Volume 282, number 2, 341–346 FEBS 09684 May 1991

ADONIS 001457939100393Z

G-proteins in etiolated *Avena* seedlings

Possible phytochrome regulation*

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Received 18 January 1991; revised version received 26 February 1991

The molecular mechanism of light signal transduction in plants mediated by the photosensor phytochrome is not well understood. The possibility that phytochrome initiates the signal transduction chain by modulating a G-protein-like receptor is examined in the present work. Etiolated *Avena* seedlings contain G-proteins as examined in terms of the binding of GTP as well as by cross-reaction with mammalian G-protein antibodies. The binding of GTP was regulated in vivo by red/far-red light. The possible involvement of G-proteins in the phytochrome-mediated signal transduction in etiolated *Avena* seedlings has been implicated from the study of the light regulated expression of the *Cab* and *phy* genes.

Avena sativa; GTP-binding protein; Photoregulation; Signal transduction

1. INTRODUCTION

Light plays a critical role in the development of plants. The photosensor molecule for the red/far-red reversible morphogenetic and developmental responses of plants is phytochrome [1,2]. The molecular mechanism of phytochrome in light signal transduction processes in plants is largely unknown, although involvement of calcium ions and the phosphorylation of proteins in the photo-signal transduction processes have been proposed in plant systems [3,4,5]. In animal signal-transduction, G-proteins play a pivotal role. These proteins belong to a highly conserved family of proteins that couple receptors to various effector systems [6]. At present, 2 families of GTP-binding proteins have been characterized; (i) heterotrimeric GTP-binding proteins (G-proteins) composed of α, β and γ-subunits (e.g. transducin), and (ii) small molecular weight GTP-binding proteins (20–30 kDa; e.g. *ras* oncogenes) [7]. Modulation of G-protein activity (GTP binding and/or its hydrolysis) by receptor-catalysis and covalent modification with bacterial toxins via ADP-ribosylation [6,8] is particularly relevant to the present study.

The presence of GTP-binding proteins in plants has been reported in crude extracts of *Lemna paucicostata* [9], in spinach thylakoid membranes [10], in zucchini hypocotyl fraction [11] and in the plasma membrane of several plants [12]. DNA sequences homologous to the oncogenes *myb*, *ras* and *src* have been detected in *Zea mays* [13], and 2 genes, one homologous to the *ras*-related gene family (*ara*) and the other to the G-protein α subunit (*GPA1*) have been recently cloned in *Arabidopsis thaliana* [14,15]. It is noteworthy that over-expression of the yeast *RAS2* gene in *Nicotiana* affects the cell viability and mitotic division [16]. The presence of GTP-binding proteins has also been reported in green algae [17,18]. In the present paper, we describe results from the study of the relationship between phytochrome and its possible effector G-protein(s) in the light signal transduction in *Avena*.

2. MATERIALS AND METHODS

2.1. *Plant material*

Avena sativa L. seedlings (cv Garry oat; Agriculver Co., Trumansburg, NY) were grown in the dark on moist vermiculite for 3.5 days at 25°C and harvested under dim green light as described previously [19]. Etiolated plant extract was prepared from fresh tissue mixed in extraction buffer (25 mM PIPES, pH 6.4, 0.24 mM EDTA, 1 mM $MgCl_2$, and 1 mM PMSF) using a ratio of 0.75 g of tissue per ml of extraction buffer. The mixture was homogenized in an Ultra

* Dedicated to Professor W. Haupt on the occasion of his 70th birthday.

Abbreviations: *BSA*, bovine serum albumin; *Cab*, chlorophyll a/b-binding protein gene; DTT, dithiothreitol; GTP-binding proteins or G-proteins, guanine nucleotide binding proteins; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PBS, phosphate buffered saline with 0.9% NaCl in 10 mM sodium phosphate, pH 7.2); *phy*, phytochrome gene; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate.

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Turrax-T25 homogenizer for 1 min at maximum speed (24 000 rpm) and centrifuged for 20 min at 5 000 × g. The supernatant, containing cytosolic and membrane fractions, was used as plant extract. Protein content was determined by using the Bio-Rad protein assay based on the Bradford method.

2.2. *GTP-binding assay*

Assay for the binding of [^{35}S]GTPγS to GTP-binding proteins was performed according to a modification of the previously described method [20]. Reaction mixtures (200 μl final volume) contained *Avena* seedling extract (50–200 μg protein) in 20 mM PIPES, pH 6.4, 0.1 mM EDTA, 0.1 M NaCl, 1.5 mM $MgCl_2$, and 0.1 mM PMSF. The reaction was initiated by adding 1 μCi of [^{35}S]GTPγS, 1320 Ci/mmol (NEN Research Products, USA) and was carried out at 25°C for 30 min. The reaction was stopped by adding 800 μl of a charcoal solution containing 7.5% (w/v) activated charcoal, 1% (w/v) BSA, 0.2% (w/v) dextran and 20 mM PIPES, pH 6.4. Samples were centrifuged at 14 000 × g for 5 min; to 700 μl of supernatant, 300 μl of charcoal solution was added and centrifuged as above. The resulting supernatant (700 μl) was mixed with 8 ml of scintillation cocktail (Research Products International Corp., USA) and the radioactivity was counted. The assays were performed under dim green light.

2.3. *SDS Polyacrylamide gel electrophoresis*

Slab gel electrophoresis was performed by the method of Doucet and Trifaró [21] using a 10%–20% linear gradient running gel and a 4% stacking gel. Samples were prepared by adding 25 μl of sample buffer (100 mM Tris-HCl, pH 6.7, 4% SDS, 100 mM mercaptoethanol, 10% glycerol and 0.01% Bromophenol blue) to 75 μl of sample and boiling for 5 min. After electrophoresis the gel was stained with Coomassie brilliant blue R-250, dried into Whatman 3MM paper and subjected to autoradiography on Kodak X-Omat AR film. A DuPont intensifying screen was used for gels containing ^{32}P.

2.4. *Western blot analysis*

The seedling proteins contained in the crude extract were separated by SDS-PAGE on a 10%–20% polyacrylamide gradient gel as described above. The proteins were electrophoretically transferred to nitrocellulose membranes. Transfer buffer containing 192 mM glycine, 25 mM Tris base and 20% methanol was used. Transfer was achieved at 100 mV for 12–16 h at 4°C. After transferring the proteins, the lane containing molecular weight markers was removed and stained with 0.5% Ponceau red (dissolved in 1% acetic acid) and the rest of the membrane was immersed in incubation solution containing 5% BSA in PBS for 2 h. Primary antibodies, anti-*ras* p21, GA/1 or SW/1 raised against animal G-proteins (NEN Research Products, USA) were diluted 1:500 in incubation buffer, added to the membrane and then incubated overnight. The membranes were washed 3 times with PBS containing 0.05% Nonidet-P40 and twice with PBS. For detection, protein A-horseradish peroxidase conjugate was used at a dilution of 1:1000, and incubated for 2 h. The membrane was then washed as described above. The blot was developed by placing the membrane in substrate solution containing 25 mg diaminobenzidine, 340 mg imidazole and 50 μl of H_2O_2 in 50 ml PBS.

2.5. *ADP-ribosylation of GTP-binding proteins*

Bacterial toxin was activated prior to use under the following conditions: for the stock solution, 5 mg of cholera toxin was dissolved in 0.5 ml of 100 mM potassium phosphate buffer, pH 8, and dialyzed against the same buffer. After dialysis, 0.5 ml of 100 mM DTT was added and the solution was allowed to stand for 2 h at room temperature. The reaction mixture for the ADP-ribosylation of GTP-binding proteins (75 μl) contained 200 mM potassium phosphate, pH 8.0, 20 mM thymidine, 5 mM DTT, 2 mM $MgCl_2$, 80 μg of seedling proteins in crude extract, 5 μg of thiol-activated toxin and 5 μCi [^{32}P]NAD^+, 800 Ci/mmol (NEN Research Products, USA). The reactions were started by the addition of [^{32}P]NAD^+. The samples were incubated for 60 min at 25°C and the reactions were stopped with 25 μl of electrophoresis sample buffer.

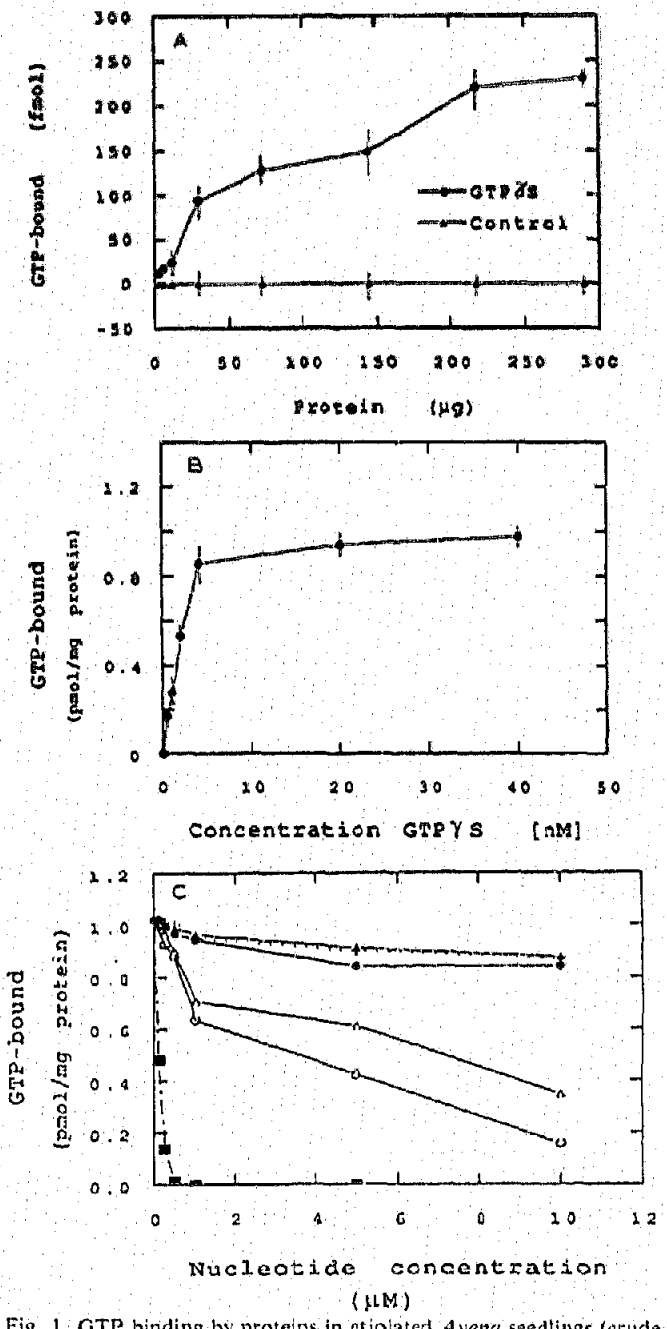

Fig. 1. GTP binding by proteins in etiolated *Avena* seedlings (crude extract). (A) Binding of GTP to GTP-binding proteins was measured in crude extract from etiolated *Avena* seedlings. The samples were incubated with 1 μCi of [^{35}S]GTPγS and the radioactivity counted in a control without crude extract was deducted from the sample counts. As a separate control, the binding of [^{35}S]GTPγS to crude extract boiled for 5 min was carried out ('control' in panel A). (B) Saturation of the binding of [^{35}S]GTPγS was measured in samples containing 175 μg of crude extract proteins as a function of increasing amounts of the nucleotide. A sample without protein containing the same amount of [^{35}S]GTPγS was used as a control and was deducted from the sample counts. (C) The binding of [^{35}S]GTPγS was measured in etiolated *Avena* seedling extract (150 μg of protein) by using the standard assay containing 0.4 nM [^{35}S]GTPγS. Nucleotides GTP (○), ATP (△), UTP (●), CTP (▲), GDP (+) and GDPβS (■) were added to the assay at the indicated concentrations and the binding of [^{35}S]GTPγS was measured. Mean value ± SE of 2 independent experiments are indicated.

2.6. *Isolation of RNA and Northern blot analysis*

Avena RNA was extracted from each sample of etiolated seedlings by using a RNAgents™ total RNA isolation kit from Promega (USA). The RNA concentration was determined by measuring the absorbance at 260 nm. RNA samples (30 μg) were separated in a formaldehyde-agarose gel and blotted onto a nylon membrane (Hybond-N⁺, Amersham) as described in [22]. The blots were prehybridized at 42°C for 6 h in 5×SSC, 5×Denhardt's solution, 0.45 mg/ml denatured salmon sperm DNA, 0.1% SDS and 30% or 50% formamide (for *Cab* and *phy*, respectively). Hybridization was performed for 48 h in the same solution containing 0.5 μg of ^{32}P-labeled DNA probe. The filters were washed for 20 min in 2×SSC and 0.1% SDS, once at room temperature and 3 times at 42°C.

The probes used for the detection of *Cab* mRNA was the *Pst*I fragment of the clone pAB96, a heterologous probe from *Pisum sativum* [23]. *phy* mRNA was detected using *Pst*I fragments of the clone pAP3.2, a homologous probe from *Avena sativa* [24]. The probes were labelled by nick translation (Amersham, USA).

3. RESULTS

The assay for the binding of [^{35}S]GTPγS, a non-hydrolyzable analog of GTP, to GTP-binding proteins has been used to characterize these proteins in animal cells and more recently in plant systems [9,20]. We have used this assay to study the presence of GTP-binding proteins in etiolated oat seedlings. Binding of GTPγS is approximately proportional to the amount of protein contained in the assay extract from the oat seedlings (Fig. 1A) and is saturable at low nucleotide concentrations (Fig. 1B). Binding of nucleotide was not observed when the plant extract was boiled for 5 min (Fig. 1A). GTP and ATP effectively competed with GTPγS for binding to the GTP-binding proteins in oat seedling extract (Fig. 1C). The binding of GTPγS was also strongly inhibited by the mammalian G-protein inhibitor GDPβS (Fig. 1C).

For immunological detection of GTP-binding proteins in plant extract, GA/1 antibody, a rabbit antiserum with specificity for the GTP binding site that recognizes common α-subunits, was used. By Western blot analysis, GA/1 antibody showed high specificity for a 24 kDa protein (Fig. 2A), while no specific reaction was observed when anti-*ras* p21 or antibodies against common β-subunit were used (SW/1) (data not shown). A 24 kDa protein from oat seedling was more specifically ADP-ribosylated by cholera toxin in the presence of GTP than in the presence of GDP or in the absence of added nucleotides (Fig. 2B). Although other

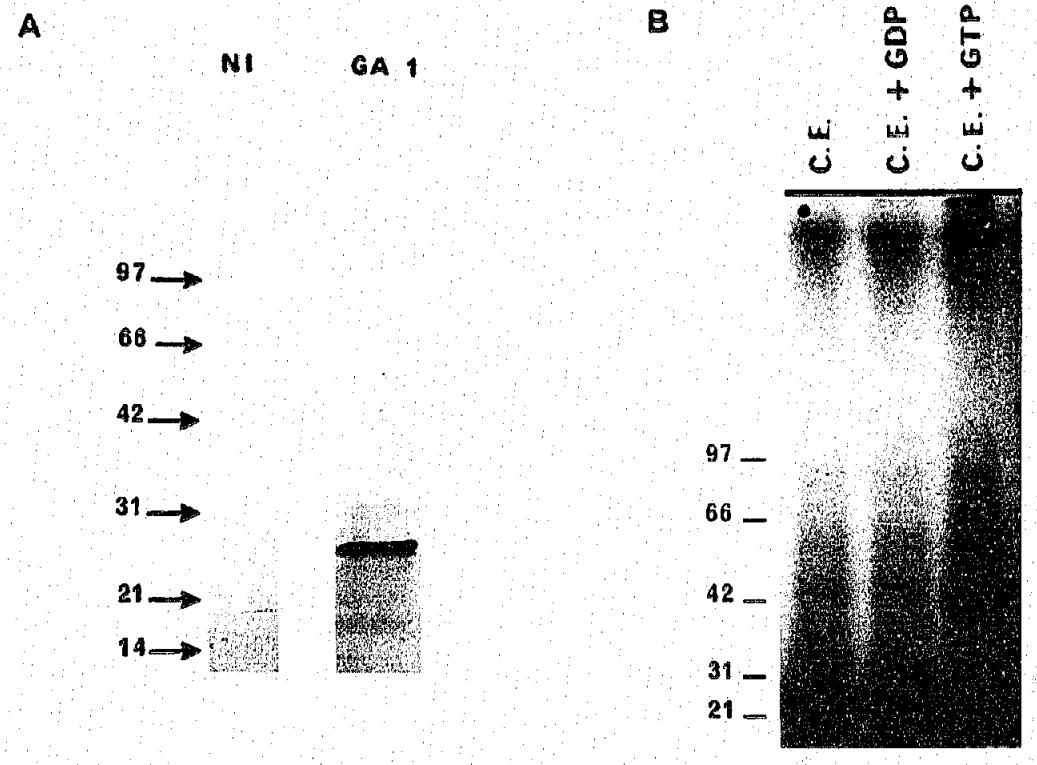

Fig. 2. Immuno-characterization and ADP-ribosylation of etiolated *Avena* seedling proteins. (A) Western blot analysis. The proteins contained in the plant extract from *Avena* seedlings were separated by SDS-PAGE using a 10%–20% linear gradient gel, transferred to nitrocellulose membrane and incubated with non-immune serum (NI) or GA/1 antibody (80 μg in 25 ml PBS) at 4°C overnight. The complex was detected with protein A conjugated to horseradish peroxidase and diaminobenzidine. Molecular weight markers were stained with Ponceau red and are shown on the left of the figure. (B) ADP-ribosylation stimulated by cholera toxin. *Avena* seedling extracts containing 80 μg of protein were incubated with activated-cholera toxin (50 μg/ml) and 5 μCi [^{32}P]NAD⁺ for 60 min at 30°C in the conditions described in the text, in the presence of GDP (3 mM) or GTP (3 mM). The [^{32}P]ADP-ribosylated proteins were analyzed by SDS-PAGE in a 10%–20% gradient gel, dried onto nitrocellulose paper and submitted to autoradiography for 5 days. A DuPont intensifying screen was used for the autoradiogram.

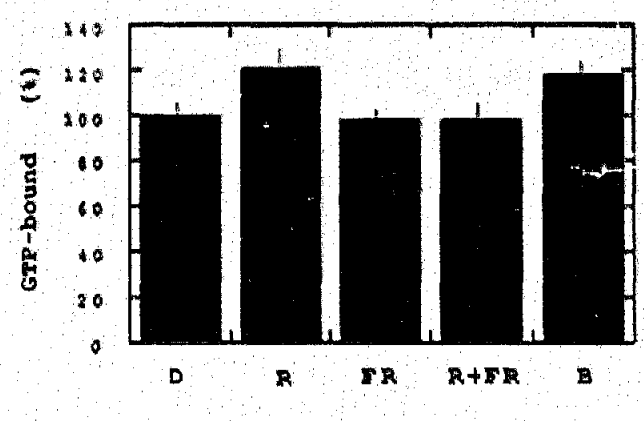

Fig. 3. Effect of different light conditions on the binding of GTP. Etiolated oat seedlings grown in the dark for 3.5 days (D) were irradiated for 5 min under the following conditions: red light (R, General Electric gold fluorescent lamp F40 GO, 8 mW/m²); far-red light (FR, Kodak safelight filter 20, 4 mW/m²); red followed by far-red light (R + FR); and blue light (B, Edmund Scientific filter, 1.2 mW/m²). After irradiation, the plants were harvested and the crude extracts prepared. The binding of GTP to the extract proteins was measured under dim green light as described in the text. Mean value ± SE of 2 independent experiments are indicated. 100% binding corresponds to 0.83 pmol of GTP/mg of protein.

proteins present in the crude extract were also ADP-ribosylated to a much less significant extent, the effect was also sensitive to the presence of GTP. These effects were not due to overloading of the samples on the gel because the Coomassie blue pattern did not show these differences (data not shown).

The binding of [^{35}S]GTPγS to GTP-binding proteins was stimulated by 21% in plants that were irradiated for 5 min with red light before homogenization (Fig. 3). This stimulatory effect was negated by far-red light. The red light stimulation was abolished when plants irradiated with red light were immediately subjected to far-red light. The red/far-red responses are typical of phytochrome-mediated reactions. Irradiation with blue light also stimulated the binding of GTP.

These results suggest that a G-protein-based signal cascade could be involved in phytochrome-mediated responses in vivo. In order to test this possibility, we examined the effect of cholera toxin on well-characterized phytochrome-regulated gene expressions in etiolated oat seedlings, namely the *Cab* and *phy* genes [1]. The *Cab* and *phy* genes are positively and negatively regulated, respectively, by red light (Fig. 4A). If the ex-

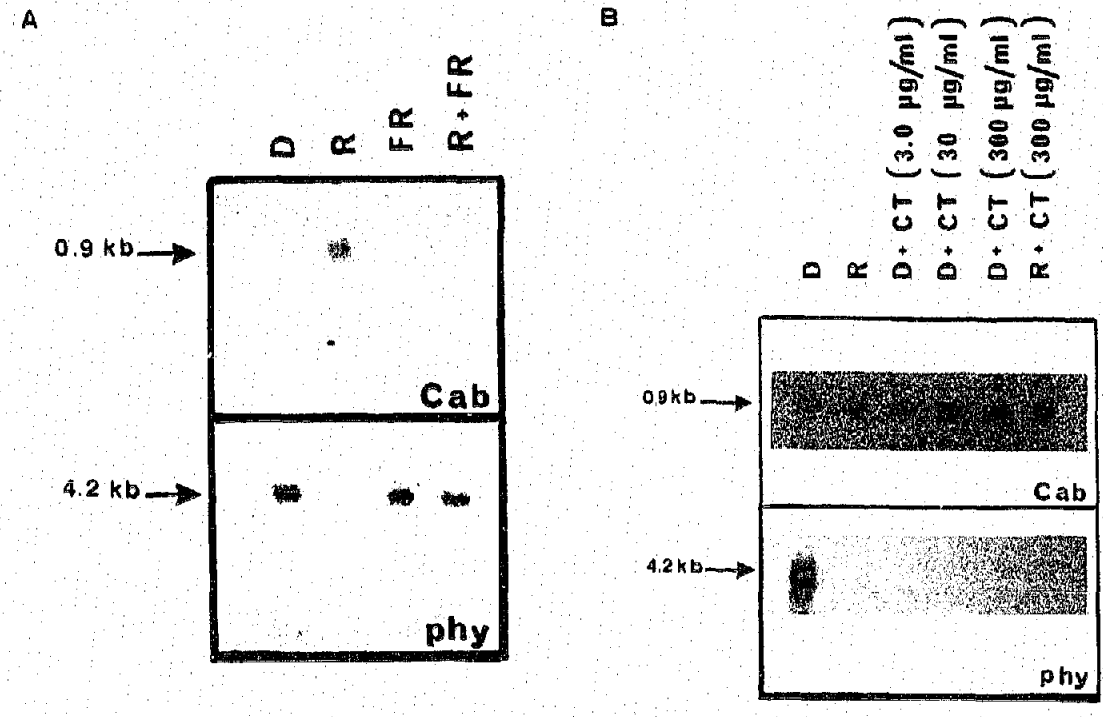

Fig. 4. Effect of cholera toxin on the light-regulated expression of Cab and phy genes. Etiolated oat seedlings (3.5-day-old) were collected, cut into 1–2 mm pieces and washed with 20 mM HEPES-KOH buffer, pH 7.0. Samples containing 1 g of tissue were incubated with 2.5 ml of HEPES buffer in darkness for 2 h at room temperature, in the absence (plant control capable of light regulation but not treated with cholera toxin, panel (A) and lines 1 and 2 in panel (B) or presence of activated cholera toxin (CT) at the concentrations indicated. After this treatment, the samples were kept in the dark (D) or irradiated with red (R), far-red (FR) or red + far-red light (R + FR) for 5 min and incubated again in darkness for 5 h before the start of the RNA isolation. RNA samples (30 µg) were analyzed by Northern blot as described in the text. Autoradiograms were exposed for 12 h for the *phy* blot and 120 h for the *Cab* blot. Longer time exposure of the *phy* blot did not show any additional bands.

pression of these genes is regulated by phytochrome (thus light) via a G-protein cascade, cholera toxin would affect the level of their gene expression. Using this approach, it is also possible to determine whether the effect of cholera toxin is due to a modification in the signal transduction chain or in the transcription step itself. Etiolated oat seedlings were incubated in different doses of cholera toxin during a 2 h dark incubation. The plants were then kept in the dark (dark control), irradiated with red light (light control), or kept in the dark for 5 h in the presence of activated cholera toxin. *Cab* expression was up-regulated by cholera toxin, whereas the *phy* gene was down-regulated (Fig. 4B). Thus, remarkably, cholera toxin can regulate the expression of these genes in the absence of a light signal.

4. DISCUSSION

The data presented in Figs 1 and 2 demonstrate that GTP-binding proteins are present in etiolated *Avena* seedling, as assayed by the method of Hasunuma et al. [9,20] and by Western blot. The nucleotide competition experiments suggest that, in contrast to mammalian G-proteins, the *Avena* GTP-binding proteins have less specificity for GTP, and ATP could compete for the binding domain. This specificity is similar to the results observed in *Lemna paucicostata* [9].

The GA/1 antibody showed a high specificity for a 24 kDa protein, in contrast to other antibodies used. This protein has a molecular weight similar to the protein encoded by the *ara* gene in *Arabidopsis thaliana* which is homologous to the *ras*-related gene family [15]. In addition, a light-dependent 24 kDa G-protein has recently been detected in the eyespot of *Chlamydomonas reinhardtii* which also immunoreacts with common α-subunit antisera [18]. Cholera toxin specifically ADP-ribosylated the 24 kDa protein (Fig. 2B). *The SDS-PAGE of both experiments (Figs. 2A and 2B) were performed with the same gel system.* The mobility of the molecular weight markers was linear when represented on the log scale for the molecular weight. Resolution of the gel was sufficient to establish the identity of the 24 kDa band in Figs 2A and 2B.

Since the ADP-ribosylation of the 24 kDa protein was sensitive to the presence of GDP or GTP, it can be suggested that the toxin target is indeed a GTP-binding protein. It is possible that there is a slow turnover between the active and inactive forms, depending on the rate of GTP-binding and GTPase activity of the protein(s). This would account for the observation that ADP-ribosylation is higher in the presence of GTP as the proportion of the active form of G-protein(s) is greatest in this case. Although ADP-ribosylation of small G-proteins in animal cells by cholera toxin has not been reported, rho proteins (a small G-protein family) can be ADP-ribosylated by a botulinum toxin [25].

Since red light stimulated GTP binding in etiolated *Avena* seedling in vitro, and far-red light abolished the light stimulated GTP binding (Fig. 3), it is possible that G-protein(s) are involved in the phytochrome-mediated light signal transduction in *Avena*. At first glance, a 21% light-stimulation of GTP binding appears to be small. However, this amount of light stimulation may be physiologically significant, since phytochrome may activate only a G-protein involved in light-signal transduction, while the bulk of the G-protein pool is not light-activatable. Interestingly, blue light also stimulated the binding of GTP to its receptor(s) in the extract by ca 17% (Fig. 3). The observed stimulation in GTP binding can be explained in terms of both phytochrome and blue light receptors, since phytochrome itself significantly absorbs the blue light used. Secondly, the phenomenon observed, i.e. GTP binding, may not be due to the so-called high irradiance reaction (HIR), thus requiring a low fluence for the observed blue light effect. Recently, a blue light-dependent GTP-binding protein has been detected in pea plasmalemma [26]. It is unknown if the blue light effect in *Avena* is due to a G-protein similar to the pea protein.

The hypothesis that the phytochrome-mediated light signal transduction is mediated by the activation of a G-protein is supported by the effect of cholera toxin on the expression of *Cab* and *phy* genes, shown in Fig. 4B. Covalent modification of GTP-binding proteins by cholera toxin is known to induce the inhibition of GTPase and stabilizes the protein in its active form [6,8,27]. It is significant that cholera toxin elicits a positive expression of the *Cab* gene, while the *phy* gene is down-regulated. Thus, cholera toxin essentially duplicated the light response patterns of these 2 gene expressions. We were not able to find a good negative control for this experiment, since available genes typically used as controls are also regulated by hormones (processes also regulated by GTP-binding proteins in animal systems). In this regard, it is significant that *phy* gene is down-regulated by cholera toxin, thus serving as a negative control to the up-regulation of *Cab* gene. The involvement of a GTP-binding protein in phytochrome-mediated responses in plants is also supported by an observation reported in etiolated wheat protoplast. Bossen et al. [28] observed that the Ca^{2+}-dependent, red light-induced swelling of mesophyll protoplasts was inhibited by GDPβS, and in darkness or after control irradiation with far red, GTPγS induced the swelling to the same extent as after red light treatment.

In conclusion, the present results suggest that a G-protein(s) is a signal transducing component following the phototransformation of phytochrome from its Pr to Pfr form. The observation that phytochrome accelerates the inositolphospholipid turnover via Ca^{2+} ion mobilization [29] and activates a protein kinase [30] is consistent with the present hypothesis. At present, we are not able to indicate whether or not the detected

small G-protein in etiolated *Avena* seedlings, which can be ADP-ribosylated to cholera toxin, is responsible for the described effect. Clearly, more study is warranted to explore the hypothesis presented here. Work is in progress to establish the role of G-proteins in phytochrome-mediated signal transduction in general and that of a 24 kDa small G-protein in particular.

Acknowledgements: We thank Dr Koji Hasunuma for his help with the GTP-binding assay, Dr Nam-Hai Chua and Dr Peter Quail for the pAB86 and pAP3.2 samples, respectively. We also thank Dr Robert J. Spreitzer for his help with the gene expression experiments. This work was supported in part by the U.S.P.H.S. NIH grant GM 36956.

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