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PHYTOCHROME FROM GREEN PLANTS:

ASSAY, PURIFICATION, AND CHARACTERIZATION

Progress Report

for the period:

July 1, 1987 – June 10, 1991

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CONTENTS

REPRINTS (5 ATTACHED)

not attached

PROGRESS REPORT

I. Introduction

This funding period was directed at developing an in-depth molecular analysis of the low-abundance, 118,000 M_r green-tissue phytochrome that had at that time been relatively recently identified as being distinct from the better characterized 124,000 M_r phytochrome abundant in etiolated tissue. The specific objectives as stated in the original proposal were:

a. To generate monoclonal antibodies specific to the 118,000 M_r green-tissue phytochrome.

b. To develop additional and improved procedures to permit progress toward the ultimate goal of purifying green-tissue phytochrome to homogeneity.

c. To initiate an alternative approach to determining the structural properties of green-tissue phytochrome by isolating and sequencing cDNA clones representing the 118,000 M_r green-tissue polypeptide in *Avena*. This approach is based on and will test the hypothesis that the 118,000 M_r polypeptide is encoded by a gene(s) distinct from those encoding etiolated-tissue 124,000 M_r phytochrome.

d. To utilize any such 118,000 M_r phytochrome specific cDNA clones as hybridization probes to begin to investigate the structure, organization, and regulation of the corresponding gene(s) in *Avena*.

e. To begin to investigate the possible presence in other higher plant and algal species of sequences homologous to the 118,000 M_r *Avena* polypeptide using the *Avena* clones as hybridization probes.

Most of these objectives have been accomplished, at least in principle, although the major breakthrough establishing that phytochrome is encoded by a multigene family came from the use of *Arabidopsis* rather than *Avena*. Similarly, much of the characterization subsequent to this discovery has been performed in *Arabidopsis* and rice as model dicot and monocot systems, respectively, rather than *Avena*.

II. Biogenesis of *Avena* Phytochrome

Using antibodies that detect both the 124- and 118-kDa *Avena* polypeptides, we quantified the relative levels of the two species during germination and early seedling growth in light and darkness. Both molecular species are present at about equal levels in the mature, quiescent embryo. During seedling development, the 124 kDa polypeptide accumulates $\geq$ 200-fold in darkness and is labile in the light, whereas the 118 kDa species remains at about initial levels and is unaffected by light. These data verify the well-established dark-accumulation and Pfr-lability of the 124 kDa etiolated-tissue phytochrome and suggest that the 118 kDa species is relatively stable in the Pfr form, a property of Type 2 phytochrome. See reprint, Tokuhisa and Quail (1987).

III. Partial Purification of 118 kDa Phytochrome from Green *Avena*

We wished to develop a purification scheme for the low abundance 118 kDa phytochrome (0.003% of soluble cellular protein) for two principal reasons. First, to generate at least partially purified preparations sufficiently enriched to enable monoclonal antibody production. Second, ultimately to perform biochemical characterization of homogeneous preparations. Although we have not yet obtained purified 118 kDa phytochrome, in approaching this problem we established three main points. First, green *Avena* tissue contains a different set of protease activities (thiol protease-like) than etiolated tissue (serine protease-like). Thus, PMSF used in prior studies is ineffective in preventing phytochrome proteolysis in green tissue extracts, whereas iodoacetamide and leupeptin are effective. Second, confusion in the literature regarding the molecular mass of green *Avena* phytochrome results at least partly from the fact that this molecular species undergoes changes in electrophoretic mobility under the different electrophoretic conditions used in different laboratories. Third, we have developed a procedure employing a novel chromatographic step which provides preparations with up to 200-fold enrichment of 118 kDa phytochrome free of proteolytic activity. See reprint, Tokuhisa and Quail (1989). Although this biochemical approach remains a viable one, we have chosen for the time being to focus our efforts on molecular approaches following the successful cloning of multiple phytochrome sequences (see below).

IV. Molecular Cloning of Multiple Phytochromes

A. *Avena*

We invested considerable effort over a 2 year period attempting to identify cDNA clones corresponding to the 118 kDa polypeptide from green *Avena* tissue. Over this period, a total of 8 cDNA libraries ranging up to 6×10^6 plaques in size from green *Avena* shoots were constructed in λgt11 or λzap vectors and screened using a number of strategies. Initially, we screened these expression libraries using both polyclonal and monoclonal antibodies previously generated against 124 kDa etiolated tissue phytochrome and known to recognize the 118 kDa band. A large number of positive clones obtained in this way were shown to be false positives upon further analysis using various techniques. We also screened these libraries using a synthetic oligonucleotide which we constructed based on a report from Dr. Lee Pratt's laboratory (7). Dr. Pratt has reported identifying a 6-amino acid sequence representing the epitope recognized by his "universal" monoclonal antibody that has been shown to react with green tissue phytochrome. We reasoned that the corresponding 18-nucleotide probe should hybridize to green- as well as etiolated-tissue phytochrome cDNA sequences. Unfortunately, the redundant probe that we synthesized recognized such a large number of clones that it was impossible to analyze them for true phytochrome sequences. Finally, we analyzed a series of positive clones identified by double-screening with antibodies and existing phytochrome hybridization probes without success.

B. *Arabidopsis*

Southern blot analysis of *Arabidopsis* genomic DNA with a *phyA* coding region probe under low stringency conditions provided the first indications of the presence of up to five divergent phytochrome genes in this species. We have isolated and fully sequenced cDNAs for three of these genes, designated *phyA*, *phyB*, and *phyC*, and shown that they are equally divergent from each other, exhibiting only 50% amino acid sequence identity. The *phyA* sequence has greater identity (≥ 65%)

with all available *phyA* sequences from other angiosperms, including monocots and dicots, than it does with the *Arabidopsis phyB* and *phyC* sequences. It appears therefore that the three phytochrome subfamilies diverged before the divergence of monocots and dicots. Because, where available, the *phyA* cDNA sequences correspond precisely to the etiolated tissue phytochrome amino acid sequences determined by microsequencing of purified preparations, it can be concluded that *phyA* genes encode Type 1 phytochrome apoproteins. By implication then, *phyB* and *phyC* must encode non-Type 1 phytochrome. See reprint, Sharrock and Quail (1989). We have also isolated and partially characterized genomic clones for each of the *phyA*, *phyB*, and *phyC* genes (R. Sharrock and P. Quail, unpublished) and in collaboration with Dr. E. Meyerowitz at CalTech have mapped these genes to chromosomes 1, 2 and 5, respectively.

C. Rice

Because of its diploid, reasonably sized genome and the regenerability of transformed plants, we have decided to utilize rice as our model monocot system for future phytochrome characterizations. Thus far, we have isolated a series of non-*phyA* genomic and cDNA clones from this species and have characterized the *phyB* sequence in some detail. This monocot polypeptide sequence is more closely related to the *Arabidopsis phyB* apoprotein (73% identity) than to all other available *phy* sequences, providing evidence that phytochrome in most, if not all, angiosperms is likely to be encoded by a small multigene family. See reprint, Dehesh *et al.* (1991). Preliminary genomic Southern blot and sequencing data indicate that rice, like *Arabidopsis*, probably has up to five divergent *phy* genes (K. Dehesh and P. Quail, unpublished).

D. *Mesotaenium*

We made several successive attempts during this funding period to clone *phy* sequences from the single celled green alga *Mesotaenium* known to exhibit phytochrome responses. Southern blot analysis of *Mesotaenium* genomic DNA indicated only very weakly hybridizing bands with all *phyA*, *phyB*, and *phyC* probes available, and attempts to screen a genomic library with these clones produced no algal *phy* sequences. Likewise, a more recent attempt using a PCR protocol with a nested series of degenerate oligonucleotides from highly conserved regions of the angiosperm sequences was also unsuccessful. Whether, these failures are due to technical problems or highly divergent algal phytochrome is unclear at present.

V. Type-Specific Monoclonal Antibody Production

The availability of *phyB* and *phyC* sequences has provided the opportunity to examine the behavior of the proteins they encode. Initially, we invested considerable effort in attempting to generate polyclonal antisera to synthetic peptides chosen to represent unique stretches of the individual phytochrome A, B, and C polypeptides. However, while all antisera tested show strong reactivity with the corresponding original synthetic peptides, for undetermined reasons they do not react with the full-length polypeptide. We therefore decided to overproduce the full-length polypeptide for each phytochrome type in *E. coli* and generate monoclonal antibodies. We have now identified a set of such antibodies which are specific for the individual phytochrome polypeptides encoded by *phyA*, *phyB*, and *phyC* in *Arabidopsis* (Fig. 1). We have also generated monoclonal

antibodies against rice phytochrome B which are type-specific but which cross-react with *Arabidopsis* phytochrome B (Fig. 2).

VI. Regulation of Phytochrome mRNA and Protein Levels

A. mRNA Levels

Using gene-specific probes, we have shown in both *Arabidopsis* and rice that, whereas *phyA* mRNA levels are negatively light regulated, *phyB* transcript levels are constitutive, unaffected by light. *Arabidopsis phyC* is also constitutively expressed. These results are consistent with *phyB* and *phyC* encoding Type 2 phytochromes and indicate that the regulatory mechanisms controlling *phy* gene expression have been conserved in parallel with the respective coding regions. See reprints, Sharrock and Quail (1989), Dehesh *et al.* (1991).

B. Phytochrome Protein Levels

The availability of type-specific monoclonal antibodies against *Arabidopsis* phytochrome B and phytochrome C polypeptides has enabled us to address the question of whether or not these molecules are labile or stable in the light *in vivo*. Whereas phytochrome A shows the expected decline in *Arabidopsis* seedlings transferred to light, phytochrome B remains constant (Fig. 3). Differences in proteolytic digestion patterns *in vitro* following red or far-red light irradiations of *Arabidopsis* extracts establish that the phytochrome B molecule does indeed carry a chromophore and undergoes photoreversible conformational changes (D. Somers and P. Quail, data not shown). Thus, phytochrome B exhibits Pfr stability *in vivo*, a characteristic of Type 2 phytochrome. The data for phytochrome C are less definitive thus far because of weak and variable immunoblot signals. Although the data in Figure 3 suggest relative stability of phytochrome C, other experiments indicate a decline in levels in the light (data not shown).

VII. Physiological Roles of Individual Phytochromes

A. Antisense

We have initiated studies aimed at selectively decreasing the levels of phytochromes A, B, and C using expression of antisense transcripts in transgenic *Arabidopsis*. Thus far, we have generated four sets of primary transformants (R0) containing antisense encoding sequences driven by the 35S CaMV promoter and directed either at the 5' untranslated exons plus intron, the coding region or the 3' untranslated region of the *Arabidopsis phyA* gene, or at the coding region of the *Arabidopsis phyB* gene. R1 transgenic seedlings and plants will be examined in the near future for altered phenotype and for expression of *phyA* and *phyB* mRNAs and protein.

B. Phytochrome B Deficiency in the *hy-3* Mutant of *Arabidopsis*

When the *Arabidopsis phyA*, *phyB*, and *phyC* clones were isolated, an initial survey of the corresponding transcript levels in the long hypocotyl (*hy*) mutants of *Arabidopsis* indicated that the *hy-3* mutant has reduced *phyB* mRNA levels (R. Sharrock and P. Quail, unpublished). We have now

verified this observation at the protein level using phytochrome B specific monoclonal antibodies (Fig. 4). It will be of interest to determine whether the *hy-3* mutant lacks an end-of-day FR response considered to be under the control of physiologically defined Type 2 or stable phytochrome (6).

VIII. Functional Dissection of Phytochromes B and C

A. Overexpression of Rice *phyB* and *Arabidopsis phyB* in Transgenic *Arabidopsis*

Prior to demonstrating that the *Arabidopsis hy-3* mutant is deficient in phytochrome B, we initiated a set of experiments directed at testing whether overexpression of *phyB* in *Arabidopsis* would induce a phenotype that could be used to dissect the functional domains of phytochrome B by site-directed *in vitro* mutagenesis. This strategy is working well for *Avena phyA* in a separately funded program in this laboratory (1). Thus far, we have been able to demonstrate that 35S CaMV promoter-driven overexpression of either rice or *Arabidopsis* full-length *phyB* sequences in transgenic *Arabidopsis* results in a short hypocotyl phenotype in light grown seedlings (Figs. 5, 6, 7, 8). The data establish that both monocot and dicot *phyB* polypeptides form spectrally and biologically active phytochrome in the dicot cells and provide a system for examining mutagenized *phyB* sequences. The phenotype is similar to that induced by *Avena phyA* overexpression (1) suggesting that, superficially at least, both types of phytochrome can affect similar cellular processes. We have established homozygous lines of short-hypocotyl phytochrome B overexpressors for further studies (Fig. 7).

B. *In Vitro* Production of Spectrally Active Phytochrome B and Phytochrome C from Polypeptides Synthesized in *E. coli*

In parallel with the above investigations, we have initiated studies to examine the structural properties of mutagenized phytochrome polypeptides *in vitro* following overexpression in *E. coli*. In these studies, we have exploited the pioneering observations of Lagarias and colleagues who showed that *Avena* phytochrome A apoprotein synthesized in an *in vitro* transcription-translation system forms a photoreversible holoprotein with phycocyanobilin, a phytochrome-chromophore analog (5). In preliminary experiments, we have produced full-length *Arabidopsis* phytochrome A, B, and C polypeptides in *E. coli* and found that a soluble fraction obtained from the bacterial cells is capable of forming a fully photoreversible product with phycocyanobilin in each case (Fig. 9) We do not know at this time why the phytochrome C spectrum is relatively weak, but it is reproducible. These data provide direct evidence that the polypeptides encoded by the *Arabidopsis phyB* and *phyC* cDNAs are capable of forming stable adducts with a tetrapyrrole in an apparently autocatalytic manner similar to that observed for *phyA* apoproteins. We are thus in a position to monitor the effects of various site-directed mutations on *in vitro* assembly and spectral properties of the B and C members of the photoreceptor family in parallel with their behavior in transgenic plants.

IX. Summary Statement: Contributions of Research Effort

This research has provided the first direct, molecular evidence for the existence of multiple, divergent species of phytochrome in plants. The conceptual framework and molecular tools that have emerged from this work open up new opportunities for phytochrome researchers to address a broad spectrum of questions of central importance to the field. In particular, the identification of an

Arabidopsis mutant deficient in phytochrome B, and the establishment of transgenic *Arabidopsis* and *in vitro* assembly systems for functional dissection of the various molecular species of the photoreceptor will provide access to the twin questions of the physiological roles and mechanism of action of individual phytochrome family members in the plant.

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XI. Figure Legends

Fig. 1. Type-specific monoclonal antibodies discriminate between *Arabidopsis* phytochromes A, B, and C. *Arabidopsis* phytochrome apoproteins A (track 1), B (track 2), and C (track 3) were overexpressed from their respective full-length cDNAs in *E. coli* and subjected to immunoblot analysis. Identical blots were probed with monoclonal antibodies (McAb) generated against *Avena* phytochrome A (top panel), *Arabidopsis* phytochrome B (middle panel), and *Arabidopsis* phytochrome C (bottom panel). The *Arabidopsis* antibodies were generated against phytochrome B and C apoproteins overexpressed in *E. coli*.

Fig. 2. Monoclonal antibodies directed against rice phytochrome B recognize rice and *Arabidopsis* phytochrome B but not phytochrome A or C. Rice phytochrome B (track 4), and *Arabidopsis* phytochromes A (track 1), C (track 2), and B (track 3) were overexpressed in *E. coli* and subjected to immunoblot analysis using a monoclonal antibody against rice phytochrome B. The antibody was generated against full-length rice phytochrome B apoprotein overexpressed in *E. coli*.

Fig. 3. Immunoblot analysis of the levels of *Arabidopsis* phytochrome A (top panel), B (middle panel), and C (bottom panel) in etiolated seedlings before (D) and after exposure to continuous light for 8 and 24 h as indicated. Identical blots of seedling extracts were probed with the three monoclonal antibodies analyzed in Fig. 1. The levels of the different phytochromes relative to one another cannot be deduced from this blot because the relative staining intensities between antibodies are not quantitative in this experiment.

Fig. 4. The *hy-3* long-hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. Immunoblot analysis of the levels of phytochromes A (top panel) and B (bottom panel) in etiolated wild type (W) and *hy-3* (h) *Arabidopsis* seedlings before (D) and after exposure to 6 and 24 h of red light as indicated. Identical blots were probed with the monoclonal antibodies directed against phytochromes A and B analyzed in Fig. 1. The levels of phytochromes A and B relative to each other cannot be deduced directly from the relative staining intensities shown.

Fig. 5. R1 transgenic *Arabidopsis* seedlings overexpressing rice (top) or *Arabidopsis* (bottom) phytochrome B segregate for hypocotyl length when grown in the light. Full-length rice or *Arabidopsis phyB* cDNAs under control of the 35S CaMV promoter were introduced into *Arabidopsis* using *Agrobacterium*-mediated root transformation. R1 seedlings from selfed primary transformants (R0) of single transgenic lines were analyzed for height segregation. The taller seedlings rising up above the background lawn of short seedlings are indistiguishable from wild type *Arabidopsis*.

Fig. 6. The segregation ratio is 3:1 in light-grown R1 transgenic *Arabidopsis* seedlings overexpressing phytochrome B. The frequency distribution of hypocotyl lengths is plotted for transgenic *Arabidopsis* seedlings generated as in Fig. 5 following transformation with (A) the empty T-DNA vector; (B) the rice *phyB* cDNA; and (C) the *Arabidopsis phyB* cDNA. Absolute numbers of short and long hypocotyls, and of kanamycin resistant (KanR) and sensitive (KanS) seedlings determined in separate experiments are boxed.

Fig. 7 Phytochrome B expression in homozygous transgenic *Arabidopsis* lines. Homozygous lines were established from both short and tall (= normal sized) transgenic seedling populations analyzed in Fig. 6, and light-grown homozygous seedlings were subjected to immunoblot analysis of

phytochrome B levels. Lane 1: Rice phytochrome B apoprotein overexpressed in *E. coli* (ER); Lane 2: Extract of tall line from rice phytochrome B transformation (RBO); Lane 3: Extract of short line from rice phytochrome B transformation (RBO); Lane 4: Extract of empty vector trangenic line (EV); Lane 5: Extract of short line from *Arabidopsis* phytochrome B transformation (ABO); Lane 6: Extract of tall line from *Arabidopsis* phytochrome B transformation (ABO); Lane 7: *Arabidopsis* phytochrome B apoprotein overexpressed in *E. coli* (EA). Identical blots were probed (A) with a monoclonal antibody directed against rice phytochrome B (see Fig. 2); and (B) with a preabsorbed polyclonal serum prepared against *E. coli*-synthesized rice phytochrome B. The polyclonal serum was preabsorbed with *Arabidopsis* phytochrome B produced in *E. coli* (i.e., the sample in Lane 7) to remove antibodies that cross-react with the *Arabidopsis* phytochrome B, while leaving antibodies specific to rice phytochrome B. The short hypocotyl phenotype is fully correlated with high levels of either rice (Lane 3) or *Arabidopsis* (Lane 5) phytochrome B expression. Spectral measurements and controlled *in vitro* proteolysis experiments indicate that the overexpressed phytochrome B molecules carry a fully active chromophore (data not shown). Endogenous *Arabidopsis* phytochrome B levels are barely detectable in the crude extracts used in these experiments.

Fig. 8. The short hypocotyl phenotype associated with rice and *Arabidopsis* phytochrome B overexpression is light dependent. EV = empty vector homozygous line; RBO = homozygous rice phytochrome B overexpressor; ABO = homozygous *Arabidopsis* phytochrome B overexpressor. This result establishes that photochemically and biologically active rice and *Arabidopsis* phytochrome B cause the phenotype.

Fig. 9. Self-assembly of photochemically active phytochrome *in vitro*. Soluble *Arabidopsis* phytochrome A, B, and C apoproteins, extracted following overexpression in *E. coli*, were incubated with purified phycocyanobilin chromophore according to Lagarias and coworkers (2, 3, 4, 5). Difference spectra were measured using sequential irradiations with red (R) and far-red (FR) light. Although weak, the *phyC* signal is reproducible and reversible.

FIG. 1

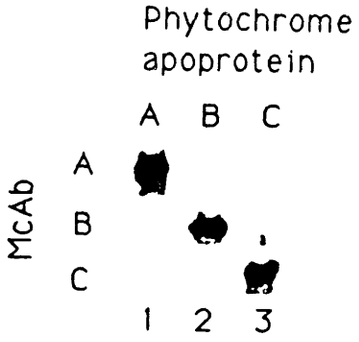

Phytochrome
apoprotein
A B C
McAb
A
B
C
1 2 3

FIG. 2

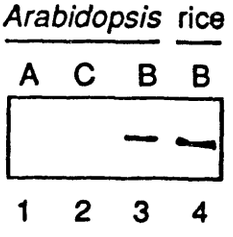

Arabidopsis
rice
A C B B
1 2 3 4

FIG. 3

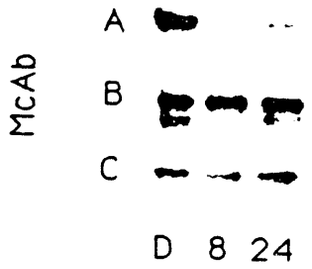


FIG. 4

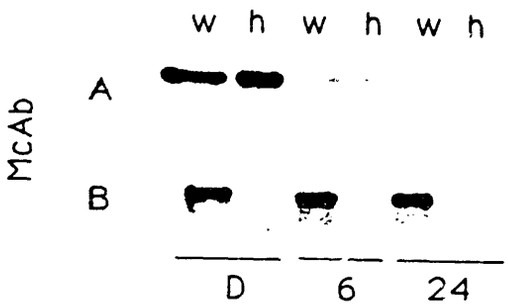


FIG. 5

A

B

FIG. 6

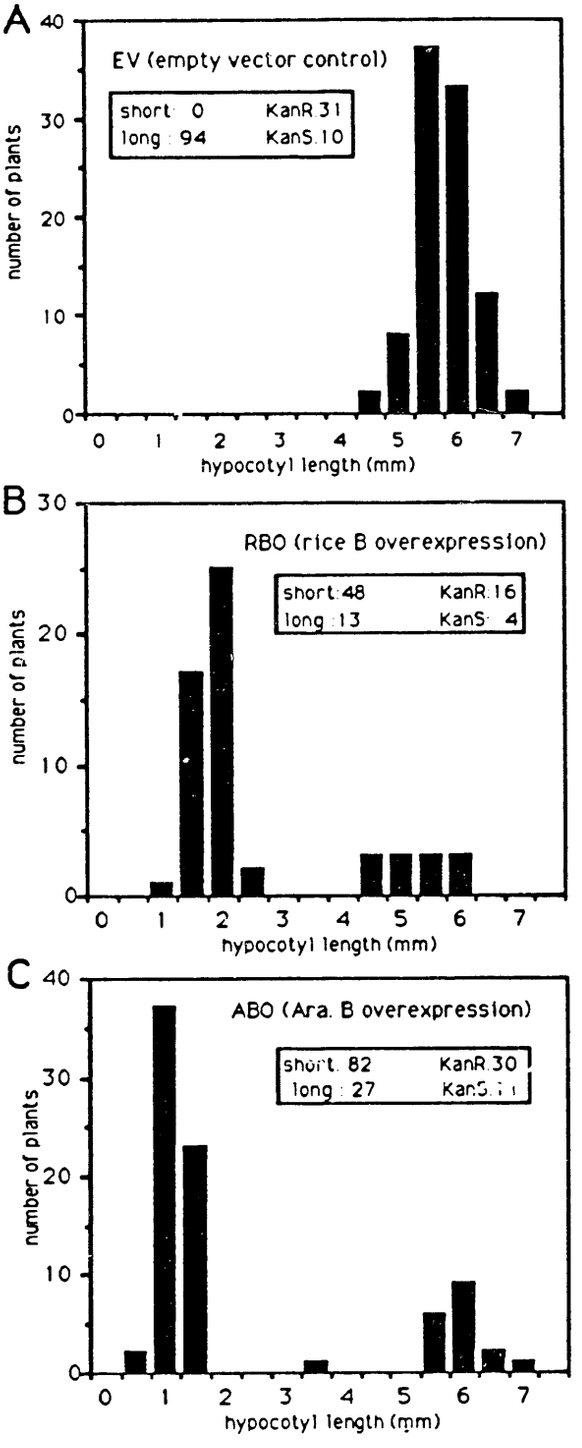

A
EV (empty vector control)
short: 0
long : 94
KanR.31
KanS.10
number of plants
hypocotyl length (mm)
B
RBO (rice B overexpression)
short:48
long :13
KanR.16
KanS: 4
number of plants
hypocotyl length (mm)
C
ABO (Ara. B overexpression)
short. 82
long : 27
KanR.30
number of plants
hypocotyl length (mm)

FIG. 7

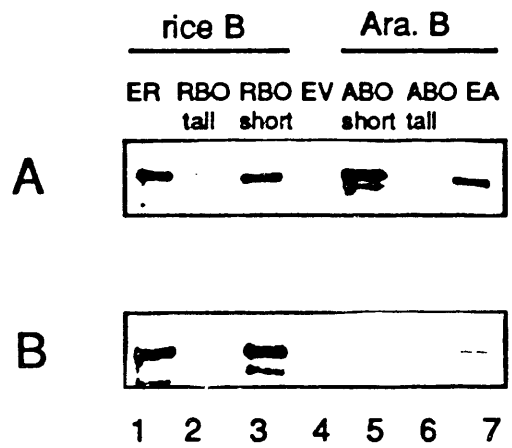

rice B
Ara. B
ER
RBO tall
RBO short
EV
ABO short
ABO tall
EA
A
B
1 2 3 4 5 6 7

FIG. 8

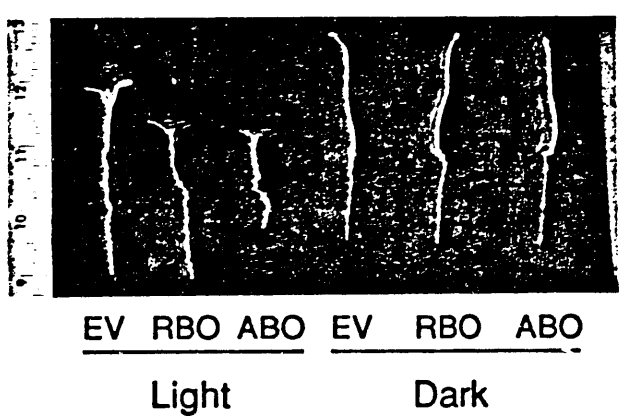
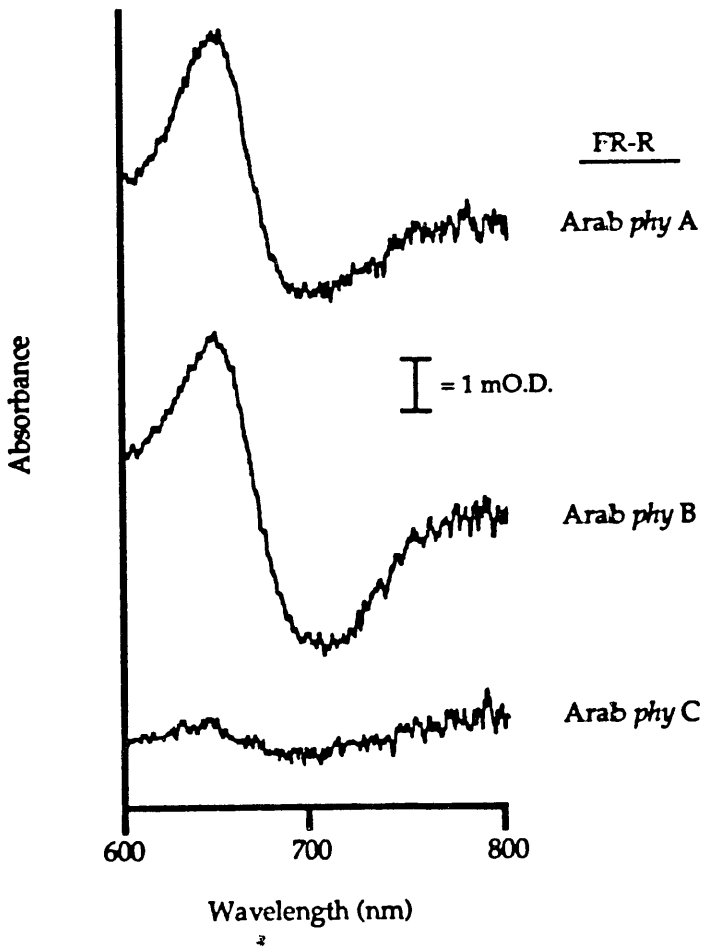

EV RBO ABO EV RBO ABO
Light
Dark

FIG. 9

Arabidopsis A,B, and C phytochromes self-assemble *in vitro*



XII. Publications Attributable to DOE Funding

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