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A Common Genetic Basis to the Origin of the Leaf Economics Spectrum and Metabolic Scaling Allometry

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Abstract

Many facets of plant form and function are reflected in general cross-taxa scaling relationships. Metabolic scaling theory (MST) and the leaf economics spectrum (LES) have each proposed unifying frameworks and organizational principles to understand the origin of botanical diversity. Here we test the evolutionary assumptions of MST and the LES using a cross of two genetic variants of *Arabidopsis thaliana*. We show that there is enough genetic variation to generate a large fraction of variation in the LES and MST scaling functions. The progeny sharing the parental, naturally occurring, allelic combinations at two pleiotropic genes exhibited the theorized optimum ¾ allometric scaling of growth rate and intermediate leaf economics. Our findings: (i) imply that a few pleiotropic genes underlie many plant functional traits and life histories; (ii) unify MST and LES within a common genetic framework; and (iii) suggest that observed intermediate size and longevity in natural populations originates from stabilizing selection to optimize physiological trade-offs.

Introduction

Since Julian Huxley (1932) showed that traits covaried with each other according to simple mathematical relationships, understanding covariation of traits within integrated phenotypes has been a central focus of comparative biology (Gould 1966; Coleman *et al.* 1994). Organismal size is a central trait in biology and influences how numerous traits and ecological processes, and dynamics covary (Niklas 1994). The dependence of a given biological trait, Y, on organismal mass, M, is known as allometry (Huxley 1932). Allometric relationships are characterized by 'power laws' where traits vary or scale with M as:

$$Y = Y_0 M^{\theta} \qquad (1)$$

where θ is the scaling exponent and Y_0 is a normalization constant that may be characteristic of a given genotype or taxon. A sampling of *intra*- and *inter*-specific data reveals that the central tendency of θ often approximates quarter-powers (Niklas 1994; e.g., 1/4, 3/4, 3/8, etc.), although for any given relationship considerable variation may exist (Glazier 2005; Price *et al.* 2007) and the 'canonical' value of θ is still debated (Riisgard 1998; Kolokotrones *et al.* 2010), notably within vascular plants (Reich *et al.* 2006; Enquist *et al.* 2007b; Mori *et al.* 2010).

In addition to allometric scaling, other scaling relationships between traits have also been reported. For example, the trade-offs that govern the carbon and nutrient economy of plants appear to generate trait covariation functions that are also approximate power-laws (Reich *et al.* 1997; Westoby *et al.* 2002). Indeed, the nexus of trait correlations that makes up the leaf economics spectrum (LES) reflects the fundamental trade-off between the rate of acquisition of resources and lifespan (Charnov 1993; Reich *et al.* 1997; Wright *et al.* 2004; Shipley *et al.* 2006; Blonder *et al.* 2011). The LES describes how multiple physiological and morphological leaf traits, including net photosynthetic rate, dry mass per area (LMA), longevity, and nitrogen (N) concentration, covary across vascular plant taxa. This spectrum of covariations reflects the fact that leaves with long lifespan require more structural investment (associated with high LMA, reduced CO_2 permeability and light intensity inside the leaf), and a low mass-based photosynthetic and respiration rate (Kikuzawa 1991; Reich *et al.* 1997; Wright *et al.* 2004; Blonder *et al.* 2011). Conversely, high rates of photosynthesis are characterized by low LMA values. Further, low LMA leaves are more vulnerable to herbivory and physical damages (Kikuzawa 1991; Westoby *et al.* 2002). The LES appears to be universal across biomes and has been applied to understand functional variation in scaling relationships at whole-plant (Baraloto *et al.* 2010) and community (Kikuzawa & Lechowicz 2006) levels.

Metabolic scaling theory (MST) posits that various scaling exponents in biology – most notably, the scaling of whole plant metabolism (B) and growth rate (dM/dt) with M – are the result of natural selection on the scaling of whole-plant resource use. In particular, MST hypothesizes that for volume-filling vascular networks, natural selection will act to maximize the scaling of whole-organism resource uptake but simultaneously minimize the scaling of vascular transport resistance (West *et al.* 1999a). As a result, values of θ will tend to cluster around 'quarter-powers' so that $dM/dt \propto B \propto M^{3/4}$. However, in making this assumption, MST implicitly assumes that there is potential variation in θ and that this variation is heritable (Enquist & Bentley 2012). Indeed, elaborations of MST openly state that selection is expected to act on θ (Price *et al.* 2007; Olson *et al.* 2009) but we know of no examples showing a clear genetic basis to the scaling exponents highlighted by MST.

Similarly to MST, explanations for the LES are framed in the context of how selection optimizes the trade-off between investment for organ longevity and return on investment in carbon and nitrogen (Kikuzawa 1991; Westoby *et al.* 2000). Because of the physiological linkages between the traits that govern leaf economics, the global variation of many of the LES traits have been hypothesized to be under the control of a common genetic mechanism (Chapin *et al.* 1993). Consistent with this hypothesis, several pleiotropic genes underlying many continuous traits related to plant development, physiology and growth have been identified in Arabidopsis (e.g. McKay *et al.* 2003; Masle *et al.* 2005; Fu *et al.* 2009; Mendez-Vigo *et al.* 2010) and other species (e.g. Poorter *et al.* 2005; Edwards *et al.* 2011). The evolutionary importance of pleiotropic genes in explaining observed coordinated changes in covarying traits has been intensively debated (e.g. Pavlicev & Wagner 2012). Because of the difficulty of measuring traits related to carbon fixation (but see Edwards *et al.* 2011; Flood *et al.* 2011), the genetic bases underlying plant life histories and the LES remained to be elucidated. Thus, the role of pleiotropic genes and genetic constraints in shaping the evolutionary dynamics of plant functional diversity is unclear (Donovan *et al.* 2011).

Arguments for the origin of the scaling relationships described by the LES and MST have not been tested. In particular, they make two implicit evolutionary assumptions. First, they assume that there is variation in the subsidiary traits underpinning scaling relationships. Secondly, they assume that subsequent Darwinian selection on scaling relationships occurs at the *intra*-specific level. However, studies that have assessed the predictions and generality of the LES and MST have mainly been conducted at the *inter*-specific level. Here, we test the evolutionary assumptions of botanical scaling theory. We characterized the scaling of carbon and nutrient economics and the allometric scaling of growth rate across numerous Arabidopsis genotypes spanning 3 orders of magnitude in size.

We utilized a powerful high-throughput phenotyping platform (Granier *et al.* 2006) to grow a population of recombinant inbred lines or RILs under strictly controlled environmental conditions. Two pleiotropic quantitative trait loci (QTL) with major effects (*EDI* and *FLG*) have been identified through the analysis of plant development and life history in these RILs (Alonso-Blanco *et al.* 1998b; El-Assal *et al.* 2001; Doyle *et al.* 2005; Fu *et al.* 2009). Allelic variability in these genes leads to a corresponding diversity in the timing of flowering, the rate of leaf production and the general pattern of vegetative growth (Mendez-Vigo *et al.* 2010).

We hypothesize that variation in life history, in particular the time to reach reproductive maturity, has important consequences for the lifetime carbon and nutrient budget at the leaf and whole-plant levels. As a result, selection should act on the scaling of carbon and nutrient budgets via the traits that underlie their physiological rates and life histories.

Material and methods

Plant material

We analyzed genetic variability in leaf economics and the scaling of plant growth across the RILs previously generated from a cross between Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi) (Alonso-Blanco *et al.* 1998a), two accessions that derived from contrasted locations. We also selected near isogenic lines (NILs) and targeted mutants to confirm the quantitative trait loci (QTL) identified from the genetic analysis and test candidate genes, respectively. NILs were chosen from the population previously developed by introgressing genomic regions Cvi into L*er* (Keurentjes *et al.* 2007). The NIL LCN 1-2.5 (NASC code N717045; Cvi-$EDI_{\mathrm{L}er}$) carries a Cvi fragment at the top of chromosome I and was selected to confirm the *EDI* locus. LCN 5-7 (N717123; Cvi-$FLG_{\mathrm{L}er}$) carries a Cvi fragment in the middle of chromosome V and was selected to confirm the *FLG* locus. Genetic and molecular studies have identified two candidate genes of the regulatory pathway of circadian clock as major contributors of *EDI* and *FLG* effects: *CRY2*, a gene coding a blue-light receptor (El-Assal *et al.* 2001), and *HUA2*, a gene coding a transcription factor of the AGAMOUS pathway (Doyle *et al.* 2005), respectively. We selected two knock-out mutants to investigate the candidate gene *CRY2*: one in Col-4 background (N3732; $cry2_{\mathrm{Col}}$) and one in L*er* background (N108; $cry2_{\mathrm{L}er}$). To investigate the candidate gene *HUA2*, we selected a knock-out mutant of *HUA2* in Col-0 (N656341; $hua2_{\mathrm{Col}}$). The choice of Col background was motivated by the collection of mutants available in stock centers.

Growth conditions

We performed two experiments utilizing the PHENOPSIS automated growth chamber (Granier *et al.* 2006). The PHENOPSIS facility maintains constant growing environment and allows for the precise temporal monitoring and automated measurements of 504 potted plants. In Experiment 1, we phenotyped the parental accessions (L*er* and Cvi; $n = 8$ replicates) and 120 RILs ($n = 4$) selected from the 162 available lines (See Appendix S1 in Supporting Information). Plants were grown in four randomized blocks. In Experiment 2, we phenotyped the two parental accessions ($n = 8$), 16 RILs ($n = 6$) spanning the range of trait variability observed in Experiment 1, the NILs ($n = 7$), and the mutants and associated wild-types (both $n = 10$). All detailed growing and meteorological conditions are given in Appendix S1 and Table S1 therein, in Supporting Information.

Measurements of plant traits

The total projected leaf area of the rosette (RA, cm^2) was determined every 2 to 3 days from zenithal images of the plants. A sigmoid curve was fitted for each plant following:

$$RA = \frac{a}{1+e^{\left(\frac{(d-d_0)}{b}\right)}} \qquad (1)$$

where d is the number of days after emergence of the firsts two true leaves, a is the maximum vegetative rosette area, d_0 is the time when $a/2$ leaf area has expanded and b is related to the maximum rate of leaf production. The maximum rate of leaf expansion (R_{max}, m^2 d^{-1}) was calculated from the first derivative of the logistic model at d_0 as $R_{max} = a/(4b)$.

Photosynthesis was measured at flowering and under growing conditions using a whole-plant chamber prototype designed for Arabidopsis by M. Dauzat (INRA, Montpellier, France) and K.J. Parkinson (PP System, UK) and connected to an infrared gas analyzer system

(CIRAS 2, PP systems, USA). To insure plant gas exchange was not corrupted by soil respiration, we sealed the soil surface with four layers of plastic film. The flowering stem was detached from the rosette before measurement to record leaf gas exchange only. Whole-plant photosynthetic rate was expressed on a dry mass basis (nmol g^{-1} s^{-1}).

All plants were harvested after photosynthetic measurements. Each rosette was cut, wrapped in moist paper and kept at 4 °C overnight in darkness to achieve complete rehydration. Leaf blades were then separated from their petiole and scanned for area measurements. Next, both were oven-dried at 65 °C for 72 h and their dry weight was determined. Aboveground plant dry mass (M, mg) was determined as the sum of dry mass of petioles and blades. Total leaf area (cm^2) was determined as the sum of individual leaf blade areas. Leaf dry mass per area (LMA, g m^{-2}) was calculated as the ratio of dry mass and total leaf area. Assuming that LMA did not vary over time during the period of maximum expansion rate, we calculated maximum absolute growth rate (G, g dry mass d^{-1}) from R_{max} and LMA. In order to obtain sufficient dried material for chemical analyses, leaf blades and petioles were ground together to determine N concentration by mass spectrometry (EA2000, Eurovec, Isoprime, Elementar). Leaf lifespan was estimated from the oldest active leaf that showed some signs of senescence at harvest from the daily pictures of the 16 RILs in Experiment 2. This estimation was used to test the relationship between age at flowering and leaf lifespan (See Appendix S2). Traits were measured on each individual, except N concentration which was measured on a single replicate in Experiment 1 and on three replicates in Experiment 2. Phenotypic data are stored in the PHENOPSIS database (see Appendix S1).

Statistical analyses

We first assessed the allometric relationship between aboveground dry mass (M) and maximum absolute growth rate (G) across all RILs by fitting a linear model: $\log_{10}(G) = \log_{10}(b_0) + b_1\log_{10}(M)$. Inspection of the residuals from this model revealed a significant departure from linearity (Figs S1 and S2). Next, following Kolokotrones *et al.* (2010), we fit a nonlinear quadratic model: $\log_{10}(G) = \log_{10}(b_0) + b_1\log_{10}(M) + b_2(\log_{10}(M))^2$, using the Generalized Estimation Equation (*gee* package in the statistical program R 2.12). The slope θ_q of the quadratic at any given M value was calculated as the derivative of the quadratic function $\theta_q = b_1 + 2b_2\log_{10}(M)$.

Broad-sense heritability (H^2) of each trait was estimated as the ratio of (among – within) lines (RILs) to total (among + within) variance components with replicate plant within RIL treated as random effect (R/*nlme* package).

We used 144 AFLP markers spanning all the genome to perform a QTL analysis of all traits by composite interval mapping (R/*qtl* package). For each trait, 5%-level significance threshold for QTL LOD scores were calculated following 1000 permutations. Here, this threshold did not exceed 2.9. Relationship QTLs (rQTLs) were detected by testing the allelic effect on the major axis slope of the allometric relationship at each locus (Tisné *et al.* 2008; Pavlicev & Wagner 2012; Fig. S3).

Results

Across the RILs, we observed a considerable amount of trait variation. All of the morphological, physiological and growth-related traits showed significant between-line variance ($P < 0.001$) despite the weak differences between the parental accessions L*er* and Cvi (Fig. 1 and Table S2). Interestingly, the range of variation in these traits was often a considerable fraction of the global variation in these traits (see Fig. S4). Broad sense heritabilities ranged from 0.68 (LMA) to 0.89 (plant dry mass) (Table 1). Such high heritability values reflect the important role of genetics in determining the observed trait variation, and also point to the low environmental variability within the PHENOPSIS automaton (e.g. lack of significant block effect for all traits (all $P > 0.10$)).

Our results show that, in accordance with MST predictions, the maximum absolute plant growth rate (G), across all RILs, scaled to the ¾-power of plant dry mass (M) (Fig. 2; $G = 6.32M^{0.76}$; $R^2 = 0.96$). However, a quadratic model better fitted to the allometric relationship so that as plant mass increases, there is a progressive shallowing of the allometric exponent, θ (Figs S1 and S2). However, as we show below, this curvilinearity was generated by a shift in scaling exponent across RILs.

Next, we determined if there was a genetic basis to the observed variation in allometric scaling. We performed a QTL detection for the allometric growth exponent, θ_q, estimated for each RIL by fitting the quadratic model, and a rQTL analysis of the relationship scaling. These two analyses identified two loci that control variation in the allometric exponent (LOD score > 2.9; Figs 3A and S3) and exhibit additive effects. These loci were: *EDI*, located at the top of chromosome 1 (CI = [5; 11] cM), and *FLG* in the middle of chromosome 5 (CI = [37; 45] cM). Their additive effect explained 68% of the total variability in θ_q (Table 1; Fig 3A and Fig. S5). As previously found through the dissection of Arabidospis' life history (Alonso-Blanco *et al.* 1998b; Mendez-Vigo *et al.* 2010), these two QTLs were also the major determinants of age at flowering (Fig. 3B), indicating that variation in θ_q is also associated with life history variation. We found that the subsets of RILs carrying the parental combinations at *EDI* /*FLG* loci (parental types; i.e. L*er*/L*er* and Cvi/Cvi) shared a *common* allometric slope ($P = 0.34$) that did not differ significantly from ¾ ($\theta = 0.77$; CI = [0.74; 0.80]; Fig. 2). However, the recombinant types at *EDI* /*FLG* loci displayed either significantly higher (Cvi/L*er*; $\theta = 0.89$; CI = [0.85; 0.94]) or significantly lower (L*er*/Cvi; $\theta = 0.61$; CI = [0.58; 0.65]) scaling exponents (both $P < 0.001$; Fig. 2). Our analysis revealed no epistatic interactions between *EDI* and *FLG* ($P > 0.05$ except for N concentration, see Fig. S5).

A strong pattern of covariation was found across RILs between the physiological and morphological traits involved in the leaf economics spectrum, LES. We found that mass-based net photosynthetic rate and N concentration were positively correlated, whereas they were negatively correlated with age at flowering and LMA (Table 1; Fig. 4). Our genetic analysis revealed that *EDI* and *FLG* are also major pleiotropic QTLs with additive effects that explained 63%, 56%, 60% and 35% of the variability in age at flowering, LMA, mass-based photosynthetic rate and N concentration, respectively (Table 1; Figs 3B and S5). As a result, we observed strong correlations between these traits and the allometric exponent, θ_q (Table 1). Values of θ_q were positively correlated with variation in traits related to carbon fixation (photosynthetic rate and N concentration) and negatively correlated with the traits related to organ longevity (age at flowering and LMA). Together these results demonstrate that differing allelic combinations at the *EDI* and *FLG* loci result in plants displaying significant differences in leaf economics (Figs 4 and S6) with concomitantly significant changes in metabolic exponent (Figs 2 and 4). Nonetheless, each of the parental types did not exhibit significant changes in θ_q and each was characterized by the predicted ‘optimal’ ¾-power allometric scaling of growth rate and intermediate LES strategies. In contrast, recombinant types showed extreme LES and MST phenotypes characterized by either strongly hastened or delayed flowering life histories. These extremes in life history are characterized by either increased or decreased LES traits and steeper or shallower allometric exponents, respectively (Fig. 2).

The role of *EDI* and *FLG* in controlling the allometric scaling of plant growth and the traits that underlie leaf economics was confirmed in Experiment 2. A high reproducibility of the phenotypes was observed among the 16 RILs grown in both experiments (correlations between trait values $r_{Spearman} > 0.93$ and $P < 0.001$). Across these 16 RILs, we observed significant differences in LES traits (Fig. S7) and allometric slopes (Fig. S8) according to the allelic combination at *EDI* and *FLG* loci. Although the values of the exponent θ_q varied from 1.33 to 0.57, the values of the parental types were again not significantly different from 0.75 ($P > 0.35$ in both parental types; Fig. S8), as observed in Experiment 1. Moreover, the introgressions of the Cvi chromosomal region carrying *EDI* or *FLG* into L*er* significantly hastened (Cvi-$EDI_{\mathrm{L}er}$) or delayed flowering (Cvi-$FLG_{\mathrm{L}er}$), respectively (Fig. 5 and Table S2), with an associated decreased or increased plant size, growth rate, LMA, photosynthetic rate and *N* concentration in a coordinated way (Fig. 5 and Table S2). For the 16 RILs grown in Experiment 2, we found a highly significant relationship between the lifespan of the oldest senescing leaf and age at flowering ($R^2 = 0.86$; $P < 0.001$; Fig. S9) indicating that at least in this population, age at flowering is a reasonable proxy for mean lifespan of the first leaves.

Lastly, we investigated the candidate genes, *CRY2* and *HUA2* as major contributors of *EDI* and *FLG* effects, respectively. The $hua2_{\mathrm{Col}}$ KO-mutant displayed significant changes in leaf economics ($P < 0.05$ for all traits; Table S2 and Fig. 5), whereas the *CRY2* ($cry2_{\mathrm{L}er}$ and $cry2_{\mathrm{Col}}$) KO-mutants displayed strong differences in age at flowering and less difference in photosynthetic rate, LMA and N concentration (Table S2 and Fig. 5). We found no difference in the phenotypes of $cry2_{\mathrm{L}er}$ and $cry2_{\mathrm{Col}}$, suggesting that the genetic background did not influence our results. Finally, the effects of *CRY2* and *HUA2* on growth strategy were confirmed since NILs and mutants displayed significant changes in plant mass but no changes in growth rate, indicating a departure from the allometric relationship.

Discussion

In this paper we assessed several of the implicit assumptions of MST and the LES. We demonstrated that a few genes can generate a large fraction of variation in MST exponents and LES traits. Within Arabidopsis, these genes appear to be responsible for constraining the covariation of the leaf economics and the allometric scaling of plant growth. Based on our findings we propose a novel conceptual framework that links the principles of MST to the LES.

Our findings support two central evolutionary assumptions of MST. First, MST implicitly assumes that selection can act on metabolic scaling exponents. In other words, there is genetic variation in metabolic scaling that selection can act upon. Interestingly, as previously observed for *inter*-specific metabolic allometric scaling of mammals (Kolokotrones *et al.* 2010) and plants (Enquist *et al.* 2007b; Mori *et al.* 2010) the relationship between whole-plant growth rate and plant biomass across RILs was curvilinear and not a pure power-law. This decrease in allometric exponent within increased size is also consistent with the decline in relative growth rate or RGR with size observed in other species (Poorter *et al.* 2005; although these RGR studies have not typically controlled for allometric effects on RGR). Importantly, our results also show that the observed allometric curvilinearity was primarily due to a mixing of different exponents across genotypes. In other words, genetic variation for the metabolic growth exponent resulted in a curvilinear 'inter-RIL' scaling allometry. Second, the subsets of inbred lines carrying the parental (naturally occurring) allelic combinations at two specific QTLs shared a *common* allometric exponent centered on

¾, whereas the recombinant types displayed higher and lower scaling exponents than the canonical '¾' hypothesized by MST (Fig. 2). These findings are consistent with a core MST assumption that 'quarter-power' scaling is the outcome of stabilizing selection on metabolic allometries (Enquist *et al.* 2007a). Interestingly, recombinant types were characterized by strongly hastened or delayed flowering, as well as increased or decreased photosynthetic rates, LMA, and N concentration, respectively (Fig. 4 and Fig. S5). Together, these findings suggest a tight coupling between life history, LES traits, and MST.

As stated by Wright *et al.* (2004), "*leaf lifespan describes the average duration of the revenue stream from each leaf constructed*". However, whole-plant growth rates and competitive ability depend not only on the photosynthetic rate of individual leaves, but also on the geometry and dynamics of a plant's canopy, and the pattern of energy allocation among all organs (Givnish 1988). We argue that, at least for annual plants in which all the leaves die almost simultaneously during the final stage of reproduction, whole-plant functioning should be tightly coupled to the lifespan of the plant (Charnov 1993). Indeed, a strong correlation between plant age at flowering and leaf longevity was found in this study and in the literature (Appendix S2 and Fig. S9). Although the comparison with the interspecific GLOPNET data (Wright *et al.* 2004) is limited due to the differences in the levels of measurement – leaf versus whole-plant level in this study –, the ratio of interquartile range for photosynthesis and LMA showed that our data span 70% and 55% of the variation in these traits, respectively (Fig. S4). In addition, the observed variation in the scaling exponents of growth rate within the RILs captures most of the variation in allometric exponents observed worldwide (Price *et al.* 2007). Measurements of plant growth and photosynthetic rate at the canopy level integrate the changes in architectural constrains associated with size, such as leaf shape and leaf overlapping. Hence, these measurements reflect the physiological trade-offs and the variation in leaf morphology such as LMA, occurring at the whole-plant level. In this view, we argue that the changes in rosette architecture are likely also associated with the nexus of traits and allometric covariation that we observed. In particular, departure from space-filling branching for light interception, is likely the reason why we observe departure from the ‘allometrically ideal’ MST ¾-power scaling of plant growth (Price *et al.* 2007).

The effects of the QTLs responsible for the variation in the scaling relationships were confirmed in the targeted NILs for which a coordinated change in the traits related to the leaf economics was observed (Fig. 5 and Table S2). In most relationships we find that the parental accession L*er* was closer to the parental accession Cvi (intermediate positions) than to the NILs (extreme positions). This is probably due to the opposite and counterbalancing effects of *EDI* (e.g. Cvi allele decreases size and age at flowering whereas it increases photosynthetic rate and N concentration) and *FLG* (e.g. Cvi allele increases size and age at flowering whereas it decreases photosynthetic rate and N concentration). Two genes, *CRY2* and *HUA2* have been shown to be the major contributors of *EDI* and *FLG* pleiotropic effects, respectively (Fu *et al.* 2009). Our results show that a single amino acid Val-to-Met replacement in the Cvi allele of *CRY2* and a premature codon stop in the L*er* allele of *HUA2* cause a cascade of large changes across numerous leaf physiological traits, and in the scaling of plant metabolism. This shift in metabolic scaling associated with the effects of *HUA2* is consistent with the change in the rate of leaf production reported by Mendez-Vigo *et al.* (2010). The Cvi ecotype carries a rare allele of *CRY2*, unique over more than 100 sequenced ecotypes (El-Assal *et al.* 2001), whereas the L*er* allele of *HUA2* is identified as common only in ecotypes from UK and Central Europe (Doyle *et al.* 2005; Wang *et al.* 2007). Moreover, Cvi is an unusual accession from the Cape Verde Islands which exhibit peculiar climatic conditions. Although contrasted phenotypes could be expected in the Cvi accession, we observed ‘allometric ideal’ ¾ exponent, intermediate timing of flowering and intermediate leaf economics in both parental types, despite the climatic differences in the parental sites of origin. We argue that these findings are in accordance with Metcalf and Mitchell-Olds (2009) who hypothesized that selection to optimize the size at reproduction without sacrificing leaf and whole-plant functioning has likely resulted in an intermediate timing of reproduction. This explanation does not necessarily imply that flowering time is the target of natural selection but rather that there are integrated physiological trade-offs linking life history, leaf economics and plant allometry.

Our results also appear consistent with predictions from the ‘Selection, Pleiotropy and Compensation’ (SPC) model of Pavlicev and Wagner (2012). Specifically, this Dobzhansky-Muller view of evolutionary dynamics states that within isolated or semi-isolated populations differing allelic associations of pleiotropic genes with major effects on life history and physiology underlie trait covariation patterns and are possibly responsible for deleterious changes in metabolic scaling. In artificially-generated RILs, the allelic association of a few genes with major effects often leads to remarkably extreme phenotypes. However, these extreme phenotypes likely would not be successful in nature compared to naturally occurring genotypes due to hybrid breakdown (Bomblies *et al.* 2007). Specifically, the observed ¾ scaling exponent could be then maintained by selection because crosses between populations create hybrid breakdown. Nonetheless, despite the strong genetic effect depicted by the high heritabilities observed here, we strongly suggest that future tests of the evolutionary role of pleiotropy in maintaining allometric scaling and life history trade-offs utilize transplant experiments in the field. The massive collection of Arabidopsis accessions that are currently genotyped or sequenced (e.g. Hancock *et al.* 2011) offer a promising tool to further explore the genetic diversity, and elucidate the evolutionary and ecological links between variation in climate and the traits that define leaf economics and metabolic allometry.

Genetic constraints, which occur when the genes controlling many correlated traits have antagonist effects, have also been proposed to shape the LES by restricting the genetic variation for each trait combination (Reich *et al.* 1999; Donovan *et al.* 2011). Using a mutant approach we show clear evidence that silencing the pleiotropic genes underlying the LES did not result in aberrant (i.e. out of the RILs pattern) or non-viable phenotypes but instead resulted in a coordinated adjustment of all physiological leaf traits. This result suggests that the LES is ‘hardwired’ into the genome. Specifically, due to direct pleiotropic effects or indirect physiological linkages, *CRY2* and *HUA2* constrain the space of possible trait values so as to avoid a change in one trait without a change in other correlated traits. Differences between phenotypes of NILs and mutants (such as between Cvi-*FLG*$_{\mathrm{L}er}$ and *hua2*$_{\mathrm{Col}}$) can be explained by *i*) the effect of the genetic background, *ii*) the contrasted effects of silencing one gene in KO-mutants versus carrying a natural variant of this gene in NILs, or *iii)* the effects of other genes in the introgressed regions. As suggested by the differences in the phenotypes of *cry2*$_{\mathrm{Ler}}$ and Cvi-*EDI*$_{\mathrm{L}er}$, unknown genes, linked to *CRY2* and *HUA2* in *EDI* and *FLG* respectively, could contribute to the QTL effects. For instance, *HUA2* has been shown to be mediated by the effect of a co-locating QTL, *FLC*, that acts as a positive regulator of *HUA2* effects (Mendez-Vigo *et al.* 2010). Together these findings suggest that genetic constraints limit the range of leaf trade-offs to a spectrum of covariations, but selection on major pleiotropic genes could arise inside the spectrum for a plant to take advantage of, depending on the environment, different optimal combinations of leaf economics.

We propose that, in general, across environmental gradients selection will act on leaf economics traits to select for genotypes that maintain an approximate ¾-power scaling of growth, but yet different LES trait values and thus result in the local adaptation of populations (Mitchell-Olds & Schmitt 2006; Alonso-Blanco *et al.* 2009). This does not necessarily imply that selection, in certain environments, could result in different values of the allometric exponent (Price *et al.* 2007) but rather is consistent with the general argument made by both LES and MST that, ultimately, botanical scaling relationships are the outcome of natural selection (West *et al.* 1999b; Enquist *et al.* 2007a). If the same pleiotropic mechanism is general across Embryophytes then multiple *intra*- and *inter*-specific scaling relationships at the leaf and whole-plant levels could be tightly linked to genetic variability in few genes.

MST has been criticized on empirical, statistical, and theoretical grounds (e.g. Riisgard 1998; Glazier 2005; Reich *et al.* 2006) in part because of the difficulty in testing its basic assumptions (Enquist & Bentley 2012). Our study, for the first time, tests several of the fundamental evolutionary assumptions that underlie MST. Similarly, by translating the trade-offs between structural investment for longevity and return on investment in carbon and nitrogen, the LES has been hypothesized to be the outcome of natural selection to optimize leaf carbon balance within a given environment (Reich *et al.* 1999; Blonder *et al.* 2011; Donovan *et al.* 2011). Our results show that leaf economics and variation in metabolic allometries, at least in Arabidopsis, are intimately linked through the effects of key genes. Together, these findings support Chapin's (1993) hypothesis that variation in leaf and other plant metabolic traits have a common genetic underpinning and that evolutionary filtering of a small number of antagonistic pleiotropic genes could be at the origin of many botanical scaling relationships.

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SUPPORTING INFORMATION

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Table 1. Correlations between traits, heritabilities and percentage of variation explained by the loci *EDI* and *FLG* in the recombinant inbred lines. Pearson's correlations (lower matrix). Broad-sense heritabilities (H^2). Plant dry mass (M); allometric exponent (θ_q); leaf dry mass per area (LMA). No epistatic interactions were found between *EDI* and *FLG* ($P > 0.05$) except for N concentration (see Supporting Information). Data from Experiment 1.

	M	Growth rate	θ_q	Age at flowering	Photosynthetic rate	LMA	H^2 (%)	EDI (%)	FLG (%)
M							0.89	23.8	21.4
Growth rate	0.98						0.84	25.8	19.5
θ_q	-0.98	-0.96					0.90	33.8	21.9
Age at flowering	0.96	0.91	-0.97				0.82	26.8	23.1
Photosynthetic rate	-0.92	-0.86	0.94	-0.95			0.80	29.3	19.1
LMA	0.94	0.93	-0.94	0.93	-0.93		0.68	25.2	21.3
N concentration	-0.60	-0.53	0.66	-0.67	0.72	-0.66	-	19.1	16.4

Figure legends

Figure 1. Variation of physiological and growth-related traits in the *A. thaliana* L*er* × Cvi RIL population. (**A**) plant dry mass (*M*); (**B**), growth rate; (**C**), mass-based photosynthetic rate; (**D**), N concentration; (**E**) age at flowering and (**F**) leaf dry mass per area (LMA). Bars are means ± *se* for each RIL ($n = 4$ except for N concentration $n = 1$) and for the parents (L*er* and Cvi; arrows; $n = 8$ except for N concentration $n = 1$). Data from Experiment 1. Lines ordered by increasing plant dry mass.

Figure 2. *EDI* and *FLG* effects on the allometric scaling of plant growth. (**A**) Regression lines and equations (standardized major axis) of the relationships between aboveground plant dry mass (*M*) and growth rate shown across individuals for the four sub-populations defined by the two loci *EDI* and *FLG*. Parental types Cvi/Cvi (yellow squares) and L*er*/L*er* (green circles), and recombinant types Cvi/L*er* (blue upward triangles) and L*er*/Cvi (red downward triangles) at the two loci *EDI*/*FLG*, respectively. (**B**) Density distributions and box-and-whisker plots of the local allometric exponent θ_q according to the allelic combination at the two QTLs (same colors used). Vertical dotted line: expected θ value (0.75) of allometric exponent following MST predictions. Asterisks represent significant differences from 0.75 ($P < 0.001$). Data from Experiment 1.

Figure 3. QTL analysis of the allometric exponent of plant growth and of the traits underlying the leaf economics. Likehood value of a QTL presence at the specified position along the five chromosomes (LOD score) for (**A**) the allometric exponent of plant growth (θ_q), and (**B**) the traits underlying the leaf economics. LMA: leaf dry mass per area. Dotted lines: maximum significance threshold across traits (2.9). Data from Experiment 1.

Figure 4. ***EDI*** **and** ***FLG*** **effects on the patterns of correlation between the traits underlying the leaf economics in the** ***A. thaliana*** **L*****er*** **× Cvi RIL population.** Each point is the mean value of four replicates per RIL (except for N concentration, $n = 1$). Parental types Cvi/Cvi (yellow squares) and L*er*/L*er* (green circles), and recombinant types Cvi/L*er* (blue upward triangles) and L*er*/Cvi (red downward triangles) at the two loci *EDI*/*FLG*, respectively. LMA: leaf dry mass per area. Bivariate relationships are shown on 2D plans (grey dotted symbols). See Table 1 for correlation statistics. Data from Experiment 1.

Figure 5. **QTL confirmation and validation of** ***CRY2*** **and** ***HUA2*** **as major contributors of the variation in leaf economics and scaling allometry of plant growth in Arabidopsis.** Projections of mean ± sd (n = 3-10) trait values of NILs, KO-mutants and wild-types (Experiment 2) in the patterns of leaf economics (**A-E**) and allometric scaling relationships (**F**) observed across RILs (Experiment 1, grey points). NILs are Cvi fragments introgressed into L*er* at the top of chromosome I (Cvi-$EDI_{\mathrm{L}er}$; red plus sign) and in the middle of chromosome V (Cvi-$FLG_{\mathrm{L}er}$; red cross). $cry2_{\mathrm{L}er}$ (red circle) and $cry2_{\mathrm{Col}}$ (blue point up triangle) are KO-mutants of *CRY2* in L*er* (red filled circle) and Col (blue filled triangle) genetic backgrounds, respectively. $hua2_{\mathrm{Col}}$ (blue point down triangle) is a KO-mutant of *HUA2* in Col background. Cvi (red point up triangle). Leaf dry mass per area (LMA); plant dry mass (*M*).

Figure 1.

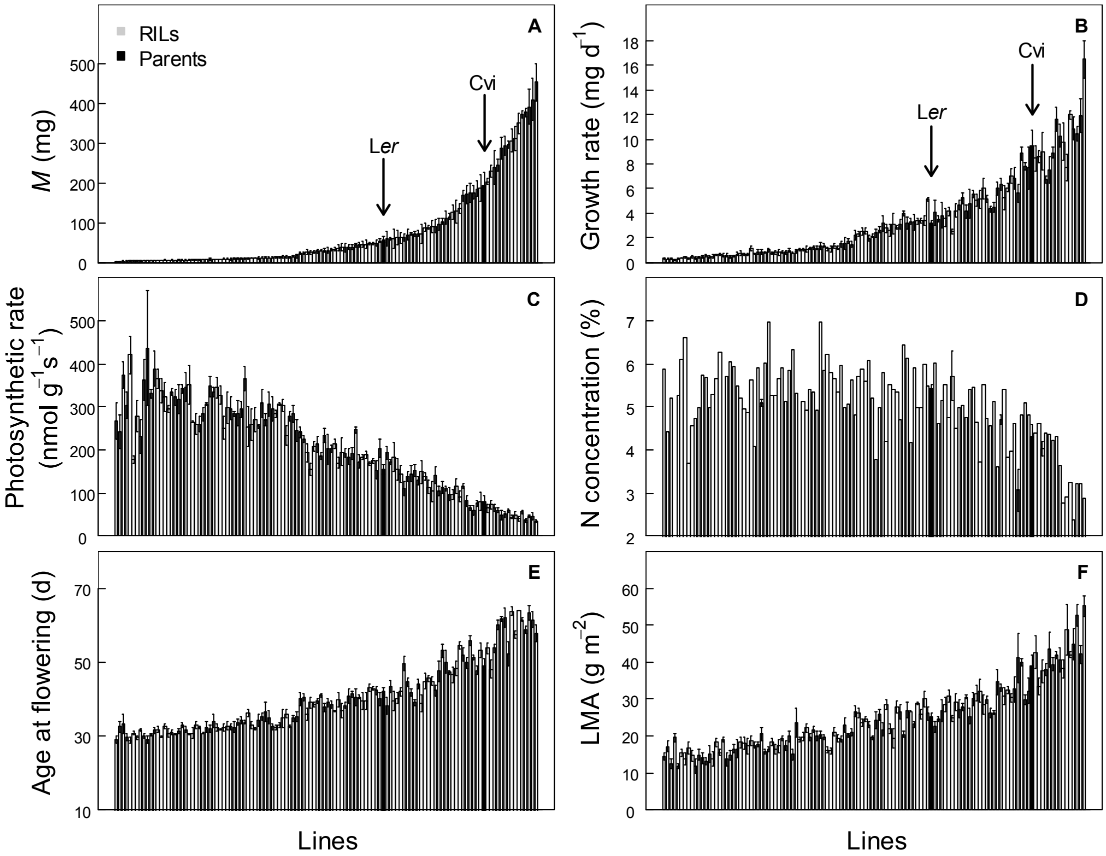

RILs
Parents
A
M (mg)
0
100
200
300
400
500
Ler
Cvi
B
Growth rate (mg d⁻¹)
0
2
4
6
8
10
12
14
16
18
Ler
Cvi
C
Photosynthetic rate (nmol g⁻¹s⁻¹)
0
100
200
300
400
500
D
N concentration (%)
2
3
4
5
6
7
E
Age at flowering (d)
10
30
50
70
F
LMA (g m⁻²)
0
10
20
30
40
50
60
Lines
Lines

Figure 2.

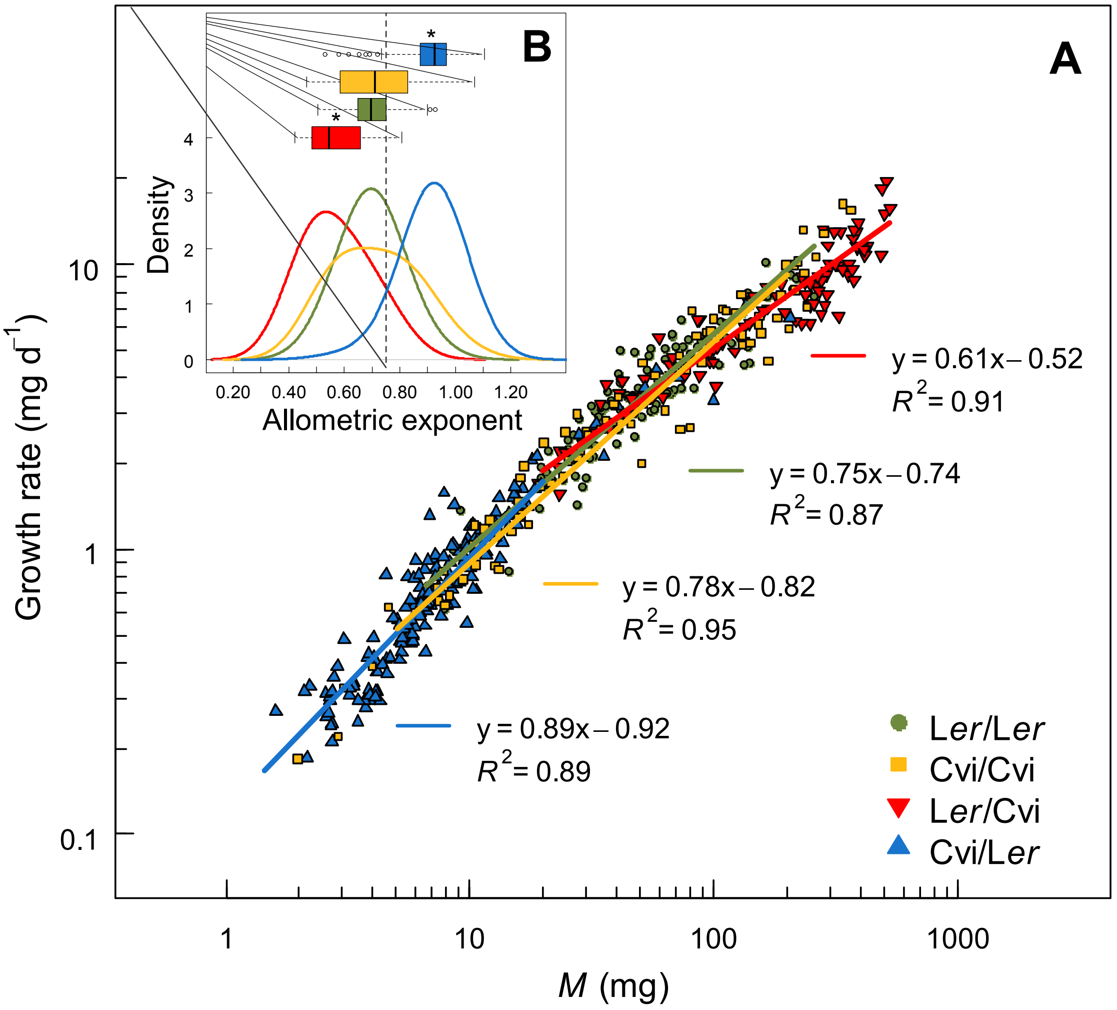

A
B
*
*
Density
4
3
2
1
0
0.20
0.40
0.60
0.80
1.00
1.20
Allometric exponent
Growth rate (mg d^{-1})
10
1
0.1
1
10
100
1000
M (mg)
y = 0.61x – 0.52
R^2 = 0.91
y = 0.75x – 0.74
R^2 = 0.87
y = 0.78x – 0.82
R^2 = 0.95
y = 0.89x – 0.92
R^2 = 0.89
L*er*/L*er*
Cvi/Cvi
L*er*/Cvi
Cvi/L*er*

Figure 3.

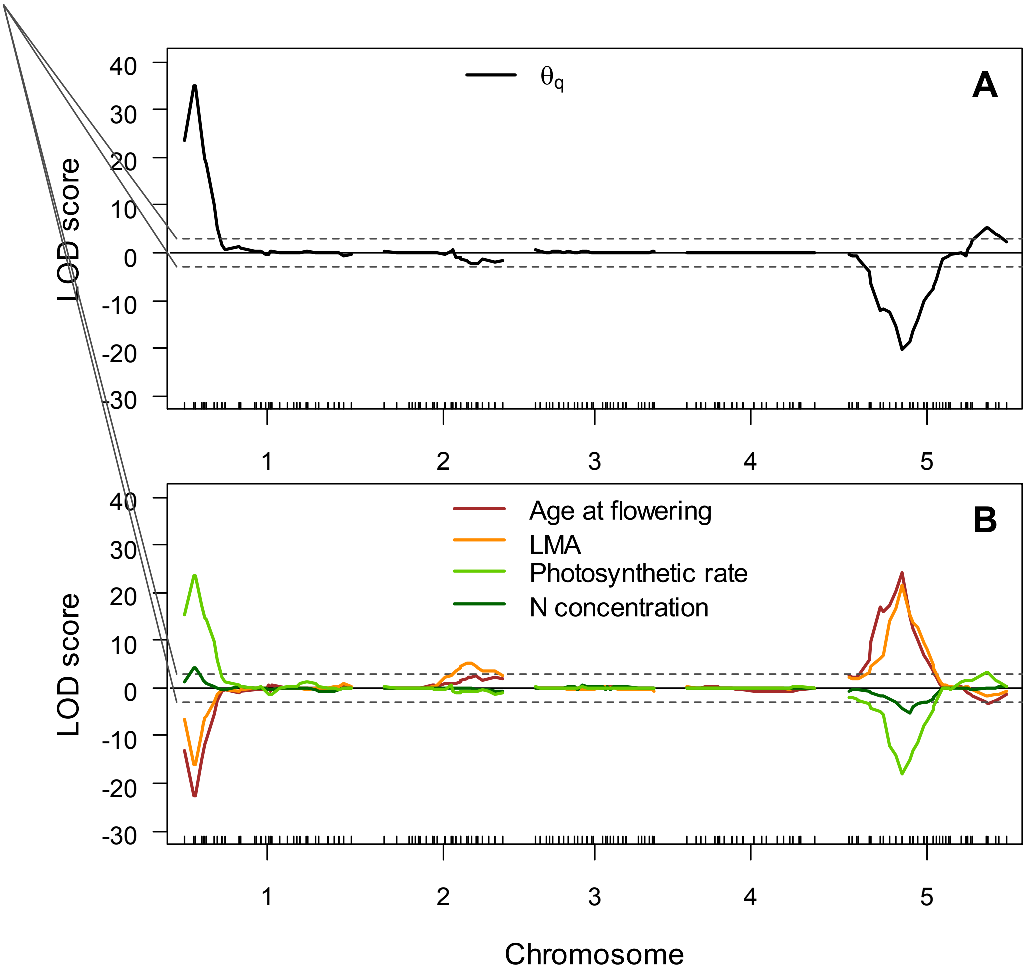

θ_q
A
LOD score
40
30
20
10
0
-10
-20
-30
1
2
3
4
5
Age at flowering
LMA
Photosynthetic rate
N concentration
B
Chromosome

Figure 4.

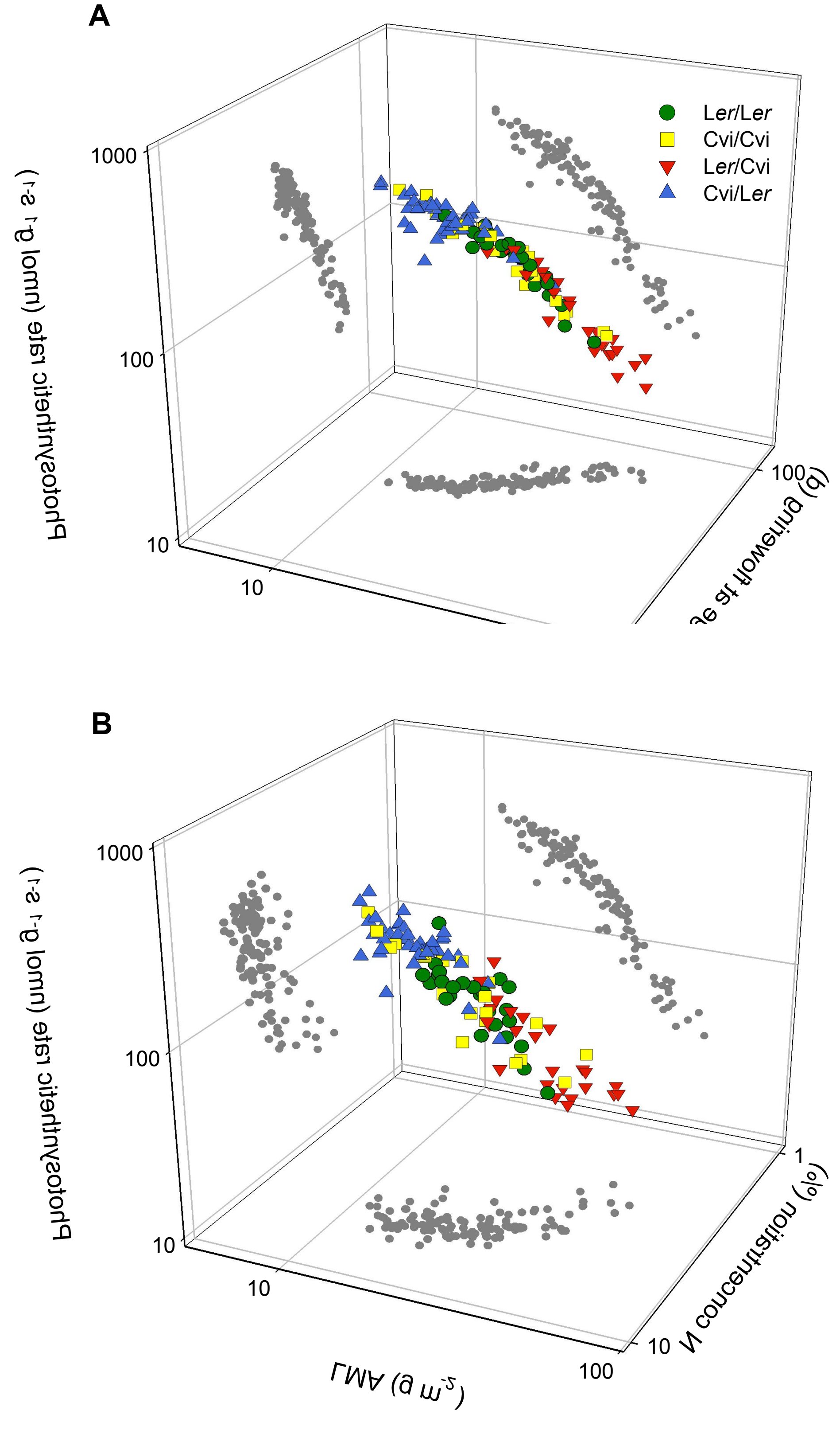
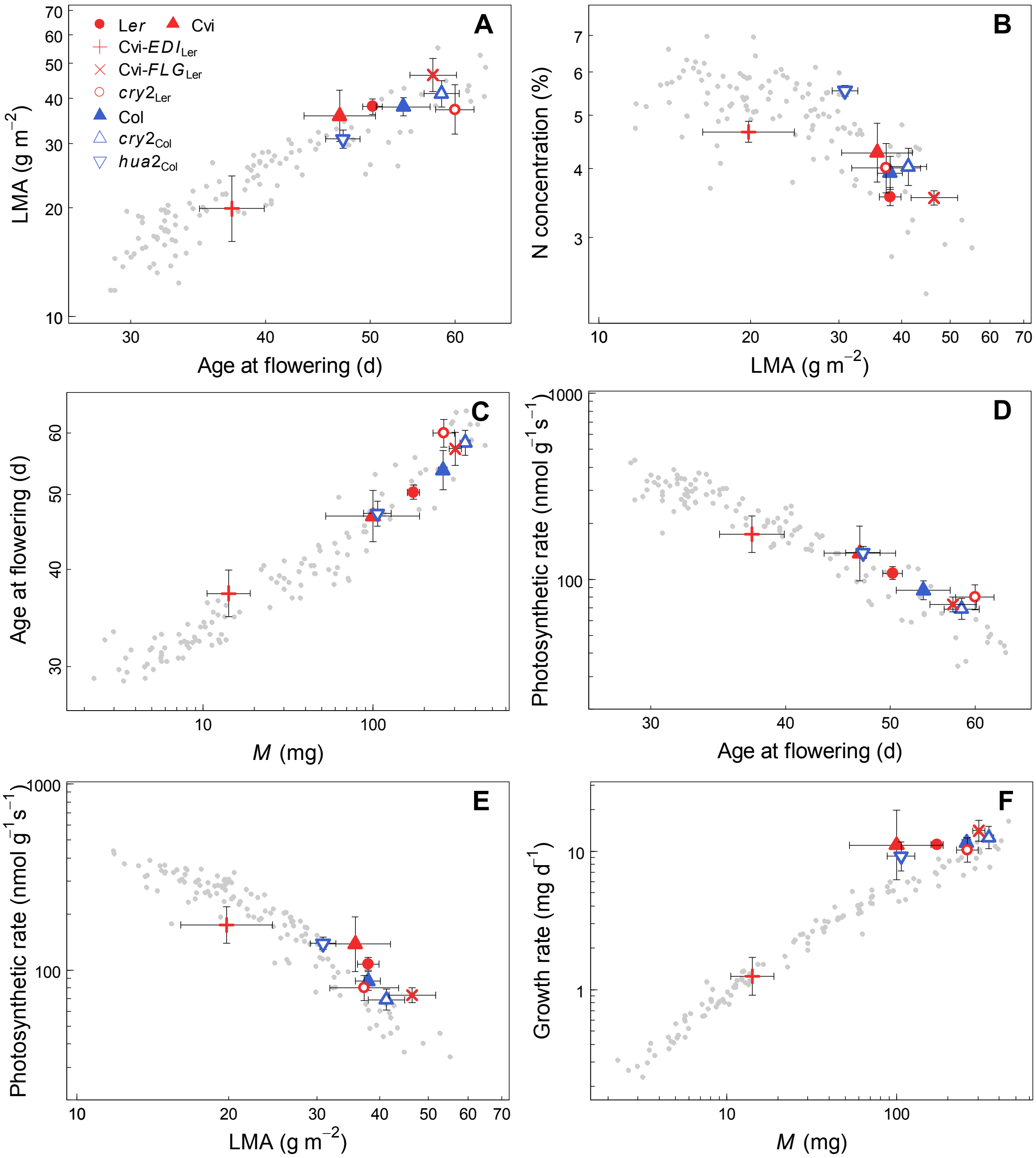

A
Ler/Ler
Cvi/Cvi
Ler/Cvi
Cvi/Ler
Photosynthetic rate (nmol g-1 s-1)
1000
100
10
10
100
Age at flowering (d)
B
Photosynthetic rate (nmol g-1 s-1)
1000
100
10
10
100
LMA (g m-2)
1
10
N concentration (%)

Figure 5.


A
LMA (g m^{-2})
70
60
50
40
30
20
10
30
40
50
60
Age at flowering (d)
Ler
Cvi
Cvi-EDI_{Ler}
Cvi-FLG_{Ler}
$cry2_{Ler}$
Col
$cry2_{Col}$
$hua2_{Col}$
B
N concentration (%)
7
6
5
4
3
10
20
30
40
50
60
70
LMA (g m^{-2})
C
Age at flowering (d)
60
50
40
30
10
100
M (mg)
D
Photosynthetic rate (nmol g^{-1}s^{-1})
1000
100
30
40
50
60
Age at flowering (d)
E
Photosynthetic rate (nmol g^{-1}s^{-1})
1000
100
10
20
30
40
50
60
70
LMA (g m^{-2})
F
Growth rate (mg d^{-1})
10
1
10
100
M (mg)