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The Rice Phytochrome Gene: Structure, Autoregulated Expression, and Binding of GT-1 to a Conserved Site in the 5′ Upstream Region

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We have isolated and characterized both cDNA and genomic clones encoding the apoprotein of rice phytochrome. The mRNA produced from this gene is expressed at a low level in etiolated leaves. Following a flash of red light, the steady-state mRNA level decreases within 15 minutes, and is barely detectable after 2 hours. This effect is partially reversed by far red light demonstrating autoregulation of phytochrome mRNA levels. Nuclear run-on experiments show that this effect is exerted on transcription of the phytochrome gene. In etiolated plants, phytochrome mRNA is twofold higher in leaves than in roots, whereas the reverse is true in fully green plants where phytochrome mRNA accumulates despite illumination of the leaves. DNA gel blots and screening of libraries indicate the presence of only a single gene, allowing convenient study of the autoregulatory phenomenon for a specific phytochrome gene. Gel retardation analysis using a fragment from the 5′ upstream region reveals that GT-1 is present in nuclear extracts of etiolated rice leaves and binds to sites conserved between rice and oat phytochrome genes.

INTRODUCTION

Light plays a critical role in the life of plants, providing not only energy for photosynthesis but also stimuli for growth and development. A number of regulatory photoreceptors have evolved to provide information on the quality, quantity, and spatial direction of light (see Kendrick and Kronenberg, 1986). The best characterized of these is phytochrome (Furuya, 1987). In darkness, phytochrome is synthesized as the physiologically inactive red-absorbing form, Pr. Upon illumination with red light, Pr is converted to the active far red absorbing form, Pfr, which can be transformed back to Pr by far red light. These interconvertible forms provide the plant with a cellular switch that can convert information on spectral quantity and quality into a suitable response.

Initially, phytochrome was characterized in terms of its morphological responses, such as flowering and germination (Shropshire and Mohr, 1983). More recently it has become clear that phytochrome causes dramatic changes in molecular events, most notably gene expression (for reviews see Silverthorne and Tobin, 1987; Cuozzo et al., 1988; Nagy et al., 1988a). Initially, phytochrome-induced increases in the steady-state level of mRNAs ("photophilic" expression), such as the genes encoding the chlorophyll *a/b* binding protein (Cab) and small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) have been demonstrated (see Kuhlemeier et al., 1987a). This was followed by the use of run-on transcription assays to reveal that phytochrome acts on these genes at the level of transcription (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984; Mosinger et al., 1985). To define the transcriptional components more accurately, the 5′ regions of these genes have been analyzed by in vitro mutagenesis followed by transfer to, and analysis in, transgenic plants (Cuozzo et al., 1988). This has led to the identification of light-responsive elements (LREs), which are short sequences that mediate transcriptional induction by white light, and in some cases phytochrome. In the case of the oat photoreceptor gene (Quail et al., 1987), phytochrome down-regulates the expression of its own gene ("photophobic" regulation) at the level of transcription. A similar effect has also been observed for pea phytochrome (Otto et al., 1984).

To complement our own studies on photophilic gene expression mediated by phytochrome (Nagy et al., 1988a), we have chosen to investigate the mechanisms underlying photophobic transcription of the rice phytochrome gene. We anticipate that, by comparing the components of the different pathways for up- and down-regulated expression, e.g. *cis*-acting DNA sequences and *trans*-acting protein factors, we will be able to determine the shared elements and at what point the pathways diverge. Ultimately, the task at hand is to deduce the chain of events leading from light perception to altered transcription.

Rice was chosen as a model system for several reasons.

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Rice is one of the most important food crops, serving as the major staple for almost two billion people (Swaminathan, 1984). Previous studies have shown that phytochrome has a dramatic effect on flowering in rice (Ikeda and Ishida, 1980) and the spectroscopic properties of rice phytochrome have been well characterized (Pjon and Furuya, 1968). We demonstrate here that rice also provides an excellent model system for studying phytochrome effects on gene transcription. The rice phytochrome gene is single copy, allowing for the first time the demonstration of autoregulation on a single gene, rather than a gene family. Autoregulation occurs at the level of transcription and is sensitive to very low levels of Pfr. Interestingly, the nuclear factor GT-1, which has been shown previously to bind LREs of photophilic genes (Green et al., 1987, 1988), also binds to the upstream sequence of the rice phytochrome gene. The binding site is highly conserved between rice and oat (Hershey et al., 1987) phytochrome genes, which suggests that this may be a functional interaction. Thus, the possibility exists that a binding protein involved in the transcription of a light-induced gene (Green et al., 1988) may also be important in the photophobic expression of a phytochrome gene.

RESULTS

Isolation of a Rice Phytochrome cDNA Clone

To initiate our studies on down-regulation by phytochrome, we synthesized two 60-mer oligonucleotides that span the chromophore binding region of the oat phytochrome cDNA clone, AP3 (Hershey et al., 1985). A cDNA library was then constructed from polyA$^+$ RNA prepared from etiolated rice leaves and screened in duplicate with each oligomer. Several cDNA clones corresponding to rice phytochrome were isolated and characterized, the longest of which, *cphy5*, is shown in Figure 1. This clone is 3.5 kb long, but is not full-length and terminates at the 5′ end about 250 bp downstream from the ATG initiation codon. Sequence analysis reveals that a PstI site present just 3′ of the TAA termination codon allows the generation of a 250-bp PstI-EcoRI fragment containing only a 3′-untranslated region. This fragment can then be used as a gene-specific probe, given the lower levels of conserved bases normally found in the 3′-untranslated region.

Autoregulation of Phytochrome mRNA Levels

Previous studies on the autoregulation of phytochrome mRNA have shown that the magnitude varies greatly among different plant species. For example, a large effect is seen in oats (Colbert et al., 1985) and pea (Otto et al., 1984), but only a modest decline in phytochrome mRNA is seen in zucchini (Lissemore et al., 1987). Therefore, we wanted to ensure that rice shows a sufficiently dramatic response. Figure 2A shows a slot blot analysis of polyA$^+$ RNA isolated from etiolated rice leaves given different light treatments. The RNA was probed with the 3′ PstI-EcoRI cDNA fragment. Phytochrome mRNA accumulates in etiolated leaves, but after either 4 hr of continuous white light or a 1-min red light pulse followed by 4 hr of darkness, there is a substantial decrease in phytochrome mRNA abundance (see Figure 2A). Initially we had conducted our experiments at 22°C to 24°C and had obtained a very poor response (data not shown). Only at 30°C did we observe such a large effect. This may reflect the normal physiological growth conditions for this Asiatic rice cultivar. To test whether the decline in mRNA following a red light pulse is due to phytochrome, we also illuminated the plants with red light followed by far red or far red alone (see Figure 2A), and returned them to darkness for 4 hr. Far red light can partially abrogate the effect of red light, whereas far red light alone elicits a slight decline. Taken together, these results demonstrate that phytochrome causes a decrease in the abundance of its own mRNA and the sensitivity to the low amounts of Pfr present after far red light treatment indicates this is a very low fluence response. Similar results have been obtained for the etiolated oat phytochrome gene family (Colbert et al., 1985).

To define further the phytochrome-induced decrease in mRNA abundance, we investigated the kinetics of this response by using the highly sensitive technique of RNase protection assays (Melton et al., 1984). In Figure 2B etiolated leaves were given either a 1-min red light pulse and returned to darkness or were exposed to continuous white

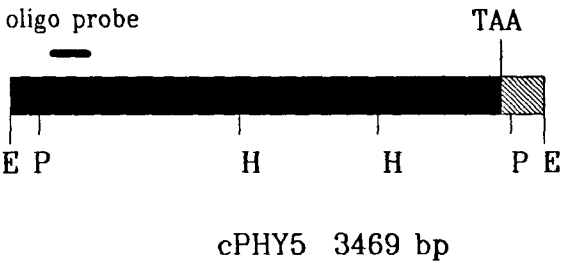

Figure 1. Physical Map of a Rice Phytochrome cDNA Clone, *cphy5*.

The map shows the approximately 3.5-kb EcoRI fragment excised from a λ-gt11 phage. Restriction sites used as entry points to sequence the clone are shown. The black bar represents the protein coding sequence and the hatched bar, the 3′-untranslated region. Translation termination codon position is indicated by TAA. The line labeled "oligo probe" shows the region of hybridization between the clone and the two 60-mer oligonucleotides used to screen the cDNA library. The two EcoRI sites are derived from linkers used in generating the library. E, EcoRI; H, HindIII; P, PstI.

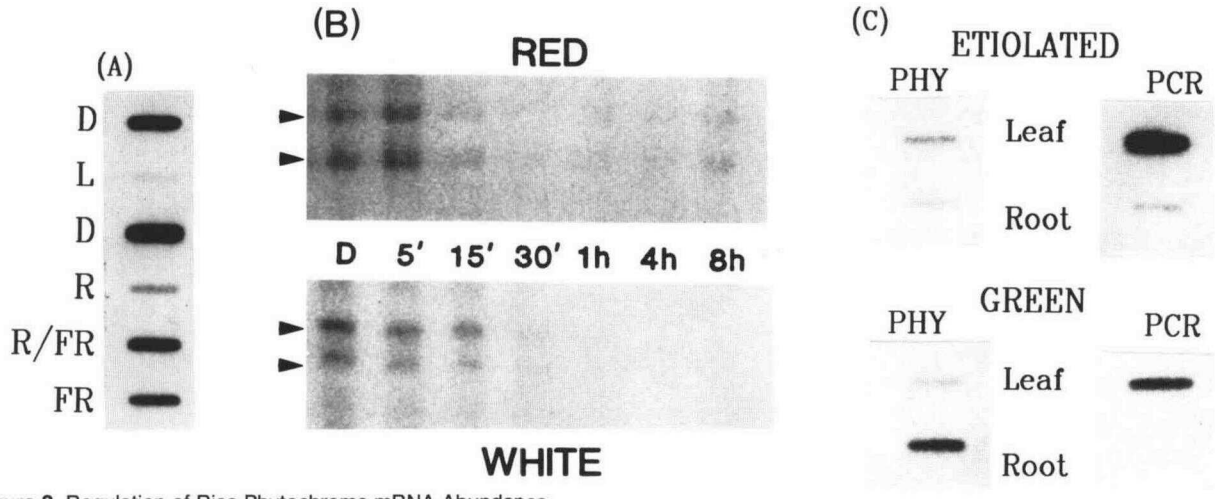

Figure 2. Regulation of Rice Phytochrome mRNA Abundance.

(A) Autoregulation of rice *cphy5* mRNA in young rice seedlings. Hybridization to polyA$^+$ RNA isolated from etiolated leaves (D) that had been treated with continuous white light (L), a red flash (R), red followed by far red (R/FR), or far red alone (FR). The probe used was a PstI-EcoRI DNA fragment encoding 3′-untranslated region. Each slot contained 5 μg of polyA$^+$ RNA.

(B) Kinetics of red and white light induced decrease in *cphy5* mRNA abundance. Total RNA isolated from etiolated leaves that were either red flashed and returned to darkness, or exposed to continuous white light, were analyzed at different times by RNase protection assays. The two bands that are protected are due to clipping at a poly A tract in the probe by endoribonuclease activity. Fifty micrograms of total RNA were used for hybridization in each lane.

(C) Expression of *cphy5* mRNA in leaves and roots of etiolated and greenhouse-grown rice seedlings. Total RNA was analyzed for *cphy5* mRNA by using an antisense RNA probe corresponding to the DNA probe used in **(A)**. PCR refers to hybridization of mRNA encoding protochlorophyllide reductase to a rice cDNA probe. The panel for PHY hybridization from green tissue was exposed for 48 hr, whereas the other panels were exposed for 16 hr. Ten micrograms of RNA were loaded in each slot.

light of the same intensity. Initially, both light treatments elicit a very rapid decline detectable within 15 min following the red or white light and after 30 min the phytochrome mRNA is barely detectable. The red pulse produces a transient decrease in mRNA which begins to reaccumulate after 8 hr. Continuous white light, however, causes a constant decline. This difference is probably due to the continuous conversion of Pr to Pfr under white light, whereas the red pulse produces a defined amount of Pfr, which turns over, allowing derepression of the gene. This is one of the most rapid phytochrome responses observed, and confirms that rice is a useful plant to study this phenomenon.

To date, nothing has been reported on the presence of phytochrome mRNA in different plant organs. Total cell RNA was extracted from leaves and roots of both etiolated and fully green plants, and analyzed in slot blots using a gene-specific RNA probe. The results of such a hybridization experiment are shown in Figure 2C. As a control we also hybridized an identical set of filters with a cDNA encoding protochlorophyllide reductase (PCR), a gene that in rice is expressed highly in the dark and repressed by phytochrome (S. Kay, unpublished results). In etiolated seedlings, phytochrome mRNA is present at a twofold higher level in leaves than in roots, whereas in green tissue the converse is true. The panel for green tissue was exposed three times longer than that for etiolated tissue to demonstrate the very low, but detectable, levels of phytochrome mRNA in green leaves. When the same RNAs are analyzed for PCR mRNA, leaf specificity is observed for both etiolated and green tissue. The observation that phytochrome mRNA is present in roots raises the question of the role of phytochrome in this organ.

Autoregulation of Phytochrome mRNA Occurs at the Transcriptional Level

When a change is observed in the steady-state level of an mRNA in response to a stimulus, two possible mechanisms may be involved: an alteration in transcription rate or a change in mRNA stability (or both). To investigate which of these mechanisms contributes to the decline in phytochrome mRNA, we performed nuclear run-on experiments, the results of which are shown in Figure 3. Nuclei were isolated from leaves of either etiolated plants, plants flashed for 1 min with either red or far red and returned to darkness for 15 min, or fully green leaves. Radiolabeled

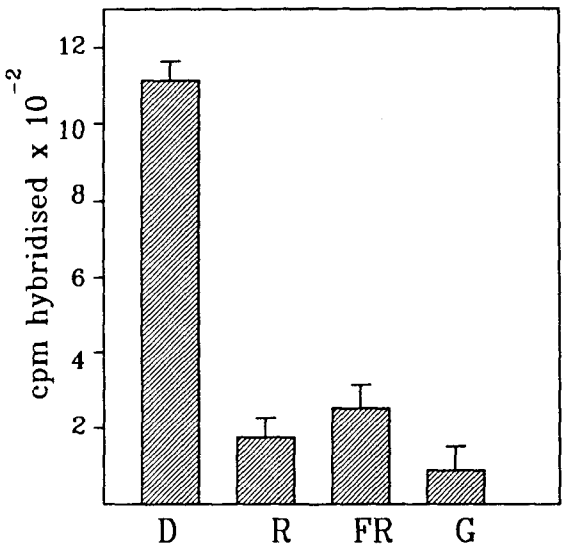

Figure 3. Autoregulation of Rice Phytochrome Gene Transcription.

Nuclear run-off assays were used to analyze the changes in transcription rate of the *cphy5* gene in nuclei isolated from etiolated leaves (D), red flashed (R), far red flashed (FR), or from fully green leaves (G).

transcripts synthesized from the nuclei in vitro were then hybridized to filters bearing an excess of single-stranded DNA containing the antisense strand of the *cphy5* 3′ end. After quantitation and normalization, the number of counts per minute bound to the filter is a reflection of the phytochrome gene transcription rate at the time of isolation. Clearly, 15 min following the red pulse a dramatic decline in transcription is observed, to about 15% of the dark level. A far red pulse elicits a similar response, with only 22% of the dark transcription rate, whereas in green leaves the gene is repressed to about 7% of the dark rate. Although at best this technique can only give an indirect measure of transcription, it is clear that the down-regulation occurs at this level. Despite repression of the gene by its own product, phytochrome transcription is occurring at a low but significant rate in green leaves. Repression of transcription of the oat phytochrome multigene family has also been demonstrated recently (Lissemore and Quail, 1988).

Isolation and Characterization of the Single Copy Genomic Clone Encoding Rice Phytochrome

To extend our studies on the organization and regulation of the rice phytochrome gene, we constructed a rice (cultivar IR36) genomic library that was screened using the entire *cphy5* insert (see Figure 1). Ten independent genomic clones were isolated and shown to be identical and partially overlapping by restriction analysis (data not shown). The physical map of a fragment from one of these clones, *phy18*, is shown in Figure 4A. The region that was sequenced is about 10 kb and includes 1.9 kb of the 5′ upstream sequence and 300 bp of the 3′ downstream sequence. The mRNA is encoded by 6 exons separated by 5 introns, with an intron being present in both the 5′- and 3′-untranslated regions. The intron residing in the 5′ leader is 2.6 kb, which is one of the longest introns characterized to date for a plant gene. The intron/exon boundaries are well preserved between rice and one of the oat genes, *phy3* (Hershey et al., 1987), although the intron sizes vary most notably in the 5′- and 3′-untranslated regions, where they are significantly longer in rice.

Screening of the genomic library (whose complexity is approximately equal to three genomes of rice) at either high or low stringency produced many independent clones of the same gene. To confirm that a single copy of the rice phytochrome gene is present per haploid genome, we performed nuclear DNA gel blots using different probes. Figure 4B shows a typical result when the 3′ probe (see Figure 1) is used, producing the expected single bands for each of the three enzymes used. If the same blot is stripped and reprobed with a 95-mer oligonucleotide that covers a highly conserved region of the chromophore attachment site, the hybridizing bands are consistent with the presence of a single gene (see Figure 4B, right panel). As this result was obtained under conditions of low stringency, and taken together with the library screenings, we can conclude that only a single rice phytochrome gene is detectable by hybridization methods. This is in sharp contrast to oat, where at least four genes are present and expressed in etiolated tissue (Hershey et al., 1985) and immunological data suggest other more divergent forms of phytochrome may be present (Abe et al., 1985; Shimazaki and Pratt, 1985; Tokuhisa et al., 1985).

The entire 9.9-kb EcoRI-XbaI fragment of *phy18*, which also incorporates *cphy5*, was sequenced and part of this sequence from −300 to −1 is shown in Figure 4C. This 5′-flanking sequence contains some interesting motifs. A long TATA box is present between −31 and −20, which consists of three copies of TATT. Another striking AT-rich region is the 42-bp repeat of $(TA)_{21}$ that resides between −290 and −249. Several homologies exist between regions of *phy18* and the core binding site (GGTTAA) of GT-1, a nuclear protein that binds to the LRE of the pea *rbcS-3A* gene (Green et al., 1987, 1988). These are present between −1285/−1266 and −242/−220, the latter being very well conserved between rice and *phy3* of oat. Another evolutionary conserved box is 14 nucleotides of a GC-rich sequence residing between −165/−152 that is perfectly matched between rice and oat (−95/−82, Hershey et al., 1987).

The mapping of the 5′ end of the phytochrome transcript is shown in Figure 4D. Due to the low abundance of phytochrome mRNA, conventional mapping techniques using end-labeled probes or primer extension were unsuc-

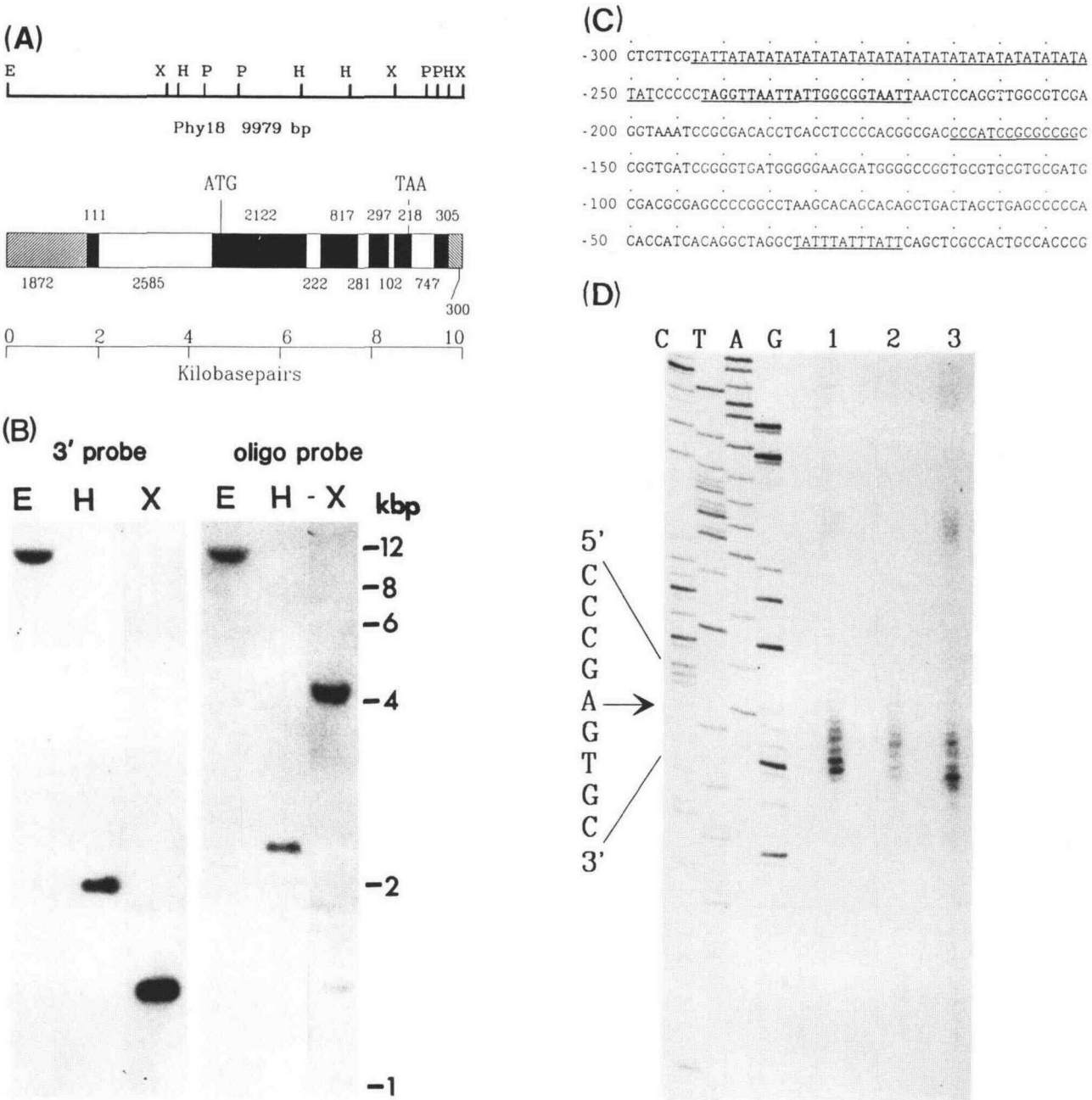

Figure 4. Isolation and Characterization of a Genomic DNA Clone Encoding the Single Copy Rice Phytochrome Gene.

(A) Physical map of the rice phytochrome genomic clone, *phy18*. Restriction sites shown refer to entry points for sequence analysis. Black bars represent exons, white bars represent introns, and hatched bars are 5′ upstream and 3′ downstream sequences. Numbers on top of the map are in base pairs and designate the length of each exon and those underneath for introns and flanking sequences. Enzymes are as in Figure 1 except X, XbaI.

(B) DNA gel blot of rice DNA. The 3′ probe was that used in Figure 2A and the oligo probe was a 95-mer covering the chromophore attachment domain. High stringency was used for the 3′ probe and low stringency for the oligonucleotide probe. The faint 1.5-kb XbaI band in the right oligo panel is due to residual hybridization from the 3′ probe.

(C) Partial sequence of *phy18*. The sequence from −300 to −1 is shown. Underlined sequences are shown in the text. The double GT-1 core motifs from −242 to −220 are shown in bold and underlined.

(D) Mapping of the transcription start site of *phy18*. S1 nuclease-resistant DNA fragments are shown in lanes 1 to 3, and GATC are the respective lanes of a dideoxy sequencing ladder. Ten micrograms of etiolated leaf polyA$^+$ RNA were used in each lane. S1 nuclease concentrations were: lane 1, 500 units/ml.; lane 2, 1000 units/ml.; lane 3, 2000 units/ml.

cessful. Therefore, we used single-stranded, uniformly labeled antisense DNA probes that were hybridized under aqueous conditions for S1 nuclease protection experiments. In this way, probes of very high specific activity can be used, as hybridization times are short (Burke, 1984). We performed an S1 nuclease titration (see Figure 4D, tracks 1 to 3), which demonstrates that the small group of bands present are due to S1 "end-nibbling" (Green and Roeder, 1980) rather than true multiple start sites in vivo. The uppermost band that could be detected corresponds to an A and is designated as the transcriptional start site. The borders of the large 5′ intron were also mapped in this way, as our cDNA clone did not span this region (data not shown).

Binding of GT-1 to the 5′ Upstream Region

To test whether GT-1 is present in rice nuclei and binds to the conserved site at −242/−220, we performed gel retardation experiments. Protein extracts from etiolated rice leaf nuclei are incubated with a radiolabeled probe (−441 to −204) in the presence of different cold competitors and analyzed in agarose gels. Figure 5A, track 1, shows the free probe migrating near the bottom of the gel, whereas addition of nuclear extract causes retarded mobility (Figure 5B, track 2). If 50 ng of cold probe are included in the reaction, the binding is effectively competed, indicating the specificity of this band (track 3). However, if the reaction includes 10 ng of an unlabeled fragment containing 4 copies of the pea *rbcS-3A* box II sequence, a GT-1 binding site (Green et al., 1987), efficient competition is also seen (track 4). Conversely, 20 ng of a mutated box II fragment that does not bind GT-1 (Green et al., 1988), does not compete for binding with the rice phytochrome probe (track 5). These data demonstrate that GT-1 is present in etiolated rice nuclear extracts and binds to the fragment containing the box II core sequences. The homology between this site and the one present in the oat *phy3* gene (Hershey et al., 1987), is shown in Figure 5B. Out of the 23 nucleotides aligned, 20 are homologous and this sequence represents two GT-1 binding cores (GGTTAA and GGTAAT, Green et al., 1988) with a distance of 14 nucleotides from the first critical GG to the next.

DISCUSSION

As a first step toward dissection of the divergent pathways leading to phytochrome-regulated gene expression, we have characterized the down-regulation of rice phytochrome gene transcription. Rice offers several advantages for studying this system—in particular, a single gene that demonstrates a large and rapid phytochrome-mediated decrease in transcription. Also, the comparison of the rice

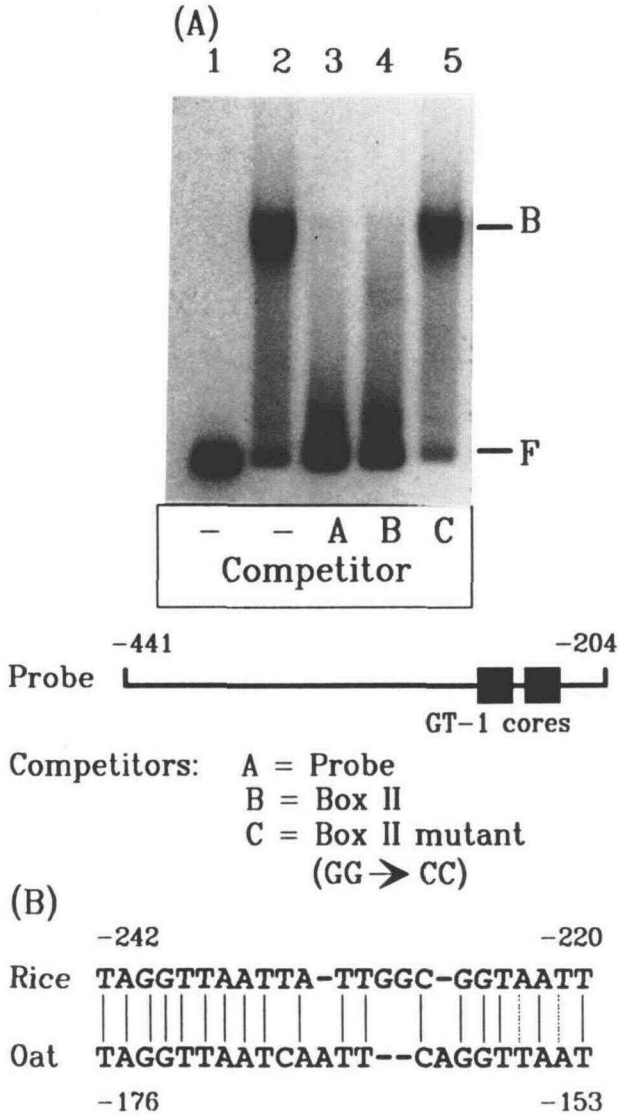

Figure 5. GT-1 Binds to a Site That Is Conserved Between the 5′ Upstream Regions of Rice and Oat Phytochrome Genes.

(A) Gel retardation analysis of rice nuclear extracts and a fragment of the phytochrome 5′ upstream region. The probe was incubated in the presence (lanes 2 to 5) or absence (lane 1) of a rice nuclear extract. Cold competitor DNA was also added as follows: lane 3, 50 ng; lane 4, 10 ng; lane 5, 20 ng. Box II refers to an 84-mer oligonucleotide containing four copies of the pea *rbcS-3A* BoxII sequence (a GT-1 binding site). The mutant is identical except the double Gs in the core binding site have been replaced by double Cs. The two GT-1 core binding site sequences present on the probe fragment are shown.

(B) Sequence alignment of rice and oat phytochrome 5′-flanking sequences reveal strong homology for the two GT-1 core binding sites. The dotted lines between some base pairs are nucleotide substitutions that do not significantly affect GT-1 binding.

phy18 to the oat *phy3* (Hershey et al., 1987) 5′-flanking sequence should facilitate the definition of *cis* and *trans* elements involved in the phytochrome response.

The down-regulation event itself is intriguing when placed in a broader biological context. One might have expected that the regulation of a major higher plant photoreceptor would be well conserved between species. This is evidently not the case, as rice, oat (Colbert et al., 1985), and pea (Otto et al., 1984) all show a large rapid effect, whereas zucchini and maize do not (Lissemore et al., 1987). Therefore, it is difficult to assess the physiological significance of this process. Although species may vary in their response at the mRNA level, both monocots and dicots studied exhibit destruction of Pfr at the protein level (Quail et al., 1973), regardless of changes in mRNA concentration. Perhaps regulation of the mRNA level has evolved to provide additional ways of controlling the Pr/Pfr ratio under different conditions, instead of relying entirely on proteolysis. It is interesting to note that higher plants have chosen to negatively regulate the amounts of two important photoreceptors, phytochrome and PCR (Apel, 1981; Kay and Griffiths, 1983). In the case of phytochrome, one might argue that the etiolated (form I) type phytochrome accumulates in the initial etiolated state of the shoot and triggers photomorphogenesis, after which it is degraded. In addition, evidence has been provided that the dark destruction of Pfr following irradiation may be a mechanism for assessing the length of the dark period (Lissemore and Quail, 1988). In the case of PCR, it has been proposed that negative regulation is best viewed in the context of light/dark cycles, where accumulation of the enzyme during the night may have a role in chloroplast maintenance and turnover of chlorophyll binding proteins (Griffiths et al., 1985; Nagy et al., 1988b).

Another insight into the different mechanisms used for phytochrome up- or down-regulated gene expression may come from studying this process in different organs, and eventually in different cell types. In this context it is interesting that phytochrome is expressed in roots, as little is known about its function there (Pratt, 1986). Some physiological studies have provided evidence that phytochrome may be involved in auxin transport in rice (Furuya et al., 1969) and geotropism (Tepfer and Bonnett, 1972). The expected finding of phytochrome mRNA in the roots of etiolated rice seedlings (see Figure 2C) is consistent with the spectroscopic detection of the pigment in this organ by Pjon and Furuya (1968). However, the presence of phytochrome mRNA in roots of green seedlings has not been observed previously, demonstrating that, although the leaves of the same plant have been illuminated and have greatly reduced amounts of phytochrome mRNA (and transcription), the signal that caused this reduction is not transmitted to roots buried in the soil. Preliminary experiments indicate that illumination of roots from green plants causes a slow decline in mRNA level (S. Kay, unpublished data). It will be interesting to determine whether or not the same factors regulate phytochrome mRNA abundance in roots and leaves.

The detection of a single gene encoding etiolated (form I) phytochrome in rice has allowed us easily to focus our studies on a single gene. However, in etiolated oats (Hershey et al., 1985), at least four genes are expressed, and immunological data suggest the presence of a "green tissue" (form II) type phytochrome (Shimazaki and Pratt, 1985; Tokuhisa et al., 1985). In pea, immunological and peptide mapping data also suggest multiple forms of the protein (Abe et al., 1985; Konomi et al., 1987). In zucchini, only a single copy of the form I gene is expressed (Lissemore et al., 1987) and DNA gel blots indicate two or more genes are present in tomato (Sharrock et al., 1988). However, it is not clear whether these multiple forms are functionally distinct or in some cases whether they are different gene products or result from posttranslational modifications. It is attractive to propose the existence of functionally distinct pools of phytochrome encoded by evolutionary divergent genes. Although our data demonstrate the existence of a single form I gene in rice, a form II gene may be present that is insufficiently homologous to allow detection at the nucleotide level. Experiments will need to be conducted at the protein level to test whether a form II type phytochrome is present in rice.

It is now clear both from this study as well as others (Quail et al., 1987; Lissemore and Quail, 1988) that the photophobic expression of phytochrome mRNA in rice and oats is largely due to a decrease in transcription. The earlier report by Quail et al. (1987) using run-on transcription assays for the oat genes demonstrated only a partial decrease in hybridizing transcripts. This led to a model proposing that differential mRNA stability may play a large role in regulating this decline. However, the more elegant experiments performed later (Lissemore and Quail, 1988) using single-stranded coding sequence probes demonstrated a rapid and large decline in run-on transcription following a pulse of red light. Taken together with our results, it would appear that the major factor influencing phytochrome mRNA abundance is transcription rather than mRNA stability. However, a contribution from mRNA stability cannot be ruled out. Even if there is no difference in the rate of mRNA degradation between light and dark, the rapid decline in mRNA for phytochrome following the transcriptional shutoff implies that it is an exceptionally unstable message. Previously, we have shown that transgenic plants may be used to address the question of mRNA turnover (Nagy et al., 1988b). In that case, when the wheat Cab-1 gene is transferred to tobacco, a transcriptional shutoff occurs around noon and, subsequently, the Cab-1 mRNA declines in the afternoon. When the Cab-1 flanking sequences are fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and transferred to tobacco, the same transcriptional shutoff occurs, but the CAT mRNA

declines more rapidly. This demonstrates that, under these conditions in the transgenic tobacco leaves, CAT mRNA is significantly less stable than Cab-1 mRNA.

The elucidation of the components involved in the transcriptional regulation requires analysis of the *cis*-acting DNA elements and the *trans*-acting protein factors that mediate this response. As we are particularly interested in comparing the mechanisms and pathways for photophilic and photophobic gene expression, it is very interesting to find GT-1 binding to the upstream region of the rice phytochrome gene. Green et al. (1987, 1988) have demonstrated previously that this protein in pea specifically recognizes the light-responsive elements Box II and Box III (and their homologs) of the pea *rbcS-3A* gene. Furthermore, mutation of the two critical Gs in the core binding site (GGTTAA) eliminates both GT-1 binding in vitro and detectable expression in vivo of the *rbcS-3A* gene when only 170 bp of the 5′-flanking sequences are present (Green et al., 1988). Although GT-1 binding is correlated with positive expression of the *rbcS-3A* gene, its detailed role in vivo remains obscure. The binding sites for GT-1 (boxes II and III) overlap with both positive and negative elements (Kuhlemeier et al,. 1987b, 1988; Green et al., 1988) and are further complicated by redundancy. To determine its role in the transcription of the rice phytochrome gene, we will have to define the binding sites further by DNase I footprinting and test activity and mutation of these sites in vivo. The conservation of the double GT-1 cores within the upstream regions of both rice and oat *phy3* genes suggests that binding may be functional in vivo. Note that, although Hershey et al. (1987) indicated the presence of 3 "GT" boxes in the oat *phy3* gene, these are neither GT-1 consensus binding sites nor conserved between rice and oat. Future experiments will determine the role, if any, of common elements shared by both photophobic and photophilic regulatory pathways.

METHODS

Plant Material

Rice *Oryza sativa* IR36 were imbibed and germinated in the dark and grown for 5 to 6 days at 30°C on moist vermiculite. At this stage the major part of the material harvested is primary leaf, containing a small amount of coleoptile. For green tissue, seedlings were grown in a growth chamber at 30°C for 7 days under a 12-hr light (1000 $\mu E/m^2/sec$)/12-hr dark cycle. Leaves were harvested and immediately frozen in liquid nitrogen. Roots from either etiolated or green seedlings were harvested in the dark by washing off the vermiculite in distilled water and then frozen. All dark manipulations were performed under a dim green safe light. For phytochrome experiments, plants were given either red (1 min), far red (5 min), or combinations of both and then returned to darkness for the specified times. For white light experiments, etiolated tissue was placed under fluorescent lamps (100 $\mu E/m^2/sec$) for the specified times. All other details of light sources and filters have been described previously (Nagy et al., 1988c).

RNA Isolation and Analysis

Total cell RNA and polyA$^+$ RNA were isolated as described previously (Nagy et al., 1988c). RNA slot blot analysis was performed on nitrocellulose filters by standard methods (Nagy et al., 1988c). For RNase protection assays, the 3′ PstI-EcoRI fragment of *cphy5* was subcloned into pBluescript SK-(Stratagene) and ^{32}P-RNA was synthesized using T7 polymerase according to the manufacturer's instructions. Hybridization and RNase digestion conditions were as described (Melton et al., 1984). The same RNA probe was used in slot blot analysis. For mapping the 5′ transcription start site of *phy18*, a single-stranded template (in the RNA sense) was annealed with an oligonucleotide (+91 to +110) and a single-stranded probe was synthesized, annealed to polyA$^+$ RNA, and S1 digested as described (Burke, 1984). The protected probe was analyzed on a 6% sequencing gel against a dideoxy sequence ladder.

Isolation of Nuclei and Transcription Assays

Nuclei were isolated from rice leaves as described (Green et al., 1987, 1989). Run-on transcription assays and hybridization to filters bearing single-stranded antisense 3′ PstI-EcoRI DNA were as described (Berry-Lowe and Meagher, 1985). Filters were normalized by subtracting background hybridization to nitrocellulose discs bearing single-stranded vector DNA. Experiments were performed twice in triplicate. Filters were cut and counted by liquid scintillation.

Construction of cDNA and Genomic Libraries

Double-stranded cDNA was synthesized from polyA$^+$ RNA prepared from etiolated rice leaves by the RNase H method (Gubler, 1987). Following methylation, size fractionation, and linkering, the cDNA was cloned into the EcoRI site of λ gt11 (Jendrisak et al., 1987). The library was screened in duplicate using the two 60-mer oligonucleotides described in the text. High molecular weight DNA was prepared from purified nuclei by lysis with sarkosyl/SDS and CsCl gradient centrifugation. A genomic library was prepared from Sau3A partially digested DNA (15 kb to 25 kb; Kaiser and Murray, 1986) cloned into λ DASH (Stratagene) and amplified once on *Escherichia coli* strain LE392. The library was screened using the entire *cphy5* insert.

DNA Sequence Analysis

The entire *cphy5* insert (3.5 kb) as well as the EcoRI-XbaI *phy18* subclone (9.9 kb) were sequenced by overlaps on both strands using the dideoxy method with single-stranded templates (Barnes, 1987). Templates were prepared in bluescript plasmids, and sequencing start points were generated either by specific "primer jumping" (Barnes, 1987) or by exoIII/mung bean nested deletions (Stratagene). Some sequence data was generated by using modified T7 DNA polymerase (Sequenase, US Biochemicals). All entry

and analysis of sequence data was performed with the Hitachi DNASIS software package (Hitachi USA/ LKB).

Genomic DNA Gel Blots

Nuclear DNA (5 μg) was digested with the appropriate enzymes and separated and transferred to nitrocellulose filters by standard procedures. Hybridization to the labeled 3′ probe was performed in 6 X SSC, 50% formamide at 42°C, followed by washing in 0.1 X SSC at 65°C. After exposure, the probe was removed by boiling in water for 10 min. The filter was then rehybridized with a labeled oligonucleotide probe (95-mer, +3676 to +3770) in 6 X SSC, 25% formamide at 37°C, with the most stringent wash at 50°C in 2 X SSC. Autoradiography was for 8 hr in both cases.

Gel Retardation Assays

Nuclear protein extracts were prepared from etiolated rice leaves as described (Green et al., 1989). Gel retardation experiments were performed essentially as described previously (Green et al., 1987, 1989). The Box II and Box II mutant competitors used have been described (Green et al., 1988).

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REFERENCES

Abe, H., Yamamoto, K.T., Nagatani, A., and Furuya, M. (1985). Characterization of green tissue-specific phytochrome isolated immunochemically from pea seedlings. Plant Cell Physiol. **26,** 1387–1399.

Apel, K. (1981). The protochlorophyllide holochrome of barley. Phytochrome induced decrease of translatable mRNA coding for the NADPH:protochlorophyllide oxidoreductase. Eur. J. Biochem. **120,** 89–93.

Barnes, W.M. (1987). Sequencing DNA with dideoxyribonucleotides as chain terminators: Hints and strategies for big projects. In Guide to Molecular Cloning Techniques, S.L. Berger and A.R. Kimmel, eds (San Diego: Academic Press), pp. 538–555.

Berry-Lowe, S.L., and Meagher, R.B. (1985). Transcriptional regulation of a gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean tissue is linked to the phytochrome response. Mol. Cell. Biol. **5,** 1910–1917.

Burke, J. F. (1984). High sensitivity S1 mapping with single stranded [^{32}P]DNA probes synthesized from bacteriophage M13mp templates. Gene (Amst.) **30,** 63–68.

Colbert, J. T., Hershey, H. P., and Quail, P. H. (1985). Phytochrome regulation of phytochrome mRNA abundance. Plant Mol. Biol. **5,** 91–101.

Cuozzo, M., Kay, S. A., and Chua N.-H. (1988). Regulatory circuits of light-responsive genes. In Temporal and Spatial Regulation of Plant Genes, D.P.S. Verma and R.B. Goldberg, eds (Vienna: Springer-Verlag), pp. 131–146.

Furuya, M., ed (1987) Phytochrome and Photoregulation in Plants. Proceedings of the Yamada Conference XVI, (Tokyo: Academic Press).

Furuya, M., Pjon, C.-J., Fujii, T., and Ito, M. (1969). Phytochrome action in *Oryza sativa* L. III. The separation of photoperceptive site and growing zone in coleoptiles, and auxin transport as effector system. Dev. Growth Diff. **11,** 62–68.

Gallagher, T.F., and Ellis, R.J. (1982). Light-stimulated transcription of genes for two chloroplast polypeptides in isolated pea leaf nuclei. EMBO J. **1,** 1493–1498.

Green, M.R., and Roeder, R.G. (1980). Definition of a novel promoter for the major adenovirus-associated virus mRNA. Cell **22,** 231–242.

Green, P.J., Kay S.A., and Chua, N.-H. (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. EMBO J. **6,** 2543–2549.

Green, P.J., Yong, M.-H., Cuozzo, M., Kano-Murakami, Y., Silverstein, P., and Chua, N.-H. (1988). Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation of the *rbcS-3A* gene. EMBO J, **7,** 4035–4044.

Green, P.J., Kay, S.A., Lam, E., and Chua N.-H. (1989). *In vitro* DNA footprinting. In Plant Molecular Biology Manual, Supplement I, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic), in press.

Griffiths, W.T., Kay, S.A., and Oliver, R.P. (1985). The presence and photoregulation of protochlorophyllide reductase *in vitro*. Plant Mol. Biol. **4,** 13–22.

Gubler, U. (1987). Second strand cDNA synthesis: mRNA fragments as primers. In Guide to Molecular Cloning Techniques, S.L. Berger and A.R. Kimmel, eds (San Diego: Academic Press), pp. 330–334.

Hershey, H.P., Barker, R.F., Idler, K.B., Lissemore, J.L., and Quail, P.H. (1985). Analysis of cloned cDNA and genomic sequences for phytochrome: Complete amino acid sequences for two gene products expressed in etiolated *Avena*. Nucleic Acids Res. **13,** 8543–8559.

Hershey, H.P., Barker, R.F., Idler, K.B., Murray, M.G., and Quail, P.H. (1987). Nucleotide sequence and characterization of a gene encoding the phytochrome polypeptide from *Avena*. Gene (Amst.) **61,** 339–348.

Ikeda, K., and Ishida, S. (1980). Floral response of rice cultivars

grown in early seasonal culture practice. Rep. Tokai Br. Crop Sci. Soc. Jpn. **87**, 17–20.

Jendrisak, J., Young, R.A., and Engel, J.D. (1987). Cloning cDNA into lambda gt10 and gt11. In Guide to Molecular Cloning Techniques, S.L. Berger and A.R. Kimmel, eds (San Diego: Academic Press), pp. 359–370.

Kaiser, K., and Murray, N.E. (1986). The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In DNA Cloning Vol. 1, A Practical Approach, D.M. Glover, ed (Oxford: IRL Press), pp. 1–48.

Kay, S.A., and Griffiths, W.T. (1983). Light induced breakdown of NADPH:protochlorophyllide reductase *in vitro*. Plant Physiol. **72**, 229–237.

Kendrick, R.E., and Kronenberg, G.H.M. (1986) eds. Photomorphogenesis in Plants (Dordrecht, The Netherlands: Martinus Nijhoff).

Konomi, K., Abe, H., and Furuya, M. (1987). Changes in the content of phytochrome I and II apoproteins in embryonic axes of pea seeds during imbibition. Plant Cell Physiol **28**, 1443–1451.

Kuhlemeier, C., Green, P.J., and Chua N.-H. (1987a). Regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. **38**, 221–257.

Kuhlemeier, C., Fluhr, R., Green, P.J., and Chua, N.-H. (1987b). Sequences in the pea *rbcS-3A* gene have homology to constitutive mammalian enhancers but function as negative regulatory elements. Genes Dev. **1**, 247–255.

Kuhlemeier, C., Cuozzo, M., Green, P.J., Goyvaerts, E., Ward, K., and Chua, N.-H. (1988). Localization and conditional redundancy of regulatory elements in *rbcS-3A*, a pea gene encoding the small subunit of ribulose-bisphosphate carboxylase. Proc. Natl. Acad. Sci. USA **85**, 4662–4666.

Lissemore, J.L., and Quail, P.H. (1988). Rapid transcriptional regulation by phytochrome of the genes for phytochrome and chlorophyll a/b binding protein in *Avena sativa*. Mol. Cell. Biol. **8**, 4840–4850.

Lissemore, J.L., Colbert, J.T., and Quail, P.H. (1987). Cloning of cDNA for phytochrome from etiolated Cucurbita and coordinate photoregulation of the abundance of two distinct phytochrome transcripts. Plant Mol. Biol. **8**, 485–496.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**, 7035–7056.

Mösinger, E., Batschauer, A., Schafer, E., and Apel, K. (1985). Phytochrome control of *in vitro* transcription of specific genes in isolated nuclei from barley. Eur. J. Biochem. **147**, 137–142.

Nagy, F., Kay, S.A., and Chua, N.-H. (1988a). Gene regulation by phytochrome. Trends Genet. **4**, 37–42.

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

Nagy, F., Kay, S.A., and Chua, N.-H. (1988c). Analysis of gene expression in transgenic plants. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic), pp. B4, 1–29.

Otto, V., Schafer, E., Nagatani, A., Yamamoto, K.T., and Furuya, M. (1984). Phytochrome control of its own synthesis in *Pisum sativum*. Plant Cell Physiol. **25**, 1579–1584.

Pjon, C.-J., and Furuya, M. (1968). Phytochrome action in *Oryza sativa* L. II. The spectrophotometric versus the physiological status of phytochrome in coleoptiles. Planta (Berl.) **81**, 303–313.

Pratt, L.H. (1986). Localization within the plant. In Photomorphogenesis in Plants, R.E. Kendrick and G.H.M. Kronenberg, eds (Dordrecht, The Netherlands: Martinus Nijhoff), pp. 61–82.

Quail, P.H., Schaefer, E., and Marme, D. (1973). Turnover of phytochrome in pumpkin cotyledons. Plant Physiol. **52**, 124–127.

Quail, P.H., Gatz, C., Hershey, H., Jones, A., Lissemore, J.L., Parks, B.M., Sharrock, R.A., Barker, R.F., Idler, K., Murray, M.G., Koorneef, M., and Kendrick, R.E. (1987). Molecular biology of phytochrome. In Phytochrome and Photoregulation in Plants, M. Furuya, ed (Tokyo: Academic Press), pp. 23–37.

Sharrock, R.A., Parks, B.M., Koorneef, M., and Quail, P.H. (1988). Molecular analysis of the phytochrome deficiency in an *aurea* mutant of tomato. Mol. Gen. Genet. **213**, 9–14.

Shimazaki, Y., and Pratt, L.H. (1985). Immunochemical detection with rabbit polyclonal and mouse monoclonal antibodies of different pools of phytochrome from etiolated and green *Avena* shoots. Planta (Berl.) **164**, 333–344.

Shropshire W., Jr., and Mohr, H., eds (1983) Photomorphogenesis. Encyclopedia of Plant Physiology, New Series, 16A and 16B, (Berlin: Springer Verlag).

Silverthorne J., and Tobin, E.M. (1984). Demonstration of transcriptional regulation of specific genes by phytochrome action. Proc. Natl. Acad. Sci. U.S.A. **81**, 1112–1116.

Silverthorne, J., and Tobin, E.M. (1987). Phytochrome regulation of nuclear gene expression. BioEssays **7**, 18–23.

Swaminathan, M.S. (1984). Rice. Sci. Am. January, 81–93.

Tepfer, D.A., and Bonnett, H.T. (1972). The role of phytochrome in the geotropic behaviour of roots of *Convolvulus arvensis*. Planta (Berl.) **106**, 311–324.

Tokuhisa, J.G., Daniels, S.M., and Quail, P.H. (1985). Phytochrome in green tissue: Spectral and immunochemical evidence for distinct molecular species of phytochrome in light grown *Avena sativa* L. Planta (Berl.) **164**, 321–332.

NOTE ADDED IN PROOF

The entire sequence of *phy18* will appear in the EMBL, Genbank, and DDJB Nucleotide Sequence Databases under the accession number X14172.