Redox rhythm reinforces the circadian clock to gate immune response

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Recent studies have shown that in addition to the transcriptional circadian clock, many organisms, including Arabidopsis, have a circadian redox rhythm driven by the organism’s metabolic activities7–9. It has been hypothesized that the redox rhythm is linked to the circadian clock, but the mechanism and the biological significance of this link have only begun to be investigated5–7. Here we report that the master immune regulator NPR1 (non-expressor of pathogenesis-related gene 1) of Arabidopsis is a sensor of the plant’s redox state and regulates transcription of core circadian clock genes even in the absence of pathogen challenge. Surprisingly, acute perturbation in the redox status triggered by the immune signal salicylic acid does not compromise the circadian clock but rather leads to its reinforcement. Mathematical modelling and subsequent experiments show that NPR1 reinforces the circadian clock without changing the period by regulating both the morning and the evening clock genes. This balanced network architecture helps plants gate their immune responses towards the morning and minimize costs on growth at night. Our study demonstrates how a sensitive redox rhythm interacts with a robust circadian clock to ensure proper responsiveness to environmental stimuli without compromising fitness of the organism.

Life on Earth has evolved the circadian clock to anticipate diurnal and seasonal changes4. This ‘scheduling’ mechanism coordinates biological processes to reduce random energy expenditures and increase fitness. In Arabidopsis, daily light-dark cycles are divided into three interlocked transcription–translation feedback loops (TTFLs): the core loop, the morning loop, and the evening loop. The core loop consists of three transcriptional factors: the morning-phased CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and the evening-phased TIMING OF CAB EXPRESSION 1 (TOC1). CCA1/LHY and TOC1 are repressors of each other’s expression8,9. Besides the TTFL circadian clock, non-transcriptional redox oscillations exist in all domains of life, including Arabidopsis3. Even though redox rhythm was shown to influence the TTFL clock, how these two oscillatory systems are linked molecularly, and what the biological significance of having two oscillatory systems is, remain largely unknown.

To begin addressing these questions, we examined the daily changes in the reduction–oxidation coenzymes NADPH and NADP⁺ in Arabidopsis under constant light and found them to display circadian rhythms (P < 10⁻⁴), with NADPH peaking before subjective dawn and NADP⁺ peaking before subjective dusk (Fig. 1a, b). Moreover, their ratio also oscillated in a circadian manner (Extended Data Fig. 1). These data support the existence of widespread metabolic and redox rhythms in plants beyond the previously reported oscillations of oxidized peroxiredoxin, H₂O₂, and catalases7,10. It is known that the plant immune-inducing signal salicylic acid (SA) can alter the cellular redox to trigger defence gene expression11. We found that under constant light, treating plants with SA could significantly perturb NADPH and NADP⁺ rhythms as well as their ratio (Fig. 1a, b and Extended Data Fig. 1), indicating that the redox rhythm is sensitive to external perturbations.

We next examined whether this SA-triggered redox rhythm perturbation could be transduced to the circadian clock by first focusing on the evening-phased TOC1, which is responsive to many environmental factors13. Using quantitative PCR (qPCR), we observed significant increases in amplitude and average expression of TOC1 upon SA treatment (Fig. 1c). Similar results were observed using a transgenic line carrying a reporter of the TOC1 promoter fused to luciferase (TOC1p:LUC)14 (Fig. 1d, e and Extended Data Fig. 2a). Strikingly, the period of the TOC1p:LUC expression rhythm did not change, regardless of whether SA was applied at subjective dawn (Fig. 1d) or dusk (Fig. 1e).

To study the effect of endogenous SA, which oscillates in a circadian manner15, on the clock, we crossed the TOC1p:LUC reporter into the SA biosynthesis mutant, sid2 (SA induction–deficient)2. We found that the amplitude and average expression of TOC1 were significantly reduced in sid2 and this phenotype was rescued upon treatment with exogenous SA (Extended Data Fig. 2b). Our results indicate that endogenous SA plays a part in the redox rhythm that modulates the amplitude and average expression of the circadian clock.

SA-induced redox changes can lead to reduction of the master immune regulator, NPR1, the release of NPR1 monomer for nuclear translocation, defence gene induction12, and subsequent degradation mediated by the nuclear SA receptors NPR3 and NPR4 (ref. 17). To test whether the SA-mediated regulation of TOC1 is through NPR1, we crossed TOC1p:LUC into the npr1 mutant18. We found that the mutation not only dampened the basal expression of TOC1 but also abolished the SA-triggered increases in expression regardless of the time of treatment (Fig. 2a and Extended Data Fig. 3a–c).

We hypothesized that NPR1 is an intrinsic regulator of TOC1 in response to the rhythmic accumulation of the endogenous SA15. Through western blotting, we indeed found a circadian oscillatory pattern for the NPR1 monomer (P < 0.01) with a peak at night (Fig. 2b and Extended Data Fig. 4a). Therefore, oscillation in the endogenous SA level may drive the rhythmic nuclear translocation of NPR1 to regulate the circadian clock genes. To test this hypothesis, we used mutants of cytoplasmic-localized thioredoxins (TRX), trx-h3 and trx-h5, in which NPR1 nuclear translocation is largely impaired19. We found that both the basal rhythm of TOC1p:LUC and its responsiveness to SA were diminished in trx-h3 trx-h5 (Fig. 2c and Extended Data Fig. 5a), suggesting the requirement of NPR1 nuclear translocation in regulating TOC1 expression. Besides SA, glutathione-reduced ethyl ester (GSHmee), a redox-altering reagent20, could also enhance TOC1 expression in an NPR1-dependent manner (Extended Data Fig. 5b), suggesting that NPR1 is a general redox sensor in modulating this clock gene.

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Figure 1 | SA disrupts redox rhythm but boosts TOC1 expression without changing its period.  

- a, c NADPH (a), NADP⁺ (b), and TOC1 messenger RNA (mRNA) (c) in plants after application of water (CK) or SA at 0 h under constant light (LL). White and grey bars represent subjective days and nights, respectively. Data are mean ± s.e.m. (n = 3; t-test; ***P < 0.001).

NPR1 is a transcription cofactor of the TGA class of transcription factors in SA-induced defence gene expression²⁵. Using a yeast one-hybrid assay, six *Arabidopsis* TGAs were found to have strong binding affinities to the TOC1 promoter at the two TGA-binding sites (TBS) (Fig. 2d). To confirm this in planta, we mutated TBS in the TOC1p:LUC reporter (TOC1p (TBSm):LUC) and transformed it into *Arabidopsis*. We found that these mutations significantly inhibited transcription of the reporter (P < 0.001), indicating that TGAs are transcription activators of TOC1 (Fig. 2e). A direct role that NPR1/TGA plays in regulating TOC1 expression was further confirmed through chromatin immunoprecipitation (ChIP) in which association of NPR1 to TBS in the TOC1 promoter was significantly enhanced upon SA induction (Fig. 2f).

TOC1 is unlikely to be the only clock gene regulated by NPR1, because lowering the TOC1 level shortens the clock period whereas elevating the level lengthens the period²²,²³. However, no such perturbation was observed in npr1 (Extended Data Fig. 3d) or after SA treatment (Fig. 1d, e). Moreover, SA treatment at dawn should have caused an immediate induction in TOC1 expression instead of a 12-h delay (Fig. 1d). To systematically search for other NPR1-targeted clock genes, we performed mathematical modelling using the P2012 circadian model²¹ under the assumption that NPR1 is also a transcriptional activator of other clock genes (X and Y in Fig. 3a) (see Methods and Extended Data Figs 6 and 9 for details).

We first optimized the P2012 model to fit the TOC1 expression in npr1 (Fig. 2a), which was a single parameter fit (that is, basal expression in the absence of functional NPR1). The heat map of the best least-squares fit showed a characteristic ‘crosshair’ pattern centred on PSEUDO-RESPONSE REGULATOR 7 (PRR7) (Fig. 3b), indicating that the basal regulation of PRR7 by NPR1 best explains the unchanged TOC1 period in npr1 (Extended Data Fig. 6a, b). This prediction was verified using qPCR in which PRR7 transcript levels in npr1 were found to be significantly lower than wild type (WT) (Fig. 3c). The second fitting for SA-induction data involved multiple parameters. We used our fixed basal expression parameter and NPR1 western data (Fig. 2b and Extended Data Fig. 4) to fit the TOC1 expression from Fig. 2a. The resulting heat map showed a ‘crosshair’ pattern for LHY/CCA1 (Fig. 3d), suggesting that either one or both of these genes is responsive to SA through the function of NPR1. Using qPCR, we found that while CCA1 and EARLY FLOWERING 3 (a negative control) did not respond to SA, the amplitude of LHY expression was significantly elevated by SA (P < 0.05) (Extended Data Fig. 6c–e) as predicted by our model. This result was further confirmed using the LHY:pLUC reporter (Fig. 3e). Consistently, the amplitude of basal LHY expression was reduced in npr1 (P < 0.05) whereas that of CCA1 remained unchanged (Extended Data Fig. 6f, g). Because LHY is an antagonist of TOC1 in the clock, induction of LHY by SA explains the delayed increase in TOC1 after SA treatment at dawn (Extended Data Fig. 6i, j) when LHY has its highest expression. This balanced network architecture of NPR1 regulating both the morning-phased LHY and the evening-phased TOC1 (Fig. 3a) strengthens the clock when the redox rhythm is perturbed.

To investigate the effect of reinforced circadian clock on plant immunity, we examined SA-induced resistance against bacteria in a toc1 mutant and found it to be more sensitive to induction than WT and npr1 (Fig. 4a). While TOC1 negatively regulates resistance against bacteria, CCA1 and LHY have been reported to positively regulate resistance against bacteria and oomycetes²⁴,²⁵, timing immunity for the morning when temperature and humidity are the most favourable for infection²⁵. We hypothesized that SA/NPR1-mediated induction of both morning and evening components of the circadian clock plays a
They were mainly defence-related genes (Fig. 4d). In contrast, a larger number of genes with higher induction by SA treatment were identified (Fig. 4b). We next performed microarray to investigate the time-of-day-specific sensitivity globally (GSE61059) (patterns of representative genes verified by qPCR shown in Extended Data Fig. 4a). We found more genes showing higher induction by morning SA treatment than the evening treatment (like PR1) (Fig. 4c). They were mainly defence-related genes (Fig. 4d). In contrast, a larger number of genes appeared to be more repressed after the evening treatment and they were enriched in plant growth and development (Fig. 4d). Furthermore, promoter analysis of the differentially induced genes in the morning showed significant enrichments for both cis-elements bound by CCA1/LHY and TOC1, and those of the differentially repressed genes in the evening had significantly enriched cis-elements bound by CCA1/LHY (Extended Data Fig. 7c, d). Collectively, these data strongly support our hypothesis that acute perturbation in redox rhythm caused by SA treatment leads to increased expression of both positive and negative regulators of defence towards the morning may also be a mechanism (Fig. 4e) but induced a severe loss in fresh weight (Fig. 4f). The uncropped version is provided for the TOC1 gene using 5S0NPR1-GFP (in npr1-1) plants. Data are mean ± s.e.m. (n = 3; Tukey’s multiple comparisons test; P < 0.0001).
We propose that in Arabidopsis the daily redox rhythm is intrinsically linked with the basal expression of the circadian clock through NPR1 (Fig. 4h and Extended Data Fig. 8). Perturbation in redox rhythms caused by SA during pathogen challenge is sensed by NPR1 to trigger defence gene expression and to reinforce the circadian clock. The wiring of NPR1 to defence genes as well as to the clock shows how plants gate their immune responses towards the morning to anticipate infection while minimizing fitness costs on plant growth, which occurs mainly at night.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions M.Z., W.W., S.K., and J.M. performed the experiments and statistical analysis. S.K. and N.E.B. identified additional links of NPR1 to the circadian clock through mathematical modelling. X.D. supervised the project. M.Z., W.W., S.K., and J.M. performed the experiments and statistical analysis. S.K. and N.E.B. identified additional links of NPR1 to the circadian clock through mathematical modelling. X.D. supervised the project.
METHODS

No statistical methods were used to predetermine sample size.

Plant materials. The TOC1p:LUC (Col-0), LHYp:LUC (Col-0), and CAT2p:LUC (Col-0) seeds were provided by D. R. McClung and the toc1-h10 mutant by S.-H. Wu. Mutants of npr1-1/d, npr1-2, sid2 (ref. 16), trh-4H (ref. 19), and trh-5H (ref. 19) were used to cross with the luciferase reporter lines. 35S::NPR1::GFP (in nap-1)12 plants were used in ChIP experiments. To generate CAT2p:LUC, CAT2p:LUC homozygous lines and different T1 lines of TOC1p:LUC and TOC1p::LUC (TOC1 promoter with mutated TGA-binding sites), WT CAT3, CAT2, and TOC1 promoters and mutated TOC1 promoter (amplified using QuikChange Lighting Multi Site-directed mutagenesis kit (Agilent Technologies) were cloned into the pDONR207 vector (Invitrogen) through the Gateway LR recombination reaction and transferred to the destination vector pGBW235 (ref. 32) through the Gateway LR recombination reaction (Invitrogen). Agrobacterium-mediated transformation of Arabidopsis was performed as previously described using WT plants33. Homozygous T3 lines of CAT2p:LUC, CAT3p:LUC, and different T1 lines of TOC1p:LUC and TOC1p::LUC (TOC1 promoter mutated in the first TGA-binding site) were selected and used for the luciferase imaging experiment. All primer sequences used for making the transgenic constructs are listed in Extended Data Table 1.

NADPH and NADPH measurement. Three-week-old WT (Col-0) plants grown in soil under diurnal condition (12 h light/12 h dark) were treated with water or 1 mM SA at subjective dawn and samples were collected every 4 h for 2 days under constant light conditions. NADPH and NADPH were measured according to the manufacturer’s protocols. All primer sequences used for yeast one-hybrid assay (Y1H) are listed in Extended Data Table 1.

Luciferase activity measurement. Plants grown in soil with under 12 h light/12 h dark cycles for 3 weeks were sprayed with 2.5 mM luciferin (Gold Biotechnology) for 20 min. To test the effect of SA or GSHmee, 1 mM SA (Sigma)/3 mM GSHmee (Invitrogen) and the Gateway BP reaction. The entry clones were recombined into destination vectors pMW#2 (Invitrogen) and pMW#3 (Invitrogen). Mutagenesis of the TOC1 promoter was performed using a QuikChange Lighting Multi Site-directed mutagenesis kit (Agilent Technologies) according to the instruction manual. TOC1p_Mut1 (the TOC1 promoter mutated in the first TGA-binding site), TOC1p_Mut2 (the TOC1 promoter mutated in the second TGA-binding site), and TOC1p_Mut1+2 (the TOC1 promoter mutated in both TGA-binding sites) were cloned into destination vectors pMW#2 and pMW#3 through the Gateway cloning kit (Invitrogen). The coding sequences of TGAs were cloned into pDONR207 and subsequently transferred into pDEST-AD by the Gateway LR reactions. Transformations of constructs into the yeast strain YM4271 was performed as previously described34. β-Galactosidase reporter activities were measured using ONPG as the substrate35 and normalized to the control with an empty vector pDEST-AD. All primer sequences used for yeast one-hybrid assay (Y1H) are listed in Extended Data Table 1.

ChIP. Three-week-old soil-grown 35S::NPR1::GFP (in nap-1)12 plants were treated with either water (CK) or 1 mM SA at dusk and samples were collected 3 h after treatment. ChIP was performed as described previously34. Immunoprecipitation was performed using a polyclonal antibody against GFP (Ab290, Abcam) and Dynabeads Protein G (Invitrogen). The purified ChIP samples were subject to qPCR using primer pairs for the promoter region (−639 to −589 base pairs (bp) upstream of the start codon) and the coding region (+753 to +803 bp downstream of the start codon) of TOC1. Fold of enrichment was calculated using the comparative Ct method36 using the input samples as normalizers. All primer sequences used for ChIP are listed in Extended Data Table 1.

The mathematical model of the Arabidopsis circadian clock. We applied the P2012 plant circadian model from the Plant Systems Modelling portal37 to elucidate new connections between SA signalling and known plant circadian genes. This numerical ordinary differential equation model in MATLAB consists of 32 ordinary differential equations and includes transcription terms for ten genes, which are LHY/CCA1, PSEUDO-RESPONSE REGULATOR 9, 7, 5 (PRR9, PRR7, PRR5), TOC1, EARLY FLOWERING 4, 3, 1 (ELF4, ELF3, LUX ARRHYTHMOS (LUX), GIGANTEA (GI) and ABA receptor (ABAR). The 133 parameters in P2012 were previously fitted to multiple data sets in various light–dark photoperiods, different genetic backgrounds, and ABA signalling. It is important to note that the P2012 model was designed to understand and predict changes in period and phase when perturbed by genetic or environmental variations. The model does not aim to reflect the exact transcriptional profiles or the absolute protein concentrations.

It was recently shown that plant circadian models exhibit a ‘period overshoot’ when transitioning from DD to LL cycles38. This period overshoot introduced a constant phase delay in the DD data relative to the LL data. Since this phenomenon was not observed in the experimental data, it is an artefact of the mathematical model. For example, the P2012 model predicts that TOC1 mRNA peaks at ZT18 (that is, 6 h after subjective light-to-dark transition) under LL conditions39 instead of the real peak time at ZT12. Moreover, our experiments indicate that TOC1 expression is higher in mock- and SA-treated plants, which is known to exhibit delays40. This delay was deduced to be 2 h because the luciferase reporter peaked at ZT14 (Fig. 1d) whereas the TOC1 mRNA peaked at ZT12 (Fig. 1c).

To take this 2-h delay and the ‘period overshoot’ in the model into consideration, we empirically measured a 4-h delay between simulation TOC1 mRNA levels and our luminescence data. This total 4-h delay was inferred by aligning the second peak after the LD to LL transition in our luciferase experiments (38 h) and TOC1 mRNA in the model (42 h). We subsequently used this 4-h delay to correctly align and fit the P2012 simulation TOC1 mRNA to our experimental luciferase data.

Addition of NPR1 regulation to the circadian clock model. While keeping the original P2012 parameter fits, we added NPR1 as a transcriptional activator of TOC1, as it has been shown experimentally. We also added NPR1 as a transcriptional activator of two additional clock genes (‘query pair’). Our goal was to systematically determine which query pair best fit our measured TOC1p:LUC expression in WT and nap1-3 in mock- and SA-treated plants. We multiplied the P2012 transcriptional synthesis term of TOC1 and each gene in the query pair by...
their own NPR1-dependent regulatory function \( F(t) \) (that is, non-competitive activation). Each regulatory function \( F \) has the form

\[
F(t) = n_0 + n_i + \frac{[\text{NPR1}]}{[\text{NPR1}]} + K_d
\]

where \([\text{NPR1}]\) is the NPR1 monomer concentration over time, \( n_i \) is the basal, NPR1-independent transcription level of the gene of interest, \( n_0 \) is the maximum NPR1-activated transcription level of the gene, and \( K_d \) is the effective DNA-binding dissociation constant for the gene. The \([\text{NPR1}]\) monomer levels for mock-treated and SA-treated plants were taken from western blot data in Fig. 2b and Extended Data Fig. 4. The NPR1 data for the mock-treated and SA-treated plants were then averaged, normalized, and linearly interpolated to serve as an input function for modelling (Extended Data Fig. 9a, b).

**Least-squares fitting of the TOC1p:LUC data.** For every query pair and the TOC1 gene, we optimized \( n_0, n_i, K_d \) parameters (among the nine parameters, six are independent; see below) to give the best least-squares fit of the TOC1 mRNA profile in the model to the patterns of TOC1p:LUC expression in WT and npr1-3 to 24 h in our experiments. We fitted 3-day-long P2012 model and the use of the reporter, we started fitting at 28 h, which corresponds to 24 h in our experiments. To account for the 4-h delay inherent to ‘period-overshoot’ in the TOC1 mRNA profile. To circumvent the waveform issue, we first calculated the waveform, we first calculated the (ratio) of the different nodes (that is, WT with and without SA treatment, and npr1), we ran nonlinear least-squares fit from 15 random starting points. We found that again, in general, they converged to the same global optimum (Extended Data Fig. 9f, g). Figure 3d shows a cross-hair pattern centred on LHY/CCA1, suggesting that LHY/CCA1 activation by the induced, arrhythmic NPR1 levels during SA treatment should counteract the effect of TOC1 induction. We noted that the best-fit solution from a TOC1-only case shows immediate TOC1 induction after SA treatment (Extended Data Fig. 6i) instead of the observed delay until dusk. However, if we added LHY as an NPR1 target in addition to TOC1, the modelling results best fit our experimental data (Extended Data Fig. 6j). Consistently, LHY expression was found to be induced by SA (Fig. 3e), but reduced in the npr1 mutant (Extended Data Fig. 6k).

**Limitations of the model.** There are limitations to our model. First, our model was only fitted to the expression of one gene (TOC1p:LUC) under three different conditions (that is, WT with and without SA treatment, and npr1). Second, our model combines LHY/CCA1 into one gene and cannot resolve the experimental differences that we observed in those genes (Fig. 3e and Extended Data Fig. 6c, d). Third, our model for the SA-induction data pre-sets \( PRR7 \) to maximum expression even without SA treatment (that is, \( K_d = 0 \)) (Extended Data Fig. 9g). This is unlikely to be an accurate reflection of a real physiological state. Last, all our experiments and modelling were done under constant light conditions and the additive (acute) light activation terms are effectively 0 because the hypothetical protein responsible for light activation has decayed to 0. These additive light terms should not be affected by our assumption of non-competitive activation by NPR1. Uncovering the proper relationship between the light-dependent terms and the NPR1-dependent terms (that is, competitive versus non-competitive activation) would require experiments under different circadian conditions. This is outside of the scope of the current paper. Even though the modelling approach correctly predicted \( PRR7 \) and LHY as direct targets of NPR1, it had mixed results with ELF3, which is not a direct target. Our model predicted ELF3 to decrease after SA treatment and to increase in the npr1 mutant. The lack of induction in ELF3 by SA (Extended Data Fig. 6e) is consistent with the model prediction. However, the significantly decreased expression of ELF3 observed in npr1 (Extended Data Fig. 6f) was not in agreement with the model. This discrepancy suggests there are other links between NPR1 and the circadian clock that the current model cannot capture. A future model should be fitted to ELF3p:LUC, which would be an informative constraint.

**Code availability.** The MATLAB code, which was used to fit the modified P2012 plant circadian clock model to our TOC1 luciferase data, is available upon request. A final SBML version of the modified P2012 model with our best-fit parameters can be downloaded from the BioModels Database (MODEL1506010000).

**Bacterial infection.** Three-week-old plants grown in soil were pre-treated with water or 1 mM SA at ZT12 and infiltrated 24 h later with *Pseudomonas syringae* pathovar *maculicola* (Psm) ES4326 (absorbance \( A_{600 nm} = 0.001 \) as previously described21. Bacteria were either grown in LB medium or in solidified LB medium, with SA, Psm, Psm infiltrated, and SA infiltrated. Bacterial growth was measured 3 days after inoculation.

**Microarray analysis.** To test time-of-day-specific sensitivity to SA, 3-week-old soil-grown plants were transferred to constant light condition 1 day before treatment. Water or 1 mM SA was applied in the subjective morning (ZT24) or evening (ZT36). The fifth and sixth leaves were sampled 0 and 3 h after treatment. RNA was extracted, amplified, labelled, and hybridized to ATH1 GeneChip (Affymetrix) as previously reported38. The arrays were normalized with RMA and centred to median. Two-way ANOVA (\( P < 0.05 \)) and Student’s \( t \)-test with multiple comparison correction (\( P < 0.05, 0.05, 0.001 \) were used) to identify genes that were significantly more induced or more repressed by water or SA when treated at ZT24 or at ZT36. Athana program (http://www.bioinfor

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statistical significance. Enriched gene ontology categories were identified using BiNGO (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html).

Fresh/dry weight measurement. Three-week-old soil-grown WT (Col-0) plants were transferred into constant dark condition or normal diurnal condition at dusk. After 36 h, water (control) or 1 mM SA was applied. Two days after treatment, pictures were taken and fresh/dry weight was measured.

Statistical analysis. Statistical analysis used GraphPad Prism 6 (GraphPad Software). All the centre values shown in the figures are means of technical (Figs 1a, b and 2d and Extended Data Fig. 1) or biological (all other figures where applicable) replicates. Experiments were repeated twice for Fig. 1a, b and CAB2p:LUC in Fig. 4g. All other experiments were repeated three times where applicable. Harmonic regression (\(Y = a\sin(\pi t/12) + b\cos(\pi t/12) + c\)) followed by ANOVA test was used to identify statistically significant oscillation. The null hypothesis was that all data across different time points were sampled from the same normal distribution. Student’s t-test with multiple comparison correction was performed to identify statistically significant differences between mock and treated samples. Two-way ANOVA was used to assess significant interactions between genotype and treatment or between time of treatment and treatment. Significant interactions suggested the effect of the treatment was dependent on genotype or time of treatment. Tukey’s multiple comparisons test was performed to identify the orders of samples that were significantly different from each other. All statistical tests were two-sided tests where applicable.

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Extended Data Figure 1 | Circadian oscillation of the NADPH/NADP⁺ ratio. NADPH/NADP⁺ ratios in 3-week-old soil-grown plants derived from Fig. 1a, b. Water (CK) or 1 mM SA was applied at 0 h. Data are mean ± s.e.m. (n = 3). White bars represent subjective days and grey bars represent subjective nights. Harmonic regression analysis suggests significant circadian oscillation of water-treated NADPH/NADP⁺ ratio (P < 0.0001).
Extended Data Figure 2 | The effects of exogenous and endogenous SA on TOC1 expression. a, Luciferase activity measurements using the TOC1p:LUC plant extracts. Relative luciferase activity of the fifth and sixth leaves from 3-week-old soil-grown TOC1p:LUC plants. Water (CK) or 1 mM SA was applied at ZT24. LL, constant light. a.u., arbitrary unit. Data are mean ± s.e.m. (n = 6 biological replicates; t-test; ***P < 0.001). b, TOC1p:LUC activity rhythms in 3-week-old soil-grown WT and sid2 plants treated with water (CK) or 1 mM SA at subjective dusk (black arrow) (mean ± s.e.m., n = 8 plants). White bars represent subjective days and grey bars represent subjective nights. The bar graphs represent the estimates of amplitude and average expression of TOC1p:LUC, respectively (mean ± s.e.m.). The letters above the bars indicate statistically significant differences between groups at P < 0.05 (Tukey’s multiple comparisons test). NS, non-significant (two-way ANOVA, non-significant interaction between genotype and treatment). This experiment was repeated three times with similar results.
Extended Data Figure 3 | NPR1 regulates the amplitude and average expression of TOC1p:LUC. a, TOC1p:LUC activity rhythms in 3-week-old soil-grown WT and npr1-3 plants treated with water (CK) or 1 mM SA at subjective dusk (black arrow) (mean ± s.e.m.; n = 6 plants). LL, constant light. a.u., arbitrary unit. White bars represent subjective days and grey bars represent subjective nights. The bar graphs show the estimates of amplitude and average expression level (mean ± s.e.m.; two-way ANOVA; *P < 0.05; ****P < 0.0001). b–d, Estimates of amplitude (b), average expression (c), and period (d) of TOC1p:LUC in WT and npr1-3. Data are mean ± s.e.m. (t-test; ****P < 0.0001). These experiments were repeated three times with similar results.
Extended Data Figure 4 | The abundance of NPR1 monomer under constant light conditions. NPR1 monomer (M) abundance in 3-week-old soil-grown plants without treatment (a; uncropped version of Fig. 2b) and after 1 mM SA treatment at 0 h (b) under constant light (LL) conditions. NPR1 protein were detected using western blot after non-reducing SDS–PAGE (a, b). NPR1 monomer protein was quantified using the non-specific band (*) as a loading control (b; mean ± s.e.m., n = 3 biological replicates). O, NPR1 oligomer. White bars represent subjective days and grey bars represent subjective nights.
Extended Data Figure 5 | Redox perturbations affect the amplitude and average expression of TOC1p:LUC in an NPR1-dependent manner. a, TOC1p:LUC activity rhythms in 3-week-old soil-grown WT and trx-h3 trx-h5 (trx-h3 h5) (mean ± s.e.m., n = 6 plants). LL, constant light. White bars represent subjective days and grey bars represent subjective nights. The bar graphs show the estimates of amplitude and average expression (mean ± s.e.m.; t-test; ****P < 0.0001). b, TOC1p:LUC activity rhythms in 3-week-old soil-grown WT and npr1 plants treated with water (CK) or 3 mM GSHmee at subjective dusk (black arrow) (mean ± s.e.m., n = 8 plants). The bar graphs represent the estimates of amplitude and average expression of TOC1p:LUC, respectively (mean ± s.e.m.). The letters above the bars indicate statistically significant differences between groups at P < 0.01 (Tukey’s multiple comparisons test). **P < 0.01; ****P < 0.0001 (two-way ANOVA). These experiments were repeated three times with similar results.
Extended Data Figure 6 | Model prediction and validation. a, Comparison of best-fit solutions for the TOC1-only and the TOC1-and-PRR7 coupling in npr1. LL, constant light. White bars represent subjective days and grey bars represent subjective nights. b, Addition of PRR7 coupling improves the fitness and mostly rescues the short period phenotype of the TOC1-only model (mean ± s.e.m.; n = 715, n is degree of freedom derived from nonlinear regression). c–e, The transcript levels of CCA1 (c), LHY (d), and ELF3 (e) in WT plants after water (CK) or 1 mM SA treatment. f–h, The transcript levels of CCA1 (f), LHY (g), and ELF3 (h) in WT and npr1 plants. The expression was normalized to UBQ5 (c–h). The bar graphs show the estimates of amplitude and average expression level, respectively (c–h; mean ± s.e.m.; n = 3 biological replicates; t-test; *P < 0.05; ***P < 0.001; ****P < 0.0001). i, j, Comparison of best-fit solutions for NPR1 activation of TOC1-only (i) and NPR1 activation of TOC1 and LHY/CCA1 (j) after SA treatment.
Extended Data Figure 7 | Validation and analysis of microarray data.

a, b, The transcript levels of *CML40* (a) and *AT4G33960* (b) in 3-week-old soil-grown plants 0 or 3 h after application of 1 mM SA either in the subjective morning (ZT24) or in the subjective evening (ZT36) normalized to *UBQ5* under constant light conditions. Data are mean ± s.e.m. (n = 3 biological replicates; two-way ANOVA; ***P*, 0.001; ****P*, 0.0001). c, d, Enrichment of *cis*-elements affecting time-of-day-specific sensitivity to induction. Promoter analysis of genes that were more induced by SA when treated at ZT24 (c) or more repressed by SA when treated at ZT36 (d). The heat maps show the average expression levels based on the microarray. Circadian correlation coefficients were extracted from Diurnal (http://diurnal.mocklerlab.org/diurnal_data_finders/new). Yellow represents a high value or a target of CCA1/LHY or TOC1. Blue represents a low value or not a target of CCA1/LHY or TOC1. X represents a gene that was more induced by SA when treated at ZT24 (c) or more repressed by SA when treated at ZT36 (d). Arrows represent activation. Blocked arrows represent repression. *P* values were determined on the basis of hypergeometric distribution.
Extended Data Figure 8 | NPR1 senses and transduces redox signals to trigger transcriptional reprogramming. SA-triggered redox changes induce the oligomer-to-monomer switch of NPR1. The monomer then enters the nucleus and upregulates both defence genes and clock genes through interaction with TGA transcription factors.
Extended Data Figure 9 | Technical details for model fitting. a, Normalized NPR1 monomer abundance in mock-treated samples. The blue line presents the mean values from Fig. 2b, where the value at 48 h (marked with an open star) was inferred to be the same as that at 0 h. The red line represents the smoothened values used for modelling by averaging over 2 days to create a 1-day trace, which was then repeated over 2 days. The smoothened data were normalized, such that the time average of NPR1 was equal to 1. LL, constant light. White bars represent subjective days and grey bars represent subjective nights. b, SA-treated NPR1 monomer abundance. NPR1 monomer abundance after SA treatment from Extended Data Fig. 4b was normalized so that 0 h has the same value as the corresponding mock-treated NPR1 monomer level. On the basis of the assumption that the SA induction lasted for 2 days, the value of the last time point was inferred to be equal to the basal level (marked with an open star). c, Coefficient of variation (CV) of least-squares residual $\Sigma$ for 15 different, random initial parameters for the model fitting of $npr1$ data. d, Coefficient of variation of $n_b^*$ for 15 different, random initial parameters for the model fitting of $npr1$ data. e, Optimal $n_a^*$, $K_d^*$ exhibit a linear relationship. log($\Sigma$) was plotted as a function of $n_a$ and $K_d$ for mock-treated TOC1-only coupling (no query pairs). A ‘low’, linear $\Sigma$ region is evident and is described by a simple analytical linear relationship, $n_b^* = 0.5689 h^{-1}$. f, Coefficient of variation of $\Sigma$ for 15 different, random initial parameters for the model fitting of SA-treated data. g, Coefficient of variation of $K_d^*$ for 15 different, random initial parameters for the model fitting of SA-treated data.
## Extended Data Table 1 | Primer sequences

| Purpose | Primer name | Sequences |
|---------|-------------|-----------|
| Transgenic plants | TOC1p_F | GGGGACAAGTTTGTACAATAAAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TOC1p_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TOC1p_Mut1 | ATATTTTCTCCAAGAGTCGGCTGCTTTCCT |
| | TOC1p_Mut2 | TTTTTATTTGCAAGGACTCTTGCTGCTGCTCAA |
| | CAT3p_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | CAT3p_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | CAT2p_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | CAT2p_R | GGGGACAACCTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| Y1H | TOC1p_P4p1R_F | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TOC1p_P4p1R_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TOC1p_Mut1 | ATATTTTCTCCAAGAGTCGGCTGCTTTCCT |
| | TOC1p_Mut2 | TTTTTATTTGCAAGGACTCTTGCTGCTGCTCAA |
| | TGA1_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA1_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA2_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA2_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA3_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA3_R | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA4_F | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA4_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA5_F | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA5_R | GGGGACAACCTTTGTACAAAGAAAAGGCTGGCTTGGTCATTGTTTTCATTGGCTCAATC |
| | TGA6_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA6_R | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA7_F | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| | TGA7_R | GGGGACAAGTTTGTACAAAGAAAAGCAGGCTTAGATCGTCGGCTGCTCAACAA |
| qPCR | TOC1_qP_F | AATAGTAAATCCAGGCGCAATTTTCTTC |
| | TOC1_qP_R | CTTCAAATCTAGTTTCTCCTGGCT |
| | LHY_qP_F | CGCTGTTCCGGTCTGCTGGCT |
| | LHY_qP_R | TGTACAGCAGGGCAATGAGCAGT |
| | PRR7_qP_F | CAGTCCAAGGAGCGCTTCTC |
| | PRR7_qP_R | CAGTCCAAGGAGCGCTTCTC |
| | CCA1_qP_F | CGAGGCGCAGCTCAGGGTTT |
| | CCA1_qP_R | CGAGGCGCAGCTCAGGGTTT |
| | ELF3_qP_F | TGCCAAATACTGGTCAAAGGA |
| | ELF3_qP_R | TGCCAAATACTGGTCAAAGGA |
| | PR1_qP_F | TGGGAACAGCATCAGGCAAGCT |
| | PR1_qP_R | TGCCAAATACTGGTCAAAGGA |
| | WRKY40_qP_F | ACAAGAAGTACAGCTGAGAAGAAGAC |
| | WRKY40_qP_R | ACAAGAAGTACAGCTGAGAAGAAGAC |
| | CML40_qP_F | GAGCCAAACAGAGAGAAGTAT |
| | CML40_qP_R | GAGCCAAACAGAGAGAAGTAT |
| | AT4G33960_qP_F | CGTCGAGAGAAGGAGAAGAGGAG |
| | AT4G33960_qP_R | CGTCGAGAGAAGGAGAAGAGGAG |
| | UBQ5_qP_F | GACGCTCTCATGCTC |
| | UBQ5_qP_R | GACGCTCTCATGCTC |
| ChIP | TOC1 ChIP promoter_F | TGTCGAGAGAAGGAGAAGAGGAG |
| | TOC1 ChIP promoter_R | AGCTTTAATGAGGAGGACTTCGG |
| | TOC1 ChIP coding region_F | GACGCAAGAGGAAACAGGTCCTG |
| | TOC1 ChIP coding region_R | GACGCAAGAGGAAACAGGTCCTG |