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GENETICS OF DROUGHT ADAPTATION IN *ARABIDOPSIS THALIANA* II. QTL ANALYSIS OF A NEW MAPPING POPULATION, KAS-1 × TSU-1

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Despite compelling evidence that adaptation to local climate is common in plant populations, little is known about the evolutionary genetics of traits that contribute to climatic adaptation. A screen of natural accessions of *Arabidopsis thaliana* revealed Tsu-1 and Kas-1 to be opposite extremes for water-use efficiency and climate at collection sites for these accessions differs greatly. To provide a tool to understand the genetic basis of this putative adaptation, Kas-1 and Tsu-1 were reciprocally crossed to create a new mapping population. Analysis of F_3 families showed segregating variation in both $\delta^{13}C$ and transpiration rate, and as expected these traits had a negative genetic correlation ($r_g = -0.3$). 346 RILs, 148 with Kas-1 cytoplasm and 198 with Tsu-1 cytoplasm, were advanced to the F_9 and genotyped using 48 microsatellites and 55 SNPs for a total of 103 markers. This mapping population was used for QTL analysis of $\delta^{13}C$ using F_9 RIL means. Analysis of this reciprocal cross showed a large effect of cytoplasmic background, as well as two QTL for $\delta^{13}C$. The Kas-1 × Tsu-1 mapping population provides a powerful new resource for mapping QTL underlying natural variation and for dissecting the genetic basis of water-use efficiency differences.

KEY WORDS: **Adaptation, *Arabidopsis thaliana*, carbon isotope ratio, correlated traits, drought avoidance, drought escape, drought tolerance, transpiration, water-use efficiency.**

Over 200 years of research on the genetics of ecological races within plant species provides overwhelming support for climate as a selective pressure to which populations locally adapt. Ecological genetics studies have revealed that traits underlying such adaptation are quantitative and influenced by multiple genes and the environment. Despite this rich history of research, there remain several fundamental questions to be answered. In particular, information is lacking on the identity and function of genes

responsible for adaptation. Characterization of the loci underlying natural variation is the holy grail of ecological and functional genomics (Feder and Mitchell-Olds 2003). Understanding the genes underlying adaptation is needed for both theoretical and applied questions in ecology and evolution (Lynch and Walsh 1998; Barton and Keightley 2002). These include longstanding questions such as the debate over micro- versus macromutation (Barton and Turelli 1989; Orr and Coyne 1992; Orr 1998; Barton and Keightley 2002; Orr 2005) as well as more current question regarding the relative roles of cis- versus trans-acting mutations. Here we focus on drought adaptation, because drought stress is a major consequence of climate, and a number of candidate traits may contribute to drought adaptation in plants (Richards 1996).

Water availability is fundamental to almost all aspects of plant physiology and water deficits have imposed strong and recurring selective pressure in the evolution of the diversity of plant form and physiology observed today (Stebbins 1952; Ehleringer and Monson 1993; Bohnert et al. 1995; Bray 1997). Soil water availability and atmospheric demand, interacting with temperature, are fundamental determinants of plant distribution, abundance, and productivity worldwide (Walter 1964; Whittaker 1975; Boyer 1982). Crop yields are commonly reduced by water limitations to less than half of potential yields (Boyer 1982; Gleick 1998). Because of the fundamental role of water on plant growth and survival (Comstock and Ehleringer 1992), habitat differences in water availability are predicted to lead to genetic differences in drought tolerance among populations (Stebbins 1952). Although little is known regarding genetic control of these traits in natural populations (McKay et al. 2003), dry environments have been shown to often select for higher water-use efficiency (Dudley 1996; Heschel et al. 2002).

Adaptation to optimize water use for maximum yield or fitness for a given water availability is complex (Schulze 1986; Ludlow and Muchow 1990; Passioura 1996; Chaves and Oliveira 2004; Heschel and Riginos 2005). In predictably wet habitats plants should spend water liberally, maximizing growth and fitness, whereas in drier habitats, minimizing water use at the cost of growth is one way to maximize fitness. The great variety of mechanisms involved in plant acclimation and adaptation to local soil water availability and atmospheric demand include phenological, developmental, morphological, molecular, and physiological traits (Passioura 1996; Araus et al. 2002; McKay et al. 2003; Monneveux et al. 2006; Yue et al. 2006). Plant growth, survival, and reproduction under limited water availability and/or high atmospheric demand involve both acclimation (adjustment of development, morphology, and physiology through inducible responses) and adaptation (heritable differences that allow some varieties to be more fit when water availability is limited relative to demand, due to constitutive differences or to better acclimation). Ludlow (1989) described three general strategies (suites of mechanistically related traits) plants use to cope with water limitation: dehydration tolerance, dehydration avoidance, and drought escape. Dehydration tolerance refers to plants in dry environments that survive internal water deficits (Scott 2000). Dehydration avoidance involves maintaining internal water status in a dry environment by minimizing water loss and/or maximizing water uptake. Finally, drought escape is attained through a short life cycle (e.g., annuals) or growing season (e.g., drought deciduous), allowing plants to grow and reproduce before the environment becomes dry.

Here we focus on drought avoidance, specifically water-use efficiency. Plants with C_3 photosynthesis face a fundamental trade-off between acquiring CO_2 for growth versus losing water, because CO_2 uptake (photosynthesis, A) and water loss (transpiration rate, E) both occur through stomata. The efficiency with which plants fix CO_2 relative to their rate of H_2O loss is called water-use efficiency (WUE = A/E). In water limited environments high WUE can allow drought avoidance, and have fitness and yield benefits. However, increasing WUE, when constitutive, often comes at the loss of fast growth when water is readily available (Heschel and Riginos 2005). WUE can be estimated by $\delta^{13}C$ of plant tissue, which provides a time-integrated index of WUE (Farquhar et al. 1982a,b, 1989; Dawson et al. 2002). $\delta^{13}C$ is the ratio of the amount of ^{13}C to ^{12}C isotope in a sample relative to a standard. Variation in $\delta^{13}C$ can be generated by variation in either or both photosynthetic capacity or stomatal conductance. $\delta^{13}C$ has been successfully used to investigate the role of WUE in drought adaptation in many studies (e.g., Comstock and Ehleringer 1992; Dawson et al. 2002; Condon et al. 2004; Comstock et al. 2005). Dehydration avoidance is achieved both through phenotypically plastic stomatal regulation (Buckley and Mott 2002; Comstock 2002), as well as constitutive differences in stomatal conductance or photosynthetic capacity evolved among accessions, ecotypes, or cultivars (Richards 1996; Geber and Dawson 1997; McKay et al. 2001; Araus et al. 2002; Condon et al. 2004; Juenger et al. 2005). For example, breeding via artificial selection on $\delta^{13}C$ under well-watered conditions has resulted in over 10% yield increases in drought stressed field plots for a newly released wheat cultivar (Rebetzke et al. 2001; cultivar Drysdale; www.csiro.au). In contrast, wheat cultivars with high yield under irrigation environments show reduced WUE, resulting largely from constitutively higher stomatal conductance (Fischer et al. 1998).

Although much is known about which trait values might be optimal for a particular environment, little is known regarding the genetics and evolution of drought adaptation. Crop breeders have focused a deserving amount of attention to understanding the genetic and physiological basis of the differences in drought adaptation among cultivars (Lebreton et al. 1995; Passioura 1996; Richards 1996; Thumma et al. 2001; Araus et al. 2002; Price et al. 2002; Teulat et al. 2002; Chaves et al. 2003; Reymond et al. 2003;

Yue et al. 2006), yet identifying the causal polymorphisms underlying this variation is limited by a lack of genomic information in many crops. In contrast, in *Arabidopsis. thaliana*, the plant with the most genomic tools to facilitate QTL cloning, research has focused on understanding the molecular basis of drought acclimation (Yamaguchi-Shinozaki and Shinozaki 1994; Uno et al. 2000; Kreps et al. 2002; Seki et al. 2002; Shinozaki et al. 2003; Bray 2004; Kawaguchi et al. 2004; Fujita et al. 2005), but unlike crops, little attention has been paid to constitutive differences among accessions that may reflect adaptation, or at least the variation needed for adaptation to evolve. To date, a few studies have examined candidate drought adaptation traits in *A. thaliana*, using both natural accessions (Masle et al. 1993; Neinhuis et al. 1994; McKay et al. 2003), mapping populations and near isogenic lines (NILs; McKay et al. 2003; Hausmann et al. 2005; Juenger et al. 2005; Masle et al. 2005). Juenger et al. (2005) identified two QTL of substantial effect causing an accession from the Cape Verde Islands (Cvi) to have lower drought avoidance than Landsberg erecta (L*er*), and further efforts to identify the loci underlying these QTL are ongoing (Juenger et al. 2006). There are few obvious candidate genes for drought avoidance, in part due to the lack of physiological annotation of the *A. thaliana* genome. Masle et al. (2005) recently showed that mutagen induced loss-of-function mutations at the ERECTA locus have large effects on transpiration efficiency, $\delta^{13}C$, stomatal density, and mesophyll cell proliferation. To date there is no evidence that naturally occurring variants at the ERECTA locus underlie differences in drought avoidance among accessions. It is likely that mutations at many loci may lead to variation in whole-plant physiology, and thus additional QTL could be identified by examining crosses of more extreme parents. For annuals like *A. thaliana* the relative adaptive importance of traits related to escape, avoidance, and tolerance depends on the characteristics of the growth environment (Passioura 1996; Araus et al. 2002).

Given its clear importance, studies elucidating the molecular genetic basis of adaptation to water availability in both crops and model systems are critical to understanding plant function (Araus et al. 2002; Condon et al. 2002; Chavez, et al. 2003; Chaves and Oliveira 2004; Condon et al. 2004; Lanceras et al. 2004; Comstock et al. 2005; Yue et al. 2006). Here we describe the creation of a new mapping population, a cross between Kas-1 and Tsu-1, accessions from among the driest (Kas -1, Kashmir, India) and wettest (Tsu-1, Japan) habitats in the species range. This mapping population shows variation in all three strategies of drought adaptation and we present QTL analysis for a focal dehydration avoidance trait, stable carbon isotope composition of plant tissue ($\delta^{13}C$). We are using this new population to begin to answer the following questions: How many genes underlie quantitative traits involved in drought adaptation and what are the magnitudes of their effects? How extensive are nonadditive gene interactions? How genetically independent are physiological components of drought adaptation?

Materials and Methods

NATURAL VARIATION

Thirty-nine natural accessions (see table 1 of McKay et al. 2003) selected from the native range of *A. thaliana* in Europe, Asia, and northern Africa, were grown in a common garden experiment in a greenhouse at UC Davis (see McKay et al. 2003 for details). Four replicates of each genotype were planted (see greenhouse below) and evaluated for variation and covariation in $\delta^{13}C$ and leaf water content. The genetic correlation between $\delta^{13}C$ and leaf water content was estimated as the standard Pearson product-moment correlation among accession means. Climatic data from the accession sites were obtained from New et al. (2000).

DEHYDRATION RESISTANCE AND WATER RELATIONS OF KAS-1 × TSU-1

To investigate fitness consequences of terminal drought, 30 replicates of both Kas-1 and Tsu-1 were grown in potting mix under growth chamber conditions (described below) and a terminal drought treatment was imposed 15 days after germination. The survival was scored 8 and 16 days after watering ceased. To further explore the physiological differences between Tsu-1 and Kas-1, we planted six reps of each in potting mix and water was withheld for 17 days. Plants were sampled at various stages of slow dehydration over the 17-day period to determine leaf water potential (using end-window Peltier thermocouple psychrometers, Merrill, J R D Specialty Equipment, located in Logan, UT) and relative water content. Methods of gas exchange measurement of stomatal conductance (g_s) and leaf internal CO_2 concentration (C_i) are described below.

KAS-1 × TSU-1 MAPPING POPULATION

Two accessions, representing the most extreme values for $\delta^{13}C$ in McKay et al. (2003), Kas-1 (CS 903), and Tsu-1 (CS 1640) were crossed reciprocally and advanced to the F_9 via single seed descent, resulting in 148 RILs with Kas-1 cytoplasm and 196 recombinant inbred lines (RILs) with Tsu-1 cytoplasm. Aracon growth containers (Lehle Seeds, Round Rock, TX) were used to isolate individual lineages and optimal conditions were used to facilitate healthy plant growth and vigorous seed production. Care was taken to not impose selection on fitness or phenological traits throughout the breeding program. At the F_9, all RILs were grown in a growth chamber and entire rosettes were harvested for DNA extraction.

GENOTYPING AND MAPPING

We screened a large panel of the hundreds of the available microsatellites in *A. thaliana* (Bell and Ecker 1994; Symonds and

Lloyd 2003; arabidopis.org) to detect loci polymorphic between the Tsu-1 and Kas-1 parents and chose 48 loci with easily distinguished alleles for RIL genotyping. Microsatellites were amplified in 8-μl multiplexed reactions, each targeting a set of three loci. Primer sets were arrayed for multiplexing according to annealing temperature compatibility and an expectation of nonoverlapping fragment sizes. Each primer pair included a forward primer fluorescently labeled with HEX (1 per set) or 6-FAM (2 per set) (Integrated DNA Technologies, Coralville, IA). Multiplexing was accomplished using a Qiagen Multiplex PCR kit (Valencia, CA), scaled for smaller reactions, but otherwise used according to the manufacturer's recommendations. Five to 20 ng of genomic DNA in 0.8 μl served as template in each reaction. A standard thermocycling regime was implemented for all primer sets, optimized only for primer-appropriate annealing temperatures. This consisted of an initial denaturation and hot-start activation at 95°C for 15 min, then 30 cycles of 94°C for 30 sec, 50–61°C for 90 sec, and 72°C for 60 sec. A final extension at 60°C for 30 min was performed. PCR products were diluted in sterile water, at ratios ranging from 1:1 to 1:10, and 1 μl of the dilution was mixed with a custom size standard (Symonds and Lloyd 2004) and Hi-Di Formamide (Applied Biosystems, Foster City, CA). Fragments were electrophoresed on an ABI 3730 sequencer (Applied Biosystems). Size determinations and genotype assignments were made using GeneMarker 1.30 software (Softgenetics, State College, PA). Forty-six microsatellite genotypes were obtained for 258 of the RIL.

To increase marker density, single nucleotide polymorphisms (SNPs) between Tsu-1 and Kas-1 were identified by sequencing genomic regions from these parents, or by testing a large number of SNPs based on the *A. thaliana* sequence database (http://walnut.usc.edu) (Nordborg et al. 2005). Suitable SNPs were screened by the SNPlex Genotyping System (Applied Biosystems), which allows for multiplexing of 48 SNPs against each biological sample. In this technique, allele- and locus-specific probes are first hybridized to the loci containing the SNPs and linked via an Oligonucleotide Ligation Assay (Applied Biosystems). This ligation product was then enzymatically purified and PCR amplified. These amplified products were hybridized to a universal set of dye-labeled fragments called Zipchute Mobility Modifiers (Applied Biosystems), which carry the genotype information and were detected by capillary electrophoresis. SNPlex products were run on an ABI 3730 DNA Analyzer (Applied Biosystems) and analyzed by GeneMapper version. 4.0 (Applied Biosystems). Custom Taqman® SNP Genotyping Assays (Applied Biosystems) were also used to screen several remaining SNPs. The SNP loci were amplified in a Peltier Engine Tetrad 2 Thermal Cycler in 5 μl reaction volumes of 2.0 μl DNA, 2.5 μl 2X ABsolute QPCR Rox Mix (ABgene, Thermo Fisher Scientific, Rockford, IL), 0.125 μl custom Taqman probe/primer mix, and 0.375 μl water. Cycling conditions included a hot-start at 95°C for 10 min, followed by 35 cycles of denaturation (92°C for 15 sec), and annealing/extension (60°C for 1 min). Following cycling, an endpoint plate read was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the data were analyzed by SDS 2.2.1 (Applied Biosystems). Fifty-six SNP genotypes were obtained for all 346 recombinant inbred lines.

The software package Joinmap (Stam 1993) was used for the grouping and initial ordering of markers on linkage groups. A preliminary map was imported into the software program R/qtl using the R statistical package (Broman et al. 2003). The preliminary Tsu-1 × Kas-1 map was subsequently screened for genotyping and data-management errors, marker orders were confirmed with additional likelihood "ripple" analyses, and marker positions were reestimated using joint maximum-likelihood algorithms. Several characteristics of the population were explored including patterns of segregation distortion and nonsyntenic linkage. The estimated order and position of the markers on each chromosome were consistent with the location of each marker on the physical map of Columbia.

PHENOTYPING AND GROWTH CONDITIONS FOR F_3

Plants were grown in 175-mL capacity pots (Supercell conetainers, Stuewe and Sons, Corvallis, OR). These pots were filled with UC Davis sunshine mix (peat potting soil and vermiculite) with 0.625 mL of Osmocote slow release fertilizer. Soil was packed tightly into tubes and then tubes were placed in standing water until saturated. Next, Conetainers (Stuewe and Sons) were capped by modified, sealed, 50-mL centrifuge tubes to adapt to the cuvette used for gas exchange measurements. Five seeds of a given genotype 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 days at 4°C. Once planted, tubes were randomized into racks, covered with black plastic, and 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 $m^{-2}s^{-1}$ at the level of the soil. Relative humidity was not directly controlled, but ambient RH in the chamber typically ranged 50/75 (day/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.

After 21 days, we began measuring transpiration rate (E) during the light period using a custom-made whole-plant cuvette with the LI-6400 photosynthesis system (LiCor Inc., Lincoln, NE) as described by McKay et al. (2001). More details on gas

exchange methods are provided in Juenger et al. (2005). Each replicate plant was measured at least 10 times and repeated measures were averaged to obtain a single phenotypic value per plant. On the day of measurement plants were harvested, rosette leaves were separated, and subsequently scanned using a desktop scanner. Images were analyzed for projected leaf area using a series of macros in Photoshop (Adobe, Seattle, WA) and Scion Image (Scion, Frederick, MD). 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. The entire rosette of each harvested plant was used for carbon isotope analysis (see below). Phenotypic data from this experiment were analyzed with standard GLM in SAS incorporating genotype and blocking factors (SAS Institute 1997).

PHENOTYPING AND GROWTH CONDITIONS FOR F_9

Six replicate complete blocks of all 346 RILs were screened for δ^{13} C. Each replicate block consisted of seven 50-well planting flats filled with potting soil as above, soaked in water, and thoroughly misted before planting. Each well was planted with 3–6 seeds; racks were covered and placed at 4°C for 5 days. Following cold stratification, the racks were moved to the greenhouse and heavily misted for the first week to encourage germination. Plants were bottom watered for optimal growth for the duration of the experiment. Germination date was recorded daily and approximately 5 days after germination each well was thinned to the first germinated seedling. After approximately 2 weeks of growth in a long-day greenhouse, rosettes were harvested, immediately weighed (wet weight), and then freeze-dried and weighed again (dry weight).

Dried plant tissue was processed for carbon stable isotope analysis. We subsampled 10–20 mg of crushed leaf material from each individual into a 2-mL microfuge tube containing 4 ball bearings, then shook for 1 min on a paint shaker. The result was a uniform powder, from which two subsamples (1 mg and 3 mg) from each plant were analyzed, to reduce measurement error (For plants where multiple subsamples were analyzed, the average was used for analysis.). Samples were combusted and then purified using cryogenic traps. Purified CO_2 was analyzed for isotope ratios on a dual-inlet mass spectrometer at the Stable Isotope facility at UC Davis. Data are presented as carbon isotope ratios relative to the PDB standard (R_{PDB}), where δ^{13}C (‰) = $(R_S/R_{PDB} - 1) \times 1000$ (Hubick et al. 1986). We do not convert these data to carbon isotope discrimination, because that requires the assumption that the δ^{13}C of the ambient air is known and constant. We measured the δ^{13}C of the ambient air in all of our growth conditions and found that it varies greatly and unpredictably over time.

QUANTITATIVE GENETIC ANALYSIS

QTL mapping was conducted on RIL line means obtained as the average of three independent randomized blocks. We completed both one- and two-dimensional interval-mapping analyses using the Rqtl program of the R Statistical package (Broman et al. 2003) [http://www.biostat.jhsph.edu/kbroman/qtl/]. The Rqtl program is a flexible software package allowing a diversity of QTL mapping approaches. Here, we implemented interval mapping using the Haley–Knot regression algorithm at 2-cM steps across the *A. thaliana* genome. Given the reciprocal nature of our design, we included a cytoplasmic covariate in our interval mapping scans. We used 1000 permutations of the dataset to empirically derive significance thresholds corresponding to an experiment-wise Type 1 error rate, $\alpha = 0.05$ (Churchill and Doerge 1994; Doerge and Churchill 1996). One unique feature of the Rqtl framework is that it directly incorporates multiple QTL models and scans for epistatically interacting QTL. See Juenger et al. (2005) for additional details of our mapping methods.

Results

PHENOTYPIC VARIATION AMONG ACCESSIONS

The climate of the accession sites differed greatly in both precipitation and temperature, with the Tsu site of origin (Tsushima, Japan) having high water availability throughout the growing season and the Kas site of origin (Kashmir, India) having very limited precipitation inputs during the growing season (Fig. 1). A greenhouse screen of 39 natural accessions under well-watered conditions revealed large variation among accessions in both δ^{13}C and leaf water content (Fig. 2). In addition, the covariation of these traits revealed a strong, negative correlation ($r = -0.69$), where plants that have a less negative δ^{13}C (higher WUE) also have lower constitutive water content (Fig. 2). L*er* and Col had the 1st and 3rd highest water content, respectively. Kas-1 had the least negative value for δ^{13}C (indicating high WUE), and was among the accessions with the lowest water content. Tsu-1 had average water content and was among the accessions with more negative values for δ^{13}C (indicating lower WUE). These two lines were subject to a more-detailed analysis of gas exchange, survival, and leaf water relations, as a function of drought stress.

COMPARING WATER RELATIONS OF TSU-1 and KAS-1

Under well-watered conditions, Tsu-1 had higher water content than Kas-1 (Fig. 2), but both accessions approached the same water content (dry weight basis) when drought stressed. We measured leaf water potential and leaf relative water content on replicates of each accession as plants were exposed to droughted soils. These data are depicted as Höfler diagrams (Lambers et al. 1998) and show that relative water content (RWC) was much lower

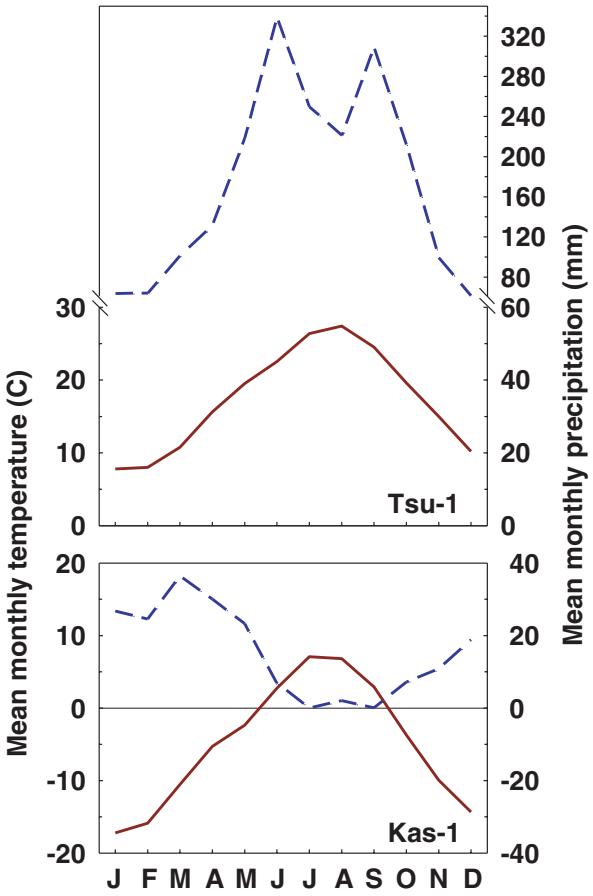

Figure 1. Climadiagrams (following Walter and Leith 1960) for the sites of origin of Tsu-1 and Kas-1. Mean monthly temperature (solid line) and precipitation (dashed line) are plotted such that when the precipitation line is below the temperature line water deficit is indicated, on average, whereas, if the precipitation line is above the temperature line soil moisture is predicted to be abundant (see Walter and Leith 1960; Walter 1964). Data source was New et al. (2000). The abscissa represents 12 months of the year from January through December.

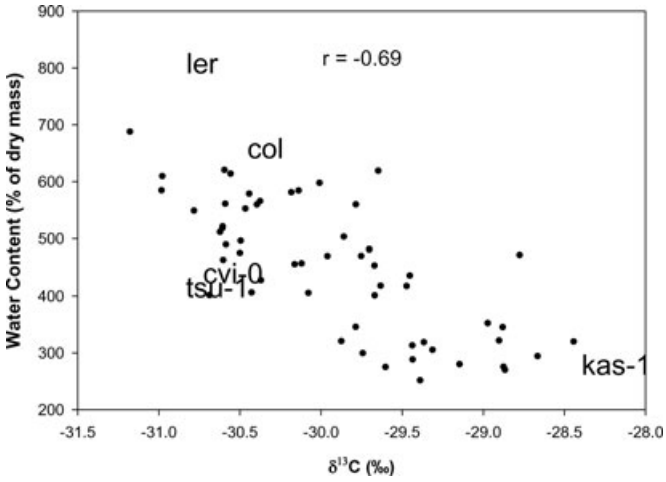

Figure 2. Spectrum of genetic (co)variation for the ratio of water to dry mass (water content) and carbon isotope composition ($\delta^{13}C$) in a sample of 39 *A. thaliana* accessions. The negative genetic correlation ($r = 0.69$) of these traits is highly significant. Points shown are genotype means from plants grown in a greenhouse as described in McKay et al. (2003). Parents of existing mapping populations (Col, L*er*, and Cvi) and the parents of our new mapping population (Kas-1 and Tsu-1) are labeled. See McKay et al. (2003) for a list of the other accessions.

at wilting in Tsu-1 plants (Fig. 3A) when compared with Kas-1 (Fig. 3B), although leaf water potential at wilting was only slightly lower in Tsu-1. The data on both absolute water content and RWC indicated that Tsu-1 dehydrated more than Kas-1 before loosing turgor. Kas-1 was a very good dehydration avoider and its leaf RWC did not decrease nearly as much before wilting (Fig. 3B). An independent experiment using terminal drought revealed Kas-1 had significantly higher survival than Tsu-1, both 8 days and 16 days after drought was imposed (Fig. 3C). Finally, when well-watered, Tsu-1 had significantly higher stomatal conductance (g_s) and leaf internal CO_2 concentration (C_i) than Kas-1, based on whole-plant gas exchange measurements (Fig. 3D, E). The lower C_i of Kas-1 is mainly due to lower g_s; Kas-1 has lower photosynthetic capacity than Tsu-1. These differences are consistent with the $\delta^{13}C$ observed in the accessions (Fig. 2).

THE KAS-1/TSU-1 LINKAGE MAP AND RIL POPULATION

Kas-1 and Tsu-1 were crossed reciprocally and 346 RILs were advanced to the F_9 generation—148 and 198 lines carry the cytoplasm of the Kas-1 and Tsu-1 parents, respectively. These lines were genotyped using 103 genetic markers to create a linkage map spanning 463 cM of the *Arabidopsis* genome (Fig. 4A). The average intermarker distance for this map was 4.7 cM with several modest gaps (24 cM, middle of chromosome II; 21 cM, top of chromosome IV; 21.7 cM, middle of chromosome V). We are currently closing these gaps with the addition of a small number of targeted SNPs. We found low residual heterozygosity (less than 1.5%) for genotyped markers in the F_9 generation. The expectation in an RIL population is that half of the RI genotypes should carry Tsu-1 alleles and half Kas-1 alleles. We found good agreement for this globally with 50.5% of the observed genotypes corresponding to Kas-1 homozygotes and 49.5% corresponding to Tsu-1 homozygotes. Nonetheless, several genomic regions exhibited significant segregation distortion despite the conservative use of a Bonferroni correction for multiple testing and the exclusion of markers with a high degree of missing data. In particular, several marker clusters exhibited consistent distortion across a broad region of chromosome I (11–38 cM, favoring Kas-1 alleles) and to a lesser degree regions of chromosome III (63–73 cM,

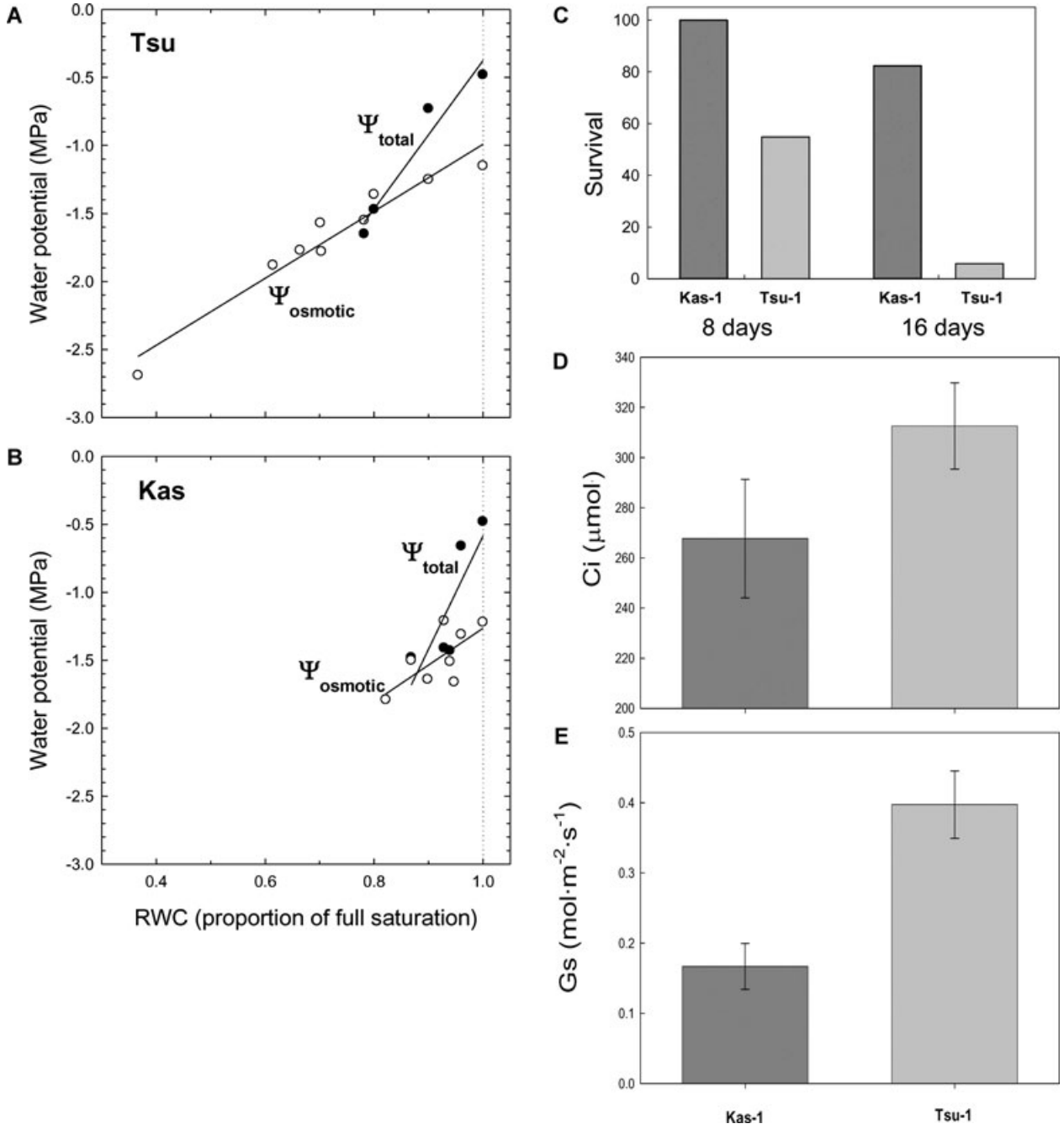

Figure 3. **Whole-plant physiology of water relations in Tsu-1 and Kas-1. (A, B) Höfler diagrams showing how leaf water potential and leaf relative water content of each accession co-varies as plants are exposed to droughted soils. Each point is the mean value for an individual plant harvested at different degree of soil drought. These data show the Tsu-1 accession dehydrates more before losing turgor than the Kas-1 accession. The Kas-1 accession, on the other hand, is a very good dehydration avoider and the relative water content (RWC) does not decrease nearly as much as in Tsu-1 before wilting. (C) Survival of terminal drought as a result of physiological tolerance differences between Tsu-1 and Kas-1. (D, E) show constitutive differences in gas exchange traits under well-watered, high light conditions. Tsu-1 has higher leaf internal CO_2 concentration (C_i) than Kas-1, which is largely due to higher stomatal conductance (g_s) under all conditions studied.**

favoring Tsu-1 alleles), chromosome IV (52–55 cM, favoring Tsu-1 alleles), and chromosome V (9 cM, favoring Kas-1 alleles). The extent of distortion ranged from a low of 33% to a high of 68% Kas-1 homozygotes. The underlying cause of the observed distortion is unknown but likely resulted from natural selection for particular alleles or allele combinations in the early generation of the breeding process, despite our efforts to minimize selection during the inbreeding of the lines.

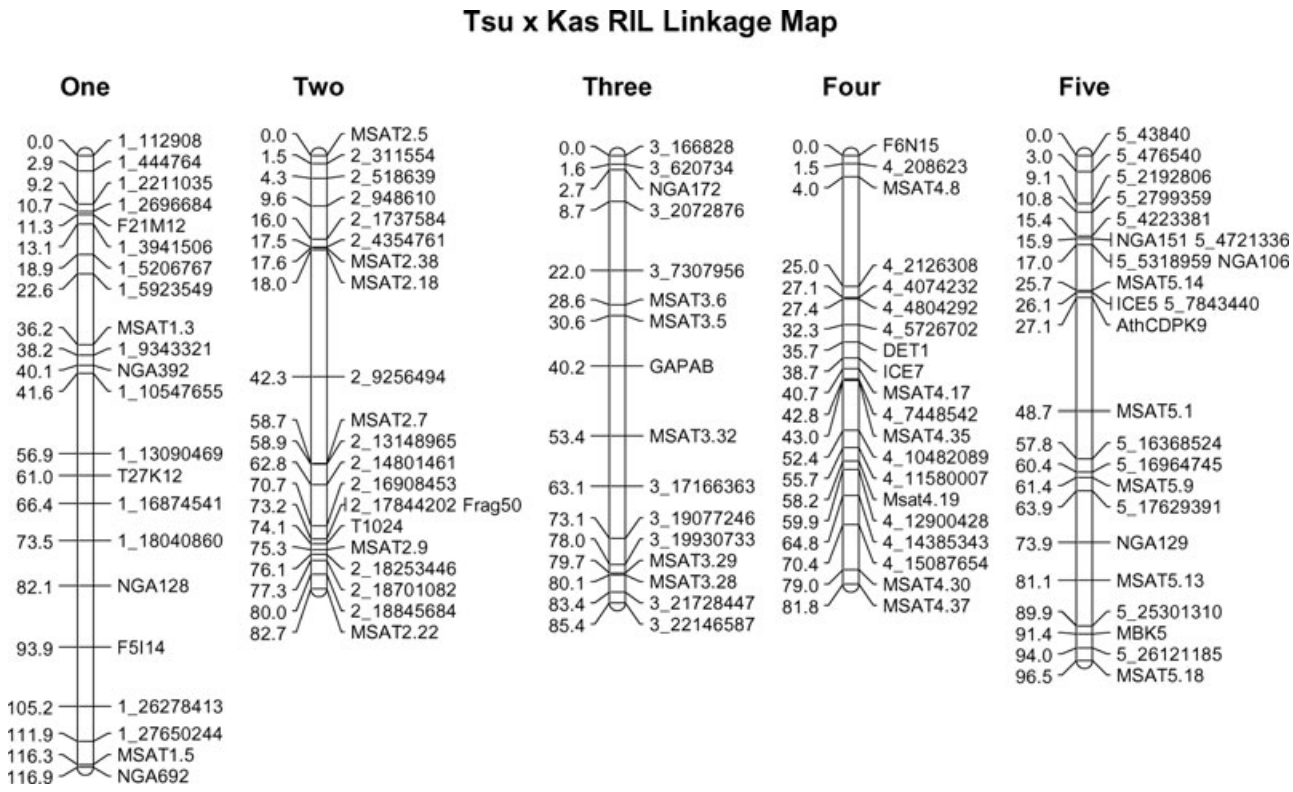

Figure 4. The markers used and location on the linkage map. All markers were in the order expected from blasting primer sequences against the Col genome.

TRAIT VARIATION IN THE RILS

Measurement of $\delta^{13}C$ and instantaneous transpiration rate in a replicated growth chamber screening of the F_3 families (which were further inbred six generations to create the RILs) showed large variation in both traits. As expected, these traits showed a negative genetic correlation ($r_g = 0.30$, $P < 0.0001$) suggesting that variation in stomatal conductance was at least one of the causes of the genetic variation in $\delta^{13}C$. Similarly, variation in leaf water content and $\delta^{13}C$ was measured in replicates of the F_9 RILs grown in a high light greenhouse. As found in the natural accessions (Fig. 2), these two traits had a significant negative correlation. Plants with less negative $\delta^{13}C$ values (higher WUE) also had lower constitutive water content (dry weight basis). However the correlation was weaker in the RILs than in the accessions, suggesting that several loci acting in linkage contributed to the strong correlation observed among accessions.

QTL MAPPING OF $\delta^{13}C$ IN THE F_9

We detected significant impacts of the reciprocal crossing design on $\delta^{13}C$ phenotypes collected from the RIL panel (Table 1). RIL containing Kas-1 cytoplasm had significantly more negative (lower WUE) measures of $\delta^{13}C$ relative to RIL containing the Tsu-1 cytoplasm. Cytoplasmic substitution resulted in a 0.11 ± 0.04 per mil change in $\delta^{13}C$ (Fig. 5A). In addition, two QTL were detected with considerable effects on $\delta^{13}C$. A QTL localized to the telomere of Chromosome IV (0–4.6 cM confidence interval) exhibited an additive effect (2a) of 0.15 ± 0.04 per mil (Fig. 5B; where 2a equals the difference in KK and TT homozygote means) and explained approximately 7.6% of the variation in $\delta^{13}C$. Similarly, a QTL occurring on the top of Chromosome V (0–27 cM) exhibited an additive effect (2a) of 0.08 ± 0.04 per mil (Fig. 5C) and explained approximately 3.2% of the variation in $\delta^{13}C$. In both cases, QTL effects were such that substituting a Kas-1 allele resulted in increased WUE.

Table 1. ANOVA table corresponding to multiple QTL model testing for cytoplasmic and QTL effects and their interactions.

Source	Num df	Den df	*F*-value	*P*-value
Cytoplasm (Cyto)	1	232	6.87	0.0093
F6N15 (Chrom4)	1	232	14.85	0.0002
5_2799359 (Chrom5)	1	232	4.35	0.0380
Cyto × Chrom4	1	232	1.74	0.1881
Cyto × Chrom5	1	232	0.46	0.4987
Chrom4 × Chrom5	1	232	0.03	0.8540
Cyto × Chrom4 × Chrom5	1	232	2.49	0.1162

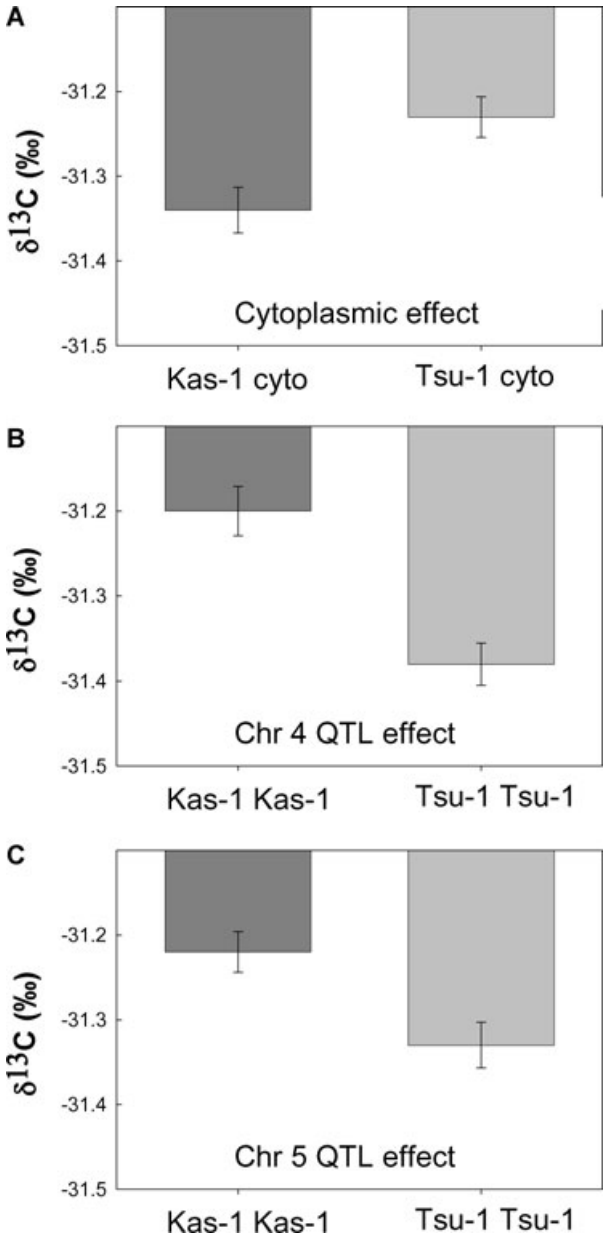

Figure 5. (A) The phenotype effect of the cytoplasmic background on carbon isotope composition ($\delta^{13}C$). (B, C) The phenotype of homozygotes at the QTL at the top of chromosome 4 and, the QTL at the top of chromosome 5.

Discussion

WATER RELATIONS OF TSU-1 AND KAS-1

We screened species-wide variation in *A. thaliana* for $\delta^{13}C$ and water relations and selected extreme accessions as parents for genetic mapping. The selected parents, Kas-1 and Tsu-1, show large differences in survival of terminal drought. The superior survival of the Kas-1 parent can be attributed to the superior drought avoidance, as this accession has lower constitutive stomatal conductance than Tsu-1 and, as a result of turgor loss at high leaf RWC, rapidly reduces leaf water loss and maintains a higher degree of leaf hydration at wilting. Tsu-1 retains turgor to lower leaf RWC and slightly lower water potential causing it to continue dehydrating. These patterns are consistent with the higher water use, more rapid flowering, and lower WUE of Tsu-1 based on gas exchange, $\delta^{13}C$, and growth measurements (this study; McKay et al. 2003). Further analyses of constitutive and induced differences in both whole-plant physiology and gene expression between these parents will provide candidate networks underlying these differences.

TRAIT VARIATION IN THE RILS

Most of the data and conceptual theory on trait covariation (syndromes of correlated traits) in plant ecophysiology is based on comparisons among species (Lambers et al. 1998). Although such data can reveal interesting patterns, it does not provide a mechanistic explanation. Genetic studies, where recombination is used to break up linkage disequilibrium among traits (and polymorphic loci), are necessary to distinguish the degree to which selection, drift, and pleiotropy contribute to the evolution of individual traits and correlated suited of traits thought to be important in adaptation.

Substantial variation was observed in leaf water content, $\delta^{13}C$, and transpiration rate in populations derived from the Tsu-1 and Kas-1 parents. In addition, significant genetic correlations were detected among these traits. In the F_3 families, $\delta^{13}C$ was negatively correlated with transpiration rate, which suggests at least some of the variation in $\delta^{13}C$ is due to stomatal limitations on C_i.

In both natural accessions and the RILs, water content was genetically correlated with δ^{13} C, such that plants with higher WUE had lower water content. The reasons for this deserve further attention from both functional and evolutionary perspectives. This correlation was weaker in the RILs than in the natural accessions, suggesting that multiple loci in linkage contribute to this correlation among accessions as opposed to one pleiotropic locus. This correlation is mirrored by the differences between Kas-1 and Tsu-1. Under well-watered conditions, Kas-1 plants have higher WUE and lower water content than Tsu-1 plants. Future efforts will focus on QTL mapping of both traits in this population to determine if pleiotropic loci contribute to the correlation.

QTL MAPPING

The cytoplasmic effects on $\delta^{13}C$, a novel finding for *A. thaliana*, are consistent with results from sunflower (Lambrides et al. 2004). Such effects of cytoplasm are not surprising as much of the biochemistry of photosynthesis is controlled by chloroplast gene products (Susek and Chory 1992). An interesting result from QTL mapping in this reciprocal cross was the finding of antagonistic effects between the cytoplasm and nuclear genomes. Although the Kas-1 parent was always found to have higher WUE than

Tsu-1, RILs with the Kas-1 cytoplasm showed the opposite effect. This analysis shows that loci in both the nuclear genome and the cytoplasm harbor the functional divergent alleles. For the nuclear genome, both of the δ^{13}C QTL that we identified are in the expected direction, the Kas-1 allele increases WUE (Fig. 5), however, the Kas-1 cytoplasm showed the opposite effect (Fig. 5). This suggests that selection on photosynthetic traits may involve some antagonistic interactions between the chloroplast and nuclear genomes. Although it has long been known that the subunits of the photosynthetic complex are endcoded by both cholorplast and nuclear genes, genomic approaches are just beginning to understand how transcription and translation of these two genomes is coordinated (Woodson and Chory 2008). Finding QTL that map to both genomes shows that both harbor functional variation. Future research can investigate how this variation manifests itself, both in terms of the genetic network controlling photosynthesis, and in terms of how this covariation responds to selection.

Unfortunately, recombination mapping to localize the QTL in the cytoplasm is not feasible, but biochemical and (co)expression data may provide candidates in either the chloroplast or mitochondrial genomes. Future efforts of QTL mapping in this populations will focus on cytoplasm $\times$ QTL interaction (Table 1), which if found may localize the interaction to a location in the nuclear genome and in turn may help to narrow the list of chloroplast or mitochondrial candidate loci.

Two QTL were detected for δ^{13}C. Both δ^{13}C QTL map near the locations of loci identified as flowering time genes, *FRIGIDA* (*FRI*) on the top of chromosome 4 and *FLC* on the top of chromosome 5. This and previous results showing that these loci harbor natural allelic variation that effects both δ^{13}C and flowering time (McKay et al. 2003) make these intriguing candidate genes for underlying the evolution of drought escape or drought avoidance. We used the method of typing for deletions as described by Johanson et al. (2000) and Stinchcombe et al. (2004) to determine *FRI* functionality. Based on this screening Kas-1 has a functional allele at *FRI* and Tsu-1 has a nonfunctional allele. This is consistent with previous findings of McKay et al. (2003) showing that functional *FRI* alleles increase WUE, as the Kas-1 allele at this QTL did. We also used the method of Caicedo et al. (2004) to determine the haplotype group at *FLC* for each parent; both Kas-1 and Tsu-1 had the same (B) haplotype at *FLC*. Although *FRI* is promising as a candidate gene, there are thousands of loci within the confidence intervals of these QTL and determining which is the causal polymorphism (and whether it is cis or trans-acting) will require further fine mapping and genetics. We are currently capturing Kas-1 alleles at these QTL in the Tsu-1 background to begin fine mapping these QTL. We are also measuring drought avoidance (flowering time) and dehydration tolerance traits in this mapping population to look at trait covariation and pleiotropy.

That both of our δ^{13}C QTL map to flowering time loci provides further evidence that components of adaptation are not genetically independent (McKay et al. 2003). Flowering time is the most well-investigated ecological trait in *A. thaliana*. This is motivated by the ecological significance of flowering, as well how easy it is to phenotype and the high heritability of the trait. To date, the study of variation in flowering time is based on a florigen model, where loci turn flowering on and off. Although originally identified as "flowering time genes," subsequent work has shown that many of these loci, including *FRI* and *FLC* also effect WUE and gas exchange (McKay et al. 2003; Christman et al. 2008). From a perspective of whole-plant physiology, flowering time is a very downstream trait, and therefore we can expect that any mutations that affect the ability to sense the environment or acquire and allocate resources might ultimately effect flowering time. Although flowering time has important fitness consequences in *A. thaliana* (and many other species) it is important to realize that every mutation affecting natural variation in flowering time is not necessarily maintained by natural selection on flowering time. One possibility suggested by our data is that population differences in flowering time may result from selection on alleles that affect both drought physiology and flowering time. Although the genetic tools for *A. thaliana* are outstanding, basic details concerning the ecology of natural populations are lacking. For example, although it is tempting to try to correlate phenotypes from a common garden with climate data, it first needs to be established what months of the year the populations are experiencing the climate in a vegetative state. The use of climadiagrams can provide an educated guess (Fig. 1). Ultimately, detailed studies of selection in natural populations are needed to determine whether patterns variation in flowering results from selection on flowering time per se, or from selection in drought-related traits, which in turn fixes alleles with pleiotropic effects on flowering time.

With regard to the genetics of adaptation, results from this study represent a limited sample, but fit into a larger dataset emerging in the last two decades. Overall, these QTL studies suggest that infinitesimal model, although mathematically convenient, is not an accurate model of the genetics of adaptation. Data may be more consistent with Orr's (1998) predicted exponential distribution, with mutations of large effect being fixed as populations are far from the optimal phenotype and then subsequent mutations of smaller effect providing a fine-tuning. Ultimately, this question must be attacked empirically, across many species and many populations within species. Once QTL are identified to a causal polymorphism, sequence data from individual QTL and flanking regions can be compared to the forthcoming genome sequence of *Arabidopsis lyrata* and *Capsella rubella* to determine of the ancestral state of each locus. For each derived mutation, polymorphism data could be used to estimate the relative age of each allele. This would provide a more comprehensive view of

adaptation, and a test of Orr's model, by comparing the effect sizes along the course of an adaptive walk. This is particularly feasible in model systems such as *A. thaliana* where large amounts of genomic data are now being collected on a population scale (Nordborg et al. 2005; Clark et al. 2007).

In summary we describe a new mapping population and use it to locate two nuclear genomic regions that contribute to whole-plant physiological differences between Kas-1 and Tsu-1. The mapping population represents a unique resource. Novel attributes of this population are: (1) it is large (346 lines), (2) it is based on a reciprocal cross that allows screens of cytoplasmic and cytoplasmic × nuclear interactions, (3) it is based on extreme natural genotypes within the species, and (4) it has been genotyped to produced a high-density linkage map. The Kas × Tsu mapping population shows large variation in whole-plant physiology and water relations. The new RIL mapping population will be used to confirm and determine the physiological basis of the QTL identified in this study, as well as distributed to the stock center for other researchers' use.

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