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Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis thaliana*: $\delta^{13}C$, stomatal conductance and transpiration efficiency

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ABSTRACT

Water limitation is one of the most important factors limiting crop productivity world-wide and has likely been an important selective regime influencing the evolution of plant physiology. Understanding the genetic and physiological basis of drought adaptation is therefore important for improving crops as well as for understanding the evolution of wild species. Here, results are presented from quantitative trait loci (QTL) mapping of flowering time (a drought escape mechanism) and carbon stable isotope ratio ($\delta^{13}C$) (a drought-avoidance mechanism) in *Arabidopsis thaliana*. Whole-genome scans were performed using multiple-QTL models for both additive and epistatic QTL effects. We mapped five QTL affecting flowering time and five QTL affecting $\delta^{13}C$, but two genomic regions contained QTL with effects on both traits, suggesting a potential pleiotropic relationship. In addition, we observed QTL–QTL interaction for both traits. Two $\delta^{13}C$ QTL were captured in near-isogenic lines to further characterize their physiological basis. These experiments revealed allelic effects on $\delta^{13}C$ through the upstream trait of stomatal conductance with subsequent consequences for whole plant transpiration efficiency and water loss. Our findings document considerable natural genetic variation in whole-plant, drought resistance physiology of *Arabidopsis* and highlight the value of quantitative genetic approaches for exploring functional relationships regulating physiology.

Key-words: *Arabidopsis*; drought; quantitative trait loci; stable carbon isotopes; water deficit; water-use efficiency.

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INTRODUCTION

A central question in plant biology is the role and importance of physiological traits in plant adaptation and performance under diverse environmental conditions. Water limitation is a fundamental determinant of plant distribution and abundance, is one of the most important factors limiting crop productivity world-wide, and has probably been an important selective regime influencing the evolution of plant morphology, development, and physiology. Nearly all terrestrial plants are exposed to drought stress at varying times and to different degrees during their life cycle as water is fundamental to almost all aspects of plant physiology (Stebbins 1952; Bohnert, Nelson & Jensen 1995; Bray 1997). Plants are thought to have evolved numerous strategies for coping with limited water availability including changes in phenological, developmental, and physiological traits (Schulze *et al*. 1987; Ludlow 1989; Ehleringer & Monson 1993; Ingram & Bartels 1996; Passioura 1996; Geber & Dawson 1997; Ackerly *et al*. 2000; Araus *et al*. 2002). Nonetheless, we know relatively little about the natural genetic variation underlying these strategies or the genes involved in drought adaptation in nature

For most plants, drought avoidance is achieved primarily through regulation of stomatal conductance in response to soil and atmospheric water deficits (Cohen 1970; Cowan 1982; Schulze 1986; Dawson & Ehleringer 1993; Meinzer 1993). Stomatal conductance can be optimized over the long-term through developmental changes in stomata density or by short-term changes in stomatal opening and closing. Stomatal closure can serve as a rapid and effective drought-avoidance response. However prolonged stomatal closure is not sustainable as stomatal CO_2 uptake is also reduced and will ultimately limit photosynthetic assimilation and growth (Farquhar & Sharkey 1982; Schulze *et al*. 1987). A high ratio of carbon gained per unit water lost through transpiration, namely high water-use efficiency (WUE), is hypothesized to confer a fitness advantage under

drought stress (Cohen 1970; Dudley 1996; McKay *et al*. 2001), but may come at a cost of reduced growth rate or delayed phenology in more favourable environments (Geber & Dawson 1990, 1997; Condon & Hall 1997; McKay, Richards & Mitchell-Olds 2003). Understanding the genetic basis of WUE is therefore important for improving crops as well as for understanding the evolution of wild species.

WUE can be assessed instantaneously by measuring leaf gas exchange or, as a time integrated measure, by calculating the stable carbon isotope ratio, $\delta^{13}C$, of leaves or sugars of C_3 plants (Farquhar, Ehleringer & Hubick 1989). Variation in $\delta^{13}C$ reflects differences in the partial pressure of CO_2 inside the leaf and can be generated by variation in either photosynthetic biochemistry or stomatal constraints on diffusion of CO_2 to the leaf interior and chloroplast (Farquhar *et al*. 1989). As such, $\delta^{13}C$ has been successfully used to investigate the role of WUE in drought adaptation in a growing number of studies (Comstock & Ehleringer 1992; Ehleringer, Hall & Farquhar 1993; Virgona & Farquhar 1996; Martin, Taur & Kin 1999; reviewed in Dawson *et al*. 2002). There is also a great deal of interest in improving crop performance through direct selection on $\delta^{13}C$ (Rebetzke *et al*. 2002; Araus *et al*. 2002). For example, artificial selection on $\delta^{13}C$ in wheat has resulted in higher yielding cultivars (Rebetzke *et al*. 2002).

A variety of molecular approaches have identified candidate genes that might influence drought avoidance and WUE in crop and model plant systems (Ingram & Bartels 1996; Cushman & Bohnert 2000; Haake *et al*. 2002; Seki *et al*. 2002). However, many of these candidate loci involve loss-of-function alleles (e.g. inability to synthesize ABA) resulting in impaired growth and reproduction even under very benign growth chamber conditions. Many candidate genes have also been identified through differential gene expression studies where experimentally imposed cellular water-deficit results in up- and down-regulation of gene expression (Seki *et al*. 2002). Unfortunately, the functions of many of these genes are unknown and not all desiccation-induced genes are expected to be involved in adaptive or tolerance responses (Bray 2002). It is unclear the degree to which loci detected through these methods will also be responsible for naturally occurring variation (McKay *et al*. 2003) and knowledge of loci underlying natural variation in drought-related whole-plant traits like WUE and $\delta^{13}C$ would be valuable for breeding applications.

Quantitative trait loci (QTL) mapping is a complementary approach useful for identifying the genes underlying naturally occurring variation in traits such as those conferring drought adaptation. QTL mapping allows one to statistically identify chromosomal regions containing genetic factors contributing to variation in a polygenic trait (Lynch & Walsh 1998; Alonso-Blanco & Koornneef 2000). Several QTL that affect $\delta^{13}C$ have been identified in tomato, rice, barley and soybean (Martin *et al*. 1989; Nienhuis *et al*. 1994; Specht *et al*. 2001; Teulat *et al*. 2002; Price *et al*. 2002). In *A. thaliana,* we recently reported significant genetic variation in $\delta^{13}C$ among accessions (McKay *et al*. 2003), mapped preliminary QTL for $\delta^{13}C$ (Hausmann *et al*. 2004), and showed that two loci responsible for natural variation in flowering time (*FRIGIDA* and *FLOWERING LOCUS C*) also have pleiotropic effects on $\delta^{13}C$ (McKay *et al*. 2003). We have also found an association between ecotype $\delta^{13}C$ values and the climatic conditions of their native habitats, suggesting that natural variation in $\delta^{13}C$ may reflect adaptation to different climates.

Here, we confirm and extend our earlier studies with additional QTL mapping. Furthermore, we fine-map two $\delta^{13}C$ QTL using near-isogenic lines to further characterize their physiological effect and to explore the functional relationships regulating whole-plant, drought resistance physiology.

MATERIALS AND METHODS

Plant material – recombinant inbred lines

We used 162 recombinant inbred lines (RILs) generated from a reciprocal cross between Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi) accessions (Alonso-Blanco *et al*. 1998b) to map QTL. The parental accessions for this mapping population are derived from climatically divergent locations (Northern Europe and Tropical Cape Verde Island) and differ in both flowering time and $\delta^{13}C$ – the Cvi accession flowers slightly later and has a lower (more negative) $\delta^{13}C$ (lower WUE) when compared with the L*er* accession. These lines are available from the *Arabidopsis* stock centre (*http://arabidopsis.org*) as accession number cs22000. We constructed a linkage map using 111 genetic markers. The RIL genotype at each marker locus was obtained from the published data available from the *Arabidopsis* stock centre. The linkage map was constructed using markers genotyped in at least 80% of the sampled lines. The map position of each marker (*d* cM) was estimated from the observed recombination frequencies (*r*) using the Kosambi mapping function as implemented by the software MAPMAKER 3.0 (Lander *et al*. 1987). These analyses provided a unique position for each marker that did not differ in order from the published *Arabidopsis* RIL linkage maps.

Plant material – near-isogenic lines

Two QTL regions were investigated with near-isogenic lines (NILs) that captured Cvi alleles in a homogeneous L*er* background. RIL Cvl-134 from the L*er*/Cvi RIL population was chosen for generating a NIL for the QTL detected on the bottom of chromosome II [delta-2.1]. This RIL contained a Cvi introgression at the lower arm of chromosome II (11.2 Mbp – end) along with three additional introgressions on chromosomes I and IV. Following procedures similar to those of Alonso-Blanco *et al*. (2003) and Bentsink *et al*. (2003 and references therein) using backcrosses to L*er*, further selfed generations, and marker-assisted selection, a homozygous line containing a single Cvi introgression on the end of chromosome II (~17.9 Mbp – end) was selected. We refer to this NIL as 'NIL delta-2.1'.

A NIL previously developed from RIL Cvl-49 and L*er* by Bentsink *et al*. (2003) (NIL26) captured the $\delta^{13}C$ QTL detected on chromosome III [delta-3.1]. This NIL contains a single Cvi introgression at the top of chromosome III (end- approximately 0.79 Mbp) and for the purposes of this paper, we refer to it as 'NIL delta-3.1'.

Plant growth conditions – QTL mapping experiment

Replicate plants were grown under standard glasshouse conditions using Promix BT™ potting soil and 164-mL Conetainer™ pots (Stuewe & Sons, Corvallis, OR). Individual Conetainers™ were racked in 61 × 30-cm trays at half the possible density (49 plants per tray – skipping every other position). Long-day photoperiod conditions (16 h light/8 h dark) were obtained with supplemental high-intensity discharge lamps and during daytime PPFD was maintained at ≥1000 μmol m^{-2} s^{-1}. Glasshouse temperature was approximately 18–21 °C. Several seeds were initially planted in each Conetainer™, cold/wet stratified for 7 d at 4 °C, and subsequently thinned to a single replicate individual at the first true leaf stage. Plants were kept well-watered, such that the soil surface of each pot was continuously damp and soil conditions were optimal for *A. thaliana* growth.

We used a randomized incomplete block design including the two parental lines and 162 RILs derived from the L*er* × Cvi mapping population. Twelve replicate plants from each of the 164 lines were randomly arrayed across individual Conetainer™ trays that each contained 49 plants – we considered each tray an incomplete block (164 lines × 12 replicates = 1968 plants). Flowering time was recorded by daily inspection of the plants and was recorded as the number of days since flowering began in the mapping population. This experiment was conducted from June to August 2000.

QTL analyses

We mapped QTL using the *Pseudomarker* multiple-QTL framework presented by Sen & Churchill (2001). This methodology relies on a Monte Carlo imputation algorithm to simulate multiple versions of complete genotype information on a dense genome-wide grid. The authors refer to these imputed genotypes as 'pseudomarkers'. This pseudomarker grid is scanned using both one and two QTL models at each position across the genome and evidence for a QTL or a QTL–QTL (epistatic) interaction is determined using robust one- and two-dimensional permutation tests.

We used a series of 256 Monte Carlo imputations to estimate the missing marker data at genotyped locations as well as to infer the genotype of 'pseudomarkers' at 2 cM intervals across the *A. thaliana* genome. This fine-scale analysis was implemented because the L*er* × Cvi linkage map contained several modest gaps on chromosome V. Here, the residual sum of squares corresponding to a particular model was calculated by averaging the residual sum of squares over the imputations. For technical reasons, the average is not a simple arithmetic mean (see Appendices C and F in Sen & Churchill 2001). Additional details of our model selection and QTL mapping strategy were presented in an earlier paper (Juenger *et al*. 2005).

We estimated the additive effect of each QTL as half the difference in the phenotypic means for the two homozygous genotypes at a locus. The sign of the additive effect corresponds to the direction of the effect of alleles from the Cvi parent: positive values indicate that alleles from Cvi increased trait values while negative values indicate that Cvi alleles decreased trait values. We estimated the proportion of the total genetic variance ($\%V_G$) explained by each QTL by dividing the sums of squares for each significant marker by the total corrected model sums of squares from full QTL models in PROC GLM in SAS (SAS Institute 1997). We estimated the proportion of genetic variance explained by interacting QTL as the difference in the adjusted r^2 of additive GLM models versus those incorporating interactions. We plotted the posterior probability distribution of the QTL locations under the final model to locate the genomic position of QTL. In the case of linked or interacting QTL, we plotted the two-dimensional posterior probability distribution under a multiple-QTL model. Under complex genetic models (e.g. multiple-linked QTL, epistasis) strict confidence intervals are difficult to define. As a result, the confidence intervals presented for several QTL (delta-1.1, delta-5.1, delta-5.2) should be taken as approximations.

We fit models of the main and interactive effects of all significant markers detected in the *Pseudomarker* analysis (Lynch & Walsh 1998) using SAS Proc Mixed (SAS Institute 1997). In this framework, marker–marker interaction represents epistasis (Hausmann *et al*. 2004; Juenger *et al*. 2005). We also tested for nuclear–cytoplasmic interactions for all QTL and QTL pairs identified with *Pseudomarker* using the above approach. We estimated genetic correlations (r_G) among traits as the standard Pearson product-moment pairwise correlation between the breeding values for each trait. The significance of each genetic correlation was determined using a *t*-test after a *Z* transformation of the correlation coefficient.

NIL analyses

We used a randomized complete block design with six replicates of each NIL and the recurrent parent L*er* in a study of whole-plant physiology (three blocks, each with two replicates of each genotype). Plants were grown in Conetainers™ filled with a 1 : 1 mixture of fritted clay (Turface Pro-League®; Profile Products LLC, Buffalo Grove, IL, USA) and peat-based potting mix. These Conetainers™ were capped by modified, sealed, 50-mL centrifuge tubes to adapt to the cuvette used for gas exchange measurements. Seeds were planted into moist soil in a small hole in the top of the centrifuge tube cap and then cold stratified in the dark for 7 d at 4 °C. The plants germinated and

grew in a high light growth chamber (370 μmol m^{-2} s^{-1} PPFD). Photoperiod was 12 h light/12 h dark, where the lights ramped up and down for the first and last hour of light and the temperature cycled 23/13 °C light/dark. Germination was scored daily and used to schedule gas exchange measurements on plants of exactly equal age. After 21 d, we began measuring photosynthesis (A) and transpiration (E) during the middle of the light period using a custom-made whole-plant cuvette with the LiCor 6400 photosynthesis system (LiCor Inc., Lincoln, NE, USA) as described by McKay *et al*. (2001). In the cuvette the leaf temperature was 26 ± 1 °C and PPFD was 315 μmol m^{-2} s^{-1}. Input CO_2 was constant at 400 μmol mol^{-1}, so depending on the plant, the sample CO_2 ranged from 378 to 393 μmol mol^{-1} and VPD ranged from 1.65 to 2.0 kPa. Each replicate plant was measured at least 10 times on each of two measurement periods over a 6-d period. Thus, a minimum of 20 repeated measures were averaged to obtain a single phenotypic value per plant. At the time of each measurement a digital photo was taken of each plant and analysed for projected leaf area using a series of macros in PHOTOSHOP (Adobe, Seattle, WA, USA) and SCION IMAGE (Scion, Frederick, MD, USA). Stomatal conductance (g_s) was calculated according to the LiCor 6400 manual (LiCor Inc. 2003) and expressed on a projected leaf area basis (Donahue, Poulson & Edwards 1997). Leaf temperature was measured on one leaf of each rosette during gas exchange measurements with a fine wire thermocouple and boundary layer resistance was kept minimal by vigorous mixing of cuvette air. After the second set of measures, plants in two blocks were harvested and total leaf area and dry mass were obtained for these plants. The entire rosette of each harvested plant was then used for carbon isotope analysis (see below). Phenotypic data from this experiment were analysed with standard GLM in SAS incorporating genotype and blocking factors (SAS Institute 1997). When significant genotype effects were detected, the pattern was further explored using Dunnett one-tailed *post hoc* analyses.

To further evaluate water use in the NILs, we conducted a whole-plant transpiration efficiency (TE) experiment. Individual plants were grown in 250-mL plastic cups, each filled with a standard mass of 1 : 1 fritted clay and Promix BT™ potting soil mix. We measured field capacity of the soil mix following a 24-h gravitational drain of saturated soil. Each cup was covered with parafilm and sealed with a plastic lid that had a 6-mm diameter hole for the plant. Eight replicates of each NIL and L*er* were planted in TE containers and cold stratified in the dark for 7 d at 4 °C. The plants germinated and grew in a growth chamber (200 μmol m^{-2} s^{-1} PPFD) in a randomized block design. Photoperiod was 16 h light/8 h dark and the temperature cycled 23/18 °C (light/dark). We weighed each container every 2 d during the experiment – then additional water was added with a syringe to bring the soil in each container back to 90% of field capacity. Total water use was summed for the 35-d growing period and all plants were flowering and setting fruit at the experiment's completion, when above-ground dry biomass and $\delta^{13}C$ were measured. We assessed evaporative loss from TE containers using 'blanks' lacking an *Arabidopsis* plant. Total evaporation from blank containers was < 4% of the average total water lost from pots in the experiment. Transpiration efficiency of each plant was calculated as mg dry above-ground biomass divided by ml of water transpired. As evaporative losses were extremely low, we did not correct estimates of transpired water for evaporative loss. These data were analysed using a randomized block ANOVA with GLM in SAS incorporating genotype and blocking factors (SAS Institute 1997).

To confirm differences in stomatal conductance, we also examined the rate of water loss from detached rosettes. Whole rosettes of 20-day-old-plants prior to bolting were cut off from their root system, weighed immediately for fresh weight, and then weighed every 20 min for 3.7 h. Ten replicates of each NIL and 20 replicates of L*er* were screened. Plants had grown in a randomized block design in 5-cm-square pots with Promix BT™ soil using the same protocol and growing conditions as for the TE experiment, except plants were watered as needed to ensure optimal *Arabidopsis* growth.

For growth rate assessments we used a randomized complete block design with 24 replicates of each NIL and the recurrent parent (two blocks, each with 12 replicates of each genotype). Plants were grown using the same protocol and conditions as for the water loss experiments. Pots were shuffled within blocks each morning to homogenize environmental variation. We scored date of seed germination and collected digital images of growing rosettes on days 12, 14, 16, 18, and 20 of the experiment. These images were analysed for projected leaf area as described for the gas exchange measurements. All plants were harvested on day 21, rosettes were weighed for fresh mass, leaves were separated, and total leaf area was estimated using a flat-bed scanner.

Univariate repeated measure ANOVAs were used to analyse both the water loss and growth rate experiments using the GLM procedure of SAS (SAS Institute 1997).

Carbon isotope analyses

Above-ground biomass was analysed for carbon isotope ratio ($\delta^{13}C$) of all RIL. As $\delta^{13}C$ of large numbers of samples is an expensive phenotype to obtain, we used a pooling scheme to derive estimates of $\delta^{13}C$ for each experimental line. For the initial QTL analysis, five replicate plants were randomly chosen from each inbred line. The above-ground portions of these plants were pooled and ground for approximately 1 min in a Krups Type 203 Household Coffee Grinder to reach a flour-like consistency. A subsample was taken from this homogeneous mixture and placed in a screw-type capsule with one stainless steel ball pestle and ground to a fine powder by shaking for 2 min in a Wig-L-Bug™ (Dentsply International, Surrey, UK). Five milligrams of powder from each line were placed in a tin capsule and $\delta^{13}C$ determined at the

Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. For the NIL experiments, we harvested all leaves from four of the replicates. These were freeze dried and ground to a fine powder in centrifuge tubes with ball-bearing pestles. Two milligrams of powder from each sample were loaded into a tin capsule and analysed at the UC Davis Stable Isotope Facility. Data are presented as carbon isotope ratios relative to the V-PDB standard (R_{PDB}), where $\delta^{13}C\ (‰) = (R_S/R_{PDB} - 1) \times 1000$. These values are expressed per mil (‰). As the $\delta^{13}C$ of CO_2 in the air in the various experiments, especially those in the growth chambers, was not assured to be constant and equal to –8‰, we chose to use the isotopic composition of the samples ($\delta^{13}C$) rather than discrimination, $\Delta^{13}C$ (Farquhar *et al*. 1989).

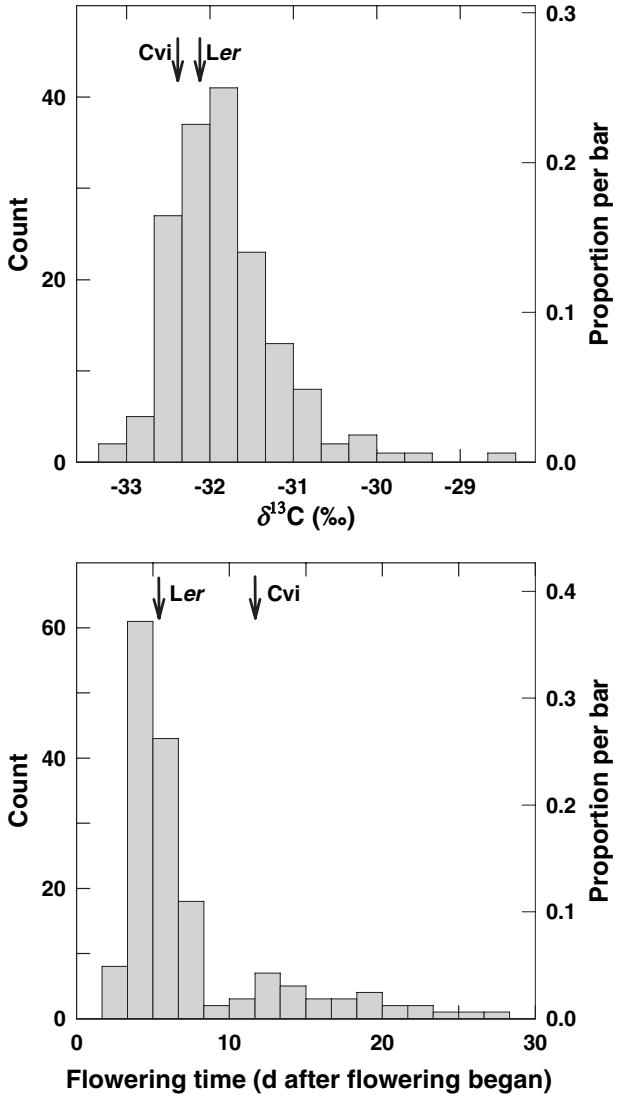

Figure 1. Frequency distributions of $\delta^{13}C$ and flowering time for the L*er* × Cvi RIL population. Arrows correspond to the parental line means and flowering time is expressed as days after flowering was first observed in the population.

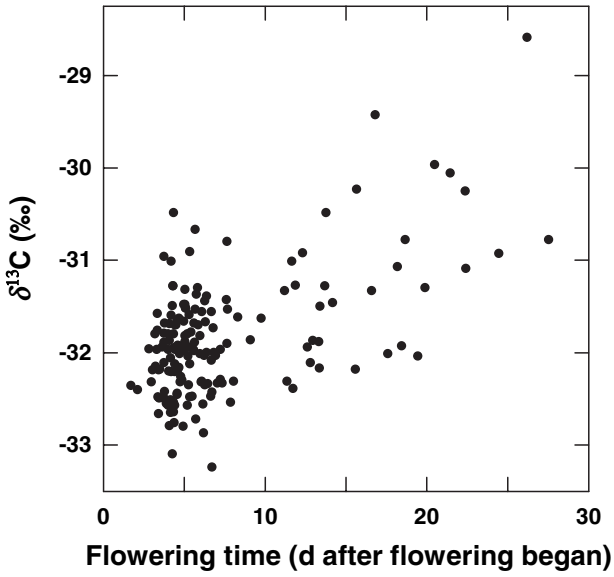

Figure 2. Bivariate relationship between $\delta^{13}C$ and flowering time in 162 L*er* × Cvi RILs. Flowering time is expressed as days after flowering was first observed in the population.

RESULTS

Quantitative genetic analysis

The L*er* × Cvi RIL population displayed considerable transgressive segregation for both $\delta^{13}C$ and flowering time. There were approximately 4- and 17-fold increases in variation for flowering time and $\delta^{13}C$ in the RIL population compared with the differences between the parents (Fig. 1). We observed a 4.65‰ difference in $\delta^{13}C$ and a 26 d difference in flowering time between the extreme RILs, indicating very large differences in whole-plant physiology among the RILs. Broad sense heritability for flowering time was estimated as 0.86. We were unable to estimate the broad sense heritability of $\delta^{13}C$ as we lacked data on its within-RIL variance since we used biomass pooled from all replicates per line to obtain a single $\delta^{13}C$ value for each RIL. We found a significant positive genetic correlation (r_G) between $\delta^{13}C$ and flowering time ($r_G = 0.47$). Early flowering genotypes have decreased and late flowering genotypes increased $\delta^{13}C$ (Fig. 2).

QTL analyses

A variety of methods have been used to localize the genomic regions underlying complex polygenic variation (see Broman 2001 for a recent review). Here, we implement a novel Bayesian QTL mapping strategy that incorporates multiple-QTL models and genome-wide scans for both additive and epistatic QTL effects.

Table 1 lists the QTL detected in this experiment. Each $\delta^{13}C$ QTL is designated by the identifier 'delta' followed by the chromosome and a unique number corresponding to the order of the QTL along the linkage group. Several

Table 1. Results of QTL analyses in the L*er* × Cvi population using two-dimensional genome scans

QTL	Trait	Chromosome	Position (cM)	Marker	Additive effect 2*a* (SE)	%V_G
EDI –cry2*	flowering time	I	9	AXR-1	–2.30 (0.75)	4.7
delta-1.1	δ^{13}C	I	24	GD.86C	–0.36 (0.08)	6.5
delta-2.1	δ^{13}C	II	78	EC.235 L-Col/247C	–0.42 (0.8)	8.9
FT-3.1	flowering time	III	0	DF.77C	+0.66 (0.54)	<1
delta-3.1	δ^{13}C	III	0	DF.77C	–0.41 (0.08)	8.3
FLF – FLC*	flowering time	V	18	BH.180C	+5.08 (0.59)	29
delta-5.1	δ^{13}C	V	34	DF.184 L-Col	+0.35 (0.11)	4.0
*FLG**	flowering time	V	39	GH.121 L-Col	+4.12 (0.58)	23
delta-5.2	δ^{13}C	V	46	AD.129 L-Col	+0.31 (0.10)	3.4
*FLH**	flowering time	V	107	DF.119 L	+1.21 (0.53)	2.3

The sign of the additive effects indicate the direction effect of Cvi alleles. For each QTL, the chromosome on which it resides, the estimated position, the associated genetic marker, the additive genotypic effect (2*a*) with its SE, and the proportion of the total genetic variance it explained in the full QTL model (%V_G) is given. *QTL detected in this study that overlap with previously described QTL using the nomenclature of Alonso-Blanco *et al*. (1998a).

flowering time and δ^{13}C QTL detected in this study overlap with those identified previously (Alonso-Blanco *et al*. 1998a, b; Ungerer *et al*. 2003; Hausmann *et al*. 2004; Juenger *et al*. 2005) – we use the published nomenclature for the flowering time QTL but acknowledge the difficulty of comparing QTL results across experiments. One unique flowering time QTL was identified and is designated by the identifier 'FT' followed by chromosome and ordering indicators. QTL presented in Table 1 were significant at the empirically determined threshold value corresponding to $P = 0.05$ based on permutation.

We detected five QTL affecting flowering time and five QTL affecting δ^{13}C (Fig. 3). Two flowering time QTL overlapped in genomic position with two δ^{13}C QTL (Table 1). This co-localization may result from pleiotropic relationships between these two traits or be due to physical linkage. The proportion of the total genetic variance explained by additive QTL (%V_G) ranged from 1 to 29%, and averaged 9.2%. Additive effects (2*a*) corresponded, on average, to 2.67 d in flowering time and 0.38‰ difference in δ^{13}C.

We detected significant QTL–QTL interactions for the traits measured. A very strong interaction occurred between two QTL located on chromosome V (*FLF* × *FLG*), which explained approximately 12% of the total genetic variation in flowering time. This QTL interaction has been observed in a number of studies of the L*er* × Cvi mapping population (Alonso-Blanco *et al*. 1998a, b; Ungerer *et al*. 2003; Hausmann *et al*. 2004; Juenger *et al*. 2005) – *FLF* and *FLG* QTL are likely caused, respectively, by polymorphisms in *FLOWERING LOCUS C* (*FLC*) (El-Assal *et al*. 2003) and *AERIAL ROSETTE 1* (*ART*) (Poduska *et al*. 2003). We detected interchromosomal epistasis between two δ^{13}C QTL (delta-1.1 × delta-5.1), which explained approximately 2.5% of the total genetic variance in δ^{13}C (Fig. 4). We found no cytoplasmic effect or nuclear–cytoplasmic interactions for either flowering time or δ^{13}C phenotypes.

Confidence intervals for delta-5.1 and delta-5.2 QTL are broadly overlapping. Despite similar localization, support for two δ^{13}C QTL on chromosome V comes from the pattern of epistasis observed. In this case, models incorporating QTL interaction are significant for markers near LOD peaks for delta-5.1 but not delta-5.2. Under complex genetic interactions, the precise positions of QTL are difficult to define and therefore the presence of and localization of delta-5.1 should be taken as preliminary until confirmed with additional fine-mapping.

Whole-plant physiological analyses

Genome-wide QTL scans are a quick and relatively straightforward means for coarse localization of genetic factors influencing a complex trait. In general, QTL studies using the existing RIL mapping populations in *A. thaliana* assign the positions of QTL to genetic intervals ranging from 5 to 50 cM, corresponding (on average) to 1.2–12 Mb of DNA (Koornneef, Alonso-Blanco & Vreugdenhil 2004). The accuracy of the mapping depends primarily on the magnitude of QTL effects, the genetic architecture of QTL (e.g. the occurrence of epistasis or linked QTL), and the heritability of the traits under study.

Fine-scale localization and, ultimately, identification of the molecular variants accounting for natural variation represent major challenges. One step toward molecular identification of QTL is the development of NILs that capture particular QTL in an isolated homogeneous background. NILs facilitate detailed phenotyping as they are immortal, easily replicated, and provide a well-controlled genetic background. These features increase the statistical power of detecting subtle QTL effects and evaluating traits that are particularly difficult to phenotype.

We utilized NILs containing Cvi alleles at two δ^{13}C QTL [delta-2.1 and delta-3.1] introgressed into a homogeneous L*er* nuclear and cytoplasmic background. NIL delta-2.1 and NIL delta-3.1 contain approximately 1.8 and 0.79 Mb of the Cvi genome, respectively. This span of genomic DNA corresponds to several hundred genes in each interval. We screened replicates of each NIL and L*er* (the recurrent

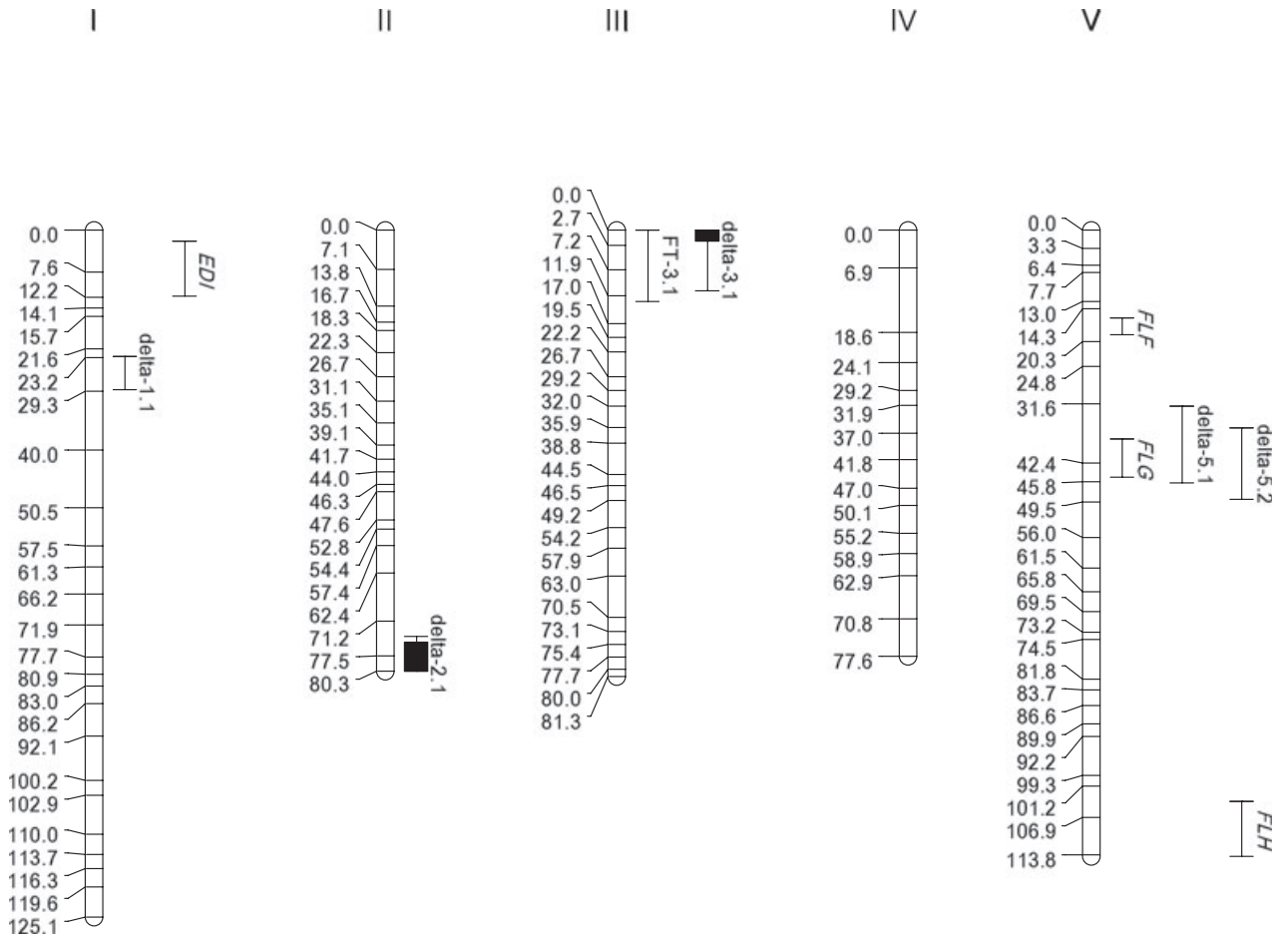

Figure 3. QTL positions for $\delta^{13}C$ and flowering time on each of the *Arabidopsis* linkage groups. Each bar represents the 95% confidence interval surrounding a QTL position as determined from the area under the posterior probability density of QTL locations. Solid black bars represent approximate positions of NIL introgressions for NIL delta-2.1 and NIL delta-3.1 intervals. Genomic positions are presented in cM units at each of the genetic markers used in the QTL mapping study.

parent) for $\delta^{13}C$, whole-plant gas-exchange rates, plant water loss rates, and growth characteristics in growth chamber experiments. We detected significant differences between each NIL and L*er* for $\delta^{13}C$ ($P < 0.05$), confirming and further localizing these QTL (Fig. 5). Additive QTL effects ($2a$) were estimated at – 0.83 and – 0.72‰ for delta-2.1 and delta-3.1, respectively.

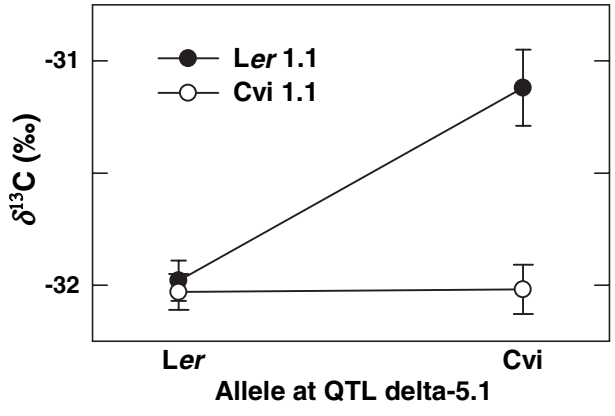

Figure 4. Epistatic interaction between delta-1.1 and delta-5.1 QTL.

We used a custom-made whole-plant gas exchange cuvette (McKay *et al.* 2001) and the LiCor 6400 to evaluate the physiological impact of Cvi alleles at delta-2.1 and delta-3.1. There was a significant difference between each NIL and L*er* for g_s ($P < 0.05$; Fig. 5), but no differences for A ($P > 0.50$). These results support the hypothesis that delta-2.1 and delta-3.1 alleles affect $\delta^{13}C$ through changes in stomatal control of CO_2 diffusion to the leaf interior, although internal conductance differences or feed-backs, not assessed with the gas exchange measurements, cannot be excluded. Additive QTL effects for g_s were estimated at 0.17 mol H_2O m^{-2} s^{-1} for both NIL lines.

To evaluate the impact of g_s differences on plant performance, we conducted a transpiration efficiency (TE) experiment using a closed-container growth system. Again, we found significant differences between each NIL and L*er* in TE ($P < 0.0001$, Fig. 5). We observed a 16.2 and 22.5% decrease in TE for NIL delta-2.1 and NIL delta-3.1 relative to L*er*, respectively. We found a significant positive relation-

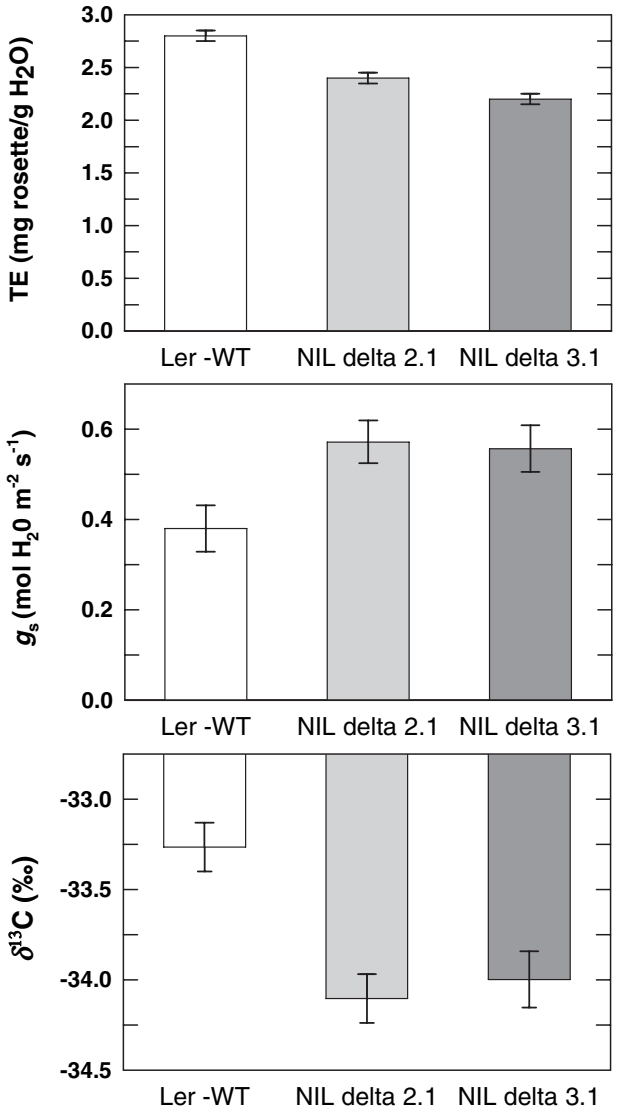

Figure 5. Phenotypic effects of NIL delta-2.1 and NIL delta-3.1 for δ^{13}C, stomatal conductance, and transpiration efficiency. Each bar represents the mean ± 1 SE (n = 5–8) for each genetic line. In each case, both NILs are significantly different from the recurrent parent L*er* ($P < 0.05$) in the expected direction.

ship between δ^{13}C and TE among replicate plants within the TE experiment (Fig. 6).

To further characterize the NILs, we measured water loss rates from whole rosettes over time after they were severed from their root systems. Variation in the rate of water loss on excised plants is likely the result of constitutive differences in initial g_s (prior to stomatal closure), the sensitivity of stomatal closure to water deficit, and cuticular conductance. We detected strikingly different rates of water loss from detached rosettes for each NIL when compared to L*er* (Fig. 7). We present the results of the repeated measures analysis of variance for delta-2.1 in Table 2 (the pattern of significant is virtually identical for delta-3.1 and so is not included). At the end of the 220-min experiment, detached rosettes from L*er*, NIL delta-2.1, and NIL delta-3.1 had lost

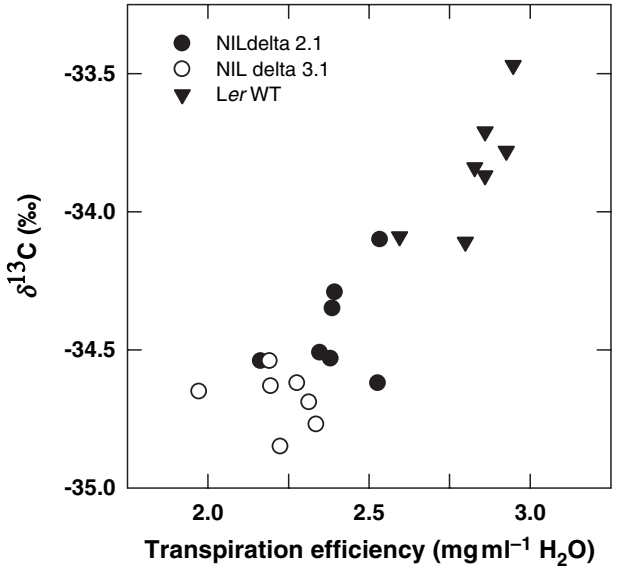

Figure 6. Transpiration efficiency (TE) and δ^{13}C among seven replicates each of NIL delta-2.1, NIL delta-3.1, and L*er*. As expected the two traits are positively correlated (r = 0.8, $P < 0.00000005$).

approximately 56, 68 and 70% of their initial fresh weight, respectively. In general, the NILs exhibit a more rapid rate of immediate water loss (within 100 min) followed by parallel responses through the later time intervals.

Finally, we investigated the impact of these QTL on overall plant growth. We measured the growth rate of rep-

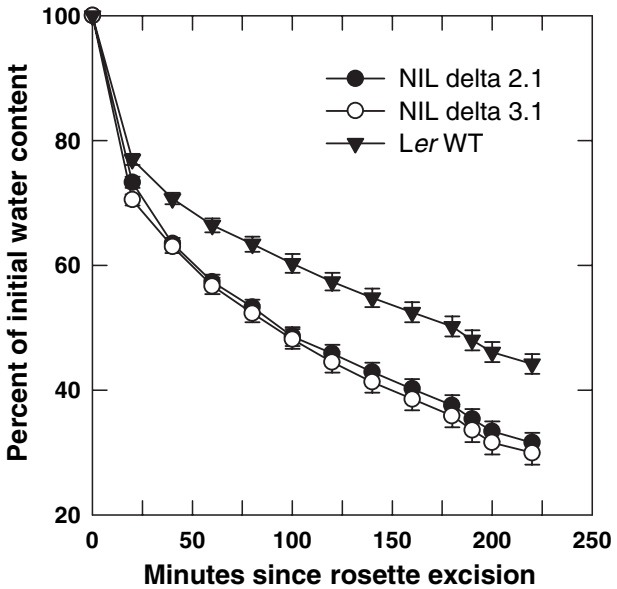

Figure 7. Time course of water loss from detached rosettes of NIL delta-2.1, NIL delta-3.1, and L*er*. Each point represents the mean ± 1 SE (n = 10 for each NIL, n = 20 for L*er*) at 20 min intervals.

Table 2. Univariate repeated measures ANOVA of the rate of water loss in detached rosettes of NIL delta-2.1 compared to L*er*

Source	DF	Type III SS	Mean square	*F*-value	*P*-value
Between subjects					
Line	1	0.63	0.63	31.27	< 0.0001
Block	1	0.07	0.07	3.33	0.0858
Error	17	0.34	0.02		
Within subjects					
Time	12	7.09	0.59	1839	< 0.0001
Time × Line	12	0.10	0.01	25.82	< 0.0001
Time × Block	12	0.01	0.00	2.73	0.0913
Error (Time)	204	0.07	0.00		

licate plants for each NIL and L*er* in terms of leaf area production over 3 weeks of vegetative growth. We found no significant difference in leaf area relative growth rates among NIL delta-2.1, NIL delta-3.1, or L*er* when plants were grown under well-watered conditions (in all cases, $P > 0.25$).

DISCUSSION

Most physiological traits, like most phenotypes of ecological and agricultural significance, are quantitative and generally influenced by multiple genes and environmental variation. In the past, the segregation of multiple genes, environmental effects, and their interactions prevented simple determination of genotype from phenotypic data (Falconer & Mackay 1996; Lynch & Walsh 1998). Statistical genetic studies have focused, alternatively, on population level patterns of genetic variance for polygenic traits. This approach has been useful for evolutionary biology and crop breeding, but does not offer a functional characterization of the actual genes underlying polygenic variation. Until recently, geneticists simply lacked adequate methods for evaluating the effect of polygenes. Therefore we know relatively little about the genetic details of these traits or their impact on plant physiology (Ehleringer & Monson 1993; Arntz & Delph 2001). This study is based on an integrated investigation of physiology and quantitative genetics in *Arabidopsis thaliana* using a set of RILs derived from genetically diverse parental ecotypes. Our goal is to characterize QTL underlying constitutive differences in physiology between natural accessions of *Arabidopsis* that have evolved under conditions differing in water availability.

We found significant genetic variation for both flowering time and δ^{13}C in the L*er* × Cvi mapping population and identified 10 genomic regions affecting these traits. Our mapping identified five QTL with effects on δ^{13}C [delta-1.1, delta-2.1, delta-3.1, delta-5.1, delta-5.2]. Cvi alleles from three of the five QTL made δ^{13}C values more negative, indicating decreased WUE relative to the L*er* ecotype. The fact that both the Cvi and L*er* parental ecotypes contained plus and minus alleles for δ^{13}C provides a general explanation for the observed transgressive segregation in the RIL population. Three of the δ^{13}C QTL detected in this study (delta-2.1, delta-3.1, delta-5.1) were previously identified in similar experiments utilizing both wet and dry experimental conditions (Hausmann *et al*. 2004). Hausmann *et al*. (2004) detected delta-2.1, delta-3.1, delta-5.1 and a novel QTL on chromosome III at approximately 22.2 cM. Interestingly, the QTL corresponding to delta-5.1 was detected only under wet conditions and through its interaction with the novel QTL on chromosome III. Differences between our previous experiments and those presented in this study could reflect either difference in statistical power, differing environmental conditions in each experiment, or the presence of QTL–environment interaction. Additional experiments will be needed to further explore the role of environmental conditions and QTL–environment interactions on δ^{13}C.

Interestingly, in this study, two δ^{13}C QTL were located within the genomic position of flowering time QTL. This may indicate a functional or pleiotropic relationship between these traits. A number of studies have reported a positive genetic correlation between δ^{13}C and flowering in C_3 annuals, suggesting a genetically based tradeoff between avoidance of and escape from drought (McKay *et al*. 2003). These patterns are further supported by a remarkably strong genetic correlation ($r_G = 0.98$) between date of first flowering and δ^{13}C among 39 *Arabidopsis* ecotypes grown in a common garden (McKay *et al*. 2003). Further characterization of loci affecting both traits, as well as those with unique effects on δ^{13}C, will provide further insight into the mechanisms driving the strong correlation observed in nature.

We utilized NILs to confirm and characterize two of the δ^{13}C QTL identified in our study. Here, a small interval of the Cvi genome capturing either delta-2.1 or delta-3.1 was introgressed into a homogeneous L*er* genomic background. In both cases, NIL studies verify lower δ^{13}C values for genotypes with Cvi alleles at either QTL. Furthermore, each NIL exhibited significantly higher g_s, as measured with whole-plant gas exchange. Cvi alleles result in less resistance to the escape of water vapour from plant leaves through stomata. The effect is consistent with increased g_s leading to increased c_i with a subsequent decrease in δ^{13}C values. Using the photosynthesis model of Farquhar *et al*. (1980) and assuming the NILs have the photosynthetic properties of L*er*, the calculated magnitude of change in δ^{13}C is very close to what would be expected if g_s is the only

limit on internal CO_2 partial pressure. At a whole-plant level, we detected a corresponding decrease in TE in both NILs relative to L*er*. Finally, we observed increased rates of water loss from NIL genotypes compared to L*er* in rosettes detached from their root system. NIL containing Cvi alleles at two $\delta^{13}C$ QTL exhibit increased g_s, decreased TE, and altered ability to limit the rate of water loss. These results suggest that genes underlying the observed physiological effects may play important roles in controlling stomatal closure during leaf dehydration as well as during gas exchange under well-watered conditions.

It is reasonable to expect some effects of these (or other) $\delta^{13}C$ QTL to be due to differences in A, given the link between A, g_s, and c_i. Increased g_s is expected to increase c_i and increased A is expected to lower c_i (Farquhar & Sharkey 1982). Consideration of indirect (feedback) influences through A or internal conductance differences may complicate our interpretations. For example, experimental evidence suggests that A can affect g_s as well as c_i (Wong, Cowan & Farquhar 1979). We plan to further characterize causal relationships between whole-plant physiological parameters and genetic variation in $\delta^{13}C$ with more comprehensive gas exchange measurements and analysis of A–c_i curves, fluorescence, and limitations due to internal conductances (Bernacchi *et al*. 2002).

We clearly detected whole-plant physiological effects ($\delta^{13}C$, g_s, TE) of the identified genomic regions captured by the NILs. Although no differences were detected in growth rate under well-watered conditions, this is not surprising. Typically advantages of increased WUE are seen in dry environments (Dudley 1996; Rebetzke *et al*. 2002; Condon *et al*. 2004). Conversely, increased g_s may be detrimental under increased soil or atmospheric water deficit. For example, constitutive increases in g_s through either increased stomatal density or stomatal opening may result in reduced survivorship or growth rates in the face of water deficit. We are currently exploring the TE and competitive ability of these NILs under a variety of soil moisture and competitive conditions. These efforts will shed some light on the ecological impacts of genetic variation in whole-plant physiology in *A. thaliana*.

In summary, we have identified and characterized several genomic regions that contribute to flowering time and whole-plant physiological differences observed between L*er* and Cvi. Genome-wide screens for $\delta^{13}C$ followed by detailed phenotyping of NILs provide some of the first data evaluating the natural genetic variation regulating physiology in *A. thaliana*. A concerted effort integrating quantitative genetics, whole-plant physiology, and genomic approaches hold promise for the positional cloning of these loci. Ultimately, these efforts will help to elucidate the factors affecting plant performance under environmental stress in both crop and ecological settings.

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