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PHYTOCHROMES IN PHOTOSYNTHETICALLY COMPETENT PLANTS

PROGRESS REPORT

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SCIENTIFIC BACKGROUND

INTRODUCTION

Plants utilize light as a source of information in photomorphogenesis and of free energy in photosynthesis, two processes that are interrelated in that the former serves to increase the efficiency with which plants can perform the latter. Only one pigment involved in photomorphogenesis has been identified unequivocally, namely phytochrome. The thrust of this proposal is to investigate this pigment and its mode(s) of action in photosynthetically competent plants.

Phytochrome is a dimeric chromoprotein with a linear tetrapyrrole chromophore attached via a thioether bond to a specific cysteine residue in each monomer (Vierstra and Quail, 1986). It is synthesized in an inactive, red-light-absorbing form (Pr; $A_{\max} \approx 660-670$ nm) that upon absorption of light is photoconverted to an active, far-red-light-absorbing form (Pfr; $A_{\max} \approx 730$ nm). Similarly, when Pfr absorbs light it is photoconverted back to Pr. As a result, the proportion of phytochrome present as Pfr is a function of the wavelength distribution of incident radiant energy, especially as modified by other green plants (Smith and Whitelam, 1987). Thus, not only does phytochrome mediate de-etiolation as a seedling emerges from the soil, but it modulates growth and development of plants throughout their life span (Kendrick and Kronenberg, 1986; Pratt and Cordonnier, 1989). For example, plants effectively compete for available solar energy by increasing elongation growth in response to the presence of other green plants, whether above or beside them (e.g., Smith and Whitelam, 1987; Ballaré *et al.*, 1990; Smith *et al.*, 1990), as though they were trying to escape from the shade of the other plants and thereby capture a greater proportion of incident radiation.

Although phytochrome plays an important role in modifying the photosynthetic capacity of green plants, little is known about this chromoprotein in such plants for two reasons. (1) It is up to two orders of magnitude less abundant than in etiolated tissue. (2) The chlorophyll in light-grown plants prevents spectrophotometric assay of phytochrome. Thus, only phytochrome from etiolated tissues has been characterized in detail. Nevertheless, it has long been hypothesized that phytochrome in light- and dark-grown plants might differ from one another (e.g., Hillman, 1967). Consequently, it has been important to determine whether different populations of phytochrome do exist. Until recently (Tokuhisa and Quail, 1983), however, there has been no substantial characterization of phytochrome from green plants. Since then, it has become clear that the phytochromes predominating in green and etiolated tissues are different proteins deriving from different genes. Moreover, we have most recently discovered that there are at least two phytochromes in green oats, both of which differ from that which predominates in etiolated tissue (Wang *et al.*, 1990; Pratt *et al.*, 1991). It is therefore evident that much remains to be learned about phytochrome in photosynthetically competent plants, including the most basic information possible — how many phytochromes there are. Presumably because of difficulties associated with purification of phytochrome from green plants, however, very few research groups are working with the chromoprotein from such tissues.

Etiolated- and green-plant phytochromes — definitions. The phytochromes that predominate in dark- and light-grown plants will be referred to as 'etiolated-' and 'green-plant phytochromes,' respectively. These terms do not imply that either etiolated or green plants contain only one type of phytochrome. Evidence indicates, in fact, that this is not the case (Tokuhisa and Quail, 1987; Konomi *et al.*, 1987). Etiolated- and green-plant phytochromes are the type I and type II phytochromes of Abe *et al.* (1985). As discussed in the Progress Report, it is now also evident that green-plant phytochrome itself, at least in oat, consists of two types. Specific reference to these two will be via their apparent monomer sizes (i.e., '125-kDa' or '123-kDa' green-oat phytochrome).

RATIONALE AND LONG TERM OBJECTIVE

Other groups are working with green-plant phytochrome, in particular those of Quail and Furuya. Quail's is using *Arabidopsis* as a source of green-plant phytochrome genes (Sharrock and

Quail, 1989), while Furuya's is working with pea (Abe *et al.*, 1989). Rather than compete directly with these groups, we propose developing complementary information utilizing our own background and tools developed over the past several years. Our background includes extensive experience with both biochemical and biophysical investigations of phytochrome, while the tools include not only antibodies, but also both genomic and cDNA clones for oat and tomato phytochromes. In this section, I will describe the rationale behind each of the three immediate objectives of this proposal and will summarize our long term objective towards which these immediate objectives are oriented.

How many phytochromes? As already noted, green-plant phytochrome differs from the etiolated-plant phytochrome that had long been characterized (Tokuhisa *et al.*, 1985; Shimazaki and Pratt, 1985; Abe *et al.*, 1985). It thus became necessary to reevaluate prior work with phytochrome. Within the past year, we have discovered that there is yet a third phytochrome, at least in oats (Wang *et al.*, 1990; Pratt *et al.*, 1991). Again, it becomes necessary to re-evaluate prior work. This situation is reminiscent of that related to the growing size of the phytochrome monomer, which required that at each step along the way much work had to be repeated, and earlier work reevaluated. Rather than continue to define the phytochrome system repeatedly, it therefore seems best to invest resources now in a rigorous effort to establish the total number of phytochromes that exist in a representative dicotyledon and monocotyledon.

Model systems. A second objective derives from the need to utilize model systems that offer an optimal compromise among criteria for working with phytochrome, as discussed below (page 4). It is self evident that systems chosen two or more decades ago were selected when little consideration was given to molecular and/or genetic approaches. It is not surprising, therefore, that oat would now make a poor choice, because of its hexaploid genome and inability to be stably transformed. Care should be taken, however, that newly selected systems will be useful in an integrative sense, permitting work at all levels, and from as many perspectives as possible. Thus, a major aspect of this proposal will be the selection and development of both a dicotyledonous and a monocotyledonous model system, with the immediate emphasis being on exploitation of the dicotyledonous system for reasons to be explained below (pages 8, 10). It is these model systems that will be used for achieving objective 1.

Expression system for green-plant phytochromes. The third objective is to develop what amounts to an expression vector for making relatively large quantities of green-plant phytochromes, and to begin characterization of the green-plant phytochromes they produce. These phytochromes are otherwise present at such low levels that their independent characterization *in vitro* is exceptionally difficult, if not effectively impossible.

Long term objective. Our long term objective is to characterize phytochrome and its functions in photosynthetically competent plants from molecular, biochemical and cellular perspectives. It is anticipated that others will continue to contribute indirectly to these efforts at the physiological level. The ultimate goal will be to develop this information from a comparative perspective in order to learn (1) whether the different phytochromes have significantly different physicochemical properties, (2) whether they fulfill independent functions and if so what these different functions are, and (3) how each of the different phytochromes acts at primary molecular and cellular levels.

IMMEDIATE OBJECTIVES

- 1) Determine the full repertoire of the phytochrome gene family in both a selected monocotyledon and a selected dicotyledon.
- 2) Exploit tomato as a model dicotyledon in which to investigate phytochrome and its biology in a photosynthetically competent organism.
- 3) Isolate green-rice phytochrome coding regions, express them at a high level in tomato, and initiate physicochemical characterization of the chromoproteins they produce.

BACKGROUND AND PROGRESS TO DATE

Phytochrome-mediated morphogenesis in light-grown plants

Phytochrome as a sensor of light quality. Plant growth and development is modified in several ways via phytochrome when it senses a change in wavelength distribution, monitored primarily as the ratio of red to far-red light (Smith, 1986). The parameter that has received the greatest attention has been the rate of internode elongation. Phytochrome has two independent effects on stem extension rate: a rapid, transient effect, with a lag time of about 10 min, and a more persistent effect that is smaller in magnitude. The rapid effect is apparently mediated by phytochrome in the stem itself (Morgan *et al.*, 1980; Child and Smith, 1987), while the persistent effect is mediated by phytochrome in the leaves (Casal and Smith, 1988). Whether these two effects result from differences in transduction chains, or from different phytochromes in the two tissues is an intriguing question. The work proposed here should provide information bearing on this question as it relates to characterization of multiple green-plant phytochromes. As pointed out by Smith (1986), it will be necessary to understand green-plant phytochromes much better than is now the case in order to gain a fuller understanding of how plant growth and development is regulated by incident radiant energy.

Mutants. Recent evidence obtained with mutants is consistent with the existence of at least two phytochromes (Kendrick, 1988). Of special interest here is a tomato mutant at the *aurea* locus, which accumulates in etiolated seedlings less than 5% as much phytochrome as wild type (Parks *et al.*, 1987), even though it contains normal levels of phytochrome mRNA (Sharrock *et al.*, 1988). Both molecular and physiological characterizations of this mutant are consistent with the hypothesis that it is defective in etiolated-, rather than green-, plant phytochrome (Adamse *et al.*, 1988b; López-Juez *et al.*, 1990). Conversely, Adamse *et al.* (1987, 1988a) have proposed that a long hypocotyl mutant of cucumber is deficient in green-plant phytochrome, although recent evidence indicates this might not be the case (Nagatani *et al.*, 1989). Additionally, investigation of long hypocotyl mutants of *Arabidopsis* that fail to accumulate spectrophotometrically detectable phytochrome in etiolated seedlings (Koornneef *et al.*, 1980; Parks *et al.*, 1989; Chory *et al.*, 1989a) has led to the conclusion that phytochromes in etiolated and light-grown plants play different roles (Chory *et al.*, 1989a). Thus, initial data obtained with mutants are consistent with the suggestion that plants contain at least two phytochromes, each of which contributes differently to the overall regulation of photomorphogenesis. It will be essential, as proposed here, to define fully the number of different phytochromes before proceeding much further with the analysis of mutants such as these. Not to do so would prevent one from making critical use of them, because without this information, and without probes specific for each of the phytochromes, it will be impossible to correlate organismal data with what happens at molecular and cellular levels.

Etiolated-plant phytochrome

As already noted, virtually all that we know about phytochrome derives from characterization of etiolated-plant phytochrome, which will serve as a frame of reference to which green-plant phytochrome will be compared (see Pratt and Cordonnier, 1989, for additional information and references). Each monomer of etiolated-plant phytochrome has a molecular mass of *ca.* 120–127 kDa. Genomic and cDNA clones have been isolated and sequenced for phytochrome from oat, rice, pea, zucchini, maize and *Arabidopsis* (see Appendix Fig. 2 for references). Considerable information has also been obtained about the structure/function relationships of its protein moiety, and much is known about its photoconversion pathways and photoequilibria. Etiolated-plant phytochrome undergoes three reactions in addition to that which leads to its biological activity. Following photoconversion of Pr to Pfr, the rate of turnover of etiolated-plant phytochrome increases markedly. This so-called destruction of phytochrome results from enhanced proteolytic degradation of its apoprotein. A second reaction is the nonphotochemical reversion of Pfr back to Pr. The third is the red-light-induced pelletability and sequestering of phytochrome as Pfr, which results from the photoreversible redistribution of this chromoprotein within the cell.

Green-plant phytochromes

Properties. Monomer sizes of green- and etiolated-plant phytochromes are similar (Abe *et al.*, 1985; Tokuhiya *et al.*, 1985; Fig. 4 in Wang *et al.*, 1990). Both etiolated- and at least 123-kDa green-oat phytochrome behave as a dimer in solution (Cordonnier *et al.*, 1986b; Tokuhiya and Quail, 1989). While green- and etiolated-pea phytochromes have essentially identical Pr — Pfr absorbance difference spectra (Abe *et al.*, 1985), green- and etiolated-oat phytochromes differ significantly (Tokuhiya *et al.*, 1985; Figs. 3, 4 in Pratt *et al.*, 1990a). The 123-kDa green-oat phytochrome undergoes a terminal proteolytic cleavage, which results in an 8-kDa mass decrease and which occurs much more rapidly than does the N-terminal cleavage of etiolated-oat phytochrome (Cordonnier *et al.*, 1986b). In addition, the 123-kDa green-oat phytochrome undergoes a Zn^{2+} -induced mobility shift during SDS PAGE, as though it might be a Zn^{2+} -binding protein, although this point is far from being established (Wang *et al.*, 1990). As in the case of etiolated-plant phytochrome, the Pr forms of 123- and 125-kDa green-plant phytochromes behave as though they were soluble, cytoplasmic proteins. Apart from this limited information, almost nothing is known about green-plant phytochromes. Virtually nothing is known about the proportion of phytochrome present as Pfr at photoequilibrium in red light or about the pathways or kinetics of its photointerconversions. Similarly, essentially nothing is known about the nonphotochemical reactions of green-plant phytochromes. Based upon physiological and other data, it is thought that they neither undergo rapid Pfr-mediated destruction (e.g., Brockmann and Schäfer, 1982) nor reversion (Smith, 1986), although direct and unequivocal tests of these assumptions remain to be made. And, it is not known whether green-plant Pfr undergoes either red-light-enhanced pelletability and/or sequestering. Objectives 1 and 3 are intended to serve as essential first steps in resolving these and other questions, objective 1 by defining the different phytochromes with which one must work independently, and objective 3 by providing the individual phytochromes in sufficient quantity for their characterization.

Different genes. Etiolated- and at least one green-plant phytochrome derive from different genes, as indicated by the four partial amino acid sequences that Abe *et al.* (1989) obtained for green-pea phytochrome. It appears likely that all four sequences probably belong to the same gene product, comparable to that putatively produced by the *phyB* gene of *Arabidopsis* (Sharrock and Quail, 1989; see Pratt *et al.*, 1991, for discussion). A 19-residue sequence has also been obtained for a peptide derived from 125-kDa green-oat phytochrome, leading to the comparable conclusion that it derives from a different gene than that which encodes etiolated-oat phytochrome (Fig. 2 in Pratt *et al.*, 1991). It is presently unknown whether 123-kDa green-oat phytochrome derives from yet a third gene, although this seems likely given recent data of Sharrock and Quail (1989) describing three phytochrome (*phy*) genes in *Arabidopsis*. One of these three (*phyA*) is closely related to other etiolated-plant phytochrome genes while the other two (*phyB* and *phyC*) are divergent, not only from *phyA*, but also from each other. Expression of *phyA* is like that of other etiolated-plant phytochrome genes, while expression of *phyB* and *phyC* is what would be expected if they were green-*Arabidopsis*-phytochrome genes. It is tempting to assume that *phyB* and *phyC* encode phytochromes homologous to the 125-kDa and 123-kDa green-oat phytochromes. This assumption, however, remains to be tested. Work described below is intended to resolve this question.

Model systems for characterization of green-plant phytochromes

Criteria. The exceptionally low abundance of phytochrome in green plants, together with the need to work with relatively large quantities of etiolated tissue for many applications, severely limits choices of model systems. While as noted oat would not now be selected, its ability to yield large quantities of both etiolated and light-grown tissues remains an important criterion. In addition, it is important to select plants that are diploid, readily transformable, have a small genome, are genetically well characterized, are of agronomic significance, are in use as model systems for other applications, and are at least somewhat characterized with respect to phytochrome-mediated physiology.

Tomato as a model dicotyledon. While *Arabidopsis* is an excellent model system, it suffers here because it cannot provide the etiolated tissue that will be needed, as our own experience has documented (Chory *et al.*, 1989a,b). Pea is a second possibility, especially since phytochrome is better characterized from this plant than from any other dicotyledon. Nevertheless, it is not readily transformable, which is a fatal flaw. And, while cauliflower, which has been reported to contain substantial amounts of phytochrome in its achlorophyllous inflorescence, also appears useful, at least superficially, it too has serious limitations. At least some attempts to extract phytochrome have been unsuccessful (personal communications), and unlike seedlings the inflorescence is not readily amenable to experimental manipulation. We have instead chosen tomato as the ideal compromise (see Nevins and Jones, 1987, for discussion and references). (1) It is readily transformable and regenerable. (2) Among such plants, it is unique in that its seed are large, thereby readily providing even kilogram quantities of etiolated tissue. (3) Its genome is small (1–2 pg per haploid genome), contains little repetitive DNA, is diploid, and is distributed on only 12 chromosomes (Rick, 1974; Zamir and Tanksley, 1988). (4) An extensive RFLP map is being developed (Zamir and Tanksley, 1988). (5) A large number of tomato mutants are in the public domain (Rick, 1974, 1987). (6) It is a useful recipient of transgenic phytochrome, even from a monocotyledon (Boylan and Quail, 1989). (7) Several physiological responses under phytochrome control have already been described in tomato (*e.g.*, Piringner and Heinze, 1954; Mancinelli *et al.*, 1966; Mancinelli, 1990). (8) At least one phytochrome-related mutant (*aurea*) has been described and characterized (see page 3). (9) In collaboration with Dr. Michel Caboche, we are planning to develop in tomato a selectable marker that will permit identification of phytochrome structural gene mutants and mutants in a transduction chain leading from Pfr action to modulation of gene transcription. (10) Tomato is of agronomic importance and is in widespread use as a model system for several applications. Thus, development of methods important to present and future work with this plant will be going on in many laboratories, thereby facilitating our own progress.

Rice as a model monocotyledon. Two obvious candidates exist for a monocotyledonous model system: rice and maize. Each is diploid, each is genetically well characterized, each is readily amenable to biochemical investigations of phytochrome, and each is in widespread use. Rice is the preferred system, largely because of its 10-fold smaller genome and because of the extensive resources currently being put into its development as a model system (Swaminathan, 1986; Swaminathan and Oka, 1990). Because rice is not yet readily transformable in non-expert hands (*e.g.*, Transformation Techniques session in Swaminathan and Oka, 1990), except for objective 1 and as a source of green-plant phytochrome genes for objective 3, our primary emphasis at present will be on exploitation of tomato as a model system.

Preliminary results

Phytochrome clones. In recent years we have developed, independently and in collaboration with CIBA-GEIGY, both genomic [λ DASH (oat), λ FIXII (tomato), λ GEM-12 (tomato)] and cDNA [2 λ gt11 (oat), λ ZAP (oat), λ ZAPII (tomato)] libraries. The cDNA libraries were prepared from size-enriched poly(A)⁺-RNA isolated from both etiolated and green seedlings. From these libraries we have already obtained numerous cDNA and genomic clones for etiolated-oat phytochrome, as well as five genomic clones for tomato phytochrome. Experience gained in the preparation and screening of these libraries will facilitate construction (when necessary) and screening of libraries to obtain new clones.

Transformation. The graduate student for whom support is being requested (Mr. Bernard Hauser) was responsible for the preparation of a tomato genomic library and for the isolation of tomato phytochrome clones. He intends to complete this work by identifying, isolating, and characterizing clones for each of the tomato phytochrome genes (objectives 1 and 2). He has also initiated transformation of UC82B tomato, achieving 5 to 10% efficiency with *Agrobacterium*-mediated transformation of cotyledons, followed by regeneration.

Probes. Nucleic acid probes are readily available from existing clones. Both polyclonal and monoclonal antibodies specific to monocotyledonous phytochrome are available (Cordonnier and Pratt, 1982; Cordonnier *et al.* 1984, 1985; Pratt *et al.*, 1990b). Conversely, we have already prepared polyclonal antibodies to pea, zucchini and lettuce phytochrome (Cordonnier and Pratt, 1982), and have 17 MAbs to pea and 9 to soybean phytochrome (Cordonnier *et al.*, 1984, 1986a; unpublished data). Among these antibodies are several that recognize tomato phytochrome well, and some that are specific for tomato, as opposed to monocotyledonous, phytochrome.

PCR and cloning phytochrome genes. We are developing a PCR-based strategy for amplifying and cloning low abundance DNA, either genomic or derived from mRNA. Because we propose to make extensive use of this strategy, it will be presented in detail below (page 7).

SIGNIFICANCE

Conversion of solar to chemical energy via photosynthesis is unique because it is an indefinitely renewable and nonpolluting resource. The importance of understanding photosynthesis itself is therefore self evident. It is equally important, however, that we also understand the fundamental sensory mechanisms that influence the efficiency with which plants capture radiant energy and use it in photosynthesis. Phytochrome is the best understood photosensory system in plants and, by extrapolation, knowledge about phytochrome in photosynthetically competent plants will be exceptionally important with respect to optimizing the creation of new biomass. The work proposed here will contribute significantly to our understanding of phytochrome and how it functions in photosynthetically competent plants.

While the importance of phytochrome control of morphogenesis has not been as extensively investigated in the field as it has been in the laboratory, enough information is available to indicate its practical significance. For example, Kasperbauer and Karlen (1986) have shown that field-grown wheat detects the density at which it is grown via phytochrome-sensed changes in the red to far-red ratio of light incident upon them, and that they respond by modifying partitioning of photosynthate, as do plants in the laboratory (Keiller and Smith, 1989). Similarly, field-grown soybean detects in addition to its density whether it is in North-South or East-West rows, again via phytochrome-sensed changes in red to far-red light ratios (Kasperbauer *et al.*, 1984; Kasperbauer, 1987). The result is a significant effect on seed (and therefore crop) yield. Hunt *et al.* (1989) and Decoteau *et al.* (1989) more recently indicate that via phytochrome plants can even sense and respond to the wavelength distribution of light reflected upwards from the soil surface. For example, the yield of fresh-market tomatoes is in part a function of the color of ground cover over which they are grown. There can be little doubt, therefore, that phytochrome plays a central role in regulating the efficiency with which plants create biomass and partition it to harvestable tissue.

Apart from this direct contribution to better understanding of photomorphogenesis in light-grown plants, the work proposed here involves applications of PCR technology to the characterization of genes that are only weakly expressed. Work proposed here should make this methodology more readily available for other, comparable applications. Thus, our work will not only contribute to a discrete scientific problem, but in doing so will develop methodology with broader application.

Proprietary material.
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