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Effect of *Vrn-1*, *Ppd-1* genes and earliness *per se* on heading time in Argentinean bread wheat cultivars

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ABSTRACT

Predicting phenology, in particular heading time, is crucial to avoid and/or minimize risk of damage of frost, drought and high temperatures during grain filling. Although some of the major genes controlling development, associated with photoperiod and vernalization responses, were identified, the association between the molecular characterization of those genes and photoperiod sensitivity, vernalization responses and earliness *per se* has been poorly studied. The present study was conducted to determine the effects of photoperiod and vernalization genes (and their allelic combination characterized by molecular approach) on heading time and its correlation with the phenological parameters determined by field experiments in a wide range of Argentine bread wheat commercial cultivars. Additionally, the association between photoperiod and vernalization responses with earliness *per se* was analyzed. Molecular characterization showed that most of the commercial Argentine wheat cultivars available in the market correspond to spring growth habit with dominant insensitive photoperiod alleles (SI) followed by spring habit sensitive to photoperiod (SS), while winter insensitive (WI) habit represented a minority group. All genotypes included in the present study (even those classified as SI and WI) were photoperiod sensitive when that trait was quantified from a physiological analysis as the slope of the relationship between duration of a particular phase and mean photoperiod sensed during the period between emergence and heading. SI showed lower photoperiod sensitivity than SS and WI, without clear differences between both later groups. In all cases, photoperiod sensitivity was the main attribute that determined the differences in time to heading even when vernalization requirements were not completely fulfilled in the WI. The genotypes with different photoperiod and vernalization allele combinations showed a wide range of duration of earliness *per se*. However, differences in earliness *per se* did not show any particular association with the groups classified by molecular markers for photoperiod and vernalization. The information included in the present study can be used to build a gene-based model for predicting phenology. However, the variations in photoperiod and/or vernalization sensitivity within the same allelic combination could still determine mismatching in the prediction of the models based on Ppd-1 and Vrn-1 genes.

1. Introduction

The importance of wheat, which currently provides 21% of the food calories and 20% of the protein to more than 4.5 billion people in 94 developing countries, is based on the wide range of environments (climates and soils) in which can be cultivated. However, in the future the challenge for wheat breeders is to increase the rate progress in genetic yield potential (and also reduce the gap between actual and potential yields) to match future demand for wheat due to the growing of global population. In this scenario, the understanding of the genetic basis of traits governing adaptability will be crucial for developing wheat varieties specifically adapted to different environments, and thereby ensuring maximum crop production (Ortiz-Ferrara et al., 1995; Foulkes et al., 2011).

The wide adaptability of wheat is largely governed by three genetic systems – vernalization response, photoperiod sensitivity, and earliness *per se* (Herndl et al., 2008) – that act together to determine flowering time and hence the basic adaptation of a genotype for a particular environmental condition (Worland and Sayers, 1996; Worland et al., 2001). Flowering time is crucial for wheat adaptation to avoid frost and heat damage and drought during the critical period and also during grain filling.

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Vernalization is defined as the acquisition or acceleration of the ability to flower (and initiate heading) by a chilling treatment (Chouard, 1960; Fu et al., 2005). This feature determines the growth habit: winter wheats, with high vernalization requirement; and spring wheats, with low or absence of vernalization requirement. In wheat, has been demonstrated that that vernalization requirement is controlled by at least four genes designated *Vrn-1*, *Vrn-2*, *Vrn-3* and *Vrn-4* (Yan et al., 2003, 2004b, 2006; Yoshida et al., 2010; Distelfeld et al., 2009) being *Vrn-1* the major determinant of the vernalization requirement in wheat and barley (Pugsley, 1971; Trevaskis, 2010). The *Vrn-1* gene encodes a MADS-box transcription factor related to the Arabidopsis meristem identify genes AP1/FRUITFULL (Yan et al., 2003), which is essential for the transition from the vegetative to reproductive stage in wheat (Shitsukawa et al., 2007). Presence of insertions or deletions in regulatory regions (promoter, first intron) of the three homoeologous genes found in hexaploid wheat (*Vrn-A1*, *Vrn-B1*, *Vrn-D1*), are associated with dominant alleles for spring growth habit (Yan et al., 2004a; Fu et al., 2005; Shcherban et al., 2012). Different combinations of *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* spring (dominant) alleles are the most common sources of spring growth habit among landraces and commercial cultivars of polyploid wheat around the world (Fu et al., 2005; Iqbal et al., 2007, 2010; Nowak and Kowalczyk, 2010; Yan et al., 2004a; Zhang et al., 2008).

In relation to photoperiod, wheat can be described as a quantitative long day photoperiod response and photoperiod sensitivity can be defined as the slope of the relationship between duration of a particular phase and mean photoperiod sensed during that particular phase. Photoperiod insensitive wheats flower independently of day length and can be grown to maturity in long or short day environments once vernalization requirement has been satisfied. Natural variation in the response to photoperiod is mainly determined by allelic differences in the *Ppd-1* gene, a member of the pseudoresponse regulator (PRR) gene family (Turner et al., 2005). Beales et al. (2007) showed that the widely used photoperiod-insensitive *Ppd-D1a* allele (photoperiod-insensitive alleles are given an "a" suffix) has a 2-kb deletion upstream of the coding region which is associated with the mis-expression of the gene and induction of the floral activator FT in short days. More recently, causal mutations in the *Ppd-A1a* and *Ppd-B1a* alleles have been described (Wilhelm et al., 2009; Diaz et al., 2012).

The duration of the phenological phases is also affected by earliness *per se* which is defined as the variation in flowering observed once photoperiod and vernalization requirements were fully satisfied. The variability in earliness *per se* is important to fine-tune flowering time and to be exploited to maximize yield potential in different environments (Lewis et al., 2008). Previous mapping and segregation analyses have shown that vernalization requirement and photoperiod sensitivity are mainly regulated by major genes (Law and Worland, 1997), whereas earliness *per se* is more polygenic. For example, 19 meta-QTLs for earliness *per se* were detected in different chromosomal regions of the wheat genome considering European winter germplasm (Griffiths et al., 2009). Unfortunately, and in contrast with vernalization and photoperiod response pathways, no gene/s associated with earliness *per se* have been cloned so far, and only *Eps-1*, located on the distal region of *Triticum monococcum* chromosome $1A^mL$ (Lewis et al., 2008; Faricelli et al., 2010), and *Eps-3A*^{m}, in the long arm of *T. monococcum* chromosome 3A (Gawroński and Schnurbusch, 2012) have been fine mapped and phenotypically associated not only with early flowering but also with spike development.

The different alleles of adaptation genes here described generate a wide spectrum of vernalization requirements, photoperiod response and, to a lesser degree, earliness *per se*, to adapt varieties to a wide range of growing regions and sowing times. The understanding of the differential sensitivity to the attributes that regulates the length of the phase (photoperiod, vernalization and earliness *per se*) is crucial to expose the critical period during which yield is determined to the most favorable environmental conditions (Miralles and Slafer, 1999; Miralles et al., 2007). Phenology prediction (and simulation) for different genotypes in response to different attributes that regulate the length of different phases (vernalization, photoperiod and earliness *per se*) are characterized by field trials using different sowing dates, locations and years (Miralles et al., 2007). However, that approach is time consuming and expensive as they require an important manpower and a permanent characterization throughout the time to include the new genotypes that appear in the commercial market. Other possibility is to predict phenology by a gene-based model from the allelic combination of vernalization and photoperiod genes (Zheng et al., 2013) and determine how genes combination interact with other less studied genes as "earliness *per se*". Thus, the present study was conducted to determine the effect of photoperiod, vernalization and earliness *per se* on heading time based on a gene composition in a wide range of Argentine bread wheat commercial cultivars.

2. Materials and methods

2.1. Plant material

A set of 32 bread wheat cultivars released in Argentina between 1994 and 2007 was used in this study. Seed stocks were provided by the INTA Marcos Juarez Wheat Germplasm Bank (Marcos Juarez, Argentina).

2.2. Experiments and measurements

Two experiments (experiments 1 and 2) were conducted to investigate the influence of the *Vrn-1* and *Ppd-1* genes on heading time (and its association with earliness *per se*).

Experiment 1 was designed to expose the cultivars to a wide range of environmental conditions during three sowing seasons. Seven sowing dates (between April 25th and July 25th sowing every 15 days) were carried out to produce changes in heading time in the 32 selected commercial wheat cultivars. Seeding rates were standardized in all sowing based on seed size to 300 seeds m^{-2}. Cultivars were grown in the experimental field of the INTA Marcos Juarez Experimental Station (32°42′ S, 62°07′ W, 114 m.a.s.l.) during 2008, 2009 and 2010 growing seasons. The soil is a typical Argiudol, class I, from the Marcos Juarez series, which is dark, deep and well drained and with an almost nil slope, with a loamy loam texture. Trials were carried out under irrigation and adequate nutrients applied. To avoid water stress during the cycle, supplementary irrigation was applied with the aim of achieving around 600 mm of water available to the crop, including rainwater and water available in the soil at planting. Supplementary irrigation was distributed during the whole crop cycle. Fertilization was adequate to obtain ca. 35 ppm of phosphorus (i.e. 91 kg P per ha) and 200 kg of nitrogen per hectare. Thus, a mix of monoammonium phosphate (N: 12%, P: 22.5%, K: 0%) and urea (N: 46%, P: 0%, K: 0%) was incorporated into the soil 1 week before the first sowing date. Plant pathogens and pests were prevented by chemical treatments, and weeds were removed by hand to avoid any negative effect of hormonal herbicides on crop development. The trials were conducted as randomized complete block designs with two replications (plot size was 5.0 m long by 7 rows wide). Heading time was measured in calendar and degree days from emergence until 50% of the spike was completely emerged from the flag leaf in 50% of the plants (Zadoks et al., 1974). Duration of the phase between emergence and heading (measured in calendar days and thermal units, using base temperature of 0 °C) was plotted against mean photoperiod of

the phase and a linear regression was fitted to determine the intercept (theoretical maximum duration of the phase) and the slope of the regression that represent the photoperiod sensitivity measured in days h^{-1} and $^{\circ}Cd\,h^{-1}$.

Experiment 2: In this experiment the same selected 32 wheat cultivars of the experiment 1 were grown considering vernalization requirements and two different photoperiod regimes. Seeds were germinated in pots filled with an inert substrate (i.e. vermiculite) and, after 24 h, they were transferred to a cold chamber (4–6 °C, 8 h day length) for 6 weeks. Vernalized seedlings were transplanted to 5 L-pots – 18 cm high and 23 cm internal diameter – (three seedlings per pot, two pots per cultivar) which were placed in a field (sowing date July 10th) under (i) natural photoperiod (12.5 h mean photoperiod) and (ii) long-day conditions (natural photoperiod extended up to 20 h photoperiod with low intensity – 25 W – incandescent lamps to fulfill photoperiod saturation requirements. Pots were organized in a randomized complete block design (RCB) with two replicates and heading time was measured again in calendar and thermal units (°Cd) from emergence until 50% of the spike was completely emerged from the flag leaf in 50% of the plants (Zadoks et al., 1974). The earliness *per se* (EPS) was measured as the duration of the emergence–heading phase assuming that long day conditions (20 h photoperiod) and fully vernalized conditions (6 weeks) saturated the photoperiod and – probably – vernalization requirements, respectively. The genotypes vernalized and exposed to long day conditions were classified by EPS according to the duration of the emergence–heading phase. The classification of the genotypes in relation to the duration of EPS was determined arbitrarily using the least significant differences (LSD) of the ANOVA. Thus, genotypes were classified as early (E), medium (M) and late (L) duration for that phase (see Table 1).

2.3. DNA extraction and marker screening

Total DNA was extracted from fresh leaves of single plants using a fast, small-scale DNA isolation procedure based on Weining and Langridge (1991). PCR markers developed by Yan et al. (2004a,b) and Fu et al. (2005) were used to detect previously described spring and winter alleles in *Vrn-A1*, *Vrn-B1* and *Vrn-D1* genes. Presence of photoperiod insensitive and sensitive alleles in *Ppd-D1*, *Ppd-B1* and *Ppd-A1* genes was detected using PCR markers described in Beales et al. (2007), Diaz et al. (2012) and Wilhelm et al. (2009), respectively. The PCRs were performed in 25 μl aliquots using a thermal cycler PTC-100 (MJ Research). The reaction buffer contained 100 ng of template DNA, 1 × Taq polymerase buffer (Promega Corp., Madison, WI), 1.0 U Taq DNA polymerase (Promega), 0.2 mM of each deoxynucleotide, 0.2 μM of each primer (synthesized by Alpha DNA, Quebec H4C 3N9, Canada) and 1.5 mM of $MgCl_2$. Primer names, sequences, target alleles and cycling conditions varied depending on the different PCR markers used in this study according to previous references (Yan et al., 2004b; Fu et al., 2005; Beales et al., 2007; Diaz et al., 2012; Wilhelm et al., 2009). In all cases, PCR products (10 μl each) were run on 2% agarose gels, stained with ethidium bromide [0.5 g/L] and visualized with UV. Cultivars Sonora 64 (*Vrn-A1a*, *vrn-B1*, *Vrn-D1*) (*Ppd-D1a*, *Ppd-B1a*), ProINTA Granar (*Vrn-A1a*, *Vrn-B1*, *vrn-D1*), Sinvalocho (*Vrn-A1b*), Klein Rendidor (*vrn-A1*, *vrn-B1*, *vrn-D1*), Cappelle Dezprez (*Ppd-D1b*, *Ppd-B1b*) and the durum wheat Kofa (*Ppd-A1a*) were used as molecular checks

Table 1
Molecular (*Vrn-1* and *Ppd-1* alleles) and phenotypic (earliness *per se* – EPS) variation in cultivars used in this study.

Genotype	*Vrn-1* alleles[a]			VR[b]	*Ppd-1* alleles[a]			PR[c]	EPS[d]
ACA 801	*vrn-A1*	*Vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Short
ACA 901	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
BAGUETTE 10	*Vrn-A1b*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Long
BAGUETTE 11	*Vrn-A1b*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Long
BAGUETTE 13	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Long
BAGUETTE 9	*Vrn-A1b*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Long
BIOINTA 1001	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
BIOINTA 1002	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Medium
BIOINTA 1003	*Vrn-A1a*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
BUCK PUELCHE	*Vrn-A1a*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
CRONOX	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Medium
INIA CÓNDOR	*Vrn-A1a*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
KLEIN FLECHA	*Vrn-A1a*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
KLEIN TAURO	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Short
KLEIN ZORRO	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
ONIX	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Long
ACA 315	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Short
BAGUETTE 20	*Vrn-A1a*	*vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Medium
BIOINTA 3000	*Vrn-A1a*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Short
BIOINTA 3004	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Short
BUCK ARRIERO	*Vrn-A1a*	*Vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Medium
BUCK BAQUEANO	*vrn-A1*	*vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Medium
BUCK GUAPO	*vrn-A1*	*Vrn-B1*	*vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Medium
KLEIN CARPINCHO	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Medium
KLEIN GAVILAN	*Vrn-A1a*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Short
KLEIN GUERRERO	*vrn-A1*	*Vrn-B1*	*Vrn-D1*	S	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*	S	Short
BAGUETTE 21	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Long
BIOINTA 2004	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Medium
BUCK RANQUEL	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1a*	I	Medium
PROINTA PUNTAL	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Medium
SRM NOGAL	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*	I	Medium
THEMIX	*vrn-A1*	*vrn-B1*	*vrn-D1*	W	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*	I	Long

[a] Nomenclature used for spring/winter (in gray) *Vrn-1* vernalization alleles and insensitive/sensitive (in gray) *Ppd-1* photoperiod response alleles is based on Yan et al. (2004a,b), Fu et al. (2005), Beales et al. (2007) and Diaz et al. (2012).
[b] VR: vernalization requirements/growth habit, W = winter, S = spring.
[c] PR: photoperiod response, I = insensitive, S = sensitive.
[d] EPS: earliness *per se*.

based on previously known *Vrn-1* and/or *Ppd-D1* allele/s constitution (Fu et al., 2005; Beales et al., 2007; Diaz et al., 2012; Wilhelm et al., 2009).

2.4. Statistical analysis

Statistical differences in heading time considering groups based on *Vrn-1*, *Ppd-1* and EPS data were tested by ANOVA analyses using InfoStat software (Di Rienzo et al., 2010). When these analyses revealed significant differences, the mean values of each group were compared using Fisher test ($\alpha = 0.05$).

3. Results

3.1. Environmental conditions

Radiation and temperature conditions during the experimental years are shown in the supplementary figure (Fig. S1). During the 2008 growing season daily mean temperature was warmer during the second third of May, July and from October onwards (with peaks over 5 °C) than the historical mean. In 2009, the daily temperatures showed peaks of 5 °C warmer than historical temperatures during April and May and ending of August; and cooler temperatures respect to the historical records in the third of July. During the 2010 growing season, July was cooler than the historical temperatures, but the rest of the period was similar to the historical temperatures (Fig. S1).

Regarding the daily radiation, during the 2008 growing season, in general records were similar sometimes higher than the historical radiation, except in July and the second third of October where radiation was lower than the historical mean. In 2009 daily radiation was higher than the historical records, except in the first third of September where it was 2.3 MJ/m^2 lower than the historical mean. The 2010 growing season was the most variable year, as it shows higher and lower peaks than the historical mean during the whole crop cycle.

3.2. Molecular (vernalization and photoperiod) and phenotypic (earliness per se) characterization

From the PCR markers used in the present study, genotypes were characterized as (i) spring (26 genotypes) and winter (6 genotypes) in relation to allelic variants detected into the *Vrn-A1*, *Vrn-B1* and *Vrn-D1* genes and as (ii) sensitive (10 genotypes) and insensitive (16 genotypes) according to allelic combinations observed in the *Ppd-D1* and *Ppd-B1* genes combination as *Ppd-A1* only exhibited sensitive *Ppd-A1b* allele (Table 1). In relation to EPS classification, genotypes were arbitrary classified as short, medium and long earliness *per se* according to ±1 LSD deviations (3.06 days) of the average (53 days) calculated by the ANOVA (Table 1 and Fig. 3). Taking account earliness *per se*, and the criteria indicated above, 7 genotypes were classified as short, 17 as medium, and seven as long earliness *per se* duration (Table 1).

3.3. Variation in photoperiod sensitivity

Genotypes showed important variation in photoperiod sensitivity when measured in days, as well as in degree days. In fact, photoperiod sensitivity for the emergence (E)–heading (Hd) phase ranged from 30 to 50 days h^{-1} when the length of the phases was measured in calendar days and from ca. 230 to 450 °Cd h^{-1} when duration of the E–Hd phase was measured in degree days (Fig. 1). The fact that the relationship between intercept of the regression and photoperiod sensitivity (i.e. the slope of the relationship between the duration of the phase and the average photoperiod sensed during that period) fit to a common line, suggests that all

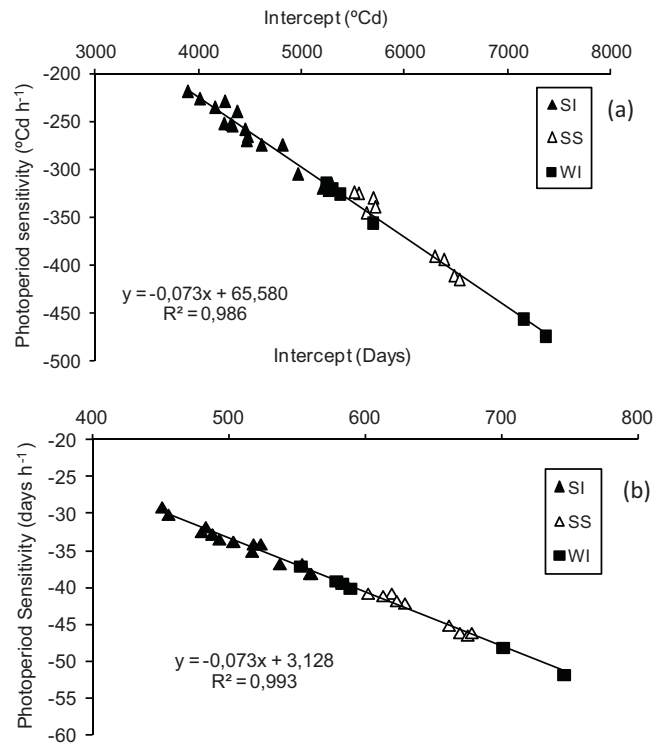

Fig. 1. Relationship between photoperiod sensitivity (measured in days – days h^{-1} and degree days – °Cd h^{-1}) between emergence and heading and the intercept of the linear function between the duration of the phase from emergence to heading (in days and degree days) and the average photoperiod of that phase. Cultivars were classified according to alleles combinations in spring sensitive (SS filled triangles), spring insensitive (SI empty triangles) and winter insensitive (WI squares).

the variation among genotypes in the length of the phase was associated with photoperiod sensitivity. Thus, the higher the photoperiod sensitivity (expressed in negative values as the phase from E to Hd become shorter as photoperiod is extended) the higher the intercept, demonstrating that photoperiod sensitivity is the driving force of the changes in the duration of the phase E–Hd (Fig. 1).

When the genotypes were discriminated in Fig. 1 by the molecular characterization of *Vrn-1* and *Ppd-1* genes (see Table 1), the spring (carrying at least one "spring"allele within *Vrn-1* homoeologs, with null or low vernalization requirements) and insensitive (carrying at least one "insensitive" allele within *Ppd-1* homoeologs, with low photoperiod response) genotypes (SI) showed the lowest photoperiod sensitivity, while those genotypes characterized as spring and sensitive (carrying triple combination of "sensitive" alleles within *Ppd-1* homoeologs) (SS) showed consistently higher photoperiod sensitivity than SI. The genotypes characterized by molecular markers as winter (carrying triple combination of "winter" alleles within *Vrn-1* homoeologs) insensitive (WI) registered a wide range in the values of photoperiod sensitivity ranging from ca. −55 to −35 days h^{-1} (Fig. 1b). However, with the exception of two genotypes (DM Themix and Baguette 21), that showed the sharpest reduction in the length of the E–Hd phase per hour of photoperiod (i.e. the highest negative values for photoperiod sensitivity), the rest of the genotypes in general recorded intermediate values of photoperiod sensitivity between SI and SS (see Fig. 1). Supporting this data, significant effects ($p < 0.0001$) of *Ppd-1*/*Vrn-1* groups (SI, SS and WI) over the phase E–Hd were observed across all sowing dates. From the E–Hd phase SI genotypes showed an average of 101 days (TT 1318 °Cd), WI showed 112 days (TT 1498 °Cd) and SS registered an average of 115 days (TT 1553 °Cd) (Table S1). Additionally a highly significant interaction ($p < 0.0001$) between *Ppd-1*/*Vrn-1* groups and sowing dates was detected (Table S1).

3.4. Interaction between vernalization requirements and photoperiod sensitivity

Fig. 2 shows the relationship between the duration of the emergence to heading phase in days (Fig. 2a and c) and thermal time (Fig. 2b and d), when genotypes were sown under field conditions (corresponding to date of emergence July 29 in Exp. 1) during 2008 (*y* axis), and the duration of the same phase when genotypes were vernalized (6 weeks) and after that exposed to extended (Fig. 2a and b) or to natural photoperiod (Exp. 2, Fig. 2c and d) coinciding in the emergence date with the field experiment sown in July 10 (emergence July 29) for the same growing season. It is important to highlight that as the emergence date was the same in both experiments, the average photoperiod for the E–Hd phase was almost the same in both experiments (i.e. 12.7 and 12.5 h in Exp. 1 and Exp. 2, respectively). When duration of E–Hd phase was compared in vernalized and extended photoperiod conditions to the non-vernalized and natural photoperiod, with the same emergence date, the range of days to heading was the same for the SI, SS and WI groups, Thus, when vernalization and photoperiod requirements were fulfilled the range of duration of "earliness *per se*" was similar in the three groups (Fig. 1a and b). On the other hand, when E–Hd phase was measured under field conditions (i.e. natural photoperiod) two groups were evident in the *y* axis. The SS group showed longer durations for the E–Hd phase than the SI and WI when that phase was measured in days (Fig. 2a) as well as in thermal time (Fig. 2b) under field conditions (natural photoperiod, without vernalization). However, when the duration of the E–Hd phase for the genotypes grown under natural photoperiod (without vernalization) was plotted against the duration of the same phase when genotypes were vernalized and grown under natural photoperiod, all points fell on the 1:1 ratio. However, the SS registered longer duration than the SI and WI when E–Hd phase was measured in days and thermal time (Fig. 2c and d). The fact that all genotypes were fell on the 1:1 line, suggests that vernalization requirements were in general well satisfied when sowing was around middle July, at least for the environmental conditions (temperature) occurred during 2008 when cultivars were sown under natural conditions.

3.5. Earliness per se

The duration of the E–Hd phase measured in the *x* axis of Fig. 2a and b indicate the earliness *per se* that represent the shortest duration of the E–Hd phase as both vernalization and photoperiod were fulfilled. The genotypes were classified, according to the criteria indicated in Section 2, in three different groups: short (7 genotypes), medium (18 genotypes) and long (7 genotypes) earliness *per se* according to the general mean of heading time (53 days) ± the least significant difference (3.06 days) (Fig. 3). Unfortunately, not all *Ppd-1*/*Vrn-1* groups included genotypes with the three categories of EPS (for example WS). Because of this, the ANOVA analysis was performed within each *Ppd-1*/*Vrn-1* group (Table S2). EPS categories showed a highly significant effect ($p < 0.0001$) on E–Hd phase in each *Ppd-1*/*Vrn-1* group. No significant interactions between EPS and sowing dates were detected in SI ($p = 0.9519$) and SS groups ($p = 0.7763$) (Table S2). However, a significant interaction between

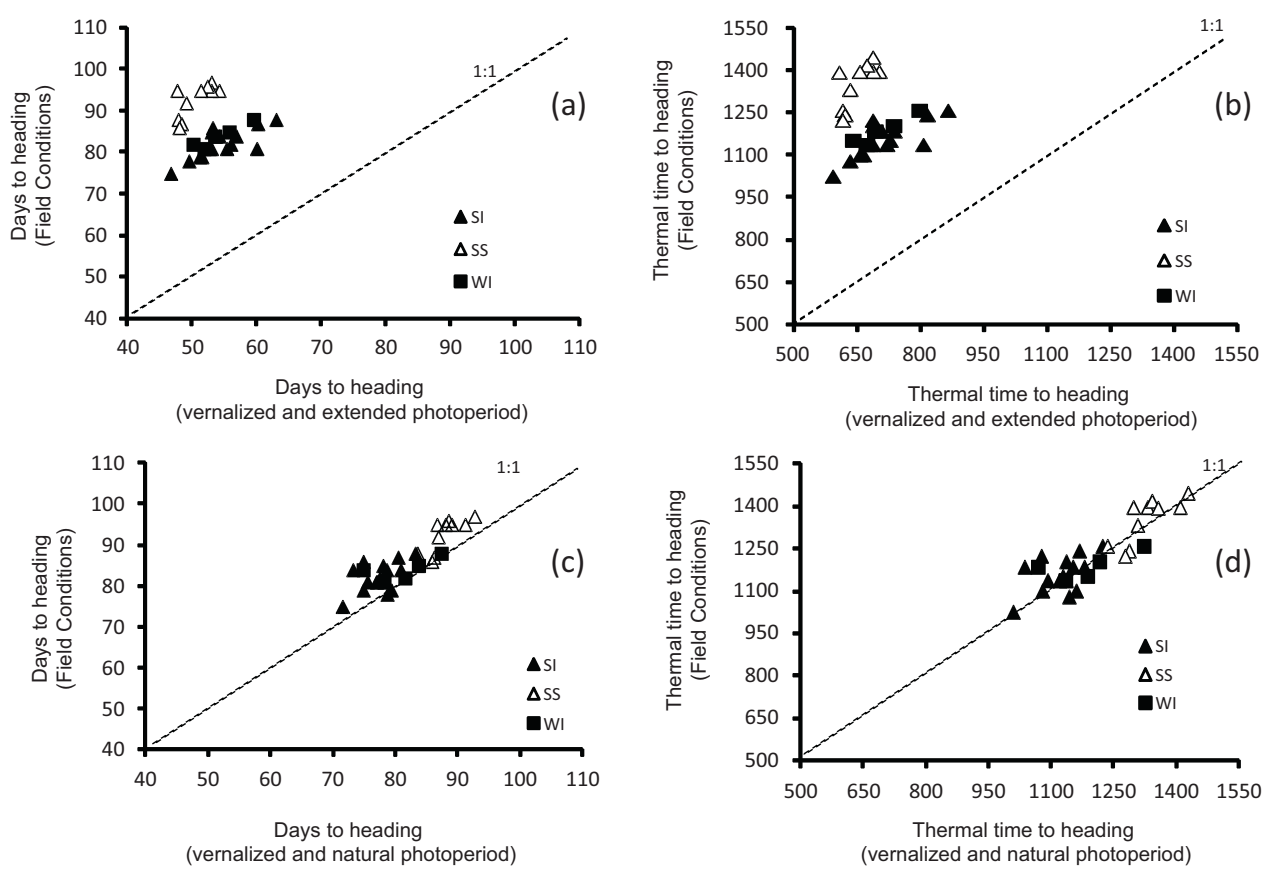

Fig. 2. Relationship between time to heading when cultivars were sown under field conditions (corresponding to sowing date of July 10th) and when vernalized and extended photoperiod of 20 h, measured in days (a) and degree days (b). Panels c and d represent the relationship between duration up to heading (from emergence) for the same sowing date (i.e. July 10th with similar average photoperiod for the emergence–heading phase) with (*y* axis) and without (*x* axis) vernalization requirements fulfilled. For cultivars classification, see Table 1 and Fig. 1.

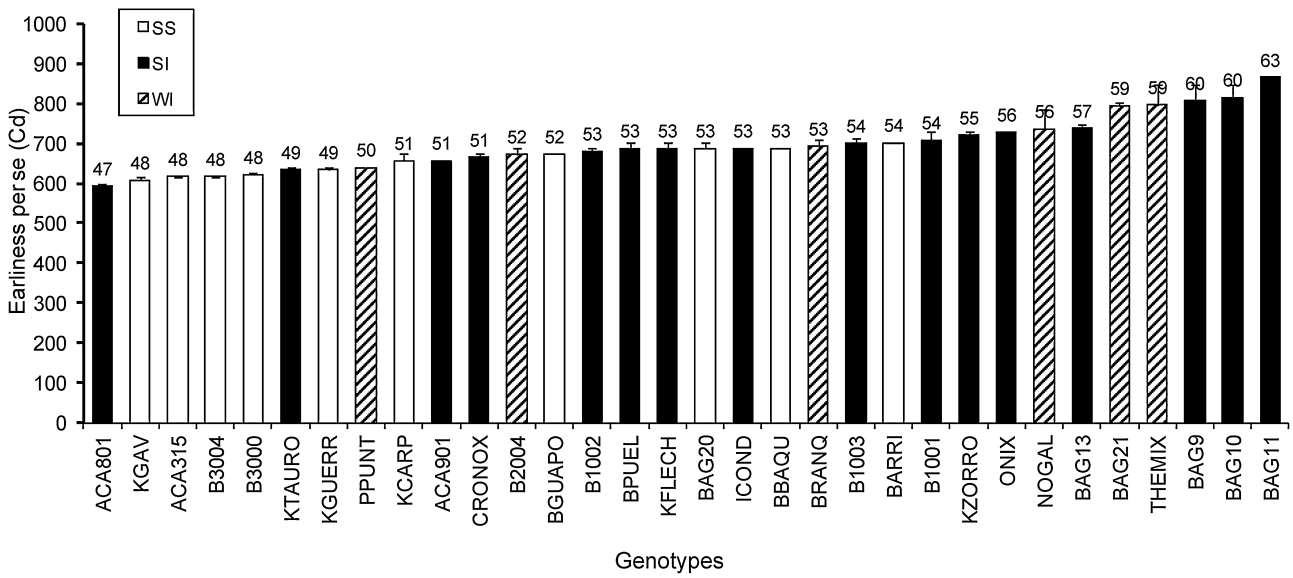

Fig. 3. Earliness *per se* (i.e. the minimum duration of the emergence–heading phase) measured in degree days (°C in *y* axis) and in days (values over the columns) for SS (empty bars), SI (filled bars) and (WI diagonal filled bars). Vertical bars within each column indicate the standard error of mean.

EPS and sowing date was observed in WI group ($p < 0.0001$) (Table S2).

Unlike what was observed when the genotypes were grown under the field conditions; earliness *per se* did not show any particular association in its length with the groups classified by molecular markers. The genotypes with different photoperiod and vernalization allele combinations (Table 1) showed a wide range of duration of earliness *per se* in days (between 47 and 63 days) as well as in thermal time (from 600 to 850 °Cd) (Figs. 2 and 3). As stated above, a clear pattern associated with the allelic combination of Ppd and Vrn genes and the duration of the earliness *per se* was not identified. Thus, some genotypes of the SI and WI groups showed greater values of earliness *per se* (less precocity) than those of the SS group which in average showed shorter values of earliness *per se* than SI and WI (Fig. 3). No relationship was found between EPS and photoperiod sensitivity although as was previously highlighted, the longest duration of EPS was recorded in some genotypes classified as SI and WI (Fig. S2).

4. Discussion

The present study demonstrated that, at least for the wide range of cultivars included in this study, most of the variations in cycle duration up to heading were associated with photoperiod sensitivity responses more than with changes in the threshold photoperiod. No association between photoperiod sensitivity and earliness per was found. Although, photoperiod sensitivity was in general lower in those cultivars classified molecularly as "insensitive" than those classified as "sensitive" all cultivars reduced the E–Hd phase when photoperiod was extended by delay in sowing dates. The range of variation in photoperiod sensitivity in genotypes classified as winter insensitive was variable depending on the degree of satisfaction of vernalization requirements and the differences in EPS or a combination of both factors.

4.1. Dominant Vrn-1 and Ppd-1 alleles in Argentinean bread wheat

Most of the commercial wheat cultivars in Argentina are sown between end of fall (late maturity cultivars) and beginning of winter (early maturity cultivars) to take advantage of rain water stored in the soil between late summer and early fall, and to avoid the risk of frost damage around flowering and high temperatures during grain filling. Appendino et al. (2003) showed that most of Argentinean wheat cultivars possessed spring growth habit. Fu et al. (2005) characterized a set of 67 cultivars released in Argentina between 1930 and 2004 using molecular markers for *Vrn-1* finding 62 cultivars (92.5%) carrying at least one spring allele (and spring growth habit), and only five cultivars possessed the triple combination of winter alleles *vrn-A1*/*vrn-B1*/*vrn-D1* associated with winter growth habit (7.5%). A more recent molecular survey considering a larger (102) but more recent (>80% released between 1999 and 2010) set of cultivars confirmed spring growth habit (89%) as the best fit for the wheat production area in Argentina (Vanzetti et al., 2013). The same study also reflected a high frequency of photoperiod insensitive alleles *Ppd-D1a* and/or *Ppd-B1a* alleles (72%) regarding *Ppd-D1b*/*Ppd-B1b* allele combination associated with photoperiod sensitivity (28%). Based on this data, dominant combinations of vernalization and photoperiod response alleles in wheat cultivars from Argentina were: Spring-Insensitive (62%) > Spring-Sensitive (27%) > Winter-Insensitive (10%) > Winter-Sensitive (1%) which mostly agree with SI, SS and WI genotype classification used in our study.

Typical winter-photoperiod sensitive (WS) wheats are normally grown in environments with a longer growing season than that is explored by most of the wheat cultivars sown in Argentina wheat belt. For example, cultivars with 145–165 days to heading in UK environments (more than 50° N latitude, Griffiths et al., 2009) are longer than the 90–100 days to heading observed in late flowering cultivars of the present study (see Fig. 2) or respect to the later flowering cultivars that are sown in the South East of Buenos Aires Province (37° S latitude) that could extend their cycles to heading up to 120–130 days. In the Argentinean wheat belt delays in heading time will probably penalize yield by exposing grain-filling phase to high summer temperatures, similar to which has been observed in Southern Europe (Worland et al., 2001; Isidro et al., 2011). Thus, it is important that genotypes, as sowing date is delayed, adjust the length of the cycle to expose the critical period (i.e. immediately previous to flowering) to the best environmental conditions avoiding the high temperatures during grain filling.

4.2. Earliness per se groups are related with germplasm origin

Since 1999 several breeding companies in Argentina have been incorporating germplasm from Europe (mainly France) due to its excellent adaptation to the Argentinean wheat belt and high yield potential. Additionally, Vanzetti et al. (2013) showed that cultivars introduced from Europe exhibited a different genetic structure respect to the traditional cultivars from Argentina. In the present study most cultivars classified as long EPS (5/7) are introductions from France suggesting a different set of adaptation genes/alleles for this trait, compared with traditional (and CIMMYT) germplasm, mostly associated with short EPS.

4.3. From phenotypic characterization to a gene-based model to predict heading time

Results of the present study demonstrated that most of the variations in cycle duration up to heading were associated with photoperiod sensitivity responses more than changes in the threshold photoperiod. In fact, Fig. 1 showed that the higher the photoperiod sensitivity the higher the intercept confirming that photoperiod sensitivity changed in parallel with the intercept confirming that the driven force to modify the heading time in different sowing dates was photoperiod sensitivity. Miralles et al. (2007) analyzed 20 different wheat commercial cultivars (10 representing early maturity and the other 10 corresponding to late maturity cultivars) and showed that most of the variations in the phenological phases previous to flowering time were associated with changes in photoperiod sensitivity without significant variations in the threshold photoperiod which was around of 13.5 h of photoperiod. Although the lower photoperiod sensitivity was observed in the present work in those cultivars molecularly classified as "spring insensitive" (SI) respect to the sensitive cultivars (SS); from an ecophysiological point of view those cultivars should be classified as "low photoperiod sensitivity" (as the range of reductions of the E–Hd phase was between 30 and 45 days h^{-1} of photoperiod extension). The opposite could be applied for the "spring sensitive" cultivars (SS) as revealed higher photoperiod sensitivity than SI but also evidenced a wide range of variation (i.e. between 40 and 50 days of reduction per hour of photoperiod extension). On the other hand, the group of cultivars classified as WI showed, in general, ranges of photoperiod sensitivities similar than that classified as SI. However, some cultivars with vernalization requirements (as was stated above) increased photoperiod sensitivity due to (i) the vernalization requirements were not completely satisfied and (ii) differences in EPS or a combination of both factors. These results suggest that other minor genes (beyond the major genes described in the present paper) are involved in the duration of the phases. González et al. (2005) showed that the time to heading depended on the *Ppd* constitution of the lines, as in that experiment the duration of the E–Hd phase was in decreasing order: ppd > *Ppd-B*1 > *Ppd-D*1 (*Ppd-D1b*/*Ppd-B1b*/*Ppd-A1b* > *Ppd-B1a* > *Ppd-D1a*). However, the response to photoperiod of the phases prior to heading (i.e. emergence to the beginning of stem elongation and from that stage to heading) depends on the *Ppd* locus present. González et al. (2005) showed that *Ppd-D1* was insensitive to photoperiod when photoperiods extensions were applied after beginning of stem elongation while *Ppd-B1* and *ppd* were sensitive, evidencing the complexity (and the interaction between phenological phases) of the responses of the photoperiod genes in different crop phases. Although the present study did not discriminate the duration of the phases prior to heading it is possible to hypothesize, regarding the differences in the allelic composition of the genotypes, that the response of the pre-heading phases to photoperiod is different among the genotypes. Explaining the variability would be important to establish the relative duration of the phases and the effects on the setting of grain number and thereby in yield (García et al., 2011).

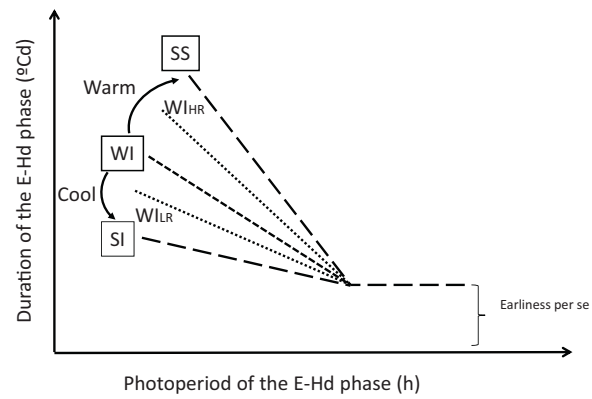

Fig. 4. Schematic model of the duration of the phase up to heading (measured in degree days) in response to average photoperiod of the phase for SS, SI and WI genotypes. The scheme shows the variations in photoperiod sensitivity in the WI depending on the environmental temperature (cool or warm) associated with the satisfaction of vernalization requirements.

Based on experimental data, a schematic model of the behavior of the different molecular groups (*Ppd-1* and *Vrn-1*) and EPS over the duration of the phase E–Hd was proposed (Fig. 4). The model represents the relationship between the duration of the emergence (E)–heading (Hd) phase (°C) (*y* axis, Fig. 4) and photoperiod (hours) during that phase (*x* axis, Fig. 4). In the model the genotypes of the SS group are represented with the highest photoperiod sensitivity for the E–Hd phase. Conversely, the genotypes corresponding to the SI group are characterized with the lowest photoperiod sensitivity and, in a wide range of photoperiod values, the genotypes molecularly classified as SI have shorter duration of the E–Hd phase respect to the SS, difference that is reduced as photoperiod is increased. The WI group shows, in general, intermediate photoperiod sensitivity with respect to the SI and SS groups, and the slope of the relationship between duration and photoperiod length changes according to vernalization requirements and the environments at which are exposed to fulfill partially or completely those vernalization requirements. Unfortunately the WS group could not be incorporated into the model due to the absence of these genotypes in the Argentinean wheat germplasm.

The information included in the present study can be used to build a gene-based model for predicting phenology in wheat as in the general picture, genotypes with different allelic combinations for *Ppd-1* and *Vrn-1* can be classified in groups with different photoperiod sensitivity (and thereby different duration for one particular developmental phase). This approach was recently applied to the APSIM model that was modified to incorporate gene effects in heading time (Zheng et al., 2013). However, the fact that genotypes included into each particular group (e.g. SI) had variations in photoperiod sensitivity could determine mismatching in the predictions given by the model for one particular genotype. Another, additional problem associated with the precision of the gene-based model, arises from those genotypes with vernalization requirements (WI and WS), as the degree to which vernalization requirements are fulfilled, or not, depends on (beyond the requirement of the cultivar) the particular environmental conditions (i.e. temperature) occurring during each particular growing season. Including genetic variability from present and additional genes involved in vernalization and photoperiod pathways not included in this study like *Vrn-2* (Yan et al., 2004a), *Vrn-3* (Yan et al., 2006), *Vrn-4* (Yoshida et al., 2010), will probably improve the prediction of phenology in a model based on gene combinations. In the case

of photoperiod response, main genes *Ppd-1* (*Ppd-D1*), Ppd-2 (*Ppd-B1*) and *Ppd-3* (*Ppd-A1*) have already been included in the analysis; however the occurrence of additional alleles in *Ppd-1* affecting photoperiod response cannot be discarded (Nishida et al., 2013). A similar caveat may apply to *Vrn-1*, for example, as Shcherban et al. (2012) detected novel spring alleles in the *Vrn-B1* locus using Russian germplasm.

The EPS was the other source of variation in heading among the genotypes and could not be associated with *Ppd-1* and *Vrn-1* allelic combinations. Miura and Worland (1994) and Lewis et al. (2008), found that EPS genes control development rate, independent of vernalization and day-length responses. Moreover, the polygenic nature of EPS (Griffiths et al., 2009) may show different scenarios of interactions with environment, for example, Lewis et al. (2008) observed that the effect of *Eps*-A^m1 on heading time is modulated by temperature, which is not clear in the case of *Eps*-3A^m (Gawroński and Schnurbusch, 2012). More recently, Kamran et al. (2013) observed that earliness *per se* and flowering time QTLs interact in an additive fashion with photoperiod insensitive gene *Ppd-D1a*.

In summary, the present study marks a further step toward the molecular/phenotypic elucidation of main components of heading control (vernalization, photoperiod response and *Eps*) considering Argentinean bread wheat germplasm as model, but further challenges remain to be overcome to design a reliable gene-based model to predict phenology.

5. Conclusions

From the results obtained in the present study it is possible to conclude that: (i) the main attribute determining differences in heading time of cultivars used in the present study was the sensitivity to photoperiod, (ii) most of the variation in heading time could be explained by allelic combinations of Ppd-1 and Vrn-1 genes. However, even those cultivars classified molecularly as "insensitive" to photoperiod showed reductions in the E–Hd phase, suggesting that additional Ppd genes could be affecting photoperiod sensitivity response, (iii) a minor but significant variation in heading time associated with EPS was also observed under controlled and field conditions (independent from Ppd-1 and Vrn-1) and there was not association between photoperiod sensitivity and EPS.

The development of near-isogenic lines differing in Ppd-1 and Vrn-1 alleles combined with earliness *per se* (short and long EPS materials) and bi-parental mapping populations segregating for similar traits can be a valuable tool to precisely measure the agronomic performance of combinations of adaptation genes in specific environments in order to identify the superior ones for breeding purposes.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at
http://dx.doi.org/10.1016/j.fcr.2013.12.023.

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