Wheat pre-anthesis development as affected by photoperiod sensitivity genes (*Ppd-1*) under contrasting photoperiods

Thomas I. Pérez-Gianmarco[A,B,F], *Gustavo A. Slafer*[A,C,D] *and Fernanda G. González*[B,E]

[A]Department of Crop and Forest Sciences, University of Lleida, Av. Rovira Roure 191, 25198 Lleida, Spain.

[B]CITNOBA, CONICET-UNNOBA. Monteagudo 2772, B2700KIZ Pergamino, Buenos Aires, Argentina.

[C]ICREA (Catalonian Institution for Research and Advanced Studies), Spain.

[D]AGROTECNIO (Centre for Research in Agrotechnology), University of Lleida, Av. Rovira Roure 191, 25198 Lleida, Spain.

[E]EEA INTA Pergamino. Ruta 32, Km 4,5, B2700XAC Pergamino, Buenos Aires, Argentina.

[F]Corresponding author. Email: t.perezgianmarco@conicet.gov.ar

Fine tuning wheat phenology is of paramount importance for adaptation. A better understanding of how genetic constitution modulates the developmental responses during pre-anthesis phases would help to maintain or even increase yield potential as temperature increases due to climate change. The photoperiod-sensitive cultivar Paragon, and four near isogenic lines with different combinations of insensitivity alleles (*Ppd-A1a*, *Ppd-B1a*, *Ppd-D1a* or their triple stack) were evaluated under short (12 h) and long (16 h) photoperiods. Insensitivity alleles decreased time to anthesis and duration of the three pre-anthesis phases (vegetative, early reproductive and late reproductive), following the *Ppd-D1a* > *Ppd-A1a* > *Ppd-B1a* ranking of strength. Stacking them intensified the insensitivity, but had no additive effect over that of *Ppd-D1a*. The late reproductive phase was the most responsive, even exhibiting a qualitative response. Leaf plastochron was not affected but spikelet plastochron increased according to *Ppd-1a* ranking of strength. Earlier anthesis resulted from less leaves differentiated and a fine tuning effect of accelerated rate of leaf appearance. None of the alleles affected development exclusively during any particular pre-anthesis phase, which would be ideal for tailoring time to anthesis with specific partitioning of developmental time into particular phases. Other allelic variants should be further tested to this purpose.

Additional keywords: final leaf number, insensitivity alleles, ontogenesis, phenology, primordia dynamics, spikelet number.

The duration of phases leading to wheat anthesis date, and hence yield potential, are regulated by daylength (photoperiod). Genes controlling the response to photoperiod (*Ppd-1*): (i) provided varying magnitudes of insensitivity; (ii) were not particularly associated to duration – photoperiod sensitivity – of any specific phase; and (iii) showed no additive effect when stacked. Further investigation with different allelic variants should be conducted to tailor time to anthesis and duration of each particular phase to improve wheat yield potential facing climate change.

Introduction

The phenological pattern determining anthesis date plays a major role in wheat (Triticum aestivum L.) adaptation (Worland 1996; Snape et al. 2001) maximising grain yield for a given environment. This is because phenology of the crop defines resource capture and stress avoidance opportunities during the pre- and post-anthesis periods of yield generation (Fischer 1975; Evans 1978). For instance, rising global temperatures because of climate change (IPCC 2014) are predicted to reduce yield by 6% per °C of temperature increment, based solely on accelerated developmental rates (Asseng et al. 2015; García et al. 2015), particularly during pre-anthesis.

Wheat undergoes three phenophases before reaching anthesis: the vegetative phase (VP) from seed imbibition to floral initiation, when leaf primordia are differentiated; the early reproductive phase (ERP) from then to the differentiation of the terminal spikelet (TS), when spikelet primordia initiation takes place; and the late reproductive phase (LRP) from TS to anthesis, when florets develop within the spikelets while the stems and spikes grow (Slafer 2012). Manipulating the duration of these phases, i.e. increasing duration of LRP, which includes the pre-anthesis critical period for yield generation, might be an avenue to raising wheat yield potential (Slafer et al. 1996, 2001; Miralles et al. 2000; González et al. 2003, 2005a, 2011), helping to overcome part of the predicted yield losses due to global warming.

From physiological studies exposing wheat to contrasting photoperiod environments, it has been suggested that photoperiod sensitivity of each phenophase might be under, at least partially, independent genetic control (Halloran and Pennell 1982; Slafer and Rawson 1994a; Slafer et al. 1996, 2001; González et al. 2002), which would allow for manipulation of sensitivity to photoperiod of a particular phenophase – and with it, its duration – without significantly affecting duration of other phases. Studies on the differences in rates of development comparing isogenic lines for *Photoperiod-1* (*Ppd-1*, photoperiod sensitivity genes) have been far less common (Foulkes et al. 2004; González et al. 2005b; Matsuyama et al. 2015), and cases in which the effects of these alleles included the study of developmental processes in each of these phases – instead of simply time to heading or anthesis – are almost non-existent (González et al. 2005b). Such studies are critical in understanding the impact that particular *Ppd-1* genes have on developmental processes occurring in each phenophase, which is relevant to design the best genetic combination to optimise adaptation and yield potential.

The *Ppd-1* genes are a homeoallelic series of loci located in short arms of chromosome 2 of the A, B and D genomes (Scarth and Law 1983, 1984*): Ppd-A1*, *Ppd-B1* and *Ppd-D1* respectively (McIntosh et al. 2003). Early investigations suggested these are the main source of variation in response to photoperiod amongst wheat genotypes (Law et al. 1978; Scarth and Law 1984). The wild-type allele, *Ppd-1b*, is associated with photoperiod sensitivity (i.e. late flowering under short days) whereas semi-dominant mutations, *Ppd-1a*, provide levels of insensitivity to photoperiod (Bentley et al. 2011; Shaw et al. 2012). More recent investigations deploying near isogenic-lines (NILs) confirmed the multi-allelism proposed by Scarth and Law (1984) and suggested a ranking of insensitivity for time to anthesis as being *Ppd-D1a* > *Ppd-A1a* > *Ppd-B1a* (e.g. Díaz et al. 2012; Shaw et al. 2012; Bentley et al. 2013). Response to photoperiod has been reported to be controlled as well by other genes in the long arm of chromosome 6B (Islam-Faridi et al. 1996), chromosome 3D, possibly 3A and 3B too (Miura and Worland 1994) and 1A and 1B (Law et al. 1998). More recently, a report located a Ppd-B2 in the short arm of chromosome 2B (Khlestkina et al. 2009). Their effect, however, is relatively small when compared with that of *Ppd-1* genes.

As mentioned above, there are few studies showing the effects of *Ppd-1* on physiological process during the pre-anthesis phases. Further, the studies that do exist have provided inconsistent results (see revision in tables 1 and 2 in work by González et al. 2005b). For instance, *Ppd-D1* has been associated with duration of the three pre-anthesis phases (González et al. 2005b), or mainly with the early phases, VP+ERP, but without impact on LRP (Foulkes et al. 2004), or only during ERP (Scarth et al. 1985). Ppd-B1 has been associated with duration of ERP (Scarth et al. 1985; González et al. 2005b), or VP+ERP with less or no impact on LRP (Whitechurch and Slafer 2002; Matsuyama et al. 2015). Finally, as far as we are

aware there have been no reports on effects of *Ppd-A1* on these different phases.

Similarly, we could find no published reports on primordia differentiation dynamics in response to differences in duration of pre-anthesis phases using isogenic lines for *Ppd-1* genes. However, Scarth et al. (1985) using chromosomes substitution lines observed that *Ppd-D1a* did not modify the final number of leaves nor the rate of spikelet initiation resulting in less spikelets per spike due to reduced ERP duration. For *Ppd-B1a*, rate of leaf primordia differentiation was not altered resulting in less leaves produced, but spikelet primordia differentiation rate was increased. Together with a shorter duration of ERP, it resulted in no change in spikelet count per spike (Scarth et al. 1985). The effect of insensitivity alleles on the number of spikelets per spike has not been studied to any great extent. A few reports agreed on differences between the most extreme phenotypes to be of ~2 to 3.5 spikelets per spike, both on growth chambers (Scarth et al. 1985) and field conditions (González et al. 2005b; Matsuyama et al. 2015). Likewise, the impact of *Ppd-1* genes on the leaf appearance rate (LAR) seems to have been only exceptionally considered so far (González et al. 2005b). This is relevant as LAR, together with final leaf number, determines the time to flag leaf appearance, which largely determines time to anthesis.

In this study, we assessed the individual effects of *Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a* and their triple stacking not only on time to anthesis and final number of leaves, but also on duration of different pre-anthesis phenophases (VP, ERP and LRP), dynamics of leaf and spikelet primordia differentiation, and leaf appearance rate.

Materials and methods

Experiments, treatments and design

We conducted two independent experiments at the University of Lleida (Catalonia, Spain) in 2015. In each of these experiments, treatments consisted of the factorial combination of five wheat (Triticum aestivum L.) genotypes and two contrasting photoperiod conditions. The genotypes were the wild type with only Ppd-1b alleles, Paragon (a spring cultivar, Winfield et al. 2010), and four NILs for *Ppd-1* genes (Table 1), kindly provided by the John Innes Centre (UK). *Ppd-1a* alleles from GS-100, Chinese Spring and Sonora 64 (*Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a* respectively) were introgressed into the photoperiod-sensitive cultivar Paragon by crossing with each of these genotypes, and then backcrossing with Paragon as the recurrent parent to develop BC4 families. The detailed procedure is described by Bentley et al. (2011). Photoperiod treatments consisted of two contrasting regimes: one with relatively short days (12 h light/12 h dark or neutral day, henceforth referenced to as short photoperiod or SP), and another with long days (16 h light/8 h dark, hereafter long photoperiod or LP), which were applied in individual growth chambers in consecutive runs. Care was taken to ensure same daily incident radiation (~2.36 MJ m–2 day–1) for both treatments by turning off some of the lamps in the chamber set with long days. Temperature in both photoperiodic conditions and in both experiments was constant at 16°C. Each experiment was arranged as a completely randomised design: all genotypes were equally distributed between chambers and randomly set within them. Number of replicates depended on the response variable (see below).

Plants were grown in pots (235 mL) filled with a 7:3 mixture of peat and manure-based soil amendment. One seed per pot was sown after being coated with the recommended dose of insecticide and fungicide, then pots were irrigated and left at room temperature until seedling emergence. Fifty per cent more pots than those required to fully fill the chambers were sown for each genotype to select for the experiments those with evenly emerged plants, starting the experiment at seedling emergence. The number of pots per genotype ranged from 38 to 54 depending on *Ppd-1* genetic constitution and photoperiod treatment, i.e. the short photoperiod and the genotypes expected to be more sensitive had more pots than the others, to allow for more dissections. The pot was considered the experimental unit. Twice a week pots were rearranged inside the chambers to avoid border effects. Macro- (P, K) and micronutrients (B, Cu, Fe, Mn, Zn) were applied once at the beginning of the experiments, adding 0.01 mL of Manvert's 0-17-19 per pot with irrigation water as supplement. Each pot was periodically irrigated using an automatic drenching gun at a fixed dose. Insects

and diseases were prevented spraying insecticides and fungicides.

Measurements, response variables and analyses

In each experiment, eight plants per genotype within each photoperiod treatment were identified and labelled immediately after seedling emergence (EM). In these plants, we determined the stages of flag leaf emergence (FL) and anthesis (AN) (Zadoks et al. 1974). Also, we recorded periodically (2–3 times a week) the number of leaves appeared on the main shoot (Haun 1973) from EM to FL, when the final leaf number (FLN) was counted. At AN, when the experiment finished, the number of spikelets per spike was also recorded. Therefore, for all these traits there were eight replicates per genotype × photoperiod combination in each of the experiments.

Thermal time from EM to each particular stage was computed using 0°C as base temperature. To estimate leaf appearance rate (LAR) a linear model relating the cumulative number of appeared leaves with thermal time from EM was fitted considering all the observations in each genotype × photoperiod combination (Eqn 1, b, LAR). The segmental linear model was fitted instead (Eqn 2) when evidence of lack of linear fit was detected through the analysis of the distribution of residuals. In this case, early leaves appeared at a faster rate (b) than the late-appearing leaves (d), being (c) the timing when the change of rate occurred.

$$y = a + bx, \quad (1)$$

$$y = a + bx\ (x \leq c) + bc\ (x > c) + d\ (x - c)\ (x > c). \quad (2)$$

For each experiment, two plants per genotype × photoperiod treatment were randomly sampled two or three times a week depending on developmental rate for each genotype × photoperiod combination, and dissected under binocular microscope. In each case, from EM to TS, we counted the number of primordia and determined the stage of development of the apex (Kirby and Appleyard 1981). Following each sampling, the remaining plants were rearranged to keep a canopy-like structure within the growth chamber. The cumulative number of primordia was related to thermal time by fitting a segmental linear model for estimation of primordia differentiation rates also by Eqn 2 but with different interpretation of parameters: 'b' and 'd' mostly represent leaf and spikelet primordia differentiation rates, respectively, whereas 'c' indicates the timing of change in primordia differentiation rates. Model adequacy was tested by using replicates test (GraphPad Prism ver. 6.00). Time to beginning of reproductive development (or floral initiation, FI) was estimated a posteriori, as the moment when the first reproductive primordium (collar, i.e. the first one in excess of FLN) was initiated for each plant. Duration of pre-anthesis phases was calculated as the difference in thermal time between the following stages: VP = FI–EM, ERP = TS–FI and LRP = AN–TS.

Analyses of variance were performed to assess the effects of experiments, main factors genotypes and photoperiod treatments, and the interactions genotype × photoperiod and experiment × genotype × photoperiod. Means of response variables were compared by Tukey's test ($\alpha = 0.05$) when found to significantly differ from one another using Infostat (Di Rienzo et al. 2015). Regression analyses were performed with GraphPad Prism ver. 6.00. The 95% confidence interval was used to determine statistical significance of differences amongst means for LAR, primordia differentiation rates and timing for change in these rates.

Results

Analyses of variance consistently showed that the main effects of both photoperiod and genotype were highly significant for all traits (Table 2). Their interaction, although of a lower impact than the main factors, was also highly significant (Table 2), as expected when growing in contrasting photoperiods genotypes produced to differ in photoperiod sensitivity. In contrast, the differences between the two independent experiments was not significant for most traits, and for the few cases in which it was, differences between experiments were negligible compared with those among genotypes or between photoperiod regimes (mean squares of the effects of the experiments represented at most 7.6 and 2.3% of the genotype and photoperiod mean squares, respectively, Table 2). Also, the triple interaction (genotype × photoperiod × experiment) was always extremely small, beyond of non-significant (Table 2). This implies that the minor differences between experiments did not alter the effects of the main factors nor that of the genotype × photoperiod interaction. Therefore, means across experiments were used to describe each genotype × photoperiod

performance in terms of duration of phases and number of leaves and spikelets, and data from both experiments were fitted together when describing leaf appearance or primordia differentiation dynamics.

In the genotypes with at least one *Ppd-1a* allele, all plants developed normally until anthesis regardless of the photoperiodic condition. In contrast, in the genotype with the three *Ppd-1b* alleles, Paragon, some plants reached anthesis normally whereas others failed to develop towards that stage, particularly under short photoperiod when only 31% of the plants reached anthesis (25% in experiment 1 and 38% in experiment 2). The plants that did not reach anthesis by the end of the experiment (~2300°C d after seedling emergence), would have not reached it either should the experiments had lasted longer, as they showed stalled post-TS development (see Fig. S1, available as Supplementary Material to this paper). Thus, most of the plants of Paragon exhibited a qualitative response to photoperiod during LRP, whilst the response was quantitative for the earlier phases of development.

In the rest of the results of this paper, we concentrated on the quantitative differences. For that purpose, the LRP and the whole period EM–AN for Paragon were analysed considering only the plants that developed normally until AN.

Time to anthesis, final leaf number and leaf appearance rate

Depending on photoperiod treatments, genotypes differed for the complete pre-anthesis cycle duration: Paragon reached AN significantly later than the NILs possessing insensitivity alleles in both short and long photoperiod, though the difference was rather large (>1000°C d later than the triple insensitive NIL) under short photoperiod but relatively marginal (~200°C d) under long photoperiod (Fig. 1). Differences amongst NILs with insensitivity alleles were statistically significant only in short days. Under such condition, the strength of the alleles for producing insensitivity to photoperiod was *Ppd-D1a* > *Ppd-A1a* > *Ppd-B1a*. When the three Ppd-1a alleles were introgressed together the insensitivity was the strongest – although the difference with *Ppd-D1a* was statistically significant only with $\alpha = 0.10$.

Duration of the cycle from EM to AN was related to both duration from EM to flag leaf appearance, FL (R^2 = 0.99, $P < 0.001$) and duration of peduncle elongation, FL to AN, albeit much less strongly (R^2 = 0.43, $P = 0.04$). Genotypes differed little amongst them for duration of peduncle elongation, as values ranged from ~270 to 335°C d under short photoperiod and from ~225 to 280°C d when photoperiod was long. In contrast, very large differences were observed amongst genotypes for duration from EM to FL, ranging from ~832 to 1921°C d under short photoperiod and from ~771 to 913°C d under long photoperiod. FLN was highly and positively associated with duration EM–FL (R^2 = 0.93, $P < 0.001$), mainly setting time to FL appearance. The ranking of strength amongst Ppd-1a alleles for the durations of FL–AN and EM–FL was similar to that observed on the duration of the EM–AN phase.

Duration from EM to FL comes as result of final leaf number (FLN) and the rate at which those leaves appear (leaf appearance rate, LAR, which determines the phyllochron). In nine out of the 10 combinations of genotypes and photoperiod conditions, the dynamics of leaf appearance was adequately fitted by a linear regression (Fig. 2). In contrast, when data from Paragon under 12 h of photoperiod were fitted with a linear regression the distribution of residuals was not at random (Fig. 2, inset on the left panel), indicating the appropriateness of a segmental linear regression in this case. When this bi-linear regression was fitted, it was clear that late leaves (from ~the seventh onwards) appeared at a significantly lower rate than the early leaves (Fig. 2). Each of the *Ppd-1a* alleles, and their triple combination, accelerated the LAR significantly, in either long or short photoperiod. The magnitude of such effect was minor in long photoperiod (phyllochron ranged from 122°C d in the triple insensitive to 132°C d in Paragon; Fig. 2, right panel) and much more noticeable in short photoperiod (from 128°C d in the triple insensitive to 163°C d in the early leaves and 200°C d in the late leaves of Paragon, Fig. 2).

Considering the 10 combinations of genotype × photoperiod, FLN was negatively associated with LAR (R^2 = 0.69 $P = 0.003$; Fig. 3). However, the overall relationship was strongly driven by the response to photoperiod of each genotype (Fig. 3). Within each photoperiod regime differences between genotypes in LAR were independent of those in FLN, except for

Paragon under short photoperiod. This cultivar under short photoperiod exhibited both higher FLN and lower LAR than the NILs (Fig. 3). Thus, when analysing the effects of *Ppd-1a* alleles, compared with the triple insensitive, under short photoperiod they varied little in FLN (~0.7 leaves) and their differences in LAR explained most differences in EM–FL (Fig. 4, $R^2 = 0.99$, $P = 0.022$). When including Paragon, the difference in EM–FL duration was simultaneously due to an increased FLN and decreased LAR (Fig. 4, $R^2 = 0.97$, $P = 0.017$ and $R^2 = 0.99$ $P = 0.075$ respectively). The curvilinear trend indicates that the increase in EM–FL was much larger than the decrease in LAR, whilst this was not the case for the relationship with FLN, which followed a linear trend (Fig. 4). This implies that the large delay in FL in Paragon under short photoperiod was chiefly due to the effect of the sensitivity alleles on FLN, complemented by a relatively minor change in LAR. Under long photoperiod, differences in EM–FL were significant only between Paragon and the genotypes with insensitivity alleles, yielding non-significant relationships with LAR and FLN (Fig. 4, $R^2 = 0.36$, $P = 0.400$; $R^2 = 0.19$, $P = 0.561$ respectively).

Duration of vegetative, early and late reproductive phases

The insensitivity alleles sped up developmental rates of all phases under short photoperiod, thus shortening VP, ERP and LRP for genotypes carrying any of them alone or their triple combination (Table 2; Fig. 5 bottom panels). Under long photoperiod, the effects of *Ppd-1a* alleles were milder than under short photoperiod, but still significant at least when comparing the extreme cases of Paragon and the NIL carrying the triple insensitivity (Fig. 5 top panels).

Under short photoperiod, Paragon showed the longest duration of VP, followed by P(CS-2B) and P(GS-100-2A). The latter had similar VP duration to P(S64-2D) and the triple insensitive NIL (Fig. 5 bottom-left panel). This means that for the duration of VP *Ppd-A1a* and *Ppd-D1a* had similar strength, and *Ppd-B1a* was the weakest allele. Under long photoperiod, although noticeably reduced, differences in duration of VP were still detected when comparing the triple insensitive and P(GS-100-2A) to Paragon (Fig. 5 top-left panel).

For ERP, it was again observed that under short photoperiod Paragon was the longest (~540°C d) and NILs carrying *Ppd-D1a*, *Ppd-A1a* and the three insensitivity alleles were the shortest. Although the NIL carrying the *Ppd-B1a* tended to show an intermediate duration (similar to that observed in VP), it was not statically different from the other NILs (Fig. 5 bottom-middle panel). Long photoperiod treatment lessened the differences amongst genotypes. Even though significant differences in duration of the ERP were detected between P(S64-2D) and Paragon, the actual difference was rather minor (~70°C d) and there was no difference at all between Paragon and the NIL with the triple insensitivity (Fig. 5 top-middle panel).

Single and triple *Ppd-1a*-bearing combinations also shortened LRP, and the magnitude of their effect was greater than for previous VP and ERP phases (Fig. 5, right panel). Although this shortening was significant for every genotype with at least one *Ppd-1a*, there was a differential effect depending on the particular alleles involved. The ranking in LRP duration under short photoperiod was identical to that of the whole period to anthesis: Paragon > P(CS-2B) > P(GS-100-2A) > P(S64-2D) ≅ Triple Insensitive (Fig. 5 bottom-right panel). Under long photoperiod, only the triple stacking of insensitivity alleles shortened the LRP significantly (reducing it by ~200°C d) compared with Paragon (Fig. 5 top-right panel).

Every genotype responded to photoperiod increase by shortening all of the three pre-anthesis phases, although only for P(CS-2B) – the NIL with the weakest allele – and Paragon – with all three *Ppd-1b* alleles – was such response always statistically significant. Also, the magnitude of such response was greater for the LRP than for earlier phases.

Leaf and spikelet number

Genotypes carrying any combination of insensitivity alleles produced significantly less primordia than Paragon under short photoperiod (Table 3); whereas the differences were less clear and inconsistent under long photoperiod (Tables 2, 3). The effect of insensitivity alleles on the number of primordia was due to reductions in both vegetative (leaves) and reproductive (spikelets) primordia.

Differences in FLN were detected amongst genotypes grown at 12 h photoperiod, as even P(CS-2B), the NIL that evidenced the weakest insensitivity in time to anthesis, produced fewer leaves than Paragon but more than the triple insensitive genotype; whereas

P(S64-2D) and P(GS-100-2A) were intermediate amongst the genotypes but did not differ significantly from either of them (Table 3). Under long photoperiod, most of such differences disappeared, as the FLN ranged only from six to seven across all genotypes. Furthermore, the slight differences were not clearly related to the *Ppd-1* allele composition: there were no differences between Paragon and either the triple insensitive or P(S64-2D), whereas the genotype that had the weakest *Ppd-1a* allele in terms of phenology, P(CS-2B), produced the lowest FLN (Table 3).

Similarly, under short photoperiod the number of spikelets initiated was reduced by the introgression of insensitive alleles, with Paragon and the triple insensitive genotype showing the highest and lowest number of spikelets, respectively (Table 3). Differences were much smaller, and not significant, when grown in 16 h photoperiod, although with a consistent trend for genotypes carrying at least one *Ppd-1a* allele having fewer spikelets than Paragon (Table 3). It is noteworthy that, considering photoperiod response as the difference between primordia production under short vs long photoperiod, every NIL responded when FLN was the response variable, but only Paragon significantly did so for the number of spikelets.

To assess the importance of phase duration in determining number of structures achieved, a linear regression was fitted to the relationship between number of structures differentiated during a particular phase and its duration (Fig. 6). FLN was significantly related to the duration of the VP (Fig. 6a). Although the relationship was strongly influenced by a single data-point, Paragon under short photoperiod, it was still significant if that data-point were excluded from the analysis (R^2 = 0.71, P = 0.004), mainly due to the photoperiod treatments and the interaction with genotypes. Differences in FLN were completely unrelated to duration of VP amongst genotypes under long photoperiod, even when including Paragon (R^2 = 0.17, P = 0.495). In contrast, under short photoperiod differences amongst all genotypes in FLN were related to their differences in duration of VP (R^2 = 0.98, P = 0.002) (Fig. 6a). When photoperiod treatments are compared within each genotype, differences in FLN were mostly related to differences in duration of VP; i.e. the response to photoperiod of each of the lines in terms of duration of VP translated in a parallel response of FLN (Fig. 6a).

The relationship between the number of spikelets per spike and duration of ERP was much weaker than that between FLN and VP. Not only did the overall relationship have a lower coefficient of determination (R^2 = 0.82, Fig. 6b) but it also strongly depended upon the single response of Paragon to photoperiod, as removal of this particular data-point rendered the relationship non-significant (R^2 = 0.25, P = 0.175). The change in duration of ERP between short and long photoperiod within each genotype did not translate in differences in spikelets per spike (Fig. 6b), except for Paragon in which the shortening of the ERP when grown under long photoperiod was followed by a reduction, albeit small, in number of spikelets.

Primordia differentiation dynamics

The relationships between the cumulative number of primordia differentiated in the apex from emergence to TS and thermal time were always bi-linear (Fig. 7). For the presentation and discussion of these results we assumed the first slope represented the leaf primordia differentiation rate and the second slope, the spikelet initiation rate. Comparing timing of FI in Fig. 2 with the arrowheads in Fig. 7 reveals that all leaves were initiated at the rate represented by the first slope and that most of the spikelets were initiated at the rate of the second slope (although a few initial spikelets were differentiated at the same rate of the leaf primordia).

The leaf primordia differentiation rate was similar amongst all genotypes and photoperiods (Fig. 7; Table 4). Averaging across all genotypes in both photoperiods, these rates represented a leaf plastochron (interval between differentiations of two consecutive primordia) of 51.4 ± 6.5°C d. In contrast, genotypes differed in the timing when the change in rate of primordia differentiation occurred. This timing was also affected by photoperiod treatments. The lines with insensitivity alleles advanced the timing of this change in short photoperiod compared with Paragon, whilst long photoperiod advanced this timing in Paragon and P(CS-2B) (Table 4). For the rest of genotypes with Ppd-1a alleles long photoperiod effect was not significant, but a consistent trend to advance this timing was observed (Fig. 7; Table 4).

The spikelet initiation rate was modified by the interaction between genotypes and photoperiod treatments. It increased under long compared with short photoperiod only for Paragon and P(CS-2B), whereas for the other genotypes the differences were not significant. When grown under 16 h photoperiod, this rate was similar amongst all genotypes, averaging across them a spikelet plastochron of 16.3 ± 1.0°C d. Adjustment in the rate at which reproductive primordia were differentiated when the ERP was shortened, due to either photoperiod or *Ppd-1a* constitution under short days, allowed for the – at least, partial – compensation on final number of spikelets.

Discussion

Time to anthesis and duration of pre-anthesis phases

The response to short photoperiod was not only quantitatively large but also qualitative. For the most sensitive genotype, Paragon, only a small proportion (approximately one third) of plants reached AN when grown under short days. The plants that did not reach AN had their development stalled after TS. It has been previously reported that not a single plant of Paragon reached anthesis when grown under 10 h photoperiod (after 120 days of experiment) (Bentley et al. 2011). Other authors reported similar responses when strongly sensitive cultivars were exposed to short photoperiod (Pugsley 1966; Halse and Weir 1970; Slafer and Rawson 1996) or non-inductive vernalising conditions (González et al. 2002). The noteworthy fact that the qualitative response occurred during post TS formation was in agreement with previous results (e.g. Slafer and Rawson 1996; for sensitivity to photoperiod; González et al. 2002; for sensitivity to vernalisation). This indicates that sensitivity to environmental cues might increase as development progress, which was also clear with the quantitative response observed in those plants that did develop until anthesis (see below).

Considering the quantitative response, i.e. restricting the analysis to the plants of Paragon that did reach AN, different strength in terms of AN hastening was observed for each insensitivity allele, being the effect of *Ppd-D1a* > *Ppd-A1a* > *Ppd-B1a*. This is in agreement with (i) the ranking proposed by latest investigations evaluating the same alleles, also using NILs with Paragon background (Díaz et al. 2012; Shaw et al. 2012; Bentley et al. 2013), and (ii) with the conclusion by Langer et al. (2014) who determined that *Ppd-D1a* is the allele with strongest effect in time to anthesis in European wheat.

Although major differences in EM–AN duration were associated with changes in both FLN and LAR, fine tuning of AN date was still accomplished by changes in LAR, even when no significant differences in FLN could be detected. Insensitivity alleles accelerated LAR of leaves emerging before the seventh – following the ranking stated above – in both short and long photoperiods, which shortened EM–FL duration beyond the effect of these alleles on FLN, something that, as far as we are aware, has never been reported before for the particular effects of *Ppd-1a* alleles. This simultaneous effect on FLN and LAR reveals that time to AN could be coarsely adjusted by changes in FLN and fine-tuned by changes in developmental rates given by further changes in LAR. The result that *Ppd-1a* alleles reduced FLN agrees with González et al. (2005b), whereas our description of their effect on LAR contrasts with their report: they found no impact of *Ppd-D1a* and *Ppd-B1a* on phyllochron of early emerging leaves.

Every *Ppd-1a* allele shortened each of the pre-anthesis phenophases, VP, ERP and LRP under short photoperiod, following a very similar ranking of magnitude to that observed for the whole cycle to AN. To the best of our knowledge, no previous records exist relating to *Ppd-A1a* effects on duration of pre-anthesis phenophases, but we found both agreeing and conflicting results for *Ppd-D1a* and *Ppd-B1a* effects in preceding literature. For *Ppd-D1a*, González et al. (2005b) also found effects on all three phases, whereas others reported effects only on duration of ERP or during EM–TS phase (Scarth et al. 1985; Foulkes et al. 2004). Similarly, whereas *Ppd-B1a* has been found to shorten EM–TS (Whitechurch and Slafer 2002; Matsuyama et al. 2015) or even only ERP (Scarth et al. 1985; González et al. 2005b), no previous reports on it shortening LRP, as it did here, have been found.

In addition, our results not only showed that *Ppd-1a* alleles reduce the duration of all pre-anthesis phases, but also that the most responsive was LRP. Under short photoperiod, this increase in responsiveness was so critical that the late reproductive phase

exhibited a qualitative response to photoperiod in most of the plants of the most sensitive genotype. In contrast, all of the plants showed a quantitative response for the VP and ERP. The few plants that developed normally to anthesis showed a large increment of the duration of LRP. Even under long photoperiod, in which durations of VP and ERP were somewhat affected, LRP was much longer in Paragon than in the NILs with *Ppd-1a* alleles. This stronger responsiveness of LRP than earlier phases agrees with physiological experiments in which sensitive cultivars were subjected to different photoperiods (e.g. Slafer and Rawson 1995). Not only did we prove that all of the pre-anthesis phases (VP, ERP or LRP) were responsive to the action of *Ppd-1a* alleles, but also that responsiveness to them differ throughout the cycle, as it does to photoperiod (Slafer and Rawson 1994a).

Contrasting results from previous reports on *Ppd-1a* effects may be due to (i) the use of whole chromosomes substitution lines (Scarth and Law 1984; Scarth et al. 1985; Whitechurch and Slafer 2002) in which too many other genes might have affected the results; (ii) the possible interaction with different degrees of vernalisation satisfaction when using winter-habit cultivars (Foulkes et al. 2004; González et al. 2005b); and/or (iii) interactions between *Ppd-1* and the genetic background of the material on which *Ppd-1* have been tested on (Kiss et al. 2014). Sources of variation in the ranking mentioned – beyond the stated above – could be different alleles for a given locus, i.e. the functional polymorphism in *Ppd-B1* (Tanio and Kato 2007; Nishida et al. 2013) or copy number variation in *Ppd-B1* as well (Beales et al. 2007; Bentley et al. 2011; Díaz et al. 2012; Shaw et al. 2012; Nishida et al. 2013; Muterko et al. 2015; Matsuyama et al. 2015).

Finally, we found no evidence of any particular *Ppd-1* allele affecting developmental rates of any particular pre-anthesis phases. Previous physiological studies proposed that duration of different pre-anthesis phases could be manipulated, given their photoperiodic sensitivity seemed to be under independent genetic control (Slafer et al. 1996, 2001; González et al. 2002). In this experiment, none of the tested alleles, affected developmental rates exclusively during any particular pre-anthesis phase, which would be ideal for tailoring time to anthesis with specific partitioning of developmental time into particular phases. Three main systems controls wheat development to anthesis *Vrn*, *Eps* and *Ppd* genes, of which Ppd-1 is only a part (Stelmakh 1997; Kamran et al. 2014). In the present work, only some combinations of *Ppd-1* were studied. Other loci of minor impact have been found to affect photoperiod sensitivity (Law et al. 1998; Cockram et al. 2007; Khlestkina et al. 2009) to anthesis; the impact of them on particular pre-anthesis phases remains unknown. As research continues on the genetic controls of anthesis in general and on photoperiod sensitivity in particular, new combinations of *Vrn*, *Eps* and *Ppd* genes will be made available to further test this hypothesis.

Number of primordia differentiated in the apex and their dynamics

Insensitivity alleles differently affected leaf and spikelet primordia differentiation rates. They had no effect on leaf differentiation rate and, consequently, their effect on FLN largely reflected those on VP duration. This coincides with the only other known report on effects of *Ppd-1* genes on primordia differentiation (Scarth et al. 1985) comparing *Ppd-B1a* and *Ppd-B1b* chromosome substitution lines. The leaf plastochron values we determined (~50°C d leaf^{-1}) was within the range of those previously reported in the literature (Evans and Blundell 1994; Miralles and Richards 2000; González et al. 2002). In addition, this is in line with physiological models of response of FLN to environmental factors. These models assume that leaf plastochron would be insensitive to photoperiod whilst the period of leaf differentiation (VP) would be sensitive, so that the relative change in duration of VP would be paralleled by the same relative change in FLN (e.g. Miglietta 1989; Slafer and Rawson 1994b; Slafer 2012).

Insensitivity alleles did, however, accelerate spikelet differentiation when ERP was shortened, as well as they hastened the timing to change of rate in primordia differentiation. Thus, even with ERP being shorter, a partial compensation in number of spikelets was observed. These findings coincide with the only other known report of *Ppd-1* genes on primordia dynamics (Scarth et al. 1985) in the case of *Ppd-D1a*, but not for *Ppd-B1a*, for which they found full compensation for number of spikelets. This is of particular interest as breeding to optimise pre-anthesis phases duration could then enlarge LRP at

the expense of ERP (Slafer et al. 2001) without negatively affecting the number of spikelets per spike, a numerical component of yield.

The spikelet plastochron was also in accordance with previous reports (Rahman 1980; Scarth et al. 1985; Evans and Blundell 1994; Miralles and Richards 2000; González et al. 2002), averaging 18.5°C d spikelet^{-1}. Also, as therein described, spikelet plastochron was reduced when exposed to longer photoperiods for all genotypes but the triple insensitive, albeit significant responses (~–2.5°C d spikelet^{-1} h) were only detected for the most sensitive ones, Paragon and P(CS-2B). As a result, there was much less variation in the number of structures produced, owing mainly to remaining variations in duration of ERP.

Generally, shortening ERP affected spikelet number per spike, and fewer spikelets were associated with hastened development, i.e. shorter differentiation phases (please refer to Fig. 6b). That can be the result of either greater insensitivity (Ppd-1a alleles' effect) or higher photoperiod hastening development. González et al. (2005b) observed a similar relationship for *Ppd-1a* NILs on two different winter backgrounds in field conditions, and spikelet number per spike ranged from ~20 (*Ppd-1b* genotype) to 16.4 or 17.6 for insensitive genotypes. Matsuyama et al. (2015) found their most sensitive genotype to produce 17.7 to 18.7 spikelets per spike depending on year and site combination, whereas insensitive genotypes produced 15.5 spikelets per spike. Scarth et al. (1985), working with chromosome substitution lines, show a decline of nearly three spikelets when introgressing a chromosome with *Ppd-D1a* in Chinese Spring background. Average spikelet count was however considerably higher than in the present study, possibly due to much shorter photoperiod (8 h), even though plants were grown in a growth chamber at constant 18°C.

Linking the insensitive phenotype to the role of Ppd-1a in the flowering pathway

The effects of insensitivity alleles (*Ppd-1a*) on phasic development and rates of leaf and spikelet initiation were comparable to that of longer, more inductive photoperiods, the magnitude of such effect being dependent on the strength of the allele (González et al. 2005b). Considering recent molecular studies of the flowering pathway, and particularly how *Ppd-1* genes interact with it, this seems unsurprising. Mutant alleles (*Ppd-1a*) show altered patterns of expression of the mutated gene, promoting high transcript levels – as long days would – throughout the dark period, which is associated with elevated, flower-inducing *TaFT1* (wheat's orthologue of *FLOWERING LOCUS T*) transcript levels even under non-inductive photoperiod (Turner et al. 2005; Beales et al. 2007; Díaz et al. 2012; Shaw et al. 2012). As a result, in the present study we found that the NIL with the triple combination of all *Ppd-1a* alleles showed little variation between photoperiods in either phase duration, number of primordia differentiated at the apex or the rate at which they are initiated and expanded (leaves). In contrast, the performance of the rest of the genotypes was very similar to that of the triple insensitive NIL when in long photoperiods. Also, it was under short photoperiod that *Ppd-1a* effects (and differences among genotypes) were the most notorious.

By no means was anthesis or any of the phenophases or other developmental processes described in the NIL carrying *Ppd-1a* in all three genomes hastened to the same extent as the sum of the three single allele's individual effect. Although the triple insensitive genotype consistently showed to have the strongest insensitivity, it was difficult to find significant differences between it and P(S64-2D), carrying the strongest single allele. This has been previously recognised by Shaw et al. (2012) – for heading date only – when working with single, double and triple NILs of the same origin. Although they detected a direct relationship between increasing number of insensitivity alleles and *TaFT1* expression levels, flowering was not always accordingly hastened –in strong double and triple insensitive genotypes, grown under 10 h photoperiod. They suggested a rate-limiting process downstream of *TaFT1*. The same mechanism may be explaining not only anthesis date, but also all the other traits we measured in the present study. Shaw et al. (2012) showed that *Ppd-1* transcription products are not genome-specific as of their downstream targets, i.e. *Ppd-1a* mutations on any given genome regulate the expression of downstream targets at all three genomes. Thus, high transcript levels from a single 'strong' *Ppd-1a* allele might already upregulate *TaFT* to levels that saturate the response observed. The lack of strong additive effects among *Ppd-1a* observed in the present paper, i.e. no further response was observed by stacking alleles on duration of subphases or number of

structures generated (leaves and spikelets) is in line with this molecular model.

Conclusion

In conclusion, *Ppd-1a* alleles hastened anthesis both under short and long photoperiod, each providing different levels of insensitivity. The ranking on strength of the insensitivity alleles for anthesis was *Ppd-D1a* (Sonora 64) > *Ppd-A1a* (GS-100) > *Ppd-B1a* (Chinese Spring). All of the pre-anthesis phases (VP, ERP or LRP) were sensitive to the action of *Ppd-1a* alleles, but not equally so. Unlike what is commonly assumed, i.e. that early phases might be more sensitive, the magnitude of the effects of these alleles increased with advances in development. The increment in sensitivity was so critical that the late reproductive phase exhibited a qualitative response in the most sensitive genotype under short photoperiod, whilst previous phases only exhibited quantitative responses. Furthermore, photoperiod insensitivity alleles may affect time to anthesis not only through their effects on FLN but also through an additional, fine-tuning adjustment, through effects on phyllochron. These effects are not trivial as they may be responsible for the quantitative response to photoperiod of the late reproductive phase. Stacking of *Ppd-1a* alleles intensified the insensitivity, but the cumulative effect was far from being additive. We also showed that every combination of either one or three *Ppd-1a* alleles, on Paragon background, responded to photoperiod; the magnitude of the response varying according to the strength of the alleles. None of the tested alleles affected developmental rates exclusively during any particular pre-anthesis phase, which would be ideal for tailoring time to anthesis with specific partitioning of developmental time into particular phases. The effect of other allelic variants should be further tested to this purpose.

Conflicts of interest

The authors declare no conflicts of interest.

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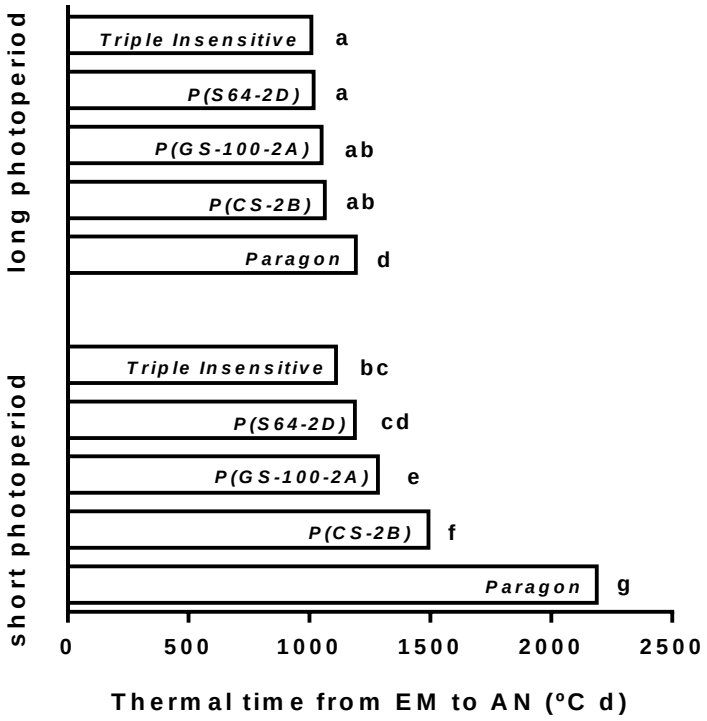

Fig. 1. Duration of the whole pre-anthesis cycle. Thermal time from seedling emergence (EM) to anthesis (AN) for Paragon and each of the NILs in both photoperiods. The same letters at the end of the bar indicates that the difference was not statistically significant between genotypes and across photoperiod treatments (Tukey, $\alpha = 0.05$). Data are means of two independent experiments.

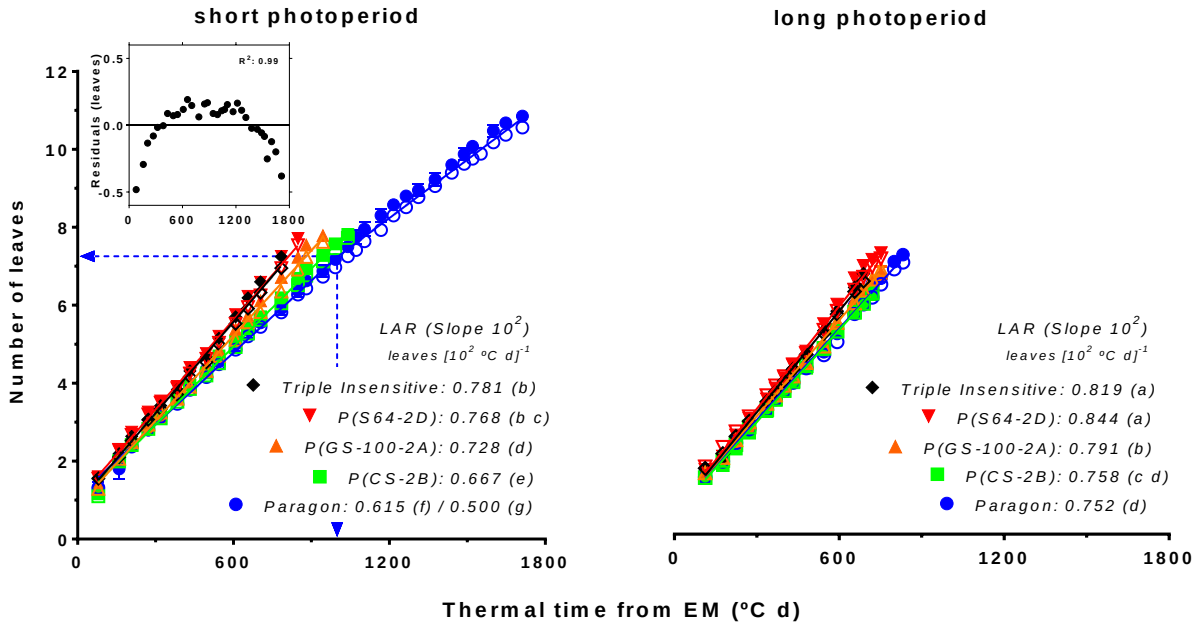

Fig. 2. Leaf appearance dynamics. Relationship between leaves appeared on the main shoot in experiments 1 and 2 (closed and open symbols respectively) and thermal time from emergence (EM). The error bars in data-points stand for the corresponding s.e. Slopes (inset, bottom right) indicate leaf appearance rates (LAR, leaves $(10^2$ °C d$)^{-1}$). Lines were fitted by either linear or segmental-linear regression, being $R^2 > 0.97$ ($P < 0.001$) for all. Shared letters between any two LARs indicate that the CI95 of parameter estimation (slopes) overlapped. The arrowed dotted line indicates the number of leaves (ordinate) appeared and time (abscissa) when the change in slope for Paragon in short days occurred. The relationship between the residuals of the linear regression between the number of leaves and thermal time (inset, top left) justified the need for a segmental-linear regression in this case.

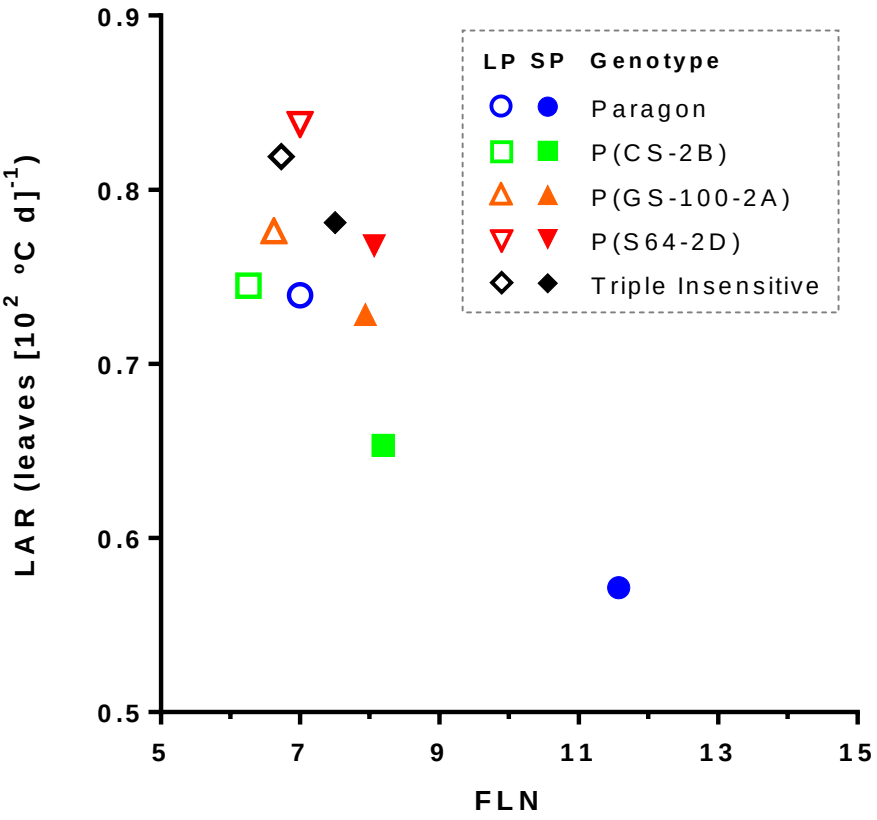

Fig. 3. Relationship between leaf appearance rate (LAR) and final leaf number (FLN) under long (LP) and short photoperiod (SP). LAR for Paragon in short photoperiod was calculated as the weighted average of LAR values for early and late appearing leaves.

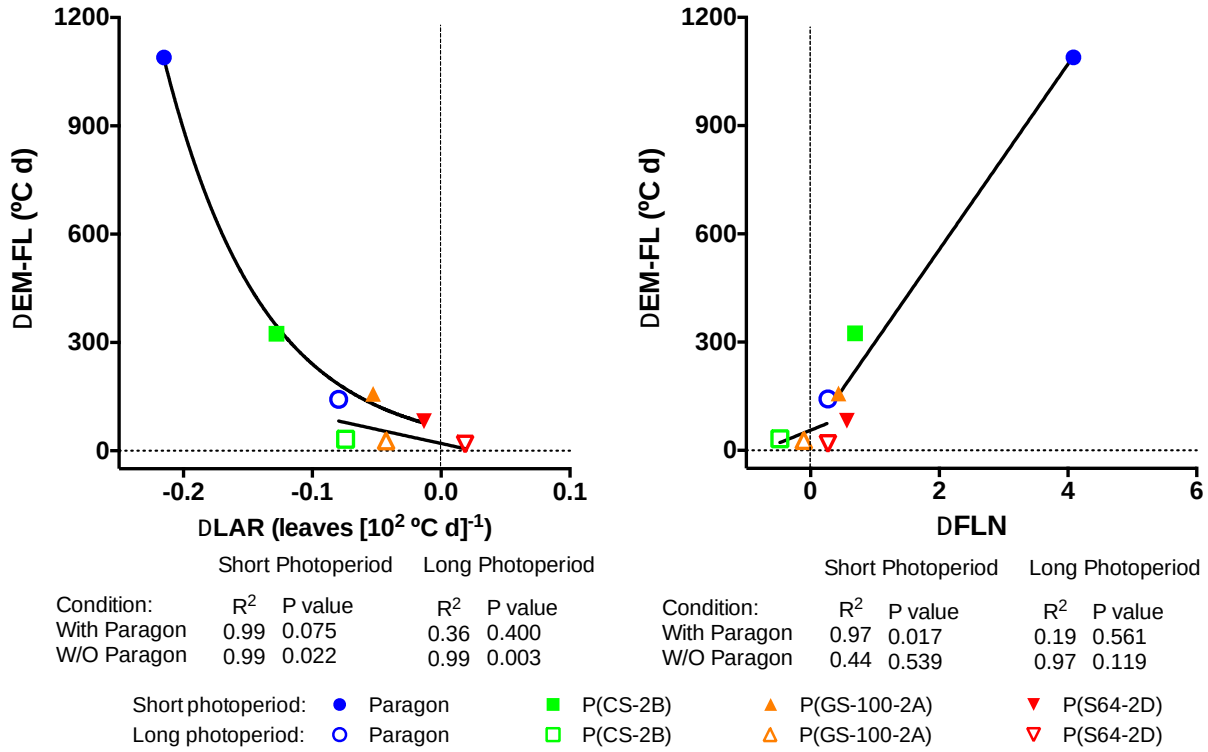

Fig. 4. Differences in thermal time from seedling emergence to flag leaf appearance (EM–FL) between each genotype and the triple insensitive NIL plotted against differences in either leaf appearance rate (LAR, left panel) or final leaf number (FLN, right panel). Regressions (exponential in the case of the relationship with LAR under short photoperiod, linear in the three other cases) were fitted for each photoperiod treatment including and excluding Paragon, but only the lines from the ones including Paragon are shown.

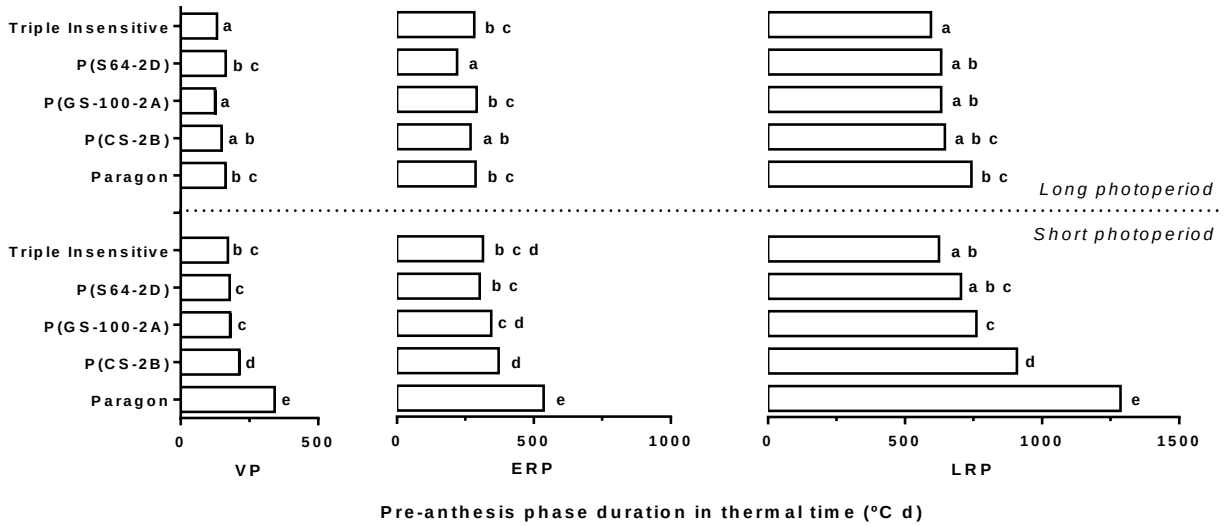

Fig. 5. Pre-anthesis phases duration, defined as vegetative phase (VP, left panel), early reproductive phase (ERP, central panel) and late reproductive phase (LRP, right panel). Bars with no shared letters for a specific phase are significantly different (Tukey $\alpha = 0.05$). Each bar shows the average of two independent experiments.

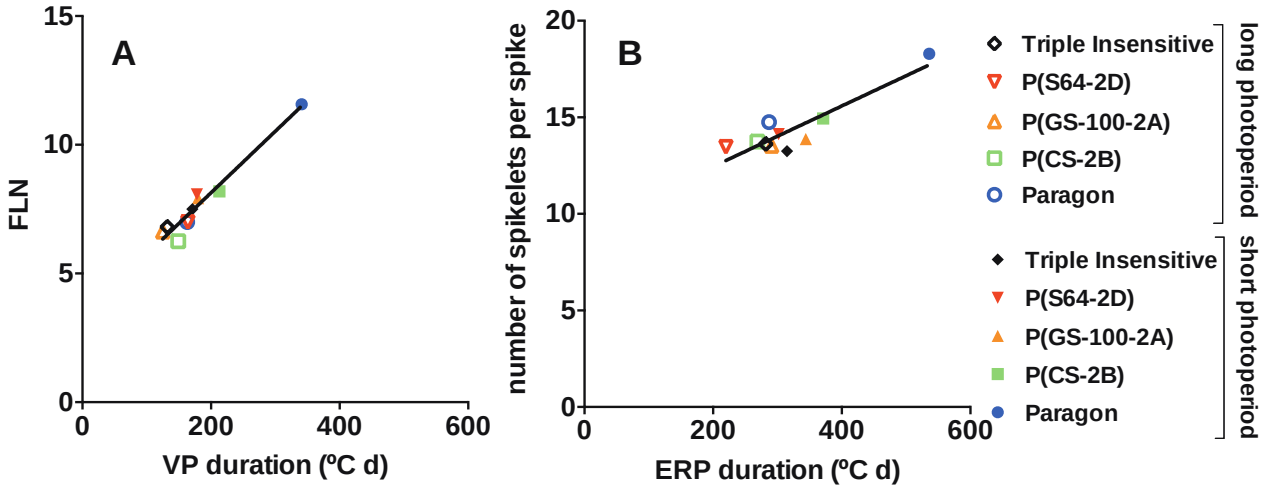

Fig. 6. Relationship between number of structures differentiated and the duration of the phase during which they were differentiated. (*a*) Final leaf number (FLN) and duration of the vegetative phase (VP), (R^2 = 0.95, P < 0.001); and (*b*) number of spikelets per spike and duration of the early reproductive phase (ERP); (with Paragon 12 h, R^2 = 0.825, P < 0.001; without Paragon 12 h, R^2 = 0.25, P < 0.175).

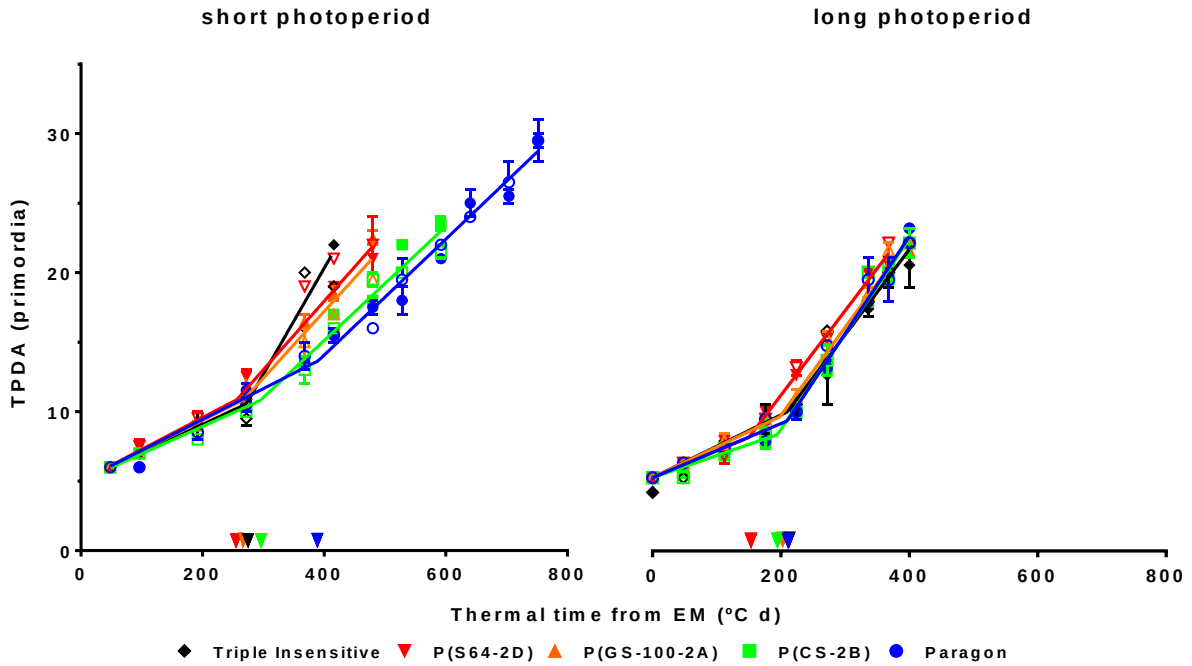

Fig. 7. Primordia differentiation dynamics. Relationship between total primordia number differentiated in the apex (TPDA) and thermal time from EM in both short and long photoperiod (left and right panels). Each data-point is the average from each experiment (1, closed symbols and 2, open symbols). Error bars for each data-point stand for s.e. (when not visible it was smaller than the diameter of the symbol). Data-points for each genotype and photoperiodic condition were fitted with a segmental linear regression ($R2 > 0.98$, $P < 0.001$ in all cases). The model fitted yielded two primordia differentiation rates: a slower first slope – mostly for leaves – and second faster one – mostly for spikelets. Arrowheads indicate timing of change of rate for each case.

Table 1. Allelic composition for *Ppd-1* of each genotype

The A, B and D genomes of the five genotypes compared in the study: Paragon (a spring cultivar with the three sensitive alleles), and its four NILs: Paragon (P) with *Ppd-A1a*, *Ppd-B1a*, or *Ppd-D1a*, and with the three of them together (triple insensitive). The donors of the insensitive alleles were GS-100, Chinese Spring and Sonora 64, respectively. a: photoperiod-insensitive allele; b: photoperiod-sensitive allele

Genotype	Allelic composition		
Paragon	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1b*
P(GS-100-2A)	*Ppd-A1a*	*Ppd-B1b*	*Ppd-D1b*
P(CS-2B)	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*
P(S64-2D)	*Ppd-A1b*	*Ppd-B1b*	*Ppd-D1a*
Triple insensitive	*Ppd-A1a*	*Ppd-B1a*	*Ppd-D1a*

Table 2. Mean squares for the main effects of experiment (Exp), photoperiod (Phot) and genotype (Gen) and interactions Gen × Phot and Gen × Phot × Exp for ANOVA tests performed on durations of phases and on number of structures produced in the apex of the main shoot

Durations considered were those of the cycle from seedling emergence (EM) to anthesis (AN) or to terminal spikelet (TS) and of each pre-anthesis phases (vegetative phase (VP), early reproductive phase (ERP) and late reproductive phase (LRP)). Numbers of structures considered were total number of primordia differentiated at the apex (TPDA) and its components: final leaf number (FLN) and spikelets per spike (SPKLTS SPK^{-1}). On the right of each mean square it is indicated whether the effect was statistically significant: (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant

Source of variation	Duration (10^{-3} °C d)					Structures produced (leaves and/or spikelets)		
	EM–AN	EM–TS	VP	ERP	LRP	TPDA	FLN	SPKLTS SPK^{-1}
Experiment	83.5 ***	3.2 *	0.2 ns	2.0 ns	10.0 *	0.5 ns	0.2 ns	0.1 ns
Photoperiod	4514.2 ***	302.8 ***	49.5 ***	107.5 ***	429.7 ***	334.2 ***	136.9 ***	43.2 ***
Genotype	1091.4 ***	69.7 ***	13.7 ***	24.6 ***	199.9 ***	118.7 ***	22.6 ***	41.2 ***
Gen × Phot	511.2 ***	42.7 ***	8.1 ***	14.6 ***	94.4 ***	62.4 ***	16.9 ***	15.6 ***
Gen × Phot × Exp	3.4 ns	0.4 ns	0.1 ns	0.5 ns	2.0 ns	1.0 ns	0.2 ns	1.1 ns

Table 3. Final leaf number (FLN), number of spikelets per spike (SPKLTS SPK^{-1}) and total number of primordia differentiated in the apex (TPDA) for each genotype under long (16 h) or short (12 h) photoperiod

Different letters within columns indicate statistically significant differences amongst genotypes and photoperiods (Tukey $\alpha = 0.05$)

Photoperiod	Genotype	FLN (leaves)	SPKLTS SPK^{-1} (spikelets)	TPDA (primordia)
16 h	Triple Insensitive	6.8 b	13.6 abc	20.4 ab
	P(S64-2D)	7.0 bc	13.5 ab	20.5 ab
	P(GS-100-2A)	6.6 ab	13.5 ab	20.1 a
	P(CS-2B)	6.3 a	13.8 abc	20.0 a
	Paragon	7.0 bc	14.7 bc	21.7 b cd
12 h	Triple Insensitive	7.5 cd	13.3 a	20.8 ab
	P(S64-2D)	8.1 e	14.1 abc	22.2 cd
	P(GS-100-2A)	7.9 de	13.9 abc	21.9 bcd
	P(CS-2B)	8.2 e	14.9 c	23.1 d
	Paragon	11.6 f	18.3 d	29.9 e

Table 4. Leaf primordia differentiation rate (LPDR), spikelet primordia differentiation rate (SPDR) and timing of change of rate (ToCR)

The three variables (LPDR, SPDR and ToCR) were parameters estimated from the segmental linear regression between number of primordia differentiated in the apex and thermal time from emergence (EM) for each genotype when grown under long (16 h) or short (12 h) photoperiod. Shared letters within columns indicate that the CI95 of the parameter estimation overlapped.

Photoperiod	NIL	LPDR(leaves (°C d)$^{-1}$)	SPDR (spikelets (°C d)$^{-1}$)	ToCR (°C d from EM)
16 h	Triple Insensitive	0.021 a	0.058 a	210 abc
	P(S64-2D)	0.018 a	0.058 a	150 a
	P(GS-100-2A)	0.021 a	0.060 a	198 ab
	P(CS-2B)	0.015 a	0.065 a	195 ab
	Paragon	0.018 a	0.066 a	210 bc
12h	Triple Insensitive	0.020 a	0.076 a	272 bcd
	P(S64-2D)	0.022 a	0.049 ab	256 abcd
	P(GS-100-2A)	0.021 a	0.048 ab	267 bcd
	P(CS-2B)	0.019 a	0.041 b	294 cd
	Paragon	0.022 a	0.041 b	390 d

Supplemental Figure 1: Detail on the qualitative response of Paragon to short photoperiod. Photographs of spikes show post-terminal spikelet stalled development in Paragon plants grown under 12 h photoperiod (top and bottom left). Bottom-right picture shows detail on aborted apical spikelets seen on these spikes. Photographs were taken at the end of the experiment, c. 2300ºC d after seedling emergence –when plants that were not developing further were dissected. Segments in each panel are equivalent to 1 cm.