Circadian rhythms of ethylene emission in Arabidopsis

Circadian Rhythms of Ethylene Emission in Arabidopsis[1[w]]

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Ethylene controls multiple physiological processes in plants, including cell elongation. Consequently, ethylene synthesis is regulated by internal and external signals. We show that a light-entrained circadian clock regulates ethylene release from unstressed, wild-type Arabidopsis (*Arabidopsis thaliana*) seedlings, with a peak in the mid-subjective day. The circadian clock drives the expression of multiple *ACC SYNTHASE* genes, resulting in peak RNA levels at the phase of maximal ethylene synthesis. Ethylene production levels are tightly correlated with *ACC SYNTHASE 8* steady-state transcript levels. The expression of this gene is controlled by light, by the circadian clock, and by negative feedback regulation through ethylene signaling. In addition, ethylene production is controlled by the *TIMING OF CAB EXPRESSION 1* and *CIRCADIAN CLOCK ASSOCIATED 1* genes, which are critical for all circadian rhythms yet tested in Arabidopsis. Mutation of ethylene signaling pathways did not alter the phase or period of circadian rhythms. Mutants with altered ethylene production or signaling also retained normal rhythmicity of leaf movement. We conclude that circadian rhythms of ethylene production are not critical for rhythmic growth.

Since the discovery of ethylene production in plants in the 1930s, researchers have tried to elucidate mechanisms governing ethylene formation. A major breakthrough was the completion of the enzymatic pathway for ethylene biosynthesis 50 years later (for review, see Yang and Hoffman, 1984). Shortly thereafter, the first genes encoding ethylene biosynthetic enzymes were cloned (Sato and Theologis, 1989; Van Der Straeten et al., 1990; Hamilton et al., 1991; Spanu et al., 1991). With the use of tomato (*Lycopersicon esculentum*) and especially Arabidopsis (*Arabidopsis thaliana*) as model plants, molecular biological and genetic analysis has shed light on many physiological processes involving ethylene (Abeles et al., 1992; Somerville and Meyerowitz, 2002). In higher plants, the enzymes for ethylene biosynthesis are encoded by gene families. The members of these families are differentially responsive to various ethylene-inducing factors, including wounding, fruit ripening, pathogen infections, auxins, and cytokinins (for review, see Fluhr and Mattoo, 1996).

In Arabidopsis, there are 12 genes in the family of enzymes that produces the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC), one of which, *ACC SYNTHASE 3* (*ACS3*), is a pseudogene (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004). ACS1 is not functional as an ACS (Liang et al., 1992). ACS10 and ACS12 do not function as ACSs either, but as aminotransferases (Yamagami et al., 2003). Many of the *ACS* genes are regulated on the transcriptional level. *ACS2* transcription in leaves is switched off when tissues mature (Rodrigues-Pousada et al., 1993; in this paper the gene was designated *ACS1*). *ACS4* can be induced by auxins (Abel et al., 1995). ACS5 is regulated by cytokinins that cause stabilization of the protein (Chae et al., 2003). *ACS6* is induced by ozone, wounding, auxins, and ethylene (Vahala et al., 1998; Tian et al., 2002).

ACC oxidases (ACOs), which catalyze the conversion of ACC to ethylene, belong to a large family of dioxygenases containing at least 17 members. Nevertheless, only two of them have been functionally characterized (Gomez-Lim et al., 1993; Raz and Ecker, 1999).

[1] This work was supported by a Biotechnology and Biological Science Research Council (graduate studentship to S.C.T.), by the Fund for Scientific Research (Flanders; grant nos. G.0281.98, WO.004.99, and G.0345.02 to D.V.D.S.), by the European Community Training and Mobility of Researchers Programme, and by the Gatsby Charitable Foundation, the Royal Society, and the Biotechnology and Biological Science Research Council (grants to A.J.M.).

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[w]The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.042523.

Only two mutants in ethylene biosynthesis genes have been described: *eto2* and *eto3* (Kieber et al., 1993). The genes affected in these mutants are highly similar: *ACS5* in *eto2* and *ACS9* in *eto3* (Vogel et al., 1998; Chae et al., 2003). In contrast, a plethora of mutants involved in ethylene signaling has been described. These have been invaluable in the elucidation of the ethylene signal transduction pathway during the last decade. There are mutants for the five ethylene receptors, ETR1, ETR2, ERS1, ERS2, and EIN4, and also for some of the downstream components (Guzmán and Ecker, 1990; Chang et al., 1993; Kieber et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998). The receptors interact with CTR1, a MAP kinase kinase kinase protein, and MAP kinases further transduce the signal (Clark et al., 1998; Ouaked et al., 2003). EIN2 acts downstream of this MAP kinase cascade (Alonso et al., 1999). The signal goes into the nucleus via EIN3-like proteins and ends in transcriptional control of response genes (Chao et al., 1997). The stability of the EIN3 protein is a key controlling element in ethylene signaling (Guo and Ecker, 2003; Potuschak et al., 2003).

The circadian clock drives 24-h biological rhythms of many processes in higher plants (McClung, 2001), including rhythms of hypocotyl elongation and vertical leaf movement (Dowson-Day and Millar, 1999) and the expression of about 6% of the transcriptome in light-grown Arabidopsis seedlings (Harmer et al., 2000). In some plant species, ethylene production was shown to be regulated in a circadian manner. In *Sorghum bicolor*, ethylene peaks during the day (Finlayson et al., 1998). In *Chenopodium rubrum*, ACC levels showed circadian fluctuation (Machackova et al., 1997). Cotton (*Gossypium hirsutum*) and *Stellaria longipes* also have rhythmic ethylene emanation. In these plants, the process is related to rhythmicity in ACC synthase and ACO activity, respectively (Rikin et al., 1984; Kathiresan et al., 1998).

The molecular clock mechanism has recently been characterized, based on genetic approaches in Arabidopsis (for review, see Eriksson and Millar, 2003; Hayama and Coupland, 2003). The current model includes the *myb*-domain DNA-binding proteins LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), which are expressed around dawn (Schaffer et al., 1998; Wang and Tobin, 1998), and the atypical response regulator *TIMING OF CAB EXPRESSION 1* (TOC1; Millar et al., 1995; Strayer et al., 2000), which is expressed in the late day to early night. The *toc1-1* mutation shortens the period of circadian rhythms without altering seedling morphology in white light (Millar et al., 1995; Somers et al., 1998). More severe loss-of-function *toc1* alleles can cause arrhythmia, probably by preventing the expression of *CCA1* and related genes (Alabadi et al., 2001; Mas et al., 2003). In contrast, constitutive expression of *CCA1* causes exaggerated hypocotyl elongation and overt arrhythmia (Wang and Tobin, 1998), probably due to direct repression of *TOC1* gene expression (Alabadi et al., 2001).

Here, we show that a light-entrained clock, the mechanism of which includes *CCA1* and *TOC1*, controls ethylene production. Mutations in ethylene signaling do not affect the phase or the period of circadian ethylene production. The main control point for ethylene production in Arabidopsis is the synthesis of the precursor ACC. The rhythmic emanation of ethylene was correlated with *ACS8* transcript levels. In addition, our data suggest that light controls accumulation of this transcript and that negative feedback regulation of this gene through ethylene signaling is superimposed on the endogenous circadian regulation.

RESULTS

Light-Entrained Circadian Regulation

We tested for circadian regulation of ethylene evolution by growing vials of seedlings under opposite photoperiods (12 h light/12 h dark [LD 12, 12] or 12 h dark/12 h light [DL 12, 12]) before measuring their ethylene evolution together under constant light. Levels of ethylene were high enough to be reliably measured by the time of radicle emergence, 48 to 72 h after sowing (data not shown). Ethylene measurements were initiated 3 d after sowing, when essentially all seed had germinated; the amplitude of the hypocotyl elongation rhythm is greatest at this stage of development (Dowson-Day and Millar, 1999). Ethylene evolution was rhythmic, with a peak at subjective midday and a trough in the middle of the subjective night (Fig. 1A). The rhythmic amplitude ranged from 2- to 4-fold. The phase of the rhythm in each vial was determined by the preceding light-dark cycle, indicating that the rhythms were light entrained and were not caused by fluctuations in the measurement system. Biomathematical analysis estimated periods close to 24 h under constant light for the three wild-type Arabidopsis accessions tested: C24, Columbia (Col-0; Table I), and Landsberg *erecta* (S.C. Thain and A.J. Millar, unpublished data).

Rhythms of ethylene evolution from seedlings grown in LD (12, 12) persisted for up to 4 d in constant darkness (Fig. 1B). The rhythmic patterns were more variable than under constant light, so it was not possible to compare period length in light with that in darkness. The progressive increase in ethylene evolution after 48 h in Figure 1B also was not always that pronounced. These light-entrained rhythms, which persisted in constant conditions with periods close to 24 h, indicate that ethylene evolution is controlled by the circadian clock in Arabidopsis.

The effect of daylength on the rhythm was also studied. In 6-d-old seedlings, the phase of the ethylene production was shifted later by 2 to 4 h in LD (16, 8) entrained plants compared with LD (12, 12) entrained plants (Fig. 1C). Consequently, ethylene production peaked around subjective midday in both cases.

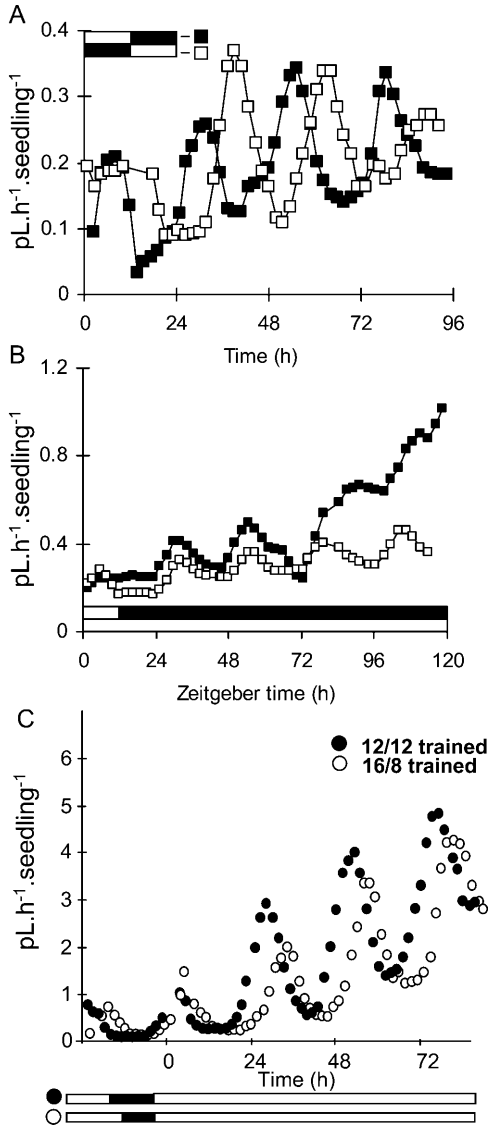

Figure 1. Circadian rhythms of ethylene evolution in Arabidopsis. Arabidopsis seedlings were grown on sterile agar medium. Ethylene was measured in gas-tight vials using laser-based photoacoustic detection. A, Groups of wild-type C24 seedlings were entrained to three cycles of LD (12, 12) or reversed cycles, DL (12, 12), then transferred to constant light and tested for ethylene release. Culture A (black symbols) entered constant light at time −12 h, and culture B entered constant light at 0 h (white symbols). Upper inset indicates the predicted light-dark cycle and symbol for each culture. B, Groups of wild-type C24 seedlings were grown for 3 d in LD (12, 12), then transferred to constant light at 0 h (white symbols) or to constant darkness at 12 h (black symbols) and tested for ethylene release. Zeitgeber time is, by convention, the time in hours since the last dark-light transition. C, Influence of daylength on circadian ethylene production in Arabidopsis. Groups of wild-type Col-0 seedlings were grown for 6 d in LD (12, 12; black symbols) or LD (16, 8; white symbols) and tested for ethylene release during one LD cycle followed by constant light. White box, light interval; black box, dark interval.

TOC1 and *CCA1* Control the Ethylene Rhythm

Arabidopsis mutants and misexpression lines have been identified that alter the timing of several rhythmic processes, including rhythms of hypocotyl elongation and gene expression. The cognate, wild-type gene products are thought to be components of the circadian clock, such as TOC1 and CCA1. We tested ethylene evolution in mutant and wild-type seedlings to determine whether *TOC1* and *CCA1* also control the ethylene rhythm (Fig. 2). As previously reported (Millar et al., 1995), the *toc1-1* seedlings had wild-type morphology (data not shown). The absolute levels of ethylene produced and the amplitude of the circadian rhythm were very similar to those of wild-type seedlings (Fig. 2A). There was a clear change in the time lag between the peaks of the *toc1-1* and wild-type rhythms on successive days, indicating that the plants had different periods. The mean period calculated for *toc1-1* was significantly shorter than wild type (Table I). *CCA1-ox* seedlings had elongated hypocotyls under our conditions (data not shown), as previously reported (Wang and Tobin, 1998). *CCA1-ox* seedlings gave approximately 2-fold higher levels of ethylene evolution from the start of the experiment, with no hint of rhythmicity (Fig. 2B).

Circadian Rhythms Depend on Rhythmicity of *CCA1* But Not on Ethylene Signaling

We have previously observed a circadian rhythm of hypocotyl elongation and cotyledon movement in Arabidopsis seedlings under constant light (Dowson-Day and Millar, 1999). We tested this rhythm in *CCA1-ox* seedlings that were germinated and grown in LD (12, 12) for 2 d, then transferred into constant light of low intensity. Wild-type hypocotyls elongated most rapidly around subjective dusk (about 10 h after dawn), when cotyledons were raised up to the highest angle (Supplemental Fig. 1, available at www.plantphysiol.org). Hypocotyl elongation was arrested for several hours around subjective dawn, when cotyledons were lowered. In the *CCA1-ox* plants, hypocotyls elongated

Table I. *Circadian rhythms in ethylene from Arabidopsis seedlings*

Ethylene evolution data collected as described in Figure 2 were analyzed using FFT-NLLS (see "Materials and Methods"). Mean periods were very significantly different, $P < 0.001$ in Student's *t* test. Col, Columbia; Period, variance-weighted mean period; SEM, variance-weighted SE of the mean; Rhythmic, number of samples with rhythmic periods in the circadian range (15–35 h); −, no circadian period detectable.

Genotype	Accession	Period	SEM	Rhythmic	Samples	Experiments
		h			*n*	*n*
Wild type	C24	24.4[1]	0.21	5	5	3
toc1-1	C24	22.5[1]	0.15	5	5	2
Wild type	Col	24.3	0.24	5	5	3
CCA1-ox	Col	–	–	0	4	1

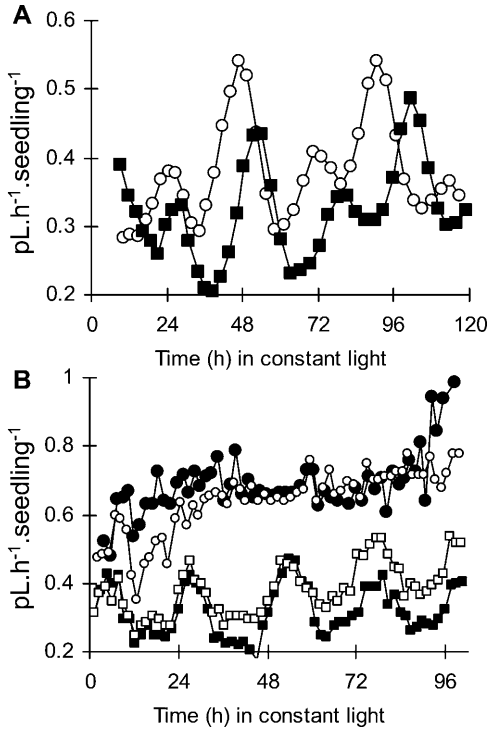

Figure 2. Aberrant ethylene rhythms in clock mutants. Ethylene rhythms were measured under constant light as described in Figure 1. A, *toc1-1* seedlings (white circles) exhibited a short period rhythm compared to the wild-type parent (black squares). B, Ethylene release was arrhythmic in *CCA1-ox* (circles) but rhythmic in Col-0 (squares). White and black symbols show two representative examples from the data analyzed in Table I.

at a constant rate similar to the maximum rate of wild-type plants, without detectable growth arrests (Supplemental Fig. 1). Mathematical analysis confirmed the arrhythmia of *CCA1-ox* (Table II; Supplemental Fig. 2).

Ethylene is known to influence cell elongation, raising the possibility that the ethylene rhythm causes rhythmic growth. Rhythmic hypocotyl elongation (Dowson-Day and Millar, 1999) was not disturbed in ethylene-insensitive mutants (Fig. 3A). In plants that lack a pulvinus, such as Arabidopsis, the circadian rhythm of leaf angle is driven by petiole elongation (Engelmann and Johnsson, 1998). Rhythmic leaf movement in constant light was unaffected by mutations in ethylene signaling and biosynthetic pathways, including *etr1-1*, *ein4-1*, and *eto2-1* (Fig. 3B). Though circadian period is variable among leaf traces, no differences in circadian timing were observed among the mutants. The rhythmic expression of *CAB* (Fig. 3, C and D) and *PHYB* (data not shown) genes, revealed by the *CAB:LUC* (Millar et al.,1995) or *PHYB:LUC* reporter genes, was unaffected by pulsed or continuous ethylene treatments. The *ctr1-1* mutation, which greatly enhances signaling and confers a dwarfed phenotype, was crossed into the *CAB:LUC* background. The mutant seedlings within the segregating F_2 progeny all displayed rhythmic luminescence under constant light, with the same mean period as their wild-type siblings (*ctr1-1*: 24.3-h period, SEM 0.2 h, $n = 8$; wild type: 24.6-h period, SEM 0.1 h, $n = 15$).

Ethylene Biosynthesis Genes Are under Circadian Control

Using reverse transcription (RT)-PCR, we investigated whether there was a correlation between the circadian ethylene production and the transcript patterns of ACSs and ACOs, and the putative ACOs At1g04350 and At5g63600. We sampled seedlings at subjective midday and midnight, the time points corresponding to the maximal amplitudes in circadian cycling of ethylene production and studied the steady-state levels of mRNA over 3 d.

The expression of *ACS8* clearly followed the pattern of ethylene emanation, both in continuous light and in continuous darkness (Fig. 4). It is noteworthy that the differences between peak and trough transcript levels of *ACS8* diminish over time in darkness. In continuous light, *ACS5* and *ACS9* had a similar, though less pronounced, expression pattern with transcript levels on subjective midday higher than those at the subsequent subjective midnight (Fig. 4A). Interestingly, *ACS2* appeared to have an inverted expression pattern. Steady-state mRNA levels of the other ethylene biosynthesis enzymes did not follow the rhythm of ethylene production.

For several genes, we observed differences in plants transferred to continuous light, compared with those transferred to continuous dark. When plants were put in continuous darkness after 6 d of entrainment, *ACS5* and *ACS8* expression levels increased (Fig. 4B). *ACS8* was the only gene for which the rhythm persisted in continuous dark. The steady-state messenger level of a putative ACO gene (*At1g04350*) was repressed in darkness.

Table II. *Circadian rhythms of hypocotyl elongation in Arabidopsis*

Seedling growth rhythms were monitored as described in Figure 3 and analyzed using FFT-NLLS (see "Materials and Methods"). Period, Arithmetic mean period; SD, arithmetic SD; Rhythmic, number (and percentage) of seedlings with robust rhythmic periods in the circadian range (15–35 h).

Genotype	Period	SD	Rhythmic *n*	Seedlings	Experiments
	h		%	*n*	*n*
Col-0 Wild type	24.2	2.16	25 (89)	28	15
CCA1-ox 034	29.4	3.41	6 (25)	24	7
CCA1-ox 038	22.7	6.01	4 (18)	22	7

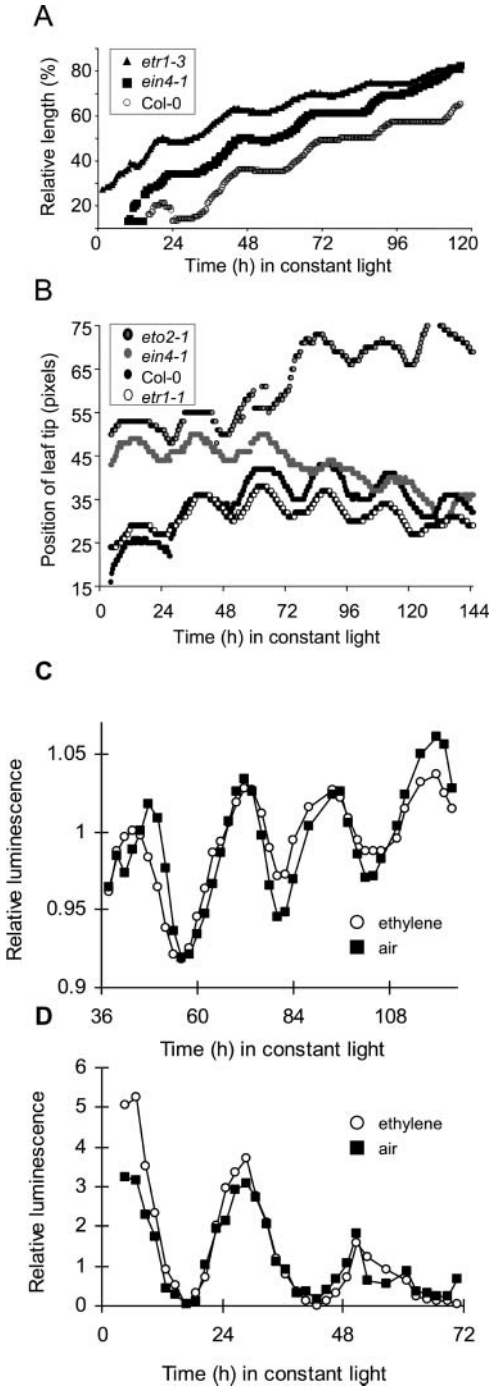

Figure 3. Ethylene signaling does not affect circadian rhythms. A, Rhythmic elongation of hypocotyl growth in *etr1-1*, *ein4-1*, and Col-0 seedlings. Symbols are detailed in the legend. B, Rhythmic movement of the leaf tip was assayed in *etr1-1*, *ein4-1*, *eto2-1*, and Col-0 seedlings. Symbols are detailed in the legend. Exogenous ethylene and ethylene signaling mutations do not affect circadian rhythms of gene expression. C and D, Wild-type seedlings carrying the *CAB:LUC* reporter gene (Millar et al., 1995) were grown in LD (12, 12) cycles for 5 d, transferred to constant white light at time 0, and treated with ethylene. Rhythms of in vivo luminescence were assayed by low-light video imaging. C, A 12-h treatment of 10 μL L^{-1} ethylene during the subjective night (time 12–24 h) does not alter the phase of *CAB:LUC* expression. Seedlings were imaged from 36 h to exclude any acute effects of ethylene treatment. D, The continuous presence of ethylene (20 μL L^{-1} from time 0) does not affect rhythmic *CAB:LUC* expression. Ethylene-treated samples, white symbols; air-treated controls, black symbols.

Since ACS8 appears the most likely candidate to cause circadian ethylene production, we analyzed the ethylene emanation from SALK_066725 line, which has its T-DNA insertion in the coding region of the *ACS8* gene, close to the C-terminal end. Hence, the ACS8 protein is predicted to miss the last 15 amino acids. After 4 d of entrainment under LD (12, 12), the ethylene production in seedlings was followed in continuous light. The pattern of the SALK_066725 line did not show any significant or reproducible difference from the wild type (Supplemental Fig. 3). Also, the plants did not show a constitutive triple response in the dark (data not shown).

Permanent Presence of a High Concentration of ACC Dampens the Amplitude of Rhythmic Ethylene Production

Earlier research has established *eto2* as an ethylene overproducer (Kieber et al., 1993; Vogel et al., 1998). The overproduction in this mutant is due to a mutation in the C-terminal end of ACS5. Consequently, the enzyme lacks the ultimate 12 amino acids, resulting in a hyperstable protein (Chae et al., 2003). We tested which influence the *eto2* mutation exerts on transcript levels of ethylene biosynthesis genes. In continuous light, *ACS5* transcript accumulation in the *eto2* background had a weakly rhythmic pattern. When transferred to continuous dark, the rhythmicity disappeared (Fig. 4). This corresponded with a dampening in the rhythm of the elevated ethylene production in *eto2* mutants (Fig. 5A), while the transcript accumulation pattern of *ACS5* was similar to that for the wild type (Figs. 4 and 5B). The pattern for *ACS8* transcript accumulation was unaffected by the *eto2* mutation (compare Figs. 4A and 5B), although the ethylene production was highly increased in the mutant (compare Figs. 2A and 5A). Together, these data suggest a role for the mutated *ACS5* in the circadian ethylene production of *eto2,* possibly implying that the activity of the truncated ACS5 protein in *eto2* is regulated by the clock, through a yet unknown mechanism. The dampening in ethylene rhythm in continuous darkness coincided with a lower expression of the *ACO* gene *At1g04350* than in the wild type (Fig. 5C).

Col-0 plants were grown on medium containing 50 μM ACC for 6 d in 12-h-light/12-h-dark rhythm. After transfer to continuous light or to continuous dark, ethylene levels were followed during 2.5 d (Fig. 6A). In both cases there was little or no diurnal fluctuation in ethylene production. This suggests that circadian ACO activity is not the main cause for the large fluctuations in ethylene production. As plants grew older, there was a gradual decrease in ethylene emanation in permanent darkness. It was also striking that the production levels were lower than in the *eto2* mutants, grown on a medium without ACC. This indicates that

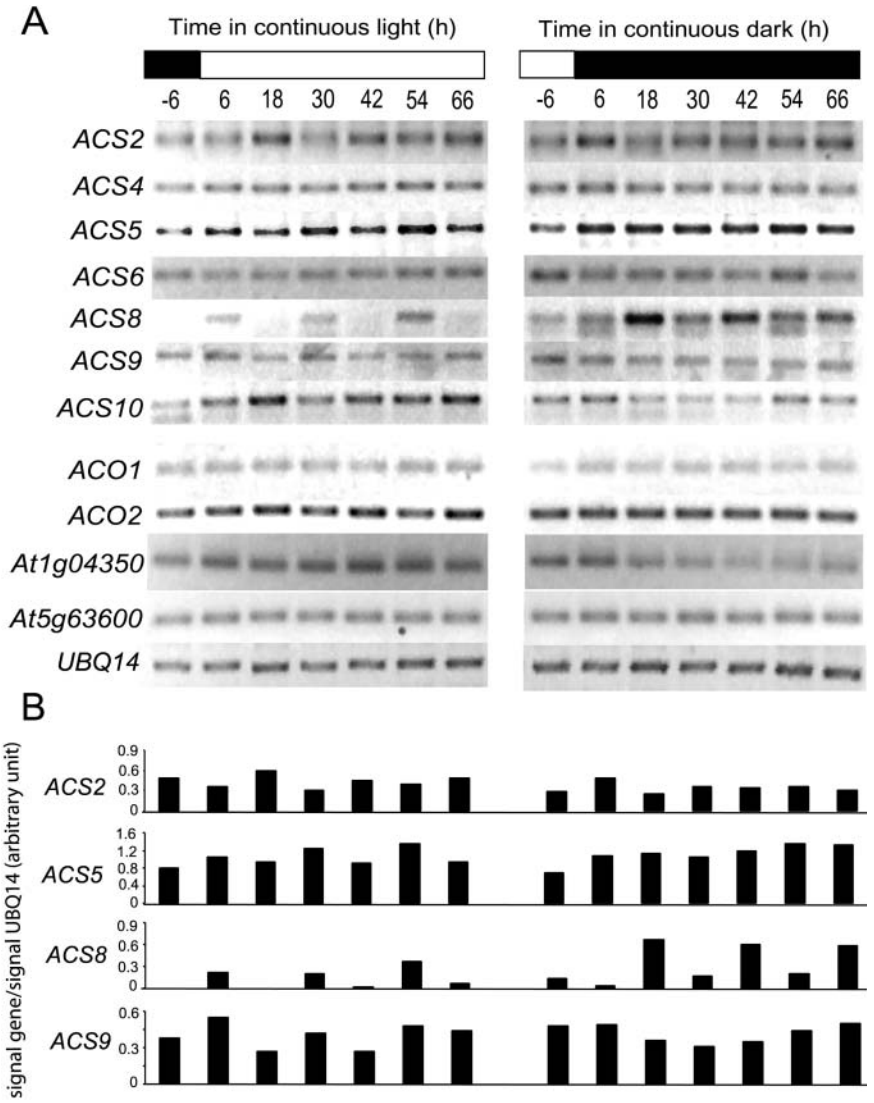

Figure 4. Steady-state transcript levels of ethylene biosynthesis and *UBQ14* genes as visualized on gel after RT-PCR. A, On the left half, seedlings entrained for 6 d in LD (12, 12) and transferred to continuous light (black bar followed by white bar). On the right, seedlings entrained for 6 d in LD (12, 12) and transferred to continuous dark (white bar followed by black bar). Time points are indicated in hours after the start of continuous conditions. B, Intensity values of the transcript levels as detected in section A of genes that are regulated in phase or anti-phase with the ethylene production rhythm. Values are normalized to the values of *At5g63600*. The bars correspond to the time points indicated in section A.

permanent presence of saturating levels of ACC puts a serious restraint on ethylene biosynthesis.

RT-PCR analysis revealed that most *ACS* genes of plants grown on ACC had a similar pattern of expression as in the nontreated wild type, including *ACS5* and *ACS8* (Fig. 6B; data not shown). Like in *eto2* plants, a similar repression was found in the *ACO* RNA (*At1g04350*), which was virtually absent in darkness in the presence of ACC. The repression of the latter gene in darkness thus coincides with, and therefore may be related to, the gradual decrease of ethylene production.

Feedback Regulation via Signaling Affects Ethylene Release But Has Little Effect on Circadian Rhythm of Ethylene Production

We tested whether the ethylene-insensitive mutants *etr1-3*, *ein4-1*, and *ein2-1* had defects in circadian ethylene production. Therefore, we measured ethylene emanation of seedlings transferred to either continuous light (Fig. 7A) or continuous dark (Fig. 7B). Both in continuous light and dark, the rhythm persisted, and compared to wild type, more ethylene was released. For instance, *etr1-3* and *ein2-1* produced at least 20-fold more ethylene than the wild type. This indicates that ethylene signaling does not interfere with circadian regulation of ethylene biosynthesis. Moreover, *ACS8* transcripts in *ein2-1* plants accumulated to a higher level than in wild type at the peak and trough time points of ethylene production in both continuous light and dark (Fig. 7C). We did not detect a difference in steady-state mRNA levels between wild type and *ein2-1* for the other ethylene biosynthesis genes we tested (data not shown).

DISCUSSION

Light and the Circadian Clock Control Transcript Levels of Ethylene Biosynthesis Genes

Ethylene biosynthesis is regulated at several steps. Controls at the levels of formation of both ACC and

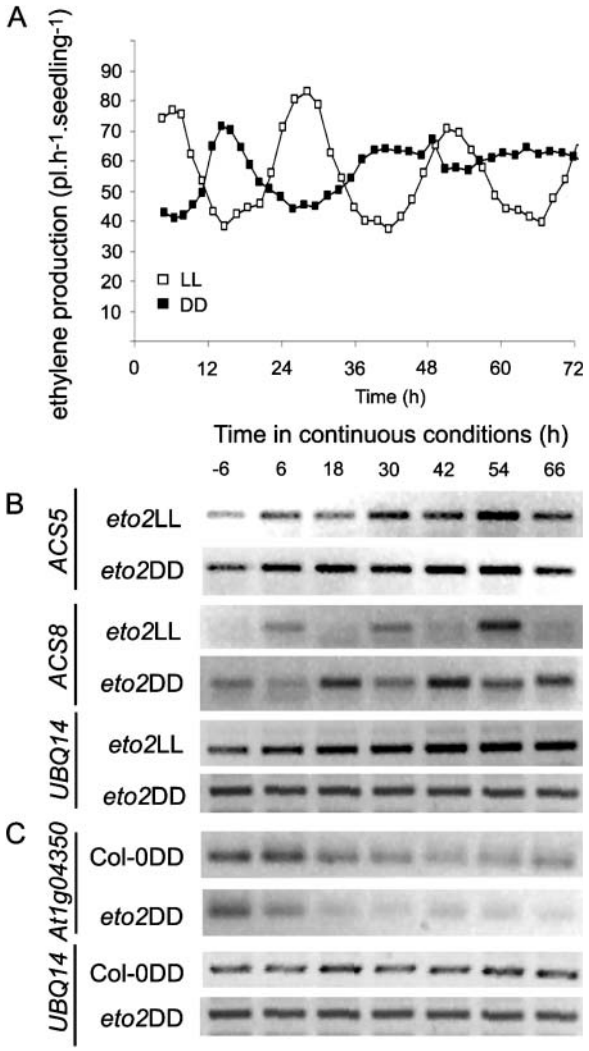

Figure 5. A, Ethylene production levels in *eto2* mutant seedlings. Six-day-old seedlings were transferred to continuous light or darkness. White squares, continuous light (LL); black squares, continuous dark (DD). B and C, RT-PCR visualization of expression levels of relevant ethylene biosynthesis genes in the *eto2* mutant and in wild type Col-0 seedlings. Six-day-old seedlings were transferred to continuous light or darkness. LL, continuous light; DD, continuous dark.

ethylene have been reported, respectively catalyzed by ACS and ACO. Depending on the species and on the environmental conditions either one of these steps can be of crucial importance (Kathiresan et al., 1996, 1998; Machackova et al., 1997; Finlayson et al., 1999).

Arabidopsis seedlings displayed a robust, circadian rhythm of basal ethylene levels in light and darkness (Fig. 1). However, when treated with exogenous ACC, the rhythm in circadian ethylene emanation was severely dampened, indicating that ACOs may not be responsible for the rhythm. Also, the family of *ACO* and *ACO*-like genes in Arabidopsis consists of over 17 members. Therefore, it is possible that cycling *ACO*s are redundant with noncycling family members or *ACO*s cycling out of phase with the ethylene production rhythm (Harmer et al., 2000; Fig. 4).

A minor role for ACO in determining basal ethylene production appears of a more general nature, as indicated by tobacco plants that overexpress ACO yet do not overproduce ethylene, whereas ACS overexpressors do (Knoester et al., 1997). In addition to *ACS8*, transcript accumulation of *ACS5* was higher in darkness. Although circadian rhythms were observed for the latter gene in continuous light, they were not detectable in continuous dark (Fig. 4). Consistent with this, in *eto2* the rhythm in ethylene production disappears in continuous dark. At the same time, it can explain the

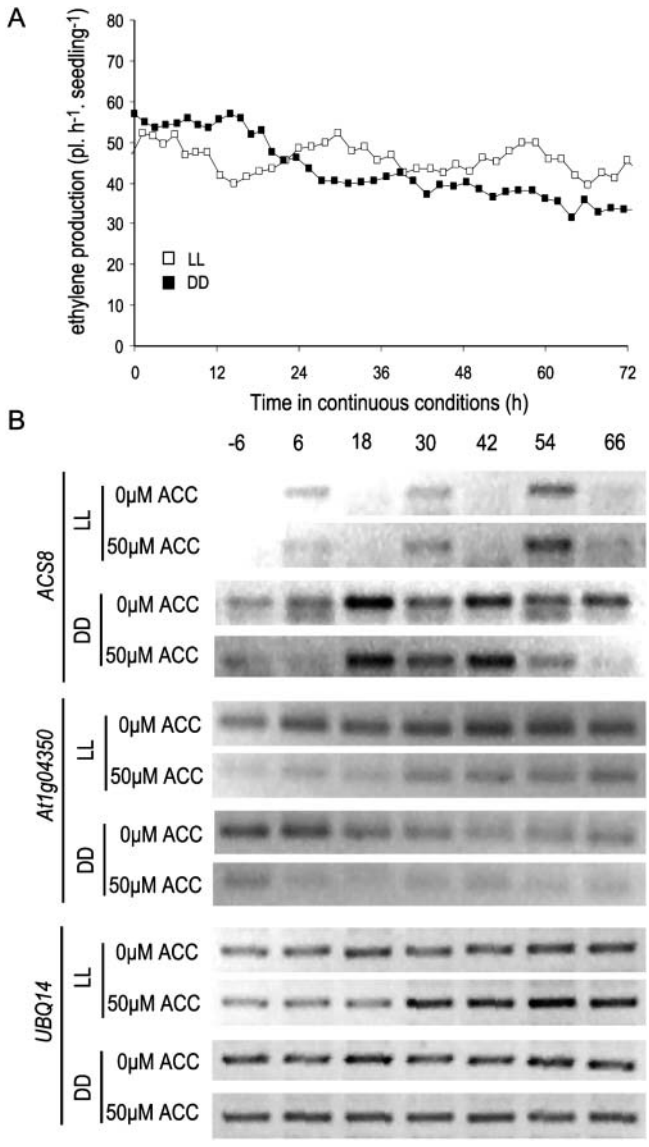

Figure 6. A, Effects of exogenous ACC on ethylene production in Col-0 plants transferred to continuous light (white squares) or continuous dark (black squares). Plants were grown for 6 d (at $T = 0$ h) on medium without or containing 50 μM of the ethylene precursor ACC. Under the conditions used, the ethylene levels in control plants fall below detection limit. B, RT-PCR products of biosynthesis genes in ACC treated or nontreated wild-type plants. Plants were grown for 6 d (at $T = 0$ h) on medium without or containing 50 μM of the ethylene precursor ACC.

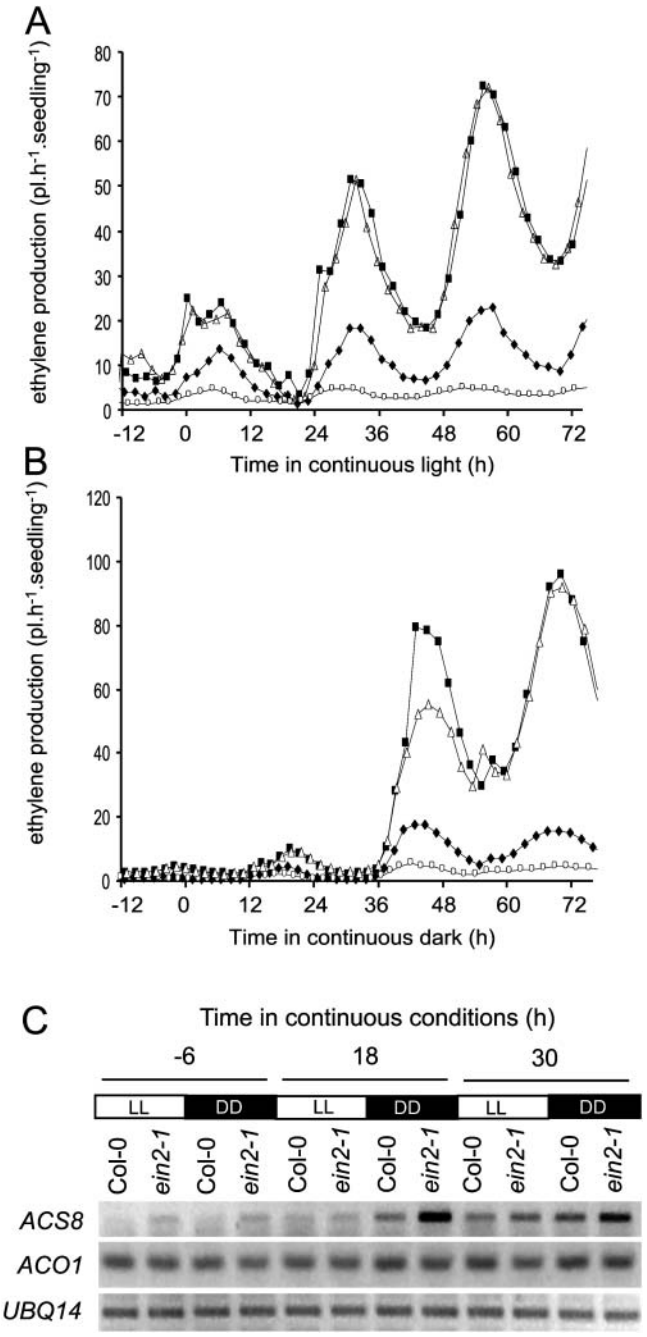

Figure 7. Ethylene emanation in ethylene-insensitive Arabidopsis mutants. Plants were 12/12 trained for 6 d and transferred to continuous light (A) or continuous dark (B). Data for a representative experiment are shown. The experiments were repeated at least three times. Black squares, *etr1-3*; white triangles, *ein2-1*; black diamonds, *ein4-1*; white circles, Col-0. C, RT-PCR analysis of ethylene biosynthesis and *UBQ14* control genes in *ein2-1* and Col-0 plants. Seedlings were entrained under LD (12, 12) for 6 d and then transferred to continuous conditions. Numbers indicate the time (in hours) after transfer to continuous conditions. LL, Constant light starting at time 0; DD, Constant dark starting at time 0.

higher ethylene production in etiolated (skotomorphogenic) compared to light-grown *eto2* seedlings (Kieber et al., 1993). In this mutant, ACS5 is hyperstable and consequently may be the main cause for ethylene overproduction (Chae et al., 2003). These observations also indicate that there is a separate input of light and the circadian clock into ACC synthase gene transcription. Although our data indicate a correlation between transcript levels of some *ACS* genes and ethylene production, it remains possible that regulation of ethylene biosynthesis occurs at the level of protein modification.

Together, the data suggest that, as in other organisms, control of vegetative ethylene production in Arabidopsis is predominantly regulated at the level of ACC synthesis.

However, there are situations in which *ACO* regulates ethylene production. When ACC is supplied in high amounts to Arabidopsis seedlings that were transferred to continuous dark, we observed a gradual decrease in ethylene production, contrasting with the situation in continuous light. This response was highly similar to the one observed in *C. rubrum* (Machackova et al., 1997). In our experiments, the decrease in ethylene emanation coincided with a decrease in transcripts of putative *ACO*s. Therefore, ACOs may limit ethylene biosynthesis in this situation.

Feedback Control of Ethylene Biosynthesis Coincides with ACS8 Transcript Accumulation But Does Not Involve the Circadian Clock

Using accumulation of ethylene in gas-tight vials, it was shown previously that ethylene-insensitive mutants overproduce ethylene (Guzman and Ecker, 1990; Roman et al., 1995). However, the mechanism behind it remains unknown. Some exaggerated phenotypes result from defects in temporary arrest in responses that are usually under circadian regulation. For instance, a lack of growth arrest at subjective dawn in *elf3* mutants and *CCA1* overexpressors overrules circadian control of hypocotyl elongation, thus resulting in a long hypocotyl phenotype. We found that the ethylene-insensitive mutants *ein4-1*, *etr1-3*, and *ein2-1* overproduced ethylene but did not disturb the rhythm. Therefore, feedback control of ethylene biosynthesis does not involve the circadian clock.

Feedback control of ethylene production was previously suggested to be dependent of the activity of biosynthesis enzymes rather than gene transcription (Lee et al., 1996). However, we have shown an increase in the transcript of *ACS8* in plants that are mutated in EIN2, a component that acts downstream of the ethylene receptors (Fig. 7). Therefore, it is possible that this gene is a main control point for feedback regulation of ethylene biosynthesis. At the same time, *ACS8* is also the most clearly circadian-regulated ACS (Fig. 4). A previous microarray study indicated that among the ACC synthases, *ACS8* is 20-fold up-regulated by exogenous auxin, which is 3 to 4 times more than

the classical auxin-responsive *ACS6* and *ACS4* genes (Abel et al., 1995; Vahala et al., 1998; Tian et al., 2002). It may be that the fluctuation of *ACS8* is dependent of the circadian regulation of auxin content, since they both peak at subjective midday (Jouve et al., 1999). Analysis of the *ACS8* promoter region showed that the latter contains an element (CAANNNNATC) that is necessary for circadian regulation of light harvesting complex proteins (chlorophyll *a*/*b*-binding proteins [CAB]) in tomato (Piechulla et al., 1998). Moreover, in Arabidopsis, *CAB* gene expression has a daylength-dependent phase shift similar to the phase shift in ethylene production (Millar and Kay, 1996; Fig. 1C), which may imply a common regulatory mechanism. *ACS8* may thus be a direct target of the circadian clock. Also, transcripts of *ACS4* and *ACS6* do not show a rhythmic pattern, which could indicate that these genes are under other control mechanisms than auxin alone. However, if this is not the case, it is less likely that auxin is at the basis of the rhythms in *ACS8* transcript accumulation.

Together, the data indicate that *ACS8* transcript levels are controlled by light and shade (Vandenbussche et al., 2003), by auxin (Tian et al., 2002), by the circadian clock, and by ethylene signaling. Furthermore, the ACS8 protein belongs to a clade of ACC synthases that contain a C-terminal motif that is thought to be responsible for protein stability (Chae et al., 2003). Nevertheless, a C-terminal T-DNA insertion in the *ACS8* gene, which deletes 15 amino acids at the C terminus, did not cause a change in ethylene production. A similar mutation in *ACS5*, which abolishes the C-terminal 12 amino acids (named *eto2*), causes hyperstability of the ACS5 protein and severe ethylene overproduction. Considering the fact that ACS8 also has a significantly high enzyme activity (Yamagami et al., 2003), this suggests that control of ACS8 may not involve C-terminal dependent regulation of protein degradation.

The Function of Rhythmic Ethylene Production Remains Obscure

The function of ethylene rhythms has not been conclusively determined in any species. Higher mean levels of ethylene frequently correlate with greater overall cell elongation (e.g. Finlayson et al., 1998; Cox et al., 2003), and ethylene likewise increases hypocotyl elongation in light-grown Arabidopsis (Smalle et al., 1997). Consistent with this, mean ethylene production levels were positively correlated with overall hypocotyl elongation in our experiments, being wild type in the *toc1-1* mutant, which has normal morphology (Millar et al., 1995; Somers et al., 1998; Mas et al., 2003) but somewhat elevated in the *CCA1-ox* lines, which have long hypocotyls (Wang and Tobin,1998). However, maximal ethylene production around midday (Fig. 1) was out of phase with the peak of hypocotyl elongation and the increase in leaf angle, which occur 4 to 6 h later in the subjective evening (Fig. 3; Dowson-Day and Millar, 1999). Direct ethylene effects on hypocotyl growth, in contrast, are reported within 15 min (Abeles et al., 1992). Mutations that affect ethylene signaling had no effect on leaf movement rhythms or rhythmic hypocotyl growth (Fig. 3). Ethylene signaling pathways are clearly not important for the rhythmicity of elongation, though the mutations, in the case of *etr1-1*, abolish ethylene responses (Hall et al., 1999; Ouaked et al., 2003). Ethylene does

Table III. *Primer combinations for RT-PCR analysis*

Gene	Primer Set	No. Cycles	T_m
			°C
ACS2 (At1g01480)	5′-AGATCGTCGAGAAAGCATCTG-3′ 5′-GAAGAGGTGAGTGTGGTGAC-3′	30	56
ACS4	5′-GTTTACGAAGTGAAGCTCAAC-3′ 5′-GTCTCATCAATCATGTTCGCG-3′	30	56
ACS5	5′-GCGGCAAGTCTCAAGAGGA-3′ 5′-TTCTGGGCTTGTTGGTAAGC-3′	30	54
ACS6	5′-CTGAATCTATTGTCTAAAATCGC-3′ 5′-ACGCATCAAATCTCCACAAAG-3′	30	55
ACS8	5′-GTCCAGTTTCGGTCTAATCTC-3′ 5′-ATAGGTGTCTCATGTCAACCC-3′	28	55
ACS9	5′-TCGGTTTACCAGGTTTTCGC-3′ 5′-ACACGAGTTTCTTCTGACGAA-3′	28	55
ACS10	5′-ACAGGCAGAGATTGCAGAG-3′ 5′-ACTGAAACAGATACGGAACC-3′	30	55
ACO (At5g63600)	5′-CCTGTCTACTGAAAACCCTC-3′ 5′-GTCTCCTTGAACAATTCATCA-3′	30	56
ACO (At1g04350)	5′-GCATTCACTAAAATTATACA-3′ 5′-CAAATAAGTAAACCATTTCCT-3′	28	56
ACO1 (At1g05010)	5′-GATCTGCTGTGCGAGAATCTC-3′ 5′-TAAATAACCCTTCTCTAAACC-3′	28	56
ACO2 (At1g62380)	5′-CCAGCTACTTCGCTTGTCGAG-3′ 5′-GTCTCTACGGCTGCTGTAGGA-3′	28	56

not provide significant input signals to the circadian clocks controlling gene expression, as neither ethylene treatments nor ethylene signaling mutants altered the circadian period or phase of rhythms in gene expression or organ growth. The similarity of rhythmic ethylene production across plant species argues for some adaptive value, but not all rhythms in signaling molecules are necessarily significant for signaling. Circadian regulation might also confer adaptive value through the temporal coordination of potentially conflicting metabolic functions, for example limiting ethylene biosynthesis in unstressed plants to a phase of predictably high substrate availability.

MATERIALS AND METHODS

Plant Materials

Col-0, C24, *eto2*, *ctr1-1*, *ein4-1*, *etr1-3*, and *ein2-1* lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC) or Arabidopsis Biological Resource Center (Columbus, OH). All mutants are in Col-0 background. The *toc1-1* mutant is in the *CAB:LUC* background (NASC stock N3756; Millar et al., 1995). *CCA1-ox* lines 034 and 038 in Col-0 have been described (Wang and Tobin, 1998). Seeds were sown and plants were grown under sterile conditions as described (Millar et al., 1995; Smalle et al., 1997). The T-DNA insertion line SALK_066725 was obtained from NASC. This line has the T-DNA insert in an exon at position 1,365 of 1,410 bp of the cDNA and thus is predicted to miss the last 45 bp or 15 amino acids at protein level (for more details regarding this line, we refer to the SIGNAL Web site, http://signal.salk.edu/cgi-bin/tdnaexpress?JOB=TEXT&TYPE=GENE&QUERY=At4g37770). The offspring of the received T3 seeds were checked for homozygosity by PCR amplification using two left border primers (LBa and LBb; Alonso et al., 2003) and a gene specific primer 5′-TTCCTCGGGTTCACGGTCGTG-3′.

Measurement of Rhythms

Circadian rhythms of hypocotyl extension and cotyledon movement were monitored and analyzed using FFT-NLLS, as described (Dowson-Day and Millar, 1999). The robustness of a rhythm was measured as the Relative Amplitude Error (RAE), which varies between 0 (perfect sine wave) and 1 (rhythm not significant). Circadian rhythmicity was defined as a rhythm with a period in the range of 15 to 35 h, with RAE less than the wild-type average RAE plus two SDS (cutoff in Supplemental Fig. 1 is 0.48), similarly to Hicks et al. (1996). Circadian rhythms of *LUC* luminescence were monitored by low-light video imaging essentially as described (Millar et al., 1995).

Gas Measurements

Per line approximately 300 seedlings were grown in a 10-mL vial for 3 (Fig. 1, A and B) or 6 d (all other figures) on Murashige and Skoog medium containing 3% Suc, in LD (12, 12) at 22°C, 35 μmol m^{-2} s^{-1} of photosynthetic photon flux density and 60% relative humidity, unless stated otherwise. Ethylene was measured after accumulation. Every 1.8 h, the vials were flushed at a flow rate of 1 L h^{-1} and ethylene was measured with a photo-acoustic detector (Bijnen et al., 1996). *eto2* and ACC-treated Col-0 seedlings were measured in a flow through system, without accumulation, as levels of ethylene were high enough for on-line detection.

Transcript Analysis

Seedlings were grown on Murashige and Skoog medium containing 3% Suc at 22°C in a 65% relative humidity and under 45 μmol m^{-2} s^{-1} photosynthetic photon flux density. After 6 d in LD (12, 12), they were put in continuous light. Two independent biological replicates were performed and a representative experiment is shown. Plant material was harvested at the respective time points and frozen at −80°C. RNA was prepared using Qiagen RNeasy (Qiagen, Hilden, Germany). RNA was treated with Dnase amplification grade (GibcoBRL, Life Technologies, Rockville, MD). To check the purity of the cDNA, a negative control for *ACS8* and *UBQ14* was checked by performing a PCR on a RT minus reaction.

We performed a semiquantitative analysis of steady-state transcript levels using an RT-PCR with gene specific primers. PCR mixtures were made according to the manufacturer's protocol (Invitrogen Carlsbad, CA). All PCRs were done in a Mastercycler (Eppendorf, Hamburg, Germany). Cycles were run as follows: 30″ at 95°C, 35″ at hybridization temperature (T_m), and 30″ at 72°C. A list of gene specific primers and reaction conditions is given in Table III. Separation of the PCR products was done on a 1% agarose gel. DNA was stained with EtBr in the gel. Normalization was performed after band intensity determination using ImageJ software (http://rsb.info.nih.gov/ij/).

ACKNOWLEDGMENTS

We are grateful to Dr. Alex Webb and members of the chronobiology group at the University of Warwick for numerous discussions and assistance with imaging experiments. S.C.T., L.J.L., F.H., and A.J.M. established the ethylene assay and circadian regulation in Arabidopsis; S.C.T., F.V., L.J.L., F.H., A.J.M., and D.V.D.S. tested circadian regulation in Arabidopsis mutants; Z.Y.W. and E.T. produced the *CCA1-ox* lines; M.J.D.D., S.C.T., and A.J.M. assayed the growth and luciferase rhythms; F.V., L.J.L., F.H., and D.V.D.S. tested ACC effects on ethylene rhythms; and F.V. and D.V.D.S. tested circadian gene regulation.

Received March 18, 2004; returned for revision July 29, 2004; accepted August 13, 2004.

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