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Original Article

Plasticity to simulated shade is associated with altitude in structured populations of *Arabidopsis thaliana*

Javier F. Botto

IFEVA, Facultad de Agronomía, Universidad de Buenos Aires y Consejo Nacional de Investigaciones Científicas y Técnicas, Av. San Martín 4453, C1417DSE, Ciudad de Buenos Aires, Argentina

ABSTRACT

Plants compete for photosynthesis light and induce a shade avoidance syndrome (SAS) that confers an important advantage in asymmetric competition for light at high canopy densities. Shade plasticity was studied in a greenhouse experiment cultivating *Arabidopsis thaliana* plants from 15 populations spread across an altitudinal gradient in the northeast area of Spain that contain a high genetic variation into a reduced geographical range. Plants were exposed to sunlight or simulated shade to identify the range of shade plasticity. Fourteen vegetative, flowering and reproductive traits were measured throughout the life cycle. Shade plasticity in flowering time and dry mass was significantly associated with the altitude of population origin. Plants from coastal populations showed higher shade plasticity indexes than those from mountains. The altitudinal variation in flowering leaf plasticity adjusted negatively with average and minimum temperatures, whereas dry mass plasticity was better explained by negative regressions with the average, maximum and minimum temperatures, and by a positive regression with average precipitation of the population origin. The lack of an altitudinal gradient for the widest number of traits suggests that shade light could be a driver explaining the distribution pattern of individuals in smaller geographical scales than those explored here.

Key-words: altitudinal gradient; light; local adaptation; phenotypic plasticity; phytochromes; shade avoidance syndrome.

INTRODUCTION

Plants need resources such as water, nutrients and light to grow. Under dense vegetation, light is a limited resource and competition for light can strongly influence the success of a plant (Pierik & de Wit 2013). Plants have evolved sophisticated mechanisms mediated by phytochromes that allow them to detect the early presence of neighbouring plants and to initiate developmental adaptive strategies that avoid shading before the canopy is closed (Ballaré *et al*. 1990). The most significant changes in the red/far-red (R/FR) ratios occur when daylight is reflected or transmitted by green vegetation. Absorption of red (R) and blue photons by chlorophyll and carotenoids results in a selective enrichment of far-red (FR) photons, reducing the R/FR ratios perceived by the plant tissues. As a result of changes in the light spectrum, plants display the shade avoidance syndrome (SAS), a set of physiological responses that increase vegetative structures such as stems, petioles and hypocotyls, accelerate flowering, and reduce seed number and size (Casal 2012).

In *Arabidopsis thaliana* and other species, phytochrome B (phyB) is the main phytochrome, and phyD and phyE contribute secondarily, mediating the SAS. In open environments, the Pr, the inactive form of the phytochromes located in the cell cytoplasm, migrates to the nucleus when it absorbs photons of R light and phototransforms to Pfr. In the nucleus, the accumulated Pfr form interacts and degrades PIFs (phytochrome interacting factors) through the proteosome, leading to growth inhibition by the deactivation of gene expression (Lorrain *et al*. 2008). In opposition, the shade light converts Pfr to Pr form that no longer interacts with PIFs. These proteins will thus rapidly re-accumulate, promoting the expression of early shade genes such as *PIL1*, *ATHB2*, *HFR1* and *PAR1* inducing cell elongation responses (Lorrain *et al*. 2008; Hornitschek *et al*. 2012). In addition, the full expression of SAS requires other photomorphogenic regulators such as COP1 (McNellis *et al*. 1994; Pacín *et al*. 2013), SPA (Rolauffs *et al*. 2012) double B-Box proteins (Crocco *et al*. 2010; Gangappa *et al*. 2013) and bHLH/HLH transcription factors (Hao *et al*. 2012).

The hypothesis of adaptive plasticity predicts that the phenotype of shade avoidance induced by low R/FR ratios has a better fitness in dense canopies but is penalized at low densities (Schmitt *et al*. 1995). Because the light is a critical resource for plants, the SAS confers an important advantage in asymmetric competition for light at high densities. However, in the absence of competition, allocation of resources to height at the expense of leaves, roots and branches may reduce growth and reproduction, and elongated stems may have a greater risk of mechanical damage (Casal & Smith 1989; Schmitt & Wulff 1993). Natural variation is a prerequisite for the evolution of phenotypic plasticity (Via & Lande 1985) Nucleotide polymorphims at photoreceptor genes underlie natural variation for plant light responses (references). (Aukerman *et al*. 1997; El-assal *et al*. 2001; Maloof *et al*. 2001; Balasubramanian *et al*. 2006). PhyB

Correspondence: J. F. Botto. Fax: (54-11)4514-8730; e-mail: botto @agro.uba.ar

is the principal photoreceptor responsible for red light and shade avoidance responses and is proposed to be the gene responsible for several QTL found when plants grow under shade (Borevitz *et al*. 2002; Botto *et al*. 2003; Botto & Coluccio 2007; Kasulin *et al*. 2013). By the analysis of the phyB sequence in 33 *A. thaliana* accessions, Filiault *et al*. (2008) found 14 non-synonymous polymorphisms with at least one of them responsible for the phenotypic variation observed in seedlings exposed to red light. PIF4 polymorphisms are also supposed to be associated with internode length of inflorescence and reproductive timing and fitness under shade (Brock *et al*. 2010). Natural variation at *ELF3*, a gene involved in the circadian clock, was clearly associated with a function for shade avoidance in *A. thaliana*. In fact, a single amino acid change in the *ELF3* gene is responsible for the natural variation mediating cell elongation growth (Coluccio *et al*. 2011) and flowering time (Jiménez-Gómez *et al*. 2010) between two contrasting accessions originated in Bayreuth (Bay, Germany) and Shahdara (Sha, Tajikistan).

Genetic diversity and structure analysis in more than 6000 wild genotypes from different world regions, at global and regional scales, suggest several major events in *A. thaliana* demographic history in Europe (Nordborg *et al*. 2005; Platt *et al*. 2010). In particular, high diversity has been described in the Mediterranean Peninsulas compared with Central and Northern Europe (Beck *et al*. 2008; Picó *et al*. 2008). The largest diversity has been found in the Iberian Peninsula, whose strong geographical structure has prompted the hypothesis of multiple Iberian glacial refuges with differential contribution to the colonization of Europe (Picó *et al*. 2008). The structure of northeastern Iberian populations is important and contains huge genetic variation across an altitudinal gradient, suggesting that they may be locally adapted (Montesinos-Navarro *et al*. 2009, 2011; Wolfe & Tonsor 2014). These *A. thaliana* populations grow in two contrasting climatic environments: maritime lowland coastal area characterized by cool temperatures and moderate rainfall in the winter, low rainfall and high maximum temperatures in spring and summer; and mountainous areas with higher rainfalls and lower minimum temperatures in winter and a prolonged cool and wet spring. Interestingly, Montesinos-Navarro *et al*. (2011) found that the phenotypes of these populations are associated with a climatic gradient defined by altitudinal clines. Working with northeastern Iberian populations, Tonsor's group showed that biomass, leaf number, flowering time and seed weight increase, whereas translocation of resources to the root, vegetative growth and number of seeds decrease with the altitude of the population origin. These life strategies favour the selection of individuals for rapid life cycle in Mediterranean regions near the sea, avoiding typical warm dry summer periods, and long life cycles in individuals growing in the mountains that help to maximize growth, cold tolerance in the winter and late flowering.

The eco-physiological basis of the shade plasticity variation remains obscure. Some studies have found a significant correlation between light sensitivity and the latitude of accession location, suggesting that light phenotypic variation could be a results of genotype adaptation to a latitudinal gradient (Maloof *et al*. 2001; Stenøien *et al*. 2002; Kasulin *et al*. 2013). Stenoien *et al*. (2002), working with 10 Norwegian populations of *A. thaliana* collected in a narrow geographical range, found a latitudinal cline in response to light: the northern genotypes are more responsive than southern populations to R or FR continuous light during seedling de-etiolation. Furthermore, the hypocotyl elongation response to an FR pulse at the end of the day, a laboratory treatment that simulates shade avoidance, was positively associated with the increase of latitude for European accessions collected between 15° and 65° (Kasulin *et al*. 2013). However, in other studies, hypocotyl and flowering shade response correlations with latitude were missing (Botto & Smith 2002; Filiault & Maloof 2012).

The strong structure of northeastern Iberian populations, containing a wide range of genetic diversity within a narrow geographical range, is an ideal system for testing hypotheses associated with the SAS. To have a better understanding about the drivers of shade avoidance plasticity, we designed a greenhouse experiment using the northeastern Iberian populations of *A. thaliana*. We evaluated the range of variation of vegetative, flowering and reproductive traits in response to simulated shade to answer the following questions:

1 What is the range of phenotypic variation to R/FR ratios in structured populations?
2 Is the expression of shade plasticity traits associated with an altitudinal gradient?
3 If the previous question is yes, what are the climatic drivers explaining this variation?

MATERIALS AND METHODS

Genetic material

Sixty genotypes from 15 populations of *A. thaliana* originated from the Northeast area of Spain were used in this study. These populations were collected in different locations defined by an altitudinal gradient (Montesinos-Navarro *et al*. 2009). In this area, the rainfall increases and high spring temperatures and minimum winter temperatures decrease with the altitude (Montesinos-Navarro *et al*. 2011).

Culture conditions and light treatments

Seeds were sown in transparent plastic boxes on a 0.8% agar solution. The boxes were placed in darkness at 5 °C for 1 week to break dormancy. After that, the boxes with seeds were placed in a chamber with continuous white light for another week to induce uniform germination and the development of seedlings with well-developed green cotyledons and radicle. Then, seedlings were vernalized for 2 weeks in a light chamber under non-inductive short-day conditions (8 + 16 h light, dark) at 5 °C before transplanting. The seedlings were transplanted on 27 August 2011 to 7 × 4 cm pots (height × diameter) with a substrate of vermiculite, perlite and peat in a ratio of 30:30:10. After that, the plants were grown with natural radiation and controlled temperature in a

greenhouse at IFEVA, Faculty of Agronomy, University of Buenos Aires (34°35′S, 58°29′W), Buenos Aires, Argentina. The pots with plants were watered with Hoagland's solution (20 mL of Hakaphos Compo Red solution in 5 L of water).

After a week of transplanting, the plants were exposed to sunlight or simulated shade, a treatment that consisted of sunlight plus lateral FR light mimicking neighbouring plants (Rondanini *et al*. 2014). The FR light was provided by two banks equipped with nine incandescent reflector lamps of 40 W each and a red acetate filter with two filters of blue acrylic Paolini (1 m long × 0.25 m wide) of 2 mm thick placed in front of the plants. To avoid the increase of temperature by the lamps, transparent bottles with water were placed between lamps and filters along with two fans that allowed ventilation. Plants grown in simulated shade received R/FR ratios ranging from 0.07 to 0.12 (Supporting Information Table S1). Two lines of plants were located in front of the Paolini filters. The pots with plants were rotated every week to randomize light differences into the treatment. The sunlight treatment consisted of a similar experimental design without the addition of FR light. The lateral R/FR ratio was 0.65 (Supporting Information Table S1). The sunlight on the top of the plants was similar between both light conditions: the average photosynthetically active radiation (PAR) was nearly 500 μmol m^{-2} s^{-1} at noon for sunny days and the R/FR ratios ranged between 0.94 and 1.02 (Supporting Information Table S1). During the experiment, the daily average temperature ranged between 20.5 and 30.4 °C (Supporting Information Fig. S1). PAR and R/FR ratios were measured with a model Spectroradiometer SPECTROSENSE2 / 2 + Meter, Skye Instruments Ltd. (Powys, UK). The temperature was measured with a digital maximum and minimum thermometer (TFA, Wertheim-Reicholzheim, Germany).

Traits and shade plasticity index

Fourteen vegetative, flowering and reproductive traits were measured during the experiment: length and width of leaf, petiole length, leaf angle, rosette diameter and height, flowering time as the number of leaves or days at flowering, length and diameter of primary axis, number of basal axes, number of secondary axes on the primary inflorescence, seed weight (100 seeds) and above-ground dry mass. The leaf angle was taken in the first and second week after starting the light treatments with a goniometer consisting of a protractor and a weight to mark the normal. The angle formed between the normal and the tallest petiole leaf was estimated. Vegetative traits were measured every week during the 28 d after the beginning of light treatments. Above-ground dry mass was assessed at the end of the trial (23/12/11) by placing the harvested aerial parts (including flowering axes) in an oven at 80 °C for 72 h and then the dried material was weighed with a precision balance. Seed yield, as the total weight of seeds, was not included in the analysis because heat stress increased towards the end of the experiment (Supporting Information Fig. S1), producing higher flower mortality at the extreme of inflorescences in the late flowering individuals compared with the earlier flowering individuals.

A shade plasticity index for each individual and trait was estimated as the difference between sunlight and simulated shade relative to sunlight as follows:

$$\text{Shade plasticity index} = 1 + [(\text{Simulated shade} - \text{Sunlight})/\text{Sunlight}]$$

Shade plasticity indexes higher than 1 indicate that simulated shade increases the response, and values lower than 1 indicate that simulated shade reduces the response with respect to sunlight. Values close to 1 mean that the individuals have low shade plasticity in contrast with higher or lower indexes, which means that the individuals display strong shade plasticity.

Experimental design and statistical analysis

The experimental design was a randomized-block factorial design of two factors: population (P) consisted of 15 populations distributed along an altitudinal gradient, and light (L) consisted of two light conditions: sunlight and simulated shade. For each light condition, eight replicates for population were established and each population was represented by four genotypes. Data were statistically analysed by two-factor ANOVA including P, L, P × L and B (block) factors. Paired comparisons by Bonferroni test were included when the P × L interaction factor was significant. Genetic correlations for vegetative and reproductive traits within and between light treatments were estimated. Univariate regression analyses were performed to evaluate clinal population differentiation between shade plasticity indexes and altitude or climatic parameters.

Because the SAS includes several morphological and developmental traits rather than any single factor, multivariate analysis was performed. The suite of SAS traits was treated as a group testing the effect of light of all the measured traits ($F = 14.57$, $P < 0.0001$). The overall clinal population differentiation between shade plasticity indexes and altitude was evaluated using a multivariate analysis of variance (MANOVA) of all the measured traits as dependent variables and altitude of population origin as the independent variable ($F = 1.96$, $P < 0.0001$). In addition, a principal component analysis (PCA) was conducted to represent the complexity of data matrix in two principal axes. All measured traits for each individual were included in the analysis as dependent variables. For graphical representation, population and light were introduced as classification factors. Statistical analyses were performed using the statistical program Infostat (http://www.infostat.com.ar/).

RESULTS

Time-course responses to simulated shade for vegetative traits

Six vegetative traits (length and width of lamina, petiole length and leaf angle, and diameter and height of rosette) were measured during the four weeks after the beginning of light treatments to study the effect of simulated shade on the

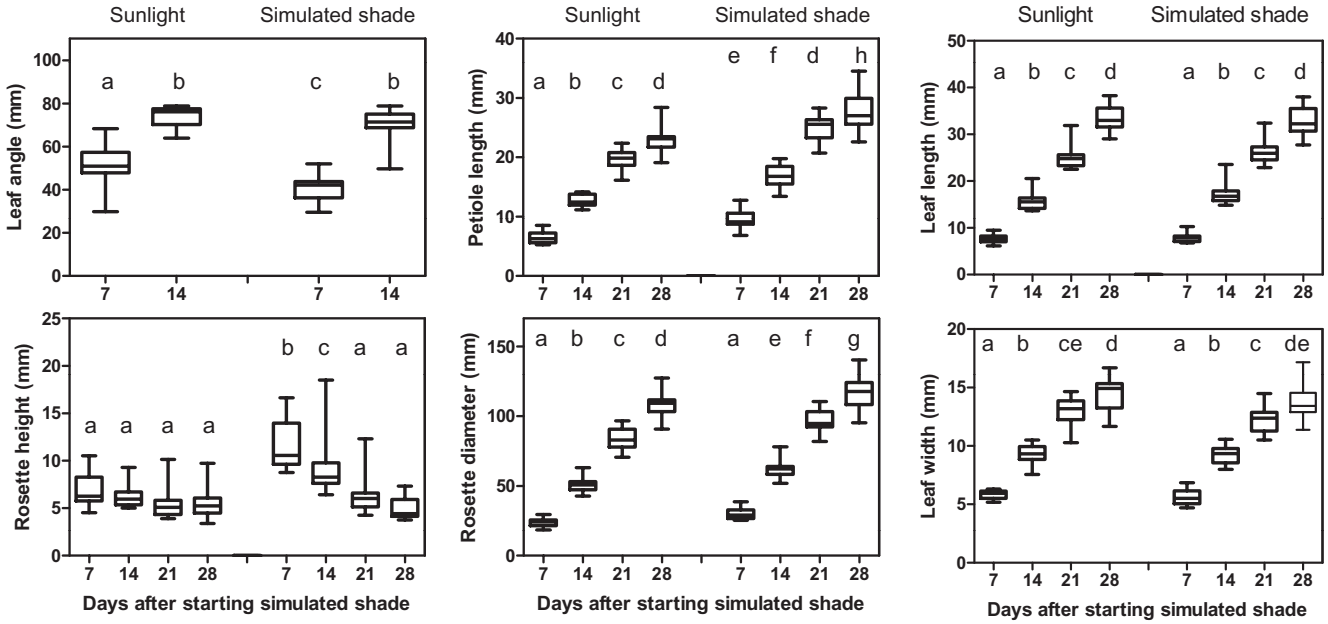

Figure 1. Time-course response to simulated shade for vegetative traits. Response to sunlight and simulated shade was calculated as the average of 15 Iberian populations at each date. The lines outside the box plot graphs indicate the minimum and maximum values for sunlight and simulated shade between 7 and 28 d after the starting of light treatments. Means were compared by Tukey's test ($P < 0.05$) after one-way ANOVA.

time-course of vegetative growth. To increase the robustness of the analysis, the average response was estimated for the 15 populations in each light condition and date. The time-course growth was affected by simulated shade in four traits: leaf angle, petiole length, and height and diameter of rosette (Fig. 1). Simulated shade increased the erect position of the young leaves and rosette height at the starting of the experiment (Fig. 1, first and second weeks). Furthermore, the shade light increased significantly the petiole length and rosette diameter during the first month of the experiment (Fig. 1). The length and the width of the leaves also increased systematically during the first month but no significant differences were found between light treatments (Fig. 1).

Reaction norms to simulated shade

The average expression of the six vegetative traits with the exception of leaf length differed significantly among populations and light treatments (Fig. 2, Supporting Information Table S2, see P and L factors). Simulated shade altered the vegetative phenotype in different intensities increasing the petiole length and the rosette height, and reducing the leaf angle with respect to the normal. In addition, simulated shade reduced marginally the leaf width and increased the rosette diameter in most of the populations (Fig. 2 and Supporting Information Table S3). No significant effects were detected for the population by light interaction factor in any of the six vegetative traits (Fig. 2 and Supporting Information Table S2).

Flowering was affected by population and light factors. As expected, the simulated shade accelerated flowering. The light factor was more sensitive for the number of leaves than for the number of days at flowering (Fig. 3 and Supporting Information Table S2). The population by light interaction effect was not significant for leaves and days at flowering. After flowering, six reproductive traits were measured. Population and light effects were significant for the number of basal axes, length of the principal axis, above-ground dry mass and seed weight. The population by light interaction factor was significant for the length of principal axis and seed weight (Fig. 3 and Supporting Information Table S2). In these traits, plants from the ARU population showed significantly longer axes under simulated shade compared with sunlight (45.6 cm versus 31.4 cm, respectively), and the individuals from the VDM population produced significantly lighter seeds under sunlight compared with simulated shade (2.7 mg/100 seeds versus 3.6 mg/100 seeds, respectively). These differences disappeared in other populations, suggesting different light sensitivities to the same light signal (Supporting Information Table S3). Although the population factor was significant for the principal axis diameter and the number of secondary axes, the light factor did not significantly affect these reproductive traits (Fig. 3 and Supporting Information Table S2).

Genetic correlations for traits within and between light treatments

Least-squares means of vegetative, flowering and reproductive traits were used to estimate Pearson's product correlations and genetic variance-covariance matrices within each light environment. Independently of the light factor, stronger positive correlations were found among leaf length with other vegetative traits such as leaf width, petiole length and

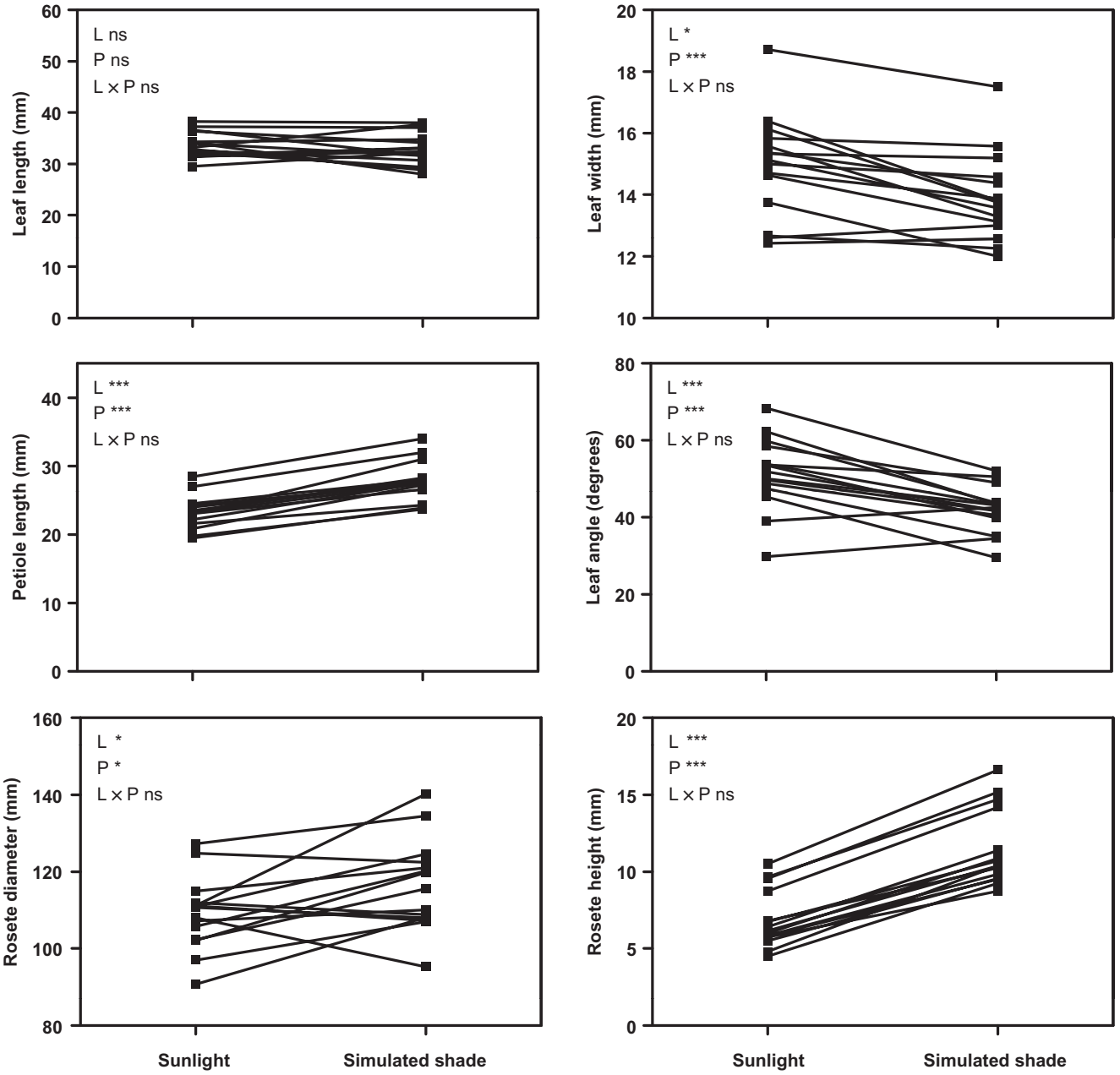

Figure 2. Reaction norms to simulated shade for vegetative traits. Each point represents the average response to sunlight and simulated shade for each population. On the top of each graph is indicated the output of the ANOVA for each independent variable (L = light, P = population) and the interaction between L × P. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. ns, not significant.

rosette diameter, and also between flowering time (leaves or days) with leaf angle, number of secondary axes and above-ground dry mass (Table 1). Some positive correlations were only found in plants cultivated in simulated shade. For example, plants with higher above-ground dry mass produced wider flowering axes and heavier seeds (Table 1).

A lower number of negative correlations were also found. For both light conditions, the rosette height showed a negative correlation with flowering (days or leaves) and inflorescence length (Table 1). Interestingly, the rosette height was negatively correlated with fitness traits such as dry biomass and seed weight, specifically in plants cultivated under simulated shade (Table 1).

Population origin is the principal driver of the phenotypic variation

Multivariate analysis was applied for all the measurements of individuals corresponding to 15 populations and 14 traits in sunlight and simulated shade. The principal component

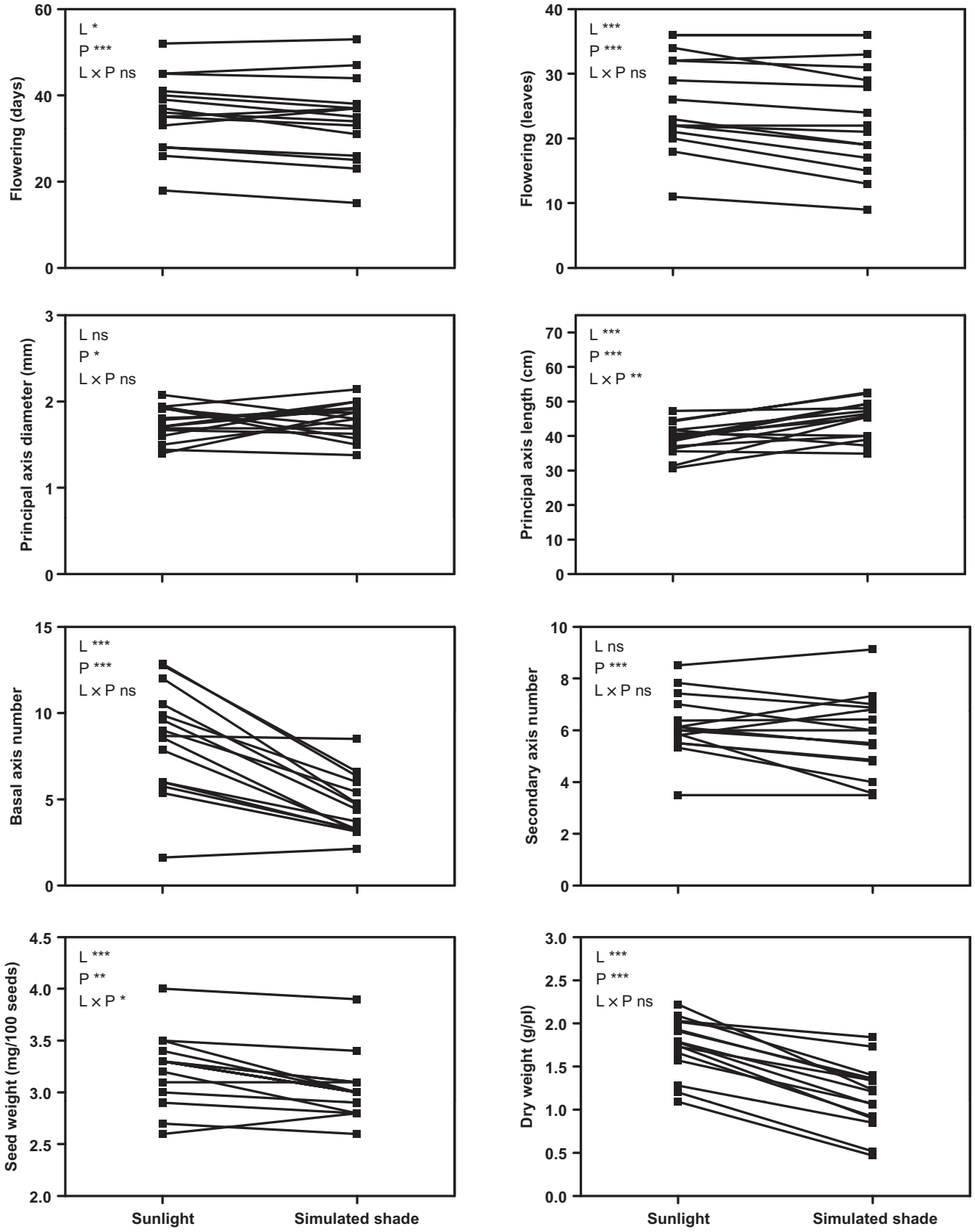

Figure 3. Reaction norms to simulated shade for flowering and reproductive traits. Each point represents the average response to sunlight and simulated shade. On the top of each graph is indicated the output of the ANOVA for each independent variable (L = light, P = population) and the interaction between L × P., $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. ns, not significant.

Table 1. Genetic correlation matrix of vegetative, flowering and reproductive traits from *Arabidopsis thaliana* plants exposed to sunlight and simulated shade

	LL	LW	PL	LA	RD	RH	FD	FL	PAL	PAD	SAN	AN	DW	SW
LL	–	0.55***	0.51***	−0.16	0.78***	0.4***	−0.23*	−0.08	−0.18	0.2*	−0.11	0.31**	0.42***	0.15
LW	0.69***	–	0.37***	−0.09	0.44***	0.16	0.001	0.03	−0.19	0.19*	−0.01	0.12	0.16	0.2
PL	0.64***	0.59***	–	−0.06	0.58***	0.05	0.1	0.14	−0.21*	0.12	0.009	0.31**	0.28**	0.07
LA	−0.08	−0.01	0.05	–	−0.13	−0.58***	0.34***	0.39***	0.1	−0.02	0.28***	−0.31***	0.03	0.13
RD	0.91***	0.71***	0.82***	−0.05	–	0.29**	−0.04	0.06	−0.15	0.15	0.004	0.22*	0.42***	0.1
RH	0.06	0.09	−0.16	−0.46***	−0.03	–	−0.57***	−0.48***	−0.2*	−0.04	−0.31***	0.27**	−0.01	−0.08
FD	−0.13	−0.11	0.06	0.38***	−0.06	−0.47***	–	0.9***	0.17	0.15	0.66***	−0.34**	0.34***	0.3*
FL	−0.02	−0.04	0.12	0.4***	0.04	−0.43***	0.92***	–	0.18	0.25**	0.67***	−0.26**	0.42***	0.35***
PAL	0.04	−0.1	0.14	0.17	0.11	−0.28**	0.16	0.21*	–	0.22*	0.25**	−0.11	0.21*	−0.08
PAD	0.13	0.13	0.3**	0.09	0.25**	−0.19	0.24*	0.3**	0.4***	–	0.31**	−0.06	0.018	0.03
SAN	−0.08	−0.01	0.13	0.09	0.01	−0.11	0.55***	0.54***	0.2*	0.46***	–	−0.23*	0.34***	0.26*
AN	0.2	0.13	0.28**	−0.02	0.25**	−0.04	−0.1	−0.1	0.04	0.02	−0.22*	–	0.28**	−0.15
DW	0.1	0.09	0.14	0.19	0.17	−0.35***	0.68***	0.68***	0.28**	0.34***	0.39***	0.19	–	0.18
SW	−0.17	−0.19	−0.16	0.44***	−0.15	−0.49***	0.35**	0.35**	0.29*	0.17	0.22	0.09	0.39***	–

Sunlight (above the diagonal) and simulated shade (below the diagonal). Significant correlations are indicated by *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).
AN, basal axe number; DW, above dry weight; FD, flowering in days; FL, flowering in leaves; LA, leaf angle; LL, leaf length; LW, leaf width; PAL, principal axe length; PAD, principal axe diameter; PL, petiole length; RD, rosette diameter; RH, rosette height; SAN, secondary axe number; SW, seed weight.

Figure 4. Multivariate analysis for all the traits from plants of 15 populations cultivated in sunlight and simulated shade. Principal component analysis (PCA) was conducted to reduce the complexity of the data matrix in two eigenvectors. All the measured traits for each individual were included in the analysis as dependent variables and population and light as classification factors. Numbers indicate populations as 1: PIN, 2: RAB, 3: SAL, 4: BAR, 5: HOR, 6: ARU, 7: COC, 8: BOS, 9: MUR, 10: VDM, 11: ALE, 12: PAL, 13: BIS, 14: VIE, and 15: PAN. For additional references on traits names, see Table 1.

analysis (PCA) reduced the variability of the data in a lower dimensional space than the original space of variables. The first and the second dimensions of the PCA (CP1 and CP2) explained 34 and 19.6% of the data variability (Fig. 4). The first axis ordered the cases following a pattern that was very similar to the geographical gradient of the population origin. The biplot representation allowed the identification of the variables (arrows in the graph) that determine the location of the cases in this gradient: with some exceptions, vegetative traits and coastal populations were grouped together on the left side of the first axis, and flowering and reproductive traits together with populations originating from the mountains were grouped on the other side. Light was the secondary factor explaining the observations principally on the second axis. On the upper side of the graph appeared the cases associated with plants cultivated in simulated shade while in the bottom were grouped the cases of plants exposed to sunlight with the exception of BOS population BOS population (Fig. 4, 8:shade).

Shade plasticity for flowering and dry mass is associated with an altitudinal cline

The shade plasticity was estimated in six vegetative and eight flowering and reproductive traits for each population as the

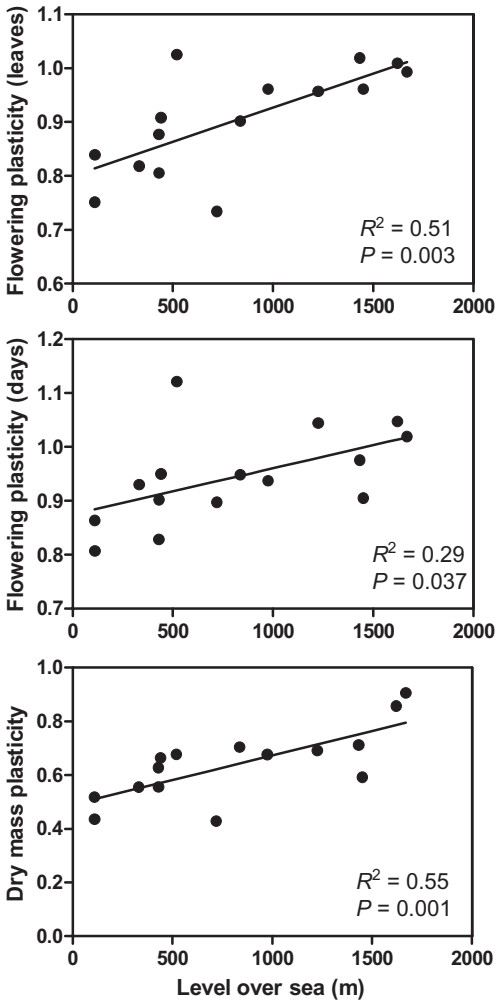

Figure 5. Clinal variation associated with altitude for flowering and above-ground mass plasticity to simulated shade. Fitting regression lines are presented with R^2 and P values indicating significant regression with respect to zero. Each point represents the average plasticity response for each population estimated as the difference between simulated shade and sunlight relative to sunlight. Values closed to 1 mean that the population has null or low plasticity in opposition to higher or lower indexes than 1 indicating that populations display strong shade plasticity.

average response of the individuals. To test whether the shade plasticity is associated with the altitude of the place of population origin, regression analyses were performed for each trait. In those traits associated significantly with an altitudinal pattern, climatic parameters were examined in order to explain this variation. The number of leaves or days at flowering and above-ground dry mass plasticity indexes showed a clear and significant regression with the altitude of origin (Fig. 5). The distribution of shade plasticity indexes was adjusted to a regression line that differed from the horizontal (Fig. 5, $P = 0.003$ for leaves at flowering, $P = 0.037$ for days at flowering and $P = 0.001$ for above-ground dry mass). Individuals from coastal areas showed a higher plasticity to shade than those plants from mountain locations that displayed null or reduced shade plasticity (Fig. 5). Shade plasticity indexes for other vegetative and reproductive traits were not associated with the altitude of population origin (Supporting Information Figs S2 & S3). Furthermore, flowering leaf plasticity index showed a significant regression with average and minimum temperatures (Fig. 6), but not with maximum temperature neither average precipitation of the place of population origin (Supporting Information Fig. S4). In other words, individuals from coastal areas that experience higher average and minimum temperatures showed higher flowering shade plasticity than those individuals from mountains sites. The shade plasticity of above-ground dry mass showed a negative regression with temperatures (average, minimum and maximum) and a positive regression with the average precipitation (Fig. 6), but not with the distribution of precipitations in autumn and spring (Supporting Information Fig. S4). It means that higher dry mass plasticity index was associated with plants from coastal areas growing with higher temperatures and lower average precipitation than those from mountainous areas. No significant regressions were found for the flowering day plasticity index and the six climatic parameters evaluated (Supporting Information Fig. S5).

DISCUSSION

Shade response in vegetative, floral and reproductive traits was studied in 15 populations of *A. thaliana* spread across an altitudinal transect in the northeastern area of Spain. These populations contain a high genetic diversity and are characterized by a strong population structure (Picó *et al.* 2008; Montesinos-Navarro *et al.* 2011). Vegetative traits such as petiole length, leaf width, diameter and height of rosette, and angle of insertion of the leaf were significantly affected by changes in R/FR ratios. The simulated shade produced plants with narrower lamina, larger petioles and rosette diameter, and also more erected leaves at early developmental stages compared with those plants cultivated under sunlight (Figs 1 and 2). In other species close to *Arabidopsis*, like rapeseed plants, it has also been observed that low R/FR ratios induce dramatic shade avoidance responses. Shade signals increase the leaf length but not the leaf width, and produce elevated leaf angles in early stages of the development of a spring rapeseed hybrid (Rondanini *et al.* 2014). It is well known that the low R/FR ratios of the reflected light provide early warnings of the presence and proximity of neighbouring plants, allowing the initiation of development adaptive strategies to avoid shading before the canopy is closed (Ballaré *et al.* 1990). Furthermore, a huge natural variation for shade avoidance responses was documented in a representative panel of *Arabidopsis* accessions (Botto & Smith 2002). Interestingly, natural variation at the *ELF3*, a circadian clock gene, is responsible for the shade avoidance variation for hypocotyl length elongation, leaf angle movement (Coluccio *et al.* 2011) and flowering response (Jiménez-Gómez *et al.* 2010) between Bay and Sha accessions of *A. thaliana*. The altered shade elongation response and leaf movement in Sha accession was associated to a rare alanine by valine

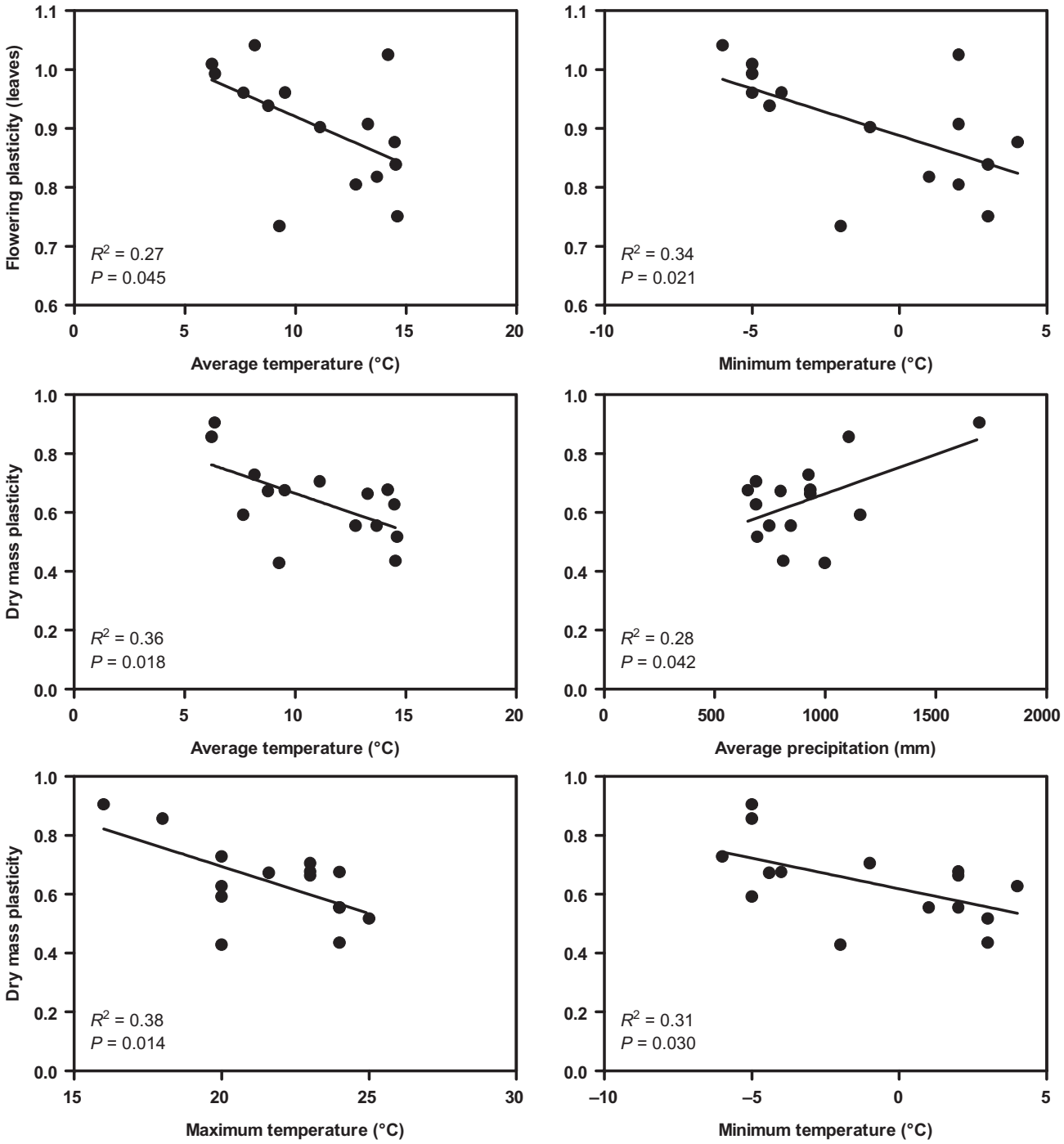

Figure 6. Climatic parameters associated with clinal variation for leaf flowering and above-ground mass plasticity to simulated shade. Plasticity traits are represented as function of the climatic parameters associated to each population. Fitting regression lines are presented with R^2 and P value indicating significant regression with respect to zero. For other references, see Fig. 5.

substitution that alters ELF3-Sha circadian rhythms of leaf movements and clock gene expression (Coluccio *et al*. 2011; Anwer *et al*. 2014).

Shade light accelerates flowering response in *A. thaliana* plants. Some studies show that the number of leaves at flowering is a more sensitive trait than bolting time (Fig. 3, Callaghan and Pigliucci, 2002; Botto & Coluccio 2007). In *Arabidopsis*, low R/FR ratios accelerate flowering by enhancing the expression of *FLOWERING LOCUS T* (FT), the gene involved in the induction of flowering by long days (Halliday *et al*. 2003). Callaghan and Pigliucci (2002) found that flowering time was accelerated by shade under field conditions but not when *Arabidopsis* plants where grown in a greenhouse with the presence of grass neighbours. However,

working with a wide range of natural variation, flowering time in response to low R/FR ratios was accelerated either when plants were cultivated in a light chamber (Botto & Smith 2002) or in a greenhouse (Botto & Coluccio 2007).

Reproductive traits were also affected by shade. Low R/FR ratios produced taller inflorescences and reduced the number of flowering axes, plant biomass and seed weight (Fig. 3). The effect of light on the inflorescence length was dependent upon the population. Interestingly, the ARB population produced longer axes under simulated shade ($P \times L$ interaction, $P = 0.0022$) but these differences disappeared in other populations, suggesting that shade sensitivity depends upon the origin of the population. Although cell elongation is stimulated by shade, Brock *et al*. (2010) found that most of the accessions of *A. thaliana* cultivated in low density in a greenhouse developed taller inflorescences than those growing in crowded stands. The authors interpreted these odd results as a limitation of translocation resources from leaves to fruits. Furthermore, branching is inhibited by low R/FR ratios. Loss of phyB function leads to a reduced branching by a down-regulation of the expression of auxin genes (Su *et al*. 2011; Reddy & Finlayson 2014). Molecular and pharmacological assays suggest that the active form of phyB suppresses auxin signalling to promote branching (Reddy & Finlayson 2014). Furthermore, in the present study, the simulated shade reduced above-ground dry mass and seed weight (Fig. 3), and these results are in accordance with previous evidence demonstrating that environments with resource limitation, such as low R/FR ratios, reduce plant growth and productivity (Sultan 2000).

It is well known that climatic variables have important consequences for the geographical distribution of individuals and species. Shade plasticity indexes of flowering time and above-ground dry mass were significantly associated with the altitude of collection place (Fig. 5). However, other plasticity indexes of vegetative and reproductive parameters did not show clinal variation associated with the altitude (Supporting Information Figs S2 & S3). Interestingly, plants from coastal populations showed higher plasticity to shade than mountainous populations, suggesting different light sensitivities according with the population origin. In fact, low R/FR ratios accelerated flowering and reduced the plant biomass more dramatically in coastal populations than in mountainous populations. Furthermore, some climatic parameters were significantly associated with shade plasticity indexes of some traits. For example, the flowering leaf index showed a significant correlation with the average temperature and the minimum temperature of population origin, whereas the shade plasticity index for dry mass was better explained by the pattern of variation in temperatures and the average precipitation (Fig. 6). Northeastern Iberian populations of *A. thaliana* show strong demographic and genetic patterns defined by the altitude of origin, with mountain populations less genetically diverse than coastal populations (Montesinos-Navarro *et al*. 2009; Picó 2012). The drivers of this altitudinal cline are associated with colder winter temperatures and wetter and longer springs in mountain areas (Montesinos-Navarro *et al*. 2009; Gomaa *et al*. 2011). Accordingly, the patterns of evolutionary diversification in these structured populations can be influenced by the plasticity to light. As predicted by the ecological theory, adaptation through natural selection will not occur as readily for genetically distinct coastal populations because individuals are more plastic and produce phenotypes more appropriate to different local environments. Conversely, mountain ecotypes, in which individuals express limited plasticity, would be predicted to show greater response to local selection regimes and therefore greater genetic divergence (Sultan 2000).

The altitudinal patterns in shade plasticity found for flowering leaf and biomass were obtained from plants cultivated in optimal growth conditions. We should be cautious in generalizing the conclusion of this work to other suboptimal environmental conditions. In fact, the expression of the shade avoidance plasticity can be limited by microenvironmental variation in water availability in seedlings from natural populations of *Impatiens capensis* (Huber *et al*. 2004). The authors found that local seedling density was a poor predictor of selection on shade avoidance traits as a consequence of the unpredictability of water availability, particularly in dry microsites that may affect the costs and benefits of expressing shade avoidance (Huber *et al*. 2004).

The results of this work illustrate clearly that the shade plasticity for flowering and dry biomass shows clinal variation associated with altitude in structured populations of *Arabidopsis* originating in the northeast area of Spain. Ecotype differences in response to shade signals were also documented for *Stellaria longipes* adapted to two ecological environments. In concordance with the results shown here, the prairie ecotype responds quickly to low R/FR ratios elongating their ramets as an adaptation to growth in dense vegetation stands, in contrast to the alpine ecotype that displays dwarf phenotypes with resistance to wind but unresponsive to shade signals allowing adaption to areas of sparse vegetation where abiotic stresses predominate (Sasidharan *et al*. 2008). Furthermore, the shade light, changing also across a micro-environmental context, may be a driver explaining the distribution patterns of individuals in smaller geographical scales than those explored here. New experimental approaches should be undertaken to test this hypothesis. To evaluate this idea, it is necessary to work with a bigger collection of populations correctly described both in their geographical positions and types and environments (woodland, scrubland, anthropic, prairie, etc.), as well as having detailed descriptions of the environmental conditions of the collection site (radiation, light quality, etc.). The atlas of ecological and climatic information along with genetic databases of each individual and their corresponding phenotype may help to identify the underlying genes that express the enormous plasticity documented in the SAS. Deciphering the genetic and molecular basis of phenotypic plasticity is a challenge to understand how plants function, and it is essential to understand the evolutionary forces operating in the adaptation of species to a changing environment (Alonso-Blanco & Méndez-Vigo 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Average, minimum and maximum temperature during the experiment.

Figure S2. Shade plasticity in vegetative traits regressed with altitude.

Figure S3. Shade plasticity for flowering and reproductive traits regressed with altitude.

Figure S4. Climatic parameters not associated with clinal variation for leaf flowering and above-ground dry mass to simulated shade.

Figure S5. Climatic parameters not associated with clinal variation for flowering day to simulated shade.

Table S1. PAR (400–700 nm), R (650–670 nm), FR (720–740 nm), Blue (460–480 nm) irradiance and R/FR ratios measured at 2 and 5 weeks after the starting of the light treatments.

Table S2. ANOVA testing the effect of population and light (sunlight versus simulated shade) for vegetative, flowering and reproductive traits for 15 structured populations of *A. thaliana*.

Table S3. Mean, SD and n values for each trait and light condition in each population.