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Arabidopsis sirtuins and poly(ADP-ribose) polymerases regulate gene expression in the day but do not affect circadian rhythms

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Abstract

Nicotinamide-adenine dinucleotide (NAD) is involved in redox homeostasis and acts as a substrate for NADases, including poly(ADP-ribose) polymerases (PARPs) that add poly(ADP-ribose) polymers to proteins and DNA, and sirtuins that deacetylate proteins. Nicotinamide, a by-product of NADases increases circadian period in both plants and animals. In mammals, the effect of nicotinamide on circadian period might be mediated by the PARPs and sirtuins because they directly bind to core circadian oscillator genes. We have investigated whether PARPs and sirtuins contribute to the regulation of the circadian oscillator in Arabidopsis. We found no evidence that PARPs and sirtuins regulate the circadian oscillator of Arabidopsis or are involved in the response to nicotinamide. RNA-seq analysis indicated that PARPs regulate the expression of only a few genes, including *FLOWERING LOCUS C*. However, we found profound effects of reduced *sirtuin 1* expression on gene expression during the day but not at night, and an embryo lethal phenotype in knockouts. Our results demonstrate that PARPs and sirtuins are not associated with NAD regulation of the circadian oscillator and that *sirtuin 1* is associated with daytime regulation of gene expression.

KEYWORDS
Arabidopsis thaliana, circadian clock, Nicotinamide-adenine dinucleotide, poly(ADP-ribose) glycohydrolase, poly(ADP-ribose) polymerases, sirtuins

1 | INTRODUCTION

Plant life is heavily influenced by the cycles of light, dark and temperature caused by the rotation of the planet on its axis, resulting in rhythmic outputs modulated by the circadian clock (Webb, Seki, Satake, & Caldana, 2019). Metabolism is an important output of the circadian system in plants, with the circadian oscillator regulating the expression and activity of the components of many metabolic pathways, including photosynthesis and starch metabolism (Dodd et al., 2005; Graf, Schlereth, Stitt, & Smith, 2010; Lu, Gehan, & Sharkey, 2005), nutrient assimilation (Gutierez et al., 2008), redox homeostasis (Lai et al., 2012) and secondary metabolism (Kerwin et al., 2011). However, metabolism has recently become recognized also to act as a crucial input regulating the circadian oscillators of both plants and animals. For example, in Arabidopsis, the period of the circadian oscillator is regulated by sugars (Frank et al., 2018; Haydon et al., 2013) and nicotinamide (Dodd et al., 2007; Mombaerts et al., 2019). Sugars fluctuate as a consequence of carbon homeostasis and photosynthesis (Webb

et al., 2019), whereas nicotinamide is a breakdown product of NAD acting as a substrate for post-translational modifications including poly(ADP-ribosyl)ation and protein deacetylation, and for the production of the Ca^{2+} agonist cyclic ADP-ribose (cADPR; Hunt, Lerner, & Ziegler, 2004). Nicotinamide lengthens circadian period in all organisms tested with proposed modes of action being through inhibition of poly(ADP-ribose) polymerases (PARPs), sirtuins (SRTs), ADPR cyclases, the reduction of H3K4me3 accumulation, target of Rapamycin (TOR) and the action of BIG, a protein of unknown function (Asher et al., 2008; Asher et al., 2010; Dodd et al., 2007; Hearn et al., 2018; Malapeira, Crhak Khaitova, & Mas, 2012; Mombaerts et al., 2019; Nakahata et al., 2008; Zhang et al., 2019).

In mammals, PARP1 participates in the phase entrainment of peripheral clocks (Asher et al., 2010) and mice lacking PARP1 have a phase shift in the timing of complex formation between the circadian oscillator components, CLOCK/BMAL1 and PER, and the entrainment of the peripheral circadian clock in the liver to inverted feeding cycles was significantly delayed (Kumar & Takahashi, 2010). PARPs also play roles in DNA repair, maintenance of genomic stability, transcription, chromatin structure, cell cycle and telomere length, energy metabolism and cell death (Krishnakumar & Kraus, 2010; Schreiber, Dantzer, Ame, & de Murcia, 2006). In plants, PARPs play a crucial role in the innate immune responses because *parp1parp2* double mutant Arabidopsis have compromised immune gene activation and enhanced susceptibility to pathogen infections (Feng et al., 2015). Decreased levels of PARP activity by chemical inhibition or genetic down-regulation have been correlated with increased tolerance to abiotic stresses including oxidative, drought and heat stress (De Block, Verduyn, De Brouwer, & Cornelissen, 2005; Vanderauwera et al., 2007) but *parp* loss-of-function mutants are not affected in abiotic stress responses (Rissel et al., 2017). Therefore, the role of PARPs in plant abiotic stress responses is still an open question and their effect on circadian function in plants has not been reported.

PARP enzymatic activity is counteracted by the enzyme poly(ADP-ribose) glycohydrolase (PARG), which hydrolyses PAR polymers and releases ADP-ribose subunits. Consistent with a role for PARPs in immune responses in plants, both PARG1 and PARG2 are required for stress responses to *Botrytis cinerea* in tomato and an increase in ADP-ribose polymer levels was observed in response to avirulent *Pst* DC3000 (Adams-Phillips, Briggs, & Bent, 2010). Unlike the PARPs, PARG activity has been shown to regulate the circadian oscillator of Arabidopsis because the *TEJ1* mutation in *PARG1* has long free-running circadian period (Panda, Poirier, & Kay, 2002).

Sirtuins, like the PARPs, are NADases whose enzymatic activity is also inhibited by nicotinamide. Sirtuins are NAD^+-dependent protein deacetylases homologous to the yeast Sir2 protein, which is responsible for heterochromatin formation in yeast (Imai, Armstrong, Kaeberlein, & Guarente, 2000; Landry et al., 2000; Smith et al., 2000). In mammals, Sirtuin 1 (SIRT1) is closely associated with core circadian oscillator components. SIRT1 activity is rhythmic and cultured mouse cells null for *SIRT1* or transfected with *SIRT1* siRNA had reduced amplitude of the *BMAL1:LUC* circadian oscillator reporter, suggesting SIRT1 is required to maintain the magnitude of oscillator gene expression (Asher et al., 2008).

We have found evidence that nicotinamide affects the Arabidopsis circadian oscillator through its action on ADPR cyclase and the inhibition of Ca^{2+} signalling (Abdul-Awal et al., 2016; Dodd et al., 2007; Hearn et al., 2018; Martí Ruiz et al., 2018; Mombaerts et al., 2019). Here, we have tested the counter hypothesis that members of the Arabidopsis *PARP* and *SRT* gene families participate in circadian regulation and that this might explain the mode of action of nicotinamide in the circadian system. We performed this study because of the important role of the PARPs and SRTs in mammalian systems and the potential that this might be a conserved function between the circadian systems across Kingdoms. Using a combination of genetic and transcriptome analysis, we find that the PARPs and SRTs are unlikely to be involved in circadian regulation in Arabidopsis and do not appear to explain the response of the Arabidopsis circadian oscillator to nicotinamide. These data define a major regulatory difference between the plant and mammalian circadian oscillators. We identify a function for PARPs in photoperiodic responses through the regulation of the flowering time regulator *flowering locus C* (*FLC*). We demonstrate that SRT1 function might have been obscured in previous analysis of Arabidopsis because *srt1* knockouts are embryo lethal, which we conclude might be related to a profound effect of *srt1* knockdown on gene expression during the day. Together, these studies investigate how NADases that can regulate gene expression can affect plant responses.

2 | MATERIAL AND METHODS

2.1 | Plant material and growth conditions

Surface sterilized *Arabidopsis thaliana* seeds were sown directly onto half strength Murashige and Skoog (MS; Duchefa Biochemie, Netherlands), 0.8% Bacto Agar (BD). Stratification at 4°C in the dark was for 2 days, before transfer to Sanyo (UK) growth cabinets (19–22°C, 100 μmol m^{-2} s^{-2}; 12 hr light/12 hr dark).

T-DNA lines used in this investigation are described in Table S1. Genotyping was carried out according to the instructions at http://signal.salk.edu/tdnaprimers.2.html using the primers listed in Table S2. Left border PCR products were sequenced to determine the precise locations of the T-DNA inserts.

For *srt1-4 heterozygous* (*hete*) and *srt1-4 hete x 2–1* mutants, we performed genotyping by PCR to identify heterozygous plants for each experiment. *SRT1* artificial miRNAs were designed using the MicroRNA Designer tool of the WMD3 Web site (Ossowski, Schwab, & Weigel, 2008) and were introduced by transformation as described below.

2.2 | Leaf movement imaging and analysis

A. thaliana seeds were sown individually, with an approximately 1 cm gap in between each seed. To promote hypocotyl elongation, plants were grown under the same condition as described above in individual 5 cm tall cardboard boxes for 10–12 days. Individual seedlings were cut out in 1 cm^2 blocks of agar with a scalpel and transferred to

vertical standing 25-well 10 cm^2 culture dishes (Sterilin, UK) in constant white light (100 μmol m^{-2} s^{-2}; Dodd et al., 2007). Circadian rhythms of leaf movement were recorded using time-lapse image capture from an array of video cameras controlled by Metamorph software (Molecular Devices LLS). Images were captured every 20 min for 7 days. Leaf position data were extracted from images using Metamorph software. Rhythmic traces were analysed using Biological Rhythms Analysis Software System (BRASS; Southern & Millar, 2005) software package, which obtains period estimates and relative amplitude error (RAE) values using a fast-Fourier transform non-linear least squares (FFT-NLS) method. The first 24 hr of data were excluded from analysis and movements were considered rhythmic if they had a period between 15 and 35 hr.

2.3 | Photoperiodic flowering time screening

Sixteen plants per line were sown into soil trays with 24 cells with the positions being allocated randomly. The seeds were stratified for 3 days at 4°C before transfer to 20°C and 100 μmol m^{-2} s^{-1} in either long day (LD) conditions (16 hr L:8 hr D) or short day (SD) conditions (8 hr L: 16 hr D). Seed trays were moved regularly to prevent any positional effects on plant growth. The time to bolt and the number of rosette leaves at the time of bolting were recorded. Bolting time was measured as the number of days from sowing when the inflorescence stem was approximately 5 mm tall.

2.4 | Transformation of Arabidopsis with pCCA1:LUC$^+$

Agrobacterium tumefaciens (Gv3101) transformed with appropriate constructs (Ti plasmid: pPCVH containing *LUC*$^+$; helper plasmid: pM90RK; *CCA1:LUC* binary vector), a gift from A. Millar (University of Edinburgh; O'Neill et al., 2011) were inoculated into 5 ml Luria Bertani cultures containing gentamycin (25 μg ml^{-1}) and ampicillin (50 μg ml^{-1}) 2 days before transformation. After cultures were left overnight at 30°C, 2.5 ml was sub-cultured into a 50 ml culture and then grown at 30°C overnight. The culture was generously painted onto whole plants (leaves, secondary stems and inflorescences) with an autoclaved paintbrush. Plants were covered in a cling film tent for 2 days to increase humidity.

2.5 | Measurement of circadian rhythms using luminescent circadian clock reporter gene fusions

Seeds were sown on to 0.5 MS 0.8% agar in PVC tubing rings (0.7 mm diameter) in clusters of 2–5 seeds for imaging of luciferase. Plants were grown under the same condition as described above for 10–12 days before imaging. Twenty-four and 48 hr before imaging seedlings were dosed with 50 μl of 2 mM luciferin, the substrate of luciferase. Plants were imaged using Photek ICCD225 photon counting cameras using IFS32 software. Automated images were captured every hour for 800 s for luciferase imaging, or for 1,500 s every 2 hr for imaging of aequorin. To analyse images, captured regions were drawn on a pseudo-coloured image around the sites of clusters using IFS32 software, with the bright field image used as a reference. Total photon counts (luminescence) per image per unit time were extracted. Period estimates were obtained using BRASS software.

2.6 | Delayed chlorophyll fluorescence

Seeds were sown in clusters of approximately 20 seeds, one cluster per well in a 10 cm square 25 well plate. The dividing walls of the plate were covered in black electrical tape, to reduce cross contamination of signal between wells. After 10–12 days of entrainment, plates were transferred into a NightSHADE LB 985 photon counting camera (Berthold Technologies), controlled by IndiGO software. Plants were grown in the camera box for one light/dark cycle before switching to constant light conditions for imaging. Illumination in the camera was maintained by light emitting diodes (LEDs) set to 24% of maximum emission for red (660 nm) and blue (470 nm) wavelengths to provide light intensity 50–70 μmol m^{-2} s^{-2} at plant height. Delayed fluorescence (DF) was measured at a wavelength of 560 nm for 60 s after illumination every hour for 5 days (Gould et al., 2009). Images produced were analysed by IndiGO software, background corrected and detrended. Background corrected data were analysed using BRASS.

2.7 | Quantitative real-time polymerase chain reaction

RNA was extracted from 40 mg fresh weight of 2-week-old seedlings using a Qiagen (Manchester, UK) RNeasy plant mini kit with on-column DNase treatment as per manufacturer's instructions. RNA quality and quantity were measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific). cDNA (500 ng) was produced using Thermo Scientific RevertAid kit with oligo $(dT)_{18}$ primers as per manufacturer's instructions. Primers for quantitative real-time polymerase chain reaction (qRT-PCR) were designed using Primer3 or NCBI Primer-BLAST. A list of qRT-PCR primers used is provided (Table S2). qRT-PCR was performed with SYBR Green PCR kits (Qiagen), which include premixed HotStarTaq DNA polymerase, SYBR Green I dye and dNTPs. Samples were analysed in a Qiagen Rotor-Gene Q cycler and melt curve analysis was also performed. qPT-PCR data were normalized by delta–delta Ct method. Each experiment was performed independently three times providing three biological replicates and three technical replicates were performed within each experiment.

2.8 | RNA sequencing

For RNA-seq analyses, Col-0, *parp1-2*, *parp2-1*, *parp3-1*, *parp1-2x2-1x3-1*, *srt1-4 hete*, *srt2-1 and srt1-4 hete x srt2-1* mutant

seedlings were grown for 2 weeks in the conditions used for the other experiments. Three independent biological replicate seedlings were collected at ZT6 and ZT18 representing the day and night samples.

RNA was extracted using the same method as for the qRT-PCR. RNAseq libraries were generated using the Illumina TruSeq RNA Prep Kit v2 (Illumina, San Diego, CA) and sequenced by BGI-Hongkong (Hong Kong, China) on an Illumina Hiseq 4,000 with 100 base pair paired-end reads. The sequencing data were provided demultiplexed, pre-filtered and with the adaptor sequences trimmed off. Quality Checks (QC) on the data were performed using the fastQC software v0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.9 | RNA sequencing alignment, quantification and differential expression

The RNA-seq reads were aligned against *Arabidopsis thaliana* TAIR10 transcriptome models using bowtie2 (Langmead & Salzberg, 2012) with the -very-sensitive preset. Transcript quantification was performed by the eXpress software v1.5.1 (Roberts & Pachter, 2013) using default parameters. The transcript counts were used as input for the R/Bioconductor package baySeq v2.6.0 (Hardcastle & Kelly, 2010) to perform differential expression analysis. This package was run using default parameters and with a false discovery rate (FDR) of 0.05. For each condition (day, night), each mutant was compared with Col-0.

Publicly available datasets from studies of differential expression related to light signalling and a control unrelated to light signalling were downloaded from the European Bioinformatics Institute (EBI) for comparison with the results for our *srt1-4 hete* mutant. These external datasets are listed in Table S3. The differentially expressed transcripts were divided into a list of up-regulated and a list of down-regulated transcripts. Then, the overlap between the up- and down-regulated lists for *srt1-4 hete* and each of the corresponding up- and down-regulated lists for the five comparisons was identified. The R/Bioconductor package GeneOverlap v1.24.0 (Shen, 2020) was used to determine whether the overlap was statistically significant. Because the internal and external datasets do not contain exactly the same genes, the parameter 'genome size' was given as the number of gene identifiers common to both data sets. For the comparison between *srt1-4 hete* in the day and the 'white light' (WL) data from EBI, the genes in each of the four overlaps were tested for enrichment of Gene Ontology (GO) terms (Ashburner et al., 2000) using the R/Bioconductor package GOstats v2.54.0 (Falcon & Gentleman, 2007) with GO.db v3.11.4 (Carlson, 2020a) and org.At.tair.db v3.11.4 (Carlson, 2020b).

2.10 | Other statistical analysis

The Student's *t* test was used to compare differences between two groups. Where there were more than two groups, a one-way ANOVA was used. A two-way ANOVA was performed in experiments where both genotypes and conditions were tested. Non-parametric alternatives were used when assumptions for parametric tests were not met. Sigmaplot (Systat Software, Inc.) was used to perform these statistical analyses. Data are presented as the mean plus and minus the standard deviation.

3 | RESULTS

3.1 | NADase inhibitors lengthen the period of circadian rhythms

We confirmed previous reports that nicotinamide increases circadian period in Arabidopsis (Dodd et al., 2007; Mombaerts et al., 2019). The period of the circadian rhythms of leaf position of untreated Col-0 was 24.5 ± 1.5 hr, which increased to 26.0 ± 0.5 hr, $p < .001$ in the presence of 50 mM nicotinamide (Figure 1a,b). Similarly, using *clock associated 1* (*CCA1*) fused to the luciferase reporter gene (*CCA1:LUC*), we measured an increase of circadian period from 23.7 ± 0.3 hr to 26.0 ± 0.5 hr following treatment with 50 mM nicotinamide (Figure 1c,d).

To investigate if PARPs might be mediating the effect of nicotinamide on the circadian oscillator, we measured the response to 3-Methoxybenzamide (3 MB), an inhibitor that has been reported to be more specific than nicotinamide for PARP activity (Chen, Shall, & Ofarrell, 1994). 3 MB increased the period of circadian oscillations of *CCA1:LUC* luminescence (Figure 2a,b; untreated 25.2 ± 0.6 hr, DMSO solvent control 25.4 ± 0.7 hr, 1 mM 3 MB 26.0 ± 0.6 hr, 2 mM 3 MB 27.1 ± 0.7 hr $p < .001$).

3.2 | *PARP* mutants do not have altered circadian rhythms

The sensitivity of circadian rhythms to nicotinamide and 3 MB might suggest a role of PARPs in the regulation of the Arabidopsis circadian oscillator, which prompted us to investigate the regulation of *PARP* transcripts and to isolate mutants in the three known *PARP*-encoding genes in Arabidopsis. None of the transcripts encoding PARPs in Arabidopsis oscillated with a circadian dynamic (Figure S1). We were unable to detect *PARP3* by qRT-PCR due to low abundance.

Homozygous T-DNA insertion alleles were identified for the *parp1-2* (*At4G02390*; *SAIL line 1250B03*), *parp2-1* (*At2G31320*; *GABI line 380E06*) and *parp3-1* (*At5G22470*; *SAIK line 108092*) mutants (Figure S2a and Table S1) These were combined to generate a *parp1-2x2-1x3-1* triple mutant (Figure S2b) in which we could not detect transcripts of *PARP1*, *2* or *3* (Figure S2c). We found no evidence that PARP activity contributes to circadian regulation as measured by leaf movement analysis (Figure 3a–d) or the measurement of circadian rhythms of delayed chlorophyll fluorescence (DF; Figure S3) because circadian periods were indistinguishable from wild-type background (leaf movement Col-0 24.4 ± 1.0 hr, *parp1*-2 25 ± 1.3 hr, Col-0 25.0 ± 1.4 hr, *parp2*-1 25.3 ± 1.1 hr, Col-0 24.1 ± 0.9 hr, *parp3*-1 24.3 ± 1.0 hr $p > .05$ Figure 3; DF Col-0 23.8 ± 0.9 hr, *parp1-2* 22.6

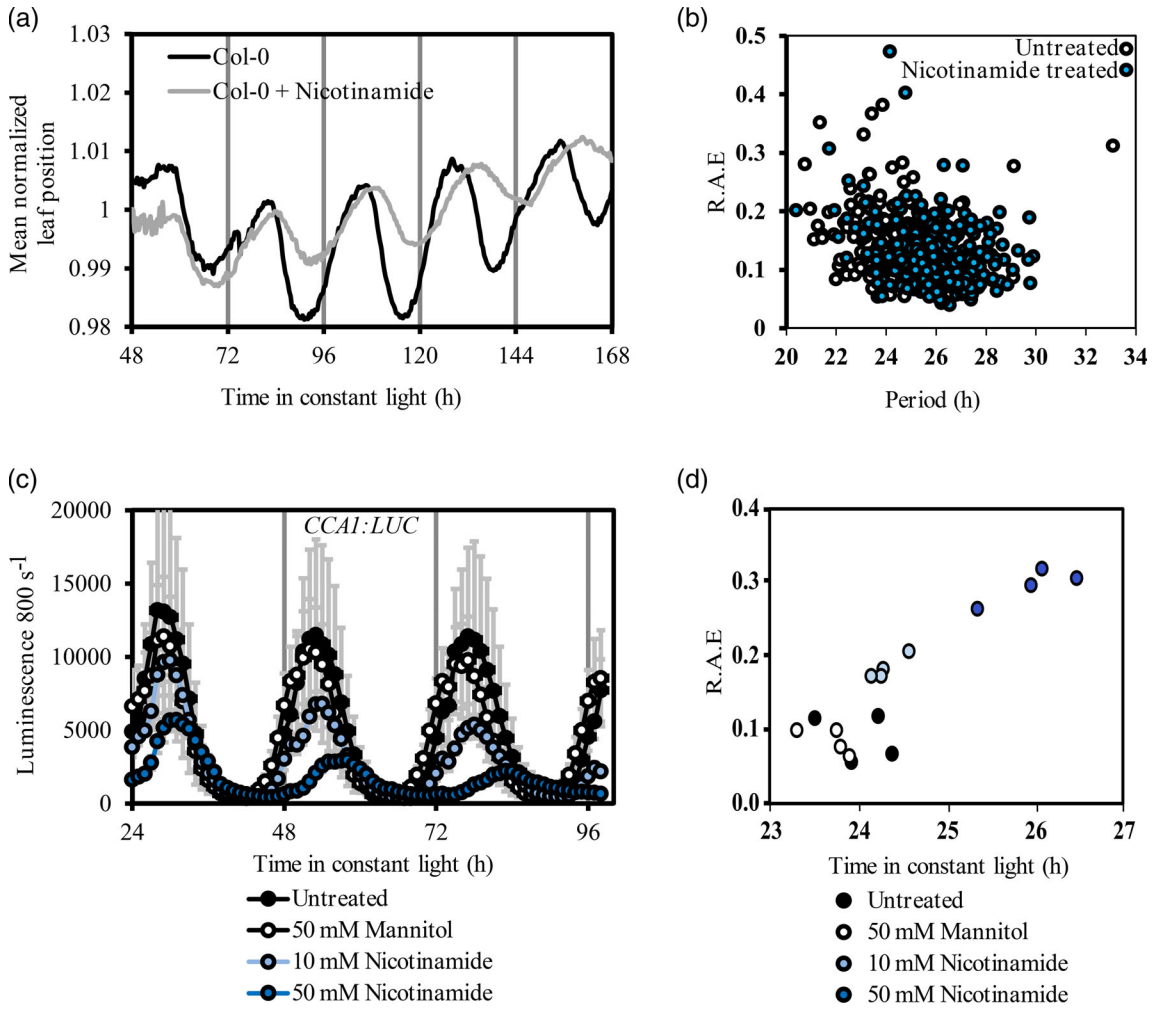


FIGURE 1 Nicotinamide lengthens the period of circadian rhythms. (a) Mean Y pixel positions of leaves of Col-0 in constant light (60–80 μmol m^{-1} s^{-1}) with or without two 50 mM nicotinamide treatments 1 and 2 days before imaging. (b) Individual FFT-NLLS period estimates and RAE values for circadian rhythms of leaf movement, data combined from separate experiments, untreated n_{rhy} = 218, nicotinamide treated n_{rhy} = 292 (c) Rhythms of *CCA1:LUC* luminescence in constant light. Seedling clusters were dosed with 50 μl 5 mM luciferin plus or minus nicotinamide every 12 hr, the points represent mean of n_{rhy} = 4 for each treatment. Error bars represent standard deviation. N_{rhy} = the number of rhythmic plants as estimated by FFT NLS (d) Individual FFT-NLLS period estimates and RAE values for *CCA1:LUC* luminescence, data combined from separate experiments. FFT-NLLS, fast-Fourier transform non-linear least squares; RAE, relative amplitude error

± 2.7 hr, Col-0 23.2 ± 0.7 hr, *parp2*-1 23.7 ± 1.0 hr, Col-0 23.8 ± 0.9 hr, *parp3-1* 23.9 ± 1.4 hr $p > .1$). We can exclude genetic redundancy between the three known PARP-encoding genes because the triple mutant was also without effect on leaf movement (Col-0 23.8 ± 1.5 hr, *parp1-2x2-1x3-1* 24.6 ± 1.9 hr, $p = .190$; Figure 3d) and DF (Col-0 23.5 ± 0.6 hr, *parp1-2x2-1x3-1* 24.0 ± 0.9 hr, $p = .029$; Figure S3d).

3.3 | Loss of *SRT1* function causes embryo lethality

To investigate if sirtuin activity contributes to the response of the circadian oscillator to nicotinamide, we identified four different alleles of *SRT1* (At5G55760) T-DNA insertion mutants (*srt1-1*;SALK_086287, *srt1-2*;SALK_001493, *srt1-3*;SALK_064336 and *srt1-4*;SAIL_552_E02; Figure 4a and Table S1). However, qRT-PCR, demonstrated that three of the lines had little effect on the abundance of *SRT1* transcripts compared to Col-0 (*srt1-1*, *srt1-2* and *srt1-3*; Figure 4b). For this reason, we proceeded only to investigate the T-DNA insertion mutant *srt1-4* (SAIL_552_E02) with the insert located 98 bp from the start of exon 5, which resulted in reduced expression of *SRT1* (Figure 4a,b).

We were unable to isolate homozygous T-DNA lines of *srt1-4* because it caused an embryo lethal phenotype, which was not present in heterozygotic mutants (Figure 4c). To confirm embryo lethality is due to the absence of *SRT1*, we generated complementation lines with a native promoter and the full-length *SRT1* gene. All of the complementation lines had normal embryo development, which along with

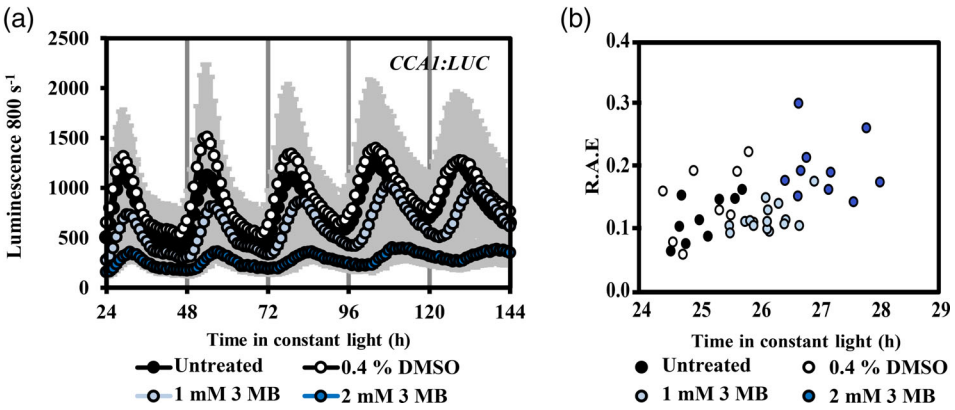


FIGURE 2 3 MB is a modifier of circadian clock period. (a) 3 MB treatment applied in the media lengthens the period of *CCA1:LUC* rhythms in Col-0 seeds in constant light. Untreated, DMSO n = 10, 1 mM 3 MB n = 15 and 2 mM 3 MB n = 10. (b) Individual FFT-NLLS period estimates and RAE values for *CCA1:LUC* luminescence, data combined from separate experiments. FFT-NLLS, fast-Fourier transform non-linear least squares; RAE, relative amplitude error

a lack of an embryo phenotype in heterozygotes suggests that it is loss of *SRT1* that affects embryo development, rather than a dominant negative effect of the T-DNA insertion (Figure 4c,d). Both *SRT1* and *SRT2* are highly expressed in siliques and flowers, which determine embryo development (Figure S4). Because full knock out of *SRT1* was embryo lethal, for the rest of the investigation we used heterozygous *srt1-4* plants, which were knockdowns, having less than half the expression of *SRT1* compared to the Col-0 background (Figure 4b).

We confirmed that the *srt2-1* (At5G09230; SALK_149295; Table S1) mutant (previously described in Wang et al., 2010) has an insert located 38 bp from the start of exon 2 and reduced expression of *SRT2* (Figure 4a,b).

3.4 | *Sirtuin* mutants do not have altered circadian rhythms

SRT1 and *2* transcript abundance was weakly rhythmic in 24 hr of constant light (Figure S1) but we found no evidence that sirtuins participate in the regulation of circadian period in Arabidopsis. Neither the *srt2-1* knock out, the *srt1-4 SRT1* heterozygous knock down or double mutants of those had any significant effect on the circadian period of leaf movement rhythms (Figure 3e–g, Col-0 24.1 ± 2.2 hr, *srt1-4 SRT1* 24.7 ± 2.4 hr, *srt2-1* 24.0 ± 1.6 hr; *srt1-4 SRT1 x 2–1* 24.2 ± 3.4 hr, $p > .3$). Similarly, knock down of *SRT1* and knockout of *SRT2* was without effect on circadian rhythms of delayed chlorophyll fluorescence (Figure S3e,f, Col-0 24.2 ± 3.1 hr, *srt1-4 hete* 24.4 ± 3.3 hr, *srt2-1* 24. ± 0.6 hr, $p > .4$).

3.5 | *parg1-1* is a long circadian period mutant

The lack of effect of *PARP* and *SRT* mutants on circadian period might be unexpected because the *tej-1* mutation of *PARG1*, which is predicted to encode a poly(ADP-ribose) glycohydrolase that counteracts PARP activity has been reported to increase the period of circadian rhythms in Arabidopsis (Panda et al., 2002). We, therefore, reinvestigated whether lesions in *PARG1* can affect circadian period. As reported previously, *tej-1* (a point mutation that results in a change of the glycine at position 262 to a glutamic acid) increases circadian period (Figure S5a, C24 26.8 ± 0.6 hr, *tej*-1 30.5 ± 2.64 hr, $p = .006$). Having confirmed the phenotype of the *tej-1* mutant, we next sought to confirm whether the phenotype of *tej-1* was due to a lesion in *PARG1*. We obtained a second allele of *PARG1* (At2g31870), we named as *parg1-1* (SALK_147805, NASC; Figure 5a). When transformed with the *CCA1:LUC* reporter, *parg1-1* mutants had a slightly longer circadian period of luminescence (Figure S5b, Col-0 24.5 ± 0.6 hr, *parg1*-1 24.8 ± 0.4 hr, $p = .021$). The mean period in circadian rhythms of leaf movement was 1–1.5 hr longer than wild type (Figure 5b, Col-0 24.3 ± 1.0 hr, *parg1-1* 25.4 ± 1.3 hr, $p < .001$). Similarly, circadian rhythms in DF were long period in the *parg1-1* mutant (Figure 5c, Col-0 23.4 ± 0.9 hr, *parg1-1* 25.0 ± 1.1 hr, $p = .011$). We also isolated insertion mutants of *PARG2* (At2g31865), *parg2-1* (GK072_B04, NASC), to determine if this close gene family member might also contribute to circadian regulation (Figure 5a). The *parg2-1* mutant by contrast had weak to no effect on circadian rhythms of leaf movement (Figure 5d, Col-0 24.5 ± 0.7 hr, *parg2-1* 24.8 ± 0.6 hr, $p = .209$) or delayed chlorophyll fluorescence (Figure 5e, Col-0 24.23 ± 3.06 hr, *parg2*-1 23.2 ± 0.8 hr, $p = .667$). Thus, the effects of the *PARG1* mutant are specific to that member of the gene family.

3.6 | Nicotinamide acts through a pathway independent of *PARP1*, *2* and *3*, *PARG2*, and *SIRTUIN 1* and *2*

We have previously proposed that nicotinamide regulates circadian period in Arabidopsis through inhibition of ADPR cyclase activity (Dodd et al., 2007). Here, we tested a counter hypothesis that nicotinamide regulates circadian clocks through the inhibition of PARP or

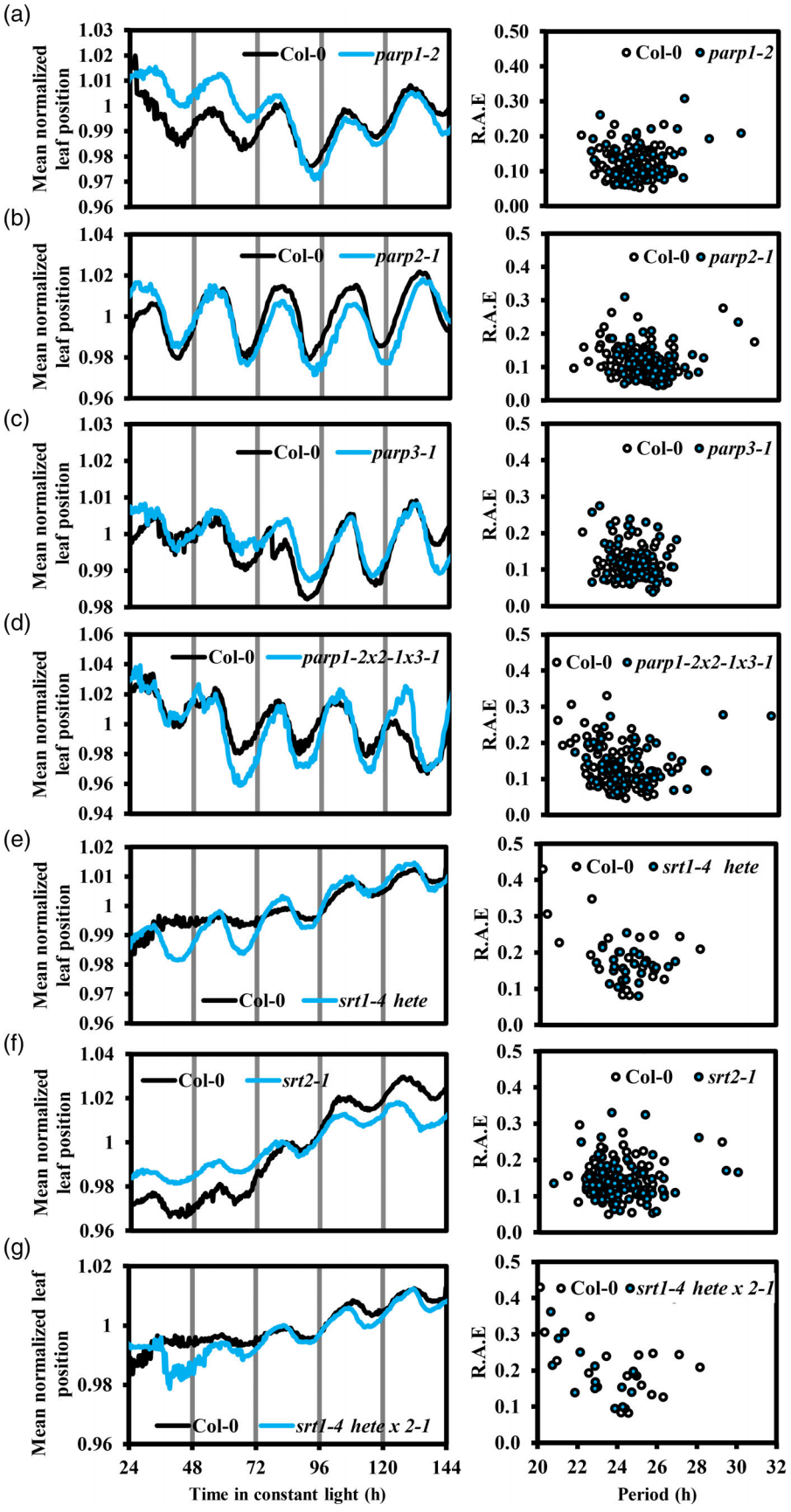


FIGURE 3 *PARP* and *SRT* mutations do not affect the circadian period of leaf movement. Circadian rhythms of leaf movement in constant light (LL) of *parp* and *sirtuin* mutants from a single representative experiment and individual FFT-NLLS period estimates and RAE values from combined separate experiments. Col-0 was used as same control for e and g. (a) Col-0 n_{rhy} = 87, *parp1-2* n_{rhy} = 75 (b) Col-0 n_{rhy} = 92, *parp2-1* n_{rhy} = 94 (c) Col-0 n_{rhy} = 50, *parp3-1* n_{rhy} = 53 (d) Col-0 n_{rhy} = 63, *parp1-2x2-1x3-1* n_{rhy} = 54 (e) Col-0 n_{rhy} = 18, *srt1-4 hete* n_{rhy} = 33 (f) Col-0 n_{rhy} = 76, *srt2-1* n_{rhy} = 105 (g) Col-0 n_{rhy} = 18, *srt1-4 hetex2-1* n_{rhy} = 19. Error bars represent standard deviation. N_{rhy} = the number of rhythmic plants as estimated by FFT NLS. FFT-NLLS, fast-Fourier transform non-linear least squares; RAE, relative amplitude error

SRT activity. We reasoned that in knock downs of *PARP* or *SRT* that nicotinamide would be less effective if its mode of action in the circadian system was associated with the activity of the encoded proteins. We found no evidence that PARPs or sirtuins are the target for

nicotinamide to affect circadian rhythms. The period of circadian rhythms of leaf movement in the *parp1-2*, *parp2-1* and *parp3-1* single mutants was longer in the presence of nicotinamide in comparison to untreated controls (Figure 6 a,b,c; circadian period estimates for each genotype minus and plus 50 mM nicotinamide follow: *parp1-2* 24.8 ± 2.1, 25.3 ± 2.4 hr, p = .002; *parp2-1* 25.0 ± 0.8 hr, 26.1 ± 1.1 hr, p = .007; *parp3-1* 24.5 ± 2.1 hr, 25.7 ± 0.8 hr, p = .001). Circadian rhythms of leaf movement in the *parp1-2x2-1x3-1* mutant also had a long period with the addition of nicotinamide (Figure 6d; *parp1-2x2-1x3-1* 25.5 ± 1.0 hr, *parp1-2x2-1x3-1* 50 mM nicotinamide 26.8 ± 1.2 hr, two-way ANOVA, $P_{(genotype)}$ = 0.146, $P_{(treatment)}$ < .001). We also confirmed the lack of interaction between PARPs and nicotinamide by examining the circadian regulation of transcript abundance (Figure 7). Nicotinamide treatment delayed the peak time of *CCA1*, *PRR7* and *TOC1* expression by 4 hr in both Col-0 and *parp1-2x2-1x3-1* with no difference between the genotypes (p > .2).

Sirtuins also are not required for the response to nicotinamide. *srt1-4 hete and srt2-1* mutants had a significantly longer period in the presence of nicotinamide (Figure 6e–g circadian period estimates for each genotype minus and plus 50 mM nicotinamide follow: *srt1-4 hete* 24.7 ± 0.9 hr, *srt1-4 hete* 25.7 ± 0.8 hr, two-way ANOVA, $P_{(genotype)}$ = 0.521, $P_{(treatment)}$ = 0.05; *srt2-1* 24.8 ± 1.3 hr, 25.5 ± 1.7 hr, two-way ANOVA, $P_{(genotype)}$ = 0.717, $P_{(treatment)}$ = 0.002; *srt1-4 hetex2-1* 24.2 ± 0.7 hr, 25.0 ± 0.5 hr, two-way ANOVA $P_{(genotype)}$ = 0.9, $P_{(treatment)}$ = 0.013).

We also examined the effects of nicotinamide in the PARG mutants; while these are not expected to be targets, there is a possibility of interaction because PARG reverses the effects of PARP. Nicotinamide treatment lengthens the period of circadian rhythms of leaf movement in *parg1-1* mutants (Figure 6h, *parg1-1* 25.37 ± 1.5 hr, *parg1-1* + nicotinamide 26.59 ± 1.2 hr, p < .001) and *parg2-1* (Figure 6i, *parg2-1* 25.00 ± 1.7 hr, *parg2-1* + nicotinamide 26.1 ± 1.1 hr, p < .001). Taken together, we found no evidence that nicotinamide regulates the circadian period of Arabidopsis through the action of PARPs, PARGs or sirtuins.

3.6.1 | PARP can affect FLC expression and flowering time in Arabidopsis Arabidopsis

Since we found no evidence that PARPs and SRTs are implicated in circadian regulation, we performed RNAseq to investigate their wider role. We performed these measurements under 12 hr L/12 hr D light

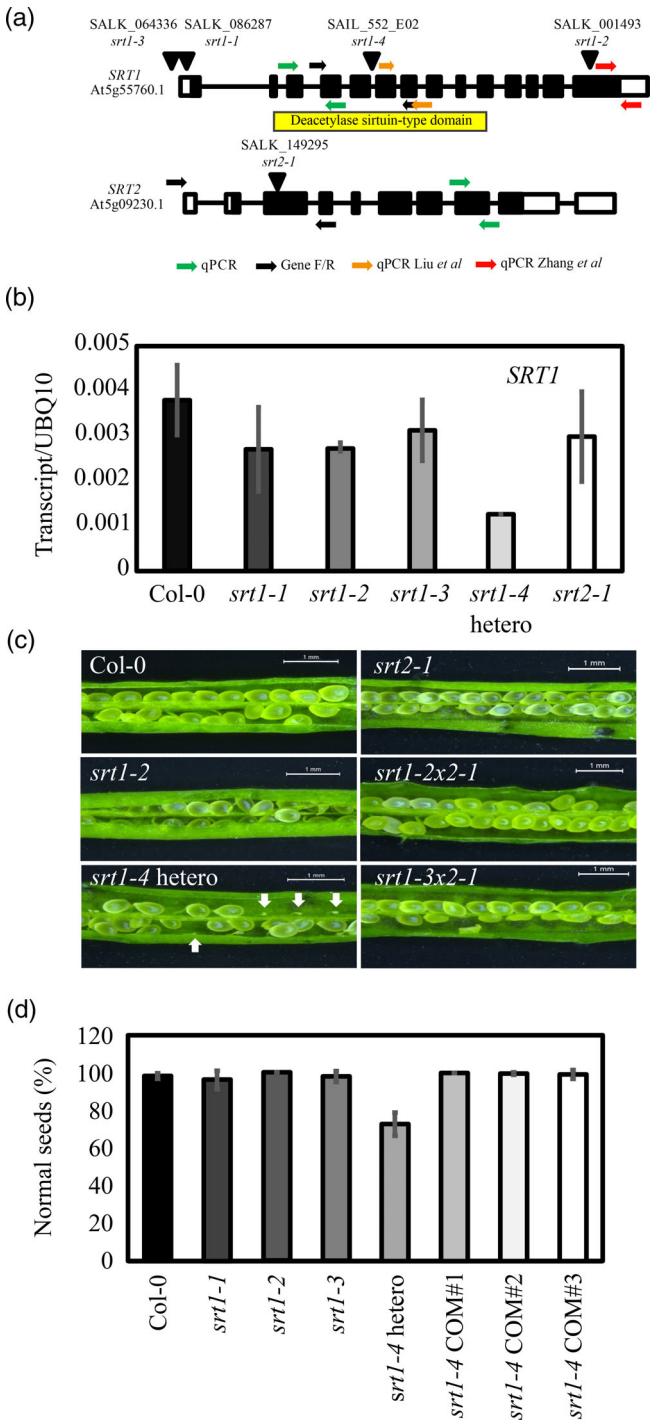


FIGURE 4 *srt1-4* insertion line has an embryo lethal phenotype. (a) Gene structures of *SRT1* and *SRT2*. Confirmed insertion sites are indicated. Positions of relevant primer pairs are indicated. Gene F/R (black arrows) = primers positioned to flank insertion site, qPCR (green arrows) = primers positioned downstream of insertion site. Orange arrows indicate qPCR primers used by Liu et al. (2017) and red arrows indicate qPCR primers used in the study of Zhang et al. (2018) (b) Relative gene expression of *SRT1* in *srt1-1*, *srt1-2*, *srt1-3*, *srt1-4 hete* and *srt2-1* measured by quantitative real-time polymerase chain reaction. N = 3 (c) Seeds of Arabidopsis *SRT1* mutant alleles and Col- 0 background. *srt1-4 heterozygous* mutant has aborted seeds (white arrows) that are not observed in Col-0 background or *srt1-2*, *srt2-1*, *srt1-2x2-1* and *srt1-3x2-1*. White bar indicates 1 mm. (d) Percentage seed set in *srt1* mutants and three *srt1-4* complementation lines (COM). (Col-0 n = 516, *srt1-1* n = 542, *srt1-2* n = 509, *srt1-3* n = 501, *srt1-4 hete* n = 322, *srt1-4* Complementation line #1 n = 602, *srt1-4* Complementation line #2 n = 609 and *srt1-4* Complementation line #3 n = 586)

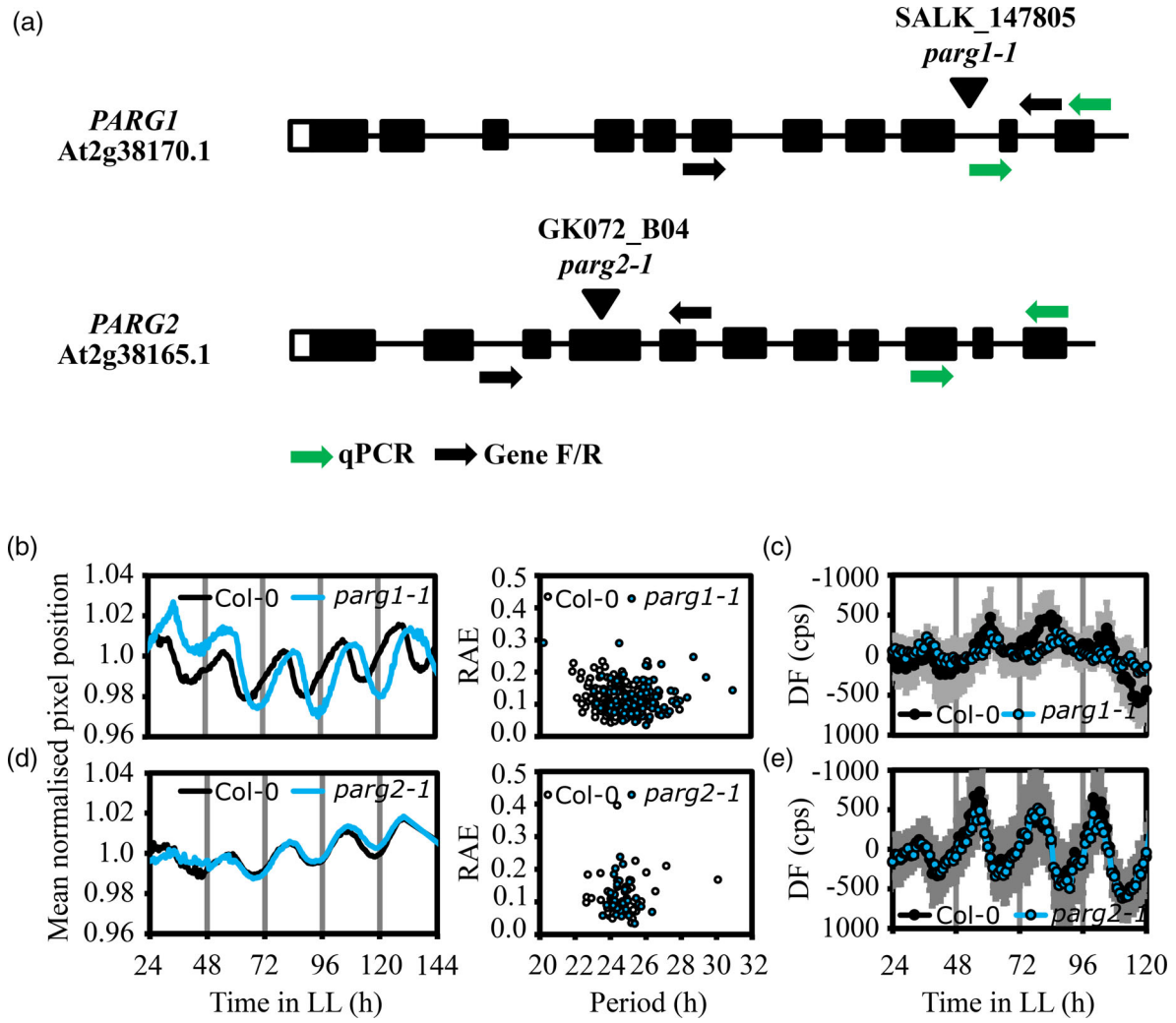
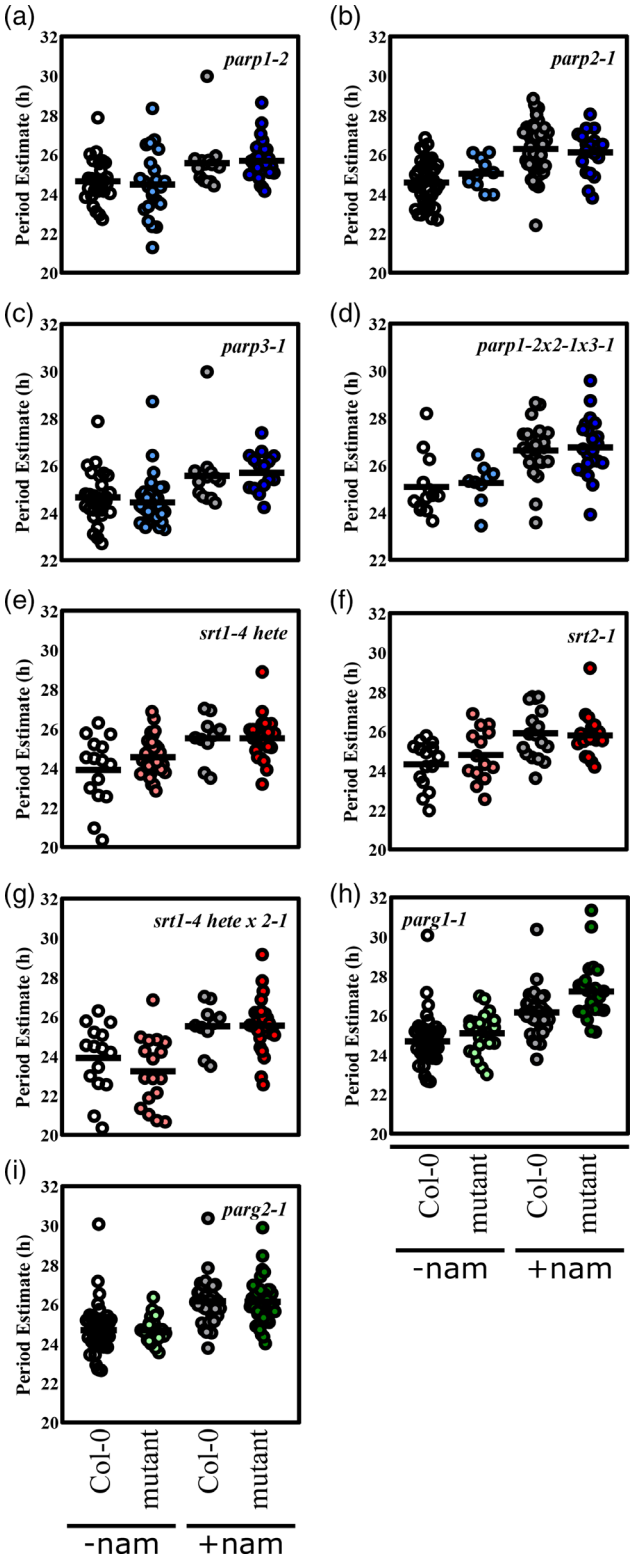


FIGURE 5 *parg 1–1* lengthens circadian period. (a) Gene structures and confirmed insertion sites of *PARG1*, with *parg1-1* (SALK_147805) allele, *PARG2* and *parg2*-1 (GK072_B04). Positions of relevant primer pairs are indicated. Gene F/R (black arrows) = primers positioned to flank insertion site, qPCR (green arrows) = primers positioned downstream of insertion site. (b–e) Circadian rhythms in constant light of leaf movement and delayed chlorophyll fluorescence in *parg1-1* (b, c), *parg2-1* (d, e) and Col-0 background. (b, d) Mean Y pixel positions of leaf movement from a single representative experiment and individual FFT-NLLS period estimates and RAE values. (c, e) Delayed chlorophyll fluorescence. Data are combined from separate experiments, (a) Col-0 n_{rhy} = 158, *parg1-1*n_{rhy} = 160 (b) Col-0 n_{rhy} = 10, *parg1-1*n_{rhy} = 9. (c) Col-0 n_{rhy} = 83, *parg2-1*n_{rhy} = 84 (d) Col-0 n_{rhy} = 12, *parg1-1*n_{rhy} = 11. Error bars represent standard deviation. N_{rhy} = the number of rhythmic plants as estimated by FFT NLS. FFT-NLLS, fast-Fourier transform non-linear least squares; RAE, relative amplitude error

cycles, sampling in the middle of the day (ZT 6) and night (ZT18) to capture temporal regulation of gene expression. In *parp* mutants, only 11 genes were mis-regulated in all the *parp* mutants in the day (number of mis-regulated genes: *parp1-2* day 27, night 57, *parp2-1* day 43, night 39, *parp3-1* day 79, night 44 and *parp1-2x2-1x3-1* day 227, night 41; Figure 8a,b and Table S4). To attempt to confirm RNAseq data, we performed qRT-PCR to measure the transcript abundance of several genes that were found to have similar changes in all the *parp* lines. Genes were chosen representing no change (*PRR7*), a decrease (*GP2*, *SUS4*), a moderate increase (*CCA1*) or a large increase (*AHA8*) in abundance. The independent qRT-PCR experiments were consistent with the effects measured by RNAseq (Figure S6).

We were surprised that so few genes were differentially expressed in the *PARP* mutants and, therefore, we tested for any physiological consequences of the change in expression of the few genes we detected. We focused on *FLC*, because this was significantly up-regulated in all *PARP* mutants in the day and night, except *parp3-1* at night where the transcript counts were higher but the FDR did not reach statistical significance (FDR = 0.14). It is *PARP1* and *2* that are expressed in vegetative tissue (Figure S1), which might explain why *parp3-1* had less effect on *FLC* abundance than mutations in *PARP1* and *2*. Firstly, we confirmed that *FLC* is differentially expressed in *PARP* mutants by qRT-PCR of independent samples of the mutant plants (Figure 8c,d). These data are strongly indicative that PARP activity can regulate *FLC* gene expression. We found that the effects

on *FLC* might be meaningful because the elevation of the flowering suppressor was associated with a delay in the days to flowering and an increase of the number of leaves at flowering in the *parp* mutants and this was specific to short days (Figure 8e,f). The flowering phenotype of the *parp* mutants is, therefore, consistent with the molecular phenotype as identified by the RNAseq, supporting the conclusion that PARP activity is not a major regulator of gene expression, at least in stress-free conditions, but can affect *FLC* expression and flowering time.



3.7 | Sirtuin 1 affects gene expression during the day

In contrast to the PARPs, alterations to sirtuin expression had a profound effect on the transcriptome. In *srt1-4* heterozygous plants, 6,490 genes were differentially expressed in the day (sampling at ZT 6) compared to Col-0 (Figure 9a and Table S5). This effect was mostly specific to the day, because at night the transcripts of only 49 genes were differently expressed (sampling at ZT 18; Figure 9a and Table S5). Our results suggest that the sirtuin 1 might have a very important role in daytime gene expression. To further investigate this hypothesis, we compared our transcription profiles with publicly available light signalling- and sucrose-related transcriptomes (white light, red light, UV-B and sucrose; Table S3) and a control transcriptome unrelated to light signalling (heat and salt) downloaded from the EBI (https://www.ebi.ac.uk/gxa/). There were highly significant overlaps between *SRT1*-regulated transcript sets and those regulated by light and sucrose, with *SRT1* expression affecting the gene expression in the opposite direction to light and sucrose signals (Figure 9b, Tables S6 and S7). A wide range of GO terms were associated with day and *SRT1* regulation many of which can be associated with light signalling and growth (Table S7). These data indicated that *SRT1* might positively regulate transcripts activated in the day by light signalling and light induction of sugar production by photosynthesis. In contrast, *srt2-1* had a relatively small effect on gene expression, with only 235 transcripts at day and 97 at night being differentially expressed

FIGURE 6 Nicotinamide increases the period of circadian rhythms of leaf movement in *parp*, *srt* and *parg* mutants. Circadian period of leaf movement in constant light in Col-0 and mutant lines with and without dosing with 50 mM nicotinamide (nam) 1 and 2 days before the start of imaging. Col-0 background minus (white) or plus nicotinamide (grey). *PARP* mutants are blue, sirtuin mutants are red and *PARG* mutants are green. Within each plot the darker colour shade represents the mutant line treated with nicotinamide. All data are shown with the mean indicated by a line. (a) Col-0 n_{rhy} = 25, *parp1-2* n_{rhy} = 22, Col-0 + nic n_{rhy} = 12, *parp1-2* + nic n_{rhy} = 27 (b) Col-0 n_{rhy} = 22, *parp2-1* n_{rhy} = 11, Col-0 + nic n_{rhy} = 25, *parp1-2* + nic n_{rhy} = 20 (c) Col-0 (as for (a)) *parp3-1* n_{rhy} = 27, *parp3-1* + nic n_{rhy} = 14 (d) Col-0 n_{rhy} = 16, *parp1-2x2-1x3-1* n_{rhy} = 9, Col-0 + nic n_{rhy} = 23, *parp1-2x2-1x3-1* + nic n_{rhy} = 22 (e) Col-0 n_{rhy} = 12, *srt1-4 hete* n_{rhy} = 33, Col-0 + nic n_{rhy} = 10, *srt1-4 hete* + nic n_{rhy} = 21 (f) Col-0 n_{rhy} = 15, *srt2-1* n_{rhy} = 14, Col-0 + nic n_{rhy} = 17, *srt2-1* + nic n_{rhy} = 18 (g) Col-0 (as for (e)), *srt1-4 hete* n_{rhy} = 19, *srt1-4 hete* + nic n_{rhy} = 29 (h) *parg2-1* n_{rhy} = 31, *parg2-1* + nic n_{rhy} = 41 (i) Col-0 (as for (h)), Col-0 n_{rhy} = 48, *parg1-1* n_{rhy} = 22, Col-0 + nic n_{rhy} = 36, *parg1-1* + nic n_{rhy} = 21. N_{rhy} = the number of rhythmic plants as estimated by FFT NLS

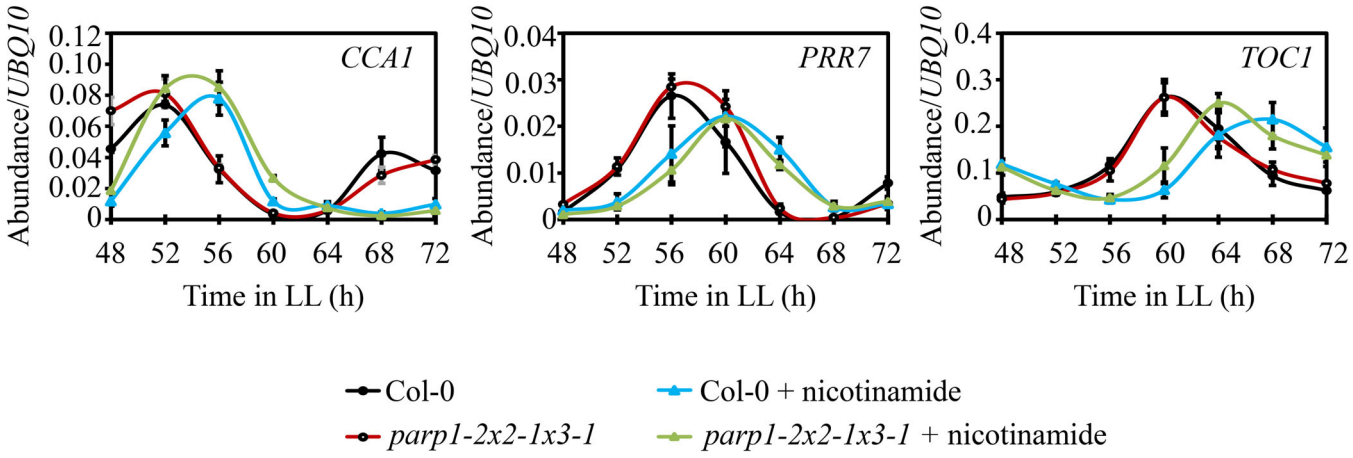


FIGURE 7 Circadian rhythms of circadian oscillator gene expression in *parp1-2x2-1x3-1* mutants are delayed in the presence of nicotinamide. Plants were entrained for 9 days in 12L/12D before transfer into constant white light. On day seven, plants were transferred onto treatment media (10 mM nicotinamide or untreated control). Tissue samples (three biological replicates) were harvested every 4 hr in the third cycle of constant light. Abundance measured using quantitative real-time polymerase chain reaction was normalized to *UBQ10*. *CCA1*, *TOC1* and *PRR7* peak abundance is delayed by nicotinamide treatment in both genotypes. Error bars represent standard deviation

(Figure 9a and Table S5). The dramatic effect of reduced *SRT1* expression on transcript abundance was confirmed by analysis in *srt1-4 hete x srt2-1*, which had an overlap of 4,143 differentially expressed transcripts with the *srt1-4 hete* single mutant (Figure 9a).

To confirm the results of the RNAseq, we performed the experiment again three times with new biological material for each experiment and a selected subset of genes to be measured by qRT-PCR to compare to the findings of the RNAseq. *At2G36970* (UDP-Glycosyltransferase superfamily protein) was selected because it has a large decrease in abundance in RNAseq in the mutant. *AT5G55580* (Mitochondrial transcription termination factor family protein) and *AT5G55540* were selected to represent transcripts that had smaller changes in abundance. *AT5G62720* (tornado 1), *AT4G11830* (phospholipase D gamma 2) and *AT2G15880* (Leucine-rich repeat [LRR] family protein) were selected representing large increases in abundance in *srt1-4 hete* in the RNAseq data (Table S5 and Figure S7). The qRT-PCR independently confirmed the RNAseq experiment findings about the effects on gene expression during the day, showing that *AT2G36970*, *At5G55540* and *AT5G55580* had reduced expression and *AT5G62720*, *AT4G11830* and *AT2G15880* were increased in expression in the *srt1-4 hete* mutant during the day (Figure S7a,b). Similarly, the qRT-PCR confirmed the findings of the effects on gene expression that we measured at night with RNAseq, demonstrating reduced expression of *AT2G36970*, *AT5G55580*, *At5G55540*, *AT5G62720* and increased expression at night of *AT4G11830* and *AT2G15880* in *srt1-4 hete* (Figure S7c,d).

We also examined the abundance of these transcripts by qRT-PCR in *SRT1* artificial microRNA lines, which also knocked down *SRT1* expression (Figure S8a). We generated nine independent microRNA lines of which lines 5 and 10 had the greatest effect on *SRT1* transcript abundance, similar to *SRT1* abundance in *srt1-4 hete* (Figure S8a). Possibly the embryo lethal effects of complete *SRT1* knock out explain why we did not recover lines with greater reduction in *SRT1* than that seen in *srt1-4 hete*. All the transcripts had similar patterns of abundance between *srt1-4 hete* and *SRT* microRNA lines in the day, though in line with their reduced effect on *SRT1* expression compared to *srt1-4 hete*, the microRNA lines had less of an effect on the abundance of the transcripts (Figure S7a). The trend of similar but reduced effects of the microRNA lines compared to *srt1-4 hete* was also found at night (Figure S7c). For *AT2G36970*, *AT5G55580*, *AT5G62720* expression was reduced in *srt1-4 hete* and in the microRNA lines, and for *AT4G11830* and *AT2G15880* expression was increased in *srt1-4 hete* and the microRNA lines (Figure S7c). The exception being *AT5G5540*, for which at night there was little effect of the microRNA lines, but a strong effect of *srt1-4 hete*. Thus, in independent experiments, we confirmed that *srt1-4 hete* and micro RNA lines had similar effects on transcript abundance and the degree of effect was in line with the abundance of the *SRT1* transcript. This demonstrates that the effects on the transcriptome are due to changes in *SRT1* expression.

To investigate further the regulation of gene expression by *SRT1* in the light, we performed qRT-PCR to measure light signalling gene expression in RNA extracted from *SRT1* artificial microRNA lines and *srt1-1*, *srt1-2*, *srt1-3*, *srt1-4 hete* and *srt2-1* mutants (Figure S8a). We chose several light signalling related genes (*AT2G37678*, *FHY1 far-red elongated hypocotyl 1*, *AT5G02200 FHL far-red elongated hypocotyl 1 like*, *AT2G42870 PAR1 phy rapidly regulated 1*, *AT1G70290 TPS8 trehalose-6-phosphatase synthase 8*). There was good correspondence between the effects of *srt1-4 hete* and *SRT1* microRNA lines on the expression of these light-regulated signalling transcripts (Figure S8b). *srt1-4 hete* and microRNA lines increased the expression of all of the selected light signalling genes confirming that *SRT1* affects their abundance (Figure S8b), as had been found in the RNAseq (Figure S8c).

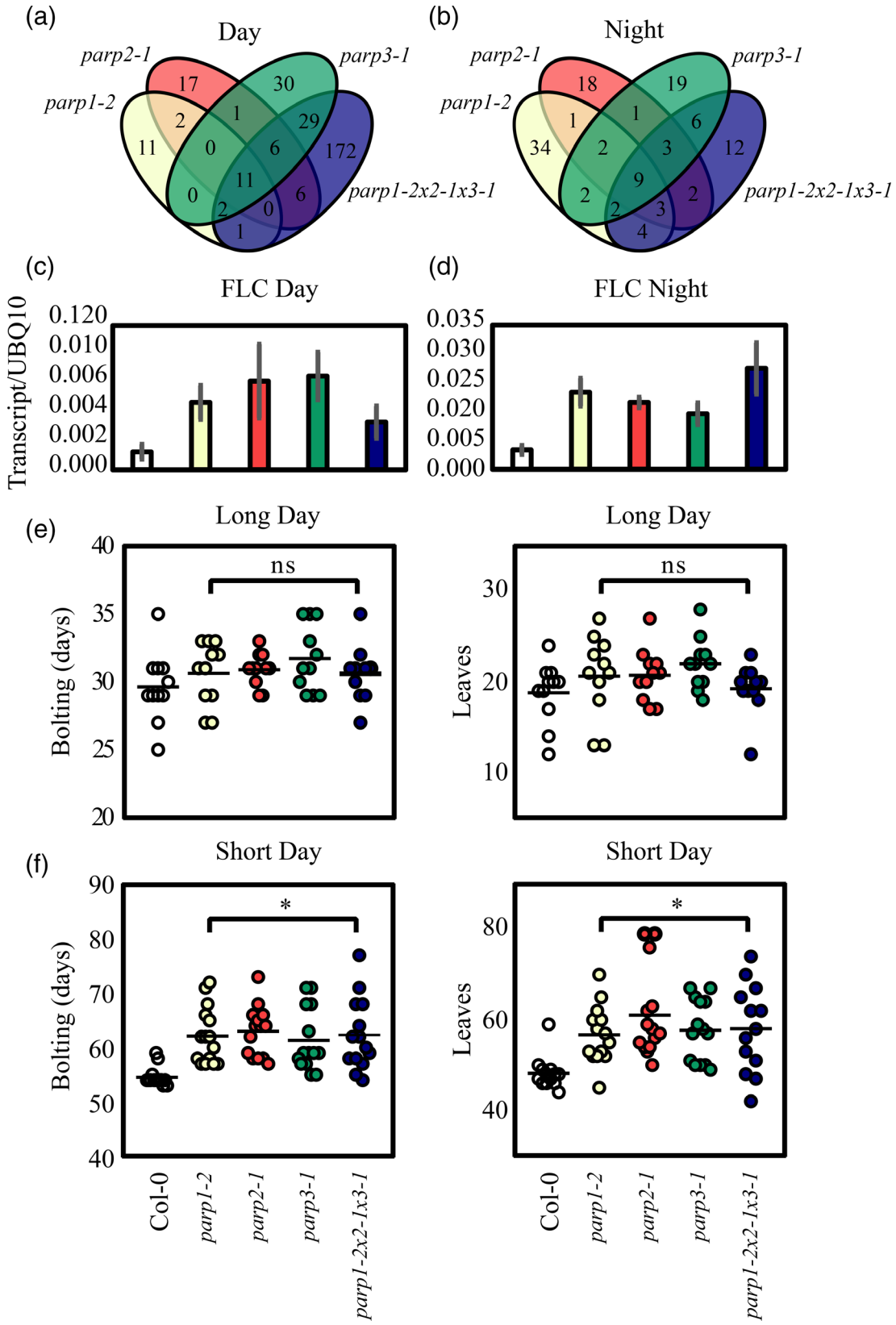


FIGURE 8 Legend on next page.

4 | DISCUSSION

Understanding of the mechanisms that regulate circadian timing has been enhanced greatly in the past few decades with the discovery of post-transcriptional and post-translational modifications, chromatin remodelling and cytosolic events. We have been investigating how cellular metabolism, an important output of the circadian clock, might reciprocally communicate with the circadian clock to modify circadian

clock behaviour. To this end, we screened a group of NAD^+-related mutants for circadian phenotypes. This study was motivated by the extensive literature concerning the function of the PARPs and SRTs in regulating the circadian clock of mammals and the effect of nicotinamide being common between the plant and mammalian systems (Asher et al., 2010; Dodd et al., 2007; Nakahata et al., 2008; Nakahata, Sahar, Astarita, Kaluzova, & Sassone-Corsi, 2009).

We found no evidence that mutation of the *PARPs* or *SRTs* affected circadian period, the response to nicotinamide or affected specifically the expression of circadian oscillator genes. Our finding that the effects of nicotinamide on the circadian oscillator of plants are not associated with inhibition of the PARP and SRT NADases means that the mechanisms by which NAD regulates the circadian clock of Arabidopsis might be different to that which occur in mammals (Asher et al., 2010; Dodd et al., 2007; Nakahata et al., 2008, 2009). We cannot exclude the possibility that unidentified members of the *PARP* and *SRT* gene families could be targets for nicotinamide. Based on these findings and our previous data, we favour alternative explanations for the effects of nicotinamide on the circadian oscillator.

Nicotinamide is an inhibitor also of ADPR cyclase, which generates the Ca^{2+} agonist, cADPR (Abdul-Awal et al., 2016), and we have measured circadian oscillations of cADPR in Arabidopsis (Dodd et al., 2007). Furthermore, we reported that Ca^{2+} affects circadian period through an interaction with *TOC1* by CALMODULIN-LIKE 24 (CML24; Martí Ruiz et al., 2018). Ca^{2+} is sensed in the circadian oscillator by CML24 and the effects of mutations in *CML24* on circadian period are not additive to the effects of nicotinamide, which is consistent with the effect of nicotinamide being to abolish the Ca^{2+} signal through inhibition of ADPR cyclase activity (Martí Ruiz et al., 2018). However, nicotinamide has a greater effect on circadian period than mutation of *CML24* alone, suggesting that either there are other Ca^{2+} sensors in the circadian system or nicotinamide has other targets, such as reduction of H3K4me3 accumulation (Malapeira et al., 2012) and the action of BIG (Hearn et al., 2018). TOR seems to be required for the response of the circadian oscillator to nicotinamide, and it has been proposed this might be due to altered energy production by the mitochondria (Zhang et al., 2019). The mitochondria are affected by and regulate Ca^{2+} dynamics and therefore a dual role for TOR and Ca^{2+} signalling in regulation of the circadian oscillator due to mitochondrial responses to nicotinamide is plausible (Bravo-Sagua et al., 2017).

We also found a PARP inhibitor 3 MB increases circadian period similarly to nicotinamide (Figure 2). However, 3 MB is a structural analogue of nicotinamide, and therefore may also target enzymes other than PARP, which bind NAD^+ or nicotinamide. In contrast, the use of another chemical inhibitor of PARP activity, thymidine, did not affect the period length or amplitude of circadian rhythms of *CCA1:LUC* bioluminescence (Malapeira et al., 2012). Overall, based on these observations, there is no strong evidence for the role of PARPs in mediating the effect of nicotinamide on clock function in Arabidopsis and the specificity of the drugs used is questionable, as different drugs targeting PARPs have conflicting effects on circadian period. While PARPs do not affect the circadian oscillator, we found evidence that they affect seasonal timing through the regulation of *FLC* expression and flowering time.

The lack of a role for the PARPs is at first sight surprising given the reproducible role of *PARG1* in setting circadian period (Figure 5). Panda et al. (2002) predicted that *parp* mutants would have reduced circadian period due to the period reducing effect of the PARP inhibitor 3 AB (3-aminobenzamide); however, our data indicate that the chemical inhibition of PARP may have multiple effects and may act independently of PARP. The mechanism by which PARG activity affects circadian period is unknown. PARG activity is unlikely to affect circadian period through alterations in gene expression because the *tej* mutant did not affect the expression of oscillator genes sufficiently to account for the ~2 h change in period (Panda et al., 2002). It has been assumed that the effect of *tej* must be mediated through effects on PARP, possibly causing PARP to be perpetually automodified due to lack of counteracting PARG activity. Automodification of PARP was proposed due to the high luciferase activity in *tej* mutants (Panda et al., 2002). However, the lack of an effect of the PARP mutants on circadian period might suggest an alternative explanation independent of the function of PARPs. It is possible that other proteins encoded in the Arabidopsis genome, which have ADP-ribosyl transferase activity, might be responsible for counteracting PARGs. For example, the SRO (SIMILAR TO RCD-ONE) family of plant-specific proteins have the conserved PARP catalytic domain, however, these have been found

FIGURE 8 *FLC* is mis-expressed in *parp* mutants, which have delayed flowering in short day photoperiods. (a, b) Venn diagrams of the number of transcripts differentially expressed (FDR < 0.05) as measured by RNAseq in *parp1-2*, *parp2-1*, *parp3-1* and *parp1-2x2-1x3-1* mutants compared to Col-0 background in (a) day at ZT6 and (b) night at ZT18. (c,d) Transcript abundance of *FLC* measured by quantitative real-time polymerase chain reaction (qRT-PCR) in Col-0, *parp1-2*, *parp2-1*, *parp3-1* and *parp1-2x2-1x3-1* in the (c) day and (d) night. qRT-PCR normalised to *UBQ10*. Seedlings were grown under 12 hr L/12 hr D in white light on 1/2 MS agar plates for 2 weeks and sampled during the day (ZT6) and night (ZT18). Growth, harvesting, RNA extraction and qRT-PCR analysis for *FLC* were performed independently of the RNAseq experiments. Error bars represent the standard deviation. $n = 3$. (e, f) Flowering time as measured by the emergence of bolt and numbers of rosette leaves at bolting of Col-0, and *parp1-2*, *parp2-1*, *parp3-1*, *parp1-2x2-1x3-1* under (e) long (16 hr L:8 hr D) or (f) short day (8 hr L:16 hr D). The number of rosette leaves and bolting time were recorded when the emerging bolt was 5 mm high. Dots represent the individual plants and the black horizontal bars the mean. In long photoperiods $n = 12$ for each genotype and in short photoperiods $n = 15$ for each genotype. The Kruskal–Wallis one-way analysis of variance was used to determine if there was any overall difference among the five genotypes. Dunn's method was then used to test the significance of pairwise comparisons between Col-0 and each mutant. Single asterisks indicate $p \leq .05$. Col-0 (white) *parp1-2* (yellow), *parp2-1* (red), *parp3-1* (green) and *parp1-2x2-1x3-1* (blue)

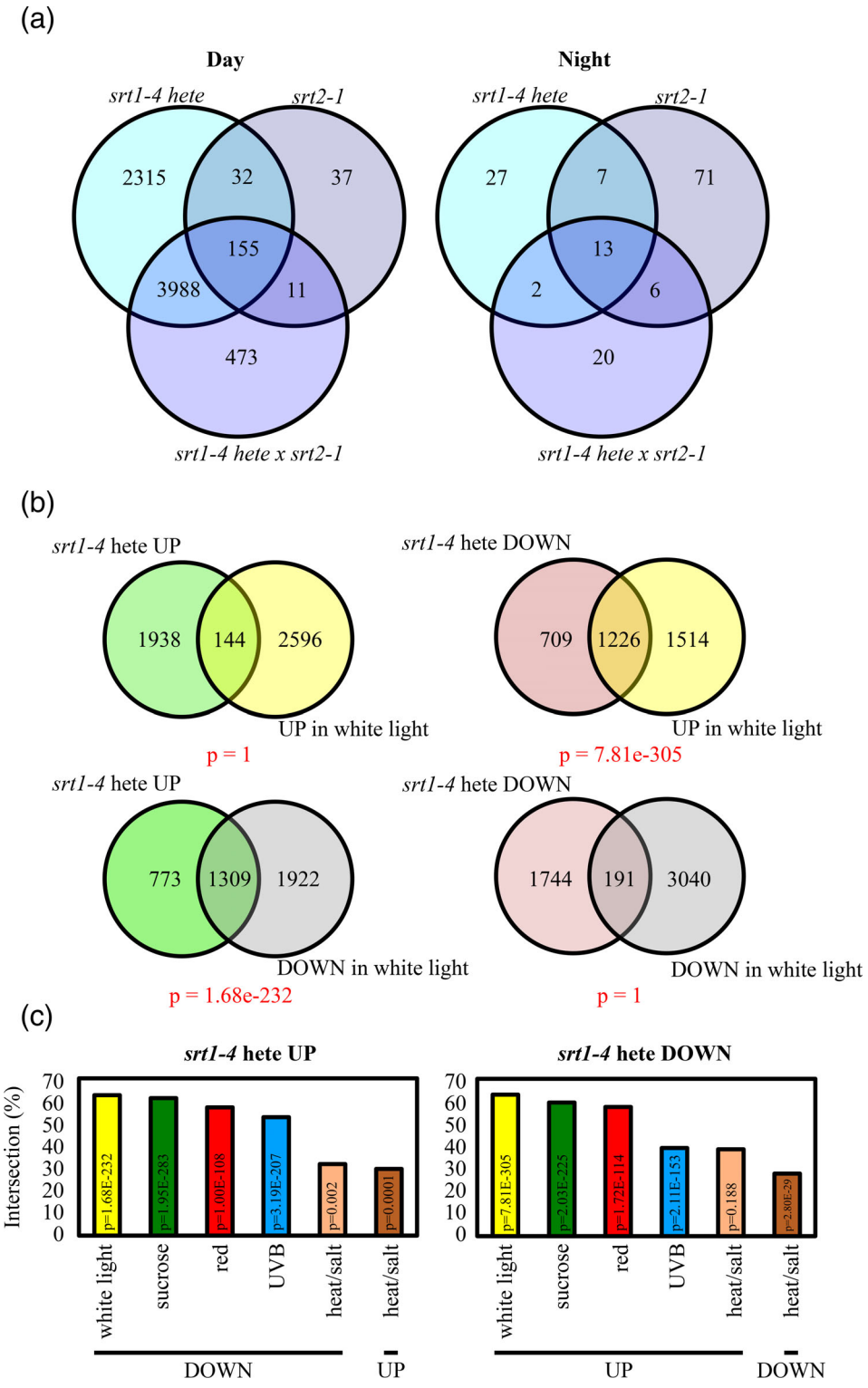


FIGURE 9 *srt1-4 hete* affects the accumulation of transcripts regulated in the day. Reduced expression of *SRT1* in a *srt1-4* heterozygous mutant has a profound effect on the abundance of transcripts in the day. (a) Venn diagram showing the number of differentially expressed genes (FDR < 0.05) in *srt1-4 hete*, *srt2-1* and *srt1-4 hete x 2–1* mutants compared to Col-0 background in the day and the night as measured by RNAseq. Lists of differently expressed genes are given in Table S5. (b) Venn diagrams showing the overlap between lists of up- and down-regulated genes in the *srt1-4 hete* mutant at ZT6 and in the 'white light' transcriptome from the European Bioinformatics Institute Gene Expression Atlas (Table S3). There is a high degree of correspondence between transcripts up-regulated in *srt1-4 hete* in the day and transcripts that are down-regulated in a white light treatment. Similarly, transcripts that are up-regulated by white light are often in lower abundance in the mutant. (c) The number of differentially expressed genes is common between the *srt1*-4 heterozygous mutants and plants treated with treatments of white, red light and UV-A light, 1% sucrose or combined heat and salt stress, expressed as a percentage of the total number of differentially expressed genes in *srt1-4* heterozygous mutants (see methods and Table S3 for the data sets used). Significance levels for interactions are shown in the bars. The differentially expressed genes in the *srt1-4* heterozygous mutants are listed in Table S5

not to possess ADP-ribosyl transferase activity (Jaspers et al., 2010; Wirthmueller et al., 2018).

We also found no evidence for the role of sirtuins in modulating clock behaviour in Arabidopsis. Neither *srt* single nor double mutants had significantly different circadian periods to wild type in assays of leaf movement or DF (Figure 3 and Figure S3). This suggests that unlike mammals, sirtuins are not regulators of the circadian oscillator in Arabidopsis. Sirtuins are intimately associated with central circadian clock components in mice (Asher et al., 2008; Chang & Guarente, 2013; Nakahata et al., 2008, 2009). In mammals, there is significant evidence that aspects of nicotinamide effects might be mediated through sirtuins, due to the long period of rhythms in locomotor activity seen in brain-specific *sirt1* mutants (Chang & Guarente, 2013). Furthermore, resveratrol, an activator of SIRT1

(Lagouge et al., 2006), causes a shortened period of circadian rhythms in locomotor activity of grey mouse lemur (Das, Mukherjee, & Ray, 2010; Pifferi, Dal-Pan, Menaker, & Aujard, 2011) and increased circadian clock gene expression in Rat-1 fibroblast cells (Oike & Kobori, 2008). In Arabidopsis, acetylation has been established as a regulatory mechanism controlling circadian clock gene expression (Farinas & Mas, 2011; Malapeira et al., 2012; Song & Noh, 2012) and flowering time by modifying chromatin acetylation at the *FLC* locus (Ausin, Alonso-Blanco, Jarillo, Ruiz-Garcia, & Martinez-Zapater, 2004; He, Michaels, & Amasino, 2003; Kim et al., 2004; Xiao et al., 2013). As acetylation is evidently involved within the circadian clock in Arabidopsis at a transcriptional level (Farinas & Mas, 2011; Malapeira et al., 2012; Perales & Mas, 2007), other non-NAD^+-dependent deacetylases must be responsible. A corepressor protein TOPLESS (TPL) has been found to interact with *PRR7*, *9* and *5* at the promoter regions of *CCA1* and *LHY* to repress transcription (Wang, Kim, & Somers, 2013). This was found to require histone deacetylase activity; treatment with Trichostatin A (TSA) disrupts this repression and histone deacetylase 6 (HDA6) forms a complex with TPL and PRR9 in vitro (Wang et al., 2013).

Further evidence that the effect of nicotinamide is independent of sirtuin-like activity is provided by the finding that H3K56ac was decreased in nicotinamide treated plants, opposite to the expectation if deacetylation is a nicotinamide-sensitive activity (Malapeira et al., 2012). C646, an acetylase inhibitor, phenocopies the effects of nicotinamide also suggesting that sirtuin-like deacetylase activity is not a regulator of the Arabidopsis circadian oscillator (Malapeira et al., 2012). Interestingly, nicotinamide treatment increases histone acetylation at the *VIN3* locus and induces *FLC* repression and flowering (Bond, Dennis, Pogson, & Finnegan, 2009). However, *VIN3* expression was not altered in sirtuin mutants, which suggested the effect of nicotinamide on *VIN3* expression also was not mediated by sirtuins.

We found profound effects of *SRT1* on transcript abundance only during the day (Figure 9). Knockdown of *srt1* affected the abundance of nearly one-third of the transcriptome in the day but not at night. The transcripts affected were strongly associated with light and sugar signalling pathways and less so with those associated with heat and salt stress (Figure 9). This was confirmed in microRNA lines and by using qRT-PCR in independent experiments. Our finding that *SRT1* can have such profound effects on the transcriptome in the day and that complete knock out of *SRT1* is embryo lethal has not been reported in previous studies, possibly because other investigations have focused on lines with less strong effects on *SRT1* expression.

Liu et al. (2017) found that the T-DNA insertion mutant line *srt1-2* (SALK_001493) has weak effects on *SRT1* expression and that RNAi lines had stronger effects on *SRT1* expression and metabolism. From RNAi and overexpression studies, Liu et al. (2017) concluded that SRT1 interacts with Arabidopsis cMyc-Binding Protein 1 (AtMBP-1), which is a transcriptional repressor to regulate AtMBP-1 targets resulting in altered gene expression and metabolism. We also found that *srt1-2* has weak or no effects on *SRT1* expression, and that RNAi was more effective in reducing *SRT1* transcript abundance (Figure 4b; Figure S8a). Zhang, Wang, Ko, Shao, and Qiao (2018) reported that *srt1-1* and *srt1-2* completely abolished *SRT1* expression, resulting in phenotypes associated with ethylene responses. The qPCR primers used by Zhang et al. (2018) were down stream of the *srt1-2* T-DNA insert representing the end of the CDS, whereas we used primers upstream of the *srt1-4* insertion site in the deacetylase domain and Liu et al. (2017) used primers in the same domain downstream of the location of the *srt1-4* insert (Figure 4a). Possibly the location of the *srt1-1*, *srt1-2* and *srt1-3* at the extreme 5′ and 3′ ends of the gene accounts for their limited effect on transcript abundance. The location of *srt1-4* in the middle of the gene might account for its greater effect on expression and therefore phenotype. It is clear that the *srt1-1* and *srt1-2* mutants make transcripts encoding an intact deacetylase domain, which might explain why in our hands and those of Liu et al. (2017), the *srt1-1* and *srt1-2* mutants had weak or no phenotypes.

Furthermore, we found that complete knock out of *SRT1* by insertion of a T-DNA in the acetylase domain in the *srt1-4* mutants was embryo lethal, which was not reported by Zhang et al. (2018) for *srt1-1* and *srt1-2*, which suggests the lines used in the study of Zhang et al. (2018) were not abolished in *SRT1* function. Based on our findings that *srt1-4* homozygous plants are embryo lethal and that *srt1-4* heterozygous plants have lower expression of *SRT1* than RNAi lines and other T-DNA alleles, we conclude that the full extent of the effects of *SRT1* have been obscured previously by investigation in lines that have little or no effect on the expression of the *SRT1* deacetylase domain (*srt1-1*, *srt1-2*) and possibly weaker RNAi knock down lines. The profound regulation of gene expression we find in *srt1-4* heterozygous lines, and the associated embryo lethal effects of the *srt1-4* homozygous lines, is associated with the loss of transcripts encoding the deacetylase domain.

The strong correspondence between the transcripts misregulated in plants with reduced *SRT1* and those regulated by light and sugars (Figure 9) and the opposite direction of the transcript regulation by *srt1-4* to the regulation by light signals (Figure 9) could suggest that *SRT1* participates in the regulation of transcripts by pathways activated by light signalling and the light regulation of photosynthesis. Our data suggest that SRT1 is required for the correct regulation of gene expression during the day but independent of a function in the circadian oscillator.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Jun Hyeok Kim, Laura J. Bell, Xiao Wang, Rinuckshi Wimalasekera: Performed the experimentation. **Hugo P. Bastos, Krystyna A. Kelly:**

Performed the bioinformatics. **Matthew A. Hannah, Alex A. R. Webb:** Conceived the research and obtained funding. **Jun Hyeok Kim, Laura J. Bell, Alex A. R. Webb:** Wrote the paper.

DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

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

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