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Evidence for some common signal transduction events for opposite regulation of nitrate reductase and phytochrome-I gene expression by light

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Abstract

We have explored the possible involvement of the phosphoinositide (PI) cycle and protein kinase C (PKC) in the phytochrome (Pfr)-mediated light signal transduction pathway using nitrate reductase (NR) and phytochrome-I (PhyI) genes as model systems. We have shown earlier that phorbol myristate acetate (PMA) completely replaces the red light effect in stimulating nitrate reductase activity and transcript levels in maize. In this paper, we present detailed evidence to show that PMA mimics the red light effect and follows similar kinetics to enhance NR steady-state transcript accumulation in a nitrate-dependent manner. We also show that PMA inhibits *phyI* steady-state transcript accumulation in a manner similar to red light, indicating that a PKC-type enzyme(s) may be involved in mediating the light effect in both cases. Serotonin or 5-hydroxytryptamine (5-HT), a stimulator of PI turnover, was also found to mimic the red light effect in enhancing NR transcript levels and inhibiting *phyI* transcript accumulation, indicating the role of the PI cycle in generating second messengers for regulating the two genes. These results indicate that phytochrome-mediated light regulation of NR and *phyI* gene expression may involve certain common steps in the signal transduction pathway such as the PI cycle and protein phosphorylation by a PKC-type enzyme.

Introduction

Higher plants have evolved well developed photosensory and signal transduction mechanisms to monitor the quality and quantity of the light signal and to adjust their growth and development through regulated gene expression throughout their life cycle. While the regulation of various light-responsive genes has been extensively studied [15, 52], knowledge regarding the molecular mechanism of light signal transduction is still limited.

Light perception is known to be initiated by at least three different types of photoreceptors of which phytochromes are the best characterized and are known to mediate several light-regulated responses in higher plants [14, 31]. Phytochromes exist in two inter-convertible forms, Pr and Pfr, which absorb red and far-red light, respectively. The Pfr form is considered to be mainly responsible for most physiological end-responses mediated by phytochrome, though the Pr form of PhyB has been recently implicated in inhibiting the negative gravitropic response of the *Arabidopsis*

shoot [24]. After the realization that multiple phytochrome genes exist in higher plants (five of them cloned in *Arabidopsis*, *phyA–E*), they have been grouped into two classes, *phyI* (*phyA*) and *phyII* (*phyB–E*) [13, 46]. *PhyI* is most abundantly expressed in etiolated plants and is strongly repressed by light whereas the isoforms of *phyII* are photostable and are constitutively expressed at low levels, regardless of the light regime [31, 56]. The negative photocontrol of *phyI* gene expression is particularly true in monocots such as oat [8], rice [19] and maize [7], though the degree of the light-induced decline varies depending upon which plants were tested. In dicots, however, the situation is more complicated due to the existence of multiple transcription start sites within their *phyI* genes, resulting in the production of multiple *phyI* transcripts [14]. Thus, the single-copy *phyI* gene in pea produces three different transcripts with varying degrees of photoinhibition and photoreversibility [41, 54]. The expression of *phyI* RNA3 in pea is probably constitutive or even slightly light-enhanced [54], whereas in cucumber, only a transient down-regulation of *phyI* mRNA abundance in etiolated cotyledons by white light has been reported [53].

The signal transduction mechanism by which *phyI* gene expression is down-regulated by Pfr is not understood, except the report that a G-protein might mediate this process [39].

Unlike *phyI* gene expression, which is negatively regulated by light, the expression of nitrate reductase (NR), a key enzyme in the nitrogen metabolism of higher plants, is strongly up-regulated by light, in the presence of nitrate [15, 33, 50]. We have shown earlier that in etiolated maize leaves, NR is regulated by light via phytochrome and that light and nitrate have independent, yet synergistic effects on the *de novo* synthesis of the enzyme [35, 45]. Using PMA, a phorbol ester known to stimulate PKC in animal systems, we have recently suggested that a PKC-type enzyme may be involved in transmitting the phytochrome signal, since PMA completely replaced the light effect in bringing about optimum expression of NR activity [4] and transcript levels [44] in the dark. We have also shown recently that serotonin (5-hydroxytryptamine or 5-HT), a compound known to stimulate PI turnover in animal systems also replaces red light effect in stimulating PI turnover and increasing NR activity in maize [5], indicating that PI cycle may be involved in generating second messengers for transducing the light signal.

In this paper, we have examined the effect of PMA and 5-HT on the steady-state transcript levels of NR and *phyI* genes in maize. We show that these compounds mimic the red light effect in stimulating NR gene expression and inhibiting *phyI* gene expression in the dark, indicating that a PKC-type enzyme, probably activated by metabolites of the PI cycle may be involved in bringing about opposite regulation of these two genes by light.

Materials and methods

Plant material and growth conditions

Seeds of *Zea mays* cv. Ganga-5 were obtained from National Seeds Corporation, New Delhi, India. Seeds were washed thoroughly, soaked in water overnight and again washed with deionized or distilled water. Seeds of uniform size were grown in plastic trays on moist paper in total darkness in an incubator and watered daily for 9 days at 27 ± 1 °C. Fully opened primary leaves of uniform size were excised from the seedlings of similar morphology for experimentation. All the manipulations during growth and treatments were done under green safe light.

Light sources and treatments

Red light was obtained from four 100 W tungsten lamps filtered through CBS 650 filter (Carolina Biological Supply Co., USA, emission maximum 650 nm). The intensity of red light at the plant level was 8 μE m^{-2} s^{-1}. Etiolated, excised leaves were floated on either water or nitrate (60 mM KNO_3) and exposed to a saturating pulse of red light for 5 min and were subsequently incubated

in the dark along with their respective dark controls. PMA was prepared as a 100 ng/ml stock in 1.25% DMSO (both from Sigma, USA) and used at a final concentration of 5 ng/ml along with, or without nitrate (60 mM KNO_3) in the dark. 5-HT (Sigma) was added directly into the treatment solution (either water or nitrate) to a final concentration of 30 mM. The concentrations of PMA and 5-HT used in this study were decided on the basis of earlier studies on NR activity conducted in this laboratory [4, 5]. All the manipulations were done in green safe light (0.04 $\mu E\ m^{-2}\ s^{-1}$) which was obtained by filtering the light from a cool fluorescent tube through several layers of green cellophane paper (emission maximum 500 nm).

RNA isolation

After the treatments, leaves were frozen in liquid nitrogen and processed for total RNA isolation by guanidine-HCl-phenol-chloroform extraction method [25]. Final RNA pellets were dissolved in formamide to prevent degradation during handling [6]. The RNA samples were quantified by spectrophotometry and their quality was verified by agarose gel electrophoresis [40].

Dot blot/northern blot hybridization

Dot blots were generated by using 20 μg of total RNA per sample as described earlier [44]. For northern blotting, 30 μg each of the RNA samples were denatured under similar conditions, electrophoresed on 1.2% agarose 6% formaldehyde gels using MOPS buffer essentially as described [40]. Blotting was performed by using Duralon UV membranes and a Posiblot pressure blotting apparatus (Stratagene, USA) as per the instructions of the manufacturer. Both dot blots and northern blots were crosslinked under UV using Stratalinker (Stratagene, USA) and baked for 2 h at 80 °C before hybridization. The transfer of RNA on the northern blots was verified by methylene blue staining of the blots [17]. The cDNA probes for maize NR and *phyI* genes were prepared from the clones obtained from Prof. Wilbur Campbell [16] and Dr Peter Quail [7] respectively. The clones were amplified, plasmids isolated and inserts purified from agarose gels as described [40]. Radiolabelled DNA probes were prepared from these cDNA fragments by random primer extension method and were purified by the spin column method using Sephadex G-50 [34]. The specific activity of the probe ranged between 0.8 and 2×10^9 cpm/μg. Prehybridization of the blots was performed for 1 h at 65 °C in a solution containing 0.5 M NaCl, 0.1 M NaH_2PO_4, 0.1 M Tris Base, 2.0 mM EDTA, 1% SDS and 100 μg/ml denatured salmon sperm DNA at 65 °C in a rotating rack hybridization oven (either from GFL, Germany or Robin Scientific, USA). Denatured probe was added subsequently (10^6 cpm/ml) and hybridization was allowed to continue for 12–16 h. Washing of the blots was performed at least thrice in 10 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 1% SDS at 65 °C followed by a rinse at room temperature in the same buffer without SDS. Autoradiography was performed by using Kodak X-ray films and two intensifying screens at −70 °C for appropriate periods of time. Films were developed using Kodak developer and fixer materials.

Data analysis and presentation

The autoradiograms were scanned using Ultroscan XL densitometric scanner (Pharmacia-LKB, USA). Scanning was performed at 523 nm covering the entire area of the hybridization signal. The baseline was defined as an average of 16 lowest data points in each scanning lane. The areas under the peaks (arbitrary units) were calculated and used for plotting the data. Each experiment was repeated at least twice and only a representative set of data has been shown. The number of repetitions as well as variations in the data, if any, have been indicated in the text.

Results

Effect of PMA on NR transcript levels

Initially, the kinetics of NR transcript accumulation were analyzed under red light and PMA treatment conditions. Etiolated leaves were excised and floated either on water or 60 mM KNO_3 with or without 5 ng/ml of PMA and incubated in the dark. As controls, leaves were irradiated with red light for 5 min either in the presence or absence of nitrate and transferred to the dark. Samples were collected at 1, 2, 4 and 8 h and steady-state transcript levels of NR were analyzed. Figure 1A shows the autoradiograms obtained after dot blot hybridization and Fig. 1b shows the plots based on densitometric scanning of the autoradiogram.

It is clear from the data presented in these figures that PMA mimicked the red light effect in enhancing NR transcript levels in the presence of nitrate. The kinetics of NR transcript accumulation were also similar to that of red light. Data from four independent experiments indicated that at the peak level of induction (i.e., 2 h), PMA could bring about 4- to 7-fold increase of NR transcript in the presence of KNO_3 when compared to the uninduced (dark + water) levels. When compared to the peak levels attainable with red light and nitrate, the extent of increase in NR transcript levels in the presence of PMA generally varied between 65 to 100%. PMA alone (in the absence of nitrate) was incapable of enhancing the RNA levels, much the same as under red light conditions.

These results were further verified in a northern blot hybridization experiment using RNAs isolated from leaves exposed to red light or treated with PMA and incubated for 2 h in the dark, either in the presence or absence of nitrate. The results of this experiment (Fig. 2) once again demonstrated that PMA mimicked the red light effect in enhancing nitrate-induced NR transcript levels. Further, this experiment indicated that the transcripts induced in PMA and red light were of the same size and that therefore, they represented the same NR gene.

Effect of duration of PMA treatment

In the experiments described above, PMA was present in the medium continuously whereas red light was given as a 5 min pulse. To verify whether

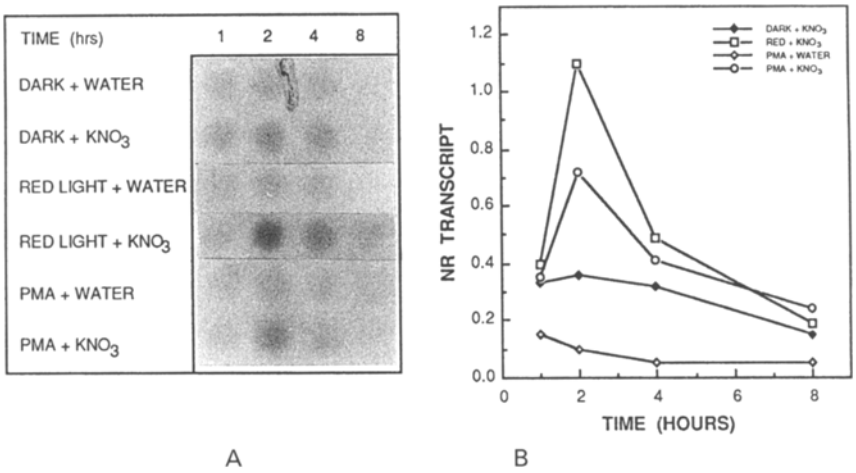

Fig. 1. Effect of PMA on the kinetics of NR transcript accumulation. A representative set of data from four independent experiments has been shown. Etiolated leaves were floated either on water or 60 mM KNO_3 with or without 5 ng/ml of PMA. Appropriate dark/red light controls were maintained. Samples were collected at 1, 2, 4 and 8 h and steady-state transcript levels were analyzed. A. Autoradiograms obtained after total RNA dot blot hybridization. B. The plots based on densitometric scanning of the autoradiograms. The scanning data, obtained as areas under the peaks (arbitrary units) were plotted against time (h of treatment).

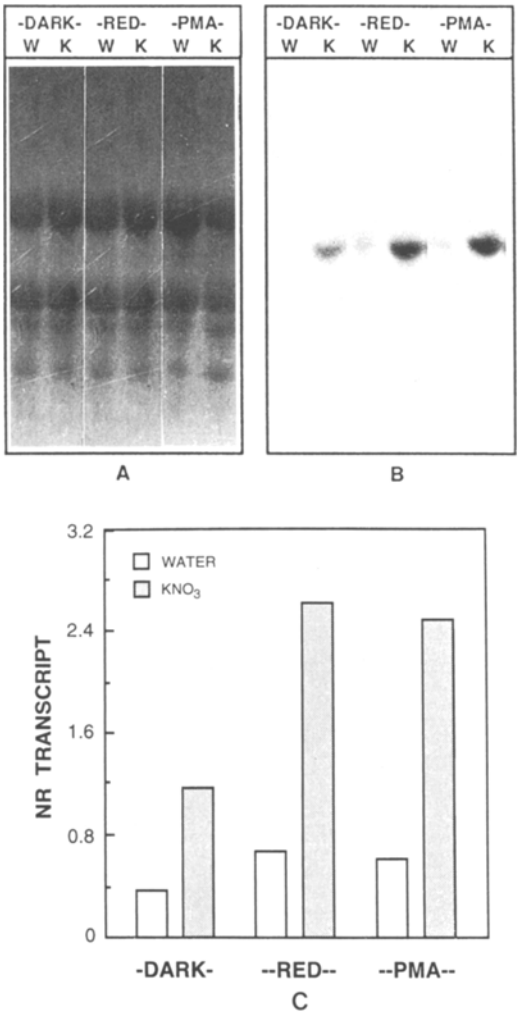

Fig. 2. Effect of PMA on NR transcript levels. Experimental conditions were the same as described for Fig. 1. The duration of treatment was 2 h. Part A shows the methylene blue-stained northern blot, Part B shows the autoradiogram obtained after hybridization of the blot and Part C shows the plots based on densitometric scanning of the autoradiogram (W, water; K, KNO_3).

continuous presence of PMA was essential to mimic the red light effect as well as to standardize the conditions for optimum PMA effect, different durations of PMA treatment were tested. For this purpose, PMA was given either for 5 min or for 2 h following which leaves were rinsed with distilled water and nitrate was supplied. A control of continuous PMA + nitrate was maintained apart from the red light + nitrate control. Steady-state transcript levels were analyzed from these samples after 2 h of incubation in nitrate regardless of the duration of PMA treatment. The northern blot hybridization data presented in Fig. 3 indicates that PMA given for 5 min was not as effective as that of 2 h, irrespective of whether

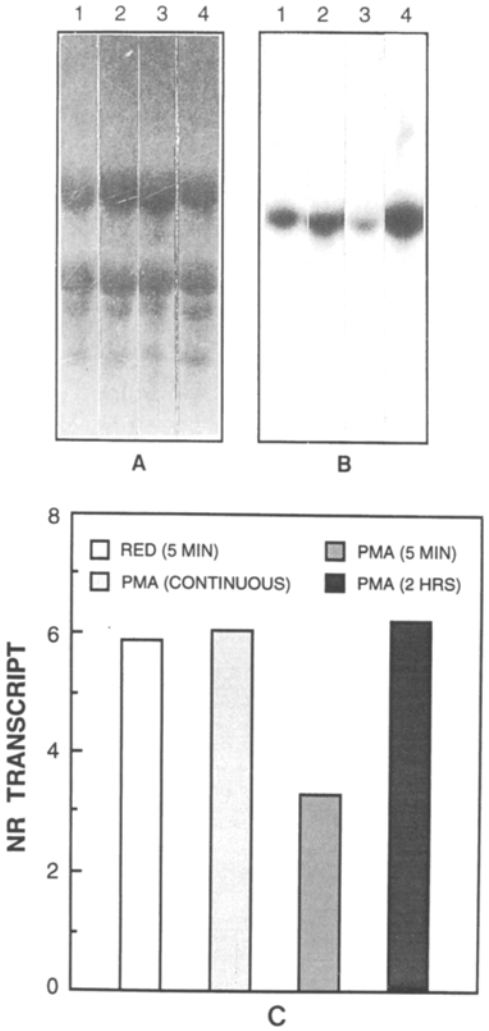

Fig. 3. Effect of duration of PMA treatment on NR transcript levels. Etiolated leaves were floated on 60 mM KNO_3, exposed to red light for 5 min and subsequently incubated in the dark for 2 h (lane 1). PMA treatment was given either continuously (along with nitrate), or for 5 min or 2 h before nitrate treatment (lanes 2, 3 and 4 respectively). Steady-state transcript levels were analyzed from these samples after 2 h of nitrate treatment, regardless of the duration of PMA treatment. A. Methylene blue-stained northern blot. B. The autoradiogram obtained after hybridization of the blot. C. The plots based on densitometric scanning of the autoradiogram.

it was supplied along with, or prior to nitrate treatment. However, once nitrate was supplied, continued presence of PMA along with nitrate does not seem to be essential for optimum effect. In this respect, again, PMA seems to mimic the red light effect.

Effect of PMA on phyI *transcript levels*

In order to examine whether PMA has a similar red light mimicking effect on other light regulated genes as well, experiments were conducted on *phyI* gene expression which is known to be negatively regulated by light. The kinetics of *phyI* steady-state transcript accumulation in the dark, red light and PMA (continuous) treatment conditions were analyzed by total RNA dot blot hybridization and densitometric scanning of the autoradiogram. The results of this experiment (Fig. 4) indicated that PMA mimics red light effect in inhibiting *phyI* transcript accumulation. However, the kinetics of inhibition by PMA was slower as compared to red light. Data from three independent experiments indicated that at 8 h, *phyI* transcript levels decreased by 50–65% in the leaves irradiated with red light, as compared to dark controls.

A comparison of the effect of PMA on NR and *phyI* transcript accumulation was made after 2 h of red light/PMA treatment (with nitrate) by hybridizing the same blot with both the probes (Fig. 5). The data clearly indicates that PMA had opposite effects on NR and *phyI* transcript accumulation in a manner that is similar, if not identical to red light.

Effect of 5-HT on NR and phyI *transcript levels*

In an attempt to verify the role of PI cycle in generating second messengers that are required for the activation of a PKC-type enzyme, the effect of 5-HT on NR and *phyI* transcript accumulation was examined. It was found that just as in the case of PMA, 5-HT also mimicked the light effect in increasing NR transcript levels (in the presence of nitrate) and decreasing *phyI* transcript levels (Fig. 6). A comparison of data from four independent experiments indicated that the transcript levels in 5-HT-treated samples varied by a maximum of 20% relative to red-light controls.

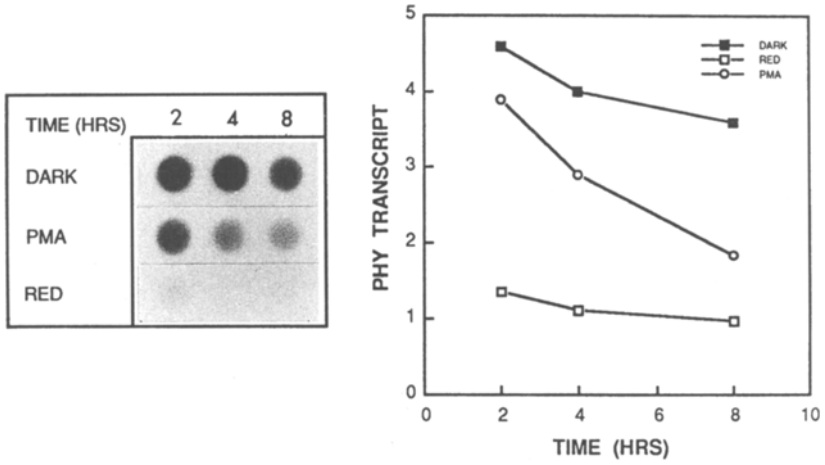

Fig. 4. Effect of PMA on *phyI* transcript levels. A representative set of data from three independent experiments is shown. Etiolated leaves were floated on water and exposed to red light for 5 min or incubated in PMA (5 ng/ml). They were subsequently incubated in the dark along with a dark control. Tissue samples were collected after 2, 4 and 8 h and processed for *phyI* steady-state transcript level analysis. A. Autoradiogram obtained after total RNA dot blot hybridization. B. The plots based on densitometric scanning of the autoradiogram.

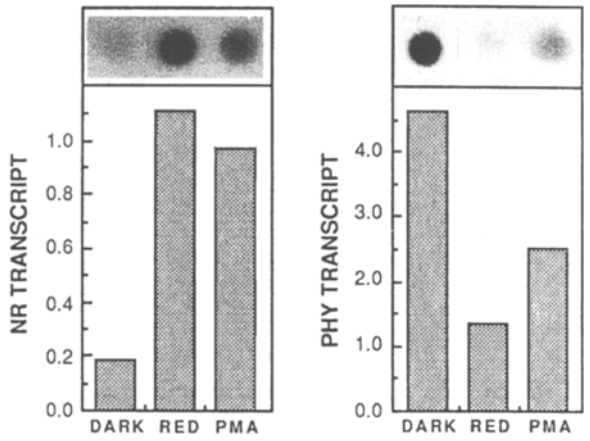

Fig. 5. Comparison of the effect of PMA on NR and *phyI* transcript levels. Etiolated leaves were floated on 60 mM KNO_3 and treated with red light irradiation (5 min) or PMA (5 ng/ml). The samples were incubated in the dark for 2 h and steady-state transcript levels were analyzed by total RNA dot blot hybridization using labelled NR and *phyI* probes sequentially. The hybridization signals were quantified by densitometric scanning of the autoradiograms and areas under the peaks (arbitrary units) were plotted against their respective treatments. The autoradiogram strips corresponding to the respective bars are also shown.

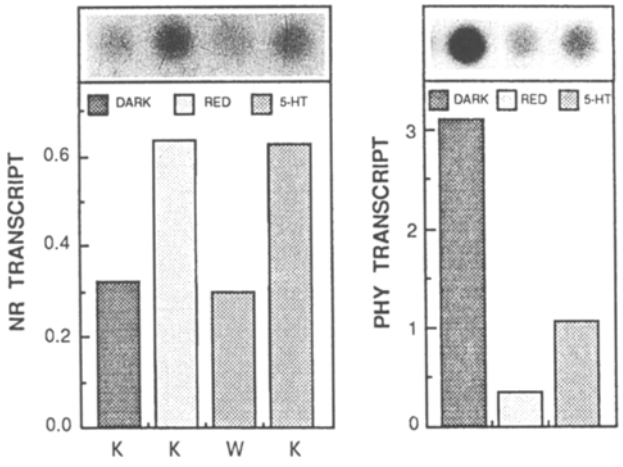

Fig. 6. Effect of serotonin (5-HT) on NR and *phyI* transcript levels. A representative set of data from four independent experiments has been shown. Etiolated leaves were floated on serotonin (30 mM) prepared in water (W) or KNO_3 (K) and incubated in the dark for 4 h. Appropriate dark and red-light controls (without serotonin) were maintained. Tissue samples were collected after 4 h and processed for NR and *phyI* steady-state transcript analysis by total RNA isolation and dot blot hybridization. The hybridization signals were quantified by densitometric scanning of the autoradiograms and areas under the peaks (arbitrary units) were plotted against their respective treatments.

These results indicate that PI cycle does play an important role in the early events of light signal transduction leading to the opposite regulation of NR and *phyI* gene expression.

Discussion

The mechanism of phytochrome phototransduction has attracted considerable attention in recent years [10, 56]. Phytochrome responses are generally known to be mediated by calcium [36, 55], cGMP [3], protein kinases [23, 34, 49, 50] or protein phosphatases [47] leading to activation of *trans*-acting factors [20, 42] that regulate the transcription of specific genes [22]. The initial events of the signal transduction chain are mediated by G proteins [3, 28, 37–39] and phosphoinositides [12]. However, a complete signal transduction pathway for any given phytochrome-regulated gene still remains to be elucidated.

The sheer number of responses regulated by phytochrome photosensory mechanism necessitates the assumption that common signal transduction pathways must exist for at least some genes. It was recently demonstrated that activation of G-proteins alone accounts for the regulation of expression of all the genes responsible for full chloroplast development and anthocyanin biosynthesis and that it is brought about by calcium- and/or cGMP-mediated pathways [3]. Similarly, after the realization that protein phosphorylation is a major event in light signal transduction [34], a functional relationship between phytochrome phototransduction and protein phosphorylation in higher plants has been suggested [10, 56] based on the recent insights obtained from lower organisms [1, 43]. It was even suggested that multiple protein kinases may account for multiple signalling pathways [56].

We have been exploring the various possible events in the phytochrome-mediated light signal transduction pathway using nitrate reductase and phytochrome genes as model systems. We have demonstrated earlier by red/far-red photoreversibility experiments that the phosphorylation status of cellular proteins changes in a light-

dependent manner in *Sorghum bicolor* [11]. We have also shown that PMA, an analogue of diacylglycerol (DAG) and a known stimulator of protein kinase C in animal systems, completely replaces red-light effects in stimulating nitrate reductase activity [4] and transcript levels in maize [44]. This effect was not due to enhanced nitrate accumulation in the presence of PMA, nor due to DMSO, the solvent in which the PMA solution was prepared [4]. The data on the effect of PMA on NR transcript levels were, however, preliminary. In this paper, we present detailed evidence to show that PMA does mimic red light in enhancing NR transcript levels, indicating the possible involvement of a PKC-type enzyme in the Pfr-mediated regulation of NR gene expression by light.

PMA has been used by others earlier, to study the involvement of PKC in plants [2, 30]. The presence of phorbol ester, calcium and phospholipid-dependent protein kinases has also been reported from rice [21, 26], wheat [29] and maize [4]. Recently, a PKC-type enzyme was partially purified from *Brassica campestris* [27] whereas in maize, it was purified to homogeneity (Chandok and Sopory, in preparation). A maize cDNA which has extensive homology to a bovine brain PKC inhibitor-coding gene has also been isolated recently [48].

Our data on the effect of PMA on *phyI* transcript levels clearly indicate that PMA mimics the effect of red light in inhibiting the accumulation of *phyI* transcripts in the dark. However, the kinetics of inhibition seems to be slower, unlike that of NR in which PMA and red light followed similar kinetics. The cause of this difference in kinetics is not clear at present and needs further experimentation. Nevertheless, it is important to note the indication from our data that a PKC-type enzyme may be involved in the Pfr-mediated down-regulation of *phyI* gene expression by light.

The fact that PMA had opposite effects on NR and *phyI* gene expression in a manner that is strikingly similar, if not identical to red light has interesting implications for explaining signal transduction. It is possible to envisage a situation in which the initial events of light signal transduction are common for the two oppositely regulated genes and divergence in the pathway is brought about by differential phosphorylation of *trans*-acting factors by PKC-type enzyme(s). It may be useful to examine other light-regulated genes from this perspective and address the question as to how many genes are regulated via PKC.

Since PMA is an analogue of DAG, a product of the PI cycle, a common signal transduction mechanism for the light regulation of NR and *phyI* genes should also have the PI cycle in common. This was verified by using 5-HT to stimulate PI turnover in the dark in our system. 5-HT is a neurotransmitter and a known stimulator of the PI cycle [51]. Even in maize, it was demonstrated in this laboratory that 5-HT acts in a manner similar to red light to enhance the uptake of calcium [9] as well as to alter the levels of various PI species [5]. Moreover, 5-HT was found to mimic the red light effect to enhance NR activity in the presence of nitrate in our system [5]. In the present study, our results on the effect of 5-HT on NR and *phyI* transcript accumulation in the dark clearly indicate that 5-HT could mimic red light effect in enhancing NR transcript accumulation and inhibiting *phyI* transcript accumulation (Fig. 6), suggesting that modulation of PI turnover is also a common event in the Pfr-mediated light signal transduction chain that controls the expression of these two genes. Our recent findings that lithium, an inhibitor of the PI cycle, interferes with the light regulation of NR as well as *phyI* transcript accumulation lend further support to these results and our studies on the effect of cholera toxin on NR gene expression indicate that a G-protein may mediate this process [32]. The involvement of a G-protein in the regulation of *phyI* gene expression has already been reported [39].

On the basis of the above findings, we propose a model (Fig. 7) which suggests that the light signal passes through Pfr, G protein, PI cycle and PKC, leading to the phosphorylation of *trans*-acting actors that bring about opposite regulation of NR and *phyI* gene expression. We are currently engaged in testing this model in further detail.

It is obvious, therefore, that apart from the

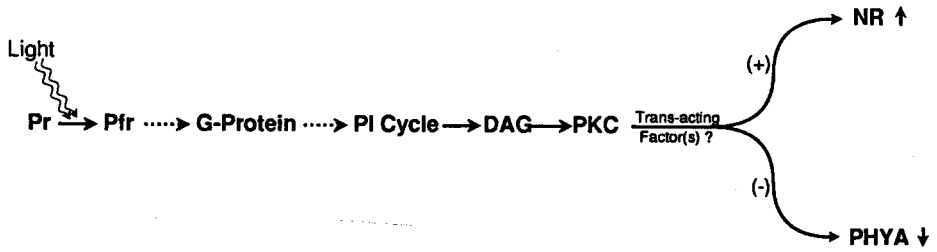

Fig. 7. Model depicting a common mechanism of signal transduction for the opposite regulation of NR and *phyI* gene expression by light (DAG, diacylglycerol, PKC, protein kinase C).

signalling pathways mediated by calcium/calmodulin [28, 36, 55] and cGMP [3], PI-mediated signalling mechanisms also exist in plants. Further, G protein may not be the only common point in phytochrome signal transduction. We propose that divergence in signal transduction could also be brought about at the protein phosphorylation stage, involving phosphorylation-dependent activation/inactivation of specific *trans*-acting factors by PKC-type enzyme(s).

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