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An Arabidopsis Cell Culture With Weak Circadian Rhythms Under Constant Light Compared With Constant Dark Can Be Rescued by ELF3

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ABSTRACT

Callus and cell suspension culture techniques are valuable tools in plant biotechnology and are widely used in fundamental and applied research. For studies in callus and cell suspension cultures to be relevant, it is essential to know if the underlying biochemistry is similar to intact plants. This study examined the expression of core circadian genes in Arabidopsis callus from the cell suspension named AT2 and found that the circadian rhythms were impaired. The circadian waveforms were like intact plants in the light/dark cycles, but the circadian expression in the AT2 callus became weaker in the free-running, constant light conditions. Temperature cycles could drive the rhythmic expression in constant conditions, but there were novel peaks at the point of temperature transitions unique to each clock gene. We found that callus freshly induced from seedlings had normal oscillations, like intact plants, suggesting that the loss of the circadian oscillation in the AT2 callus was specific to this callus. We determined that neither the media composition nor the source of the AT2 callus caused this disruption. We observed that *ELF3* expression was not differentially expressed between dawn and dusk in both entrained, light–dark cycles and constant light conditions. Overexpression of *AtELF3* in the AT2 callus partially recovers the circadian oscillation in the AT2 callus. This work shows that while callus and cell suspension cultures can be valuable tools for investigating plant responses, careful evaluation of their phenotype is important. Moreover, the altered circadian rhythms under constant light and temperature cycles in the AT2 callus could be useful backgrounds to understand the connections driving circadian oscillators and light and temperature sensing at the cellular level.

1 | Introduction

Circadian clocks are biological mechanisms maintaining a 24-h period regardless of external cues (McClung 2006). Circadian clocks exist in a wide range of organisms, from bacteria to mammals and plants (Jabbur, Zhao, and Johnson 2021). Robust clocks result in improved growth and fitness under environmental changes (Dodd et al. 2005; Green et al. 2002; Hotta et al. 2007; Yerushalmi and Green 2009; Webb et al. 2019). The three key characteristics of the circadian clocks used to distinguish circadian-regulated phenotypes from other responses to environmental cues are (1) the rhythm has an approximate 24-h period, (2) the rhythms can be reset by the environmental signals, and (3) the rhythmic period is consistent across a set of temperature ranges (McClung 2006). The simplified clock model comprises inputs, central oscillators, and outputs (Harmer 2009). When plants perceive changes in the inputs such as light and temperature, the core oscillators

adjust their expression to regulate rhythmic outputs such as leaf movement, stomatal opening, hypocotyl elongation, and flowering time (Barak et al. 2000; Dowson-Day and Millar 1999; Engelmann, Simon, and Phen 1992; Somers et al. 1998).

Arabidopsis clock proteins form complex transcription-translation feedback loops (Nakamichi 2011; Harmer 2009). Key proteins in these circadian loops are CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and TIMING OF CAB EXPRESSION 1 (TOC1) (Alabadí et al. 2001; Strayer et al. 2000). The morning-expressed proteins CCA1 and LHY redundantly repress the expression of the evening gene, *TOC1* (Alabadí et al. 2001), and vice versa (Perales and Más 2007; Pruneda-Paz et al. 2009). Another key component in the clock system is the evening complex (EC) which consists of LUX ARRHYTHMO (LUX), EARLY FLOWERING 3 (ELF3), and ELF4 (Nusinow et al. 2011). This complex accumulates at dusk and represses the expression of *PSEUDORESPONSE REGULATOR 9* (*PRR9*), a CCA1/LHY repressor, creating a negative feedback loop with the morning genes (Dixon et al. 2011; Chow et al. 2012; Helfer et al. 2011; Herrero et al. 2012). The clock components directly or indirectly interact with each other, resulting in a complex network to control output genes in the circadian-related pathways such as *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB2*) in photosynthesis (Kay 1993; Millar et al. 1995), *FLAVIN-BINDING*, *KELCH REPEAT*, *F-BOX PROTEIN 1* (*FKF1*) in flowering (Imaizumi et al. 2003), and *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) in plant growth (Choi and Oh 2016).

ELF3, a component of the EC, is a multifunctional protein involving both light and temperature signaling (McWatters et al. 2000; Covington et al. 2001; Hicks, Albertson, and Wagner 2001; Jung et al. 2020). ELF3 regulates the gating of light inputs to the clock and light resetting of the clock (McWatters et al. 2000; Covington et al. 2001; Hicks, Albertson, and Wagner 2001). Through interaction with PHYTOCHROME B (PHYB), ELF3 modulates light input to the clock (Kolmos et al. 2011; Liu et al. 2001). ELF3 is required for temperature entrainment; the *elf3* mutant maintains rhythmic expression in thermocycles but does not exhibit a 24-h circadian rhythm in either constant light or temperature (Thines and Harmon 2010). In addition to arrhythmia in free-running conditions, loss of ELF3 has several phenotypes, including the lack of a light gating response and altered photomorphogenesis (McWatters et al. 2000; Covington et al. 2001; Hicks, Albertson, and Wagner 2001; Zagotta et al. 1996). A prion-like domain (PrD) of ELF3 functions as a thermosensor in which a variation in the length of the polyQ region inside the PrD leads to different thermal responsiveness (Jung et al. 2020). These multiple roles of ELF3 in circadian regulation of light and temperature signaling pathways suggest that it is an integrator between the clock and environmental cues.

Callus systems have been used to generate transgenic reporters, allowing a quick evaluation of many genotypes compared to transforming intact plants (Xu, Xie, and McClung 2010). To induce callus from explants in vitro, two plant growth regulators, auxin and cytokinin, are used (Grafi and Barak 2015). Callus cells have different gene expression profiles than other tissues (Tanurdzic et al. 2008; He et al. 2012; Du et al. 2019; Shim et al. 2020; K. Lee, Park, and Seo 2018). About 138 circadian-related genes, including *CCA1*, *LHY*, *TOC1*, and *ELF3*, were differentially expressed in Arabidopsis callus compared to intact leaves, suggesting that changes in clock gene expression were important for the establishment and maintenance of callus (K. Lee, Park, and Seo 2016).

Plant cell suspension cultures have been successfully used to investigate the components and kinetics of the circadian clock, supporting the idea that this system could be a powerful tool for understanding intracellular circadian regulation (W.-Y. Kim, Geng, and Somers 2003). However, some reports show a variation in the circadian rhythm between intact plants and callus and cell suspension systems (W.-Y. Kim, Geng, and Somers 2003; Nakamichi et al. 2003, 2004; Xu, Xie, and McClung 2010; Sai and Johnson 1999). *Brassica napa* callus is considered to have functional clocks based on the observation that *CCA1* expression was entrained by both photocycles and thermocycles (Xu, Xie, and McClung 2010). However, in this callus, the period of *CCA1* was consistently longer than the cotyledon movement period in intact seedlings. In Arabidopsis, the cell suspension T87 had robust circadian clocks as the expression of *CCA1* and *TOC1* was rhythmic under constant light and dark similar to intact plants (Nakamichi et al. 2003; 2004). Although another line of Arabidopsis cell suspension maintained rhythmic *CCA1* and *TOC1* mRNA levels under free-running conditions, the period was significantly more extended than in intact plants (W.-Y. Kim, Geng, and Somers 2003). Therefore, our study aimed to characterize the circadian oscillation in the Arabidopsis callus obtained from the cell suspension named AT2 (Tanurdzic et al. 2008). Examining multiple transcriptional reporters, we found that the AT2 callus lacked circadian oscillations in constant light conditions. This loss of oscillations in constant light is specific to the AT2 callus compared to freshly derived callus. We determined the possible factors contributing to oscillation patterns in the AT2 callus and freshly generated callus. Finally, we determined the effect of *AtELF3* overexpression on circadian oscillations in the AT2 callus.

2 | Materials and Methods

2.1 | Plant Growth Conditions

Arabidopsis AT2 cell suspension culture was provided by Dr. Linda Hanley-Bowdoin, North Carolina State University, Raleigh, NC, USA. This cell line was established from leaves of the *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) (Tanurdzic et al. 2008; Wheeler et al. 2020; T.-J. Lee et al. 2010; Concia et al. 2018). Cells were maintained as described in Tanurdzic et al. (2008) with small modifications. Cells were grown in Gamborg's B5 (GB5) medium (Gamborg, Miller, and Ojima 1968) supplemented with 2.5-mM (2-[*N*-morpholino]ethanesulfonic acid) hydrate (MES), 0.5-μg/mL 2,4-dichlorophenoxyacetic acid (2,4-D), and 3% (w/v) sucrose (pH 5.8) (Tanurdzic et al. 2008). Cells were shaken at 160 rpm under 12/12-h light/dark (LD) cycles with 75–80 $\mu mol/m^2/s$ of light intensity. Cells were subcultured weekly by transferring 5-mL cell suspension to 50-mL fresh media.

CCA1::LUC (Pruneda-Paz et al. 2009), *LHY::LUC* (Baudry et al. 2010), *TOC1::LUC* (Alabadí et al. 2001), and *FKF1::LUC* seeds were sterilized and plated on 1/2 MS medium (Murashige and Skoog 1962) with 0.4% (w/v) agar. Seeds were stratified at 4°C for 3 days and then moved to a growth chamber set to 23°C and LD. Seedlings (1–2 weeks old) on 1/2 MS agar plates were used in the time-course bioluminescence assay.

2.2 | *Agrobacterium* Transformation and Initiation of AT2 Transformed Callus

The 7-day-post-subculture AT2 cells were precultured in a transformation medium (GB5 medium supplemented with 2.5-mM MES, 2.7-μM NAA, 0.23-μM kinetin, and 3% (w/v) sucrose) for 2 days. *Agrobacterium tumefaciens* strain GV3101 containing *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* were cocultured with AT2 cells supplemented with 100-μM acetosyringone for 2 days. Transfected cells were then washed and grown in transformation media containing 250-μg/mL timentin with shaking for 3 days. Transformed cells were plated on a selective medium (GB5 medium supplemented with 2.5-mM MES, 2.7-μM 1-naphthaleneacetic acid [NAA], 0.23-μM kinetin, 250-μg/mL timentin, 3% [w/v] sucrose, and 0.8% [w/v] agar) for 2 weeks. The 50-μg/mL gentamicin was used to select *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC*. Selected calli were transferred to fresh selective agar plates every 2 weeks to maintain their growth. Transformed calli used in the time-course bioluminescence assay were grown on selective agar plates for 7–12 days after subculture.

2.3 | Generating the Overexpression of *AtELF3* in AT2 Transformed Callus

Gateway cloning technology was used to generate the *35s::AtELF3* construct. The *AtELF3* coding sequence (CDS) in the pENTR vector (Pruneda-Paz et al. 2014) was cloned into the 35s-promoter pB7WG2 vector (Karimi, Inzé, and Depicker 2002) via LR reaction (Gateway LR Clonase II Enzyme mix, Invitrogen, USA). *A. tumefaciens* strain GV3101 carrying *35s::AtELF3* vector was used to infect *CCA1::LUC*, *LHY::LUC*, and *TOC1::LUC* AT2 calli as described above. Transformed calli containing both *35s::AtELF3* and clock reporters were selected on the selective medium supplemented with 50-μg/mL gentamicin and 10-μg/mL glufosinate-ammonium for 2 weeks. Selected calli were subcultured to fresh selective medium every 2 weeks. The transformed calli on the selective medium were then used in the time-course bioluminescence imaging.

2.4 | Callus Induction From Arabidopsis Tissues and Seedlings

CCA1::LUC, *LHY::LUC*, and *FKF1::LUC* seeds were sterilized in 50% (v/v) bleach and 0.02% (v/v) Triton X-100 and placed on a callus induction medium. We used two recipes of callus induction media reported in Barkla, Vera-Estrella, and Pantoja (2014) and Sello et al. (2017). The Barkla medium was MS medium supplemented with 1-μg/mL 2,4-D, 0.05-μg/mL BA, 3% (w/v) sucrose, and 0.8% (w/v) agar (pH 5.7) (Barkla, Vera-Estrella, and Pantoja 2014). The Sello medium was MS medium supplemented with 0.5-μg/mL 2,4-D, 0.25-μg/mL benzylaminopurine (BA), 3% (w/v) sucrose, and 0.8% plant agar (pH 5.5) (Sello et al. 2017). Seeds were stratified at 4°C for 3 days, and the plates were then moved to a growth chamber with 23°C and LD. Callus was generated from seedlings within 3 weeks. Calli were subcultured to fresh media every 2 weeks to maintain their growth. In the bioluminescence assay, calli were grown on the Barkla or Sello agar plates for 7–12 days after the previous subculture.

To generate calli from different plant tissues, *CCA1::LUC* seeds were sterilized as described above and placed on the 1/2 MS medium with 0.4% (w/v) agar. Seeds were stratified at 4°C for 3 days and then moved to a growth chamber with the condition described above. Hypocotyls, first leaves, and roots were isolated from 7-day-old seedlings and placed on Barkla callus induction medium (Barkla, Vera-Estrella, and Pantoja 2014). Calli induced from those tissues were subcultured to fresh medium every 2 weeks. The 7–12 day-after-subculture calli on Barkla medium were used in the bioluminescence assay.

2.5 | Luciferin Spraying and Time-Course Bioluminescence Assay

Calli and seedlings were sprayed with 250-μg/mL D-luciferin (GoldBio, USA) in 0.01% (v/v) Triton X-100. The plates were placed in the growth chamber installed with a CCD camera (Eagle V 4240 Scientific CCD camera, Raptor Photonics). The camera was controlled by Micro-Manager software (Edelstein et al. 2014), and images were captured with a 20-min exposure time every 2 h to detect bioluminescence. For the LD condition, the light was on at 8 a.m. (circadian time 0; ZT0) and off at 8 p.m. (ZT12). To transition from LD to the free-running conditions, the lights were kept on starting at 8 p.m. (ZT12) for continuous light (LL), and the lights were kept off starting at 8 a.m. (ZT0) for continuous darkness (DD). In the temperature cycles (HC), daytime temperatures were kept at 23°C for 12 h and nighttime temperatures were 12°C.

The luminescence signal of luciferase from each callus or seedling was measured as mean gray values (total intensity in the selected area divided by the number of pixels) using ImageJ software (Schneider, Rasband, and Eliceiri 2012). The mean gray value was subtracted by the mean gray value of the background to obtain the normalized intensity. To calculate a relative normalized intensity, the normalized intensity at individual time points was divided by the maximum normalized intensity in that time series. Relative normalized intensity was then linearly detrended on BioDare2 (biodare2.ed.ac.uk) (Zielinski et al. 2014). Rhythmicity test (BD2 eJTK method) on BioDare2 was used to select only rhythmic data (p value < 0.05 with Benjamini–Hochberg correction) for making time-series plots (Hutchison et al. 2015). Periods, phases, and relative amplitude error (RAE) were estimated by a Fourier transform–nonlinear least squares (FFT-NLLS) method on BioDare2 (Zielinski et al. 2014).

Statistical analysis and data visualization were performed in R Version 4.0.5. Wilcoxon rank-sum test was used to compare means of period, phase, and amplitude between

treatments: ns = not significant (p value > 0.05), * = significant at p value ≤ 0.05, ** = significant at p value ≤ 0.01, *** = significant at p value ≤ 0.001, and **** = significant at p value ≤ 0.0001.

2.6 | RNA Sequencing (RNA-Seq) Data Processing

We obtained RNA-Seq data of AT2 cell suspension from Dr. Linda Hanley-Bowdoin (PRJNA412215 and PRJNA412233 on NCBI SRA) and RNA-Seq data of Col-0 seedlings from Grinevich et al. (2019) (PRJNA488799 on NCBI SRA) to compare the read counts of clock genes between the AT2 cell suspension and seedlings. The cell suspension was maintained as described in T.-J. Lee et al. (2010). The AT2 cell suspension was grown in LL and DD for 7 days, and the cells were then harvested with three biological replicates (T.-J. Lee et al. 2010). However, the time of day the cells were harvested was not reported. The Col-0 seedlings used for comparison were grown in LL and harvested at subjective dawn and dusk as described in Grinevich et al. (2019). FastQC (Version 0.11.8) (Andrews 2010) was used to quality check reads before and after adapter trimming by BBDuk (in BBMap Version 38.34) (Bushnell 2014; BBMap Guide 2016). Reads were aligned to the Arabidopsis reference genome TAIR10 (Berardini et al. 2015) using Hisat2 (Version 2.2.0) (D. Kim et al. 2019). Read counts were obtained by featureCounts from the Rsubread package (Version 2.0.1) (Liao, Smyth, and Shi 2014). Low read counts were filtered out by the command *filterByExpr()* from EdgeR (M. D. Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012). The read counts were normalized by the median of ratio method in DESeq2 (Version 1.26.0) to obtain normalized gene expression (Love, Huber, and Anders 2014). The heat maps of normalized read counts and percent rank of gene expression were generated using the R package "pheatmap" in R Version 3.6.3.

2.7 | Identification of Single Nucleotide Polymorphisms (SNPs) in the AT2 Cell Suspension

Whole-genome reads were aggregated together using the following studies that use *A. thaliana* suspension culture (PRJNA412215 and PRJNA412233 on NCBI SRA). STAR (Version 2.5.3a) was used to align reads. STAR's 2-pass mapping was used with default parameters (Dobin et al. 2013). Picard (Version 2.10.2; https://github.com/broadinstitute/picard) was used to order bam files and mark duplicate reads. Finally, GATK's HaplotypeCaller (Version 3.7; https://software.broadinstitute.org/gatk/) was used to call variants. Variants were filtered with FS > 30 and QD > 2. Sequence alignment was visualized on IGV Version 2.8.13 (J. T. Robinson et al. 2011).

2.8 | RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

To compare the expression of clock genes between the AT2 cell suspension and intact plants, 7-day-old AT2 cell suspension and 2-week-old Col-0 seedlings were harvested at ZT8 and ZT12 and flash-frozen in liquid nitrogen before storing at −80°C. To compare the expression of clock genes between LD and LL in the AT2 callus, 7-day-old AT2 callus were harvested at ZT0 and ZT12. Total RNAs were extracted from 100 mg of plant tissues by RNeasy Plant RNA Mini Kit (Qiagen, USA). One nanogram of ArrayControl RNA Spikes (Invitrogen, USA) was added during tissue homogenization. DNase I treatment (Roche, USA) was performed on columns to remove DNA. The total RNA was measured by the NanoDrop Lite Spectrophotometer (Thermo Fisher, USA). For comparing gene expression in the AT2 cell suspension and Col-0, 500 ng of total RNA was used in cDNA synthesis with iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, USA) while 700 ng of total RNA from the AT2 callus in LD and LL was used as an input. cDNAs were then 1:4 diluted in nuclease-free water prior to the qPCR. The qPCR reaction contained 5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), 1 μL of 5-μM forward primer, 1 μL of 5-μM reverse primer, 2-μL diluted cDNA, and 1 μL of nuclease-free water. The qPCR was performed on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA) under the following conditions: initial denaturation at 95°C for 30 s and the 35 cycles of denaturation (95°C for 5 s) and annealing (60°C for 30 s). Melt curve analysis (65°C°C–95°C with a 0.5°C increment) was done at the end of qPCR cycles. Only samples with a single discrete peak in the melt curve analysis were kept for the gene expression analysis. Four technical replicates were performed for each cDNA sample in each target gene. Relative normalized expression (ΔΔCq) was determined by CFX Maestro 1.1 software (Bio-Rad, USA). External RNA spikes and three housekeeping genes *ISOPENTENYL DIPHOSPHATE ISOMERASE 2* (*IPP2*), *ACTIN 2* (*ACT2*), and *TUBULIN 2/3* (*TUB2/3*) were used as reference genes to compare gene expression between the AT2 cell suspension and intact plants. Only external RNA spikes and *IPP2* were used as references to compare gene expression between LD and LL in the AT2 callus. Primers used in the study were listed in Table S1. The two-sample t test was used to compare the means between two time points. Statistical analysis and plot making were performed in R Version 4.0.5.

3 | Results

3.1 | AT2 Callus Showed a Rhythmic Expression in LD but Weaker Oscillation in the Free-Running Condition

We aimed to observe whether Arabidopsis AT2 callus has a functional circadian clock by generating the calli carrying *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* via *Agrobacterium*-mediated transformation (Figure S1A–E). Gentamicin was used to select transformants. To ensure that the changes we observed were not due to the selective antibiotic, we compared the expression of *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* in the AT2 callus grown on the selective and regular media under LD (Figure S2 and Table S2). There was no significant difference in waveforms as *CCA1::LUC* and *LHY::LUC* were highly expressed in the morning (ZT1–ZT5) while *TOC1::LUC*, and *FKF1::LUC* peaked at night (ZT13–ZT19) (Figure S2). The expression of clock genes in calli on both media had an approximately 24-h period with similar phases and RAE (Figure S2E–H and Table S2), indicating that gentamicin did not affect the expression of the circadian clock genes in LD. Therefore, we decided to maintain the AT2 callus on gentamicin in further experiments.

Plant growth regulators are the key to switch cell fate in tissue culture systems (Grafi and Barak 2015). The types and concentrations of plant growth regulators used in the tissue culture system could affect the rhythms in the callus. In addition, if coordination across tissues is required for coordinated expression and proper timing of gene expression in plants, the callus with limited cellular organization may have disruptions in its rhythmic expression of circadian genes. Therefore, we examined the expression of *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* in the AT2 callus and intact seedlings under LD (Figures 1 and S3). The expression waveforms of *CCA1::LUC* and *LHY::LUC* in the AT2 callus cells under the LD were similar to those in intact plants (Figures 1A,B and S3A,B). However, the peaks of *TOC1::LUC* and *FKF1::LUC* in AT2 callus cells were broader than those in seedlings (Figures 1F,G and S3F,G). A phase difference in LD was detected between the AT2 callus and seedlings for *CCA1*, *TOC1*, and *FKF1* (Figure S2E–H and Table S2). However, the seedlings were grown in media without sucrose, while the AT2 callus requires sucrose in the media. The periods of all reporters in callus and seedlings were in the 24-h range, with a slightly longer period in callus than in seedlings (Figures 1 and S3 and Tables S3 and S4). This suggested that

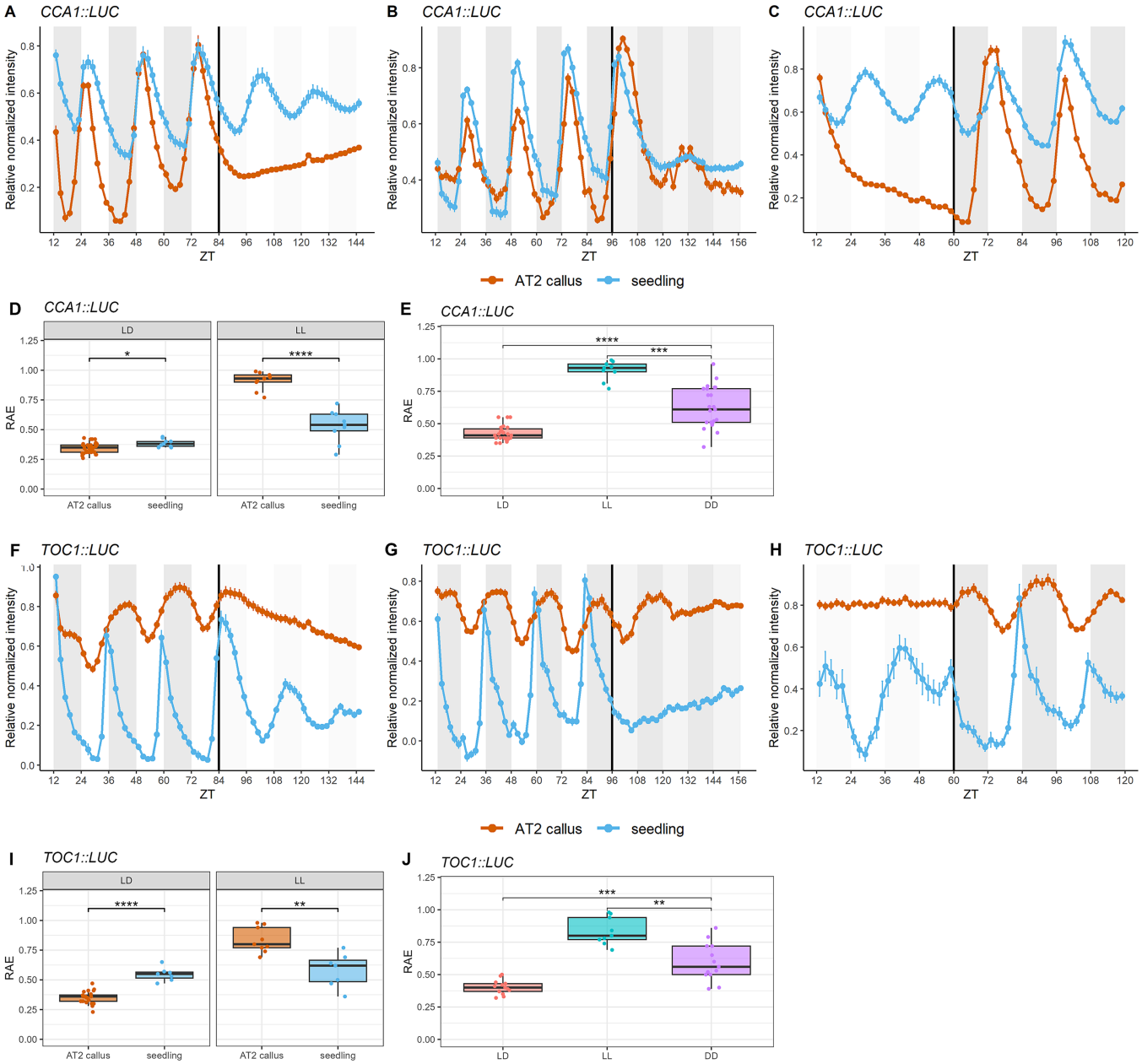


FIGURE 1 | AT2 callus had an altered circadian oscillation. (A–C and F–H) Bioluminescence assay of (A–C) *CCA1::LUC* and (F–H) *TOC1::LUC* in the AT2 callus and seedlings under (A,F) LD for 72 h and subsequent LL for 60 h, (B,G) LD for 84 h and subsequent DD for 60 h, and (C,H) LL for 48 h and subsequent LD for 58 h. The calli were grown on GB5 medium supplemented with 3% (w/v) sucrose and 50-μg/mL gentamicin for selection. Seedlings were grown on 1/2 MS medium without sucrose. Data are mean ± SEM. $n = 15–35$ calli and $n = 6–32$ seedlings (see Tables S2–S5 for specific n for each reporter). (D,I) Relative amplitude error (RAE) of *CCA1::LUC* and *TOC1::LUC* in the AT2 callus and seedlings calculated from oscillations in LD and LL for 48 h. (E,J) RAEs of *CCA1::LUC* and *TOC1::LUC* in the AT2 callus calculated from oscillations in LD, LL, and DD for 48 h. The "LL" AT2 callus boxplots in panels (E) and (J) are the same as those in panels (D) and (I), respectively.

while under entraining conditions of LD cycles, the overall cascade and timing of clock gene expression in the AT2 callus was similar to that in seedlings.

Circadian-controlled rhythms are sustained under free-running conditions (Somers et al. 1998; Dowson-Day and Millar 1999; Engelmann, Simon, and Phen 1992; Harmer 2009). To determine if the AT2 callus maintained rhythmic expression in free-running conditions, we measured the bioluminescence of *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* in AT2 callus under LL and DD at constant temperatures (Figures 1A,B,E,F,G,J and S3A,B,E–G,J). In LL, there were either very weak or no detectible rhythmic oscillations of *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* in the AT2 callus in contrast to seedlings where their expression persisted (Figures 1A,D,F,I and S3A,D,F,I and Table S3). The RT-qPCR results from the AT2 callus grown in LD and LL for 7 days confirmed that *CCA1*, *LHY*, *TOC1*, *ELF4*, and *GI* had significantly altered rhythmicity under LL compared to LD (Figure S4A–C,E,G). Surprisingly, unlike the expected expression pattern in seedlings, *ELF3* showed no difference in expression between dawn and dusk in both LD and LL. At these two time points, *LUX* was differentially expressed only in LL (Figure S4D,F). In summary, the robust rhythmic expression of the core clock genes in the AT2 callus was lost in LL, and the differences in expression observed between ZT0 and ZT12 are reduced for most core clock genes.

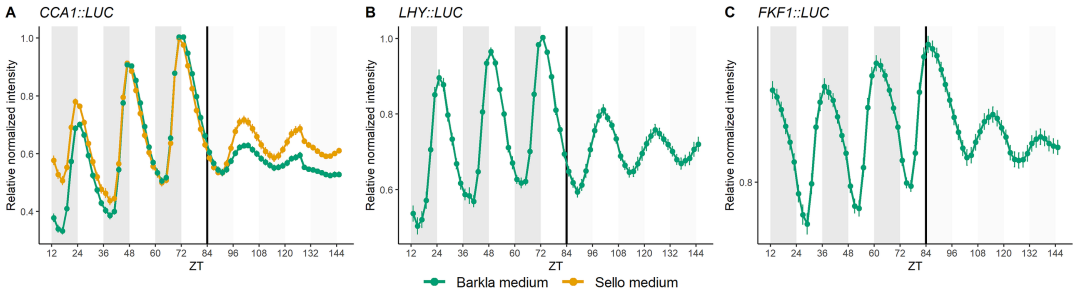
We observed the expression of clock genes in DD which is another type of free-running condition. In the AT2 callus, the clock gene expression persisted under DD with a longer period and phase shift compared to LD conditions (Figures 1B,E,G,J and S3B,E,G,J and Table S4). The rhythmic expression in the AT2 callus was more pronounced in DD than LL with a lower RAE in DD than in LL and a phase in DD closer to the expected phase (Figures 1E,J and S3E,J and Table S4). In seedlings, which are grown without sucrose, the clock genes rapidly lost their rhythmicity under DD (Figures 1B,G and S3B,G). This loss of circadian oscillations in seedlings grown in DD without sucrose is expected as the circadian oscillation usually dampens faster in DD than in LL possibly due to reduced phototransduction to input the clocks in darkness (Millar et al. 1995; Johnson et al. 1995). Therefore, although the AT2 callus maintained rhythmic expression in DD, this is a weak rhythm compared to the LL expression in seedlings. This indicates that the AT2 callus has a circadian oscillator that can function, albeit poorly, in free-running conditions, but the loss of rhythms in LL conditions suggests a disruption in the AT2 circadian oscillator compared to intact seedlings.

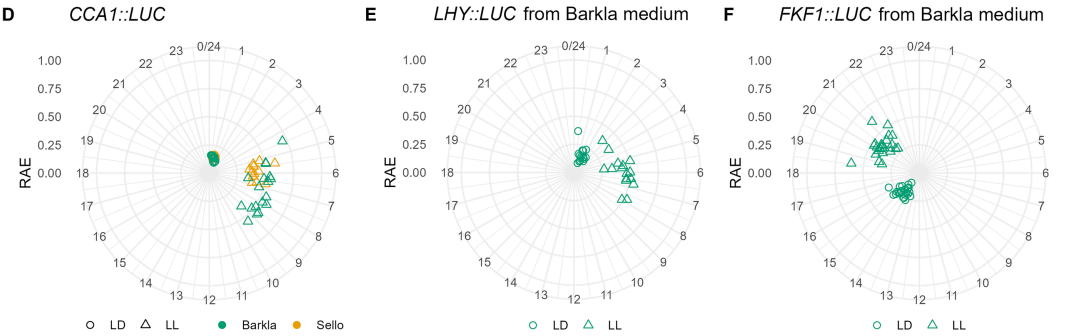
Because the AT2 callus displayed a rhythmic expression of core clock genes under LD and DD but not in the LL condition, it was possible that the light acts as a signal in LL to drive the expression. To examine this, we measured the bioluminescence of *CCA1::LUC*, *LHY::LUC*, *TOC1::LUC*, and *FKF1::LUC* transferred from LL to LD in the dark period (Figures 1C,H and S3C,H). Seedlings were able to maintain the rhythmic expression in both LL and the subsequent LD. As observed above, there was no rhythmic expression in the AT2 callus under LL. However, the AT2 callus resumed rhythmic expression of these clock genes in the first dark period and immediately returned to the rhythmic waveforms observed in LD. For example, *CCA1::LUC* and *LHY::LUC* showed a constant decrease in LL, but once in the dark, the expression levels gradually increased and peak in the light period (Figures 1C and S3C). This is in contrast to the seedlings that maintain their waveform after the dark transition. In the AT2 callus, the expression of the evening genes *TOC1::LUC* and *FKF1::LUC* started rising right after the dark transition, while in seedlings, the transition caused a sudden decrease in luminescence (Figures 1H and S3H). The periods of all reporters except *LHY::LUC* in the 2 days of LD after LL in AT2 callus were significantly longer than those in seedlings (Table S5). The recovery of the expected expression pattern in the dark suggests that light is disrupting the expression of clock promoters in the AT2 callus.

We found that the AT2 callus had altered circadian oscillation in LL. However, several publications show that the circadian clocks run normally in some callus tissue (W.-Y. Kim, Geng, and Somers 2003; Nakamichi et al. 2003, 2004; Xu, Xie, and McClung 2010; Sai and Johnson 1999). We wondered whether the altered circadian rhythms were specific to the AT2 callus. We induced calli from *CCA1::LUC*, *LHY::LUC*, and *FKF::LUC* seedlings on two callus induction media, one from Barkla, Vera-Estrella, and Pantoja (2014) and another from Sello et al. (2017) (Figure S1F–I). Both recipes used the same plant growth regulators (2,4-D as auxin and BA as cytokinin) but at different concentrations. We measured the bioluminescence under LD and found that *CCA1::LUC* calli induced by both media exhibited a similar period and phase of expression (Figure 2A,D). We observed the expression of clock genes under LL and found that calli derived from both recipes exhibited sustained oscillations in LL (Figure 2). The phase of expression in callus induced by both media under LL was more variable compared to LD, possibly due to the increased peak breadth in LL (Figure 2D–F). This peak broadening and reduced amplitude are consistent with the observed transition from LD to LL in seedlings (Figures 1A and S3A). The Barkla medium contained 1-μg/mL 2,4-D and 0.05-μg/mL BA, so the auxin-to-cytokinin ratio was 1:20 (Barkla, Vera-Estrella, and Pantoja 2014). The Sello medium contained lower auxin but higher cytokinin (0.5-μg/mL 2,4-D and 0.25-μg/mL BA) than the Barkla medium, and the ratio of auxin to cytokinin was 1:2 (Sello et al. 2017). Compared to the clock gene expression in the AT2 callus and consistent with prior publications, the results indicated that the weak circadian rhythms under LL in the AT2 callus is not inherent to the hormones used to generate the callus tissue.

3.2 | HC Can Recover the Circadian Oscillations of Clock-Associated Reporters in the AT2 Callus Under Constant Light Conditions

Light and temperature are two primary signals that plants use to integrate their internal clock to environmental changes (Devlin and Kay 2001; McClung, Salomé, and Michael 2002). As constant light eliminated the rhythmic waveforms of clock genes in the AT2 callus, we wanted to determine if HC could recover rhythmic expression in LL. We measured the bioluminescence of *CCA1::LUC* and *FKF1::LUC* in the AT2 callus, seedlings, and callus derived from seedlings under constant 23°C (HH) and 23°C/12°C HC in LL (Figure 3A,B,D,E). In seedlings and callus derived from seedlings, the transition to HC increased the





medium	LD	LL
Barkla	0.99 ± 0.45 (n=18)	7.24 ± 1.43 ****
Sello	1.10 ± 0.25 (n=12)	5.93 ± 0.60 ***

LD	LL
1.72 ± 0.59 (n=16)	5.84 ± 1.29 ****

LD	LL
14.68 ± 0.69 (n=24)	20.39 ± 0.84 ****

FIGURE 2 | Callus derived from seedlings had normal circadian oscillation. (A–C) Bioluminescence assay and (D–F) phases and RAEs of (A,D) *CCA1::LUC*, (B,E) *LHY::LUC*, and (C,F) *FKF1::LUC* in calli derived from seedlings on Barkla (green) and Sello (yellow) media under LD for 72 h and subsequent LL for 60 h. Both media contained 3% (w/v) sucrose. Data in panels (A)–(C) are mean ± SEM. The radii of the circular plots in panels (D)–(F) represent RAEs. The tables under the circular plots show mean ± SD of the phases. Paired-sample Wilcoxon test was used to compare the means between LD and LL.

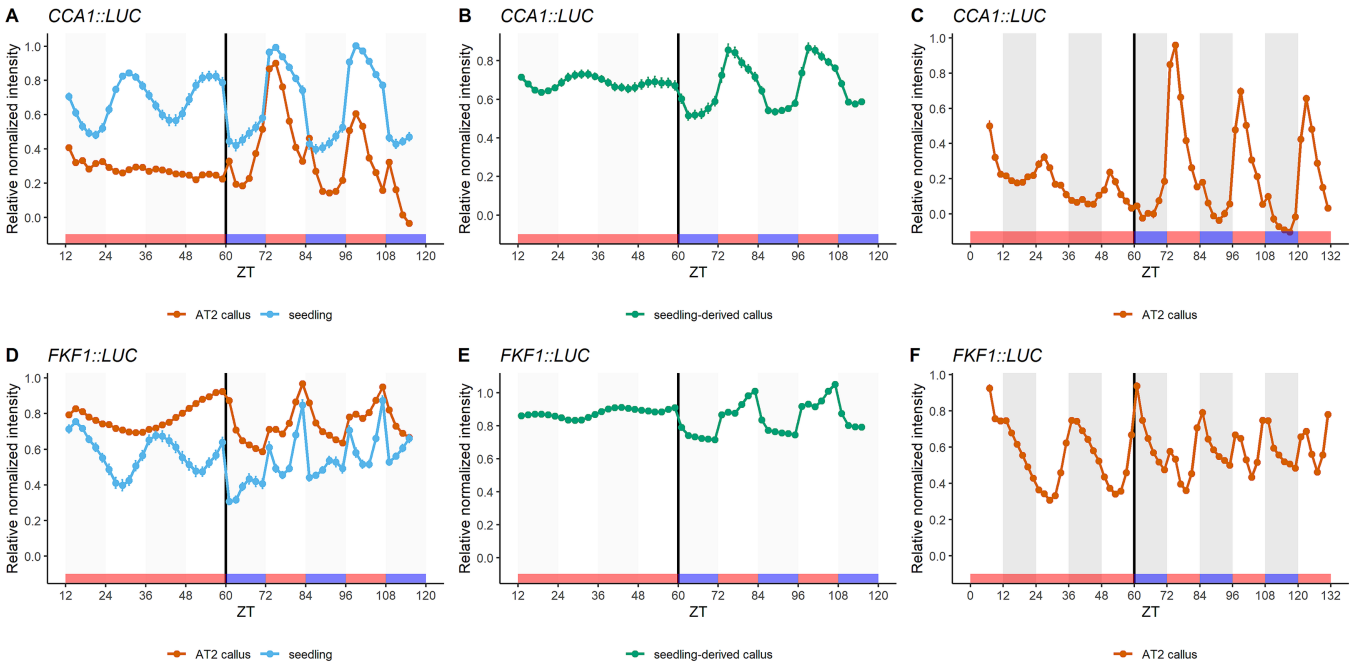


FIGURE 3 | AT2 callus showed a peak response to temperature changes. (A,B,D,E) Bioluminescence assay of (A,B) *CCA1::LUC* and (D,E) *FKF1::LUC* in (A,D) AT2 callus versus seedlings and (B,E) calli derived from seedlings on Barkla medium under LLHH for 48 h and LLHC for 54 h. The AT2 calli were grown on GB5 medium supplemented with 3% (w/v) sucrose and 50-μg/mL gentamicin for selection. Seedlings were grown on 1/2 MS medium without sucrose. Calli derived from seedlings were grown on Barkla medium containing 3% (w/v) sucrose. Calli and seedlings were entrained in LDHH for at least 7 days prior to LLHH. Data are mean ± SEM. $n = 26–36$ AT2 calli, $n = 13–30$ seedlings, and $n = 12–19$ calli on Barkla medium. (C,F) Bioluminescence assay of (C) *CCA1::LUC* and (F) *FKF1::LUC* in the AT2 callus under LDHH for 54 h and LDHC for 70 h. Data are mean ± SEM. $n = 36$ calli.

amplitude of *CCA1::LUC* (Figure 3A). HC were able to recover the rhythmic expression of *CCA1::LUC* and *FKF1::LUC* in the AT2 callus under LL (Figure 3A,D). Additionally, in the AT2 callus, a small increase in *CCA1::LUC* level was detected when the temperature was shifted from warm to cool temperatures (23°C°C–12°C) (Figure 3A). This small peak was not detectable in seedlings or callus derived from seedlings (Figure 3A,B). In contrast, *FKF1::LUC* expression was induced at the transition from cool to warm temperature (12°C°C–23°C) in the AT2 callus, seedlings, and callus induced from seedlings (Figure 3D,E). These results indicate that entrainment by HC can recover the rhythmic expression of these core clock genes, although there are slight differences in their expression waveforms in the presence of these thermocycles.

We next tested whether the temperature-responsive minor peaks observed in the AT2 callus persisted in the presence of both cycling light and temperatures by observing the expression of *CCA1::LUC* and *FKF1::LUC* under light and HC (LDHC) (Figure 3C,F). Both clock genes still showed two peaks, one "time of day" peak, with a phase consistent with the single peak in LD conditions, and a second, temperature-responsive peak in LDHC (Figure 3C,F). As the temperature-responsive peak appeared in both LD and LL, this suggested that this peak was specific to temperature changes and was not influenced by the constant light conditions. Overall, the expression of the clock genes examined in the AT2 callus showed that the rhythmic waveform not detectable in constant light could be recovered by the addition of HC.

3.3 | Growth Media and Explant Source Did Not Contribute to the Altered Circadian Rhythms in the AT2 Callus

The AT2 callus was grown on maintenance media that contains different auxin and cytokinin (NAA and kinetin) compared to the media used to induce callus from seedlings (2,4-D and BA). As auxin and cytokinin affect the rhythmicity of clock genes in intact plants (Covington and Harmer 2007; Zheng et al. 2006). We tested whether plant growth regulators used in callus induction media could recover the circadian oscillation in the AT2 callus. We transferred the AT2 callus containing *CCA1::LUC* to the Barkla medium and cultured them for a month. *CCA1::LUC* oscillation in the AT2 callus on both media was similar as they showed approximately 24-h period and similar RAE (Figure 4A–C). However, the phase of *CCA1::LUC* in the AT2 callus on Barkla medium was more variable than the callus on its original medium (Figure 4B). In constant light, the *CCA1::LUC* oscillation was not detectable in the AT2 callus on either media, indicating that the culture medium was not the cause of the disrupted LL circadian oscillations in the AT2 callus (Figure 4C).

The AT2 cell suspension was initiated from Col-0 leaf tissues while the callus we compared it to was generated from whole seedlings which were composed of various tissue types. We wanted to evaluate if the original tissue source affects the circadian oscillation in the callus because different organs exhibit a variation in robustness and precision of circadian oscillations

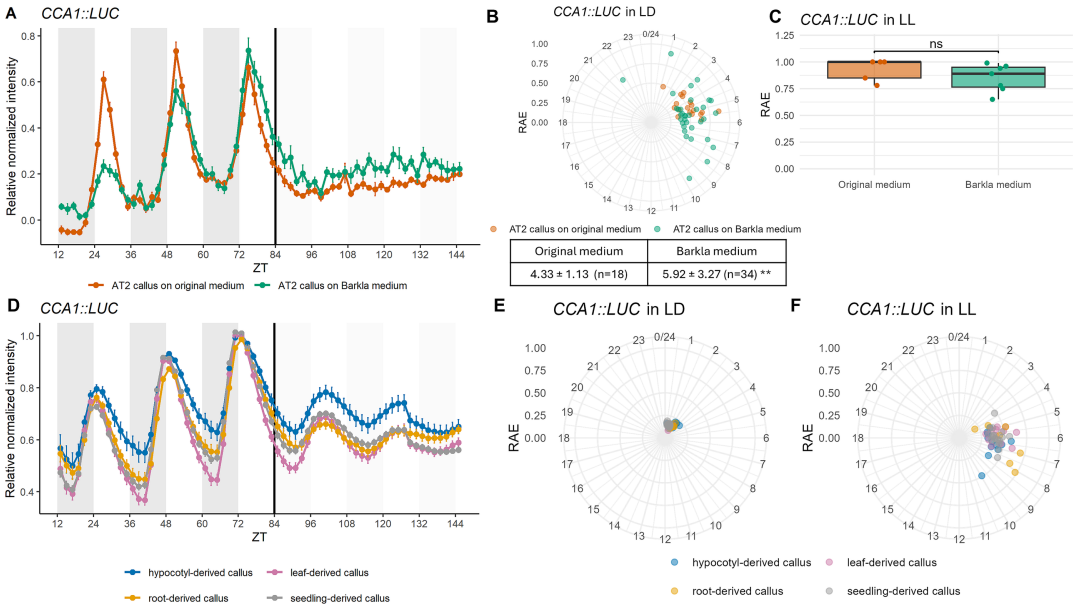


FIGURE 4 | Expression of *CCA1::LUC* reporter was not affected by growth medium or source tissue. (A) Bioluminescence assay of *CCA1::LUC* expression in the AT2 callus grown on the original GB5 medium ($n = 18$) and Barkla medium ($n = 34$) under LD for 72 h and subsequent LL for 60 h. Data are mean ± SEM, and n indicates the number of calli. Both GB5 and Barkla media contained 3% (w/v) sucrose. (B) Phases and RAEs of *CCA1::LUC* in the AT2 callus grown on the original GB5 medium and Barkla medium under LD for 48 h. The radii of the circular plot represent RAEs. (C) RAEs of *CCA1::LUC* in the AT2 callus grown on the original GB5 medium and Barkla medium under LL for 60 h. Two-sample Wilcoxon test was used to compare the means between LD and LL. (D) Bioluminescence assay of *CCA1::LUC* expression in calli derived from hypocotyls ($n = 12$), leaves ($n = 12$), roots ($n = 7$), and seedlings ($n = 18$) on Barkla medium under LD for 72 h and subsequent LL for 60 h. Data are mean ± SEM, and n indicates the number of calli. (E,F) Phases and RAEs of *CCA1::LUC* in calli derived from hypocotyls, leaves, roots, and seedlings on the Barkla medium under (E) LD and (F) LL for 48 h. The radii of the circular plot represent RAEs.

(Takahashi et al. 2015). We produced calli from hypocotyl, leaves, and roots using the Barkla medium and measured *CCA1::LUC* expression under LD and LL along with the callus derived from seedlings (Figure S1F–I). The calli from different tissues showed similar *CCA1::LUC* waveforms and phases in LD (Figure 4D,E and Table S6). In the subsequent LL, calli derived from various tissues also maintained their rhythmic oscillations with shorter periods in LL than LD (Figure 4D,F and Table S9). This is consistent with prior reports that callus cells form a meristematic state that is similar to developmental lateral root formation despite of types of tissues used for generating callus (Sugimoto, Jiao, and Meyerowitz 2010; Fan et al. 2012; Ikeuchi, Sugimoto, and Iwase 2013; Atta et al. 2009). These results, consistent with prior literature, suggest that the loss of rhythmic gene expression in constant light in the AT2 callus is not due to growth medium and the explants used to generate the callus.

3.4 | Polymorphisms and Altered Expression of Circadian Clock Genes Are Observed in AT2 Cells

Another possible mechanism for the observed loss of rhythmicity in LL conditions is the presence of genomic mutations in the AT2 cells acquired during their maintenance in the pluripotent state. Such mutations could change gene expression at transcription or protein levels. As the time-course luciferase imaging could only determine the expression of a few circadian-associated genes in the AT2 cells, we analyzed RNA-Seq data previously performed on the AT2 cell suspension lines grown in LL and DD to evaluate the expression of 15 circadian-associated genes. As a baseline, we compared expression levels of the circadian-associated genes to RNA-Seq data from Col-0 seedlings grown in LL at ZT0 and ZT12 (Grinevich et al. 2019) (Figure S5A). Most of these 15 circadian-associated genes peaked in expression either near ZT0 or ZT12 in Col-0 seedlings (Figure S5A). Because there was no record of the time of day when the AT2 cell suspension was harvested, it is not possible to evaluate their expression patterns from these data alone. However, overall, the total counts of most clock genes were substantially lower in both LL and DD in the AT2 cell suspension than at either ZT0 or ZT12 in Col-0 seedlings (Figure S5A). Only *PRR5*, *TOC1*, and *GI* had similar levels in the cell suspension and seedlings (Figure S5A). The expression of *CCA1* and *ELF4* in the AT2 callus were 19 times and 5 times lower than the lowest expression of those genes in seedlings at AM and PM, respectively (Figure S5A). In addition to the difference in phases we would expect with these circadian-associated genes, we calculated the percent rank of gene expression to determine the ranks of clock genes among expressed genes in Col-0 seedlings and AT2 cell suspension. In Col-0 seedlings, the core clock genes such as *CCA1*, *LHY*, *PRR7*, *PRR9*, *TOC1*, *LUX*, *ELF3*, and *ELF4* were ranked in the top 20% at their highest time of expression (Figure S5B). Even at the time when they are expected to be at their trough of expression, many circadian-associated genes remain in the top 50% of gene expression by counts (Figure S5B). However, in the AT2 cell suspension, those genes were ranked as very lowly expressed with many in the lowest 30% of expressed genes in both data sets (Figure S5B). This suggests that the expression of clock genes was not robust in the AT2 cell suspension.

We used the RNA-Seq data from the AT2 cell suspension to identify SNPs in the clock genes listed in (Nakamichi 2011) (Figure 5A and Table S7). We identified mutations in *CASEIN KINASE II BETA CHAIN 3* (*CKB3*), *LIGHT-REGULATED WD* (*LWD1*), *LOV KELCH PROTEIN 2* (*LKP2*), *GI*, and *ELF3*. SNPs in *CKB3* and *LWD1* occurred in the 3′ UTR while the SNP in *LKP2* resulted in no amino acid changes (Table S7). There were two SNPs in *GI*, one causing no changes in amino acid and another leading to a premature stop. The premature stop was at Amino Acid Position 1156 in the last exon which was close to the true stop codon (Position 1174). In the literature, the *gi-5* mutant has a base deletion in the last exon, which alters the last eight amino acids and adds 27 more amino acids at the C terminus (Fowler et al. 1999). The *gi-5* mutant has similar gene expression as wild-type plants and shows a mild alteration in phenotype (late flowering only in the long-day condition) (Fowler et al. 1999; Mishra and Panigrahi 2015).

An SNP (T to C) was also observed in the second exon of the *ELF3* gene resulting in a change in Amino Acid Position 291 (Figure 5A and Table S7). CUG encoding leucine was changed to CCG encoding proline in the AT2 cell suspension (Figure 5A). The ELF3 protein is rich in serine, proline, and glutamine, and it does not share any domains that could be found in other protein families (Hicks, Albertson, and Wagner 2001). The ELF3 protein is divided into four regions based on amino acid richness: an acidic region (206–320), a proline-rich region (440–540), a threonine-rich region near the C terminus (636–652), and glutamine repeats or polyQ (544–585) in the region that has been recently predicted as a PrD (430–609) (Hicks, Albertson, and Wagner 2001; Jung et al. 2020). The tertiary structure of ELF3 protein has not been constructed. In AlphaFold prediction, L291 is in a region of very low prediction confidence (Figure S6A), so we cannot predict how this amino acid change would impact protein folding (Jumper et al. 2021; Varadi et al. 2024). We did sequence alignment to determine how conserved Leucine 291 was among plant species (Figure S6B, Table S8, and Supporting Information S1). Leucine 291 was found mostly in dicot species (BrELF3a, VvELF3a, VvELF3, PtELF3a, and PtELF3b). GmELF3 had isoleucine and CpELF3 had phenylalanine instead of leucine. One rice ELF3 homolog had leucine like AtELF3, but other homologs in monocot species and lower plants (moss and spikemoss) had no conserved amino acids at this position, and in fact, many had a deletion of about 15 amino acids in the region surrounding Leucine 291. The *ELF3* gene from *Brachypodium distachyon* and *Setaria viridis* is able to rescue defects in Arabidopsis *elf3* mutant even though there is a lack of sequence conservation at Leucine 291 and nearby amino acids (Huang et al. 2017). This suggests that changes in protein sequences might not strongly affect the ELF3 expression or function.

With the RNA-Seq data, we are unable to detect mutations in the noncoding regulatory regions. However, the RNA-Seq indicated that the expression of circadian clock genes in the AT2 cells was different from intact plants. We performed RT-qPCR to examine the expression of clock-associated genes in the AT2 cells at dawn and dusk (ZT0 and ZT12) as previously mentioned in Section 3.1 (Figure S4). The expression of *GI* in AT2 callus cells was significantly different between dawn and

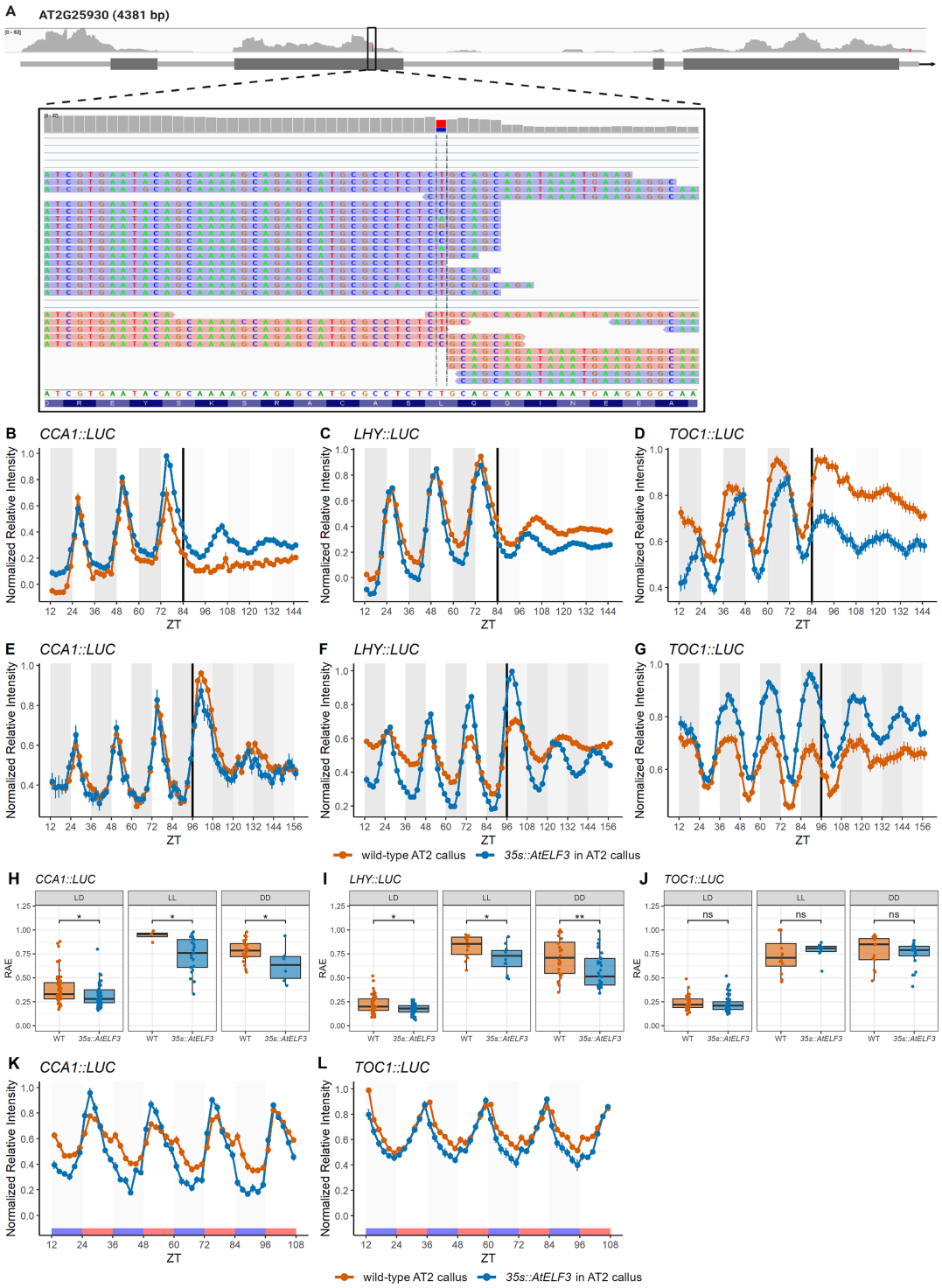


FIGURE 5 | The overexpression of *AtELF3* partially restored the circadian oscillations in the AT2 callus. (A) Location of the SNP on the second exon of the ELF3 gene in the AT2 cell suspension. The sequences were visualized on IGV Version 2.8.13. (B–G) Bioluminescence assay of (B,E) *CCA1::LUC*, (C,F) *LHY::LUC*, and (D,G) *TOC1::LUC* in the wild-type and AtELF3-overexpressing AT2 calli under (B–D) LD for 84 h and subsequent LL for 96 h and (E–G) LD for 96 h and subsequent DD for 96 h. Both calli were grown on GB5 medium supplemented with 3% (w/v) sucrose and antibiotics for selection. Data are mean ± SEM. $n = 18–35$ wild-type calli and $n = 10–30$ AtELF3-overexpressing calli. (H–J) RAEs of (H) *CCA1::LUC*, (I) *LHY::LUC*, and (J) *TOC1::LUC* in the wild-type and AtELF3-overexpressing AT2 calli under LD, LL, and DD for 48 h. Two-sample Wilcoxon test was used to compare the means between wild-type and AtELF3-overexpressing AT2 calli. (K,L) Bioluminescence assay of (K) *CCA1::LUC* and (L) *TOC1::LUC* in the wild-type and AtELF3-overexpressing AT2 calli under LLHC for 96 h. Data are mean ± SEM. $n = 18–36$ individual wild-type callus groups and $n = 12$ AtELF3-overexpressing calli.

dusk in LD, but this difference diminished under LL similar to other clock genes with no SNPs (*CCA1*, *LHY*, *TOC1*, and *ELF4*) (Figure S4A–C,E,G). This suggests that the observed SNPs in *GI* might not affect *GI's* expression. Only *ELF3* in the AT2 callus was not significantly cycling in both LD and LL (Figure S4D).

For comparison, intact seedlings show a significant difference in the expression of *ELF3* and all other tested circadian-associated genes even at two closer time points, ZT8 and ZT12 (Figure S7). *ELF3*, as a member of the EC, increases in expression between ZT8 and ZT12 in seedlings, consistent with its peak expression at this time (Figure S7D). However, in the AT2 cells, the induction *ELF3* is not observed between these two time points (Figure S7D). In *elf3* knockout plants, circadian rhythms were abolished in the free-running conditions but maintained rhythmic in LD cycles (Hicks et al. 1996; Reed et al. 2000; Thines and Harmon 2010), similar to the pattern we observed in the AT2 callus. Therefore, based on the SNPs in the ELF3 CDS and the altered expression patterns, we hypothesized that either misexpression of *ELF3* or the mutation in the CDS could be the reason behind the altered circadian rhythm in the AT2 callus.

3.5 | *AtELF3* Overexpression Recovered the Circadian Oscillation in the AT2 Callus

To test the role of ELF3 in the disrupted circadian expression in the AT2 callus, we overexpressed *AtELF3* in the AT2 callus and measured *CCA1::LUC*, *LHY::LUC*, and *TOC1::LUC* expression under LD and free-running conditions (LL and DD) (Figure 5B–J). The expression of all three clock genes under LD in wild-type and At*ELF3*-overexpressing calli were similar. The periods of *CCA1::LUC*, *LHY::LUC*, and *TOC1::LUC* in the *AtELF3*-overexpressed callus were in the 24-h range, suggesting that AT2 callus maintained the 24-h LD rhythms with the *AtELF3* overexpression (Figure 5B–J and Table S9). In constant light, the *AtELF3*-overexpressing callus recovered the oscillations of *CCA1::LUC* and *LHY::LUC*, showing reduced RAE compared to wild type (Figure 5B–D,H–J). The reporter genes were rhythmically expressed in the AT2 callus under DD, and the overexpression of *AtELF3* improved the oscillations of *CCA1::LUC* and *LHY:: LUC*, by reducing the RAE and the variation of the phase of expression and (Figure 5E,F,H,I and Table S9). There was no significant effect on *TOC1::LUC* in either LL or DD. We also tested whether the *AtELF3*-overexpressing callus displayed the temperature-responsive peak in HC (Figure 5K,L). The *CCA1::LUC* temperature-responsive peak was reduced in the *AtELF3*-overexpressing callus compared to wild type (Figure 5K). However, *TOC1::LUC* exhibited a temperature-responsive peak in response to the shift from cool to warm temperatures (12°C to 23°C) in both wild-type and *AtELF3*-overexpressing calli (Figure 5L). The results in the free-running conditions and HC indicate that proper expression of a wild-type *AtELF3* was able to partially recover normal oscillations in the AT2 callus.

4 | Discussion

4.1 | The AT2 Callus System had Altered Circadian Oscillations

Callus and cell suspension culture are valuable tools in plant basic research and industrial application (Efferth 2019). However, reports vary on if callus have a functional circadian clock (Nakamichi et al. 2003, 2004; K. Lee, Park, and Seo 2016). Therefore, we investigated the oscillation of four circadian-associated genes *CCA1*, *LHY*, *TOC1*, and *FKF1* in the AT2 callus under diel cycles and free-running conditions. We found that the expression period of those genes was normal under LD. However, the phase of expression in LD was different between AT2 callus and seedlings for *CCA1*, *TOC1*, and *FKF1* (Figures 1 and S3F–J and Table S2). While this difference could be due to the differences between the growth media, for *CCA1*, a similar difference in phase is observed in LD when AT2 callus are compared to callus from freshly derived seedlings (Table S6). CCA1 expression in AT2 callus has a peak of expression 2 h after freshly derived seedling callus grown in the same media (Figure 4B and Table S6). Changes in phase with no change in period have been previously described for *outofphase1* (*oop1*) a PHYB truncation mutant and the null PHYB mutation *phyB-9* (Salomé et al. 2002). Thus, changes in phase in LD might be a potential phenotype for initial screens to evaluate callus from different sources for circadian defects.

The rhythmic expression was reduced in LL but maintained in DD albeit with lengthening periods. In LL, many calli showed RAE close to 1, suggesting poor or no robust oscillation under these conditions (Figures 1, 5, and S3, Figure S3). However, we did observe differences between the transcripts. For example, while *CCA1* and *FKF1* showed a complete loss of rhythmic activity, *LHY* and *TOC1* did have a trace of rhythmic activity (Figure 5C,D). In DD, the rhythmic expression was maintained for a longer period, and the phase was delayed compared to the LD-grown AT2 callus and seedlings under LL (Figures 1, 5, and S3 and Tables S2–S4). Callus needs sucrose as an external carbon source, and this could contribute to the maintenance of rhythmic expression under DD (Thorpe and Meier 1973). In Arabidopsis seedlings, the addition of sucrose to growth media results in a shortening period in constant light but helps maintain the robust rhythms in the constant dark condition (Knight, Thomson, and McWatters 2008; Dalchau et al. 2011; Haydon et al. 2013). In the AT2 callus, we observed a significant effect on oscillations, especially in constant light, suggesting that the sucrose in the media is not the only factor in the disrupted rhythmicity of the AT2 callus. Loss of robust rhythms in LL indicates that the AT2 callus has a disrupted circadian clock.

To evaluate if the loss of rhythms in AT2 callus cells was unique to this line or a result of the growth conditions, including medium composition or callus source tissues, we generated fresh callus from seedlings. Unlike the AT2 callus, the seedling-generated callus from all tissue sources tested maintained oscillations in LL (Figure 4D and Table S6). These oscillations persisted on different media suggesting that the poor rhythmicity in LL was unique to the AT2 callus and not the medium composition. The observed rhythmicity in callus for these reporters is consistent with prior publications (W.-Y. Kim, Geng, and Somers 2003; Nakamichi et al. 2003, 2004; Xu, Xie, and McClung 2010; Sai and Johnson 1999). However, we observed shorter period lengths of *CCA1::LUC* in constant conditions in callus derived from all tissues than in intact seedlings. If this effect persists for other circadian reporters, this could be relevant for understanding how the cellular organization or identity contributes to circadian rhythms.

The weak rhythm we observe in the AT2 callus has been previously observed in Arabidopsis cell lines. Nakamichi et al. (2003) observed rhythmic expression of the *PRRs* genes (*PRR1*/*TOC1*, *PRR5*, *PRR7*, and *PRR9*) in the T87 Arabidopsis cell line under LD and DD but not in LL (Nakamichi et al. 2003), a phenotype similar to what we observed with the AT2 callus. However, a

year later, the same group reported that the T87 cell line had a functional clock because the expression of *CCA1::LUC* and *TOC1::LUC* was rhythmic under both LL and DD (Nakamichi et al. 2004). They suggested that the growth phase could affect the observation of circadian rhythms in the cell suspension. They used the callus grown on agar plates for over 2 weeks in the first publication but used fresher callus (3 days on agar plates) in the experiment where the cells showed rhythmic expression. To ensure that the growth phase was not an issue with the rhythmicity, we grew the AT2 callus on the agar plates for 7–12 days before imaging to ensure that the AT2 callus and cell suspension were still in the exponential phase during this period (Figure S8).

We evaluated if the response of the AT2 callus to temperature changes, another circadian input, was altered compared to intact seedlings. In the AT2 callus and callus derived from seedlings, the morning gene *CCA1* showed a small peak when the temperature decreased at dusk (22°C°C–12°C), but this temperature-responsive peak did not occur in intact seedlings (Figure 3A). However, the evening gene *FKF1* showed a similar temperature response when the temperature rose in the morning (12°C°C–22°C) in both callus and seedlings (Figure 3D). To our knowledge, these temperature-responsive peaks to the daily HC have not been reported in intact plants so far (Somers et al. 1998; Salomé and McClung 2005; McWatters et al. 2000). However, Kusakina, Gould, and Hall (2014) showed that there was a second peak of *TOC1::LUC* occurring at the dark-to-light transition in seedlings grown in LD cycles at constant temperatures (12°C, 17°C, and 27°C), and the peak was enhanced as temperature increased (Kusakina, Gould, and Hall 2014). They observed the expression of *CCA1::LUC*, *LHY::LUC*, and *CAB2::LUC* and found a small peak showing up at the transition from light to dark under low temperatures (17°C and 12°C) (Kusakina, Gould, and Hall 2014). In our results, we found the second peak at the transition of temperature in both LD and LL, indicating that these peaks are specifically temperature responsive and persist in constant light conditions despite the lack of rhythmicity in constant light. This induction of the circadian clock genes by temperature responses can be studied in callus to improve our understanding of temperature signaling in plants.

We examined the ability of the AT2 callus to resume the rhythmic gene expression when returned to LD to evaluate if the circadian clock persists and the rhythm is masked under LL. We saw that when the callus was returned to the dark, *CCA1* expression in the AT2 callus and seedlings decreased slightly and then began rising in the middle of the night to peak, as expected for an intact clock, at the dawn of the next light period (Figure 1C). The expression of *TOC1* in the AT2 callus increased right after the dark period. In seedlings, *TOC1* started increasing at 4 h before the dark period, and darkness immediately shut down the *TOC1* expression (Figure 1H). This completely opposite response of *TOC1* expression to the dark after growth in LL between seedlings and the AT2 callus suggests that the AT2 callus could provide a novel means to examine circadian connections in the AT2 callus. The responsiveness of *CCA1* and *TOC1* expression to the dark after growth in LL suggests that either LL is masking an underlying circadian rhythm or that the transition from light to dark serves as an input to the circadian oscillator in the AT2 callus. The experiments performed here cannot distinguish between these two possibilities.

4.2 | Callus Induction Media and Types of Explant Did Not Alter Circadian Oscillation

In our attempt to uncover the cause of the loss of rhythmicity in the AT2 callus, we discovered that the medium composition and the tissue sources of the callus did not significantly affect the rhythmic expression of the core circadian genes in freshly induced callus. The disrupted rhythms were unique to the AT2 callus. Callus induced from seedlings had circadian expression similar to seedlings (Figure 2). The callus induction medium contains auxin and cytokinin, which are known to affect circadian rhythms (Covington and Harmer 2007; Zheng et al. 2006; Hanano et al. 2006). Auxin decreases the amplitude of the circadian rhythm while cytokinin delays the phase in Arabidopsis seedlings (Covington and Harmer 2007; Zheng et al. 2006; Hanano et al. 2006). We found that the callus induced by two different concentrations of 2,4-D and BA exhibited a similar rhythmic expression pattern, indicating that this difference in hormones did not contribute to the loss of circadian oscillation in the AT2 callus under LL (Figures 2 and 4A).

We also found that calli induced from hypocotyls, leaves, and roots showed a similar expression pattern compared to callus from whole seedlings, suggesting that initial tissues did not affect the circadian rhythms in the callus (Figure 4D–F). Several studies indicated that the circadian clock function is tissue and cell specific (Thain et al. 2002; Takahashi et al. 2015; Endo et al. 2014; Román et al. 2020; Gould et al. 2018; James et al. 2008; Greenwood et al. 2019). At the tissue level, the root clock is less robust and precise than the shoot clock (Thain et al. 2002; Takahashi et al. 2015; Chen et al. 2020). Aerial and belowground tissues are composed of different cell types and experience different environments which result in altered oscillations (Sorkin and Nusinow 2021). Signaling molecules such as sucrose and ELF4 transmit circadian information to synchronize the clocks across tissues (Chen et al. 2020; James et al. 2008). It is possible that explants are transformed to callus via the same molecular mechanism regardless of types and concentrations of auxin and cytokinin or tissue source. As callus is considered a unique type of plant tissue based on gene expression profiles (Tanurdzic et al. 2008; He et al. 2012; Du et al. 2019; Shim et al. 2020; K. Lee, Park, and Seo 2018), cellular reprogramming during callus formation could resynchronize the clocks between cells, making callus cells exhibit similar circadian rhythms regardless of the origin of explants. Importantly, the seedling-like rhythms obtained in callus, independent of callus source or media, indicate that freshly derived callus can be a useful tool for studying circadian regulation.

4.3 | ELF3 Recovered the Circadian Defect in the AT2 Callus

We hypothesized that the difference in rhythmic gene expression between the freshly derived callus and the AT2 callus could be due to one or more mutations accumulated in the AT2 cell suspension. We examined the RNA-Seq data and found that the expressions of clock genes were relatively low compared to intact plants in LD and free-running conditions. We found that, unlike in seedlings, the expression of *ELF3* was not significantly different between dawn and dusk in both LD and LL, and there was one SNP in the second exon of *ELF3* which led to

leucine-to-proline mutation at Position 291. *ELF3* is a key circadian component involved in light and temperature signaling, regulation of flowering, and thermomorphogenesis (Box, Emma Huang, and Domijan 2015; Zagotta et al. 1996; Jung et al. 2020; McWatters et al. 2000; Nusinow et al. 2011).

The protein sequence alignment indicates that Leucine 291 is not conserved among species (Figure S6B). Monocot ELF3 proteins lack Leucine 291 and nearby amino acids, but they can complement the functions of ELF3 in Arabidopsis *elf3* knockout (Huang et al. 2017). This suggested that changes in Leucine 291 may not strongly impact the functions of ELF3. However, we did not assess whether the SNP affects protein function as we do not know the folding of ELF3 protein. Previous *ELF3* studies showed that *elf3* knockout exhibited arrhythmic *CAB2* and *CCR2* in LL but not in DD (Hicks, Albertson, and Wagner 2001; Covington et al. 2001; Reed et al. 2000). But Thines and Harmon (2010) found that the *elf3* knockout had no rhythmic *TOC1*, *CCR2*, and *FKF* expression in both LL and DD (Thines and Harmon 2010). The authors commented that the rhythmic expression in the previous publication could be driven by the last light cycles prior to free-running conditions (Thines and Harmon 2010).

Overexpressing *AtELF3* in the AT2 callus restored circadian expression of *CCA1::LUC* and *LHY::LUC* in the free-running conditions and HC, resulting in rhythms similar to that in seedlings (Figure 5). Overexpression of *AtELF*3 in seedlings resulted in increased robustness of circadian rhythms in both LL and DD (Covington et al. 2001). Despite a reduced minor peak of *CCA1::LUC* in the *AtELF3*-overexpressed callus under HC, we found that the minor temperature-responsive peak of *TOC1::LUC* still persisted, suggesting that some subtle differences remain between the *ELF3*-overexpressing AT2 cells and seedlings in circadian gene expression. Both mammalian and Drosophila clocks show reduced rhythmicity under constant light. In mammalian systems, the cells of the SCN become desynchronized, resulting in reduced rhythmic locomotor activity and longer periods (Ohta, Yamazaki, and McMahon 2005). Mutations in intracellular signaling pathways recover the rhythms in constant light (Hughes et al. 2015). In Drosophila, the circadian clock is arrested in constant light due to the continual activation of the photoreceptor CRY2 (Dapergola et al. 2021). Like the AT2 callus, rhythms under LL are restored by HC (Yoshii et al. 2005; Glaser and Stanewsky 2005; Lamaze et al. 2022). Mutations in JETLAG (JET) increase rhythmic behavior under constant light (Koh, Zheng, and Sehgal 2006). JET is an F-box protein that targets the key Drosophila circadian component, TIMELESS, for degradation. The Drosophila jetlag mutation provides insights into how light connects to the Drosophila clock (Lamaze et al. 2022). In the AT2 callus, ELF3 plays an opposite role. The loss of *elf3* shows reduced rhythms in Arabidopsis, and in the AT2 callus, we observe that ELF3 overexpression restores rhythms in constant light (Hicks et al. 1996; Reed et al. 2000; Thines and Harmon 2010). Although we cannot yet determine if the arrhythmic AT2 callus is due to the desynchronization of the individual cells or a stopped clock, these findings suggest that the changes that have accrued in the AT2 callus might provide mechanisms for understanding the connections between light and the circadian clock.

Overall, this study shows that the expression of circadian clock genes in the AT2 callus is driven by light and temperature signals, but the AT2 callus exhibits weak circadian oscillations in constant light conditions. The overexpression of *AtELF3* in the AT2 callus restores rhythmic expression in LL. Rhythmic expression of clock genes was robust in freshly made calli in both LD and LL despite induction medium composition and explant source tissues. This suggests that callus could be useful in clock studies, but caution should be employed to ensure that the callus or cell suspension cultures do not lose their rhythmic expression.

Author Contributions

C.J.D. conceptualized the experiments. K.L. grew callus and plants for luciferase imaging, analyzed bioluminescence data and RNA-Seq data, and performed RT-qPCR. J.S.D. identified SNPs from the RNA-Seq data. All authors participated in writing the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequencing data that support the findings presented here are previously published works available at NCBI SRA PRJNA412215, PRJNA412233, and PRJNA488799.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.