REVEILLE1, a Myb-like transcription factor, integrates the circadian clock and auxin pathways

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The circadian clock modulates expression of a large fraction of the *Arabidopsis* genome and affects many aspects of plant growth and development. We have discovered one way in which the circadian system regulates hormone signaling, identifying a node that links the clock and auxin networks. Auxin plays key roles in development and responses to environmental cues, in part through regulation of plant growth. We have characterized REVEILLE1 (RVE1), a Myb-like, clock-regulated transcription factor that is homologous to the central clock genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). Despite this homology, inactivation of RVE1 does not affect circadian rhythmicity but instead causes a growth phenotype, indicating this factor is a clock output affecting plant development. *CCA1* regulates growth via the bHLH transcription factors PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5, but RVE1 acts independently of these genes. RVE1 instead controls auxin levels, promoting free auxin production during the day but having no effect during the night. RVE1 positively regulates the expression of the auxin biosynthetic gene *YUCCA8* (*YUC8*), providing a mechanism for its growth-promoting effects. RVE1 is therefore a node that connects two important signaling networks that coordinate plant growth with rhythmic changes in the environment.

growth control | hypocotyl | yucca

Circadian rhythms are approximately 24-h rhythms in physiology or behavior that are generated by an endogenous clock. Circadian rhythms persist in constant environmental conditions; however they can be entrained or set by environmental cues like light/dark or temperature cycles (1). In plants, these rhythms regulate myriad processes including leaf and cotyledon movement, growth, photosynthesis, and timing of the transition to flowering (2). A functional circadian clock provides an adaptive advantage, perhaps by predicting fluctuations in the external environment (3, 4). In all model systems studied, these self-sustained rhythms are generated by a cell-autonomous central oscillator which regulates the expression of many genes involved in metabolic and physiological functions (1). Microarray studies show that nearly one third of *Arabidopsis* genes are circadian regulated, with peak expression at different times of day (5, 6).

In plants, the central oscillator is composed of interlocking feedback loops with both positive and negative transcriptional regulators (1). The central loop is thought to consist of three genes, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION* (*TOC1*). CCA1 and LHY are morning-phased transcription factors with a single Myb-like domain containing a distinctive SHAQKYF motif (7, 8). They bind a motif, termed the evening element (EE; AAAATATCT), in the *TOC1* promoter to negatively regulate *TOC1* expression. *TOC1*, an evening-phased gene, in turn positively regulates the expression of *CCA1* and *LHY* through an unknown mechanism, thus forming the core clock negative feedback loop (7–9). The EE motif is overrepresented in the promoters of evening-phased genes and when multimerized is sufficient to confer rhythmic, evening-phased expression on a reporter gene (10, 11). These data suggest CCA1 and LHY directly negatively regulate expression of many evening-phased, clock-regulated genes. In addition, CCA1 and LHY may directly positively regulate expression of some clock-controlled, day-phased genes (4, 12, 13). Overexpression of *CCA1* or *LHY*, or of two homologous genes, *EARLY-PHYTOCHROME-RESPONSIVE1*/*REVEILLE7* (*EPR1*/*RVE7*) and *CIRCADIAN1*/*REVEILLE2* (*CIR1*/*RVE2*), disrupts clock-regulated gene expression (7, 8, 14–16).

Mutation of clock genes, in addition to disrupting circadian rhythmicity, can alter other traits such as the photoperiodic control of flowering time and light regulation of growth (2, 17). Elongation of the embryonic stem, or hypocotyl, is influenced by the circadian clock, by multiple plant hormones, and by light (17, 18). One key regulator of growth is auxin, the first-identified plant hormone. In addition to promoting cell elongation in response to environmental cues, auxin plays important roles in plant patterning. Auxin has different effects in different organs; auxin overproducers have long hypocotyls but short roots relative to wild type (19) while plants with decreased levels of auxin have shorter hypocotyls and fewer lateral roots than wild type (20).

The circadian and auxin signaling networks interact in multiple ways, although the underlying mechanisms are unknown. Levels of bioactive auxin are circadian-regulated in mature plants while seedling sensitivity to auxin is also clock-controlled (21, 22). Auxin-induced genes are more often clock-regulated than expected by chance, and the timing of peak clock-regulated expression of these genes coincides with times of maximal hypocotyl growth (5, 22, 23).

In the present study we investigate a functional link between the circadian clock and auxin signaling pathways. We focus on RVE1, a Myb-like transcription factor homologous to CCA1 and LHY. We show that, unlike CCA1 and LHY, RVE1 does not act within the plant central clock. Instead, RVE1 regulates hypocotyl growth by regulating free auxin levels in a time-of-day specific manner. We identify *YUCCA8* (*YUC8*), a gene involved in auxin biosynthesis, as a downstream target of RVE1, providing a mechanism for the auxin phenotypes. Thus, RVE1 is a node that connects the circadian and auxin networks, providing a mechanistic link between two important signaling pathways.

Results

RVE1 Is a Clock Output But Not a Clock Component. The EE has been implicated in the regulation of hundreds of clock-regulated genes

Author contributions: R.R., J.S., M.A.J., and S.L.H. designed research; R.R., J.S., M.A.J., I.S., and S.L.H. performed research; R.R., J.S., M.A.J., I.S., Y.C., C.R.A., Y.Z., and K.L. contributed new reagents/analytic tools; R.R., J.S., M.A.J., I.S., C.R.A., K.L., and S.L.H. analyzed data; and R.R. and S.L.H. wrote the paper.

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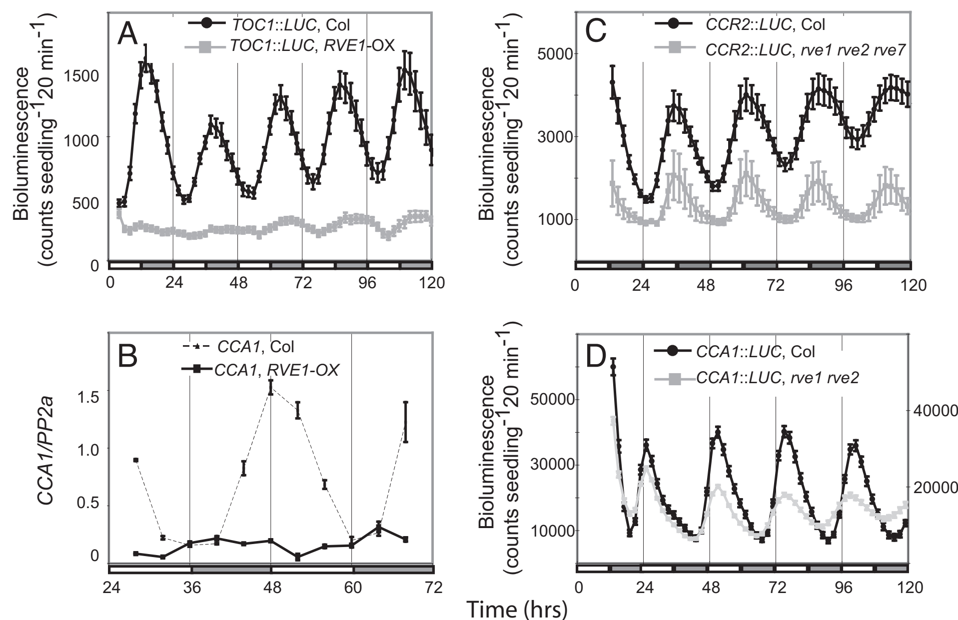

Fig. 1. Rhythmic gene expression is perturbed in *RVE1-OX* but not *rve1* mutant plants. Seedlings were entrained in 12-h light/12-h dark cycles for 6 days before transfer to either constant red (*A*, *C*, and *D*) or white light (*B*). (*A*) Average luciferase activity of wild-type and *RVE1-OX* plants expressing *TOC1*::*LUC*; (*C*) of wild-type and *rve1 rve2 rve7* plants expressing *CCR2*::*LUC*; and (*D*) of wild-type and *rve1 rve2* mutants expressing *CCA1*::*LUC*. In (*D*), luciferase activity in the loss-of-function mutants is graphed on the secondary *y*-axis for clarity. (*B*) Expression of *CCA1* in wild-type and *RVE1-OX* plants as determined using qRT-PCR. Values are expressed relative to *PP2a*. White and gray boxes on the *x* axes represent subjective day and subjective night, respectively. Each data point is the average of 25–30 seedlings for the luciferase experiments. Error bars, ± SE. These data are representative of at least two independent experiments.

and is sufficient to drive evening-phased clock-regulated gene expression (9–11). To identify transcription factors that might mediate this evening-phased gene expression, we performed a yeast one-hybrid screen using a conserved EE sequence as bait. We identified a candidate that interacts strongly with the wild-type EE, but does not bind a mutant EE that does not confer rhythmic gene expression (Fig. S1 *A* and *B*). This clone encodes RVE1 (At5g17300), a SHAQKYF-type Myb-like transcription factor previously found to bind the EE in a protein microarray study (24). RVE1 belongs to a family of 11 proteins all containing a single Myb-like domain followed by a proline-rich region; well-known members of this family include CCA1 and LHY (16) (Fig. S2 *A* and *B*). The closest homologs to RVE1 are CIR1/RVE2, EPR1/RVE7, and RVE7-like, the latter encoding a protein very similar to RVE7 (14–16). RVE1, RVE2, and RVE7 proteins share limited homology with each other outside of the Myb-like and proline-rich repeats (30% identity and 40% similarity); this homology is not seen between RVE1 and the other proteins in this family.

Like CCA1 and LHY, expression of RVE1, RVE2 and RVE7 is clock-regulated with peak transcript abundance near subjective dawn (Fig. S2*C*) (8, 13–16, 24). This suggests these genes may be important in morning-phased clock-regulated processes and prompted us to name the *REVEILLE* family accordingly. Plants expressing luciferase under the control of the *RVE1* promoter show peak luciferase activity at subjective dawn, confirming that activity of the *RVE1* promoter is clock-regulated (Fig. S3*E*).

To further investigate the interaction between RVE1 and the EE, we performed electrophoretic mobility shift assays using recombinant RVE1 protein. Competition experiments revealed that recombinant RVE1 and CCA1 have similar binding affinity for the EE, explaining the similar ability of these proteins to induce EE-driven β-galactosidase activity in yeast (Fig. S1 *B*–*D*). Consistent with their similar DNA binding activities, the amino acid sequences of these proteins are 80% identical and 94% similar within the Myb-like region.

Constitutive overexpression of CCA1, LHY, RVE2/CIR1, or RVE7/EPR1 affects the circadian clock (7, 8, 14, 15). We therefore overexpressed RVE1 in plants carrying different luciferase reporters to examine its effects on clock-regulated gene expression. Constitutive expression of RVE1 causes low and arrhythmic reporter gene activity in *TOC1*::*LUC*, *CCR2*::*LUC*, and *CAB2*::*LUC* plants, (Fig. 1*A* and Fig. S3 *A* and *B*). Similarly, quantitative real-time PCR (qRT-PCR) assays showed that expression of the core clock-associated genes *CCA1*, *GIGANTEA* (*GI*), and *LUX ARRYTHMO* (*LUX*) is low and arrhythmic in *RVE1-OX* plants (Fig. 1*B* and Fig. S3 *C* and *D*). Similar wide-ranging effects on clock-regulated gene expression are seen in plants overexpressing CCA1, LHY, or RVE2/CIR1; in contrast, overexpression of RVE7/EPR1 disrupts expression of only a subset of clock-regulated genes (7, 8, 14, 15).

Overexpression of CCA1 represses mRNA levels of *LHY*, *RVE7/EPR1*, and *RVE2/CIR1* (7, 14, 15). Similarly, we found that luciferase activity in *RVE1*::*LUC* plants is low and arrhythmic in plants that overexpress *CCA1* (Fig. S3*E*). Thus genes in the *RVE1* clade are repressed by *CCA1*, either directly or indirectly.

Although these overexpression phenotypes implicate both *RVE1* and *RVE2/CIR1* in central clock function, *rve1*, *rve2/cir1*, and *rve7/epr1* single mutants do not exhibit altered regulation of core clock-associated genes (15, 25). Reasoning that RVE1, RVE2, and RVE7 may act close to the core clock in a redundant manner, we examined clock-regulated gene expression in *rve1 rve2* and *rve1 rve2 rve7* mutants. No full-length mRNA encoding the targeted genes can be detected in the T-DNA alleles that we used, suggesting these are all loss-of-function mutants (Fig. S4 *A* and *B*). Moreover, no *RVE7-like* message can be detected in *rve1 rve2 rve7* seedlings, suggesting this gene is not expressed in seedlings (Fig. S4 *A* and *B*). We did not observe any alterations in the period or phase of luciferase activity in *rve1 rve2* or *rve1 rve2 rve7* mutants expressing this reporter gene driven by the *CCR2*, or *CCA1* promoters (Fig. 1 *C* and *D*). However, luciferase activity is low in these mutants, perhaps due to partial silencing of the reporter transgenes (26, 27). Consistent with this suggestion, mRNA levels and clock regulation

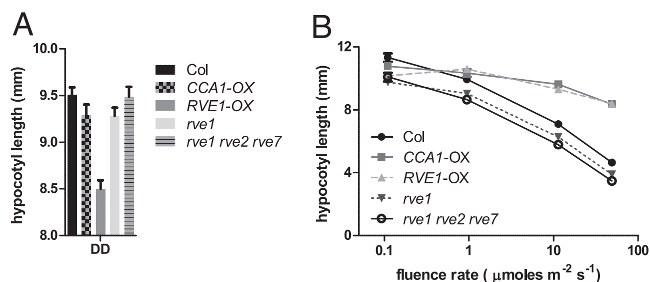

Fig. 2. *RVE1* regulates seedling growth. Seedlings were grown in either constant darkness or SD (8-h white light/16-h dark) for 6 days and hypocotyl lengths of the seedlings were then measured. (*A*) Mean hypocotyl lengths of etiolated plants. (*B*) Mean hypocotyl lengths of plants grown in different fluence rates of white light. Error bars, ± SE; $n = 20–30$. Similar results were obtained in at least three independent experiments.

of two output genes, *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) and *PIF5* are similar to wild type in *rve1 rve2 rve7* mutants (Fig. S5 *C* and *D*). Together, these data suggest that RVE1, RVE2, and RVE7 do not function close to the circadian oscillator but rather are exclusively clock outputs.

RVE1 Affects Hypocotyl Elongation. Most clock mutants have defects in light regulation of hypocotyl growth (17). Overexpression of CCA1, LHY, or RVE2/CIR1 causes elongated hypocotyls, while overexpression of RVE7/EPR1 has no effect on hypocotyl growth (7, 8, 14, 15). We therefore investigated the effects of loss- and gain-of-function *RVE1* alleles on hypocotyl growth in white light. At fluence rates greater than 1 μmol m^{-2} s^{-1}, overexpression of RVE1 or CCA1 causes hypocotyls to be significantly elongated relative to wild type ($P < 0.01$, Student's *t* test) (Fig. 2*B*). However, at very low fluence rates (0.1 μmol m^{-2} s^{-1}) and in etiolated plants, overexpression of RVE1 but not CCA1 causes hypocotyls to be significantly shorter than those of wild type ($P < 0.0005$, Student's *t* test) (Fig. 2 *A* and *B*).

We next examined the hypocotyl lengths of *rve1* mutants. Light-grown *rve1* and *rve1 rve2 rve7* plants have significantly shorter hypocotyls than wild type at all fluence rates tested ($P < 0.0005$, Student's *t* test; Fig. 2*B*). The hypocotyls of *rve2* or *rve7* single mutants are not significantly different from wild type (14, 15). However, at fluence rates above 10 μmol m^{-2} s^{-1}, *rve1 rve2 rve7* triple mutants are slightly but significantly shorter than *rve1* single mutants ($P < 0.05$, Student's *t* test), suggesting that RVE2 and/or RVE7 also play minor roles in this process. Transformation of *rve1* mutants with a genomic copy of *RVE1* resulted in plants with slightly longer hypocotyls than wild type, suggesting that RVE1 function is dosage-dependent (Fig. S4*C*). RVE1 is therefore a clock output with a role in growth regulation in *Arabidopsis*.

Mutation of photoreceptor signaling or circadian components can cause similar light-dependent growth phenotypes when monitored in an end-point assay. However, plants with defects in these different pathways can be distinguished by monitoring hypocotyl growth using time-lapse photography (18). We compared hypocotyl growth kinetics of wild-type, *RVE1-OX*, and *CCA1-OX* plants maintained in abnormally short light/dark cycles (Fig. S6). In 4-h light/4-h dark cycles, wild-type plants grow rapidly in only one out of every three dark intervals, resulting in a 24-h rhythm in hypocotyl growth (Fig. S6*A*), demonstrating growth regulation by both clock and light signaling (18). In contrast, both *CCA1-OX* and *RVE1-OX* plants show an increase in growth rate during every dark interval (Fig. S6 *B* and *C*). This is very different from the behavior of light signaling mutants, which are relatively unresponsive to light/dark transitions in this assay (18). This suggests that the growth phenotypes seen in *CCA1-OX* and *RVE1-OX* are caused by disruption of circadian rather than light signaling pathways.

RVE1-Mediated Growth Control Does Not Require PIF4 and PIF5. Overexpression of CCA1 causes increased expression of PIF4 and PIF5, two clock-regulated bHLH transcription factors that promote hypocotyl elongation (18). Given the similar growth phenotypes of plants overexpressing CCA1 or RVE1, we investigated the relationship between RVE1 and PIF4 and PIF5. We first examined the expression levels of PIF4 and PIF5 in *RVE1-OX* and *rve1 rve2 rve7* plants using qRT-PCR. As seen for other clock-regulated genes, *PIF4* and *PIF5* mRNA levels are arrhythmic in *RVE1-OX* plants (Fig. S5 *A* and *B*). Interestingly, although these transcripts damp at peak levels in *CCA1-OX* (18), they damp at low-to-intermediate levels in *RVE1-OX*. In *rve1 rve2 rve7* seedlings, *PIF4* and *PIF5* message levels are indistinguishable from wild type (Fig. S5 *C* and *D*). These data indicate that the short hypocotyls seen in *rve1 rve2 rve7* plants are not due to decreased expression of PIF4 and PIF5 and suggest that the increased hypocotyl elongation in *RVE1-OX* may be independent of these bHLH factors as well.

To test the latter hypothesis, hypocotyl elongation was assessed in wild-type and *pif4 pif5* plants transformed with a 35S::*RVE1* vector. Overexpression of RVE1 greatly increases hypocotyl elongation in both wild-type and *pif4 pif5* plants (Figs. 2*B* and 3 *A* and *B*), indicating that PIF4 and PIF5 are not required for enhanced hypocotyl growth in response to RVE1. In a parallel experiment, *PIF5-OX* and *RVE1-OX* plants were crossed and hypocotyl elongation in the F1 progeny was compared to the parental lines. Plants overexpressing both PIF5 and RVE1 have longer hypocotyls than plants overexpressing only one of these genes (Fig. 3*C*), indicating that their growth effects are either additive or synergistic. Considered together, the gene expression and genetic interaction data suggest that RVE1, PIF4, and PIF5 promote growth via different pathways.

***RVE1* Increases Auxin Levels.** A well-known mediator of plant growth is the hormone auxin. The predominant natural auxin is thought to be indole acetic acid, or IAA. Auxin overproducers have distinctive phenotypes, including tall hypocotyls in the light, short hypocotyls in the dark, short roots, and epinastic cotyledons and leaves (19). Notably, *RVE1-OX* plants have similar phenotypes (Figs. 2 and 3, and Fig. S7*I*) prompting us to examine auxin responses in both *rve1* and *RVE1-OX* plants. Treatment of wild-type plants with low concentrations of exogenous auxin causes enhanced hypocotyl elongation, while treatment with higher concentrations inhibits hypocotyl growth (Fig. 4 *A* and *B*). In contrast, all concentrations of IAA tested inhibit hypocotyl elongation in *RVE1-OX* (Fig. 4*A*), suggesting these plants may be overproducing auxin.

We next examined the responsiveness of *rve1* mutants to exogenous auxin. Low doses of IAA cause a strong increase in hypocotyl elongation in *rve1* seedlings, completely rescuing the short-hypocotyl phenotypes relative to wild type (Fig. 4*A*; $P < 10^{-6}$ without IAA and $P > 0.3$ at all IAA doses tested; Student's *t* test). These data suggest that the short hypocotyls of *rve1* plants are due to auxin deficiency.

Since plants mutant for or overexpressing RVE1, CCA1 or LHY share many hypocotyl phenotypes, we investigated the roles of CCA1 and LHY in auxin-mediated stem growth. As seen for *RVE1-OX*, treatment of *CCA1-OX* plants with exogenous auxin causes a decrease in hypocotyl elongation, suggesting these plants may be auxin overproducers (Fig. 4*B*). A loss-of-function allele of *CCA1* was not available in the Col background; however, since *cca1* and *lhy* mutants have similar short-hypocotyl phenotypes we examined the responsiveness of *lhy-20* to exogenous auxin (28, 29). Although *lhy-20* shows increased hypocotyl elongation in response to IAA, these mutants are shorter than wild type at all IAA concentrations tested [Fig. 4*B*; statistically significant at concentrations less than 20 μM ($P < 0.05$, Student's *t* test)]. Similarly, IAA treatment of *cca1 lhy* mutants does not rescue hypocotyl lengths to those of the wild-type Ler controls (Fig. S8). Thus the short hypocotyl phenotype of *rve1* plants appears to be caused by a

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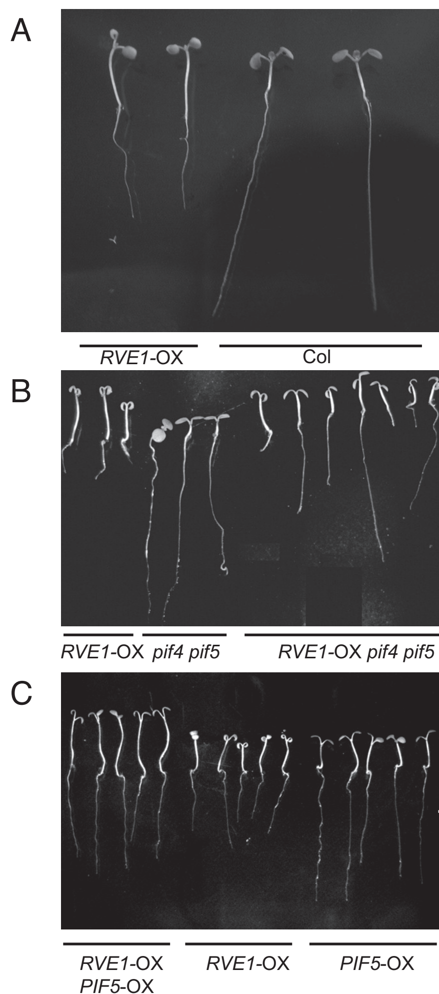

Fig. 3. The *RVE1-OX* hypocotyl phenotype does not require PIF4 and PIF5. Seedlings were grown in SD as described in the legend for Fig. 2. (*A*) Col and *RVE1-OX* seedlings. (*B*) *pif4 pif5* plants, wild-type plants transformed with *35S*::*RVE1* (T1 generation), and *pif4 pif5* plants transformed with *35S*::*RVE1* (T1 generation). (*C*) Plants overexpressing RVE1, PIF5, or both (these plants are the F1 of a cross between the *RVE1-OX* and *PIF5-OX* lines shown in the figure). Similar results were obtained in at least two independent experiments.

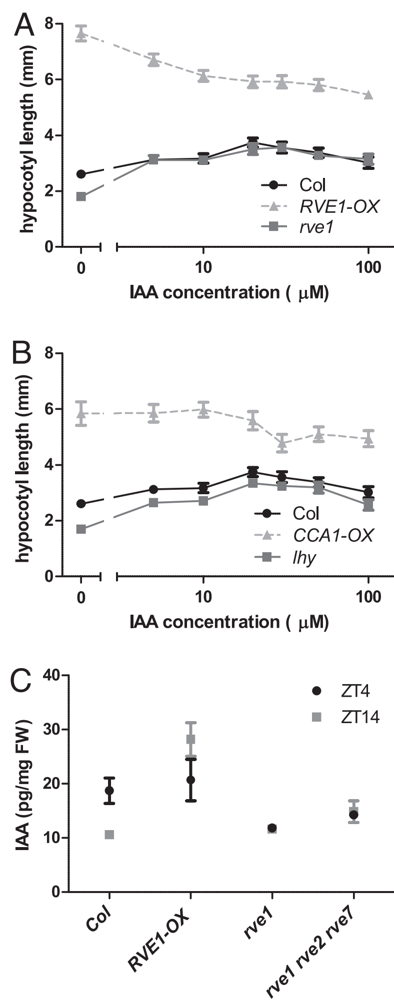

Fig. 4. RVE1 regulates diurnal rhythms in free auxin levels. (*A* and *B*) Seedlings were grown on MS medium in SD for 7 days. (*A*) Wild-type, *RVE1-OX*, and *rve1* plants were treated with the indicated doses of IAA. (*B*) Wild-type, *CCA1-OX*, and *lhy-20* plants were treated with the indicated doses of IAA. (*C*) Levels of free IAA were determined in wild type, *RVE1-OX*, *rve1*, and *rve1 rve2 rve7* plants. Plants were grown on MS medium in SD for 10 days. Error bars, ± SE; $n = 20$ (*A* and *B*) and $n = 5$ (*C*). Similar results were obtained in four (*A* and *B*) or two (*C*) independent experiments.

different molecular mechanism than underlying the *lhy* and *cca1 lhy* phenotypes.

Next, we examined the levels of free IAA in wild-type, *rve1*, *rve1 rve2 rve7*, and *RVE1-OX* seedlings grown in light/dark cycles. Wild-type plants harvested during the day have higher IAA levels than those harvested during the night (Student's *t* test; $P = 0.01$) (Fig. 4*C*). In contrast, plants mutant for or misexpressing RVE1 do not show diurnal variations in IAA levels. *RVE1-OX* plants have higher levels of IAA than wild type during the night, while *rve1* and *rve1 rve2 rve7* mutants have lower levels of IAA than wild type during the day (Fig. 4*C*). The time by genotype interaction is highly significant (two-way analysis of variance, $P = 0.006$), indicating that rhythmic *RVE1* expression is important for diurnal variations in free IAA concentration. RVE1 increases levels of active auxin during the day, consistent with the effects of exogenous IAA on hypocotyl growth in *RVE1-OX* and *rve1* plants.

We next wished to identify possible RVE1 targets that might affect auxin production. RVE1 and CCA1 have very similar DNA binding affinity in vitro, as do CCA1 and LHY (Fig. S1) (11). We reasoned that RVE1 targets might also be up-regulated in plants overexpressing *LHY*. We therefore examined previously published expression profiling data to identify genes involved in auxin production that are altered in *LHY-OX* (23) (data were accessed from the Diurnal web site: http://diurnal.cgrb.oregonstate.edu/). Interestingly, *YUC2*, *YUC5*, and *YUC8* all appear to have increased expression levels in *LHY-OX*, with the largest difference seen for *YUC8* (Fig. S9).

YUCCA gene expression is very tissue specific (30). Therefore, to investigate whether RVE1 might regulate the expression of *YUC2*, *5* or *8*, we examined β-glucuronidase (GUS) activity in wild-type and *RVE1-OX* plants expressing *GUS* under the control of these promoters. We found no obvious differences in GUS activity between *RVE1-OX* and Col plants expressing *YUC2*::*GUS* or *YUC5*::*GUS*. In contrast, we did find reproducible differences in GUS activity in wild-type and *RVE1-OX* seedlings expressing *YUC8*::*GUS*.

Wild-type plants expressing *YUC8*::*GUS* show prominent staining in the root vasculature and quiescent center but not in the meristematic zone of the root tip (Fig. S7 *A* and *C*). There is little GUS activity in the aerial portions of wild-type seedlings, with the exception of occasional very faint staining in leaf and cotyledon

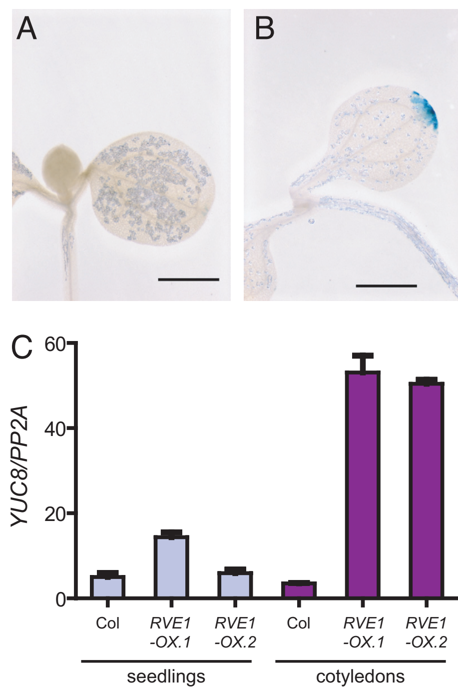

Fig. 5. YUC8 expression is increased in *RVE1-OX* plants. (*A* and *B*) Transgenic *YUC8*::*GUS* plants were grown on MS media for 10 days in SD and then stained for GUS activity. (*A*) Col, (*B*) *RVE1-OX*. (Scale bars, 1 mm.) $n = 15–17$. (*C*) Wild-type and *RVE1-OX* plants were grown as above and harvested at ZT14. RNA was extracted from intact seedlings or dissected cotyledons and YUC8 expression determined using qRT-PCR. Values are expressed relative to *PP2a*. Similar results were obtained in at least two independent experiments.

hydathodes (Fig. 5*A*). In seedlings overexpressing *RVE1*, GUS activity is greatly enhanced in a tissue-specific manner, with increased staining in cotyledon hydathodes, at the root-shoot junction, and in the meristematic zone of the root tip (Fig. 5*B* and Fig. S7 *B* and *D*). To confirm the increased expression of *YUC8* in *RVE1-OX* plants, we performed qRT-PCR on RNA extracted from whole seedlings and from dissected cotyledons. In agreement with the GUS staining data, YUC8 expression is greatly increased in the cotyledons of *RVE1-OX* plants relative to wild type (Fig. 5C). In contrast, much smaller differences in YUC8 expression are seen in samples extracted from intact *RVE1-OX* and wild-type seedlings (Fig. 5*C*).

Consistent with the possibility that RVE1 directly regulates YUC8 expression, the expression patterns of these genes overlap. GUS staining in *RVE1*::*GUS* plants is seen throughout young seedlings, with the strongest signal in root and shoot vasculature, hydathodes, young leaves, lateral root primordia, and young root tips (Fig. S7 *E–H*). We therefore examined the ability of RVE1 to regulate *YUC8* expression in a transient system. Co-expression of RVE1 with *YUC8*::*GUS* in *N. benthamiana* leaves reduces normalized glucuronidase activity relative to a vector-only control (Fig. S10). In contrast, co-expression of *YUC8*::*GUS* with *RVE1-VP64*, in which four VP16 activation domains are fused to RVE1, increases normalized glucuronidase activity in this assay (Fig. S10). Together, these data indicate that RVE1 regulates YUC8 expression.

Discussion

RVE1, CCA1, and LHY Play Distinct Roles in the Circadian Clock. We have isolated and characterized RVE1, a morning-phased transcription factor with homology to the known core clock components CCA1 and LHY. We show that overexpression of RVE1 disrupts clock-regulated gene expression, as has previously been reported for CCA1, LHY, RVE2/CIR1, and EPR1/RVE7 (7, 8, 13–15). However, neither single *rve1* nor triple *rve1 rve2 rve7* loss-of-function mutants have circadian phenotypes, when assayed either at 21 °C (Fig. 1; Fig. S5) or at 28 °C, a stressful condition for *Arabidopsis*. This suggests that these *RVE* genes are primarily clock outputs and that the disruption in rhythmic gene expression seen in *RVE1-OX* is likely due to neomorphic effects (31). The similar in vitro binding affinities of RVE1 and CCA1 to the EE (Fig. S1) suggest that when RVE1 is expressed at abnormally high levels or in inappropriate tissues it may be able to assume central clock functions normally specific to CCA1 and/or LHY (7, 8). Perhaps when expressed at high levels RVE1 is able to bind to regulatory co-factors that usually interact only with CCA1 or LHY.

RVE1 Promotes Accumulation of Free Auxin. *rve1*, *cca1*, and *lhy* mutants all have short hypocotyls in both constant light and light/dark cycles (Fig. 2 and Fig. S8) (28), suggesting these Myb-like genes may play similar roles in regulation of seedling growth. However, detailed analysis suggests this is not the case: the *rve1* hypocotyl phenotype, but not those of *lhy* or *cca1 lhy*, can be fully rescued by the addition of exogenous IAA (Fig. 4 *A* and *B*, and Fig. S8). Together with the reduced auxin levels seen in *rve1* mutants (Fig. 4*C*), this suggests *rve1* mutants are solely deficient in auxin production while *lhy* and *cca1 lhy* mutants have a defect in auxin responsiveness or in another growth-regulatory pathway. Consistent with the latter possibility, CCA1 positively regulates two transcription factors, PIF4 and PIF5, that promote hypocotyl elongation (18). In contrast, RVE1 does not strongly up-regulate PIF4 or PIF5 expression (Fig. S5), and growth promotion by RVE1 does not depend upon these factors (Fig. 3). Therefore RVE1 modulates plant growth through regulation of auxin levels while CCA1 and LHY likely control growth via different mechanism(s), such as regulation of PIF4 and PIF5 expression.

YUC8, a gene implicated in auxin biosynthesis, is up-regulated in *RVE1-OX* plants (Fig. 5), providing a possible mechanism underlying the changes in free auxin levels seen in *RVE1-OX* and *rve1* plants (Fig. 4*C*). Increased *YUC8* expresssion may be due to direct action of RVE1. An EE motif, to which RVE1 binds with high affinity (Fig. S1), is located 817 base pairs upstream of the *YUC8* transcriptional start site. In addition, RVE1 is able to repress *YUC8* promoter activity in a transient assay (Fig. S10). Such opposite effects of regulatory factors on gene expression in transient and stable assays have been previously reported (32).

In some circadian microarray experiments, *YUC8* appears to be clock-regulated with a peak phase of expression during the subjective day, while in other experiments it appears to be arrhythmic (22, 23, 25) (http://diurnal.cgrb.oregonstate.edu/); this discrepancy may be due to the low levels of *YUC* gene expression in all microarray experiments. The apparent day-phase of expression of *YUC8* would be consistent with it being activated by RVE1 (a morning-phased gene) (Fig. S2*C*) and our observation that IAA levels are higher during the day than at night (Fig. 4*C*).

RVE1 Function Is Tissue-Specific. *YUC8* expression in *RVE1-OX* plants is strongly enhanced relative to wild type only in a few tissues (Fig. 5 and Fig. S7 *A–D*) even though the viral promoter driving *RVE1* expression is active throughout young seedlings. Similarly, although as young plants *RVE1-OX* have phenotypes typical of auxin overproducers (Fig. 3 and Fig. S7*I*), as adults they do not show the enhanced apical dominance usually seen in plants with excessive auxin. This suggests that other, spatially and temporally regulated factors are required for RVE1 accumulation or function. This interpretation is supported by the opposite effects of RVE1 on *YUC8* expression in stable and transient assays (Fig. 5*C* and Fig. S10).

Consistent with YUC8 being an auxin biosynthetic enzyme, GUS activity in *YUC8:GUS* plants is observed in root tips, a known site of auxin synthesis, and in hydathodes, an area with active auxin signaling (33–35) (Fig. 5*A* and *B*, and Fig. S7 *A–D*). *RVE1* expres-

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sion is strong in these tissues but is also seen in many other organs (Fig. S7 *E–H*). This is consistent with additional, tissue-specific factors being required for RVE1 promotion of *YUC8* expression. Similarly, precise expression of *YUC4* in inflorescences is likely achieved via the combined action of the widely expressed *NGATHA* genes and unidentified tissue-specific factors (36).

Interacting Networks Regulate Seedling Growth. Rhythmic growth of seedlings is regulated by many factors, including the circadian clock, light, and hormones such as auxin, ethylene, gibberellins, and brassinosteroids (17). Genes regulated by these hormones are more likely to be clock-regulated than expected by chance and, intriguingly, the times of maximal growth rate of the young stem coincide with the times of peak circadian expression of many clock- and hormone-regulated genes (5, 22, 23). This suggests the clock may help regulate plant growth by coordinating the activity of multiple hormone pathways.

In previous work, we showed that the circadian clock modulates both endogenous auxin signaling and plant responses to exogenous auxin (22). We now demonstrate that RVE1 is a clock-regulated transcription factor that promotes *YUC8* expression and is essential for diurnal rhythms in auxin levels. Thus RVE1 is a node that connects the auxin and circadian signaling networks, providing a mechanistic link between the circadian system and hormone signaling.

Materials and Methods

Plant Materials and qRT-PCR Assays. Details on the construction of the binary vectors, the mutants used in this study, and qRT-PCR protocols may be found in the *SI Text*.

Luciferase Imaging. Seeds were plated on Murashige and Skoog (MS) (MP Biomedicals) media containing 0.8% agar (Sigma A1296) and 3% sucrose (EMD Chemicals). After 6 days of growth in 12-h light (50 μmol m^{-2} s^{-1})/12-h dark cycles (22 °C; illumination provided by cool white fluorescent bulbs), luciferase activity was monitored in constant red light as previously described (26).

Hypocotyl Growth Assays. Seedlings were grown on MS medium (0.8% agar and 3% sucrose) at 22 °C either in darkness or short days (SD; 8-h white light (50 μmol m^{-2} s^{-1})/16-h dark) for 6 days. For the exogenous IAA assay, seedlings were grown on MS media (0.8% agar) for 2 days, transferred to media containing IAA, then grown for 4 days in cool white fluorescent light (40 μmol m^{-2} s^{-1}) passed through a yellow filter (Ridout Plastics, ACRY 22080, 125C). Seedlings were transferred to transparencies, scanned and measured using the application ImageJ (http://rsb.info.nih.gov/ij).

GUS Staining. Seedlings were grown on MS media (0.8% agar and 3% sucrose) in SD at 22 °C for 10 days. Whole seedlings were stained in 0.2% Triton X-100, 50 mM phosphate buffer (pH 7.2), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (Biosynth Ag) for 13 h and then cleared with an ethanol series.

Auxin Quantification. Seedlings were grown on Whatman filter paper placed on MS media (0.8% agar and 3% sucrose) in SD (22 °C) for 10 days. Whole seedlings were collected 4 and 14 h after dawn (ZT4 and ZT14) and frozen in liquid nitrogen. Ten seedlings were pooled per sample and five biological replicates were harvested for each time point. Free IAA was quantified as previously described (37) with minor modifications.

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