New Arabidopsis Recombinant Inbred Lines (Landsberg *erecta* × Nossen) Reveal Natural Variation in Phytochrome-Mediated Responses[1]

Teresa M. Alconada Magliano[2], Javier F. Botto, A. Veronica Godoy[3], V. Vaughan Symonds, Alan M. Lloyd, and Jorge J. Casal*

IFEVA, Facultad de Agronomía, Universidad de Buenos Aires y Consejo Nacional de Investigaciones Científicas y Técnicas, 1417 Buenos Aires, Argentina (T.M.A.M., J.F.B., J.J.C.); and University of Texas, Department of Molecular, Cell, and Developmental Biology, Austin, Texas 78712 (A.V.G., V.V.S., A.M.L.)

We used 52 Arabidopsis (*Arabidopsis thaliana*) accessions and developed a new set of 137 recombinant inbred lines between Landsberg *erecta* (L*er*) and Nossen (No-0) to explore the genetic basis of phytochrome-mediated responses during deetiolation. Unexpectedly, most accessions showed weak or moderate hypocotyl growth and cotyledon unfolding responses to pulses of far-red light (FR). Crosses between Columbia and No-0, two accessions with poor response, segregated seedlings with unfolded cotyledons under pulsed FR, suggesting the occurrence of accession-specific loci in the repression of morphological responses to weak light signals. Confirming the latter expectation, mapping of responses to pulsed FR in the L*er* × No-0 lines identified novel loci. Despite its weak response to pulsed FR, No-0 showed a response to continuous FR stronger than that observed in L*er*. By mapping the differential effect of pulsed versus continuous FR, we identified two high-irradiance response loci that account for the steeper response to continuous FR in No-0. This underscores the potential of the methodology to identify loci involved in the regulation of the shape of signal input-output relationships. Loci specific for a given phytochrome-mediated response were more frequent than pleiotropic loci. Segregation of these specific loci is predicted to yield different combinations of seedling responsivity to light. Such flexibility in combination of responses is observed among accessions and could aid in the adjustment to different microenvironments.

Some fluctuations of the light environment tightly correlate with the occurrence of conditions that impose a challenge to plant survival such as seasonal changes that result in extreme temperatures, organ emergence out of the soil, or competition with neighbor individuals. Subtle light signals, including small changes in photoperiod throughout the year, the transition between darkness and very low fluences of light reaching the top millimeters of the soil, and small reductions in the red light (R) to far-red light (FR) ratio caused by light reflected by neighbors, actually anticipate stressful conditions. Plants are able to perceive these signals, which are translated into regulation of developmental plasticity (Casal et al., 2004). Not surprisingly, fitting developmental decisions to these signals requires an intricate network of molecular players.

The use of Arabidopsis (*Arabidopsis thaliana*) mutants has been the primary approach in the search for players in light signaling. Mutant screens have led to the discovery of the photoreceptors phytochrome A (phyA; Whitelam et al., 1993), phytochrome B (phyB; Koornneef et al., 1980; Reed et al., 1993), cryptochrome 1 (Koornneef et al., 1980; Ahmad and Cashmore, 1993), and cryptochrome 2 (cry2; Koornneef et al., 1980; Guo et al., 1998). However, a complementary approach to mutant analysis has been the exploration of natural allelic variation. The use of recombinant inbred lines (RILs) between the accessions Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi) helped to identify an allele of the *CRY2* gene with distinctive kinetic properties, which accelerates flowering under short days (El-Assal et al., 2001); the screening of different Arabidopsis accessions revealed that Le Mans-2 carries a rare allele of the *PHYA* gene that stabilizes the light-labile PHYA protein and impairs the response to FR (Maloof et al., 2001). Additionally, we have identified several quantitative trait loci (QTL) in RILs derived from crosses between L*er* and Columbia (Col; Yanovsky et al., 1997) and between L*er* and Cvi (Botto et al., 2003) that affect the very-low-fluence response (VLFR) of phyA. Notably, one of these QTLs corresponds to the blue-light photoreceptor cry2, which affects seedling morphology in the absence of blue light (Botto et al., 2003).

phyA mediates two different types of response, the VLFR and the high-irradiance response (HIR). The

[1] This work was supported by the University of Buenos Aires (grant nos. G021 to J.J.C. and G013 to J.F.B), by the Agencia Nacional de Promoción Científica y Tecnológica (grant nos. PICT 11631 to J.J.C and PICT 10765 to JFB), and by the National Science Foundation (grant no. 0114976 to A.M.L.).

[2] Present address: INIBIOLP, Instituto de Investigaciones Bioquímicas La Plata, Facultad de Ciencias Médicas, Universidad de La Plata, calle 60 y 120, 1900 La Plata, Argentina.

[3] Present address: Universidad Nacional de Mar del Plata, Instituto de Investigaciones Biológicas, 7600 Mar del Plata, Argentina.

* Corresponding author; casal@ifeva.edu.ar; fax 5411–4514–8730.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.059071.

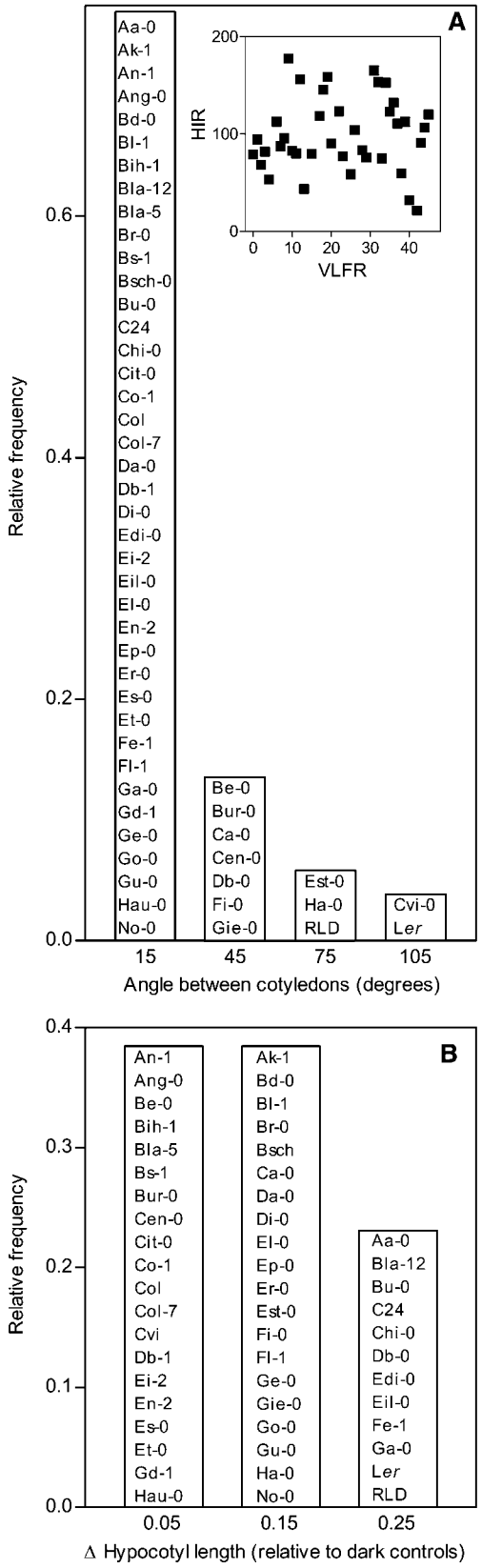

Figure 1. Variability of Arabidopsis accessions in the VLFR of cotyledon unfolding (A) and hypocotyl growth inhibition (B). The VLFR is calculated as the difference between dark controls and seedlings exposed to hourly FR pulses (cotyledon angle in darkness = 0 for all the accessions). The inset shows the lack of genetic correlation between VLFR and HIR of cotyledon angle of different accessions. Heritability was 68% for the VLFR of cotyledon unfolding, 94% for the VLFR of hypocotyl growth, and 70% for the HIR of cotyledon unfolding.

VLFR is saturated by a brief exposure to R or FR, which establishes a very small proportion of phyA in its Pfr form (Botto et al., 1996; Shinomura et al., 1996). In the case of hypocotyl growth or cotyledon unfolding, these pulses have to be repeated (e.g. 1 pulse/h) to obtain a visible response. The HIR requires either continuous FR or a frequency of FR pulses well beyond that necessary to saturate the VLFR, is specific for FR, and requires higher fluence rates than the VLFR (Casal et al., 2000). Specific domains of the phyA molecule (Casal et al., 2002; Yanovsky et al., 2002), FHY3 (Yanovsky et al., 2000; Wang and Deng, 2002), and PHYTOCHROME KINASE SUBSTRATE 1 and 2 (Lariguet et al., 2003) differentially affect VLFR and HIR. These discrete response modes of phyA are important under different ecological conditions. The VLFR is important, for instance, for the germination of seeds briefly exposed to light during soil tillage (Scopel et al., 1991). The HIR is critical for seedling survival under dense canopies (Yanovsky et al., 1995). So far, despite the identification of several VLFR QTLs, we have been unable to find any QTL affecting the HIR in the same RIL populations (Yanovsky et al., 1997; Botto et al., 2003). This could reflect a reduced variability for HIR in the RILs examined so far and/or an intrinsic limitation of the methodology to map such QTLs. Since continuous FR saturates the VLFR and causes an HIR, the actual HIR is calculated as the difference between continuous FR and hourly pulses of FR, which saturate the VLFR but do not initiate the HIR. This derived trait could therefore be more difficult to map.

Here, we report on the genetic variability among Arabidopsis accessions in VLFR and on the construction of a new set of RILs derived from a cross between L*er* and Nossen (No-0). L*er* was originally collected from Poland and No-0 from Germany. There are several reasons for this choice. First, while exploring variability among different accessions, we observed that L*er* and No-0 differed not only in VLFR but also in HIR. This provided an excellent opportunity to identify novel QTLs, particularly those controlling HIR. Second, since we had already used L*er* × Col and L*er* × Cvi RILs (Yanovsky et al., 1997; Botto et al., 2003), the availability of a third set of RILs sharing the L*er* parental line could be useful to investigate the degree of conservation of polymorphisms and to aid fine mapping. Third, since both accessions are frequently used in laboratory studies, the availability of L*er* × No-0 RILs would provide rapid means to investigate the genetic basis of polymorphisms between densely characterized accessions.

RESULTS

Variability among Arabidopsis Accessions in the Response to FR Pulses

We investigated the variability of the response of hypocotyl growth and cotyledon unfolding to hourly pulses of FR in 52 Arabidopsis accessions available at the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus). This light treatment induces weak responses mediated by phyA (Yanovsky et al., 1997) and is therefore ideal to explore the genetic control of sensitivity to light. Based on previous experiments showing poor responses in Col and significant responses in L*er*, Cvi, and RLD (Yanovsky et al., 1997; Casal et al., 2002; Botto et al., 2003), our prediction was that the accessions showing poor responses were the exception to the general pattern. The latter proved wrong as the vast majority of the accessions showed weak VLFR of cotyledon unfolding and weak to moderate VLFR of hypocotyl growth. The accessions with strong VLFR of hypocotyl growth did not necessarily show strong VLFR of cotyledon unfolding (compare with Fig. 1, A and B), and we observed no significant overall genetic correlation between both effects ($r^2 = 0.01$; $P > 0.4$).

We investigated in further detail the differences between L*er* and No-0, an accession frequently used as wild-type background and phenotypically representative of the majority under pulses of FR. No-0 seedlings are taller than those of L*er* in darkness (Fig. 2A) and show reduced inhibition of hypocotyl growth and cotyledon unfolding in response to pulses of FR (calculated Pfr/P = 10%; Fig. 2, B and C). The difference between L*er* and No-0 increased with the Pfr/P provided by hourly R/FR pulses, reaching a maximum for a calculated Pfr/P = 10% (Fig. 2, B and C). This corresponds to the phyA-mediated VLFR (Yanovsky et al., 1997). At higher Pfr/P, the response curves remained parallel, indicating no obvious differences in the phyB-mediated low-fluence response (LFR).

Most accessions show reduced cotyledon unfolding under pulses of FR, suggesting that common and/or accession-specific loci could be involved in repression of this VLFR. Before initiating the effort to obtain L*er* × No-0 RILs, we evaluated the potential of No-0 as a source of QTLs involved in VLFR different from those already identified as polymorphic between L*er* and Col. We observed seedlings with higher cotyledon angle under pulses of FR in the F_2 generation of Col × No-0 than in the parental lines (Fig. 3). This pattern of segregation suggests that at least partially different loci could be responsible for the reduced cotyledon unfolding in Col and No-0 compared to L*er*.

In addition to the VLFR, phyA also mediates the HIR, which requires sustained (continuous or very frequent) excitation with FR at higher fluence rates than those required to saturate the VLFR phase with hourly light pulses (Casal et al., 2000). The accessions showing stronger VLFR did not necessarily exhibit stronger HIR (Fig. 1A, inset). Actually, although No-0 showed a reduced VLFR of hypocotyl growth inhibition (Fig. 2B), its HIR was stronger (note steeper slope, Fig. 4A). The different slope was detectable in the linear phase of the log fluence rate response curve (i.e. below 10 μmol m^{-2} s^{-1}), and this argues against a physically constrained HIR as a consequence of enhanced VLFR in L*er* compared to No-0. L*er* reached maximum cotyledon unfolding at 10 μmol m^{-2} s^{-1} of

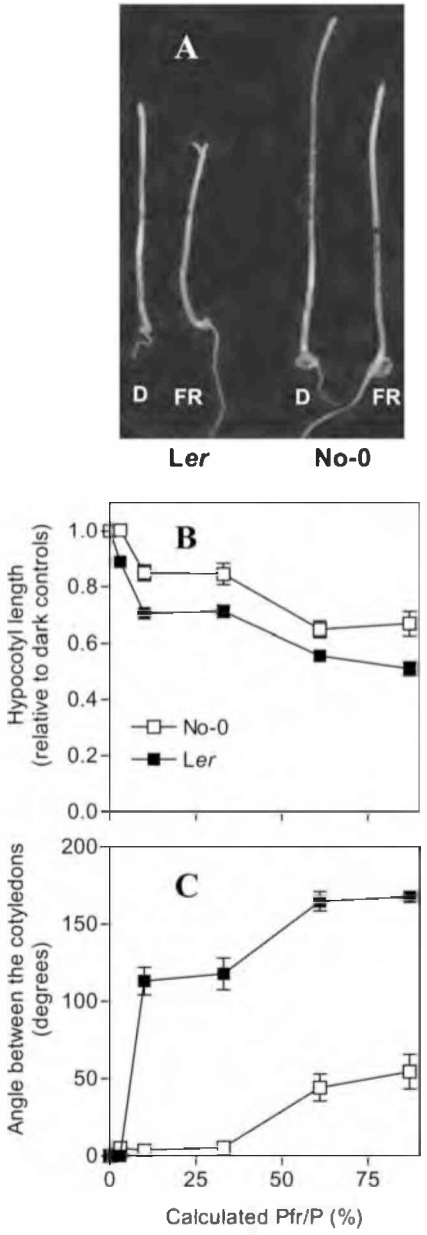

Figure 2. Reduced VLFR of hypocotyl growth and cotyledon unfolding in No-0 compared to L*er*. A, Representative seedlings of each accession grown in darkness (D) or under hourly pulses of FR. B, Hypocotyl length response to the proportion of Pfr (Pfr/P) established by hourly pulses of R/FR mixtures. C, Cotyledon angle response to Pfr/P. Data are means and SE of at least 17 replicate boxes (i.e. 170 seedlings).

continuous FR, and at lower fluence rates the average slope of the cotyledon angle-fluence rate response was similar for both accessions (Fig. 4B). The analysis of additional accessions showed that the enhanced HIR of hypocotyl growth in lines with reduced VLFR of the same process was neither exclusive to No-0 (see Eppenheim, Ep-0, in Fig. 4C) nor obligatory (see Achkarren, Ak-1, in Fig. 4C). Taken together, these observations suggest that at least partially independent loci control VLFR and HIR.

Generation of L*er* × No-0 RILs

To investigate in further detail some of the aforementioned differences among accessions, we generated a mapping population of 137 F_9 lines obtained by the single-seed descent method after a cross between the parental lines. A linkage map of polymorphisms between L*er* and No-0 was constructed with 46 microsatellite markers and Mapmaker (Lander et al., 1987; Fig. 5). The resulting average distance between markers was 11.3 cM, the maximum distance was 20.7 cM (between markers NF19K23 and nga111 in chromosome I), and the minimum distance was 1.50 cM. The average residual heterozygosity was 0.42%, very close to the expected value for F_9 lines (0.39%). Despite random selection of the plants to produce successive generations, some regions of the genome deviated significantly ($P < 0.05$) from the expected 1:1 segregation ratio of L*er* and No-0 alleles (Fig. 5). The ratio was largely below 1:1.8, which is expected not to affect QTL analysis (Clerkx et al., 2004, and refs. therein), except for a small region at the top of chromosome III (marker nga172) and another region in the lower arm of chromosome IV (between markers nga8 and msat4.37). Thus, QTLs mapping to the latter region should be considered with caution (none is reported here).

To illustrate the potential of these RILs, we mapped several QTLs that reflect variability in vegetative growth. In plants grown under short days in a glasshouse, we identified three QTLs controlling leaf

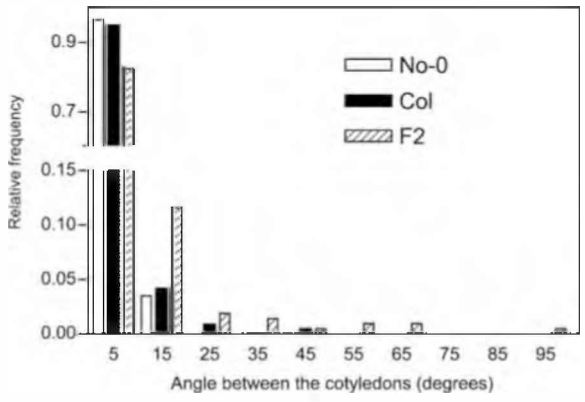

Figure 3. Segregation of cotyledon unfolding under hourly pulses of FR in the F_2 generation of a cross between No-0 and Col. Number of seedlings, F_2 = 216; Col = 245; No-0 = 86.

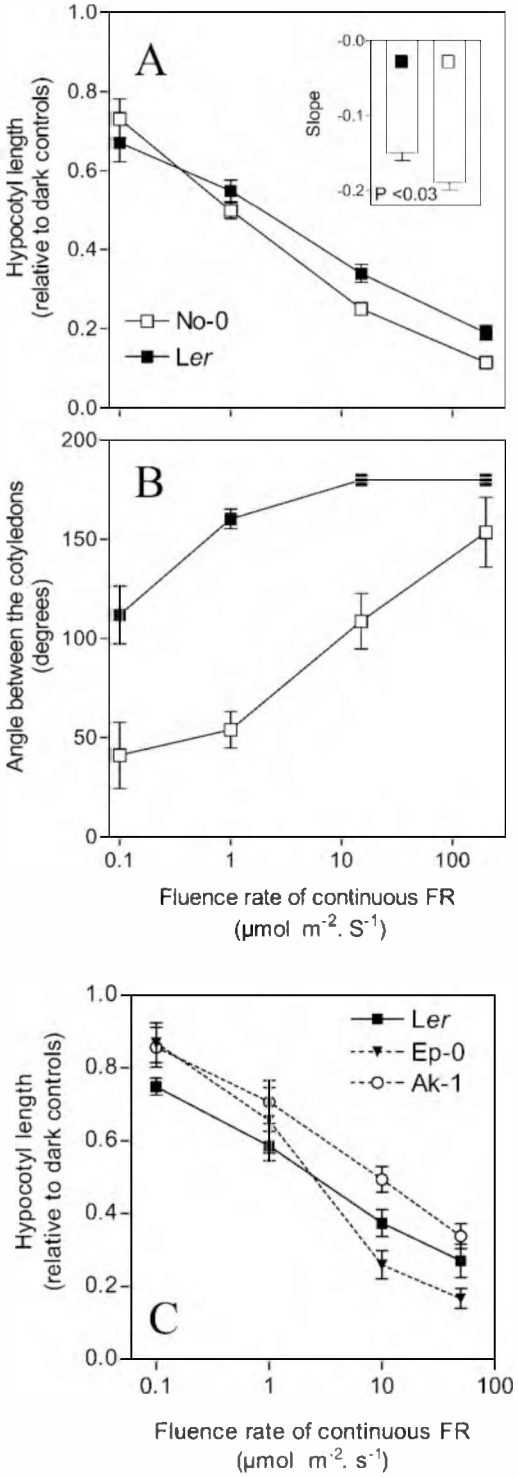

Figure 4. Enhanced HIR of hypocotyl growth in No-0 compared to L*er*. A, Hypocotyl length response to the fluence rate of continuous FR. The inset shows the average slope across the tested range of fluence rates and the results of Students' *t* test to compare the slopes. B, Cotyledon angle response to the fluence rate of continuous FR. C, Fluence response curves of hypocotyl length in Ep-0 and Ak-1 compared to L*er*. Data are means and SE of at least 12 (A and B) or three (C) replicate boxes.

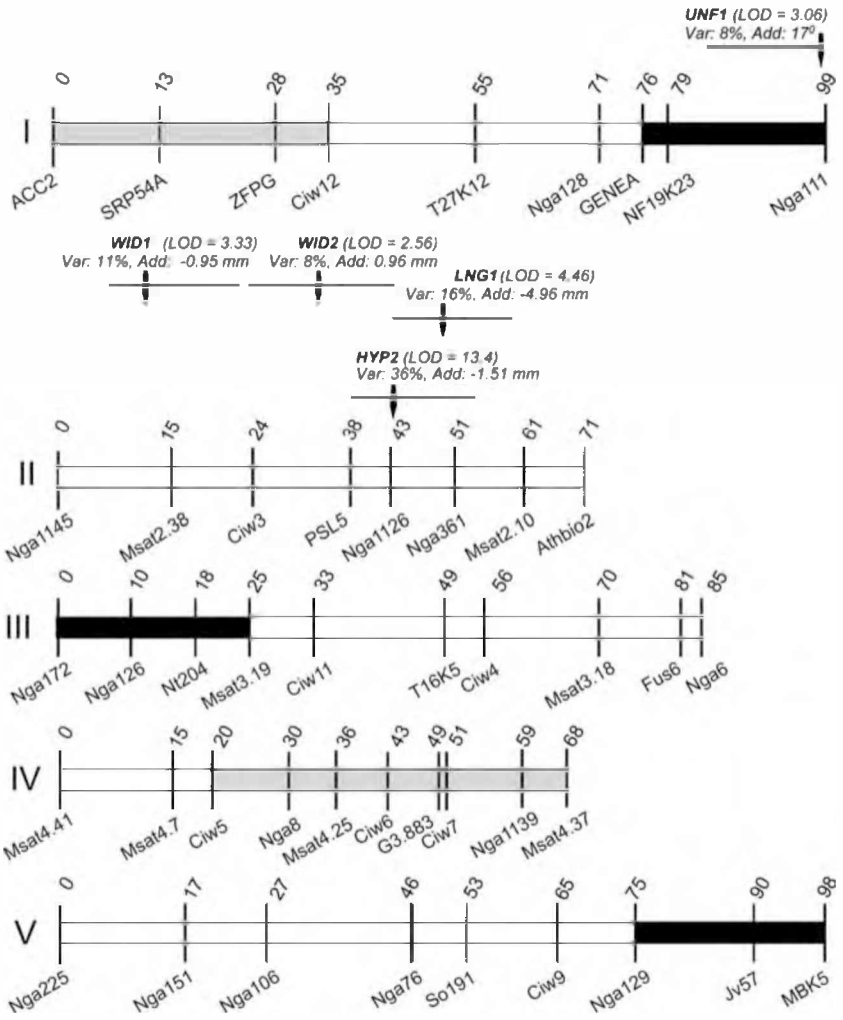

Figure 5. The L*er* × No-0 linkage map. Gray areas indicate regions with higher ($P < 0.05$) proportion of L*er* alleles and black areas indicate regions with higher proportion of No-0. The locations of QTLs affecting leaf lamina and petiole length (*LNG*), leaf lamina width (*WID*), hypocotyl length (*HYP2*), and cotyledon unfolding (*UNF1*), their 2-LOD support intervals, percent of accounted variability (Var), and additive effect (Add, L*er* minus No-0) are indicated.

dimensions, all in chromosome II (Fig. 5). *LNG* affects petiole length and leaf lamina length and apparently overlaps with ju-*LSI2*/ad-*PSI1*, mapped in RILs derived from a L*er* × Col-4 cross (Pérez-Pérez et al., 2002). *WID1* and *WID2* affect leaf lamina width (Fig. 5).

VLFR, LFR, and HIR in L*er* × No-0 RILs

Seedlings of the L*er* × No-0 RILs were grown under hourly pulses of FR, hourly pulses of R, or continuous FR (2 μmol m^{-2} s^{-1}), or in full darkness. In 4-d-old seedlings, we identified a QTL that collocates with *HYP2*, a QTL that accounts for the longer hypocotyl in No-0 compared to L*er* under different light or dark conditions, previously mapped using L*er* × Cvi RILs (Borevitz et al., 2002; Botto et al., 2003; Fig. 5). In L*er* × No-0, we also mapped *UNF1*, a QTL that caused enhanced cotyledon unfolding in L*er* than No-0 under continuous FR (Fig. 5). *HYP2* and *UNF1* are not VLFR-, LFR-, or HIR-specific. To investigate such specific QTLs, the VLFR was calculated as the differences between hypocotyl length or cotyledon angle in darkness versus hourly FR and the HIR as the difference between hourly and continuous FR. The LFR was calculated as the difference between hourly FR and R. Hypocotyl growth responses showed normal distribution and significant transgression (Fig. 6A). This was also the case for the LFR and HIR of cotyledon unfolding (Fig. 6B). For the VLFR of cotyledon unfolding, the extreme values corresponded to the parental lines and the distribution of RILs deviated significantly from a normal distribution (Fig. 6B). However, since similar mapping results were obtained for transformed and untransformed data of the latter trait we present mapping results based on untransformed data for the six traits.

Mapping of *VLF*, *LFR*, and *HIR* QTLs

We identified two QTLs affecting VLFR, two affecting HIR, and one affecting LFR (Fig. 7). One of the alleles affecting VLFR maps close to marker nga225 in chromosome V, a region where we had identified *VLF2* in L*er* × Col RILs (Yanovsky et al., 1997). As observed for L*er* × Col, the L*er* allele enhanced the VLFR of hypocotyl growth inhibition and cotyledon unfolding

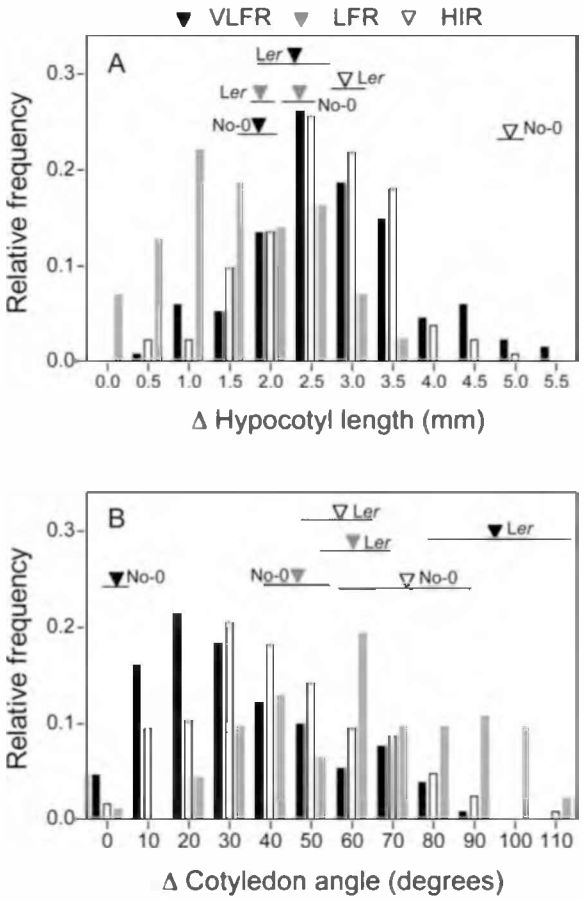

Figure 6. VLFR, LFR, and HIR in L*er* × No-0 RILs. A, Distribution of mean VLFR, LFR, and HIR of hypocotyl growth inhibition. B, Cotyledon unfolding. Heritability ranged between 33% and 38%. The means of the parental lines are indicated.

compared to No-0 (Fig. 7). Thus, this locus was tentatively named *VLF2*. A second QTL involved in VLFR maps close to the ciw3 marker in chromosome II and was named *VLF8* (Fig. 7) because it had not been mapped in L*er* × Col (Yanovsky et al., 1997) or L*er* × Cvi (Botto et al., 2003). *VLF8* enhanced the VLFR of hypocotyl growth inhibition and cotyledon unfolding in the RILs bearing the L*er* allele.

The *HIR1* QTL mapped close to the marker ACC2 at the top of chromosome I and caused enhanced HIR of cotyledon unfolding in the lines carrying the No-0 allele (Fig. 7). The *HIR2* allele mapped close to the nga1126 marker in chromosome II and enhanced the HIR of hypocotyl growth in the lines carrying the No-0 allele (Fig. 7). The *LFR1* locus mapped close to the marker nga1126 in chromosome II and enhanced cotyledon unfolding in the lines bearing L*er* alleles (Fig. 7).

Mapping of *HIR1*, *HIR2*, and *LFR1* is based on the difference between seedlings grown under FR pulses and seedlings grown under continuous FR or pulsed R. Since the FR pulses induce VLFR, we evaluated the possibility that these derived QTLs were mathematical artifacts created as a reaction to the presence of *VLF* loci. We conclude that *HIR1*, *HIR2*, and *LFR1* are genuine QTLs for the following reasons. First, *HIR1* and *LFR1* do not overlap with any locus involved in VLFR (maximum log of the odds [LOD] scores for cotyledon unfolding VLFR in the 2-LOD support

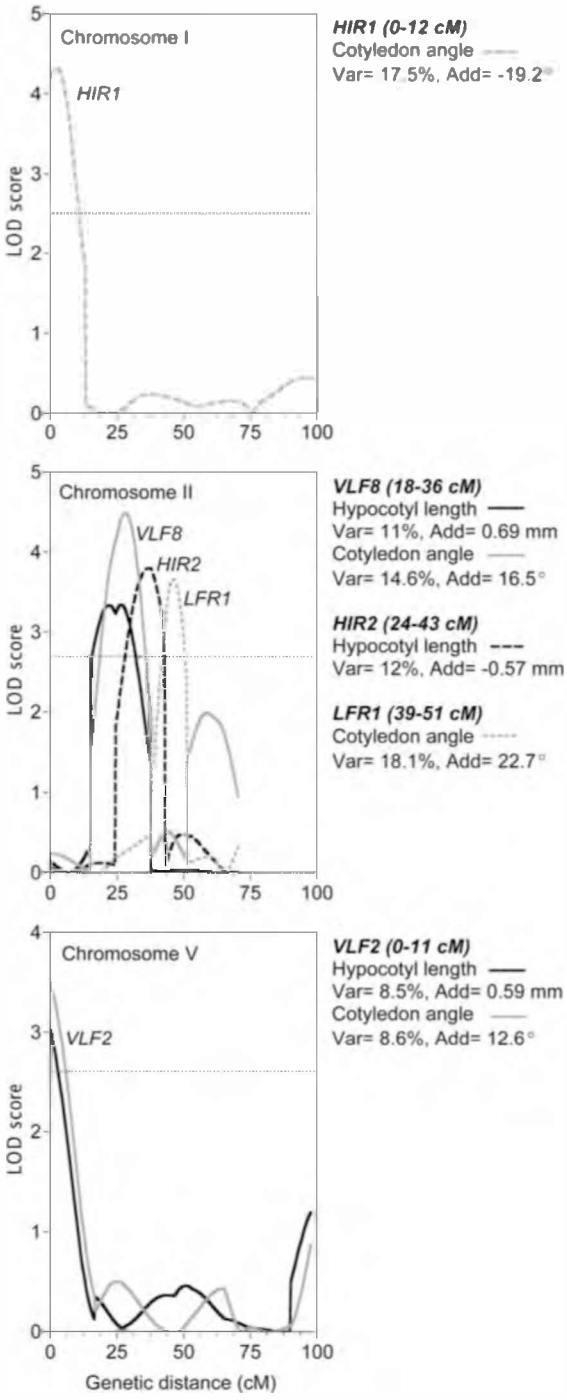

Figure 7. Likelihood plot of the QTLs affecting VLFR, LFR, and HIR of hypocotyl growth and cotyledon unfolding. The horizontal line represents the LOD threshold. The 2-LOD support interval, the percentage of accounted variation (Var), and the additive effect (Add) are indicated for each QTL.

interval were 0.1 and 0.5, respectively). Second, *HIR2* partially overlaps with *VLF8* but *VLF1*, another QTL with comparable LOD score for hypocotyl growth (3.3 and 3.0, respectively) and based on the same set of data (Fig. 7), had no associated QTL involved in HIR (maximum LOD score for HIR in the 2-LOD support interval of *VLF1* = 0.2). Thus, the occurrence of an *HIR* locus is not a necessary consequence of the presence of a *VLF* locus. Third, to minimize (dilute) the contribution of a VLFR component to the response to continuous FR without having to subtract the effect of FR pulses, we used 10 rather than 2 μmol m^{-2} s^{-1} of continuous FR. We mapped the difference in hypocotyl length between the seedlings grown in darkness and those grown under 10 μmol m^{-2} s^{-1} FR. Following this alternative procedure, any contribution of *VLF8* should play against finding *HIR2* because their effects have opposite signal and are not discriminated by the calculation. Despite this unfavorable protocol, a QTL indicating stronger inhibition by continuous FR in No-0 than L*er* was mapped close to the previous *HIR2* (Fig. 8). The small shift in location is likely to reflect the fact that the difference between darkness and 10 μmol m^{-2} s^{-1} FR still includes the diluted VLFR component playing against *HIR2* in the vicinity of *VLF8*. Thus, *HIR2* can be identified by a protocol that does not require subtracting the effect of hourly FR pulses.

DISCUSSION

To investigate the genetic basis of sensitivity to light, we compared the response of Arabidopsis accessions to pulses of FR and mapped QTL for light responses in a newly generated set of RILs between the L*er* and No-0 accessions. RILs derived from parents of divergent locations have allowed the identification of loci that denote adaptation to divergent geographical locations such as photoperiod (El-Assal et al., 2001). L*er* and No-0 are of relatively close geographic origins but they still contain significant variability as revealed by the leaf morphology, hypocotyl growth, and cotyledon unfolding QTLs reported here (Figs. 5 and 7) and the trichrome density and auxin response QTLs that will be reported elsewhere. Interestingly, L*er* and No-0 also occupied distant positions in the analysis of hypocotyl growth response to continuous light of different wavelengths (Maloof et al., 2001) and in microarray gene expression studies (Wang et al., 2002). The L*er* × No-0 RILs have already been donated and will become available through the ABRC stocks.

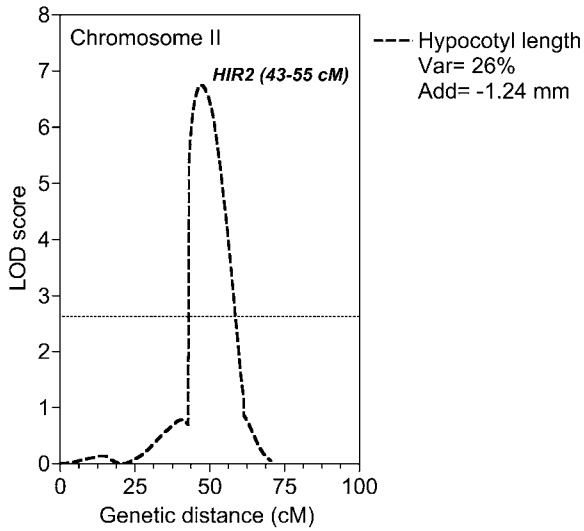

Figure 8. Identification of *HIR2* does not require incorporating hourly FR data in the calculations. Likelihood plot of the QTL affecting the difference in hypocotyl length between seedlings grown in darkness or under 10 μmol m^{-2} s^{-1}. The distribution of the data was normal (S = 1.45). The horizontal line represents the LOD threshold. The 2-LOD support interval, the percentage of accounted variation (Var), and the additive effect (Add) are indicated.

Previous reports describe the variability of Arabidopsis accessions in hypocotyl length under continuous light (Maloof et al., 2001; Botto and Smith, 2002). To explore the variability in sensitivity, here we focused on the response of two morphological traits (cotyledon unfolding and hypocotyl growth) to pulses of FR. The classical VLFRs to a single pulse of light are well documented in Arabidopsis for seed germination (Botto et al., 1996; Shinomura et al., 1996) and photosynthetic gene expression (Hamazato et al., 1997). A significant VLFR induced by pulses repeated with low frequency had also been reported for hypocotyl growth inhibition and cotyledon unfolding in L*er*, RLD, and Cvi, and even hypocotyl growth in No-0, while these VLFR are very weak in Col (Yanovsky et al., 1997; Casal et al., 2000, 2002; Botto et al., 2003). The results presented here represent a broader sampling and demonstrate that most accessions show a weak VLFR for cotyledon angle and a weak to moderate VLFR for hypocotyl growth (Fig. 1). The VLFR for cotyledon unfolding is weak in both Col and No-0 compared to L*er* but the F_2 generation of Col × No-0 yielded seedlings with open cotyledons under pulses of FR. This suggests the presence of accession-specific loci involved in the repression of morphological responses to weak light signals. We mapped two QTLs affecting VLFR in L*er* × No-0 (Fig. 7). One of these loci maps to the 0-to-11-cM region of chromosome V with *VLF2* (Yanovsky et al., 1997) and was named accordingly. L*er*, compared to No-0 or Col alleles of *VLF2*, enhance the VLFR of hypocotyl growth and cotyledon unfolding. In addition, we identified *VLF8*, a QTL in the 18-to-36-cM region of chromosome II. The L*er* allele of *VLF8* enhances the VLFR of hypocotyl growth and cotyledon unfolding compared to the No-0 allele. *VLF1*, which was previously found to be polymorphic between L*er* and Col and L*er* and Cvi (Yanovsky et al., 1997; Botto et al., 2003), and *VLF3* through *VLF7*, found to be polymorphic between L*er* and Cvi (Botto et al., 2003), were not mapped in the L*er* × No-0 RILs. Different accessions appear to achieve repression of VLFR by both shared and different loci,

underscoring their potential as a source of genetic variability.

We mapped one QTL affecting LFR and two QTLs affecting HIR (Fig. 7). *LFR1* maps to the 39-to-51-cM region of chromosome II. Compared to No-0, the L*er* allele at this locus enhances the difference in cotyledon unfolding induced by pulses of R and pulses of FR. *HIR1* locates to the 0-to-12-cM region of chromosome I, and No-0 alleles at this locus enhanced the HIR of cotyledon unfolding compared to L*er*. *HIR2* locates to the 24-to-43-cM region of chromosome II and No-0, compared to L*er*, alleles at this locus enhanced the HIR or hypocotyl growth. The contrasting L*er*/No-0 allelic effects of *VLF8* and *HIR2* account, at least in principle, for the differential FR fluence-rate response curves of the parental accessions (Fig. 4A). Many genes respond differentially to continuous FR in L*er* and No-0 (Wang et al., 2002). *HIR1* and *HIR2*, respectively, overlap with 11 genes (including the auxin-responsive protein IAA17 locus, At1g04250) and nine genes where the effect of continuous FR shows at least a 2-fold difference between L*er* and No-0. The *HIR1* locus also includes the *HFR1/RSF1/ REP1* gene, which has large effects on seedling deetiolation under FR (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). The *LFR1* locus includes *BAS1*, a cytochrome P450 (CYP72B1) involved in the regulation of phyB-mediated responses (Neff et al., 1999). The *VLF8* region includes *PIL5*, a negative regulator of phyA signaling (Oh et al., 2004). These genes are among the candidates to account for the polymorphism between L*er* and No-0. *VLF8*, *HIR2*, and *LFR* were given different names because they affect different physiological responses but, given the mapping intervals of these QTLs, we cannot exclude that they correspond to less than three different genes.

We have successfully mapped QTLs based on the differential response observed under two light conditions that differ either in the duration of the light exposure (for *HIR* loci) or the established proportion of Pfr (for the *LFR* locus). This indicates that RILs may be a powerful tool to map loci involved in regulating the shape of signal input-output relationships. Such loci could be difficult to identify if a single input at a time (a single light treatment in the case reported here) was mapped. Mutant screening protocols are based on the analysis of the phenotype under a given environment. Loci involved in the regulation of dose response curves may be more readily identified with the use of RILs, where a given genotype can be characterized under different conditions.

After the analysis of three sets of RILs (L*er* × Col, L*er* × Cvi, and L*er* × No-0), we have found no locus with significant effects in the same direction for more than one response mode (VLFR, LFR, HIR). For *VLF6/ CRY2*, the alleles that enhance VLFR reduce LFR, and this locus is considered to operate upstream of the phyA VLFR-signaling branch that down-regulates phyB signaling (Botto et al., 2003). *VLF1*, *VLF3*, *VLF4*, *VLF5*, *VLF6*, *VLF7*, *HIR1*, *HIR2*, and *LFR1* are specific either for hypocotyl growth or cotyledon unfolding, while only *VLF2* and *VLF8* affect both morphological traits (Yanovsky et al., 1997; Botto et al., 2003; Fig. 7). Thus, loci specific for a phytochrome response mode and a physiological process appear to be more frequent than pleiotropic loci. The occurrence of loci with specific effects may be required to yield a highly flexible network controlling plant shape in response to different light signals. Segregation of these loci can originate plants with different ranges of sensitivity to light for different physiological processes. The lack of correlation between the VLFR of hypocotyl growth and the VLFR of cotyledon unfolding or between the VLFR and the HIR of cotyledon unfolding of different accessions (Fig. 1) is consistent with this model.

MATERIALS AND METHODS

Generation of RILs

A set of 137 RILs were generated by single-seed descent to the F_8 generation from a segregating F_2 population derived from a cross between the laboratory strain L*er* (kindly provided by Maarteen Koornneef) and the accession No-0 (ABRC CS1394). Plants were grown in a growth chamber at 22°C under continuous fluorescent-white light. Seeds were bulked at the F_9 generation. L*er* originates from Northern Europe (Rédei, 1992). No-0 originates from Halle, Germany (51°N, 13°E–14°E), an area with an altitude of 200 to 300 m, where average spring/autumn temperatures and monthly precipitation are 5°C to 6°C/9°C to 10°C and 30 to 40/30 to 40 mm, respectively (http://www.arabidopsis.org/abrc/catalog/natural_accession).

Experimental Settings and Measurements

Fifteen seeds of each of the accessions (ABRC) or RIL were sown on 0.8% agar-water in clear plastic boxes (42 × 35 mm^2 × 20 mm to compare L*er* and No-0, and 215 × 85 mm^2 × 20 mm when multiple lines were involved) and incubated in darkness at 6°C for 3 d. Chilled seeds were given a saturating pulse of R and incubated in darkness at 22°C for 24 h. One-day-old seedlings were exposed to hourly pulses of R, FR, or R plus FR mixtures (3 min, 15–40 $\mu mol\ m^{-2}\ s^{-1}$; these fluence rates saturate the response to the pulses) or to continuous FR (fluence rates between 0.1 and 200 $\mu mol\ m^{-2}\ s^{-1}$) for 3 d, whereas control seedlings remained in darkness. Details of light sources, spectral distribution, and Pfr/P calculations were as described earlier (Yanovsky et al., 2000). Hypocotyl length was measured to the nearest 0.5 mm with a ruler and the largest 10 seedlings of each box (i.e. one replicate) were averaged. The angle between the cotyledons was measured with a protractor in the same seedlings used for length measurements and the 10 values obtained per box were also averaged before statistical analysis.

Genetic Mapping

We used 46 PCR-based markers (Fig. 5) to detect simple sequence length polymorphisms between L*er* and No-0 found in The Arabidopsis Information Resource database (http://www.arabidopsis.org) or in Loudet et al. (2002). Genomic DNA from the 137 F_9 RIL lines was extracted either from whole 10-d-old seedlings or from one or two rosette leaves from 2- to 3-week-old plants. The material was transferred to an Eppendorf tube and ground using a plastic pestle. Then, 150 μL of 2× cetyl-trimethyl-ammonium bromide extraction buffer (2% cetyl-trimethyl-ammonium bromide; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 100 mM Tris-HCl, pH 8.0; 1% polyvinyl-polypyrrolidone; and 0.2% β-mercaptoethanol) was added following vortexing for 15 s. Another 150 μL of extraction buffer was added and the sample was incubated 2 h at 65°C. Next, 300 μL of chloroform/isoamyl alcohol (24:1) was added, and the sample was well shaken and centrifuged 2 min. The aqueous phase was transferred to a clean Eppendorf tube. DNA was precipitated, washed, and dissolved in 100 μL of Tris-EDTA. DNA was diluted 10 times before being used for PCR reactions.

Thirty-nine markers were screened in similar PCR reactions (10 μL reaction mix in 96-well plates containing 4 μL of DNA; 200 μM of each dNTP, 1.5 mM $MgCl_2$; 0.6 μM primer, 1× PCR reaction buffer [Invitrogen, Carlsbad, CA], and 1 unit Taq Polymerase [Invitrogen]). For some markers, 2.0 mM Mg_2Cl was used. Amplification was performed using an Eppendorph Mastercycler gradient (1 cycle, 2 min at 95°C; 30 cycles, 45 s denaturation at 94°C, 45 s annealing at 57°C; 1 min elongation at 72°C; the cycles were followed by a final elongation at 72°C for 7 min). Ten microliters of loading buffer were added to the amplification reactions. Five microliters of this mix were loaded on 3% or 4% high-resolution agarose gels (MetaPhor, Bio Whittaker, Walkersville, MD) containing 50 μg/mL ethidium bromide and run for 3 h at 100 V. Markers ACC2, CIW5, NGA8, NT204, MSAT4.37, MSAT4.41, and MSAT 4.7 were screened by using a tailed-PCR scheme. Each forward primer was 5′ tailed with the M13 forward sequence and used in conjunction with a standard reverse primer and a FAM-6-labeled M13 primer. The M13 and reverse primers were equimolar and the forward primer was used at 15-fold lower concentration. Cycling was the same as described above, except that the annealing temperature was 52°C. Following confirmation of amplification success on standard agarose gels, 0.5 μL of each reaction was combined with 9.5 μL formamide and 0.5 μL size standard, and separated and sized on an ABI 3100 DNA analyzer.

Mapping and QTL Analysis

Mapmaker/EXP 3.0 (Lander et al., 1987) was used to construct the linkage map. Linkage groups were verified with a minimum LOD = 3 and a maximum distance = 50 cM (Kosambi function). Marker segregation data for the 137 RILs will become publicly available at http://www.arabidopsis.org/.

All RILs were grown simultaneously in each box. The experiments were repeated on three to five occasions (one box per experiment) and QTL analysis was based on the phenotypic mean of each RIL. The S statistic (Qstats package of QTL Cartographer; S. Wang, C.J. Basten, and Z.-B. Zeng [2001–2004] Windows QTL Cartographer 2.0. Department of Statistics, North Carolina State University, Raleigh, NC [http://statgen.ncsu.edu/qtlcart/WQTLCart.htm]) was used to test the normality of the distributions.

The composite-interval mapping (Zeng, 1994) procedure of QTL Cartographer (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) was used for QTL analysis. QTL cofactors were initially selected by using forward-backward stepwise multiple regression. Mapping was conducted with a walking speed = 0.5 cM and a window size = 3 cM. LOD thresholds for each trait were calculated with 5,000 permutations (Doerge and Churchill, 1996) and ranged between 2.4 and 2.68 ($P = 0.05$).

Received December 28, 2004; revised February 1, 2005; accepted February 2, 2005; published May 20, 2005.

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