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Sumoylation of the Plant Clock Transcription Factor CCA1 Suppresses DNA Binding

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Running title: Sumoylation of CCA1

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ABSTRACT (300 WORDS)

In plants, the circadian clock regulates the expression of a third of all transcripts and is crucial to virtually every aspect of metabolism and growth. We now establish sumoylation, a post-translational protein modification, as a novel regulator of the key clock protein CCA1 in the model plant Arabidopsis. Dynamic sumoylation of CCA1 is observed *in planta* and confirmed in a heterologous expression system. To characterise how sumoylation might affect the activity of CCA1, we investigated the properties of CCA1 in a wild-type plant background in comparison to *ots1 ots2*; a mutant background showing increased overall levels of sumoylation. Neither the localisation nor the stability of CCA1 was significantly affected. However, binding of CCA1 to a target promoter was significantly reduced in chromatin-IP experiments. *In vitro* experiments using recombinant protein revealed that reduced affinity to the cognate promoter element is a direct consequence of sumoylation of CCA1 that does not require any other factors. Combined, these results suggest sumoylation as a mechanism that tunes the DNA binding activity of the central plant clock transcription factor CCA1.

KEYWORDS

Sumoylation, *Arabidopsis thaliana*, CCA1, post-translational modification (PTM), DNA binding.

INTRODUCTION

Circadian rhythms are driven by an intrinsic biological timekeeping system, the circadian clock, which exists in animals, plants, fungi, and even certain prokaryotes (Zhang and Kay, 2010). In plants, clock defects can decrease both carbon fixation and biomass production by about half (Dodd et al., 2005), highlighting the importance of circadian timekeeping for food crop productivity. The circadian clock drives the rhythmic expression of about 30% of all genes in the model plant *Arabidopsis thaliana* (Covington et al., 2008). This daily transcriptional reprogramming is achieved through a set of 'clock

genes' that indirectly regulate their own expression via a complex circuitry of Transcriptional-Translational Feedback Loops (TTFLs). The TTFL system in Arabidopsis contains a crucial morning-phased transcription factor complex of CIRCADIAN CLOCK ASSOCIATED (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). The CCA1/LHY complex represses expression of the evening-phased gene *TIMING OF CAB2 EXPRESSION 1 (TOC1)* and is thought to activate the expression of other clock genes such as *PSEUDO-RESPONSE REGULATOR 9 (PRR9)* (Farre et al., 2005). However, recent studies have found that CCA1/LHY also repress the PRR-genes (Kamioka et al., 2016). TOC1 and PRR9 proteins feed back to repress *CCA1* and *LHY* expression. *CCA1/LHY* expression is indirectly activated by a so-called Evening Complex (EC), which represses *PRR9* expression (Hsu and Harmer, 2013).

In all rhythmic organisms, timekeeping depends on post-translational modification of TTFL proteins, to tune the expression and localisation dynamics of clock proteins and turn the clock system into a 24-hr oscillator. Phosphorylation is the most characterised post-translational modification on circadian clock proteins in any organism, but it is evident that other modifications dynamically regulate clock proteins and might be equally important for clock function. For example, the cellular machinery that modifies proteins by the covalent attachment of the Small Ubiquitin-related MOdifier (SUMO) is necessary for clock function in mammals (Cardone et al., 2005; Lee et al., 2008), is fully conserved across eukaryotes (Johnson, 2004), and has now been shown in this issue of JBR to also influence timekeeping in the model plant Arabidopsis. In this study, we identified the crucial clock transcription factor CCA1 as a target of sumoylation. Although sumoylation is an abundant modification, very few examples exist (especially in plants) of functional consequences on specific targets. Our results show that reduced DNA binding affinity is a direct effect of CCA1 sumoylation, providing a striking new example of the effect of sumoylation on a specific target protein.

MATERIALS AND METHODS

All chemicals were obtained from Sigma Aldrich unless otherwise stated. Restriction enzymes were from New England Biolabs. H_2O means double-distilled water. All oligonucleotides used in this study are listed in Table S1, and all novel DNA constructs reported here were verified by sequencing. All results presented are representative of at least two independent experiments.

Plant lines, genotyping, and growth conditions

The Arabidopsis Columbia-0 plant lines *ots1 ots2* (Conti et al., 2008); *CCA1pro:*CCA1-3xHA-YFP *cca1* (Yakir et al., 2009), here referred to as CCA1-3HY, were described previously. CCA1-3HY was crossed with *ots1 ots2* to give CCA1-3HY *ots1 ots2*. F1-F3 progeny were selected for T-DNA insertions and transgenes by PCR-based genotyping. Soil-grown plants were grown under long day conditions (16 hr light / 8 hr dark) at 22°C unless otherwise stated. Seedlings were grown on half strength Murashige and Skoog media without vitamins (0.5 MS) at 21°C in 100 μmol $\times$ m^{-2} $\times$ s^{-1} white light.

HA-immunoprecipitation

For HA-pulldowns of sumoylated CCA1, leaf material was homogenised in liquid nitrogen and ground in extraction buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM DTT, 10% glycerol, 0.2% NP-40, 0.1% Triton X-100, 20 mM *N*-ethylmaleimide (NEM), 50 μM MG132 and 50 μM PR-619, Complete protease inhibitor tablet (Roche), PhosStop cocktail (Roche), 1% PVPP). Cell debris was removed by centrifugation and filtration through a 0.22 μm filter. The crude extract was incubated with anti-HA antibody-conjugated agarose beads (Roche #11815016001) for 4 h rotating at 4°C. Beads were pelleted and washed four times in extraction buffer. Bound protein was eluted from the beads in LDS protein loading buffer (Invitrogen) at 70°C. Proteins were separated on a 4-12% polyacrylamide gels (Invitrogen Novex) and transferred to nitrocellulose membranes using the iBlot system (Invitrogen).

Primary antibodies were anti-HA-tag (1:5000, abcam #ab20084), anti-SUMO1/2 (1:2000-5000, abcam #ab5316), and anti-Ubiquitin (1:1000, Millipore #04-263). An important note is that the results in Fig. 1 are consistently observed with batch numbers GR143233-1, GR38587-1, and GR272641-1 of the SUMO antibody, but not with batches GR94030-1 and GR244508-1. SuperSignal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific) was used to visualize hybridized bands according to the manufacturer's instructions.

Heterologous sumoylation of CCA1

An *E. coli* sumoylation system (Okada et al., 2009) was used for heterologous sumoylation of CCA1 with a His-tag or a MBP-tag. A culture of BL21 (DE3) cells expressing pCDFDuet-*AtSUMO1*-(GG or AA)-*AtSCE1*, pACYCDuet-*AtSAE1a-AtSAE2* and pET28a-*CCA1-His/MBP* was grown ~3 hours to OD_{600} 0.8 at 37°C. Overnight expression at 17°C was induced by adding 200 µM IPTG.

For MBP-tag purification, cells were pelleted and resuspended in 60 ml ice-cold wash buffer (1x PBS, 1x Complete protease inhibitor (Roche), 1 mM DTT, 20 mM $MgSO_4$), aliquoted into 10 ml tubes, and flash frozen after the addition of 0.1 % Triton. Per experiment, two tubes were thawed by rotation at room temperature in the presence of lysozyme (1 mg/ml) and benzonase nuclease (0.5 µl/ml, Merck Millipore). When lysed, the cell extract was cleared by centrifugation for 60 min at 50228 *g*. To the supernatant, 0.4 ml amylose beads was added and incubated rotating in cold room for ~1 hour. Beads were pelleted and washed in wash buffer 2 (HBS-EP+ buffer, 1 mM TCEP, 1x Roche Complete protease inhibitors). Washed beads were pelleted and eluted in 5 ml elution buffer (wash buffer, 20 mM maltose) for 10 min. Beads were removed by centrifugation and the supernatant was dialysed overnight to remove the maltose. For His-tag purification, the samples were pelleted and snap frozen. Samples were thawed on ice and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1% Triton X100, 5% glycerol, 1 mM DTT, 1x Protease inhibitor without EDTA (Roche), 1 mM PMSF, pH 8.0) with 1mg/ml

lysozyme. The cells were further lysed by sonication. RNase A and DNase I were added to reduce the viscosity. Cell debris was pelleted by centrifugation at 20,000 g for 20-30 min or 4,000 g for 60 min at 4°C. The supernatant was collected for His-purification. Ni-NTA agarose beads were equilibrated in lysis buffer. The protein extract was applied to the beads and the mix was incubated 1-2 hours rotating at 4°C. The beads were pelleted by centrifugation at 100 g at 4°C for 1 min, and the supernatant was removed. The beads were washed 3-5 times in wash buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 20mM imidazole 1x Protease inhibitor without EDTA (Roche), 1 mM PMSF, pH 8.0) before eluting the proteins from the beads with elution buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 250mM imidazole, pH 8.0). A second aliquot of elution buffer was added to the beads, they were heated to 96°C for 10 min, and the eluate was collected.

For blotting, (MBP- and His-purified) protein samples were separated on 4-12% polyacrylamide gels (Invitrogen Novex) and transferred to nitrocellulose membranes using the iBlot system (Invitrogen). The primary antibody was anti-T7-tag (1:15000, Novagen).

Protein identification by mass spectroscopy

The His-purified protein samples were concentrated by TCA-precipitation. 1 volume ice cold 20% TCA was added to the sample and incubated on ice for 30 min. Protein was pelleted by centrifugation at 17,000 g at 4°C for 15 min. The pellet was washed in ice cold acetone and centrifuged 5 min at 17,000 g at 4°C twice, then air dried. The pellet was resuspended in 1 x LDS sample buffer (Invitrogen) with 100 mM DTT and separated on a 4-12% polyacrylamide gel (Novex) and stained with SimplyBlue Safestain (Invitrogen). The bands of interest were excised and prepared for mass spectrometric analysis. Gel slices were shrunk by twice adding 200 µL methanol for 10 min and replacing it with 200 µL 50 mM ammonium bicarbonate (ABC) for 10 min. 200 µL methanol was added a third time, replaced by 100 µL 20 mM DTT and the samples were incubated at 60°C for 30 min. After shrinking the gel pieces in

methanol again, 100µL 55mM NEM was added and incubated at room temperature for 60 min to block cysteine residues. After incubating the gel pieces twice in methanol, they were digested overnight with 20 µL trypsin in 80 µL 50 mM ABC. The supernatant was saved, and additional peptides were extracted by incubating the gel pieces 15 min in 80 µL 1% FA, followed by 80 µL 1% FA in 50% methanol, and 80 µL methanol twice. The solutions were combined, dried in a speedvac and resuspended in methanol for LC-MS/MS analysis. LC-MS/MS data was analysed with MASCOT. The SUMO Interaction Motif at CCA1 amino acids 598-593 was identified using the online GPS-SUMO tools available at http://sumosp.biocuckoo.org/online.php.

Site-directed mutagenesis and cloning of hepta-mutant

The putative sumoylation sites (K117, K124, K393, K483, K559, K595, K598) identified by mass spectrometry were mutated from lysine to arginine by site-directed mutagenesis. The primers, containing a single nucleotide mutation, were designed using Quickchange software (Agilent Technologies). Template DNA was removed by *DpnI* digestion from the PCR product, which was transfected to OneShot *E. coli* cells (Invitrogen). The N-terminal triple mutant of *CCA1* was generated by successive site-directed mutagenesis reactions of K117, K124 and K393 in one pET28a-*AtCCA1* plasmid (pET28a-*CCA1*-Nterm) and the quadruple C-terminal mutant containing substitutions of K483, K559, K595 and K598 in another pET28a-*AtCCA1* plasmid (pET28a-*CCA1*-Cterm). The two parts of CCA1 were combined through restriction digest at the *Age*I and *Xho*I sites and subsequent T4 DNA ligation to generate the hepta-mutant.

Protoplast assays

For confocal imaging vectors, the *CCA1pro:CCA1-YFP* DNA sequence was amplified from a vector obtained from Laszlo Bodnar (Hungarian Academy of Sciences), adding the restriction sites *Not*I and *Stu*I

and this product was ligated into pOmega+ to give *CCA1pro:*CCA1-YFP. For localisation studies of SUMO fusions, the coding sequences of *SUMO1* (At4g26840), *SUMO2* (At5g55160), *SUMO3* (At5g55170) were amplified to add *Bam*HI sites at the 5' and 3' ends and the fragment was introduced at the *Bam*HI site, yielding a N-terminal fusion protein of SUMO to CCA1 in the pOmega *CCA1pro:CCA1-YFP* plasmid. Over-expresser constructs were created by replacing the CCA1 promoter sequence with the *35S* promoter sequence amplified from pCambia *35Spro:*NanoLUC kindly provided by Andrew Millar (University of Edinburgh), using PCR primers to introduce *Not*I and *Bam*HI sites that allowed cloning into the pOmega vector set. The YFP controls *CCA1pro:YFP* and *35Spro:YFP* were generated by excising the coding sequences of *SUMO* and *CCA1* with *Bam*HI and *Hind*III, blunting the linearised vectors (NEB E1201S) and re-ligation with T4 DNA ligase. Protoplasts for confocal imaging were generated as described in (Hansen and van Ooijen, 2016), transfected with 30-40 μg plasmid and resuspended in 1 ml W5 solution (Hansen and van Ooijen, 2016) before being transferred to a 6-well plate pre-treated with 1% BSA in W5 solution. The protoplasts were kept in 30 μmol m^{-2} s^{-1} white light overnight at 21°C. A 35 μl aliquot of protoplasts was imaged on a Leica SP5 in photon counting mode with a 20x objective. Excitation wavelength was 514 nm for detection of YFP-signal and 594 nm for chloroplast autofluorescence detection. The images were analysed in Fiji (Schindelin et al., 2012).

For circadian gene expression assays in protoplasts, techniques were modified from (Kim and Somers, 2010) identically to what is detailed in the accompanying manuscript.

Cell-free protein degradation assay

The cell-free protein degradation assay was adapted from (Más et al., 2003). Col-0, *ots1 ots2*, CCA1-3HY, and CCA1-3HY *ots1 ots2* adult plants grown at 12 h light/ 12 h dark were sampled at dawn. Plant material was homogenised in liquid nitrogen and protein extracted in extraction buffer (50 mM Tris–HCl pH7.4, 300 mM NaCl, 10 mM MgCl$_2$, 5 mM DTT, 5 mM ATP, 0.2% NP-40, 0.1% Triton X-100). Cell

debris was removed by centrifugation and filtration through a 0.22 μm filter. Protein content was quantification by Bradford assays. The samples were transferred to a room temperature water bath and at the indicated times aliquots were transferred to fresh tubes containing 4xLDS containing DTT and stored at -20°C immediately. Samples were separated on 4-12% polyacrylamide gels (Invitrogen Novex), transferred to nitrocellulose membranes using the iBlot system (Invitrogen), and detected using an anti-HA antibody (1:5000, abcam #ab20084).

ChIP analyses

Chromatin immunoprecipitation (ChIP) was based on procedures described by (Nagel et al., 2015). Briefly, 0.5 g 3-week-old seedlings were submerged in 1% formaldehyde, and vacuum (-70kPa) was drawn for 2x 15 min. Glycine was added to a final concentration of 0.127 M to quench the crosslinking and vacuum (-70kPa) was drawn for a further 5 min. Seedlings were washed once in ice cold PBS and snap frozen in liquid N_2. Tissue was ground in nuclei extraction buffer (100 mM MOPS pH 7.6, 10 mM $MgCl_2$, 250 mM sucrose, 5% Dextran T-40, 2.5% Ficoll 400, 40 mM β-mercaptoethanol, 20 mM NEM, 50 μM MG132, 50 μM PR-619, 1x Complete protease inhibitor (Roche), 1 x PhosStop (Roche)), filtered through nylon mesh, and the nuclei were pelleted by centrifugation at 10,000 g at 4°C. The nuclei were lysed in nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS) and diluted in ChIP dilution buffer (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA pH 8, 0.01% SDS, 20 mM NEM, 50 μM MG132, 50 μM PR-619, 1x Complete protease inhibitor (Roche), 1x PhosStop (Roche)) before the DNA was sheared by sonication (Diagenode BioRuptor Plus). The samples were further diluted and Triton X-100 was added to give a concentration of 1.1%. Cellular debris was removed by centrifugation and samples were pre-cleared with Protein G agarose beads (Invitrogen). 1/50 of the samples was kept as input before the remainder was split in two. 20 μg GFP antibody (abcam, ab290) was added to one aliquot, the other was used as mock. Samples were incubated for 5 hrs rotating at 4°C,

Protein G beads were added and the samples were incubated for 2 hrs. The beads were collected by centrifugation and washed once in a series of ice-cold wash buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA pH 8, 500 mM NaCl), LiCl buffer (250mM LiCl, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and 0.5x TBE. DNA was precipitated from the input samples by ethanol precipitation. 10% Chelex resin was added to input and IP samples and the samples were incubated at 95°C for 10 min, followed by Proteinase K treatment for 30 min at 55°C. DNA was purified from the supernatant using a PCR purification Kit (Qiagen). DNA was quantified by qRT-PCR with primer pairs listed in Table S1. Abundance of DNA in the IP samples was calculated as % of Input = $(2^{-\Delta Ct})$ x 100%.

Electrophoretic mobility shift assay

Biotin-labelled DNA duplexes *PRR9* or *mutPRR9* (Table S1) were prepared by mixing the biotin-labelled forward oligonucleotide and the reverse oligonucleotide to an equimolar concentration of 4 nM and incubating the mixture at 55°C. Purified recombinant MBP-tagged CCA1 or sumoylated MBP-tagged CCA1 were added to the binding reaction mix (0.4 nM biotin-labelled DNA duplex, 1µg/µl poly (dI•dC), 12mM HEPES, 50mM KCl, 20% glycerol, 1x protease inhibitor (50 µg/ml TPCK, 50 µg/ml TLCK, and 0.6 mM PMSF) at 1 nM, 10 nM or 45 nM of protein. After incubation at room temperature the samples were separated on a native PAGE gel (4% acrylamide/bis-acrylamide (37.5:1), 0.5 x TBE, 0.1% ammonium persulfate (APS), 0.08% TEMED) at 70V at 4°C. Protein-DNA complexes and free DNA duplexes were transferred to a nylon membrane. The DNA was crosslinked to the nylon membrane by UV irradiation (315 nm). The biotin-labelled DNA duplexes were detected using the commercial Chemiluminescent Nucleic Acid Detection Module Kit (Thermo #89880) and exposed to X-ray films (Thermo Scientific).

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) was performed as published previously (O'Neill et al., 2011) with minor modifications to reflect the higher sensitivity of the Biacore T200 (GE Healthcare) used in this study. Biotinylated oligonucleotides were immobilised on a pre-treated Streptavidin chip to 3 Response Units by injection of 1 nM for at 5 µl/min. Reverse oligonucleotides were bound by injecting 10 µM at 5 µl/min until saturation. One flow cell was always kept blank for reference subtractions. Protein and buffers were filter-sterilised before use; dilutions of protein were made in HBS-EP+ buffer (GE Healthcare), injected at 75 µl/min for 90 seconds, and dissociated for 600 seconds. Surfaces were regenerated with 3 cycles of 60 seconds of regeneration buffer (0.1% SDS, 3 mM EDTA) in between each protein run. Steady-state affinity analyses were performed to deduce K_D using BiaEvaluation software (GE Healthcare) using the standard one-site binding model. Chi-squared values are reported for all fits (any value below 2 indicates a reliable fit). Curves were exported and plotted in GraphPad Prism.

RESULTS

CCA1 is sumoylated *in vivo*

Targets of sumoylation are predominantly nuclear localised, and often function in DNA transcription, chromatin modification, and other nuclear-related processes (Miller et al., 2010). TTFL timekeeping in plants involves numerous proteins that fit that description. Dynamic post-translational regulation of CCA1 is known to affect its function (Yakir et al., 2009), and it belongs to the MYB-like superfamily of transcription factors that are particularly overrepresented among sumoylated proteins (Miller et al., 2010). To investigate whether CCA1 is modified by SUMO *in vivo*, we employed Arabidopsis plants that express a fusion protein of CCA1 with a triple HA-tag and the YFP epitope from the native CCA1 promoter (Yakir et al., 2009; referred to in this manuscript as CCA1-3HY). This transgene rescues the

period effect of the *cca1* mutation (Yakir et al., 2009). Plants were grown in light-dark cycles, harvested over a time series spanning the CCA1 expression window, and subjected to immunoprecipitation in an extraction buffer inactivating SUMO protease activity. The circadian accumulation of CCA1 was confirmed using immunoblotting with an anti-HA antibody (Fig. 1A, middle panel), and dynamic sumoylation of CCA1 was observed over this time series using an antibody against SUMO isoforms 1 and 2 (anti-SUMO; upper panel). To test whether sumoylation of CCA1 relies on the light-dark cycle, this experiment was repeated under circadian conditions of constant light and temperature. Under these conditions, both rhythmic accumulation of CCA1 as well as rhythmic sumoylation of CCA1 persisted (Fig. 1B) even though total sumoylation remained stable over this experiment (Supplemental Figure S1A).

In either condition, multiple distinct bands are visible using a SUMO antibody on CCA1 pulldowns (Fig. 1), at molecular weights that are consistent with either SUMO modification at multiple sites, or SUMO chain formation, or SUMO modification(s) combined with additional modifications.

Identifying sumoylation sites in CCA1

To identify potential sumoylation sites in CCA1, a recombinant expression system was employed to allow easy production of larger quantities of CCA1. Previously, a system was reported to reconstitute part of the Arabidopsis sumoylation pathway in *E. coli*, which has no endogenous sumoylation pathway (Okada et al., 2009). Recombinant T7- and His-tagged CCA1 protein was expressed in this system to verify that the protein contains sumoylation sites and to subsequently identify these by mass spectrometry. As a positive control, the Arabidopsis transcription factor MYB30 was included, a known sumoylation target (Zheng et al., 2012). Target proteins were expressed along with two variants of SUMO; an intact conjugatable SUMO (+) or, as a negative control, an unconjugatable SUMO (-) lacking the double glycine conjugation motif (Okada et al., 2009). Upon immunoblotting using anti-T7 antibody,

CCA1 and MYB30 were detected in His-tag affinity purified protein extracts. Clear sumoylation of the positive control MYB30 was observed (Fig. 2A) using intact SUMO (+) and not in negative controls (-), indicating that the heterologous co-expression system worked as expected. Sumoylation of CCA1 was evident in this assay, verifying that CCA1 can indeed be sumoylated.

Identification of sumoylation sites was attempted by mass spectrometric analysis. CCA1 was sumoylated in *E. coli* using a variant of SUMO suitable for mass spectrometric detection. In this variant, the threonine immediately upstream of the double glycine motif has been substituted with an arginine to introduce a trypsin cleavage site. In liquid chromatography tandem mass spectrometry analysis (LC MS/MS), lysines modified with this SUMO variant are distinguishable by the double glycine modification. The CCA1 peptides detected in two independent experiments covered between 13.9% and 41.7% of the lysines in the full amino acid sequence (Fig. 2B). In total, 7 sumoylation sites were detected (Figure 2B and 2C). To validate whether any of these lysines were the predominant sumoylation sites, point mutations from lysine to arginine were introduced. Neither single mutant showed a decrease in sumoylation in *E. coli* (data not shown), and therefore several sites were mutated in a single construct: either the 3 most N-terminal, the 4 most C-terminal, or all 7 lysines. No obvious differences in sumoylation were detected in any of these protein variants, suggesting that these sites do not account for the bulk of sumoylation detected on CCA1 and that additional sumoylated lysines have remained undetected by our mass spectrometric analyses.

Functional relevance of CCA1 sumoylation

The most unequivocal way of testing the contribution of differential sumoylation of CCA1 to the circadian clock system would be to complement the *cca1 lhy* double mutant with wild-type CCA1 versus a non-sumoylatable version of CCA1. Since it was not possible to generate a non-sumoylatable version of CCA1 (Fig. 2B), we tested instead the characteristics of the CCA1 protein *in planta* in the wild-type

background compared to a SUMO protease double mutant (*ots1 ots2*). In this background, increased

global sumoylation has been reported (Conti et al., 2008, Fig. S1B) as well as a long-period circadian

phenotype (see accompanying paper). To test the expression profile of CCA1 target promoters in this

background, a protoplast assay was used (Kim and Somers, 2010). Whilst the long period phenotype was

consistent across reporters, the altered expression levels suggest that increased global sumoylation affects

more than one point in the complex clock network (Supplemental Figure S1C and D), at least as reported

in this protoplast assay.

Sumoylation can regulate the localisation, stability, or activity of a protein, as well as its

intermolecular interactions (Wilkinson and Henley, 2010; Mazur and van den Burg, 2012). CCA1

accumulates in the nucleus at subjective dawn (Yakir et al., 2011), and we firstly tested the effects of

sumoylation on this nuclear localisation. YFP-tagged CCA1 was expressed in protoplasts of wild-type

plants (Col-0) versus *ots1 ots2* using either the native, circadian CCA1 promoter or the constitutive 35S

promoter. No difference in CCA1 localisation was observed between the genotypes with either promoter

(Fig. 3A), suggesting that changes to overall sumoylation levels do not influence localisation of CCA1.

To verify that result, the effects of N-terminal fusion of SUMO isoforms to the CCA1 protein were

studied, as expression of a fusion protein of SUMO with a target protein can mimic constitutive

sumoylation of that target (Georges et al., 2011). No difference in localisation was observed between

CCA1 and SUMO-CCA1 (Fig. 3B). In conclusion, neither the predominant nuclear localisation nor the

detectable amount of protein that remains in the cytoplasm were differential between genotypes or CCA1

constructs, supporting the notion that either CCA1 sumoylation is not differential in this *ots1 ots2*

background, or sumoylation does not have a major influence on CCA1 protein localisation.

Secondly, sumoylation can affect target protein stability (Miura et al., 2009; Zheng et al., 2012)

and the dynamics of several clock components were previously demonstrated to be fine-tuned through

regulation of their stability (Más et al., 2003; Song and Carré, 2005; Kim et al., 2007; Para et al., 2007;

Baudry et al., 2010; van Ooijen et al., 2011)**.** To test whether changes in global sumoylation affect CCA1 stability, the CCA1-3HY line was crossed to the *ots1 ots2* background to allow comparative stability assays. Total protein lysates from both lines were generated at the peak of CCA1 accumulation, and CCA1 protein stability was analysed by immunoblotting (Fig. 4A and B). No significant changes to protein stability were observed between the two genetic backgrounds, indicating either that CCA1 sumoylation levels are not altered in the *ots1 ots1* background, or that sumoylation has no major influence on CCA1 stability.

Thirdly, sumoylation can regulate the specific activity of target proteins involved in transcription (Lee et al., 2008; Miura et al., 2009; Zheng et al., 2012; Li et al., 2013). The main activity of CCA1 in the clock is to inhibit or activate the expression of genes through binding to a conserved promoter element called the 'evening element', found in CCA1-controlled gene promoters such as the *PRR9* promoter (Hsu and Harmer, 2013). ChIP experiments were carried out to elucidate whether binding activity of CCA1 to the *PRR9* promoter sequence was differential between wild-type and *ots1 ots2* backgrounds. Seedlings were harvested at dawn, the samples were cross-linked, and the abundance of the *PRR9* promoter sequence in the immunoprecipitated CCA1 complexes was analysed using qRT-PCR. A greater enrichment was consistently observed in the wild-type compared to *ots1 ots2* background (Fig. 5A and Supplementary Fig. S1E). Non-specific binding was not observed in negative control Col-0 plants lacking the CCA1-3HY transgene, nor in no-antibody controls, and no signal was detected on control DNA fragments (actin). Combined, this verifies that reduced binding of CCA1 to the *PRR9* evening element is observed in the SUMO protease-defective line *ots1 ots2* compared to control plants.

Sumoylation of CCA1 directly supresses DNA binding

A limitation of the approach taken above is that it remains unclear whether (part of) the changes to overall clock dynamics or even to CCA1 promoter binding in the *ots1 ots2* background are due to potentially

differential sumoylation of CCA1 directly, or from altered sumoylation of additional proteins involved in the intricate clock feedback system. The only unequivocal way to establish whether direct effects exist for CCA1 sumoylation, is to use purified recombinant CCA1 protein in binding assays without any other proteins present. Sumoylated and non-sumoylated CCA1 protein (as in Fig. 2) was purified from *E. coli* and compared in *in vitro* DNA binding assays. To test whether sumoylation has a direct effect on DNA binding, biotin-labelled oligonucleotide duplexes containing the evening element of the *PRR9* promoter (*PRR9ee,* Fig. 5B) or a mutated negative control probe (*mutPRR9*) were added to multiple concentrations of sumoylated or control CCA1 protein. DNA duplexes and protein-DNA complexes were resolved in an electrophoretic mobility shift assay (EMSA). At a highest protein concentration (45 nM, Fig. 5C), all *PRR9ee* probe was bound to either sumoylated or non-sumoylated CCA1, indicating that both can bind DNA. However, at intermediate CCA1 protein concentrations (10 nM), more free probe remained in the reactions with sumoylated compared to non-sumoylated CCA1. At low protein concentrations (1 nM), bound probe was only detected in reactions containing non-sumoylated CCA1. This demonstrates that the binding affinity of CCA1 to the evening element in the *PRR9* promoter is reduced by sumoylation of CCA1. The shift indicative for a protein-DNA complex was not observed with negative control probe, verifying the binding of CCA1 to the evening element is specific in this assay.

To confirm this result and to quantify the difference in DNA binding affinity, Surface Plasmon Resonance (SPR) analyses were conducted. Biotinylated DNA duplexes of *PRR9ee* and *mutPRR9ee* were immobilised on streptavidin-coated SPR chip surfaces, and binding of a wide concentration range of recombinant protein was analysed. Consistent across a protein concentration range, reduced binding of sumoylated CCA1 was observed versus control CCA1 (Fig. 5D). To quantify the difference in affinity to the two heterologous proteins, the steady-state affinity was fitted to the binding curves (Fig. 5E). Non-specific binding to the *mutPRR9ee* probe was low and identical between the two protein samples. The

binding constant K_D in the control protein was lower (0.9 nM) than in the sumoylated CCA1 protein (1.7 nM) (Fig. 5F), indicating that sumoylation of CCA1 directly reduces DNA binding.

DISCUSSION

Phosphorylation of CCA1 by CK2 affects circadian period length and this phosphorylation is necessary to form the dimers required for DNA binding (Daniel et al., 2004). Here we demonstrated that CCA1 is additionally modified by sumoylation (Fig. 1). It is important to note that *in vivo* and *in vitro* (Fig. 5), only a small amount of the total CCA1 pool is sumoylated, yet function of the total pool is significantly affected. In fact, this is perfectly consistent with all previous observations. Without exceptions, the effects of sumoylation on target proteins are mediated by only a small percentage of the total pool of that particular target protein *in vivo*. This phenomenon is referred to as the 'SUMO enigma' (Lee et al., 2008; Wilkinson and Henley, 2010), first coined in 2005 (Hay, 2005). Especially enigmatic, this phenomenon occurs not only *in vivo* but also *in vitro*: sumoylation of a small percentage of the mammalian RNA binding protein La produced in *E. coli* results in a striking change to the binding activity as detected by electrophoretic mobility shift (Kota et al., 2016). In this respect, CCA1 appears to be a typical sumoylation target both *in vivo* and *in vitro*. Like CCA1, La is additionally controlled by CK2 phosphorylation. Interplay between phosphorylation and sumoylation is a widespread mechanism for modulating the activity or function of a protein (Gareau and Lima, 2010; Nukarinen et al., 2017).

In plants, phosphorylation-dependent sumoylation was demonstrated on the brassinosteroid-responsive transcription factor CESTA (Khan et al., 2014), and on the master regulator of plant immunity, NPR1 (Saleh et al., 2015). Sumoylation is predominantly a nuclear modification (Miller et al., 2010), and within the nucleus, sumoylation of CESTA induces the assembly of nuclear bodies that are proposed to promote protein-protein interactions required for the brassinosteroid response (Khan et al., 2014). Although sumoylation does not affect the predominantly nuclear localisation of CCA1 (Fig. 3), it could

still have effects on sub-organellar localisation. Confocal imaging of the mammalian CLOCK/BMAL1 transcription factor complex revealed that SUMO is required for localisation to nuclear bodies within the nucleus (Lee et al., 2008). Further investigation into the associations between phosphorylation and sumoylation of CCA1 could reveal whether these modifications are co-dependent and act as a regulatory mechanism of transcriptional regulation by CCA1 within the nucleus.

CCA1 binds specifically to the highly conserved Evening Element promoter sequence, but the resultant phase of expression is dependent on flanking DNA sequences (Harmer and Kay, 2005). Furthermore, recent ChIP-Seq studies identified additional binding motifs included morning-phase specific sequences (Nagel et al., 2015). How CCA1 is directed towards one or the other motif is still poorly understood, but could result from differential protein-protein interactions that might be affected by sumoylation (Ouyang et al., 2009; Wilkinson and Henley, 2010; Saleh et al., 2015). Although our studies were limited to the effects of sumoylation on the binding of CCA1 to the Evening Element (Fig. 5), it will now be useful to identify differential effects on binding to other sites (either directly or through differential interactions with co-regulators). Clearly, given the complexity of a) the feedback loop structure b) post-translational regulation of clock proteins and c) the number of CCA1 binding elements, a complicated pattern is bound to emerge. Elucidating the full role of sumoylation on these aspects would ultimately require detailed mathematical modelling to predict the poorly understood difference between resultant expression phase as well as repression or induction.

Sumoylation regulates timekeeping in plants (see accompanying paper), and we now established that the crucial plant transcription factor CCA1 is a sumoylation target. Whilst it is possible that part of the overall *in planta* effect of sumoylation mutants on the clock is via CCA1, circumstantial evidence exists that sumoylation modulates additional clock proteins. Firstly, the evening-phased clock components EARLY FLOWERING 3 (ELF3) and GIGANTEA (GI) were identified as SUMO targets (Miller et al., 2010; López-Torrejón et al., 2013). However, both results were obtained upon exposure to

non-physiological heat stress, and a functional role has not been demonstrated in either case. Sumoylation does functionally influence red light signalling via PhyB and photomorphogenesis via COP1, but whether these events influence timekeeping is not evident (Sadanandom et al., 2015; Lin et al., 2016). Based on these studies and the results presented here, it remains unclear whether any percentage of the overall clock defect in the *ots1 ots2* background is mediated via CCA1. What we have unequivocally shown however, is that the central plant clock transcription factor CCA1 undergoes dynamic sumoylation, which directly alters the binding affinity to the evening element. The CCA1 protein binds to the promoter of >1500 genes in Arabidopsis (Nagel et al., 2015; Kamioka et al., 2016), indicating that these observations could have wide consequences for the growth and health of plants.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

NOTES

Supplemental Figure S1, Data Tables S1 and S2 are available for download at the website.

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Fig. 1: The clock transcription factor CCA1 is sumoylated *in planta*

Plants were samples at the indicated times under Light : Dark cycles (LD; *A*) or upon transfer to constant light (LL; *B*). In both, time is hours since the last dawn. CCA1-3HY was immunoprecipitated and detected on immunoblot using anti-HA antibody (mid panel). Sumoylation of CCA1 was detected with anti-SUMO (upper panel). Input protein loading control in lower panel.

Fig. 2: CCA1 is sumoylated in *E. coli* on several lysine residues

A) CCA1 and positive control MYB30 protein was co-expressed with the Arabidopsis sumoylation machinery and wild-type (+) SUMO or nonconjugateable SUMO (–) in *E. coli,* and His-tag affinity purified protein was detected on immunoblots using an anti-T7 tag antibody. Asterisks indicate the expected molecular weight band of sumoylated target protein, arrowheads indicate the non-sumoylated bulk of the protein. B) Sumoylation sites identified by MS in two independent experiments each with 4 replicates. C) Schematic of CCA1 protein with lysines indicated by blue lines and observed sumoylated lysines by yellow lines. A predicted SUMO Interaction Motif (SIM) is also indicated. The amino acid number is indicated above and below the schematic.

Fig. 3: CCA1 localization is not altered in the *ots1 ots2* mutant background

A) CCA1-YFP fusion protein or control YFP protein were expressed from the indicated promoters in the Col-0 and *ots1 ots2* backgrounds, and imaged by confocal microscopy. B) N-terminal fusion to SUMO isoforms does not change localisation of CCA1. Expression of CCA1 in Col-0 protoplasts as fusion proteins with C-terminal YFP tag and N-terminal SUMO isoforms as indicated, expressed from the CCA1 promoter (left) or 35S promoter (right), and imaged by confocal microscopy. Individual YFP (middle panel) and chlorophyll (lower panel) channels are provided in grey scale, and as an overlay in colour (upper panel). Scale bar = 10 μm.

Fig. 4: CCA1 protein stability is not altered in the *ots1 ots2* mutant background

A) Stability of CCA1-3HY protein was analysed in *ots1 ots2* mutant and control background plants. Aliquots of total protein extracts were sampled at the indicated time points and immunoblotted. Representative blots of three biological replicates. B) Densitometry data of blots in (A) and 2 additional biological replicates (mean value +/- SEM. Paired t-test; P-value = 0.5).

Fig. 5: DNA binding of CCA1 is reduced in the *ots1 ots2* mutant background *in vivo* and directly by sumoylation *in vitro*

A) Indicated plant lines were harvested at dawn and subjected to chromatin immunoprecipitation of CCA1-3HY along with negative controls without antibody. PRR9 promoter sequences were detected using qRT-PCR along with negative controls of the actin sequence (mean +/- SEM of 4 technical replicates, ordinary one-way ANOVA with Dunnett's multiple comparisons test. *P*-value: <0.0001 (****); <0.05 (*)). B) The evening element in the *PRR9* promoter sequence (*PRR9ee*) is highlighted in blue. Four nucleotides were mutated (*mutPRR9ee*) to act as a negative control for CCA1 binding (underlined; *mutPRR9ee*). C) Electrophoretic mobility shift assay (EMSA) of biotinylated DNA duplexes of the *PRR9* evening element (*PRRee*) or a mutated negative control (*mutPRRee*) with three concentrations of sumoylated or non-sumoylated CCA1 protein (1 nM, 10 nM, and 45 nM). Arrows indicate free, unbound probe and probe bound in protein-DNA complexes. D) Surface Plasmon Resonance (SPR) experiments detecting the binding of sumoylated or non-sumoylated CCA1 protein to the *PRR9* evening element sequence during a 90-second interaction (0-90 sec) at the indicated protein concentrations, along with the following dissociation (90-270 sec). E) Surface Plasmon Resonance analysis of recombinant sumoylated (blue) and non-sumoylated CCA1 (black) protein, binding to the wild-type evening element (EE; circles) or mutated sequence (triangles, mutEE). The lines indicate the steady-state affinity fits to the data points.

F) Binding constant K_D as determined by steady-state affinity analyses of the data in (E). Chi-squared values are provided, indicating goodness of fits.

Figure 1
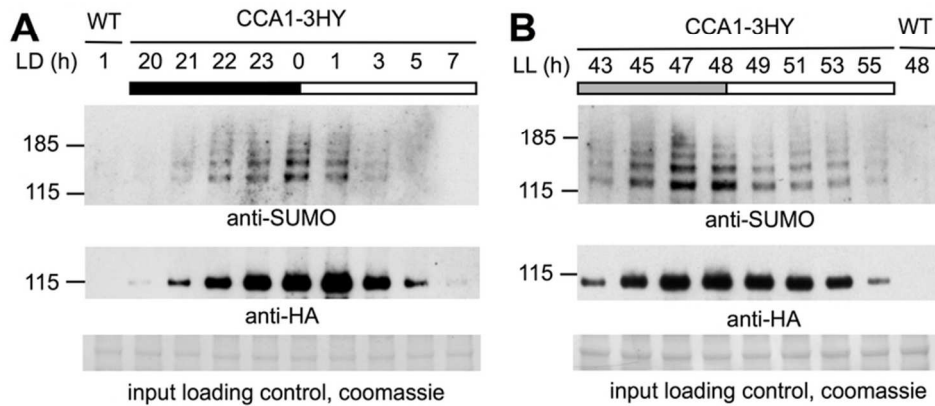
Fig. 1: The clock transcription factor CCA1 is sumoylated in planta
Plants were samples at the indicated times under Light : Dark cycles (LD; A) or upon transfer to constant light (LL; B). In both, time is hours since the last dawn. CCA1-3HY was immunoprecipitated and detected on immunoblot using anti-HA antibody (mid panel). Sumoylation of CCA1 was detected with anti-SUMO (upper panel). Input protein loading control in lower panel.

78x38mm (300 x 300 DPI)

Figure 2

A

| | MYB30 | CCA1 | N-term triple | C-term quadruple | hepta-mutant |

SUMO: + − (MYB30), + − (CCA1), + − (N-term triple), + − (C-term quadruple), + − (hepta-mutant)

50 − kDa

−80 kDa

anti-T7

B

Run	Technical replicate	Lysines detected	Lysine coverage (%)	Sumoylated lysines
1	1	12	33.3	K483, K559, K595, K598
	2	15	41.7	K483, K559, K595, K598
	3	5	13.9	K595
	4	10	27.8	K559, K595
2	1	5	13.9	K483, K559, K595
	2	5	13.9	K483, K559, K595
	3	7	19.4	K393, K483
	4	15	41.7	K117, K124, K393, K483

C

K117, K124, K393, K483, K559, K595, K598, SIM 589-593

N- ———— -C

Fig. 2: CCA1 is sumoylated in E. coli on several lysine residues
A) CCA1 and positive control MYB30 protein was co-expressed with the Arabidopsis sumoylation machinery and wild-type (+) SUMO or nonconjugateable SUMO (−) in E. coli, and His-tag affinity purified protein was detected on immunoblots using an anti-T7 tag antibody. Asterisks indicate the expected molecular weight band of sumoylated target protein, arrowheads indicate the non-sumoylated bulk of the protein. B) Sumoylation sites identified by MS in two independent experiments each with 4 replicates. C) Schematic of CCA1 protein with lysines indicated by blue lines and observed sumoylated lysines by yellow lines. A predicted SUMO Interaction Motif (SIM) is also indicated. The amino acid number is indicated above and below the schematic.

154x154mm (300 x 300 DPI)

Figure 3

A

CCA1pro:CCA1-YFP | CCA1pro:YFP | 35Spro:CCA1-YFP | 35Spro:YFP

Col-0 | ots1 ots2 | Col-0 | Col-0 | ots1 ots2 | Col-0

Overlay

YFP

Chlor.

B

CCA1 promoter | 35S promoter

CCA1-YFP | SUMO1-CCA1-YFP | SUMO2-CCA1-YFP | SUMO3-CCA1-YFP | CCA1-YFP | SUMO1-CCA1-YFP | SUMO2-CCA1-YFP | SUMO3-CCA1-YFP

Overlay

YFP

Chlor.

Fig. 3: CCA1 localization is not altered in the ots1 ots2 mutant background
A) CCA1-YFP fusion protein or control YFP protein were expressed from the indicated promoters in the Col-0 and ots1 ots2 backgrounds, and imaged by confocal microscopy. B) N-terminal fusion to SUMO isoforms does not change localisation of CCA1. Expression of CCA1 in Col-0 protoplasts as fusion proteins with C-terminal YFP tag and N-terminal SUMO isoforms as indicated, expressed from the CCA1 promoter (left) or 35S promoter (right), and imaged by confocal microscopy. Individual YFP (middle panel) and chlorophyll (lower panel) channels are provided in grey scale, and as an overlay in colour (upper panel). Scale bar = 10 μm.

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Figure 4

Fig. 4: CCA1 protein stability is not altered in the ots1 ots2 mutant background
A) Stability of CCA1-3HY protein was analysed in ots1 ots2 mutant and control background plants. Aliquots of total protein extracts were sampled at the indicated time points and immunoblotted. Representative blots of three biological replicates. B) Densitometry data of blots in (A) and 2 additional biological replicates (mean value +/- SEM. Paired t-test; P-value = 0.5).

50x15mm (300 x 300 DPI)

Figure 5

Fig. 5: DNA binding of CCA1 is reduced in the ots1 ots2 mutant background in vivo and directly by sumoylation in vitro

A) Indicated plant lines were harvested at dawn and subjected to chromatin immunoprecipitation of CCA1-3HY along with negative controls without antibody. PRR9 promoter sequences were detected using qRT-PCR along with negative controls of the actin sequence (mean +/- SEM of 4 technical replicates, ordinary one-way ANOVA with Dunnett's multiple comparisons test. P-value: <0.0001 (****); <0.05 (*)). B) The evening element in the PRR9 promoter sequence (PRR9ee) is highlighted in blue. Four nucleotides were mutated (mutPRR9ee) to act as a negative control for CCA1 binding (underlined; mutPRR9ee). C) Electrophoretic mobility shift assay (EMSA) of biotinylated DNA duplexes of the PRR9 evening element (PRRee) or a mutated negative control (mutPRRee) with three concentrations of sumoylated or non-sumoylated CCA1 protein (1 nM, 10 nM, and 45 nM). Arrows indicate free, unbound probe and probe bou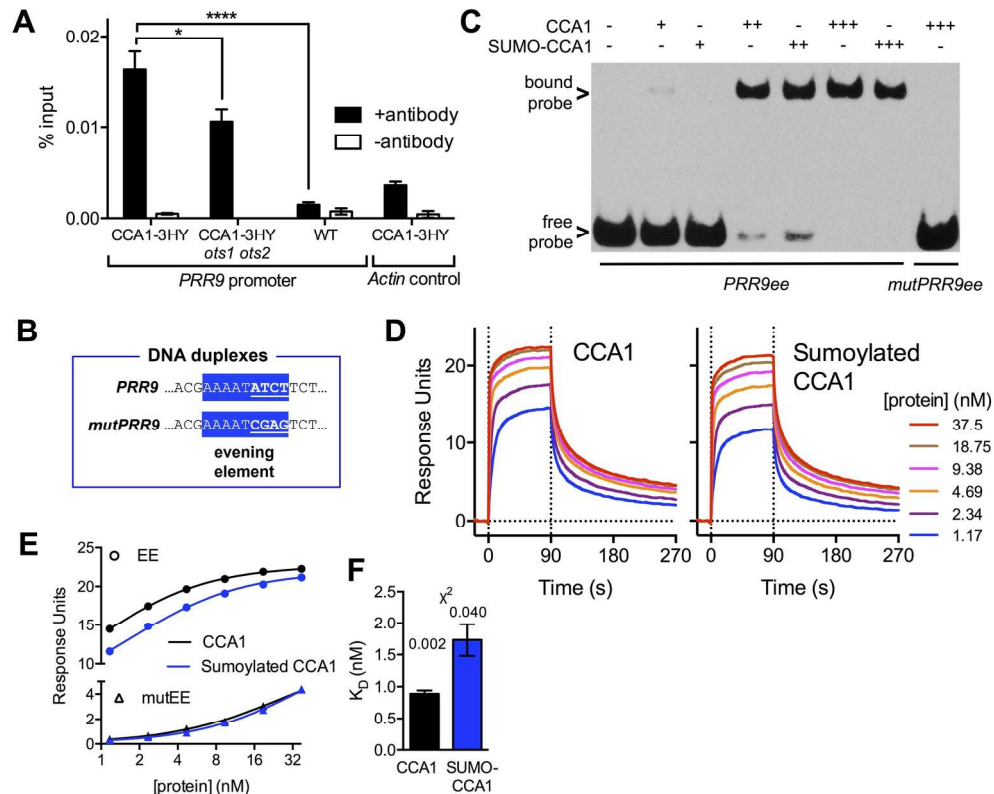nd in protein-DNA complexes. D) Surface Plasmon Resonance (SPR) experiments detecting the binding of sumoylated or non-sumoylated CCA1 protein to the PRR9 evening element sequence during a 90-second interaction (0-90 sec) at the indicated protein concentrations, along with the following dissociation (90-270 sec). E) Surface Plasmon Resonance analysis of recombinant sumoylated (blue) and non-sumoylated CCA1 (black) protein, binding to the wild-type evening element (EE; circles) or mutated sequence (triangles, mutEE). The lines indicate the steady-state affinity fits to the data points. F) Binding constant KD as determined by steady-state affinity analyses of the data in (E). Chi-squared values are provided, indicating goodness of fits.

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Figure S1

A CCA1-3HY wt

LL (h) 43 45 47 48 49 51 53 55 48

185
115
80

anti-SUMO

115 ← CCA1

anti-HA

B

ots1 ots2
Col-0
CCA1-3HY ots1 ots2
CCA1-3HY

15 ← Free SUMO

C

CCA1pro:LUC GIpro:LUC PRR9pro:LUC

Luminescence (RLU)

Time in LL (h)

— Col-0
— ots1 ots2

D

Circadian period (h)

CCA1 GI PRR9

E

% input

**** *

■ +antibody
□ -antibody

CCA1-3HY   CCA1-3HY   WT   CCA1-3HY
            ots1 ots2

PRR9 promoter   Actin control

F

CCA1   - + - ++ - +++ - +++
SUMO-CCA1   - - + - ++ - +++ -

bound probe >

free probe >

PRR9ee   mutPRR9ee

G

χ^2

0.012 0.023

K_D (nM)

CCA1   SUMO-CCA1

Supplementary Fig. 1: Supporting experimental results
A) As indicated by western blotting of total protein lysates, global sumoylation levels do not vary over the duration of the expression window of CCA1, indicating differential sumoylation shown in main Figure 1B is specific to CCA1. B) A clear decrease in free SUMO is observed in the *ots1 ots2* background compared to the wild-type background, and this difference persists when crossed to the CCA1-3HY line. C) Expression of *CCA1pro:*LUC and CCA1 target promoters reported by *GIpro:*LUC and *PRR9pro:*LUC in the *ots1 ots2* background compared to wild-type indicate that long period rhythms (D) are observed in all. C-D are derived from protoplast assays upon transient expression of the indicated reporter. An independent biological replicate experiment of the ChIP (E), EMSA (F), and SPR data (G) in main Figure 5 are presented to verify reproducibility.

245x317mm (300 x 300 DPI)

Table S1: Oligonucleotides used in this study.

Use	Name	Sequence (5'-3')	References
Genotyping	SALK Lba1	tggttcacgtagtgggccatcg	(Alonso et al., 2003)
	OTS1 LP	gatgatgcaaggaggctagtg	This study
	OTS1 RP	cgacaagaagtggtttagacc	(Conti et al., 2008)
	OTS2 LP	gcttcttccggtttaaaccac	
	OTS2 RP	tttttcttctggcgactcatg	This study
	CCA1 LP	tttgtgtttgcagaggtttg	
	CCA1 RP	ctataaacgaagcacacaatct	
	CCA1 LB	gatgcactcgaaatcagccaattttagac	(Green and Tobin, 1999)
	CCA1-HA fwd	cgggaagagggaagtcagaa	This study
	CCA1-HA rev	gcaattcgaccctcgtcaga	
Cloning constructs for confocal imaging	CCA1pro_fwd	tcgcggccgcatttagtcttctacccttcatgc	This study
	YFP_rev	tcaggcctttacttgtacagctcgtccatgc	
	fwd SUMO1	tcggatccatgtctgcaaaccaggaggaaga	
	REV SUMO1	agggatccgccaccagtctgatggagcatc	
	fwd SUMO2	tcggatccatgtctgctactccggaagaaga	
	REV SUMO2	gaggatccaccaccagtctgatgaagcatt	
	fwd SUMO3	tcggatccatgtctaaccctcaagatgaca	
	REV SUMO3	gaggatccaccaccactcatcgcccgg	
	fwd SUMO-CCA1	tcgaatccaagctgattttg	
	fwd NotI-35Spro	tcgcggccgctgagactttcaacaaaggg	
	rev 35SproBamHI	agggatcctgttctctccaaatgaaatgaac	
Sequencing constructs for confocal imaging	fwd-seq1	aacagttttcccaatgccat	
	rev-seq1	tgaccctgaagttcatctgc	
	fwd-seq2	tttatgcttccggctcgtat	
	fwd-seq2	ggaatctttatcgaatccaagc	
	fwd SUMO-CCA1	tcgaatccaagctgattttg	
	rev SUMO-CCA1	gtatatggcttccgagtcta	
qPCR	PRR9 fwd	aggaccacctccaccgaatc	(Portolés and Más, 2010)
	PRR9 rev	aacaaagcgggccttcact	
	ACT2 fwd	cgtttcgctttccttagtgtta	(Pruneda-Paz et al., 2009)
	ACT2 rev	agcgaacggatctagagactc	
Oligos for EMSA and SPR	Bi-PRR9_fwd	Biotin-cgatcacaaccacgaaaatatcttctcagagaaagaaga	(O'Neill et al., 2011)
	PRR9_rev	tcttctttctctgagaagatattttcgtggttgtgatcg	
	Bi-mutPRR9_fwd	Biotin-cgatcacaaccacgaaaatcgagtctcagagaaagaaga	
	mutPRR9_rev	tcttctttctctgagactcgattttcgtggttgtgatcg	
Point mutation of CCA1	CCA1-K117R-fwd	gaagtggaacgatccttatgtcaa**G**aacgggtgtgaat	This study.
	CCA1-K117R-rev	attcacacccgtt**C**ttgacataaggatcgttccacttc	
	CCA1-K124R-fwd	aacgggtgtgaatgatggaa**G**agagtcccttgga	
	CCA1-K124R-rev	tccaagggactct**C**ttccatcattcacacccgtt	
	CCA1-K393R-fwd	actcagaggatgttgaaaataagagta**G**accagtttgtcatgagc	
	CCA1-K393R-rev	gctcatgacaaactggt**C**tactcttattttcaacatcctctgagt	
	CCA1-K483R-fwd	cctcgtcagacacagac**C**tccatggatcggttata	
	CCA1-K483R-rev	tataaccgatccatgga**G**gtctgtgtctgacgagg	
	CCA1-K559R-fwd	ttcctctactcattagc**C**ttgaagcatctaatccgattcc	
	CCA1-K559R-rev	ggaatcggattagatgcttcaa**G**gctaatgagtagaggaa	
	CCA1-K595R-fwd	ccgtttgggatct**C**tctgttccacatgaatgataggatt	
	CCA1-K595R-rev	aatcctatcattcatgtggaacaga**G**agatcccaaacgg	
	CCA1-K598R-fwd	aaccgcatccgt**C**tgggatctttctgttccacatga	
	CCA1-K598R-rev	tcatgtggaacagaaagatccca**G**acggatgcggtt	
	CCA1-K595R-fwd_2	tcc**t**atcattcatgtggaacaga**G**agatcccagacg	Note: K595R in CCA K598R
	CCA1-K595R-rev_2	cgtctgggatct**C**tctgttccacatgaatgat**a**gga	Note: K595R in CCA K598R
Seq. of point mutations	T7-fwd	taatacgactcactatagggg	
	CCA1seq_rev	ctcaagacccgtttagaggc	

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Table S2: Identification of sumoylated lysines on CCA1

Run, rep	Sequence (sumoylated lysine bold)	Observed relative mass	Calculated relative mass	Mascot ions score	Expectation value
1-1	ISSNITDPWK	1273.6312	1273.6302	34.62	0.00035
	NTGFLGIGLDASK	1405.7233	1405.7201	24.94	0.0032
	ILNNNPIIHVEQK	1644.9054	1644.8947	27.67	0.0017
	ILNNNPIIHVEQKDPK	1985.0864	1985.0694	25.42	0.0029
1-2	ISSNITDPWK	1273.633	1273.6302	34.35	0.00037
	NTGFLGIGLDASK	1405.7216	1405.7201	25.46	0.0028
	ILNNNPIIHVEQK	1644.8963	1644.8947	29.67	0.0011
	ILNNNPIIHVEQK	1644.8964	1644.8947	35.47	0.00028
	ILNNNPIIHVEQKDPK	1985.0654	1985.0694	39.78	0.00011
	ILNNNPIIHVEQKDPK	1985.0764	1985.0694	21.83	0.0066
1-3	ILNNNPIIHVEQKDPK	1985.0782	1985.0694	21.4	0.0072
1-4	NTGFLGIGLDASK	1405.7243	1405.7201	33.06	0.00049
	ILNNNPIIHVEQK	1644.896	1644.8947	32.8	0.00052
	ILNNNPIIHVEQKDPK	1985.0782	1985.0694	24.97	0.0032
2-1	LVIWINGDK	1170.6404	1170.6397	29.47	0.0011
	ISSNITDPWK	1273.6286	1273.6302	32.58	0.00055
	NTGFLGIGLDASK	1405.714	1405.7201	30.98	0.0008
	ILNNNPIIHVEQK	1644.8886	1644.8947	20.24	0.0095
2-2	LVIWINGDK	1170.6397	1170.6397	26.63	0.0022
	ISSNITDPWK	1273.6272	1273.6302	33.75	0.00042
	NTGFLGIGLDASK	1405.7172	1405.7201	35.27	0.0003
	ILNNNPIIHVEQK	1644.8863	1644.8947	23.4	0.0046
	ILNNNPIIHVEQK	1644.8895	1644.8947	41.86	6.50E-05
	ILNNNPIIHVEQK	1644.8915	1644.8947	21.04	0.0079
2-3	GYNGLAEVGK	1120.5454	1120.5513	23.62	0.0043
	DKPLGAVALK	1124.655	1124.6553	22.68	0.0054
	ISSNITDPWK	1273.6267	1273.6302	52.11	6.20E-06
	SKPVCHEQPSATPESDAK	2080.942	2080.9484	24.32	0.0037
2-4	LVIWINGDK	1170.6402	1170.6397	28.16	0.0015
	GYNGLAEVGKK	1248.6454	1248.6462	23.57	0.0044
	ISSNITDPWK	1273.6288	1273.6302	30.18	0.00096
	TGVNDGKESLGSEK	1533.7282	1533.7271	20.9	0.0081
	SKPVCHEQPSATPESDAK	2080.937	2080.9484	35.65	0.00027
	KTGSGTILMSKTGVNDGK	1922.9704	1922.9731	49.66	1.10E-05
	KTGSGTILMSKTGVNDGK	1922.9775	1922.9731	45.44	2.90E-05