Plant, Cell and Environment (2006) **29**, 1673–1685 doi: 10.1111/j.1365-3040.2006.01538.x

Phytochromes A1 and B1 have distinct functions in the photoperiodic control of flowering in the obligate long-day plant *Nicotiana sylvestris*

ZHI-LIANG ZHENG[1,2], ZHENBIAO YANG[2], JYAN-CHYUN JANG[1] & JAMES D. METZGER[1]

[1]*Department of Horticulture and Crop Science, The Ohio State University, 2021 Coffey Road, Columbus, OH 43210, and* [2]*Department of Plant Cellular and Molecular Biology, The Ohio State University, 1060 Carmack Road, Columbus, OH 43210, USA*

ABSTRACT

The obligate long-day plant *Nicotiana sylvestris* with a nominal critical day length of 12 h was used to dissect the roles of two major phytochromes (phyA1 and phyB1) in the photoperiodic control of flowering using transgenic plants under-expressing *PHYA1* (SUA2), over-expressing *PHYB1* (SOB36), or cosuppressing the *PHYB1* gene (SCB35). When tungsten filament lamps were used to extend an 8 h main photoperiod, SCB35 and SOB36 flowered earlier and later, respectively, than wild-type plants, while flowering was greatly delayed in SUA2. These results are consistent with those obtained with other long-day plants in that phyB has a negative role in the control of flowering, while phyA is required for sensing day-length extensions. However, evidence was obtained for a positive role for *PHYB1* in the control of flowering. Firstly, transgenic plants under-expressing both *PHYA1* and *PHYB1* exhibited extreme insensitivity to day-length extensions. Secondly, flowering in SCB35 was completely repressed under 8 h extensions with far-red-deficient light from fluorescent lamps. This indicates that the dual requirement for both far-red and red for maximum floral induction is mediated by an interaction between phyA1 and phyB1. In addition, a diurnal periodicity to the sensitivity of both negative and positive light signals was observed. This is consistent with existing models in which photoperiodic time measurement is not based on the actual measurement of the duration of either the light or dark period, but rather the coincidence of endogenous rhythms of sensitivity – both positive and negative – and the presence of light cues.

Key-words: critical day length; floral induction; floral stimulus; photoperiodism.

Correspondence: James D. Metzger. Fax: +1 614 292 7162; e-mail: metzger.72@osu.edu

*Present address: Department of Biological Science, Lehman College, The City University of New York, New York, NY 10468, USA.

INTRODUCTION

The timing of flowering is a critical component in the success of angiosperms. Flower initiation must occur early enough in the growing season so that seed production is completed before the onset of unfavourable conditions. Flowering must also be synchronized among individuals of a population if outcrossing is to occur to a significant extent. In many plant species the timing of flowering is controlled by environmental cues such as photoperiod and temperature.

Day-length information is sensed through photoreceptors in the leaves, activating the production and transport of graft-transmissible floral stimuli and inhibitors that control the transition of the shoot apical meristem from the vegetative to the reproductive state (Zeevaart 1976; Thomas & Vince-Prue 1997; Boss *et al*. 2004). The primary photoreceptors involved in photoperiodic timing mechanisms are the phytochromes (Thomas & Vince-Prue 1997), and the cryptochrome family of blue-light photoreceptors (Mockler *et al*. 1999; Botto *et al*. 2003; El-Assal *et al*. 2003). Physiological studies have led to the general conclusion that there is a diurnal fluctuation in the requirement for the presence or absence of Pfr for flowering in both long-day plants (LDP) and short-day plants (SDP). Generally speaking, in SDP high levels of Pfr during the day promote flowering, but become inhibitory in the middle of the inductive dark period. LDP, on the other hand, are the mirror image of SDP in that high levels of Pfr are usually inhibitory to flowering during the early part of day-length extension but become promotive later (Zeevaart 1976; Thomas & Vince-Prue 1997). The paradox that the presence of Pfr can be either promotive or inhibitory to flowering led to the hypothesis that two different pools of phytochrome with differential stabilities were involved in the photoperiodic timing mechanisms (Takimoto & Saji 1984).

The subsequent discovery (Mathews & Sharrock 1997; Pratt *et al*. 1997) of the existence of multiple species of phytochrome encoded by a gene family (*PHYA* through *PHYE* in *Arabidopsis*) demonstrated that different pools of phytochrome do indeed exist *in vivo*. Moreover, considerable evidence has accumulated indicating individual

phytochromes have distinct, albeit in some cases overlapping, functions in many aspects of photomorphogenesis, including photoperiodically controlled flowering (Quail *et al*. 1995; Smith 1995; Thomas & Vince-Prue 1997; Whitelam & Delvin 1997).

The flowering responses in mutants deficient in phyA, phyB or other phytochromes, as well as transgenic plants ectopically expressing different phytochrome genes, have supported the notion that multiple phytochromes are involved in the photoperiodic control of flowering (Whitelam & Delvin 1997). It has been suggested that phyA is required for sensing the day-length extension in LDP *Arabidopsis* and pea (Johnson *et al*. 1994; Weller, Murfet & Reid 1997). Furthermore, phyA controls the synthesis or transport of a flower inhibitor in pea (Weller *et al*. 1997, 2001). On the other hand, phyB is inhibitory to flowering in *Arabidopsis* and pea, both LDP and the SDP sorghum (Reed *et al*. 1994; Weller *et al*. 1995; Childs *et al*. 1997). In addition, the disruption of short day (SD)-induced tuber formation in antisense PHYB potato plants led to the suggestion that phyB also controls the production of a graft-transmissible flowering inhibitor (Jackson *et al*. 1996; Jackson & Thomas 1997). However, this does not appear to be the case for phyB-mediated SD suppression of flowering in pea (Weller *et al*. 2001).

Considerable progress has been made identifying genetic loci involved in the signalling pathways leading to flowering and elucidating their genetic relationships (Boss *et al*. 2004). The genes *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) are obligatory components of the photoperiod pathway leading to flowering in *Arabidopsis* (Boss *et al*. 2004). Transfer to long day (LD) results in CO-mediated induction of *FT* in the leaves, the site of perception of day length. *FT* mRNA is then transported to the apex where activation of *SOC1* and other downstream genes leads to flowering. Apparently *FT* mRNA is (or a component of) the elusive florigen (Ayre & Turgeon 2004; Huang *et al*. 2005; Yoo *et al*. 2005).

At least in *Arabidopsis*, the roles of phyA and phyB in photoperiodic timing mechanisms are manifested through an antagonistic regulation of the stability of CO resulting in a diurnal fluctuation in CO abundance (Valverde *et al*. 2004). In this model, phyB promotes degradation of CO early in the photoperiod, but as the day progresses, the inhibitory effects of phyB wane. Simultaneously, a stabilizing effect of phyA becomes more pronounced thereby leading to the accumulation of CO and the subsequent induction of *FT* (Valverde *et al*. 2004). Nevertheless, the mechanisms by which phyB and phyA regulate CO stability remain unclear. Furthermore, this model does not address which aspect of the photoperiod is actually measured; e.g. do plants measure the duration of the light period or of the dark period as commonly believed (Lang 1965)?

In some respects, there is a degree of ambiguity associated with studies on the photoperiodic control of flowering in quantitative LDP such as *Arabidopsis*. Plants with quantitative photoperiodic requirements will flower under non-inductive photoperiods because other floral pathways are activated (Boss *et al*. 2004). This is especially true in situations in which light signals mediated by phytochrome promote flowering by simultaneously activating both the photoperiodic (Valverde *et al*. 2004) and light-quality pathways (Mockler *et al*. 2003). Under such circumstances, it is difficult to discern how phytochrome is involved in photoperiodic time measurement. The use of a species with an obligate photoperiodic requirement provides a significant advantage in these types of studies. In this study, the obligate LDP *Nicotiana sylvestris* was chosen as a model plant to dissect the functions of individual phytochromes in the photoperiodic timing mechanisms. Using transgenic plants with altered expression levels of the *PHYA1* or *PHYB1* genes, we have gained additional insights about the physiological roles of type-A and type-B phytochromes in the photoperiodic control of flowering.

MATERIALS AND METHODS

Plant material and general growth conditions

Seeds of wild-type (WT) and transgenic *N. sylvestris* were germinated in plug flats of $22 \times 22 \times 35$ mm cells filled with a commercial soil-less media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA). One-month-old plants were transplanted into 3.8 L plastic pots, transferred to a greenhouse with a temperature set point of 25 °C and grown under 8 h photoperiods (SD) until the initiation of the experimental treatments. SDs were obtained by completely enclosing the bench with one-layer-cotton blackout cloth (F.C. Gloeckner, Inc., Harrison, NY, USA), which in turn was covered with a layer of XLS-Obscura Revolux A/B plastic weave containing a reflective outer surface (Cravo Equipment, Ltd, Brantford, Ontario, Canada) from 1600 to 0800 h. During the main photoperiod, supplemental light from high-intensity discharge lamps (photosynthetic photon flux at bench top level = 100 μmol m^{-2} s^{-1}) was provided when the intensity of sunlight outside of the greenhouse fell below 300 W m^{-2}.

Throughout the experiment, plants were fertilized three times a week with a 20 N-4.4 P-16.6 K complete liquid fertilizer (Scotts-Sierra Horticultural Products) at a rate of 200 mg L^{-1} N.

Construction of antisense PHYA1 and sense PHYB1 expression cassettes

A 2.3 kb EcoRI fragment of a partial *Nt-PHYA1* cDNA (Adam *et al*. 1993), which shared 62% homology at the nucleotide level with the corresponding *Nt-PHYB1* region (Kern *et al*. 1993), was blunt-ended and ligated in the antisense orientation into the SmaI site of the pUC18 vector, resulting in the pUA2.3. The 2.3 kb BamHI/SacI fragment containing the antisense *PHYA1* from pUA2.3 was then subcloned into BamH1/SacI sites in the binary vector pBI121 with a CaMV 35S promoter and NOS terminator. The resulting plasmid was designated as pAA2.3.

For the sense *PHYB1* cassette under the control of a single 35S promoter, a 4 kb BamHI/SacI fragment of a full-length *Nt-PHYB1* cDNA sharing 59% nucleic acid sequence homology with *PHYA1* (Adam *et al*. 1993; Kern *et al*. 1993) was directly cloned into BamHI/SacI sites of pBI121, resulting in the pSB. Construction of the enhanced *PHYB1* expression cassette pESB, with dual CaMV35S enhancers is described elsewhere (Zheng *et al*. 2001).

Transformation and phenotypic screening of transgenic tobacco plants

The resulting plasmids pAA2.3, pSB and pESB were transferred into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method (An *et al*. 1993). *Agrobacterium*-mediated transformation was described by Horsch *et al*. (1985), except that plants were generated from leaf discs cultured on MS medium containing BA (1 mg L^{-1}) and NAA (0.1 mg L^{-1}), and selection was based on carbenicillin (500 mg L^{-1}) and kanamycin (100 mg L^{-1}).

Putative lines under-expressing *PHYA1* and lines over- or cosuppressing *PHYB1* were identified by the altered hypocotyl length when grown for 12–14 d under continuous white light (Wc), red light (Rc), far-red light (FRc), or darkness (Dc). Seeds were spread on one-half-strength MS agar medium without sucrose, placed in darkness at 4 °C for 2 d followed by Wc from fluorescent lamps for 1–2 d to synchronize germination, and then transferred to growth chambers at 24 °C with Wc, Rc or FRc. Wc was obtained from 20 W cool-white fluorescent lamps that provided an approximate photon flux (400–700 nm) of 36 μmol m^{-2} s^{-1} to the seedlings. Rc was obtained by wrapping 20 W cool-white fluorescent tubes with two layers of R19S red cellulose plastic (Barbizon Light, Woburn, MA, USA); the photon flux (400–700 nm) was 3 μmol m^{-2} s^{-1}. FRc was obtained by filtering light from a 250 W halogen spotlight through a CBS-750 Far Red filter (Carolina Biological Supply Co., Burlington, NC, USA), at a photon flux (700–750 nm) of about 2 μmol m^{-2} s^{-1} to the seedlings; there was negligible radiation at wavelengths shorter than 700 nm.

T_1 transgenic lines showing seedling phenotype of longer hypocotyls under FRc (for antisense *PHYA1* transformants) and longer or shorter hypocotyls under Rc (for sense *PHYB1* transformants) were selected, grown to maturity in the greenhouse in which natural photoperiods were extended to 14 h by providing supplemental light from high-discharge lamps from 0600 to 1000 h and from 1600 to 2000 h in order to induce flowering, and selfed to obtain T_2 seeds. Homozygous T_2 seeds were identified by uniform kanamycin resistance and exhibition of the appropriate seedling phenotypes under the same light conditions used in the initial selection process.

PCR analysis

PCR was used to amplify the tobacco *PHYA1* and *PHYB1* cDNA to identify putative transgenic plants. Genomic DNA was extracted from young leaves of the T_2 homozygous lines by the CTAB-method (McGarvey & Kaper 1991). For *PHYA1*, two primers were used to amplify a 0.68 kb genomic DNA fragment and a 0.48 kb transgenic cDNA fragment. The sequence of the 22-mer forward primer AA1 was 5′-CGT GAG TAA AAT GTT GGA ATT A-3′, and the sequence of the 22-mer reverse primer AA2 was 5′-CTT CGC ACA CTC TTG ACC AGT C-3′. For PHYB1, two primers were used to amplify a 1.2 kb cDNA fragment. The forward primer SB1 (5′-GAA TGG TAT ACG CTT TAC AA-3′) was specific for Nt-PHYB1 cDNA, while the reverse primer SB2 was based on the pBI121 vector sequence: 5′-TCC AGC CGA ATT CCC CGA TA-3′. PCR was conducted using Taq DNA polymerase (Gibco BRL; Grand Island, NY, USA) under the following conditions for 30 cycles: 94 °C, 1 min; 52 °C (for *PHYA1*) or 55 °C (for *PHYB1*), 1 min; 72 °C, 1 min. An additional 7 min extension at 72 °C was performed after the last cycle.

RNA gel-blot analysis

Total RNA was extracted from 10-day-old dark-grown seedlings or young leaves of light-grown plants under dim green light, using the method described by Logemann, Schell & Willmitzer (1987). Ten micrograms of total RNA were fractionated on a 1.2% formaldehyde agarose gel and then transferred onto a nylon membrane. The probe was labelled by PCR (Sambrook, Fritsch & Maniatis 1989). The primers AA1 and AA2 used for PCR screening of *PHYA1* antisense plants were also used to label a 0.48 kb *PHYA1* cDNA probe that shared 57% nucleic acid sequence homology with *PHYB1* cDNA. The *PHYB1* probe was a 0.3 kb *PHYB1* cDNA fragment (showing about 48% nucleotide sequence identity with *PHYA1* gene) generated using a forward primer SB1 used in PCR screening of transgenic plants and a reverse primer BP2 (5′-ACC TGT AGT ATT CTC ACT TG-3′). PCR labelling was carried out using the same conditions as described above except α (^{32}P)-dATP was incorporated. The hybridization was performed as described (Sambrook *et al*. 1989).

Photoperiodic treatments

Experiments designed to examine the response of plants to various photoperiodic treatments were carried out in the greenhouse. Plants received a basic 8 h photoperiod as described before. Various light treatments were imposed during the subsequent 16 h dark period. Lamp systems providing different red to far-red ratios (R:FR; photon flux 655 nm to 665 nm photon^{-1} flux 725 nm to 735 nm) were suspended approximately 1.5 m above each bench, but underneath photoperiodic blackout cloth. Two different light sources were used: (1) 30 W cool-white fluorescent lamps (Flu) that provided light with a bench-level photon flux (400–800 nm) of approximately 4 μmol m^{-2} s^{-1} and an R:FR of 5.9; and (2) 60 W tungsten-filament lamps (TF) that provided light with a bench-level photon flux (400–800 nm) of approximately 8 μmol m^{-2} s^{-1} and an R:FR of 0.7. Photon irradiances and R:FR ratios were measured

using a Model LI-1800 Portable Spectroradiometer (Li-Cor Inc., Lincoln, NE, USA). The number of days from the start of the treatments to the appearance of flower buds was used as a quantitative measure of flowering.

Grafting experiments

Scions of the WT and the various transgenic lines of *N. sylvestris* were grafted onto stocks of a day-neutral cultivar of *Nicotiana tabacum* L., 'Wisconsin 38'. Plants for scions and stocks were grown under SD. *N. tabacum* stocks were prepared by removing the top 1–2 cm portion of the stem, followed by complete defoliation. All axillary buds were removed except one that was located about 5 cm from the top of the stem; this bud was allowed to grow out as an 'indicator' shoot for presence of flower promoting or inhibitory substances originating from the *N. sylvestris* scions (Lang, Chailakhyan & Frolova 1977). The *N. sylvestris* scions were prepared using the top 2–3 cm of the rosette from which all leaves greater than 5 cm were removed. The scions were then cleft-grafted onto the stocks and secured by wrapping a 2-cm-wide strip of Parafilm (American National Can, Neenah, WI, USA) around the scion–stock junction. The grafted plants were transferred to a standard mist-propagation house under natural light conditions for 2 weeks until a graft union had formed; the day length during this period of the grafting process was about 9.25 h. The grafted plants were transferred to a greenhouse and maintained under SD. After 56 d, five grafts of various *N. sylvestris* genotypes were subjected to an 8 h day-length extension with light from TF lamps, while five grafts were maintained under SD. During the entire experimental period, leaves from the *N. tabacum* indicator shoot were regularly removed when they reached 10 cm in length.

Experimental design and statistical treatment of data

All greenhouse experiments employed a randomized complete block design with multiple locations. The data are presented as means and standard deviations of the number of days until flower formation (appearance of visible flower buds). For statistical analysis, data were subjected to one-way analysis of variance following reciprocal transformation to ensure homogeneous variances (Roberts *et al*. 1997) and significantly different means separated using Tukey's pair-wise comparison test at $P \leq 0.05$. Greenhouse experiments were performed three times (twice and once during the winter and summer months, respectively) except for the grafting experiments, which were performed twice, both during the winter months of two successive years.

RESULTS

Generation and characterization of transgenic plants under-expressing PHYA1

There are two *PHYA* genes, *PHYA1* and *PHYA2*, in the tetraploid *N. tabacum* (Adam *et al*. 1993, 1997). Because *N. tabacum* is derived from the two parental diploid species, *N. sylvestris* and *Nicotiana tomentosiformis*, the two *PHYA* genes may have individually originated from each of the parental species, or they may have existed in both species. A preliminary work (Zheng 1999) using specific primers for the 5′ upstream regions of the two genes to amplify genomic DNA from *N. sylvestris* and *N. tomentosiformis* showed that *PHYA1* and *PHYA2* are derived from *N. sylvestris* and *N. tomentosiformis*, respectively. Thus, generation of transgenic plants that under-produce phyA was based on the sequence of the *N. tabacum PHYA1* gene because it has the closest sequence similarity to the *N. sylvestris* gene.

Plants of *N. sylvestris* that under-produce phyA1 were generated using *Agrobacterium*-mediated transformation with a partial cDNA *PHYA1* clone oriented in the antisense direction. Selection based on kanamycin resistance provided a total of 11 putative independent T_0 lines transformed with the antisense *PHYA1* construct; these lines were designated as SUA (*N. sylvestris* under-expressing *PHYA1*). Seedlings of the T_1 progeny were then screened for altered hypocotyl length under different light conditions. The T_1 lines SUA2, SUA7 and SUA11 had longer hypocotyls under FRc than WT seedlings, but exhibited normal hypocotyl phenotypes under Dc, Rc and Wc deficient in wavelengths longer than 700 nm (Fig. 1a). PCR analysis of genomic DNA from young leaves of homozygous T_2 plants showed the *PHYA1* transgene was incorporated into the genome of each of the three SUA lines (Fig. 1b). RNA blot analysis of dark-grown seedlings confirmed that expression of antisense *PHYA1* suppressed, but did not eliminate, the expression of the endogenous *PHYA1* gene (Fig. 1c) without affecting *PHYB1* gene expression (Fig. 1d). All three transgenic lines were apparently the result of one T-DNA insertion based on 3:1 segregation ratio of kanamycin-resistant to kanamycin-sensitive individuals (data not shown). Thus, the elongated hypocotyl phenotype of seedlings grown under FRc in the three SUA lines was a result of the down-regulation of *PHYA1* gene expression.

Generation and characterization of transgenic plants over-expressing or cosuppressing PHYB1

As is the case for *PHYA*, *N. tabacum* contains two *PHYB*-like genes, *PHYB1* and *PHYB2* (Kern *et al*. 1993), and because it appears likely that PHYB1 is also derived from *N. sylvestris* (Zheng 1999), it was used to generate transgenic plants in this species. A total of 13 T_0 lines independently transformed with the sense *PHYB1* construct pESB and one line (SOB1) transformed with pSB were obtained. Lines displaying segregation of individuals with short and long hypocotyls under Rc were designated as SOB (*N. sylvestris* over-expressing *PHYB1*). Seedlings from T_2 homozygous lines SOB1, 35, 36, 40 and 50, which had one T-DNA insertion based on a 3:1 segregation ratio of kanamycin resistance (data not shown), were subjected

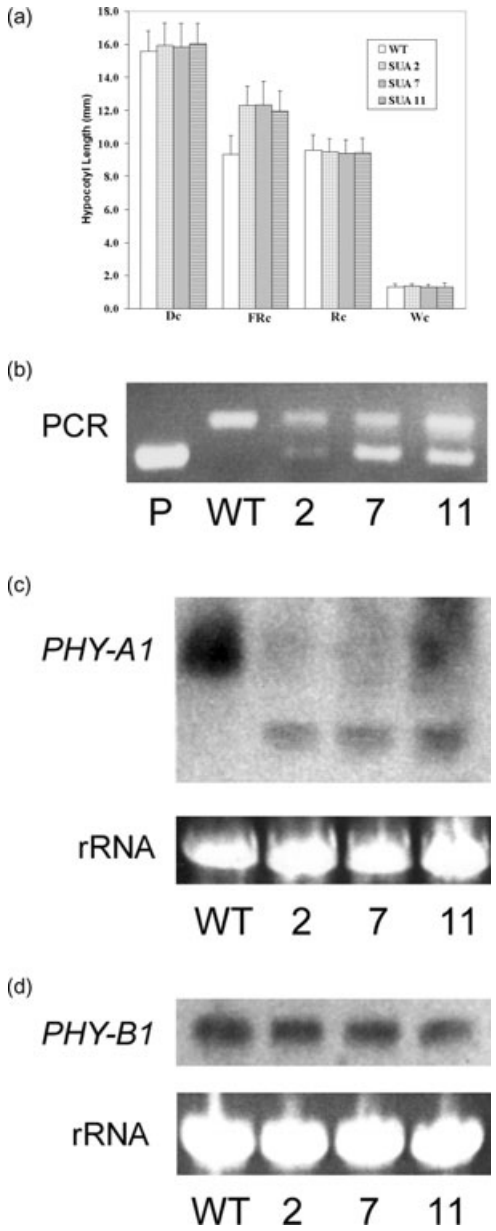

Figure 1. Molecular analysis of three putative phyA1 underproducing lines of *Nicotiana sylvestris*. (a) Comparison of hypocotyl lengths of seedlings of wild-type (WT) and three independent T_2 homozygous *N. sylvestris* underexpressing *PHYA1* (SUA) lines grown under various light conditions for 7 d. Data represent the mean of 40 seedlings; vertical bars represent the standard deviations. Dc, Rc, FRc, Wc: continuous dark, red, far-red and white light from fluorescent lamps, respectively. (b) PCR amplification of both antisense *PHYA1* cDNA (0.48 kb) and endogenous *PHYA1* genomic DNA (0.68 kb) containing an intron. P, plasmid pAA2.3. (c) RNA blot analysis using a 0.48 kb *PHYA1* cDNA probe. Both the antisense RNA (lower band) and the endogenous *PHYA1* mRNA (upper band) were hybridized. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. (d) RNA blot analysis using a 0.3 kb *PHYB1* cDNA probe. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA.

to Dc, Wc, Rc and FRc. Typical of phyB-mediated responses, FRc had no effect on seedling growth of any line. Under Rc and Wc, four of the five SOB lines exhibited shorter hypocotyls and well-separated cotyledons. The remaining line, SOB35, had longer hypocotyls than WT under Rc, Wc and Dc (Fig. 2a). Moreover, the cotyledons of this line only partially separated when grown under Wc and Rc (data not shown). Because this phenotype is similar to that expected for a plant deficient in phyB, SOB35 represented a potential line in which the endogenous *PHYB1* gene was down-regulated through cosuppression.

PCR analysis indicated the *PHYB1* transgene was incorporated into the genomes of the five SOB lines (Fig. 2b). RNA blot analysis confirmed that the expression of sense *PHYB1* resulted in higher *PHYB1* mRNA levels in all of the SOB lines except for SOB35 in which both endogenous and transgene expression were suppressed (Fig. 2c), indicating cosuppression was indeed the basis for the observed phenotype of SOB35 (Meyer & Saedler 1996). Therefore, this line was redesignated as SCB35 (*N. sylvestris* cosuppressing *PHYB1*). Expression of the *PHYA1* gene was not altered in SCB35 or the four SOB lines (Fig. 2d), consistent with a lack of an effect by FRc on either the hypocotyl or the cotyledon phenotypes.

Generation and characterization of transgenic plants in which *PHYA1* and *PHYB1* are simultaneously down-regulated

Flowering T_3 homozygous SUA2 and SCB35 plants were crossed to produce progeny in which both *PHYA1* and *PHYB1* were simultaneously down-regulated. PCR analysis showed that both the antisense *PHYA1* gene and the sense *PHYB1* gene were incorporated into the genomes of the progeny regardless of which genotype served as the pollen source (Figs 3a & b, respectively). Moreover, the seedling phenotype of the progeny from the crosses indicated a functional deficiency in the perception of both R and FR light. Hypocotyls of seedlings of the crosses were longer than WT when grown under either Rc or FRc (Fig. 3c), a characteristic similar to *phyAphyB* double mutants in *Arabidopsis* (Smith, Xu & Quail 1997).

There are similarities in the roles of *PHYA1* and *PHYB1* in the photoperiodic control of flowering in *N. sylvestris* to those of *PHYA* and *PHYB* in other long-day plants

The effect of altered expression of *PHYA1* and *PHYB1* on the flowering responses to different photoperiods was compared. Because of space considerations, one line of each transgenic genotype was used in all experiments: SUA2, SCB35, SOB36 and SUA2 × SCB35. Plants of the four transgenic lines and WT were maintained SD, or subjected to 10, 12 or 16h photoperiods by extending the main 8 h

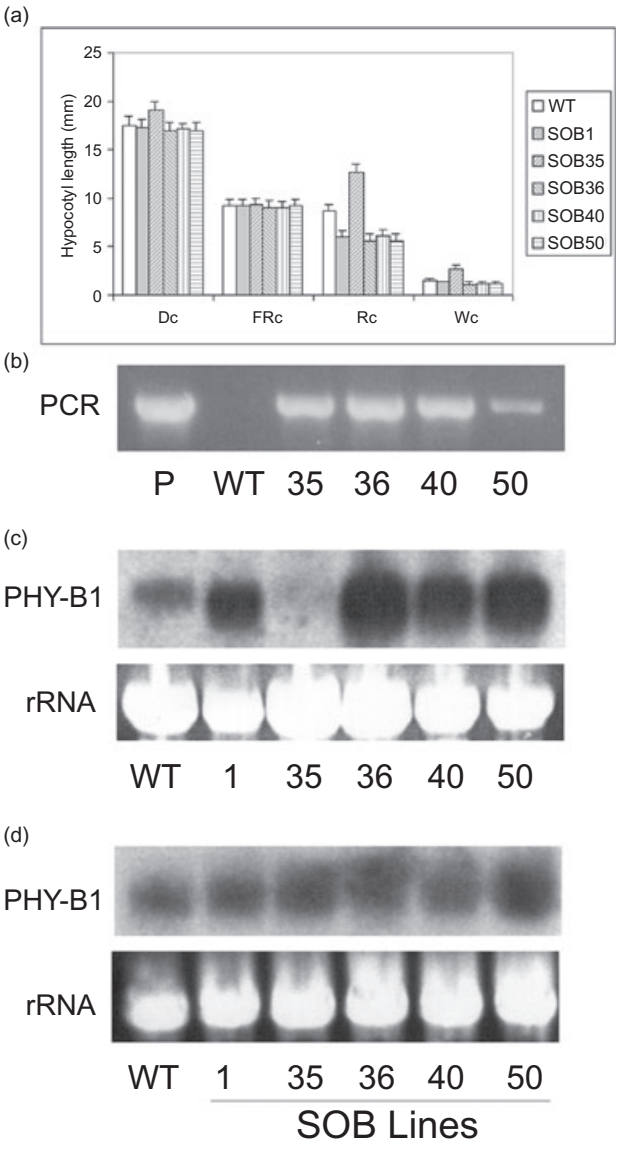

Figure 2. Molecular analysis of five *Nicotiana sylvestris* lines transformed with the *Nicotiana tabacum PHYB1* gene. (a) Comparison of hypocotyl lengths of seedlings of wild-type (WT) and five T_2 homozygous *N. sylvestris* overexpressing *PHYB1* (SOB) lines grown under various light conditions for 7 d. Data represent the mean of 40 seedlings; vertical bars represent the standard deviations. Dc, Rc, FRc, Wc: continuous dark, red, far-red and white fluorescent light, respectively. (b) PCR amplification of *PHYB1* genomic DNA (1.2 kb). P, plasmid pESB; WT, wild-type genomic DNA. (c) RNA blot analysis using a 0.3 kb *PHYB1* cDNA probe. Both the transgenic RNA (slightly lower position) and endogenous *PHYB1* mRNA (slightly higher position) were hybridized. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. (d) RNA blot analysis using a 0.48 kb *PHYA1* cDNA probe. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. The reduction of *PHYB1* expression and seedling phenotype of SOB35 was apparently the result of the cosuppression of the endogenous *PHYB1* gene; hence this line was renamed SCB35.

photoperiod with 2, 4 or 8 h of TF. Under SD, none of the genotypes flowered even after nearly 6 months following the initiation of the photoperiodic treatments when the plants were about 9 months old (Table 1). Extending the total photoperiod from 8 to 10, 12, or 16 h with TF promoted flowering by different degrees in WT, SCB35, SOB36 and SUA2.

All genotypes remained vegetative under SD (Table 1). Increasing duration of TF extensions resulted in a concomitant reduction in flowering times, the magnitude of which was dependent on genotype. Altering the level of *PHYB1* expression changed the sensitivity of plants to day lengths close to the critical day length (Table 1). Compared with WT, flowering was significantly delayed in SOB36, especially under a 2 h TF extension. In contrast, SCB35 flowered substantially earlier than WT. For example, a 2 h TF extension was as effective in promoting flowering in SCB35 as a 4 h TF extension was for WT (Table 1). However, there were no significant differences in the flowering times of WT, SOB36 or SCB35 under 8 h TF extensions (Table 1).

A reduction in the expression of *PHYA1* substantially delayed flowering compared with WT under all three TF

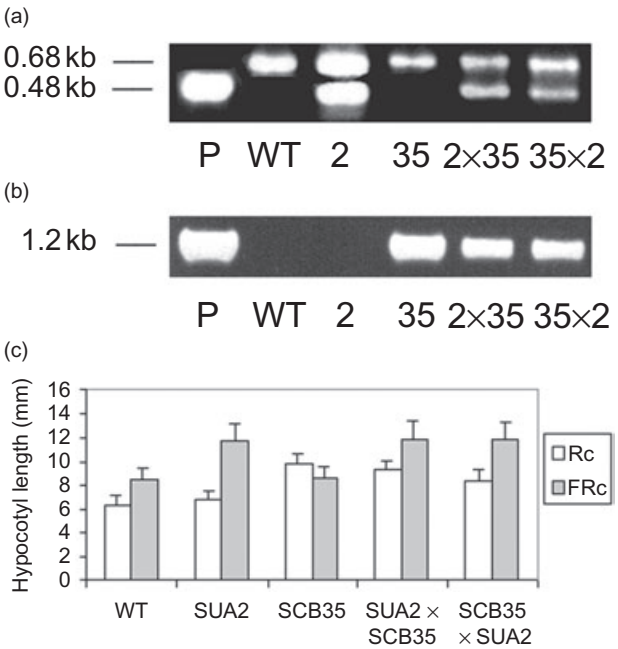

Figure 3. Molecular analysis of the F_1 progeny from two crosses between SCB35 and SUA2. (a) PCR amplification of both antisense *PHYA1* cDNA (0.48 kb) and endogenous *PHYA1* DNA (0.68 kb) containing an intron. (b) PCR amplification of the sense *PHYB1* cDNA (1.2 kb). Because the reverse primer was based on the vector sequence, no amplification of WT DNA occurred. (c) Comparison of the hypocotyl length of seedlings of WT, SUA2, SCB35, and the progeny of two crosses between SCB35 and SUA2 lines grown under continuous red (Rc) and far-red light (FRc) conditions for 7 d. Values represent the average lengths of 30 hypocotyls and vertical bars represent the standard deviations. WT, wild-type; 2, SUA2; 35, SCB35; 2 × 35, SUA2 × SCB35 in which SUA2 was the pollen source; 35 × 2, SCB35 × SUA2 in which SCB35 was the pollen source.

Table 1. Flowering responses of transgenic plants under various photoperiods

	Duration of day-length extension with tungsten filament light (total day length)			
Genotype	0 h (8 h; SD)	2 h (10 h)	4 h (12 h)	8 h (16 h)
	Days until flower formation			
Wild type	> 160	81 ± 9	40 ± 1	21 ± 2
SOB36	> 160	> 160	48 ± 5	24 ± 2
SCB35	> 160	41 ± 5	29 ± 2	22 ± 1
SUA2	> 160	> 160	87 ± 6	64 ± 1
SUA2 × SCB35	> 160	> 160	> 160	83 ± 6
	Duration of day-length extension with fluorescent light (total day length)			
Genotype		2 h (10 h)	4 h (12 h)	8 h (16 h)
		Days until flower formation		
Wild type		> 160	103 ± 13	59 ± 2
SOB36		> 160	98 ± 15	60 ± 1
SCB35		> 160	> 160	> 160
SUA2		> 160	> 160	87 ± 12
SUA2 × SCB35		> 160	> 160	> 160

Plants grown under short-day conditions (8 h natural light) were subjected to day-length extensions from low intensity light from tungsten filament or fluorescent lamps for 0 (short-day control), 2, 4 or 8 h. Values represent the means (± standard deviations) of the number of days from initiation of the day-length extension treatment until flower formation. Each treatment contained five plants.

extensions. Even under 8 h TF extensions, SUA2 flowered about 6 weeks later than WT, SOB36 and SCB35 (Table 1). In total, the results are consistent with other LDP and SDP in that phyB tends to be inhibitory to flowering under sub-optimal or non-inductive photoperiods, while phyA is required for sensing day-length extensions in LDP (Childs *et al*. 1997; Thomas & Vince-Prue 1997; Lin 2000; Weller *et al*. 2001). Surprisingly, none of the plants of the SUA2 × SCB35 cross-flowered when subjected to 2 or 4 h TF extensions. Even under 8 h TF extensions, SUA2 × SCB35 flowered nearly 3 weeks after SUA2. Apparently, a reduction in *PHYB1* expression enhanced the delay in flowering caused by lower *PHYA1* expression levels, rather than counteracting it.

Spectral composition of the day-length extension and the flowering response

In general, day-length extensions that are most effective in promoting flowering in LDP are those containing R and FR light mixtures (Thomas & Vince-Prue 1997). The roles of the two phytochromes in the perception of these two light signals were investigated by subjecting plants of the various genotypes to 2, 4 or 8 h day-length extensions of light from TF or Flu lamps which had R:FR ratios of 0.7 and 5.9, respectively. Day-length extensions of FR-deficient Flu were far less effective than TF in promoting flower formation (Table 1). This, along with the observation that day-length extensions of FR alone were totally ineffective in promoting flowering (Zheng 1999), is consistent with many other species in which day-length extensions most effective in promoting flowering in LDP are those containing mixtures of R and FR light (Thomas & Vince-Prue 1997).

No genotype flowered under 2 h Flu extensions. Under 4 h Flu extensions, flowering was observed only in WT and SOB36, which were 8 and 7 weeks, respectively, later than plants of both genotypes subjected to 4 h TF extensions (Table 1). WT plants flowered about 6 weeks later using day-length extensions with Flu than with TF (Table 1). Whereas over-expression of *PHYB1* resulted in a delay of flowering of SOB36 compared with WT with TF day-length extensions, there was no difference in flowering times between WT and SOB36 with Flu extensions (Table 1).

Quite unexpectedly, none of the SCB35 plants flowered by the end of the 160-day-treatment period when subjected to 2, 4 or 8 h Flu extensions (Table 1). Thus, despite the fact that the Pfr form of phyB is generally considered to be inhibitory to flowering in other LDP (Lin 2000), SCB35 is paradoxically less sensitive to day-length extensions containing negligible FR than either WT or SOB36. This indicates that *PHYB1* has an additional, positive role in flowering of *N. sylvestris* as well.

Although the *PHYA1* under-producing line, SUA2, exhibited reduced sensitivity to day-length extensions with TF, flowering was even more delayed under FR-deficient day-length extensions (Table 1). Flowering was observed only under 8 h Flu extensions and was over 3 weeks later than SUA2 plants subjected to 8 h TF extensions (Table 1). This is consistent with results from investigations on the quantitative LDP *Arabidopsis* (Johnson *et al*. 1994) and pea (Weller *et al*. 1995) demonstrating FR promotion of flowering is mediated by phyA. However, FR must act in

conjunction with R because day-length extensions with FR alone failed to promote flowering in any genotype (Zheng 1999).

Additional insight on the interaction of phyB and phyA on a functional level was obtained by examining the flowering response in plants of the cross SUA2 × SCB35 subjected to day-length extensions with different R:FR ratios. Of all the genotypes, flowering was delayed the most in SUA2 × SCB35 (Table 1). In fact, plants of this genotype flowered only when the day length was extended with TF. Under these conditions, plants of the SUA2 × SCB35 flowered, on average, 19 and 61 d later than SUA2 and SCB35, respectively (Table 1). Rather than counteracting the late flowering phenotype in SUA2, the reduction in *PHYB1* magnified the negative effects of decreased *PHYA1* expression on flowering. One interpretation of this observation is that *PHYB1* has more than one role in the control of flowering that includes both the generally accepted negative function (Thomas & Vince-Prue 1997; Lin 2000; Mockler *et al*. 2003) and a positive one as well, at least in *N. sylvestris*.

The level of phytochrome signalling affects the apparent critical day length

The results mentioned earlier suggest that the level of expression of either *PHYA1* or *PHYB1* may be important in defining the critical day length in this species. In a preliminary work, Zheng (1999) determined the critical day length of the WT line of *N. sylvestris* used in this study to be nominally 12 h. This value was the shortest photoperiod under which all plants produced visible flower buds within 60 d of the initiation of the photoperiodic treatment. In a subsequent work in which the observation period was extended from 2 to 6 months, we found that flowering will occur under 10 or 11 h photoperiods, although considerably late and inconsistent. However, WT plants grown under 8 h photoperiods do not flower even after 1 year. (Zheng 1999; J.D. Metzger, unpublished observations). Thus, although the 12 h value for the critical day length of the WT line is somewhat arbitrary, it does serve as a useful benchmark for making physiological comparisons with other genotypes.

To further examine the roles of *PHYA1* and *PHYB1* in defining the critical day length, plants of all genotypes except SOB36 were grown for 10 weeks under SD and transferred to a greenhouse section maintained under natural photoperiods from 1 January (day length = 9.3 h) to second week of April (day length = 13 h). Although the differences in flowering times between the genotypes were not as pronounced under natural photoperiods (Fig. 4) as when days were extended with TF (Table 1), similar trends were observed. The earliest flowering genotype was SCB35, with the average date of flower formation occurring on 1 March, while WT plants flowered about 2 weeks later (Fig. 4). Both SUA2 and SUA2 × SCB35 flowered in the second week of April, some 4 weeks after SCB35.

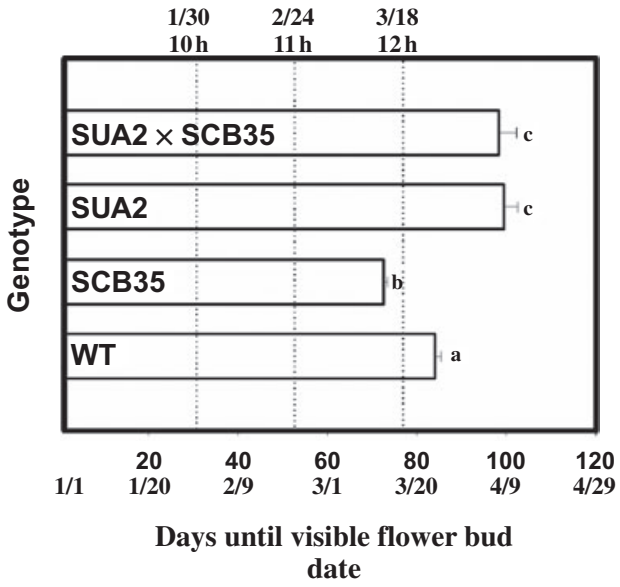

Figure 4. The comparative flowering response of *Nicotiana sylvestris* genotypes that under-produce either phyA or phyB to naturally increasing photoperiods from January to April. Plants were grown for 10 weeks under short-day conditions in a growth chamber and then transferred on 1 January (day length = 9.3 h) to a greenhouse section without supplemental light. Columns with different letters are significantly different at $P \leq 0.05$ level of confidence as determined by Tukey's pair-wise comparison test following reciprocal transformation of the means.

Under natural light conditions, the additional negative effect of simultaneously under-expressing both the *PHYA1* and *PHYB1* genes that occurred when plants were subjected to day-length extensions TF was not observed (Table 1 and Fig. 4). This is probably the result of the fact that there was incomplete suppression of *PHYA1* and *PHYB1* gene expression in SUA2 and SCB35, respectively (Figs 1 & 2), which resulted in plants with decreased sensitivity, rather than a complete loss of the ability to respond to light signals. It is likely that some of the decreased sensitivity was compensated by the higher light intensities when plants were maintained under natural photoperiods. Nevertheless, a reduction in the expression level of *PHYA1* did result in a significant lengthening of the critical day length, while reducing *PHYB1* expression shortened the critical day length.

Diurnal fluctuations in the flowering response to light cues

Physiological studies have shown that there is a diurnal fluctuation in the sensitivity to light cues in both SDP and LDP. Night-break experiments with the LDP *Hyoscyamus niger* (Hsu & Hamner 1967), *Sinapis alba* (Kinet *et al*. 1973) and *Lolium temulentum* (Vince-Prue 1975) have shown that there is a circadian rhythm in the ability of a night break to promote flowering. We investigated the physiological roles of phytochromes A and B in these rhythms of

Table 2. The effect of timing of a 2 h night break on flower formation in transgenic plants

	Genotype				
Time of night break	Wild type	SOB36	SCB35	SUA2	SUA2 × SCB35
	Days to flower formation				
1600–1800 h	121 ± 17	> 150	74 ± 6	> 150	> 150
1800–2000 h	71 ± 10	87 ± 4	40 ± 4	> 150	> 150
2000–2200 h	54 ± 5	79 ± 7	36 ± 4	> 150	> 150
2200–0000 h	39 ± 3	44 ± 4	34 ± 3	128 ± 10	> 150
0000–0200 h	35 ± 3	41 ± 6	31 ± 0	112 ± 16	> 150
0200–0400 h	34 ± 3	38 ± 8	32 ± 1	119 ± 24	> 150
0400–0600 h	66 ± 12	79 ± 4	41 ± 7	> 150	> 150
0600–0800 h	> 150	> 150	> 150	> 150	> 150

Plants grown under short-day conditions (8 h natural light) were subjected daily to 2 h 'night breaks' with low intensity light from tungsten-filament lamps at various times during the 16 h dark period. Values represent the means (± standard deviations) of the number of days from initiation of the night break treatments until flower formation. Each treatment contained five plants.

sensitivity by subjecting plants of the five genotypes to 2 h night breaks with TF at various times during the 16 h night such that all plants received a total of 10 h of light each day. Table 2 shows that there was a definite periodicity in the sensitivity to night breaks. With the exception of SUA2 × SCB35, which did not flower regardless of the timing of the night break, maximum sensitivity of all other genotypes was observed when the night breaks occurred between 2200 and 0400 h. Night breaks imposed during this period of maximum sensitivity resulted in little or no difference in the flowering times of WT, SCB35 and SOB36, while SUA2 flowered about 4 months later. In contrast, a 2 h night break was least effective when imposed immediately before or after the main 8 h photoperiod (Table 2).

Although there were no significant differences in the flowering responses of WT, SOB36 and SCB35 to the night breaks in the middle of the dark period (i.e. the 6 h period beginning 6 h and ending 12 h after termination of the main photoperiod), altering expression levels of *PHYB1* did have a substantial effect on flowering times when the night breaks were imposed at other times during the 16 h dark period. When the night break was imposed at 1600–1800 h (immediately after the main 8 h photoperiod), no flower formation was observed in SOB36 plants. Even though both WT and SCB35 eventually flowered, SCB35 flowered nearly 7 weeks earlier than WT (Table 2). However, the differences between the three genotypes gradually diminished as the timing of the night break approached the middle of the 16 h dark period when maximum sensitivity occurred and no differences in the flowering responses between genotypes were observed. Twelve hours after the start of the dark period (0400 h), plants of all three genotypes became less sensitive to the night break, with SOB36 and SCB35 exhibiting the greatest and least delay in flowering, respectively. None of the genotypes flowered when the night break was imposed just prior to the beginning of the main 8 h light period (Table 2). In total, these results indicate that there is a diurnal fluctuation in the inhibitory action of phyB1, which is at a maximum during the day and progressively declines until a minimum is reached sometime between 14 and 20 h after the beginning of the main light period.

SUA2 plants flowered only when the night breaks were applied between 2200 and 0400 h, which corresponded to the timing of the peak of maximum response observed in the other genotypes; however, flower formation in SUA2 was considerably delayed (Table 2). This is consistent with the observation that transformation with the antisense *PHYA1* gene resulted in substantial, but incomplete, suppression of *PHYA1* (Fig. 1c). Furthermore, SUA2 plants flowered when the night breaks coincided with the period during which *PHYB1* inhibition was at its apparent minimum (Table 2). This may indicate that the timing of the maximum in the flowering response of SUA2 simply reflects the minimum in the cycle of inhibitory action of *PHYB1*. The existence of a second rhythm of light sensitivity with a positive or promotive role in flowering is also possible. Evidence for such a rhythm, although not incontrovertible, is the observation that none of the genotypes, including SCB35, flowered either in SD (Tables 1 & 2) or when the night break was applied at 0600 h (Table 2), despite suppression of *PHYB1* expression. Conversely, SUA2 did not flower when the night break was imposed at 1800 or 2000 h, during which *PHYB1* inhibition of flowering was clearly reduced to different degrees in the other three genotypes (Table 2), suggesting that the positive actions of *PHYA1* and *PHYB1* in LD-induced flowering also exhibit a diurnal cycle of activity, but are 180° (or nearly so) out of phase with the diurnal cycle of *PHYB1* inhibition.

Phytochrome control of graft-transmissible flower promoting and inhibitory substances

From the results of the grafting experiments using *N. sylvestris* scions as donors and a day-neutral tobacco (*N. tabacum*) cultivar as the 'indicator' stocks, Lang and coworkers concluded that photoperiodic control of

Table 3. Flower formation in grafts between various transgenic lines of *Nicotiana sylvestris* and a day-neutral cultivar of *Nicotiana tabacum* (var. Wisconsin 38)

	Short day			Long day		
Scion genotype	Scion	Indicator	Control	Scion	Indicator	Control
	Days to flower formation					
Wis38	43 ± 3	44 ± 3	–	–	–	–
Wild type	> 140	> 140	> 140	101 ± 5	89 ± 2	85 ± 1
SCB35	> 140	> 140	> 140	100 ± 6	90 ± 3	84 ± 1
SUA2	> 140	> 140	> 140	> 140	> 140	136[a]
SUA2 × SCB35	> 140	> 140	> 140	> 140	> 140	> 140

[a]Only one control SUA2 plant flowered during the experimental period.
Scions derived from plants of one of four genotypes of *N. sylvestris* were cleft-grafted onto stock plants of *N. tabacum* var. Wisconsin 38 under short-day conditions. After 56 d, five graft combinations with each genotype were transferred to long days (8 h natural light plus 8 h day-length extension from tungsten-filament lamps), and five were maintained under short-day conditions. For comparison purposes, five non-grafted plants of each *N. sylvestris* genotype from the group of plants used for grafting were included in each photoperiodic treatment ('control'). Values represent the means ± standard deviations of the number of days from the day that plants were grafted until the flower formation in both the scion and the indicator shoot of the stock plant.

flowering in *N. sylvestris* is mediated by the production and transport from the leaves of a graft-transmissible floral inhibitor and promoter under non-inductive and inductive photoperiods, respectively (Lang *et al*. 1977; Lang 1989). Inasmuch as *PHYB1* has an inhibitory role in flowering, it is reasonable to infer that this inhibition is mediated through *PHYB1* control of production and/or transport of the inhibitor.

Possible phytochrome control of transmissible substances that inhibit flower formation was investigated by using the method developed by Lang *et al*. (1977) to assay for the presence of flowering inhibitors in *N. sylvestris*. In this system, the scion, which serves as the source of putative inhibitory and promotive flowering signals, is grafted onto a defoliated stock plant with all but one of its axillary buds removed. This bud is allowed to grow out to serve as an assay or 'indicator' shoot for the production of graft-transmissible flowering signals. The stock is a day-neutral line of tobacco. Flowering time in the indicator shoot from a control graft combination containing a scion from the same day-neutral line serves as a comparative measure by which to ascertain the presence or absence of inhibitors originating from the scion.

In the present work, scions from the various genotypes of *N. sylvestris* were grafted onto indicator stocks derived from the day-neutral tobacco cultivar 'Wisconsin 38' (Wis38). The graft combinations were then subjected to either SD or LD (SD + 8 h TF extension) (Table 3). Under SD, both the scions and the indicator shoots flowered rapidly in Wis38/Wis38 control graft combinations. In contrast there was no flower formation in the WT/Wis38 graft combinations under SD. However, both the WT scion and the Wis38 indicator shoot flowered 45 and 33 d after the transfer to LD. Consistent with the conclusions of Lang *et al*. (1977), the suppression of flowering in the day-neutral Wis38 indicator shoots in SD can be interpreted in terms of production and transport of a flowering inhibitor by the WT *N. sylvestris* scions. However, there was no difference in the flowering response of the Wis38 indicator shoots in the graft combinations with WT and the SCB35 *N sylvestris* donor scions (Table 3). This, along with the fact that the Wis38 indicator shoot in a graft combination with SUA2 × SCB35 did not flower under either SD or LD, indicates that altering phyB1 levels has no effect on the production or transport of the putative flowering inhibitor, as similarly suggested by Weller *et al*. (2001) for pea.

There is more evidence that *PHYA1* rather than *PHYB1* regulates some aspect of the transmission of flowering signals: flowering in the Wis38 indicator shoots was only observed under LD and if *PHYA1* was fully expressed in the scion (Table 3). Transfer to LD resulted in flowering of both the indicator shoot and the scion of the SCB35/Wis38 and WT/Wis38 graft combinations, while no flowering was observed in the SUA2/Wis38 and the SUA2 × SCB35/Wis38 combinations. There are two equally plausible explanations for these results. One interpretation is that *PHYA1* suppresses a flowering inhibitor as proposed for pea (Weller *et al*. 2001). However, the fact that the Wis38 indictor shoot flowered only when the donor scion flowered argues against this hypothesis and instead suggests that *PHYA1* mediates the LD-induced production of a promoter that is required for flowering.

DISCUSSION

Roles of *PHYA1* and *PHYB1* in the photoperiodic control of flowering

In many respects, *PHYA1* and *PHYB1* function in a similar manner in the obligate LDP *N. sylvestris* as their homologs in *Arabidopsis* and pea, both quantitative LDP. In all three species, phyA is clearly required for sensing day-length extensions (Johnson *et al*. 1994; Bagnall *et al*. 1995; Weller

et al. 1997) while phyB represses flowering under SD or suboptimal LD (Reed *et al*. 1994; Bagnall *et al*. 1995; Weller *et al*. 1995).

As has been observed in many LDP (Thomas & Vince-Prue 1997), day-length extensions with mixtures of R and FR light were most effective in promoting flowering in all *N. sylvestris* genotypes; FR alone was not sufficient for flower induction (Zheng 1999), while FR-deficient Flu evoked a much weaker response than TF (Table 1). The dual R and FR requirement for maximum induction is an indication that, as in many other LDP, the day-length extension is sensed by a phytochrome-mediated high irradiance response (HIR) mechanism (Mancinelli & Rabino 1978; Thomas & Vince-Prue 1997).

This raises the question of whether a single photoreceptor senses both R and FR signals. It is assumed that phyA1 is the FR sensor for flower induction because it mediates FR-induced inhibition of hypocotyl growth (Fig. 1a) and because phyA appears to be the sensor mediating FR-induced flowering in *Arabidopsis* (Johnson *et al*. 1994), and FR-containing day-length extensions in pea (Weller *et al*. 1997, 2001). It remains unclear, however, if phyA1 is also the receptor for red light required in the day-length extension in *N. sylvestris*. On the one hand, the HIR action spectrum can be explained in terms of the kinetics of the photochemical and dark reactions of a single phytochrome species (Wall & Johnson 1983), but such models do not easily explain the observation that phyB1-deficient SCB35 plants did not flower when subjected to day-length extensions of Flu, but flower early with TF (Table 1). Even plants of the phyA1 under-producing genotype SUA2, flowered, albeit extremely late, under FR-deficient day-length extensions with Flu (Table 1). This indicates that phyB1 has an essential positive role in sensing day-length extensions in *N. sylvestris* that contrasts with its apparent negative role when the photoperiods are less than the critical day length. Similar to its photosensory functions in seedling development, phyB1 likely mediates R-induced processes in the light-grown mature plants; in other words, PfrB1 is the active form in the flower-induction process. Additional evidence for separate but essential roles of the two phytochrome species in sensing day-length extensions is the observation that SUA2 × SCB35 flowered 7 weeks later than SUA2 and nearly 15 weeks later than SCB35 when plants were subjected to 8 h day-length extensions with TF (Table 1). We conclude that the promotion of flowering by day-length extensions is the result of a functional interaction between phyA1 and phyB1. In addition, we presume that the requirement for a mixture of R and FR is a reflection of the fact that Pfr and Pr forms of phyB1 and phyA1, respectively, mediate the promotion of flowering by day-length extensions.

The fact that SCB35 flowers as early as WT under 8 h TF day-length extensions and yet did not flower under Flu extensions of the same length (Table 2) seems at first glance to be a direct contradiction of this model. But this can be explained as follows. Although flowering in all genotypes was induced by 8 h day-length extensions of either TF or Flu (exception: SUA2 × SCB35), TF, which contains a high proportion of FR (R:FR = 0.7), was much more effective than Flu with an R:FR ratio of 5.9 (Table 1). Because the promotive effects of FR are quantitatively dependent on the absolute amounts of the Pr form of phyA1 (PrA1), Flu is therefore substantially less effective than TF in inducing flowering. Likewise, reducing the expression of *PHYA1* will result in lower absolute PrA1 levels in SUA2, which flowers later than WT, regardless of the light source. The fact that flowering in SUA2 is not completely suppressed under TF or even Flu extensions is likely due to incomplete suppression of *PHYA1* expression (Fig. 1c), although the redundant action of another phytochrome cannot be completely ruled out.

The component of the flowering response to day-length extensions mediated by phyB1 will be related to the absolute level of the Pfr form of this phytochrome (PfrB1). However, the minimum level of PfrB1 required for flower induction must be low because under an 8 h TF day-length extension, SCB35 flowered at the same time as WT (Table 1). The paradox of the failure of SCB35 to flower with 8 h Flu day-length extensions can be explained by the fact that under FR-deficient conditions, the combined levels of PfrB1 and PrA1 are below some critical threshold required for LD-induced flower induction.

The phyB requirement for sensing day-length extensions has not been observed in *Arabidopsis* (Goto, Kumagai & Koornneef 1991; Halliday, Thomas & Whitelam 1997) or pea (Weller *et al*. 2001). This apparent discrepancy may be due to the fact that, in contrast to *N. sylvestris*, both of these species are quantitative LDP that ultimately flower in SD. In other words, plants with a quantitative LD requirement will flower in SD because other floral pathways such as the autonomous or light-quality pathways (Boss *et al*. 2004) are activated. In obligate LDP, such as *N. sylvestris*, these alternate floral pathways may be greatly delayed or perhaps non-existent.

Photoperiodic timing mechanisms in *N. sylvestris*: what is actually measured?

Photoperiodic timing mechanisms in LDP are thought to be based on a circadian rhythm of sensitivity to light. A general model to explain the involvement of circadian rhythms is the external coincidence model in which there is a daily cycle in the sensitivity to light such that flowering is promoted when the light stimulus coincides with a light-sensitive phase of a circadian cycle (Thomas & Vince-Prue 1997). The results presented in Table 2 are generally consistent with the observations of other LDP and with the external coincidence model, but suggest additional insights. For example, it appears that there are two rhythms of light sensitivity opposite in action and 180° out of phase with one another with respect to the timing of maximum sensitivity to light. One of these rhythms is inhibitory and is mediated by PfrB1. Maximum sensitivity in the inhibitory rhythm to light occurs during the period just prior to the initiation of the main-light period ('dawn') and most likely continues for

at least several hours. However, by 8 h after the beginning of the main-light period, sensitivity to light begins to decline, and reaches a minimum 14–20 h (Table 2). The second rhythm of sensitivity to light is responsible for promotion of flowering and is mediated by a combination of PrA1 and PfrB1; maximum sensitivity to light in this rhythm occurs during the same period when inhibitory rhythm is the least sensitive (Table 2). Thus, a major conclusion of this model is that photoperiodic time measurement is not based on the actual measurement of the duration of either the light period or, as generally believed, the dark period (Lang 1965). This physiological model is generally consistent with the model for molecular basis of photoperiodic control of flowering in *Arabidopsis* proposed by Valverde *et al*. (2004): the phyB-mediated inhibitory and phyA-promotive rhythms in *N. sylvestris* correspond to the diurnal fluctuation in phyB-induced degradation and phyA-mediated stabilization of CO, respectively.

The physiological mechanisms underlying the control of flowering by the two rhythms are not known, but grafting experiments (Table 3) indicate that graft-transmissible signals are likely involved. As in the case of pea (Weller *et al*. 2001), the inhibitory rhythm apparently does not involve phyB1-modulated transport or synthesis of a graft-transmissible inhibitor (Table 3). In contrast, there is a more compelling evidence for a role of the promotive rhythm in regulating the production (or transport) of a substance or substances that play a pivotal role in photoperiodically induced flowering, because flower formation in the Wis38 indicator shoots was only observed when the *N. sylvestris* scion flowered (Table 3). During the receptive phase of the promotive rhythm, light signals may induce the suppression of a floral inhibitor as postulated for pea (Weller *et al*. 2001). On the other hand, an equally plausible explanation is that maintenance of the vegetative state in SD is the result of the lack of a promoter, rather than the presence of an inhibitor. By inference this flowering-promoting signal could be the product of the *N. sylvestris* ortholog of *Arabidopsis* FT (Ayre & Turgeon 2004; Huang *et al*. 2005).

The existence of two rhythms, opposite in action and out of phase also provides an explanation for the physiological basis of the critical day length: this will be the time after plants sense the beginning of the day when the activity of the promotive rhythm exceeds that of the inhibitory rhythm, which for WT under our experimental conditions is about 12 h (Zheng 1999). Variations in the sensitivity of the two rhythms to light signals can lead to subtle, but significant shifts in the critical day length. For example, reduction in *PHYB1* expression resulted in the shortening of the critical day length in SCB35 plants maintained under natural photoperiods, while the apparent critical day length was longer than WT when PHYA1 expression was inhibited in SUA2 plants (Fig. 4). Thus, clinal variations in critical day lengths for flowering (McMillan 1974) and bud dormancy in trees (Håbjørg 1978) may be based in part on genetic variation in the sensitivity of light-signal perception.

ACKNOWLEDGMENTS

This research was supported by funds from the Kiplinger Endowment for Floriculture. Salary and additional research support was provided by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. This paper is manuscript HCS 05-24 and is based on a portion of a dissertation submitted by Z.-L. Z in partial fulfilment of the requirements for the PhD degree in horticulture. The authors would like to thank Eva Adam and Ferenc Nagy for providing us with *Nt-PHYA1* and *PHYB1* cDNA clones and unpublished *PHYA2* partial genomic sequences. We are also grateful to Fang Cheng Gong for providing us with the pJITΔ2 vector. We are also indebted to Elizabeth Hunt and Marcia Feller for their help in the culture of plants and execution of the greenhouse experiments.

REFERENCES

Adam E., Deak M., Kay S., Chua N-H. & Nagy F. (1993) Sequence of a tobacco (*Nicotiana tabacum*) gene coding for type-A phytochrome. *Plant Physiology* **101,** 1407–1408.

Adam E., Kozma-Bognar L., Schaffer E. & Nagy F. (1997) Tobacco phytochromes: genes, structure and expression. *Plant, Cell and Environment* **20,** 678–684.

An G., Ebert P.R., Klee H., Horsch R.B. & Fraley R.T. (1993) Binary vectors. In *Plant Molecular Biology Manual* (eds S.B. Gelvin, R.A. Schilperoort & D.P.S. Verma), pp. A1: 1–19. Kluwer Academic Publishers, Amsterdam, the Netherlands.

Ayre B.G. & Turgeon R. (2004) Graft transmission of a floral stimulant derived from *CONSTANS*. *Plant Physiology* **135,** 2271–2278.

Bagnall D.J., King R.W., Whitelam G.C., Boylan M.T., Wagner D. & Quail P.H. (1995) Flowering responses to altered expression of phytochrome in mutants and transgenic lines of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* **108,** 1495–1503.

Boss P.K., Bastow R.M., Mylne J.S. & Dean C. (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. *Plant Cell* **16,** 18–31.

Botto J.F., Alonso-Blanco C., Garzarón I., Sánchez R. & Casal J.J. (2003) The Cape Verde Islands allele of cryptochrome 2 enhances cotyledon unfolding in the absence of blue light in Arabidopsis. *Plant Physiology* **133,** 1–10.

Childs K.L., Miller F.R., Cordonnier-Pratt M.-M., Pratt L.H., Morgan P.W. & Mullet J.E. (1997) The sorghum photoperiod sensitivity gene, Ma_3, encodes a phytochrome B. *Plant Physiology* **113,** 611–619.

El-Assal S., Alonso-Blanco C., Peeters A.J.M., Wagemaker C., Weller J.L. & Koorneef M. (2003) The role of cryptochrome 2 in flowering of Arabidopsis. *Plant Physiology* **133,** 1504–1516.

Goto N., Kumagai T. & Koornneef M. (1991) Flowering responses to light-breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long-day plant. *Physiologia Plantarum* **83,** 209–215.

Håbjørg A. (1978) Photoperiodic ecotypes in Scandinavian trees and shrubs. *Melding Norges Landbrukshøgskole* **71,** 1–20.

Halliday K.J., Thomas B. & Whitelam G.C. (1997) Expression of heterologous phytochromes A, B or C in transgenic tobacco plants alters vegetative development and flowering time. *Plant Journal* **12,** 1079–1090.

Horsch R.B., Fry J., Hoffman N.L., Wallroth M., Eichholtz D., Rogers S.G. & Fraley R.T. (1985) A simple and general method for transferring genes into plants. *Science* **223,** 496–498.

Hsu J.C.S. & Hamner K.C. (1967) Studies on the involvement of

an endogenous rhythm in the photoperiodic response of *Hyocyamus niger*. *Plant Physiology* **42,** 725–730.

Huang T., Böhlenius H., Eriksson S., Parcy F. & Nilsson O. (2005) The mRNA of the *Arabidopsis* gene *FT* moves from leaf to shoot apex and induces flowering. *Science* **309,** 1694–1696.

Jackson S. & Thomas B. (1997) Photoreceptors and signals in the photoperiodic control of development. *Plant, Cell & Environment* **20,** 790–795.

Jackson S.D., Heyer A., Dietz J. & Prat S. (1996) Phytochrome B mediates the photoperiodic control of tuber formation in potato. *Plant Journal* **9,** 159–166.

Johnson E., Bradley M., Harberd N.P. & Whitelam G.C. (1994) Photoresponses of light-grown phyA mutants of *Arabidopsis*: phytochrome A is required for the perception of daylength extensions. *Plant Physiology* **105,** 141–149.

Kern R., Gasch A., Deak M., Kay S.A. & Chua N.-H. (1993) *phyB* of tobacco, a new member of the phytochrome family. *Plant Physiology* **102,** 1363–1364.

Kinet J.M., Bernier G., Bodson M. & Jaqmard A. (1973) Circadian rhythms and the induction of flowering in *Sinapis alba*. *Plant Physiology* **51,** 598–600.

Lang A. (1965) Physiology of flower initiation. In *Encyclopedia of Plant Physiology, Old Series*, Vol. 15 (ed. W. Ruhland), pp. 1380–1535. Springer, Berlin, Germany.

Lang A. (1989) Nicotiana. In *CRC Handbook of Flowering*, Vol. 6 (ed. A.H. Halevy), pp. 427–483. CRC Press, Boca Raton, FL, USA.

Lang A., Chailakhyan M.K. & Frolova I.A. (1977) Promotion and inhibition of flower formation in a dayneutral plant in grafts with a short-day plant and a long-day plant. *Proceedings of the National Academy of Sciences of the USA* **74,** 2412–2416.

Lin C. (2000) Photoreceptors and regulation of flowering time. *Plant Physiology* **123,** 39–50.

Logemann J., Schell J. & Willmitzer L. (1987) Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163,** 16–20.

McGarvey P. & Kaper J.M. (1991) A simple and rapid method for screening transgenic plants using the PCR. *Biotechniques* **11,** 428–432.

McMillan C. (1974) Photoperiodic responses of *Xanthium strumarium* (Compositae) introduced and indigenous in eastern Asia. *Botanical Magazine (Tokyo)* **87,** 261–269.

Mancinelli A.L. & Rabino I. (1978) The 'high irradiance responses' of plant photomorphogenesis. *Botanical Reviews* **44,** 129–180.

Mathews S. & Sharrock R.A. (1997) Phytochrome gene diversity. *Plant, Cell & Environment* **20,** 666–671.

Meyer P. & Saedler H. (1996) Homology-dependent gene silencing in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 23–48.

Mockler T.C., Guo H.W., Yang H.Y., Duong H. & Lin C.T. (1999) Antagonistic actions of Arabidopsis cryptochromes and phytochrome B in the regulation of floral induction. *Development* **126,** 2073–2082.

Mockler T., Yang H., Yu X., Parikh D., Cheng Y.-C., Dolan S. & Lin C. (2003) Regulation of photoperiodic flowering by *Arabidopsis* photoreceptors. *Proceedings of the National Academy of Science of the USA* **100,** 2140–2145.

Pratt L.H., Cordonnier-Pratt M.M., Kelmenson P.M., Lazarova G.I., Kubota T. & Alba R.M. (1997) The phytochrome gene family in tomato (*Solanum lycopersicum* L.). *Plant, Cell and Environment* **20,** 672–677.

Quail P.H., Boylan M.T., Parks B.M., Short T.W., Xu Y. & Wagner D. (1995) Phytochromes: photosensory perception and signal transduction. *Science* **268,** 675–680.

Reed J.W., Nagatani A., Elich T.D., Fagan M. & Chory J. (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. *Plant Physiology* **104,** 1139–1149.

Roberts E.H., Summerfield R.J., Ellis R.H., Craufurd P.Q. & Wheeler T.R. (1997) The induction of flowering. In *The Physiology of Vegetable Crops* (ed. H.C. Wien), pp. 69–99. CABI Publishing, Cambridge, MA, USA.

Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Smith H. (1995) Physiological and ecological function within the phytochrome family. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 289–315.

Smith H., Xu Y. & Quail P.H. (1997) Antagonistic but complementary actions of phytochromes A and B allow optimum seedling de-etiolation. *Plant Physiology* **114,** 637–641.

Takimoto A. & Saji H. (1984) A role of phytochrome in photoperiodic induction: two-phytochrome-pool theory. *Physiologia Plantarum* **61,** 675–682.

Thomas B. & Vince-Prue D. (1997) *Photoperiodism in Plants*, 2nd edn. Academic Press, San Diego, CA, USA.

Valverde F., Mouradov A., Soppe W., Ravenscroft D., Samach A. & Coupland G. (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303,** 1003–1006.

Vince-Prue D. (1975) *Photoperiodism in Plants*. McGraw-Hill, New York, NY, USA.

Wall J.K. & Johnson C.B. (1983) An analysis of phytochrome action in the 'High Irradiance Response'. *Planta* **159,** 387–397.

Weller J.L., Nagatani A., Kendrick R.E., Murfet I.C. & Reid J.B. (1995) New *lv* mutants of pea are deficient in phytochrome B. *Plant Physiology* **108,** 525–532.

Weller J.L., Murfet I.C. & Reid J.B. (1997) Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. *Plant Physiology* **114,** 1225–1236.

Weller J.L., Beuchamp N., Kerckhoffs L.H.J., Platten J.D. & Reid J.B. (2001) Interaction of phytochromes A and B in the control of de-etiolation and flowering in pea. *Plant Journal* **26,** 283–294.

Whitelam G.C. & Delvin P.F. (1997) Roles of different phytochromes in Arabidopsis photomorphogenesis. *Plant, Cell and Environment* **20,** 752–758.

Yoo S.K., Chung K.S., Kim J., Lee J.H., Hong S.M., Yoo S.J., Yoo S.Y., Lee J.S. & Ahn J.H. (2005) *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in Arabidopsis. *Plant Physiology* **139,** 770–778.

Zeevaart J.A.D. (1976) Physiology of flower formation. *Annual Review of Plant Physiology* **27,** 321–348.

Zheng Z. (1999) Distinct functions of type-A and type-B phytochromes in photoperiodic control of flowering in Nicotiana sylvestris and biotechnological applications of phytochrome transgenes in chrysanthemum. PhD Dissertation, Ohio State University, Columbus, OH, USA.

Zheng Z-L., Yang Z., Jang J-C. & Metzger J.D. (2001) Modification of plant architecture in chrysanthemum through ectopic expression of a tobacco phytochrome B1 gene. *Journal of the American Society for Horticultural Science* **126,** 19–26.

Received 6 December 2005; received in revised form 29 March 2006; accepted for publication 8 April 2006

