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A functional genomics approach reveals CHE as a novel component of the Arabidopsis circadian clock

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Abstract

Transcriptional feedback loops constitute the molecular circuitry of the plant circadian clock. In Arabidopsis, a core loop is established between CCA1 and TOC1. Although CCA1 directly represses *TOC1*, the TOC1 protein has no DNA binding domains thus suggesting it cannot directly regulate *CCA1*. Here, we established a functional genomic strategy that led to the identification of CHE, a TCP transcription factor that binds specifically to the *CCA1* promoter. CHE is a clock component partially redundant with LHY in the repression of *CCA1*. The expression of *CHE* is regulated by CCA1, thus adding a CCA1/CHE feedback loop to the Arabidopsis circadian network. Because CHE and TOC1 interact, and CHE binds to the *CCA1* promoter, a molecular linkage between TOC1 and *CCA1* gene regulation is established.

The circadian system provides an adaptive advantage by allowing the anticipation of daily changes in the environment (1, 2). For example, in plants, the resonance between the internal time-keeper and the environmental cues enhances their fitness and survival (3, 4).

Circadian networks are comprised of multiple positive and negative factors organized in interlocked autoregulatory loops (1, 2). In *Arabidopsis thaliana* (Arabidopsis), the molecular wiring of these regulatory circuits is mainly based on transcriptional feedback loops (5, 6). The core clock stems from the reciprocal regulation between the evening-phased pseudoresponse regulator TIMING OF CAB EXPRESSION1 (TOC1) and the two morning-expressed MYB transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (7). CCA1 and LHY are DNA binding proteins that repress *TOC1* expression through direct binding to its promoter region (7, 8). In contrast, no functional domains have been recognized in the TOC1 protein (9), and direct regulators of *CCA1* or *LHY* expression have never been identified (5, 6, 10).

Functional redundancies amongst gene families provide barriers for identifying clock-related transcription factors by classic forward genetic screens. Thus, to uncover direct regulators of *CCA1/LHY* we implemented an alternative genomics approach where the yeast one-hybrid

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Supporting online material www.sciencemag.org
Materials and methods
Figs. S1 to S13
Table S1
References

system was used to screen a collection of transcription factors, for their binding to the *CCA1/LHY* regulatory regions (Fig. S1). The high throughput design of this approach, termed “Promoter Hiking”, allowed us to test several tiled-fragments for each promoter, thus minimizing the negative effect that the distance of the *cis*-elements to the TATA box has over transcriptional activation in yeast (11). Because most clock components identified to date exhibit a circadian pattern of mRNA expression, we created a comprehensive library of circadian-regulated transcription factors (Table S1) (12). We identified a transcription factor that belongs to the class I TCP (TB1, CYC, PCFs) family (13) (At5g08330) hereafter referred to as CCA1 HIKING EXPEDITION (CHE). CHE specifically binds to the *CCA1* promoter fragment encompassing nucleotides -363 to -192 (Fig. 1A) but not to any of the *LHY* promoter fragments tested (Fig. 1B and S2). Consistent with this result a motif present in the promoter fragment bound matches the consensus class I TCP-binding site (GGNCCCAC) (14). The binding of CHE to the TCP-binding site in *CCA1* promoter (GGTCCCAC) was confirmed by electrophoretic mobility shift assays (Fig. 1C), and was further corroborated using the yeast one-hybrid system with a synthetic TCP-binding site trimer (Fig. S2).

To address whether CHE regulates *CCA1 in vivo*, we performed assays in Arabidopsis protoplasts where the *CCA1* promoter activity was monitored with a CCA1: :LUC+ reporter construct. The overexpression of CHE using a 35S: :CHE effector construct caused a reduction of the luciferase activity, suggesting that CHE functions as a repressor of *CCA1* (Fig. S3). This possibility is consistent with the observation that *CHE* mRNA levels oscillate 9 hours out of phase with *CCA1* transcript (Fig. 1D). Moreover, we demonstrate that CHE protein accumulates in the nuclei (Fig. 1 E-F), and that both CHE and CCA1 exhibit a similarly broad tissue expression pattern (Fig. 1 G-H). If CHE act as a repressor through binding to the TCP-binding site, mutations in this motif should result in increased *CCA1* promoter activity. This hypothesis was confirmed by using promoter: :LUC+ fusions (Fig. 1I and S4), indicating that the TCP-binding site mediates a repressor function. Altogether these results support the notion that CHE directly represses *CCA1* promoter activity in a TCP-binding site specific manner. To investigate this interaction *in vivo*, transgenic seedlings overexpressing green fluorescent protein-tagged CHE (Fig. S5A-B) were used to perform chromatin immunoprecipitations (ChIP). This assay revealed a CHE-specific enrichment of *CCA1* promoter fragments containing the TCP-binding site (Fig. 1J and S5C), indicating that CHE binds to the *CCA1* promoter *in vivo*.

The proper regulation of *CCA1* is required to maintain normal clock function (15, 16), thus we hypothesized that any factor modulating *CCA1* expression may be part of the Arabidopsis circadian network. To analyze whether CHE has a role in the Arabidopsis oscillator, we overexpressed CHE in transgenic plants carrying a CCA1: :LUC+ reporter construct (Fig. S6A). Consistent with its role as a repressor, elevated levels of *CHE* result in an overall reduction of the *CCA1* promoter activity (Fig. 2A). The luciferase levels oscillate with a period close to 24 h (Fig. S6B) but the peak of expression exhibits a phase advance (Fig. 2A-B), suggesting that the constitutive expression of *CHE* leads to an earlier repression of *CCA1* at the beginning of the subjective day.

To further characterize the role of CHE in the Arabidopsis clock, we isolated two independent T-DNA insertion lines with reduced *CHE* expression levels, named *che-1* and *che-2* (Fig. S7A-B). These mutant seedlings were crossed to the CCA1: :LUC+ reporter background, and the resulting lines were used to evaluate the clock function under constant light conditions. Consistent with the function of CHE as a transcriptional repressor, both T-DNA insertion lines displayed an overall increase in *CCA1* promoter activity (Fig. 2C). However, no significant change in the period length or phase of the luciferase expression was observed (Fig. 2C and S7C), suggesting the presence of redundant repressor activities that may counteract the effect of reduced *CHE* expression on the clock function. The Arabidopsis genome encodes twelve other TCP transcription factor genes that contain a DNA binding domain similar to CHE (Fig. S8A-B) suggesting they could also bind to the TCP-binding site. To test if this motif is targeted by repressor activities other than the one caused by CHE (potentially another TCP), we transformed *che* mutant seedlings with CCA1: :LUC+ constructs and compared the promoter activities in the presence or absence of a functional TCP-binding site. In this condition, similar luciferase levels of were obtained with the native or mutant promoter constructs (Fig. 2D and S9A to C) indicating that *CCA1* promoter activity is affected in a similar fashion by mutations in the TCP-binding site or by the reduction of *CHE* expression levels. This result suggests that CHE is the main repressor acting through the TCP-binding site. Therefore, the repressor activity redundant with CHE, if any, should occur through binding to other *CCA1* promoter motifs. LHY inhibits *CCA1* expression (17) probably through direct binding to a CCA1-binding site (18) (AGATTTTT) located 479 nucleotides upstream of the TCP-binding site. Hence, we reasoned that the repressor effects of LHY and CHE could be redundant. Consistent with this hypothesis the pace of the clock in *che/lhy* double mutant lines exhibited a significantly shorter period length compared to the *lhy* single mutant (Fig. 2E), suggesting that LHY is at least partially redundant with CHE or that it regulates the expression of a CHE redundant-factor. We conclude that CHE functions within the Arabidopsis circadian system as a repressor of *CCA1*.

Reciprocal feedback loops are a common feature in clock regulatory networks (2). Analysis of the *CHE* promoter sequence revealed a CCA1-binding site located between nucleotides -956 and -949 (AAAAATCT), suggesting that CCA1 and LHY could reciprocally modulate *CHE* expression. In fact, although the expression of *CHE* oscillated in wild-type seedlings, it was constantly elevated at peak levels in a *cca1/lhy* double mutant background (Fig.3A). Moreover, electrophoretic mobility shift assays indicate that CCA1 and LHY specifically bind to the CCA1-binding site in CHE promoter (Fig. 3B and S10). These results support the notion that CCA1 and LHY repress *CHE* expression through direct binding to the CCA1-binding site of *CHE* promoter. The binding of CCA1 to the *CHE* promoter was further confirmed *in vivo*, by performing ChIP assays with transgenic seedlings expressing green fluorescent protein-tagged CCA1 (Fig. 3C and S11). The mutual regulation observed between CCA1 and CHE establishes a transcriptional feedback module within the Arabidopsis core clock network.

The molecular basis of the regulation of *CCA1/LHY* expression by TOC1 remains unclear. The mechanism appears to be more complex than what was originally thought, due to the

lack of DNA binding domains in TOC1 protein (9), and that either reduced or elevated TOC1 levels lead to low *CCA1/LHY* mRNA expression (7, 19). It has been proposed that TOC1 could regulate the expression or protein turnover of a direct regulator of *CCA1/LHY*, or that TOC1 could regulate transcription by interacting with a transcription factor specific for *CCA1/LHY* promoters. Therefore, the discovery of CHE as a direct regulator of *CCA1* provides the opportunity to investigate if CHE is part of the mechanism by which TOC1 regulates the expression of *CCA1*. There is a spatiotemporal coincidence in the expression of CHE and TOC1. Both proteins are nuclear localized (Fig. 1F and (9)), and their expression is broadly distributed in Arabidopsis seedlings (Fig. 1G and (20)), reaching maximum levels at similar times of the day (ZT 9 and ZT13, respectively) (Fig. 1D and (9)). Consistently, when using the yeast two-hybrid system, we observed a direct protein-protein interaction between TOC1 and the N-terminal domain of CHE (Fig. 4A). To confirm this interaction *in vivo*, we performed co-immunoprecipitations using CHE- and TOC1-tagged proteins transiently co-expressed in *Nicotiana benthamiana* leaves. In these experiments, TOC1 was only detected in the samples where CHE had been immunoprecipitated (Fig. 4B), indicating that CHE and TOC1 directly interact *in planta*, and therefore, that CHE may recruit TOC1 to the *CCA1* promoter.

To test whether TOC1 binds *in vivo* to the *CCA1* promoter region encompassing the TCP-binding site, seedlings expressing a yellow fluorescent protein tagged-TOC1 under the regulation of its endogenous promoter were used to perform ChIP experiments. Analysis of the DNA recovered after immunoprecipitation revealed a specific enrichment of *CCA1* promoter fragments containing the TCP-binding site (Fig. 4C and S12), indicating that TOC1 is linked to the *CCA1* promoter *in vivo*. A direct interaction of TOC1 with the *CCA1* promoter was investigated by electrophoretic mobility shift assays and the yeast one-hybrid system, but no binding was detected using these approaches (data not shown), suggesting that TOC1 is unable to bind directly to the *CCA1* promoter. Thus, TOC1 could be recruited to the *CCA1* promoter through the interaction with CHE, or other *CCA1* promoter binding proteins bound to *cis*-elements close to the TCP-binding site. Alternatively, a specific post-translational modification or co-factor could be required for TOC1 to bind directly to the DNA.

The pace of the clock is particularly sensitive to changes in TOC1 activity or concentration, but the period only varies in correlation with TOC1 dosage when TOC1 expression is above endogenous levels (21). Therefore, we reasoned that a sensitized background where TOC1 levels are moderately elevated would be the best scenario to explore whether there is a functional interaction between TOC1 and CHE. The overexpression of *CHE* in this context resulted in a significant shortening of the period compared to the parental line (Fig. 4D and Fig. S13). These results indicate that the interaction between CHE and TOC1 exert a biologically meaningful and opposite effect over the control of the period length *in vivo*. Thus, CHE may not merely recruit TOC1 to the *CCA1* promoter, but seems antagonistic to TOC1 function as well. For example, high levels of CHE could sequester TOC1 and interfere with its interaction with other transcription factors bound to the *CCA1* promoter.

We have applied a yeast one-hybrid genomic strategy towards the discovery and molecular characterization of CHE, which is a component of the Arabidopsis clock that negatively

regulates *CCA1* expression. *CHE* and *CCA1* exhibit a reciprocal regulation, which uncovers a feedback loop at the core of Arabidopsis circadian network. Our data indicate that CHE directly interacts with TOC1 and that both proteins are associated with the same region of *CCA1* promoter, establishing a molecular link between TOC1 protein levels and *CCA1* expression (Fig. 4E). The "Promoter Hiking" strategy, as presented here, is limited by the use of a circadian-regulated transcription factor library. Although for our purposes this was an effective enrichment, the collection screened only represents about 10% of all the transcription factors encountered in Arabidopsis, suggesting that additional regulators of *CCA1* are yet to be discovered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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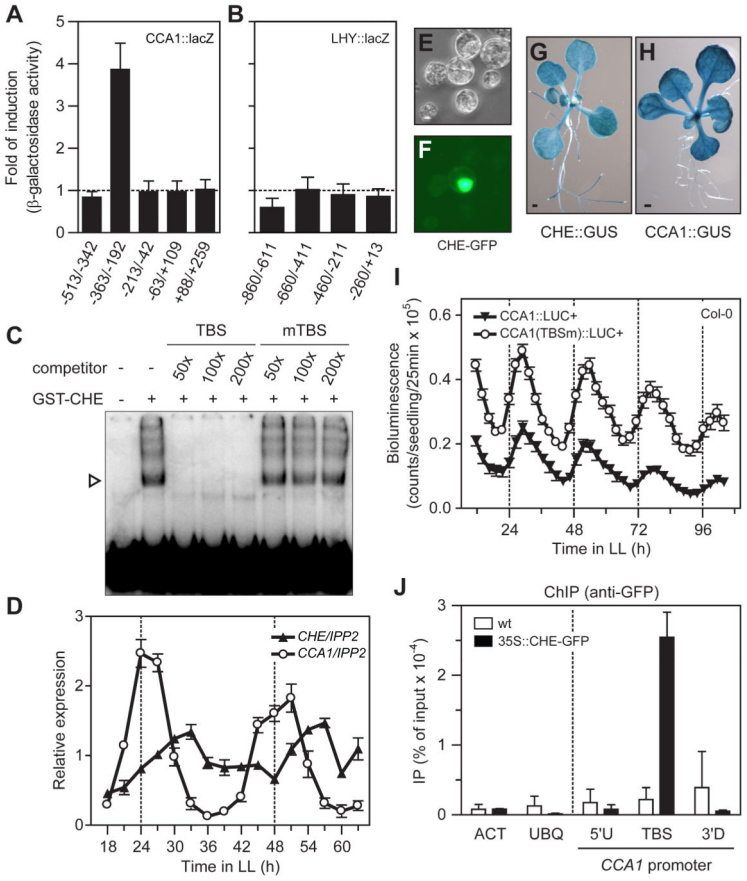

Fig. 1. CHE is a novel *CCA1* promoter binding protein

(A and **B)** Interaction of CHE to different regions of *CCA1* (A) and *LHY* (B) promoters in yeast. Bars represent the fold of induction in β-galactosidase activity for each of the DNA fragments indicated (n=6). **(C)** Binding of CHE to the TCP-binding site (TBS) in *CCA1* promoter determined by electrophoretic mobility shift assay. The DNA/CHE complex (indicated by the arrowhead) was competed by the addition of unlabeled wild-type (TBS) or mutant (mTBS) probes. **(D)** *CHE* and *CCA1* expression in Col-0 wild-type seedlings growing under constant light (LL). mRNA levels were normalized to *IPP2* expression (n=3). **(E** and **F)** Subcellular localization of CHE in Arabidopsis protoplasts. Visible light (E), GFP channel (F). **(G** and **H)**. CHE and CCA1 expression patterns determined by histochemical staining for GUS activity in CHE: :GUS (G) and CCA1: :GUS (H) transgenic seedlings. Scale bars, 0.5 mm. **(I)** Effect of mutations within the TCP-binding site (mTBS) on *CCA1* promoter activity. Promoter: :luciferase constructs [CCA1: :LUC+ and CCA1(mTBS): :LUC +] were transformed into Col-0 seedlings. Luciferase activity was determined in T1 lines (n=27). **(J)** Binding of CHE to *CCA1* promoter *in vivo.* ChIP assays were performed with 35S: :CHE-GFP or wild-type CCA1: :LUC+ (wt) seedlings. Immunoprecipitated DNA was quantified by real-time PCR with primers specific for the TCP-binding site in *CCA1* promoter (TBS), and for control regions (5′U, 3′D, ACT, UBQ) (12). Results were normalized to the input DNA (n=4). Values represent means ± SEM [(A), (B), (D), (I), (J)].

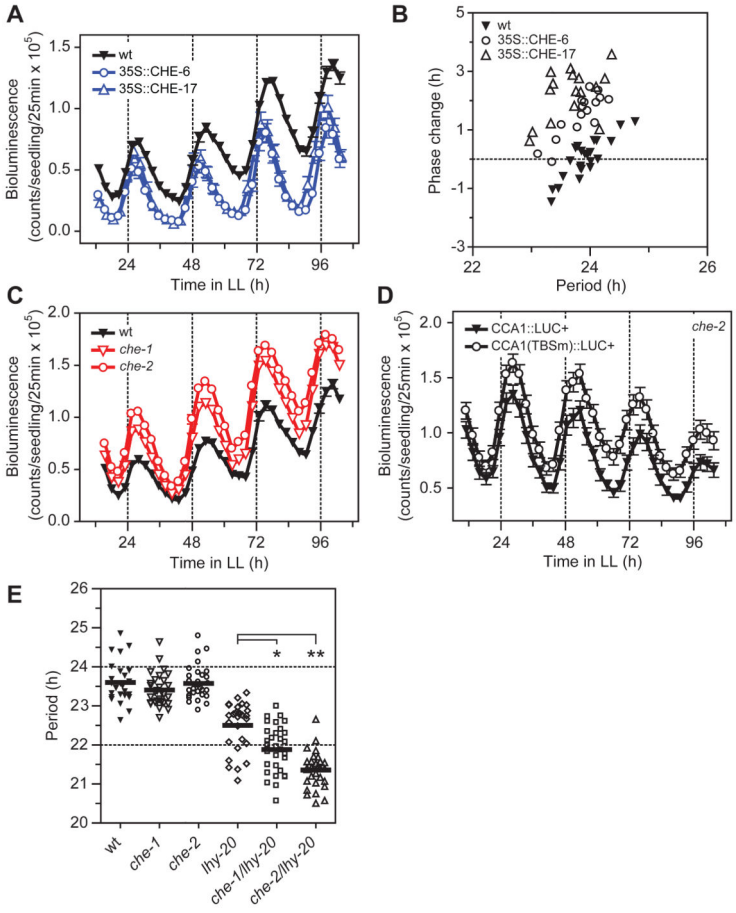

Fig. 2. CHE is a clock component directly repressing *CCA1* promoter activity

(A and **C)** Bioluminescence analysis of CCA1: :LUC+ expression in *CHE* overexpression lines (35S: :CHE) (n=20) (A) and *che* T-DNA insertion lines (*che-1* and *che-2*) (n=45) (C). Wild-type (wt) traces correspond to CCA1: :LUC+ seedlings. **(B)** Phase change of luciferase expression in wild-type CCA1: :LUC+ (wt) and the 35S: :CHE lines shown in (A). Each symbol represents one seedling. **(D)** Effect of mutations within the TCP-binding site (mTBS) on *CCA1* promoter activity in a *che* mutant background. Promoter: :luciferase constructs [CCA1: :LUC+ and CCA1(mTBS): :LUC+] were transformed into *che-2* seedlings. Luciferase activity was determined in T1 lines (n=37) **(E)** Period estimates of luciferase expression in wild-type CCA1: :LUC+ (wt), *che* and *lhy* T-DNA insertion lines (*che-1*, *che-2* and *lhy-20*), and *che/lhy* double mutants (*che-1/lhy-20* and *che-2/lhy-20*). Each symbol represents one seedling and the line is the average period value (*p<0.0005, **p<0.0001). Values represent means ± SEM [(A), (C), (D)].

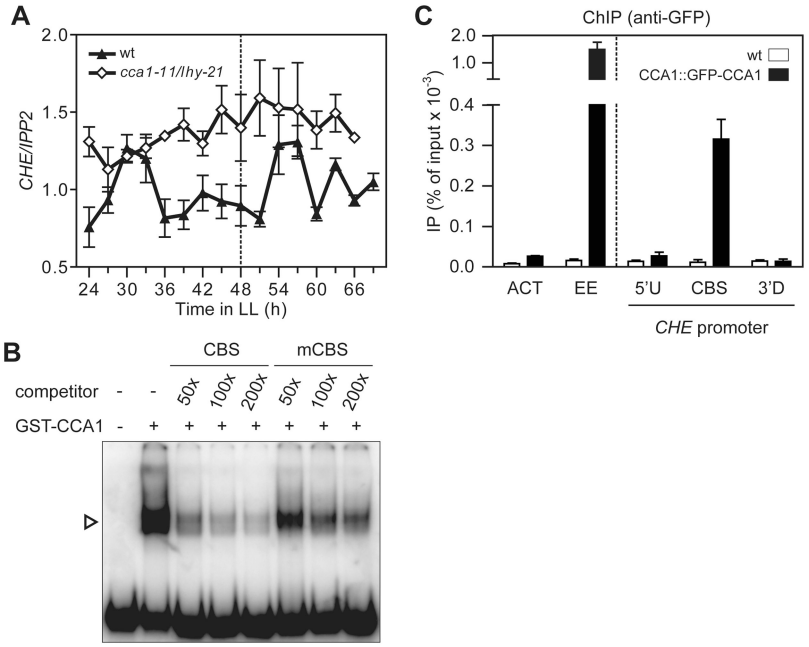

Fig. 3. *CHE* expression is regulated by CCA1 and LHY

(A) Expression of *CHE* in wild-type Ws (wt) and *cca1-11/lhy-21* double mutant seedlings growing in constant light (LL). mRNA levels were normalized to the expression of *IPP2* (*CHE/IPP2*) (n=3). **(B)** Binding of CCA1 to the CCA1-binding site in *CHE* promoter determined by electrophoretic mobility shift assay. The DNA/CCA1 complex (indicated by the arrowhead) was competed by the addition of unlabeled wild-type (CBS) or mutant (mCBS) probes. **(C)** Binding of CCA1 to *CHE* promoter *in vivo.* ChIP assays were performed with CCA1: :GFP-CCA1 or wild-type Ws (wt) seedlings. Immunoprecipitated DNA was quantified by real-time PCR with primers specific for the CCA1-binding site in *CHE* promoter (CBS), and for control regions (ACT, EE, 5′U, 3′D) (12). Results were normalized to the input DNA (n=3). Values represent means ± SEM [(A) and (C)].

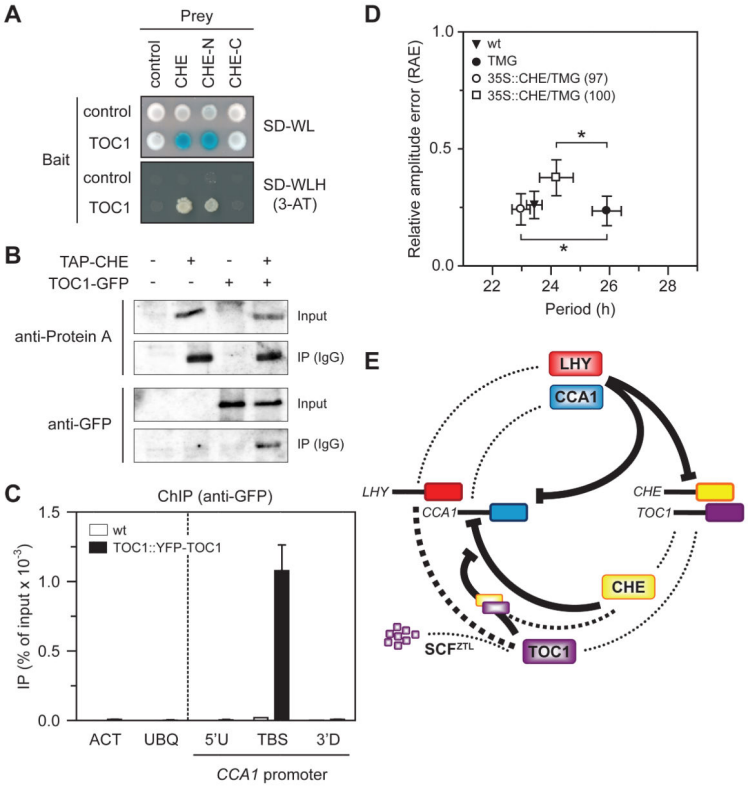

Fig. 4. CHE interacts with TOC1

(A) Interaction between CHE and TOC1 proteins in yeast. SD–WL medium is used for the selection of bait and prey proteins where a β-galactosidase overlay assay was performed to visualize the interaction. SD-WLH (3-AT) medium is used for the auxotrophic selection of bait and prey protein interactions. **(B)** Co-immunoprecipitation assay between TOC1 (YFP-TOC1) and CHE (TAP-CHE) expressed in tobacco leaves. The results are representative of four independent experiments. **(C)** Binding of TOC1 to the *CCA1* promoter *in vivo.* ChIP assays were performed with TOC1: :YFP-TOC1 or wild-type CAB2: :LUC (wt) seedlings. DNA was quantified by real-time PCR with primers specific for the TCP-binding site in *CCA1* promoter (TBS), and for control regions (5′U, 3′D, ACT, UBQ) (12). Results were normalized to the input DNA. Values represent means ± SEM (n=4). **(D)** Genetic interaction between CHE and TOC1. CHE was overexpressed in the TOC1: :YFP-TOC1 (TMG) background (35S: :CHE/TMG). Period and relative amplitude error estimates of CAB2: :LUC (wt) expression were determined in T3 seedlings (*p<0.0001). Values represent means ± SD (n=20). **(E)** Model for the proposed role of CHE in the Arabidopsis clock. At dawn high levels of CCA1 and LHY repress *CHE* and their own expression. CHE levels rise as the day progresses to maintain *CCA1* at a minimum. By the end of the day TOC1 antagonizes CHE resetting the cycle.