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23. A multicomponent mineral assemblage of approximately constant proportions (for example, brucite + analcime + Fe-Mg smectite + olivine) is a possible alternative explanation for the observed position of the 2.3- and 2.5- $\mu\text{m}$  features and their relative strengths. However, alteration assemblages are typically compositionally variable. Especially over the  $10^5 \text{ km}^2$  considered here and in light of the 3.4- and 3.9- $\mu\text{m}$  bands, a fixed-proportion multicomponent mixture is less plausible geologically than a contribution from a single component, namely Mg carbonate.
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#### Supporting Online Material

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SOM Text

Figs. S1 and S2

Table S1

References

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## The Circadian Clock in *Arabidopsis* Roots Is a Simplified Slave Version of the Clock in Shoots

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The circadian oscillator in eukaryotes consists of several interlocking feedback loops through which the expression of clock genes is controlled. It is generally assumed that all plant cells contain essentially identical and cell-autonomous multiloop clocks. Here, we show that the circadian clock in the roots of mature *Arabidopsis* plants differs markedly from that in the shoots and that the root clock is synchronized by a photosynthesis-related signal from the shoot. Two of the feedback loops of the plant circadian clock are disengaged in roots, because two key clock components, the transcription factors CCA1 and LHY, are able to inhibit gene expression in shoots but not in roots. Thus, the plant clock is organ-specific but not organ-autonomous.

Many organisms have circadian clocks that temporally regulate their physiology and behavior and contribute to fitness (1–3). The eukaryotic clock involves gene expression feedback loops, with both negative and positive elements, and cytosolic signaling

molecules (4–7). In the model plant *Arabidopsis*, the clock mechanism is thought to include at least three interlocking feedback loops (5, 8, 9). The central loop comprises two partially redundant MYB domain transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which inhibit expression of a pseudo-response regulator TIMING OF CAB EXPRESSION1 (TOC1) (also known as PSEUDO-RESPONSE REGULATOR1, PRR1), whereas TOC1 activates expression of CCA1 and LHY by an unknown mechanism (5, 10–12). In the morning-phased loop, CCA1 and LHY activate the expression of PSEUDO-RESPONSE REGULATOR7 (PRR7)

and PSEUDO-RESPONSE REGULATOR9 (PRR9) (13, 14); the evening-phased loop involves TOC1 and GIGANTEA (GI) (see legend to fig. S12 for further information). These conclusions are based on experiments using whole seedlings grown in the presence of sucrose, without consideration of organ specificity. Yet, one major function of the plant clock involves the temporal partitioning of metabolic pathways via the control of output gene expression (15), and metabolism is inherently organ-specific. We therefore analyzed the circadian clock separately in shoots and roots of mature, hydroponically grown *Arabidopsis* plants (16).

Following transfer of plants from 12 hours light/12 hours dark (LD) to constant light (LL), *LHY* and *CCA1* transcripts continued to oscillate in both shoots and roots for three full cycles, with some damping (Fig. 1A and fig. S1), as determined by quantitative real-time reverse transcription polymerase chain reaction (qPCR). Notably, the period was some 2 hours longer in roots than in shoots; analysis of LHY protein (fig. S2) gave a similar result. *PRR9* and *PRR7* transcripts oscillated in both organs, with the time of peak expression later in roots than in shoots (fig. S3). *TOC1* transcripts in shoots oscillated in LL, in antiphase to those of *CCA1* and *LHY*, as expected. In marked contrast, *TOC1* transcripts in roots dipped slightly during the first subjective day in LL, then remained at a high level without oscillations (Fig. 1B and table S1). In shoots, oscillations in TOC1 protein were detectable for at least two cycles, whereas in roots TOC1 was present, with little variation, for 72

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hours in LL (fig. S2). The differences in *LHY*, *CCA1*, and *TOC1* expression between shoots and roots were also observed with microarray analysis (fig. S4). Furthermore, the other genes implicated in the central oscillator, namely *GI*, *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*), and *PSEUDO-RESPONSE REGULATOR 3* and *5* (*PRR3* and *PRR5*), all behaved similarly to *TOC1*; their transcripts oscillated in shoots but not in roots (fig. S5). Analysis of qPCR data for genes in the central clock with the modified cosinor analysis program COSOPT (17) confirmed that only *CCA1*, *LHY*, *PRR7*, and *PRR9* behaved rhythmically in roots and that the period in roots was longer than that in shoots (table S1). We also studied expression patterns in constant darkness (DD) (fig. S6). Oscillations in *CCA1* and *LHY* transcripts were detectable in both

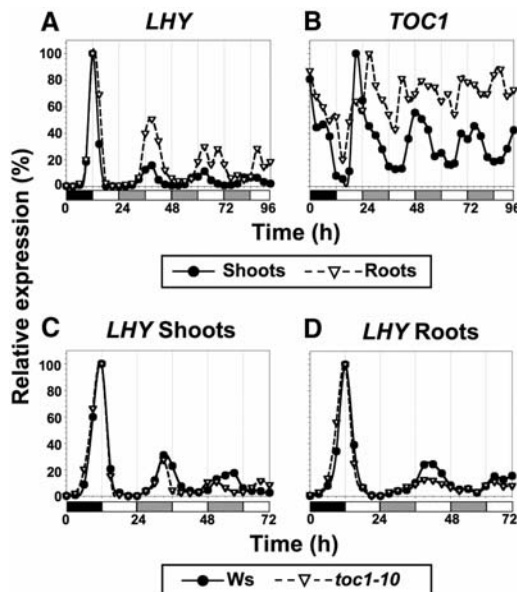
shoots and roots for three cycles. In shoots, the period was appreciably longer in DD than LL, in accord with Aschoff's rule (18), but in roots the periods in LL and DD were similar (tables S1 and S2). In DD, *TOC1* transcripts showed a low amplitude rhythm in shoots, but no clear rhythmic behavior in roots.

These data show that, in roots, the morning-phased loop of the clock operates, but the genes of the central and evening-phased loops are decoupled from oscillations in *CCA1* and *LHY* expression. To determine whether *TOC1* is necessary for the clock in roots, we examined the *toc1-10* null mutant, which has a short-period phenotype in seedlings (8). Compared with the wild-type (*Ws-0*), the *toc1-10* mutation shortens the period of transcript oscillations for both *LHY* (Fig. 1, C and D) and *CCA1* (fig. S1) in shoots, but not in roots. Thus *TOC1* does not contribute to the period of the root clock in mature plants. Consistent with previous work on seedlings (19), the phase of the circadian rhythm in *Ws-0* shoots, as judged by the timing of maximum expression of *LHY*, is earlier than that in *Col-0*. However, this is not observed in roots, again pointing to a fundamental difference in clock mechanism between the organs. It is surprising that the decoupling of the central and evening-phased loops from the morning-phased loop in roots requires *PRR7* and/or *PRR9*, as both *TOC1* and *GI* transcripts regain rhythmicity in roots of the *prp7*, *prp9* double mutant (fig. S7).

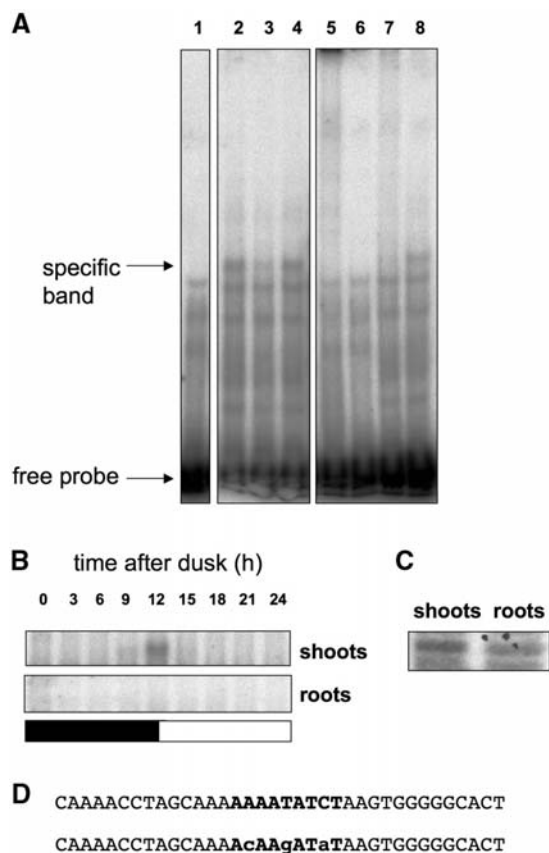
The evening-phased expression of many *Arabidopsis* genes, including *TOC1*, *GI*, other central clock genes, and many output genes such as *CHALCONE SYNTHASE* (*CHS*), is thought to be caused at least partly by the binding of the morning-expressed proteins *CCA1* and/or *LHY* to evening elements (EEs) (the sequence AAAATATCT) in the promoters of these genes, resulting in inhibition of expression (15). We therefore analyzed the expression of output genes regulated by EEs. For those genes expressed in both shoots and roots, transcript abundance in LL was rhythmic in shoots but not in roots (fig. S5), and root transcript levels were much closer to the corresponding peak values in shoots than to the trough values. A previous study using seedlings grown in the light on sucrose (20) reported that the *toc1-1* mutation altered the period of *CHS* expression in roots. However, *CHS* is not expressed in mature roots grown hydroponically (fig. S5), and the roots of plants grown in this way evidently differ physiologically from those of light-grown seedlings. For *TOC1* (Fig. 1) and *GI* (not shown), root transcript levels in LL defaulted to the highest level seen in LD cycles. Thus, our data suggest that *CCA1* and *LHY* do not cause EE-mediated inhibition of gene expression in mature roots. This behavior is not confined to *Arabidopsis*, because circadian expression of the soybean protein kinase gene *GmPPCK4* was observed in shoots but not in roots (21).

To test the hypothesis that *CCA1* and *LHY* do not cause EE-mediated inhibition of gene

**Fig. 1.** *TOC1* does not contribute to the root clock in constant light. (A and B) Gene expression in (A) *LHY* and (B) *TOC1* was monitored over 24 hours in LD and then 72 hours in LL in *Arabidopsis Col-0* by qPCR in shoots and roots. Estimates of period and the probability that the best-fit rhythm has a significant amplitude were made with COSOPT and are given in table S1. (C and D) *LHY* expression was monitored in (C) shoots and (D) roots in *Arabidopsis Ws-0* and *Arabidopsis toc1-10* by qPCR over 24 hours in LD and then 48 hours in LL. The levels of *LHY* transcripts in *Ws-0* and *toc1-10* were similar. Black bar, dark; white bars, light; day or subjective day; hatched bars, light, subjective night.



**Fig. 2.** Shoots, but not roots, contain an EE-binding protein expressed around dawn. (A) Specificity of the probe. Lane 1, labeled probe alone; lane 2, labeled probe plus shoot extract prepared at dawn; lane 3, as lane 2 plus unlabeled competitor probe; lane 4, as lane 2 plus unlabeled mutant competitor; lane 5, as lane 1 plus antiserum to *LHY* diluted 1:100; lane 6, as lane 1 plus antiserum to *LHY* diluted 1:1000; lane 7, as lane 2 plus antiserum to *LHY* diluted 1:100; lane 8, as lane 2 plus antiserum to *LHY* diluted 1:1000. (B) EE binding across a diurnal cycle. Shoot and root samples harvested at 3-hour intervals in an LD cycle were incubated with labeled probe. White bar, day; black bar, night. (C) *LHY* doublet bands (see also fig. S10) from shoot and root samples harvested at dawn and prepared for mobility shift analysis are shown by Western blotting. (D) Sequences of the probes: top, wild-type *CAT3* EE probe, EE in bold; bottom, mutated probe, the altered bases are in lower case.



expression in roots, we sought to detect EE-binding proteins using mobility shift assays. We used a 36-bp sequence from the promoter of the *CATALASE3* (*CAT3*) gene in a region that confers evening-phased expression (22) (Fig. 2D). Shoot extracts prepared at dawn contained a component able to bind this sequence. Binding was substantially reduced by addition of unlabeled competitor oligonucleotide but not by a mutated competitor (Fig. 2A). No binding was detected with mutated radiolabeled probe. Preincubation of extracts with LHY-specific antiserum prevented binding of the probe (Fig. 2A), but two control antisera had no effect. Antiserum to CCA1 had no effect (not shown), but this does not rule out the involvement of CCA1 in the EE-binding complex; for example, the epitopes recognized by CCA1-specific antiserum might be hidden in a putative CCA1/LHY/DNA complex. Extracts were prepared from shoot and root samples at 3-hour intervals across a normal diurnal cycle. Binding was detected in the shoot samples only around dawn, which matched the expression profile of *CCA1* and *LHY*, but no binding was detected in the root samples (Fig. 2B). The shoot and root samples prepared at dawn contained comparable amounts of LHY protein (Fig. 2C). Thus LHY, alone or in combination with other components, can specifically bind the EE in shoots but not in roots.

We, therefore, predicted that fewer genes would display rhythmicity in roots than in shoots. Indeed, applying COSOPT with the criterion for significant rhythmicity of  $p\text{MMC-}\beta < 0.05$  to microarray data, 13.7% of shoot genes were scored rhythmic, but only 3.2% of root genes. Examples of rhythmic root genes are shown in fig. S8, including the MYB genes *RVE1* and *RVE8* (At5g17300 and At3g09600, respectively). The time of peak expression of these genes, like that of *CCA1* and *LHY*, is delayed in roots relative to shoots, consistent with control by the root-specific clock.

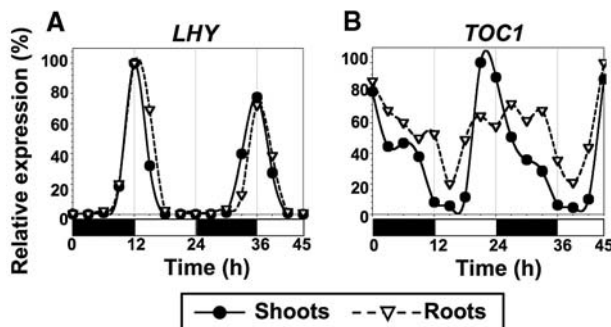
In contrast to LL, the expression profiles of *LHY* and *CCA1* in shoots and roots in LD were exactly in phase (Fig. 3A and fig. S9). *TOC1* transcripts in shoots showed a marked peak around dusk with a later shoulder and a trough during the first half of the day. However, *TOC1* transcripts in roots were relatively steady across most of the diurnal cycle, with a modest 3-fold

decline 3 hours into the day, compared with the 20-fold change seen in shoots (Fig. 3B). The abundance of LHY, CCA1 and TOC1 proteins closely followed the transcript abundances (fig. S10). Transcript profiles for *PRR9*, *PRR7*, *GI*, and *ELF4* in shoots and roots in LD were also exactly in phase, though *ELF3* transcripts oscillated only in shoots (fig. S9). Thus, the expression of several clock genes is synchronous in shoots and roots in LD but not in LL.

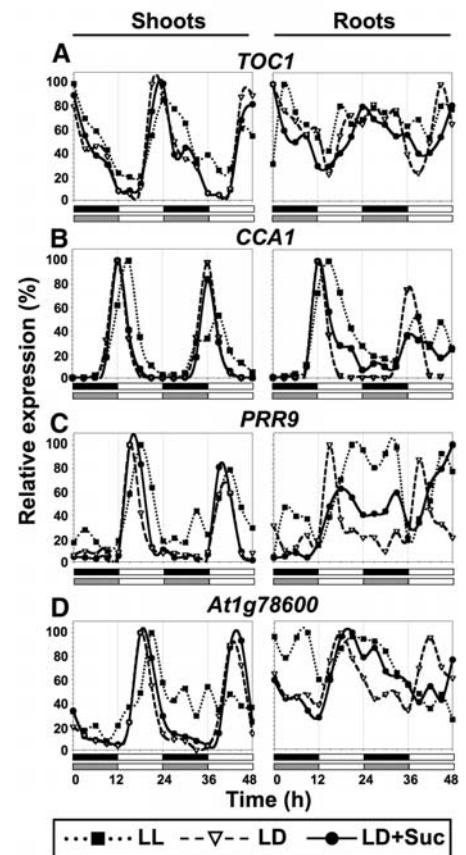
This finding demonstrates that a signal is transmitted between the organs in LD conditions, probably from shoots to roots. It seems unlikely that the signal is direct perception of light by the roots because the expression of *CCA1* and *LHY* commences before dawn in LD cycles. We reasoned that the signal might be related to shoot metabolism. We first tested whether the addition of sucrose to the hydroponic medium could selectively affect the root clock (Fig. 4). Provision of 2.5 mM sucrose at dusk in LD delayed and extended the next expression of *CCA1* and *PRR9* in roots, but not in shoots, and resulted in an expression pattern like that observed in LL (Fig. 4, B and C). A similar effect was observed with *LHY* and with the output gene At1g78600, which encodes a zinc finger protein (Fig. 4D). In contrast, this treatment did not affect the expression of *TOC1* (Fig. 4A) in roots; nor did it have a significant effect on the expression of these genes in shoots (Fig. 4). Provision of palatinose, a nonmetabolizable sucrose analog, had no effect. Thus, exogenous sucrose can prevent the entrainment of the root clock by LD cycles, acting as an anti-zeitgeber for roots. We then showed that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a specific inhibitor of photosynthetic electron transport, progressively disrupts operation of the root clock. It curtails rhythmic expression of *CCA1*, *LHY*, and *PRR9* in roots but does not affect their expression in shoots (fig. S11). Thus, a synchronizing signal between shoots and roots depends on photosynthesis and can be antagonized by sucrose. This signal may result from diurnal fluctuations in the supply of carbohydrate to the roots, although the precise mechanism remains unclear, and the existence of additional signals cannot yet be ruled out.

We draw two main conclusions from this work and propose the scheme shown in fig. S12.

**Fig. 3.** The shoot and root clocks are synchronized in diurnal cycles. Gene expression in (A) *LHY* and (B) *TOC1* was monitored over 45 hours in LD in *Arabidopsis* Col-0 by qPCR in shoots and roots. Black bars, dark; white bars, light. The data shown are from one representative experiment. Means and SEM from three replicate experiments are shown in fig. S13.



First, rhythmic expression of many of the genes associated with the plant circadian clock is not required for circadian rhythmicity in roots. Indeed, in roots, the circadian clock appears to run on only one of the loops in the current model and drives rhythmic expression of only a restricted set of genes. This organ specificity of the clock machinery is achieved through the failure of the MYB protein LHY to complex with DNA and cause EE-mediated inhibition of gene expression in roots. Whether CCA1 is involved in this protein: DNA complex remains to be determined. In mammalian systems, some clock components are dispensable in particular cell types (23, 24), but the plant root clock exhibits an extreme version of organ specificity. Second, the circadian clock is synchronized between shoots and roots only in LD cycles. Contrary to previous thinking (25), there must be communication between the clocks of different plant organs. In mammals, restricted food availability can reset the circadian



**Fig. 4.** Sucrose perturbs expression of clock genes in roots but not shoots. Gene expression in (A) *TOC1*, (B) *CCA1*, (C) *PRR9*, and (D) *At1g78600* was monitored in *Arabidopsis* Col-0 by qPCR over 48 hours in LD, in LL, and in LD after addition of 2.5 mM sucrose to the hydroponic medium at dusk. Black bars, dark; white bars, light, day or subjective day; hatched bars, light, subjective night. The data shown are from one representative experiment. Means and SEM from three replicate experiments are shown in fig. S14.

clock in peripheral tissues, but not the master clock in the suprachiasmatic nucleus (26, 27), and there is increasing evidence of links between diet, metabolism, and the clock (28, 29). Similarly, our data show that in plants a photosynthesis-related signal, possibly sucrose or a derivative, can affect setting of the clock in roots but not in shoots. In summary, the plant clock is organ-specific but not organ-autonomous.

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Materials and Methods

Figs. S1 to S14

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References

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## A Conserved Molecular Framework for Compound Leaf Development

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Diversity in leaf shape is produced by alterations of the margin: for example, deep dissection leads to leaflet formation and less-pronounced incision results in serrations or lobes. By combining gene silencing and mutant analyses in four distantly related eudicot species, we show that reducing the function of *NAM/CUC* boundary genes (*NO APICAL MERISTEM* and *CUP-SHAPED COTYLEDON*) leads to a suppression of all marginal outgrowths and to fewer and fused leaflets. We propose that *NAM/CUC* genes promote formation of a boundary domain that delimits leaflets. This domain has a dual role promoting leaflet separation locally and leaflet formation at distance. In this manner, boundaries of compound leaves resemble boundaries functioning during animal development.

Leaves of seed plants can be simple, with a single leaf blade, or compound when divided into distinct leaflets (1, 2). Additionally, margins of both simple and compound leaves can elaborate less-pronounced incisions such as serrations or lobes. Regardless of the final shape, leaves are initiated as simple primordia from the shoot apical meristem. Primordia of

compound leaves maintain an organogenic region at their margin from which leaflet primordia emerge (1, 2). Two different pathways have been recruited to promote this organogenic activity during the multiple independent origins of compound leaves in seed plants. One pathway involves expression in the primordia of compound leaves of class 1 homeodomain *KNOTTED1*-like (*KNOXI*) transcription factors that were initially identified for their role in maintenance of meristem identity (3–5). This pathway is active in a wide range of flowering seed plants, including *Solanum lycopersicum* and *Cardamine hirsuta*. A second pathway involving the *UNIFOLIATA* (*UNI*) gene is found in *Pisum sativum*, which does not express *KNOXI* genes in the leaf primordium. *UNI* encodes a member of the *LEAFY* (*LFY*) family of transcription factors, initially identified for its role in floral meristem identity (6, 7). Despite progress in understanding what promotes the organogenic potential of compound leaves, the mechanistic basis of leaflet formation and delimitation is less clear. The generation of activity maxima of auxin, a small indolic hormone, is

one such mechanism that facilitates initiation and separation of both leaves at the shoot apical meristem and leaflets from the rachis (8–10). Other key regulators of organ initiation and delimitation are the *NAM/CUC3* genes, which are members of a large evolutionarily conserved family of plant transcription factors that are subdivided into *NAM* (*NO APICAL MERISTEM*) and *CUC3* (*CUP-SHAPED COTYLEDON3*) clades (11–14). They are expressed in the boundary of organ primordia, where they repress growth to allow organ separation (15). In addition, they are involved in meristem establishment via their activation of *KNOXI* expression (16).

Because previous work showed that *AtCUC2* is required for *Arabidopsis* leaf serration (17), we hypothesized that *NAM/CUC3* genes could have a broader role in leaf dissection. To test this hypothesis, we analyzed the function of *NAM/CUC3* genes in a selection of five eudicots with compound leaves (*Aquilegia caerulea*, *S. lycopersicum*, *S. tuberosum*, *C. hirsuta*, and *P. sativum*) that show contrasting phylogenetic positions, genetic controls and patterning of leaflet development, dissection of leaflet margins, and leaflet specialization (Fig. 1, A, E, I, M, and R, and fig. S1) (18). We cloned 11 *NAM/CUC3* genes from these species, and phylogenetic analysis showed that they group either into *NAM* (*AcNAM*, *SINAM*, *StNAM*, *PsNAM1*, *PsNAM2*, *ChCUC1*, and *ChCUC2*) or *CUC3* (*AcCUC3*, *StCUC3*, *PsCUC3*, and *ChCUC3*) clades (fig. S2). The *NAM/CUC3* genes had a typical expression pattern in the boundary domain at the base of organ primordia, a pattern that is complementary to the cell proliferation marker *HISTONE H4* (fig. S3). This suggested conserved roles in defining boundary and organ separation by local repression of cell proliferation.

To determine whether the *NAM/CUC3* genes have a role in defining compound leaf morphology, we examined their expression during leaf development. A similar expression pattern was

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