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Dark and Circadian Regulation of mRNA Accumulation in the Short-Day Plant *Pharbitis nil*[1]

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The developmental transition of the meristem from vegetative to reproductive growth is controlled by the cyclic alternation of light and darkness in photoperiodic plants. Photoperiod is perceived in the leaves or cotyledons, where a flower-inducing signal is produced and transmitted to the apex. To begin to understand the molecular basis of the photoperiodic induction of flowering, we investigated changes in gene expression at the level of mRNA abundance that occur in association with dark induction of flowering in the short-day species *Pharbitis nil*. Several cDNAs were isolated that corresponded to mRNAs whose abundance is altered after the transition to darkness. The pattern of increase in mRNA levels corresponding to one cDNA clone, PN1, showed a dark-induced maximum at 8 h of darkness, whereas a second clone, PN9, showed a dark-induced accumulation of mRNA with peak levels at 12 to 16 h of darkness. When plants were held in continuous darkness, both PN1 and PN9 exhibited rhythmic patterns of mRNA accumulation with an approximate circadian periodicity, suggesting that their expression is under the control of an endogenous clock. The observed pattern of expression of PN1 and PN9 in cotyledon tissue was unusual in that darkness rather than light promoted mRNA accumulation, which is a temporal pattern of expression distinct from that of several other *Pharbitis* genes, including *Cab*, *PsaG*, and actin, whose mRNAs were most prevalent or equally prevalent in the light. Brief illumination of an inductive dark period by a red light night break strongly inhibited the accumulation of both PN1 and PN9 mRNA. The expression of both PN1 and PN9 was spatially regulated in that mRNA transcripts were detected in the cotyledons and stems, but not the roots, of photoperiodically competent seedlings. Both PN1 and PN9 appeared to be present as single-copy genes in the *Pharbitis* genome. Sequence analysis has not determined the identity of these genes. Overall, the accumulation of mRNAs corresponding to both PN1 and PN9 closely paralleled the process of photoperiodic floral induction in *P. nil*, but a clear involvement with this process cannot be established from our findings because of the difficulty of separating photoperiodic events from other light-regulated processes, especially those involved in photosynthesis, such as *Cab* gene expression. These results identify the products of circadian-regulated genes in photoreceptive tissue of *P. nil* and support the concept that circadian-regulated gene expression interacting with darkness may be involved in the regulation of photoperiodically controlled physiological processes, including flower induction.

Flowering involves the developmental transition of the shoot meristem from vegetative to reproductive growth. In photoperiodic species of flowering plants, this developmental transition is regulated by the absolute duration of light and darkness in a 24-h cycle (Garner and Allard, 1920; Vince-Prue, 1975; Salisbury, 1982). It is well established that the leaf is the principal site of photoperiodic perception and that phytochrome is the primary photoreceptor involved in photoperiodic flowering responses. Many physiological studies have led to the hypothesis that inductive processes occurring in photoperiodically sensitive foliar tissue lead to the synthesis of a flower-promoting signal, the floral stimulus, that is translocated to target meristem cells of the shoot apex where floral evocation is triggered (Lang, 1965; Evans, 1971; Zeevaart, 1976). In this case, evocation of floral initiation in the shoot apex is controlled by inductive processes in the leaf and, therefore, involves interorgan signaling mediated by a translocatable chemical messenger.

It is reasonable to assume that a series of reactions leads to production of a floral stimulus in photoperiodically induced tissues. Regulation of these processes by photoperiod may occur at a number of levels, including at the level of gene activation. In most other developmental transitions in plants, including floral initiation, flower development, and fruit ripening, expression of novel genes appears to be involved (Lincoln et al., 1987; Bowman et al., 1989; Kelly et al., 1990; Melzer et al., 1990). Thus, it is possible that an altered pattern of gene expression may be a fundamental component of the mechanism regulating photoperiodic floral induction. Previous research has attempted to assess the possible contribution of changes in gene expression to floral induction using inhibitors of RNA and protein synthesis and, more recently, by examining changes in mRNA composition associated with floral induction (for review, see O'Neill, 1992). A single quantitative change of in vitro translation products of mRNA isolated from cotyledons of *Pharbitis nil* was specifically associated with photoperiodic floral induction (Lay-Yee et al., 1987). Similar efforts also using *P. nil* failed to detect even these few changes in mRNA composition (Ono et al., 1988; Bassett et al., 1991), indicating that if changes in mRNA composition accompany floral induction, they must involve regulation of a relatively few and/or low-abundance transcripts. Alternatively, because mRNA populations were analyzed at only a single time, after 16 h of dark treatment, it is possible that the timing of the experimental sampling was not optimal to detect transient but critical changes in mRNA

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Abbreviations: CL, continuous light; FI, fractional induction; NB, night break.

composition that might accompany dark induction of flowering.

Recently, it has been recognized that circadian rhythms regulate expression of a number of light-regulated plant genes, notably nuclear-encoded photosynthetic genes, and in certain cases, this regulation occurs at the level of gene transcription (Kloppstech, 1985; Piechulla and Gruissem, 1987; Guilano et al., 1988; Nagy et al., 1988; Piechulla, 1988; Meyer et al., 1989; Taylor, 1989; Millar and Kay, 1991). Phase shifting by red light or temperature of the circadian rhythm of cycling mRNA levels has also been reported, strongly suggesting control of gene expression by the biological clock (Tavladoraki et al., 1989; Piechulla and Riesselmann, 1990). Because all rhythmically regulated genes characterized to date are also light regulated, it has been suggested that light regulation of gene expression may reflect the interaction of light with an endogenous circadian rhythm (reviewed by Rosbash and Hall, 1989).

These concepts are consistent with a model of light and circadian regulation of photoperiodic phenomena in plants. It is well established that photoperiodic floral induction is regulated by light interacting with underlying endogenous rhythms (Bünning, 1936; Hamner and Takimoto, 1964; reviewed by Vince-Prue, 1986), yet few investigators have analyzed changes in gene expression in relation to photoperiodic floral induction over a 24-h cycle. A single report describes rhythmic changes in mRNA levels associated with photoperiodic induction of flowering in the LDP *Sinapis alba* (Cremer et al., 1991).

A major goal of our research on the photoperiodic control of flowering is to identify molecular components of the mechanism of induction. The experimental system we have used is *P. nil* Choisy strain Violet (Japanese morning glory), a qualitative SD species that has been well characterized with regard to the physiology and biochemistry of photoperiodic floral induction (Imamura, 1967; Takimoto, 1967; Vince-Prue and Gressel, 1985). The photoperiodic timing mechanism of this SDP (Takimoto and Hamner, 1965; Spector and Paraska, 1973; Lumsden et al., 1982; Vince-Prue and Lumsden, 1987; Heide et al., 1988) and the role of phytochrome in its photoperiodic responses (Evans and King, 1969; Vince-Prue et al., 1978; Takimoto and Saji, 1984; Vince-Prue, 1989; Thomas, 1991) are also perhaps the most extensively characterized of all photoperiodic species. Unlike other SD photoperiodic species, *Pharbitis* becomes fully responsive to photoperiodic treatment to induce flowering within a few days after germination (Marushige and Marushige, 1963a; Imamura and Marushige, 1967), and flowering in seedlings is induced by exposure of the cotyledon to a single dark period (Imamura and Takimoto, 1955). In addition, the inductive effects of dark treatment can be diminished and under certain conditions completely nullified by brief irradiation with red light (NB) given 8 h into the dark period (Takimoto and Ikeda, 1960a; Takimoto and Hamner, 1965). Thus, NB treatment provides a rigorous negative control for experimental treatments that induce flowering.

To identify and critically assess the function of molecular components of the mechanism controlling photoperiodic floral induction, it will be necessary to isolate genes encoding functional steps in this process. Toward these goals, we have cloned mRNAs whose expression changes during a dark treatment that induces flowering in *Pharbitis* seedlings, with the long-term goal of assessing the functions of the corresponding genes by modification of their expression in transgenic plants.

MATERIALS AND METHODS

Plant Material

Pharbitis nil Choisy strain Violet (Japanese morning glory) was used for all experiments. Seed of this *Pharbitis* strain was originally obtained from Marutane Co., Ltd. (Kyoto, Japan), and consisted of seed produced by open-pollinated, field-grown plants. A population of approximately 50 individual plants exhibiting uniform germination, vigorous growth habit, and a strong flowering response to a single inductive treatment was selected for seed increase under greenhouse conditions. Subsequently, the seed line has been maintained as an inbred population for 10 generations at the University of California, Davis. Seed from the 9th and 10th generations were used in the experiments described in this paper.

Seedling Preparation and Growth

Seedlings were prepared according to a routine schedule as follows. Seeds were scarified in concentrated sulfuric acid for 45 min at room temperature on a stir plate, rinsed well for 15 min under a continuous stream of deionized water, and then allowed to imbibe in aerated, distilled water for 8 h. Seeds that had imbibed were placed on moist germination paper in plastic boxes and held in a growth chamber under continuous fluorescent light (250 μmol m^{-2} s^{-1}; Phillips VHO/EW 185 W/1500 mA) at 28°C until germination was complete. When the emerging radical was approximately 5 mm in length, the germinated seeds were planted in trays containing a modified University of California standard soil mixture. When planted, seeds were spaced apart using a planting grid to avoid the shading of cotyledons by adjacent seedlings, an important consideration for light-interruption experiments. Following watering, trays were transferred to the growth chamber (27 ± 2°C) where seedlings emerged during an 8-h period. Any late-germinating seedlings were removed from the trays to obtain a uniformly developed population for experimental treatment. The 1st d of seedling emergence and growth has been designated as "d 1" in our experiments. Photoperiodic treatments were routinely initiated at 4:30 PM (0 h) on postgermination d 6 except in the seedling development studies. In the latter case, treatments were initiated at 4:30 PM (0 h) using seedlings varying in age from 1 to 6 d old from the time of germination.

Photoperiodic Treatments

All seedlings used in these experiments received pretreatment in continuous white light in a controlled environment growth chamber. Light was provided by a combination of cool-white fluorescent light (Phillips VHO/EW 185 W/1500 mA) and incandescent bulbs. Light intensity was determined at plant height using a Li-Cor quantum sensor (model Li-190SB) and ranged from 250 to 275 μmol m^{-2} s^{-1} during the

experimental series. Seedlings were subjected simultaneously to one of the following five treatments (see Fig. 1): (a) CL, 24 h light (LL); (b) LD, 16 h of light plus 8 h of dark (L16:D8); (c) SD, 8 h of light plus 16 h of dark (L8:D16); (d) FI, 12 h of light plus 12 h of dark (L12:D12); (e) NB, 8 h of light plus 16 h of dark with 10 min of red light interruption at 8 h into the dark period (L8:D8:NB:D8). Temperature during the dark was 23 ± 2°C.

For the NB treatment, a red light interruption of the 16-h dark period was accomplished as follows. A small growth chamber consisting of two separate compartments (5,400 cm^2) was designed to simultaneously administer an NB treatment and a treatment requiring uninterrupted darkness (SD, FI, LD) to two flats of seedlings each. Both compartments were sealed to give complete interior darkness. One chamber equipped with four 3-foot cool-white fluorescent tubes (Phillips F30T12/CW/RS) was fitted with a 3-mm-thick translucent red plexiglass sheet (Acrylite GP, color 210–0; CYRO Industries, Mt. Arlington, NJ) as a light filter. Spectral properties of the filter were determined between 400 and 800 nm using a Beckman DU-64 Scanning Spectrophotometer (Beckman Instruments, Palo Alto, CA). Transmission of light was maximum between 600 and 700 nm of the visible wavelength spectrum with no transmission below about 600 nm. Given these spectral characteristics and the fact that the fluorescent light source is a poor emitter of far-red light, these conditions provided a good source of red light. Red light was applied for precisely 10 min at an intensity of 16.25 μmol m^{-2} s^{-1} (average value) beginning at the 8th h of darkness for NB treatment.

At the end of each treatment, seedling tissue was harvested in complete darkness directly into liquid nitrogen, transferred to light-proof plastic containers, and stored at −80°C for later extraction of RNA. For each experimental time, a minimum of 10 plants was returned to the growth chamber for an additional 2 to 3 weeks of growth in CL. After 10 d, the effectiveness of each treatment in inducing flowering was determined by examining each node with the aid of a dissecting microscope. Flowering was assessed by determining the number of flower buds per plant, the percentage of flower buds/total buds, the percentage of plants possessing flower buds, and the percentage of plants with terminal flower buds.

For extended time-course studies, experiments were conducted as follows. After pretreatment in CL, treatment group plants were transferred to darkness for up to 48 h or held in CL. The time immediately prior to the transfer to darkness has been designated as the 0 h point. Plants were removed from the dark at 4-h intervals from 4 to 48 h and returned to CL as experimental controls for evaluation of flowering response at each time. During dark treatment, tissue was sampled in the dark at 4-h intervals by harvest directly into liquid nitrogen as previously described. Plants in the CL group were sampled at 12-h intervals during a 48-h period, beginning at 0 h and ending at 48 h. Flowering was assessed on 10 plants from each experimental time.

Seedling Development and Photoperiodic Competence

Groups of seedlings ranging in age from d 1 to d 6 postgermination were examined for development and dissected into cotyledons, stems (hypocotyl, plumule, petioles), and roots. These were frozen in liquid nitrogen and stored at −80°C for later use in RNA extraction. At the same time, groups of developing seedlings were given an SD treatment to determine their photoperiodic competence at daily intervals for 6 d.

Heat-Shock Treatment

Heat-shock treatments were given during a continuous 12-h dark treatment (FI) at 25 ± 2°C. At 2-h intervals, groups of plants were transferred to a completely dark, heated growth incubator to receive a 2-h 42°C heat-shock treatment. This was followed by return to darkness at 25°C for the duration of the 12-h dark period. Soil temperature was measured and reached 42°C by the middle of the 2-h heat treatment. Flowering was assessed on 10 plants from each heat-shock group and compared with the response of those given FI treatment at normal temperature.

RNA Isolation

RNA was isolated as previously described (O'Neill et al., 1990). Poly(A)$^+$ RNA was subsequently isolated by oligo(dT)-cellulose chromatography (Pharmacia) as originally described by Aviv and Leder (1972) and stored as a precipitate at −80°C for later use. Typical total RNA yields from cotyledon tissue was 100 μg g^{-1} fresh weight of tissue, with a poly(A)$^+$ RNA yield of approximately 1.5% of total RNA.

cDNA Library Screening

Poly(A)$^+$ RNA (5 μg) isolated from dark-induced cotyledon tissue was used to construct a cDNA library by a vector-primer method as previously described (O'Neill, 1989). The cDNA library consisted of approximately 6×10^6 independent transformants with cDNA inserts ranging in size from 200 to 2500 bp. For primary screening, approximately 5×10^4 colonies were screened by differential hybridization using ^{32}P-labeled cDNA probes as follows. Bacterial colonies were plated directly onto ten 132-mm nitrocellulose filter discs (Millipore HATF No. 14250 filters) at a density of 5×10^4 colony-forming units per plate, creating a set of primary master filters. Two sets of replica filters were prepared from the master filters by replica plating. The duplicate filters were probed simultaneously with cDNA probes prepared from control and experimental probes as described below.

Prehybridization, hybridization, and filter washing were carried out following standard procedures as previously described (O'Neill et al., 1990). The "plus" (experimental) and "minus" (control) cDNA probes were synthesized to high specific activity in the presence of [^{32}P]dATP (6000 Ci $mmol^{-1}$; Amersham) using 1 μg of poly(A)$^+$ RNA, Moloney murine leukemia virus reverse transcriptase (Pharmacia LKB Biotechnology, Inc.), and oligo(dT)$_{12-18}$ (1 μg mL^{-1}) as primer (Pharmacia). The control probe was synthesized using poly(A)$^+$ RNA isolated from noninduced cotyledon tissue of similar age to that used for isolating induced poly(A)$^+$ RNA. The experimental probe was synthesized using a combination of poly(A)$^+$ RNA isolated from cotyledon tissue photoperiod-

ically induced by 12 and 14 h of dark treatment. Equal amounts of ^{32}P-labeled cDNA probe were added to 2 mL of hybridization solution, heat denatured, and then added to the hybridization solution prior to adding the prehybridized filters. Approximately 6×10^6 cpm mL^{-1} of hybridization solution were used to screen 50,000 colonies in 75 mL of hybridization solution.

Differentially hybridizing cDNA clones were identified by comparing the autoradiograms of replica filters probed with either the control probe or experimental probe. Several rounds of primary screening were carried out in succession. Candidate colonies were picked and screened by secondary and tertiary screening following the same procedure as used for primary screening. Only a few cDNA clones were identified that showed a pattern of differential expression correlated with floral induction. Two of these cDNA clones, PN1 and PN9, are described in this report.

RNA Gel Blot Hybridization Analysis

RNA was fractionated by electrophoresis in formaldehyde agarose gels (Nevins and Wilson, 1981) and transferred from agarose gels to nitrocellulose or nylon membrane (Schleicher & Schuell) as described by Thomas (1983). RNA gel blots were probed with ^{32}P-labeled DNA sequences representing the coding regions of individual cDNA clones or PCR DNA products. The cDNA inserts used as probes were isolated from the plasmid by electrophoresis in low-melting temperature agarose (Sambrook et al., 1989) and labeled to high specific activity with [^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). The cDNA inserts for PN1 and PN9 were approximately 350 and 450 bp, respectively. Prehybridization, hybridization, and washing of filters was carried out as previously described (O'Neill et al., 1990). Autoradiography was carried out at −80°C using preflashed Kodak XAR-5 film and a single intensifying screen (Cronex Lightning Plus, DuPont). The hybridization signal was quantified from autoradiographs using the Bio Image system (Millipore Corp., Ann Arbor, MI) analytical imaging instrument equipped with a video camera, digital image processing, and data processing. Software used was Visage release 4.6K on a SPARC station computer system (UNIX). For the extended dark time course and NB experiments, relative hybridization signal was expressed relative to the maximum signal on each individual autoradiograph.

DNA Sequence Analysis

Nucleotide sequence analysis, predicted amino acid sequence analysis, and data base homology searching was carried out using PC/Gene sequence analysis software, release 6.6 (IntelliGenetics, Inc.).

RESULTS

Physiology of Flowering

Nodal Patterns of Flowering

Extensive research has been conducted on the photoperiodic sensitivity and flowering response of *P. nil* strain Violet (Imamura, 1967; Takimoto, 1967; Vince-Prue and Gressel, 1985). In general, this species produces flowers sequentially in axillary positions along the stem following induction. Following a strong induction, the shoot apical meristem also becomes florally evoked, resulting in a terminal flower, after which growth ceases. In our experiments, plants were examined at each node for the presence or absence of a flower bud. The two cotyledonary nodes remained vegetative in most experiments as expected, having been formed prior to treatment. The first true leaf node (node 1) also remained vegetative in most plants, reflecting a loss of responsiveness with developmental age (King and Evans, 1969; Owens and Paolillo, 1986). For this reason, node 1 is not included in our determination of flowering response. The second leaf node was the most responsive to dark induction, in agreement with earlier reports for *Pharbitis* (Imamura and Marushige, 1967). Above node 2, flowers formed in axillary positions usually up to node 7, followed by the formation of a terminal flower bud usually at node 8 but ranging up to node 10.

Photoperiodic Responses

The response of 6-d-old *Pharbitis* seedlings to various photoperiodic treatments is shown in Figure 1. Seedlings received either CL, LD, FI, SD, or NB treatment. Both CL and LD treatments failed to induce flowering at even a single node, whereas FI and SD treatments resulted in more than 50 and 100% flowering, respectively, when assessed on the

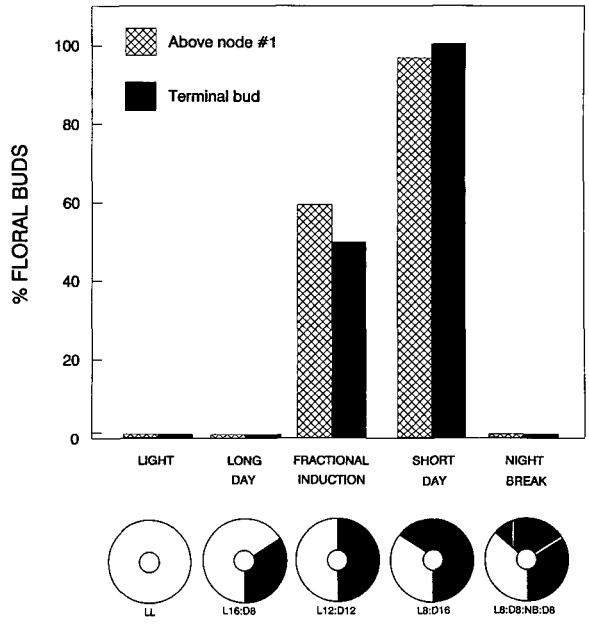

Figure 1. The flowering response of *Pharbitis* seedlings to photoperiodic treatment. Seedlings (6 d old) grown in CL received either CL, LD, FI, SD, or NB treatment as described in "Materials and Methods." Flowering response was assessed on the basis of the percentage of floral buds above node 1 (hatched bars) and of terminal floral buds (solid bars). The absolute duration of light (L) and dark (D) in a 24-h cycle and the timing of the NB treatment are schematically presented below each bar.

basis of either the number of floral buds or the number of plants with terminal flower buds. The application of 10 min of red light at 8 h into the dark period of a 16-h dark treatment (NB) completely abolished flowering at all nodes in this experiment. Thus, NB treatment resulted in full inhibition of flowering, making it a suitable control (in addition to CL and LD treatments) over inductive treatments such as SD or FI.

In detailed studies of the duration of dark required to induce flowering under our conditions, no flowering was observed until darkness of 10 h duration was exceeded. The first floral node, usually at node 2, was induced by 11 h of darkness. This amounted to only one or two nodes per treatment group (about 1% floral buds). After 12 h of darkness, 50% flowering was consistently observed, and after 14 h of darkness, maximum induction had occurred, such that no additional promotion of flowering was observed by 16, 18, and 20 h of darkness (data not shown). Thus, approximately 10.5 h of darkness corresponds to the critical night length for flower induction under our experimental conditions.

Development of Photoperiodic Competence

To relate changes in gene expression to physiological responses associated with photoperiodic sensitivity, it was necessary to establish the pattern of developmental sensitivity in the seed line used in our investigations. Therefore, *Pharbitis* seedling growth patterns during seedling development were examined in relation to the appearance of photoperiodic sensitivity. The results of this analysis are shown in Figure 2, where the response of different-age seedlings to a single SD treatment is shown. Development of photoperiodic sensitivity at 3 d after germination was correlated with cotyledon expansion and hypocotyl growth but not with root growth, lateral root initiation, or hypocotyl hook opening. It is interesting that the development of photoperiodic sensitivity correlates with the downward movement of the cotyledons, which were still appressed together in a vertical position on d 2 but fully open on d 3.

Perturbation of Induction by Heat Shock

To examine the timing of critical processes that occur during dark induction, 6-d-old seedlings were pretreated in CL and transferred to darkness at 23°C. At the indicated intervals, groups of plants were given a heat-shock treatment of 42°C for 2 h in the dark and then returned to darkness at 23°C. Following 12 h total of darkness, all plants were returned to the growth chamber and maintained under CL for an additional 2 weeks, after which time flowering was assessed. The results of the heat-shock treatment of *Pharbitis* seedlings during an FI treatment are shown in Figure 3, which indicates that control plants receiving only an FI treatment and no heat shock formed 2.5 flower buds per plant. Plants receiving heat shock between 0 to 2 and 2 to 4 h of darkness were not noticeably reduced in their flowering response, but after 4 h of darkness, the application of heat shock was disruptive to induction, being most effective in inhibiting flowering after 8 h of dark, with no flowering at all if heat shock was applied between 10 and 12 h of darkness. Heat-shock treatment did not noticeably perturb subsequent growth and development of the plant. Although there are alternate interpretations, these results suggest that heat-shock-sensitive processes involved in flower induction are initiated at 4 h and reach their maximum level by 10 to 12 h of darkness.

Identification of mRNAs Associated with Photoperiodic Floral Induction

The results of the heat-shock experiment directed our strategy for differential screening of a *Pharbitis* cDNA library to isolate genes specifically involved in floral induction. We reasoned that critical inductive processes, including gene

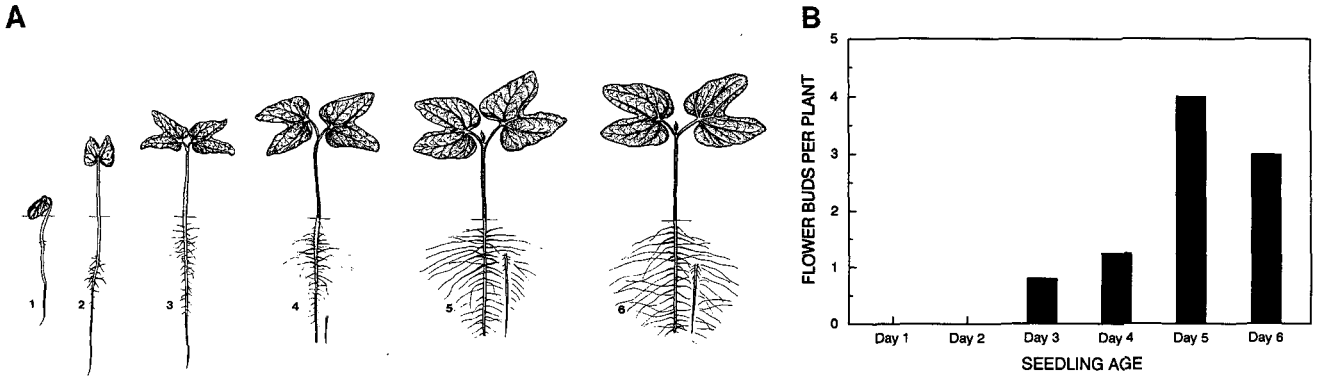

Figure 2. Development of photoperiodic competence as determined by flowering response of seedlings of different ages. A, *Pharbitis* seedling growth patterns during development from d 1 to d 6 postgermination. B, Flowering response to a single SD treatment. In A, groups of seedlings ranging in age from 1 to 6 d postgermination were examined for physical characteristics during development. These drawings represent accurate illustrations of representative seedlings chosen from the same samples used for the experimental treatments in B and are drawn to scale, shown on a relative basis. In B, groups of seedlings grown in CL were given SD treatment to determine their photoperiodic competence at daily intervals for 6 d. Flowering response was assessed as the number of flower buds per plant at d 1 though d 6 postgermination.

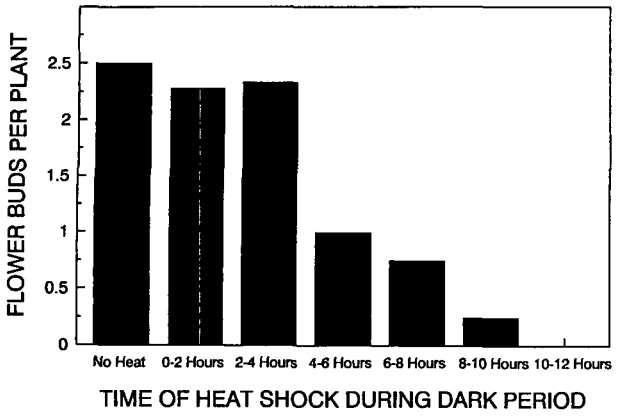

Figure 3. Perturbation of floral induction in *Pharbitis* by heat shock. Following growth in CL, 6-d-old seedlings were transferred to darkness for an FI treatment. At different intervals, groups of plants were given a 42°C heat-shock treatment in the dark for 2 h and then returned to darkness at a normal temperature. Following 12 h of total darkness, all plants were returned to growth in CL for assessment of floral induction. Flowering response was assessed as the average number of flower buds per plant as a function of time of heat-shock application during the 12-h dark period.

activation and expression, must be occurring during the 8- to 12-h "window of darkness," the time of maximum heat-shock sensitivity. This represents a time much earlier than the 16-h time we had investigated previously (O'Neill, 1989). In the findings reported here, a CL control cDNA probe was used in combination with a 12-h dark experimental cDNA probe to detect changes in mRNA transcript levels during the critical window of darkness. A second combination of probes consisting of the same control probe in combination with a 14-h dark experimental cDNA probe was also used for library screening to detect later changes in mRNA abundance.

Primary screening by differential hybridization of a *Pharbitis* cDNA library, representing the mRNA population present in photoperiodically induced cotyledon tissue, resulted in the isolation of a number of cDNA clones whose abundance appeared to be altered upon photoperiodic treatment by SD. Some members of this group of cDNA clones isolated by differential screening showed either an increase or decrease in mRNA abundance after transfer to darkness.

To determine whether any of these clones were likely candidates for those involved in floral induction or, instead, could be identified as light-regulated genes involved in photosynthesis and related metabolic processes, the nucleotide sequence of approximately 30 cDNA clones was determined in an attempt to determine the identity of the corresponding gene and its product. In some cases, the nucleotide sequence and its predicted amino acid translation resulted in a positive identification of the corresponding gene (Table I). For example, cDNA clones representing a number of nuclear-encoded genes involved in photosynthesis were identified, including *Cab*, *RbcS*, Rubisco activase, *PsaD*, *PsaE*, *PsaG*, *PsaH*, and *PsbR*. We also identified sequences not related to photosynthesis, including those encoding Ser carboxypeptidase, Gln synthase, high mobility group 1 DNA-binding protein (Zheng et al., 1993), and peptidyl-prolyl *cis-trans* isomerase. In other cases, the nucleotide sequence did not correspond to a previously characterized gene. This was the case for PN1, PN9, PN13, and PN31. The latter cDNA clone has a predicted polypeptide that is rich in His residues at the 5′ end of the coding sequence, a feature that is also found in a number of His-rich proteins involved in cell fate determination and developmental regulation (LaRosa and Gudas, 1988; Tomlinson et al., 1988; Weigel et al., 1989). Based on preliminary analysis of expression at the level of mRNA, a number of cDNA clones, including PN1, PN9, PN38 (*Cab*), and PN100 (*PsaG*), were selected for further characterization.

The two cDNA clones PN1 and PN9 represent cDNAs of 0.44 and 0.45 kb, respectively. Because PN1 hybridized to a single mRNA transcript of approximately 1.0 kb on an RNA gel blot, this indicated that the PN1 cDNA was not a full-length clone. DNA gel blot analysis revealed that PN1 is a single-copy gene (data not shown). PN9 hybridized to a single transcript of approximately 0.45 kb on an RNA gel blot, indicating that the PN9 cDNA encoded the complete PN9 mRNA. As with PN1, the PN9 cDNA corresponded to a single-copy gene (data not shown).

Dark- and Circadian-Regulated Transcripts

Four cDNA clones, PN1, PN9, *Cab* (PN38), and *PsaG* (PN100), were the focus of additional characterization to

Table I. *P. nil cDNA clones representing nuclear-encoded mRNAs isolated by differential hybridization of an induced cotyledon cDNA library*

cDNA Clone	cDNA Insert	Identity of Corresponding Gene
	kb	
PN1	0.44	Unknown[a]
PN9	0.45	Unknown[a,b]
PN11	0.73	HMG1[b]
PN12	0.56	RNA-binding protein[c]
PN13	0.4	Ser carboxypeptidase I precursor[c]
PN20	0.5	PSI reaction center subunit VI precursor (*PsaH*)[b]
PN22	0.8	Chl *a*/*b*-binding protein precursor (*Cab*)[b]
PN23	0.5	NADH-plastoquinone oxidoreductase subunit[c]
PN25	0.4	PSII 10-kD polypeptide precursor (*PsbR*)[b]
PN29	0.6	PSI reaction center subunit II precursor (*PsaD*)[b]
PN31	0.75	His-rich protein[b]
PN33	0.7	Gln synthase leaf isozyme precursor[c]
PN38	0.75	Ribulose bisphosphate carboxylase small chain precursor (*RbcS*)[b]
PN100	0.46	PSI reaction center subunit V precursor (*PsaG*)[b]
PN112	0.6	Peptidyl-prolyl *cis-trans* isomerase (*PPiase*)[b]
PN182	0.5	PSI reaction center subunit IV precursor (*PsaE*)[b]
ACTIN	1.06	Actin[d]

[a] Identity not determinable from sequence information. [b] Full-length coding sequence. [c] Partial coding sequence. [d] PCR product.

correlate patterns of mRNA accumulation with photoperiodic treatment. The products of both the *Cab* and *PsaG* genes have known functions in photosynthesis. *Cab* represents the light-harvesting Chl *a/b*-binding protein, whereas *PsaG* represents a low mol wt polypeptide associated as a peripheral component of PSI. The patterns of mRNA abundance following the transition to darkness and during an extended dark period or during parallel treatment in CL during the same time was determined for each of these clones.

Seedlings were pretreated by growth in CL and then transferred to darkness on the evening of the 6th d. Following the transition to darkness, levels of PN1 mRNA changed dramatically, as shown in Figure 4A. Within 4 h of the light-to-dark transition, PN1 mRNA abundance increased markedly, reaching a maximum level after approximately 8 h of darkness. Quantitative analysis of the increase indicated that PN1 mRNA increased 2-fold by 4 h and 4-fold by 8 h of darkness (Fig. 4B). After 12 h of darkness, PN1 mRNA transcripts declined from their peak abundance to levels only 2-fold higher than predark levels and reached the predark levels by 16 h of darkness. Thus, levels of PN1 mRNA were elevated between 4 and 12 h of darkness, approximately coincident with attainment of the critical night length for induction of flowering in our system.

The pattern of mRNA abundance for PN1 also appeared to be under the control of a circadian rhythm (Fig. 4A). Beginning approximately 24 h after the initial increase in PN1 transcript (at 4 h), a second increase in PN1 mRNA levels occurred, and they reached a second peak at 36 h after the light-to-dark transition. The two peaks of mRNA abundance in the dark were separated by 28 h, a time course approximating a free-running circadian rhythm, perhaps released upon the transition to darkness. After 48 h of continuous dark treatment, PN1 mRNA declined to its lowest level. This low level was approximately 5-fold lower than expression in the light at time 0 h (Fig. 4B).

A similar pattern of change was observed for PN9 mRNA levels (Fig. 4A). The time course of dark induction was slightly later and less pronounced. As compared to PN1, the levels of PN9 mRNA were relatively higher in the light, and PN9 mRNA levels did not increase as suddenly or to such an extent during the first 4 h of darkness. Instead, transcript levels first showed an increase at 8 h of darkness (nearly 2-fold), reaching about a 3-fold increase by 12 to 16 h of darkness (Fig. 4B). A second peak was observed at 36 h of darkness, approximately 24 h after the first peak, again suggesting a circadian regulation of PN9 mRNA accumulation (Fig. 4).

Both PN1 and PN9 transcripts declined to levels below 0-h levels after 20 h in darkness, reaching lowest levels by 48 h of darkness, suggesting that some component of their expression is regulated by light as well. However, the observed dark-induced increases in mRNA abundance were unique to these two cDNA clones, with the exception of one other cDNA clone, PN11. This latter clone also showed an increase in mRNA levels in darkness, but in the case of PN11, the first and second mRNA peaks occurred later than those of PN1 or PN9, at 20 h and 48 h, respectively (Zheng et al., 1993). Thus, maximum PN11 transcript levels did not occur until after 20 h of darkness, a pattern of expression clearly

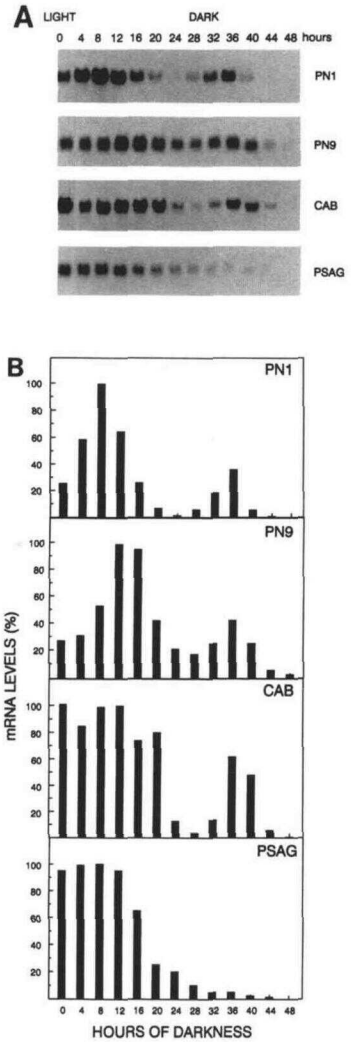

Figure 4. Analysis of *Pharbitis* seedling mRNA levels during extended dark treatment. A, RNA blot hybridization analysis of mRNA levels of PN1, PN9, *Cab*, and *PsaG* during an extended dark treatment. Seedlings were grown in CL for 6 d and then transferred to darkness at 0 h for up to 48 h. At the hours indicated, plants were harvested for analysis of mRNA levels as described in "Materials and Methods." Each lane contained 30 μg of cotyledon total RNA. Flowering was assessed at each time and was 50% by 12 h of darkness and 100% by 16 h of darkness. B, Analysis of relative mRNA hybridization signals for PN1, PN9, *Cab*, and *PsaG* during extended dark treatment. Relative mRNA levels were calculated from computerized image analysis of hybridization signals on autoradiographs (as shown in A) as described in "Materials and Methods." The mRNA levels are expressed as percentages of the maximum level of each transcript obtained in each individual hybridization experiment.

displaced in dark time from that of PN1 and PN9, whose mRNAs had already cycled up and down to their lowest levels by this time.

To provide a comparison with a gene of known function, we identified the *P. nil Cab* gene as a well-characterized gene known to be regulated by light and an endogenous rhythm (Piechulla, 1988; Meyer et al., 1989; Millar and Kay, 1991).

The pattern of *Cab* mRNA abundance was also examined during the same dark time course as for PN1 and PN9, as shown in Figure 4. As has been previously reported, *Cab* mRNA was present at high levels in light-grown cotyledon tissue (Fig. 5). Unlike PN1 and PN9, *Cab* mRNA was not significantly induced above light-grown levels following the transfer to darkness. *Cab* mRNA levels showed a broad maximum from 4 to 20 h of darkness. Furthermore, darkness did not significantly reduce the high levels of *Cab* mRNA until after 20 h of darkness, suggesting continued transcription in the dark or relatively high mRNA stability. Rhythmicity of expression was also observed for *Cab* in that a second transitory peak in mRNA level was observed at 36 to 40 h, indicating a periodicity of transcript accumulation with an approximately circadian interval. Analysis of mRNA maxima indicates that the first and second maxima of *Cab* mRNA levels occurred approximately 6 h later in the dark than that of PN1.

The pattern of mRNA abundance for *PsaG* provided another useful point of contrast, as shown in Figure 4. Transcript levels were not significantly affected by transfer to darkness, maintaining approximately the same levels of abundance during the first 12 to 16 h of darkness. However, after an initial lag, levels declined continuously in the dark and failed to show the second transitory peak with a circadian periodicity suggestive of a rhythmic pattern of expression as was observed for PN1, PN9, PN11 (Zheng et al., 1993), and *Cab*. Several other cDNA clones, including PN12, which encodes peptidyl-prolyl *cis-trans* isomerase (S.D. O'Neill, unpublished results), showed the same nonrhythmic decline and lack of immediate response to dark transfer (data not shown). Actin mRNA levels were relatively stable, showing only minor fluctuations during the 48-h extended dark time course, and did not exhibit increases or rhythmic oscillations in mRNA levels (data not shown).

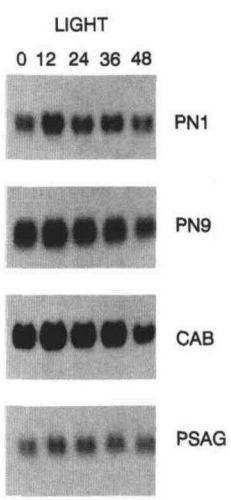

Figure 5. RNA blot hybridization analysis of mRNA levels of PN1, PN9, *Cab*, and *PsaG* during CL treatment. Seedlings were grown in CL for 6 d in parallel with those used for the extended dark experiment. At 0 h, samples were collected at 12-h intervals for up to 48 h. At the hours indicated, plants were harvested for RNA extraction and analysis of mRNA levels as described in "Materials and Methods." Each lane contained 30 μg of cotyledon total RNA.

A parallel analysis of expression in CL was also conducted to determine whether a rhythmic pattern of mRNA abundance could be detected in CL, as shown in Figure 5. Light levels in our experiments were optimal for this species and, therefore, would not be expected to result in arrhythmia. Plants were grown for 6 d in CL and then at 0 h were maintained in CL for up to 48 h and sampled at 12-h intervals, starting at 0 h. In contrast to the analysis after transfer to darkness, only slight variation was observed in transcript levels for all cDNA clones held in CL, with the maximum variation at any time not exceeding a 0.5-fold increase relative to 0-h levels.

NB Inhibition

Interruption of the inductive dark period by a brief irradiation with red light at the approximate middle of a 16-h dark period completely inhibits floral induction in *Pharbitis* seedlings (Fig. 1). Such NB treatment has been used extensively as an experimental tool to investigate the mechanism of photoperiodic floral induction (Hamner and Bonner, 1938; reviewed in O'Neill, 1992). This treatment was used here to test further the correlation of PN1 and PN9 mRNA levels with photoperiodic flowering response, because we reasoned that genes specifically involved in the photoperiodic floral inductive process should also be responsive to NB treatment. The nature of the response to NB cannot be predicted with certainty in advance because NB could result in a repression of gene activation or, alternatively, could cause a phase shift in rhythmic regulation of mRNA abundance.

The NB experiment was conducted by comparing the response of cotyledon tissue treated with three durations of darkness (16, 20, or 24 h) with tissue receiving the same duration of darkness but also receiving an NB at the 8th h of darkness. In this experiment, the flowering response was as follows: partial induction after 16 h and full induction by 20 and 24 h (data not shown). No flowering occurred in NB-treated plants. The effect of NB treatment on mRNA levels for PN1, PN9, *Cab*, and actin are shown in Figure 6. For both PN1 and PN9, NB treatment resulted in decreased levels of mRNA transcript at all times. A similar response was observed for *Cab* expression. Several other clones, including PN11 and PN12, were also examined and either showed an opposite response to NB as in the case of PN11 (Zheng et al., 1993) or failed to show any change in transcript level as observed for PN12 (data not shown). Hybridization to the actin probe demonstrated equal reactivity and equal amounts of RNA in all samples, thus supporting the conclusion that the observed differences in transcript levels were the result of NB treatment.

Developmental Regulation and Tissue Specificity

P. nil seedlings become photoperiodically competent 3 d after germination, suggesting that some component of the process of photoperiodic perception or the response system is not fully elaborated until that time of development (Fig. 2). We examined the levels of PN1 and PN9 mRNA in the

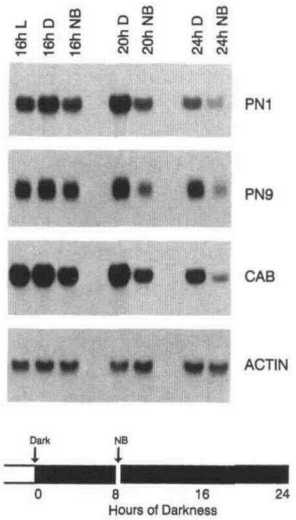

Figure 6. The effect of NB treatment on dark-induced mRNA levels of PN1, PN9, *Cab*, and actin. Control seedlings were treated with three durations of darkness (D) (16, 20, or 24 h), and NB-treated seedlings received the same durations of darkness but the darkness was interrupted at the 8th h by a 10-min NB with red light as described in "Materials and Methods." For the 16-h point only, a CL (L) control was also examined. Each lane contained 30 μg of total RNA isolated from cotyledon tissue. The black and white bar at the bottom of the figure schematically presents the light/dark conditions and the timing of the NB.

seedling each day after germination to determine whether the temporal pattern of mRNA accumulation in the cotyledons paralleled the development of photoperiodic competence. At the same time, we also examined the tissue specificity of expression to determine whether localization patterns were consistent with these two genes being candidates for involvement in the processes of floral induction. Figure 7 shows the time course of accumulation of PN1 and PN9 mRNA in the major tissues of the seedling during its growth, development, and acquisition of photoperiodic competence. The patterns of abundance of mRNAs corresponding to both clones were similar in that mRNA transcript was not detected in root tissue and only at low levels in stem tissue but was most prevalent in the cotyledon tissue. Thus, the two mRNAs are localized only to photoresponsive tissue; however, because these same tissues are also photosynthetic, this level of analysis in itself cannot distinguish between possible involvement in photoperiodic as opposed to photosynthetic processes. The developmental appearance of the two transcripts preceded the acquisition of photoperiodic competence by 1 d when the cotyledons were still folded together in an upright position (Fig. 2A). A parallel analysis of *Cab* gene expression indicated a parallel pattern of mRNA accumulation with PN1 and PN9 (Fig. 7). This result is not surprising, because development of photoperiodic competence parallels development of photosynthetic competence in *Pharbitis*.

DISCUSSION

The photoperiodic responses of *P. nil* strain Violet, the Japanese morning glory, have been extensively investigated. Under LD conditions, *P. nil* remains strictly vegetative, but following an SD treatment, the transition to reproductive growth is initiated, resulting in the production of floral primordia in the axils of successive nodes (Imamura and Takimoto, 1955; Imamura and Marushige, 1967; King and Evans, 1969; Owens and Paolillo, 1986). In response to a strong induction, the shoot apex produces a terminal flower leading to a cessation of further growth (Imamura and Marushige, 1967).

For our studies of the mechanism of photoperiodic floral induction, we have developed an inbred line of *P. nil* strain Violet. Physiological studies of the response of individual plants to photoperiodic treatments have established a general profile of response for our particular system. This was an important goal of our research because *Pharbitis* is unusually sensitive to treatment, and, therefore, critical variations between laboratories can occur. In terms of the nodal pattern of flowering, *Pharbitis* seedlings subjected to a single SD were induced to flower at successively higher axillary nodal positions until terminal flowering caused growth to cease. Nodes 2 and 3 were the most responsive to photoperiodic induction. The flowering response to darkness was minimal after 11 h of darkness, amounting to only 1% of floral nodes. Flowering increased dramatically with an additional 1 h of darkness (12 h dark total) and generally resulted in about 50% flowering. The response was saturated by 14 h of darkness (maximal induction). Our results are consistent with those previously reported by Imamura and Takimoto (1955) but differ slightly from those of Imamura (1967), who determined a critical night length of 9 to 10 h.

Development of Photoperiodic Sensitivity

During development, *Pharbitis* seedlings develop the capacity to respond to photoperiodic induction. *Pharbitis* may be exceptional as an SDP in being responsive to photoinduction in the early seedling stage when only a pair of cotyledons are present (Kujirai and Imamura, 1958; Marushige and

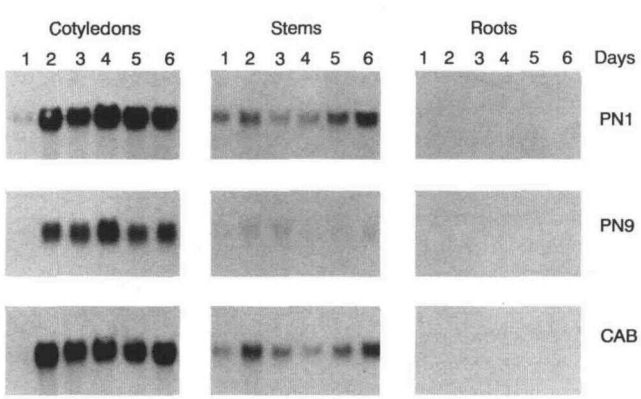

Figure 7. Temporal and spatial patterns of expression of PN1, PN9, and *Cab* mRNA during early seedling growth and development. At daily intervals from the time of germination (given as days postgermination), seedlings were dissected into cotyledons, stems, and roots for analysis of mRNA levels corresponding to the PN1, PN9, and *Cab* genes as described in "Materials and Methods." Each lane contained 30 μg of total RNA.

Marushige, 1963a, 1966). The attainment of photoperiodic sensitivity has been correlated with photomorphogenic differentiation of the cotyledon, establishment of an axial growth system, and establishment of photosynthetic capacity (Marushige and Marushige, 1963b, 1966). In our experiments, *Pharbitis* seedlings were unresponsive to SD treatment until 3 d of postgerminative development had occurred. By this time, both PN1 and PN9 are fully expressed in photoresponsive tissue, especially the cotyledons; thus, their expression appears to precede photoperiodic competence by 1 d.

In *Pharbitis*, it has also been shown that, in addition to the cotyledons, the plumule is also a photoperiodically responsive tissue because NB treatment to the plumule inhibits floral induction (Gressel et al., 1980). Thus, the expression of both PN1 and PN9 in stem tissue, which includes the plumular region, is consistent with their potential role in the photoperiodic mechanism. Because photoperiodic tissue is also photosynthetic tissue, these results cannot eliminate a role for these genes in photosynthesis.

Heat-Shock Perturbation of Photoperiodic Induction

The importance of transcriptional activation in the photoperiodic induction of flowering remains unestablished (reviewed by O'Neill, 1993). We sought a method of perturbation of floral induction that might provide indirect support for the hypothesis that transcriptional or posttranscriptional processes, including protein synthesis, are involved. We took advantage of the well-characterized "heat-shock response" to manipulate the inductive process in *Pharbitis* seedlings. The heat-shock response is an evolutionarily conserved molecular mechanism of gene activation by heat, resulting in recruitment of the transcriptional and translational machinery of the cell to the production of heat-shock proteins, disrupting synthesis of other mRNAs and proteins (Nagao et al., 1990; Gurley and Key, 1991; Vierling, 1991).

Heat-shock treatment of *P. nil* seedlings during dark induction caused an inhibitory effect on flowering, with maximum inhibition occurring in plants treated with heat between 8 and 12 h after the beginning of dark treatment. The marked depression in the flowering response earlier, between 4 and 8 h of darkness, also suggested that key preparatory processes occur during this time as well. These results differ from those of Nakayama (1958), who previously reported a reduction in flowering response by exposure of *Pharbitis* seedlings to high temperature (36°C). However, in this earlier report maximum inhibition occurred 2 h later, between 12 and 14 h of darkness, whereas exposure at 0 to 6 h actually promoted flowering. The difference in our results is most likely the consequence of different experimental conditions, which can strongly affect the response of this extremely sensitive species. A similar inhibition by high temperature was also reported for another SDP, *Xanthium* (Salisbury, 1963).

In our heat-shock experiments, the pattern of high-temperature sensitivity closely parallels the pattern of dark requirement for the inductive dark process as reported by Takimoto and Ikeda (1960b), who determined that in *Pharbitis* seedlings the third phase of a 16-h dark period (8–12 h) was the most light-sensitive (or dark-dependent) phase of the entire dark period. The coordinate inhibition by heat and light of the dark inductive process, being maximal between 8 and 12 h of darkness, suggests that this window of dark time is when the essential and perhaps final biochemical reactions are taking place that lead to the production of the floral stimulus. By analogy to the effect of heat shock in other higher eukaryotic systems, we speculated that the perturbation of flowering by heat shock may be mediated through a negative effect on normally occurring dark-induced gene transcription, although a primary effect at the level of translation cannot be ruled out in our experiments. We also reasoned that, if this were correct, then the critical changes in gene transcription must be occurring earlier than we had previously studied (O'Neill, 1989). Based on these considerations, we focused our differential screening efforts on this critical window of darkness.

Identification of mRNAs Associated with Photoperiodic Timing in *P. nil*

Based on extensive data indicating that regulation of photoperiodic floral induction involves darkness interacting with endogenous rhythms, we considered that mRNAs functionally associated with floral induction should (a) be regulated by darkness, (b) demonstrate a circadian rhythm in their regulation of abundance, and (c) be affected by NB treatment. Among many cDNAs representing mRNAs isolated from dark-treated *Pharbitis* cotyledons, we identified PN1 and PN9, whose pattern of expression exhibits these three features, including positive regulation by darkness, cycling of abundance with a periodicity of approximately 24 h, and reduction of abundance by NB. Based on the above criteria, PN1 and PN9 represent candidates for involvement in the process of floral induction, although a role in some other photoperiodically controlled or photosynthetic process cannot be ruled out by our experiments.

The involvement of circadian rhythms in light-regulated gene expression of plants is widely recognized (Kloppstech, 1985; Guilano et al., 1988; Nagy et al., 1988; Piechulla, 1988; Meyer et al., 1989; Millar and Kay, 1991). In the majority of studies of circadian-regulated gene expression in plants, light rather than darkness is the external signal influencing the amplitude of cycling mRNA levels (Guilano et al., 1988; Taylor, 1989). Indeed, darkness was observed to have a negative effect on the amplitude (Guilano et al., 1988) and transcriptional regulation of expression (Taylor, 1989) of *Cab* genes in tomato and maize, respectively.

In general, less attention has been paid to understanding the role of darkness as a night signal in plants. In studies in which the effect of darkness on mRNA levels has been studied, only a single time following the transition to darkness has been examined (Warm, 1984; Lay-Yee et al., 1987; Ono et al., 1988; Bassett et al., 1991). In this study, we examined the pattern of mRNA abundance in the light and dark for up to 48 h and obtained a detailed analysis of the temporal pattern of expression during darkness for a number of *Pharbitis* genes. For PN1, we observed that dark, rather than light, activates or promotes the maximum expression of this gene and that mRNA levels appear to be under the control of a circadian rhythm. The effect of an NB in altering the expression of the PN1 gene would argue that light represses mRNA

accumulation or, alternatively, that light phase shifts the rhythm controlling the expression of PN1 as it does the photoperiodic flowering response rhythm (Lumsden et al., 1986). The same could be true for PN9, although the higher level of abundance of this mRNA in the light than was observed for PN1 makes the interpretation of its pattern of expression less clear. The expression of the *Cab* gene was similar to that of PN1 and PN9 in terms of the observed rhythmically controlled mRNA levels and response to NB treatment. However, it differed significantly from PN1 and PN9 in that its highest expression was observed in light-grown tissue, and, furthermore, it did not respond to dark transfer by a dramatic increase in mRNA levels as did PN1. Therefore, although there are expected parallels among PN1, PN9, and *Cab*, there are also significant differences in the responses of these genes to darkness.

CONCLUSIONS

Overall, the results indicate that in *P. nil* a class of mRNAs exists that have the following properties: (a) their expression at the level of mRNA abundance is regulated by photoperiodic change, specifically the transition to darkness or by darkness itself, although light activation may also be involved; (b) they show rhythmic expression with a periodicity approximating a 24-h circadian rhythm; (c) their accumulation is inhibited by NB treatment; and (d) their expression is localized to photoperiodically sensitive tissues where inductive processes occur. These results in themselves do not provide conclusive evidence that PN1 and PN9 are involved in regulation of floral inductive processes but support the concept that circadian-regulated gene expression interacting with darkness could be involved in the regulation of photoperiodically controlled physiological processes, including flower induction.

Although *P. nil* has not been successfully transformed and regenerated, it is readily infected by *Agrobacterium* (Araki et al., 1989) and recently has been completely regenerated to flowering-size plants from immature embryos (Jia and Chua, 1992; Li and O'Neill, 1993). Thus, it should be possible in the future to use genetic transformation of *Pharbitis* to critically assess the function of genes such as PN1 and PN9 and their possible involvement in the photoperiodic induction of flowering.

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LITERATURE CITED

Araki T, Hirana H, Naito S, Komeda Y (1989) Introduction of foreign genes into *Pharbitis nil* calli using a vector derived from *Agrobacterium* pTi. Plant Cell Rep **8:** 259–262

Aviv H, Leder P (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA **69:** 1408–1412

Bassett CL, Mothershed CP, Galau GA (1991) Polypeptide profiles from cotyledons of developing and photoperioically induced seedlings of the Japanese morning glory (*Pharbitis* [*Ipomoea*] *nil*). J Plant Growth Regul **10:** 147–155

Bowman JL, Smyth DR, Meyerowitz EM (1989) Genes directing flower development in *Arabidopsis*. Plant Cell **1:** 37–52

Bünning E (1936) Die endogene Tagesrhythmik als Grundlage der photoperiodischen reaktion. Ber Dtsch Bot Ges **54:** 590–607

Cremer F, Van de Walle C, Bernier G (1991) Changes in mRNA level rhythmicity in the leaves of *Sinapis alba* during a lengthening of the photoperiod which induces flowering. Plant Mol Biol **17:** 465–473

Evans LT (1971) Flower induction and the florigen concept. Annu Rev Plant Physiol **22:** 365–394

Evans LT, King RW (1969) Role of phytochrome in photoperiodic induction of *Pharbitis nil*. Z Pflanzenphysiol Bodenk **60:** 277–288

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Garner WW, Allard HA (1920) Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J Agric Res **18:** 553–606

Gressel J, Zilberstein A, Porath D, Arzee T (1980) Demonstration with fiber illumination that *Pharbitis* plumules also perceive flowering photoinduction. *In* J DeGreef, ed, Photoreceptors and Plant Development. Antwerpen University Press, Antwerpen, The Netherlands, pp 525–530

Guilano G, Hoffman NE, Ko K, Scolnick PA, Cashmore AR (1988) A light-entrained circadian clock controls transcription of several plant genes. EMBO J **7:** 3635–3642

Gurley WB, Key JL (1991) Transcriptional regulation of the heat shock response: a plant perspective. Biochemistry **30:** 1–12

Hamner KC, Bonner J (1938) Photoperiodism in relation to hormones as factors in floral initiation and development. Bot Gaz **100:** 388–431

Hamner KC, Takimoto A (1964) Circadian rhythms and plant photoperiodism. Am Nat **98:** 295–322

Heide OM, King RW, Evans LT (1988) The semidian rhythm in flowering response of *Pharbitis nil* in relation to dark period time measurements and to a circadian rhythm. Physiol Plant **73:** 286–294

Imamura S (1967) Photoperiodic induction and the floral stimulus. *In* S Imamura, ed, Physiology of Flowering in *Pharbitis nil*. Japanese Society of Plant Physiologists, Tokyo, Japan, pp 120–138

Imamura S, Marushige Y (1967) The morphology and development of floral primordia. *In* S Imamura, ed, Physiology of Flowering in *Pharbitis nil*. Japanese Society of Plant Physiologists, Tokyo, Japan, pp 7–13

Imamura S, Takimoto A (1955) Photoperiodic responses in Japanese morning glory, *Pharbitis nil* Chois, a sensitive short day plant. Bot Mag Tokyo **68:** 235–241

Jia S-R, Chua N-H (1992) Somatic embryogenesis and plant regeneration from immature embryo culture of *Pharbitis nil*. Plant Sci **87:** 215–223

Kelly AJ, Zagotta MT, White RA, Chang C, Meeks-Wagner DR (1990) Identification of genes expressed in the tobacco shoot apex during the floral transition. Plant Cell **2:** 963–972

King RW, Evans LT (1969) Timing of evocation and development of flowers in *Pharbitis nil*. Aust J Biol Sci **22:** 559–572

Kloppstech K (1985) Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. Planta **165:** 502–506

Kujirai C, Imamura S (1958) Über die photoperiodische Empfindlichkeit der Kotyledonen von *Pharbitis nil* Chois. Bot Mag Tokyo **71:** 408–416

Lang A (1965) Physiology of flower initiation. *In* W Ruhland, ed, Encyclopedia of Plant Physiology, Vol XV, Part I. Springer-Verlag, New York, pp 1380–1536

LaRosa G, Gudas LJ (1988) Early retinoic acid-induced F9 teratocarcinoma stem cell gene *ERA-1*: alternate splicing creates transcripts

for a homeobox-containing protein and one lacking the homeobox. Mol Cell Biol **8:** 3906–3917

Lay-Yee M, Sachs RM, Reid, MS (1987) Changes in cotyledon mRNA during floral induction of *Pharbitis nil* strain Violet. Planta **171:** 104–109

Li J, O'Neill SD (1993) Somatic embryogenesis and plantlet regeneration in *Pharbitis nil* (abstract No 543). Plant Physiol **102:** S-97

Lincoln JE, Cordes S, Read EL, Fischer RL (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA **84:** 2793–2797

Lumsden P, Thomas B, Vince-Prue D (1982) Photoperiodic control of flowering in dark-grown seedlings of *Pharbitis nil* Choisy. The effect of skeleton and continuous light photoperiods. Plant Physiol **70:** 277–282

Lumsden PJ, Vince-Prue D, Furuya M (1986) Phase shifting of the photoperiodic flowering response rhythm in *Pharbitis nil* by red-light pulses. Physiol Plant **67:** 604–607

Marushige K, Marushige Y (1963a) Photoperiodic sensitivity of *Pharbitis nil* seedlings of different ages in special reference to growth patterns. Bot Mag Tokyo **76:** 92–99

Marushige K, Marushige Y (1963b) Changes in the growth and photoperiodic sensitivity of *Pharbitis nil* seedlings in relation to low intensity light. Bot Mag Tokyo **76:** 181–190

Marushige K, Marushige Y (1966) Photoperiodic sensitivity with respect to metabolic patterns of cotyledons in *Pharbitis nil*. Bot Mag Tokyo **76:** 142–148

Melzer S, Majewski DM, Apel K (1990) Early changes in gene expression during the transition from vegetative to generative growth in the long-day plant *Sinapis alba*. Plant Cell **2:** 953–961

Meyer H, Thienel U, Piechulla B (1989) Molecular characterization of the diurnal/circadian expression of the chlorophyll a/b binding proteins in leaves of tomato and other dicotyledonous and monocotyledonous plant species. Planta **180:** 5–15

Millar AJ, Kay SA (1991) Circadian control of *cab* gene transcription and mRNA accumulation of *Arabidopsis*. Plant Cell **3:** 541–550

Nagao RT, Kimpel JA, Key JL (1990) Molecular and cellular biology of the heat-shock response. Adv Genet **28:** 235–274

Nagy F, Kay SA, Chua N-H (1988) A circadian clock regulates transcription of the wheat *cab-1* gene. Genes Dev **2:** 376–382

Nakayama S (1958) Studies on the dark process in the photoperiodic response of *Pharbitis* seedlings. Sci Rep Tohoku Univ Fourth Ser **24:** 137–183

Nevins JR, Wilson MC (1981) Regulation of adenovirus-2 gene expression at the level of transcription termination and RNA processing. Nature **290:** 113–118

O'Neill SD (1989) Molecular analysis of floral induction in *Pharbitis nil*. *In* E Lord, G Bernier, eds, Plant Reproduction: From Floral Induction to Pollination, The American Society of Plant Physiologists Symposium Series, Vol 1. American Society of Plant Physiologists, Rockville, MD, pp 19–28

O'Neill SD (1992) The photoperiodic control of flowering: progress toward understanding the mechanism of induction. Photochem Photobiol **56:** 789–801

O'Neill SD (1993) Changes in gene expression associated with floral induction and evocation. *In* BR Jordan, ed, The Molecular Biology of Flowering. C.A.B. International Press, Wallingford, Oxon, UK, pp 69–92

O'Neill SD, Tong Y, Spörlein B, Forkmann G, Yoder JI (1990) Molecular genetic analysis of chalcone synthase in *Lycopersicon esculentum* and an anthocyanin-deficient mutant. Mol Gen Genet **224:** 279–288

Ono M, Okazaki M, Harada H, Hirofumi U (1988) *In vitro* translated polypeptides of different organs of *Pharbitis nil* Chois strain Violet under flower-inductive and non-inductive conditions. Plant Sci **58:** 1–7

Owens V, Paolillo DJ (1986) Effect of aging on flowering of the axillary buds of *Pharbitis nil*. Am J Bot **73:** 882–887

Piechulla B (1988) Plastid and nuclear mRNA fluctuations in tomato leaves—diurnal and circadian rhythms during extended dark and light periods. Plant Mol Biol **11:** 345–353

Piechulla B, Gruissem W (1987) Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. EMBO J **6:** 3593–3599

Piechulla B, Riesselmann S (1990) Effect of temperature alterations on the diurnal expression pattern of the chlorophyll *a/b* binding proteins in tomato seedlings. Plant Physiol **94:** 1903–1906

Rosbash M, Hall JC (1989) The molecular biology of circadian rhythms. Neuron **3:** 387–398

Salisbury FB (1963) Biological timing and hormone synthesis in flowering of *Xanthium*. Planta **59:** 518–534

Salisbury FB (1982) Photoperiodism. Hortic Rev **4:** 66–105

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Spector C, Paraska JR (1973) Rhythmicity of flowering in *Pharbitis nil*. Physiol Plant **29:** 402–405

Takimoto A (1967) *Pharbitis nil* Chois. *In* LT Evans, ed, The Induction of Flowering: Some Case Histories. Cornell University Press, Ithaca, NY, pp 90–115

Takimoto A, Hamner KC (1965) Studies on red light interruption in relation to timing mechanisms involved in the photoperiodic response of *Pharbitis nil*. Plant Physiol **40:** 852–854

Takimoto A, Ikeda K (1960a) Studies on the light controlling flower initiation of *Pharbitis nil*. VII. Light-break. Bot Mag Tokyo **37:** 341–347

Takimoto A, Ikeda K (1960b) Studies on the light controlling flower initiation of *Pharbitis nil*. VIII. Light-sensitivity of the inductive dark process. Bot Mag Tokyo **73:** 468–473

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

Tavladoraki P, Kloppstech K, Argyroudi-Akoyunoglou J (1989) Circadian rhythm in the expression of the mRNA coding for the apoprotein of the light-harvesting complex of photosystem II. Plant Physiol **90:** 665–672

Taylor WC (1989) Transcriptional regulation by a circadian rhythm. Plant Cell **1:** 259–264

Thomas B (1991) Phytochrome and photoperiodic induction. Physiol Plant **81:** 571–577

Thomas PS (1983) Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol **100:** 255–266

Tomlinson A, Kimmel BE, Rubin GM (1988) *rough*, a *Drosophila* homeobox gene required in photoreceptors R2 and R5 for inductive interactions in the developing eye. Cell **55:** 771–784

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 579–620

Vince-Prue D (1975) Photoperiodism in Plants. McGraw Hill, London

Vince-Prue D (1986) The duration of light and photoperiodic responses *In* RE Kendrick, GHM Kronenberg, eds, Photomorphogenesis in Plants. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp 269–305

Vince-Prue D (1989) The role of phytochrome in the control of flowering. Flowering Newslett **8:** 3–14

Vince-Prue D, Gressel J (1985) *Pharbitis nil*. En. Japanese Morning Glory. *In* AH Halevy, ed Handbook of Flowering, Vol IV. CRC Press, Boca Raton, FL, pp 47–81

Vince-Prue D, King RW, Quail PH (1978) Light requirement, phytochrome and photoperiodic induction in flowering of *Pharbitis nil* Chois. II. A critical examination of spectrophotometric assays of phytochrome transformations. Planta **141:** 9–14

Vince-Prue D, Lumsden PJ (1987) Inductive events in the leaves: time measurement and photoperception in the short-day plant, *Pharbitis nil*. *In* JG Atherton, ed, Manipulation of Flowering. Butterworth, London, pp 255–268

Warm E (1984) Changes in the composition of *in vitro* translated leaf mRNA caused by photoperiodic flower induction of *Hyoscyamus niger*. Physiol Plant **61:** 344–350

Weigel D, Jürgens G, Küttner F, Seifert E, Jäckle H (1989) The homeotic gene *fork head* encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. Cell **57:** 645–658

Zeevaart JAD (1976) Physiology of flower formation. Annu Rev Plant Physiol **27:** 321–348

Zheng CC, Bui AQ, O'Neill SD (1993) Abundance of an mRNA encoding a high mobility group DNA-binding protein is regulated by light and an endogenous rhythm. Plant Mol Biol **23:** 813–823