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Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the *Mimulus guttatus* species complex

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Summary

- Species with extensive ranges experience highly variable environments with respect to temperature, light and soil moisture. Synchronizing the transition from vegetative to floral growth is important to employ favorable conditions for reproduction. Optimal timing of this transition might be different for semelparous annual plants and iteroparous perennial plants.
- We studied variation in the critical photoperiod necessary for floral induction and the requirement for a period of cold-chilling (vernalization) in 46 populations of annuals and perennials in the *Mimulus guttatus* species complex. We then examined critical photoperiod and vernalization QTLs in growth chambers using F_2 progeny from annual and perennial parents that differed in their requirements for flowering.
- We identify extensive variation in critical photoperiod, with most annual populations requiring substantially shorter day lengths to initiate flowering than perennial populations. We discover a novel type of vernalization requirement in perennial populations that is contingent on plants experiencing short days first. QTL analyses identify two large-effect QTLs which influence critical photoperiod. In two separate vernalization experiments we discover each set of crosses contain different large-effect QTLs for vernalization.
- *Mimulus guttatus* harbors extensive variation in critical photoperiod and vernalization that may be a consequence of local adaptation.

Introduction

In flowering plants, the timing of the transition from vegetative growth to flowering is a critical life history trait. Many plants display precise coordination of flowering to seasonal changes, so that vegetative and reproductive phases are tailored to local climate and edaphic conditions. The synchronization of flowering time promotes simultaneous flowering of individuals within a population to increase pollination and fertilization success, and also ensures that seeds develop, disperse and germinate at appropriate times. Seasonal cues play a large part in regulating the transition from vegetative growth to reproduction, in particular, photoperiod, temperature and the duration of winter chilling (vernalization) (Rathcke & Lacey, 1985; Michaels & Amasino, 2000; Weinig *et al.*, 2002; Lempe *et al.*, 2005; Li *et al.*, 2006; Kim *et al.*, 2009; Wilczek *et al.*, 2009). Over evolutionary time, the response to these cues can be shaped by local climatic conditions resulting in distinct flowering times in different environments (Stinchcombe *et al.*, 2004; Scarcelli *et al.*, 2007; Verhoeven *et al.*, 2008; Sandring & Ågren, 2009).

Annual or perennial life history strategies can be adaptive responses to the environment, and theoretical models predict that the annual habit evolves in response to unpredictable conditions and arid environments, where chances of long-term survival are low (Stearns, 1976). Annual, semelparous plants initiate flowering only once and complete their life cycle in 1 yr. Iteroparus perennials, however, cycle repeatedly through vegetative and flowering phases in synchrony to the changing seasons. Perennial plants might also delay flowering to ensure that plants have acquired sufficient biomass before flowering to allow them to sustain the perennial life cycle. Thus, one might expect that annuals and perennials respond in fundamentally different ways to seasonal fluctuations in temperature or light. A key component to understanding how populations have adapted to their environment is to identify natural genetic variation that corresponds with local climatic conditions, where systematic correlation of phenotypes with environmental gradients can indicate adaptation. In the semelparous *Arabidopsis thaliana*, latitudinal and altitudinal clines in flowering time correspond to local climatic factors (Maloof *et al.*, 2001; Stinchcombe *et al.*, 2004; Hoffmann *et al.*, 2005; Lempe *et al.*, 2005; Méndez-Vigo *et al.*, 2011). However, it is unclear how semelparous annuals and iteroparous perennials might respond differently to gradients in photoperiod and temperature.

The genetic control of the flowering transition is exceptionally well characterized in the model annual plant *Arabidopsis thaliana*.

In *Arabidopsis*, rapid flowering is facilitated by long days as mediated by the photoperiod pathway (Koornneef *et al.*, 1998; Levy & Dean, 1998), and by prolonged exposure to cold through the vernalization pathway (Johanson *et al.*, 2000; Simpson & Dean, 2002). Under inductive long days, transcription of *FLOWERING LOCUS T* (*FT*) is induced by CONSTANS (CO), which itself is regulated by light and the circadian clock (Suárez-López *et al.*, 2001; Valverde *et al.*, 2004). Once induced, FT protein travels from the leaf via phloem to the shoot apical meristem, where it induces expression of meristem identity genes like *APETELA1* (*AP1*) and *LEAFY* (*LFY*) which promote the transition from vegetative to floral growth (Liljegren *et al.*, 1999; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Jaeger & Wigge, 2007). Natural selection has acted on allelic variation in a range of genes in the flowering time pathways resulting in extensive quantitative variation in flowering time among natural accessions of *A. thaliana*, including two broad types, 'winter-' and 'spring-annuals'. These differences are largely related to climatic conditions and reflect adaptive differences in flowering time. The genetic mechanisms controlling flowering time in response to day length tend to be well conserved across the flowering plants (Hayama & Coupland, 2004), so progress in understanding the molecular foundation in *Arabidopsis* is generally transferrable to other species.

For temperate plants, a requirement for vernalization ensures that plants over-winter and flower in the appropriate season. Unlike the photoperiod pathway, the molecular foundation of vernalization is unlikely to be conserved across flowering plants as it has arisen many times by convergent evolution (Amasino, 2010). In both cereals and *Arabidopsis* the vernalization requirement is a result of a repressor of flowering that is repressed during cold exposure; however, these are unrelated proteins in the two taxa (Dennis & Peacock, 2009; Amasino, 2010). In both monocots (e.g. barley and wheat) and dicots (e.g. *Arabidopsis*), strains that require vernalization will flower very late, even under inductive days. In the vernalization-requiring *Arabidopsis* strains, flowering is repressed by *FRIGIDA* (*FRI*)-mediated transcription of *FLOWERING LOCUS C* (*FLC*). FLC binds directly to floral promoting genes *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) to repress their transcription (Michaels *et al.*, 2005). Prolonged exposure to cold reduces transcription of *FLC*, enabling the shoot apical meristem to undergo the floral transition (Michaels & Amasino, 1999). Typically, the vernalized state is mitotically stable through epigenetic modifications. However, vernalization may not affect all meristems identically. For example, in the perennial, iteroparious relative of *Arabidopsis*, *Arabis alpina*, vernalization renders some shoot apical meristems (SAM) competent to flower but some SAMs remain vegetative for another season of growth (Wang *et al.*, 2009).

The yellow monkeyflowers of the *Mimulus guttatus* (Phrymaceae) species complex are well suited to studies of variation in flowering time and flowering responses to environmental cues in annuals and perennials. The group contains populations and species that live in habitats that vary widely in elevation, latitude and edaphic conditions. *Mimulus guttatus* comprises distinct populations that contain individuals that are small, selfing and facultatively annual, and others that are larger, invest heavily in vegetative growth, and are perennial. The annual populations thrive in a Mediterranean climate, characterized by a wet autumn, winter and spring, followed by intense summer drought. The perennial populations of *M. guttatus* are protected from the summer drought by growing in soils that remain wet year round due to their proximity to springs or rivers. Research shows that members of this species complex have adapted to environments that vary in the seasonal availability of water (Kian & Hamrick, 1978; Vickery, 1978; Hall & Willis, 2006; Lowry *et al.*, 2008), and adaptive differences in flowering time may be a key drought avoidance strategy (Wu *et al.*, 2010).

Here, we tested for natural variation in traits related to flowering within a collection of 46 *Mimulus* populations that encompass a range of latitudes, altitudes and life histories in the *M. guttatus* species complex by imposing different photoperiod and vernalization treatments. Our goal was to quantify variation in flowering time related traits in populations from diverse habitats across the geographic range of the species. We then selected phenotypically divergent populations and created F_2 mapping populations to identify QTLs contributing to the photoperiod and vernalization differences between annual and perennial populations of *M. guttatus*. The major goals of this study were to: (1) characterize variation in photoperiod and vernalization responses across the *M. guttatus* species complex; and (2) discover QTLs associated with the variation in photoperiod and vernalization within the annual and perennial *M. guttatus*. Understanding the genetic architecture of natural variation in photoperiod and vernalization is an important step in understanding the diversity of flowering responses that is part of local adaption in the *M. guttatus* species complex.

Materials and Methods

Study species and population lines

The yellow monkeyflowers of the *Mimulus guttatus* species complex (sect. *Simiolus*, Phrymaceae) are a phenotypically diverse, yet broadly interfertile group of wildflowers with their center of diversity in western North America (Vickery, 1978; Beardsley *et al.*, 2004). The group comprises species with a variety of life history, developmental and physiological traits that enable them to occupy a broad range of habitats, ranging from coastal sand dunes to montane meadows, serpentine barrens and copper mine tailings (Wu *et al.*, 2008, and references therein). Previous experiments have shown that all plants will flower fairly rapidly when grown under long days in the glasshouse without a need for cold exposure. Moreover, we have noticed that individuals from some populations will flower under very short days. We have also observed that, for some populations, when we start plants growing in short days and move them to long days, they remain vegetative and will not flower. This suggests that there is some memory of short days that is inhibiting flowering.

We assembled a collection of four individuals from each of 46 different populations that span the geographic range and life

history, mating and edaphic conditions of species in the *M. guttatus* species complex (Fig. 1 and Table 1). These included populations from coastal perennial, inland perennial and annual populations of *Mimulus guttatus* DC that have been recognized as distinct taxonomic groups (Pennell, 1947; Lowry *et al.*, 2008): the self-fertilizing copper-tolerant *M. cupriphilis* Macnair; granite-endemic and high-altitude selfing *M. laciniatus* Gray; the selfing, serpentine *M. pardalis* Pennell; the self-fertilizing *M. nasutus* Greene; and the serpentine endemic *M. nudatus* Greene (Table 1). The edaphic specialist species occur on thin-soiled rocky outcrops that experience even earlier onset of summer drought than *M. guttatus* annual populations. Whenever possible, we chose populations so that the edaphic specialists were paired with a geographically nearby annual *M. guttatus*, and the perennial *M. guttatus* were paired with an annual *M. guttatus*, so that any trends are not the result of differences in overall geographical distribution (see Fig. 1). The classification of *M. guttatus* as either annual or perennial rests largely on the degree of investment in vegetative structures. Annual plants are diminutive, with small leaves and thin stems and have fibrous roots. Perennial plants have much larger leaves, produce rhizomes or stolons, and have thick stems. In the field, annuals die in summer due to lack of water, while perennials persist. These distinctions have been well characterized elsewhere (Hall *et al.*, 2006; Lowry *et al.*, 2008).

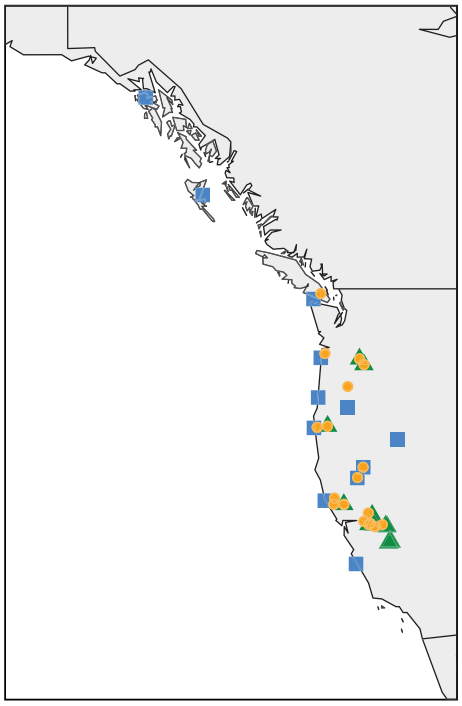

Fig. 1 Map of Western North America showing locations of populations used in this study. Orange circles are annual *Mimulus guttatus*, blue squares are perennial *M. guttatus*, and green triangles are other annual *Mimulus* species, including *M. cupriphilis, M. laciniatus, M. nasutus, M. nudatus* and *M. pardalis.* See Table 1 for full list of population locations.

Growth chamber experiments

Photoperiod In order to investigate the effect of critical photoperiod on flowering, we grew individuals in eight separate growth chamber treatments, that included day lengths of 8-, 10-, 11-, 12-, 13-, 14-, 15- and 16-h. All growth chambers were held constant at 21°C. To maximize our use of limited growth chamber space, we opted to use 46 different populations with one individual from each of four inbred maternal families per population (for a total of 184 plants per treatment). This allowed us to focus on differences between populations but still establish within-population variation. To minimize maternal effects, samples for this study originated as seeds from plants that had undergone at least two generations of self-fertilization in the glasshouse at Duke University. Seeds were planted into 10-cm (4-inch) pots filled with moist Fafard 4P potting mix, and stratified in the dark at 4°C for 1 wk. Pots were then moved into their assigned growth chamber treatment at the Duke University Phytotron. All flats were bottom-watered every day. The positions of pots within flats, and flats within the chamber, were randomized every 5 d. Individuals were monitored for germination date and the date of first flowering. If plants did not flower 14 wk after germination, we recorded them as nonflowering.

For each population, we determined the critical photoperiod required for flowering as the minimum photoperiod in which at least 50% of individuals flowered. We analysed the effects of life history, latitude and altitude (and their interactions) on critical photoperiod using general linear models in SAS (PROC GLM, SAS v9.2; SAS Institute, Cary, NC, USA). All factors were treated as fixed effects. Nonsignificant factors were removed by stepwise backward elimination. We also examined these same effects on the number of days to flowering under 16-h photoperiods, which was selected because all populations flowered at this photoperiod.

Vernalization We tested whether plants required prolonged exposure to cold (vernalization) to overcome the short-day inhibition of flowering, following our preliminary observations that plants grown under short days and then exposed to long days remained vegetative. For all populations that did not flower under the shortest day length (8-h) after 14 wk, we randomly assigned individuals within populations to one of two treatments. We moved half of the plants into a cold (4°C) 8-h day length growth chamber, where they were maintained for 6-wk, and then moved to a warm (21°C) 16-h growth chamber (this treatment tests whether plants flowered with vernalization following the short-day inhibition of flowering). The other half of the plants was moved into a warm (21°C) 16-h day length growth chamber immediately following being in the short-day warm (21°C) 8-h chamber (this treatment tests whether plants flower under long days without vernalization after short-day inhibition of flowering). We recorded whether plants flowered (and the time to flowering) for each of the treatments.

We analysed the requirement for vernalization using a generalized linear model in SAS (PROC GENMOD, SAS v9.2; SAS Institute). The data were fit to a binomial distribution with

Table 1 Populations in the *Mimulus guttatus* species complex used in the experiments investigating variation in critical photoperiod and vernalization

Species	Life history	Population	Latitude (N)	Longitude (W)	Altitude (m a.s.l.)
M. guttatus	Annual	CCC	37°58′19″	120°37′49″	270.36
M. guttatus	Annual	CCG	45°42′26″	121°21′30″	30.48
M. guttatus	Annual	COL	40°07′08″	121°29′09″	1524
M. guttatus	Annual	CSS	38°51′42″	122°24′55″	637.95
M. guttatus	Annual	HCG	42°32′41″	123°30′09″	243.84
M. guttatus	Annual	HNT	38°03′51″	120°63′58″	504.14
M. guttatus	Annual	IM	44°24′03″	122°08′57″	1432.56
M. guttatus	Annual	LMC	38°51′50″	123°05′02″	306.02
M. guttatus	Annual	MCC	37°87′37″	120°45′43″	304.80
M. guttatus	Annual	MCN	37°54′72″	120°43′44″	256.03
M. guttatus	Annual	MED	37°48′53″	120°18′42″	304.80
M. guttatus	Annual	OBR	38°69′43″	123°02′75″	91.44
M. guttatus	Annual	PTH	37°54′49″	119°48′70″	1414.88
M. guttatus	Annual	RGR	42°29′21″	124°12′30″	82.29
M. guttatus	Annual	RH	38°51′27″	122°24′48″	687.93
M. guttatus	Annual	SAM	45°57′33″	123°40′46″	556.87
M. guttatus	Annual	SBG	45°26′13″	121°03′34″	238.96
M. guttatus	Annual	SKZ	48°46′96″	123°57′17″	116.43
M. guttatus	Annual	SLP	37°87′37″	120°45′43″	304.8
M. guttatus	Annual	WKR	39°96′50″	120°66′57″	1868.42
M. guttatus	Perennial	ALA	58°00′26″	135°44′55″	18.89
M. guttatus	Perennial	BCB	36°03′46″	121°35′31″	4.57
M. guttatus	Perennial	BOG	41°55′25″	118°48′21″	1307.9
M. guttatus	Perennial	BOB	48°31′42″	124°27′03″	7.62
M. guttatus	Perennial	CH	40°06′09″	121°30′00″	1371.6
M. guttatus	Perennial	DUN	43°53′35″	124°08′16″	7.62
M. guttatus	Perennial	IMP	44°24′03″	122°09′02″	1573.38
M. guttatus	Perennial	OPB	42°27′50″	124°25′22″	7.62
M. guttatus	Perennial	OSW	45°45′39″	123°57′56″	4.57
M. guttatus	Perennial	SWB	39°02′09″	123°41′25″	4.57
M. guttatus	Perennial	TSG	53°25′07″	131°54′56″	4.57
M. guttatus	Perennial	WKQ	39°96′42″	120°66′06″	1902.26
M. cupriphilis	Annual	MCNC	37°54′72″	120°43′44″	257.56
M. laciniatus	Annual	DNK	37°05′11″	119°13′10″	1851.66
M. laciniatus	Annual	PETE	37°03′35″	119°22′12″	1256.69
M. laciniatus	Annual	SHL	37°08′68″	119°18′39″	1594.41
M. laciniatus	Annual	SNB	37°02′32″	119°24′38″	1003.40
M. laciniatus	Annual	TIGR	37°48′72″	119°30′35″	2587.45
M. laciniatus	Annual	WLF	37°50′49″	119°35′63″	2395.42
M. nasutus	Annual	CCN	45°42′26″	121°21′30″	28.96
M. nasutus	Annual	HCN	42°32′41″	123°30′09″	243.84
M. nasutus	Annual	MEN	37°80′89″	120°30′16″	289.56
M. nasutus	Annual	SBN	45°26′13″	121°03′34″	242.93
M. nudatus	Annual	CSH	38°51′40″	122°24′55″	646.18
M. pardalis	Annual	PARD	37°53′50″	120°23′40″	440.44

See Fig. 1 for map locations of each population.

logit-link function. We examined the fixed effects of life history, latitude and altitude (and their interactions). Nonsignificant factors were removed by stepwise backward elimination.

Quantitative trait locus mapping

To understand the genetic basis of the variation in photoperiod and vernalization responses we initiated QTL mapping studies. We found extensive diversity in response to photoperiod and vernalization within *M. guttatus*, largely distinguishing the annual and perennial populations (Fig. 2). Thus for this part of the project, we focused on variation within *M. guttatus*. The mapping population for the photoperiod experiment involved the parent populations LMC (a typical annual population that requires at least an 11-h day length to flower) and a geographically paired perennial population SWB (a typical perennial population requiring 15-h day lengths to flower). We crossed a highly inbred line from each population to create an F_1 hybrid. This hybrid was then selfed to produce a large number of recombinant F_2 progeny. Seeds from 992 F_2 SWB × LMC individuals, and 32 of each parent, were sown in 10-cm (4-inch) pots filled with moist Fafard 4P potting mix, and stratified in the dark at 4°C for 1 wk. Pots were then moved into a 13-h day growth chamber (constant 21°C) at Duke University. The positions of flats were randomized weekly, and plants were monitored for flowering. The experiment was terminated after 4 months.

Species	Life history	Population	Critical photoperiod 8 10 11 12 13 14 15 16	Flower without vernalization	Flower with vernalization	Mean (SE) days to flower at 16-hr
M. cupriphilis	annual	MCNC				20.33 (1.2)
M. laciniatus	annual	SNB				18.25 (0.48)
M. laciniatus	annual	PETE				17.75 (0.48)
M. laciniatus	annual	SHL		?	?	21.5 (3.5)
M. laciniatus	annual	DNK		?	?	19.33 (0.33)
M. laciniatus	annual	WLF				16.5 (0.29)
M. laciniatus	annual	TIGR		?	?	17 (1)
M. nasutus	annual	CCN				17 (0)
M. nasutus	annual	SBN				18.5 (0.29)
M. nasutus	annual	HCN				25 (2.35)
M. nasutus	annual	MEN				20 (0.58)
M. nudatus	annual	CSH				20 (0.58)
M. pardalis	annual	PARD				33 (0)
M. guttatus	annual	SKZ				20.5 (0.5)
M. guttatus	annual	SLP				24.75 (1.75)
M. guttatus	annual	MCN				19 (0.58)
M. guttatus	annual	CCG				20 (0.58)
M. guttatus	annual	CCC				20.25 (0.48)
M. guttatus	annual	LMC				21 (0.58)
M. guttatus	annual	HNT				21.75 (1.8)
M. guttatus	annual	OBR				20.5 (0.96)
M. guttatus	annual	MCC				21.33 (1.45)
M. guttatus	annual	MED				22.5 (1.66)
M. guttatus	annual	CSS				21.25 (0.25)
M. guttatus	annual	SBG		?	?	23.75 (1.89)
M. guttatus	annual	BOB		?	?	23.75 (1.11)
M. guttatus	annual	RH		?	?	22 (0)
M. guttatus	annual	HCG				24.75 (1.75)
M. guttatus	annual	SAM				23.33 (2.4)
M. guttatus	annual	RGR		?	?	24.67 (0.88)
M. guttatus	annual	IM				24.75 (2.39)
M. guttatus	annual	WKR		?	?	28.33 (1.67)
M. guttatus	annual	COL				29 (2.12)
M. guttatus	perennial	BOG				41.5 (2.5)
M. guttatus	perennial	WKQ		?	?	29.25 (1.44)
M. guttatus	perennial	DUN				37 (1)
M. guttatus	perennial	ALA				31.75 (1.38)
M. guttatus	perennial	SWB				32.75 (2.17)
M. guttatus	perennial	BCB				36.25 (2.25)
M. guttatus	perennial	OPB				37 (1.87)
M. guttatus	perennial	IMP				30.67 (2.73)
M. guttatus	perennial	OSW				45 (3)
M. guttatus	perennial	CH				42 (9.06)
M. guttatus	perennial	TSG				30 (0)

Fig. 2 Photoperiod (hours of light per 24-h day) and vernalization (6 wk duration of cold) required to induce flowering in growth chambers in one population of *Mimulus cupriphilis,* six *M. laciniatus* populations, four *M. nasutus* populations, one *M. nudatus,* one *M. pardalis*, 22 annual *M. guttatus* populations and 11 perennial *M. guttatus* populations. Solid grey indicate that 50% or more of individuals flowered, hatched areas indicate that <50% of individuals flowered. White (empty) cells indicate that no individuals flowered. Question marks signify populations that were not included in a particular treatment. See text for details on the vernalization experiments. The far-right column shows the mean number of days post-germination required for flowering under 16-h of light. Populations are ranked by their tendency to flower under short day lengths, except *M. laciniatus* populations, which are ranked by increasing altitude.

For the vernalization experiments we created two separate F_2 mapping populations: the first population was from a cross between two annual populations (LMC and IM), the second from a cross between two perennial populations (BOG and DUN). In each case one parent population did not require vernalization to flower following exposure to short days (the first one listed) and the other parent population required vernalization. For each cross, we used a highly inbred parental line from each

population, and crossed them to create an F_1 hybrid. A single F_1 plant was then selfed to create a population of F_2 plants used for mapping. Seeds from 960 F_2 LMC × IM, 864 F_2 BOG × DUN and 24 from each of the four parents, were sown using the same methods as above. Plants were maintained in a growth chamber with 8-h days for 6 wk (constant 21°C), and then switched to 16-h days (constant 21°C). Plants were monitored for flowering for 4 months subsequent to the long-day switch. The two vernalization mapping experiments were carried out simultaneously in the same walk-in growth chamber.

Mapping using bulk segregant analysis and next-gen sequencing We used a bulk segregant analysis (BSA) to identify genomic regions containing loci affecting our trait of interest (photoperiod or vernalization sensitivity). For each phenotypic trait, we created two pools (bulks) of individuals: those that flowered, and those that remained vegetative. For each bulk we collected a single flowering bud or small leaf from 160 flowering plants and 160 vegetative plants. We pooled tissue for 32 individuals into a single 15-ml tube and froze it at −80°C until we were ready for extraction. We ground tissue with liquid nitrogen using a mortar and pestle. We used 0.2 g of this tissue pooled across the tubes of tissue within a flowering class for a total of 1 g of ground tissue. This meant that for each of the three experiments (one photoperiod experiment and two vernalization experiments) we had two sets of ground tissue containing one bulk of plants that flowered and one bulk of plants that remained vegetative. We used a modified CTAB extraction protocol to extract genomic DNA (Kelly & Willis, 1998). Each pool of genomic DNA was sequenced on an Illumina GAII machine using single-end reads at the Duke University Genome Sequencing and Analysis Core Resource.

Statistical analysis

Bulk Segregant Analysis (BSA) has long been used to quickly but crudely map major QTLs using traditional markers, and with genomic techniques that rapidly quantify allele frequencies at densely spaced markers, QTLs can be mapped with much greater precision (Magwene *et al.*, 2011). We began by aligning each Illumina read file to the *Mimulus guttatus* reference genome (www.phytozome.net) using BWA (Li & Durbin, 2010), and used SAMtools (Li *et al.*, 2009) to create a pileup file of the combined read files for each experiment. We ignored positions with < 4× coverage and if only one or more than two alleles segregate at a given site. Using the allele counts at each SNP for all pileup files, we used a sliding window analysis with window sizes of 50 SNPs to calculate the frequency of SNPs from each parent (B. K. Blackman *et al.*, unpublished). We used a sliding window approach to accommodate for low coverage and low read counts for any given SNP. Differences in allele frequencies between the two bulks are expected to be close to zero at neutral unlinked markers, while allele frequency differences will increase in markers closely linked to the underlying QTLs. We considered an allele frequency difference of 0.2 as the threshold to select potential markers linked to QTLs, which were further validated by additional PCR-based markers (details below). For the LMC × SWB photoperiod experiment, we only sequenced the flowering pool for the bulk segregant analysis and looked for regions of the genome that were enriched for alleles from the flowering parent (LMC).

Bulk segregant analysis does not provide information about the phenotypic effects of each QTL genotype, since it is based solely on differences in allele frequencies. In order to determine the genotypic effects at each QTL, we followed up on the bulk segregant analysis by genotyping either 192 or 384 random individuals (both flowering and nonflowering) from each F_2 mapping population for markers known to occur in the QTL region. We first screened the inbred parental lines for polymorphism using exon-primed intron-crossing (EPIC) markers derived from expressed sequence tags (ESTs). Polymorphism was evaluated in terms of variation in the length of PCR products, which is typically caused by indel variation in the introns. The development of these markers is outlined elsewhere (Fishman *et al.*, 2008) and primers can be found at the website (http://www.mimulusevolution.org). The PCR products were subjected to capillary electrophoresis and fragment analysis on an ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA). The size of the amplified fragments was scored using the program GeneMarker (SoftGenetics, State College, PA, USA). We estimate the relative effect of QTL by calculating the proportion of variation in flowering explained by the three genotypes, and assessed its significance using a χ^2 test of association. We calculated the genotypic value of the three genotypes at each marker as the proportion of individuals with that genotype that flowered. For traits that involved more than one large QTL, we assessed epistatic interactions using loglinear models in SAS (PROC CATMOD; Stokes *et al.*, 2000) and modeled the probability of either flowering or being vegetative as a function of genotype at each locus.

Results

Growth chamber experiment

Photoperiod and flowering time We found tremendous variation in the critical photoperiod required for flowering (Fig. 2). Some of the edaphic specialists, like *Mimulus cupriphilis*, *M. nudatus* and some of the annual *M. guttatus* populations

Table 2 Summary of general linear models of the influences on critical photoperiod and vernalization on flowering in the *Mimulus guttatus* species complex

Source of variation	Critical photoperiod	Vernalization
Life history	$F_{1,166} = 93.75$***	$F_{1,105} = 96.77$***
Altitude	$F_{1,166} = 16.46$***	$F_{1,105} = 3.99$*
Life history × Altitude	$F_{1,166} = 11.96$***	

Latitude (and its interactions) were considered initially, but then excluded from the analyses because they did not affect the dependent variable significantly.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

flowered with as little as 8 h of light. In contrast, the perennial populations typically required much longer day lengths for floral induction. Overall, there was a significant effect of life history, altitude and a life history × altitude interaction on critical photoperiod (Table 2). In general the edaphic specialists and annual populations flowered under shorter day lengths than the perennial *M. guttatus* (annual: 11.72 ± 0.15; perennial: 14.63 ± 0.26 (LS Mean ± SE); $F_{1,166} = 93.75$, $P < 0.0001$), and populations at higher elevations required longer photoperiods ($F_{1,166} = 16.46$, $P < 0.0001$). The significant life history × altitude interaction arises because annual populations at low elevations flower significantly earlier than those at high elevations – the three annual populations requiring the longest photoperiods (IM, WKR and COL) are all high-altitude populations. For perennial populations there is no effect of altitude on critical photoperiod. There is no significant effect of latitude or any of its interactions on critical photoperiod.

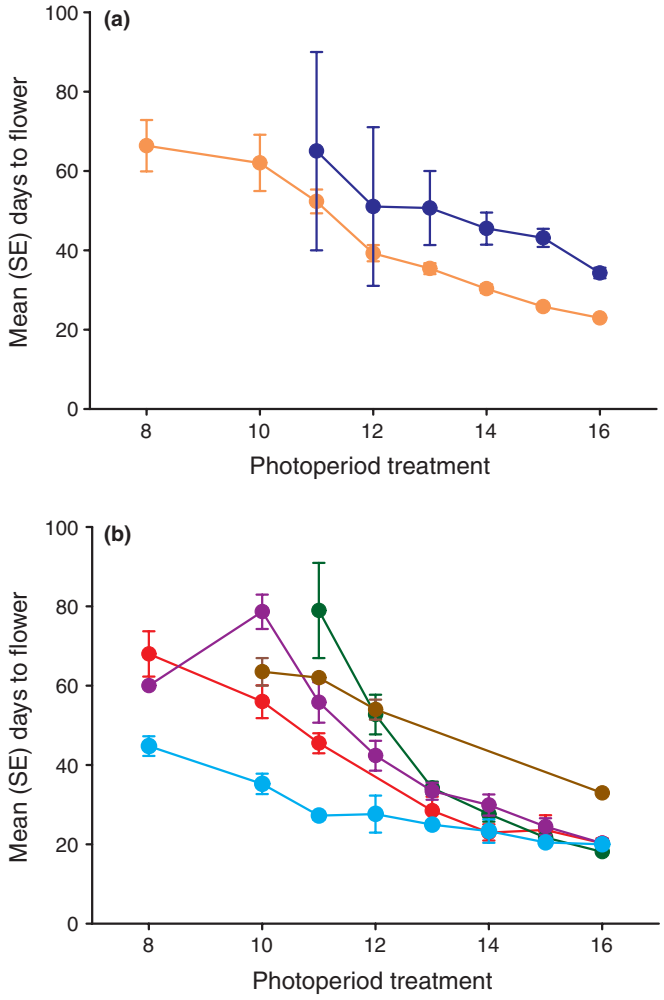

Fig. 3 Relation between photoperiod treatment and mean (SE) days-to-flower for (a) *Mimulus guttatus* annual (orange) and perennial (blue) populations and (b) populations from six edaphic specialist species in the *Mimulus guttatus* species complex – *M. cupriphilis* (red), *M. laciniatus* (green), *M. nasutus* (purple), *M. nudatus* (blue), *M. pardalis* (brown).

The edaphic specialists and annual *M. guttatus* also flower much more rapidly than perennial *M. guttatus*. The number of days to flowering (at 16 h photoperiod) is significantly lower for the annual populations than the perennial populations (annual: 22.87 ± 0.69 d; perennial: 34.20 ± 0.92 d (LS Mean ± SE); $F_{1,105} = 96.77$, $P < 0.0001$). There was a weak significant effect of altitude ($F_{1,105} = 3.99$, $P < 0.05$), but no effect of latitude or any interactions. Although quite variable, there is a linear decrease in the number of days to flowering as photoperiod increases (Fig. 3a), so that all plants flower more rapidly under longer day lengths. Also, at the shorter photoperiods there is substantially more variation in days-to-flower between lines within a population, and between populations within each species or life history group (Fig. 3b).

Vernalization When grown under 16-h days all plants flower rather quickly (Fig. 2). There appears to be no vernalization requirement like that found in *Arabidopsis* or wheat, where strains that require vernalization flower very late under long days if they do not first experience cold. However, for the first time, we discovered a vernalization requirement in the *M. guttatus* complex. This type of vernalization – contingent on experiencing short days first – is quite distinct from the universal requirement that has been studied in other species. Furthermore, almost all of the variation in whether plants required vernalization to flower after experiencing short days could be explained by life history (Fig. 2; Table 2). All of the annual populations (except for the high-altitude IM and COL) and none of the perennial populations (except for BOG) flowered without cold treatment (main effect of life history $\chi^2 = 18.93$, $P < 0.0001$). Interestingly, BOG is unique amongst the perennial populations in that it occurs in a hot-spring environment where it may not experience cold temperatures. There was a weakly significant effect of altitude, and no effect of latitude or any of their interactions on vernalization requirement. All individuals flowered if they were exposed to a 6-wk cold treatment before experiencing long days (Fig. 2).

Quantitative trait locus analyses

Critical photoperiod QTL In order to commence mapping QTLs underlying the variation in critical photoperiod, we grew 32 LMC plants, 32 SWB plants and 992 F_2 individuals in a growth chamber with a 13-h day length. Consistent with our earlier photoperiod experiment, all of the LMC plants flowered within 3–4 wk of germination, and none of the SWB plants flowered after 4 months. Of the F_2 plants, 326 individuals flowered (32.86%). This distribution of flowering in the F_2s suggest that critical photoperiod has a fairly simple genetic basis. We identified two putative QTLs in the LMC × SWB F_2 mapping population affecting flowering under 13-h days on the basis of allele frequency differences in the bulk segregant analysis (Supporting Information Fig. S1). Both QTLs are on Linkage Group 8 and correspond to two previously identified pleiotropic QTLs in a different *M. guttatus* annual × perennial cross (IM × DUN; Hall *et al.*, 2006; Hall *et al.* 2010). Although the two QTLs are on the

Table 3 Marker details and effect sizes for QTLs in *Mimulus guttatus* critical photoperiod and vernalization mapping experiments

Experiment	Marker	Linkage group	Position (bp)	Proportion of variation explained by genotype	Test of association	Probability of flowering for G_{11} (flowering parent allele)	Probability of flowering for G_{12}	Probability of flowering for G_{22} (nonflowering parent allele)
Photoperiod	MgSTS675	8	2 044 482	0.39	$\chi^2_2 = 145.53$***	0.736	0.50	0.025
LMC × SWB	MgSTS76	8	22 971 814	0.17	$\chi^2_2 = 63.96$***	0.620	0.25	0.063
Vernalization	Mgv1a24042	8	23774162	0.20	$\chi^2_2 = 42.67$***	0.879	0.468	0.270
LMC × IM	Mgv1a022347	11	14 640 430	0.46	$\chi^2_2 = 116.06$***	1.00	0.407	0.063
Vernalization	MgSTS230	6	18 948 887	0.16	$\chi^2_2 = 41.63$***	0.214	0.546	0.879
BOG × DUN	MgSTS122	5	16 644 667	0.09	$\chi^2_2 = 21.18$***	0.769	0.611	0.288

For each significant QTL, the marker that explained the largest proportion of variation is listed.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

same linkage group, they are essentially unlinked to each other because recombination between markers in the two regions is 0.4. In univariate analyses, the first QTL explains 39% of the variation in critical photoperiod (details in Table 3), and the second accounts for 17% of the variation. In both cases, the heterozygote is intermediate between the two homozygotes in the proportion of individuals flowering. There was a significant epistatic interaction between the two loci (LG8a × LG8b: $\chi^2_4 = 15.20$; $P < 0.005$; Fig. 4a). Additionally, individuals that were homozygous for the flowering parent allele at both loci almost always flowered (19/20 = 0.95 probability; Fig. 4a) and individuals that were homozygous at both markers for the nonflowering parent allele almost never flowered (1/43 = 0.02 probability of flowering).

Vernalization QTL We performed two sets of mapping experiments for the vernalization experiments, the first with F_2s created from two annual parents (LMC and IM) and the second with F_2s from two perennial parents (BOG and DUN). Consistent with our previous study on vernalization, all of the 24 LMC parent plants flowered within 3–5 wk (mean: 27 d) of the transition to long days, and none of the IM parents flowered. Of the 960 F_2 plants, 509 plants flowered (53%) with a mean ± SE of 30.39 ± 0.31 (min 15 d, max 56 d) to flower following the switch to long days. Twenty-three of the 24 BOG parent plants flowered (96%), and took an average of 44 d to flower, and 2 of the 24 (8%) DUN parents flowered after an average of 52 d. Of the 864 BOG × DUN F_2 plants, 672 plants flowered (78%). They took a mean ± SE of 40.96 ± 0.36 (min 23 d, max 65 d) to flower after the switch to long days. The distributions of flowering in both sets of F_2s suggest a fairly simple genetic basis.

In each mapping population we discovered different QTLs underlying the requirement for flowering (Fig. S2). In LMC × IM, we discovered a large-effect QTL on LG 8 (in the same region as the LG 8b QTL from the photoperiod experiment) and a broad region of interest on LG 11. Based on univariate marker analysis, the QTL on LG 8 explains at least 20% of the variation, with individuals homozygous for the flowering parent allele having a probability of 0.88 of flowering (Table 3). In LG 11, we examined three markers in the regions where the peaks were most elevated (see Fig. S2). These three markers all showed a highly significant association with flowering. Both of the two markers with greatest effect explained upwards of 45% of the variation, and 100% of the individuals homozygous for the flowering-parent allele at marker *mgv1a022347m* (LG11) flowered (Table 3). For both QTLs, heterozygotes are intermediate between the two homozygotes for flowering. There is evidence for epistasis between the two QTLs (LG8 × LG11: $\chi^2_4 = 11.56$; $P < 0.05$; Fig. 4b). However, the distributions of phenotypes for the two-locus genotypes (Fig. 4b) suggests that the genotype at the QTL on LG 8 is most relevant when individuals carry at least one allele for the nonflowering parent at the QTL on LG 11.

In the BOG × DUN F_2s we found two significant QTLs – one on LG 5 and one on LG 6, acting in opposite directions. The QTL on LG 5 explains *c.* 9% of the variation in flowering, with individuals homozygous for the flowering parent allele having a probability of 0.77 of flowering. The QTL on LG 6 is negative – that is individuals homozygous for the nonflowering parent allele flowered with a probability of 0.88. There is no evidence for epistasis between the two regions (LG5 × LG6: $\chi^2_4 = 3.81$; $P = 0.4$; Fig. 4c).

Discussion

We discovered substantial variation across the *Mimulus guttatus* species complex for critical photoperiod and also identified a novel type of vernalization requirement for the transition from vegetative growth to flowering. Most of the variation lay between the early flowering edaphic specialist species and the life history ecotypes of *M. guttatus,* although in some cases, the variation in critical photoperiod was associated with the altitude of the populations (e.g. longer photoperiods required by the high alpine *M. guttatus* annual populations IM, COL and WKR, and within *M. laciniatus*). Within *M. guttatus*, the high-altitude annual IM population and the perennial populations were the only ones that required vernalization to overcome the short-day inhibition of flowering. We then showed that the difference in critical photoperiod between an annual and perennial population can be explained by a few large-effect QTLs, and differences in vernalization requirement between two annual populations and two perennial populations involve different QTLs, both of large effect.

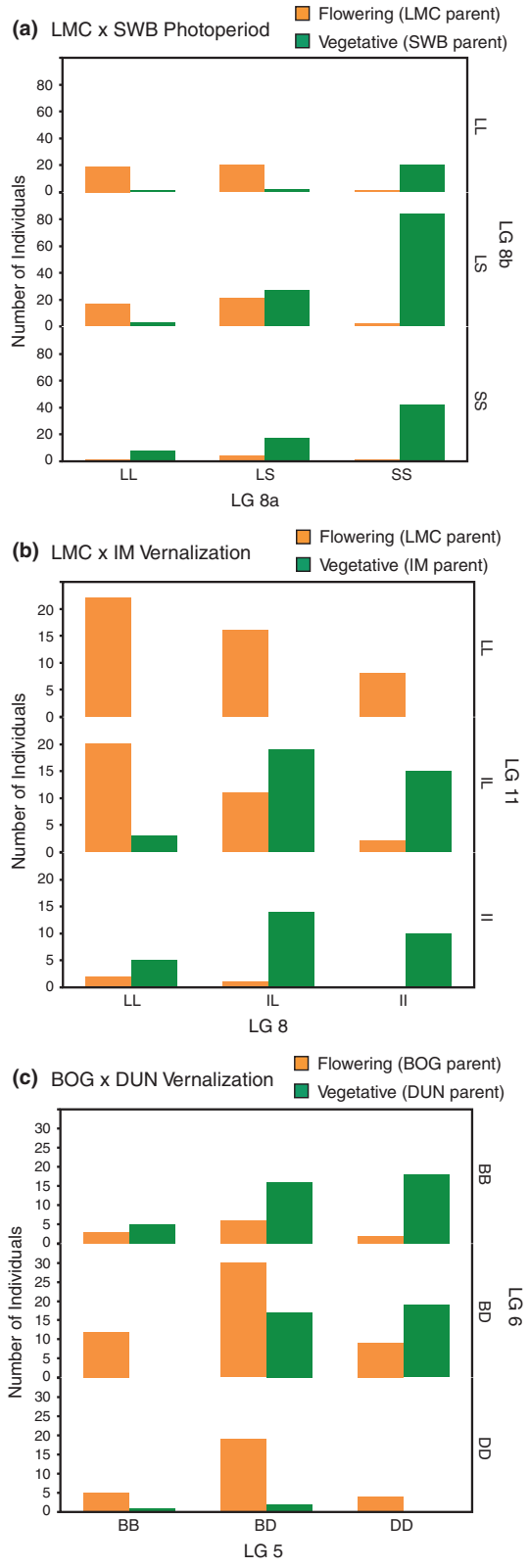

Fig. 4 Number of individuals (of those genotyped) that flowered (orange) or remained vegetative (green) for each of the 2-locus genotypes for the two significant QTLs in *Mimulus guttatus* for (a) LMC × SWB photoperiod experiment at 13 h; (b) LMC × IM vernalization experiment and (c) BOG × DUN vernalization experiment. See text for details on the significance of the epistatic interaction between the 2 QTLs.

Responses across the *M. guttatus* species complex

Temperate plants often use photoperiod as a reliable cue to signal the time to transition to flowering. In *Arabidopsis*, a latitudinal cline in flowering time and critical photoperiod ensures that populations that vary latitudinally flower during the appropriate season (Caicedo *et al.*, 2004; Stinchcombe *et al.*, 2004). Unlike this situation, our data show no evidence of a latitudinal effect of critical photoperiod on days-to-flower, although with more systematic and focused sampling within life history types a latitudinal cline might be evident. We do, however, find a significant effect of altitude (high-altitude populations flower later), which is closely related to the timing of snowmelt. The variation in critical photoperiod across the 46 populations is best explained by a combination of life history differences and fine-scale local microclimate. The annual selfing species (*M. nudatus*, *M. laciniatus*, *M. curpriphilis*, *M. pardalis* and *M. nasutus*) tend to occur in drier microhabitats and more rapidly draining soils than their *M. guttatus* counterparts.

The annual populations of the *M. guttatus* species complex thrive in a Mediterranean climate, characterized by a wet autumn, winter and spring, followed by summer drought. The timing of summer drought is driven largely by soil characteristics, where the thin soils of copper mines and serpentine sites dry out more rapidly. Even when the selfing species occur sympatrically with *M. guttatus*, they can be found on thin-soiled rocky outcrops embedded within a wet meadow. The perennial populations of *M. guttatus* are shielded from the summer drought by growing in soils that are wet all year round due to their proximity to springs or rivers. The ability to flower under shorter photoperiods means that the annual populations can flower and produce viable seeds before the onset of drought-induced senescence. In rapidly drying conditions, early spring flowering is expected to maximize the time available for reproduction and be selected as a drought-escape mechanism (Wu *et al.*, 2010).

In much of western North America, climate change models predict that growing seasons will shift earlier and become shorter due to warmer temperatures, reductions in rain and snowfall, earlier snow melt, and more prolonged and intense droughts (USGCRP Report 2009). In species like *Mimulus* with broad geographic distributions, these changes in growing season are somewhat paralleled by current variation in the onset of drought or winter due to differences in altitude, latitude and edaphic conditions throughout the range. The result is a cline in flowering time and critical photoperiod that may be driven by strong selection in each environment. Studies of natural populations in other groups have found a diversity of alterations in the flowering time gene network that underlie clinal adaptive variation (Etterson, 2004; Lempe *et al.*, 2005; Böhlenius *et al.*, 2006; Izawa, 2007). Understanding the phenotypic and genetic differences of populations varying in local edaphic conditions can inform our understanding of the response of populations through time.

One of the implications of the different critical photoperiods of populations and/or closely related species is that reproductive asynchrony can cause prezygotic isolation between sympatric populations. Although different critical photoperiods may be

due to selection for adaptation to microclimate, it might also promote reproductive isolation if flowering phenologies no longer overlap, facilitating local adaptation by preventing gene flow. This is particularly apparent for the sympatric pairs of *M. guttatus* and *M. nasutus* (e.g. populations MED and MEN, HCG and HCN, and SBG and SBN – see Fig. 1 for overlapping *M. guttatus* annual (orange circle) and other *Mimulus* annual (green triangle) populations), where the *M. nasutus* population consistently flowers under shorter days than the *M. guttatus* population. Martin & Willis (2007) found that divergent flowering phenologies between sympatric *M. guttatus* and *M. nasutus* (MED and MEN, respectively) reduced the chance for F_1 hybridization by as much as 70%. The mean flowering date for *M. nasutus* was 10 April, while that for *M. guttatus* was 28 April. At this site, this difference represents *c.* 40 min of day length. Fenster *et al.* (1995) state that the proximate cause of earlier flowering in *M. nasutus* is not known, but it may be the result of more rapid flower development, or differential flowering responses to environmental cues such as day length. Our data strongly supports the latter hypothesis.

Critical photoperiod for flowering in *M. guttatus*

We found substantial variation for critical photoperiod among the annual populations of *M. guttatus*, with almost continuous variation in the number of hours of light required to induce flowering. However, within a single population critical photoperiod acts as a threshold character with very little variation between individuals. It is currently unclear whether different genes are involved in the different populations. If the same genes are involved, it may be that adaptive alleles successively replace each other at a single locus to produce an allelic series resulting in continuous variation in this threshold character.

Interestingly, there is much less variation in critical photoperiod between the different perennial populations, suggesting that they may harbour less allelic variation.

QTL analysis of a nearly sympatric pair of annual and perennial populations (LMC × SWB) indicates that two QTLs on LG 8 underlie almost all of the variation in critical photoperiod. The two critical photoperiod QTLs occur in the same broad chromosomal region as QTLs for flowering time in a different mapping population in *M. guttatus* grown under long days (IM × DUN; Hall & Willis, 2006). At this point, these chromosomal regions are very large and neither one of these traits has been fine mapped, so there might be multiple genes in each QTL. However, the pattern suggests that the genetic architecture underlying days-to-flower might be related to the genetic architecture for critical photoperiod. In our study of 45 populations from across the *M. guttatus* complex, we found that if populations flowered under short days, they also tend to flower more rapidly (Fig. 3), suggesting that the two traits are also phenotypically correlated.

The first QTL region on LG 8 overlaps with a known inversion polymorphism that distinguishes annual and perennial populations (Lowry & Willis, 2010). In their study, Lowry & Willis (2010) demonstrate that the inversion polymorphism affects flowering time divergence and other morphological traits in the same cross between LMC and SWB, both in the glasshouse and using NILs in the field. They did not study critical photoperiod, but our data suggest that this inversion region underlies variation in critical photoperiod that distinguishes this sympatric annual and perennial population. However there are not obvious candidate genes from the *Arabidopsis* flowering time pathway that occur in this inverted region, but putative homologs of *GAI* (Gibberellic Acid Insensitive – mgv1a024641m), *VRN1* (Reduced Vernalization 1 – mgv11b018073m) and *SVP* (Short Vegetative Phase – mgv1a021820m) occur in the other region (LG 8b).

The discovery of a vernalization requirement in *M. guttatus*

We discovered a novel requirement for cold chilling in *M. guttatus*, which is contingent on plants first experiencing short days. As far as we know, this type of short-day dependent vernalization requirement is unique in the angiosperms. Before this experiment, we had not explored vernalization in *Mimulus*, because plants grown in constant long-day conditions show no requirement for chilling to accelerate flowering. In perennial *M. guttatus* flowering is inhibited under short days, and the repression is maintained even when plants move into inductive long days. This suggests that there is a memory of short days that requires a period of cold-chilling to inactivate.

Interestingly, in our two mapping populations (one comprised a cross between two perennials, and the other a cross between two annuals), we found different QTLs for vernalization. This might suggest that the vernalization pathway is quite flexible and that there are different ways to achieve the same phenotype. In *A. thaliana*, the ancestral state is vernalization-requiring even when plants are grown under long days. Rapid-flowering types have arisen independently several times from loss-of-function mutations (Johanson *et al.*, 2000), with various adaptive explanations for their origin (Brock *et al.*, 2009; Scarcelli & Kover, 2009; Wilczek *et al.*, 2009). Presumably the ancestral state in *M. guttatus* is a perennial, requiring vernalization to flower (although this is currently unknown). This might suggest that the annual IM population has retained the vernalization requirement, perhaps because it is under snow during the short days of spring. For the rest of the rapidly flowering annuals there may have been selection against the cold-chilling requirement as it might prevent individuals that germinate in the early spring under short days from flowering the same season.

The clear difference in vernalization requirement between annual and perennial *M. guttatus* may be adaptive, because low-elevation annuals are selected to flower in the spring under shorter days, whereas perennials will benefit from switching from flowering to vegetative growth as day length shortens in the fall, and continue vegetative growth until the following summer when flowering is optimal (i.e. overwinter before flowering). The precise manner in which this may be adaptive is not yet entirely clear, and of course our studies were conducted in discrete controlled environments that do not involve all of the complexities of the cyclical nature of the seasons. Further studies in growth

chambers and in native field sites should shed light on these issues and might also reveal whether there is variation in the duration of cold required.

In *Arabidopsis*, varietal differences in the requirement for cold-chilling before flowering are largely the result of allelic variation at *FLC* and/or *FRI*. Direct homologs of *FRI* and *FLC* are absent in *Mimulus*. In one of our mapping populations (LMC × IM) we detected a highly significant QTL in the region containing *FLC*-like genes in the *MADS AFFECTING FLOWERING* clade (LG 11 – e.g. mgv1a014602m), which is epistatic on the QTL at LG 8. Similar major epistatic behaviors have been described for *Arabidopsis* genes *FLC* and *FRI* and for wheat genes *VRN1* and *VRN2* (Andrés & Coupland, 2012). It remains unknown if the epistatic behavior of *M. guttatus* resembles the epistasis in wheat or *Arabidopsis* because it is not yet known if the epistatic locus (LG11) is a flowering promoter (like wheat *VRN1*) or a repressor (like *Arabidopsis FLC*). The QTL on LG 8 in the LMC × IM cross occurs in the genomic region that contains genes that show homology to the *Arabidopsis* genes *GAI, VRN1* and *SVP* (mgv1a024641m, mgv11b018073m and mgv1a021820m, respectively). In *Arabidopsis*, *VRN1* functions in stable repression of the floral repressor *FLC* in the vernalization pathway (Levy *et al.*, 2002), while *SVP* binds to *FT* and *SOC1* (the same targets as *FLC*; Lee *et al.*, 2007), and *GAI* represses gibberellic acid signaling and interferes with flowering, particularly under short days (Wilson *et al.*, 1992).

In the BOG × DUN population we found two different QTLs – a positive one on LG 5 and a negative one on LG 6. It is still unclear to us the action of the negative QTL on LG 6, and why BOG parents that carry these alleles flower, while the F_2 plants with the same alleles rarely flower (Fig. 3c). Perhaps there are other uncharacterized QTLs of more minor effect that are interacting with these genes, so that the two detected QTLs are not sufficient to explain the behavior of the parents. The QTL on LG 6 colocalizes with genes that show homology to *FT* (mgv1a023027m) and *FLD* (mgv1a001229m.) *FT* is a well-characterized floral promoter, and *FLD* has been implicated in the transition from rosette to inflorescence (Yang & Chou, 1999). The QTL on LG 5 lies in the genomic region that includes genes which show homology to *SPA2* (mgv1a000578m), *GA1* (mgv1a024771m) and *GA2* (mgv1a006406m) – all genes that have been implicated in the *Arabidopsis* flowering pathway. Clearly, however, QTL co-localization with candidate genes requires further fine mapping and subsequent experimental verification.

Differences between flowering in annual and perennial populations

Regulation of seasonal patterns of vegetative growth and flowering in perennial plants is much more poorly understood than in annuals and studies have mostly been restricted to agriculturally important species, and generally describe the involvement of homologs of *A. thaliana* genes in regulating perennial-specific traits related to flowering (Albani & Coupland, 2010). In a recent study of *Arabis alpina*, a perennial relative of *A. thaliana*, chromatin modifications of H3 increase during vernalization but do not persist after vernalization, causing repeated seasonal cycles of repression and activation of *PEP1* (ortholog of *FLC*) transcription that cause the characteristic cyclical perennial life history. Apical meristems of the main and axillary shoots that are present before vernalization produce flower buds during the cold period that develop into flowers when temperatures increase. Meristems that originate after the onset of vernalization remain vegetative (Wang *et al.*, 2009). This might be key, because the perennial strategy requires differential behavior of meristems on a single plant so that some remain in the vegetative state while others undergo the floral transition, or inflorescence meristems must revert back to vegetative growth after flowering (Tooke *et al.*, 2005).

The timing of floral initiation, and the return to vegetative development after flowering, are major determinants of life history strategy and distinguishing features between annual and perennial *M. guttatus*. In our large survey of critical photoperiod and vernalization requirement in the *M. guttatus* species complex, the greatest variation lay between life history strategies. Our QTL analyses indicate that for both critical photoperiod and vernalization, large-effect QTLs underlie the intraspecific divergence. Furthermore, most of these genomic regions contain genes that show homology to known *Arabidopsis* flowering time genes. The ability to map differences in the seasonal cues used by annuals and perennials in intraspecific crosses provides great promise for identifying the key features and genes that characterize life history strategies. Along with other studies focusing on perennial plants (Böhlenius *et al.* 2006; Wang *et al.*, 2009; Anderson *et al.*, 2011), we are beginning to achieve a greater understanding of the mechanisms involved in perennial flowering.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 QTL results from BSA analysis for critical photoperiod in LMC × SWB mapping population.

Fig. S2 QTL results from BSA analysis for vernalization requirement in (a) IM × LMC and (b) BOG × DUN mapping populations.

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