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Light Regulation of Nitrate Reductase Gene Expression in Maize Involves a G-Protein

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This paper reports three lines of evidence to demonstrate the presence of heterotrimeric G-proteins in maize and their involvement in the regulation of nitrate reductase gene expression by light: (1) Southern blot analysis of maize genomic DNA using a human Ha-*ras* cDNA probe revealed specific bands indicating the presence of G-protein (α subunit) gene(s) in maize. Northern blot analysis of maize total RNA using the same probe revealed that the putative Gα gene(s) is transcriptionally active. (2) Western blots containing purified plasma membrane proteins from maize leaves showed specific binding of γ [^{35}S]-labeled GTP in a red light-dependent manner, indicating the involvement of G-proteins in mediating the light signal. The size of the putative Gα gene product (~45 kDa) indicates that it may be a heterotrimeric G-protein. (3) Cholera toxin mimicked the effect of red light to enhance the transcript levels of nitrate reductase (NR), indicating that G-proteins may mediate light regulation of NR gene expression.

Key Words: **nitrate reductase; G-protein; phytochrome; signal transduction; maize.**

G-proteins have been reported from several plants in the recent years by GTP binding studies, immunoblotting using heterologous antibodies and ADP ribosylation by cholera and pertussis toxins. Both heterotrimeric G-proteins and small GTP-binding proteins are known, and many of them have been cloned and sequenced in the recent years [1, 2]. Genomic and cDNA clones encoding the heterotrimeric Gα subunits have been isolated from *Arabidopsis thaliana*, tomato [2], Lotus japonicus [3], soybean [4] and rice [5, 6]. Similarly, cDNA clones for the Gβ subunit genes from maize and *Arabidopsis* have been reported [7]. However, Gγ subunit is yet to be identified in plants, and the purification or cloning of a Gα subunit from maize is yet to be reported. Heterotrimeric G-proteins have been implicated in a variety of plant responses to signals such as light, hormones and pathogens [2], as well as in the regulation of guard cell K^+ channels [8]. Light regulates a variety of physiological and developmental responses in plants by modulating the expression of a large number of genes [9]. The involvement of G-proteins in phytochrome phototransduction has been demonstrated by the ability of plasma membrane proteins to bind GTP in a red-far red reversible manner [10, 11]. Other studies have demonstrated the involvement of G-proteins in the regulation of specific genes [12–14]. However, the association of G-proteins with specific upstream and downstream events of signal transduction remains to be established, just as the complete signaling mechanism for any single gene in plants is yet to be elucidated.

We have been using nitrate reductase as a model system to study the light (phytochrome) signal transduction mechanism that leads to the regulation of gene expression in maize. Nitrate reductase (NR), the initiating enzyme of plant nitrogen metabolism, is among the first substrate-inducible enzymes known in plants and is one of the best studied enzymes of the nitrate assimilatory pathway [15]. The role of light in the regulation of NR gene expression has been reviewed recently [16]. We have shown earlier that NR is under the control of phytochrome in maize, and that light and nitrate have independent effects on the *de novo* expression of the enzyme [17, 18]. Further characterization of the phytochrome-mediated light signaling pathway for NR regulation revealed the involvement of phosphoinositide cycle [19] and a protein kinase C (PKC) type enzyme inducible by PMA [20–22]. In this paper, we examined the role of G-proteins in mediating the regulation of NR gene expression by phytochrome.

Abbreviations used: NR, nitrate reductase; Pfr, active phytochrome; *phy*, phytochrome gene; *cab*, chlorophyll a,b binding protein gene.

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MATERIALS AND METHODS

Plant material and light regimes. Excised leaves were used from 9-day-old etiolated and nitrogen-starved seedlings of maize (*Zea mays* cv. Ganga 5), grown on moist paper in plastic trays at 27 ± 1°C. Red light was given as a saturating 5-min pulse from four 100-W tungsten lamps filtered through a CBS-650 filter (Carolina Biological Supply Co., U.S.A.; emission maximum 650 nm). The intensity of red light at the plant level was 1.47 Wm^{-2}. All the manipulations were done in dim green light (0.01 Wm^{-2}) obtained by filtering the light from a cool fluorescent tube through several layers of green cellophane (emission maximum 500 nm).

Gene probes. The gene probes used in this study were derived from Maize NR cDNA clone and human normal Ha-*ras* clone, kindly provided by Prof. Wilbur Campbell (USA) Dr. Patil, CCMB (India) respectively, as recombinant plasmids. They were transformed and amplified in *E. coli* DH5α cells, plasmids isolated from them, cDNA inserts purified by standard methods [23] and used as probes.

Southern/Northern blot hybridization with Ha-ras probe. Maize genomic DNA was isolated from 9-day-old etiolated leaves as described [24] and digested separately with *Eco*RI and *Hin*dIII. The digests were electrophoresed on a 1% agarose gel (20 μg per lane) using TAE buffer at 2–3 V/cm and processed for Southern blotting onto a Genescreen plus (DuPont NEN, U.S.A.) nylon membrane as described [23] and used for hybridization. Maize total RNA was isolated by guanidine thiocyanate method [25] and electrophoresed (μg per lane) on denaturing gels containing 1.2% agarose, 6% formaldehyde and 1× Mops buffer, and processed for Northern blotting onto a Genescreen Plus nylon membrane as described [23]. Radiolabeled DNA probes were prepared from purified human Ha-*ras* cDNA fragment using [^{32}P] labeled dCTP supplied by BARC, Bombay (India), and a random primer labeling kit from NEN, U.S.A., as per the instructions of the manufacturer. The specific activity of the purified probe was 0.8×10^9 cpm/μg. Since Ha-*ras* was a heterologous probe, hybridization of both DNA and RNA blots was carried out at 25°C in a buffer that contained 6× SSC, 0.5% SDS, 5× Denhardt's reagent, 50% formamide, and 10 mM EDTA apart from denatured salmon sperm DNA (100 μg/ml) and Ha-*ras* probe (10^6 cpm/ml). The filters were initially washed twice in 2× SSC and 0.5% SDS at RT, followed by two changes in 1× SSC and 0.5% SSC at 45°C and a final wash with 1× SSC at RT, before processing for autoradiography using intensifying screens.

GTP binding assay. Plasma membrane proteins were purified from etiolated, red light-irradiated maize leaves and a dark control, by an aqueous two-phase procedure and tested by vanadate-sensitive ATPase activity assay [26]. The proteins were estimated by Bradford's microassay method [27] and subjected to SDS–PAGE using 5% stacking gel and 10% resolving gel [28]. One hundred micrograms of protein was loaded per lane in duplicate sets for this purpose. The separated proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, U.S.A.) by electroblotting [29] using Transphor-Power Lid (Hoefer Scientific, USA) at 30 V for 16 h at 4°C. The transfer was verified by staining with 0.5% (w/v) Ponceau-S in 1% (v/v) acetic acid, destained by rinsing in distilled water and used for GTP binding experiments. The blot was incubated at RT in 10 ml of 25 mM Hepes buffer containing 10 mM $MgCl_2$, 1 mM DTT and 2 mCi/ml of γ-[^{35}S]-GTP (obtained from DuPont NEN, U.S.A.) for 30 min. As a control, γ-[^{32}P]-ATP (obtained from BARC, Bombay) was used for the duplicate set. The blots were washed in the same buffer containing 0.3% Tween 20 but without the radiolabeled nucleotides. They were dried at 60°C for 30 min, dipped in 20% (w/v) PPO in toluene for fluorography, redried and autoradiography was performed.

Cholera toxin treatment and NR transcript analysis. Cholera toxin (Sigma, U.S.A.) was activated by incubation at 30°C for 1 h in a buffer containing 50 mM Hepes (pH 8.0), 20 mM DTT, 1 mg/ml BSA and 0.125% SDS. Etiolated maize leaves were excised and floated on either water or 60 mM KNO_3 containing activated cholera toxin (CT, 25 μg/ml) and incubated in the dark. Appropriate dark and red light (5-min pulse irradiation) controls were maintained. Tissue samples were collected after 2 h of induction as standardized for NR earlier [19] and processed for total RNA isolation [25]. Total RNA dot blots were generated using a Schleicher and Schuell (U.S.A.) manifold blotting apparatus and Genescreen plus (DuPont NEN, U.S.A.) nylon membrane as per the instructions of the manufacturer. The samples (20 μg RNA per dot) were prepared in 6% formaldehyde and 50% formamide and denatured at 60°C for 30 min, snap-cooled and loaded for blotting. Radiolabeled DNA probes were prepared from purified NR cDNA fragment as described above for Ha-*ras* probe. The specific activity of the probe was 2×10^9 cpm/μg. The dot blots were prehybridized for 1 h 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. Denatured probe was added subsequently (10^6 cpm/ml hybridization fluid) and hybridization was allowed proceed for 12 h. The filters were washed at least three times at 65°C in 10 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 2 mM EDTA and once at RT in the same buffer without SDS, and autoradiography was performed. The autoradiograms were scanned using Pharmacia-LKB "Ultroscan XL" scanner (U.S.A.) at

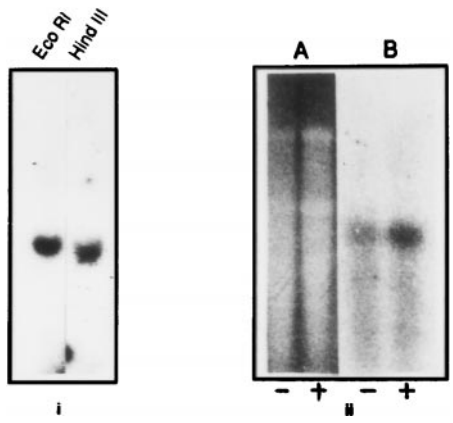

FIG. 1. Evidence for the presence of a functional, putative Gα gene in maize. (i) Southern blot hybridization analysis: Maize genomic DNA was digested with the enzymes indicated, electrophoresed on a 1% agarose gel, transferred onto Genescreen Plus membrane by capillary blotting and hybridized to a ^{32}P-labeled human Ha-*ras* cDNA probe as described under Materials and Methods. (ii) Northern blot hybridization analysis: Etiolated cut leaves were floated on water (−) or 60 mM KNO_3 (+) for 4 h and processed for total RNA isolation. The RNA was subjected to agarose gel electrophoresis in duplicate sets. One set (A) was verified by ethidium bromide staining and the other set (B) was processed for capillary blotting and hybridization analysis with a radiolabeled human Ha-*ras* cDNA probe as described under Materials and Methods.

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 average values from two independent experiments were used for plotting the data.

RESULTS AND DISCUSSION

A number of studies have implicated GTP-binding proteins in light-stimulated signaling pathways, using GTP analogues and bacterial toxins. Both phytochrome and cryptochrome, the photoreceptors for red/far red light and blue light respectively, have been shown to operate via G-proteins [2]. For example, G-proteins have been implicated in phytochrome (Pfr)-mediated regulation of *cab* and *phy* gene expression in etiolated *Avena* seedlings and dark maintained soybean suspension cultures [10, 13] and swelling of etiolated wheat protoplasts [30, 31]. Similarly, G-proteins have been implicated in Pfr-dependent protein phosphorylation in the nuclei preparations from etiolated *Avena* seedlings [32]. In alfalfa, Pfr-dependent GTP binding activity was reported in the plasma membranes of etiolated protoplasts by red/far-red photoreversibility experiments [11]. In tomato, regulation of multiple phytochrome responses by the agonists and antagonists of G-proteins was demonstrated in a unique single cell assay system developed by using the hypocotyl cells from the PhyA deficient *aurea* mutants of tomato [12, 14].

In the present study, three types of experiments were conducted to investigate the presence of G-proteins in maize and their possible involvement in Pfr-mediated regulation of NR gene expression by light. They are (a) Southern and Northern blot hybridizations using human Ha-*ras* cDNA probe; (b) GTP binding assays *in vitro* using Western blots of plasma membrane proteins purified from dark grown and red light-irradiated leaves; and (c) Analysis of NR steady-state transcript levels with and without cholera toxin in the dark.

Maize has a transcriptionally active Gα gene. To examine the possible role of G-proteins in light signal transduction and NR gene regulation in the present study, the presence of functional gene(s) coding for GTP-binding proteins in maize was examined initially. Southern blot hybridization analysis of maize genomic DNA using a human Ha-*ras* probe revealed specific, single band signals in the autoradiogram (Fig. 1, part i) under low stringency hybridization and washing conditions, which allow the retention of probe in the homology range of >60%. Using the same probe, Northern blot hybridization with maize total RNA under similar conditions also revealed specific a single band signal in the range 1.2–1.5 kb as estimated with reference to the rRNA bands (Fig. 1, part ii). It may code for a protein large enough to be identified with the

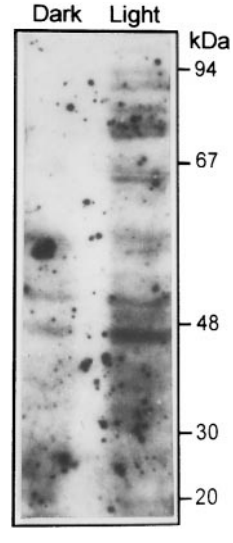

FIG. 2. Light-dependent GTP binding of plasma membrane proteins. Purified and tested preparations of plasma membranes from etiolated, red light-irradiated and dark control leaves were obtained and the proteins were separated on a 10% SDS polyacrylamide gel (100 μg per lane) and transferred onto nitrocellulose membrane by electroblotting. The positions of molecular weight markers were identified by Ponceau-S staining of the blot. The blot was incubated with γ [^{35}S]-labeled GTP, washed and autoradiography performed as described under Materials and Methods. The positions of the molecular weight markers were identified by Ponceau-S staining of the blot.

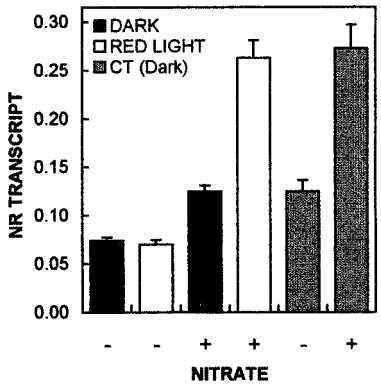

FIG. 3. Effect of cholera toxin on NR transcript levels. Etiolated cut leaves were floated on either water (−) or 60 mM KNO_3 (+) containing activated cholera toxin (CT, 25 μg/ml) and incubated in the dark. Appropriate dark and red light controls were maintained. Tissue samples were collected after 2 h of induction and processed for total RNA isolation and dot blot hybridization analysis using a radiolabeled homologous NR cDNA probe as described under Materials and Methods. The hybridization signals were quantified by densitometric scanning of the autoradiogram and the areas under the peaks (arbitrary units) were averaged from two independent experiments and plotted against their respective treatments.

α-subunit of the heterotrimeric G-protein family, since the predicted molecular weights of all the putative small GTP binding proteins known in plants fall in the range 22–24 kDa, whereas those of the putative Gα subunits of the heterotrimeric G-proteins are usually above 40 kDa [2]. These results indicate the presence of a transcriptionally active, putative Gα-coding gene in maize, which shares significant homology with the human *ras* oncogene. Interestingly, nitrate seems to enhance the putative Gα transcript level in our system (Fig. 1, part ii). Nitrate is known to act as a signal to regulate the expression of the genes of nitrogen and carbon metabolism [33], but there is no evidence for external modulation of Gα gene expression in plant literature so far. It would be interesting to examine this aspect in detail.

Maize plasma membrane proteins bind GTP in a red-light dependent manner. To verify whether light-induced signal transduction involves phytochrome-dependent binding of GTP in maize, *in vitro* GTP-binding assay was performed using a Western blot of purified and tested plasma membrane proteins and γ-[^{35}S]-GTP. The autoradiogram clearly shows red-light dependent binding of GTP by plasma membrane proteins, with the strongest signal in the 45 kDa range (Fig. 2). The dark control lane shows very little specific binding, if any. As a control for GTP, γ-[^{32}P]-ATP was used and no binding was observed (data not shown). These results are in broad agreement with the earlier biochemical evidence for the presence of heterotrimeric G proteins in maize root plasma membranes [34], and the molecular cloning of a G-protein β subunit gene in maize [7]. It is possible, though not proven, that the 45-kDa band corresponds to the protein coded by the transcript shown in the northern blot mentioned above (Fig. 1, part ii), considering the close molecular weight match between the transcript and the protein under question. The red light-dependent binding of GTP in our system indicates that it may be a Pfr-mediated response. This is consistent with earlier studies in alfalfa [11].

Cholera toxin mimics red light to stimulate NR gene expression in the dark. To examine whether phytochrome regulation of NR gene expression is mediated by a G-protein, the effect of cholera toxin on NR steady state transcript levels was studied by total RNA dot blot hybridization using a homologous NR cDNA probe. If a stimulatory G-protein is involved in transducing the phytochrome signal to enhance NR gene expression, cholera toxin would be expected to mimic the red light signal in bringing about the same response in the dark. As is evident from the data presented in Fig. 3, cholera toxin could mimic the effect of red light in enhancing the transcript levels of NR in the presence of nitrate almost to the same extent as obtainable under red light irradiation conditions. Variation in the amount of CT used (25 and 50 μg/ml) did not significantly alter the results, and the kinetics of NR induction by CT and red light were similar (data not shown). These results indicate that G-proteins are involved in Pfr-mediated regulation of NR gene expression. Cholera toxin has been used by others earlier to study the role of G-proteins in phytochrome regulation of plant gene expression [10, 32]. The detection of an ADP ribosylation factor (ARF) in plants [35] makes it possible to rely on *in vivo* ADP ribosylation by cholera toxin for these experiments. Interestingly, apart from mimicking the light effect, cholera toxin also causes slight increase in NR transcript levels even in the absence of nitrate, indicating that the toxin mimicked the effect of nitrate independently. Similar independent effects were obtained using serotonin and lithium ions in our analysis of the downstream signaling events, based on which we have recently reported that light and nitrate act through independent signaling pathways to regulate NR gene expression [36]. However, a further characterization of nitrate signaling is needed to delineate the pathways clearly.

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