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Analysis of Genes Negatively Regulated by Phytochrome Action in *Lemna gibba* and Identification of a Promoter Region Required for Phytochrome Responsiveness[1]

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As a step to understanding how the photoreceptor phytochrome acts to change the transcription of specific nuclear genes in *Lemna gibba*, we wish to compare promoter elements involved in negative regulation by phytochrome with those involved in positive regulaion. We have isolated three genes negatively regulated by phytochrome, designated *NPR* (negatively phytochrome regulated) genes (P.A. Okubara, E.M. Tobin [1991] Plant Physiol 96:1237-1245), and we have now sequenced two of these. The promoters of both contain some sequence motifs that are identical with motifs from other genes. We used a transient assay in *L. gibba* to demonstrate that approximately 1.7 kb pairs of the *NPR*1 promoter and 1.1 kb pairs of the *NPR*2 promoter could confer negative phytochrome regulation to a luciferase reporter gene. Deletion analysis of the *NPR*2 promoter showed that sequences between −208 and −82 from the transcription start were necessary for negative phytochrome regulation. However, this region was not sufficient to confer negative regulation by phytochrome to another promoter. Additionally, we noted that this region showed no similarity to a region identified as important for the negative regulation of the oat *phyA* promoter (W.B. Bruce, X.-W. Deng, P.H. Quail [1991] EMBO J 10:3015-3024), but it does contain a sequence element found in several other kinds of genes, including ones positively regulated by phytochrome. The deduced amino acid sequences of NPR1 and NPR2 were found to share similarities with many abscisic acid-induced or seed-abundant proteins. Thus, these genes, like other phytochrome-regulated genes, might respond to multiple regulatory signals.

Phytochrome is the photoreceptor involved in the transcriptional regulation of a number of specific genes in various species during development. Most such genes that have been studied show increased expression in response to phytochrome action, but a number of genes have been shown to be negatively regulated by phytochrome, including genes for protochlorophyllide reductase and for phytochrome itself (reviewed by Watson, 1989; Gilmartin et al., 1990; Thompson and White, 1991). We have reported the isolation of three distinct cDNA clones that represent mRNAs whose transcription is negatively regulated by phytochrome in the aquatic monocot *Lemna gibba* (Okubara and Tobin, 1991). These genes have been designated as NPR (negatively phytochrome regulated) genes. Phytochrome action has also been reported to cause reduction in the level of Asn synthetase mRNA in pea (Tsai and Coruzzi, 1991) and of β-tubulin mRNA in soybean (Bustos et al., 1989), oat, and barley (Colbert et al., 1990), suggesting that transcription of the corresponding genes might also be negatively regulated by phytochrome.

The molecular mechanisms by which phytochrome acts to affect transcription remain largely unknown. However, the analyses of the promoters of genes for Chl *a/b* proteins (*cab* genes) (Castresana et al., 1988; Schindler and Cashmore, 1990), the small subunit of Rubisco (*rbcS* genes) (Giuliano et al., 1988; Green et al., 1988; Manzara et al., 1991), chalcone synthase (*chs* genes) (Schulze-Lefert et al., 1989; Weisshaar et al., 1991), and phytochrome (*phy* genes) (Kay et al., 1989; Dehesh et al., 1990; Bruce et al., 1991) have indicated that common *cis*-regulatory elements are shared not only among phytochrome-regulated genes but also with genes that are not under phytochrome control.

One of these elements, originally called the G box (Giuliano et al., 1988), appears to serve as the promoter element for ABA responsiveness (Marcotte et al., 1989; Mundy et al., 1990) and is also found in promoters of genes induced by wounding, anaerobic conditions, or UV light (summarized by Schindler and Cashmore, 1990; Oeda et al., 1991; Weisshaar et al., 1991). A second common motif, the GATA box, occurs in the regulatory regions of *cab* (Lam and Chua, 1988; Schindler and Cashmore, 1990), cauliflower mosaic virus 35S (Lam and Chua, 1988), and *rbcS* (Manzara et al., 1991) genes. An AT-rich, factor-binding sequence has also been found in the promoters of *rbcS* genes (Datta and Cashmore, 1989; Manzara et al., 1991). Several GT elements have been identified and may have different functions. One is necessary for rice *phyA* transcription (Dehesh et al., 1990), and another, in a pea *rbcS* gene, is associated with light responsiveness (Green et al., 1988). The GT element differs from the sequence element in *Lemna rbcS* genes that interacts with a light-dependent binding activity called LRF-1 (Buzby et al., 1990).

Negative phytochrome regulation of the oat *phyA* gene is

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Abbreviations: act::GUS, rice actin promoter fused to the bacterial *uidA* gene encoding β-glucuronidase; FR, far-red light; GUS, β-glucuronidase; Lea, late embryogenesis abundant; Luc, luciferase; NPR genes, negatively phytochrome regulated genes; PCR, polymerase chain reaction; R, red light; rab, rice ABA-induced; SV40, simian virus 40.

abolished upon mutation of an element called RE1 (Bruce et al., 1991). RE1 can bind nuclear factors isolated from either dark-grown seedlings or from seedlings given an R treatment, suggesting that repression of the transcription of this gene by phytochrome does not involve a change in factor binding. Other factors interact with regions of the *phyA* promoter, including the GT-1 factor that binds to the GT element upstream of RE1, but their regulatory roles remain to be defined. The presence of a few elements in many types of promoters suggests that regulation is the result of interactions between arrays of common elements and that regulation is specified in part by the composition and the arrangement of the elements. The existence of a distinct phytochrome-responsive consensus element has not been demonstrated so far.

To contribute to the understanding of how phytochrome acts to decrease transcription, we have now further characterized the two most highly transcribed of the *Lemna NPR* genes, *NPR*1 and *NPR*2. We have used a transient assay to define a region of the *NPR*2 promoter that is necessary for its phytochrome responsiveness in *Lemna*. Furthermore, we have found by comparison of predicted amino acid sequences that the *NPR* genes of *Lemna* are homologous to genes regulated by ABA, desiccation, and cold treatment in other plants.

MATERIALS AND METHODS

Plant Material

Etiolated *Lemna gibba* L. G-3 were cultured aseptically in liquid E medium supplemented with 3 μM kinetin under a regimen of 2 min of R every 8 h, as described previously (Rolfe and Tobin, 1991). For microprojectile bombardment, colonies were gently arranged in a 4-cm diameter monolayer on E medium plates containing 1.25% Phytagar (Gibco), under a dim green safelight. Each monolayer consisted of about 60 colonies. After the plants were plated, they were given 10 min FR and then placed in darkness for 20 to 22 h before bombardment for experiments with the NPR1 and NPR2 promoters and for 48 h for the NPR2-ΔAB19 experiments.

RNase Protection Analysis

The *NPR*1 transcription start was determined by RNase protection analysis. The transcription template for making the antisense RNA consisted of an *Sph*I-*Cfr*10I NPR1 fragment (−429 to +252 of the NPR1 gene) in pGEM3Z. After the plasmid was linearized at the unique *Hin*dIII restriction site, the RNA was transcribed from the T7 promoter as recommended by Promega, except that UTP was substituted for CTP. The probe included 41 nucleotides of the pGEM3Z vector, and it was radiolabeled to a specific activity of 2×10^8 cpm μg^{-1} template, using [α-^{32}P]UTP (800 Ci/mmol; Amersham). Probe was annealed to 5 or 20 μg of total RNA obtained from etiolated plants placed in darkness for 24 h and then treated with RNase A (Worthington) and RNase T1 (BRL) as described by Zinn et al. (1983). An RNA ladder (0.24–9.5 kB; BRL) was radiolabeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) using T4 polynucleotide kinase (BRL) and run beside the DNA-sequencing ladder to verify equivalent migration rates of RNA and DNA for size determination.

S1 Nuclease Analysis

The *NPR*2 transcription start site was determined by S1 nuclease mapping (Sambrook et al., 1989). Single-stranded *NPR*2 DNA probe, spanning −300 (*Ssp*I restriction site) to +108 (*Nco*I restriction site), was radiolabeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) and T4 polynucleotide kinase (BRL) to a specific activity of 8×10^4 cpm μg^{-1}. Probe DNA was isolated by electrophoresis in 1% agarose, concentrated by precipitation in ethanol, and then incubated in the presence of 10 μg of total *Lemna* RNA at 62°C for 34 h. Hybridization products were treated with 200 $\mu g\ mL^{-1}$ of nuclease S1 (Pharmacia) at 37°C for 30 min. Maxam and Gilbert (1980) chemical cleavage reactions were performed on aliquots of the radiolabeled probe and fractionated by electrophoresis along with the S1 nuclease protection products on a denaturing 6% acrylamide sequencing gel.

Nucleotide Sequencing

*NPR*1 and *NPR*2 gene sequences were obtained by dideoxynucleotide sequencing (Sequenase DNA Sequencing Kit, United States Biochemical) of denatured double-stranded DNA templates (Sambrook et al., 1989) using T7 or SP6 primers (Promega) or oligonucleotide primers complementary to various regions of the genes. *NPR*1-specific primers were CCACGTAACGACACGCGTCGAA, CATGGTATCAAAGCCCAAG, AAGAGCTGAGACGCTCCGGAAG, GAGACGAGTACCGGGAATC, and TTCGACGCGTGTCGTTACGTGG. NPR2-specific primers were GCCTGCTTCGGCTATT, TGACGTTGCCTCAAT, TCTCCTCGGACATCAGAA, TCGCAGGTGCAGCAAT, GTTCACCACCAGTCTG, and ACTTTTCAGACGGTCC. Portions of the *NPR*1 and *NPR*2 sequences were obtained at the DNA Core Sequencing Facility, University of California, Los Angeles, CA.

Nucleotide and Amino Acid Sequence Comparisons

The GenBank/EMBL nucleotide sequence data base and the GenPept, Swiss-Protein, and National Biomedical Research Foundation amino acid sequence data bases were searched using the sequence comparison programs of Pearson and Lipman (1988) and Sequence Analysis Software Programs of the Genetics Computer Group, University of Wisconsin (version 7.0, 1991). Additional searches for homologies to short sequences were performed with the DNA/Protein Sequence Analysis Programs of Pustell (version 4.0; Pustell and Kafatos, 1982).

Promoter Fusion and Deletion Constructs

To study the promoters in a transient assay, translational fusions of *NPR*1 and *NPR*2 were made with a *Luc* reporter gene. A 2.1-kb fragment of the *NPR*1 gene (containing 1.7 kb of the promoter and extending to +431 in the transcript) and a 1.2-kb fragment of the *NPR*2 gene (containing 1.1 kb of the promoter and extending to +108 in the transcript)

were ligated to pDR102, a pUC12-based plasmid carrying the firefly *Luc* gene and 3′-untranslated region of the *Agrobacterium tumefaciens* nopaline synthase gene (Riggs and Chrispeels, 1987). Both the *NPR*1 and *NPR*2 fragments and pDR102 DNA were treated with DNA polymerase I large fragment (BRL) in the presence of deoxynucleotides before ligation. These constructs are designated as the full-length promoter constructs NPR1 and NPR2.

For the *NPR*2 promoter deletions, a pair of annealed synthetic oligonucleotides,

TACAAGCTTGAGGCC
  GTTCGAACTCCGGAT,

containing *Hin*dIII and *Stu*I cleavage sites, was inserted at the *Nde*I restriction site in pDR101, 210 bp upstream of the inserted *NPR*2 fragment. This modified NPR2::Luc construct is called −1138 in Figure 6. To obtain unidirectional 5′ to 3′ deletions, 25 μg of plasmid DNA was digested with 40 units of *Hin*dIII (Promega), and the 5′-unpaired bases were complemented using DNA polymerase I large fragment in the presence of α-thiodeoxynucleotides (*Exo*III/Mung Bean Deletion Kit; Stratagene). Five-microgram amounts of DNA were treated with 40 units of *Stu*I (Promega) and then with 400 units of exonuclease III (Stratagene) at 30°C from 60 to 240 s. Unpaired bases were removed by digestion with mung bean nuclease (Stratagene) before religation with T4 DNA ligase (BRL).

An additional *NPR*2 promoter deletion, −82, was made by PCR amplification of the −208 construct with oligonucleotide primers CCAGCTCAGGTGTCATCACCGAATCCA (spanning −82 to −62 of NPR2) and CCTTATGCAGTTGCTCTCCAG (complementary to +116 to +136 of the *Luc* gene; de Wet et al., 1987). The 250-bp PCR product was digested with *Xba*I restriction enzyme for insertion between the *Hin*dIII and *Xba*I sites of *pDR101*. *pDR101* was digested with *Hin*dIII and the 5′ overhang was made flush with DNA polymerase I large fragment. To complement a 5′ dA added to the amplified DNA by Taq polymerase during PCR, a dT was added to *Hin*dIII-treated pDR101 by incubation with Taq polymerase and thymine triphosphate at 70°C for 2 h.

An NPR2-ΔAB19 promoter chimera was constructed by inserting a 171-bp portion of the *NPR*2 promoter (from −259 to −89) into a *Hin*dIII cleavage site at −182 in pAB19Δ-183/104. pAB19Δ-183/104 is a translational fusion of the *Lemna cab*AB19 promoter (from 1600 nucleotides upstream of the transcription start) to the *Luc* gene of pDR101, in which the sequence from −183 to −104 of AB19 was deleted (Kehoe, 1992).

All recombinant plasmids were mobilized in *Escherichia coli* HB101 (Sambrook et al., 1989). Sequences of all constructs, including promoter deletion end points and PCR-generated inserts, were confirmed by dideoxynucleotide sequencing.

Microprojectile Bombardment and Light Treatments

Plated colonies of *L. gibba* were given a 10-min FR illumination and placed in complete darkness for 1 d before introduction of NPR::Luc constructs by bombardment with the Biolistics PDS-1000/He particle gun (Bio-Rad). NPR::Luc DNA was mixed with act::GUS reference DNA before precipitation onto 1.0-μm gold particles (Bio-Rad). Each precipitation was done with 6 μg of NPR::Luc DNA and 2 μg of act::GUS DNA. One-third of the DNA-coated gold was used in each bombardment. Controls consisted of gold precipitated without any DNA. Optimal reporter gene activity was obtained with 1100-p.s.i. rupture discs, with the macrocarrier placed 6 mm from the stopping screen, and with bombarded samples placed 61 mm from the macrocarrier.

For consistency with early experiments using the charge-driven particle gun (cf. Rolfe and Tobin, 1991), all plants received 2 min of FR within 10 to 90 s of bombardment. After this treatment, plants were either immediately given 2 min of R or 2 min of R plus 2 min of FR or given no further light treatment and placed in darkness for 18 to 19 h before harvesting and assaying reporter gene activities. Bombardments were carried out under a dim green safelight.

Biochemical Assays

Plants were homogenized in 0.4 to 0.55 mL of ice-cold buffer (de Wet et al., 1987) containing 200 mM phosphate buffer, pH 7.8, 1 mM DTT, 0.5 mM PMSF (Sigma), using motorized Kontes glass homogenizers. Crude homogenates were clarified by centrifugation for 10 min at 4°C at 14,000*g*. Two 100-μL portions were assayed for Luc activity essentially as described by de Wet et al. (1987), except that the final concentration of ATP was 10 mM instead of 5 mM. Luc activity was measured as relative light units (1 relative light unit = 10 photons) using a luminometer (Tropix model ILA-911). High-incident background readings were avoided by performing the Luc assays in a dimly lit room. GUS activity was measured from 300 μL of clarified homogenate as described by Jefferson (1987), using 4-methyl umbelliferyl glucuronide (Sigma) as the substrate. Methyl umbelliferone product was quantitated with a DNA fluorometer (Hoefer model TKO 100). Protein was quantitated with Bio-Rad Protein Assay Dye Reagent. Background activities from plants shot with gold only were subtracted from both the Luc and GUS values. The relationship between Luc and GUS activity was determined over multiple experiments, and the adjusted mean Luc activity values were determined for each construct under each illumination treatment by analysis of covariance. The mean values were normalized to that of the full-length construct kept in the dark or, for Figure 7, to the maximum average value. Sample sizes for each value ranged from 6 to 14. Differences between the adjusted mean Luc activities were tested for significance by the *t* test.

RESULTS

*NPR*1 and *NPR*2 Genes

To analyze the promoters of *NPR* genes, we have obtained nucleotide sequences for two such genes from *Lemna*, *NPR1* and *NPR*2. Genomic clones corresponding to the *NPR* cDNAs were obtained from a Charon 35 library (Okubara and Tobin, 1991). The nucleotide sequence of *NPR*1, a composite of the genomic and a partial cDNA clone, is shown in Figure 1, with the overlapping sequence underlined. The transcription

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-779   TTCTGCCATGCATCATTCATCATATTTAGGTCATGAAATGATGCCCTTTAGTAGCTATG
                       *
-720  ATAGGAAAACAAATTCTGTCGCGCACCATTCATCATATTTGGGGCCATCTTCATTCTTGA
                                      *
-660  GAGGGAGGGTCCTCTTTGAGGAAGGATAATTTATCTTGGTTTCAGAGAGCATACTTGAAG
-600  TGGGTGCCAACTTGGTTGAGCAGTGAAACTAGACATAAAGATTACATGATGTTCTTGGTG
-540  GTCGTAATATGTCAAATAGATGGTATGCTAAAAAACAGTTAAAACTAGATGAGAAGATTG
                                                        a
-480  TGAAAGACTGATTTGCCAGAGAAAATCTAGTAGATATTTATTGGAGTTGGCCTAGCATGT
                                      b
-420  GGGGCAAGTGCATGCTTGAATGCCCCCTGATCATGGGTAGATGTCAGCCACAAGGAGCCC
-360  AATAAACCTGGGCTTTGATACCATGTTAAATAAAAAATATATATGCCTCATATATATGAT
-300  CTATAAACTCCTAAATTGCATCTAAACACTCGATGGAAGAAGGGTGAAATCAGTTTATCG
-240  ATTTAACTAAATTAATTTATTTTGGGGGTTTTTTTGGCTTTTAAAATGGGTTTGAAGATA
-180  ACTCTCTCTCTCTCTCCCCCCGCCCATGCAAAGAGGTCGGCAATTTTAGATAAAGACGTC
                                  c             d
-120  CATTTTTTCGACGCGTGTCGTTACGTGGCGAAACGTCGTGGAAGGACGAGTCTTTGAGGG
                              e
 -60  CACGCGTCAGACTATCGTGGTCATCCTCTGCCTACTTATATCCCTCTGGTCTTCTTCTTC
                                            f
  +1  AGCAGCAGTCCATTGGAGAATCTACTTCCTGTTGAAATCCACTTCAGAGGCTAGATTTTC
                                                  g
                    ●●
 +61  AAGGTTCTAATCTAGGGTTTTGAGCTCTCTCTCTCTCTCTCTCTCCTTCCGTTACTTA
                             ●●
+121  GATCGTGGTTTTGCAGGTGTGAGAAAAGTTTCGATAATGGCGGAGATGAGAGACGAgtac
                                             M  A  E  M  R  D  E
+181  gggaatcccctccgccagACCGACGAGTTCGGCAACCCCATCGGCGCCTCGGCGATGGGC
                         P  T  S  S  A  T  P  S  A  P  R  R  W  A
+241  GCGGGCCAGCAGGCCGGCGGATACGGCGCGGGGCCAAGCAAGGCCCCGGCGGATACGGTG
       R  A  S  R  P  A  D  T  A  R  G  Q  A  R  P  R  R  I  R  C
+301  CGGGGCAGCAGGCCGGCGGATACGGTGCGGACAGCAGGCCGGCGGATACGGTGCGGGGCA
       G  A  A  G  R  R  I  R  C  G  Q  Q  A  G  G  Y  G  A  G  Q
+361  GCAGGCCGGCGGATACGGCGCCGGGCAGCAGGCCGGCCAGCAAGAGCTGAGACGCTCCGG
       Q  A  G  G  Y  G  A  G  Q  Q  A  G  Q  Q  E  L  R  R  S  G
+421  AAGCTCAAGCTCGAGCTCGgtacgttctttttctttcatgaaattcactgggtagagag
       S  S  S  S  S  S
+481  agaaagtgtttatcactctagaaaatgtgatcgagTCGGAGGATGACGGGATGGAGGGA
                                          S  E  D  D  G  M  G  G  R
+541  GGAGGAAGAAGAAGGGCATCATGGAGAAGATCAAGGATAAGATGCCGGGGCAGAAGTCGG
        R  K  K  K  G  I  M  E  K  I  K  D  K  M  P  G  Q  K  S  E
+601  AGCAGCATCATCACGAACCAGGATACGCTCATCAGAGGCCGACCCACGTTGAGCCGGAGA
        Q  H  H  H  E  P  G  Y  A  H  Q  R  P  T  H  V  E  P  E  K
+661  AGAAGGGGATAATGGAGAAGATAAAGGAGAAGCTGCCGGGCCATAACTGAGGGGAAGCTG
        K  G  I  M  E  K  I  K  E  K  L  P  G  H  N  -
+721  GAGACGGAGCTCTAGTTTAGCGGTGCAACTCTTTCGGCTTGCTGTCTACTTGTCTAAATA
+781  TGTCGTTCGTTAGCGTTTTTTTTATCTTTGGAATTGTAATAACCAGCCGCTAAGAACTAC
+841  TTCTCGCCGCCGGCTTCATGCTGGTGATTTATGTGGCTATACTATCTGCTTTATCTCTC
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Figure 1. Nucleotide sequence and deduced amino acid sequence of *NPR*1 (EMBL accession no. X64327). The sequence of both the genomic clone and the partial cDNA clone are shown. The extended underlining indicates the overlap between the genomic and cDNA clones, and the cDNA sequence continues for 104 nucleotides (to +899) beyond the end of the genomic clone. The transcription start site is designated +1. Similarities to other sequences are underlined: *, repeats; a, tomato *rbcS* elements; b, *NPR2*; c, SV40 core enhancer; d, CAAT; e, G box of Em1a, rab 16A; f, TATA box; g, *NPR2*; ●●, 3′-intron junction splice consensus.

start site was determined by RNase protection analysis as shown in Figure 2 and is indicated as +1. A putative TATA box is located at −25, and a CAAT box occurs at −140. Other features of possible interest in the sequence are detailed in the legend to Figure 1.

RNase protection analysis also demonstrated the presence of three smaller protection products (S.A. Williams and E.M. Tobin, unpublished data), suggesting that there are least three introns in the 5′ portion of the *NPR*1 gene. The 3′ termini of introns 1 and 2 are indicated with circled asterisks; the 5′ splice junctions of these introns have not been defined. For intron 3, several possible 5′-splice consensus sequences (Joshi, 1987a) are present, but the one at +177 would conserve a sole initiation codon at +157 and is, therefore, proposed as the upstream splice junction. Intron 3 is represented by lowercase letters in Figure 1. A fourth intron is deduced from sequence comparisons, discussed in the following section. A full-length cDNA clone would be needed to define the introns unequivocally.

Because the *NPR*1 cDNA sequence extends downstream of the genomic DNA sequence (Fig. 1), the size of the mRNA is predicted to be >615 nucleotides, and the two exons that flank the fourth intron are predicted to be 260 nucleotides and >400 nucleotides. These values are consistent with those obtained by S1 nuclease analyses: 720 nucleotides for the mRNA; 260 nucleotides and 490 nucleotides for the exons (Okubara, 1992).

The nucleotide sequence of *NPR*2 is shown in Figure 3. Two S1 nuclease protection products originated at consecutive G and T nucleotides (Fig. 2). The G is designated as the start of transcription (+1). A TATA motif occurs 31 bases upstream of the transcription start site. Of three possible initiation codons (at +16, +91, and +109), the one at +109 is situated in the most favorable context according to Kozak (1987) and is postulated to be the start of translation. Putative polyadenylation signals AAUAAA and UGUGUUU (Joshi, 1987b) are encoded at +457 and +508, respectively, in the *NPR*2 gene. The *NPR*2 message is, therefore, expected to be about 460 nucleotides, which is in close agreement with the size of 500 nucleotides determined by S1 nuclease analysis (Okubara, 1992). The absence of introns in the deduced open reading frame of *NPR*2 is also consistent with S1 nuclease analysis.

There is an unexpected but extensive sequence similarity between a portion of the 3′-flanking region of *NPR*2, spanning nucleotides +744 to +928, and a sequence encoding the chloroplast RNA polymerase β-subunit (*rpo*B; Ohme et al., 1986). Within this 185-nucleotide region of similarity, 150 nucleotides of *NPR*2 (approximately 80%) are identical with those of *rpo*B.

Deduced Amino Acid Sequences of NPR1 and NPR2

The translated amino acid sequence of *NPR*1 is shown in Figure 1. The deduced 151 amino acids encode a protein of approximately 18 kD, which contains two blocks of significant similarity to those of several ABA-induced or seed-abundant mRNAs, including Lea mRNAs, rab mRNAs, and dehydrin mRNAs. These similarities are summarized with references in Figure 4. A translational fusion of the *NPR*1 promoter in this reading frame to a *Luc* gene resulted in Luc expression in a *Lemna* transient assay, whereas the other two reading frame fusions gave no expression (discussed in a following section). Therefore, we are confident that the reading frame shown in Figure 1 is correct.

The conserved region also has led us to postulate the presence of a fourth intron from +440 to +515. In fact, a perfect 5′-intron splice junction consensus (Shapiro and Senapathy, 1987) is present at +440, and a putative 3′-splice junction consensus at +515 extends the region of similarity

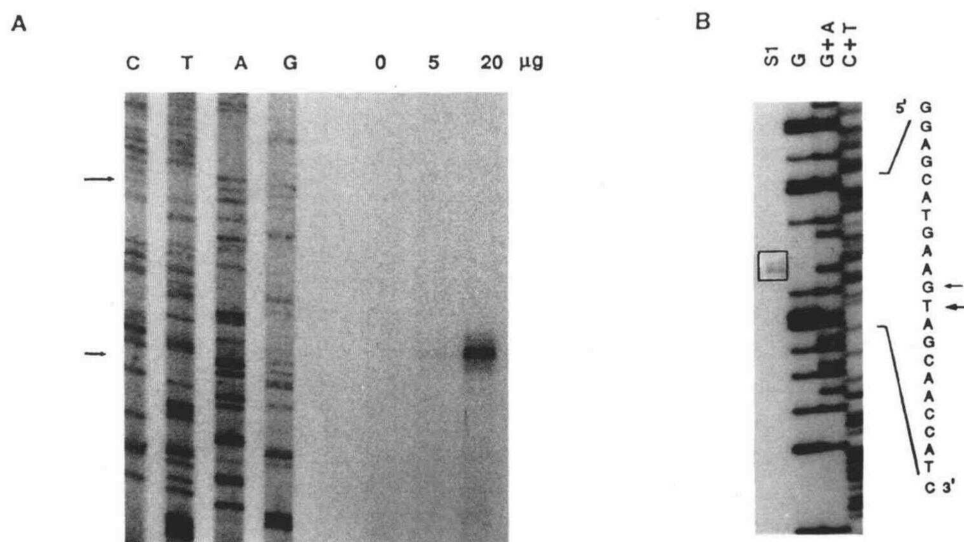

Figure 2. Mapping of the transcription start sites of *NPR*1 (A) and *NPR*2 (B). A, RNase protection mapping of the *NPR*1 transcription start site. The amount of total RNA in each reaction is indicated. The dideoxynucleotide sequencing ladders are on the left (lanes C, T, A, and G). The lower arrow indicates the sequencing product that comigrates with the protected product. The upper arrow indicates the actual transcription start site at an A, 41 nucleotides upstream, taking into account the 41-nucleotide pGEM leader present on the sequencing ladder but absent from the protected product. B, S1 nuclease mapping of the NPR2 transcription start site (lane S1) and Maxam-Gilbert cleavage reactions of the end-labeled noncoding strand probe (G, G+A, and C+T). The arrows indicate the positions of the protection products on the coding strand of the probe. Autoradiography was done at −70°C for 18 and 108 h. For clarity, S1 nuclease protection products obtained from the 108-h exposure are substituted for those of the 18-h exposure and boxed. The sequence is shown at the right with the terminal nucleotide indicated by an arrow.

found for *NPR*1 and the other translated sequences. Intron 3 appears to interrupt a third block of amino acid identity (DEYGNP) in NPR1 that is found in the N-terminal regions of other deduced proteins.

Two and one-half tandem repeats of the amino acid sequence GQQAGGYGA occur in NPR1 from nucleotide position +329 to +381. Two copies of a smaller Arg-rich repeat, RRIRCG, are also present at +287 and at +314.

The deduced amino acid sequence of the *NPR*2 gene (Fig. 3) is predicted to encode a protein of 103 amino acids, with a mol wt of approximately 13,000. The translated amino acid sequence of NPR2 has some regions of substantial similarity to Lea proteins of soybean (Chen et al., 1992) and cotton (Baker et al., 1988), as shown in Figure 4B. On the basis of amino acid sequence, these Lea proteins appear to constitute a different class from those shown in Figure 4A.

NPR1 and NPR2 Promoter Regions Contain Motifs Found in Other Eukaryotic Genes

The 5′ regions of *NPR*1 and *NPR*2 contain G box-like elements found in *rbcS* genes from pea, in ABA-responsive Em genes of wheat and *rab* genes from rice, and in anaerobiosis-responsive *Adh* genes of maize and *Arabidopsis*. Similarities to elements identified by footprint analyses in other genes and their locations within the *NPR* genes are summarized with references in Table I. Another similarity of particular interest is the presence in *NPR*2 of an 8-base region of identity to part of a conserved element, called box Y, found at −117 in three *Lemna rbcS* genes. This element is located at −108 in the *NPR*2 promoter. Other motif similarities include a conserved element in tomato *rbcS* genes and an SV40 core enhancer element in *NPR*1, and, in *NPR*2, the hexameric core motif of the wheat histone H3 gene, an AT-rich element from *rbcS* genes, and two copies of a different AT-rich core motif found in a gene encoding the γ-subunit of Gln synthetase from French bean.

The 5′-flanking regions of both *NPR*1 and *NPR*2 contain two previously unidentified common sequences: ATCCACTTTC(A/T)GA is located at +37 in *NPR*1 and at −862 in *NPR*2, and a second sequence, AATCAGTTT, is found at −253 and −483 in *NPR*1 and *NPR*2, respectively. *NPR*2 contains three copies of a 13-bp sequence, AACCGGCCGGCTC, located at −421, −384, and −267.

*NPR*1 and *NPR*2 5′ Regions Confer Negative Phytochrome Regulation to a Reporter Gene in a Transient Expression Assay

To test the ability of each promoter to confer negative phytochrome regulation in a transient expression system, translational fusions of 2.1 kbp of *NPR*1 promoter and 5′-coding region and 1.2 kbp of *NPR*2 promoter and 5′-coding region were made to the firefly *Luc* gene and introduced into *Lemna* by microprojectile bombardment (Bruce et al., 1989; Rolfe and Tobin, 1991). Translational fusions of the *NPR*1 promoter to the *Luc* reporter gene were made in all three reading frames, but only one fusion resulted in Luc expression:

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-1138   GAATTCTTTCGGAGAAAATGGATTGATGCAAGACTACAAATAGAAGAAAATAATAAAA
-1080  TGAAATTTTCATTCATGTTGCCTTCTCATGCCATCAATTCTCCAGGCAAAATTGAGGATT
-1020  AAATTGAATTGAAATCTATGAAGTCATACAAGATCAGAAGAACTCTGGATTTGAAGCCAT
 -960  CTGACAGAGCCTAAAGTCGCCGATTGCTGCACCTGCGAGAGAAGACTCGATAATCAACCG
 -900  TAGAAGAAGAAGAAAAATCAGCAACAACCGAAATTTAGATCCACTTCTGATCGTTCTCCC
                                             a
 -840  AAAATTAAGATGCAGAGACACGGAAAACAGCAAGGATCAAGCAATTATCGAGCATTTACG
 -780  GCACTAGAGACGGAGAAATCATACCAGGCGGGATCGGATCTTGGAAAGAACCGAAACACT
 -720  CCGCATGAACACCATTTCGCCGAGGACCGTCTGAAAAGTTGAGACACGGAATCGGCTAGA
 -660  AAGATCAGCCGTTGGCATGATTTGGAAGATCATCAGACGCGGAATCAGAAATAGAACAGG
 -600  TAAGAAGAGATCGGAGATAGAAAAACAGTAATGAAAACCTTCCTACAGTCAATTCCACCG
 -540  TATACCTGGATCCTTCTGATGTCCCGAGGAGAAAACGACCGGATGCAAGATTGAAGGAAT
 -480  CAGTTTTGAGAGAGAGAGCGAGCAAGAGAGAGAGAGAGAGACGACTCTCTCCAGGTTA
         b             c
 -420  ACCGGCCGGCTCGAAGCGTGCAAATCTAAGCACGCGAACCGGCCGGCTCTGTATACAACG
            *                               *
 -360  GATGGCAAAATCGTAATTTGACTAGTTCTATATTTTATTTCGTAAAAAACCCACAAAAAT
                                      d
 -300  ATTTTTATCGCAAAATAATACTTATTTTCGCTGAACCGGCCGGCTCGATGCGGGAGAATC
         e                                   *
 -240  GAAGCAATTGAGGCAACGTCATAATTTCCTCAATTTTTATATTTCTATTTCATAAAATTC
                        f
 -180  CCCAAAAATAGTTTTATATCAAATAATAAATCTCACATTTAATTCAGACTTTTGTATTTAT
 -120  TTAATGTTACACGTGGAGGAAATCCAGAAGCCGGGGGACAGGTGTCATCACCGAATCCAC
         g    h     i
  -60  GCGGCAGAATCGCAGCCTTCCCAGGGCGCTATAAATAGCCGAAGCAGGCCGGAGCATGAA
                                      j
   +1  GTAGCAACCATCATCATGCAGACGGGGAAGACAGCAGCGGCGACGGTGAAGGAGAAGGCG
  +61  GCCAACGTGGCCGCCTCGGCGCAGTCGGGCATGGAGAAGACGAAGGCCATGGCGCAGGAG
                                                        M  A  Q  E
 +121  AAGGCGGAGAAGATGAGGGCCCACACGCCGGAAGACAAGGGGATCGCGGCGGAGAGGAAG
        K  A  E  K  M  R  A  H  T  P  E  D  K  G  I  A  A  E  R  K
 +181  CAGCAGATGATGGAGCAGGCGGAGCTCAACAAGCAGCAGGCGAAGGAGGAGAACGCCGCG
        Q  Q  M  M  E  Q  A  E  L  N  K  Q  Q  A  K  E  E  N  A  A
 +241  CAGAAGGAGCAGGCCCGCGCGGCGACCGTGCCGGGCACACGGCGCCGCCGCGGCACGGCC
        Q  K  E  Q  A  R  A  A  T  V  P  G  T  R  R  R  R  G  T  A
 +301  GCGTGGCGCACGCCAGCCCCATCGGCACGGCCACCGGCACCGGGCGGGCCGCACGGCGCA
        A  W  R  T  P  A  P  S  A  R  P  P  A  P  G  G  P  H  G  A
 +361  CAACCCGCTGAGCGGTGGGACCCAGACTGGTGGTCAACGCCCGGAGGTGGGTACACCTGA
        Q  P  A  E  R  W  D  P  D  W  W  S  T  P  G  G  G  Y  T  -
 +421  TGTGAATGAAGGGAAAGGGGGTTGACTTTTATGGGAATAAATTGAGAGGAATGTGTGTCA
                                           m
 +481  AGTGGCTAGTTGTGCTGTTTGTAAGCTTGTGTTTGAGTAGAAGTTGAGAATGTCTGCAGC
                                   n
 +541  TTGTGTGTAAAACTAGATAATGCTTTATGGGTTACCTCTCTCTCTCGCTCTCTCGCTCTC
 +601  TCCCCCCCCCTCGCTCTATCTATCTCTCCCCCTCTTACTCTTACCCTCCCTCTCTCTCTC
 +661  CCTCAGCTCTATCTATCTATCTATCTCTCTATCTCTCGCCAGAGAGAGAGAGAGAGAGAG
 +721  AGAGAGAGAGAGATTTATCACCAAAGTACAGGTTGTGCAGGCTTGATAACGTCAAGATTT
 +781  CATGACTATTGCGTGGGAACAGGTTCACCTTTGATGCAATTTCCGTTTCCAATAGTTTTC
 +841  TAATAGAGCCTCTCTCATTCGTTTCTTAGCATAATGATATGAATATGGGTTTAAATGAGT
 +901  TGTAATATGTAGTGTCAAGCAGTTCCTAGGCATAACCATCAAGGTGAGTAGGGATCTTAA
 +961  AGATTGAATAACATAATATT
```

Figure 3. Nucleotide sequence and deduced amino acid sequence of *NPR2* (EMBL accession no. X64145). The start of transcription is indicated as +1. Similarities to other sequences are underlined: *, Repeats; a and b, *NPR1*; c, Em2, rab 21 (Marcotte et al., 1989); d and g, AT-rich element (Forde et al., 1990); e, AT-1 box (Datta and Cashmore, 1989; Manzara et al., 1991); f, histone H3 gene core motif (Tabata et al., 1991); h, *rbcS* G box (Giuliano et al., 1988); i, *rbcS* box Y (Buzby et al., 1990); j, TATA box; k, Em1 and rab 16A G box (Marcotte et al., 1989; Mundy et al., 1990); m and n, polyadenylation signals (Joshi, 1987b).

```
AGC TCA AGC TCG ATC GAC CTG GAA GAC
 S   S   S   S   I   D   L   E   D
```

The underlined portion indicates *NPR1* sequence. In this reading frame, the *NPR1* construct has a deduced amino acid sequence found to be conserved in other proteins.

Act::GUS DNA (McElroy et al., 1990) was used as an internal reference and co-bombarded with NPR::Luc DNA. Although such a reference construct was not found to be necessary for evaluating *rbcS* deletion constructs (Rolfe and Tobin, 1991), it helped to reduce the variability among bombardments with the NPR fusion constructs. Promoter function was quantitated as described in "Materials and Methods." Figure 5 shows the results of experiments with both *NPR1* and *NPR2* full-length promoter constructs. The expression of the reporter gene from both constructs was relatively high in

```
   Protein    AA#
A.
   NPR1       79   RSGSS-SSSSSEDDGMGGRRKKKGIMEKIKDKMPGQK
   rab 16A    62   RSGSS-SSSSSEDDGMGGRRKK-GIKEKIKEKLPGGN
   rab 16B    70   RSGSSSSSSSSEDDGMGGRRKK-GIKEKIKEKLPGGN
   B18        59   RSGSS-SSSSSEDDGMGGRRKK-GIKEKIKEKLPGGH
   M3         74   RSGSS-SSSSSEDDGMGGRRKK-GIKEKIKEKLPGGH
   RAB-17     75   RSGSS-SSSSSEDDGMGGRRKK-GIKEKIKEKLPGGH
   pcC6-19    81   RSGSSSSSSSSESDGEGGRRKK-GMKDKMKEKLPGGH
   pcC27-04   46   RSNSS-SSSSSEDDGQGGRRKK-PIKEKVKEKLPGGA
   TAS14      71   RSDSS---SSSEDDGEGGRRKK-GLKEKIMEKMPGQH
   RSLEA2     99   RSGSS-SSSSSEDDGQGGRRKK-SIKDKIKDKLGGGK

   NPR1       135  EKKGIMEKIKEKLPG-HN*
   rab 16A    147  EKKGIMDKIKEKLPG-QH*
   rab 16B    148  EKKGFMDKIKEKLPG-QH*
   rab 16D    135  EKKGIVDKIKEKLPG-QH*
   B18        209  EKKGIMDKIKEKLPG-QH*
   M3         150  EKKGIMDKIKEKLPG-QH*
   RAB-17     152  EKKGIMDKIKEKLPG-QH*
   pcC6-19    138  EKKGIMDKIKEKLPGGQH*
   pcC27-04   101  EKKGVMEKIKEKLPG-QH*
   TAS14      114  EKKGMMDKIKDKIPG-MH*
   RSLEA2     164  EKKGILEKIKEKLPGHHNHHP*

B.
   NPR2         3  QEKAE--KMRA--n18--EQAELNKQQAK
   SOYLEA      34  QEKAE--RMIA--n18--NQAELDKQAAR
   D-113       23  QEKVD--QMKT--n18--EDAELRKQEAR
   D-7         29  KEKAEAAKQKT

   NPR2        51  AAT-VPGTRR
   D-113      141  AASNNAGTRR
```

Figure 4. Sequence similarities of regions of the deduced amino acid sequences of NPR1 (A) and NPR2 (B) to those derived from genes from other species. The compared sequences are derived from: rab 16A-D, a family of ABA-induced mRNAs from rice (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1990); B18, a representative dehydration-induced mRNA from barley; M3, a dehydration-induced mRNA from maize (Close et al., 1989); RAB-17, a member of the rab 16 family (Vilardell et al., 1990); pcC6-19 and pcC27-04, ABA-induced mRNAs from Craterostigma (Piatkowski et al., 1990); TAS14, a salt stress- and ABA-induced mRNA from tomato (Godoy et al., 1990); RSLEA2, a Lea mRNA from radish (Raynal et al., 1990); SoyLEA, a Lea mRNA from soybean, encoding an 18-kD polypeptide (Chen et al., 1992); D-7 and D-113, ABA-inducible Lea mRNAs from cotton (Baker et al., 1988). AA#, Amino acid number.

Table I. *Similarities in NPR1 and NPR2 to protein-binding elements from other genes*

Motif	Gene	Position in NPR1	Position in NPR2	Reference
ACGTCA	Histone H3 (wheat)		−225	Tabata et al., 1991
TACACGTGGC	*rbcS* G box (pea)		−113	Giuliano et al., 1988
ACGTG(G/C)CG	Em1 G box (wheat)	−99	+63	Marcotte et al., 1989
	rab16A G box (rice)			Mundy et al., 1990
CCACG	*Adh* G box (maize and *Arabidopsis*)	−96, −45, +123	−109, −64, +66	Ferl and Nick, 1987
				Ferl and Laughner, 1989
GTGGAGGA	*rbcS* Box Y (*Lemna*)		−108	Buzby et al., 1990
TATTT(T/A)AT	Gln synthetase γ-Subunit (French bean)		−330, −123	Forde et al., 1990
CGAGCAG	Em2 (wheat)		−462	Marcotte et al., 1989
ATGCAAAG	SV40 core enhancer	−155		Davidson et al., 1986
AGATGAGG	*rbcS* gene family (tomato)	−494		Manzara et al., 1991
AATATTTTTATT	*cab*, *rbcS* (various)		−303	Datta and Cashmore, 1989

plants that received 2 min of FR immediately after bombardment, but the expression was reduced by approximately 50% for both *NPR*1 and *NPR*2 in plants that were treated with 2 min of R. A subsequent treatment with FR could reverse the effect of the R treatment. Thus, both *NPR*1 and *NPR*2 promoter sequences (1.7 and 1.1 kb, respectively) could confer negative phytochrome regulation to a reporter gene in a homologous transient expression assay.

NPR2 Sequences between −208 and −82 Are Involved in Negative Regulation

To localize regions within the *NPR*2 promoter that are involved in the negative response to phytochrome action, progressive 5′ truncations of the *NPR*2 promoter were fused to *Luc* and tested in the transient expression assay described above. Figure 6 shows the levels of expression and phytochrome responsiveness for four such constructs. All values were normalized to that of the full-length −1138 construct kept in the dark. Negative phytochrome-dependent regulation was still observed with the promoter truncated to −208. Further deletion of the promoter to −82 left significant Luc expression, but no significant phytochrome-dependent decrease in expression was observed. Therefore, we conclude that the region between −208 and −82 is required for phytochrome responsiveness.

To determine whether sequences between −208 and −82 could act independently of adjacent sequences to confer negative regulation, we replaced a 70-bp portion of a *Lemna cab* gene promoter (AB19; Karlin-Neumann et al., 1985) involved in positive phytochrome regulation (Kehoe, 1992) with a 171-bp segment of the *NPR*2 promoter, from −259 to

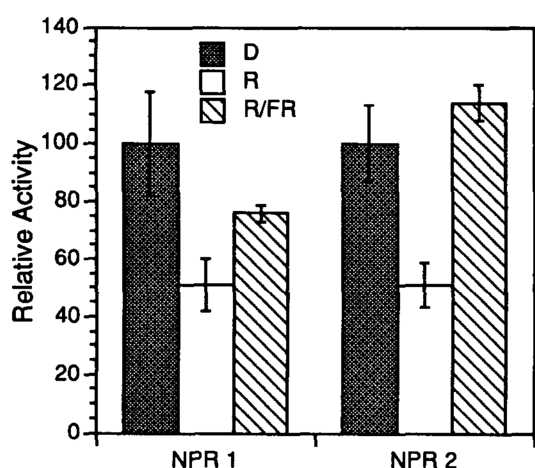

Figure 5. *NPR*1 and *NPR*2 5′-promoter sequences confer phytochrome regulation to a Luc reporter gene in a transient expression assay. Relative activity is defined in "Materials and Methods." All plants were given 2 min of FR immediately after bombardment. Further treatments were given immediately: D, Darkness; R, 2 min of R; R/FR, 2 min of R + 2 min of FR. The bars show the SE for six NPR1 bombardments and 11 NPR2 bombardments.

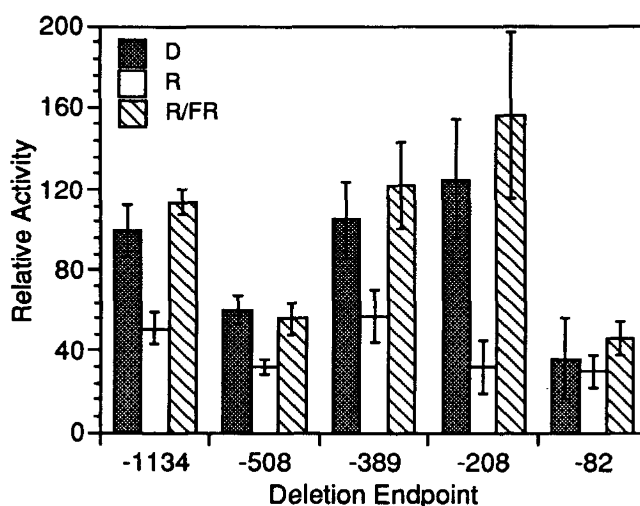

Figure 6. Deletion analysis of the *NPR*2 promoter fused to a *Luc* reporter gene. The numbers along the x axis refer to the 5′ end of the promoter relative to the transcription start. Relative activity is defined in "Materials and Methods." Light treatments were as described in Figure 5. The bars show the SE for 7 to 11 bombardments.

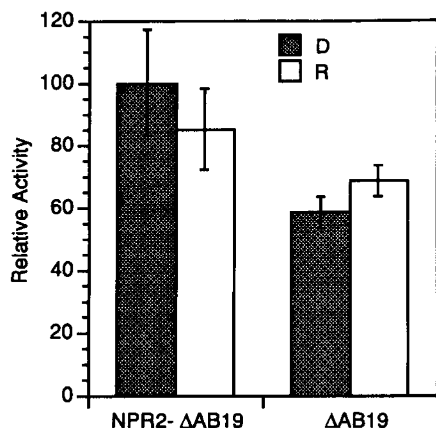

Figure 7. Expression of a *Luc* fusion construct containing the region from −259 to −89 of the NPR2 promoter within an internal deletion of a *cab* promoter. ΔAB19 refers to pAB19Δ-183/104, described in "Materials and Methods." NPR2-ΔAB19 contains −259 through −89 of the NPR2 promoter inserted at the −182 *Hin*dIII restriction site of pAb19Δ-183/104. Relative activity is defined in "Materials and Methods." Light treatments were as described in Figure 5. The bars are the SE for 11 to 12 bombardments.

−89. This construct was tested in the transient expression assay. As shown in Figure 7, the presence of the *NPR2* segment conferred no significant negative (or positive) phytochrome regulation to the AB19::Luc construct from which the endogenous positive regulatory region had been deleted. We conclude either that sequence information located between −208 and −82 in the *NPR2* promoter is not sufficient to confer negative phytochrome regulation in the transient expression assay or that the action of such a sequence is disrupted in the context of the chimeric promoter. In fact, the addition of this region actually increased the expression of the reporter gene over that of the initial deletion construct.

DISCUSSION

The *NPR1* gene described here represents one of a two- or three-member gene family in *Lemna* (Okubara and Tobin, 1991). The *NPR1* cDNA sequence and the corresponding genomic sequence have 100% identity, strongly supporting the likelihood that this *NPR1* gene encodes the *NPR1* cDNA. In any case, the activity of the *NPR1* construct used in the transient assay has demonstrated that this gene can be regulated by phytochrome. *NPR2* is a single-copy intronless gene in *Lemna*. The transcription initiation site of the *NPR2* gene differs from the consensus nucleotide A reported for other plant genes (Joshi, 1987a).

The region of the *NPR2* promoter, −208 to −82, that was required for negative phytochrome regulation contains at least three motifs found in other genes (Table I). The TACACGTGGA at −113 is nearly identical with a G box element in an *rbcS* gene of pea (TACACGTGGC). This sequence is overlapped by a second region, GTGGAGGAA at −108, that matches eight consecutive bases found in the 5′ portion of the *L. gibba rbcS* box Y consensus (GTGGAGGAG). Because the box Y consensus is distinct from the LRF-1 binding site of the *rbcS* 5B gene identified by in vitro footprint analysis (Buzby et al., 1990), the functional significance of the box Y similarity is unknown. An AT-rich sequence at −123 in *NPR2* is identical with a protein-binding region of a gene encoding the γ-subunit of Gln synthetase in French bean (Forde et al., 1990). A similar AT-rich element was reported to function as a TATA box in a phytochrome gene from pea (Tomizawa et al., 1989), but there is no evidence that this is the case for either the Gln synthetase subunit or the *NPR2* gene.

The similarities between the deduced amino acid sequences of *NPR1* and *NPR2* and those of a number of ABA-induced or seed-specific mRNAs (reviewed by Dure et al., 1989; Skriver and Mundy, 1990; Galau and Close, 1992) have raised the possibility that the *NPR* gene products have a role in desiccation or water stress. However, the importance of such roles seems unlikely under our growth conditions on liquid medium, and, therefore, we postulate that the *NPR* gene products have another or additional functions. Both *NPR1* and *NPR2* promoters have G box-like elements shown to be involved in ABA induction of *rab* genes (Mundy et al., 1990) and embryogenesis-related genes of wheat (Guiltinan et al., 1990). Preliminary experiments have shown that ABA can increase the levels of expression of these genes (S.A. Williams and E.M. Tobin, unpublished data). If induction by phytochrome is independent of induction by other environmental signals, then the *NPR* gene products might be required at more than one developmental stage or physiological condition (cf. Eyal et al., 1992). Neither *NPR1* nor *NPR2* 5′ regions contain the negative phytochrome-dependent response element RE1, identified in the oat *phyA* gene (Bruce et al., 1991).

An unexpected finding was that deduced amino acid sequences derived from a 3′-flanking region of the *NPR2* gene contain several extensive blocks of identity with the chloroplast RNA polymerase β-subunit encoded by the chloroplast *rpoB* gene. However, four to seven termination codons occur in this region in each of the three reading frames, indicating that a functional open reading frame is not present. The significance of this extensive identity is unknown.

The reduction of *NPR1*- and *NPR2*-mediated reporter gene expression by R in the transient assay was similar to the effect of R on endogenous *NPR1* and *NPR2* nuclear transcript levels in nuclear run-on transcription experiments (Okubara and Tobin, 1991). We conclude that the 5′ regions of both genes substantially account for the phytochrome-dependent regulation.

In comparison to the −1138 and −389 *NPR2* promoter constructs, the −508 promoter resulted in a significant reduction of reporter gene expression, without loss of phytochrome-dependent regulation. One possible interpretation of these data is that activating sequences are located upstream as well as downstream of −508 and that a "silencer" is present between −508 and −389. These results might also be explained by the unmasking of cryptic elements or by altered DNA topology in the deletion constructs.

The *NPR2* promoter segment spanning −259 to −88 contains the region required for negative phytochrome regulation defined in the transient expression assay but did not confer negative phytochrome regulation when used in place of a positive promoter region in the *Lemna* AB19 promoter (Fig.

7). One possibility for the absence of phytochrome responsiveness in this construct is that the *NPR2* segment is substantially larger than the region of the AB19 promoter it replaces and that spacing between regulatory elements within the *NPR2* segment and adjacent sequences may be critical for either negative or positive regulation (cf. Gilmartin and Chua, 1990). It is also possible that phytochrome action requires both sequences in the *NPR2* −208 to −82 segment and sequences downstream of −82 that are not present in the AB19 promoter.

More rigorous definition of the functional elements of the *NPR1* and *NPR2* promoters will help determine whether the similarities they share have significance in vivo. Although the deletion analysis suggested that there is a negative element involved in phytochrome responsiveness, both promoters also contain ubiquitous G box-like motifs and other elements found in positively phytochrome-regulated genes. Therefore, the action of phytochrome on differential gene transcription might be specified at the level of regulatory factor binding or complex formation rather than solely through the presence of a negative phytochrome element. In addition, further analysis of the NPR promoters might reveal novel regulatory elements or factors.

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LITERATURE CITED

Baker J, Steele C, Dure L III (1988) Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Mol Biol **11:** 277–291

Bruce WB, Christensen AH, Klein T, Fromm M, Quail PH (1989) Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. Proc Natl Acad Sci USA **86:** 9692–9696

Bruce WB, Deng X-W, Quail PH (1991) A negatively acting DNA sequence element mediates phytochrome-directed repression of *phyA* gene transcription. EMBO J **10:** 3015–3024

Bustos MM, Guiltinan MJ, Cyr RJ, Ahdoot D, Fosket DE (1989) Light regulation of β-tubulin gene expression during internode development in soybean (*Glycine max* [L.] Merr.). Plant Physiol. **91:** 1157–1161

Buzby JS, Yamada T, Tobin EM (1990) A light-regulated DNA-binding activity interacts with a conserved region of a *Lemna gibba rbcS* promoter. Plant Cell **2:** 805–814

Castresana C, Garcia-Luque I, Alonso E, Malik VS, Cashmore AR (1988) Both positive and negative regulatory elements mediate expression of a photoregulated *CAB* gene from *Nicotiana plumbaginafolia.* EMBO J **7:** 1929–1936

Chen Z-Y, Hsing YC, Lee P-F, Chow T-Y (1992) Nucleotide sequences of a soybean cDNA encoding a 18 kilodalton late embryogenesis abundant protein. Plant Physiol **99:** 773–774

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol Biol **13:** 95–108

Colbert JT, Costigan SA, Zhao Z (1990) Photoregulation of β-tubulin mRNA abundance in etiolated oat and barley seedlings. Plant Physiol **93:** 1196–1202

Datta N, Cashmore AR (1989) Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell **1:** 1069–1077

Davidson I, Fromental C, Augereau P, Wildeman A, Zenke M, Chambon P (1986) Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. Nature **323:** 544–548

Dehesh K, Bruce WB, Quail PH (1990) A trans-acting factor that binds to a GT-motif in a phytochrome gene promoter. Science **250:** 1397–1399

de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol **7:** 725–737

Dure L III, Crouch M, Harada J, Tuan-Hua DH, Mundy J, Quatrano R, Thomas T, Sung ZR (1989) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol **12:** 475–486

Eyal Y, Sagee O, Fluhr R (1992) Dark-induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene. Plant Mol Biol **19:** 589–599

Ferl RJ, Laughner BH (1989) *In vivo* detection of regulatory factor binding sites of *Arabidopsis thaliana* Adh. Plant Mol Biol **12:** 357–366

Ferl RJ, Nick HS (1987) *In vivo* detection of regulatory factor binding sites in the 5′-flanking region of maize Adh1. J Biol Chem **262:** 7947–7950

Forde BG, Freeman J, Oliver JE, Pineda M (1990) Nuclear factors interact with conserved A/T-rich elements upstream of a nodule-enhanced glutamine synthetase gene from French bean. Plant Cell **2:** 925–939

Galau GA, Close TJ (1992) Sequences of the cotton group 2 LEA/RAB/dehydrin proteins encoded by *Lea* 3 cDNAs. Plant Physiol **98:** 1523–1525

Gilmartin PM, Chua N-H (1990) Spacing between GT-1 binding sites within a light-responsive element is critical for transcriptional activity. Plant Cell **2:** 447–455

Gilmartin PM, Sarokin L, Memelink J, Chua N-H (1990) Molecular light switches for plant genes. Plant Cell **2:** 369–378

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA **85:** 7089–7093

Godoy JA, Pardo JM, Pintor-Toro JA (1990) A tomato cDNA inducible by salt stress and abscisic acid: nucleotide sequence and expression pattern. Plant Mol Biol **15:** 695–705

Green PJ, Yong M-H, Cuozzo M, Kano-Murakami Y, Silverstein P, 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

Guiltinan MJ, Marcotte WR Jr, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250:** 267–271

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Joshi CP (1987a) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res **15:** 6643–6653

Joshi CP (1987b) Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. Nucleic Acids Res **15:** 9627–9640

Karlin-Neumann GA, Kohorn BD, Thornber JP, Tobin EM (1985) A chlorophyll a/b-protein encoded by a gene containing an intron with characteristics of a transposable element. J Mol Appl Genet **3:** 45–61

Kay SA, Keith B, Shinozaki K, Chye M-L, Chua N-H (1989) The rice phytochrome gene: structure, autoregulated expression, and binding of GT-1 to a conserved site in the 5′ upstream region. Plant Cell **1:** 351–360

Kehoe DM (1992) Phytochrome regulation of *Lemna gibba cab*AB19 transcription. PhD thesis. University of California, Los Angeles

Kozak M (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res **15:** 8125–8148

Lam E, Chua N-H (1989) ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in *cab* promoters. Plant Cell **1:** 1147–1156

Manzara T, Carrasco P, Gruissem W (1991) Developmental and organ-specific changes in promoter DNA-protein interactions in the tomato *rbcS* gene family. Plant Cell **3:** 1305–1316

Marcotte WR Jr, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the Em gene of wheat. Plant Cell **1:** 969–976

Maxam AM, Gilbert W (1980) Squencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol **65:** 499–560

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell **2:** 163–171

Mundy J, Chua N-H (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J **7:** 2279–2286

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice rab gene. Proc Natl Acad Sci USA **87:** 1406–1410

Oeda K, Salinas J, Chua N-H (1991) A tobacco bZip transcription activator (TAF-1) binds to a G-box-like motif conserved in plant genes. EMBO J **10:** 1793–1802

Ohme M, Tanaka M, Chunwongse J, Shinozaki K, Sugiura M (1986) A tobacco chloroplast DNA sequence possibly coding for a polypeptide similar to E. coli RNA polymerase beta-subunit. FEBS Lett **200:** 87–90

Okubara PA (1992) The characterization of four genes negatively regulated by the action of white light or red light in *Lemna gibba*. PhD thesis. University of California, Los Angeles

Okubara PA, Tobin EM (1991) Isolation and characterization of three genes negatively regulated by phytochrome action in *Lemna gibba*. Plant Physiol **96:** 1237–1245

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA **85:** 2444–2448

Piatkowski D, Schneider K, Salamini F, Bartels D (1990) Characterization of five abscisic acid-responsive cDNA clones isolated from the dessication-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. Plant Physiol **94:** 1682–1688

Pustell J, Kafatos FC (1982) A convenient and adaptable package of DNA sequence analysis programs for microcomputers. Nucleic Acids Res **10:** 51–59

Raynal M, Gaubier P, Grellet F, Delseny M (1990) Nucleotide sequence of a radish cDNA clone coding for a late embryogenesis abundant (LEA) protein. Nucleic Acids Res **18:** 6132

Riggs CD, Chrispeels M (1987) Luciferase reporter gene cassettes for plant gene expression studies. Nucleic Acids Res **15:** 8115

Rolfe SA, Tobin EM (1991) Deletion analysis of a phytochrome-regulated monocot *rbcS* promoter in a transient assay system. Proc Natl Acad Sci USA **88:** 2683–2686

Sambrook J, Fritsch EF, Maniatis T (1989) Mapping RNA with nuclease S1. *In* Molecular Cloning, A Laboratory Manual, Ed 2 Vol 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schindler U, Cashmore AR (1990) Photoregulated gene expression may involve ubiquitous DNA binding proteins. EMBO J **9:** 3415–3427

Schulze-Lefert P, Dangl JL, Becker-Andre M, Hahlbrock K, Schulz W (1989) Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. EMBO J **8:** 651–656

Shapiro MB, Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res **15:** 7155–7174

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Tabata T, Nakayama T, Mikami K, Iwabuchi M (1991) HBP-1 and HBP-2: leucine zipper-type transcription factors of wheat. EMBO J **10:** 1459–1467

Thompson WF, White MJ (1991) Physiological and molecular studies of light-regulated nuclear genes in higher plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 423–466

Tomizawa K, Sato N, Furuya M (1989) Phytochrome control of multiple transcripts of the phytochrome gene in *Pisum sativum*. Plant Mol Biol **12:** 295–299

Tsai F-Y, Coruzzi G (1991) Light represses transcription of asparagine synthetase genes in photosynthetic and nonphotosynthetic organs of plants. Mol Cell Biol **11:** 4966–4976

Vilardell J, Goday A, Freire MA, Torrent M, Martinez MC, Torne JM, Pages M (1990) Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. Plant Mol Biol **14:** 423–432

Watson JC (1989) Photoregulation of gene expression in plants. *In* S-D. Kung, CJ Arntzen, eds, Plant Biotechnology. Butterworths, Boston, pp 161–205

Weisshaar B, Armstrong GA, Block A, da Costa e Silva O, Hahlbrock K (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J **10:** 1777–1786

Yamaguchi-Shinozaki K, Mundy J, Chua N-H (1990) Four tightly-linked rab genes are differentially expressed in rice. Plant Mol Biol **14:** 29–39

Zinn K, DiMaio D, Maniatis T (1983) Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34:** 865