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cis-Acting Elements Involved in Photoregulation of an Oat Phytochrome Promoter in Rice

Wesley B. Bruce[1] and Peter H. Quail[2]

University of California, Berkeley/United States Department of Agriculture, Plant Gene Expression Center, 800 Buchanan Street, Albany, California 94710

Phytochrome negatively regulates the transcription of its own *phyA* genes. High levels of Pfr, the active, far-red-light absorbing form of phytochrome, repress *phyA* transcription; low Pfr levels result in derepression. We have utilized microprojectile-mediated gene transfer to identify regions of an oat *phyA3* gene involved in this autoregulation. Chimeric constructs containing various deletion and sequence substitution mutants of the oat *phyA3* gene fused to a chloramphenicol acetyltransferase reporter (*phyA3*/CAT) have been introduced into etiolated rice seedlings by particle bombardment. Low Pfr concentrations induce high *phyA3*/CAT expression, whereas high Pfr represses activity to near basal levels. Removal of *phyA3* sequences 3′ to the transcription start site reduces expression about fivefold, suggesting that intron 1 of the *phyA3* gene may be required for high activity. The degree of high-Pfr-imposed repression is unaffected by any of a series of deletions or sequence substitutions in the *phyA3* promoter, thus providing no evidence of any Pfr-activated negative elements. In contrast, 5′ and internal deletions identify a minimum of three major positive promoter elements, designated PE1 [−381 base pairs (bp) to −348 bp], PE2 (−635 bp to −489 bp), and PE3 (−110 bp to −76 bp) that are necessary for high-level expression in low-Pfr cells. The data indicate that PE1 and PE2 are functionally redundant, but that PE3 is required in conjunction with either PE1 or PE2 for activity. PE3 contains a sequence element that is highly conserved between monocot *phyA* promoters, indicative of a critical role in *phyA* expression.

INTRODUCTION

Light is a critical environmental signal for the normal growth and development of plants, and a set of regulatory photoreceptors has evolved to monitor this signal (Kendrick and Kronenberg, 1986; Furuya, 1987). The best characterized of these photoreceptors, phytochrome, is responsible for mediating a large number of the light-induced morphogenic changes that occur in plants. Such responses are inducible by red (R) light, which converts the phytochrome molecule to its active, far-red-light-absorbing form, Pfr, and, with certain exceptions (Kendrick and Kronenberg, 1986), are reversible by far-red (F) light, which converts the photoreceptor back to its inactive, red-light-absorbing form, Pr. Clear evidence has accumulated in recent years that in performing its regulatory function, phytochrome modulates the expression of a number of nuclear genes, either in a positive or negative manner (Tobin and Silverthorne, 1985; Kuhlemeier et al., 1987; Lissemore and Quail, 1988; Nagy et al., 1988; Benfey and Chua, 1989). However, the molecular mechanism by which this regulation occurs is unknown.

A number of laboratories are approaching this problem using gene-transfer and in vitro DNA-binding techniques to define *cis*-acting elements and *trans*-acting factors responsible for light-regulated gene expression (Green et al., 1987, 1988; Kuhlemeier et al., 1987, 1988; Castresana et al., 1988; Giuliano et al., 1988; Nagy et al., 1988; Benfey and Chua, 1989; Datta and Cashmore, 1989; Dean et al., 1989). Evidence has been obtained for the involvement of an array of sequence motifs and DNA binding activities in nuclear extracts, primarily for the positively regulated chlorophyll *a*/*b*-binding protein (*cab*) and ribulose bisphosphate carboxylase small subunit (*rbcS*) genes (Green et al., 1987, 1988; Kuhlemeier et al., 1987, 1988; Castresana et al., 1988; Giuliano et al., 1988; Datta and Cashmore, 1989). The most in-depth study thus far has identified a conserved GT-containing element in *rbcS* genes as being involved in phytochrome-regulated expression (Kuhlemeier et al., 1988; Benfey and Chua, 1989), and has detected in nuclear extracts a factor, GT-1, that interacts with this element in a sequence-specific manner (Green et al., 1987, 1988). However, no light-induced changes in the binding of any nuclear factors to promoter elements have been documented. Moreover, a recent report from Lam et al. (1989) that phytochrome activation of *cab* and *rbcS* genes re-

[1] Current address: Plant Molecular Biology Center, Montgomery Hall 325, Northern Illinois University, DeKalb, IL 60115-2861.
[2] To whom correspondence should be addressed.

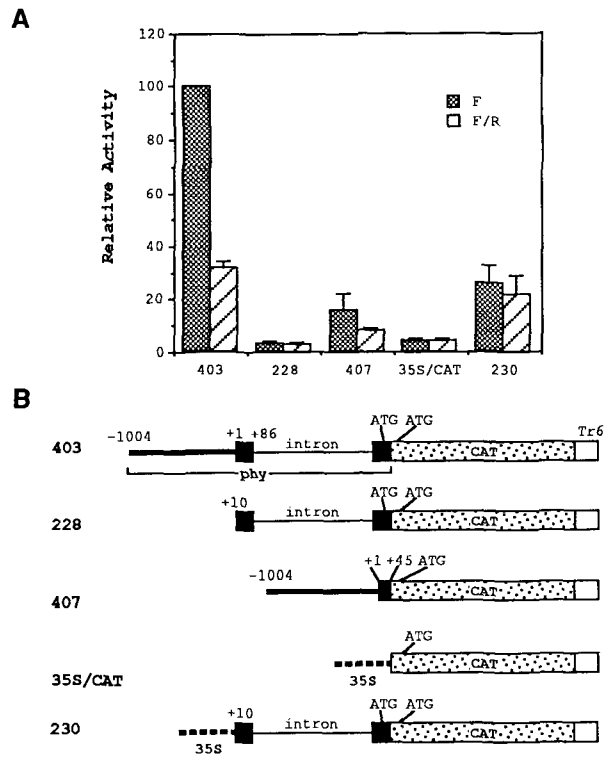

Figure 1. Sequences 3′ to the Transcription Start Site of the Oat *phyA3* Gene Enhance Expression in Chimeric Constructs.

(A) Relative CAT activities of designated chimeric constructs introduced into 2-day-old etiolated rice seedlings by particle bombardment. After bombardment, seedlings were irradiated with F light (low Pfr) or F followed by R light (high Pfr) and returned to the dark for 24 hr before extraction. All values have been corrected for a promoterless CAT construct, pUC19:CAT (Bruce et al., 1989), and are the result of at least four independently grown samples. Error bars are SEM.

(B) Schematic of the various chimeric CAT constructs used in **(A)** (see Methods). *phy* refers to oat *phyA3* sequences, Tr6 refers to the polyA addition site from gene 6a of *Agrobacterium* T-DNA (An, 1987).

quires protein synthesis raises the possibility that these genes respond only indirectly to the primary phytochrome transduction signal by way of regulated expression of an intermediary gene, such as one encoding a *trans*-acting factor (see also Lissemore and Quail, 1988).

We have chosen to utilize the autoregulatory control that phytochrome exerts over the expression of its own *phyA* genes in monocots, (Colbert et al., 1985; Colbert, 1988; Lissemore and Quail, 1988; Christensen and Quail, 1989; Kay et al., 1989a) as a model system for exploring this photoreceptor's mechanism of action. The phytochrome polypeptide is encoded by a minimum of three distinct genes designated *phyA*, *phyB*, and *phyC* in *Arabidopsis* (Sharrock and Quail, 1989) and probably all angiosperms (K. Dehesh, J. Tepperman, and P. Quail, unpublished data). *phyA* transcription in oats is high in dark-grown tissue where only Pr is present but is repressed in less than 5 min from Pfr formation in the presence or absence of protein synthesis (Lissemore and Quail, 1988). These data indicate that all essential transduction pathway components preexist in the cell before phytochrome photoconversion and that *phyA* genes, therefore, are regulated directly by the primary phytochrome signal. The duration of transcriptional repression in the dark after a pulse of light is proportional to the initial Pfr pool size established by the pulse: R light establishes a large pool (86% Pfr) that maintains repression for >12 hr, whereas F light, given either alone or after R light, establishes a small pool (about 1% Pfr) that results in derepression in <3 hr (Lissemore and Quail, 1988). Derepression occurs when the Pfr concentration drops below some saturation level as a result of the rapid turnover rate of this form in the cell.

Functional analysis of the promoter elements involved in this autoregulated expression of *phyA* genes became possible for the first time recently (Bruce et al., 1989) through the development of the high-velocity microprojectile-mediated gene transfer procedure (Klein et al., 1987, 1988, 1989). We have used this procedure in a transient expression configuration to demonstrate that a 1-kb *phyA3* promoter from oat is regulated in parallel with endogenous *phyA* genes after particle-mediated introduction of chimeric oat *phyA3*/chloramphenicol acetyltransferase (CAT) constructs into etiolated rice seedlings (Bruce et al., 1989). This protocol exploits the derepression that occurs during 24 hr of darkness after pulse irradiations to demonstrate phytochrome-regulated expression of the introduced construct. Rapid derepression (high CAT activity) after a terminal F light pulse (low-Pfr tissue) and slow derepression (low CAT activity) after a terminal R light pulse (high-Pfr tissue) is indicative of phytochrome control (Bruce et al., 1989). We report here the effects of various 5′ deletions, internal deletions, and sequence substitutions on the phytochrome-regulated expression of the oat *phyA3*/CAT gene in rice cells.

RESULTS

Downstream *phyA3* Sequences Contribute to *phyA3*/CAT Expression

Because of reports that introns can substantially modulate the expression of genes in which they reside (Callis et al., 1987; Lee et al., 1989; Oard et al., 1989), we included sequences 3′ to the transcription start site in the initial chimeric constructs used in testing oat *phyA3* promoter activity (Bruce et al., 1989). These downstream sequences

encompass the first exon (85 bp of untranslated leader), the first intron (1.2 kb), and 75 bp of the second exon (the remaining 60 bp of the untranslated leader +15 bp of coding sequence) fused in frame to the CAT reporter gene (Hershey et al., 1987), as shown in Figure 1, clone 403. Although the promoterless construct (clone 228) shows that these sequences are alone incapable of supporting significant levels of expression (Bruce et al., 1989) (Figure 1), we wished to examine their influence on promoter-driven expression. Figure 1A shows that the activity of clone 407, which contains the 1-kb *phyA3* promoter but lacks the region from +45 bp to +1377 bp, is reduced fourfold to fivefold relative to the wild-type clone 403, with retention of photoresponsiveness. Conversely, comparison of the activity of clone 35S/CAT (cauliflower mosaic virus 35S transcript promoter-CAT fusion) with that of clone 230 indicates that the oat *phyA3* sequence from +10 bp to +1377 bp enhances the expression driven by the heterologous 35S promoter by fivefold with no evidence of photoregulation (Figure 1). Statistical analysis of the data in this and subsequent figures using the Student-Newman-Keuls Means Separation Test and/or the Wilcoxon Rank Sum Test shows that all values that have nonoverlapping error bars are significantly different at the 95% confidence level. Taken together, the data in Figure 1 indicate that *phyA3* sequences 3′ to the transcription start site (+10 bp to +1377 bp), although apparently having no independent capacity to support transcription nor to confer photoresponsiveness, are able to significantly enhance expression driven by either homologous or heterologous promoters. Because of this general enhancement effect, all subsequent experiments reported here utilize constructs that include the 3′ downstream sequences present in clone 403 (Figure 1).

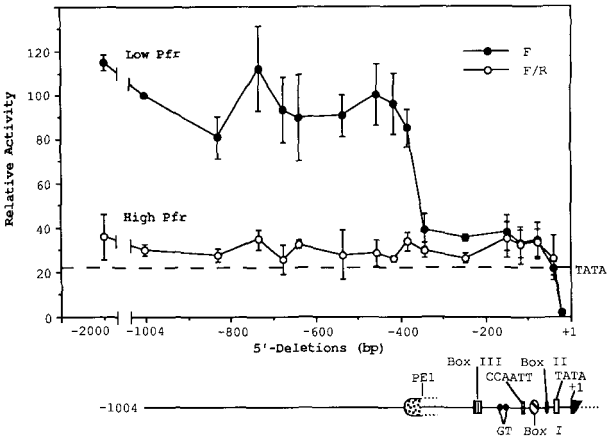

Figure 2. 5′ Deletion Analysis of the Oat *phyA3* Promoter.

Various 5′-terminal deletions of the oat *phyA3*/CAT gene were introduced into 2-day-old etiolated rice seedlings by particle bombardment. Seedlings were then irradiated with a pulse of either F light (low Pfr) or F followed by R light (high Pfr) and returned to the dark for 24 hr before extraction and measurement of relative CAT activity. Endpoints for the 5′ deletions are numbered from the start of transcription. The dashed line (– – –) designated TATA refers to the basal activity driven by the TATA box alone (see text). All values are corrected for the promoterless clone 228 (see Figure 1) and made relative to the clone 403 (1004 bp) set at 100. These values are the result of five independently grown samples. Error bars are the SEM. A schematic of the 5′-flanking region of the oat *phyA3* gene is shown below the graph. Box I, Box II, Box III, and GT refer to conserved sequences that have been identified by sequence comparison in the 5′-flanking regions of oat, rice, and maize *phyA* genes (Christensen and Quail, 1989; Kay et al., 1989a, 1989b). TATA and CCAATT are canonical sequences present in most eukaryotic promoters (Breatnach and Chambon, 1981; Ephrussi et al., 1985). PE1 is a positive promoter element identified by the results of the 5′ deletion series.

5′ Deletion Analysis Identifies a Positive Element (PE1) in the *phyA3* Promoter

The schematic in Figure 2 indicates the locations of various motifs within the first 250 bp upstream of the transcription start site of the oat *phyA3* gene that have been identified by sequence comparison as being conserved in monocot *phyA* promoters (Hershey et al., 1987; Christensen and Quail, 1989; Kay et al., 1989a, 1989b). To begin to approach the question of the functional significance of these motifs, and other regions of the 5′-flanking DNA, to the regulation of *phyA3* expression, we have examined the effects of progressive 5′-terminal deletions on *phyA3* promoter activity in low-Pfr (F) and high-Pfr (F/R) tissue (Figure 2). The data show that the activity and photoresponsiveness of the "standard" 1-kb promoter (Bruce et al., 1989; and clone 403, Figure 1) are not altered by additional sequences up to 2 kb upstream, nor by deletion of sequences down to −415 bp. It is concluded, therefore, that all elements necessary for autoregulated expression of the oat *phyA3* gene are located within the first 415 bp upstream of the transcription start site.

Deletion of all 5′-flanking DNA to −41 bp, immediately upstream of the TATA box, resulted in a level of residual activity that was about 20% of the maximum expression of the 1-kb promoter exhibited in low-Pfr tissue (Figure 2). Because the removal of the TATA motif, either by further deletion to −22 bp (Figure 2) or by sequence substitution in the context of the full-length 1-kb promoter (see Figure 3), reduced activity to background levels, it is clear that the TATA box alone is responsible for 20% of the activity observed in this transient expression system (dotted line in Figure 2 and all subsequent figures).

Conversely, the data show that positive sequence elements that in low-Pfr tissue enhance the level of activity above the basal TATA level lie upstream of this motif. The deletion profile shows that at least the 5′ boundary of one

such positive element, designated PE1, is located between −381 bp and −348 bp from the transcription start site (Figure 2). Deletion of this region reduced activity from maximum expression to the basal TATA level in a single step, indicating that PE1 is critical to full activity, at least in the context of the −381 deletion.

High Pfr levels repressed the activity of all 5′ deletions almost to the basal TATA level (Figure 2). The extent of down-regulation was 20-fold to 50-fold relative to the maximum activity in low-Pfr tissue when the basal TATA level was subtracted. These data indicate that high Pfr levels strongly repress the activity of the upstream positive elements in the *phyA3* promoter. The results also indicate that the repressive action of high Pfr levels is not mediated exclusively by negative sequence elements upstream of deletion −381 because any deletion removing such elements would be expected to restore activity to maximal levels in the presence of high Pfr, and this did not occur (Figure 2). The data do not, however, preclude the possibility of redundant negative elements both upstream and downstream of −381.

Sequence Substitutions Provide No Evidence of a Major Role for a GT Element in the *phyA3* Promoter

The presence of a number of conserved sequence motifs in the 5′-flanking DNA of oat, maize, and rice *phyA* genes (Hershey et al., 1987; Christensen and Quail, 1989; Kay et al., 1989a, 1989b) suggests the possibility of functional significance. We have targeted the role of two of these motifs here, using sequence-substitution mutations in the context of the 1-kb promoter. Figure 3 shows that alterations in the Box III sequence (Christensen and Quail, 1989) were without detectable effect on the 1-kb promoter. Alteration of the 3′ unit of a pair of tandem GT motifs, on the other hand, appears to have caused a minor reduction in low-Pfr-tissue expression without affecting high-Pfr repression (Figure 3).

Internal Deletions Identify Two Additional Positive Elements (PE2 and PE3) in the *phyA3* Promoter

Figure 4 shows that an extensive series of internal deletions within the 1-kb promoter had little, if any, significant effect on the extent of repression by high Pfr levels. Thus, these data fail to identify any negative elements whose deletion would release the promoter from Pfr-imposed repression.

In contrast, two more positive elements, designated PE2 and PE3, become apparent. Clones 422 and 423 (Figure 4A) confirmed the activity of PE1 (−381 to −348) and demonstrated that sequences between −1004 and −635 are unable to compensate for PE1 deletion. The restoration of full activity in low-Pfr tissue by clones 427 and 428,

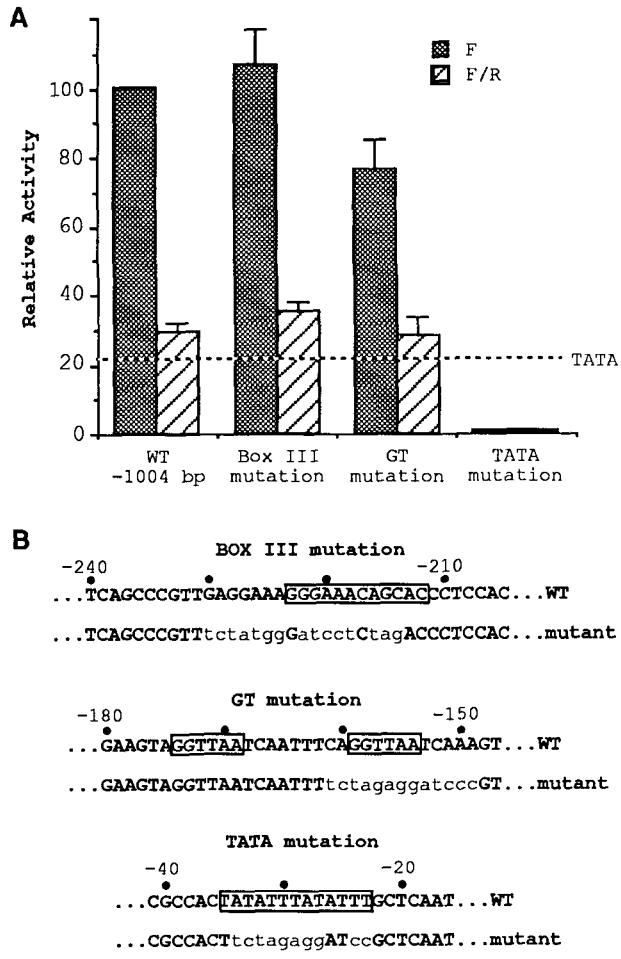

Figure 3. Effects of Sequence-Substitution Mutations in the TATA, GT, and Box III Motifs on Expression of the Oat *phyA3* Promoter.

(A) Relative activities of various chimeric oat *phyA3*/CAT constructs introduced into 2-day-old etiolated rice seedlings by particle bombardment. After bombardment, seedlings were irradiated with a pulse of either F light (low Pfr) or F followed by R light (high Pfr) and returned to the dark for 24 hr before extraction and measurement of the relative CAT activity. Dotted line (- - -) indicates the basal TATA-driven activity. All values are the result of at least four independently grown samples. Error bars are SEM.
(B) Schematics of constructs analyzed in **(A)**. WT refers to the standard 1-kb promoter construct (clone 403, Figure 1). The sequences of clone 403 that were mutated in each motif are shown in lower-case letters.

however, indicates the presence of another positive element, PE2, between −635 and −489 that can substitute fully for the deleted PE1 (Figure 4A). Conversely, clones 420 and 424, which lack PE2, confirmed the 5′ deletion results (Figure 2), indicating that PE1 is fully active in the

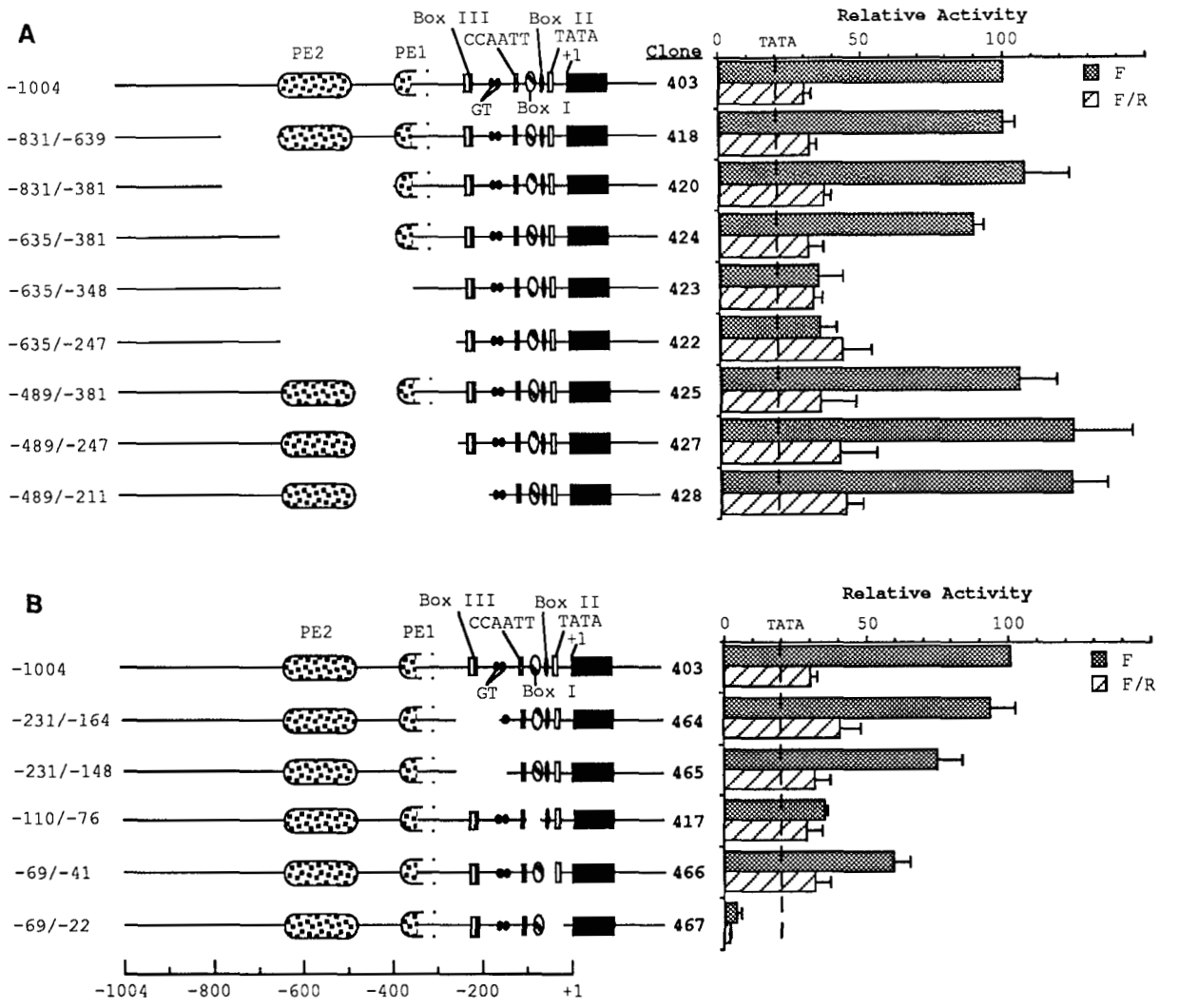

Figure 4. Effects of Internal Deletions in the Oat *phyA3* Promoter on Expression.

Various internal deletion mutations of the promoter region of the oat *phyA3*/CAT gene were introduced into etiolated rice seedlings by particle bombardment. Seedlings were then irradiated with a pulse of either F light (low Pfr) or F followed by R light (high Pfr) and returned to the dark for 24 hr before extraction and measurement of relative CAT activity. The vertical dashed line (– – –) indicates the basal activity driven by the TATA box alone. All values are corrected for the promoterless clone 228 (see Figure 1) and made relative to the wild-type clone 403 set at 100. These values are the result of at least four independently grown samples. Error bars are SEM. Schematic representations of the wild-type (clone 403) and various mutant *phyA3* promoter constructs are shown with deletion endpoints in base pairs to the left. Box I, Box II, Box III, and GT refer to conserved sequence motifs present in the 5′-flanking regions of oat, maize, and rice *phyA* genes (Christensen and Quail, 1989; Kay et al., 1989a, 1989b). CCAATT and TATA are canonical eukaryotic promoter motifs. PE1 and PE2 are positive promoter elements identified here.

(A) Internal deletion constructs affecting the TATA distal region of −831 bp to −211 bp.

(B) Internal deletion constructs affecting the TATA-proximal region of −231 bp to −22 bp.

absence of PE2. Thus, PE1 and PE2 are functionally redundant but not additive in their activities.

Deletion of sequences that include Box III and the 5′ member of the tandem GT motifs (clone 464) had no significant impact on expression (Figure 4B). Additional removal of the 3′ GT motif (clone 465) caused partial reduction in activity (Figure 4B), consistent with the results of sequence substitution (Figure 3). Deletion of sequences

that include the conserved Box I (Christensen and Quail, 1989; Kay et al., 1989a, 1989b) revealed the presence of a third major positive element, designated PE3, in this region (clone 417, Figure 4B). In addition, the removal of Box II (Christensen and Quail, 1989) and associated sequences (clone 466) caused partial reduction in activity (Figure 4), possibly indicative of another minor positive element. However, it should be noted that, in addition to removing sequences, internal deletions alter spacing and that this potentially becomes an increasingly important factor the closer deletions are made to the TATA box. Clone 467 confirmed that removal of the TATA element reduces activity to background levels (Figure 4B).

DISCUSSION

Three major regions of the oat *phyA3* gene examined here affect its expression: (1) sequences 3′ to the transcription start site, (2) the TATA element, and (3) 5′-flanking sequences upstream of the TATA box.

The sequences 3′ to the transcription start site that were included in previously tested *phyA3*/CAT chimeric constructs (Bruce et al., 1989) are shown here to enhance CAT expression driven by either homologous *phyA3* or heterologous 35S promoters by about fivefold. The observation that this enhancement occurred without conferral of photoresponsiveness on the 35S promoter indicates that these sequences do not contain elements that respond to Pfr regulation. Likewise, a promoterless construct (clone 228) showed that these 3′ sequences have no independent capacity to support significant levels of expression. Precedence in other monocot gene systems (Callis et al., 1987; Klein et al., 1988; Lee et al., 1989; Oard et al., 1989; Vasil et al., 1989) suggests that the intron present in the downstream *phyA3* sequences may be primarily responsible for this effect. These previous studies with introns from maize *adh*, bronze, and sucrose synthetase genes indicate that splicing may in some way enhance the steady-state levels of mature transcripts produced, independent of the promoter used. However, because the region of the *phyA3* gene tested here also included the *phyA3* translation initiation site, it remains to be determined whether this might also be involved.

The observation that a sequence substitution mutation of the TATA motif completely eliminated expression supported by the 1-kb promoter establishes that this motif is absolutely required for significant transcriptional activity of the *phyA3* gene. Although the necessity of the TATA box for plant gene expression is generally inferred from experience with other eukaryotic systems, this point has apparently only rarely been directly established using sequence-substitution mutations (Bandyopadhyay et al., 1989; Czarnecka et al., 1989). The reason for the observation that the TATA motif alone supports about 20% of the maximum activity exhibited by the 1-kb *phyA3* promoter is unknown, but one possibility is that the potentially high number of construct copies delivered per cell by the microprojectile procedure might greatly elevate what would otherwise be a very low basal level of expression. It seems unlikely that the TATA box alone normally supports 20% of the maximum expression of endogenous *phyA* genes in the cell because run-on transcription experiments have shown that the level of expression in the fully repressed state in high-Pfr tissue is only a few percent of maximum (Lissemore and Quail, 1988). The fact that in this study high-Pfr levels repressed the activity of the 1-kb *phyA3*/CAT promoter to levels approaching the basal TATA activity, and that the basal TATA-driven activity was unaffected by Pfr levels (−41-bp 5′ deletion; Figure 2), is consistent with this supposition. It seems reasonable to conclude, therefore, that the extent of differential expression over and above the TATA-driven level reflects the true extent of phytochrome regulation. Indeed, when basal TATA-driven activity is subtracted, the extent of transcriptional repression in the 1-kb *phyA3* promoter by high Pfr levels is 20-fold to 50-fold, consistent with the extent of autoregulation of endogenous *phyA* transcription (Lissemore and Quail, 1988).

As implied in the above discussion, the data presented here establish that sequences upstream of the TATA box in the *phyA3* promoter contain the elements responsible both for enhanced expression above the TATA-supported level and for Pfr-imposed repression of this enhanced expression. Three major positive elements (PE) active in low-Pfr tissue have been identified. PE1 (−381 to −348) and PE2 (−635 to −489) are functionally redundant and nonadditive in their activities because deletion of either alone had no effect on expression or photoresponsiveness. In contrast, PE3 (−110 to −76) is required in conjunction with PE1 or PE2 for maximum expression. These data indicate that a two-component positive element system is responsible for transcriptional activation of the *phyA3* promoter in the derepressed state, and that the upstream elements PE1 and PE2 are functionally interchangeable in this process. Inspection of the sequences within the regions containing the three elements reveals no obvious homologies between them. There is no evidence, therefore, that the same *trans*-acting factors bind to any of these elements. Likewise, there is no evidence of sequence homologies between PE1 and PE2 and other *phyA* genes (Hershey et al., 1987; Sato, 1988; Christensen and Quail, 1989; Kay et al., 1989a, 1989b), nor previously identified regulatory elements in other genes. The PE3 region, in contrast, contains a sequence, designated Box I (Christensen and Quail, 1989), that is highly conserved in the *phyA* genes of maize (19/22) and rice (14/22). Indeed, the first 14 bp of this motif are identical in the rice and oat genes. Because the probability of this 14-bp sequence occurring randomly is only once in a haploid rice genome, it is highly likely that this motif is an integral component of

the PE3 element. Based on these combined observations, it is tempting to speculate that *phyA* promoters may be modular with an array of different upstream *cis*-acting sequences having been recruited in different plants and in different locations within the promoters to serve the same transcriptional activation function, but with the downstream element being strictly conserved and possibly exclusive to *phyA* promoters.

In addition to PE1, PE2, and PE3, there is evidence for possible minor positive roles for two other sequences with homologies to previously described motifs. Deletion and sequence-substitution mutations of the tandem GT elements (−174 to −153), which have core sequences identical to *rbcS* Box II motifs (Green et al., 1987, 1988; Kuhlemeier et al., 1988; Nagy et al., 1988; Benfey and Chua, 1989), caused a partial decrease in activity in low-Pfr tissue (Figures 3 and 4B). Similarly, deletion of a *phyA3* Box II motif (−63 to −52) (unrelated to *rbcS* Box II) that is conserved between oat, maize, and rice *phyA* promoters (Christensen and Quail, 1989) also resulted in a partial reduction in activity (Figure 4B). A third motif, Box III (−223 to −212) (Christensen and Quail, 1989), could be removed or substituted without effect (Figures 3 and 4A). However, because all these mutations were generated in the 1-kb promoter, it remains possible that functionally redundant elements elsewhere in the flanking DNA might mask more dramatic effects of removing or altering these motifs. Conversely, because internal deletions inevitably alter spacing, it is possible that detrimental effects unrelated to the removed sequence may occur, especially where the deletion is in the TATA proximal region (Ptashne, 1986; Maniatis et al., 1987), as is the case for the Box II region (−69 to −41).

The identity of the element(s) responsible for the strong repression of *phyA3* expression by Pfr remains unknown. The present data cannot distinguish between the two formal possibilities: (1) that Pfr alters the properties of a positive-element-binding factor(s), thereby negating its/their capacity to bind or activate transcription; and (2) that Pfr alters the properties of a negative-element-binding factor(s), thereby activating its/their capacity to repress transcriptional activation by the positive factors/elements [see Levine and Manley (1989) for review]. If the second of these alternative mechanisms is operative, at least one such negative *cis*-acting element must be located downstream of −381 bp because deletion of sequences upstream of this point does not lead to derepression in the presence of high Pfr as would otherwise be expected (Figure 2).

METHODS

Plasmid Constructions

All enzymes used in plasmid constructions were from New England Biolabs, Inc., unless otherwise stated, and used according to the manufacturer's specifications. Construction of clones 403, 228, p35SCAT, and pUC19:CAT have been previously described (Bruce et al., 1989). Construction of clone 407 involved a Bal31 (Bethesda Research Laboratories)-generated 3′ deletion from the SpeI site (+470 bp; Hershey et al., 1987) of clone 403 to +45 bp (within the first exon) followed by BamHI linker addition. The resultant HindIII-BamHI fragment (−1004 bp to +45 bp) replaced the HindIII-BglII fragment (−1004 bp to +1377 bp) of clone 403. The resulting plasmid, clone 407, lacks the intron and the start of translation of the oat phytochrome gene. Clone 230 was constructed by ligating the PstI-XbaI fragment from pBI221 (Jefferson, 1987) containing the cauliflower mosaic virus 35S promoter into the same site of clone 228 immediately upstream of the +10-bp 5′ deletion endpoint of the phytochrome gene.

5′ Deletions

Construction of the −2000 bp, −830 bp, −639 bp, and −116 bp 5′ deletions of the oat *phyA3*/CAT gene involved the convenient restriction endonuclease sites XbaI, DraI, BglII, and SalI, respectively (Hershey et al., 1987). The 5′ deletions −381 bp, −348 bp, −247 bp, −148 bp, and −76 bp were constructed by Bal31 deletion from the HindIII site of clone 403, followed by BamHI linker addition. The −735 bp, −675 bp, −535 bp, −455 bp, −415 bp, −41 bp, and −22 bp 5′ deletions were constructed using a method described by Higuchi et al. (1988) involving the polymerase chain reaction procedure.

Internal Deletions and Sequence Substitutions

The internal deletion constructs using the −831 bp, −635 bp, and −489 bp 3′ deletion endpoints were generated using the restriction endonuclease sites DraI, BglII, and ClaI, respectively, followed by fusion to various 5′ deletion constructs as indicated. Internal deletion constructs using the −231 bp, −150 bp, −110 bp, −69 bp, and −35 bp 3′ deletion endpoints were generated by Bal31 digestion from the KpnI site (+89 bp) of clone 403, followed by BamHI linker addition and ligation to various 5′ deletions. The sequence-substitution constructs were generated by fusion of 3′ deletions, constructed by the modified polymerase chain reaction procedure (Higuchi et al., 1988) to existing 5′ deletions, as designated.

Bombardment, Irradiation, and Enzyme Assay

Microprojectile bombardment, irradiations, and enzyme assays were as previously described (Bruce et al., 1989). In brief, 2-day-old, dark-grown rice seedlings were exposed to a standard 3-hr white light period during which they were bombarded once with tungsten particles coated with a mixture of two plasmids: one containing one of the CAT constructs and the other a ubiquitin/luciferase (internal standard) plasmid (ubi/LUC), pAHC18 (Bruce et al., 1989) in a 3:1 molar ratio. The seedlings were then given a saturating F-light pulse only or an F-light pulse immediately followed by an R-light pulse before incubation in the dark for 24 hr at 26°C. The shoots of the rice seedlings were then extracted

and assayed for CAT and luciferase activities. All CAT values (determined as percent chloramphenicol conversion) were normalized to the corresponding luciferase value (measured in the same extract), corrected for the promoterless clone 228 activity (unless otherwise stated), and expressed as percentage of clone 403 (1-kb promoter) activity.

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