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Different Red Light Requirements for Phytochrome-Induced Accumulation of *cab* RNA and *rbcS* RNA

Abstract. *For several species of plants the abundance of those transcripts encoding the chlorophyll a/b binding protein (*cab *RNA) and the small subunit of ribulose-1,5-biphosphate carboxylase-oxygenase (*rbcS *RNA) has been established as being under the control of phytochrome. However, this conclusion does not take into account the various types of phytochrome control based on both the fluence of red light necessary to induce the response and the ability of far red light either to induce or to reverse the response. The fluence of red light necessary to induce the accumulation of* rbcS *RNA was found to be 10,000 times greater than that necessary to induce the accumulation of* cab *RNA. Furthermore, far red light alone was capable of inducing the accumulation of* cab *RNA. It is possible, therefore, that developing pea buds accumulate* cab *RNA before* rbcS *and that* cab *RNA is not subject to the normal end-of-day signals affecting many phytochrome responses.*

The abundance of those transcripts encoding the light-harvesting chlorophyll a/b protein complex (*cab* RNA) and the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS* RNA) is regulated by phytochrome in pea (*1*, *2*), mung bean (*1*), *Lemna* (*3*), and barley (*4*). Excitation of the phytochrome system similarly affects the abundance of several other nuclear- and chloroplast-encoded transcripts: phytochrome in oats (*5*), reduced nictotinamide adenine dinucleotide phosphate: protochlorophyllide oxidoreductase in barley (*6*), the large subunit of *rbc* protein in pea (*1*) and mung bean (*1*), a 17-kilodalton chloroplast polypeptide in pea (*7*), photogene in pea (*1*) and mustard (*8*), and approximately ten other nuclear-encoded transcripts in pea (*1*) and five others in mung bean (*1*).

In several of these studies, multiple pulses of red light or dark-adapted rather than dark-grown plants were used. In all the studies pulses of red light having extremely large fluences were used to elicit the phytochrome response. Furthermore, time-course studies (*1*, *2*) were designed on the basis of these large fluences, and a relation was suggested between them and the apparent development of the plant. Because land plant seedlings are normally exposed to a logarithmic gradient of red light as they grow up through the soil (*9*), knowing the fluence of red light at which the transcripts of phytochrome-induced genes begin to show altered abundance would presumably be useful in studies of normal seedling development in the soil.

Two kinds of phytochrome responses are defined by the fluence of red light at which they are initiated and by the abili-

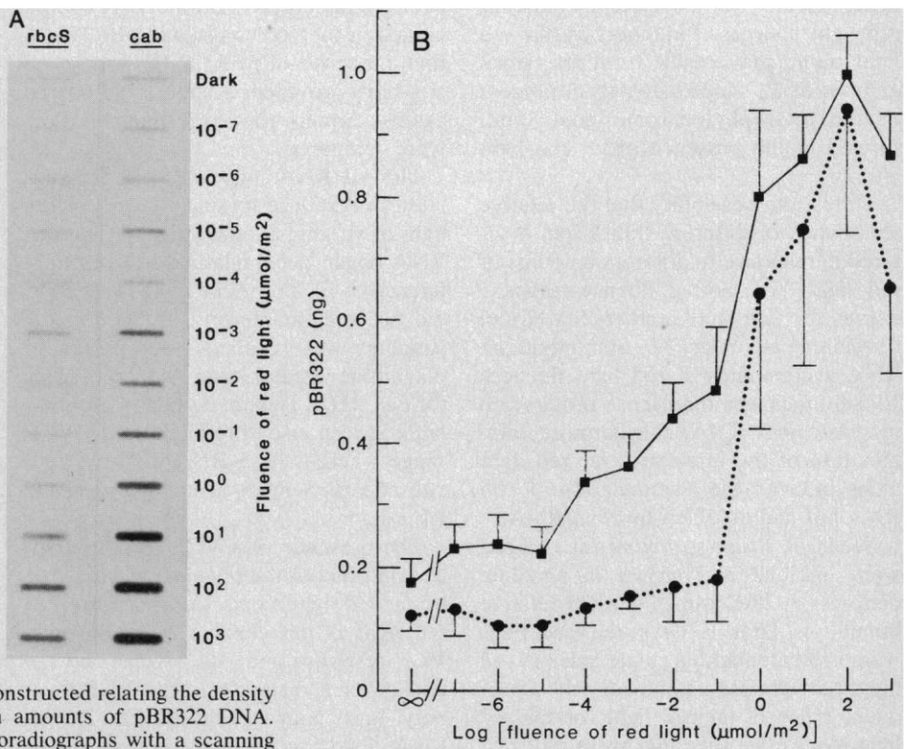

Fig. 1. Red light fluence response of *cab* RNA and *rbcS* RNA. Seeds of *Pisum sativum* var. Alaska were imbibed for 5 hours at 28°C in absolute darkness, planted on water-saturated Kimpac (Kimberly-Clarke), and maintained in absolute darkness at 28°C and 85 percent relative humidity for 6 days. Sets of seedlings were irradiated at each of the fluences indicated. Times for irradiations (μmol/m^2) were as follows: 10^{-7} to 1, 0.1 second; 10, 1 second; 10^2, 10 seconds; 10^3, 100 seconds. The red light source has been described (*12*). All seedlings were returned to darkness for 24 hours. Apical buds were harvested onto ice under dim green light in a 4°C cold room, quickly frozen in liquid nitrogen, and stored at −70°C until the RNA was extracted. Extracted RNA was fixed to nitrocellulose filters. The cDNA probes for *cab* RNA (pAB96) and *rbcS* RNA (pSS15) (*14*) were nick-translated and used to probe the blots. Hybridization conditions have been described (*1*); wash conditions were 0.3 × standard saline citrate at 65°C. A dilution series of the plasmid vector pBR322 DNA was also present on each slot blot. Hybridization of the pBR322 sequences in the probes to the pBR322 DNA on the blot provided a set of concentration standards on each autoradiograph. A standard curve was constructed relating the density of the autoradiograph images to the known amounts of pBR322 DNA. Density was determined by scanning the autoradiographs with a scanning densitometer (Hoeffer). The relative abundance of *cab* RNA and *rbcS* RNA was determined by comparing the density of the experimental slots to the standard curve. Each point represents three to five independent experiments. In each experiment approximately 100 pea buds were used per point. (A) An example of the slot blots used to calculate the fluence response curves shown in (B) for *cab* RNA (■) and *rbcS* RNA (●).

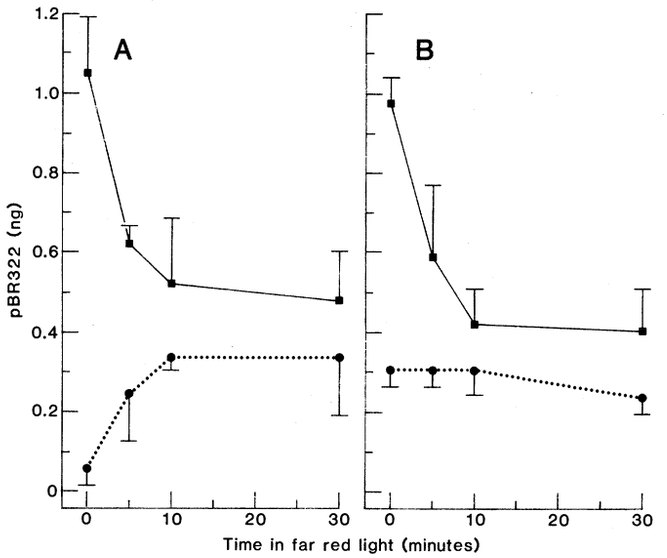

Fig. 2. Far red responses of *cab* RNA and *rbcS* RNA. Seedlings were grown, buds harvested, RNA extracted, and hybridizations performed as for Fig. 1. However, seedlings were irradiated with either far red light alone (1.25×10^{-7} J cm^{-2} sec^{-1}) for 0, 5, 10, or 30 minutes (●) or with a pulse of red light (10^3 μmol/m^2 in 100 seconds) and subsequent far red treatment (■). (A) *cab* RNA, (B) *rbcS* RNA.

ty of far red light to reverse or to induce the response (*10*). The low-fluence (LF) response to red light has a threshold of approximately 1 μmol/m^2 and is fully reversible by far red light. The very low fluence (VLF) response to red light has a threshold of approximately 10^{-4} μmol/m^2—four orders of magnitude fewer photons than the threshold of the LF response. This fluence of red light converts approximately 0.1 percent of the phytochrome population to P_{fr} (the biologically active form of the molecule). The VLF response is not reversible by far red light; indeed, it is inducible by most far red light sources. Induction by far red light alone may result from the small amount of P_{fr} (approximately 3 percent of the total phytochrome population) formed in the presence of far red light (*11*).

To test the possibility that the relative abundance of different transcripts is altered in response to different amounts of red light, we plotted fluence-response curves for *cab* RNA and *rbc*S RNA in developing pea buds. We found that *cab* RNA accumulates at red light fluences 10,000 times less than those required to increase *rbcS* RNA. Furthermore, illumination of the buds with far red light alone induced the accumulation of *cab* RNA but had no effect on *rbcS* RNA.

Seeds of *Pisum sativum* var. Alaska were imbibed and grown in absolute darkness at 28°C and 85 percent relative humidity. After 6 days the seedlings were irradiated with a single pulse of red light, red light and an immediately subsequent pulse of far red light, or far red light alone. The seedlings were returned to total darkness for 24 hours, and the buds were then harvested onto ice in dim green light in a 4°C cold room. The green safe light (*12*), red light (*12*), and far red light sources (*13*) have been described.

Harvested buds were quickly frozen in liquid nitrogen and stored at −70°C before RNA extraction. Total RNA was extracted, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled complementary DNA (cDNA) probes as described (*1*) except that a Minifold II slot-blot apparatus (Schleicher and Schuell) was used instead of a dot-blot apparatus (*2*). The probes used to measure the amounts of *cab* RNA and *rbcS* RNA, pAB96 and pSS15, respectively, have been described (*14*). Both cDNA's were cloned in the vector plasmid pBR322. A dilution series of pBR322 DNA on each slot-blot provided a set of standards against which the experimental slots were compared.

The *cab* RNA and *rbcS* RNA accumulated in response to single pulses of red light of varying fluence (Fig. 1). The *cab* RNA began accumulating at a red light fluence of 10^{-4} μmol/m^2. The *rbcS* RNA did not accumulate until a fluence of 1 μmol/m^2 was reached—four orders of magnitude greater than that necessary for *cab* RNA. Hence, *cab* RNA accumulated in response to red light in the VLF range whereas *rbcS* RNA did not; both *cab* and *rbcS* RNA accumulated in the LF range.

The presence of a VLF response can be confirmed if the response is inducible by far red light alone. Consequently, far red light is not capable of reversing a VLF response and will reverse the LF portion of a response to the VLF level only. In such an experiment (Fig. 2), *cab* RNA clearly accumulated in response to far red light alone, and far red reversal of the red response did not result in the dark level but rather in that produced by the VLF response alone (Fig. 2A).

A similar experiment was conducted for *rbcS* RNA (Fig. 2B). Far red light alone had little if any effect on the abundance of *rbcS* RNA. Furthermore, the effect of red light was fully reversible by far red light. These results confirm the absence of any significant VLF effect on the accumulation of *rbcS* RNA.

That *cab* RNA accumulated in response to the VLF range of red light as well as in response to far red light may have several implications for the developing plant. Because red light in the VLF range is present deeper in the soil than red light in the LF range (*9*), *cab* RNA may start to accumulate very early in the developing seedling, possibly before *rbcS* RNA accumulation and certainly before chlorophyll accumulation. The possibility of accumulation in far red light as well as the inability of far red light to reverse the VLF response indicates that *cab* RNA accumulation may not be affected by the normal end-of-day signals that affect other phytochrome responses.

The accumulation of *cab* RNA before *rbcS* RNA and the continuous accumulation of *cab* RNA appear to contrast with the inability of the translation product to accumulate in the absence of continued chlorophyll accumulation (*15*). However, since chlorophyll will not accumulate in the absence of *cab* protein, it would seem desirable for the protein to be present before the onset of chlorophyll synthesis. The accumulation of some *cab* RNA under conditions in which chlorophyll is not synthesized (that is, the VLF range of red light) might be important to ensure the presence of *cab* protein as soon as chlorophyll synthesis is induced.

In a study of barley leaves developing in white light (*16*), *cab* RNA was seen to accumulate earlier than *rbcS* RNA. Because these leaves developed in relatively high fluences of continuous white light, the mechanism resulting in the accumulation of *cab* RNA before that of *rbcS* RNA was probably not fluence-dependent. Two such different developmental schemes as those found in pea buds and barley leaves seem to ensure the accumulation of *cab* RNA before chlorophyll synthesis and *rbcS* RNA accumulation.

It is likely that changes in transcription are at least partly responsible for the observed accumulation of these transcripts. Treatment of pea leaves with white light has been shown to increase the ability of subsequently isolated nuclei to incorporate precursers into *cab* RNA and *rbcS* RNA (*17*). A similar

observation has been made (*18*) for the LF phytochrome responses of *cab* RNA and *rbcS* RNA in *Lemna*.

There are approximately 6 to 12 copies each of *cab* and *rbcS* in *P. sativum* var. Alaska (*19*, *20*). In petunia, these gene families comprise several subfamilies distinguishable by the sequence immediately 3′ to the coding region (*21*). If such subfamilies exist in pea, it is possible that different light responses or responses in different tissues (or both) are the result of transcription by different copies of the gene. Hence, for the *cab* genes one copy or subfamily may be responsible for the VLF accumulation while another may be responsible for the LF accumulation. The cDNA clones used in our study could not differentiate among transcripts originating from the different copies of the genes (*20*). Studies of the individual genes must await the isolation and characterization of clones capable of discriminating among the different copies.

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Hemoglobin I Mutation Encoded at Both α-Globin Loci on the Same Chromosome: Concerted Evolution in the Human Genome

Abstract. *Genetic analysis of an individual expressing an unexpectedly high level of hemoglobin I, an α-globin structural mutant, reveals that the mutation is present at both the α1- and the α2-globin gene loci. Kindred analysis confirms that the two affected genes are located in cis. The most likely explanation for this finding is that a recent conversion event occurred within the human α-globin gene cluster.*

The structures of the two adjacent α-globin genes within individual mammalian species are highly homologous despite the significant divergence of α-globin gene structure among the mammalian species during 400 million years of evolution (*1–9*). This parallel development of gene structure during evolution, referred to most commonly as concerted evolution (*5*), appears to be a general pattern shared by a number of linked (*4–7*, *10*) as well as unlinked gene families (*11*). The gene conversion events that result in concerted evolution occur at a high frequency within the α-globin clusters of primates (*5–9*). Comparison of the human and chimpanzee α-globin gene sequences suggests that at least one gene conversion event has occurred in the two human α-globin genes since the divergence of humans and chimpanzees 4 million years ago (*9*). We report here a more recent gene conversion in the human α-globin gene cluster. This conversion, unlike the one previously described, results in a parallel change in the structure of the α-globin protein encoded at both α-globin loci. We have identified an individual whose two adjacent α-globin genes, α1 and α2, encode the same α-globin protein mutation—the lysine at position 16 of the amino acid sequence is replaced with glutamine (α^{16Glu}). Delineation of this genotype supports the concept of concerted evolution in the human genome and clarifies the genetic basis of the α^{16Glu} structural hemoglobinopathy.

The two human α-globin genes, α1 and α2, are expressed equally at the protein level. This conclusion is based on studies of individuals heterozygous for α-globin structural mutations. In such individuals, one of the four α-globin genes encodes the mutant, and this structural variant is usually expressed at 25 percent of total α (*12*). If the function of one, two, or three of the normal α-globin genes in such individuals is lost because of α-thalassemia (*13*), the amount of the variant can approach 33, 50, or 100 percent of total α-globin, respectively (*14–16*). We now describe an individual in whom the level of the α-globin mutant α^{16Glu} cannot be explained by interaction of α-thalassemia with a single mutant locus. The concentration of hemoglobin I (HbI) ($\alpha_2^{16Glu}\beta_2$) in this otherwise normal black woman constitutes 65 percent of total hemoglobin (*17*, *18*). Southern blot analysis (*19*) (data not shown) revealed that her DNA contains three α-globin genes. One chromosome contains the normally arranged α1- and α2-globin genes (*4*, *20–22*). The homologous chromosome contains only a single α-globin gene, as a result of a rightward type α-thal-2 deletion (*4*, *23*, *24*). Her genotype is thus αα/−α. This woman's daughter, who has mild α-thalassemia (hemoglobin, 11.1; mean corpuscular volume, 74.2), is homozygous for the α-thal-2 chromosome (−α/−α). Since she has no HbI and has inherited her mother's α-thal-2 chromosome, the α gene on the α-thal-2 chromosome of the mother must encode normal α-globin. Therefore, the α^{16Glu} mutation must reside on the mother's chromosome that contains two α genes.

The high (65 percent) level of globin variant expression in this case could arise by several mechanisms. If there is only one α^{16Glu} locus in the mother, then a mutation that increases some combination of α^{16Glu} transcriptional output, translational efficiency, and messenger RNA (mRNA) stability could account for her 65 percent level of variant. Alternatively, if all three genes are expressed equally at the protein level, the 65 percent HbI would suggest the presence of two α^{16Glu} genes. Although generation of such an arrangement by two identical point mutations is extremely unlikely, the extensive regions of homology surrounding the α genes (*4*, *8*, *25*) could lead to a gene conversion event (*5*, *7*, *8*, *25*) that could produce two adjacent α^{16Glu} genes.

We used the technique of hybrid-selected translation (*26–28*) to identify the locus of the α^{16Glu} mutation on the structurally intact chromosome. The α1- and α2-globin mRNA's were separately isolated by hybridizing total reticulocyte RNA to nitrocellulose paper containing plasmid DNA specific for α1- or α2-globin mRNA. Since the structural divergence between these two mRNA's is limited to the 3′ nontranslated region (*7*, *29*), we used recombinant complementary DNA (cDNA) plasmids containing