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Title: Sumoylation contributes to timekeeping and temperature compensation of the plant circadian clock

Running title: Sumoylation and temperature compensation

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ABSTRACT (300 WORDS)

The transcriptional circadian clock network is tuned into a 24-hour oscillator by numerous post-translational modifications on the proteins encoded by clock genes, differentially influencing their sub-cellular localisation or activity. Clock proteins in any circadian organism are subject to post-translational regulation, and many of the key enzymes, notably kinases and phosphatases, are functionally conserved between the clocks of mammals, fungi, and plant. We now establish sumoylation, the post-translational modification of target proteins by the covalent attachment of the small ubiquitin-like modifier protein SUMO, as a novel mechanism regulating key clock properties in the model plant Arabidopsis. Using two different approaches, we show that mutant plant lines with decreased or increased levels of global sumoylation exhibit shortened or lengthened circadian period, respectively. One known functional role of sumoylation is to protect the proteome from temperature stress. The circadian clock is characterised by temperature compensation, meaning that proper timekeeping is ensured over the full range of physiologically relevant temperatures. Interestingly, we observed that the period defects in sumoylation mutant plants are strongly differential across temperature. Increased global sumoylation leads to undercompensation of the clock against temperature and decreased sumoylation to overcompensation, implying that sumoylation buffers the plant clock system against differential ambient temperature.

KEYWORDS

Sumoylation, *Arabidopsis thaliana*, temperature compensation, post-translational modification (PTM), circadian period.

INTRODUCTION

Post-translational modification instantly alters the characteristics of target proteins and provides organisms with a rapid means to respond to changes in their surrounding environment without having to

realign transcription or translation to that change. Circadian timekeeping is dependent on post-transcriptional and post-translational regulation of clock gene products to tune the transcriptional system into a 24-hour oscillator (Gallego and Virshup, 2007; Mehra et al., 2009; Seo and Mas, 2014).

Like in other higher clades of circadian organisms, the most abundant post-translational modification identified to date in the plant circadian clock is phosphorylation (Hsu and Harmer, 2013; Choudhary et al., 2015). Phosphorylation can alter the stability of clock proteins and alter their function (Fujiwara et al., 2008). Interestingly, the role of post-translational modifications in timekeeping appears to be well-conserved. For example Casein Kinase (CK) 1 and 2 are involved in circadian regulation in mammals and fungi (Lee et al., 2009; Querfurth et al., 2011). Both kinases were recently implicated in timekeeping in the simplistic proto-clock system of the green algae *Ostreococcus tauri* (O'Neill et al., 2011; van Ooijen et al., 2013a; van Ooijen et al., 2013b; Le Bihan et al., 2015). In plants, phosphorylation of CCA1 by Casein Kinase (CK) 2 affects its binding to target promoters (Daniel et al., 2004; Portolés and Más, 2010).

Increasing evidence implicates several additional PTMs in timekeeping in various model organisms (van Ooijen and Millar, 2012). For example, sumoylation functions as a regulator of the mammalian clock through dynamic modification of BMAL1 (Cardone et al., 2005; Lee et al., 2008). Recent proteomic datasets suggest that sumoylation in plants often co-regulates function of target proteins with phosphorylation (Nukarinen et al., 2017). Sumoylation is the modification of proteins by the Small Ubiquitin-related MOdifier, SUMO; a reversible and highly dynamic modification that is conserved throughout the eukaryote lineage (Melchior, 2000). SUMO is a ~10 kDa protein that structurally resembles ubiquitin and requires maturation by SUMO proteases before attachment to a target protein (Ha and Kim, 2008). SUMO is attached covalently to a lysine residue in the substrate protein, in an ATP-dependent reaction involving an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (Fig. 1) (Gareau and Lima, 2010). Whilst E1 and E2 are vital enzymes (Saracco et al., 2007), E3 is

biochemically not strictly required for the formation of a covalent bond between SUMO and substrate (Desterro et al., 1999). However, *in vitro* and *in vivo* studies show that it does facilitate and enhance binding (Kagey et al., 2003; Miura et al., 2009; Okada et al., 2009). Sumoylation is reversible by deconjugation of SUMO from a substrate protein, by the same SUMO proteases required for maturing SUMO (Chosed et al., 2006; Colby et al., 2006; Gareau and Lima, 2010).

Abiotic stress causes substantial increases in the level of sumoylation and the overall number of sumoylated proteins (Kurepa et al., 2003; Saracco et al., 2007; van den Burg et al., 2010; Miller et al., 2010). Upon heat stress, SUMO conjugation rapidly increases, reducing the overall level of free SUMO. The modification is only transient and sumoylation levels return to normal a few hours after heat shock (Kurepa et al., 2003; Saracco et al., 2007). Increased sumoylation in response to stress is thought to be an important mechanism by which cells can reprogram transcription to cope with a changed environment (Yoo et al., 2006; Tomanov et al., 2013). In plants, many abiotic stress responses are gated by the circadian clock (reviewed in Spoel and van Ooijen, 2013). Plants respond to milder changes in temperature leading to altered growth and development in a process called thermomorphogenesis (Quint et al., 2016). Remarkably, several of the proteins involved in thermomorphogenesis have been identified as sumoylation targets (Conti et al., 2014; Sadanandom et al., 2015; Tan et al., 2015; Lin et al., 2016), indicating that SUMO plays a role not only in regulating response to temperature extremes, but also within the temperature range not regarded as 'stressful'.

The circadian clock is characterised by temperature compensation, which means that the free running period of the clock remains stable over a wide range of physiologically relevant temperatures (Pittendrigh, 1954; Kondo et al., 1993; Somers et al., 1998; O'Neill and Reddy, 2011). Temperature compensation is a defining feature of the circadian clock and though the underlying mechanism(s) is/are not fully elucidated, it is clear that it involves transcriptional, post-transcriptional, and post-translational regulation. In plants, temperature compensation has been shown to involve the clock genes *CCA1, LHY,*

RVE8, PRR7, PRR9, and *GI,* covering all expression phases of a full 24h cycle (Gould et al., 2006; Salomé et al., 2010; Rawat et al., 2011). Additionally, several genes involved in light sensing and photoperiodic responses are involved in temperature compensation (Edwards, 2005; Edwards et al., 2006; Ito et al., 2012; Gould et al., 2013; Nagel et al., 2014; Edwards et al., 2015). At the post-translational level, phosphorylation and in particular CK2 activity, are known regulators of timekeeping and temperature compensation (Sugano et al., 1998; Daniel et al., 2004; Baker et al., 2009; Lee et al., 2009; van Ooijen et al., 2013a). Inhibition of phosphorylation through mutations or chemical treatment compromises temperature compensation in plants, mammals, and fungi (Mehra et al., 2009; Portolés and Más, 2010; Zhou et al., 2015). In this manuscript, we report our investigations into a possible role of sumoylation in circadian timekeeping, and notably, given the known roles of sumoylation in temperature responses, the potential link between sumoylation and temperature compensation of circadian rhythms.

MATERIALS AND METHODS

Plant lines, genotyping, and growth conditions

The Columbia-0 plant lines *ots1 ots2* (Conti et al., 2008); *siz1-2* (Miura et al., 2005); *sum1-1* (Saracco et al., 2007); SUM1-OX, and *sum1-1 35S:amiR*-SUM2 (van den Burg et al., 2010) were described previously. The reporter line *CCA1pro*:LUC*,* in Col-*0* background was kindly provided by Karen Halliday (University of Edinburgh).

*CCA1pro:*LUC Col-0 was crossed with *ots1 ots2* and *siz1* to give *CCA1pro:*LUC *ots1 ots2* and *CCA1pro:*LUC *siz1*. F1-F3 progeny were genotyped by PCR to confirm T-DNA insertions (SIZ1 LP 5'-gagctgaagcatctggttttg-3' and RP 5'-cacgacagatgaagcattgtg-3' (Miura et al., 2007), OTS1 RP 5'-cgacaagaagtggtttagacc-3' (Conti et al., 2008), and LP 5'-gatgatgcaaggaggctagtg-3', OTS2 LP 5'-gcttcttccggtttaaaccac-3' and RP 5'-tttttcttctggcgactcatg-3', and SALK Lba1 5'-tggttcacgtagtgggccatcg-3'

(Alonso et al., 2003)) and presence of transgene (CCA1prom 5'-tccatttccgtagcttctggtctc-3', LUCrev 5'-gccttatgcagttgctctcc-3'). Soil-grown plants were grown under long day conditions (16 hr light / 8 hr dark) at 22°C using 60-80 µmol × m^{-2} × s^{-1} white LED tube light (Impact, T8). Seedlings were grown on half strength Murashige and Skoog media without vitamins and sucrose at 21°C in 100 µmol × m^{-2} × s^{-1} white tube light (Phillips, Alto II).

Luminescent imaging

The protoplast transfection vector system for rapid circadian imaging was provided by David Somers (Kim and Somers, 2010) and experiments were carried out at 20ºC under constant blue (470 nm) and red (630 nm) LED light as described previously (Hansen and van Ooijen, 2016). For imaging of seedlings, 5-6-day old seedlings were sprayed with luciferin (5 mM luciferin (Biosynth AG), 10 mM Tris pH 8, 0.01% Triton), transferred to constant light conditions (50 µmol × m^{-2} × s^{-1} 630 nm red LED light and 25 µmol × m^{-2} × s^{-1} 470 nm blue LED light) at 15°C, 21°C or 27°C, and imaged every 2 h with a CCD camera (Hamamatsu). Images were analysed with MetaMorph software. All Circadian parameters described in this paper were determined using nonlinear regression analysis in GraphPad Prism, similar to what is published in (Putker et al., 2017): A centered fifth-order polynomial fit is performed to remove trend and create a residuals plot of the data on which a circadian damped cosine wave is fitted to determine period length using the user-defined equation: Y= (m*X) + amplitude*exp(-k*X)*cos(((2*π*(X-phase))/period)) where X is time, Y is signal, k is the decay constant (such that 1/k is the half-life), which is constrained to >0. Initial values are chosen automatically (m: 1; phase: 1; period: 24). The range of time that is analysed for both fits is set based on visual inspection of raw data, and usually excludes the first part of any time series where traces can be erratic, as well as the last part that is often dampened. A sample dataset analysed as above can be obtained as a Prism file from the corresponding author on request.

RESULTS

Sumoylation tunes circadian rhythms in plants

Besides the several SUMO isoforms itself, the sumoylation machinery involves the enzymatic activities of a SUMO Activating Enzyme, a SUMO Conjugating Enzyme, SUMO Ligases, and specific SUMO Proteases (Fig. 1). We subjected existing Arabidopsis lines defective in these activities to a cell-based clock assay to rapidly determine if sumoylation could be important for timekeeping. Leaf mesophyll cells were isolated from adult wild-type and mutant plants, and these so-called protoplasts were transfected with a construct carrying the clock-regulated *CCA1* promoter driving expression of the firefly luciferase protein (*CCA1pro:LUC*) for longitudinal imaging of circadian rhythms. This assay provides a rapid and reliable means of assessing clock defects (Kim and Somers, 2010; Hansen and van Ooijen, 2016) in any line where the mutant phenotype does not affect protoplast viability. Most mutant or transgenic lines tested exhibited no or only a minor period difference to wild-type plants (Fig. 2A and B), presumably reflecting the known functional redundancy between isoforms and enzymes within the sumoylation machinery (Saracco et al., 2007) and/or the well-documented capacity of the circadian clock network to buffer against perturbations (Zhang and Kay, 2010). However, a double mutant of the two isoforms of SUMO protease *OVERLY TOLERANT TO SALT* (*ots1 ots2*) exhibited a marked lengthening in circadian rhythms. The *ots1 ots2* mutant exhibits an increased level of overall sumoylation (Conti et al., 2008), suggesting that sumoylation might be a delay mechanism within the clock network. To verify that result, the *ots1 ots2* line was crossed to the stably transgenic, rhythmically luminescent clock marker line *CCA1pro:LUC*. Indeed, a comparison of the resulting luminescent signal reveals that increased global sumoylation affects circadian period length (Fig. 3A). We hypothesised that if increased overall sumoylation slows circadian rhythms, decreased global sumoylation might have the opposite effect. Decreased global sumoylation is observed in the SUMO ligase mutant *siz1* (Miura et al., 2005), which could not reliably be tested in the protoplast assay due to its dwarfed phenotype that affects protoplast

viability. The *siz1* line was therefore crossed to the *CCA1pro:LUC* line to analyse clock defects on a whole-plant level. Circadian rhythms in the resulting line revealed that reduced overall levels of sumoylation are indeed associated with a short circadian period (Fig. 3B), supporting the notion that SUMO ligase SIZ1 and SUMO proteases OTS1/OTS2 exhibit opposing effects in tuning the circadian clock in plants.

Temperature compensation is affected by sumoylation

Given the general role of sumoylation in temperature responses, the effects of *siz1* and *ots1 ots2* on circadian period were analysed at moderate cold and warm conditions. Seedlings of *CCA1pro:LUC siz1*, *CCA1pro:LUC ots1 ots2*, and the parent line *CCA1pro:LUC* Col-*0* were grown at 21ºC for 5-6 days under light-dark cycles, and then transferred to luminescent imaging conditions of constant light at either 15ºC or 27ºC. Remarkably, whilst a long-period circadian phenotype was very evident in the *CCA1pro:LUC ots1 ots2* at 15ºC (Fig. 4A), there was no significant difference in circadian period at 27ºC (Fig. 4A). This result suggests that desumoylation is required for proper temperature compensation at colder temperatures. To test whether the opposite is true for SUMO conjugation, *CCA1pro:LUC* expression in the *siz1* background was compared to the parent line at 15 and 27º C. Astonishingly, we observed no significant period difference at 15º C whilst a strong period shortening effect was observed at 27°C (Fig. 4B). This result shows that in contrast to SUMO protease activity, SUMO ligase activity is especially important for circadian timekeeping at higher temperatures.

The effects of reduced SUMO ligase and protease activity are better visualised if the quantified period differences are plotted relative to those observed in wild-type plants (Fig. 5). Biochemical reactions proceed faster at higher temperatures, except those that are temperature-compensated such as the circadian system. The clock in the *siz1* background is undercompensated at higher temperatures (i.e. speeds up with temperature increase), indicating that SUMO ligase activity is required for temperature

compensation at high temperatures. The clock in the *ots1 ots2* background is undercompensated at the lower temperatures (i.e. slows down with decreased temperature), indicating that SUMO protease activity is required for temperature compensation at low temperatures.

DISCUSSION

The data in figures 1-3 imply that sumoylation contributes to the tuning of circadian period, differentially over moderate temperature ranges. Combined, these data suggest sumoylation is a delay mechanism under normal lab conditions of ~21º C. To compensate clocks against temperature, SIZ1 activity (i.e. SUMO ligase activity (Miura et al., 2005)) negates the speeding that higher temperatures would have, whereas OTS1/2 activity (i.e. SUMO protease activity (Conti et al., 2008)) negates the slowing effect of decreased temperature. It is important to note that although reported differences to controls in this study are relatively small, the true effect of these enzymes on the clock and temperature compensation might well be greater as the several additional SUMO ligases and proteases that have been identified might balance mutant phenotypes. However, no significant period changes were observed upon mutation of both predominant SUMO isoforms nor upon overexpression of the only known conjugating enzyme (Figure 2) in a protoplast assay system at 21 ºC. The authors deem it likely that future studies using stably transgenic clock marker lines in additional mutant backgrounds will reveal clock phenotypes in these mutants that, too, are dependent on environmental conditions: the data in Figure 5 suggest that had the original screen been carried out at 27 or 15 ºC, the *ots1/ots2* or *siz1* mutant backgrounds, respectively, might not have displayed their phenotype. A second important note is that temperature might not be the only environmental factor that influences the overt phenotypes of sumoylation mutants. In the supplementary files of a previous study (Jin et al., 2008), the effects of the siz1 mutation on circadian rhythms in red light versus blue light were compared. The authors of that study concluded that there were no changes in circadian period length, although no statistical analyses were reported. Performing these analyses using

the published period estimates, standard deviations, and replicate numbers (Supplementary Figure 1), we find period differences, while biologically small, that are statistically significant for some genotypes, and, crucially, depend on light conditions. Raw data are only provided for the red light experiment, but based on the published period estimates, a slight period shortening was observed at 21 ºC under blue light: not dissimilar to results presented here. Our current results suggest that this effect might have been more pronounced if experiments were carried out at slightly lower temperatures. Unfortunately, both sets of results are difficult to compare as in the current study results were gathered under red ánd blue light. It is however an interesting avenue for further study to elucidate if the phenotype of the *siz1* mutation and other sumoylation mutants depend on light spectrum as well as temperature.

It is not likely that the effects of sumoylation mutants on the clock are mediated via differential sumoylation of a single target. Indeed, a small number of clock-relevant proteins have already been reported to be sumoylated (Miller et al., 2010; López-Torrejón et al., 2013; Lin et al., 2016). To obtain a general idea of clock-relevant targets that might be subject to sumoylation, we compared the identified SUMO targets from a proteome-wide screen (Miller et al., 2010) with known circadian-expressed transcripts (Covington et al., 2008). From the 297 known SUMO targets for which transcript expression profiles were available, 43% (127) are encoded by a circadian expressed transcript (Fig. 5A and Supplementary Table 1), which is more than the ~30% that would be expected by chance. A GO analysis (Mi et al., 2017) of the 127 SUMO targets that have circadian transcripts returned the protein classes 'transcription factor', and 'nucleic acid binding' as the most frequent (Fig. 5B). A GO-term enrichment analysis comparing the 127 to the set of circadian transcripts returned: helicase activity, negative regulation of transcription (DNA templated), negative regulation of gene expression, and chromatin and chromosome organisation as some of the most enriched terms. Not surprisingly, cellular compartments within the nucleus were enriched, including nucleolus, chromosome and nucleoplasm (Supplementary Table 2-4). Interestingly, histone H2B is part of the overlapping dataset. H2B is conserved in the eukaryote linage,

and sumoylation was observed not only in plants but also in yeast and humans (Nathan et al., 2006; Vertegaal et al., 2006). Modification of H2B by ubiquitin E3 ligases is required for proper timekeeping in both Arabidopsis and mammals (Himanen et al., 2012; Tamayo et al., 2015). Mining existing datasets could point to more proteins on which regulatory sumoylation is functionally conserved, or to proteins with a conserved function in timekeeping. However, not every clock-relevant protein is encoded by a rhythmically expressed transcript, nor does every rhythmically expressed transcript have a product that is involved in timekeeping. Detailed biochemical experimental studies will now be necessary to identify the target proteins through which sumoylation affects timekeeping and, more importantly, to characterise the effect that sumoylation has on the biochemical properties of these target clock proteins.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

NOTES

Supplemental Data Figure 1 and Tables S1-4 are available for download at the website.

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FIGURE LEGENDS

Fig. 1: The sumoylation pathway

A) The precursor SUMO is C-terminally cleaved by SUMO proteases. Mature SUMO is bound by SUMO Activating Enzyme (SAE) and subsequently transferred to SUMO Conjugating Enzyme (SCE). Ligation to a lysine residue can be facilitated by SUMO ligases. One or more lysine residues on a target protein can be sumoylated by a single or a chain of SUMO. Modification by SUMO can be reversed by SUMO proteases. The SUMO machinery enzymes investigated in this study are indicated in bold.

Fig. 2: Sumoylation affects timekeeping in plants

A) Transient expression of *CCA1pro:*LUC in protoplasts from the indicated mutant backgrounds imaged in constant light at 20ºC. SUMO isoforms are indicated (SUM1, SUM2); SCE1 is Sumo Conjugating Enzyme 1; OTS1/2 is Overly Tolerant to Salt homolog 1 or 2; amiR stands for artificial micro-RNA. The *ots1 ots2* data is scaled to the right y-axis. B) Circadian period as calculated from the traces in (A) (Mean value +/- SEM, n = 7 biological replicates for all genotypes except *ots1 ots2* (n = 6). Significant differences from Col-*0* were determined by ordinary one-way ANOVA with Dunnett's multiple comparisons test. *P*-value: >0.05 (n.s.), <0.01 (**), <0.0001 (****)). Result has been replicated in three independent biological replicate experiments.

Fig. 3: SUMO protease and ligase activities have opposing effect on circadian period

Circadian gene expression as reported by *CCA1pro:*LUC was analysed in the Col-*0* background (black traces) compared to the *ots1 ots2* (A) or *siz1* (B) mutant backgrounds at 21ºC. Resulting luminescent traces (left) revealed significant changes to the period (right) of circadian rhythms (mean value +/- SEM of the indicated number of biological replicates; comparisons between mutant data and wild-type using

unpaired t-tests; *P*-values as indicated). Result has been replicated in three independent biological replicate experiments.

Fig. 4: Circadian rhythms in *ots1 ots2* and *siz1* at high and low temperatures

Seedlings of CCA1pro:LUC *ots1 ots2* (A-B) and CCA1pro:LUC *siz1* (C-D) compared to the parent line CCA1pro:LUC Col-*0* imaged at 27°C (top) or 15°C (bottom) in constant light over 4 days. Luminescent traces revealed significant changes to the period of circadian rhythms (mean value +/- SEM of the indicated number of biological replicates; comparisons between mutant data and wild-type using unpaired t-tests; *P*-values as indicated). Result has been replicated in two independent biological replicate experiments.

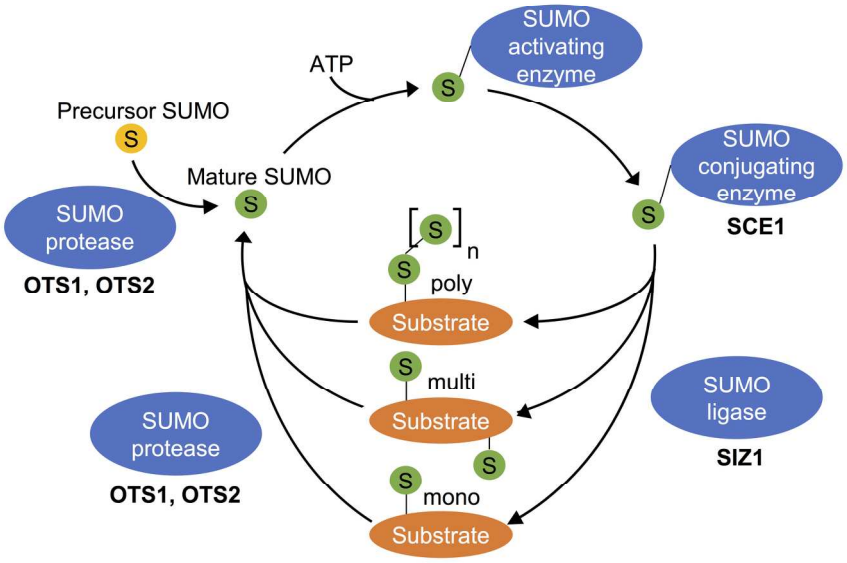
Fig. 5: Differential effects on the circadian period of mutants of the SUMO machinery across a range of physiologically relevant temperatures

CCA1pro:LUC *ots1 ots2* and CCA1pro:LUC *siz1* period difference from parent line at 27°C, 21°C, and 15°C, from traces in Fig. 3 and 4. Mean of difference in period is provided +/- SEM, significant differences were determined by t-tests between the mutant and the parent line for each temperature. *P*-value: >0.05 (n.s.), <0.01 (**), <0.001 (***), <0.0001 (****). Result has been replicated in two (15 and 27ºC) or three (21ºC) independent biological replicate experiments.

Fig. 6: Overlap between sumoylation targets and circadian transcripts

A) Venn diagram of overlap between sumoylated proteins identified by Miller et al. (2010) and all circadian transcripts in the dataset from Covington et al. (2008). B) Protein classes associated with the overlapping dataset assigned using Panther. Panther protein class IDs indicated in brackets.

Figure 1



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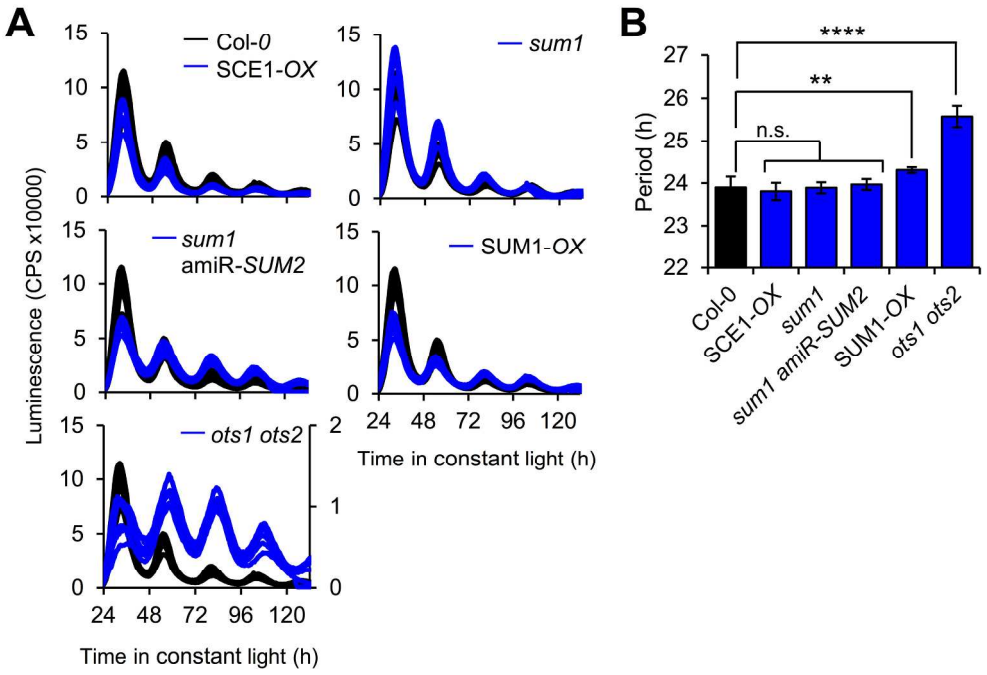
93x62mm (600 x 600 DPI)

Figure 2



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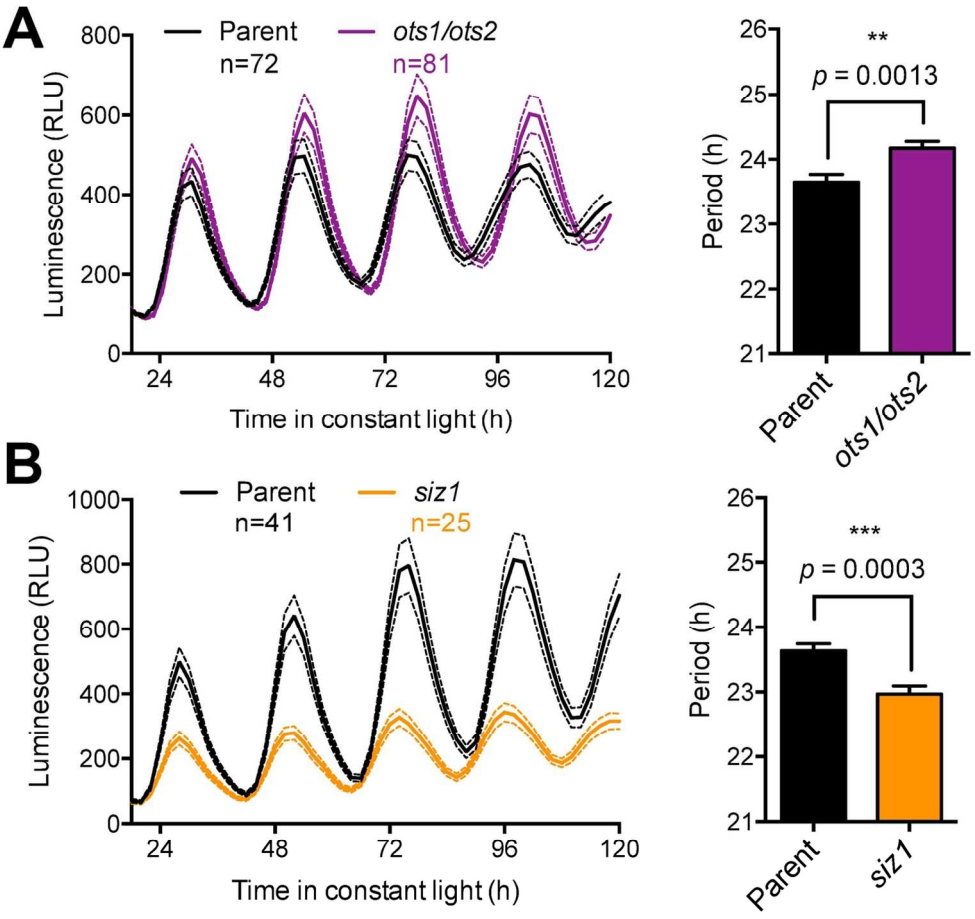
116x87mm (600 x 600 DPI)

Figure 3



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133x141mm (300 x 300 DPI)

Figure 4

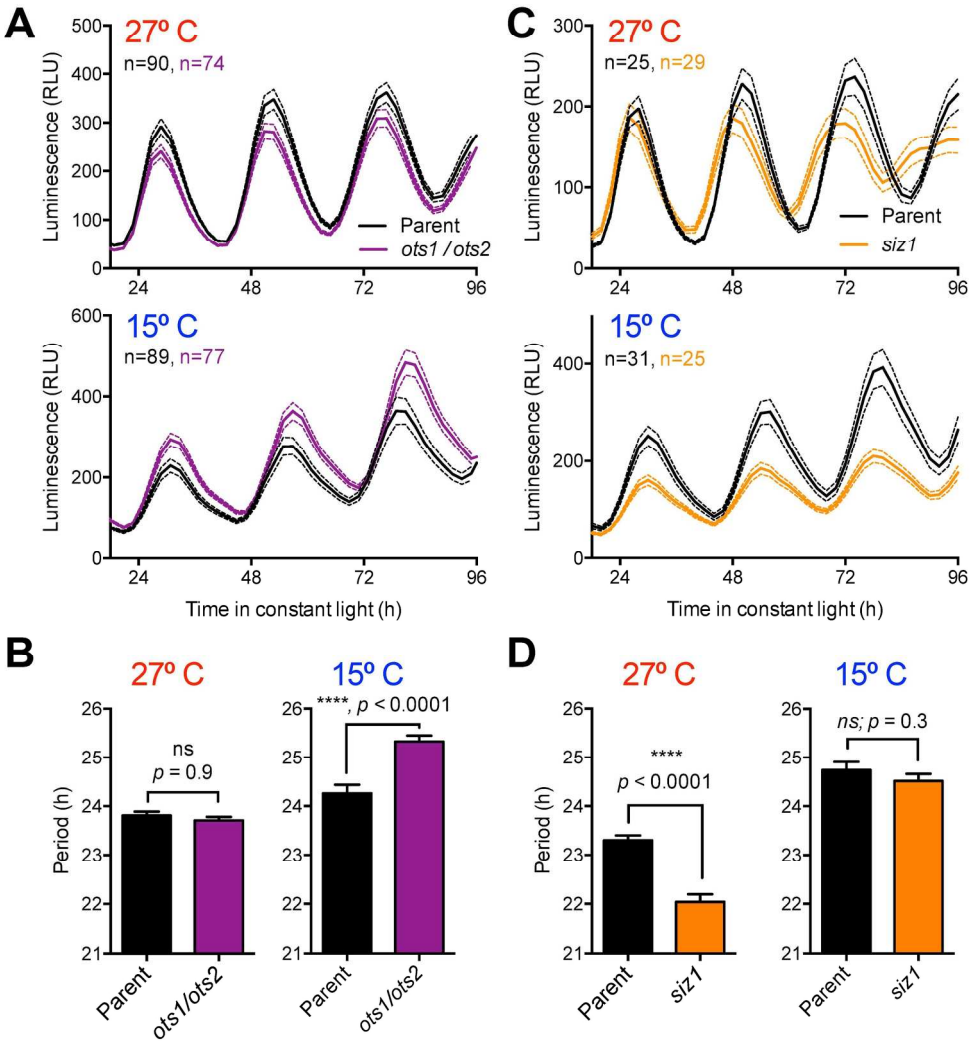
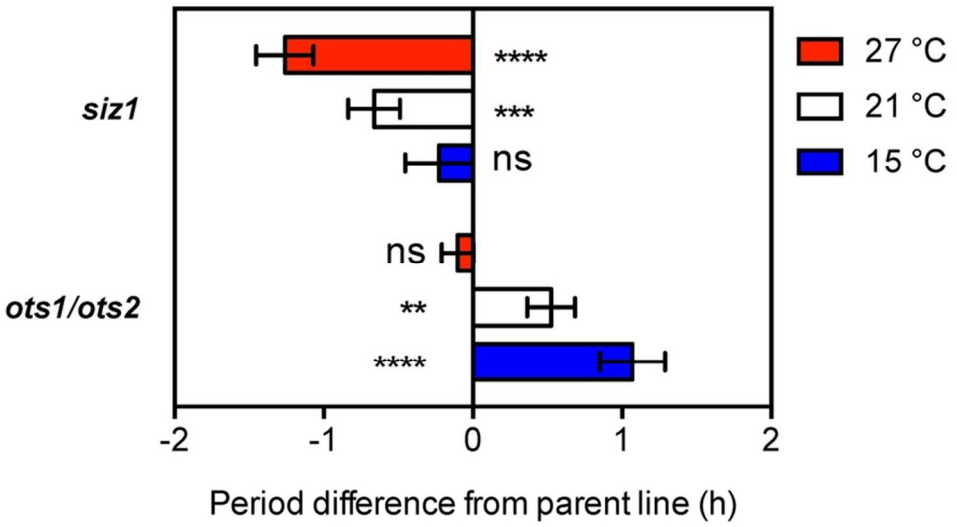


Fig. 4: Circadian rhythms in ots1 ots2 and siz1 at high and low temperatures ‼ ┼ Seedlings of CCA1pro:LUC ots1 ots2 (A-B) and CCA1pro:LUC siz1 (C-D) compared to the parent line CCA1pro:LUC Col-0 imaged at 27°C (top) and 15°C (bottom) in constant light over 4 days. Luminescent traces revealed significant changes to the period of circadian rhythms (mean value +/- SEM of the indicated number of biological replicates; comparisons between mutant data and wild-type using unpaired t-tests; P-values as indicated). ‼ ┼

189x214mm (300 x 300 DPI)

Figure 5



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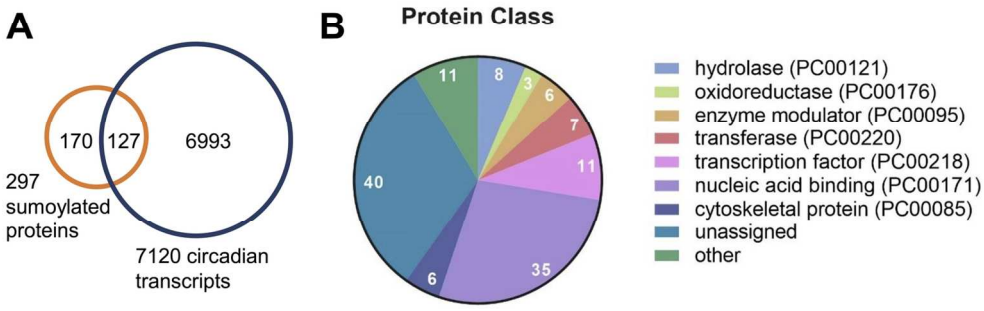
78x53mm (300 x 300 DPI)

view

Figure 6



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67x26mm (600 x 600 DPI)

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Table S1: Locus identifiers of sumoylated proteins (Miller at al. 2010) with circadian transcripts (Covington et al. 2008)

AT1G04410	AT2G24100	AT3G15880	AT4G38890	AT5G12250
AT1G04820	AT2G25170	AT3G15970	AT5G02500	AT5G13950
AT1G07890	AT1G54440	AT3G16830	AT5G04290	AT5G14170
AT1G08130	AT1G56110	AT2G25450	AT5G08130	AT5G15020
AT1G09770	AT1G60670	AT2G25930	AT3G16857	AT5G16270
AT1G13440	AT1G62380	AT2G26230	AT3G20770	AT5G22330
AT1G13940	AT1G63470	AT2G28080	AT3G22520	AT5G23610
AT1G14740	AT1G63660	AT2G32700	AT3G23690	AT5G25060
AT1G15730	AT1G64980	AT2G34900	AT3G42660	AT5G25220
AT1G15750	AT1G70410	AT2G38470	AT3G45980	AT5G26210
AT1G17210	AT1G71040	AT2G41500	AT3G57870	AT5G27650
AT1G18450	AT1G74560	AT2G42300	AT3G58120	AT5G28300
AT1G19700	AT1G76380	AT2G43970	AT4G00730	AT5G41410
AT1G20696	AT1G79730	AT2G44440	AT4G02400	AT5G42520
AT1G24020	AT1G80480	AT2G45460	AT4G02480	AT5G44800
AT1G25540	AT1G80930	AT3G01500	AT4G03430	AT5G47220
AT1G26665	AT2G01250	AT3G01540	AT4G11660	AT5G49530
AT1G27400	AT2G03340	AT3G06480	AT4G17490	AT5G53180
AT1G27650	AT2G06510	AT3G06580	AT4G19880	AT5G55160
AT1G33240	AT2G16600	AT3G09440	AT4G20400	AT5G59830
AT1G44770	AT2G17340	AT3G10030	AT4G20890	AT5G61600
AT1G48380	AT2G18300	AT3G10250	AT4G22670	AT5G62190
AT1G48920	AT2G18750	AT3G12580	AT4G23680	AT5G65630
AT1G50410	AT2G20310	AT3G13670	AT4G25100	
AT1G50670	AT2G21660	AT3G13920	AT4G28610	
AT1G52360	AT2G22430	AT3G14120	AT4G32551	

150x167mm (300 x 300 DPI)

Table S2: Enrichment of GO molecular function terms

GO molecular function	# of circadian transcripts	# of SUMO targets	Expected # of SUMO targets	Fold enrichment	+/-	P value
structural constituent of cytoskeleton	17	5	0.31	16.09	+	0.0175
helicase activity	34	6	0.62	9.65	+	0.0432
DNA binding	475	33	8.69	3.8	+	1.94E-08
nucleic acid binding	814	41	14.88	2.75	+	8.04E-07
organic cyclic compound binding	1623	52	29.68	1.75	+	0.00877
heterocyclic compound binding	1616	52	29.55	1.76	+	0.00771
transcription factor activity, sequence-specific DNA binding	366	21	6.69	3.14	+	0.0033
nucleic acid binding transcription factor activity	366	21	6.69	3.14	+	0.0033
protein binding	959	41	17.54	2.34	+	9.11E-05
Unclassified	2006	41	36.68	1.12	+	0

61x22mm (600 x 600 DPI)

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Table S3: Enrichment of GO cellular component terms

GO cellular component	# of circadian transcripts	# of SUMO targets	Expected # of SUMO targets	Fold enrichment	+/-	P value
nucleolus	103	9	1.88	4.78	+	0.0376
nuclear lumen	181	17	3.31	5.14	+	1.22E-05
intracellular organelle lumen	230	18	4.21	4.28	+	7.03E-05
organelle lumen	230	18	4.21	4.28	+	7.03E-05
membrane-enclosed lumen	230	18	4.21	4.28	+	7.03E-05
nuclear part	227	19	4.15	4.58	+	1.11E-05
nucleus	1995	72	36.48	1.97	+	1.6E-08
intracellular non-membrane-bounded organelle	353	20	6.45	3.1	+	0.00205
non-membrane-bounded organelle	353	20	6.45	3.1	+	0.00205
cytosol	625	26	11.43	2.28	+	0.0174
integral component of membrane	1212	4	22.16	< 0.2	-	1.93E-04
intrinsic component of membrane	1280	4	23.4	< 0.2	-	5.51E-05
membrane part	1386	6	25.34	0.24	-	2.46E-04
membrane	2176	20	39.79	0.5	-	0.0145
Unclassified	1580	35	28.89	1.21	+	0

77x35mm (600 x 600 DPI)

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Table S4: Enrichment of GO biological process terms

GO biological process	# of circadian transcripts	# of SUMO targets	Expected # of SUMO targets	Fold enrichment	+/-	P value
meristem development	46	7	0.84	8.32	+	0.0329
tissue development	108	10	1.97	5.06	+	0.0437
negative regulation of transcription, DNA-templated	47	7	0.86	8.15	+	0.0377
regulation of biological process	1188	43	21.72	1.98	+	0.00511
biological regulation	1290	43	23.59	1.82	+	0.0417
regulation of metabolic process	690	39	12.62	3.09	+	1.13E-07
regulation of cellular process	1073	40	19.62	2.04	+	0.00634
regulation of cellular metabolic process	642	37	11.74	3.15	+	2.36E-07
regulation of cellular biosynthetic process	566	37	10.35	3.58	+	6.35E-09
regulation of biosynthetic process	574	37	10.5	3.53	+	9.56E-09
regulation of cellular macromolecule biosynthetic process	548	36	10.02	3.59	+	1.17E-08
regulation of macromolecule biosynthetic process	549	36	10.04	3.59	+	1.23E-08
regulation of macromolecule metabolic process	617	38	11.28	3.37	+	1.76E-08
negative regulation of gene expression	88	10	1.61	6.21	+	0.00765
regulation of gene expression	584	38	10.68	3.56	+	3.42E-09
regulation of nitrogen compound metabolic process	590	37	10.79	3.43	+	2.12E-08
regulation of nucleobase-containing compound metabolic process	520	36	9.51	3.79	+	2.6E-09
regulation of primary metabolic process	605	37	11.06	3.34	+	4.38E-08
regulation of RNA metabolic process	511	36	9.34	3.85	+	1.57E-09
regulation of RNA biosynthetic process	504	35	9.22	3.8	+	5.29E-09
regulation of nucleic acid-templated transcription	504	35	9.22	3.8	+	5.29E-09
regulation of transcription, DNA-templated	503	35	9.2	3.81	+	5E-09
chromatin organization	62	8	1.13	7.06	+	0.0278
chromosome organization	82	11	1.5	7.34	+	0.00052
response to metal ion	170	13	3.11	4.18	+	0.0211
transcription, DNA-templated	404	30	7.39	4.06	+	5.2E-08
cellular macromolecule biosynthetic process	642	37	11.74	3.15	+	2.36E-07
macromolecule biosynthetic process	659	37	12.05	3.07	+	4.91E-07
macromolecule metabolic process	1434	50	26.22	1.91	+	0.00159
organic substance biosynthetic process	1165	40	21.3	1.88	+	0.0457
cellular biosynthetic process	1118	40	20.44	1.96	+	0.0173
cellular macromolecule metabolic process	1297	47	23.72	1.98	+	0.00134
nucleic acid-templated transcription	404	30	7.39	4.06	+	5.2E-08
RNA biosynthetic process	405	30	7.41	4.05	+	5.52E-08
RNA metabolic process	568	37	10.39	3.56	+	7.04E-09
nucleic acid metabolic process	626	41	11.45	3.58	+	2.76E-10
nucleobase-containing compound metabolic process	756	42	13.82	3.04	+	2.77E-08
organic cyclic compound metabolic process	964	42	17.63	2.38	+	4.49E-05
cellular nitrogen compound metabolic process	1001	45	18.3	2.46	+	4.23E-06
heterocycle metabolic process	861	42	15.74	2.67	+	1.59E-06
cellular aromatic compound metabolic process	908	42	16.6	2.53	+	7.82E-06
nucleobase-containing compound biosynthetic process	471	31	8.61	3.6	+	4.49E-7
organic cyclic compound biosynthetic process	645	32	11.79	2.71	+	1.89E-04
heterocycle biosynthetic process	557	31	10.18	3.04	+	2.37E-05
cellular nitrogen compound biosynthetic process	709	35	12.96	2.7	+	4.61E-05
aromatic compound biosynthetic process	583	31	10.66	2.91	+	6.69E-05
gene expression	686	39	12.54	3.11	+	9.55E-08
Unclassified	2110	39	38.58	1.01	+	0

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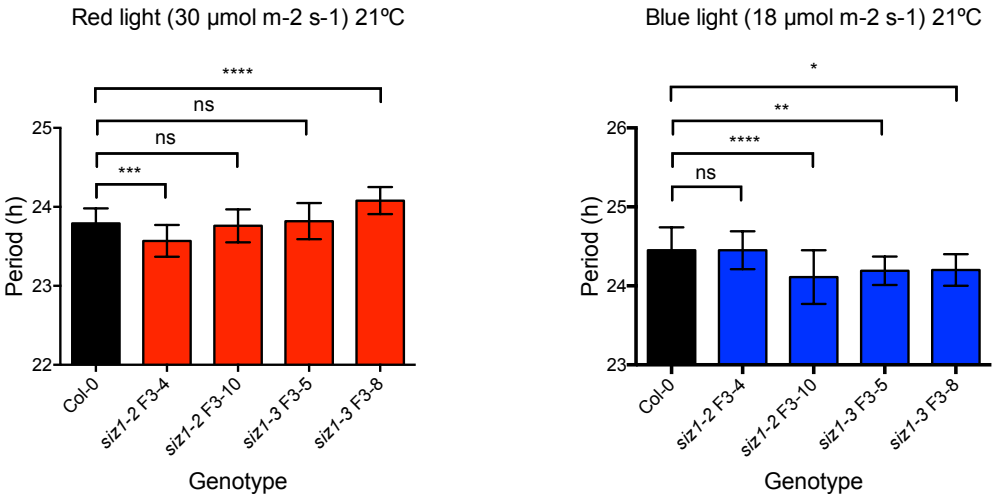

Supplemental Figure S1:
Analysis of the *siz1* mutant line period estimates reported in Jin *et al.* 2008
Ordinary one-way ANOVA with multiple comparisons to the Col-0 wild-type control, using the period, SD, and replicate numbers as provided in Jin *et al.*, 2008 (Table S1 in that paper). Small but in some cases significant differences are observed that depend on genotype as well as light conditions.

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