Theor Appl Genet (2011) 123:397–409
DOI 10.1007/s00122-011-1592-x

ORIGINAL PAPER

Genetic architecture of the circadian clock and flowering time in *Brassica rapa*

P. Lou · Q. Xie · X. Xu · C. E. Edwards · M. T. Brock · C. Weinig · C. R. McClung

Received: 27 January 2011 / Accepted: 31 March 2011 / Published online: 20 April 2011

Abstract The circadian clock serves to coordinate physiology and behavior with the diurnal cycles derived from the daily rotation of the earth. In plants, circadian rhythms contribute to growth and yield and, hence, to both agricultural productivity and evolutionary fitness. *Arabidopsis thaliana* has served as a tractable model species in which to dissect clock mechanism and function, but it now becomes important to define the extent to which the *Arabidopsis* model can be extrapolated to other species, including crops. Accordingly, we have extended our studies to the close *Arabidopsis* relative and crop species, *Brassica rapa*. We have investigated natural variation in circadian function and flowering time among multiple *B. rapa* collections. There is wide variation in clock function, based on a robust rhythm in cotyledon movement, within a collection of *B. rapa* accessions, wild populations and recombinant inbred lines (RILs) derived from a cross between parents from two distinct subspecies, a rapid cycling Chinese cabbage (ssp. *pekinensis*) and a Yellow Sarson oilseed (ssp. *trilocularis*). We further analyzed the RILs to identify the quantitative trait loci (QTL) responsible for this natural variation in clock period and temperature compensation, as well as for flowering time under different temperature and day length settings. Most clock and flowering-time QTL mapped to overlapping chromosomal loci. We have exploited micro-synteny between the *Arabidopsis* and *B. rapa* genomes to identify candidate genes for these QTL.

Communicated by C. Quiros.

P. Lou, Q. Xie and X. Xu contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-011-1592-x) contains supplementary material, which is available to authorized users.

P. Lou · Q. Xie · X. Xu · C. R. McClung (✉)
Department of Biological Sciences, 6044 Gilman Laboratories, Dartmouth College, Hanover, NH 03755, USA
e-mail: c.robertson.mcclung@dartmouth.edu

C. E. Edwards · M. T. Brock · C. Weinig
Department of Botany, University of Wyoming, Laramie, WY 82071, USA

Present Address:
X. Xu
Hebei Key Laboratory of Molecular Cell Biology, College of Biological Sciences, Hebei Normal University, Shijiazhuang 050016, Hebei, China

Introduction

Circadian rhythms are endogenously generated and self-sustaining, and exhibit periods of ~24 h. These rhythms are temperature compensated, meaning that the period is maintained relatively constant over a broad range of temperatures (Johnson et al. 2004). Circadian rhythms are widespread from cyanobacteria to mammals and allow the coordination of biological functions with the environmental oscillation of day and night imposed by the earth's rotation (Zhang and Kay 2010). The ability to use a circadian clock to anticipate the transitions associated with light/dark cycles confers a fitness advantage as shown in cyanobacteria, *Arabidopsis*, and chipmunks (DeCoursey et al. 2000; Dodd et al. 2005; Graf et al. 2010; Green et al. 2002; Michael et al. 2003; Ouyang et al. 1998; Yerushalmi et al. 2011). In *Arabidopsis*, biomass and yield are adversely affected in clock mutants or when the environmental period deviates from 24 h (Dodd et al. 2005). This has been attributed to adverse effects on photosynthetic

rates (Dodd et al. 2005) and also to suboptimal starch utilization during the night (Graf et al. 2010). In addition, the analysis of allopolyploids and hybrids in *Arabidopsis* has suggested that clock function may contribute to hybrid vigor (Ni et al. 2009). These results impel the study of the genetics and physiology of circadian rhythms in crops to develop tools of use for increasing yield.

The *Arabidopsis* clock is composed of multiple interlocked feedback loops (Harmer 2009; McClung and Gutiérrez 2010; Pruneda-Paz and Kay 2010). Two single Myb domain transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), together with TIMING OF CAB EXPRESSION 1 (TOC1) form a central feedback loop. CCA1 and LHY also participate in the second loop as positive regulators of *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) and *PRR9,* which encode negative regulators of *CCA1* and *LHY* (Nakamichi et al. 2010). TOC1 participates in a third loop as a negative regulator of GIGANTEA (GI) and possibly PRR5 (Harmer 2009). Little is known of how variation in these components contributes to functional differences in clock function among natural populations. Several studies have identified allelic variation in clock function, including period length and temperature compensation, based on QTL analysis in *Arabidopsis* (Darrah et al. 2006; Edwards et al. 2006; Edwards et al. 2005; Michael et al. 2003; Swarup et al. 1999), and a number of known clock genes have been suggested as candidates underlying some of these QTL.

It is important to address the extent to which the circadian clock model elucidated in *Arabidopsis* will apply to other plants. Orthologs to *Arabidopsis* clock genes have been identified in a number of species, and phylogenetic analysis suggests that the common ancestor of monocots and eudicots had the components to construct clocks consisting of multiple interlocked feedback loops (McClung 2010; Takata et al. 2010).

Brassica species include a number of crops adapted to a broad range of agricultural and climatic conditions. *Brassica* and *Arabidopsis* lineages have only recently, ~43 Mya, diverged (Beilstein et al. 2010). Extensive studies on the syntenic relationships between *Arabidopsis* and *Brassica* at different levels have revealed that conserved colinearity is found in several but not in all genomic regions (Cheung et al. 2009; Kim et al. 2007, 2009; Parkin et al. 2005; Schranz et al. 2006; Trick et al. 2009). *B. rapa* orthologs of the *TOC1*/*PRR* clock genes have been identified by comparative genomics with *Arabidopsis* (Kim et al. 2007), but functional studies of the genetic and molecular bases of natural variation in circadian clock function are in their infancy. Quantitative trait loci (QTL) for cotyledon movement have been detected in *B. oleracea* (Salathia et al. 2007), and we have recently described a circadian rhythm in cotyledon movement in *B. rapa* and shown that *B. rapa* tissue culture expresses circadian rhythms in gene expression (Xu et al. 2010). Here, we identify natural variation in clock function as well as correlated effects on flowering time among natural accessions and cultivars of *B. rapa* and identify QTL for these traits. *B. rapa*, like *B. oleracea*, displays a robust circadian rhythm in cotyledon movement over a broad range of temperatures, allowing the identification of loci that contribute to period length and temperature compensation. In addition, we identify QTL for flowering time and explore the correlation between circadian clock function and flowering time.

Materials and methods

Brassica rapa cultivars, accessions and recombinant inbred lines

Genotypes used in this study include a collection of 50 *B. rapa* accessions, provided by Genetic Resources Centre (CGN) in the Netherlands that includes traditional cultivars and breeding materials from different latitudes (Fig. S1 and Table S1). In addition, eight wild *B. rapa* populations were sampled from southern California (Fig. S2). Finally, we used a *Brassica rapa* mapping population, BraIRRI (*Brassica rapa* International Reference Recombinant Inbred), consisting of 159 RILs derived from a cross between a rapid cycling Chinese cabbage (ssp. *pekinensis*) genotype (IMB211; ♀ parent) and an annual Yellow Sarson seed oil (ssp. *trilocularis*) genotype (R500; ♂ parent). The F1 generation was selfed and the progeny were advanced to the S6 generation by single seed descent (Iniguez-Luy et al. 2009). A linkage map of 1125 cM and a mean distance between marker loci of 5.7 cM was used. However, for the current work the north–south orientations of Chr. A5, A8, A9 and A10 are inverted relative to Iniguez-Luy et al. (Iniguez-Luy et al. 2009) to be consistent with the orientation defined by Parkin et al. (Parkin et al. 2005).

Plant growth conditions for cotyledon movement measurement

Seeds were sown in a standard soil mixture (Pro-mix "BX," Premier, Rivière-du-Loup, Québec, Canada) in 1.5-inch diameter pots and stratified at 4°C for 4 days to synchronize germination, then released into growth chambers with 12-h light (120 μmol m^{-2} s^{-1} white light provided by fluorescent and incandescent bulbs)/12-h dark cycles at constant 18°C temperature for 5–7 days, until most individuals had fully emerged cotyledons. Ten individual plants per RIL were grown in a completely randomized

design. For the cotyledon movement assay, white polystyrene balls were glued to the end of the cotyledon blade to improve the tracking of cotyledon position (Salathia et al. 2007), and then seedlings were transferred into a growth chamber under continuous white light at different (12, 18 or 24°C) temperatures to determine the effects of temperature on circadian parameters. Q_{10} is the increase in rate (the inverse of period) for a 10°C increase in temperature and was calculated as $Q_{10} = (1/\text{period}_{T+10})/(1/\text{period}_T)$, where period_{T+10} is the period at temperature $T + 10$ and period_T is the period at temperature T. Seedlings were imaged using a Lariion Digital Video Surveillance System (http://www.lariion.com) (Xu et al. 2010). Images were recorded every 10 min for at least 5 days. Cotyledon position was recovered from the time series images using nktrace (Onai et al. 2004) and Metamorph software (Molecular Devices).

Growth conditions and trait estimates for flowering time

One individual of each of the RILs and the two parental genotypes were grown in each of six growth chamber compartments. For all growth chambers, light intensity during light cycles was maintained at a photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1} and the vapor pressure deficit was maintained below 1.7. The growth chambers were set to the following conditions: (1) WL: warm temperature (24°C), long photoperiod (14 h/10 h light/dark cycles); (2) CL: cool temperature (12°C), long photoperiod (14 h/10 h light/dark cycles); and (3) CS: cool temperature (12°C), short photoperiod (10 h/14 h light/dark cycles). These treatments simulate conditions encountered by crop and wild populations. Each of these treatments occupied two of the six growth chambers at one time, and this was temporally replicated three times so that a total of six replicates of each genotype were grown in each of the three treatments. The treatments rotated among growth chambers such that each treatment occupied each growth chamber compartment once.

For each genotype, three seeds were planted in 360 cm^3 pots filled with 1 ml of Osmocote 18-6-12 N–P–K fertilizer (Scotts Miracle Grow, Marysville, OH, USA), and a 50/50 combination of metromix 200 soil (Sun-Gro Horticulture, Vancouver, BC, Canada) and Fafard custom mix (Conrad Fafard Inc., Agawam, MA, USA). After planting, the seeds were lightly covered with vermiculite and then cold/dark stratified for 3 days at 4°C. The pots were then placed into growth chambers set to the three treatments, with genotypes fully randomized within each chamber. Once seeds had germinated, seedlings were thinned to one plant closest to the center of the pot. Plants were watered regularly to maintain moist soil conditions.

Plants were checked daily for bolting, which was defined as the date when buds 1 mm in length were visible. Once a plant had bolted, it was checked each morning for flowering. After flowering, each plant was checked daily at 2 p.m. for the presence of a mature flower; this additional measure of flowering was collected because some genotypes aborted several buds before producing the first viable flower. To estimate phenology, we recorded planting to first mature flower (PtoF), and bolting to flowering (BtoF).

Statistical analysis

Circadian clock parameters were analyzed from leaf movement data using fast Fourier transform non-linear least squares (FFT-NLLS) (Plautz et al. 1997) in BRASS software (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). For the estimation of mean circadian periods, results were restricted to period values within the range of 18–32 h; values outside that range were considered to be non-circadian and excluded from further analysis. Phase estimates were normalized for differences in period and are presented in circadian time (CT = phase/period × 24 h). Amplitude measures the distance that a cotyledon moves in the y-axis over one period; amplitude therefore estimates the magnitude of the cotyledon movement but does not represent an estimate of amplitude of the circadian oscillator.

We carried out separate statistical analyses on the following four data sets: (1) circadian data from the CGN accessions, (2) circadian data from the wild California populations, (3) circadian data from the RILs, and (4) flowering-time data from the RILs. For each of these four data sets, we used restricted maximum likelihood to estimate the random effect of genotype on each phenotypic trait in each treatment (PROC MIXED, SAS ver. 9.2). The variance components estimated from this analysis were used to estimate broad-sense heritability for each trait, V_G/V_P, where V_G is the among-genotype variance component in each treatment and V_P is the sum of all variance components for a trait in each treatment.

We next carried out analysis of variance to evaluate the fixed effect of treatment and the random effects of genotype and the genotype × treatment interaction (PROC MIXED, SAS ver. 9.2). While a fully randomized design was used to measure circadian traits, the growth chamber experiment that measured phenological traits was spatially and temporally blocked. Chamber nested within treatment and temporal block were therefore included as an additional factor in the model for phenological traits; however, the blocking factors were never significant and are not reported further. The preceding models were also used to estimate variance components attributable to each factor and the genotypic least-square means of each trait in each

treatment, the latter of which were used in QTL mapping (see "QTL analysis" below). To determine the source of significant genotype × treatment interactions, we removed one treatment at a time from the data set and re-ran the two-way ANOVA model.

Using the genotypic estimates from above, bivariate correlations were estimated between circadian clock parameters and the geographic origin of the 50 accessions, between clock parameters and Q_{10}, and between clock parameters and flowering-time parameters (SPSS Version18).

QTL analysis

The linkage map used in this study consisted of 224 RFLP and microsatellite markers covering ten linkage groups, with an average marker density of 5.7 cM/marker (Iniguez-Luy et al. 2009). Composite interval mapping in QTL Cartographer (Wang et al. 2007) was utilized for all QTL mapping. Genome-wide significance thresholds were estimated using 1,000 permutations in QTL cartographer with a type-I error rate of 0.05. The output from QTL Cartographer was further verified using the fitQTL model in the R/QTL software package. We also performed analyses of the epistatic interactions on each trait using the scantwo procedure in R/QTL (R Development Core Team 2009). The final QTL results are graphically displayed including 1-LOD and 2-LOD support intervals using Mapchart 2.1 (Voorrips 2002). We considered QTL to co-localize when their 2-LOD support intervals overlapped.

For individual QTL, we tested for significant QTL × treatment interactions. For these analyses, we evaluated the fixed effects of treatment, the genotype at the marker nearest to each detected QTL and the marker × treatment interaction on the genotypic values of each trait (PROC GLM, SAS ver. 9.2). To differentiate the effects of temperature, for instance, on the expression of circadian QTL, these analyses were carried out for all pairwise combinations of treatments in which the QTL was detected (for example, if the QTL was detected in 12°C, we evaluated QTL × treatment interactions across 12/18°C and across 12/24°C).

Results

Natural variation in circadian clock parameters within *Brassica rapa*

To investigate the natural variation of circadian clock function within *B. rapa*, we measured clock parameters by cotyledon movement using three different genetic materials: 50 *B. rapa* accessions including breeding material and landraces, 8 wild populations collected from southern California, USA, and a population of 159 RILs derived from parents representing two subspecies, *pekinensis* and *trilocularis*.

The 50 accessions represent different morphotypes and geographic origins primarily from Europe (Table S1; Fig. S1). The 50 accessions exhibited considerable variation in circadian period at each of the three temperatures tested (Table S2), with period length ranging from ~22 to 28 h and with the majority of accessions showing a period length of 25–26 h (Fig. 1a and S3). There was very little difference in average period length between 12 and 18°C, but periods were shorter by about 1 h at 24°C (Fig. 1a; significant effect of treatment, Table S3).

Among the 50 accessions, there was considerable variation in phase, defined as the peak or acrophase of the cotyledon movement rhythm, within each treatment (Table S2), and the distributions in phase responded to temperature, with the earliest phases at 24°C and the latest phases at 18°C (Fig. 1c; significant effect of treatment, Table S3). Amplitude, defined as one-half the peak to trough distance of cotyledon movement, does not refer to amplitude of circadian oscillator cycle, but instead gives an indication of the magnitude of cotyledon movement. The amplitude was weakest at 24°C (Fig. 1b and S3), which apparently approached the upper temperature limit at which this rhythmic output could be detected. We asked if there was evidence for systematic variation in clock parameters with geographic location, but detected no significant correlation between clock parameters and either latitude or longitude.

For the eight wild populations from southern California, USA (Fig. S2), we detected significant (or marginally significant) effects of population on period at 12 and 18°C ($p = 0.04$ and 0.07, respectively), but not at 24°C (Table S2). It is also worth noting that there was substantial phenotypic variation within each population, with differences in period ranging from as little as 1.0 h to as much as 4.3 h at all three temperatures (Fig. S4).

There was considerable variation in clock parameters between the parents of the RIL collection, oil type Yellow Sarson (R500) and Rapid Cycling line (IMB211) at each of the three temperatures tested (Fig. 1d–f and S5). The period of the cotyledon movement rhythm of IMB211 was 1–2 h longer than that of R500 at each temperature. Correspondingly, RILs differed significantly in period length (Table S2), and the variation in period among the RILs showed transgressive inheritance, ranging from ~22 to 27 h (range of 5.3 h) at 12°C, from ~22 to 28 h (range of 5.8 h) at 18°C, and from ~21 to 28 h (range of 6.7 h) at 24°C (Fig. 1d and S5).

Each of the clock parameters showed a continuous distribution at all three temperatures, demonstrating multigenic control of the clock function in *B. rapa* and

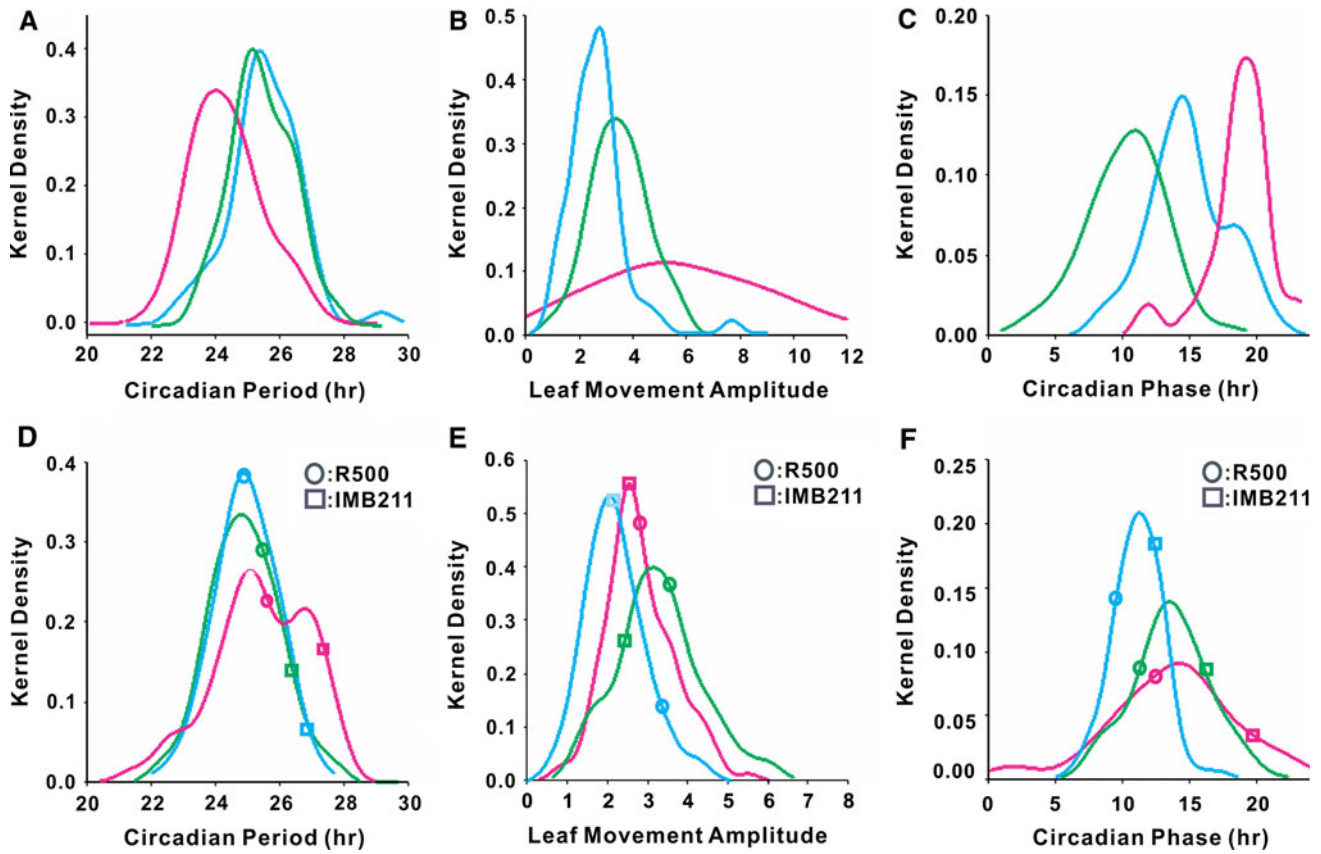


Fig. 1 Distribution of clock parameters among accessions and recombinant inbred lines. Kernel density, a non-parametric estimate of frequency (Parzen 1962; Rosenblatt 1956), of the circadian parameters period (**a**, **d**), amplitude (**b**, **e**) and phase of the oscillation (**c**, **f**), as determined by cotyledon movement, among natural accessions (**a**–**c**) and RILs (**d**–**f**). Cotyledon movement was measured at three temperatures: 12°C (*cyan*), 18°C (*green*), and 24°C (*magenta*)

suggesting that there are multiple period-shortening and period-lengthening loci (Fig. 1a, d; and S5). As noted, the IMB211 period was consistently longer than that of R500 and that of most RILs, suggesting that R500 alleles tend to shorten period. In general, the ranges in period were greater among the RILs (6.5 h) than among the accessions (5 h) or the wild California populations (4 h), which may suggest a homeostatic effect of natural selection acting against phenotypic extremes. Phase and amplitude also showed transgressive variation at all temperatures (Fig. 1e, f; and S5), but expressed values both higher and lower than the parental ones, suggesting that both parents contain loci that increased or decreased these phenotypes. For circadian phase, the IMB211 alleles tended to have a lagging (greater phase value) effect and for amplitude, while the R500 alleles tended to increase the amplitude of movement.

Natural variation in temperature compensation within *B. rapa*

We tested the responsiveness of all the *B. rapa* genotypes to three different temperatures (12, 18 and 24°C) in order to characterize temperature compensation. All the genotypes expressed robust rhythms in cotyledon movement at 12 and 18°C, but a number of lines failed to express rhythms in cotyledon movement at 24°C. Among the 50 accessions period generally shortened with increasing temperature, as alluded to above, whereas in the RILs period generally lengthened with increasing temperature. The wild California populations showed the longest period at higher temperatures of 18 and 24°C (Fig. 2).

Although period varied with temperature, all the lines examined showed strong temperature compensation. Q_{10}, the temperature coefficient, is the change in rate in response to an increase of 10°C, and is ~ 2 for typical biochemical reactions. Most of the RIL lines and accessions showed Q_{10} between 0.9 and 1.2 and the wild California populations showed a narrow range in Q_{10} of 0.9–1.0, indicating that clock function was fairly constant between 12 and 18°C (Fig. 3). Q_{10} values calculated for 12 versus 24°C and for 18 versus 24°C were consistent with those for 12 versus 18°C. Because a number of the genotypes were arrhythmic at 24°C, we have emphasized Q_{10} values calculated for the lower range of temperatures.

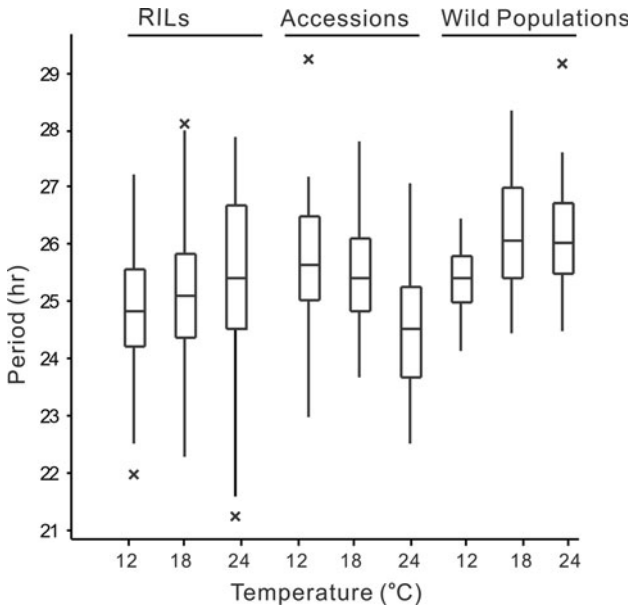


Fig. 2 Response to temperature in clock parameters of RILs, accessions and wild populations The *box* spans the interquartile range (25–75%) of the values in the variate, so the middle 50% of the data lie within the *box*, with a *horizontal line* indicating the median. *Whiskers* extend beyond the ends of the *box* as far as the minimum and maximum values. *X* indicates variate outside of boundary (95%)

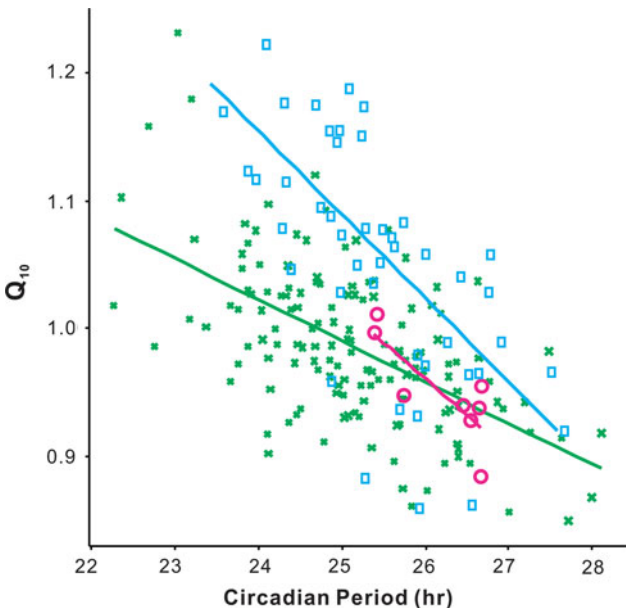


Fig. 3 Relationship of temperature compensation with period length. Temperature response (Q_{10}) of circadian period from 12 to 18°C plotted against period at 18°C. RILs *green X*, accessions *cyan squares*, wild populations *magenta circles*

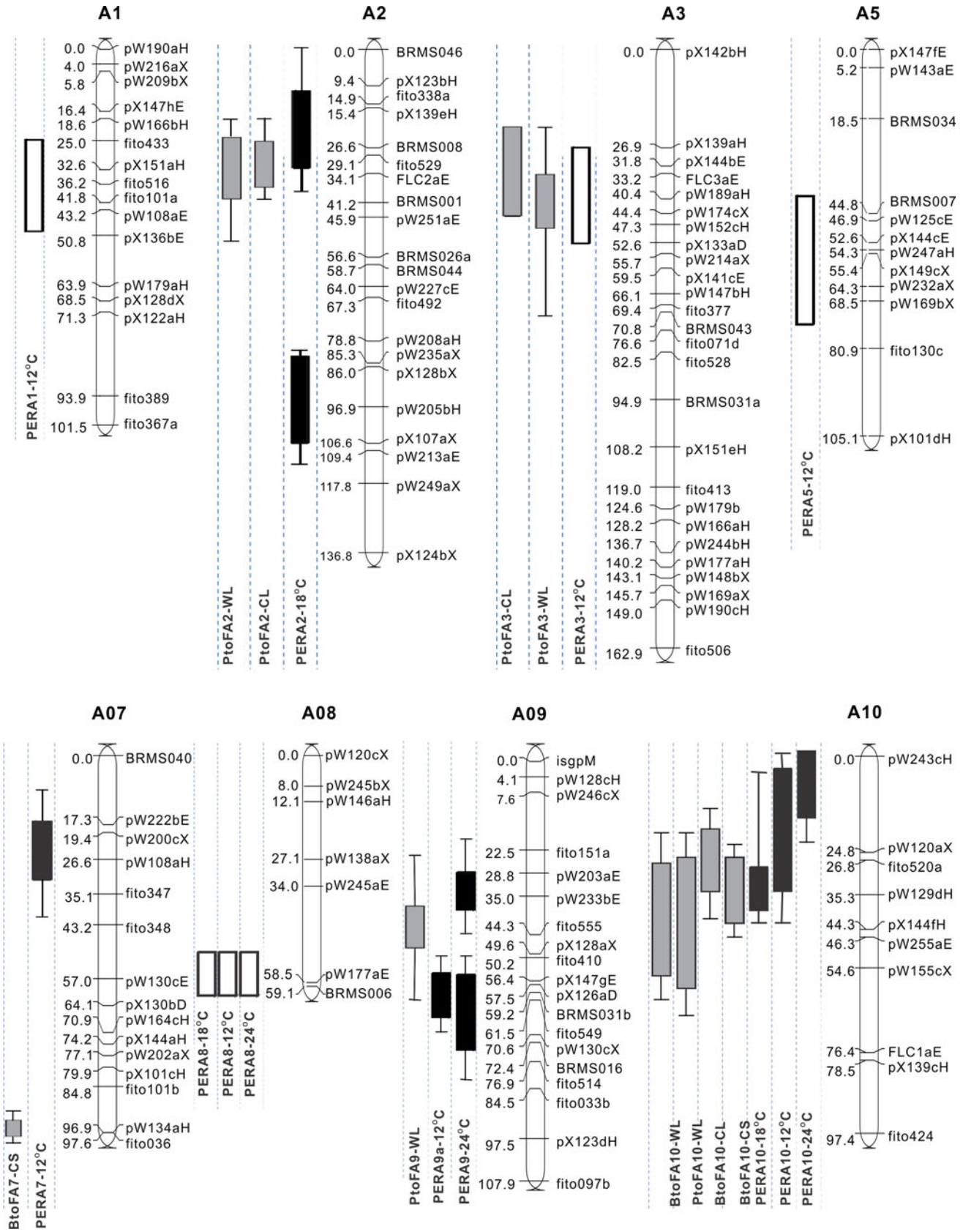
There is a negative correlation between Q_{10} and period in RIL populations ($r = -0.60$, $p < 0.001$) and accessions ($r = -0.62$, $p < 0.001$). We observed a more negative correlation in wild California populations ($r = -0.80$, $p = 0.31$), although this correlation was not statistically significant because of small sample size. Q_{10} was not correlated with geographic origin of the accessions.

For the accessions and RILs, the genotype × treatment interaction for circadian period was significant (Table S3), indicating that genotypes differed in the response of circadian period to increasing temperatures. Despite the significant genotype × treatment interaction, most genotypes, including the RIL parents (IMB211 and R500), the accessions and the wild populations, showed relatively little change in period across the three temperatures (Fig. S6A and S7A, Table S4). However, some genotypes differed from this pattern; for example, several genotypes showed similar period lengths at low and high temperatures, but either slightly longer or shorter periods at the intermediate temperature (Fig. S6, E and F, S7E). Other genotypes showed a slight period shortening at higher temperatures (Fig. S6, D and G, S7, D and G), but in every case the Q_{10} was less than 1.2, indicating that temperature compensation was maintained. Only one accession, a Chinese cabbage (CC-048), showed a progressive shortening of period with increasing temperature, suggestive of a loss of temperature compensation (Fig. S6, I); however, the Q_{10} for this accession was 1.18, indicating that period length was nevertheless compensated. A number of genotypes showed a slight period lengthening with increasing temperature (Fig. S6, B and C, Fig. S7B), which was most pronounced for one RIL (RIL109; Fig. S6H) and for one accession (a vegetable turnip variety from the former Soviet Union, VT-007; Fig. S7H). This is consistent with weak overcompensation, as described in some long-period mutants in *Neurospora crassa* (Gardner and Feldman 1981; Mehra et al. 2009), *Drosophila melanogaster* (Huang et al. 1995; Matsumoto et al. 1999) and *Arabidopsis* (Salomé et al. 2010).

QTL analysis of clock parameters and temperature compensation

We detected QTL for circadian period and amplitude in the RIL populations at each temperature (Fig. 4; Table 1). QTL were named PER for period and AMP for amplitude, followed by the chromosome position of the QTL.

Fig. 4 Period and phenological (flowering-time) quantitative trait ▶ loci. Location of quantitative trait loci (QTL) of clock parameters and flowering time detected in the *Brassica rapa* IRRI RIL population at different environmental settings. LOD profiles are graphically displayed using Map Chart, including the LOD minus 1/LOD minus 2 support interval to approximate a 95% confidence interval. *Black bars* QTL for clock parameters, *open bar* nonsignificant QTL for clock parameters, *gray bars* QTL for flowering time. QTL nomenclature: PtoF, planting to first mature flower; BtoF, bolting to flowering; WL, warm (24°C) long (14-h light) days: CL, cool (12°C) long (14 h light) days; PER, circadian period at the indicated temperature


A1
0.0 pW190aH
4.0 pW216aX
5.8 pW209bX
16.4 pX147hE
18.6 pW166bH
25.0 fito433
32.6 pX151aH
36.2 fito516
41.8 fito101a
43.2 pW108aE
50.8 pX136bE
63.9 pW179aH
68.5 pX128dX
71.3 pX122aH
93.9 fito389
101.5 fito367a
PERA1-12°C
A2
0.0 BRMS046
9.4 pX123bH
14.9 fito338a
15.4 pX139eH
26.6 BRMS008
29.1 fito529
34.1 FLC2aE
41.2 BRMS001
45.9 pW251aE
56.6 BRMS026a
58.7 BRMS044
64.0 pW227cE
67.3 fito492
78.8 pW208aH
85.3 pW235aX
86.0 pX128bX
96.9 pW205bH
106.6 pX107aX
109.4 pW213aE
117.8 pW249aX
136.8 pX124bX
PtoFA2-WL
PtoFA2-CL
PERA2-18°C
A3
0.0 pX142bH
26.9 pX139aH
31.8 pX144bE
33.2 FLC3aE
40.4 pW189aH
44.4 pW174cX
47.3 pW152cH
52.6 pX133aD
55.7 pW214aX
59.5 pX141cE
66.1 pW147bH
69.4 fito377
70.8 BRMS043
76.6 fito071d
82.5 fito528
94.9 BRMS031a
108.2 pX151eH
119.0 fito413
124.6 pW179b
128.2 pW166aH
136.7 pW244bH
140.2 pW177aH
143.1 pW148bX
145.7 pW169aX
149.0 pW190cH
162.9 fito506
PtoFA3-CL
PtoFA3-WL
PERA3-12°C
A5
0.0 pX147fE
5.2 pW143aE
18.5 BRMS034
44.8 BRMS007
46.9 pW125cE
52.6 pX144cE
54.3 pW247aH
55.4 pX149cX
64.3 pW232aX
68.5 pW169bX
80.9 fito130c
105.1 pX101dH
PERA5-12°C
A07
0.0 BRMS040
17.3 pW222bE
19.4 pW200cX
26.6 pW108aH
35.1 fito347
43.2 fito348
57.0 pW130cE
64.1 pX130bD
70.9 pW164cH
74.2 pX144aH
77.1 pW202aX
79.9 pX101cH
84.8 fito101b
96.9 pW134aH
97.6 fito036
BtoFA7-CS
PERA7-12°C
A08
0.0 pW120cX
8.0 pW245bX
12.1 pW146aH
27.1 pW138aX
34.0 pW245aE
58.5 pW177aE
59.1 BRMS006
PERA8-18°C
PERA8-12°C
PERA8-24°C
A09
0.0 isgpM
4.1 pW128cH
7.6 pW246cX
22.5 fito151a
28.8 pW203aE
35.0 pW233bE
44.3 fito555
49.6 pX128aX
50.2 fito410
56.4 pX147gE
57.5 pX126aD
59.2 BRMS031b
61.5 fito549
70.6 pW130cX
72.4 BRMS016
76.9 fito514
84.5 fito033b
97.5 pX123dH
107.9 fito097b
PtoFA9-WL
PERA9a-12°C
PERA9-24°C
A10
0.0 pW243cH
24.8 pW120aX
26.8 fito520a
35.3 pW129dH
44.3 pX144fH
46.3 pW255aE
54.6 pW155cX
76.4 FLC1aE
78.5 pX139cH
97.4 fito424
BtoFA10-WL
PtoFA10-WL
BtoFA10-CL
BtoFA10-CS
PERA10-18°C
PERA10-12°C
PERA10-24°C

We identified a total of six significant and four additional marginally significant ($p = 0.075$) period QTL in the RIL population; collectively, these QTL explained 55.8, 34.8 and 38.9% of the genetically determined variation among the RILs at 12, 18 and 24°C, respectively. We detected no significant QTL for circadian phase in this study.

The major period QTL, PERA10, was observed at all three temperatures and explained from 11.6 to 14.2% of the variation in period length, depending on the treatment; the R500 allele shortened the period, consistent with the shorter period of R500 at all three temperatures (Table 1). A second QTL, PERA8, was also observed at all three temperatures, with the R500 allele increasing the period. However, the expression of many QTL showed temperature specificity (i.e., the QTL × environment interaction was significant; Table 1). Even when the interaction effect was not formally significant, QTL mapped in only one environment may well be temperature-specific, given the missing genotypic information for some RILs and the generally low power to test for environmental interactions. Several QTL were mapped only in a single environment; for example, two QTL, PERA2a and PERA2b, were detected only at 18°C, with the R500 alleles increasing the period length. The observed QTL effects are consistent with a model of multigenic inheritance in which period is determined by the additive effects of multiple period-lengthening and period-shortening alleles. Four QTL, PERA1, PERA3, PERA5 and PERA7, were specific to low (12°C) temperature and one QTL, PERA9b, was specific at high (24°C) temperature. One QTL, PERA9a, was detected at both 12 and 24°C, suggesting sensitivity to temperature extremes.

QTL for flowering time in different environmental settings

Flowering time, measured both as planting to first mature flower (PtoF) and bolting to flowering (BtoF), was accelerated by increased day length and higher temperature (Table S2). Among the RILs, there was a strong genotypic component to this variation, as indicated by the highly significant ($p < 0.0001$) V_G values (Table S2). Flowering-time QTL were mapped in the RIL populations grown under three different conditions in growth chambers: cool short days (CS; 12°C, 10 h light/14 h dark), cool long days (CL; 12°C, 14 h light/10 h dark) and warm long days (WL; 24°C, 14 h light/10 h dark). A total of six QTL were mapped in the RIL population for flowering time under different temperature and day length settings, with some of the QTL showing temperature or day length specificity (Table 2). Two flowering-time QTL, PtoFA2 and PtoFA3, were detected only in long days, BtoFA7 was detected only in short days, and PtoFA9 and PtoFA10 were detected only at high temperature. The major flowering-time QTL, BtoFA10, explained from 13 to 18% of the variation and was observed in all conditions for bolting to flowering. In all temperature and day length settings, the R500 alleles delayed flowering time, consistent with the relative flowering times of the parents (IMB211 is a rapid cycler).

Correlations between circadian traits and flowering time

The magnitude of genotypic correlations between circadian period and flowering time was generally small, but

Table 1 Summary of circadian period QTL detected in the *B. rapa* IRRI population

QTL	CHR	12°C				18°C				24°C				QTL × E
		LOD	INT (POS)	VAR	ADD	LOD	INT (POS)	VAR	ADD	LOD	INT (POS)	VAR	ADD	
PERA1	*A1*	*2.4*	*NA (35)*	*5.3*	*0.2*									
PERA2a	A2					2.7	12–24 (15)	8.1	0.3					
PERA2b	A2					2.9	85–112 (95)	8.7	0.4					
PERA3	*A3*	*2.3*	*NA (45)*	*4.6*	*0.2*									
PERA5	*A5*	*2*	*NA (55)*	*4*	*0.2*									
PERA7	A7	4.3	8–42 (34)	10.6	−0.3									12°C/24°C *
PERA8	*A8*	*2.2*	*NA (58)*	*6.3*	*0.2*	*2.1*	*NA (58)*	*6.4*	*0.3*	*2*	*NA (58)*	*6*	*0.3*	
PERA9a	A9	3.8	66–74 (67)	10.8	−0.3					2.6	56–75 (61)	9.6	−0.5	18°C/24°C *
PERA9b	A9									3	31–40 (36)	11.2	−0.5	18°C/24°C *
PERA10	A10	5.1	25–45 (34)	14.2	−0.4	4	0–40 (11)	11.6	−0.5	3.4	0–17 (4)	12.1	−0.6	

QTL for circadian period (PER) of the cotyledon movement rhythm are numbered by chromosomal location. *CHR* chromosome in the *Brassica* A genome; *LOD* LOD score of the QTL. *INT/POS* ± 2-LOD QTL interval (peak position) (cM); *NA* not applicable, because the LOD score of the QTL was non-significant; *VAR* % variation explained by QTL; *ADD* additive effects of the QTL (in h). Positive values indicate that the IMB211 allele is associated with increased value of period. QTL values in italics are not statistically significant but are recorded because significant QTL were detected at similar positions in other conditions; *QTL × E* QTL that demonstrate significant QTL × E across treatment pairs. *$p < 0.05$

Table 2 Summary of flowering-time QTL detected in *B. rapa* IRRI population

QTL	CHR	CL				CS				WL				
		LOD	INT (POS)	VAR	ADD	LOD	INT (POS)	VAR	ADD	LOD	INT (POS)	VAR	ADD	QTL × E
PtoFA2	A2	3.2	18–39 (31)	9.8	2.2					2.6	18–50 (31)	7.7	1.9	
PtoFA3	A3	2.5	29–52 (44)	7.6	1.8					3.0	29–79 (48)	9	2.0	
BtoFA7	A7					5.2	90–97 (97)	15.5	0.9					WL/CS ***
PtoFA9	A9									3.0	27–63 (46)	9.1	2.0	
PtoFA10	A10									3.1	19–65 (40)	9.2	2.1	
BtoFA10	A10	5.2	13–41 (28)	15.3	0.8	5.1	22–45 (31)	15.2	1.0	4.6	19–61 (48)	13.3	0.7	

QTL for planting to first mature flower (PtoF) or bolting to flowering (BtoF) are numbered by chromosomal location; *CHR* chromosome in the *Brassica* A genome, *CL* cool long days (12°C, 14-h light/10-h dark), *CS* cool short days (12°C, 10-h light/14-h dark), *WL* warm long days (24°C, 14-h light/10-h dark), *LOD* LOD score of the QTL, *INT (POS)* ± 2-LOD QTL interval (peak position) (cM), *VAR* % variation explained by QTL, *ADD* additive effects of the QTL (days). Positive values indicate the R500 allele is associated with increased value. *QTL × E* QTL that demonstrate significant QTL × E across treatment pairs.*** $p < 0.005$

significant (Fig. S8). Period was negatively correlated with flowering time (BtoF) in cool, long days ($r = -0.24$, $p = 0.004$) as well as in cool, short days ($r = -0.16$, $p = 0.054$). This correlation was non-significant in warm long days (Fig. S9). Despite weak genotypic correlations, a number of circadian period QTL and flowering-time QTL co-localized (Fig. 4). For example, PERA10 overlapped with PtoFA10/BtoFA10, and the marginal QTL, PERA3, co-localized with PtoFA3. In addition, PERA2 overlapped with PtoFA2, although the peaks of these two QTL were separated by ~15 cM, making it unlikely that they result from variation at a single locus.

Discussion

Significant progress has been made in characterizing the molecular genetic basis of the circadian clock in model organisms such as *Arabidopsis*, *Neurospora crassa*, *Drosophila melanogaster* and *Mus musculus*. Much less is known about the genetic basis of quantitative variation in clock function in natural populations and non-model systems. In this study, we investigated natural variation in circadian clock function and flowering time in *Brassica rapa*. We observed wide variation (5-6 h) in circadian period length among cultivated accessions and among RILs at all three temperatures (Fig. 1, 2). This is greater than the range seen among *Brassica oleracea* populations (Salathia et al. 2007), but may simply reflect larger sample sizes permitting the identification of more extreme variants. There was more variation in period length among the RILs than among the accessions, and there was little variation among natural populations. This is consistent with natural and artificial selection reducing the frequency of extreme phenotypes, although it is important to acknowledge that we sampled only a small number of natural populations from a restricted geographic region, and broader sampling might reveal considerably more variation in circadian phenotype. Among the RILs, there are several extreme short-period lines. For example, RIL18 showed a period of less than 22 h, which is considerably shorter than the extremes among the accessions and wild populations. Consistent with the importance of wild-type circadian function for fitness in a diurnal environment (Dodd et al. 2005; Graf et al. 2010; Green et al. 2002; Yerushalmi et al. 2011), this extreme short-period line shows reduced growth vigor, slow development, reduced seed set and low survival rate in greenhouse environments under natural photoperiods, although it must be emphasized that we have no direct evidence establishing a causal relationship between short circadian period and the growth, survival and reproductive phenotypes.

We observed no correlation between circadian parameters and geographic origin, unlike the case in *Arabidopsis* (Michael et al. 2003). This may reflect the difference in evolutionary histories, as *Arabidopsis* is an inbreeding wild species, whereas *B. rapa* is an outcrossing crop that has been subject to strong human manipulation for a long time, which may have obscured any relationship between period and geography. It is also worth noting that a latitudinal cline was found in flowering time among the subset of *Arabidopsis* accessions carrying the FRI^{+} allele, but not among the subset carrying *fri* loss of function alleles (Stinchcombe et al. 2004). So, it is possible that relationships between clock parameters or flowering time and geographic origin may exist within restricted genotypic subsets.

We observed a correlation between period and temperature compensation/sensitivity. In each population, period and Q_{10} were inversely related (Fig. 3). Nonetheless, in every case the Q_{10} fell within a narrow range between 0.8 and 1.2, indicating that the *B. rapa* clock is very well buffered against temperature changes, at least across this range of temperatures. However, we observed that fewer

lines exhibited robust rhythmicity at higher temperature. This could suggest that clock function is compromised at higher temperatures in this cool-weather crop, but it could also simply indicate that the overt rhythm of cotyledon movement is compromised even when clock function persists relatively unperturbed. Distinguishing between these possibilities will require the development of alternate assays for rhythmicity, such as gene expression. For example, robust circadian rhythmicity was detected at 24°C with a transcriptional *CCA1 promoter:LUCIFERASE* fusion expressed in tissue culture derived from *B. rapa* hypocotyl explants taken from R500, IMB211 and a number of the RILs (Xu et al. 2010), suggesting that the *B. rapa* clock is functional at 24°C, at least in tissue culture.

Among the RILs, we identified five major QTL controlling circadian period (Fig. 4, Table 1). Consistent with significant genotype × environment interaction effects, only one of the period QTL, PerA10, was detected at all three temperatures, which suggests that QTL effects are environment-specific. Such QTL × environment interactions could also account for the constant Q_{10} because different QTL apparently regulate period in different temperatures. This is consistent with the observations in *Arabidopsis* that the specific loci responsible for temperature compensation are only evident at specific temperatures. For example, *LHY* is more important than *CCA1* for period length determination at high temperatures and vice versa (Gould et al. 2006), and *PRR7* and *PRR9* are important for period determination at high temperature but are dispensable at low temperature (Salomé et al. 2010). For flowering time, only one major QTL, BtoFA10, was detected at all temperature and day length combinations, again consistent with strong QTL × environment effects. Interestingly, these two QTL, PerA10 and BtoFA10, partially overlap at higher temperatures and completely overlap at 12°C (cool) in long days (Fig. 4).

The genotypic correlation observed between circadian period and flowering time ($r = -0.24$, $p = 0.004$) and the co-localization of period and flowering-time QTL (Fig. 4) could indicate a common locus underlying the two phenotypes, but could also result from tight linkage of two distinct loci or from epistasis between flowering-time genes and circadian-rhythm genes. Consistent with a hypothesis of pleiotropy in our quantitative-genetic analyses, molecular genetic studies have shown that the circadian clock is an important regulator of flowering via its role in the photoperiodic pathway (Imaizumi 2010; Turck et al. 2008). Considerable evidence indicates that flowering time is under selection in wild populations and is an important trait selected during crop domestication and improvement (e.g., Blackman et al. 2010; Buckler et al. 2009; Stinchcombe et al. 2004; Wilczek et al. 2009). With regard to fitness consequences of the clock, studies to date have emphasized on the role of the clock in the regulation of photosynthesis and starch utilization in experimental genetic material such as mutants carrying loss-of-function alleles of clock genes as well as transgenic lines overexpressing clock genes (Dodd et al. 2005; Graf et al. 2010). Regulation of flowering time by quantitative variation in the clock may also contribute to the adaptive significance of the clock in natural populations and crops.

Since its divergence from *Arabidopsis*, the *B. rapa* genome has undergone triplication and segmental rearrangement, so typically genes are present in up to three homeologous copies in the *B. rapa* genome (Park et al. 2005; Schranz et al. 2002; Trick et al. 2009). However, because of gene loss following duplication, the *B. rapa* genome contains at most only approximately twice the number of genes as in *Arabidopsis* (Mun et al. 2009). The pattern of segmental duplications of *Arabidopsis* segments within the *B. rapa* genome has been described (Cheung et al. 2009; Parkin et al. 2005; Schranz et al. 2006) and permits the identification of candidate genes for most of the QTL we have identified (Fig. 5). At least three of the period QTL identified in this study co-localize with period QTL identified in *B. oleracea* (Salathia et al. 2007): PERA2b co-localizes with the *B. oleracea* O2 period QTL, PERA3 with *B. oleracea* O4 and PERA10 with *B. oleracea* O9. In each case, one or more candidate genes, based on synteny with *Arabidopsis*, can be proposed (Fig. 5), although the low resolution of our QTLs compromises our confidence in these proposals.

With the exception of BtoFA7, all the flowering-time QTL at least partially overlap with a circadian period QTL, which generally supports the observed phenotypic correlations between circadian clock function and photoperiodic flowering time. PerA2a and PtoFA2 partially overlap near the top of A2. Two strong candidate genes map to this region. One, *FLC2*, has also been observed in other flowering-time QTL studies in *B. rapa* (Li et al. 2009; Lou et al. 2007; Schranz et al. 2002; Zhao et al. 2007) and is a strong candidate for the PtoFA2 QTL. In *Arabidopsis*, *FLC* was also identified as a period QTL and has been shown to contribute to period length (Edwards et al. 2005, 2006; Salathia et al. 2006; Swarup et al. 1999), making *FLC2* a candidate for PerA2a as well. However, a second strong candidate, *PRR7*, also maps to this region. *PRR7* is known to affect period length and has been suggested to underlie a period QTL on *Arabidopsis* chromosome 5 (Edwards et al. 2005; Michael et al. 2003). In cereals, *PRR7* orthologs have been shown to be important determinants of photoperiodic flowering (Beales et al. 2007; Turner et al. 2005). In *Arabidopsis*, *PRR7* together with *PRR5* and *PRR9* encode partially redundant positive regulators of flowering time (Nakamichi et al. 2007), making *PRR7* a candidate for PtoFA2 as well. Of course, different genetic loci may also

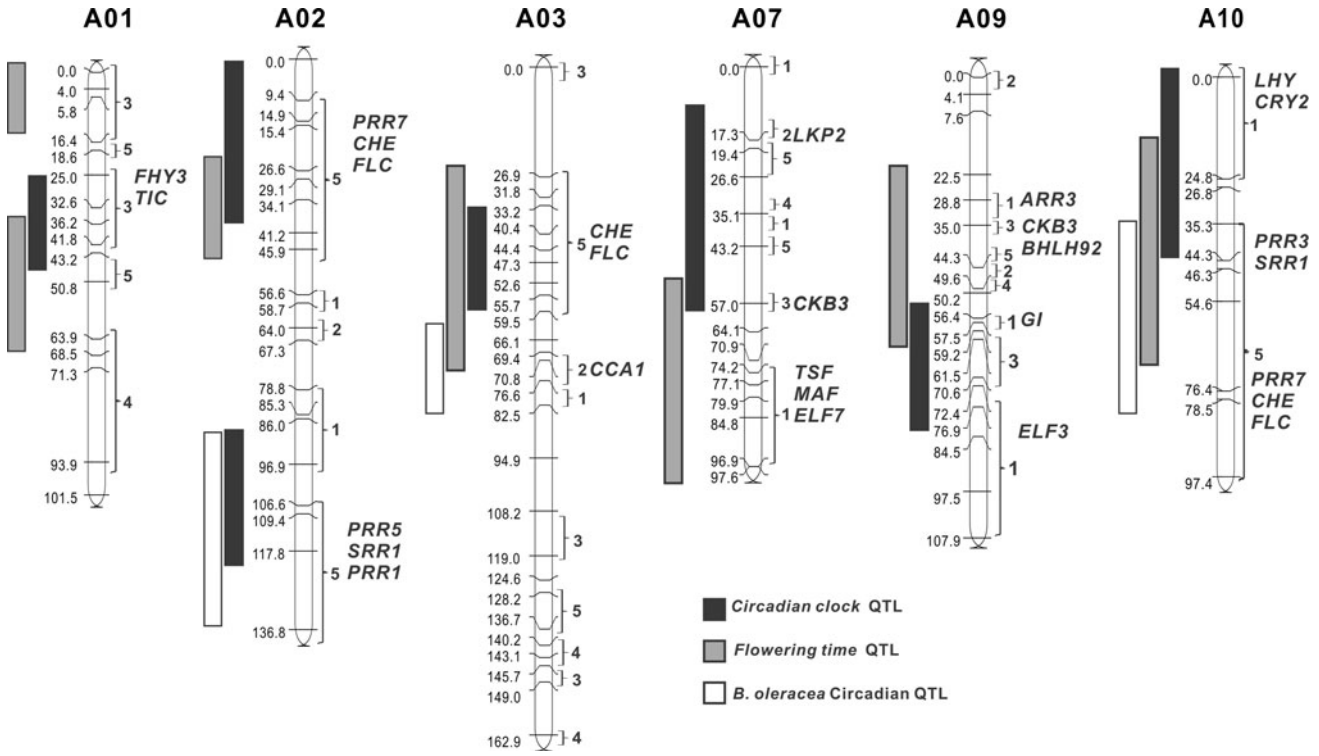


Fig. 5 Candidates for period and phenological (flowering-time) quantitative trait loci. Overview of *Brassica rapa* QTL for circadian rhythms and flowering time, with candidate *B. rapa* loci based on synteny with the *Arabidopsis* genome (Parkin et al. 2005; Schranz et al. 2006). *B. oleracea* circadian QTL (Salathia et al. 2007) co-localizing with *B. rapa* circadian QTL are indicated for comparison

underlie QTL that affect clock period and flowering time, and a more detailed analysis will be required to identify the causal loci. Efforts to identify genes responsible for the QTL are ongoing, using both classical genetics (development of near isogenic lines and heterogeneous inbred families) as well as molecular methods. Toward this latter approach, it is exciting that an excellent TILLING population of mutants in the transformable accession R-o-18 has been recently described (Stephenson et al. 2010), which will permit transgenic analysis of candidates for specific QTL by phenotypic rescue.

Of relevance to plant fitness and the role of the circadian clock in adaptive evolution, we have also observed that period is strongly correlated with many ecophysiological traits at both the genotypic and QTL levels in *B. rapa* (data not shown). For instance, quantitative variation in period is correlated with estimates of net carbon assimilation (A), stomatal conductance (g_s), transpiration (E), nitrogen concentration (per unit leaf area) and $\Delta^{13}C$ a long-term proxy for water-use efficiency (Donovan and Ehleringer 1994; McKay et al. 2003; Rebetzke et al. 2008; Seibt et al. 2008). Period QTL co-localize with QTL for ecophysiological traits on all linkage groups. These results provide some of the first evidence for a link between quantitative variation in the clock and plant ecophysiology. Further, the observations support the functional hypothesis that the adaptive significance of the clock lies in part in its regulation of ecophysiological traits. With regard to agronomy, as the genetic basis of circadian rhythms and flowering time becomes clearer, we hope to identify loci and markers useful in breeding *Brassica* vegetables with improved growth and development in different latitudes, growing seasons and climates, thereby improving production and yield of these important food crops.

Acknowledgments This study was supported by a National Science Foundation grant (IOS 0605736) to C.R.M., C.W. and R.M. Amasino. We thank Fede Iñiguez-Luy (Temuco, Chile) for *Brassica rapa* IRRI RILs and linkage maps and the Centre for Genetic Resources, the Netherlands (CGN), for *Brassica rapa* accessions.

References

Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A Pseudo-Response Regulator is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). Theor Appl Genet 115:721–733

Beilstein MA, Nagalingum NS, Clements MD, Manchester SR, Mathews S (2010) Dated molecular phylogenies indicate a Miocene origin for *Arabidopsis thaliana*. Proc Natl Acad Sci USA 107:18724–18728

Blackman BK, Strasburg JL, Raduski AR, Michaels SD, Rieseberg LH (2010) The role of recently derived FT paralogs in sunflower domestication. Curr Biol 20:629–635

Buckler ES, Holland JB, Bradbury P, Acharya C, Brown P, Browne C, Ersoz E, Flint-Garcia S, Garcia A, Glaubitz J, Goodman M, Harjes C, Guill K, Kroon D, Larsson S, Lepak N, Li H, Mitchell S, Pressoir G, Peiffer J, Rosas M, Rocheford T, Romay M, Romero S, Salvo S, Villeda H, da Silva H, Sun Q, Tian F, Upadyayula N, Ware D, Yates H, Yu J, Zhang Z, Kresovich S, McMullen M (2009) The genetic architecture of maize flowering time. Science 325:714–718

Cheung F, Trick M, Drou N, Lim YP, Park JY, Kwon SJ, Kim JA, Scott R, Pires JC, Paterson AH, Town C, Bancroft I (2009) Comparative analysis between homoeologous genome segments of *Brassica napus* and its progenitor species reveals extensive sequence-level divergence. Plant Cell 21:1912–1928

Darrah C, Taylor BL, Edwards KD, Brown PE, Hall A, McWatters HG (2006) Analysis of phase of LUCIFERASE expression reveals novel circadian quantitative trait loci in *Arabidopsis*. Plant Physiol 140:1464–1474

DeCoursey PJ, Walker JK, Smith SA (2000) A circadian pacemaker in free-living chipmunks: essential for survival? J Comp Physiol A 186:169–180

Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AAR (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630–633

Donovan LA, Ehleringer JR (1994) Carbon-isotope discrimination, water-use efficiency, growth, and mortality in a natural shrub population. Oecologia 100:347–354

Edwards KD, Lynn JR, Gyula P, Nagy F, Millar AJ (2005) Natural allelic variation in the temperature compensation mechanisms of the *Arabidopsis thaliana* circadian clock. Genetics 170:387–400

Edwards KD, Anderson PE, Hall A, Salathia NS, Locke JCW, Lynn JR, Straume M, Smith JQ, Millar AJ (2006) *FLOWERING LOCUS C* mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. Plant Cell 18:639–650

Gardner GF, Feldman JF (1981) Temperature compensation of circadian period length in clock mutants of *Neurospora crassa*. Plant Physiol 68:1244–1248

Gould PD, Locke JCW, Larue C, Southern MM, Davis SJ, Hanano S, Moyle R, Milich R, Putterill J, Millar AJ, Hall A (2006) The molecular basis of temperature compensation in the *Arabidopsis* circadian clock. Plant Cell 18:1177–1187

Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. Proc Natl Acad Sci USA 107:9458–9463

Green RM, Tingay S, Wang Z-Y, Tobin EM (2002) Circadian rhythms confer a higher level of fitness to *Arabidopsis* plants. Plant Physiol 129:576–584

Harmer SL (2009) The circadian system in higher plants. Annu Rev Plant Biol 60:357–377

Huang ZJ, Curtin KD, Rosbash M (1995) PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. Science 267:1169–1172

Imaizumi T (2010) *Arabidopsis* circadian clock and photoperiodism: time to think about location. Curr Opin Plant Biol 13:83–89

Iniguez-Luy FL, Lukens L, Farnham MW, Amasino RM, Osborn TC (2009) Development of public immortal mapping populations, molecular markers and linkage maps for rapid cycling *Brassica rapa* and *B. oleracea*. Theor Appl Genet 119:31–43

Johnson CH, Elliott J, Foster R, Honma K-I, Kronauer R (2004) Fundamental properties of circadian rhythms. In: Dunlap JC, Loros JJ, DeCoursey P (eds) Chronobiology: biological timekeeping. Sinauer, Sunderland, pp 67–105

Kim JA, Yang T-J, Kim JS, Park JY, Kwon S-J, Lim M-H, Jin M, Lee SC, Lee SI, Choi B-S, Um S-H, Kim H-I, Chun C, Park B-S (2007) Isolation of circadian-associated genes in *Brassica rapa* by comparative genomics with *Arabidopsis thaliana*. Mol Cells 23:145–153

Kim H, Choi SR, Bae J, Hong CP, Lee SY, Hossain MJ, Van Nguyen D, Jin M, Park BS, Bang JW, Bancroft I, Lim YP (2009) Sequenced BAC anchored reference genetic map that reconciles the ten individual chromosomes of *Brassica rapa*. BMC Genomics 10:432

Li F, Kitashiba H, Inaba K, Nishio T (2009) A *Brassica rapa* linkage map of EST-based SNP markers for identification of candidate genes controlling flowering time and leaf morphological traits. DNA Res 16:311–323

Lou P, Zhao J, Kim JS, Shen S, Del Carpio DP, Song X, Jin M, Vreugdenhil D, Wang X, Koornneef M, Bonnema G (2007) Quantitative trait loci for flowering time and morphological traits in multiple populations of *Brassica rapa*. J Exp Bot 58: 4005–4016

Matsumoto A, Tomioka K, Chiba Y, Tanimura T (1999) *tim*^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343–4354

McClung CR (2010) A modern circadian clock in the common angiosperm ancestor of monocots and eudicots. BMC Biol 8:55

McClung CR, Gutiérrez RA (2010) Network news: prime time for systems biology of the plant circadian clock. Curr Opin Genet Dev 20:588–598

McKay JK, Richards JH, Mitchell-Olds T (2003) Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits. Mol Ecol 12:1137–1151

Mehra A, Shi M, Baker CL, Colot HV, Loros JJ, Dunlap JC (2009) A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell 137:749–760

Michael TP, Salomé PA, Yu HJ, Spencer TR, Sharp EL, Alonso JM, Ecker JR, McClung CR (2003) Enhanced fitness conferred by naturally occurring variation in the circadian clock. Science 302:1049–1053

Mun J-H, Kwon S-J, Yang T-J, Seol Y-J, Jin M, Kim J-A, Lim M-H, Kim JS, Baek S, Choi B-S, Yu H-J, Kim D-S, Kim N, Lim K-B, Lee S-I, Hahn J-H, Lim YP, Bancroft I, Park B-S (2009) Genome-wide comparative analysis of the *Brassica rapa* gene space reveals genome shrinkage and differential loss of duplicated genes after whole genome triplication. Genome Biol 10:R111

Nakamichi N, Kita M, Niinuma K, Ito S, Yamashino T, Mizoguchi T, Mizuno T (2007) *Arabidopsis* clock-associated Pseudo-Response Regulators PRR9, PRR7 and PRR5 coordinately and positively regulate flowering time through the canonical CONSTANS-dependent photoperiodic pathway. Plant Cell Physiol 48:822–832

Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua N-H, Sakakibara H (2010) PSEUDO-RESPONSE REGULATORS 9, 7 and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. Plant Cell 22:594–605

Ni Z, Kim E-D, Ha M, Lackey E, Liu J, Zhang Y, Sun Q, Chen ZJ (2009) Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. Nature 457:327–331

Onai K, Okamoto K, Nishimoto H, Morioka C, Hirano M, Kami-ike N, Ishiura M (2004) Large-scale screening of *Arabidopsis* circadian clock mutants by a high-throughput real-time bioluminescence monitoring system. Plant J 40:1–11

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci USA 95:8660–8664

Park JY, Koo DH, Hong CP, Lee SJ, Jeon JW, Lee SH, Yun PY, Park BS, Kim HR, Bang JW (2005) Physical mapping and microsynteny of *Brassica rapa* ssp. *pekinensis* genome corresponding

to a 222 kbp gene-rich region of *Arabidopsis* chromosome 4 and partially duplicated on chromosome 5. Mol Gen Genomics 274:579–588

Parkin IAP, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics 171:765–781

Parzen E (1962) On estimation of a probability density function and mode. Ann Math Stat 33:1065–1076

Plautz JD, Straume M, Stanewsky R, Jamison CF, Brandes C, Dowse HB, Hall JC, Kay SA (1997) Quantitative analysis of *Drosophila period* gene transcription in living animals. J Biol Rhythms 12:204–217

Pruneda-Paz JL, Kay SA (2010) An expanding universe of circadian networks in higher plants. Trends Plant Sci 15:259–265

R Development Core Team (2009) R: A language and environment for statistical computing. Vienna, Austria

Rebetzke GJ, Condon AG, Farquhar GD, Appels R, Richards R (2008) Quantitative trait loci for carbon isotope discrimination are repeatable across environments and wheat mapping populations. Theor Appl Genet 118:123–137

Rosenblatt M (1956) Remarks on some nonparametric estimates of a density function. Ann Math Stat 27:832–837

Salathia N, Davis SJ, Lynn JR, Michaels SD, Amasino RM, Millar AJ (2006) FLOWERING LOCUS C-dependent and -independent regulation of the circadian clock by the autonomous and vernalization pathways. BMC Plant Biol 6:10

Salathia N, Lynn JR, Millar AJ, King GJ (2007) Detection and resolution of genetic loci affecting circadian period in *Brassica oleracea*. Theor Appl Genet 114:683–692

Salomé PA, Weigel D, McClung CR (2010) The role of the *Arabidopsis* morning loop components CCA1, LHY, PRR7 and PRR9 in temperature compensation. Plant Cell 22: 3650–3661

Schranz ME, Quijada P, Sung SB, Lukens L, Amasino R, Osborn TC (2002) Characterization and effects of the replicated flowering time gene *FLC* in *Brassica rapa*. Genetics 162:1457–1468

Schranz M, Lysak M, Mitchell-Olds T (2006) The ABC's of comparative genomics in the Brassicaceae: building blocks of crucifer genomes. Trends Plant Sci 11:535–542

Seibt U, Rajabi A, Griffiths H, Berry JA (2008) Carbon isotopes and water use efficiency: sense and sensitivity. Oecologia 155:441–454

Stephenson P, Baker D, Girin T, Perez A, Amoah S, King GJ, Østergaard L (2010) A rich TILLING resource for studying gene function in *Brassica rapa*. BMC Plant Biol 10:62

Stinchcombe JR, Weinig C, Ungerer M, Olsen KM, Mays C, Halldorsdottir SS, Purugganan MD, Schmitt J (2004) A latitudinal cline in flowering time in *Arabidopsis thaliana* modulated by the flowering time gene *FRIGIDA*. Proc Natl Acad Sci USA 101:4712–4717

Swarup K, Alonso-Blanco C, Lynn JR, Michaels SD, Amasino RM, Koornneef M, Millar AJ (1999) Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. Plant J 20:67–77

Takata N, Saito S, Saito CT, Uemura M (2010) Phylogenetic footprint of the plant clock system in angiosperms: evolutionary processes of Pseudo-Response Regulators. BMC Evol Biol 10:126

Trick M, Kwon SJ, Choi SR, Fraser F, Soumpourou E, Drou N, Wang Z, Lee SY, Yang TJ, Mun JH, Paterson AH, Town CD, Pires JC, Lim YP, Park BS, Bancroft I (2009) Complexity of genome evolution by segmental rearrangement in *Brassica rapa* revealed by sequence-level analysis. BMC Genomics 10:539

Turck F, Fornara F, Coupland G (2008) Regulation and identity of Florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol 59:573–594

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The Pseudo-Response Regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science 310:1031–1034

Voorrips RE (2002) MapChart: Software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wang S, Basten CJ, Zeng Z (2007) Windows Qtl Cartographer 2.5. N.C. State University, Bioinformatics Research Center, USA

Wilczek AM, Roe JL, Knapp MC, Cooper MD, Lopez-Gallego C, Martin LJ, Muir CD, Sim S, Walker A, Anderson J, Egan JF, Moyers BT, Petipas R, Giakountis A, Charbit E, Coupland G, Welch SM, Schmitt J (2009) Effects of genetic perturbation on seasonal life history plasticity. Science 323:930–934

Xu X, Xie Q, McClung CR (2010) Robust circadian rhythms of gene expression in *Brassica rapa* tissue culture. Plant Physiol 153:841–850

Yerushalmi S, Yakir E, Green RM (2011) Circadian clocks and adaptation in *Arabidopsis*. Mol Ecol 20:1155–1165

Zhang EE, Kay SA (2010) Clocks not winding down: unravelling circadian networks. Nat Rev Mol Cell Biol 11:764–776

Zhao J, Paulo M-J, Jamar D, Lou P, Van Eeuwijk F, Bonnema G, Vreugdenhil D, Koornneef M (2007) Association mapping of leaf traits, flowering time, and phytate content in *Brassica rapa*. Genome 50:963–973