In the flowering plant *Arabidopsis thaliana*, significant progress has been made in defining the molecular mechanism of circadian clock operation in plants. The central oscillator that has been uncovered is composed of at least ten gene-products, including CCA1, LHY, PRR9, PRR7, PRR5, TOC1 (or PRR1), LUX (or PCL1), ELF3, ELF4, and GI, which all together constitute a multi-looped transcriptional circuitry that generates robust and free-running circadian rhythms. This clock transcriptional circuitry has the capacity to integrate the external cues of light and temperature in order to not only accurately maintain the central oscillator functions in response to ever-changing seasonal conditions in natural habitats but also properly control a variety of output pathways. It has been postulated that CCA1/LHY, PRR9, and GI are implicated in light responses in the clock transcriptional circuitry. Temperature has two mechanistic impacts on the plant oscillator system. In a process referred to as temperature compensation, oscillator period resists changes in ambient temperature. On the other hand, in a process termed entrainment, temperature can act as a resetting cue. Molecular mechanisms underlying these temperature-related characteristics of the core clock genes might be relevant to the fundamental oscillator functions. Here, we further show that the recently identified *LNK1* light-inducible and clock-controlled gene, which actually has a robust peak at daytime, is induced also by warm-night through the EC nighttime repressor in a manner very similar to *PRR7*, which is also night light-inducible daytime gene. Based on these findings, a hypothetical view is proposed with regard to the temperature entrainment of the central oscillator.

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conditions. As pointed out previously, it might be puzzling that LUX is a target gene of the EC and is induced by a warm temperature that inactivates the EC nighttime repressor containing the gene product of LUX itself. However, it is conceivable that the induced LUX transcription factor may play a specific role in regulating a certain set of output genes without the need to form the EC under these conditions. It is also conceivable that the induced LUX transcription factor may compensate the EC inactivation by a temperature upshift. In any case, these temperature-associated characteristics of the core clock genes might be relevant to the fundamental oscillator functions such as temperature compensation and/or entrainment. Furthermore, transcription of another EC-target, the output PIF4 gene, is modulated through the same thermoregulatory mechanism, thereby leading to the PIF4-dependent temperature-adaptive photoperiodic regulation of hypocotyl elongation.

During the last decade, a number of clock-associated genes have been reported for A. thaliana based on the fact that a miss-expression and/or loss-of-function mutation of each gene result in an altered property of circadian rhythm. Hence, we wanted to look for additional EC-targeted and thermo-regulated clock-associated genes. In this study, we focused on the RVE8, LNK1, and JMJ30 (also known as JMJDS) genes, not only because the expression of these newly identified genes are directly controlled by the central oscillator, thereby showing robust free-running rhythms with their peaks at the daytime, but also because the clock functions are markedly compromised in their loss-of-function mutants. Among them, here we show that the LNK1 night-light-inducible and clock-regulated gene is also induced by warm-night through the EC nighttime repressor.

**Transcription of LNK1 is Able to Respond to Changes in Ambient Temperature Specifically at Dark Period**

To examine temperature responsiveness of the recently identified clock-associated genes, RVE8, LNK1, and JMJ30, in response to changes in growth-compatible ambient temperatures, wild-type (WT) seedlings (accession Col-0) were grown at 22 °C in light/dark cycles, and the temperature was raised to 28 °C at eight different zeitgeber time (ZT) points including the daytime and nighttime, as schematically shown in Figure 1 (top). After 3 h, RNA samples were prepared and temperature responsiveness of RVE8, LNK1, and JMJ30 was examined (Fig. 1A, D, and G, red). As a control, samples prepared from plants grown continuously at 22 °C were also analyzed (Fig. 1A, D, and G, green). The diurnal expressions of these genes robustly oscillated. RVE8 showed a peak at morning, LNK1 at daytime, whereas JMJ30 displayed its peak at evening. It was then revealed that the transcription of LNK1 was markedly upregulated following the temperature upshift, specifically during the dark period (red arrows in Figure 1D), while RVE8 and JMJ30 did not respond at any time (Fig. 1A and G). To examine these phenomena more closely, seedlings grown at 22 °C were upshifted to 28 °C at ZT = 12 (early night) and ZT = 18 (midnight), respectively, and the temperature responses were followed at 1 h or 2 h intervals. Indeed, LNK1 was upregulated in response to the temperature upshift at both ZT = 12 and ZT = 18 (Fig. 1E and F). Other genes (RVE8 and JMJ30) appeared to be insensitive to changes in temperature (Fig. 1B, C, H, and I). Furthermore, the expression of LNK1 was downregulated in response to a temperature downshift, when seedlings grown at 28 °C were downshifted to 16 °C at ZT = 18 (Fig. 2A). Taken together, it was concluded that the expression of LNK1 is reversibly regulated in response to changes across a range of growth-compatible temperatures, specifically during the dark period. Essentially the same properties were seen for LNK2 (a homolog of LNK1), although less strikingly (Fig. 2B). These properties are essentially same as those previously reported for a set of core clock genes, PRR9, PRR7, GI, and LUX.14

**Temperature Response of LNK1 is Gated through the Clock Function**

The above results suggested that the temperature response of LNK1 is gated in a time-of-day (or dark period) specific manner through the clock function. To address this issue, we compared the temperature response of LNK1 grown under light/dark (LD) cycles with that grown in continuous darkness (DD), as schematically illustrated (Fig. 3, top). First, as a control, a biologically independent experiment was replicated in LD and seedlings were subjected to temperature upshift at ZT = 15 (Fig. 3A). Then, seedlings grown at 22 °C under LD were transferred to DD, and exposed to 28 °C at appropriate timings during the first subjective daytime (Fig. 3B) and nighttime (Fig. 3C). LNK1 responded to the temperature upshift at the subjective nighttime in DD as well as in LD (Fig. 3C), but not at the subjective daytime (Fig. 3B). These results suggested that the free-running circadian clock gates temperature signals in such a manner that the timing of temperature responses is confined to strictly during the dark period. This idea was further confirmed by analyzing the temperature response of LNK1 under continuous light (LL) conditions (Fig. 3, bottom). Indeed, LNK1 responded to the temperature upshift even in LL at the subjective nighttime (Fig. 3E).

**The EC Nighttime Repressor is Implicated in the Temperature Responsiveness of LNK1**

It was previously shown that the EC nighttime repressor is a common factor for the temperature responses of PRR9, PRR7, GI, and LUX.14 To see whether this is the case also for LNK1, the temperature response of LNK1 was examined through employing a set of EC loss-of function mutants, namely, elf3–8, elf4–2, and pcl1–1, in addition to wild-type Col-0 (Fig. 4A to D). These seedlings were upshifted from 22 °C to 28 °C at ZT = 18, as described above. Compared with Col-0 (Fig. 4A), the expression of LNK1 in elf3–8 was constitutively high both before and after the temperature upshift (Fig. 4B). As a result, the temperature responsiveness of LNK1 was apparently abolished in this mutant. Essentially the same phenotypes were seen in both elf4–2 and pcl1–1 mutant seedlings.
Figure 1. (Upper part, A, D, and G) Temperature responses of a set of clock genes. Seedlings (Col-0) were grown at 22 °C for 8 d in light/dark cycles, and the growth temperature was upshifted to 28 °C at different zeitgeber time (ZT) points, as indicated schematically. RNA samples were prepared after incubation for 3 h, and levels of transcripts were determined by qRT-PCR (red). RNA samples were also prepared from control plants grown continuously at 22 °C (green). Relative expression levels are shown as mean values ± SD (n = 3). The values were normalized to the maximum value of the samples at 22 °C. The shaded period corresponds to the dark. (Lower part, B, E, F, H, and I) Expression of a set of clock genes following temperature upshift. Seedlings (Col-0) grown at 22 °C under light/dark cycles were upshifted to 28 °C at ZT = 12 (B, E, H) and ZT = 18 (C, F, I), and the temperature responses of a set of indicated clock genes were examined. Values were normalized to the initial ones, and relative expression levels are shown as mean values ± SD (n = 3). Detailed methods were described previously.\textsuperscript{14} The LNK1 primers for qRT-PCR used are 5'-GTCTTGGAAA CGAGGCCCA GCTTG-3' and 5'- GGAGCAGCAT CAGGAAACACC-3'.\textsuperscript{17}
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In other words, the temperature response of LNK1 is severely compromised in the set of EC loss-of-function mutants. Hence, it was suggested that the EC nighttime repressor is crucially and commonly implicated in the mechanism underlying the temperature responses of not only a set of core clock genes (PRR9, PRR7, GI, and LUX) but also the clock-associated LNK1 gene.

The EC Nighttime Repressor Binds to the LNK1 Promoter

To test the possibility that the EC nighttime repressor binds to the LNK1 promoter, we conducted chromatin immunoprecipitation (ChIP) assays by employing a set of transgenic lines each carrying an appropriate composite transgene, namely, ELF3pro-ELF3-YFP and LUXpro-LUX-GFP, both of which have been established previously.\(^{20,21}\) These transgenic lines have been successfully used for ChIP assays, the results of which demonstrated that the consensus LUX-binding site (designated LBS) is 5′-GAT(T/A)CG-3′, and that PRR9, PRR7, GI, LUX, and PIF4 are the direct targets of the EC.\(^{14,20-23}\) The promoter sequence of LNK1 contains several perfect or near-perfect LBSs. Based on this fact, we selected appropriate candidate regions (amplicons designated a to d) in addition to a negative control amplicon designated cs which is derived from the coding-region of LNK1, as schematically shown (Fig. 4, middle). The amplicons a and b contain a single and a double LBS, respectively. The amplicons c and d contain a single near-perfect LBS (GATTCT), respectively. First, we conducted ChIP assays by employing ELF3pro-ELF3-YFP with special reference to these amplicons. ELF3-YFP efficiently binds to the region confined by the amplicon c, which is located around the transcription start site of LNK1 (Fig. 4E). The result was confirmed by the replicated experiment with biologically independent samples (Fig. 4F). Concomitantly, ChIP assay employing LUXpro-LUX-GFP was also performed (Fig. 4F). Both ELF3-YFP and LUX-GFP preferentially bind to the same region located near the LUX transcription start site. These results are compatible with the idea that the LNK1 gene is a direct target of the EC nighttime repressor, although ChIP assay employing an appropriate ELF4 probe still remains to be performed. Taken together with the genetic evidence shown in Figure 4A–D, it was concluded that the EC nighttime repressor is essential for the temperature-dependent repression of LNK1 as well as a set of core clock genes (PRR9, PRR7, GI, and LUX).
The essence of this and previous studies was schematically summarized in Figure 5A. We showed that a set of core clock genes (PRR9, PRR7, GI, and LUX), a clock-associated light-inducible gene (LNK1), and a pair of clock output genes (PIF4 and PIF5) are common targets of the EC nighttime repressor. Growth-compatible temperature signals (e.g., changes in temperature of Δ6 °C, and differences in steady-state temperature from 16 °C to 28 °C) feed into the clock transcriptional circuitry through a common pathway in which a warm temperature antagonizes EC repressor-activity, whereas a cool temperature stimulates it. Consequently, these target genes are commonly regulated at the level of transcription, depending on ambient temperatures in such a way that a warmer temperature more efficiently induces their transcription specifically during the dark period, whereas a cooler temperature more strongly represses them. As noted previously,14 we do not know whether the DNA-binding of EC to the target promoters itself is inhibited by a warm temperature, or a warm temperature inhibits the repressor ability of EC without affecting its DNA-binding ability. Although it was suggested that the EC nighttime repressor
Ambient temperature affects the fundamental clock functions at several aspects. The revealed EC-mediated and temperature-regulated signaling network of the clock transcriptional circuitry might impact strongly on these clock functions (see Figure 5A). (1) In a process referred to as temperature compensation, the oscillator period resists changes in ambient temperature. This mechanism ensures a constant oscillation period of about 24 h within a wide range of ambient temperatures. On the other hand, in a process termed entrainment, temperature can act as a resetting cue. Indeed, temperature fluctuations as small as Δ4 °C within a day can reset the plant circadian oscillator.6-8 The EC-dependent coordinate thermoregulations of LUX, GI, LNK1, PRR9, and PRR7 might be implicated in the mechanisms underlying temperature compensation and/or entrainment. (2) It was previously shown that a 1 h light-pulse at the nighttime effectively induced the transcription of LNK1 that has a robust peak normally at the daytime.17 Interestingly, it was also noted that the same characteristics (i.e., night light-responsiveness) were seen for PRR7 and GI as well.17 Taken together with the results in this study, these light and warm temperature-inducible genes might integrate coordinately both light and temperature signals at the core oscillator to keep track of (or entrain) seasonal changes in photo-cycles and thermo-cycles. This might allow the central oscillator to regulate a
variety of output pathways in order to properly control the plant development and/or flowering time in response to changes in photoperiods and ambient temperatures. Indeed, another EC-controlled output gene, PIF4, is crucially involved in the photoperiod- and temperature-dependent control of hypocotyl elongation and flowering time (see Figure 5).\textsuperscript{24-27} In any case, the results of this study will shed new light on the longstanding problems with regard to the impact of ambient temperature on the plant circadian clock, as further discussed. It may be worth noting that recent studies showed that the temperature-dependent alternative splicing of certain clock genes (e.g., \textit{CCA1} and \textit{LHY}) results in modulation of their transcriptional profiles, which occur usually at an extremely low temperature (e.g., 4 °C).\textsuperscript{28,29} The temperature-induced events reported in this and previous studies are most likely not relevant to such effects of alternative splicing on the transcriptional circuitry of clock genes, because the events are observed within a range of growth-compatible moderate temperatures.

A Proposed Hypothesis

Finally, we would like to consider with regard to the above implications in further detail (Fig. 5B). For the dark period-dependent temperature responses of both the evening (e.g., \textit{GI} and \textit{LUX}) and daytime genes (e.g., \textit{LNK1} and \textit{PRR7}), the temperature-sensitive EC nighttime repressor plays a crucial role, as demonstrated in this and previous studies.\textsuperscript{18} A warmer temperature at dusk is indicative of a prolonged evening. Accordingly, the evening genes, \textit{GI} and \textit{LUX}, should remain expressed. The same signal at dawn is indicative of the coming sunrise. Accordingly, the daytime genes, \textit{LNK1} and \textit{PRR7}, should promptly start to be expressed. As schematically shown, the time interval of phases of the evening and daytime genes would be shortened in a warmer day (Fig. 5B, left). This means that a warmer ambient temperature would be indicative of short-nights (or long-days). Vice versa, the time interval of phases of the evening and daytime genes would be lengthened in a cooler day (Fig. 5B, right). This means that a cooler ambient temperature, which activates the EC nighttime repressor, would be indicative of long-nights (or short-days). It is worth mentioning that light signal during the subjective night, which is indicative of the sunrise, also induces the early expression of \textit{PRR7} as well as \textit{LNK1}.\textsuperscript{17} Taken together, it is tempting to hypothesize that through these EC-mediated regulatory mechanisms, temperature and light signals are integrated coordinately at the core oscillator to properly track seasonal changes in photo-cycles and thermo-cycles. This idea is consistent with the proposed functions of LNKs.\textsuperscript{19} Together with the recent and related studies from other groups,\textsuperscript{10,13,30,31} our results will shed new light on the longstanding temperature-associated subjects in this field.

Disclosure of Potential Conflicts of Interests

There were no potential conflicts of interests to disclose.

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