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Insight into a Physiological Role for the EC Night-Time Repressor in the Arabidopsis Circadian Clock

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Regular Paper

Life cycle adaptation to seasonal variation in photoperiod and temperature is a major determinant of ecological success of widespread domestication of *Arabidopsis thaliana*. The circadian clock plays a role in the underlying mechanism for adaptation. Nevertheless, the mechanism by which the circadian clock tracks seasonal changes in photoperiod and temperature is a longstanding subject of research in the field. We previously showed that a set of the target genes (i.e. *GI*, *LNK1*. *PRR9* and *PRR7*) of the Evening Complex (EC) consisting of LUX–ELF3–ELF4 is synergistically induced in response to both warm-night and night-light signals. Here, we further show that the responses occur within a wide range of growth-compatible temperatures (16–28°C) in response to a small change in temperature (Δ4°C). A dim light pulse (<1 μmol m^{-2} s^{-1}) causes the enhanced effect on the transcription of EC targets. The night-light pulse antagonizes against a positive effect of the cool-night signal on the EC activity. The mechanism of double-checking external temperature and light signals through the EC nighttime repressor might enable plants to ignore (or tolerate) daily fluctuation of ambient temperature within a short time interval in their natural habitats. Taken together, the EC night-time repressor might play a physiological role in tracking seasonal variation in photoperiod and temperature by conservatively double-checking both the light and temperature conditions. Another EC target output gene *PIF4* regulating plant morphologies is also regulated by both the temperature and light stimuli during the night. Hence, the EC night-time repressor is also implicated in a physiological output of the PIF4-mediated regulation of morphologies in response to seasonal variation in photoperiod and ambient temperature.

Keywords: *Arabidopsis thaliana* • Circadian clock • Light response • Temperature response • Transcriptional regulation.

Abbreviations: CCA1, CIRCADIAN CLOCK ASSOCIATED 1; EC, Evening Complex; ELF3, EARLY FLOWERING 3; ELF4, EARLY FLOWERING 4; GI, GIGANTEA; LHY, LATE ELONGATED HYPOCOTYL; LNK1, NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE 1; LUX, LUX ARRHYTHMO; PCL1, PHYTOCLOCK 1; PIF4, PHYTOCHROME-INTERACTING FACTOR 4; PRR, PSEUDO RESPONSE REGULATOR; qRT-PCR, quantitative real-time PCR; TOC1, TIMING OF CAB EXPRESSION 1; ZT, Zeitgeber time.

Introduction

In the flowering plant *Arabidopsis thaliana*, significant progress has been made in defining the molecular mechanism underlying circadian clock operation. The central oscillator that has been uncovered is composed of mainly three classes of transcriptional regulators (McClung 2011, Nagel and Kay 2012, Carre and Veflingstad 2013, Hsu and Harmer 2014, Sanchez and Yanovsky 2013, and references therein). (i) Morning-phased components are composed of CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL). (ii) Day-phased components include two members of a small PRR (PSEUDO RESPONSE REGULATOR) family, namely PRR9 and PRR7. Another small LNK (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE) family of day-phased components has recently been reported (see Hsu and Harmer 2014). (iii) Evening-phased components include PRR5, TOC1 (TIMING OF CAB EXPRESSION 1), GI (GIGANTEA), LUX (LUX ARRHYTHMO), ELF3 (EARLY FLOWERING 3) and ELF4. Over the past decade, several models have been proposed to describe the clock system in terms of multiple interacting transcriptional loops involving these core clock genes (Locke et al. 2006, Pokhilko et al. 2010, Pokhilko et al. 2012, Pokhilko et al. 2013, Fogelmark and Troein 2014, and references therein). The circadian clock transcriptional circuitry thus revealed is too complex to understand intuitively the role of individual genes. Here, we sought to clarify the roles of the so-called Evening Complex (EC) night-time repressor, which is composed of the three evening-phased components (LUX, ELF3 and ELF4).

Originally, two independent groups identified *LUX* (also known as *PCL1*) through forward genetics as a circadian clock-associated gene, which encodes a Myb domain-containing putative transcription factor (Hazen et al. 2005b, Onai and Ishiura 2005). It was then demonstrated that LUX acts during the night as an autorepressor for its own transcription through directly binding a specific DNA sequence within the promoter (Helfer et al. 2011, Nusinow et al. 2011). LUX forms a complex with ELF3 and ELF4, both of which were uncovered more than a decade ago as crucial factors for the control of photoperiodic flowering time, and then it was revealed that a number of loss-of function mutants of these genes display severe defects in the clock function per se (Doyle at al. 2002, Kikis et al. 2005, Kolmos et al. 2009, Thines and Harmon 2010, Dixon et al. 2011 Kolmos et al. 2011). Indeed, the EC night-time repressor acts as a repressor for *PRR9* (Chow et al. 2012). It was recently shown that

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GI, *LNK1* and *PRR7* are also targets of the EC night-time repressor (Mizuno et al. 2104a, Mizuno et al. 2014b). The EC night-time repressor also negatively regulates the clock output gene *PIF4* (Helfer et al. 2011, Nusinow et al. 2011), which plays an important role in hypocotyl elongation of young seedlings (Niwa et al. 2009, Kunihiro et al. 2011, Nomoto et al. 2012, Nomoto et al. 2013). In short, the EC night-time repressor plays an essential role in forming the circadian clock transcriptional circuitry, which is central to the clock.

Light and temperature are the two predominant external cues entraining the circadian clock. Hence, the circadian clock must have the capacity to integrate these signals into the transcriptional circuitry in order to track seasonal variation in photoperiod and ambient temperature properly. Clarification of the underlying mechanism has been a major subject in the field. In this respect, we recently reported that temperature signals feed into the clock transcriptional circuitry through the EC night-time repressor, so that its downstream target clock genes, including *GI*, *LNK1*, *PRR9* and *PRR7*, are commonly regulated at the transcriptional level during the night in response to both moderate changes in temperature and differences in steady-state growth-compatible temperatures (Mizuno et al. 2104a, Mizuno et al. 2014b). A warmer temperature inhibits EC function more, so that the expression of these target genes is up-regulated in response to the warm-night signal. We further reported that a night-time light signal also inhibits the EC night-time repressor, thereby resulting in up-regulation of the same set of EC target genes (Mizuno et al. 2104c). Here, by extending these findings, we provide evidence that the EC night-time repressor in *A. thaliana* plays a physiological role in tracking seasonal variation in photoperiod and ambient temperature by conservatively double-checking both the external temperature and light conditions. Furthermore, we show that the EC night-time repressor is implicated in a physiological output of the PIF4-mediated regulation of plant morphologies in response to seasonal variation in photoperiod and ambient temperature.

Results and Discussion

A set of EC target genes is induced in response to simultaneous stimuli of both warm-night and night-light signals

We examined the transcriptional profiles of some core clock genes including *GI*, *LNK1*, *PRR9* and *PRR7* to determine their responses to both temperature and light signals during the night (**Fig. 1**). Seedlings of *A. thaliana* (accession Columbia, Col-0) were grown for 7 d at 22°C under 12 h light/12 h dark cycles, and the growth temperature was up-shifted to 28°C at midnight [i.e. Zeitgeber time (ZT) 18]. Concomitantly, the seedlings were exposed to white light [photosynthetic photon flux density (PPFD) 80 μmol m^{-2} s^{-1}). RNA samples were prepared at intervals and quantified by quantitative real-time PCR (qRT-PCR; **Fig. 1**, red filled circles). As controls, (i) RNA samples were prepared from seedlings grown continuously in the dark (**Fig. 1**, filled circles), (ii) seedlings were exposed to light at 22°C without the temperature up-shift (**Fig. 1**, green rectangles) or (iii) they were exposed to the warm temperature of 28°C in continuous darkness (**Fig. 1**, orange triangles). As previously shown (Mizuno et al. 2014c), here we reproduced the intriguing phenomenon that transcription of EC target genes (*GI*, *LNK1*, *PRR9* and *PRR7*) was exponentially up-regulated only when seedlings were simultaneously exposed to both external stimuli (**Fig. 1A–D**). It has already been shown that these enhanced responses of *GI*, *LNK1*, *PRR9* and *PRR7* to both the temperature and light signals during the night are severely compromised in a set of loss-of-function EC mutants (i.e. *elf3*, *elf4* or *lux*), but not in other clock mutants (e.g. *cca1lhy*, *prr975*, *toc1* or *gi*). It has also been reported that chromatin immunoprecipitation assays showed that *PRR7* (PRR9), *GI* and *LUX* are direct targets of the night-time repressor (Chow et al. 2012, Mizuno et al. 2014a, Mizuno et al. 2014b, Mizuno et al. 2014c). These results are consistent with the idea that these responses at the transcriptional level are regulated through the EC night-time repressor. Taken together, we propose the view as to the function of the EC night-time repressor, as shown schematically in **Fig. 1I**. Briefly, both the warm-night and night-light signals negatively modulate EC activity, so that an exponential burst of transcription of the EC target genes is specifically observed only when these signals are simultaneously fed into the night-time repressor. It was also shown that other clock genes (e.g. *PRR5*, *ELF3*, *ELF4* and *TOC1*) did not significantly respond to the stimuli.

Enhanced responses occur within a wide range of growth-compatible temperatures

We then wanted to determine whether such responses occur within a wide range of growth-compatible temperatures. To examine this point, three different conditions were applied to the next experiments; namely, temperature up-shifts from 16 to 20°C, from 20 to 24°C and from 24 to 28°C (**Fig. 2A–F**; **Supplementary Fig. S1**). The results showed that the responses occur within a wide range of growth-compatible temperatures (16–28°C), and suggest that the EC night-time repressor is able to respond to a small up-shift of temperature. To replicate the results biologically, we carried out another experiment, in which a temperature up-shift from 16 to 22°C was also applied (**Supplementary Fig. S2**).

A night-time-light pulse inhibits the EC activity

In the experiments of **Figs. 1** and **2**, seedlings were exposed to light throughout the time course to induce the EC target genes. However, a 20 min night-time light pulse was sufficient for significant induction of the EC target genes (**Fig. 3A, C**, red open circles). Then seedlings of Col-0 were grown for 7 d at 22°C under 12 h light/12 h dark cycles, and the growth temperature was up-shifted to 28°C at ZT18, and, after 30 min incubation, they were exposed to a light pulse (30 min). The result showed that a 30 min night-light pulse together with the warm-night signal results in a prolonged induction of the EC target genes (**Fig. 3B, D**; **Supplementary Fig. S3**), indicating that a pulse of night-light is sufficient to inhibit the EC activity. These results were confirmed by another biologically independent experiment (**Supplementary Fig. S4**).

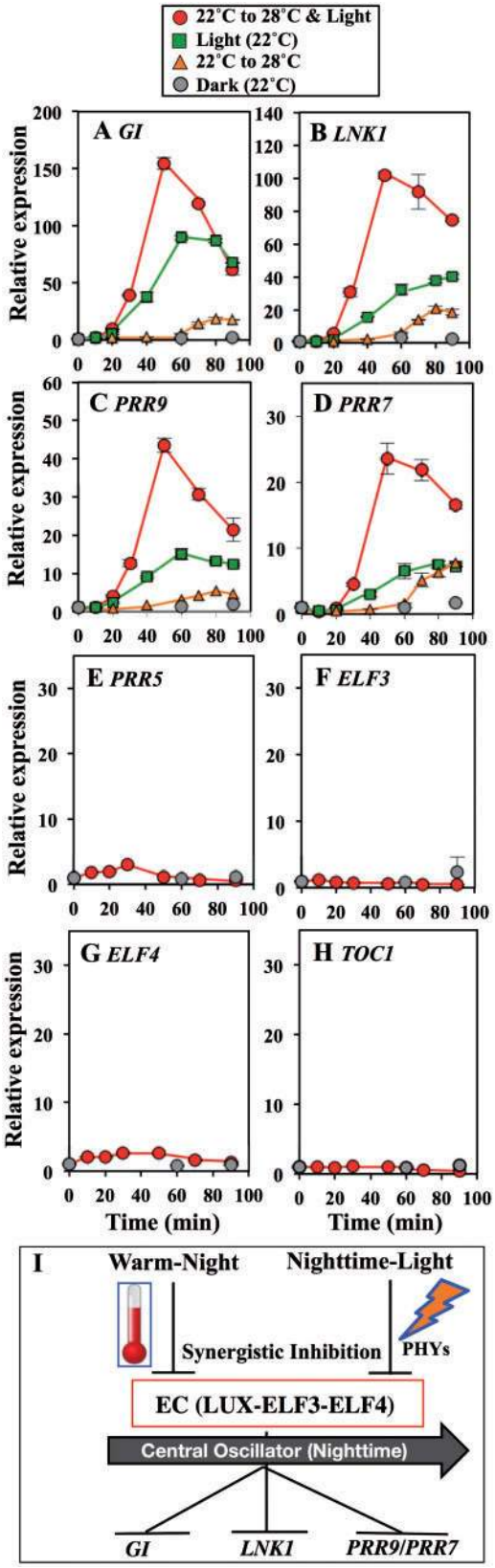

Fig. 1 Effect of a warm-night (22–28°C) and night-time light on exponential induction of EC target genes. Seedlings of Col-0 were grown for 7 d at 22°C under 12 h light/12 h dark cycles, and the growth temperature was up-shifted to 28°C at midnight (ZT18). Concomitantly, the seedlings were exposed to white light (80 μmol m^{-2} s^{-1}). RNA samples were prepared at the indicated intervals and quantified by qRT-PCR (red filled circles). The indicated clock genes were analyzed for their responses to the stimuli during the night. Relative expression levels are shown as mean values ± SD ($n = 3$). Three other control experiments were carried out, as indicated at the top. Results are explained schematically at the bottom. This illustration was adapted from our previous paper (Mizuno et al. 2014c) with slight modifications.

A dim light signal also inhibits the EC activity

In the above experiments, we used relatively bright light (80 μmol m^{-2} s^{-1}). However, we wished to test whether or not a dim light signal also regulates the EC activity, because the dim light signal of dusk and/or dawn might affect the EC activity during seasonal changes in day/night cycles. Hence, we cut the night-light intensity by >99% to see whether the resulting dim light (0.56 μmol m^{-2} s^{-1}) was sufficient to induce the EC target genes during the night. These experiments were conducted at three different growth temperatures: 28, 22 and 16°C. Indeed, this level of dim light was sufficient to induce the EC target genes, regardless of growth temperature, although the induction levels were lower than those induced by bright light (**Fig. 4**). Then seedlings of Col-0 were grown for 7 d at 22°C under 12 h light/12 h dark cycles, and the growth temperature was up-shifted to 28°C at ZT18 and, after 30 min incubation, they were exposed to a dim light pulse. The results showed that simultaneous exposure to both stimuli, a dim night-light pulse (30 min) and warm-night (28°C) signals, results in an advanced induction of the EC target genes (**Fig. 5**). These results were confirmed by another experiment, which was carried out under slightly different conditions with use of a prolonged dim light (90 min) (**Supplementary Fig. S5**). Essentially the same experiment as in **Fig. 5** was also performed at the later timing of ZT22, verifying the above view (**Supplementary Fig. S6**).

A dim night-light signal antagonizes the positive effect of a cool-night signal on EC activity

We previously showed that the effect of ambient temperature on EC activity is reversible in the sense that a temperature down-shift (e.g. from 26 to 18°C) activates the EC night-time repressor, thereby resulting in prolonged inhibition of its targets (Mizuno et al. 2014a), as reproduced in the results of **Fig. 6A** and **C**. Interestingly, this effect of temperature down-shift on *LNK1* and *PRR7* was abolished when seedlings were concomitantly exposed to dim night-light (**Fig. 6B, D**; **Supplementary Fig. S7**). Furthermore, a 60 min dim night-light pulse appeared to be sufficient to antagonize the effect of temperature down-shift on EC activity (**Fig. 6E–H**). These results were confirmed through another independent experiment (**Supplementary Fig. S8**). Essentially the same experiment was also performed at the later timing of ZT21.5, verifying the above view (**Supplementary Fig. S9**).

In short, the results of **Figs. 5** and **6** indicated that night light always results in the induction of EC target genes, while the effect of night-time temperature on EC activity is bidirectional (i.e. both activation and inhibition), so that the concomitant

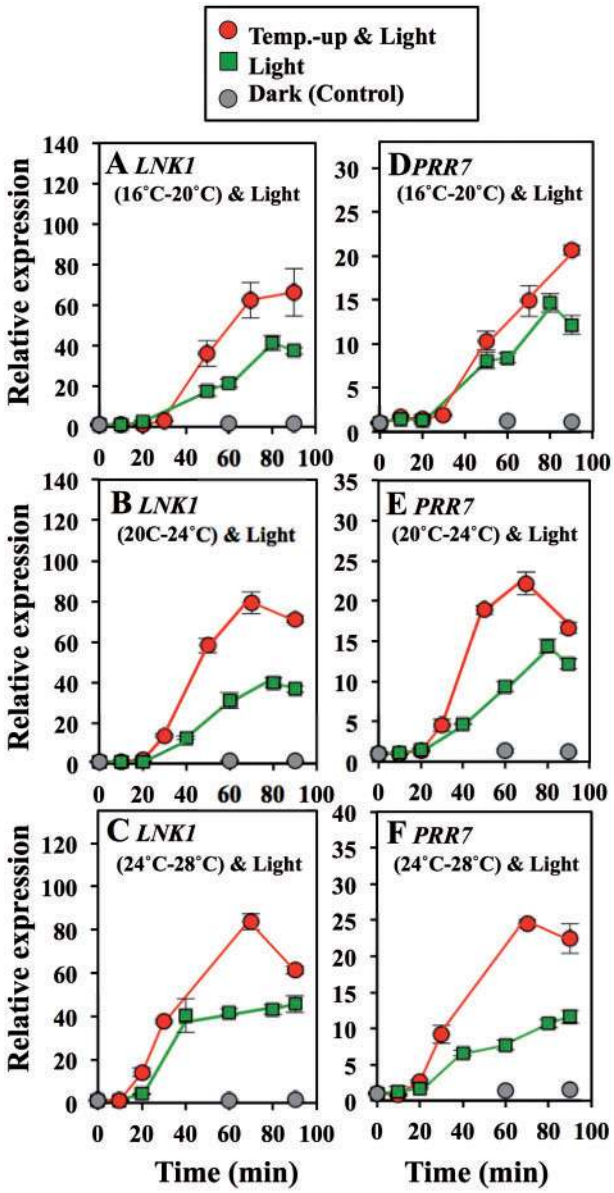

Fig. 2 Responses of *LNK1* and *PRR7* occurred within a wide range of growth-compatible temperatures (16–28°C) in response to a small change in temperature (Δ4°C) only when seedlings were simultaneously exposed to night-time light. For other details, see the legend to **Fig. 1**.

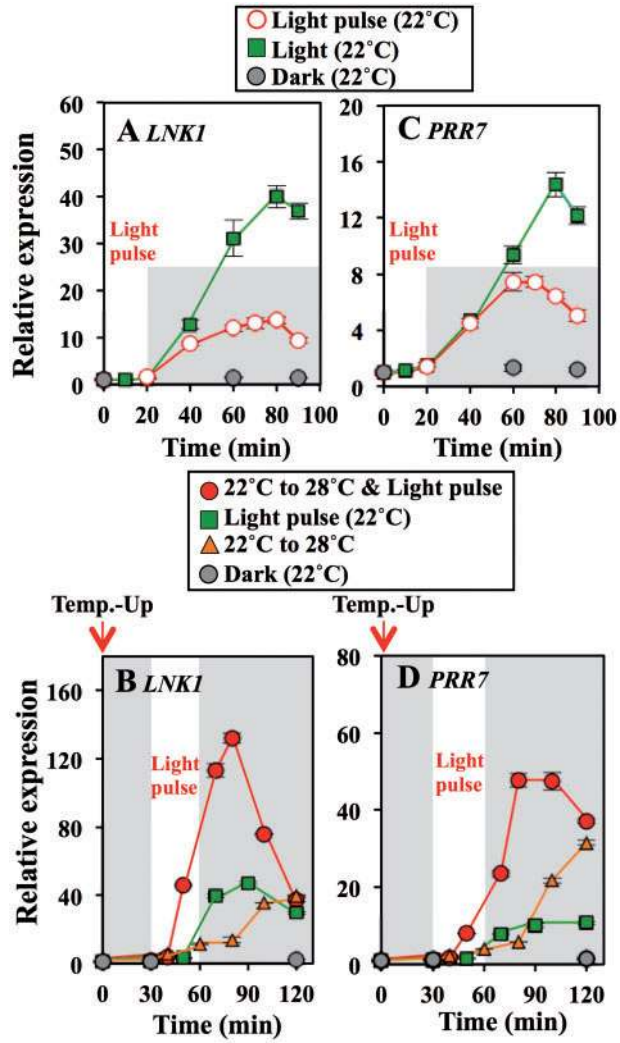

Fig. 3 A light pulse (30 min) is sufficient to trigger an effect on exponential expression of EC targets (*LNK1* and *PRR7*). For other details, see the legend to **Fig. 1**.

external stimuli of warm nights and night light result in advanced expression of the EC target genes, whereas a cool-night signal induces delayed expression of these clock genes only when seedlings were not exposed to light during the night.

Insight into the physiological role of the EC night-time repressor

General consideration with regard to seasonal changes in photoperiod and temperature. Nagoya, Japan is located at 35°10′N and 136°55′E (north latitude and east longitude) (**Fig. 7**, middle panel). With regard to the range in photoperiod in this area, the longest day (summer solstice) is approximately 14.5 h, while the shortest day (winter solstice) is 9.5 h (purple profile, data from http://eco.mtk.nao.ac.jp). The change in average lowest temperature (i.e. night-time temperature) of each month of 2014 is also shown (green profile, data from http://www.data.jma.go.jp). Accordingly, the growth-compatible season for *A. thaliana* in Nagoya would be from the middle of March to the end of October (black horizontal arrow). This period could be divided into three seasons: Season I (red arrows), during which both the daytime and ambient temperature rapidly increase by about 65 min of daytime per month and 5°C of ambient temperature per month; and Season II (blue arrows), during which both the daytime and ambient temperature quickly decrease by about 65 min of daytime per month and 6°C per month. During the period between these seasons, the daytime decreases, while the temperature increases. However, the changes in both the photoperiod and ambient temperature during this period are relatively modest compared with Seasons I and II. Taken together, we considered that the internal circadian clock must properly track external changes in the photoperiod and ambient temperature, particularly in Seasons I and II.

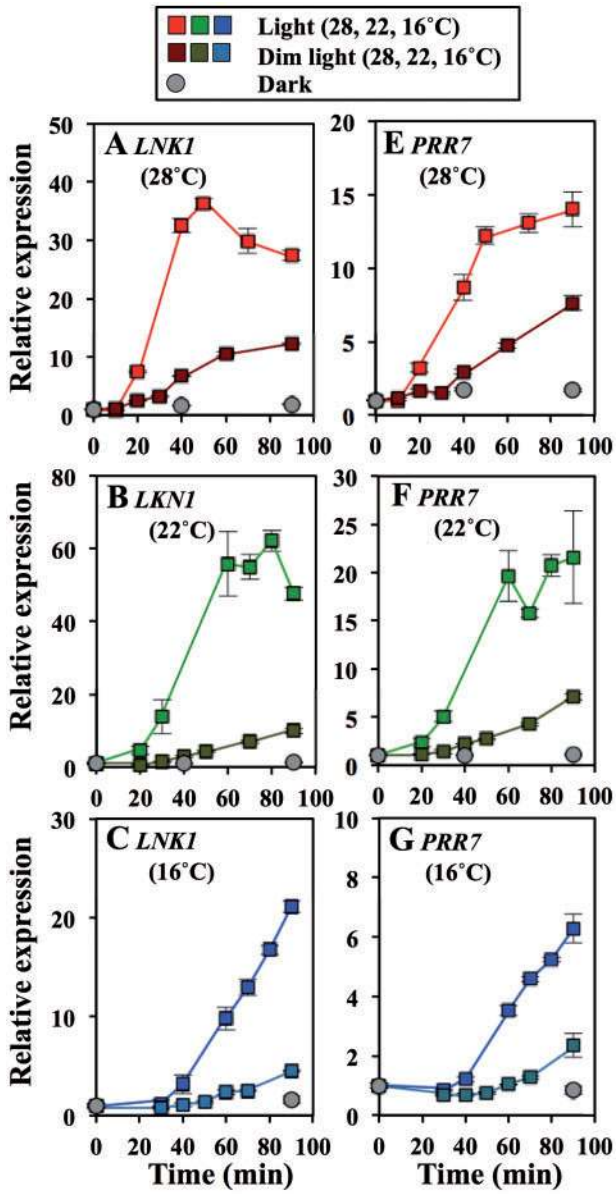

Fig. 4 A dim light pulse (<1 μmol m^{-2} s^{-1}) is sufficient to induce the expression of EC targets (*LNK1* and *PRR7*). For other details, see the legend to **Fig. 1**.

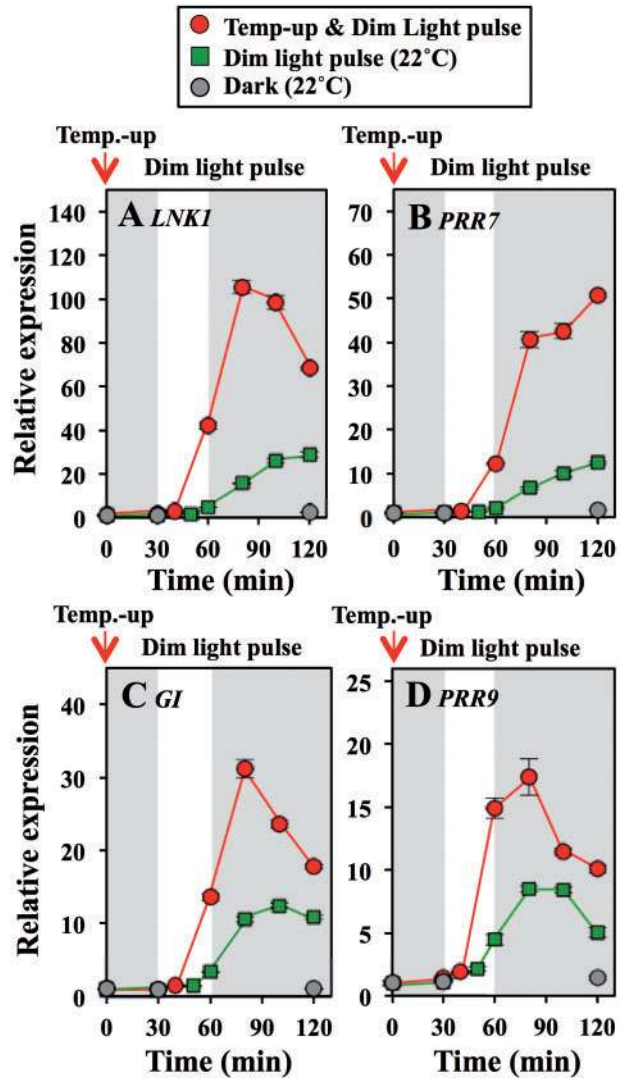

Fig. 5 A dim light pulse (0.56 μmol m^{-2} s^{-1} for 30 min) is sufficient to trigger a effect on expression of EC targets (*LNK1* and *PRR7*). For other details, see the legend to **Fig. 1**.

Problems in the real world. As considered above, changes in both photoperiod and temperature during Seasons I and II appear to be the signals to entrain the internal oscillator in order to track the change in season. However, the real world is more complex than those changes considered above, because temperature markedly fluctuates (increases or decreases) within a few days (**Supplementary Fig. S10**). The plant circadian clock must ignore this type of daily variation in temperature within a short interval. The results of this study suggest that the EC night-time repressor may successfully satisfy this demand through the mechanism by which the clock is conservatively double-checking not only the temperature signal but also the light signal. In other words, the variation in external temperature alone seems not to be a sufficient signal to entrain the internal oscillator in the sense that light conditions should be properly fulfilled concomitantly. Another problem emerges from the question of how a plant is able to measure changes in photoperiod. Plants might measure the hours of sunshine as the photoperiod during a given day. Again, the real world is more complex, because the hours of sunshine dramatically change within a few days depending on the weather (**Supplementary Fig. S11**). Hence, bright sunlight appears not to be the external light signal which entrains the internal oscillator. In this respect, we showed that a dim light signal also regulates the EC activity only when the temperature signal is properly fed concomitantly. Hence, the findings of this study might be relevant to the physiological role of the EC night-time repressor in the natural habitat.

The EC appears to play a role in adapting to the real world. In **Fig. 7** (upper and lower panels), typical transcriptional profiles of *PRR7* under conditions of both long days (16 h light, red profile) and short days (8 h light, blue profile) are schematically

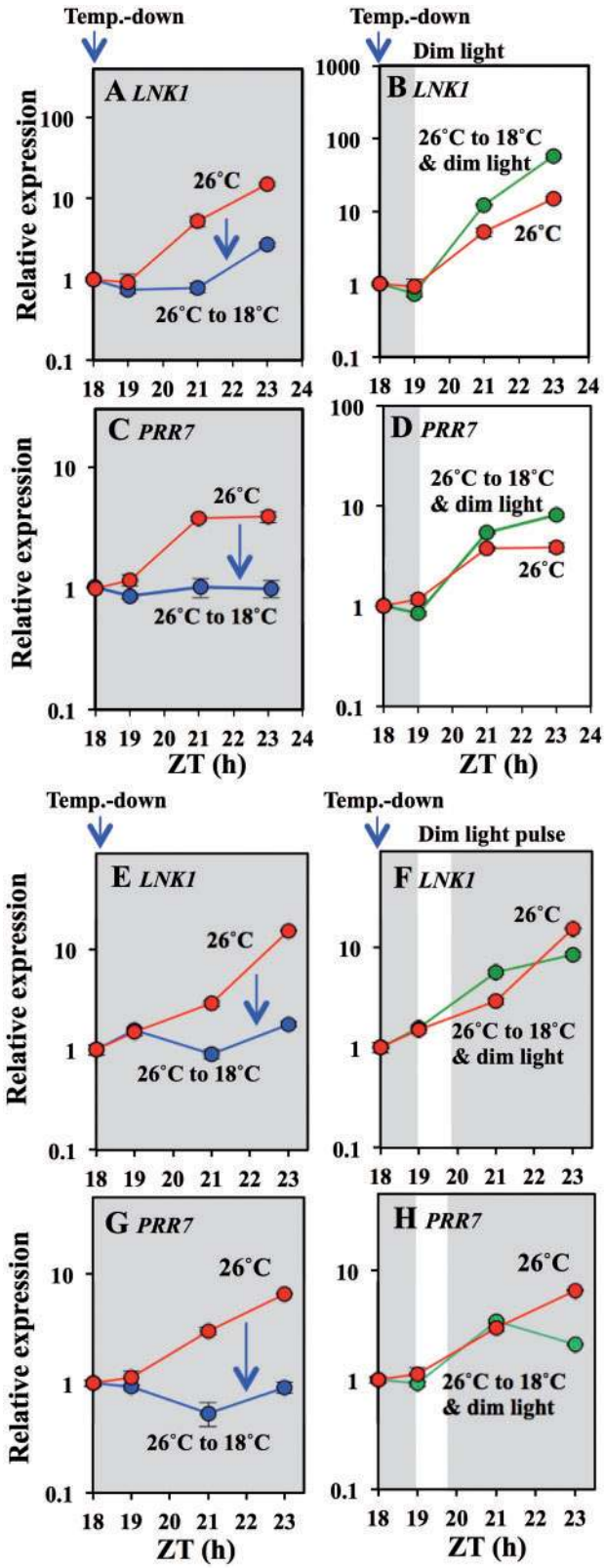

Fig. 6 A dim night-time light pulse (0.56 μmol m^{-2} s^{-1} for 60 min) antagonizes the positive effect of the cool-night (26–18°C) signal on EC activity. Temperature down-shift resulted in repression of EC target genes (*LNK1* and *PRR7*) (blue vertical arrows). This temperature effect was abolished by concomitant exposure to a dim night-time light pulse (right panels). For other details, see the legend to **Fig. 1**.

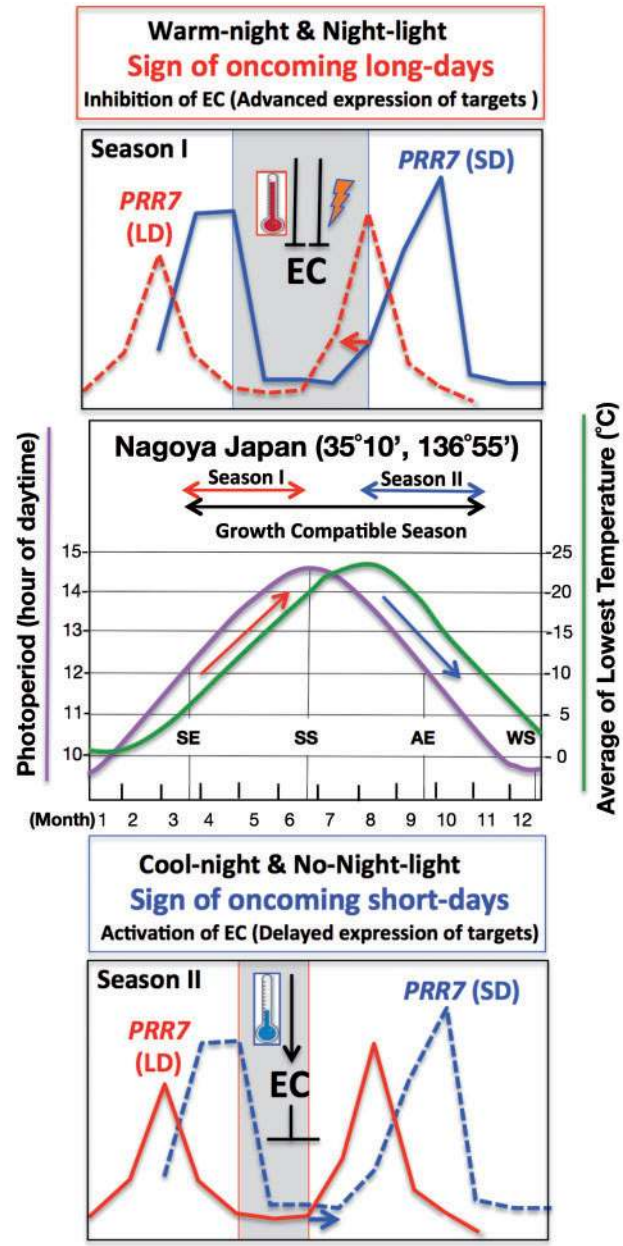

Fig. 7 Hypothetical view of the ecological function of the EC night-time repressor. The results of this study together suggest that the EC night-time repressor plays a role in properly tracking seasonal variation in photoperiod and ambient temperature by conservatively double-checking both the temperature and light stimuli (i.e. warn night/night-time light or cool night/no night-time light) in *Arabidopsis thaliana*. Only the relevant dark period is indicated with shadowing, and other dark periods are not indicated for clarity. Other details are given in the text.

illustrated (data from http://diurnal.mocklerlab.org). The beginning of the night was superimposed to allow recognition more intuitively of oncoming changes in length of the night (horizontal red and blue arrows). As shown in the upper panel of **Fig. 7**, the results of this study suggest that the EC night-time repressor double-checks both warm-night and night-light signals to conservatively advance the expression of its targets

including *PRR7* to adapt to the anticipated long days of Season I. As shown in the lower panel of **Fig. 7**, the results of this study suggest further that the EC night-time repressor again double-checks both cool-night and no-night-light signals to conservatively delay the expression of its targets to adapt to the anticipated short days of Season II. Based on these considerations, the findings in this study are compatible with the idea that the EC night-time repressor plays a role at least in part in tracking these seasonal changes in the photoperiod and ambient temperature. In this respect, we do not know the mechanism by which light and temperature signals simultaneously inhibit the EC night-time repressor. Clarification of such mechanisms is the next interesting subject, as recently addressed also by others (Box et al. 2015).

The EC target output gene *PIF4* is controlled in the same manner

To gain further insight into the ecological role of the EC night-time repressor, it was of interest to examine whether the well-established EC target output gene *PIF4* is controlled at the transcriptional level in response not only to warm-night/dim night-light pulse but also to cool night/no night light. Hence, the same experiments conducted for the clock genes (i.e. those shown in **Figs. 5** and **6**) were carried out for *PIF4* and its homologous *PIF5* gene (**Fig. 8**, top panel). The expression of both *PIF4* and *PIF5* was exponentially enhanced when seedlings were exposed to both the stimuli of warm nights and night light (**Fig. 8A, B**). Furthermore, the expression of *PIF4* was inhibited by a temperature down-shift (from 26 to 18°C), while this phenomenon was abolished through exposure to a dim night-light pulse (**Fig. 8C, D**). Hence, the concomitant stimuli of warm nights and night light result in advanced expression of the EC target genes, whereas the cool-night signal without night light induces delayed expression of *PIF4*.

It is well known that hypocotyl elongation is regulated in a manner dependent on variation in both photoperiod and temperature (**Supplementary Fig. S12**). We previously proposed a coincident mechanism in which the photoperiod- and temperature-dependent modulation of *PIF4* expression is central to the photoperiod- and temperature-dependent regulation of hypocotyl elongation (Niwa et al. 2009, Kunihiro et al. 2011, Nomoto et al. 2012, Nomoto et al. 2013). In the mechanism, the short-day- and warm-night-promoted elongation of hypocotyls is best explained by an advanced expression of *PIF4* into the dark period, in which the PIF4 protein is stably and actively accumulated (Nomoto et al. 2013).

The results of **Fig. 8** together with the above notions led us to the idea that this EC-mediated modulation of *PIF4* expression during the night might be particularly relevant to seasonal variation in plant morphology. To gain insight into this, Col-0 (wild type) and *pif4-101* (a loss-of-function mutant) were grown under both long days (16 h light/8 h dark) and short days (8 h light/16 h dark) at 28, 22 and 16°C (**Fig. 9**). They were grown until the same number (5–6) of leaves had apparently developed on each plant in order to standardize their developmental stage (**Fig. 9**). The morphology of Col-0 leaves (petiole length and blade area) varied significantly depending on the photoperiod and growth temperature. Growth in warm temperature or a short photoperiod resulted in long petioles and small blades. However, such variation was diminished or abolished in the case of *pif4-101* (for example, compare the morphology of Col-0 and *pif4-101* plants grown under long days at 28°C). These results are consistent with the idea that PIF4 is at least in part implicated in seasonal variation in plant morphology. In other words, the observed EC-mediated modulation of *PIF4* expression during the night (**Fig. 8**) appears to be relevant to seasonal variation in plant morphology (**Fig. 9**). These results support the idea that EC-regulated *PIF4* is at least in part implicated in seasonal variation in plant morphology in natural habitats. This proposed view is consistent with the recent finding that *ELF3* is a crucial determinant of natural variation in thermo-responsiveness among a number of Arabidopsis accessions (Box et al. 2015).

Concluding remarks

Life cycle adaptation to seasonal variation in photoperiod and ambient temperature is a major determinant of the widespread ecological success of flowering plants. The findings in this study are compatible with the idea that the EC night-time repressor plays a role at least in part in tracking these seasonal changes in the photoperiod and ambient temperature. This is an intrinsic property of the circadian clock, which must somehow be linked to physiological outputs; otherwise, the sophisticated circadian clock would be useless. In this respect, it has long been established that the circadian clock is involved in the life cycle adaptation to photoperiodic variation in flowering season (Johansson and Staiger 2014, Song et al. 2015, and references therein). In the underlying mechanism, for instance, ELF3 plays an important role together with GI (Yu et al. 2008). The results of this study support the extended view that the EC night-time repressor plays a role in regulating seasonal variation in plant morphology through modulating *PIF4* expression. PIF4 is also involved in warm temperature-induced flowering in short-day conditions (Kumar et al. 2012). The EC night-time repressor appears to be central to life cycle adaptation to seasonal variation not only in the timing of reproduction but also in the morphology during vegetative growth. Taken together, these conceptual views proposed in this study are schematically summarized in **Fig. 10**.

Materials and Methods

Plant materials and growth conditions

The *A. thaliana* Col-0 ecotype was mainly used in this study. The functionally null *pif4-101* mutant has been described previously (Lorrain et al. 2008). Seeds were surface sterilized and stratified at 4°C for a few days, and then they were germinated and grown on a 0.3% (w/v) gellan gum plate containing Murashige and Skoog medium (pH is adjusted to 5.7) and 1.0% (w/v) sucrose in climate-controlled growth chambers. Growth temperatures were varied from 16 to 28°C, depending on the design of the experiment. Seedlings were grown in white fluorescent light (80 μmol m^{-2} s^{-1}) under 12 h light/12 h dark photoperiod cycles, unless otherwise noted. They were also exposed to white fluorescent light (80 μmol m^{-2} s^{-1}) during the night. When necessary, the light intensity was reduced (0.56 μmol m^{-2} s^{-1}) by using a natural density filter.

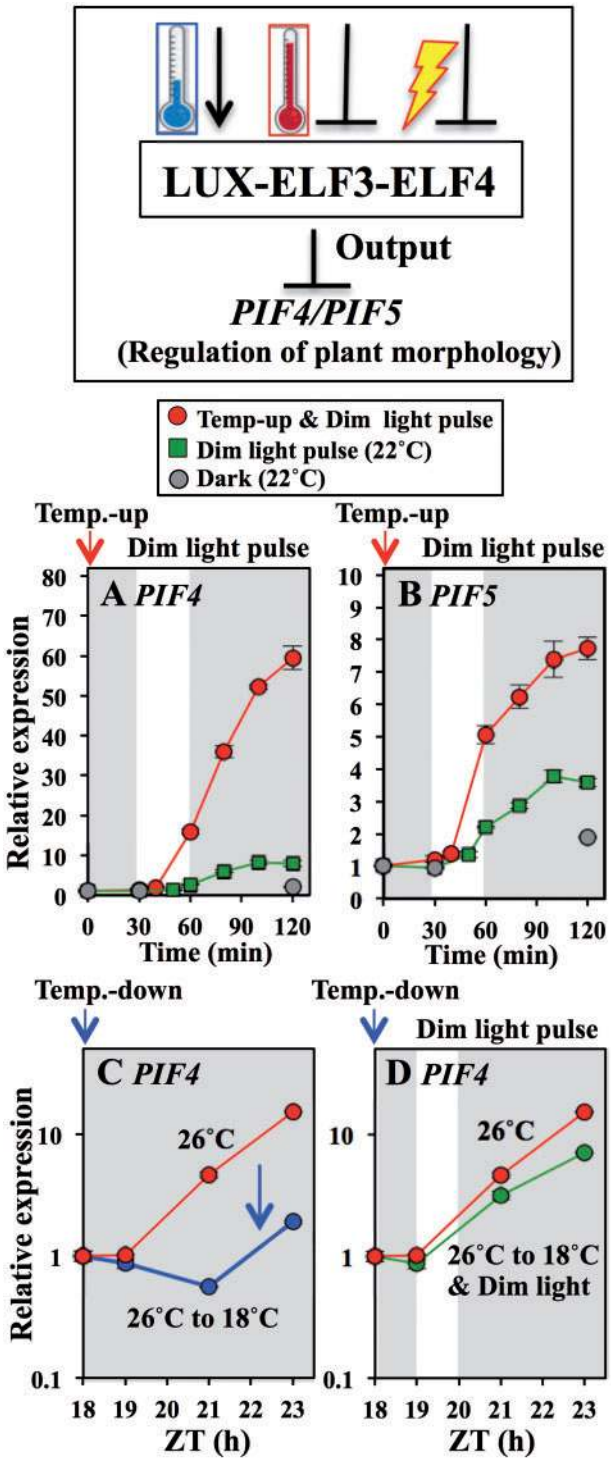

Fig. 8 Expression of *PIF4* is also modulated by the EC night-time repressor. For details, see the legends to **Figs. 5** and **6**.

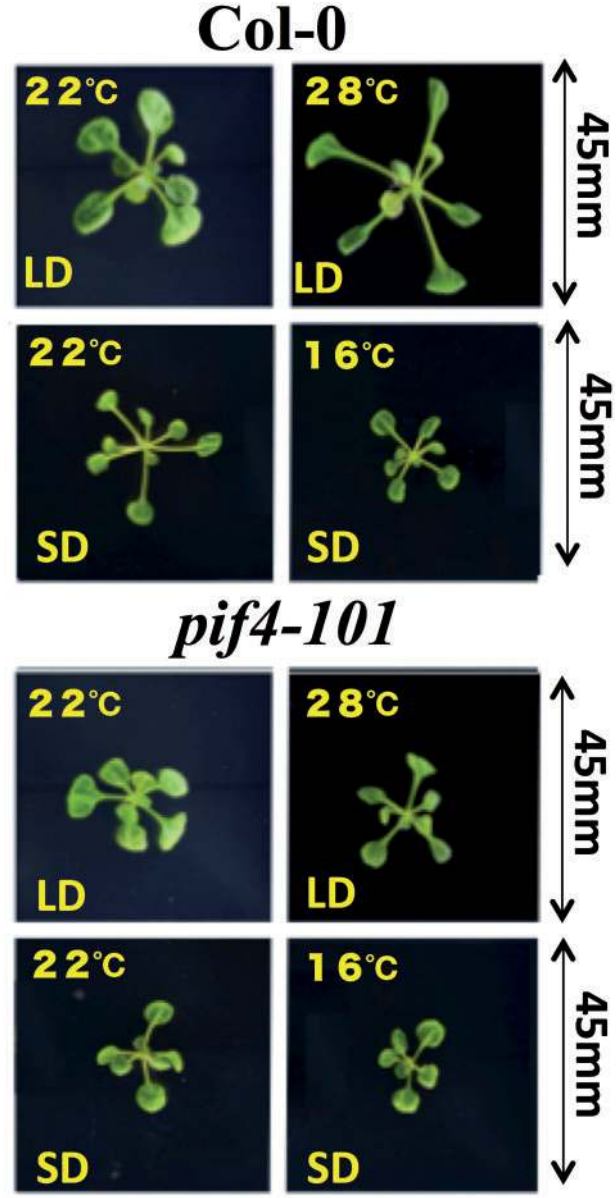

Fig. 9 *PIF4* is implicated in the regulation of plant morphology in response to seasonal variation in photoperiod and ambient temperature. Col-0 (wild type) and *pif4-101* (loss-of-function mutant) were both grown under long-day (16 h light/8 h dark) and short-day (8 h light/16 h dark) cycles at 28, 22 and 16°C. They were grown until the same number (5–6) of leaves had apparently developed on each plant in order to standardize their developmental stage and then photographed at an appropriate time depending on the growth conditions.

Preparation of RNA and qRT-PCR

Total RNA was purified from frozen plant materials (the aerial part of 7- or 8-day-old seedlings) with the RNeasy plant mini kit (Qiagen). To synthesize cDNA, RNA (1 μg of each) was converted to cDNA with ReverTra Ace (Toyobo) and an oligo(dT) primer. The synthesized cDNAs were amplified with SYBR Premix Ex Taq II (TAKARA) and the primer set for each target gene, and analyzed by using a Stepone Plus™ Real-Time PCR System (Life Technologies). The primer sets used in this study have already been presented in previous reports (Mizuno et al. 2014a, Mizuno et al. 2014b). The following standard thermal cycling program was used for all PCRs: 95°C for 120 s, 40 cycles of 95°C for 10 s, and 60°C for 60 s. The Ct value for individual reactions was determined by analysis of raw fluorescence data (without baseline correction) using the freely available software PCR Miner (Zhao and Fernald 2005; http://www.miner.ewindup.info). Based on the comparative Ct method, the relative expression level was calculated. The *APX3* gene encoding an ascorbate peroxidase isozyme was used as an internal reference.

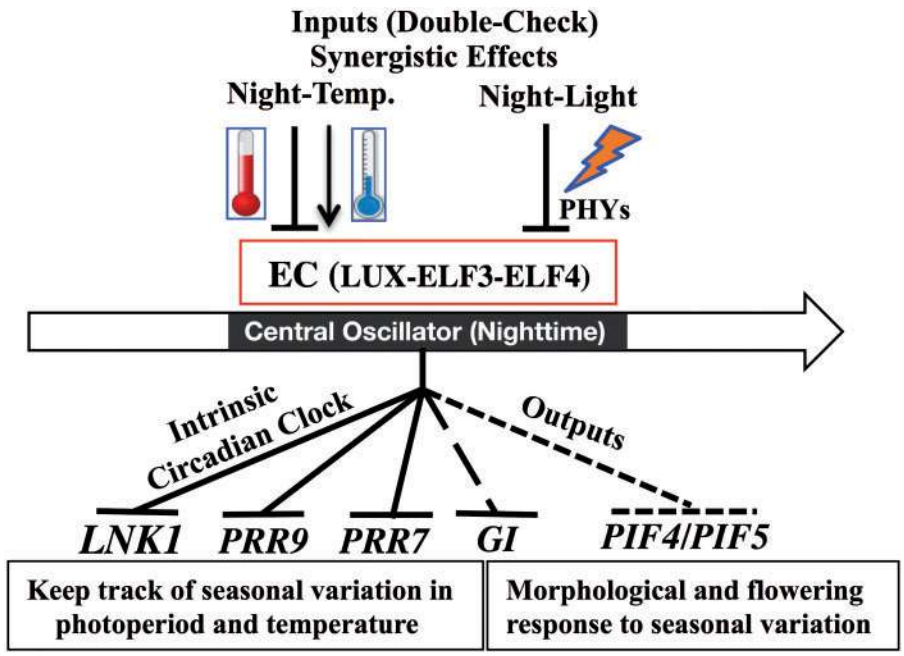

Fig. 10 Schematic representation of the views proposed in this study as to the EC night-time repressor-mediated circadian clock transcriptional network. For this illustration, the original illustration reported in previous studies (Mizuno et al. 2014a, Mizuno et al. 2014b, Mizuno et al. 2014c) was improved on the basis of the new findings in the present study. Note that the intrinsic clock component GI plays also important roles in diverse outputs. Hence, GI together with PIF4 isindicated by a broken line to emphasize this fact. Other details are given in the text.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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