Genomics 113 (2021) 2989–3001

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Polyphenol oxidase genes as integral part of the evolutionary history of domesticated tetraploid wheat

Francesca Taranto [a,1,*], Giacomo Mangini [b,1,**], Monica Marilena Miazzi [c], Piergiorgio Stevanato [d], Pasquale De Vita [e]

[a] National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), 80055 Portici, NA, Italy
[b] National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), 70126 Bari, BA, Italy
[c] Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, 70126 Bari, Italy
[d] DAFNAE, Università degli Studi di Padova, 35020 Legnaro, Italy
[e] Council for Agricultural Research and Economics (CREA), Research Centre for Cereal and Industrial Crops (CREA-CI), 71122 Foggia, Italy

ARTICLE INFO

Keywords:
Triticum turgidum
Polyphenol oxidase
SNP
Breeding
Haplotype

ABSTRACT

Studying and understanding the genetic basis of polyphenol oxidases (PPO)-related traits plays a crucial role in genetic improvement of crops.

A tetraploid wheat collection (*T. turgidum* ssp., TWC) was analyzed using the 90K wheat SNP iSelect assay and phenotyped for PPO activity. A total of 21,347 polymorphic SNPs were used to perform genome-wide association analysis (GWA) in TWC and durum wheat sub-groups, detecting 23 and 85 marker-trait associations (MTA). In addition, candidate genes responsible for PPO activity were predicted. Based on the 23 MTAs detected in TWC, two haplotypes associated with low and high PPO activity were identified. Four SNPs were developed and validated providing one reliable marker (IWB75732) for marker assisted selection. The 23 MTAs were used to evaluate the genetic divergence ($F_{ST} > 0.25$) between the *T. turgidum* subspecies, providing new information important for understanding the domestication process of *Triticum turgidum* ssp. and in particular of ssp. *carthlicum*.

1. Introduction

Tetraploid wheat accessions (2n = 4× = 28; AABB genome) have played a fundamental role in the history of agriculture and human civilization as the progenitors of modern durum and bread wheat varieties. Wild and domesticated ancestors possess many desirable agronomic traits that could be useful in durum wheat breeding programs to improve durum wheat yield and quality [1]. All members belong to the *Triticum turgidum* L. species but only durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) is the most widely grown tetraploid wheat. Pasta, couscous, and bulgur are the main products of durum wheat in the world and their qualities depends on the characteristics of the raw material. Grain protein content, vitreousness, together with the gluten strength and the yellow semolina color, represented the main breeding targets for decades [2]. Semolina and pasta color are affected by an equilibrium between carotenoid biosynthesis and carotenoid degradation during processing phases [3,4]. This last process has been principally attributed to antioxidant enzymes, such as the lipoxygenases (LOXs) and peroxidases (PODs), that can cause the loss of yellow color in semolina and pasta [5–8].

Recently, the polyphenol oxidases (PPOs) were included among the oxidative enzymes related to carotenoid degradation and browning flour [6,9]. In particular, PPO enzymes are the most important factor that determines the rate and degree of enzymatic browning of flour/semolina [10]. Understanding the enzymatic browning process is necessary to control it effectively and to obtain high-quality product that is acceptable to consumers.

In plants, PPOs are nuclear-encoded enzymes which contain two

* Corresponding author at: National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), via Giovanni Amendola 165/A, 70126, Bari, BA, Italy.
** Corresponding author at: National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), via Giovanni Amendola 165/A, 70126, Bari, BA, Italy.
E-mail addresses: francesca.taranto@ibbr.cnr.it (F. Taranto), giacomo.mangini@ibbr.cnr.it (G. Mangini).
[1] Authors contributed equally to this work

https://doi.org/10.1016/j.ygeno.2021.06.030
Received 29 December 2020; Received in revised form 14 April 2021; Accepted 23 June 2021
Available online 26 June 2021

copper (Cu) binding sites (CuA and CuB) that catalyze the oxidation of several *o*-diphenols (monophenolase activity; E.C. 1.14.18.1) which occur naturally in durum wheat grain into highly reactive *o*-quinones (diphenolase activity; E.C. 1.10.3.2) [11,12]. The resulting quinones further react non-enzymatically with other phenolic compounds, amino acids, and proteins, and form brown-colored complexes called melanins [13]. PPOs are in the lumen of the thylakoid membrane, whereas phenolic compounds are confined to the vacuoles [14]. Both are localized primarily in the bran layer of the wheat kernel [15] although very low levels of PPO are observed in the germ [16]. Thus, the enzymes can come into contact with their substrate only if cells are disrupted, such as during tissue damage (i.e. milling) [4,17] . Therefore, in wheat, endogenous PPO activity and phenolic content increase with the flour extraction rate [18] causing undesirable browning or darkening of pasta products and reducing consumer's willingness to buy them [19].

While the biochemical reactions catalyzed by PPOs are well known, the physiological role of these enzymes is poorly understood. Most modern varieties show a lower PPO activity than old varieties and domesticated/wild accessions [20]. This might suggest (or expect) that breeding directly improves this quality trait. Indeed, the reduction of the PPO level in wheat has only become a goal of genetic improvement in recent years, so it could be probably an indirect effect due to the selective pressure exerted on other traits of interest [21].

Actually, PPO activity plays an important role in plant defence mechanisms which can help the plant withstand stress conditions and develop adaptation strategies that enhance plant resilience [22,23]. Studies showed that levels of PPO activities change in response to biotic and abiotic stresses in several crop species [24–28], including wheat [11], although the underlying mechanisms have not yet been clarified.

Correlations of PPO activity with quality traits and/or plant defence may be confounded by the complexity of PPO gene families.

Numerous studies have been conducted to understand the genetic basis of PPO in wheat [29–31]. PPO genes located on homoeologous group 2 play a major role in the PPO activity [13,32–38]. A series of QTLs with minor effects were also detected on chromosomes 3, 5, 6 and 7 in bread and durum wheat [2,29,38,39].

PPO genes on the homoeologous group 2 are arranged in two paralogous families that are about 10 cM apart, named *Ppo-1* (*Ppo-A1*, *Ppo-B1,* and *Ppo-D1*) and *Ppo-2* (*Ppo-A2*, *Ppo-B2,* and *Ppo-D2*) [31]. Prior works indicate that *Ppo-A1* allelic variation has the greatest effect on kernel PPO activity [35,36,40–42], due to an in/del of 191-bp in the first intron that determines alternative splicing of pre-mRNAs, which in turn influences the level of PPO activity [35,43]. These in/del polymorphisms of 191-bp have been used to develop several functional markers to discover allelic variants related to variations in PPO activity [35,37,44]. Later, a few additional markers were developed to detect allelic variants for the remaining *Ppo-1* and *Ppo-2* genes [31,45].

The emergence of new genotyping technologies, based on the acquiring genomic data at a whole-genome scale, allow to generate millions of the single nucleotide polymorphisms (SNPs) and to develop markers based on single base changes in DNA [46–48]. Genome-wide association studies (GWAS) are very helpful in characterizing the nature of gene variation in crops and assembling an extensive catalog of SNPs in candidate genes in association with agronomic and quality traits that are the major goals of wheat breeding [2,49–52].

The statistical power to detect associations largely depends on the sample size and composition; thus, the use of collections, composed by varieties, domesticated and wild accessions, allows fine-scale identification of the genomic regions affecting a trait [53] and provides new insights into crop breeding history [50]. Recently, SNPs identified by GWAS, have been associated with PPO genes, making the marker-assisted selection (MAS) process more efficient because of the availability of high-density genetic maps [2,38,54].

With this in mind, we aimed to dissect the genetic basis of PPO activity in *T. turgidum* by identifying loci associated with polyphenol oxidases, and suitable candidate genes involved in their expression and regulation. Herein, we elucidate the role of PPOs during the domestication and breeding history of *Triticum turgidum* ssp. The exploration of the genetic diversity for PPO activity in tetraploid wheats could be a useful strategy to identify new SNP markers, as well as a way to understand the effects of the distribution and adaptive significance of allelic variants in PPO loci in *Triticum turgidum* L. ssp. This would make the PPO gene family very useful for studying adaptive processes to natural and artificial selection, which could be considered an integral part of the evolutionary history of domesticated durum wheat.

In this study, the genetic variation of PPO activity has been extensively investigated by testing a large tetraploid wheat collection, consisting of wild, domesticated and durum wheat accessions. To distinguish the role of PPO genes during domestication and breeding, we conducted a comprehensive review of genetic loci controlling PPO activity using two association panels consisting of total tetraploid wheat collections and modern durum wheat cultivars. From MTAs we searched for candidate genes in chromosome regions tightly linked to QTLs for those traits exploiting the reference durum wheat genome. We used this information to identify and validate new "functional gene markers" to facilitate in enhancing the selection efficiency for PPO activity in durum wheat breeding programs.

2. Material and methods

2.1. Plant materials and phenotypic data

In this study, a collection of 220 tetraploid wheat accessions (*T. turgidum* L., 2n = 4× = 28; AABB genome), classified into seven subspecies according to van Slageren (1994), [55], was considered (Table S1). The collection included *Triticum turgidum* L. ssp. *durum* (Desf.) (no. 122), ssp. *turanicum* (Jakubz.) (khorasan wheat, no. 20), ssp. *polonicum* (L.) Thell. (polish wheat, no. 19), ssp. *turgidum* (rivet wheat, no. 19), ssp. *carthlicum* (Nevski) (no. 12), ssp. *dicoccun* (Schrank) Thell., (*dicoccum*, no. 18), and ssp. *dicoccoides* (Korn. Ex Asch. & Graebn.) Thell. (no. 10). For this study, all genetic materials were grouped in two association panels, the first included the whole tetraploid wheat collection (TWC) whereas, the second only the durum wheat subgroup (DWC). Previous studies, conducted on the same genetic materials, showed a higher variability in PPO activity in the TWC compared to DWC [17,26].

Experimental field trials were carried out at Valenzano (Bari, Italy) during three growing seasons (2008–09, 2009–10 and 2011–12) using a randomized complete block design with three replications and plots consisting of 1-m rows, 60 cm apart, with 50 germinating seeds per plot. The grain samples were analyzed for PPO activity as described by Mangini et al. [44]. In brief, thirty seeds from each plot were incubated in 5 mL of 0.01 M disodium tyrosinate solution (pH 11.0) at 37 °C for 19 h, and after removal of the seeds, the absorbance of the solution was measured at 405 nm by a UV/visible spectrophotometer (Amersham Pharmacia Biotec, Uppsala, Sweden).

Heading date (HD) and plant height (PH) data were collected during the growing seasons 2009, 2010 and 2012. Heading date (HD) was recorded in each field trial as the number of days from 1 March to 50% ear-emergence. Plant height (PH) was measured at complete maturity of plants, and expressed in cm.

The analyzed data were the mean values recorded over the three study years per each genotype (Table S1). The year-by-genotype interactions were estimated using Least square means (LSMean) function in the R package *lsmeans*. Correlation analysis (*r*) was carried out between the three years of PPO activity and the means of PPO activity, HD and PH using the R package *corr.test* with the Pearson method.

2.2. Genotype data

Genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method [56]. The DNA samples were genotyped with the Illumina® iSelect 90K wheat SNP assay [57] at

TraitGenetics GmbH, Gatersleben, DE. Markers were ordered according to the physical map of Svevo durum wheat [53] available at https://www.interomics.eu/durum-wheat-genome.

SNP filtering was carried out based on the following criteria: minor allele frequency (> 1%) and call rate (> 90%) using the SNP and Variation Suite (SVS) software package v.8.4.0 (Golden Helix Inc). All of the downstream analyses were carried out on two distinct datasets: *i*) the TWC (220 genotypes) and *ii*) the DWC (122 genotypes).

Principal component analyses (PCA) and Kinship matrix ("Centered_IBS" method) were calculated using the Trait Analysis by aSSociation, Evolution, and Linkage (TASSEL) software [58].

Linkage disequilibrium (LD) was calculated using adjacent pairs analysis markers performed in the SNP and Variation Suite (SVS) software package (version 8.4.0, Golden Helix Inc.). The LD decay distance was determined across the whole genome using a threshold of $r^2 = 0.20$ [50,53]. The LD decay was visualized by plotting each marker pair against their distance (bp), and by fitting a nonlinear regression based on Remington (2001) model [59].

2.3. GWAS and candidate genes

Association tests were independently run-on single year data and on LSMeans of the three growing seasons both in TWC and DWC. A Mixed Linear Model (MLM) was run using TASSEL v. 5.0 software [58]. Two models were used: *i*) the population structure (PCA) as the fixed effect and a Kinship (K) matrix as the random effect (MLM + PCA + K) and *ii*) the model with only a K matrix (MLM + K). Additionally, the Genomic Association and Prediction Integrated Tool (GAPIT) [58,60] was used to confirm the associations derived from the durum wheat subset. The significance level of the Marker-Trait Associations (MTAs) was corrected with two methods, the Bonferroni multiple testing correction ($p$value < 0.05, $-\log10p > 5.63$) and by adjusting the p-value with the False-Discovery-Rate (FDR < 0.05). Threshold levels of Bonferroni correction $-\log10p > 5.0$ and FDR < 0.05 were used to indicate suggestive MTAs. The additive effect allele was estimated as the difference in the average value of the two allele effects at a locus [58].

To determine the effects of adaptation traits (heading date and plant height) on the MTAs for PPO activity, GWA was re-conducted by using HD and PH as covariates.

Gene annotation of the significant MTAs was performed using the Svevo durum wheat high-confidence gene models (https://www.interomics.eu/durum-wheat-genome). The confidence size interval of SNP flanking regions was determined based on LD decay.

2.4. Functional annotation and gene ontology enrichment analyses

Proteins included in the confidence interval of MTAs were functionally classified with Mercator annotation pipeline v4 http://www.plabipd.de/portal/mercator-sequence-annotation.

To identify Gene Ontology (GO) terms (Biological Processes; BPs) associated with positional candidate genes detected in TWC and DWC, and to find the over-representation of a given GO term in a subset in comparison with the genome-wide background frequency, enrichment analyses were performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/) with default parameters. The significance of over-representation was determined using the hyper geometric distribution followed by the Bonferroni correction for multiple testing (corrected $p \leq 0.05$).

2.5. Haplotype network and divergent loci

All MTAs detected in the TWC were used to construct a haplotype network using a Minimum Spanning method in PopART [61]. Based on the haplotypes identified, a box plot representation of the PPO activity variation was displayed using R software v. 4.0.2. To verify if MTAs found associated with PPO activity in the whole collection were divergent among *Triticum turgidum* populations (i.e. divergent selection at single loci: a selection that acts in contrasting directions in two or more populations [62]), the Fixation index F_{ST} at individual SNP loci was estimated using the Weir and Cockerham formula [63] by pairwise comparisons as implemented in SVS v. 8.4.0.

2.6. SNP marker assay validation

Ten accessions for each haplotype were selected to validate the SNP associated with PPO activity in the TWC. SNP marker assay validation was conducted on the Pyromark Q48 platform (Qiagen, Germany) according to the manufacturer's instructions. PCR and sequencing primers were developed using the PyroMark Assay Design Software 2.0.1.15 (Qiagen). PCR amplification was performed using the PyroMark PCR Kit (Qiagen). The following thermal cycling conditions were used: 15 min at 95 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C and 10 min at 72 °C. Sequence analysis was performed using the PyroMark Q48 Autoprep software (Version 2.4.2) in the Allele Quantification mode.

3. Results

3.1. Association mapping in TWC and DWC

The TWC was analyzed with the 90K iSelect genotyping array including 81,587 gene-associated SNPs developed by [57]. A total of 21,347 SNP markers were retained for the GWA analysis after filtering. The genome-wide analysis was conducted both in TWC and DWC association panels, using an equal set of SNPs.

To minimize type I errors (i.e. false positive associations), the GWAS was performed testing two MLM models: the MLM + K model that included the Kinship matrix and MLM + K + PCA incorporating also the population structure estimated by PCA analysis. Both models showed very similar results, nevertheless, the MLM + K model, which minimizes the false positives, was adopted in this study for downstream analysis (Fig. S1).

A total of 23 significant MTAs was detected on chromosomes 2A, 2B, 3A, 4B, and 7A in TWC (Fig. 1, Table S2). The SNPs on 2A, 2B, and 3A were found in all seasons (2009, 2010, and 2012), while the remaining ones on the 4B and 7A were confirmed in two years (2009 and 2012). All MTAs were confirmed using also LSMean values (Table S2). In these regions, two strong signals were detected on chromosomes 2A and 2B. Sixteen and five SNPs were included in two regions spanning 4.4 Mb and 5.5 Mb on chromosomes 2A and 2B, respectively (Table S2). As reported in Table 1, the most significant SNPs were IWB62504, IWB29311, and IWB14315, physically mapped on chromosomes 2A, 2B, and 3A, respectively. Among these, both IWB62504 and IWB29311 explained the highest proportion (18%) of the phenotype variation with an additive effect of −0.19 and 0.19, respectively, in 2009 and 2010. The SNP IWB14315 explained a phenotype variation ranging from 11% (2012) to 16% (2009 and 2010) with an additive effect of −0.17 (2009), −0.18 (2010) and − 0.13 (2012).

A further GWAS was performed in the DWC which revealed the contribution of other putative MTAs with minor effects. A higher number of MTAs were found in the DWC than in the TWC. A total of 85 significantly associated SNPs were detected on all chromosomes, except for 6A (Fig. 1, Table 1, Table S2). We considered significant MTAs those detected in at least two years and using LSMean. The highest number of MTAs was on chromosome 2A (17) and chromosome 7A (8). One SNP was detected on chromosomes 1A, 1B, and 3B (Table S2). The regions on chromosomes 2A and 2B overlapped with those associated in TWC, confirming the IWB62504 and IWB29311 markers as the most significant SNPs in the phenotypic expression of PPO activity. Interestingly, the strongest signal detected in DWC was on chromosome 4A and was confirmed in all three years, albeit with different levels of significance. A total of seven MTAs were detected on chromosome 4A with the peak

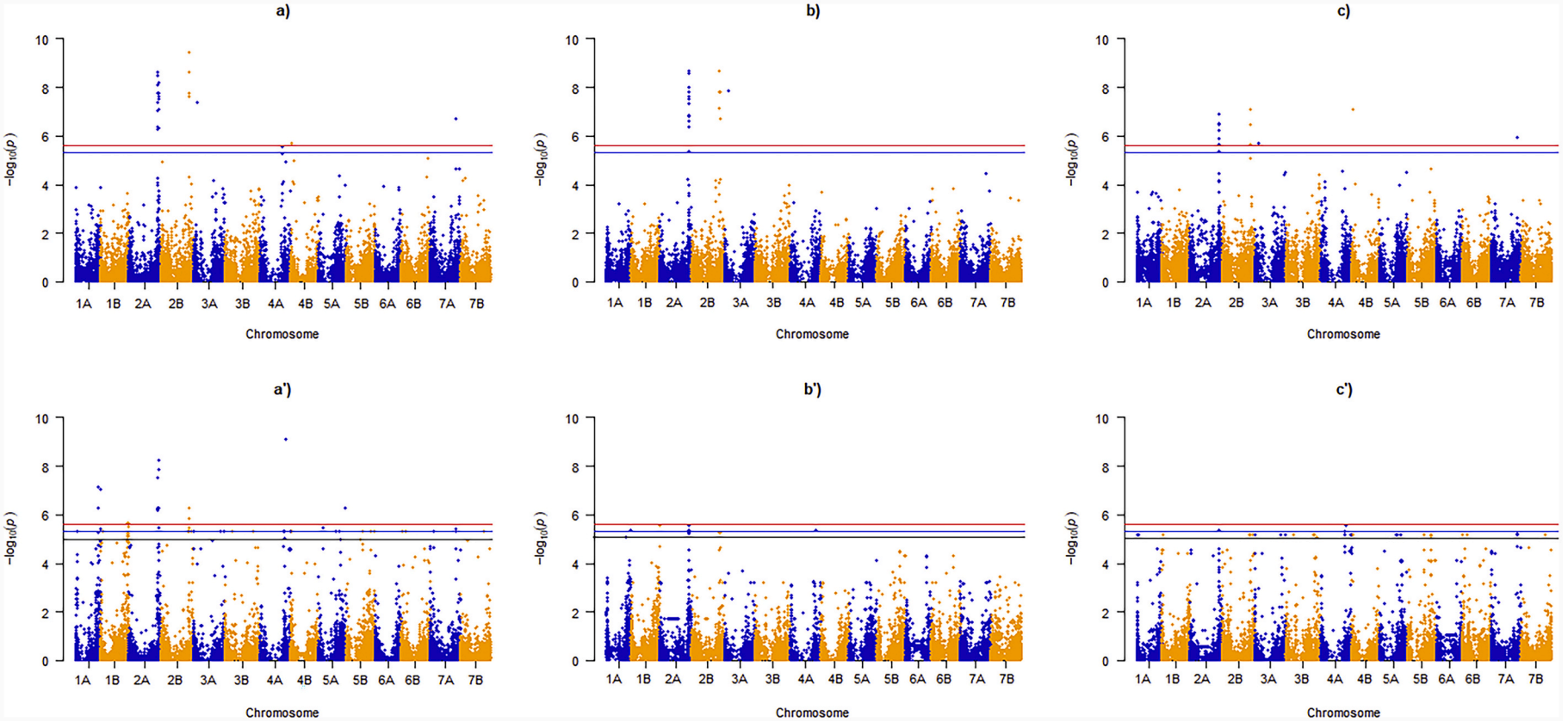

Fig. 1. Manhattan plots showing GWAS results using the MLM + K model for PPO activity. GWAS was carried out using 21,347 SNP markers and two sets of accessions during three growing seasons: whole collection (a = 2009, b = 2010 and c = 2012) and durum wheat collection (a' = 2009, b' = 2010 and c' = 2012). X-axis represents SNP positions across the entire durum wheat genome by chromosome according to Svevo genome assembly and the y-axis is the negative logarithm p-value: -log10 (p) of each SNP. The red line indicates the threshold of significance (Bonferroni adjusted p-value = 0.05). The blue line indicates the significance threshold (Bonferroni adjusted p-value = 0.10). The black indicates the "suggestive" MTAs (False Discovery Rate adjusted p-value = 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Genome wide significant MTAs identified for the PPO activiy using the model MLM + K. The MTAs were reported for three years (2009, 2010 and 2012) in a tetraploid wheat collection (TWC) and in durum cultivar sub-group (DWC). The markers with the higher -log10$_{(p\text{-value})}$ within the associated genomic regions were considered and reported as the marker peak.

SNP id	SNP name	Chromosome	Position		Allele	Years								
			cM[a]	Mb[b]		2009			2010			2012		
						LOD	Add	R^2	LOD	Add	R^2	LOD	Add	R^2
TWC (220)														
IWB62504	RAC875_rep_c119619_54	2A	154.6	706.48	A/C	7.78	0.19	0.18	7.79	0.19	0.18	5.65	0.15	0.13
IWB29311	Excalibur_c907_1643	2B	137.9	677.24	T/C	7.78	−0.19	0.18	7.79	−0.19	0.18	5.65	−0.15	0.13
IWB14315	CAP7_c9218_89	3A	160.5	89.00	C/T	7.37	−0.17	0.16	7.84	−0.18	0.16	5.69	−0.13	0.11
IWB21548	Excalibur_c10113_1138	4B	28.5	26.17	C/T	5.70	−0.20	0.13	–			7.10	−0.21	0.16
IWB55547	RAC875_c23665_68	7A	n.a	624.48	G/A	6.73	0.28	0.13	–			5.94	0.24	0.11
DWC (122)														
IWB31932	GENE-0507_285	1A	31.4	28.55	T/G	5.33	0.37	0.19	–			5.18	0.46	0.19
IWB6488	BS00011892_51	1A	n.a.	575.08	A/G	7.07	0.22	0.24	5.38	0.19	0.19	–		
IWB61685	RAC875_rep_c107325_644	1B	n.a.	44.65	A/G	5.33	0.37	0.19	–			5.18	0.46	0.19
IWB62079	RAC875_rep_c111730_97	1B	162.5	676.90	A/G	5.53	−0.13	0.19	5.57	−0.13	0.19	–		
IWB62504	RAC875_rep_c119619_54	2A	154.6	706.48	A/C	6.27	0.19	0.19	5.29	0.16	0.19	–		
IWB29311	Excalibur_c907_1643	2B	137.9	677.24	T/C	6.27	0.19	0.19	5.29	0.16	0.19	–		
IWB22595	Excalibur_c1529_1369	3A	n.a.	4.33	T/C	5.33	0.37	0.19	–			5.18	0.46	0.24
IWB25409	Excalibur_c34069_520	3A	n.a.	631.01	T/C	5.33	0.37	0.19	–			5.18	0.46	0.24
IWB75323	tplb0062h15_59	3B	n.a.	179.79	A/C	5.33	0.37	0.19	–			5.18	0.46	0.19
IWB25408	Excalibur_c34069_487	3B	123	665.12	A/G	5.33	0.37	0.19	–			5.18	0.46	0.24
IWB80091	wsnp_Ku_c13768_21859275	4A	80.8	618.66	A/G	9.10	−0.35	0.19	5.35	−0.25	0.19	5.54	−0.32	0.19
IWB77304	wsnp_Ex_c23248_32488191	4B	n.a.	37.52	T/C	5.33	−0.37	0.19	–			5.18	−0.46	0.19
IWB75412	wsnp_BE443187A_Ta_2_3	5A	256	411.14	T/C	5.33	−0.37	0.19	–			5.18	−0.46	0.19
IWB8223	BS00040350_51	5B	n.a.	402.76	C/T	5.33	−0.37	0.20	–			*5.18*	*−0.46*	*0.23*
IWB66058	TA006116–0815	5B	131.6	583.07	A/G	5.33	−0.37	0.20	–			*5.18*	*−0.46*	*0.19*
IWB47927	Kukri_c8239_987	6B	25.2	22.62	T/C	5.33	0.37	0.19	–			5.18	0.46	0.19
IWB28246	Excalibur_c63900_724	7A	140.9	621.13	T/C	5.33	0.37	0.19	–			5.18	0.46	0.19
IWB58381	RAC875_c478_1364	7B	113.4	576.84	A/C	5.33	0.37	0.19	–			*5.18*	*0.46*	*0.19*

LOD = (−log10(pvalue). The threshold was calculated using the Bonferroni correction at p-value pvalue <0.05. The MTSs detected using Bonferroni correction at p-value <0.10 and FDR < 0.05 (suggestive MTAs) were showed respectively underlined and in italics.
Add = Additive effect estimated as mean of difference between SNP allele effect.
R^2 = Phenotypic variation explained.
[a] Genetic position from Maccaferri et al. (2014). [b] Physical position based on the durum wheat reference genome Svevo (Maccaferri et al. 2019).

identified by the most significant SNP IWB80091 ($-\log_{\text{p-value}} = 9.10$) (Table 1).

Since the mean values of PPO activity were significantly and positively correlated with HD ($r = 0.63$ and $r = 0.43$, in TWC and DWC respectively) and PH ($r = 0.57$ and $r = 0.42$, in TWC and DWC respectively) (Table S3),

GWA analysis was also performed adjusting for covariates (HD + PH). All MTAs previously detected on the TWC were confirmed, with LOD ranging from 8.23 (IWB52510 and IWB22408) to 5.81 (IWB21548) and R^2 from 0.17 to 0.11 (Table S4). For the DWC, only four markers

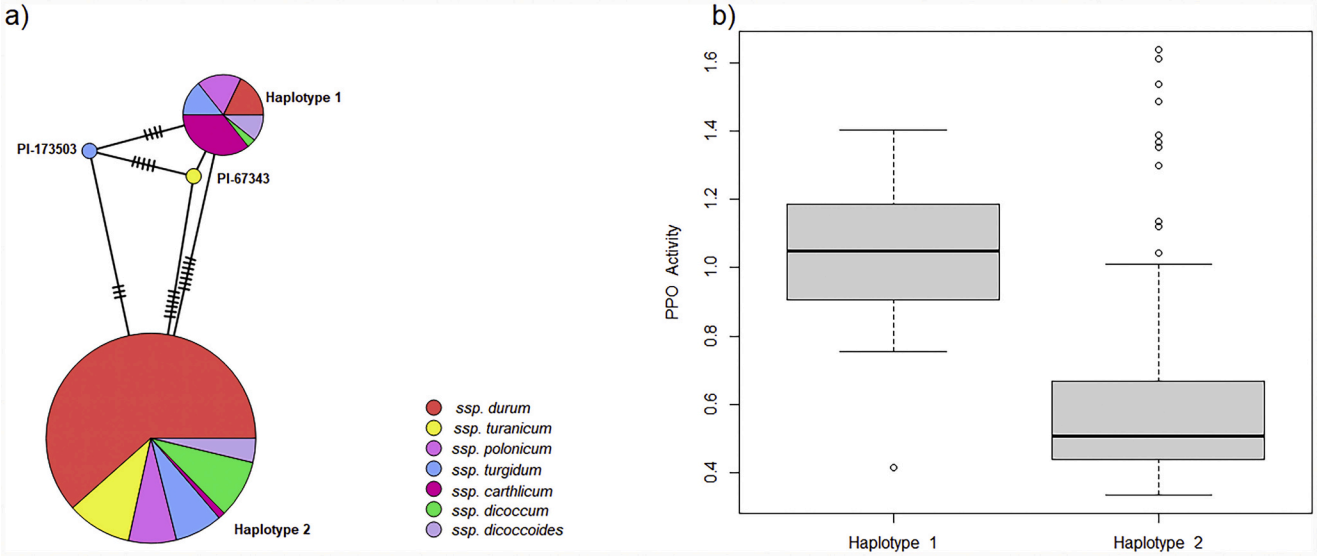

Fig. 2. Haplotype analysis performed on the 220 tetraploid wheat genotypes. a) Minimum spanning networks was constructed with PopART for the two haplotypes detected using the 21 MTAs on chromosomes 2A and 2B, generated by GWAS. The size of a circle indicates the relative frequency of sequences belonging to a particular haplotype type. Hatch marks along the branches indicate the numbers of mutations. Each color indicates a different subspecie of *Triticum turgidum*. Two genotypes have a different haplotype. b) Boxplot showing the PPO activity mean for the two haplotypes detected by PopART analysis.

were found significantly associated. The MTAs were located on chromosome 2A (IWB2708, IWB38412 and IWB80904) and 4A (IWB80091), with the highest LOD (6.38) and R^2 (0.23) detected for IWB80091 and IWB2708, respectively.

3.2. Haplotype network and divergent loci

The twenty-three MTAs found associated in the TWC were used to construct a Minimum Spanning tree (Fig. 2a). Eighteen genotypes were removed from the analysis due to the presence of heterozygous SNPs. The network distinguished all accessions in two main haplotypes, named 'Haplotype1' and 'Haplotype2', with only two accessions that showed a unique haplotype (PI-173503, ssp. *turgidum* and PI-67343, ssp. *turanicum*) (Table S1).

The Haplotype1 occurred with a lower frequency (13.9%) than Haplotype2 (85.1%) in the whole collection. The Haplotype1 included most domesticated accessions belonging to ssp. *carthlicum* (10 accessions, representing 35% of the total), and none of the ssp. *turanicum*. Five out of one hundred and twenty-two durum wheat cultivars showed Haplotype1 as well as an accession of ssp. *dicoccum* (one out seventeen). The Haplotype2 group included almost all durum wheat cultivars, all *turanicum* accessions, and the remaining genotypes belonging to the remaining *T. turgidum* subspecies.

The average PPO activity within three years was calculated based on two haplotype groups (Fig. 2b). The boxplots showed a clear difference in PPO activity between the two groups, with an average value of 1.05 and 0.51 for Haplotype1 and Haplotype2, respectively. Therefore, Haplotype1 and Haplotype2 can be related to high and low levels of PPO activity, respectively, although there were several outliers for Haplotype2 which mainly consist of *dicoccum* accessions.

The twenty-one MTAs detected on chromosome 2A and 2B in the whole collection were used to estimate the genetic divergence between the subspecies of *T. turgidum* (Fig. S2, Table S5).

The F_{ST} index, calculated in pairs between the subspecies, highlighted the strongest genetic divergence between the *carthlicum* and the other free-threshing subspecies (excluding the *turgidum* and *polonicum* ssp.). For these pairwise comparisons, the F_{ST} values for all the SNP markers considered were higher than 0.7 except for IWB12761, IWB34556, and IWB38411 markers on chromosome 2A and the IWB60022 marker on chromosome 2B. On the contrary, the pairwise comparisons between *durum* versus *dicoccum*, *carthlicum* vs *dicoccoides*, and *carthlicum* vs *turgidum* showed F_{ST} mean values lower than 0.6 for all markers, with some exceptions. The lower F_{ST} values were from comparisons *durum* vs *dicoccum* and *turanicum*, *turanicum* vs *dicoccum*, and *polonicum* vs *turgidum* and *dicoccoides*.

3.3. Allelic quantification in two haplotype datasets

Four out of 23 SNPs generated by GWA analysis on TWC were quantified via pyrosequencing to confirm allelic discrimination between Haplotype1 and Haplotype2. The analysis was carried out on two validation sets of accessions for each haplotype (Table 2). Sequences of primers for pyrosequencing assays are available in Table S6. The IWB75732 marker distinguished Haplotype1 from Haplotype2. The allele frequencies were higher than 0.90 and 0.81 in Haplotype1 (G) and Haplotype2 (A), respectively. The allele frequencies of marker IWB29311 identified Haplotype1 (A) with an average high percentage (89.2). The same result was not obtained for Haplotype2 (G), that showed a large allele frequency variability in the tested accessions. The other two markers (IWB59867, IWB79388) showed lower allele frequencies within each haplotype.

3.4. Linkage disequilibrium and candidate genes

LD analysis was performed both for the TWC and DWC. The plots of the LD estimates (r^2) as a function of physical distance indicated a decay of the LD of ± 1.5 Mb and ± 1.8 Mb for the TWC and DWC, respectively, using a critical value of $r^2 = 0.20$ (Fig. S3).

All the 23 and 85 MTAs which had associations with PPO activity in TWC and DWC, respectively, were used to identify the positional candidate genes (CGs) within confidence intervals (CI) estimated based on LD decay. Based on the physical position of the CGs on the chromosomes, we investigated whether the significantly associated regions were new, the same or close to those previously identified. Also, we speculated on candidate genes with known biological functions directly or indirectly regulating the developmental processes of the trait under

Table 2
Allele frequencies of four MTAs which associated with PPO activity in TWC. The pyrosequencing allelic quantification was tested in two validation datasets based on haplotype.

Accession	*Triticum turgidum* ssp.	Chromosome 2A						Chromosome 2B	
		IWB75732		IWB59867		IWB79388		IWB29311	
		A (%)	G (%)	G (%)	T (%)	A (%)	G (%)	A (%)	G (%)
Haplotype_1									
PI-306549	*polonicum*	5.20	94.8	7.28	64.87	0	7.57	93.00	7.00
PI-352487	*polonicum*	7.59	92.41	7.64	75.3	0	8.83	89.03	10.97
PI-352489	*polonicum*	8.70	91.30	9.01	64.44	0	20.16	87.71	12.29
PI-341391	*turgidum*	8.34	91.66	5.48	79.96	0	54.31	90.00	10.00
PI-115816	*carthlicum*	7.48	92.52	9.58	72.57	0	59.3	83.86	16.14
PI-585018	*carthlicum*	6.29	93.71	8.62	79.42	0	30.83	86.49	13.51
PI-346783	*dicoccoides*	7.25	92.75	2.65	92.42	0	42.4	89.49	10.51
PI-355459	*dicoccoides*	6.87	93.13	7.83	65.21	0	63.86	88.2	11.80
Timilia	*durum*	5.49	94.51	10.32	89.68	0	65.83	94.36	5.64
MG-4387	*dicoccum*	9.40	90.60	6.05	75.05	0	15.4	89.82	10.18
Haplotype_2									
Capeiti-8	*durum*	96.96	3.04	48.03	12.85	82.15	3.47	4.36	88.37
Appulo	*durum*	94.48	5.52	41.88	25.63	58.34	5.01	4.18	85.66
Creso	*durum*	95.26	4.74	26.05	27.54	79.1	2.74	4.14	25.78
Latino	*durum*	92.85	7.15	62.24	10.51	82.62	3.28	3.55	30.83
Saragolla	*durum*	95.38	4.62	56.72	12.08	23.78	6.12	7.23	77.39
Kamut	*turanicum*	95.43	4.57	52.30	15.15	38.48	5.01	2.97	86.85
CLTR-11390	*turanicum*	93.86	6.14	45.55	16.28	42.55	4.22	7.13	79.53
Ofanto	*durum*	96.4	3.60	17.72	32.55	91.06	2.93	2.58	24.40
Simeto	*durum*	88.3	11.70	71.95	8.52	58.51	11.45	2.08	65.50
Svevo	*durum*	81.76	18.24	78.07	7.82	89.26	10.74	3.62	27.84

investigation.

According to the LD decay, two homologous regions were found on chromosomes 2A and 2B, both in TWC and DWC (Table S7, Table S8). The two most significant SNPs, IWB62504 and IWA572, were located upstream and downstream of the two transcripts TRITD2Av1G261300 (*Ppo-A1*) (~ 170 kb) and TRITD2Av1G261390 (*Ppo-A2*) (~ 37 kb), both annotated as polyphenol oxidase genes on chromosome 2A. A homologous region was identified on chromosome 2B, where the SNPs IWB29311 and IWB60022 were found proximal (114 kb) to the polyphenol oxidase gene (TRITD2Bv1G224170). A zinc finger (*Ran-binding*) gene was found proximal (159 kb) to *Ppo-A1*.

Moving to the flanking homologous regions on chromosome 2A and 2B, we observed two clusters of acidic endochitinase genes involved in defence against chitin-containing fungal pathogens (Table S7, Table S8). Other stress response and plant defence genes such as peroxidase (Chr. 2A TRITD2Av1G261030; Chr. 2B TRITD2Bv1G245780 and TRITD2Bv1G223720), pectinesterase (Chr. 2A TRITD2Av1G261160; Chr. 2B TRITD2Bv1G223930), pectin lyase (Chr. 2A TRITD2Av1G261370 and TRITD2Av1G261510; Chr. 2B TRITD2Bv1G224130), nucleotide-binding site and leucine-rich repeat (NBS-LRR) disease resistance protein (Chr. 2A TRITD2Av1G260790 and TRITD2Av1G260950; Chr. 2B TRITD2Bv1G223210 and TRITD2Bv1G223640), toll/interleukin-1 receptor (TIR)-NBS-LRR disease resistance proteins (Chr. 2A TRITD2Av1G260830; Chr. 2B TRITD2Bv1G223370), ABC transporter (Chr. 2A TRITD2Av1G260620; Chr. 2B TRITD2Bv1G223060), RPM1-interacting protein (Chr.2A TRITD2Av1G260740; Chr. 2B TRITD2Bv1G223180), and benzyl alcohol O-benzoyltransferase (Chr. 2A TRITD2Av1G262080) were also annotated.

In addition, genes involved in the biosynthesis of amylopectin such as soluble starch synthase genes (Chr. 2A TRITD2Av1G261450 and Chr. 2B TRITD2Bv1G224370) were physically annotated proximal to polyphenol oxidase genes on chromosome 2A (260 kb) and 2B (1 Mb).

The other three regions which were investigated in TWC showed candidate genes with minor effects on chromosomes 3A, 4B, and 7A (Table S7). Among these, IAA-alanine resistance protein (Chr. 3A TRITD3Av1G040790; Chr.7A IAA-amino acid hydrolase ILR1), auxin-responsive protein (Chr. 3A TRITD3Av1G041110), serine/threonine protein phosphatase 2A regulatory (Chr. 3A TRITD3Av1G041140), and photosystem II CP47 reaction center protein (Chr. 7A TRITD7Av1G233400) were the most relevant.

In the DWC, the most significant region was identified on chromosome 4A, flanking the SNP IWB80091. Relevant candidate genes, involved in the biological process 'late endosome to vacuole transport', were Ras-related protein (TRITD4Av1G216410), vacuolar protein sorting-associated protein (TRITD4Av1G216510), and charged multivesicular body (TRITD4Av1G216710) (Table S8). In addition, a copper ion-binding protein (TRITD4Av1G217630) and a cluster of ATP-dependent zinc metalloprotease FTSH proteins were identified.

Several minor associated regions were identified by GWA analysis in DWC (Table S8). Candidate genes related to splicing mechanisms were found such as 'pre-mRNA-splicing factor ISY1' (Chr. 1A) and 'arginine/serine-rich splicing factor' (Chr. 1A, 1B and 3A). Cu-transporting ATPase (*RAN1* gene) was detected on chromosome 5B (TRITD5Bv1G135700) (Table S8) and 7B (TRITD7Bv1G184110), as well as genes involved in oxidation pathways as lysine demethylase, cytochrome oxidases, succinate dehydrogenase, and oxoglutarate Fe-dependent oxygenase.

Finally, high-affinity nitrate transporter was located distal to peroxidase (750 kb), and glutamate dehydrogenase was close to copper-transporting ATPase (713 kb) on chromosome 7B.

3.5. Protein classification and annotations, and Gene Ontology Enrichment (GO) analysis

The candidate genes identified in the TWC (Table S7) were classified and annotated based on the proteins of the *Oryza sativa* pathways (Fig. S4). The proteins were placed into twenty-seven bins that describe the biological contexts/concepts. The most represented bin was annotated as 'RNA-processing' (11.7%), of which 'RNA processing.pre-mRNA splicing. U2-type-intron-specific major spliceosome.U1 (16.4%)' was the major component. The bins 'protein modification' (10.9%), 'cell-cycle-organization' (8.3%), 'RNA-biosynthesis', (8.2%), 'protein-homeostasis' (7.8%) and 'photosynthesis' (7.5%) were also represented. A minor part of the proteins (<1%) were classified in 'polyamine-metabolism', 'multi-process-regulation', 'enzyme-classification', and 'nutrient-uptake'.

GO enrichment analysis in the regions flanking the MTAs on chromosomes 2A and 2B was also performed to better understand if genes of interest were more associated to certain biological functions or processes. Two GO terms were over-represented (FDR $\leq$ 0.01) (Table 3) and corresponded to the molecular functions: "hydrolase activity, acting on glycosyl bonds" (p = 7.1e-05) and "hydrolase activity, hydrolyzing O-glycosyl compounds" (p = 6.2e-05). Both GO terms referred to the cluster of acidic endochitinase genes.

For DWC, as previously described (i.e. paragraph 1.1) the strongest association was detected on chromosome 4A (Table 1). Therefore, we selected the candidate genes in the confidence interval of the marker IWB80091 (LOD > 9) to explore the functional implications of the statistically related genes with PPO activity. We found 13 GO terms that were over-represented (FDR $\leq$ 0.1) (Table 3). The biological processes corresponding to the lowest p-values were: "metalloendopeptidase-activity" (p = 1.30E-08) and "metallopeptidase-activity" (p = 1.70E-07). Other relevant over-represented categories included: "cellular-component-biogenesis" (p = 5.70E-05), and "macromolecular-complex-assembly" (p = 7.80E-05).

4. Discussion

4.1. Polyphenol oxidase in the domestication process of Triticum turgidum ssp

Polyphenol oxidase enzymes are involved in different biological processes in plants [11,12]. As all oxidative enzymes, they play an essential role in plant defence against biotic and abiotic stresses, but otherwise, they indirectly reduce the quality of the wheat end products due to an undesirable browning of external layers of the kernel [9,27,28].

The complex mechanisms underlying the activity and the regulation of PPO enzymes are poorly understood [64] and represent a limit of breeding in wheat improvement. Understanding the biological processes in which PPOs are involved became pivotal to develop and/or select varieties that satisfy both aspects. For that reason, the exploration, and exploitation of *Triticum turgidum* ssp. can provide a great opportunity to discover useful alleles and haplotypes related to PPO kernel activity. In previous works, we highlighted the wide phenotypic variation in PPO activity within the current TWC [20,44]. In the present work, the PPO activity data were used to perform GWA analysis to increase the knowledge of genes located in key regions for PPO activity. The use of the two association panels provided novel findings on the effects produced by domestication process in TWC and by breeding strategy in DWC on PPO activity.

In the TWC, two strong GWA signals were detected near the genes responsible for PPO activity in the TWC on chromosome 2A included the paralogous *Ppo-A1* and *Ppo-A2* genes [30,31] located at 217 kb apart. The homoeologous region was identified on chromosome 2B in correspondence with the *Ppo-B2* gene mapped by Taranto et al. [31]. These results confirmed the presence of the PPO genes on chromosomes 2A and 2B and their involvement in PPO activity.

The MTAs detected on chromosome 2A and 2B were also used to estimate the genetic divergence among the *T. turgidum* ssp. There was a strong genetic divergence between *carthlicum* vs *durum*, *turanicum,* and *dicoccum* subspecies (Fig. S2, Table S5). Laidò et al. [65], using the same

Table 3
Gene ontology (GO) enrichment analysis performed on the set of candidate genes identified in the region (± 1.5 Mb) flanking the MTAs detected on chromosome 2A and 2B (TWC), and in the region ((± 1.8 Mb) of the marker IWB80091 (DWC). Ontology: F = molecular function; B = biological process; C = cellular component.

GO term	Ontology	Description	# genes in input list	# genes in Svevo reference genome	p-value	FDR
TWC						
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	10	961	3.40E-04	0.011
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	10	945	3.90E-04	0.013
DWC						
GO:0065003	P	Macromolecular complex assembly	7	208	7.80E-05	0.013
GO:0044085	P	Cellular component biogenesis	9	344	5.70E-05	0.013
GO:0043933	P	Macromolecular complex subunit organization	7	226	1.30E-04	0.015
GO:0070271	P	Protein complex biogenesis	5	117	2.70E-04	0.015
GO:0022607	P	Cellular component assembly	7	241	1.90E-04	0.015
GO:0006461	P	Protein complex assembly	5	117	2.70E-04	0.015
GO:0008652	P	Cellular amino acid biosynthetic process	6	223	8.00E-04	0.039
GO:0004222	F	Metalloendopeptidase activity	8	87	1.30E-08	2.80E-06
GO:0008237	F	Metallopeptidase activity	8	121	1.70E-07	1.80E-05
GO:0005856	C	Cytoskeleton	6	173	2.10E-04	0.006
GO:0044430	C	Cytoskeletal part	6	170	1.90E-04	0.006
GO:0005874	C	Microtubule	5	110	2.00E-04	0.006
GO:0015630	C	Microtubule cytoskeleton	5	152	8.80E-04	0.019

tetraploid wheat collection highlighted that the *carthlicum* accessions formed a distinctive group from the other tetraploid wheat subspecies. The divergence between *carthlicum* and the other *T. turgidum* ssp., based on the F_{ST} index, was corroborated by the two haplotypes identified in the TWC with SNPs associated with PPO activity. The Haplotype1 was represented entirely by *carthlicum* accessions and few other genotypes with high PPO activity. Most studies have shown that these free-threshing tetraploid wheats evolved from the natural selection of cultivated emmer [66,67]. Durum wheat was certainly evolved from domesticated emmer (*T. turgidum* ssp. *dicoccum*) in the eastern Mediterranean region due to the adaptation to the local ecological conditions [68]. A similar hypothesis can apply to the other tetraploid subspecies such as polish wheat, rivet wheat, and khorasan wheat. These species might have emerged due to agro-ecological pressures too. Another possibility of species diversification is inter-ploidy introgression like Persian wheat (*T. turgidum* L. ssp. *carthlicum*) which is believed to have segregated from a cross between domesticated emmer and *T. aestivum* [69], (Kuckuck [70], cited according to Matsuoka [71]). Morphologically, Persian wheat is similar to *T. aestivum* so that initially was classified as hexaploid species [72]. Kuckuck [70] found hexaploid wheat accessions showing the ssp. *carthlicum*-like morphology called *T. aestivum* ssp. *carthlicoides* and proposed that *carthlicum* could have been originated from spontaneous hybridization between ssp. *carthlicoides* and emmer wheat. Takumi and Morimoto [73] also suggested that introgression between related species with different ploidy levels has played an important role in wheat subspecies differentiation. Based on a comparison of the *carthlicoides Wknox1b* allele with other alleles in related species, they suggested an alternative hypothesis about the origin of ssp. *carthlicum*. The study revealed that ssp. *carthlicum* showed the same allele as ssp. *dicoccoides* for the *Wknox1b* 5th-to-6th exon, suggesting that *carthlicum* originated from interploidy hybridization between wild emmer wheat and ssp. *carthlicoides*, and that ssp. *dicoccoides* could be one of the parents of *carthlicum*. Our data based on SNP markers at the PPO loci on chromosomes 2A and 2B seem to support this last hypothesis as proposed by Takumi and Morimoto [73]. Pairwise subspecies F_{ST} values revealed as the ssp. *carthlicum* was more similar to ssp. *dicoccoides* than durum wheat and some other free-threshing species supporting the possible origin from hybridization between wild emmer rather than domesticated emmer wheat.

In addition to the major PPO genes identified on chromosome 2 of TWC, LD analysis allowed us to explore adjacent genomic regions to suggest other genes of interest that might help us to understand the evolutionary history of tetraploid wheats and their post-domestication diversification. Adaptive and/or resistance genes were in strong LD with PPO genes, confirming the key role of PPOs in the modulation and regulation of biotic and/or abiotic stress response [27,28,67]. Among these genes, two clusters of acidic endochitinase genes were identified by GO enrichment analysis.

In plants, chitinase plays a role in such a mechanism of divergent biological functions [74] These enzymes are glycosyl hydrolases responsible for the hydrolysis of the chitin polymer a structural component found in the cell walls of fungi and insects [75]. Some studies demonstrated that change in PPO gene expression affected the regulation of transcriptional pathogen-related gene expression, such as chitinase and peroxidase [28,76]. In wheat, it has been demonstrated that chitinases are important components of plant defence against fungal pathogens, such as *Fusarium culmorum* [77], *Fusarium graminearum* [78,79] and powdery mildew [80]. In addition, rice plants under salt, drought, high and low temperature stresses as well as infection by fungus and bacterium showed acidic endochitinase upregulated, confirming the relationship between chitinase and stresses [81].

Other genes such as GDSL-like lipase/acylhydrolase, peroxidase, pectinesterase, polyphenol oxidase, ABC transporter ATP-binding protein/permease, and pectin lyase proteins increase their expression at the same time under drought-stressed in plants, as demonstrated by [28,82]. QTLs for biotic stresses were also annotated in the regions flanking PPO genes on chromosome 2A and 2B [83–85].

One of the most interesting findings of this study derived from the analysis of the annotated proteins in the regions flanking the MTAs on chromosome 2A, 2B, 3A, 4B, and 7A detected in TWC. The most representative bin was that of 'RNA-processing', with a specific distinctness on 'Pre-mRNA splicing by the U2-type spliceosome'. Although the regulatory mechanisms of PPOs are still poorly understood, it is instead known that the variation in PPO activity can be regulated by alternative splicing mechanisms [28]. Sun et al. [35,43] attempted to elucidate the splicing mechanisms underlying the change in expression of PPO activity in wheat. An in/del of 191-bp, in the I intron of the *Ppo-A1* gene, was associated with high/low PPO activity, respectively. Therefore, as well as the PPO genes, in the future, it will be necessary to take into account genes that participate in the steps of pre-mRNA splicing that take place in the spliceosome, such as zinc finger (Ran-binding) protein and PRPF18 (pre-mRNA-splicing-factor18, TRITD4Bv1G010970) flanking the IWB62504 and IWB21548 on chromosome 2A and 4B, respectively. Recently, in *Arabidopsis thaliana,* it has been suggested that the *RanBP2* zinc finger domain is a chloroplast editing factor [86] that could be involved in a post-transcriptional event such as regulation of splicing [87]. In addition, the PRPF18 protein may promote splicing at weak, non-canonical splice sites, with a large part of cases of intron retention and a preponderance of altered 3′ splice sites [88].

4.2. PPO activity and durum wheat breeding

The GWA analysis performed on the DWC association panel provided results in part overlapping to those obtained with the TWC (homologous group 2), some of which were completely new. Indeed, QTLs with minor effects were previously mapped to homoeologous groups 3, 4, 5, 6 and 7 [3,29,39,40,89–91].

A strong durum GWA signal was found on chromosome 4A, suggesting that this region, while not including PPO genes, may have a possible role in the expression/regulation of PPO activity. Exploring the region flanking the SNP marker IWB80091 on chromosome 4A, "metalloendopeptidase-activity" and "metallopeptidase-activity" candidate genes were found. A copper ion-binding protein (TRITD4Av1G217630) was found closed to IWB80091, confirming the role of Cu- binding sites in PPO activity. Our findings were also confirmed in bread wheat by [38] as our SNP IWB80091 was mapped in the overlapped region where they found the marker IWB47707 in linkage with a Cu ion-binding protein, considered as candidate gene for participation in PPO activity on chromosome 4A. Polyphenol oxidases are Cu-containing metalloenzymes that are constituted of two highly conserved Cu-binding sites [9]. The copper ion-binding protein on chromosome 4A could be a trans-acting element involved in a regulation mechanism of the PPO genes on chromosome 2A and 2B. This relationship is still poorly studied; however, there are evidence that a specific group of the stress-responsive miRNAs target a number of genes encoding Cu-containing proteins, modulating Cu homeostasis in plants [28,92,93]. The role of miRNAs in regulation of PPOs has been demonstrated in populus [94], grapevine [95], banana [93], *salvia* [96], and monocots [93] .

These studies elucidated one of the possible mechanisms of regulation of the expression of PPO genes in plants. Therefore, based on our results we can hypothesize that two possible mechanisms, underlying the regulation of PPO genes in wheat, are related to alternative splicing and to the presence of Cu-proteins which are targets of the Cu-microRNAs based.

Two MTAs (IWB58381 and IWB8223) were annotated inside and near (1.4 Mb) to the genes Cu-transporting ATPase *RAN1* on chromosomes 7B (TRITD7Bv1G184110) and 5B (TRITD5Bv1G135700), respectively. The discovery of these regions corroborates the idea of the complex mechanisms underpinning the expression of PPO genes, opening up new scenarios and suggesting strong candidate genes as copper ion-binding and Cu-transporting ATPase *RAN1* to be validated by functional analysis in the future.

By enlarging the chromosomal region around the Cu ion-binding protein gene we found semi-dwarfing (*Rht-A1*) and lipoxygenase (*LOX-A1*) genes in strongly LD (Fig. 3) as well as the SNPs associated with PPO activity on chromosome 4B, which were in LD with *Rht-B1* and *LOX-B1* genes, as previously highlighted by [97]. These results, together with those observed by GWA analysis using HD and PH as covariates, may support the hypothesis suggested by Taranto et al. [50] that the selective pressure exerted by breeders during the last century to reduce plant height at the *Rht* locus in durum wheat has also indirectly influenced the expression of PPO and LOX activity. In like manner, other regions potentially involved in the expression of traits of agronomic interest were found on chromosome group 2, where the PPO genes are (Fig. 3). In particular, ethylene responsive factor (*ERF*) and soluble starch synthase (starch branching enzyme II - *SSIIb*) genes, in LD with *Ppo-A1*, *Ppo-A2,* and *Ppo-B2* genes, were involved in plant architecture and grain yield, respectively. Some ERFs have been related to gibberellin acids (GA) in regulating plant height [98,99], increasing yield production, and other beneficial traits [100]. In our study, we found that the ERFs genes, were located on chromosome group 2, in the region where the plant height, heading date, and 1000 kernels weight QTLs were previously identified (Fig. 3).

The SNPs associated with *SSIIb* genes suggested a further hypothesis about the indirect selection of PPO genes during artificial selection. It is well known that increasing amylose and resistant starch was an important target for both durum and common wheat breeding programs [101–103]. The strong LD between *SSIIb and PPO* genes on chromosomes 2A and 2B, may suggest that the selection for the starch composition genes might have indirectly influenced the selection of specific variants of the PPOs genes in modern cultivars.

SNPs associated with PPO activity were also found in LD with early flowering (ELF), flowering time (FT), and photoperiod sensitivity (PPD) genes located on chromosomes 1B, 3A, and 5B, respectively [50]. Flowering time was one of the most crucial target traits in durum breeding programs due to its high correlation with the final grain yield

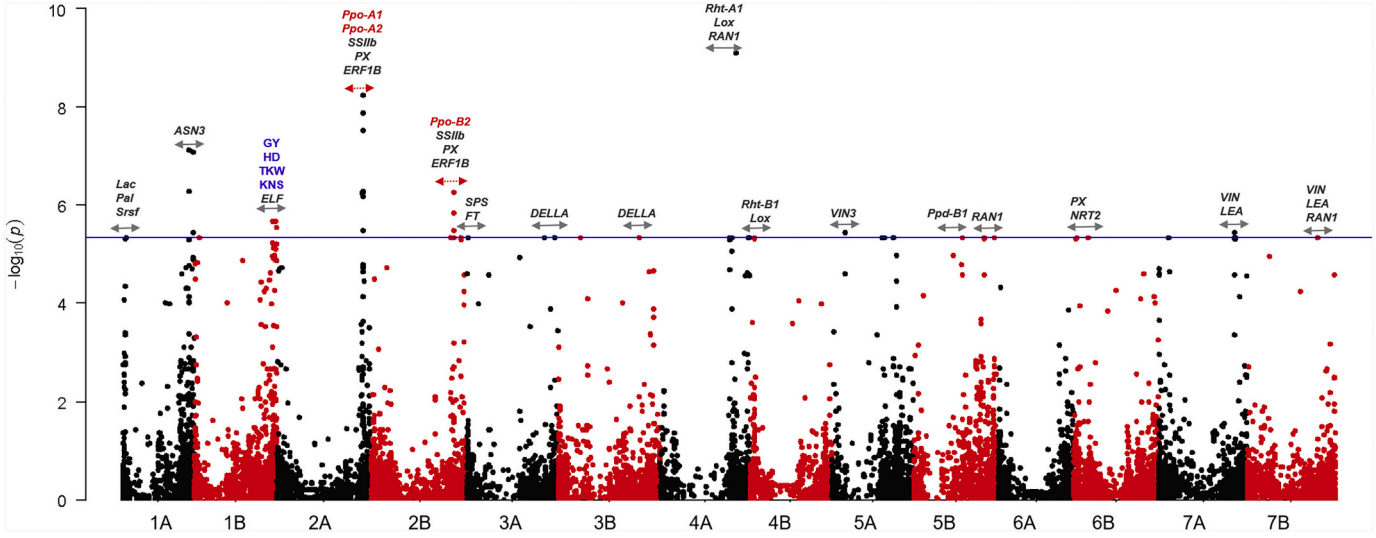

Fig. 3. The Manhattan plot shows the results of GWA analysis performed both in tetraploid (TWC) and durum (DWC) collections. Svevo chromosomes and -log10p are indicated on the x and y axes, respectively. The blue line indicates the threshold of significance (Bonferroni adjusted p-value = 0.05). Gene name abbreviation: Lac, Laccase; Pal, Phenylalanine ammonia-lyase; SRSF, Ser/Arg (*SR*)-rich splicing factor; ASN3, asparagine synthetase 3; ELF, Early flowering; Px, peroxidase; ERF1, Ethylene Responsive Factor; FT, Flowering locus T; SPS, Sucrose-Phosphate Synthase; DELLA, proteins are nuclear repressors of plant gibberellin responses; VIN, neutral invertase and vacuolar invertase; NRT2, nitrate transporter; Rht, reduced height; Ran, GTP-binding nuclear protein; Ppo, polyphenol oxidase; Lox, lipoxygenase; Ppd, photoperiod sensitivity; LEA, Late embryogenesis abundant. QTL name, abbreviation (in blue): GY, grain yield; TKW, thousand kernel weight; HD, heading date; KNS, grains per spike. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[104]. Similar considerations could be applied to SNPs of PPO activity in LD with nitrogen metabolism-related genes detected on chromosome 1A and 6B (*TaASN3* and *NRT2*), respectively [50]. Overall, it is interesting to note that genes associated with PPO activity were in LD with key genes of plant architecture as early flowering selection and plant height, and grain yield and nitrogen metabolism [105–107].

4.3. SNP markers for PPO activity in durum wheat breeding

The availability of information on SNP-trait associations can be conveniently used to assist breeding programs [108]. In our study, based on the GWAS results, four pairs of SNP markers were developed based on the MTA information and validated via pyrosequencing to confirm allelic discrimination between the two haplotypes associated with low and high PPO activity.

The rapid detection of allelic variation and identification of haplotypes responsible for variation in PPO activity can play a crucial role in wheat quality and resistance improvement. Among the four SNPs, the marker IWB75732, localized on chromosome 2A, was able to discriminate significantly between the two haplotypes and it can be considered an accurate SNP marker for discriminating genotypes with high and low PPO. To date, only gene markers have been developed to distinguish between high and low PPO in wheat [31,35,37,44]. Consequently, the IWB75732 provides reliable haplotypes for MAS in durum wheat PPO activity.

5. Conclusion and perspective

The GWA analysis conducted in this study reveals the important role played by PPO genes both in the evolutionary and domestication process of the species of the genus *Triticum* supporting the Yu et al. [109] hypothesis verified in rice (*Oryza sativa*), in which PPOs were considered an example of selection driving the differentiation among domesticated varieties. The strong genetic and phenotypic differentiation between Haplotype1 and Haplotype2 supported the idea that PPO genes were closely related to natural and artificial selection pressure, respectively. The fact that PPO genes are strongly linked to clusters of adaptive and/or resistance genes supports the idea that these genomic regions controlled and affected the adaptation of the plants to environmental stresses. In this context, it emerged that there are only a few studies that highlight the different origins of the *carthlicum* subspecies and its potential in starting new breeding programs as a source of novel disease sources, such a novel donor of useful genes. Indeed, *T. turgidum* ssp. *carthlicum* was understudied and largely untapped, so a better evaluation of their germplasm may provide us an important source for abiotic and biotic stress tolerance.

Our results add new information about the breeding process of durum wheat and on the causes that favored the fixation of PPO alleles for low PPO activity in modern varieties. Finally, the GWA analysis was useful to develop functional markers that could be used in the future marker-assisted selection breeding programs for PPO activity.

Data availability statement

The filtered VCF file has been uploaded to the Mendeley data repository (https://doi.org/10.17632/rt2gmzbvmz.1).

Author contributions

Conceptualization = F.T., G.M. and PDV; Correlation, GWA, F_{ST}, Haplotype and Go enrichment analysis = F.T.; Candidate genes analysis = F.T., M.G. and M.M.M; Pyrosequencing analysis = P.S.; Funding acquisition = P.D.V.; Data curation and Investigation = F.T., G.M., M.M.M., P.S., P.D.V.; Resources = F.T., G.M. and PDV; Supervision = F.T. and PDV; Roles/Writing - original draft = F.T. and PDV; Writing - review & editing = F.T., G.M., M.M.M., P.S., P.D.V.

Conceptualization = F.T., G.M. and PDV; Correlation, GWA, F_{ST}, Haplotype and Go enrichment analysis = F.T.; Candidate genes analysis = F.T., M.G. and M.M.M; Pyrosequencing analysis = P.S.; Funding acquisition = P.D.V.; Data curation and Investigation = F.T., G.M., M.M.M., P.S., P.D.V.; Resources = F.T., G.M. and PDV; Supervision = F.T. and PDV; Roles/Writing - original draft = F.T. and PDV; Writing - review & editing = F.T., G.M., M.M.M., P.S., P.D.V.

Funding

This research was funded within the project “INNOGRANO” (F/0593/00/X32) HORIZON 2020 PON I&C 2014–2020 by the Italian Ministry of Economic Development (MISE).

Author statement

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Acknowledgements

The authors wish to thank Dr. Robert J. Elshire for scientific English language editorial assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.06.030.

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Update

Genomics

Volume 114, Issue 1, January 2022, Page 465

DOI: https://doi.org/10.1016/j.ygeno.2021.12.006

Genomics 114 (2022) 465–465

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Corrigendum

Corrigendum to "Polyphenol oxidase genes as integral part of the evolutionary history of domesticated tetraploid wheat" [Genomics 113 (2021) 2989–3001]

Francesca Taranto [a,1,*], Giacomo Mangini [b,1,**], Monica Marilena Miazzi [c], Piergiorgio Stevanato [d], Pasquale De Vita [e]

[a] National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), 80055 Portici, NA, Italy
[b] National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), 70126 Bari, BA, Italy
[c] Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, 70126 Bari, Italy
[d] DAFNAE, Università degli Studi di Padova, 35020 Legnaro, Italy
[e] Council for Agricultural Research and Economics (CREA), Research Centre for Cereal and Industrial Crops (CREA-CI), 71122 Foggia, Italy

The authors regret that there are two misspelled marker names (SNP ID) in the text and tables. The correct name of the SNP ID "IWB75732" is "IWA572". This substitution should be considered in the Abstract, in Table 2, in the paragraphs "3.3. Allelic quantification in two haplotype datasets" and 4.3. "SNP markers for PPO activity in durum wheat breeding", and in the Supplementary Tables S2, S4 and S5.

The correct name of the SNP ID "IWB79388" is "IWA5463". This substitution should be considered in the paragraph 3.3. "Allelic quantification in two haplotype datasets", Table 2, Table S2, S4 and S6.

The authors would like to apologise for any inconvenience caused.

DOI of original article: https://doi.org/10.1016/j.ygeno.2021.06.030.

* Correspondence to: T. Francesca, National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), Via Università 133, 80055 Portici, NA, Italy.

** Correspondence to: M. Giacomo, National Research Council (CNR), Institute of Biosciences and Bioresources (CNR-IBBR), via Giovanni Amendola 165/A, 70126 Bari, BA, Italy.

E-mail addresses: francesca.taranto@ibbr.cnr.it (F. Taranto), giacomo.mangini@ibbr.cnr.it (G. Mangini).

[1] These authors contributed equally.

https://doi.org/10.1016/j.ygeno.2021.12.006

Available online 27 December 2021