LWD–TCP complex activates the morning gene CCA1 in Arabidopsis

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A double-negative feedback loop formed by the morning genes CIRCADIAN CLOCK ASSOCIATED1 (CCA1)/LATE ELONGATED HYPOCHOTYL (LHY) and the evening gene TIMING OF CAB EXPRESSION1 (TOC1) contributes to regulation of the circadian clock in Arabidopsis. A 24-h circadian cycle starts with the peak expression of CCA1 at dawn. Although CCA1 is targeted by multiple transcriptional repressors, including PSEUDO-RESPONSE REGULATOR9 (PRR9), PRR7, PRR5 and CCA1 HIKING EXPEDITION (CHE), activators of CCA1 remain elusive. Here we use mathematical modelling to infer a co-activator role for LIGHT-REGULATED WD1 (LWD1) in CCA1 expression. We show that the TEOSINTE BRANCHED 1-CYCLOIDEA-PCF20 (TCP20) and TCP22 proteins act as LWD-interacting transcriptional activators. The concomitant binding of LWD1 and TCP20/TCP22 to the TCP-binding site in the CCA1 promoter activates CCA1. Our study reveals activators of the morning gene CCA1 and provides an action mechanism that ensures elevated expression of CCA1 at dawn to sustain a robust clock.
The operation of the circadian clock allows organisms to synchronize the internal biological activities with the external 24-h rhythmic light/dark or temperature cues. Plants with a defective circadian clock have reduced fitness. The circadian clock in Arabidopsis is mainly driven by a double-negative feedback loop formed by the morning-phased CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)/LATE ELONGATED HYPOCOTYL (LHY) and the evening-phased TIMING OF CAB EXPRESSION1 (TOC1) (refs 2–5). In the current model of the Arabidopsis circadian clock, the key driving force for the daily oscillation is based on the repressilatory machinery that uses a repressor of a repressor as an activator to increase the expression of clock genes. In this model, CCA1 is a transcriptional repressor functionally overlapping with LHY for suppressing TOC1 in the morning. Reciprocally, TOC1 binds to the CCA1 promoter to suppress CCA1 expression in the evening. In addition to TOC1, multiple repressors including PSEUDO-RESPONSE REGULATOR9 (PRR9), PRR7, PRR5 and CCA1 HIKING EXPEDITION (CHE) suppress CCA1 at different times of the day. Although a repressor-based system can oscillate, the robustness could be enhanced by the integration of a positive feedback loop. One such positive-feedback loop in the Arabidopsis circadian clock is formed by LIGHT-REGULATED WD1 (LWD1) and PRR9 (ref. 14). More recently, an activator co-activator system consisting of REVEILLE8 (RVE8) and NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATEDs (LNKs) could directly target the promoters of evening genes, PRR5 and TOC1, to activate their expression. However, although the light-activated expression of CCA1 prompts the synchronization of an oscillating clock, activators for CCA1 expression at dawn are still unknown.

Here, our mathematical models show that the simultaneous activation of PRR9 and CCA1 by LWD1 in the clock system allows for more precise predictions of clock behaviours in both wild-type plants and clock mutants. We further demonstrated that by direct interaction with two transcription factors, TEOSINTE BRANCHED 1–CYCLOIDEA–PCL20 (TCP20) and TCP22, LWD1 could associate with the CCA1 promoter in vivo. TCP20 and TCP22 are new clock proteins capable of binding to the TCP-binding site (TBS) in the CCA1 promoter. Without LWDs, the binding of TCP20 and TCP22 to CCA1 promoter fails to activate CCA1. This study first discloses an activator–coactivator complex triggering CCA1 expression in the morning.

**Results**

The dual activation of PRR9 and CCA1 by LWD1. LWD1 can activate PRR9 (ref. 14), one of the transcriptional repressors of CCA1 (ref. 10). However, in contrast to the increased CCA1 level one would expect in the lwd1 lwd2 double mutant with reduced PRR9 expression, CCA1 expression is also much reduced in lwd1 lwd2 (ref. 14). We previously showed that LWD1 can directly target promoters of multiple clock genes, including a weak association with CCA1 at ZT0 (ref. 14). This finding led us to hypothesize that, in addition to activating PRR9, LWD1 can also activate CCA1. To determine the plausibility of such interaction, we used mathematical models to examine clock performance/robustness when LWD1 activates only PRR9 (Model I) or both PRR9 and CCA1 (Model II) (Fig. 1a). Among 2.4 × 10⁸ random parameter sets examined, 1,004 met our criteria of producing oscillation in both the wild type and lwd1 lwd2 mutant (Supplementary Note 1) for Model II as compared with only 27 of 3 × 10⁸ random parameter sets for Model I. Thus, despite having two more parameters to search for in Model II (Supplementary Note 1), the chance of obtaining parameter sets that met our criteria was significantly greater when LWD1 activates both PRR9 and CCA1. Also, only in the circadian system with LWD1 activating both PRR9 and CCA1, but not just PRR9, could the simulation reveal short period lengths of cca1 and toc1 mutants and the long period length of prr9 mutant (Fig. 1a). Including the activation of CCA1 by LWD1 increases the plausibility and robustness of the clock oscillation.

To account for the possible limitations inherent to these simplified models, we also assessed the impact of LWD1 on PRR9 alone or both PRR9 and CCA1 by integrating the regulation into a more comprehensive and established model. We chose the model by Pokhilo et al., involving many known clock genes, which can replicate the multiple genetic perturbations observed in the

**Figure 1 | LWD1 functions as an activator in the circadian clock.**

(a) Proposed models for the role of LWD1 in activating the Arabidopsis clock system via PRR9 (Model I) or both PRR9 and CCA1 (Model II). The box plots show period lengths simulated under the representative genetic perturbations (prr9, cca1 or toc1) in these two models, with the wild-type model period being 24 h. The period lengths were calculated from a good majority of the parameter sets obtained (See Supplementary Note 1 for details). Horizontal lines are medians, box edges are interquartile range and whiskers are minimum and maximum. (b) LWD1 associates with the CCA1 promoter in vivo. Diagram shows the gene structure of CCA1. Transcriptional start and exons are marked with arrow and boxes. Black boxes illustrate the coding region. Amplicons ‘a’ and ‘b’ for ChIP-qPCR assays are marked by horizontal black bars. ChIP assays involved use of the anti-LWD1 antibody at ZT21. UBC21 was a negative control. Data are mean ± s.d. (n = 3 technical replicates). Asterisk indicates that LWD1 preferentially binds with the amplicon ‘b’ in the wild type (Student’s t test; *P < 0.001). Similar results were observed in three independent experiments.
quantitative PCR (ChIP-qPCR) demonstrated a clear association examined whether LWD1 activated CCA1 reported previously16. These behaviours included the advanced maintained the perturbed clock behaviours similar to those reported previously16. These behaviours included the advanced phases of TOC1/EVENING COMPLEX and elevated levels of TOC1 mRNA in the cca1 lhy mutant (left panel in Supplementary Fig. 1c), and increased and decreased CCA1/LHY levels in toc1 and in TOC1ox, respectively (right panel in Supplementary Fig. 1c). Therefore, the integrity of the Pokhilko model could be maintained after incorporating LWD1 as an activator of both PRR9 and CCA1.

Our mathematical simulation provided a new hypothesis for LWD1 in clock regulation via CCA1 activation. We next examined whether LWD1 activated CCA1 by binding to the CCA1 promoter. Results of chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) demonstrated a clear association of LWD1 with the CCA1 promoter at ZT21, preceding the peak expression of CCA1 (Fig. 1b), which implies the bona fide direct regulation of CCA1 by LWD1.

LWD1 interacts with TCP20 and TCP22. Although LWD1 has no recognizable DNA binding domain, it has 5 WD-repeats that constitute a propeller structure frequently considered a protein–protein interaction interface19. Therefore, LWD1 may be recruited to the CCA1 promoter by interacting with transcription factors that remain to be identified. We used yeast two-hybrid screening to identify LWD1-interacting transcription factor(s) from a prey library of ~1,400 Arabidopsis full-length transcription factors20. TCP transcription factors were identified as potential interacting proteins of LWD1. An initial screen revealed four class I TCPs (TCP19, –20, –22 and CHE (also known as TCP21)) and TCP3 belonging to class II TCPs (Supplementary Table 1). TCP20 and TCP22 were the predominant interacting partners of LWD1 by their dominant presence (>98%) in the positive clones sequenced (Supplementary Table 1). Both LWD1 and LWD2 could interact with TCP20/TCP22 (Fig. 2a), which is consistent with the overlapping functions of LWD1 and LWD2 (ref. 21). Bimolecular fluorescence complementation assay (BiFC) confirmed that TCP20/TCP22 interacted with full-length LWD1 in Arabidopsis nuclei (Fig. 2b) but not truncated LWD1 with deletion of the fourth and fifth WD-repeats and expressed at a comparable level (Fig. 2b; Supplementary Fig. 2). The interactions between TCP20/TCP22 and LWD1 but not truncated LWD1 under the control of their native promoters were further validated in Arabidopsis seedlings with luciferase complementation imaging assays (Fig. 2c).

Like other Arabidopsis short-period mutants3,5, the lwd1 lwd2 mutant has an early-flowering phenotype21. The protein–protein interaction between LWDs and TCP20/TCP22 suggested that TCP20/TCP22 might function with LWDs in flowering time control. Indeed, tcp20-2 (ref. 22), tcp20-4 (ref. 22) (Supplementary Fig. 3a), and tcp22-1 (ref. 23) single mutants and the tcp20 tcp22 double mutant show early transition from the vegetative to reproductive stage under long-day conditions (16-h light/8-h dark) (Fig. 2d and Supplementary Fig. 3b). The early-flowering phenotype of tcp mutants and the interaction between TCP and LWD proteins imply that TCP20/TCP22 also functions in the photoperiodic pathway.

TCP20 and TCP22 are new clock components. The expression of CCA1 is greatly dampened under constant dark condition21 and can be quickly induced by light signals24. To test whether TCP20 and TCP22 are required for CCA1 activation by light, seedlings of the wild type and tcp20 tcp22 were first grown under a 12-h light/12-h dark cycle for 10 days and then transferred to constant dark (DD) to minimize the CCA1 expression. The light-induced and rhythmic expression of CCA1 was triggered by 1 h of light treatment at DD24. The wild type showed peak CCA1 expression at DD45 (the following circadian cycle), but the expression amplitude was reduced in tcp20 tcp22 (Fig. 3a), which indicates that TCP20 and TCP22 are required for the full activation of CCA1 by light. We next investigated whether the activator role of TCPs on CCA1 expression results from a circadian rhythm after a full 24-h rhythm or is a direct regulation by examining the immediate function of TCP20 on the CCA1 promoter. We fused TCP20 with the ligand-binding domain of glucocorticoid receptor (TCP20-GR) and expressed it under the control of a 35S promoter. With dexamethasone (DEX) treatment, TCP20-GR immediately enhanced the expression of the reporter gene luciferase (LUC2) driven by the CCA1 promoter in Arabidopsis seedlings (Fig. 3b), which implies a direct activator role of TCP20 on CCA1 expression.

To examine whether TCP20 and TCP22 regulate the circadian period length of CCA1, we introduced pCCA1::LUC2 into tcp single and double mutants by crossing with the reporter line in a wild-type background14. Consistent with the transcript levels, the expression of pCCA1::LUC2 was greatly reduced in tcp mutants (Fig. 3c and Supplementary Fig. 3c). Mutations in TCP20 or TCP22 shortened the period lengths by 0.7 h as compared with the wild type (Fig. 3c and Supplementary Fig. 3c). Under entrainment condition with a broadened morning peak, the expression of pCCA1::LUC2 was reduced in the tcp mutants as compared with the wild type (Supplementary Fig. 4). This finding is consistent with the short period phenotype, thus an advanced phase of CCA1 expression in tcp mutants. Of note, the short-period and early flowering phenotypes were not further exaggerated in the tcp20 tcp22 double mutant (Figs 3c and 2d). Therefore, TCP20 and TCP22 function cooperatively rather than redundantly in activating clock genes, possibly because TCP20 and TCP22 can exist as heterodimers25.

The low activity of the CCA1 promoter of tcp20-2 and tcp22-1 could be partially rescued by expressing pTCP20::TCP20-Flag and pTCP22::TCP22-Flag in the complementation lines, respectively (Supplementary Fig. 5a). The early-flowering phenotypes could also be complemented (Supplementary Fig. 5b). The degree of complementation was positively correlated with levels of TCP20-Flag or TCP22-Flag proteins in multiple independent lines (Supplementary Fig. 5c).

Like the activity of other clock genes identified previously, that of TCP20 and TCP22 promoters oscillated rhythmically under continuous light (LL) in the wild type (Fig. 3d). The transcript levels of TCP20 and TCP22 also oscillated slightly under the light/dark cycle (Fig. 3e). Both the promoter activities and transcript levels of TCP20 and TCP22 were enhanced in the cca1 lhy mutant (Fig. 3e), so the expression of TCP20/TCP22 was likely repressed by CCA1/LHY. Consistent with the repressor role of CCA1 on TCP20/TCP22, the peak CCA1 protein level at dawn4 coincided with the expression troughs of TCP20 and TCP22 (Fig. 3e). Thus, TCP20 and TCP22 are new clock genes under...
feedback regulation of the morning genes CCA1/LHY in the Arabidopsis circadian clock.

A canonical CCA1-binding site (CBS) and an evening element (EE) were found in the upstream sequences of TCP20 (−779 to −772) and TCP22 (−732 to −725), respectively, relative to the translational start site. We examined whether TCP20 and TCP22 are direct targets of CCA1 in the cca1-1 pCCA1-HA-gCCA1 complementation line. The complementation line accumulated a morning-phased HA-CCA1 protein as endogenous CCA1 (Supplementary Fig. 6a). ChIP-qPCR results showed clear binding of HA-CCA1 to the EE-like region (−549 to −543) in the CCA1 promoter and EE (−728 to −720) in the TOC1 promoter in vivo as reported26. However, HA-CCA1 did not seem to bind to TCP20 or TCP22 promoters (Supplementary Fig. 6b). Similarly, two recent genome-wide surveys of CCA1 target genes did not reveal TCP20 or TCP22 (refs 27,28). Thus, CCA1 indirectly represses the expression of TCP20 and TCP22.

TCP20 and TCP22 directly activates the CCA1 promoter. CHE, also known as TCP21, represses CCA1 via TBS (GGTCCCAC, −564 to −557 relative to the translational start site)12, which is present in a region of the CCA1 promoter bound by LWD1 (Fig. 1b). We used ChIP-qPCR assays to examine whether TCP20/TCP22 associated with the TBS of the CCA1 promoter by using anti-Flag or TCP22-specific antisera with a tcp20-2 tcp22::TCP20-Flag complementation line or wild-type plants. Only DNA fragment b containing TBS bound by LWD1 (Fig. 1b). We used ChIP-qPCR assays to examine whether TCP20/TCP22 associated with the TBS of the CCA1 promoter by using anti-Flag or TCP22-specific antisera with a tcp20-2 tcp22::TCP20-Flag complementation line or wild-type plants. Only DNA fragment b containing TBS bound by LWD1.

TCP20 and TCP22 interact with clock proteins LWD1/LWD2 and regulate flowering time. (a) LWD1/LWD2 and TCP20/TCP22 interact on yeast two-hybrid assay. Positive interactions between LWD1/LWD2 and TCP20/TCP22 were shown by the growth on the selection medium without Trp, Leu and His (SD-WLH + 2 mM 3-AT) for the reporter gene HIS3. Bimolecular fluorescence complementation (BIFC) assay (b) and luciferase complementation imaging assay (c) showed that the co-expression between LWD1, but not truncated LWD1 (ΔLWD1), and TCP20/TCP22 reconstituted the activity of fluorescence protein (green signal) and luciferase. For luciferase complementation imaging (LCI) assay, one representative image from three independent experiments is shown. Data are mean ± s.e. (n = 3). Asterisks indicate that LCI activity from the interactions of TCP20/TCP22 with LWD1 is significantly higher than that with ΔLWD1 (Student’s t test; *P < 0.05). (d) Early flowering of tcp20-2, tcp22-1, and tcp20 tcp22 mutants under long-day conditions. Data are mean ± s.d. (n ≥ 10). Asterisks indicate that mutant plants flowered significantly earlier than wild-type plants (Student’s t test; *P < 0.01). Ten to twelve plants for each genotype were planted for scoring for each biological replicate. Similar results were observed in three independent experiments. Scale bars, 10 μm.
enriched in the immunoprecipitated protein–chromatin complex from the complementation line (Fig. 4a) and wild-type plants (Supplementary Fig. 7). These results indicate a direct binding of TCP20/TCP22 to the CCA1 promoter in Arabidopsis.

Electrophoresis mobility shift assay showed that TCP20 and TCP22 could form a protein–DNA complex with a 50-bp fragment containing TBS (−584 to −535 of the CCA1 promoter relative to the translational start site) (Fig. 4b). As well, the formation of the TCP–TBS protein–DNA complex could be compromised only in the presence of excess and unlabelled TBS competitor but not the mutated TBS (mTBS) competitor (Fig. 4b), which suggests specific binding of TCP20/TCP22 to

![Graph a](image)

![Graph b](image)

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TBS in vitro. We further investigated the transcriptional activator role of TCP20 and TCP22 by binding to the TBS in plant cells. To simplify the regulation contributed by TBS, the same 50-bp CCA1 promoter fragment was used to assay the regulatory role of TCP20 and TCP22 on TBS in the protoplast transient system. The transcriptional activator activity of TCP20 and TCP22 relied on the presence of TBS in this promoter fragment (Fig. 4c). Consistently, the TCP20-dependent activation of the 634-bp CCA1 promoter was significantly compromised when TBS was mutated (Supplementary Fig. 8a). As expected, the activation was specific for the CCA1 promoter but not LHY promoter (Supplementary Fig. 8b), on which no putative TBS could be identified. Thus, TCP20 and TCP22 activate the expression of CCA1 via direct and specific binding to the TBS in the CCA1 promoter. For two functionally redundant genes such as CCA1 and LHY in the circadian clock, their differential regulation by TCPs may increase the flexibility and tunability of the circadian clock.

TCP20 and TCP22 activate CCA1 in an LWD1-dependent manner. Consistent with the activator roles of TCP20 and TCP22 on CCA1, CCA1 promoter activity was significantly greater in multiple independent transgenic lines overexpressing TCP20 (TCP20ox) or TCP22 (TCP22ox) than in wild-type plants (Fig. 5a and Supplementary Fig. 9a). To address whether interacting with LWDs was crucial for TCP20 and TCP22 to activate CCA1, we investigated the functional dependency of TCP20 and TCP22 on LWDs by comparing the CCA1 promoter activity in TCP20 or TCP22-overexpressing lines in the wild type or in lwd1 lwd2 backgrounds. Overexpression of TCP20 or TCP22 failed to enhance the CCA1 promoter activity in lwd1 lwd2 (Fig. 5b and Supplementary Fig. 9b), even though TCP20 and TCP22 levels were comparable in the wild type and lwd1 lwd2 (Supplementary Fig. 9c). Thus, TCP20 and TCP22 functioned as activators of CCA1 only in the presence of LWDs. Consistent with this notion, the trans-activation of TBS-containing promoter by TCP20 was significantly compromised in protoplasts from the lwd1 lwd2 mutant (Supplementary Fig. 10).

We next examined whether this lack of CCA1 activation in lwd1 lwd2 was due to a compromised binding of TCP20 and TCP22 to the TBS in the CCA1 promoter. TCP20 and TCP22 could still associate with the TBS in the CCA1 promoter with or without LWDs (Fig. 5c). Our results also showed comparable association of LWD1 with the TBS in the CCA1 promoter in the wild type and tcp20 tcp22 double mutant (Fig. 5d), so LWD1 could still be recruited to the CCA1 promoter in tcp20 tcp22, possibly via other transcription factors. In-depth yeast two-hybrid assays revealed additional LWD1- and LWD2-interacting class I TCPs, including TCP7, TCP8, TCP9, TCP14, TCP15 and TCP23 (Supplementary Fig. 11a), which were distributed in various clades in a phylogenetic tree of class I TCPs (Supplementary Fig. 11b). Among the members in the same clade containing TCP20 and TCP22, TCP14 and TCP23 could also activate CCA1 expression in a protoplast transient assay (Supplementary Fig. 11c), which suggests that additional class I TCPs have activator roles for CCA1. This finding may explain why the expression of CCA1 was not completely compromised in tcp20 tcp22 (Fig. 3a,c).

LWD1 protein accumulates throughout the day (Supplementary Fig. 12), thereby rendering accessibility in interacting with both activators (TCP14, TCP20, TCP22 and TCP23) and the repressor (CHE; Supplementary Table 1) of CCA1 in the TCP superfamily. Future in-depth studies of LWDs are needed to uncover their regulatory roles at different times of the day via different interacting transcription factors and/or target genes.

Collectively, although TCP20/TCP22 could directly target the TBS in the CCA1 promoter, the binding of TCP20/TCP22 to the CCA1 promoter was insufficient for CCA1 activation without the concomitant binding of LWDs. The interaction with LWDs likely confers TCP20/TCP22 with transcriptional activator activity. Therefore, LWDs are the co-activators of TCP20 and TCP22 for the transcriptional activation of CCA1 (left panel in Fig. 5e).

Discussion

Transcriptional oscillation can be steadily produced by the combination of three repressors in a repressillator system, however, the amplitude and robustness of biological oscillations could be better maintained when the system contains interlocked positive and negative feedback loops. In the Arabidopsis circadian clock, LWD1 and PRR9/PRR7 constitute a positive feedback loop. The expression of the clock co-activator LWD1 was significantly reduced in prr9 and prrr7 mutants. Without the full capacity of LWD1 as the co-activator, CCA1 expression amplitude at dawn could be not maintained, which explains the much-reduced CCA1 expression peak in the higher-order CCA1 repressor mutants (prr9 prr7 and prrr7 prr7 toc1). Previous mathematical modelling of clock gene expression in Arabidopsis was mostly based on repressors in the negative regulatory loops. The regulation of CCA1 expression revealed only transcriptional repressors, including PRR9, PRR7, PRR5, TOC1 and CHE. Of note, CHE represses CCA1 by binding to TBS (ref. 12). We provide direct evidence that the LWD–TCP complex could activate and maintain the robust rhythm of CCA1 by also binding to TBS. Thus, TCP family members could positively or negatively regulate CCA1, and TBS is a target for both negative (CHE) and positive (TCP20 and TCP22) regulators.

Although LNKs function as co-activators of RVEs for the expression of PRR5 and TOC1 (ref. 15), they can antagonize RVEs’ positive regulation of anthocyanin biosynthesis genes at a
specific time during the day\textsuperscript{32}, which indicates a time-dependent switch of roles of LNKs on RVEs toward different target genes. The consecutive expression of the repressors PRR9, PRR7, PRR5, TOC1 and CHE (refs 8–12,16), followed by the activators TOC1 and CHE (refs 8–12,16), followed by the activators CCA1\textsuperscript{7} and LHY (refs 17,18). The plants were briefly dried and ground to powder in liquid nitrogen. Five hundred \( \mu \)l powder was then lysed in 0.8 ml cold nuclei lysis buffer (50 \( \mu \)M HEPES pH 7.5, 150 mM NaCl, 0.5% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1 mM PMF, 50 \( \mu \)M MG135 and 1 \( \times \) protease inhibitor cocktail (Roche, Monheim, Germany)). The lysate was filtered through 100 \( \mu \)m nylon mesh (Calbiochem, La Jolla, CA) in a 2 ml centrifuge tube, and sonicated in an ice bath with the use of Bioruptor (Diagenode, Liege, Belgium) set at high power and 15-s ON/15-s OFF for seven times, each with 5 min. A one-tenth volume of the sonicated lysate was saved for the input fraction control. The LWD1 or TCP22-associated chromatin complexes were immunoprecipitated by incubating the lysate with 3 \( \mu l \) rabbit polyclonal antisera generated with recombinant LWD1 (amino acid residues 1–73) or a polypeptide comprising residues 361–375 with 3 \( \mu l \) rabbit polyclonal antisera generated with recombinant LWD1 (amino acid residues 1–73) or a polypeptide comprising residues 361–375.

Chromatin immunoprecipitation quantitative PCR assay. To detect direct target genes of endogenous LWD1 or TCP22, chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assays were performed by using 18-d-old wild-type, bsd1 bsd2 or tcp20 tcp22 mutants grown under 16-h light/8-h dark on half-strength MS medium. Tissues were harvested at the indicated ZT points. Four hundred plants were treated with 35 ml cross-linking buffer (0.4 M sucrose, 10 mM Tris–HCl, pH 8.0 and 1% formaldehyde) under vacuum for four times, each with 5 min. Glycine was added to a final concentration of 125 mM to quench the excess formaldehyde and to terminate the cross-linking reaction under vacuum for twice, each for 2.5 min, followed by rinsing with 50 ml deionized water for three times. The plants were briefly dried and ground to powder in liquid nitrogen. Five hundred \( \mu \)l powder was then lysed in 0.8 ml cold nuclei lysis buffer (50 \( \mu \)M HEPES pH 7.5, 150 mM NaCl, 0.5% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1 mM PMFS, 50 \( \mu \)M MG135 and 1 \( \times \) protease inhibitor cocktail (Roche, Monheim, Germany)). The lysate was filtered through 100 \( \mu \)m nylon mesh (Calbiochem, La Jolla, CA) in a 2 ml centrifuge tube, and sonicated in an ice bath with the use of Bioruptor (Diagenode, Liege, Belgium) set at high power and 15-s ON/15-s OFF for seven times, each with 5 min. A one-tenth volume of the sonicated lysate was saved for the input fraction control. The LWD1 or TCP22-associated chromatin complexes were immunoprecipitated by incubating the lysate with 3 \( \mu l \) rabbit polyclonal antisera generated with recombinant LWD1 (amino acid residues 1–73) or a polypeptide comprising residues 361–375 (PNQQASENGGDDKK) of TCP22 for overnight at 4°C. The immunoprecipitated chromatin complexes were purified by incubating with 50 \( \mu l \) slurry of Protein A Sepharose (GE, 17-5280-01) pre-equilibrated with 1 mg ml\(^{-1}\) rabbit polyclonal antisera generated with recombinant LWD1 (amino acid residues 1–73) or a polypeptide comprising residues 361–375 (PNQQASENGGDDKK) of TCP22 for overnight at 4°C. The immunoprecipitated chromatin complexes were purified by incubating with 50 \( \mu l \) slurry of Protein A Sepharose (GE, 17-5280-01) pre-equilibrated with 1 mg ml\(^{-1}\) salmon sperm DNA and 1 mg ml\(^{-1}\) BSA for 3 h at 4°C.

For HA-tagged CCA1-ChIP, a DNA fragment encoding three tandem repeats of HA-tag was inserted in front of the start codon of a CCA1 genomic fragment spanning from –1,418 (relative to the translational start site) to the stop codon to generate the CCA1 complementation construct into BambHI and KpnI-digested pCAMBIA1390. For Flag-tagged TCP20 or TCP22-ChIP, the overexpressing lines were generated by inserting the coding region of TCP20 or TCP22 into Ncol and BamHI sites of a modified version of pEarleyGate100 (ref. 35) to generate...
CaMV35S (35S) promoter-driven Flag-TCP20 or Flag-TCP22 under wild-type or lwd1 lwd2 background. ChIP analyses were performed by using 50 ml Sigma monoclonal anti-HA agarose antibody (A2095) or Sigma monoclonal anti-Flag M2 affinity gel (A2225) for HA-CCA1 or Flag-TCP20 and Flag-TCP22. The affinity beads bound chromatin complexes were washed with 850 ml nuclei lysis buffer for three times, LNDET buffer (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, pH8.0) for three times and TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0) for three times. Chromatin complexes were eluted from the beads with use of 200 ml elution buffer (1% SDS, 0.1 M NaHCO₃) followed by the digestion with 2.5 ml Proteinase-K (20 mg ml⁻¹, Invitrogen, Carlsbad, CA) for overnight at 65 °C. DNA released from chromatin complexes was then purified by use of a QIAEXII gel purification kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instruction. Primer sequences used for PCR amplification are listed in Supplementary Table 2. Independent biological replicates of ChIP-qPCR results are shown in Supplementary Fig. 14.

**Yeast two-hybrid analysis.** LWD1-interacting proteins were screened in a prey library of 1,400 transcription factors by yeast two-hybrid assay. Protein–protein interactions were re-confirmed by using a GAL4-based yeast two-hybrid system (MATCHMAKER GAL4 two-hybrid system, Clontech). Empty pDBLeu (Invitrogen) and pEXP-AD502HA plasmids were used as negative bait and prey controls, respectively. The coding region of LWD1 or LWD2 was cloned into the XmaI and NolI sites of pDBLeu. The pEXP-AD502HA vector is a modified

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**Figure 5 | LWD1 is the co-activator of TCP20 for the expression of CCA1 at dawn.** (a) pCCA1::LUC2 expression is increased in TCP20-overexpressing lines. (b) Overexpression of TCP20 fails to increase the expression of CCA1 promoter in lwd1 lwd2. Data are mean ± s.e. (n = 6–8 seedlings for each independent line; see the corresponding data for TCP20ox in Supplementary Fig. 9a,b). Similar results were observed in three independent experiments. (c) TCP20/TCP22 associate with the CCA1 promoter in vivo independent of LWDs. Asterisk indicates that overexpressed TCP20/TCP22 in WT or lwd1 lwd2 backgrounds preferentially binds with amplicon ’b’ (Student’s t test; * P < 0.005). (d) LWD1 associates with CCA1 promoter independent of TCP20/TCP22. Data are shown as mean ± s.d. from one representative experiment (n = 3 technical replicates). Asterisk indicates that LWD1 preferentially binds to the amplicon ‘b’ in both WT or tcp20 tcp22 (Student’s t test; * P < 0.001). Similar results were observed in three independent experiments. (e) A diagram of LWD1 and TCP20/TCP22 co-activating the expression of CCA1.
version of the pExp-AD502 (Invitrogen) plasmid. Briefly, the T7-3A-MS derived from pGADT7 (Clontech) was inserted into the SalI and NotI sites of the pExp-AD502 vector. Coding regions of TCP26, 7, 8, 9, 11, 14, 15, 16, 20, 22 and 23 were cloned into pExp-AD502HA. SD-WL (-Tpr -Leu) plates were used to select for the presence of both bait and prey vectors, and SD-WLH (-Tpr -Leu -His) plates with 3-AT (3-amino-1,2,4-triazole) were used to select for interactions between the bait and prey proteins. All primers for these constructs for yeast two-hybrid assays are in Supplementary Table 2.

Bimolecular fluorescence complementation imaging (BiFC) assay. The LWD1 full-length coding region or truncated variant lacking amino acids 236–290 (ALW/D1) was subcloned into hygG-VYNE(R)-pGPTVII, and the coding regions of TCP20 or TCP22 were subcloned into kanII-SCYE(R)-pGPTVII (ref. 36). All primers of these constructs for BiFC are in Supplementary Table 2. Venus137, fused LWD1 or ALW/D1 with translationally expressed with SCP3PA fused TCP20, TCP22, or CHE in Arabidopsis seedlings by the AGROBEST method37. Three days after infection, a GFP filter set was used for detecting the fluorescence signals of SCP3PA and Venus by Zeiss LSM 780 confocal microscopy.

Luciferase complementation imaging assay. Luciferase complementation imaging assays were performed with constructs pLWD1::LUC2-LWD1, pLWD1::NLC2-LWD1, pTCP20::LUC2-TCP20 and pTCP22::LUC2-TCP22 in Arabidopsis seedlings by AGROBEST37. Coding regions of LWD1 (ALW/D1) and TCP20/TCP22 were fused with firefly luciferase NLC2 (+1 to +1,248, relative to the translational start site) and CLUC2 (+1,194 to +1,650), respectively, and expressed under the control of their native promoters, LWD1 (-1,068 to −1), TCP20 (-2,564 to −1), and TCP22 (-2,000 to −1).

Flowering time determination. Arabidopsis seeds were sown on stratified and stratified at 4°C for 3 or 4 days at 4°C and then grown under long-day (16-h light/8-h dark) conditions at fluence rate 80–100 μmol m−2 s−1 at 22°C. The number of rosette leaves ≥ 5 mm long was recorded for each plant when the primary florescence reached 5 cm above the rosette leaves, this phenotype was repeated at least three times.

RNA preparation and quantitative real-time PCR. Plants grown under conditions at 65°C were excised by leaf tissue powder from 10 plants with 70% of mature leaves. The RNA in the liquid phase was precipitated overnight at 4°C, the RNA pellet was washed by 70% ethanol, briefly air-dried, and then dissolved in diethylpyrocarbonate (DEPC) water. The RNA was quantified by measuring the absorbance at 260 nm. DNA contamination was determined by using 1 μg of RNA to 2.0 μl of 4,4′-diaminobenzidine (DAB) solution. The RNA samples were reverse transcribed to cDNA with SuperScript III Reverse transcriptase (Invitrogen). The qPCR assay was performed using the LightCycler system (Roche) with the iQ SYBR Green Supermix (Bio-Rad) and the following primers:

- **TCP20**: 5'-ATGGAGAAGAGAACAGAAGG-3’ (forward) and 5'-AGCAGAGGTAGGCTTGACA-3’ (reverse).
- **TCP22**: 5'-ATGGAGAAGAGAACAGAAGG-3’ (forward) and 5'-AGCAGAGGTAGGCTTGACA-3’ (reverse).
- **UBQ10**: 5’-AATATCCCAACAGCATGCAAGG-3’ (forward) and 5’-TTGGGTTGCTTTTGAGAACC-3’ (reverse).

The expression level of TCP20 or TCP22 was determined by the comparative C(T) method relative to the expression level of UBQ10 as the internal control. The obtained Ct values were used to determine the fold change in gene expression.

Electrophoretic mobility shift assay. The coding region of TCP20 or TCP22 was cloned into the pET2a (+) vector (Novagen) to generate GST-tagged TCP20 or TCP22. Recombinant GST-TCP20 and GST-TCP22 were expressed in *Escherichia coli* BL21(DE3) and purified by using glutathione-affinity agarose beads (GE) following the manufacturer’s protocol. To generate radioactive probes used in electrophoretic mobility shift assay (EMSA), two oligonucleotides for the wild-type or mutated TBS sequence with 10-nt complementary 3’-ends were annealed at a final concentration of 50 μM for each oligonucleotide in 10 μl buffer. The partially annealed DNA were filled-in and radiolabeled for 30 min at 37°C in a 10 μl reaction mixture comprised 25 μCi of (α-32P)dCTP (specificity equals to 3,000 Ci mmol; Perkin-Elmer). 2.5 units Klenow fragment of DNA polymerase 1 (New England Biolabs), labelling buffer (200 μM HEPES, 50 mM MgCl2, 10 mM MgCl2, 20 μM for each of dATP, dTTP and dGTP). The oligonucleotides used to generate radiolabeled DNA probes and unlabelled competitors are listed in Supplementary Table 2. EMSA was performed by mixing 0.05 pmol of the probe with purified recombinant protein in the binding buffer (35 mM KCl, 15 mM HEPES 7.5, 0.4 mM EDTA, 6% glycerol, 50 μg ml−1 poly(dI-dC)) with or without competitors at room temperature for 10 min. DNA–protein complexes were separated by a 4.5% Tris/Borate/EDTA polyacrylamide gel under 0.5 × Tris-Glycine buffered electrophoresis system, and fixed in 10% acetic acid for autoradiography detection.

**Transient expression in Arabidopsis protoplasts.** DNA fragments relative to the translational start site of CCA1 including 50-bp (−584 to −535) fused with 35S promoter was inserted into p35S::NOS-CP (Gateway cloning vector containing the wild-type or mutated TCP binding site (TBS or mTBS), respectively, and LHY promoter (−1,661 to −1) (ref. 14) were ligated to the upstream of LUC2 in the LUC2-modified pD301 vector to generate reporter constructs in protoplasts. The coding regions of GFP, LWD1, TCP14, TCP20 or TCP23 were tagged with epitope HA and sub-cloned into the p35S::EFP (ref. 43) vector for transient overexpression under the control of the 35S promoter. For transient assay in Arabidopsis protoplasts, 150 leaves of 3-wk-old plants grown under 12-h light/12-h dark condition were to isolate mesophyll protoplasts by the sandwich method. Briefer, the leaves were digested in the enzyme solution (1% cellulase R10, 0.25% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM CaCl2, 5 mM MgCl2, 0.1% BSA) to obtain 2.5 × 106 mesophyll protoplasts for transfections. An amount of 2.5 × 106 protoplasts was co-transfected with 2 μg reporter construct, 5 μg 35S::TCP(I)-HA as the effector and 10 μg pB221 (35S:GUS) as the transcription control. Transfected cells were incubated at 22°C overnight and harvested for LUC2 and GUS reporter assays.

Phylogenetic analysis. The phylogenetic analysis of full-length protein sequence classification of 1 TCP members was performed with the one-click mode of the web-based phylogeny tool Phylogeny.fr (http://www.phylogeny.fr). Sequence data for genes referred to in this study can be found in the Arabidopsis Genome Initiative data library with the following locus identifiers: L1WI (At1g12990), L1WI (At2g26490), C1A1 (At1g66830), LHY (At1g01060), PRR9 (At5g2810), PRR5 (At1g35200), TCP1 (At5g13870), TOC1 (At5g13270), TCP2 (At3g23820), TCP7 (At1g58100), TCP8 (At2g45580), TCP11 (At3g37000), TCP14 (At3g47620), TCP15 (At1g69690), TCP16 (At3g65150), TCP19 (At5g19100), TCP20 (At2g70100), TCP21/CHE (At5g83300), TCP23 (At5g72800), UBQ10 (At4g65320) and UBC21 (At5g27660).

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information Files or are available from the corresponding author upon request.

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