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Isolation and Characterization of Three Genes Negatively Regulated by Phytochrome Action in *Lemna gibba*[1]

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ABSTRACT

We have isolated three distinct cDNA clones from *Lemna gibba* representing mRNAs that increase in abundance during dark treatment. All three mRNAs showed reduced expression in response to red or white light. These mRNAs range from approximately 680 to 800 nucleotides in length and thus encode relatively small proteins (maximum relative molecular weight 17,000 to 19,000). The genes corresponding to these dark-abundant mRNAs are designated NPR (negatively phytochrome regulated) 1, 2, and 3. Differences in the rapidity of mRNA accumulation during dark treatment were observed for each of the genes in both mature green plants and in etiolated plants. Differences in accumulation pattern were also observed in etiolated plants, depending on whether the plants received a far-red light treatment prior to darkness. Transcription of all three genes, assayed in nuclei isolated from either green or etiolated plants, increased during dark treatment. In etiolated plants, a single 2 minute red light treatment caused a detectable decrease in the transcription of the genes after the dark treatment, and 10 minutes of far-red light given immediately after the red light resulted in a reversal of the effect of red light. Additionally, treatment of the plants with far-red light prior to darkness resulted in greater rates of transcription of the NPR genes. Therefore, we conclude that phytochrome action results in decreased transcription of these NPR genes. Each of the NPR mRNAs are encoded by one to two genes.

Development, growth, and metabolism in higher plants involves complex pathways leading from external stimuli reception or endogenous triggers to biochemical, cellular, and ultimately organismal responses. Normal development requires that processes be both activated and inhibited at the appropriate times and in the appropriate cells or tissues. Indeed, analyses of DNA regulatory sequences indicate the presence of both positive and negative regulatory elements upstream of several plant genes, including the *cab*[2] genes, which encode the major light-harvesting protein of PSII (2, 28); genes encoding the small subunit of ribulose-1,5 bisphosphate carboxylase (12, 35); a phytohemagglutinin gene (21); and genes for chalcone synthase (6) and for phenylalanine ammonia-lyase (16). A gene might be positively regulated in one cellular or biochemical context and negatively regulated in a different context by other molecular mechanisms, including the differential binding of activators and repressors to a set of regulatory elements (14, 20), through a regulatory protein that can function as both activator and repressor (14, 27), or through a *cis*-acting element that functions as both enhancer and silencer (*e.g.* 5, 12, 14, 27).

Phytochrome is an important developmental photoreceptor that affects numerous and diverse physiological and biochemical processes in higher plants. Phytochrome can have an important role in seed germination, suppression of stem elongation, flowering, and senescence. A number of cellular and biochemical processes have been reported to be regulated by phytochrome action, including rapid changes in membrane permeability (9, 15), changes in calcium ion levels (22), and changes in the activity of many enzymes (reviewed in refs. 13 and 25). One major biochemical change that can be affected by phytochrome action is the level of specific RNAs. Phytochrome might regulate specific mRNA levels by acting either on transcription or on posttranscriptional processes. Phytochrome action has been shown to cause transcriptional increases in the *cab* genes and in genes for the small subunit of ribulose 1,5-bisphosphate carboxylase (reviewed in refs. 7 and 36) and phospho*enol*pyruvate carboxylase (29). Phytochrome action also can cause an increase in mRNA levels of chalcone synthase and ferredoxin (reviewed in ref. 36), glutamine synthetase (23, 30), nitrate reductase (18), and thionins (19). Negative regulation of gene expression by phytochrome has been demonstrated for several phytochrome genes and for an NADPH-protochlorophyllide oxidoreductase gene (4, 34, reviewed in 36), and for an asparagine synthetase gene (7). Recently, negative regulation of β-tubulin mRNA by white light or red light has also been demonstrated (1, 3).

To understand how phytochrome causes changes in transcription in higher plants, it is important to examine not only genes that are transcriptionally activated but also genes that show reduced transcription in response to the action of phytochrome. Therefore, we have isolated three distinct genes from *Lemna gibba* that are negatively regulated by phytochrome and have characterized the transcriptional responses of these genes to red and far-red light.

MATERIALS AND METHODS

Growth and Harvest of Plants

Lemna gibba L. G-3 was grown on liquid E medium (31) in continuous W or under an IR regimen consisting of 2 min

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[2] Abbreviations: *cab*, Chl *a*/*b*-protein; NPR, negatively phytochrome-regulated; FR, far-red light; IR, intermittent red light; R, red light; W, white light.

R every 8 h as previously described (31). IR plants were grown on E medium supplemented with 3×10^{-6} M kinetin (Sigma K-2875). FR treatment of W-grown plants consisted of 10 min of FR given immediately before placement in darkness. For nuclei isolation and run-on transcription experiments, IR-grown plants were given 10 min of FR immediately following the final R treatment and just prior to placement in the dark. IR-grown plants for Northern analyses were given 10 min FR 2 h after receiving the final R treatment. W-grown plants were harvested in laboratory light and IR-grown plants were harvested under a dim green safelight. Dark-treated plants were harvested in complete darkness for RNA isolation and under a dim green safelight for isolation of nuclei. W-grown *Lemna* cultures were 2.5 to 3 weeks old and IR-grown cultures were 4 to 5 weeks old at the time of harvest.

cDNA Library Construction and Screening for Negatively W-Regulated mRNAs

RNA was isolated from *Lemna* grown either in continuous W or in IR, then placed in darkness for 4 d. Poly(A) RNA was obtained from both kinds of RNA by fractionation on an oligo (dT) cellulose column (10) and combined in equal amounts for cDNA synthesis. cDNA was synthesized essentially as described by Huynh *et al.* (8). First strand synthesis was carried out with 100 to 120 ng/μL of poly(A) RNA and 20 units/μL Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories) in the presence of 40 ng/μL oligo$(dT)_{12-18}$ (Pharmacia) and 200 ng/μL random primers [$pd(N)_6$ (Pharmacia)] at 37°C for 1 h. Second strand synthesis was carried out using 0.16 units/μL *Escherichia coli* DNA polymerase (Boehringer Mannheim) and 0.012 units/μL RNase H (Bethesda Research Laboratories) at 14°C for 2 h. Efficiency of strand synthesis was estimated by incorporation of radiolabeled [α-^{32}P]dGTP. Doubled-stranded cDNA was treated with T4 DNA polymerase (0.03 units/μL, Bethesda Research Laboratories), polynucleotide kinase (0.45 units/μL, Boehringer Mannheim), and *Eco*RI methylase (0.4 units/μL, New England Biolabs) before ligation to *Eco*RI oligonucleotide linkers (GGAATTCC, Colloborative Research). cDNAs ranging in length from 0.3 to 1.2 kilobase pairs were size-selected by elution from a 3 mm (i.d.) × 175 mm Sepharose 4B column. cDNAs were cloned into *Eco*RI-cleaved Lambda Zap I phage vector DNA (1 μg, Stratagene). Ligation products were packaged with Gigapack Plus 10 packaging extracts (Stratagene) and mobilized by transfection into *E. coli* strain BB4 (24). The titer of recombinants was estimated on the basis of the colorimetric isopropyl-β-D-thiogalactopyranoside assay (24, Stratagene Lambda Zap I protocol).

Plaque lifts for differential screening were done by the method of Benton and Davis (24). cDNA probes were made using either 2.5 μg poly(A) RNA from W-grown plants or 2.5 μg poly(A) RNA from IR-grown plants given 10 min FR prior to a 4 d dark treatment. The cDNA was labeled with 250 μCi (1 Ci = 3.7×10^{10} Bq) of [α-^{32}P]dCTP (DuPont-NEN, 800 Ci/mmol) to a specific activity of 2.5 to 5×10^7 cpm/μg. Hybridizations were carried out with 10^6 cpm/mL radiolabeled cDNA in 50% formamide, 5× SSC (24), 5× Denhardt's solution (24), 250 μg/mL herring sperm DNA, sheared and denatured, 50 mM sodium phosphate, pH 7.0, and 0.1% (w/v) SDS at 42°C for 3 d. Approximately 10^4 recombinants were screened. Excision of cloned cDNAs from the phage vector and transformation of *E. coli* strain XL1-Blue was done as recommended by Stratagene. Rescued plasmids were mobilized into *E. coli* strain HB101 (24) for subsequent use. cDNA clones selected in the screen were further tested by Northern analysis to identify those that represented mRNAs negatively regulated by red light.

Northern Blot Analyses

For identification of NPR clones, relative abundances of the mRNAs in light-grown and dark-treated plants were determined by Northern blot analysis. cDNA inserts were excised from the vector using *Eco*RI or *Pvu*II restriction endonuclease, isolated by low gel temperature agarose electrophoresis and purified by adsorption onto silica beads (GeneClean, BIO 101, Inc., La Jolla, CA). Isolated cDNAs were labeled by nick translation (Bethesda Research Laboratories Nick Translation Kit) using [α-^{32}P]dCTP (DuPont-NEN, 800 Ci/mmol) to a specific activity of 5×10^7 cpm/μg. Ten micrograms of total RNA (17) isolated from W-grown, IR-grown, and dark-treated plants given 10 min FR prior to 4 d darkness was fractionated on 1% agarose gels containing 6% formaldehyde (24), then transferred to Zeta Probe nylon membrane by capillary blotting in 10× SSC and hybridized 42°C for 60 to 72 h in 330 mM sodium phosphate, pH 6.9, 2.5 mM EDTA, and 400 μg/mL herring sperm DNA as recommended by the supplier (Bio-Rad). Filters were washed three times in 2× SSC, 0.1% SDS at room temperature, then three times in 0.1× SSC, 0.1% SDS at 57 to 58°C.

For characterization of NPR mRNA accumulation, total RNA was isolated from IR-grown *Lemna,* with and without 10 min FR treatment, or from plants grown in W light. Plants were placed in darkness for up to 5 d prior to harvest and RNA isolation. Total RNA was subjected to electrophoresis as described above and transferred to Zeta Probe nylon membrane in 50 mM NaOH for 2.5 h as recommended by Bio-Rad Laboratories. The cDNA portions of the plasmids were excised from vector DNA with *Bam*HI plus *Hin*dIII (NPR1), *Pvu*II (NPR2), or *Pst*I plus *Sal*I (NPR3). Probes consisted of 10 to 25 ng gel-purified cDNA inserts radiolabeled by random primer extension (24) using 30 μCi [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol). Specific activity of probes were 10^8 to 2×10^9 cpm/μg. Hybridization was conducted for 25 to 42 h at 42°C. Washes were done as described above or in single successive washes of 2× SSC, 0.1% SDS and 0.5× SSC, 0.1% SDS at room temperature, followed by 0.1× SSC, 0.1% SDS at 57 to 58°C. Autoradiography was done with preflashed Kodak XAR-5 x-ray film and Cronex Quanta IIB or Quanta III intensifying screens (DuPont) at −70°C for up to 10 d. Quantitation of autoradiographs was done using a BBC microcomputer and Hoefer GS300 scanning densitometer.

Construction of *Lemna* Genomic Library

A genomic library of *Lemna gibba* was constructed in the lambda phage vector Charon 35 (24). Genomic DNA from 3-week-old W-grown *Lemna* cultures was isolated (17) and

digested to completion with *Bam*HI restriction endonuclease (10 units/μL, Bethesda Research Laboratories), or partially digested with *Bam*HI and *Sau*3A I restriction endonucleases (8 units/μL, Bethesda Research Laboratories). Digestion products were pooled and fractionated on 10 to 40% sucrose gradients (24). DNA fragments of 15 to 20 kilobase pairs in size were recovered for cloning into *Bam*HI-cleaved Charon 35 vector DNA. Ligation products were packaged with Gigapak Plus Packaging Extract (Stratagene).

Isolation of NPR Genomic Clones and Subclones

E. coli strain K802 (24) was transfected with packaged ligation products. Approximately 4×10^5 plaque-forming units were screened (24). cDNA probes were made as described in "Northern Blot Analysis." Two genomic clones of NPR1, four clones of NPR2, and two clones of NPR3 were subsequently mapped using restriction enzymes and by partial restriction endonuclease digestion of 5′ end-labeled DNA (24). Genomic DNA restriction fragments that hybridized to nuclear transcripts from dark-treated *Lemna* were subcloned into plasmid vectors pBR322 or pGEM-3Z (Promega) for use as probes in nuclear run-on transcription assays.

AB30R and Lg106 Clones

A 1.3 kilobase pair *Sst*I-*Hin*dIII genomic *Lemna* AB30 (*cab*) DNA fragment was excised from SP65AB30, an existing clone (11), and ligated to pGEM3Z vector DNA. The new clone, called AB30R, carried approximately 60 base pairs of 5′ untranslated region of the AB30 gene as well as the entire coding and 3′ untranslated regions. About 300 base pairs of 5′ flanking DNA have been removed in AB30R to eliminate potential hybridization to transcripts not of the *cab* gene family. The isolation of a genomic clone of *Lg106*, a *Lemna* gene of unknown function, was described previously (17).

Nuclear Run-On Transcription Assays

Lemna nuclei and *in vitro* radiolabeled transcripts were obtained by a modified method of Luthe and Quatrano (17). Purified transcripts were resuspended in phosphate-EDTA hybridization buffer at a uniform concentration of 2 to 4×10^6 cpm/mL. For use as probes for NPR-specific transcripts, cloned NPR genomic DNA (approximately 60 ng each per assay) was excised from vector DNA by restriction endonucleases, partitioned on agarose gels, then transferred to Zeta Probe nylon membrane by the method of Southern (24). Prehybridization and hybridization were conducted in 50% formamide, 240 mM NaCl, 13 mM Na_2HPO_4, 1.3 mM EDTA, and 100 to 200 μg/mL *E. coli* transfer RNA (Sigma R-1753) as recommended by Bio-Rad Laboratories, at 55°C for 21 to 28 h. Filters were washed in 2×, 0.5×, and 0.1× SSC with 0.1% SDS as described under "Northern Blot Analysis," except the final wash was done at 55°C. Hybrid-selected transcripts were localized by autoradiography at −70°C for up to 3 d. The transcripts were quantitated by liquid scintillation counting (Ecoscint cocktail, National Diagnostics, Manville, NJ) of uniform sections cut from the filters.

Genomic Southern Analysis

Lemna genomic DNA (17) was digested with *Bam*HI or *Hin*dIII (10 units/μL, Bethesda Research Laboratories). A 2 to 4 μg sample of each digest was subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to cDNA fragments radiolabeled by random primer extension as described in "Northern Blot Analysis." Autoradiography was carried out at −70°C for 4 to 7 d.

RESULTS

Isolation of NPR cDNA Clones

Thirty-four phage cDNA clones corresponding to mRNAs more abundant in dark-treated *Lemna* than in W-grown plants were obtained by differential screening of a cDNA library. Four of these clones, identified from Northern blot analysis, represented mRNAs that were more abundant in dark-treated plants than in IR-grown plants. Of these, two clones with no apparent nucleotide sequence similarity hybridized to identical restriction endonuclease-generated DNA fragments in phage clone blots (data not shown). It is possible that these two cDNAs are encoded by adjacent but unrelated genes located on the same genomic fragment. However, the cDNAs were 500 base pairs and 170 base pairs in length, and could represent different portions of the same 720 nucleotide mRNA (NPR3). Both cDNAs hybridized to a 720 nucleotide mRNA in Northern blots, and the shorter of the two cDNAs hybridized a subset of genomic fragments that showed homology to the longer cDNA in genomic Southern blots; therefore, it is likely that the clones either represent the same gene or two members of a gene family. The longer of the two cDNAs was used in subsequent studies. Thus, cDNA clones representing at least three distinct gene families were obtained, and were designated NPR1, NPR2, and NPR3. The expression of the corresponding genes was further characterized to determine if regulation was occurring at the transcriptional level and if the regulation was attributable to phytochrome action.

Accumulation of NPR mRNAs in Darkness

Figure 1 shows time courses of accumulation of NPR1, NPR2, and NPR3 mRNAs from plants grown either in W (Fig. 1A) or in IR (Fig. 1B). All three mRNAs were barely detectable in Northern blots of total RNA from plants grown in continuous W. The mRNAs increased in these plants during 5 d of darkness. Various exposures of the autoradiograph shown in Figure 1A were quantitated for Figure 2A. In IR-grown plants given 10 min FR prior to darkness, detectable increases in the levels of NPR1 and NPR2 were observed within 6 h darkness, and within 12 h darkness for NPR3 (Fig. 1B). A maximum level of NPR2 mRNA was reached between 2 and 6 h in the dark. The transient decrease in mRNA levels at 2 h darkness, also seen in a second experiment, could not be explained by unequal amounts of total RNA in the gels used for the blots. The mature NPR1, NPR2, and NPR3 mRNAs are approximately 800, 680, and 720 nucleotides, respectively.

Under the IR regimen, *Lemna* plants were yellow in color

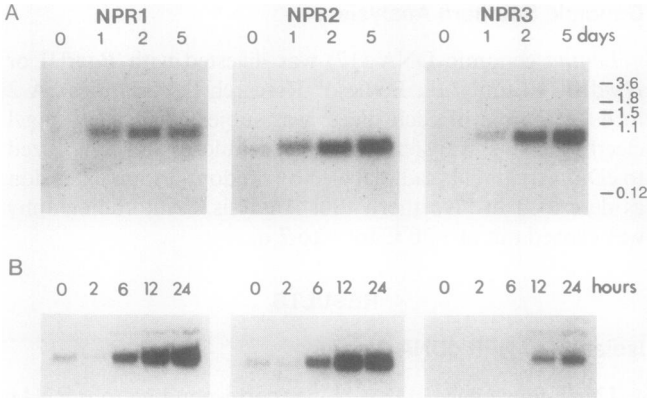

Figure 1. Northern blot analyses of NPR mRNA accumulation during 5 d darkness in W-grown plants (A) and 24 h darkness in IR-grown plants given 10 min FR prior to the dark (B). Ten micrograms of total RNA was used per time point. A, Specific activities of the probes were 2.2×10^5 cpm/ng, 7.6×10^5 cpm/ng, and 1.6×10^5 cpm/ng for NPR1, NPR2, and NPR3, respectively. Autoradiograph of NPR1 and NPR3 was exposed for 7 d at −70°C and that of NPR2 at −70°C for 10 d. Size markers (nucleotides $\times 10^3$) correspond to *Lemna* ribosomal RNAs. B, Specific activities of the probes were 2.1×10^6 cpm/ng for NPR1 and NPR3 and 1.8×10^5 cpm/ng for NPR2. The autoradiograph was exposed for 5 d at −70°C.

and contained undifferentiated plastids, hence were in an etiolated state of development (31). W-grown *Lemna* were green and contained fully-differentiated plastids. To determine how the NPR mRNAs accumulated during dark treatment in both types of plants, the relative mRNA levels from plants placed in darkness 0 to 5 d were obtained from Northern blot analyses.

The mRNA levels of all three NPR genes increased during a 5 d dark period in both mature W-grown and etiolated IR-grown plants (Fig. 2). After 1 d (24 h) in darkness, the level of NPR1 mRNA reached a maximum in W-grown and in IR-grown plants given 10 min FR (Fig. 2A, B). In contrast to NPR1, the levels of NPR2 and NPR3 mRNA in W-grown *Lemna* increased between 2 d and 5 d of dark treatment. In IR-grown plants with or without FR, however, both mRNAs appeared to have reached a steady-state level by 2 d in the dark (Fig. 2B, C). In almost every case, a small amount of mRNA was detectable in plants grown under both the W or IR regimens prior to dark treatment.

FR irradiation given to etiolated plants prior to dark treatment generally accelerated the accumulation of NPR mRNAs. NPR1 mRNA abundance reached maximum levels at 1 d, about 24 h more rapidly in IR-grown plants given FR as compared to plants without FR (Fig. 2B, C). FR treatment resulted in a similar change in the accumulation of NPR2 mRNA. Although FR did not appear to alter the time required for NPR3 mRNA to reach steady-state levels in IR-grown plants, it increased the relative amount of the message that accumulated after 1 d in darkness.

The temporal changes in mRNA levels of each of the genes during the dark treatment were found to be consistent in subsequent Northern analyses. The relative RNA levels were determined directly from densitometry scan data and cor-

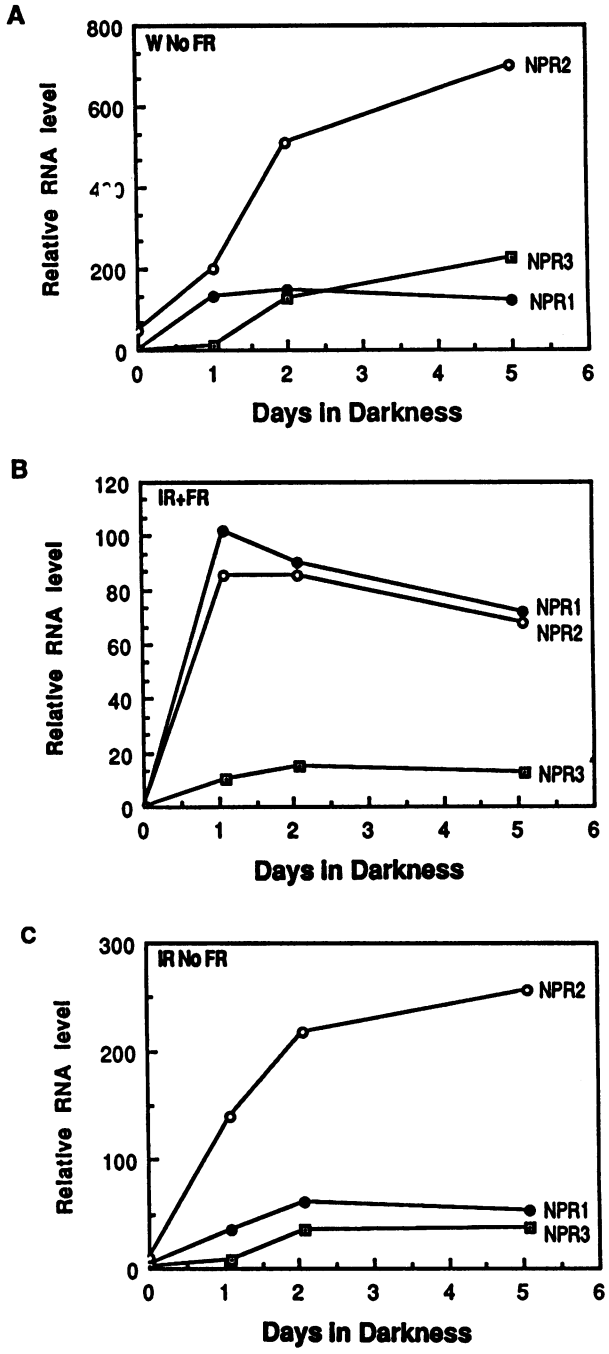

Figure 2. Accumulation of NPR1, NPR2, and NPR3 mRNAs during a 5-d dark treatment period. A, RNA from plants grown in continuous W without FR treatment prior to darkness (W No FR). B, RNA from plants grown in IR with an FR treatment prior to darkness (IR + FR). C, RNA from IR-grown plants without FR prior to darkness (IR No FR). Time in darkness for IR-grown plants is indicated with respect to the final R treatment. Ten micrograms of total RNA was used per time point. Relative RNA levels represent arbitrary optical transmittance values obtained by densitometric scanning of Northern blot autoradiographs from a single representative experiment.

rected for exposure time and the amount of radioactivity incorporated into each probe. However, the units shown are arbitrary, and the relative RNA levels cannot be directly compared between experiments.

Transcriptional Regulation of NPR Genes

To determine if the increases in NPR mRNAs observed during dark treatment were due to changes in transcription, *in vitro* nuclear run-on transcription assays were performed. Relative levels of transcription of each of the NPR genes in nuclei isolated from IR-grown *Lemna* during a dark treatment are shown in Figure 3A–C. For all three NPR genes, increases in the amounts of radiolabeled transcripts hybridizing to cloned genomic sequences occurred between 6 and 12 h of darkness.

Genomic clones were used for hybrid selection of the transcripts because they are longer than the corresponding cDNA clones, and thus more extensive hybridization of the labeled transcripts could be obtained. The NPR1 and NPR2 genomic clones contained entire transcribed regions; the NPR3 genomic clone contained approximately two-thirds of the transcribed region, estimated from hybridization to nuclear transcripts and S1 analyses (data not shown). The complete nucleotide sequences of the NPR genes are not yet known. Therefore, the radioactivity incorporated into transcripts could not be used to determine quantitatively rates of transcription.

FR treatment of IR-grown *Lemna* resulted in a greater and more rapid increase of transcription during the dark treatment. After 12 h of darkness, the rates of transcription of all three NPR genes were consistently higher in plants given FR than in plants placed in the dark without FR. These results suggested that the biologically active form of phytochrome (P_{fr}) acts to inhibit the transcription of the NPR genes. Because plants used for these experiments were grown under uniform conditions, and nuclear run-on transcription assays for both FR-treated and untreated plants were done simultaneously, the transcription rates obtained for a specific NPR gene with and without FR could be directly compared.

Cab transcription was measured to observe the effects of the light treatments on a gene positively regulated by phytochrome, and *Lg106* was used as a standard for light- and phytochrome-independent transcriptional regulation. Expression of the *cab* gene family was monitored with a genomic DNA clone (AB30R). The level of transcription of the *cab* genes increased 2 h after a final R treatment followed immediately by FR (Fig. 3D). The rate of transcription appeared to

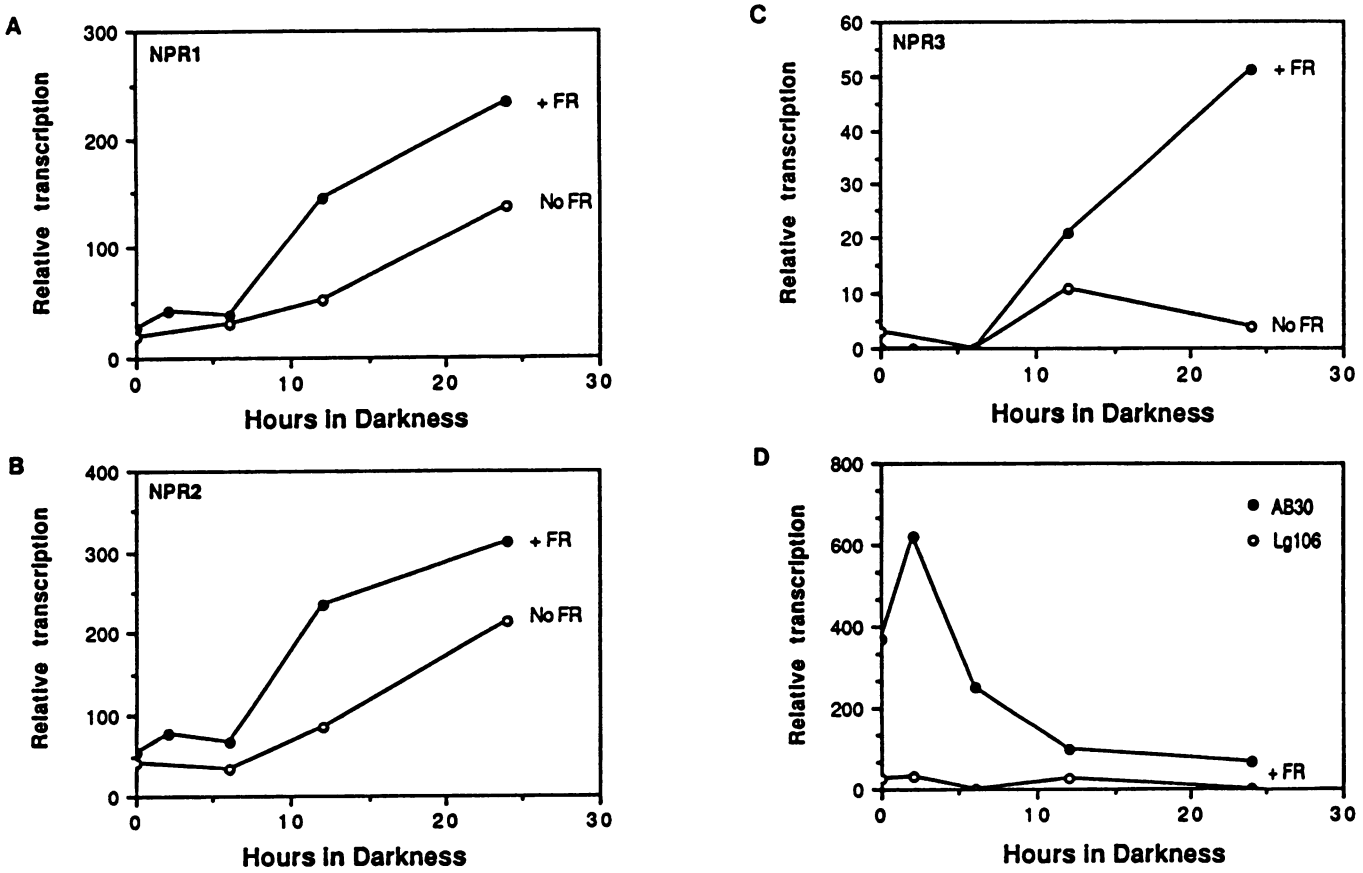

Figure 3. Transcription of NPR1 (A), NPR2 (B), NPR3 (C), and *cab* and *Lg106* (D) during a 24-h dark treatment period. *In vitro* radiolabeled nuclear transcripts were obtained from IR-grown plants placed in darkness for up to 24 h immediately after the final R treatment. Plants were placed in the dark without FR (No FR) or given FR prior to darkness (+ FR). The cpm obtained in hybrid-selected transcripts from a single experiment is expressed as Relative transcription.

reach a minimum level by 12 h of darkness (Fig. 3D). Although the *Lg106* level was relatively low compared with the other transcripts, virtually no variability in *Lg106* transcription was observed in autoradiographs under all the light treatments used here (17 and Figs. 3D, 4D, 5D), so normalization to *Lg106* was not necessary in each experiment.

Variation in sample measurements was also determined by quantitating the amounts of NPR1, NPR2, and NPR3 hybrid-selected transcripts obtained in three separate nuclei preparations (Table I). For this experiment, IR-grown plants were given 10 min FR, placed in darkness for 4 d, given 2 min of R, then returned to the dark for 2 h. Run-on transcription assays of all three nuclei preparations were performed simultaneously. The average deviation for the NPR gene transcripts ranged from about 7 to 12%, and from 11 to 16% for the *Lg106* and AB30R transcripts.

R-Induced Reduction of NPR Transcription

The levels of NPR transcription obtained after a 4 d dark treatment could be reduced by a single 2 min R treatment for each of the genes (Fig. 4A–C). The reduction of transcription could be observed within 2 h after the R treatment, and after 4 h, the levels of transcription were about 50% of the dark-treated plants receiving no R. As expected for *cab*, a transient increase in transcription was observed in nuclei from IR-grown plants 2 to 4 h after the R treatment (Fig. 4D).

Regulation of NPR Transcription by Phytochrome

To determine whether phytochrome was involved in the transcriptional regulation of the NPR genes, the effect on transcription of either a single R treatment or single R followed by FR was examined. The R-induced decrease in transcription was reversible by FR given immediately after the R for all three of the NPR genes (Fig. 5A–C). Nuclei were isolated from plants 4 h after the light treatments. Levels of transcription were normalized to the rates obtained in plants placed in darkness for 4 d, but receiving no light treatment. The results of two separate experiments were averaged and the ranges are shown by the vertical bars. Relative levels of transcription of each of the NPR genes decreased between 40 and 60% in response to a single R treatment. Furthermore, FR given immediately after the R resulted in a substantial

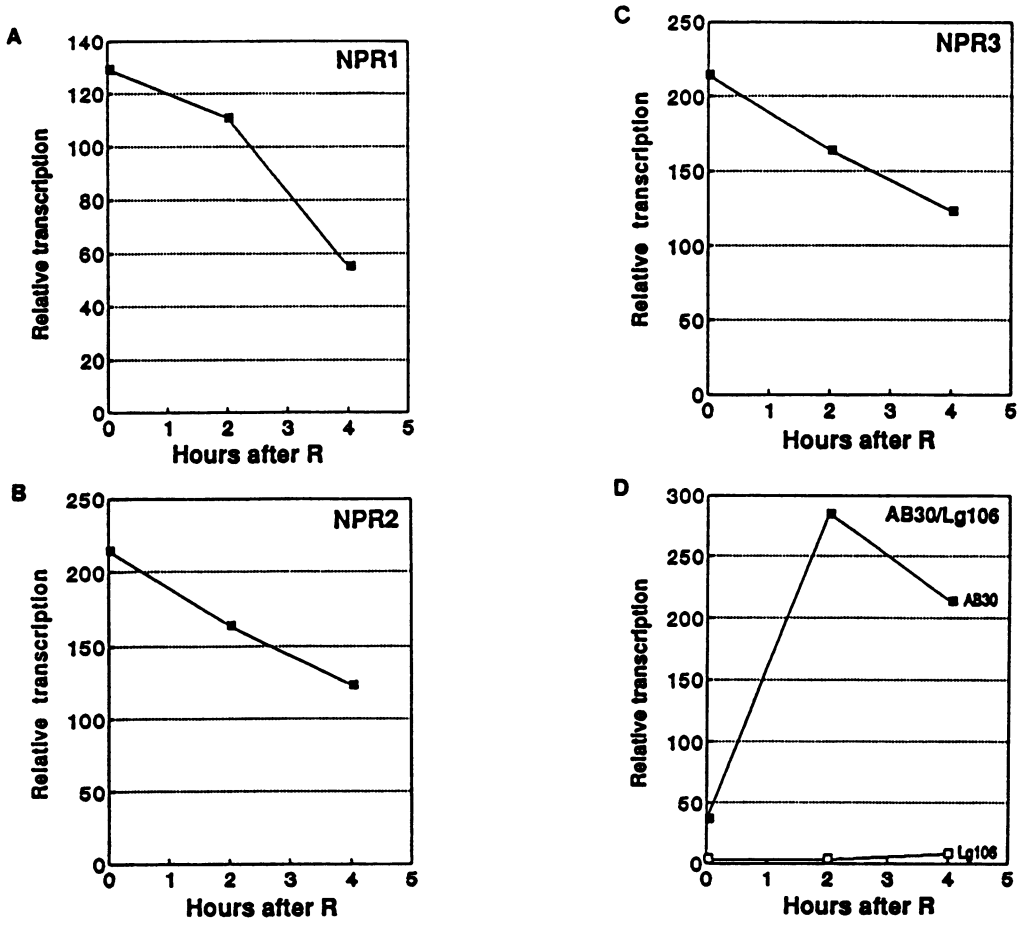

Figure 4. Transcription of NPR1 (A), NPR2 (B), NPR3 (C), and *cab* and *Lg106* (D) following R treatment. IR-grown plants were given 10 min FR, placed in darkness for 4 d, given 2 min of R, then returned to darkness. *In vitro*-radiolabeled transcripts were isolated from nuclei obtained prior to R treatment (0 h), or 2 and 4 h after R treatment. The cpm obtained in hybrid-selected transcripts from a single representative experiment is expressed as Relative transcription.

reversal of the R-induced reduction in transcription. Thus, phytochrome is involved in the regulation of NPR gene transcription. The R-induced transcriptional increase of the *cab* genes was reversed by FR (Fig. 5D).

Sizes of NPR Gene Families

Southern blot analysis was used to estimate the sizes of the NPR gene families. The results shown in Figure 6 suggest that NPR1 and NPR3 are encoded by two genes in *Lemna gibba,* and that NPR2 is encoded by a single gene. Additional supporting evidence for this estimation is provided by restriction enzyme mapping of genomic subclones of the NPR genes (data not shown) and the partial sequences of the cDNA clones. NPR1 genomic clones containing the coding region and approximately 3 kilobases each of 5′ and 3′ flanking sequences have no *Bam*HI or *Hin*dIII restriction sites. This suggests that each of the genomic fragments that hybridize to the NPR1 cDNA represent two different copies of the gene. Four independently isolated NPR2 genomic clones were identical on the basis of restriction site mapping and were represented by a single fragment in the genomic Southern blot. Therefore, it is probable that NPR2 is present in a single copy. NPR3 genomic clones and the NPR3 cDNA contain a *Hin*dIII site. However, *Bam*HI does not cleave an isolated

Table I. *Variation in in Vitro Run-On Transcription Assays*

IR-grown plants were given 10 min FR, placed in darkness for 4 d, then given 2 min of R. Nuclei were isolated from plants harvested 2 h after R. Counts obtained in hybrid-selected transcripts were averaged from three separate nuclei preparations radiolabeled in concurrent nuclear run-on transcription assays. Average values are shown ± SD.

Probe	Average cpm	Relative Deviation
		%
NPR1	81 ± 8	7
NPR2	174 ± 19	8
NPR3	42 ± 7	12
Lg106	15 ± 2	11
AB30R	125 ± 26	16

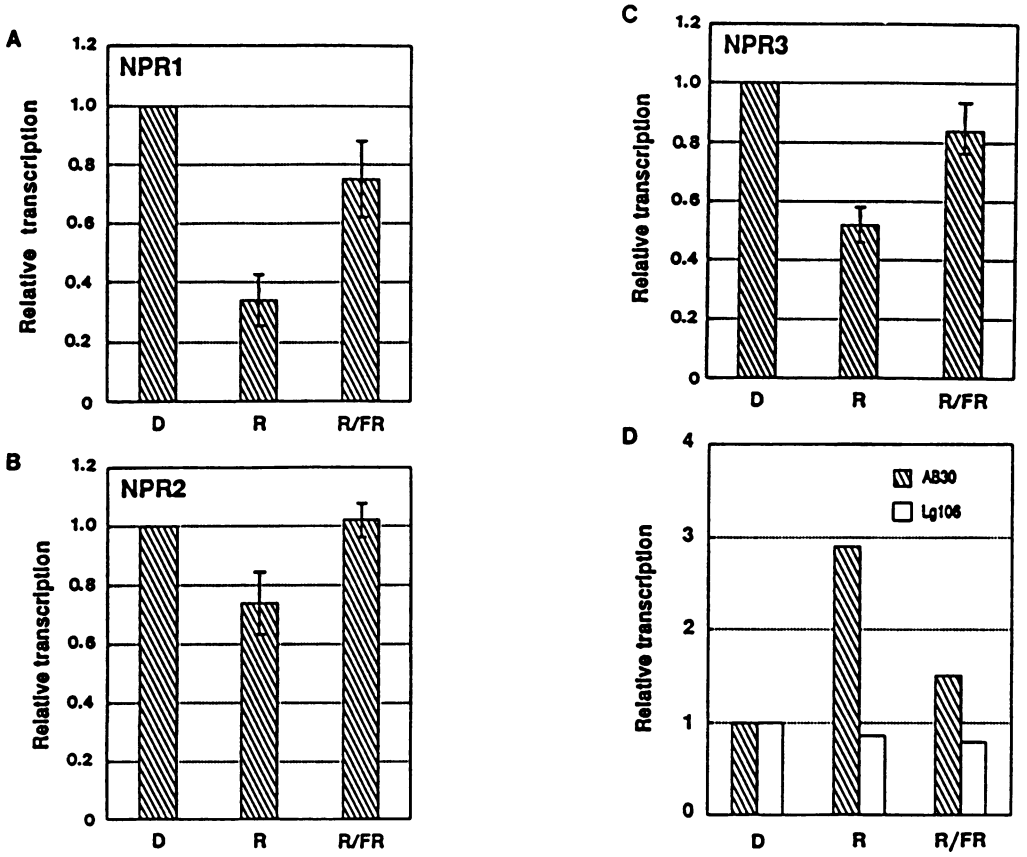

Figure 5. Phytochrome regulation of NPR gene transcription. IR-grown plants were given 10 min FR, placed in darkness for 4 d (D), then given either 2 min R (R) or 2 min R followed immediately by 10 min FR (R/FR). Nuclei were isolated 4 h after R and R/FR treatments. Transcription is shown relative to the D levels. A–C, The bars for NPR transcription represent the range above and below the average of two separate experiments. D, *cab* and *Lg106* transcription from a single representative experiment.

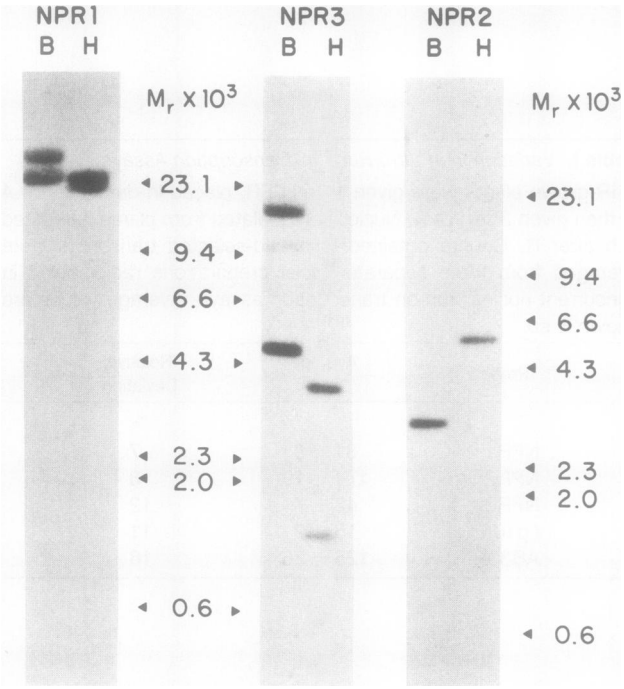

Figure 6. Southern blot of *Lemna* genomic DNA probed for sequences homologous to the NPR1, NPR2, and NPR3 cDNAs. Four micrograms of genomic DNA digested with either *Bam*HI (B) or *Hin*dIII (H) restriction endonuclease was loaded in each lane for NPR1 and NPR3, and 2 μg of DNA was loaded in each lane for NPR2. Autoradiography was carried out at −70°C for 5 d.

genomic clone containing the coding region of NPR3 and approximately 1.8 kilobases each of 3′ and 5′ flanking sequences. The two *Hin*dIII fragments that hybridize to the NPR3 cDNA in the genomic Southern blot might represent two portions of a single gene, but considering that two hybridizing genomic DNA fragments also result from *Bam*HI digestion, it is likely that there are at least two separate NPR3 genes in *Lemna*. This is substantiated by the presence of a number of restriction sites in the NPR3 cDNA that are not present in one of the NPR3 genomic clones.

DISCUSSION

We have isolated three distinct cDNA clones from *Lemna gibba* that represent mRNAs that are more abundant in dark-treated than in light-grown plants. We have also demonstrated that transcription of the corresponding genes, NPR1, NPR2, and NPR3, is negatively regulated by the action of phytochrome. After a dark treatment that resulted in increased transcription of these genes, a single R treatment was sufficient to reduce their transcription in IR-grown plants. FR given immediately after the R restored the levels of transcription to near those observed in dark-treated plants.

In W-grown *Lemna,* transcriptional increases for NPR1 and NPR2 were detectable as early as 6 h in darkness (32), but in IR-grown plants, increases in transcription were reproducibly observed after longer (12 h) periods in the dark. The reason for the difference in rapidity of the transcriptional increases is not understood at this time. However, it might be attributable to basic differences in the general transcriptional capability of etiolated plants as compared with mature, fully green plants, to the ability of each kind of plant to respond at the transcriptional level to changes in light (day/night), to the amount or species of the phytochrome in each kind of plant, and/or to differences in the molecular pathway of phytochrome action on the transcription of the NPR genes.

Despite apparent differences in the timing of the transcriptional increase of the NPR genes in W- and IR-grown *Lemna,* both kinds of plants exhibited an enhancement of transcription in response to FR given at the onset of darkness. In experiments reported previously, 10 min FR given to W-grown plants enhanced the transcription of all of the NPR genes (32). Likewise, in IR-grown plants given 10 min FR prior to dark treatment, marked increases in the levels of NPR gene transcription were observed after 12 h darkness. Because such a treatment returns phytochrome predominantly to the P_r form and presumably allows a more rapid release of the NPR genes from negative regulation by phytochrome action, we infer that phytochrome is involved in regulating the expression of these genes in green plants as well as in etiolated plants. The time in darkness required for an observable effect of FR suggests that either the factor(s) involved in the negative regulation of the NPR genes is fairly stable or that the synthesis or accumulation of factors involved in the activation of NPR transcription occurs slowly.

FR treatment also resulted in accelerated accumulation of NPR mRNA. Whether Pfr exerts an inhibitory role mainly at the transcriptional level or whether there is also a posttranscriptional component in the regulation of the NPR mRNAs has not yet been determined.

The amount of transcription measured for NPR3 was consistently lower than for NPR1 and NPR2. Increases in NPR1 and NPR2 transcription were detectable within 2 to 6 h of dark treatment, whereas an increase in NPR3 transcription was not detectable for at least 6 h of darkness. It is possible that NPR3 transcription was increasing concomitantly with that of the other two genes, but due to low amounts of NPR3 transcripts, early changes in transcription could not be visualized.

The 2 to 4 h duration over which changes in NPR gene transcription were observed in etiolated *Lemna* in response to R contrasts with the more rapid R-induced transcriptional changes observed within 15 to 30 min for other genes (reviewed in ref. 36). It is also of interest to note that the negative regulation of the NPR genes by R observed in etiolated plants could not be demonstrated in green plants (data not shown). In particular, 2 min R was sufficient to reduce NPR gene transcription in dark-treated etiolated but not in mature green plants. The basis for this difference is not known at present. However, other instances of differences between green and etiolated plants with respect to phytochrome action or prevalent species of phytochrome have been reported (6a, 26, 33). The involvement of a blue light receptor in NPR transcription in green plants could also account for such a result.

The NPR mRNAs are all significantly smaller than those for phytochrome, protochlorophyllide reductase, β-tubulin, or asparagine synthetase, other genes that are negatively reg-

ulated by phytochrome. Therefore, the NPR genes are unique with respect to other such genes known to date.

Note Added in Proof

NPR 1, 2, and 3 were originally designated NR 11, 18, and 300, respectively (32), and they are the same genes cited by these earlier names in **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

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