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ORIGINAL RESEARCH

Enhancement of Arabidopsis growth by non–24 hour day–night cycles

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Abstract

Plant yield can be increased by matching the internal circadian rhythms with the external light and dark cycle (circadian resonance). The circadian resonance reported in the past was analyzed under light–dark cycles with 20-, 24-, or 28-hr periods; however, the mechanism for circadian resonance is still debatable due to the experimental time schedules in previous studies being few in number and widely separated. By analyzing the yield of *Arabidopsis thaliana* grown under eight different external light and dark periods, we found that the yield increased when the external cycle was 22 and 26 hr instead of 24 hr. Time course RNA-seq analysis determined that seedling circadian clock genes had a free-running period of 22 $\pm$ 1 hr. Furthermore, a group of genes with 25- to 26-hr period rhythms were also observed in the seedlings with a 22- $\pm$ 1-hr period as their circadian clock. We propose that resonance that occurred by matching the expression time of a group of genes with the 25- to 26-hr cycle and providing an external day–night cycle of 25 to 26 hr was one factor that caused the yield increase.

KEYWORDS

Arabidopsis thaliana, circadian resonance, free-running period, light–dark cycle

1 | INTRODUCTION

Plant biomass or productivity is influenced by a combination of genetic traits and environmental conditions. For example, under circadian control, starch degradation continues throughout the night until the next dawn and is necessary for maintaining plant productivity (Graf et al., 2010). The circadian clock is a timekeeping system with about a 24-hr oscillation that governs the circadian rhythms of many physiological processes allowing plants to adapt to day–night cycles. Circadian rhythms have three characteristics: self-sustaining oscillation under continuous conditions, temperature compensation of the free running period, and entrainment to environmental time cues such as light and temperature. Entrainment activity enables the plant circadian clock to synchronize with external cycles. Therefore, plants having 24-hr internal rhythms can be entrained by an external day–night cycle with a non-24-hr period (e.g., 20 or 28 hr) (Dodd et al., 2014). In addition, *toc1-1* plants that have short periods and *ztl* plants that have long periods can synchronize to a 24-hr light and dark cycle (Dodd et al., 2014); however, a positive effect on growth is observed when the period length of the free running rhythm and the external light and dark cycle coincide (circadian resonance), (Golombek & Rosenstein, 2010; Ouyang et al., 1998). For example, the *toc1* Arabidopsis mutants have a short period and exhibit greater survival and a higher chlorophyll content than *ztl* plants that have a long period when grown in a growth chamber with a 20-hr period; under 28-hr growth conditions, the fitness of *ztl* plants increases compared with that of the *toc1* mutants (Dodd et al., 2005). In a previous report, circadian resonance was analyzed under light and

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dark cycles for periods of 20, 24, and 28 hr (Dodd et al., 2005). We analyzed circadian resonance and yield after varying the external light and dark periods more precisely. In our study, growth was faster in *Arabidopsis thaliana* grown in external light and dark cycles of 22 or 25 and 26 hr in comparison with plants grown in a 24-hr cycle. By transcriptome analysis, a gene group with a 25- to 26-hr expression period has been found that is distinct from a gene group with a circadian clock period.

2 | MATERIALS AND METHODS

2.1 | Plant growth under various T-cycles

A schematic diagram showing the light/dark cycle growth conditions of *A. thaliana* (Col-0) is shown in Figure S7. Seeds were sown on plates of 1/2-strength Murashige and Skoog (MS) gellan gum medium (Murashige & Skoog, 1962) containing of .5% ($w \cdot v^{-1}$) sucrose and stratified in the dark at 4°C before sowing. The seedlings were grown under continuous light (photosynthetic photon flux density [PPFD] of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by a light-emitting diode (LED) lamp (Plant Flec, Nippon Medical & Chemical Instruments Co., Ltd.) at 22°C for 5 days in a growth chamber (LPH-410SPCS, Nippon Medical & Chemical Instruments Co., Ltd.). The seedlings were then transferred to different growth chambers whose photoperiods were 20 hr (T20), 21 hr (T21), 22 hr (T22), 23 hr (T23), 24 hr (T24), 25 hr (T25), 26 hr (T26), 27 hr (T27), or 28 hr (T28). Two independent experiments were performed using randomly assigned growth chambers. The ratio of the light duration to the dark duration was fixed at 1:2 (e.g., 8 hr light: 16 hr dark for T24). The seedlings were grown for about 3 weeks until the cumulative time of the light treatment reached a total of 244 hr, that is, the sum of 120 hr (5 days) in constant light and 124 hr after being transferred to growth chambers set with varying photoperiods. The dry weights (mg) of the plants were then determined with a micro analytical balance, BM-20 (A&D Company, Ltd) after they were dried at 60°C for 48 hr. Statistical analysis was conducted using ezr (Kim, 2020).

2.2 | RNA sequencing (RNA-seq) analysis

A schematic diagram showing the light/dark cycle growth conditions of *A. thaliana* (Col-0) for RNA-seq analysis is shown in Figure S8. Seeds were sown on plates of 1/2-strength MS gellan gum containing .5% ($w \cdot v^{-1}$) sucrose after being stratified in the dark at 4°C before sowing. Seedlings were subsequently grown under conditions in which the ratio of the light duration to the dark duration was maintained at 1:1 (12-hr light: 12-hr dark) for 2 weeks in a growth chamber (LPH-410SPCS, Nippon Medical & Chemical Instruments Co., Ltd.). Light with a PPFD of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$, blue (450 nm), green (550 nm), and red (660 nm) was provided by an LED lamp (Plant Flec, Nippon Medical & Chemical Instruments Co., Ltd.). At the end of 2 weeks, plants were transferred to clean room (MCU-H4545W-DRP, SANYO Electric Co., Ltd.) for 1 day with constant light conditions. The light sources used were blue (450 nm) and red (660 nm) (Shibasaki, Inc., Saitama, Japan) with a PPFD of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 24 hr, aerial parts of the seedlings were sampled at 2-hr intervals for 72 hr. The temperature was maintained at 22°C. Total RNA was isolated using a RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality of the RNA samples was checked via an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The quantity of the RNA sample was determined by a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA-seq libraries were prepared with a TruSeq Stranded mRNA Sample Prep Kit (Illumina KK). Sequencing reads were obtained by the single-read method (50 bp) with an Illumina HiSeq 2000 (Illumina KK) high-throughput sequencing system. The RNA sequence datasets used for calculating gene expression length periods are available at the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession numbers DRP004324 (Bio Project; PRJDB5719 at http://trace.ddbj.nig.ac.jp/BPSearch/bioproject?acc=PRJDB5719, https://ddbj.nig.ac.jp/DRASearch/study?acc=DRP004324). The quality of all reads per sample was checked by FastQC and mapped to the Araport10 annotation models via RNA-seq by expectation maximization (RSEM; Li & Dewey, 2011) with Bowtie2 software (Langmead & Salzberg, 2012). The number of reads after chastity filtering each sample is shown in Table S1. Via these processes, the expression level data of the contigs for each sample were ultimately obtained. Finally, the reads per kilobase per million mapped reads were calculated. The expression levels throughout the time course were normalized by comparing the total read lengths and the number of sequencing reads. The normalized expression level was subsequently calculated as the value of the expression level minus the average expression level, divided by the standard deviation.

2.3 | Estimation of period length for gene expression

METACYCLE (meta2d, Wu et al., 2016 and https://cran.r-project.org/web/packages/MetaCycle/index.html) was used to detect genes whose expression oscillates. From the transcriptome time course data, this method can be used to identify genes that are expressed at a high level and to determine their periodicity. METACYCLE provides an integrated analysis using three different cycling detection algorithms (meta2d; integrated ARSER, JTK-CYCLE and LS). Genes whose expression was high and periodic were selected by setting a cut-off false discovery rate (FDR) controlled q-value $\geqq .05$ for the results of meta2d. We used a single biological replicate for this approach, but the procedure is fine because we got good fitting models to the data, as suggested previously (Hughes et al., 2017).

2.4 | Gene expression analysis using the diurnal database

Circadian gene expression data from *A. thaliana* were retrieved from the Diurnal database (http://diurnal.mocklerlab.org) (Mockler et al., 2007). We obtained circadian gene expression time course data

from two datasets: LL12(LDHH) and LL23(LDHH). The abbreviation LL indicates that Arabidopsis seedlings were sampled under continuous light and heat. LDHH is the abbreviation for the environmental conditions before sampling in which Arabidopsis seedlings were grown with a 24-h photoperiod (light 12 hr, dark 12 hr) and continuous heat. In other words, both datasets were from RNA of Arabidopsis grown under continuous light and continuous heat after initially being on LDHH (Edwards et al., 2006; Harmer et al., 2000; Peng et al., 2015). The period length for gene expression was calculated by METACYCLE (meta2d; integrated ARSER, JTK-CYCLE and LS). Genes whose expression was high and periodic were selected by setting a cut-off of the FDR controlled *q*-value ≥.05 for the results of meta2d.

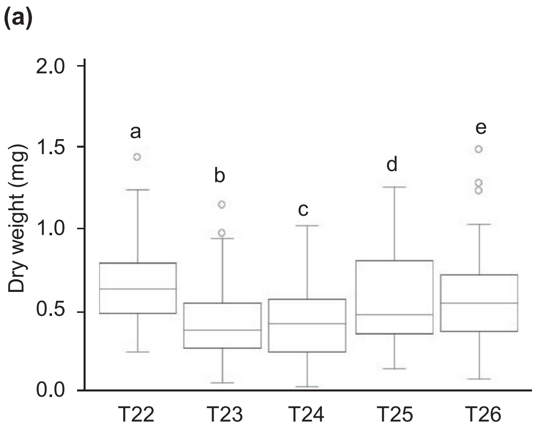
3 | RESULTS

3.1 | Plant growth under various T-cycles

To investigate the effects of external period length on biomass, growth of *A. thaliana* (Arabidopsis) accession *Col-0* was tested under various non-24-hr treatment cycles (T-cycles) with a 1:2 ratio of light: dark durations (e.g., 8 hr light/16 hr dark for T24 [control], Figure 1). The temperature was continuously 22°C in the experiments. We measured Arabidopsis biomass accumulation under five different T-cycles (T22, T23, T24, T25, and T26). The test was performed twice for reproducibility. Our tests using five different T-cycles revealed that the median dry weights of plants cultivated under T22 and T26 were higher than that of plants cultivated under T24 conditions (Figure 1a,b). Notably, the median dry weight accumulated under T22 was 1.5 (Figure 1a) and 1.7 (Figure 1b) times as much as that of plants grown under T24 conditions. Additionally, the median dry weight accumulated under T26 was 1.3 (Figure 1a) and 1.6 (Figure 1b) times as much as that under T24 conditions. The results of an experiment shown in Figure 1a, and the results from four other different T-cycles (T20, T21, T27, and T28) were integrated into Figure S9. This analysis showed that the dry weights of Arabidopsis grown in T21, T22, T25, and T26 conditions were greater than those grown in T24 conditions, perhaps suggesting that non-24-hr cycles from the environment enhanced Arabidopsis biomass under our conditions. We also hypothesized that matching the external 21- to 22-hr and 25- to 26-hr cycles and the internal 21- to 22-hr and 25- to 26-hr cycles enhanced biomass accumulation.

3.2 | Free-running period of the circadian clock

To explore the possible molecular mechanism that enhances biomass accumulation in T21, T22, T25, and T26 and not in T24, we wished to understand the pace of the circadian clock under the conditions we used (Figure 2). Arabidopsis seedlings were grown under the T24 cycles for 14 days and were then transferred into continuous light conditions for 1 day. Seedlings were sampled for 72 hr at 2-hr intervals, totally 36 samples, followed by transcriptome analysis. Due to the low number of reads (less than one million reads per library), we could not use data from one sample (corresponding to 34 hr in Table S1) to estimate the period length. Thus, to estimate the gene expression period length, we evaluated 18 RNA samples collected at 4-hr intervals (Table S2) using meta2d in the analytical tool



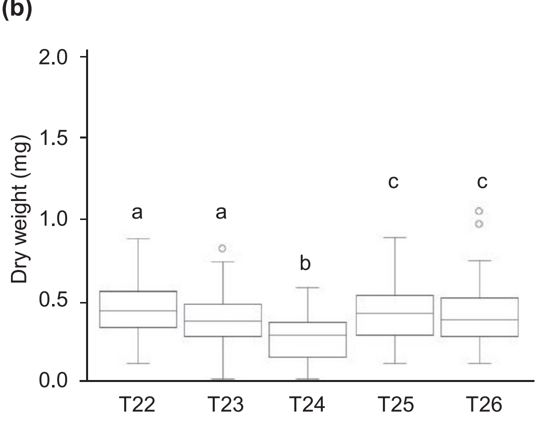


FIGURE 1 Dry weight of Arabidopsis growth under different T-cycles. *Arabidopsis thaliana* (Col-0) were sown on plates, after which the seedlings were grown under continuous light in a growth chamber for 5 days. The seedlings were then transferred to different chambers whose photoperiods were 22 hr (T22), 23 hr (T23), 24 hr (T24), 25 hr (T25), 26 hr (T26), under light–dark cycles. The ratio of light period durations to dark period durations was fixed at 1:2. The seedlings were grown until the accumulated time of the light treatment reached 244 hr. Dry weight (mg) represents the median. Box plots: Centerlines show the medians; box limits indicate the 25th and 75th percentiles; circles above and below the whiskers indicate outliers outside the 10th and 90th percentiles. Two growing tests were performed repeatedly and resulted in boxplot in (a) and (b). (biological replicates in (a); $n = 100$ for T22, $n = 53$ for T23, $n = 54$ for T24, $n = 55$ for 25, $n = 54$ for T26, biological replicates in (b); $n = 90$ for T22, $n = 90$ for T23, $n = 87$ for T24, $n = 84$ for 25, $n = 90$ for T26). Different letters indicate statistically significant differences determined by ANOVA with a post-hoc Tukey test ($p < .05$). For this experiment, environmental conditions other than the duration of the light and dark cycles were constant

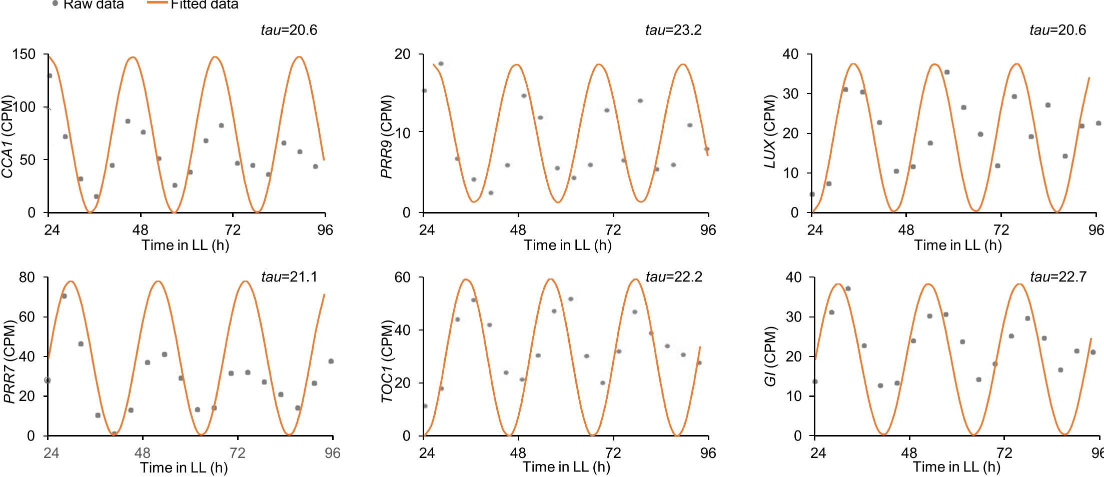


FIGURE 2 Free-running periods of clock-associated genes. Arabidopsis seedlings were grown under the T24 cycle (light 12 hr/dark 12 hr) for 14 days and transferred into continuous light conditions for 1 day. Seedlings were sampled for transcriptome analysis at 4-hr intervals for 72 hr. Counts per million reads (CPM) of circadian clock-associated genes are plotted as filled circles. Line charts schematically indicate the model cosine curves that had the highest correlation value with the raw data (filled circles). Tau indicates the period length of the cosine curves. *CCA1* (AT2G46830), *PRR7* (AT5G02810), *PRR9* (AT2G46790), *TOC1* (AT5G61380), *LUX* (AT3G46640), and *GI* (AT1G22770) are clock-associated genes

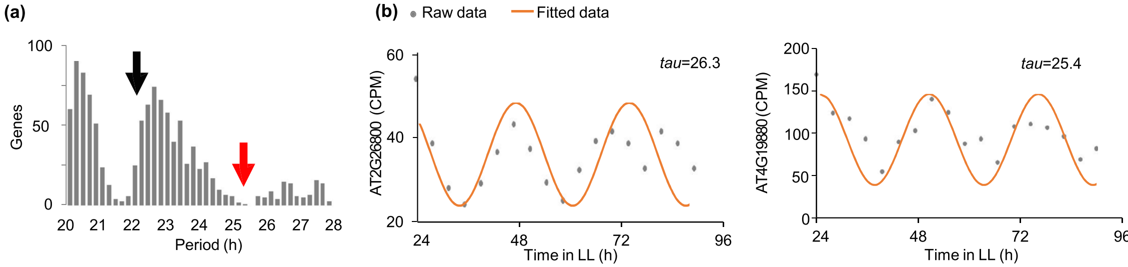


FIGURE 3 Genes that exhibit a free-running period different from the circadian clock. The expression period lengths of the oscillatory genes were estimated by meta2d. The genes whose FDR controlled *q*-value was lower than .05 were identified as oscillatory 1,095 genes. The frequency of oscillatory genes was indicated for each estimated period length (a). The set of 306 genes that oscillated with about a 22-hr period contains clock-associated genes (a, black arrow). Another group of oscillatory genes (56 genes) with a period of about 25.5 hr is shown with a red arrow (a). The raw data and the data fitted to a model cosine curve of two representative genes are shown in (b)

METACYCLE package (Wu et al., 2016). The output parameters for all transcripts of METACYCLE (meta2d; integrated ARSER, JTK and LS) are provided in Table S7. Free running periods of circadian clock genes, such as *CCA1*, *PRR7*, *PRR5*, *TOC1*, *LUX*, and *GI*, were about 22 hr (Figure 2). Further, genes that are directly regulated by circadian clock genes (PRR5; AT5G24470 [Nakamichi et al., 2012], PRR7; AT5G02810 [Liu et al., 2013], PRR9; AT2G46790 [Liu et al., 2016], LHY; AT1G01060 [Adams et al., 2018], CCA1; AT2G46830 [Nagel et al., 2015], GI; AT1G22770 [Kim et al., 2012; Nohales et al., 2019], and TOC1; AT5G61380 [Huang et al., 2012]) or by the circadian-associated protein complex (EC; Evening Complex [Ezer et al., 2017]) oscillated with a period of about 22 hr (Figure S2). Table S3 enumerates the number of target genes for each circadian clock gene. Therefore, in this experimental condition, the circadian clock oscillated with a period of about 22 hr. In addition, the circadian clock cycle coincided with the environmental cycle (circadian resonance) under the T22 conditions (Figure 1a,b).

3.3 | Estimation of the oscillation period length for expression of genes that possibly provide photosynthetic advantages

As background for increasing the biomass in T25 and that in T26 conditions, genes that exhibit periodicity in autonomous expression have been comprehensively identified (Figure 3). The period lengths of genes whose expression oscillated were estimated by meta2d. Genes whose FDR controlled *q*-values were less than .05 were selected as oscillated genes. As a result, 1,095 genes were identified as oscillatory genes, 306 of which oscillated with about a 22-hr period (Figure 3a, black arrow). These 306 genes belong to a gene group regulated by the circadian clock as shown in Figure 2. There was also a group of oscillatory genes (56 genes) with a period of about 25.5 hr (red arrow in Figure 3a). Descriptions of these 56 genes with AGI locus codes and the analytical results from meta2d are shown in Table S4. Briefly, the 56 gene set was enriched in genes associated

with cellular metabolic processes (GO:0044237) and metabolic processes (GO:0008152) (Table S5). As examples, the raw data for two representative genes (*AT2G26800* and *AT4G19880*) out of the 56 oscillatory genes and their data fitted to the model cosine curve are indicated in Figure 3b. The results show that expression of the two representative genes oscillated with a period of about 25.5 hr. Additionally, *AT2G26800* and *AT4G19880* were not on target list for clock-associated genes (Figure S2). In the T25 and T26 conditions of Figures 1a,b, biomass possibly increased by overlapping the rhythm with a period length different from the circadian clock and with the cycle length of the external light and dark cycles.

4 | DISCUSSION

Circadian resonance is an increase in productivity that occurs when the circadian clock cycle coincides with the external light–dark cycle, a phenomenon known in plants and photosynthetic bacteria (Dodd et al., 2005; Ouyang et al., 1998). In the current study, circadian resonance was analyzed under conditions that set the external time period more precisely. As a result, higher productivities were shown when plants were cultivated with an external light–dark cycle matching the circadian clock period length (shorter than a 24-hr period) (Figures 1 and 3a). Previous studies (Dodd et al., 2005; Graf et al., 2010) showed more robust growth peaking when plants were subjected to 24-hr T-cycles (T24) than T20 and T28. In our study, plants grew better in T20 than they did in T24 (Figure S9). When grown in culture on the medium used in this study and with equal durations of day and night, plants under T28 conditions bolted at the sampling time and could not be compared with other T-cycles. Therefore, short-day conditions were used for sampling during vegetative growth in all T-cycles. This difference in the ratio of light to dark periods may also explain the difference from the study reported by Dodd et al. (2005). It is possible that differences in growth conditions such as distinct T-cycles and growth media may influence plant productivity. In addition, the primary reason for productivity differences is that the periods of the clock genes were estimated to be approximately 22 hr in our study (Black arrows in Figure 3a). In other words, T20, rather than T24 conditions possibly increased productivity due to circadian resonance in our study. Although higher productivity was also shown when plants were cultivated with an external light–dark cycle longer than the circadian clock cycle length (about a 25.5-hour cycle) (Figure 1a,b). In addition, transcriptome analysis revealed a group of genes that oscillate at longer periods (about 25.5 hr) than the circadian clock cycle (Figure 3). The 56 gene set that oscillated with a frequency of about 25.5 hr was enriched in genes involved in metabolic processes (Table S5). The resulting enrichment analysis of the 56 genes further supported our ideas that resonance occurred when the expression time for a group of genes matched the 25- to 26-hr cycle and that providing an external day–night cycle of 25 to 26 hr was one of the factors responsible for the yield increase. To confirm whether the longer -period genes were not simply "noisy genes," Figure S1a shows a plot of the estimated period against FDR for 1,095 oscillatory genes. The FDR values for 20, 21, and 28 cycles were significantly different from those of the 22-, 23-, 24-, 25-, 26-, and 27-hr cycles, suggesting noisier rhythms for the 20, 21, and 28 cycles (Figure S1a). In addition, Figure S1b represents a plot of the estimated period against the relative amplitudes for 1,095 oscillatory genes. The amplitudes of all periodic categories were not significantly different (Figure S1b). In other words, the larger FDR of the 20-, 21-, and 28-hr cycles did not come from the amplitude. A plot without the FDR controlled *q*-value cut-off (Figure S1c) is also provided. This trend shows how well the current period-fitting algorithm is fitting. However, we cannot deny the possibility that the observed period variation is a technical problem caused by the period-fitting algorithm. Such a longer period of oscillating gene expression was also found in microarray data (IDs; LL12_LDHH and LL23_LDHH; see Section 2). From the microarray data, genes that exhibit periodicity in autonomous expression were comprehensively identified (Figures S3 and S4). As a result, 3,645 genes for LL23 and 1962 genes for LL12 were identified as oscillatory genes. Most of the clock-regulated genes such as *CCA1*, *PRR7*, *PRR9*, *TOC1*, and *LUX* oscillated in a cycle shorter than 24 hr in LL23 or LL12 (Figures S5 and S6). There were 2,826 genes whose expression oscillated with shorter than a 24-hr period in LL23 (Figure S3a, black arrow), and 1,467 genes had a shorter than 24-hr period in LL12 (Figure S4a, black arrow). The circadian clock genes had a relatively shorter period under the light conditions used in this study (Figure 2) than those in LL23 (Figure S5) or LL12 (Figure S6). This result might be related to a difference in light intensity as reported by Somers et al. (1998). There were also groups of oscillatory genes in LL23 (819 genes) and LL12 (495 genes) with periods longer than 24 hr (red arrows in Figures S3a and S4a). As examples, the raw data for two representative genes (*AT2G26510* and *AT5G21105*) out of the 819 oscillatory genes with periods longer than 24 hr in LL23 and their data fitted to the model cosine curve are shown in Figure S3b. The raw data for two representative genes (*AT5G05890* and *AT4G20410*) out of the 495 oscillatory genes with periods longer than 24 hr in LL12 and their data fitted to the model cosine curve are shown in Figure S4b. We propose that when this internal approximately 25.5-hr rhythm coincides with the external light–dark cycle, there will be a positive effect on seedling productivity. Among the long-period genes extracted by this study, there were genes whose relationships with previously reported circadian clock genes were unknown, for example, *AT2G26800* and *AT4G19880* (Figure 3b). In addition, eight genes identified as long-period genes in our transcriptome experiment were classified as rhythmic by two other microarray analyses (Diurnal; LL12_LDHH and LL23_LDHH). We hypothesize that these eight genes have relatively stable and rhythmic expression. Four out of the eight genes were not on target lists for clock-associated genes: *CCA1*, *PRR7*, *PRR5*, *PRR9*, *TOC1*, and *GI* or the clock-associated protein complex: *EC* (Table S6). The longer than 24-hr periodic genes identified in our study, for example, *AT5G52640* in Table S6, may be driven by a cycle other than the circadian cycle as described above. Thus, there are two possible explanations for our results: one possibility is that the presence of short-cycle genes, including clock-regulated genes, explains the higher biomass produced

under short T-cycles, and the other possibility is that photosynthesis-related long-cycle genes may support growth under long T-cycles. It cannot be excluded, however, that productivity can be increased if the circadian clock is entrained to a slightly longer period than the external light–dark cycle. The longer 24-hr cycle found in the environment could be an important finding for understanding how an internal cycle of about 25.5 hr is generated. For example, a cyclic 24.8-hr gravity change coming from the sun and moon can influence periodic root growth (Barlow, 2015; Barlow & Fisahn, 2012; Gallep et al., 2017). It will be intriguing to characterize the determinants that produce an internal cycle that is longer than a circadian-associated cycle and to define its relationship to unknown external factor cycles by investigating gene expression patterns or the phenotypes of loss-of-function mutants.

Additionally, we compared the rhythmic genes reported previously (Edwards et al., 2006; Harmer et al., 2000) with those identified in our study using GO term enrichment analysis (https://www.arabidopsis.org/tools/go_term_enrichment.jsp). Among the significant patterns, genes involved in photosynthesis (e.g., GO:0015979, *p*-value 2.29E-17) and carbon metabolism (e.g., GO:0015977, *p*-value 1.03E-2) were overrepresented, among a result consistent with earlier findings (Edwards et al., 2006; Harmer et al., 2000). As cited in a previous study (Edwards et al., 2006), not all of the important clock-affecting genes are rhythmically regulated at the transcript level. Our study identified a similar enrichment result for rhythmic genes as those found in previous studies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, software, validation: Y.Y. Formal analysis: Y. Y and K.T. Investigation: Y.Y. Resources: K.T. Data curation: Y.Y. Writing – original draft preparation: Y.Y. Writing – review & editing: Y.Y. Visualization: Y.Y. Supervision: K.T. Project administration: K.T. Funding acquisition: K.T. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

All available data are contained within the article or appear in the supporting information. The following are available online: RNA-seq data at https://ddbj.nig.ac.jp/DRASearch/study?acc=DRP004324 and at http://trace.ddbj.nig.ac.jp/BPSearch/bioproject?acc=PRJDB5719. MetaCycle package is available on the CRAN repository (https://cran.r-project.org/web/packages/MetaCycle/index.html) and GitHub (https://github.com/gangwug/MetaCycle).

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