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MECHANISM FOR THE SELECTIVE CONJUGATION OF UBIQUITIN TO PHYTOCHROME

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The long term goal of this project is to understand at the molecular level how phytochrome functions and how intracellular proteins are degraded. The purpose of this research was to characterize the form-dependent degradation of phytochrome as a model system for the study of selective protein breakdown. Phytochrome exists in two photo-interconveretible forms, a red-absorbing Pr form and a far-red absorbing Pfr form. It is synthesized as Pr with a half-life of > 100h but upon photoconversion to Pfr, the half-life of the chromoprotein decreases to 1-2h. Recent evidence indicates that selective breakdown of phytochrome in etiolated oat seedlings occurs by a ubiquitin-dependent proteolytic pathway. Ubiquitin is a 76 amino acid eukaryotic protein that is covalently ligated to proteins destined for catabolism and serves as recognition signal for proteases specific for ubiquitin-protein conjugates.

In an attempt to understand why Pfr and not Pr is recognized by the ubiquitin pathway, we characterized ubiquitin-phytochrome conjugates (Ub-P) with respect to their kinetics of accumulation, localization within the cell, and sites of ubiquitin attachment. In accord with our hypothesis that Pfr is degraded by Ub-P intermediates in etiolated oat we found that: (i) accumulation of Ub-P was dependent on the amount of Pfr degraded; (ii) using pulse chase studies with light, Ub-P turnover was much faster than Pfr (20 min versus 80 min); (iii) degradation of "cycled Pr" like that of Pfr also coincides with Ub-P formation and; (iv) Ub-P were located primarily in the pelletable fraction of phytochrome which is the form preferentially lost during Pfr degradation (Jabben *et al.*, 1989a).

We also examined Pfr degradation in etiolated seedlings from a variety of other plant species (corn, rye, pea and zucchini squash) for their ability to form Ub-P during Pfr degradation. This approach was based on the assumption that a common mechanism is used in plants to degraded the photoreceptor. Like oats, these species also formed Ub-P during Pfr degradation and lost Ub-P rapidly after photoconversion of Pfr to Pr (Jabben *et al.*, 1989b). Thus, Ub-P formation appears to be a general phenomemon during Pfr degradation.

In an attempt to map the attachment sites of ubiquitin to phytochrome, Ub-P were partially purified from red-light irradiated oat seedlings and characterized (Shanklin *et al.*, 1989). It is possible that the identification of such sites will help explain why Pfr is selectively conjugated and thus explain the form dependence of phytochrome degradation. While Ub-P appear to have spectral properties similar to unmodified phytochrome, its native size is substantially larger (600 versus 360 kDa) suggesting that the attached ubiquitins extend significantly from the chromoprotein's surface. Both proteolytic mapping techniques and immunorecognition studies with a library of phytochrome monoclonal antibodies identified several possible sites for ubiquitin attachment (Shanklin *et al.*, 1989). One site of interest is between residues 747 and 830 because it is adjacent to domain(s) that become more exposed after photoconversion of Pr to Pfr. Ubiquitin attachment to phytochrome as with all target proteins requires access to a free lysyl epsilon amino group. Several lysine-containing domains within this region are conserved

among phytochromes from different species which may represent conserved ubiquitin ligation sites.

While characterization of the structure and pool dynamics of Ub-P will provide strong correlative evidence concerning the relationship of ubiquitin conjugation with Pfr degradation, it will not provide definitive proof. To provide such proof and allow us to more precisely define ubiquitin attachment sites, a transgenic system for phytochrome degradation was developed. This system involved the insertion of an intronless etiolated oat phytochrome gene attached to the CaMV 35S promoter into tobacco using *Agrobacterium* transformation vectors. With this system, we were able to produce high levels of the oat chromoprotein that is functional in tobacco (Keller *et al.*, 1989). Such expression resulted in over a five fold increase in spectrally active chromoprotein in etiolated and green tissues. Interestingly, this over expression led to an altered phenotype of the transgenic tobacco, with the plants now attaining an exaggerated "light-grown" phenotype. Phenotypic changes include, darker greener leaves, semi-dwarfism, reduced apical dominance, delayed leaf senescence, and insensitivity to the spectral quality of light (Keller et al.,1989; Cherry *et al.*, 1991; McCormac *et al.*, unpublished). Despite elevated levels of most photosynthetic enzymes, the phytochrome overexpressing plants actually had lower photosynthetic rates as a result of physical limitations for CO_2 difusion with the leaf (Sharkey *et al.*, 1991). The functional nature of the oat protein demonstrated that (i) the mechanism for producing active Pfr can recognize phytochrome apoproteins from widely divergent species (moncot protein in a dicot plant); (ii) etiolated phytochrome can function in in green plants; and (iii) the levels of phytochrome maintained by a delicate balence of synthesis and degradation are important for the correct morphogenic response of plants to light. We are studying several of these changes further because they provide valuable information as to the functions of phytochrome.

Biochemical analyses of the oat phytochrome synthesized in tobacco indicated that the molecule is identical to that present in oat (Cherry *et al.* 1991). Spectra of both Pr and Pfr are unaltered as measured by difference spectroscopy. The oat chromo-protein exist as a dimer of 290 kDa under non-denaturing conditions. Preliminary evidence indicated that the oat and tobacco chromproteins do form heterodimers suggesting that the regions involved in dimer formation are conserved between the two forms. Kinetic studies indicated that etiolated tobacco rapidly degrades its own Pfr via a ubiquitin-dependent proteolytic pathway. Upon conversion of Pr to Pfr, tobacco also recognizes oat phytochrome and rapidly degrades it with the concomitant synthesis of oat Ub-P (Cherry *et al.*, 1991). The apparent half-life of oat Pfr is approx. 4 h while that of tobacco Pfr is approx. 1 h. Whether this apparent decreased rate of degradation indicates that the ubiquitin system in tobacco does not recognize oat Pfr as efficiently as tobacco Pfr or reflects the strong synthetic capacity of the CaMV 35S promoter driving oat phytochrome transcription is unknown. It appears that tobacco can recognize and degrade oat Pfr through the formation of Ub-P.

By altering the oat gene using site directed mutagenesis, it should now be possible to identify sites required for this form dependent catabolism by the ubiquitin system. Moreover because oat phytochrome is functional in tobacco, it should be possible to use this bioassay to define the domains on phytochrome required for phytochrome action. Taking this approach, we have generated a variety of deletion mutations in the oat phytochrome and inserted them into tobacco. Mutations include both N- and C-terminal deletions and various combinations of the two (Figure 1). The constructs, NA and NACE, represent the 120- and 60-kDa "large" and "small" phytochromes previously characterized in the literature that are photointerconvertible. Work is

now in progress characterizing these transgenic plants for the synthesis of the truncated proteins, rapid Pfr degradation, and alterations in tobacco morphology.

The new DOE grant will place greater emphasis on the transgenic system because of its implications in understanding phytochrome's mode of action in addition to elucidating the methods for selective phytochrome degradation.

PUBLICATIONS DERIVED FROM PREVIOUS DOE GRANT
Grant Number: DE-FG02-88ER13968

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2. Jabben, M, J Shanklin and RD Vierstra (1989a) Ubiquitin-phytochrome conjugates: Pool dynamics during in vivo phytochrome degradation. *J. Biol. Chem.* 264: 4998-5505.
3. Jabben, M, J Shanklin and RD Vierstra (1989b) Red light-induced accumulation of ubiquitin-phytochrome conjugates in both monocots and dicots. *Plant Physiol.* 90: 380-384
4. Shanklin, J, M Jabben and RD Vierstra (1989) Partial purification and peptide mapping of ubiquitin-phytochrome conjugates from oat. *Biochemistry* 28: 6028-6034.
5. Cherry, JR, D Hondred, JM Keller, HP Hershey, and RD Vierstra (1990) Use of transgenic plants to study phytochrome domains involved in structure and function. IN: *NATO-ASI: Properties and Biological Action of Phytochrome.* (B Thomas ed) Springer-Verlag (in press).
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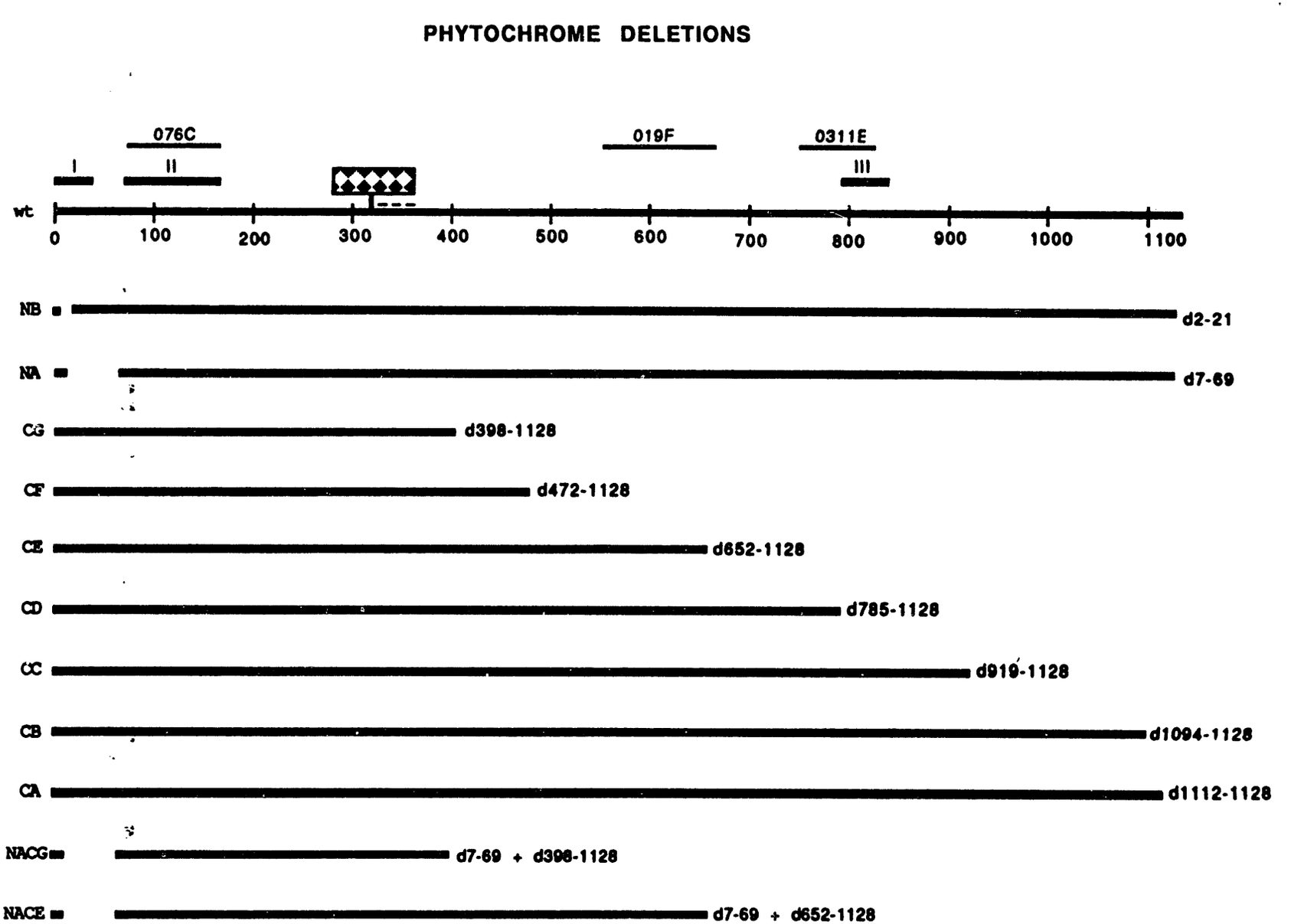
7. Cherry, JR, HP Hershey, and RD Vierstra (1991) Characterization of Tobacco Expressing Oat Phytochrome: Domains Responsible for the Rapid Degradation of Pfr Are Conserved between Monocots and Dicots. *Plant Physiol.* 96: 775-785.
8. Sharkey, TD, TL Vassey, PJ Vanderveer, and RD Vierstra (1991) Carbon Metabolism Enzymes and Photosynthesis in Transgenic Tobacco (*Nicotiana tabacum* L.) Having Excess Phytochrome. *Planta* (in press).

DISCLAIMER

Figure 1


PHYTOCHROME DELETIONS
076C
019F
0311E
I
II
III
wt
0
100
200
300
400
500
600
700
800
900
1000
1100
NB
d2-21
NA
d7-69
CG
d398-1128
CF
d472-1128
CE
d652-1128
CD
d785-1128
CC
d919-1128
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d1094-1128
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NACG
d7-69 + d398-1128
NACE
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