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Natural allelic variation identifies new genes in the *Arabidopsis* circadian system

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Summary

We have analysed the circadian rhythm of *Arabidopsis thaliana* leaf movements in the accession Cvi from the Cape Verde Islands, and in the commonly used laboratory strains Columbia (Col) and Landsberg (*erecta*) (L*er*), which originated in Northern Europe. The parental lines have similar rhythmic periods, but the progeny of crosses among them reveal extensive variation for this trait. An analysis of 48 L*er*/Cvi recombinant inbred lines (RILs) and a further 30 L*er*/Col RILs allowed us to locate four putative quantitative trait loci (QTLs) that control the period of the circadian clock. Near-isogenic lines (NILs) that contain a QTL in a small, defined chromosomal region allowed us to confirm the phenotypic effect and to map the positions of three period QTLs, designated *ESPRESSO, NON TROPPO* and *RALENTANDO*. Quantitative trait loci at the locations of *RALENTANDO* and of a fourth QTL, *ANDANTE*, were identified in both L*er*/Cvi and L*er*/Col RIL populations. Some QTLs for circadian period are closely linked to loci that control flowering time, including *FLC*. We show that *flc* mutations shorten the circadian period such that the known allelic variation in the MADS-box gene *FLC* can account for the *ANDANTE* QTL. The QTLs *ESPRESSO* and *RALENTANDO* identify new genes that regulate the Arabidopsis circadian system in nature, one of which may be the flowering-time gene *GIGANTEA*.

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Introduction

The circadian system is an endogenous biological timer controlling a wide range of rhythmic processes, all of which maintain rhythmic periods close to 24 h under constant environmental conditions (Lumsden and Millar, 1998; Millar, 1999; Sweeney, 1987). Circadian rhythms in *Arabidopsis thaliana* include rhythmic leaf movements (nyctinasty, Engelmann *et al.*, 1992) and hypocotyl elongation (Dowson-Day and Millar, 1999); the rhythmic opening of stomata (Somers *et al.*, 1998b; Webb, 1998); and the transcription of a number of genes including chlorophyll *a*/*b*-binding protein (*CAB* or *LHC*) genes (reviewed by Fejes and Nagy, 1998).

The biochemical timer that underlies circadian rhythms under constant (or 'free-running') conditions is known as the circadian oscillator. The oscillator mechanism is thought to depend on a 24 h molecular cycle in the activity of the products of a small number of genes, typified by the *frequency* gene in the fungus *Neurospora crassa*, and the *period* gene in the fruit fly *Drosophila melanogaster*. The expected number of these 'clock genes' ranges from two or three to about six, depending on the species. The first known examples were identified by mutations that altered the circadian period or abolished circadian rhythms, in *Synechococcus*, *Neurospora*, *Drosophila* and the mouse; others have been identified by interspecific homology (reviewed by Dunlap, 1993; Dunlap, 1999; Golden *et al.*, 1998; Young, 1998). A similar mutant class in Arabidopsis has been identified by direct screening for period defects (Millar *et al.*, 1995a). The *timing of CAB expression* (*toc1*) mutant, for example, has a 21 h period for *CAB* transcription and other rhythmic markers, compared to the wild-type period of 24.5 h (Millar *et al.*, 1995a; Somers *et al.*, 1998b). The two arhythmic mutants *late elongated hypocotyl*, *lhy* (Schaffer *et al.*, 1998) and *early flowering 3*, *elf3* (Anderson *et al.*, 1997; Hicks *et al.*, 1996; Millar, 1998) were identified indirectly in genetic screens for aphotoperiodic flowering. A further arhythmic mutant was identified as a transgenic line that overexpressed the gene *circadian clock-associated 1*, *CCA1* (Wang and Tobin, 1998). Circadian rhythm defects are expected in some aphotoperiodic mutants, because a circadian system is thought to be required for the measurement of day length that underlies photoperiodism (Coupland, 1998; Koornneef *et al.*, 1998; Millar, 1999; Thomas and Vince-Prue, 1996). It is not yet clear whether any of the circadian rhythm mutations identifies components of the central oscillator in Arabidopsis; *ELF3* appears to affect the oscillator indirectly

via interaction with a phototransduction pathway (Anderson *et al.*, 1997; Hicks *et al.*, 1996; Millar, 1998).

Forward genetic screens have been essential tools in the identification of circadian oscillator components, in each of the model systems studied to date (reviewed by Dunlap, 1993; Dunlap, 1999; Hall, 1995). The requirement to monitor each candidate mutant over several days makes such brute-force screens extremely labour intensive, although heavy experimental mutagenesis procedures can reduce the number of individuals that must be screened. Allelic differences can occur throughout the genomes of naturally occurring ecotypes, as well as in lines that have been artificially selected for particular phenotypic traits. Such genetic variation was first tested for effects on circadian rhythms by Bünning, working with commercial lines of the bean *Phaseolus coccineus* that he had selected for contrasting circadian periods in the rhythm of leaf movement (Bünning, 1935). Selection experiments in *Drosophila pseudoobscura*, in contrast, modified the circadian phase of developmental markers but affected the circadian period very little (reviewed by Hall and Kyriacou, 1990). The genes responsible for the rhythm alterations in these examples remain unknown. Natural isolates of *D. melanogaster* have been shown to exhibit period differences that can be accentuated under high or low temperatures (Sawyer *et al.*, 1997). Well characterized polymorphisms at the *period* (*per*) locus confer much of the variation in circadian period, and a latitudinal cline in the major *per* alleles suggests that these polymorphisms are subject to selective pressure (Costa *et al.*, 1992; Rosato *et al.*, 1997).

The analysis of naturally occurring genetic variation has been greatly facilitated by the methods of quantitative trait locus (QTL) mapping (Jansen, 1996; Tanksley, 1993). This is particularly true in 'immortal' recombinant populations such as recombinant inbred lines (RILs; Koornneef *et al.*, 1997), which allow disparate phenotypic data sets to be directly compared both to each other and to a single genetic map. The location and effect of the putative QTLs can be confirmed by the analysis of near-isogenic lines (NILs), which differ in the alleles in only a small region of an otherwise homogeneous genetic background; the most useful NILs are introgression lines in a reference background. Isolation of a single QTL in an NIL is referred to as the 'mendelization' of the QTL, because the QTL effect then behaves as a mutation at a single locus when segregation is subsequently obtained in the progeny of these lines.

Putative QTLs that affect the period of the circadian clock have been reported to date only in the mouse: these were identified based on the analysis of small numbers of RILs and have not yet been confirmed in NILs (Hofstetter and Mayeda, 1998; Hofstetter *et al.*, 1995; Mayeda *et al.*, 1996). The example of *Drosophila per* indicates that some of these QTLs may identify central components of the circadian oscillator. Recombinant inbred line populations in Arabidopsis have been used to identify putative QTLs that affect flowering time, trichome number, pathogen resistance and other traits (Alonso-Blanco *et al.*, 1998a; Buell and Somerville, 1997; Larkin *et al.*, 1996). Natural alleles that have been isolated in NILs can have phenotypic effects as strong as those of mutations identified by mutagenesis, and might represent novel genes that were not identified by conventional genetic screens (Buell and Somerville, 1997; Clarke and Dean, 1994; Sanda and Amasino, 1996). This confirms the power of QTL mapping to analyse complex genetic regulation in cases where suitable genetic variation exists.

We have mapped QTLs that affect the circadian period of leaf movement in two Arabidopsis RIL populations. Two of the putative QTLs may have been identified in both RIL populations, and three QTLs have been confirmed in NILs. Some of these loci identify new genetic components that regulate the circadian system of Arabidopsis.

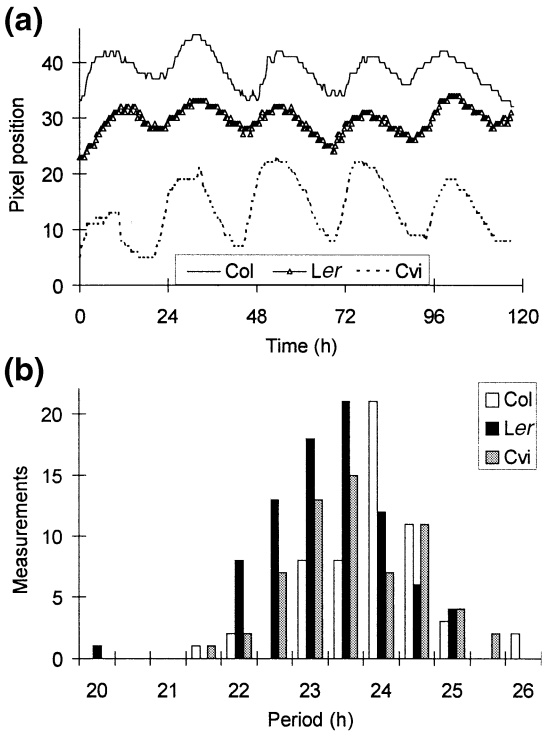

Figure 1. Leaf-movement rhythms in lines Col, L*er* and Cvi.
(a) Representative data traces showing the position of leaves of Col (solid line), L*er* (triangles) and Cvi (dashed line) obtained by image processing. Plants were maintained in constant white light and images recorded every 20 min.
(b) Distribution of circadian periods for individual data traces of Col (open bars), L*er* (solid bars) and Cvi (grey bars), such as those in (a). The total numbers of periods shown are 64 (Col), 83 (L*er*) and 62 (Cvi). Period bins are labelled with the upper bound.

Results

Leaf movements in Ler, Col, Cvi and their RI lines

We monitored the circadian rhythm of leaf position in the first pair of primary leaves in plants of accessions L*er*, Col, Cvi, in 30 of the L*er*/Col RILs (Lister and Dean, 1993) and in 48 of the L*er*/Cvi RILs (Alonso-Blanco *et al.*, 1998b). Figure 1 shows that the L*er,* Col and Cvi parents exhibited robust rhythmic movements with mean circadian periods close to 23 h in L*er*, and only slightly longer in Col (23.7 h) and Cvi (23.2 h). The variation in period estimates from individual plants was similar in all three parental lines (arithmetic SD = 0.82 to 0.89 h). The mean periods of L*er* and Col populations were significantly different, though the period distributions for individuals overlap (Figure 1b).

The waveform of the leaf movements was modified by the obvious differences in growth habit. The short petioles of *erecta* mutant leaves (Torii *et al.*, 1996) reduced the amplitude of the rhythmic movements but resulted in distinct rhythms that were easily scored (Figure 1a). The longer petioles of *ER*+ parental lines had the potential to give higher-amplitude traces, but with an increased probability that overlap between successive leaves would obscure the video imaging data.

Each of the RIL populations included transgressive lines with mean periods longer and shorter than the parents (Figure 2). The transgression was particularly marked in the L*er*/Cvi population (Figure 2a). This indicates that the parental lines contained a balance of different period-lengthening and period-shortening alleles at several loci.

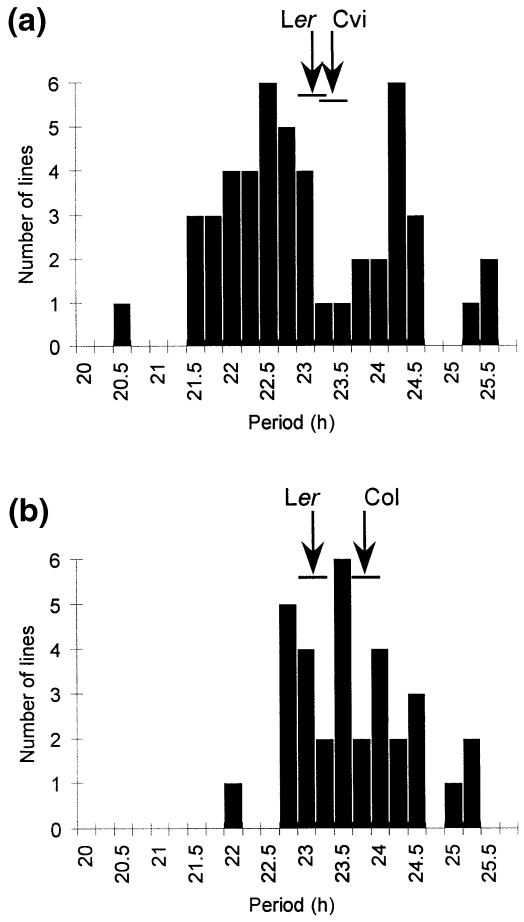

Figure 2. Distribution of mean circadian periods for RILs.
Derived from (a) Cvi/L*er* and (b) Col/L*er*. Data are means for 10–20 data traces per line. The mean periods of parental controls are marked with arrows; horizontal lines indicate 95% confidence intervals. Period bins are labelled with the upper bound.

QTL mapping for circadian period in Ler/Cvi and Ler/Col RILs

The mean phenotypic values of the RILs were used for QTL analysis in order to identify the number, effect and genetic map position of the loci controlling circadian period variation. The MQM method (van Ooijen and Maliepaard, 1996) of the MapQTL program identified four putative QTLs in the L*er*/Cvi population (Figure 3). Each locus had only a moderate effect on period: the strongest effects of 0.9–1.1 h (14–22% of the phenotypic variance) were localized towards the top of chromosome I and at the top and the bottom of chromosome V. We named these loci *ESPRESSO* (*ESP*), *ANDANTE* (*AND*) and *RALENTANDO* (*RAL*), respectively, for the effects of the Cvi alleles. A putative QTL on chromosome II was named *NON TROPPO* (*NOT*), for the weak, period-shortening effect of the Cvi allele. Together, the additive effects of these four QTLs accounted for 69% of the phenotypic variation. A genome-wide search failed to reveal any significant two-way interactions and a simple, additive model predicted the periods of the parental lines with reasonable accuracy, although other, minor QTLs and/or more complex interactions among the major QTLs may be present.

The analysis of the L*er*/Col population identified two putative QTLs. A period-lengthening effect of about 1 h produced by the Col allele was located at a similar position to *AND* at the top of chromosome V, and was named *ANOTHER ANDANTE* (*AAN*) (Figure 4). Col alleles shortened period by about 0.7 h at a locus closely linked to *RAL* at the bottom of chromosome V, whereas the *RAL*-Cvi allele lengthened the period (Figure 3). Mutants of circadian clock genes characteristically exhibit both period-lengthening and period-shortening alleles (Dunlap, 1993), so we named this QTL *GHARI* (*GHA*, 'time'). The additive effects of *AAN* and *GHA* accounted for 58% of the phenotypic variation. The data from so few lines did not lend significant support to any models incorporating epistatic interactions among the QTLs, and a simple additive model for these two loci accounted for the period difference between the parental lines and for the periods of

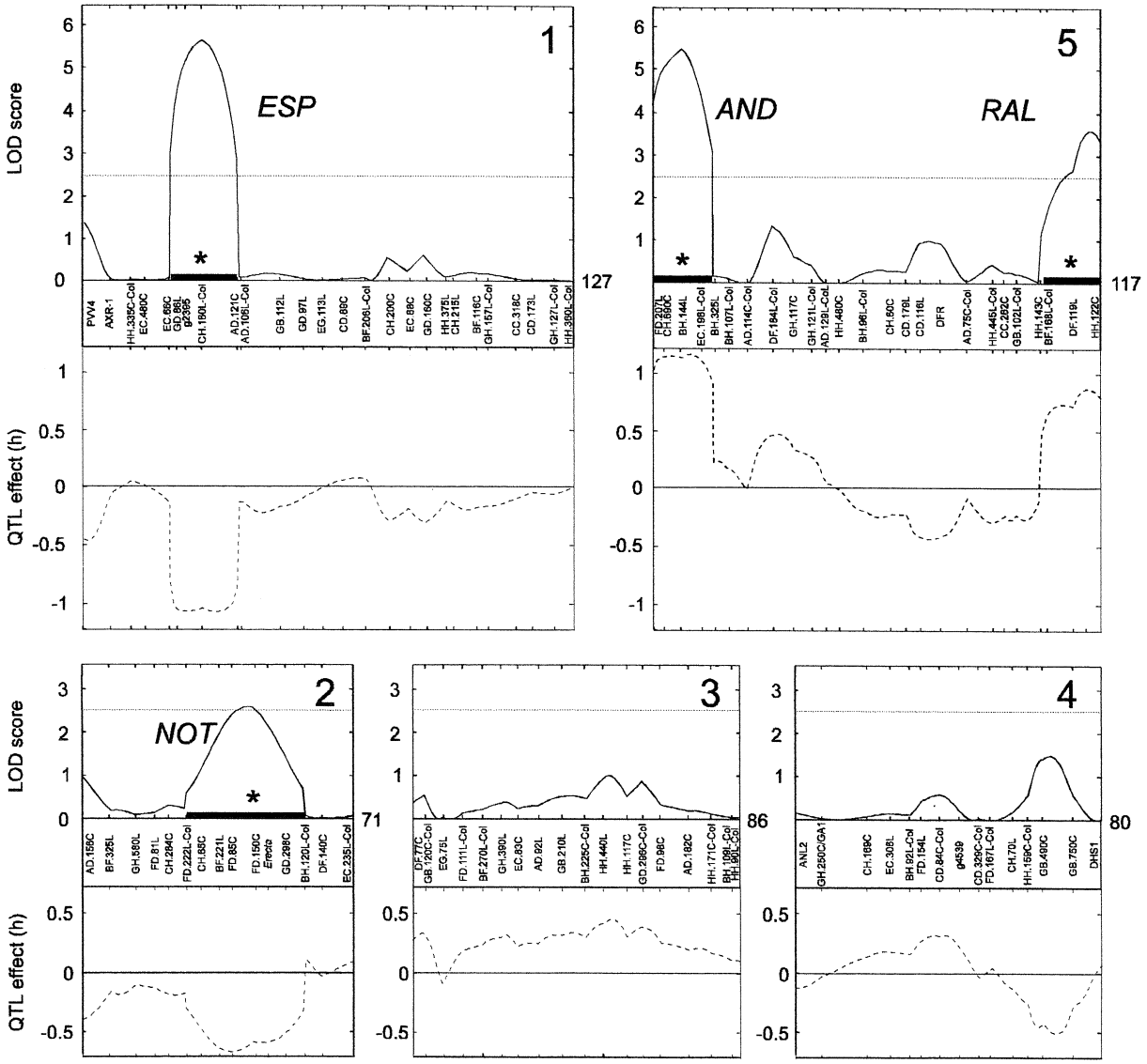

Figure 3. Genetic mapping of circadian period QTLs in the L*er*/Cvi RIL population.
The QTL likelihood and QTL effect maps for each chromosome were obtained using the MQM procedure of MAPQTL, fixing one marker cofactor per putative QTL (see Experimental procedures). Selected markers used as cofactors in the analysis are indicated with asterisks. The abscissas correspond to map positions in centimorgans; the chromosome number is indicated in the upper-right corner. Horizontal dashed lines indicate the 2.4 LOD score threshold. QTL effects were estimated as the mean difference in period between two RIL genotypic groups, comparing the Cvi allele effect to the L*er* effect (i.e. twice the additive allele effects). Putative QTL designations are indicated in italics. Filled boxes on the genetic maps span the 2-LOD support intervals at each QTL.

the most transgressive RILs. Further QTLs with small effects might contribute to the variation, but these could not be mapped with the current data set.

Characterization of the period-altering alleles

In order to confirm the identity of the *ESP, RAL* and *NOT* QTLs from the L*er*/Cvi lines, near-isogenic lines (NILs) containing small Cvi chromosomal regions introgressed into a L*er* genetic background were tested for circadian period in multiple, replicated experiments (Figure 5 and Table 1).

ESPRESSO (*ESP*: chromosome I, position » 30 cM). The position of *ESP* was confirmed by the analysis of three NILs containing Cvi segments of different length (NILs 42, 45 and 105) at the top of chromosome I. The largest region (NIL 42) shortened the period by 1.3 h, as expected if it contained *ESP*-Cvi allele (Figures 3 and 5). Cvi F_1 progeny of a cross between this NIL and L*er* had an intermediate period, suggesting that the QTL effect was semidominant

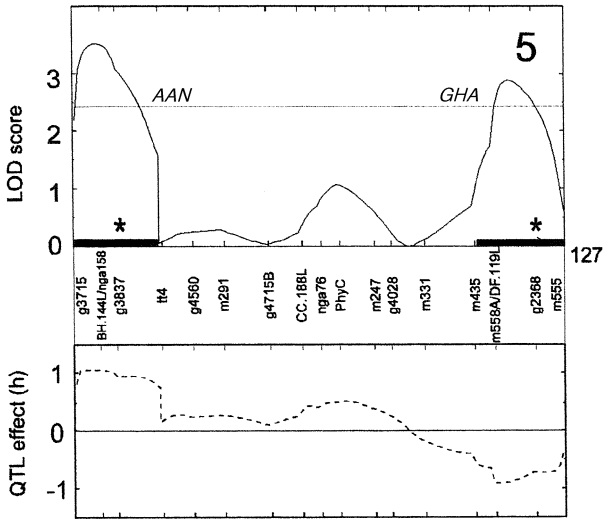

Figure 4. Genetic mapping of circadian period QTLs in the L*er*/Col RIL population.
The QTL likelihood and QTL effect maps were obtained as described for Figure 4. The two putative QTLs were both identified on chromosome 5, so no other chromosomes are shown. Selected markers used as cofactors in the analysis are indicated with asterisks. Horizontal dashed lines indicate the 2.4 LOD-score threshold. QTL effects were estimated as the mean difference in period between two RIL genotypic groups, comparing the Col allele effect to the L*er* effect (i.e. twice the additive allele effects). Putative QTL designations are indicated in italics.

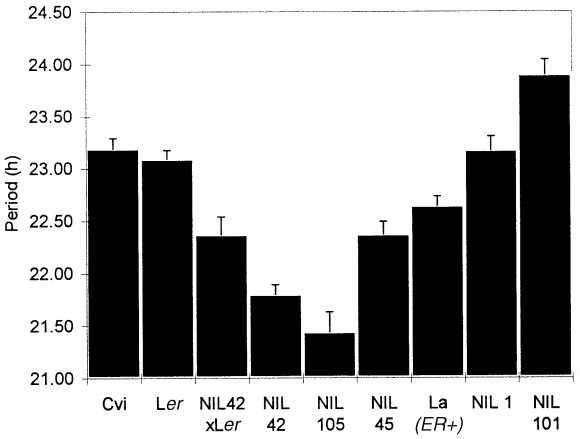

Figure 5. Effects on circadian period of isolated QTLs.
The mean circadian period of a range of NILs and the SEM were estimated as described in Experimental procedures. The lines affected the following chromosomes: chromosome I, NILs 42, 105, 45; chromosome II, Landsberg *ER+*, La(*ER+*); chromosome V, NILs 1, 101. For graphical genotypes, see Table 1.

(Figure 5). A derivative region containing only a proximal portion of the Cvi interval, below the *PHYA* marker (NIL 105), conferred the same period-shortening effect (Figure 5). A second NIL, derived from NIL 42 and containing an overlapping, distal portion (NIL 45), had a 0.7 h effect that was statistically significantly different from the effect of the proximal interval (Figure 5). The *ESP* region delimited by the proximal Cvi interval may therefore contain two loci. One of these maps above the recombination breakpoint of the distal NIL 45 (to a region between markers *PHYA* and g2395; *AtDB*) and confers the period effect of NIL 45. The other QTL maps below this breakpoint (between g2395 and AD121.C/nga392). Linkage between the two QTLs might have affected the original estimates of map position and period effects such that only the larger of these QTLs was detected.

Non troppo (*NOT*; chromosome II, position » 45 cM). The *NOT* QTL was mapped slightly above or overlapping the *ER* locus. The QTL effect could have been due to segregation for *ER+/er*, given the pleiotropic effects of *er* and the limited resolution of mapping with only 48 lines. The circadian period of a line containing *ER+* in a Landsberg background (Fransz *et al.*, 1998) was close to the predicted QTL effect (Figure 5), indicating that the *erecta* mutation probably contributes to the *NOT* QTL.

Leaf architecture and growth habit clearly differed among the RILs, so we measured the petiole of the longest leaf at the time of flowering in all 162 L*er*/Cvi RILs. The major effect on petiole length was closely linked to the *er* mutation, which shortened the petiole. The effects of *er* were confirmed in the *ER+* line (data not shown).

ANDANTE (*AND*; chromosome V, position » 8 cM). The *AND* QTL was located close to the flowering-time QTL *FLF*, which segregates in the L*er*/Cvi RIL population, and to the *FLC* gene, which segregates in the L*er*/Col RILs (Jansen *et al.*, 1995; Sanda and Amasino, 1996). L*er* behaves as if it carries a defective *flc* allele (Koornneef *et al.*, 1994; Michaels and Amasino, 1999; Sanda and Amasino, 1996; Sheldon *et al.*, 1999). *FLF* behaves similarly to *FLC*, and variation at these loci is likely to be allelic (Alonso-Blanco *et al.*, 1998a). We measured the leaf-movement period of *FLC* null mutants in the Columbia-*FRI* background (Michaels and Amasino, 1999) in order to test the effect of the *FLC* gene on the circadian system. The period of the null *FLC* alleles was significantly shorter than the parental Columbia-*FRI*, indicating that the *AND* and *AAN* QTLs may be allelic with *FLC*/*FLF* (Table 2).

RALENTANDO (*RAL*; chromosome V, position » 95 cM). The *RAL* QTL was close to the map position of the flowering-time QTL *FLH* that was previously mapped in the L*er*/Cvi population (Alonso-Blanco *et al.*, 1998a) and of the circadian rhythm gene *TOC1* (Millar *et al.*, 1995a; Somers *et al.*, 1998b). We tested the circadian period of two NILs, one of which (NIL 1) contained *FLH-Cvi* in a Cvi region of about 10 cM at the bottom of chromosome V (Alonso-Blanco *et al.*, 1998a) and the other of which (NIL 101) carried a 24 cM Cvi region above and overlapping the NIL 1 region. Only the upper Cvi region conferred the expected 0.8 h period lengthening, indicating that *RAL* is contained within the 20 cM Cvi region that is present in NIL 101, but absent from NIL 1 (Figure 5). Molecular mapping of NIL 1 indicated that it contained Cvi alleles at a locus

Table 1. Circadian period of leaf movement in Cvi, L*er* and Cvi/L*er* NILs

Name	Genotype	Period (h)	SE	*n*	*P*
Cvi		23.19	0.10	103	L*er*: > 0.2
L*er*		23.09	0.08	209	–
NIL 42 × L*er* F_1		22.37	0.16	60	L*er*: < 0.001; NIL42: < 0.01
NIL 42		21.80	0.09	137	L*er*: < 0.001; NIL45: < 0.001
NIL 105		21.44	0.19	17	L*er*: < 0.001; NIL45: < 0.001
NIL 45		22.37	0.12	76	L*er*: < 0.001
La(*ER*+)		22.64	0.09	122	L*er*: < 0.001
NIL 101		23.90	0.14	65	L*er*: < 0.001
NIL 1		23.17	0.13	93	L*er*: > 0.4

Mean circadian periods of a range of NILs were estimated and compared as described under Experimental procedures.
SEs are based on the analysis of 16 experiments with all period estimates. The significance levels of *t*-tests comparing the mean periods to other genotypes are indicated (*P*). Vertical bars indicate the genotype of the five chromosomes: open bar, L*er*; filled bar, Cvi; hatched bar, *ER*+ introgression.

Table 2. Circadian period of leaf movement in *FRI-Col* and derived *flc* mutant lines (Michaels and Amasino, 1999)

Name	Period (h)	SE	*n*	*P*
FRI-Col	24.23	0.14	53	–
flc-2	23.37	0.22	45	< 0.001
flc-3	23.44	0.17	52	< 0.001
flc-4	23.75	0.17	43	< 0.05

Period estimation and statistical comparisons were performed as described in Experimental procedures.
Significance levels of *t*-tests comparing the mean periods to the period of *FRI*-Col are indicated (*P*).

immediately proximal to the *LFY3* marker (see Experimental procedures), and also at all loci distal to *LFY3* (Alonso-Blanco *et al.*, 1998a). The original mapping of the clock mutation *toc1-1* placed it close to *LFY3* (Millar *et al.*, 1995a); recent data have confirmed this position and refined it to a location just distal to *LFY* (C.A. Strayer and S.A. Kay, unpublished results). *TOC1* therefore lies within the NIL 1 region, whereas *RAL* does not. This indicates that *RAL* is a new clock-affecting gene in Arabidopsis, which is not allelic with *TOC1* (Figure 5).

Discussion

We present an analysis of the natural variation for circadian period in the Arabidopsis accession from the Cape Verde Islands (Cvi) and the laboratory strains L*er* and Col. The method of QTL mapping and the available RIL populations allowed us rapidly and economically to locate the genes responsible for this variation. Our analysis has implicated at least three new genes in the circadian system, one of which is most likely to be the flowering-time locus *FLC*.

Homoeostasis of the circadian system

Both formal arguments (Pittendrigh, 1960; Pittendrigh, 1993) and experimental data (Highkin and Hanson, 1954; Yan *et al.*, 1998) indicate that a circadian system confers the greatest selective advantage when the period of the system approximates to the period of the environmental day/night cycle. This suggests that circadian periods should be maintained close to 24 h by stabilizing selection. The observed periods of circadian leaf movement in Arabidopsis range from about 23 h in L*er*, to close to 25 h in C24 (Dowson-Day and Millar, 1999; Millar *et al.*, 1995a), in agreement with this expectation. RILs derived from L*er* × Col and particularly from L*er* × Cvi crosses show a much wider period range (Figure 2). This transgressive variation indicates that the circadian period is regulated by different period-shortening and period-lengthening alleles at a number of loci. Our QTL analysis identified some of these loci in each population. The lengthening effects of *RALENTANDO* and *ANDANTE* in Cvi are balanced by the shortening *ESPRESSO* and *NON TROPPO* alleles, whereas in Col the lengthening of *AAN* is balanced by period shortening at *GHA*. Our RIL data are adequately explained by a simple, additive model of gene interactions, without epistasis.

Relatively rapid rates of molecular evolution have been observed in some portions of the genes encoding circadian oscillator components in other species, such as *per* and *frq* (Costa and Kyriacou, 1998; Lewis and Feldman, 1996; Merrow and Dunlap, 1994; Regier *et al.*, 1998). If this observation holds in plants, it suggests that genes encoding oscillator components may give rise to QTLs, possibly including the QTLs that we describe. The period of a free-running circadian rhythm reflects not only the operation of the oscillator, but also its modification by several mechanisms, notably by light input and temperature compensation. The light-input pathways function to

synchronize the phase of the circadian oscillator with 'local' time in the light/dark cycle, but they also modify the free-running period depending on the quality and fluence rate of ambient light (Johnson *et al.*, 1998). Photoreceptors of the phytochrome and cryptochrome families transduce red and blue light-input signals, respectively, to the Arabidopsis circadian oscillator (Millar *et al.*, 1995b; Somers *et al.*, 1998a). Loss-of-function mutants in single genes encoding the photoreceptor apoproteins have no period phenotype in white light, owing to the functional redundancy among these pathways (Millar *et al.*, 1995b; Somers and Kay, 1998). They are unlikely to be identified by any genetic screen under white light (Millar and Kay, 1997). Mutations in shared regulatory components, such as the *de-etiolated* and *constitutively photomorphogenic* genes (*DET1 and COP1*), do affect period under constant white light (Millar and Kay, 1997; Millar *et al.*, 1995b), but none of our QTLs maps to these loci.

Temperature compensation describes the homoeostasis of the circadian period over the physiological temperature range. The temperature compensation mechanisms that have been described to date are properties of central oscillator components (Hall, 1997; Liu *et al.*, 1998). These properties are modified by alleles at the *period* locus that are polymorphic in *Drosophila* strains collected in southern and northern Europe (Peixoto *et al.*, 1998; Sawyer *et al.*, 1997). The parents of the L*er*/Cvi RIL population originate at very different latitudes (about 50°N and 15°N, respectively), so this population might be expected to contain variation for temperature compensation similar to that in *Drosophila*. A subset of the QTLs that we have identified may therefore be involved in temperature compensation and possibly in central functions of the plant circadian oscillator.

Identity of the QTLs

RAL and *GHA* are the only circadian period QTLs that are closely linked to a gene previously reported to affect circadian rhythms, namely *TOC1.* The *RAL* QTL from Cvi is absent from an NIL that contains *TOC1* (Figure 5), so this QTL identifies a new gene that controls the circadian period and is located within 20 cM proximal to *TOC1*. The *GHA* QTL from Col maps very close to *RAL* but has the opposite effect on period. If *GHA* is allelic with *RAL*, then the Col, L*er* and Cvi alleles at this locus are all different. It remains possible that *GHA* is an allele of a different clock-regulating gene in this region, such as *TOC1*.

All of the QTLs described are linked to genes that control flowering time. Such co-localization at 10 cM resolution could arise by chance linkage, because about 80 loci with some effect on flowering time have been described, throughout the Arabidopsis genome (reviewed by Koornneef *et al.*, 1998; Levy and Dean, 1998). Many of the mutations that affect photoperiodic control are thought to have functions specific to photoperiodism (Coupland, 1998; Koornneef *et al.*, 1998; Millar, 1999). Such genes are not expected to affect the circadian system, although feedback from the photoperiodic system to the circadian oscillator remains possible. Two types of mutation have been shown to affect both circadian period and flowering time. Simple models of photoperiodic control suggest that the measurement of day length that underlies photoperiodism requires both photoreceptors and circadian timing (Coupland, 1998; Koornneef *et al.*, 1998; Millar, 1999; Thomas and Vince-Prue, 1996). A QTL that affects the circadian clock could alter period directly, and flowering time indirectly, via the photoperiodic mechanism. The *toc1-1* mutation has the phenotypes expected of such a clock defect (Millar *et al.*, 1995a; Somers *et al.*, 1998b). Alternatively, a QTL that affected a phototransduction pathway might give rise to flowering time and circadian period alterations as independent, secondary effects. The expected phenotypes occur in some phytochrome and cryptochrome mutants, for example, because their signalling pathways control the floral transition (Cashmore, 1998; Guo *et al.*, 1998; Whitelam and Devlin, 1997) and also mediate light input to the circadian clock (Millar *et al.*, 1995b; Somers *et al.*, 1998a).

The *ESP* QTLs on chromosome I are located overlapping or just distal to three QTLs that affect flowering time in various Arabidopsis accessions, and to the *GI* gene which participates in the photoperiodic control of flowering (Koornneef *et al.*, 1998; Levy and Dean, 1998). There is a minor QTL at the map location of *GI* in the L*er*/Cvi RILs, which overlaps with the lower *ESP* QTL and affects flowering time but showed no significant QTL effect on photoperiodic control (Alonso-Blanco *et al.*, 1998a). It is possible that this genetic variation at *GI* contributes to one of the *ESP* QTLs. The flowering-time locus *EDI* is contained within the shorter Cvi region (in NIL 45; Alonso-Blanco *et al.*, 1998a), indicating that the lower QTL at *ESP* is not allelic with *EDI. NOT,* the minor circadian period QTL on chromosome II, can be almost fully explained by the period difference between the L*er* parent and the La(*ER+*) line (Figure 5). The *erecta* mutation therefore seems to be principally responsible for the small effect on period. *ER* is strongly expressed in the petioles of 5- to 10-day-old seedlings (Yokoyama *et al.*, 1998); the *er* mutation might affect the leaf movement rhythm via indirect effects on petiole growth. The *erecta* allele present in L*er* is a point mutation that does not cause drastic rearrangement of the *ER* locus (Torii *et al.*, 1996). However, rare allelic differences remain between L*er* and *ER+* (Fransz *et al.*, 1998) so it is possible that other genes linked to *ER* might contribute to the QTL effect.

Flowering-time QTLs have also been described in a cluster around the *RAL* and *GHA* QTLs on the lower arm of

chromosome V (Koornneef *et al.*, 1998; Levy and Dean, 1998). Some of the flowering-time QTLs may be allelic with *RAL* and *GHA*, or with *TOC1* as described above. It will now be of great interest to determine whether *GHA*, like *RAL*, maps to a location distinct from *TOC1.*

The *FLC, CO*, *FY* and *HY5* genes are located overlapping or just proximal to the circadian period QTLs *AND* and *AAN* at the top of chromosome V, and flowering time QTLs have been mapped to the same region (Koornneef *et al.*, 1998; Levy and Dean, 1998). The flowering time QTL *FLF* is very likely to be allelic with *FLC*, based on its phenotypic effects and interaction with the *FLC*-interacting gene *FRI* (Alonso-Blanco *et al.*, 1998a). The approximate 95% support intervals for *AND* and *FLF* overlap at two markers, EC198L.Col and BH.325L (Alonso-Blanco *et al.*, 1998a), which are predicted to span the region containing the cloned *FLC* gene in Col (Alonso-Blanco *et al.*, 1998b; Michaels and Amasino, 1999; *AtDB*). The L*er* alleles of *AND* and *AAN* cause an identical period-shortening effect relative to the Cvi and Col alleles, respectively. If these QTLs are allelic, this suggests that Cvi and Col may share an allele that differs from L*er*. L*er* is known to carry a recessive allele of *flc*, whereas Cvi and Col carry dominant *FLC* alleles (Alonso-Blanco *et al.*, 1998a; Koornneef *et al.*, 1994; Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Three null *flc* alleles derived from mutagenesis shorten the circadian period by about 0.8 h (Table 2). The effect of the *FLC*/*flc* allelic variation is therefore sufficient to account for the *AND* and *AAN* QTLs, strongly suggesting that these QTLs identify a new function for *FLC* as a regulator of the circadian clock.

FLC is a member of the MADS-box multigene family of transcription factors and putative transcription factors (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). MADS-box transcription factors have not previously been implicated in regulating the circadian clock, although this large gene family includes genes that can affect flowering time (Chung *et al.*, 1994; Mandel and Yanofsky, 1995) and genes that are regulated by photoperiod (Hempel *et al.*, 1997). *FLC* is unlikely to have a unique function at the centre of the circadian system, because deletion of the *FLC* locus altered the circadian period by 1 h at most (Table 2). MADS-family proteins might directly control the expression of central components of the circadian clock, but with sufficient functional redundancy to compensate for the absence of FLC. Alternatively, the regulators of flowering time may affect the clock by an unexpected feedback mechanism.

QTL mapping as an efficient means to locate genes of interest

Brute-force screens for circadian rhythm mutants require each mutagenized individual to be monitored for at least several days, or up to 1 week. Almost all circadian rhythm mutants in all species have nevertheless been identified by this highly discriminating procedure (Dunlap, 1993; Hall, 1995). The leaf-movement assay for circadian period in Arabidopsis is typical of these time-consuming experiments: each set of plants is tested by 1 week of video imaging, and the number of video cameras strictly limits sample throughput. For example, we tested only 48 RILs from the L*er*/Cvi RIL population compared to the 8000 M_2 seedlings analysed in the screen that identified *toc1-1* (Millar *et al.*, 1995a). Nevertheless, we have identified several major QTLs and confirmed their effects either in NILs or by identification of similar QTLs in the L*er*/Col population. Therefore QTL analysis in Arabidopsis is an efficient means of identifying genes involved in complex regulatory networks where labour-intensive assays may be required to quantify the phenotypes of interest.

Experimental procedures

Plant materials

The L*er*/Cvi RILs (Alonso-Blanco *et al.*, 1998b), NIL 1 and NIL 45 (Alonso-Blanco *et al.*, 1998a), and the La(*ER*+) line (Fransz *et al.*, 1998) have been previously described, as have the L*er*/Col RILs (Lister and Dean, 1993). NIL 1 carries the Cvi alleles at CAPS markers T6B16 (position within 1 cM proximal to *LFY3,* C.A. Strayer and S.A. Kay, unpublished results) and MQB2, but the L*er* allele at EG7F2 (*AtDB*). NIL 101 was introgressed from the RIL CVL125 after crossing to L*er*, and carries Cvi alleles at markers EG7F2 and MQB2, but L*er* alleles at nga129 and CH.124C (Alonso-Blanco *et al.*, 1998b; *AtDB*). NIL 42 carries Cvi alleles in a 35–40 cM region at the top of chromosome I, with a recombination breakpoint above nga392 and AD.121C (Alonso-Blanco *et al.*, 1998b; *AtDB*), both of which carry the L*er* alleles. NIL 105 was introgressed from a back-cross progeny of NIL 42 to L*er*, which carried L*er* alleles at the distal markers *PVV4*, *AXR1 and PHYA* but the Cvi allele at g2395 (≈ 27 cM). All map positions refer to the L*er*/Cvi map (Alonso-Blanco *et al.*, 1998b).

Growth and imaging conditions

Seed was surface-sterilized and sown in rows on MS agar medium (Murashige and Skoog, 1962) with 3% sucrose. After cold pretreatment, the seeds were germinated under L/D (12/12) of 88 μmol m^{-2} sec^{-1} cool white fluorescent light, at 21.5°C ± 0.5°C. After 7 days, agar blocks carrying a single seedling were transferred to a 25-well square tissue-culture dish, placed vertically before a monochrome video camera. The seedlings were imaged in the Kujata system under 50–60 μmol m^{-2} sec^{-1} continuous cool white fluorescent light, at 21–22°C, as described previously (Dowson-Day and Millar, 1999; Millar *et al.*, 1995a).

Period data analysis

Leaf movement data were analysed by the Fast Fourier Transform non-linear least squares program (FFT–NLLS; Plautz *et al.*, 1997), as described (Dowson-Day and Millar, 1999). The circadian period of each RIL was estimated as the variance-weighted mean (Millar

et al., 1995b) of the most significant period within the circadian range (15–35 h), from 10 to 20 leaf-movement traces per genotype. A total of approximately 700 data traces were analysed for the L*er*/Cvi RILs, for example, with 150 000 data points contributing to period estimation. NIL data are mean periods derived from two to eight independent experiments. The data were analysed using REML (Patterson and Thompson, 1971) in the statistical package GENSTAT 5 (Payne *et al.*, 1993). REML can be thought of as a generalization of analysis of variance to unbalanced designs. Data were weighted for analysis by the reciprocal of the estimated variance of the circadian period for the trace, which was derived from FFT–NLLS (as in Millar *et al.*, 1995a). The data were analysed with each line taken as a fixed effect, and experiment and trace within experiment as random effects. Waveforms in the data that are unusually close to the sinusoidal can give very low estimated standard errors from FFT–NLLS and thus gain disproportionate weight in the variance-weighted means. The analysis was repeated with revised weights that were derived by adding 0.1 to the original, estimated standard errors, in order to reduce the effects of such rare estimates. The results of the analysis were not altered by this procedure, and the data presented are weighted using the original estimates. The significance of differences between pairs of genotypes was assessed by *t*-tests, using standard errors of the differences (SEDs) derived from REML (Patterson and Thompson, 1971), rather than the SEs of individual means given in Tables 1 and 2.

QTL analysis

The significance of the phenotypic variance in the L*er*/Cvi and L*er*/Col RIL populations was found by ANOVA to be extremely significant ($P < 0.0001$ in each case). In order to map QTLs in these populations, we used 99 and 81 previously mapped markers, respectively (Alonso-Blanco *et al.*, 1998b, *AtDB*; Lister and Dean, 1993). The markers span most of the genome at intervals of 5–15 cM. The computer programme MAPQTL (van Ooijen and Maliepaard, 1996) was used to identify and locate the putative QTLs. Single-marker analysis using the Kruskal–Wallis test, interval mapping (IM) and multiple QTL model (MQM) mapping methods identified putative QTLs in the same genomic regions, at threshold values of $P < 0.005$ (Kruskal–Wallis) and log of the likelihood odd ratio (LOD) score of 2.4 (IM and MQM). The LOD profiles were obtained with the MQM procedure, using one marker cofactor for each putative QTL except for the one under analysis. The cofactor associated with the putative QTL under analysis was removed in a window as large as the 2-LOD support interval, which therefore was of variable size depending on the QTL. Only the *NOT* QTL was significant in MQM but not in IM or single-marker analyses. Two-way interactions between QTLs were tested among all pairwise combinations of the mapping markers, using the programme EPISTAT (Chase *et al.*, 1997) at a significance threshold of $P < 0.001$.

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