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Night-Break Experiments Shed Light on the Photoperiod1-Mediated Flowering[1[OPEN]]

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Plants utilize variation in day length (photoperiod) to anticipate seasonal changes. They respond by modulating their growth and development to maximize seed production, which in cereal crops is directly related to yield. In wheat (*Triticum aestivum*), the acceleration of flowering under long days (LD) is dependent on the light induction of *PHOTOPERIOD1* (*PPD1*) by phytochromes. Under LD, *PPD1* activates *FLOWERING LOCUS T1* (*FT1*), a mobile signaling protein that travels from the leaves to the shoot apical meristem to promote flowering. Here, we show that the interruption of long nights by short pulses of light ("night-break" [NB]) accelerates wheat flowering, suggesting that the duration of the night is critical for wheat photoperiodic response. *PPD1* transcription was rapidly upregulated by NBs, and the magnitude of this induction increased with the length of darkness preceding the NB. Cycloheximide abolished the NB up-regulation of *PPD1*, suggesting that this process is dependent on active protein synthesis during darkness. While one NB was sufficient to induce *PPD1*, more than 15 NBs were required to induce high levels of *FT1* expression and a strong acceleration of flowering. Multiple NBs did not affect the expression of core circadian clock genes. The acceleration of flowering by NB disappeared in *ppd1*-null mutants, demonstrating that this response is mediated by *PPD1*. The acceleration of flowering was strongest when NBs were applied in the middle of the night, suggesting that in addition to *PPD1*, other circadian-controlled factors are required for the up-regulation of *FT1* expression and the acceleration of flowering.

Plants can anticipate diurnal and seasonal fluctuations in their environment and adjust their growth and development to coincide with favorable conditions. In flowering plants, reproductive development must be optimally timed to minimize the risk of damage to sensitive floral organs by late frosts or early high temperatures. The correct timing of this transition is a major determinant of reproductive success and, in cereal crops such as wheat (*Triticum aestivum*), of grain yield. Therefore, an improved understanding of the regulation of flowering time can contribute to the development of crop varieties better adapted to diverse environments.

Photoperiodic flowering responses vary in different species; short-day (SD) plants and long-day (LD) plants exhibit accelerated flowering in SD and LD, respectively, while day-neutral plants exhibit similar flowering profiles irrespective of day length (Garner and Allard, 1920, 1923). Plants possess complex regulatory mechanisms to perceive and respond to changes in photoperiod, which ensure that flowering occurs only under inductive conditions. The regulation of flowering time by photoperiod is best understood in Arabidopsis (*Arabidopsis thaliana*), a LD plant where the photoperiodic response is mainly controlled by CONSTANS (CO). *CO* expression is regulated by the circadian clock and peaks in the late afternoon and evening (Suárez-López et al., 2001). The CO protein is destabilized and degraded in darkness, ensuring that CO activity is restricted to LD photoperiods, when the peak of *CO* transcription coincides with external light (Valverde et al., 2004). Under these conditions, CO directly activates *FLOWERING LOCUS T* (*FT*) expression (Valverde et al., 2004; Tiwari et al., 2010). *FT* encodes a mobile signaling protein (florigen) with homology to members of the PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN family, which is synthesized in the leaves and travels to the shoot apical meristem via the phloem (Corbesier et al., 2007), where it binds the promoter of the meristem identity gene *APETALA1* and induces flowering (Wigge et al., 2005).

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S.P. and J.D. initiated the project; S.P., H.L., L.S., C.L., and J.D. designed the experiments; S.P., L.S., J.C., and H.L. performed the experiments; S.P. wrote the first draft of the manuscript; all authors contributed to data analysis and revision of the manuscript.

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Similarly, *FT* homologs in grass species such as rice (*Oryza sativa; Heading date3a* [*Hd3a*]) and wheat (*FT1* or *VERNALIZATION3*) function as flowering promoters. Upon arrival at the shoot apical meristem, FT-like proteins form part of a hexameric floral activation complex that binds the promoters of MADS-box meristem identity genes, inducing flowering development (Taoka et al., 2011; Li et al., 2015). Overexpression of *FT1* in transgenic wheat plants results in an early-flowering phenotype, even under noninductive SD conditions, whereas plants carrying loss-of-function mutations in *FT1* exhibit a late-flowering phenotype (Lv et al., 2014).

CO plays a role in the photoperiodic response in several grass species, such as rice (Hayama et al., 2003), sorghum (*Sorghum bicolor*; Yang et al., 2014), and barley (*Hordeum vulgare*; Campoli et al., 2012; Alqudah et al., 2014). However, these species also possess an additional photoperiod pathway that is not present in Arabidopsis, in which the *PHOTOPERIOD1* (*PPD1*) gene plays a central role. *PPD1* (named *PRR37* in rice and sorghum) encodes a member of the PSEUDO RESPONSE REGULATOR (PRR) protein family and is homologous to the Arabidopsis circadian clock genes *PRR3* and *PRR7* (Farré and Liu, 2013). The duplication that originated *PRR3* and *PRR7* in Arabidopsis and *PRR37* and *PRR73* in the grasses are independent, and therefore their subfunctionalization is independent (Farré and Liu, 2013). In Arabidopsis, *PRR3* and *PRR7* encode components of the circadian clock, and their disruption alters the expression of other clock genes (Farré et al., 2005). By contrast, variation in *PPD1*/*PRR37* in the grasses has no impact on the periodicity of the circadian response (Murphy et al., 2011; Shaw et al., 2012). These results suggest that after its duplication in the grass lineage, *PPD1* evolved as a photoperiod gene that functions as an output of the circadian clock.

Most natural variants in the photoperiodic response in wheat are associated with deletions in the promoters of *PPD-A1* (Wilhelm et al., 2009) or *PPD-D1* (Beales et al., 2007) or with differences in *PPD-B1* copy number (Díaz et al., 2012). The promoter deletions in the *Ppd-A1a* or *Ppd-D1a* alleles are associated with the misexpression of *PPD1* during the night, the induction of *FT1*, and the acceleration of flowering under SD (Beales et al., 2007; Wilhelm et al., 2009). Plants carrying these alleles still flower earlier under LD than under SD and, therefore, will be referred to as "reduced photoperiodic response" alleles (rather than as "photoperiod-insensitive" alleles).

The acceleration of flowering by *PPD1* requires its transcriptional activation by light, which is mediated by two members of the phytochrome family, PHYB and PHYC (Chen et al., 2014; Pearce et al., 2016). The phytochromes absorb light maximally in the red (R) and far-red (FR) spectrum and exist as two interchangeable isoforms, the inactive R light absorbing Pr form synthesized in the cytoplasm and the active FR light absorbing Pfr form that is translocated to the nucleus (Nagatani, 2004; Rockwell et al., 2006). Upon arriving in the nucleus, Pfr phytochromes interact with bHLH proteins known as PHYTOCHROME-INTERACTING FACTORS (PIFs), which initiates a cascade of light-regulated signaling pathways (Leivar and Monte, 2014). During darkness and upon exposure to FR light, Pfr phytochromes revert to the inactive Pr form.

Despite the molecular characterization of some of the components of the *PPD1*-dependent flowering pathway in wheat, there are still large gaps in our knowledge of the mechanisms involved in the light regulation of *PPD1* and *FT1* and in the perception of photoperiodic differences. In this study, we characterized the response of wheat when exposed to short pulses of light during the long nights of SD photoperiods, which are referred to as night-breaks or NBs hereafter. NB experiments have the advantage of modifying photoperiods without changing the total hours of light received by the plant.

NBs cause significant delays in flowering when applied to SD plants grown under SD (Hamner and Bonner, 1938; Coulter and Hamner, 1964; Lumsden and Furuya, 1986; Ishikawa et al., 2005). The greatest inhibition of flowering (henceforth NBmax) occurs when NBs are applied in the middle of the night (Thomas and Vince-Prue, 1997). These observations demonstrate that the duration of the night is critical to regulate flowering time in many SD plants and that the NB response can be characterized as a transient period of sensitivity to light that inhibits flowering. In rice, a single NB was sufficient to inhibit flowering in SD via the PHYB-mediated transcriptional repression of *Hd3a* (Ishikawa et al., 2005; Ishikawa et al., 2009). These observations are consistent with the external coincidence model of flowering, according to which flowering is induced when external light and internal oscillating circadian signals coincide (Bünning, 1936; Pittendrigh and Minis, 1964).

In this study, we show that NBs accelerate flowering in wheat plants grown under SD and that the response is strongest in the middle of the night. Using *ppd1*-null mutants, we demonstrate that this response is mediated by *PPD1*. We also show that although *PPD1* transcription is rapidly induced within 1 h of exposure to a single NB, multiple NBs are required for induction of *FT1* to high levels and for early flowering. Finally, we show that the magnitude of *PPD1* induction in response to NBs increases in accordance with the length of darkness preceding the light signal and that this induction is dependent on active protein synthesis during darkness.

RESULTS

NBs Induce *PPD-B1* and *FT1* Expression and Accelerate Flowering in Photoperiod-Sensitive Wheat

Near-isogenic plants of the tetraploid wheat variety Kronos carrying the photoperiod-sensitive *Ppd-A1b* allele (henceforth Kronos-PS) head rapidly (average 52.8 d) in a LD photoperiod (16 h light at 22°C/8 h dark at 17°C) but exhibit large delays in heading date (>150 d; Fig. 1A) when grown in a SD photoperiod (8 h light/16 h dark).

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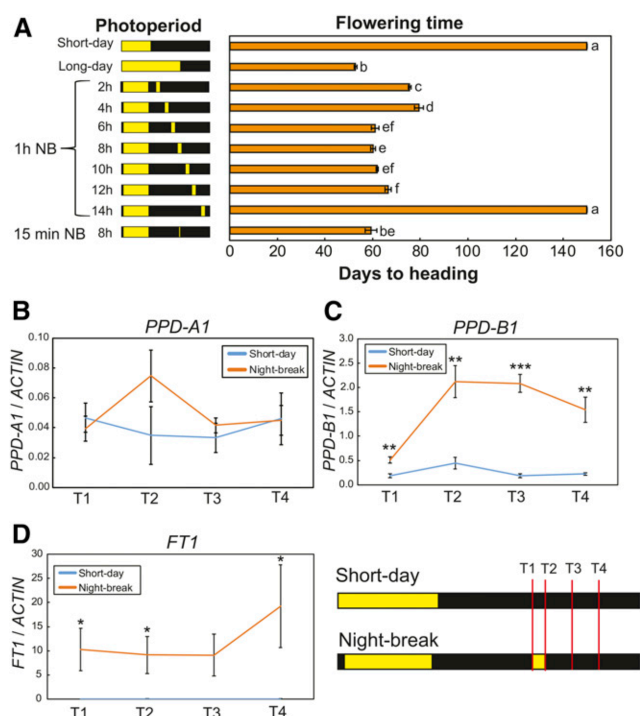

Figure 1. Effect of NB on heading time and the expression of flowering time genes in Kronos-PS (*Ppd-A1b*) plants. A, Heading time of plants grown from germination in SD, LD, and NB conditions. Plants were exposed to 1 h NBs after 2 to 14 h of darkness as indicated. In one experiment, a 15 min NB was applied after 8 h of darkness. Heading time is reported as days to heading ± SE ($n > 5$). Different letters beside the bars denote a significant difference between treatments ($P < 0.05$, Tukey's test). B to D, Expression of flowering time genes in the leaves of 6-week-old plants grown since germination in SD and NBmax conditions. B, *PPD-A1*; C, *PPD-B1*; D, *FT1* expression reported in fold-*ACTIN* levels. Kronos-PS plants were grown for 6 weeks either in SD or in NB conditions with the pulse of light applied after 8 h of darkness. Samples were harvested immediately before the NB (T1), immediately after the NB (T2), then 3 (T3) and 5 h (T4) after the start of the NB. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$.

When the long nights of SD were interrupted by 1 h pulses of white light at different points of the night (NB), flowering of the Kronos-PS plants was accelerated (Fig. 1A).

The timing of the NB had a strong effect on heading date, with a maximum acceleration (NBmax) when the NB was applied in the middle of the night (after 8 h of darkness). Under these conditions, plants headed just 7 d later than those grown in a LD photoperiod (Fig. 1A). NBs applied either earlier or later than this point had a weaker effect on heading date, although among plants exposed to NBs after 6, 8, or 10 h of darkness, heading date was not significantly different ($P > 0.05$; Fig. 1A). NBs of 15 min given after 8 h of darkness were equally effective in accelerating flowering as 1 h NBs applied at the same time (Fig. 1A).

To characterize the transcriptional responses associated with accelerated flowering in NB, we compared the expression levels of selected flowering time genes in 6-week-old plants grown since germination under NBmax conditions with those maintained in a SD photoperiod. Because allelic variation at the *PPD1* loci can affect the expression of each homeolog separately, we measured *PPD-A1* and *PPD-B1* transcript levels using homeolog-specific assays. For all other targets, quantitative reverse transcription (qRT)-PCR assays that amplify both A and B homeologs were used (Supplemental Table S1).

In SD-grown Kronos-PS plants, *PPD-A1* and *PPD-B1* expression levels remained low throughout the night, and *FT1* transcripts were not detected at any of the analyzed time points (Fig. 1, B–D). In plants grown in NB conditions from germination, *PPD-A1* transcript levels doubled in response to NB, but this homeolog was expressed at very low levels in all assayed time points (<0.1-fold *ACTIN*; Fig. 1B). By contrast, *PPD-B1* transcript levels were approximately 20-fold higher than *PPD-A1* before NB (time point T1) and 26-fold higher after NB (time point T2), suggesting that the *PPD-B1* homeolog contributes the majority of *PPD1* transcripts in photoperiod-sensitive tetraploid wheat. This result is consistent with a previous study in the hexaploid wheat variety Paragon, where *PPD-B1* accounted for 90% of all *PPD1* transcripts (Shaw et al., 2012).

PPD-B1 expression was significantly higher ($P < 0.01$) in NB than in SD conditions at all time points and was rapidly upregulated by NB, peaking between 1 h and 3 h after the start of the NB (Fig. 1C). *FT1* transcript levels were significantly higher in NB conditions (~10- to 20-fold *ACTIN*) than in SD ($P < 0.05$) and showed increased expression 5 h after the start of the NB (Fig. 1D). Even before exposure to NB, *FT1* transcript levels were significantly higher in plants grown under NB since germination than in those grown under SD (T1 time point; Fig 1D).

FLOWERING LOCUS T2 (*FT2*) and *VERNALIZATION1* expression levels were also elevated in plants grown in NB, while *FLOWERING LOCUS T3* (*FT3*) expression was reduced in comparison to SD-grown plants (Supplemental Fig. S1, A–C). These results show that the transcriptional regulation of these flowering time genes in NB is similar to their regulation in LD photoperiods (Lv et al., 2014).

The *Ppd-A1a* Allele with Reduced Sensitivity to Photoperiod Reduces the NB Effect on Heading Time

We next studied the NB response in wheat plants carrying the *Ppd-A1a* allele, which confers reduced sensitivity to photoperiod by accelerating flowering in SD (Wilhelm et al., 2009). Under LD, these plants headed 26.2 d earlier ($P < 0.001$) than under SD, showing that they still have some sensitivity to photoperiod (Fig. 2A). However, the differences in heading date between LD and SD were greatly reduced when compared to the differences in Kronos-PS lines grown under the same conditions (>95 d delay; Fig. 1A). This reduced sensitivity to photoperiod was also reflected in the NB response. Plants grown since germination in different NBs showed accelerated flowering compared to SD, but the timing of the NB had less impact on heading date than in the Kronos-PS lines (Fig. 2A).

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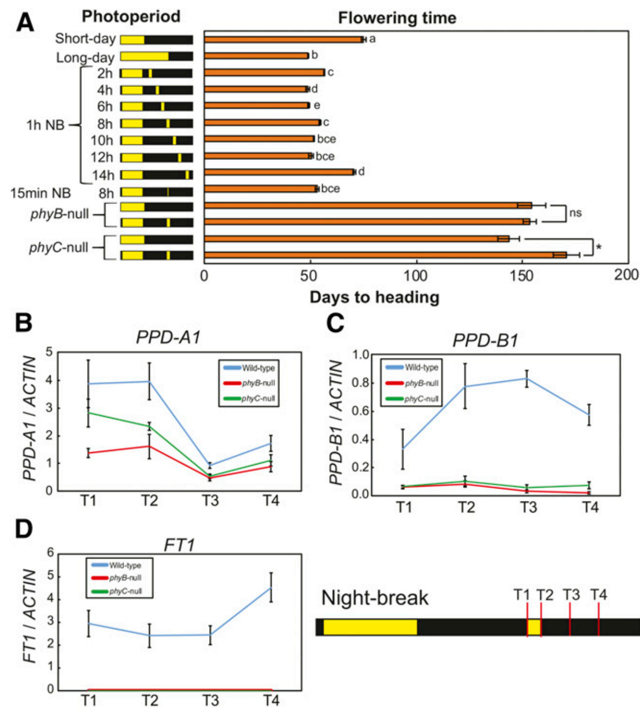

Figure 2. NB response in Kronos plants carrying the *Ppd-A1a* allele conferring reduced sensitivity to photoperiod with either wild-type or mutant *PHYB* (*phyB*-null) or *PHYC* alleles (*phyC*-null). A, Heading time of wild type, *phyB*-null, and *phyC*-null mutants in SD and LD conditions and in response to NBs applied at different times of the night (2–14 h, as indicated). Different letters beside the bars denote a significant difference between treatments ($P<0.05$, Tukey's test). Expression levels of (B) *PPD-A1*, (C) *PPD-B1*, and (D) *FT1* in response to NBs in the leaves of 6-week-old wild-type, *phyB*-null, and *phyC*-null plants reported in fold-*ACTIN* levels. Plants were grown for 6 weeks in NB conditions with the pulse of light applied after 8 h of darkness. Sampling time points for expression analysis are shown at the bottom of the figure and are also described in the legend of Figure 1. $^{*}P < 0.05$. ns, Not significant.

In 6-week-old *Ppd-A1a* plants grown since germination in NBmax conditions, *PPD-A1* transcript levels during the night were approximately 10-fold higher than those of *PPD-B1* (T1 time point, wild-type genotype; Fig. 2, B and C), confirming previous results (Wilhelm et al., 2009). *FT1* was also expressed at high levels before the NB (~3-fold *ACTIN*) in these plants (Fig. 2D). Following NB, *PPD-A1* expression was reduced but remained higher than *PPD-B1* (>1-fold *ACTIN*) in all time points assayed (Fig. 2B). Both *PPD-B1* and *FT1* exhibited similar induction profiles in response to NB (Fig. 2, C and D) as in Kronos-PS lines (Fig. 1, C and D).

Acceleration of Flowering by NBs Requires Both PHYB and PHYC

The acceleration of flowering in LD photoperiods requires the activity of both PHYB and PHYC (Chen et al., 2014; Pearce et al., 2016), so we next tested the NB response in Kronos mutants carrying nonfunctional copies of both homeologs of *PHYB* (*phyB*-null) or of *PHYC* (*phyC*-null). All plants in this experiment carried the *Ppd-A1a* allele conferring reduced sensitivity to photoperiod. Under SD photoperiods, both *phyB*-null and *phyC*-null mutants exhibited very late-flowering phenotypes when compared to the wild-type control line (Fig. 2A). Exposure to NB had no effect on heading date in *phyB*-null mutants and resulted in a 27.2 d delay in heading in the *phyC*-null mutants ($P = 0.018$; Fig. 2A).

We next studied the transcriptional responses of *PPD1* and *FT1* in these mutants following NB. *PPD-A1* was expressed in both *phyB*-null and *phyC*-null mutants at all time points but was significantly lower in the mutants than in the wild-type plants at time points T2 and T3 ($P < 0.05$; Fig. 2B). Transcript levels of *PPD-B1* were significantly lower in both mutants when compared to the wild type at time points T2, T3, and T4 ($P < 0.001$; Fig. 2C) and did not increase in response to NB. Despite the relatively high transcript levels of *PPD-A1* in the *phyB*-null and *phyC*-null mutants at several time points (Fig. 2B), *FT1* was not expressed in either mutant (Fig. 2D). These results demonstrate that both PHYB and PHYC are required for the induction of *PPD-B1* and *FT1* by NB.

Acceleration of Flowering by NBs Is Partially Suppressed by FR Light Treatment

Phytochromes are activated and inactivated following exposure to R and FR light, respectively, so we tested the effects of FR light treatment on the NB response. Kronos-PS plants were grown under two different conditions from germination. In one chamber, plants were exposed to a 1 h NB after 8 h of darkness, and in the other chamber, plants were exposed to the same conditions except that the 1 h NB was followed by a 30 min pulse of FR light. Plants exposed to FR light exhibited a delay of 8.9 d in heading date when compared to control plants, but the difference was not significant ($P = 0.19$; Fig. 3A).

One possible reason for the mild effect of this FR treatment on heading date could be that the exposure to 1 h of white light was sufficient for the irreversible activation of downstream genes or proteins in the flowering induction pathway before the FR light inactivation of the phytochromes. To test this possibility, we applied the NBs as 15 1-min pulses of white light intercalated either with 15 1-min periods of darkness (control chamber) or 15 1-min pulses of FR light (FR chamber). Application of the NB using this protocol was less effective in accelerating heading than when the NB was given as a 1 h block of white light, but the FR treatment had a proportionally larger effect and significantly delayed heading date (average 22.5 d delay, $P = 0.012$; Fig. 3A). At the transcriptional level, *PPD-B1* expression was significantly reduced only by the pulsed FR treatment ($P < 0.01$ in the latter three time-points; Fig. 3, B and C). These results suggest that despite the absolute requirement of PHYB and PHYC function for the NB response, the FR light conditions used in these experiments were not sufficient to abolish the NB response completely.

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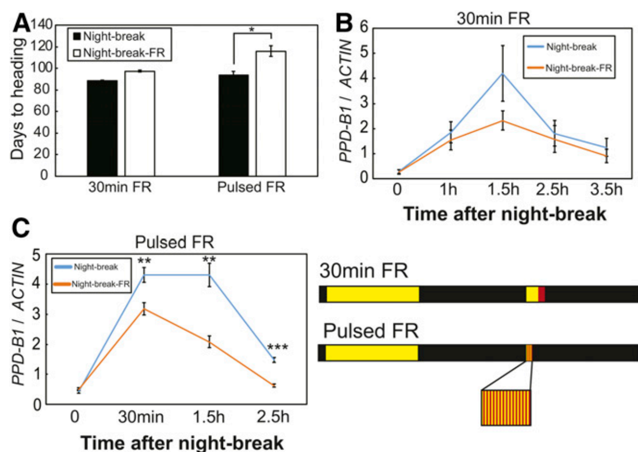

Figure 3. Effect of FR light treatment on heading time and expression of *PPD-B1*. A, Heading time of Kronos-PS plants in response to FR treatment. FR light was applied either as a 30 min block immediately after a 1 h NB (30 min FR) or as 15 1-min pulses of white light intercalated with 15 1-min pulses of FR light (pulsed FR). *PPD-B1* expression in B, 30 min FR or C, pulsed FR treatment. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Acceleration of Flowering by NBs Requires PPD1

To determine the importance of *PPD1* for the NB response, we compared wild-type and *ppd1*-null mutant lines lacking all functional copies of *PPD1* in the photoperiod-sensitive hexaploid variety Paragon (Shaw et al., 2013) and in the tetraploid line Kronos-PS (developed in this study; see "Materials and Methods"). We first measured heading date in these lines when grown in SD or LD conditions since germination. Under SD conditions, neither the wild type nor the *ppd1*-null mutants of either variety flowered within 150 d, when the experiment was terminated (Fig. 4A). Under LD conditions, the *ppd1*-null mutants headed 60 d and 34 d later than the wild type Kronos-PS and Paragon-PS lines respectively (Fig. 4A).

In a separate experiment using slightly different conditions (plants were first grown under SDs for 4 weeks before moving to NBmax conditions), we compared the effect of NBmax in photoperiod-sensitive and *ppd1*-null mutant lines. Kronos-PS and Paragon-PS plants headed on average at 73 d and 91 d under NB, respectively, but neither *ppd1*-null line flowered within 150 d, when the experiment was terminated (Fig. 4B). These results demonstrated that *PPD1* plays a major role in the effect of NB and LD on heading time.

We next assayed *PPD-B1* and *FT1* transcript levels in Kronos-PS and *ppd1*-null plants at four time points, including dusk, when these flowering time genes are normally expressed at high levels under LD (Chen et al., 2014). Plants were grown in SDs for 4 weeks, then either maintained in SD conditions or moved to NBmax conditions for 6 weeks. In Kronos-PS plants, *PPD-B1* transcript levels were upregulated 1 h and 3 h after the start of the NB (similar to Figs. 1C and 2C) and to even higher levels at dusk (Fig. 4C). In the Kronos-PS plants kept under SD, *PPD-B1* transcript levels were not upregulated during the night but showed an increase at dusk, although the levels were significantly lower ($P = 0.04$) than in plants that were exposed to multiple NBs (Fig. 4C). As expected, *PPD-B1* transcripts in the Kronos *ppd1*-null mutants were not detected in either SD or NB conditions, confirming the specificity of the qRT-PCR primers used in this assay (Fig. 4C). Consistent with previous results, *FT1* transcripts were undetected in Kronos-PS plants under SD but were highly upregulated in NB conditions at all time points ($P < 0.001$; Fig. 4D). However, in the *ppd1*-null mutant, *FT1* transcripts were not detected in any sampled time points, including dusk, under either SD or NB conditions.

PPD1 and *FT1* Respond Differently to NBs

To investigate the effects of single NBs on the transcriptional activation of *PPD1* and *FT1* at a greater resolution during the initial hour of the NB, we grew Kronos-PS plants for 4 weeks in SD photoperiods before exposing them to a single NB. Immediately before exposure to the NB, *PPD-B1* expression was very low (< 0.1-fold *ACTIN*) but increased rapidly between 30 min and 1 h (Fig. 5A). Transcript levels peaked 3 h after the start of the NB before falling 2 h later (5 h time point; Fig. 5A). The effect of NB on *PPD-B1* expression at 1, 3, and 5 h after NB, was similar to the pattern observed in 6-week-old plants grown under NB since germination (Fig. 1C). We also observed a similar *PPD-B1* transcriptional profile in response to a 15 min NB (Fig. 5B). However, the rapid

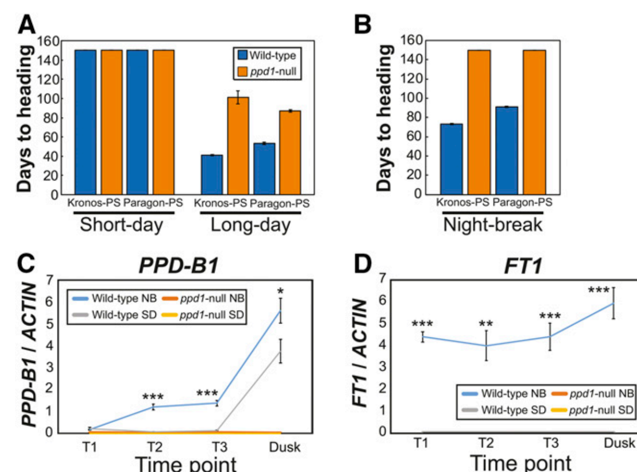

Figure 4. NB response in *ppd1*-null plants. A, Heading date of wild type and *ppd1*-null mutants grown since germination in SD or LD conditions. B, Heading date of wild type and *ppd1*-null mutants in NB conditions. Plants were grown in SD conditions for 4 weeks before being transferred to NBmax conditions. Isogenic lines in tetraploid Kronos and hexaploid Paragon backgrounds were used. All experiments were terminated at 150 d. Expression of *PPD-B1* (C) and *FT1* (D) in fold-*ACTIN* levels in Kronos-PS and Kronos *ppd1*-null plants grown for 10 weeks in SD conditions or for 4 weeks in SD conditions followed by 6 weeks in NBmax conditions. Samples were harvested immediately before the NB (T1), immediately after the NB (T2), then 3 h after the start of the NB (T3) and dusk of the following day. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$.

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induction of *PPD-B1* was not reflected in *FT1* transcript levels, which remained low at all assayed time points following a single NB (Fig. 5, A and B).

Six-week-old Kronos-PS plants grown since germination under NB exhibited significantly increased expression of both *PPD-B1* and *FT1* in the darkness before the NB (time point T1 in Fig. 1, C and D), suggesting that transcripts of these genes may accumulate in response to multiple NBs. We therefore measured *PPD-B1* and *FT1* expression levels in response to increasing numbers of NBs by assaying transcript levels immediately before the NB. While *PPD-B1* transcript levels were slightly elevated in samples exposed to more than three NBs relative to plants maintained in SD conditions, we found no consistent evidence of increased *PPD-B1* expression in response to increasing numbers of NBs (Figs. 4C and 5C). In contrast, transcript levels of *FT1*, which were undetectable after the first three consecutive NBs, showed a gradual increase after six NBs and a large increase after 18 NBs (Fig. 5C). We also tested the expression levels of *ZCCT2* (the functional *VERNALIZATION2* copy in Kronos), a LD repressor of *FT1* (Yan et al., 2004; Distelfeld et al., 2009), and found no transcriptional changes with increasing number of NBs (Fig. 5C).

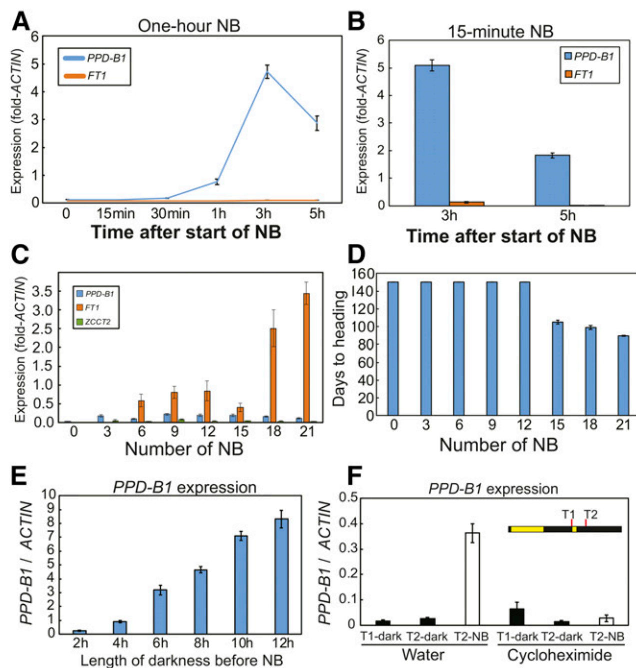

Figure 5. Transcriptional response of *PPD-B1* and *FT1* to single and multiple NBs. *PPD-B1* and *FT1* expression levels in Kronos-PS plants in response to a single 1 h (A) or 15 min (B) NB. C, Transcription of *PPD-B1*, *FT1*, and *ZCCT2* and (D) heading time of plants exposed to different numbers of NBs. Kronos-PS plants were grown in SD conditions for 4 weeks, exposed to different numbers of NBs after 8 h of darkness and returned to SD conditions thereafter. E, Effect of length of darkness before a single NB on the induction of *PPD-B1*. Kronos-PS plants were grown for 4 weeks in SD and exposed to a single NB at different stages of the night. Leaf tissue was harvested 3 h after the start of the NB. F, Effect of cycloheximide treatment on *PPD-B1* induction in response to NB (4-week-old Kronos-PS plants in water or cycloheximide hydroponic solutions). Half of the plants were kept in darkness, and half were exposed to a 1 h NB. Leaf tissue was harvested immediately before the NB (T1) and 3 h after the start of the NB (T2). All expression data are presented in fold-*ACTIN* levels.

The larger increases in *FT1* transcript levels after 18 NB were associated with accelerated heading time (Fig. 5D). Plants exposed to 21 NB were the earliest to head (average 88.7 d), followed by plants exposed to 18 NB (99 d) and 15 NB (105.8 d; Fig. 5D). Plants exposed to fewer than 15 NBs did not head within 150 d (Fig. 5D). To analyze spike development in these latter samples, we dissected one plant from each class and measured spike length. Plants that received zero, three, or six NBs were at the early stages of spike development (<5 mm), whereas those that received nine NB were more advanced (>15 mm; Supplemental Fig. S2).

The gradual acceleration of heading time in plants exposed to increasing number of NBs was similar to the acceleration observed after increasing numbers of LD (Supplemental Fig. S3). However, the effect of LD exposure was stronger than the effect following NB exposure. Fewer LDs than NBs were required to start the acceleration of flowering and the acceleration was stronger after 20 LD (Supplemental Fig. S3) than after 21 NB (Fig. 5D). This finding is consistent with our previous experiment showing that plants grown under LD since germination flowered ~7 d earlier than those grown in NBmax conditions since germination (Fig. 1A).

PPD-B1 Induction by NB Requires Active Protein Synthesis during Darkness

We next tested the effect of the timing of the NB on *PPD-B1* induction by exposing SD-grown plants to a single NB at different times of the night. We hypothesized that maximal induction of *PPD-B1* would coincide with the strongest acceleration of heading date (i.e. after 8 h of darkness). This hypothesis proved to be incorrect and, instead, we found that *PPD-B1* was induced to progressively higher levels in accordance with the duration of the dark period preceding the NB (Fig. 5E).

We first thought that the gradual accumulation of inactive Pr phytochromes in the nucleus resulting from dark reversion could explain the increased *PPD-B1* induction with longer periods of darkness. However, plants treated with FR light immediately before a NB applied after 2 h of darkness did not exhibit increased *PPD-B1* expression (Supplemental Fig. S4). This result suggested that the accumulation of Pr phytochromes in the nucleus was not responsible for the progressive induction of *PPD-B1* with extended dark periods.

We then thought that *PPD-B1* induction could be associated with the de novo synthesis of phytochromes or other intermediate proteins during darkness. To test this hypothesis, we grew Kronos-PS plants in hydroponic solution and treated half of them with cycloheximide to block protein synthesis and left half of the plants untreated as a control. Consistent with previous results, control plants maintained in darkness showed no

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induction of *PPD-B1*, whereas those exposed to a single NB exhibited strong up-regulation of *PPD-B1* expression 2 h after the NB (time point T2; Fig. 5F). The induction of *PPD-B1* in response to NB was abolished in plants treated with cycloheximide (Fig. 5F), which demonstrates that the expression of *PPD1* in response to light requires active protein synthesis during darkness. This experiment was performed twice with identical results.

Multiple NBs Did Not Alter the SD Entrainment of Circadian Clock Genes

Finally, we tested whether exposure to multiple NBs was sufficient to change the SD entrainment of the circadian clock core genes. Kronos-PS plants were grown for 7 weeks in SD or for 4 weeks in SD followed by 3 weeks in NBmax conditions. After 7 weeks, all plants were moved to free-running conditions (constant light and temperature) for 24 h and then leaves were sampled at 4-h intervals during an additional 24-h period of free-running conditions. The expression profiles of *PRR59*, *PRR95*, *PRR73*, *CIRCADIAN CLOCK ASSOCIATED1*, *GIGANTEA* (*GI*), and *TIMING OF CAB EXPRESSION1* showed no significant differences between SD- and NB-grown plants at all sampled time points (Supplemental Fig. S5, A–F). These results suggest that NBs have a limited impact on the expression profiles of the core circadian clock genes. In the same experiment, *PPD-B1* transcript levels were higher in the plants previously grown under NB than in the plants previously grown under SD (Supplemental Fig. S5G). The differences were particularly large at the subjective dusk in agreement with the results observed in Figure 4C. *FT1* transcript levels were high at all times in the samples from the plants grown under NB but were almost undetectable in the plants previously grown under SD (Supplemental Fig. S5H). This result confirmed that 2 d under continuous light are insufficient for the up-regulation of *FT1*.

DISCUSSION

NB Responses in SD and LD Plants

Many studies using NBs to characterize the effects of changing photoperiods on flowering time focused on SD plants, mainly because the inhibition of flowering by NB was found to be a simpler system of study than the acceleration of flowering by NB in LD plants (Thomas and Vince-Prue, 1997). Our characterization of the NB response in wheat highlights some of the similarities and differences between these two systems.

In many SD plants, flowering is inhibited by NB and in rice; this effect is associated with the suppression of *Hd3a* (orthologous to *FT*) transcription (Ishikawa et al., 2005). When rice plants are moved from NB back to inductive SD photoperiods, this inhibition is lost and *Hd3a* expression returns to high levels. In wheat, NBs also affect the expression of *FT1* and flowering time, although these responses are reversed. These results suggest SD and LD plants both respond to NB through regulatory mechanisms acting on *FT* expression. The opposite effect of NB on *FT* expression and flowering in rice and wheat is likely determined by the opposite roles of PPD1 (= PRR37 in rice and sorghum) in different grass species. In LD grasses, such as wheat and barley, PPD1 induces *FT1* and accelerates flowering (Turner et al., 2005; Shaw et al., 2013), whereas in SD grasses, such as sorghum and rice, PRR37 suppresses *FT*-like genes and delays flowering (Murphy et al., 2011; Koo et al., 2013).

The NB responses in SD and LD grasses also differ in their response to FR light after the NB. In some SD plants, the suppression of flowering by a single R light NB is completely reversible by immediate exposure to FR light (Downs, 1956; Cathey and Borthwick, 1957). In wheat, we found that a single FR exposure after NB had a limited effect on heading time (Fig. 3A). One-minute pulses of FR after 1-min pulses of white light were more effective (22.5 d delay in heading), but did not completely abolish the acceleration of heading by NB (Fig. 3A). The partial effect of FR light on the NB acceleration of flowering is consistent with previous results in the LD grass barley (Downs, 1956).

Finally, rice and wheat differ in the role of PHYC in the NB response. In rice, the NB response is completely abolished in plants carrying *PHYB* loss-of-function mutations but is unaffected by similar mutations in *PHYC* (Ishikawa et al., 2005, 2009). By contrast, the NB response in wheat is abolished in both the *phyB*-null and *phyC*-null mutants (Fig. 2). The different roles of PHYC on NB parallel the different roles played by this phytochrome in the photoperiodic response in wheat and rice. PHYC is a positive regulator of flowering time in some temperate grasses such as wheat, barley, and *Brachypodium distachyon* (Nishida et al., 2013; Chen et al., 2014; Woods et al., 2014) but has limited or no effect on flowering time in rice and Arabidopsis (Monte et al., 2003; Takano et al., 2005; Hu et al., 2013). These results suggest PHYC plays a more critical role in the photoperiod and NB response in the LD temperate grasses than in other plant species.

Multiple NBs Are Required for Flowering Induction in Wheat

Whereas a single NB is sufficient to repress flowering in rice (Ishikawa et al., 2005) and promote flowering in *Lolium temulentum* cv Ceres (Evans, 1958), multiple LDs are required to accelerate flowering in many temperate grasses (Heide, 1994). Most temperate grasses show some acceleration of flowering after being exposed to 4 to 8 LD photoperiods, but full saturation of this response requires 12 to 16 d of exposure to LD (Heide, 1994). These results are consistent with our observations for wheat, where 6 to 10 LDs induced a mild acceleration in flowering, but the greatest acceleration in flowering was seen in plants exposed to 12 or more LDs (Supplemental Fig. S3). The acceleration in heading time in response to increasing numbers of NBs was similar to that observed in response to increasing numbers of LDs,

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but the effects were smaller and at least 15 NBs were required to initiate the acceleration of flowering (Fig. 5D). These results are consistent with the existence of a *PPD1*-independent photoperiod pathway, which may be more responsive to LDs than to NBs.

In Arabidopsis, the induction of the transition from the vegetative to the reproductive apex also requires cycles of *FT* induction repeated over several days. However, while 4 to 5 LDs are sufficient to saturate the acceleration of flowering in Arabidopsis more than 20 LDs are required in wheat (Krzymuski et al., 2015). Possible explanations for the requirement of multiple NBs or LDs to induce *FT1* in wheat include a gradual accumulation of a flowering promoter, a gradual reduction of a flowering repressor, or a gradual change in epigenetic marks in some of the involved genes. No correlation was detected between the number of NBs and transcript levels of *ZCCT2* (a repressor of *FT1*), suggesting that this gene is not critical for the observed changes in *FT1* in this genetic background (Fig. 5C). Similarly, *PPD1* transcript levels did not increase in response to multiple NBs, indicating that the putative accumulating factor is unlikely to be a regulator of *PPD1* transcription. However, it is still possible that the number of NBs affect the levels of active PPD1 protein. To test this hypothesis, we have initiated the generation of transgenic wheat plants expressing an HA-tagged PPD1 protein. It is also possible that proteins other than PPD1 also play a role in the regulation of *FT1* in response to multiple NBs.

The Induction of *PPD-B1* by Light Increases during the Night

Interestingly, we found that the magnitude of *PPD-B1* induction by NBs was proportional to the length of darkness preceding the NB. This phenomenon appears to be unrelated to the accumulation of Pr phytochrome protein arising from dark reversion, since exposure to FR light prior to NBs (expected to increase the pool of Pr phytochromes) had no effect on the subsequent induction of *PPD1* by light (Supplemental Fig. S4). Instead, we found that treating plants with cycloheximide during the night abolished the NB up-regulation of *PPD1* (Fig. 5F), which suggests that the induction of *PPD1* by light is dependent on active protein synthesis during darkness.

One possibility is that the de novo synthesis of Pr isoforms of PHYB and/or PHYC during darkness is correlated with the strength of *PPD1* induction. During longer periods of darkness, newly synthesized PHYB and PHYC proteins would accumulate to higher levels, so that subsequent light signals would result in stronger induction of *PPD1*. An alternative possibility is the de novo synthesis and dark accumulation of a PHYB/PHYC-induced transcription factor required for the activation of *PPD1*. This may include one or more PIFs, which have been shown to act as coactivators of light-induced genes in some cases (Leivar and Monte, 2014). Additional experiments will be required to test these hypotheses and to identify the dark-synthesized protein responsible for the increased activation of *PPD1* with longer periods of darkness.

The Effect of NB on Flowering Time Is Likely Gated by One or More Circadian Clock-Regulated Genes

Despite the stronger NB induction of *PPD1* following longer periods of darkness, *PPD1* transcript levels were not directly correlated with heading date. The greatest effect of NB on heading date was observed when the NB was timed to coincide with the middle of the night, even though *PPD1* transcript levels were lower at this point than after NBs applied later in the night. This dependence on the time of the night suggests that PPD1 activity may be gated by circadian clock-regulated genes. The existence of a gating mechanism is also supported by the fact that although *PPD1* transcription is induced during the light phases of both SD and LD, *FT1* transcription is only observed under LD photoperiods (Beales et al., 2007; Wilhelm et al., 2009). Furthermore, rhythmic sensitivities for NB-induced flowering have been observed in other LD grasses (Thomas and Vince-Prue, 1997). *L. temulentum* cv Ceres plants, which are induced to flower by a single LD cycle, showed two phases of high sensitivity to NB when SD-grown plants were moved to constant darkness. The first phase occurred between 4 and 8 h from the start of the darkness period, and the second one was approximately 20 to 24 h later, suggesting the involvement of a circadian rhythm in the control of flowering in *L. temulentum* (Périlleux et al., 1994). Similar experiments would be challenging to perform in wheat because of the requirement for multiple NBs to induce flowering.

It is tempting to speculate that the regulation of *FT1* expression by PPD1 may function in a manner analogous to the regulation of *FT* by CO in Arabidopsis. In Arabidopsis, *FT* is induced only in LD conditions when the transcriptional peak of *CO* coincides with light, which is required to stabilize the CO protein (Valverde et al., 2004). In wheat, *FT1* induction and flowering may be determined by the coincidence of an external signal (light activation of *PPD1* transcription by PHYB/PHYC) with an internal rhythm mediated by the circadian clock.

In addition to this putative role in gating the effect of *PPD1*, the circadian clock is known to be involved in the regulation of *PPD1* expression (Beales et al., 2007; Chen et al., 2014; Alvarez et al., 2016). Plants carrying loss-of-function mutations in *EARLY FLOWERING3* (a component of the evening complex in the circadian clock) exhibited elevated expression of *PPD1* and earlier flowering under both LD and SD (Alvarez et al., 2016). Similar results were observed in *LUX ARRHYTHMO* mutants in diploid wheat, another component of the evening complex (Gawrónski et al., 2014). These results suggest that in the temperate cereals, the evening complex of the circadian clock acts as a transcriptional repressor of *PPD1* (Campoli et al., 2013; Alvarez et al., 2016). Interestingly, two LUX-binding sites (GATWCG; Chow et al., 2012) are present in the *PPD1* promoter, including one in the region deleted in the *Ppd-A1a* allele.

In barley, changes in photoperiod have been shown to have rapid effects on the expression of circadian clock genes (Deng et al., 2015). However, we did not observe significant changes in the expression profiles of

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any of the core circadian clock genes after 21 NBs, suggesting that changes in the clock played a limited role in the induction of flowering by NBs (Supplemental Fig. S5). Moreover, the strong delay in heading time observed in the Kronos and Paragon *ppd1*-null mutants under NBmax demonstrated that *PPD1* is the major driver of the acceleration of heading time by NBs. This does not rule out the possibility that the circadian clock may play an important role in the regulation of the intermediate steps between *PPD1* and *FT1* induction or in the *PPD1*-independent photoperiod pathway in the temperate grasses.

A Working Model for the *PPD1* Regulation of the Photoperiod Response in Wheat

In this study, we show that while a single NB as short as 15 min in duration is sufficient to induce *PPD1*, the peak of expression is not observed until 3 h after the NB (Fig. 5A). This timeline of events suggests that additional molecular steps may be involved in the transcriptional activation of *PPD1* following the initial short exposure to white light. NB responses have previously been shown to be rapid, and red-light NBs of 2 min were shown to be sufficient to accelerate flowering (Downs, 1956; Cathey and Borthwick, 1957).

The short length of the light pulse required to trigger the NB response is consistent with a role of the phytochromes in the initial steps of the NB response. In Arabidopsis, conversion of phytochromes from Pfr to Pr forms occurs within 5 min of exposure to high radiance R light, and 2 min of R light treatment is sufficient to initiate the phosphorylation of PIFs, which are direct targets of activated phytochromes (Park et al., 2004; Shen et al., 2007, 2008; Al-Sady et al., 2008). Phosphorylated PIFs are targeted for degradation by the 26S proteasome, triggering downstream transcriptional responses within 15 min of the light signals (Hwang and Quail, 2008). The time lag between the light application and the up-regulation of *PPD1* transcript levels (Fig. 5A) suggests the existence of intermediate molecular steps. Based on the involvement of wheat PHYB and PHYC in the light activation of *PPD1* transcription (Chen et al., 2014; Pearce et al., 2016) and the known interactions between phytochromes and PIFs in Arabidopsis, we hypothesize that the degradation of one or more PIFs acting as *PPD1* transcriptional repressors may be involved in the light activation of this gene. A putative PIF binding site is present within the region of the *PPD1* promoter that is deleted in the *Ppd-A1a* allele (which shows expression during the night). According to this hypothesis, the application of FR after NB reduces Pfr levels and limits the degradation of this putative PIF(s), thereby maintaining some transcriptional repression of *PPD1* (Fig. 3C).

Although NBs do not perfectly mimic the LD response, there are several similarities between the two processes, particularly in the *PPD1*-dependent photoperiodic response. Both processes are dependent on the PHYB/PHYC-mediated light activation of *PPD1*, both processes require multiple inductive cycles to accelerate flowering, and in both NBs and in plants carrying the *Ppd-A1a* allele, expression of *PPD1* during the night is associated with accelerated flowering. Based on these similarities and on previous studies, we propose a tentative working model for the *PPD1*-dependent photoperiodic regulation of flowering in wheat (Fig. 6). According to this model, flowering is accelerated only when the light-induced expression of *PPD1* coincides with the expression and/or activity of one or more circadian-regulated factor(s) required for the induction of *FT1*. Under LD, but not under SD, *PPD1* expression coincides with the putative additional factor, inducing *FT1* expression (Fig. 6, A and B). When NBs are applied in the middle of the night, light-induced *PPD1* expression coincides with a peak of the putative additional factor, resulting in maximal activation of *FT1* and early flowering (Fig. 6C). Although NBs applied earlier or

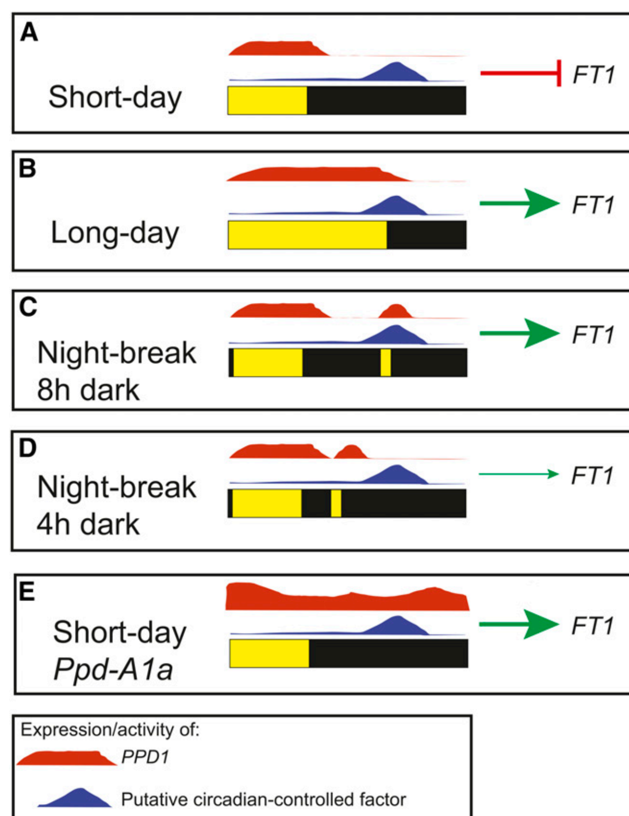

Figure 6. Working model for the photoperiod-mediated induction of flowering in wheat. The induction of *FT1* and acceleration of flowering occurs maximally when light-induced *PPD1* expression coincides with the activity of a factor regulated by the circadian clock. A, In SD, *PPD1* expression falls during darkness before the peak in activity of a putative component, unlike in LD (B), where *PPD1* expression coincides with this peak, inducing *FT1* and flowering. *PPD1* expression also coincides with this component when NB are given in the middle of the night (C), whereas NB applied earlier (D) result in reduced overlap of *PPD1* with this putative factor and have less impact on flowering time. E, The *Ppd-A1a* allele conferring reduced sensitivity to photoperiod is misexpressed during darkness, resulting in the activation of *FT1* and flowering, even in SDs.

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later than this point still result in the induction of *PPD1*, these NBs no longer coincide with a peak of the putative circadian-regulated factor required for the activation of *FT1*. In Arabidopsis, the sensitivity of the flowering response to the induction of *FT* expression is most effective when *FT* is artificially induced during the evening and early night (Krzymuski et al., 2015), suggesting that the timing of *FT* induction can also carry information relevant to the acceleration of flowering.

Other studies support the hypothesis that the timing of *PPD1* induction is critical for flowering. In wheat plants carrying the *Ppd-A1a* allele conferring reduced sensitivity to photoperiod, *PPD1* is expressed during darkness (Turner et al., 2005; Beales et al., 2007; Wilhelm et al., 2009). Therefore, even in noninductive SD photoperiods, *PPD1* expression coincides with the peak activity of the putative circadian-regulated factor required for the activation of *FT1* and the induction of flowering (Fig. 6E). This last result suggests that no light stimuli are required to induce *FT1* and flowering when *PPD1* is misexpressed during the night. However, in both the *phyB*-null and *phyC*-null mutants, the relatively high transcript levels of *PPD-A1a* were insufficient to induce *FT1*. A possible explanation for this observation is that *PHYB* and *PHYC* are important for some of the intermediate molecular steps required for the *FT1* up-regulation by *PPD1*. The altered expression of the core clock genes in the *phyC*-null mutant may also contribute to this effect (Chen et al., 2014).

The putative additional factor required for *FT1* induction is likely to be regulated by the circadian clock, with its expression or activity peaking between 6 and 10 h after dusk under a SD photoperiod of 16 h of darkness. This putative factor could function to stabilize or activate the PPD1 protein or be an additional factor that acts either in a complex with PPD1 or downstream of PPD1 to activate *FT1*. Alternatively, PPD1 may activate a protein that degrades a repressor of FT1 or induce epigenetic changes in *FT1* or other intermediate genes. The identification of this clock-regulated putative factor involved in the PPD1 activation of *FT1* is an outstanding question of the PPD1-mediated photoperiodic response in wheat.

CONCLUSION

In this study, we demonstrated that the duration of the dark period rather than of the light period is critical for the photoperiodic response in wheat. We showed that *PPD1* plays a central role in the acceleration of heading time by NBs and that this response requires functional *PHYB* and *PHYC* genes and active protein translation during darkness. In wheat, the induction of *FT1* was greater with increasing numbers of NBs or LDs, suggesting the gradual accumulation of a signal with additional NBs or LDs. The timing of the NB induction of *PPD1* also affected the intensity of flowering acceleration, suggesting that the *PPD1* induction of *FT1* may be gated by circadian clock-regulated genes. In summary, the NB experiments described here provide valuable insights into the regulatory mechanisms controlling wheat photoperiodic response.

MATERIALS AND METHODS

Plant Materials

Experiments were performed using the tetraploid durum wheat (*Triticum turgidum* subsp. *durum*) variety Kronos and the hexaploid bread wheat (*Triticum aestivum* subsp. *aestivum*) variety Paragon. Kronos has a spring growth habit and carries the *Ppd-A1a* allele, which has a deletion in the promoter region conferring reduced sensitivity to photoperiod by accelerating flowering under SD (Wilhelm et al., 2009). We developed a near-isogenic Kronos line carrying the wild-type photoperiod-sensitive *Ppd-A1b* allele (Kronos-PS), which flowers very late under SD. This line was developed by crossing Kronos with the tetraploid durum variety Langdon (which carries the *Ppd-A1b* allele) and performing three backcrosses using Kronos as the recurrent parent as described previously (Pearce et al., 2013). We also developed a second near-isogenic line of Kronos with no functional *PPD1* alleles, designated hereafter as *ppd1*-null. We first introgressed a deletion of *PPD-B1* from a Paragon γ-ray mutant (Shaw et al., 2013) into Kronos by three backcrosses. We then intercrossed this line with a Kronos TILLING line carrying a mutation encoding a premature stop codon in the *PPD-A1* coding region (W154*). This mutation eliminates 514 of this protein's 668 amino acids, including the highly conserved CCT domain that has been shown to be essential for photoperiod response (Turner et al., 2005). This mutant line was first backcrossed twice to Kronos to reduce background mutations. In the F2 progeny, we selected plants homozygous for the two mutations using molecular markers. The *PPD-A1* mutant allele was detected using a dCAPs marker. PCR was performed (35 cycles of 94°C 20 s, 55°C 30 s, 72°C 30 s) using forward primer AACGAGCTTAAGAACCACTG and reverse primer TATAATAATCACACACGTTG to amplify a 215-bp fragment. This fragment was digested with *Bsr*I. Plants homozygous for the nonfunctional *PPD-A1* allele showed a digestion pattern of 195/20 bp when run on a 1% agarose gel, whereas PCR products amplified from plants homozygous for the wild-type allele were not digested and showed an intact 215-bp band. To detect the *PPD-B1* deletion, we used a Taqman assay as described previously (Díaz et al., 2012). F4 lines from two independent crosses were used in this study. We also included the photoperiod-sensitive hexaploid variety Paragon and its isogenic *ppd1*-null mutant carrying deletions or loss-of-function mutations in all three *PPD1* homeologs, which has been described previously (Shaw et al., 2013).

The *phyB*-null and *phyC*-null mutants were obtained by combining nonfunctional mutations in the A and B homeologs of each gene in Kronos lines carrying the *Ppd-A1a* allele (Chen et al., 2014; Pearce et al., 2016).

Growth Conditions

All experiments were performed in controlled environment conditions using either PGR15 or E7/2 growth chambers (Conviron), which were located in the same room. During the lights-on period, the growth chambers were set at 22°C, but the first and last hour of this lights-on period were set at 20°C to provide a more gradual change between temperatures. Night temperatures were set at 17°C. All PGR15 chambers used similar metal halide and high-pressure sodium light configurations, and lights were set to the same intensity in all experiments (~260 μM m^{-2} s^{-1}; Conviron setting 2 for both types of lamps). Lights were on for 8 h in SD experiments and 16 h in LD experiments. In NB experiments, the lights were on for 7 h and then for 1 h in the middle of the night, making the same total of 8 h of light. For the experiment testing the effect of the timing of the NB, the 1 h light break was applied 2, 4, 6, 8, 10, 12, and 14 h after dusk.

Experiments applying FR light were performed in dual E7/2 growth chambers fitted with fluorescent lamps (250 μM m^{-2} s^{-1}) supplemented with two strips of LED lights emitting FR wavelength light (~720 nm at 100% intensity). These experiments used the same growing conditions (22°C day/17°C night, including a 1-h transition period at 20°C). In the first experiment, the 1 h NB applied in the middle of a 16 h night (17°C) was followed by a 30 min pulse of FR light at 100% intensity. In the second experiment, the NBs consisted of 15 1-min pulses of white light intercalated with either 1 min of darkness (control) or 1 min of FR at 100% intensity. Heading date was measured as the number of days from germination until complete emergence of the main spike.

To determine the effect of different number of NBs or LDs on heading time, Kronos-PS plants were grown under SD conditions for 4 weeks before plants were transferred to LD or NB conditions. Plants were moved back to SD conditions after the indicated number of days. The experiments were terminated after 150 d.

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For the circadian time course experiment, Kronos-PS plants were grown in SD conditions for 4 weeks, and then half of the plants were transferred to NBmax conditions (15 min NB after 8 h of darkness), while the remaining plants were maintained under SD conditions. These photoperiods were maintained for 21 d, when both chambers were switched to free-running conditions (constant light and temperature). After 24 h in free-running conditions, leaf tissues were collected from six biological replicates at 4 h intervals for an additional 24-h period.

Cycloheximide Hydroponic Experiment

One week after germination, Kronos-PS seedlings were transplanted into a hydroponic system with Hoagland solution (Hoagland and Arnon, 1950) and grown under SD conditions for 4 weeks. The Hoagland solution was changed twice per week. Immediately before the beginning of the night of the 28th day in SD conditions, two batches of six plants were transferred to 1-liter beakers containing water (control), and two batches of six plants were transferred to a beaker with 500 μM cycloheximide (CHX). Plants from one water and one CHX batch were left in darkness (no-NB), while plants from the other water and CHX batches were exposed to a 1 h pulse of white light after 6 h of darkness. Leaf tissues were harvested at two time points: immediately before the start of the NB (T1) and 2 h after the end of the NB (T2).

qRT-PCR

Expression analysis was performed using the middle of the leaf blade of the most recently fully emerged leaf from each plant, which were harvested and frozen immediately in liquid nitrogen. When sampling plants in the dark, harvests were performed as quickly as possible with no supplemental lighting in the room to avoid exposure to external lights. To study the effect of increasing numbers of NB, leaf samples were collected immediately before the NB from plants exposed to different numbers of NB (i.e. the three-NB sample was collected in the dark immediately before exposure to the fourth NB).

Harvested leaves were ground to a fine powder in liquid nitrogen, and RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich). One microgram of cDNA was synthesized from RNA using the High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Each qRT-PCR reaction used 10 ng of cDNA and 1× VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix), with the exception of the circadian clock experiment, where PowerUP SYBR Green qPCR master mix (Thermo Fisher) was used.

Primers were designed in regions conserved between the A- and B-genome homeolog, except for *PPD-A1* and *PPD-B1*, for which homeolog-specific primers were used. Primers for all target and control genes have been described previously, and full details are provided in Supplemental Table S1. qRT-PCR reactions were performed using a 7500 Fast Real-Time PCR system (Applied Biosystems), except from the circadian experiment, which were performed using a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The endogenous control gene for all reactions was *ACTIN*. Transcript levels are expressed as linearized fold-*ACTIN* levels calculated by the equation $2^{(ACTIN\ \mathrm{CT}\ -\ TARGET\ \mathrm{CT})} \pm \mathrm{SE}$ of the mean and represent the ratio between the initial number of molecules of the target gene and the number of molecules of *ACTIN* (scales are comparable across genes and experiments).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ■■■.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of NB on the expression of flowering time genes in the leaves.

Supplemental Figure S2. Phenotype of Kronos-PS plants exposed to different numbers of NB.

Supplemental Figure S3. Heading time of plants exposed to different numbers of LDs.

Supplemental Figure S4. Effect of FR treatment prior to NB on *PPD-B1* expression.

Supplemental Figure S5. Circadian expression profiles of core clock and flowering time genes in Kronos-PS.

Supplemental Table S1. qRT-PCR primers used in this study.

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Supplementary materials

Night-break experiments shed light on the *PPD1*-mediated photoperiodic response in wheat

Figure S1: Effect of night break (NB) on the expression of flowering time genes in the leaves. Kronos-PS plants were grown since germination either under short day or NBmax conditions for six weeks. Samples were harvested immediately before the NB (T1), immediately after the NB (T2), 3 h after (T3) and 5 h after (T4) the start of the NB. Transcript levels of (A) *FT2*, (B) *FT3* and (C) *VRN1* are presented as fold-*ACTIN*. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.0001$.

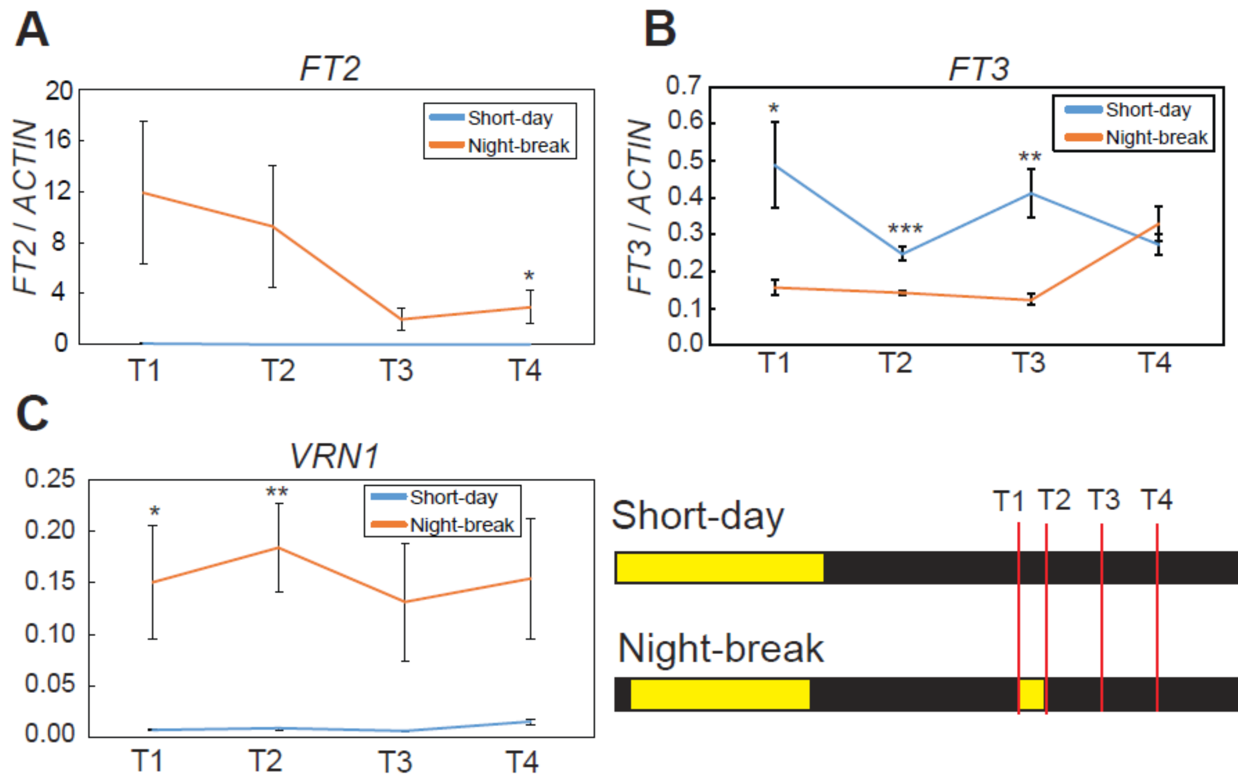

Figure S2: Phenotype of Kronos-PS plants exposed to different numbers of NB. Kronos-PS plants were grown in SD conditions for four weeks, then exposed to different numbers of 1 h NBs after 8 h darkness and transferred back to SD thereafter. Photos were taken when plants were 91 d old. One plant from each of the 0-9 NB treatments was dissected to show the developmental stage of the spike. Bar = 0.5 mm for 0-6 NB samples and 2 mm for 9 NB sample.

Figure S3: Heading time of plants exposed to different numbers of LDs. Kronos-PS plants were grown in SD conditions for four weeks, exposed to different numbers of LDs and returned to SD conditions thereafter.

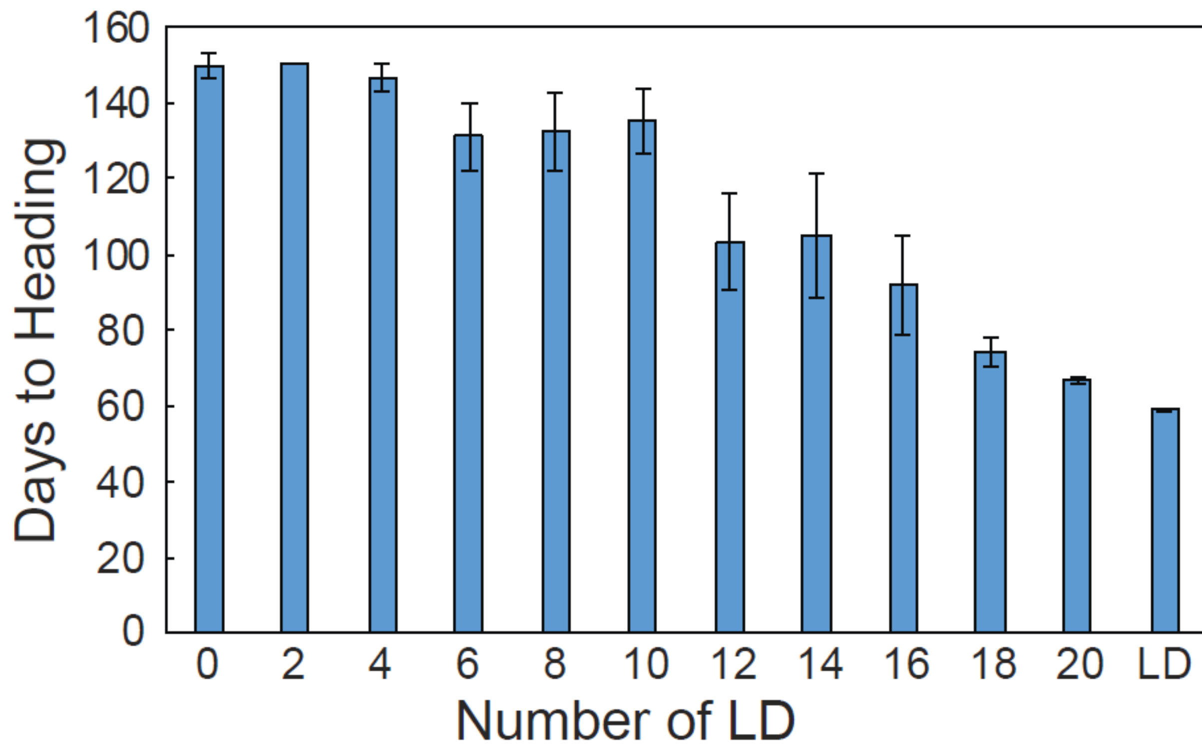

Figure S4: Effect of FR treatment prior to NB on *PPD-B1* expression. Kronos-PS plants were grown in SD for four weeks then exposed to a single NB after 2 h darkness. In one chamber, a 30 min pulse of FR light preceded the NB. Leaf tissues were harvested 1 h and 3 h after the start of the NB. *PPD-B1* transcript levels are presented as fold-*ACTIN*.

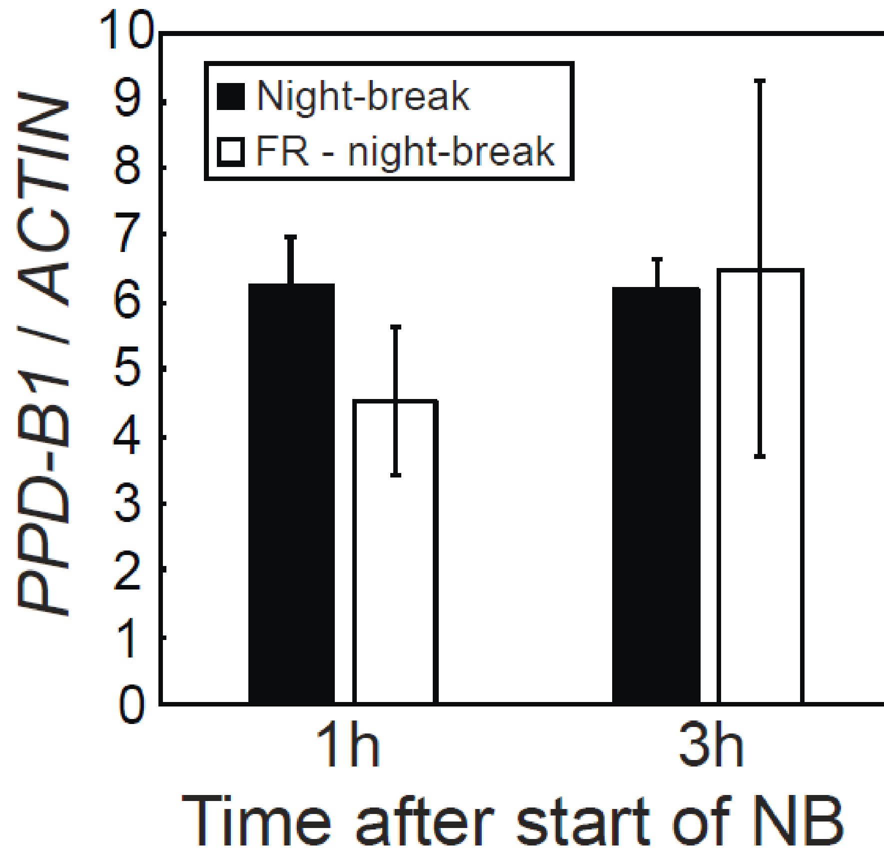

Figure S5: Circadian expression profiles of core clock and flowering time genes in Kronos-PS. Plants were grown for seven weeks in SD or for four weeks in SD followed by three weeks in NBmax conditions. After seven weeks, all plants were moved to free running conditions for 24 h and samples were collected every 4 h under continuous light (0 h = subjective dawn, 8h = subjective dusk). Transcript levels of core clock genes (A) *PRR59*, (B) *PRR95*, (C) *GI*, (D) *PRR73*, (E) *TOC1*, (F) *CCA1* and flowering time genes (G) *PPD-B1* and (H) *FT1* are presented as fold-*ACTIN*. * = $P < 0.05$, ** = $P < 0.01$.

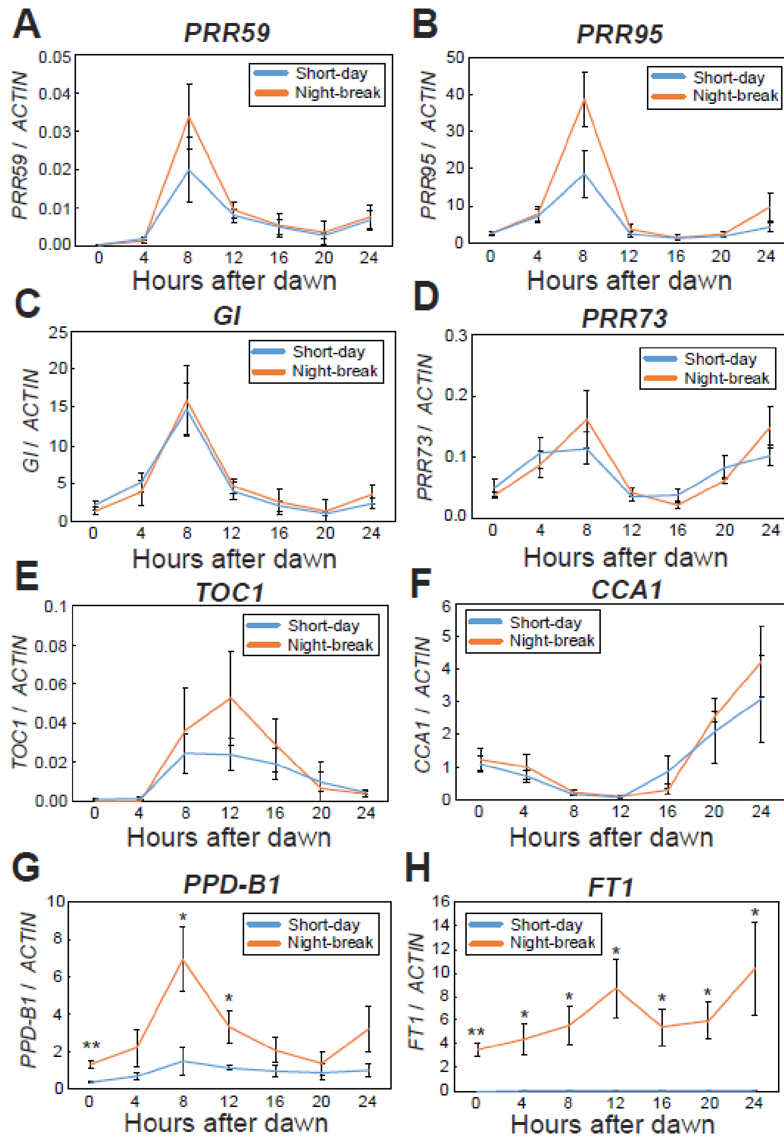

Table S1: Primers used in this study.

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
PPD-A1	AGACAAGGCTGATGAAACGA	CGATGGATTGACCAAACTG	Shaw et al., 2012
PPD-B1	AAGACAAGGTTGATGACGTGA	GAGGGATTGATCACGTTGG	Shaw et al., 2012
FT1	CAGCAGCCCAGGGTTGAG	ATCTGGGTCTACCATCACGAGTG	Yan et al., 2006
FT2	TTTCTACACGCTGGTGATGG	GTGACCAGCCAGTGCAAGTA	Yan et al., 2006
FT3	TCGGCCAAGAGCTTGTAGTT	AAATTGTGTCGCACATCTGG	Lv et al., 2014
VRN1	AAGAAGGAGAGGTCACTGCAGG	GGCTGCACTGCCGCA	Yan et al., 2006
ZCCT2	CCACCATCGTGCCATTCT	CCCACCATCATCTCTGTATCAA	Distelfeld et al., 2009
PRR59	GCGTAACTTATGGCAACAT	CTGAGCATCACTTTCCTC	Chen et al. 2014
PRR95	GACTATGGCAGATCAGAGGAC	TGAGCATCACCAGCGTTACC	Chen et al. 2014
GI	TCTGGATTGCTCGAGATGAC	AAGCTTCACCGTCGACAA	Chen et al. 2014
TOC1	GCTCATACGCCACCAAGA	ACCACACATTCCGCGT	Shaw et al. 2012
CCA1	CCTGGAATTGGAGATGGAGA	TGAGCATGGCTTCTGATTTG	Campoli et al. 2012
PRR73	CGATGCAGCACCGAG	GCTTTGCTGTGCCTCACT	Shaw et al. 2012
ACTIN	ACCTTCAGTTGCCCAGCAAT	CAGAGTCGAGCACAATACCAGTTG	Distelfeld et al., 2009

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