Discussion
DISCUSSION

One of the pigment that perceives light, and controls morphogenetic processes, is phytochrome (Mohr, 1972). The molecular mechanism by which phytochrome regulates various metabolic and morphogenetic processes is still elusive. The present study was undertaken to unravel the primary steps triggered by phytochrome and a possible relationship, if any, of these early events to enzyme stimulation. Of the primary events, turnover of phosphoinositides and protein phosphorylation was studied. For enzyme stimulation, phytochrome regulated and substrate (nitrate) inducible enzyme, nitrate reductase, was taken as a model system (Rao et al., 1980, 1983). This system, which is described briefly in the following paragraph, is interesting as the coupling between the formation of light induced signal and its utilization for affecting the response can be separated on the time scale (Sharma and Sopory, 1984).

In the dark grown etiolated maize leaves, at the time of light irradiation, nitrate is withheld and it is supplied only after a lapse of certain time. It was found that if the irradiation was followed by a dark period of 2 hrs, NR stimulation was maximum. It was shown earlier (Sharma and Sopory, 1984) and has now been confirmed by dot blot
hybridization using cDNA probe of NR that during the period after red light irradiation, mRNA for NR is not produced. It was logical then to conclude that during the ensuing dark period following red irradiation, which converts Pr to Pfr, a 'signal' would be produced which is utilized later by nitrate to stimulate NRA. This system therefore enabled us to look for the early changes induced by Pfr without these getting coupled to the end response i.e stimulation of NR. In an earlier work from this laboratory it was shown that one of the early response induced by Pfr is to modulate the calcium fluxes (Das and Sopory, 1985). This response was mimicked by serotonin, a compound known to affect PPI cycle in animals. In view of this, our first question was, does Pfr affect PPI cycle in maize and if so, whether it has any correlation with enzyme stimulation.

**SIGNAL GENERATION**

*Phytochrome affects the turnover of poly phosphoinositides at kinase/phospholipase level*

Phosphatidylinositol is known to undergo rapid turnover (Hokin, 1985) leading to the formation of PIP and PIP$_2$. The release of IP$_3$ and DAG through breakdown of PIP$_2$ appears to be a key event for initiating several biochemical processes (Berridge and Irvine, 1984; Nishizuka, 1984a&b; Downes and Michell, 1985). IP$_3$ affects the release of endogenous calcium, and DAG activates protein kinase which is known to transduce the signal.
Presence of inositol phosphates and their role during seed germination was shown by Biswas et al. (1978) who proposed a metabolic cycle involving glucose 6-phosphate and myo-inositol phosphates. A number of other roles of inositol have also been implicated in plant systems (Loewus and Loewus, 1983). However, recently several investigators have suggested that inositol turnover may also be involved in the signal transduction chain in plants (Boss and Moore, 1989; Irvine, 1990; Moore et al., 1990). Not only most of the components of PPI cycle and the enzyme have now been reported from plant sources (Morre et al., 1990) but even the exogenous factors like light and hormones have been shown to affect PPI cycle (Morse et al., 1987 b; Memon and Boss, 1990).

In the present work we have tested if Pfr regulates PPI cycle or not. We have measured the level of PI, PIP and PIP₂ in dark grown tissue and after red light/ far-red light irradiation. The measurements were done by labelling the phosphoinositides in separate experiments with both ⁳²P and [³H]-myo-inositol.

One of the basic criteria of a response to be a Pfr mediated is to satisfy the red and far-red light reversibility. Interestingly, the changes in the level of PIP₂ were found to fulfill this need. After the red light irradiation the level of PIP₂ increased and this effect was
reversed if red light irradiation was followed by far-red light irradiation. There was no change when far-red light irradiation was given alone. Also, if the red light irradiation was followed by dark period of 10 min, a decrease in PIP_2 level was noticed. In the leaves irradiated with red light followed by 1 hr dark period, the level of PIP_2 decreased to less than the dark control, and yet at the same time the level of PIP did not increase. It could be inferred that the decrease in PIP_2 level could not be due to an increase in PIP phosphatase activity. The control therefore appears to be at PIP kinase and phospholipase level. This shows that one of the initial events triggered by Pfr is to affect PIP kinase/phosphatase activity in order to increase PIP_2 level.

In earlier studies Morse et al. (1987 b) had shown that light induced an increase in the inositol phosphates and decrease in the PIP_2 levels. Melin et al. (1987) found that phospholipase C was more active in dark leading to a decrease in the PIP_2 levels in dark, as compared to light. This seems to be true in the present system also, as the level of PIP_2 decreased in the dark period after red light irradiation. It is, however, not clear as to why under longer durations of red light irradiation the increase in PIP_2 was less. It could be that under these conditions a steady state level is maintained between the synthesis and
the degradation of PIP$_2$.

**Turnover of polyphosphoinositides could be initial event in enzyme stimulation**

Having established that one of the initial events triggered by Pfr is to affect the turnover of PPI cycle, the next question was, if this change has any bearing on the stimulation of nitrate reductase. Although, other exogenous factors have been shown to affect the turnover of PPI cycle (Ettlinger and Lehele, 1988; Memon and Boss, 1990; Morse et al., 1987 a&b; Strasser et al., 1986), its relationship with a biochemical response has not been established.

As reported earlier one of the initial response of Pfr in the system was to increase the influx of calcium and this effect was mimicked by 5-HT (serotonin) (Das and Sopory, 1985). Based on this observation involvement of PPI cycle in Pfr mediated responses was suggested. Serotonin was later shown to effect the gravity response in roots (Perdue et al., 1988). It is known to activate hydrolysis of polyphosphoinositol in animal cell membranes, releasing inositol triphosphate and diacyl glycerol as second messengers. In view of this, experiments were carried out to check

i. If in plants 5-HT affects PPI cycle?

ii. Whether this effect is similar to Pfr effect?
iii. If 5-HT can replace the requirement of light in the stimulation of NR as it did in case of calcium uptake.

The results obtained in the present study showed that 5-HT affected PPI turnover in dark grown etiolated maize leaves and the overall changes monitored were similar to those obtained after red light irradiation. The level of PIP2 increased even after incubation of leaves in 5-HT for 5 min. The longer incubations in 5-HT resulted in decrease in the PIP2 level. It is clear, that the red light irradiation and 5-HT treatment affected the turnover of PPI cycle by first increasing the level of PIP2. Therefore in both cases the initial site of action could be at PIP kinase level. In animal system an agonist is known to affect phospholipase C activity which results in immediate decrease of PIP2 and thereby resulting in an increase in IP3 and DAG (Nishizuka, 1984b). Unlike this, the effect of both, red light and 5-HT, seems to be at the level of PIP kinase/phosphatase. The increase in the level of PIP2 decreases later probably, through phospholipase C thus releasing second messengers which could amplify the signals.

Since 5-HT could affect the turnover of PPI cycle in a manner comparable to red light irradiation, the next interest was to look for a correlation between changes in PPI levels and phytochrome mediated stimulation of NRA. When
tested 5-HT was also found to mimic the effect of red light irradiation to stimulate NRA in dark grown tissues. This means that 5-HT could replace the requirement of light for the stimulation of NRA. However, a difference between the 5-HT and red light treatment was also noticed. This was evident from the experiments where 5-HT treatment and red light irradiation was given for 5 min and after a dark period of 2 hrs, nitrate was added. In case of red light irradiation, NRA showed linear increase till 12 hrs whereas in the case of 5-HT, NRA was maximally stimulated at 4 hrs and then declined and reached basal level by 8 hrs (Fig. 9). This suggested that the red light induced signal was found to be more stable than one induced by 5-HT. It was observed that red light together with 5-HT was not able to sustain the NRA as long as it was sustained in case of red light alone. Therefore, it appears that the 5-HT induced 'signal' could not be stabilized any longer even in the presence of red light irradiation. Though the initial event (PIP₂ increase) triggered by red light and 5-HT seems to be similar and also the end result, i.e. the stimulation of NRA, there seems to be some difference in their mode of transmission and the nature of the signal. It seems possible that red light, mediating its effect through phytochrome, affects some processes other than turnover of PPI cycle also whereas 5-HT effect may be specific to processes leading to change in PPI turnover. This could be
the reason that the 'signal' generated by 5-HT does not remain stable as long as red light induced signal does. Another difference between 5-HT and red light mediated stimulation of NR was that 5-HT effect was found to be faster as it did not require the following dark period for the generation of the signal. When 5-HT and KNO₃ were given together, NR was stimulated more than when 5-HT treatment was followed by dark period. This was unlike red light effect where the following dark period was required; most probably for optimal generation and amplification and for "storage and stabilization" of the signal.

There are couple of other points which need mention. It was found that the levels of PI, PIP, and PIP₂, increased by treatment of 5-HT for 5 min while the longer duration of 5-HT (2 hrs) decreased the PIP and PIP₂ levels. It could be possible that longer duration of 5-HT inhibited PIP kinase activity, thereby decreasing PIP₂ level, and yet at the same time whatever PIP₂ was present was further utilized to continue the cycle thus resulting in decreased PIP₂ level. The increase in the PI level in this case could be due to inhibition of PI kinase activity while conversion of PIP₂ back to PI would continue or there could be other pathway to supply PI (discussed under section involvement of other phospholipids in the generation of second messengers). These observations need further analysis. However, it may be
mentioned that a longer treatment with 5-HT also resulted in less stimulation of NRA.

To ascertain further that the changes in polyphosinositide have some correlation with NRA stimulation, lithium, which is known to inhibit the turnover of PPI cycle in animals was used in the present study. In animal system lithium is known to inhibit the conversion of inositol-1-phosphate to inositol thereby limiting the supply of inositol for the continuation of the cycle (Drummond, 1988; Hallcher and Sherman, 1980). Lithium has been used in plant system earlier (Bagga et al., 1987; Perdue et al., 1988; Bossen et al., 1990). Hartmann and Pfaffmann (1990) suggested that lithium affects PPI cycle by inhibiting inositol monophosphatase which in turn affects PIP_2 level. When lithium was given for 1 hr, levels of PI, PIP, PIP_2 decreased. The results showed that it did inhibit red light stimulated PIP_2 accumulation suggesting that probably it acts at some other steps also besides affecting inositol monophosphatase. Lithium action appears to be at the multiple sites rather than at one site as suggested in animals (Drummond, 1988; Avissar et al., 1988). As lithium could decrease the levels of PPIs the interest was to see what happens to NRA in case when lithium is given. When used at different concentrations, lithium inhibited red light stimulated NRA. Whether this effect of lithium is due to
its action on PPI turnover alone can not be concluded. Nevertheless, when it was added soon after red light irradiation in the ensuing dark period or after a gap of 1 hr lithium did block the generation of signal by Pfr. If lithium was given after 1 hr of red light irradiation, inhibition was much less as compared to the situation when lithium was given immediately after red light irradiation. This could be due to the reason that some signal may have already escaped by 1 hr in order to stimulate NRA. Earlier it was found that photoreversibility was lost after 30 minutes and it was assumed that 'signal' moves out at this time and is then stored (Sharma and Sopory, 1984).

To confirm if lithium inhibition of NRA was due to its effect on PPI cycle thereby limiting the inositol level, reversibility of lithium inhibition by myo-inositol was checked. It was found that myo-inositol could reverse the inhibition partially. This effect was not noticed when mannitol was added. Perdue et al. (1988) were not able to get any reversal by myo-inositol. These results show that probably lithium inhibition of PPI cycle in plants does not result in immediate total depletion of myo-inositol.

**TRANSDUCTION OF SIGNAL**

From the work discussed above it seems that Pfr affects PPI cycle and it may be involved in light stimulation of NR. If that is so, the next question is to understand how the
messengers generated by PPI cycle transduce the signal. Is it through calcium or PKC type enzyme.

Through calcium

Requirement of calcium in phytochrome mediated signal transduction has been suggested by many workers (Poovaiah and Reddy, 1987; Bossen et al., 1988, 1990, 1991; Tretyn et al., 1991). Turnover of PPI has also been shown to be correlated with the changes in calcium level (Das and Sopory, 1985; Morse et al., 1989, see, Tretyn et al., 1991). This possibility was also checked in the present study thinking that lithium inhibition could affect the cytosolic calcium levels as (i) myo-inositol could not totally reverse lithium inhibition (Fig. 14). (ii) Lithium decreased IP$_3$ levels (Fig. 18) and (iii) PA levels also decreased (Fig. 4a & 17). PA is also known to act as Ca$^{2+}$ channel.

When tested it was found that at concentration of 10 mM the inhibition of NRA by lithium was totally reversed by calcium to the red light level. This suggests that in plants, besides affecting inositol monophosphatase, lithium could be blocking Ca$^{2+}$ uptake or its release machinery also, either directly or indirectly, and that calcium may be involved in signal transduction pathway. This is also supported from the experiments where dark grown leaves when incubated in the presence of exogenous calcium, showed 40% increase in NRA. However, it was never stimulated to the
same extent to which red light irradiation does, therefore there could be other reasons for this but if leaves were irradiated with red light and simultaneously incubated in Ca\(^{2+}\) there was a decrease in the NRA. This inhibition, however was noticed only if exogenous Ca\(^{2+}\) concentration was increased beyond 2 mM. These results indicated that Ca\(^{2+}\) is an important component of signal chain but not the only one. This also shows that Pfr may have multiple effects. It not only regulates turnover of PPI cycle and thereby effecting calcium homoeostasis but probably also affects uptake of Ca\(^{2+}\) as was reported earlier (Das and Sopory, 1985). This could be the reason that in leaves irradiated with red light even 5 mM Ca\(^{2+}\) was inhibitory whereas in dark grown leaves, exogenous calcium was stimulatory. In the former case high levels of Ca\(^{2+}\) would be accumulated in short period of time through enhanced uptake and through release from endogenous pool (via involving PPI cycle) and thereby the system showed inhibition. Since in dark, exogenous Ca\(^{2+}\) did not totally replace light effect it could be inferred that either release from endogenous pools is a requirement or some other second messengers released by turnover of PPI cycle, like DAG, may also be involved in light mediated signalling in this system. Changes in level of IP\(_3\) and PA levels by 5-HT and inhibition by lithium suggests that these may be helpful in maintaining the calcium levels to meet the optimal requierment for the present system. PA has been shown to
promotes Ca\textsuperscript{2+} entry into cells (Salmon and Honeyman, 1980; Putney et al., 1980; Ohsaki et al., 1981) or help in the mobilization of intracellular calcium (Murayama and Ui, 1985; Moolenaar et al., 1986).

Through DAG stimulated kinase.

From the results obtained in the present work and as already discussed above, it follows that Pfr mediated signal transduction chain involves PPI turnover and calcium could not totally replace light effect in the stimulation of NRA whereas 5-HT could. In animal systems it is known that one of the product of PIP\textsubscript{2} breakdown is diacyl glycerol which is involved in the stimulation of PKC for effecting sustained responses (Nishizuka, 1984). The major gap in the evidence for a signal转duceng system in plants, analogous to animals, is the absence of compelling evidences that protein kinase C exists in plants.

Different groups (Elliot and Skinner, 1986, Schafer et al., 1975, Elliot and Kokke, 1987a\&b) have shown the presence of phospholipid and calcium dependent kinase and suggested the possibility of the presence of PKC type enzyme in plants. Olah and Kiss (1986) have shown PMA stimulated kinase activity in wheat. Park and Chae (1990) proposed that phytochrome action in oat protoplasts is associated with PKC-dependent protein phosphorylation. Participation of PKC in the regulation of phytochrome controlled
processses has also been postulated by Bossen et al. (1990) and Haas et al. (1991). Although the involvement of PKC in Pfr mediated response has been suggested yet strong evidence for its role in stimulating any biochemical event and affecting gene expression is still lacking.

Since the indications were already there for the involvement of PPI turnover in Pfr mediated stimulation, it led us to check if stimulation of NRA via PPI turnover could be through DAG (lipid) stimulated PKC. Therefore, for this purpose PMA, an anologue of DAG, was tested. PMA has been used earlier to see the involvement of PKC in plants (Olah and Kiss, 1986; Park and Chae, 1990; Bossen et al., 1990).

Incubation of etiolated leaves for 5 min with PMA was sufficient to increase NRA, irrespective of whether the treatment was followed by 2 hrs of dark period before the addition of nitrate or not. It was found that after red light irradiation, a dark period of 2 hrs was essential to elicit maximum NR stimulation following nitrate addition (Fig. 3). Therefore, it can be suggested that for the stimulation of NR by PMA, the ensuing dark period is not required. Longer duration of PMA down regulated the NR stimulation. These results suggest that Pfr may not be acting like PMA by directly affecting the kinase activity. It may be mediating its effect through steps preceeding kinase activity which could be through PPI turnover as
discussed above. Although there are some similarities in the stimulation of NRA by PMA and Pfr, their mode of action could be different. Since PMA stimulated NRA in absence of light, and there were some indications for the presence of PMA dependent kinase activity in plants (Olah and Kiss, 1986), we attempted to purify DAG or PMA stimulated kinase activity from dark grown leaves.

Following the protocol given in materials and methods, and taking advantage of employing a lipid (PS) affinity chromatography, we were able to obtain a fraction that showed stimulation in kinase activity by PS, calcium and OAG. The kinase was eluted from the affinity column at 0.1 mM Ca$^{2+}$ concentration. In animal systems such kinases have been shown and get eluted with EGTA. This showed that the kinase from the plant system has some properties different from the animal kinase. This peak fraction showed two bands on SDS and native gels. The molecular weight of the two bands were 55 and 68 kDa.

Although different reports exist in the literature on the presence of lipid stimulated kinase in plants yet their classification as a PKC type is still a matter of discussion (Harmon, 1990). In plants till today none of the lipid-stimulated protein kinase has been purified to homogeneity. These protein kinases also differ from PKC; either they are all activated by Ca$^{2+}$ alone or unlike PKC, activated by
phospholipid in the absence of \( \text{Ca}^{2+} \). Activation by \( \text{Ca}^{2+} \) alone indicates that either these kinases differ from PKC in their mode of regulation, or that a contaminating calcium dependent protein kinase activity exists as suggested by Harmon et al. (1987). Since none of these enzymes is pure, chances of contaminating kinases cannot be ignored. Another possible explanation for activation by \( \text{Ca}^{2+} \) could be that the enzyme preparation may not be totally free of contaminating phospholipids, thereby making the addition of phospholipids unnecessary. Also the possibility that the PKC in plants may not be exactly analogous to animal PKC cannot be excluded. Moreover, during purification in the present system elution of major activity was by low \( \text{Ca}^{2+} \), whereas in case of animals where major peak comes in EGTA elution, suggesting that the present protein could be of PKC type where as its properties may be different from the plant protein kinases discussed above as well with the animal protein kinase C.

Since we also obtained a peak activity in affinity chromatography that was stimulated more by \( \text{PS} + \text{Ca}^{2+} + \text{PMA} \), we got a strong indication that this kinase may be a PKC type. Our results with binding studies with \( ^{3}\text{H}-\text{PMA} \) further supports this argument. Earlier, absence of specific binding for \( ^{3}\text{H}-\text{PMA} \) in soybean callus or crown gall T37 callus tobacco were considered as negative
indications for a PKC type enzyme even though the kinase activity was stimulated by PS (Elliot and Skinner, 1986). In present case, the enzyme has shown binding to $[^3H]$-PMA in the presence of PS and Ca$^{2+}$, suggesting that PKC type enzyme has binding site for PMA.

One interesting property of PS+OAG stimulated kinase in the present study was its fluorescence spectrum which indicated that the enzyme is rich in tyrosine residue. It has been reported that phospholipid and Ca$^{2+}$ dependent kinases may undergo autophosphorylation (Elliot and Kokke, 1987a). Experiments with the autophosphorylations and blotting by using phosphotryosine antibodies also indicated that both bands of 55 and 68 kDa seemed to be autophosphorylated at tyrosine residue(s).

Although this kinase autophosphorylates itself it also phosphorylated other substrates. We found that it can phosphorylate histone at Ser residues. The autophosphorylation at Tyr and phosphorylation of histone as substrate has given rise to a question that whether kinase has more affinity to Ser residues (in case histone was used as substrate) or Tyr (as it autophosphorylates itself at Tyr residue) or can phosphorylate both with equal efficiency, as shown for one kinase recently (Featherstone and Russell, 1991). The experiments done with synthetic substrate rich in Tyr showed that with our kinase
the percent phosphate incorporated was double in case of synthetic (Tyr) substrate. It could be inferred that this kinase can also phosphorylate other substrates at Ser and Tyr as was shown for the other PKC by Featherstone and Russell (1991).

The results from present study show that OAG/PMA stimulated kinases are present in plants and these may be involved in Pfr signal transduction. This inference is similar to those of Bossen et al. (1990) where PMA induced swelling of the protoplasts (Bossen et al., 1990) while H-7 inhibited red light induced swelling of etiolated wheat protoplasts, and Haas et al. (1991), who found significant decrease in the phytochrome-mediated, Ca\(^{2+}\) dependent germination of *Dryopteris filix-mas* L by using an inhibitor of PKC, staurosporine. Both groups strongly suggested that PKC type enzyme may involved in Pfr mediated responses as a component of signal transduction chain. How Pfr would affect PKC activity is not clear. It could be indirect via release of DAG by stimulating PPI cycle or as indicated by others the kinases may be have affinity for Pfr. It has also been suggested that phytochrome itself could act as a substrate for protein kinase. Wong et al. (1986) showed that mammalian PKA phosphorylated both Pr and Pfr, whereas PKC phosphorylated intensively the Pr form. Together with this the phosphorylation of the N-terminus of purified oat
phytochrome was stimulated by polycation-stimulated kinase (Wong et al., 1989; McMichael and Lagarius, 1990). This increases further the probability of involvement of PKC type enzyme and its role in regulation of phytochrome mediated response(s).

Through protein phosphorylation

Whether Pfr affects PKC type enzyme or other kinases directly or indirectly, one has to visualize that the signal will have to be further transduced via phosphorylation of substrates of these kinases. In our system we checked whether red light and PMA affect phosphorylation status of different polypeptides, by doing in vivo and in vitro phosphorylations.

From the in vivo phosphorylation studies, changes in level of polypeptide by PMA and red light suggested that there could be some similarities in stimulating the kinase by these two factors and thereby affecting the phosphorylation. Moreover it supported further that PMA stimulated kinase which is present in the system can phosphorylate endogenous proteins as substrate. Interestingly some of the polypeptides that were phosphorylated by PMA (5 min) were also (on molecular weight basis) phosphorylated to the same extent, when leaves were kept in dark period of 1 hr after red light irradiation. It could be assumed that these polypeptides play a role in case
of dt dark period, for signal transmission and storage. The difference in the extent of phosphorylation of different polypeptide under the same treatment suggests that phosphorylation of different polypeptides may be via different kinases. This needs further analysis.

Changes in phosphorylation status of polypeptides were also observed in case when phosphorylation was done in vitro. The extent of phosphorylation was found to be changed by red light. In the present system calcium was found to phosphorylate only specific polypeptides however the status of phosphorylation was different than what was observed in case of red light, suggesting that system needs factors other than calcium to bring out the specificity of Pfr action. Changes in phosphorylation pattern by Pfr has been reported by different groups (Otto and Schafer, 1988; Dooshi et al., 1992). Although, the exact nature of substrate is unknown yet these reports together with the present results increase the possibility that phosphorylation/ dephosphorylation being one of the step in Pfr mediated signal transduction.

FAR-END OF SIGNAL CHAIN

Gene expression

It has been shown in a number of instances that Pfr mediated stimulation of proteins is regulated at the level
of transcription (Silverthrone and Tobin, 1984; Mosinger et al., 1985; Fluhr et al., 1986). Light regulatory cis elements have been identified at the 5' upstream region of a few genes which confer light responsiveness even on reporter genes (Kuhlmeier et al., 1987 b). It has also been shown that there are number of specific transacting factor which bind to different LRE and interestingly binding of some of them have been shown to be dependent on their phosphorylation/dephosphorylation status (Datta and Cashmore, 1989; Sarokin and Chua, 1992; Klimczak et al., 1992).

Presently we do not have genomic clone of NR of maize and therefore studies related to promoter analysis are lacking. However, we have a partial cDNA clone which was used to check if red light irradiation and PMA treatment affect the transcript level of NR. It was found that in response to red light and PMA the transcript level increased in the presence of nitrate. However, the level of transcripts in PMA treated leaves was less. In leaves treated with red light first and then followed by dark period (without nitrate), transcript levels were detected only if nitrate was supplied. This increase was much less if the leaves had been kept in darkness throughout and not exposed to short irradiation of red light. The question that arises is whether red light and PMA stimulation of
transcript level of NR is mediated via a similar mechanism or not.

Different mechanisms have been proposed in different systems, by which PMA affects gene expression (Guilfoyle, 1989). PMA regulatory sequence has been identified by different workers in PMA regulated genes in animal systems (Angel et al., 1987; Chiu et al., 1987 Imbra and Karin, 1987). DNA footprinting analysis of PMA responsive genes indicate that a nuclear protein specifically binds to TPA regulatory element. This factor shown to be identical to the AP-1 transcription factor (Angel et al., 1987; Lee et al., 1987). The resistance of the induction and binding responses to protein synthesis inhibitors suggests that a post-translational mechanism also operates in TPA-responsive genes. A probable mechanism regulating TPA gene induction is the phosphorylation of AP-1 by protein kinase C (Angel et al., 1987; Lee et al., 1987). Besides AP-1, other transcription factors identified are AP-2 (Imagawa et al., 1987; Mitchell et al., 1987) and NF-kB (Lenardo et al., 1987; Sen and Baltimore, 1986). The results from these studies suggest that the transcription factor, AP-1, is at the receiving end of the transmission elicited by phorbol esters. This pathway starts at the plasma membrane and terminates with the post-translational modification of this specific transcription factor. Phosphorylation of other
transcription factors might also be catalyzed by protein kinase C and some of these, along with AP-1, could be involved in gene expression. It is difficult to interpret from the present results, that in what way PMA is affecting the gene expression of NR. However, from the sequence analysis of NR promoter of Arabidopsis, synthesized by using PCR technique, it was found that some of the known TPA elements from TPA responsive genes share 70-80% homology with the sequences found in NR promoter. Whether these elements are functional in plant systems needs to be analyzed.

Enzyme modulation

Since after PMA treatment transcript level of NR was lower as compared to red light irradiation and yet in the presence of PMA, the NRA was equivalent to light irradiated leaves, it was thought whether PMA could affect the NRA. It could be possible that PKC type kinase could be using NR as a substrate. This means that NR could be present in both phosphorylated and dephosphorylated state. Although we have no evidence if PMA acts this way, we only tested if NR at all gets phosphorylated in vivo. In vivo labelling with $^{32}$P and purification of NR showed that it in fact exists in phosphorylated form. This is an interesting finding. Recently, Cambell's group have also given indications that this may be so (Huber et al., 1992). However, any
correlation between enzyme activity and phosphorylation status needs to be established. And of course in vitro studies with purified kinase and NR should be done to support further that this PMA stimulated kinase is involved in NR regulation.

GENERATION OF SECOND MESSENGERS BY PHOSPHOLIPIDS OTHER THAN POLYPHOSPHOINOSITIDES AS POSSIBLE ALTERNATE ROUTE IN Pfr MEDIATED RESPONSE(S)

It has been shown that besides PIP₂ breakdown DAG can also be produced by affecting the phosphatidylcholine and phosphatidyl ethanolamine turnover (Exton, 1990). This possibility has been checked by looking at changes in PC and PE levels after red light irradiation. Decrease in level of PC and PE suggests that lipids other than phosphoinositides could also play an important role in signalling by providing second messengers like DAG. This has been suggested for animal systems (Exton, 1990).

In addition to PC and PE, the level of PA also changed. The decrease in the level of PA induced by red light suggests that this may be one of the ways phytochrome acts. PA can get converted to PI or DAG. Changes in the level of PA during red light and dark could also be important in maintaining the calcium, PI and DAG levels.

Although the initial changes in PIP₂ and the changes in the NRA suggest that the initial and the last effect of Pfr
has some similarities, but changes in the levels of other lipids together with polyphosphoinositides may be important for the continuous supply of second messengers. For example, in 5-HT treatment the signal was not stable for long time and NR decreased very fast compared to red light irradiated leaves. Another important difference in case of red light and 5-HT is the changes in the level of PA. In case of 5 min treatment of 5-HT the decrease in the level of PA was less as compared to red light 5 min. This together with the observation that there was an increase in the level of PIP₂ and IP₃, suggests that the action of 5-HT in affecting PPI turnover may be similar but the replenishment of PI for the PPI cycle to continue in case of 5-HT appears to be via IP₃-IP₂ pathway whereas in case of red light it appears to be replenished via PA.

As the level of PI did not change whereas PIP₂ level increased and IP₃ level in decreased red light irradiated tissue, it suggests that the first action of light was at PIP kinase to increase PIP₂ which will be utilized in the dark period, as in dark the phospholipase C is more active. Therefore the chances of replenishment of PI in case of red light by PPI turnover seems to be less, thereby increasing the probability of replenishment of PI via PA route. This type of mechanism for replenishment of phosphoinositides was also suggested by Hokin (1985). Detailed analysis of the
other end product, DAG, may give better picture for the presence of such a possibility in plants.

Although it is too early to say what are the actual second messengers that are involved in Pfr mediated signal transduction, yet it is reasonable to suggest that Pfr affects the turnover of PPI cycle which could be one of the initial early biochemical step involved in phytochrome mediated signal transduction via PKC type enzyme. Alteration of PPI cycle may, however, not be the only signalling pathway for Pfr regulated developmental responses.

Overall conclusion

From the data obtained in the present work it follows that there is a relation between the fast responses triggered by phytochrome with the slower responses like enzyme stimulation. Also it seems that phytochrome initiates its action at more than one site. The working hypothesis that we are contemplating for the mechanism of phytochrome action at the level of phosphoinositide cycle is given in a schematic diagram in Fig. 47.

In dark grown tissues the PLC is active and it increases the level of IP3 and DAG. The DAG thus produced is converted to PA through the action of DAG kinase. IP3 is converted back to IP2 ---> IP1 ---> I and finally in
conjunction with CDP-choline enters the PPI cycle again. A kind of steady state level is maintained in this cycle under darkness.

Once plants perceive light through phytochrome and enough Pfr is formed to initiate action, the following changes seem to get triggered immediately. Due to activation of PIP kinase and decrease in the activity of PLC, the level of PIP_2 increases. The level of PC/PE decreases and so also the level of PA.

In ensuing period, PIP_2 will be degraded to IP_3 and DAG. The net result of all these changes seem to be to build up DAG level. This increase/change in DAG level may not only be a quantitative effect but may also result in a qualitative change in the nature of fatty acids. Simultaneously, as we have seen earlier, the level of endogenous Ca^{2+} may also be altered; either through net increase in influx or release from endogenous pools! A change in the scenario in the cell follows due to change in DAG and Ca^{2+} and this in turn would activate a different set of protein kinases (either Ca^{2+} dependent, Ca^{2+}/Cam dependent or Ca^{2+}/lipid dependent). Accordingly either phosphorylation of different set of polypeptides or enhanced phosphorylation level of polypeptides would lead a changed pattern of gene expression. The role of phosphorylation in gene expression has been shown in some systems.
This hypothesis on signal transduction has its origin in animal literature however, we now show that this mechanism in a modified way is probably operative in plants too. However, one main question that needs to be addressed to is to explain how phytochrome, which is considered to be mainly cytosolic protein brings about its initial effects at the level of membranes. More work surely is needed to find out the exact relationship between one event with the next step in the signal transduction pathway.