Plant Physiol. (1997) 114: 1225–1236

Pea Mutants with Reduced Sensitivity to Far-Red Light Define an Important Role for Phytochrome A in Day-Length Detection[1]

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In garden pea (*Pisum sativum* L.), a long-day plant, long photoperiods promote flowering by reducing the synthesis or transport of a graft-transmissible inhibitor of flowering. Previous physiological studies have indicated that this promotive effect is predominantly achieved through a response that requires long exposures to light and for which far-red (FR) light is the most effective. These characteristics implicate the action of phytochrome A (phyA). To investigate this matter further, we screened ethylmethane sulfonate-mutagenized pea seedlings for FR-unresponsive, potentially phyA-deficient mutants. Two allelic, recessive mutants were isolated and were designated *fun1* for *FR unresponsive*. The *fun1–1* mutant is specifically deficient in the PHYA apoprotein and has a seedling phenotype indistinguishable from wild type when grown under white light. However, *fun1–1* plants grown to maturity under long photoperiods show a highly pleiotropic phenotype, with short internodes, thickened stems, delayed flowering and senescence, longer peduncles, and higher seed yield. This phenotype results in large part from an inability of *fun1–1* to detect day extensions. These results establish a crucial role for phyA in the control of flowering in pea, and show that phyA mediates responses to both red and FR light. Furthermore, grafting and epistasis studies with *fun1* and *dne*, a mutant deficient in the floral inhibitor, show that the roles of phyA in seedling de-etiolation and in day-length detection are genetically separable and that the phyA-mediated promotion of flowering results from a reduction in the synthesis or transport of the floral inhibitor.

The phytochromes are a family of biliprotein photoreceptors that absorb predominantly in the R and FR regions of the spectrum and mediate a diverse range of developmental responses to light (Quail et al., 1995; Smith, 1995; Furuya and Schäfer, 1996). Higher plant phytochromes are encoded by a small gene family, which in Arabidopsis consists of five genes (*PHYA* to *PHYE*) in four subfamilies; A, B(D), C, and E (Sharrock and Quail, 1989; Clack et al., 1994). The differentiation of subfamilies A, B, and C from a single ancestral phytochrome is thought to have occurred before or soon after the emergence of higher plants, and subsequent duplication and divergence within each subfamily appears to have taken place to a varying extent depending on the species (Mathews et al., 1995). In tomato (*Lycopersicon esculentum* L.) at least 5 phytochrome genes are expressed, and as many as 13 phytochrome-related sequences may be present in the genome (Hauser et al., 1995). *PHYA* is strongly expressed in dark-grown plants and encodes the well-characterized light-labile phytochrome that is the predominant phytochrome in etiolated tissue. Expression of *PHYA* is strongly repressed by light, in contrast to the other *PHY* genes, which are expressed at a much lower level in etiolated tissue and are not markedly light-regulated (Clack et al., 1994).

Although all phytochromes studied to date absorb both R and FR light, recent evidence indicates that individual members of the phytochrome family have specialized functions. This evidence has been obtained predominantly from the study of mutants deficient in specific phytochromes. R light absorbed by phyB-type phytochromes induces seed germination (Shinomura et al., 1994) and promotes and maintains seedling de-etiolation (Somers et al., 1991; McCormac et al., 1993; Reed et al., 1993; van Tuinen et al., 1995b), whereas the absorption of FR light by phyB results in the suppression of these responses. In contrast, phyA promotes seed germination and seedling de-etiolation in response to both R and FR light, and has a much lower fluence threshold than phyB (Parks and Quail, 1993; Reed et al., 1994; van Tuinen et al., 1995a; Botto et al., 1996; Shinomura et al., 1996).

These differences in spectral sensitivity are also reflected in the responses of transgenic *PHYA*- or *PHYB*-overexpressing plants (McCormac et al., 1993). It therefore appears that in the control of de-etiolation, phyA and phyB can act in concert or antagonistically, depending on their relative levels of expression and on the ratio of R to FR light in the incident light. This may also be the case for the control of the transition to flowering, which in the LD plant Arabidopsis is inhibited by phyB (Goto et al., 1991; Halliday et al., 1994) and, under some circumstances at least, is promoted by phyA (Johnson et al., 1994).

[1] This paper was supported in part by an Australian Postgraduate Research Award to J.L.W. and a grant to J.B.R. from the Australian Research Council.

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Abbreviations: FR, far-red; LD, long day(s); phyA, phytochrome A; phyB, phytochrome B; R, red; SD, short day(s); WFL, light from cool-white fluorescent tubes; WI, white light from incandescent globes.

We have previously reported the characterization of pea (*Pisum sativum* L.) mutants deficient in phyB (Weller et al., 1995) and in the synthesis of the phytochrome chromophore (Weller et al., 1996, 1997). phyB-deficient pea mutants retain promotion of de-etiolation by FR light, implying that at least one other phytochrome in addition to phyB is necessary for normal light-regulated development. We report here on the isolation and initial characterization of a pea mutant specifically deficient in phyA. In addition to displaying a FR-light-insensitive seedling phenotype, this mutation has a profoundly pleiotropic effect on the phenotype of the mature plant, which is related to an inability to detect day length. We also present evidence that in pea the pathway for phyA action in the mature plant is distinct from that in de-etiolating seedlings, and in part involves the action of an as yet unidentified inhibitory influence that can be transmitted across a graft union.

MATERIALS AND METHODS

Growth Conditions and Plant Husbandry

Four different growing environments were used for the experiments described below. Plants used for immunoblotting (Fig. 3), in vivo spectrophotometry (Table I), and pulse experiments (Fig. 2D) were grown at RIKEN (Wako, Japan) in growth cabinets at 25°C. These plants were grown in water-saturated, drained vermiculite. All other plants were grown at Hobart (Australia) in the greenhouse, in the phytotron, or in growth cabinets, in a 1:1 mix of dolerite chips and vermiculite topped with potting mix. For mutant screening, seedling de-etiolation experiments, and segregant selection, plants were grown in growth cabinets at 20°C. In the Hobart greenhouse plants received an 18-h photoperiod, which was comprised of a natural photoperiod extended before dawn and after dusk with mixed WFL and WI. This environment was used as general-purpose LD space for all genetic experiments and for physiological experiments presented in Figures 4, 5, 6, and 9. In the Hobart phytotron, plants received an 8-h photoperiod of natural daylight in a greenhouse before transfer to night compartments maintained at 16°C, where various light treatments were administered. This environment was used for photoperiod and grafting experiments (Figs. 7, 8, and 10). Standard Hobart and RIKEN light sources were employed as described by Weller et al. (1997).

All plants grown to maturity received nutrient solution once weekly. Lateral shoots were regularly excised from all plants grown in the flowering experiments. Only main shoots were scored, and counting of nodes began with the cotyledons as node 0. Stem length was variously measured as the length between two nodes x and y.

Table I. *In vivo spectrophotometric measurement of phytochrome in phytochrome-deficient mutant seedlings*

Phytochrome content was measured in etiolated seedlings after 5 d of growth in complete darkness, or after an additional 4-h exposure to R light (17 μmol m^{-2} s^{-1}). Values are expressed as the mean ± SE of four samples. Percentages within parentheses indicate phytochrome content relative to etiolated wild-type seedlings.

Genotype	$\Delta A_{(730-800\ nm)}$	
	Dark	4 h of Red Light
Wild type	20.7 ± 1.2	3.0 ± 0.3 (14.5)
fun1-1	0.6 ± 0.2	0.5 ± 0.1 (2.4)
pcd2	0.2 ± 0.1	0.2 ± 0.1 (<1.0)
lv-5	18.9 ± 2.1	2.8 ± 0.1 (13.5)

Plant Material, Mutagenesis, and Mutant Screening

Approximately 1500 seeds of wild-type pea (*Pisum sativum* L. cv Torsdag) were allowed to imbibe in 1 L of a 1% (v/v) solution of ethylmethane sulfonate for 6 h at room temperature (approximately 18°C). After rinsing for an additional hour in running water, seeds were planted in standard potting mix. These M_1 plants were grown to maturity under an 18-h photoperiod in the greenhouse. M_2 seeds from individual plants were harvested, planted in plastic tote boxes, and grown under 8 μmol m^{-2} s^{-1} FR light for selection of FR-light-unresponsive mutants. Each family was represented by no more than eight individuals. After 7 d the tote boxes were transferred to the greenhouse, where selected mutants were allowed to de-etiolate for several days before transplantation to pots. Except where specified, the *fun1-1* mutant material used in physiological experiments was bulked from mutant segregants in the F_3 of the second successive back-cross of the original AF140 line to cv Torsdag. The mutant line K218 (*dne*) was isolated following ethylmethane sulfonate mutagenesis of cv Torsdag by Uzhintseva and Sidorova (1979), and characterized by King and Murfet (1985).

Phytochrome Detection and Quantitation

The immunochemical detection of PHYA and PHYB followed a procedure described previously (Weller et al., 1995) and employed the monoclonal antibodies mAP5 (anti-PHYA; Nagatani et al., 1984) and mAT5 (anti-PHYB; López-Juez et al., 1992). Spectrophotometric measurements of phytochrome were made according to the method described by Weller et al. (1995).

Grafting

Grafting experiments employed a wedge-grafting technique. Plants used for stocks were grown one plant per pot for 11 d from the time of sowing, at which time the fourth internode (between nodes 4 and 5) was fully expanded in all plants. Plants were decapitated between nodes 4 and 5 and a longitudinal slit of about 1 cm was cut down the center of the stem to receive the scion. Plants used for scions were planted more deeply than usual (3 to 4 cm below the soil surface) to provide a greater length of epicotyl from which to prepare the graft wedge. These plants were grown for 6 d from the time of sowing, and were decapitated at the epicotyl 0.5 to 1 cm above the cotyledonary node. A graft wedge was prepared from the cut end of the scion and introduced into the stock. The graft union was secured by a small rubber band, and pots were then

enclosed in plastic bags to maintain high humidity during establishment of the graft. Lateral shoots arising from the stock were excised daily.

RESULTS

Isolation of FR-Light-Unresponsive Mutants

Approximately 6000 M_2 seedlings representing 1100 M_1 families were screened under continuous FR light (8 μmol m^{-2} s^{-1}). Wild-type pea plants grown under these FR light conditions were similar in total stem length to plants grown in darkness, but showed decreased internode length, increased leaflet expansion, and an increased rate of node production. We isolated several mutants that failed to respond to the FR light, exhibiting the long internodes, unexpanded leaves, and apical hook typical of pea seedlings grown in complete darkness.

Reduced sensitivity to FR light can result from a deficiency in the synthesis of the phytochrome chromophore phytochromobilin (Parks and Quail, 1991; Terry and Kendrick, 1996; Weller et al., 1996, 1997), as well as from mutations that specifically affect phyA. However, mutants deficient in phytochromobilin are also insensitive to R light (Koornneef et al., 1980, 1985; Weller et al., 1996, 1997). To exclude such mutants, we screened the M_3 progeny of the FR-light-insensitive lines under R light. This secondary screen identified a relatively normal response to R light in two of the FR-light-insensitive mutant lines (AF140 and AF188). The phenotypes of these mutants under FR light are shown in Figure 1A. When grown to maturity under our standard greenhouse conditions (18-h photoperiod), both lines exhibited a short-internode, late-senescing phenotype. Complementation testing under FR light showed the two mutants to be allelic (Fig. 1B), and AF140 was selected for further investigation.

Grown under FR light, the F_2 generations of backcrosses of AF140 and AF188 to their parental line cv Torsdag segregated into two classes (Fig. 1B). In both crosses, the ratio of wild type to FR-light-unresponsive mutant segregants was in close accordance with a 3:1 ratio (for AF140, 30:12, $\chi^2_{(3:1)} = 0.29$, $P > 0.5$; for AF188, 30:7, $\chi^2_{(3:1)} = 0.72$, $P > 0.3$). This indicated that in both cases the mutant phenotype resulted from a recessive mutation at a single locus, which we have designated *fun1*, for FR *un*responsive. Preliminary mapping data have placed the *fun1* locus 28 units below the *k* locus in linkage group II (J.L. Weller, unpublished data). In F_2 progeny of the same crosses, grown to maturity under our standard greenhouse conditions, the reduced internode length and delayed senescence seen in mature AF140 (*fun1–1*) and AF188 (*fun1–2*) plants showed perfect cosegregation, indicating that these are most likely to be additional pleiotropic effects of mutation at the *fun1* locus.

fun1 Mutant Seedlings Are Specifically Insensitive to FR Light

When grown in complete darkness, the *fun1–1* mutant did not differ significantly from dark-grown wild-type plants, either in terms of internode length or leaf expansion (Fig. 2, A and B). *fun1–1* plants grown in FR light also did not differ significantly from dark-grown wild-type plants in either respect (Fig. 2, A and B), indicating that the *fun1–1* mutant is completely lacking in the ability to respond to continuous FR light. However, *fun1–1* seedlings were virtually indistinguishable from wild type when grown under WFL (Fig. 2C), and were also very similar to wild type under R light. Under blue light, the *fun1–1* mutant showed similar internode elongation to wild type, but showed substantially reduced leaf expansion. A specific insensitivity to FR light and the lack of a clear phenotype in WL are characteristic of mutants in Arabidopsis and tomato that are deficient in functional phyA (Parks and Quail, 1993; Whitelam et al., 1993; van Tuinen et al., 1995a).

We also examined the R/FR reversibility of de-etiolation in the *fun1–1* mutant in comparison with the phyB-deficient *lv-5* mutant (Fig. 2D). In wild-type seedlings the de-etiolation induced by brief pulses of R light showed partial FR light reversibility. As previously reported, phyB-deficient plants were less responsive to the R light pulses due to selective loss of the FR-light-reversible component

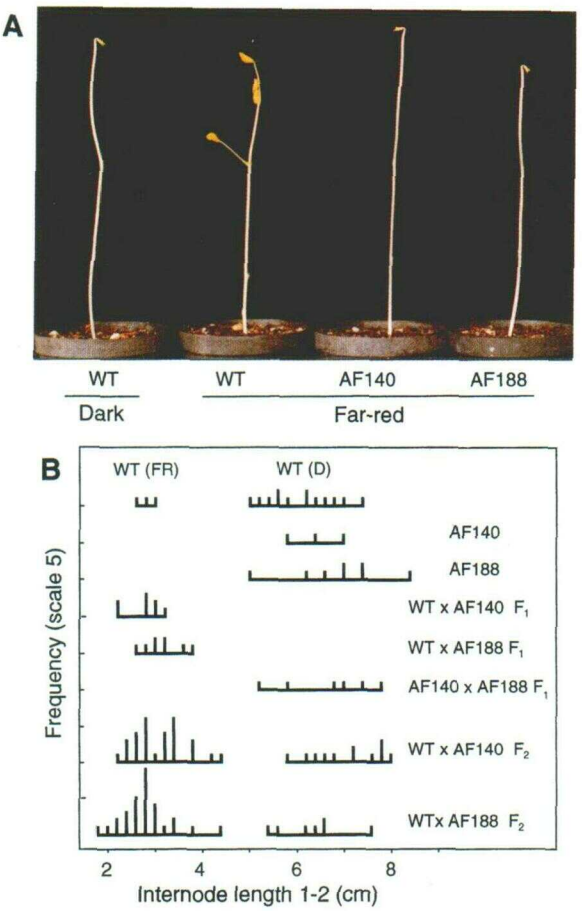

Figure 1. Isolation of pea mutants with reduced response to FR light. A, Phenotype of FR-light-unresponsive mutant lines AF140 and AF188 compared with that of wild-type plants (WT). B, Genetic analysis of mutant lines AF140 and AF188. All plants were grown for 7 d at 20°C under continuous FR light (8 μmol m^{-2} s^{-1}), with the exception of WT(D), wild-type plants grown in complete darkness.

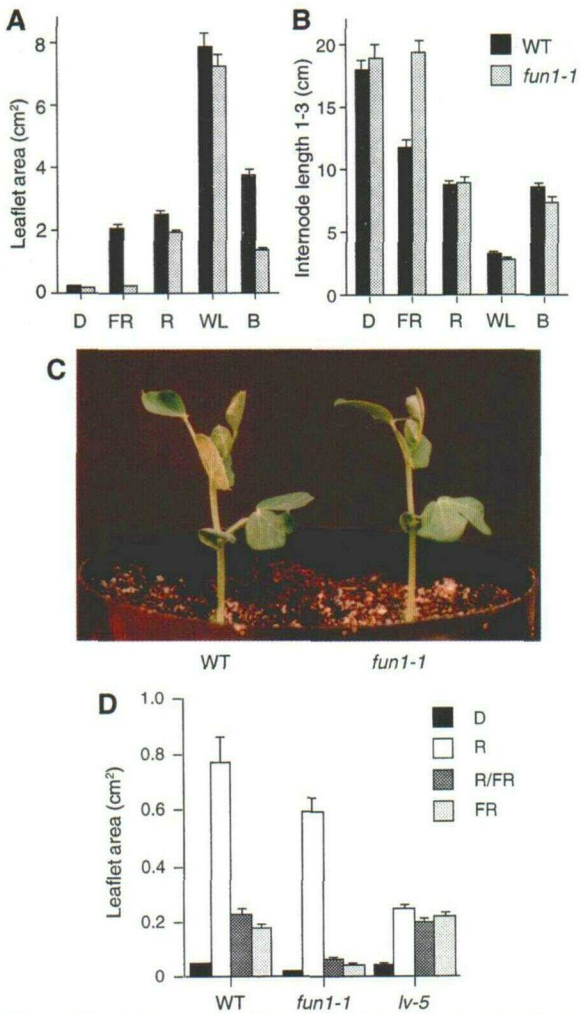

Figure 2. De-etiolation of wild-type (WT) and *fun1–1* mutant seedlings grown under continuous monochromatic light. Seedlings were grown at 20°C in complete darkness (D) or under continuous FR light (8 μmol m^{-2} s^{-1}), R light (R; 20 μmol m^{-2} s^{-1}), blue light (B; 10 μmol m^{-2} s^{-1}), or white light (WL; 150 μmol m^{-2} s^{-1}). A, Leaflet area estimated as the product of the length and width of a leaflet from leaf 3. B, Stem length between nodes 1 and 3. C, Phenotype of 10-d-old wild-type and *fun1–1* seedlings grown under white light. D, R/FR reversibility of de-etiolation in wild-type, *fun1–1*, and *lv-5* seedlings. Plants were grown at 25°C and given saturating pulses of R light (17 μmol m^{-2} s^{-1}), FR light (12 μmol m^{-2} s^{-1}), or R light followed by FR light (R/FR) at 4-h intervals, or were maintained in complete darkness (D) for 8 d from the time of sowing. Error bars represent SE; n = 12 to 15.

(Weller et al., 1995). *fun1–1* seedlings were also somewhat less responsive than wild type to the R light pulses. However, in contrast to *lv-5* seedlings, de-etiolation in *fun1–1* mutant seedlings showed normal FR light reversibility, but was substantially reduced in response to treatments terminating with FR light.

fun1 Mutants Are Deficient in phyA

To determine whether phyA was selectively affected in the *fun1–1* mutant, we examined PHYA and PHYB apoprotein levels in the mutant by immunoblotting. Figure 3 shows clearly that the 121-kD PHYA apoprotein is undetectable in crude protein extracts from etiolated *fun1–1* seedlings. In contrast, PHYB, which was detected by the monoclonal antibody mAT5 as two bands of 115 and 117 kD, and which is lacking in *lv* mutants (Weller et al., 1995), is present at a normal level in *fun1–1*. An examination of phytochrome levels in etiolated *fun1–1* seedlings by in vivo difference spectrophotometry revealed that the *fun1–1* mutant possesses only about 2.5% of the photoreversible phytochrome present in the wild type (Table I). This indicates that phyA accounts for at least 97.5% of the spectrally active phytochrome in etiolated pea seedlings.

After a 4-h R light treatment, phytochrome in wild-type seedlings was depleted to about 15% of the dark level, whereas the level in *fun1* seedlings was not appreciably altered. These results indicate that phyA makes up about 80% of the phytochrome pool present in seedlings that have been grown in the dark and then exposed to 4 h of R light. However, this is almost certainly an overestimate of the proportion of phyA in fully de-etiolated plants, because phytochrome in wild-type pea seedlings can be depleted below the level present after 4 h of R light (J.L. Weller, unpublished data). In comparison, the phyB-deficient *lv-5* mutant has levels of photoreversible phytochrome that are not significantly different from wild type, indicating that the contribution of phyB to the stable pool is very small.

phyA Deficiency Confers a Highly Pleiotropic, Short-Internode, Late-Flowering Phenotype

As mentioned above, mutant *fun1* seedlings were virtually indistinguishable from wild type when grown for up to 12 d under continuous WFL (Fig. 2C). However, when grown in our standard greenhouse conditions under a natural photoperiod extended to 18 h with a mixture of WI

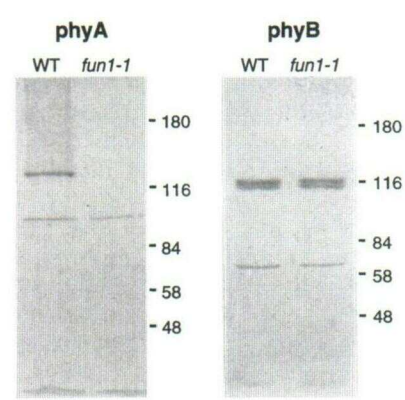

Figure 3. Immunoblot detection of PHYA and PHYB apoprotein in crude protein extracts from 5-d-old etiolated wild-type cv Torsdag (WT) and *fun1–1* seedlings. The positions and molecular masses (in kilodaltons) of prestained markers (Sigma) are indicated. PHYA is detected as a single band of approximately 121 kD by monoclonal antibody mAP5 (Nagatani et al., 1984), whereas PHYB is detected as two bands of 115 and 117 kD by monoclonal antibody mAT5 (López-Juez et al., 1992). Lanes were loaded on an equivalent fresh weight basis.

and WFL, *fun1* plants exhibited a striking phenotype (Fig. 4A) with a reduction in internode length of up to 50% relative to wild type. The onset of this short-internode phenotype was monitored in more detail by measuring lengths of individual internodes in *fun1* plants grown in the greenhouse. Under these conditions, internodes in the *fun1* mutant were slightly longer than those of the wild type until about node 6, beyond which a dwarfing effect of *fun1* gradually became apparent (Fig. 5A).

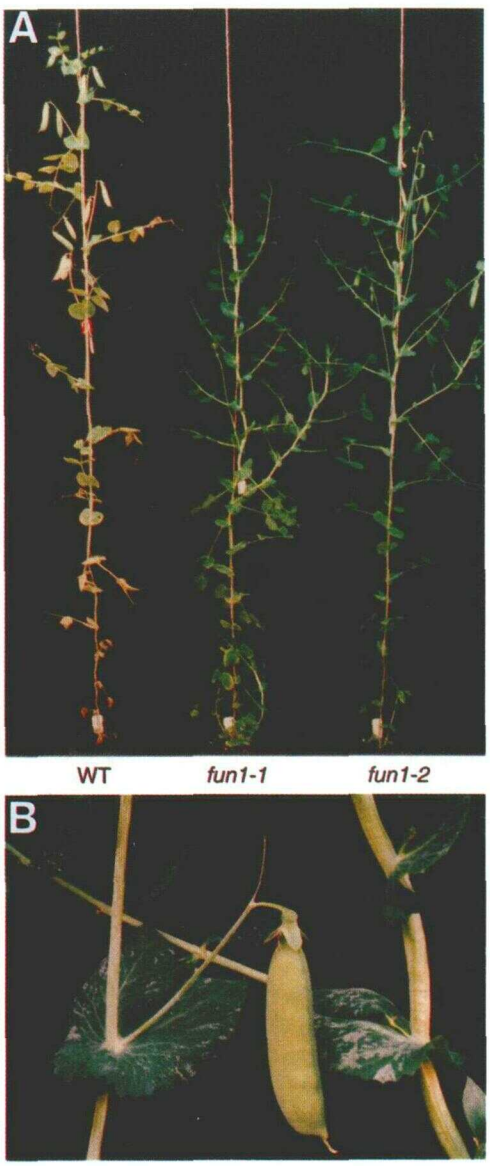

Figure 4. Phenotype of mature *fun1* plants. A, Representative wild-type cv Torsdag (WT), *fun1–1*, and *fun1–2* plants grown under an 18-h photoperiod. The mutants show reduced internode length, increased lateral branching from aerial and basal nodes, and delayed flowering and senescence relative to wild type. Plants are approximately 8 weeks of age. B, Comparison of stem morphology at the node of flower initiation in wild-type (left) and *fun1–1* (right) plants grown under an 18-h photoperiod. The *fun1–1* mutant internodes show reduced length, increased thickness, and transverse banding. Note also the aborted flower initials in the axils of the *fun1* plant.

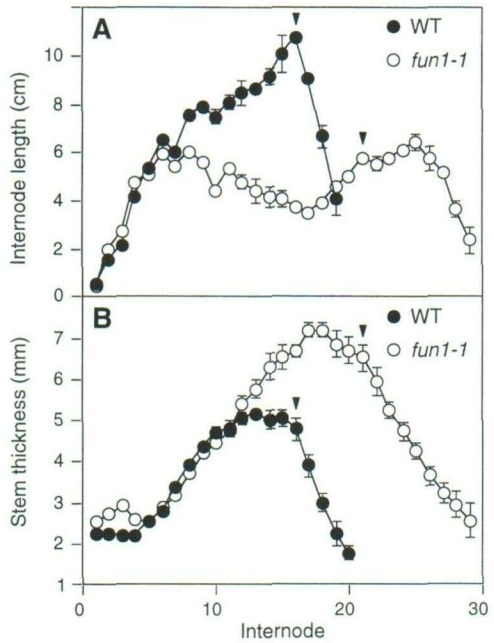

Figure 5. Ontogenetic development of the *fun1* stem phenotype. A, Internode length. B, Internode thickness. Internode length and maximum thickness were measured for each internode in wild type (WT) and *fun1–1* segregants in the F_2 of the second back-cross of AF140 to wild-type cv Torsdag. Plants were grown under an 18-h photoperiod in the greenhouse. The node of flower development for each genotype is indicated by a small arrowhead. Bars represent SE; $n = 6$.

The reduction in internode length in *fun1* plants was associated with a thickening of the stem (Fig. 5B), which commenced at about node 12 and became particularly severe several internodes below the node of flower initiation (Fig. 4B). Later internodes often took on a yellowish, succulent appearance and showed prominent transverse banding (Fig. 4B). The severity of the stem phenotype gradually diminished once regular pod set was established.

Wild-type and *fun1* plants also differed in other respects. Whereas wild-type cv Torsdag plants do not show any lateral branching when grown under our standard greenhouse (LD) conditions, both *fun1* mutants branched strongly both from basal and aerial nodes (Fig. 4A). Differences between wild-type and *fun1–1* plants are shown in Table II. The *fun1–2* mutant showed a phenotype similar to *fun1–1* for all of the characteristics listed. When grown under LD conditions, wild-type plants flowered at about node 16. Flowers developed on relatively short peduncles and set strongly, with plants producing 5 to 6 flowering nodes and 20 to 30 seeds before apical arrest (Table II). However, in *fun1–1* plants flower initials did not appear until about node 19 and aborted for several nodes (Fig. 4B), not developing into mature flowers until about node 24 (Table II).

The first flower to develop on *fun1–1* plants opened much further behind the apical bud and on a much longer peduncle than in the wild type (Table II). Most striking was the large delay in apical arrest and senescence of *fun1* plants, which produced four times as many reproductive

Table II. *Phenotypic characteristics of fun1–1 plants grown in LD conditions*

Characteristics were measured in wild-type (n = 76) and *fun1-1* (n = 28) segregants in the F_2 of a cross between wild type cv Torsdag and AF140. All plants were grown under an 18-h photoperiod in standard greenhouse conditions.

Characteristic	Genotype	
	Wild type	*fun1–1*
Internode length 1–12 (cm)	80.4 ± 0.8	56.9 ± 1.0
Node of flower initiation (NFI)	16.5 ± 0.1	19.0 ± 0.3
Node of flower development (NFD)	16.5 ± 0.1	24.1 ± 0.8
Leaf/flower relativity index[a]	0.65 ± 0.02	1.92 ± 0.09
Peduncle length at NFD (centimeters)	10.4 ± 0.2	18.7 ± 0.8
Total nodes at apical arrest (TN)	22.1 ± 0.2	41.2 ± 0.6
Reproductive nodes (TN-NFI+1)	5.7 ± 0.2	23.2 ± 0.7
Seed yield (seeds/plant)	29.6 ± 0.6	71.1 ± 2.4

[a] The number of leaves expanded above the first open flower at the time of opening. This is a measure of the commitment of resources to vegetative relative to reproductive development, and is the negative of flower/leaf relativity defined by Murfet (1982).

nodes before apical arrest and gave two to three times the yield (60–100 seeds) of wild-type plants (Table II). In some cases, *fun1* plants grew for more than 6 months before senescence, whereas wild-type plants senesced and dried after about 3 months. The *fun1* mutations also appeared to alter leaf development, conferring a characteristic buckled appearance to leaflets (Fig. 6A), suggestive of a greater rate of expansion of interveinal photosynthetic tissue relative to vascular tissue. Finally, *fun1* leaves developed to a greater degree of complexity, with some leaves developing four pairs of leaflets and thus exceeding the maximum of three pairs seen in wild-type plants grown under the same LD conditions (Fig. 6B).

phyA Is Necessary for the Detection of Photoperiod Extensions

The phenotypic syndrome exhibited by *fun1* mutant plants in LD conditions was very similar to that shown by wild-type plants grown in SD conditions. Under SD conditions, wild-type plants show delayed flower initiation, retarded flower and fruit development, longer peduncles, delayed transition to more complex leaf pattern, and delayed onset of apical senescence (Barber, 1959; Murfet, 1982, 1985). We therefore considered that *fun1* plants might be unable to detect the difference between LD and SD conditions. To test this possibility, wild-type and *fun1–1* plants were grown under our standard phytotron SD (8 h of natural daylight) and LD conditions (8 h of natural daylight extended with 16 h of WI). The low fluence rate used in the extension (10 μmol m^{-2} s^{-1}) excludes any substantial contribution to total photosynthetically active radiation.

Figure 7A shows that *fun1* plants grown in LD conditions were in fact very similar in appearance to wild-type plants grown in SD conditions. Wild-type plants developed longer internodes and flowered earlier in response to a photoperiod extension, whereas *fun1* plants were essentially unresponsive to the extension, showing a similar growth habit in both LD and SD conditions. The lack of response in *fun1* is also manifest in other characteristics, both reproductive and vegetative. These include stem elongation and senescence (Fig. 7B), as well as branching, peduncle length, and seed number (data not shown). These results indicate that phyA is the primary phytochrome responsible for the detection of a FR-rich photoperiod extension in pea.

Since *fun1* seedlings are specifically insensitive to FR light (Fig. 2), we next considered whether the inability of *fun1* plants to detect a photoperiod extension was restricted to extensions employing FR-rich light. However, *fun1* plants were similarly unresponsive to 16-h extensions with low-intensity (10 μmol m^{-2} s^{-1}, Fig. 8) or high-intensity (150 μmol m^{-2} s^{-1}, data not shown) WFL, which contains essentially no FR light. In fact, a promotive effect of the WI relative to the WFL extension was observed. At least in terms of stem elongation, it is possible that this difference might represent an end-of-day FR light response mediated by phyB, because it is eliminated by the *lv-1* mutation (Fig. 8). Regardless of this small difference, it appears that in pea phyA is active in the detection of low-fluence-rate photoperiod extensions at both high and low R:FR ratios. Therefore, the apparent FR-light specificity seen for phyA-induced de-etiolation in pea seedlings does not necessarily exist for other photoresponses.

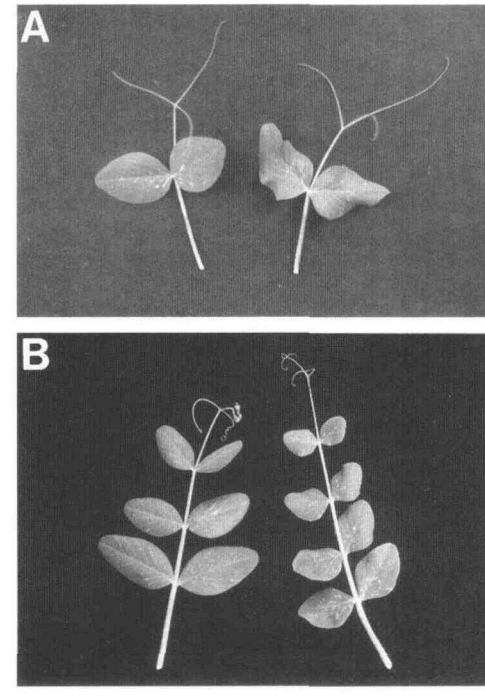

Figure 6. Altered leaf morphology in *fun1* plants. A, Leaf taken from node 6 of representative wild-type (left) and *fun1–1* (right) seedlings grown under an 18-h photoperiod. B, Comparison of maximum leaf complexity seen in wild-type (left) and *fun1–1* (right) plants grown under an 18-h photoperiod. Leaves were taken from the node of flower initiation.

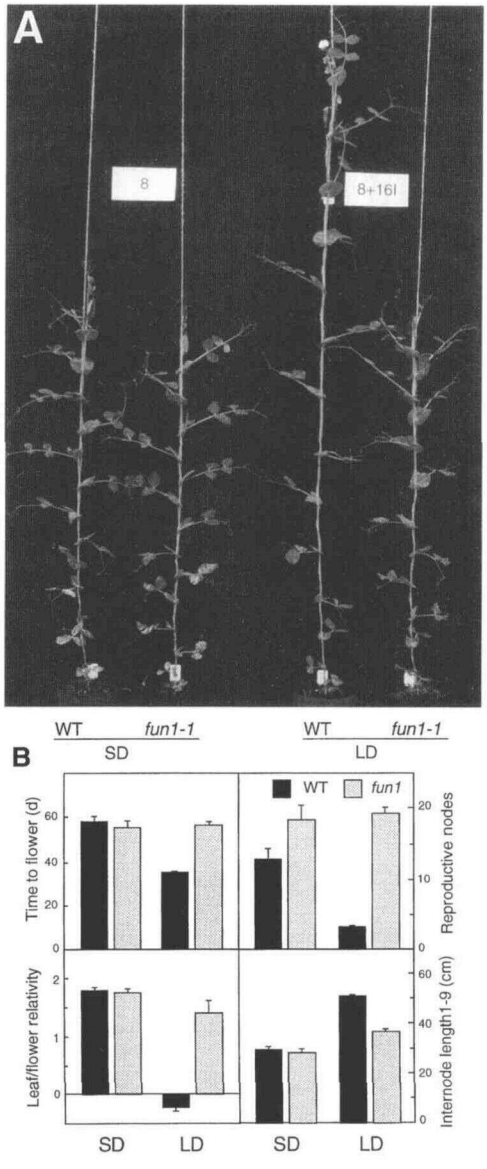

Figure 7. Response of *fun1–1* to a day extension. Wild-type cv Torsdag (WT) and *fun1–1* plants were grown from the time of sowing under an 8-h photoperiod of natural daylight either with (LD) or without (SD) a 16-h extension with weak incandescent light (10 μmol m^{-2} s^{-1}). A, Representative plants at approximately 6 weeks of age. B, Quantification of characters associated with flowering, senescence, and elongation. Bars represent SE; n = 8 to 12.

dne, an Early Flowering, Photoperiod-Insensitive Mutant, Is Epistatic to *fun1* for Flowering

The pleiotropic photoperiod response in pea has been extensively studied over many years (e.g. Barber, 1959; Murfet, 1971a, 1971b, 1971c), and progress has been reviewed thoroughly on several occasions (Murfet, 1977, 1985). Central to this work has been the characterization of the mutants *sn*, *dne*, and *ppd*, which are early flowering and day-neutral, having lost the capacity for delay of flowering under noninductive, SD conditions (Barber, 1959; Murfet, 1971a, 1971b; King and Murfet, 1985; Arumingtyas and Murfet, 1994). The *fun1* mutant is the converse of these mutants in that it is also day-neutral, but is unable to promote flowering under inductive conditions. One of the most interesting aspects of the *sn*, *dne*, and *ppd* mutants is that they have readily demonstrable graft-transmissible effects on flowering and other characteristics associated with the photoperiod response (Barber and Paton, 1952; Murfet, 1971c; Murfet and Reid, 1973; King and Murfet, 1985; Taylor and Murfet, 1996).

Studies of these mutants have formed the basis of a model for the photoperiod response in pea that invokes the action of a graft-transmissible substance that is produced in SD conditions and acts to delay flowering and promote vegetative development (Murfet 1971c, 1977, 1985). The genes *SN*, *DNE*, and *PPD* are thought to be involved in the synthesis of the inhibitor, as mutations at any of the three loci result in a loss of a graft-transmissible flower-inhibitory effect. Inhibitor production can be reduced by low-temperature treatment (part of the basis of the vernalization effect; Reid and Murfet, 1975) and by exposure to light, in a manner consistent with phytochrome action (Reid and Murfet, 1977).

We considered that expression of the *fun1* phenotype under LD conditions might in part result from an inappropriately high level of inhibitor production, and that *sn*, *dne*, and *ppd* might therefore be largely epistatic to *fun1* in the control of flowering and senescence. To address this possibility, we examined the epistatic relationships of *fun1* and *dne* in the F_2 of a cross between AF140 (*fun1–1*) and K218 (*dne*). Both mutants were derived from wild-type cv Torsdag. Preliminary experiments showed that *dne* seedlings did not differ substantially from wild type under FR light, whereas mature *dne* plants flowered distinctly earlier than

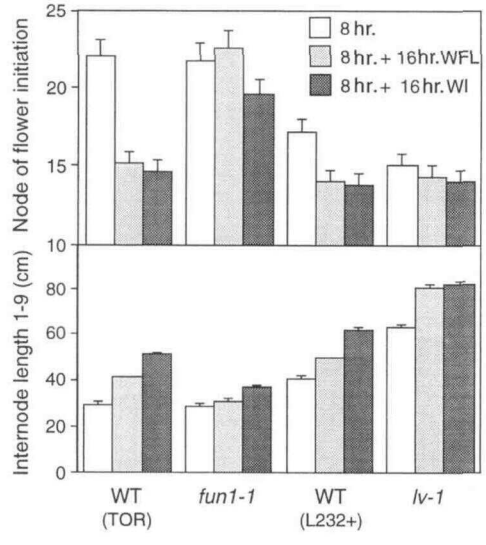

Figure 8. Response of *fun1–1* to day extensions with a low or high R:FR ratio. Wild-type cv Torsdag (WT) and *fun1–1* plants were grown from sowing in an 8-h photoperiod of natural daylight with or without a 16-h extension of either WI (10 μmol m^{-2} s^{-1}; R:FR = 0.6) or WFL (10 μmol m^{-2} s^{-1}; R:FR = 4.8) The *lv-1* mutant (Weller et al., 1995) and its corresponding wild-type line ($L232^+$) were included for comparison. Bars represent SE; n = 8 to 12.

wild type under LD conditions (data not shown; King and Murfet, 1985). F_2 seedlings were therefore grown for 7 d under continuous FR light to identify *fun1* mutant segregants, and were then transferred to standard greenhouse LD conditions to identify *dne* segregants.

As expected, *fun1 dne* double mutants were identified as a discrete class of FR-light-unresponsive, early flowering segregants, with the segregation of 57 wild-type:18 *fun1*:17 *dne*:4 *fun1 dne* plants corresponding closely to the expected 9:3:3:1 ratio (χ^2=0.89, P > 0.3). The data shown in Figure 9 confirm that *dne* has no effect on FR-light-induced deetiolation, but is completely epistatic to *fun1* in control of the node of flowering. In addition, *dne* partly overcame the short internode phenotype of *fun1* (Fig. 9D). Taken together, these results indicate that many of the observed effects of phyA in the mature plant under LD conditions require *DNE* activity for expression, and therefore result from an abnormally high level of the floral inhibitor.

fun1 Has a Graft-Transmissible Inhibitory Effect on Flower Initiation

In the event that *fun1* plants do have an abnormally high inhibitor level in LD conditions, as suggested by the interaction of *fun1* and *dne*, it should be possible to demonstrate directly a graft-transmissible flower inhibitory effect of *fun1*. To examine this possibility, we compared the ability of wild-type and *fun1* stocks to delay flowering in wild-type and *dne* scions held in LD conditions. Because we were interested in demonstrating a light-dependent difference between wild-type and *fun1* stocks, we grafted scions to leafy stocks with two fully expanded foliage leaves rather than to the stock epicotyls, as in our previous grafting studies (e.g. King and Murfet, 1985).

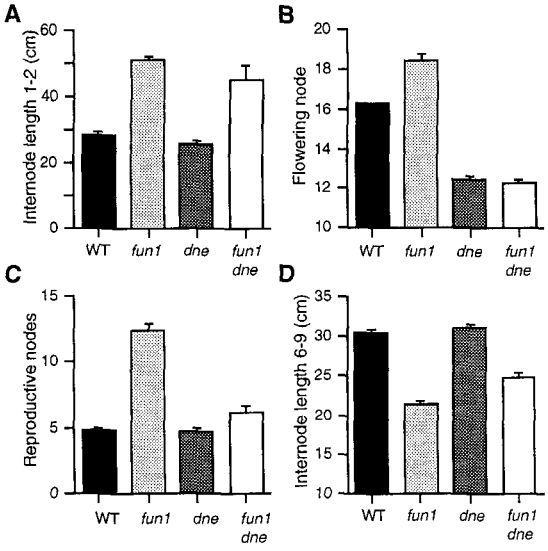

Figure 9. Interaction of *fun1–1* with *dne*. An F_2 population from the cross AF140 (*fun1–1*) × K218 (*dne*) was grown under continuous FR light (8 μmol m^{-2} s^{-1}) for 7 d from the time of sowing and then transferred to an 18-h photoperiod until senescence. *fun1–1 dne* double-mutant segregants were identified as described in the text. A, Stem length between nodes 1 and 2. This internode was fully expanded in all plants when transferred out of FR light. B, Node of flower development. C, Number of reproductive nodes at apical arrest. D, Stem length between nodes 6 and 9. Values given are mean values for all segregants in each class with n = 57, 18, 17, and 4 for wild type (WT), *fun1–1*, *dne*, and *fun1–1 dne* segregants, respectively. Bars represent SE.

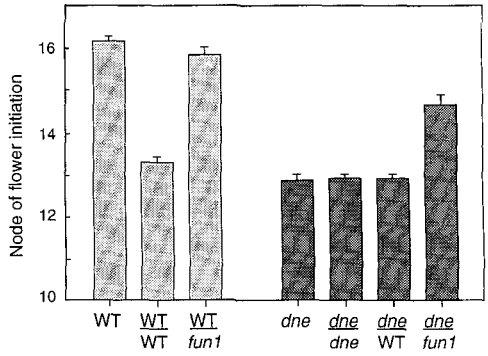

Figure 10. Graft-transmissible inhibition of flowering by *fun1–1* stocks in LD conditions. Epicotyls of 6-d-old seedlings (scions) were wedge-grafted into the fourth internode of 11-d-old seedlings (stocks) in the genotype combinations indicated. All plants were grown in a 24-h photoperiod of 8 h of daylight extended with 16 h of weak incandescent white light (10 μmol m^{-2} s^{-1}). Bars represent SE; n = 8 to 12. WT, Wild type.

Figure 10 shows that self-grafted wild-type or *dne* plants flowered at about node 13, similar to ungrafted *dne* controls, and consistent with the flowering node observed for other inhibitor-deficient mutants with an otherwise similar genetic background for flowering (Taylor and Murfet, 1996). The earlier flowering of the wild-type self-grafts compared with ungrafted wild-type plants results from the fact that the scions were excised above their cotyledons, which are a major source of inhibitor when buried (Murfet and Reid, 1974), and that the 11-d-old wild-type stocks have lost the capacity to produce inhibitor under these LD conditions (Reid, 1979). Leafy wild-type stocks were unable to delay flowering in *dne* mutant scions, whereas *fun1* stocks clearly inhibited flowering in both wild-type and *dne* scions. As expected, self-grafted *fun1* controls flowered much later than any of the other plants (node of flower initiation, 21.6 ± 0.5), a result that is consistent with the continued production of inhibitor in both stock and scion. These results establish that *fun1* stocks maintained under strongly inductive conditions can still transmit a significant inhibitory influence across a graft union.

DISCUSSION

Role of phyA in Pea Seedling Photomorphogenesis

Screening of mutant pea seedlings under continuous FR light has resulted in the isolation of two allelic mutants (*fun1–1* and *fun1–2*) that show a dramatically reduced response to continuous FR light (Figs. 1 and 2). The *fun1–1* mutant has reduced levels of spectrophotometrically detectable phytochrome (Table I) and is strongly deficient in the PHYA apoprotein (Fig. 3). These results indicate that the altered photomorphogenesis displayed by the *fun1* mu-

tant lines results from a deficiency in functional phyA and, therefore, that phyA is the predominant phytochrome mediating FR-light-induced de-etiolation responses in pea. In contrast, the essentially wild-type appearance of *fun1* seedlings grown under continuous WFL and R light (Fig. 2) suggests that phyA is largely dispensible for pea seedling photomorphogenesis under these conditions. However, phyA clearly mediates a component of the response to R light pulses (Fig. 2D) that is retained in phyB-deficient *lv* mutants (Weller et al., 1995). This suggests that R light can induce de-etiolation through both phyA and phyB, but that under continuous R light, the loss of phyA can be largely compensated for by phyB or some other phytochrome(s).

In all of these respects, the *fun1* mutants are very similar to phyA-deficient mutants in Arabidopsis (Parks and Quail, 1993; Whitelam et al., 1993; Reed et al., 1994) and tomato (van Tuinen et al., 1995b), suggesting that phyA has a similar function in de-etiolating seedlings of all three species. However, one feature distinguishes *fun1* mutant seedlings from phyA-deficient mutants in Arabidopsis and tomato: in these species, phyA-deficient mutants have somewhat longer hypocotyls than wild type when grown under blue light (Whitelam et al., 1993; van Tuinen et al., 1995b), showing that phyA contributes to the inhibition of stem elongation under blue light.

The *fun1–1* mutant showed no such increase in elongation under blue light, despite a substantial reduction in leaf expansion (Fig. 2, A and B). This result may be interpreted in three ways: First, phyA may mediate the promotion of leaf expansion but not the inhibition of stem elongation in pea. Second, phyA may mediate both responses, but inhibition of elongation might be masked by the action of a blue light receptor at the relatively high fluence rates used. Third, phyA may mediate both responses, but the *fun1–1* allele used might be leaky, and inhibition of elongation might require a higher threshold level of phyA activity than the promotion of leaf expansion. However, leakiness is unlikely to be an adequate explanation, because the phytochrome chromophore-deficient *pcd1 pcd2* double mutant also retains normal inhibition of stem elongation under blue light, and is no less sensitive than either the *pcd1* or *pcd2* single mutants (Weller et al., 1997). These results therefore demonstrate a role for phyA in the promotion of leaf expansion under blue light, and imply a role for a blue light receptor in the inhibition of elongation. However, this leaves open the question of whether phyA is also active in the control of elongation under blue light.

Role of phyA in Mature Plants

phyA deficiency has previously been reported to have little effect on the appearance of mature, white-light-grown Arabidopsis and tomato plants (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; van Tuinen et al., 1995b). This is true for *fun1* seedlings grown under continuous white light at a high R:FR ratio (Fig. 2, A and B), but under light with some FR light content, mutant seedlings are slightly elongated relative to wild type (Fig. 5A). More striking is the fact that under our LD greenhouse conditions, older *fun1* plants show a profoundly pleiotropic phenotype, with reduced stem elongation, thickened stems, increased branching, delayed flowering, retarded fruit development, and delayed apical senescence (Figs. 4 and 5; Table II). This phenotype is very similar to that displayed by wild-type plants grown under noninductive SD conditions, and represents a developmental orientation away from reproduction in favor of vigorous vegetative growth. It reflects the fact that *fun1* plants are unable to respond to a photoperiod extension of either a low (Fig. 7) or high (Fig. 8) R:FR ratio, and are thus essentially day-neutral, flowering late in both SD and LD conditions.

It was shown previously that the main determinant of the photoperiod response in pea is a high-irradiance-response-like reaction that is stimulated most effectively by light of low R:FR ratio (Reid and Murfet, 1977). In contrast, treatment regimens that employ brief exposures to light (e.g. given as end-of-day FR light or night breaks) appear much less effective for the promotion of flowering in pea, at least when the photoperiod is given as natural daylight (Reid and Murfet, 1977; J.L. Weller, unpublished data). The results presented in this study clearly establish phyA as the main phytochrome responsible for daylength detection in pea under our standard growth conditions, and show that phyA and phyB have opposing effects on flower initiation (Fig. 8; Weller and Reid, 1993). They also reinforce previous observations that the photoperiod response in pea involves not only control of flower initiation but also of flower development and a variety of other pleiotropic characteristics (Murfet, 1985). However, the way in which phyA and phyB may be involved in other commonly studied flowering photoresponses (e.g. responses to night breaks, end-of-day FR light, and daytime R:FR ratio) has yet to be determined for the pea system.

The dominance of a high-irradiance-response-like promotive response is also observed for other LD plant species (Vince-Prue, 1994), and a possible role for phyA in the mediation of this response has been discussed since multiple phytochromes were first distinguished (Carr-Smith et al., 1989; Thomas, 1991). Arabidopsis is currently the only species for which the effects of phyA deficiency on flowering have been reported. The loss of phyA in Arabidopsis results in a reduced promotion of flowering in response to an FR-light-rich photoperiod extension (Johnson et al., 1994), which is consistent with our results from pea. However, the two species appear to differ in their response to an extension with WFL, which contains negligible FR light. Flowering in phyA-null Arabidopsis mutants is promoted to a normal extent by an extension with WFL (Johnson et al., 1994), whereas a WFL extension given to the *fun1* mutant was even less effective than a FR-light-rich extension (Fig. 8), regardless of whether low- or high-fluence-rate WFL was used.

These results initially suggest that in pea, promotion of flowering by day extensions with WFL is dependent only on phyA, whereas in Arabidopsis another photoreceptor must also be active. However, the photoperiod response in both pea and Arabidopsis is subject to modification by other genes not directly related to photoperiod perception, which may in some cases mask the action of specific phytochromes. For example, phyB-deficient Arabidopsis mu-

tants in the early, Landsberg *erecta* background do not respond to differences in R:FR, but show a clear response when combined with mutants such as *co* that magnify the response to changes in R:FR (Halliday et al., 1994). Similarly, in pea the introduction of the dominant *HR* allele uncovers a large differential sensitivity to changes in R:FR not seen in *hr* backgrounds such as cv Torsdag (Reid, 1982). Transfer of phyA-deficient mutants to these later-flowering backgrounds may allow a more detailed comparison of the roles of phyA in the two species.

Beyond the reports of altered flowering mentioned above, pleiotropic effects of phyA deficiency on the development of mature Arabidopsis plants have not been described. This may be because phyA has a different role in this species, or because the effects of phyA deficiency are less pronounced and have been overlooked. In many species, the transition to flowering is associated with more general changes in plant growth, which reflect a redirection of assimilates away from vegetative growth toward reproductive organs (Vince-Prue, 1975; Wallace et al., 1993). Pleiotropic effects of the photoperiod might be expected to be evident in any species exhibiting photoperiodic control of flowering. Consistent with this view, the photoperiod is known to influence a range of morphological characters in Arabidopsis in addition to flowering. For example, Martínez-Zapater et al. (1995) report that the inflorescences of wild-type Arabidopsis plants grown in SD conditions are more highly branched and have shorter internodes than those of plants grown in LD conditions, and that photoperiod also has heterochronic effects on rosette leaf morphology.

Two Pathways for phyA Action in Pea

One of the more intriguing conclusions to be drawn from the *fun1* mutant phenotype is that in pea, phyA acts to inhibit elongation in de-etiolating seedlings, but acts to promote stem elongation in older plants (Figs. 2 and 5). These opposite effects suggest that phyA may act through at least two discrete signal transduction pathways. The interaction of the *fun1* and *dne* mutants (Fig. 9) confirms the presence of two genetically separable pathways, respectively controlling seedling de-etiolation and photoperiodism. The *DNE* gene product is dispensible for phyA-mediated de-etiolation but is required for phyA-mediated promotion of flowering and associated reproductive and vegetative changes. In a formal genetic sense, *DNE* therefore defines a novel branch point in the transduction of signals from phyA. Although genetically defined branch points in phyA signal transduction have been identified previously (Johnson et al., 1994; Barnes et al., 1996), our study provides the clearest distinction to date between the effects of phyA on de-etiolation and on photoperiod detection.

Our results also provide insight into the way in which phyA promotes flowering. Since *DNE* is required for expression of the flower-promoting activity of phyA (Fig. 9) and is known to control the synthesis or transport of a graft-transmissible inhibitor of flowering (King and Murfet, 1985), it appears that promotion of flowering by phyA is achieved by a reduction in the level of this same inhibitor. This conclusion is supported more directly by evidence from grafting experiments (Fig. 10). Although the identity of this inhibitor is currently unknown, its effects have been well characterized (Murfet, 1985), and two other genes, *SN* and *PPD*, are also known to be involved in its production (Barber and Paton, 1952; Murfet, 1971b, 1971c; Taylor and Murfet, 1996). Mutant alleles at these loci are therefore expected to interact with *fun1* in a manner very similar to that found for *dne*.

Genes with analogous action to the *SN*, *DNE*, and *PPD* group have also been identified in the related LD species *Lathyrus odoratus* (Ross and Murfet, 1985a) and *Lens culinaris* (J.L. Weller and I.C. Murfet, unpublished data), and *trans*-specific grafts have suggested that the inhibitory influence is transmissible among these three species (Ross and Murfet, 1985b; J.L. Weller and I.C. Murfet, unpublished data). The multiple phenotypic effects of the inhibitor have led to the suggestion that its primary role may be to direct assimilate flow (Reid and Murfet, 1984; Murfet, 1985; Beveridge et al., 1992; Wallace et al., 1993). Regardless of the nature of the inhibitor, these results imply that phyA acts to control flowering in a similar manner in all three species.

It would be interesting to examine whether similar effects of phyA are also found in other more distantly related LD plant species. Genes similar to *SN*, *DNE*, and *PPD* (i.e. genes required for the inhibition of flowering and associated vegetative changes under noninductive conditions) have also been identified in SD plants such as the legumes *Phaseolus vulgaris* (Wallace et al., 1993) and *Glycine max* (Cober et al., 1996). This suggests that an inhibitor similar to that in pea may also function in these species, although the grafting experiments necessary to demonstrate such an activity have yet to be performed.

The importance of the phytochrome photoreceptor family in the control of flowering by photoperiod is well established (Vince-Prue, 1994). However, the mechanism by which phytochrome alters flowering has long been a matter of considerable debate, with two main hypotheses upholding important causal roles for specific flowering "hormones" and for assimilate distribution (Bernier, 1988). Whether either or both of these hypotheses is correct remains to be determined. However, the characterization of the *fun1* mutant presented here clearly links the action of a specific phytochrome (phyA) with a physiologically defined flower inhibitor, the primary role of which may be to regulate assimilate redistribution. The *fun1* mutant thus promises to serve as another important tool for use in further studies of the way in which light influences flower initiation and the commitment to reproductive development.

ACKNOWLEDGMENTS

We thank Akira Nagatani for providing anti-pea phytochrome monoclonal antibodies, Matthew Terry, John Ross, and Scott Taylor for comments on the manuscript, and Rob Warren, Ian Cummings, and Tracey Jackson for help with plant husbandry. We also thank Dick Kendrick at RIKEN for his advice and generous support.

Received April 25, 1997; accepted May 7, 1997.

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