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RESEARCH ARTICLE

The Cucumber Long Hypocotyl Mutant Lacks a Light-Stable PHYB-like Phytochrome

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A novel cDNA sequence homologous to a phytochrome B (*phyB*) gene that was isolated in a library from tobacco tissue has been used in an *Escherichia coli* expression system to raise anti-phytochrome B (anti-PHYB) polypeptide-specific monoclonal antibodies. The specificity of these antibodies has been tested by cross-reactivity against purified pea light-labile type 1 and light-stable type 2 phytochromes, with some antibodies reacting with the type 2 and none with the type 1 phytochromes. One such antibody, monoclonal mAT1, has been employed to analyze the phytochrome molecular species present in a photomorphogenic long hypocotyl (*lh*) mutant of cucumber. The results indicated that the mutant contains wild-type levels of the light-labile type 1 phytochrome polypeptide (PHYA), which has an apparent molecular mass of ~120 kD, but shows <1% (detection limit) of a light-stable polypeptide recognized by mAT1 in wild-type seedlings. This protein, not detectable in the *lh* mutant, has the properties of light-stable type 2 phytochrome, has an apparent molecular mass of 116 to 117 kD, and remains at constant levels under continuous low-fluence-rate red light. Therefore, we conclude that the *lh* mutant lacks at least one type 2 phytochrome-like polypeptide, most probably a *phyB* gene product. The correlation between the lack of this protein and the deficiency or absence of physiological responses to a light-stable phytochrome species in this mutant helps to identify the physiological roles played by the products of different subfamilies within the phytochrome gene family.

INTRODUCTION

Plant development is the result of the expression of endogenous programs (genes) and their modulation in response to environmental signals. Light is one of the most important signals modifying development with the aid of several photomorphogenic receptors, including phytochromes, blue/UV-A receptors, and a UV-B receptor (Liscum and Hangarter, 1991; Thomas and Johnson, 1991). Phytochrome is a dimeric biliprotein photoreceptor (Thomas and Johnson, 1991). Irradiation of previously dark-grown seedlings with light, most effectively in the red region of the spectrum, converts the chromoprotein to its biologically active form. Light also reverts the active form back to the inactive red light–absorbing one, most effectively in the far-red region of the spectrum. Responses under the control of this photoreceptor are in general red/far-red reversible (Shropshire and Mohr, 1983; Kendrick and Kronenberg, 1986; Furuya, 1987, 1989). The formation of the active phytochrome form in dark-grown tissue triggers phytochrome destruction (Cherry et al., 1991) and the down regulation of the expression of its own gene, now known as *phyA* (Lissemore and Quail, 1988). Simultaneously, many other genes are positively or negatively regulated by active phytochrome (Thompson and White, 1991).

Early physiological studies revealed responses that could be explained only on the basis of a phytochrome stable in the light (for review, see Furuya, 1989). Both a light-labile and a light-stable phytochrome pool could be detected by spectrophotometry (Brockmann and Schäfer, 1982). Subsequently, immunochemical investigations described two different phytochrome types in oat and pea (Abe et al., 1985; Hilton and Thomas, 1985; Shimazaki and Pratt, 1985; Tokuhisa et al., 1985). An important step forward was the finding that both phytochrome types differ in their amino acid sequence in pea and are, therefore, the products of different genes (Abe et al., 1989). The classically known light-labile phytochrome, predominant in etiolated tissue, decays in the light to levels that are

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very low in pea (Abe et al., 1985) or undetectable in oat (Pratt et al., 1991). This phytochrome has been named type 1 (Furuya, 1989). On the other hand, the light-stable phytochrome, which was revealed in light-grown tissue and described first immunochemically, was named type 2. In this article, we use the term type 2 to refer to any phytochrome whose level is principally light stable, as opposed to type 1, which is in principle labile in the light (even if a small fraction of this same molecular species is differently regulated in a particular compartment or tissue). It is increasingly clear, from work with oats, that there are several type 2 phytochromes (Wang et al., 1991). Therefore, green peas contain simultaneously type 1 and type 2 phytochromes (Konomi et al., 1987), whereas in green oats two type 2 but no type 1 phytochromes can be detected (Wang et al., 1991).

Another important discovery has been that at least three, and possibly five, different phytochrome (*phy*) genes exist in Arabidopsis: the three are *phyA*, *phyB*, and *phyC* (Sharrock and Quail, 1989), with *phyA* coding for phytochrome type 1 (Dehesh et al., 1991), *phyB* potentially coding for the predominant phytochrome type 2, and *phyC* potentially coding for another type 2 phytochrome polypeptide that is 10 to 20 times less abundant than the one encoded by *phyB* (Quail et al., 1991; Somers et al., 1991). Divergence of their sequence suggests that they represent subfamilies in the phytochrome gene family. Recently, *phyB* from rice has been sequenced (Dehesh et al., 1991), showing that *phyA* and *phyB* genes exist both in dicot and monocot species (Hersey et al., 1985; Sharrock et al., 1986; Kay et al., 1989; Sharrock and Quail, 1989). Whereas the two phytochromes described in green oats differ immunochemically between themselves and from the one predominant in etiolated seedlings (Wang et al., 1991), it is not known whether they are products of different genes. We will refer here to phytochrome as the spectrally active chromoprotein, whereas the usage of phytochrome gene product (PHY) will be restricted to the phytochrome apoprotein.

Much is known about the way phytochrome regulates the expression of different genes during the de-etiolation or first dark-to-light transition of a seedling (Thompson and White, 1991); nevertheless, the main role or evolutionary advantage of red/far-red reversible phytochrome in nature has been proposed to be the detection of vegetational shade (Smith, 1986) and proximity of other plants. In shade or plant-reflected light, the high levels of far-red radiation reduce the amount of the active phytochrome, which results in the complex "shade-avoidance response" (Smith, 1986). This response involves increased stem elongation, as well as additional changes in photosynthate partitioning and leaf performance, increase in apical dominance, and acceleration of flowering and senescence (López-Juez et al., 1990a).

Two photomorphogenic mutants that are physiologically very different from each other are the aurea (*au*) of tomato (Koornneef et al., 1985; Adamse et al., 1988b) and the long hypocotyl (*lh*) of cucumber (Adamse et al., 1987, 1988a); their characterization has led to the suggestion of separate functions for the different molecular species of phytochrome (for review, see Kendrick and Nagatani, 1991). It is of interest to ascertain whether such closely related proteins have separate biological roles. An end-of-day, far-red light, stem-growth reaction was exhibited by the *au* mutant (Adamse et al., 1988b; López-Juez et al., 1990b) but was absent in the *lh* mutant (Adamse et al., 1987, 1988a; López-Juez et al., 1990a). This end-of-day far-red treatment depletes active phytochrome during the subsequent dark period and mimics the action of vegetational shade-light. The *lh* mutant also failed to respond to continuous far-red light added to the hypocotyl during the light period (Ballaré et al., 1991). Furthermore, the complex shade-avoidance response has been illustrated by the end-of-day response of wild-type cucumber and appeared to be already saturated in the *lh* mutant (López-Juez et al., 1990a); it was suggested as being the specific role of a type 2 phytochrome. Recently, a small but significant response of the *lh* mutant to changes in the ratio of active phytochrome has been detected (Whitelam and Smith, 1991). In contrast to *lh*, the *au* mutant exhibits its most dramatic phenotype at the time of seedling de-etiolation (Adamse et al., 1988b).

To date, it remains unclear whether the *lh* mutant is a photoreceptor-deficient mutant or not. In vivo spectrophotometric studies (Adamse et al., 1988a; Peters et al., 1991) indicate that the bulk of spectrophotometrically detectable phytochrome in etiolated seedlings, of both the wild type and the *lh* mutant, undergoes rapid decay under red light, reaching a low stable level. This level in *lh* tissue is approximately half of that in the wild type, suggesting that a type 2 phytochrome is missing in the mutant. However, this observation apparently contradicts an earlier immunological study (Nagatani et al., 1989), in which similar amounts of total phytochrome (no distinction being made between molecular species) were extracted from de-etiolated wild-type and *lh* mutant seedlings.

Recently, in one of our laboratories, researchers (R. Kern, M. Deak, S.A. Kay, and N.-H. Chua, unpublished observations) have initiated the isolation and characterization of the different *phy* cDNA clones and the genes present in the tobacco genome. In this article, we report the use of a partial cDNA clone from tobacco, homologous to *phyB* in Arabidopsis and rice, in an *Escherichia coli* expression system, to produce protein for use as an antigen. Our aim has been to obtain monoclonal antibodies specific to PHYB (the phytochrome B polypeptide) and to use them to characterize the molecular species of phytochrome present in the *lh* mutant.

RESULTS

Production and Initial Characterization of Monoclonal Anti-Tobacco Phytochrome B Antibodies

Screening of a tobacco cDNA library resulted in the identification of a clone (pA2) encoding a DNA sequence related to phytochrome. The homology of the new *phy* gene was much higher to *phyB* from Arabidopsis and rice than to *phyA* sequences of any other related plant. The cDNA clone contained only the 3′ end of the coding region, as the predicted amino

Table 1. Sequence Similarity between the Polypeptides Used to Raise Antibodies against PHYA and PHYB, and Those Predicted for Several *phy* Genes[a]

phy Sequence	Tobacco PHYB[b] (mAT1-7)	Pea PHYA[c] (mAP5)
Rice *phyB*[d]	78.0	51.5
Arabidopsis *phyB*[e]	75.9	52.1
Rice *phyA*[f]	47.7	64.5
Pea *phyA*	51.0	100.0
Zucchini *phyA*[g]	51.8	78.1
Arabidopsis *phyA*[e]	51.2	79.1
Arabidopsis *phyC*[e]	48.0	52.0

[a] Percentage of sequence similarity between polypeptides calculated using the PROSIS 6.00 program of Hitachi Inc. (Tokyo).
[b] GenBank accession number M65023.
[c] Sato (1988).
[d] Dehesh et al. (1991).
[e] Sharrock and Quail (1989).
[f] Kay et al. (1989).
[g] Sharrock et al. (1986). (Zucchini is closely related to cucumber.)

acid sequence started at a position homologous to 489 for Arabidopsis and 490 for rice, and coded for the complete C-terminal region: 677 amino acids or ~58% of the full-length predicted polypeptide. Table 1 shows the percentage of the similarity of the predicted amino acid sequence of the tobacco clone (GenBank accession number M65023) with amino acid sequences deduced from several *phy* genes. These results support the observation of Dehesh et al. (1991) for Arabidopsis and rice; the *phyB* genes seem to have been even more conserved during evolution than *phyA* genes. The fact that the tobacco sequence is more similar to that of monocot rice than to the dicot Arabidopsis is probably due to the incompleteness of the tobacco clone, which codes only for the C-terminal region of the polypeptide.

Using the procedures of Tomizawa et al. (1991), developed to express pea *phyA* in *E. coli*, we transformed bacterial cells with the tobacco *phyB* cDNA clone. Because this clone codes for the C-terminal region of the protein, where homology among *phy* genes is low (Sharrock and Quail, 1989; Dehesh et al., 1991), antibodies against this *phyB* gene product are likely to be PHYB specific. The transformant of *E. coli* formed inclusion bodies during 24-hr growth in the presence of the inducer (see Methods). Figure 1 shows that these inclusion bodies contained large amounts of an inducer-promoted polypeptide, with a relative mobility of 72 kD in polyacrylamide gels after SDS-PAGE. The polypeptide was specifically recognized in immunoblot analysis by polyclonal antibodies raised in rabbit against total phytochrome from etiolated peas (Figure 1). To confirm the phytochrome nature of this polypeptide, inclusion body proteins were probed with the monoclonal antibody mAP5, which was raised against phytochrome from etiolated peas (Nagatani et al., 1984), and mAP11, which is specific for the dominant type 2 pea phytochrome (Konomi et al., 1987).

As Figure 1 shows, only mAP11 recognized the 72-kD polypeptide, showing it contains at least one epitope in common with a type 2 pea phytochrome. This polypeptide was used for immunization of mice and subsequent production of several monoclonal antibodies capable of staining the recombinant PHYB-homologous fragment in immunoblots (Figure 1). Figure 2 shows how one antibody specifically recognized a protein of ~117 kD in tobacco extracts. The antibody-producing hybridoma clone was named mAT1, for monoclonal anti-tobacco phytochrome antibody 1. The type 1 antibody mAP5 recognized a light-labile protein of 120 kD, reflecting the size of most type 1 phytochromes in other plant species ranging between 116 and 127 kD (Parks et al., 1987). Table 1 shows the high homology between the phytochrome largely dominant in the preparation used to raise mAP5 and other PHYA polypeptides from dicot species. The several smaller bands that are also

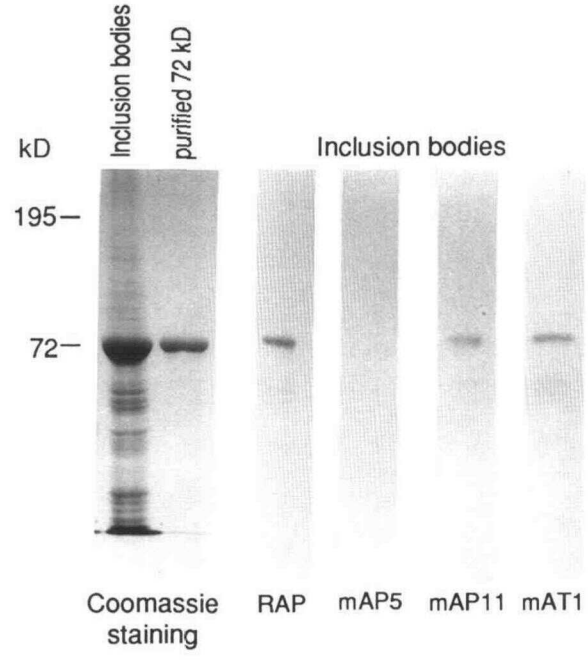

Figure 1. Reaction of mAT1 and Anti-Pea Phytochrome Antibodies with a Recombinant Tobacco PHYB Homologous Fragment Produced in *E. coli* Cells.

E. coli cells transformed with pA2 produced inclusion bodies. These inclusion bodies were purified, and the proteins in the preparation were fractionated (first lane) by SDS-PAGE using 7.5% (w/v) acrylamide concentration and then were stained with Coomassie blue. The second lane contains purified 72-kD polypeptide from the same preparation that was used to produce monoclonal antibodies. One microgram of 72-kD protein was loaded per lane (overload to show purity, resulting in apparently higher relative mobility). Four additional lanes were used for immunoblot analysis of inclusion bodies containing 25 ng (RAP, mAP5, mAP11 lanes) or 5 ng (mAT1 lane) of the 72-kD protein. The blots were stained using rabbit anti-etiolated pea phytochrome polyclonal antibodies (RAP), monoclonal antibodies anti–pea phytochrome type 1 (mAP5), type 2 (mAP11), or one of the newly raised antibodies against the 72-kD tobacco PHYB fragment (mAT1).

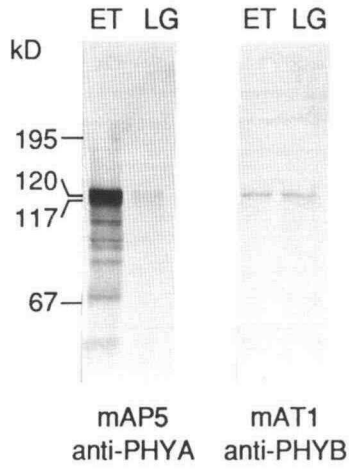

Figure 2. Recognition of PHYA and PHYB-like Phytochrome Polypeptide in Tobacco Extracts by Immunoblotting Using mAP5 and mAT1 Antibodies.

Tobacco phytochrome–enriched extracts were prepared as described in Methods. The proteins were fractionated in a 6.5% (w/v) acrylamide gel, electroblotted, and stained with mAP5 to recognize PHYA, and mAT1 to localize a PHYB homologous polypeptide. ET, tobacco etiolated seedlings; LG, light-grown seedlings that had received a 10-min red light pulse every 2 hr for 24 hr. Each lane contains extract corresponding to 150 mg fresh weight of tissue.

stained with mAP5 are most probably products of partial proteolysis because they undergo identical decay in the light. Because mAT1 recognizes an apparently smaller, light-stable protein, which reflects type 2 phytochrome characteristics, and was raised against a *phyB* gene product, we will refer to it during this report as PHYB (polypeptide product of the *phyB* gene), operationally being the mAT1-detectable phytochrome polypeptide. Likewise, PHYA will be referred to as the *phyA* gene product, operationally being the mAP5-detectable phytochrome polypeptide labile in the light.

Figure 3 illustrates further tests of the new antibodies raised against the tobacco PHYB fragment that complement the data in Figure 1. Two of the new clones, mAT2 and mAT5, cross-react with purified type 2 pea phytochrome, which was kindly provided by Hiroshi Abe (Abe et al., 1989), and none of them cross-reacts with purified native type 1 (Lumsden et al., 1985). Therefore, at least these two antibodies recognize a type 2 phytochrome.

Molecular Species of Phytochrome Present in the *lh* Mutant

We used two antibodies to discriminate between the two species of phytochrome in enriched protein extracts of the *lh* mutant and wild-type seedlings of cucumber. Figure 4 shows that in both dark-grown wild-type and *lh* mutant tissue, mAP5 detects a band with a relative molecular mass of ~120 kD. As expected, this band was strongly reduced in extracts from seedlings after a 24-hr intermittent red light treatment, consistent with the light lability of PHYA. Other minor bands in the blot probably correspond to degradation products of phytochrome because they increased with the prolonged handling of the samples necessary for protein concentration (data not shown). Dilution series indicated that after the light treatment for de-etiolation, this phytochrome molecular species was roughly 2% of that in dark-grown tissue (data not shown).

In contrast, mAT1 only recognized a protein of 116 to 117 kD in extracts from wild-type tissue that appears to be unaffected by light conditions (Figure 4). A different PHYB-raised antibody, mAT2, recognized a band with identical mobility to the one identified by mAT1 in immunoblots from light-grown, wild-type cucumber extracts. In addition, mAT2 reacted against a faint band from the cucumber extracts with the same mobility as that stained by mAP5. This faint band was detectable in higher amounts in etiolated tissue (Figure 4). Because this protein is most likely the type 1 protein, this weak cross-reactivity gives additional confirmation that the band specifically detected by mAT1 has some conserved epitopes with PHYA, identifying it as a phytochrome polypeptide. The remaining three new antibodies showed a similar staining pattern to mAT1 with extracts of the cucumber wild type and *lh* mutant. Data in Figures 3 and 4 indicate that at least mAT2, and probably mAT5, recognizes different epitopes compared to the rest of the mAT antibodies. The recognition of slightly different patterns of proteolysis products, inadvertently generated during the preparation of the seedling extracts, and the differential

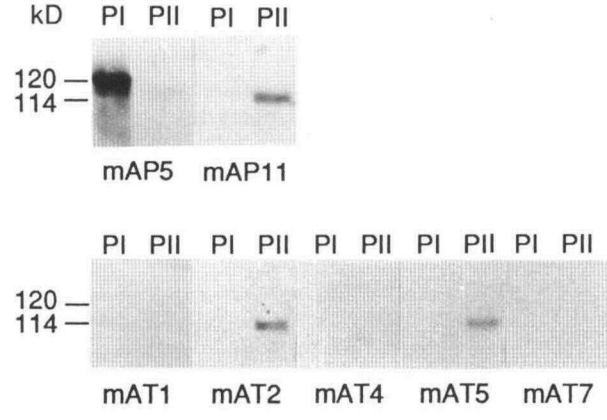

Figure 3. Cross-Reactivity of the mAT Antibodies to Purified Type 1 and Type 2 Pea Phytochrome.

Samples of authentic pea phytochrome, containing 10 ng of type 1 (PI) or 2.5 ng type 2 (PII, a gift from H. Abe) as estimated by spectrophotometry, were fractionated in a 7.5% (w/v) overrun polyacrylamide gel, electroblotted onto nylon, and stained using mAP5 (for PHYA), mAP11 (for type 2 phytochrome), and the mAT antibodies. Because type 2 phytochrome probably has a low extinction coefficient (Abe et al., 1989), the real amounts of PI and PII are approximately equivalent (see Methods).

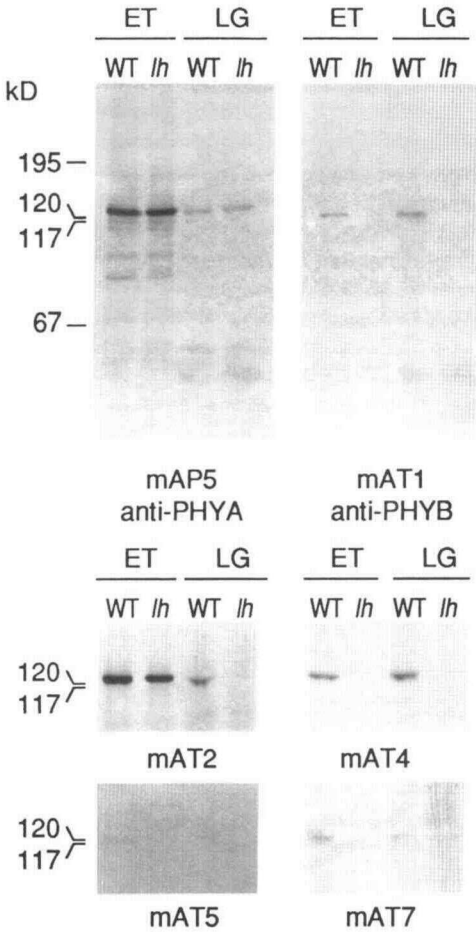

Figure 4. Analysis of PHYA and PHYB-like Polypeptide in the Crude Extract from Wild-Type and *lh* Mutant Cucumber Seedlings by Immunoblotting.

Proteins from phytochrome-enriched extracts of cucumber wild type (WT) and *lh* mutant were analyzed by immunoblotting, as in Figure 2. Cucumber etiolated (ET) and light-grown (LG) seedlings were used. The light treatment was as in Figure 2. Proteins in each lane correspond to 25 mg fresh weight of tissue.

cross-reactivity toward a polypeptide from a range of plant species are additional indications that these antibodies recognize at least two, and probably three, different epitopes on the PHYB-like protein (data not shown). The sharing of at least two epitopes between the original recombinant tobacco PHYB C-terminal fragment and the protein recognized by mAT1 in wild-type cucumber extracts argues for this protein also being PHYB. Nevertheless, we cannot exclude the possibility of it being another phytochrome showing a PHYB-type homology, or even being more than one protein with identical mobility in polyacrylamide gels. From these data, we tentatively conclude that both wild-type and *lh* mutant plants contain similar amounts of PHYA, but only the wild type contains at least one type 2 phytochrome, most probably PHYB, in detectable levels.

To rule out the possibility that PHYB-like polypeptide was not recognized in the *lh* phytochrome-enriched extracts because of different solubility or extractability characteristics, direct total protein extraction of mature green leaves into boiling electrophoresis sample buffer was performed (see Methods). Figure 5 shows that the mAT1 antibody recognized a protein in wild-type tissue, but no staining was observed in the mutant extract.

Figure 6 shows the minimum detectable levels of the phytochrome sharing PHYB homology in the cucumber extracts. A band could be recognized by mAT1 in a 1:30 dilution of the wild-type seedling extract, or 3% of the amount of PHYB in wild-type tissue. Furthermore, a very faint band can still be seen in the 1:90 dilution of the wild-type extract, with no staining at all in the extract from the *lh* mutant. We therefore conclude that *lh* seedling shoots contain <1% of the amount of the PHYB-like protein present in the corresponding wild-type tissue.

The amount of PHYB in wild-type extracts was estimated with reference to the immunostaining of known quantities of the purified recombinant 72-kD tobacco polypeptide expressed in *E. coli*, which corresponds to ~60% of the total PHYB molecular mass. Under conditions that extract soluble proteins, we obtained ~50 ng of PHYB-like protein per gram fresh weight of dark- or light-grown tissue in the cotyledons plus upper one-third of the hypocotyl where PHYB is most abundant (E. López-Juez, A. Nagatani, and M. Furuya, unpublished observations). This means that we extracted between 25 and 100 μg of PHYB per gram of protein in our preparations, depending on whether plants were grown in dark or light. The variation

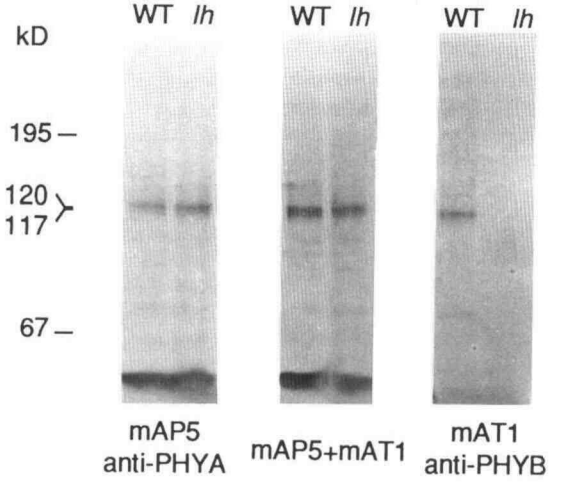

Figure 5. Detection of PHYA and PHYB-like Polypeptides in Hot SDS Extracts of the Wild-Type and *lh* Mutant Cucumber Green Leaves.

Hot SDS sample buffer protein extracts were prepared from fully light-grown mature leaves of wild-type (WT) and *lh* plants, as described in Methods. The extracts were analyzed by immunoblotting as in Figure 2. The antibodies used in each case are indicated beneath the figure. The concentration of mAP5 antibody was 0.7 μg/mL.

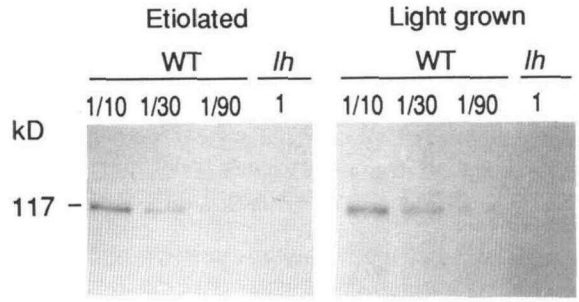

Figure 6. Detectability of PHYB-like Polypeptide in the Wild-Type and *lh* Mutant Cucumber Seedling Extracts.

Extracts of etiolated and light-grown wild type (WT) cucumber diluted to 1:10, 1:30, or 1:90 of their original concentration and corresponding undiluted (1) *lh* mutant extracts were analyzed by immunoblotting, as given in Figure 2, using the mAT1 antibody to determine the detection limit of PHYB-like protein in these extracts.

was found partly because total protein levels increased in the light. Because the mAT1 antibody reactivity is expected to be similar or weaker to cucumber PHYB-like protein than to the original antigen against which it was developed, this estimation must be regarded as a minimum value.

Effect of Light on PHYA and PHYB-like Content in Wild-Type and *lh* Mutant Plants

We attempted to obtain an approximate estimate of levels of PHYA and PHYB-like protein during phytochrome decay in the light using mAP5 and mAT1 in immunoblots. Figure 7 shows the results: the band recognized by mAP5 exhibits decay that is more or less completed within 4 hr, by which time destruction of the type 1 phytochrome pool has probably reached an equilibrium with resynthesis (Peters et al., 1991). Although not totally clear in this particular blot, the average of all the analyses showed indistinguishable decay kinetics in the wild type and the *lh* mutant, which argues that the band corresponds to the type 1 *phyA* gene product. On the other hand, Figure 7 shows that the anti-PHYB antibody recognizes a protein that is essentially light stable in wild-type seedlings, indicating that the PHYB homologous protein has the characteristics of a type 2 phytochrome.

Figure 8 shows that mAT1 recognizes a polypeptide that has an apparent molecular mass ~3 to 4 kD smaller than PHYA detected by mAP5, helping to further distinguish between these phytochrome species.

DISCUSSION

Because multiple phytochrome molecular types exist in a single plant species (Abe et al., 1989; Sharrock and Quail, 1989; Wang et al., 1991), efforts have been made to identify similar or different roles for the different types. The analysis of transgenic plants either overexpressing or repressing by antisense methods these specific genes may ultimately be required before the situation is resolved. However, properly characterized monogenic photoreceptor mutants have proved very useful to this end (Adamse et al., 1988a, 1988b; Chory et al., 1989; López-Juez et al., 1990a, 1990b; for reviews, see Furuya, 1989; Kendrick and Nagatani, 1991).

The aim of this work was to characterize the *lh* mutant of cucumber in this respect because it had been previously proposed that it lacks a light-stable type of phytochrome response or the type 2 photoreceptor itself (Adamse et al., 1988b; Peters et al., 1991) and that this lack results in a plant exhibiting a saturated shade-avoidance response (López-Juez et al., 1990a). The results reported here provide direct evidence that the *lh* mutant is a photoreceptor mutant and contains <1% of the wild-type levels of at least one type 2 phytochrome, the *phyB*-like gene product.

The relationship between different phytochrome types, characterized at the level of proteins (type 1 and type 2), genes (*phyA*, *phyB*, and *phyC* at least), and physiological pools (light-labile/light-stable) remains obscure (Furuya, 1989). From these and previous data, some conclusions can be drawn. Similarity of amino acid composition (Vierstra and Quail, 1986) and red light down regulation of its gene expression (Sharrock and Quail, 1989) has led to the acceptance of the idea that the most thoroughly studied, type 1 phytochrome is identical to the product of *phyA* genes, which have homologies of between 60 and 79%. Nevertheless, this statement has usually been avoided. In addition, the degradation kinetics of our operationally defined PHYA band are consistent with those of the bulk phytochrome present in etiolated cucumber measured by spectrophotometry: a half-life of 30 min at 25°C, reaching a stable level after 4 hr of continuous low-fluence-rate red light (Peters et al., 1991).

The first protein sequence data of the most abundant type 2 phytochrome in green pea (Abe et al., 1989) suggest homology with the product of the *phyB* gene in Arabidopsis (Sharrock and Quail, 1989). Two of our PHYB-raised antibodies cross-react specifically with pea type 2 phytochrome. The *lh* mutant, deficient in a spectrophotometrically detectable type 2 phytochrome (Peters et al., 1991), appears to lack at least a phytochrome polypeptide that corresponds to PHYB. It is certainly probable that type 2 phytochrome from light-grown tissue results from a combination of different species, but the correlation between data in this article and spectrophotometric measurements on the mutant (Peters et al., 1991) and the fact that Abe et al. (1989) found PHYB homology for type 2 phytochrome from light-grown pea suggest that the phytochrome B species should be quantitatively dominant. It seems unlikely that our new antibody mAT1 stains a phytochrome species different from PHYA or PHYB. We cannot rule out the possibility of one or more different phytochrome species being absent in the mutant. Nevertheless, as mentioned in Results, our antibodies recognize at least two different epitopes in the

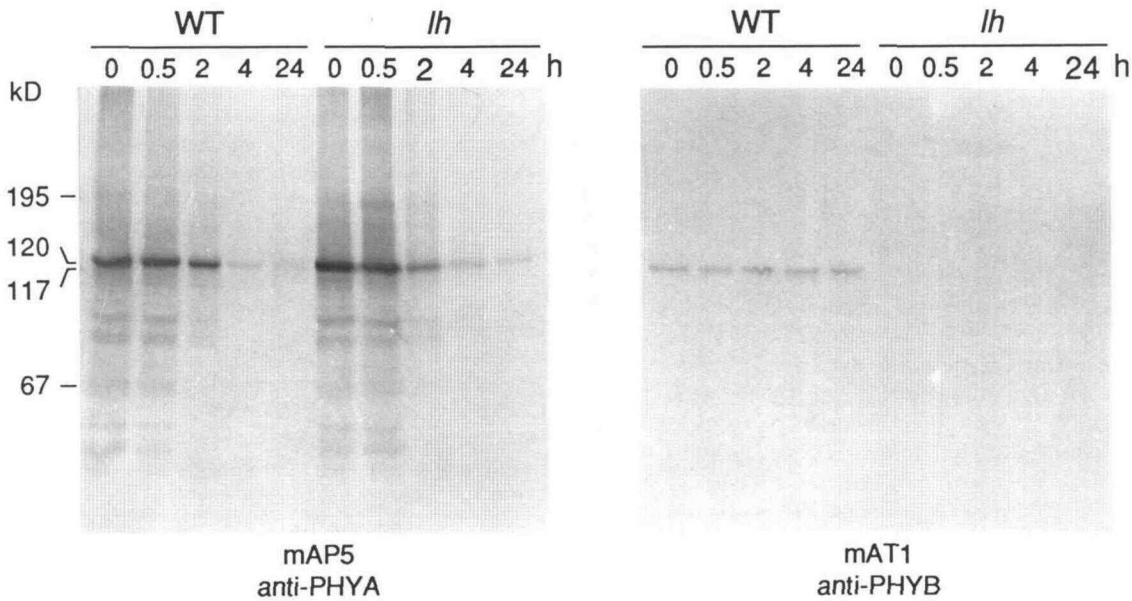

Figure 7. Difference in Light Stability of PHYA and PHYB-like Polypeptides in the Wild-Type and *lh* Mutant Cucumber Seedlings Monitored with mAP5 and mAT1 Antibodies.

Cucumber wild-type (WT) and *lh* mutant seedlings, previously grown in the dark, were exposed to a low-fluence-rate red light treatment. At the times indicated above each lane in hr (h), extracts were prepared from the seedlings. These extracts were analyzed by immunoblotting as shown in Figure 2. Each lane contains proteins equivalent to 50 mg fresh weight of tissue from a pooled sample of two independent experiments.

same polypeptide, relating it to the protein against which the antibodies were raised: that is, against a PHYB C-terminal fragment. The quantitative predominance of phytochrome B species in green tissue, although not as strong an argument, gives some additional support to the conclusion that the polypeptide that mAT1 fails to recognize in the *lh* mutant is PHYB.

Furthermore, in our hands the detected PHYB-like polypeptide has a slightly smaller apparent molecular mass than PHYA,

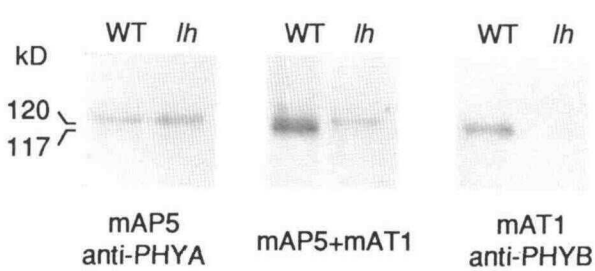

Figure 8. Difference in Apparent Molecular Mass of PHYA and PHYB Polypeptides in Cucumber.

Extracts from cucumber wild-type (WT) and *lh* mutant seedlings submitted to the light treatment, as shown in Figure 2, were fractionated by SDS-PAGE using a 10% (w/v) acrylamide gel, electroblotted, and stained to detect PHYA with mAP5 or PHYB with mAT1 or both mAP5 and mAT1 antibodies. The concentration of mAP5 antibody (0.7 μg/mL) was chosen to obtain equivalent staining intensity of the two protein bands resolved.

despite the fact that it arises from a *phyB* coding region, whose polypeptide would be predicted to be slightly larger than PHYA (Sharrock and Quail, 1989). It has similarly been reported that the most abundant type 2 phytochrome extracted from light-grown tissue exhibits a slightly lower apparent molecular mass than type 1 phytochrome: 6 kD lower both in oat (Tokuhisa and Quail, 1989) and pea, in which it shows homology to predicted PHYB (Abe et al., 1989). Nevertheless, apparent molecular sizes of phytochromes have been shown to vary according to experimental conditions (Cordonnier et al., 1986), and, in oats, for example, it is possible that two 124- and 118-kD species (Tokuhisa and Quail, 1989) are equivalent to two 125- and 123-kD species (Wang et al., 1991).

To our knowledge, no direct information has been published on the expression of *phyC* or other *phy* genes and the possible physiological roles of their products. PHYA appears to behave normally in the *lh* mutant, indicating that there is no reason to believe that the mutation is involved in some general pathway (such as chromophore biosynthesis) essential for the production of all phytochromes. However, we cannot absolutely exclude the possibility that putative phytochrome C or others are missing in the mutant.

Because the *lh* mutation was induced by γ-ray irradiation (Van der Knaap and de Ruiter, 1978), a deletion resulting in the complete absence of the *phyB* gene or parts of it might have occurred. Experiments are currently in progress to study this possibility. Other hypothetical reasons for the deficiency of PHYB in the *lh* mutant include a point mutation of *phyB* gene

leading to the production of an unstable polypeptide, missense mutation altering the reading frame, a mutation or deletion in the regulatory region preventing the expression of the gene, or the deficiency of a type-specific prosthetic group or post-translational modification step, resulting in an apoprotein that is rapidly turned over.

The absence of a type 2 *phy* gene product correlates with the *lh* phenotype that is apparently almost saturated for the shade-avoidance response, even though the PHYA polypeptide appears to be normal and present in both the *lh* mutant and wild-type plants. Interestingly, *phyB* exhibits a degree of conservation among two dicot (Arabidopsis and tobacco) and one monocot (rice) species that is even slightly higher than that of *phyA* (Sharrock and Quail, 1989; Dehesh et al., 1991; data in Table 1), suggesting an important physiological function for this distinctive phytochrome species. The phytochrome species deficient in the *lh* mutant appears to play a role in the control of plant morphology, involving not only stem elongation but also many other developmental responses, ranging from changes in leaf performance to apical dominance (López-Juez et al., 1990a). However, the comparison of the photosynthetic performance on a chlorophyll basis and the similarity of leaf soluble protein levels and of the chlorophyll *a*/*b* ratio between the *lh* mutant and wild type make it unlikely that this phytochrome species plays a dominant role in regulation of the expression of ribulose bisphosphate carboxylase small subunit, chlorophyll *a*/*b* binding protein, or other photosynthesis-related genes (López-Juez et al., 1990a). This result is in contrast to the tomato *au* mutant that is deficient in the bulk type 1 pool (Sharrock et al., 1988; López-Juez et al., 1990b). Therefore, further characterization of this and other mutants will assist in unraveling the roles played by products of the different subfamilies within the phytochrome gene family in plant development.

METHODS

Production of Antibodies to the PHYB C-Terminal Fragment

Escherichia coli XL1/Blue was transformed with plasmid pA2, and the transformants were grown on Luria-Bertani medium (standard techniques according to Sambrook et al., 1989), at 30°C with slow shaking, in the presence of 10 mM isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque, Kyoto, Japan), to induce expression of the *phyB* sequence under the control of the *lacZ* promoter. After 24 hr, the *E. coli* cells were found to contain inclusion bodies, which were purified according to Marston (1987). The purified inclusion body fraction was resolved by preparative SDS-PAGE according to Laemmli (1970), using prestained molecular mass standards (SDS 7B markers; Sigma). The apparent molecular mass of these prestained markers was recalibrated against standard and low molecular weight nonstained electrophoresis calibration kits (Bio-Rad and Pharmacia LKB Biotechnology Inc., respectively). A phytochrome (PHY) homologous fragment was detected as a 72-kD, isopropyl-β-D-thiogalactopyranoside–induced band that was stained in immunoblots by rabbit polyclonal antibodies anti-pea type 1 phytochrome. The 72-kD band was then sliced from gels and eluted with an electroelution kit (Atto, Tokyo, Japan) according to manufacturer's instructions, using an elution buffer of 20 mM glycine and 2.5 mM Tris. The protein was quantified through Coomassie Brilliant Blue G 250 staining of a dilution series on polyacrylamide gels.

BALB/c mice were immunized subcutaneously four times at 2-week intervals with 20 μg of protein in elution buffer to which 300 mM NaCl was added and mixed 1:1 with Freund's complete adjuvant. One week after an intraperitoneal boost injection, the spleen was collected, and fusion to murine myeloma cells was carried out as described previously (Nagatani et al., 1983). The dot blot technique with 0.1 μg of protein per dot was used for the screening of hybridoma cell lines (Nagatani et al., 1987). Specificity of the antibodies was confirmed by immunoblotting, as described below. Seven hybridoma lines produced antibodies that recognized a light-stable band, the size of phytochrome, in tobacco extracts. Five of them also reacted with cucumber extracts. After cloning of the line corresponding to mAT1, hybridoma were grown in 50-mL flasks in RPMI 1640 culture medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA), and the supernatants of this culture medium were used as the antibody preparation for immunoblotting. Hybridoma culture supernatants were also used for monoclonal antibodies mAT2, mAT4, mAT5, and mAT7.

During the characterization of the new monoclonal antibodies, samples of authentic phytochrome type 1 (Lumsden et al., 1985) and type 2 (Abe et al., 1989) were used. The original samples were quantified spectrophotometrically using the known extinction coefficient for type 1 phytochrome in both cases. Different amounts of type 1 and type 2 were employed for electrophoresis to compensate for the fact that type 2 probably has a reduced extinction coefficient (Abe et al., 1989).

Plant Materials and Growing Conditions

For tobacco extracts, 200 mg of tobacco cv SR1 seeds were surface-sterilized and sown on wet filter paper in Plantcon plastic boxes (Flow Laboratories). After 24 hr of imbibition in the dark, germination was induced by a 10-min red light pulse. Seedlings were grown at 25°C in the dark for 7 more days (etiolated seedlings) or 6 days plus 24 hr under a de-etiolation treatment (light-grown seedlings, see below).

The *lh* mutant and near isogenic wild-type cucumber (*Cucumis sativus*) plants used in this study have been described previously (Adamse et al., 1987) and are shown in Figure 9. The mutant fails to respond to end-of-day far-red light pulses, a stable phytochrome response that triggers a shade-avoidance response in the wild type, but has the appearance of a plant already saturated for this response (López-Juez et al., 1990a). The mutant was originally isolated in the progeny of γ-ray irradiated cucumber plants of complex hybrid origin, which were believed to be an interspecific cross (Van der Knaap and de Ruiter, 1978). Fifteen cucumber seeds were surface-sterilized and grown as the tobacco seeds, but did not require a red light pulse to induce germination. Seedlings were grown at 25°C for 5 days in darkness (etiolated) or 4 days in darkness plus 24 hr under the de-etiolation treatment (light grown).

The de-etiolation treatment consisted of 10-min red light pulses of 3 W m^{-2} every 1 hr and 50 min of darkness, for 24 hr. This treatment minimized chlorophyll synthesis and change both in morphology of the seedling and in the pattern of proteins resolved by SDS-PAGE. Red light was obtained from "daylight" white fluorescent tubes (FL20.S; Toshiba, Tokyo, Japan) in combination with a red acrylic filter (Shinkolite A 102; Mitsubishi Rayon, Tokyo, Japan). For the kinetic experiment of phytochrome destruction (Figure 5), continuous red light was used, at 25°C and an irradiance of 1 W m^{-2}.

Figure 9. Phenotype of the Cucumber *lh* Mutant and Its Isogenic Wild-Type Plant with and without End-of-Day Far-Red Light Treatment.

(**Left**) Cucumber *lh* mutant.
(**Right**) Wild type.
Ten-day-old seedlings were treated for 20 days under a daily regime of 14 hr of white fluorescent light (25 W m^{-2}) per 10 hr of darkness with (FR) or without (D) 20-min far-red light exposure at the end of the day. The treatment with far-red light mimics the response exhibited to vegetational shade-light.

Preparation of Tissue Extracts

The upper half (tobacco) or one third (cucumber) of the seedlings (hypocotyl, including cotyledons) were harvested and irradiated on ice with 5-min red light exposure. This converts ~87% of total phytochrome present to the far-red light–absorbing form, which is more resistant to proteolysis. After freezing in liquid nitrogen, 2 g original weight of tissue plus 0.2 g of insoluble polyvinylpyrrolidone (Polyclar AT; Gokyo-Sangyo Co., Osaka, Japan) were added to 2 mL of phytochrome extraction buffer A (Grimm and Rüdiger, 1986), also containing 52 mM 2-mercaptoethanol. The buffer contains various protease inhibitors. After homogenizing using a blender (Physcotron, Niti-on Co., Tokyo, Japan) at full speed for 1 min, the extract was filtered through four layers of cheesecloth and centrifuged at 1000*g* for 10 min. Phytochrome in the supernatant was further concentrated. The supernatant was carefully collected without the upper lipid layer, polyethyleneimine was added to a final concentration of 0.04% w/v (tobacco) or 0.1% (cucumber), the extract was vortexed, and the precipitation of acidic materials was allowed for 15 min. After centrifugation at 12000*g* for 10 min, phytochrome in the supernatant was precipitated by adding either 25% (weight/initial volume) solid ammonium sulfate, in several steps, or 0.725 volumes of saturated ammonium sulfate solution (equivalent final concentration). Precipitation was allowed for 1 hr, and the precipitate was collected by centrifugation at 12000*g* for 15 min, directly resuspended into electrophoresis sample buffer (Laemmli, 1970), and dissolved by boiling for 2 min. When the extracts were stored, they were also in sample buffer. Protein was quantified by Bio-Rad protein assay, using 1 μL of sample per milliliter of reagent, and 1 μL of sample buffer in the blank.

Direct hot SDS–sample buffer extraction was as follows: 100-mg portions of fully light-grown leaves were dipped into liquid nitrogen, transferred to a microcentrifuge tube also containing liquid nitrogen, and partially homogenized. One hundred microliters of 2 × sample buffer at 90°C was added immediately after nitrogen evaporation. The sample was vortexed and allowed to stand for 2 min at 90°C, rehomogenized with a Physcotron for 10 sec, and boiled for 2 min, transferred on ice, and centrifuged at 12000*g* for 2 min; the supernatant was immediately loaded onto the polyacrylamide gel.

All procedures were performed at 4°C under very dim green light as described by Nagatani et al. (1989).

Immunoblotting

After separation by SDS-PAGE according to Laemmli (1970), proteins were electroblotted onto a nylon filter (Fineblott; Atto, Tokyo, Japan) in 100 mM Tris, 192 mM glycine, 20% (v/v) methanol. The membranes were blocked in a series of Tris buffer/saline Tween (TBST) solutions, all containing 20 mM Tris, pH 7.5, and varying Tween-20 and NaCl concentrations, and are as follows: first 2% (v/v) Tween, 500 mM NaCl for 3 min, followed by 15 min in 0.05% Tween, 500 mM NaCl, and 10 μg/mL rabbit IgG (Sigma), and then 10 min final wash in 0.05% Tween and 150 mM NaCl. Incubation with the primary antibody was in 50 mM Tris, pH 7.5, 200 mM NaCl, and 1% (w/v) fat-free milk powder. Unless stated, the following dilutions were used: 2.5 μg/mL of mAP5 to stain type 1 phytochrome (Nagatani et al., 1984), 2.5 μg/mL of mAP11 for type 2 phytochrome (Konomi et al., 1987), or a 1:1 dilution of hybridoma culture supernatant for all the mAT antibodies. This incubation was at 4°C overnight, followed by 1 hr at room temperature. After washing three times with TBST, as at the end of blocking, membranes were incubated with a 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Protoblot kit; Promega) for 1 hr at room temperature, washed, and stained for alkaline phosphatase according to the manufacturer's instructions.

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