

CROPS AND SOILS RESEARCH PAPER

Effect of *Ppd-1* genes on durum wheat flowering time and grain filling duration in a wide range of latitudesC. ROYO^{1*}, S. DREISIGACKER², C. ALFARO³, K. AMMAR² AND D. VILLEGAS¹¹ IRTA (Institute for Food and Agricultural Research and Technology), Field Crops Program, Rovira Roure, 191, E-25198 Lleida, Spain² CIMMYT (International Maize and Wheat Improvement Center), 06600 Mexico, DF, Mexico³ INIA (Instituto de Investigaciones Agropecuarias), Centro Regional de Investigación Rayentué, Casilla 13, 2940000 Rengo, Chile

(Received 24 September 2014; revised 17 March 2015; accepted 30 April 2015; first published online 4 August 2015)

SUMMARY

Understanding the effect of genetic factors controlling flowering time is essential to fine-tune crop development to each target environment and to maximize yield. A set of 35 durum wheat genotypes of spring growth-habit involving different allelic combinations at *Ppd-A1* and *Ppd-B1* genes was grown for 2 years at four sites at latitudes ranging from 19°N to 41°N. The emergence-flowering period was reduced from north to south. The frequency in the collection of the insensitive allele GS-105 at *Ppd-A1* was greater (34%) than that of allele GS-100 (20%). Genotypes that flowered earlier due to the presence of alleles causing photoperiod insensitivity extended their grain-filling period, but less than the shortening in flowering time. The effect of the allele conferring photoperiod sensitivity at *Ppd-A1* was stronger than that at *Ppd-B1* (*Ppd-A1b* > *Ppd-B1b*). The effect of photoperiod insensitivity alleles was classified as GS-100 > GS-105 > *Ppd-B1a*. The phenotypic expression of alleles conferring photoperiod insensitivity at *Ppd-A1* increased at sites with average day length from emergence to flowering lower than 12 h. An interaction effect was found between *Ppd-A1* and *Ppd-B1*. Differences between allelic combinations in flowering time accounted for c. 66% of the variability induced by the genotype effect, with the remaining 34% being explained by genes controlling earliness *per se*. The shortest flowering time across sites corresponded to the allelic combination GS-100/*Ppd-B1a*, which reduced flowering time by 11 days irrespective of the *Ppd-A1b*/*Ppd-B1b* combination. The current study marks a further step towards elucidation of the phenotypic expression of genes regulating photoperiod sensitivity and their interaction with the environment.

INTRODUCTION

Maximizing plant yield potential in any given environment requires optimizing the use of water, nutrients and radiation, and avoiding negative effects from any type of stress during the vegetative and grain-filling periods. This can only be achieved by growing varieties with a flowering time and life-cycle duration suited to the environmental conditions. In wheat, flowering time is a critical stage as it defines the duration of spike formation and therefore the allocation of resources

to seed production, and marks the beginning of the grain-filling period. The trade-off between resource allocation and stress avoidance is also of primary importance: for example, brief episodes of high temperature (>32–36 °C) coinciding with a critical period of only 1–3 days around wheat anthesis can greatly reduce seed set and yield (Wheeler *et al.* 2000). Therefore, setting the optimum flowering time for a target environment is essential, not only to enhance grain yield but also to permit full expression of end-use quality genetic potential. Manipulation of flowering time has always been a major objective in wheat breeding programmes. Understanding its underlying genetic control and the

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environmental effect on its expression is crucial to fine-tune phenology for a particular set of environmental conditions for optimum and stable performance.

Wheat flowering time is controlled mainly by three groups of loci, two of which interact with environmental factors, namely photoperiod sensitivity genes (*Ppd*) and vernalization requirement genes (*Vrn*) (Distelfeld *et al.* 2009). The third group of loci, controlling 'narrow-sense earliness' or 'earliness *per se*' (*Eps*), act on the developmental rate independently of vernalization and photoperiod (Scarth & Law 1984).

Vernalization is the acquisition or acceleration of a plant's ability to flower by exposure to cold (Chouard 1960). According to the vernalization requirements, wheat is classified as having a winter or spring growth habit. Winter wheat has a considerable vernalization requirement but spring wheat may be insensitive or only partly sensitive to vernalization. Vernalization requirement is mainly controlled by the *Vrn-1* genes. Durum wheat contains a homologous copy of *Vrn-1*, designated *Vrn-A1* and *Vrn-B1* and located on the long arms of chromosomes 5A and 5B, respectively (Yan *et al.* 2004; Fu *et al.* 2005). As compared with hexaploid wheat, the major elite durum wheat gene pools show no major vernalization requirements (spring wheat), while functionally variant alleles are present at main loci for the photoperiod-sensitive response (Clarke *et al.* 1998).

Photoperiod-sensitive wheat is stimulated to flower only on exposure to long-days, provided that any requirement for vernalization is met, and flowering is delayed under short days. In spring-habit wheat, photoperiod-sensitive types cannot be grown as an overwinter crop in tropical or low-latitude areas, since the day length requirement would not be satisfied in a short enough time-frame to produce a commercially viable crop (Worland & Snape 2001). Photoperiod-insensitive wheat flowers independently of day length and can be grown to maturity in long- or short-day environments. This is a particular advantage in warm and dry climates, as early flowering varieties are able to fill the grain prior to the onset of the high temperatures and drought stress that occur late in the season (Worland & Snape 2001). Photoperiod sensitivity in durum wheat is determined at the *Ppd-A1* and *Ppd-B1* loci, located on chromosomes 2AS and 2BS, respectively (Laurie 1997).

The intensive selection for photoperiod insensitivity in modern wheat was an important factor in the success of the 'Green Revolution' cultivars with the 'shuttle-breeding' strategy (selecting plants in segregating populations shuttling generations between two highly contrasting environments but both of short

cycle duration), resulting in the selection of early types, most of them with little to no photoperiod sensitivity, with adaptation to a broad range of temperate agricultural environments. This allowed the Mexican semi-dwarf wheat to spread to millions of hectares around the world (Borlaug 1995). First implemented by Norman Borlaug, this shuttle-breeding approach still represents the cornerstone of the wide-adaptation breeding strategy used by the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico. Yield advantages resulting from photoperiod insensitivity have been estimated at over 35% in Southern European environments and 15% in Central Europe (Worland 1996). The effects of breeding on flowering time of durum wheat during the 20th century have been described in Spain, where a reduction of 8 days from sowing to flowering was estimated (Álvaro *et al.* 2008), and in Italy, where the reduction was 2 days (Álvaro *et al.* 2008), and the rate of flowering time has been reported to advance 5 °C per year (Motzo & Giunta 2007). Flowering dates vary widely among durum wheat landraces, according to their area of origin. A recent study demonstrated that the number of days to heading and flowering of Mediterranean landraces increased steadily from the warmest and driest zone of origin to the coldest and wettest one (Royo *et al.* 2014).

Photoperiod insensitivity in durum wheat results from mutations in the *Ppd-1* genes on the A or B genomes. By convention, alleles conferring photoperiod insensitivity are assigned by an 'a' suffix (e.g. *Ppd-A1a*, McIntosh *et al.* 2003), while wild-type alleles are given a 'b' suffix. The basis and degrees of photoperiod insensitivity have been insufficiently characterized in durum wheat. Wilhelm *et al.* (2009) found two large deletions within the *Ppd-A1* gene in durum wheat (1027 and 1117 base pair (bp) deletion designated as alleles 'GS-100' and 'GS-105, respectively), which remove a common region from the wild-type sequence. The presence of either deletion accelerated flowering, which led to the conclusion that these deletions are the likely causal basis of photoperiod insensitivity in tetraploid wheat (Wilhelm *et al.* 2009). A quantitative trait locus (QTL) associated with *Ppd-A1a* significantly reducing heading date was detected by Maccaferri *et al.* (2008) in a recombinant inbred line population derived from the cross 'Kofa' ('GS-100' allele) × 'Svevo' ('GS-105' allele), suggesting that these alleles decrease photoperiod sensitivity to different degrees. As both mutations are predominant in modern durum wheat but absent from wild tetraploid wheat, it has been suggested that the *Ppd-A1*

insensitive alleles arose by mutation during domestication (Bentley *et al.* 2011). The *Ppd-B1* locus was originally mapped by Hanocq *et al.* (2004) and Mohler *et al.* (2004) in bread wheat and was confirmed by Maccaferri *et al.* (2008) in durum wheat. Beales *et al.* (2007) found several polymorphisms within *Ppd-B1* genes of hexaploid wheat, including five single-nucleotide polymorphisms (SNPs) and a retrotransposon insertion. However, none corresponded to photoperiodic response, implying that the critical mutation causing the allelic difference of the *Ppd-B1* gene has not been found. Diaz *et al.* (2012) found the changes in flowering time to be associated with increased copy number and not with specific sequence polymorphism in bread wheat. Nishida *et al.* (2013) reported a novel mutation in the 5' upstream region of *Ppd-B1*, suggesting that an allelic series of photo-period-insensitive mutation exists in hexaploid wheat.

The third component regulating flowering time, *Eps*, is characterized by a polygenic inheritance, and 20 meta-QTLs associated with it have been identified on chromosomes 1B, 3A, 3B, 4A, 4B, 5B, 6A and 6B (Griffiths *et al.* 2009; Kamran *et al.* 2013). Although the effects of *Eps* are considered relatively small, it can cause measurable variations in flowering date independently from the effect of major genes such as *Ppd* or *Vrn* (Van Beem *et al.* 2005).

The objective of the current study was to examine the effect of allelic variants at *Ppd-A1* and *Ppd-B1* genes on the length of durum wheat developmental periods, namely, emergence to flowering and flowering to physiological maturity, across a wide range of northern temperate latitudes. The relative effect of putative *Eps* was also evaluated.

MATERIALS AND METHODS

Plant material

Thirty-five spring durum wheat (*Triticum turgidum* L. var. *durum*) genotypes were used in the study. Thirty resulted from a divergent selection process within the offspring of crosses between parents with contrasting flowering time. Five late-flowering genotypes (Durabon, Megadur, 2716–25·94·01, 2805–49·94·02, 2905–13·93·04) from the breeding programme of the University of Hohenheim (Germany) were crossed with five early-flowering advanced lines (Sooty_9/Rascon_37, Cado/Boomer_33, Dukem_12/2*Rascon_21, Guanay and Snitan) from the CIMMYT-Mexico programme. The F₁, F₂ and F₃ populations were advanced

in bulk at CIMMYT. From each F₄ population, an early-flowering and a late-flowering plant were selected in order to capture the maximum range for time to flowering. From generations F₅ to F₇, selected lines were selfed, purified and increased at the Institute for Food and Agricultural Research and Technology (IRTA) in Spain. At generations F₈ and F₉, the seed of fixed lines with contrasting flowering dates was used in field experiments. The collection also included two sister lines derived from the cross CF4-JS40/3/Stot//Altar84/Ald, and three well known commercial cultivars with varying flowering dates that were used as controls: Mexa (early-flowering in Mexico and Spain), Simeto (late-flowering in Mexico and medium-to late-flowering in Spain) and Anton (late-flowering in both countries).

Molecular characterization

The selected genotypes were analysed with a set of molecular markers. Leaf tissue from five to ten plants per plot was collected in the field and DNA was extracted using the cetyltrimethyl ammonium bromide modified procedure (Saghai-Marooif *et al.* 1984) described at <http://repository.cimmyt.org/xmlui/handle/10883/3221>.

Sequence-tagged sites (STS), simple sequence repeats (SSR) and SNP markers associated with identified polymorphisms in durum wheat were utilized first. Subsequently, additional markers for known bread wheat alleles were tested (Table 2).

The genotypes were initially characterized for the *Vrn-1* and *Vrn-3* genetic loci (*Vrn-A1*, *Vrn-B1* and *Vrn-B3*) to determine the spring or winter growth habit. Dominant spring alleles due to variation in the promoter and intron-1 region of the *Vrn-A1* locus were identified utilizing the gene-specific STS markers described by Yan *et al.* (2004) and Fu *et al.* (2005). In addition, the presence of an SNP was tested for in Exon 4 of *Vrn-A1*, identified so far only in bread wheat (Diaz *et al.* 2012). Deletion alleles affecting the vernalization response in the intron-1 region of *Vrn-B1* and *Vrn-B3*, respectively, were detected as described in Fu *et al.* (2005), Yan *et al.* (2006) and Chu *et al.* (2011).

For *Ppd-A1*, two SNP KASP assays were applied to detect the 1027 bp 'GS-100' type and 1117 bp 'GS-105' type deletions in durum wheat (Wilhelm *et al.* 2009). Furthermore, the genotypes were tested for the presence of the bread wheat 1·2 kb insertion (cvar Chinese Spring was used as a control) and 306 bp deletion (cvar Cappelle-Desprez as a control) at *Ppd-A1*, respectively (Beales *et al.* 2007). For *Ppd-B1*, linked

SSR markers *gwm148* and *gwm257* as described in Hanocq *et al.* (2004) were primarily utilized. Gene-specific KASP assays determining truncated copies, transposon-junction and allele specific SNPs observed in cvar 'Sonora64' (containing three copies of *Ppd-B1*), cv. 'Chinese Spring' (carrying four copies of *Ppd-B1*) and cvar 'Cheyenne' (carrying one copy of *Ppd-B1*) were tested to determine whether similar allele variation exists in durum wheat (Diaz *et al.* 2012). Copy number variation of *Ppd-B1* alleles could not be identified at the time the genotypic analyses were made. Following Beales *et al.* (2007), the photoperiod-insensitive allele was designated as *Ppd-1a*. The alternative allele, which was assumed to infer some photoperiod sensitivity, was arbitrarily designated *Ppd-1b*.

The polymerase chain reaction (PCR) assay reaction mixture in single 10 µl reactions used to amplify all primers contained final concentrations of 1 × Buffer with Green Dye (Promega Corp., USA), 200 µM deoxy-nucleotide triphosphates, 1.2 mM magnesium chloride, 0.25 µM of each primer, 1U of DNA polymerase (GoTaq@Flexi, Promega Corp., Cat. # M8295) and 50 ng of DNA template. The PCR profile was 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 54–60 °C for 2 min (dependent on the primer), and 72 °C for 2 min. The amplified products were separated on 1.2% agarose gels in tris-acetate/ethylene-diamine-tetraacetic acid (TAE) buffer. The SNP polymorphisms were scored using Kompetitive Allele Specific PCR (KASP) reagents (<http://www.lgcgenomics.com>) in reactions containing 2.5 ml water, 2.5 ml 2 × KASPar Reaction mix, 0.07 ml assay mix and 50 ng of dried DNA with a PCR profile of 94 °C for 15 min activation time followed by 20 cycles of 94 °C for 10 s, 57 °C for 5 s and 72 °C for 10 s and followed by 18 cycles of 94 °C for 10 s, 57 °C for 20 s, and 72 °C for 40 s. Fluorescence was read as an end point reading at 25 °C.

Experimental field setup

Field experiments were conducted in 2007 and 2008 at two sites in Spain: Lleida in the north (Spain-North 41° 38'N), and Jerez de la Frontera in the south (Spain-South 37°0'N), and two locations in Mexico: Ciudad Obregon in the north (Mexico-North 27°21'N) and El Batán (Texcoco) in the Central Mexican Highlands (Mexico-South 19°31'N). The experiments were arranged in randomized complete block designs with three replications and plots of 12 m². Sowing density was adjusted at each site in order to obtain an approximate plant density of 450 spikes/m². Plots were

managed according to the common cultural practices at each site, and were maintained free of weeds, diseases and pests. Three experiments were planted in autumn (from 19 November to 22 December) and the fourth, the one established in Mexico-South, was planted in spring (from 18 to 28 May) for a summer crop cycle. Irrigation was provided during the whole cycle in the Mexico-North site (full irrigation) and when necessary to avoid water stress (Fig. 1) in the other three, mostly rainfed, sites (Spain-North, Spain-South and Mexico-South).

Data recording

The following developmental stages were determined on the central part of each plot according to the Zadoks scale (Zadoks *et al.* 1974): growth stage (GS) 10 (emergence), GS 65 (flowering or anthesis) and GS 87 (physiological maturity, indicated by the loss of green colour in the spike peduncles). A plot was considered to have reached a given developmental stage when at least 50% of the plants exhibited the stage-specific phenotypic characteristics. Daily maximum and minimum temperatures and rainfall were obtained from weather stations located in the experimental fields or at a distance <3 km from them. Photoperiod was calculated with the model proposed by Forsythe *et al.* (1995), as a function of latitude and Julian day, and including the civil twilight (when the centre of the sun is 6° below the horizon). Accumulated photoperiod (ACP, h) from emergence to flowering and from flowering to maturity was calculated by summing the daily photoperiod during each development period. The effects of *Eps* on the length of the emergence-flowering period were estimated in South Mexico, assuming that the long photoperiod existing at this site (c. 14 h) saturated photoperiod requirements. Allelic combination GS-100/*Ppd-B1a* was used as reference as it led to the shortest time to flowering. For each genotype (*i*) carrying other allelic combinations, *Eps* was calculated as:

$$Eps_i = TF_i - (TF_{aci} - TF_{I0I})$$

where TF_i = time to flowering of genotype *i*, TF_{aci} = average time to flowering of allelic combination carried by genotype *i*, and TF_{I0I} = average time to flowering of allelic combination GS-100/*Ppd-B1a* (I0I).

Statistical analysis

After checking for homogeneity of error variances, analyses of variance were carried out using the GLM

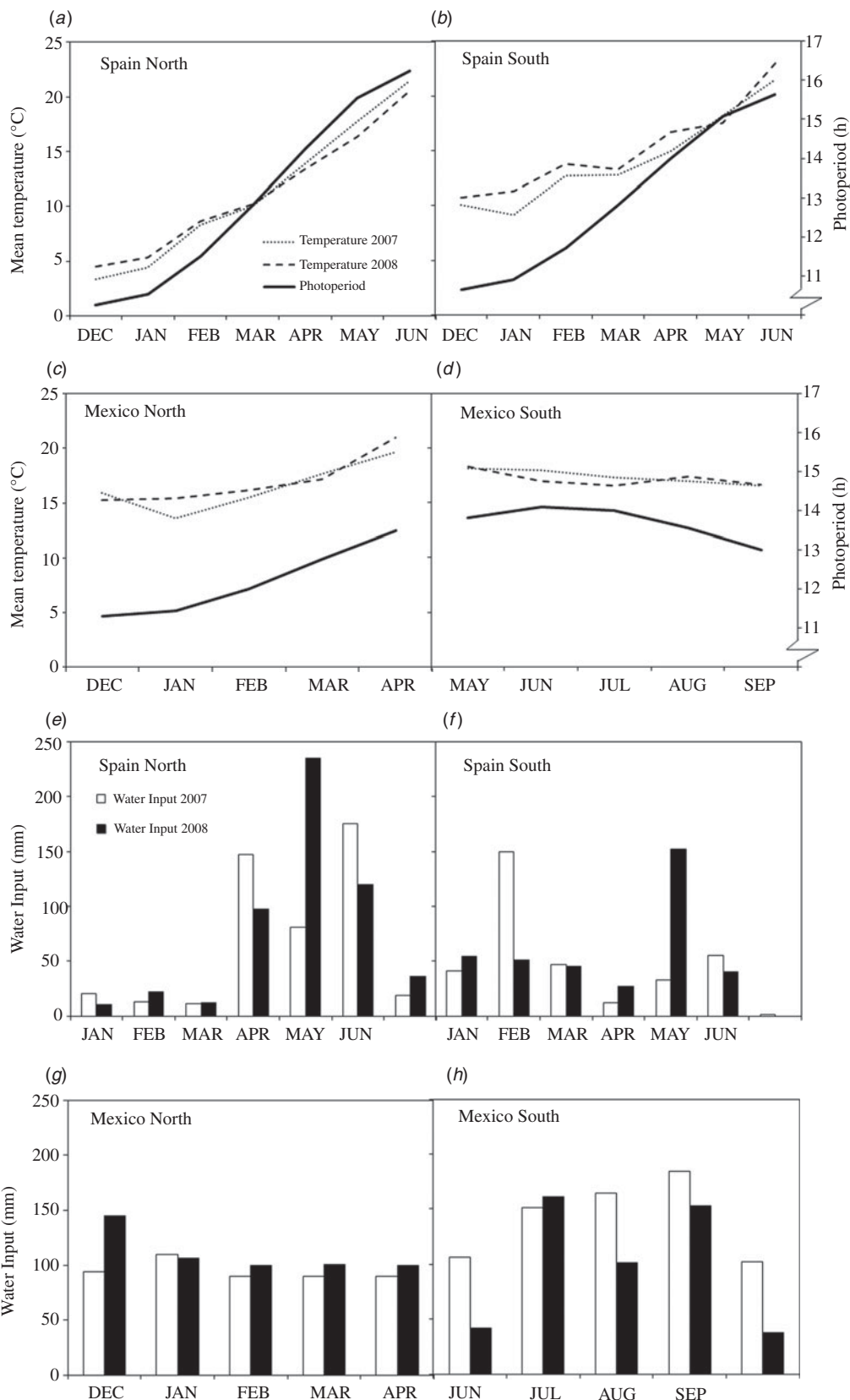


Fig. 1. Environmental conditions prevailing during the experiments conducted in 2007 and 2008 at two contrasting sites in Spain and Mexico. (a–d): Mean temperatures and photoperiod. (e–h): Water input (rainfall + irrigation).

Table 1. Allelic combinations for *Ppd-A1*, *Ppd-B1* and *Vrn-A1* genes present in a collection of 35 durum wheat genotypes obtained through a divergent selection process for flowering time, acronyms used and frequencies within the collection

<i>Ppd-A1</i>		<i>Ppd-B1</i>		<i>Vrn-A1</i>		Allelic combination acronym	Number of lines
Allele*	Photoperiod response	Allele	Photoperiod response	Allele	Vernalization response		
<i>Ppd-A1b</i>	Sensitive	<i>Ppd-B1b</i>	Sensitive	<i>Vrn-A1c</i>	Spring	SS	7
<i>Ppd-A1b</i>	Sensitive	<i>Ppd-B1a</i>	Insensitive	<i>Vrn-A1c</i>	Spring	SI	9
GS-105 <i>Ppd-A1a</i>	Insensitive	<i>Ppd-B1b</i>	Sensitive	<i>Vrn-A1c</i>	Spring	I5S	6
GS-105 <i>Ppd-A1a</i>	Insensitive	<i>Ppd-B1a</i>	Insensitive	<i>Vrn-A1c</i>	Spring	I5I	6
GS-100 <i>Ppd-A1a</i> †	Insensitive	<i>Ppd-B1b</i>	Sensitive	<i>Vrn-A1c</i>	Spring	I0S	1
GS-100 <i>Ppd-A1a</i>	Insensitive	<i>Ppd-B1a</i>	Insensitive	<i>Vrn-A1c</i>	Spring	I0I	6

* Nomenclature described in Wilhelm *et al.* (2009).

† Discarded from statistical analyses due to uniqueness in present collection.

procedure of the SAS statistical package (SAS Institute Inc. 2009), with a fixed-factor model. The sum of squares of the genotype and its interactions were partitioned into differences between allelic combinations and differences within each of them. The mean square (MS) of the 'Between allelic combinations' effect was tested with the sum of residuals of the analysis of variance (ANOVA) for the 'within' effects. Means of the allelic combinations for the two periods, emergence-flowering and flowering-maturity, were compared according to a least significant difference (LSD) test at $P < 0.05$.

A linear regression model was fitted to the relationship between ACP in the emergence-flowering period and the number of days of the same period. The relationship between the length of the emergence-flowering and flowering-maturity periods was assessed through the calculation of the Pearson correlation coefficients using the mean data of genotypes across environments ($n = 34$).

RESULTS

Molecular characterization

The molecular characterization (Tables 1 and 2) revealed that all of the 35 genotypes were spring types, carrying the dominant allele *Vrn-A1c* with a deletion in intron-1 of *Vrn-A1* (Yan *et al.* 2004) and the recessive alleles *vrn-B1* and *vrn-B3* (Fu *et al.* 2005; Yan *et al.* 2006).

Three alleles were identified at *Ppd-A1* (Table 1). Sixteen out of the 35 genotypes carried the allele *Ppd-A1b* conferring photoperiod sensitivity, while

the alleles 'GS-105' and 'GS-100' were identified in 12 and 7 genotypes, respectively. For *Ppd-B1*, the wild-type allele conferring photoperiod sensitivity (*Ppd-B1b*) was identified in 14 genotypes, while the mutation conferring photoperiod insensitivity (*Ppd-B1a*) was identified in 21 genotypes using the linked SSR markers. Allele polymorphisms that were identified in bread wheat (the SNP in Exon 4 of *Vrn-A1*, Indels in *Ppd-A1*, truncated copies, transposon-junctions and SNP variations in *Ppd-B1*) were not observed in this set of durum wheat (Table 2).

The genotypes were classified into *Ppd-A1*–*Ppd-B1* allelic combinations (Table 1). Given the low frequency of the allelic combination GS-100/*Ppd-B1b* – identified only in the control variety Mexa – this combination was removed from the statistical analysis. The controls Simeto and Anton had the allelic combination identified as SI in Table 1.

Environmental and genetic effects on crop development

The four experimental sites have been characterized previously in terms of their environmental variables Villegas *et al.* (2015) and the growing conditions during the two evaluation years are summarized in Fig. 1. Yearly average temperatures increased from north to south. Total water input ranged from 294 to 583 mm, including irrigation, but did not result in any measurable water stress in any of the experiments. The largest variation in photoperiod amplitude during the growth cycle, calculated as the difference between days of maximum and minimum photoperiod, corresponded to Spain-North with 6.05 h. This amplitude

Table 2. Molecular marker summary

STS/SSR marker								
Marker name	Gene	Forward primer	Reverse primer	Product size	Reference			
wms148	<i>Ppd-B1</i>	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACCAAA	<i>Ppd-B1a</i> (Ins) = 164 bp	Hanocq et al. (2004)			
wms257	<i>Ppd-B1</i>	AGAGTGCATGGTGGGACG	CCAAGACGATGCTGAAGTCA	<i>Ppd-B1a</i> (Ins) = 195 bp	Hanocq et al. (2004)			
<i>VRN-A1F</i> , <i>VRN1R</i>	<i>Vrn-A1</i>	GAAAGGAAAAATTCTGCTCG	TGCACCTTCCCCGCCCCAT	<i>vrn-A1/Vrn-A1c</i> = 484 bp, <i>Vrn-A1a</i> = 715 bp	Yan et al. (2004)			
<i>Ex1/C/F</i> , <i>Intr1/A/R3</i>	<i>Vrn-A1</i>	GTTCTCCACCGAGTCATGGT	AAGTAAGACAACACGAATGTGAGA	<i>Vrn-A1</i> = 522 bp	Fu et al. (2005)			
<i>Intr/B/F</i> , <i>Intro1/B/R3</i> , <i>Intro1/B/R4</i> , <i>Ex1/B/F3</i>	<i>Vrn-B1</i>	<i>Intr/B/F</i> : CAAGTGGAAACGGTTAGGACA <i>Ex1/B/F3</i> : GAAGCGGATCGAGAACAAGA	<i>Intro1/B/R3</i> : CTCATGCCAAAAATTGAAGATGA <i>Intro1/B/R4</i> : CAAATGAAAAGGAATGAGAGCA	<i>Vrn-B1</i> = 709 bp	Fu et al. (2005), Chu et al. (2011)			
<i>VRN4-B-INS-F</i> , <i>VRN4-B-INS-R</i>	<i>Vrn-B3</i>	CATAATGCCAAGCCGGTGAGTAC	ATGTCTGCCAATTAGCTAGC	<i>Vrn-B3</i> = 1200 bp	Yan et al. (2006)			
SNP marker								
Marker ID	Gene	Primer Allele FAM	Primer allele VIC	Primer common	FAM allele	VIC allele	Comment	Reference
wMAS000027	<i>Ppd-B1</i>	GACGTTATGAACGCTTGCCA	CCGTTTTCGCGGCCTT	GGGTTCTGTCGGGAGCTGT	Insertion (A)	Wildtype (T)	Chinese Spring truncated copy assay (retrotransposon exon 7)	Beales et al. (2007)
wMAS000028	<i>Ppd-B1</i>	CGTGAAGAGCTAGCGATGAACA	TGGGCACGTTAACACACCTTT	Null	A		Sonora 64 variant, dominant	Diaz et al. (2012)
wMAS000029	<i>Ppd-A1</i>	CATTAGTTTCTTTTGTTTCTGCA	CAATCAGATCAGCAGCTCGAAC	CCTGAAGTCAGAGATATGCAGCAAC	Insertion (A)	Wildtype (C)	Cappelle-Desprez type deletion (303-bp exon5-intron5-exon6)	Beales et al. (2007)
wMAS000030	<i>Ppd-A1</i>	CCAGTATCTTTAGATGCACCATGC	GCCGGCGGCTAAAAGG	CTATACAATGCTAAAGTCGCACAT	Wildtype (C)	Deletion (G)	GS-100 type deletion (promoter region)	Wilhelm et al. (2009)
wMAS000031	<i>Ppd-A1</i>	GGGGACCAATACCGCTCG	CGTTTGGTGGTGGACGGG	GAAACAGAGGGTGGTTTGAAT	Wildtype	Deletion	GS-105 type deletion (promoter region)	Wilhelm et al. (2009)
wMAS000034	<i>Vrn-A1</i>	CAACTCCTTGAGATTCAAAGATTCAAG	GCAACTCCTTGAGATTCAAAGATTCAAA	CATCCTGCATCTGCAGGCATCTC	C	T	Exon 4 SNP for long/short vernalization requirement: Jagger (C) / 2174 (T)	Diaz et al. (2012)

Table 3. Percentage of the sum of squares (% SS) of the analysis of variance (ANOVA) for the number of days of developmental periods of 34 durum wheat genotypes grown at two contrasting sites in Spain and Mexico in 2007 and 2008

Source of variation	D.F.	Emergence–flowering	Flowering–maturity	Emergence–maturity
Site	3	86.77	16.55	91.30
Year	1	1.96	21.16	0.00**
Site × year	3	4.89	30.72	4.41
Genotype	33	4.36	11.47	2.38
Between allelic combinations	4	2.89	5.90	1.08
Within SS	6	0.45	0.53	0.25
Within SI	8	0.43	1.44	0.19
Within I5S	5	0.35	1.32	0.11
Within I5I	5	0.05	1.24	0.18
Within I0I	5	0.19	1.04	0.57
Genotype × site	99	1.54	8.44	0.89
Site × between allelic combinations	12	0.49	4.32	0.19
Site × within SS	18	0.14	0.51	0.11
Site × within SI	24	0.46	1.03	0.29
Site × within I5S	15	0.12	0.62	0.09
Site × within I5I	15	0.13	0.71	0.08
Site × within I0I	15	0.20	1.25	0.13
Genotype × year	33	0.05	1.77	0.15
Genotype × site × year	99	0.24	4.72	0.42
Rep (site × year)	16	0.01*	0.54	0.08
Residual	528	0.18	4.63	0.37
Total	815			

The genotype effect and the environment × genotype interaction are partitioned into differences between allelic combinations and differences within each of them (see Table 1 for allelic combination acronyms).

%SS values without symbol are significant at $P < 0.001$, * $P < 0.01$, ** $P > 0.05$.

decreased sharply with decreasing latitude, to a minimum of 1.15 h in Mexico-South, which was the only site with decreasing photoperiod because of spring planting, while at the remaining sites the photoperiod increased during the growth cycle (Fig. 1).

In each experiment, plants of all plots emerged on the same day. The ANOVA results are presented in Table 3. The environmental effects (site, year and site × year) accounted for the largest proportion of the variability in crop phenology (Table 3). The site effect was the most important in explaining the length of the emergence–flowering and emergence–maturity periods. Genotype mean values across experiments are shown in Table 4. For the duration of emergence–flowering differences between allelic combinations accounted for 66.3% of the variability due to genotype, with the remaining 33.7% being explained by differences within combinations (deduced from Table 3). The genotype effect accounted for 11.5% of variation in the flowering–maturity period. Differences between allelic combinations accounted for 51.4% of the variation

induced by the genotype effect for the duration of this period. For the total cycle length, the emergence–maturity period, allelic combinations accounted for c. 45.4% of the genotype effect (deduced from Table 3).

The genotype × site interaction accounted for the largest portion of the genotype × environment interaction for all the variables measured. The site × between allelic combinations interaction was significant for the three periods and accounted from c. 21.3 to 51.2% of the variance explained by the genotype × site interaction, and from 0.19 to 4.32% of the total variance of the model. The interactions between site and the variability within allelic combinations were significant in all cases ($P < 0.001$) and explained between 0.08 and 1.25% of the total variance of the models (Table 3).

Main effect of allelic variation at Ppd-A1

Genotypes carrying allele *Ppd-A1b* (conferring photoperiod-sensitivity) had on average a longer time to flowering than those carrying any of the two alleles

Table 4. Mean values across sites and years \pm s.e. of the number of days for developmental periods of 34 durum wheat genotypes grown at four sites of varying latitude during 2 years

Genotype	Emergence–lowering	Flowering–maturity	Emergence–maturity
Allelic combination: <i>Ppd-A1b/Ppd-B1b</i>			
Line 1	113 \pm 5.1	40 \pm 1.2	153 \pm 4.6
Line 2	107 \pm 4.6	41 \pm 1.7	148 \pm 4.6
Line 3	104 \pm 5.5	43 \pm 1.3	147 \pm 4.9
Line 4	103 \pm 5.0	42 \pm 1.3	145 \pm 4.8
Line 5	102 \pm 5.6	43 \pm 1.4	145 \pm 5.1
Line 6	101 \pm 5.6	44 \pm 1.5	145 \pm 5.2
Line 7	101 \pm 5.8	43 \pm 1.6	144 \pm 5.3
Allelic combination: <i>Ppd-A1b/Ppd-B1a</i>			
Line 8	110 \pm 5.1	39 \pm 1.1	150 \pm 4.7
Line 9	110 \pm 5.3	41 \pm 1.2	151 \pm 4.7
Line 10	108 \pm 4.8	40 \pm 1.1	148 \pm 4.7
Line 11	106 \pm 4.8	40 \pm 1.3	146 \pm 4.5
Control Anton	105 \pm 5.7	42 \pm 1.2	147 \pm 5.1
Line 12	104 \pm 5.8	41 \pm 1.4	145 \pm 5.2
Line 13	104 \pm 5.7	41 \pm 1.4	145 \pm 5.5
Line 14	103 \pm 5.6	44 \pm 1.6	147 \pm 5.2
Control Simeto	99 \pm 4.3	45 \pm 1.2	144 \pm 4.2
Allelic combination: <i>GS-105/Ppd-B1b</i>			
Line 15	106 \pm 5.4	40 \pm 1.3	146 \pm 5.1
Line 16	104 \pm 5.6	43 \pm 1.3	147 \pm 5.2
Line 17	99 \pm 5.8	46 \pm 1.6	144 \pm 5.2
Line 18	98 \pm 5.8	45 \pm 1.3	143 \pm 5.2
Line 19	97 \pm 5.0	44 \pm 1.3	141 \pm 4.8
Line 20	96 \pm 5.5	47 \pm 2.0	143 \pm 5.5
Allelic combination: <i>GS-105/Ppd-B1a</i>			
Line 21	98 \pm 5.7	45 \pm 1.7	144 \pm 5.2
Line 22	98 \pm 4.9	46 \pm 1.8	144 \pm 5.0
Line 23	97 \pm 4.9	42 \pm 1.3	139 \pm 5.0
Line 24	96 \pm 5.2	48 \pm 2.1	144 \pm 5.2
Line 25	95 \pm 5.3	44 \pm 1.2	139 \pm 5.1
Line 26	94 \pm 5.8	46 \pm 1.2	140 \pm 5.6
Allelic combination: <i>GS-100/Ppd-B1a</i>			
Line 27	98 \pm 4.2	48 \pm 2.3	146 \pm 4.9
Line 28	97 \pm 4.8	48 \pm 1.7	145 \pm 4.9
Line 29	96 \pm 5.2	47 \pm 2.1	143 \pm 5.6
Line 30	94 \pm 5.2	46 \pm 1.4	140 \pm 5.2
Line 31	92 \pm 5.8	46 \pm 1.6	139 \pm 5.6
Line 32	90 \pm 5.4	43 \pm 1.5	133 \pm 5.7

conferring photoperiod insensitivity (Fig. 2a). Allelic variants at *Ppd-A1a* reduced the duration of the emergence-flowering period. This reduction was higher in Spain-South and Mexico-North than in Spain-North or Mexico-South (Table 5). Emergence-flowering time reductions from 7 to 12 days were observed in Spain-South and Mexico-North in the genotypes carrying allele GS-105 compared with those carrying the sensitive allele, while in Spain-North and

Mexico-South the reductions ranged between 3 and 5 days. Similarly, the reduction in the duration of the emergence-flowering period caused by allele GS-100 at *Ppd-A1a* when compared with *Ppd-A1b* were more than double in Spain-South and Mexico-North (11–19 days) than in Spain-North and Mexico-South (5–9 days) (Table 5). In order to examine the divergences in the expression of *Ppd-A1a* alleles at contrasting sites during emergence-flowering, the relationship

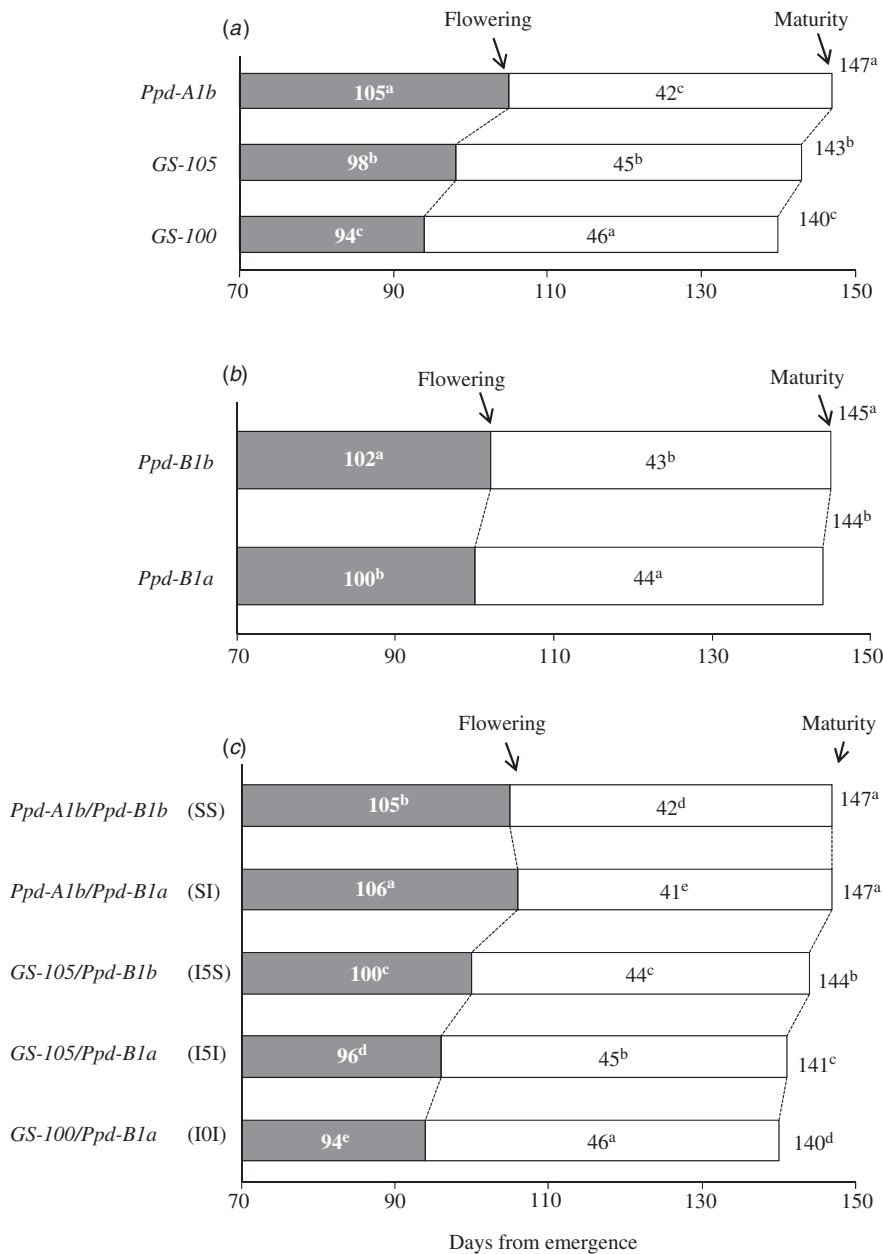


Fig. 2. Average number of days corresponding to different developmental stages of 34 durum wheat genotypes grown for 2 years at four sites at different latitudes: (a) contrast based on allelic composition at *Ppd-A1*; (b) contrast based on allelic composition at *Ppd-B1* and (c) contrast based on allelic composition at both loci.

between the length of this period and the environmental variables recorded at each site were investigated. The results showed that differences in emergence-flowering caused by alleles at *Ppd-A1a* were larger at sites with an average photoperiod below 12 h, as shown in Fig. 3.

The comparison of the reduction in flowering time associated with the two mutations at *Ppd-A1* causing photoperiod insensitivity showed that allele *GS-100* had a consistent stronger effect than allele *GS-105*

(Fig. 2a and Table 5). The effect of *Ppd-A1* alleles on the duration of the flowering-maturity period was in the opposite direction and to a lesser extent than that observed for time to flowering (Fig. 2a). On average, alleles *GS-105* and *GS-100* extended the flowering-maturity period by 3 days and 4 days, respectively, when compared with the wild type, but their effect was not consistent across sites and years (Table 5). Given that the effect of *Ppd-A1* alleles conferring photoperiod insensitivity was greater in

Table 5. Main effects of alleles at *Ppd-A1* and *Ppd-B1* loci on the number of days of developmental periods of 34 durum wheat genotypes grown at four sites of varying latitude during 2 years

Allele	Photoperiod response	D.F.	Spain-North		Spain-South		Mexico-North		Mexico-South	
			2007	2008	2007	2008	2007	2008	2007	2008
Days emergence–flowering										
<i>Ppd-A1</i>										
<i>Ppd-A1b</i>	Sensitive	15	148	121	117	118	101	97	68	70
GS-105	Insensitive	11	145 (–3)	118 (–3)	110 (–7)	108 (–10)	89 (–12)	87 (–10)	63 (–5)	66 (–4)
GS-100	Insensitive	5	139 (–9)	114 (–7)	106 (–11)	99 (–19)	88 (–13)	84 (–13)	60 (–8)	65 (–5)
	S.E.D.		0.33	0.27	0.23	0.25	0.52	0.54	0.31	0.22
<i>Ppd-B1</i>										
<i>Ppd-B1b</i>	Sensitive	12	148	120	114	115	96	92	66	68
<i>Ppd-B1a</i>	Insensitive	20	144 (–4)	117 (–3)	112 (–2)	109 (–6)	94 (–2)	90 (–2)	65 (–1)	68 (0)
	S.E.D.		0.25	0.21	0.17	0.19	0.41	0.42	0.24	0.17
Days flowering–maturity										
<i>Ppd-A1</i>										
<i>Ppd-A1b</i>	Sensitive	15	33	45	40	49	32	43	49	42
GS-105	Insensitive	11	35 (+2)	47 (+2)	44 (+4)	54 (+5)	35 (+3)	51 (+8)	49 (0)	42 (0)
GS-100	Insensitive	5	39 (+6)	50 (+5)	47 (+7)	61 (+12)	33 (+1)	50 (+7)	49 (0)	41 (–1)
	S.E.D.		0.41	0.37	0.48	0.32	0.65	0.73	0.68	0.53
<i>Ppd-B1</i>										
<i>Ppd-B1b</i>	Sensitive	12	33	46	42	51	33	46	50	42
<i>Ppd-B1a</i>	Insensitive	20	36 (+3)	47 (+1)	43 (+1)	54 (+3)	33 (0)	48 (+2)	48 (–2)	41 (–1)
	S.E.D.		0.32	0.29	0.37	0.25	0.50	0.56	0.53	0.41
Days emergence–maturity										
<i>Ppd-A1</i>										
<i>Ppd-A1b</i>	Sensitive	15	181	166	157	167	133	140	117	112
GS-105	Insensitive	11	180 (–1)	165 (–1)	154 (–3)	162 (–5)	124 (–9)	138 (–2)	112 (–5)	108 (–4)
GS-100	Insensitive	5	179 (–3)	164 (–2)	153 (–4)	160 (–7)	121 (–12)	134 (–6)	109 (–8)	106 (–6)
	S.E.D.		0.36	0.39	0.44	0.24	0.54	0.61	0.66	0.53
<i>Ppd-B1</i>										
<i>Ppd-B1b</i>	Sensitive	12	181	166	156	166	129	138	116	110
<i>Ppd-B1a</i>	Insensitive	20	180 (–1)	164 (–2)	155 (–1)	163 (–3)	127 (–2)	138 (0)	113 (–3)	109 (–1)
	S.E.D.		0.28	0.30	0.34	0.19	0.42	0.47	0.51	0.41

Numbers in parentheses are the difference from the sensitive allele within each period and locus.

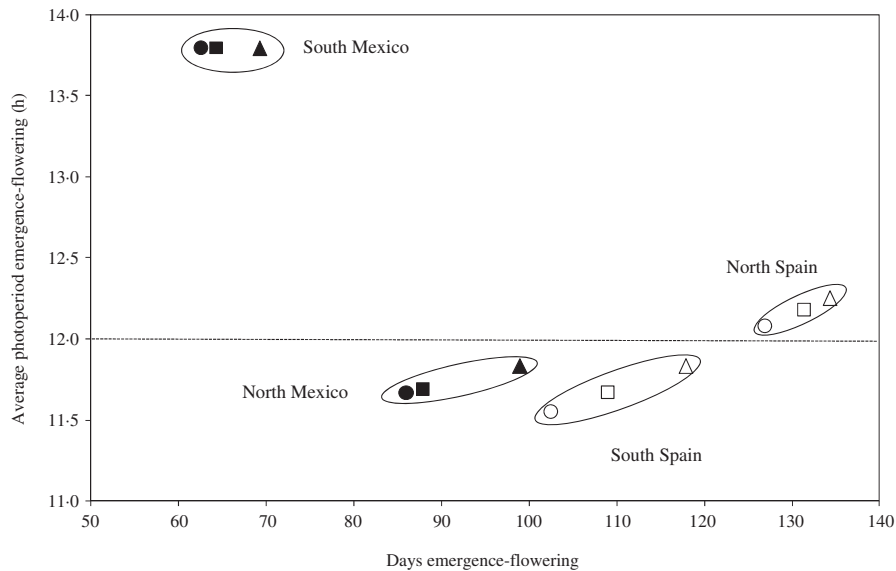


Fig. 3. Relationship between the number of days from emergence to flowering and average daily photoperiod in the same period. Each point represents the mean value of a set of durum wheat genotypes carrying the same allele at the *Ppd-A1* locus grown in field experiments in 2007 and 2008 at four sites. Triangle = *Ppd-A1b* conferring photoperiod sensitivity; square = *Ppd-A1a* GS-105 and circle = *Ppd-A1a* GS-100 conferring photoperiod insensitivity. Open and solid symbols correspond to Spanish and Mexican sites, respectively.

shortening the time to flowering than extending the flowering-maturity period, their effect amounted to a net reduction of total cycle length (Fig. 2a). However, the intensity of their effect depended on the site \times year interaction (Table 5).

Main effect of allelic variation at *Ppd-B1*

The comparison of the two allelic variants at *Ppd-B1* revealed that, on average, the allele *Ppd-B1a* (conferring photoperiod insensitivity) reduced the duration of the emergence-flowering period by 2 days compared with the allele *Ppd-B1b* (Fig. 2b). This effect was consistent in all sites and years except in Mexico-South 2008 (Table 5).

Photoperiod insensitivity conferred by *Ppd-B1a* caused a slight lengthening of the mean flowering-maturity period across sites (Fig. 2b), but the effect was much more consistent in Spain than in Mexico (Table 5). Compared with the wild type, the presence of allele *Ppd-B1a* reduced total cycle length by 1 day on average consistently at all sites and years, except in Mexico-North 2008.

Allelic combinations at *Ppd-A1* and *Ppd-B1*

The comparison of the mean values of cycle length across sites showed that the genotypes carrying the allelic combination *Ppd-A1b/Ppd-B1a* (SI) had the

longest emergence-flowering duration and the shortest flowering-maturity (Fig. 2c). However, when site \times year interactions were considered, differences between combination SI and the wild type (SS) were not always statistically significant (Table 6).

Combinations involving the non-wild type at *Ppd-A1* (I5S, I5I, I0I) resulted in a significant reduction of the emergence-flowering period, a lengthening of the flowering-maturity period and a net shortening of the total cycle length, to extents that were dependent on the allelic composition at *Ppd-B1* (Fig. 2c). However, in Mexico-South differences between combinations were low (Table 6). The effect of allele GS-105 depended on the allele present at *Ppd-B1*, as the flowering of genotypes carrying the sensitive allele at *Ppd-B1* (I5S) were delayed 4 days, on average, compared with those carrying the insensitive allele (I5I) (Fig. 2c). This effect was consistent at all sites and years except Mexico-South in 2008 (Table 6). On the other hand, genotypes carrying the combination I5I slightly lengthened their grain filling period on average by 1 day when compared with those carrying combination I5S. Net total cycle length was decreased on average by 3 days in genotypes carrying the combination I5I in comparison with those carrying combination I5S (Fig. 2c).

The shortest time to flowering corresponded to the allelic combination I0I (Fig. 2c), but differences with combination I5I were not statistically significant in

Table 6. Interaction effects of the allelic combination at *Ppd-A1* and *Ppd-B1* loci on the number of days for developmental periods of 34 durum wheat genotypes grown at four sites of varying latitude during 2 years

Allelic combination	Acronym	D.F.	Spain-North		Spain-South		Mexico-North		Mexico-South	
			2007	2008	2007	2008	2007	2008	2007	2008
Days emergence–flowering										
<i>Ppd-A1b/Ppd-B1b</i>	SS	6	148	121	117	118	100	96	67	70
<i>Ppd-A1b/Ppd-B1a</i>	SI	8	148 (0)	120 (–1)	118 (+1)	119 (+1)	102 (+2)	97 (+1)	69 (+2)	71 (+1)
<i>GS-105/Ppd-B1b</i>	I5S	5	147 (–1)	119 (–2)	111 (–6)	111 (–7)	92 (–8)	89 (–8)	64 (–3)	66 (–4)
<i>GS-105/Ppd-B1a</i>	I5I	5	142 (–6)	117 (–4)	109 (–8)	105 (–13)	86 (–14)	85 (–11)	62 (–5)	66 (–4)
<i>GS-100/Ppd-B1a</i>	I0I	5	139 (–9)	114 (–7)	106 (–11)	99 (–19)	87 (–13)	84 (–12)	60 (–7)	65 (–5)
S.E.D.			0.39	0.33	0.27	0.30	0.63	0.65	0.37	0.27
Days flowering–maturity										
<i>Ppd-A1b/Ppd-B1b</i>	SS	6	33	46	40	50	32	43	51	43
<i>Ppd-A1b/Ppd-B1a</i>	SI	8	34 (+1)	45 (–1)	40 (0)	47 (–3)	32 (0)	43 (0)	49 (–2)	41 (–2)
<i>GS-105/Ppd-B1b</i>	I5S	5	34 (+1)	47 (+1)	43 (+3)	52 (+2)	35 (+3)	49 (+6)	50 (–1)	42 (–1)
<i>GS-105/Ppd-B1a</i>	I5I	5	36 (+3)	47 (+1)	44 (+4)	56 (+6)	35 (+3)	53 (+10)	48 (–3)	42 (–1)
<i>GS-100/Ppd-B1a</i>	I0I	5	39 (+6)	50 (+4)	47 (+7)	61 (+11)	33 (+1)	51 (+7)	49 (–2)	41 (–2)
S.E.D.			0.49	0.45	0.58	0.39	0.78	0.88	0.82	0.64
Days emergence–maturity										
<i>Ppd-A1b/Ppd-B1b</i>	SS	6	181	167	157	168	132	139	118	112
<i>Ppd-A1b/Ppd-B1a</i>	SI	8	182 (+1)	165 (–2)	158 (+1)	166 (–2)	134 (+2)	140 (+1)	118 (0)	112 (0)
<i>GS-105/Ppd-B1b</i>	I5S	5	181 (0)	166 (–1)	154 (–3)	163 (–5)	127 (–5)	138 (–1)	114 (–4)	108 (–4)
<i>GS-105/Ppd-B1a</i>	I5I	5	178 (–3)	164 (–3)	153 (–4)	161 (–7)	121 (–11)	138 (–1)	110 (–8)	108 (–4)
<i>GS-100/Ppd-B1a</i>	I0I	5	178 (–3)	164 (–3)	153 (–4)	160 (–8)	120 (–12)	135 (–4)	109 (–9)	106 (–6)
S.E.D.			0.43	0.46	0.53	0.29	0.65	0.73	0.80	0.63

For each site, year and period, numbers in parentheses are the differences from the combination of sensitive alleles at both loci (SS).

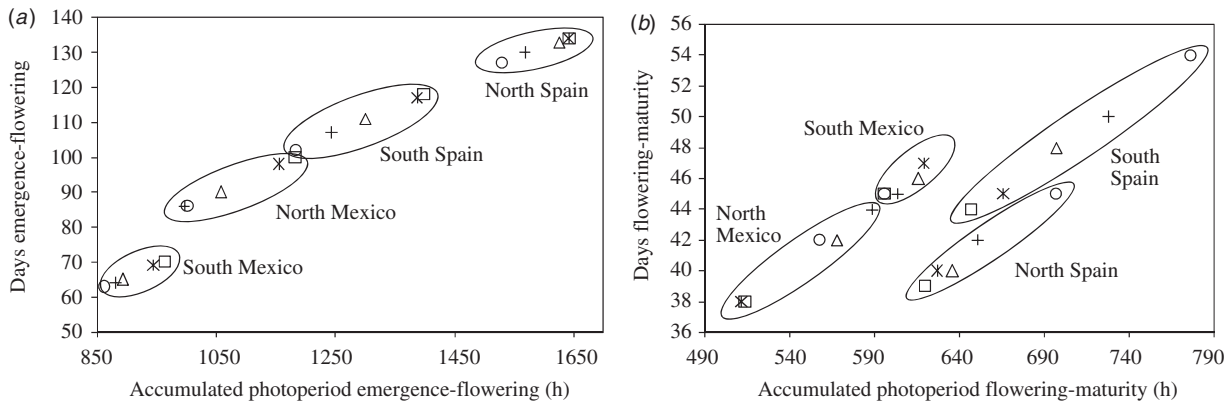


Fig. 4. Relationships between accumulated photoperiod and (a) number of days from emergence to flowering, (b) number of days from flowering to maturity. Each value represents the mean of 34 durum wheat genotypes carrying the same allelic combination for the *Ppd-A1* and *Ppd-B1* genes grown in field experiments in 2007 and 2008 at four sites with contrasting latitude. * = SS, □ = SI, Δ = I5S, + = I5I, ○ = I0I, see Table 1 for allelic combination acronyms.

Mexico-North (Table 6). In addition, combination I0I resulted in a slightly longer grain filling period than combination I5I, and a slightly shorter total cycle length (Fig. 2c).

The relationship between ACP and the number of days before and after flowering is shown in Fig. 4. For the duration of the emergence-flowering period, the interaction between photoperiod and allelic combination was quantitative in nature, with all the site clusters showing the allelic combinations SS and SI with the highest ACP, and allelic combination I0I with the lowest ACP. The allelic combinations I5S and I5I had intermediate ACP values within clusters in most cases. As shown in Table 6, the spring sowing-time in Mexico-South resulted in reduced differences in time to flowering between the allelic combinations. A linear model ($y = 0.0904x - 10.374$; $R^2 = 0.97$; $P < 0.001$) was properly fitted to the relationship between ACP and the number of days from emergence to flowering ($n = 20$, points shown on Fig. 4a). Figure 4b shows a substantial interaction between the flowering-maturity period and allelic combination groups at different sites. A negative relationship was found between the duration of the emergence-flowering and flowering-maturity periods ($r = -0.77$, $P < 0.001$).

Earliness *per se*

In the ANOVA (Table 3), the sums of squares for variation within allelic combinations were highly significant ($P < 0.001$), indicating an important source of variation in the duration of all developmental phases independently of the allelic composition at the *Ppd-*

1 loci, and *Vrn* loci since the latter were fixed. This suggests the presence of *Eps* effects. Although significant in a few cases, Mexico-South had the smallest effects of allelic combinations at the *Ppd-1* loci because the day length theoretically exceeded the photoperiod requirements for any spring wheat. At this site the average day length from emergence to flowering across genotypes was 13.8 ± 0.01 h, and for the day of flowering it was 13.7 ± 0.10 h. Under these conditions, a wide range of flowering times was observed within each allelic combination. Differences in flowering date between the earliest and the latest genotype were 17, 14, 8, 14 and 18 days for allelic combinations SS, SI, I5S, I5I and I0I, respectively (Fig. 5).

DISCUSSION

The current study focused on the effects of allelic variants for the photoperiod response genes *Ppd-A1* and *Ppd-B1* on flowering time and cycle duration in a set of 34 genotypes. All genotypes revealed the same spring growth habit at *Vrn-1* based on published molecular markers. Differentiating interactions between *Ppd-1* and *Vrn-1* alleles, frequently mentioned in the literature (Casao *et al.* 2011; Turner *et al.* 2013), should not be expected in the present set of genotypes. Furthermore, the high synchrony in time to full emergence between the genotypes used provides confidence that the differences in phenology observed in the current study were not affected by variations in germination or crop establishment capacities.

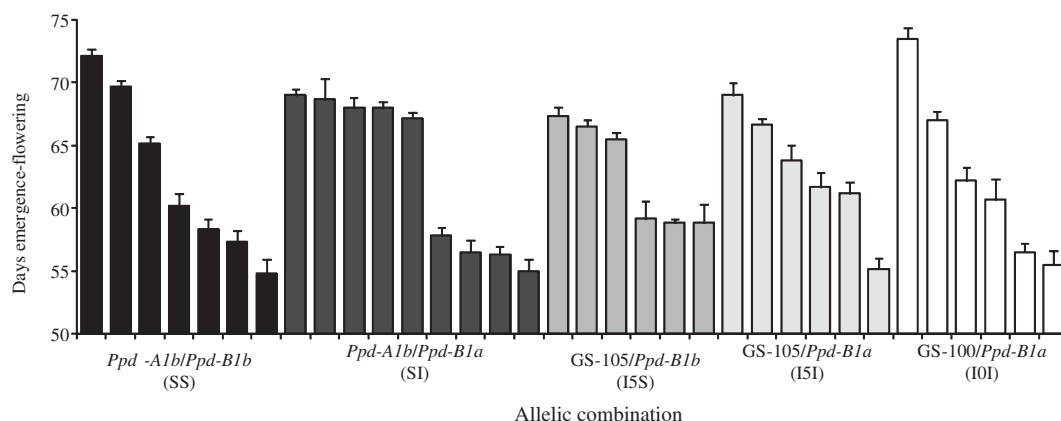


Fig. 5. Estimation of earliness *per se* (i.e. minimum duration of the emergence-flowering date) measured in days, for durum wheat genotypes carrying different allelic combinations at *Ppd-A1* and *Ppd-B1* genes. Data are means of experiments conducted in the South of Mexico during 2 years. Vertical bars indicate the s.e. of mean.

The frequency of the insensitive allele GS-105 in the population was higher than that of allele GS-100 (34 and 20%, respectively), in agreement with the preponderance of allele GS-105 in advanced germplasm from Europe and CIMMYT reported by Bentley *et al.* (2011). It has been speculated that the mutation resulting in allele GS-105 preceded the one resulting in allele GS-100, with GS-105 being detected in old landraces, while GS-100 has been found only in a few modern varieties to date (Bentley *et al.* 2011). Mutations within the gene sequence of *Ppd-B1* have been reported (Beales *et al.* 2007; Shaw *et al.* 2012), but none corresponded to photoperiodic response, implying that the critical mutation causing the allelic difference of the *Ppd-B1* gene has not been found. The role of copy number variation of *Ppd-B1* has been described recently in bread wheat (Diaz *et al.* 2012) and Nishida *et al.* (2013) reported a novel mutation in the 5' upstream region of *Ppd-B1*.

The mutations described by Nishida *et al.* (2013) and allelic variants found in the bread wheat varieties were not found in the current study using durum wheat elite germplasm. However, the overall number of parents in the current study was low. A more extensive allele mining study in durum wheat of *Ppd-A1* and *Ppd-B1* is therefore warranted. The marker *gwm148* linked to *Ppd-B1* in several studies including durum wheat (Hanocq *et al.* 2004; Mohler *et al.* 2004; Maccaferri *et al.* 2008) showed polymorphism in the current study. Cane *et al.* (2013) reported the relationship of *gwm148* with the one-copy and two-copy alleles in bread wheat. However, quantitative techniques to identify copy number variants of the *Ppd-B1* alleles will be investigated further.

The previously published allelic combinations in durum wheat at *Ppd-A1* and *Ppd-B1* were present in the current collection. The relatively balanced representation of alleles conferring photoperiod sensitivity (46% at *Ppd-A1* and 40% at *Ppd-B1*) and their variants conferring photoperiod insensitivity confirm the suitability of the present germplasm collection for addressing the objectives of the study.

Photoperiod, or period of daylight every 24 h, is dependent on latitude and season (Lee 1970). Previous papers have shown that latitude integrates a number of variables affecting wheat development, among which the most important are photoperiod (Laurie *et al.* 1995), temperature (Craufurd & Wheeler 2009) and their interaction (Hemming *et al.* 2012). With the aim of testing the current germplasm under field conditions and a wide range of photoperiods, experiments were conducted at four sites with latitudes ranging from 19° to 41°N, and average day length from sowing to flowering between 11.8 and 14.2 h, and from flowering to maturity between 13.3 and 15.8 h Villegas *et al.* (2015). Although each site corresponded to a different latitude, the site effect in the current study involved not only differences in day length, but also other environmental (e.g. temperature, water input and soil type) and agronomic (e.g. dose and type of fertilizers and sowing dates) variations, which should provide more robustness to the average differences in phenology observed between *Ppd-1* allelic combinations.

The results of the ANOVA showed that the site effect was the most important factor explaining variability in time to flowering and total cycle length. The year effect, which included seasonal differences

in environmental variables and small variations in agronomic conditions, and the site \times year interaction had a much smaller effect than the site itself. This result suggests that differences between sites in photoperiod and temperature (associated with the latitude) were probably the most important variables in explaining phenological differences between sites. This statement is supported by the results of a recent study involving the same experiments, which showed that photoperiod and temperature jointly explained c. 77% of environmental variation between sites, and that day length from sowing to flowering steadily increased from north to south, while day length from flowering to maturity followed the opposite trend Villegas *et al.* (2015). The spring planting in Mexico-South had important implications on the results obtained, as discussed below.

The ANOVA results also showed that grain filling duration depended largely on the year and the site \times year interaction. This observation is in accordance with the reported relatively low heritability of grain filling duration, and the large environmental influence on this trait (Egli 2004; Royo *et al.* 2006).

The strong linear relationship between ACP and number of days from emergence to flowering shown in Fig. 4a demonstrated a consistent trend of flowering time regulation associated with the different allelic combinations. Within the range of latitudes and with the genotypes used in the current study, time from emergence to flowering ranged from 63 to 134 days and accumulated photoperiod until flowering from 863 to 1642 h.

The fraction of the genotype effect explained by differences between allelic combinations was c. 66%. As all genotypes studied had a spring growth habit and no significant interaction with vernalization requirement was expected, this result provided a quantitative estimate of the relative effect of allelic variation at the *Ppd-1* loci in explaining genotypic variance for time to flowering. However, it also suggested that other genetic factors determined c. 34% of the genotypic variance and also interacted significantly with the site. This non-*Ppd-1* related variation is supported by the fact that there were statistically significant differences in all traits within all allelic combinations, suggesting the involvement of *Eps*, in accordance with previous studies reporting the influence of minor genes on bread wheat flowering time (Griffiths *et al.* 2009; García *et al.* 2011).

Genotype variability for the emergence-flowering period was greater in the presence of the *Ppd-A1b*

allele. It may be hypothesized that, within genotypes carrying the sensitive allele, other genes implied in regulation of flowering time have a higher phenotypic expression than when they are present in combination with the insensitive allele. Shaw *et al.* (2012) reported that bread wheat genotypes with three mutations had a phenology similar to that of genotypes with the two mutations with the strongest effect. In the current study, it was hypothesized that the *Ppd-A1* gene was powerful enough to cause a rate of earliness close to the maximum in durum wheat. This could be an evolutionary effect based on polyploidization that could explain the small differences observed between genotypes with *Ppd-A1a*.

In the current study, the longest time to flowering was recorded consistently in allelic combinations carrying the wild-type allele *Ppd-A1b*, which resulted in an average delay of flowering of 3 days compared with allelic combinations involving the wild-type allele at the other locus, *Ppd-B1b*. These results suggest a stronger effect of the alleles conferring photoperiod sensitivity at *Ppd-A1* than that at *Ppd-B1* (*Ppd-A1b* > *Ppd-B1b*). Similarly, alleles causing insensitivity at *Ppd-A1* (GS-105 and GS-100) had a stronger effect in reducing flowering time than the allele at *Ppd-B1* (*Ppd-A1a* > *Ppd-B1a*). The current results are in agreement with those recently reported in hexaploid wheat by Shaw *et al.* (2012), who classified the photoperiod insensitivity alleles according to their effect on flowering date as *Ppd-D1a* > *Ppd-A1a* > *Ppd-B1a*, when *Ppd-A1a* is the GS-100 durum wheat variant. Other studies in bread wheat ranked the relative strength of these genes differently. Scarth & Law (1984) speculated that *Ppd-B1a* may have a stronger effect than *Ppd-A1a*, and this in turn would be stronger than *Ppd-D1*. Worland *et al.* (1998) suggested that *Ppd-D1* confers greater precocity, followed by *Ppd-B1*, with the *Ppd-A1* gene having a smaller effect. A greater effect of *Ppd-B1a* than *Ppd-D1a* of hexaploid wheat was reported by Tanio & Kato (2007), who suggested that there may be different alleles conferring insensitivity in the B genome, with different effects on earliness. A recent study suggests that copy number in addition of diverse mutations have different effects on the date of anthesis (Diaz *et al.* 2012).

The current results suggest that the effect of allele *Ppd-B1a* depended on the allele present at *Ppd-A1*. In presence of allele *Ppd-A1b* the effect of the *Ppd-B1a* gene was minor. However, in the presence of *Ppd-A1a*, *Ppd-B1a* reduced flowering time by 4 days

in comparison with the wild-type allele at the same locus. This tendency was observed in all the experiments except that carried out at Mexico-South in 2008, where differences were not statistically significant. These results point out the interaction between genes *Ppd-A1* and *Ppd-B1*. Tanio & Kato (2007) described an incomplete dominance and interaction between genes *Ppd-B1* and *Ppd-D1* similar to that observed in the current study between genes *Ppd-A1* and *Ppd-B1*.

Among the two allelic combinations causing photoperiod insensitivity at *Ppd-A1*, GS-105/*Ppd-B1a* and GS-100/*Ppd-B1a*, the latter resulted in average genotypes flowering 2 days earlier on average. Moreover, genotypes carrying allele GS-100 independent of the allele at *Ppd-B1* flowered, on average, 4 days before those carrying allele GS-105. However, the latter group included different alleles at *Ppd-B1* and the former only those carrying allele *Ppd-B1a*. The tendency was consistent in all experiments, suggesting that allele GS-100 had a stronger effect than GS-105. These results are in agreement with those reported by Bentley *et al.* (2011), who found that allele GS-100 conferred earlier flowering than GS-105, and are also consistent with previous observations in durum wheat (Clarke *et al.* 1998; Maccaferri *et al.* 2008; Wilhelm *et al.* 2009).

Allelic variants at *Ppd-1* genes causing photoperiod insensitivity had an opposite, but more modest effect on grain filling period when compared with their effect on flowering time. Consequently, a consistent negative relationship was found between time to flowering and grain filling duration. This relationship could be attributed to the environmental conditions prevailing at the end of the growth cycle (acceleration of senescence due to heat), but also to the presence of genetic factors inducing earlier flowering in combination with elongating grain filling duration, such as the QTL regulating *Eps* (*QFlt.dms-5B.1*) recently identified by Kamran *et al.* (2013). Bogard *et al.* (2011) also found co-location of QTLs responsible for grain filling duration and anthesis date in bread wheat. As a result, total cycle length was reduced slightly, following the same general trends observed for time to flowering. Early-flowering genotypes have their spike growth phase under lower temperatures, resulting in a higher grain number per unit of land (Ratjen *et al.* 2012). This represents a stronger sink force, which has been associated with accelerated senescence (Gan 2007; Fois *et al.* 2009), as could be the case of the earliest genotypes in the current study.

The current results showed that photoperiod affected the expression of alleles at *Ppd-A1*. At the Spain-North site, where mean photoperiod from emergence to flowering was 12.2 h, differences in flowering time between genotypes carrying different allelic combinations were statistically significant but small. In Spain-South and Mexico-North, where photoperiod from emergence to flowering averaged 11.7 h, these differences increased. In Mexico-South, with decreasing photoperiod from emergence to flowering and a 13.79 h average day-length, total cycle length was greatly shortened, and average differences between allelic combinations groups were reduced. Based on the current results, it appears that the phenotypic expression of *Ppd* increases at sites with average pre-flowering day length lower than 12 h, which is in agreement with the results of Kumar *et al.* (2012), who reported a critical photoperiod of 12 h for bread wheat phenotypes to manifest the genetic contribution of *Ppd-D1*. Wilhelm *et al.* (2009) also reported that the expression of *Ppd-A1a* increases under short days.

Earliness *per se* genes have been reported to be strong enough to induce earlier flowering, even in the presence of *Vrn* and *Ppd* genes (Van Beem *et al.* 2005). It can be assessed only in conditions which minimize the effects of *Vrn/Ppd* related variations. The Mexico-South site, with its spring planting and 14 h day length during the whole pre-flowering period, provided such an environment and the opportunity to assess *Eps* within the current collection. Since *Ppd* effects were still significant at this site, they were properly deducted for *Eps* calculations, according to Ortiz Ferrara *et al.* (1998). The significance of *Ppd* effects in Mexico-South is not surprising given that plants carrying the allele GS-100 have been recently reported to be still photoperiod-sensitive (Chen *et al.* 2014).

Effectively, a wide range of *Eps* effect was recorded, given that differences between the latest and the earliest genotypes reached at that site 19 days from emergence to flowering. This was the same effect than the one between allelic combinations recorded in Spain-South in 2008, where *Ppd* effects were maximized. These results suggest an important role of *Eps* in the germplasm used in the current study. In addition, no relationship was found between allelic combination and the effect of *Eps*, as all the allelic combination groups showed a wide range of time to flowering. These results are consistent with those reported by Gomez *et al.* (2014) in Argentinean bread wheat

cultivars, and those for heading time reported by Gawronski & Schnurbusch (2012) describing the effect of the *Eps-3A^m* under glasshouse conditions. Miura & Worland (1994) found that the effect of *Eps* in reducing heading date was independent of environmental stimuli, but Lewis *et al.* (2008) reported significant interactions between *Eps-A^m1* alleles and temperature, suggesting that the effect of some *Eps* genes may vary depending on the environment. It has been reported recently that natural variation for the Phytochrome C gene (a light-responsive activator of *Ppd-1* genes) may also be associated with wheat adaptation to different latitudes (Chen *et al.* 2014). The findings of the current study open further approaches for the identification and study of new genes regulating *Eps* in durum wheat.

CONCLUSION

The results obtained in the current study lead to the conclusion that in durum wheat: (i) *Ppd-A1a*, conferring photoperiod insensitivity, had a stronger effect on flowering time than *Ppd-B1a*; (ii) allele GS-100 had a stronger effect than GS-105, so according to their effect on flowering date the effect of photoperiod insensitivity alleles could be classified as *Ppd-A1a* (GS-100) > *Ppd-A1a* (GS-105) > *Ppd-B1a*; (iii) interaction existed between genes *Ppd-A1* and *Ppd-B1*; (iv) the phenotypic expression of photoperiod genes increased at sites with mean day length from emergence to flowering lower than 12 h; (v) the effect of *Ppd-A1* alleles on flowering date was greater than the effect on grain filling period but this differed among alleles; and (vi) estimation of *Eps* effects revealed great variation in time to flowering independent of allele variants at *Ppd-1*.

The current study is the first one showing the phenotypic effect of *Ppd-1* genes in durum wheat under field conditions at a range of latitudes, thus being a significant contribution to the elucidation of the phenotypic expression of genetic factors controlling flowering time in durum wheat.

This study was partially funded by INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria) Spain under projects RTA2009-00085 and RTA2012-00011, and project AGL2012-37217. C Alfaro was recipient of a PhD grant from INIA (Instituto de Investigaciones Agropecuarias) Chile. We thank the contribution of Dr Luis F. García del Moral (University of Granada), Dr María del Mar

Cátedra (Consejería de Agricultura, Pesca y Medio Ambiente, Spain) and Mr. Chafik Harrathi. Thanks are also given to Dr Kling from Hohenheim University and to Dr Wolfgang Pfeiffer (formerly durum breeder at CIMMYT, now at CIAT-Columbia) for providing parental germplasm and resulting segregating populations for the current study.

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