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Flowering Responses to Altered Expression of Phytochrome in Mutants and Transgenic Lines of *Arabidopsis thaliana* (L.) Heynh.[1]

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The long-day plant *Arabidopsis thaliana* (L.) Heynh. flowers early in response to brief end-of-day (EOD) exposures to far-red light (FR) following a fluorescent short day of 8 h. FR promotion of flowering was nullified by subsequent brief red light (R) EOD exposure, indicating phytochrome involvement. The EOD response to R or FR is a robust measure of phytochrome action. Along with their wild-type (WT) parents, mutants deficient in either phytochrome A or B responded similarly to the EOD treatments. Thus, neither phytochrome A nor B exclusively regulated flowering, although phytochrome B controlled hypocotyl elongation. Perhaps a third phytochrome species is important for the EOD responses of the mutants and/or their flowering is regulated by the amount of the FR-absorbing form of phytochrome, irrespective of the phytochrome species. Overexpression of phytochrome A or phytochrome B resulted in differing photoperiod and EOD responses among the genotypes. The day-neutral overexpressor of phytochrome A had an EOD response similar to all of the mutants and WTs, whereas R EOD exposure promoted flowering in the overexpressor of phytochrome B and FR EOD exposure inhibited this promotion. The comparisons between relative flowering times and leaf numbers at flowering of the overexpressors and their WTs were not consistent across photoperiods and light treatments, although both phytochromes A and B contributed to regulating flowering of the transgenic plants.

The light environment is perceived by plants via several photoreceptors, including phytochrome. These photoreceptors allow detection of changes in light quality, intensity, and duration, which regulate the timing of flowering and many other developmental processes. Phytochrome plays a major photoregulatory role in flowering (see summary by Vince-Prue, 1975). It exists as more than one molecular species and, recently, in *Arabidopsis thaliana* (L.) Heynh., five genes encoding apo-phytochromes A to E have been identified (Sharrock and Quail, 1989; Clack et al., 1994). Each of the phytochromes A, B, and C is present in both dark- and light-grown plants, although in different amounts (Somers et al., 1991). The polypeptides encoded by the *PHYA*, *PHYB*, and *PHYC* genes are immunologically distinct (Somers et al., 1991; Wagner et al., 1991), and it has been suggested that the different members of the phytochrome family have differing functional roles (see reviews by Smith and Whitelam, 1990; Whitelam and Harberd, 1994). Phytochrome A is light labile and appears to be most relevant to de-etiolation phenomena involving HIRs to FR (Boylan and Quail, 1991; Whitelam et al., 1992; Dehesh et al., 1993; Parks and Quail, 1993). Phytochrome B, by contrast, is light stable and involved with phenomena, such as shade avoidance and EOD FR responses (Whitelam and Smith, 1991; Parks and Quail, 1993).

With photoreceptor-deficient mutants of Arabidopsis, a lack of phytochrome A delays flowering in low-intensity incandescent light-extended days (Johnson et al., 1994), but flowering is somewhat earlier in the mutant *phyB-1* (Goto et al., 1991), which has a deficiency in phytochrome B (Nagatani et al., 1991; Reed et al., 1993). This latter response with loss of a photoreceptor is not easy to explain unless the Pfr form of phytochrome B mediates a floral inhibition, whereas phytochrome A Pfr could be responsible for floral promotion. However, the vegetative growth habit for mutants can be quite atypical with altered plant morphology, a slow rate of leaf production relative to the WT (cf. Goto et al., 1991), and large (2-fold) reductions in leaf Chl content (Wester et al., 1994) so that effects on flowering could be quite indirect. Overexpression of phytochrome A and B genes in transgenic Arabidopsis allows a complementary approach to the use of mutants for the examination of the role of phytochrome(s) in the control of flowering of a LDP, notwithstanding the difficulties associated with evaluating the role of overexpressed phytochrome in transgenic plants (Cherry and Vierstra, 1994). In a manner analogous to the

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Abbreviations: *ABO*, *RBO*, phytochrome B overexpression lines; EOD, end of day; *F*, fluorescent photosynthetic photoperiod; *f*, fluorescent daylength extension; FR, far-red light; HIR, high-irradiance response; *i*, incandescent daylength extension; *phyA*, *phyB*, phytochrome A- or phytochrome B-deficient mutants; R, red light; WT, wild type; *13K7*, *21K15*, phytochrome A overexpression lines.

mutants, phytochrome overexpression modifies morphology, and this may also indirectly affect flowering.

For analysis of phytochrome regulation of photoperiodic processes, particularly in quantitative LDPs such as Arabidopsis, LD exposure should involve conditions that do not alter the photosynthetic input relative to that of plants maintained under short days. This objective is achieved here in three ways: (a) with exposure to brief (10 min) EOD R or FR in short days; (b) photoperiod extension of the main photosynthetic short day with low fluence but prolonged exposure to incandescent lamps that provide an optimal phytochrome setting for many LDPs (Evans et al., 1965; Lane et al., 1965) (such exposure to low fluences from incandescent lamps contributes minimally to photosynthesis [up to 10% during 16 h and mostly 2–5%] and should not boost apical Suc content [King and Evans, 1991]); and (c) use of a prolonged photosynthetic and photoperiodic condition but with contrasting R:FR ratios. Here more extreme R:FR ratios have been used than were used previously (Bagnall, 1993).

Treatments a and c in particular establish photobiological conditions that more directly test for phytochrome responses. However, prolonged FR-enrichment (case c) could give a small, additional photosynthetic enhancement (approximately 8% for *Pisum*; Chow et al., 1990).

Here we have examined the flowering responses of Arabidopsis to various R and FR treatments in combination with lines over- and underexpressing the phytochrome A or B proteins. Both phytochromes appear to be important in the LD flowering response of Arabidopsis. We have also taken care to define and, if possible, to eliminate any photosynthetic effects on flowering of Arabidopsis since these can be quite substantial (Bagnall, 1992; Bernier et al., 1993) and have been confounding factors in many past studies.

MATERIALS AND METHODS

Plant Material

Experiments were conducted with mutants of *Arabidopsis thaliana* (L.) Heynh. that were deficient in phytochrome in the early-flowering ecotype Landsberg *erecta*, *phyA-1* (Whitelam et al., 1993) and *phyB-1* (seed kindly provided by M. Koornneef, Agricultural University, Wageningen, The Netherlands); in the late-flowering ecotype Columbia, *fhy-3* (a phytochrome A-defective line; Whitelam et al., 1993) and *phyB-9* (seed kindly provided by J. Chory, Salk Institute, San Diego, CA; Reed et al., 1993); and in the late-flowering Nossen and the progeny of a backcross between Nossen and *phyB-1* in Landsberg (seed kindly provided by R. Sharrock, Montana State University, Bozeman; see Wester et al., 1994). Phytochrome nomenclature is according to that of Quail et al. (1994).

The overexpression lines in these experiments had all been transformed with phytochrome cDNAs under the control of the cauliflower mosaic virus 35S promoter to overexpress phytochrome in the Nossen ecotype. The phytochrome A overexpression lines are designated as *21K15* (Whitelam et al., 1992) and *13K7* (Boylan and Quail, 1991) and phytochrome B overexpression lines are designated as *ABO* and *RBO* (Wagner et al., 1991). Line *13K7* contains 16 times more phytochrome A than its WT parent when grown in the light (Boylan and Quail, 1991), whereas *RBO* and *ABO* were found to have 3- to 5-fold (*RBO*) or 18- to 30-fold (*ABO*) more phytochrome B when grown in the light (Wagner et al., 1991).

EOD Experiments

The importance of light quality at the EOD was assessed for plants growing in short days. At the end of each 8-h day, the plants were moved from fluorescent lamps (R:FR ratio at 660:730 nm = 5.8, PPFD = 200 μmol m^{-2} s^{-1}) to darkness immediately or after a brief (15 min) exposure to low-fluence light from incandescent lamps (R:FR at 660:730 nm = 0.8, PPFD = 10 μmol m^{-2} s^{-1}). Other experiments utilized 10-min exposures to broadband R or FR sources. The FR source was light from tungsten floodlamps filtered through 2 cm of water and one layer of FR plastic (Westlake Plastics, Lenni, PA) (R:FR = 0.05; 9.2 W m^{-2}, 700–750 nm), with or without a following exposure to R wavebands from Philips TL 40W/15 red fluorescent lamps (R:FR = 61.5; 2.7 W m^{-2}, 640–690 nm). Temperature was reduced to 20°C in these FR/R EOD experiments to slow the flowering response.

Photoperiod Extension Experiments

The plants were grown aseptically in test tubes in the presence of 1.5% Suc, as described previously (Bagnall 1992, 1993), in cabinets with an air temperature of 23°C with an 8-h photosynthetic period using light from fluorescent lamps at 200 μmol m^{-2} s^{-1} PPFD, followed by daylength extension with low-intensity (10 μmol m^{-2} s^{-1} PPFD) light from incandescent or fluorescent bulbs. In the photoperiod transfer experiment, all plants were present in the same growth cabinet during the 8-h of *F*, and each day after *i*, ranging from 15 min to 16 h, groups of plants were transferred to a dark room with the same temperature (23°C). The plants treated for 24 h (8*F* + 16*i*) remained in the growth cabinet continuously, whereas all other plants experienced 30 s of low-intensity (3–5 μmol m^{-2} s^{-1}) fluorescent light (R:FR ratio at 660:730 nm = 5.8) when transferred to the dark room. In initial experiments on the overexpressors (data not shown), separate cabinets were used so that transfers were not necessary, but only two daylengths could be examined at one time and potentially there could have been between-cabinet flowering differences due to factors other than daylength.

Response to Light Quality of Continuous Light

Studies with two extreme R:FR ratios utilized plants grown in a 3:1 mixture of potting compost:sand. Seeds were soaked for 4 d at 4°C and then germinated and grown under continuous white fluorescent light (96 μmol m^{-2} s^{-1}) at 18 to 20°C. The low and high R:FR ratio cabinets were described by Keiller and Smith (1989) and subsequently by Whitelam et al. (1992) and gave R:FR ratios of 0.07 versus 8.61.

Measurement of Flowering

Time to flower has been recorded as the time from sowing of the seed to first petal appearance. This is a robust measure of rate of flowering (inverse of time). The various photobiological treatments did not begin until seedling emergence, and prior germination was always completed in less than 1 d.

As a second measure of flowering, both rosette and cauline main-shoot leaf number were determined during and at the end of the experiment. The relationship between leaf number and time to flower may vary, and when they are not similar it is an important indicator of one of the following developmental aberrancies: (a) early or normal time to visible floral initiation at a large final leaf number; (b) late visible floral initiation at a small or normal, final leaf number; (c) slow rates of leaf production indicative of some environmental or physiological limitation on growth; or (d) early but nonvisible floral initiation (i.e. small, final leaf number) but late bolting and flowering.

Effects of leaf number on total photosynthetic leaf area could lead to anomalous and indirect hastening (a) or delay (b) in time to flowering via a change in assimilate availability, which is known to affect flowering time in Arabidopsis (Bagnall, 1992; Bernier et al., 1993). With a slower leaf production rate (c) an indirect effect of photosynthesis on flowering must also be considered especially for the altered-phytochrome, *phyB* mutants, which may have no more than 50% of WT leaf Chl (Wester et al., 1994) and a much slower leaf production rate (Goto et al., 1991).

In this study we detected no aberrant flowering responses associated with responses a and b above, and we highlight the potential problems with *phyB* mutants (c). As for potential early, nonvisible floral initiation (d) this did not occur in our studies with Arabidopsis, although it has been reported by Downs and Thomas (1982) for photoperiodic and light quality effects on flowering of *Hyoscyamus niger*. In our studies there were no discernible differences in leaf production rates between the various photoperiodic treatments (D.J. Bagnall and R.W. King, unpublished observations), and time to flower was always directly correlated with rosette leaf number (this work; Bagnall, 1993). Thus, late flowering occurred at a high leaf number and was a true indication of a late vegetative to floral transition. Errors are given as the SE or as error bars (2× SE). Replicate numbers ranged from 12 to 20 plants unless shown otherwise.

RESULTS

EOD Light Quality, Flowering, Growth, and Phytochrome-Deficient Mutants

phyA or *phyB* or mutants possessing a reduced-phytochrome A-elongation response (*fhy3*) all flower faster after 10 min of FR at the end of an 8-h fluorescent day compared to untreated plants or those subjected to FR followed by R EOD (Tables I and II). These clear phytochrome, photoreversible responses of the mutants were qualitatively similar to those of the WTs. Notwithstanding that both *phyA* and *phyB* were faster to flower after FR EOD, the *phyB-1* hypocotyl lengths were identical in all EOD treatments (Table II). Thus, although *phyB-1* exhibited a classical null elongation response to FR (Nagatani et al., 1991; Robson et al., 1993), flowering was promoted by FR and this promotion was photoreversible, i.e. 10 min of R reversed the FR promotion.

The loss of phytochrome B resulted in faster flowering than WT in both the Nossen (Fig. 1) and Columbia (Table I) backgrounds, although it was difficult to detect in Landsberg (Table II), notwithstanding that other research has shown Landsberg to be slow to flower compared with its *phyB* mutants (Goto et al., 1991; Reed et al., 1994; D.J. Bagnall, unpublished data). However, the responses of *phyB* mutants to EOD treatments were consistent across all three background genotypes (Columbia, Landsberg [Tables I and II], and Nossen [data not shown]), with FR promoting flowering in both WT and mutants.

EOD Light Quality, Flowering, and Phytochrome Overexpression

Terminating an 8-h day of fluorescent light exposure (R:FR = 5.8, PPFD = 200 μmol m^{-2} s^{-1}) with 10 min of low-fluence light from either incandescent lamps (R:FR = 0.8), an FR source (R:FR = 0.05), or FR followed by R (R:FR = 61.5) resulted in significant changes in flowering times for Nossen, *ABO*, and *13K7* (Table III). The response to a brief, incandescent exposure was intermediate, with FR reversing the response to an R-rich main photoperiod (*8F*) and, in turn, 10 min of R completely reversing the FR response. This clear photoreversibility establishes that both overexpressed phytochrome A and B can act via EOD phytochrome responses to regulate flowering. Furthermore, the pattern of the response of *13K7*, with FR promoting flowering, matches that of Nossen and the various phytochrome mutants, whereas *ABO* exhibited an inverse

Table I. *Flowering of Arabidopsis genotypes fhy-3 (a putative phytochrome A transduction mutant with reduced elongation response to FR), phyB-9 (a phyB), and Columbia (WT for both mutants) after various low-fluence EOD light treatments*

The temperature was 20°C. Values are means ± SE.

Light Treatment	Days to Flower			Total Leaf No.		
	Columbia	*fhy3*	*phyB-9*	Columbia	*fhy-3*	*phyB-9*
8F	82.4 ± 2.0	43.1 ± 2.0	51.7 ± 3.4	25.5 ± 1.0	15.1 ± 0.7	13.3 ± 1.4
8F + 10 min FR	42.6 ± 2.6	33.6 ± 1.5	38.2 ± 1.5	11.0 ± 0.7	8.4 ± 0.4	7.8 ± 0.5
8F + 10 min FR + 10 min R	71.0 ± 4.7	46.2 ± 2.8	46.9 ± 3.7	21.4 ± 1.5	14.7 ± 0.9	10.5 ± 1.0

Table II. *Flowering and hypocotyl elongation of Arabidopsis genotypes phyA-1 and phyB-1 (phytochrome-deficient mutants) and Landsberg (WT) after various low-fluence EOD light treatments*

The temperature was 20°C. Values are means ± SE.

Light Treatment	Days to Flower			Total Leaf No.			Hypocotyl Length		
	Landsberg	*phyA-1*	*phyB-1*	Landsberg	*phyA-1*	*phyB-1*	Landsberg	*phyA-1*	*phyB-1*
8F	37.2 ± 0.8	38.7 ± 0.9	34.5 ± 0.6	11.5 ± 0.3	12.0 ± 0.3	8.9 ± 0.2	5.6 ± 0.3	3.8 ± 0.3	9.9 ± 0.3
8F + 10 min FR	26.4 ± 0.2	26.0 ± 0.5	26.5 ± 0.4	9.4 ± 0.2	9.6 ± 0.3	7.0 ± 0.1	7.2 ± 0.3	7.7 ± 0.4	9.8 ± 0.2
8F + 10 min FR + 10 min R	35.8 ± 0.6	37.7 ± 1.2	36.0 ± 0.5	11.2 ± 0.3	12.5 ± 0.5	9.0 ± 0.2	5.6 ± 0.2	4.9 ± 0.3	9.5 ± 0.3

set of responses, with R promoting and FR delaying flowering.

Photoperiodic Extension and Phytochrome-Deficient Mutants

Phytochrome mutants showed altered photoperiodic responses compared to their WT parents in a classical, photoperiod experiment, in which an 8-h fluorescent (photosynthetic) day was extended with low irradiance from incandescent lamps. The loss of phytochrome A in the mutant *phyA-1* resulted in slightly later flowering relative to the WT Landsberg in long days terminated with low-intensity incandescent extensions (Fig. 1). Similarly, the *fhy3* genotype, which is a putative phytochrome A signal transduction mutant, was slightly late in incandescent-terminated long days (Fig. 1) and was slightly early in short days relative to Columbia.

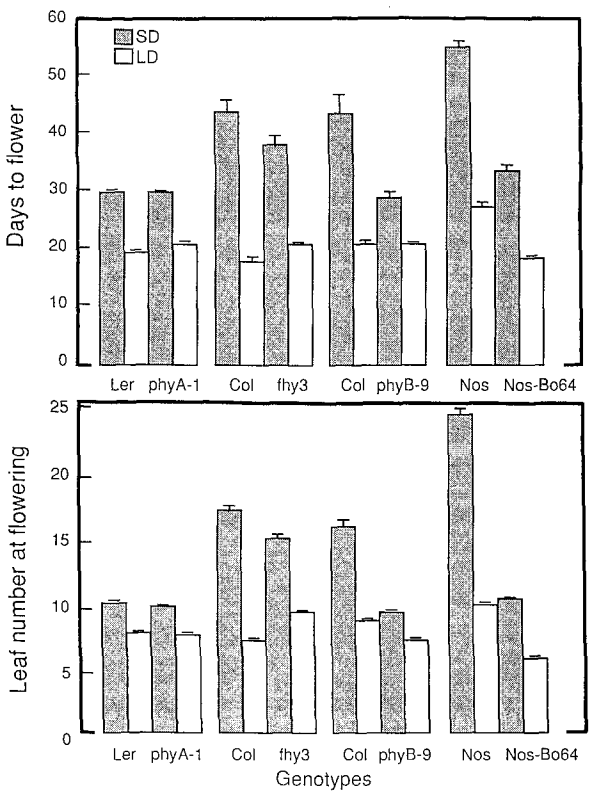

Figure 1. Time to flower of Arabidopsis WT and mutants in short days (*8F* terminated by 15 min of incandescent light) and long days (*8F* followed by 8 h of incandescent light).

The loss of phytochrome B from both Columbia and Nossen resulted in faster flowering in short days, as had previously been observed in *phyB-1* in Landsberg (Goto et al., 1991). In Nossen, the absence of phytochrome B also resulted in faster flowering in long days. In both backgrounds, the phytochrome B-deficient mutants retained an LD flowering response, which was proportional to but less than that observed in the respective WT.

Comparisons of leaf number at flowering are reported as total rather than as rosette leaf number in these experiments because elongated internodes and petioles of *phyB* mutants (Nagatani et al., 1991; Robson et al., 1993) result in a noncompact rosette that is difficult to distinguish from cauline leaves. In these experiments slow leaf production rates in *phyB* Arabidopsis mutants with respect to WT (Fig. 1) limit the usefulness of comparing leaf number at flowering.

Photoperiodic Extension and Phytochrome Overexpression

Exposure of the Arabidopsis ecotype Nossen to a range of *i* provided a comprehensive assessment of flowering. Long days clearly resulted in faster flowering (Fig. 2). Daylength extensions were given at low irradiances from incandescent lamps, so their possible photosynthetic contribution was minimal (2–10% of the input of an 8-h day).

The effect of phytochrome overexpression on photoperiodic response was dramatic. Phytochrome A overexpression in line *13K7* led to early flowering in all daylengths and approached day neutrality. By contrast, line *ABO* retained most of the photoperiodic response that was observed in the WT (Fig. 2), although it was generally earlier than Nossen. In further, more limited experiments, qualitatively similar results were obtained in individual cabinets at three photoperiods, although despite continuous monitoring of conditions there could have been slight differences between cabinets in PPFD (<25 μmol m^{-2} s^{-1}) and temperature (<0.5°C). Where cabinets are not replicated in experiments running for 2 months, such small cabinet-to-cabinet variation will result in significant differences in flowering that are not due to photoperiod. However, by manually transferring plants each day as in the experiment in Figure 2, only a single light and dark cabinet was required, thus minimizing potential extraneous, environmental effects.

There were parallel effects of daylength on rosette leaf number at flowering and on time to flower; *13K7* flowered

Table III. *Flowering of Arabidopsis with a photosynthetic short day of 8 h from fluorescent (8F) (200 μmol m^{-2} s^{-1}) followed by various low-fluence EOD light treatments*

The genotypes *13K7* and *ABO* are, respectively, phytochrome A and B overexpression lines in the Nossen background. The temperature was 20°C. Values are means ± SE.

Light Treatment	Days to Flower			Rosette Leaf No.		
	Nossen	*13K7*	*ABO*	Nossen	*13K7*	*ABO*
8F	73.3 ± 0.8	47.8 ± 1.1	32.3 ± 0.6	21.7 ± 0.9	15.4 ± 0.5	11.2 ± 0.4
8F + 10 min FR	53.2 ± 1.0	32.7 ± 0.5	53.2 ± 1.0	14.4 ± 0.6	8.8 ± 0.2	14.4 ± 0.6
8F + 10 min FR + 10 min R	74.8 ± 1.1	44.1 ± 0.9	34.2 ± 1.1	21.4 ± 0.7	13.9 ± 0.4	12.2 ± 0.5
8F + 10 min *i*	68.4 ± 1.7	38.4 ± 0.6	48.5 ± 1.7	17.1 ± 0.6	11.4 ± 0.3	14.1 ± 0.4

early and responded little to photoperiod and it flowered with fewer leaves than Nossen or *ABO* (Fig. 2b versus Fig. 2a). As will be discussed later, compared with Nossen, the transgenic lines show a difference in early leaf production that is unrelated to their photoperiodic flowering responses.

When daylength was extended with prolonged exposures to incandescent lamps (FR-like EOD), *ABO* retained a daylength response most like Nossen (Fig. 2). However, 15-min and 3-h incandescent exposures after an 8-h short day gave somewhat earlier flowering of *ABO* than Nossen (Fig. 2). By contrast, fluorescent (R-rich) terminations to either 8- or 16-h photoperiods resulted in early flowering (Table IV). As shown in Table III, these findings fit the EOD R/FR photoreversible effects of phytochrome on flowering of *ABO*, although possibly only in short days.

Phytochrome Overexpression, Plant Height, and Leaf Production

The transgenic phytochrome-overexpressing lines were much shorter than the WT in all conditions (Table V) as reported earlier by Boylan and Quail (1991) and Whitelam et al. (1992). By contrast, there was an increase in leaf number, area per leaf, and total leaf area for transgenic plants exposed to only 8 h of high irradiance daily (Table V), although in continuous high irradiance leaf size was similar for all genotypes (Table V). Between the two photoperiod treatments (Table V) there was a 3-fold difference in PPFD, so in a further experiment an 8-h main photosynthetic period was utilized, along with low-fluence incandescent extensions. There was no photoperiodic effect on leaf numbers, but at flowering the transgenic plants had produced more leaves, a response that reflects an early onset of leaf initiation during the first 7 d from imbibition. Later, from 7 to 14 d, and over photoperiods ranging from 8 to 24 h, there were no significant differences in leaf production rate (phyllochron) for the three lines (data not shown).

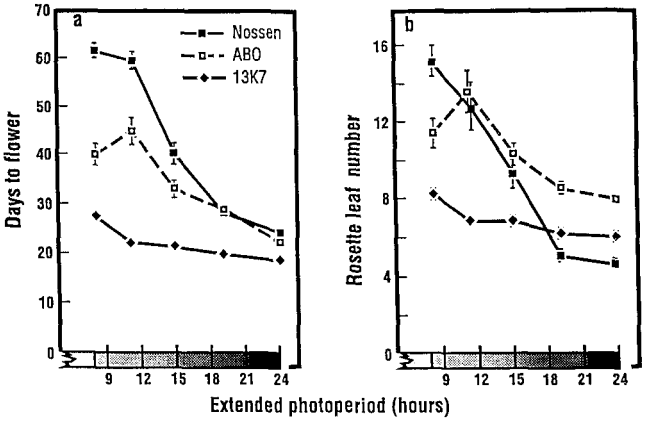

Figure 2. Photoperiodic regulation of flowering in Arabidopsis. a, Days to flower; b, rosette leaf number at flowering. The phytochrome overexpressing lines *ABO* and *13K7* should be compared to Nossen as the control or WT background. All plants were present in the same cabinet during the fluorescent, photosynthetic period of 8 h (200 μmol m^{-2} s^{-1}). During the subsequent low PPFD *i* (10 μmol m^{-2} s^{-1}), the plants were transferred daily into a dark room after either 15 min or 3, 7, or 12 h. Values are means ± SE.

The consequences of greater early leaf production in the phytochrome overexpressors are revealed in Figure 3 in which leaf numbers at flowering (taken from Fig. 2b) are plotted against days to flowering for all photoperiods (taken from Fig. 2a). For flowering time the responses to photoperiod were parallel across lines, whether they flowered early at a low leaf number or later with a higher leaf number. However, the early production of true leaves and their greater area (Table V), as a consequence of phytochrome overexpression, was carried through to give a fixed additional number of rosette leaves at flowering in the transgenics relative to Nossen. Interpretation of genotype comparisons like those in Figure 2 are facilitated by including both rosette leaf number and days to flower data. Flowering time must be a primary measure and, when comparing genotypes, it is essential to determine leaf number so that growth aberrancies can be identified (see "Materials and Methods").

Flowering may be promoted by photosynthetic input, where either PPFD (Bagnall, 1992) or the duration of a photosynthetic light period are increased. In a preliminary experiment with the strain Landsberg *erecta*, grown in soil, its flowering time was directly related to photosynthetic input (PPFD) (70–200 μmol m^{-2} s^{-1}). However, when seedlings were grown on agar that included 1.5% Suc, time to flower did not vary over the same range of PPFD, and flowering occurred as early as in seedlings grown in soil at the highest PPFD (D.J. Bagnall, unpublished results). Thus, in the present study, because the seedlings can utilize Suc from the medium, photosynthetic input is probably not important for flowering. For our growth conditions, genotypic differences in leaf area (Table V) also appear to be

Table IV. *Flowering of Arabidopsis with a photosynthetic short day of 8 h from fluorescent lamps (8F) and 16 h of darkness (16 D) or 8F that was extended with 8 h of low PPFD from fluorescent lamps (8f)*

Values are means ± SE for replicate numbers between 20 and 35 plants. Each treatment value is the mean from two separate experiments. Both SD and LD treatments differ from those in Figure 1 by not terminating with an incandescent exposure. Rosette leaf numbers at flowering changed in parallel with time to flower. The temperature was 23°C.

Genotype	Days to Flower	
	SD (8F:16D)	LD fluorescent (8F:8f:8D)
Nossen (WT)	51.5 ± 1.9	43.0 ± 2.1
13K7	27.8 ± 0.5	24.6 ± 0.5
ABO	25.8 ± 0.4	26.6 ± 0.4

unimportant for flowering time, since *ABO* could flower as late as Nossen but with as great a leaf area at 14 d as *13K7*, which flowered early (Fig. 2).

Phytochrome Overexpression and Flowering Response to Differences in R:FR

To further examine the effects of light quality on flowering, extremes in the R:FR balance were imposed in continuous (i.e. 24 h) photosynthetic irradiances of 96 μmol m^{-2} s^{-1} PPFD. The results presented in Table VI show that the transgenic phytochrome A overexpression plants (*21K15* and *13K7*) flowered early in high R:FR treatments compared to WT; flowering of the phytochrome B-overexpressing lines occurred as late as the WT, Nossen. All genotypes, including Nossen, were significantly faster flowering in low R:FR ratios. In further experiments utilizing less extreme R:FR ratios (R:FR 1.1–3.2) and photoperiods of 15 or 24 h, differences between lines were smaller, with flowering occurring earlier than shown in Figure 2. Furthermore, only Nossen flowered earlier under FR-enriched light conditions (data not shown). Overall, based on the findings in Figure 2 and Table VI, overexpression of phytochrome A results in day-neutral, very rapid flowering in response to differences in daylength and R:FR, whereas overexpression of phytochrome B may not greatly affect flowering relative to Nossen.

DISCUSSION

Phytochrome regulation of the LD flowering response of Arabidopsis has been examined here using a combination of genetic and photobiological manipulations. FR enrichment was most effective whether given as incandescent photoperiod extensions (Fig. 2), as prolonged exposure to extreme R:FR ratios (Table VI), or as EOD exposure to brief R or FR (Tables I–III). This enhanced flowering with FR enrichment confirms earlier evidence for Arabidopsis (Brown and Klein, 1971; Martinez-Zapater and Sommerville, 1990; Bagnall, 1993; Karlsson et al., 1993). Similar FR response is also known for other LDP (Evans et al., 1965; Lane et al., 1965; see reviews by Deitzer, 1984; Thomas, 1993; Vince-Prue, 1975, 1994). EOD R/FR photoreversible promotion of flowering is characteristic of phytochrome action and is a novel finding for Arabidopsis. Another LDP, *Lemna gibba*, shows EOD R/FR photoreversible flowering responses (Oota and Hoshino, 1979), but other LDPs may not (Evans, 1976).

Is Phytochrome B Involved in EOD Responses of Flowering as It Is for Elongation?

The use of molecular and genetic approaches to alter phytochrome species/content has further confirmed its role in the regulation of flowering of Arabidopsis (Figs. 1 and 2; Tables I–III and VI). However, a satisfactory model of phytochrome control of flowering still needs to be developed. In discussing flowering responses of LDPs, Thomas (1993) focused on the light stability of phytochrome B and the converse for phytochrome A. Thus, the differences in flowering response between FR-HIR and R/FR EOD would mirror earlier findings for hypocotyl elongation (Nagatani et al., 1991, 1993; Dehesh et al., 1993; McCormac et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), where FR-HIR were shown to be associated with phytochrome A and R/FR EOD with phytochrome B. Here we have confirmed the lack of EOD R/FR photoreversibility of hypocotyl elongation in the phytochrome B mutant (Table II). By contrast, the same phytochrome B mutant seedlings showed clear EOD R/FR photoreversible regulation of flowering (Table II). There are at least two explanations for these novel differences between EOD R/FR response for hypocotyl elongation and flowering: (a) phytochrome species other than A or B are important for flowering and (b) phytochrome species can substitute for each other in their regulation of flowering but not for hypocotyl elongation.

Considering a above, we are currently examining the flowering responses of *phyA,phyB* double mutants and searching for flowering mutants of phytochromes C, D, or E of Arabidopsis. The second explanation (b above) involving substitution between phytochromes A and B (Table II)

Table V. *Growth response at 14 d after imbibition of Nossen, ABO, and 13K7 exposed to 8 or 24 h of 200 μmol m^{-2} s^{-1} photosynthetic light per day*

The temperature was 23°C. Values are means ± SE.

Genotype	8 h			24 h		
	Height	Leaf No.	Leaf area	Height	Leaf No.	Leaf area
	mm		*mm^2*	*mm*		*mm^2*
Nossen	12.2 ± 0.8	5.2 ± 0.1	12.0 ± 0.9	14.0 ± 1.0	7.6 ± 0.2	142 ± 5
13K7	8.1 ± 0.8	6.4 ± 0.2	26.6 ± 1.1	7.4 ± 0.6	8.2 ± 0.6	147 ± 23
ABO	6.6 ± 0.3	6.1 ± 0.1	29.2 ± 2.1	8.8 ± 0.4	8.6 ± 0.4	146 ± 5

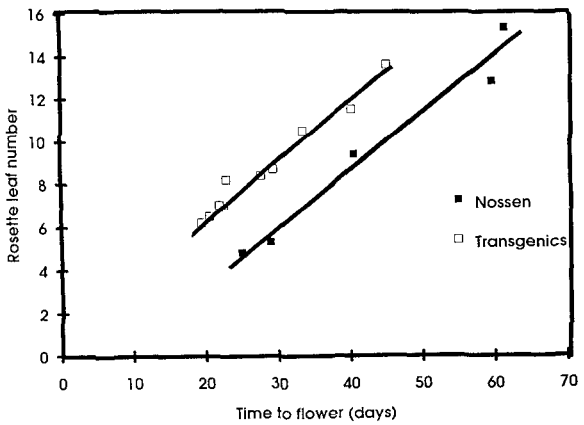

Figure 3. Relationship between leaf number at flowering and days to flower for data presented in Figure 2.

requires two reasonable assumptions: that total Pfr is critical irrespective of its genetic origin and that, unlike hypocotyl growth, flowering of an LDP is optimal with low Pfr settings (see earlier discussion and Evans et al., 1965; Lane et al., 1965). Thus, despite its instability in light, there might have been sufficient phytochrome A in 8-h short days to allow it to contribute an EOD R/FR response in mutants lacking phytochrome B (Table II), whereas phytochrome B would operate normally in the phytochrome A mutant. Such substitution may be an extreme example of the overlapping roles of phytochrome A and B in hypocotyl elongation (Nagatani et al., 1993; Parks and Quail, 1993) and seed germination (Shinomura et al., 1994).

Use of Phytochrome Overexpression to Identify Its Role in Flowering

To understand how flowering responses of phytochrome overexpression lines complement those of phytochrome-deficient mutants requires resolution of the question of which phytochrome(s) are involved. The similar R/FR responses of WT and overexpressed phytochrome A genotype (Table III), but earlier flowering in the transgenics, implies that phytochrome A is limiting and, perhaps, to the extent of being unimportant in the WT, since phytochrome A mutants are not much different from WT in flowering time (Fig. 1). The reversal in the pattern of R/FR response with phytochrome B overexpression may reflect excessive expression of phytochrome B (up to 30-fold; Wagner et al., 1991). Further studies are needed to determine the level of overexpression that results in such a switch. A likely explanation is that the 10-min FR exposure presumably removes most of the Pfr by its photoconversion to Pr, and the results in Table III could indicate that, in *ABO*, Pfr is active during the 16-h dark period. For *13K7* and WT, the converse explanation could be invoked, i.e. that Pfr is active during the main photoperiod. However, this hypothesis is an oversimplification, especially given the well-documented evidence of a requirement for low levels of Pfr during the long day for many LDPs (Deitzer, 1984). It would be interesting, nevertheless, if with transgenic plants we could convert an LDP into a SDP, as some of the light quality responses of the transgenic *ABO* are reminiscent of those of SDPs (Salisbury, 1965).

Some Limitations of the Use of Mutants and Transgenic Plants

Irrespective of whether phytochrome A, B, or C, D, and E are important for flowering, it is well known that mutants in phytochrome B flower early both in Arabidopsis (Fig. 1; Tables I and II; Goto et al., 1991; Reed et al., 1992, and refs. therein) and in *Pisum sativum* (Weller and Reid, 1993). However, such earliness of flowering in phytochrome B-deficient mutants may be an indirect and separate response to loss of phytochrome B. For example, there was no relationship between the extent of R/FR photoreversibility in EOD responses (Tables I and II) and early flowering. The EOD phytochrome responses appear to be independent of the separate "earliness" effects of the loss of phytochrome B. Also, for phytochrome A mutants EOD R/FR photoreversibile regulation of flowering was clear, despite the flowering occurring later than WT (Johnson et al., 1994), unchanged (Table II; Reed et al., 1994), or earlier in a potential phytochrome A transduction mutant (Table I). Apparently there are independent (i.e. pleiotropic) effects of phytochrome mutation on flowering; the EOD responses are distinct from another more variable effect of the mutation on flowering time. Many aspects of growth of Arabidopsis are altered in phytochrome mutants (Goto et al., 1991; Wester et al., 1994; Tables I and II) that could lead to such secondary early or late flowering responses.

As an aside, caution must be exercised in the interpretation of experiments with Arabidopsis, in which photosynthetic inputs may differ. For the transgenic lines in some photoperiods, there were differences in leaf number and area and, hence, in photosynthetic potential (Table V). This should not have been problematic when sugar was supplied in the medium, as here. Also, the data for effects of daylength treatments on flowering time versus leaf number (Fig. 2) could be seen as parallel lines (Fig. 3), which implies that there was no interaction between phytochrome and photosynthetic input, a conclusion also reached by King and Evans (1991) from comparison of photoassimilate

Table VI. *Flowering of Arabidopsis in extreme low or high R:FR conditions in a continuous (24-h) photosynthetic (96 μmol m^{-2} s^{-1}) exposure*

Rosette leaf numbers at flowering changed in parallel with time to flower. The temperature was 18 to 20°C. Values are means ± SE.

Genotype	Days to Flower	
	High R:FR (ratio 8.61)	Low R:FR (ratio 0.07)
WT (Nossen)	28.8 ± 0.7	18.8 ± 0.8
Transgenic		
Phytochrome A		
21K15	24.0 ± 1.0	18.1 ± 0.7
13K7	23.7 ± 2.1	17.6 ± 0.7
Phytochrome B		
ABO	28.0 ± 1.2	19.9 ± 1.1
RBO	29.2 ± 1.8	20.5 ± 1.1

level and photoperiodic response in the LDP *Lolium temulentum*. On the other hand, these two inputs (photoperiodic versus photosynthetic) have often been confounded (Mozley and Thomas, 1995), so that the only generalization possible has been that photosynthetic and true photoperiodic responses influence flowering of LDPs but that photosynthetic input, although essential, may not have been sufficient for flowering (Friend, 1969; Bodson et al., 1977; Lejeune et al., 1993).

Overall, our studies provide only the beginning of an approach to understanding the role of phytochrome in flowering of LDPs. Considering the extensive literature on photoperiodism and flowering it remains possible that in day-neutral lines or in SDPs the different light-stable and -labile phytochromes interact with or substitute for each other. However, there may be further inputs by other phytochromes (e.g. C, D, and E). Other photoreceptors may also be important for photoperiodic control, especially given the large promotion of flowering by blue wavelengths as observed by Brown and Klein (1971). Furthermore, we have not taken account of rhythmic and other time-dependent changes in phytochrome-mediated responses of Arabidopsis to R and FR wavelengths (Deitzer, 1984).

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