Keeping time in the dark: Potato diel and circadian rhythmic gene expression reveals tissue-specific circadian clocks

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Abstract
The circadian clock is an internal molecular oscillator and coordinates numerous physiological processes through regulation of molecular pathways. Tissue-specific clocks connected by mobile signals have previously been found to run at different speeds in Arabidopsis thaliana tissues. However, tissue variation in circadian clocks in crop species is unknown. In this study, leaf and tuber global gene expression in cultivated potato under cycling and constant environmental conditions was profiled. In addition, we used a circadian-regulated luciferase reporter construct to study tuber gene expression rhythms. Diel and circadian expression patterns were present among 17.9% and 5.6% of the expressed genes in the tuber. Over 500 genes displayed differential tissue specific diel phases. Intriguingly, few core circadian clock genes had circadian expression patterns, while all such genes were circadian rhythmic in cultivated tomato leaves. Furthermore, robust diel and circadian transcriptional rhythms were observed among detached tubers. Our results suggest alternative regulatory mechanisms and/or clock composition is present in potato, as well as the presence of tissue-specific independent circadian clocks. We have provided the first evidence of a functional circadian clock in below-ground storage organs, holding important implications for other storage root and tuberous crops.

KEYWORDS
circadian clock, diel, gene expression, luciferase luminescence, Solanum tuberosum L. (potato), tissue-specific

INTRODUCTION

Daily environmental changes, such as light and temperature cycles, have led organisms to time molecular and physiological activities at specific times of day. For example, C3 plants open and close their stomata during the day and night, respectively, and solar tracking species such as sunflower follow the course of the sun during the day returning to an easterly direction at night (Meidner & Willmer, 1993; Vandenbrink et al., 2014). Many organisms have adapted internal molecular oscillators or circadian clocks, composed of interlocked transcriptional and post-translational feedback loops with groups of genes expressed at specific times of day, to anticipate these environmental changes and attune their physiology to their environment. In plants, numerous physiological processes are regulated by the circadian clock including photosynthesis (Dodd et al., 2014), carbohydrate metabolism (Graf et al., 2010), defense responses (Butt et al., 2020).
and flowering time (Shim et al., 2017). Diel and circadian transcriptomes have been profiled in seedlings and leaves of numerous plant species (Doherty & Kay, 2010), and demonstrate a pervasive role of the clock in gene expression.

The vast majority of circadian clock studies have been performed in whole Arabidopsis thaliana seedlings without differentiation of above or below-ground tissues. Several studies have demonstrated there is a functional circadian clock in A. thaliana roots with many of the core circadian clock genes expressed in this tissue (Bordage et al., 2016; Voß et al., 2015). Differences in circadian period (the amount of time to complete one cycle), phase (the timing of the cycle), and amplitude (the intensity of the cycle) have been observed between A. thaliana shoots and roots using luciferase reporter assays (Bordage et al., 2016; Chen et al., 2020; Greenwood et al., 2019). In addition, some studies reported altered circadian rhythms in A. thaliana roots upon disruption of the clock in the shoot apical meristem (Takahashi et al., 2015) with mobile signals moving from the shoot to the root to coordinate the pace of each clock (Chen et al., 2020). However, little is known about circadian control in other types of heterotrophic organs.

Cultivated potato (Solanum tuberosum L.) is a highly heterozygous, vegetatively propagated tetraploid (2n = 4x = 48) and the fourth most produced food crop globally (http://www.fao.org/faostat/en/#home). Potato and tomato (Solanum lycopersicum L.) are estimated to have diverged 6 million years ago (Wang et al., 2014) and share similar domestication histories (Hardigan et al., 2017; Lin et al., 2014). During domestication and improvement efforts, a lengthening of the circadian period was observed for tomato, while potato retained a short circadian period (the amount of time to complete one cycle), phase (the timing of the cycle), and amplitude (the intensity of the cycle) have been observed between A. thaliana shoots and roots using luciferase reporter assays (Bordage et al., 2016; Chen et al., 2020). In addition, some studies reported altered circadian rhythms in A. thaliana roots upon disruption of the clock in the shoot apical meristem (Takahashi et al., 2015) with mobile signals moving from the shoot to the root to coordinate the pace of each clock (Chen et al., 2020). However, little is known about circadian control in other types of heterotrophic organs.

We analyzed gene expression in cultivated potato leaf and tuber tissues in both cycling and constant light conditions. Differences in circadian period, phase, and amplitude between the tissues were examined and genes displaying differentially rhythmic expression patterns were identified. Both core circadian clock and tuberization genes were investigated. Using potato luciferase reporter lines, transcriptional rhythms in tubers and detached tubers were examined.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

The round white tetraploid chipping S. tuberosum cultivar “Atlantic” was used for all studies and maintained through vegetative propagation. For the time course transcriptional profiling experiments, Atlantic was grown in Suremix soil (Michigan Grower Products, Galesburg, MI) in 4-inch pots in a BioChambers TPC-19 growth chamber under 340-μmol m⁻² s⁻¹ light intensity under 12-h light/dark 22°C/18°C (LD/HC) cycles for 52 days. Afterwards, replicated leaf and tuber tissues were harvested every 4 h (ZT 0, 4, 8, 12, 16, 20, 24) for 24 h under the same cycling conditions (LD/HC samples). ZT0 samples were sampled in the dark and ZT12 samples in the light. For the LL transcriptomic samples, replicated leaf and tuber tissues were harvested every 4 h for 48 h under constant light and temperature (22°C) conditions (ZT 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60). Tubers remained in the soil in the dark for both the LD/HC and the LL experiments. For the luciferase reporter assays, transgenic Atlantic lines were grown in Green’s Grade soil (Profile, Buffalo Grove, IL) in 4-inch pots in the same BioChambers TPC-19 growth chamber under 340-μmol m⁻² s⁻¹ light intensity under 12-h light/dark 22°C/18°C cycles for 6 weeks before analysis.

### 2.2 | Library preparation and sequencing

Three biological replicates of leaf and tuber samples were harvested from the LD/HC and LL time course experiment. For each replicate, a 1.27-cm diameter cork borer was used to collect two leaf punches from the penultimate leaflet on the third leaf from the top of the plant, which were then flash frozen in liquid nitrogen. For tubers, a .64 cm diameter cork borer was used to collect one core each from three tubers per plant; the periderm was removed prior to flash freezing in liquid nitrogen. RNA was isolated from the leaf and tuber samples using hot phenol as described previously (The Potato Genome Sequencing Consortium, 2011) and DNase-treated with TURBO DNase (Invitrogen, Waltham, MA). DNase-treated RNA samples were sent to the Genomics Facility at Cornell University (http://www.biotech.cornell.edu/brc/genomics-facility) where Lexogen QuantSeq 3’ mRNA-sequencing FWD libraries (Vienna, Austria) were constructed and sequenced on an Illumina NextSeq 500 instrument (San Diego, CA, USA) to obtain 86 nucleotide (nt) single end reads.
2.3 Calculation of expression abundances and preferential tissue expression

Details for the calculation of expression abundances from the potato 3’ mRNA-seq data and the analysis of tomato mRNA-sequencing (mRNA-seq) reads generated previously (Müller et al., 2016) are in Methods S1. To test for tissue-specific expression and obtain normalized gene counts, DESeq2 (v1.22.2) (Love et al., 2014) was used. Potato leaf and tuber samples, and tomato leaf samples, were separately processed with the DESeq2 “rlog” function to obtain gene counts normalized for sequencing depth, RNA composition, and variance stabilization (Tables S1–S4). Genes with a rlog > 0 in at least one sample were classified as expressed. To test for outliers in replicates, Pearson’s correlation coefficients (PCC) were calculated using the R “cor” function. One potato leaf sample harvested at 20 ZT had PCC values below .9 with the other two biological replicates and was removed from the dataset (Figure S1). Potato leaf and tuber samples were tested for preferential tissue expression using an alpha level of .01. Genes with an adjusted p value < .01 and a log2-fold change >2 or log2-fold change < -2 were considered differentially expressed.

2.4 Identification of rhythmic gene expression

We identified cycling genes using normalized gene counts for the potato samples with the JTK_CYCLE algorithm in MetaCycle (v1.2.0) (Wu et al., 2016) and with ECHO (v4.0) (De Los Santos et al., 2020). ECHO uses an extended harmonic oscillator to identify rhythms, which have changes in amplitude over time (De Los Santos et al., 2020). The gene-wise period range was limited to 17–29 h for both LD/HC and LL experiments. Results between the JTK CYCLE and ECHO algorithms were combined following the methods of MetaCycle where p values are combined with Fisher’s Method, period lengths are averaged, and the circular mean is calculated for the phases (Wu et al., 2016); relative amplitude was obtained from MetaCycle. This combined data set is referred to as “JTK_ECHO,” and genes with an FDR adjusted p value < .05 and with period length differences <3 h between JTK and ECHO were considered to display rhythmic gene expression. Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder & Horvath, 2008) was also used to identify time-dependent expression patterns independently of fitting a model to the data. Independently for LD/HC and LL, a coefficient of variance (COV = .05) filter was used first to remove genes with unvarying gene expression patterns and WGCNA (v1.69) (Langfelder & Horvath, 2008) then performed with the COV filtered gene sets (referred to as COV). The parameters used for WGCNA were soft power (12 for LD/HC, 9 for LL), tree cut height (.99 or .975), and tree merge height (.4). To define WGCNA modules in each condition with rhythmic expression patterns and compare cycling detection approaches, we first placed genes not grouped into a co-expression module in a catch-all “grey” module and any module displaying double the percentage of JTK_ECHO significant genes compared to the catch-all “grey” module was considered a “cycling module.” This threshold of enrichment was determined by the manual evaluation of the eigengene patterns to determine a value that leads to an eigengene cyclic trace across the different conditions and tissues. The catch-all “grey” module was not used in further analyses.

Cytoscape (Shannon et al., 2003) was used to visualize the WGCNA networks with the files generated using the WGCNA “exportNetworkToCytoscape” function with an adjacency value threshold of .5 for calling a network edge.

2.5 Species and tissue comparisons

To compare the genes identified as displaying rhythmic gene expression between potato and tomato, orthologous groups were identified using OrthoFinder (v2.5.1) (Emms & Kelly, 2019) with the representative protein models of A. thaliana (TAIR10) (Lamesch et al., 2012), Solanum pennellii (Bolger et al., 2014), S. lycopersicum (ITAG4.1) (Hosmani et al., 2019), and S. tuberosum (DMv6.1) (Pham et al., 2020). A. thaliana core circadian clock genes were identified from the literature and orthologs were assigned via visual inspection of the OrthoFinder orthologous group gene tree for each orthologous group. The gene tree displayed sequence similarity relationships and enabled identification of corresponding orthologs. To characterize time-dependent differences in expression patterns between leaf and tuber tissues, we used DiPALM (v1.1) (Greenham et al., 2020). The parameters are in Table S5 and details in Methods S2.

2.6 Quantification of RT-qPCR abundances

RNA (500 µg, same material used for RNA sequencing) wasreverse transcribed into cDNA and subsequently used as a template for quantitative real-time PCR (qRT-PCR) as described previously (Poliner et al., 2015). Primers were designed using IDT PrimerQuest (Integrated DNA Technologies, Coralville, IA) to amplify regions that are conserved between all Atlantic gene isoforms and to span one exon-exon boundary within the gene for all intron-containing genes (Table S6). Relative expression was determined using the comparative Ct method with the average Ct values of Soltu.DM.06G026290 and Soltu.DM.03G006780 as an internal control.

2.7 Agrobacterium-mediated transformation

Atlantic plants were stably transformed with the StGH3-LUC2 construct (Figure S2) (see Methods S3 for details) via agrobacterium-mediated transformation as previously described (Li et al., 1999). Transgenic plants were first identified by rooting and growth on MS media containing the antibiotic kanamycin. DNA from putative transformants was tested for the presence of NPTII using primers specific to NPTII (Table S6). A second confirmation used a forward primer from the StGH3 fragment and the reverse from the LUC2 gene to amplify across the promoter-LUC2 junction (Table S6).
2.8 | Imaging luciferase luminescence

Imaging was performed on 6-week old plants using an imaging system described previously (Liu et al., 2016) at 22°C and 70μmol m⁻² s⁻¹ light intensity provided by Heliospectra RX-30 (Heliospectra, Chicago, IL). The light spectrum has been previously published (Hardigan et al., 2017). With the exception of the leaf to be imaged, above-ground shoot tissue was wrapped in a screen material to prevent obstruction of the imaging plane during the assay (Figure S3). For tubers, on the day of the assay, tubers of *S.GH3-LUC2* lines were placed on top of the soil still attached to the stolon; detached tubers were removed from their stolon. The leaf and tubers to be imaged were then sprayed with 5-mM D-Luciferin (Gold Biotechnology, St. Louis, MO). Images were taken every 2 h for 4 days as described previously (Liu et al., 2016). Images from the first 16 h after spraying with D-luciferin were excluded from downstream analysis. To detrend the raw luminescence data, a moving mean and standard deviation were calculated every 10 images using “TTR” (v0.23-6). The raw luminescence values were then subtracted from the moving mean and divided by the moving standard deviation and normalized between 0 and 1. BioDare2 (Zielinski et al., 2014) was used with the NFT NLLS algorithm and no detrending to calculate the period and phase values.

3 | RESULTS

3.1 | Heterotrophic tuber tissue displays diel and circadian expression patterns

Little is known about the role of the circadian clock in heterotrophic, below-ground plant tissues compared to autotrophic leaf tissue, with studies to date mainly limited to *A. thaliana* roots (Bordage et al., 2016; Chen et al., 2020; Greenwood et al., 2019). To determine if diel and circadian rhythmic gene expression was present in potato in leaf and tuber tissues, we profiled global gene expression of *S. tuberosum* cv. “Atlantic” (referred to here as Atlantic) leaf and tuber tissues at the bulking stage, sampling every 4 h under cycling light/dark and temperature (LD/HC), and constant light and temperature (LL) for 24 and 48 h, respectively (Tables S1–S4). Plants were grown on soil and therefore tubers were in the dark for all time points for both the LD/HC and the LL experiments. A total of 22,985 and 19,988 genes were expressed in leaf and tuber tissue, respectively.

Between 16% and 64% of significant cycling JTK_ECHO genes were present in WGCNA modules displaying cyclic expression patterns. Within rhythmic modules, JTK_ECHO significant genes were more highly expressed compared to genes that were not detected as cycling (p value < 2.2E−16), suggesting that WGCNA is able to identify lowly expressed genes with rhythmic expression patterns. We therefore extended the definition of “cycling” genes to include all genes present in WGCNA modules with rhythmic expression patterns regardless of JTK_ECHO significance (see Section 2 for details). The addition of genes significantly co-expressed with genes identified as cycling using model fitting algorithms will also help define co-regulatory expression networks regulating oscillatory activities.

In the leaf, a total of 10,276 genes (44.7% of expressed genes) and 7416 genes (32% of expressed genes) were diel and circadian rhythmic, respectively, with 52.7% of the circadian genes also displaying diel rhythms (Figure 1a, Figure 2a and Table 1). In the tuber, a total of 3574 genes (17.9% of expressed genes) and 1129 genes (5.6% of expressed genes) were diel and circadian rhythmic, respectively, with 12.8% of the circadian genes also displaying diel rhythms (Figures 1b and 2b and Table 1). We identified 1682 genes that had diel oscillations in both leaves and tubers, and 454 genes that had free running rhythms in both tissues (Figures 1c and 2c).

In both tissues, genes functioning in photosynthesis, transcription, translation, signal transduction, and cytoskeletal organization were enriched among diel and circadian rhythmic genes (Table S7), similar to what has been found in other photosynthetic organisms (Ferrari et al., 2019). Several primary metabolic pathways and processes were enriched for diel and circadian rhythmic gene expression among leaf and tuber tissue. For example, genes functioning in photosynthesis (GO:0015979; adj. p value < 1E−14) and related light harvesting pathways (GO:0009768; GO:0009765; GO:0019684) were highly enriched (adj. p value < 1E−18) among shared genes with diel and circadian rhythmic genes. All genes annotated with these GO terms were preferentially expressed in the leaf tissue, with an average log2-fold difference (lfd) > 6 (Table S8), indicating that while these genes display rhythmic gene expression patterns in the tuber, they are lowly expressed in that tissue. Indeed, genes preferentially expressed in leaf tissue (lfd > 2) were enriched for diel and circadian rhythms in both the leaf and tuber (p value < 2E−14), suggesting independent mechanisms regulate spatial and temporal expression patterns.

Functional gene enrichment analyses indicate that growth and stress responses are diel-regulated in potato tubers. Tuber LD/HC Module 1/red (Figures 1a and 1c and Table S7), with a peak of expression in the afternoon, is enriched for genes involved in microtubule-based movement (GO:0007018; adj. p value < 8.8E−16) and the cell cycle (GO:0007049; adj. p value < 2.8E−29). In this module, we found cyclins and cyclin dependent kinases that did not display tissue-specific differences at the expression level. Tuber LD/HC Module 2/blue contains a group of tightly co-expressed genes with high amplitude that is enriched in protein folding (GO:0006457; adj. p value < 1.8E−23) and response to heat (GO:0009408; adj. p value < 3.7E−28). These genes have a peak expression in the middle of the day, coinciding with increases in temperature under field conditions as the day progresses. Tuber LD/HC Module 3/purple is enriched in genes involved in sulfate assimilation (GO:0000103; adj. p value < 1.1E−5). In Arabidopsis leaves, genes involved in sulfate assimilation have been shown to cycle under constant light (Harmer et al., 2000) and as we observed for photosynthesis related genes, most of these sulfate assimilation related genes were preferentially expressed in leaves.
3.2 Many circadian clock genes do not display circadian rhythmic expression

To determine if core circadian clock genes have diel and circadian rhythmic expression in potato leaf and tuber tissues, potato orthologs of *A. thaliana* clock genes were identified. The number of paralogs present for each gene were consistent with previous analyses (Bombarely et al., 2016) with the exception of *StPRR3*, for which we identified two copies, and *StELF4*, for which we identified three copies (Table S9). One of the *StELF4* paralogs, *StELF4c*, was not expressed. While *SLHY, StPRR5, StPRR7*, and *StPRR9* are present as a single copy, *StGI, StTOC1, StLUX*, and *StELF4* have two expressed copies, and *StELF3* has three copies (Bombarely et al., 2016). We also identified six *StRVE* and four *StLNK* orthologs (Figure S4 and Table S9). Most core clock genes had higher average expression in leaf tissue than in tubers (Figure S4), with many significantly preferentially expressed in leaf tissue, including LHY and all LNK orthologs (Figure S4). Only *StRVE3/*5, *StPRR3a, StPRR5, StTOC1b, StELF4a*, and *StLUXa* had mean expression levels similar between leaf and tuber tissues (Figure S4).

In leaf tissue, diel oscillations in gene expression, similar to those previously identified (Hancock et al., 2014; Morris et al., 2014), were found among all core clock orthologs, with the exception of *StRVE1c* (Figures 3 and S4). In contrast, although several clock genes had diel expression patterns in the tuber, the amplitude of the oscillations was strongly reduced in comparison to the leaf for all the genes analyzed (Figure S4). For clock-related genes displaying diel oscillations in both tissues, most had no or small phase differences between leaves and tubers.

In contrast to the strong diel rhythms found in potato leaves, very few clock-associated genes displayed circadian rhythmic expression in either the leaf or tuber (Figures 3 and S5). In the leaf, *JTK_ECHO* algorithm detected free running oscillations in gene expression for only eight clock-associated genes (*StCRY2, StELF3b, StFHY3, StPRR3b, StPRR7, StSRR1a, StSTN7*, and *StLNK2*). Two other genes appeared to have cyclic RNA content in the leaf (*StRVE4/8 and StTOC1a*) by visual inspection of the expression traces (Figure 3). No clock-associated genes was detected as cycling by *JTK_ECHO* in tuber tissue (Table S9) although some genes appear to show some degree of oscillations by visual inspection, such as *StFHY3* (Figures 3 and S5).
JTK_ECHO FDR adjusted p values of the top 100 genes with strongest cycling patterns in potato leaves were all lower than for the ones for potato clock associated genes in that tissue (Figure 4). Figure 3c shows one of the strongest leaf and one of the strongest tuber cycling genes as a reference, indicating that robust oscillations are possible in potato. Finally, we compared the percent of genes cycling among all genes and among clock associated genes (Table 1). Under LD conditions, we observed a higher percentage of cycling of clock associated genes in both leaf and tuber tissues when compared to all expressed genes, however, a lower percentage was observed under constant light conditions, indicating a specific effect on free running oscillations.

We also measured gene expression patterns of several clock associated genes in our samples using real time quantitative PCR (RT-qPCR). In the LL experiment gene expression values display high variability between and among time points, as well as between detection methods (Figure S6). However, all the genes tested had cyclic expression under LD conditions and both RNA-seq and RT-qPCR traces were similar (Figure S6), indicating that the method of detection did not have a major influence on rhythmic expression patterns. The increased variability in the LL data might be partially responsible for the lack of rhythm detection.

A previous study determined diel and circadian expression of clock associated genes in cultivated tomato leaves (Müller...
et al., 2016). For a direct comparison with our potato experiment, tomato leaf mRNA-seq raw data was processed and analyzed as for potato. Tomato and potato leaf tissue displayed very similar phases of expression for putative clock genes in LD/HC conditions (Figure 3 and Table S9). In tomato leaves, all core clock genes were circadian rhythmic by JTK_ECHO (FDR adjusted p value < .05) and although several of the analyzed genes had a very low amplitude under constant light, similar to potato (Figures 4 and S7). We had measured period differences between cultivated tomato and potato in delayed fluorescence rhythms (Hardigan et al., 2017). The average period length of clock and photoperiod associated genes determined by JTK_ECHO was 25.7 h in leaf tissue, in contrast to the tomato average of 31.1 h (t test p value < .05) (Table S9). Although period determination on these short transcriptome time courses has statistical limitations, this analysis is consistent with the period differences between these cultivated species.

### 3.3 | Photoperiod sensing and tuberization genes display low rates of rhythmicity under constant light conditions

The circadian clock has been proposed to regulate photoperiod control of tuberization in landraces of the group *S. tuberosum* Group (Gp) Andigena (Kloosterman et al., 2013). In the current model, the putatively clock controlled GIGANTEA (StGI) and FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (StFKF1) mediate the degradation of CYCLIC DOP FACTOR 1 (StCDEF1) in leaves under long day conditions, leading to increased CONSTANS (StCO) expression, which in turn leads to a decrease in the FLOWERING LOCUS T (FT) paralog SELF PRUNING 6A (StSP6A) expression via StSP5G and repression of tuberization (Hannapel et al., 2017) (Figure 5). Although able to tuberize under long day conditions, short photoperiods are still able to accelerate tuberization of modern potato cultivars (Kittipadukal et al., 2012).
Cycling robustness among putative clock genes in potato. The FDR adjusted p value from the JTK_ECHO analyses in leaves of putative potato clock associated genes (Potato Clock, 64 genes), the top 100 strongest cycling genes in potato (Potato Top 100), and tomato clock associated genes (Tomato Clock, 22 genes).

In our experiments under LD/HC conditions, the expression of StGlA, StGlB, StFKF1, and StCDF1 cycled strongly leaves, and StCDF1, StGlA, and StGlB expression also cycled substantially in tubers (JTK_ECHO FDR adjusted p value < .01) (Figure S8), as has been observed in other cultivated potatoes (Hancock et al., 2014). Under LL conditions, we only detected weak free running rhythms for StGlA in tubers, but no significant 24-h free running rhythms of StFKF1 or StCDF1 according to our cycling criteria (Figures 5 and S9).

Potato has three homologs of the CO gene that are arranged in tandem. It was previously reported that StCO2 (Soltu. DM.02G030300) is lowly expressed in S. tuberosum Gp Andigena (Abelenda et al., 2016) and was not detected in our study. The expression of StCO genes was low or undetectable in tubers (Figures 5 and S8). In spite of the potential lack of regulation by StCDF1 due to the presence of a predicted truncated protein in the Atlantic cultivar (Hardigan et al., 2017), the expression of StCO1 and StCO2 cycled strongly under LD/HC, with the peak occurring at dawn as previously described in genotypes with weak photoperiod sensitivity (JTK_ECHO FDR adjusted p value < .001) (Hancock et al., 2014; Morris et al., 2014). We did not observe free running oscillations of StCO1, but visual inspection indicates that StCO2 leaf expression cycled in LL although it was not detected as cycling by our statistical analyses (Figures 5 and S9). StphyB and StphyF are needed for the stabilization of StCO1 during the first half of the light period under long day conditions (Abelenda et al., 2016; Zhou et al., 2019), and we observed significant gene expression oscillations of their genes under LD/HC conditions, with peak expression at dawn (JTK_ECHO FDR adjusted p value < .001) but no strong oscillations under free running conditions (Figures S8 and S9).

The RNA of FT-like genes in potato had very low amplitude oscillations with a bimodal expression pattern under light/dark cycles, similar to what was observed in other studies and was not detected as cycling by JTK_ECHO (Figure S8) (Hancock et al., 2014; Morris et al., 2014). The expression of StSPSG and StSPSG-like in leaves was low, as expected under tuber inducing conditions (Figure S8). In contrast, StSP6A was preferentially expressed in leaf tissue (lfd = 4.8), which might be due to tissue-specific differences in StSPSG expression. We did not detect free running oscillations in either StSPSG or StSP5-like expression (Figure S9). StBEL5 (BEL1-LIKE PROTEIN 5) promotes the expression of StSP6A and StCDF1, leading to early tuberization (Sharma et al., 2016). In Gp Andigena, tuber-inducing conditions have been associated with significant diel oscillations of StBEL5 expression (Kloosterman et al., 2013). Although we did not detect strong diel oscillations of StBEL5 RNA in Atlantic, its RNA abundance cycled in the tuber under LL conditions (JTK_ECHO FDR adjusted p value < .001) (Figures S9 and 5).

Sucrose availability is a key signal for tuber formation in potato and the plasma membrane sucrose transporters StSUT4 (Chincinska et al., 2008, 2013) and StSWEET11 (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER 11) (Abelenda et al., 2019) influence StSP6A expression and tuberization time. In turn, it has been shown that StSP6A modulates StSWEET11 activity (Abelenda et al., 2019). In our experiments, StSUT4 was slightly higher expressed in tubers relative to leaves (lfd = −1.7). In diel conditions, we observed strong differences in the timing of expression of StSUT4 in the two tissues, with a peak at dawn in leaves, while the RNA peaked at the end of the day in tubers (Figure S8). Although not detected as cycling in our analysis weak oscillations in leaf StSUT4 RNA were apparent under LL conditions (Figure 5) (Chincinska et al., 2008). StSWEET11 expression was significantly higher in the leaves than in the tubers (lfd = 7.0) (Figure S8). Although we did not detect strong diel oscillations of StSWEET11 RNA (Figure S8), its expression displayed free running oscillations in the tuber tissue (JTK_ECHO FDR adjusted p value < .01) (Figure 4).

### 3.4 | Differential rhythmic gene expression patterns between leaf and tuber tissue

As we observed some tissue-specific differences in clock and tuberization-related gene expression, and differences in cycling properties have been observed among both plants and animals (Bordage et al., 2016; Greenwood et al., 2019; Litovchenko et al., 2021), we investigated wider tissue dependent effects in our transcriptome data. To identify specific genes and pathways that displayed time-dependent differences between the leaf and tuber, DiPALM (Greenham et al., 2020) was used to detect changes in the correlation of gene expression patterns (Table S10). We selected 7487 genes with a COV > .05 in LD/HC conditions for both the leaf and tuber, of which, a total of 571 genes displayed tissue-specific expression patterns under diel conditions (Figure 6). A total of 107 genes displayed different gene expression patterns between the tissues for the LL conditions out of 7,025 genes with a COV > .05. Only four genes displayed differences under both LD/HC and LL conditions (Figure 6b).
Most of the genes with different expression patterns in LD/HC between leaves and tubers had striking tissue-specific differences in phase (Figure 6a). Co-expression analysis showed that these genes were either expressed at dawn in the tuber and at ZT 8 in the leaf or vice versa (Figure 6a).

One of the modules displaying differences in phase between leaf and tuber tissue under LD/HC conditions was enriched for water transport related genes (GO:0006833; adj. \( p \) value = 8.93E−05) (Table S10). For example, a total of four plasma membrane intrinsic proteins, which belong to a family of aquaporins located in the plasma membrane (Higuchi et al., 1998), were present in Module 1/red (Figure 6a). In the leaf, these genes are phased at dawn while in the tuber they peaked later in the light period. Over 250 genes were connected to these plasma membrane intrinsic proteins in the leaf diel co-expression network, with eight genes connecting all four aquaporins, directly or indirectly (Figure 6c). These eight genes included two dawn-phased core circadian clock orthologs, StLHY and StRVE4/8, which were connected directly to three of the four aquaporins. The aquaporins were preferentially expressed in the leaf (\( lfd > 2.7 \)), as were StLHY and StRVE4/8 (Figure S4). Although the peak of expression of StLHY does not differ between the tissues, the expression of StRVE4/8 peaks later in the tuber than in the leaf, which could explain the delayed expression in the tuber of the aquaporin genes (Figure S4).

3.5 Detached tubers display robust transcriptional circadian rhythms

To test if circadian rhythms in the tuber are independent from above-ground tissues, we investigated attached- and detached-tuber rhythms using luciferase reporter lines. Soltu.DM.02G8028070 is annotated as a Gretchen Hagen 3 (hereafter referred to as StGH3) auxin-responsive gene, which is a ubiquitous gene family rapidly induced upon plant treatment with auxin (Hagen & Guilfoyle, 2002). We selected this gene because in the tuber, StGH3 expression cycles under both diel and constant light conditions in our transcriptome experiments, in which tubers remained in the soil (Figure 7a). To test for diel and circadian transcriptional rhythms in attached and detached tubers, the promoter of StGH3 was cloned into a firefly luciferase vector and luminescence was measured in two independently stably transformed 6-week old lines. In leaves, this reporter led
to cyclic luminescence only under LD/HH conditions but not in constant light (Figure S10). Both attached and detached tubers displayed robust transcriptional rhythms after 4 days when exposed to either LD/HH, LL, or LD/HC conditions, with a higher variation present in the tuber circadian rhythms for line #52 (Figures 7b and S10). Under LD/HH cycles, the median circular phase for the attached and detached tubers was 20.91 and 18.55 h, respectively (Figure 7c). This phase of the bioluminescence rhythms is similar, although slightly delayed, to the phase of StGH3 RNA oscillations (Figure 7a). Attached tubers displayed a higher variability of phase distribution than detached tubers (Figure 7c). Due to our current technical constrains, tubers do not remain in the dark during imaging. It is possible that the presence of both direct light and internal signals from leaves in attached tubers led to this increased variation. These results initially indicate that all primary cis-regulatory components were captured in the promoter sequence and that there were no major differences in gene expression oscillations between tubers in the soil in our RNA-seq experiment and tubers exposed to the light/dark cycles in the luciferase imaging experiment. Under constant light, the median period was 22.10 and 23.33 h for the attached and detached tuber, respectively, which are similar to the period determined for Atlantic using delayed fluorescence (Figure 7d) (Hardigan et al., 2017). We also measured luminescence rhythms in tubers in constant dark (DD) (Figure 7d). No free running rhythms in either attached or detached tubers were detected in DD and constant temperature. The absence of rhythms was not due to a negative effect of lack of light on luciferase activity, since 18°C/22°C temperature cycles were able to restore rhythmic luminescence in both attached and detached tubers in constant dark. StGH3 RNA content in the tuber was rhythmic in our transcriptome experiment, in which tubers were kept in the soil (dark) but the leaves where in the light (Figure 7a). We could not reproduce these conditions using luminescence measurements due to technical limitations. However, our experiments indicate that signals from the leaves are required for maintaining rhythms in the tuber in the dark in the absence of temperature oscillations.

4 | DISCUSSION

Through profiling global gene expression over time in potato leaf and tuber tissue, we report on the first circadian rhythmic gene expression study in the below-ground heterotrophic tuber tissue. Potato leaves had similar percentages of genes displaying rhythmic expression as reported for A. thaliana seedlings, where 27.5% and 14.9% of all genes cycled under LD/HC or LL (after LD/HC entrainment) respectively (Michael et al., 2008). Diel expression patterns were present in the tuber despite residing in the soil with little to no exposure to light fluctuations. In contrast, only 4.1% and .39% of all genes were
identified as having diel expression patterns in grape (Vitis vinifera L.) berries (Rienth et al., 2014) and immature maize (Z. mays L.) ears (Hayes et al., 2010). A total of 4.9% of all genes displayed circadian expression patterns in A. thaliana lateral root primordia (Voß et al., 2015) grown exposed to continuous light, which is similar to the percentage of circadian rhythmic genes identified in the tuber, which resided in the soil. Our tuber luminescence reporter experiments using plants with both tubers and leaves exposed to either constant light or constant darkness, indicate that information from the above ground tissues is needed to maintain oscillations in the tubers in the dark (Figure 7).

Furthermore, our observations of a de-coupling between tissue-specific average expression level and rhythmic expression patterns is similar to what has been reported in flies (Litovchenko et al., 2021) and suggests alternative mechanisms regulate temporal and spatial gene expression. Moreover, most genes displaying strong tissue-specific differences in the time of expression under LD/HC did not display significant differences in constant light (Figure 6), indicating...
that environment driven signals play a key role in determining the phase of gene expression in different tissues.

4.1 Weak oscillations lead to challenges in the identification of cyclic expression patterns

Our visual analysis of clock and photoperiod indicate that there are still limitations on the methodology of the determination of cyclic gene expression using JTK_ECHO and WGCNA for transcriptomic studies. There are genes such as StCO2 or StRVE4/8 that are not classified as rhythmic in leaf tissue although oscillations appear to be there (Figures 3 and 5). Other genes, such as StSWEET1a or PIF7, which are only classified as rhythmic via the WGCNA module criteria, do not display clearly visible ~24-h free running oscillations (Figures S5 and S9). The use of 2-h resolution time courses is recommended for rhythmic analysis of transcriptome data (Hughes et al., 2017), although that adds to a significant cost increase, it might reveal a more accurate determination of cycling patterns in species with low amplitude oscillations, such as cultivated potato.

4.2 Gene expression analyses indicate diel control of growth and stress responses in tubers

We observed similar enrichments for primary plant pathways among genes displaying rhythmic expression patterns in potato leaves as found in other time course gene expression studies in both land plants and other photosynthetic organisms (Ferrari et al., 2019; Filichkin et al., 2011; Higashi et al., 2016; Müller et al., 2020; Poliner et al., 2015; Zones et al., 2015). For example, we observed light harvesting related genes as well as translation related genes among those with cycling gene expression. Although no diel oscillations of tuber size have been detected when tubers are kept in the soil (Pérez-Torres et al., 2015), we found an enrichment of genes associated with cell division and the cell cycle within the cycling genes in tubers in diel conditions (Figures 1 and S7). We did not find enrichment of cell cycle genes under LL conditions in the tuber but several cyclin and cyclin dependent kinase genes were present in cycling modules (Table S7). These results indicate that the cell cycle in tuber tissue might be under circadian control as has been observed in young Arabidopsis seedlings (Fung-Uceda et al., 2018).

Elevated temperatures are detrimental for tuber formation in potato (Levy & Veilleux, 2007) and stress related genes were co-expressed in the tuber under LD/HC conditions. One of those genes is heat shock cognate protein 70-1 (HScP70; Solu1,DM.04G007430), which had been identified as part of a QTL that improves tuber yield at slightly elevated temperatures (Traperro-Mozos et al., 2018). Furthermore, the putative clock component StTOC1a is involved in the regulation of tuberization at elevated temperatures in cultivated potatoes (Hancock et al., 2014; Morris et al., 2019), suggesting a role of the circadian clock in tuber heat responses.

4.3 Putative circadian clock components display weak free running gene expression rhythms in potato

In angiosperms, most core circadian clock orthologs display free running rhythmic expression patterns in both leaf and heterotrophic tissues, (Covington et al., 2008; Filichkin et al., 2011; Higashi et al., 2016; Müller et al., 2016; Panter et al., 2019; Voß et al., 2015). However, in cultivated potato only about a quarter of the analyzed putative clock associated genes had significant cycling RNA levels under constant light in leaves and only two were detected as cycling in tubers. Since there are genes displaying significant oscillations under constant light (Figures 3 and 4), we do not believe that the lack of rhythmicity is caused by desynchronization among plants. Weak free running rhythms of gene expression have also been observed in cultivated tomato when compared to its wild relative, and it has been hypothesized that the tomato circadian clock was weakened during domestication and improvement efforts (Müller et al., 2016). However, putative clock associated genes in cultivated potato appear to have overall even weaker rhythms than in cultivated tomato (Figure 4). We had previously observed that the cultivated potato short circadian period appeared to be selected for during domestication and that StRVE3/5, StLNK3/4, StELF4a, and StELF4b had signatures of selection during both potato domestication and improvement (Hardigan et al., 2017). A similar weakening of the circadian clock might have occurred in cultivated potato compared to wild species and future studies using wild potatoes will be needed to confirm this hypothesis. In spite of weak RNA oscillations and low expression of putative clock components, potato tubers exhibit a significant number of circadian rhythmic expression patterns and are able to maintain oscillations when detached from the mother plant, indicating the presence of an independent self-sustained circadian oscillator (Figure 2, Figure 7). Similar results have been found A. thaliana roots (Bordage et al., 2016; Greenwood et al., 2019) and may signify a more universal phenomena of tissue-specific circadian clocks in plants. Alternative regulatory mechanisms (e.g., post-translational regulation) may be present in the tuber to drive gene expression among downstream genes. Tissue-specific differences in clock gene expression and tissue-specific effects of clock mutants have been observed in A. thaliana (Endo et al., 2014; James et al., 2008; Nimmo et al., 2020; Takahashi et al., 2015). An improved description of tissue-specific circadian regulatory networks will be needed for a better understanding of the role of the clock in plant growth and development.

4.4 Circadian input into tuber development

It has been assumed that the circadian clock is involved in the regulation of the photoperiod control of tuberization in potato. Similar to the putative clock components genes, we observed few tuberization related genes with free running oscillations. CO genes are a key component of the integration of circadian and light signals for photoperiod sensing (Shim et al., 2017). We observed strong leaf diel oscillations for both StCO1 and StCO2, however, only observed LL rhythms for
StCO2 in cultivated potato (Figure 5, Figure S9). Atlantic contains a truncated StCDF1 allele lacking the C-terminus domain involved in the interaction with StGI/StFKF1 (Hardigan et al., 2017; Kloosterman et al., 2013), which is expected to limit StCDF1 protein oscillations, stabilizing it at high levels throughout the day, and leading to low StCO1 and StCO2 expression (Kloosterman et al., 2013). The occurrence of RNA diel and circadian oscillations for StCO2 suggests StFKF1/StGI independent clock mechanisms additionally regulate StCDF1 expression in potato. Moreover, as other modern cultivars, Atlantic also contains a non-truncated StCDF1 allele (Hardigan et al., 2017) and its role in regulating StCO1 and StCO2 expression remains to be investigated. Finally, sucrose and sucrose transporters play a crucial role in impacting tuberization time and tuber development (Abelenda et al., 2019; Chincinska et al., 2008, 2013). Constant light oscillations of leaf StSUT4 RNA in leaves of cultivated potato had been reported and we observed weak oscillations under those conditions for StSUT4, as well as free running rhythms for StSWEET11 in tubers (Figure 5). Thus, sucrose transport may represent an additional pathway through which the circadian clock regulates tuberization in potato.

5 | CONCLUDING COMMENTS

Our study expands the resources on cyclic gene expression in crops and in particular provides an important dataset for studying circadian regulation in heterotrophic organs. We show that potato tubers are able to maintain free running transcriptional rhythms, even detached, in the absence of strong rhythmicity in the RNA abundance of clock components. This observation raises questions not only on the role of transcriptional regulation in circadian control but also on the specific role of the clock in the regulation of tuberization.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

GMH, AF, CRB, and EF designed experiments. GMH, DZ, AF, KA, JPH, and EF performed experiments and/or analyzed data. DD, CRB, and EF prepared the manuscript.

CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT

The raw sequencing data are available from NCBI at the following BioProject: PRJNA744935. Below is the private link to review the data, which will be made publicly available upon publication. Normalized expression data, lists of cycling genes, and results from other analyses are available via supplementary tables.

https://dataview.ncbi.nlm.nih.gov/object/PRJNA744935?reviewer=sk3vm3ek1nju3nreh3s1cn56k

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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