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Research

Modeling development and quantitative trait mapping reveal independent genetic modules for leaf size and shape

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Summary

- Improved predictions of fitness and yield may be obtained by characterizing the genetic controls and environmental dependencies of organismal ontogeny. Elucidating the shape of growth curves may reveal novel genetic controls that single-time-point (STP) analyses do not because, in theory, infinite numbers of growth curves can result in the same final measurement.
- We measured leaf lengths and widths in *Brassica rapa* recombinant inbred lines (RILs) throughout ontogeny. We modeled leaf growth and allometry as function valued traits (FVT), and examined genetic correlations between these traits and aspects of phenology, physiology, circadian rhythms and fitness. We used RNA-seq to construct a SNP linkage map and mapped trait quantitative trait loci (QTL).
- We found genetic trade-offs between leaf size and growth rate FVT and uncovered differences in genotypic and QTL correlations involving FVT vs STPs. We identified leaf shape (allometry) as a genetic module independent of length and width and identified selection on FVT parameters of development.
- Leaf shape is associated with venation features that affect desiccation resistance. The genetic independence of leaf shape from other leaf traits may therefore enable crop optimization in leaf shape without negative effects on traits such as size, growth rate, duration or gas exchange.

Introduction

Plant fitness in natural populations and yield in agricultural fields are influenced by ontogenetic contingencies – that is, by the history and shape of developmental growth curves (Diggle, 1997; Weinig & Delph, 2001). Developmental variation can have dramatic effects on fitness and yield, and consequently should be an important target for natural and artificial selection (Dobzhansky, 1956; Ritchie *et al.*, 1998). Characterizing the genetic controls and environmental dependencies of organismal ontogeny may lead to better predictions of fitness and yield than final estimates alone (Hammer & Jordan, 2007). Further, elucidating the shape of developmental growth curves may reveal genetic controls that single-time-point analyses cannot because, in theory, there are an infinite number of growth curves that can result in the same final measurement. However, relatively few studies incorporate the entirety of organismal ontogeny. In part this is because studying developmental variation adds not only significant time and cost to experiments, but also complexity to data analysis.

Mathematical modeling is one means to manage complex developmental data. Rather than considering development as a series of discrete, single-time-point measurements, organismal development can be viewed as a continuous and continuously changing trait. Such traits are commonly referred to as function-valued traits (FVT), because mathematical modeling is used to describe the values of these traits as a function of time (Jaffrézic & Pletcher, 2000; Kingsolver *et al.*, 2001; Wu & Lin, 2006). FVT modeling has several advantages over traditional approaches to developmental analyses. FVT modeling effectively increases statistical power by eliminating the need for factors that account for repeated measurements (Griswold *et al.*, 2008; Stinchcombe & Kirkpatrick, 2012). When model parameters are treated as traits for quantitative trait locus (QTL) mapping purposes, no novel statistical methods are necessary for analyzing the data (Xiong *et al.*, 2011). FVT parameters can be used to estimate the effects of selection while repeated measures of a trait over time are typically highly correlated and violate the assumptions of multivariate regression used in selection analyses (Lande & Arnold, 1983). FVT modeling is also an effective data reduction step: complex logistic growth curves can be described with just a few parameters. However, unlike data reduction, FVT modeling does not imply that data or precision are lost. In fact, FVT integrate across development enabling extraction of novel, composite variables such as growth rates and durations, which may

influence fitness and be under different genetic controls than final size.

Traits that show continued development or growth over time benefit from FVT modeling. Examples of evolutionary and agroecologically relevant traits include fruit and flower production, which are important components of reproductive output, and vegetative traits that can contribute to carbon fixation and resources available for eventual reproduction (Stinchcombe *et al.*, 2010). Herbivory and disease resistance over time have also been modeled as FVT (Pilson, 2000; Roux *et al.*, 2010). Using an FVT approach to describing leaf development throughout ontogeny may improve estimates of quantitative genetic features such as heritabilities and can be used to test for significant covariances with other traits. In relation to leaf traits, FVT enable modeling not only of leaf length and width, but also change in leaf shape by examining the relationship between length and width over time. It remains an open question whether such allometric features are directly (mathematically or genetically) related to growth curves of individual component traits. Further, leaf allometry is commonly correlated with venation patterns that affect gas-exchange traits important to crops such as desiccation resistance (Nicotra *et al.*, 2011), whereas leaf growth rates may affect yield in leaf crops; hence understanding the genetic similarity vs independence of different leaf traits is relevant to crop improvement.

As is the case for traits measured at a single-time-point, significant associations between FVT model parameters and other traits may provide insights into mechanisms of growth and reproductive output. We hypothesize that leaf growth rates (or allometric changes) correlate with resource availability or other aspects of development, because leaf growth rates and shapes could be affected by (or affect) net carbon assimilation. Although the transition to flowering is commonly mediated by environmental cues such as day length (Guo *et al.*, 1998) and temperature (Johanson *et al.*, 2000; Blazquez *et al.*, 2003), plants may need to attain sufficient resources before becoming competent to respond to these signals (Méndez & Obeso, 1993). Thus, flowering time may be related to rates of vegetative growth. Growth rate, duration or allometry may also be affected by developmental regulators such as the circadian clock, which gates the diurnal timing of cell and organ elongation (Nozue *et al.*, 2007). Because FVT incorporate the dynamic genetic and environmental determinants of growth, FVT parameters may have stronger (or simply different) genetic correlations and underlying loci than single-time-point measures.

In order to investigate the genetic architecture of organ-level developmental dynamics and compare these to organism-level performance, we measured leaf development in a mapping population of *Brassica rapa* recombinant inbred lines (RILs). *B. rapa* is a widely cultivated crop species domesticated for seed oil content, vegetable turnips and leaf crops (Zhao *et al.*, 2005). Consequently *B. rapa* has a rich history of artificial selection, breeding for crop improvement and diversification (Annisa & Cowling, 2013; Guo *et al.*, 2014). Additionally, weedy populations of *B. rapa* are of interest as a model for evolutionary ecology and, because of gene flow between weedy populations and cultivated fields, are also agriculturally relevant (Adler *et al.*, 1993; Snow *et al.*, 1999). To test for agroecologically relevant environmental contingencies, we grew the RILs in field settings that differed in density. Specifically, we generated FVT parameters for leaf length, width and allometry, and examined the genetic architecture of these traits. We address the following questions: (1) are FVT parameters for leaf length, width or allometry genetically similar to or distinct from single-time-point (STP) measurements, as estimated from genetic correlations and QTL; (2) Do leaf FVT co-vary with leaf-level photosynthetic capacity (A_{max}), stomatal conductance (g_s), and water-use efficiency (WUE), flowering time or circadian parameters? (3) Are FVT parameters for leaf length and width genetically correlated with allometry parameters? (4) Do associations between allometry parameters and physiological or phenological traits resemble those observed for leaf length or width parameters, as would be expected if leaf length, width and allometry are controlled by the same genetic mechanisms? and (5) Are model parameters for leaf development related to fitness?

Materials and Methods

Species description

Brassica rapa L. (Brasssicaceae) is an annual to biennial herbaceous crop that was domesticated in Eurasia. This study was conducted on Recombinant Inbred Lines (RILs) derived from R500, an oil seed variety of *B. rapa* that has been cultivated for > 3000 yr in India (Hinata & Prakash, 1984), with IMB211, a line derived from the Wisconsin Fast Plant (WFP) line. The WFP line was produced by selection for early flowering and short generations for 10 generations. IMB211 was derived from this line by intercrossing WFPs and selecting for self-compatibility and high fecundity (Williams & Hill, 1986). In comparison with IMB211, R500 flowers later, attains a larger size and greater biomass, and allocates more resources to seed production. IMB211 and R500 were crossed to produce an F_2 generation. Plants from the F_2 generation were advanced by selfing and single-seed descent for eight generations to produce RILs predicted to be > 99% homozygous (Iniguez-Luy *et al.*, 2009). Thereafter, the RILs were propagated by bulking (Brock & Weinig, 2007). This experiment includes 119 RILs, the R500 parent and a representative of IMB211.

Experimental design and data collection

In 2012, the RILs were germinated in the University of Wyoming Glasshouse (41°19′11″N, 105°33′34″W, 2218 m elevation) in 4″ peat pots filled with soil from the field and fertilized with 15 ml Osmocote (Scotts Company LLC, Marysville, OH, USA). Pots were topped with 1 cm LP5 potting soil (Sun Gro Horticulture, Agawam, MA, USA). Seeds were planted 5–10 mm deep and covered with vermiculite. To examine the effects of shade on leaf development, two density treatments were employed: uncrowded (UN) with one plant per pot and crowded (CR) with five plants per pot. For the UN treatment, three seeds were planted in the center of the pot. For the CR treatment, three seeds were planted in each of five locations: the center of the pot and near each corner using a grid that spaced planting locations 1.5 cm apart. All plants in a pot were the same genotype (RIL).

After germination, seedlings were thinned to the appropriate density. In the CR treatment, the single central plant was designated the focal individual. If the central plant did not germinate, an alternative plant was designated the focal individual. CR treatment pots with fewer than three germinants were dropped from the study.

Once the cotyledons had fully expanded, all plants were transplanted into the field into multiple blocks with each pot 18 cm apart. Blocks consisted of either UN or CR pots, and each block contained one replicate of each RIL as well as R500 and a representative of the IMB211 parent. Eight CR and eight UN blocks (for a total of 2128 pots) were located randomly in the field, and pot location within each block was randomized. Plants were irrigated to field capacity, and a rotating regime of systemic (Pasada 1.6F (Makhteshim Agan of North America, Inc, Raleigh, NC, USA) and Marathon (Bayer CropScience Ag, Monheim am Rhein, Germany)) and nonsystemic (Sevin (GardenTech Palatine, IL, USA) or Monterey Garden Insect Spray (Lawn and Garden Products Inc., Fresno, CA USA)) pesticides were applied as needed. Temperature data were recorded every 5 s in the Glasshouse and field using a series of Onset® Hobo® data loggers and a Campbell Scientific (Logan, UT, USA) CR23X data logger equipped with a Vaisala (Helsinki, Finland) HMP-50 sensor. Temperature data were used to produce hourly and daily averages, as well as hourly and daily minimums and maximums, for degree day calculations, which used a *B. rapa*-specific base value of 0.96°C (Vigil *et al.*, 1997).

Leaf length (*LL*) and leaf width (*LW*) were measured on the second epicotylar leaf two to three times per week from leaf emergence until leaf senescence. Leaf lengths were measured from the leaf base to tip, and widths were recorded at the widest part of the lamina. Plants were scored daily for germination and three times per week for bolting and flowering. We estimated fitness from a subset of plants (for practical reasons related to labor) by harvesting five CR and three UN blocks after plants had senesced. For each harvested pot, the numbers of plants per pot, 'good fruit' (filled with mature seed) and seeds from two representative fruits were counted. These data were used to generate good fruit per plant (number of good fruit/number of plants per pot; hereafter 'fruit') and seed per plant (average seed in a fruit × good fruit per plant; hereafter, 'seed').

Physiological data [photosynthetic capacity (A_{max}), stomatal conductance (g_s) and water-use efficiency (WUE; A_{max}/g_s)] were collected from plants grown in the same field in 2010 (as described in Edwards & Weinig, 2011). In Edwards *et al.* (2012), a severe drought treatment was applied to these RILs in 2010. Yet, physiological traits among drought and control treatments were relatively highly correlated, and the rank order among RILs was largely maintained. Furthermore, correlations between physiological traits measured in 2010 and the same traits measured in a subset of the 2012 RILs ranged from 0.73 to 0.91 ($P < 0.01$ for all correlations), suggesting that for this RIL set, genotypic values for gas exchange collected in one field season are highly representative of those collected in another season. Circadian data were collected from plants grown in a growth chamber as described in Lou *et al.* (2012).

Data analysis

Modeling Leaf development was modeled by fitting leaf length and width to a logistic growth curve optimized with a Least Squares approach using the Levenberg–Marquardt algorithm and the closed form solution for the following differential equations:

$$\frac{d}{dt}LL = rLL\left(\frac{LL_{Lmax} - LL}{LL_{Lmax}}\right) \quad \text{Eqn 1}$$

and

$$\frac{d}{dt}LW = rLW\left(\frac{LW_{Lmax} - LW}{LW_{Lmax}}\right) \quad \text{Eqn 2}$$

where r estimates growth rate and $Lmax$ estimates maximum size. Duration of growth (d) is the time the first observation occurred after growth reached 95% of $Lmax$. Data from UN and CR treatments were modeled independently. Model parameters are compared to single-time-point (STP) measures, which represent the last actual data point recorded.

The *LL* and *LW* traits were quality controlled. Obviously erroneous data were removed or replaced with the average of the surrounding data points for that plant. Individual plants that had too few data points, that developed oddly compared to plants from the same line (for instance CR plants that were suppressed), or for which the *LL* or *LW* measures did not capture the entire growth curve were excluded. After quality control, all genotypes were represented by a minimum of three plants per treatment.

Leaf allometry was modeled using a standard power-law equation (Eqn 3), which was used to describe allometric growth as early as the 19th century and is still extensively used today (Snell, 1892). The allometric model generates a relationship between leaf length and leaf width and implicitly includes developmental time according to the formula:

$$LW = a(LL)^b \quad \text{Eqn 3}$$

where a is a scaling factor relating *LL* to *LW*, and b describes the curvature of the relationship. When $b > 1$, leaf width increases proportionately faster than length (positive allometry); the reverse is true when $b < 1$ (negative allometry). When $b = 1$, the ratio of width to length is constant, a condition called isometric allometry (Gould, 1966).

Heritability For all phenology, fitness and leaf traits Best Linear Unbiased Predictions (BLUPs) of genotypic means and errors were generated and broad-sense heritabilities were calculated independently for CR and UN treatments using PROC MIXED in SAS 9.2 while controlling for line and block (Supporting Information Tables S1 and S2). BLUPs for A_{max} and g_s were re-estimated from the original raw data (C. E. Edwards *et al.*, unpublished) to control for line, block and IRGA ID. Circadian rhythm BLUPs (Lou *et al.*, 2012) were also re-estimated. In a similar set of models, we combined CR and UN datasets to test

for the main effects of treatment, line and block, as well as random effects of block nested within treatment, treatment, line and line-by-treatment interactions (SAS 9.2 PROC MIXED, Cary, NC, USA).

Genetic correlations Bivariate genetic correlations were estimated using genotypic means, and the procedure *cor.test* for Pearson's product moment correlations in R (R Core Team, 2014). A Bonferroni correction for multiple tests was applied.

QTL analyses QTL analyses were performed in R/QTL (Broman *et al.*, 2003) using a highly resolved RNA-seq based SNP map with 1273 informative genomic bins ('markers') distributed across the 10 *B. rapa* chromosomes with an average distance of 0.79 cM. Genotypic bins were delineated by genotyping 124 RILs at > 65K SNP positions. SNPs were identified by a samtools/bcftools-based analysis using > 355 million mapped 44-bp RNA-seq reads with an average depth across the transcriptome of 2.6 reads per RIL. Because only a fraction of genes are expressed, the actual coverage for expressed genes is significantly higher (M. F. Covington *et al.*, in prep.). Single QTL were identified using the SCANONE interval mapping function at 1 cM resolution with estimated genotyping errors of 0.001 and Haley-Knott regression (Broman & Sen, 2009). Epistatic interactions were identified via SCANTWO interval mapping at 2 cM resolution using the EM algorithm for identifying maximum likelihoods (Broman *et al.*, 2003; Broman & Sen, 2009). All 95% significance thresholds were obtained using 1000 permutations. Significant QTL identified from SCANONE and SCANTWO functions were used to seed an initial model to explore more complicated genetic architecture. Model space for each trait was searched using an iterative process (FITQTL, REFINEQTL and ADDQTL functions using 1000 imputations at 1 cM resolution with estimated genotyping errors of 0.001). After each iteration, nonsignificant QTL were dropped from the model and significant QTL were added. QTL and their 1.5 LOD intervals are displayed using MAPCHART2.0 (Voorrips, 2002). QTL-by-environment interactions for QTL with additive effects were assessed using a series linear regression models (*lm*) and two-way ANOVAs to asses each significant marker individually in R (Fox & Weisberg, 2011). A *P*-value < 0.05 for the type III *F*-values of treatment-by-QTL interactions was considered evidence of QTL by environment interactions.

Selection analyses Selection analyses followed Lande & Arnold (1983). Genotypic means were standardized to a mean of zero and a standard deviation of one, and estimates of relative fitness were generated by dividing the genotypic mean of 'seed' for each line by the grand mean. Linear and quadratic regression models were constructed separately for the UN and CR treatments, and selection on the length (*LL_Lmax*, *LL_r*, *LL_d*), width (*LW_Lmax*, *LW_r*, *LW_d*), and width and allometry (*LW_Lmax*, *LW_r*, *LW_d* and *b*) of leaves (R Core Team, 2014) was assessed independently. For pairs of traits that were highly auto-correlated, one trait was dropped from the model (therefore STP and *a* are not included in any models). Indirect selection on leaf morphology mediated by phenology was assessed by re-running each model including a significantly correlated aspect of phenology (bolt-to-flower for LL and LW; flowering time for allometry) and comparing it to the previous model.

Results

Modeling

For all FVT models, there were sufficient data to support all aspects of the growth curves modeled, and the models fit well to the data (Fig. 1 for example model fits).

Genotypic means and heritabilities

There were significant genotype effects for all phenotypic traits in both the CR and UN treatments (Table 1). The main effect of treatment was never significant, although there was a nonsignificant trend for allometry such that *b* was slightly > 1 in UN ($b = 1.15$) but < 1 in CR ($b = 0.80$). Line-by-treatment interactions were significant for all leaf growth (*r*, *d*, *Lmax*) traits and fitness estimates, but not for allometry parameters or phenology (Table 1).

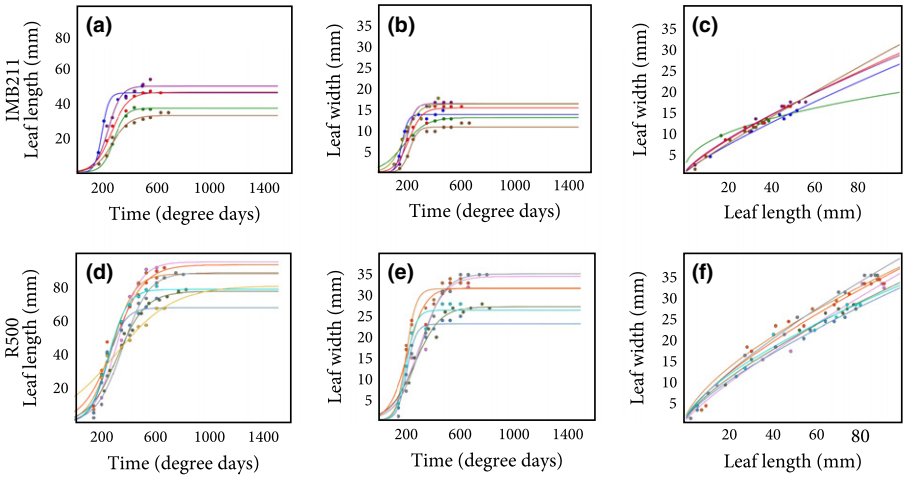

Fig. 1 Data and least-squares optimized logistic growth models for uncrowded *Brassica rapa* RILs (a–c, IMB211 representative; d–f, R500) for leaf length (a, d), leaf width (b, e) and allometry (c, f). Colors indicate individual replicate plants within a recombinant inbred line (RIL). Circles, actual recorded data; lines, logistic growth curves fitted to the data. Note that the color designating a replicate is consistent across plots such that the same plant will have the same color in all three plots.

Table 1 Block, environment (Treat), genotypic (Line) and genotype-by-environment effects and heritabilities in *Brassica rapa* recombinant inbred lines (RILs)

		Random effects (*Z*-values)					Heritability (H^2)	
Trait	Model	Block (Treat)	Treat	Line	Line × Treat	Residual	UN (%)	CR (%)
LL_r	$t_{(1.62)} = 557.88$ ***	2.28 *	0.07 NS	4.02 ***	3.36 **	26.42 ***	28.30	15.38
LL_Lmax	$t_{(1.8)} = 39.52$ **	2.21 *	0.54 NS	6.39 ***	3.65 ***	26.43 ***	53.31	36.41
LL_d	$t_{(1.83)} = 47.75$ **	2.22 *	0.38 NS	5.76 ***	2.59 **	25.21 ***	38.18	25.01
LLSTP	$t_{(2.47)} = 49.38$ ***	2.32 *	0.35 NS	6.52 ***	2.99 **	26.47 ***	44.11	33.18
LW_r	$t_{(1.5)} = 463.34$ ***	1.73 *	0.42 NS	4.12 ***	2.07 *	26.72 ***	17.15	11.37
LW_Lmax	$t_{(1.64)}$=28.49 **	1.92 *	0.64 NS	6.74 ***	3.25 **	26.74 ***	53.26	45.79
LW_d	$t_{(1.67)} = 43.68$ **	1.59 NS	0.54 NS	5.44 ***	2 *	25.88 ***	27.30	21.83
LWSTP	$t_{(1.75)} = 30.29$ **	2.03 *	0.63 NS	6.88 ***	2.69 **	26.78 ***	54.36	46.32
a	$t_{(1.42)} = 17.16$ *	2.25 *	0.43 NS	5.86 ***	. NS	26.75 ***	25.09	21.90
b	$t_{(2.76)} = 55.93$ ***	2.19 *	0.16 NS	6.39 ***	. NS	26.75 ***	27.89	26.35
Germination	$t_{(16.2)} = 21.73$ ***	2.7 **	. NS	5.39 ***	0.09 NS	30.17 ***	10.79	8.32
Bolt	$t_{(7.53)} = 45.97$ ***	2.31 *	0.35 NS	7.64 ***	1.02 NS	29.02 ***	66.77 (67.64)	62.10 (65.24)
Flower	$t_{(133)} = 95.41$ ***	1.74 *	. NS	7.66 ***	0.75 NS	28.79 ***	66.44 (66.47)	66.74 (70.02)
Bolt to flower	$t_{(1.21)} = 34.1$ **	2.32 *	0.47 NS	5.34 ***	0.34 NS	28.52 ***	16.67 (16.65)	10.80 (11.48)
Fruit	$t_{(1.02)} = 1.7$ NS	2.1 *	0.68 NS	4.06 ***	6.05 ***	24.75 ***	59.34	16.02
Seed	$t_{(1.02)} = 1.66$ NS	2.1 *	0.67 NS	3.68 ***	5.87 ***	24.36 ***	55.63	20.10

All phenology components are listed in degree days with heritabilities for calendar days listed in parenthesis.
UN, uncrowded; CR, crowded; *LL*, leaf length; *LW*, leaf width; *r*, growth rate; *d*, growth duration (degree days); *STP*, single-time-point measurement at maximum size (mm); *a*, a parameter of allometry; *b*, a parameter of allometry; bolt, time to bolting; flower, time to flowering; bolt to flower, the interval between bolting and flowering; fruit, average good fruit per plant; seed, average seed per plant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

Broad-sense heritabilities were, with the exception of flowering time, always higher for UN compared to CR plants (Table 1). This difference is most striking for fitness characters, where heritabilities where between two and four times higher for UN plants compared to CR plants (Table 1). Heritabilities for model parameters estimating *Lmax* tended to be somewhat higher than the STP measures of maximum size in both UN and CR conditions (Table 1). For leaf width, heritabilities were similar for *LW_Lmax* and LWSTP (Table 1). Among leaf traits, *r* had the lowest heritability, with the exception of *UNLL_r*, which had a heritability of 28.3% (Table 1). Heritabilities for *d* and for allometry were intermediate (between 21.8% and 38.4%; Table 1) between *Lmax* and *r*.

Genetic correlations

Genotypic correlations among and between leaf parameters and nonleaf traits were similar across treatments (compare Figs S1 and S2). For the purpose of exploring detailed trait associations, correlations between uncrowded leaf width traits, allometry FVT and a subset of nonleaf traits are presented (Fig. 2).

Modeled estimates of *LW_Lmax* and LWSTP were, not surprisingly, almost perfectly correlated with one another (Fig. 2), suggesting that from a genetic perspective these are nearly identical traits and that the models are generally a good fit to the data. *LW_d* was less strongly positively correlated with LWSTP in UN (relative to *LW_Lmax)*, whereas *LW_r* was negatively correlated with LWSTP (Fig. 2); the magnitude of these correlations was reduced in CR (Fig. S2).

Parameters of the function describing leaf allometry (*a* and *b*) were highly negatively correlated (Fig. 2). Although allometry describes the relationship between leaf length and leaf width, *a* and *b* were never correlated with model parameters for either *LL* or *LW* (Figs 2, S1, S2). This counterintuitive result occurred because although *LL_Lmax* and *LW_Lmax* anchor the end point of the line describing allometry, there are many different ways of achieving that endpoint. Consequently, we identify allometry as an independent property of leaves not captured by either length

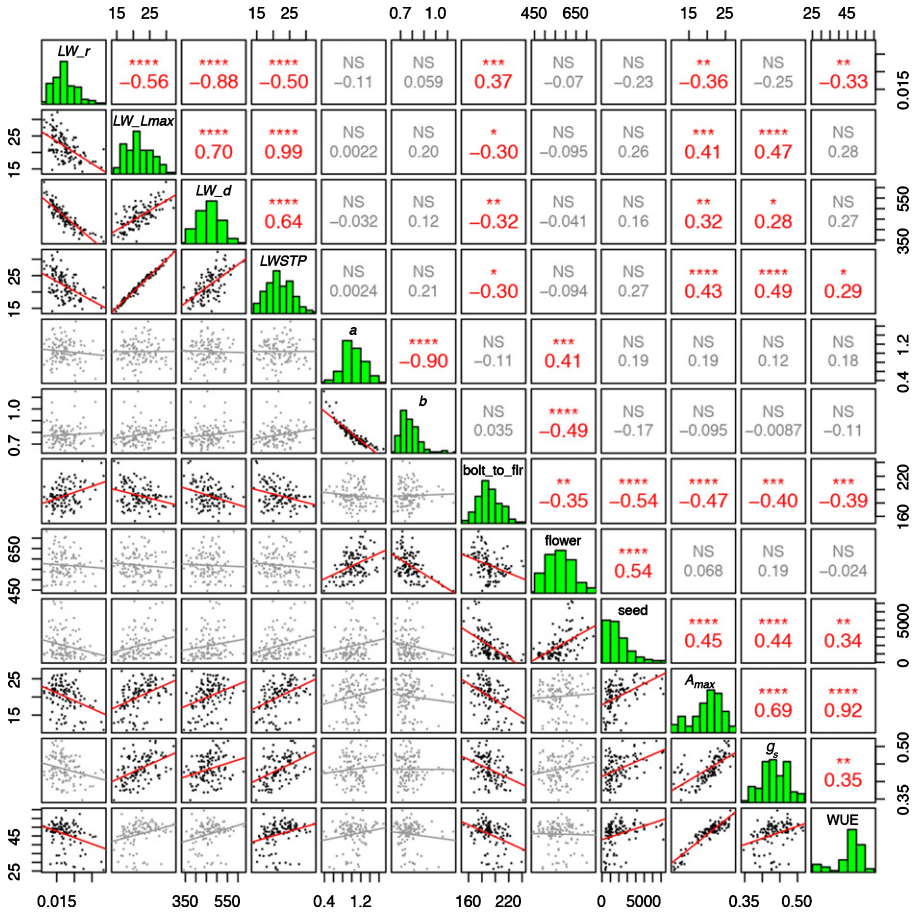

Fig. 2 Bonferroni-corrected genetic correlations for uncrowded leaf width model parameters, allometry, phenology, fitness and physiology in *Brassica rapa* recombinant inbred lines (RILs). The strong correlations between models estimates of maximum leaf width (*LW_Lmax*, in mm) and single-time-point measures of final leaf size (STP, in mm) indicate that the developmental models are a good fit for the data. However, these correlations are < 1 indicating that modeling leaf development captures genetic aspects of plant growth that *STP* alone does not. Maximum leaf width is negatively correlated with growth rate (*LW_r*, in degree days), a dynamic that is the result of a genetic trade-offs between growth rate and growth duration (*LW_d*, in degree days). Note that linear estimates of leaf growth are not correlated with parameters describing leaf developmental allometry (*a* and *b*). Furthermore, these suites of traits have different patterns of correlations with phenology and physiology. bolt_to_flr, the interval between bolting and flowering (degree days); flower, time to flowering (degree days); seed, number of good seed produced; A_{max}, photosynthetic rate (μmol CO_2 m^{-2} s^{-1}); g_s, stomatal conductance (mol H_2O m^{-2} s^{-1}); WUE, water-use efficiency (A_{max}/g_s). NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

or width. It is also noteworthy that *a* and *b* are uncorrelated with the STP, indicating that dynamic aspects of allometry over ontogeny are uncorrelated with final leaf sizes.

Leaf and phenological traits are often correlated, but parameters of linear leaf growth are associated with different aspects of phenology than estimates of allometry. *a* and *b* are correlated with days to bolting (Fig. S2) and days to flowering time but no other nonleaf traits (Fig. 2), whereas *LL* and *LW* parameters are not correlated with bolting or flowering time, but instead are correlated with the interval between bolting and flowering (Figs 2, S1, S2). These differences in correlations support the hypothesis that linear leaf parameters are genetically distinct from allometry parameters. The phenological correlations are consistent with negative correlations between *LW_r* and *LW_Lmax*. Specifically, leaf growth rates (*LW_r*) were negatively correlated with the interval between bolting and flowering, whereas *LW_d* and *LW_Lmax* were positively correlated with the time between bolting and flowering (Fig. 2).

Photosynthetic capacity (A_{max}) was positively correlated with *LW_d*, *LW_Lmax*, LWSTP and seed, but negatively correlated with *LW_r* (Fig. 2). Although *LW_r* and stomatal conductance (g_s) were not correlated, *LW_Lmax*, *LW_d* and LWSTP were positively correlated with g_s (Fig. 2). The same pattern of associations was observed for *LL* (Fig. S1). Allometry parameters are not correlated with gas exchange. Although not the primary focus of our experiment, it is notable that gas-exchange traits were not correlated with flowering time but were significantly correlated with the interval from bolting to flowering (Fig. 2).

Circadian measures of period and phase at 18°C (PER18, Phase18) were never significantly correlated with parameters of the leaf developmental models, allometry, phenology, fitness or physiology (Figs S1, S2). The lack of correlation between circadian rhythms and any field-measured traits may reflect the fact that these datasets were collected in different environments. Circadian rhythms were not included in additional analyses.

Additive QTL

QTL mapping revealed a total of 112 QTL with between 1 and 9 significant QTL for each combination of trait and environment (Fig. 3; Table S3). However, because the 1.5 LOD support limits indicate that many QTL colocalize, an alternative interpretation is that we identified a minimum of 12 independent genomic regions that could be responsible for the genotypic variation observed. Each of the 112 individual additive QTL explained 1.41%-33.33% of variation in the RILs (Table S3). Fewer QTL were identified for traits in the CR treatment vs the UN treatment (44 vs 54), consistent with higher H^2 for UN traits compared to CR traits (Table 3). This trend was driven by allometry (8 UN QTL vs 4 CR QTL) and phenology (15 UN vs 6 CR QTL).

Additive QTL were distributed across all ten *B. rapa* chromosomes, although chromosomes 4 and 8 harbored relatively few QTL (Fig. 3; Table S3). As expected based on the extremely strong correlations, STP and *Lmax* often colocalize (e.g. QTL located on chromosomes 1, 2, 3, 6 and 7). However, some QTL appear to affect the parameters *r* and *d* and occasionally even *Lmax* without associated effects on STP. For instance, a QTL on chromosome 5 affects *CRLW_r* and *CRLL_r* but does not colocalize with QTL affecting STP (Fig. 3; Table S3). Similarly, a QTL for *UNLL_r* and *UNLW_d* on chromosome 10 does not have significant effects on STP. A QTL on chromosome 3 positively affects *UNLW_r* but negatively affects *UNLW_Lmax, UNLW_d* and UNLWSTP, a pattern that is also repeated for the leaf length FVT parameters in CR. Despite the strong genetic correlations between STP and *Lmax* traits, QTL on chromosomes 4 and 10 affect *CRLL_Lmax* but do not colocalize with QTL affecting STP (Fig. 3; Table S3). Notably, the higher H^2 of (UN) leaf FVT compared to STP results in a greater power to detect FVT QTL. Therefore, locations harboring only STP QTL likely do not have pleiotropic effects on FVT (i.e. type II error does not cause failure to detect effects on FVT).

As expected based on the lack of genetic correlations, QTL for *a* and *b* are often distinct from QTL for STP or *r*, *d* and *Lmax* (e.g. QTL for *UN_a* and *UN_b* on chromosomes 1, 9 and 10, as well as QTL for *UN_a* on chromosome 8, are independent of other leaf FVT QTL in the same treatment). These independent QTL indicate that the genetic architecture underlying allometry differs from STP and linear leaf parameters. However, in a few cases, *a* and *b* do colocalize with *Lmax* within the same treatment (e.g. on chromosome 10) even though allometry and *Lmax* are not genetically correlated.

Consistent with genetic correlations between leaf traits and phenology, *UNLL_Lmax* and *UNLL_r* QTL colocalize with QTL for the interval from bolting to flowering (on chromosome 3; Fig. 3; Table S3). The majority of leaf length and width QTL are independent of QTL for bolting or flowering time (44 QTL are independent vs 7 that colocalize). By contrast, QTL for *a* and *b* colocalize with QTL for degree days to bolting and flowering (e.g. 4 overlapping allometry QTL on chromosome 10), consistent with the significant genetic correlations among these traits. These QTL on chromosome 10 affecting allometry (*b*), bolting and flowering time in both treatments explain a significant percentage of the variance and are among the largest effect QTL that were mapped, as each QTL explains 20.60–38.62% of genotypic variation.

QTL-by-environment interactions (QTL × E)

Although there are differences in the number and location of significant QTL detected across environments, tests for QTL × E only detected significant environmental interactions for QTL

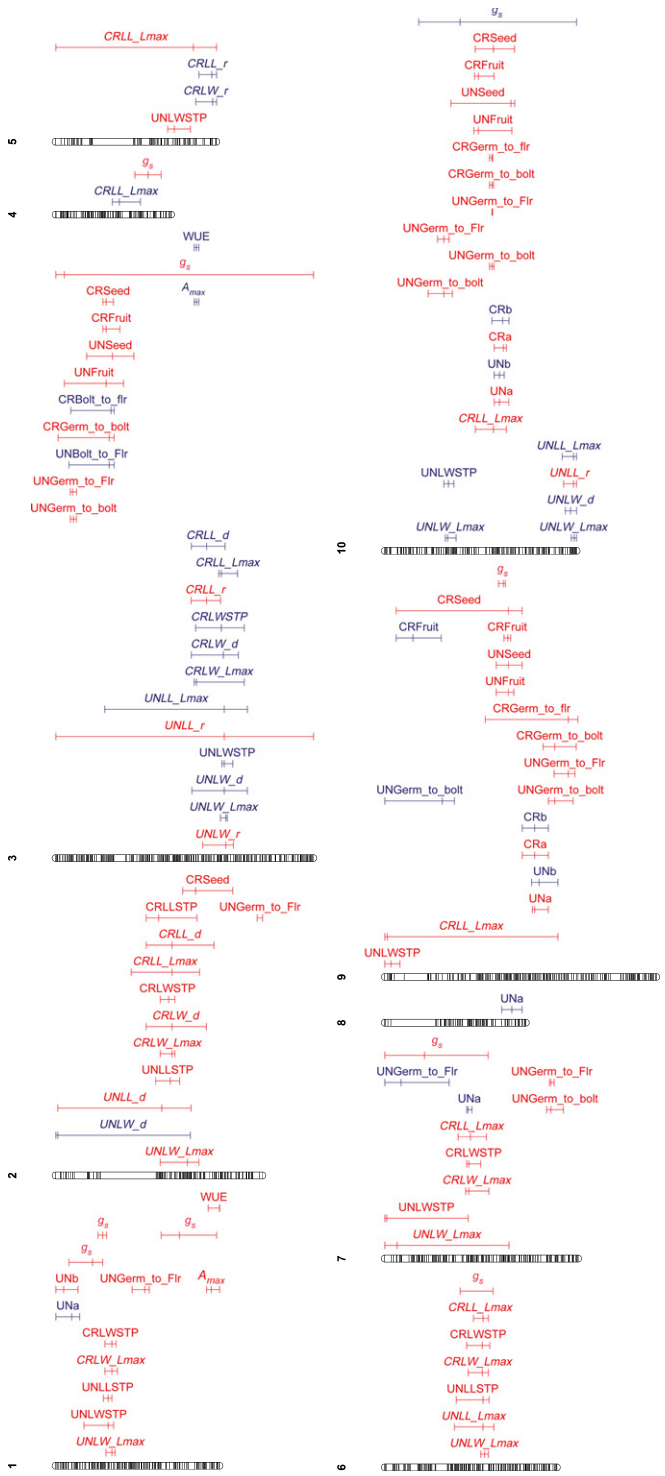

Fig. 3 RNA-seq-based single nucleotide polymorphism linkage map of *Brassica rapa*. Twelve hundred and seventy-three markers are roughly evenly distributed across 10 chromosomes. Marker gaps correspond to centromeres or ancient centromeres, which are areas of low expression. Additive quantitative trait loci (QTL) and 1.5-LOD support limits are shown. Positive QTL (with respect to IMB211) are red and negative QTL are blue. Overlapping 1.5-LOD support limits are interpreted as evidence for colocalization of QTL. UN, uncrowded; CR, crowded; *LW*, leaf width; *LL*, leaf length; *Lmax*, maximum estimated size (mm); *r*, growth rate; *d*, growth duration; STP, single-time-point measures of final leaf size (mm); *a*, parameter of allometry; *b*, parameter of allometry; Germ_to_Flr, time from germination to flowering; Germ_to_bolt; time from germination to bolting; Bolt_to_Flr, interval between bolting and flowering; Fruit, average number of good fruit; seed, average number of seed; A_{max}, photosynthetic rate (μmol CO_2 m^{-2} s^{-1}); g_s, stomatal conductance (mol H_2O m^{-2} s^{-1}); WUE, water-use efficiency (A_{max}/g_s).

Table 2 Marker names for quantitative trait loci (QTL) that have significant environmental interactions in *Brassica rapa* recombinant inbred lines (RILs)

Trait	Marker name	$F_{(Df\_num, Df\_den)}$	*P*-value	UN variance (%)	CR variance (%)
Good fruit	A03_4610765	$F_{(1,230)} = 3.35$	$P = 0.037$	10.20	11.97
	A09_14638518	$F_{(1, 232)} = 11.1$	$P = 0.001$	13.06	NS
	A10_11112220	$F_{(1,232)} = 7.12$	$P = 0.008$	12.66	15.47
Seed	A03_5429398	$F_{(1,230)} = 3.62$	$P = 0.028$	10.00	NS
	A03_4610765	$F_{(1,232)} = 8.21$	$P = 0.005$	NS	14.77
	A10_13413567	$F_{(1,232)} = 4.69$	$P = 0.031$	NS	13.01
	A10_14464079	$F_{(1,232)} = 5.32$	$P = 0.022$	11.63	NS

UN, uncrowded; CR, crowded; NS, not significant.

associated with fitness: fruit (Fig. S3A,B; Table 2) and seed (Figs S3C–3F; Table 2). The relative paucity of QTL × E is consistent with comparatively lower magnitude of G × E interactions relative to genotype effects in the ANOVA (Table 1).

Epistatic interactions

We identified epistatic interactions among QTL for leaf model parameters including *LW_Lmax*, LWSTP, *LL_Lmax*, allometry and phenology (Table 3; Figs S4, S5). Epistatic QTL affecting the linear leaf and allometry parameters differed from those for LWSTP (Fig. S4). All of the epistatic QTL, except for a QTL for *UNLW_Lmax* on chromosome 9, also have significant additive effects. In general the effect size of epistatic QTL (1.17 = 14.1%) was less than additive QTL. One notable exception was the interaction between loci on chromosomes 7 and 8, which explained 14.5% of variation in *UN_a* (Table 3).

Selection analyses

Selection analyses were performed to estimate the strength of selection on leaf width, length and allometry parameters independently using linear models and fully balanced quadratic models; quadratic terms did not improve model fit and are not reported. Results were also similar between UN (Table 4) and CR (Table S4) settings, and results for UN are presented. Relative seed number was used as an estimate of fitness. In the model for growth curve parameters, *LW_r* and *LW_d* were under negative selection ($\beta' = -0.38$ and -0.39, respectively), and *LW_Lmax* was under positive selection ($\beta' = 0.27$). Selection upon allometry was marginally significant. When the interval between bolting and flowering was included in linear *LW* models, *LW_r* and *LW_d* were no longer significant, indicating that selection on these traits was indirect and mediated by strong negative selection on the interval between bolting and flowering ($\beta' = -0.38$). Similarly, including phenology in selection models for allometry removed the marginally significant negative selection on *b*, demonstrating that these effects were likely indirect and mediated by strong positive selection on flowering time ($\beta' = 0.48$; Table 4).

Discussion

The evolution of organismal diversity is dependent on variation in the genetic architecture of traits. Trait data are often collected at a single-time-point without considering developmental differences among individuals. However, fitness is influenced by the entirety of organismal ontogeny, and genetic and environmental factors may affect the process by which a given endpoint is reached and not just the endpoint itself. We utilized a function valued trait (FVT) modeling approach to examine the genetic architecture of leaf development and assess the importance of collecting ontogenetic data. Furthermore, because many aspects of plant ontogeny are phenotypically plastic (Sultan, 2000; Diggle, 2002; Baker *et al.*, 2014), we examined leaf development in crowded and uncrowded environments. To explore the potential importance of leaf development for evolution and crop improvement, we tested for associations between leaf developmental genetic data and both leaf-level physiological data and whole

Table 3 Quantitative trait loci (QTL) with epistatic effects in *Brassica rapa* recombinant inbred lines (RILs)

Trait	Treatment	*n*	LOD	Variance (%)	Chromosome	Start marker	Start position	QTL marker	QTL position	End marker	End position
LW_Lmax	UN	124	5.1	6.7	6	loc51	54.9	Loc53	56.9	Loc55	58.9
					9	17139415	100.1	17382954	101.7	17508299	102.9
LWSTP	UN	124	4.3	7.6	5	21765900	61.1	loc63	64.6	22647157	73.1
					9	1100290	0.14	490546	3.4	loc8	8.1
LL_Lmax	CR	118	3.3	5.8	7	loc39	39.2	12838696	45.7	loc54	54.2
					3	16879608	86.7	16926246	88.0	18644298	96.6
		118	5.0	8.9	4	loc30	30.1	11531882	33.8	14089896	45.0
					9	1100290	0.1	952470	1.4	loc92	92.1
		118	1.7	2.8	4	loc30	30.1	11531882	33.8	14089896	45.0
					10	10765113	49.1	13413567	58.7	14176461	65.6
Allometry (*a*)	UN	118	7.0	14.5	7	12487828	43.7	12722269	44.5	13060999	46.5
					8	20354325	62.2	20903550	67.6	loc73	73.1
Germination to Flowering	UN		1.3	1.2	1	10259189	43.8	17216013	50.5	20267483	52.9
					3	1519411	7.9	1627346	9.1	loc11	11.1

UN, uncrowded; CR, crowded; *LL*, leaf length; *LW*, leaf width; STP, single-time-point measure at final size (mm); *_Lmax*, estimated final size (mm); *a*, a parameter of allometry.

Table 4 Selection analyses for the uncrowded treatment in *Brassica rapa* recombinant inbred lines (RILs)

Model	Traits	Beta (no phenology)	Beta (with phenology)
Length	*LL_Lmax*	0.41***	0.19*
	LL_r	−0.08	−0.21
	LL_d	−0.21	−0.32*
	Bolt-to-flower	NA	−0.41***
Width	*LW_Lmax*	0.27*	0.27**
	LW_r	−0.38*	−0.2
	LW_d	−0.39*	−0.15
	Bolt-to-flower	NA	−0.38***
Allometry and Width	*b*	−0.14†	0.08
	LW_Lmax	0.29**	0.29***
	LW_r	−0.28†	−0.23
	LW_d	−0.30	−0.26†
	flowering	NA	0.48***

***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; †, $P<0.1$; *LL_Lmax*, maximum estimated leaf length; *LL_r*, leaf length growth rate; *LL_d*, leaf length growth duration; bolt-to-flower, the interval between bolting and flowering; *LW_Lmax*, maximum estimated leaf width; *LW_r*, leaf width growth rate; *LW_d*, leaf width growth duration; *b*, a parameter of the function describing the ontogenetic allometric relationship between leaf length and width.

plant features including circadian rhythms, phenology and fitness.

From logistic growth curves, we estimated parameters of leaf development including growth rates, duration and maximum leaf size (*Lmax*) as well as allometry parameters (*a* and *b*). The strong positive correlation between our estimates of *Lmax* and single-time-point (STP) measures of final leaf size indicate that our models accurately portray leaf development. However, as a previous study comparing single-time-point (STP) and FVT estimates found (Ma *et al.*, 2002), FVT modeling enabled us to detect additional quantitative trait loci (QTL) that we could not with STP (in particular multiple QTL for *Lmax* at the bottom of chromosome 10). Duration (*d*), was less strongly correlated with STP, whereas growth rate (*r*), was negatively correlated with STP; and the magnitude of these correlations was also reduced in the crowded (CR) relative to uncrowded (UN) conditions. These weaker (and for *r*, negative) correlations indicate that modeling FVT captures dynamic aspects of developmental genetics that STP alone does not, and thereby reveals novel genetic controls of growth that could not be predicted directly from STP. The allometry parameters, *a* and *b*, were likewise uncorrelated with STP. In support of the view that leaf growth and allometry parameters are distinct from STP, leaf growth and allometry parameters often map to genomic regions (QTL) not associated with STP QTL (Fig. 3).

Leaves exhibit deterministic growth in most eudicots (Bell & Bryan, 2008) and are the primary pre-flowering photosynthetic organ of *B. rapa*. Indeed, we found that leaf growth was finite and genotype-specific. Additionally, there were genetic trade-offs between leaf growth rates, duration and final sizes. Although fast-growing leaves are typically larger than slow-growing leaves (Ridge *et al.*, 1986; Poorter & Remkes, 1990), we found a strong negative correlation between leaf growth rates and final sizes (Figs 2, S1, S2). This negative correlation is driven by duration of growth: fast-growing leaves stop growing earlier in development than slow-growing leaves (Figs 2, S1, S2). To explore this relationship further, we compared our parameters of leaf development to genotypic means of leaf-level physiological traits. A single leaf can be viewed as both a carbon source (by fixing carbon via photosynthesis) and a carbon sink (as carbon is used in structural aspects of leaf growth) (Turgeon, 1989). Typically, faster growth rates are associated with higher photosynthetic capacities (Reich *et al.*, 1997; Saied *et al.*, 2003). However, we observed fast-growing leaves with a lower per-area photosynthetic capacity (A_{max}) than larger, slow-growing leaves (Figs. 1, S1, S2). One mechanistic explanation is that these fast-growing leaves reached their final size early, and transitioned from carbon sink to carbon source earlier than slower growing leaves, allowing them to serve as net carbon sources when integrating across ontogeny.

Independently of leaf size, leaf shape can influence leaf temperature (Vogel, 2009), and consequently photosynthetic capacity (Kobza & Edwards, 1987; Lin *et al.*, 2012) and eventual plant performance. To examine leaf shape, we assumed a simplified and ovate form (although there is variation in (1) leaf lobes, (2) petiole wings, and (3) stipules), and utilized a power-law function to model the allometric relationship between leaf length and width as an FVT throughout development (Snell, 1892; Schlichting & Pigliucci, 1998). The function describes the line made by plotting corresponding leaf lengths and leaf widths and is specified by two parameters, *a* and *b* (Eqn 3), which are highly correlated ($-0.92 < r < -0.90$). In our study species and experimental settings, we did not see a significant relationship between either *a* or *b* and any leaf-level physiological parameters (Figs 1, S1, S2). Somewhat unexpectedly, we also observed no significant genetic correlation between parameters describing ontogenetic allometry and any of the developmental parameters associated with leaf length or width (*r*, *d* or *Lmax*) (Figs 1, S1, S2). Consistent this observation, one quarter of additive allometry QTL did not colocalize with any aspect of leaf length or width, and allometry QTL never colocalized with leaf growth rates or durations (Fig. 2; Table S3). These counterintuitive results can be explained mathematically and biologically. Throughout development *b* is influenced by the ratio of *LW_r* to *LL_r*. During early development, *LL* and *LW* approach zero and contribute relatively little to *b*. During later development, it is the ratio of the rate that *LL* and *LW* approach their maximums that influence *b* rather than any individual trait (Notes S1 (Eqns S1–S8)). Biologically this means that if leaf shapes remain constant as leaf sizes increase, the line describing allometry will be straight and have a slope of one. If this were the case, we might expect to see genetic correlations between *a* and *b* and leaf lengths and widths. Instead, leaves of crowded and uncrowded plants both changed shape as they grew larger. Consequently the allometric relationship between leaf length and width is curvilinear, and the correlations between leaf shape, and individual growth rates, durations and final sizes break down.

Our results indicate that leaf allometry constitutes a genetically determined module which is independent from linear leaf

developmental parameters as well as single-time-point measures. In part, this is because allometry model parameters and underlying QTL are associated neither with estimates of leaf size and growth (Raff & Sly, 2000; Edwards & Weinig, 2011) nor many of their underlying QTL. In addition, allometry and linear leaf parameters had strikingly different patterns of correlation with whole plant estimates of phenology and fitness. Leaf length and width parameters were never correlated with either time to bolting or flowering; instead they were genetically correlated with the interval between bolting and flowering. Parameters describing allometry had the exact opposite pattern: they were highly genetically correlated with bolting and flowering, but not with the interval between bolting and flowering (Figs 1, S1, S2). Alternative approaches to defining modularity focus on high degrees of association within modules (von Dassow & Munro, 1999; Bolker, 2000). Indeed, in our study components of allometry are more highly correlated with each other than with other aspects of leaf development. Likewise, linear leaf FVT parameters were also all more highly correlated with each other than with allometry.

Tight integration within modules can be viewed as an evolutionary constraint on traits within that module (Klingenberg, 2010). However, loose genetic associations between modules can also facilitate evolutionary change and novelty (Ackerly & Donoghue, 1998; Wagner *et al.*, 2007). In *B. rapa*, there are multiple potential advantages to disassociating leaf shape and size. For instance, narrower leaves may avoid self-shading (Yocum & McKee, 1970; Reed *et al.*, 1993; Chitwood *et al.*, 2012). By contrast, shorter, more circular leaves have a smaller perimeter-to-area ratio and may be relatively protected from damage that preferentially effects leaf margins such as freezing (Prášil & Zámečník, 1998). Perhaps more importantly, leaf shape may be related to leaf venation (Jones *et al.*, 2009). Because minor veins are responsible for considerable hydraulic resistance and leaf margins are far from main veins, leaf margins wither when exposed to strong winds such as those at our field site (Yapp, 1912; Nicotra *et al.*, 2011). In fact, we observed dry, dead leaf margins on many of our leaves. Because allometry and leaf length and width are genetically independent, it should be possible to select for a less rounded leaf where leaf margins are in closer proximity to the midrib and may suffer less from desiccation without affecting leaf length or width, and therefore without jeopardizing leaf growth rates, sizes or photosynthetic capacity.

In order to more fully understand the genetic architecture underlying leaf development and whole plant dynamics, we tested for epistatic effects. Although we found relatively few epistatic QTL compared to additive QTL, the effects of epistasis were occasionally quite strong. In particular, the interaction between QTL for *UN_a* on chromosomes 7 and 8 explained 14.5% of the total variation in *UN_a*, or about the same amount as any individual additive QTL for *UN_a* (Table S3). Interestingly, although we found epistatic effects for final leaf measures (STP) and estimates (*Lmax*), we did not detect any epistatic effects for aspects of leaf growth or duration (Table 3). Unlike many species where dominance relationships and epistatic effects cannot be directly inherited, selfing or biparental inbreeding in *B. rapa* allows for direct transmission of beneficial epistatic interactions, making them a potential target for selection and crop improvement.

We also tested whether (and which) leaf-level traits were environmentally sensitive and associated with fitness. Unlike other studies (Weiner & Thomas, 1992; reviewed in Schmitt & Wulff, 1993), we found no main effect of treatment. Instead, we found significant genotype-by-environment interactions for all linear leaf traits and fitness traits, but not parameters of allometry or phenology (Table 1). Although QTL-by-environment interactions are typically considered common (reviewed in Des Marais *et al.*, 2013), when we examined the effect of environment on individual QTL, we found significant QTL-by-environment interactions only for fitness traits (Table 2).

In addition to assessing novel aspects of genetic architecture, FVT approaches are also advantageous for looking at natural selection, as they can reduce data from many auto-correlated repeated measures into one (or a few) traits, thereby enabling selection analyses. We found that *r*, *d* and *Lmax* were under selection. In particular, selection favored reduced values for the rate and duration of growth after accounting for selection for *Lmax*. For leaf width, selection on rate and duration was mediated by flowering interval, and selection favored a faster reproductive transition from bolting to flowering. Thus, reproductive developmental rate affects selection on vegetative growth rates and patterns (*r* and *d*). Leaf allometry (*b*) experienced weak selection that was again mediated by a reproductive trait – days to flowering.

Taken together, our results demonstrate that modeling leaf development reveals novel genetic trade-offs, bivariate associations and controls for aspects of leaf development. Similar to other studies, which find that FVT modeling is superior to parametric and repeated-measures analyses for QTL mapping (Griswold *et al.*, 2008; Xiong *et al.*, 2011), we conclude that FVT modeling of developmental trajectories is an improvement upon single estimates of final organ size. We draw on advantages of FVT modeling, such as data reduction and novel trait identification to uncover developmental modules and estimate selection on leaf phenotypes. Yet, other features of FVT modeling are relevant to predicting the evolutionary dynamics of complex traits. The ordered pattern of growth as a function of time allows for estimation of a continuous genetic (co)variance function ($\mathscr{G}$) that describes genetic variance in size and covariances between size at every pair of time points. $\mathscr{G}$ provides increased power to estimate the effect of selection on population means across generations (Stinchcombe & Kirkpatrick, 2012). Future avenues of research include estimating $\mathscr{G}$ and utilizing a Bayesian framework to draw upon the information content of the entire dataset simultaneously.

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Supporting Information

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

Fig. S1 Bonferroni-corrected genetic correlations in the uncrowded treatment for *Brassica rapa* (recombinant inbred lines, RILs).

Fig. S2 Bonferroni-corrected genetic correlations in the crowded treatment for *Brassica rapa* RILs.

Fig. S3 The effects of environment (UN, uncrowded; CR, crowded) and quantitative trait loci (QTL) interactions on fitness in *Brassica rapa* RILs.

Fig. S4 Map of significant epistatic QTL in *Brassica rapa* RILs.

Fig. S5 The magnitude and direction of significant epistatic interactions among QTL in *Brassica rapa* RILs.

Table S1 *Brassica rapa* genotypic means for previously unpublished traits used in this study

Table S2 Standard errors for genotypic means in *Brassica rapa*

Table S3 Location, LOD scores and percentage variance explained for significant additive QTL in *Brassica rapa* RILs

Table S4 Selection analyses for the crowded treatment in *Brassica rapa* RILs

Notes S1 Equations describing the derivation of the allometric function, demonstrating that neither *a* nor *b* need necessarily be correlated with either leaf length or width parameters.

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