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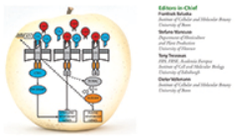

Plant Signaling & Behavior

ISSN: (Print) 1559-2324 (Online) Journal homepage: https://www.tandfonline.com/loi/kpsb20

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To cite this article: Yan Wang, Yumei Qin, Bin Li, Yuanyuan Zhang & Lei Wang (2020): Attenuated TOR signaling lengthens circadian period in *Arabidopsis*, Plant Signaling & Behavior

To link to this article: https://doi.org/10.1080/15592324.2019.1710935

PLANT SIGNALING & BEHAVIOR
https://doi.org/10.1080/15592324.2019.1710935

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SHORT COMMUNICATION

Attenuated TOR signaling lengthens circadian period in *Arabidopsis*

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ABSTRACT

By timing many diel rhythmic events, circadian clock provides an adaptive advantage for higher plants. Meanwhile, circadian clock displays plasticity and can be entrained by the external environmental cues and internal factors. However, whether cellular energy status can regulate circadian clock is largely unknown in higher plants. The evolutionarily conserved TOR (target of rapamycin) signaling among eukaryotic organisms has been implicated as an integrator for cellular nutrient and energy status. Here, we demonstrated that chemically blocking electron transport chain of mitochondrial can lengthen the circadian period. Similarly, chemical inhibition of TOR activity by Torin 1, a specific inhibitor for TOR kinase, and knockdown of *TOR* transcript levels significantly elongate the circadian period as well. Our findings imply that TOR signaling may mediate energy status-regulated circadian clock in plants, and the reciprocal regulation between the circadian clock and TOR signaling might be an evolutionary mechanism for fitness and adaptation in plants.

ARTICLE HISTORY
Received 10 December 2019
Revised 25 December 2019
Accepted 26 December 2019

KEYWORDS
TOR signaling; circadian period; cross talk; *Arabidopsis*

Introduction

Circadian clock times numerous key biological processes to the appropriate time of day or season in higher plants, including hypocotyl growth, pathogen defense, flowering time, and leaf senescence.[1-5] Hence, a proper circadian phase, which is mainly determined by the circadian period, provides growth fitness for higher plants by facilitating the endogenous rhythms to match the external day–night cycle.[6,7] Intriguingly, the circadian period and phase are not fixed in high plants; instead, they are dynamically adjusted by the external light and temperature cues and internal signals such as plant hormones and photosynthetic products.[8,9]

In photosynthetic organisms, TOR (target of rapamycin) signaling had been shown to be a central integrator of nutrient, energy, and stress status to modulate a broad spectrum of cellular processes, especially including cell proliferation in root meristem zone.[10,11] The close reciprocal intersections between TOR signaling and circadian clock had been implicated in other eukaryotic organisms including mammals,[12-14] but in plants, the crosstalk between circadian clock and TOR signaling still remains largely elusive. Very recently, we demonstrated that PRRs-TZF1-TOR molecular axis meditated a novel circadian output pathway to regulate cell proliferation activity in root meristem through integrating transcriptional and post-transcriptional mechanisms.[15] In this mechanism, we demonstrated that pseudo response regulator (PRR) proteins act as crucial circadian hubs to regulate cell proliferation activity in the root meristem, via acting as positive regulators of TOR signaling by directly repressing *Tandem Zinc Finger 1* (TZF1)*, a processing body localized RNA-binding protein. We further found that TZF1 protein binds to* TOR *mRNA through its tandem zinc finger motif to affect the stability of* TOR *mRNA.*[15] *However, whether TOR signaling can feedback-regulate circadian clock is still an open question.*

In this study, we utilized chemical inhibition and knockdown of *TOR* transcript levels to attenuate TOR signaling and found the circadian period could be dramatically lengthened by the compromised TOR signaling. We also demonstrated that chemically blocking the electron transport chain in mitochondrial could lengthen circadian period as well, indicating that energy status can regulate circadian clock in the plant via TOR signaling. Collectively, our findings uncovered that attenuated TOR signaling could significantly lengthen the circadian period in *Arabidopsis*, and suggest that the reciprocal crosstalk between circadian clock and TOR signaling may be evolutionarily conserved in plants.

Results and discussion

The reciprocal regulation between TOR signaling and the circadian clock has been documented in a number of eukaryotic organisms. In mammals, ribosomal S6 protein kinase 1 (S6K1), one of mTOR-effector kinase, is required for BMAL1 to associate with translational machinery and promote protein synthesis.[12] In Drosophila, TOR signaling can gate the nuclear accumulation of TIMELESS.[14] Our recent findings demonstrated that PRR-TZF-TOR molecular module shapes root architecture by coordinating clock outputs with cellular metabolism in higher plants. Nonetheless, whether TOR signaling

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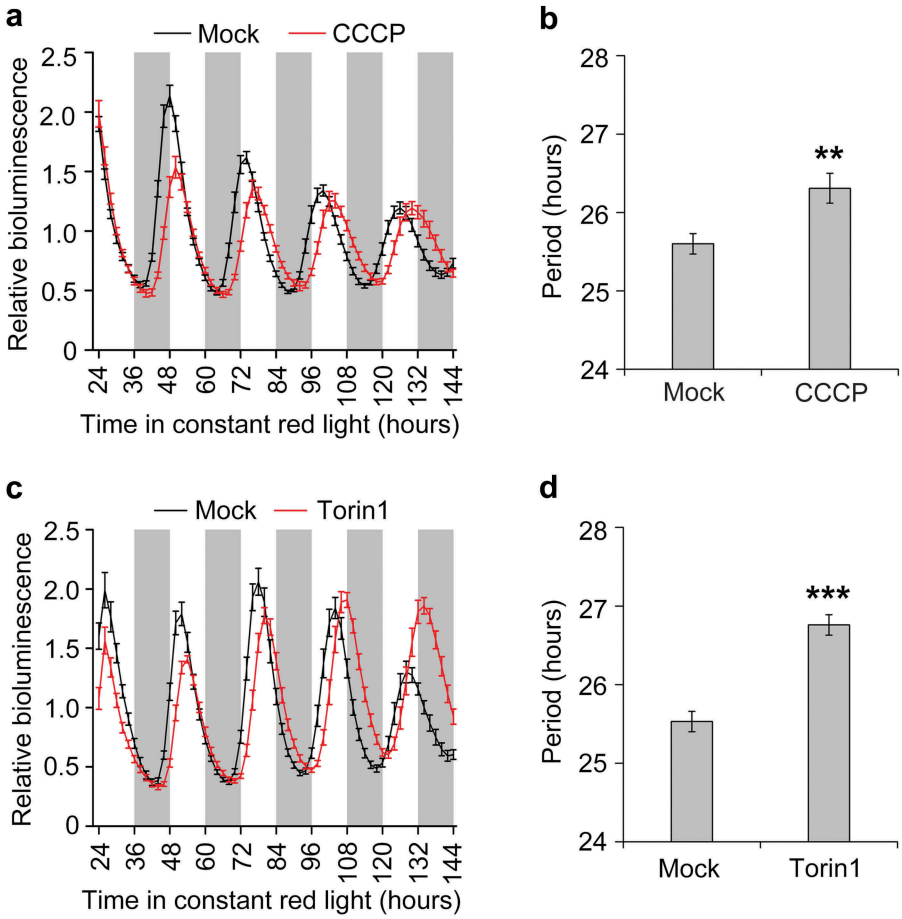

Figure 1. Inhibition of TOR signaling elongates the circadian period.

(A) Bioluminescence traces of *CCA1pro: LUC* reporter lines with or without 2 mM CCCP treatment (CCCP: carbonyl cyanide m-chlorophenylhydrazone, a mitochondria uncoupler). Data represent mean ± SEM (n = 21). The experiments were performed with three biological repeats with similar results. (B) Period length estimation of (A). CCCP treatment lengthens circadian period for about 0.71 h (n = 21). (C) Bioluminescence traces of *CCA1pro: LUC* reporter lines under constant red light, in the absence or presence of 40 μM Torin 1, a potent and selective TOR inhibitor. Data represent mean ± SEM (n = 22). (D) Period length estimation of (C). Torin 1 lengthened the circadian period for about 1.23 h in *Arabidopsis* seedlings (n = 22). In (A)–(D), 7-d seedlings grown on 1/2 MS medium with 1.5% sucrose under 12-h light/12-h dark and were placed in 96-well plates with liquid 1/2 MS medium plus 1.5% sucrose. CCCP or Torin 1 was added to the medium at a final concentration of 2 μM or 40 μM, respectively. After 1-d treatment, the luminescence images of the seedlings were captured under constant red light. In (B) and (D), the asterisks indicate the significant difference by *t*-test (**$P < .01$ and ***$P < .001$).

can feedback-regulate circadian clock is still largely unknown in higher plants.

Previously, we observed the lesser of *TOR* messenger RNA in the roots of *TZF1 OE* plants; however, we failed to find a dramatic circadian period change in *TZF1 OE* plants. We only found a slightly increased median value of circadian period in *TZF1 OE* plants,[15] which could be explained by the reduced degree of *TOR* mRNA which is not sufficient to cause the evident change of circadian period, as *TZF1 OE* is still fertile with late flowering,[16] while significant knockdown of TOR caused embryolethal phenotype.[10] Alternatively, we cannot rule out the possibility that the circadian phenotype was assessed with a circadian reporter in the aerial part of plants, where the role of TZF1 in the regulation of circadian clock might be masked by other downstream targets in an organ-specific manner.

As TOR signaling is an integrator for energy status, to directly investigate whether TOR signaling could feedback-regulate circadian clock, we initially treated 7-d-old seedlings containing the *CCA1pro:LUC* reporter with carbonylcyanide m-chlorophenylhydrazone (CCCP),[10] a mitochondrial uncoupler which could block upstream energy relays of TOR signaling. Strikingly, we found that CCCP could elongate the circadian period for about 0.91 h (Figure 1A,B), compared to the mock-treated plants, indicating that endogenous energy status might affect circadian clock in higher plants. To further investigate whether this phenomenon is caused by reduced TOR signaling, we directly inhibited TOR activity by using Torin 1, which has been shown as a potent TOR inhibitor.[10] Consistently, we found the circadian period was lengthened for about 1.1 h in the presence of Torin 1 (Figure 1C,D), suggesting that chemically blocking TOR signaling may regulate circadian speed. Notably, we designed an artificial microRNA of *TOR* as the previously adopted method.[17] Although we were only able to recover the weak amiTOR lines in T2 progeny (Figure 2E), the circadian period was lengthened to over 0.4 h (Figure 2A–D). Moreover, we crossed a well-established chemical induced RNAi line of TOR with Col-0 harboring *CCA1:LUC*, and examined its circadian phenotype with or without estradiol induction.

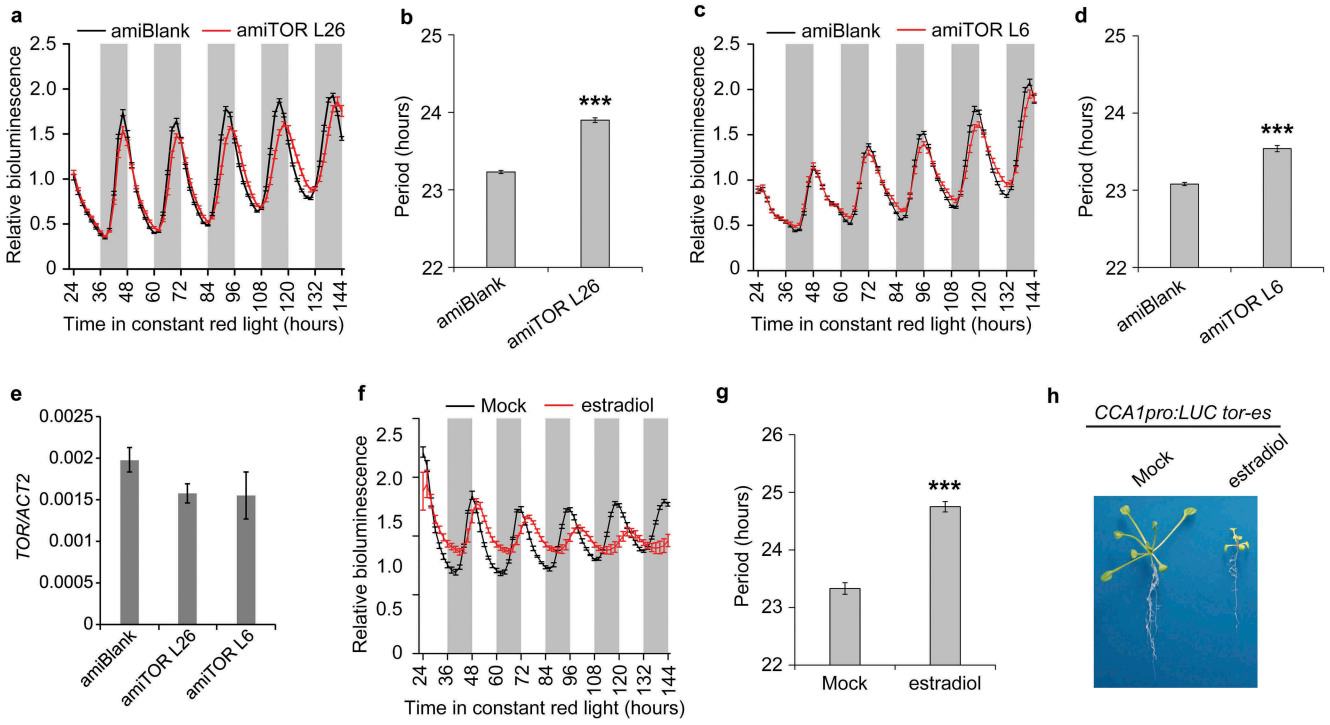

Figure 2. Knockdown *TOR* expression level lengthened the circadian period.

(A and C) Bioluminescence trace of the amiRNA-TOR transgenic lines containing *CCA1pro:LUC* reporter. (B and D) The circadian period of the amiRNA-TOR transgenic lines and the control lines. L6 and L26 represent two independent transgenic lines of *amiRNA-TOR*. In (A and B), data represent mean ± SEM of 20 plants. In (C and D), data represent mean ± SEM of 14 plants. (E) The transcript level of *TOR* in two *amiRNA-TOR* transgenic lines. Data represent mean ± SD of three technical replicates. The experiments were performed twice with similar results. The gene expression level was normalized with *the ACT2* gene. (F) Bioluminescence traces of *CCA1pro:LUC* reporter in *tor-es* plants under constant red light. Data represent mean ± SEM of 11 plants. Col-0 *CCA1pro:LUC* was crossed with *tor-es* to generate the *CCA1pro:LUC tor-es* lines. 5-d seedlings grown in MS with 3% sucrose under 12-h light/12-h dark condition were transferred to fresh MS with 3% sucrose and 10 μM estradiol for 3 d, and then the luminescence images were captured under constant red light. The experiments were performed with three biological repeats with similar result. (G) Period length estimation of (F). (H) The phenotype of the seedlings after estradiol treatment in (F). The experiments were performed with three biological repeats. In (B), (D), and (G), the asterisks indicate the significant difference by *t*-test (*** $P < .001$).

Strikingly, we found the estradiol-treated seedlings display a significantly longer circadian period than mock-treated seedlings (Figure 2F–H) in the normal growth condition with 1.5% sucrose. Together, we concluded that attenuated TOR signaling, by chemically inhibiting its activity or knockdown of its transcript level, could lengthen the circadian period in *Arabidopsis*. Thus, we propose that Glc-TOR signaling is not only a novel circadian output, but also a feedback regulator on the circadian clock, supporting a notion of TOR signaling-circadian clock close crosstalk in plants. Meanwhile, another report also suggested that TOR signaling mediates metabolite-regulated circadian clock in *Arabidopsis*.[18] Taken together, these findings unequivocally uncovered that TOR can feedback-regulate circadian clock in *Arabidopsis*.

Nonetheless, there are a few questions remaining to be addressed in the future. First, whether TOR signaling regulates circadian clock in organ or tissue-specific manner needs to be resolved. Previously, we reported that PRRs can affect TOR signaling in the root; however, the long hypocotyl in *prr579* mutant is not due to the reduced TOR signaling,[15] instead by affecting the abundance and activity of Phytochrome Interacting Factors (PIFs).[19-22] Given PIFs were also shown to mediate sugar-regulated circadian clock,[23,24] and it thus would be interesting to examine whether TOR signaling is involved in the organ- or tissue-specific regulation of the circadian clock.[25-27] Second, as TOR is a nuclear localized kinase,[10] how mitochondrial energy status could be perceived or transmitted to TOR signaling should be another attracting question. It is conceivable that one or more potential retrograde signaling pathways from mitochondrial to the nucleus may exist. Especially, the lower energy status may cause mitochondrial stress as well; thus, it would be desired to determine whether the role of TOR signaling in the regulation of circadian clock is through the dysfunction of mitochondria. Third, the underlying molecular mechanisms by which TOR signaling feedback-regulates circadian clock will be of great interest for future investigation. It is reasonable that, as a kinase, TOR protein may physically interact with core circadian components, subsequently affecting the circadian period by regulating the stability or activity of its interacting core clock components. Finally, whether the reciprocal regulation between the circadian clock and TOR signaling occurs in major crops, which may benefit crop yield in an ever-changing climate, is warranted to be explored in the future.

Materials and methods

Plant materials and plasmid construction

Laboratory-stored Col-0 harboring *CCA1:LUC* was used for wild type. To make a construct of amiRNA, the amiRNA was

designed by the Web MicroRNA Designer algorithm (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi), and primers were designed as previously described,[17,28] as listed in Supplemental Table S1. The construct of amiTOR was generated with former methods driven by *the CsVMV* promoter. After floral dipping-mediated transformation and screening based on hygromycin resistance, 10-d-old T2 transgenic seedlings, grown on 12-h light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/12-h dark photocycles on the half-strength Mruashige and Skoog (MS) medium plus 3% sucrose, were used for circadian phenotype analysis under constant red light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$). To generate *CCA1pro: LUC tor-es* lines, Col-0 CCA1:LUC was crossed with *tor-es* line, and F1 seedlings were used for assessing the circadian phenotype with or without estradiol, as noted.

Bioluminescence assays and circadian rhythm analysis

For chemical treatment with Torin 1 and CCCP, the seedlings were placed in 96-well plates with an indicated concentration in liquid MS medium, with the concentration as noted. The image acquisition was taken by a CCD camera (LN/1300-EB/1, Princeton Instruments, USA). Luminescence images were then processed and quantified by MetaMorph software. Data were imported into the Biological Rhythms Analysis software system (BRASS v2.14, available from www.amillar.org) and analyzed with the Fourier-transform nonlinear least-squares suite of programs. Period lengths are reported as variance-weighted periods ± SE, which were estimated using bioluminescence data with a time window from 24 h to 144 h.

Acknowledgments

We thank Prof. Yan Xiong from Fujian Agricultural and Forestry University for *tor-es* seeds.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by National Key Research and Development Program of China [2016YFD0100600] and National Natural Science Foundation of China [No. 31570292].

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