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The Sorghum Photoperiod Sensitivity Gene, Ma_3, Encodes a Phytochrome B[1]

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The Ma_3 gene is one of six genes that regulate the photoperiodic sensitivity of flowering in sorghum (*Sorghum bicolor* [L.] Moench). The ma_3^R mutation of this gene causes a phenotype that is similar to plants that are known to lack phytochrome B, and ma_3^R sorghum lacks a 123-kD phytochrome that predominates in light-grown plants and that is present in non-ma_3^R plants. A population segregating for Ma_3 and ma_3^R was created and used to identify two randomly amplified polymorphic DNA markers linked to Ma_3. These two markers were cloned and mapped in a recombinant inbred population as restriction fragment length polymorphisms. cDNA clones of *PHYA* and *PHYC* were cloned and sequenced from a cDNA library prepared from green sorghum leaves. Using a genome-walking technique, a 7941-bp partial sequence of *PHYB* was determined from genomic DNA from ma_3^R sorghum. *PHYA*, *PHYB*, and *PHYC* all mapped to the same linkage group. The Ma_3-linked markers mapped with *PHYB* more than 121 centimorgans from *PHYA* and *PHYC*. A frameshift mutation resulting in a premature stop codon was found in the *PHYB* sequence from ma_3^R sorghum. Therefore, we conclude that the Ma_3 locus in sorghum is a *PHYB* gene that encodes a 123-kD phytochrome.

The transition from vegetative to reproductive growth is the result of the activation of genes responsible for inflorescence and floral organ formation. These genes, which control apex identity and floral organ morphogenesis, are strictly regulated, since their improper expression results in abnormal flowers and inflorescences (Okamuro et al., 1993; Veit et al., 1993). The initial activation of these genes is usually the result of environmental cues that indicate an appropriate time to flower. The mechanisms by which environmental factors activate inflorescence and floral organ production are complex and many genes are known to be involved in the transduction of environmental signals that regulate flowering (Bernier et al., 1993; Coupland, 1995).

Of all of the environmental factors that are sensed by plants, daylength is probably the most important in inducing flowering. The phenomenon whereby daylength regulates flowering is referred to as photoperiodism. Photoperiodic control of flowering allows plants to coordinate their reproduction with the environment or with other members of their species. An understanding of the effect of daylength on reproductive development has agronomic importance because the ability to alter flowering time allows the cultivation of a species in environments that differ greatly from the one in which it originally evolved.

Our understanding of photoperiodism has historically relied upon a physiological examination of the phenomenon. Recently, genetic analysis of floral induction has provided new insights into this process. In the LD plant *Arabidopsis thaliana* a series of genes has been recognized that influences flowering time, and these genes have been categorized into six phenotypic groups based on earliness or lateness in flowering in response to short days, long days, and vernalization; (Coupland, 1995). The existence of these separate phenotypic classes suggests the existence of several pathways that regulate photoperiod sensitivity. A similar process has been used in the study of photoperiodic sensing in the SD grass, sorghum (*Sorghum bicolor* [L.] Moench). A series of six maturity genes has been recognized to alter flowering time in sorghum: Ma_1, Ma_2, Ma_3, Ma_4, Ma_5, and Ma_6 (Quinby, 1967; F.R. Miller, unpublished data). The first four maturity genes cause long days to inhibit flowering but allow early flowering under short days. Of these four genes, mutations at Ma_1 cause the greatest reduction in sensitivity to long days. Mutations at Ma_2, Ma_3, and Ma_4 generally have a more modest effect on sensitivity to long days (Quinby, 1967). However, even sorghum with the recessive alleles ma_1, ma_2, or ma_3 flower later under long days than under short days (Pao and Morgan, 1986). The genes Ma_5 and Ma_6 represent a special case because only when they are both present in the dominant form will they very strongly inhibit floral initiation regardless of daylength (F.R. Miller, unpublished data). The sorghum photoperiod mutants constitute a phenotypic class that corresponds to one of the early-flowering phenotypic classes of *A. thaliana*. Mutations in any of the sorghum maturity genes or the *A. thaliana* genes *ELF*, *ESD*, *COP1*,

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Abbreviations: cM, centimorgans; LD, long-day; QTL, quantitative trait loci; RACE, rapid amplification of cDNA ends; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment-length polymorphism; RI, recombinant inbred; RT, reverse transcriptase; SD, short-day.

and *PHYB* cause decreased sensitivity to inhibitory photoperiods (Coupland, 1995).

It is interesting that mutations in sorghum Ma_3 and *A. thaliana PHYB* both reduce sensitivity to noninductive daylengths, since we hypothesized that Ma_3 codes for PhyB. This hypothesis was based on work done with the drastic, pleiotropic mutation ma_3^R. Sorghum plants with ma_3^R have a greatly reduced photoperiod sensitivity compared with Ma_3 plants and will flower early under any photoperiod (Pao and Morgan, 1986; Childs et al., 1995). The phenotype caused by the ma_3^R mutation is characteristic of mutants that are known to lack PhyB: *phyB* (*A. thaliana*), *ein* (*Brassica rapa*), *lh* (*Cucumis sativus*), and *tri* (*Lycopersicon esculentum*) (Devlin et al., 1992; López-Juez et al., 1992; Reed et al., 1993; van Tuinen et al., 1995). Because of the similarities of ma_3^R sorghum to these mutants known to lack PhyB, it was our hypothesis that the Ma_3 locus encodes a *PHYB* gene (Childs et al., 1991, 1992, 1995).

To provide evidence that Ma_3 is synonymous with *PHYB*, we have mapped *PHYA*, *PHYB*, *PHYC*, and Ma_3-linked molecular markers. We sequenced the three phytochrome genes and demonstrate that ma_3^R sorghum contains a mutation in *PHYB*. The ability of PhyB to regulate flowering similarly in both a LD dicot and a SD monocot is discussed.

MATERIALS AND METHODS

The cultivars of *Sorghum bicolor* (L.) Moench used in these experiments were 58M (ma_3^R), 100M (Ma_3), BTx 623, and IS 3620C. 58M and 100M were grown in a greenhouse and crossed to produce heterozygous F_1 plants, which were grown and self-pollinated to produce F_2 seed. F_2 plants were grown in the field and scored for days-to-flowering. All F_2 plants were self-pollinated. F_3 family rows were grown to confirm the phenotype of early-flowering F_2 plants and to differentiate heterozygous and homozygous late-flowering F_2 plants. For mapping within this Ma_3/ma_3^R segregating population, reserved seed from F_3 families were planted in a growth room, and DNA was extracted from the combined tissue of several members within each family.

Identification of Ma_3-Linked RAPD Markers

DNA was extracted and RAPD-PCR was performed as described by Pammi et al. (1994). Four-hundred-eighty 10-mer primers (Operon Technologies, Alameda, CA) were used to screen for RAPDs. RAPD-PCR products were radiolabeled by the incorporation of [^{32}P]dCTP in the reaction. Radiolabeled products were separated on sequencing gels that were dried on blotting paper and exposed to film. Polymorphisms were detected by visual examination of the autoradiographs.

Polymorphic DNA bands were extracted from the dried acrylamide gel by incubating at 37°C for 12 h with elution buffer consisting of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS (Ogden and Adams, 1987). The DNA was precipitated with ethanol after the addition of tRNA and then cloned into T-tailed pBluescript II (SK+) (Stratagene) (Holton and Graham, 1991).

Cloning of *PHYA* and *PHYC*

Degenerate primers corresponding to the conserved amino acid sequences DIPQA and ACEFL were created to allow PCR amplification of partial phytochrome genomic sequences from sorghum DNA (Pratt et al., 1995). The products from this reaction were cloned into pBluescript II (KS−), and individual clones were sequenced. Truncated clones derived in this manner, which corresponded to *PHYA* and *PHYC*, were used to screen a λgt10 cDNA library created using RNA isolated from green sorghum leaf tissue (Sambrook et al., 1989). cDNA clones for *PHYA* and *PHYC* were subcloned into pBluescript (SK+) and sequenced.

phyB Sequencing

To obtain the sequence of *PHYB*, the DIPQA and ACEFL primers were used to amplify by PCR a 359-bp *PHYB* fragment from sorghum that was cloned into pBluescript II (KS−). From the sequence of this clone, a gene-specific primer was made that was used in a 3′ RACE reaction using cDNA from light-grown ma_3^R sorghum (Frohman et al., 1988). cDNA was synthesized from 1 μg of poly(A) RNA using a dT-AMP primer (5′-GCTCGAGTC GACCGCTTT TTTTTTTTT-3′) in a 24-μL reaction containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 2 mM each dNTP, 15 pmol of dT-AMP primer, 10 units of RNasin, and 10 units of avian myeloblastosis virus RT incubated at 42°C for 2 h. The gene-specific primer (5′-CTCCTCGGC AATGAAGTT GT-3′, nucleotides 801–820 [nucleotide numbering refers to the nucleotides of the final sequences submitted to GenBank]) and a 3′ specific primer, AMP (5′-GCTCGAGTC GACCGC-3′), were used in a PCR reaction with the first-strand cDNA and resulted in a 1.3-kb fragment that was cloned into T-tailed pBluescript (SK+). This clone was sequenced and found to be homologous to *PHYB*. This partial clone is referred to as pBSB18. Because the same size fragment was obtained by 3′ RACE when using cDNA from either Ma_3 or ma_3^R sorghum (data not shown), it was assumed that the truncated 3′ RACE product was due to internal annealing of the dT-AMP primer. Later, the region of *PHYB* corresponding to pBSB18 was amplified from genomic DNA, and the PCR product was directly sequenced to confirm the accuracy of the 3′ RACE clone.

To obtain additional sequence 5′ and 3′ to the initial 1.7 kb of sequence, a genome-walking technique was used (Siebert et al., 1995). Briefly, genomic DNA from ma_3^R sorghum was digested in separate reactions with blunt-end endonucleases: *Dra*I, *Eco*RV, *Pvu*II, *Sca*I, and *Sma*I. The ends of the DNA in each digested pool were ligated to an adaptor that had been created by the annealing of two oligonucleotides: adaptor L (5′-CTAATACGA CTCACTATA GGGCTCGAG CGGCCGCCC GGGCAGGT-3′) and adaptor S (5′-PO_3-ACCTGCCC-NH_2-3′). Long PCR was then performed using a commercial enzyme mix (Elongase,

Life Technologies) and adaptor- and gene-specific primers. The procedure involved two rounds of PCR in which the products from the first reaction were diluted 1:100 and used in the second reaction. Thermocycling conditions consisted of an initial 1-min, 94°C denaturation followed by 35 (primary PCR) or 20 (secondary PCR) cycles of denaturation for 30 s at 94°C, annealing/extension for 6 min at 68°C, and a final extension of 15 min at 68°C. Nested primers were used for each set of primary and secondary reactions (Table I). The products from the secondary PCR reaction were precipitated with ethanol and resuspended for loading onto a 1% low-melting-point agarose gel. After electrophoresis and visualization under UV, the predominant band resulting from each adaptor-ligated pool of DNA was excised and purified from the agarose using Qiaquick spin columns (Qiagen, Chatsworth, CA). The PCR products were then sequenced directly.

Sequencing

Initially, sequencing of plasmid clones was done using Sequenase version 2.0 (United States Biochemical). For the majority of the sequencing, sequence data were generated using either the ABI 373a or the ABI 377 with samples prepared with the ABI Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer).

RT-PCR

RT-PCR was done as described by Ohtsuki et al. (1994). RNA was extracted from both green and etiolated leaves (Childs et al., 1995). RNA and cDNA were prepared as described above except that for cDNA synthesis 1 μg of poly(A) RNA was used and 2 μM random hexamers (Pharmacia) were substituted for the oligo(dT)-AMP primer. Amplification reactions were similar to the RAPD reactions except that cDNA corresponding to 10 ng of initial poly(A) RNA was used as the target DNA, no [^{32}P]dCTP was used, and 1.3 μM of each of two gene-specific primers were included (for *PHYA*: 5′-GCTGAGATC ACCAAACCT GG-3′, nucleotides 1000–1019 and 5′-GCATCCTTC TGTTGTCAT CC-3′, nucleotides 1891–1872; for *PHYB*: 5′-TGTCATGGG GACTCAACA GG-3′, nucleotides 1156–1173 and 5′-CATCTCTCT TGCTACAGA GC-3′, nucleotides 1599–1570; for *PHYC*: 5′-CTGGTCGTCT GCCATCATAC-3′, nucleotides 1395–1414 and 5′-CGTGGATGCA TCTTCCTGCC-3′, nucleotides 1929–1948). The reaction products were separated by electrophoresis on a 0.8% agarose gel and visualized with UV light.

Mapping

An RI population derived from a cross of cultivars BTx 623 and IS 3620C was used for the mapping of markers onto a genomic map of 1462 cM (Y. Peng, K.S. Schertz, and G.E. Hart, unpublished data). This population consisted of 137 F_6 and F_7 lines and has been used for the creation of a genomic map comprising primarily sorghum and *Zea mays* RFLP markers. Several plants from each family were grown, and harvested tissues from each family were combined for DNA extractions.

For RFLP mapping, the *PHYB* partial clone pBSB18, a 476-bp *Bgl*II fragment of the the *PHYC* cDNA clone, and clones of the Ma_3-linked markers were used in Southern hybridizations. Southern blots prepared after the digestion of DNA from each of the RI lines with either *Hin*dIII or *Xba*I were kindly provided by Dr. Gary Hart. Probes were prepared by random primer labeling (Feinberg and Vogelstein, 1983), and hybridization and washing were as described by Sambrook et al. (1989).

A single-nucleotide polymorphism that distinguished BTx 623 from IS 3620C was found in a region corresponding to the very 3′ terminus of the *PHYA* cDNA clone (nucleotide 3559). This polymorphism resulted in the creation of a *Dde*I restriction site in the IS 3620C parent that was absent in the BTx 623 parent. DNA from each of the RI lines was used in PCR reactions to amplify the region containing the site of this polymorphism (primers: 5′-TGTTGGTGG CTCTGTTG-3′, nucleotides 3333–3349; and 5′-TGCCAGTAC AACTTTCC-3′, nucleotides 3782–3766). The resulting 449-bp products were digested with *Dde*I, electrophoresed on a 3% agarose gel (Metaphor, FMC, Rockland, ME), and visualized under UV.

Mapping Data Analysis

The RI population used for mapping has been used previously to create a detailed genetic map of sorghum (Y. Peng, K.S. Schertz, and G.E. Hart, unpublished data). Our data were incorporated into this existing map using Map-

Table I. *List of primer sets used for flanking PCR to obtain PHYB gene sequence*

Primer Name[a]	Sequence	Nucleotides
Adaptor primers	5′-GGATCCTAAT ACGACTCACT ATAGGGC-3′	n.a.[b]
	5′-AATACGACTC ACTATAGGGC TCGAGCGGC-3′	n.a.
B2	5′-TCTGTAGCAA GAGAGATGGT TCGGTTGATAG-3′	1804 to 1834
	5′-GATGGTTCGG TTGATAGAGA CAGCAACAGTAC-3′	1818 to 1849
B3	5′-GACCGCCAAC ACAAGGAGCA AAATGGATGG-3′	3200 to 3229
	5′-AGCAAAATCC ATGGTAAATC CATTGGCGCC-3′	3216 to 3245
B4	5′-AAGCTGCAAT TCCATGTTGA GCTGCAGCCC-3′	936 to 907
	5′-GCAGCCCAGA TGCCTGCATG AGAAACTCGC-3′	913 to 884
B5	5′-ACCATCATAC TGTACAGTAA CTGGCATGCG-3′	4257 to 4286
	5′-ACTGTACAGT AACTGGCATG CGTCTGGCTG-3′	4265 to 4294

[a] The adaptor primers were common to each set of nested reactions. The primer names refer to the section of the gene amplified by that primer set (see Fig. 2). [b] n.a., Not applicable.

maker Macintosh version 2.0 (Lander et al., 1987). Our markers were assigned to an existing linkage group using the "near" command and then placed in the most appropriate order using the "try" command. The "ripple" command confirmed marker order and indicated that markers had been placed with a LOD > 3. Recombination distances were determined with the Kosambi function (Kosambi, 1944).

RESULTS

The near-isogenic cultivars 100M (Ma_3) and 58M (ma_3^R) were screened for RAPD markers using 480 random 10-mers. Because of the near-isogenic nature of these two cultivars, only 21 RAPD markers were found to differentiate the two cultivars (data not shown). A population segregating for Ma_3 and ma_3^R was made by crossing 100M and 58M. Forty F_3 families were used to determine that three RAPD markers cosegregated with Ma_3. Two of these Ma_3-linked RAPD markers, *op-k20a* and *op-k20c*, were cloned.

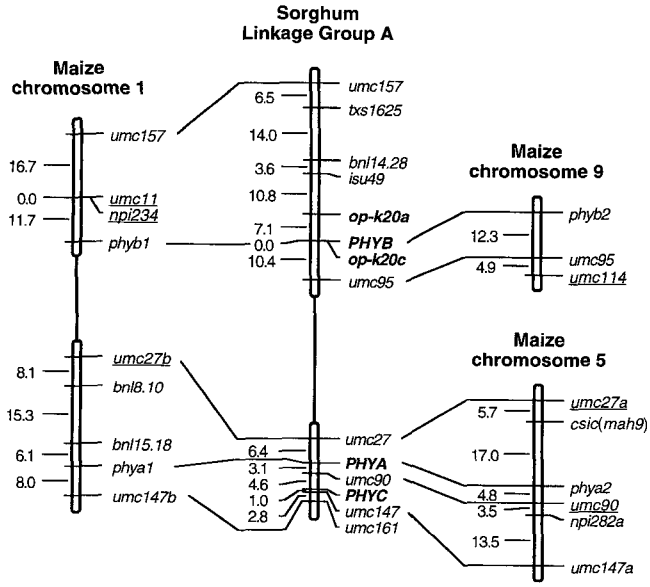

Figure 1. Linkage maps showing positions of *PHYA*, *PHYB*, *PHYC*, and Ma_3-linked markers and showing the synteny between *S. bicolor* and *Z. mays* linkage groups. Gene-specific markers for *PHYA*, *PHYB*, and *PHYC* and Ma_3-linked markers *op-k20a* and *op-k20c* were mapped in a sorghum RI population that has been used previously to create a map of RFLP markers (Y. Peng, K.S. Schertz, and G.E. Hart, unpublished data). Data from *PHYA*, *PHYB*, *PHYC*, and the Ma_3-linked markers were incorporated into the RFLP map. The *Z. mays* linkage groups shown were created for each linkage group by using data provided to Agricultural Genome Information Server's MaizeDB by Brookhaven National Laboratory. For *Z. mays* chromosomes 1 and 9, data were from Brookhaven National Laboratory May 93 maps created from a C159 × TX303 RI population (Burr et al., 1988). For *Z. mays* chromosome 5, data were used from a Brookhaven National Laboratory May 93 map made from a T232 × CM37 RI population (Burr et al., 1988). Markers that are underlined were shown by Phillips et al. (1992) to be linked to QTL for flowering time in *Z. mays*. The numbers in the diagram indicate cM distances between markers. For the *Z. mays* linkage groups presented, only the markers shown were used to create the maps shown.

Table II. *Comparison of derived amino acid sequences from sorghum phytochromes A and C and partial phytochrome B sequences*

The percent identity values were obtained by comparing derived protein translations using the Bestfit program from the Wisconsin Package (Genetics Computer Group, Madison, WI).

Phytochromes	PhyA	PhyC	Partial PhyB
		% identity	
Maize PhyA	96.5	52.3	50.3
Rice PhyA	88.2	53.1	50.5
Rice PhyB	50.2	53.0	91.1
Arabidopsis PhyA	64.4	56.4	53.5
Arabidopsis PhyB	49.0	51.9	75.0
Arabidopsis PhyC	50.5	59.4	51.7
Arabidopsis PhyD	49.1	51.8	71.8
Arabidopsis PhyE	45.8	49.1	58.6

Ma_3 was then mapped in an RI population of 137 lines resulting from a cross between BTx 623 and IS 3620C using *op-k20a* and *op-k20c* as probes to detect RFLPs. The Ma_3-linked RAPD markers mapped within 11.6 cM of each other on sorghum linkage group A (Fig. 1) (Y. Peng, K.S. Schertz, and G.E. Hart, unpublished data).

We determined the sequences of cDNA clones of *PHYA* and *PHYC*. The *PHYA* and *PHYC* clones are 3805 and 3755 bp in length, respectively. The open reading frames of these two clones are 3393 and 3405 bp long, respectively. A 7941-bp sequence of *PHYB* was determined from genomic DNA from ma_3^R sorghum. Based on comparison with *PHYB* from *Oryza sativa*, the sorghum sequence begins within 500 bp of the 5′ end of the gene and extends to 1076 bp past the end of the stop codon. The gene is made up of at least four exons and three introns (Fig. 2). The partial *PHYB* sequence encodes 1036 amino acids.

The identification of the sequences as *PHYA*, *PHYB*, and *PHYC* was based on a comparison of the amino acid sequences of the encoded proteins to known phytochromes by using the Bestfit program from the Wisconsin Package

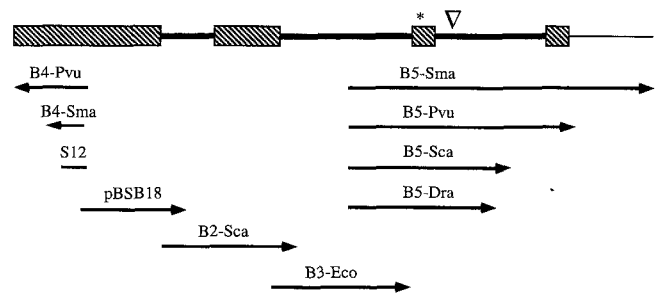

Figure 2. Diagram of the *PHYB* gene and the mutation that is present in ma_3^R. Diagram of the *PHYB* gene showing exons, introns, and the untranslated region. Boxes, exons; thick lines, introns; thin line, the untranslated region; *, the position of the point mutation found in ma_3^R sorghum; ∇, the area within the last intron that is highly homologous to noncoding regions found in several other sorghum genes. The arrows indicate steps used in walking across this gene. The primers used for each step in the walk are indicated in Table I. The fragment S12 shows the position of the 359-bp fragment used to begin the sequencing of this gene.

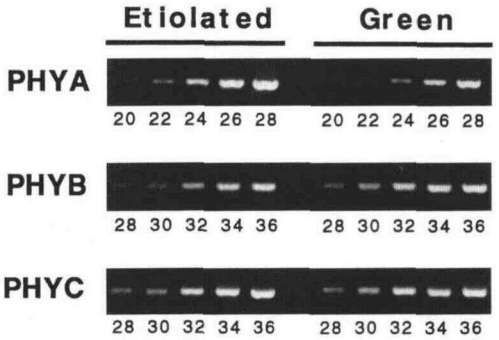

Figure 3. Expression levels of *PHYA*, *PHYB*, and *PHYC* in etiolated and green leaf tissues from *phyB*-1 (ma_3^R) sorghum. RNA was extracted from etiolated and green leaves and used for the preparation of first-strand cDNA. RT-PCR using the first-strand cDNA was performed using gene-specific primers and various numbers of cycles. The numbers below each band indicate the number of PCR cycles used to generate that band.

(Genetics Computer Group) (Table II). The sorghum *PHYA*-derived protein sequence is most similar to the other monocot PhyA sequences. The partial sorghum PhyB amino acid sequence has a 91.1% identity with the *O. sativa* PhyB. Although the sorghum *PHYC*-encoded protein sequence has only a 59.4% identity with *A. thaliana* PhyC, it is even less similar to the other phytochromes.

100M (Ma_3)

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TTCCTTGAAACTTGCTCTGCTTGTGAGAAACAGATGTCCAA
F  L  E  T  C  S  A  C  E  K  Q  M  S  K
                                        ▽
GATTGTTAAGGACGCCACTCTCCAAAGTATTGAGGACGGCT
 I  V  K  D  A  T  L  Q  S  I  E  D  G  S

CTTTGGTACTTGAGAAAAGTGAGTTTTCTTTTGGAGACGTT
  L  V  L  E  K  S  E  F  S  F  G  D  V
   ◆
ATGAATGCTGTTGTCAGCCAAGCAATGTTATTGTTGAGGGA
M  N  A  V  V  S  Q  A  M  L  L  L  R  E

GAGGGATTTACAACTT
 R  D  L  Q  L
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58M (ma_3^R)

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TTCCTTGAAACTTGCTCTGCTTGTGAGAAACAGATGTCCAA
F  L  E  T  C  S  A  C  E  K  Q  M  S  K
                                        ▽
GATTGTTAAGGACGCCACTCTCCAAAGTATTGAGGACGGCT
 I  V  K  D  A  T  L  Q  S  I  E  D  G  S

CTTTGGTACTTGAGAAAAGTGAGTTTTCTTTTGGAGACGTT
  L  V  L  E  K  S  E  F  S  F  G  D  V
   ◆
ATGATGCTGTTGTCAGCCAAGCAATGTTATTGTTGAGGGAG
M  M  L  L  S  A  K  Q  C  Y  C  STP

AGGGATTTACAACTT
```

Figure 4. Nucleotide and amino acid sequences from a region of *PHYB* from 100M (Ma_3) and 58M (ma_3^R) sorghum. RT-PCR was used to amplify the region surrounding the ma_3^R mutation from poly(A) RNA isolated from the green leaf tissue. ◆, The point of divergence in the two sequences. The sequence obtained lacks the intervening intron that is found in the genomic sequence. The portion of sequence given here begins at nucleotide 3216 and ends at nucleotide 5073. The intron splice site (▽) joins nucleotides 3295 and 4975. Nucleotide numbering refers to nucleotides of the final sequence submitted to GenBank.

As an additional test of the identities of the sorghum phytochrome genes, RT-PCR was used to examine the abundance of *PHYA*, *PHYB*, and *PHYC* mRNAs in etiolated and green leaves from ma_3^R sorghum (Fig. 3). The level of *PHYA* transcripts was much higher in etiolated than in green leaves. The levels of *PHYB* and *PHYC* mRNA were similar in these two cases. The expression of *PHYA* was also much greater than that of *PHYB* or *PHYC* in either tissue (Fig. 3; data not shown).

Gene-specific probes of *PHYB* and *PHYC* were used to map these genes as RFLP markers in the RI mapping population. A cleavable amplified polymorphic site in *PHYA* was used to map *PHYA* in the RI population (Konieczny and Ausubel, 1993). All three phytochrome genes mapped to sorghum linkage group A (Fig. 1) (Y. Peng, K.S. Schertz, and G.E. Hart, unpublished data). This observation documents an unpublished report cited by Lin et al. (1995) noting that all three sorghum phytochrome genes mapped to the same chromosome, *PHYA* and *PHYC* mapping within 7.7 cM of each other (Fig. 1), and *PHYB* is 137.2 cM from *PHYA* on the same linkage group. *PHYB* and Ma_3-linked *op-k20c* map to the same position of linkage group A (Fig. 1).

The expected translation of the PhyB gene from ma_3^R sorghum indicates a prematurely terminated protein compared with the amino acid sequence from *O. sativa* PhyB (Dehesh et al., 1991). To determine if the premature termination could correspond to the ma_3^R mutation, a 508-bp region of the open reading frame surrounding this site was amplified from Ma_3 and ma_3^R sorghum using RT-PCR. The amplified products were of the expected size and did not contain the 1.6-kb intron that is present in the genomic sequence. A 1-bp deletion was detected 30 bp upstream from the termination codon in the mutant (Fig. 4). The absence of this deletion would result in a protein comparable to that of PhyB from *O. sativa*.

Analysis of the third intron indicated that a region of 220 to 310 bp (Fig. 2; nucleotides 5550–5860) has a high degree of identity (approximately 70%) to sequences found adjacent to other sorghum genes encoding a Hyp-rich glycoprotein, NADP-malate dehydrogenase, PEP carboxylase, and lipid transfer protein. The location of the homologous sequences within the other sorghum genes varies from the 5′ promoter region, to an intron, and to a region past the transcription stop site (data not shown).

DISCUSSION

Photoperiod sensitivity is controlled by at least six known genes in sorghum: Ma_1, Ma_2, Ma_3, Ma_4, Ma_5, and Ma_6 (Quinby, 1967; F.R. Miller, unpublished data). One of our long-term goals is to isolate these genes to understand better their roles in photoperiod sensing in sorghum. We report here that one gene, Ma_3, encodes the apoprotein of phytochrome B. This is the first sorghum gene affecting photoperiod sensitivity that has been isolated and characterized.

The evidence that Ma_3 is synonymous with *PHYB* is several-fold. There are many physiological similarities be-

tween ma_3^R sorghum and other plants known to contain *PHYB* mutations. Like other *PHYB* mutants, ma_3^R sorghum is relatively insensitive to photoperiod, displays an elongated growth pattern, has a reduced chlorophyll content, and lacks a deetiolation response to high-irradiance red light (Childs et al., 1991, 1992, 1995; Goto et al., 1991; Devlin et al., 1992; López-Juez et al., 1992; McCormac et al., 1993; Reed et al., 1993; van Tuinen et al., 1995). In this report, two Ma_3-linked markers and *PHYB* are shown to map to the same location in the sorghum genome (Fig. 1). Further proof that Ma_3 codes PhyB was obtained by sequencing *PHYB* from ma_3^R sorghum that lacks a 123-kD phytochrome predominating in green seedlings (Childs et al., 1992). If *PHYB* codes for the 123-kD sorghum phytochrome, then the sequence of *PHYB* from ma_3^R should possess a mutation that would cause the absence of PhyB. In fact, a frameshift mutation in the 3′ terminus of *PHYB* in ma_3^R sorghum was found (Figs. 2 and 4). The mutation results in a premature stop codon shortly after the mutation. This mutation is not present in *PHYB* from Ma_3 sorghum (Fig. 4). The portion of PhyB in ma_3^R sorghum that is not translated is presumed to contain regions that are necessary for dimerization and biological activity (Cherry et al., 1993; Edgerton and Jones, 1993).

Similar mutations have been demonstrated in *PHYB* of *A. thaliana*, and these *A. thaliana* mutants (37-5 and 49-8) also lack immunologically detectable PhyB (Wagner and Quail, 1995). Although we have been unable to obtain a small portion of the 5′ end of the *PHYB* gene, it is demonstrated here that the sequence that we have obtained is expressed. Using RT-PCR, a portion of the *PHYB* transcript was amplified from both Ma_3 and ma_3^R sorghum. The amplified product not only contains the mutation found in the ma_3^R genomic sequence, but it lacks a 1.6-kb intron that would have been amplified if genomic DNA had been used in the reaction. Because of the physiological similarities between ma_3^R sorghum and known *PHYB* mutants, because Ma_3-linked markers map with *PHYB*, and because a frameshift mutation occurs in the *PHYB* gene of phytochrome-deficient ma_3^R plants, we conclude that Ma_3 is synonymous with *PHYB*. Given that the identity of this sorghum maturity gene is now known, Ma_3 and ma_3^R will now be referred to as *PHYB* and *phyB*-1, respectively.

In the course of this work, we obtained two full-length phytochrome cDNA clones, *PHYA* and *PHYC*, and a partial *PHYB* genomic sequence from sorghum. The sequence of *PHYB* was resolved by utilizing a new technique called genome walking, which involved sequencing the products of PCR amplification of DNA flanking a known sequence. We are confident that the use of this technique resulted in the determination of contiguous sequence for four reasons. First, long gene-specific primers were used to anchor the amplification reaction to the site in the genome in which we were interested (Table I). Thus, the likelihood of an unrelated region of the genome being amplified using such a specific primer was minimal. Second, the gene-specific primers were designed so that the resulting amplification products overlapped with known sequence. This allowed us to determine that each step of a genome walk was related to the starting point of that walk. Third, PCR was performed using a polymerase mix that contained a small amount of a polymerase with 3′ to 5′ exonuclease proofreading activity. Such activity can reduce the incorporation of inappropriate bases by 10-fold compared with polymerases without proofreading activity (Eckert and Kunkel, 1991; Scott et al., 1994). Finally, PCR products were directly sequenced to further reduce the likelihood of polymerase-induced errors being incorporated into the final sequence. For these reasons, we conclude that the sequence that we have reported as *PHYB* is accurate and represents a single contiguous region of the sorghum genome.

The isolation of *PHYA*, *PHYB*, and *PHYC* allowed a determination of their map positions, which indicated that only *PHYB*, and not *PHYA* or *PHYC*, could be Ma_3 (Fig. 1). Additionally, the sequencing of these genes allowed their identification. Derived amino acid sequence comparisons indicate that sorghum *PHYA* and *PHYB* are most similar to other monocot *PHYA*s and *PHYB*, respectively (Table II). There are no full-length monocot *PHYC*s to which the sorghum *PHYC* can be compared. However, of the phytochromes from *A. thaliana*, sorghum *PHYC* is most similar to *A. thaliana PHYC* (Table II). Additionally, phylogenetic analysis indicates that the sorghum and *A. thaliana PHYC*s are more closely related to each other than to any other phytochrome (data not shown). To further confirm that these sorghum phytochrome genes have been identified correctly, the expression of the genes was examined in etiolated and green leaves of ma_3^R sorghum using RT-PCR (Fig. 3). Sorghum *PHYA* mRNA level was reduced in green but not in etiolated leaves. *PHYB* and *PHYC* mRNA levels were similar in both etiolated and green leaves. These results are consistent with the expression of *PHYA*, *PHYB*, and *PHYC* in other plants (Dehesh et al., 1991; Clack et al., 1994). Based on amino acid similarities and expression patterns, we conclude that we have correctly identified these sorghum genes as *PHYA*, *PHYB*, and *PHYC*, and we note that this is the first report of a complete *PHYC* sequence from a monocot.

It is now possible to state that the aberrant phenotype of *phyB*-1 sorghum is due to the lack of a 123-kD PhyB. This means that in both monocots and dicots, PhyB controls shoot elongation, chlorophyll content, and red-light-high-irradiance-response-mediated deetiolation (Childs et al., 1991, 1992, 1995; Goto et al., 1991; Devlin et al., 1992; López-Juez et al., 1992; McCormac et al., 1993; Reed et al., 1993; van Tuinen et al., 1995). This degree of conservation of developmental regulation between monocots and dicots is not surprising given that photoperception is so important to sessile, photosynthetic organisms that begin their lives below ground. However, it is surprising to find that PhyB deficiency in both SD sorghum and LD *A. thaliana* causes relative photoperiod insensitivity and early flowering (Pao and Morgan, 1986; Halliday et al., 1994; Childs et al., 1995). This observation can be explained if PhyB plays a common role in photoperiod sensing in both plants. Thomas and Vince-Prue (1995) suggest that a light-dominant (LD) response pathway and a dark-dominant (SD) response pathway coexist in plants to regulate photo-

periodism through the actions of phytochromes, and each pathway is coupled to the circadian oscillator but is out of phase with the other by 12 h. Whereas both response pathways are proposed to exist in most plants, only one would usually predominate, although either could be manifest under the proper conditions. This hypothesis offers an explanation of why PhyB regulates photoperiodism similarly in plants that respond oppositely to daylength. PhyB might activate or be a component of the SD response pathway, which is dominant in sorghum but latent in *A. thaliana*. The absence of PhyB in either plant would allow inappropriate activation of the SD mechanism under all daylengths. This hypothesis could also explain the nature of the quantitative class of photoperiodic plants, of which sorghum and *A. thaliana* are members. The existence of LD and SD pathways for inducing flowering would ensure that flowering would eventually take place regardless of the photoperiodic environment in which a plant finds itself. Additionally, the presence of multiple mechanisms to promote or inhibit flowering may provide an adaptive advantage to plants and explain how the various ecotypes of *A. thaliana* can flower at appropriate times at widely divergent latitudes. We believe that the photoperiodically insensitive phenotype of *phyB*-1 sorghum and *phyB A. thaliana* is consistent with the hypothesis that there are two photoperiod response pathways (Thomas and Vince-Prue, 1995).

Additional evidence exists supporting the presence of common means of inducing flowering in sorghum and *A. thaliana*. Far-red end-of-day treatments hasten flowering in both species. Additionally, GAs are involved in flowering in both sorghum and *A. thaliana*. In sorghum, GA levels fluctuate diurnally (Foster and Morgan, 1995). PhyB deficiency disrupts the rhythm of GA levels and results in an increase in GA concentrations. GA metabolism inhibitors can prevent flowering in *phyB*-1 sorghum and in *PHYB* sorghum grown under short days (Lee et al., 1995). However, exogenous GA_3 can only partially mimic the effect of short days when given to plants grown under long days (Morgan and Quinby, 1987; Lee et al., 1995). In *A. thaliana*, PhyB deficiency does not affect GA concentrations, but it does increase the sensitivity to GAs (Reed et al., 1996). The mutation *ga1* in *A. thaliana* prevents GA synthesis and completely abolishes flowering when plants are grown under short days. Plants with *ga1* will only flower under short days if provided exogenous GA_3 (Wilson et al., 1992). Clearly, GAs are important for flowering of both sorghum and *A. thaliana*, at least under short days.

It is also possible that PhyB does not interact with any LD or SD clock-related mechanism but responds directly to daylength itself. PhyB acting in the high-irradiance response mode could cause the buildup of a flowering inhibitor during the day. The concentration of this inhibitor would be greater under long days than under short days. The lack of PhyB would prevent the formation of the inhibitor under all photoperiods and hasten flowering. This model would still allow for the existence of a clock-related photoperiod sensor that would stimulate or inhibit flowering independently of PhyB. Such a model could also explain how *phyB*-1 sorghum still retains some photoperiod sensitivity (Childs et al., 1995). This model is consistent with the known behavior of *A. thaliana*. Given that *ga1* mutants flower under long days but not under short days, it is probable that the promotion of flowering under long days is distinct from that under short days. Thus, in *A. thaliana* there may also be distinct inhibitors of flowering under short days.

Several phytochrome actions are known to be involved in photoperiod sensing, and these separate actions may be mediated by phytochromes other than PhyB (Takimoto and Saji, 1984; Halliday et al., 1994; Johnson et al., 1994). *PHYA* and *PHYC* may also be involved in photoperiod sensitivity. In fact, PhyA-deficient *A. thaliana* lacks the response to far-red daylength extensions that normally hasten flowering (Johnson et al., 1994). It is therefore reasonable to assume that variations in phytochrome genes in addition to *PHYB* may be used by plants to regulate their photoperiodic responses. Now that these genes have been sequenced and mapped in sorghum and other cereals, their relationship to other maturity genes can be assessed. Figure 1 shows the synteny between sorghum chromosome 1 and *Z. mays* chromosomes 1, 5, and 9. The observations reported here both confirm and extend to *Z. mays* chromosome 9 the synteny reported by Paterson et al. (1995) for these same linkage groups. Moreover, Cordonnier-Pratt et al. (1994) have mapped *PHYA*, *PHYB*, and *PHYC* to loci at corresponding positions on rice chromosome 3, which together with the data reported here (Fig. 1) illustrates the synteny between sorghum linkage group A and rice chromosome 3. In addition, Phillips et al. (1992) have identified markers linked to QTLs that affect photoperiod sensitivity in *Z. mays*. Several of the QTL-linked markers that they have identified fall very near the phytochrome genes of *Z. mays* (Fig. 1). Some of the parents used by Phillips et al. (1992) may contain alleles of phytochrome that differ in their effect on photoperiod sensitivity. Additional analysis using *PHYA*-, *PHYB*-, and *PHYC*-specific markers in the *Z. mays* populations might clarify the roles of *PHYA* and *PHYC* in photoperiod sensitivity. A recent study in sorghum included phytochrome genes as markers in an analysis of QTLs that affect maturity (Paterson et al., 1995). No flowering QTLs were associated with phytochromes in sorghum in the single cross analyzed. The failure to find phytochrome-associated flowering QTLs in that study indicates either that the population used did not contain significant variation at these loci or that the overriding effect of the locus, which drastically affected photoperiod sensitivity (putatively Ma_1), obscured any contribution made by the phytochromes.

A small region of the last intron in the *PHYB* gene was found to have significant homology with sequences near or in several other sorghum genes. It is possible that this sequence represents a regulatory element such as the 3′ enhancer recently found in the immunoglobulin heavy chain gene from *Rattus rattus* (Mocikat et al., 1995). Alternatively, the region of the last intron from *PHYB* may simply be a footprint from a transposon or retrovirus excision event.

The work presented here is part of our continuing effort to understand the mechanism of photoperiod sensing in sorghum. As part of that effort we have undertaken to isolate genes that are known to be involved in photoperiodic control of flowering. We have demonstrated that Ma_3 is synonymous with *PHYB* and that mutations in *PHYB* can reduce photoperiod sensitivity in sorghum. It is now possible to state that photoperiod sensitivity in sorghum is controlled by at least six genes: Ma_1, Ma_2, *PHYB*, Ma_4, Ma_5, and Ma_6. The similar effects on flowering of mutations in *PHYB* from sorghum and *A. thaliana* leads to the conclusion that there are common pathways in the control of flowering in LD and SD plants. The degree to which these pathways have been conserved among higher plants remains to be determined.

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