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Light-sensitive plasticity genes in *Arabidopsis thaliana*: Mutant analysis and ecological genetics

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ABSTRACT

To explore how altered plasticity genes affect whole-plant phenotypes and plant fecundity, we conducted an experiment with isogenic lines of *Arabidopsis thaliana*, representing a wild type and seven photomorphogenic mutants. We exposed all genotypes to a 3×3 factorial array of light intensity and water availability. We documented altered plasticity to light intensity not only for mutants insensitive to blue wavelengths, but also for mutants insensitive to red and far-red wavelengths. Our results are consistent with models of co-action between these two distinct photoreceptory systems. Photomorphogenic mutants showed altered plasticity to light intensity not only for seedling and rosette morphology, but also for the duration of the vegetative and reproductive phases. Fruit production and two other performance traits showed no significant variation for plasticity to light intensity, but the means of all three performance traits differed significantly among genotypes. Notably, mean fruit production was much lower in the wild type than in the blue-insensitive and far-red insensitive mutants. The high fruit production of these mutants declined with decreasing water availability. In contrast, the low fruit production of the wild type was quite stable with declining water. However, we found only marginally significant variation among genotypes for the water-mediated plasticity of fruit production. Our comparisons of photomorphogenic mutants and wild-type plants suggest that there may be significant costs associated with the photoreceptory systems. However, the enhanced fitness of mutant genotypes may not be realizable in natural conditions, where very low water availability or curtailed growing seasons may be common.

Keywords: cryptochrome, epistasis, flowering time, life-history evolution, phenotypic plasticity, photomorphogenesis.

INTRODUCTION

Plants in natural habitats experience a variety of environmental resources and signals (Bazzaz, 1996, p. 29). Responses to these are mediated, in part, by sets of plasticity genes – regulatory loci that directly receive environmental signals, often exerting pleiotropic effects on suites of traits (Schlichting and Pigliucci, 1995; Pigliucci, 1996). Responses to complex environmental heterogeneity may also be indirect, owing to inevitable variation in plant size or growth rate, to fundamental energetic or developmental trade-offs, or to the timing and

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length of the growing season (Sultan, 1995; Mitchell-Olds, 1996; Van Tienderen *et al.*, 1996). In this paper, our interest is in the plasticity generated by complex (i.e. multifactorial) environments, how this complexity is dealt with by a series of candidate plasticity genes, and the fitness consequences of pleiotropy and epistasis of environmental receptors.

Detailed molecular genetic studies have already thoroughly characterized numerous light-sensitive plasticity genes (e.g. photoregulatory loci such as phytochrome A and B or the cryptochromes, which are blue light-sensitive photoreceptors: Smith, 1995; Ahmad and Cashmore, 1996; Guo *et al.*, 1998). Such genes are essential to photomorphogenesis, a sequence of plastic developmental events, from de-etiolation of the seedling (Quail *et al.*, 1995), to regulation of flowering time (Mozley and Thomas, 1995; Bagnall *et al.*, 1996), to senescence (Nooden *et al.*, 1996).

There is ample evidence that natural genetic variation for light-mediated plasticity exists within and among natural plant populations (Winn and Evans, 1991; Schmitt, 1993; Sultan and Bazzas, 1993; Andersson and Shaw, 1994; Wulff *et al.*, 1994; Dudley and Schmitt, 1995, 1996; Van Tienderen and Van Hinsberg, 1996; Nicotra *et al.*, 1997). Nevertheless, we rarely know which specific loci are effecting such variation. It is still not clear what the relationship is, if any exists, between mutants indicated in the laboratory and variants found in natural populations. However, there have been recent reports of allelic variation for regulatory loci (H. Smith, personal communication; M. Purrugganan, personal communication) and of variation in the expression of regulatory loci in both animal and plant populations (Krebs and Feder, 1997; Downs *et al.*, 1998). By paralleling studies of natural variation with studies of photomorphogenic mutants, important insights can be gained about plant development, perhaps leading to a greater unity of molecular genetic, evolutionary and ecological perspectives (Callahan *et al.*, 1997).

Photomorphogenic mutants can be considered genotypes with altered plasticity genes (Pigliucci, 1996), whether the particular mutation results in constitutive expression of a phenotype normally induced by the environment, or in an ability to respond plastically (Schmitt *et al.*, 1995). Studies of mutants that functionally dissect plastic responses will inform us about the long-term evolution of natural phenotypic plasticity (Pigliucci and Schmitt, 1999). Specifically, mutants offer an excellent and perhaps unique opportunity to probe at least two issues very relevant to evolutionary ecology.

First, non-lethal mutations in plasticity genes make it possible to examine ecologically relevant pleiotropic effects across the entire life cycle. In an environment where a plastic phenotype is typically not induced, we can discern whether a wild-type plant with normal plasticity genes has reduced fitness relative to a mutant plant with disabled plasticity genes. Such evidence can provide very direct insight into possible costs of phenotypic plasticity, especially those relating to signal acquisition or the maintenance of regulatory mechanisms (De Witt *et al.*, 1998).

Second, non-lethal mutations in plasticity genes can be examined under multiple environmental conditions, to discern how wild-type and mutant plants differ in their ability to integrate environmental signals or resources. Analogous to genetic co-variation (for example, owing to pleiotropy), co-variation of environmental factors may exist, which is certainly the case with light spectral quality and light intensity (Smith, 1994). Variation in light intensity may, in turn, be correlated with other factors such as temperature or water availability. Environmental co-variation has been incorporated into theoretical models of the evolution of phenotypic plasticity, specifically those that distinguish between the environment of development (that induces a plastic response) and the environment of

selection (Gavrilets and Scheiner, 1993). It is also plausible that environmental co-variation has the potential to act as a *limit* to adaptive phenotypic plasticity (regardless of possible *costs* of plasticity due to pleiotropy). This is because plastic phenotypes may match poorly their environments if selection is unable simultaneously to optimize plasticity to two (or more) distinct but correlated environmental factors (De Witt *et al.*, 1998).

To begin to examine 'complex' environments, the experiment presented here investigates plasticity to two rather than just a single environmental factor. Environmental variables (light and water availability) and genotypes were chosen, in part, based on a previous study that examined pleiotropy of photomorphogenic genes by comparing plasticities of wild-type and mutant lines (Pigliucci and Schmitt, 1999). This previous study both corroborated and expanded evidence regarding the pleiotropic effects of such genes. For example, even when grown in non-shaded conditions (i.e. high R : FR), *phyB* (also known as *hy3*) mutants abnormally and inappropriately over-elongate hypocotyls, reduce branching and accelerate floral induction (i.e. shade avoidance: Halliday *et al.*, 1994; Reed *et al.*, 1994). In these same conditions, *hy4* mutants abnormally elongate hypocotyls, but *increase* branching and *delay* flowering (Mozley and Thomas, 1995; Bagnall *et al.*, 1996). Despite such differences, *phyB* mutants and wild types achieve similar fruit production, and *hy4* mutants actually have strongly enhanced fruit production. Why might mutant genotypes (and their associated abnormal phenotypes) achieve similar or even enhanced fitness relative to the wild type? To what extent does such a result reflect controlled experimental environments that are benign and simple relative to natural ecological conditions?

In nature, light and myriad other environmental factors may vary more or less independently or in concert, with light and water availability likely to have a fairly regular and positive correlation. We therefore conducted the experiment described here using three levels of light intensity (all with high R : FR ratios) arranged factorially with three levels of water availability (achieved by varying the frequency of watering). Relative to our earlier study (Pigliucci and Schmitt, 1999), we expanded the collection of genotypes to include a standard isogenic wild-type line (the Landsberg *erecta* strain of *Arabidopsis thaliana*) and seven mutant genotypes. These represent single and double mutants lacking the red-sensitive light-stable phytochrome B, the far-red sensitive light-labile phytochrome A, and the blue-sensitive photoreceptor HY4, also referred to as cryptochrome 1. We examined the entire life cycle, from seedling development through fruit maturation.

First, we ask whether mutants show altered patterns of light- and water-mediated plasticity for a suite of traits, including known light-sensitive traits, life-span traits and performance traits. Next, we ask whether and how two double mutant genotypes differ from the wild type and from their corresponding single mutant genotypes. It is well known that *A. thaliana* possesses multiple sets of photoreceptors, and both molecular geneticists and physiologists have begun analysing epistatic interactions among them. Thus far, the evidence suggests that genes from the same gene family often operate in a partially divergent but partly overlapping (or redundant) manner (Reed *et al.*, 1994; Quail *et al.*, 1995). Also, co-action may occur between genes from different families (Mohr, 1994; Casal and Boccalandro, 1995; Guo *et al.*, 1998). Although a full dissection of interactions among photoreceptors is beyond the scope of this study, it is important to consider to what extent such interactions may be relevant from the perspective of evolutionary ecology.

Finally, to explore the possibility that plasticity genes may have pleiotropic effects on fitness, we paralleled our earlier study (Pigliucci and Schmitt, 1999) and included fruit production, an important component of fitness. We also ask how wild-type and mutant

patterns of light-mediated plasticity affect the ability of morphological, life-span and performance traits to respond plastically to variation in water availability (i.e. if there are constraints imposed by the response to one environmental factor over the plasticity to another factor).

MATERIALS AND METHODS

Plant material

In *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), germination and seedling development are followed by a period of vegetative growth as a basal rosette. Plants switch quite abruptly from the vegetative to the reproductive stage, producing one or several elongating and potentially branching inflorescences. This transition from vegetative to reproductive growth, called 'bolting', effectively divides the life cycle into two distinct phases. Phenological habit varies among ecotypes, and may be winter annual (i.e. over-wintering as a rosette and bolting in the early spring), ephemeral spring or fall annual (i.e. growing vegetatively, bolting and senescing very quickly), or longer-lived summer annual (Napp-Zinn, 1985). Native populations are found throughout Europe and western Asia; diverse naturalized populations occur in temperate North America and east Asia. A number of 'domesticated' laboratory lines have been developed for studying the molecular genetic basis of plant physiology and development. These appear to be derived from short-lived spring ephemeral ecotypes (Redei, 1992; King *et al.*, 1993; Zhang and Lechowicz, 1994; Pigliucci and Schlichting, 1995). In all wild ecotypes and laboratory lines, flowers and fruits develop acropetally. The mating system is predominantly selfing (Abbott and Gomes, 1989). Approximately 30–60 small seeds dehisce from each fruit at maturity.

In this study, we used eight isogenic lines, including one wild type and seven mutant genotypes (Table 1). Seven of these were obtained from the *Arabidopsis* Information Management System (AIMS) at Michigan State University (http://aims.cps.msu.edu/aims/): one cryptochrome mutant (a single-locus mutant), five phytochrome mutants (four single-locus

Table 1. The seven mutants and the wild-type lines used in the current study[a]

Genotype	Allele	Description	AIMS no.	Reference
phyA (weak)	*phyA*-203	lacks phytochrome A	CS6221	Reed *et al.* (1994)
phyA (strong)	*phyA*-201	lacks phytochrome A	CS6219	Reed *et al.* (1994)
phyB (weak)	*phyB*-7	lacks phytochrome B	CS6215	Koornneef *et al.* (1980)
phyB (strong)	*phyB*-8	lacks phytochrome B	CS6216	Koornneef *et al.* (1980)
phyA–phyB	*phyA*-201; *phyB*-5	lacks phytochromes A and B	CS6224	Reed *et al.* (1994)
hy4	*hy4*-1	lacks blue receptor	CS70	Koornneef *et al.* (1980)
hy4–phyA	*hy4*; *phyA*-201	lacks blue receptor and phyA	courtesy of Brian Parks	
Landsberg *erecta* (wild type)		wild type	CS6220	Redei (1992)

[a] All lines except the *hy4–phyA* double mutant were obtained from the AIMS (*Arabidopsis* Information Management System) collection at Michigan State University (http://aims.cps.msu.edu/aims/).

mutants and one double mutant) and the wild-type Landsberg *erecta* (the background for six of the mutant lines). We included a 'strong' and a 'weak' mutant allele of *phyA* and *phyB*, with the *phyA–phyB* double mutant carrying two 'strong' mutant alleles. (In the double mutant, the *phyA* allele is identical to the strong *phyA* single mutant. The double mutant's *phyB* allele, although not identical to the *phyB* single mutant, is classified as a strong allele. Comparisons between the single *phyB* strong mutant and the *phyA–phyB* double mutant are therefore tentative.) Finally, Brian Parks, at the University of Wisconsin-Madison, provided a *phyA–hy4* double mutant that carries the same *hy4* allele as AIMS' *hy4* single mutant, but a third and distinct *phyA* strong allele. This eighth line is in a RLD/Columbia rather than a Landsberg background. Comparisons between this double mutant and corresponding *phyA* and *hy4* single mutants (and the Landsberg 'wild type') must therefore be interpreted with prudence. These cautionary statements notwithstanding, the different backgrounds are genetically very similar to each other, derived from a common Landsberg ancestor collected from a natural population (Redei, 1992).

Growth conditions

We exposed the wild type and the seven mutant lines to all combinations of two environmental factors, light intensity and water availability. We conducted the experiment in the Plant Growth Facility at the University of Tennessee, Knoxville, on two growth racks, each with three shelves illuminated by up to six high-intensity (115 W) fluorescent light tubes. To manipulate light, we turned combinations of tubes on or off and altered the distance between lights and plants. Only light intensity varied; light quality and photoperiod were held constant (i.e. high red : far red ratio and 16 h photoperiod). The treatments were *high light*, 362 $\mu mol \cdot m^{-2} \cdot s^{-1}$; *medium light*, 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$; and *low light*, 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$. With a LI-1800 portable spectroradiometer, we verified that light intensity and light quality were similar across the two growth racks. Within a light treatment, we imposed a rotating schedule of watering every 24, 36 or 48 h, when flats were bottom-watered to a depth of 2 cm.

Seeds were kept moist in the dark for a week at 8°C to stimulate and synchronize germination (Pigliucci *et al.*, 1995), then planted onto moist soil (Fafard #2). To prevent mortality, we did not begin imposing watering treatments until seedlings had developed their first true leaves (day 12). The experiment continued until senescence of all reproductive individuals (day 66).

Experimental design

Four individuals of each mutant or wild-type line (genotypes) were planted in individual pots and then assigned at random to a flat. Next, watering regimes were randomly assigned to flats, and flats randomly assigned to shelves. Finally, light treatments were randomly assigned to each shelf. Given this restricted randomization (a split–split plot; Kuehl, 1994), light treatment is a whole plot, watering treatment is a subplot, and genotype is nested within sub-subplots. Additionally, the entire experiment was blocked (on two light racks, each with three shelves and a total of nine flats). This 'generalized randomized block design' is effectively similar to standard randomized block designs, but four rather than a single experimental unit were assigned to each block × light × water × genotype combination (Addelman, 1969). Such a design minimizes empty cells due to individual plant mortality. The experiment involved 576 plants overall, with complete measurements obtained for 555

plants and no empty cells. To minimize position effects, we re-randomized flat positions within shelves and the light treatments assigned to a shelf biweekly. We also re-randomized the position of pots within flats on day 24, approximately midway through the experiment.

Trait measurements

Based upon our knowledge of the *A. thaliana* life cycle, we selected a suite of seven traits to capture various aspects of the whole-plant phenotype. These included two light-sensitive traits (known to be sensitive to light quantity or quality): hypocotyl length and leaf number at bolting. Hypocotyl length was employed originally to isolate the mutants used for this study and is also a very early character in the ontogeny of *A. thaliana.* Unfortunately, it is difficult to measure hypocotyl length precisely in a non-destructive manner. We used digital calipers and recorded length to the nearest 0.1 mm. By counting leaf number at bolting, we assessed meristem allocation to vegetative growth. We also included two life-span traits describing the duration of two distinct phases of the life cycle. Vegetative duration is the number of days between germination and bolting (i.e. commencement of the reproductive phase); reproductive duration is the number of days between bolting and dehiscence of the first mature fruit. Finally, we included three performance traits: rosette diameter at senescence (to the nearest millimetre), total inflorescence length (including all branches on single or multiple inflorescences, to the nearest centimetre) and total fruit production (i.e. fruit number). To verify that these seven traits are not subject to strong multi-collinearity and to validate these three broad groupings, we used Systat 6.0 to conduct a principal components analysis, using correlations derived from all complete observations in the experiment ($n = 555$) (Systat, 1996). We then examined factor loading of each individual trait onto principal components with eigenvalues >1 (Table 2).

Statistical analyses

Univariate analyses of variance (ANOVA) permitted a detailed examination of individual traits affected by interactions among the three experimental factors: light intensity, watering frequency and mutant line (hereafter referred to as light, water and genotype). To prevent seedling mortality, we did not commence watering treatments until plants had produced

Table 2. Factor loadings of individual traits onto principal components (PCs) with eigenvalues >1 (**boldface** indicates variables with the highest weight on each component)

	PC1	PC2	PC3
Hypocotyl length	–0.387	**0.728**	0.069
Leaves at bolting[a]	**0.676**	–0.495	0.094
Vegetative duration	0.207	**–0.613**	**0.682**
Reproductive duration[a]	–0.305	0.484	**0.758**
Rosette diameter	**0.774**	0.295	–0.006
Total inflorescence length[a]	**0.822**	0.458	0.085
Fruit production[b]	**0.699**	0.409	0.001

[a] Square-root-transformed; [b] log-10-transformed.

their first leaves. We therefore fit a separate split-plot ANOVA to examine the effects of light and genotype on hypocotyl length; this model includes only two error terms (i.e. whole-plot and subplot: Table 3). For the other six traits, we used an ANOVA for a three-factor split–split plot design involving three error terms. Whole-plot error is generated by the randomization of light treatments to shelves and across the two blocks. Subplot error is generated by the randomization of water treatments within each light level and across the two blocks. Sub-subplot error results from randomization of replicated genotypes across and within the block × light × water treatment combinations (Tables 4–6).

Because we were interested in how specific mutations alter wild-type patterns of plasticity to light and water, we focused on tests of light × genotype and water × genotype interactions. Before proceeding with the tests of the split–split plot model, we examined the second-order light × water × genotype interaction term, which estimates genetic variation among the genotypes for plasticity to light × water combinations; this interaction was tested over the sub-subplot error term. First-order interactions are properly tested and interpreted only when this second-order interaction is non-significant (or non-crossing), which was the case in our study. Light × genotype and water × genotype interactions were then tested over sub-subplot error; in contrast, the water × light interaction was tested over the subplot error. When these interaction terms are non-significant or non-crossing, main effects of genotype, water and light could be tested, respectively, over the sub-subplot, subplot and whole-plot error terms. The effect of block, accounting for variation between the two growth racks, was tested over the whole-plot error term. Even when non-significant, interaction terms and the block term were maintained in all models. Throughout these hierarchical analyses, we treated light, water and genotype as fixed factors. Variances for error terms, interaction terms and main effects were estimated using the Multivariate General Linear Hypothesis (MGLH) feature of Systat (version 6.0 for Windows 95: Systat, 1996). Mean squares and *F*-ratios are reported, and associated *P*-values are evaluated using a sequential Bonferroni procedure (Rice, 1989). To better meet assumptions of normality and

Table 3. Type III mean squares, *F*-ratios and *P*-values for split-plot ANOVA for hypocotyl length[a]

Source of variation	d.f.	Hypocotyl length		
		MS	*F*-ratio	*P*-value
Block	1	9.9383	0.7575	0.4759
Light treatment	2	35.6093	2.7143	0.2692
Whole-plot error	2	13.119		
Genotype	7	**35.9268**	**39.7665**	**0.0001**
Genotype × light	14	**1.9526**	**2.1613**	**0.0082**
Subplot error	533	0.9034		

[a] To prevent seedling mortality, we did not commence watering treatments until plants had produced their first leaves. It was therefore necessary to fit a different model to examine the effect of light and genotype on this trait. Effects significant at the $P < 0.05$ level are indicated in **boldface**. The significant light × genotype interaction indicates that plasticity to light varies among the genotypes in the study.

homogeneous variances, we square-root-transformed leaf number at bolting and reproductive duration, and log-transformed total inflorescence length and fruit production.

Where analyses of variance indicated genetic variation for trait plasticity to either of the two environmental factors (i.e. significant genotype × light or genotype × water interaction terms), or genetic variation for trait means (i.e. significant genotype effect), we conducted *post-hoc* pairwise comparisons using Tukey's HSD procedure for multiple comparisons (Ott, 1988). This facilitated specific contrasts of trait means, either within a given treatment (Figs 1, 2) or averaged across treatments (Figs 3, 4). Comparisons of particular interest were between the wild-type and the single and double mutants, between strong and weak alleles, and between single mutants and associated double mutants.

RESULTS

The principal components analysis (Table 2) showed that the first principal component correlated strongly with performance traits (fruit number, inflorescence length, rosette diameter) and with leaf number at bolting. The third principal component correlated strongly with the two life-span traits (vegetative duration and reproductive duration). The second principal component encompassed variation in hypocotyl length and vegetative duration. (Interestingly, these traits were correlated negatively: longer hypocotyls were associated with shorter vegetative duration and vice versa.)

In the univariate analyses of variance, the second-order genotype × light × water interaction was non-significant for all six traits (Tables 4–6). Both light-sensitive traits (hypocotyl length, leaf number at bolting) (Tables 3 and 4) and both life-span traits

Table 4. Type III mean squares, *F*-ratios and *P*-values for split–split plot analyses of variance for number of leaves at bolting[a]

		Leaf number at bolting		
Source of variation	d.f.	MS	*F*-ratio	*P*-value
Block	1	0.9703	0.7911	0.4676
Light treatment	2	0.9227	0.7523	0.5707
Whole-plot error	2	1.2266		
Water treatment	2	0.0919	0.1378	0.8739
Water × light	4	0.8873	1.3303	0.3587
Subplot error	6	0.6669		
Genotype (G)	7	**9.4630**	**90.5591**	**0.0001**
Genotype × light (L)	14	**0.2599**	**2.4870**	**0.0020**
Genotype × water (W)	14	0.1718	1.6437	0.0644
G × L × W	28	0.1254	1.1997	0.2237
Sub-subplot error	474	0.1045		

[a] **Boldface** indicates effects significant at the $P < 0.05$ level after a sequential Bonferroni procedure corrected for multiple comparisons (across the six traits in Tables 4–6). The significant light × genotype interaction indicates that, for this trait, plasticity to light varies among the genotypes in the study.

Table 5. Type III mean squares, *F*-ratios and *P*-values for split–split plot analyses of variance for life-span traits[a]

		Vegetative duration			Reproductive duration		
Source of variation	d.f.	MS	*F*-ratio	*P*-value	MS	*F*-ratio	*P*-value
Block	1	274.6125	1.3575	0.3641	2.3731	2.5088	0.2541
Light treatment	2	497.5627	2.4596	0.2891	5.7753	6.1055	0.1407
Whole-plot error	2	202.2959			0.9459		
Water treatment	2	35.8493	0.5239	0.6170	0.2006	0.3438	0.7222
Water × light	4	68.4948	1.0010	0.4748	0.6874	1.1782	0.4076
Subplot error	6	68.7272			0.5834		
Genotype (G)	7	**99.4567**	**11.7603**	**0.0001**	**2.7146**	**48.6374**	**0.0001**
Genotype × light (L)	14	**32.4117**	**3.8325**	**0.0001**	**0.1220**	**2.1865**	**0.0076**
Genotype × water (W)	14	13.3296	1.5762	0.0820	0.0320	0.5727	0.8867
G × L × W	28	8.0359	0.9502	0.5407	0.0587	1.0520	0.3949
Sub-subplot error		8.4570			0.0558		

[a] **Boldface** indicates effects significant at the $P < 0.05$ level after a sequential Bonferroni procedure corrected for multiple comparisons (across the six traits in Tables 4–6). Sub-subplot error degrees of freedom for vegetative duration = 474; for reproductive duration = 470. The significant light × genotype interactions indicate that, for these traits, plasticity to light varies among the genotypes in the study.

(Table 5) showed a light × genotype interaction. For all three performance traits, neither the light × genotype nor the water × genotype interaction was significant, but there were differences among the genotypes in the means of all three performance traits (Table 6). Fruit production, an important performance trait, was marginally sensitive to watering frequency (Table 6). The results of the univariate analyses of variance and multiple comparisons are discussed below and diagrammed in two sub-groups, according to genotype: (1) wild type, weak and strong *phyB* single mutants, weak and strong *phyA* single mutants, and *phyA–phyB* double mutants (right-hand side of Figs 1 and 2); (b) wild type, *hy4* single mutant, weak and strong *phyA* single mutants, and *hy4–phyA* double mutant (left-hand side of Figs 1 and 2).

phyA and *phyB* single mutants and the *phyA–phyB* double mutant

In the *phyA* weak and strong mutants, hypocotyl length (Fig. 1A) and reproductive duration (Fig. 2C) did not differ significantly from the wild type in any light treatment (i.e. *phyA* mutants did not show shifts in either trait means or trait plasticities). Relative to the wild type, both of the *phyA* mutants had significantly reduced leaf number at bolting in all three light treatments (Fig. 1C). Also, both had a vegetative duration very similar to the wild type in high and medium light, but significantly briefer in low light (Fig. 2A). Finally, the weak and strong *phyA* mutants were very similar to each other for both trait means and trait plasticities (Figs 1–4).

Hypocotyl length was consistently longer in both *phyB* mutants relative to the wild type, but the significance of these differences was not consistent across all light treatments

Table 6. Type III mean squares, *F*-ratios and *P*-values for split–split plot analyses of variance for performance traits[a]

		Rosette diameter			Inflorescence length			Fruit production		
Source of variation	d.f.	MS	*F*-ratio	*P*-value	MS	*F*-ratio	*P*-value	MS	*F*-ratio	*P*-value
Block	1	20.3364	1.9265	0.2995	0.0550	0.3358	0.6208	0.0023	0.0138	0.9171
Light treatment	2	11.3292	1.0733	0.4830	0.2108	1.2865	0.4374	0.7762	4.6269	0.1777
Whole-plot error	2	10.5559			0.1639			0.1678		
Water treatment	2	11.5012	3.2388	0.1112	0.7786	5.4350	0.0450	1.0716	5.7341	0.0405
Water × light	4	2.3528	0.6626	0.6404	0.2076	1.4489	0.3256	0.3702	1.9812	0.2166
Subplot error	6	3.5511			0.1433			0.1869		
Genotype (G)	7	**25.9724**	**42.6212**	**0.0001**	**0.9403**	**38.3157**	**0.0001**	**1.2585**	**33.4153**	**0.0001**
Genotype × light (L)	14	0.6423	1.0540	0.3980	0.0282	1.1485	0.3127	0.0623	1.6554	0.0618
Genotype × water (W)	14	1.0574	1.7352	0.0460	0.0389	1.5831	0.0800	0.0745	1.9790	0.0178
G × L × W	28	0.9250	1.5179	0.0453	0.0251	1.0215	0.4369	0.0485	1.2873	0.1510
Sub-subplot error	470	0.6094			0.0450			0.0377		

[a] **Boldface** type indicates effects significant at the $P < 0.05$ level after a sequential Bonferroni procedure corrected for multiple comparisons (across the six traits in Tables 4–6). There is limited variation among genotypes for the plasticity of performance traits to both light and water as indicated, respectively, by non-significant light × genotype and water × genotype interactions. There is, however, substantial variation among genotypes for trait means.

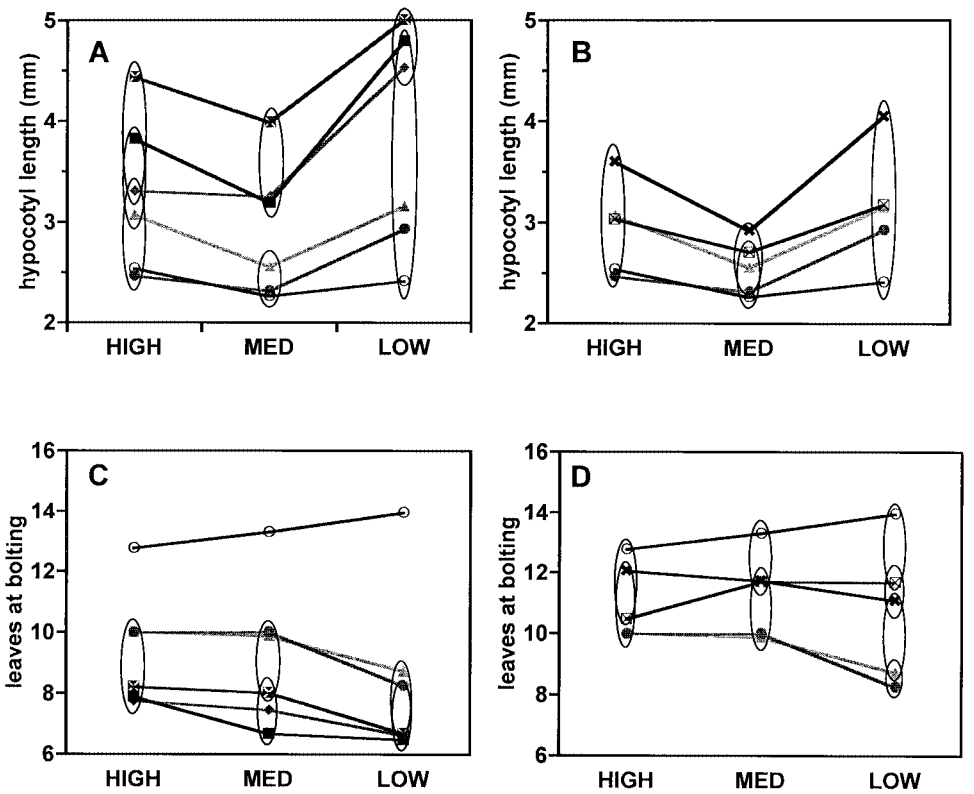

Fig. 1. Reaction norms to light availability for two light-sensitive traits: hypocotyl length (A, B) and leaf number at bolting (C, D). Left-hand diagrams (A, C) include wild type (○), *phyB* strong mutants (■), *phyB* weak mutants (◆), *phyA* strong mutants (●), *phyA* weak mutants (▲) and the *phyA–phyB* double mutant (⊠). Right-hand diagrams (B, D) include wild-type, *phyA* strong mutant, *phyA* weak mutant, *hy4* mutant (✖) and the *phyA–hy4* double mutant (⊠). Ovals indicate genotype means within a given light treatment that did not differ significantly.

(Fig. 1A). In both *phyB* single mutants, leaf number at bolting was significantly reduced in all light treatments (to 6–8 leaves *vs* 13–14 leaves in the wild type) (Fig. 1C). Relative to the wild type, the *phyB* weak and strong mutants had significantly shortened vegetative duration in low light only; in high and medium light, their vegetative duration was, respectively, essentially identical or only slightly (and not significantly) prolonged (Fig. 2A). In the *phyB* weak mutant, reproductive duration was always significantly longer than that of the wild type (by 3–7 days); in the *phyB* strong mutant, reproductive duration was significantly longer (by 1–2 days) in low and medium but not in high light (Fig. 2C). Although there was no significant genotype × light interaction for rosette diameter or inflorescence length (Table 6), there were significant differences in trait means between the wild type and the *phyB* mutants. Relative to the wild type, *phyB* weak mutants had smaller rosettes and *phyB* strong mutants had shorter inflorescences (Fig. 3). Finally, although neither the *phyB* weak nor the *phyB* strong mutant differed significantly from the wild type, the *phyB* weak mutants did produce more fruits than the *phyB* strong mutants (Fig. 4).

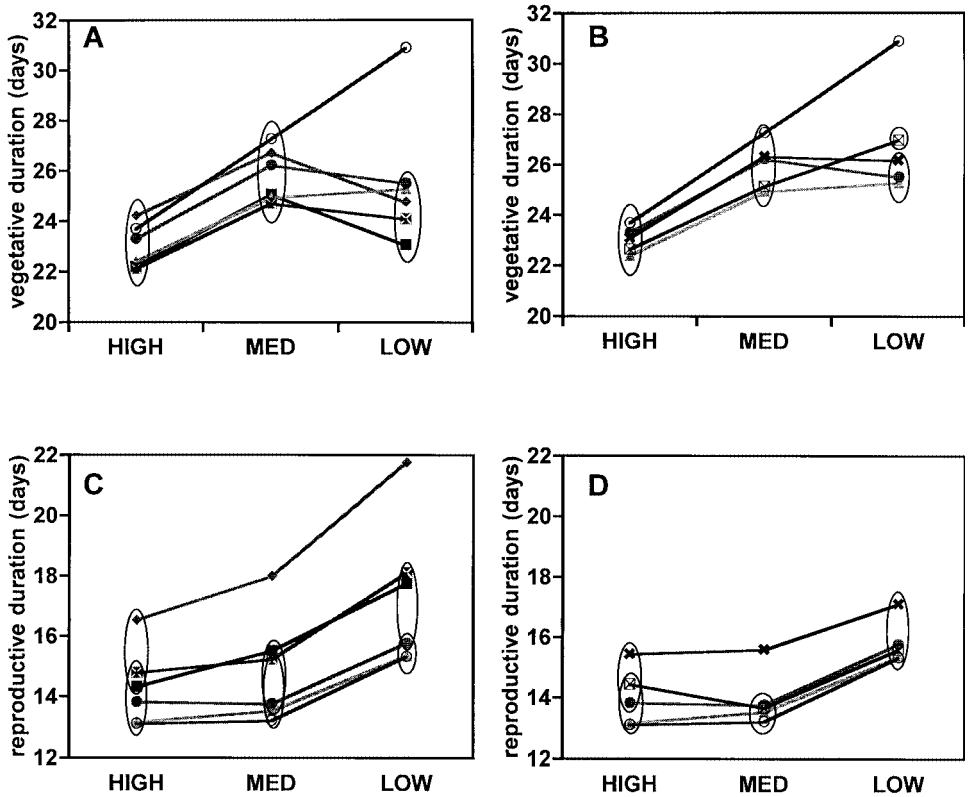

Fig. 2. Reaction norms to light availability for two life-span traits: vegetative duration (days to bolting) (A, B) and reproductive duration (days between bolting and fruit maturation) (C, D). Format, symbols and ovals as in Fig. 1.

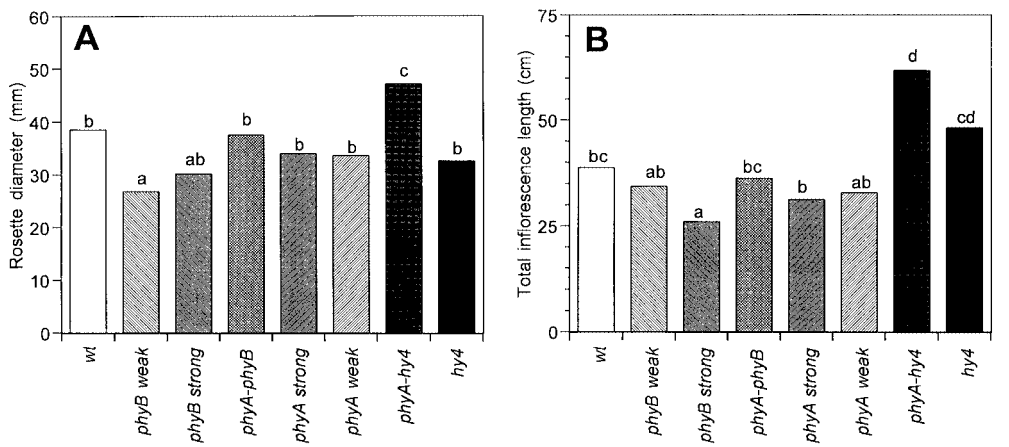

Fig. 3. Mean rosette diameter (A) and mean total inflorescence length (B) for each genotype, averaged across all light and water treatments. Letters indicate genotype means that did not differ significantly. wt = wild type.

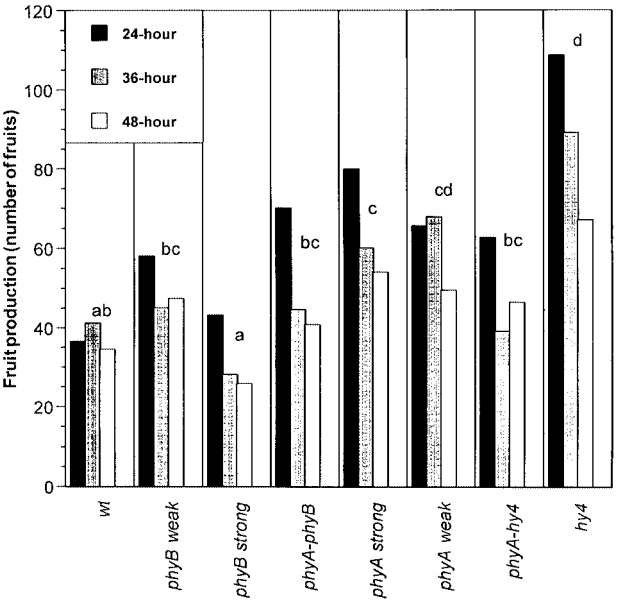

Fig. 4. Mean number of fruits produced for each genotype, in each of the three watering frequency treatments. Fruit production differed significantly among the three watering treatments, but there was no significant genotype × water interaction. Letters indicate genotype means (across all three water treatments) that did not differ significantly. wt = wild type.

Although the *phyA–phyB* double mutants and *phyB* strong mutants do not share identical *phyB* alleles, both alleles are classified as strong (Table 1). We found that the effects of strong mutations in the PHYB locus had a similar effect, regardless of whether we were examining single or double mutants. Both had similar hypocotyl lengths and number of leaves at bolting (Fig. 1A,C), and also showed very similar light-induced changes in vegetative duration and reproductive duration (Fig. 2A,C). They did not differ in the plasticity of performance traits, but they did differ significantly in mean inflorescence length (Fig. 3B) and mean fruit production (Fig. 4).

The *hy4* single mutant and the *phyA–hy4* double mutant

Mean hypocotyl length differed slightly between *hy4* and the wild type in medium light only. Otherwise, hypocotyl length, mean rosette diameter and mean inflorescence length did not differ significantly between *hy4* single mutants and the wild type (Figs 1B, 3A,B). However, mean fruit production was significantly greater (nearly double) in *hy4* mutants (Fig. 4). For other traits, the *hy4* mutants differed from the wild type in plasticity. Under low light, the *hy4* mutants bolted with significantly fewer leaves than the wild type (Fig. 1D) and had a shortened vegetative duration (to about 24 days *vs* 30 days in the wild type) (Fig. 2B). In medium and high light, differences were slight and non-significant (Fig. 2B). Reproductive duration was 2–3 days longer in *hy4* single mutants than in the wild type in high and medium but not in low light (Fig. 2D). The *hy4* mutants produced more fruits than the

wild type under all three watering frequencies; *hy4* mutants also showed a general trend of declining fruit production with less frequent watering. Interestingly, however, wild-type plants maintained similarly low fruit production across all water treatments (Fig. 4). In the ANOVA for fruit production, however, the genotype × water interaction was only marginally significant and was not robust to a Bonferroni procedure that corrects for multiple tests (Rice, 1989).

Although *hy4* mutants and the wild type were similar in terms of mean rosette diameter and inflorescence length, the *phyA–hy4* double mutants had broader rosettes and longer inflorescences (Fig. 3A). Also, although the *hy4* single mutants had fewer leaves at bolting, greater reproductive duration and enhanced fruit production (see above), the *phyA–hy4* double mutants did not differ from the wild type (Figs 1D, 2D, 4). In sum, the relationships among the wild type, *phyA* and *hy4* single mutants, and *phyA–hy4* double mutants were remarkably inconsistent, varying among traits and among environments.

DISCUSSION

Although research has been conducted with photomorphogenic mutants in *Brassica rapa* (Devlin *et al.*, 1992; Schmitt *et al.*, 1995), cucumber, tomato (Whitelam and Smith, 1991; Casal *et al.*, 1994; Ballare *et al.*, 1995; Casal, 1996) and other species (Koornneef and Kendrick, 1994), *A. thaliana* has been studied most frequently and most extensively in this respect. Previous studies of this model species have documented numerous light-sensitive pleiotropic effects across seedling to flowering adult stages, apparently regulated by phytochrome or cryptochrome (i.e. HY4).There is also some evidence that photomorphogenic loci influence the timing of flowering (Mozley and Thomas, 1995; Bagnall *et al.*, 1996) and possibly other aspects of the plant life span (Nooden *et al.*, 1996). Only a handful of studies have attempted to investigate effects on performance traits, including fitness components (Schmitt *et al.*, 1995; Pigliucci and Schmitt, 1999).

The three parts of this discussion are structured around the basic questions presented in the Introduction. First, we briefly discuss the altered plasticity to light exhibited by photomorphogenic mutants as discussed in the literature and as further documented by our experiment. Second, we address some of the complexities that may arise from epistatic interactions or co-action among photoreceptors. In the final section, we consider two specific types of pleiotropic effects: (1) effects on a key fitness component (fruit production) and (2) effects on responses to another environmental factor (water availability).

Light-mediated development and plant life span

The light-stable form of phytochrome (PHYB in *A. thaliana*) has been implicated not only in seedling germination, seedling de-etiolation, hypocotyl and cotyledon development (Quail *et al.*, 1995), but also in earlier flowering induced by shifted R : FR ratios ('the ultimate expression of a shade-avoidance response': Halliday *et al.*, 1994) or by manipulated photoperiod (Reed *et al.*, 1994; Mozley and Thomas, 1995). Consistent with this idea that PHYB normally permits a plant to forego the 'shade-avoiding' phenotype in white light, *phyB* mutants in our study abnormally elongated hypocotyls and flowered with fewer rosette leaves under all light intensities. As we discuss in the next section, differences in other traits may be less simple to explain because PHYB may interact with PHYA in an antagonistic but complementary fashion (Reed *et al.*, 1994; Quail *et al.*, 1995), and also

because PHYB may co-act with HY4 (Koornneef and Kendrick, 1994; Mohr, 1994; Casal and Boccalandro, 1995).

The light-labile form of phytochrome (PHYA in *A. thaliana*) has its own distinct functions in a similar range of events from seedling to flowering (Reed *et al.*, 1994; Quail *et al.*, 1995). Previous work has shown that the light-sensitivity of PHYA in *A. thaliana* seedlings is in response to high irradiance in far-red wavelengths (HIR: Koornneef and Kendrick, 1994; Quail *et al.*, 1995; Smith, 1995; Botto *et al.*, 1996). This is consistent with the observation that *phyA* mutants have essentially normal phenotype in red or white light, leading to the tentative conclusion that PHYA is not essential to plants grown in white light (Whitelam *et al.*, 1993). Our results are partly consistent with this idea; *phyA* mutants and the wild type had similar hypocotyl elongation, inflorescence elongation and reproductive duration. Moreover, the *phyA–phyB* double mutants were essentially similar to associated *phyB* strong single mutants in terms of hypocotyl elongation, leaf number at bolting, and vegetative and reproductive duration. However, we observed significant differences between *phyA* mutants and the wild type in terms of vegetative duration and leaf number at bolting. Changes in leaf number at bolting were significant in all light treatments; changes in vegetative duration were significant only in low light. Thus, under white light (and long photoperiods), PHYA almost certainly plays some role in the timing of floral induction, perhaps acting in concert with PHYB or with blue-sensitive photoreceptors (Reed *et al.*, 1994; Mozley and Thomas, 1995; Quail *et al.*, 1995; Guo *et al.*, 1998; see below).

The blue photoreceptor (HY4 or CRY1 in *A. thaliana*) also has a similarly wide range of effects (Koornneef *et al.*, 1980; Ahmad and Cashmore, 1996). When grown in white light and compared with the wild type, *hy4* mutants have only slightly elongated hypocotyls (Koornneef *et al.*, 1980; Koornneef and Kendrick, 1994). Mutants also have elongated petioles and leaves (Jackson and Jenkins, 1995), altered anthocyanin accumulation (Ahmad *et al.*, 1995; Lin *et al.*, 1996), changed photosynthetic rates and chloroplast composition (Walters and Horton, 1995), abnormal flavonoid biosynthesis (Fuglevand *et al.*, 1996; Lin *et al.*, 1996), and reduced sensitivity of flowering time to length of day (Mozley and Thomas, 1995; Bagnall *et al.*, 1996; Lin *et al.*, 1996). Consistent with previous studies, we found only very slight differences in hypocotyl length between *hy4* mutants and the wild type in white light. Extending previous studies, we found that increasing intensity of white light resulted in a convergence of *hy4* mutants and the wild type in terms of leaf number at bolting and vegetative duration.

Epistasis, co-action and other physiological complications

In low intensities of white light, we discerned some divergence of *phyB*, *phyA* and *hy4* mutants from the wild type (Figs 1B, 2A). Specifically, *phyB* mutants had altered plasticity to light intensity for the durations of the vegetative and reproductive stages. Under medium and high light, both weak and strong *phyB* mutants flowered at the same time as the wild type, but under low light they flowered earlier (Fig. 2A). In contrast, under low light, the *phyB* strong and weak mutants (and the *phyA–phyB* double mutants) had a longer reproductive duration than the wild type. Moreover, although the *phyB* weak mutants had a much longer reproductive duration than the wild type in all light treatments, the difference between the weak and strong *phyB* mutants was statistically significant only under medium and low light (this was also the case for differences between the *phyB* strong mutants and the wild type). As with the *phyB* mutants, we discerned a shorter vegetative duration in the weak

and strong *phyA* mutants relative to the wild type in low light only. Finally, we also observed that number of leaves at bolting and vegetative duration were similar in *hy4* mutants and the wild type under high and medium light, but were significantly reduced in *hy4* mutants under low light (Figs 1B, 2A).

Mozley and Thomas (1995) have proposed an integrated model of development in which PHYB tends to delay flowering indirectly, by promoting vegetative growth, while PHYA and HY4 are both directly involved in promoting earlier flowering. Their model predicts that *phyB* mutants should flower earlier and with fewer leaves under white light and long photoperiods, while *hy4*, *phyA* and *phyA–hy4* double mutants should flower later than the wild type. Our results confirmed the former but not the latter prediction. We observed *phyA* single mutants flowering with fewer leaves than the wild type and, in low light only, a shorter vegetative duration (i.e. bolting after fewer days). Also in low light only, the *hy4* mutants had fewer leaves at bolting and a shorter vegetative duration. The *phyA–hy4* double mutants did not differ from the wild type in leaf number at bolting in any light treatment, but they did differ from the wild type (and from the *hy4* single mutant) for vegetative duration in low but not in high or medium light. The *phyA–hy4* double mutants also showed a distinctly intermediate phenotype in low but not in medium or high light, with a vegetative duration that was shorter than that of the wild type but longer than that of the *hy4* and *phyA* single mutants.

In long photoperiods, it is likely that aspects of floral induction (number of leaves at bolting, days prior to bolting, or both) are affected by co-action between HY4 and PHYB (or PHYA). Any form of co-action between phytochromes and HY4 is likely to be sensitive to light intensity for several reasons. First, the blue-sensitive photoreceptor is probably involved in 'photon counting' rather than gauging the balance between two different wavelengths (Smith, 1994). Second, because the blue receptor makes plants more responsive to the action of phytochromes (Koornneef and Kendrick, 1994; Mohr, 1994; Ahmad and Cashmore, 1996), it is indirectly involved in sensing and responding to red and far-red wavelengths. Third, although blue- and red-sensitive photoreceptory systems may overlap to some extent, they may also act in a partially independent manner, with some potential to substitute or compensate for one another (cf. Koornneef and Kendrick, 1994; Reed *et al.*, 1994; Yanovsky *et al.*, 1995). In our study, the divergence of *phyB*, *phyA* and *hy4* mutants from the wild type did increase with diminishing intensity of white light for many but not all traits. This is consistent with the idea that there may be co-action between the light-quality sensing role played by phytochromes and the photon-counting role of HY4.

Our ecologically minded approach was not intended as a means of devising or testing models of specific co-actions among photoreceptors. In fact, by including a large suite of traits (including some expressed very late in the life cycle), rather inconsistent results emerge. Such results lead to the conclusion that photoreceptor function (and co-action) may be quite specific, changing not only with developmental stage (i.e. seedling development *vs* influorescence development), but also with respect to specific traits. This rather general conclusion contrasts with the often very specific models that emerge from studies examining single traits or developmental stages (e.g. Casal and Boccalandro, 1995).

Pleiotropic effects of photomorphogenic loci in complex environments

Our ecological approach to the study of photomorphogenic loci involves examining multiple traits, including fitness components and plasticity to environmental factors

other than light (in our case, water availability). A handful of past studies have taken similar routes. For example, seedlings of *phyA* mutants and *A. thaliana* died prematurely because they failed to de-etiolate under deep shade, as wild-type seedlings do (Yanovsky *et al.*, 1995). This is a very direct reduction of fitness due to altered plasticity genes.

Van Tienderen *et al.* (1996) examined whether mutations in regulatory loci might have indirect (i.e. pleiotropic) impacts on fitness components. They imposed high and low nutrient treatments on constitutive and photoperiod-sensitive late-flowering mutants of *A. thaliana*. In low nutrients, fruit production was quite similar in the late-flowering mutants and the wild type. In high nutrients, the later-flowering mutants eventually exceeded the earlier-flowering wild type. In the field, high nutrient conditions may thus counter selection for earlier flowering due to other environmental factors (e.g. risk of drought, competition, mowing, or grazing late in the season), allowing late-flowering mutations to persist in some natural populations. In general, multiple environmental factors make it much more likely that mismatches between plastic phenotypes and the environment will arise. Such mismatches can seriously limit the evolution of adaptive syndromes of plasticity (De Witt *et al.*, 1998), beyond the possible costs directly associated with plasticity.

For examining costs associated with plasticity (De Witt *et al.*, 1998; Scheiner and Berrigan, 1998), mutants with altered plasticity are an excellent tool. In a previous study, comparisons of wild-type *A. thaliana* with *phyB* and *hy4* mutants revealed numerous pleiotropic effects (Pigliucci and Schmitt, 1999). This previous study examined plasticity to both light quality and light intensity. In both high light and 50% neutral shade, *hy4* mutants had longer hypocotyls and more leaves at bolting, whereas *phyB* mutants had shorter hypocotyls, fewer leaves at bolting and a shorter vegetative duration. The plasticity of these traits to light quantity was similar in the wild type and in the *phyB* mutants. Despite their altered phenotypes, *phyB* mutants and the wild type were comparable in both environments for an important fitness component, fruit production. In contrast, *hy4* mutants had higher fruit production in both environments. These results suggest that there may be little or no costs associated with some plasticity genes (e.g. PHYB), but high costs associated with others (e.g. HY4). The present study paralleled this previous study and focused on seedling development, leaf number at bolting, vegetative duration and overall fruit production; we also examined reproductive duration.

We corroborated that *phyB* mutants initiate bolting with many fewer leaves than the wild type; under low light intensity, we found that they also flower earlier. Also, *phyB* mutants have a prolonged reproductive duration relative to the wild type; to our knowledge, our study is the first to report this effect. Moreover, a longer reproductive phase may be involved in permitting the *phyB* weak and strong mutants to produce comparable numbers of fruits relative to the wild type.

Under high and medium light, *hy4* mutants and the wild type converged; under low light, they differed, with *hy4* mutants having fewer leaves at bolting. This contrasts with our previous study. Consistent with our previous study, however, we found greatly enhanced fruit production in *hy4* mutants. We also found that *phyA* weak and strong mutants had enhanced fruit production relative to the wild type. The enhanced fecundity of these mutant genotypes suggests that the intact photoregulatory genes found in wild type plants may have a high cost (De Witt *et al.*, 1998; Scheiner and Berrigan, 1998). Could plasticity mediated by water availability or some other factor offset these costs in natural environments? We

found that fruit production declined with decreasing watering frequency in the *hy4* and *phyA* mutants (Fig. 4); in contrast, wild-type plants maintained consistently low fruit production under all watering frequencies (Fig. 4). In statistical tests, mutants and the wild type did not differ significantly in their plasticity to the watering treatments (although the genotype × water interaction for fruit production was marginally significant before correcting for multiple tests) (Table 6). If water availability declines to lower levels in the field than it did in our study, as may be the case in areas exposed to strong light, the apparently 'rushed' and less prolific reproductive phase of wild-type plants may be advantageous. Moreover, as in the study of Van Tienderen *et al.* (1996) and in other research conducted by our laboratory (M. Pigliucci and E. Marlow, in prep.), factors that curtail the growing season may also favour the wild-type 'strategy'.

CONCLUSIONS

By taking this more ecologically minded approach, our study yielded novel insights. First, we uncovered a previously unreported effect of the PHYB photoreceptor – it may be a very direct and important regulator of the reproductive phase. We point out that PHYB and other photoreceptor loci will certainly exert numerous and cumulative pleiotropic effects as the life cycle of *A. thaliana* reaches its later stages. Moreover, the context or 'internal environment' of the large, adult phenotype may considerably complicate the analysis of any genes or signal transduction pathways operating during those stages. These realities make it difficult to attribute directly altered phenotypes to a single gene or a small number of genes, but the differences in reproductive duration in the *phyB* mutants were quite striking and merit further study.

Second, we were able to examine an important fitness component (fruit production) and its sensitivity to combinations of light intensity and water availability. Mutant genotypes with altered plasticity of the vegetative–reproductive transition were those that tended to reduce fruit production in line with reduced water availability. The genotype × water interaction for fruit production, although non-significant after correcting for simultaneous tests, was marginally significant. Relative to the field, the 'drought' imposed by our least-frequent watering regime may be quite benign. To incorporate more stressful watering frequencies into studies of this type, it may be necessary to examine not only fruit production (i.e. fecundity) but also survivorship (Biere, 1995; Yanovsky *et al.*, 1995).

Finally, examining complex environments that involve heterogeneity of two or more factors (i.e. light and water) naturally makes for more complex results that are more difficult to interpret. From an ecological point of view, however, it is critical to understand the development of whole-plant phenotypes in complex environments. Photoreceptor mutants are excellent tools for examining the vegetative–reproductive transition ('flowering time'), its plasticity, the complex genetic basis of that plasticity, and the possible limits and costs associated with that plasticity. This is especially true when comparisons of mutants and the wild type persist through the entire life cycle rather than concluding at the seedling stage or at floral induction, and when mutants are placed in more complex and ecologically realistic environments – in growth chambers and ultimately in the field. Through ecological and evolutionary studies of mutant genotypes, we believe it is possible to attain a more complete understanding of light-regulated development and other forms of phenotypic plasticity in *A. thaliana* and other plant species.

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