprinting of the positive clones suggests that *Lpx-B1.1* and *Lpx-B3* are less than 103-kb apart, whereas *Lpx-B1.2* is further apart from them. In the A genome a partially deleted copy of *Lpx-1 (Lpx-A1_like*) was found, colocalizing within a 42 kbp region with *Lpx-A3,* confirming that in both genomes these two genes are close to each other. The knowledge of the physical location of these two genes is important to understand the evolution of this family but also has practical implications since closely linked genes are difficult to separate by recombination. This may limit the number of *Lpx* allele combinations that can be obtained and affect the selection of optimal *Lpx* allele combinations for pasta quality improvement.

2. Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum,* genomes AABB) constitutes the cereal of preference for semolina and pasta production. The main quality factors for pasta production include a high grain protein content, strong gluten and bright yellow color. This last parameter is mainly determined by the concentration of carotenoid pigment content in the grain. Carotenoids pigments are not only important to satisfy consumer's preferences but also to improve the nutritional value of pasta. Carotenoid pigments act as antioxidant compounds, reducing oxidative damage to biologic membranes by scavenging peroxide radicals (Bast et al., 1996). 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 and 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 (Tróccoli et al., 2000).

Lipoxygenases (LPXs; linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a family of enzymes widely distributed in plants and animals. They are nonheme iron-containing dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene system leading to unsaturated fatty acid hydroperoxides. LPX generate a wide variety of products depending on the lipid substrate, the site of oxygen incorporation and the stereospecificity of the reaction.

The bleaching of dough and pasta products is the result of a coupled oxidation of pigments due to the free radical species generated by fatty acids oxidation (Siedow, 1991). β-carotene acts as an inhibitor of LPX activity, preventing semolina

68 bleaching (Lomnitski et al., 1993; Trono et al., 1999). Additionally, the enzymatic
69 cleavage of hydroperoxides results in off-flavors (Shibata, 1996).
70 The identification of the chromosome location of the different LPX isoforms was
71 initially determined using Chinese Spring nulli-tetrasomic lines (AABBDD). LPX
72 zymograms of these lines mapped the LPX1 and LPX2 isozymes to locus *Lpx-1* on
73 chromosome 4 (*Lpx-A1, Lpx-B1* and *Lpx-D1*) and *Lpx-2* on chromosome 5 (*Lpx-*
74 *A2, Lpx-B2* and *Lpx-D2*), respectively (Hart and Langston, 1977). Southern
75 hybridization with maize-based *Lpx* probes confirmed the chromosome location of
76 these genes (Li et al., 1999). *Lpx* loci were further mapped using a RIL population
77 in chromosomes 4A, 5A and 5B (Li et al., 1999). In tetraploid wheat (AABB), the
78 *Lpx-1* locus was mapped on chromosome 4B (Nachit et al., 2001; Hessler et al.,
79 2002; Zhang et al., 2008). Carrera et al. (2007) reported a duplication/deletion of
80 this locus, the resulting loci were designated *Lpx-B1.1* and *Lpx-B1.2*. No evidence
81 of the presence of *Lpx-1* locus on the A genome has been reported so far in
82 tetraploid wheat (Carrera et al., 2007). The *Lpx-3* locus was identified on both
83 genomes, but could only be mapped on the A genome (Zhang et al., 2008).
84 The linkage among *Lpx* loci in durum wheat could not be established since the
85 *Lpx-1* and *Lpx-3* were not mapped on the same chromosome in any of the current
86 available wheat maps. In barley, they were mapped 1 cM apart on the short arm of
87 chromosome 4HS (van Mechelen et al., 1999).
88 The main purpose of this work was to study the physical organization of the *Lpx-1*
89 and *Lpx-3* loci in durum wheat. We used the tetraploid durum wheat BAC library
90 constructed from the var. Langdon (Cenci et al., 2003) to determine the

91 approximate distances between some of the *Lpx* loci and to determine if functional
92 copies of the different loci exist in the different genomes.
93
94

3. Experimental

3.1. Plant material

Plant nuclear DNA was extracted from leaves of the varieties Kofa and Langdon and breeding line UC1113, following a CTAB protocol (CIMMYT). DNA was quantified through a VersaFluor Fluorometer (BIORAD) and diluted for PCR reactions.

3.2. BAC library screening

A BAC library constructed from the tetraploid wheat (AABB) *Triticum turgidum* ssp *durum.* cv. Langdon (Cenci et al., 2003), was screened using a mix including barley *Lox-A* and *Lox-B* cDNAs probes. The average size of the BAC clones in this library is 131 kb and it has a genome coverage of near 5X for each genome (99.4 % probability of finding any desired gene). Probes were heat denatured, PCR labeled and purified through commercial columns (ProbeQuant® G-50 Amershan). Hybridization was carried out overnight at 42°C. Membranes were washed three times and visualized by autoradiography. Positive BAC clones were picked and amplified using standard laboratory plasmid isolation protocols and commercial kits (Wizard Plus SV Minipreps DNA Purification System, Promega). Purified BAC concentration ranged from 30-100 ng/μl.

3.3. PCR procedures

Genomic DNA and BAC clones were amplified by PCR. Different primer combinations based on barley and wheat lipoxygenase sequences were used to

amplify preferentially wheat sequences orthologous to barley *LoxA* or *LoxB* genes, as previously reported in Carrera et al. (2007). Primers LOXA L (CTGATCGACGTCAACAAC) and LOXA R (CAGGTACTCGCTCACGTA), collectively called LOXAL/R, differentially amplify *Lpx-1* (wheat ortholog of barley *LoxA)* over the *Lpx-2* and *Lpx-3* (wheat orthologs of barley *LoxC* and *LoxB*, respectively). Primers LOXB L (CACGATAACTTCATGCCAT) and LOXB R (ACTCCTCCAGCTCCTTGT), collectively called LOXBL/R, were used to differentially amplify *Lpx-3*, as described in Carrera et al. (2007). Additionally two primer pairs (with a common left primer) were designed to amplify an *Lpx-A1* pseudogene discovered in this study: LpxLike1R (GTACGGGTAATCCGACACCA), LpxLike2L (TCCGAGTTCCTGCTCAAGAC) and LpxLike2R (CATGCACGTTCCAATCGTAT).

Amplification reactions were performed in a BIORAD thermal cycler in a 25 μl reaction mixture. Each reaction consisted of 200 mM dNTPs, 1.5mM $MgCl_2$, 100nM of each primer, 1U of Taq polymerase (Promega) 1 μl of each BAC dilution, leading to a final concentration of near 20 ng/ reaction. PCR amplification conditions were as follow: 3 min at 94°C; 5 touchdown cycles (-1 °C each) of 45 s at 94°C, 45 s at 60–55°C and 1 min at 72°C. After that, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C followed by final extension step of 10 min at 72°C.

For sequencing purposes, PCR products were cloned into 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- amplicillin XGal - IPTG plates. White colonies were picked and plasmids plus insert were amplified and purified. The presence of the PCR fragment was checked by restriction profile with the enzyme *EcoR*I. Three clones per PCR reaction were sequenced in SIGYSA (Castelar). Sequences alignments were performed using the software BioEdit 7.0 Sequence Alignment Editor (Hall, 1999). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/).

3.4. BAC fingerprinting

High quality DNA from the BAC clones (~1 μg/μl) was obtained using the commercial kit BACMAX (Epicentre) following the manufacturer protocol. Then, 1 μg of BAC DNA was restricted with *Hin*dIII at 37°C during 4 hours. Electrophoresis was performed in 1% agarosa gels during five hours at 50 mV in 0.5X TBE including two commercial size standards (λ/H*in*dIII and λ/*Eco*RI+*Hin*dIII, PB-L, Quilmes, Argentina). Agarose gels were stained with ethidium bromide, visualized under UV light and digitalized both with a Kodak Easy share Z7590 zoom digital camera and with a transilluminator coupled to software for the analysis of gel images.

3.5. Phylogenetic tree construction

Sorghum and *Brachypodium* lipoxygenase genes were screened from genomic databases (www.*Brachypodium*.org and Gramene) using *LpxB1.1* sequence as a probe. The relation of the sequences identified in rice, sorghum and *Brachypodium* with the barley ones was established from the phylogenetic trees. Trees were

performed using the Neighbor-Joining method (Saitou and Nei, 1987) and were drown 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 Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

4. Results

4.1. Identification of *Lpx* sequences within the BAC library.

The screening of the Langdon BAC library with the *Lox-A* and *Lox-B* cDNAs probe pool yielded 14 positive clones (Table 1). Given the 5.1 fold genome coverage of the library, four unlinked genes are expected to yield an average of 20 BAC clones.

4.2. Identification of *Lpx* genes present in the different BAC clones

To obtain genomic sequences of the *Lpx* genes from the same germplasm used to construct the BAC library, genomic DNA was extracted from young leaves of the wheat var. Langdon. Genomic DNA was amplified by PCR using different gene specific primers that differentially amplify the three *Lpx* loci. In addition, DNA from the var. Kofa and breeding line UC1113 was used as controls since its amplification has been well characterized before (Carrera et al., 2007). Using the primer pair LOXAL/R, two bands of 900 and 1000 bp were amplified from Langdon DNA (Fig. 1a). In agarose gels, this amplification profile was indistinguishable from the one obtained with the line UC1113, whereas the higher band was absent in Kofa, as was previously reported by Carrera et al. (2007). PCR amplifications with primer pairs LOXBF/R yielded a single band of 1000 bp (Fig. 1b). Here, no polymorphism could be deduced from the agarose gel electrophoresis profile among Langdon, UC1113 and Kofa.

We used the same *Lpx* primers to screen the selected BAC clones (Table 1). Twelve clones showed amplification products with LOXAF/R primer pair (Fig. 1c). It is interesting to notice that half of them amplified the higher band and the others the lower one, whereas the presence of both bands was no detected in any BAC

197 (Fig. 1c), suggesting that these two loci must be further apart than the distance
198 spanned by single BAC clones.
199 The primer pair LOXBF/R yielded amplification products in 8 BAC clones (Table 1).
200 As was observed with genomic DNA, no differential amplification was observed
201 among BAC clones. Based on the sequences of *LpxA3* (DQ474242 and
202 DQ474244) and *LpxB3* (DQ474243) loci, a *Sal*I restriction site was identified in the
203 *LpxA3* sequence. This polymorphic restriction site was used to assign four of the
204 *Lpx3* BAC clones to the A genome (chromosome 4A) and four to the B genome
205 (chromosome 4B). Sequence analysis of LOXBF/R bands confirmed the data
206 obtained through this CAP marker.
207 We found that some of the BAC clones carrying *Lpx-A3* also yielded amplification
208 products with primer pair LOXAF/R (*Lpx1*). This was unexpected since previous
209 results showed that these primers preferentially amplified the *Lpx-B1* locus.
210 However, in nullisomic tetrasomic line N4B (lacking *Lpx-B1*) the LOXAF/R primers
211 yielded new bands suggesting that in the absence of the preferred target, these
212 primers have the ability to amplify *Lpx-1* sequences from the other genomes
213 (Carrera et al., 2007). To test this hypothesis, we sequenced the two fragments
214 amplified with LOXAF/R. The sequences from the larger band found in the B
215 genome BACs were identical to those previously obtained for *Lpx-B1.1* from
216 UC1113 (DQ474240). Two different sequences were identified from the BACs that
217 yielded the smaller amplification product. The sequence from the fragments
218 amplified from BACs 314-D7 and 595-G8 was identical to the *Lpx-B1.2*
219 (DQ474241) locus. These two BACs did not yielded amplification products with the
220 primers for *Lpx-3* genes (Table 1). In contrast, amplifications products were

obtained with both the LOXAF/R (915-bp fragment, Fig. 2A) and LOXBF/R primers (*Lpx-A3* gene) from BACs 187-M9, 657-K12, 626I15, and 691-K22 (Table 1).

Sequence analysis of the 915-bp LOXAF/R amplification product (GenBank accession number FJ518909) showed that it is likely a partial *Lpx-A1* pseudogene, which will be referred hereafter as *Lpx-A1_like.* Alignments of this sequence with known barley and wheat lipoxygenase genes showed that *Lpx-A1_like* fragment amplified with the LOXAF/R primers includes a piece of exon 2 (90% similar to barley LOXA L35931, orthologous to wheat *Lpx1*), a 375-bp region with no homology to other sequences except for a partial MITE (miniature inverted transposable element, 82-bp, Icarus), and a 359-bp region including the complete exon 8 and the start of exon 9 (Fig. 2B). This last segment lacks the intron 8 observed in the orthologous regions in the *Lpx-B1* genes (DQ474240 and DQ474241). The region of *Lpx-A1_like* similar to exon 8 also differs from the functional *Lpx-B1* sequences by the presence of two deletions (14 and 15 bp long) and several SNPs.

We then designed primers based on exon 2 and exon 4 of the available sequence from a barley LOXA cDNA. These primers were able to amplify sequences from Langdon genomic DNA and from *Lpx-B1.1* and *Lpx-B1.2* containing BAC clones, but no amplification was observed in the BAC clones carrying *Lpx-A1_like* (data not shown) thus confirming the existence of deletions within this sequence.

The presence of the *Lpx-A1_like* sequence was also confirmed in Langdon, Kofa and UC1113 genomic DNAs, through PCR amplification and sequencing using two primer pairs based on the *Lpx-A1_like* sequence, Lpxlike2L/ Lpxlike2R and Lpxlike2L/ Lpxlike1R (Fig. 2A).

245

246 **4.3. Physical mapping and fingerprinting analysis**

247 The BAC s including *Lpx1* and *Lpx3* genes were organized into two separate
248 contigs using *Hind*III fingerprints (Fig. 3a and b). The first contig included seven
249 BAC clones (average size 117-kb) from the B genome (Fig. 3B). Based on the
250 presence of *Lpx-B1.1* and *Lpx-B3* genes alone or in combination in different BAC
251 clones (Fig. 3B) and the estimated sized of shared fragments among BAC clones it
252 was possible to establish that the *Lpx-B1.1* and *Lpx-B3* genes are 40- to 103-kb
253 apart.

254 The other contig, including the *Lpx-A1_like* sequence and *Lpx-A3,* included four
255 BAC clones (average size 116,4-kb). Based on the *Hind*III fingerprint, it was
256 possible to establish that loci *Lpx-A1_like* and *Lpx-A3* are included within a
257 genomic region not larger than 42- kb.

258

5. Discussion

The genetic localization of *Lpx* genes and their role on pasta and semolina color have been the focus of several recent studies. QTL analysis established that more than 50% of the variation in lipoxygenase activity was attributable to the *Lpx-B1* (Hessler et al., 2002; Carrera et al., 2007). The two genes found at this locus, *Lpx-B1.1* and *Lpx-B1.2*, are 95% identical suggesting a relatively recent duplication. The deletion of the *Lpx-B1.1* copy has been associated with lower lipoxygenase activity and improved pasta color both in a segregating population and in a screening including different durum varieties (Carrera et al., 2007; Zhang et al., 2008). This result suggests that the number of *Lpx1* copies might be important for the regulation of lipoxygenase activity in mature seeds.

A minor locus related to yellow color was identified on chromosome 4A (Hessler et al., 2002; Carrera et al., 2007; Zhang et al., 2008) but it is not known if it was generated by polymorphisms at the *Lpx-A1*, *Lpx-A3* or a closely linked locus. We report here the existence of another deletion affecting the A genome copy of the *Lpx1* gene.

5.1. The *Lpx-A1_like* pseudogene

The *Lpx-A1_like* is in the same BACs that include *Lpx-A3* (Table 1), which was previously mapped on chromosome 4A (Carrera et al., 2007; Zhang et al., 2008). Therefore, we can conclude that *Lpx-A1_like* is located in the same chromosome. *Lpx-A1_like* is more similar to barley *LOXA* and wheat *Lpx-B1.1* and *LpxB1.2* than to *LOXB* of wheat *Lpx3*. Therefore, we concluded that this sequence is derived

from *Lpx1*. The deletions of the complete exons 3, 4, 5, 6 and 7 indicate that this copy is not functional. In addition, the complete exon 8 has two deletions (4-bp and 14-bp) that would disrupt the reading frame, indicating that this sequence is not longer under purifying selection. This pseudogene also lacks the intron 8.

The region between Exons 2 and 8 includes a truncated MITE of the *Stowaway* class Icarus that could be implicated in some of the deletions that occurred in this region. 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).

In addition to the BAC library, the non-functional *Lpx-A1_like* sequence was also found in the variety Kofa and the breeding line UC1113 genomes, suggesting that this gene deletion might be common among the durum wheats. It would be interesting to test these primers in wild accessions of tetraploid wheat to establish if this deletion occurred before or after domestication. The absence of polymorphisms for functional *Lpx-A1* genes, may explain why no QTLs for lipoxygenase activity have been reported for this region.

The large deletions found in the *Lpx-A1_like* sequence might have also limited the ability of the barley LOX probes to hybridize with this sequence and partially explain the relatively low number of BAC clones identified during the screening of the Langdon BAC library.

5.2. Observed and predicted coverage of the Langdon BAC library

304 The wheat genome sequence is not available yet, so the best way to characterize
305 the physical organization of a gene family is using BAC libraries with appropriate
306 genome coverage. The genome coverage of the Langdon wheat BAC library was
307 initially estimated to have a 5.6-fold coverage per genome (Cenci et al., 2003). This
308 value has been later confirmed in other studies (Cenci et al., 2004). The 13 BAC
309 clones confirmed to have at least one copy of the *Lpx1* or *Lpx3* gene is slightly
310 lower than the number expected from 2 genes in two genomes (22 positive
311 clones). However, additional factors need to be considered in this calculation. The
312 first one is the duplication of the *Lpx-B1.1* and *Lpx-B1.2* which should increase the
313 number of expected clones. The second one is the partial deletion of the *Lpx-A1*
314 gene that may have resulted in a reduced detection by hybridization. The absence
315 of BACs carrying only the *Lpx-A1_like* pseudogene is in agreement with this
316 hypothesis, which will reduce the number of expected BAC clones. Finally, the *Lpx-*
317 *1* and *Lpx-3* genes are close to each other at distances that are lower than the
318 average size of the BAC clones. If the seven BAC clones including two genes are
319 counted twice, the total number of detections would be 21, which is closer to the
320 expected number based on the known coverage of the library.

321

322 **5.3. Physical organization of the *Lpx1-Lpx3* region**

323 The relative location of the *Lpx-1* and *Lpx-3* loci on the wheat chromosomes was
324 not known because polymorphisms from both loci were not found simultaneously
325 within the available mapping populations. The co-location of the *Lpx-1* and *Lpx-3*
326 genes within single BAC clones, both for the A and B genomes indicates that these

two genes (or pseudogene in the case of *Lpx-A1*) are very close to each other in both genomes.

The fingerprints of the BAC clones including the different *Lpx1* and *Lpx3* genes, and the subsequent contig assembly, showed that the *Lpx-A1_like* and *Lpx-A3* genes are located less than 43-kb apart, whereas the *Lpx-B1.1* and *Lpx-B3* are within a region that is between 40- to 147-kb. The *LpxB1.2* gene was found in BAC clones that were not connected to the *Lpx-B1.1 / Lpx-B3* contig suggesting that this gene is separated from the other two by a distance that is larger than the average BAC clone size.

The A and B genome BACs were assembled in two separate contigs using *Hind*III fingerprints. Although the A and B genomes are highly colinear (Blanco et al., 1998; Nachit et al., 2001) and their respective genes are very similar (≈95%) the degree of divergence between them is sufficient to generate separate contigs from colinear regions (Cenci et al., 2003). These different fingerprints are originated in the fast divergence of the intergenic regions of the different wheat genomes, which result in very limited conserved sequences in these regions (Wicker et al., 2003; Dubcovsky and Dvorak, 2007). The BAC clones and contigs identified in this study can now be sequenced to better understand the evolution of these chromosome regions and to add additional examples of orthologous regions between different wheat genomes.

The discovery that the *Lpx-1* and *Lpx-3* genes are so close to each other has several practical implications. First, it suggests that it would be very difficult to separate these loci by recombination if beneficial mutations or deletions are found

in repulsion in the *Lpx-1* and *Lpx-3* genes. In addition, the close proximity of these genes will complicate the generation of mutants knocking out both genes, since it will be difficult to combine independent point mutations in these two genes by recombination. Alternatively, it might be possible to eliminate both loci simultaneously using mutagens that produce large deletions, or use mutagens that generate point mutations (e.g. EMS mutagenesis) in a genetic background that already has the *Lpx-B1.1* deletion if a double mutant is desired. Since large natural deletions are frequent in polyploid wheat (Wicker et al., 2003; Dubcovsky and Dvorak, 2007) it might be also possible to find natural deletions including multiple *Lpx* genes by screening a large and diverse germplasm collection.

5.4. Comparative genome organization of the *Lpx* loci

The region of chromosome 4 including the *Lpx1* and *Lpx3* loci in wheat is orthologous to the region in rice chromosome 3 that includes three rice lipoxygenase genes designated *OsLOX1*, *OsLOX3* and *OsLOX4*. *Os*LOX1 and *Os*LOX3 predicted proteins are more similar to each other (74% identical) than to the *Os*LOX4 predicted protein (66-69%) (Fig. 4a). These three genes are located within a region of 57-kb (chromosome 3 27,999-kb to 28,056-kb). The *Os*LOX1 and *Os*LOX3 rice proteins are more similar to the barley LOXA protein (74-82% identical) than to the LPX3 protein (68% identical). On the contrary, the OsLOX4 protein is more similar to the barley LOXB protein (77% identical) than to the wheat LPX1 protein (68% identical). In sorghum, three lipoxygenase genes were found on chromosome 1, which is colinear with wheat chromosome 4. As in rice, two of

sorghum LOX proteins were more similar to barley LOXB (72 and 70%, respectively) than to barley *LOXA* (63 and 61%), whereas the other one was more similar to barley LOXA (83%) than to barley LOXB (65%) (Fig. 4b). These three sequences are located within a region of 24.5 kb based on their alignment with ESTs CN145489.1, CN148918.1, CX607208.1, CX607302.1, CN133062.1, and CN142292.1. The analysis of *Brachypodium* genome sequences, a species more closely related to wheat than rice or sorghum, revealed two lipoxygenase sequences located 13.1 kb apart (in super-contig super_0). The predicted protein of one the sequences is more similar to barley LOXA (84%) than to barley LOXB (67%), whereas the other one is more similar to LOXB (83%) than to LOXA (63%) (Fig. 4c). Both sequences are likely functional based on the alignment with ESTs DV475177.1, DN552319.1, DV478987.1, and DV482580.1).

These results suggest that the duplication that originated the *Lpx1* and *Lpx3* genes occurred before the wheat-rice-sorghum divergence and, that after this event, independent LPX1 duplications occurred in the different lineages.

6. Acknowledgments

This work was granted by CONICET/NSF (Res. Nº 0060), the Agencia Nacional de Promoción Científica y Tecnológica (PICTO08-12948 and PICT 1011) and Secretaría de Ciencia y Tecnología UNS (PGI24/A098).

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Figure captions

Fig. 1 PCR amplification with barley-based primers of durum wheat *Lpx* genes. Genomic DNA obtained from the line UC1113 (U) and the varieties Kofa (K) and Langdon (L) were amplified using the primer pairs a) LOXAF/R and b) LOXBF/R. In c), the polymorphic amplification with LOXAF/R of the BAC clones (indicated over the sampled line) is shown. The standard is the Ladder 100 bp (PB-L products) and the size in bp is shown

Fig. 2 a) *Lpx-A1_like* nucleotide sequence. LOXAF/R primers annealing sites are shown in grey. The *Lpx-A1_like* based primers designed to search for this sequences in genomic DNA are underlined; b) Schematic comparison between *Lpx-A1_like* and the closest sequences. The upper bar is showing barley LoxA cDNA exon assembly, predicted based of the corresponding rice sequence. The regions with shared identity among the four compared sequences are represented by the same color. White boxes represent introns

Fig. 3 a) Electrophoresis in agarose gel of selected BAC clones digested with *Hin*dIII. The standard sampled corresponds to λ phage digested with *Hin*dIII. The sampled BAC clones are shown over the lane. b) Schematic assembly of the obtained fragments from each clone, showing the inferred distance between loci

Fig. 4 Evolutionary relationships of barley lipoxygenases protein sequences with a) rice, b) sorghum and c) *Brachypodium*, identified in the genomic databases.

Table 1. Loci identified in the BAC clones using barley based primers for loci *LoxA* and *LoxB,* orthologous to wheat *Lpx-1* and *Lpx-3*, respectively. The first line shows the primer pair that was used to obtain the amplification products.

CLON	LOX AF/R	LOX BF/R	LOX CF/R
481-B18, 635-J17, 771-C14,	*Lpx-B1.1*	*Lpx-B3*	-
321-J7	-	*Lpx-B3*	
195-B1, 544-O18, 762-C19	*Lpx-B1.1*	-	-
314-D7, 595-G8	*Lpx-B1.2*	-	-
187-M9, 657-K12, 626-I15, 691-K22	*Lpx-A1_like*	*Lpx-A3*	-

Table 2. Data obtained from the fingerprinting analysis. The BAC clones analyzed were grouped according to the genome (A or B) from which they derive. The estimated molecular size (MW) and the number of bands obtained after *Hind*III digestion (Bands) of each clone are indicated.

Genome A			Genome B		
BAC clon	**MW**	**Bands**	**BAC clon**	**MW**	**Bands**
691-K22	87.4	20	544-O18	112.6	28
626-I15	116.4	22	762-C19	120.9	29
657-K12	117.1	22	321-J7	60.0	18
187-M9	115.9	21	635-J17	147.4	32
			195-B1	116.0	26
			771-C14	149.1	33
			481-B18	108.7	25

Figure 1

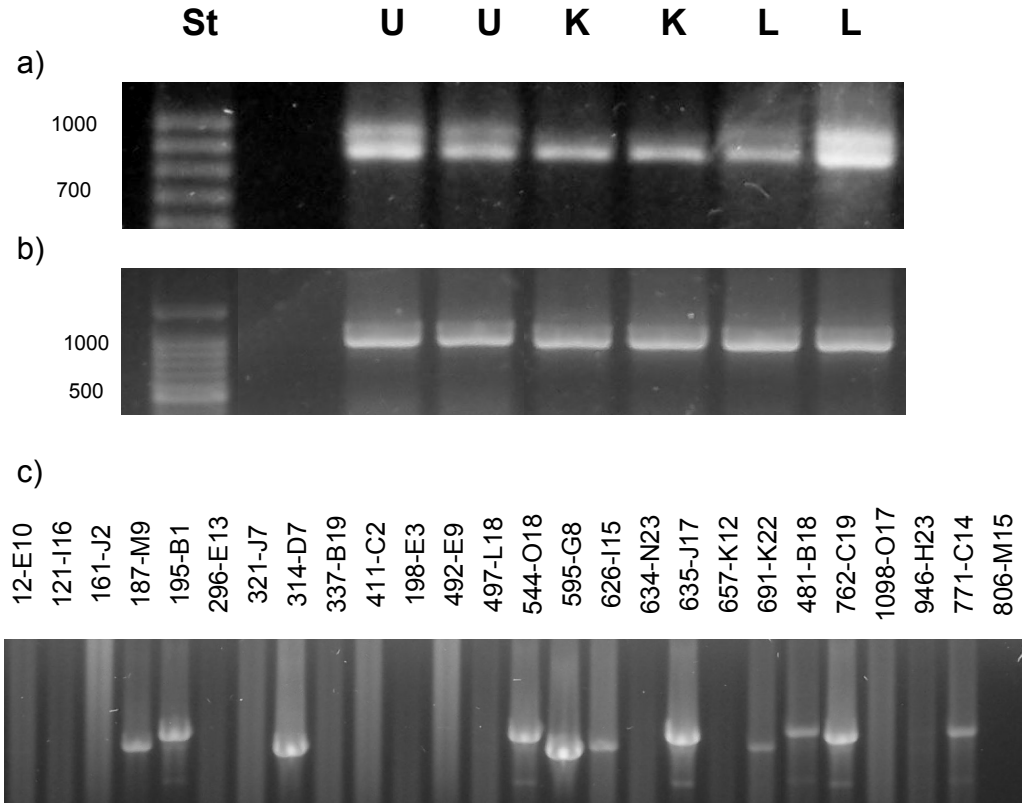

St
U
U
K
K
L
L
a)
1000
700
b)
1000
500
c)
12-E10
121-I16
161-J2
187-M9
195-B1
296-E13
321-J7
314-D7
337-B19
411-C2
198-E3
492-E9
497-L18
544-O18
595-G8
626-I15
634-N23
635-J17
657-K12
691-K22
481-B18
762-C19
1098-O17
946-H23
771-C14
806-M15

Figure 2

a)

```
   1 AATTCACTAGTGATTCTGATCGACGTCAACAACTACCACAGCTCCGAGTTCCTGCTCAAG
                                                 LpxLike2L
  61 ACCGTCACCCTCCACGACGTCCCCGGCCGCGGCAGCCTCTCCTTCGTCGCCAACTCCTGG
 121 GTCTACCCCGCCGCCAGCTACACCTACAGCCGCGTCTTCTTCGCCAACGACGTGAGTGAT
 181 CCTTTTGCCTCTCCTCTCCTTTCCTTTTCACCGGCCGGCTTCGTCATTCATGGTCATTAA
 241 GTCTTCTTTGAGATAAAATATATTTAGTGGGTGCAGAATTTATTCCGTGTTGGTAGAAAA
 301 GATAGTATGGCTAGGTGCAGCACAAGATTGAATGAAACTGGCACCGTGGCACGCTGGTAG
 361 GTGAGGAAAACTGTTGCACCATATTATCTGTCGTTGATTTAGTACAAAGTCTCATTAAAT
 421 CAGTGACGAGTACTCCCTCCATTCGGAATTACTTGTCGCGGAAATATACGTATCAAGACA
 481 TATTTTAGTTCTAGATACATCCATATCCAAGACACCTGATGTAATACGATTGGAACGTGC
                                                 LpxLike2R
 541 ATGCAGGCTGAACACTCACGCGGTGATGGAGCCGTTCGTGATCTCGACGAACCGGCACCT
 601 CAGCGTGACGCACCCGGTGCACAAGCTGCTGAGCCCGCACTACCGCGACACCATGACCAT
 661 GCAGACGCTCATCAACGCCGGCGGCATCTTCGAGATGACGGTTCGCGCTGGGGATGTCGT
 721 CGGTGGTGTACAAGGACTGGAAGTTCACCGAGCAGGGCCTGCCCGACGATCTCATCAAGA
 781 GGGGCATGGCGGTGGAGGACCCGTCGAGCCCGTACAAGGTGCGGCTGCTGGTGTCGGATT
                                                 LpxLike1R
 841 ACCCGTACGCGGCGGACGGGCTGGCGATCTGGCACGCCAtCGAGGAGTACGTGAGCGAGT
 901 ACCTGAATCACTAGT
```

b)

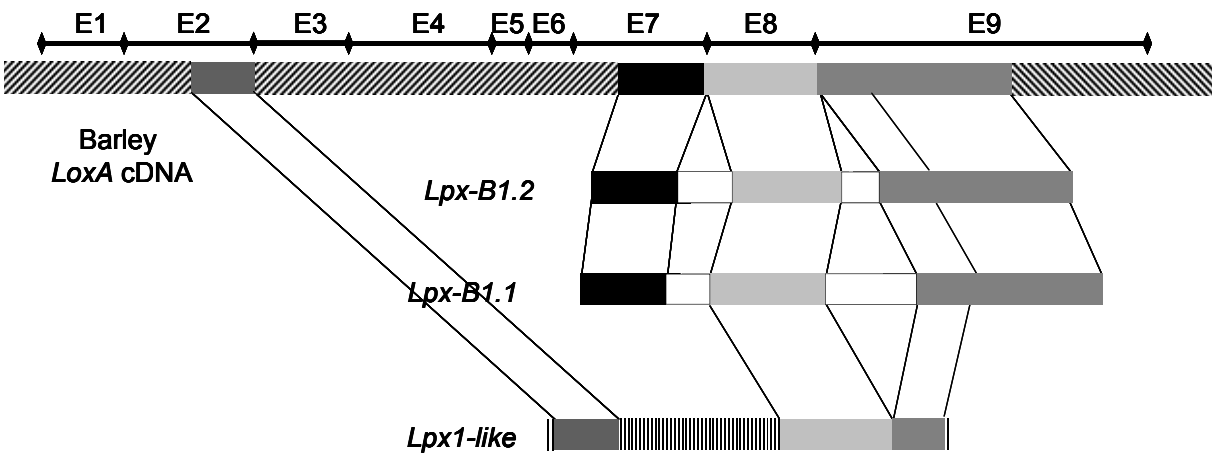


Figure 3.

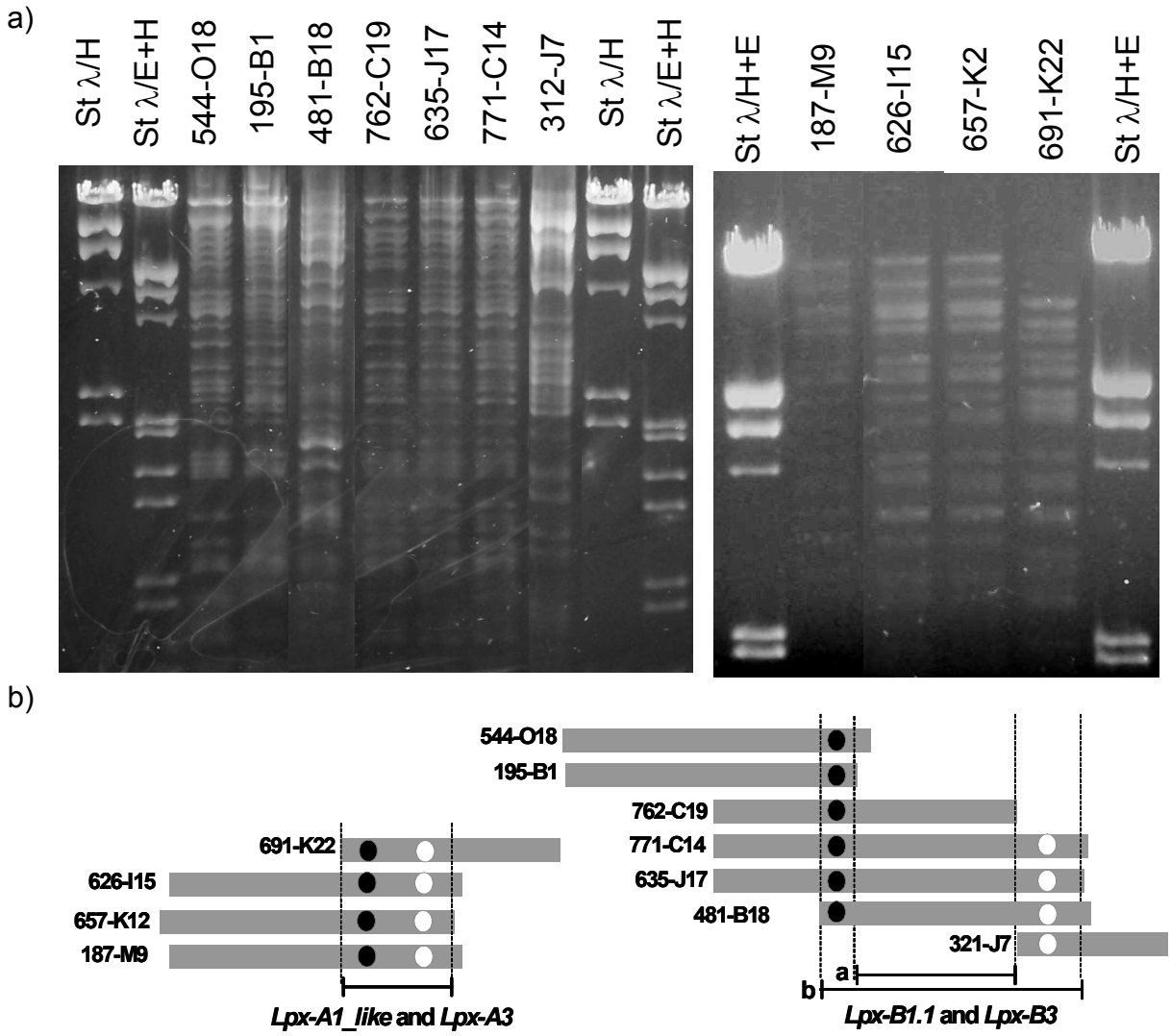
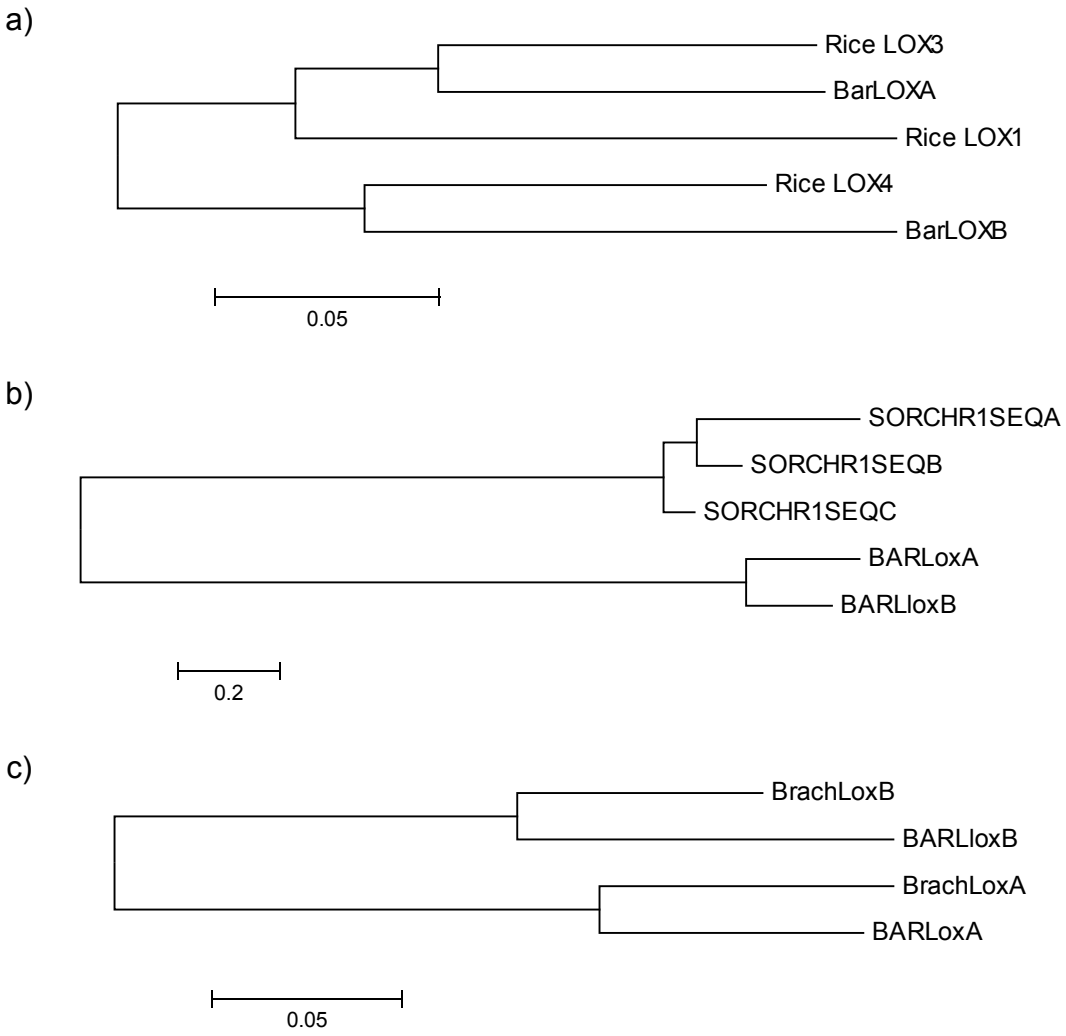

a)
St λ/H
St λ/E+H
544-O18
195-B1
481-B18
762-C19
635-J17
771-C14
312-J7
St λ/H
St λ/E+H
St λ/H+E
187-M9
626-I15
657-K2
691-K22
St λ/H+E
b)
544-O18
195-B1
762-C19
771-C14
635-J17
481-B18
321-J7
691-K22
626-I15
657-K12
187-M9
a
b
Lpx-A1_like and Lpx-A3
Lpx-B1.1 and Lpx-B3

Figure 4.


a)
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BarLOXA
Rice LOX1
Rice LOX4
BarLOXB
0.05
b)
SORCHR1SEQA
SORCHR1SEQB
SORCHR1SEQC
BARLoxA
BARLloxB
0.2
c)
BrachLoxB
BARLloxB
BrachLoxA
BARLoxA
0.05