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Journal of Cereal Science 45 (2007) 67–77

Journal of CEREAL SCIENCE

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A deletion at the *Lpx-B1* locus is associated with low lipoxygenase activity and improved pasta color in durum wheat (*Triticum turgidum* ssp. *durum*) ☆

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Received 21 April 2006; received in revised form 6 July 2006; accepted 6 July 2006

Abstract

The concentration of yellow carotenoid pigments in durum wheat grain is an important quality criterion and is determined both by their accumulation and by their degradation by lipoxygenase enzymes (*Lpx* loci). The existence of a duplication at the *Lpx-B1* locus and the allelic variation for a deletion of the *Lpx-B1.1* copy is reported. This deletion was associated with a 4.5-fold reduction in lipoxygenase activity and improved pasta color ($P<0.0001$) but not semolina color, suggesting reduced pigment degradation during pasta processing. A molecular marker for the deletion was mapped on chromosome 4B in a population obtained from the cross between durum line UC1113 and variety Kofa. A second lipoxygenase locus, designated *Lpx-A3*, was mapped on the homoeologous region on chromosome 4A and was associated with semolina and pasta color ($P<0.01$) but not with lipoxygenase activity in the mature grain. Selection for both the UC1113 allele for *Lpx-A3* and the Kofa *Lpx-B1.1* deletion resulted in a 10% increase in yellow scores for dry pasta relative to the opposite allele combination. This result indicates that the markers and the new allelic variants reported here will be useful tools to manipulate the wheat *Lpx* loci and to improve pasta color.

Keywords: Lipoxygenase; Wheat; Pasta quality; Semolina color; Carotenoid pigments; Breeding

1. Introduction

Tetraploid wheat (*Triticum turgidum* L. ssp. *durum*, $2n = 28$, genomes AABB) is used mainly to produce pasta. An important quality criterion for the durum wheat industry is a bright yellow color in the final pasta product (Troccoli et al., 2000). The color of dry and cooked pasta is the result of a balance between the accumulation of natural carotenoid yellow pigments in the seeds and of their oxidative degradation by lipoxygenase (LOX) activity. High carotenoid content in the grain enhances not only the quality of the pasta, but also its nutritional value. Carotenoids reduce the oxidative damage to biological membranes by scavenging peroxyradicals such as those involved in certain human diseases and in the aging processes and are, therefore, a valuable nutritional component of pasta products (Bast et al., 1996).

A significant proportion of the carotenoid pigments are lost during milling (approximately 8%) or during pasta processing (approximately 16%) (Borrelli et al., 1999). This large reduction in yellow pigments during pasta processing

Abbreviations: ANOVA, analysis of variance; CPC, cooked pasta color; CIMMYT, International Center for Maize and Wheat Improvement; DPC, dry pasta color; EST, expressed sequence tag; LOX, lipoxygenase; PCR, polymerase chain reaction; RCBD, randomized complete block design; RILs, recombinant inbred lines; SC, semolina color; UCD, University of California Davis.

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doi:10.1016/j.jcs.2006.07.001

is mainly due to LOX activity. LOXs are non-haem, iron-containing dioxygenases that catalyze the oxidation of polyunsaturated fatty acids (e.g. linoleic and linolenic acids) in plants, animals and microorganisms. Radical forms produced during the intermediate states of substrate peroxidation are responsible for the degradation of carotenoid pigments (Faubion and Hoseney, 1981; Siedow, 1991). A high LOX activity can also negatively affect the aroma of the end product. The hydroperoxides produced by LOX activity during dough mixing are then destroyed forming volatile compounds that can result in undesirable flavors and odors in the pasta (Siedow, 1991).

Cereal LOX genes have been studied in more detail in barley than in wheat. Two LOX isoenzymes have been purified from germinating barley grains (Doderer et al., 1992), which differ in the product they form from the linoleic acid substrate. The lipoxygenase 1 (LOX-1) isoenzyme produces 9-hydroperoxide-octadecadienoic acid whereas the lipoxygenase 2 (LOX-2) produces 13-hydroperoxide-octadecadienoic acid. LOX-1 is present in both quiescent and germinating barley grains and accounts for most of the LOX activity in mature barley grains. LOX-2 is detected only at early stages of grain development and after germination (Holtman et al., 1996; Schmitt and van Mechelen, 1997; van Mechelen et al., 1999). The LOX-1 isoform is encoded by the *LoxA* gene (GenBank L35931) located in chromosome arm 4HS, whereas the LOX-2 isoform is encoded by the *LoxC* gene (GenBank L37358) mapped on chromosome arm 5HL (van Mechelen et al., 1999). A third LOX cDNA, *LoxB* (GenBank L37359), was mapped 1-cM distal to the *LoxA* locus on barley chromosome arm 4HS (van Mechelen et al., 1999). *LoxB* mRNAs were mainly detected in germinating seeds and at much lower levels than the other two genes in the developing barley grains, and only at the onset of development (Schmitt and van Mechelen, 1997; van Mechelen et al., 1999).

Wheat LOX isoenzymes were first assigned to chromosomes 4A (*Lpx-A1*), 4B (*Lpx-B1*), 4D (*Lpx-D1*), 5A (*Lpx-A2*), 5B (*Lpx-B2*), and 5D (*Lpx-D2*) using nulli-tetrasomic lines (Hart and Langston, 1977). More recent mapping studies in tetraploid (Nachit et al., 2001) and hexaploid wheat (Li et al., 1999) confirmed the isoenzyme studies and showed that LOX genes are located in colinear regions in wheat and barley suggesting that they are orthologous. These mapping results indicate that the wheat *Lpx-2* locus corresponds to the barley *LoxC* gene (homoeologous group 5) whereas the wheat *Lpx-1* locus corresponds to barley *LoxA* or *LoxB* locus (homoeologous group 4). Hessler et al. (2002) sequenced several wheat fragments and based on their higher similarity to the barley *LoxA* gene assigned them to the *Lpx-1* locus.

In this study we show that there are also wheat sequences on homoeologous group 4 corresponding to the barley *LoxB* gene, which we propose to designate *Lpx-3*. We also describe the presence of two different genes related to the barley *LoxA* on chromosome 4B, which we will refer to as *Lpx-B1.1* and *Lpx-B1.2* hereafter. In addition, we report a deletion of the *Lpx-B1.1* copy in the variety Kofa, which results in large reduction in LOX activity and significant improvement in pasta color and an *Lpx-A3* allele associated with improved semolina and pasta color.

2. Material and methods

2.1. Plant material

This study used a single seed descent mapping population of 93 F_9 recombinant inbred lines (RILs) derived from the cross between durum wheats Kofa and UC1113. Kofa is a desert durum variety developed by Western Plant Breeders (Now WestBred, Bozeman, Montana, USA) from a population designated "DICOCCUM ALPHA POP-85 S-1". This variety has excellent pasta quality with optimal semolina and pasta color. UC1113 is a breeding line from the University of California Davis (UCD) program selected from International Center for Maize and Wheat Improvement (CIMMYT) cross CD52600 (KIFS//RSS/BD1419/3/MEXIS-CP/4/WAHAS/5/YAV79) with excellent agronomic performance but intermediate pasta color.

The 93 RILs and the two parental lines were grown in the field at the UCD (Sacramento Valley, 38° 32′ N, 121° 46′ W, Yolo Loam soils) in 2003 and 2004. In the 2003 and 2004 experiments plot sizes were 2.5×1.2 and 3.6×1.2 m, respectively. In both years the fertilization was 220 kg/ha of nitrogen (ammonium nitrate 33.5%) applied 110 kg/ha at planting and 110 kg/ha at tillering stage. The field trials were organized in a randomized complete block design (RCBD) with 3 replications. Samples from the three plots for each of the 93 RILs and the parental lines were pooled and evaluated for semolina, dry pasta and cooked pasta color at the Durum Wheat Quality Laboratory at North Dakota State University, Fargo, ND.

In addition to the RILs, a set of durum wheat varieties was analyzed to evaluate the distribution of the *Lpx-B1.1* deletion. This set included eight durum varieties from Argentina (Bonaerense INTA Cariló, Bonaerense INTA Cumenay, Bonaerense INTA Facón, ACA001, ACA003, Buck Ambar, Buck Esmeralda, Buck Topacio), five from Italy (Adamello, Ciccio, Colosseo, Duilio, Simeto), and two from CIMMYT (IAT2-65, IAT2-66). Chinese Spring nullisomic-tetrasomic lines N4AT4D, N4BT4D and N4DT4B were used to assign polymerase chain reaction (PCR) fragments to homoeologous chromosomes (Sears, 1954).

2.2. Semolina and pasta processing

Grain samples were cleaned, scoured, and tempered to 17.5% moisture (dry basis). Tempered grain was milled into semolina using a Bühler experimental mill fitted with two Miag laboratory scale purifiers (Bühler-Miag, Minneapolis, MN, USA). Semolina was hydrated to 32% moisture (dry basis), while being mixed at high speed for

4 min in a Hobart A120 mixer (Hobart Corporation, Troy, OH, USA). The hydrated semolina was placed in a mixing chamber under vacuum, and extruded as spaghetti using a DeMaCo semi-commercial laboratory extruder (DeFrancisci Machine Corp, Melbourne, FL, USA). The extruder was operated under the following conditions: extrusion temperature of 45 °C, mixing chamber vacuum with 46 cm of Hg, and auger extrusion speed of 25 rpm. The extruded spaghetti was cut to approximately 1 m, hung on a rod, and dried in a laboratory pasta drier (Standard Industries, Inc., Fargo, ND, USA) using a low temperature (40 °C) drying cycle.

2.3. Molecular methods

DNA extraction was performed from leaves of the parental lines and each of the 93 RILs as described before (Dvorak et al., 1988). Different primer combinations based on published sequences (Hessler et al., 2002; van Mechelen et al., 1999) were used to amplify preferentially each of the wheat sequences orthologous to barley *LoxA*, *LoxB*, or *LoxC* genes. Primers LOXA-L1 (CTGATCGACGTCAA CAAC) and LOXA–R1 (CAGGTACTCGCTCACGTA) were based on the barley sequences and were designed to amplify preferentially *LoxA* (*Lpx-1*) over *LoxB* or *LoxC* genes. Three additional primers were used to confirm an *Lpx-1* deletion suggested by the first pair of primers. Primers LOXA-4BSL and LOXA-4BSR were developed by Hessler et al. (2002) and primer LOXA-4BSL2 (TCTCAT CAAGAGGTACCTACTTA) was based on the *Lpx-B1* sequence (Hessler et al., 2002).

Primers LOXB-L (CACGATAACTTCATGCCAT) and LOXB-R (ACTCCTCCAGCTCCTTGT) were designed to amplify preferentially *Lpx-3* (wheat orthologues of barley *LoxB*), whereas primers LOXC-L (GACATCAA CAACCTCGAC) and LOXC-R (CGTACGGG TAGTCCTTGA) were designed to amplify *Lpx-2* (wheat orthologues of *LoxC*). Amplification reactions were perfotmed in a MJ Research termocycler model PTC 100 in a 25 µl reaction mixture. Each reaction consisted of 200 µM dNTPs, 1.5 mM $MgCl_2$, 100 nM of each primer, 1 U of *Taq* polymerase and 75–150 ng of genomic DNA as template. PCR amplification conditions were as follow: 3 min at 94 °C; 5 touchdown cycles (−1 °C each) of 30 s at 94 °C, 30 s at 60–55 °C and 45 s at 72 °C. After that, 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C followed by final extension of 10 min at 72 °C.

For sequencing, purified PCR products were cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Recombinant clones were purified and sequenced using an ABI3730 automatic sequencer. Program Primer3 (http://www.frodo.wi.mit.edu/cgi-bin/primer3/) was used for primer design. Best-fit alignments between sequences were obtained using ClustalW 1.8 (http://www.searchlauncher.bcm.tmc.edu/multialign/multi-align.html). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/).

2.4. Trait evaluation

LOX extraction and substrate preparation were performed as described by McDonald (1979) and Surrey (1964). Briefly, crude extracts were prepared using 2 g of semolina in 10 ml of cold 0.1 M sodium phosphate buffer, pH 7.0. Substrate solution was prepared as follows: Tween 20, 0.5 ml, was dissolved in 10 ml of 0.1 M borate buffer at pH 9.0 and 0.5 ml of linoleic acid (99%) was added drop by drop. Finally the solution was adjusted at pH 4.8 and 6.6 with concentrated HCl. These two pH were selected because they correspond to activity peaks of LOX isoforms (McDonald, 1979) which would be active at the pH of the pasta dough. The substrate was divided into small volumes, sealed under N_2 and maintained at −20 °C. For the LOX assays, 9 ml of linoleate substrate and 1 ml of semolina extract was used in the reaction tube with O_2 passed continuously through the reaction mixture.

Enzyme activity in samples of Kofa and UC1113 was evaluated in semolina extracts over a period of 22.5 min, taking aliquots every 2.5 min of reaction at 25 °C. The corresponding measurements in the RILs were taken at 8 min from the reaction starting point, which was determined as an appropriate time to differentiate both alleles in the previous time course. LOX activity was determined by measuring diene absorption at 234 nm with an UV2100 Shimadzu spectrophotometer. Enzyme activity was monitored through the linoleic acid hydroperoxides production and was expressed as nanomoles of hydroperoxides (ROOH) per g of semolina per minute, using a molar absorptivity 2.8×10^4/M/cm (Privett et al., 1955). Analytical tests on semolina samples were performed in triplicate and averaged.

CIE *b*-value (yellowness) of semolina, dry spaghetti, and cooked spaghetti was quantified using a colorimeter (Minolta chromameter model CR310, Minolta Corp., Ramsey, NJ). For each sample, CIE *b*-value was an average of three separate measurements. To minimize the effect of semolina particle size on CIE *b*-values, all grain samples were milled on the same mill by the same miller. Measurements for semolina color (SC) were made using a black cell (5 cm id, 0.9 cm deep) with a quartz window. Measurements for dry pasta color (DPC) were made using a black template that maintained a spaghetti depth of 1 cm. For the cooked pasta color (CPC) determinations, spaghetti (10 g, 5 cm long) was cooked for 12 min in 300 ml boiling distilled water. The water was drained and the spaghetti was allowed to cool for 5 min before the *b*-value was measured. Measurements for cooked spaghetti were made using a glass cell (7.5 cm id, 0.5 cm deep) that had a black background and a quartz glass window. The effect of the allelic variation at the LOX loci on DPC and CPC were compared.

For the evaluation of the yellow pigment content, we used a modified version of the AACC 14–50 procedure as described by Zhang et al. (2005). Pigments were extracted from 0.5 g of integral flour with three volumes

of water-saturated 1-butanol and the spectrophotometric determinations performed out at 448 nm.

2.5. Data analysis

Since the values for LOX activity at pH 4.8 and 6.6 were highly correlated in our population ($R = 0.92$ and 0.91, for 2003 and 2004, respectively), the average of both determinations was used for all statistical analyses. RILs were classified into Kofa and UC1113 alleles for the *Lpx-B1.1* and *Lpx-A3* loci using the primers described above. Differences in LOX activity, and semolina and pasta color (CIE *b*-value) between these two classes were tested using a factorial analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute Inc. 2004). The three fixed factors considered were loci *Lpx-B1.1* and *Lpx-A3* and year. The model included these three factors plus the interactions between the two loci and the interactions of each locus with year. The three-way interaction was not significant and was merged with the error. Normality of the residuals was confirmed by the Kolmogorov-Smirnov test (SAS Institute Inc., 2004).

Genetic distances between *Lpx* loci and flanking microsatellite markers were calculated with the program MapMaker (Lander et al., 1987) using the Kosambi function (Kosambi, 1944).

3. Results

3.1. Lpx-1: Wheat sequences orthologous to barley LoxA

Primers LOXA-L/R amplified two bands in UC1113 and one in Kofa (Fig. 1A). To test if the missing higher band in Kofa was the result of a point mutation in the primer annealing site or a deletion affecting a larger portion of this LOX gene, the same DNAs were tested with primers LOXA-4BS-L/4BS-R (Fig. 1B) and LOXA-4BS-L2/4BS-R (Fig. 1C) that were specific for the sequence of the upper band. These primers amplified a single fragment in UC1113 and no product in Kofa, suggesting that in the last variety a deletion is present in the LOX gene corresponding to the higher band in Fig. 1A.

Nullisomic-tetrasomic analyses showed that the two bands amplified by primers LOXA-L/R and the single fragments amplified by the other two primer pairs, where absent in the N4BT4D line but present in N4AT4D and N4DT4B, as well as in Chinese Spring (CS, Fig. 1A-C), indicating that they were both amplified from chromosome 4B. In the absence of the primary target, alternative bands were amplified in N4BT4D (Fig. 1A). The presence/absence of polymorphisms between UC1113 and Kofa detected by these three pairs of primers (Fig. 1A–C, first two lines) were completely linked in the 93 RILs, as expected from fragments amplified from the same gene. Two microsatellite markers were also mapped in the same RILs. Microsatellite locus *Xwmc617b* (Kofa 205-bp, UC1113 210-bp), previously mapped on chromosome 4B

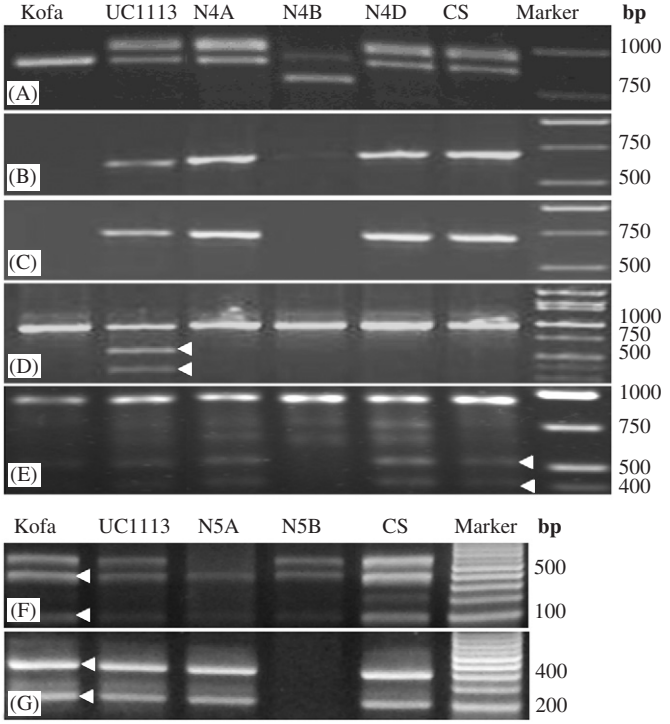

Fig. 1. Genomic segments of LOX genes obtained using primers: (A) LOXA-L/R, (B) LOXA-4BSL/4BSR, (C) LOXA-4BSL2/4BSR, (D–E) LOXB-L/R, F) LOXC-L/R, and (G) LOXC-L/18R2 followed by digestions with (D) *Hae*II, (E) *Ase*I or (F–G) *Hae*III (arrowheads indicate digestion products). Genomic DNAs from left to right are durum lines Kofa and UC1113, and hexaploid nulli-tetrasomic lines (A–E) N4AT4D (N4A), N4BT4D (N4B) and N4DT4B (N4D), and Chinese Spring (CS), (F–G) N5AT5B (N5A), N5BT5D (N5B) and CS.

(Somers et al., 2004), was mapped 13-cM distal to *Lpx-B1* whereas microsatellite locus *Xksm62* (Kofa 185-bp, UC1113 190-bp) was mapped 8-cM proximal to the LOX locus.

The sequence of the UC1113 higher band (Fig. 1A) amplified with primers LOXA-L/R (Fig. 1A) was combined with the overlapping sequence of the fragment amplified with primers LOXA-4BSL/R, and deposited as GenBank DQ474240. The predicted cDNA showed higher identity with the barley *LoxA* gene (95%) than with the barley *LoxB* (83%) or *LoxC* (83%) genes, confirming that it belongs to the *Lpx-B1* locus. The predicted partial cDNA sequence from the higher band is 100% identical to wheat expressed sequence tag (EST) BJ280227, suggesting that this LOX gene is transcribed.

The DNA sequence of the lower band (GenBank DQ474241) showed no polymorphisms between Kofa and UC1113 and was 95% identical to the genomic sequence from the higher band (98% identical at the protein level). The coding region of the lower band also showed higher identity with the barley *LoxA* gene (94%) than with the barley *LoxB* (83%) or *LoxC* (82%) genes. The predicted partial cDNA sequence from the lower band is 99.5% identical to wheat ESTs BQ743519 and CK206737 suggesting that this LOX gene is transcribed.

These results indicate that there are two *Lpx-B1* loci on wheat chromosome 4B. We propose to designate the locus corresponding to GenBank DQ474240 as *Lpx-B1.1* and the locus corresponding to GenBank DQ474241 as *Lpx-B1.2*. According to this nomenclature Kofa has a deletion in the *Lpx-B1.1* locus.

The sequence of the higher band includes a 320-bp region that is almost identical to sequence C7.1.2 published by Hessler et al. (2002), except for one SNP which is also polymorphic between their parental lines 'Jennah Khetifa' and 'Cham 1'. This region includes a complete MITE element that is absent in the lower band. The lower band includes a 193-bp region that is identical to sequence J2.2 from Hessler et al. (2002).

3.2. Lpx-2: Wheat sequences orthologous to barley LoxC

Primers designed to amplify the wheat orthologous sequences for the barley *LoxC* gene (LOXC-L LOXC-R) produced a PCR fragment of approximately 700-bp in both parental lines. Two different groups of sequences were obtained from this PCR product in parental line UC1113. These sequences were 96% identical, suggesting that they were from two different genomes.

The coding portion of these two sequences showed higher identity with the barley *LoxC* gene (95–96%) than with the barley *LoxA* (83%) or *LoxB* (77%) genes, confirming that these sequences were amplified from wheat *Lpx-2* genes.

This higher sequence similarity between wheat *Lpx-2* and the barley *LoxC* gene was paralleled by a similar chromosome location. The *LoxC* gene has been previously mapped on barley chromosome 5H (van Mechelen et al., 1999) and the two wheat sequences were assigned to wheat chromosomes from homoeologous group 5 (Fig. 1F–G). The first group of wheat sequences did not have any internal *Hae*III restriction site. After digestion of the LOXC-L/R PCR products with this restriction enzyme the undigested 700-bp fragment disappeared in nullitetrasomic line lacking chromosome 5A (Fig. 1F), indicating that this sequence corresponded to the *Lpx-A2* gene (GenBank DQ448002).

A sequence specific primer LOXC18R2 (5′-TCCACGTCATTTGTCATTGAT′-3′) was designed in the intron of the sequence carrying the *Hae*III restriction site. This genome specific primer in combination with primer LOXC-L failed to amplify any product in the nulli-tetrasomic line lacking the chromosome 5B (Fig. 1G) confirming that this second sequence corresponded to the *Lpx-B2* gene (GenBank DQ448001).

3.3. Lpx-3: Wheat sequences orthologous to barley LoxB

Primers LOXB-L/R amplified fragments of approximately 900-bp in the two parental genotypes. Two groups of sequences were detected in the *LoxB* PCR products in both parents, which were later assigned to the A and B genomes. These sequences were 97% identical and differed in length by 9-bp (approximately 920 and 930-bp) due to the presence of several polymorphic indels in the predicted intron region.

The short 920-bp fragment showed three SNPs between Kofa and UC1113. One of these SNPs determined a *Hae*II restriction site in UC1113 (GenBank DQ474244), which was absent in Kofa (GenBank DQ474242). This polymorphic *Hae*II site was used to develop a dominant Cleavage Amplification Polymorphic Sequence (CAPS) marker. After *Hae*II digestion Kofa showed only the 920–930 bp fragments, whereas UC1113 showed an undigested fragment (930-bp) and two additional fragments of 360 and 560-bp (Fig. 1D). This *Hae*II polymorphism was not present in Chinese Spring and therefore, it could not be assigned to a particular chromosome using nulli-tetrasomic lines (Fig. 1D). The *Hae*II restriction site polymorphism was mapped in the RIL population 15-cM proximal to microsatellite locus *Xgwm192b* (Kofa 135-bp, UC1113 140-bp) and 10-cM distal to microsatellite locus *Xwmc617a* (Kofa 285-bp, UC1113 265-bp) previously mapped on chromosome 4A (Somers et al., 2004). This result indicates that this sequence was amplified from chromosome 4A.

The larger 930-bp fragment showed no polymorphisms between Kofa and UC1113 and therefore could not be mapped in the RIL population (GenBank DQ474243). However, this fragment differed from the 920-bp fragment by the presence of an *Ase*I restriction site. Digestion of the LOXB-L/R PCR products with *Ase*I, showed that the digested products were absent in the nulli-tetrasomic line N4BT4D (Fig. 1E). This result showed that the sequence carrying the *Ase*I restriction site was amplified from chromosome 4B.

The 920-bp and 930-bp sequences from both Kofa and UC1113 showed higher identity with the barley *LoxB* gene (92–93%) than with the barley *LoxA* (81%) or *LoxC* (79%) genes, confirming that these sequences are orthologous to the *LoxB* gene, previously mapped in a colinear region in barley chromosome 4H (van Mechelen et al., 1999). We propose to designate the wheat locus as *Lpx-3* to be consistent with the previous wheat nomenclature. The 920-bp product mapped on chromosome 4A corresponds to the *Lpx-A3* locus (GenBanks: DQ474244, DQ474244, DQ474242) whereas the 930-bp product assigned to chromosome 4B corresponds to the *Lpx-B3* locus (GenBank DQ474243).

3.4. Association between polymorphic Lpx loci and quality traits

3.4.1. Lipoxygenase activity

To determine the optimum parameters to differentiate the LOX activity between UC1113 and Kofa, we tested the enzyme activity in function of time at pH 4.8 and 6.6 (Fig. 2A and B). At both pHs, UC1113 showed consistently higher LOX activity than did Kofa at most of the tested

times. Similar curves were observed at both pHs and, therefore, the average pH was used in all the LOX statistical analyses described below. The differences observed between the parental lines were also present among the RILs, which showed a clear bimodal distribution (Fig. 2C).

The three-way factorial ANOVA including the two mapped *Lpx* loci and years as factors showed that LOX activity in semolina was significantly affected by the *Lpx-B1.1* locus ($P<0.0001$) but not by the *Lpx-A3* locus ($P=0.89$, Table 1). In this segregating population, single way ANOVAs for each year including only the *Lpx-B1.1* locus as a factor showed that this locus explained between 57% (2003) and 53% (2004) of the variation in LOX activity. The LOX activity of the RILs carrying the *Lpx-B1.1* deletion allele from Kofa was 4.5-fold lower than the activity observed in the RILS carrying the normal UC1113 allele (Least Squares adjusted averages, Fig. 3A). A significant value ($P=0.02$) was associated with year suggesting a small environmental effect on LOX activity. No significant interactions were observed between year and loci indicating that the loci effects were consistent across years (Table 1).

To explore the possibility that the LOX activity associated with the *Lpx-B1.1* locus was actually the result of another linked locus, we performed an additional ANOVA with the loci *Xwmc617b* and *Xksm62*, which flank the *Lpx-B1.1* locus on chromosome 4B. The *F*-value for the *Lpx-B1.1* locus in the complete ANOVA model ($F=211.1$) was larger than the corresponding values for *Xwmc617b* ($F=28.4$) and *Xksm62* ($F=94.5$), suggesting that the differences in LOX activity were generated by variation at the *Lpx-B1.1* locus itself or by a closely linked gene located within the *Xwmc617b–Xksm62* interval.

3.4.2. Semolina color

A significant effect on semolina CIE *b*-values was detected for the *Lpx-A3* locus ($P<0.0001$) but not for the *Lpx-B1.1* locus ($P=0.51$, Table 1, Fig. 3B). Lines carrying the UC1113 allele at the *Lpx-A3* locus showed an adjusted average for semolina color (26.9) that was 4.7% higher than the adjusted average for Kofa (25.7).

For the 2003 samples, we also measured 1-butanol-extracted yellow pigments and found a significant correla-

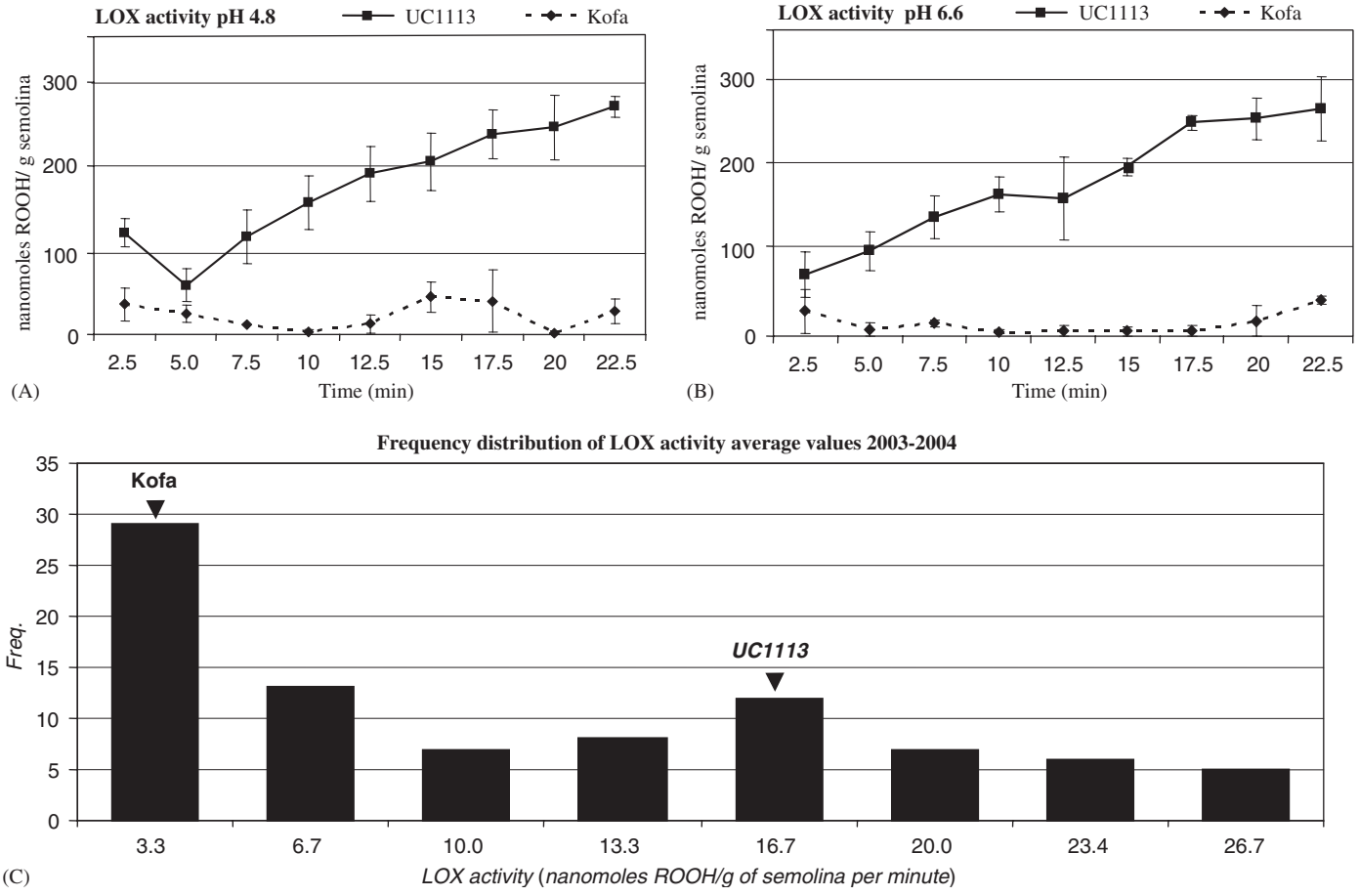

Fig. 2. (A-B) Time course of LOX activity in semolina extracts of the experimental line UC1113 (——) and the variety Kofa (- - - -) at pH 4.8 (A) and 6.6 (B). Activity is expressed as nanomoles of hydroperoxides per g of semolina. (C) Frequency distribution of the 93 lines with different levels of LOX activity measured at 8 min (average of pH 4.8 and 6.6 activity values). Activity is expressed as nanomoles of hydroperoxides per g of semolina per minute. Arrows indicate the averages of the lines with the UC1113 or Kofa *Lpx-B1.1* alleles. Note the bimodal distribution.

Table 1
Factorial analysis of variance for factors *Lpx-B1*, *Lpx-A3*, and year

Source	DF	LOX *P*	SC *P*	DPC *P*	CPC *P*
Lpx-B1(4B)	1	<0.0001***	0.5060	<0.0001***	0.0010***
Lpx-A3(4A)	1	0.8944	<0.0001***	0.0070**	0.0009***
Lpx-B1 × *Lpx-A3*	1	0.3645	0.4730	0.6269	0.3412
Year	1	0.0208*	0.8083	0.0430*	<0.0001***
Year × *Lpx-B1*	1	0.7739	0.9757	0.6249	0.0422*
Year × *Lpx-A3*	1	0.1392	0.5510	0.6789	0.6609

Probability values for LOX activity, semolina color (SC), dry pasta color (DPC), and cooked pasta color (CPC). Symbols *, **, and *** indicate $P<0.05$, $P<0.01$, and $P<0.001$ respectively.

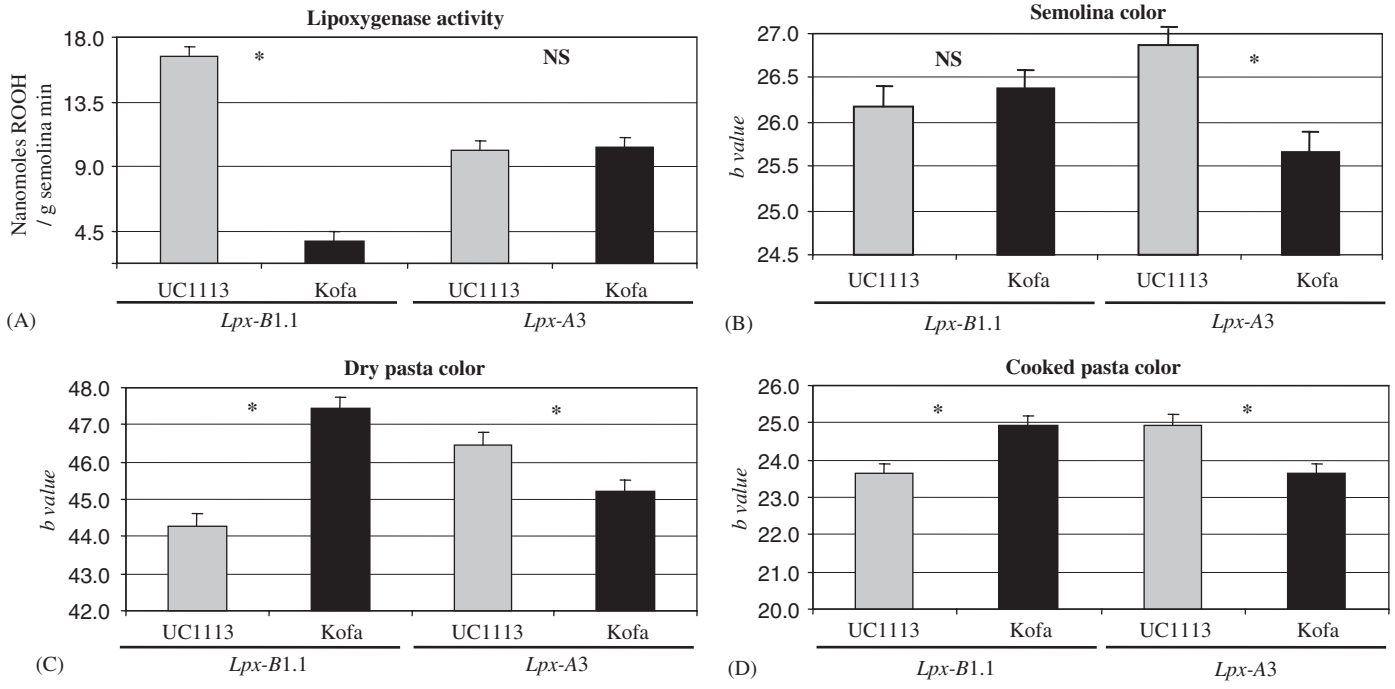

Fig. 3. Least square mean values for LOX activity (A), semolina color (B) dry pasta color (C) and cooked pasta color (D) obtained from RIL classified by the UC1113 or Kofa *Lpx* alleles. Values are averages from the two years and error bars represent one standard error of the mean. *$P<0.01$, NS = not significant difference ($P>0.05$). Activity is expressed as nanomoles of hydroperoxides per gram of semolina per min.

tion with the CIE *b*-values obtained from the same samples ($R = 0.81$). Since the two values were correlated, we used only the CIE *b*-value for the 2004 samples and for the statistical analyses.

To test if the effect on semolina CIE *b*-values of the chromosome 4A region was higher for the markers flanking the *Lpx-A3* locus, we performed an additional ANOVA with flanking loci *Xgwm192b* and *Xwmc617a*. The *F*-value for the *Lpx-A3* locus ($F = 16.9$) was larger than the corresponding values for *Xgwm192b* ($F = 5.4$) and *Xwmc617a* ($F = 6.0$), suggesting that the differences in semolina color were generated by variation at the *Lpx-A3* locus or by a closely linked gene located between *Xgwm192b* and *Xwmc617a*.

3.4.3. Dry pasta color

The dry pasta *b*-values showed significant effects for both *Lpx* loci, most likely as a result of a combination of their previous effects on both LOX activity and semolina color (Table 1). The *Lpx-A3* allele from UC1113 was associated with an increase in pasta color ($P = 0.007$), most likely as a result of the higher level of semolina pigments associated with this allele. The *Lpx-B1.1* allele from Kofa was associated with an increase in dry pasta *b*-values ($P<0.00001$), likely determined by the lower level of LOX pigment degradation during pasta processing associated with the *Lpx-B1.1* deletion (Fig. 3C).

The effects of these two loci were mainly additive, as indicated by a non-significant interaction between the two loci ($P = 0.63$). The *Lpx-A3*-UC1113/*Lpx-B1.1*-Kofa allele combination showed the highest *b*-value (adjusted mean = 47.9), whereas the opposite allele combination, *Lpx-A3*-Kofa/*Lpx-B1.1*-UC1113 showed the lowest *b*-value (adjusted mean = 43.5). The combination of the two positive alleles resulted in a 10% increase in *b*-values relative to the combination of the two negative alleles.

Table 2
Distribution of the *Lpx-B1.1* deletion among 15 durum wheat varieties and its association with LOX activity in semolina extracts at pH 4.8 and 6.6. Activity is expressed as nanomoles of hydroperoxides per gram of semolina per min using absorbance at 234 nm and a 1-cm light path cell

Variety	Origin	*Lpx-B1.1*	pH-4.8	pH-6.6
Bonaerense INTA Cariló	Argentina	Deletion	1.3	2.2
Adamello	Italy	Deletion	2.7	0.0
Bonaerense INTA Cumenay	Argentina	Deletion	2.4	0.0
ACA 001	Argentina	Deletion	12.0	4.5
ACA 003	Argentina	Deletion	3.6	0.0
Colosseo	Italy	Deletion	20.2	21.4
Ciccio	Italy	Deletion	7.8	6.0
Buck Ambar	Argentina	Deletion	12.0	7.6
Buck Topacio	Argentina	Wild type	29.6	48.7
Duilio	Italy	Wild type	29.6	30.3
Bonaerense INTA Facón	Argentina	Wild type	25.6	26.3
Buck Esmeralda	Argentina	Wild type	39.2	38.3
Simeto	Italy	Wild type	12.0	8.7
IAT2-65	CIMMYT	Wild type	20.7	18.7
IAT2-66	CIMMYT	Wild type	20.7	17.4
Average *LpxB1.1* wild type			25.3	26.9
Average *LpxB1.1* deletion			7.8	5.2
Wild type vs. deletion *P* value			0.0006	0.0017

The effect of the different years was small ($P = 0.04$) compared to the genetic effects and no significant year-genotype interactions were detected. The complete ANOVA model explained 31% of the variation in dry pasta in this population. The significance of the differences in color *b*-values at the *Lpx-A3* locus ($F = 10.5$) was larger than at the flanking loci *Xgwm192b* ($F = 4.3$) or *Xwmc617a* ($F = 4.1$). A similar result was observed for the *Lpx-B1.1* locus that showed larger differences in DPC ($F = 50.2$) than flanking markers *Xwmc617b* ($F = 26.4$) or *Xksm62* ($F = 23.9$). These results suggest that the differences in DPC were originated in variation at the LOX loci or at closely linked genes located within the interval delimited by the respective flanking markers.

3.4.4. Cooked pasta color

As expected, the cooked pasta *b*-values showed similar results to those observed for dry pasta ($R = 0.70$ over the two years). Significant increases in cooked pasta *b*-values were associated with the *Lpx-A3* allele from UC1113 ($P = 0.0009$) and the *Lpx-B1.1* allele from Kofa ($P = 0.001$), with no significant interactions.

The lines with the optimum allele combination showed cooked pasta *b*-values (adjusted average = 25.8) that were 11% higher than the *Lpx-A3*-Kofa/*Lpx-B1.1*-UC1113 combination (adjusted average = 23.2, Fig. 3D). The effect of the different years on cooked pasta was larger than the one observed in dry pasta ($P < 0.0001$) and showed a marginally significant interaction with the *Lpx-B1.1* locus ($P = 0.04$), which was the result of smaller differences between the two *Lpx-B1.1* alleles in 2004 relative to 2003. The complete ANOVA model explained 29% of the variation in cooked pasta in this population.

The difference in CPC detected at the *Lpx-A3* locus ($F = 15.3$) was more significant than the one detected at the flanking loci *Xgwm192b* ($F = 5.7$) or *Xwmc617a* ($F = 8.1$); whereas the difference associated with the *Lpx-B1.1* locus ($F = 12.5$) was more significant than the one detected at flanking markers *Xwmc617b* ($F = 7.5$) or *Xksm62* ($F = 8.6$). These results indicate that the differences in CPC were most likely originated by variation at the LOX loci or by closely linked genes located within the interval delimited by these two flanking markers.

3.5. Distribution of the Lpx-B1.1 deletion across the durum wheat germplasm

Since a highly significant decrease in LOX activity was observed in the RILs carrying the *Lpx-B1.1* deletion, we decided to explore the distribution of this deletion across a larger set of germplasm and to test its effect on LOX activity. Results from these analyses are shown in Table 2. All varieties and lines included in this survey showed either an *Lpx-B1.1* fragment of similar size (Fig. 1A, fragment #1) as the one observed in UC1113 (47%) or its absence, as observed in Kofa (53%).

Highly significant differences in LOX activity were detected between the varieties with and without the *Lpx-B1.1* deletion, both at pH 4.8 ($P < 0.0007$) and at pH 6.6 ($P < 0.002$). This result indicates that the allelic differences at the *Lpx-B1.1* locus are strongly associated with LOX activity in this set of varieties.

4. Discussion

4.1. Evolution of LOX genes in the Triticeae

The map location of the wheat LOX genes obtained in this study agrees with previous mapping studies in wheat and barley (Hart and Langston, 1977; Li et al., 1999;

Nachit et al., 2001; van Mechelen et al., 1999). The *Lpx-B1* locus was mapped on the short arm of chromosome 4B, whereas the *Lpx-A3* locus was mapped on the long arm of chromosome 4A. The different arm locations are the result of structural rearrangements that occurred in wheat chromosome 4A (Devos et al., 1995), and the two regions are orthologous. A similar result has been reported for wheat EST BE442666 (99.8% identical to the *Lpx-A3* sequences reported here), which was mapped by hybridization in bins located in the short arm of chromosomes 4B (4BS8-0.57-0.81) and 4D (4DS3-0.67-0.82) but in the long arm of chromosomes 4A (4AL13-0.59-0.66) (http://www.wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class = locus;name = CNL045BE442666–1).

The *Lpx-1* and *Lpx-3* loci map to colinear regions on chromosomes from homoeologous group 4 chromosomes but have not been mapped together in the same chromosome in any of the current wheat maps, precluding the calculation of the actual distance between these two loci in wheat. However, since wheat and barley show good colinearity within this chromosome segment (Dubcovsky et al., 1996), their relative position can be inferred from their map location in barley, where they were mapped only 1 cM apart on the short arm of chromosome 4HS (van Mechelen et al., 1999). The close linkage between these two genes is relevant for this study because, even if a significant effect on LOX activity or color is detected for one of these loci, it is not possible to determine if the effect is originated by variation at the *Lpx-1* or at the *Lpx-3* locus. Therefore, results should be interpreted with caution.

The discovery of two related copies of *Lpx-B1* on chromosome 4B, *Lpx-B1.1* and *Lpx-B1.2*, complicates even further the interpretation of the observed differences in LOX activity or semolina and pasta color. The existence of wheat ESTs more than 99.5% identical to each of these two copies suggests that both genes are transcribed and likely active. The level of divergence between *Lpx-B1.1* and *Lpx-B1.2* DNA sequences (95% for genomic DNA and 97% for the predicted cDNA) indicates that this duplication occurred close to the time of divergence of the A, B and D genomes but after the wheat-barley divergence (Ramakrishna et al., 2002). This explains why this duplication is not present in the barley genome where the three LOX genes have been reported to be single-copy members of a small gene family (van Mechelen et al., 1999).

The close proximity in time between the duplication of the *Lpx-B1.1* and *Lpx-B1.2* loci and the divergence of the different wheat genomes makes it difficult to predict if similar duplications occurred in the A and D genomes. The presence of only two additional groups of *T. aestivum* ESTs each of them 96–97% similar to *Lpx-B1.1* (CA731270 and CV761196) and *Lpx-B1.2* (CD911683 and BE516334) suggests that only the B genome has duplicated functional *Lpx-1* genes. This prediction requires experimental validation.

Hybridization of hexaploid wheat with a maize LOX probe has revealed a total of 11 bands located on chromosomes 4 and 5 (Li et al., 1999). This result is consistent with our findings of two *Lpx-1* genes in the B genome (with single or duplicated copies in the A and D genomes) and single copies of the *Lpx-2* and *Lpx-3* loci in each of the genomes (total 10 to 12 LOX genes).

4.2. Characterization of loci affecting color and LOX activity in durum wheat

We found a high correlation between LOX activity at pH 4.8 and 6.6 ($R > 0.9$), in agreement with previous studies showing appreciable LOX activity in the pH range from 4.8 to 7.5 (Barone et al., 1999). Borrelli et al. (1999) also found a significant correlation between LOX activity at pH 4.8 and 6.6 and suggested that they might be the result of the activity of the same isoenzymatic form. LOX enzymes with optimum activity within this pH range have been reported as being primarily responsible for the loss of carotenoid pigments during pasta processing, because they are active at the pH found in pasta dough (Borrelli et al., 1999). The previous observation is consistent with the effect of the *Lpx-B1.1* deletion on LOX activity and pasta color, but not on semolina color. These results indicate that the differences in pasta color associated with this locus were most likely due to differences in the degradation of the pigments during pasta processing rather than differences in the degradation of pigments during grain development.

The *Lpx-B1.1* locus seems to be an important breeding target for pasta color improvement. In our mapping population, this locus alone explained 54% of the variation in LOX activity and 25% of the variation in DPC ($P < 0.0001$). Hessler et al. (2002) also detected a significant effect of the *Lpx-B1* locus, which explained 36% of the variation in LOX activity in their mapping population. As in our study these differences were not associated with differences in semolina color (pasta color was not determined in that study).

Although the previous studies suggest that the *Lpx-B1* locus is responsible for the observed differences in LOX activity and pasta color, it is not possible to rule out the possibility that these differences were determined by allelic variation at the closely linked *Lpx-B3* locus, also located on the short arm of chromosome 4B and likely closely linked to the *Lpx-B1* locus (van Mechelen et al., 1999). However, two indirect lines of evidence suggest that *Lpx-B1* may have a more important role than *Lpx-B3* on the observed differences in LOX activity and pasta color.

First, transcript levels of the barley *LoxB* gene (orthologue of wheat *Lpx-3*) are detected mainly in germinating grains and are extremely low during grain development compared with *LoxA* and *LoxC* (Schmitt and van Mechelen, 1997; van Mechelen et al., 1999). On the contrary, *LoxA* (orthologue of wheat *Lpx-1*) accounts for most of the total LOX activity in mature barley grains (Schmitt and van Mechelen, 1997) and therefore, is the one

that can affect pigments during pasta processing. A second line of evidence supporting the more important role of the *Lpx-1* gene on LOX activity was the discovery of a deletion in the *Lpx-B1.1* locus in the parent with low LOX activity (Kofa). This deletion provides a simple explanation for the high reduction in LOX activity observed in this variety, and for the differences observed in the set of 15 varieties analyzed in this study. However, these two lines of evidence are indirect and more direct genetic experiments including lines with recombination events between the *Lpx-B1* and *Lpx-B3* loci will be necessary to determine the relative importance of these two genes on the differences in LOX activity and pasta color.

In contrast, with the 4BS locus, the 4AL *Lpx-A3* locus was not associated with differences in LOX activity in semolina but showed a significant effect on semolina color (Table 1). This result suggests that the differences in LOX activity may have occurred earlier during grain development, when they could affect the final level of carotenoid pigments in the grain. Similar examples of a relationship between the LOX genes and semolina color have been reported before (Manna et al., 1998). These authors observed a strong negative correlation ($R = -0.954$) between LOX activity at pH 10.2 and semolina yellow index in a collection of durum wheat varieties.

As in the case of the *Lpx-B1*/*Lpx-B3* loci, we do not know if the observed differences in semolina color are the result of allelic variation at the mapped *Lpx-A3* locus or at the putatively linked *Lpx-A1* locus. Unfortunately, in this case we do not have a clear polymorphism to support one hypothesis or the other. In addition, we cannot rule out the possibility that these differences in semolina color were not caused by a LOX gene, but by another closely linked gene located within the SSR markers flanking this region.

4.3. Breeding for improved pasta color

The results described above suggest that different LOX genes might have differential effects at different points of the grain development affecting both the final pigment content of the grain or its degradation during pasta processing. Therefore, different LOX loci can be targeted to obtain combinations that simultaneously increase semolina color and minimize pigment degradation during processing, determining an optimum pasta color.

In our mapping population, the larger decrease in LOX activity was associated with the *Lpx-B1.1* deletion. A preliminary screening of 15 varieties from diverse origins showed that this deletion was present in approximately half of the varieties and was significantly associated with differences in LOX activity at pH 4.8 and 6.6. The high frequency of the *Lpx-B1.1* deletion in the cultivated durum germplasm analyzed here may be the result of a strong selection pressure for increased pasta yellow pigment.

Based on the previous results, the *Lpx-B1.1* deletion seems to be a valid target for durum breeding programs aimed to improve pasta color. The LOXA-L/R primers provide a good tool to identify lines homozygous for the *Lpx-B1.1* deletion, because they also amplify *Lpx-B1.2*, which serves as a positive control of PCR amplification. Unfortunately, this is a dominant marker and cannot be used to identify heterozygous carriers of the *Lpx-B1.1* deletion. If heterozygous plants need to be selected during marker assisted selection or backcrossing, the flanking microsatellite markers identified in this study may provide a useful alternative tool.

In addition to the *Lpx-B1.1* deletion and its effect on pigment degradation during pasta processing we found allelic variation at the *Lpx-A3* locus associated with differences in semolina and pasta color. The interaction between these two *Lpx* loci was not significant, indicating that the effect of these two loci was additive and can, therefore, be combined to obtain an optimum pasta color. The best pasta color was observed in the lines combining the *Lpx-A3* allele from UC1113 (higher semolina pigment) with the *Lpx-B1.1* deletion from Kofa (lower pigment degradation during pasta processing). These lines, carrying the optimum allele combination, showed a 10% increase in dry pasta *b*-values relative to the lines with the opposite (worst) allele combination (Fig. 3C).

The CAPS marker for *Lpx-A3* designed in this study (or the flanking microsatellite loci) can be used to combine the UC1113 *Lpx-A3* allele with the Kofa *Lpx-B1.1* deletion by marker assisted selection. These molecular markers provide a valuable tool to the breeders to select durum lines with improved semolina color and lower carotenoid degradation during pasta processing, which will result in improved pasta color.

Acknowledgments

The project was supported by the National Research Initiative of USDA's Cooperative State Research, Education and Extension Service, CAP grant number 2006-55606-16629 and by NSF-CONICET AMERICAS grant 0404590 and by the Fondo para la Investigación Científica y Tecnológica (FONCyT—INTA) PICTO 12948 (Argentina).

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