and offspring, whereas, from estimates of band-sharing between unrelated individuals (Fig. 1), D values of less than 0.25 should identify adults that can be excluded as parents. The mean D value (±SE) for nestlings not excluded as offspring of their putative parents on the basis of their MHC genotypes was 0.52 ± 0.009 for male parents and 0.48 ± 0.009 for female parents. Mean D values for excluded chicks were 0.04 ± 0.007 (male parents) and 0.48 ± 0.013 (female parents). These values confirm the MHC results that only males and not females are excluded as the parents of the mismatched chicks. The probability of false inclusion of an unrelated male (assuming correct maternity) is approximately $X^{y/2}$ [U. B. Gyllensten, S. Jakobsson, H. Temrin, *ibid.* **343**, 168 (1990)] where X is the proportion of shared fragments and $y/2$ is the minimum number of paternal specific bands. For males in this population this value is always less than 2×10^{-6}, depending on the probe-enzyme combination used to generate the data.
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32. We thank J. Marsolais for technical assistance, C. Francis for statistical help, T. Bargiello, C. Benoist, A. J. Jeffreys, and D. Mathis for generously sending us probes, I. Jamieson, R. Montgomerie, and J. Quinn for comments on the manuscript, and the Molecular Population Genetics Group at Queen's University for advice and discussion. H.L.G. is the recipient of an A. P. Sloan Postdoctoral Fellowship for Molecular Studies of Evolution. Research supported by an A. P. Sloan grant to H.L.G. and B.N.W., and NSERC operating and infrastructure grants to P.T.B., P.J.W., and B.N.W.

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A Trans-Acting Factor That Binds to a GT-Motif in a Phytochrome Gene Promoter

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The regulatory photoreceptor, phytochrome, controls the expression of numerous genes, including its own *phyA* genes, which are transcriptionally repressed in response to light. Functional analysis of a rice *phyA* gene promoter, by means of microprojectile-mediated gene transfer, indicates that a GT motif, GCGGTAATT, closely related to elements in the promoters of a number of other light-regulated genes, is critical for expression. Partial complementary DNA clones have been obtained for a rice nuclear protein, designated GT-2, that binds in a highly sequence-specific fashion to this motif. Mutational analysis shows that the paired G's are most crucial to binding. GT-2 has domains related to certain other transcription factors. Northern blot analysis shows that GT-2 messenger RNA levels decline in white light although red and far red light pulses are ineffective.

PHYTOCHROME IS A BINARY MOLECULAR switch that regulates plant gene expression in response to light (*1–3*). This photoreceptor is reversibly interconvertible between its inactive Pr form and its active Pfr form by red (R) and far red (F) light. However, the molecular mechanism by which the regulatory signal is transduced from Pfr to responsive genes remains unknown (*3–7*).

We have focused on the genes for phytochrome itself to study this transduction mechanism. Transcription of *phyA* genes in monocots is repressed within 5 min of Pfr formation, and repression occurs in the absence of protein synthesis (*8–11*). These data indicate that all components necessary for transduction exist in the cell before light perception. Similarities between sequence motifs in different monocot *phyA* promoters (*8, 11, 12*) suggest that these motifs could be functionally important. These motifs include tandem GT elements related to the motif GTGTGGTTAAT that has been implicated in the phytochrome-regulated expression of a pea *rbcS* gene and that binds to a factor, designated GT-1, detected in crude nuclear extracts (*3, 5, 6*). Indeed, a factor in rice nuclear extracts binds to a rice *phyA* promoter fragment containing tandem GT elements, and the binding is completed by an oligonucleotide with the sequence GTGTGGTTAAT (*11*). We have therefore examined here the potential role of GT motifs in the autoregulated expression of the rice *phyA* gene. We also report the characterization of a partial cDNA clone encoding a protein that binds in a highly sequence-specific manner to one of two GT motifs in this promoter.

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The activity of the rice *phyA* promoter was assayed by means of microprojectile-mediated gene transfer (*13, 14*) into etiolated rice seedlings (Fig. 1). Deletion analysis showed that the −364 to +111 bp fragment used here supports the same level of expression as a fragment from −2 kb to +111 bp upstream (*15*). The wild-type promoter (Fig. 1, plasmid 702) responded reversibly to R and F light, indicating that this fragment contains the sequences necessary for autoregulation of *phyA* genes in rice cells. The TATA element alone (plasmid 717) supported expression at ~25% of the maximum wild-type level with no significant effect of light. Thus, sequences that determine differences in transcriptional activity between high-Pfr and low-Pfr cells reside between −364 and −31 bp in the rice *phyA* promoter. Deletion of both GT elements (plasmid 711), or linker-substitution mutation (*16*) of the 3′ element (plasmid 712), showed that at least the 3′ motif (−228 to −219 bp) functions as a positive element in transcriptional activation in cells with low Pfr levels (Fig. 1). The alternative, that this motif functions as a negative element that causes repression in response to high Pfr levels and derepression upon Pfr depletion, is unlikely because removal of this sequence did not lead to derepression in the presence of high Pfr levels (plasmids 711 and 712).

An oligonucleotide containing both of the tandem GT motifs from the rice *phyA* promoter (−242 to −219 bp) was used to isolate cDNA clones producing proteins that bound to these elements (*17, 18*). The insert from the largest positive clone (designated λGT-2) was used for overexpression of the protein product, GT-2, which was then examined for specificity of bind-

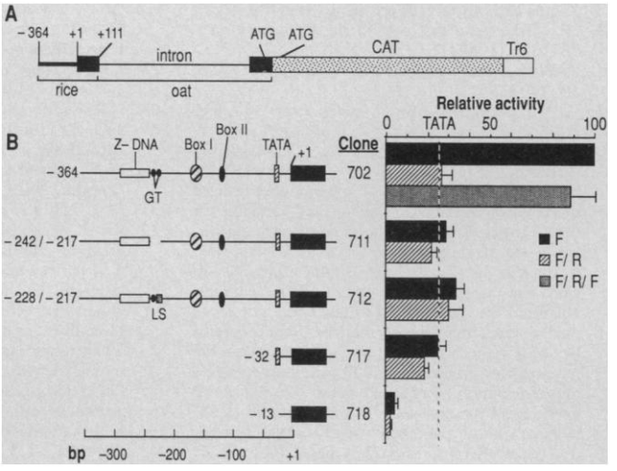

Fig. 1. Functional analysis of a rice *phyA* promoter. (**A**) Wild-type promoter-fusion construct (plasmid 702) used in gene transfer experiments (*23*). (**B**) Expression of rice *phyA* wild-type and mutant promoter fusion constructs after microprojectile-mediated transfer into etiolated rice seedlings (*14*). Mutational end points in base pairs are shown to the left of the schematics (*16, 24*). Various sequence motifs present in the 5′ flanking DNA are indicated. Box I and II are conserved sequences present in the upstream regions of oat, maize, and rice *phyA* genes (*8, 11,12*). GT refers to conserved tandem GT motifs in rice and oats. LS denotes a linker-scan mutation (*16*) replacing the 3′ GT element with the sequence CGTAGCTCGAG at positions −228 to −217 bp. Relative chloramphenicol acetyltransferase (CAT) activities (*14*) are shown to the right. Pulse irradiations given after microprojectile bombardment are indicated: far red (F; low Pfr); far red followed by red (F/R; high Pfr); and far red followed by red followed by far red again (F/R/F; low Pfr). The dashed line indicates the basal TATA-driven activity of the plasmid deleted 5′ to position −31 (plasmid 717). Each value is the result of at least four independently grown samples. Error bars are SEM.

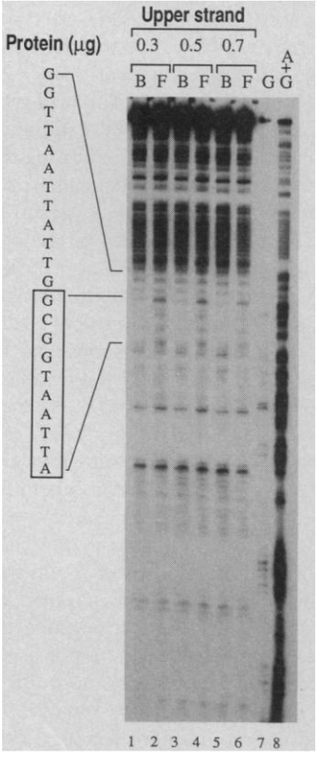

Fig. 2. Deoxyribonuclease I footprint analysis (*20*) of the specificity of binding of cloned GT-2 to the rice *phyA* promoter. A fragment from the rice *phyA* promoter (−364 to −158) was incubated with 0.3, 0.5, or 0.7 μg of protein extract from overexpressing *Escherichia coli* cells with GT-2 either in the correct (lanes 1, 3, 5) or reverse (lanes 2, 4, 6) orientation, respectively (*19*). Purine and G-specific Maxam and Gilbert (*25*) sequencing ladders of the same fragment are shown as G and A+G (lanes 7 and 8), respectively. The position of the footprint is shown in the box to the left of the lanes.

ing to the rice *phyA* promoter (*19*). Deoxyribonuclease (DNase) I footprint analysis (*20*) showed that protection by the cloned GT-2 protein was confined exclusively to the 3′ GT motif (−228 to −219 bp) (Fig. 2).

The core sequence, GGTAATT, of the 3′ GT motif in the rice *phyA* promoter differs in only two positions from the 5′ core motif, GGTTAAT (Fig. 2), which in turn is identical to both tandem GT motifs in the oat *phyA3* promoter (*12*) and the GT motif (core box II) in pea *rbcS* promoters (*3, 5, 6*) (Fig. 3A). Oligonucleotides representing the GGTTAAT-containing sequences from the pea and oat promoters bound to GT-2 at a level two orders of magnitude below that of the rice *phyA* sequence (Fig. 3A). Moreover, retention of the rice *phyA* sequence, except for a two-nucleotide change within the 3′ box to produce the GGTTAAT motif, resulted in a reduction to 30% of original binding. Pairwise nucleotide substitutions across the rice *phyA* GT sequences confirmed that the 3′ motif accounts for essentially all GT-2 binding in this region (Fig. 3B). The data define the motif GCGGTAATT as the minimal binding site for GT-2 and show that the paired G's are crucial to this interaction. The 3′ border of this binding site remains to be precisely defined, although the footprint analysis (Fig. 2) suggests that the site extends little, if any, beyond the indicated motif.

The cloned insert of λGT-2 comprises 1.7 kb with the longest open reading frame containing 334 amino acids (Fig. 4). Northern blot analysis showed that GT-2 mRNA comprises 3.8 kb (Fig. 5). Thus, although the cloned GT-2 protein obviously contains the DNA binding domain, it is likely that the GT-2 mRNA encodes additional NH_2-terminal polypeptide sequence. GT-2 has no striking sequence homology to any proteins in the databases, but it does contain a proline- and glutamine-rich domain, separate basic and acidic regions, and a segment with the potential (*21*) to form a helix-loop-helix structure (Fig. 4). These features are related to characteristics observed in various transcription factors (*22*).

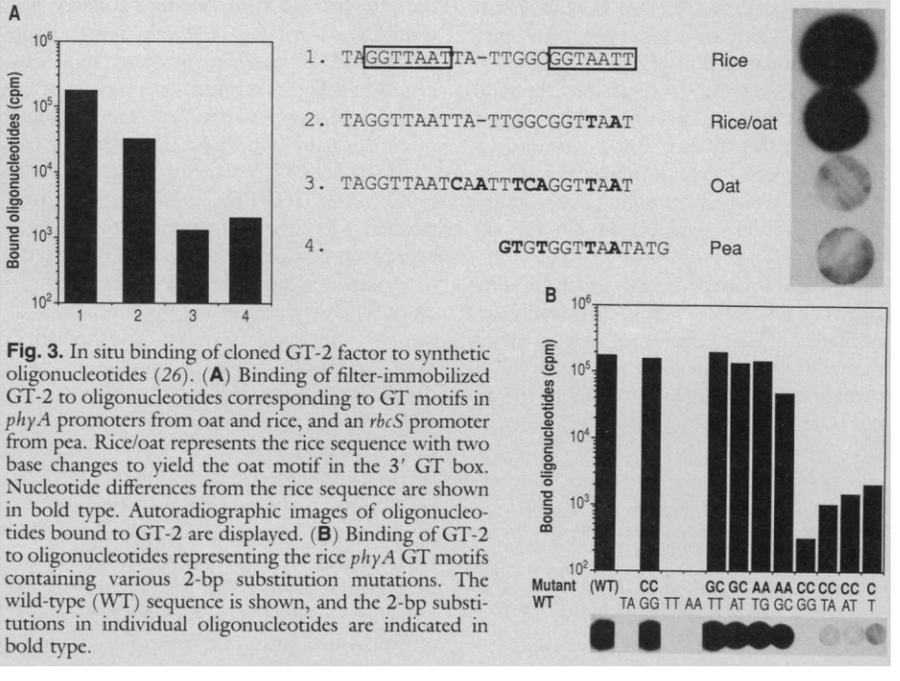

Fig. 3. In situ binding of cloned GT-2 factor to synthetic oligonucleotides (*26*). (**A**) Binding of filter-immobilized GT-2 to oligonucleotides corresponding to GT motifs in *phyA* promoters from oat and rice, and an *rbcS* promoter from pea. Rice/oat represents the rice sequence with two base changes to yield the oat motif in the 3′ GT box. Nucleotide differences from the rice sequence are shown in bold type. Autoradiographic images of oligonucleotides bound to GT-2 are displayed. (**B**) Binding of GT-2 to oligonucleotides representing the rice *phyA* GT motifs containing various 2-bp substitution mutations. The wild-type (WT) sequence is shown, and the 2-bp substitutions in individual oligonucleotides are indicated in bold type.

Fig. 4. Amino acid sequence of GT-2 (*27*) in single-letter code (*28*). Proline and glutamine residues within the first 40 amino acids are indicated by dots below and above, respectively. Two predicted (*21*) amphipathic helices, which flank a putative loop, are boxed. This region is also rich in basic residues (14/33; indicated by pluses). A segment rich in acidic residues (15/26; indicated by minuses) is underlined.

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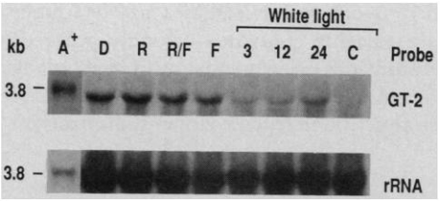
  1  PPVKQPARQHQPQPTPPPPQAAPIPAAPLQQQPPQPQHKETIHHEAVTPR
 51  RAPPTSGSSLELVPAAEQHVESGLGGGEGGSASSSRWPKTEVQALIQLRM
101  ELDMRYQETGPKGPLWEEISSGMRRIGYNRSSKRCKEKWENINKYFKKVK
151  ESNKKRPEDSKTCPYFHQLDVIYRRKHLTGGGGGGASAANVAATAIEHQN
201  PNRHEIEGKNINDNDKRKNGGGGGAQVPTSNGDTAPTTATFDVDSGMKKP
251  EDIVRELSEQPPREFTTDETDSDDMGDDYTDDGEEGEDDGKMQYRIQFQR
301  PNPGGANTAPPPATTPASAVPTSTPTSTFLAMVQ  334
```

Fig. 5. Northern blot analyses (*29*) of rice GT-2 mRNA. Lanes contain total RNA (*9*) (15 μg/lane) from seedlings that had been treated as follows: grown 7 days in the dark without irradiation (D); grown 7 days in darkness and then given saturating pulse irradiations of red (R), red followed by far red (R/F), or far red (F) light alone 3 hours before harvest; grown in darkness and then transferred to white light 3, 12, or 24 hours before harvest on day 7; or grown 7 days in continuous white light from germination (C). Poly(A)$^+$ RNA (0.3 μg/lane) was also isolated (*9*) from sample D (A$^+$). The blot was initially hybridized with ^{32}P-labeled antisense GT-2 RNA (top panel). To verify that all tracks contained equal amounts of RNA, the same blot was then rehybridized with a 28*S* ribosomal DNA probe (bottom panel).



Although the rapid, short-term repression of *phyA* transcription by phytochrome (*10, 11*) is unlikely to proceed by way of altered GT-2 gene expression, longer term effects of light on the abundance of this factor could influence the ultimate level of *phyA* expression. Northern blot analysis of total RNA showed that the amount of GT-2 mRNA declined within 3 hours of exposure of etiolated seedlings to white light and remained approximately constant for as long as 7 days in continuous light (Fig. 5). In contrast, pulses of R and F light had no effect on the amount of GT-2 mRNA up to 3 hours after exposure. The hybridization signals on these blots were somewhat weak and distorted compared to poly(A)$^+$ RNA blots because of the low abundance of the GT-2 mRNA and its comigration with ribosomal RNA (Fig. 5). The contrasting effects of continuous white light and pulses of R and F on GT-2 mRNA abundance leave open the question of the photoreceptor involved (*1*).

The correlation between the binding of cloned GT-2 to the GCGGTAATT motif in vitro and the functional activity of this motif in the rice *phyA* promoter in vivo indicates that GT-2 may function as a transcriptional activator. The fact that GT-2 binds in highly sequence-specific fashion to this motif and not to the closely related motif GTGGTTAAT present in *phyA* and *rbcS* genes distinguishes it from another binding activity, designated GT-1, which has been detected in nuclear extracts and which does bind to the GTGGTTAAT motif and its variants (*5, 6, 11*). Thus, plants may have a family of trans-acting factors capable of binding in a highly selective manner to closely related GT elements.

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18. A cDNA library was constructed from polyadenylated [poly(A)$^+$] RNA (*9*) from 9-day-old green IR36 rice seedlings. Commercial kits were used for cDNA synthesis and λgt11 cloning (Amersham). A probe containing the tandem rice *phyA* GT motifs was generated with the two complementary, synthetic oligonucleotides 5′-AATTAGGTTAATTATTGGCGGTAATT-3′ and 3′-TCCAATTAATAACCGCCATTAATTAA-5′. Each oligonucleotide (5 μg) was phosphorylated, annealed, ligated [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)], and radiolabeled with a nick-translation kit and ^{32}P (Amersham). The library was screened as described (*17*).
19. A high level of GT-2 protein expression was obtained by cloning the λGT-2 cDNA insert into a plasmid vector under control of a T7 promoter [F. W. Studier, B. A. Moffatt, *J. Mol. Biol.* **189**, 113 (1986)]. As a control, the insert was also cloned in the reverse orientation. Protein extracts from lysed cultures were analyzed on SDS-polyacrylamide gels to verify expression and stored at −70°C.
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23. The *phyA* gene used here was isolated from a λEMBL3 rice (var. IR36) genomic DNA library with the use of an oat cDNA clone pAP3.2 [H. P. Hershey, R. F. Barker, K. B. Idler, J. L. Lissemore, P. H. Quail, *Nucleic Acids Res.* **13**, 8543 (1985)]. The 5′ flanking region and first exon were identified by restriction mapping and sequencing as being identical to those of the published sequence designated rice *phy-18* (*11*). A restriction fragment from this region (−364 to +111 bp) was fused to the Kpn I site at the exon/intron junction of intron 1 of an oat *phyA*/CAT reporter construct used previously to analyze oat *phyA3* promoter activity (*14*).
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26. Bacterial cells were infected with bacteriophage λGT-2, plated on a single 15-cm petri plate and grown to confluence. Plaque-lift filters were prepared from each plate as described [R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)], air-dried, and cut into 2-cm disks. The disks were processed by guanidine hydrochloride denaturation and renaturation (*17*). Probes consisted of catenated, double-stranded oligonucleotides of the various motifs, radiolabeled with the use of a nick-translation kit and ^{32}P (Amersham). Disks were incubated with probes overnight (*17*), washed, blotted, and autoradiographed. Bound oligonucleotides were quantified by Cerenkov counting.
27. The cDNA insert of λGT-2 was subcloned into pUC118, and the nucleotide sequences of both strands were determined with a sequenase kit (U.S. Biochemical).
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30. We thank J. DeWett and V. Walbot for the rice genomic library; A. Christensen for the *ubi*-LUC construct; S. Goff and P. Oeller for technical advice; J. Tepperman, H. Hung, and S. Casper for technical assistance; and R. Wells for manuscript preparation. Supported by grants 89-37280-4800 from the U.S.D.A. Competitive Research Grants Program and DCB 8796344 from the National Science Foundation.

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