CHAPTER I

INTRODUCTION

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PHYTOCHROME

Morphogenesis in plants is susceptible to quantitative and qualitative modification by environmental factors. Light is perhaps the most important environmental factor affecting plant growth and development, where it acts in a catalytic fashion independent of its energetic participation in photosynthesis. The influence of light on morphogenesis of plants is discernible in every phase of plant life cycle right from seed germination, stem elongation, leaf expansion, root initiation, flowering, fruiting to bud dormancy and senescence. This phenomenon, where light exerts control over growth and differentiation of plants independently of its participation in photosynthesis, has been termed as "photomorphogenesis". The regulation of plant growth and development in relation to light environment is not only due to presence or absence of light but also due to the variation in the quantity and quality of light.

In any biological response evoked by light, a basic sequence of events must occur. The light must be absorbed by a specific photoreceptive molecule whose chemical reactivity is thereby changed. By virtue of its changed chemical reactivity, the photoreceptor then initiates a sequence of metabolic processes which lead ultimately to the observed developmental changes (Fig.a).

In order to fully understand photomorphogenesis it is necessary to acquire information on the chemical identity of the photoreceptors and photochemical changes that occur on light absorption. The relative effectiveness of different wavelengths of light has revealed that the

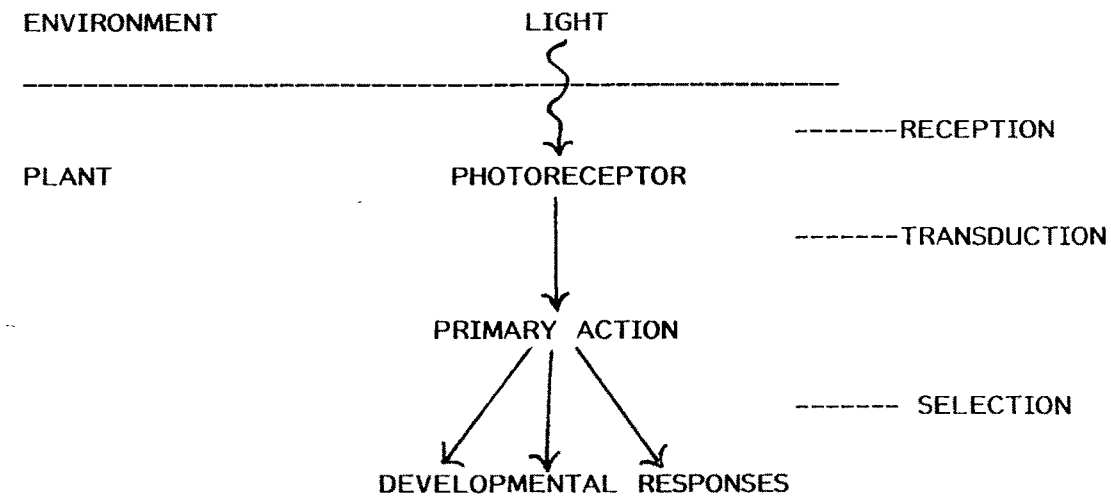


Fig.a : The partial processes of photomorphogenesis.

photomorphogenetic effects of light are largely restricted to the blue and red regions of the visible spectrum. The photoreceptor perceiving light signal in the blue region of the spectrum is yet to be characterized but is believed to be either a carotenoid or a flavin molecule (Senger, 1980). In contrast, the red light absorbing photoreceptor known as 'phytochrome' has been well characterized due to its remarkable property of existing in two spectrally different and photointerconvertible forms (Siegelman and Butler, 1965).

Phytochrome regulates the growth and development of plant at every phase of its life cycle by affecting various metabolic processes at the level of enzymes (Smith, 1975). Involvement of phytochrome in the photocontrol of enzymes was first suggested by the discovery of red (R) light inducible and far-red (FR) light reversible increase in the activity of glyceraldehyde phosphate dehydrogenase (Marcus, 1960). Since then R-FR reversibility has been observed in a wide range of metabolic processes including photosynthesis, fat degradation, starch degradation, nitrogen assimilation, nucleic acid synthesis and degradation and secondary product synthesis at the level of specific enzymes. Table-A shows some phytochrome mediated changes in enzymes.

Molecular Properties of Phytochrome :

Phytochrome is a bluish chromoprotein, which has a linear tetrapyrrole chromophore linked to its protein moiety via a thioether linkage to a cysteine residue (Lagarias and Rapoport, 1980). Phytochrome exists in two isomeric forms which differ in their spectroscopic properties. One form having absorption peak in the red region is called Pr or P_{660}

Table - A : **Phytochrome-mediated changes in enzyme activity.**

Enzyme	Plant	Effect	Lag
Intermediary metabolism			
NAD-kinase(in vitro)	Pea	+	0
Lipoxygenase	Mustard	-	0
Amylase	Mustard	+	6 h
Ascorbic acid oxidase	Mustard	+	3 h
Galactosyl transferase	Mustard	+	12 h
NAD+ -linked glyceraldehyde-3-phosphate dehydrogenase.	Bean	+	12 h
Nitrate-reductase	Pea	+	2 h
Nucleic acid and protein metabolism			
RNA-polymerase(nuclear)	Pea	+	4 h
Ribonuclease	Lupin	+	4 h
Amino acid activating enzymes	Pea	+	-
Photosynthesis and chlorophyll synthesis			
Ribulose-1,5-bisphosphate carboxylase	Bean	+	24 h
Transketolase	Rye	+	-
NADP+ -linked glyceraldehyde phosphate dehydrogenase	Bean	+	12 h
Alkaline fructose 1,6-diphosphatase	Pea	+	-
Inorganic pyrophosphatase	Maize	+	2 h
Adenylate-kinase	Maize	+	-
Succinyl CoA-synthetase	Bean	+	-
Sucrose phosphate synthase	Maize	+	4 h
Peroxisome and glyoxisome enzymes			
Peroxidase	Mustard	+	72 h
Glycollate-oxidase	Mustard	+	6 h
Glyoxylate-reductase	Mustard	+	6 h
Isocitrate-lyase	Mustard	0	-
Catalase	Mustard	0	-
Secondary product synthesis			
Phenylalanine ammonia-lyase	Pea (many others)	+	1.5 h
Cinnamate-hydroxylase	Pea	+	-

and the other form having absorption peak at 730 nm is called P_{fr} or P_{730} . These two forms are interconvertible by absorbing light at appropriate wavelengths; red light (660 nm) converts P_r to P_{fr} and and far-red light (730 nm) reverts P_{fr} to P_r (Fig.b).

There seem to be a general agreement that phytochrome is a multimer of 120,000 daltons (Rice and Briggs, 1973; Roux et al, 1975; Smith and Correll, 1975). Amino acid analysis of phytochrome shows a high content of polar as well as non polar amino acids (Rice and Briggs, 1973) suggesting that it is capable of both hydrophilic and hydrophobic interactions (Roux and Yguerabide, 1973). There are some evidences to suggest that small changes in protein conformation do occur upon phototransformation (Roux and Hillman, 1969). A tentative model for phytochrome chromophore and its binding to the protein moiety is shown in Fig.c. Very recently it has been reported that phytochrome has highly reactive sulfhydryl residues located on the portion of the protein that undergoes a conformational change on interconversion of P_r and P_{fr} (Smith and Cyr, 1988).

Of the two forms of phytochrome, P_{fr} form is regarded as the physiologically active form (Schmidt and Mohr, 1982). On the other hand, in certain cases, the ratio of P_{fr} /P total, rather than P_r or P_{fr} alone, may determine the biological action (Smith, 1981). Phytochrome exists predominatly in P_r form in plants grown under prolonged darkness, which phototransforms to P_{fr} form when the plants are exposed to light. P_{fr} is thermodynamically unstable, it disappears through a first order destruction process which is independent of light

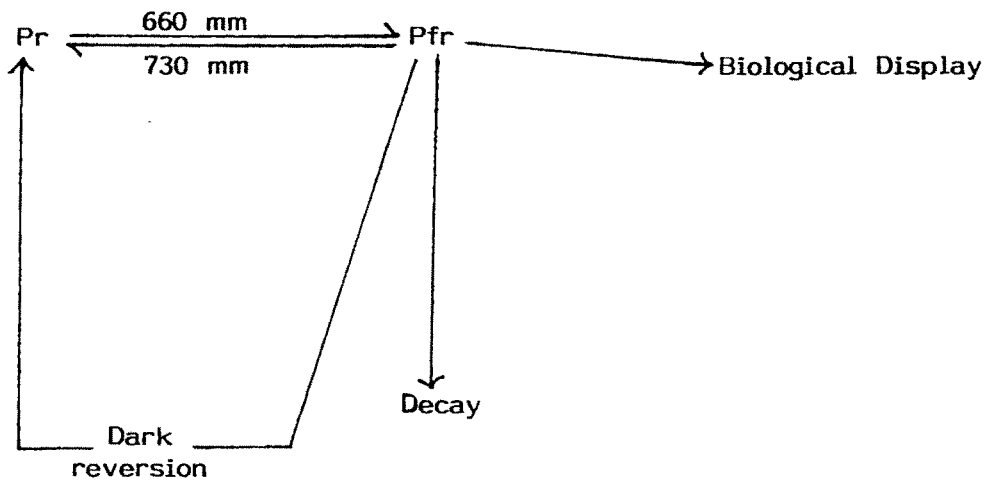


Fig. b : Interrelationship between Pr and Pfr.

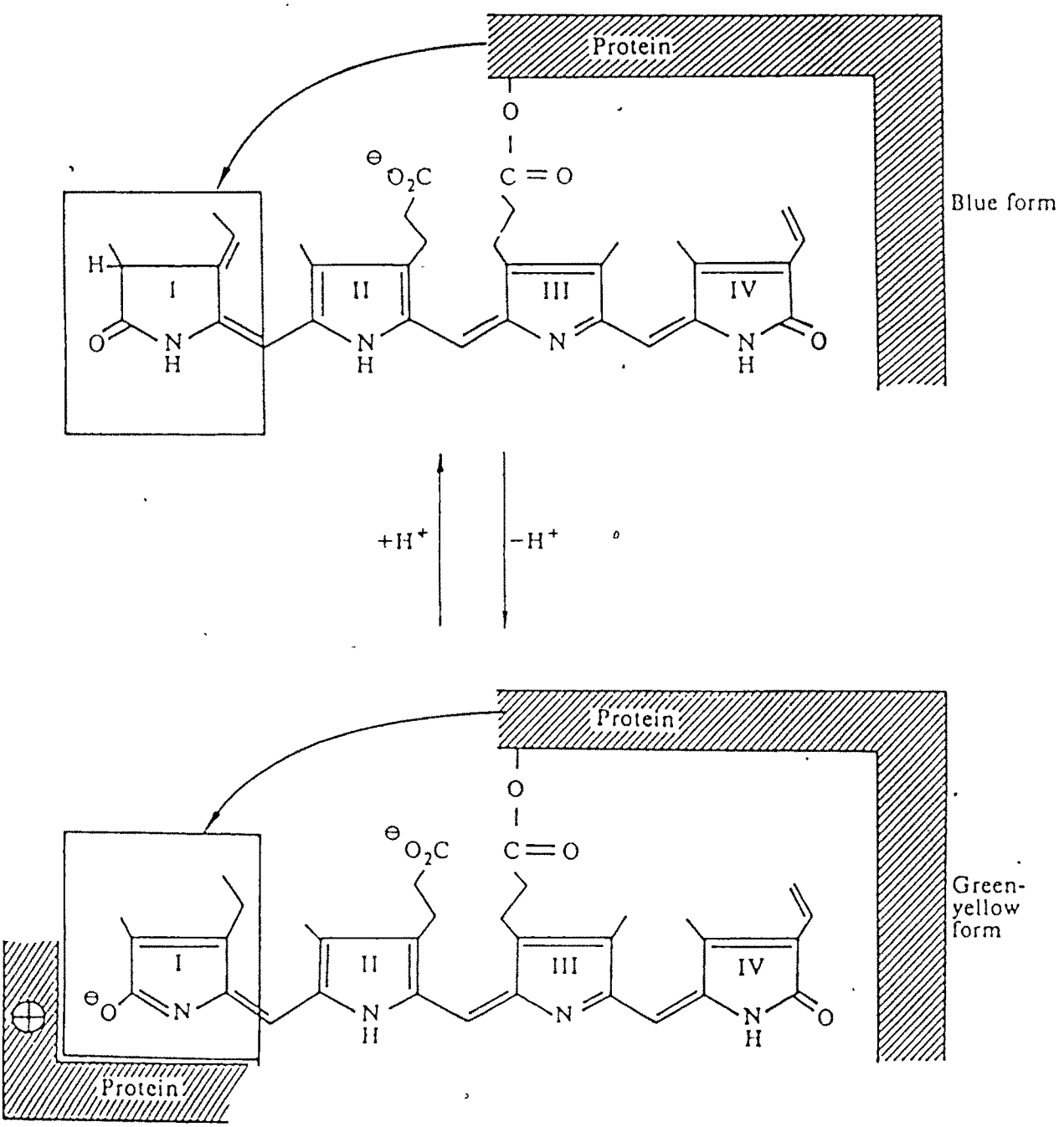


Fig.C : Model for the binding of phytochrome chromophore to the protein moiety.

or reverts to P_r by a process called "dark reversion" (Butler et al, 1963; Butler and Lane, 1964). The phenomenon of dark reversion is absent in monocots and in members of Centrospermae (Kendrick and Hillman, 1971).

Under continuous white light, the in vivo level of phytochrome decreases as a consequence of destruction until an equilibrium between the rates of synthesis and destruction is established, reaching a steady state, which is 1-3% of the initial phytochrome concentration in etiolated plants. This low steady state level of phytochrome in continuous light has been accounted for in terms of differential turnover rates of P_r and P_{fr} , where P_{fr} has a rapid rate of destruction against a background of constant rate of P_r synthesis (Sharma, 1984). Phytochrome also exerts an autoregulatory control over its own translatable mRNA level (Colbert et al, 1983).

Photochemistry of phytochrome transformation has been studied extensively both in vivo and in vitro by a variety of techniques. Phototransformation of phytochrome from P_r to P_{fr} form or in the reverse direction occurs via different pathways involving a number of short-lived intermediates (Kendrick and Spruit, 1977). Since P_r and P_{fr} forms absorb light throughout ultraviolet and visible range of the spectrum, continuous irradiation results in a photoequilibrium between P_r and P_{fr} (Butler et al, 1964). Therefore, P_{fr} /P total ratio is characteristic of wavelength and is independent of the intensity of light. Under red light it is 0.75-0.84 (Yamamoto and Smith, 1981a) while under far-red light it is 0.03-0.06 (Kendrick and Smith, 1976). Under continuous

irradiation the phytochrome molecule undergoes cycling between P_r and P_{fr} forms, the rate of which depends strongly on the intensity of irradiation.

Many attempts have been made to elucidate the physico-chemical changes occurring in the protein moiety as well as in the chromophore of phytochrome during phototransformation. No significant differences have been observed between the two forms of phytochrome in terms of size, shape, ionic charges or immunological activity, ruling out any major conformational changes (Tobin and Briggs, 1973; Cundiff and Pratt, 1975; Hunt and Pratt, 1981). However, there exist subtle differences between the two forms, as P_r and P_{fr} respond differently to various treatments. P_{fr} form is more susceptible to ammonium sulphate, urea, metal ions, sulfhydryl reagent, glutaraldehyde, etc., indicating minor changes in the conformation of the molecule during phototransformation (Smith, 1981; Schafer, 1982). P_{fr} form has higher affinity for DEAE agarose and some alkyl agaroses than P_r (Yamamoto and Smith, 1981b). These results have been taken as evidence for P_{fr} form being more hydrophobic in nature than P_r. Hahn and Song (1981) have proposed a model for the molecular basis of effectiveness of phytochrome in P_{fr} form on the basis of certain physico-chemical differences between P_r and P_{fr} forms. This model envisages that chrompophore in P_r form is rigidly held over a crevice on the surface of the molecule and on phototransformation to P_{fr} form, the chromophore orients away from the surface. This orientation of chromophore leads to exposure of the crevice having hydrophobic surface which may determine preferential interaction of P_{fr} molecule with a putative receptor to elicit a biological response.

High and Low Irradiance Reactions of Phytochrome :

Phytochrome is known to control photomorphogenesis by two reactions: (1) low irradiance reaction, and (2) high irradiance reaction. The photoresponses classified under 'low irradiance reaction' are typical phytochrome mediated responses as they show a characteristic photo-reversibility reaction, where a response triggered by red light can be reversed by far-red light, if followed subsequently. The action spectrum (quantum responsivity of a photoresponse plotted as a function of wavelength) of light induced response shows a peak at 660 nm corresponding to the absorption peak of P_r, while the action spectrum of reversion shows a peak at 730 nm corresponding to the absorption peak of P_{fr}. Further, these responses generally obey Bunsen-Roscoe reciprocity law (intensity x time = constant) (Mohr, 1972). These characteristic features of low irradiance responses lead to the belief that the photoresponses are directly modulated by the P_{fr} level *in vivo*, where the magnitude of a photoresponse increases with increasing P_{fr} level (Schmidt and Mohr, 1982). This type of quantitative relationship between P_{fr} level and the extent of photoresponse may not be apparent in all cases. For example, in cotyledons of mustard seedlings, phytochrome controls repression of lipoxygenase activity through a threshold mechanism (Oelze-Karow *et al*, 1970). The repression of lipoxygenase activity becomes apparent only when P_{fr} level is higher than 1.25% of the total phytochrome level. On the other hand, in the same cotyledon, no such threshold mechanism operates in the case of regulation of anthocyanin synthesis which is dependent on quantitative changes in the total level of P_{fr} (Schmidt and Mohr, 1982).

In contrast to these low irradiance responses, which are largely restricted to dark grown seedlings, under natural conditions where plants are exposed to alternate light and dark periods of considerable durations, phytochrome operates via 'high irradiance reaction'. The photoresponses grouped under high irradiance reaction are distinguished from low irradiance reaction on the basis of their characteristic action spectra. The action spectra of the photoresponses under prolonged irradiation with diferent wavelengths show a strong peak of action in the far-red region close to 720 nm, and also a substantial multiple peak of action in the blue region while there is no or very little effect in the red region of the spectrum (Hartmann, 1966). These phtoresponses are further characterized by the magnitude of response which is a function of irradiance either exponentially or directly. These photoresponses do not obey Bunsen-Roscoe reciprocity law and also lack the characteristic photoreversibility of the low irradiance reaction. It was found earlier (Mohr, 1972) that when given red light, the cotyledons of Sinapsis alba expand, an effect which is reversed by far-red light. However, when far-red light was given for 2-3 hrs instead of 5 min there was no reversal, in fact there was stimulation of expansion. This shows that long period of high irradiance far-red light acts like red light of short duration. This was also found to be the case for many other developmental responses (Mohr, 1972).

Mode of Phytochrome Action :

The fact that phytochrome is a protein, initially led to the idea that P_{fr} was the active form of an enzyme (Hendricks, 1959). Light induced changes in chromophore lead to changes in the conformation

of the protein which could increase the accessibility of certain active groups (e.g. -SH groups) which then allows binding with the substrate. However, there is no indication of the nature of the substrate nor is there any convincing experimental evidence that phytochrome is an enzyme (Kendrick and Frankland, 1983).

A hypothesis which satisfactorily explains both amplification and specificity of the developmental responses to phytochrome photoactivation was postulated by Mohr(1966). This hypothesis suggested a phytochrome action through the activation or repression of specific genes. Subsequent changes in enzyme levels are held to be responsible for the amplification and the specificity is determined by the differential status of the genome of the particular cell. Any direct effect of phytochrome on gene expression implies a rather slow response time, since the synthesis of new enzymes necessarily takes a finite time. However, the discovery of very rapid effects of light mediated by phytochrome e.g., the rotation of chloroplast in Mougeotia (Dreyer and Weisenseel, 1979), the leaflet closing in Mimosa pudica L. (Fondeville et al, 1966), increase in bioelectric potential of the etiolated oat coleoptiles (Jaffe, 1968) etc. which occur within few seconds or minutes consequently caused some investigators to reject the idea of an intimate relationship between the photoreceptor and genome. Thus the primary action of phytochrome in initiating photoresponses might begin right with the phototransformation of phytochrome to active form.

An idea regarding the minimum time required for the initiation of the primary action of phytochrome in a photoresponse can be obtained

by studying the kinetics of the time course of escape from reversibility (Fukshansky and Mohr, 1980). Normally the effect of a red light pulse on a photoresponse is fully reversible by a far-red light pulse, provided far-red light is given immediately after red light. However, if a dark interval is interposed between the two pulses, an escape from full reversibility is observed after certain time interval. The duration of this time interval indicates the minimum time required by phytochrome to trigger its primary action for the initiation of a particular photoresponse. Once phytochrome completes its primary action, the signal is passed on to the next component of the signal chain leading to the photoresponse. Since at this stage the signal is no longer retained within the photoreversible elements of phytochrome, the response loses its photoreversibility (Sharma, 1984).

It has been established by the kinetics of escape from photoreversibility that the primary action of phytochrome in bringing about the photoresponses is completed within a few seconds or minutes of its phototransformation, even though the manifestation of photoresponse may take place hours or even days later. For example, a brief red light pretreatment to etiolated seedlings of Sorghum vulgare stimulates chlorophyll synthesis in shoots on transfer to continuous white light. This red light effect is fully reversible by a far-red light pulse for 45 min (Oelze-Karow and Mohr, 1982). Thereafter, the escape from photoreversibility is fast and it is completed within 2 hrs indicating that the primary action of phytochrome in stimulating chlorophyll synthesis begins after 45 min of red light treatment and is over within 2 h of red light exposure. In the case of red light stimulated elongation of

maize coleoptile sections, the photoresponse escapes from photoreversibility within 45 sec of red light treatment, pointing to a rapid action of phytochrome on this response, which manifests a few hours later (Warner et al, 1981). Red light also leads to increase in cell wall extensibility in maize coleoptile sections which manifests after 20 min of irradiation. However, the kinetics of escape from photoreversibility indicates that the initial action of phytochrome is completed within 2 sec of the onset of red irradiation (Warner and Ross, 1981).

Completion of the primary action of phytochrome on photoresponses in such a short time requires an efficient coupling between the active form of phytochrome and the next component of signal chain in transducing the light generated signal. To unravel, this hitherto unknown signal chain, plant physiologists have made attempts to understand two major aspects : (a) the primary action of phytochrome at membrane level and (b) the role of secondary messengers.

(a) The primary action of phytochrome at membrane level :

It is now widely believed that the primary action of phytochrome may result from its interaction with the cellular membranes, leading to changes in their functional properties. Attempts to find a meaningful association of phytochrome with cellular membranes have been numerous since Hendricks and Borthwick (1967) first porposed that membranes were a likely candidate for the primary site of action of phytochrome. Rubinstein et al. (1969) were the first to report that a small proportion of the extractable phytochrome is associated with the crude membrane preparation. Since then, irradiation enhanced pelletability of phytochrome has been widely investigated (Quail, 1975; Marme, 1977; Pratt, 1978).

It has been shown that small amounts of phytochrome are strongly bound to membranes in etiolated Avena seedlings and that red light induces an increase in binding that is fully reversed by subsequent far- red light (Waston and Smith, 1982a,b). This binding response can only be observed under the appropriate extraction conditions i.e., if the tissue is homogenized and extracted above pH 7.5 in the absence of exogenous divalent cations. After saturation with red light, the phytochrome bound to the crude membrane fraction comprises less than 8% of the total cellular phytochrome. Below pH 7.5 in the presence of divalent cations, a second type of light induced binding response is observed (reviewed by Quail, 1975; Marme, 1977; Pratt, 1978). This binding causes upto 75% of the total phytochrome to pellet with the membranes, obscuring the much smaller light-induced variations in binding studied by Watson and Smith (1982a,b) which nevertheless contributes to the total pool of bound phytochrome (Napier and Smith, 1987a). Napier and Smith (1987b) designated the two binding responses as Association I and Association II. Association I is the binding that is readily and rapidly (in less than 5 min) reversed by FR given in vivo and Association II is the electrostatic binding that is reversed by FR but with a much longer half time (Quail et al, 1973; Pratt and Marme, 1976). The characteristics of Association I are consistent with the binding having physiological significance (Napier and Smith, 1987b). Napier and Smith (1987b) have proposed a model which explains all the binding data for saturating red light irradiations. The model is based on the assumption that P_{fr} binds to a specific membrane-bound site which is not yet identified. So far, no physiological response has been recorded that might link

the binding of phytochrome to membranes with subsequent biological action and, therefore, the significance of such binding remains uncertain.

Apart from phytochrome binding to the membrane, various studies have also shown that phytochrome phototransformation leads to modulation of membrane properties such as alterations in bioelectric potential (Jaffe, 1968: Newman and Briggs, 1972; Racusen and Satter, 1975) and ionic permeability (Satter et al, 1970, 1974, 1977; Hale and Roux, 1980). The phytochrome mediated permeability changes are not restricted to plasma membrane alone and can also be observed in organelles (Manabe and Furuya, 1974). All these observations have reinforced the view that phytochrome is either an integral part of membranes or it gets associated with them after phototransformation to active form. It is evident that in vivo the interaction of phytochrome with membranes is one of the early steps of phytochrome action, perhaps representing the primary action of phytochrome, leading to initiation of signal chain for the expression of various photoresponses.

(b) Role of secondary messengers in phytochrome action :

It is now well known that the primary action of phytochrome takes place on cellular membranes and is over within a few seconds or minutes though the manifestation of photoresponse is observed only after a few hours or days. It is, therefore, evident that additional steps or components exist in between the primary action of phytochrome and the final expression of a photoresponse, whose identity is not yet known. This component of the signal chain might contain certain specific small molecules which are freely diffusible in a cell or in between

the cells. The photoresponse may be initiated by intercellular or intracellular change in the level or transport of these molecules by phytochrome. These molecules are termed as secondary messengers.

The biochemical basis for the transduction of extracellular signals into intracellular events has been well studied in animals. Extensive information from animal system indicates that a signal (message) received on the surface of an individual cell is transmitted on to the metabolic machinery of the cell by two major signal pathways. One employs the second messenger cyclic adenosine monophosphate (cAMP) (Rasmussen and Kujima, 1983) and the other employs a combination of second messengers that include Ca^{+2} ions, inositol 1,4,5-trisphosphate and diacylglycerol (Nishizuka, 1984; Berridge and Irvine, 1984). Attempts have been made to explore the presence of both the signal pathways in plants also. Although there is convincing evidence for the presence of cAMP in higher plants, no evidence has been reported for its physiological role as a second messenger (Brown and Newton, 1981).

The importance of calcium in plant growth and development has been known for years. However, only recently evidences have accumulated to suggest that calcium acts as a second messenger in the transduction of extracellular signals in plants. If calcium is acting as a second messenger in mediating the physiological processes evoked by primary stimuli, then the following criteria must be met by calcium: (1) Cytoplasmic calcium levels must respond to stimuli from the environment and neighbouring cells. Furthermore, these changes must precede the physiological response. Because of the technical difficulties in direct

measurement of free cytosolic calcium concentration in plants, only in a few cases changes have been documented in resoponse to stimuli (Williamson, 1981; Hepler and Wayne, 1985). However, considerable indirect evidence exists in the literature indicating that cytosolic calcium levels change in response to external stimuli. (2) It must be possible to evoke a physiological response by inducing a change in cytoplasmic calcium in the absence of normal external stimuli. A number of physiological responses elicited by primary signals have been induced by manipulating cellular calcium levels in the absence of stimuli (Hepler and Wayne, 1985; Evans, 1985). (3) Cells must possess the mechanism to sense the changes in cytosolic calcium and translate them into physiological responses. The sensitivity to cytoplasmic calcium changes is conferred on numerous physiological response elements by calmodulin, a ubiquitous calcium binding protein found in animals and higher plants, and by calcium dependent enzymes (Marme, 1982). (4) Blocking the operation of a second messenger sensing system must block the physiological response to external stimuli. Inhibition of calmodulin activity by calmodulin inhibitors has been shown to inhibit some of the responses evoked by primary signals (Raghothama et al, 1985).

Since calcium has met the criteria listed above, it is now generally accepted that calcium acts as a second messenger in coupling stimulus to response. Various calcium mediated physiological processes, elicited by extracellular signals such as light, hormones and gravity have been reviewed extensively (Marme and Dieter, 1983; Hepler and Wayne, 1985; Poovaiah and Reddy, 1987).

Role of calcium in the phytochrome mediated responses was first proposed by Haupt and Weisenseel (1976). It was proposed that some phytochrome effects on cellular enzyme activities and on cell growth and development could be mediated through Ca^{+2} and Ca^{+2} dependent regulator proteins. There was no such Ca^{+2} -dependent protein known in plants at that time but a major one was soon discovered, one that was already well known in animal cells -Calmodulin.

Calmodulin has now been found in many species throughout the plant kingdom. Calmodulin from higher plants and fungi has been isolated and characterized (Charbonneau et al, 1980; Anderson et al, 1980). It's primary structure has been found to be highly conserved among eukaryotes (Klee and Vanaman, 1982; Wang and Waisman, 1979). Calmodulin is a heat stable, acidic polypeptide of 148 amino acids having four Ca^{+2} -binding sites with affinities in the micromolar range (Cheung, 1982). It occurs both in the cytosol and in organelles of cells and gets activated by only a small increase in the free Ca^{+2} concentration of its subcellular environment. In its Ca^{+2} activated form calmodulin can stimulate the activity of several Key enzymes that regulate major metabolic functions (Marme and Dieter, 1983). There may be many different Ca^{+2}-binding proteins in plants but calmodulin is by far the best known.

Using calmodulin as a specific example of Ca^{+2} dependent regulator protein, the hypothesis of Haupt and Weisenseel postulates that the photoactivation of phytochrome rapidly leads to increase in Ca^{+2} concentration in certain subcellular compartments and this in turn would activate calmodulin and calmodulin-dependent enzymes in these

compartments. Some of these enzymes could then catalyze activities that would result in photomorphogenesis (Roux et al, 1986) (Fig.d). A large number of experimental evidences have accumulated in favour of this hypothesis.

Red light triggers a large array of physiological and developmental events that require Ca^{+2}, including chloroplast rotation in Mougeotia (Dreyer and Weisenseel, 1979); spore germination (Wayne and Hepler, 1984; 1985) and cell expansion (Cooke and Racusen, 1982) in Onoclea; leaflet closure in Mimosa (Campbell et al, 1979; Campbell and Thomson, 1977; Toriyama and Jaffe, 1972); root tip adhesion in Phaseolus (Tanada, 1968; Yunghans and Jaffe, 1970), peroxidase secretion in Spinacia (Kerege et al, 1982; Penel and Greppin, 1979; 1982); membrane depolarization in Nitella (Weisenseel and Ruppert, 1977) as well as activation of NAD kinase (Anderson and Cormier,1978; Tezuka and Yamamoto, 1972) and inhibition of mitochondrial ATPase (Serlin et al, 1984). Micromolar concentrations of external Ca^{+2} are sufficient to stimulate germination (Wayne and Hepler,1984), peroxidase secretion (Sticher et al, 1981), activation of NAD kinase (Dieter and Marme, 1980) and inhibition of mitochondrial ATPase (Serlin et al, 1984). Further, treatment with calmodulin antagonists such as trifluoperazine, calmidazolium (R_{24571}), W_7 etc. has been shown to block phytochrome mediated chloroplast rotation in Mougeotia (Serlin and Roux, 1984) and spore germination in Onoclea (Wayne and Hepler, 1984) suggesting that Ca^{+2} may be binding to calmodulin and thereby leading to the response.

If all phytochrome-mediated responses are coupled to red light through Ca^{+2}, it should be possible to explain the responses in

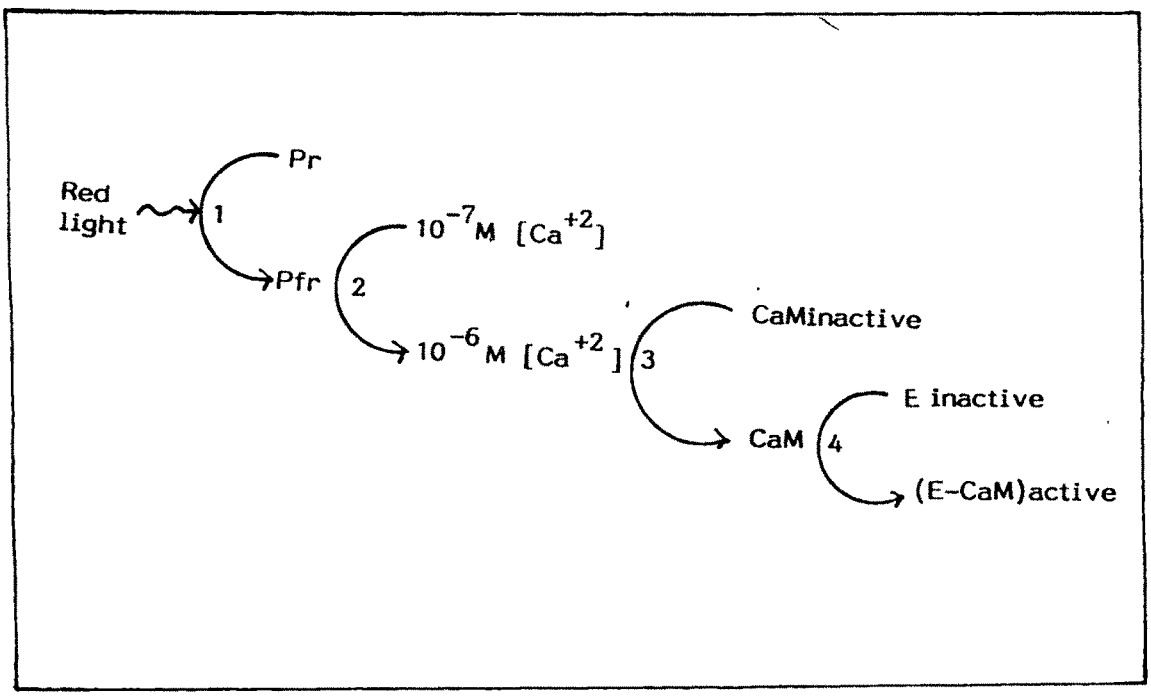


Fig. d : **Involvement of Calmodulin in phytochrome response - a proposed model.**

1. Phytochrome activation by red light irradiation;
2. Pfr -mediated increase in the cytosolic Ca^{+2} concentration;
3. Activation of Calmodulin (CaM) i.e. formation of Ca^{+2} complex with CaM;
4. Activation of CaM dependent enzymes.

which long irradiations are required or long escape times have been demonstrated. Phytochrome control solely by amplitude modulation would be impossible in these long-term responses because of the cytotoxicity of calcium. However, these responses may be regulated through sensitivity modulation (Hepler and Wayne, 1985).

In order to modulate the level of intracellular Ca^{+2}, phytochrome would have to be associated with membranes directly or indirectly. Role of phytochrome in mediating many membrane properties have been reviewed (Marme, 1977; Quail, 1980). Of particular interest is the fact that red light induces a small deplorization with a lag time of less than 1 sec (Newman, 1981; Weisenseel and Ruppert, 1977). This depolarization may be sufficient to open voltage dependent Ca^{+2}-channels and allow the ion to enter the cell. As phytochrome has been found to be localized in mitochondria (Roux et al, 1981), chloroplasts (Hilton, 1983), cytoplasmic vesicles (Saunders et al, 1984) and microsomal fractions (Watson and Smith,1982a,b) it may also induce a release of Ca^{+2} from internal organelles.

Thus, increased cellular concentration of Ca^{+2} acts as the second messenger of a stimulus, the chemical signal that initiates the response may not be Ca^{+2} ion itself but the complex between Ca^{+2} and calcium binding proteins such as calmodulin. The Ca^{+2}-calmodulin complex can act in two ways : directly on an effector system or indirectly on a regulatory system usually a protein kinase which through phosphorylation promotes or inhibits the activity of other enzymes. These two modes of action allow fast and slow responses to be mediated by Ca^{+2} and calmodulin.

A number of plant enzymes such as NAD kinase (Muto and Miyachi, 1977; Anderson et al, 1980), Ca^{+2} -ATPase (Dieter and Marme, 1981), H^{+} -ATPase (Zocchi et al, 1983; Zocchi, 1985), quinate-NAD^{+} -oxidoreductase (Ranjeva et al, 1983), adenylate cyclase (Carricarte et al, 1988) and protein kinases (Veluthambi and Poovaiah, 1984a, b; Hetherington and Trewavas, 1982; Polya and Davies, 1982) have been shown to be regulated by Ca^{+2} and calmodulin.

Protein phosphorylation and dephosphorylation is recognized as an important regulatory mechanism by which the activity of key regulatory enzymes and receptor molecules is altered within cells in response to a wide variety of external stimuli (Cohen, 1985). Calcium-Calmodulin dependent phosphorylation has been suggested as an intermediate step in transducing phytochrome effects on transcription and thereby on protein synthesis on the basis of evidence that nuclear protein phosphorylation in isolated pea nuclei is regulated by phytochrome and is dependent on calcium and calmodulin (Datta et al, 1985). Another interesting evidence demonstrates the presence of polycation-dependent protein kinase associated with purified phytochrome preparation from etiolated Avena seedlings (Wong et al, 1986). A differential phosphorylation of P_r and P_{fr} forms of phytochrome by a phytochrome associated protein kinase and also by mammalian protein kinase (cGMP dependent protein kinase and calcium activated phospholipid dependent protein kinase) has been reported (Wong et al, 1986). The differential phosphorylation of two forms of phytochrome raises the possibility that phosphorylation may play an important role in the regulation of phytochrome-mediated biochemical changes in plants.

The mechanism(s) by which primary stimuli cause changes in cytosolic Ca^{+2} level is beginning to be understood. In animal cells, it is well documented that phosphatidylinositol-4,5 -bisphosphate (PIP_2), one of the inositol phospholipids, plays an important role in transducing extracellular signals into intracellular events (Berridge and Irvine, 1984; Majerus et al, 1986). A wide variety of extracellular signals such as hormones, growth factors, neurotransmitters and light which activate cellular functions and cellular proliferation have been shown to stimulate the hydrolysis of PIP_2 by activating phospholipase C (PIP_2 phospho-diesterase) (Berridge and Irvine, 1984). This gives rise to the production of diacylglycerol (DAG) and inositol -1,4,5-trisphosphate (IP_3). While DAG remains in the membrane matrix and modulates the activity of protein kinase C, IP_3 is released into the cytosol, triggering release of Ca^{+2} from internal stores. The ensuing increase in cytosolic Ca^{+2} leads to a cascade of metabolic events, dependent on either the Ca^{+2} ion itself or on Ca: Ca^{+2} -binding protein complexes, such as Ca^{+2}: calmodulin. The events involved in the transduction of external signals are summarized in Fig.e.

Since there is a considerable analogy between plants and animals with regard to Ca^{+2} messenger system, a possible role of inositol phospholipids in signal transduction is being investigated in plants also. Although research in this area began very recently, evidence in support of the above hypothesis is steadily accumulating. Many components of the inositol phospholipid turnover system have been reported to exist in plants. The presence of polyphosphoinositides (PIP and PIP_2) has been reported in suspension cultured cells of carrot (Boss and Massel, 1985), Catharanthus roseus (Heim and Wagner, 1986), pulvini of legume

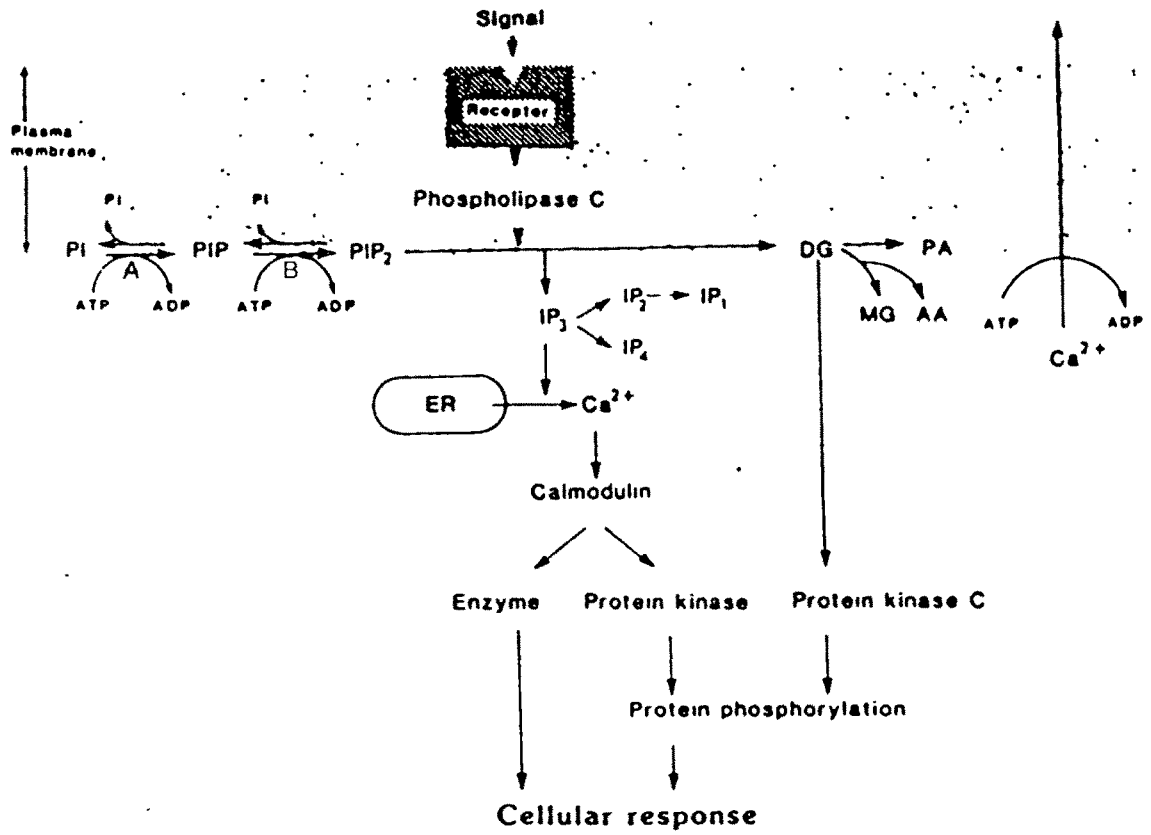


Fig. e : Schematic illustration of stimulus-induced turnover of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the role of turnover products in the Ca^{+2} messenger system.

PI, phosphatidylinositol; PIP, Phosphatidylinositol 4-phsphate; A, PI Kinase, B, PIP kinase; ER, endoplasmic reticulum; IP_4, inositol tetrakisphosphate; IP_3 inositol trisphosphate; IP_2 inositol bisphosphate; IP_1, inositol monophosphate; MG, monoglyceride; AA, arachidonic acid; PA, phosphatidic acid.

Samanea saman (Morse et al, 1986, 1987) and tomato leaf (Drobak et al, 1988). Presence of PI- and PIP-kinases have been demonstrated in membranes isolated from dark grown wheat (Sandelius and Sommarin, 1986). As in animal system, calcium-dependent-phospholipase C has also been detected in higher plants (Irvine et al, 1980; Melin et al, 1987; McMurray and Irvine, 1988). IP_3 has been demonstrated to release Ca^{+2} from mocrosomes isolated from zucchini hypocotyls (Drobak and Ferguson, 1985), corn coleoptiles (Reddy and Poovaiah, 1987) and oat roots (Schumaker and Sze, 1987) and to affect Ca^{+2} fluxes across plasma membrane in isolated carrot protoplasts (Rincon and Boss, 1986).

Das and Sopory (1985) have provided indirect evidence for the role of phosphoinositides in phytochrome mediated responses. They found that 5-hydroxytryptamine (5-HT), an animal hormone, known to stimulate the hydrolysis of PIP_2 mimics the red light mediated regulation of calcium uptake in maize protoplasts. It was suggested that phytochrome might stimulate the hydrolysis of PIP_2 whose products somehow open the Ca^{+2} channels in the membrane. Also, 5-HT has been shown to substitute for light in case of gravitropic curvature of corn roots and levels of IP_3 have been found to increase on either 5-HT or light treatment (Reddy et al, 1987). On the basis of certain preliminary evidences Morse et al. (1986) have suggested a light stimulated turnover of phosphoinositides in the pulvini of legume Samanea saman. Action of plant hormones has also been suggested to involve polyphosphoinositide signalling system. Auxin induced rapid breakdown of phosphoinositide has been observed in Catharanthus roseus (Ettlinger and Lehle, 1988) suggesting that this may initiate a signal cascade and mobilize calcium, resulting in auxin

specific response. All these reports strengthen the likelyhood that the phosphoinositide signalling system is used by higher plants also.

Phytochrome Regulation of Gene Expression :

Irrespective of the mechanism by which phytochrome signal is transduced through the cell, the terminal step of the signal transduction pathway is the alteration of gene expression. Phytochrome mediated photomorphogenesis in plants is accompanied by and may largely result from an orderly temporal and spatial pattern of protein (enzyme) synthesis (Mohr, 1983; Sharma, 1985). Control of protein synthesis by phytochrome is generally described in terms of transcription since the expression of a number of nuclear genes has been shown to be controlled by phytochrome (Tobin and Silverthorne, 1985).

Phytochrome has been shown to cause an increase in concentration of several abundant translatable mRNA in radish cotyledons (Fourcroy et al, 1985) and among them that coding for the small subunit of ribulose 1,5-bisphosphate carboxylase (Fourcroy, 1986). Phytochrome control of this enzyme has also been studied in other plants such as lemna (Stiekema et al, 1983), soyabean (Berry-Lowe and Meagher, 1985), and pea (Jenkins et al, 1983; Thompson et al, 1983). Kaufman et al. (1986) have studied the time course for accumulation of each of 12 different nuclear gene transcripts in pea buds after irradiating dark grown seedlings with a single pulse low fluence red light. Phytochrome mediated control of β-amylase synthesis is also exerted at the level of mRNA synthesis in mustard cotyledons (Sharma and Schopfer, 1987).

From these studies it is evident that phytochrome controls protein synthesis at the level of transcription. However, there are certain evidences which show additional control points, such as translation, activation and inactivation of enzymes, post transcriptional processing of m-RNA etc. at which phytochrome action can be realized. In the case of peroxidase regulation by P_{fr} , the control was apparent at a post-transcriptional level rather than at transcriptional level (Sharma et al, 1976a, b). Phytochrome mediated decrease in the activity of hydroxymethyl glutaryl coenzyme A reductase is evident within 10 min of the onset of exposure due to rapid enzyme inactivation (Brooker and Russell, 1979). However, phytochrome control of phenylalanine ammonia lyase was found to be due to the activation of enzyme (Acton and Schopfer, 1975) but later on it was reported to be due to de novo synthesis of the enzyme (Tong and Schopfer, 1976).

Recently the role of calcium in gene expression in plants has been suggested by Poovaiah and Reddy (1987) on the basis of certain indirect and circumstantial evidences :

(i) Plant hormones, whose action is believed to be mediated by Ca^{+2}, have been shown to regulate gene expression (Theologis, 1986; Guilfoyle, 1986).

(ii) The active form of phytochrome (P_{fr}) which regulates the expression of a number of genes, can modulate Ca^{+2} fluxes in plant cells (Fluhr et al, 1986; Roux et al, 1986).

(iii) A causal link between the phosphorylation of proteins and the regulation of gene expression has been implicated (Purello et al,

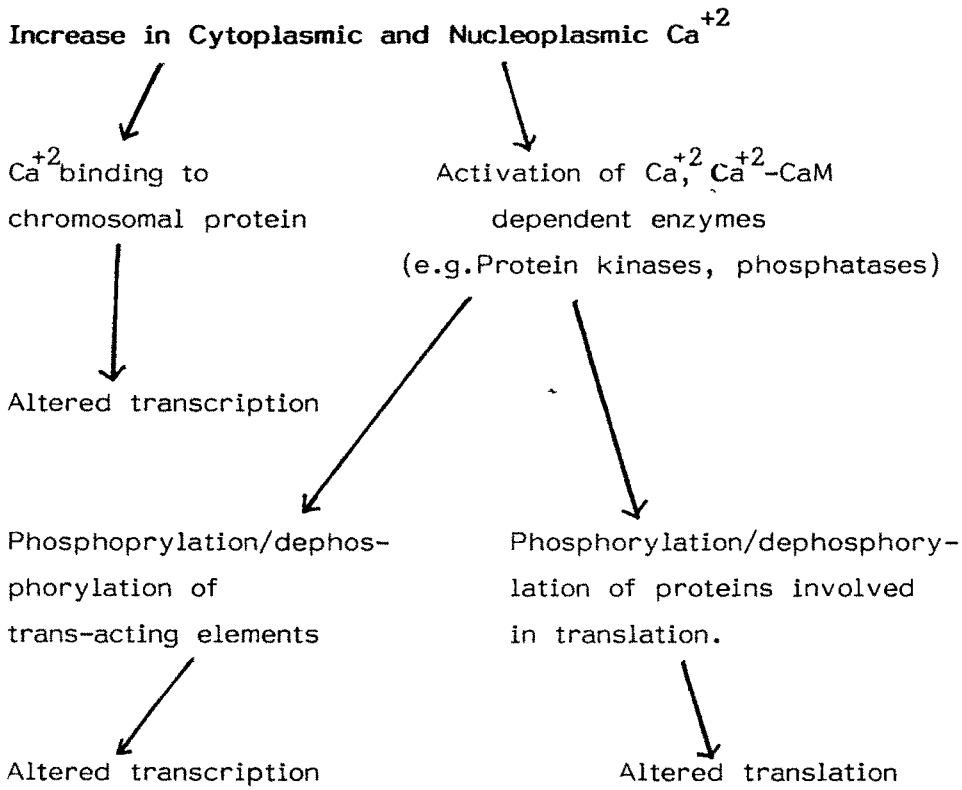


Fig. f : Schematic representation of proposed mechanisms by which calcium could regulate transcription and translation.

1983) and phosphorylation of proteins is regulated by Ca^{+2}-dependent protein kinases (Hetherington and Trewavas, 1982; Veluthambi and Poovaiah, 1984a, b; Polya and Davies, 1982).

Based on these evidences Poovaiah and Reddy (1987) proposed a mechanism by which Ca^{+2} could regulate gene expression (Fig.f).

It is apparent from the foregoing discussion that our understanding of the molecular mechanism of enzyme regulation by phytochrome is far from complete. This is further complicated by the multiplicity of control points where the action of phytochrome can be exerted, which itself can be different for different responses. Nevertheless, elucidation of the total pattern of enzyme regulation by phytochrome is a prime prerequisite for an understanding of the mechanism of the action of light on plant development.

POLYAMINES

Polyamines, spermidine and spermine and their diamine precursor putrescine represent a group of small molecular weight, organic polycations. They play vital role as modulators of a number of biological processes from enzymes activation and maintenance of ionic balance through regulation of growth and development, to mediation of hormonal action and progress of cell division cycle.

In addition to these, other amines closely related both structurally and metabolically have also been found to occur (Smith, 1972). Many analogues of polyamines occur in algae (Hamana and Matsuzaki, 1982; Maiss et al, 1982). The structures of polyamines and their related diamines are shown below :

Putrescine	$H_2N-(CH_2)_4-NH_2$
Cadaverine	$H_2N-(CH_2)_5-NH_2$
Spermidine	$H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$
Spermine	$H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$

Though, spermine was discovered as early as 1678, polyamines remained relatively unfamiliar for a long time because their significance was not known. In recent years there has been a renewed interest in these compounds due to the recognition of their possible role in various growth and developmental processes and other regulatory effects. Several reviews concerning the occurrence, metabolism and functions of polyamines in animals, micro-organisms and plants have been published (Tabor and Tabor, 1984; Smith, 1984, 1985a; Bacharach et al, 1983; Galston,

1983; Slocum et al, 1984).

Occurrence and Metabolism :

Polyamines are known to be ubiquitous in biological materials, though their amounts may vary greatly in different cells. Most of the earlier work involving plants has been centered on putrescine. Putrescine has been detected in several species of higher plants (Hiwatari, 1926; Smith, 1977). The polyamines, spermidine and spermine also occur in leaves, roots and fruits of higher plants and in especially high concentration in embryos of several grains (Smith, 1971). Cadaverine, a higher homologue of putrescine, was found in tobacco and tomato plants (Hault et al, 1970) and in Pisum, Lathyrus and Vicia seeds (Le Rudlier and Goas, 1971; Ramkrishna and Adiga, 1975a). 1,3-diaminopropane, an oxidation product of polyamines, has been detected in a variety of plants including barley (Smith, 1970) and cucumber (Flayeh et al, 1984).

The intracellular distribution of polyamines is not very clearly known. Being positively charged, they bind to polyanions and are highly soluble in aqueous media which results into a redistribution of the amines on cell disruption and homogenization. In spite of these difficulties spermidine and spermine have been shown to be present in both particulate and soluble fractions of etiolated pea epicotyls (Bagni and Serafini-Fracassini, 1974).

Biosynthesis :

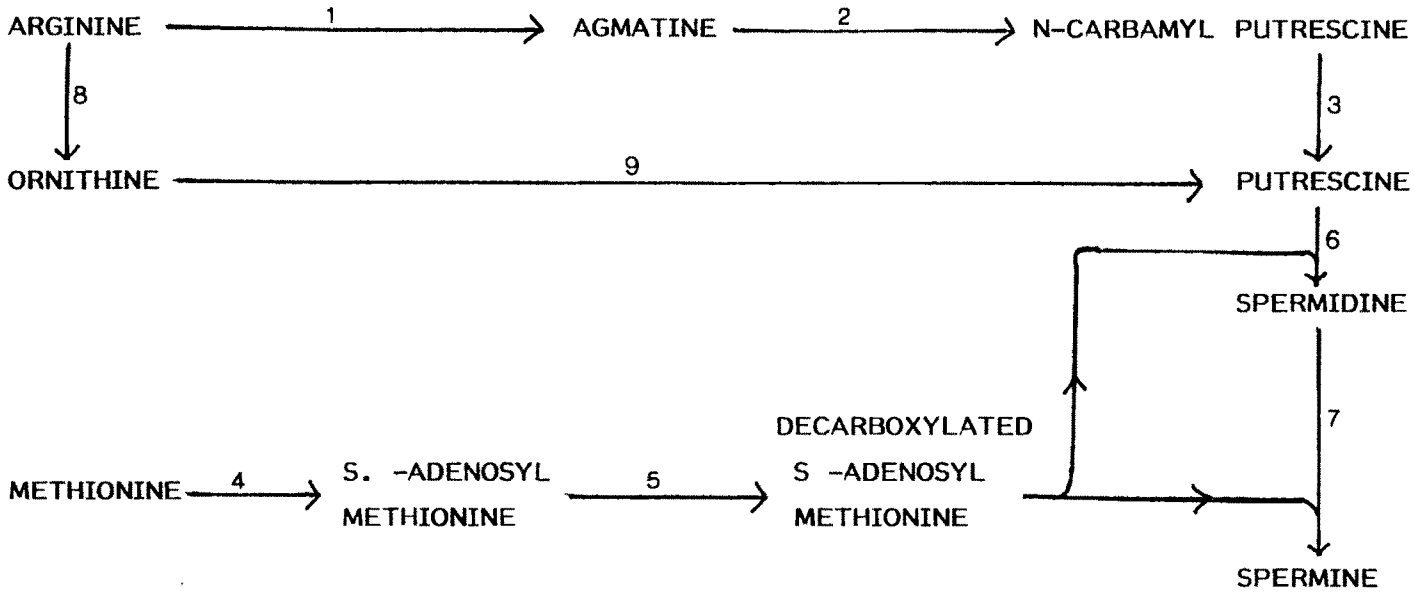
Putrescine occupies a central position in the biosynthetic pathway of polyamines as it is the precursor of both spermidine and spermine. There are two pathways operating for the biosynthesis of

putrescine (Fig.g). First is by decarboxylation of ornithine produced from arginine by arginase reaction. This pathway operates predominantly in animal systems (Heimer et al, 1979). The second pathway occurs in plants, where arginine is first decarboxylated to agmatine (Ramakrishna and Adiga, 1975b, Smith, 1979) which is converted to N-carbamyl putrescine (Yanagisawa and Suzuki, 1981) and then to putrescine (Smith, 1965). In Sesamum indicum N-carbamyl putrescine is derived from citrulline by decarboxylation (Crocomo and Basso, 1974). Even though, agmatine pathway appears to predominate in plants, the presence of ornithine decarboxylase has been reported in several plant systems suggesting that both the pathways are operative in plants (Smith, 1977; Kaur-Sawhney et al, 1982a; Flores et al, 1984).

Spermidine and spermine are synthesized from putrescine by a stepwise addition of propylamine moiety derived from the decarboxylated S-adenosyl methionine which is formed from methionine (Suresh and Adiga, 1977). Addition of propylamine moiety to putrescine for the synthesis of spermidine and spermine is catalyzed by two separate synthetases (Cohen et al, 1981). The enzymes for both the pathways have been well characterized (Adiga and Prasad, 1985).

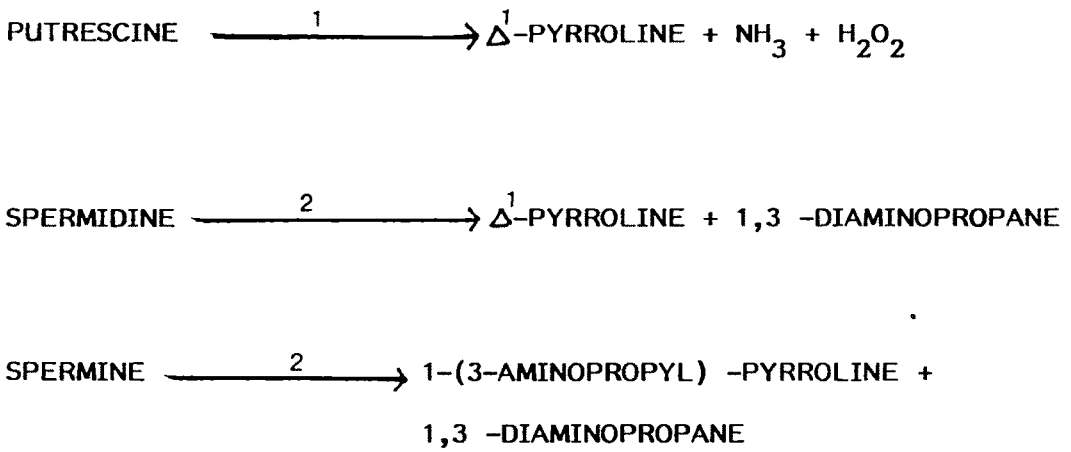
Degradation :

Major pathway for the degradation of polyamines is by oxidation, though, a number of other non-oxidative reactions such as transamination (Kim and Tchen, 1962), carbamylation (Friedman, 1957) and transamidation (Clarke et al, 1959) have also been reported. Two types of oxidases have been reported (Fig.h), an unspecific amine oxidase, generally found



1. ARGININE DECARBOXYLASE
2. AGMATINE IMINOHYDROLASE
3. N -CARBAMYLPUTRESCINE AMIDOHYDROLASE
4. S -ADENOSYLMETHIONINE SYNTHETASE
5. S -ADENOSYLMETHIONINE DECARBOXYLASE
6. SPERMIDINE SYNTHASE
7. SPERMINE SYNTHASE
8. ARGINASE
9. ORNITHINE DECARBOXYLASE

Fig. g : BIOSYNTHETIC PATHWAY OF POLYAMINES



1. DIAMINE OXIDASE
2. POLYAMINE OXIDASE

Fig. h : OXIDATIVE DEGRADATION OF POLYAMINES

in legumes, having high affinity for diamines, putrescine and cadaverine (Hill and Mann, 1968; Smith, 1977; Matsuda and Suzuki, 1981) and the other found in cereals, specific for spermidine and spermine, acting at the secondary amino group and forming 1,3-diaminopropane and pyrroline or amino-propyl pyrroline respectively from spermidine and spermine (Smith, 1971; Kaur-Sawhney et al, 1981).

Functions of Polyamines :

Most of the functions of polyamines may be attributed to their polycationic nature due to which they bind to the negatively charged molecules such as nucleic acids, proteins, neuraminic acid, phospholipids and other cellular anions.

(A) Growth and development :

Recent interest in polyamines has been due to the fact that they were shown to be associated with a variety of growth processes in almost all systems examined. In plants they are implicated in several growth regulatory functions and are being considered as a new class of plant growth regulators (Galston and Kaur-Sawhney, 1982; Galston, 1983).

The effect of polyamines on growth was first demonstrated in the dormant Helianthus tubers where spermine promoted the growth of tubers in vitro (Bertossi et al, 1965; Bagni et al, 1971). Low levels of polyamines as well as plant hormones present in dormant tubers did not permit cell proliferation. However, when polyamines were supplied exogenously the tuber cells were able to divide and grow without any other growth substances (Serafini-Fracassini et al, 1980). Higher

polyamine content may be associated with rapid tissue growth. In Phaseolus vulgaris, the spermidine and spermine levels decreased in cotyledons and increased in shoot with simultaneous and parallel changes in RNA and protein content (Bagni, 1970). The increase in plant growth was also associated with a parallel increase of ADC and/or ODC, the two key enzymes of the biosynthetic pathway of polyamines (Palavan and Galston, 1982). However, recently Felix and Harr (1987) have shown a marked increase in polyamine content in cotyledons or endosperms, rather than in hypocotyl, radicle or coleoptile in the seeds on germination.

Polyamine concentration was also found to increase in response to external stimuli which bring about an increase in growth. Application of red light to etiolated pea seedlings have been shown to cause an increase in the growth of leaf and terminal buds and a reduction in the rate of elongation. These changes in growth are paralleled by changes in the level of ADC activity and polyamine levels (Dai and Galston, 1981; Goren et al, 1982a, b). In radish seeds, polyamine enhanced embryo growth during early periods of germination by enhancing protease activity in cotyledons and hence enhanced reserve protein mobilization rather than nitrogen assimilation process (Srivastava et al, 1985).

(B) Stabilization of membranes :

Polyamines are known to bind and stabilize cell membranes (Harold, 1964) thereby altering permeability of the membrane and preventing leakage of intracellular compounds. Freshly isolated oat leaf protoplasts were stabilized against lysis by polyamines. Spermine was more effective than spermidine, which in turn was more effective than putrescine or

cadaverine (Altman et al, 1977). Naik and Srivastava (1978) and Naik et al. (1980) have shown that the efflux of betacyanin from beet root was prevented by polyamines due to stabilization of plasmalemma. Evidence for the stabilizing effect of polyamines on membranes was also obtained with apple cell culture, storage tissue from swede and spinach leaf discs. Increased ion efflux from these tissues induced by various guanidines was partially reversed on adding spermine. This reversal could also be effected with $CaCl_2$ (Srivastava and Smith, 1982a). Spermine also facilitates the inter and intra specific electrofusion of protoplast, probably as a result of its ability to stabilize the plasmalemma (Chapel et al, 1984).

Roberts et al, (1986) studied the effect of polyamines on the fluidity of microsomal membranes from primary leaves of Phaseolus vulgaris and observed that spermidine and spermine were more effective than putrescine at reducing membrane fluidity. Also at equimolar concentrations polyamines and calcium have similar effect on the mobility of membrane probes. Spermine had essentially equivalent effect on the fluidity of native membranes, heat denatured membranes and liposomes prepared from the total lipid extract of the membranes, indicating that polyamines associate with membrane lipids. These result suggest that some of the physiological effects previously attributed to exogenously added polyamines could reflect membrane rigidification rather than a true physiological response. However, a more recent study by Agazio et al. (1988) demonstrating inhibition of K^+ influx and H^+ extrusion from plasma membrane of maize root segments by exogenously supplied polyamines reflects a true physiological role.

Polyamines have been found to affect several membrane bound enzymes such as NADH-dehydrogenase (Hochstein and Dalton, 1968), membrane bound ATPase as well as peroxidase (Srivastava and Rajbabu, 1983a,b). The stabilizing effect of polyamines on membrane and membrane bound enzymes appears to be due to their association with the phospholipids (Gary-Bobo, 1970). Some studies however indicate that polyamines may also interact with -SH groups of the membrane proteins (Naik and Srivastava, 1981; Srivastava and Smith, 1982a).

(C) Macromolecules :

Several functions of polyamines at molecular level are still not defined but evidences suggest that their role is to stabilize and organize DNA and RNA structures (Zhurkin et al, 1980). Due to their polycationic nature, polyamines may bind strongly to nucleic acid. Attachment of polyamines to t-RNA was confirmed by their presence in t-RNAs isolated from pea epicotyls, spinach leaves (Cohen et al, 1969) and Helianthus tuber (Bagni et al, 1981). Polyamines are also known to be involved in the synthesis of nucleic acids (Guilfoyle and Hanson, 1973; Kaur-Sawhney et al, 1980) and proteins (Igarashi et al, 1979, 1980).

Polyamines modulate phosphorylation of various proteins which may indirectly result in the regulation of cellular enzymes (Kuehn et al, 1979; Cohen, 1980). Later, polyamine biosynthesis itself was found to be regulated through a polyamine mediated, reversible phosphorylation of ODC (Kuehn and Atmar, 1982). Polyamines stimulate phosphorylation of proteins from corn coleoptiles (Veluthambi and Poovaiah, 1984c) and

isolated pea nuclei (Datta et al, 1986). Polyamines also appear to be more directly involved in the regulation of other enzymes through various types of ionic interactions (Nakamura et al, 1972) as well as covalent binding to enzyme proteins (Folk, 1980).

(D) Senescence :

Many studies have shown that polyamines play a role in retarding senescence in both attached (Altman and Bacharach, 1981) and detached leaves (Cohen et al, 1979; Kaur-Sawhney et al, 1982b). Shih et al. (1982) showed that 1,3-diaminopropane, the oxidation product of polyamines, was responsible for delaying the senescence. Polyamines retard senescence by affecting various biochemical changes associated with the process of senescence.

Polyamines delay or prevent the rise in protease and RNAase activities alongwith chlorophyll loss in dark incubated oat leaves (Kaur-Sawhney and Galston, 1979). Studies from our laboratory have shown that polyamines decrease lipid peroxidation and maintain superoxide dismutase level during leaf senescence (Srivastava et al, unpublished observation). Polyamines were also found to retard senescence caused by exogenous ethylene (Galston, 1983). Polyamine content was found to decline during senescence and this decline was proposed to be responsible for the senescence of the tissue (Altman and Bacharach, 1981). Though polyamines have been reported to delay senescence in a number of tissues, they had no protective effect on senescence in carnation flowers (Downs and Lovell, 1986).

(E) Stress :

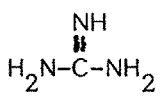
Plants respond to various stress conditions by developing protective measures against them. Under stress conditions such as osmotic stress, low pH, chilling, high salinity or mineral deficiency there is an activation of putrescine biosynthesis and a massive increase in diamine concentration (Shevyakova, 1981; Altman et al, 1982; Bagni, 1982; Young and Galston, 1973; Flores et al, 1984; Smith, 1984). In general, the concentrations of spermidine and spermine are relatively unresponsive to stress (Young and Galston, 1983; Smith, 1984). The accumulation of putrescine may be a mechanism by which the plant balances excess of H^+. Putrescine may also be protecting or reversing the membrane disruption caused by these conditions.

GUANIDINES

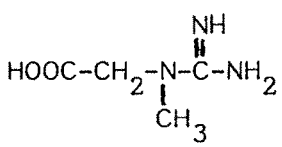
Guanidines are nitrogenous compounds of wide spread occurrence. They have been detected in fungi (Boldt et al,; 1971), animals (Pisano et al., 1963) as well as in plants (Reinbothe and Mothes, 1962).

Guanidine was first synthesized from guanine in 1861 by Strecker. It was subsequently isolated from Vicia sativa seeds by Schulze (1892). The isolation of guanidine was followed by the discovery of other guanidino compounds in plants (cf. Guggenheim, 1958). Some naturally occurring guanidines are shown below :

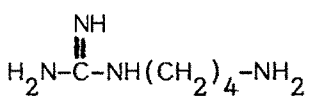
Guanidine



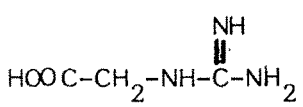
Creatine



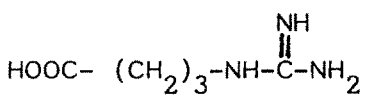
Agmatine



Gaunidino ac etic acid



Guanidino butyric acid



Mono-and di-substituted guanidines have also been detected, though fewer di-substituted guanidines are known. Di-substituted guanidines like arcaine are found in marine plants and seeds (Shibuya and Makizumi, 1953).

Crown gall tumour tissues also contain guanidines and compounds like octopine, not occurring in normal tissue (Goldmann et al., 1969; Morel, 1971). Several guanidino compounds have been detected in tobacco (Yoshida and Mitake, 1966); barley and spruce (Durzan and Steward 1967) and Douglas fir (Ebell and McMullan, 1970). The natural occurrence of 3-guanidinoproline (Gallina et al., 1971), streptolidine (Byeroft and King, 1972), and guanidino amino acid (Wakamiya et al., 1973) has been reported.

Many of the naturally occurring guanididines arise from arginine by transamidination. Transamidination is also involved in the formation of bi-guanidines like arcaine.

Besides transamidination, guanidines may also be synthesized by several other reactions. Agmatine is formed by the decarboxylation of arginine (Smith, 1963). In pine, γ-guanidinobutyric acid arises by oxidative pathways via α-keto-β-guanidinovaleric acid (Barnes, 1962). This intermediate has been found in spruce (Durzan, 1968a). Barnes (1962) observed that radioactivity from ^{14}C-1-arginine was found in the γ-aminobutyric acid moiety of γ-guanidinobutyric acid, which is consistent with the oxidative pathway, though the possibility of transamidination cannot be ruled out.

The ultimate source for monosubstituted guanidines in plants is inorganic nitrogen and guanidines were found to accumulate in response to ammonium-nitrogen (Durzan and Steward, 1967). However, Ebell and

McMullan (1970) found that the level of guanidines in Douglas fir was higher when nitrate rather than ammonium was used as fertilizer.

Guanidines are degraded to yield amines as one of the products. Thus, agmatine gave putrescine (Smith, 1969), while canavanine formed the amine canaline and guanidino acetic acid forms glycine (cf. Bidwell and Durzan, 1975).

The guanidine level has been found to vary diurnally and with the plant part examined in spruce saplings (Durzan, 1968b).

Functions of Guanidines :

Guanidines are known to inhibit mitochondrial respiration (Wilson and Bonner, 1970). They possess antilipolytic properties (Weitzel et al, 1972) and affect urate oxidase activity (Bentley and Truscoe, 1969).

Guanidines also effect metabolism in a number of ways and are used as fungicides for the prevention of apple scab (Woodcock, 1968). In white spruce callus, guanidines inhibited the assimilation of ^{14}C-urea (cf. Bidwell and Durzan, 1975).

Exposure of Valonia and Nitella to guanidines lowered the threshold of electrical excitability and produced a long series of quick action currents (Osterhout, 1942). The mitochondrial transport of sodium and potassium were also sensitive to guanidine (Gomez-Puyou et al, 1973). When the effect of octyl guanidine on the growth of intact etiolated barley seedlings was tested, it was observed that root and shoot growth was inhibited. This inhibition was partially relieved by addition of K^+ to the culture medium. Octylguanidine probably inhibits growth

of roots and shoots by interfering with the transport of K^+ across the cell membrane (Gomez-Lepe and Salmon, 1986).

Guanidine, above 0.1 ppm is toxic to plants (Kawakita, 1905), probably because it acts as a very strong organic base. High levels of guanidine, like urea, break the hydrogen bonds of macromolecules (Katz and Miller, 1972).

Some guanidines, such as N-methyl-N'-nitro-N-nitrosoguanidine (Craddock, 1968) and nitrosoarginine (Endo and Takahashi, 1973) are reported to have mutagenic activity. They react with the nitrate in food and form carcinogenic products (Archer et al, 1971).

Due to their fungicidal properties, synthetic guanidines like dodine and guazatine have been recommended for the control of a number of diseases of fruits, nuts and vegetable crops and on certain ornamental and shade trees (cf. Byrde et al, 1962). Guazatine salts are effective as cereal dressing. They are effective in the control of brown spot, blast, alternaria leaf spot in the rice, apples and other fruits.

Some of the toxicity of guanidines may be associated with an increased permeability of plant membranes (Srivastava and Smith, 1982a). They are found to increase permeability of the tonoplast surface by interacting with phospholipids of the membranes (Beatriz et al, 1979).

Guanidine salts also inhibit virus infection in plants such as the tobacco necrosis virus (Varma, 1968) as well as the infectivity of many animal viruses.

Other studies have indicated that guanidines may have an action antagonistic to that of polyamines in many tissues. Thus, they inhibit the growth of cress, barley and oat seedlings (Srivastava and Smith 1982b). This toxicity of guanidines was reversed by spermine. Guanidines have been shown to destabilize cell membranes and this effect was again reversed with the application of polyamines (Srivastava and Smith, 1982a). On the other hand, both dodine and spermine were found to retain chlorophyll and retard the senescence of detached barley leaves (Srivastava et al, 1983). Similarly, both polyamines and guanidines increase membrane bound ATPase activity in maize scuttellum (Srivastava and Rajbabu, 1983a) whereas peroxidase was activated by guanidines but inhibited by polyamines (Srivastava and Rajbabu, 1983b). Moreover, guanidines reversed the effect of polyamines but polyamines could not reverse the effect of guanidines indicating that guanidines probably have two separate binding sites on the membrane. The effects of guanidines thus appear to vary with the system studied.

From the literature surveyed above it is evident that both phytochrome and polyamines mediate plant growth and development primarily through their interaction with membranes. It is, therefore, quite likely that polyamines may play an important role in regulating phytochrome mediated responses. The aim of the present study was to investigate this by studying the phytochrome regulation of some light induced enzymes. Literature surveyed below showed that the two enzymes - glutamine synthetase, a key enzyme of nitrogen assimilation pathway, and NAD kinase, an important enzyme for producing NADP, are both regulated

by light. NAD kinase is also activated by Ca^{+2}: Calmodulin. No phytochrome regulation of glutamine synthetase is known though in one report NAD kinase has been shown to be regulated by phytochrome in in vitro system but it was not confirmed by other workers.

GLUTAMINE SYNTHETASE

Nitrogen assimilation has been reported to be higher in light than in dark (Canvin and Atkins, 1974). Light regulation of nitrate assimilation occurs at the level of various enzymes of the pathway. The reduction of nitrate to ammonia is brought about by a two step process, mediated by the enzymes nitrate reductase (NR) (Evans and Nason, 1953) and nitrite reductase (NiR) (Hageman et al, 1962). The ammonia so formed may be assimilated by any one of the two pathways, catalyzed either by glutamate dehydrogenase (GDH) or by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Fig.i). In most plant systems studied GS/GOGAT appears to be the predominant pathway at physiological concentrations of ammonia (Stewart et al, 1980).

Light mediated induction of nitrate reductase has been shown to be under phytochrome regulation in a number of tissues (Jones and Sheard, 1972, 1974, 1975; Duke and Duke, 1978; Sasakawa and Yamamoto, 1979; Rao et al, 1980; Ramaswamy et al, 1983; Moroz et al, 1984). Phytochrome may affect NR induction either by increased accessibility of nitrate or by increased synthesis of the enzyme. Phytochrome regulation of nitrite reductase has also been reported (Rao et al, 1981).

Glutamine synthetase, a key regulatory enzyme of the ammonia assimilation pathway, is also likely to be regulated by light especially in chlorophyllous tissue since a net increase in GS activity has been reported during the greening of etiolated plant tissues (Guiz et al, 1979; Evstigneeva et al, 1981; Hirel et al, 1982; Nishimura et al, 1982; Canovas et al, 1986). However, so far no report is available on the

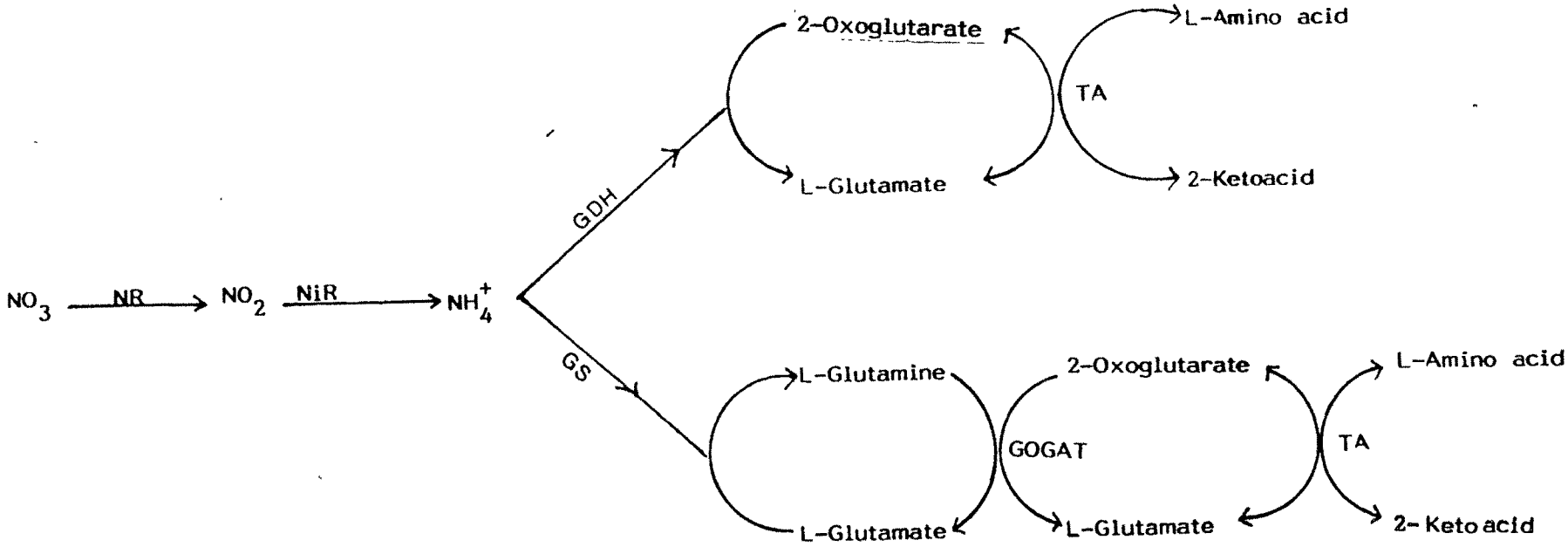


Fig. i : Nitrate assimilation pathway

phytochrome mediated regulation of GS.

GS is found to occur throughout the plant and animal kingdom and has been studied from a wide variety of sources. It catalyzes the reaction

$$ATP + L\text{-Glutamate} + NH_3 \xrightarrow{Mg^{+2}} L\text{-Glutamine} + ADP + Pi.$$

In addition to the biosynthetic reaction, GS can also utilize hydroxylamine as substrate, producing γ-glutamyl-hydroxamate (Varner, 1960).

$$ATP + L\text{-Glutamate} + NH_2OH \xrightarrow{Mn^{+2}} \gamma\text{-Glutamylhydroxamate}$$

GS can also catalyse a γ-glutamyl transfer reaction with glutamine as its substrate, resulting in the formation of γ-glutamylhydroxamate. The transferase activity seems to be several times higher than synthetase and is often used for quick measurements of GS in crude preparations (Shapiro and Stadtman, 1970).

The enzyme seems to be distributed in all parts of the plant but the activity differs markedly from one tissue to another. In general, specific activity of GS is higher in shoots than in roots, except in the case of species that assimilate a large quantity of nitrogen in the roots (Lee and Stewart, 1978). The enzyme activity is higher in developing and germinating seedlings as well as young expanding leaves but decreases with leaf senescence (Storey and Beevers, 1978).

The subcellular distribution of GS has been studied by several investigators (Wallsgrove et al, 1979, Mann et al, 1979). The enzyme is present in the cytosol as well as in the chloroplasts of the cell.

The cytosolic and chloroplastic forms from barley have similar molecular weights (Mann et al, 1979) but differ in their pH optima and stability. Since then two distinct isoenzymes of GS, with specific kinetic and regulatory properties, have been identified in green tissues of several higher plants (McNally et al, 1983). The first isoform (GS_1), present in the cytosol, is probably involved in ammonia recycling during photorespiratory nitrogen cycle and in ammonia assimilation in dark (Hirel and Gadal, 1980a; Ito et al, 1978; Mann et al, 1980). The second isoform (GS_2), located in etioplasts (Hirel and Gadal, 1980b) and chloroplasts (Hirel and Gadal, 1980a; Mann et al, 1979), is specific for ammonia assimilation inside the plastids.

GS studied from all sources has a very low Km for ammonia, in the range of 1-2 x 10^{-5} M(O'Neal and Joy, 1974) which supports the view that GS plays a major role in ammonia assimilation at the physiological concentration of ammonia.

GS forms a locus of control over the input of ammonia into the organic form, and is regulated by several factors. Studies on the ammonia assimilating enzymes in lemna show a reciprocal pattern of control over GS and GDH (Rhodes et al, 1976). GS is active at low ammonia and high energy level, while GDH is active at high ammonia and comparatively low energy state. Consistent with this hypothesis is the observation that ADP and AMP competitively inhibit GS in rice roots (Kanamori and Matsumoto, 1972), pea leaves (O'Neal and Joy, 1975) and in lemna leaves (Stewart and Rhodes, 1977).

GS has also been found to be susceptible to a variety of

metabolites. Kapoor and Bray (1968) and Kapoor et al. (1968) showed that GS from Neurospora crassa is inhibited by glycine and histidine, as well as anthranilic acid, which derive their amino group from glutamine. They suggested that a system of cumulative feed back inhibition operates in N. crassa. The effect of amino acids, is however, variable. Kanamori and Matsumoto (1972) found no inhibition of rice root GS with any of the amino acids but Stewart and Rhodes (1977) reported that a mixture of amino acids inhibit GS in lemna.

Various intermediates of metabolic pathways are also reported to affect GS activity. Carbamoyl phosphate inhibits GS from many sources (O'Neal and Joy, 1975) but lemna GS is not affected (Stewart and Rhodes, 1977). On the other hand, nitrate and 2-oxoglutarate activate the enzyme while pyruvate and glyoxylate inhibit it in pea seeds and rice roots (Kanamori and Matsumoto, 1972). These effects are also observed in lemna leaf enzyme (Stewart et al, 1980).

Various cellular metabolites altering GS activity do so either by reversibly deactivating the enzyme or by dissociating it into less active monomers or by conformational modification of the enzyme. Reversible deactivation of the enzyme has been shown in E. coli and other microorganisms (Stadtman et al, 1970) but not in higher plants. It involves adenylation of the enzyme catalysed by an adenyl transferase. The modified enzyme has lower catalytic activity and is more susceptible to feed-back inhibition. GS may exist in various states of adenylation, depending upon ATP levels in the cell. Sims (1976) has extensively studied GS activity in yeast and found that it is controlled by a reversible dissociation of active octamer into tetramers.

GS has also been suggested to be subject to conformational modification. Rhodes et al. (1975, 1976) observed that GS in lemna is reversibly inactivated when the plants are grown in ammonium or when ammonium is supplied to nitrate grown plants. Reactivation of the enyzme occurs on transfer of the plants to light or in vitro incubation with ATP, glutamate and reduced glutathione. They suggested that conformational changes in the enzyme in relation to thiol groups on the subunits lead to an active or inactive GS. Chloroplastic GS may also be regulated similarly, thus providing a means for the integration of assimilatory carbon and nitrogen metabolism.

Several studies indicate that the end product, glutamine, controls the levels of not only GS and GDH but also NR and NiR (Stewart and Rhodes, 1977; Rhodes et al, 1976). It is suggested that a group of ammonia utilizing enyzmes in plants share a common regulatory metabolite as in the case of fungi and bacteria.

Apart from being regulated by various metabolites, GS is also expected to be regulated by light (Canvin and Atkins, 1974). GS has been reported to increase during the greening of the etiolated pumpkin cotyledons (Evstigneeva et al, 1981; Nishimura et al, 1982), rice leaves (Hirel et al, 1982) and tomato leaves (Canovas et al, 1986). The increase in GS activity in rice leaves occurs in the chloroplast compartment where as cytoplasmic GS activity decreases (Hirel et al, 1982). The changes in GS have sometimes been explained on the basis of feed-back inhibition by amino acids, whose concentration has been shown to change with transition from dark to light in pea leaves (Bauer et al, 1977) and sunflower roots (Knight and Weissman, 1982). This

does not appear to be valid since the inhibitory effect of amino acids on GS in vitro is very small in tomato leaves (Canovas et al, 1984). In lemna minor, a decrease in GS activity has been reported to occur after transferring the plants from light to dark (Rhodes et al, 1976).

The exposure of plant tissue to light appears to cause two primary events, increase in cell reducing power and phosphate bond energy. Transition from dark to light changes the energy charge inside the chloroplast from ca.0.3 to 0.6 in the dark to 0.8 in the light (Halliwell, 1981; Stitt et al, 1982; Pradet and Raymond, 1983) and tomato leaf GS shows marked differences within this range (Canovas et al, 1986).

Besides the energy charge, number of sulfhydryl groups in the GS also regulate its activity. Purified GS from tomato leaves has two sulfhydryl groups in each monomer and the activity of the enzyme is inhibited by p-CMB (Canovas et al, 1986). During the greening of the tissue, the reducing power of photosynthetic process can be used to maintain the sulfhydryl groups in the reduced form through a thioredoxin/ thioredoxin-ferredoxin reductase system (Buchanan et al, 1979). However, in rice leaves, the changes reported in GS may also be explained by changes in the synthesis of the enzyme (Hirel, et al, 1982).

Thus increase in GS activity by light exposure may be due to increased energy charge, increased reducing power as well as due to new synthesis of the enzyme.

NAD KINASE

Nicotinamide coenzymes i.e. NAD and NADP exist in both oxidized and reduced states and function as carriers in oxidation reduction reactions. Nicotinamide coenzymes occur in various plant tissues at such a low concentration that they could be rate limiting for specific enzyme reactions (Yamamoto, 1963). Both the coenzymes have physiologically different roles. NAD is reduced to NADH in a large number of catabolic dehydrogenase reactions and this NADH is reoxidized by the mitochondrial respiratory chain to yield useful chemical energy. On the other hand, NADPH is formed from NADP by a relatively small number of dehydrogenase reactions and is used mainly as a source of reducing power for biosynthetic reactions. The nicotinamide coenzymes may be considered to be a self maintaining system (Yamamoto, 1970). Accordingly the pattern of nicotinamide coenzymes may rapidly change with the variation of environmental factors such as oxygen and light. A high NADP +NADPH level occurs under aerobic conditions (Yamamoto, 1966a). Illumination with a high intensity of white light increases NADP level in chlorella (Oh-hama and Miyachi, 1959) and higher plants (Ogren and Krogman, 1965). This increase in NADP may be related to the efficiency of photosynthesis since NADP is a terminal electron acceptor of photosynthetic electron transport chain.

NADP is synthesized by the phosphorylation of NAD, catalyzed by the enzyme NAD kinase (AMP : NAD 2' -phosphotransferase EC 2.7.1.23) in presence of ATP and Mg^{+2}. Unlike the yeast NAD-kinase, plant enzyme does not phosphorylate NADH to NADPH and the reduced NAD is a potent

inhibitor of plant NAD kinase (Yamamoto, 1966b, 1970). Accordingly the slowness of NADH oxidation in plant tissues due to anaerobic conditions results in the inhibition of plant NAD kinase.

Increase in NADP level on illumination to white light is postulated to be due to increase in NAD kinase activity (Oh-hama et al, 1963). Tezuka and Yamamoto (1972) have shown that in cotyledons of 5 day old germinating seeds of Pharbitis nil, 5 min of red light and subsequent 15 min of darkness raises NADP level. The 5 min of far-red light decreases the red light effect and subsequent red light again raises NADP level. Further incubation in dark for 2 hr after the red light illumination decreases NADP level gradually to the dark control. These results suggest a close relation of phytochrome action to NADP synthesizing and/or decomposing systems (Fig. j).

Phytochrome control of NAD kinase is reported in the cell free extracts of six day old upper most internode including terminal bud of Pisum sativum (Tezuka and Yamamoto, 1972). When the assay mixture containing NAD, ATP, Mg^{+2} and partially purified phytochrome preparation having NAD kinase was illuminated successively with red light for 5 min, far red light for 5 min and the second red light for 5 min and the mixture was incubated for 30 min at 30°C in the dark to allow NADP formation showed that NAD kinase activity is stimulated by red light and subsequent far-red light reverses the red light effect. The Km value for NAD was 1.84 mM in dark and 0.9 mM under red light. Thus P_{fr} form of phytochrome may contribute to the decrease of Km value for NAD by introducing a conformational change in the enzyme.

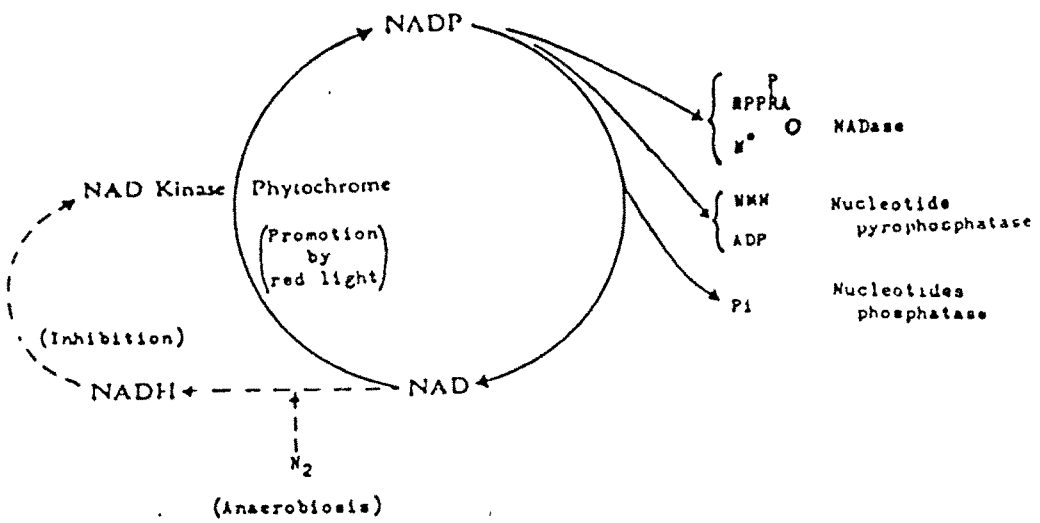


Fig. j : Enzymatic device related to variation of NADP level.

However, Hopkins and Briggs (1973) could not demonstrate any significant increase in NAD kinase activity in partially purified preparation from apical shoots of Pisum sativum on exposure to red light.

Apart from being photoregulated, Muto and Miyachi (1977) reported the presence of an activator of NAD kinase in several species of higher plants. Anderson and Cormier (1978) subsequently showed that the activation of NAD kinase requires free Ca^{+2} and that the activator is a protein. Anderson et al. (1980) purified the activator to homogeneity from plant sources and identified it as calmodulin. Comparision of the properties of calmodulin isolated from plant and animal sources showed that they are strikingly similar proteins (Anderson et al, 1980). A variety of physical measurements on both mammalian (Klee, 1977) and plant (Anderson et al, 1980) calmodulins have shown a Ca^{+2} dependent conformational change in calmodulin. For calmodulin activation of NAD kinase these relationships have been illustrated as follows where Ca^{+2}-calmodulin* indicates the active conformation of calmodulin (Jarrett et al, 1980).

$$Ca^{+2} + \text{Calmodulin} \rightleftharpoons Ca^{+2}\text{-calmodulin} \rightleftharpoons Ca^{+2}\text{-calmodulin}^*$$

$$Ca^{+2}\text{-calmodulin}^* + \underset{\text{(inactive)}}{\text{NAD kinase}} \rightleftharpoons Ca^{+2}\text{-calmodulin-}\underset{\text{(active)}}{\text{NAD kinase}}$$

This mechanism is analogous to the calmodulin dependent activation of several enzymes of mammalian origin (Wang and Waisman, 1979).

Light dependent conversion of NAD to NADP occurs in the chloroplast and over 90% of the NAD kinase activity is located in the

chloroplast of pea, wheat, sunflower, crabgrass and foxtail whereas in corn only about 5% of the total NAD kinase was found to be associated with the chloroplast fraction (Muto et al, 1981). NAD kinase in dark grown corn coleoptiles has also been found to be localized on outer mitochondrial membrane (Dieter and Marme, 1984). Both chloroplastic and mitochondrial NAD kinases were dependent upon Ca^{+2} -calmodulin for its activity (Jarrett et al, 1982; Dieter and Marme, 1984). Calmodulin has been found to be present in the stroma of pea chloroplasts at a concentration of 0.1 μM which is in excess of that needed to saturate the NAD kinase (Jarrett et al, 1982). Muto (1982) has reported similar observations with wheat chloroplast. In both pea and wheat, only 1% of the total cellular calmodulin is found in the chloroplasts whereas about 95% is found in cytosolic fraction. These results suggest that plant calmodulin may also be involved in regulatory events associated with the cytosolic fraction and/or other cellular organelles (Cormier et al, 1985).

Dieter (1986) studied the photoregulation of NAD kinase in corn coleoptiles and showed that light irradiation of intact corn seedlings leads to an increase in NADP without any change in the properties of NAD kinase. Calmodulin content also remains same in both dark and light grown corn coleoptiles. Thus, neither the enzyme nor calmodulin level seems to be altered by the light irradiation. Since light irradiation had no effect on the mitochondrial NAD kinase in vitro, it was suggested that the light dependent increase of NADP in vivo can not be due to a photoregulation of the enzyme. Based on these and other experimental evidences Dieter (1986) proposed a hypothesis for the light dependent regulation of NAD kinase (Fig.k).

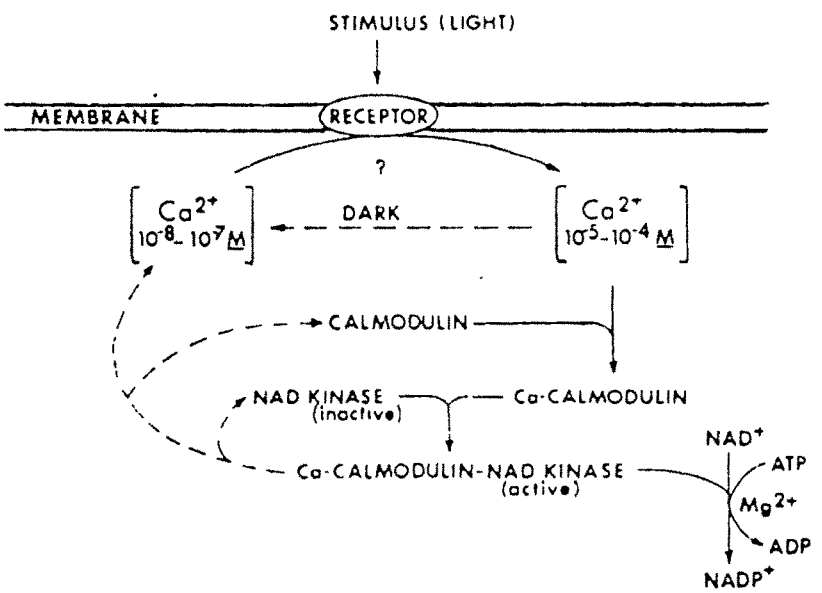


Fig. k : Model for the involvement of Ca^{+2} and calmodulin during stimulus-response coupling in plants.

It is proposed that light increases the cytoplasmic free calcium concentration via an alteration of the enzyme activity of the calcium pump or via change in the properties of calcium channels. The increase of the free calcium concentration in the cytoplasm leads consequently to the formation of the "active" Ca^{+2} -calmodulin complex. The Ca^{+2} calmodulin complex binds to the NAD kinase in its inactive form and this binding leads to the activation of the enzyme and consequently an increase in NADP level.