Phytoene synthase 1 (Psy-1) and lipoxygenase 1 (Lpx-1) Genes Influence on Semolina Yellowness in Wheat Mediterranean Germplasm

Roberto Parada 1, Conxita Royo 2, Agata Gadaleta 3, Pasqualina Colasuonno 3, Ilaria Marcotuli 3, Iván Matus 4, Dalma Castillo 4, Adriano Costa de Camargo 1,5, Jorge Araya-Flores 1, Dolors Villegas 2 and Andrés R. Schwember 1,*

1 Departamento de Ciencias Vegetales, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Santiago 306-22, Chile; Roberto.paradasalazar@gmail.com (R.P.); adrianoesalq@gmail.com (A.C.d.C.); j.araya.f@gmail.com (J.A.-F.)
2 Sustainable Field Crops Programme, Institute for Food and Agricultural Research and Technology (IRTA), 25191 Lleida, Spain; Conxita.Royo@irta.cat (C.R.); dolors.villegas@irta.cat (D.V.)
3 Department of Agricultural and Environmental Science, University of Bari Aldo Moro, 70121 Bari, Italy; agata.gadaleta@uniba.it (A.G.); pattybiotec@yahoo.it (I.C.); i.marcotuli@gmail.com (I.M.)
4 Instituto de Investigaciones Agropecuarias (INIA), Centro Regional de Investigación Quilamapu, Chillán 426, Chile; imatus@inia.cl (I.M.); dalma.castillo@inia.cl (D.C.)
5 Laboratory of Antioxidants, Nutrition and Food Technology Institute, University of Chile, Santiago 7810000, Chile
* Correspondence: aschwember@uc.cl

Received: 9 June 2020; Accepted: 25 June 2020; Published: 30 June 2020

Abstract: Phytoene synthase 1 (Psy1) and lipoxygenase 1 (Lpx-1) are key genes involved in the synthesis and catalysis of carotenoid pigments in durum wheat, regulating the increase and decrease in these compounds, respectively, resulting in the distinct yellow color of semolina and pasta. Here, we reported new haplotype variants and/or allele combinations of these two genes significantly affecting yellow pigment content in grain and semolina through their effect on carotenoid pigments. To reach the purpose of this work, three complementary approaches were undertaken: the identification of QTLs associated to carotenoid content on a recombinant inbred line (RIL) population, the characterization of a Mediterranean panel of accessions for Psy1 and Lpx-1 genes, and monitoring the expression of Psy1 and Lpx-1 genes during grain filling on two genotypes with contrasting yellow pigments. Our data suggest that Psy1 plays a major role during grain development, contributing to semolina yellowness, and Lpx-1 appears to be more predominant at post-harvest stages and during pasta making.

Keywords: durum wheat; grain carotenoids; semolina yellowness; landraces; phytoene synthase 1; lipoxygenase 1

1. Introduction

Wheat is one of the most important cereal crops worldwide [1,2], as about 725 million tons of grain are produced globally every year [3]. Durum wheat (Triticum turgidum L. ssp. durum) is a tetraploid species, with genomes A and B, used in a variety of food products, including couscous and bulgur, but mostly semolina, the raw material for pasta manufacturing [4–6]. Durum represents about 8% of the total wheat cultivated area and 5% of global wheat production [7]. In the twentieth century, major breeding programs were focused on improving the durum productivity traits of wheat, such as grain yield and biotic and abiotic stress resistance. In this century, the attention on food quality over quantity has switched the research aims to increasing wheat nutritional value, estimated
through different parameters like protein content, water absorption, and flour color. The latter is due to carotenoid pigments, which have an enormous importance for nutritional value in human health [8].

Yellow pigment content (YPC) is one of the most important quality features of durum wheat grain [6,9], due to the positive correlation existing between yellowness and pasta quality [10], which is associated with a higher consumer acceptance [2]. For this reason, improving YPC is a main goal in most durum wheat breeding programs [2,11], particularly considering that the competition in the pasta market has made this trait even more important [12], especially after the legal ban of the use of artificial coloring in pasta production in certain countries in Europe [13], which has strengthened the role of durum wheat breeding programs for YPC enhancements.

The carotenoid content in the wheat grain is mainly composed of lutein and small amounts of zeaxanthin and β-cryptoxanthin [14,15]. Lutein is a compound that contributes to the organoleptic quality of pasta (yellowness) [14]. A high carotenoid content in pasta enhances its nutritional value due to the cell membrane protective role of carotenoids against oxidative damage [4], by reducing the effective concentration of free radicals in the cells [9,16]. Grain and semolina yellowness depend on several factors, including the endogenous carotenoid content of kernels, grinding rate, the pasta-making conditions, and the oxidative degradation of enzymes, in which lipoxygenases (LOX) play a major role [9,17]. Thus, breeding programs should aim to produce cultivars with high endogenous carotenoid pigments and low oxidative activity [10].

Although grain yellowness is genetically controlled [9,18,19], it is a very complex polygenic trait [20,21]. Quantitative trait loci (QTLs) for YPC have been found across all wheat chromosomes. A complete and comprehensive review of the QTLs and genes (with the specific accession numbers) that affect YPC is reported in Colasuonno et al. [7].

The enzyme phytoene synthase (PSY) catalyzes the rate-controlling step in the synthesis of phytoene from geranylgeranyl diphosphate. Psy1-A1 and Psy1-B1 encoding phytoene synthase 1 are major QTLs for YPC in chromosomes 7A and 7B, respectively [22]. There is considerable information regarding allelic variation in these genes related to grain yellowness variation in wheat. Several authors had reported and developed markers in both Psy1-A1 and Psy1-B1 that are able to select cultivars for high yellowness levels in both durum and common wheat [22–28]. Three allelic variants at Psy1-A1 have been reported, including Psy1-A1a (low semolina yellowness), Psy1-A1l (intermediate yellowness), and Psy1-A1o (high yellowness) [29]. However, a previous study of our group that involved 155 Mediterranean landraces and 20 modern cultivars of diverse origin associated the presence of the allele Psy1-A1l with the highest values of semolina yellowness and the presence of Psy1-A1a with the lowest values [30]. In that study, no differences between Psy1-B1a and Psy1-B1b were identified as being related to semolina yellowness.

Nevertheless, a high carotenoid content and a high endosperm yellowness do not guarantee high yellowness content in pasta products. During pasta processing, the oxidative degradation of carotenoids can occur, leading to the bleaching of the end-products. Even though there are several enzymes contributing to this effect, including peroxidases and polyphenol oxidases, lipoxygenases are the main players [6,7,9,11,31]. Lipoxygenase enzymes catalyze the addition of molecular oxygen across the cis, cis-1,4 pentadiene system to produce the corresponding hydroperoxides [32]. In plants, lipoxygenases are found in leaves, seedlings, and seeds. LOX activity produces reactive oxygen species (ROS), originating from fatty acid oxidation, and these radicals can produce the oxidation and degradation of carotenoids [10]. In durum wheat, there are different lipoxygenase genes and alleles contributing to the variation in pasta yellowness [11,32]. Hessler et al. [31] sequenced in durum wheat several fragments of lipoxigenase-1, which was reported to be responsible for LOX activity in barley seeds [33], and based on the similarity to the LoxA gene between the two species, these sequences were assigned to the Lpx-1 locus in durum wheat [31]. De Simone et al. [34] reported different levels of Lpx-1 and Lpx-3 transcripts at maturity between cultivars with contrasting LOX activities, meanwhile, Lpx-2 transcripts were absent at this stage. The Lpx-B1 locus is located on the short arm of chromosome 4B [16,31,32] and five related genes and allele sequences have been reported,
Lpx-B1.1a (Genbank HM126466), Lpx-B1.1b (Genbank HM126468), and Lpx-B1.1c (Genbank HM126470) for the Lpx-B1.1 locus and Lpx-B1.2 (Genbank HM126467) and Lpx-B1.3 (Genbank HM126469) [4,31,32]. QTL analyses in durum wheat showed that 36–54% of the variation in LOX activity is attributable to Lpx-B1 [4,31]. Additionally, Verlotta et al. [32] identified three different combinations between the alleles for Lpx-B1.1 and the Lpx-B1.2 and Lpx-B1.3 genes, named haplotypes I (Lpx-B1.1b and Lpx-B1.3), II (Lpx-B1.1a and Lpx-B1.2), and III (Lpx-B1.1c and Lpx-B1.2), in which haplotypes I, II, and III showed high, intermediate, and low levels of functional Lpx-B1 transcripts and enzymatic activity, respectively. Thus, understanding LOX activity in mature durum wheat kernels is critical for semolina yellowness and end-products derived from this cereal.

This study was conducted to identify new allele variants and/or allele combinations significantly affecting YPC in semolina through their effect on the synthesis and degradation of carotenoid pigments in grain and semolina. For this purpose, three complementary approaches were undertaken: the identification of QTLs associated with carotenoid content on a recombinant inbred line (RIL) population, the characterization of a Mediterranean panel of accessions for Psy1 and Lpx-1 genes, and monitoring the expression of Psy1 and Lpx-1 genes during grain filling in two genotypes with contrasting YPC.

2. Results

2.1. Detection of QTLs for Carotenoid Content in the RIL Population

2.1.1. Phenotyping

The ANOVA of the yellow index (YI) data assessed in the RIL population revealed that all factors in the analysis (genotype (G), environment (E), and G × E interaction) were statistically significant (p < 0.01, data not shown). The parental lines differed significantly in YI values in the three years, with cv. “Saragolla” showing consistently higher values than “02-5B-318” (Table 1). The RIL values for YI appeared normally distributed and showed ranges of 5.38, 6.39, and 5.86 in 2015, 2016, and 2017, respectively (Table 1). The broad-sense heritability of YI ranged from 0.85 to 0.88 in the three testing years, highlighting that the phenotype was largely due to a genotypic effect (Table 1).

Table 1. Yellow index (YI ± SE) values of the parental lines and the Recombinant Inbred Line (RIL) population grown in Valenzano for three years. C.V., coefficient of variation; σ²C, genetic variance; h²B, broad-sense heritability.

| Year | Mean 2015 | Mean 2016 | Mean 2017 |
|------|-----------|-----------|-----------|
| Parent “02-5B-318” | 10.06 ± 0.27 | 10.37 ± 0.51 | 10.39 ± 0.25 | 10.29 ± 0.33 |
| Parent “Saragolla” | 13.89 ± 0.11 | 14.21 ± 0.26 | 14.80 ± 0.42 | 14.25 ± 0.37 |
| RIL mean | 12.79 ± 1.03 | 12.75 ± 0.94 | 13.41 ± 1.71 | 13.09 ± 1.23 |
| RIL range | 10.77–16.15 | 11.13–17.52 | 11.78–17.64 |
| C.V. | 3.26 | 3.01 | 3.06 |
| σ²C | 1.02 | 1.06 | 0.98 |
| h²B | 0.85 | 0.88 | 0.87 |

2.1.2. QTL Analysis and Identification of Carotenoid Genes

As a starting point for the identification of QTL regions, the genetic map developed for the RIL population by Giancaspro et al. [35] was used as a reference for the analysis. The composite interval mapping (CIM) method was employed for QTL analysis. Putative QTLs for YI are listed in Table 2. Eight QTLs for YI (with LOD > 3) were mapped on chromosomes 2B (one), 4A (one), 4B (three), 5A (two), and 7A (one). The percentage of phenotypic variation (R²) for YI explained by individual QTLs ranged from 10% to 59%. The major QTL was detected on chromosome 2B, flanked by the markers IWB12724 and IWB11333 (IWB32245 as the closest marker). This QTL was
significant within the three years and across them and explained from 30% to 59% of YI variations. The QTLs on chromosomes 5A (interval IWA1258-IWB2888) and 7A (interval IWB73689-IWB25891) were statistically significant in all cases, accounting from 12% to 21% of the phenotypic variation. The QTL on chromosome 4B (IWB6397-IWB44213) was significant in two environments, accounting for 13% of YI variation. The QTLs on chromosomes 4A (IWB12722-IWB14501), 4B (IWB73832-IWB11928; IWB71402-IWB53932), 5A (IWB65257-IWB35711), and 7A (IWB49929-IWB8841) were significant in one environment with $R^2$ values ranging between 10 and 20%. Table 2 summarizes the location of QTLs on the genetic map, their LOD scores, and the closest markers flanking the region with the respective associated marker.

In order to verify the possible presence of candidate genes for the yellow index trait, all single nucleotide polymorphism (SNP) markers present in the detected QTL regions were studied. These SNP markers, mapped by Giancaspro et al. [35], were subjected to BLASTn analysis (based on percentage identity) to verify that the sequences have a high percentage of homology with the biosynthesis genes of the carotenoid pigments. All gene sequences used as queries were derived from Colasuonno et al. [7]. The analysis allowed for the identification of the lipoxygenase gene ($Lpx$) in the QTL region on chromosome 4B (IWA103 marker located in the 4B-2 QTL region, Table 2), confirming the key role of this chromosome in trait control. For the other key genes involved in the catabolic and biosynthetic pathway of the carotenoid pigment, no polymorphic markers were present in the analyzed regions.

2.1.3. Expression Profile of Lpx Genes on Leaves

The relationships between Lpx and yellow index were analyzed in the leaf tissue of the bread wheat accession “02-5B-318” and the durum wheat cv. “Saragolla”, characterized by low and high YIs, respectively. The Lpx genes mapped on chromosomes 4A, 4B, 5A, and 5B were considered in the expression study in order to analyze each homoeologous Lpx gene. Total RNA was extracted from kernels, and quantitative real-time PCR (qPCR) was carried out with genome-specific primers. Significant statistical differences were observed in the three homoeologous forms in the two parental lines. High expression levels and significantly different expression values ($p < 0.05$, t-test) were detected between genotypes “02-5B-318” and “Saragolla” (0.67 and 1.58, respectively) for the Lpx gene on chromosome 5B, indicating that the Lpx allele present in cv. “Saragolla”, the parent with a high YI, was more active in the accumulation of yellow pigment that the allele present in “02-5B-318”, the parent with a low YI (Figure 1). Furthermore, significant differences ($p < 0.05$) were also observed for Lpx genes located on homologous group 4, showing the positive contribution to the trait of the “02-5B-318” parent, with normalized expression data of 1.0 for both the A and B isoforms compared to 0.32 and 0.47 for the 4A and 4B genes of cv. “Saragolla”.

2.2. Characterization of the Mediterranean Panel for Psy1-A1 and Lpx-B1

A previous study of our team identified the allele composition of the Psy1-A1 gene as a reliable indicator of semolina b* value [30]. To gain more insight into the genomic background of this gene, allele variants of Psy1-A1 were mapped in the whole population, analyzed herein, and the results are shown in Table 3. Similar allele frequencies were found for the alleles Psy1-A1a and Psy1-A1o, but Psy1-A1l predominated (Table 4). Semolina b* values were significantly lower for genotypes harboring the Psy1-A1a allele in comparison to the ones carrying the alleles Psy1-A1l or Psy1-A1o, which showed similar values (Table 4).
Table 2. Composite interval mapping results estimated for yellow index (YI) from RIL lines derived from the cross “02-5B-318” x “Saragolla” tested at Valenzano in 2015, 2016, and 2017.

| Chr | Linkage Group | Marker Interval | Associated Marker | Associated Candidate Gene | Position (cM) | Valenzano 2015 | Valenzano 2016 | Valenzano 2017 | Mean Across Environments |
|-----|---------------|-----------------|-------------------|--------------------------|---------------|---------------|---------------|---------------|--------------------------|
|     |               |                 |                   |                          | Add | LOD | R²     | Add | LOD | R²     | Add | LOD | R²     | Add | LOD | R²     |
| 2B  | 2B-4          | IWB12724-IWB11333 | IWB32245          |                         | 114.50 | -96.0 | 21.4 | 59.0 | -52.0 | 9.2 | 30.0 | -71.0 | 11.8 | 36.0 | -66.0 | 12.8 | 38.0 |
| 4A  | 4A-2          | IWB12722-IWB14501 | IWB68425          |                         | 184.10 | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| 4B  | 4B-2          | IWB73832-IWB11928 | IWB73831          | Lpx                      | 2.00  | -    | -    | -    | -    | -    | -    | -    | -    | 20.0 | 5.0  | 10.0 | -    | -    |
| 4B  | 4B-3          | IWB71402-IWB53932 | IWB15007          |                          | 35.50  | 36.0 | 4.6  | 18.0 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| 4B  | 4B-4          | IWA6397-IWB44213 | IWB7473           |                          | 38.70  | -    | -    | -    | -    | -    | -    | -    | -    | 35.0 | 3.6  | 13.0 | 32.0 | 3.8  |
| 5A  | 5A-3          | IWB65257-IWB35711 | IWA850            |                          | 46.10  | 39.0 | 5.7  | 20.0 | -    | -    | -    | -    | -    | -    | -    | -    | -    |
| 5A  | 5A-4          | IWA1258-IWB25888 | IWA1258           |                          | 0.00  | 41.0 | 5.7  | 21.0 | 34.0 | 4.4 | 15.0 | 35.0 | 3.5 | 12.0 | 33.0 | 3.9  | 14.0 | -    |
| 7A  | 7A-2          | IWB73689-IWB25891 | IWB72199          |                          | 75.20  | 36.0 | 4.5  | 17.0 | 33.0 | 4.3 | 15.0 | 45.0 | 5.7 | 19.0 | 42.0 | 6.0  | 20.0 | -    |
Primer pairs outlined in Table 5 allowed the identification of five combinations (haplotypes) between allele variants of \( Lpx-B1.1, Lpx-B1.2, \) and \( Lpx-B1.3 \) genes (Table 3). Three of them correspond to those previously described by Verlotta et al. [32], i.e., haplotypes I (\( Lpx-B1.1b \) and \( Lpx-B1.3 \)), II (\( Lpx-B1.1a \) and \( Lpx-B1.2 \)), and III (\( Lpx-B1.1c \) and \( Lpx-B1.2 \)), but two new ones with a low frequency in the population, named haplotypes IV and V following the nomenclature of Verlotta et al. [32], were found for the first time in the present study (Table 6). Haplotype IV, which showed the combination of \( Lpx-B1.1b \) and \( Lpx-B1.2 \), was identified in three landraces from Spain, Cyprus, and Morocco, whereas haplotype V, formed by the combination of \( Lpx-B1.1a \) and \( Lpx-B1.3 \), was detected in four landraces, two French, one Syrian, and one Spanish (Table 3). Alignments were made between the obtained amplicons and the corresponding sequences for \( Lpx-B1.1a, Lpx-B1.1b, Lpx-B1.1c, Lpx-B1.2, \) and \( Lpx-B1.3 \), showing no presence of new genes or allele sequences within the population evaluated (data not shown).

Large variability was identified in haplotypes I and II, with \( b^* \) values ranging from 12.38 to 25.78, and 10.32 to 23.49, respectively. These haplotypes were the most frequent within the Mediterranean panel (Table 6). The results of the ANOVA and the comparison of the mean \( b^* \) values of the five haplotypes showed no statistical differences among them (Table 6). Interestingly, all modern genotypes harbored haplotype II (Table 3). When the genotypes carrying haplotype II were segregated in “haplotype II landraces” and “haplotype II modern cultivars”, significant differences appeared between them, as the latter encompassed the cultivars with higher levels of yellowness (Figure 2).
Table 3. Molecular characterization for Phytoene synthase 1 (Psy1-A1) and Lipoxygenase 1 (Lpx-B1), and yellow index value of the genotypes in the Mediterranean panel. See Table 8 for code interpretation.

| Code  | Psy1-A1 | Lpx-B1.1a | Lpx-B1.1b | Lpx-B1.1c | Lpx-B1.2 | Lpx-B1.3 | Lpx-B1 Haplotype | b* Pirque 2017 | b* Chillán 2017 | b* Chillán 2018 | Psy1-A1/Lpx-B1 Allele Combination |
|-------|---------|-----------|-----------|-----------|----------|----------|--------------------|---------------|----------------|----------------|-----------------------------------|
| DW024 | a       | ✓         | ✓         | ✓         | I        | 14.45    | 13.84              | 13.11         | 1              | 1              |                                 |
| DW113 | a       | ✓         | ✓         | ✓         | I        | 15.15    | 16.04              | 15.24         | 1              | 1              |                                 |
| DW001 | l       | ✓         | ✓         | ✓         | ✓         | 19.30    | 21.04              | 21.65         | 2              | 2              |                                 |
| DW002 | l       | ✓         | ✓         | ✓         | ✓         | 16.89    | 18.71              | 20.59         | 2              | 2              |                                 |
| DW006 | l       | ✓         | ✓         | ✓         | ✓         | 18.78    | 18.79              | 20.09         | 2              | 2              |                                 |
| DW014 | l       | ✓         | ✓         | ✓         | ✓         | 18.80    | 18.64              | 20.10         | 2              | 2              |                                 |
| DW019 | l       | ✓         | ✓         | ✓         | ✓         | 18.20    | 17.03              | 15.23         | 2              | 2              |                                 |
| DW020 | l       | ✓         | ✓         | ✓         | ✓         | 21.74    | 21.41              | 20.93         | 2              | 2              |                                 |
| DW022 | l       | ✓         | ✓         | ✓         | ✓         | 18.80    | 18.81              | 18.52         | 2              | 2              |                                 |
| DW025 | l       | ✓         | ✓         | ✓         | ✓         | 13.88    | 13.38              | 12.38         | 2              | 2              |                                 |
| DW032 | l       | ✓         | ✓         | ✓         | ✓         | 20.34    | 21.70              | 22.70         | 2              | 2              |                                 |
| DW034 | l       | ✓         | ✓         | ✓         | ✓         | 19.87    | 19.54              | 20.62         | 2              | 2              |                                 |
| DW037 | l       | ✓         | ✓         | ✓         | ✓         | 17.27    | 17.42              | 18.99         | 2              | 2              |                                 |
| DW043 | l       | ✓         | ✓         | ✓         | ✓         | 19.02    | 18.34              | 23.12         | 2              | 2              |                                 |
| DW056 | l       | ✓         | ✓         | ✓         | ✓         | 17.44    | 17.52              | 16.98         | 2              | 2              |                                 |
| DW058 | l       | ✓         | ✓         | ✓         | ✓         | 19.39    | 18.25              | 21.84         | 2              | 2              |                                 |
| DW061 | l       | ✓         | ✓         | ✓         | ✓         | 16.57    | 16.42              | 17.39         | 2              | 2              |                                 |
| DW067 | l       | ✓         | ✓         | ✓         | ✓         | 18.95    | 19.45              | 20.99         | 2              | 2              |                                 |
| DW071 | l       | ✓         | ✓         | ✓         | ✓         | 16.96    | 17.66              | 17.61         | 2              | 2              |                                 |
| DW074 | l       | ✓         | ✓         | ✓         | ✓         | 17.81    | 18.36              | 18.19         | 2              | 2              |                                 |
| DW083 | l       | ✓         | ✓         | ✓         | ✓         | 21.27    | 20.15              | 21.10         | 2              | 2              |                                 |
| DW084 | l       | ✓         | ✓         | ✓         | ✓         | 18.93    | 17.34              | 14.87         | 2              | 2              |                                 |
| DW090 | l       | ✓         | ✓         | ✓         | ✓         | 14.60    | 16.60              | 17.23         | 2              | 2              |                                 |
| DW092 | l       | ✓         | ✓         | ✓         | ✓         | 14.57    | 14.76              | 15.96         | 2              | 2              |                                 |
| DW093 | l       | ✓         | ✓         | ✓         | ✓         | 18.34    | 18.97              | 18.89         | 2              | 2              |                                 |
| DW096 | l       | ✓         | ✓         | ✓         | ✓         | 16.65    | 16.44              | 18.49         | 2              | 2              |                                 |
| DW099 | l       | ✓         | ✓         | ✓         | ✓         | 17.31    | 20.29              | 18.39         | 2              | 2              |                                 |
| DW103 | l       | ✓         | ✓         | ✓         | ✓         | 20.77    | 22.06              | 23.82         | 2              | 2              |                                 |
| DW111 | l       | ✓         | ✓         | ✓         | ✓         | 18.41    | 18.11              | 19.29         | 2              | 2              |                                 |
| DW115 | l       | ✓         | ✓         | ✓         | ✓         | 16.33    | 15.85              | 16.32         | 2              | 2              |                                 |
| DW121 | l       | ✓         | ✓         | ✓         | ✓         | 15.89    | 16.98              | 16.94         | 2              | 2              |                                 |
| DW124 | l       | ✓         | ✓         | ✓         | ✓         | 24.17    | 23.17              | 24.38         | 2              | 2              |                                 |
| DW126 | l       | ✓         | ✓         | ✓         | ✓         | 16.77    | 13.23              | 16.91         | 2              | 2              |                                 |
| DW127 | l       | ✓         | ✓         | ✓         | ✓         | 16.59    | 18.49              | 18.16         | 2              | 2              |                                 |
| DW130 | l       | ✓         | ✓         | ✓         | ✓         | 18.91    | 17.91              | 17.04         | 2              | 2              |                                 |
| DW139 | l       | ✓         | ✓         | ✓         | ✓         | 22.78    | 22.87              | 22.78         | 2              | 2              |                                 |
| DW149 | l       | ✓         | ✓         | ✓         | ✓         | 22.25    | 21.93              | 22.74         | 2              | 2              |                                 |
| DW160 | l       | ✓         | ✓         | ✓         | ✓         | 25.70    | 25.44              | 25.78         | 2              | 2              |                                 |
| DW162 | l       | ✓         | ✓         | ✓         | ✓         | 17.17    | 14.98              | 18.64         | 2              | 2              |                                 |
| DW166 | l       | ✓         | ✓         | ✓         | ✓         | 16.59    | 17.27              | 17.94         | 2              | 2              |                                 |
| DW172 | l       | ✓         | ✓         | ✓         | ✓         | 19.36    | 17.13              | 21.12         | 2              | 2              |                                 |
| DW012 | o       | ✓         | ✓         | ✓         | ✓         | 17.41    | 18.36              | 18.94         | 3              |                |                                 |
## Table 3. Cont.

| Code  | Psy1-A1 | Lpx-B1.1a | Lpx-B1.1b | Lpx-B1.1c | Lpx-B1.2 | Lpx-B1.3 | Lpx-B1 Haplotype | b* Pirque 2017 | b* Chillan 2017 | b* Chillan 2018 | Psy1-A1/Lpx-B1 Allele Combination |
|-------|---------|-----------|-----------|-----------|----------|----------|------------------|----------------|----------------|----------------|----------------------------------|
| DW015 | o       | ✓         | ✓         | ✓         | I        |          |                  | 20.23          | 20.00          | 20.89          | 3                                 |
| DW017 | o       | ✓         |          | ✓         | I        |          |                  | 23.71          | 23.63          | 24.06          | 3                                 |
| DW028 | o       | ✓         |          |          | I        |          |                  | 21.32          | 22.79          | 20.49          | 3                                 |
| DW038 | o       | ✓         |          |          | I        |          |                  | 16.36          | 14.56          | 21.02          | 3                                 |
| DW080 | o       | ✓         |          |          | I        |          |                  | 16.95          | 18.37          | 20.49          | 3                                 |
| DW082 | o       | ✓         |          |          | I        |          |                  | 18.38          | 19.82          | 18.38          | 3                                 |
| DW086 | o       | ✓         |          |          | I        |          |                  | 16.41          | 12.59          | 14.04          | 3                                 |
| DW129 | o       | ✓         |          |          | I        |          |                  | 23.41          | 21.60          | 21.73          | 3                                 |
| DW039 | a       | ✓         | ✓         |          | I        |          |                  | 18.91          | 18.29          | 17.96          | 4                                 |
| DW011 | a       | ✓         | ✓         |          | II       |          |                  | 12.94          | 16.76          | 12.83          | 4                                 |
| DW013 | a       | ✓         | ✓         |          | II       |          |                  | 18.54          | 15.89          | 15.89          | 4                                 |
| DW060 | a       | ✓         | ✓         |          | II       |          |                  | 19.69          | 19.04          | 21.45          | 4                                 |
| DW069 | a       | ✓         | ✓         |          | II       |          |                  | 14.94          | 15.99          | 15.95          | 4                                 |
| DW070 | a       | ✓         | ✓         |          | II       |          |                  | 14.44          | 16.46          | 18.51          | 4                                 |
| DW072 | a       | ✓         | ✓         |          | II       |          |                  | 13.54          | 12.40          | 10.32          | 4                                 |
| DW110 | a       | ✓         | ✓         |          | II       |          |                  | 14.18          | 12.77          | 14.66          | 4                                 |
| DW134 | a       | ✓         | ✓         |          | II       |          |                  | 14.56          | 15.18          | 15.18          | 4                                 |
| DW136 | a       | ✓         | ✓         |          | II       |          |                  | 16.29          | 14.03          | 14.03          | 4                                 |
| DW138 | a       | ✓         | ✓         |          | II       |          |                  | 17.68          | 18.83          | 18.57          | 4                                 |
| DW148 | a       | ✓         | ✓         |          | II       |          |                  | 14.60          | 15.19          | 17.52          | 4                                 |
| DW154 | a       | ✓         | ✓         |          | II       |          |                  | 16.16          | 15.91          | 15.91          | 4                                 |
| DW191 | a       | ✓         | ✓         |          | II       |          |                  | 17.84          | 18.47          | 19.00          | 4                                 |
| DW003 | l       | ✓         | ✓         |          | II       |          |                  | 14.71          | 12.98          | 14.59          | 5                                 |
| DW004 | l       | ✓         | ✓         |          | II       |          |                  | 16.61          | 18.21          | 15.96          | 5                                 |
| DW010 | l       | ✓         | ✓         |          | II       |          |                  | 16.45          | 16.71          | 17.20          | 5                                 |
| DW016 | l       | ✓         | ✓         |          | II       |          |                  | 18.90          | 15.92          | 15.92          | 5                                 |
| DW018 | l       | ✓         | ✓         |          | II       |          |                  | 22.26          | 20.09          | 20.29          | 5                                 |
| DW021 | l       | ✓         | ✓         |          | II       |          |                  | 19.02          | 20.94          | 18.80          | 5                                 |
| DW027 | l       | ✓         | ✓         |          | II       |          |                  | 17.23          | 18.35          | 19.73          | 5                                 |
| DW033 | l       | ✓         | ✓         |          | II       |          |                  | 17.76          | 18.22          | 18.48          | 5                                 |
| DW041 | l       | ✓         | ✓         |          | II       |          |                  | 18.66          | 18.63          | 20.02          | 5                                 |
| DW042 | l       | ✓         | ✓         |          | II       |          |                  | 16.26          | 15.42          | 15.14          | 5                                 |
| DW044 | l       | ✓         | ✓         |          | II       |          |                  | 16.91          | 17.62          | 19.78          | 5                                 |
| DW046 | l       | ✓         | ✓         |          | II       |          |                  | 16.03          | 17.01          | 17.19          | 5                                 |
| DW048 | l       | ✓         | ✓         |          | II       |          |                  | 16.65          | 16.84          | 17.79          | 5                                 |
| DW052 | l       | ✓         | ✓         |          | II       |          |                  | 18.32          | 16.38          | 19.72          | 5                                 |
| DW054 | l       | ✓         | ✓         |          | II       |          |                  | 18.41          | 20.04          | 21.38          | 5                                 |
| DW062 | l       | ✓         | ✓         |          | II       |          |                  | 19.19          | 19.57          | 20.15          | 5                                 |
| DW068 | l       | ✓         | ✓         |          | II       |          |                  | 16.52          | 18.67          | 19.59          | 5                                 |
| DW091 | l       | ✓         | ✓         |          | II       |          |                  | 20.19          | 21.24          | 20.03          | 5                                 |
| DW094 | l       | ✓         | ✓         |          | II       |          |                  | 14.28          | 14.29          | 16.99          | 5                                 |
| DW095 | l       | ✓         | ✓         |          | II       |          |                  | 16.25          | 19.70          | 19.68          | 5                                 |
| DW104 | l       | ✓         | ✓         |          | II       |          |                  | 20.19          | 20.92          | 21.87          | 5                                 |
| DW117 | l       | ✓         | ✓         |          | II       |          |                  | 20.47          | 21.83          | 18.68          | 5                                 |
| Code    | Psy1-A1 | Lpx-B1.1a | Lpx-B1.1b | Lpx-B1.1c | Lpx-B1.2 | Lpx-B1.3 | Haplotype | b\(^*\) Pirque 2017 | b\(^*\) Chillan 2017 | b\(^*\) Chillan 2018 |
|---------|---------|-----------|-----------|-----------|-----------|-----------|-----------|---------------------|----------------------|----------------------|
| DW128   | 1       | ✓         | ✓         |           |           |           | II        | 21.38               | 20.81                | 5                    |
| DW131   | 1       | ✓         |           |           |           |           | II        | 15.32               | 15.80                | 16.96               |
| DW132   | 1       | ✓         |           | ✓         |           |           | II        | 20.85               | 22.23                | 21.63               |
| DW133   | 1       | ✓         |           | ✓         |           |           | II        | 22.08               | 21.94                | 5                    |
| DW137   | 1       | ✓         |           | ✓         |           |           | II        | 21.08               | 21.09                | 5                    |
| DW144   | 1       | ✓         |           | ✓         |           |           | II        | 20.71               | 20.96                | 5                    |
| DW145   | 1       | ✓         |           | ✓         |           |           | II        | 20.04               | 20.73                | 21.04               |
| DW146   | 1       | ✓         |           |           |           |           | II        | 19.56               | 20.17                | 5                    |
| DW150   | 1       | ✓         |           | ✓         |           |           | II        | 21.72               | 22.59                | 21.82               |
| DW151   | 1       | ✓         |           | ✓         |           |           | II        | 19.97               | 20.75                | 19.68               |
| DW158   | 1       | ✓         |           | ✓         |           |           | II        | 15.26               | 16.90                | 15.98               |
| DW161   | 1       | ✓         |           |           |           |           | II        | 17.05               | 18.35                | 18.18               |
| DW163   | 1       | ✓         |           |           |           |           | II        | 17.76               | 15.35                | 19.48               |
| DW165   | 1       | ✓         |           | ✓         |           |           | II        | 15.32               | 15.87                | 18.20               |
| DW167   | 1       | ✓         |           |           |           |           | II        | 16.93               | 16.32                | 15.03               |
| DW168   | 1       | ✓         |           |           |           |           | II        | 14.30               | 14.75                | 17.65               |
| DW170   | 1       | ✓         |           |           |           |           | II        | 15.68               | 15.25                | 16.79               |
| DW171   | 1       | ✓         |           |           |           |           | II        | 17.57               | 18.54                | 19.06               |
| DW174   | 1       | ✓         |           |           |           |           | II        | 18.45               | 18.86                | 21.54               |
| DW175   | 1       | ✓         |           |           |           |           | II        | 20.01               | 20.17                | 21.70               |
| DW176   | 1       | ✓         |           |           |           |           | II        | 19.48               | 20.32                | 20.87               |
| DW177   | 1       | ✓         |           |           |           |           | II        | 20.58               | 20.70                | 21.53               |
| DW187   | 1       | ✓         |           |           |           |           | II        | 21.73               | 21.68                | 21.33               |
| DW189   | 1       | ✓         |           |           |           |           | II        | 18.83               | 21.21                | 20.10               |
| DW190   | 1       | ✓         |           |           |           |           | II        | 23.49               | 22.93                | 23.33               |
| DW192   | 1       | ✓         |           |           |           |           | II        | 19.72               | 22.65                | 20.16               |
| DW023   | o       | ✓         |           |           |           |           | II        | 20.75               | 21.73                | 21.78               |
| DW078   | o       | ✓         |           |           |           |           | II        | 22.54               | 23.17                | 21.33               |
| DW085   | o       | ✓         |           |           |           |           | II        | 15.06               | 13.65                | 14.31               |
| DW098   | o       | ✓         |           |           |           |           | II        | 16.97               | 17.91                | 18.14               |
| DW116   | o       | ✓         |           |           |           |           | II        | 15.75               | 16.82                | 16.90               |
| DW152   | o       | ✓         |           |           |           |           | II        | 16.29               | 18.05                | 18.34               |
| DW059   | I       | ✓         | ✓         |           |           |           | III       | 14.05               | 14.98                | 13.27               |
| DW008   | o       | ✓         | ✓         |           |           |           | III       | 18.45               | 19.84                | 19.24               |
| DW047   | I       | ✓         | ✓         |           |           |           | IV        | 16.34               | 15.98                | 16.54               |
| DW122   | I       | ✓         | ✓         |           |           |           | IV        | 15.52               | 18.46                | 18.00               |
| DW147   | I       | ✓         | ✓         |           |           |           | IV        | 19.10               | 19.56                | 20.24               |
| DW035   | I       | ✓         | ✓         |           |           |           | V         | 15.47               | 15.45                | 17.32               |
| DW040   | I       | ✓         | ✓         |           |           |           | V         | 20.00               | 18.36                | 20.38               |
| DW111   | I       | ✓         | ✓         |           |           |           | V         | 17.06               | 17.87                | 17.42               |
| DW114   | I       | ✓         | ✓         |           |           |           | V         | 15.31               | 16.09                | 16.86               |
Table 4. Allele variants of the \(Psy1-A1\) gene identified in the Mediterranean durum wheat panel and associated \(b^*\) values.

| Allele | Number of Genotypes | Frequency (%) | \(n^a\) | Minimum | Maximum | Mean ± SE\(^b\) |
|--------|---------------------|--------------|--------|---------|---------|----------------|
| \(Psy1-A1a\) | 16 | 12.4 | 44 | 10.32 | 21.45 | 16.00 ± 0.35\(^b\) |
| \(Psy1-A1l\) | 97 | 75.2 | 282 | 12.38 | 25.78 | 18.66 ± 0.15\(^a\) |
| \(Psy1-A1o\) | 16 | 12.4 | 48 | 12.59 | 24.06 | 18.89 ± 0.43\(^a\) |

\(^a\) Data from Table 3, \(b^*\) values for Pirque 2017, Chillán 2017, and Chillán 2018. \(^b\) Different uppercase letters correspond to significantly different values after one-way ANOVA and Tukey’s test (\(p < 0.05\)).

Table 5. List of primers used to genotype the Mediterranean panel for \(Psy1-A1\) and \(Lpx-B1\).

| Primer Name | Sequence (5’ → 3’) | Gene/Allele | Product Length (bp) | Reference |
|-------------|---------------------|-------------|---------------------|-----------|
| \(Psy1-A1\_STS_F\) | GTG GAT ATT CCC TGT CAG CATC GCC TCC TCG AAG AAC ATC CTC | \(Psy1-A1o - Psy1-A1a\) | 897 - 1089 - 1776 | Singh et al. [29] |
| \(qrtPsy1\_F\) | GCCAGGGGTGACCTGACCT | \(Psy1\) | 117 | Rodriguez-Suarez et al. [36] |
| \(qrtPsy1\_R\) | CTTTGCTGAATCTGGTCTAGTCA | | | |
| \(qrt\_Lpx-B1\_F\) | TCAAC TTCGGCCAGTACCATT | \(Lpx-B1\) | 177 | This study |
| \(qrt\_Lpx-B1\_R\) | CCAACAGCGATGGCCATGAT | | | |
| \(Lpx-B1.1a\_F\) | GCA GGC CCT CGA AAG CAA CAG GC GCC CCT TAA CCT GCC GTA CTC G | \(Lpx-B1.1a - Lpx-B1.1b\) | 1320 - 1246 | Verlotta et al. [32] |
| \(Lpx-B1.1a\_R\) | CCT GGT TGG TGG GCC GTG GGG CCC CTC CTT GGG GGC GCC GTA CTC G | \(Lpx-B1.1c\) | 1558 | |
| \(Lpx-B1.2\_F\) | GAA CCG AGA CTT GAG AGG GTG CTC CTT GAC C GTG CTC CCA GGT GTG GGG GTA GAC C | \(Lpx-B1.2 - Lpx-B1.3\) | 1785 - 1799 | This study |
| \(Lpx-B1.2\_R\) | | | | |

Table 6. \(Lpx-B1\) haplotype polymorphisms identified in the Mediterranean panel and average \(b^*\) values across Pirque 2017, Chillán 2017, and Chillán 2018. Differences in \(b^*\) values were not statistically significant (\(p > 0.05\)).

| Haplotype | Allelic Combination | N. of Genotypes | Frequency (%) | \(n^a\) | Minimum | Maximum | Mean ± SE |
|-----------|---------------------|-----------------|--------------|--------|---------|---------|---------|
| I         | \(Lpx-B1.1b\) and \(Lpx-B1.3\) | 50              | 39.1         | 149    | 12.38   | 25.78   | 18.74 ± 0.23 |
| II        | \(Lpx-B1.1a\) and \(Lpx-B1.2\) | 69              | 53.9         | 198    | 10.32   | 23.49   | 18.28 ± 0.18 |
| III       | \(Lpx-B1.1c\) and \(Lpx-B1.2\) | 2               | 1.6          | 6      | 13.27   | 19.84   | 16.64 ± 1.17 |
| IV        | \(Lpx-B1.1b\) and \(Lpx-B1.2\) | 3               | 2.3          | 9      | 15.52   | 20.24   | 17.75 ± 0.57 |
| V         | \(Lpx-B1.1a\) and \(Lpx-B1.3\) | 4               | 3.1          | 12     | 15.31   | 20.38   | 17.30 ± 0.48 |

\(^a\) Data from Table 3, \(b^*\) values for Pirque 2017, Chillán 2017, and Chillán 2018.

Figure 2. \(Lpx-B1\) haplotype \(b^*\) values. Genotypes from the Mediterranean panel were grouped according to their \(Lpx-B1\) haplotype and a distinction was made between modern cultivars and landraces. One-way ANOVA and Tukey’s post hoc test were performed to find significant differences (\(p < 0.05\)).
To further dissect the population into smaller groups and get more information on the combinations between allele variants at different loci, we named the combinations between the three Psy1-A1 allele variants and the five Lpx-B1 haplotypes identified in this study. Eleven different combinations were obtained (named 1 to 10), and since eight of the nine modern genotypes harbored combination 5, the modern/landrace distinction was applied to it (Table 7). As expected, groups carrying Psy1-A1a (1 and 4) showed the lowest b* values. Allele combinations 2, 4, and 5 harbored approximately 80% of the population. By separating genotypes carrying combination 5 into modern cultivars and landraces, modern cultivars exhibited the highest b* values, which were significantly higher than the b* values of landraces from allele combination 5. The highest levels of yellowness (b* values over 19.0) were recorded for allele combinations 3 and 8 and in modern cultivars, without significant differences between them (Table 7).

Table 7. Frequency of the Psy1-A1 + Lpx-B1 allele combinations identified in the Mediterranean panel and yellow index (b* values) across Pirque 2017, Chillán 2017, and Chillán 2018.

| Allelic Combination | Composition       | Number of Genotypes | Frequency (%) | n | Minimum | Maximum | Mean ± SEb |
|---------------------|-------------------|---------------------|---------------|---|---------|---------|------------|
| 1                   | Haplotype I + Psy1-A1 | 2                   | 1.6           | 6 | 13.11   | 16.04   | 14.64 ± 0.43E |
| 2                   | Haplotype I + Psy1-All | 39                  | 30.5          | 116| 12.38   | 25.78   | 18.83 ± 0.25C |
| 3                   | Haplotype I + Psy1-A1 | 9                   | 7.0           | 27 | 12.59   | 24.06   | 19.25 ± 0.61ABC |
| 4                   | Haplotype II + Psy1-A1 | 14                  | 10.9          | 38 | 10.32   | 21.45   | 16.22 ± 0.39E |
| 5 Modern            | Haplotype II + Psy1-All | 41                  | 32.0          | 118| 12.96   | 22.59   | 18.41 ± 0.28BCD |
| 6                   | Haplotype II + Psy1-A1 | 8                   | 6.3           | 24 | 18.45   | 23.49   | 20.89 ± 0.28A |
| 7                   | Haplotype III + Psy1-All | 1                   | 0.8           | 3  | 13.27   | 14.98   | 14.10 ± 0.49BCD |
| 8                   | Haplotype III + Psy1-A1 | 1                   | 0.8           | 3  | 18.45   | 19.84   | 19.18 ± 0.40BCD |
| 9                   | Haplotype IV + Psy1-All | 3                   | 2.3           | 9  | 15.52   | 20.24   | 17.75 ± 0.57BCD |
| 10                  | Haplotype V + Psy1-All | 4                   | 3.1           | 12 | 15.31   | 20.38   | 17.30 ± 0.45BCDE |

a Data from Table 3, b* values for Pirque 2017, Chillán 2017, and Chillán 2018. b Means with different uppercase letters correspond to significantly different values after one-way ANOVA and Tukey’s post hoc test (p < 0.05).

2.3. Expression Levels of Psy1 and Lpx-B1 Genes during Grain Filling in Genotypes with Contrasting YI

The transcription levels of Psy1 and Lpx-B1 genes during grain filling were assessed in two landraces that showed consistently high (DW028) and low (DW011) YI values in each of the three environments (Table 3). The results showed that DW011 had no statistical difference in Lpx-B1 gene expression levels during the grain-filling period, and Psy1 had a peak at 28 days post anthesis (DPA) with a subsequent decrease in its expression (Figure 3A). The high yellowness line (DW028) exhibited no statistical expression differences for the Lpx-B1 gene, similarly to DW011, but Psy1 showed an increase in expression from 14 to 49 DPA, peaking at 42 and 49 DPA (Figure 3B). When the Psy1 expression levels between the low and high yellowness genotypes were compared, they did not show statistical differences until 42 and 49 DPA, when DW028 had 7.5 and 5.8 times higher expression than DW011, respectively (Figure 3C). In the case of Lpx-B1, DW011 had 1.8 and 2.1 times higher expression than DW028 at 14 and 21 DPA, respectively, they showed no differences at 28 and 35 DPA, and the trend reverted at 42 and 49 DPA, when DW028 had 9.3 and 9.9 times higher expression than DW011 (Figure 3D).
Yellowness is a major trait determining durum wheat quality for pasta making purposes. Psy1 and Lpx-1 have been pointed out as key enzymes predominately involved in carotenoid synthesis and degradation, respectively. Several studies reported many QTLs for yellow index spread all over the wheat genome [7], but to our knowledge no previous research has been reported simultaneously analyzing the two genes, the diversity of genetic materials, and expression profiles. Among them, two different QTLs were mapped on the long arm of chromosome group 7, co-localized with the phytoene synthase 1 (Psy1) and aldehyde oxidase 3 (AO3) genes, respectively [23,37–39]. While the Psy1 involvement in YPC has been deeply studied, the role of the AO3 gene in carotenoid accumulation needs to be elucidated. AO isoforms are key enzymes for abscisic acid (ABA) biosynthesis [40,41]. The plant AO family is composed of proteins with a high similarity in sequences, but different subunit compositions and substrate preferences. AO isoforms have been largely characterized in Arabidopsis and are composed of four isoforms [42].

In the current study, the Lpx gene was studied in a RIL population developed by crossing two genotypes, “02-BIL-318” and “Saragolla”, which consistently differ in their yellow coloring, thanks to its association with a QTL located on chromosome 4B. The localization on chromosome 4B of the Lpx gene was confirmed even in other research studies [32,43]. As reported from other authors [32,44,45], the linkage analysis highlighted not only the connection between this gene and the QTL, but also confirmed the key function of this gene in the oxidation of carotenoids. Thus, only for the isoforms mapped on chromosome group 4 was it deduced that the genotypes with a low content of carotenoid pigments were characterized by a greater catabolic activity. Carrera et al. [4] showed how the role of

**Figure 3.** Relative expression of Psy1 and Lpx-B1 genes during the grain filling stage in high and low yellowness Mediterranean genotypes. (A), relative expression in a low yellowness genotype during grain filling. (B), relative expression in a high yellowness genotype during grain filling. (C), comparison of the relative expression of Psy1 between high and low yellowness genotypes during grain filling. (D), comparison of the relative expression of Lpx-B1 in high and low yellowness genotypes. Two-way ANOVA and Tukey’s post hoc test were performed ($p < 0.05$). Upper and lower case are used to compare DPAs for each gene or genotype, an asterisk is used to denote statistical significance between conditions at each DPA. DPA, days post anthesis. All bars represent the mean of three biological and technical replicates ($n = 9$).

3. Discussion

Yellowness is a major trait determining durum wheat quality for pasta making purposes. Psy1 and Lpx-1 have been pointed out as key enzymes predominately involved in carotenoid synthesis and degradation, respectively. Several studies reported many QTLs for yellow index spread all over the wheat genome [7], but to our knowledge no previous research has been reported simultaneously analyzing the two genes, the diversity of genetic materials, and expression profiles. Among them, two different QTLs were mapped on the long arm of chromosome group 7, co-localized with the phytoene synthase 1 (Psy1) and aldehyde oxidase 3 (AO3) genes, respectively [23,37–39]. While the Psy1 involvement in YPC has been deeply studied, the role of the AO3 gene in carotenoid accumulation needs to be elucidated. AO isoforms are key enzymes for abscisic acid (ABA) biosynthesis [40,41]. The plant AO family is composed of proteins with a high similarity in sequences, but different subunit compositions and substrate preferences. AO isoforms have been largely characterized in Arabidopsis and are composed of four isoforms [42].

In the current study, the Lpx gene was studied in a RIL population developed by crossing two genotypes, “02-BIL-318” and “Saragolla”, which consistently differ in their yellow coloring, thanks to its association with a QTL located on chromosome 4B. The localization on chromosome 4B of the Lpx gene was confirmed even in other research studies [32,43]. As reported from other authors [32,44,45], the linkage analysis highlighted not only the connection between this gene and the QTL, but also confirmed the key function of this gene in the oxidation of carotenoids. Thus, only for the isoforms mapped on chromosome group 4 was it deduced that the genotypes with a low content of carotenoid pigments were characterized by a greater catabolic activity. Carrera et al. [4] showed how the role of
lipoxygenase on carotenoid degradation occurs in the process of pasta making and not during wheat grain development, which is in agreement with our own results.

The distribution of the Lpx-B1 gene family and the Psy1-A1 alleles were studied in a large durum wheat population comprising 128 Mediterranean landraces and modern cultivars, in order to link their presence and distribution to semolina yellowness.

The allelic variant Lpx-B1.1c was present in only two of the 128 genotypes analyzed in the current study, and they had very different levels of yellowness (DW059 and DW008, Table 3), even though both carried the Psy1-A1 alleles that are related to high levels of carotenoid synthesis (Table 4, [29]), and they are supposed to have low levels of LOX activity [4,32].

Verlotta et al. [32] made an association between LOX activity and haplotype types on wholemeal extracts, simulating the pH conditions (pH 6.6) during pasta processing, in which lipoxygenases are expected to be most active. In addition, Carrera et al. [4] reported that between 8 and 16% of the total carotenoid content is lost due to LOX activity during pasta processing.

Previous studies have not found relationships between LOX activity and yellow color in semolina [31,34]. In these studies, it was reported that in the steps from whole meal to semolina, and from semolina to pasta, there is a marked reduction in YPC, especially in genotypes having high LOX activity. LOX activity apparently becomes more important at the time of wheat processing, not before, so it makes more sense not to have found a relationship between the presence of the alleles and b* values. Additionally, when LOX activity was studied with different pH, it was reported that in varieties with high LOX activity, there is apparently more than one active LOX isoform, since the decrease in activity as the pH increases is not as pronounced as for cultivars with low LOX activity. In addition, a well-defined peak of maximum LOX activity was observed, which decayed rapidly when altering the pH, suggesting that in these cases a single isoform is produced, determining the LOX activity in wholemeal durum wheat. The authors found a relationship between LOX activity in semolina and yellowness in pasta. The presence of transcripts is not entirely consistent with the LOX activity reported in their work, so post-transcriptional mechanisms could be playing a role in the modulation of LOX activity. Transcript levels also associated with LOX activity in certain durum wheat varieties may be due to Lpx-1 and Lpx-3, while in others, only to Lpx-1. In addition, Verlotta et al. [32] reported the possibility of the existence of more allelic variants for Lpx-B1.2 and Lpx-B1.3 that were not detected in the current work with the specific primers used. Further, we can speculate that the differential Lpx-1 gene expression identified during the vegetative stage in this study, but not during grain filling, probably may not affect YPC levels, but post-transcriptional mechanisms, durum wheat processing conditions, and/or other allelic variants for Lpx-1 do influence semolina yellowness.

The expression levels of Psy1 and Lpx-B1 genes were analyzed during grain filling using two Mediterranean durum wheat genotypes with contrasting levels of yellowness compiled and purified at IRTA: DW011 (low yellowness; b* = 14.18 ± 1.29) and DW028 (high yellowness; b* = 21.71 ± 0.55). Psy1 exhibited a peak of expression at 42 and 49 DPA in the high yellowness landrace that could result in higher levels of grain phenotypic yellowness (Figure 3A,B). In the present study, no expression differences during grain filling were encountered for Lpx-B1 in both contrasting genotypes. The high yellowness genotype had 7.5 and 5.8 times higher expression levels for Psy1 than the low yellowness landrace at 42 and 49 DPA (Figure 3C). Interestingly, our group previously studied 12 modern durum wheat genotypes that showed greater expression of Psy1-A1, which specifically peaked at 35 DPA. At this time, Psy1-A1 was 21-fold more highly expressed in the high yellowness genotypes relative to the low yellowness genotypes [46]. The results of the present study suggest that not only is the particular allele of Psy1 responsible for the determination of grain yellowness (Psy1-A1l, Table 4), but its expression levels may also play a role in this trait (Figure 3). In addition, the higher expression levels of Psy1 of the landrace studied occurred late in grain development, while the modern durum genotypes had their peaks earlier (35 DPA), which may distinctly influence grain yellowness.

Regarding DW011, it is associated with haplotype II for Lpx-B1 (Table 3) and group 4 for Lpx-B1/Psy1-A1 (mean b* value 16.22 ± 0.39; Table 7), while DW028 is categorized as haplotype I for...
Lpx-B1 (Table 3) and group 3 for Lpx-B1/Psy1-A1 (mean b* value 19.25 ± 0.61; Table 7). There may be a relationship between the expression levels for Psy1 in the late grain filling stages and b* value. In the case of Lpx-B1, the DW011 had 1.8 and 2.19 times higher expression levels at 14 and 21 DPA than DW028, but this last genotype exhibited 9.36 and 9.97 higher expression levels at 42 and 49 DPA than the low yellowness landrace. Since Lpx-B1 encodes for lipoxygenase, lower gene expression levels were expected in the high yellowness genotypes, in fact, if the ratio Psy1/Lpx-B1 expression levels are compared at 49 DPA, the low yellowness genotype showed a ratio of 41.75 and the high yellowness genotype had a ratio of 24.17. Considering that the b* values for DW011 and DW028 are 14.18 ± 1.29 and 21.71 ± 0.55, respectively (Table 3), our result suggests that the expression of Psy1 had a greater influence than Lpx-B1 on b* values, but protein quantification and activity assays are required to draw more definitive conclusions.

Finally, high levels of carotenoid pigments in wheat kernels have important positive implications for human health since they are antioxidant compounds and precursors of vitamin A. Identifying the role of the main carotenoid genes in durum wheat and the specific alleles present in each cultivar can allow the development of superior cultivars through marker-assisted breeding programs. Molecular markers associated with Lpx-1 are currently being effectively used for marker-assisted selection in durum wheat breeding programs to improve YPC in different parts of the world, including the United States [11] and Canada [2].

4. Materials and methods

4.1. Plant Material and Experimental Setup

A set of 135 F_6-7 RILs was developed by the Department of Environmental and Territorial Sciences, University of Bari, Italy (DISAAT) through a single seed descendant (SSD) method, as described by Giancaspro et al. [35], and used for QTL analysis. The parents were the bread wheat accession “02-5B-318”, derived from the Chinese cv. “Sumai-3”, characterized by a low YI, and the durum wheat cv. “Saragolla”, characterized by a high YI. The parents and the RIL population were grown in Valenzano (Metropolitan City of Bari) for three years (2015, 2016, and 2017) using a randomized complete block design with four replications. Each plot consisted of 1-m rows, 30 cm apart, with four g of seeds sown in each plot and supplemented with nitrogen (10 g/m^2).

A Mediterranean durum wheat panel, including 119 landraces and nine modern cultivars (provided by IRTA, Table 8), was used to assess the relationship between Psy1-A1 and Lpx-B1 genetic composition and YI. This panel, which is described in detail in Nazco et al. [47], was grown in Chile during the 2016–2017 and 2017–2018 cropping seasons in Chillán (37°09′02.53″ S; 72°01′04.35″ W) and during the 2016–2017 cropping season in Pirque (33°40′00″ S; 70°35′23″ E). Experimental designs were randomized complete blocks with three replicates and plots of three 1-m rows and 0.2-m inter-row spacing, planted at a seed rate of 220 kg/ha. Fertilizers were applied at a dose of 23 kg N/ha as urea (46% N), 27 kg N/ha + 69 kg P_2O_5/ha as diammonium phosphate (18% N, 46% P_2O_5), and 60 kg KCl/ha of potassium chloride (60% KCl) before sowing, and additionally 284 kg N/ha were applied as urea (46% N) at tillering. Plots were irrigated when necessary to prevent water deficit. Weeds, aphids, and fungal diseases were chemically controlled. Plots were manually harvested at ripening and the grain obtained was used for YPC determination. The expression of Psy1-A1 and Lpx-B1 genes during the grain filling period was monitored in two landraces with contrasting grain yellowness: DW011, cv. “Heraldo del Rhin” (b* = 14.18 ± 1.29, Table 3) and DW028, cv. “IG-92967” (b* = 21.71 ± 0.55, Table 3).
Table 8. Genotypes included in the Mediterranean panel. LR, landraces.

| Code   | Type | Genotype             | Origin      | Code   | Type | Genotype             | Origin      |
|--------|------|----------------------|-------------|--------|------|----------------------|-------------|
| DW001  | LR   | Ariniegro de Tenerife | Spain       | DW091  | LR   | 9923                 | Lebanon     |
| DW002  | LR   | Basto Duro           | Spain       | DW092  | LR   | 9929                 | Lebanon     |
| DW003  | LR   | Blanco de Corella    | Spain       | DW093  | LR   | 9935                 | Lebanon     |
| DW004  | LR   | Blanquillo           | Spain       | DW094  | LR   | Abu Fashit           | Israel      |
| DW005  | LR   | Cardeval de Salamanca| Spain       | DW095  | LR   | D-2                  | Egypt       |
| DW006  | LR   | Colorado de Jerez    | Spain       | DW096  | LR   | Maghoussa            | Morocco     |
| DW008  | LR   | Fartó                | Spain       | DW098  | LR   | Red Beard            | Morocco     |
| DW009  | LR   | Grego de Balares     | Spain       | DW099  | LR   | Safra Jerash         | Jordan      |
| DW010  | LR   | Gros de Cerdàta      | Spain       | DW103  | LR   | Louri APS            | Tunisia     |
| DW011  | LR   | Heraldo del Rihn     | Spain       | DW104  | LR   | Souri                | Tunisia     |
| DW012  | LR   | Pintet               | Spain       | DW110  | LR   | 1F1                  | Egypt       |
| DW013  | LR   | Pisana cathayueca    | Spain       | DW111  | LR   | Beladi Rouge         | France      |
| DW014  | LR   | Raspinegro Canario   | Spain       | DW113  | LR   | lumillo              | France      |
| DW015  | LR   | Raspinegro de Alcalá Guadaira | Spain | DW114 | LR | Trigo Glutinoso | France |
| DW016  | LR   | Recio de Almería     | Spain       | DW115  | LR   | 9918                 | Lebanon     |
| DW017  | LR   | Verdial              | Spain       | DW116  | LR   | 9918                 | Lebanon     |
| DW018  | LR   | Tchiptan             | Bulgaria    | DW117  | LR   | Hourah               | Lebanon     |
| DW019  | LR   | Looz Zul            | Bulgaria    | DW121  | LR   | Maghoussa Amizmiz    | Morocco     |
| DW020  | LR   | Vosko                | Cyprus      | DW122  | LR   | Horari               | Jordan      |
| DW021  | LR   | IG-82549             | Cyprus      | DW124  | LR   | Harani Autuma        | Jordan      |
| DW022  | LR   | Carlantino           | Italy        | DW126  | LR   | Horari Howawi        | Jordan      |
| DW023  | LR   | Cicerolo             | Italy        | DW127  | LR   | Zogbieh Sutra        | Jordan      |
| DW024  | LR   | IG-83905             | Italy        | DW128  | LR   | Belgrade 9           | Serbia      |
| DW025  | LR   | IG-83920             | Italy        | DW129  | LR   | Zogbieh Safra        | Jordan      |
| DW027  | LR   | IG-92895             | Algeria      | DW130  | LR   | Enith                | Israel      |
| DW028  | LR   | IG-92627             | Algeria      | DW131  | LR   | Julith               | Israel      |
| DW032  | LR   | IG-95812             | Syria        | DW132  | LR   | 248-VII/7            | Macedonia   |
| DW033  | LR   | IG-95841             | Syria        | DW133  | LR   | 259-VII/12           | Macedonia   |
| DW034  | LR   | IG-95847             | Syria        | DW134  | LR   | 256-19               | Montenegro  |
| DW035  | LR   | IG-95931             | Syria        | DW136  | LR   | 441-1X/97            | Croatia     |
| DW037  | LR   | IG-96851             | Creta        | DW137  | LR   | VII/13-X11           | Macedonia   |
| DW038  | LR   | Alonso               | Spain        | DW138  | LR   | VII/18-X24           | Macedonia   |
| DW040  | LR   | Azulejo de Villa del Río | Spain | DW139 | LR | Safra Maan | Jordan |
| DW041  | LR   | Blancal              | Spain        | DW144  | LR   | 196/71               | Macedonia   |
| DW042  | LR   | Blanquillo de Borbar | Spain       | DW145  | LR   | 1575                 | Serbia      |
| DW043  | LR   | Claro de Balazote    | Spain        | DW146  | LR   | II/4                 | Macedonia   |
| DW044  | LR   | Entrelaga de Montijo | Spain        | DW147  | LR   | Cobros               | Morocco     |
| DW045  | LR   | Farto canfrino       | Spain        | DW148  | LR   | Haj Mouline           | Morocco     |
| DW046  | LR   | Rubio de Mejadas     | Spain        | DW149  | LR   | 26                   | Jordan      |
| DW047  | LR   | Rubio de Montijo     | Spain        | DW150  | LR   | Mavranzi             | Greece      |
| DW048  | LR   | Russo                | Spain        | DW151  | LR   | Rapsani              | Greece      |
| DW052  | LR   | Senatore Capelli     | Italy         | DW152  | LR   | Giza 2               | Egypt       |
| DW054  | LR   | Hymera               | Italy         | DW154  | LR   | 33                   | Montenegro  |
| DW056  | LR   | Azirizah 17/45       | Italy         | DW158  | LR   | Rubio enlargado d' Atlemejo | France |
| DW058  | LR   | Bailla Falso         | Italy         | DW160  | LR   | Hatt                 | Israel      |
| DW059  | LR   | Marques              | Portugal      | DW161  | LR   | Tripshiyo            | Libya       |
| DW060  | LR   | Mindum               | Turkey        | DW162  | LR   | 2751                 | Egypt       |
| DW061  | LR   | Raposinho            | Portugal      | DW163  | LR   | JM-3987              | Israel      |
| DW062  | LR   | Reading              | Egypt         | DW165  | LR   | MG26429              | Egypt       |
| DW067  | LR   | Anafil               | Portugal      | DW166  | LR   | 18/71                | Serbia      |
| DW068  | LR   | Español              | Portugal      | DW167  | LR   | 28                   | Egypt       |
| DW069  | LR   | Deazasette           | Portugal      | DW168  | LR   | 31                   | Egypt       |
| DW070  | LR   | Durazo Rijo Glablo   | Portugal      | DW170  | LR   | Mishrikii            | Egypt       |
| DW071  | LR   | Amaren Digalenta Preta | Portugal | DW171 | LR | Cigalsh               | Egypt       |
| DW072  | LR   | Alentejo             | Portugal      | DW172  | LR   | Hamira               | Tunisia     |
| DW074  | LR   | BGE-018192           | Turkey        | DW174  | LR   | Ancalei              | Spain       |
| DW078  | LR   | BGE-018354           | Turkey        | DW175  | LR   | Arment               | France      |
| DW080  | LR   | BGE-019263           | Turkey        | DW176  | LR   | Astigui              | Spain       |
| DW082  | LR   | BGE-019265           | Turkey        | DW177  | LR   | Boabidil             | Spain       |
| DW083  | LR   | BGE019266            | Turkey        | DW178  | LR   | Senadur              | Spain       |
| DW084  | LR   | BGE-019270           | Turkey        | DW189  | LR   | Sala                  | Spain       |
| DW085  | LR   | Tremosol             | Portugal      | DW190  | LR   | Trimor               | Spain       |
| DW086  | LR   | Lebeiro de grao escuro | Portugal | DW191 | LR | Vitron               | Spain       |
| DW090  | LR   | Zoco Yebel Hevil     | Morocco       | DW192  | LR   | Vitrone              | Spain       |

4.2. Detection of QTLs for Carotenoid Content in the RIL Population

The identification of QTLs associated with YI was carried out based on the genetic map previously developed by Giancaspro et al. [35]. QTL detection was performed in QGene 8.3.16 using composite interval mapping (CIM), as proposed by Zeng [48]. The association between marker and trait was considered significant when one or more markers showed a $-\log_{10}(p) \geq 3.0$, determined by modified Bonferroni correction. The contributions of “02-5B-318” and “Saragolla” were highlighted by a positive and negative sign, respectively.
Graphical representations of linkage groups and QTLs were carried out using MapChart 2.2 software (SolarWinds, Austin, TX, USA). Genes located in the QTL regions were identified by blasting the SNP sequences from Wang et al. [49] against the annotated *Triticum* (NCBI) and contig (URGI) sequences.

In order to pick primer combinations to use for the *Lpx* gene expression analysis, the sequences and the gene models were downloaded from the Svevo [50] and Chinese Spring [51] genome databases. The *Lpx* genes were located on chromosome groups 4 and 5 (4A: TRITD4Av1G195350; 4B: TRITD4Bv1G010710; 5A: TRITD5Av1G200190; 5B: TRITD5Bv1G195300). Primer combinations were designed in the conserved region of the first exon for all genes, considering dissimilarities between the two homologous copies (Table 9). The genetic materials used for the quantitative real-time PCR analyses were collected from leaves at the seedling stage belonging to the wheat cultivars “Saragolla” and “02-5B-318”, characterized by high and low values of YI, respectively.

**Table 9.** Primer pairs used for *Lpx* gene expression analysis in the RIL population.

| Gene Name | Primer Orientation | Sequence (5′ → 3′) | Product Length (bp) |
|-----------|--------------------|--------------------|---------------------|
| *Lpx*-4A  | Fw                 | CAG TTC CAG ACC ATC CTC GG | 229 |
|           | Rv                 | TGT AGG GCA TCT TCA CCG G    |
| *Lpx*-4B  | Fw                 | ATC CTG TCC AAG CAC TCC TC | 240 |
|           | Rv                 | CAG CCC TTT TCT GCC GTC    |
| *Lpx*-5A  | Fw                 | GAG GTC TGG CAC GCG ATC | 171 |
|           | Rv                 | CAC GGT GTG CAT CTT GGC C   |
| *Lpx*-5B  | Fw                 | CAA GAT GCA GAC GGT GCC | 217 |
|           | Rv                 | GGT GAT GGT GAG GAT AAA GCC |

Total RNA was extracted from the grain tissue of both genotypes using the RNeasy Plant Mini Kit (QIAGEN®) (Germantown, MD, USA) and checked on 1.5% denaturing agarose gel. All RNA samples were the same concentration (1 µg) and were reverse transcribed into double stranded cDNA with a QuantiTect Reverse Transcriptase Kit (QIAGEN®). Data were normalized, as previously described by Marcotuli et al. [52], using three reference genes (ADP-RF, RLI, and CDC), which have a stability value, calculated with NormFinder software, of 0.035 [52].

Quantitative real-time PCR was carried out using Cyber® GREEN in the CFX96TM real-time PCR system (BIO-RAD) following the protocol described by Marcotuli et al. [52]. A series of six scalar dilutions were carried out to determine the primer amplification efficiency, while the specificity of the amplicons was confirmed by the following steps: a single band on the agarose gel (2% w/v) and a single peak in the melting curves and the sequence of the amplified fragments (3500 Genetic Analyzer, Applied Biosystems).

The qRT-PCR data for genes were derived from the mean values of three independent amplification reactions carried out on the parental lines (“Saragolla” and “02-5B-318”) of the RIL population.

Three reference genes were used to normalize data (cell division control AAA superfamily of ATPases, CDC; ADP-ribosilation factor, ADP-RF; RNase L inhibitor-like protein, RLI [53,54]). All calculations and analyses were performed, as reported by Marcotuli et al. [52], using CFX Manager 2.1 software (Bio-Rad Laboratories), applying the ΔΔCt method. Standard deviations were used to normalize values, and ANOVA and LSD tests were used to underline significant differences between the genotypes.

### 4.3. Psy1-A1 and Lpx-B1 Genotyping in the Mediterranean Panel

Plants were grown in the greenhouse, in triplicate, in a mixture of peat and perlite in a 2:1 ratio, without fertilization, until they reached the two to three leaves stage, in order to extract DNA with the CTAB protocol [55], with minor modifications. One hundred milligrams of leaves were crushed to powder in the presence of liquid nitrogen and 1 mL of CTAB buffer and 0.2% β-mercaptoethanol and...
2% PVP were added. The mixture was incubated at 65 °C for 30 min, mixing it gently every 5 min. Then, 1 mL of chloroform and isooamyl alcohol in a ratio of 24:1 were added, mixed by vortexing, and centrifuged at 13,000 rpm for 10 min. Then, the upper phase was extracted and mixed with an equal volume of isopropanol and incubated overnight at −20 °C. Then, the mixture was centrifuged at 13,000 rpm at 4 °C for 10 min and the pellets were air dried at 37 °C, before adding 50 μL of ultrapure water (Invitrogen) (Waltham, MA, USA). Each DNA sequence was quantified by QUBIT 3.0 (Life Technologies) (Carlsbad, CA, USA) and the integrity was checked by agarose electrophoresis.

Regarding Psy1-A1, 112 out of 128 genotypes used in this study were previously characterized by Campos et al. [30]. The rest of the population was genotyped with the marker PSY1-A1_STS (Table 5) and PCR conditions used by Singh et al. [29], allowing the discrimination between the alleles Psy1-A1a, Psy1-A1b, and Psy1-A1o, using the previously extracted DNA.

In order to amplify the Lpx-B1 genes and different alleles [4,32], the primer pairs in Table 5 were employed in this study. The PCR conditions for every sequence were as follows: Lpx-B1.1a (HM126471) and Lpx-B1.1b (HM126473) (there is a 74 bp difference between both amplicons that allows differentiation in agarose electrophoresis): one cycle of 1 min at 94 °C; 35 cycles of 30 s at 94 °C followed by 10 s at 68 °C, 1 min 25 s at 72 °C, and one cycle of 7 min at 72 °C. Lpx-B1.1c (HM126475): one cycle of 1 min at 94 °C; 35 cycles of 30 s at 94 °C followed by 20 s at 67 °C, 1 min 35 s at 72 °C, and one cycle of 7 min at 72 °C. Lpx-B1.2 (HM126472) and Lpx-B1.3 (HM126469) (there is a 76 bp difference between both amplicons that allows differentiation in agarose electrophoresis): one cycle of 1 min at 94 °C, 35 cycles of 30 s at 94 °C followed by 10 s at 62 °C, 1 min 50 s at 72 °C, and one cycle of 7 min at 72 °C. Amplicon identity was checked by sequencing at Macrogen Inc. (Korea). Haplotypes were named following Verlotta et al. [32] nomenclature.

4.4. Psy1 and Lpx-1 Gene Expression during Grain Filling

The expression of Psy1 and Lpx-1 genes during grain filling was monitored through qPCR. For total RNA extraction, 100 mg of grain tissue were used at each grain developmental stage from 14 DPA to 49 DPA, in triplicate, for genotypes DW011 (low yellowness) and DW028 (high yellowness). RNA extraction was performed based upon the Furtado [56] protocol, with some modifications. The two-step RNA extraction involved the use of TRIzol™ Reagent (ThermoFisher Scientific, Carlsbad, CA, USA), and the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Düren, Germany). Firstly, seed tissue was pulverized in a mortar and pestle in the presence of liquid nitrogen and 1.5 mL of TRIzol Reagent™ were added. The samples were then centrifuged at 12,000× g and 4 °C for 10 min. Subsequently, the upper phase (~750 μL) was recovered, mixed with 300 μL of chloroform and agitated through inversion for 3 min, and then centrifuged for 15 min at 12,000× g and 4 °C. The upper phase was then recovered in a new tube, and from this step onwards, the NucleoSpin® RNA Plant Kit was used according to the manufacturer’s instructions. Finally, RNA was eluted with 30 μL RNase-free H₂O. RNA integrity was verified on 2% agarose gel and visualized with GelRed® staining (Biotium (company name), Ferment, CA, USA), and RNA was quantified using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific (company name), Carlsbad, CA, USA). First strand cDNA synthesis was obtained from 4 μg of total RNA using SuperScript™ II (ThermoFisher Scientific (company name), Carlsbad, CA, USA) and oligo dT primers, following the manufacturer’s instructions.

The relative expression analyses were carried out using the SensiFast™ SYBR® No-ROX kit (Bioline (company name), United Kingdom). For each reaction, 50 ng of cDNA were used and tested for the expression of the genes Psy1 (primers qrtPsy1_F and qrtPsy1_R, Table 5) and Lpx-1 (primers qrtLpx_B1_F and qrtLpx_B1_R, Table 5) using the ADP ribosylation factor as a reference gene [53]. All primers were designed using the Amplifix and the NCBI primer blast, and they are further characterized in Table 5. The efficiency and CT value of each reaction were determined using LinRegPCR V2018.0 [57], using a common threshold and an individual fit. The relative expression of each gene was calculated using the Pfaffl method [58] with a calibrator value of 0, and then the relative
expression was divided by the lower value in each group of data. Three biological and three technical replicates were used at each developmental stage.

4.5. Yellowness Determination

Yellow intensity was assessed in the two germplasm panels in wholegrain flour through the yellow index (YI) [11]. L*, a* and b* coordinates in the Munsell color system were taken using D65 lightning. A reflectance colorimeter (CR-400, Konica-Minolta, Japan) equipped with a filter tri-stimulate system was used for these determinations.

4.6. Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 8.2.1. GenStat (version 18, VSN International Ltd., Hemel Hempstead, UK), which was used to carry out the ANOVA in the RIL population on the YI to identify how much of the variation was attributed to genotype. Broad-sense heritability (h\textsuperscript{2}B), was estimated, accounting the genetic variance, \(\sigma^2_G\), the genotype x environment interaction variance, the residual variance, the number of environments (in this case three), and the number of replicates per line (in this case three replicates) [52,59].

5. Conclusions

Semolina yellowness is a key aspect determining durum wheat end products. Understanding the synthesis and degradation of carotenoid pigments is a vital aspect of durum wheat breeding programs aiming to produce superior cultivars with higher yellowness. In the current study, eight QTLs related to yellowness were identified, with different degrees of involvement in YI variation, and \(Lpx\) was linked to a QTL found in chromosome 4B, which showed lower expression in a high yellowness cultivar in comparison to a low yellowness wheat.

The \(Lpx-B1\) characterization in a landrace and modern wheat Mediterranean population allowed the identification of novel allele combinations (haplotypes IV and V), and by adding a \(Psy1-A1\) characterization, 11 different combinations of groups appeared, revealing different levels of YI, the modern group being the one with higher levels of yellowness, harboring \(Psy1-A1l, Lpx-B1.1a,\) and \(Lpx-B1.2.\) Besides, by comparing high and low yellowness landraces, it is possible to suggest that, during grain development, \(Psy1\) plays a major role contributing to semolina yellowness, and \(Lpx\) is suggested to be more predominant at post-harvest stages and during pasta making. The practical applications of our work in durum wheat breeding programs relate to the use of marker-assisted selection for \(Psy1\) alleles showing higher transcript abundance and greater semolina yellowness, whose genotypes could be specifically evaluated and selected at 42 DPA. In addition, studying whether the high yellowness \(Psy1\) alleles and transcript abundance could be identified at earlier vegetative stages (i.e., leaves) of the plant would be very useful to reduce the selection times without waiting for grain filling. Furthermore, the utilization of biotechnological approaches to elevate the \(Psy1\) transcripts at late grain development stages would be valuable to potentially increase semolina yellowness. Finally, this study highlights the applicability of markers linked to the \(Lpx-1\) gene in marker-assisted selection programs.

Author Contributions: Conceptualization, R.P., C.R., A.G. and A.S.; Writing—Original Draft Preparation, R.P., C.R., A.G., J.A.-F., and A.S. Final Review and Editing, R.P., C.R., A.G., P.C., I.M. (Ilaria Marcotuli), I.M. (Iván Matus), D.C., A.C.d.C., J.A.-F., D.V., A.R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico - FONDECYT Regular (grant n° 1161298), FONDECYT postdoctorado (grant n° 3180432), PRIMA 2019 project “CEREAALMED” (Italy), and AGL2012-37217 from MINECO, Spain. The CERCA Programme/Generalitat de Catalunya (http://cerca.cat/) also supported this research.

Conflicts of Interest: The authors declare no conflict of interest.
Abbreviations

Psy  Phytoene Synthase  
Lpx  Lipoxygenase  
RIL  Recombinant Inbred Line  
QTL  Quantitative Trait Locus  
YPC  Yellow Pigment Content  
LOX  Lipoxygenase (protein)  
ROS  Reactive Oxygen Species  
YI  Yellow Index  
CIM  Composite Interval Mapping  
SNP  Single Nucleotide Polymorphism  
qPCR  Quantitative Real-Time PCR

References

1. Ficco, D.B.M.; Mastrangelo, A.M.; Trono, D.; Borrelli, G.M.; De Vita, P.; Fares, C.; Beleggia, R.; Platani, C.; Papa, R. The colours of durum wheat: A review. Crop Pasture Sci. 2014, 65, 1–15. [CrossRef]
2. Randhawa, H.S.; Asif, M.; Pozniak, C.; Clarke, J.M.; Graf, R.J.; Fox, S.L.; Humphreys, D.G.; Knox, R.E.; DePauw, R.M.; Singh, A.K.; et al. Application of molecular markers to wheat breeding in Canada. Plant Breed. 2013, 132, 458–471. [CrossRef]
3. Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/worldfoodsituation/cpdb/en/ (accessed on 23 November 2019).
4. Carrera, A.; Echenique, V.; Zhang, W.; Helguera, M.; Manthey, F.; Schrager, A.; Picca, A.; Cervigni, G.; Dubcovsky, J. A deletion at the Lpx-B1 locus is associated with low lipoxygenase activity and improved pasta color in durum wheat (Triticum turgidum ssp. durum). J. Cereal Sci. 2007, 45, 67–77. [CrossRef]
5. Gale, K.R. Diagnostic DNA markers for quality traits in wheat. J. Cereal Sci. 2005, 41, 181–192. [CrossRef]
6. Troccoli, A.; Borrelli, G.M.; De Vita, P.; Fares, C.; Di Fonzo, N. Mini review: Durum wheat quality: A multidisciplinary concept. J. Cereal Sci. 2000, 32, 99–113. [CrossRef]
7. Colasuonno, P.; Marcotuli, I.; Blanco, A.; Maccaferri, M.; Condorelli, G.E.; Tuberosa, R.; Parada, R.; de Camargo, A.C.; Schwember, A.R.; Gadaleta, A. Carotenoid pigment content in durum wheat (Triticum turgidum L. var durum): An overview of quantitative trait loci and candidate genes. Front. Plant. Sci. 2019, 10, 1347. [CrossRef]
8. Sommer, A.; Davidson, F.R. Assessment and control of vitamin A deficiency: The anecy accords. J. Nutr. 2002, 132, 2845S–2850S. [CrossRef] [PubMed]
9. Borrelli, G.M.; Troccoli, A.; Di Fonzo, N.; Fares, C. Durum wheat lipoxygenase activity and other quality parameters that affect pasta color. Cereal Chem. J. 1999, 76, 335–340. [CrossRef]
10. Borrelli, G.M.; De Leonardis, A.M.; Fares, C.; Platani, C.; Di Fonzo, N. Effects of modified processing conditions on oxidative properties of semolina dough and pasta. Cereal Chem. J. 2003, 80, 225–231. [CrossRef]
11. Borrelli, G.M.; Trono, D. Molecular approaches to genetically improve the accumulation of health-promoting secondary metabolites in staple crops—A case study: The Lipoxygenase-B1 genes and regulation of the carotenoid content in pasta products. Int. J. Mol. Sci. 2016, 17, 1177. [CrossRef] [PubMed]
12. Dexter, J.E.; Marchylo, B.A. Recent trends in durum wheat milling and pasta processing: Impact on durum wheat quality requirements. In Proceedings of the International Workshop on Durum Wheat, Semolina, and Pasta Quality: Recent Achievements and New Trends, Montpellier, France, 27 November 2000.
13. Hare, R. Agronomy of the Durum Wheats Kamilarioi, Yallaro, Wollaro and EGA Bellaroii. Available online: http://http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0007/6364/Agronomy-of-the-durum-wheats---Primefact-140-final.pdf (accessed on 18 June 2020).
14. Hentschel, V.; Kranl, K.; Hollmann, J.; Lindhauer, M.G.; Bohm, V.; Bitsch, R. Spectrophotometric determination of yellow pigment content and evaluation of carotenoids by high-performance liquid chromatography in durum wheat grain. J. Agric. Food Chem. 2002, 50, 6663–6668. [CrossRef] [PubMed]
15. Adom, K.K.; Sorrells, M.E.; Liu, R.H. Phytochemical profiles and antioxidant activity of wheat varieties. J. Agric. Food Chem. 2003, 51, 7825–7834. [CrossRef] [PubMed]
16. Garbus, I.; Carrera, A.D.; Dubcovsky, J.; Echenique, V. Physical mapping of durum wheat lipoygenase genes. *J. Cereal Sci.* 2009, 50, 67–73. [CrossRef]

17. Porceddu, E.; Blanco, A. Evolution of durum wheat breeding in Italy. In *Proceedings of the International Symposium on Genetics and Breeding of Durum Wheat*; Porceddu, E., Qualset, C.O., Damania, A.B., Eds.; CIHEAM: Bari, Italy, 2014; Volume 110, pp. 157–173.

18. Clarke, F.R.; Clarke, J.M.; McCaig, T.N.; Knox, R.E.; DePauw, R.M. Inheritance of yellow pigment concentration in seven durum wheat crosses. *Can. J. Plant Sci.* 2006, 86, 133–141. [CrossRef]

19. Magallanes-Lopez, A.M.; Ammar, K.; Morales-Dorantes, A.; Gonzalez-Santoyo, H.; Crossa, J.; Guzman, C. Grain quality traits of commercial durum wheat varieties and their relationships with drought stress and glutenins composition. *J. Cereal Sci.* 2017, 75, 1–9. [CrossRef]

20. Roncallo, P.F.; Cervigni, G.L.; Jensen, C.; Miranda, R.; Carrera, A.D.; Helguera, M.; Echenique, V. QTL analysis of main and epistatic effects for flour color traits in durum wheat. *Euphytica* 2012, 185, 77–92. [CrossRef]

21. Patil, R.M.; Oak, M.D.; Tamhankar, S.A.; Sourdille, P.; Rao, V.S. Mapping and validation of a major QTL for yellow pigment content on 7AL in durum wheat (*Triticum turgidum* L. ssp. *durmum*). *Mol. Breed.* 2008, 21, 485–496. [CrossRef]

22. Patil, R.; Oak, M.; Deshpande, A.; Tamhankar, S. Development of a robust marker for *Psy*-1 homoeologs and its application in improvement of yellow pigment content in durum wheat. *Mol. Breed.* 2018, 38, 136. [CrossRef]

23. Zhang, W.; Dubcovsky, J. Association between allelic variation at the Phytoene synthase 1 gene and yellow pigment content in the wheat grain. *Theor. Appl. Genet.* 2008, 116, 635–645. [CrossRef]

24. Zhang, F.; Chen, F.; Wu, P.; Zhang, N.; Cui, D. Molecular characterization of lipoygenase genes on chromosome 4BS in Chinese bread wheat (*Triticum aestivum* L.). *Theor Appl Genet.* 2015, 128, 1467–1479. [CrossRef]

25. Zhai, S.; Li, G.; Sun, Y.; Song, J.; Li, J.; Song, G.; Li, Y.; Ling, H.; He, Z.; Xia, X. Genetic analysis of phytoene synthase 1 (*Psy1*) gene function and regulation in common wheat. *BMC Plant Biol.* 2016, 16, 228. [CrossRef] [PubMed]

26. He, X.Y.; Zhang, Y.L.; He, Z.H.; Wu, Y.P.; Xiao, Y.G.; Ma, C.X.; Xia, X.C. Characterization of phytoene synthase 1 gene (*Psy1*) located on common wheat chromosome 7A and development of a functional marker. *Theor. Appl. Genet.* 2008, 116, 213–221. [CrossRef] [PubMed]

27. He, X.; Wang, J.; Ammar, K.; Peña, R.J.; Xia, X.; He, Z. Allelic variants at the and loci in durum wheat and their associations with grain yellowness. *Crop Sci.* 2009, 49, 2058. [CrossRef]

28. He, X.Y.; He, Z.H.; Ma, W.; Appels, R.; Xia, X.C. Allelic variants of phytoene synthase 1 (*Psy1*) genes in Chinese and CIMMYT wheat cultivars and development of functional markers for flour colour. *Mol. Breed.* 2009, 23, 553–563. [CrossRef]

29. Singh, A.; Reimer, S.; Pozniak, C.J.; Clarke, F.R.; Clarke, J.M.; Knox, R.E.; Singh, A.K. Allelic variation at *Psy1*-A1 and association with yellow pigment in durum wheat grain. *Theor. Appl. Genet.* 2009, 118, 1539–1548. [CrossRef]

30. Campos, K.M.; Royo, C.; Schulthess, A.; Villegas, D.; Matus, I.; Ammar, K.; Schwambeter, A.R. Association of phytoene synthase *Psy1*-A1 and *Psy1*-B1 allelic variants with semolina yellowness in durum wheat (*Triticum durum* L. var. *durum*). *Euphytica* 2015, 207, 109–117. [CrossRef]

31. Hessler, T.G.; Thomson, M.J.; Benschler, D.; Nachit, M.; Sorrells, M. Association of a lipoygenase locus, *Lpx-B1*, with variation in lipoygenase activity in durum wheat seeds. *Crop Sci.* 2002, 42. [CrossRef]

32. Verlotta, A.; De Simone, V.; Mastrangelo, A.M.; Cattivelli, L.; Papa, R.; Trono, D. Insight into durum wheat *Lpx-B1*: A small gene family coding for the lipoygenase responsible for carotenoid bleaching in mature grains. *BMC Plant Biol.* 2010, 10, 263. [CrossRef]

33. Holtman, W.L.; Van Duijn, G.; Sedee, N.; Douma, A.C. Differential expression of lipoygenase isoenzymes in embryos of germinating barley. *Plant Physiol.* 1996, 111, 569–576. [CrossRef]

34. De Simone, V.; Menzo, V.; De Leonardis, A.M.; Fico, D.B.M.; Trono, D.; Cattivelli, L.; De Vita, P. Different mechanisms control lipoygenase activity in durum wheat kernels. *J. Cereal Sci.* 2010, 52, 121–128. [CrossRef]

35. Giancaspro, A.; Giove, S.L.; Zito, D.; Blanco, A.; Gadaleta, A. Mapping QTLs for fusarium head blight resistance in an interspecific wheat population. *Front. Plant Sci.* 2016, 7, 1381. [CrossRef] [PubMed]
36. Rodriguez-Suarez, C.; Mellado-Ortega, E.; Hornero-Mendez, D.; Atienza, S.G. Increase in transcript accumulation of Psy1 and e-Lcy genes in grain development is associated with differences in seed carotenoid content between durum wheat and tritordeum. *Plant Mol. Biol.* 2014, 84, 659–673. [CrossRef]

37. Blanco, A.; Colasuonno, P.; Gadaleta, A.; Mangini, G.; Schiavulli, A.; Simeone, R.; Digesì, A.M.; De Vita, P.; Mastrangelo, A.M.; Cattivelli, L. Quantitative trait loci for yellow pigment concentration and individual carotenoid compounds in durum wheat. *J. Cereal Sci.* 2011, 54, 255–264. [CrossRef]

38. Colasuonno, P.; Gadaleta, A.; Giancaspro, A.; Nigro, D.; Giove, S.; Incerti, O.; Mangini, G.; Signorile, A.; Simeone, R.; Blanco, A. Development of a high-density SNP-based linkage map and detection of yellow pigment content QTLs in durum wheat. *Mol. Breed.* 2014, 34, 1563–1578. [CrossRef]

39. Colasuonno, P.; Marcotuli, I.; Lozito, M.L.; Simeone, R.; Blanco, A.; Gadaleta, A. Characterization of aldehyde oxidase (AO) genes involved in the accumulation of carotenoid pigments in wheat grain. *Front. Plant Sci.* 2017, 8, 863. [CrossRef] [PubMed]

40. Fang, J.; Chai, C.; Qian, Q.; Li, C.; Tang, J.; Sun, L.; Huang, Z.; Guo, X.; Sun, C.; Liu, M.; et al. Mutations of genes in synthesis of the carotenoid precursors of ABA lead to pre-harvest sprouting and photo-oxidation in rice. *Plant J.* 2008, 54, 177–189. [CrossRef] [PubMed]

41. Zdunek-Zastocka, E. The activity pattern and gene expression profile of aldehyde oxidase during the development of Pismum sativum seeds. *Plant Sci.* 2010, 179, 543–548. [CrossRef]

42. Seeo, M.; Koiwai, H.; Akaba, S.; Komano, T.; Oritani, T.; Kamiya, Y.; Koshiba, T. Abscisic aldehyde oxidase in leaves of Arabidopsis thaliana. *Plant. J.* 2000, 23, 481–488. [CrossRef]

43. Maccaferri, M.; Cane’, M.A.; Sanguineti, M.C.; Salvi, S.; Colalongo, M.C.; Massi, A.; Clarke, F.; Knox, R.; Pozniak, C.J.; Clarke, J.M.; et al. A consensus framework map of durum wheat (Triticum durum Desf.) suitable for linkage disequilibrium analysis and genome-wide association mapping. *BMC Genom.* 2014, 15, 873. [CrossRef]

44. Mares, D.; Aw, C. Mapping components of flour and noodle colour in Australian wheat. *Australian J. Agric. Res.* 2001, 52, 1297–1309. [CrossRef]

45. Garbus, I.; Soresi, D.; Romero, J.; Echenique, V. Identification, mapping and evolutionary course of wheat lipoxygenase-1 genes located on the A genome. *J. Cereal Sci.* 2013, 58, 298–304. [CrossRef]

46. Vargas, V.H.; Schulthess, A.; Royo, C.; Matus, I.; Schwember, A.R. Transcripts levels of Phytoene synthase 1 (Psy-1) are associated to semolina yellowness variation in durum wheat (*Triticum turgidum L.* ssp. durum). *J. Cereal Sci.* 2016, 68, 155–163. [CrossRef]

47. Nazco, R.; Villegas, D.; Ammar, K.; Peña, R.J.; Moragues, M.; Royo, C. Can Mediterranean durum wheat landraces contribute to improved grain quality attributes in modern cultivars? *Euphytica* 2011, 185, 1–17. [CrossRef]

48. Zeng, Z.B. Precision mapping of quantitative trait loci. *Genetics* 1994, 136, 1457–1468.

49. Wang, Z.; Li, H.; Zhang, D.; Guo, L.; Chen, J.; Chen, Y.; Wu, Q.; Xie, J.; Zhang, Y.; Sun, Q.; et al. Genetic and physical mapping of powdery mildew resistance gene MHILT in Chinese wheat landrace Hulutou. *Theor. Appl. Genet.* 2015, 128, 365–373. [CrossRef] [PubMed]

50. Durum Wheat Genome (cv. Svevo). Available online: https://www.interomics.eu/durum-wheat-genome (accessed on 6 June 2017).

51. Alaux, M.; Rogers, J.; Letellier, T.; Flores, R.; Alfama, F.; Pommier, C.; Mohellibi, N.; Durand, S.; Kimmel, E.; Michotey, C.; et al. Linking the International Wheat Genome Sequencing Consortium bread wheat reference genome sequence to wheat genetic and phenomic data. *Genome Biol.* 2018, 19, 111. [CrossRef]

52. Marcotuli, I.; Gadaleta, A.; Mangini, G.; Signorile, A.M.; Zacheo, S.A.; Blanco, A.; Simeone, R.; Colasuonno, P. Development of a high-density SNP-based linkage map and detection of QTL for beta-glucans, protein content, grain yield per spike and heading time in durum wheat. *Int. J. Mol. Sci.* 2017, 18, 1329. [CrossRef] [PubMed]

53. Paolacci, A.R.; Tanzarella, O.A.; Porceddu, E.; Ciaffi, M. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol. Biol.* 2009, 10, 11. [CrossRef] [PubMed]

54. Gimenez, M.J.; Piston, F.; Atienza, S.G. Identification of suitable reference genes for normalization of qPCR data in comparative transcriptomics analyses in the Triticeae. *Planta* 2011, 233, 163–173. [CrossRef] [PubMed]

55. Doyle, J.J. Isolation of plant DNA from fresh tissue. *Focus* 1990, 12, 13–15.

56. Furtado, A. RNA extraction from developing or mature wheat seeds. In *Cereal Genomics;* Humana Press: Totowa, NJ, USA, 2014; Volume 1099, pp. 23–28.
57. Ramakers, C.; Ruijter, J.M.; Deprez, R.H.; Moorman, A.F. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 2003, 339, 62–66. [CrossRef]

58. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001, 29, e45. [CrossRef] [PubMed]

59. Marcotuli, I.; Houston, K.; Schwerdt, J.G.; Waugh, R.; Fincher, G.B.; Burton, R.A.; Blanco, A.; Gadaleta, A. Genetic diversity and genome wide association study of β-glucan content in tetraploid wheat grains. *PLoS ONE* 2016, 11, e0152590. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).