

SHORT COMMUNICATION



PRR9 and *PRR7* negatively regulate the expression of EC components under warm temperature in roots

Li Yuan^{a,b}, Yue Hu^a, Shilin Li^a, Qiguang Xie^{ORCID}^a, and Xiaodong Xu^{ORCID}^a

^aState Key Laboratory of Crop Stress Adaptation and Improvement, School of Life Sciences, Henan University, Kaifeng, China; ^bCollege of Life Sciences, Hebei Normal University, Shijiazhuang, China

ABSTRACT

Circadian clock operates autonomously in each cell and drives the approximately 24-h rhythm in individual tissues and organs. It is known that the evening complex (EC) components and *GI* are required for ambient temperature perception and thermomorphogenesis in higher plants. Our previous study found that *PRR9* and *7* are required for the lengthened period of the circadian rhythm in roots, and they are responsible for the temperature overcompensation in shoots. However, the molecular mechanism of the circadian clock, especially in different tissues, in response to temperature oscillations remains largely unknown. Here, we studied the transcript levels of EC genes and *GI* of the *prp7 prp9* mutant shoots and roots in response to 22°C or 28°C, respectively. The results showed that *PRR9*, *7* in roots inhibited the transcripts accumulation of *ELF3*, *ELF4*, and *LUX* at 28°C. In addition, loss-of-function of both *PRR9* and *7* caused an increase in *GI* expression at 22°C, but warm temperature of 28°C limited the negative effect of *PRR9*, *7* on *GI* in roots. Our findings proposed a temperature-dependent molecular basis for root-specific circadian clock and indicated the critical role for *PRR9*, *7* in negatively regulating *ELF3*, *ELF4*, *LUX*, and *GI* in the circadian gating of thermoresponse.

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Circadian clock, as a time-keeping mechanism, drives a large variety of physiological rhythms to synchronize with cyclic changes of environmental zeitgebers, such as light and temperature.¹ The five members of Pseudo-Response Regulators (PRRs) family are expressed sequentially from morning to evening during a 24-h day, and function as negative regulators of morning-phased *CCA1*, *LHY*, and *RVE8* in the transcriptional feedback loops of the circadian clock.² The long periodicity in the *prp7 prp9* mutant continues to lengthen as the ambient temperature rises to 30°C, demonstrating a temperature overcompensation phenotype.^{3,4} In addition, temperature compensation in the *prp7 prp9* mutants is related to the increased expression of *CCA1* and *LHY* at warm temperature. *CCA1* and *LHY* mainly act as transcription repressors in the circadian negative feedback loops, and their targets include *PRR5*, *PRR1/TOC1*, and *ELF3-ELF4-LUX* evening complexes (EC) genes. EC components perceive the changes in ambient temperature, and warm temperature inhibits EC gene expression; and as a result, *ELF3* regulates thermoresponsive growth and flowering.^{5–7} Whereas the relationship between *PRR9*, *7* and *ELF3-ELF4-LUX* complex in thermoresponse and temperature compensation of the circadian clock remains largely unknown.

Circadian rhythms run autonomously in individual organ, apical tissue, and single cell of plants.^{8–10} Period length in the roots is much longer than that in the shoot. The core oscillators in the shoot apex and vasculature, like in the central clock of mammals, play a dominant role in the perception of photosynthetic signals and orchestrates the entire circadian network

in whole plant.^{9,11–13} In a recent study, we found that the detached hairy roots of soybean perceive the intensity of red and blue light, and respond to discontinuous light or temperature pulses applied within the 24 h, which cause the phase shift of circadian rhythm.¹⁴ This finding implied that the root clock independently responds to external zeitgebers. We also found protein–protein interactions (PPI) between core oscillators present an approximately 24-h rhythms in both shoots and roots, and the period length of PPI rhythm in roots is longer than that in the shoots.¹⁵ What is more, mutation of *PRR9* and *PRR7* caused lengthened period in roots to be insignificant, indicating that *PRR9*, *7* is required for autonomous root clock. However, at 28°C warm temperature, *prp7 prp9* mutant has shown overcompensation only in the shoots, but not in the roots.¹⁵ In summary, we speculate that there are more circadian components involved in the molecular mechanism for temperature signal feeding into root clock.

Here, to explore the molecular architecture of the circadian clock in the temperature responses, we investigated the transcriptional feedback loops mediated by shoot- or root-specific expression of *PRR9* and *PRR7*. Shoots and roots of *prp7 prp9* mutant and wild-type plant were isolated and transferred to continuous light, 22°C or 28°C, respectively. Samples were collected during the second free-running cycle, and then transcripts enrichment of *ELF3*, *ELF4*, and *LUX* were analyzed by qRT-PCR. All the three EC complex genes showed higher expression in roots, at both 22°C and 28°C, than that in shoots (Figure 1). The result suggested that, like *PRR9* and *7*, the EC complex has a specific effect on the circadian clock in the roots.

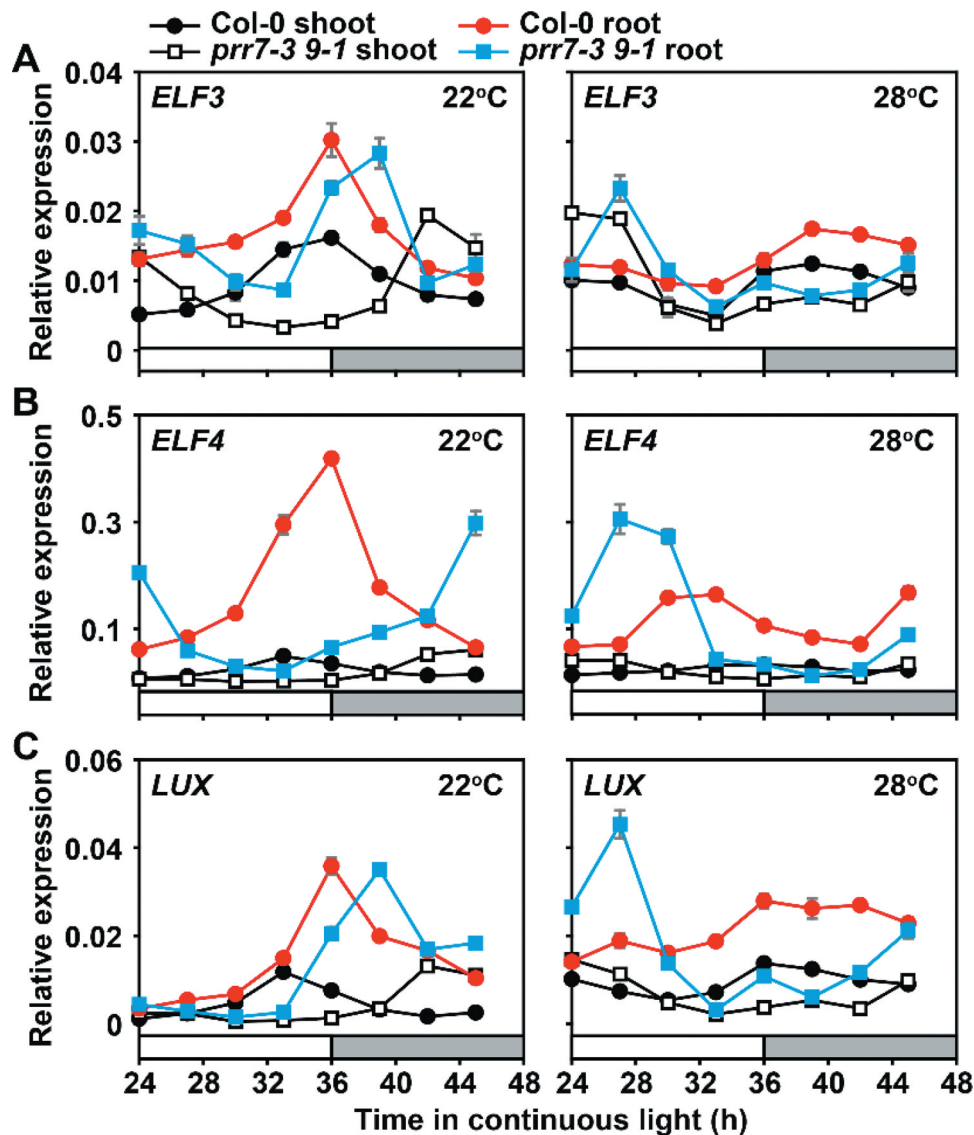


Figure 1. PRR9, 7 in roots inhibit EC complex gene expression under warm temperature. Transcript accumulation of *ELF3* (a), *ELF4* (b), and *LUX* (c) were examined in *prrr7 prr9* mutant and wild-type plants. The seedlings were grown under 12:12 LD cycles at 22°C for 14 days. After the shoots and roots were separated, they were transferred to 22°C or 28°C, respectively, under constant light conditions. Samples were harvested every 3 h over a 24-h cycle for RNA extraction and qRT-PCR analysis. The reference gene *IPP2* was used in the quantitative expression analysis. The data are presented as the mean \pm SEM of three technical replicates from one of three independent biological experiments; all experiments yielded congruent results. White or gray bars represent subjective day or subjective night, respectively.

In addition, at ZT24–48 of the constant light and temperature conditions, the peaks of the EC gene expression in the *prrr7 prr9* mutant moved to the daytime, especially the peaks in the roots at 28°C appeared near ZT27. We speculated that the long-period phenotype of the *prrr7 prr9* double mutant under the free-running conditions is a cause of the peaking shift of the circadian expression pattern.

Further analysis found that at 22°C, the peak expression of *ELF3*, *ELF4*, and *LUX* in the *prrr7 prr9* mutants delayed significantly under free-running conditions, but the overall accumulation or amplitude of the EC-related gene mRNA in the mutant and wild-type were similar in the 24-h day, both in shoots (black circle vs. empty square) or roots (red circle vs. blue square) (Figure 1). Under the 28°C condition, the peak expression of *ELF3* in the shoots of the *prrr7 prr9* mutant increased slightly, but the overall expression levels of *ELF4* and *LUX* were similar to those of the wild type in the 24-

h day. However, in roots, the peak expression of *ELF3*, *ELF4*, *LUX* in the *prrr7 prr9* was higher than that of the wild type (Blue square vs. red circle). This result indicated that PRR9, 7 in roots inhibited the *ELF3*, *ELF4*, *LUX* expression at warm temperature. Recently, the expression of PRR9, 7, 5, and *TOC1* were analyzed at 22°C or 28°C separately,¹⁵ and the results showed that PRR9 accumulation at 28°C was more than that at 22°C observed only in root tissues. Collectively, we provide the hypothesis that the expression of PRR9 increases in roots at warm temperature, thus inhibiting the accumulation of EC genes.

Evening-phased GIGANTEA (GI) modulate the *TOC1* and PRR5 accumulation in the evening by stabilizing ZEITLUPE (ZTL) protein,¹⁶ but the GI–ZTL interaction becomes extremely weak at warm temperature.¹⁷ The *GI* mRNA slightly increased as the temperature increased from 17°C to 27°C.¹⁸ Compared to 22°C, the *gi* mutant has a shorter period than the

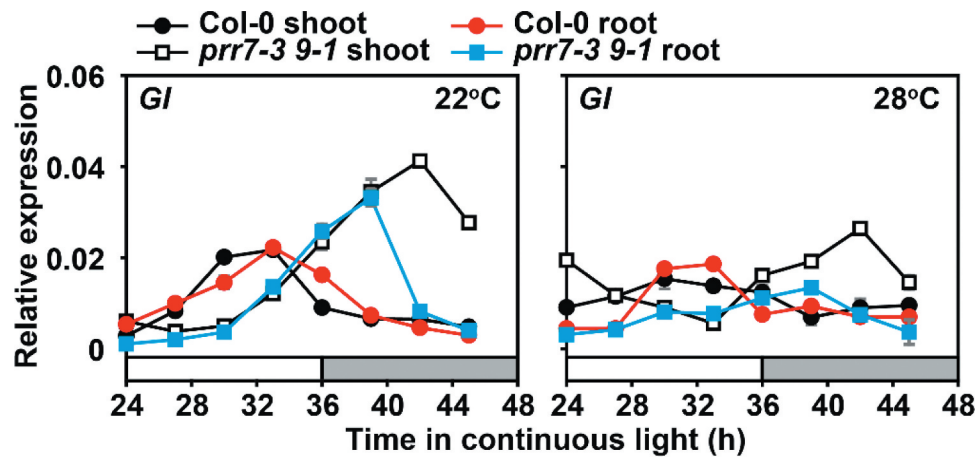


Figure 2. The negative regulation of PRR9, 7 in roots on *GI* is weakened at warm temperature. Transcript levels of *GI* were analyzed at 22°C and 28°C, respectively. *Arabidopsis* seedlings of the *prrr7 prrr9* mutant and wild-type plants were grown under 12:12 LD cycles at 22°C for 14 days. After the shoots and roots were separated, they were transferred to 22°C or 28°C, respectively, under constant light conditions. Samples were harvested every 3 h over a 24-h cycle for RNA extraction and qRT-PCR analysis. The reference gene *IPP2* was used in the quantitative expression analysis. The data are presented as the mean \pm SEM of three technical replicates from one of three independent biological experiments; all experiments yielded congruent results. White or gray bars represent subjective day or subjective night, respectively.

wild type at both 12°C and 27°C, indicating that *GI* accounts for temperature compensation. In order to determine the morning oscillators' regulation of *GI* in the interlocked loops, we examined *GI* transcript accumulation in the *prrr7 prrr9* mutant (Figure 2). Compared with 22°C, the amplitude of *GI* rhythmic expression in wild-type and mutant shoots decreased at 28°C; however, there was no significant difference in overall transcript accumulation in shoots and roots in the 24-h day. But it is worth noting that at 22°C, the increase in *GI* expression in the *prrr7 prrr9* mutant was greater than that at 28°C during the subjective night. This result indicated that the inhibitory effect of PRR9, 7 on *GI* was weakened at warm temperature. According to the RNA-seq data that has been studied, *GI* expression is up-regulated in the *prrr975* mutant.¹⁹ ChIP-seq data also indicate that PRR7 may bind to the *GI* promoter region.²⁰ Therefore, we speculated that PRR7 potentially directly inhibited the expression of *GI*. In addition, PRR9 and PRR7 may also indirectly regulate *GI* expression through CCA1/LHY and other circadian components. To verify the above hypothesis, the transcription activation experiment in transient transfected protoplasts and EMSA can be used to further analyze the mechanism of PRR9 and PRR7 in regulating the circadian responses to the temperature. In addition, Salomé et al. used the whole *Arabidopsis* seedlings to analyze the rhythmic expression of *GI* during ZT72-96 at 12°C, 22°C, and 30°C.⁴ In this study, we used the detached shoots and roots to analyze the tissue specificity of the rhythmic expression during ZT24-48 at 22°C or 28°C. Comparing the results of the two studies, it is consistent that compared with 22°C, *GI* expression in the *prrr7 prrr9* mutant was down-regulated at 28°C or 30°C, and the *GI* amplitude was reduced in both wild-type and the mutant. The difference is that at 30°C, the expression of *GI* increases in the wild-type whole plant; at 28°C, the *GI* peak expression decreases in the wild-type shoots, the trough value increases based on the rhythmic expression trace, and there were no significant changes in the roots during the 24-h day.

It is known that the products of the photosynthesis from shoots and leaves,⁸ and the EC complex component ELF4 protein²¹ regulate the root clock through transportation, thus maintaining the synchronization of the circadian rhythms between the organs. But this mechanism is not necessary for the self-sustained circadian rhythms in the roots. When the plant was intact, the circadian rhythm of the root was longer than that of the shoots; but when the shoot and root were separated, the circadian rhythm of the root was still longer than that of the shoots,^{9,15,22} which indicated that the root clock independently drives the circadian rhythm and own a lengthened period not entirely dependent on the signal transmission of vasculature and leaves. In addition, the plant root system, like the shoots on the ground, also experiences daily and seasonal changes of environmental temperature. In this study, in transcriptional feedback loops of root-specific clock, PRR9 and PRR7 inhibited the expression of evening-phased genes, *ELF3*, *ELF4*, *LUX*, and *GI*, at a warm temperature of 28°C. The proposed molecular model may function to modulate the self-sustained circadian rhythm and the temperature compensation in roots. In addition, warm temperature affects the inhibitory effect of PRR9, 7 on *GI* expression, forming a regulatory pathway of the circadian clock in anticipation of the thermocycles. Yet the genetic interactions between PRR9, 7 and *GI* in the temperature compensation and thermomorphogenesis need to be further determined.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Qiguang Xie  <http://orcid.org/0000-0001-7477-1965>Xiaodong Xu  <http://orcid.org/0000-0002-8795-7651>

Authors contribution

X.X., Q.X. conceived the project and wrote the article. L.Y. performed most of the experiments, L.Y., Y.H., S.L. performed the reference gene analysis. L.Y., Q.X., and X.X. analyzed the data.

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