Natural variation in light sensitivity of *Arabidopsis*

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Because plants depend on light for growth, their development and physiology must suit the particular light environment. Plants native to different environments show heritable, apparently adaptive, changes in their response to light[1,2]. As a first step in unraveling the genetic and molecular basis of these naturally occurring differences, we have characterized intraspecific variation in a light-dependent developmental process—seedling emergence. We examined 141 *Arabidopsis thaliana* accessions for their response to four light conditions, two hormone conditions and darkness. There was significant variation in all conditions, confirming that *Arabidopsis* is a rich source of natural genetic diversity. Hierarchical clustering revealed that some accessions had response patterns similar to known photoreceptor mutants, suggesting changes in specific signaling pathways. We found that the unusual far-red response of the Lm-2 accession is due to a single amino-acid change in the phytochrome A (PHYA) protein. This change stabilizes the light-labile PHYA protein in light and causes a 100-fold shift in the threshold for far-red light sensitivity. Purified recombinant Lm-2 PHYA also shows subtle photochemical differences and has a reduced capacity for autophosphorylation. These biochemical changes contrast with previously characterized natural alleles in loci controlling plant development, which result in altered gene expression or loss of gene function[3–9].

Light influences almost all aspects of plant development from germination to flowering. Vascular plants sense light using a suite of photoreceptors specific for different wavelengths: phytochromes[10] for red and far-red light, cryptochromes[11] for blue and UV-A and phototropins[12] for specialized blue responses. To investigate natural variation in light response, we assayed the simple process of seedling emergence. When a seed germinates in the dark, it is etiolated: the hypocotyl (embryonic shoot) extends and the cotyledons (embryonic leaves) remain folded. Light causes de-etiolation, a developmental process that allows the formation of an optimal body plan for the transition from heterotrophic to photoautotrophic physiology. During de-etiolation, hypocotyl elongation is inhibited, the cotyledons unfold and chloroplast development begins. As hypocotyl elongation is quantitative and shows an inverse log-linear relationship with the light received, hypocotyl length can be used as a quantitative assay for light sensitivity.

We obtained 141 *A. thaliana* accessions from around the Northern hemisphere and measured their hypocotyl lengths in seven different conditions (Web Table A). To examine general light-responsiveness, we grew seedlings in white light. To examine variation in specific photoreceptor pathways, we measured hypocotyl lengths in blue, red and far-red light. We also assayed hypocotyl variation that was independent of light sensitivity by

Table 1 • Summary statistics and genetic correlations

a Summary statistics

	White	Blue	Red	Far red	GA	BRZ	Dark
Mean of lines (mm)	6.71	6.10	9.93	5.15	7.85	5.40	12.14
Coefficient of variation	0.22±0.03	0.22±0.03	0.20±0.03	0.25±0.07	0.23±0.03	0.23±0.03	0.14±0.02
Heritability (H_2^B)	0.75±0.05	0.57±0.1	0.74±0.05	0.65±0.14	0.79±0.05	0.73±0.05	0.58±0.07
Minimum line mean (mm)	2.22	3.77	4.80	2.31	3.41	3.28	6.77
Maximum line mean (mm)	10.82	10.26	14.88	12.20	14.15	8.97	16.12
Average measured	15.3	15.5	11.5	13.0	16.0	15.8	10.3
Total measured	2189	2214	1638	1862	2285	2266	1478

b Genetic correlations

	White	Blue	Red	Far red	GA	BRZ	Dark
Blue	0.72±0.16						
Red	0.77±0.13	0.53±0.19					
Far red	0.59±0.17	0.70±0.15	0.46±0.17				
GA	0.86±0.13	0.71±0.13	0.72±0.11	0.50±0.2			
BRZ	0.62±0.15	0.73±0.13	0.47±0.18	0.59±0.22	0.54±0.15		
Dark	0.47±0.18	0.73±0.15	0.33±0.22	0.54±0.15	0.44±0.17	0.69±0.14	

a, Summary statistics for accession survey. Mean of lines, mean hypocotyl length of accessions in each condition. Coefficient of variation, s.d. expressed as a proportion of mean hypocotyl length. Heritability (broad sense), proportion of variance explained by between-line differences. Minimum/maximum line mean, minimum and maximum mean hypocotyl length found in any accession. Average measured, mean number of hypocotyls measured per accession in each condition. Total measured, total number of hypocotyls measured in each condition. Coefficient of variation and heritability are shown ±95% confidence interval half-widths. ***b***, The genetic correlations (r_{GE}) between environments are shown, ±95% confidence interval half-widths. Values of 1, 0, and –1 would indicate perfect, no, and inverse correlation respectively.

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growing plants in the dark. In addition, we examined variation in response to two hormones that are known to interact with the light-signaling pathway: gibberellins (GAs) and brassinosteroids[10]. We investigated the effects of GAs by adding GA_3 to the growth medium of seedlings grown in white light. We probed the effects of brassinosteroids with a biosynthesis inhibitor, brassinazole (BRZ)[13]. BRZ causes dark-grown plants to adopt a light-grown morphology; we therefore assayed the effects of BRZ on dark-grown plants.

We found substantial variation in hypocotyl lengths across all conditions (Table 1*a* and Fig. 1*a*–*g*), indicating that selection or genetic drift (or both) have caused accessions to diverge. If these differences are at least partially due to selection, we would expect to detect some correlation between the light environment from which the accessions were originally collected and their light response. Indeed, we found a modest inverse correlation between latitude and hypocotyl length in white light, with accessions from latitudes closer to the equator on average being taller in our light conditions than those collected further north (Fig. 1*h*, r=–0.41, P=1.3×10^{-5}). To remove light-independent effects, we regressed white-light hypocotyl length on dark-grown length. We then examined correlation between latitude and the residuals from this regression (which represent the light-responsive component) and found that the correlation was still significant (r=–0.35, P=2×10^{-4}), although reduced. In contrast, there was no significant correlation of latitude with dark-grown hypocotyl length (P=0.119). Together, these analyses show that there is a correlation between light response and latitude of origin. It is unlikely that this correlation is due to population stratification and drift, because there is very little geographic genetic structure in *A. thaliana* (ref. 14 and J. O. Borevitz *et al.*, unpublished observations). Consistent with this, there is no obvious association between hypocotyl length and longitude (Fig 1*i*). Our data suggest that an environmental factor that varies with latitude has selected for differences in hypocotyl length (or a correlated character). One possible factor is light intensity itself. In the absence of differences in atmospheric factors such as cloud cover, light is more intense at lower latitudes[15], although the daily light sum in summer can be higher at higher latitudes. Indeed, we have found that there is a complex relationship between the effects of light

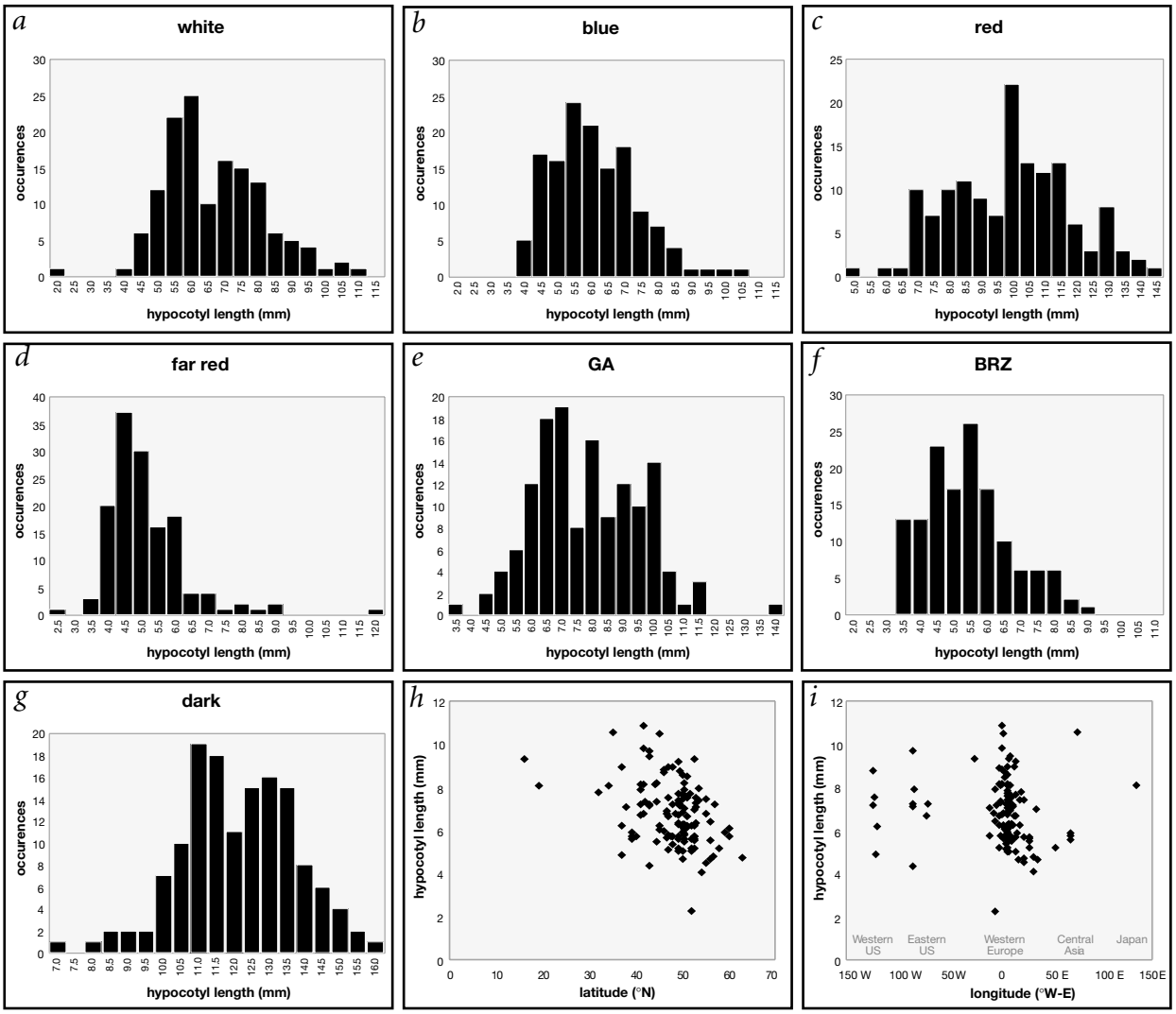

Fig. 1 Variation between different strains in response to light and hormones. ***a*–*g***, Histograms showing the distribution of mean hypocotyl lengths among accessions for each condition examined: *a*, 35 microeinsteins (μE) m^{-2} s^{-1} white light with a R/FR ratio of 1.3; *b*, 4 μE m^{-2} s^{-1} blue light; *c*, 35 μE m^{-2} s^{-1} red light; *d*, 0.5 μE m^{-2} s^{-1} far-red light; *e*, Dark; *f*, 35 μE m^{-2} s^{-1} white light with 30 μM GA_3 in the medium; *g*, Dark with 0.7 μM BRZ in the medium. ***h***, Correlation between hypocotyl length in white light and latitude. ***i***, Hypocotyl length in white light plotted versus longitude of orgin.

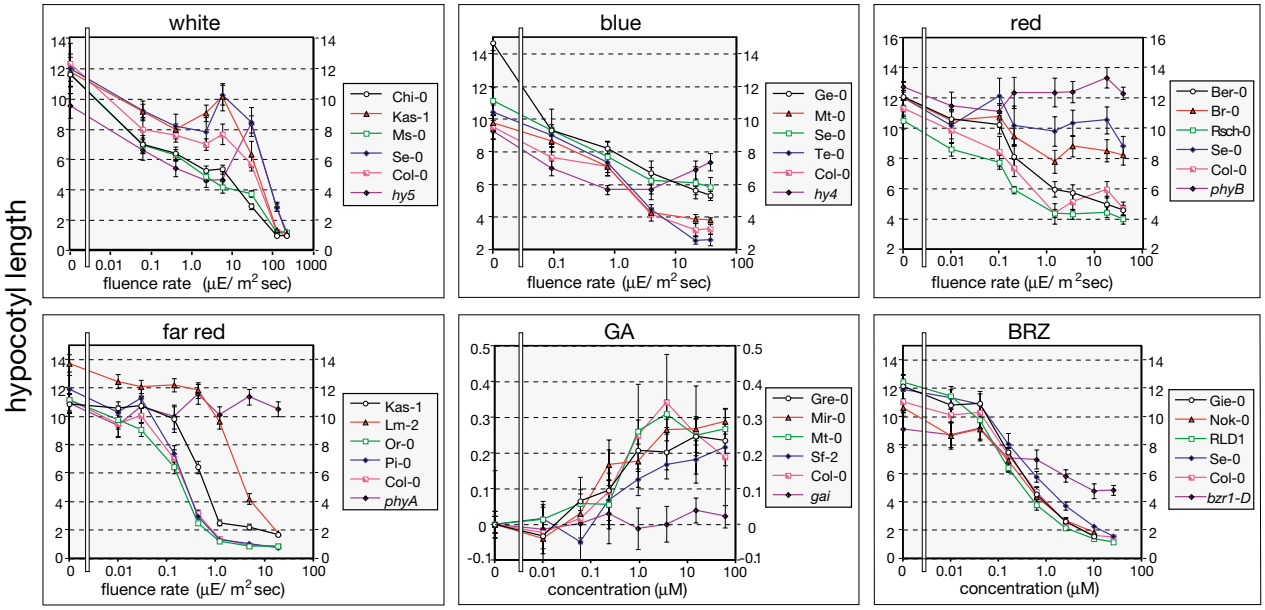

Fig. 2 Fluence response and hormone dose–response curves. Mean hypocotyl lengths are plotted against increasing light fluence or hormone concentration. The *y*-axis represents hypocotyl length in mm (except for GA, where the difference from the no-hormone treatment, in log mm, is shown). The *x*-axis represents light fluence or hormone concentration. Error bars indicate 95% confidence intervals. For hormone dose–response, two accessions with the largest and smallest residual deviation from a regression analysis against white (for GA) or dark (for BRZ) were used. For each graph, a reduced-response mutant specific for that condition is shown in purple.

intensity and day length on hypocotyl elongation (J.N.M., D.W., J.C., unpublished results). Nevertheless, it seems reasonable to speculate that plants at lower latitudes will compensate for higher light intensity by being less sensitive to light.

Variation in hypocotyl length could either be caused by changes in light- and hormone-signaling pathways or be due to general changes in embryonic or seedling development, such as the number of hypocotyl cells[16]. We used two approaches to distinguish between these possibilities. First, we assessed environment-independent effects by examining genetic correlation between hypocotyl lengths in different conditions (Table 1*b*). We found some correlation across all conditions, indicating changes in pathways that are independent of environmental inputs (or correlated changes in environmental response pathways). Most conditions, however, showed correlations substantially less than one, suggesting that there is variation in specific environmental-response pathways.

To further examine whether there was variation in light and hormone sensitivity across a range of light fluences or hormone concentrations, we selected two of the shortest and two of the tallest accessions from each treatment and measured their response to eight different levels of light or hormone (Fig. 2). For each condition, we included the laboratory strain Col-0 and a mutant with reduced response specific for the particular condition. In the case of white, red, far-red and blue light, the tall lines were significantly less sensitive to light than the short lines, validating our initial screen with only a single fluence rate to identify variation in light sensitivity. The

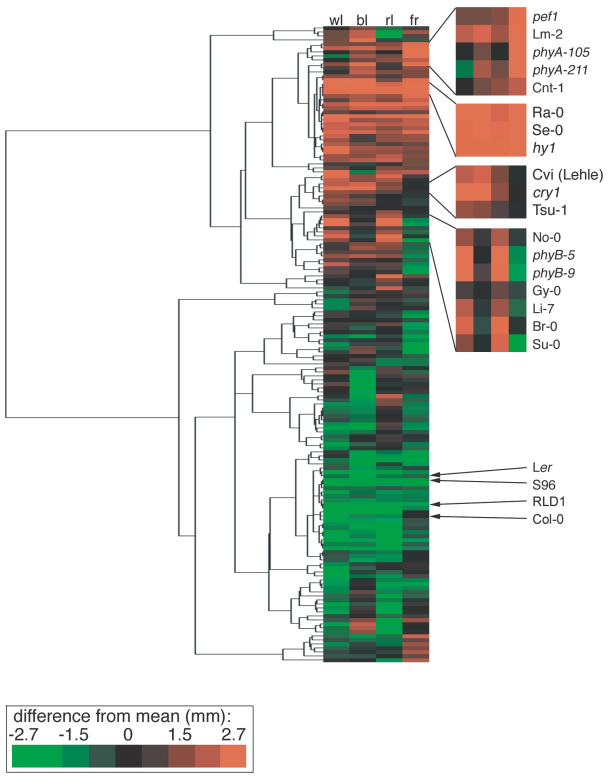

Fig. 3 Hierarchical cluster analysis of light response among accessions. Hierarchical clustering was used to group accessions that showed similar responses across the different light environments. Each row represents one accession; each column represents one light condition. The color of the rectangles indicates hypocotyl length for each accession in a particular condition. Black indicates a length equal to the average length of all strains in that condition, red is taller than average and green is shorter than average. Four clusters around known light-signaling mutants are enlarged to show accessions that may have changes in particular light-signaling pathways. *PHYA, PHYB* and *CRY1* encode photoreceptors for far-red, red and blue light, respectively. *PEF1* is required for far-red light response. *HY1* is needed for synthesis of the chromophore for phytochromes (mutants reviewed in ref. 10). The strain 'Cvi (Lehle)' is genetically distinct from that used to generate recombinant inbred lines with Ler[30]. Arrows point to some commonly used laboratory strains. See Fig. 1 legend for fluence rates used.

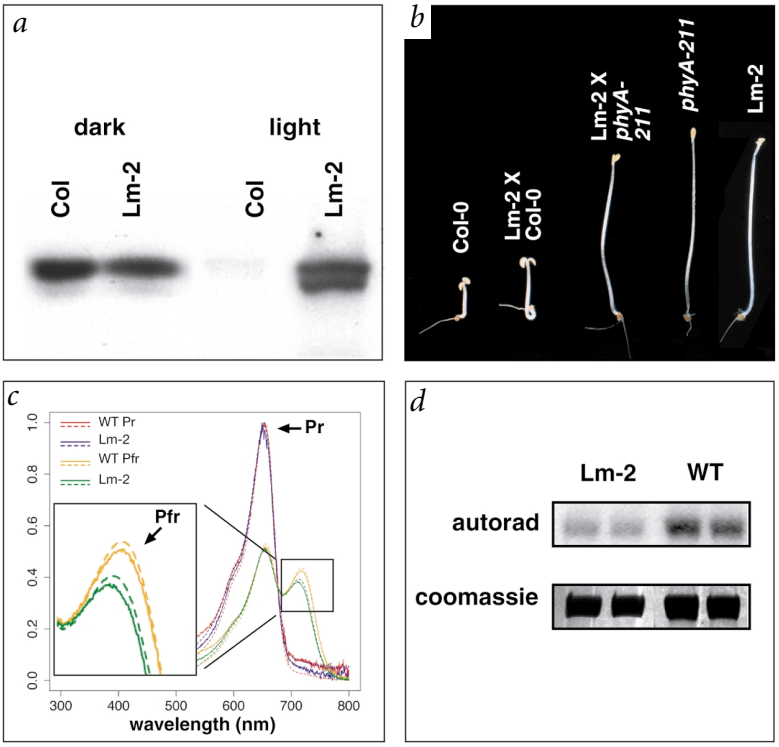

Fig. 4 Characterization of Lm-2. ***a***, Western blot of PHYA extracted from dark-grown seedlings or dark-grown seedlings exposed to white light for 8 h before harvesting. ***b***, Representative far red–grown seedlings from complementation test, grown at 0.5 μE m^{-2} s^{-1}. ***c***, Absorption spectrograph. Dashed lines indicate replicate samples from independent protein preps. *y*-axis shows absorbance in arbitrary units. Each sample was normalized relative to its Pr maximum. The inset shows an enlargement of the region containing the Pfr peaks. ***d***, Autoradiograph and Coomassie staining of autophosphorylation assay. Phosphoimager analysis showed that Lm-2 *Avena* PHYA was phosphorylated to only 40% of wildtype levels.

situation was less clear for hormone response. The accessions selected from the GA experiment had different heights in the absence of hormone, making hormone-induced changes harder to interpret. To overcome this complication, we compared the relative change caused by GA. After log transformation and subtraction of the no-hormone control, two of four lines were different in their GA response. In contrast, for BRZ, only one of the strains initially selected turned out to be different from the others over a range of concentrations.

Different photoreceptors are required for responses to different wavelengths of light. To investigate whether the patterns of variation in the accessions were caused by variation in specific photoreceptor pathways, we used hierarchical clustering of accessions and photoreceptor mutants to identify broad patterns of light response (Fig. 3). We found that some accessions showed new patterns of response, whereas others clustered with known light-signaling mutants. To test the possibility that some of the latter accessions varied in the expression or activity of the photoreceptors themselves, we examined photoreceptor levels in these accessions. Using western blot analyses, we did not detect any prominent changes in the amount of PHYB, CRY1 or CRY2 in plants grown either in the dark or under the light condition specific for the receptor under investigation (data not shown). We found one accession (Lm-2), however, with altered amounts of PHYA (Fig. 4*a*). Lm-2 clustered with *phyA* mutants and the PHYA signaling mutant *pef1* (ref. 10) and was approximately 100-fold less sensitive than Col-0 to far-red light (Figs. 2 and 3). In dark-grown seedlings, PHYA protein is stable and its levels are high, but it is normally rapidly degraded upon exposure to light[17]. In contrast, amounts of Lm-2 PHYA remained high in light (Fig. 4*a*). Light normally has two effects on PHYA: it triggers activation leading to short hypocotyls and causes protein degradation; both of these seem to be affected in Lm-2, suggesting that PHYA-Lm-2 is generally less responsive to light. When we crossed the Lm-2 strain to a *phyA-211*–null mutant in the Col-0 background, Lm-2 failed to complement *phyA-211*, consistent with a change in PHYA causing the reduced far-red sensitivity of Lm-2 (Fig. 4*b*). Indeed, sequence analysis revealed that PHYA-Lm-2 differs from PHYA-Col-0 by one amino acid; a conserved methionine at position 548 is changed to threonine (in addition, two silent base-pair substitutions were found). This methionine is conserved in all higher plant phytochromes. To determine if it has a similar role in other phytochromes, we made an analogous change in the *Arabidopsis* gene *PHYB*. We found that 'PHYB-Lm-2' showed reduced responsiveness to low-fluence red light but was unimpaired in sensing high-fluence red light (Fig. 5), confirming that the Met548Thr substitution causes altered light sensitivity. This finding demonstrates that Met548 is generally important for modulating light response in different phytochrome family members.

To better understand the effects of the Lm-2 variant on phytochrome function, we examined spectral absorption properties and kinase activity using purified, recombinant *Avena* PHYA and compared this to *Avena* PHYA carrying the Lm-2 substitution. Phytochromes can exist in two photoconvertible forms: Pr, with maximum absorbance in red light and Pfr, with maximum absorbance in far-red light. The absorption spectrum of *Avena* PHYA-Lm-2 Pr was indistinguishable from wildtype *Avena* PHYA. The absorption maximum of Lm-2 Pfr, however, was red-shifted approximately 6 nm and the Pfr peak height was slightly reduced relative to the Pr form (Fig. 4*c*), suggesting that the Lm-2 change affects photochemical properties of phytochrome.

The carboxy terminus of phytochrome has homology with bacterial histidine kinases[10] and is important for modulating phytochrome activity. In addition, phytochromes have light-regulated serine–threonine kinase activity[10]. We tested whether *Avena* PHYA-Lm-2 might have altered kinase activity and found that it showed lower levels of autophosphorylation (Fig. 4*d*). Taken together, our results suggest that Lm-2 differs from Col-0 in its response to far-red light because of a change in the biochemical properties of the PHYA protein. Notably, Met548 is neither in the chromophore-binding domain nor in the histidine kinase–related domain of phytochrome, yet it seems to affect the properties of both. The change is in the 'hinge' region separating the light-sensing domain from the rest of the protein. It is thus possible that the Lm-2 variant alters cross-talk between the sensing and output portion of the protein, rather than having a direct structural effect on either.

We have found substantial natural variation in the hypocotyl elongation response of *A. thaliana* seedlings. Some of this variation apparently results from changes in light-signaling pathways, whereas other variation may come from changes in hard-wired developmental programs. Using cluster analysis, we have identified accessions that are candidates for having

changes in known photoreceptor pathways. We have confirmed this supposition by showing that an accession with reduced far-red sensitivity contains a change in the *PHYA* coding sequence, which in turn alters the biochemical properties of the protein and causes an altered response to light. A cryptochrome photoreceptor variant with altered biochemical properties has just been described in the accompanying paper by El-Assal *et al.*[18]. These two findings contrast with previously characterized natural variants at other developmental control loci in plants, where the variation was found to be in RNA expression levels or due to gene lesions causing loss of function[3–9]. Notably, the two natural variants affecting protein function both occur in photoreceptors. Perhaps the pleiotropic nature of these proteins favors changes affecting a subset of protein function over lesions disrupting overall gene expression or function.

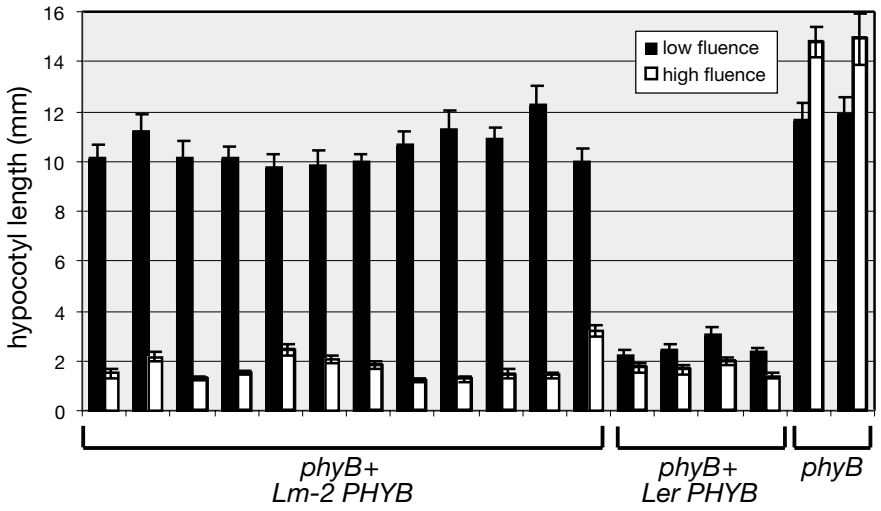

Fig. 5 The Lm-2 change affects PHYB sensitivity to red light. Each low-fluence, high-fluence pair represents data from one independent, homozygous T3 line (12 *phyB-5+35S:Lm-2 PHYB* and 4 *phyB-9+35S:Ler PHYB* lines were examined; *phyB-9* was used as a control). High-fluence: 57 μE m^{-2} s^{-1}, low-fluence: 0.3 μE m^{-2} s^{-1}.

Methods

Plant material, growth conditions and analysis. Treatment of plants and growth conditions is described in Web Note A. A list of accessions used and their calculated heights can be found in Web Table A.

***PhyB* transgenics.** We isolated total RNA from *A. thaliana* ecotype Ler using Trizol reagent (Gibco–BRL), amplified *PHYB* using Turbo *Pfu* (Stratagene) and cloned the product into pGEM-T (Promega) to create pJM59. We then cloned *PHYB* into pCHF3, a binary vector containing the constitutive 35S promoter from cauliflower mosaic virus, yielding pJM61. We used site-directed mutagenesis by PCR to introduce the desired 'Lm-2' change into pJM59 and create pJM66 and then cloned a *Bsr*G1–*Mlu*I restriction fragment containing the mutated region into pJM61 to create pJM68. We checked all constructs by sequencing after PCR amplification. We carried out *Agrobacterium* transformation of pJM61 and pJM68 as previously described[19]. We plated homozygous *phyB-9*/pJM68 and *phyB-5*/pJM61 T3 lines as well as *phyB-9* in duplicate using our standard conditions (Web Note A), except that one plate for each T3 line was transferred to high red light (57 μE m^{-2} s^{-1}) and the other to low red light (0.3 μE m^{-2} s^{-1}).

Statistical analysis. We carried out statistical analysis in R (ref. 20) using the nlme package[21]. For each condition we determined the best linear unbiased predictor (BLUP) of the line means using a linear mixed-effects model. The model treated line and plate effects as random. Broad-sense heritability (H_2) was calculated as between-line variance (V_G) divided by total variance (the sum of between-line, between plate, and within-line (error) variance). To calculate the genetic correlation between environments, r_{GE}, we used $cov_{12}/(\sigma_{L1}\sigma_{L2})$ where cov_{12} is the covariance in line means, corrected for plate effects, and σ_{L1} and σ_{L2} are genetic standard deviations (s.d.) in line means from the lme model[22]. We calculated the coefficient of genetic variation (CV_G) for each environment by taking the genetic s.d. (σ_{L1}) and dividing it by the intercept of line means from the lme model (for each condition). Confidence intervals for H_2, r_{GE}, and CV_G were calculated using a (n–1) jack-knife procedure on 141 replicates.

Cluster analysis. For each accession or mutant line, we used log hypocotyl measurements to calculate the BLUP (in R, see above) to avoid heterogeneity of variances between light conditions. We then mean-centered the BLUPs for each light condition and carried out hierarchical cluster analysis using the program Cluster[23] with an uncentered correlation matrix and average linkage clustering. We generated a self-organizing map (SOM) before hierarchical clustering to determine the best orientation of the tree nodes. The resulting trees were displayed using Treeview software[23].

Immunoblotting. We grew approximately 200 seedlings per accession (Web Note A). We collected seedlings in green light, placed them in 1.5-ml microcentrifuge tubes and weighed them. We ground seedlings for 10 s using a plastic pestle (Kontes) attached to an electric drill, added 1/2 volume of 2 × SDS loading buffer and then ground the seedlings for an additional 10 s. We boiled the samples for 150 s, briefly vortexed them, boiled them for another 150 seconds, spun them for 5 min at full speed in a microfuge and loaded 10 μl on a 4–20% polyacrylamide gradient Tris-Glycine gel (Novex). We carried out PAGE and electroblotting using standard procedures. We used antibodies against a subunit of the vacuolar ATPase[24] to determine equal loading. Antibodies against CRY1 and CRY2 (ref. 25) were used to examine Buc-0, Cvi-L, Col-0, Ler, Cvi-k and Flo-0. Antibodies against PHYA[26] were used to examine An-1, Cnt-1, Kas1-1, Lm-2, Mrk-0, Ra-0, Se-0, Ste-0, Col-0, Ler-0 and Cvi-1. Antibodies against PHYB[27] were used to examine Br-0, Edi-0, Gre-0, Li-7, Mt-0, Mv-0, Su-0, Van-0, Wt-1, Col-0, Ler-k and Cvi-k. We used 5% milk powder/TBST as block and detected primary antibody binding using horseradish peroxidase–conjugated secondary antibodies (BioRad) and Pico West Substrate (Pierce). All extractions and blots were repeated at least twice.

Phytochrome purification. We used standard PCR methods to engineer the Lm-2 Met-to-Thr change into pPICAsPhyA, a *Pichia pastoris* expression vector containing an *Avena PHYA* cDNA (provided by J.C. Lagarias). We electroporated the resulting plasmid, pJM69 and pPICAsPhyA into strain GS115 according to the supplier's directions (Invitrogen). We carried out strain growth, induction and purification essentially as described (ref. 28 and Invitrogen instructions). We made spectroscopic measurements on a Varian Cary Bio50 spectrophotometer using a multi-cell holder so that measurements could be made on different samples simultaneously. Kinase assays were done as described[29], but were incubated for 20 min and analyzed using a Molecular Dynamics Storm phosphorimager and ImageQuant software. Quantification results were the same with or without background correction. Because the biochemical differences that we saw were subtle, we took care to induce and purify both wildtype and Lm-2 phytochrome at the same time, using the same buffers and reagents. All assays were repeated at least twice on independent preps with similar results.

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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