Fig1. Transcriptomic analysis identified the reciprocal regulatory genes of CCA1 and LHY.

(A) The ChIP-seq data sets of CCA1 (refer...) and LHY (...) were re-evaluated to characterize the genes bound by CCA1 or LHY. The Venn diagram shows the overlap of the three datasets and the number of targets specific for each dataset. (B)The differentially-expressed genes (DEGs) in cca1 lhy mutant () were re-evaluated and overlapped with the combined dataset in Fig 1A. (C) Gene ontology analysis of …Log fold change values are shown. (D) … (E)...

CCA1 and LHY are core clock genes with much legacy data available, so we collected the ChIP-seq data of CCA1 and LHY from multiple research and re-evaluate them (Fig. 1A). The previous studies usually focus on the overlapping genes of multiple datasets, such as the 304 genes in our case, it turns out to be common targets of CCA1 and LHY or the stringent target genes that appear in various tests (??), but it missed many genes that are specific targets of either CCA1 or LHY and those conditionally regulated target genes. So we combined the three data sets of the ChIP-seq results and got 8473 target genes of either CCA1 or LHY, then overlapped it with the differentially-expressed genes (DEGs) in the *cca1 lhy* mutant compared to wildtype and got 556 genes, which are supposed to be those genes regulated and targeted by either CCA1 or LHY (Fig. 1B). The Gene annotation showed some canonical roles of CCA1 and LHY, such as response to light and circadian rhythm process, interestingly, multiple abiotic stress processes, such as response to cold, water deprivation, wounding, and salt, appeared in the gene annotations (Fig. 1C). So our analysis revealed that CCA1 or LHY are involved in multiple stress-related processes, and abiotic stress genes are important outputs of CCA1 and LHY. To further mine the transcriptomic datasets, we used the yeast one screening data against CCA1 promoter, which were screened against a transcription factors library in our lab, and obtained 14 genes that are output genes of CCA1 or LHY and can bind the promoters of CCA1 (Fig. 1D).  Interestingly, three ABFs, key transcription factors in the ABA signaling pathway, appear in the 14 genes, which is consistent with our findings that CCA1 and LHY are involved in multiple stress processes.

Fig 2. The expression and stress-responsiveness of *ABF3* are regulated by the circadian clock

(A) ABF1 and ABF3 oscillate in the diurnal condition. qRT-PCR analysis of gene expression of ABF1 and ABF3 in wild type (Col-0) in the diurnal condition. Col-0 was grown in ½ MS under 12-h:12-h LD cycles for 10 d and collected samples every 3 hours as indicated in the diagram. The ACT7 gene was analyzed as an internal control. Error bars, SDs of three biological replicates.

(B-C) The expression of ABF1 and ABF3 are regulated by LHY and/or CCA1. qRT-PCR analysis of ABF1 and ABF3 gene expression in Col-0, LHY-OX, and *cca1 lhy* in the diurnal condition. The seeds were grown in ½ MS under 12-h:12-h LD cycles for 10 d and collected samples every 3 hours as indicated in the diagram. The ACT7 gene was analyzed as an internal control. Error bars, SDs of three biological replicates.

(D) The experiment schema for investigating time-dependent stress responsiveness. Col-0 wildtype was entrained in ½ MS under 12-h:12-h LD cycles for 8 d before releasing to LL condition. ABA or NaCl was treated separately at ZT2 (AM treatment) and ZT10 (PM treatment), and samples were collected for a time course (0, 0.5h, 1h, and 2h).

(E-F) q-RT-PCR analysis of gene expression in response to ABA (E) or NaCl (F). ABA or NaCl treatment was applied as indicated in (D). The expression of *ABF3* was quantified using *ACT7* gene as an internal control. Error bars, SDs of three biological replicates.

(G-H) The quantified gene induction rate in response to ABA (G) or NaCl (H). The gene expression levels in the morning or in the afternoon in response to ABA (G) or NaCl (H) were normalized by dividing with its respective levels at 0h.

Out of the four ABFs in Arabidopsis, the transcription of *ABF1* and *ABF3* oscillate significantly in the diurnal conditions, with ABF3 peaks in the morning and ABF1 peaks near dusk (Fig. 2 A). The transcriptomic data suggests the regulation of *ABF1* and *ABF3* by CCA1 or LHY (Fig. 1 D, E), we verified it with q-RT-PCR, and the expression of *ABF1* and *ABF3* are altered in LHY-OX and *cca1-1 lhy-20* (Fig. 2 B, C). *ABFs* can be induced by abiotic stress and mediate the stress responses (reference…) and we tested if it’s under the regulation of the circadian clock. We entrained Arabidopsis seedlings in diurnal conditions for eight days and then applied the treatment of NaCl or ABA at different times of the day, ZT2 and ZT10, and kept the seedlings in the free-running long light conditions, so the duration of stress treatment was all with the light on (Fig. 2 D). The expression of *ABF3* can be induced by ABA both in the morning and afternoon (Fig. 2 E) and after normalization to the pre-treatment level, the induction rate is much higher in the afternoon than in the morning (Fig. 2G). Similar to ABA, NaCl treatment's induction rate is also higher in the after than in the morning (Fig. 2 F, H). So the stress responsiveness of ABF3 is gated by the circadian clock. ABF3 is….(look at my presentation to steve)

Fig3. The seed germination rate under NaCl is regulated by CCA1 and LHY

(A-C) Seed germination analysis of Col-0, abf1234, LHY-OX, *lhy-20*, *cca1-1* and *cca1-1 lhy-20* under normal conditions (MS medium) or under salinity (MS medium containing 120 mM NaCl). Seed germination rates were quantified from the second day to the 8th day after sowing in the 16-h:8-h LD cycles. Error bars, SDs of three biological replicates, with at least 40 seeds per genotype in each replicate. A crop of the representative figures on the 6th day is shown in Fig A.

The transcriptomic data has shown CCA1 and LHY are involved in multiple stress processes (Fig 1. A, B, C), to verify the role of CCA1 and LHY in regulating abiotic stress responses, we checked the seed germination under salinity as a proxy. Under the normal condition, all the seeds, including ABF knockout mutant *abf1234*, CCA1 knockout? mutant *cca1-1*, LHY knockdown? mutant *lhy-20*, double mutant *cca1-1* *lhy-20,* and  LHY-OX, germinated normally, at a similar rate as Col-0 (Fig 3. A, B, C). However, with the treatment of NaCl, *abf1234* had a better germination rate than Col-0,  whereas *cca1-1* and *lhy-20* had a compromised germination and *cca1-1* *lhy-20*  double mutant had an additively lower germination (Fig 3. A, B, C). LHY-OX showed a similarly low level of germination as *cca1-1* *lhy-20*  double mutant, suggesting the normal expression of CCA1 and LHY is critical for seed germination under salinity. CCA1 and LHY can form heterodimer or homodimer respectively, the alike phenotype of LHY-OX as *cca1-1* *lhy-20* mutant may be due to the abnormal function of CCA1 and LHY heterodimer or the excess LHY competed out CCA1 from homodimer.

Fig4. ABF3 regulates the expression of core clock genes and targets LHY

(A-D) ABF3 regulates the expression of core clock genes in Arabidopsis seedlings. qRT-PCR analysis of gene expression of *CCA1* (A), *LHY* (B), *TOC1* (C), and *PRR9* (D) in Col-0, ABF3-OX and *abf1234*. The indicated genotypes were grown in ½ MS under 12-h:12-h LD cycles for 10 d, then released to free-running LL conditions. The seedlings were harvested every 3 hours, as indicated in the diagram. The ACT7 gene was analyzed as an internal control. Error bars, SDs of three biological replicates.

(E) Promoter analysis of LHY promoter. Red circles indicate ACGTG (ABRE core sequence). P1-P9 indicate primer pairs used for ChIP-qPCR.

(F) ChIP results show that ABF3 binds to the promoters of *LHY* in vivo. 12-day-old Col-0 and pABF3-ABF3-YPET transgenic plants were treated with 1 μM ABA for 2 h before harvesting samples. Chromatin fragments (∼500 bp) were immunoprecipitated by anti-GFP beads (IP) or without immunoprecipitation (input) The precipitated DNA was analyzed by qRT-PCR using primer pairs indicated in Fig E. The level of binding was calculated as the ratio between IP and input, normalized to that of ACT as an internal control. Error bars, SDs of three biological replicates.

Since ABF3 hits in the CCA1 promoter yeast one-hybrid screening (Fig. 1 D, E), we analyzed the promoter of CCA1 and LHY and identified several ABRE core sequences (Fig.4 E and supplement fig…) . ABRE motifs are bound by ABFs and critical for the expression of the ABA-responsive genes (refer to … and add more). To confirm the regulation of ABF3 on CCA1 and LHY, we performed q-RT-PCR with ABF3-OX and *abf1234* mutant. The Arabidopsis seedlings were grown in diurnal conditions for 10 days and harvested the samples in free-running conditions for transcripts analysis. The expression patterns of CCA1, LHY, TOC1, and PRR9 were altered in the ABF3-OX and *abf1234* mutant. Specifically, the peak of CCA1 and LHY were down-regulated in ABF3-OX while up-regulated in *abf1234* mutant, consistent with the previous findings that cca1 and lhy mutant held long circadian period (reference …) and CCA1-OX and LHY-OX held short period (reference…).  In order to verify ABF3 directly target CCA1 or LHY, we performed Chromatin Immunoprecipitation (ChIP) using pABF3-ABF3-YPET (refer to …). ABF3 can bind the promoters of both CCA1 and LHY (suppliment fig…) Moreover, we tested whether the DNA binding activity of ABF3 is modulated by abiotic stress. We treated ABF3 transgenic lines with or without ABA before collecting samples for ChIP and analyzed them with a set of primers covering the promoter of LHY. Compared to Col-0, ABF3 can highly bind the promoter area containing ABRE core sequences, indicating ABF3 binds the promoter of LHY through ABRE core sequences. Furthermore, the ABA treatment enhanced the DNA binding activity of ABF3, particularly at the primer region of P3 and P4, so the ABA signal can strengthen the binding activity of ABF3 toward the LHY promoter, probably by inducing the phosphorylation of ABF3.

Fig5. ABF3 regulates the circadian period in a stress-responsive manner

(A-B) Bioluminescence analysis of *pLHY*: LUC expression in Col-0 and two ABF3-OX transgenic lines. The indicated genotypes were grown in ½ MS under 12-h:12-h LD cycles for 7d, then released to free-running LL conditions and detected by a bioluminescence reader. Period and Amplitude values were generated by FFT-NLLS. Only the seedlings for which the algorithm retrieves normal period length are represented on the plot. The quantified circadian period was shown (B).

(C)  The experiment schema for checking the circadian period with stress treatment. Arabidopsis seedlings were entrained in ½ MS under 12-h:12-h LD cycles for 8 d,  then ABA or NaCl treatment was applied before releasing to LL condition. reader…

(D) The circadian period of LHY-Luc and ABF3-OX/LHY-Luc under stress treatment as indicated in (C). Values are shown as means ± SEM; n = 12.

ABFs hit in the CCA1 promoter yeast one-hybrid screening (Fig 1. D, E), so ABFs are likely to regulate the expression of CCA1 and the circadian clock. Due to the redundancy of ABFs, we overexpressed ABF3 in a circadian clock reporter line LHY-LUC, which works well to evaluate the circadian pattern in our lab. The overexpression of ABF3 caused a significantly short period of LHY-LUC (Fig 4. A, B) and dampened the amplitude (supplement fig….), indicating ABF3 can regulate the circadian clock in Arabidopsis. ABF3 is involved in various abiotic stress responses, such as ….. and is a major transcription factor in the ABA signaling pathway, so we explored if abiotic stress is the upstream signal of ABF3 to regulate the circadian clock. We entrained Arabidopsis seedlings in the diurnal conditions for 8 days and applied the treatment of ABA or NaCl or MS () control in the free-running conditions and then detected the circadian activity (Fig 4. C). For the report line LHY-LUC, ABA treatment had a minor effect on the circadian period whereas NaCl treatment caused a significantly short period. For the ABF3-OX in LHY-LUC, the treatment of NaCl decreased the circadian period i.e. enhanced the short-period phenotype of ABF3-OX compared to LHY-LUC. Surprisingly, the ABA treatment led to a severely short period in ABF3-OX/LHY-LUC(Fig 4. D), quite different from the minor effect of ABA treatment on LHY-LUC, so ABF3 overexpression artificially exaggerated the effect of ABA treatment.

1. recap the results
2. discuss the period phenotype of Col-0 vs ABF3-OX with and without Stress. Particularly the short period of ABF3-OX with NaCl. Explain: it’s artificial effect rather than biological significance
3. discuss the importance of circadian gating stress-responsiveness: restraint or curb the excess expression in the morning
4. discuss the biology importance of ABF3 binding to LHY promoter in response to ABA:
5. discuss the dynamic reciprocal regulation between CCA1/LHY and ABF3

"The role of CCA1 and LHY in regulating abiotic stress responses through ABF3-mediated ABA-signaling pathway"

"Keeping Time to Survive: The Crucial Role of CCA1 and LHY in Plant Adaptation to Abiotic Stress"