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BIG regulates dynamic adjustment of circadian period in *Arabidopsis thaliana*

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Short Title:

BIG dynamically adjusts the circadian period

One-sentence summary:

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BIG contributes to the dynamic adjustment of the circadian period to establish the correct phase of daily rhythms in Arabidopsis.

Author contributions

TH, MJH, MAH and AW devised the study. TH, AA, CS, RM and MM conducted the experiments. TH, MM, FLT, and AW wrote the manuscript.

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Abstract

Circadian clocks drive rhythms with a period near 24 hours, but the molecular basis of the circadian period's regulation is poorly understood. We previously demonstrated that metabolites affect the free-running period of the circadian oscillator of *Arabidopsis thaliana*, with endogenous sugars acting as an accelerator and exogenous nicotinamide acting as a brake. Changes in circadian oscillator period are thought to adjust the timing of biological activities through the process of entrainment, in which the circadian oscillator becomes synchronised to rhythmic signals such as light and dark cycles, as well as changes in internal metabolism. To identify molecular components associated with the dynamic adjustment of circadian period, we performed a forward genetic screen. We identified Arabidopsis mutants that were either period insensitive to nicotinamide (*sin*) or period oversensitive to nicotinamide (*son*). We mapped *son1* to *BIG,* a gene of unknown molecular function

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that was previously shown to play a role in light signalling. We found that *son1* has an early entrained phase, suggesting that the dynamic alteration of circadian period contributes to the correct timing of biological events. Our data provide insight into how dynamic period adjustment of circadian oscillators contributes to establishing a correct phase relationship with the environment, and they show that BIG is involved in this process.

Introduction

The circadian clock is an endogenous oscillator that in *Arabidopsis thaliana* consists of nuclear and cytosolic feedback loops. It is often considered that the circadian oscillator runs with a period of 24-hour but the circadian period is plastic, depending on environmental conditions. For example, in diurnal organisms such as Arabidopsis (*Arabidopsis thaliana*), the circadian clock has a reduced period with increased light intensity (Aschoff 1960). This is commonly referred to as Aschoff's rule and was the foundation for the model of parametric entrainment that describes how the circadian oscillator synchronises with environmental cycles (Aschoff 1960). We have discovered that exogenous application of two common metabolites also regulates circadian period in Arabidopsis. Sucrose reduces circadian period under dim light conditions (Haydon et al., 2013), whereas nicotinamide makes the circadian clock run more slowly, with a period near 27 h (Dodd et al., 2007).

The way in which circadian clocks regulate and adjust circadian period is unknown. We refer to this ability of the circadian clock to adapt to the environmental conditions as dynamic adjustment of circadian period. To investigate this dynamic adjustment,

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we have used nicotinamide as a tool that increases circadian period. Previously, we have proposed that nicotinamide affects circadian period through its action as an antagonist of Ca^{2+} signalling (Dodd et al., 2007). There is circadian regulation of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) in mesophyll cells (Marti et al., 2013), and this encodes information about light intensity and quality (Xu et al., 2007; Love et al., 2004). In Arabidopsis, circadian regulation of $[Ca^{2+}]_{cyt}$ is driven by the second messenger cyclic adenosine diphosphate ribose (cADPR) under the control of the morning oscillator gene *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1*) (Xu et al., 2007; Dodd et al., 2007). Nicotinamide, the by-product of cADPR synthesis, inhibits both cADPR accumulation (Dodd et al., 2007) and ADPR cyclase activity (Abdul-Awal et al., 2016). There is no gene in Arabidopsis with homology to any of the known ADPR cyclases (Hunt et al., 2007). However, the existence of a completely novel ADPR cyclase in the green lineage cannot be ruled out, as many cyclases are yet to be characterised at the genetic level in mammals (Masuda et al., 1997).

Nicotinamide increases circadian period in all organisms tested, including Arabidopsis (Dodd et al., 2007), mouse (Asher et al., 2008) and Ostreococcus (O'Neill et al., 2011). In animals, nicotinamide has been hypothesised to affect both circadian period and amplitude through inhibition of poly-ADP-ribose polymerase (PARPs; Ramsey et al., 2008) or SIRTUINs (Asher et al., 2008). Similar to ADPR cyclase, SIRTUINS are enzymes belonging to the NADase superfamily, that release nicotinamide as a by-product with ADPR production. However, consistent with the effect of nicotinamide on circadian period being due to inhibition of ADPR cyclase, a knock out mutation of *CD38*, the main mammalian ADPR cyclase, causes a long circadian period in mice (Sahar et al., 2011).

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We have used nicotinamide as a tool to understand the potential mechanisms that regulate the dynamic adjustment of circadian period and to determine how nicotinamide regulates the circadian clock. We have performed a forward genetic screen to identify loci that affect the sensitivity of the circadian oscillator to nicotinamide. Previous genetic analysis of the circadian system has focused on identification of components of the circadian oscillator through screens for a short or long circadian period in constant light (Millar et al., 1995; Panda et al., 2002; Somers et al., 2000; Hazen et al., 2005) or constant darkness (Kevei et al., 2007; Hong et al., 2010; Martin-Tryon et al., 2007; Ashelford et al., 2011). We have taken a different approach by screening for mutations that are affected in their ability to change circadian period in response to altered conditions. We predicted that such a screen might identify genes involved in the response to nicotinamide and, more importantly, genes that participate in the dynamic adjustment of circadian period. We report the mapping-by-sequencing of an Arabidopsis mutant that is oversensitive to the effect of nicotinamide on circadian period, and identification of the causal mutation in the gene *BIG*. Phenotypic and genotypic analysis of the mutant indicate a wider role for *BIG* in the dynamic adjustment of circadian period. We tested the hypothesis that mutations in this gene that affect dynamic adjustment of free-running period also affect the entrained phase. We find that this dynamic adjustment of circadian period is associated with establishing the correct phase relationship with the environment. Our data therefore identify a genetic component required for the correct regulation of circadian period and suggest that circadian period is not fixed at 24 hours, thus permitting entrainment to different photoperiods. Our screen has provided important

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insight into how circadian clocks entrain to environmental cycles and therefore how plants tell the time.

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Results

Forward genetic screen identifies mutants that are compromised in their ability to adjust circadian period in response to nicotinamide

To identify mutants with an altered response of circadian period to nicotinamide, we mutated a Ws-2 dual reporter line with ethyl methanesulphonate (EMS), which generates A-G and C-T transitions in base sequence. This line carries both the *CHLOROPHYLL A/B BINDING PROTEIN2 promoter:LUCIFERASE*$^{+}$ (*CAB2:LUC*$^{+}$; Hall et al., 2003) and CaMV 35S promoter*:APOAEQUORIN* (*35S:AEQ*; Xu et al., 2007) reporters. The EMS population was initially screened in the M2 generation for period and amplitude of *CAB2:LUC*$^{+}$ in the presence of 10 mM nicotinamide. We used the *CAB2:LUC*$^{+}$ reporter because this had previously been used to study the effect of nicotinamide on the Arabidopsis circadian clock (Dodd et al., 2007). By measuring the behaviour of circadian clock output in *CAB2* we could examine the consequence of the entire oscillator dynamics, which is not possible when measuring the behaviour of a single oscillator component. This screen of 16,000 M2 plants identified 372 putative mutants. These mutants were categorized as: i<u>S</u> period <u>I</u>nsensitive to <u>N</u>icotinamide *(sin), i<u>S</u>* period <u>O</u>versensitive to <u>N</u>icotinamide (*son*), or *i<u>S</u>* <u>A</u>mplitude insensitive to <u>N</u>icotinamide (*san*), based upon a circadian period that was outside of 2 standard deviations of Ws-2 circadian period (*sin* <24.0 h, *son* >26.1 h) or amplitude (*san* >0.40 or <0.18) of *CAB2:LUC*$^{+}$ in the presence of 10 mM nicotinamide. The nature of the M2 screen meant that in addition to nicotinamide response mutants, it was possible that mutations affecting free-running period could have also been selected.

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We performed a rescreen of the M3 to confirm initial mutants and exclude those that were just free-running circadian period mutants. In the M3 screen, wild-type Ws-2 plants responded to 20 mM nicotinamide with an increase in circadian period of *CAB2:LUC*$^{+}$ from 23.9 ± 0.2 h to 26.2 ± 0.3 h and amplitude reduced from 1.1 ± 0.04 normalised luminescence counts (n.c.) to 0.6 ± 0.03 n.c. (Figure 1a). Sixty-three mutants were confirmed by the rescreening of the M3 generation which also allowed the exclusion of false positives from the M2 screen (Figure 1b, Supplemental Table S1).

Twenty-five mutants were confirmed for the *sin* phenotype with either no significant period increase in the presence of 20 mM nicotinamide, or with a reproducibly smaller increase in period than Ws-2 (Supplemental Table S1). Sixteen mutants were confirmed for the *son* phenotype with significantly greater period in the presence of 20 mM nicotinamide compared to Ws-2 (Supplemental Table S1). Similarly, 25 *san* mutants were confirmed to have either no significant decrease in amplitude in response to nicotinamide, or significantly smaller amplitude than Ws-2 (Supplemental Table S1).

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The strongest phenotypes (Figure 1c) were seen in *son1* with a nicotinamide-induced circadian period increase of 6.02 ± 0.75 h (*son1* H_2O: 22.6 ± 0.1 h, 20 mM NAM: 28.6 ± 0.7 h, $p<0.01$ T=8.05), *sin1* with no period increase (*sin1* H_2O: 25.2 ± 0.3 h, 20 mM NAM: 25.0 ± 0.3 h, p=0.19 T=0.92) and *san11* which had a circadian period increase of 1.3 h but with a rising amplitude of *CAB2:LUC*$^+$ compared to damping amplitude in wild type (*san11* H_2O: 1.08 ± 0.03 n.c., 20 mM NAM: 1.05 ± 0.0 n.c., T=0.83 p=0.21). The *san* lines all had very low amplitude compared to Ws-2

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Figure 1

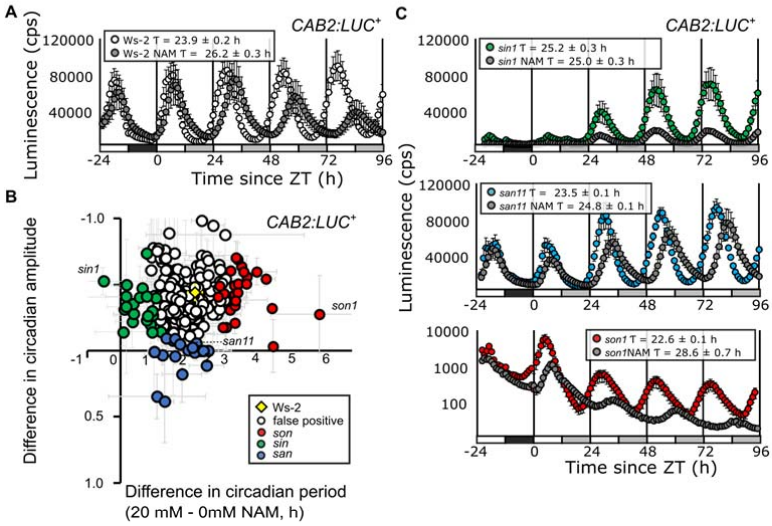

Figure 1. A forward genetic screen separates period and amplitude effects of nicotinamide. (A) *CAB2:LUC*$^+$ rhythms in wild-type Ws-2 in the presence or absence of 20 mM nicotinamide (NAM) in one entraining 12:12 light dark cycle (Black and white bars) and four days in constant light (white and grey bars). Mean FFT-NLLS period estimates are shown ± SEM (n = 8). **(B)** Free-running circadian period and amplitude difference of M3 plants in a forward genetic screen for the effect of 20 mM nicotinamide on circadian oscillations of *CAB2:LUC*$^+$. Period insensitive mutants (*sin*) are indicated in green, period oversensitive mutants (*son*) in red and amplitude sensitive mutants (*san*) in blue. Plants with no detectable nicotinamide-response phenotype in the screen of the M3 population are shown in white, and mean wild-type Ws-2 ± SEM from all experiments (n = 64) is shown overlaid in yellow. Data are pooled from eight separate experiments. (C) *CAB2:LUC*$^+$ rhythms in *sin1*, *son1* and *san11* mutants (labelled in **B**) in the presence or absence of 20 mM nicotinamide in one entraining 12:12 light dark cycle and four days in 70 µmol m^{-2} s^{-1} constant light (n = 8). Data is representative of two independent experiments in the M3 generation.

in the absence of nicotinamide, making the phenotypes difficult to measure robustly and map in segregating populations. Therefore, our laboratory has focussed our attention on the *sin* and *son* period mutant classes.

Dose response curves demonstrated that *son1* was hypersensitive to nicotinamide, with significant increases in circadian period with addition of 1 mM nicotinamide (Supplemental Figure S1a; ANOVA: F=6.87 df=15 p=0.02), whilst Ws-2 circadian period of *CAB2:LUC*$^+$ did not vary significantly until addition of 10 mM nicotinamide

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(ANOVA: F=7.85 df=19 p<0.01). *sin1* was hyposensitive to nicotinamide, as there was no variation in circadian period of *CAB2:LUC*$^+$ between 0.1 mM and 20 mM nicotinamide (Supplemental Figure S1b; ANOVA: F=2.15 df=18 p=0.11). The mutants were backcrossed twice to the parental Ws-2 line carrying *35S:AEQ* and *CAB2:LUC*$^+$ for mapping. Here we describe our findings of *son1,* the first mutant that we have mapped from the population, which has the strongest phenotype of all those identified.

son1 **maps to a mutation in a splice acceptor in** ***BIG***

We mapped the causal mutation for *son1* using a mapping population of 25 BC_1F_2 plants clearly displaying the mutant phenotype and sequenced pooled DNA to 50-fold coverage. SHOREmap analysis (Schneeberger et al., 2009) using a sliding window of allele frequency identified a region on the long arm of chromosome 3 where no recombination had occurred (Figure 2a). Underlying this region was a 750 kb interval containing eight SNPs, with three mutations at positions 433767, 474568 and 697938 predicted to cause functional changes to gene products (Figure 2b). We confirmed the existence of these SNPs using dCAPs analysis and Sanger sequencing, and fine-mapped the mutation by analysing the segregation pattern in the BC_2F_3 using the M3 screening conditions (Supplemental Figure S2, Figure 2c). When the segregation pattern of the SNPs was compared with the segregation of the *son1* phenotype, the SNP on chromosome 3 at position 433767 was the only SNP that segregated with the *son1* phenotype in the BC_2F_3 (Figure 2c; Supplemental Figure S2, e-f). The wild-type and heterozygous 3:433767 populations were not distinct from one another, with wild type period difference (plus and minus

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Figure 2

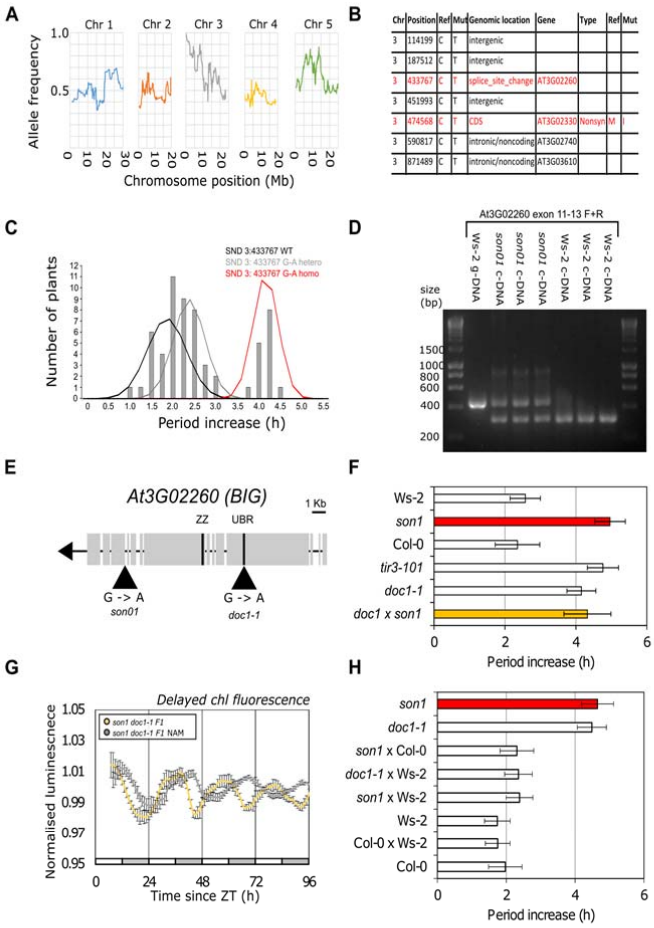

Figure 2. A causal mutation in BIG underlies *son1*

(A) SHOREmap backcross mapping by sequencing analysis of *son1* generated from 25-fold coverage Illumina sequence data obtained from 25 BC_1F_2 individual plants with the son1 phenotype. Individual chromosomes are shown separately with allele frequency sliding window generated with a moving average of 50 Kb. A region with allele frequency of one is found on the long arm of chromosome 3. **(B)** SNPs found on Chromosome 3 in son1 with allele frequency of 1.0. Mutations highlighted in red are predicted to cause a functional change in gene product. **(C)** BC_2F_3 segregation of *son1* phenotype with mutation at 3:433767. Period difference in the presence of 20 mM nicotinamide was calculated by FFT-NLLS and plotted rounded to nearest 0.2 h. Each line consisted of 8 biological replicates, 45 lines were genotyped and phenotyped. Mean and SD for each 3:433767 G-A genotypic sub-population (Wt, heterozygous, and homozygous) were used to plot normal distributions overlaid onto period histogram. **(D)** RT-PCR of *BIG* exons 11 – 12 showing the effect of *son1* on BIG transcript isoforms. Lane 1 has Ws-2 genomic DNA product of predicted size 458bp. Lane 2-4 have *son1* c-DNA products of 314and 458 bp. Lane 5-7 have Ws-2 c-DNA product of 316bp. 1 Kb Ladder annotated with fragment sizes is shown. Independently isolated BC_2F_3 pedigrees were used. **(E)** Gene structure of *At3G02260* (*BIG*), the potential UBR (CRD1) and ZZ zinc finger domains and positions of *son1* and *doc1-1* mutations are labelled. **(F)** Circadian period difference between the presence and absence of 20 mM nicotinamide for delayed chlorophyll fluorescence rhythms in Ws-2, *son1*, Col-0, *doc1-1* and *son1 doc1-1* F1. Period estimates calculated using FFT-NLLS analysis (n = 10). Data are representative of two independent experiments. **(G)** Delayed chlorophyll fluorescence rhythm for *son1* x *doc1-1* F1 in the presence or absence of 20 mM nicotinamide (NAM) across four days in constant light. White and grey bars show subjective day and night. Mean ± SEM shown for n = 10. Data are representative of two independent crosses. **(H)** Circadian period difference between the presence and absence of 20 mM nicotinamide for delayed chlorophyll fluorescence rhythms in of Col-0, Ws-2, Col-0 x Ws-2 F1, *son1*, *doc1*, *son1* x ws-2 F1 and *doc1* x Ws-2 F1. Period estimates calculated using FFT-NLLS analysis (n = 10).

197 nicotinamide) of 1.9 ± 0.5 and heterozygous period difference of 2.5 ± 0.4 h

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compared to the homozygous 3:433767 period difference of 4.3 ± 0.3 h.

This SNP resulted in a G-A transition causing a mutation in the 3' splice acceptor site of exon 12 of *At3G02260* (Figure 2d). *At3G02260* encodes BIG, a callosin-like protein of 5098 amino acids and unknown molecular function (Gil et al., 2001). The M3 line carrying *son1* is slightly short period (Figure 1). This short period phenotype in the M3 generation was reproducible but not significant (period diff = 0.53, $p>0.05$, Supplemental Figure S3). However, the short period phenotype was not present in the M4 generation, or in the BC_1F_3 (Supplemental Figure S3) or BC_2F_3 (Supplemental Figure S2), indicating that the phenotype was not linked to the *son1* phenotype after backcrossing to Ws-2 and that the *son1* mutation does not cause a classical circadian period phenotype

To test the effect of the 3:433767 mutation on transcript splicing in the *son1* mutant, PCR products were amplified from cDNA using primers spanning exon 11 - 12 of *BIG* in three independent BC_2F_3 pedigrees. In addition to the 316 bp product amplified from wild-type cDNA (Figure 2d, lane 6-8), an additional product was amplified from *son1* mutants (Figure 2d, lane 3-5) that was of equivalent size to the 458 bp PCR product amplified from wild-type genomic DNA (Figure 2d lane 2), indicating that it represented an unspliced transcript. Sequencing of both *At3G02260* splice variants in *son1* demonstrated that there was a G-A transition corresponding to 3:433737 in both products (Supplemental Figure S4). The smaller fragment was 2 bp smaller than the Ws-2 product, with a second AG immediately downstream of the first being used as a splice acceptor instead, whilst the larger 458 bp product contained the full sequence of intron 11 - 12 suggesting it is retained in *son1*, due to

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222 inefficient splicing. Thus, the G-A 3:433767 causes both the production of an
223 unspliced transcript, and the use of a cryptic splice site in *son1,* both of which result
224 in frameshifts and are predicted to cause premature stop codons.

225

226 To confirm that the *son1* phenotype was due to the G to A transition in *BIG*, we
227 assessed the response to nicotinamide in mutants in *BIG* identified from previous
228 mutant screens, *dark over-expresser of cab1-1* (*doc1-1)* (Li et al., 1994) and *auxin*
229 *transport inhibitor response 3* (*tir3-101)* (Ruegger et al., 1997), using delayed
230 chlorophyll fluorescence (Gould et al., 2009). *doc1-1* has an increase in
231 photosynthesis-related gene expression, including *CAB* genes, in etiolated seedlings
232 in the dark (Li et al., 1994; Gil et al., 2001) caused by a G-A transition resulting in a
233 Cys to Thr amino acid substitution in the first cysteine rich domain (CRD-1, also
234 known as a UBR box). *tir3*-101 is reported to have impaired polar auxin transport
235 giving rise to a dwarf phenotype (Ruegger et al., 1997; Prusinkiewicz et al., 2009).
236 Both *doc1-1* and *tir3-101* were oversensitive to nicotinamide compared to their
237 respective wild types (Figure 2f, Supplemental Figure S5; Col-0: 2.9 ± 0.5 h, *doc1-1*:
238 4.5 ± 0.4 h, *tir3-101* 4.4 ± 0.9 h). The increased response of circadian period in the
239 three different *son1*, *doc1-1* and *tir3-101* alleles of *BIG* suggested that the mutations
240 in *BIG* are causal for the nicotinamide oversensitive phenotype, and none have a
241 circadian period phenotype in constant high light.

242 As confirmation, we tested whether *son1* is allelic to *doc1-1.* The *doc1-1 son1* F1
243 plants had significantly greater period increase in the presence of nicotinamide (4.3 ±
244 0.7 h) than either Ws-2 (1.7 ± 1.2 h, T=2.22, df=19, $p<0.05$) or Col-0 (2.4 ± 0.6 h,
245 T=2.16, df=19, $p<0.05$), and were not statistically different to either *doc1-1* (4.2 ± 0.4

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h; T=0.22, df=19, p=0.42) or *son1* (4.9 ± 0.4 h, T=0.81, df=19, p=0.21) in the presence of 20 mM nicotinamide (Figure 2f - g). To control for ecotype or dominance effects we analysed delayed fluorescence in the presence and absence of nicotinamide for F1 of crosses between *son1* and Ws-2, *son1* and Col-0 and *doc1* and Ws-2 (Figure 2h, Supplemental Figure S6). These crosses all behaved as wild-type, and had circadian period increases that corresponded to the heterozygous BC_2F_3 on the segregation analysis (Figure 2c). This demonstrates that *doc1-1* is allelic to *son1* and that *BIG* regulates sensitivity of the circadian oscillator to nicotinamide.

Having established that *son1* and *doc1-1* are both nicotinamide-oversensitive for circadian period, we tested whether *son1* plants exhibit the *doc1* phenotype of increased photosynthesis related gene expression in etiolated seedlings in the dark (Li et al., 1994; Gil et al., 2001). *CAB2:LUC*$^+$ expression was higher in etiolated seedlings of *son1* than wild type in constant dark (DD) (Supplemental Figure S7 a-c), indicating that *son1* also had a dark-over expresser of *CAB* phenotype consistent with allelism to *doc1-1.* Similar to *doc1-1,* higher *CAB2* expression in constant dark was not associated with premature de-etiolation (Supplemental Figure S7 d-e).

To test if BIG could be part of the transcriptional feedback loops of the oscillator we looked at the transcript profile for *BIG* in the publicly available diurnal transcriptomic datasets under long and short days (Supplemental Figure S8). *BIG* does not oscillate in either long or short photoperiods in two separate 48 h microarray experiments (Endo et al., 2014; Mockler et al., 2007). The abundance of *BIG* transcript also does not appear to be regulated by the circadian oscillator, with no detectable oscillations using JTK cycle (p>0.05) in a circadian transcriptome taken over 48 hours in constant conditions (Dalchau et al., 2010). The lack of circadian or diel changes in

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BIG transcript abundance suggests BIG is not part of transcriptional feedback loops in the circadian oscillator.

son1 affects circadian $[Ca^{2+}]_{cyt}$ signalling

In addition to increasing circadian period, nicotinamide abolishes circadian regulation of $[Ca^{2+}]_{cyt}$, potentially through inhibition of the ADPR cyclase activity that generates the Ca^{2+} agonist cADPR (Dodd et al., 2007; Abdul-Awal et al., 2016). We investigated the effect of *son1* on Ca^{2+} signalling using the 35S:AEQ reporter. In Ws-2 there was sinusoidal circadian regulation of $[Ca^{2+}]_{cyt}$ which had an estimated period of 24.2 ± 0.9 h and RAE of 0.3 ± 0.0 (Figure 3a). *son1* affected the circadian regulation of $[Ca^{2+}]_{cyt,}$ leading to a non-sinusoidal oscillation with an increasing basal level and dampening over time. This resulted in fast Fourier transform-non-linear least squares (FFT-NLLS) analysis estimating the rhythm as only weakly rhythmic with an RAE of 0.5 ± 0.1 (period = 23.1 ± 0.2 h; Figure 3a). Both *son1* and Ws-2 circadian $[Ca^{2+}]_{cyt}$ signals were inhibited by 20 mM nicotinamide (Figure 3b; Ws-2: RAE = 0.7 ± 0.1; *son1:* 0.7 ± 0.1).

Because we have proposed previously that circadian regulation of $[Ca^{2+}]_{cyt}$ arises from cADPR-mediated Ca^{2+} release, we measured the activity of ADPR cyclase in wild type and mutant. *son1* had significantly higher ADPR cyclase activity compared to Ws-2, ($p=0.01$) in the middle of the photoperiod representing peak $[Ca^{2+}]_{cyt}$ (Figure 3c). $[Ca^{2+}]_{cyt}$ was also elevated at the same time point in *son1* compared to Ws-2 (Figure 3d; $p<0.05$). This effect was more pronounced after 72 h in constant light, where the LL 35S:AEQ data had previously indicated there to be a much higher basal level of $[Ca^{2+}]_{cyt}$ ($p<0.05$). $[Ca^{2+}]_{cyt}$ at both time points was reduced by incubation with 20 mM nicotinamide (Figure 3d). Thus, although nicotinamide

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Figure 3

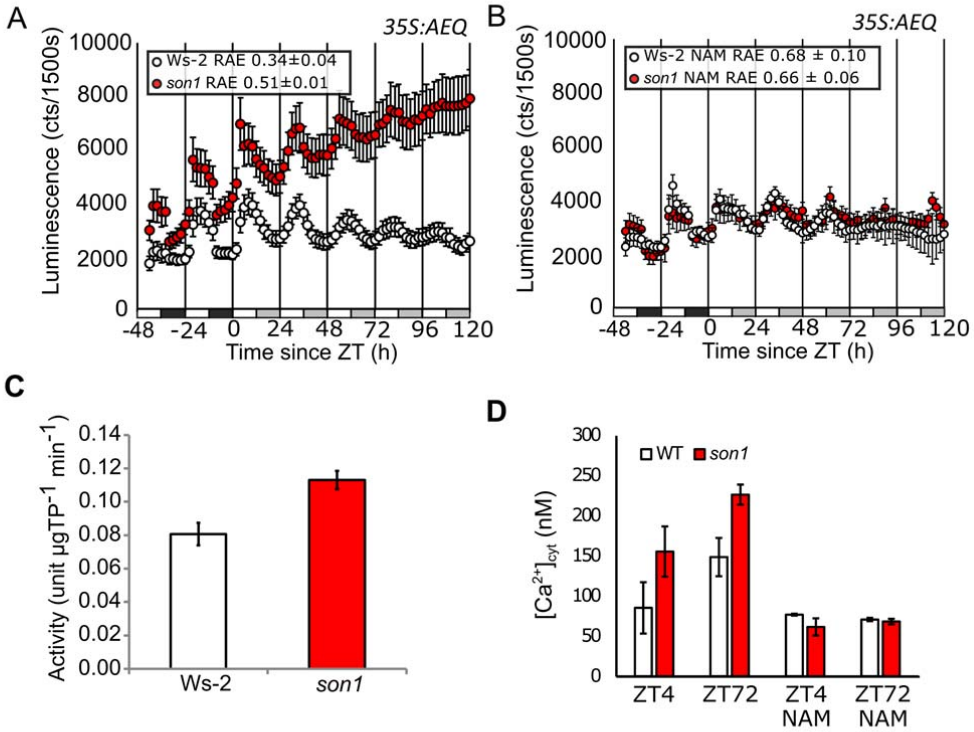

Figure 3. *son1* affects circadian $[Ca^{2+}]_{cyt}$ signals

Bioluminescence (photon counts/1500 seconds) from *son1* and Ws -2 expressing *35S:AEQUORIN* across two light dark cycles and five days in constant 70 µmol m^{-2} s^{-1} white light grown on 20 mM mannitol **(A)** or 20 mM nicotinamide **(B)**. Mean ± SEM, n = 8. Data are representative of three independent experiments in the BC_2F_3 generation. **(C)** ADPR cyclase activity measured using NGD assay at ZT4 in 70 µmol m^{-2} s^{-1} white light from 3-4 week old Ws-2 (white) and *son1* (red) seedlings, mean of 3 biological replicates shown with SEM. **(D)** $[Ca^{2+}]_{cyt}$ measured at ZT 4 and ZT72 in constant 70 µmol m^{-2} s^{-1} white light from 11 and 14 day old Ws -2 (white) and *son1* (red) seedlings respectively , n=12 . Data are representative of three independent experiments in the BC_2F_3 generation.

reduced $[Ca^{2+}]_{cyt}$ to similar concentrations in wild-type and *son1*, the change in $[Ca^{2+}]_{cyt}$ in *son1* was greater as untreated plants have higher $[Ca^{2+}]_{cyt}$, indicating that the $[Ca^{2+}]_{cyt}$ increase in *son1* might be ADPR cyclase-dependent.

son1 affects circadian oscillator gene expression

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Circadian clocks evolved to provide competitive advantage in light and dark cycles and therefore, to investigate the role of *BIG* in the daily timing of Arabidopsis we examined the effect of *son1* on oscillator gene transcript abundance in light and dark cycles and in constant light. As our phenotype was based on the *CAB2* gene, we measured abundance of *CCA1*, a main circadian regulator of *CAB2*, and also the direct regulators of *CCA1* – *TOC1*, *PRR7* and *CHE*. *son1* affected circadian oscillator transcript levels in light dark cycles. The expression of *CCA1* immediately before dawn was higher in *son1* compared to Ws-2 (Figure 4a; $p<0.01$) which corresponded to a reduction in *TOC1* expression immediately before dusk (Figure 4a; $p=0.01$) and with a significant reduction in *CHE* expression at both dawn and dusk in *son1* (Figure 4a; $p<0.01$). We also measured the expression of *CCA1*, *TOC1*, *CHE* and *PRR7* in constant light across a 48 h time course in *son1* and Ws-2 in the presence and absence of 20 mM nicotinamide and estimated circadian period using JTK-cycle (Hughes et al., 2010). We performed this to confirm the *son1* phenotype at the level of gene expression and identify if there were any changes in gene expression between mutant and wild-type in the absence of nicotinamide (Figure 4b). In Ws-2, nicotinamide treatment significantly reduced the peak expression of all the genes in the first cycle ($p<0.05$). Nicotinamide also significantly reduced peak *CCA1* and *PRR7* transcript levels in *son1,* however there was no significant change in *TOC1* and *CHE* at any time point. In *son1*, *CCA1* and *PRR7* rhythms had an increased circadian period in the presence of nicotinamide compared to Ws-2, with period of 28 h in *son1* ($p<0.001$) but 24 h in wild-type ($p<0.001$). *CHE* was not rhythmic with JTK-cycle in either Ws-2 ($p=1$) or *son1* ($p=0.08$). *TOC1* was rhythmic with JTK-cycle in Ws-2 with period of 24 h ($p<0.05$) but not rhythmic in *son1* ($p=0.16$). Thus, the *son1* phenotype can be seen in rhythms

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Figure 4

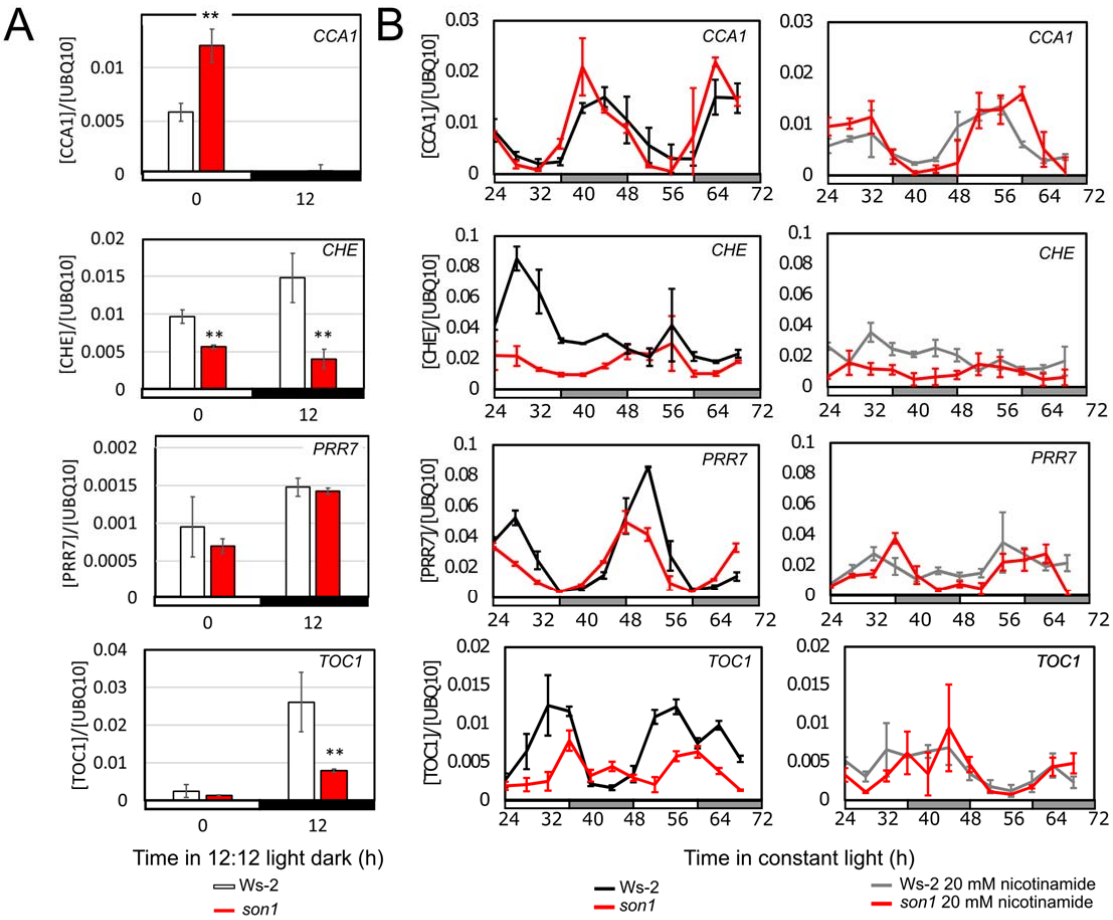

Figure 4. *son1* affects circadian clock gene expression in LD and constant light

(A) *CCA1*, *PRR7*, *TOC1* and *CHE* expression from *son1* (red) and Ws-2 (white) samples harvested immediately preceding dawn (ZT0) and dusk (ZT12).

(B) *CCA1*, *PRR7*, *TOC1* and *CHE* expression from Ws-2 and *son1* in the absence (left) and in the presence of 20 mM nicotinamide (right) across 48 hours in constant 70 µmol m^{-2} s^{-1} light from ZT24 – ZT72. Relative expression of genes normalised to UBQ10f expression is given ± standard deviation [n=3]. Plants were grown as clusters of 5 plants for 11 days in light dark cycles prior to experiment.

of *CCA1* and *PRR7*, but in the presence of nicotinamide rhythms of *CHE* and *TOC1* were suppressed with *TOC1* also being suppressed in *son1* in the absence of nicotinamide.

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son1 affects dynamic period adjustment of the circadian oscillator to regulate the entrained phase

As *son1* is compromised in the ability to regulate changes in circadian period in response to nicotinamide, we tested whether it was also affected in its ability to adjust period correctly to other stimuli. Response to light is the most well characterised dynamic adjustment of the circadian period and is described by Aschoff's rule (Aschoff 1960). We tested the hypothesis that *son1* might be compromised in the ability to regulate circadian period at different light intensities by performing a fluence response curve (Figure 5a).

There was no difference between the period length of *CAB2:LUC*$^{+}$ rhythms in Ws-2 and *son1* at 100 µmol m^{-2} s^{-1} light (Ws-2: 23.2 ± 0.1 h, *son1*: 23.0 ± 0.1 h,) which was the intensity of light used for entrainment, indicating again that *son1* is not a circadian period mutant. However, *son1* had a significantly shorter circadian period compared to wild type under low fluence rates (Figure 5a, Supplemental Figure S9): under 5 µmol m^{-2} s^{-1} light *son1* had a period of 26.6 ± 0.8 h and Ws-2 had a period of 29.0 ± 0.3 h ($p<0.01$, Figure 5b). This indicates that *son1* cannot properly regulate circadian period in response to changes in light intensity. A similar phenotype was detected in *doc1-1*. Under 5 µmol m^{-2} s^{-1} light *doc1-1* had a circadian period of *CAB2:LUC*$^{+}$ of 25.7 ± 0.1 h and Col-0 had a period of 29.2 ± 0.2 h ($p<0.01$, Supplemental Figure S9).

Having previously established that *son1* affects the expression of circadian clock genes in a light and dark cycle, we next investigated the effect of *son1* on the entrained phase to investigate the potential roles of *BIG* in the daily timing of Arabidopsis. Wild-type Ws-2 had a typical phase shift of later phase with increasing

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Figure 5

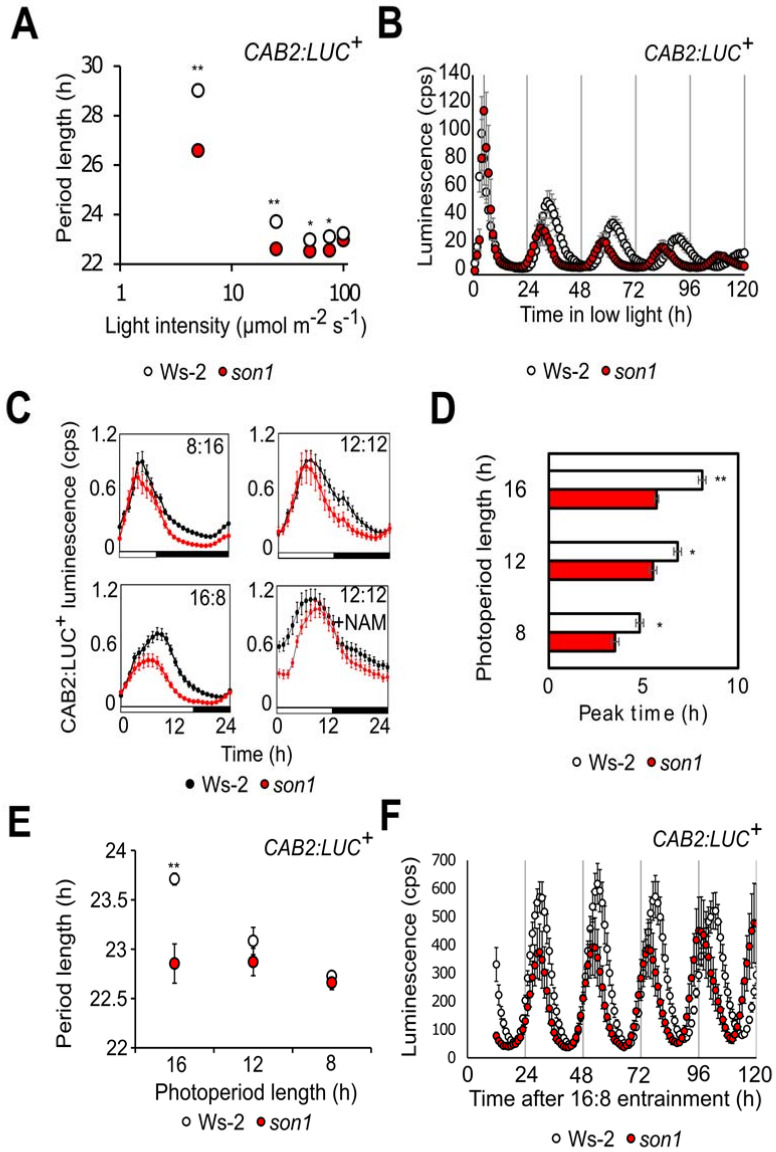

Figure 5. *son1* affects dynamic circadian period adjustment by light and photoperiod
(A) Fluence response curve for circadian period of *CAB2:LUC*$^+$ in Ws-2 and *son1* estimated in equal mix red and blue light (Mean ± SEM, n = 8-12). Data are pooled from three independent experiments. **(B)** *CAB2:LUC*$^+$ rhythm of *son1* and Ws-2 assayed over five days in constant 5 µmol m^{-2} s^{-1} equal mix red and blue light (Mean ± SEM, n = 8). Data are representative of two independent experiments in the BC_2F_3 generation. In Figure 4a the small error bars are obscured by symbols. **(C)** *CAB2:LUC*$^+$ luminescence (counts s-1) from *son1* (red) and Ws-2 (black) seedlings grown in 8:16, 12:12 and16:8 photoperiods. Plants grown in 12:12 were treated with media supplemented with or without 20 mM nicotinamide (NAM) 2 days prior to entrainment in camera chamber (Mean ± SEM, n=8). Plants were grown in entrainment conditions since germination, and transferred to camera chamber one day before imaging, maintaining the same entrainment regime. Data are representative of three independent experiments. **(D)** Peak time of *CAB2:LUC*$^+$ from LD cycles in **(C)**.
(E) Photoperiod response curve for circadian period of *CAB2:LUC*$^+$ in Ws-2 and *son1* estimated in equal mix 80 µmol m^{-2} s^{-1} red and blue light (Mean ± SEM, n = 8). Data are pooled from two independent experiments. Plants were entrained in either photoperiods of either 16, 12 or 8 hours prior to transfer to constant light. **(F)** *CAB2:LUC*$^+$ rhythm of *son1* and Ws-2 entrained in 16:8 LD cycles and released into constant light for five days (Mean ± SEM, n = 8).

352 photoperiod (Figure 5c, peak at 4.8 ± 0.2 h (8:16), peak at 6.8 ± 0.2 h (12:12) peak

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353 at 8.1 ± 0.2 h (16:8). By contrast, *son1* was an early phase mutant (Figure 5c-d,
354 peak at 3.5 ± 0.2 h (8:16), peak at 5.5 ± 0.2 h (12:12) peak at 5.7 ± 0.1 h (16:8).
355 These data demonstrate that *BIG* is required for correct circadian entrainment.
356 Lastly, we measured the effect of nicotinamide on entrained phase under 12:12
357 (Figure 5c), and found that it caused a phase delay of *CAB2:LUC*$^{+}$ peak expression
358 of 1 h in Ws-2 and 3 h in *son1* (Ws-2: ZT 7.8 ± 0.2, *son1*: ZT 8.8 ± 0.2), consistent
359 with the effect of nicotinamide on free running-period in both backgrounds.

360

361 Finally, having identified that BIG regulates dynamic adjustment of circadian period
362 and that it is required for correct circadian entrainment, we wanted to investigate
363 whether oscillator period is associated with entrainment and whether the effect of
364 *BIG* on phase could be involved in this regulation. To do this, we studied whether
365 entrainment photoperiod affects free-running period in Ws-2 and *son1* (Figure 5e).
366 The results showed that there is a relationship between length of entrainment
367 photoperiod and the length of circadian period in Ws-2 (Figure 5e). However, this
368 relationship was lost in *son1*, whose circadian period was not affected by the
369 duration of the photoperiod during entrainment. As a result of this, *son1* did not have
370 significantly shorter free-running period of *CAB2:LUC*+ compared to Ws-2 when
371 released from entrainment cycles of 8:16 and 12:12 (*son1*: 22.6 ± 0.07 h (8:16), 22.8
372 ± 0.14 h (12:12); Ws-2: 22.7 ± 0.04 h (8:16), 23.1 ± 0.14 h (12:12). However, when
373 plants were entrained in 16:8 *son1* had free-running period of 22.7 ± 0.2 h, an hour
374 shorter than Ws-2 (23.7 ± 0.06 h, $p<0.05$; Figure 5f). This shows that the
375 photoperiod-determined entrained phase of the circadian clock affects the free-
376 running period in constant light, and *son1* does not adjust circadian period correctly
377 in 16:8. We performed the same series of experiments but with circadian free-run in

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constant darkness, in the presence of sucrose to sustain the oscillation of *CAB2:LUC*$^{+}$ (Dalchau et al., 2011). Similar to the result in constant light, we saw that in wild-type plants free-running period length increased with longer entraining photoperiod (Supplemental Figure S10), and for plants entrained in 16:8 *son1* had significantly shorter free-running period than wild-type (Ws-2: 27.0 ± 0.4 h; *son1*: 25.7 ± 0.5 h; $p<0.05$).

Thus, *son1* cannot correctly adjust period and has impaired phase in response to photoperiod. Collectively, these data demonstrate that nicotinamide targets a pathway involved in establishing the phase relationship between the circadian oscillator and the external environment and that *BIG* contributes to the correct timing of physiology in light and dark cycles, through regulating the pace of the oscillator.

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Discussion

Using a forward genetic screen, we found that *BIG* is a regulator of the dynamic adjustment of circadian period and phase. The period of the circadian oscillator is not fixed to 24 hours, but instead is a dynamically plastic phenotype and dependent on environmental conditions. Typically, experimentalists measure circadian period in constant conditions that allow the circadian oscillator to free run. In these constant conditions, the period of the Arabidopsis circadian oscillator decreases with increasing light intensity (Somers et al., 1998a), temperature (Salome et al., 2010) and sucrose (Haydon et al., 2013) and increases with nicotinamide (Dodd et al., 2007). We have identified a nicotinamide over-sensitive phenotype resulting from a mutation in *BIG*. *son1* is allelic to *doc1-1,* a previously characterised mutation in *BIG* confirming that *BIG* is a regulator of the sensitivity of the circadian oscillator to nicotinamide.

Whilst NAD is an abundant metabolite, we do not suggest that cellular nicotinamide derived from NAD breakdown directly regulates the pace of the circadian oscillator as part of the normal functioning of the plant. Instead, we consider nicotinamide as a probe that can be used to understand the potential mechanisms by which the circadian oscillator dynamically adjusts circadian period. Previously, we proposed that nicotinamide affects circadian period through the inhibition of ADPR cyclase activity and therefore the production of cADPR, which is a Ca^{2+} agonist (Dodd et al., 2007; Abdul-Awal et al., 2016). Our demonstration that mutations in *BIG* affecting the sensitivity of the circadian oscillator to nicotinamide also affect the regulation of $[Ca^{2+}]_{cyt}$ are supportive of the hypothesis that nicotinamide regulates circadian period

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through a Ca^{2+}-sensitive mechanism. *son1* has higher $[Ca^{2+}]_{cyt}$ and ADPR cyclase activity than wild-type, and that increased $[Ca^{2+}]_{cyt}$ is nicotinamide-sensitive. This might indicate that increased effect of nicotinamide on circadian period is related to the altered $[Ca^{2+}]_{cyt}$ in the mutant. However, we do not exclude the possibility of additional Ca^{2+}-insensitive modes of action of nicotinamide on the circadian system (Malapeira et al., 2012).

Animal homologues of BIG, UBR4/p600 in mammals and Calossin/Pushover in Drosophila, are confirmed calmodulin-binding proteins (Xu et al., 1998; Nakatani et al., 2005; Belzil et al., 2013) and have been proposed to act as part of a Ca^{2+} sensing/signalling mechanism. In mammalian neurons, UBR4, calmodulin and calmodulin-dependent protein kinase IIα form a complex upon glutamate-induced Ca^{2+} entry through NMDA receptors or inositol trisphosphate receptor-mediated Ca^{2+} release from the ER (Belzil et al., 2013). Since BIG has a putative calmodulin binding domain (Yap et al., 2000), it is tempting to speculate that this could also play a role in Ca^{2+} signalling, although the interacting molecular players will be different in plants.

BIG was originally identified as a light signalling regulator (Li et al., 1994), and was later shown to also control multiple hormone signalling pathways (Kanyuka et al., 2003), including auxin transport (Guo et al., 2013), and has recently been implicated in CO_2-induced stomatal closure (He et al., 2018). The precise biochemical functions of BIG are unknown but mutations in *Pushover* and knockout or down-regulation of UBR4 also produce pleiotropic phenotypes (Richards et al., 1996; Sekelsky et al., 1999; Yager et al., 2001; Nakatani et al., 2005; Belzil et al., 2014). BIG, UBR4 and Pushover contain a zinc finger-like domain, the UBR box, found in ubiquitin E3

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438 ligases specific to the N-end rule for targeted protein degradation (Gil et al., 2001;
439 Tasaki et al., 2005; 2009). The N-end rule is a conserved pathway in which proteins
440 are targeted for destruction dependent on their N-terminal residue and has diverse
441 roles in different organisms (Bachmair et al., 1986; Gibbs et al., 2014). Whilst UBR4
442 is required for degradation of model and physiological N-end rule substrates, it
443 contains no HECT or RING domains and hence is considered unlikely to act as an
444 E3 ligase in isolation, rather, it may act as a substrate (N-degron) recognition subunit
445 of a complex (Tasaki et al., 2005; 2009). It is not known whether BIG belongs to an
446 E3 ligase complex or whether it has intrinsic E3 ligase activity. Direct evidence for
447 the ability of the recombinant UBR box of mammalian UBR4 to bind N-degrons is
448 lacking (Tasaki et al., 2009) but previous bioinformatics analysis identified a ZZ
449 domain in BIG (Gil et al., 2001). The ZZ domain is structurally and evolutionarily
450 related to the UBR box (Kaur and Subramanian, 2015) and has recently been shown
451 to bind N-degrons in the autophagic adaptor protein p62 (Cha-Molstad et al., 2017).
452 There is a precedent for regulation of circadian period through control of protein
453 turnover since a double mutant lacking two ubiquitin-specific proteases UBP12 and
454 UBP13 exhibits a short period circadian clock phenotype (Cui et al., 2013). Thus,
455 one potential mode of action of BIG on the circadian oscillator is through a role in
456 protein degradation, but further study will be required to confirm or reject this
457 hypothesis.

458 The effect of the *son1* mutation on levels of $[Ca^{2+}]_{cyt}$ was greater during the night or
459 subjective night than during the day or subjective day. This is indicative of a time-
460 dependent effect of BIG in the circadian system. Similarly, the *doc1-1* allele of *BIG*
461 specifically affects the expression of *CAB* and other photosynthetic genes at night,
462 rather than in the day. These data suggest that *BIG* acts at night in the circadian

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463 system. Previous studies have demonstrated that *BIG* plays a role in conveying light
464 information, and partially suppresses the phenotype of *phytochromeA* and
465 *phytochromeB* mutations on hypocotyl length (Kanyuka et al., 2003). Thus, *BIG* may
466 be involved in conveying light signalling for circadian entrainment. However, it is
467 likely that *BIG* regulates period or entrainment more widely, due to the effect of *son1*
468 on both nicotinamide period lengthening, and photoperiod regulation of period,
469 indicating that *BIG* has a further role outside of light signalling.

470 Time-dependent effects on the circadian oscillator are also sometimes associated
471 with entrainment, which is the matching of the phase and period of the oscillator with
472 that of the external photoperiod. Synchronisation of the circadian oscillator through
473 entrainment ensures that cellular events occur at the right time of day and ensures
474 that the circadian oscillator can track dawn and dusk as they change through the
475 year. This is essential to co-ordinate whole organism responses as circadian period
476 is different between organs (Takahashi et al., 2015), and is age dependent (Kim et
477 al., 2016). We found that *son1* has an early entrained phase in long day cycles,
478 suggesting an impact on entrainment. The early phase of *son1* and the reduced
479 ability to dynamically alter circadian period to light and nicotinamide might be related
480 through parametric entrainment. The inability of *son1* to adjust period depending on
481 entrainment photoperiod strongly suggests this. A previous study demonstrated that
482 tissue-specific changes in circadian period are accompanied by corresponding
483 changes in entrained phase (Takahashi et al., 2015). The effect of photoperiod on
484 the entrained phase of the oscillator has been widely reported (Yeang 2015, Millar
485 and Kay 1996, and Millar et al., 2015). Importantly, Millar et al., 2015 report that the
486 circadian mutant *cca1 lhy* has the same phase under 8:16, 12:12 and 16:8
487 photoperiods and thus is unable to adjust phase to entrainment photoperiod unlike

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488 the wild-type which had a 2.6 h difference. This is similar to the result we find here
489 for *son1* which has the same phase under 12:12 and 16:8 photoperiods. Unlike
490 *CCA1,* the transcript of *BIG* does not oscillate either in light dark cycles or in
491 constant light, and shows no modulation by photoperiod. This indicates that *BIG* is
492 not part of the transcription-based oscillator loops.

493 When previously identified Arabidopsis circadian mutants are viewed in the context
494 of phenotypic plasticity to light, they can be assigned to one of four categories
495 (Supplemental Table S2). Mutants can have a constitutive effect on circadian period
496 at all intensities of light and the mutation therefore has no effect on dynamic plasticity
497 of the circadian oscillator. Alternatively, mutants might have no plastic response to
498 light, appearing insensitive with period unchanging at all light intensities. Finally,
499 using the conventions in the literature (Martin-tryon et al 2008) we have defined
500 mutations as hyposensitive, with is a shallow response curve to light, or
501 hypersensitive in which the response curve is steep. Ten mutations do not affect
502 dynamic adjustment to either red or blue light, including four mutations that do not
503 affect the response to both wavelengths: the *toc1*-1 allele (Somers et al., 1998a),
504 *cry2-1* (Somers et al., 1998b), *fio1-1* (Kim et al 2008) and *tej* (Panda et al., 2002).
505 There are eight mutations reported to cause insensitivity to either red or blue light
506 including *prr7-11* to red light (Farre et al., 2005) and *gi-200* (Martin-Tryon et al.,
507 2007) to both red and blue light. Seven mutations cause hypersensitivity to either red
508 or blue light including *toc1-2* (Martin-Tryon and Harmer 2008), *lwd1 lwd2* (Wang et
509 al., 2011), and light signalling mutants *phyA-201* and *cry1-1* (Somers et al., 1998b).
510 The "hypersensitivity" in terms of the effect of light on circadian period for *phyA-201*
511 and *cry1-1* is caused by a very steep fluence response curve due to the inability to
512 sense low light intensities. However, only three reported mutations cause

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hyposensitivity to light. *rve4 rve6 rve8* (Gray et al., 2017) and *phyB-1* (Somers et al., 1998b) confer hyposensitivity to red light, and *prr7-3* to blue light (Farre et al., 2005). The phenotype of *son1* for the white light fluence response curve is also hyposensitive. However, as shown in Supplemental Table S2, *rve4 rve6 rve8* (Gray et al., 2017) and *phyB-1* (Somers et al., 1998b) both have long period phenotypes in addition to hyposensitivity phenotypes whereas *son1* has no period phenotype under the light intensity used for the initial entrainment in 12:12 (Figure 5; Supplemental Figure S3). Thus, the phenotype of *son1* indicates a function in adjusting period to stimuli, rather than being a core oscillator component, as under normal conditions there is no evidence for it being an oscillator component, since period defects are conditional, and the transcript abundance does not oscillate. The short period of *son1* after entrainment only to long days, or through maintenance in constant low light (Figure 5) demonstrates that the effect of *son1* is conditional on environmental input, suggesting that BIG is associated with regulation of plastic period of the oscillator by environmental signals, rather than acting as a core oscillator component. There is variability in the reported phenotypes of *prr7* mutants, with them being described as long period (Farre et al., 2005) or wild type (Nakamichi et al., 2005; Seki et al., 2016). This and the hyposensitivity to light suggest that *prr7* mutants might also have a defect in plasticity similar to *son1* in terms of responses to light. The mechanisms might be different because PRR7 is an oscillator component, whilst there is no evidence for BIG being so.

Alterations in circadian period are thought to be required for entrainment though there is not yet a consensus on how this is achieved. It is envisaged that changes in circadian period are a result of phase adjustment of the oscillator. For example, a phase advance will reduce the period of the cycle in which the advance occurred by

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an amount equal to the phase advance (Johnson, 1992). Additionally, changes in the velocity of the oscillator can affect period. Whilst changes in period are associated with entrainment, it is not known if this is due to changes in velocity, phase or both and whether these occur continuously or discontinuously (Daan, 2000). Our discovery of a mutant that is specifically compromised in the ability to dynamically alter circadian period and has altered entrained phase provides a tool to study the mechanism of entrainment and the pathways of this essential feature of the circadian oscillator. The study of how the circadian clock establishes a correct phase relationship with the environment is essential to understand the role of the circadian oscillator in the plant, because the timing of events within the diel cycle constitute the likely evolutionary pressure that resulted in the emergence and optimisation of circadian clocks.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (Arabidopsis) ecotype Wassilewskija-2 (Ws-2) carrying *CHLOROPHYLL A/B BINDING PROTEIN2:LUCIFERASE*$^{+}$ *(CAB2:LUC*$^{+}$*)* (Hall et al., 2003) and transformed with *CaMV 35S:AEQUORIN (35S:AEQ)* was described previously (Xu et al., 2007). *doc1-1* (Gil et al., 2001) was obtained from Nottingham Arabidopsis Seed Stock Centre (Arabidopsis.org). *tir3-101* was a gift from Ottoline Leyser (Sainsbury Laboratory at Cambridge University, Cambridge, UK). Plant growth on agar or soil was as described previously (Xu et al., 2007).

Mutagenesis

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562 Ws-2 seeds homozygous for *CAB2:LUC*$^+$ and *35S:AEQ* were mutagenized using
563 ethyl methane sulphonate (EMS; Sigma, UK). Seeds were suspended in 150 mM
564 EMS 0.1% (v/v) KCl for 4 hours in an atmosbag (Sigma, UK). Seeds were washed
565 three times in 100 mM sodium thiosulphate (Fisher, UK) before overnight
566 stratification at 4 ^{o}C and sowing on soil at a density of 10 seeds per 4 cm^2 of soil.
567 Ten percent of the M1 seedlings had regions of chlorosis, indicative of EMS-induced
568 alterations to the genomic sequence. Seeds were harvested in 10 plant M2 pools.
569 100 seeds were screened from 160 pools, with a total of 16,000 M2 seeds screened.

570

571 **Circadian phenotyping**

572 *Luciferase imaging.* CAB2:LUC$^+$ luminescence was imaged from either clusters of 10
573 seedlings or individual seedlings (Haydon et al., 2017). Nicotinamide treatment was
574 applied by transferring membranes (1µm, Sefar) with seven-day-old seedlings to 10
575 mM nicotinamide-containing media. Clusters of plants were transferred to
576 nicotinamide-containing plates at seven days old using a sterile toothpick, lifting
577 plants under the hypocotyls. Treatment with luciferin and imaging with a Nightshade
578 CCD camera and imaging chamber (Berthold, UK) mounted with an 18 mm lens was
579 as described in Haydon et al (2017). Where the effect of light intensity was
580 investigated, the assay plates were covered with combinations of the following
581 neutral density filters: Lee Technical Filter #211 (Lee filters, UK) and Roscolux #397,
582 #97, #98 (Rosco, USA). Light intensity was measured using a Skye Quantum Sensor
583 (Skye instruments limited, Wales).

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585 *Delayed chlorophyll fluorescence imaging.* Delayed chlorophyll fluorescence was
586 measured from excised leaves of 28-day-old plants. Leaves were excised at the
587 petiole and transplanted to fresh media in 25 well plates at dawn. The camera
588 chamber was supplied with constant RB LED light at 70 µmol m^{-2} s^{-1} and was cooled
589 to 20 ^{o}C. Measurements were automated and data extracted using IndiGO software
590 (Berthold). Delayed chlorophyll fluorescence measurements were taken by acquiring
591 luminescence for 60 seconds immediately following illumination.

592 *Aequorin bioluminescence imaging*. Aequorin bioluminescence was imaged from
593 clusters of 15 seedlings as described in Hearn and Webb (2014).

594

595 **Genetic Mapping**

596 *Segregation analysis*. Crosses were made with paternal Ws-2 and maternal mutant.
597 BC_1F_2 seedlings were screened as individual seedlings for circadian period of
598 CAB2:LUC^+ on 10 mM nicotinamide. BC_2F_3 seedlings were screened as clusters of
599 seedlings for circadian period of CAB2:LUC^+ in the presence or absence of 20 mM
600 nicotinamide.

601 *Mapping by sequencing*. Genomic DNA was extracted from 20-day-old plants using
602 the Qiagen Plant Maxi Kit and quantified using a nanodrop. Sequencing libraries
603 were prepared using Ilumina Tru-seq. DNA was sequenced by VIB nucleomics
604 (Ghent, Belgium) using an Illumina Hiseq 2000. Paired-end reads supplied in fastq
605 format were trimmed using Fast X0.0.13 to remove reads with Q<20 or read length
606 <35bp. Adapters were removed using cutadapt 1.2.1. Reads were further filtered to
607 remove those with greater than 90% A content (poly-A reads), all ambiguous reads
608 containing an N in any position, reads with <Q25, and artefact reads using FastX

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0.0.13 and ShortRead 1.20.0. Contaminant reads were removed by discarding reads that aligned to phix_illumina using Bowtie 2.1.0. Sequencing data in fastq format can be obtained from NCBI SRA (ncbi.nlm.nih.gov/sra) under accession SRP119118. Paired-end reads were aligned to the TAIR10 reference genome (Arabidopsis.org) using Bowtie2 v.2.0.2 (Langmead 2010). SNP calling was performed using SAMtools 0.1.18 mpileup and bcftools (Li 2011). Vcf files were converted to SHORE format using SHOREmap 2.1 convert. Allele frequency estimation and plots were generated using SHOREmap backcross. The Ws-2 parental strain and Ws-2 1001 genomes project (http://1001genomes.org/data/MPI/MPIcollab2011/releases/current/strains/Ws-2/) were used for background correction for BC_1F_2 in SHOREmap backcross. SNPs with background frequency <16 were discarded. The workflow was automated in a pipeline using bpipe 0.9.8.5. (Supplemental Table S3). Sliding allele frequencies were generated for SNPs based on the R statistic in SHOREmap.

SNP verification with dCAPS and Sanger sequencing. Genomic DNA was extracted from 300 µg plant material using the Plant Mini Kit (Qiagen). DNA was eluted into 150 µl of dH_2O (Sigma, UK). dCAPS was used to verify and genotype SNPs in wild-type, BC_1F_2 pools and BC_2F_3 pedigrees. Primers and restriction enzymes used for dCAPS were as follows, product sizes once amplicons had been digested are given in brackets: AT3G02260 F: TTAACATGTAATGTATTCCTCTGCA R: TCCAGTTTCCTCGTTACTGAC HindIII 300 bp (276 bp, 24 bp), AT3G02330 F: GAGATTTCGTGACCTGGAACG R: GCATCTCTCGAATAAGCTCTAATG TasI 300 bp (276 bp, 24 bp), AT3G03070 F: CTAGTCGGCAATCACACCG R: TTTCAGAAATGAACAATTCCCTGT BsmI 300 (275, 25). PCR reagents were purchased as part of the Biotaq Kit (Bioline, UK) or as part of the Phusion

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634 Polymerase Kit (NEB). PCR products were purified using the QIAquick PCR
635 Purification Kit (Qiagen) and quantified using a nanodrop 2000 (Thermo Scientific).
636 HindIII (Fisher Scientific), TasI (NEB) and BsmI (NEB) reactions were prepared for
637 100 µg DNA in their optimal buffers as specified in their instructions. Restriction
638 digestions were run in a Darwin thermocycler for 4 hours at 37 ^{o}C (HindIII) or 72 ^{o}C
639 (TasI, BsmI). Restriction enzyme reactions were deactivated by addition of 4 M TRIS
640 pH 8.4 purple loading dye (Bioline). Digested and undigested products were run on
641 2.5% and 4% fine molecular biology grade agarose (Bioline) 1x TAE buffer for
642 resolution of small fragments. Hyperladder 100 bp (Bioline) was used for size
643 comparison. Gels were imaged using a transilluminator controlled by GeneSnap
644 software with 80 s exposure. Alternatively, purified PCR products were Sanger
645 sequenced using reverse primers as the sequencing primers. Sequencing was
646 performed by Source Bioscience. Sequencing of SNPs was accepted if the
647 chromagraph had a quality score greater than 20.

648 **Isolation of RNA, determination of size and abundance**

649 *RNA extraction and reverse transcription.* RNA was extracted using RNeasy® Plant
650 Mini Kit (Qiagen) and RNase free DNase set (Qiagen). RNA was double eluted into
651 30 µl RNase-free H_2O. cDNA was generated from RNA using RevertAid First Strand
652 cDNA Synthesis Kit (K1622; Fermentas) using 0.5 µg RNA in a 10µl reaction
653 volume.

654 *RT-PCR and RT-qPCR*. Primers were generated using NCBI-primer BLAST as
655 follows: *AT3G02260* F: GATGGTGAAGCTACTGAGCCT R:
656 CTTCAGCTGGCTCCATAGCA (predicted product size for gDNA 458 bp and cDNA
657 316 bp), UBQ10 F: GGCCTTGTATAATCCCTGATGAATAAG R:

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AAAGAGATAACAGGAACGGAAACATAGT, CCA1 F: GATGATGTTGAGGCGGATG R: TGGTGTTAACTGAGCTGTGAAG, TOC1 F: TCTTCGCAGAATCCCTGTGAT R: GCTGCACCTAGCTTCAAGCA, PRR7 F: GGAAACTTGGCGGATGAAAA R: CGAGGGCGTTGTTCTGCT, CHE F: TCCACCGGAAATGGTTTTTG R: GGCGGAAGCTTGCTGTTG. RT-PCR was performed using the PCR settings and electrophoresis described above. RT-qPCR was performed as previously described (Haydon et al., 2013).

Cytosolic-free calcium measurements

Plants grown on agar plates for 11 days were transferred to cuvettes and dosed with coelenterazine to determine the free Ca^{2+} as described in Marti et al., (2013).

Nicotinamide guanine dinucleotide (NGD) assay of ADPR cyclase activity

ADPR cyclase activity was measured using the NGD assay as described in Abdul-Awal et al., (2016) from 3- to 4-week-old plants grown on agar plates. Rosette tissue (5-10 g) pooled from at least 25 rosettes was harvested as a single biological replicate. Data were collected from three biological replicates.

Estimation of circadian parameters

Data were analysed using the BRASS plug-in for MS excel (http:// www.amillar.org) to carry out Fast Fourier Transform Non-Linear Least Squares (FFT-NLLS) analysis and manual phase estimation (Plautz et al., 1997). Rhythms were analysed for at least three cycles in constant light after the first 24 hours. FFT-NLLS was performed with period limits between 18 and 35 hours at 95% confidence level. Phase was calculated using the BRASS peak time analysis function. Rhythms in RT-qPCR and microarray time courses were analysed using JTK-cycle (Hughes et al., 2010) with period limits between 20 and 32 hours.

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682 **Microarray analysis**

683 Microarray datasets were downloaded from array express (E-GEOD-19271 and E-
684 GEOD-50438) and the DIURNAL long day and short day expression sets.

685

686 **Statistical tests**

687 Two-sample T-tests, single-factor ANOVA and Chi-squared statistical tests were
688 performed using MS Excel. Probability of rejecting the null hypothesis (p), calculated
689 T-, F-, or Chi-squared statistic (T, F, x^2) and degrees of freedom (df) are quoted in
690 the text for each analysis in the form (T=n df=n p=n).

691 **Accession numbers**

692 Sequence data for genes used in this study can be found in the Arabidopsis Genome
693 Initiative or GenBank/EMBL databases under the following accession numbers:
694 locus identifiers: BIG (AT3G02260), CAB2 (AT1G29920), CCA1 (AT2G46830),
695 TOC1 (AT5G61380), CHE (AT5G08330), PRR7 (AT5G02810), ZTL (AT5G57360).
696 Sequencing data in fastq format can be obtained from NCBI SRA
697 (ncbi.nlm.nih.gov/sra) under accession SRP119118.

698

699 **Supplemental Materials**

700 **Supplemental Figure S1. Dose response of circadian period to nicotinamide in**
701 **Ws-2, *sin1* and son1.**

702 **Supplemental Figure S2. *son1* segregates with 3:433767 in BC_2F_3**

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Supplemental Figure S3. *son1* plants do not have a circadian period phenotype in the absence of nicotinamide

Supplemental Figure S4. Sequencing of cDNA for *son1* fragments

Supplemental Figure S5. The effect of nicotinamide on delayed chlorophyll fluorescence rhythms in *son1*, *doc1-1* and *tir3-101*

Supplemental Figure S6. Allelism of *son1* and *doc1* is not due to ecotype differences.

Supplemental Figure S7. *doc1-1* phenotype in *son1*

Supplemental Figure S8. *BIG* expression does not oscillate in long or short day photoperiods or in constant light

Supplemental Figure S9. Circadian rhythms of CAB2:LUC^{+} in *BIG* mutants under different light intensities.

Supplemental Figure S10. *son1* does not adjust period due to photoperiod entrainment

Supplemental Table S1 – Results of an M3 forward genetic screen for the effect of nicotinamide on the circadian clock

Supplemental Table S2 - *Arabidopsis thaliana* circadian clock genes with circadian and entrainment phenotypes.

Supplemental Table S3 – bpipe script for mapping by sequencing.

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Figure legends

Figure 1. A forward genetic screen separates period and amplitude effects of nicotinamide. (A) *CAB2:LUC*$^+$ rhythms in wild-type Ws-2 in the presence or absence of 20 mM nicotinamide (NAM) in one entraining 12:12 light dark cycle (black and white bars) and transferred into four days in constant light (white and grey bars) at dawn (Zeitgeber [ZT] 0). Mean FFT-NLLS period estimates are shown ± SEM (n = 8). **(B)** Free-running circadian period and amplitude difference of M3 plants in a forward genetic screen for the effect of 20 mM nicotinamide on circadian oscillations of *CAB2:LUC*$^+$. Period-insensitive mutants (*sin*) are indicated in green, period-oversensitive mutants (*son*) in red and amplitude-sensitive mutants (*san*) in blue. Plants with no detectable nicotinamide-response phenotype in the screen of the M3 population are shown in white, and mean wild-type Ws-2 ± SEM from all experiments (n = 64) is shown overlaid in yellow. Data are pooled from eight separate experiments. **(C)** *CAB2:LUC*$^+$ rhythms in *sin1*, *son1* and *san11* mutants (labelled in **B**) in the presence or absence of 20 mM nicotinamide in one entraining 12:12 light

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748 dark cycle and four days in 70 μmol m^{-2} s^{-1} constant light (n = 8). Data are
749 representative of two independent experiments in the M3 generation.

750

751

752 **Figure 2. A causal mutation in BIG underlies *son1***

753 **(A)** SHOREmap backcross mapping by sequencing analysis of *son1* generated from
754 25-fold coverage Illumina sequence data obtained from 25 BC_1F_2 individual plants
755 with the *son1* phenotype. Individual chromosomes are shown separately with allele
756 frequency sliding window generated with a moving average of 50 kb. A region with
757 allele frequency of one is found on the long arm of chromosome 3. **(B)** SNPs found
758 on chromosome 3 in *son1* with allele frequency of 1.0. Mutations highlighted in red
759 are predicted to cause a functional change in gene product. **(C)** BC_2F_3 segregation of
760 *son1* phenotype with mutation at 3:433767. Period difference in the presence of 20
761 mM nicotinamide was calculated by FFT-NLLS and plotted rounded to nearest 0.2 h.
762 Each line consisted of eight biological replicates, 45 lines were genotyped and
763 phenotyped. Mean and SD for each 3:433767 G-A genotypic sub-population (Wt,
764 heterozygous, and homozygous) were used to plot normal distributions overlaid onto
765 period histogram. **(D)** RT-PCR of *BIG* exons 11-12 showing the effect of *son1* on
766 *BIG* transcript isoforms. Lane 1 has Ws-2 genomic DNA product of predicted size
767 458bp. Lanes 2-4 have *son1* c-DNA products of 314 and 458 bp. Lanes 5-7 have
768 Ws-2 c-DNA product of 316bp. 1 Kb ladder annotated with fragment sizes is shown.
769 Independently isolated BC_2F_3 pedigrees were used. **(E)** Gene structure of
770 *At3G02260* (*BIG*), the potential UBR and ZZ type zinc finger domains and positions
771 of *son1* and *doc1-1* mutations are labelled. **(F)** Circadian period difference between

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the presence and absence of 20 mM nicotinamide for delayed chlorophyll fluorescence rhythms in Ws-2, *son1,* Col-0, *doc1-1* and *son1 doc1-1* F1. Period estimates calculated using FFT-NLLS analysis (Mean ± SE shown, n = 10). Data are representative of two independent experiments. **(G)** Delayed chlorophyll fluorescence rhythm for *son1* x *doc1-1* F1 in the presence or absence of 20 mM nicotinamide (NAM) across four days in constant light. White and grey bars show subjective day and night. Mean ± SEM shown for n = 10. Data are representative of two independent crosses. **(H)** Circadian period difference between the presence and absence of 20 mM nicotinamide for delayed chlorophyll fluorescence rhythms of Col-0, Ws-2, Col-0 x Ws-2 F1, *son1, doc1*, *son1* x ws-2 F1 and *doc1* x Ws-2 F1. Period estimates calculated using FFT-NLLS analysis (Mean ± SEM shown, n = 10).

Figure 3. *son1* affects circadian $[Ca^{2+}]_{cyt}$ signals

Bioluminescence (photon counts/1500 seconds) from *son1* and Ws-2 expressing *35S:AEQUORIN* across two light dark cycles and five days in constant 70 µmol m^{-2} s^{-1} white light grown on 20 mM mannitol **(A)** or 20 mM nicotinamide (NAM) **(B)**. Mean luminescence ± SEM shown, n = 8. Data are representative of three independent experiments in the BC_2F_3 generation. **(C)** ADPR cyclase activity measured using NGD assay at ZT4 in 70 µmol m^{-2} s^{-1} white light from 3-4 week old Ws-2 (white) and *son1* (red) seedlings, mean of three biological replicates shown with SEM. **(D)** $[Ca^{2+}]_{cyt}$ measured at zeitgeber (ZT) 4 and ZT 72 in constant 70 µmol m^{-2} s^{-1} white light from 11 and 14 day old Ws-2 (white) and *son1* (red) seedlings respectively, n=12. Data are representative of three independent experiments in the BC_2F_3 generation.

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Figure 4. *son1* affects circadian clock gene expression in light dark cycles and constant light

(A) *CCA1, PRR7, TOC1* and *CHE* expression from *son1* (red) and Ws-2 (white) samples harvested immediately preceding dawn and dusk. ** represents significance at $p<0.01$ with T-test. Relative expression of genes normalised to UBQ10f expression is given ± standard deviation [n=3].**(B)** *CCA1*, *PRR7*, *TOC1* and *CHE* expression from Ws-2 and *son1* in the absence (left) and in the presence of 20 mM nicotinamide (right) across 48 hours in constant 70 µmol m^{-2} s^{-1} light from ZT24–ZT72. Relative expression of genes normalised to UBQ10f expression is given ± standard deviation [n=3]. Plants were grown as clusters of five plants for 11 days in light dark cycles prior to experiment.

Figure 5. *son1* affects dynamic circadian period adjustment by light and photoperiod

(A) Fluence response curve for circadian period of *CAB2:LUC*$^+$ in Ws-2 and *son1* estimated in equal mixed red and blue light (Mean ± SEM, n = 8-12). Data are pooled from three independent experiments. **(B)** *CAB2:LUC*$^+$ rhythm of *son1* and Ws-2 assayed over five days in constant 5 µmol m^{-2} s^{-1} equal mixed red and blue light (Mean ± SEM, n = 8). Data are representative of two independent experiments in the BC_2F_3 generation. In Figure 4a, the small error bars are obscured by symbols. **(C)** *CAB2:LUC*$^+$ luminescence (counts s^{-1}) from *son1* (red) and Ws-2 (black) seedlings grown in 8:16, 12:12 and 16:8 photoperiods. Plants grown in 12:12 were treated with media supplemented with or without 20 mM nicotinamide (NAM) 2 days

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prior to entrainment in camera chamber (mean ± SEM, n=8). Plants were grown in entrainment conditions from germination and transferred to the camera chamber one day before imaging, maintaining the same entrainment regime. Data are representative of three independent experiments. **(D)** Peak time of *CAB2:LUC*$^{+}$ from LD cycles in (C). Mean peak time of CAB2:LUC+ ± SEM plotted, n=8). ** indicates P<0.05 with T-test.

(E) Photoperiod response curve for circadian period of *CAB2:LUC*$^{+}$ in Ws-2 and *son1* estimated in equal mixed 80 µmol m^{-2} s^{-1} red and blue light (Mean ± SEM, n = 8). Data are pooled from two independent experiments. Plants were entrained in either photoperiods of either 16, 12 or 8 hours prior to transfer to constant light. **(F)** *CAB2:LUC*$^{+}$ rhythm of *son1* and Ws-2 entrained in 16:8 LD cycles and released into constant light for five days (Mean ± SEM, n = 8).

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