retained both *lon-2* alleles after sexual transformation (Fig. 2L).

Cross-progeny that lost GFP expression after sexual transformation contained only the maternal *lon-2* allele (14 animals from 5 experiments) (Fig. 2M, lane 4). In contrast, untransformed GFP-positive siblings (20 animals from 5 experiments) (Fig. 2M, lane 1) and the few transformed cross-progeny that retained GFP (7 animals from 5 experiments) (Fig. 2M, lane 3) contained both the maternal and paternal *lon-2* alleles. Thus, most sexually transformed animals appeared to have lost all or most of the paternal X chromosome.

The loss of one X chromosome was substantiated by mating transformed cross-progeny with hermaphrodites homozygous for the X-linked mutations *dpy-8* and *unc-6*. Males with two X chromosomes should have yielded 100% wild-type hermaphrodite offspring. Instead, the majority of transformed XX cross-progeny yielded approximately 50% males with the mutant dumpy (Dpy) and uncoordinated (Unc) phenotype (Table 1, males 1 to 10 and 13 to 15), as expected of XO males. Moreover, even transformed GFP-positive males (males 13 to 15) did not transmit the transgene to their progeny, suggesting that the germ line had lost an X chromosome. A few transformed animals sired offspring with distorted sex ratios (males 11 and 12), perhaps indicating that only some germ nuclei lacked an X chromosome.

As with transformation of sexual phenotype, X-chromosome loss occurred only in cross-progeny. Under all tested conditions, self-progeny, both homozygous (210 of 210, $n = 10$ experiments) and heterozygous (70 of 70, $n = 10$ experiments) for the GFP-containing X chromosome expressed GFP and transmitted it in a Mendelian fashion to progeny from self-fertilization or mating (*13*).

Our findings reveal a plasticity in the sexual phenotype and genotype of *C. elegans* that is dependent on mating. The underlying mechanisms are still unknown, but they may be related to substances produced by bacteria at specific growth phases (*16*). The extensive washes of larvae in the absence of food, for the experiments, suggest that starvation may also play a role.

The relationship between chromosome loss and sexual transformation is unclear. The preferential loss of the paternal chromosome suggests that the two X chromosomes of L1 and L2 XX cross-progeny larvae are not equivalent. It also supports earlier reports of differences in histone modifications between the hermaphrodite and male X chromosomes (*17*) that persist in the embryo (*18*). Thus, environmental signals could induce the loss of the paternal X chromosome from XX cross-progeny, resetting the ratio of X chromosomes to sets of autosomes (the X:A ratio) to cause male development. Alternatively, environmental signals may override the X:A ratio to cause sexual transformation. HER-1 (*19*) and SEX-1 (*20*), proteins in part of the sex determination pathway predicted to be involved in signal transduction, are good candidates for transducing environmental cues to sexual cell-fate decisions. A small number of cross-progeny have phenotypes that suggest disruption of the dosage compensation pathway in log-phase medium (*13*), indicating that bacterial metabolites may alter the activity of genes in this pathway. Proteins of the dosage compensation pathway are homologous to those that regulate chromosome segregation (*21*) and are therefore good candidates for effecting X-chromosome loss.

The selective advantage of generating excess males on log-phase OP50 is unclear. An excess of cross-progeny develop as hermaphrodites in nonconditioned medium (Fig. 1C), suggesting that XO larvae may also switch sexual phenotype. Little is known of the natural history of *C. elegans*, and such transformation may be adaptive, allowing cross-progeny to optimize their sexual development to suit food availability and thus exploit more diverse ecological niches. Sexual reproduction also confers phenotypic plasticity to *Daphnia* and aphids by yielding cold-resistant eggs (*22*). We therefore propose that one short-term advantage of sexual reproduction (*3*) is to confer a greater degree of plasticity to progeny, thereby accelerating evolutionary change.


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23. We thank E. Haag, S. Kuersten, Q. Mitrovich, J. Kimble, the *C. elegans* Genetic Center, and the Goodwin, Pilgrim, and Anderson labs. Supported by grants from the National Sciences and Engineering Research Council of Canada to D.P. and the NIH (grant no. GM51836) to E.B.G.


**Supporting Online Material**

www.sciencemag.org/cgi/content/full/302/5647/1046/DC1
Materials and Methods
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12 June 2003; accepted 23 September 2003

# Enhanced Fitness Conferred by Naturally Occurring Variation in the Circadian Clock

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**Natural variation in clock parameters is necessary for the circadian clock to contribute to organismal fitness over a broad geographic range. Considerable variation is evident in the period, phase, and amplitude of 150 *Arabidopsis* accessions, and the period length is correlated with the day length at the latitude of origin, implying the adaptive significance of correctly regulated circadian timing. Quantitative trait loci analysis of recombinant inbred lines indicates that multiple loci interact to determine period, phase, and amplitude. The loss-of-function analysis of each member of the ARABIDOPSIS PSEUDO-RESPONSE REGULATOR family suggests that they are candidates for clock quantitative trait loci.**

The circadian clock optimizes the relationship, or phase angle, between biological activities and the dawn and dusk, thereby allowing specific biological activities to occur at precise times of day, or phases (*1*). Hallmarks of the circadian clock are that it persists without environmental time cues and it maintains a period of about 24 hours, which is theorized to enhance fitness (*2*). Indeed, an intact circadian clock confers greater fitness in *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Tamias striatus* (chipmunk) (*3*–

5). In *Synechococcus elongatus* (a cyanobacterium), free-running period affects fitness (*6*). The *Drosophila* clock component PERIOD displays a robust latitudinal cline in the length of a Thr-Gly repeat, where the southern variant better maintains a 24-hour period at higher temperatures (*7*). Apparently, natural variation of the circadian clock can contribute to variations in fitness within specific environments (*8*).

We surveyed clock-mediated leaf movement for natural variation of three circadian parameters—period, phase, and amplitude—in a global collection of 150 *A. thaliana* accessions. The period is the time required to progress through one circadian cycle, the phase is the time when the leaves are maximally pointing upward, and the amplitude is the vertical distance that a leaf travels. Circadian parameters were evaluated by fast Fourier transform nonlinear least squares (*9*). Figure 1A illustrates two *Arabidopsis* accessions that display both period and phase differences. As a result of these differences, the maximal leaf position occurs at two distinct times of day in conditions of continuous light and temperature. This collection of accessions shows considerable variation in period (22.0 to 28.5 hours), phase (10.1 to 20.4 hours), and amplitude (14.13 to 113.90 arbitrary units) (Fig. 1, C, E, and G; table S1). One accession, Sierra Alhambra (Sah-0), displayed no circadian clock–mediated leaf movement (statistically and visually; table S1).

Because the primary function of a circadian oscillator is to synchronize an organism with its specific environment, circadian parameters may show environmental dependence. We observed a positive relationship between the period of clock-mediated leaf movement and the recorded latitude of the *Arabidopsis* accessions, particularly at high (>50°) latitudes (fig. S1D). In *Arabidopsis*, latitudinal clines have been observed in sensitivity to light (*10*) and in rosette and seed size (*11*). The relationship between latitude and day length is curvilinear; accordingly, we calculated day length at the latitude of origin and observed a highly significant correlation between day length and period (Fig. 2A). There were no other significant correlations of any clock parameter (period, phase, or amplitude) with day length or with the latitude, longitude, or altitude of the accessions (fig. S1).

The striking correlation of period with day length suggests the adaptive significance of circadian timing, despite the recent spread and lack of genetic structure in *Arabidopsis* populations (*12*). However, this observation was made in plants that were grown at 22°C, which may be warmer than the temperature that northern accessions normally encounter. Similar period lengthening has been observed in higher latitude strains of *Drosophila auraria* (*13*). Why would an organism have a circadian clock with a period other than 24 hours? At higher latitudes, plants must continue to accurately entrain to the 24-hour day, despite the sharp increase in day length during the spring. In accordance with Aschoff's rule, pacemakers with periods of >24 hours enhance the ability to track dawn (*13*). Therefore, periods deviating from 24 hours should enhance seasonal acuity, particularly at high latitudes.

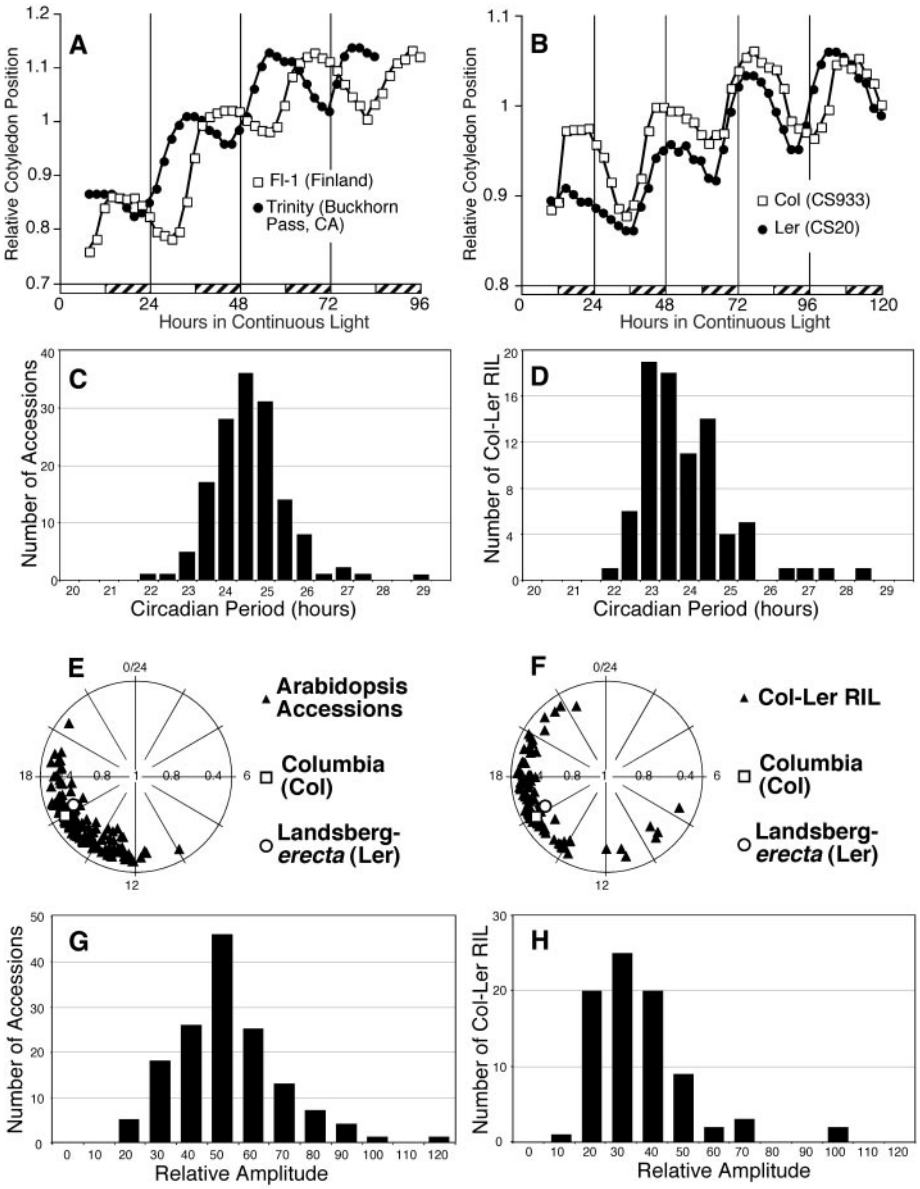


**Fig. 1.** Natural variation in the *Arabidopsis* circadian clock. (**A**) Average leaf-movement rhythms ($n$ = 6) for two *Arabidopsis* accessions, Fl-1 (period: 25.57 hours; phase: 17.63 hours) and Trinity (period: 23.79 hours; phase: 10.10 hours). (**B**) Average leaf-movement rhythms ($n$ = 6) for the Col-Ler RIL parental lines, Col (period: 24.40 hours; phase: 16.02 hours) and Ler (period: 23.16 hours; phase: 16.57 hours). (**C** and **D**) Circadian period for (C) 150 *Arabidopsis* accessions and (D) 76 Col-Ler RILs. (**E** and **F**) Phase plotted against the strength of the rhythm for (E) 150 *Arabidopsis* accessions and (F) 76 Col-Ler RILs. Phase is normalized to period length and plotted on a 24-hour circadian clock face. Strength of the rhythm, expressed as relative amplitude error (RAE), is plotted along the radius with the strongest rhythms (RAE = 0) at the outer edge of the circle and weakest rhythms (RAE = 1) at the center. (**G** and **H**) Amplitude of the leaf movement rhythms for (G) 150 *Arabidopsis* accessions and (H) 76 Col-Ler RILs. Data represent at least two independent experiments.

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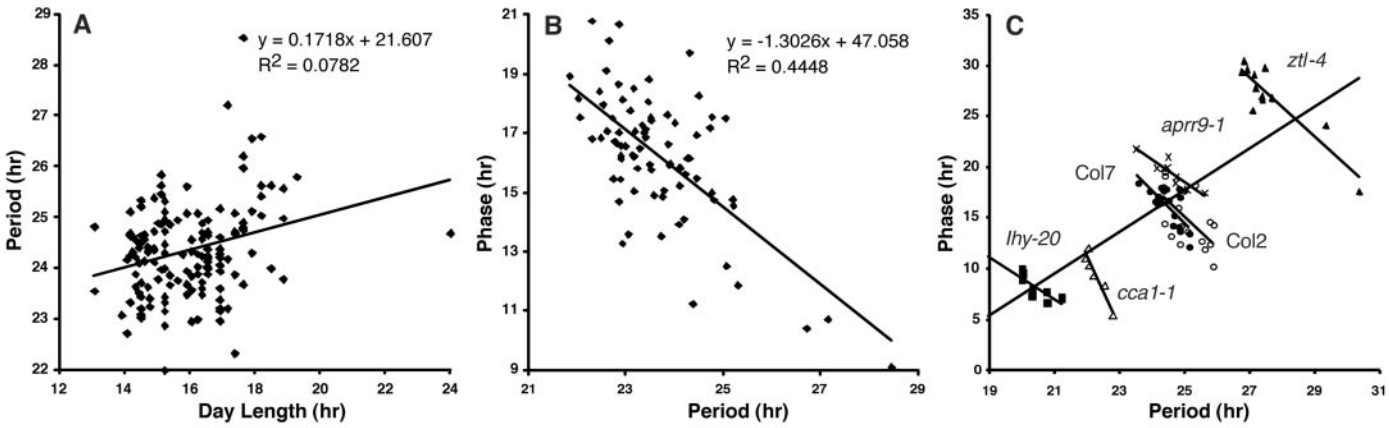


**Fig. 2.** (**A**) Period is positively correlated with day length at the geographic site of origin among 150 *Arabidopsis* accessions. Day lengths at each latitude were calculated for the vernal equinox (21 March) and at monthly intervals through the summer solstice (21 June, the data presented). In each case, the correlation of day length with period was highly significant ($P < 0.001$). (**B**) Period and phase are negatively correlated in the Col-Ler RIL. (**C**) Period and phase are positively correlated among different clock mutants (regression line with positive slope) but are negatively correlated among individuals within a genotype. The *cca1-1* was previously described (*30*). *lhy-20* and *ztl-4* are previously uncharacterized T-DNA insertion alleles. *ztl-4* (Salk 012440) has the T-DNA inserted in the second exon after the first nucleotide of codon 570. *lhy-20* (Salk 031092) has the T-DNA inserted within the third intron, after nucleotide 622 (relative to the translational start). *lhy-20* [period (±SD): 20.30 ± 0.64 hours; phase (±SD): 8.40 ± 1.46 hours], solid squares; *cca1-1* (period: 22.30 ± 0.33 hours; phase: 9.29 ± 2.34 hours), open triangles; Col isogenic wild type for *aprr9-1* (period: 24.49 ± 0.41 hours; phase: 16.13 ± 1.83 hours), solid circles; Col parent for *lhy-20* and *ztl-4* (period: 25.11 ± 0.57 hours; phase: 14.75 ± 2.76 hours), open circles; *aprr9-1* (period: 24.59 ± 0.58 hours; phase: 19.39 ± 1.43 hours), x; *ztl-4* (period: 27.64 ± 1.09 hours; phase: 26.92 ± 3.52 hours), solid triangles.

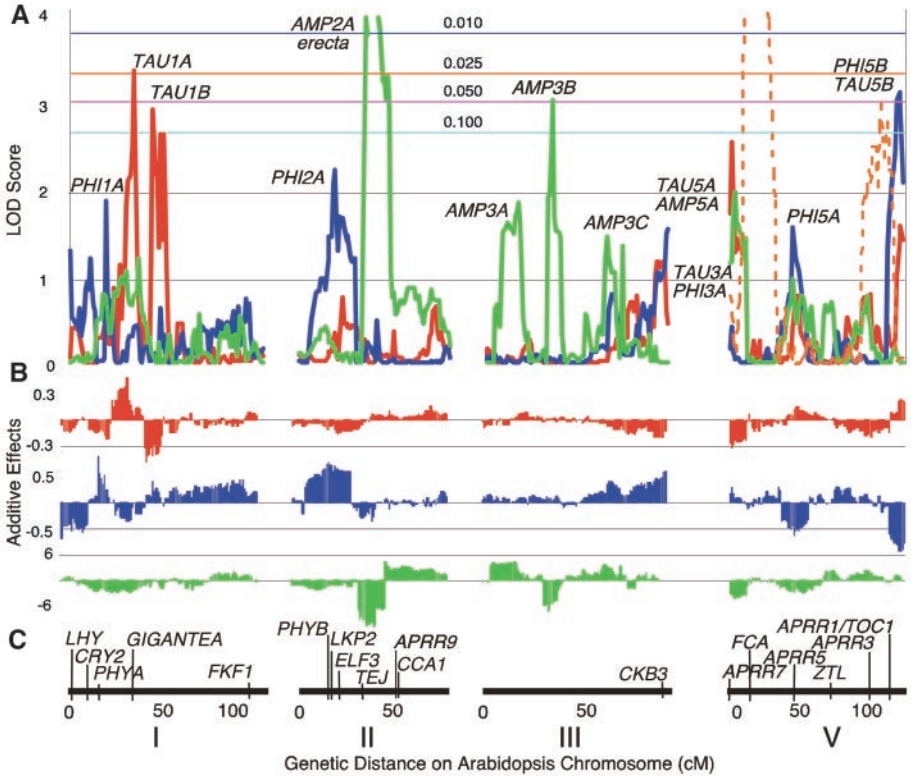


**Fig. 3.** QTL map for circadian loci in Col-Ler RILs. (**A**) Likelihood of odds (LOD) scores for circadian period (*TAU*; red), phase (*PHI*; blue), and amplitude (*AMP*; green) plotted against the *Arabidopsis* chromosomes I, II, III, and V. Confidence levels 0.100 (green), 0.050 (pink), 0.025 (orange), and 0.010 (dark blue) were established with 1000 permutations with QTL cartographer software package. (**B**) Additive effects (+, Col; –, Ler) for period (red), phase (blue), and amplitude (green). (**C**) Map positions of genes relevant to the *Arabidopsis* circadian clock. cM, centiMorgans.

How might natural selection act on period length when the period is normally entrained to a 24-hour cycle? One might suspect that the entrained phase, not the period in constant environment, would be of adaptive significance. However, we observed no significant correlation between phase and latitude or phase and day length (fig. S1, B and E). Flowering in *Arabidopsis* is photoperiodic and the transition from vegetative to reproductive growth is accelerated by long days. We suspect that the lengthened period serves to delay the onset of flowering until later in the season; this could prove advantageous in avoiding late-spring cold weather, which is more common at higher latitudes. Delayed flowering also may reduce herbivore damage if herbivore activity were high or if *Arabidopsis* were one of the few species available as attractive forage in early spring (*14*).

To identify the loci that are responsible for natural genetic variation in circadian clock phenotypes, we took advantage of the Columbia (Col)-Landsberg-*erecta* (Ler) recombinant inbred lines (RILs) (*15*). The Col and Ler parental lines displayed only slight circadian differences (Fig. 1B; tables S1 and S2). Nonetheless, the 76 Col-Ler RILs (table S2) exhibited circadian variation for period, phase, and amplitude (Fig. 1, D, F, and H) as great as that observed among the accessions (Fig. 1, C, E, and G). Transgressive segregation, the emergence of extreme phenotypes in hybrid populations that exceed the variation found in parental lines, is common in plants (*16*) and has been noted in *Arabidopsis* for both flowering time (*17*) and circadian rhythms (*18*).

Circadian period and phase were inversely correlated in the Col-Ler RILs (Fig. 2B), although not among the collection of accessions (fig. S2). We detected no correlation between the period and amplitude or between the phase and amplitude for either population

(fig. S2, C to F). The relationship between the period and phase among RILs suggests that the segregation of the quantitative trait loci (QTL) that lengthen the period is correlated with the segregation of the QTL that advance the phase. A positive correlation between the period and phase has been observed in circadian mutants under entraining conditions (*19*, *20*) and in wild-type plants grown in altered light-dark cycles (*21*). Similarly, we observed a positive correlation between the period and phase in the population means of a number of clock mutants during continuous light (Fig. 2C), but we also observed an inverse relationship between the phase and period among individual seedlings of a given genotype, including wild-type and mutant plants with altered period and phase (Fig. 2C). The slopes of these regressions were not significantly different ($F_{5,60} = 1.08$, $P > 0.35$), but the midpoints of most regressions were significantly different (analysis of covariance; $F_{5,65} = 96.55$, $P < 0.0001$).

To locate the genes responsible for the observed natural variation, we mapped the QTL in the Col-Ler RIL population (Fig. 3). QTL studies have proven useful in confirming known clock loci as well as identifying previously unknown clock loci in *Arabidopsis* (*18*) and the mouse (*22*). Five QTL [two periods (*TAU*), one phase (*PHI*), and two amplitudes (*AMP*)] were significant ($P < 0.05$), as determined by the 1000 permutation test, and were numbered on the basis of chromosomal location: *TAU1A*, *TAU1B*, *PHI5A*, *AMP2A*, and *AMP3B* (Fig. 3A). Significant QTL account for 25.15% (*TAU1A*, 13.48%; *TAU1B*, 11.67%), 12.68% (*PHI5B*), and 42.72% (*AMP2A*, 33.56%; *AMP3A*, 9.16%) of the variation observed for the period, phase, and amplitude, respectively. Also, 10 suggestive QTL overlapped with regions of the genome that contained known circadian-associated genes (Fig. 3). By testing pairwise interactions between QTL, we detected no evidence for epistasis in our model (*23*). Period and phase QTL overlapped on the bottom of chromosome V, whereas other period QTL did not overlap with phase QTL (Fig. 3A). QTL analysis of the Col-Ler RIL population is consistent with complementary gene action, in which segregation of positive and negative alleles at multiple loci underlies transgressive segregation of the period, phase, and amplitude traits in the *Arabidopsis* circadian clock (Fig. 3B).

Period QTL, *ANDANTE* (*AND*), and *ANOTHER ANDANTE* (*AAN*) have been identified at the top of chromosome V (*18*) in the region where we observed *TAU5A*. *ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 7* (*APRR7*), a member of the *TIMING OF CAB EXPRESSION 1* (*TOC1*)/*APRR* family (*24*), maps to this region (Fig. 3C). Accumulating evidence implicates the *APRR* genes in *Arabidopsis* clock function. *TOC1*/*APRR1* was initially identified on the basis of the short period of a loss-of-function allele (*25*). mRNA abundance for each *APRR* is under clock control, with peak expression at distinct times of day (*24*). Overexpression of *APRR9* shortens the period (*26*), overexpression of *APRR1* lengthens the period (*27*) or yields arrhythmicity (*28*), and overexpression of *APRR5* reduces the amplitude for multiple rhythms (*29*). To confirm that the *APRRs* function in the circadian clock and may account for natural variation between Col and Ler, we identified loss-of-function transferred DNA (T-DNA) insertion alleles for *APRR3*, *APRR5*, *APRR7*, and *APRR9* (fig. S3). All experiments with T-DNA insertion alleles were performed against isogenic siblings to account for variation resulting from trans-

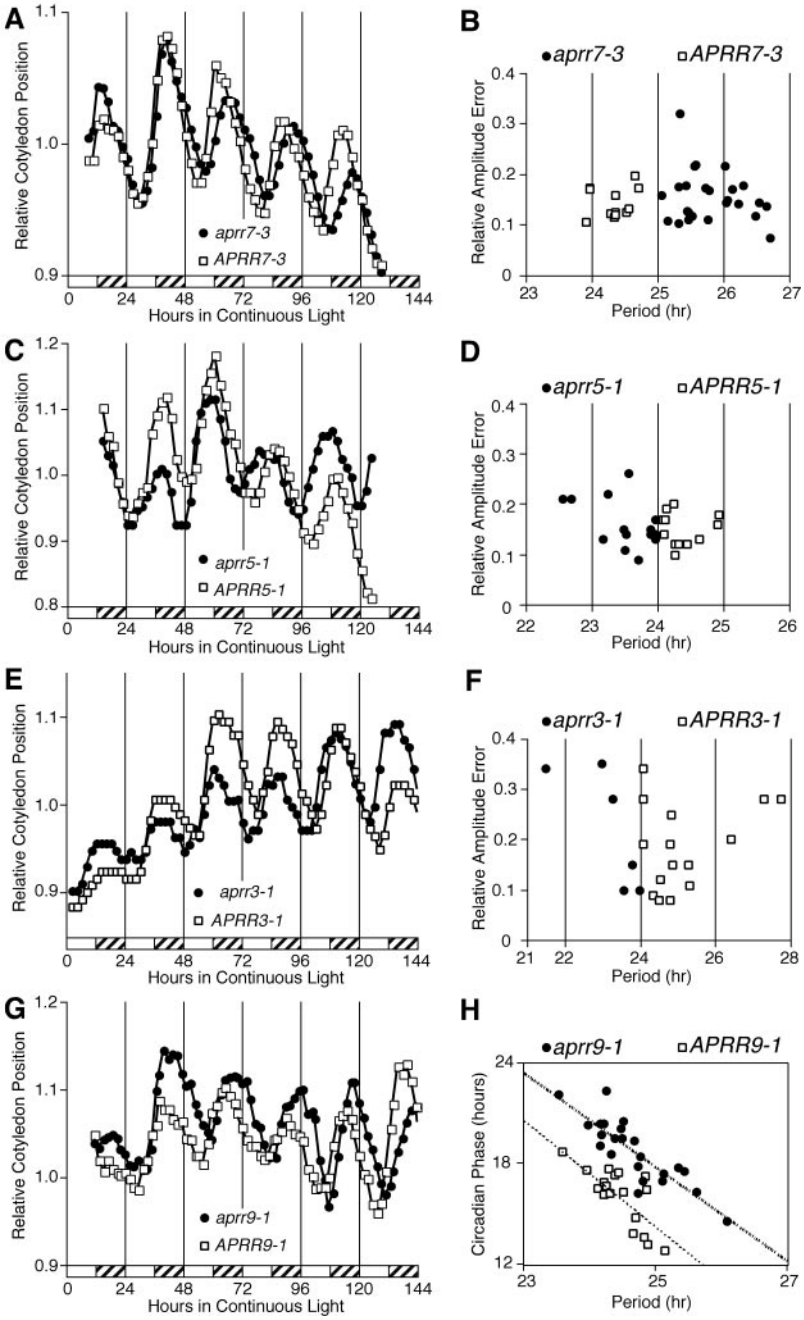


**Fig. 4.** T-DNA disruption of the *APRRs* results in defects in clock-mediated leaf movement. Clock-mediated leaf movement (**A**, **C**, and **E**) and scatter plot of period versus RAE (**B**, **D**, and **F**) for [(A) and (B)] *aprr7-3* (solid circles) and *APRR7-3* (open squares); [(C) and (D)] *aprr5-1* (solid circles) and *APRR5-1* (open squares); [(E) and (F)] *aprr3-1* (solid circles) and *APRR3-1* (open squares). Clock-mediated leaf movement (**G**) and scatter plot of period versus circadian phase (**H**) of *aprr9-1* (solid circles) and *APRR9-1* (open squares).

formation. Three *aprr7* alleles each lengthen the period of clock-mediated leaf movement by 1.5 to 2 hours without affecting the phase (Fig. 4, A and B; table S3). Two *aprr5* alleles shorten the period by 1.5 to 2 hours, again without altering the phase (Fig. 4, C and D; table S3). Similarly, two *appr3* alleles shorten the period but do not affect phase (Fig. 4, E and F; table S3). In contrast, one *aprr9* allele confers a wild-type period but a phase that lags by 4 to 5 hours (Fig. 4, G and H; table S3). Each allele affects either the period or phase, but not both, which is consistent with our observations that the period and phase are not correlated among the accessions; together, these observations show that the period and phase are under different genetic controls.

We present evidence that the period of the circadian clock in *Arabidopsis* displays a great deal of environmentally dependent natural variation. Our observation of a latitudinal cline in the period of the *Arabidopsis* circadian clock is consistent with a primary role of the circadian clock in the synchronization of an organism with its periodic surroundings. We also demonstrate transgressive segregation of clock parameters in hybrids derived from two commonly studied accessions with very similar clock parameters, which would facilitate the exploitation of new ecological niches or competition in new environments (*16*). Loci such as the *APRR* family may act as primary sources of natural variation, allowing modest complementary positive and negative effects to modulate the circadian period and phase to enhance fitness in local environments.

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31. We thank the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH), the Sendai Arabidopsis Stock Center (Sendai, Japan), and M. Jakobsson (University of Lund, Sweden) for seed stocks; M. L. Guerinot and K. Cottingham for helpful discussions; J. Borevitz for assistance with tests for epistasis; E. Tobin (University of California, Los Angeles) for providing the *cca1-1* mutation introgressed into the CO1 background; and S. Mishra for plant maintenance. Supported by grants from NSF to J.R.E. (MCB-0115103), M.A.M. (IBN-0130021), and C.R.M. (MCB-9723482 and MCB-0091008). H.J.Y. and T.R.S. were supported by Richter Undergraduate Research Fellowships, and E.L.S. was supported by a Women in Science Project Internship, administered through Dartmouth College.


**Supporting Online Material**

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Materials and Methods
Figs. S1 to S3
Tables S1 to S3
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31 January 2003; accepted 15 September 2003

# Evidence for Ozone Formation in Human Atherosclerotic Arteries

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**Here, we report evidence for the production of ozone in human disease. Signature products unique to cholesterol ozonolysis are present within atherosclerotic tissue at the time of carotid endarterectomy, suggesting that ozone production occurred during lesion development. Furthermore, advanced atherosclerotic plaques generate ozone when the leukocytes within the diseased arteries are activated in vitro. The steroids produced by cholesterol ozonolysis cause effects that are thought to be critical to the pathogenesis of atherosclerosis, including cytotoxicity, lipid-loading in macrophages, and deformation of the apolipoprotein B-100 secondary structure. We propose the trivial designation "atheronals" for this previously unrecognized class of steroids.**

Ozone is one of the most reactive chemicals known. Until our studies showed that ozone may be produced by the immune system as part of its defense strategy (*1–3*), this highly toxic oxygen allotrope had not been considered to be a product of biological systems. We reported evidence that ozone is generated during the antibody-catalyzed water-oxidation pathway and that this powerful oxidant could play a role in inflammation (*1–3*). One postulate of this work was that ozone might be generated wherever singlet ($^1\Delta_g$) oxygen ($^1O_2$*) and antibodies are juxtaposed, as occurs in most inflammatory responses.

Current concepts concerning the pathogenesis of atherosclerosis have undergone a paradigm shift: previously, the process was thought to be linked to mechanical stress in large bore arteries; now, inflammation associated with the presence of leukocytes and immunoglobulins is thought to play a central role (*4–8*). Given that all the components necessary to activate the antibody-catalyzed water-oxidation pathway are thought to be present in atherosclerotic arteries, we investigated whether ozone is produced during the evolution of human atherosclerosis and whether excised advanced plaque material could be induced to produce ozone in vitro.

We have previously demonstrated that among the oxidants thought to be associated with inflammation, only ozone oxidizes indi-

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