Molecular Ecology (2003) **12**, 1137–1151

Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits

J. K. MCKAY,*†§ J. H. RICHARDS‡ and T. MITCHELL-OLDS†
Biological Sciences, University of Montana, Missoula, USA; †Department of Genetics and Evolution, Max-Planck-Institute for Chemical Ecology, Jena, Germany; ‡Land, Air and Water Resources, University of California, Davis, USA

Abstract

We examined patterns of genetic variance and covariance in two traits (i) carbon stable isotope ratio $\delta^{13}C$ (dehydration avoidance) and (ii) time to flowering (drought escape), both of which are putative adaptations to local water availability. Greenhouse screening of 39 genotypes of *Arabidopsis thaliana* native to habitats spanning a wide range of climatic conditions, revealed a highly significant positive genetic correlation between $\delta^{13}C$ and flowering time. Studies in a range of C_3 annuals have also reported large positive correlations, suggesting the presence of a genetically based trade-off between mechanisms of dehydration avoidance ($\delta^{13}C$) and drought escape (early flowering). We examined the contribution of pleiotropy by using a combination of mutant and near-isogenic lines to test for positive mutational covariance between $\delta^{13}C$ and flowering time. Ecophysiological mutants generally showed variation in $\delta^{13}C$ but not flowering time. However, flowering time mutants generally demonstrated pleiotropic effects consistent with natural variation. Mutations that caused later flowering also typically resulted in less negative $\delta^{13}C$ and thus probably higher water use efficiency. We found strong evidence for pleiotropy using near-isogenic lines of *Frigida* and *Flowering Locus C*, cloned loci known to be responsible for natural variation in flowering time. These data suggest the correlated evolution of $\delta^{13}C$ and flowering time is explained in part by the fixation of pleiotropic alleles that alter both $\delta^{13}C$ and time to flowering.

Keywords: carbon isotope ratio, correlated traits, flowering locus c, flowering time, frigida, pleiotropy

Received 11 September 2002; revision received 24 January 2003; accepted 18 February 2003

Introduction

The study of climatic adaptation began in plants over 250 years ago (Langlet 1971; Matyas 1996), and today remains a principal theoretical and applied interest in all areas of plant biology including agriculture, ecology, evolution, functional genetics and physiology (Darwin 1859; Turesson 1922; Clausen *et al*. 1948; Comstock & Ehleringer 1992; Bohnert *et al*. 1995; Dudley 1996; Ingram & Bartels 1996; Bray 1997). As sessile organisms, plants are exposed to all extremes of their environment at some stage of their life history, and therefore ecotypic differentiation (Turesson 1922; Clausen & Heisey 1958; Linhart & Grant 1996) among populations is not surprising. In addition, reciprocal transplant and phytometer experiments demonstrate that selection coefficients for local adaptation can be quite strong (Harlan & Martini 1938; Clausen & Heisey 1958; Schemske 1984; Jordan 1991; Nagy 1997). This long history of research on the genetics of ecological races within plant species provides overwhelming support for climate as a selective pressure to which populations adapt locally. Clearly, plant populations experience spatially and temporally varying episodes of multivariate selection (Clausen & Heisey 1958; Berg 1960; Nagy 1997).

Despite compelling evidence that local adaptation is common in plant populations, little is known about the specific combinations of traits (multidimensional phenotypes) involved in adaptation to climate (Clausen & Heisey 1958; Arntz & Delph 2001). Even less is known regarding the

Correspondence: J. K. McKay. §Present address: Agronomy and Range Science & Center for Population Biology, One Shields Ave. UC Davis, Davis, CA 95616, USA. Fax: +1 530 752 4361; E-mail: jkmckay@ucdavis.edu

evolutionary genetics of traits that contribute to the process of climatic adaptation, particularly the genetic basis of trait correlations (Clausen & Heisey 1958). Genetic studies are necessary to distinguish the degree to which selection, drift, pleiotropy and recombination contribute to the evolution of individual traits and correlated suites of traits thought to be important in adaptation. Here we focus on drought, as drought stress is a major consequence of climate, and a set of candidate traits may contribute to drought adaptation in plants (Richards 1996).

Drought adaptation

Water availability imposes strong and recurring selective pressure, as it is fundamental to almost all aspects of plant physiology (Stebbins 1952; Bohnert *et al*. 1995; Bray 1997). This is evident by the fact that plant distribution and abundance in natural systems and crop yield in agricultural systems are largely determined by water availability. Plant adaptation to drought involves both phenological and physiological traits (Passioura 1996; Araus *et al*. 2002). Ludlow (1989) described three general strategies that plants have evolved to cope with drought stress: *dehydration tolerance*, *dehydration avoidance* and *drought escape*. These strategies are defined by the water status of both the plant and the environment. *Dehydration tolerance* (Ludlow 1989) refers to plants in dry environments that actually survive internal water deficits, with an extreme example being resurrection plants that tolerate losing 95% of leaf water content (Scott 2000). *Dehydration avoidan*ce (Ludlow 1989) involves maintaining internal water status in a dry environment by minimizing water loss and/or maximizing water uptake. Dehydration avoiders are exemplified by succulent CAM species and (to a lesser degree) C_3 plants. The *drought escape* strategy is attained through a short life cycle (e.g. annuals) or growing season (e.g. drought deciduous), allowing plants to reproduce before the environment becomes dry. Drought escape is often employed in agricultural breeding by selecting plants that flower and fruit early enough to avoid drought (Passioura 1996; Richards 1996; Araus *et al*. 2002) and is also common in natural populations (Fox 1990; Geber & Dawson 1997). Here we focus on the latter two strategies, dehydration avoidance (water use efficiency) and drought escape (early flowering), as these are most relevant to an annual C_3 plant.

Water use efficiency

Carbon dioxide uptake and water loss to the air (transpiration) both occur through the stomata, pores in leaves allowing gas exchange by diffusion between the environment and the mesophyll. This shared site of exchange results in a trade-off between acquiring CO_2 for growth and losing water. In habitats where plants encounter seasonal or chronic drought, selection has been shown to favour individuals able to minimize the trade-off between growth and water loss (Dudley 1996), resulting in populations adapted to drought. This trade-off has been credited in the evolution of the variety of strategies for reducing water loss among taxa (Ingram & Bartels 1996; Bray 1997), including the evolution of alternative photosynthetic pathways (CAM and C_4), which reduce the coupling of CO_2 uptake from loss of water to the air (Ehleringer & Monson 1993).

For short-lived annual C_3 plants, dehydration avoidance can be achieved by stomatal closure. This minimizes water loss and can be a rapid and effective dehydration avoidance strategy. However, stomatal CO_2 uptake is also reduced and thus stomatal closure will limit photosynthetic assimilation and growth (Schulze *et al*. 1987; Geber & Dawson 1997). The efficiency at which plants fix CO_2 relative to their rate of H_2O loss is called water use efficiency (*WUE*), and is used an indicator of the ability to which plants resolve the trade-off between CO_2 uptake and H_2O loss.

Instantaneous *WUE* can be calculated empirically as the ratio of CO_2 assimilation (A) to transpirational H_2O loss (E).

$$WUE = \frac{A}{E} = \frac{Ca\left(1 - \frac{Ci}{Ca}\right)}{1.6v} \quad (1)$$

where Ca and Ci represent the concentration of CO_2 in the ambient air and inside the leaf, respectively, and v the vapour pressure difference between the inside of the leaf and the ambient air (Condon & Hall 1997). The factor 1.6 is the ratio of diffusivity of water vapour to CO_2. Ambient air contains CO_2 comprised mainly of ^{12}C, but $\approx 1.1\%$ of atmospheric CO_2 includes the stable isotope ^{13}C (Farquhar *et al*. 1989). Plants discriminate against CO_2 comprised of the heavier ^{13}C isotope, and thus the relative abundance of ^{13}C to ^{12}C is lower in C_3 plant tissue than in source air. Because the two isotopes differ in their mass-to-charge ratio, the $^{13}C/^{12}C$ ratio of the tissue relative to a standard (henceforth carbon isotope ratio, or $\delta^{13}C$) can be quantified on a mass spectrometer, where a more negative $\delta^{13}C$ reflects greater discrimination. The degree to which plants discriminate against ^{13}C is a function of Ci (eqn 1), and therefore, other things being equal, discrimination against ^{13}C is greater with more open stomates (Farquhar *et al*. 1989; Virgona & Farquhar 1996).

For C_3 plants, $\delta^{13}C$ is a negative function of Ci/Ca and *WUE* is also a negative function of Ci/Ca under constant Ca and v (Farquhar *et al*. 1989; Condon & Hall 1997). Thus, there is an expected positive correlation between *WUE* and $\delta^{13}C$. Numerous empirical studies have confirmed this expected correlation between carbon isotope composition of C_3 plant tissues and *WUE* (Farquhar *et al*. 1989; Ehleringer & Monson 1993; Araus *et al*. 2002). Intraspecific

genetic variation in $\delta^{13}C$ is common and is often used as an indicator of variation in integrated *WUE* for plants grown in a common environment (Farquhar *et al*. 1989; Araus *et al*. 2002). Variation in $\delta^{13}C$ reflects variation in *Ci*/*Ca*, which may be due to differences in photosynthetic capacity (A_{max}) and/or stomatal conductance. Genetic differences in A_{max} may reflect differences in the capacity for carboxylation or the regeneration of ribulose bisphosphate (Farquhar & von Caemmerer 1981) and may be indicated by differences in leaf nitrogen concentration (Field & Mooney 1986; Virgona & Farquhar 1996; Lambers *et al*. 1998). In this study $\delta^{13}C$ is used as a relative index of integrated *WUE*, due to differences in stomatal conductance and/or photosynthetic capacity.

Drought escape

Flowering time is an important trait related to drought adaptation in both crops and natural systems, where a short life cycle can lead to drought escape (White 1993; Fox 1990; Araus *et al*. 2002). In many annual crops differences among genotypes in $\delta^{13}C$ are positively correlated with flowering time, suggesting that later flowering genotypes may have higher *WUE* (Hall *et al*. 1990; Craufurd *et al*. 1991; Ehdaie *et al*. 1991; Richards & Condon 1993; Richards 1996). This positive, genetically based correlation between $\delta^{13}C$ and flowering time exists across a number of hierarchical levels (among species, among populations within species and among genotypes within a population). This trade-off in drought adaptation strategies suggests three possible causes: tight linkage between $\delta^{13}C$ and flowering time loci, linkage disequilibrium generated by selection on both flowering time and $\delta^{13}C$, or pleiotropy. The latter two are most plausible; it is unlikely that physical linkage would be consistent across distantly related taxa. We investigated variation and covariation in $\delta^{13}C$ and flowering time in *Arabidopsis thaliana*, a highly selfing annual. These two traits are likely to be important in climatic adaptation and are known to vary among accessions of *A. thaliana* (Nienhuis *et al*. 1994; Koornneef *et al*. 1998).

Functional genetic variation in A. thaliana

A. thaliana has become the model angiosperm for functional genetics, generating a wealth of genomic information. Molecular population genetic studies in *A. thaliana* reveal very low levels of within-population variation, but large differences among populations at both molecular markers and functional loci (Bergelson *et al*. 1998; Mitchell-Olds 2001). These data are consistent with observations of very high levels of selfing (Abbot & Gomes 1989).

Relative to molecular data, there is a dearth of data concerning genetic variation in ecological traits, although research in this area is increasing (reviewed in Pigliucci 1998; Alonso-Blanco & Koornneef 2000). For example, genetic differences among accessions have been found in a number of traits that may be important in climatic adaptation, including: relative growth rate (Li *et al*. 1998), response to elevated CO_2 (Norton *et al*. 1995), water use efficiency (Masle *et al*. 1993; Nienhuis *et al*. 1994) and flowering time (reviewed in Koornneef *et al*. 1998; Levy & Dean 1998; Juenger *et al*. 2000). Of these, flowering time has been the subject of the most intense genetic scrutiny, permitting a candidate gene approach to examining pleiotropy. In most systems, distinguishing pleiotropy from tight linkage is a major experimental challenge (Mather & Jinks 1982). In contrast, near-isogenic and mutant knockout lines at candidate loci in *A. thaliana* provide a unique opportunity to examine the genetic basis of trait covariance (see Methods). Thus, as an experimental system to investigate the evolution of correlated traits in natural populations, *A. thaliana* has many outstanding qualities.

Candidate genes

The genetics of flowering time in *A. thaliana* has been studied intensively in the last 20 years (reviewed in Koornneef *et al*. 1998; Levy & Dean 1998). Progress in dissecting the genetics of flowering time in *A. thaliana* has benefited from a combination of mutant screens and studies of natural variation using common garden studies and quantitative trait locus (QTL) mapping (Sanda *et al*. 1997; Johanson *et al*. 2000). Of at least 54 loci found to affect flowering time by the knockout approach (Levy & Dean 1998), three loci, *FRIGIDA* (*FRI*) (Johanson *et al*. 2000), *FLOWERING LOCUS C* (*FLC*) (Sheldon *et al*. 2000; Schläppi 2001) and most recently *CRY2* (El-Assal *et al*. 2001), have been shown to be polymorphic in nature. *FRI* has been shown to have alleles of large effect on flowering time in a number of independent mapping populations and near isogenic lines (Napp-Zin 1957; Sanda *et al*. 1997; Johanson *et al*. 2000). *FRI* interacts epistatically with *FLC* to determine the time to flowering (Koornneef *et al*. 1994). Molecular analysis shows that *FLC* encodes a transcription factor, which when upregulated by *FRI*, acts as a flowering inhibitor but can be offset by cold treatment of the rosette (Sheldon *et al*. 2000; Michaels & Amasino 2001). Naturally occurring *FRI* null alleles have evolved independently multiple times (Johanson *et al*. 2000; LeCorre *et al*. 2002). These greatly reduce flowering time and the level of *FLC* mitochondrial RNA (mRNA) (Michaels & Amasino 1999) and protein (Rouse *et al*. 2002). In addition, weak and null alleles of *FLC* have been described in accessions (Michaels & Amasino 1999; Schläppi 2001) and in mutant screens for early flowering, respectively (Michaels & Amasino 2000). Functional *FLC* alleles (Sheldon *et al*. 2000) are polymorphic in their effect on flowering time and *FLC* transcript levels in a population sample (Schläppi 2001).

We first examined variation in $\delta^{13}C$ and flowering time in 39 natural accessions of *A. thaliana* collected from a wide range of climates. Second, we compared variation in $\delta^{13}C$ and flowering time among six physiological mutants. Third, we conducted a more detailed study of germination time, flowering time, $\delta^{13}C$ and per cent nitrogen of leaf tissue in four flowering-time mutants and near isogenic lines with naturally occurring alleles of flowering time QTL in two genetic backgrounds. Fourth, we controlled for plant age effects on $\delta^{13}C$ while comparing near isogenic lines (NILs). These four experiments enabled us to examine the spectrum of genetic variation and covariation in traits determining the acquisition and allocation of resources to growth and reproduction in *A. thaliana* populations adapted to differing drought regimes.

Materials and methods

Our studies included natural variation, induced phenotypic mutants and near isogenic lines. We first describe each of our four experiments separately, including the rationale, genotypes used, traits measured, where plants were grown (greenhouse or growth chamber) and statistical analyses. In the final section of the methods we provide details of growth conditions (greenhouse and growth chamber) and trait measurements for all experiments.

Natural variation

Experiment 1 consisted of 39 natural accessions (Table 1) selected from the native range of *Arabidopsis thaliana* in Europe, Asia and northern Africa, but not North America, where *A. thaliana* has only recently been introduced. Accessions were selected to represent a range of latitudes, elevations and climates. In order to examine heritable variation in potentially adaptive traits, 39 genotypes were compared in a common garden experiment in a greenhouse at UC Davis (growth conditions and phenotypic measures are described below).

Four replicates of each genotype were planted (see greenhouse below) and we tested for among-population variation and covariation in $\delta^{13}C$ and flowering time. Genotypes are considered random replicates of adaptation (Felsenstein 1985), an assumption consistent with the well-supported star phylogeny of *A. thaliana* accessions (Innan & Stephan 2000; Sharbel *et al*. 2000). The effect of genotype was first tested using the nonparametric Kruskal–Wallis test and then tested using individual trait ANOVAS. The genetic correlation between $\delta^{13}C$ and flowering time is presented as Spearman's rank correlations among genotype means. We also present estimates of r_G based on variance components estimated using the program FREESTAT (Mitchell-Olds 1986). The genetic correlation is calculated as

$$r_G = \frac{Cov(i,j)}{\sigma_i,\sigma_j} \quad (2)$$

where $Cov(i,j)$ is the genetic covariance component for traits i and j, and σ_i and σ_j are the square roots of the among genotype (accession) variance components for traits i and j (Robertson 1959; Falconer & MacKay 1996) and significance is determined by permutation (Mitchell-Olds 1986).

Mutational covariance

Experiment 2 examined mutants artificially selected for altered physiology or flowering time to determine patterns of among-trait covariance. We asked whether mutants selected for altered physiology also show altered flowering time in a direction consistent with the genetic correlation from natural populations (Experiment 1). Conversely, do mutants selected for late flowering also show less negative $\delta^{13}C$?

Physiological mutants were chosen which might alter photosynthetic capacity or stomatal conductance, which in turn affect $\delta^{13}C$ (Table 2). A*bi2-1* and *abi3-1* represent mutations at two different loci originally reported as affecting ABA sensitivity (Koornneef *et al*. 1984; Leung *et al*. 1997; Bies-Etheve *et al*. 1999). These *abi* mutants both synthesize ABA and thus germinate readily, but show reduced sensitivity of germination and seedling growth to ABA. For *abi2-1* the phenotype extends into the vegetative stage, with increased transpiration and a 'wilty' phenotype. For the other ABA insensitive mutant *abi3-1*, at the time of the experiment, the ABA phenotype was reported as being confined to the seedling stage (but see Discussion). We also examined two mutants at nuclear loci with altered chloroplast size and chloroplast number per mesophyll cell. *Arc1*, and *arc2* are mutants with chloroplasts that are 1/2, and 2 times the size of wild-type, respectively (Pyke & Leech 1992). Finally, we selected *hcs1* and *hcs2*, mutants at two loci in the Columbia background that become chlorotic at high CO_2 (Artus 1990). All of these mutants were ordered from the *Arabidopsis* stock centre in Ohio (www.arabidopsis.org). These physiological mutants were grown in the greenhouse with the natural accessions.

Four mutations affecting flowering time were already known from the literature (Table 2). We selected: *co1* and *gi* (both late flowering, vernalization insensitive) and *fve* and *ld-1* (both late flowering, vernalization responsive). All are in the Columbia genetic background, were provided by R. M. Amasino, and are described in detail by Rédei (1962), Koornneef *et al*. (1991, 1998) and Michaels & Amasino (2001).

Late flowering mutants were compared in a growth chamber to examine covariance between flowering time and carbon isotope ratio. Each genotype was replicated across two day-length treatments, treated statistically as experimental blocks: Block 1 (11 h days, three replicates)

Table 1 Thirty-nine natural accessions used in Experiment 1. Data are Accession no., name, geographical location, latitude, longitude, and elevation of collection site. Mean $\delta^{13}C$ and flowering time of each genotype in our greenhouse screening are also shown

Accession no.	Ecotype	Collection site	Lat. N	Long.	Elev (m)	$\delta^{13}C$ (‰)	Flowering time (days)
900	aa-0	Rhon, Germany	51	E 10	200	–29.7	22.7
901	ag-0	Argentat, France	45	E 1.5	100	–30.5	21.8
902	cvi-0	Cape Verde Islands	16	W 24	1200	–30.5	22.5
903	kas-1	Kashmir, India	35	E 77	1580	–28.2	31.7
904	mh-0	Muhlen, Poland	53.5	E 20.5	100	–31.2	19.0
905	ms-0	Moscow, Russia	55.5	E 38	100	–28.9	31.7
910	di-g	Dijon, France	47.5	E 5	300	–30.5	20.4
911	est	Estland, Poland	na	na	na	–30.2	19.3
915	ws-3	Wassilewskija	52	E 30	na	–31.0	21.0
916	kondara	Tajikistan	na	na	1100	–28.9	29.3
917	da(1)-12	Czechoslovakia	18	E 49	na	–30.6	20.0
922	hodja-obi-garm	Tajikistan	39	E 70.5	1800	–29.9	25.7
924	je54	Czechoslovakia	na	na	na	–30.5	22.5
926	petergof	Russia	60	E 30	na	–31.0	23.0
927	rubezhnoe-1	Ukraine	52	E 30	na	–29.8	26.7
930	sn(5)-1	Czechoslovakia	na	na	na	–30.6	19.8
931	sorbo	Tajikistan	na	na	2200	–29.0	32.0
944	an-1	Antwerpen, Belgium	51.5	E 4.5	1	–29.8	19.8
958	bch-3	Buchen, Germany	53.5	E 10.5	100	–29.7	27.0
1064	can-0	Canary Islands	28	W 15.5	700	–28.9	32.0
1102	db-1	Tenne, Germany	60	E 30	400	–30.4	20.8
1122	edi-0	Edinburgh, UK	56	W 3	100	–29.1	32.0
1126	ei-4	Eifel, Germany	50.5	E 6.5	400	–29.4	25.7
1136	en-1	Enkheim, Germany	50	E 8.5	100	–30.2	19.5
1152	et-0	Etraygues, France	44.5	E 2.5	400	–28.7	32.0
1252	jl-3	Czechoslovakia	na	na	460	–30.4	18.7
1266	ka-0	Karnten, Austria	47	E 14	900	–30.6	22.0
1374	mrk-0	Markt, Germany	49	E 9.5	200	–29.9	27.5
1454	pi-0	Pitztal (Tirol), Austria	47	E 11	1000	–29.5	24.0
1482	rd-0	Rodenbach, Germany	49.5	E 9.5	500	–30.4	22.8
1494	rsch-4	Rschew, Russia	56.5	E 34	100	–30.1	22.5
1504	sei-0	Seis am Schlern, Italy	46.5	E 11.5	1000	–30.6	19.5
1548	ta-0	Tabor, Czechoslovakia	49.5	E 14.5	400	–30.7	22.8
1630	wl-0	Wilbad, Germany	48.5	E 8.5	500	–30.6	23.3
1639	wei-1	Weiningen, Switzerland	47.5	E 8.5	na	–30.8	19.7
1640	tsu-1	Tsu, Japan	34.5	E 136.3	1	–30.6	25.5
1641	rld-2	Rschew, Russia	56.5	E 34	na	–30.6	21.5
1643	oy-1	Oytese, Norway	46.5	E 11.5	1	–29.7	22.8
6180	shahdara	Tajikistan	37.5	E 65	3400	–30.6	20.5

and Block 2 (13 h days, four replicates). These blocks were grown in the same growth chamber sequentially: immediately after Block 1 was harvested the day length of the chamber was increased from 11 to 13 h, and Block 2 was moved from the cold stratification chamber into the growth chamber. Otherwise, plant growth conditions were identical for both blocks (photoperiods).

Flowering time in *A. thaliana* is a highly plastic trait, which responds strongly to day length, seed stratification (cold treatment of imbibed seed) and vernalization (cold treatment of vegetative seedling or rosette) in many genotypes (Nordborg & Bergelson 1999). Vernalization is a well-studied environmental factor that decreases flowering time. Many mutations found to influence flowering time are classified by their response to vernalization, leading to the dichotomous classification of genotypes as winter or summer annuals. However, all diploid *A. thaliana* accessions appear capable of flowering in the absence of vernalization of the rosette (JK McKay, personal observation). Day length and light intensity are two additional environmental factors known to have a large effect on flowering time (see also Crone & McDaniel 1997). We manipulated day length in order to create environmental variance in flowering time within genetic lines, and thus

Table 2 List of mutants used in Experiment 2 to test for mutational covariance

Accession no.	Homozygous mutant allele	Genome	Described phenotype	Predicted trait effect	Reference
6172	WT – Col	Col	Early flowering		
86	WT – L*er*	L*er*	Early flowering		
23	*Abi2–1*	L*er*	Abscisic acid sensitivity	$\delta^{13}C$	Koornneef *et al*. (1984)
24	*Abi3–1*	L*er*	Abscisic acid sensitivity	$\delta^{13}C$	Koornneef *et al*. (1984)
262	*Arc1*	L*er*	50% higher chloroplast density	$\delta^{13}C$	Pyke & Leech (1992)
263	*Arc2*	L*er*	50% lower chloroplast density	$\delta^{13}C$	Pyke & Leech (1992)
6169	*Hcs1*	Col	High CO_2	$\delta^{13}C$	Artus (1990)
6171	*Hcs2*	Col	High CO_2	$\delta^{13}C$	Artus (1990)
*	*Co-1*	Col	Late flowering, vern insensitive	flowering	Rédei (1962)
*	*Gi*	Col	Late flowering, vern insensitive	flowering	Michaels & Amasino (2001)
*	*Fve*	Col	Late flowering, vern responsive	flowering	Koornneef *et al*. (1991)
*	*Ld-1*	Col	Late flowering, vern responsive	flowering	Rédei (1962)

*See Materials and methods.

Table 3 Near isogenic lines used in Experiment 3. See methods for further explanation of which alleles were introgressed into which background

Genotype	Genome	FRI	FLC	Expected phenotype	Reference
WT — Columbia	Col	fri	FLC	Early flowering	
FRI[sf2]	Col	FRI	FLC	Late flowering	Lee & Amasino (1995)
WT — L*er*	L*er*	fri	flc	Early flowering	Lee & Amasino (1995)
FRI[sf2] flc[L*er*]	L*er*	FRI	flc	Early flowering	Lee & Amasino (1995)
FRI[sf2] FLC[Col]	L*er*	FRI	FLC	Late flowering	Lee & Amasino (1995)

quantify environmentally induced (nongenetic) covariance between $\delta^{13}C$ and flowering time.

Near isogenic lines

In Experiment 3, we examined the pleiotropic effects of naturally occurring alleles at the *FRI* and *FLC* loci, which are two cloned QTL responsible for naturally occurring variation in flowering time. We used NILs consisting of the dominant, functional *FRIGIDA* allele (***FRI***) of the naturally late flowering accession San Feliu-2 (sf-2) introgressed into the homozygous background of the Columbia strain (Col) and compared it with the early flowering wild-type Col, which carries a null, recessive *FRIGIDA* allele (***fri***) (Table 3). In addition, we examine the effect of the *FLOWERING LOCUS C* genotype, by using NILs where functional alleles ***FRI*** (sf-2) and ***FLC*** (Col) are introgressed into the Landsberg *erecta* background (L*er*), which carries a null allele (***flc***) at the *FLC* locus. The genetics and molecular biology of these NILs are further described in Lee & Amasino (1995) and Michaels & Amasino (2001), and were kindly provided by R. M. Amasino.

All traits in Experiment 3 were analysed using the following model, which was the best fitting five-parameter genetic model for the flowering time data.

Trait = *FRI* + *FLC* + *FRI***FLC* + BLOCK + GENOME + error,

where genome is the wild-type background (Col or L*er*) into which the late flowering alleles were introgressed. For these NIL experiments, blocks did not represent a day-length manipulation and therefore block was considered a random effect in that analysis. Each effect was tested over the mean square error. In our model fitting we found no evidence of significant *FRI**Block, *FLC**Block or *FRI***FLC**Block interactions.

Common age at harvest

In an independent study, Experiment 4, we harvested Columbia NILs at a common age, to control for ontogenetic contributions to variation in $\delta^{13}C$. To account for within plant variation, we measured $\delta^{13}C$ separately for both root and leaf tissue. These are the same Columbia NILs used in Experiment 3 and the genetics and molecular biology of these lines are further described in Michaels & Amasino (2000). These were grown in the greenhouse as described below for Experiment 4.

Greenhouse growth conditions

Natural accessions from Experiment 1 and physiological mutants from Experiment 2 were grown together in the greenhouse at UC Davis. Three seeds of each genotype were sown into four 250 mL pots filled with a peat soil–vermiculite

mixture and 3–5 pellets of complete slow-release fertilizer. Pots were then randomized into four complete blocks. Blocks were watered, covered with plastic and then placed into a walk-in cooler at 4 °C for seed vernalization. After 5 days of cold treatment, blocks were transferred to a warm, lighted greenhouse. The average day length (natural sunlight) during the experiment was a little over 10 h, and supplemental lighting in the greenhouse was used to extend day length to 16 h throughout the experiment. Supplemental light provided PAR levels of 350 (μmol photons/m^2/s) and maximum levels during full sunlight reached 1800 (μmol photons/m^2/s^1). Relative humidity throughout the experiment ranged from 29 to 68%, with a mean of 44%. Air temperature in the greenhouse ranged from 16.8 to 24.2 °C, with a mean of 23.1 °C.

Once transferred to the greenhouse each pot was checked daily for germination. All plants were kept well watered by bottom watering trays. Once a single germination date was recorded for each pot, subsequent germinants were removed. Replicates (pots) that did not germinate in the first 2 weeks were excluded from the experiment. Plants were checked daily and when they began flowering they were harvested at the developmental stage of 1–3 open flowers.

Plants in Experiment 4 were grown in the same greenhouse, at a different time. To facilitate recovery of clean roots, plants in Experiment 4 were grown in a 1:1 mixture of sand and fritted-clay. These lines were cold stratified as imbibed seeds for 8 days and grown in a greenhouse at UC Davis in 175 mL tubular pots. Plants were fertilized with half-strength Hoagland's solution weekly (three times total).

Chamber growth conditions

Plants were grown in 175 mL tubular pots. These pots were filled with a peat soil–vermiculite mixture and 0.63 mL of complete slow-release fertilizer. Soil was packed tightly into tubes and tubes were then placed in standing water until saturated. Five seeds of a given genotype were sown directly into the soil at the centre of each tube. Once planted, tubes were randomized into the racks made to hold them, covered with black plastic and then cold treated (4 °C) in the dark for 8 days. Following cold treatment, blocks were transferred to an environmental growth chamber at 21/11 °C (day/night) with fluorescent and incandescent lighting, where PAR = 630 μmol photons/m^2/s at the level of the soil for both day-length blocks. Relative humidity ranged between 50% (day) and 75% (night). The soil in each tube was kept moist by bottom watering throughout the experiment. In addition, to insure adequate moisture for germination, tubes were hand-watered with a syringe for the first 10 days in the growth chamber, where 3 mL of water was added to the top of each tube per day. Germination was scored daily and only the first germinant per pot was retained for the experiment.

Trait screening

All plants were harvested at the initiation of flowering. All above-ground biomass was transferred to glass test tubes and oven dried at 60 °C (Farquhar *et al*. 1989). For natural accessions grown in the greenhouse, some plants flowered in as little as 18 days and produced very little biomass. For the 14 plants with less than 1 mg of above-ground biomass, all tissue was used for isotope analysis. For all other plants from the greenhouse (natural accessions and physiological mutants) the entire shoot was dried and ground (see below), and between 0.8 and 1.2 mg of each sample was transferred to a tin combustion cup for isotope analysis. In the growth chamber, where maximum PAR (600) was one-third that in the greenhouse (1800), plants took longer to flower and produced much more biomass. Dried plant tissue was processed for carbon isotope analysis, by randomly subsampling 100–200 mg of crushed leaf material from each individual into a 2-mL microfuge tube containing four ball bearings, then shaking for 1 min on a paint shaker. The result was a uniform powder, from which subsamples (1 and/or 3 mg) from each plant were analysed. In addition in the growth chamber experiment, we measured per cent mass nitrogen (%N) in the leaf tissue, which may reflect differences in biochemical photosynthetic capacity.

Samples were analysed for %N and $\delta^{13}C$ at the Stable Isotope Facility at UC Davis (http://stableisotopefacility.ucdavis.edu/). Data are presented as carbon isotope ratios relative to the PDB standard (R_{PDB}), where $\delta^{13}C(‰) = (R_S/R_{PDB} - 1) * 1000$ (Hubick *et al*. 1986). For many plants multiple subsamples were analysed, the values were highly repeatable and the average was used for analysis.

We express all values as carbon isotope ratio, $\delta^{13}C$ (lower case delta), rather than carbon isotope discrimination, $\Delta^{13}C$ uppercase delta). Although instantaneous (online) measures of $\Delta^{13}C$ are straightforward (Evans *et al*. 1986; Hubick *et al*. 1988), estimates derived from plant tissue require the assumption of a single value for the carbon isotope ratio for the air, which was not the case in our growth experiments. We sampled the air inside the growth chamber repeatedly, and found large fluctuations in the carbon isotope ratio in the source air, $\delta^{13}C_{air}$ inside the growth chamber. Thus converting to $\Delta^{13}C$ would require a temporal integration of the range of $\delta^{13}C_{air}$ weighted by maximum photosynthesis, A_{max}.

Results

Natural variation

Comparison of 39 population accessions of *Arabidopsis thaliana* revealed significant genetic differences among accessions for both of the focal drought traits, flowering time and $\delta^{13}C$. ANOVA showed a significant effect of genotype

for both flowering time ($F = 7.5$, d.f. = 38, $P < 0.00001$) and $\delta^{13}C$ ($F = 5.7$, d.f. = 38, $P < 0.00001$). The ratio of genetic to total phenotypic variation was: flowering time $V_G/V_T = 0.66$; $\delta^{13}C$ $V_G/V_T = 0.58$ (based on variance components estimated using restricted maximum likelihood). In addition, we found a strong positive genetic correlation ($r_G = 0.98$) between our measure of dehydration avoidance, $\delta^{13}C$, and flowering time, our index of drought escape (Fig. 1, upper). This genetic correlation is greater than the Spearman's correlation among genotype means (*rho* = 0.65, d.f. = 38, $P < 0.0001$) because it removes the environmental contribution to covariance, which is largely negative (see Fig. 1, lower and Discussion).

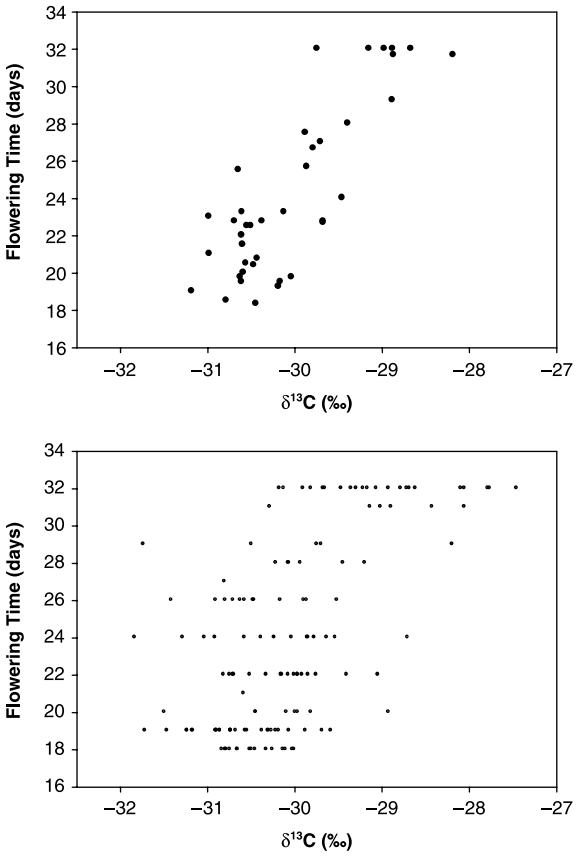

Fig. 1 Variation and covariation in flowering time and $\delta^{13}C$ among natural populations of *Arabidopsis thaliana*. (Upper) Genotype means of 39 accessions. (Lower) Individual values of the replicates. Both panels are shown to indicate the overall patterns of genetic (upper) and phenotypic (lower) (co)variance (see Results).

Mutational covariance

The mutant lines selected for altered physiology generally showed variation in $\delta^{13}C$ (significant effect of genotype in the L*er* background, d.f. = 4, $F = 22.95$, $P < 0.0001$). In contrast, we found little evidence that these induced physiological mutations had pleiotropic effects on flowering time (no significant effect of genotype). A notable exception was the *arc2* mutant which showed marginal (after controlling for multiple tests) differences from the wild-type in both $\delta^{13}C$ ($P < 0.01$) and flowering time ($P < 0.05$). The *arc2* mutant grew relatively poorly in our greenhouse conditions, flowering later, but at a smaller size and more negative $\delta^{13}C$ than the wild-type (L*er*). In addition the mutants *abi2-1* and *abi3-1* had less negative $\delta^{13}C$ than that of the wild-type, but this was not significant.

In the flowering time mutants we found strong evidence for pleiotropy. Mutations affecting flowering time did affect $\delta^{13}C$ in the direction predicted; genotypes with increased flowering time had increased (less negative) $\delta^{13}C$. The degree and direction of mutational covariation differed among flowering time mutants, with the overall pattern consistent with the genetic correlation that we found among natural accessions (Fig. 1). The mean (of both blocks) genotypic values of $\delta^{13}C$ and flowering time in Table 4 are positively correlated ($r = 0.7$); this is not significant with a sample size of 5.

For the flowering time mutants we also modified the environment between blocks by manipulating day length, and found a significant difference between 11- and 13-h

	Trait Germination time (days)		Flowering time (days)		Leaf number		$\delta^{13}C$ (‰)		Leaf nitrogen (%)	
Day length (h)	11	13	11	13	11	13	11	13	11	13
Genotype										
Col	1.0	2.3	31.3	27.7	37	24	−33.8	−33.7	4.8	1.9
Co-1	1.0	2.7	31.0	26.0	69	23	−34.8	−33.7	4.4	2.2
Gi	1.5	2.7	35.0	35.0	33	29	−33.7	−33.3	3.3	3.1
Fve	2.0	2.3	34.7	30.0	32	31	−34.8	−34.4	3.7	2.7
Ld-1	1.0	1.7	48.0	44.5	78	34	−33.6	−32.8	3.0	2.5
Standard error	0.26	0.22	1.7	1.8	6.0	1.7	0.18	0.19	0.4	0.4

Table 4 Phenotypic effects of four flowering-time mutants and Columbia wild-type (Experiment 2). Shown are means for each genotype in both blocks (photoperiods, 11 day/13 night and 13 day/11 night) and the standard error for each trait by photoperiod combination. Refer to Table 5 for main effects ANOVA, including Genotype, Photoperiod and G × E

Table 5 Statistical analysis of flowering time mutations (Experiment 2). All traits were analysed in separate univariate ANOVAS, with genotype and block (= photoperiod) considered fixed effects. Bold *P*-values indicate significance following a strict Bonferroni controlling for multiple traits

Source of variation	d.f.	Mean square	F-ratio	*P* <
Genotype				
Germination time (d)	4	0.4	0.4	0.8
Flowering time (d)	4	178.7	12.5	**0.0005**
Leaf number	4	451.8	9.6	**0.0005**
$\delta^{13}C$ (‰)	4	1.8	6.2	**0.005**
Leaf nitrogen (%)	4	3.3E-05	0.1	1.0
Photoperiod				
Germination time (d)	1	5.6	5.7	0.05
Flowering time (d)	1	43.3	3.0	0.1
Leaf number	1	2163.5	46.1	**0.00001**
$\delta^{13}C$ (‰)	1	2.0	6.8	0.05
Leaf nitrogen (%)	1	1.3E-03	5.2	0.05
$G \times E$				
Germination time (d)	4	0.455	0.5	0.8
Flowering time (d)	4	11.8	0.8	0.9
Leaf number	4	502.7	10.7	**0.0005**
$\delta^{13}C$ (‰)	4	0.24	0.8	0.6
Leaf nitrogen (%)	4	2.0E-04	0.8	0.6
Error				
Germination time (d)	19	0.99		
Flowering time (d)	17	14.21		
Leaf number	15	46.96		
$\delta^{13}C$ (‰)	20	0.30		
Leaf nitrogen (%)	20	2.6E-04		

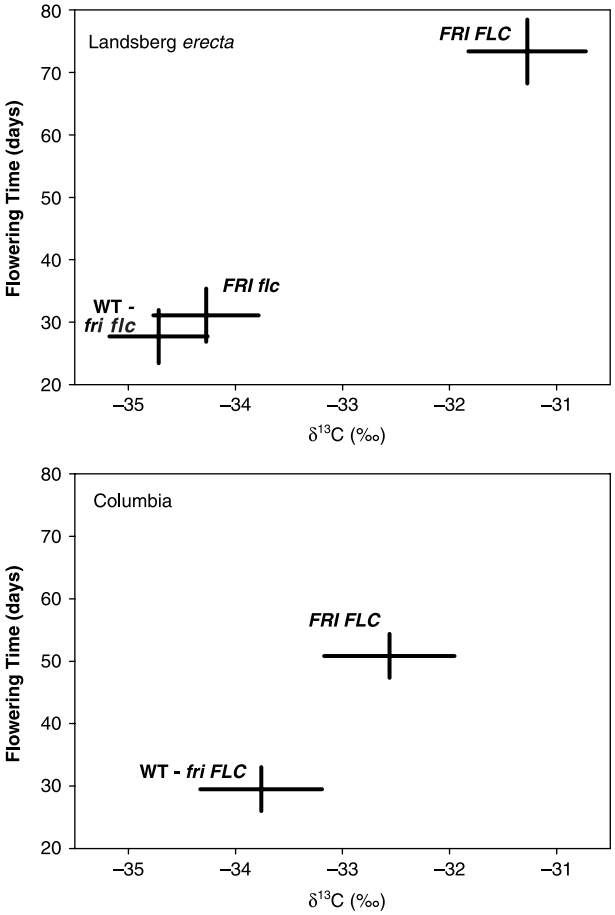

Fig. 2 Effects of *FRI* and *FLC* genotypes in two genetic backgrounds on flowering time and $\delta^{13}C$ (bars are 95% CI). See Table 3 for description of genotypes of the near isogenic lines. (Upper) The phenotype of NILs in a Landsberg *erecta* background, where the effect of the late flowering *FRI* allele depends on the genotype at the *FLC* locus (Michaels & Amasino 2000). (Lower) The phenotypic effect of the *FRI* allele in the Columbia background. Both panels are at the same scale, demonstrating a greater increase on both traits in the L*er* background.

days for leaf number. The effect of longer day length was in the direction expected, decreased number of leaves at flowering for every genotype and decreased number of days to flowering, although this latter result was not significant and did not occur in the *gi* mutant. Leaf number is often used as a surrogate for flowering time; we generally found that days to flowering and leaf number at flowering were well correlated, but there were exceptions. The *co1* mutant did not differ from the wild-type under either 11- or 13-h days, but this genotype did have significantly more leaves at flowering than the wild-type at 11-h days. Closer inspection shows that this is due to an increase in the relative number of small leaves in the *co1* mutant at 11 h. Day length also had a noticeable, but not statistically significant, effect on other measured traits in the flowering time mutants (germination time, $\delta^{13}C$ and %N in leaves), the direction of effect was consistent across genotypes (Table 4). In general, longer days increased germination time and $\delta^{13}C$, and decreased time to flowering, leaf number at flowering and %N (see Table 5 for significance tests). Within lines we found no evidence for a positive phenotypic correlation of flowering time and $\delta^{13}C$ (four of five showed a nonsignificant negative correlation) suggesting environmental covariance does not cause the pattern of positive covariance among genotypes.

Near isogenic lines

FRI and *FLC* NILs showed dramatic pleiotropic effects (Fig. 2, Table 6). The introgression of the late flowering *FRI* allele into a genotype with a functional *FLC* allele (Col) increased flowering time and also caused an increase in $\delta^{13}C$. The genetic correlation in the NILs ($r_G = 0.97$) was nearly identical to that found for the natural accessions. Our analysis (Table 6) shows that the *FRI* locus had significant effects on flowering time and $\delta^{13}C$. The *FLC* locus also had significant effects on flowering time and $\delta^{13}C$. The *FRI* × *FLC* interaction had a significant effect only

Table 6 Mixed-model ANOVA of NILs of *FRI* and *FLC* loci introgressed into isogenic Columbia and Landsberg *erecta* backgrounds (Experiment 3). The model used was the best-fitting 5-parameter model for the flowering time data. Genotype at *FRI* and *FLC*, and the interaction *FRI* × *FLC* were considered fixed effects. Block and genome (L*er* or Col) were treated as random effects. All F-ratios represent the factor mean square, tested over the error. One or more * in the *P*-values indicates significance following a strict Bonferroni controlling for multiple traits

	Germination time (days)		Flowering time (days)		$\delta^{13}C$ (‰)		Nitrogen (%)	
Source	F-ratio	*P* <	F-ratio	*P* <	F-ratio	*P* <	F-ratio	*P* <
FRI	0.29	ns	40.5	***	12.4	*	5.5	ns
FLC	1.2	ns	122.3	***	55.3	***	6.0	ns
FRI × FLC	1.2	ns	21.8	***	2.8	ns	1.6	ns
Block	68.2	***	1.0	ns	9.8	*	19.8	**
Genome	0.54	ns	52.4	***	14.0	**	0.01	ns
d.f. =	28		25		25		25	

ns, not significant, *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

on flowering time. Genetic background (Col or L*er*) also had a significant effect on flowering time and $\delta^{13}C$ (Fig. 2), consistent with the presence of additional loci affecting differences between Col and L*er* for these traits (T. Juenger, personal communication). Blocks, which in this case represent unknown environmental effects, had a significant effect on all traits but flowering time. Within NILs there was substantial plastic variation in all traits but we found no evidence for environmental or ontogenetic contributions to the positive genetic correlation of flowering time and $\delta^{13}C$.

Common age at harvest

In Experiment 4, we harvested all genotypes after 18–19 days of growth, to control for ontogenetic contributions to $\delta^{13}C$, and measured $\delta^{13}C$ separately from both root and leaf tissue to account for within plant variation. We found significant differences in $\delta^{13}C$ between root and shoot tissues, as well as overall genetic differences between Col wild-type and Col NIL *FRI* sf-2. Col NIL *FRI* sf-2 had significantly less negative $\delta^{13}C$ than the early-flowering wild-type Col. The respective means (SE) of $\delta^{13}C$ for Col, and Col *FRI* were: –29.1 (0.3) and –27.9 (0.2) for roots and –31.3 (0.3) and –30.3 (0.4) for shoots.

Discussion

Our sample of natural accessions of *Arabidopsis thaliana* displayed substantial genetic variation for ecologically important traits. We found a large positive genetic correlation between flowering time and $\delta^{13}C$ among naturally occurring ecotypes indicating a genetic trade-off between different drought adaptation strategies (Geber & Dawson 1997). This genetically based trade-off suggests three possible mechanisms: correlated selection, tight linkage and/or pleiotropy. Although it is plausible that selection on $\delta^{13}C$ and flowering time might be correlated (Geber & Dawson 1997), correlated response to selection in previous studies (Menendez & Hall 1995) provides evidence for pleiotropy or tight linkage.

We tested for pleiotropy by examining mutational covariance introduced by alleles of known effect on one trait. Most mutants had some effect on $\delta^{13}C$, whether they were physiological or flowering time mutants. This was expected for physiological mutants, but there was no evidence for pleiotropy on flowering time (see Results). These physiological mutations break up the tight positive genetic correlation observed among accessions. In addition, nearly all reduce $\delta^{13}C$. The exceptions were the ABA insensitive mutants *abi2-1* and *abi3-1*, which showed an increased $\delta^{13}C$ value. This is somewhat surprising for *abi3-1*, a transcription factor homologous to *vp-1* in maize, which mediates ABA-regulated gene expression in *A. thaliana* (Giraudat *et al*. 1992; Tamminen *et al*. 2001). In the rosette stage, *abi3-1* has wild-type stomatal behaviour and ABA sensitivity (Koornneef *et al*. 1984). However, more recent results suggest that this transcription factor is expressed in older tissue and influences chloroplast development (Rohde *et al*. 2000). Mutant alleles at this locus are now known to act in ABA-independent pathways (Boneta & McCourt 1998), and confer phenotypes in the vegetative stage (Rohde *et al*. 2002), including flowering time. Data now exist on a number of unique induced mutations at the *abi3* locus, and these data suggest a wide spectrum of (pleiotropic) effects among allelic variants at this locus (Nambara *et al*. 2002). There are now at least 37 loci known to affect abscisic acid synthesis or response; these also display varying patterns of pleiotropy (reviewed in Finkelstein *et al*. 2002).

Our growth chamber screening of late flowering mutants and their earlier flowering wild-types revealed evidence for pleiotropy, in the form of mutational covariance between $\delta^{13}C$ and flowering time (Tables 4 and 5). Three of four mutations described with a flowering time phenotype also show an effect on $\delta^{13}C$ in at least one photoperiod (Table 4). Not surprisingly, we found that the degree of pleiotropy depends on the environmental conditions (Fry 1996). We observed pleiotropy only in some flowering time mutants and not in the physiological mutants, showing that pleiotropy is far from absolute (these traits do not always covary), and depends on the particular genotypes and environments.

Stronger evidence for pleiotropy influencing adaptation comes from the genetic covariance caused by *FLC* and *FRI* alleles (Fig. 2), which are known to be responsible for natural variation in flowering time. Although these NILs

likely contain introgressed alleles other than *FRI (*sf-2) and *FLC* (col), the effect of these two introgressed regions is to increase both flowering time and $\delta^{13}C$ (Fig. 2). In addition, the results in the Landsberg *erecta* line (Fig. 2, upper) suggest the effect on $\delta^{13}C$ requires the presence of a functional *FLC* and the pleiotropic effects may be due in part to the regulation of the transcription factor *FLC*. We are currently using lines overexpressing *FLC* with a heterologous promoter (CAMV 35:s) to determine if *FLC* expression per se results in less negative $\delta^{13}C$.

In total, our data suggest that population differences in flowering time may involve alleles that pleiotropically affect drought physiology. It is reasonable to expect both flowering time and water-use physiology to experience episodes of selection. In *A. thaliana*, later flowering results in greater fecundity in some environments (Mitchell-Olds 1996). Thus, for *A. thaliana* plants exposed to drought stress, genetic differences, in both mean phenotype and the degree of plasticity of flowering time, are important components of fitness (JK McKay, unpublished data).

A positive genetic correlation between flowering time and $\delta^{13}C$ has also been shown across hierarchical levels: among species (White 1993), among genotypes and cultivars within a species, and among families within populations. Detailed data come from a number of C_3 annual crops, including barley (Craufurd *et al*. 1991), common and tepary bean (White 1993), cowpea (Menendez & Hall 1995) and wheat (Ehdaie *et al*. 1991). In cowpea, later flowering is often an unwanted correlated response to selection for reduced transpiration (Menendez & Hall 1995), suggesting pleiotropic effects of mutations that went to fixation while screening for reduced stomatal conductance or water loss. This positive genetic correlation also exists within populations, where later flowering families have greater *WUE* and less negative $\delta^{13}C$ (Geber & Dawson 1990, 1997). From a simple physiological argument, reduced stomatal conductance will result in a lower rate of carbon fixed during photosynthesis, and may explain increases in both $\delta^{13}C$ and the time required to acquire sufficient resources to flower. The scenario is probably more complicated as later flowering plants typically flower at a larger size with more leaves and have higher fecundity.

Genetic and environmental contributions to covariance

All plants made the transition to flowering in our greenhouse or growth chamber conditions without vernalization of the rosette, and at all day lengths. As expected, we found a large effect of day length on flowering time, as well as unexpected differences in other traits such as germination and percentage nitrogen. In the greenhouse (16-h days), plants of a given genotype flowered much earlier than plants in the growth chamber. Comparing Figs 1 and 2 demonstrates that overall the pattern of environmental covariance between flowering time and $\delta^{13}C$ was opposite to the genetic covariance: plants in the greenhouse experiment flowered earlier and had less negative $\delta^{13}C$ than plants grown in the growth chamber. In addition, the phenotypic correlation between flowering time and $\delta^{13}C$ was consistently lower than the genetic correlation, in our experiments (Fig. 1, lower) as well as in other species (Menendez & Hall 1995).

All plants in Experiments 1–3 were harvested at the same developmental stage (bolting), whereas in Experiment 4 they were harvested at a constant age (Coleman *et al*. 1994). It is known that age at sampling may influence $\delta^{13}C$ (Fu *et al*. 1993; Araus *et al*. 2002). We can estimate such environmental (nongenetic) contributions to trait covariance by looking within lines, where replicates within genotypes show trait differences for environmental or ontogenetic reasons. If older plants discriminate less per se, then within genotypes $\delta^{13}C$ should be correlated with later flowering. No such correlation was found within genotypes, in the natural accessions, EMS mutants or NILs. This is most clearly demonstrated in Experiment 2 (late flowering mutants), where manipulation of day length had surprisingly large effects on leaf number, flowering time and leaf nitrogen, but no significant effect on $\delta^{13}C$ (Table 5). Differences among lines are determined primarily by genetic rather than environmental covariation.

Experiment 4 shows that differences in $\delta^{13}C$ can be found in *A. thaliana* genotypes harvested at the same age. We also found large differences between root and shoot tissue, with roots having less negative $\delta^{13}C$. Our results using sequential harvests suggest that in our high light environments *A. thaliana* leaf tissue $\delta^{13}C$ is generally most negative and similar among genotypes in the first two weeks and then reaches the most positive value for a given genotype about the time of flowering (JK McKay, unpublished).

Pleiotropy and functional genetics

Mutant screening and large-scale sequencing in *A. thaliana* have identified many genes that are thought to play a role in drought adaptation. In *A. thaliana* it is now possible to identify all gene products that are expressed when exposed to drought stress, indicating that they are involved in the pathways that regulate water stress responses (Seki *et al*. 2002). These assays of gene expression can be combined with a mutant screening approach to elucidate the biochemical function of these genes (Pesaresi *et al*. 2001). Identifying the genes involved in each biochemical pathway is a tremendous task, and an important component in attempting to understand the water relations of plants (Bray 2002; Ramanjulu & Bartels 2002).

To take full advantage of these candidate genes, we are examining natural populations. This will allow elucidation of which genes, of all the possible genes in each biochemical

pathway, have been involved in the evolution of trait differences among natural populations. Using this approach it may be possible to find a subset of the estimated 26 000 loci in *A. thaliana*, at which alleles have evolved without deleterious pleiotropic effects on fitness. At the molecular level, expression of some of these traits (including drought tolerance and flowering time) is particularly well understood, permitting candidate gene approaches in population studies (Thornsberry *et al*. 2001; LeCorre *et al*. 2002). In addition, identifying loci that contribute to trait variation and are polymorphic can provide data on the genetic basis of adaptation, information that is fundamental to our understanding of evolution and biodiversity.

Mutant screens by necessity focus on a limited number of traits. Not surprisingly, we found correlated effects on traits other than those listed in the description of the mutants. This phenomenon was also demonstrated by Boyes *et al*. (2001), where five biochemical pathway mutants resulted in substantial effects on life history traits. In addition, QTL studies of nearly any trait in the Col × L*er* or L*er* × Cvi recombinant inbred lines have found some effect of the induced mutation *erecta* (JK McKay, personal observation). Other examples of pleiotropy can be found in almost every detailed description of mutants. This is expected as most mutant screens impose strong directional selection on particular traits, and minimal selection for fitness traits.

Population genetic theory suggests that most mutations of substantial effect will have pleiotropic effects on fitness (Fisher 1958). In contrast, we expect that in natural populations, natural selection has filtered the spectrum of possible mutational effects, eliminating those mutant alleles with deleterious pleiotropic effects. However, a recent mutation accumulation experiment in *A. thaliana* (Shaw *et al*. 2000), found the distribution of mutational effects symmetric (new mutations both increasing and decreasing fitness as measured in the lab). In addition, they found positive mutational covariance, where mutations either increased or decreased all traits. We found a similar pattern due to allelic variation at the *FRI* and *FLC* loci (this study). In this case, it appears that the pleiotropic effects may be due in part to the regulation of the transcription factor *FLC*.

Detailed work has shown allelic variation at the *FRI* and *FLC* loci to be responsible for naturally occurring variation in flowering time and leaf number at flowering. Later flowering appears to be the ancestral state (Johanson *et al*. 2000; LeCorre *et al*. 2002). Early flowering in natural accessions appears to have evolved to a large degree by selection for (at least eight) independent loss of function mutations at the *FRI* locus (LeCorre *et al*. 2002). In addition, weak and null alleles of *FLC* have been described in accessions (Michaels & Amasino 1999; Schläppi 2001) and in mutant screens for early flowering, respectively (Michaels & Amasino 2000). Although natural *FLC* alleles are polymorphic in their effect on flowering time and *FLC* transcript levels in a population sample (Sheldon *et al*. 2000; Schläppi 2001), this phenotypic variation may be due to *cis*-acting promoters, as the sequence of these alleles is identical (Sheldon *et al*. 2000; Schläppi 2001).

Although continuously distributed, flowering time is often analysed as a categorical trait (late or early), in which case *FRI* behaves as a dominant allele for late flowering. Categories for the dichotomy early and late are arbitrary and vary across experiments, and mask the effects of other loci. At least 20 substantial flowering time QTL have been identified in *A. thaliana* (Alonso-Blanco *et al*. 1998; Levy & Dean 1998; Juenger *et al*. 2000; Weinig *et al*. 2002) some of which map close to one or more of the over 50 candidate loci shown to affect flowering time using a knockout approach. Most recently, El-Assal *et al*. (2001) cloned a novel natural allele of *CRY2* which underlies flowering time variation. In addition there is growing evidence that QTL determining flowering time in other species may involve homologues of *A. thaliana* flowering time loci (Yano *et al*. 2000; Schranz *et al*. 2002). If natural variation is due to the repeated fixation of functional alleles at one or a few of the many loci involved in a particular pathway or network, these may be loci with minimal deleterious effects on fitness. The relative prevalence of adaptive evolution along genetic vs. ecological (selective) lines of least resistance (Schluter 1996) remains to be seen.

Here we have shown a large positive genetic correlation between flowering time and $\delta^{13}C$ among naturally occurring accessions of *A. thaliana*, indicating a genetic trade-off between different drought adaptation strategies. This genetically based trade-off suggests three possible mechanisms: correlated selection, tight linkage and/or pleiotropy. We found strong evidence for pleiotropy using near-isogenic lines of *Frigida* and *Flowering Locus C*. Naturally occurring alleles at these two flowering time loci also have significant effects on $\delta^{13}C$. These data suggest that the correlated evolution of $\delta^{13}C$ and flowering time is explained in part by the fixation of pleiotropic alleles that alter both $\delta^{13}C$ and time to flowering. At the molecular level the effect of *FLC* and *FRI* on $\delta^{13}C$ may be due, in part, to differences in levels of the transcription factor *FLC*.

Acknowledgements

We thank A Sala and D Emlen for many helpful discussions and feedback on this manuscript. RM Amasino kindly provided the flowering time mutants, near-isogenic and transgenic lines. We also thank: UCD Controlled Environment Facility; B Anderson, C Bergens, Z Cleveland, H Easlon, B Hurtle and W Mosher for help in growing and measuring plants; D Harris for carbon isotope analysis, J Fry for help on SAS proc mixed and S Dix for secretarial assistance. Partial support was provided by the Max-Planck-Gesellschaft and US National Science Foundation grants DEB 9527725 (TM-O) and IBN 9903004 (JHR).

References

Abbott RJ, Gomes MF (1989) Population genetic structure and outcrossing rate of *Arabidopsis thaliana*. *Heredity*, **62**, 411–418.

Alonso-Blanco C, El Asal SE, Coupland G, Koornneef M (1998) Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. *Genetics*, **149**, 749–764.

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science*, **5**, 22–29.

Araus JL, Slafer GA, Reynolds MP, Royo C (2002) Plant breeding and drought in C3 cereals: what should we breed for? *Annals of Botany*, **89**, 925–940.

Arntz AM, Delph LF (2001) Pattern and process: evidence for the evolution of photosynthetic traits in natural populations. *Oecologia*, **127**, 455–467.

Artus NN (1990) Two mutants of *Arabidopsis thaliana* that become chlorotic in atmospheres with enriched CO_2. *Plant, Cell and Environment*, **13**, 575–580.

Berg RL (1960) The ecological significance of correlation pleiades. *Evolution*, **14**, 171–180.

Bergelson J, Stahl EA, Dudek S, Kreitman ME (1998) Genetic variation within and among populations of *Arabidopsis thaliana*. *Genetics*, **148**, 1311–1323.

Bies-Etheve N, da Silva Conceicao A, Giraudat J *et al.* (1999) Importance of the B2 domain of the of the *Arabidopsis* ABI3 protein for Em and 2S albumin gene regulation. *Plant Molecular Biology*, **40**, 1045–1054.

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. *Plant Cell*, **7**, 1099–1111.

Boneta D, McCourt P (1998) Genetic analysis of ABA signal transduction pathways. *Trends in Plant Science*, **3**, 231–235.

Boyes DC, Zayed AM, Ascenzi R *et al.* (2001) Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. *Plant Cell*, **13**, 1499–1510.

Bray EA (1997) Plant responses to water deficit. *Trends in Plant Science*, **2**, 48–54.

Bray EA (2002) Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. *Annals of Botany*, **89**, 803–811.

Clausen J, Heisey WM (1958) Experimental studies on the nature of species IV. Genetic structure of ecological races. *Carnegie Institution of Washington Publication, 615*. Carnegie Institute, Washington, DC.

Clausen J, Keck DD, Heisey WM (1948) Experimental studies on the nature of species III. Environmental responses of climatic races of *Achillea*. *Carnegie Institute of Washington Publication 581*. Carnegie Institute, Washington, DC.

Coleman JS, McConnaughay KDM, Ackerly DD (1994) Interpreting phenotypic variation in plants. *Trends in Ecology and Evolution*, **9**, 187–190.

Comstock JP, Ehleringer JR (1992) Correlating genetic variation in carbon isotopic composition with complex climatic gradients. *Proceedings of the National Academy of Sciences of the USA*, **89**, 7747–7751.

Condon AG, Hall AE (1997) Adaptation to diverse environments: variation in water-use efficiency within crop species. In: *Ecology in Agriculture* (ed. Jackson L), pp. 79–116. Academic Press, San Diego.

Craufurd PQ, Austin RB, Acevedo E, Hall MA (1991) Carbon isotope discrimination and grain-yield in barley. *Field Crops Research*, **27**, 301–313.

Crone W, McDaniel CN (1997) Flowering response to transplanting and rooting manipulations in *Arabidopsis thaliana* (Brassicaceae). *International Journal of Plant Science*, **158**, 231–235.

Darwin C (1859) *The Origin of Species*. John Murray, London.

Dudley SA (1996) Differing selection on plant physiological traits in response to environmental water availability: a test of adaptive hypotheses. *Evolution*, **50**, 92–102.

Ehdaie B, Hall AE, Farquhar GD, Nguyen HT, Waines JG (1991) Water-use efficiency and carbon isotope discrimination in wheat. *Crop Science*, **31**, 1282–1288.

Ehleringer JR, Monson RK (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. *Annual Review of Ecology and Systematics*, **24**, 411–439.

El-Assal S, Alonso-Blanco C, Peeters AJM, Raz V, Koornneef M (2001) A QTL for flowering time in *Arabidopsis* reveals a novel allele of CRY2. *Nature Genetics*, **29**, 435–440.

Evans JR, Sharkey TD, Berry JA, Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. *Australian Journal of Plant Physiology*, **13**, 281–292.

Falconer DS, MacKay TFC (1996) *Introduction to Quantitative Genetics*, 4th edn. Longman, Essex.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, **40**, 503–537.

Farquhar GD, von Caemmerer S (1981) Electron transport limitations on the CO_2 assimilation rate of *Phasoleus vulgaris* L. In: *Proceedings of the Fifth International Congress on Photosynthesis*, Vol. 4 (ed. Akoyunoglou G), pp. 163–175. Balaban, Philadelphia.

Felsenstein J (1985) Phylogenies and the comparative method. *American Naturalist*, **125**, 1–15.

Field C, Mooney HA (1986) The photosynthesis–nitrogen relationship in wild plants. In: *On the Economy of Plant Form and Function* (ed. Givnish TJ), pp. 25–56. Cambridge University Press, Cambridge.

Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. *The Plant Cell*, S15–S45.

Fisher RA (1958) *The Genetic Theory of Natural Selection*, 2nd edn. Dover, New York.

Fox GA (1990) Drought and the evolution of flowering time in desert annuals. *American Journal of Botany*, **77**, 1508–1518.

Fry JD (1996) The evolution of host specialization: are trade-offs overrated? *American Naturalist*, **148**, S84–S107.

Fu A, Boutton TW, Ehleringer JR, Flagler RB (1993) Environmental and developmental effects on carbon isotope discrimination by two species of *Phaseolus*. In: *Stable Isotopes and Plant Carbon–Water Relations* (eds Ehleringer JR *et al.*), pp. 297–309. Academic Press, San Diego.

Geber ME, Dawson TE (1990) Genetic variation in and covariation between leaf gas exchange, morphology, and development in *Polygonum arenastrum*, an annual plant. *Oecologia*, **85**, 153–158.

Geber ME, Dawson TE (1997) Genetic variation in stomatal and biochemical limitations to photosynthesis in the annual plant *Polygonum arenastrum*. *Oecologia*, **109**, 535–546.

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *Plant Cell*, **4**, 1251–1261.

Hall AE, Mutters RG, Hubick KT, Farquhar GD (1990) Genotypic

differences in carbon isotope discrimination by cowpea under wet and dry field conditions. *Crop Science*, **30**, 300–305.

Harlan HV, Martini ML (1938) The effects of natural selection in a mixture of barley varieties. *Journal of Agricultural Research*, **57**, 189–199.

Hubick KT, Farquhar GD, Shorter R (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. *Australian Journal of Plant Physiology*, **13**, 803–816.

Hubick KT, Shorter R, Farquhar GD (1988) Heritability and genotype–environment interactions of carbon isotope discrimination and transpiration efficiency in peanut (*Arachis hypogaea* L.). *Australian Journal of Plant Physiology*, **15**, 799–813.

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 377–403.

Innan H, Stephan W (2000) The coalescent in an exponentially growing metapopulation and its application to *Arabidopsis thaliana*. *Genetics*, **155**, 2015–2019.

Johanson U, West J, Lister C *et al*. (2000) Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science*, **290**, 344–347.

Jordan N (1991) Multivariate analysis of selection in experimental populations derived from hybridization of two ecotypes of the annual plant *Diodia teres* W. (Rubiaceae). *Evolution*, **45**, 1760–1772.

Juenger TM, Purugganan M, MacKay TFC (2000) Quantitative trait loci for floral morphology in *Arabidopsis thaliana*. *Genetics*, **156**, 1379–1392.

Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W (1998) Genetic control of flowering time in *Arabidopsis*. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 345–370.

Koornneef M, Blankestijn de Vries H, Hanhart C, Soppe W, Peeters T (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. *Plant Journal*, **6**, 911–919.

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Molecular and General Genetics*, **229**, 57–66.

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiologia Plantarum*, **61**, 377–383.

Lambers H, Chapin FS III, Pons TL (1998) *Plant Physiological Ecology*. Springer, New York.

Langlet O (1971) Two hundred years of genecology. *Taxon*, **20**, 53–722.

LeCorre V, Roux F, Reboud X (2002) DNA polymorphism at the *FRIGIDA* gene in *Arabidopsis thaliana*: extensive nonsynonymous variation is consistent with local selection for flowering time. *Molecular Biology and Evolution*, **19**, 1261–1271.

Lee I, Amasino RM (1995) Effect of vernalization, photoperiod and light quality on the flowering phenotype of *Arabidopsis* plants containing the *FRIGIDA* gene. *Plant Physiology*, **108**, 157–162.

Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatase 2C involved in abscisic acid signal transduction. *Plant Cell*, **9**, 759–771.

Levy Y, Dean C (1998) The transition to flowering. *Plant Cell*, **10**, 1973–1989.

Li B, Suzuki J-I, Hara T (1998) Latitudinal variation in plant size and relative growth rate in *Arabidopsis thaliana*. *Oecologia*, **115**, 293–301.

Linhart YB, Grant MC (1996) Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics*, **27**, 237–277.

Ludlow MM (1989) Strategies of response to water stress. *Structural and Functional Responses to Environmental Stresses* (eds Kreeb KH Richter H Hinckley TM), pp. 269–281. SPB Academic, The Hague.

Masle J, Shin JS, Farquhar GD (1993) Analysis of restriction fragment length polymorphisms associated with variation of carbon isotope discrimination among ecotypes of *Arabidopsis thaliana*. In: *Stable Isotopes and Plant Carbon–Water Relations* (eds Ehleringer JR *et al*.), pp. 371–386. Academic Press, San Diego.

Mather K, Jinks JL (1982) *Biometrical Genetics*, 3rd edn. Chapman & Hall, London.

Matyas C (1996) Climatic adaptation of trees: rediscovering provenance tests. *Euphytica*, **92**, 45–54.

Menendez CM, Hall AE (1995) Heritability of carbon isotope discrimination and correlations with earliness in cowpea. *Crop Science*, **35**, 673–678.

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell*, **11**, 949–956.

Michaels SD, Amasino RM (2000) Memories of winter: vernalization and the competence to flower. *Plant, Cell and Environment*, **23**, 1145–1153.

Michaels SD, Amasino RM (2001) Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell*, **13**, 935–941.

Mitchell-Olds T (1986) Quantitive genetics of survival and growth in impatiens capensis. *Evolution*, **40**, 107–116.

Mitchell-Olds T (1996) Genetic constraints on life-history evolution: quantitative-trait loci influencing growth and flowering in *Arabidopsis thaliana*. *Evolution*, **50**, 140–145.

Mitchell-Olds T (2001) *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology and Evolution*, **16**, 693–700.

Nagy E (1997) Selection for native characters in hybrids between two locally adapted plant subspecies. *Evolution*, **51**, 1469–1480.

Nambara E, Suzuki M, Abrams S, McMarty DR, Kamiya Y, McCourt P (2002) A screen for genes that function in abscisic acid signaling in *Arabidopsis thaliana*. *Genetics*, **161**, 1247–1255.

Napp-Zin K (1957) Untersuchungen zur genetic des kältebedürfnisses bei *Arabidopsis thaliana* L. Heynh. *Zeitscrift für Indukt Abstamm Vererbungsl*, **88**, 253–285.

Nienhuis J, Sills GR, Martin B, King G (1994) Variance for water-use efficiency among ecotypes and recombinant inbred lines of *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany*, **81**, 943–947.

Nordborg M, Bergelson J (1999) The effect of seed and rosette cold treatment on germination and flowering time in some *Arabidopsis thaliana* (Brassicaceae) ecotypes. *American Journal of Botany*, **86**, 470–475.

Norton LR, Firbank LG, Watkinson AR (1995) Ecotypic differentiation of response to enhanced CO_2 and temperature levels in *Arabidopsis thaliana*. *Oecologia*, **104**, 394–396.

Passioura JB (1996) Drought and drought tolerance. *Plant Growth Regulation*, **20**, 79–83.

Pesaresi P, Varotto C, Richly E, Kurth J, Salamini F, Leister D (2001) Functional genomics of *Arabidopsis* photosynthesis. *Plant Physiology and Biochemistry*, **39**, 285–294.

Pigliucci M (1998) Ecological and evolutionary genetics of *Arabidopsis*. *Trends in Plant Science*, **3**, 485–489.

Pyke KA, Leech RM (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. *Plant Physiology*, **99**, 1005–1008.

Ramanjulu S, Bartels D (2002) Drought- and desiccation-induced modulation of gene expression in plants. *Plant, Cell and Environment*, **25**, 141–151.

Rédei GP (1962) Supervital mutants of *Arabidopsis*. *Genetics*, **47**, 443–460.

Richards RA (1996) Defining selection criteria to improve yield under drought. *Plant Growth Regulation*, **20**, 157–166.

Richards RA, Condon AG (1993) Challenges ahead in using carbon isotope discrimination in plant breeding programs. In: *Stable Isotopes and Plant Carbon–Water Relations* (eds Ehleringer JR *et al.*), pp. 451–462. Academic Press, San Diego.

Robertson A (1959) The sampling variance of the genetic correlation coefficient. *Biometrics*, **15**, 469–485.

Rohde A, De Rycke R, Beekman T, Engler G, Van Montagu M, Boerjan W (2000) ABI3 affects plastid differentiation in dark-grown *Arabidopsis* seedlings. *Plant Cell*, **12**, 35–52.

Rohde A, Prinsen E, De Rycke R, Engler G, Van Montagu M, Boerjan W (2002) PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in *Poplar*. *Plant Cell*, **14**, 1885–1901.

Rouse DT, Sheldon CC, Bagnall DJ, Peacock WJ, Dennis ES (2002) FLC, a repressor of flowering, is regulated by genes in different inductive pathways. *Plant Journal*, **29**, 183–191.

Sanda S, John M, Amasino RM (1997) Analysis of flowering time in ecotypes of *Arabidopsis thaliana*. *Journal of Heredity*, **88**, 69–72.

Schemske DW (1984) Population structure and local selection in *Impatiens pallida* (Balsamineceae), a selfing annual. *Evolution*, **38**, 817–832.

Schläppi (2001) RNA levels and activity of *FLOWERING LOCUS C* are modified in mixed genetic backgrounds of *Arabidopsis thaliana*. *International Journal of Plant Science*, **162**, 527–537.

Schluter D (1996) Adaptive radiation along genetic lines of least resistance. *Evolution*, **50**, 1766–1774.

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

Schulze ED, Robichaux RH, Grace J, Rundel PW, Ehleringer JR (1987) Plant–water balance. *Bioscience*, **37**, 30–37.

Scott P (2000) Resurrection plants and the secrets of eternal leaf. *Annals of Botany*, **85**, 159–166.

Seki M, Narusaka M, Ishida J *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant Journal*, **31**, 279–292.

Sharbel TF, Haubold B, Mitchell-Olds T (2000) Genetic isolation by distance in *Arabidopsis thaliana*: biogeography and postglacial colonization of Europe. *Molecular Ecology*, **9**, 2109–2118.

Shaw RG, Byers DL, Darmo E (2000) Spontaneous mutational effects on reproductive traits of *Arabidopsis thaliana*. *Genetics*, **155**, 369–378.

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES (2000) The molecular basis of vernalization: the central role of *FLOWERING LOCUS C (FLC)*. *Proceedings of the National Academy of Sciences of the USA*, **97**, 3753–3758.

Stebbins GL (1952) Aridity as a stimulus to plant evolution. *American Naturalist*, **86**, 33–44.

Tamminen I, Makela P, Heino P, Palva ET (2001) Ectopic expression of *ABI3* gene enhances freezing tolerance in response to abscisic acid and low temperature in *Arabidopsis thaliana*. *The Plant Journal*, **25**, 1–8.

Thornsberry JM, Goodman MM, Doebley J, Kresovich S, Nielsen D, Buckler ES (2001) Dwarf polymorphisms associate with variation in flowering time. *Nature Genetics*, **28**, 286–289.

Turesson G (1922) The genotypical response of the plant species to the habitat. *Hereditas*, **3**, 211–350.

Virgona JM, Farquhar GD (1996) Genotypic variation in relative growth rate and carbon isotope discrimination in sunflower is related to photosynthetic capacity. *Australian Journal of Plant Physiology*, **23**, 227–236.

Weinig C, Ungerer MC, Dorn LA *et al.* (2002) Novel loci control variation in reproductive timing in *Arabidopsis thaliana* in natural environments. *Genetics*, **162**, 1875–1884.

White JW (1993) Implications of carbon isotope discrimination studies for breeding common bean under water deficits. In: *Stable Isotopes and Plant Carbon–Water Relations* (eds Ehleringer JR *et al.*), pp. 387–398. Academic Press, San Diego.

Yano M, Katayose Y, Ashikari M *et al.* (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene CONSTANS. *The Plant Cell*, **12**, 2473–2483.

This paper is part of John McKay's dissertation work at the University of Montana. He is now a postdoctoral researcher at UC Davis continuing research on local adaptation and evolutionary physiology of plants. Jim Richards is a Professor at UC Davis and studies plant physiological ecology, especially aspects of water and nutrient stress. Thomas Mitchell-Olds is Director of the Max Planck Institute of Chemical Ecology. He studies the functional basis of evolutionary forces influencing ecologically important genetic variation.
